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Alexander Birbrair *Editor*

# Stem Cells Heterogeneity in Cancer

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# Stem Cells Heterogeneity in Cancer

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# Preface

This book's initial title was "Stem Cells Heterogeneity." However, due to the current great interest in this topic, we were able to assemble more chapters that would fit in one book, covering stem cell biology under distinct circumstances. Therefore, the book was subdivided into three volumes entitled: *Stem Cells Heterogeneity-Novel Concepts*, *Stem Cells Heterogeneity in Different Organs*, and *Stem Cells Heterogeneity in Cancer*.

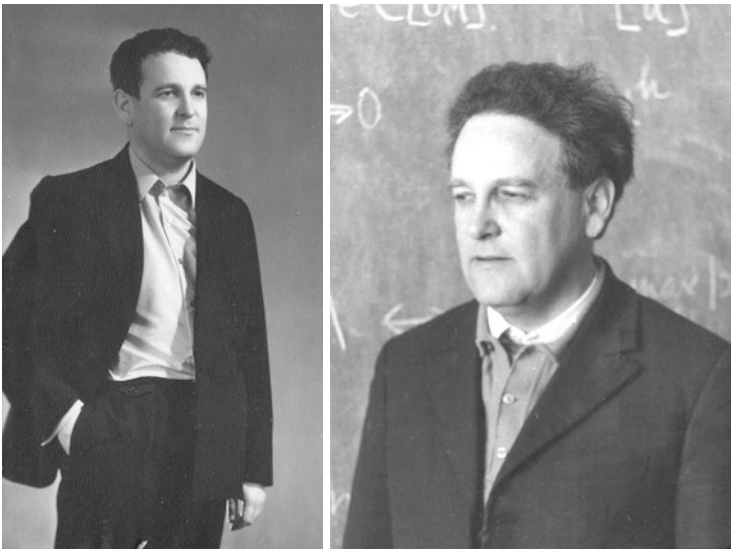
This book, *Stem Cells Heterogeneity in Cancer*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of stem cells to different cancer types. Further insights into the biology of stem cells will have important implications for our understanding of organ development, homeostasis, and disease. The authors focus on the modern methodologies and the leading-edge concepts in the field of stem cell biology. In recent years, remarkable progress has been made in the identification and characterization of stem cells in several tissues using state-of-the-art techniques. These advantages facilitated the identification of stem cell subpopulations and definition of the molecular basis of stem cell role within different organs in disease conditions. Thus, the present book is an attempt to describe the most recent developments in the area of stem cell heterogeneity which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the stem cells in cancer. Twelve chapters written by experts in the field summarize the present knowledge about stem cell heterogeneity in cancer.

Theo Mantamadiotis and colleagues from the University of Melbourne, Alice Hoy Building, discuss the heterogeneity of glioblastoma stem cells. Andreas E. Albers and colleagues from Berlin Institute of Health describe the heterogeneity in head and neck squamous cell carcinoma stem cells. D. Prabavathy and Niveditha Ramadoss from Sathyabama Institute of Science and Technology compile our understanding of small cell lung cancer stem cell heterogeneity. Caecilia Sukowati from the University of Udine updates us with what we know about heterogeneity of hepatic cancer stem cells. Joana Paredes and colleagues from the University of

Porto summarize current knowledge on the heterogeneity and plasticity of breast cancer stem cells. Mary Hendrix and colleagues from Shepherd University address the importance of melanoma stem cell heterogeneity. Hiroyuki Tomita and colleagues from Gifu University focus on the heterogeneity of colon cancer stem cells. Jiri Hatina and colleagues from Charles University introduce our current knowledge about the heterogeneity of urothelial cancer stem cells. Theodoros Karantanos and Richard J. Jones from Johns Hopkins University talk about the heterogeneity of acute myeloid leukemia stem cells and its clinical relevance. Marc G. Berger and Céline Bourgne from Clermont Auvergne University talk about the contribution of chronic myeloid leukemia as a disease model to define and study clonal heterogeneity. Dominique Heymann and colleagues from the University of Sheffield focus on osteosarcoma stem cell heterogeneity. Finally, Alain G. Zeimet and colleagues from Medical University of Innsbruck give an overview of the heterogeneity of ovarian cancer stem cells.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deepest gratitude to Veranika Ushakova, my wife, and Mr. Murugesan Tamilsevan, from Springer, who helped at every step of the execution of this project.

This book is dedicated to the memory of my grandfather Pavel Sobolevsky, PhD, a renowned mathematician, who passed away during the creation of this piece.



**My grandfather Pavel Sobolevsky z"l, PhD (March 26, 1930–August 16, 2018)**

Belo Horizonte, MG, Brazil — Alexander Birbrair
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# Chapter 1
# Multilayered Heterogeneity of Glioblastoma Stem Cells: Biological and Clinical Significance

**Daniel V. Brown, Stanley S. Stylli, Andrew H. Kaye, and Theo Mantamadiotis**

**Abstract** Glioblastoma is a primary tumor of the brain with a poor prognosis. Pathological examination shows that this disease is characterized by intra-tumor morphological heterogeneity, while numerous and ongoing genomic analysis reveals multiple layers of heterogeneity. Intra-tumor and patient-to-patient heterogeneity is underpinned by cellular, genetic, and molecular heterogeneity, which is thought to be key determinants of time to tumor recurrence and resistance to therapy. The key cell type believed to contribute to the establishment and ongoing evolution of tumor heterogeneity is a glioma stem cell (GSC) subpopulation. In this chapter, we review, highlight, and discuss controversies and clinical relevance of glioblastoma heterogeneity and its cellular basis. Characterization of how cancer stem cells (CSCs) behave is important in understanding how tumors are initiated and how they recur following initial treatment.

**Keywords** Glioblastoma · GBM · Glioma · Brain cancer · Astrocytoma · Stem cells · GSC · Heterogeneity · Plasticity · Clonal · Subclonal

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## 1.1 Introduction

The original name of the major grade IV astrocytoma, glioblastoma multiforme (GBM), describes the morphological diversity of tumor cells in histological specimens. Glioblastoma is a primary cancer of the central nervous system with limited therapeutic choices available and continues to be one of the most lethal types of tumors (Kirkpatrick et al. 2017). The diagnosis of GBM is made difficult by this heterogeneity in biopsied material (Jung et al. 2011). In addition to this gross intra-tumoral heterogeneity, genome-wide analysis of spatially distinct tumor pieces and single cells has uncovered extensive genetic diversity (Gerlinger et al. 2012; McGranahan and Swanton 2012; Sottoriva et al. 2013). Understanding the extent and source of this heterogeneity is the key to understanding why resistant subclones emerge and therapy fails for the cancer patient. The current standard of post-surgery care is radiotherapy, in combination with the oral chemotherapeutic, temozolomide (TMZ). Due to the anatomical location and the diffuse nature of GBM, complete resection of the tumor is difficult and residual malignant cells invariably cause relapse.

Another cause of this relapse has been suggested to be due to the presence of glioblastoma stem cells (GSCs) (Singh et al. 2004). A functional readout, such as tissue reconstitution is the definitive proof for true stem cell potential, but such an approach requires purification of the stem cells by fluorescent activated cell sorting (FACS), using antibodies directed at specific and unique cell surface markers. GSCs can be prospectively isolated based on the expression of the membrane-associated glycoprotein CD133, which is encoded for by the prominin-1 (PROM1) gene (Marzesco et al. 2005), although there are conflicting reports on the suitability of CD133 as a GSC marker (Kim et al. 2011; Stieber et al. 2014), since CD133 is differentially glycosylated, leading to variable epitope masking. Another putative GSC marker is CD44, which is a ligand of hyaluronic acid (HA), a major component of the extracellular matrix (Pietras et al. 2014). Based on gene expression profiling of hundreds of patient GBMs, The Cancer Genome Atlas (TCGA) analysis identified four distinct molecular subtypes: proneural (PN), classical, neural, and mesenchymal (MES) (Verhaak et al. 2010). Aside from the IDH1 mutant GBMs, there is no significant difference in long-term patient survival between these four subtypes, despite activation of different biological networks (Verhaak et al. 2010). Patient-derived GSCs grown in vitro exhibit a similar molecular classification to the parental tumor from which they originate, with two dominant cell types representing the PN and MES subtypes (Brennan et al. 2013; Phillips et al. 2006; Verhaak et al. 2010). More recently, genome-wide analysis of different regions within the same tumor or single cells derived from the same tumor demonstrated that multiple molecular subtypes exist in the same tumor mass (Patel et al. 2014; Sottoriva et al. 2013) and there appears to be a stable tumor-specific equilibrium with respect to the proportion of different molecular subtypes in a GBM tumor. Notably, there appears to be inherent plasticity in the cellular makeup or cellular heterogeneity equilibrium of GBM tumors, which can shift in response to cytotoxic therapies. Indeed, it is becoming clearer that stem cell hierarchies and heterogeneity across different

cancers may be much more plastic than previously appreciated, which makes the identification and targeting of CSCs more challenging than previously thought. This in turn means that to try and identify and target CSCs requires a deeper understanding of the molecular and genetic mechanisms regulating the cells heterogeneity. If GBM follows a stem cell model, then ablating the underlying GSCs will be sufficient to eliminate the tumor bulk (Fig. 1.1a). The distinct signaling mechanisms

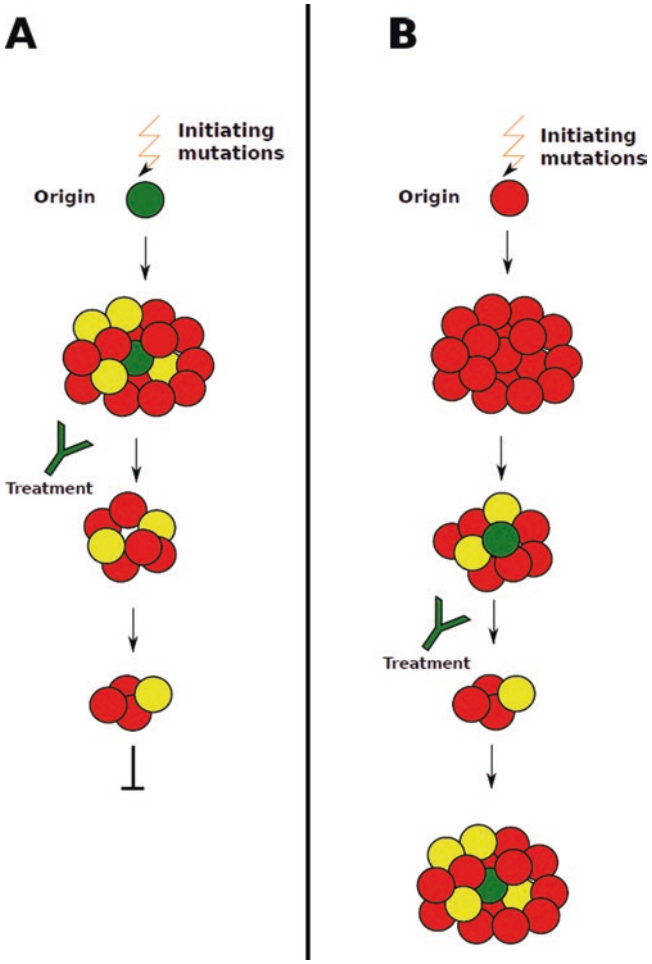


**Fig. 1.1** Models of cancer stem cell hypothesis. (**a**) The hierarchical model posits that the proliferative potential of a malignant tumor resides in a rare subpopulation (green) with a resemblance to a normal stem cell. The CSC can self-renew and differentiate into the cell types that dominate the tumor mass. Upon treatment with an agent that targets the stem cell fraction, the bulk of the tumor ultimately exhausts its replicative ability. (**b**) The plasticity model states that the stem-like subpopulation (green) arises through infrequent, stochastic dedifferentiation of cells in the tumor bulk. Upon treatment with an agent that targets the CSCs, the stem-like fraction can be regenerated by the remainder of the tumor bulk and recurrence occurs

orchestrating CSC growth, cell cycle dynamics, differentiation, dedifferentiation, and survival following cytotoxic challenges have been described in multiple classes of tumors (Magee et al. 2012; Nguyen et al. 2012).

Many CSC markers used in solid tumor biology have been adopted from studies on hematopoietic stem cells. In the hematopoietic system, only a small proportion of cells residing in the bone marrow are able to reconstitute the entire hematopoietic system of a lethally irradiated mouse (Morrison and Weissman 1994). These cells may be further subdivided into those with the ability to maintain the hematopoietic system for a lifetime (true hematopoietic stem cells) and those that could renew for a limited period of 2–3 months (transient stem cells). True hematopoietic stem cells can give rise to transient reconstituting stem cells but the reverse is not possible (Morrison et al. 1997).

The first cancer demonstrated to be driven by cancer stem cells was acute myeloid leukemia (AML) in mice (Bonnet and Dick 1997). The CD34+, CD38− hematopoietic cell subpopulation retained the ability to engraft into host immunodeficient mice and also had the ability to generate the full spectrum of cell lineages observed in the original cancer (Morrison and Weissman 1994). However, not all AML subtypes appear to follow the cancer stem cell model. In acute promyelocytic leukemia CD34+, CD38+ cells that harbor the *t*(15:17) translocation are the most tumorigenic subpopulation (Bonnet and Dick 1997). Conversely, solid tumor CSCs were first identified in breast cancer (Al-Hajj et al. 2003), followed by their identification in other cancer types, including brain (Singh et al. 2003).

## 1.2 Cancer Stem Cell Markers in GBM

Research on CSCs has typically relied on their enrichment from mixed cellular populations based on the expression of an extracellular marker or combination of markers by fluorescent activated cell sorting (FACS). Therefore, the choice of marker is critical in cancer stem cell research. The strictest criteria for CSC markers in GBM require that cells expressing the marker be enriched for clonogenic potential in vitro, be capable of differentiation into oligodendrocytic, astrocytic, and neuronal lineages, and most critically be tumorigenic at low inoculum in vivo.

## 1.3 CD133

CD133, expressed by the prominin-1 (*PROM1*) gene, was originally described as an epitope enriched in a hybridoma screen against CD34 expressing hematopoietic progenitor cells (Yin et al. 1997). The protein is a 115-kDa membrane-associated glycoprotein with no single specific molecular function. The gene is conserved in humans, mice, worms, and flies (Weigmann et al. 1997). CD133 is expressed by neural progenitor cells from human fetal brains (Uchida et al. 2000).

The expression of CD133 mRNA and protein in the adult brain also exhibit some species-specific properties (Holmberg Olausson et al. 2014). CD133 mRNA is predominately co-expressed with Olig2 in mice, in contrast to normal human tissue and GBM where CD133 mRNA is co-expressed with GFAP. In both humans and mice there is a rare subpopulation of CD133 expressing cells within the white matter component of the cortex. Unlike CD133+ cells of the SVZ (subventricular zone), this is a slowly cycling subpopulation, as shown by BrdU labelling. The expression of CD133 mRNA in GSCs is highly variable with 59% of lines expressing low to absent levels of transcript and 23% of GSCs expressing CD133 mRNA in all cells (Holmberg Olausson et al. 2014; Son et al. 2009).

Despite initial reports describing CD133 as a marker of CSCs in GBM, CD133− cells also display clonogenic potential, although at a three-fold lower efficiency compared to CD133+ cells in a mouse GBM model (Beier et al. 2007). CD133− cells were as tumorigenic as CD133+ when transplanted in a rat brain model (Wang et al. 2008). A small subpopulation of cells from the resulting tumors re-expressed CD133. Overall the data suggests that CD133 is not a universal marker of tumor initiating cells in all tissues as CD133− cells are as tumorigenic as CD133+ cells in colorectal and gastric cancer (Rocco et al. 2012; Shmelkov et al. 2008). Moreover, the variable and complex nature of CD133 expression and potential post-translational modifications means that great care must be taken when selecting and using CD133 antibodies and in the interpretation of published studies.

## 1.4 CD44

CD44 was originally identified as a receptor for hyaluronan which is a large hydrophilic polysaccharide that forms a large component of the extracellular matrix of connective tissues (Aruffo et al. 1990; Toole 2004). The interaction of CD44 and hyaluronan has been demonstrated to promote leukocyte intravasation, cell migration, and metastasis of tumors. CD44 is a large protein consisting of a molecular weight of 80–250 kDa depending on the isoform. In addition to the standard isoform which is the shortest, multiple variant isoforms of CD44 are expressed in epithelial tissues and under disease conditions. CD44 is also extensively glycosylated (Thorne et al. 2004; Zöller 2011). An alternate isoform, CD44v6 is primarily expressed during embryonic and hematopoietic development and it is upregulated in cancer and correlates with tumor grade and invasiveness (Athanassiou-Papaefthymiou et al. 2014; Günthert et al. 1991; Ruiz et al. 1995; Yu et al. 2010; Zhao et al. 2015). The CD44v6 isoform promotes interactions between hyaluronan and endothelial cells and promotes tumor invasion into the blood stream (Toole 2004).

GSCs expressing CD44 are enriched for sphere forming potential at low density (Anido et al. 2010). As few as 1000 CD44+ cells are tumorigenic in mice, whereas CD44− cells are not tumorigenic. CD44+ tumors also recapitulate the heterogeneity of the parental GBM with respect to morphology and expression of neural maturation

markers such as Sox2 and Nestin. Based on these findings, CD44 has been suggested to be a marker of CSCs in GBM (Anido et al. 2010; Jijiwa et al. 2011).

Tumors from a PDGFR overexpressing Ink4a/Arf KO mouse model of PN-GBM express CD44 only in the perivascular niche (Pietras et al. 2014). In human GBM, CD44 perivascular staining was observed only in the PN subtype. For both MES and classical subtypes, CD44 was expressed throughout the tumor bulk. This subtype-specific expression suggests that CD44 may be used as a CSC marker only in the PN subtype of tumors.

## 1.5 CD15

CD15, also known as stage-specific embryonic antigen-1 (SSEA-1) or Lewis X, is a large carbohydrate moiety expressed on the surface of embryonic cells (Capela and Temple 2002; Muramatsu 1984). The CD15 antigen is a product of the *FUT4* gene. The FUT4 protein has a role in catalyzing the fucosylation of carbohydrates at the cell surface distinct from CD15. The CD15 antigen is expressed in higher fractions of neural cells in the SVZ compared to CD133 (Son et al. 2009). CD15 is also more frequently detected by FACS in GBM tumor specimens compared to CD133, with 95% of primary GBMs containing a CD15+ subpopulation compared to 58% of GBMs containing CD133+ cells. CD15+ GSCs are more proliferative, clonogenic, and tumorigenic relative to CD15− cells (Son et al. 2009).

Lineage tracing experiments with a mixed population of CD15 positive and negative cells suggest that CD15+ cells are up to 20 times more clonogenic in vitro. This effect is more pronounced in vivo where CD15+ cells have 100-fold greater ability to generate tumors. The few tumors generated from CD15− xenografts re-express CD15 (Son et al. 2009). More recently CD15 has been identified as a marker of PN cells (Bhat et al. 2013).

## 1.6 ALDH1A3

Aldehyde dehydrogenases are a group of enzymes that catalyze the oxidation (dehydrogenation) of aldehydes (Ikawa et al. 1983). These enzymes participate in a wide variety of biological processes including the detoxification of exogenously and endogenously generated aldehydes. In hematopoietic stem cells, ALDH enzymes detoxify toxic substances such as cyclophosphamide to preserve the integrity of the stem cell compartment (Donnenberg and Donnenberg 2015).

GSCs sorted based on ALDH1A3 expression have increased sphere formation, express the stem cell markers nestin and musashi, and are capable of differentiation into multiple neural lineages (Choi et al. 2014; Rasper et al. 2010). ALDH+ cells are tumorigenic in mice at a dose of 5000 cells whereas ALDH− cells are not. These tumors are able to engraft in secondary recipients, further illustrating self-renewal

capacity (Choi et al. 2014). Expression of ALDH1A1 in GBM correlates with resistance to TMZ and treatment of GSCs with the ALDH1 inhibitor 4-diethylaminobenzaldehyde (DEAB) or knockdown of ALDH1 sensitizes GBM cells to TMZ (Schäfer et al. 2012).

## 1.7 Predictive Capacity of Cancer Stem Cell Signatures in GBM

The clinical significance of CD133 and other stem cell markers are controversial. Immunohistochemistry does not show a reproducible, statistically significant association of CD133 or CD15 expression with survival (Kim et al. 2011). This is in contrast to studies at the mRNA level demonstrating that CD133 expression is a significant negative prognostic factor for both progression-free and overall survival in GBM (Metellus et al. 2011).

A confounding feature of many gene signature analyses is the correlation of many gene expression profiles with proliferation (Venet et al. 2011). Our previous work analyzing a panel of GSC markers showed that gene co-expression modules characteristic of the GSC markers CD133 or oligodendrocyte lineage transcription factor 2 (OLIG2) were enriched in PN tumors, while a CD44 gene co-expression module was enriched in MES tumors. Cells expressing CD133 were more proliferative, cells expressing CD44 were more invasive (Brown et al. 2017), and differential expression of CD133/Olig2 or CD44 predicts response to radiotherapy (Bhat et al. 2013; Halliday et al. 2014; Meng et al. 2014). Therefore, an association of a stem cell signature with survival may instead represent the proliferative capacity of a tumor.

## 1.8 Cancer Stem Cells and the Cancer Stem Cell Model

### *1.8.1 A Cancer Stem Subpopulation in GBM*

Using in vitro culture conditions optimized for neural stem cells, a cancer stem cell subpopulation was identified in GBM (Galli et al. 2004; Singh et al. 2003). These glioma stem cells (GSCs) were able to form spheroids in suspension when plated as single cells, indicative of self-renewal capacity. GSCs were characterized by the expression of the stem cell marker CD133 and were able to establish tumors when as few as 100 cells were transplanted into immune deficient NOD-SCID mice (Singh et al. 2004). Molecular studies have further supported this stem cell origin with deep exome sequencing of normal adjacent cells from the subventricular zone uncovering a small proportion of cells with driver mutations that match the primary tumor (Lee et al. 2018).

Other studies have further subdivided GSCs into three subtypes based on malignant potential (Chen et al. 2010). Type 1 GSCs are the most malignant and type 3 the least malignant. Interestingly, intermediate type 2 cells with moderate tumorigenic potential express CD133, but types 1 and 3 do not. This suggests a hierarchy of tumor phenotypes with CD133− cells being both the most and least malignant (Chen et al. 2010). Without an extracellular marker to distinguish type 1 and 3 cells, purifying these subpopulations and characterizing them their remains challenging.

### *1.8.2 Hierarchical and Plasticity Cancer Stem Cell Models*

The cancer stem cell theory has been subject to refinement over time. The original model of CSCs, as constructed from observations in hematopoietic malignancies, presented a hierarchical model where a multipotent progenitor cell can both self-renew and differentiate into more restricted progeny (Bonnet and Dick 1997). However, genomic analysis of clinical tumor samples has uncovered substantial heterogeneity that is not consistent with the hierarchical CSC model alone (Kern and Shibata 2007; Navin et al. 2011; Shackleton et al. 2009). The hierarchical model also imposes the limitation that only the CSC fraction can initiate tumors. There are some classes of tumors that may be initiated by injection of a few or even a single cell indicating that tumorigenic potential is not rare for these cancers (Kelly et al. 2007; Quintana et al. 2008). These more recent studies were performed with more rigorous transplantation procedures using highly immunocompromised mice or syngeneic models, compared to the original studies that conceived the CSC hypothesis (Al-Hajj et al. 2003; Singh et al. 2003).

There is also evidence of interconversion from non-stem cells to stem cells. Human mammary epithelial cells (HMECs) grow adherently in culture, but a small subpopulation is able to grow in suspension (Chaffer et al. 2011). Flow cytometry analysis reveals an enrichment of stem cells over non-stem cells in the subpopulation of cells in suspension. Single-cell cloning and in vivo transplantation indicate that non-stem cell and stem cells can interconvert, albeit rarely. The development of induced pluripotent stem cells (iPSCs) has further shown that many fully differentiated cells are able to assume a stem-like state upon overexpression of three intrinsic factors: KLF4, SOX2, and OCT4 (Takahashi and Yamanaka 2006).

Maintenance of molecular subtype expression signatures in GBM is more plastic than initially believed. Gene expression profiling of matched primary tumor specimens and GSCs indicates that neural stem growth media produces a shift from a MES subtype in the original tumor to a PN identity in vitro (Baysan et al. 2014; Bhat et al. 2013). This is only partially related to growth in vitro as analysis of tumor xenografts indicates only a subset of PN shifted cell cultures can return to a MES subtype in vivo. In these instances of reversion to MES, protein expression of some mesenchymal markers is still lost.

GSCs also acquire a more G-CIMP like epigenetic configuration under in vitro growth conditions as promoter methylation analysis of 11 genes characteristic of

CIMP status reveals that cell cultures that are shifted from a MES to a PN phenotype also exhibit CpG hypermethylation, despite CIMP occurring in less than 10% of primary GBM tumors.

At present it is unclear whether this MES to PN shift is due to an initial selection of a PN subpopulation in vitro or is the result of adaptation to the microenvironment of neural stem cell medium. In addition to cell culture media, therapeutic agents and extracellular ligands are also able to reprogram PN cells to a MES cellular identity (Bhat et al. 2013; Halliday et al. 2014; Mao et al. 2013).

Plasticity between different cellular fates in normal neural stem cells is epigenetically controlled, where promoters of lineage-specific regulators are poised in a bivalent epigenetic configuration characterized by histone H3Me4 and H3Me27 marks. Epigenetic marks have been reported to change dynamically to control cell identity rather than remaining as static landmarks (Burney et al. 2013; Hu et al. 2012; Papp and Plath 2013). However, resetting the epigenetic landscape by induced pluripotent stem cell (iPSC) reprogramming fails to suppress the malignant behavior of GBM cells, suggesting mutations play a strong role in specifying the oncogenic state (Stricker et al. 2013).

### *1.8.3 Cellular Plasticity and Metastability*

Not all cancer cells can undergo a complete EMT to become mesenchymal cells. The majority of EMTs that occur in the context of cancer are not complete, such that cells remain in an intermediate or metastable cellular state (Chambers et al. 2007; Hayashi et al. 2008). This metastable state is characterized by the expression of both epithelial and mesenchymal markers.

The phenomenon of phenotype switching between cellular states has been described in melanoma cells (Hoek et al. 2008; Li et al. 2015; Verfaillie et al. 2015). Gene signature analysis of melanomas reveals two distinct clusters of tumors, proliferative and invasive (Bittner et al. 2000). The invasive melanomas have downregulated the expression of genes of the melanocyte lineage becoming more EMT-like. Cell lines from proliferative tumors grow more quickly in vitro and invasive tumors are more migratory. However, when these distinct cell lines are transplanted into mice, the resulting tumors contain cells from both proliferative and invasive states indicating bidirectional conversion between cellular states (Hoek et al. 2008). There is an interaction between oncogenic signaling and phenotype switching in melanoma (Caramel et al. 2013). Activation of *BRAF* by somatic mutation downregulates SNAIL2 and ZEB2 with concomitant upregulation of EMT promoting transcription factors, TWIST1 and ZEB1 with associated gain of migratory features.

Conversion between different cellular states has also been demonstrated and quantified in breast cancer cells (Gupta et al. 2011).

Stem-like cells in breat cancer cell cultures exhibited a cell line-specific propensity to transition into a basal or luminal state, with a preference for the dominant subpopulation in the mixed culture. Basal cells tended to self-renew, while luminal

cells exhibited a cell line specific propensity to self-renew or convert toward the basal state. This data is consistent with observations that luminal breast cancers are less efficient at dedifferentiation, compared to basal tumors (Chaffer et al. 2013).

The malignant state with associated oncogenic signaling has been proposed to increase the probability of dedifferentiation from non-stem cells to stem cells which occurs naturally in vivo, particularly in response to disruptions in tissue homoeostasis such as the healing response (Marusyk et al. 2012). This dedifferentiation mechanism has been shown to occur for GBM in vivo (Chow et al. 2011; Friedmann-Morvinski et al. 2012). Using GFAP-creER mice, knockout of the tumor suppressors PTEN and p53 in mature astrocytes results in gliomas of varying grades (Chow et al. 2011). Using synapsin I-Cre mice to overexpress oncogenic HRasV12 in neuronal cells, in combination with p53 knockdown, leads to tumors (Friedmann-Morvinski et al. 2012).

These mouse models demonstrate that GBMs may be generated from multiple brain cell lineages and tumors may arise in both proliferative and non-proliferative brain regions. This initiating cell may be an immature stem cell that differentiates to generate the multiple cell types present in GBM, or may be a GBM originating from mature cells that undergoes dedifferentiation to a precursor like state (Fig. 1.1b). If the probability of dedifferentiation is relatively high, the implication for therapy is that the entire tumor will need to be eliminated to effect a cure.

How GSC heterogeneity is driven remains an open question, although recent data suggest that GSC heterogeneity is hard-wired such that GSCs maintain their capacity for recapitulating their original heterogeneity over many cell divisions or passages, in vitro, and that treatment with cytotoxic drugs including TMZ does not disrupt this capacity (Brown et al. 2017; Sugimori et al. 2015). Notably, an elegant study using a zebrafish xenotransplant model shows that TMZ treatment drives GSC heterogeneity, as well as drug resistance (Welker et al. 2017).

### *1.8.4 Genetic Heterogeneity*

Two of the most common oncogenes activated by gene amplification in GBM are the receptor tyrosine kinases *EGFR* and *PDGFRA*. Fluorescence *in situ* hybridization (FISH) has revealed extensive heterogeneity with respect to cells within the same tumor harboring unique copy number status of these oncogenes (Liu et al. 2011; Snuderl et al. 2011; Szerlip et al. 2012). These focal amplifications of *PDGFRA* and *EGFR* are housed on small circular chromosomes which are similar to double minutes (Vogt et al. 2004). These circular chromosomes reduce in copy number in response to EGFR inhibitors, only to increase in copy number when the treatment is withdrawn (Nathanson et al. 2014).

The amount of genetic heterogeneity within a single tumor has recently been able to be investigated using advanced surgical sampling and low input sequencing methods. Sampling of multiple, spatially separated fragments of the same tumor has revealed that multiple molecular subtypes are present in the same GBM tumor

(Sottoriva et al. 2013). Tumor evolution was predicted using phylogenetic reconstruction, with gain of chromosome 7 and loss of chromosome 10 present in most samples from the same tumor, suggesting these are initiating or early events GBM tumorigenesis.

Clonal analysis of GSCs substantiates the observation of heterogeneity in clinical samples (Meyer et al. 2015). There is extensive cellular variation with respect to *PTEN* deletion and EGFRvIII amplification. Clones resistant to temozolomide are present in treatment naive tumors and recurrent tumors, which suggests out-growth of an existing resistant subclone in the GBM tumor once treatment was commenced.

Interestingly, GBMs from patients younger than 55 years with a high degree of intra-tumoral heterogeneity have an improved survival compared to GBMs that are more homogeneous (Kim et al. 2015). There is also no correlation between the number of subclonal mutations and age of the patient. This counter-intuitive observation may be due to subclonal GBMs growing more slowly, allowing more time for tumor divergence. Additionally, there may be more detrimental competition between subclones or greater exposure of neoantigens to the host immune system (Snyder et al. 2014).

### *1.8.5 Transcriptional and Molecular Heterogeneity*

Single-cell RNA-seq has revealed extensive intra-tumoral heterogeneity in GBM (Patel et al. 2014). Up to three, but never all four different molecular subtypes may be present in the same tumor. There is also evidence of hybrid states where GSC clones express a signature comprised of two molecular subtypes (Meyer et al. 2015). Individual GBM cells exhibit a broad and continuous spectrum of similarity to a CSC signature, as opposed to a rare subpopulation of cells with strong enrichment of a CSC signature. These observations, at the single-cell level, disagree with a categorical definition of GBM cells as CSCs and non-CSCs or as discrete molecular subtypes, instead suggesting a continuous set of cellular states, with the ability to transition between states. Recent studies reveal a role of micro-RNA miR-128 in bidirectional transition between PN-like and MES-like GSCs (Rooj et al. 2017). The molecular heterogeneity seen in patient GBM tissue across many studies reflects the adaptation of GSCs to metabolic requirements, exemplified by the role of Notch signaling in regulating GSC metabolism (Bayin et al. 2017).

### *1.8.6 Tumor Microenvironment*

Research over the last decade has highlighted the important role of the tumor microenvironment in the progression and maintenance of cancer (Grivennikov et al. 2010; Hanahan and Weinberg 2011; Quail and Joyce 2013). Cancer is not only a cell

autonomous disease but also a complex disruption of normal tissue homoeostasis. Solid tumors exhibit a complex 3-dimensional structure with some types of tumor consisting primarily of normal infiltrating cells (Kalluri and Zeisberg 2006). GBM tissue is also subject to intra-tumoral heterogeneous signaling, including differential activation of the PI3K and MAPK pathways, which are associated with the proliferative, invasive, and inflammatory activity of the GBM cells (Daniel et al. 2018). There is also an association between chronic inflammation and cancer, with approximately 16% of cancers attributable to infection-induced inflammation (de Martel et al. 2012). Chronic autoimmune conditions, such as inflammatory bowel disease, are associated with a greatly increased risk of developing colorectal cancer (Waldner and Neurath 2009). Conversely the long-term use of anti-inflammatory drugs such as aspirin is associated with reduced risk of colorectal cancer (Rothwell et al. 2012).

GBM tissue heterogeneity is also dependent on the biophysical conditions experienced by the tumor cells. The core of most solid tumors, especially GBM, is necrotic, surrounded by tumor cells within a peripheral hypoxic region (Fig. 1.2). For tumors to grow beyond 1 $mm^3$, new blood vessels are required to support the nutritional needs of the expanding mass (Chung et al. 2010). In the healthy brain, the level of oxygen perfusion can vary from 8% to 0.55% (Persano et al. 2012). The disorganized, leaky nature of capillaries in GBM and the competition for resources between malignant cells create a necrotic core of cells. Near this necrotic center, cells experience hypoxic conditions, where oxygen concentrations can be below 0.1%. The presence of pseudopalisading cells in GBM is suggestive of cells migrating away from a hypoxic core (Brat and Van Meir 2004; Rong et al. 2006) (Fig. 1.2). These cells express high levels of HIF1-$\alpha$ and matrix metalloproteinases (MMPs), highlighting the influence on molecular and cellular biology by these altered biophysical conditions.

### *1.8.7 Inflammation and Immune-Cell Heterogeneity Influences on Glioma Stem Cells*

The MES subtype of GBM is enriched for activation of inflammatory pathways and expression of an immune signature is predictive of poor survival (Doucette et al. 2013; Sintupisut et al. 2013). Tumor necrosis factor alpha (TNF-$\alpha$) is a major pro-inflammatory cytokine acting as a modulator of the immune system during inflammation, cell proliferation, differentiation, and apoptosis (Zelová and Hovsek 2013). TNF-$\alpha$ signaling may direct cells toward two major cellular phenotypes, cell survival and expression of pro-inflammatory genes or apoptosis and cell death. Macrophages are the major producers of TNF-$\alpha$, particularly M1-polarized macrophages. In the brain, TNF-$\alpha$ is secreted by microglia and astrocytes. TNF-$\alpha$ is a rapid inducer of the transcription factor NF-$\kappa$B, a major regulator of the inflammatory response (Baeuerle and Henkel 1994).

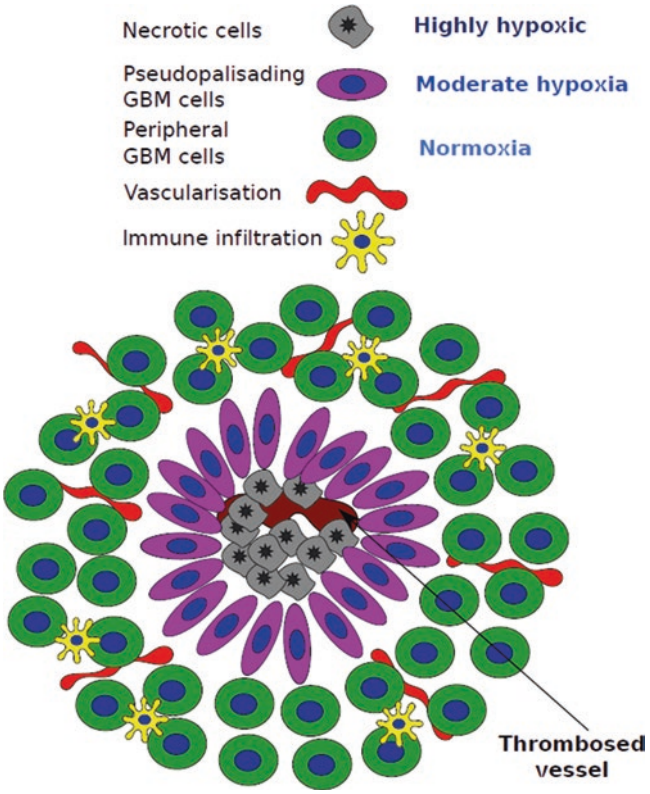


**Fig. 1.2** Tumor microenvironment in GBM. Hypoxia is extreme in the necrotic center. Pseudopalisading GBMs collectively migrate as a front of cells away from the core but are still in a moderate hypoxic microenvironment. Neo-angiogenesis at the tumor margin sustains the growth of peripheral GBM cells. Adapted from the 3-layer concentric model (Persano et al. 2011)

There is evidence that inflammatory cytokines promote cellular plasticity in GBM, such as TNF-$\alpha$ treatment induces a PN to MES shift in GSCs (Bhat et al. 2013), a phenomenon dependent on NF-$\kappa$B pathway signaling. Another study shows that within a pool of differentially drug and radiation resistant GSCs, the most resistant and aggressive GSCs were those with highest NF-$\kappa$B activity, suggesting that GSC heterogeneity fuels a diverse cell population to ensure survival in a deleterious environment (Teng et al. 2017).

## 1.9 Modelling GSC Heterogeneity

### *1.9.1 Reporter Lineage Tracing*

Beginning with early studies on *C. elegans* which followed individual cells by microscopy, the concept of following a cell through successive divisions to understand its fate is the basis of fundamental discoveries in cell biology such as apoptosis (Deppe et al. 1978). The earliest molecular approaches for lineage tracing involved transfection or transduction of cells with transgenes such as beta galactosidase or green fluorescent protein, or DNA tags (Turner and Cepko 1987). These approaches utilize limiting dilution such that only a single cell within a geographical location will express the transgene and pass it down to their progeny. Important limitations include silencing of the transgene and that the transfection/transduction process induces a stress which may perturb normal cellular lineage programs.

More sophisticated approaches include genetically engineered animal models where a transgene is endogenously expressed under the control of a tissue specific promoter (Farago et al. 2006). These systems can be modified to be under temporal control using inducible systems such as Cre-ER (Branda and Dymecki 2004). Another technical improvement on the fluorescent protein systems have been multicolor labelling strategies where multiple fluorescent protein genes are put in tandem with lox-stop-lox sequences in between. Recombination results in a random fluorescent protein being expressed enabling the tracking of multiple clones simultaneously (Snippert et al. 2010). A similar approach has been applied to DNA barcode which is randomly shuffled using Cre-lox technology (Pei et al. 2017). A highly promising technology combines CRISPR technology with single-cell RNA-seq. Self-evolving barcodes utilize CRISPR-Cas9 to progressively edit DNA barcodes or its own guide sequence over the lifetime of the lineage (Frieda et al. 2017; Kalhor et al. 2017; McKenna et al. 2016). Using the pattern of accumulated CRISPR induced errors the phylogenetic history of the cell lineage may be reconstructed. Such tracing approaches applied to mouse models of GBM may be used to determine the contribution of GSCs to the tumor. Combined with orthogonal methods such as single-cell genomics the phenotype of the GSCs may also be measured.

### *1.9.2 Single-Cell Genomics*

The original genome-wide studies of GBM profiled the tumor bulk resulting in the readout of the average gene expression profile or mutations with typical sensitivity of 1% allele frequency. Advances in molecular biology, microfluidics, and bioinformatics have enabled the high throughput analysis of thousands of individual cells in parallel (Svensson et al. 2018). Such methods have allowed the dissection of heterogeneity at an unprecedented scale. Single-cell RNA-seq (scRNA-seq) allows the discovery and classification of novel cell types and cell states. A cell state describes

the plastic movements of cells along a phenotypic continuum such as epithelial to mesenchymal or proneural to mesenchymal, in the case of GBM. Pseudotime analyses using scRNA-seq of an asynchronous population can reveal cellular trajectories along such plastic cell states (Haghverdi et al. 2016; Trapnell et al. 2014). Importantly scRNA-seq is unbiased in that it can be used to study rare GSCs in a population without the use of markers. After clustering of individual cells into subpopulations and annotation of phenotype, marker genes specific to each subpopulation may be identified enabling validation by prospective isolation and functional characterization. The high amount of amplification required for scRNA-seq introduces a large amount of technical noise. Bioinformatics approaches to extract the biological signal from this noise is a complex and ongoing effort without consensus on the best overall analytical approach (Kolodziejczyk et al. 2015).

IDH1 mutant gliomas are difficult to characterize functionally because they grow poorly in mouse models. scRNA-seq allowed the identification of a GSC subpopulation this class of tumors also (Tirosh et al. 2016). The GSCs were enriched for a proliferative gene signature which suggests these are the cells that are driving the growth of the tumor. scRNA-seq may also be used for tissue profiling to study the tumor microenvironment. Such an approach may be used to understand microglial infiltrate of GBM and how immune cells are modulating the tumor and vice versa (Muller et al. 2017). A key challenge in this approach is preparing a single-cell suspension that accurately recapitulates the tissue composition. Neurons are difficult to prepare using traditional dissociation methods. Modified dissociation methods as used in single-nucleus RNA-seq have the potential to overcome this limitation (Habib et al. 2016). Spatial transcriptomics has emerged as a novel approach to measure the expression of thousands of genes in parallel, in situ (Lein et al. 2017). Widespread adoption of this technology has the potential to illuminate the structure and organization of the GSC niche, in unprecedented detail.

Single-cell genomes are more difficult to study as typically mammalian cells contain two copies of each locus. Whole genome amplification is required which introduces biases such as allelic dropout and preferential amplification. Such methods may act as retrospective lineage tracers, by piecing together the order of acquisition of mutations in a population, the subclonal architecture and history of a tumor may be defined (Navin 2015). Such approaches can be used to delineate the contribution of different models of tumor evolution such as clonal evolution and cancer stem cells.

Single-cell epigenome methods such as ATAC-seq, Hi-C, and DNA methylation may provide an additional layer of information about gene regulation (Clark et al. 2016). The combination of these methods in the same cell termed multi-omics allows correlations between (epi)genotype and phenotype to generate mechanistic models to cellular behavior (Macaulay et al. 2017).

## 1.10 Commentary

Advances in understanding the complex mechanisms generating and maintaining glioma stem cell heterogeneity have been gaining momentum on the back of parallel advances in the experimental tools available to researchers. In vitro models and in vivo models coupled with gene editing, novel small molecule synthesis and single-cell sequencing technologies have led to findings which show that GSC heterogeneity allows these cells to survive standard cytotoxic therapies. Moreover, these therapies in fact appear to fuel further heterogeneity via molecular and cellular evolution of GSCs. However, the heterogeneity also appears to have an inherent stability or predictability which gives hope that at some point GSCs will be targetable with new generation cancer stem cell designer therapies. When we are someway to this type of therapy, GBM progression and ultimately recurrence will be better controlled resulting in extended symptom-free and overall patient survival.

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# Chapter 2
# Heterogeneity of Head and Neck Squamous Cell Carcinoma Stem Cells


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**Abstract** Current systemic cancer treatment in head and neck squamous cell carcinoma (HNSCC) is moving toward more personalized approaches such as de-escalation protocols human-papilloma-virus dependent HNSCC or application of checkpoint inhibitors. However, these treatments have been challenged by cancer stem cells (CSC), a small population within the bulk tumor, which are leading to treatment failure, tumor recurrence, or metastases. This review will give an overview of the characteristics of HNSCC-CSC. Specifically, the mechanisms by which HNSCC-CSC induce tumor initiation, progression, recurrence, or metastasis will be

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discussed. Although evidence-based treatment options targeting HNSCC-CSC specifically are still being sought for, they warrant a promise for additional and sustainable treatment options where for HNSCC patients where others have failed.

**Keywords** Head and neck squamous cell carcinoma (HNSCC) · Epidermal growth factor receptor (EGFR) · Cancer stem cell (CSC) · Immunotherapy · Epithelial-to-mesenchymal transition (EMT) · Mesenchymal-to-epithelial transition (MET) · Aldehyde dehydrogenase I · Immune response · Immune evasion · PD-1 · PD-L1 · Tumor microenvironment · CSC-directed therapeutic

## 2.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Conventional treatments including surgery, chemo- and radiotherapy have shown a curative potential in patients with early stage cancer while recurrent/metastatic (R/M) disease is mostly incurable. Cetuximab, the epidermal growth factor receptor (EGFR)-targeted therapy, has modest response rates of 10–15% as monotherapy (Vermorken et al. 2007). Also, the response rates of checkpoint inhibitors for R/M HNSCC are only 15% (Ferris et al. 2016; Chow et al. 2016). Additionally, there are no identified biomarkers for treatment response so far. In particular, the 5-year survival rates for patients with HNSCC remain stagnating at approximately 40–60% (Gregoire et al. 2010; Ferlay et al. 2015). Therefore, a better understanding of oncoimmuno-biology and the development of novel treatment strategies for HNSCC are urgently needed.

The heterogeneity of malignant cells as seen in HNSCC accounts for disease progression, recurrence, and metastasis. It has been noted that cancer stem-like cells (CSC) exist within the bulk tumor cells that drive the development of HNSCC (Qian et al. 2018; Dong et al. 2017). There are two main hypotheses for the origin of CSC: dedifferentiation of tumor cells or derivation from normal stem cells by genetic or epigenetic changes (Rycaj and Tang 2015; Shang et al. 2018). The process of epithelial-to-mesenchymal transition (EMT) allows CSC to undergo phenotypic changes whereby they become more motile (Chen et al. 2013). A recent single cell transcriptomic analysis of the pattern of primary HNSCC and lymph node metastases demonstrated that the presence of a mesenchymal subtype of malignant cells, fulfilling a partial EMT program but lacking classical EMT transcription factors, is an independent predictor of tumor invasion and metastasis (Puram et al. 2017). This new finding at a single cell level is in line with previous clinical observations of EMT on bulk tumors and strongly supports the existence of different biological subtypes of HNSCC tumor cells (Puram et al. 2017). Additionally, the interplay between CSC and tumor microenvironment is also of importance for CSC-mediated immune invasion and immune escape (Qian et al. 2015). In this review, we will describe current findings of HNSCC-CSC and discuss CSC-targeted novel therapeutic approaches.

## 2.2 Characteristics of Cancer Stem-Like Cells in HNSCC

### *2.2.1 Self-Renewal and Multi-Potency*

HNSCC-CSC are identified by expression of markers such as CD44 (Prince et al. 2007; Joshua et al. 2012), high ALDH1 activity (Clay et al. 2010; Okamoto et al. 2009), GRP78 (Wu et al. 2010), CD98 (Martens-de Kemp et al. 2013), and CD133 (Wei et al. 2009; Zhang et al. 2010). To date, CSC are hypothesized to being derived from transformed normal stem cells or dedifferentiated tumor cells (Dong et al. 2017; Rycaj and Tang 2015). A recent study analyzed genomic differences between $ALDH^+$ CSC, $ALDH^-$ tumor cells, and normal cells in four HNSCC patients (Salazar-Garcia et al. 2018). $ALDH^+$ CSC from two patients have shown potential stem cells origin while one patient had a tumor cell origin (Salazar-Garcia et al. 2018). Although the evidence of cellular origin remains sparse, in vitro studies and animal models have shown that $ALDH^+$ $CD44^+$ HNSCC cells exhibiting self-renewal properties were more tumorigenic than $ALDH^-$ $CD44^-$ cells (Krishnamurthy et al. 2010) and Oct4, Bmi-1, Wnt, Notch, and Hedgehog signaling pathways were related to CSC self-renewal (Bolos et al. 2009; Nusse 2008; Zhang et al. 2012; Chen et al. 2010; Lee et al. 2014). Using transplantation assays, CSC were found to generate cancer cells using their self-renewal capacity and trigger the differentiation into various cell types, meanwhile, they were able to undergo metastatic dissemination in HNSCC (Rycaj and Tang 2015; Chen et al. 2013). These findings lead to further investigations on how CSC promote tumor progression and facilitate therapy resistance.

### *2.2.2 Epithelial-to-Mesenchymal Transition*

EMT is commonly defined as a step during embryogenesis that drives epithelial cell phenotypes to become mesenchymal phenotypes. Once the migrated mesenchymal cells reach their destinations, they undergo a mesenchymal-to-epithelial transition (MET) process by which cells readopt epithelial phenotypes. The activation of EMT has also been confirmed to be the major culprit to the invasiveness and metastasis of several types of human malignancies in vitro and in vivo, including HNSCC (Chen et al. 2013). More importantly, accumulating evidence suggests that activation of EMT in neoplastic cells is closely related to the acquisition of CSC properties, such as the alteration of the expression of CSC-specific cell surface markers, an increased sphere-forming ability in suspension culture systems, and enhanced tumorigenicity in mice.

Mani and colleagues firstly illustrated the direct link between EMT and the gain of epithelial stem cell properties, in which they confirmed the upregulation of stem cell markers by inducing an EMT program in mammary epithelial cells and breast cancer cells (Mani et al. 2008). More recently, Driessens et al. traced CSC during the tumor growth by using a genetic labeling strategy and clonal analysis in a papilloma model, in which benign tumors are initiated, are growing, and eventually progressing into invasive tumors. In this study, tamoxifen generated yellow fluorescent

protein expression in basal tumor epithelial cells and therefore these cells could be traced over time during different stages of tumor progression. A minor population of CSC was observed being sustained in cellular hierarchy of papilloma, and served as a transient progenitor cell pool. The cell division rate of CSC was twice per day and approximately four times faster than the progenitor cell division rate in the tumor. Moreover, it was demonstrated that tumor cells in the largest clones with the highest number of divisions had contact with the stroma and endothelial cells but lost the cohesion with the residual clonal cells, and presented signs of EMT with fibroblastic-like morphology. However, the clone size and proliferative rate potential showed a different pattern of behavior in contrast to the benign papilloma, consistent with geometric expansion of a single CSC population with limited potential for terminal differentiation (Driessens et al. 2012).

Snail and Twist protein families are the main intensely studied transcription factors that promote EMT program in many types of cancers. In HNSCC cells, introduction of Snail-induced EMT properties and enhanced cellular invasion and migration, partially via direct transcriptional repression of the gene encoding E-cadherin (CDH1) and the subsequent loss of E-cadherin-mediated cell–cell adhesion (Yang et al. 2004). Moreover, Snail-induced EMT cells maintained the CSC-like properties and increased sphere formation and invasive capacities, and displayed an increased chemoresistance (Masui et al. 2014; Ota et al. 2016). We previously reported that ALDH1$^+$ HNSCC-CSC possess higher invading capacity, upregulate EMT-markers (Snail2 and Twist), and have increased expression of mesenchymal markers (alpha-smooth muscle actin and vimentin) and stemness-related transcription factors including Sox2, Nanog, and Oct3/4 in vitro (Chen et al. 2011a). Later, we examined the expression of ALDH1A1 in patients with locally advanced, metastasized HNSCC. It was interesting to find that ALDH1A1 was observed to be co-expressed with Twist1 in primary tumor and lymph node metastases, indicating the potential activation of EMT in CSC. Furthermore, another study has shown Twist1 induced HNSCC-CSC cell migration and transition from non-motile epithelial phenotype to motile mesenchymal phenotype, through activation of the Twist1-let-7i-NEDD9 axis, beyond simply suppressing E-cadherin (Yang et al. 2012). Another EMT transcription factor, ZEB1/ZEB2 has been shown to be significantly increased in CD133(+) CSC-like cells in HNSCC. Overexpression of ZEB1/ZEB2 could endow HNSCC-CD133(−) cells with enhanced sphere-forming ability, increased CD44+ cell frequency, and tumor growth capacity. In clinical samples, the levels of ZEB1/ZEB2 expression were high in metastatic lymph nodes in HNSCC tissues and patients with higher levels of ZEB1/ZEB2 often had poorer survival rates (Chu et al. 2013). Thus, EMT activation seems to be essential for the maintenance of CSC phenotypes.

Of note, certain stemness-related molecules also link the CSC phenotype and EMT program in cancer, suggesting their intimate interconnection and predicting that CSC are undergoing EMT and are responsible for metastasis and resistance to conventional therapies. Nanog and Oct3/4 are crucial CSC-related transcription factors, co-expression of Oct4 and Nanog in lung adenocarcinoma not merely induced CSC-like properties and drug resistance, but also promoted EMT through activating

another EMT-related transcription factor Slug. Conversely, double knockdown of Oct4 and Nanog suppressed Slug expression and reversed the EMT process, supporting the notion that the stemness-related signaling could control the EMT program (Chiou et al. 2010). SOX2 has an important function in stem cells and CSC maintenance; its expression is associated with increased levels of the other CSC markers ALDH1 and CD44 in pancreatic cancer cells. Importantly, SOX2 has been proven to directly bind to the Snail, Slug, and Twist promoters, imparting the CSC-like cells with an EMT phenotype (Herreros-Villanueva et al. 2013). However, low SOX2 expression is predominantly found in solid HNSCC and is associated with an advanced tumor stage (Thierauf et al. 2018). Upregulation of the proverbial CSC marker CD133 was also proven to promote the stemness properties and the tumorigenic capacity of HNSCC cells. Besides, overexpression of CD133 increased Src phosphorylation coupled with EMT transformation in HNSCC (Chen et al. 2011b). Additionally, in HNSCC cell lines that show an EMT expression profile, EMT is associated with a $CD44^{high}/EGFR^{low}$ phenotype and possibly contributes to the radioresistance response (Johansson et al. 2016). C-Met is a novel putative HNSCC-CSC marker and responsible for high chemoresistance and metastatic capabilities. Its activation by hepatocyte growth factor (HGF) through a paracrine mechanism enables HNSCC cells to acquire mesenchymal phenotypes in part through the EMT process (Sun and Wang 2011; Rothenberger and Stabile 2017). These findings indicate that CSC-related markers mediate the EMT and CSC-like properties in HNSCC.

In addition, several intracellular signaling pathways including Notch, transforming growth factor β (TGF-β)-SMAD, and canonical Wnt-β-catenin are known to contribute critically to EMT activation through influencing transcription factors and are also involved in CSC phenotype (Polyak and Weinberg 2009; Fabregat et al. 2016; Scheel et al. 2011). Notch receptors are critical regulators of stemness in HNSCC cells and most genes in the Notch pathway, such as Notch1, Notch2, and Jagged1, are upregulated in HNSCC cell lines. In biopsy specimens, when compared with normal or dysplastic tissues (Lee et al. 2016a; Hijioka et al. 2010), an upregulation of Notch4 and HEY1 expression correlated with decreased E-cadherin expression and increased expression of EMT-related factors such as vimentin, fibronectin, Twist1, and SOX2, what demonstrated that the Notch pathway promotes EMT in HNSCC (Fukusumi et al. 2018). A study has revealed that TGF-β-SMAD and Wnt-β-catenin collaborate to induce activation of the EMT program and thereafter maintain the stem cell states of non-neoplastic and neoplastic cells (Scheel et al. 2011). Consistent with this notion, suppressing the Wnt-β-catenin signaling pathway with Wnt antagonist, sFRP4, decreases the stemness and EMT phenotypes of CSC from HNSCC cell lines, and confers these cells more responsive to chemotherapeutics (Warrier et al. 2014).

Intriguingly, other novel molecules were also identified to link the EMT program and CSC phenotype in HNSCC. Hideo et al. reported that there are two CSC phenotypes in HNSCC: $CD44^{high}/ESA^{high}$ cells exhibit epithelial features (Epi-CSCs), whereas $CD44^{high}/ESA^{low}$ cells have mesenchymal features and are migratory (EMT-CSCs). Inhibition of GSK3β could induce $CD44^{high}/ESA^{low}$ cells to undergo MET to $CD44^{high}/ESA^{high}$ cells and markedly enhanced their sensitivity to 5-FU treatment

(Shigeishi et al. 2015). Other mediators of EMT and CSC phenotypes in HNSCC include c-Fos, G9a (a histone methyltransferase), S100A4, TNF receptor-associated factor 6 (TRAF6), and anterior gradient protein 2 (AGR2) (Muhammad et al. 2017; Liu et al. 2015a; Lo et al. 2011; Chen et al. 2018; Ma et al. 2015). In addition, inhibition of histone deacetylases (HDAC) could disrupt the accumulation of HNSCC-CSC cells but paradoxically induced the EMT program (Giudice et al. 2013). Together, the aforementioned studies indicate that abundant intercellular molecules and signaling pathways are involved in the induction and regulation of the EMT program and CSC phenotype in HNSCC, and ambiguity remains over their interconnection. A deeper understanding is urgently needed and critical to the future development of novel therapies directed against the CSC-mediated clinical relapse in the treatment of HNSCC.

### *2.2.3 Interaction with Tumor Microenvironment*

A large body of evidence demonstrates the interaction between CSC and the tumor microenvironment: sheltering of CSC by tumor microenvironment maintains their survival and self-renewal capacity, assists their resistance to chemoradiotherapy, and even induces the transformation of normal cells and non-tumor stem cells into CSC. In addition, CSC can adapt and even change the tumor microenvironment. In tumors, microenvironment refers to the region between tumor cells and adjacent normal tissues and is generally categorized into cellular and acellular components. These cellular components are composed of inflammatory cells, angiogenic vascular cells, and carcinoma-associated fibroblasts (CAFs). Among them CAFs are extremely heterogeneous and the precise molecular definition is still debatable. Most evidence supports that CAFs are derived from, i.e., endothelial cells, mesenchymal, and tissue-resident fibroblasts. CAFs enhance the progression of HNSCC through fibroblast-promoted EMT processes and the secretion of cytokines including VEGF, TGF-β, hepatocyte growth factor, and metalloproteinases (MMPs) (Wheeler et al. 2014; Kumar et al. 2015). More importantly, CAFs represent a crucial cellular component of the CSC microenvironment and are believed to maintain or promote certain CSC features such as EMT activation, through involvement with factors that influence both CSC and surrounding immune cells. For example, Rosenthal et al. discovered persistent elevation of TGF-β1 in CAFs from HNSCC tissues, compared to normal dermal fibroblasts and mucosal fibroblasts (Rosenthal et al. 2004). Intriguingly, TGF-β1 is a known inducer of EMT. Therefore, it can be speculated that TGF-β1-mediated EMT in CAFs will be certain to contribute to the stemness of HNSCC-CSC. Additionally, MMP9 can be secreted by CAFs and an in situ analysis demonstrated that a MMP-9 positive basal-cell-like cell layer at the invasive front of HNSCC likely contains CSC since it also expresses the putative CSC markers and there is a significant positive correlation among them (Sterz et al. 2010). Thus, these molecules represent an important CAFs-CSC crosstalk network in HNSCC and indicate a feasible direction to HNSCC therapies, even though more direct evidence should be provided to connect the CAFs and CSC in HNSCC.

It has been known for a while that vascular and perivascular niches are also supportive for the self-renewal and differentiation of CSC in HNSCC. This partially relies on secretion of important factors such as IL-6, CXCL8, and EGF by vascular endothelial cells, or suppressing the anoikis of HNSCC-CSC by activating several key endothelial cell-initiated signaling pathways (e.g., STAT3, ERK, and Akt) (Neiva et al. 2009; Campos et al. 2012). Krishnamurthy and colleagues demonstrated endothelial secreted IL-6 enhanced the self-renewal of human HNSCC-CSC, and a humanized anti-IL-6R antibody (tocilizumab) significantly inhibited the tumorigenic capacity mediated by CSC (Krishnamurthy et al. 2014). A Pearson's correlation analysis on different grades of oral squamous cell carcinoma (OSCC) found a co-localization phenomenon of several types of markers, including vascular endothelial-cadherin, CD44, and vimentin, suggesting the definite relationship among the EMT process, CSC properties, and angiogenesis in this cancer (Irani and Dehghan 2018). Another in vivo study observed that the majority of CSC located within a 100 μm radius of blood vessels in HNSCC tumors, and endothelial cell-secreted factors could induce the expression of Bmi-1 and promote the survival and self-renewal of HNSCC-CSC. Notably, specific ablation of tumor-associated endothelial cells by transduction with a caspase-based artificial death switch (iCaspase-9) gave rise to an obvious reduction of CSC numbers in xenograft tumors (Krishnamurthy et al. 2010). At present, two strategies are being designed to disrupt the crosstalk between CSC and endothelial cells. One is the anti-angiogenic therapy that lessens the tumor blood vessels and the consequent disruption of the vascular niche (Folkins et al. 2007); the other is the specific strategy targeting crucial signaling pathway that connects CSC and tumor-associated endothelial cells. Exhilaratingly, a combination therapy with cetuximab and IPI-926 (a Hedgehog signaling inhibitor) has entered Phase II clinical trial for treating the recurrent/metastatic HNSCC by interfering with CSC signaling pathways (Bowles et al. 2016).

Physiological hypoxia plays an important role in supporting the stem cell self-renewal ability of human embryonic stem cells (hESCs) and in suppressing unwanted hESCs differentiation (Xie et al. 2014). A growing body of evidence shows that hypoxia also contributes to the activation of the EMT program and maintenance of the stemness properties of CSC. In tumors, hypoxia is a common phenomenon in poorly vascularized regions of tumors and provides a non-cellular microenvironment for cancers. Cancer cells will be in a more invasive state under hypoxia and a lower oxygen often indicates a poorer tumor survival prognosis. Two similar clinical analyses on patients with locally advanced HNSCC who received postoperative radiotherapy or primary radiochemotherapy demonstrated that the expression of CSC markers (e.g., CD44 and SLC3A2) and tumor hypoxia status are potential markers for poor prognostic (Linge et al. 2016a, b). An in silico study in a HNSCC model found rapidly growing tumors with CSC in a severely hypoxic niche could not be controlled by radiotherapy alone (Marcu et al. 2016). In laryngeal cancer cell lines, hypoxic microenvironment promoted CSC-like biological properties by increasing the expression of CSC-related genes (OCT4, SOX2, and NANOG) and laryngeal CSC surface marker (CD133) (Wu et al. 2014). Duarte et al. described tumor occurrence and median tumor size were higher in mice injected with murine HNSCC cells expressing high levels of ALDH1, and this percentage was further

enriched when isolated murine CSC were cultured under hypoxic conditions (Duarte et al. 2012). Hypoxia-inducible factor 1α (HIF-1α), a central transcriptional regulator that controls the expression of most hypoxic responsive genes, is a crucial mediator for the direct connection between hypoxia and CSC and promotes the CSC formation and the resistance of tumor cells to chemo- and radio-therapy (Sasabe et al. 2007). HIF-1α is expressed earlier in HNSCC-CSC compared to non-CSC under hypoxia, and targeted inhibition of HIF-1α led to the increased sensitivity of HNSCC cells to radiation (Wozny et al. 2017). Additionally, hypoxia-induced expression of CSC markers in HNSCC cells was partially weakened by knockdown of HIF-1α (Wiechec et al. 2017). In human OSCC cells, epidermal growth factor can induce CSC-like cell properties through enhancing the EGFR/PI3K/HIF-1α axis-mediated glycolytic metabolic program (Xu et al. 2017). Interestingly, an in vitro study reported that the HNSCC-CSC proportion was highly enriched in a three-dimensional culture microenvironment, accompanied by higher tumorigenicity, metastasis, and drug resistance, indicating that additional potential factors exist to influence the tumor microenvironment (Liu et al. 2015b). Collectively, these aforementioned observations strongly indicate the variable and complex interaction of HNSCC-CSC and the tumor microenvironment. It is unquestionable that further research will certainly be required for developing more effective therapeutic strategies to eradicate HNSCC-CSC.

## 2.3 CSC-Induced Immune Response and Immune Evasion

Intratumoral cellular heterogeneity of immunogenicity has also been observed in HNSCC (Fig. 2.1). One study has shown that $ALDH^+$ CSC derived from OSCC cell lines had higher PD-L1 levels and radiotherapy augmented PD-L1 expression compared with $ALDH1^-$ cells in vitro (Tsai et al. 2017). Moreover, higher PD-L1 expression was significantly associated with ALDH1 positivity in OSCC tumors (Tsai et al. 2017). Consistently, one important recent finding is that $CD44^+$ HNSCC-CSC are less immunogenic and express higher PD-L1 at both the transcriptional and translational levels compared to the non-CSC population in an analysis of primary HNSCC tumors and patient-derived xenograft models (Lee et al. 2016b). Further, STAT3 can regulate constitutive PD-L1 expression by HNSCC-CSC and its inhibitor can decrease the PD-L1 expression. PD-L1 expression on HNSCC-CSC is inducible in response to IFNr and is associated with enhanced IFNr receptor expression and STAT1 phosphorylation. However, the decreased immunogenicity of HNSCC-CSC can be partially reversed by PD-1 blockade which suggests there are additional mechanisms of immunosuppression (Lee et al. 2016b). In a breast cancer model, CSC utilize the EMT/β-catenin/STT3/PD-L1 signaling axis by which EMT transcriptionally induces N-glycosyltransferase STT3 through β-catenin, and subsequently STT3-dependent PD-L1 N-glycosylation stabilizes and upregulates PD-L1 (Hsu et al. 2018). This effect can be suppressed by activated MET through TOP2B degradation-dependent nuclear β-catenin reduction. The downregulation of PD-L1 by MET can be seen in both CSCs and non-CSCs (Hsu et al. 2018). These data

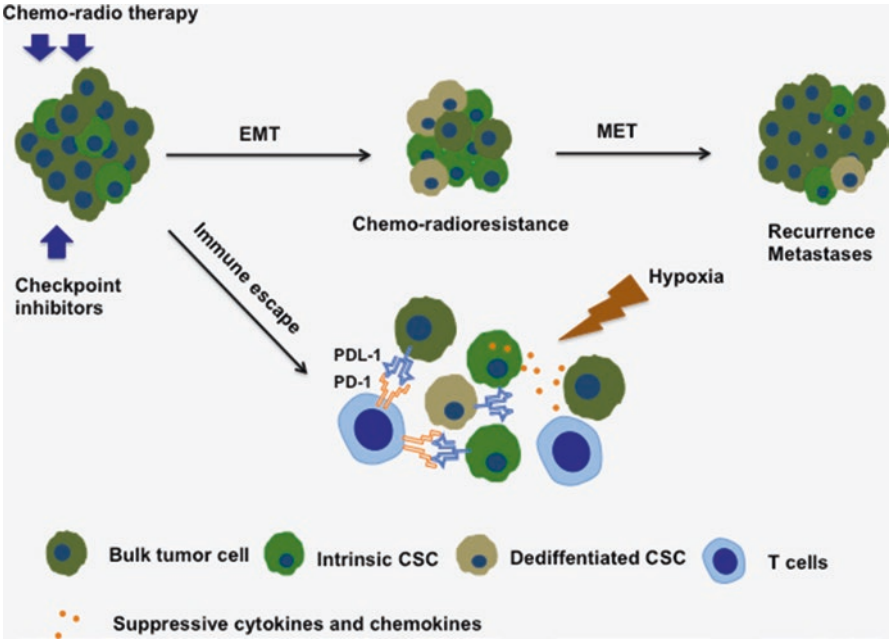


**Fig. 2.1** Role of CSC in therapy resistance. Intrinsic CSC in the tumor bulk population are more resistant to current treatment options. CSC can evade or are reinitiated after treatment leading to tumor recurrence. Novel immunotherapy options (check point inhibitors, tumor vaccines) can be compromised by immunosuppressive features of CSC secreting suppressive cytokines and chemokines

indicate that the acquisition of an EMT phenotype by CSCs aids in immune escape by the tumor. Moreover, the EMT-CSC phenotype was also found to be associated with an inhibition of CTL-mediated tumor cell lysis (Akalay et al. 2013).

In addition to the immune-suppressive phenotype of PD-L1, downregulation of MHC class I expression by CSC was also observed to support escape from immune surveillance (Liao et al. 2013; Morrison et al. 2018; Di Tomaso et al. 2010). In vitro, MHC class I expression can be increased by IFN-γ in HNSCC-CSC and cervical cancer line-derived CSC leading to improved T-cell recognition (Liao et al. 2013). In an immunocompetent murine model, mice transplanted with lung cancer CSC which were treated with IFN-γ to increase MHC class I expression prior to implantation had a significant improved tumor-free survival compared to untreated CSC (Morrison et al. 2018). In fact, these findings highlight the innate immune resistance of CSC.

We recently found increased production of cytokines and chemokines such as proinflammatory cytokine IL-6 and chemokines GRO-alpha (CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), SDF-1α (CXCR4), RANTES (CCL5), and IP-10 (CXCL10) by cervical cancer lines derived CSCs (unpublished data). These cytokines and chemokines are potent stimulators of angiogenesis and tumor cell proliferation and play an important role in tumor progression and metastasis in a variety of human cancers. Moreover, IL-6 and sIL-6R secreted from CSCs were vital to maintain the self-renewal and tumorigenic properties of CSCs in HNSCC

(Yu et al. 2013). Another study has shown that CD44⁺ HNSCC-CSC produced significantly higher levels of IL-8, granulocyte colony-stimulating factor (G-CSF), and TGF-β than the non-CSC population (Chikamatsu et al. 2011). We also observed that cervical cancer lines derived CSC exhibited a significantly higher inhibition of the production of αCD3/αCD28-stimulated immune-cell derived cytokines and chemokines (IL-2, IL-21, TNF-β, IFN-γ, TNF-α, MIP-1α, MIP-1β, and GMCSF) than non-CSC did (unpublished data). These cytokines and chemokines are mainly produced by activated T cells, which suggest a mechanism of CSC-mediated suppression of T cell-mediated immune responses. Moreover, CD44⁺ HNSCC-CSC have been shown to more strongly inhibit T-cell proliferation and suppression of Th1 responses, but also to more efficiently inhibit Treg cells and myeloid-derived suppressor cells as compared with the CD44⁻ non-CSC population (Chikamatsu et al. 2011). Taken together, these findings indicate that CSC might employ numerous escape strategies from immune attack.

## 2.4 Heterogeneous Response to Therapy and Novel CSC-Directed Therapeutic Approaches

It is well established that CSC mediate chemo- and radio-resistance as shown in both HNSCC cell lines and patients with HNSCC. Studies in cell lines have demonstrated that CSC are more resistant to standard chemo- or radiotherapy compared to non-CSC populations (Zhang et al. 2010; Reers et al. 2014; Chen et al. 2009) and Cisplatin treatment has been found to increase frequency of putative CSC (Nor et al. 2014). Poor radiotherapy outcome in HNSCC patients who were treated with accelerated platinum-based chemoradiotherapy was also found being related to CSC (Koukourakis et al. 2012). Thus, treatment strategies to overcome CSC-related therapeutic resistance are needed. Using lineage tracing and genetic ablation in a mouse model of HNSCC, Chen et al. found BMI1⁺ CSC mediated both invasive tumor growth and cervical lymph node metastasis where AP-1 played a critical role (Chen et al. 2017). In the same study, a combination of cisplatin and PTC-209 (a BMI1 inhibitor) targeting both bulk tumor cells and cisplatin resistant BMI1⁺ CSC was more effective in reducing tumor size and lymph node metastases. However, some tumor recurrences were observed after this combinational treatment suggesting other so far unknown mechanisms.

As a marker of CSC, ALDH1 activity is highly correlated with poorly differentiated tumors, lymph node metastasis, treatment resistance, and poor prognosis in HNSCC (Dong et al. 2017; Xu et al. 2012; Qian et al. 2014). There is also a growing body of evidence indicating that ALDH1 inhibitors or vaccination can induce therapeutic activity in the preclinical setting. For example, inhibition of ALDH1A1 in Cal-27 cisplatin resistant cell line successfully downregulated CSC markers, reduced CSC's migratory, self-renewal, and tumorigenic potential, and reversed the sensitivity to cisplatin treatment (Kulsum et al. 2017). Further, ex vivo cisplatin in combination with the inhibitor (NCT-501) treatment in explants from HNSCC patients has been shown to decrease proliferating cells more effectively as compared to individual treatments (Kulsum et al. 2017). Tsai et al. found that the level

of ALDH1 expression was associated with DNA methyltransferases3b (DNMT3b) expression as seen in OSCC clinical specimens and in vitro experiments (Tsai et al. 2017). The effect of radioresistance of ALDH1-positive cancer cells was attenuated using DNA hypomethylating agents as shown by decreased ALDH1 and increased DNA damages. However, the underlying mechanism is still unknown.

ALDH1 has also been utilized as a source of antigen to develop cancer vaccines (Visus et al. 2007, 2011). One approach used the $ALDH^{high}$ CSC to prime dendritic cells (DCs) as a vaccine in immunocompetent murine models of malignant melanoma and squamous cell carcinoma (Ning et al. 2012). DC-based CSC vaccine-primed CTLs and antibodies are capable to recognize and kill CSC in vitro (Ning et al. 2012). Moreover, CSC-DC vaccination after localized radiotherapy or surgical excision of established tumors successfully reduced the local tumor relapse, inhibited spontaneous lung metastases, and prolonged the survival in immunocompetent murine models (Lu et al. 2015; Hu et al. 2016). Recently, the CSC-DC vaccine combined with a dual blockade of PD-L1 and CTLA-4 in B16-F10 murine melanoma tumor model has dramatically eliminated ALDH-CSC in vivo which also resulted in fewer PD-1-CD8 T cells and CTLA-4-CD8 T cells, enhanced T-cell expansion and IFN-γ secretion, and suppressed TGF-β secretion compared to CSC-DC vaccine alone (Zheng et al. 2018). This approach has shown promise for an effective immunotherapeutic strategy with respect to the partial response of immune checkpoint responses currently seen in the clinical setting.

Metformin, an inhibitor of mitochondrial OXPHOS, has shown its anticancer properties and improved outcome in HNSCC (Sandulache et al. 2014; Stokes et al. 2018). An approach that combined metformin with curcumin treatment successfully inhibited the migratory and self-renewal of OSCC-CSC in vitro and reduced the tumor burden with improved OS in vivo (Siddappa et al. 2017). These findings warrant further investigation of the underlying mechanisms and efficacy for metformin in targeting CSC.

Taken together, preclinical data highlight several new approaches that can target HNSCC-CSC, leading to effective treatments. In addition, there remains a pressing need to developing improved strategies to overcome poor drug delivery and patient tolerability. Thus, combination therapy strategies with both anti-CSC treatment and novel nanotherapeutics have shown advantages toward curative treatment. For a detailed overview on active HNSCC-CSC targeting in nanomedicine, please refer to a review by Qian et al. (2018).

## 2.5 Conclusions

Clinically, conventional treatments including surgery, radiation, and chemotherapeutic drugs are successfully used to eliminate the bulk of cancer cells. However, the lack of targeted therapy for HNSCC-CSC spares the intrinsically resistant CSC compartment leading to regeneration of the cancer after conventional treatments. Thus, with the identification of the underlying mechanisms such as EMT and MET and potential novel approaches, therapies specifically targeting HNSCC-CSC hold great promise for curative treatments.

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# Chapter 3
# Heterogeneity of Small Cell Lung Cancer Stem Cells


**D. Prabavathy and Niveditha Ramadoss**

**Abstract** Small cell lung cancer, a subtype of lung cancer is an extremely malignant disease due to its metastases and recurrence. Patients with SCLC develop resistance to chemotherapy and the disease relapses. This relapse and resistance are attributed to the heterogeneity of SCLC. Various factors such as recurrent mutations in key regulatory genes such as TP53, RB1, and myc, epigenetic changes, and cancer stem cells contribute to the observed heterogeneity. Cancer stem cell models predict neuroendocrine origin of SCLC. Though an unambiguous established CSC marker has not been assigned, markers CD133, CD44 have been found associated with SCLC. Genetically engineered mouse models (GEMMs) allow the validation of driver mutations and are necessary for design of targeted therapy. This chapter outlines the factors contributing to SCLC heterogeneity, detection methods, and the current therapy trials.

**Keywords** SCLC · Neuroendocrine markers · CD133 · CD44 · Side population cells · Intratumor heterogeneity · TP53 · RB1 · Clonal evolution · CSC model · Lysine demethylase 1 · Notch pathway · Genetically engineered mouse model

## 3.1 Introduction

Globally lung cancer accounts for more than one-tenth of all cancer cases and is one of the most common types of cancer. The major risk factor for the development of lung cancer is smoking. Due to smoking the lung cells are exposed to carcinogens which induce extensive mutations. Mutations that accumulate in different oncogenes and tumor suppressors lead to tumor genesis in lung cancers. A range of morphological appearances and genetic aberrations observed in lung cancers

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indicate heterogeneity of the disease. Lung cancer is broadly classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The most abundant form of lung cancer is NSCLC and comprises several subclasses that include adenocarcinomas, squamous cell carcinomas, and large cell carcinomas.

SCLC represents about 15% of all lung cancer cases and can be distinguished by its neuroendocrine (NE) features (Govindan et al. 2006). SCLC shows the most rapid growth and metastasizes to distant sites of the body (Jackman and Johnson 2005). Small cell lung cancer (SCLC) is an extremely aggressive cancer that cytotoxic chemotherapies fail to exterminate. It affects >200,000 people world-wide every year with a very high mortality rate. Combination chemotherapy with cisplatin, hyperfractionated thoracic radiation, and prophylactic cranial irradiation are the standard methods of treatment for many years. Ever though SCLC recurs rapidly and <5% of patients survive 5 years. Such resistance and recurrence are attributed to the heterogeneity of SCLC (Paumier and Péchoux 2010; Rodriguez and Lilenbaum 2010).

## 3.2 A Comprehensive View of Cancer Heterogeneity

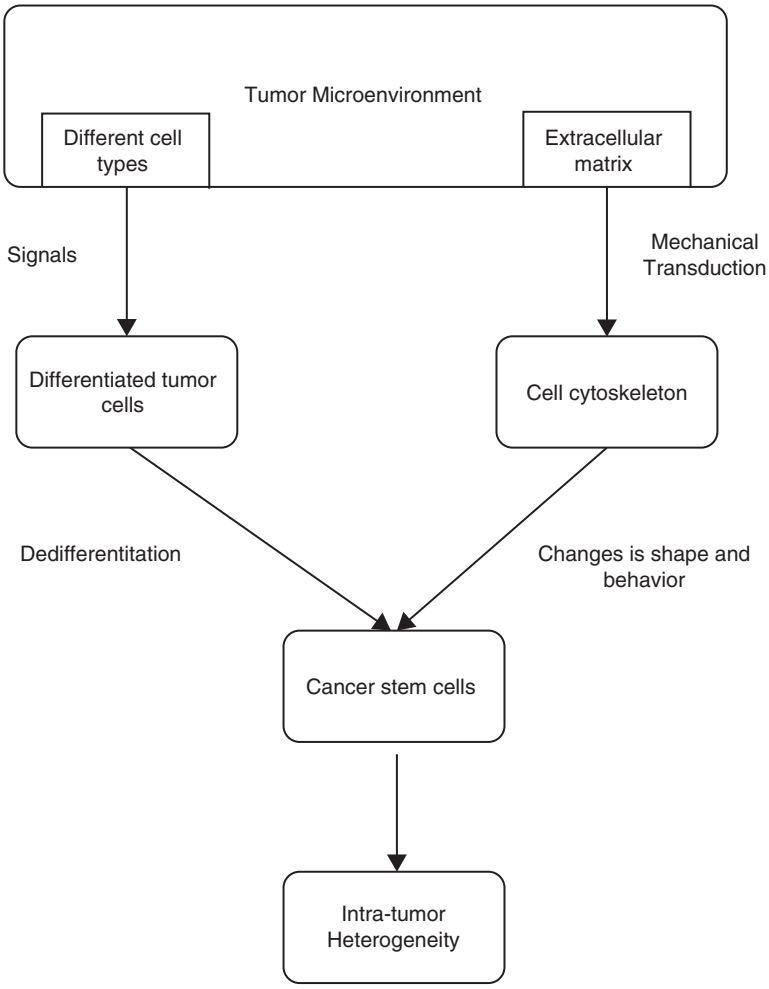
Earliest pathological reports showed variations among individual tumor cells. These variations were referred to as intratumor heterogeneity. Differences exist across individual patients presenting with cancers originating in the same organ known as intertumor heterogeneity. Advanced molecular and biochemical technologies led to a better understanding of the numerous mechanisms of both tumor heterogeneity. Genetic mutations and promoter hypermethylation within a single tumor were discovered through sequencing and methylation profiling of various tumor regions. This intratumor heterogeneity is further extended to other cell types, such as endothelial cells, infiltrating immune cells, stromal cells as well as a complex network of extracellular matrix (ECM). Tumor and microenvironment heterogeneity determine the fitness of the tumor (Table 3.1). Two models have been proposed to account for intratumor heterogeneity:

- *Clonal evolution model*—explains adaptation and selection for the fittest clones of a tumor (Greaves and Maley 2012).
- *Cancer stem cell (CSC) model*—suggests that only a subset of cancer cells possess indefinite self-renewal ability to initiate and maintain tumor growth. CSCs generate cellular heterogeneity by installing a differentiation hierarchy leading to a range of distinct cell types present within the tumor (Dick 2008a, b).

Both the theories coexist to cause tumor heterogeneity, in addition to cell origin and tumor microenvironment (Table 3.2). Major contributors of intratumor heterogeneity can be summarized as:

1. Mutations—multiple clones with genetic variations arise during tumor progression
2. Epigenetic factors—DNA methylation and histone deacetylation

**Table 3.1** Contribution of tumor microenvironment to intratumor heterogeneity

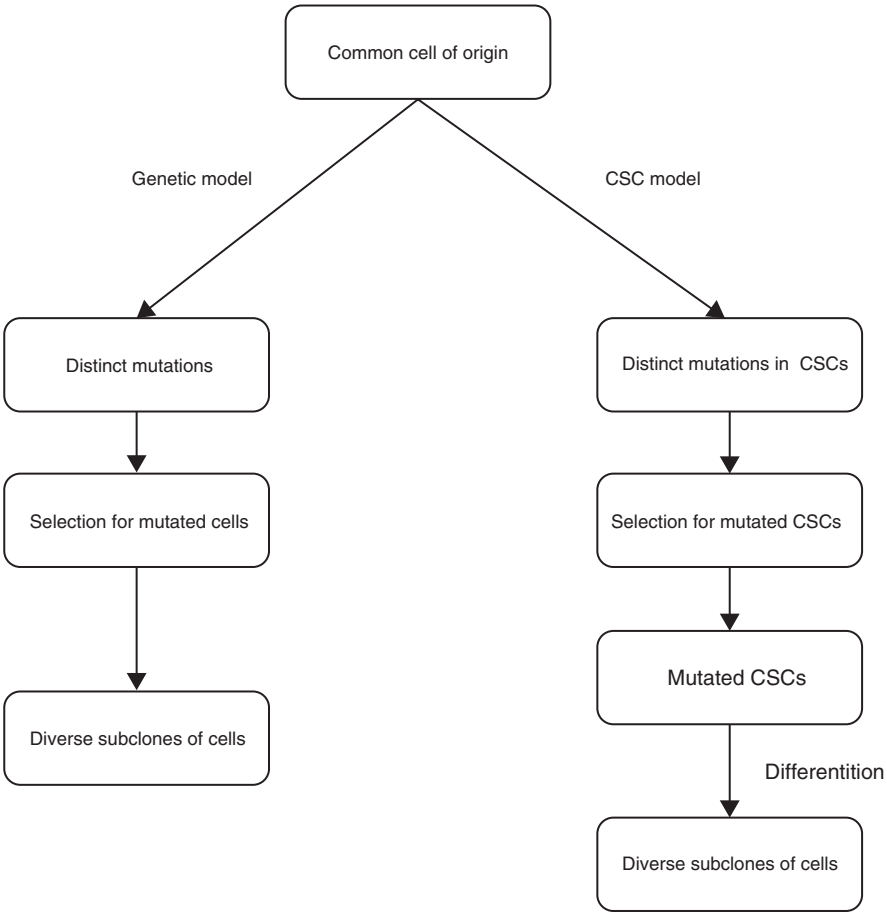



3. Role of CSCs
4. Tumor microenvironment
5. Cell of origin

### *3.2.1 Cell of Origin of Small Cell Lung Cancer (SCLC)*

Human and mouse SCLC predominately confine to the midlevel bronchioles and express a range of neuroendocrine markers, such as calcitonin-gene related peptide (CGRP) and neural cell adhesion molecule (Ncam1), and transcription factors which play important roles in neuroendocrine differentiation. These observations

**Table 3.2** Emergence of tumor subclones through genetic and CSC model




led to the hypothesis that a rare population of neuroendocrine (NE) cells is the progenitors of SCLC. In the mouse lung, microenvironments found in close proximity to neuroepithelial bodies (NEB) have been shown to maintain putative stem cell populations. However, NEB-associated pulmonary NE cells (PNECs) do not behave as stem cells. A proportion of SCLCs display a mixture of SCLC- and NSCLC-specific features suggesting for the existence of a "common" cell of origin (Sutherland and Berns 2010).

Intertumor heterogeneity of SCLC is represented by two subtypes based on the expression of neuroendocrine (NE) markers. Most SCLC tumors and cell lines express NE markers in abundance, while a 10–16% subset of cells have greatly reduced or no expression of the marker (Gazdar 2018). These subsets of cells are characterized by

1. Loss of the transcription factor NK2 homeobox 1 gene (NKX2–1) (also known as thyroid transcription factor 1)
2. Loss of Notch ligand 3 (DLL3)
3. Upregulation of the MYC gene, the Notch, HIPPO, and transforming growth factor β pathways
4. Epithelial–mesenchymal transition
5. Resistance to radiotherapy and chemotherapy.

The diagnosis and clinical outcome of therapy for SCLC is severely hindered by the two forms of heterogeneity. The "one-size-fits-all" drug cannot be functional. More targeted and personalized therapy is needed which requires understanding of the molecular mechanisms of heterogeneity (Notta et al. 2011).

## 3.3 Cancer Stem Cells in SCLC

CSCs display many features of embryonic or tissue stem cells, and typically demonstrate persistent activation of one or more highly conserved signal transduction pathways involved in the development and tissue homeostasis, including the Notch, Hedgehog, and Wnt pathways (Takebe et al. 2015). Dysregulation of these key signaling pathways plays an important role in enabling CSCs to retain their stem-like properties and are crucial for the tumorigenicity of these cells. CSCs have slower growth rates than tumor cells and are resistant to chemotherapy and/or radiotherapy. Successive therapeutic approach to cancer depends on the efficient targeting of CSCs (Medema 2013). The identification of CSC-specific markers and the targeted therapeutic eradication of CSCs remain a challenge. The selection and expansion of resistant CSC clones which evade current anti-cancer therapies result in poor responses and outcomes. Cancer stem cells in solid tumors are identified using an extensive list of markers. CSC markers derived from cell line studies or mouse xenograft models have been assumed to be valid and require stringent validation in primary human tumor samples. Few stem cell markers identified in SCLC are listed below;

### *3.3.1 CD133*

Cluster of differentiation-133 (CD133) is an established marker of hematopoietic stem cells. Eramo et al. (2008) identified CD133 as a marker of stemness SCLC tumor samples following immunohistochemical analysis. Increased tumorigenicity of CD133+ SCLC cells was also reported by Jiang et al. (2009), in addition to their identification of achaete–scute complex homolog 1 (ASCL1) as an important regulator of the stem-cell markers CD133 and aldehyde dehydrogenase (ALDH). Sarvi et al. (2014) characterized CD133 expression in H345 and H69 cell lines, in mouse

models, and human SCLCs. CD133 has been described as a CSC marker in other tumors and its expression correlated with chemoresistance to etoposide and increased tumorigenicity accompanied by increased expression of CD133 in human SCLC lung biopsy samples following chemotherapy. However, studies by Kubo et al. (2013) in a panel of six SCLC cell lines concluded that CD133 is an inadequate marker for SCLC.

### *3.3.2 CD 44*

Wang et al. (2013) established a panel of lung cancer cell lines from primary tumors and characterized a small subpopulation strongly positive for CD44 (CD44high), with the main population being weakly positive or negative for CD44. Co-expression of CD90 (CD90+) further narrowed down the putative stem cell population. This CD44 and CD90 positive subpopulation showed mesenchymal morphology, increased expression of the mesenchymal markers vimentin and N-cadherin, increased mRNA levels of the embryonic stem cell-related genes Nanog and Oct4, and resistance to irradiation compared with other subpopulations. The CD44high CD90+ subpopulation is therefore a good candidate for a CSC marker. In SCLC, it was shown that activation of CD44-MAPK-PI3K signaling results in increased expression of urokinase plasminogen activator (uPA), its receptor (uPAR) and MDR1, resulting in an enhanced invasive and multi-drug resistant cancer phenotype when treated with 5-fluorouracil (5-FU), cisplatin, and etoposide (Gutova et al. 2007).

### *3.3.3 Side Population Cells*

Side population cells (SP) are a small population of stem-like cells that exhibit a distinct low Hoechst 33342 staining pattern, due to the actions of ATP-binding cassette transporters (ABC transporters) (Zhou et al. 2001). ABC transporters are expressed in both normal stem cells and CSCs where their primary function is to exclude toxins so as to prevent cellular damage and/or cell death. Despite these cellular functions, the presence of efflux pumps in CSCs has been shown to promote drug resistance thereby reducing the efficacy of current therapies (Dean 2009). Wang et al. (2010) characterized a SP fraction in the H446 SCLC cell line and found 6.3% of SP cells by flow cytometry. They also found that SP cells were able to form tumor spheres better than non-SP cells. mRNA expression of the CSC markers ABCG2, CD133, and nucleostemin was analyzed and found to be 21.6, 7.1, and 1.02 higher than in non-SP cells, respectively. SP cells have a greater ability to form tumors when compared with non-SP cells and showed better proliferative ability and tougher viability when treated with drugs. Also, SP cells were able to differentiate into non-SP cells.

PODXL-1 and Bmi1 are markers in hematopoietic stem cells. Koch et al. (2008) studied their expression by IHC in 64SCLC samples and demonstrated that 56 sam-

ples were positive for PODXL-1 and Bmi1. They hypothesized that both could be CSC markers for SCLC.

It has been commented that SOX2 has a role in maintaining the pluripotent stem cell phenotype. There are some clinically conflicting results regarding SOX2expression, possibly either due to tumor-specific behavior of SOX2 or technical reasons. Studies had shown SOX2 protein expression was an independent marker for worse outcome in early stage lung adenocarcinoma (ADC). Wilbertz et al. (2011) showed that SOX2 expression correlates with lower grade better outcome in SCLC. SOX2 protein expression has been related to more aggressive tumors. In addition, upregulation of SOX2 enhances tumor cell proliferation and SOX2 overexpression has been shown to be essential for lung CSC function (Nakatsugawa et al. 2011).

## 3.4 Genomic Profiling of SCLC

The cancer stem cell model suggests that differences in intratumorigenic potential in patients are largely epigenetically determined. Such epigenetic differences are irreversible and distinguish tumorigenic from nontumorigenic cells for hierarchical organization of cancer. Cancer stem cells differentiate into nontumorigenic cancer cells in germ lineage cancers. Due to the robust nature of CSC markers, markers alone should not be relied upon to assess potential biological differences between tumorigenic and nontumorigenic cells; functional assays are required to confirm differences in therapy sensitivity and other biological properties. The clonal evolution model predicts that there should be genetic heterogeneity among cancer cells that leads to heterogeneity in phenotype, function, and response to therapy. Epigenetic differences are apparently layered above the genetic differences to confer additional heterogeneity. Irrespective of clonal evolution or a hierarchy of epigenetically distinct tumorigenic and nontumorigenic cells, cancers often arise and progress due to dysregulation of self-renewal pathways borrowed from normal stem cell (Shackleton et al. 2009).

### *3.4.1 Loss of Tumor Suppressors TP53 and RB1*

The hallmark of tumor development has been recurrent mutations. The genomic studies on SCLC showed extremely high frequency of mutations (Wistuba et al. 2000a). The highest mutation rate has been attributed to the exposure to mutagens in tobacco smoke (Toyooka et al. 2003). Early techniques such as karyotyping and comparative genomic hybridizations identified genomic rearrangements common to all lung cancers as well as aberrations specific for SCLC. Regions on chromosome arms 4p, 4q, 10q, 13q, 16q, and 17p showed high frequencies of loss of heterozygosity (LOH) unique to SCLC, suggesting the involvement of these genes in SCLC (Shivapurkar et al. 1999). The most striking alterations found at the individual gene

level in SCLC were uniform loss of function of the tumor suppressors TP53 and RB1. The frequency of TP53 mutations in SCLC is between 75% and 90%, indicating that the loss of this gene is an important event in the onset of SCLC development (Takahashi et al. 1989). The p53 protein is activated upon genomic stress and induces apoptosis or cell cycle arrest (Carvajal and Manfredi 2013). Loss of functional p53 due to mutations could serve as an initiating event in SCLC development (Wistuba et al. 2000b). George et al. (2015) discovered unidentified genomic rearrangements in another TP53 family member, TP73, while sequencing 110 SCLC samples. Specifically, these genomic alterations comprised the deletion of exons 2 and 3 of TP73, resulting in a known oncogenic transcript that exerts a dominant-negative function toward wild-type TP53 family.

The second tumor suppressor that is inactivated in nearly all SCLC is the retinoblastoma susceptibility gene (RB1). RB1 protein has a central role in cell cycle regulation, where it suppresses the transition of cells from G1 to S phase (Weinberg 1995). RB1 represses pluripotency in somatic cells through direct binding to pluripotency genes, Oct4 and Sox2. Loss of RB1 in SCLC was also found to be strongly associated with augmented expression of enhancer of zeste 2 (EZH2) (Coe et al. 2013). EZH2 was shown to be expressed at high levels in proliferating neural stem cells and has been implicated in neuronal progenitor maintenance. EZH2 has also been implicated in regulation of phenotypic switch between basal and secretory cells in the lung (Snitow et al. 2015). RB1 loss is thus associated with increased cell plasticity.

### *3.4.2 Amplification of Myc Proto-Oncogenes*

The MYC family proteins are transcriptional activators which drive the expression of genes contributing to cell cycle progression and developmental regulation. Transcriptional upregulation of one of the MYC proto-oncogenes—MYC, MYCN, or MYCL—has been identified in 20%–30% of SCLC cases (Krystal et al. 1988). The exact mechanism of MYC-mediated transformation in SCLC cells is not completely understood. MYC has been implicated in the control of pluripotency, self-renewal, and epithelial-to-mesenchymal transition, processes that are strongly implicated in cellular transformation (Chappell and Dalton 2013).

### *3.4.3 Role of Epigenetics in SCLC*

The molecular events during tumor initiation and progression are determined by epigenetic modifications, including DNA methylation, histone modification, and ncRNAs. These epigenetic events function as a network and contribute to the disease pathophysiology. Understanding the role of epigenetics in cancer heterogeneity will lead to a better knowledge of therapeutic approach (Dong et al. 2017).

Epigenetic changes could be regulated by mutations including chromatin modifiers and epigenetic readers (Codony-Servat et al. 2016). Methylation regulates key SCLC genes like *BCL2* overexpression and *RB1* silencing. LSD1, lysine demethylase 1, is a histone modifier that maintains the pluripotency of embryonic stem cells through demethylation of histone H3 lysine 4 (H3K4) and repression of genes controlling cell differentiation (Adamo et al. 2011). Overexpression of LSD1 has been observed in SCLC (Lv et al. 2012). Mohammad and Kruger (2016) used the GSK2879552 LSD1 inhibitor in a panel of 165 cancer cell lines representing multiple cancer cell types and found that a subset of SCLC cell lines were sensitive. GSK2879552 was cytostatic, rather than cytotoxic, resulting in delayed onset of growth inhibition. A change in the expression of genes involved in neuroendocrine differentiation was also observed. Studies on the LSD1 inhibitors demonstrated reduced cell proliferation and CSCs while promoting cell differentiation and reducing tumor growth indicating the role of LSD1 in SCLC stemness (Stewart and Byers 2015).

Mutations in the histone acetyl transferases CREBBP and EP300 and the histone methyltransferases MLL, MLL2, and EZH2 were found in independent studies at frequencies of 4–6% for each gene (Peifer et al. 2012; Ross et al. 2014; Umemura et al. 2014). Mutations in these genes could be a major source of genome-wide alterations in epigenetic regulation of SCLC.

### *3.4.4 Differential Expression of ASCL1 and NEUROD1*

Borromeo et al. (2016) revealed heterogeneity in SCLC through the lineage-specific transcription factors ASCL1 and NEUROD1. These related factors regulate largely distinct gene programs and differentially regulate key oncogenes in SCLC. Subsets of SCLC cell lines have been identified based on differential expression of the basic helix-loop-helix (bHLH) transcriptional factors ASCL1 and NEUROD1. The expression of these genes is required for initiation of tumor. NEUROD1 is also important for migratory capabilities of NEUROD1 high hSCLC cells (Osborne et al. 2013). Transcriptional targets of ASCL1 found in both mouse and human cancer models highlight ASCL1's role in regulating NOTCH signaling. Even though genetic alterations of ASCL1 and NEUROD1 have not been reported, epigenetic analysis of hSCLC cells shows that their loci are in active chromatin regions and are encompassed within super-enhancers in their respective cell lines (Christensen et al. 2014). Studies show multiple differences at the chromatin level between the ASCL1$^{High}$ and NEUROD1$^{High}$ subgroups of hSCLC. The striking differences seen between the ASCL1$^{High}$ and NEUROD1$^{High}$ subgroups of hSCLC, combined with the absence of Neurod1 in mouse lung NE cells and in the mSCLC, suggest the possibility that the heterogeneity arises from different cell types of origin (Borromeo et al. 2016).

### 3.4.5 Alterations in PI3 Pathway

In addition to activation of MYC signaling, the activation of the oncogenic phosphatidylinositol 3 kinase (PI3K) pathway has also been observed at high frequencies in SCLC (Shibata et al. 2009). It was discovered that phosphatase and tensin homolog (PTEN), an inhibitor of this pathway, is lost in a substantial fraction of SCLC cases. Using next-generation sequencing, mutations and amplifications in other members of this pathway have been identified in 20–40% of all studied SCLC tumors (Umemura et al. 2014). Activation of this pathway was shown to facilitate aberrant regulation of proliferation, survival, and migrations, giving the tumor cell a selective advantage.

### 3.4.6 Role of Signaling Pathways

The notch pathway is an intercellular signaling mechanism required for embryonic development. Key factors in the pathway are notch transmembrane receptors and delta (or delta-like) and jagged ligands. In small cell lung cancer (SCLC), 25% of inactivating mutations in the Notch family were seen in human tumors and Notch activity was associated with less tumor formation and prolonged survival in SCLC mouse models (Hassan 2018). The Notch pathway is likely involved in the regulation of the clinical behavior of SCLC, through its action on a number of biological processes such as neuroendocrine differentiation, proliferation, cell adhesion, and epithelial to mesenchymal transition (EMT). In mouse pulmonary cells, the loss of Notch expression was associated with an increase in the neuroendocrine markers achaete-scute complex homolog 1 (ASCL1). ASCL-1 regulates the expression of a series of pro-oncogenes linked with SCLC progression and survival, such as BCL2 and SOX2 (Marignol 2017, Osada et al. 2008). Lim et al. (2017) identified three types of SCLC tumors cells, are present with variable Notch signaling activity.

1. Cells that have Notch receptors only are activated by ligands on neighboring cells and lose their NE features, becoming non-NE cells
2. These non-NE SCLC cells promote the growth of NE cells that mostly harbor Notch ligands only (blue)
3. a small subpopulation that can have both Notch receptors and ligand, capable of inducing HES1 (the transcriptional target).

Hedgehog signaling regenerates CSCs by inducing epithelial to mesenchymal transition (EMT) via upregulation of the transcription factor SNAIL and the concomitant downregulation of E-cadherin. The transcriptional cascade induced by Hedgehog signaling depends on the Gli family of transcription factors through Smo. Hedgehog–Gli interaction enhances self-renewal properties and CSC plasticity either by the direct binding of Gli activator (GliA) to the promoters of SNAIL, CYCLIN D, MYC, and JAGGED2, activating the key stemness regulator BMI1.

BMI1plays a central role in the crosstalk of classical stem cell pathways and finally results in the maintenance and acquisition of CSC phenotype either by inhibiting p16 and p19 transcription or by other unidentified functions (Singh et al. 2015). Although preclinical studies have shown that Hh inhibitors block the self-renewal capacity of SCLC cells, lack of activating pathway mutations questioned the role of Hh pathway in SCLC. In particular, the existence of autocrine, ligand-dependent Hh signaling in SCLC has been disputed. In a conditional *Tp53;Rb1* mutant mouse model of SCLC, Szczepny et al. (2017) demonstrated the requirement for the Hh ligand Sonic Hedgehog (Shh) for the progression of SCLC. The conditional Shh overexpression activated canonical Hh signaling in SCLC cells, and markedly accelerated tumor progression. When compared to mouse SCLC tumors expressing an activating, ligand-independent Smo mutant, tumors overexpressing Shh exhibited marked chromosomal instability and smoothened-independent upregulation of Cyclin B1. Overexpression of Cyclin B1 induced chromosomal instability in mouse embryonic fibroblasts lacking both *Tp53* and *Rb1*. These results provide strong support for an autocrine, ligand-dependent model of Hh signaling in SCLC pathogenesis.

## 3.5 Models of SCLC

In vitro and in vivo model systems are essential for studying the role of recurrent genetic and epigenetic changes in SCLC. Cell lines are inadequate models as the tumors lose their primary characteristics due to their adaptation to culture conditions. Patient-derived xenograft (PDX) models partly overcome this drawback, as transplanted pieces of the tumor retain at least some of the heterogeneity and structural features of the primary tumor. Though cell lines and PDX models differ in their expression profiles, they harbor genetic alterations identical to those observed in the original tumor (Daniel et al. 2009). Genetically engineered mouse models (GEMMs) for SCLC have been successfully introduced as a system for validation of known driver mutations (Fig. 3.1).

Two types of preclinical mouse models, genetically engineered mouse models (GEMMs), are used

1. mutant mice develop autochthonous tumors upon targeted alterations in cancer genes,
2. mouse-derived allografts and patient-derived xenografts (PDXs) that grow in mice upon transplantation, either from tumors or from circulating tumor cells (CTCs).

The GEMMs reproduce the NE nature of the tumor and share many of the histopathological features observed in the human disease. The human tumors in models contain a high load of point mutations caused by cigarette smoke exposure. Semenova et al. (2015) review the various mice models of SCLC. Most of the SCLC models are based on site-specific Cre-Lox-mediated conditional deletion of the two

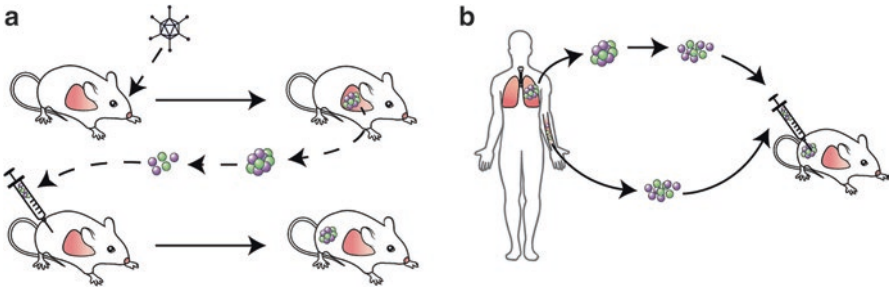



**Fig. 3.1** Modeling and studying SCLC in mice. (**a**) Deletion of *Rb* and *p53* in the lung epithelium of mice following Cre-mediated recombination of conditional alleles results in the development of SCLC. (**b**) human SCLC cells can be obtained from tumor biopsies to generate PDX in immunocompromised recipients (adapted from Shue et al. 2018)

**Table 3.3** Methods to detect heterogeneity

| Heterogeneity level | Detection methods |
|---|---|
| Phenotypic heterogeneity<br>Studies on formalin-fixed paraffin-embedded (FFPE) tissues at<br>RNA level<br>Protein level | – RT PCR, microarrays, in situ hybridization, NGS (next generation sequencing)<br>– Immunohistochemical assays<br>– LC MS, MALDI, multiplexed ion beam imaging |
| Genomic heterogeneity | – NGS<br>– Oligonucleotide microarray-based comparative genomic hybridization (array-CGH) |
| Epigenetic heterogeneity | – Methylation-specific multiple ligation-dependent probe amplification (MS-MLPA) |
| Methods applied to liquid biopsies | – CGH, NGS |

key tumor suppressors, Trp53 and Rb1, combined with either deletion of an additional suppressor (P130 or PTEN) or activation of an oncogene Mycl. In the majority of the models, cells in the lung were targeted using intranasal or intratracheal adenoviral delivery of Cre recombinase.

SCLC patients often have more CTCs than in other cancer types. Thus generation of CTC-derived explant (CDX) models (xenografts derived from CTCs) are powerful tools in longitudinal studies, and generation of large numbers of xenograft from patients (Carter et al. 2017). The use of novel technologies alongside preclinical models proves useful to identify novel subsets of SCLC cells (Table 3.3). Single-cell analyses by RNA sequencing or mass cytometry identify different stages of SCLC (Leelatian et al. 2017). CRISPR/Cas9 approaches in vivo allow faster modeling of GEMMs. A better knowledge of intratumoral and intertumoral heterogeneity from animal models and from cell lines in culture is necessary for molecular targeted therapy.

## 3.6 Targeted Therapy for SCLC

Drug discovery for SCLC has been exceptionally difficult due to the resistance to chemotherapy, which in turn is attributed to the heterogeneity of SCLC. Patients respond to the initial treatment, but the recurrence of the disease is highly frequent. In spite of poor outcome, combination chemotherapy continues to be the standard care for SCLC treatment. Currently, several investigational treatment approaches are being studied, including angiogenesis inhibition, vascular endothelial growth factor (VEGF) inhibition, molecularly targeted therapies, and immunotherapies.

Various clinical studies are underway with the present knowledge on the heterogeneity of SCLC. A comprehensive summary of the targeted therapies is tabulated (Table 3.4).

**Table 3.4** A summary of therapeutic strategies in clinical trial

| Drug | Type/mechanism | Target | Clinical trial phase | References |
|---|---|---|---|---|
| SC16LD6.5 (known as Rova-T) | Biomarker-specific antibody drug conjugate (BADC) | Delta-like protein 3 (DLL3) expressed in SCLC cells | Phase I/II | Yu et al. (2018) |
| Lorvotuzumab mertansine (IMGN901) | BADC—CD56 antibody conjugated to a microtubule inhibitor DM1 | CD56 positive SCLC cells | Phase II | Yu et al. (2018) |
| Promiximab-DUBA | Anti-CD56 hIgG1 antibody conjugated to a drug | CD56 positive SCLC cells | Preclinical assessment | Yu et al. (2018) |
| Sonidegib (LDE225), in combination with etoposide/cisplatin | Hedgehog pathway inhibition | Hedgehog pathway regulator "smoothened" | Phase I | Pietanza et al. (2016) |
| Navitoclax in combination with trametinib | Inhibits Bsl-2 and mitotic kinesin that plays a major role in mitosis | Bsl-2 and mitotic kinesin overexpressing SCLC cells | Phase I | Polley et al. (2016) |
| Irinotecan in combination with cisplatin | Inhibits DNA replication and induces apoptosis | Topoisomerase I overexpressing SCLC cells | Phase III | Rudin et al. (2008) |
| Amrubicin | Inhibits DNA replication | Topoisomerase II overexpressing SCLC cells | Phase III | Rudin et al. (2008) |
| Bevacizumab with cisplatin and etoposide | Inhibits angiogenesis and induces apoptosis | VEGF (vascular endothelial growth factor) overexpressing SCLC cells | Phase III | Rudin et al. (2008) |

(continued)

**Table 3.4** (continued)

| Drug | Type/mechanism | Target | Clinical trial phase | References |
|---|---|---|---|---|
| PRIMA-1MET | Revert mutated p53 to its wild-type form | SCLC cells that express mutated forms of p53 | Early phase clinical trials | Semenova et al. (2015) |
| PARP (poly ADP ribose polymerase) inhibitors | Inhibits DNA repair in SCLC cells after chemotherapy | PARP-I overexpressing SCLC cells | Early phase clinical trial | Semenova et al. (2015) |
| STA-8666 | Induces cell cycle arrest and apoptosis | HSP90 expressing SCLC cells | Preclinical studies | Gaponova et al. (2016) |
| Ipilimumab | Monoclonal antibody that induces the immune system to act against SCLC cells | CTLA-4, a protein receptor that downregulates the immune system | Phase II | Arcaro (2015) |
| Buparlisib | Inhibits PI3K the essential signal transduction system for oncogenes | PI3K signaling pathway | Phase I | Arcaro (2015) |
| Simvastatin | Induces cell death | Ras family proteins that regulate RTK signaling | Phase I | Arcaro (2015) |
| Imatinib, iressa, and vandetanib (administered as single agents) | Inhibits the receptors for growth factors of SCLC cells | Receptor tyrosine kinases overexpressing SCLC cells | Phase II | Arcaro (2015) |

## 3.7 Future Perspective

SCLC is a fatal malignant disease characterized by rapid recurrence. Studies over a decade have led an understanding of the heterogeneity of the disease. Emergence of novel technology and in vivo laboratory models of disease has led to the better assessment of therapeutic strategies. Yet the results are not promising as to extermination of the disease, urging further expedition for suitable biomarkers for patient stratification and the identification of synergistic combination therapies.

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# Chapter 4
# Heterogeneity of Hepatic Cancer Stem Cells


**Caecilia H. C. Sukowati**

**Abstract** Hepatocellular carcinoma (HCC) is one of the most common cancers with high mortality rate. It is a heterogeneous cancer with diverse inter- and intra-heterogeneity, also in terms of histology, prognosis, and molecular profiles. A rapidly growing evidence has demonstrated that some HCCs, if not all, were caused by the activation of the cancer stem cells (CSC), a small population within the cancer that is responsible for the initiation and maintenance of cancer growth. Until now, various populations of hepatic CSC with more than ten different phenotypical protein markers, such as CD133, CD90, EpCAM, CD24, and CD13, have been identified and validated in xenotransplantation models. They are associated with risk factors, prognosis, chemo-resistance, and metastasis. This chapter summarizes available data on different hepatic CSC markers for the development of potential future therapy.

**Keywords** Hepatocellular carcinoma · Liver cancer · Cancer stem cells · Tumor-initiating cells · Stem cells · CSC markers · CSC origin · Cancer heterogeneity · Chemo-resistance · Prognosis · Targeted therapy

## 4.1 Hepatocellular Carcinoma

### *4.1.1 Epidemiology and Risk Factors*

Recent epidemiology data Globocan 2018 of the International Agency for Research on Cancer—World Health Organization showed that liver cancer is the fifth most common cancer and the second most common cause of cancer-related death in men (Bray et al. 2018; Ferlay et al. 2019). The prognosis of this cancer is poor and the

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geographical patterns in incidence and mortality are similar (Bray et al. 2013). Hepatocellular carcinoma (HCC) accounts for about 90% of liver cancer cases, with cirrhosis as the strongest underlying condition (El-Serag 2011).

The global distribution of HCC is associated with the prevalence of its dominant risk factors. Infection of endemic hepatitis B virus (HBV) is the major cause of HCC in eastern Asia and sub-Saharan Africa for around 70%. In Europe and North America countries, hepatitis C virus (HCV) infection ranges from 50 to 70% while excessive alcohol consumption leading to alcohol steatohepatitis (ASH) contributes for around 20% of all cases (El-Serag 2011; Forner et al. 2012). Case-control studies from different countries report that chronic ethanol consumption is associated with an approximately twofold increased odds ratio for HCC (Ramadori et al. 2017). Besides the infection of hepatotropic viruses and alcohol, obesity and diabetes that commonly associated with non-alcoholic steatohepatitis (NASH) also increased the risk of HCC (Estes et al. 2018). Synergism between hepatitis virus infection and metabolic liver disease seems to worsen the course of the disease. Certain toxins and chemical agents such as aflatoxin B and vinyl chloride monomer also contribute to the progression of HCC.

### *4.1.2 Heterogeneity of HCC*

Due to its different etiological factors, as well as various genetic background and long-time development of the disease, HCC is characterized by a high phenotypic and functional heterogeneity. Variability can occur in tumors among patients (intertumoral heterogeneity) and within individual tumor (intratumoral heterogeneity). Cell morphology, molecular profile, and expression of specific markers can be used to stratify and classify discrete tumor subtypes.

Using the Edmondson–Steiner's (ES) histological morphology-based criteria, HCC can be divided into four subgroups moving from a well-differentiated HCC (Edmondson's groups 1–2) to poorly or undifferentiated HCC (ES groups 3–4) (Edmondson and Steiner 1954; Callea 1988). However, recently it was shown that from a systematic analysis of around 200 articles on histological stages (ES, WHO, and other systems), while histological grading of HCC has an important prognostic role, there is an unsatisfactory heterogeneity on the microscopic assessment of this cancer (Martins-Filho et al. 2017). Genome- and proteome-wide studies have revealed the existence of various molecular profiles that are associated with different grade of hepatocytes maturation from "hepatoblast-like" to "mature cells" signature. These molecular profiles can be used to predict the outcome of the disease, such as the expected survival or tumor recurrence (Boyault et al. 2007; Hoshida et al. 2009).

Variability is also observed in tumors within an individual, where different tumor sections may have different markers expressions. Intratumoral heterogeneity, which is unlikely to be captured from a biopsy, affects patient's outcome because a single

treatment targeting one cancer-specific pathway would spare tumor cells having distinct characteristics (Marquardt et al. 2015). An analysis from 120 tumor areas collected from 23 HCC patients, intratumor heterogeneity, comprising cells morphology, immunohistochemistry, and mutational status, was detectable in 87% of the HCC cases (Friemel et al. 2015). In line with this study, an immunostaining data of stemness markers from multifocal HCCs showed variability in the markers positivity and discrepancy in the staining degree. Interestingly, by using stemness marker Sox9, identical tumor morphology in terms of Edmondson's grading and growth pattern did not infer the same degree of immunoexpression; and the largest tumor nodule was not representative of highest IHC score (Lo et al. 2017).

From molecular aspects, the heterogeneity of HCC is even further complicated. Advances in the development of "omics" science open the possibility to integrate high-throughput data on cancer genomics, epigenomics, transcriptomics, proteomics, and metabolomics into computational and statistical frameworks. Genetic analysis by whole genome sequencing of HCC nodules showed the percentage of ubiquitous mutations to be widely varied among patients, indicating variation in the extent of intratumor heterogeneity (Xue et al. 2016).

By RNAseq screening, transcriptional deregulation was noticed in hundreds of protein-coding genes, including genes associated with drug catabolism, inflammatory responses, and cell proliferation (Jovel et al. 2018). Gene expressions global array also showed that mRNAs expression data were high heterogeneous. Differently expressed genes in HCC mainly consist of cancer-associated genes (e.g., AFP, THBS4, LCN2, GPC3, NUF2, etc.), kinases (e.g., TTK, MELK, BUB1, NEK2, BUB1B, AURKB, PLK1, CDK1, PKMYT1, PBK, etc.), and cell cycle pathways (Agarwal et al. 2017). Other frequent changes are found in telomere maintenance (telomerase reverse transcriptase (TERT)), chromatin modifiers, and inflammatory pathways (Marquardt et al. 2015).

Somatic mitochondrial (mtDNA) mutations were also a common feature in HCC and in paired non-HCC inflammatory tissues, suggesting that these tissues might not be mtDNA genetically "normal." Some mtDNA mutations may undergo positive selection during the clonal expansion (Li et al. 2018). Further, epigenetic aberrations involving DNA methylation, histone modifications, and noncoding microRNAs (miRNAs) dysregulation are associated with hepatocarcinogenesis and metastasis (Kgatle et al. 2016).

## 4.2 Hepatic Cancer Stem Cells

### *4.2.1 Definition*

Carcinogenesis (development and progression of cancer) is consisted of many steps and long-term courses from normal to malignant tissues. These factors reflect into heterogeneity within cancer "mass" as well as in the cellular functional level.

A review by Magee et al. (2012) well-summarized the sources of cellular heterogeneity in cancer through stochastic genetic and epigenetic changes (Burrell et al. 2013), environmental differences (Junttila and de Sauvage 2013), hierarchical populations (Dick 2008; Reya et al. 2001), and the combination of these factors (Magee et al. 2012).

The hierarchical cell populations or commonly known as the cancer stem cells (CSC) proposed that cancer is composed in a hierarchy and only a small population of the cells in the cancer has capacity to initiate and maintain tumor growth. Just as normal stem cells in normal tissue, CSC perform as stem cells in cancerous tissue. These cells act as the main players in the highest level of the cancer hierarchy and may still have stem cells properties.

According to CSC theory, cancer mass is assembled in heterogeneous populations of cells: malignant CSC as central populations with the capacity to divide and differentiate and partial or full-differentiated cancer cells derived from CSC that comprise the majority of cancer mass. This hierarchy model proposes that only CSC population is gifted with special and unique protective mechanisms to be responsible for the maintenance and propagation of the tumor (Ma et al. 2008a). Non-tumorigenic cells are thought to compose the bulk of tumors but have little capacity to contribute to cancer progression (Magee et al. 2012; Dick 2008).

The first conclusive evidence of CSC was demonstrated by the group of John Dick in mid-1990s in acute myeloid leukemia (AML) cells in which a CD34+/CD138− subpopulation is capable of initiating tumors in NOD/SCID mice (Lapidot et al. 1994; Bonnet and Dick 1997). After this breakthrough, many reports had demonstrated the proofs of tumor-initiating cells both in hematopoietic cancer and solid tumors. In solid tumors, the CSC have been found in breast tumor, brain tumor, colon cancer, pancreatic tumor, ovarian cancer, melanoma, lung cancer, and many others. The CSC had been identified in almost all human cancers.

Because CSC populations are important in the initiation and maintenance of the cancer, their resistance to anticancer drugs is an obstacle for the total eradication of cancer. Conventional chemotherapies may recognize and kill most of bulk (differentiated) tumor cells but spare the CSC. Therefore, to achieve a complete response in liver cancer therapy it is crucial to target the CSC first to eradicate the source of the cancer, and then the more differentiated tumor cells. Total eradication of the tumor will not only reduced the differentiated tumor size, but more importantly to prevent the reoccurrence of cancer.

### *4.2.2 Origins*

Liver, a vital internal organ with several roles in different functions, develops via a progressive series of interaction between the embryonic endoderm and nearby mesoderm. Hepatic endoderm cells, known as hepatoblast, delaminate from the epithelium, and invade the adjacent septum transversum mesenchyme (STM) to form

the liver bud. The STM then give rises to fibroblast and stellate cells of the liver, while hepatoblasts are bipotent cells that generate both cholangiocytes and hepatocytes (Zorn 2008).

In adult liver, mature hepatocytes account for over 80% of the cell population. In normal conditions, these cells remain quiescent and hardly proliferate. However, after partial hepatectomy, quiescent hepatocytes proliferate together with hepatic stellate cells and endothelial cells. This mechanism quickly undertake a serial growth to restore the original mass and the function of the liver (Mishra et al. 2009; Christ and Pelz 2013).

In severe and extensive damage, or when the regenerative capabilities of hepatocytes are compromised, the proliferation and activation of hepatic stem cell compartment is observed. Moreover, these cells rarely acquire age-related genetic defects associated with cancer induction that may imply their protective mechanism against genetic damage (Mishra et al. 2009; Roskams et al. 2003). This process, named as "ductular reaction" is the amplification and maturation of the hepatic stem/progenitors cells (HPC), located in the Canal of Hering (CoH) (Theise et al. 1999; Sell and Leffert 2008; Sell 1993), represents anatomic and physiological link between intralobular canalicular system of hepatocytes and the biliary tree (Alison et al. 2009; Russo and Parola 2011). The reside cells in CoH showed shared morphology and immunophenotype between hepatocytes and cholangiocytes. They are positive for cytokeratin CK7 and CK19, oval cell markers OV6 and OV1, neuroendocrine markers chromogranin-A, and neural cell adhesion molecule (Roskams 2006a).

Several studies have highlighted the presence of HPC in chronic liver disease, cirrhotic parenchyma, as well as in HCC tissues, both in human and animal models (Ijzer et al. 2010; Xiao et al. 2004). Moreover, a number of studies illustrated the role of bone marrow stem cells in producing hepatocytes, both in animal model and in human through cell fusion with hepatocytes. However, their precise role in liver repopulation and in cancer formation is still unclear (Lagasse et al. 2000; Sato et al. 2005; Schwartz et al. 2002).

Considering multiples steps involved in liver organogenesis and various pathogenesis of HCC, the origin of CSC in HCC is still controversial. It was shown that HCC expressing progenitor cell/ductular markers like CK19 have a more aggressive clinical course (Roskams 2006b; Uenishi et al. 2003).

Identification of the origin of cancer cells requires characterization of the normal cellular hierarchy within the given tissue and study of the stem cells niches (Anfuso et al. 2015). The source of CSC might contribute, significantly, to the variation of phenotype, severity, and prognosis of the HCC.

In 2013, the group of Thorgeirsson provided an important direct and functional evidence that human liver cancers can be derived from different hepatic cells of origin. They systematically compared liver tumorigenesis in adult hepatocytes, fetal hepatoblast, and adult HPC after controlled oncogenic transformation. Interestingly, all three lineages gave rise to tumors with varying degrees of HCC and cholangiocarcinoma (CAA) content, most hepatocytes-derived tumors were HCC, HB-derived tumors were mainly CCA, while HPC-derived tumors were anaplastic histopathology.

Moreover, all three lineages possessed CSC properties but showed different tumorigenic potential with HPC-tumors being the highest. Dedifferentiation of mature hepatocytes is a possible explanation for the observed phenotypic diversity of HCC, from well- to less-differentiated (Holczbauer et al. 2013; Zucman-Rossi et al. 2013).

The induction of chemotherapy was also found to be able to induce spherogenesis together with the expressions of pluripotency factors of bulk HCC cells grown under stem cell culture conditions showing that cancer cell itself may be an important source of CSC during tumor development (Hu et al. 2012).

Collectively these results provided a new insight regarding the origin of CSC in primary liver cancers. CSC are not just the result of oncogenic transformation of hepatic stem/progenitor cells, but might also be the result of oncogenic reprogramming of any cell types along hepatic lineage maturation (Fig. 4.1).

### *4.2.3 Methods of Identification and Isolation*

Identification and isolation of hepatic CSC are usually based on three approaches: by using a phenotypic stem cells/CSC protein marker, by isolating side population (SP), and by performing a 3-dimensional spherical population. All these approaches have to be validated in a xenograft model to be the gold standard of the tumorigenic potential of the CSC (Table 4.1).

The first evidence of CSC existence in HCC was reported in 2005 by Haraguchi and colleagues when they isolated SP cells in vitro. SP cells were found to be more resistant to anticancer drugs such as doxorubicin, 5-flouracil, and gemcitabine, compared to non-SP cells (Haraguchi et al. 2006a, b). Further, Chiba et al. demonstrated that SP cells transplantation of as few as 1000 cells successfully induced

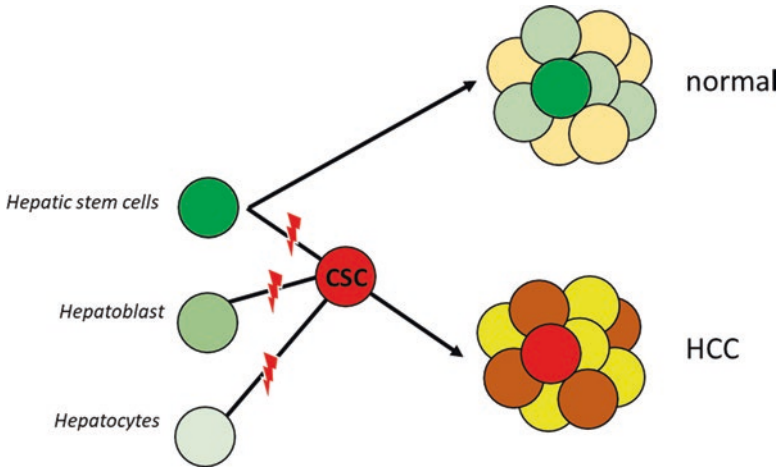


**Fig. 4.1** The concept of CSC and origin of hepatic CSC

**Table 4.1** Methods to identify and isolate hepatic CSC

| Methods | Approach | Molecular determinant |
|---|---|---|
| Surface marker | CSC marker | CD90, EpCAM, CD133, CD24, CD13, CD34, SOX9, etc. |
| Side population | Drug efflux | ABCG2 |
| Sphere formation | Clonogenicity of CSC | EpCAM, CD24, CD90 |

tumor in NOD/SCID mouse (Chiba et al. 2006) followed by another proof of the cancer stemness potential of SP population to induce metastasis (Shi et al. 2008).

The search of CSC in liver had progressed and developed by using CSC protein markers. In late 2006, CD133 was proposed to be a CSC surface marker to isolate CSC population from HCC cell lines, supported by several other reports (Ma et al. 2007, 2008b, c; Yin et al. 2007; Zhu et al. 2010).

In 2008, Yang et al. give a wider outlook and exhibit that CD90 cells isolated from hepatic cell line, primary cancer, and peripheral blood have also distinct characteristic as CSC (Yang et al. 2008a, b). In 2009, EpCAM+ HCC cells were demonstrated to display hepatic CSC-like traits including the abilities to self-renew, differentiate, and tumorigenic (Yamashita et al., 2007, 2009). Other proposed CSC markers will be discussed below.

### *4.2.4 Heterogeneity of Hepatic Cancer Stem Cells*

In many decades, the understanding that cancers are composed with heterogeneous entities has been acknowledged. A paper from Van R. Potter (1956) mentioned that the issue of uniformity versus diversity in cancer tissues is important from the standpoint of fundamental biochemistry, carcinogenesis, and chemotherapy. These heterogeneous populations within cancer contain a variety of subpopulations of cells with differing metastatic potential (Fidler 1978).

Several studies had shown that phenotypically distinct populations from a single HCC specimen had different expressions of a number of tumor-associated stem cell markers (Colombo et al. 2011; Yamashita et al. 2013; Zheng et al. 2018). These cell populations showed different cellular features, drug resistance, tumorigenic potential, and tumor-promoting function. It indicates that not only the presence of different cell populations in a single tumor but also the complex interaction between these populations. For example, Yamashita group showed that in a single primary HCC, EpCAM-positive cells had epithelial cell-like features whereas CD90-positive cells showed vascular endothelial cell-like features. Interestingly, CD90-positive cells enhanced the motility of EpCAM-positive cells when co-cultured in vitro (Yamashita et al. 2013). Different CSC subpopulations also contain distinct molecular signatures that are independently associated with prognosis (Zheng et al. 2018).

#### 4.2.4.1 Phenotypical CSC Markers

The recognition of CSC marker(s) is the easiest method to identify hepatic CSC. Stem cell marker is a specialized signaling molecule or protein receptor that mainly coating the surface of cell which allow the identification and differentiation. Because of the possible different CSC origins, wide variability of HCC risk factors, and long-term cancer development, the finding of CSC markers is heterogeneous.

Various different markers of CSC from established HCC cell lines and primary tumors had been identified and validated by in vivo xenograft assay. The use of markers protein CD133 (Prominin-1), CD90 (THY-1), EpCAM, CD24, OV-6, and CD13 (ANPEP) is the most common method to define a hepatic CSC population. The combination of these CSC markers was further used to characterize several subpopulations in a CSC population, resulting in a wide variety of CSC phenotypes (Ma et al. 2008b; Zhu et al. 2010; Yang et al. 2008a; Chen et al. 2012). Until now, at least 12 different phenotypical CSC markers had been proposed (Fig. 4.2). However, until now, no consensus on a CSC phenotype has been agreed for HCC.

**CD90 (THY-1)**

CD90 is a 25–37 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed in many cell types such as T cells, thymocytes, neurons, endothelial cells, and fibroblast. It has function as an important regulator of cell–cell and cell–matrix interaction, apoptosis, adhesion, migration, cancer, and fibrosis (Rege and Hagood 2006).

In HCC, cells expressed CD90+ had been purified from hepatic cancerous cell lines and primary cancers. The CD90+ cells, but not CD90− cells, from HCC cell lines HepG2, Hep3B, PLC, HuH-7, MHCC97L, and MHCC97H displayed tumorigenic capacity when they were injected into immunodeficient mice (Yang et al. 2008b). From primary tumors, CD90+CD45− cells from the tumor tissues and blood samples of liver cancer patients also have capacity to generate tumor nodules

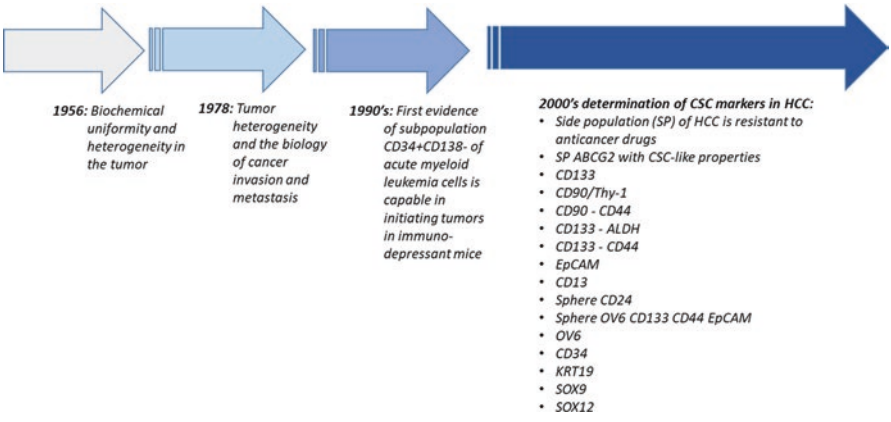


**Fig. 4.2** The search of hepatic CSC

in mice. Furthermore, CD90+CD45− cells were detectable in 90% of blood samples from cancer patients but none in normal subjects or patients with cirrhosis indicates circulating CSC in human liver cancer (Yang et al. 2008a).

In liver, CD90 expression was found highly expressed in HCC (Sukowati et al. 2013), preferably in poorly differentiated HCC and suggested to be associated with a poor prognosis (Lingala et al. 2010; Lu et al. 2011; Yu et al. 2011). Based on a gene ontology analysis, the overexpressed genes in $CD90^+$ cells from HCC were associated with inflammation, drug resistance, and lipid metabolism compared to $CD90^+$ from nontumoral liver (Ho et al. 2012). A meta-analysis of 27 studies on CSC markers expressions in HCC tissues determined that CD90 has high specificity in predicting poor differentiation HCC (Liu et al. 2015).

**CD133 (Prominin-1)**

CD133 (prominin-1) is a member of pentaspan transmembrane glycoprotein family. Human surface antigen AC133, a homologue for mouse prominin-1, was discovered by generating a monoclonal antibody to CD34+ hematopoietic stem cells isolated from fetal liver, bone marrow, and cord blood (Miraglia et al. 1997; Yin et al. 1997). Human prominin-1 consisted of 865 amino acids (aa) with a total molecule weight of 120 kDa (115 kDA in mouse). Prominin-1 has a unique structure composed of an N-terminal extracellular domain, five transmembrane domains with two large extracellular loops, and a 59 aa cytoplasmic tail. Until now, the main function of prominin-1 remains unclear (Shmelkov et al. 2005).

In HCC, CD133+ purified from cell lines SMMC-7721, Huh-7, and PLC8024 was able to induce tumors in xenograft models in contrast with their CD133− counterpart (Ma et al. 2007; Yin et al. 2007; Suetsugu et al. 2006). Following cells-directed differentiation, CD133+ cells showed a dramatic increase of angiomyogenic markers, suggesting potential to skeletal and cardiac features differentiation (Ma et al. 2007). In combination with aldehyde-dehydrogenase (ALDH), CD133+ ALDH+ cells were significantly more tumorigenic than their CD133+ALDH− or CD133−ALDH− (Ma et al. 2008b). A report exhibited that combination of CD133+CD44+ cells have extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. In vivo xenograft experiments revealed that the highly tumorigenic capacity was primarily attributed to CD133+CD44+ cells instead of their CD133+CD44− (Zhu et al. 2010).

In a meta-analysis study, CD133 was shown to have a significant role in predicting clinical outcome of the HCC patients. Its presence was also associated with poor histopathologic grade and worse survival (Ma et al. 2013a). Another study showed a consistency between prognostic value and the expression of CD133 (and CD44) both in HCC patients and in patient-derived xenograft models (Zhao et al. 2016).

**EpCAM (CD326)**

EpCAM is a another potent hepatic CSC marker. EpCAM gene encodes a carcinoma-associated antigen and is expressed on most normal epithelial cells and gastrointestinal carcinomas and functions as a homotypic calcium-independent cell adhesion molecule. The antigen is being used as a target for immunotherapy treatment of

human carcinomas. The name EpCAM derives from the original functional description as a glycoprotein epithelial cell adhesion molecule. Structurally, EpCAM is closely related to one transmembrane glycoprotein GA733-1, both are novel proteins in structure and likely function (Litvinov et al. 1997).

Yamashita et al. reported that EpCAM expression in HCC displayed a distinct molecular feature with features of stem markers, whereas HCC without EpCAM expression displayed genes with features of mature hepatocytes (Yamashita et al. 2008). The isolation of EpCAM+ cells had also been performed from both HCC cell line and primary cancers. These isolated cells had displayed hepatic CSC-like capacity including self-renewal capacity, differentiation, and tumor induction in NOD/SCID mice (Kimura et al. 2014). Furthermore, EpCAM has been shown to be a direct transcriptional target in the Wnt/β-catenin pathway that has been suggested to have an important in the self-renewal of cancer cells (Yamashita et al. 2007; Pandit et al. 2018; Khosla et al. 2017). EpCAM+ cells were highly expressed in hepatitis B and had potential anticancer drug resistance (Kimura et al. 2014). HCCs expressing EpCAM are associated with unfavorable prognostic factors and have a more aggressive clinical course than those not expressing EpCAM (Sung et al. 2016).

**OV-6 Antibody**

The OV-6 monoclonal antibody was developed in rat following treatment with hepatotoxins or hepatocarcinogens. It is a useful marker to oval cells and hepatoma cells (Dunsford and Sell 1989; Dunsford et al. 1989). The positivity of OV-6 had been observed in fetal liver and adult biliary disease, adult liver disease, and pediatric liver disease. The antigen target of OV-6, the oval cells, shared epitope with CK14 and CK19 (Libbrecht et al. 2001). Several studies have shown a progenitor cell with OV-6 reactivity in a substantial number of HCCs. These cells are thought to be derived from hepatic stem cells in the intraportal area and they have ability to differentiate into hepatocytes and biliary cells.

Isolated OV6+ tumor-initiating cells (TICs) from HCC cell lines possessed high capacity to form tumor spheroids in vitro as well as great capacity to form tumors in vivo. Further, these cells exhibited metastatic potentials. Patients with more OV6+ tumor cells were associated with aggressive clinicopathologic features and poor prognosis (Yang et al. 2012).

**CK19**

Cytokeratins (CK) are intermediate filaments forming neutral proteins found in the intracytoplasmic cytoskeleton of epithelial tissue. They provide mechanical support and play role in different additional functions of the cells. In the liver, CK19 is expressed in the bile duct, cholangiocytes, and the hepatic site of stem cells, the Canal of Hering. During liver injury, such as in cirrhosis, hepatic stem cells expand and replenish the damage of the liver parenchyma (Alison et al. 2009).

From HCC cell lines, isolated single CK19+ cells showed self-renewal and differentiation into CK19− cells, whereas single CK19− cells did not produce CK19+ cells. These cells displayed high proliferation capacity, tumorigenicity, and 5-fluorouracil resistance (Kawai et al. 2015).

**CD13 (ANPEP)**

CD13 (aminopeptidase N/ANPEP) or zinc-binding transmembrane ectopeptidase is a protein involved in the metabolism of regulatory peptides by diverse cell types. Recently, cells with CD13 phenotype had been proposed as a marker of quiescent CSC marker. These cells were found to be enriched in the SP. They had high oncogenicity and high resistant to both chemo- and radiotherapy, thanks to their capacity in preserving DNA damage through ROS modulation (Haraguchi et al. 2010; Kim et al. 2012).

In clinical samples, high expression of CD13 (CD13hi) protein was noticed in HCC with big tumor size. The CD13hi group also showed significantly earlier recurrences and shorter survival times. In a multivariate analysis, CD13hi was an independent prognostic factor for overall survival group (Yamanaka et al. 2018).

**CD24**

CD24 encodes sialoglycoprotein that is expressed in mature granulocytes and B cells and it modulates growth and differentiation signals to these cells. CD24 is also frequently overexpressed in various human cancers and is correlated with a poor prognosis. In breast cancer, the negative phenotype of CD24 (CD44+CD24−) is already established as a marker of breast CSC. However, information on CD24 in HCC CSC is still limited.

Together with CD44 variant, CD24 had been used as a phenotypic marker of spheres of cells inducted from poor and undifferentiated HCC cell lines. These spheres were more resistant to several anti-neoplastic drugs compared to their parental cells, perhaps due to high expression of ABCG2 (Hashimoto et al. 2014). CD24 was associated with the epithelial-mesenchymal transition (EMT) process and the immunity of microenvironment through Notch-related pathway (Wan et al. 2016), also in combination with CD133 (Wang et al. 2018).

**CD34**

CD34 is a transmembrane phosphoglycoprotein that was initially described in hematopoietic stem cells population. It may play a role in the attachment of stem cells to the bone marrow extracellular matrix or to stromal cells. A newly proposed hepatic CSC marker CD34 was reported to be able to induce three types of primary liver cancers: HCC, CAA, and combined HCC-CAA (Park et al. 2015a). The $CD34^+$ CSC coexpressed liver stem cell and myelomonocytic cell markers, showing a mixed phenotype, a combination of hepatobiliary stem/progenitor cells and myelomonocytic cells (Zeng et al. 2015). The CD34+ cells were able to be cultured to multiple clones without losing CSC property (Park et al. 2015b).

**SOX9**

The SOX9 (Sex determining region Y-box 9) is a transcription factor involved in the organogenesis of many organs. In the liver, SOX9 expression is confined to the bile duct that produced bipotent cells, while hepatocytes do not express SOX9. This expression pattern persists in adulthood (Kawaguchi 2013). In this 2016 study, the authors transfected HCC cell lines with SOX9 promoter. The SOX9+ cells had capability of self-renewal and differentiation into SOX9− cells. In xenograft, SOX9+ cells were able to generate tumor at a high frequency. They had also

involved in the EMT and the activation of TGFß/Smad signaling, and regulated Wnt/ß-catenin signaling. The high aggressivity of SOX9 cells was also confirmed in clinical samples (Kawai et al. 2016).

**SOX12**

The use of SOX9 as a marker of CSC in HCC was proposed in 2017. SRY-related HMG-box gene 12 (SOX12) is a member of transcription factor superfamily homologous with sex-determining gene SRY. It has been shown to play critical roles in embryonic development and cell fate determination (Dy et al. 2008). Compared to SOX12-cells, SOX12+ HCC cells generated significantly more tumor spheres in culture. Further, they were more chemo-resistant to cisplatin, were detected in circulation, and were able to form distal tumor more frequently. SOX12 appeared to functionally contribute to the stemness of HCC cells. Thus, it may be a novel marker for enriching CSC in HCC (Zou et al. 2017).

#### 4.2.4.2 Side Population

The ATP binding cassette (ABC) transporters are one of the largest families of membrane transport proteins. These proteins utilize a pair of ATP (adenosine-5′-triphosphate) molecule to export specific compounds or to flip them from inner to outer leafs of the membranes (Higgins 1992). Thus, they are responsible for translocations of various substrates such as metal ions, sugars, peptides, proteins, amino acids, and a large number of hydrophobic compounds and metabolites across the membrane barrier (Dean et al. 2001) (Fig. 4.3).

The breast cancer resistance protein (ABCG2/BCRP/ABCP/MXR) is one member of the ATP-binding cassette (ABC) transporters superfamily proteins (Dean et al. 2001). ABCG2 protein is composed of 665 aa resulting a 72 kDa protein. It has an N-terminal ATP-binding domain (NBF) and a C-terminal transmembrane domain (TMD), a structure half the size and in reverse configuration to most other ABC proteins comprising two NBFs and two TMDs (Doyle and Ross 2003; Bailey-Dell et al. 2001; Robey et al. 2009). One of the main functions of ABCG2 is related to the cell resistance to exposure of external compounds, exporting the drug out of the cells thus maintaining the intracellular drug compound below toxic level. Regarding this underlying principle, extensive studies have been carried out to find out the relationship of ABCG2 with drug resistance, especially in chemotherapy-treated cancers.

In 1997, Goodell and colleagues pioneered a technique to purify a small population of cells which is rich in stem cells. These cells, more known as side population (SP) phenotype, had capacity to export the Hoechst 33342 dye out of the cells and recognized as Hoechst$^{null/low}$ in flow cytometry instrument (Goodell et al. 1996, 1997). The SP were importantly involved in the drug efflux-related chemotherapy resistance and the SP analysis was found to be an efficient method to evaluate the functional activity of ABCG2 (Hu et al. 2008).

A study by Hu et al. showed that ABCG2 expression significantly influenced the levels of drug efflux from HCC cell lines. In resistant cancer cells, ABCG2 expres-

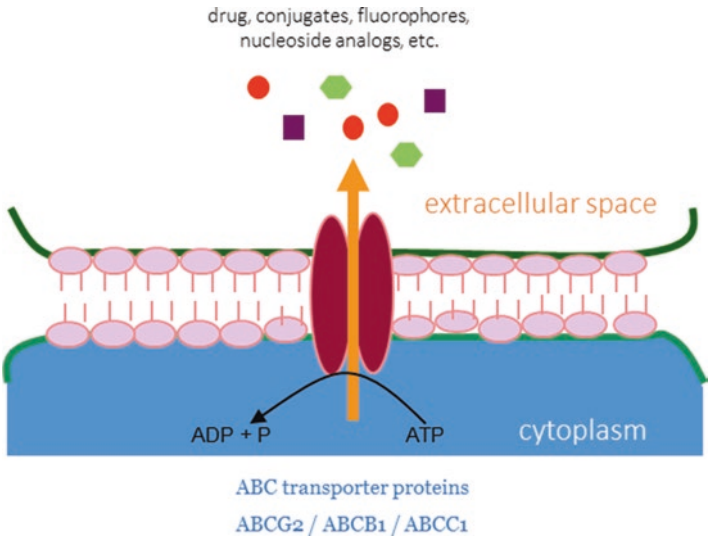


**Fig. 4.3** Function of ABC-transporters protein in drug efflux

sion is associated with many chemotherapy agents. In liver cancer cells, treatment of mitoxantrone, doxorubicin, epirubicin, and gefitinib resulted in induction of ABCG2 and low sensitivity to the drugs (Cusatis et al. 2006; Kamiyama et al. 2006; Li et al. 2007). The ABCG2 expression was found to be high (Sun et al. 2010) and was related to HCC degree of differentiation (Sukowati et al. 2012).

In HCC, various reports on SP population have been demonstrated. SP cells, sorted from HCC cell lines, harbored CSC-like traits and therapeutic-resistance (Shi et al. 2008). They also showed high proliferations, anti-apoptotic properties, and capabilities to initiate tumor formation in non-obese diabetes/severe combined immunodeficiency (NOD/SCID) mice (Mishra et al. 2009). The relative abundance of SP cells correlated directly with the metastatic potential of the HCC cell lines (Guo et al. 2016).

Further studies on ABCG2 expression in these cell lines showed that the sorted ABCG2+ cells generated both ABCG2+ and ABCG2− cells while ABCG2− cells only gave ABCG2− cells. Additionally, GATA6, an essential factor of earliest phase of hepatic development, was intensely expressed in ABCG2+ cells and C/EBPβ, a factor for late phase of liver development, was expressed more in ABCG2− cells (Zen et al. 2007).

#### 4.2.4.3 Sphere Formation

In this functional selection, CSC was defined by its clonogenic and self-renewal ability to perform spheres formation, either by single cell colony or by general three-dimensional induction from a selected HCC cell population.

By using a serum-free culture, spheroid HCC-CSC were enriched from a HCC cell line. The serum-free cultured spheroid cells demonstrated self-renewal, spheroid formation, higher EpCAM expression, increased Hoechst-33342 efflux, and upregulated Wnt/β-catenin signaling. Mice injected with CSC spheroids showed aggressive tumor initiation and growth compared with mice injected with control cells (Pandit et al. 2018).

By performing spheroid formation and subsequent single-cell cloning, subclones with enriched CSC potential was obtained from HCC cell line HepG2. The analyses in several 2D and 3D cell culture systems as well as a panel of functional assays both in vitro and in vivo, revealed that the generated subclones displayed characteristic and sustained features of tumor initiating cells. They showed highly aggressive properties related to tumor progression and metastasis. These characteristics could clearly be correlated with the expression of CSC markers that might have prognostic value in the clinical HCC setting (Muenzner et al. 2018).

### *4.2.5 Significance of Hepatic CSC Heterogeneity*

Beside the use of CSC phenotypical markers to identify and isolate the CSC, they have important clinical significances and correlate with prognosis. As mentioned above, due to the diverse risk factors, long-term disease development, and possible extrinsic exposures to lifestyles and treatment, the pattern of CSC markers are variable.

A global gene expression profiling is a potent tool to study the molecular classification based on dysregulation of the molecular event in hepatocarcinogenesis. However, considering the variation inter- and intra-individual of HCC patients, the samples size must be sufficient large to reach a statistical value and provide rather general conclusion. A good example is that based on a study of gene microarray where HCC was classified into two clusters towards prediction of survival, A (fetal hepatoblast pattern) showing a shorter survival than B (hepatocyte pattern) (Lee et al. 2004). The expression of well-known markers of hepatic oval cells, the early progenitors of adult liver stem cells, is found in the hepatoblast subtype that may arise from hepatic progenitor cells (Lee et al. 2006). In another study, high-throughput analysis showed that HCC EpCAM+ subtype had a molecular signature of hepatic progenitor cells while HCC EpCAM− subtype displayed features of mature hepatocytes. Based on the level of alpha fetoprotein (AFP), HCC could be sub-classified into four groups where EpCAM+AFP+ HCC showed a poorer prognosis compared to EpCAM−AFP− HCC (Yamashita et al. 2008).

Beside of microarray-based HCC classification, the expression of single CSC marker in HCC tissues has been used to be correlated with prognosis. Multiple clinical studies had shown that the high expression of CSC marker CD133 in HCC tissues, in particular in the cytoplasm, is correlated with poor prognosis (Chen et al. 2017; Chan et al. 2014; Sasaki et al. 2010; Ma et al. 2013a; Song et al. 2008). The CD133 cytoplasmic expression was reported to be an important risk factor for overall survival in HCC (Sasaki et al. 2010). In contrast, other studies showed that

the positive rate of CD133 in HCC was similar to that found in viral hepatitis, and not all tissues adjacent to HCC tissues were positive for this marker (Yin et al. 2007; Lingala et al. 2010). A study showed that both CD133 and EpCAM were significantly elevated after TACE treatment. However, only EpCAM, but not CD133, was predictor of tumor recurrence after transplantation (Zeng et al. 2012), leads to conclusion that the expression of CD133 alone might not sufficient to predict prognosis (Salnikov et al. 2009).

CD90 expression was found preferably in poorly differentiated HCC (Yu et al. 2011). CD90 protein was increased in HCC samples (Sukowati et al. 2013) and the overexpression correlated with age, HBV infection, and histological grade but not with alcohol or cirrhosis. Patients with highest level of CD90 expression showed the poorest prognosis (Lu et al. 2011). Furthermore, CD90+CD45− cells were detectable in 90% of blood samples from cancer patients but none in normal subjects or patients with cirrhosis without HCC suggesting circulating CSC as possible marker in human liver cancer (Yang et al. 2008a).

It had been reported that individual HCC could harbor different self-renewing tumorigenic cell types expressing a variety of morphological and phenotypical markers, karyotypic evolution, and different gene expression profile (Colombo et al. 2011). Furthermore, different HCC tissues expressed unique combinations of CSC markers and the isolated CSC using same CSC marker had unique expression profiles (Wilson et al. 2013).

### *4.2.6 Chemo- and Radio-Resistance*

The idea to target the CSC as the source of tumor has attracted researchers to develop a specific CSC-targeting therapy. However, one of the main obstacles in the drug development is the preferential resistance of the CSC against chemotherapies.

Previously, Ma et al. had demonstrated that preferential survival effect of CD133+ cells was almost completely inhibited by co-incubation of the AKT1 inhibitor with doxorubicin or 5-FU (Ma et al. 2008c). It had been demonstrated that CD13+CD133+ cells were increased drastically after the treatment of doxorubicin or 5-FU alone. Even though 5-FU inhibited CD90+ proliferating CSC and CD13-neutralizing antibody suppressed the renewal and tumorigenicity of dormant CSC CD13, the combination of both agents significantly reduced tumor growth (Haraguchi et al. 2010). Furthermore, Chiba et al. reported that 5-FU enriched CSC EpCAM+ cells and in contrast 3-deazaneplanocin A (DZnep) directly reduced these cells. The use of both DZnep and 5-FU together suppressed the tumor growth (Chiba et al. 2012).

The novel derivative of small-molecule WNT inhibitor, IC-2, has the potential to suppress liver CSC and can serve as a promising therapeutic agent to improve the prognosis of patients with HCC. IC-2 significantly reduced the CD44+ population, CD90+ HLF cells, CD133+ HepG2 cells, and EpCAM+ cells (Seto et al. 2017).

Another approach to block carcinogenesis is by immunotherapy. It was shown that CSC cells express high levels of tumor-associated antigens and major

histocompatibility complex (MHC) class I molecules (Li et al. 2016; Choi et al. 2018). Vaccination with dendritic cells loaded with SP cell lysates could induce a T cell response in vivo and suppress the tumor growth (Li et al. 2016). It was showed that EpCAM cells expressed high levels of tumor-associated antigens (TAAs) as well as MHC molecules. Pulsing dendritic cells by EpCAM peptides resulted in the efficient generation of mature dendritic cells thus enhancing T cell stimulation and generating potent cytotoxic T lymphocytes (Choi et al. 2018). To modulate the chemo-resistance with respect to ABCG2 activity, several high-throughput screening methods had identified several potential inhibitors for chemo-resistant determinant ABCG2 (Henrich et al. 2013; Antczak et al. 2014).

## 4.3 Applications and Future Perspectives

Since HCC is a heterogeneous tumor caused by various etiological factors and oncogenic transformations, personalized, tailored treatment strategies are needed for a more successful therapy. Among others aspects, attention must be given to the study of the hierarchical organization of cancers to better understand the cancer origin, cancer microenvironment, the alteration in molecular pathway, cancer prognostic types and drug administration, and chemo-resistance (Fig. 4.4).

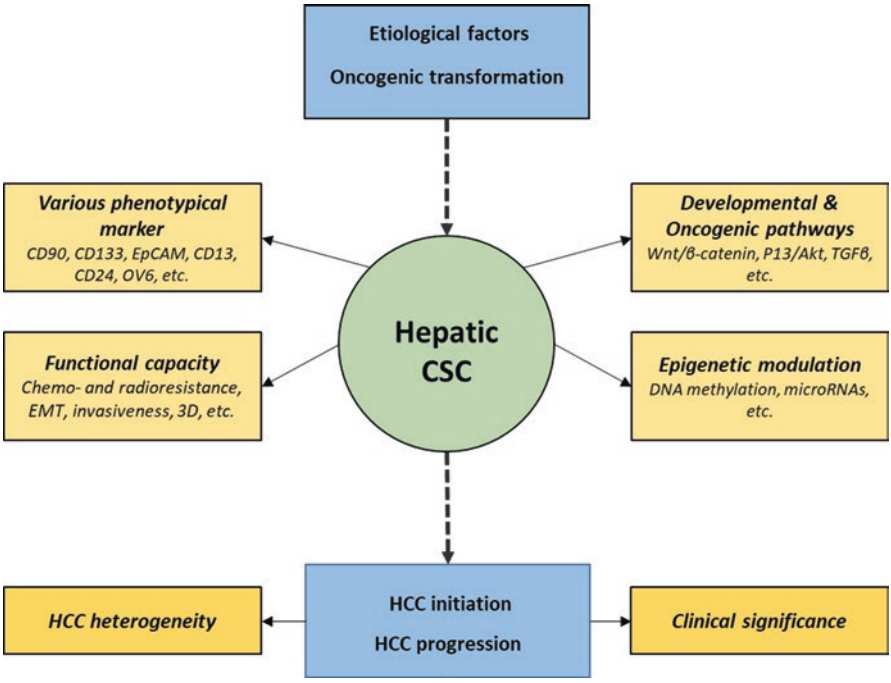


**Fig. 4.4** Important factors in the significance of the heterogeneity of hepatic CSC

Until now, the surveillance and diagnostic of HCC is frequently established by ultrasonography and imaging criteria based on the contrast enhancement pattern using computed tomography and magnetic resonance. Confirmation diagnosis based on liver biopsy is invasive, and accuracy is limited when the HCC nodule is small. Considering this, novel circulating biomarkers, such as CSC or stem cell biomarkers, will be crucial in the diagnostic, prognostic, and the development of molecular-targeted therapy in the future. If the sensitivity and specificity of these novel biomarkers will be acceptable, it will allow to obtain reliable information regarding the recurrence of disease without using expensive and potential dangerous diagnostic techniques.

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# Chapter 5
# Heterogeneity and Plasticity of Breast Cancer Stem Cells


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**Abstract** In the last 20 years, the conventional view of breast cancer as a homogeneous collection of highly proliferating malignant cells was totally replaced by a model of increased complexity, which points out that breast carcinomas are tissues composed of multiple populations of transformed cells. A large diversity of host cells and structural components of the extracellular matrix constitute the mammary tumour microenvironment, which supports its growth and progression, where individual cancer cells evolve with cumulative phenotypic and genetic heterogeneity. Moreover, contributing to this heterogeneity, it has been demonstrated that breast cancers can exhibit a hierarchical organization composed of tumour cells displaying divergent lineage biomarkers and where, at the apex of this hierarchy, some neoplastic cells are able to self-renew and to aberrantly differentiate. Breast cancer stem cells (BCSCs), as they were entitled, not only drive tumourigenesis, but also mediate metastasis and contribute to therapy resistance.

Recently, adding more complexity to the system, it has been demonstrated that BCSCs maintain high levels of plasticity, being able to change between mesenchymal-like and epithelial-like states in a process regulated by the tumour microenvironment. These stem cell state transitions play a fundamental role in the process of tumour metastasis, as well as in the resistance to putative therapeutic strategies to target these cells. In this chapter, it will be mainly discussed the emerging knowledge regarding the contribution of BCSCs to tumour heterogeneity, their plasticity, and the role that this plasticity can play in the establishment of distant

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metastasis. A major focus will also be given to potential clinical implications of these discoveries in breast cancer recurrence and to possible BCSC targeted therapeutics by the use of specific biomarkers.

**Keywords** Breast cancer · Stem cells · Breast cancer stem cells (BCSCs) · Plasticity · Stemness · EMT (epithelial-to-mesenchymal transition) · MET (mesenchymal-to-epithelial transition) · Tumour heterogeneity · Biomarkers · Therapy resistance · Metastasis · Tumour microenvironment · ECM (extracellular matrix) · Metabolism

## 5.1 Introduction

Breast cancer is the most common type of cancer and is the second leading cause of cancer-related deaths in women worldwide (Siegel et al. 2016). While improved surveillance and early detection have improved breast cancer mortality statistics over the past 30 years, there is still an unacceptably high incidence, recurrence, and mortality associated with this disease (Servick 2014). Despite recent advances in targeted therapies for ER and HER2 positive cancers, many patients still relapse and die with metastatic disease due to therapeutic resistance, which is the main cause of breast cancer death. Therefore, the ultimate goal of breast cancer researchers is to overcome therapeutic resistance in advanced stages of the disease and prevent patient recurrence (Luo et al. 2015).

The traditional view of breast cancer as a homogeneous entity of fast proliferating neoplastic cells was completely excluded in the last two decades. Several evidences revealed breast cancer as a complex disease, assumed as an anomalous mammary growing, composed by multiple cell types, including a large variety of immune and other host cells, which create the tumour microenvironment. Now, it is fully accepted that the crosstalk between all these cellular and structural components is essential for breast cancer growth and progression, contributing for the evolution of single neoplastic cells, by the accumulation of phenotypic and genetic heterogeneity.

With the acquisition of genetic/epigenetic heterogeneity, a hierarchical organization composed by neoplastic cells displaying divergent lineage markers start to be exhibited within breast carcinomas, where a fraction of tumour cells show the capacity to self-renew at the apex of this pyramid. These so-called breast cancer stem cells (BCSCs), or breast cancer initiating cells, not only drive tumourigenesis, but also seed metastasis at local and distant sites, as well as mediate therapy resistance. BCSCs also maintain high levels of plasticity, allowing them to switch between epithelial and mesenchymal states in a process epigenetically regulated by the tumour microenvironment. Actually, it is assumed that these stem cell state transitions play a fundamental role in metastasis, drug resistance, and tumour recurrence, constituting a challenge to efficient cancer therapeutics.

## 5.2 Breast Cancer Intra-Tumour Heterogeneity

It has been determined that there are multiple cancer cell clones, within a single breast carcinoma, harbouring distinct genetic and epigenetic profiles (Gerlinger et al. 2012) and which contribute to intra-tumour heterogeneity (Marjanovic et al. 2013; Prasetyanti and Medema 2017; Junttila and de Sauvage 2013; Lu et al. 2012). Breast cancer cells acquire intrinsic genetic and epigenetic aberrations in their genome (Vogelstein et al. 2013), harbouring high levels of genetic instability, a hallmark of cancer (Hanahan and Weinberg 2011). Among those mutations, some are selected as driver mutations that induce activation of oncogenic pathways and/or blockade of tumour suppressors, which will improve cell proliferation, inhibit cell differentiation, and decrease apoptosis (Stratton et al. 2009). Each subsequent mutation results in progressive "de-differentiation" and, as mutations accumulate, cells regress to a more primitive phenotype (Crabtree and Miele 2018). However, the large spectrum of factors from the tumour microenvironment is what strongly influences intra-tumour heterogeneity. Cancer cells are continuously under selective pressure as a consequence of the dynamic microenvironment, applied therapies, and attacks from the immune system (McGranahan and Swanton 2017; Colak and Medema 2014). Therefore, genetic/epigenetic alterations combined with the tumour microenvironment generate high levels of intra-tumour heterogeneity and support mammary tumour progression by conferring a competitive advantage to subsets of breast cancer cells (Prasetyanti and Medema 2017) (Fig. 5.1).

In addition to this knowledge, intra-tumour heterogeneity may also be explained by the appearance of a hierarchical cellular organization that is established during carcinogenesis, driven by a group of cancer cells displaying stem cell properties, defined by their competency to be tumorigenic, to infinitely self-renew, and to differentiate into non-stem-like cancer cells (Shibue and Weinberg 2017). When a limited number of these BCSCs, isolated by the expression of specific cell surface markers, were injected into immune-compromised mice, they showed higher ability to generate tumours with high efficiency when compared with the bulk tumour cells (Alison et al. 2011). Most importantly, tumours generated by BCSCs recapitulated the cell type heterogeneity of the primary tumour (Al-Hajj et al. 2003; Abraham et al. 2005; Liu et al. 2007; Sheridan et al. 2006; Ginestier et al. 2007a).

## 5.3 Breast Cancer Stem Cells

Tumour relapse and metastasis are the primary reasons for poor survival among breast cancer patients, despite successful resection, chemo-, radio-, or target therapy. Following their identification 15 years ago (Al-Hajj et al. 2003), BCSCs have not only been implicated in initiating and promoting primary tumour growth, but also in causing breast cancer relapse and driving metastases to distal sites. Although their specific biomarkers are still a matter of debate, it has been revealed that BCSCs are highly resistant to standard oncology therapies and are able to metastasize in a very effective way (Velasco-Velazquez et al. 2011).

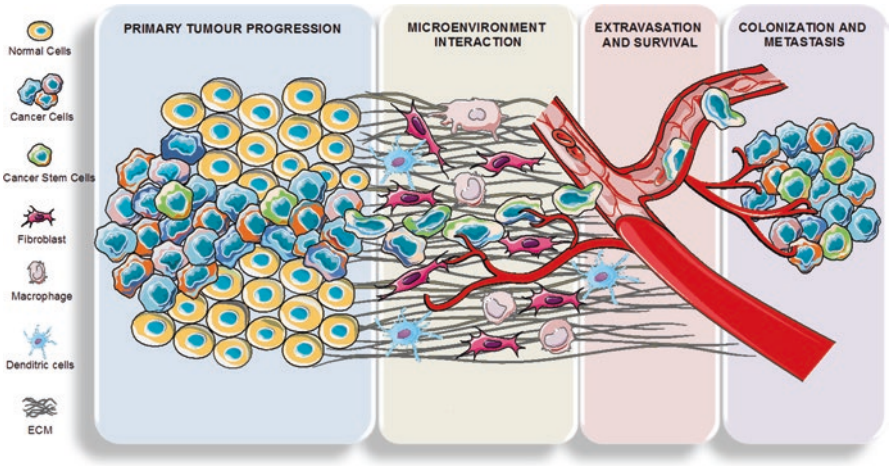


**Fig. 5.1** Graphic illustration of cancer intra-tumour heterogeneity and its role in disease progression. Tumours are a complex ecosystem containing cancer cells, as well as various infiltrating haematopoietic, stromal, and other cell types, as well as extracellular matrix components, that influence tumour behaviour. These different constituents can directly influence tumour cells, creating metabolic variations and ECM modifications that contribute to the phenotypic heterogeneity of malignant cells and that are determinant for the selection of most aggressive cancer cells. In this hierarchically organized tumour tissues, it is possible to find a subpopulation of self-renewing cancer stem cells (CSCs) that sustain the long-term clonal maintenance of the tumour itself. The appearance of these CSC pools within a tumour is strongly linked to disease progression, therapy resistance, tumour recurrence, and metastasis

### *5.3.1 Breast Cancer Stem Cell Biomarkers and Patient Prognosis*

A population of BCSCs was first isolated by fluorescence-activated cell sorting (FACS) by labelling the expression of specific cell surface markers. As few as 100 breast cancer cells expressing EpCAM, high levels of CD44, and low levels of CD24 ($EpCAM^{+}CD44^{+}CD24^{-/low}$) were able to efficiently form tumours when inoculated in immune deficient NOD/SCID mice; in contrast, over 100-fold more cancer cells without this phenotype were non-tumorigenic (Al-Hajj et al. 2003). The resulting tumours contained both stem and non-stem cancer cells, suggesting that both self-renewal and differentiation activities were present in the seeding population (Al-Hajj et al. 2003). Upon this initial report, a large number of studies have described additional biomarkers to sort the BCSC population, such as aldehyde dehydrogenase 1 (ALDH1) activity, or the expression of other cell surface markers. In a general manner, all biomarkers already described are important prognostic factors in breast cancer, being their expression associated with a significant worse patient survival when evaluated in the primary tumour:

- *EpCAM*, or epithelial cell adhesion molecule, is a transmembrane glycoprotein that mediates $Ca^{2+}$-independent homotypic cell–cell adhesion in epithelia (Litvinov et al. 1994), being involved in cell signalling (Litvinov et al. 1994; Maetzel et al. 2009), migration (Osta et al. 2004), proliferation, and differentiation (Litvinov et al. 1996). It is known that EpCAM has oncogenic potential via its capacity to upregulate c-myc and cyclins A and E (Munz et al. 2004). Moreover, since EpCAM is exclusively expressed in epithelia, it is used as a diagnostic marker for various epithelial-derived cancers or carcinomas. Its expression also plays a role in tumourigenesis and metastasis, so it can also act as a potential prognostic marker and as a possible target for immunotherapeutic strategies (Armstrong and Eck 2003).
- *CD44* is a cell surface transmembrane glycoprotein that binds hyaluronan and is involved in many cellular functions, including cellular adhesion, proliferation, survival, and differentiation (Senbanjo and Chellaiah 2017). The BCSC population demonstrates strong expression of CD44 and the presence of this glycoprotein acts to maintain their multipotency (Pham et al. 2011). Because of its elevated expression, CD44 has been also a target for BCSC therapies (Goodarzi et al. 2014; Muntimadugu et al. 2016; Phillips et al. 2006).
- *CD24*, also known as heat stable antigen (HAS), is a sialoprotein that enhances cellular adhesion, proliferation, and metastasis (Kristiansen et al. 2004). CD24 expression is typically very low or absent in BCSCs and in vitro studies demonstrated that the upregulation of CD24 inhibited stemness in breast cancer cells (Schabath et al. 2006). CD24 has also been implicated in chemoresistance in breast cancer cell lines (Bensimon et al. 2013).
- *ALDH1* is a member of the aldehyde dehydrogenase family of proteins that catalyse the oxidation of intracellular aldehydes and may have a role in early differentiation of BCSCs through its role in oxidizing retinol to retinoic acid (Tomita et al. 2016). Elevated expression of ALDH1 identifies BCSCs and correlates with poor breast cancer prognosis in receptor negative breast cancers (Ginestier et al. 2007a; Charafe-Jauffret et al. 2010). ALDH1 activity is measured by an enzymatic assay (ALDEFLUOR) and flow cytometry (Ginestier et al. 2007a). Inhibitors of ALDH1 have been examined as potential therapeutics, but efforts are hampered by the redundancy of aldehyde dehydrogenase enzymes and the lack of specificity in small molecule therapeutics (Moreb et al. 2012). Thus, $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ BCSCs show the greatest tumour-initiating capacity (Liu et al. 2014).
- *CD49f*, or α6-integrin, homodimerizes with β4-integrin to bind laminin and facilitate epithelial cell adhesion to the extracellular matrix (Radisky et al. 2002). CD49f also cooperates with signal transduction pathways to facilitate communication between the cell and the ECM. CD49f expression is associated with poor prognosis and reduced survival in breast cancer (Friedrichs et al. 1995).
- *P-cadherin*, or placental cadherin, is a cell–cell adhesion molecule, whose expression is significantly associated with poor patients' survival when evaluated in the primary breast tumour, but also in axillary lymph node metastases (Paredes et al. 2005; Vieira et al. 2017). Mechanistically, P-cadherin induces cell invasion

through E-cadherin-mediated cell–cell adhesion disruption and through the strength induced on α6β4-dependent cell–matrix adhesion (Ribeiro et al. 2010; Ribeiro et al. 2013; Vieira et al. 2014). In addition, P-cadherin expression identifies cancer cell populations with tumorigenic and stem cell properties, with a glycolytic metabolism and an acid-resistant phenotype (Vieira et al. 2012; Sousa et al. 2014).

### *5.3.2 Breast Cancer Stem Cells and Metastasis*

Cancer metastasis is a complex process requiring that disseminated cancer cells: (1) survive during long periods in the circulation under shear stress, (2) escape out of the blood vessels, and (3) invade the foreign microenvironment and proliferate in distant organs following extravasation. Indeed, although primary tumours release a large amount of cancer cells into the circulation, only a small fraction of these cells (~2%) are able to initiate growth as micrometastases and only ~0.02% of circulating tumour cells (CTCs) are estimated to form macrometastases in distant organs (Gupta and Massague 2006; Chambers et al. 2001; Cameron et al. 2000; Fidler 2003). Therefore, metastatic colonization, the last step of metastasis, appears to be the rate-limiting step of distant metastasis.

An increasing body of evidence has indicated that BCSCs, although initially identified as a subset of tumour cells with high tumorigenic properties when transplanted into immune deficient mice, are the critical cells that mediate tumour metastasis. Gene expression profiles of BCSCs show an invasive gene signature with increased metastatic potential (Liu et al. 2007). Actually, triple negative breast cancers have the highest expression of BCSC biomarkers when compared to other breast cancer subtypes (Croker et al. 2009), and the $CD44^{+}/CD24^{-/low}$ phenotype is significantly associated with poor overall survival (Liu et al. 2007; Honeth et al. 2008). The $CD44^{+}/CD24^{-/low}$ BCSC phenotype has also been found in cancer cells that have disseminated into the bone marrow of patients with breast cancer (Balic et al. 2006). In a mouse xenograft model of human triple negative breast cancer, cancer cells that metastasized to the lung showed high levels of the stem cell marker CD44 and were able to regenerate tumours following transplantation in immune suppressed mice, strongly suggesting a metastatic role of BCSCs (Liu et al. 2010).

It has been proposed that BCSCs may enter the circulation and become circulating tumour cells (CTCs) due to their capacity of anoikis resistance, with potential to metastasize to distant organs and seed metastatic lesions (Batlle and Clevers 2017). Some CTCs have high expression levels of BCSC markers (Baccelli et al. 2013). Moreover, from liquid biopsy samples of patients with luminal breast cancer, CTCs with a BCSC signature are enriched on the disease clinical progression group of patients; in contrast, CTCs with a bulk tumour signature were collected from patients who did not progress (Baccelli et al. 2013). In another study, a subset of breast cancer cells demonstrating BCSC properties, including self-renewal, cycling quiescence, asymmetric division, as well as high metastatic and invasive capabilities, was

found in the circulation of breast cancer patients (Patel et al. 2012). Together, these studies implicate that BCSCs have the ability to metastasize to distant organs where they serve as the seeds of metastatic lesions.

### *5.3.3 Breast Cancer Stem Cells and Therapy Resistance*

Besides a causal role in metastasis, a plethora of studies have also indicated that BCSCs are resistant to traditional cancer therapies, including chemotherapy and ionizing radiation in cultured breast cancer cell lines (Phillips et al. 2006; Lagadec et al. 2010; Karimi-Busheri et al. 2010; Fillmore and Kuperwasser 2008), in primary mammary tumour cells derived from mouse models of human breast cancer (Shafee et al. 2008; Diehn et al. 2009; Woodward et al. 2007), and in patient-derived tumour xenografts (Phillips et al. 2006; Yu et al. 2007; Zielske et al. 2011).

Conventional chemotherapies target and eliminate highly proliferative breast cancer cells and may be initially effective; but, over time, the surviving BCSCs cause tumour relapse (Li et al. 2008). The mechanisms of chemoresistance in BCSCs can be intrinsic, which is attributable to genetic alterations, or extrinsic, being linked to the influences from the tumour microenvironment (Rebucci and Michiels 2013).

The genetic alterations in BCSCs include aberrant expression of proteins that detoxify chemotherapy agents. For example, ABC transporters are usually expressed at high levels in BCSCs and cause cellular efflux of chemotherapeutic drugs (Hirschmann-Jax et al. 2004). BCSCs also generally have high ALDH1 expression, which metabolizes chemotherapeutic agents, such as cyclophosphamide, and thereby eliminates the chemotherapeutic toxic effects (Croker and Allan 2012).

Another major factor involved in BCSC chemoresistance is the tumour microenvironment (Crowder et al. 2014): in hypoxic environments, for example, activation of the expression of hypoxia inducible factors promotes new blood vessel formation and a quiescent phenotype in BCSCs, contributing significantly to BCSCs chemoresistance in (Mimeault and Batra 2013). Moreover, BCSCs have a distinct metabolic behaviour from differentiated bulk tumour cells, which contributes not only to tumour heterogeneity but also in cancer progression and therapeutic resistance (Snyder et al. 2018).

The intrinsic resistance of BCSCs to neoadjuvant chemotherapy in the clinical setting has also been shown in a number of studies. For example, breast cancer cells isolated from tumours treated with neoadjuvant chemotherapy compared to those rescued from chemotherapy-naive patients exhibited increased mammosphere forming activity and $CD44^{+}CD24^{-/low}$ BCSC content (Yu et al. 2007). In another study, the percentage of $CD44^{+}CD24^{-/low}$ BCSCs and mammosphere forming activity was also significantly increased after 12 weeks of treatment with chemotherapeutic agents (Li et al. 2008).

Interestingly, in a separate group of patients with HER2 amplification, treatment with HER2 and EGFR inhibitors following chemotherapy did not increase, but

rather slightly decreased the content of $CD44^+CD24^{-/low}$ BCSCs and mammosphere forming efficiency (Li et al. 2008). Since HER2 overexpression has been shown to induce BCSC activity (Korkaya et al. 2008), this study suggests that strategies combining HER2 targeting agents with chemotherapy hold the potential to overcome BCSC associated treatment resistance and get better therapeutic outcomes.

Finally, previous studies have shown that ALDH1 expression in human breast tumours is associated with poor prognosis, suggesting that $ALDH^+$ BCSCs share properties with $CD44^+CD24^{-/low}$ BCSCs in terms of metastasis/recurrence and treatment resistance (Ginestier et al. 2007a). Consistent with this observation, a clinical study examining ALDH1 expression in a cohort of primary breast cancer samples treated with chemotherapy revealed that ALDH1 positivity was significantly associated with a low pathological complete response rate and resistance to therapy (Tanei et al. 2009). Furthermore, the presence of residual $ALDH^+$ cells following new adjuvant chemotherapy was found to associate with a high recurrence rate in breast cancer (Alamgeer et al. 2014). Based on this knowledge and observations, therapies targeting BCSCs with $CD44^+CD24^{-/low}$ or $ALDH1^+$ phenotypes are on the horizon and a number of therapeutic antibodies have been proposed to target these biomarkers for the elimination of potentially metastatic BCSCs.

## 5.4 Plasticity of Breast Cancer Stem Cells

Although it was initially thought that BCSCs would be static entities at the apex of a cellular hierarchy with a unidirectional differentiation towards non-stem cancer cells, it is now recognized that these preserve high levels of plasticity, which allow them to change between distinct phenotypic states in a process epigenetically regulated by the tumour microenvironment (Liu et al. 2014; Beerling et al. 2016).

### *5.4.1 Epithelial-to-Mesenchymal Transition (EMT)*

EMT, or epithelial-to-mesenchymal transition, is a conserved morphogenetic and molecular program, during which epithelial cells suffer sequentially biochemical changes to acquire a mesenchymal phenotype (Thiery 2002). During EMT, polarized epithelial cells lose the expression of cell–cell adhesion molecules, such as E-cadherin, and acquire mesenchymal properties, such as enhanced migration, invasiveness, and increased resistance to apoptosis. Several signalling pathways activate EMT, including the ones induced by transforming growth factor beta (TGFβ), Notch, and Wnt (Lamouille et al. 2014; Liu et al. 2008; Yang et al. 2004). A number of transcription factors are activated, such as Snail, Slug, ZEB1, or Twist, which induce the EMT program by silencing E-cadherin expression at the cell surface. The loss of E-cadherin is a fundamental hallmark of full EMT (Kalluri and Weinberg 2009). Mesenchymal-like cells commonly express N-cadherin and vimentin,

adhesion, and cytoskeletal proteins that are necessary for cell migration (Kalluri and Weinberg 2009). Importantly, it has been shown that EMT is not an "all-or-nothing" process that shifts from a purely epithelial to a purely mesenchymal phenotype, but rather is a multistage process, with one or more intermediate stages (Grigore et al. 2016). These intermediate phenotypes have been referred to as partial EMT states or hybrid epithelial/mesenchymal phenotypes (Shibue and Weinberg 2017; Jolly et al. 2018).

In physiological terms, EMT is needed to control embryogenesis. During gastrulation, complete EMT is mandatory to generate fully committed mesenchymal cells that form the early mesoderm or endoderm (Thiery et al. 2009; Viebahn et al. 1995). In contrast, in the mammary gland, partial and reversible EMT is necessary during its morphogenesis (Nakaya and Sheng 2013). During puberty, mammary epithelial stem/progenitor cells, residing in the terminal end buds of the breast, transiently acquire mesenchymal features, initiate elongation and migration, thereby driving branching (Ewald et al. 2012; Micalizzi et al. 2010; Foubert et al. 2010; Kouros-Mehr and Werb 2006). However, branching morphogenesis is an exceptionally plastic process with an incomplete EMT program, since both epithelial and mesenchymal lineages are essential for normal mammary gland function (Chakrabarti et al. 2012; Watanabe et al. 2014; Choi et al. 2009; Oakes et al. 2008).

EMT also plays a major role in many types of cancer. Almost 80% of human cancers derive from epithelial tissues, including tumours of the breast (Ye and Weinberg 2015). Hyperplasia or early stage tumours express the epithelial marker E-cadherin, whereas highly aggressive primary tumours cells usually exhibit high motility and invasiveness mesenchymal features (Calabrese et al. 2004; Chaffer et al. 2016). Because tumour progression is positively associated with the acquisition of mesenchymal features, this may be an explanation for why basal and claudin-low breast cancer subtypes are highly aggressive, since these are significantly enriched in an EMT signature when compared with luminal A/B subtypes (Prat et al. 2010). Depletion of EMT-inducers in breast cancer cells, such as Twist, Snail, and Zeb, greatly inhibits metastasis induced after mammary fat pad or tail vein cellular injection (Yang et al. 2004; Guo et al. 2012; Roy et al. 2014; Tran et al. 2014; Zhang et al. 2013). Consistently, activating EMT in human breast cancer cells enhances metastatic dissemination (Yang et al. 2004).

EMT-like tumour cells are typically seen at the invasive edge of primary tumours, which are most probably the ones that eventually enter into the metastatic cascade, including intravasation, extravasation, and formation of metastases in distant organs (Kalluri and Weinberg 2009; Brabletz 2012). The roles of EMT to promote tumour cell dissemination are well supported by studies on CTCs and disseminated bone marrow tumour cells, both of which exhibiting EMT and stemness characteristics (Balic et al. 2006; Raimondi et al. 2011; Watson et al. 2007). However, although migratory cancer cells in primary tumours and CTCs have been shown to present typical EMT features, distant metastases in the majority of epithelial cancers are generally characterized as having epithelial type morphology. In some cases, metastatic tumours even show a greater degree of cellular differentiation as compared to the primary tumour (Brabletz 2012). In breast cancer, the content of $CD44^{+}CD24^{-/low}$

BCSCs in the primary tumour correlates with increased risk of distant metastasis (Shipitsin et al. 2007). However, distant metastases formed from these tumours frequently show a higher differentiation rate as manifested by increased expression of the luminal epithelial marker CD24 (Shipitsin et al. 2007).

Such paradoxical observations suggest that the EMT program activated during tumour dissemination must have been suppressed upon arrival at the site of metastasis and the reciprocal metastatic colonies at distant organs. Such dynamic EMT/MET state transitions for metastatic tumour cells may serve as the underlying driving force of metastasis and thus be harnessed for therapeutic intervention to prevent metastatic colonization.

### *5.4.2 EMT and Breast Cancer Stem Cells*

Several studies reported that BCSCs can be generated by the EMT developmental program, thereby facilitating their metastatic capacity and drug resistance (Ye et al. 2017; Scheel and Weinberg 2012). In general, the induction of EMT tends to cause an increase in expression of genes associated with "stemness", as well as an increase of CSC numbers in some tumour types, being particularly well studied in breast cancer. Induction of EMT by TGFβ in immortalized human mammary epithelial cells was sufficient to induce an enrichment of a $CD44^{+}/CD24^{-/low}$ stem cell population. This was accompanied by an increase in the formation of mammospheres, colonies in a soft agar assay, and tumourigenicity in immune deficient mice, which are all properties associated with BCSCs (Mani et al. 2008). Besides the experimental induction, natural EMT caused by endothelial cells has also been shown to increase the CSC pool (Sigurdsson et al. 2011). Finally, in normal breast tissue, overexpression of the transcription factors Slug and Sox9 was enough to push luminal lineage cells into a more stem-like state, while only Sox9 was required in basal cells that already expressed the EMT associated transcription factor Slug (Guo et al. 2012).

Concerning the effect that EMT can have on the induction of BCSCs, it is important to discuss the possible models for the cell of origin in breast cancer. Some models propose that the cell of origin should be the most stem-like cell of the natural cellular hierarchy, since BCSCs have stem-like properties including self-renewal capacity and this would require the shortest path to tumourigenesis. Other models hypothesize that the cell of origin in breast cancer is most likely a luminal progenitor cell or a unipotent luminal stem cell (Lim et al. 2009; Lindeman and Visvader 2010). If a bipotent stem cell was the cell of origin for breast cancer, one might expect similar numbers of the two cell types derived from the bipotent stem cell of the normal hierarchy. Of course, if a luminal type of cell is the most common cell of origin for breast cancer, then it would suggest that EMT could play an important role in transitioning the luminal cell of origin back to a more mesenchymal stem-like BCSC. Another alternative model suggests that the different molecular subtypes of breast cancer originate from distinct cellular compartment in the normal

mammary epithelial hierarchy (Visvader 2009). According to this hypothesis, claudin-low breast cancers originate from the most primitive mammary stem cells, while basal breast cancers originate from a luminal progenitor. This model also envisions that luminal breast cancers are derived from differentiated luminal cells.

Interestingly, a growing number of studies have been showing the extremely important role of the tumour microenvironment in the modulation of both EMT (Kumar et al. 2014; Jin et al. 2018; Hoshiba 2018) and stem cell properties (Engler et al. 2006; Karamanos 2014). Cancer cells have been shown to sense and respond to different mechanical properties of the extracellular matrix (ECM), being able to change the ECM protein expression in order to promote a rapid ECM remodelling. Several studies have identified ECM genes as critical, stage-specific, regulators of stem cell function (Morris et al. 2004; Pallafacchina et al. 2010; Fietz et al. 2012; Bi et al. 2007; Tierney et al. 2016). More specifically, ECM components are key players of the niche instructive power, supplying a microenvironment with signals deriving from cell–ECM interactions, as well as soluble and ECM-bound factors, allowing the maintenance of stem cell homeostasis (Discher et al. 2009; Peerani and Zandstra 2010; Pera and Tam 2010; Watt and Fujiwara 2011). In this way, ECM can also directly or indirectly modulate the maintenance, proliferation, self-renewal, and differentiation of BCSCs.

### 5.4.3 *Epithelial- and Mesenchymal-Breast Cancer Stem Cell States*

Two of the most widely used methods of enriching for BCSCs are sorting cells by their $CD44^{+}/CD24^{-/low}$ signature (Al-Hajj et al. 2003) or by ALDEFLUOR positivity (Ginestier et al. 2007a). However, it has been shown that these two populations of BCSCs are plastic and have the capacity to change between them (Liu et al. 2014). While both cancer cell populations show stem cell characteristics, they also have unique properties: the $CD44^{+}/CD24^{-/low}$ population shows an EMT signature, with low E-cadherin expression and high levels of vimentin, and a tendency to be quiescent; therefore, this population was labelled as EMT-BCSCs (Fig. 5.2). In contrast, the $ALDH^{+}$ population had a relatively opposite phenotype, with high expression of E-cadherin and low expression of vimentin. These cells were also more proliferative, harbouring an epithelial signature and therefore been labelled as MET-BCSCs (Liu et al. 2014). Most importantly is that the transition between these two states is likely to be critical for tumour expansion (Fig. 5.2). The EMT-BCSCs sit at the invasive edge of the tumour, where their mesenchymal features allow them to quickly move into the surrounding tissue. However, while the EMT-BCSCs allow the tumour to expand into a new territory, the proliferative MET-BCSCs likely drive tumour cell growth (Liu et al. 2014). When the conditions of the tumour microenvironment change, the two CSCs can also change between states. This is because of the extreme plasticity of tumour cells that are able to rapidly switch the transcriptional machinery to undergo MET or EMT when needed.

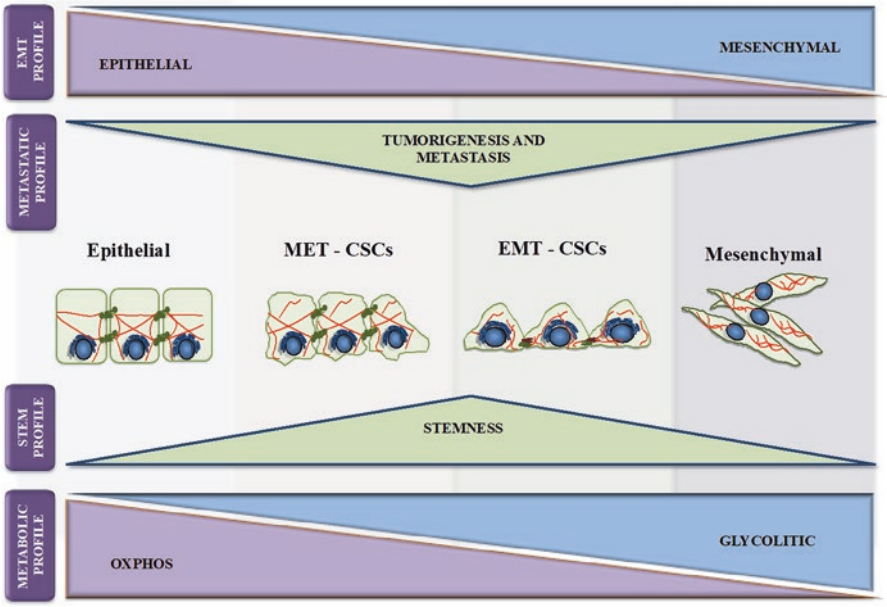


**Fig. 5.2** Schematic representation adapted from Ribeiro et al. (Ribeiro and Paredes 2014) of the different types of breast CSCs within different biological cellular processes, such as EMT, tumourigenesis and metastasis, stemness, and metabolism

Although gene expression profiling of $CD44^{+}CD24^{-}$ and $ALDH^{+}$ cell populations across different subtypes of human breast cancers indicates that they are distinct cancer cells with respective to EMT and MET gene expression signatures, both cell populations share overlapping gene signature associated with stemness (Liu et al. 2014). Together with experimental evidence demonstrating that both $CD44^{+}CD24^{-/low}$ and $ALDH^{+}$ cell populations enrich functional BCSC activities (Al-Hajj et al. 2003; Ginestier et al. 2007b), it is evident that functional BCSCs exist in inter-convertible EMT and MET states (Fig. 5.2).

This reversible, metastable epithelial–mesenchymal plasticity of BCSCs is closely connected to the current model of cancer metastasis postulating that EMT drives tumour cell dissemination and a consecutive MET drives metastatic colonization.

In the case of breast cancer, the $CD44^{+}CD24^{-/low}$ EMT-like BCSCs mediate tumour invasion towards the basal membrane and neighbouring tissues and into the blood, where they survive due to their intrinsic quiescence and anoikis resistance. After extravasation from circulation, the mesenchymal-like BCSCs form micrometastasis in distant organs, where the specific microenvironment or the metastatic niche in those sites can induce MET, which drives BCSC self-renewal and generation of macrometastasis.

Interestingly, some recent studies have suggested that induction of constitutive EMT in subpopulations of tumour cells displaying CSC properties suppressed major stem-like attributes, including anchorage-independent growth and metastatic

potential (Celià-Terrassa et al. 2012). These contradictory results could be reconciled by the model that BCSCs exist in a dynamic equilibrium between MET- and EMT-like states, where the induction of a metastable EMT program in proliferating ALDH$^+$ MET-like BCSCs will promote an imbalanced equilibrium from the MET towards the EMT state, leading to amplification of EMT BCSCs through EMT. However, induction of a permanent EMT program in ALDH$^+$ MET BCSCs by constitutive expression of EMT transcription factors, such as Snail or Twist, will break the epithelial–mesenchymal plasticity of BCSCs, leading to the formation of cells permanently locked into the mesenchymal state, where the properties of CSCs, including anchorage-independent growth and metastatic potential, are lost (Celià-Terrassa et al. 2012).

Additionally, metabolism and oxidative stress also play a role in the transition between EMT and MET states of BCSCs (Fig. 5.2). These two states were recently described as relying on distinct metabolic pathways, where epithelial-like BCSC presents an increased oxidative metabolism and a higher dependency on antioxidant response (Luo et al. 2018). Still, glycolysis enhancement, oxidative stress, and hypoxia promote the transition from mesenchymal-like to epithelial-like BCSC, demonstrating the influence of metabolism in the EMT/MET plasticity and consequently in breast cancer progression and metastasis (Luo et al. 2018). In this regard, these studies highlight the critical role of epithelial–mesenchymal plasticity in maintaining CSC characteristics.

## 5.5 Clinical Implications of Breast Cancer Stem Cells Plasticity

The CSC model suggests that, after surgical resection and/or chemo- and radiotherapy, tumour relapse can be prevented if the recurrent cancer cells are destroyed with a selective CSC targeting drug. Since CSCs are extremely aggressive, invasive, and prone to metastasis (Batlle and Clevers 2017), targeting the CSC pool could lead to a better clinical patient outcome, by reducing treatment resistance, metastasis, and tumour recurrence. However, the identification of epithelial–mesenchymal plasticity of BCSCs provided an increased level of complexity regarding the development of strategies to eliminate these cells. Since these cells frequently change between their MET and EMT states, future strategies designed to treat BCSCs have to consider their plasticity and target both phenotypic states to eliminate them in an accurate manner (Beerling et al. 2016).

Due to the connection between BCSCs and EMT, targeting the EMT process was thought to be a promising approach to treat breast cancer, especially those with high metastatic potential (Tsai and Yang 2013). Therefore, inhibitors of TGFβ-induced EMT have been already tested (Reka et al. 2011), as well as ALK5, MEK, and SRC inhibitors have been used to prevent EMT in response to EGF, HGF, and IGF-1 (Reka et al. 2011; Chua et al. 2012). Although EGFR and/or IGF1R chronic activa-

tion has been reported to promote EMT-like transition (Lo et al. 2007), cells that have undergone EMT show relative resistance to selective inhibitors of these tyrosine kinase receptors (Thomson et al. 2005; Fuchs et al. 2008; Frederick et al. 2007; Buck et al. 2008). These observations suggest that there are other signalling pathways that need to be active for the maintenance of EMT-derived cells, which should be targeted to potentially eliminate EMT-like BCSCs.

Inflammatory cytokines, like IL6 and IL8, can be also potential therapeutic targets of EMT BCSCs. High levels of IL-6 are associated with poor clinical outcome in breast cancer patients, by promoting tumourigenesis, angiogenesis, and metastasis (Korkaya et al. 2011). IL-6 has been shown to act as a direct regulator of BCSC self-renewal through Stat3 activation (Sansone et al. 2007), which in turn results in transcriptional activation of NF-kB in inflammatory cells, promoting additional release of inflammatory cytokines. Thus, a positive feedback loop between immune cells and tumour cells through IL-6 signalling is generated and further stimulates CSC self-renewal, metastasis, and therapeutic resistance. Indeed, recent studies have shown that activation of an IL6 inflammatory loop plays an important role for trastuzumab resistance of HER2+ breast cancer by expanding EMT BCSCs (Korkaya et al. 2012). Conversely, through blockade of the IL-8 receptor CXCR1, BCSCs have been successfully depleted in vitro and in NOD/SCID xenograft models (Ginestier et al. 2010).

Although approaches targeting EMT may prove to be effective by reducing EMT-like BCSCs, this strategy can be also counterproductive once tumour cells have disseminated from the primary site. As formation of distant macrometastases from disseminated tumour cells needs the reversion to a MET state, inhibition of EMT at this late stage may actually stimulate metastasis by promoting MET. Thus, for breast cancers with existing metastasis, specific strategies designed to target the metastatic niche, allowing dormant disseminated tumour cells to recover into a self-renewal MET state, may prevent the formation of new metastasis. In this regard, the BMP inhibitor, Coco, a secreted antagonist of TGFβ ligands, has been found to mediate breast cancer colonization in the lungs (Gao et al. 2012). Thus, therapeutic drugs that selectively activate BMP signalling can have the potential to inhibit CSC traits and lung colonization.

## 5.6 Conclusions and Future Directions

Over the time, standard therapies against breast cancer frequently start to fail due to molecular mechanisms of resistance, leading to disease recurrence. The recent knowledge about the role of BCSCs in breast cancer initiation, metastasis, and relapse highlight the need for developing new therapeutic strategies to eliminate these cells, in order to cure this disease. There are increasing interests in targeting the unique molecular signals that regulate BCSC maintenance and self-renewal, such as inhibiting specific cell surface markers, interference with EMT and stem signalling pathways, inhibition of drug efflux ABC transporters, impairment of the

advantageous metabolism, induction of their apoptosis and differentiation, and affecting components of the tumour microenvironment.

However, the recent evidence that BCSCs can exist in inter-convertible epithelial and mesenchymal states provided a novel model to understand how these cells contribute to metastatic disease and therapy resistance. BCSC plasticity awards them with an increased capacity for tissue invasion, dissemination, and metastatic growth at distal organs, but also suggests that targeting each one of the states alone may not be sufficient, since the targeted cell population would be rapidly regenerated by BCSCs in the alternative state. Thus, future research studies in BCSC and EMT biology will be needed, in order to simultaneously target both BCSC states to achieve maximum treatment efficacy. Robust biomarkers and elucidation of the genes and the signalling pathways that are altered in EMT-BCSCs and MET-BCSCs are required, in order to improve our understanding and to determine potential targets for novel therapies to prevent metastasis and relapse, and improve clinical outcomes.

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# Chapter 6
# Heterogeneity of Melanoma with Stem Cell Properties


**Elisabeth A. Seftor, Naira V. Margaryan, Richard E. B. Seftor, and Mary J. C. Hendrix**

**Abstract** Metastatic melanoma continues to present a significant challenge—with a cure rate of less than 10% and a median survival of 6–9 months. Despite noteworthy advances in the field, the heterogeneity of melanoma tumors, comprised of cell subpopulations expressing a cancer stem cell (CSC) phenotype concomitant with drug resistance markers presents a formidable challenge in the design of current therapies. Particularly vexing is the ability of distinct subpopulations of melanoma cells to resist standard-of-care treatments, resulting in relapse and progression to metastasis. Recent studies have provided new information and insights into the expression and function of CSC markers associated with the aggressive melanoma phenotype, such as the embryonic morphogen Nodal and CD133, together with a drug resistance marker ABCA1. This chapter highlights major findings that demonstrate the promise of targeting Nodal as a viable option to pursue in combination with standard-of-care therapy. In recognizing that aggressive melanoma tumors utilize multiple mechanisms to survive, we must consider a more strategic approach to effectively target heterogeneity, tumor cell plasticity, and functional adaptation and resistance to current therapies—to eliminate relapse, disease progression, and metastasis.

**Keywords** Melanoma · Tumor plasticity · Heterogeneity · Cancer stem cell · Nodal · Drug resistance · ABCA1 · Vasculogenic mimicry · Recurrent disease · CD133 · Metastatic disease · Prognostic biomarker

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## Abbreviations

| | |
|---|---|
| ABCA1 | ATP-Binding Cassette gene/protein, member A1 |
| BRAF | V-RAF murine sarcoma viral oncogene homolog B1 |
| BRAFi | BRAF inhibitor |
| CSCs | Cancer Stem Cells |
| DTIC | Dacarbazine |
| hESCs | Human embryonic stem cells |
| L-R | Left-Right |
| mAb | Monoclonal antibody |
| PD-1 | Programmed death 1 |
| TGFβ | Transforming growth factor β |
| VM | Vasculogenic Mimicry |

## 6.1 Introduction

It is important to recognize that metastatic melanoma continues to be a significantly deadly cancer with a cure rate of less than 10% and a median survival of 6–9 months (Song et al. 2015). One of the greatest challenges in effectively eradicating aggressive melanoma is developing therapeutic strategies that can successfully target tumor heterogeneity—comprised of subpopulations of melanoma cells (expressing various markers) that have the potential to functionally adapt to their changing microenvironment and resist various standard-of-care therapies, resulting in relapse and progression to metastasis. With these inevitable varied and changing targets, it seems prudent to consider developing approaches that are combinatorial in nature and delivered over time. This chapter is dedicated to reviewing the evidence and implications for tumor cell plasticity associated with the multipotent properties of melanoma, including those associated with embryogenesis and vasculogenesis—contributing to heterogeneous subpopulations within aggressive tumors. Most noteworthy among these properties is the cancer stem cell phenotype, which is particularly resistant to current therapies. There are new findings in the field that will be discussed relevant to targeting plasticity and drug resistance in melanoma, accompanied by a commentary on likely future directions with considerable promise. It is essential that we secure a better understanding of the evolving dynamics underlying tumor cell heterogeneity that will lead to the development of new cancer interventions.

## 6.2 Melanoma Tumor Cell Plasticity

### *6.2.1 Melanoma Multipotent Properties*

The molecular profile of aggressive melanoma cells, when originally reported, confounded our thinking about melanoma in general and raised serious questions about our ability to effectively detect and target this disease (Bittner et al. 2000;

Carr et al. 2003; Seftor et al. 2002). A high level review of these data revealed a multipotent phenotype—expressed by aggressive melanoma but not found in non-aggressive melanoma—and associated with multiple cellular types, including endothelial cells, epithelial cells, and embryonic stem cells. The co-expression of these phenotype-specific genes and proteins underlies tumor cell plasticity and presents a formidable challenge in designing rational therapies aimed at destroying a melanoma tumor with heterogeneous subpopulations (depicted in Fig. 6.1).

Most noteworthy, subsequent studies focused on understanding the functionality of these multiple phenotypes in melanoma demonstrated the remarkable ability of these aggressive tumor cells to engage in a plethora of biological activities dependent on the extracellular milieu in which they were placed. For example, melanoma cells placed in a mouse ischemic limb model formed chimeric vessels with host endothelial cells—and together reperfused the limb (Hendrix et al. 2002). In a chick neural crest embryonic model, melanoma cells were capable of following migratory cues and forming normal structures, and not tumors in this embryonic milieu (Kulesa et al. 2006). Using a zebrafish model, we observed melanoma cells directing the formation of a body axis via the secretion of a powerful embryonic morphogen (Nodal) by the tumor cells (Topczewska et al. 2006). Collectively, these findings provided new insights into the selective influence of the microenvironment on the multipotent properties of melanoma and encouraged further investigation into reversing the metastatic phenotype.

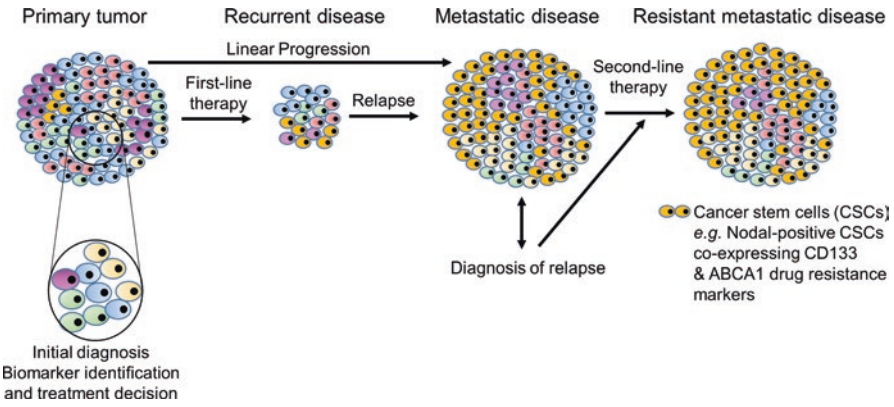


**Fig. 6.1** Melanoma tumors are comprised of heterogeneous subpopulations of tumor cells. At the time of an initial diagnosis based on a primary tumor biopsy, biomarker identification of the cellular makeup is representative of only a small portion of the tumor mass. The analysis of the cellular composition reveals specific biomarkers which help inform the best standard-of-care therapies suited for treating the tumor. With a subsequent reduction in the mass of the tumor, cells unaffected by the initial treatment remain, leading to a relapse of the tumor and progression to metastatic disease. Additional diagnoses can then lead to alternative standard-of-care therapies. Of note, cancer stem cells (CSCs), such as those expressing the embryonic morphogen Nodal, together with CD133 and ABCA1 drug resistant markers—that are present in the primary tumor, can expand and demonstrate multidrug resistance and further disease progression

### *6.2.2 Melanoma Vascular Characteristics*

Our findings regarding melanoma tumor cell vasculogenic mimicry (VM) were introduced to the scientific community in the Fall of 1999 based on a combination of molecular analysis, 3D in vitro models, and clinical findings of seminal melanoma studies (Maniotis et al. 1999; Folberg et al. 2000; Bittner et al. 2000; Hendrix et al. 2003). However, the prevailing paradigm at that time was reliant on the pioneering work of Dr. Judah Folkman pertinent to tumor angiogenesis (Folkman 1971). Specifically, the pharmaceutical industry was inspired by the groundbreaking strategy of targeting the "new" angiogenic blood vessels to growing tumors through the development of angiogenesis inhibitors—ultimately inhibiting tumor survival through nutrient deprivation (Folkman 1995). After the disappointing failure of many of the angiogenesis inhibitor clinical trials, researchers turned their attention to reevaluating the molecular evidence underlying tumor cell plasticity and drug resistance to current therapies.

Not surprisingly, our revelation that aggressive melanoma tumor cells are capable of forming vascular channels and networks—in the absence of regional endothelial cells, and can connect to traditional vessels at a tumor periphery—ignited a controversy in the field that has since led to almost universal acceptance (Leslie 2016). The 3D in vitro models demonstrated that melanoma cells expressing endothelial and stem cell markers can form de novo, perfusable, vasculogenic-like networks (Maniotis et al. 1999). Further ultrastructural analysis revealed the detailed morphological similarities and differences between the tumor cell-formed structures versus endothelial lined traditional vessels—which centered on the location of the basement membrane (Seftor et al. 2012). In both cases, evidence exists showing the passage of plasma and RBCs, and trace markers through the vasculature indicative of perfusion (Ruf et al. 2003). Also noteworthy is a more recent meta-analysis reporting tumor VM as a strong predictor of poor prognosis in cancer patients (Yang et al. 2015). Of special significance was the comparative analysis of the effects of endostatin (a classical angiogenesis inhibitor) on endothelial cell-driven angiogenesis versus melanoma tumor cell-formed VM networks. This comparison revealed the effectiveness of endostatin to inhibit angiogenesis by endothelial cells, but unable to inhibit melanoma VM (van der Schaft et al. 2004). This finding instigated further analysis of the endostatin target (integrin alpha 5-subunit) on the two cell types, which demonstrated a robust expression of the target by endothelial cells but not by melanoma tumor cells. Together, these findings portrayed aggressive melanoma as being resistant to angiogenesis inhibitor therapy and able to survive through its own perfusion pathway (Hendrix et al. 2003). These observations also inspired subsequent studies focused on the commonality of the vascular and embryonic phenotype, described in the following section.

### 6.2.3 Cancer Stem Cell Properties

Mounting evidence from myriad of molecular studies, together with a novel microgenomics approach of isolating melanoma-formed VM networks with laser capture microdissection followed by microarray analysis, collectively revealed a prominent embryonic stem cell signature with unknown functional relevance (Bittner et al. 2000; Weeraratna et al. 2002; Carr et al. 2003; Seftor et al. 2002; Demou and Hendrix 2008). More specifically, an embryonic signaling pathway, called Nodal, was found to be significantly upregulated in aggressive melanoma (but not in non-aggressive melanoma; Topczewska et al. 2006; Hendrix et al. 2007). This observation prompted the comparative analysis of human embryonic stem cells (hESCs) and melanoma, which led to the exciting discovery that aggressive melanoma reactivates the Nodal signaling pathway (Postovit et al. 2008b). In essence, we began to appreciate a commonality underlying the multipotent phenotype of melanoma—with vascular and embryonic properties, which is also associated with cancer stem cell properties (Postovit et al. 2007a).

Since this observation was the first to link Nodal expression with an aggressive form of cancer, it was imperative to understand more clearly what the underlying functional relevance of Nodal might be relevant to the progression of melanoma. Interestingly, search of the literature was somewhat confined by reports focused on developmental biology—with no helpful information available on cancer. These studies indicated that Nodal is an embryonic morphogen belonging to the TGFβ superfamily, and maintains the pluripotency of hESCs in addition to playing a critical role in axis formation and L-R patterning in the embryo (Schier and Shen 2000; Schier 2003, 2009; Saijoh et al. 2005). Nodal acts in an autocrine and paracrine manner, and has a natural and powerful inhibitor known as Lefty—also a member of the TGFβ superfamily (Schier and Shen 2000; Chen and Shen 2004; Saijoh et al. 2005). Of special interest, in humans, Nodal expression is largely restricted to embryonic tissues and is lost in most normal adult tissues. Further analysis of aggressive melanoma revealed that while Nodal is reactivated, Lefty is silenced, thereby allowing Nodal to act using a feed-forward mechanism of perpetual stimulation resulting in unregulated growth (Postovit et al. 2007b, 2008a, b; Costa et al. 2009).

An impressive body of work from our laboratory and others points to the relevance of Nodal signaling underlying the cancer stem cell (CSC) phenotype, unregulated tumor growth and metastasis, and resistance to standard-of-care therapies, which will be discussed in more detail in the next section. Furthermore, Nodal is a valuable prognostic biomarker in a variety of non-melanoma cancers associated with the aggressive phenotype, including glioblastoma, neuroblastoma, pancreatic cancer, leukemia, and cancers of the breast, prostate, ovary, colon, colorectal and gastric adenocarcinoma (De et al. 2012; Hueng et al. 2011; Lee et al. 2010; Jamil et al. 2013; Duan et al. 2015; Morrison et al. 2010; Strizzi et al. 2008, 2012; Lawrence et al. 2011; Fu and Peng 2011; Gong et al. 2014; Li et al. 2016a, b).

### 6.2.4 Targeting Plasticity and Drug Resistance in Melanoma

Using the working model of melanoma heterogeneity (illustrated in Fig. 6.1), we focused our studies on determining key markers, in addition to Nodal, that are co-expressed by aggressive melanoma tumor cells—indicative of a CSC phenotype and drug resistance. This information would be vital to our understanding of how subpopulations of melanoma escape conventional and targeted therapies and expand throughout relapse and progression.

We selected a well-characterized CSC marker, CD133, also associated with drug resistance (Lai et al. 2012)—to assess if a relationship existed between CD133-positive melanoma cells and the aggressive phenotype. Using an innovative approach with SmartFlares, heterogeneous melanoma cells were live-sorted specifically for Nodal expression, and then tested for their tumorigenic potential (Seftor et al. 2014). The functional analysis revealed robust CD133 expression associated with the Nodal-sorted melanoma cells (compared with the non-selected cells)—concomitant with significant tumor forming ability in soft agar.

An additional connection between Nodal positivity and drug resistance has been demonstrated from the molecular analysis of aggressive versus non-aggressive melanoma cells—where a greater than ninefold increase in the expression of ABCA1 is associated with the aggressive melanoma phenotype (Seftor et al. 2002). Over the past three decades, there has been a growing body of evidence demonstrating the multidrug resistance properties of ATP-binding cassette proteins, which consists of a large family of integral membrane proteins (reviewed in Gillet et al. 2007). Recent data from our laboratory show a convincing correlation between Nodal and ABCA1 expression in metastatic melanoma cells (Fig. 6.2). This observation supports the premise that Nodal-positive tumor cells inherently contain drug resistance characteristics.

Our next line of inquiry was to address whether Nodal is affected by conventional and targeted therapies in experimental melanoma in vitro and in vivo models, as well as patient clinical samples. Beginning our analyses with dacarbazine (DTIC), a conventional therapy approved by the FDA in the 1970s, we found that in several metastatic melanoma cell lines treated with varying concentrations of the drug, there were residual, viable subpopulations that were strongly positive for Nodal (Hardy et al. 2015). This finding coincided with a similar observation in patient tissues with aggressive disease—showing Nodal prominently expressed before and after DTIC treatment. Interestingly, when melanoma tumor cells were treated in a combinatorial manner with DTIC and anti-Nodal antibody, cell viability and proliferation plummeted, and apoptosis occurred.

Recognizing the importance of targeted therapy for melanoma patients and the evolution of personalized medicine, we continued our line of inquiry focused on the effects of BRAFi therapy on Nodal and clinical outcome. Our studies revealed that melanoma tumor cells treated with BRAFi in vitro had no discernable change in Nodal expression over time (Hardy et al. 2015). Most noteworthy, melanoma patients treated with various BRAFi therapy showed Nodal expression in their

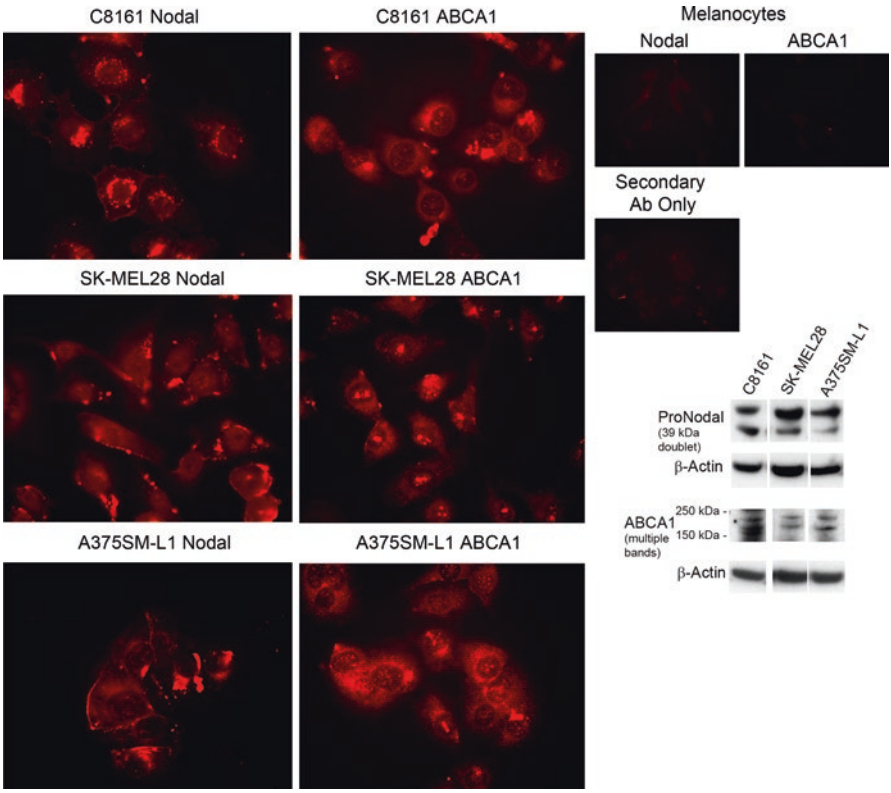


**Fig. 6.2** Aggressive melanoma cells express Nodal and ABCA1 proteins. Human metastatic melanoma cell lines C8161, SK-MEL28, and A375SM-L1 and melanocytes were treated with anti-Nodal or anti-ABCA1 antibodies followed by secondary fluorescent antibodies. Cells were viewed using a Zeiss Axioskop 2 microscope and photomicrographs taken comparing immunofluorescence staining of the melanoma cells versus melanocytes (negative control) and cells treated with secondary antibody only (antibody control; magnification 63X). All three aggressive melanoma cell lines express both Nodal and ABCA1 proteins. Furthermore, whole cell protein lysates were prepared from the C8161, SK-MEL28, and A375SM-L1 cells and Western blot analysis verified the expression of both Nodal and ABCA1 proteins at their appropriate molecular weight equivalence. β-Actin protein was used to determine the equal loading of total protein per sample per lane on the Western blot

respective tumors before, during, and after treatment, and sadly, all succumbed to their disease (Hendrix et al. 2017).

To address the possibility of targeting Nodal-positive tumor cells together with administering BRAFi therapy, we used a mouse model with tagged metastatic melanoma cells, and compared groups receiving monotherapy (of either BRAFi or anti-Nodal mAb) or a combination of both versus controls. The results clearly show the efficacy of deploying combinatorial therapy of a BRAFi plus anti-Nodal mAb, compared with monotherapy or control vehicles (Hendrix et al. 2017). These data are the first to demonstrate the promise of using a stand-of-care therapy together with a mAb to target a CSC-associated molecule—Nodal.

### 6.2.5 A Commentary on Likely Future Directions

Despite noteworthy advances in the field of melanoma research, tumor cell heterogeneity remains a formidable challenge in the design and delivery of successful therapies. As scientific studies continue to inform our knowledge base regarding unique tumor cell properties that could represent new targets for therapeutic intervention, our armamentarium for strategic approaches broadens. Particularly insightful has been the finding that aggressive melanoma expresses CSC markers such as Nodal, together with drug resistance-associated markers like CD133 and ABCA1. Certainly the promise of targeting Nodal in aggressive melanoma is emerging as a viable option to pursue in combination with standard-of-care therapy. We have not as yet had the opportunity to test whether Nodal is targeted by recent immunotherapeutic approaches, involving the programmed death 1 (PD-1) inhibitor pembrolizumab (Eggermont et al. 2018), but these studies have a high priority to pursue. In recognizing that aggressive tumors utilize multiple mechanisms to survive, our approach to effectively target melanoma heterogeneity, tumor cell plasticity, and functional adaptation and resistance to current therapies—must utilize combinatorial strategies—to eliminate relapse, disease progression, and metastasis.

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# Chapter 7
# Heterogeneity of Colon Cancer Stem Cells


**Akihiro Hirata, Yuichiro Hatano, Masayuki Niwa, Akira Hara, and Hiroyuki Tomita**

**Abstract** Colorectal cancer (CRC) remains the fourth leading cause of cancer death worldwide. Cancer stem cells (CSCs) have attracted a great deal of interest because of their potential clinical implications in a range of cancers, including CRC. CSCs were initially considered to be cell populations with well-defined phenotypic and molecular characteristics. However, accumulating evidence suggests that CSCs represent a phenotypically and functionally heterogeneous population. Recent studies also demonstrate colorectal CSCs to be dynamic rather than static populations that are continuously altered by various extrinsic factors in addition to intrinsic cellular factors such as genetic and epigenetic alterations. Thus, CSCs do not represent a fixed target population any longer, and their heterogeneous and dynamic nature present a serious problem in establishing specific therapeutic strategies. This chapter summarizes past and current literature related to CSC population heterogeneity and dynamics in CRC tissues, including evidence of the presence of distinct CSC subpopulations and signaling pathways and intra- and extra-tumoral factors involved in the regulation of CSCs in cancer tissues.

**Keywords** Colorectal cancer · Cancer stem cell · Heterogneity · Cancer stem cell marker · Metastasis · Wnt signaling · Intestinal stem cell · Reversion · Plasticity · Tumor microenvironment

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It has long been recognized that malignant cells within the same tumor display significant heterogeneity with regards to morphology, proliferative activity, and function (Heppner and Miller 1983). The cancer stem cell (CSC) concept can provide a convincing explanation of the mechanism underlying cellular heterogeneity within tumors. CSCs, also called tumor-initiating cells, are defined by their capacity to self-renew and generate diverse cells that comprise the tumor (Kreso and Dick 2014), and are thought to initiate and continually sustain tumor growth. The first evidence for the existence of CSCs came from studies on acute myelogenous leukemia (AML) in the 1990s, where a rare subset with the $CD34^+/CD38^-$ phenotype could induce leukemia in immunocompromised mice (Bonnet and Dick 1997; Lapidot et al. 1994). A decade after the identification of leukemic stem cells, the discovery of the CSC subset in breast cancers (Al-Hajj et al. 2003) expanded the concept to solid tumors and, thereafter, CSCs were identified in various solid tumors, including colorectal cancer (CRC) (Dalerba et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Since CSCs share phenotypic and molecular characteristics with their normal tissue-resident stem cells, and intestinal stem cells (ISCs) maintain a homeostatic epithelial renewal in the normal colonic tissues (Barker et al. 2007), CRC would be the most compelling model of a hierarchically organized solid tumor, with CSCs at the top. Furthermore, accumulated data on ISCs would necessarily offer clues to understanding CSCs in CRC.

CRC is the fourth leading cause of cancer death worldwide. While its occurrence is declining in the developed countries, its incidence is still rising at a rapid rate in many developing countries (International Agency for Research on Cancer 2014). The CSC concept has attracted a great deal of interest because of its potential clinical implications. A number of reports have shown that colorectal CSCs are more resistant to chemotherapy (Dylla et al. 2008; Todaro et al. 2007) and may play an essential role in recurrence following conventional anticancer treatments. Therefore, CSCs represent an attractive target for more effective therapies against CRCs.

CSCs were initially considered as a population with well-defined phenotypic and molecular characteristics. However, there are accumulating evidences that CSCs represent a phenotypically and functionally heterogeneous population, and that they are dynamic rather than static populations that are continuously altered by cell-intrinsic and extrinsic factors. In this chapter, we summarize the past and current evidences related to the heterogeneity within colorectal CSC populations and the potential mechanism of this heterogeneity (Fig. 7.1).

## 7.1 Markers of Colorectal CSCs

We first provide a brief overview of colorectal CSC markers just to facilitate the understanding of the following contents. A detailed list of the established and candidate markers for colorectal CSCs is available in our recent review article (Hatano et al. 2017).

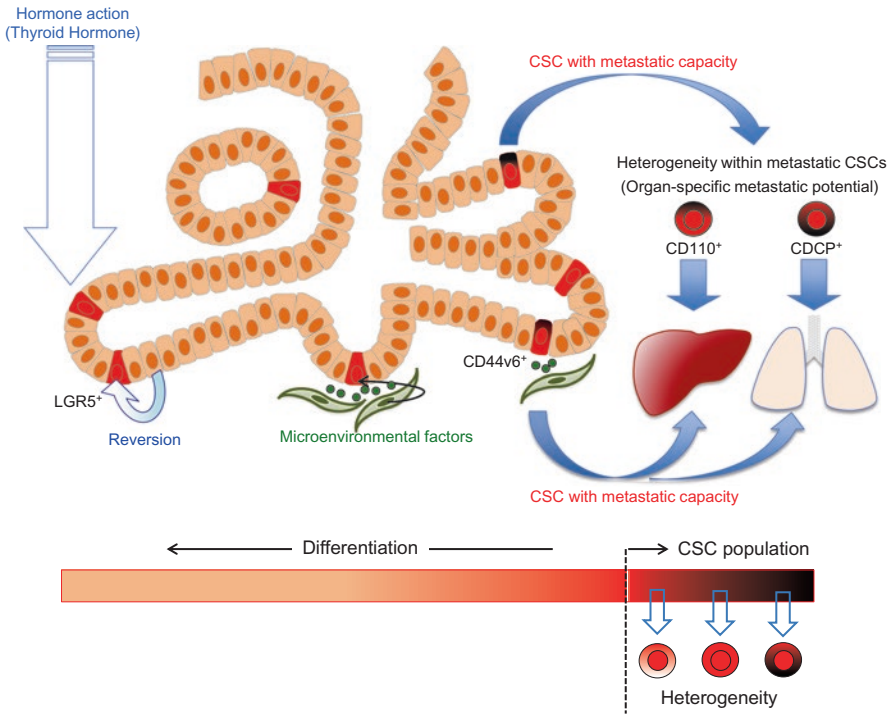


**Fig. 7.1** Heterogeneity within cancer stem cell (CSC) populations and intra- and extra-tumoral factors implicated in the regulation of CSCs. While these factors contribute to the increase in CSC numbers, they can also introduce minor and/or major changes in CSCs, thereby making CSC populations heterogeneous

Human colorectal CSCs were first identified based on CD133 expression (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). $CD133^+$ cells were consistently capable of generating tumors that resembled the original patient tumor when serially transplanted in immunocompromised mice, whereas their $CD133^-$ counterparts did not give rise to xenografts. Initial evidence of chemoresistance of colorectal CSCs was also obtained using $CD133^+$ cells; $CD133^+$ cells were largely resistant to oxaliplatin and/or 5-fluorouracil (5-FU) treatments in vitro and in vivo, whereas $CD133^-$ cells showed a high sensitivity to the treatment (Todaro et al. 2007). $CD133^+$ cells produce and utilize interleukin-4 in an autocrine manner for protection against chemotherapeutic agents (Todaro et al. 2007). To date, several markers have also been reported to identify CSCs in human CRCs, including CD44 ($EpCAM^{high}$ / $CD44^+$ and $EpCAM^{high}$ /$CD44^+$/$CD166^+$) (Dalerba et al. 2007), CD44v6 (Todaro et al. 2014), and aldehyde dehydrogenase 1 (ALDH1) (Huang et al. 2009). Notably, $CD44v6^+$ cells have been shown to generate both colonic tumors and metastasis when orthotopically injected into immunocompromised mice (Todaro et al. 2014), demonstrating that a certain type of CSCs have the capacity to initiate metastasis.

## 7.2 Heterogeneity Within Colorectal CSC Populations

CSCs were initially considered as cell populations with well-defined features, but growing evidence suggests that CSCs represent a phenotypically and functionally heterogeneous populations.

### *7.2.1 Overlap and Non-overlap Among CSC Markers*

Since the initial publication on brain tumor (Singh et al. 2004), CD133 (also known as prominin-1) has been established as a marker of CSC populations in a variety of cancers, including pancreatic cancer (Hermann et al. 2007), lung cancer (Kelly et al. 2007), and CRC (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). In addition, flow cytometric analysis has revealed that $CD133^+$ CRC cells overlap the cell fractions expressing CD44, CD29, CD24, and CD166, which have all been described to enrich colorectal CSC populations (Vermeulen et al. 2008). These findings suggest that the expression of CD133 is one of the most universal and comprehensive features of colorectal CSCs. However, as also reported in human glioblastomas (Beier et al. 2007; Joo et al. 2008; Wang et al. 2008), CD133 does not seem to specifically mark the CSC subset in CRCs. It has been shown that both $CD133^+$ and $CD133^-$ subsets from hepatic metastatic lesions of CRCs were capable of reconstituting the tumors in immunocompromised mice, and the tumors derived from $CD133^-$ cells grew at a more rapid rate (Shmelkov et al. 2008). Interestingly, further analysis revealed that $CD133^-$ cells frequently expressed CD44, a different phenotypic CSC marker, in colonospheres derived from $CD133^-$ cells (Shmelkov et al. 2008). The following finding is also indicative of the existence of CSCs phenotypically distinct from $CD133^+$ cells in CRCs; not all CRCs express CD133, but $CD44^+/EpCAM^{high}$ with CSC properties were contained even in CRCs without CD133 expression (Dalerba et al. 2007).

On the other hand, it should also be stated that CD133 and CD44 were non-mutually exclusive markers, as a partial overlap between the two cell subsets was repeatedly reported in CRCs (Dalerba et al. 2007; Haraguchi et al. 2008; Vermeulen et al. 2008). In fact, $CD133^+/CD44^+$cells induced tumors in immunocompromised mice under the condition in which the same number of $CD133^+/CD44^-$ cells failed engraftment, indicating that CD44 further enables the enrichment of CSCs within the $CD133^+$ subset (Haraguchi et al. 2008).

### *7.2.2 CSCs with Metastatic Capacity*

Distant metastasis is the predominant cause of lethality in cancer patients with CRC. Previous studies have provided a few evidences that only certain types of CSCs can metastasize to distant organs. Hermann et al. first identified a specific

subset of CSCs responsible for metastasis in human pancreatic cancer cell lines based on the cell surface expression of CD133 and CXCR4, a receptor for the chemokine CXCL12 (Hermann et al. 2007). When orthotopically injected into nude mice, both the $CD133^+/CXCR4^+$ and $CD133^+/CXCR4^-$ cells could develop tumors at the injection site, but only the $CD133^+/CXCR4^+$ cells could induce liver metastasis (Hermann et al. 2007). A subsequent study showed that the CRC cell line HCT116 also contains $CD133^+/CXCR4^+$ cells, which have a significantly higher metastatic capacity than $CD133^+/CXCR4^-$ cells (Zhang et al. 2012).

Pang et al. demonstrated that a subpopulation of colorectal CSCs expressing CD26 has both tumor-initiating and metastatic capacities (Pang et al. 2010): Orthotopic implantation of $CD133^+/CD26^+$ cells isolated from primary CRCs of a patient with hepatic metastasis (irrespective of CD44 status; both $CD133^+/CD26^+/CD44^+$ and $CD133^+/CD26^+/CD44^-$ cells) led to metastasis in the liver, following tumor formation in the cecal wall in mice, whereas their $CD26^-$ counterparts induced tumor growth only at the site of injection (Pang et al. 2010). Additionally, circulating $CD133^+/CD26^+/CD44^+$ cells were detected in portal blood after the cecal wall injection, and that the intraportal injection of $CD133^+/CD26^+/CD44^+$ cells, but not $CD133^+/CD26^-/CD44^+$ cells, led to the development of liver metastasis (Pang et al. 2010). In vitro evaluations revealed that CD26 knockdown by small interfering RNA (siRNA) reduced the migratory and invasive capacities of the $CD26^+$ cells, with a downregulation of epithelial–mesenchymal transition (EMT) markers (Pang et al. 2010). Consistent with these findings, clinical research has reported that CD26 expression is related to the poor prognosis of CRC (de la Haba-Rodriguez et al. 2002; Lam et al. 2014; Lieto et al. 2015). Interestingly, $CD26^+/CD326^-$ circulating tumor cells were proposed as prognostic markers for the recurrence of CRCs using the universal marker of circulating tumor cells recognized as epithelial specificities, CD326 (EpCAM) (Lieto et al. 2015).

In a recent study, Todaro et al. phenotypically identified colorectal CSCs with metastatic capacity based on the expression of CD44v6, a variant form 6 of CD44 (Todaro et al. 2014). $CD44v6^+$ cells were able to induce tumor growth in the gut, lung, and liver after orthotopic injection into mice, whereas their negative counterparts grew locally without forming distant metastatic lesions (Todaro et al. 2014). Interestingly, while there was a substantial overlap between $CD44v6^+$ and $CD26^+$ cells, $CD44v6^+/CD26^-$ cells showed considerable metastatic potential in the orthotopic model (Todaro et al. 2014), indicating that there is phenotypic heterogeneity even within metastatic CSCs.

A recent study suggested that the formation of metastases in certain favored target organs would be attributable in part to the diversity within metastatic CSCs. Gao et al. reported that only colorectal CRCs expressing CD110, a specific receptor for thrombopoietin, were able to colonize the liver after orthotopic implantation in immunocompromised mice, while CRCs expressing CUB-domain-containing protein 1 (CDCP1) were associated with the development of lung metastasis (Gao et al. 2013). They also confirmed that knockdown of either CD110 or CDCP1 by siRNA reduced the liver or lung metastasis burden, but had no discernible effect on primary tumor growth. In addition, they also showed that CD110 and CDCP1 would be involved in integral parts of the metastatic process, including extravasation into

the liver parenchyma and adhesion on the pulmonary endothelium (Gao et al. 2013). It is also notable that both $CD110^+$ and $CDCP1^+$ CRCs were included in the $CD133^+$ population, and there was no overlap between these cells (Gao et al. 2013), indicating that distinctly different metastatic CSC subsets are contained within $CD133^+$ CSC populations.

## 7.3 Dynamics of Colorectal CSCs

Recent studies demonstrate colorectal CSCs to be dynamic rather than static populations that are continuously altered by various factors, including genetic and epigenetic alterations, interactions between tumor cells, microenvironmental factors, hormone action, and cancer therapy. These could contribute to making colorectal CSC populations heterogeneous. This section summarizes the past and current findings related to the dynamics of colorectal CSCs and the signaling pathways and intra- and extra-tumoral factors involved in the regulation of CSCs. Although ISCs are not the main focus of this section, we have also provided a brief description of ISCs, with emphasis on the findings that offer clues to understanding CSC dynamics in CRC.

### *7.3.1 Relationship Between ISCs and Colorectal CSCs*

CSCs share phenotypic and molecular characteristics with their normal cell counterparts, tissue-resident adult stem cells. In fact, the colorectal CSC markers identified so far are also expressed by normal ISCs (Huang et al. 2009; Snippert et al. 2009). Conversely, the most established marker for murine ISCs, leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), could also serve as a marker for CSCs in human CRCs (Kemper et al. 2012). CSCs do not necessarily originate from the transformation of normal stem cells, but in the case of CRCs, the similarity could be attributable in part to the origin of CSCs. CRC develops and progresses owing to the sequential accumulation of genetic alterations, in which activation of the Wnt/β-catenin signaling pathway, via an *APC* or *β-catenin* (*CTNNB1*) mutation, marks the first step in tumor formation. Considering that intestinal epithelial cells are consistently renewed for a shorter period than that required to accumulate causative genetic alterations, it seems to be reasonable to assume that CSCs arise from long-lived ISCs. The discovery of reliable ISC markers such as *Lgr5* (Barker et al. 2007) and *B lymphoma Mo-MLV insertion region 1* (*Bmi1*) (Sangiorgi and Capecchi 2008) enables the validation of this concept. Actually, it has been demonstrated that specific activation of Wnt/β-catenin signaling in ISCs expressing *Lgr5* (Barker et al. 2009), *Bmi1* (Sangiorgi and Capecchi 2008), or *Cd133* (Zhu et al. 2009) results in adenoma formation in mice.

### 7.3.2 Convertibility of ISCs and Colorectal CSCs

ISCs would be one of the most intensively researched subjects of stem cell biology. While a previous theory assumes the existence of a single quiescent population of stem cells residing in a specific location of the intestinal crypt as typified label-retaining cells (Booth and Potten 2000), it is currently accepted that both quiescent and active ISCs coexist in distinct niches (Li and Clevers 2010). Importantly, both populations possess the capacity to self-renew and give rise to all differentiated intestinal epithelial cell types, despite having entirely different proliferative activities. *Lgr5* is the most established marker for active ISCs (Barker et al. 2007), while the quiescent ISCs have been shown to be marked with several markers including *Bmi1* (Sangiorgi and Capecchi 2008), *homeodomain-only protein* (*Hopx*) (Takeda et al. 2011), *telomerase reverse transcriptase* (*Tert*) (Montgomery et al. 2011), and *leucine-rich repeats and immunoglobulin-like domains protein 1* (*Lrig1*) (Wong et al. 2012). The active and quiescent ISCs represent functionally distinct ISC populations: actively cycling *Lgr5*$^+$ ISCs contribute to homeostatic epithelial renewal, while slow-cycling *Bmi1*$^+$ ISCs are considered as a reserve stem cell pool, based on their active contribution to regeneration after irradiation damage (Yan et al. 2012). Consistently, it has been reported that *Bmi1*$^+$ ISCs gave rise to *Lgr5*$^+$ ISCs in the small intestines of mice after the selective ablation of *Lgr5*$^+$ cells (Tian et al. 2011). Conversely, *Lgr5*$^+$ cells can give rise to *Hopx*$^+$ cells in organoid cultures (Takeda et al. 2011). These results indicate that active and quiescent ISCs can interconvert and/or replenish with each other. In addition, enterocyte progenitors can dedifferentiate into *Lgr5*$^+$ ISCs upon the depletion of *Lgr5*$^+$ cells (Tetteh et al. 2016), and similarly, secretory progenitor cells regenerate stem cell compartments that contain *Lgr5*$^+$ cells following irradiation damage (van Es et al. 2012). These findings indicated the dedifferentiation capacity of the intestinal epithelium; yet, this capacity seems limited to fated progenitors rather than terminally differentiated cells.

Recent studies showed the reversions of differentiated non-CSC populations to CSCs in CRC models. After selective ablation of *LGR5*$^+$ CSCs, *KRT20*$^+$ differentiated colon cancer cells revert to CSCs and contribute to tumor growth (Shimokawa et al. 2017). The recovery of functional *Lgr5*$^+$ CSCs after the ablation of *Lgr5*$^+$ cells was also observed in a mouse CRC model (de Sousa e Melo et al. 2017).

### 7.3.3 Regulatory Factors of Colorectal CSCs

In line with the importance of canonical Wnt signaling in both intestinal stemness and colon carcinogenesis, Wnt signaling plays a central role in the regulation of colorectal CSCs. Although other signaling pathways are also implicated in the control of colorectal CSCs, here, we have mainly focused on canonical Wnt signaling. Vermeulen et al. demonstrated that CRC cells show variable levels of Wnt activation, and only cancer cells with the highest levels of Wnt activation possess CSC property (Vermeulen et al. 2010). Our study would also support the notion that

the Wnt activity level defines colorectal CSCs. We investigated the dose-dependent effect of Wnt activation in mouse colonic epithelium by regulating the expression levels of mutant β-catenin (S33Y mutation) (Hirata et al. 2013). Higher levels of mutant β-catenin expression induced the amplification of *Lgr5*$^{+}$ cells in the colonic crypts with de novo crypt formation, whereas lower levels of its expression only enhanced cell proliferation (Hirata et al. 2013). Consistent with these findings, Ordonez-Moran et al. showed that HOXA5 induction counteracted CSC traits and prevented tumor growth and metastasis by inhibiting Wnt signaling activity in CRCs (Ordonez-Moran et al. 2015). Colorectal CRCs are regulated through the interaction between Wnt and other signaling pathways, like the case in normal ISCs. For instance, bone morphogenetic protein 4 (BMP4) promotes differentiation and apoptosis by antagonizing Wnt signaling in colorectal CSCs (Lombardo et al. 2011). In a recent study, Whissell et al. identified the transcriptional factor GATA-binding factor 6 (GATA6) as a key regulator of the Wnt and BMP signaling in colorectal CRCs (Whissell et al. 2014). GATA6 enables CSC self-renewal through the repression of BMP gene expression by competing with β-catenin/Tcf4 to bind to a regulatory region of the *BMP4* locus (Whissell et al. 2014).

The microenvironment is strongly related to the regulation of colorectal CSCs and their normal counterparts, ISCs (Medema and Vermeulen 2011). Tumor stroma undergoes dramatic changes during the process of tumor progression, which could have more complicated effects than in normal homeostatic conditions. It has been shown that microenvironmental factors play a substantial role in defining the CSC state. Hepatocyte growth factor (HGF) secreted from myofibroblasts could induce CSC property in Wnt$^{Low}$ CRC cells by enhancing Wnt signaling activity (Vermeulen et al. 2010). When co-cultured with CRC cell lines, mesenchymal stem cells can also increase the number of cancer cells with tumor-initiating capacity by producing prostaglandin E2 and cytokines that induce the activation of Wnt/β-catenin signaling (Li et al. 2012). It is possible that CSCs acquire a metastatic capacity by the actions of cytokines secreted from cancer-associated fibroblasts (CAFs) during tumor progression. As described above, the colorectal CSCs with metastatic capacity are phenotypically identified by their CD44v6 expression (Todaro et al. 2014). Cytokines secreted from CAFs such as HGF, osteopontin, and stromal-derived factor 1α enhance CD44v6 expression by activating Wnt signaling and turn non-metastatic progenitors into metastatic CSCs (Todaro et al. 2014). In addition to stromal cells, Paneth cells or cKIT$^{+}$ secretory cells constitute the niches for *Lgr5*$^{+}$ ISCs in the small intestine and colon, respectively, providing essential signals for stem cell maintenance, including Notch ligand Dll4 (Rothenberg et al. 2012; Sato et al. 2011). As Notch signaling is elevated in colorectal CSCs (Hoey et al. 2009) and antibody blockade of DLL4 reduces CSC frequency in CRCs (Hoey et al. 2009), the CSC state might be also regulated through interactions between tumor cells in CRCs.

The regulation of Wnt and BMP4 signaling pathways by activated thyroid hormones was reported in colorectal CSCs (Catalano et al. 2016). Type 3 deiodinase 3 (D3), a chief thyroid hormone T3-inactivating enzyme, is highly expressed in CD133$^{+}$ and Wnt$^{high}$ CRC populations (Catalano et al. 2016). T3 treatment induces CSC differentiation and thereby decreases the tumorigenic potential in CSCs,

accompanied by the upregulation of BMP4 and attenuation of Wnt signaling (Catalano et al. 2016). These findings suggest that colorectal CRCs could be regulated by not only local factors within or surrounding a lesion, but also by hormone action.

## 7.4 Conclusion

Although CSCs were initially considered to be cell populations with well-defined phenotypic and molecular characteristics, CSCs have been shown to be phenotypically and functionally heterogeneous and highly dynamic populations. Their heterogeneous and dynamic nature present a serious problem in establishing therapeutic strategies targeting CSCs. Considering that the stemness of CRC cells is a dynamic state that is constantly altered by various extrinsic factors in addition to intrinsic cellular factors (genetic and epigenetic alteration), a better understanding of the tumor environment would also provide new strategy for the eradication of colorectal CSC by regulating their CSC state.

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# Chapter 8
# Urothelial Cancer Stem Cell Heterogeneity


**Michaela Kripnerova, Hamendra Singh Parmar, Martin Pesta, Michaela Kohoutova, Jitka Kuncova, Karel Drbal, Marie Rajtmajerova, and Jiri Hatina**

**Abstract** Urothelial carcinoma is a tumor type featuring pronounced intertumoral heterogeneity and a high mutational and epigenetic load. The two major histopathological urothelial carcinoma types – the non-muscle-invasive and muscle-invasive urothelial carcinoma – markedly differ in terms of their respective typical mutational profiles and also by their probable cells of origin, that is, a urothelial basal cell for muscle-invasive carcinomas and a urothelial intermediate cell for at least a large part of non-muscle-invasive carcinomas. Both non-muscle-invasive and muscle-invasive urothelial carcinomas can be further classified into discrete intrinsic subtypes based on their typical transcriptomic profiles. Urothelial carcinogenesis shows a number of parallels to a urothelial regenerative response. Both of these processes seem to be dominated by specific stem cell populations. In the last years, the nature and location of urothelial stem cell(s) have been subject to many controversies, which now seem to be settled down, favoring the existence of a largely single urothelial stem cell type located among basal cells. Basal cell markers have also been amply used to identify urothelial carcinoma stem cells, especially in muscle-invasive disease, but they proved useful even in some non-muscle-invasive tumors. Analyses on molecular nature of urothelial carcinoma stem cells performed till now point to their great heterogeneity, both during the tumor development and upon intertumoral

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comparison, sexual dimorphism providing a special example of the latter. Moreover, urothelial cancer stem cells are endowed with intrinsic plasticity, whereby they can modulate their stemness in relation to other tumor-related traits, especially motility and invasiveness. Such transitional modulations suggest underlying epigenetic mechanisms and, even within this context, inter- and intratumoral heterogeneity becomes apparent. Multiple molecular aspects of urothelial cancer stem cell biology markedly influence therapeutic response, implying their knowledge as a prerequisite to improved therapies of this disease. At the same time, the notion of urothelial cancer stem cell heterogeneity implies that this therapeutic benefit would be most probably and most efficiently achieved within the context of individualized antitumor therapy.

**Keywords** Urothelium · Urothelial stem cells · Urothelial regenerative response · Lineage-tracing · Lineage-depletion · Urothelial carcinoma · Urothelial carcinoma heterogeneity · Non-muscle-invasive bladder cancer · Muscle-invasive bladder cancer · Intrinsic subtypes · Urothelial carcinoma sexual dimorphism · Urothelial carcinoma stem cells · Stemness signaling pathway · Sonic hedgehog · Wnt/β-catenin pathway · SOX-2 · STAT-3 · COX-2 · YAP-1 · Epithelial-mesenchymal transition · Epigenetic plasticity · DNMT-1

## 8.1 Cellular Heterogeneity Within Normal Urothelium

Urothelium represents a specialized pseudostratified epithelium that lines the urogenital ductal system from the ureters, through the bladder, to the proximal urethra. Its major task is to provide a practically impermeable blood-urine barrier. As is true for any other tissue, this specialized function is provided by terminally differentiated cells—superficial or umbrella cells lining the urothelial lumen. They are very large (50–100 μm), postmitotic, frequently bi- or multinucleated cells, and the blood-urine barrier function is mostly attributable to the function of highly specialized proteins—uroplakins. Assembling into a specific structure on the apical pole of umbrella cells called AUM (asymmetric unit membrane) plaques, uroplakins create an efficient barrier against water, solutes, and toxins that concentrate in the urine. In addition, adjacent superficial cells are connected by tight-junction proteins, limiting the possibility of pericellular transport. Last but not least, umbrella cells are not just a static barrier, but respond to changing bladder tonus by undergoing the regulated processes of endocytosis and exocytosis of discoidal fusiform-shaped vesicles (DFVs).

Beneath the superficial layer is one or several layers of intermediate cells, which are significantly smaller than umbrella cells (about 20 μm in diameter), sitting on a single layer of small (5–10 μm in diameter) cuboidal basal cells directly in contact with the basement membrane. Some of the intermediate cells are also in direct contact with the basement membrane, hence the notion of urothelium as transitional

epithelium. Due to these very different size relations, relative representations of these three urothelial cell types differ dramatically, basal cells being the by far most prevalent cell type (about 90%). The histological analysis of whole mount adult mouse urothelium revealed that a single superficial cell could span the area of about 40 intermediate and basal cells (reviewed in Balsara and Li 2017).

What function do intermediate and basal cells serve? A traditional view has been to regard different urothelial cell types within the context of gradual differentiation. Accordingly, basal cells would include adult stem cells, intermediate cells would constitute transit-amplifying (TA) precursor cells, and umbrella cells correspond to terminally differentiated cells (Hatina and Schulz 2012). This tentative hierarchical model proved to be rather difficult to test experimentally, for several reasons. First, the small size and the resulting very high numbers of basal cells immediately arouse the question as to which of them actually correspond to stem cells; it is barely thinkable that 90% of cells in a tissue can represent stem cells. Urothelial stem cells are thus expected to constitute just a minority of basal cells, most of the basal cells corresponding likely to TA-precursor cells. Indeed, basal cells greatly differ in terms of their relative proliferation activity, a minority of cells being quiescent over a long period of time while the majority proliferating. This became apparent when the entire population of urothelial cells were forced to incorporate a specifically modified nucleotide (bromodeoxyuridine [BrdU]) and then observed for a long time. Over 90% of basal cells diluted BrdU gradually as they divided, while only about 9% preserved it because of their quiescence. Apparently, these minor LRC (label-retaining cells) are good candidates for urothelial stem cells (Kurzrock et al. 2008).

The second problem is the unusually slow physiological turnover of adult urothelium; the umbrella cells are surprisingly long-lived, their lifespan under physiological conditions reaching about 200 days or even a year. To experimentally study the lineage succession in urothelium, researchers frequently resort to studying either prenatal urothelial development or pathophysiological regenerative processes. Indeed, despite the largely quiescent state under physiological conditions, urothelium is able to mount a surprisingly rapid regenerative response to a variety of injuries. Experiments on lineage tracing and lineage depletion, performed largely in mice exposed to various types of urothelial damage, can help decipher an intrinsic cellular hierarchy. As explained in detail in other chapters of this treatise (Chap. 1), specific loci and associated gene regulatory sequences are used to drive expression of tamoxifen-inducible Cre-recombinase (CreERT2), in order to indelibly label a certain population of cells with a marker protein, lineage succession being then derived from a gradual inheritance of this marker protein expression by cells issued from this original recombined cell population. Alternatively, Cre-recombinase can unleash the expression of the diphtheria toxin receptor gene, and, if treated with the diphtheria toxin, the targeted Cre-recombinase expressing cells are specifically eliminated (Blanpain 2013). Implicit in using the lineage tracing or lineage depletion strategies is of course a thorough knowledge of genes and proteins whose expression is restricted to specific urothelial cell populations (Fig. 8.1). Such genes

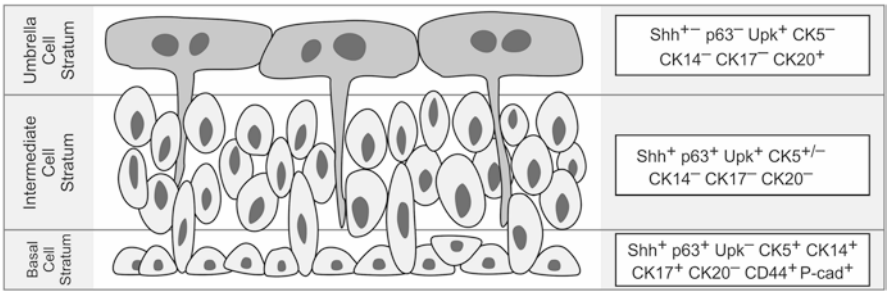


**Fig. 8.1** The expression of CK5, CD44, and P-cadherin is uniform among all basal cells, unlike of CK14, which is expressed by a relatively small fraction of them. The expression of CK14 and CK17 may become ubiquitous throughout the entire urothelium, however, and this pattern signals squamous transdifferentiation. Source: (Wang et al. 2017; Balsara and Li 2017)

and proteins can thus serve as specific lineage markers and – by the same token – as specific drivers of CreERT2 inducible recombinase in a restricted and well-defined population of urothelial cells. Lineage-tracing experiments in mouse urothelium experienced an interesting evolution, which, for a certain period of time and in fact until recently, lead to two opposing theories on the identity of adult urothelial stem cells.

One landmark study used Sonic hedgehog (Shh) as the CreERT2 driver gene and uropathogenic *Escherichia coli* infection as a type of urothelium damage to induce a regenerative response. This model helped to discover a remarkable epithelial-mesenchymal reciprocal interaction. Uropathogenic *E. coli* infection leads to a rapid exfoliation of umbrella and most intermediate cells. The remaining basal cells respond by the secretion of the signaling molecule Sonic hedgehog, which signals to the *lamina-propria* and localized fibroblasts. They are activated and signal back to the urothelium with mitogenic (Wnt 2, Wnt 4, Fgf 16) and differentiation-inducing (Bmp-4 and -5) factors; the model assumes that the former have a shorter range of action, thus primarily inducing basal cell proliferation, whereas the latter can signal to a longer distance, thus reaching newly generated intermediate cells and inducing their differentiation (Shin et al. 2014b). Using Shh as Cre-ERT2 driver in lineage depletion experiments then led to slow urothelial degeneration over a period of 8–10 months, again reflecting the slow cell turnover of normal urothelium (Shin et al. 2014a). Finally, urothelium failure and the resulting death of manipulated mice ensued, apparently because urothelial stem cells have been eliminated. This model thus seems to corroborate the traditional view of urothelial differentiation, placing urothelial stem cells among the basal cells, gradually differentiating into intermediate cells and – eventually – into umbrella cells.

As elegant as this model may appear, it was not universally accepted. The major objection was that Shh was not a basal cell-specific marker, but its expression was shared by intermediate cells (Fig. 8.1). Using Krt 5 as a Cre-ERT2 driver gene

instead seemed to yield quite a different picture (Gandhi et al. 2013). In response to a chemical damage induced by cyclophosphamide treatment applied once, twice or three times in succession, the authors did not evidence any succession of the labeled basal—intermediate—umbrella cells. But when uroplakin 2A served as a CreERT2 driver, vertical labeled units of intermediate and umbrella cells were observed. These results led the authors to form an alternative hypothesis placing two independent and autonomous cell populations, each replenished by different stem cells, into the adult urothelium. Accordingly, basal cells are one of the autonomous specific urothelial cell populations, with its own stem cells, and the intermediate cells include an independent stem cell population that self-renews and differentiates into umbrella cells. The study evidenced also an epithelial-mesenchymal crosstalk: suburothelial fibroblasts expressed enzymatic machinery to synthetize retinoic acid, which diffused to the urothelium and promoted the differentiation of umbrella cells (Gandhi et al. 2013).

This conceptual quarrel about the identity of urothelial stem cell(s) seems now to have settled down, favoring the first model. Another experiment used the more restrictive basal stem cell marker Krt 14 as a CreERT2 driver and five rounds of cyclophosphamide treatment. Vertical labeled units spanned the entire thickness of urothelium, evidencing that basal layer stem cells can differentiate into umbrella cells. In vitro explant culture experiments on Krt 14–CreERT2-driven lineage depletion corroborated the exclusive requirement of Krt 14 expressing cells for urothelial regenerative activity (Papafotiou et al. 2016).

Why did these results differ? Part of the answer might lie in technical reasons – such as different mouse strains or a subtle difference in tamoxifen application – which can influence the effectiveness of Cre-mediated recombination and be one source of result variability between different studies. Indeed, two independent groups (Papafotiou et al. 2016; Schäfer et al. 2017) repeated the lineage tracing with Krt 5-CreERT2 as a driver and evidenced what the first analysis (Gandhi et al. 2013) failed to prove: the full-thickness urothelium differentiating units.

Other reasons may have a likely biological background. First and foremost, the different studies used different types of urothelial injury, resulting in different degrees and durations of the urothelial damage. Research on other organ systems (see Chap. 4) helped to discover two important phenomena: That not every regenerative response needs to resort to the activation of respective stem cells, and that the mobilization and proliferation of transit-amplifier precursors is a preferred way to mend relatively shorter and/or milder tissue damage. Indeed, the experiments that evidenced the basally located urothelial stem cells used either prolonged chemical damage, resulting in exfoliation of upper urothelial cell layers, or full-thickness urothelial injury induced surgically. In addition, different kinds of urothelial injuries vary in terms of the degree of activation of an inflammatory response (Wang et al. 2017), which might be one factor possibly affecting stem cells as well.

Finally, the results obtained by analyzing human urothelium, based on either a common pattern of X-chromosome inactivation or shared mitochondrial mutations (Gaisa et al. 2011), also point to the linear differentiation model, with urothelial

stem cells localized among basal cells and intermediate cells serving as TA-precursors, eventually differentiating into umbrella cells.

As shown above, the knowledge of the cell population dynamics of adult urothelium has been improving recently. The same is not entirely true of embryonic and fetal urothelium, where our knowledge is still more scarce. Together with hindgut, urothelium starts to develop from endoderm around the middle of the mouse intrauterine development (i.e., from the embryonic day 10 [E10]), the whole process being regulated by the master stemness transcription factor p63 (Pignon et al. 2013). The adult hierarchical organization, with the CK 5-uniformly positive basal layer and CK 14-positive fraction of it, becomes evident only towards the end of gestation, from E16.5 (Gandhi et al. 2013; Paraskevopoulou et al. 2016). Interestingly, the CK 14-positive fraction is much higher in late embryonic and early postnatal phases, accounting for 20% and 30% of total urothelial cells, respectively, and sharply declining during further postnatal life (reaching 3.5% at 8 weeks) and adulthood (0.9% at 1 year of age) (Paraskevopoulou et al. 2016), a dynamics well reminiscent of a process of tissue aging, that is, a gradual decrease in stem cell abundance and competence. Until the appearance of the adult pattern of urothelial hierarchical organization, there are several unique stem cell populations specific for discrete stages of embryonic and fetal urothelial development. Well characterized are P-cells, which feature the unique marker profile $Foxa2^{+}$ $Upk^{+}$ $p63^{+}$ $Shh^{+}$ $CK5^{-}$. Abundant between E11 and E13, at E14 P-cells are succeeded by I-cells, characterized by the typical marker profile $Foxa2^{-}$ $Upk^{+}$ $p63^{+}$ $Shh^{+}$ $CK5^{-}$. It comes as little surprise that suburothelial-mesenchyme—derived retinoic acid is also involved in the differentiation of embryonic and fetal urothelium, as revealed by the expansion of P-cells upon the urothelial-specific expression of a dominant negative mutation in the retinoic acid receptor alpha gene and their persistence until E14 (Gandhi et al. 2013). This means that adult regenerative response and embryonic and fetal development are governed by partially overlapping molecular machineries.

There is one more – perhaps even more flagrant – example of this overlap between the embryonic and regenerative urothelium biology. Between the E14 and immediate postnatal period (i.e., with a certain overlap between the P-cell– and I-cell—dominated urothelium and adult urothelial cell hierarchy), basal and intermediate cells strongly express the developmental transcription factor Sox-9. Afterwards, its expression becomes undetectable, only in order to be reactivated in regenerating urothelium. The underlying signaling pathway is the EGFR-triggered MAPK-pathway (Ras-Raf-ERK). Two signaling events – probably acting successively – activate it in basal urothelial cells. First, urine itself contains sufficient concentrations of epidermal growth factor (EGF) and other members of this growth factor family to activate EGFR. The point is that EGFR is basally located and umbrella cells in intact urothelium provide a sufficient barrier precluding any access of urine-borne growth factors to their basally located receptors. The immediate consequence of a urothelial injury is the extensive exfoliation of the umbrella cell layer—and therefore also of this signaling barrier. Second, promptly after the injury, the remaining urothelial cells activate the autocrine expression of several members of the EGF family (especially amphiregulin, HB-EGF, epiregulin, and epigen), fur-

ther intensifying the regenerative response (Ling et al. 2011). Interestingly in this respect, phosphorylated ERK has been found as a specific molecular feature of stem cells in non-muscle-invasive papillary urothelial carcinomas (Hepburn et al. 2012); what needs to be explored is whether SOX-9 reactivation also follows and if yes, whether SOX-9 directly participated in cancer stem cell biology.

All the cases of urothelial regeneration discussed by now could resort to preexisting stem or potentially also progenitor cells that were either spared by the injury (caused by uropathogenic *E. coli* infections or various types of chemical injuries) or were available from the neighboring areas of uninjured urothelium (surgical injury). Can urothelium regenerate if urothelial stem or progenitor cells are directly damaged? A Shh-Cre- driven knockout of the gene coding for DNA-methyltransferase 1 seems to be such a case. So the answer is yes, it can, by employing a very unusual regeneration mechanism (Joseph et al. 2018). The Shh-directed *Dnmt1* knockout results in global DNA hypomethylation, which triggers DNA damage, immediately followed by the activation of the DNA damage response pathway, eventually leading to apoptosis induction. The resulting acellular gaps along the basement membrane are filled by cells originating from the Wolffian duct, which subsequently transdifferentiate into fully competent urothelial cells; tissue recombination experiments showed that embryonic mesenchyme is crucial in this process. Wolffian ducts differentiate into seminal ducts, epididymis, and vas deferens in males; in females, they regress shortly before birth. Consequently, this unusual type of urothelial regeneration can happen in both sexes practically during the whole embryonic and fetal period. Unfortunately, the Shh-Cre- *Dnmt1* knockout mice die shortly after birth, for reasons unrelated to the urogenital system. This precludes any direct analysis (even that of its existence) of this special urothelial regeneration mechanism (at least in males) during postnatal life. Therefore, any link to a somewhat similar human proliferative benign disease of the bladder known as nephrogenic adenoma of the bladder (Pavlidakey et al. 2010) could not be experimentally analyzed by now. In conclusion, depending on the type, extent, and timing of urothelial injury, stem and/or progenitor cells initiating a regenerative response can be quite heterogeneous.

## 8.2 Urothelial Carcinoma

Urothelial carcinoma represents a frequent malignancy, with a remarkable difference in terms of the incidence between the sexes, ranking as fourth most common cancer in men and 11th in women, with worldwide annual incidence exceeding 430,000 cases and mortality exceeding 160,000 cases (Antoni et al. 2017). For decades, it has been well established that urothelial carcinoma is a very heterogeneous disease, with the main histopathological discriminant being provided by the invasion into the muscle layer of the bladder. About 75% of the cases are diagnosed as non-muscle-invasive bladder cancer (NMIBC), either without any invasive properties registered at diagnosis (stage pTa) or with the invasion limited to *lamina propria* only (stage pT1). The remaining 25% are diagnosed as muscle-invasive bladder

cancer (MIBC), whose depth of invasion and extent of spread are the major determinants of clinical staging (pT2–pT4). As many as 80% of NMIBC tumors behave clinically quite indolently, but many of them have a tendency to recur, necessitating their long-term surveillance by regular cystoscopies. Not only does such a surveillance procedure cause a substantial discomfort to patients, but also repeated cystoscopies are costly: Considering a total cost from diagnosis to death, bladder cancer is the most expensive cancer type. Ten to fifteen percent of originally NMIBCs ultimately progress to MIBCs, and the most important clinical parameter to forecast this dismal behavior is tumor grade. Several grading systems have been conceived over years, the most recent being the 2004 WHO grading system. It differentiates between low- and high-grade tumors based on architectural and cytologic atypia, and, in addition to them, introduces a histopathological diagnosis of the papillary urothelial neoplasm of low malignant potential (PUNLMP); in fact, the papillary growth pattern prevails among NMIBCs. The most telling clinical variable for MIBC is, as mentioned above, the disease stage. The overall 5-year survival rate is close to 50% for all stages, but only about 5% for metastatic disease. Most MIBCs, however, do not develop from progressing NMIBCs but rather along a separate pathway with a particular high-grade superficial neoplasia termed *carcinoma* in situ (pTis, also abbreviated as CIS) as a specific precursor lesion (Kamat et al. 2016).

Not only are NMIBC and MIBC clinically and pathologically different, they also develop – according to the current consensus – along different molecular and cellular pathways, a concept known as dual-track carcinogenesis. By the molecular pathway, we mainly understand genetic and epigenetic landscape—and indeed, mutational and epigenetic changes specific to either urothelial carcinoma type have been described. Most NMIBCs rely on the mutational activation of mitogenic signaling, especially the MAPK pathway. The major mutational hit is the type 3 receptor for fibroblast growth factors (FGFR3), mutations resulting mostly in its ligand-independent activation, which might be accompanied by overexpression. Alternatively, downstream signaling molecules are changed, like Ras (activation) or Notch (inactivation); the latter has a rather specific role in bladder cancer, activating ERK-phosphatases and thereby acting as a tumor suppressor. Further mitogenic changes have been detected, like cyclin D and E2F3 gene amplifications, the latter usually in high-grade tumors. Genome stability seems to be preserved, and *p53* mutations are rare and confined to high-grade tumors as well. Their frequency is much higher in MIBC, complemented by *pRb* and *PTEN* mutations and genomic instability. Interestingly, among the most frequently mutated genes in both NMIBC and MIBC are those coding for chromatin-modifying enzymes that act epigenetically, with a surprising specificity for either tumor type. Inactivating mutations in the gene coding for H3K27 demethylase KDM6A are common in NMIBC whereas inactivating mutations in H3K4 methyltransferase MLL2 are quite common in MIBC; interestingly, both mutations seem to be mutually exclusive, suggesting common biological consequence(s). Indeed, since H3K27me3 is a repressive histone mark and H3K4me3 is an activating histone mark, both mutations could have a similar overall effect, namely that of preserving a generally silenced chromatin state (Choi et al. 2017).

The lineage-tracing and lineage-depletion experiments described in the previous section enabled us to define an additional important difference between NMIBC and MIBC, namely in terms of their respective cells of origin. The same transgenic mouse combinations as those discussed above were also exposed to urothelial-specific chemical carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) over extended periods of time. In the Shh-CreERT2 lineage-traced mice, BBN treatment led to the tumorigenic transformation of Shh-expressing cells into muscle-invasive carcinomas preceded by *carcinoma*-in-situ—like lesions, a process entirely prevented by their lineage depletion (Shin et al. 2014b). As noted above, since Shh lacks an exquisite specificity for basal urothelial cells, including stem cells, the definitive conclusion on the cell-type-specific origin of MIBC was here somewhat problematic. The same conclusion was independently drawn after a prolonged BBN treatment of Krt 5-CreERT2 lineage labeled mice, nevertheless. In contrast, Upk2-CreERT2 lineage labeled cells transformed exclusively into papillary NMIBC (Van Batavia et al. 2014). Consequently, the dual-track carcinogenic pathway outlined above should be extended to include also the different respective cells of origin for either type of urothelial carcinomas.

Interestingly, if lineage tracing was activated by tamoxifen treatment after CIS lesions have formed, a heterogeneous cellular pattern became apparent; obviously, CIS preserved the capacity of limited differentiation in form of $Shh^-$ progeny of $Shh^+$ precursor cells. Strikingly, only the $Shh^+$ cells presented tumor-initiating capacity, a crucial functional property of cancer stem cells. Apparently, Shh expression is crucial for early invasive carcinoma development.

It has been independently suggested that BBN treatment might initiate a pseudo-regenerative response, $Shh^{high}$ cells of CIS lesions might thus have a selective advantage to the surrounding normal urothelium (Shin et al. 2014b). Interestingly in this regard, the explant outgrowth of $CK14^+$ cells is uniquely dependent on β-catenin (Papafotiou et al. 2016), which is a downstream signal resulting from Shh expression within the context of urothelial regenerative response (Shin et al. 2011). As a result of this possible selective advantage, $Shh^+$ CIS-cells can spread within a (histologically normal) urothelium, occupying its large areas. Surprisingly at the first look, if the lineage-tracing onset (i.e., tamoxifen application) was further delayed, up to the invasive carcinoma stage, the Shh-induced signal was completely lost. As advantageous as the Shh expression might be at the CIS stage, it appears to be disadvantageous at the invasive carcinoma stage, and hence the selection pressure to downregulate the Shh expression later during invasive carcinoma development (Shin et al. 2014b).

What could be the biological basis of this process? As specified above, later part of the urothelial regenerative response is mediated by differentiation-inducing factors secreted by suburothelial mesenchymal stromal cells under the influence of a Shh signal. These factors, especially Bmp-4 and -5, act as a break to tumor progression (Shin et al. 2014a, b). In addition, a targeted overexpression of the constitutively active gain-of-function mutant allele of β-catenin in urothelial basal cells resulted in low-grade papillary carcinomas (Lin et al. 2013), apparently violating the above-formulated rule of cells of origin specific to different carcinoma types.

Plausibly, as beneficial as β-catenin might be for proliferation and intraurothelial spread, it might act inhibitory with regard to invasion. There presently is no consensus, however, about the exact impact of β-catenin activation in urothelial carcinogenesis. The above conclusion is thus entirely hypothetical, although some additional indices appear to support it, especially regarding the sexual dimorphism of urothelial carcinoma (see below). From another point of view, the dynamics of Shh expression could provide a clear example of heterogeneity of urothelial cancer stem cells in the course of tumor progression: In CIS, cancer stem cells feature Shh expression, while in invasive carcinomas, different markers and/or drivers characterize them.

A crucial question is whether these results of sophisticated experiments in mice are relevant for human urothelial carcinoma. First, SHH expression has been reported to be really downregulated in human MIBC, supporting the above outlined carcinogenic sequence (Shin et al. 2014b). Second, a specific bioinformatic tool has been constructed, combining stem cell-specific expression signatures of human invasive carcinoma stem cells, umbrella-basal cell-specific transcriptomic profile, NMIBC- and MIBC-specific mutations, and a variable factor of common vs distinct cell of origin. This tool was confronted with publicly available datasets including both NMIBC and MIBC cases. As a result, a better discriminating power has been achieved if distinct cells of origin were assumed, largely corroborating the mouse studies described above. Interestingly, NMIBC showed a rather perfect concordance with an expression signature characterizing expression changes in induced pluripotent stem cells, whereas, not surprisingly, MIBC-derived CSC-specific expression signatures as well as the umbrella-basal cell-specific transcriptome, were successful in identifying MIBC samples (Dancik et al. 2014). We could plausibly interpret these findings as indicating that cancer stemness is in MIBC, by and large, "inherited" from normal urothelial basal cells, while in NMIBC cancer stem cells seem to be generated de novo by dedifferentiation process—yet another clear demonstration of CSC heterogeneity in urothelial carcinoma.

## 8.3 Intrinsic Subtypes of Urothelial Carcinoma

Both NMIBC and MIBC are very heterogeneous groups—clinically, histopathologically, and molecularly. The clinical differences mostly concern an intrinsic propensity to progress on the one hand and a therapeutic response on the other. The difference in terms of a progression tendency is most dramatically expressed in NMIBCs, only a fraction of which showing a capacity to progress into muscle-invasive disease. Within both NMIBCs and MIBCs, individual tumors markedly differ also regarding their therapeutic responses. There are cases with an excellent sensitivity to Bacillus Calmette-Guérin (BCG) immunotherapy, the standard of care after the transurethral resection of NMIBC, as well as to cisplatin-based combination chemotherapy, a main systemic therapeutic option for MIBC. There are,

however, primary refractory tumors that fail to respond at all, and there are initially sensitive tumors that develop resistance during treatment (Kamat et al. 2016).

Histopathologically, especially MIBC can present a lot of variants. The most common is squamous differentiation, which may in fact refer to the differentiation plasticity of normal urothelium, where squamous metaplasia of the trigon region of the bladder is quite common; interestingly, such squamous metaplasia may aggravate upon vitamin A deficiency. Up to 60% of MIBC cases may show focal squamous changes. The consensus is to diagnose these tumors as urothelial transitional carcinoma with squamous differentiation, and to reserve the diagnosis of squamous cell carcinoma to those rare cases in which squamous histopathology extends across the entire tumor (below 5% of cases). Likewise, focal glandular differentiation is also not entirely seldom (about 6% of MIBC cases), while pure adenocarcinoma is much rarer. There are a handful of additional histopathological variants (micropapillary, plasmocytoid, nested), all of which representing a diagnostic rarity (Chan and McConkey 2015; Kamat et al. 2016).

Assessing the underlying biology of urothelial carcinoma heterogeneity has only been possible with the advent of methods for characterizing complex transcriptomes. Landmark studies, for example, on diffuse large B-cell lymphoma and breast carcinoma, revealed that histopathologically uniform tumors can be classified into subtypes that clinically behave as distinct disease entities. Strikingly, differential expression of genes implicated in the normal differentiation of original cell types – that is, B-lymphocytes and mammary epithelial cells, respectively – turned out as a major discriminator, which allowed to identify processes of stemness and differentiation as crucial for intrinsic tumor subtypes (McConkey et al. 2015). Indeed, early studies in urothelial carcinoma transcriptome clearly demonstrated that NMIBC and MIBC are molecularly distinct disorders, and – notably within the context of this book – that transcriptomes of low- and high-grade urothelial carcinomas are very different. Several groups have independently approached the issue of MIBC intrinsic molecular subtypes, their results differing in terms of both the number of subtypes recognized and their nomenclature (Choi et al. 2017); within the context of this chapter, we will adopt the classification provided by The University of Texas M.D. Anderson Cancer Center. They identified two major subtypes, basal and luminal (Choi et al. 2014). Interestingly, this classification is remarkably reminiscent of (and also biologically substantiated, far beyond the issue of nomenclature) breast cancer molecular subtypes (Damrauer et al. 2014). Basal MIBCs are enriched for basal urothelial cell markers (CD44, P-cadherin, CK5, and CK14), EGFR, and squamous markers (CK6A, CK6B, CK6C, and CK16). Bioinformatic analysis of transcription factors revealed that p63, STAT-3, HIF-1, and NF-κB were involved. A fraction of basal MIBCs showed also upregulation of proteins responsible for epithelial-mesenchymal transition (EMT—Twist 1/2, Snai2, Zeb2, and vimentin). In contrast, luminal MIBC transcriptome was enriched for CK20 (a marker of umbrella cells, but also of CIS), CD24, FOXA1 (transcriptional activator of uroplakin genes), FGFR3, PPAR-γ, and GATA-3, the last two being the major transcription factors implicated. Luminal MIBC cases were also enriched for FGFR3 activating mutations, suggesting that they could correspond to progressed NMIBC. The analo-

gous analysis of NMIBC resulted in the description of three subtypes (Hedegaard et al. 2016), termed subtype 1, 2, and 3. Subtype 2 bears the highest risk of progression. This subtype featured upregulated expression of late cell cycle genes, a feature that also turned out to be shared with luminal MIBC. The expression of CK14 was moderately increased, and subtype-2 tumors were also enriched for a CIS signature. Very interestingly, the expression of other "notorious" basal cell markers (CD44 and SHH) was rather downregulated in subtype-2 NMIBC tumors, whereas a distinct set of (bladder) cancer stem cell genes – including aldehyde dehydrogenases, CD133, nestin, and CD90 – were clearly upregulated, as was the expression of EMT factors. The expression pattern of urothelial basal and stemness genes among the intrinsic subtypes thus provides another glimpse on the heterogeneity of urothelial cancer stem cells.

Finally, a third subtype of MIBC has been revealed, called p53-like (Choi et al. 2014), after its characteristic expression profile reminiscent of wild-type p53 response genes. p53 mutations were, nevertheless, as common in p53-like tumors as in the two other subtypes, making the mechanism of specific upregulation of p53-downstream genes in this subtype rather mysterious. Notably, p53-like tumors show chemoresistance, and even initially chemosensitive basal or luminal tumors may switch to the p53-like subtype when manifesting acquired resistance. Besides p53-downstream genes, p53-like tumor-specific gene expression profile includes genes suggesting an enhanced activity of stromal cells, especially carcinoma-associated fibroblasts (CAFs). Adding to the issue of the stem-cell heterogeneity, this subtype of tumors may thus correspond to "acquired stemness" due to epithelial-mesenchymal interaction, as issue well known from the biology of normal urothelial cells and of the urothelial regenerative response (see above).

Of course, the question surfaces as to whether these subtypes are stable, and if they are, then to what extent: Are they really intrinsic, or can they change during tumor progression? A quite frequent switch to the p53-like subtype in the wake of chemotherapy could provide an example of the latter possibility. Another example was published recently. Most CIS cases could be classified as the luminal subtype, and they frequently progress to the basal subtype (Barth et al. 2018). Interestingly, as described above, this "subtype switch" coincides with a "stemness switch," from Shh-dependent to Shh-independent. The question whether this is a mere coincidence or there is a mechanistic relationship between both these switches remains open.

## 8.4 Urothelial Carcinoma Stem Cells and Their Heterogeneity

As follows from the above discussion, urothelial carcinoma could be regarded as a stem cell disease. A link between a regenerative response – another stem-cell–dominated phenomenon – and urothelial carcinoma has been recognized a long time ago (reviewed in Hatina and Schulz 2012). As heterogeneous as urothelial

carcinoma—considered as a clinical disease—clearly is, its stem cells would be as well. We can approach this heterogeneity of cancer stem cells at several levels, of which the first one may well regard their origin. As suggested above, the urothelial carcinoma stem cells might be of dual origin. In muscle-invasive tumors, which – according to the current consensus – originate from basally located urothelial stem cells, cancer stem cells are probably directly derived from normal stem cells; a lot of stemness mechanisms can thus passively pass from their cell of origin. On the other hand, superficial papillary carcinomas are believed to derive from intermediate cells. They practically always develop, however, a morphological architecture quite reminiscent of normal urothelium, including a basal layer. This similarity extends from morphological resemblance to common antigenic determinants, as evidenced by early experiments from 1980s (Dotsikas et al. 1987). This might be taken to challenge the notion of intermediate cells being the universal cell of origin for these tumors. However, if we embrace this notion of the universal cell-of-origin, the only thinkable explanation for the existence of basal-like cells in NMIBC would be that the papillary carcinoma basal layer regenerates by dedifferentiation, probably under a crucial microenvironmental influence. Therefore, paradoxically, urothelial carcinoma stem cells might appear, at least at the level of expression of certain basal cell-specific proteins, more homogeneous than they really are (see below).

It follows that basal cell markers have been always considered as good candidates to approach urothelial carcinoma stem cells. CD44 was the first (Chan et al. 2009), and it turned out to be applicable for only a fraction of tumors, but this fraction, in which $CD44^+$ cells marked serially tumorigenic (=cancer stem) cells included a single pTa tumor as well, corroborating the conclusion above. The problem with CD44 might be that its expression might become uncoupled from stemness in a large proportion of NMIBC cases, where it can become ubiquitous, or the opposite may be true and it can be entirely lost, as is rather frequent in MIBC (Sugino et al. 1996). Anyway, concentrating on those tumors, where CD44 marks probable urothelial carcinoma stem cells, a pronounced heterogeneity as to the molecular mechanism of stemness preservation has been noticed. About 5% of cancer samples had activated β-catenin in their $CD44^+$ cells, 20% expressed nuclear Bmi-1, 40% activated nuclear STAT-3, and 80% GLI-1, a transcription factor downstream of SHH. It is clear, however, that $CD44^+$ urothelial CSCs represent just one subset. Independently, another basal stem cell marker, 67 KDa laminin receptor (67LR) has been used (He et al. 2009) and comparison of specific gene expression profiles identified between $CD44^+$ and $CD44^-$ cells on one hand and $67LR^{bright}$ and $67LR^{dim}$ cells on the other hand clearly showed that these represent different stem cell subsets (Dancik et al. 2014), adding to the molecular heterogeneity discovered within $CD44^+$ cells.

With the discovery and characterization of the intrinsic subtypes of both NMIBC and MIBC (see above), another level of stem-cell heterogeneity became apparent. Strikingly, the defined intrinsic subtypes feature widespread expression (i.e., so abundant that it becomes characteristic for bulk tumors, not just in cancer stem cells after a specific enrichment procedures) of certain stemness genes. Basal MIBCs thus show the abundant expression of basal stem cell markers (CD44, P-cadherin,

CK5, and CK14) (Choi et al. 2014). If we accept the current view that a large proportion of luminal tumors derive from cluster 2 NMIBCs, similarly abundant expression of a completely different stemness genes (ALDH1A1, ALDH1A2, CD133, nestin, and CD90) (Hedegaard et al. 2016) becomes manifest. Does the widespread expression of these specific groups of stemness genes in the specific intrinsic subtypes result from stem cell expansion? Or does it rather reflect the uncoupling of expression of those genes from (still rare) urothelial cancer stem cells?

A special case of a stemness mechanism might be provided by CIS. As described above, CIS stem cells seem to be uniquely dependent on the Shh signaling. Could this explain a high proportion of $CD44^+$ urothelial cancer stem cells relying on active GLI-factors? At best for a minor part probably, as CIS lesions were not among the tumors from which $CD44^+$-urothelial CSCs were isolated and molecularly analyzed; we can perhaps only admit the simultaneous existence of CIS and a full-blown carcinoma, clinically not too rare a situation (McConkey et al. 2010). Consequently, alternative mechanisms explaining the activation of the GLI-transcription factor should be provided. For example, it has been shown that the Hedgehog pathway can be activated by chronic arsenic exposure, a well-known urothelial carcinoma carcinogen (Fei et al. 2010). The activation of nuclear STAT-3 might directly result from a stromal influence. It has been reported that urothelial cancer stem cells (in this case defined as $CD14^+$ cells) are able to actively recruit myeloid cells and promote their differentiation into tumor-associated macrophages (TAMs) (Cheah et al. 2015). TAMs signal back to cancer cells by multiple mechanisms, including the secretion of inflammatory cytokines like Interleukin-6, a known activator of STAT-3 and a factor of adverse clinical prognosis in bladder cancer (Chen et al. 2013). Even arsenic, again, could be a signal to stimulate IL-6 secretion (Luo et al. 2013). Another possible source of a signal potentially leading to STAT-3 activation could be EGFR, which is overexpressed in basal-subtype MIBC (Choi et al. 2014).

A special example of microenvironmental promotion of urothelial carcinoma stem cells can be seen with certain primary chemoresistant tumors (Kurtova et al. 2015). The chief source of the stemness-promoting signals in this case, however, is not the stromal cells but the very urothelial carcinoma cells, somewhat reminiscent of the EGF-mediated regenerative response discussed above. Well-preserved cell hierarchy plays an essential mechanistic role. Cancer stem cells are endowed with specific mechanisms of self-protection, some of which have been also experimentally exploited to purify such cells, for example, the side population assay targeting the multidrug resistance efflux pumps or Aldefluor assay® targeting aldehyde dehydrogenases (Hatina et al. 2018). Consequently, as an immediate effect of chemotherapy, CSCs survive and non-CSCs begin to die. This death of non-CSCs is accompanied by the activation of a specific gene expression program, the wound response signature. One of the prominent genes activated is *PTGS2*, coding for cyclooxygenase-2 (COX-2), the enzyme catalyzing the synthesis of prostaglandin E2. PGE2 is consequently released from the dying cells, and it signals back to the surviving CSCs, stimulating their rapid entry into cell cycle. A malicious corollary

is that the dying cells are immediately replenished by the progeny of surviving and proliferating stem cells, an elegant variation of the regenerative response discussed above, misused by the cancer to keep the cancer cell population largely constant; the tumor thus behaves as primarily refractory. One of the magics of this discovery is that the pharmacological inhibition of this specific cancer regenerative response is feasible: Celecoxib is an FDA-approved drug to inhibit COX-2, and in experiment it has been able to essentially thwart this repopulation-mediated chemoresistance, thus calling for expedite translational and clinical development (Kurtova et al. 2015).

Additional mechanisms of stemness preservation and promotion within the context of chemotherapy have been described. One attractive candidate is the Hippo-pathway downstream transcription factor YAP-1. In fact, YAP-1 has been found activated (i.e., nuclear) in a great proportion of chemoresistant urothelial carcinomas (Ciamporcero et al. 2016), and it promotes cancer stemness by directly activating SOX-2 (Ooki et al. 2017); indeed, SOX-2 has been independently described as a specific factor responsible for stemness in Aldefluor-bright cells of invasive urothelial carcinoma cell lines (Ferreira-Teixeira et al. 2015). Interestingly, the COX-2–PGE2 signaling seems to independently activate SOX-2 as well, via a let-7-miRNA-HMGA2 pathway, well characterized, for example, in sarcomas (Hatina et al. 2019): the PGE2 signal leads to the downregulation of the *let-7* miRNA gene, resulting in the derepression of HMGA2 mRNA and the subsequent activation of the *SOX-2* gene. The COX-2-PGE2 and YAP signaling pathways are connected by an intricate feedback loop, nonetheless. On the one hand, the *PTGS2* gene coding for COX-2 is the YAP-1 downstream gene. On the other hand, YAP-1 is characterized by a pronounced antiapoptotic effect—which apparently could, on its own, mediate chemoresistance (Ciamporcero et al. 2016)—but, at the same time, this apoptosis inhibition limits the PGE2 signal (PGE2 is only released from dying cells) (Ooki et al. 2017). To make the things even a little bit more complicated, the COX-2-PGE2 signaling seems to independently activate STAT-3, too (Liu et al. 2016), and both COX-2-PGE2 signals and SOX-2 seem to be also activated by arsenic (Ooki et al. 2018). The molecular biology of urothelial carcinoma stem cells is thus coined by two recurrent themes: heterogeneity and signaling convergence.

A very special case of urothelial cancer and CSC heterogeneity is provided by the sexual dimorphism of the disease. Urothelial carcinoma is about four times more common in men than in women, yet the mortality is comparable, implying that women tend to have more aggressive disease (Kamat et al. 2016). A special biological mechanism has been recently proposed to explain these differences, providing another variation on the theme of signaling pathways convergence. The key has been provided by several mouse models that could reproduce various aspects of this sexual dimorphism. One of them was based on urothelial-specific knockout of the *Foxa1* gene; FOXA1 is a transcription factor essential for urothelial differentiation, participating in the transcriptional activation of uroplakin genes, whose loss and/or mutation portend an unfavorable prognosis to bladder cancer patients (Reddy et al. 2015). Interestingly, a close relative, FOXA2 – whose expression is typical for P-cells during embryonic urothelial development (see above) and which is no more

detectable in adult urothelium – is occasionally reactivated in urothelial carcinoma, a phenomenon that could be tentatively called "FOXA-switch." *Foxa1* urothelial-specific deletion resulted in umbrella cell damage, which could initiate a regenerative response, leading to different outcomes in male and female mice: basal cell hyperplasia in the former and keratinizing squamous metaplasia in the latter. Gene expression profiling and subsequent bioinformatic analysis to reveal upstream regulatory factors pertinent for the sex-specific divergent lesions yielded β-catenin as a master gene regulatory factor for male-specific basal cell hyperplasia and p63 for female-specific squamous metaplasia (Reddy et al. 2015). Both could be regarded as stemness regulators, providing an unexpected and unique example of stem-cell heterogeneity in urothelial precancerous lesions. Recall that both of them are involved in various aspects of urothelial development and regenerative response. Strikingly, the urothelial basal cell-specific expression of a constitutively active form of β-catenin resulted in hyperplasia followed by the development of low-grade papillary tumors, with a strong male-specific predilection (Lin et al. 2013). Finally, androgen-activated androgen receptor (AR) has been found as a transcription factor closely cooperating and directly physically interacting with β-catenin (Li et al. 2013; Lin et al. 2013). Plausibly, the constant AR-mediated moderate activation of β-catenin in males could lead to an increased overall frequency of urothelial hyperplasia, some of the hyperplastic lesions transforming to papillary carcinoma. This could explain the more frequent but at the same time less aggressive disease in males. Androgen-deprivation therapy could thus constitute a biologically substantiated therapeutic option to reduce recurrence rates in male bladder cancer, as recently proposed (Izumi et al. 2014).

Returning to the urothelial-specific *Foxa1* knockout mice, they could be quite instrumental in another aspect of urothelial (cancer) stemness analysis. We have noted that high molecular weight cytokeratins (especially CK5 and CK14) could be regarded either as stem cell markers or as squamous-metaplasia markers (see above the legend to Fig. 8.1). Sexually dimorphic phenotypes of *Foxa1* urothelial-specific deletion illustrate these two aspects brilliantly. In male mice, CK14 expression is strictly restricted to basal cells of hyperplastic lesions, implying CK14 as a stem cell marker. In sharp contrast, in female lesions CK14 is expressed throughout the entire thickness of the transformed urothelium, implying CK14 as a marker of squamous metaplasia in this case (Reddy et al. 2015).

## 8.5 Urothelial Cancer Stem Cell Plasticity

The issue of cancer stem cell heterogeneity becomes even more complicated if we invoke the issue of cancer cell plasticity. As discussed above, cancer stem cells are intimately linked to a particular cancer type and its stage of progression—recall the unique biology of CIS-stem cells, which directly depend on the Sonic hedgehog signal. Moving further along the cancer progression pathway, epithelial-to-mesenchymal transition (EMT) becomes the dominant theme in cancer cell

plasticity. EMT represents an embryonic developmental program, reactivated, for example, as part of wound healing process as well as during cancer invasion and metastatic dissemination. Polarized stationary epithelial cells transform into motile mesenchymal-like cells that disengage from the (primary) tumor cell mass, invade the surrounding tissue structure, eventually reaching a blood or lymphatic vessel, enter it (intravasation), and leave it again at a secondary site (extravasation). A complex gene expression program dominates EMT, governed by a group of transcription factors, whose prominent role is to repress the *CDH1* gene coding for the principal epithelial cell adhesion molecule E-cadherin: Twist-1 and -2, Zeb-1 and -2, Slug, Snail or Prrx1. The activated genes include vimentin and especially genes underlying single-cell motility and invasiveness. There is an elegant negative feedback loop mediated by the cluster of miRNA200: These miRNAs directly target Zeb-EMT-factors, thus promoting epithelial phenotype, and the Zeb represses the expression of these miRNA genes, thus keeping EMT running. A similar effect to miRNA200 has miRNA205, except that miRNA205 does not take part in this feedback regulation. Most carcinoma metastases display overly epithelial phenotypes, however, implying an opposite transformation process (mesenchymal-to-epithelial transition, MET) once a disseminated tumor cell reaches the secondary site and is about to resume growth to establish a clinically important metastasis (Brabletz 2012).

A crucial question within the context of this chapter is: What happens with cancer stem cells along this EMT-MET cascade? During the last decade, very controversial results have been obtained (Hatina 2012). Originally, Twist-1–activated EMT has been reported to directly connect with cancer stemness in breast cancer, thus promoting these two crucial cancer phenomena at once. This mechanism seemed to be valid also for other tumor types, supporting the earlier formulated concept of migrating cancer stem cells (Mani et al. 2008; Lehmann et al. 2016). More recently, an exact opposite has been found for Prrx1-dominated EMT in breast cancer, too, where stemness (measured, like in the Twist-1 experiment above, by sphere formation, self-renewal capacity, and the typical breast cancer stem cell marker profile $CD44^{high}$ $CD24^{low}$) appeared antithetical to EMT and invasion (Ocaña et al. 2012). A great compatibility with the latter model has been convincingly demonstrated in urological tumor cell lines (both prostate and urothelial carcinoma) (Celià-Terrassa et al. 2012). Notably, there seems to be an intrinsic molecular connection between pluripotency genes and E-cadherin, and moreover, there also seems to be an intrinsic molecular antagonism between EMT-factors (Snail, Twist, and Zeb) and stemness. Invasive and motile cells produced only slowly growing and metastasis incompetent tumors whereas inoculating epithelial cell variants of the same cell lines resulted in an efficient metastatic spread. Likewise, knockdown of E-cadherin or of pluripotency factors resulted in a dramatic increase in motility and invasiveness, with a simultaneous decrease or loss of self-renewal and metastatic competence. The same was true upon overexpression of EMT-factors, with the simultaneous repression of both E-cadherin and pluripotency genes. Knockdown of EMT-factors, as expected, activated both epithelial character and stemness, both contributing to increased metastatic competence.

This study could certainly be reproached in various ways. First, stemness genes focused on involved pluripotency factors characterized in embryonic stem cells and induced pluripotent stem cells (OCT-4, SOX-2, Nanog, KLF-4 and -9, LIN28A). However, the analysis of molecular mechanisms of stemness of $CD44^+$ urothelial carcinoma stem cells failed to provide evidence of a major role of these pluripotency factors (Chan et al. 2009), and only SOX-2 was later identified as a factor underlying a fraction of invasive urothelial CSCs (Ferreira-Teixeira et al. 2015), see above. On the other hand, the analysis of a clinical impact of expression of *p63*, a typical MIBC stemness gene (represented by a specific isoform ΔNp63) largely corroborated this concept: Expression of p63 significantly positively correlated with E-cadherin expression and significantly negatively correlated with Zeb-1 and -2 EMT-factors. Importantly, tumor samples with high p63/E-cadherin expression had especially quick lethal outcomes (median overall survival of 8 months, compared with 27 months in the low p63 expression group) (Choi et al. 2012).

Another reproach could be that the results on mirror-image-like dichotomy between motility and invasion, on the one hand, and stemness and metastatic competence, on the other hand, were arrived at by largely using cell lines that were stably genetically manipulated and in which either phenotype (epithelial metastatic or motile, invasive, and poorly metastatic) has been hard-wired into the genome by the genetic manipulation implemented. As we introduced above, however, the EMT and MET processes are largely plastic in real tumors, that is, the changes are only temporary and, by inference, predominantly epigenetically determined or microenvironmentally induced. Indeed, the authors showed that coculture of both "extreme" and genetically fixed prostate cancer cell sublines (a motile and invasive subline with suppressed stemness, and an epithelial metastatic subline with high stemness and low motility, respectively) induced in the latter a transitional EMT, allowing for temporary motility at the expense of decreased stemness, which could be resumed once the cells reached the secondary site.

The above model could indeed be quite realistic. By a serendipity, we established a rather similar experimental model for urothelial carcinoma as well, with a derivative RT-112 cell line (Fig. 8.2). Strikingly, our model does not rely on any genetic manipulation at all, so we can deliberately switch between the epithelial and EMT-like phenotypes, just by modifying cell density. This switch can proceed rapidly (5–7 days), implying that the cells have to be permanently simultaneously preprogrammed to enter either direction according to the momentary signals. Importantly, it seems that there is no black-and-white picture in the relationship between stemness and EMT, implying that both epithelial and EMT states may preserve stemness, but with different underlying molecular mechanisms. Indeed, one of the characteristic features of the basal MIBC intrinsic subtype is the simultaneous overexpression of both stemness and EMT markers. At the level of gene regulation, a peculiar phenomenon explaining this type of rapid plasticity is a poised chromatin conformation, at the same moment bearing repressive and activating chromatin marks at the crucial loci (Chaffer et al. 2013).

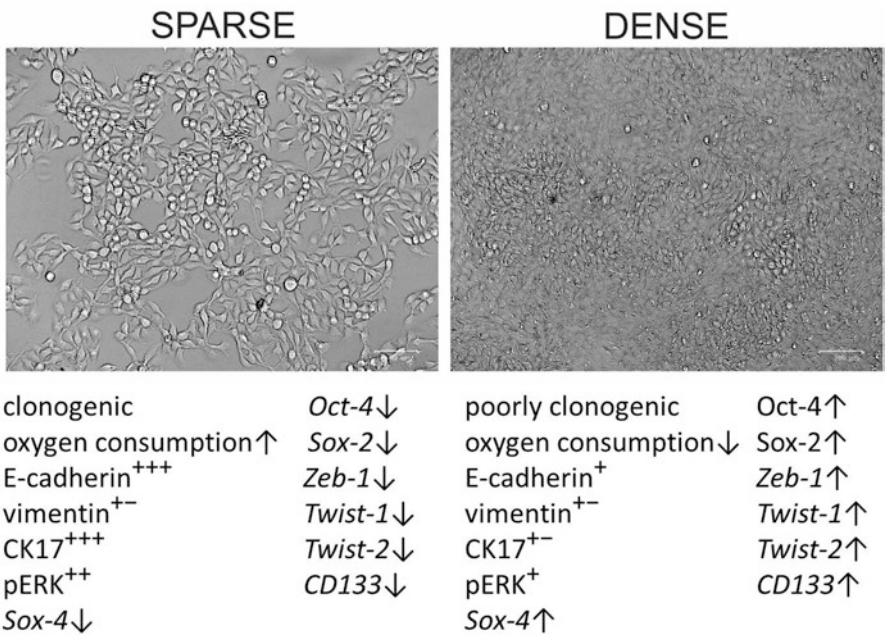


**Fig. 8.2** Density-dependent plasticity of RT-112-derivative cell line. Notice the mixed profile of stemness-related traits and stemness markers in both culture conditions

## 8.6 Epigenetic Regulation of Urothelial Carcinoma Stem Cells

We have already had the privilege to meet an epigenetic mechanism that hits urothelial stem cells really at the heart: According to all indices, the knockout of the gene coding for DNA methyltransferase 1 in the urothelium is able to target stem cells in the developing urothelium, and therefore a very special regenerative response must be resorted to, namely recruiting and reprogramming (stem) cells from the Wolffian duct (Joseph et al. 2018), see above. This particular epigenetic regulation seems also to be valid for CSCs. Indeed, DNMT1 has been published as a negative prognostic marker for urothelial carcinoma and its knockdown in two urothelial carcinoma cell lines resulted in a similar effect to that described for the embryonic urothelium, namely the activation of the DNA damage response pathway (Wu et al. 2011). There is also another plausible mechanistic explanation for the involvement of DNMT1 in cancer stemness. An analysis performed in breast cancer confirmed a crucial causal role of DNMT1 for normal mammary and breast cancer stem cells. In addition, the transcription factor ISL-1, acting as a differentiation regulator in multiple cell lineages, has been identified as a crucial mediator,

whose gene promoter had to be hypermethylated in order to preserve stemness (Pathania et al. 2015). Importantly, ISL-1 is also essentially involved in urogenital development, its mutations predisposing to classic bladder exstrophy (Zhang et al. 2017). Moreover, the hypermethylation of ISL-1 portends a significant adverse prognosis for NMIBC, predicting both recurrence and progression (Kitchen et al. 2015). These observations make the mechanism discovered for breast cancer stem cells well worth testing in urothelial carcinoma.

As previously mentioned, urothelial carcinoma is also remarkable for its high frequency of mutations in chromatin modifiers, particularly KDM6A in NMIBC and MLL2 in MIBC, both mutations promoting a repressive chromatin conformation and acting thus in the same direction as does DNMT1. While these mutational changes are per definition common to all cancer cells, a specific stem cell epigenetic mechanism has been discovered recently. Another chromatin-modifying enzyme, leading to a repressive chromatin conformation and acting as H3K9 methyltransferase – KMT1A – has been found specifically overexpressed in urothelial carcinoma stem cells. Its immediate target in promoting cancer stemness is the promoter of the gene coding for the transcription factor GATA-3, which itself acts here as the direct transcriptional repressor of the STAT-3. By repressing GATA-3 (via grafting the H3K9me3 repressive chromatin marks on its promoter), KMT1A thus indirectly activates STAT-3 expression and promotes cancer stemness (Yang et al. 2017). Recall that GATA-3 belongs to transcription factors identified as master regulators of the luminal MIBC subtype, whereas STAT-3 enjoys a similar position for basal MIBC. The just described mechanism could thus underlie the luminal-to-basal switch during urothelial cancer progression. Moreover, if we accept the direct succession of luminal MIBC from cluster 2 NMIBC (see above) that seems to be dominated by an entirely different set of stemness genes (ALDH1A1, ALDH1A2, CD133, nestin, and CD90 – Hedegaard et al. 2016), see above, then we could reconstruct the urothelial carcinoma progression pathway in terms of a stemness switch. Plausibly, in luminal MIBC, the stemness molecular mechanisms are largely inherited from cluster 2 NMIBC, and the basal stemness molecular mechanisms are actively repressed by GATA-3. Luminal-to-basal switch would then be carried out epigenetically, via the KMT1A histone methyltransferase. At present, however, this scenario remains a hypothesis.

To make even this aspect a little more complicated, a completely inverse mechanism incorporating certain biological aspects of CSCs has been discovered very recently (Puig et al. 2018). Due to their quiescence, a small proportion of cancer cells across all tumor types behave as label-retaining cells. Classic in detecting stem cells, this approach was also applied for localizing urothelial stem cells among basal cells as well, as discussed above (Kurzrock et al. 2008). In cancer, these label-retaining cells or otherwise called slow-cycling cancer cells display a very specific gene expression profile. It combines, among the activated genes, certain stemness genes (especially for pluripotency factors) and genes coding for disparate detoxification and chemoresistance mechanisms and, among the repressed genes, the major part of cell cycle progression genes and DNA-replication genes, as well as genes responsible for energy metabolism. A crucial regulator of this specific phenotype is

TET-2, catalyzing 5-methylcytosine oxidation to 5-hydroxymethylcytosine. This enzymatic reaction ushers the DNA-demethylation sequence, thereby acting in a completely opposite way to DNA-methyltransferases. Indeed, the level of 5-hmC portends a very adverse prognostic significance, especially for chemotherapy-treated patients. Although the survival analysis has been performed for colorectal carcinoma patients, quite likely it is also relevant for urothelial carcinoma, as bladder cancer represents a cancer type with the third highest proportion of high-5-hmC cases.

Obviously, the label-retaining cells, due to both their quiescence and a plethora of chemoresistance mechanisms expressed, cannot be eliminated by chemotherapy. These are exactly the cells that initiate cancer relapse, even years after an apparent cure, a picture well-known across all tumor types. Up to this point, label-retaining cells behave just like typical cancer stem cells. Thus, many results in the scientific literature describing the behavior of CSCs upon chemotherapy almost certainly actually addressed, at least in part, these cells. There is, nevertheless, one tremendous difference between true cancer stem cells on one hand and label-retaining or slow-cycling cancer cells on the other hand. The latter namely lack a self-renewal capacity. Slow-cycling cells yielded slow-cycling progeny with the same frequency as did rapidly proliferating cells (Puig et al. 2018). Slow cycling is thus an operational category, co-opting certain stemness traits and characterized by an extreme plasticity. Discovering their underlying biological mechanism opens an avenue for their pharmacological inhibition, with a potentially tremendous therapeutic impact.

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# Chapter 9
# Acute Myeloid Leukemia Stem Cell Heterogeneity and Its Clinical Relevance


**Theodoros Karantanos and Richard J. Jones**

**Abstract** The failure of complete remissions to reliably translate into cures in acute myeloid leukemia (AML) can be explained by the leukemia stem cell (LSC) paradigm, which hypothesizes that rare leukemia cells with stem cell features, including self-renewal capacity and drug resistance, are primarily responsible for both disease maintenance and relapses. Traditionally, the ability to generate AML in immunocompromised mice were how these so-called LSCs were identified. Only those rare AML cells characterized by a hematopoietic stem cell (HSC) $CD34^+CD38^-$ phenotype were believed capable of generating leukemia in immunocompromised mice, but more recently, significant heterogeneity in the phenotypes of engrafting AML cells has been demonstrated. Moreover, AML cells that engraft immunocompromised mice do not necessarily represent either the founder clone or those cells responsible for relapse. A recent study found that the most immature phenotype present in an AML was heterogeneous, but correlated with genetically defined risk groups and outcomes. Patients with AML cells expressing a primitive HSC phenotype ($CD34^+CD38^-$ with high aldehyde dehydrogenase activity) manifested significantly lower complete remission rates, as well as poorer event-free and overall survivals. AMLs in which the most primitive cells displayed more mature phenotypes were associated with better outcomes. The strong clinical correlations suggest that the most immature phenotype detectable within a patient's AML might serve as a biomarker for "clinically relevant" LSCs. The minimal residual disease state during first remission may be the optimal setting to study novel LSC-targeted therapies, since they may have limited activity against the bulk leukemia and will be utilized at lowest tumor burden as well as least tumor heterogeneity.

**Keywords** Acute myeloid leukemia · Leukemia stem cells · Heterogeneity · CD34 · CD38 · Aldehyde dehydrogenase · CD33 · CD123 · CLL-1

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## 9.1 Introduction

Acute myeloid leukemia (AML), the most common acute leukemia in adults (Jemal et al. 2010), is a clonal disorder defined by the accumulation of abnormally differentiated myeloid cells in the bone marrow. Despite the introduction of novel therapeutic approaches and significant improvements in the long-term outcomes of patients over the past two decades, AML remains a disease with an overall poor prognosis (Dohner et al. 2015; Maynadie et al. 2013); this includes high recurrence rates even in patients who achieve complete remission (CR) after induction chemotherapy (Dohner et al. 2015; Maynadie et al. 2013; Lowenberg et al. 1999). AML is a disease with complex molecular and genetic heterogeneity associated with discordant responses to chemotherapy and allogeneic blood or marrow transplantation (alloBMT) (Yanada et al. 2018; Dohner et al. 2017; Medeiros et al. 2015). However, even patients categorized in the same risk group based on their cytogenetic/molecular profiles can have highly variable outcomes (Dohner et al. 2017; Grimwade et al. 1998). The recent improvements in the molecular profiling have contributed to a better stratification of the AML patients especially in the genetically defined "intermediate-risk" group (Dohner et al. 2017; Yanada et al. 2005; Preudhomme et al. 2002; Suzuki et al. 2005). However, the persistently high relapse and death rates even in the genetically defined favorable risk group, outside of acute promyelocytic leukemia, render a better understanding of the clonal development and cellular biology of this disease, a necessity to improve clinical outcomes.

Most importantly, better understanding of the heterogeneity of AML, especially within genetically defined risk groups, should improve the prognostication of patients as well as allow earlier introduction of novel therapeutic approaches. Subclonal progression within the leukemic cell burden almost certainly explains a substantial part of the differences in AML outcomes within genetically defined subtypes (Grove and Vassiliou 2014). However, there is evidence that AML arises in various stages of hematopoietic differentiation (Klco et al. 2014), and this can contribute to the heterogeneity of the disease. Rare leukemia-initiating cells, often called leukemia stem cells (LSCs), are thought to be responsible for the maintenance of the disease as well as relapse as a result of their resistance to traditional therapies that are active against the bulk leukemia cells (Lapidot et al. 1994; Yanagisawa et al. 2016). Many recent studies have suggested that the phenotype of LSCs themselves is heterogeneous, and this is associated with differing clinical outcomes even within the same genetically defined risk groups (Yanagisawa et al. 2016; Pearce et al. 2006; Sarry et al. 2011; Quek and Otto 2016; Taussig et al. 2010; Gerber et al. 2016; Martelli et al. 2010; Dao et al. 2003; Goardon et al. 2011). Better understanding of this heterogeneity appears to be critical for the development of new strategies to target and potentially eliminate LSCs. In this chapter, we will discuss the recent evidence supporting the clinical relevance of LSC heterogeneity, as well as the novel approaches to target these cells in order to improve the outcomes of AML.

## 9.2 Phenotypic Heterogeneity of LSCs

The first clear evidence suggesting that leukemia arose from a primitive hematopoietic progenitor was published a half century ago, when Fialkow et al. demonstrated clonal hematopoiesis involving both the erythroid and myeloid lineages in patients with chronic myeloid leukemia (Fialkow et al. 1967). Later studies showed that the cells responsible for AML engraftment in NOD/SCID mice (Bonnet and Dick 1997) and long-term culture-initiating activity in vitro (Ailles et al. 1997; Blair et al. 1997) were positive for CD34 and negative for the expression of lineage markers such as CD38. Further studies supported the finding that virtually all LSCs reside in the $CD34^+CD38^-$ fraction (Kreso and Dick 2014).

However, others have found that AML cells of various differentiation phenotypes, including $CD34^+CD38^+$ and $CD34^-$, are capable of engrafting immunocompromised mice even within the same patient (Sarry et al. 2011). Interestingly, $CD34^-$ LSCs were demonstrated to have a distinct expression profile from $CD34^+$ LSCs, and this profile was enriched for a granulocyte-macrophage precursor signature (Quek and Otto 2016). Consistent with these data, Taussig et al. found that LSCs are $CD34^-$ in most nucleophosmin 1 (NPM1) mutated AML samples (Taussig et al. 2010). $CD34^+$ LSCs were generally only found in NPM1 mutated AML that contained a high number of $CD34^+$ blasts, and these AMLs additionally had higher incidence of FLT3-ITD mutations (Taussig et al. 2010). Data from our group also confirmed that LSCs in NPM1-mutated AML can be either $CD34^+$ or $CD34^-$ (Gerber et al. 2016).

## 9.3 Clinical Relevance of LSC Heterogeneity

Most of the above studies utilized engraftment of AML cells in immunocompromised mice as the gold standard measure of LSC activity. However, Pearce et al. showed that AML cells from only about half of the patients studied engrafted in NOD/SCID mice, and increasing cell dose or more permissive recipient strains did not increase the engraftment ability (Pearce et al. 2006). Interestingly, the overall survival was worse in patients whose AML engrafted in NOD/SCID mice. Thus, the ability of AML to engraft in NOD/SCID mice appeared to correlate with prognosis (Pearce et al. 2006).

Although several groups reported that the frequency (Terwijn et al. 2014; Vergez et al. 2011), or engraftability (Pearce et al. 2006), of $CD34^+CD38^-$ LSCs correlated with prognosis, many studies have questioned the clinical relevance of LSCs as classically defined by their ability to engraft immunocompromised mice. In fact, a recent study showed that AML cells that engrafted immunocompromised mice may not represent either the founder clone or those responsible for relapse (Klco et al. 2014). Thus, the mouse engraftment assay may reflect more accurately the proliferative potential of the leukemic cells and/or their interactions with the mouse microenvironment (Rombouts et al. 2000) than it does their role in disease maintenance and

relapse. These data, together with the fact that no AML subset in many patients will engraft immunocompromised mice (Pearce et al. 2006), suggest that other means for LSC identification are needed to allow them to be studied in a clinical setting.

Regardless of their phenotype or tumorigenic potential in immunocompromised mice, leukemic cells that persist after therapy [i.e., minimal residual disease (MRD)] are likely the most clinically important. Our group studied the clinical significance of the most primitive hematopoietic phenotype carrying an AML's genetic signature based on our finding that MRD is highly enriched for that primitive leukemic phenotype (Gerber et al. 2016; Kreso and Dick 2014; Gerber et al. 2012). We found that the most immature phenotype of AML was heterogeneous in terms of CD34, CD38, and aldehyde dehydrogenase (ALDH) expression (Gerber et al. 2016). As others showed, most AMLs harbored $CD34^{+}CD38^{-}$ cells (Gerber et al. 2016). In about a third of AMLs, the most primitive leukemic phenotype found was indistinguishable for primitive hematopoietic stem cells (HSCs): $CD34^{+}CD38^{-}$ and high expression of ALDH ($ALDH^{high}$); further, most of these AMLs harbored poor-risk cytogenetics or FLT3 internal tandem duplications (ITDs). $CD34^{+}CD38^{-}$ AML cells with intermediate ALDH expression ($ALDH^{int}$) were the most immature phenotype found in all core-binding factor and most intermediate-risk AMLs. The most immature phenotype in the most favorable AMLs, NPM1 as a single mutation and acute promyelocytic leukemia (APL), was usually $CD34^{+}CD38^{+}$ or $CD34^{-}$ (Gerber et al. 2016).

Not surprisingly given the strong association with poor-risk genetics, patients harboring AML cells with a primitive HSC phenotype ($CD34^{+}CD38^{-}ALDH^{high}$) displayed significantly lower event-free and overall survivals (Gerber et al. 2016). Patients whose most immature AML cells were $CD34^{-}$ displayed the best event-free and overall survivals, as others have also described (Zeijlemaker et al. 2015). Patients whose most immature AML cells had a $CD34^{+}CD38^{-}ALDH^{int}$ phenotype showed an intermediate prognosis. Thus, despite demonstrating substantial heterogeneity overall within AML patients, the most immature AML phenotypes were much more consistent within individual genetically defined risk groups (Gerber et al. 2016), and importantly correlated with outcomes.

The strong clinical correlations suggest that the most immature phenotype detectable within a patient's AML might serve as a biomarker for "clinically relevant" LSCs. Moreover, the phenotype of the LSC may be a function of the stage of hematopoietic differentiation at which the leukemogenic mutation develops. As normal $CD34^{+}CD38^{-}ALDH^{high}$ HSCs differentiate into more committed progenitors, both CD34 and ALDH expression decrease, while CD38 expression increases (Fig. 9.1a) (Gerber et al. 2016). In addition, the expression of resistance mechanisms (e.g., quiescence, efflux pumps, and detoxifying enzymes) also decreases with differentiation. The most favorable AMLs appear to arise from more differentiated progenitors ($CD34^{-}$) and the least favorable from primitive HSCs ($CD34^{+}CD38^{-}ALDH^{high}$) (Fig. 9.1b–d). The differentiation state of the AML's cell of origin also appears prognostic within genetically defined risk groups. Some NPM1-mutated AMLs and APLs appear to arise from $CD34^{+}$ progenitors and they appear to do worse than the more common $CD34^{-}$ varieties of these AMLs (Gerber et al. 2016).

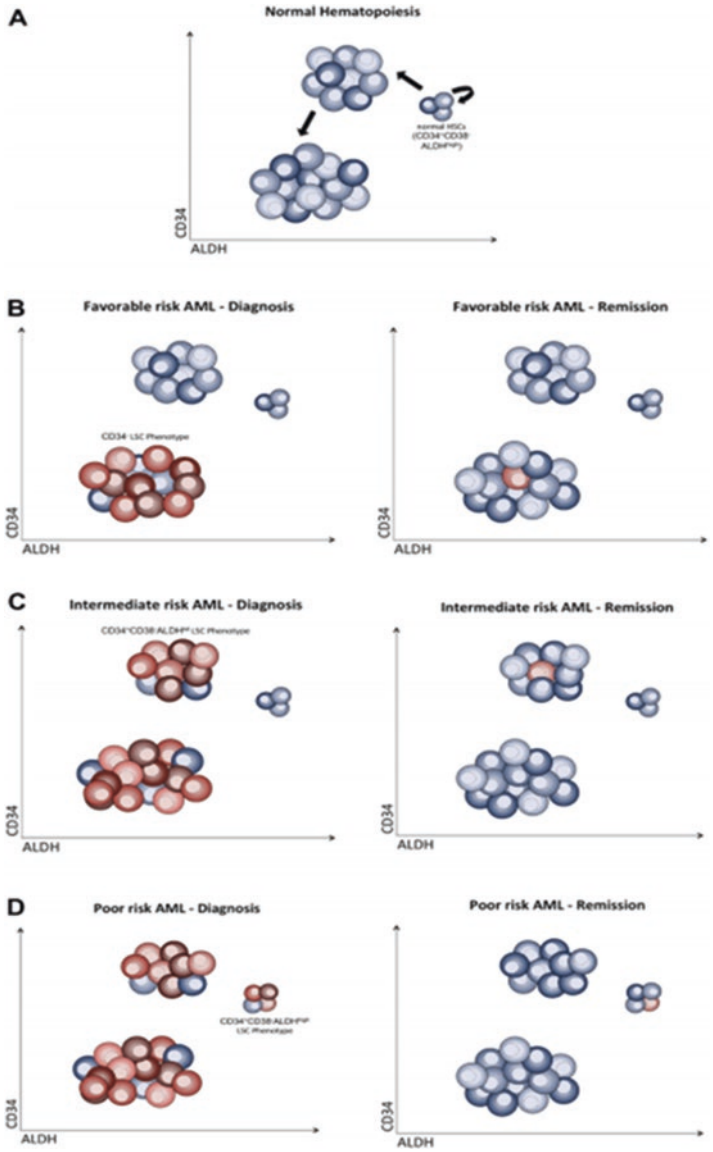


**Fig. 9.1** LSC heterogeneity as a function of the stage of hematopoietic differentiation at which the leukemogenic mutation develops. (**a**) As normal CD34+CD38–ALDH$^{high}$ HSCs (blue) differentiate into more committed progenitors, both CD34 and ALDH expression decreases. LSCs (red) are phenotypically heterogeneous, with the most favorable AMLs arising from more differentiated progenitors (CD34–) (**b**), intermediate-risk AMLs from less differentiated CD34+CD38–ALDH$^{int}$ progenitors (**c**), and the least favorable AMLs from primitive HSCs (CD34+CD38–ALDH$^{high}$) (**d**). At remission, MRD is enriched for the most immature phenotype present in the leukemia (i.e., LSCs). Reproduced from Yanagisawa B, Ghiaur G, Smith BD, Jones RJ. Translating leukemia stem cells into the clinical setting: Harmonizing the heterogeneity. Exp Hematol. 44(12):1130–1137, 2016

## 9.4 Characterization of Potential LSC Targets

In order for a LSC-based target to have clinical utility, it must not only be expressed on LSCs, but if co-expressed by any normal cells, it must also have an acceptable toxicity profile. Since studies have shown inconsistent expression of putative markers of LSCs (Quek and Otto 2016; Al-Mawali et al. 2016) probably at least in part a result of the heterogeneity of LSCs, it may also not be possible for one target to be effective for all AML patients. Although several cell-surface markers have been proposed as potential LSC-associated targets, none have yet to be definitively confirmed clinically.

### *9.4.1 CD33*

CD33 is a member of the sialic acid-binding immunoglobulin-like lectins (SIGLECS), a subset of the Ig superfamily molecules. CD33 is a myeloid marker expressed on normal hematopoietic progenitors through maturing granulocytic and monocytic cells. However, although still somewhat controversial, most data suggest that CD33 is not expressed on pluripotent HSCs (Pelosi et al. 2015). CD33 is expressed in the large majority of AMLs and usually its expression is higher on leukemic blasts than normal myeloid precursors. LSCs from some patients, but not all, have also been reported to express CD33 (Walter et al. 2012).

### *9.4.2 ALDH*

ALDH refers to a group of intracellular enzymes that share sequence homology, and are implicated in protein chaperone activity, as well as retinoids and reactive oxygen species (ROS) metabolism (Yang et al. 2017). Numerous studies have demonstrated that ALDH and particularly ALDH1 has a critical role in the biology of normal HSCs, affecting their interaction with bone marrow microenvironment, self-renewal capacity, differentiation, cellular detoxification, and drug resistance (Yang et al. 2018; Muramoto et al. 2010; Chute et al. 2006; Gasparetto et al. 2012; Singh et al. 2013). ALDH has also been involved in the development of leukemia since ALDH2 knockout in a Fanconi anemia mouse model led to the spontaneous development of acute leukemia (Langevin et al. 2011), while ALDH1A1 and ALDH3A1 deletion promotes the development of AML under NUP98-HOX10 homeodomain fusion protein treatment (Pearce et al. 2005). Despite these results suggesting that ALDH deletion could potentially promote the development of acute leukemia, high ALDH activity in AML blasts was associated with increased engraftment potential in NOD/SCID mice and worse clinical outcomes (Cheung et al. 2007). As already discussed, our group (Gerber et al. 2016; Gerber et al. 2012) and

many others (Pearce et al. 2005; Cheung et al. 2007; Ran et al. 2009; Ran et al. 2012; Hoang et al. 2015) have shown that ALDH can identify LSCs when combined with other markers.

### *9.4.3 CD123*

CD123 (IL-3 receptor alpha) is expressed in $CD34^+$ cells from various sources of hematopoietic cells including fetal liver, cord blood, peripheral blood, and bone marrow (Testa et al. 2014), and stimulation of the growth of these cells is associated with upregulation of CD123 (Sato et al. 1993). Numerous studies have evaluated the impact of CD123 expression in AML, highlighting its negative impact on clinical outcomes with AML overexpressing CD123 frequently associated with FLT3-ITD (Riccioni et al. 2009) and NPM1 mutations (Rollins-Raval et al. 2013). Several studies have focused on the role of CD123 expression in LSCs. Jordan et al. showed that $CD34^+CD38^-$ cells from most primary AML samples strongly expressed CD123 and engrafted in NOD/SCID mice, while $CD34^+CD38^-$ cells from normal marrow showed little of no CD123 expression (Jordan et al. 2000). According to a subsequent study by Testa et al., CD123 is overexpressed in 45% of AML patients but blasts with CD123 overexpression had more rapid cycling activity and showed resistance to apoptosis by growth factor deprivation along with upregulation of STAT5 signaling (Testa et al. 2002). These findings suggest that CD123 overexpression may not be uniform in AML, but it appears to be associated with stem cell features. More recently, Hwang et al. found that putative $CD34^+CD38^-CD123^+$ LSCs were detectable in about 75% of AML samples (Hwang et al. 2012) and Vergez et al. demonstrated that the proportion of these cells is predictive of progression free and overall survivals for patients with AML (Vergez et al. 2011). These data suggest that CD123 expression may be one of the most specific markers of LSCs.

### *9.4.4 CLL-1*

C-type lectin-like molecule-1 (CLL-1) is a transmembrane glycoprotein expressed only in the hematopoietic lineage and particularly in myeloid cells in the peripheral blood and marrow, but not in $CD34^+$ normal HSCs (Bakker et al. 2004). Bakker et al. demonstrated that CLL-1 is expressed in 92% of AML samples with 67% of $CD33^-$ AMLs expressing CLL-1 (Bakker et al. 2004). Subsequently, the same group showed that CLL-1 expression is present in the $CD34^+CD38^-$ compartment in 86% of AML samples with $CD34^+CD38^-CLL\text{-}1^+$ cells engrafting NOD/SCID mice with outgrowth of $CLL\text{-}1^+$ AML blasts (van Rhenen et al. 2007). More recently, Darwish et al. analyzed the expression of LSC markers in bone marrow samples from patients with AML and found that CLL-1 expression was strongly associated with poor prognosis (Darwish et al. 2016). These findings highlight that CLL-1 expression can distinguish LSCs from normal HSCs.

### *9.4.5 Other Potential LSC Markers*

CD96, also known as Tactile, is a member of Ig gene superfamily and was initially found to be expressed in activated T cells (Wang et al. 1992). In an early study, Hosen et al. found that CD96 was expressed at significantly higher levels in $CD34^{+}CD38^{-}$ AML cells compared to normal HSCs with significant variation of its expression among different AML samples (Hosen et al. 2007). Of note, only $CD96^{+}$ AML cells were capable of engrafting immunocompromised mice (Hosen et al. 2007), suggesting that CD96 is a marker associated with LSC features. More recently, Du et al. studied the expression of CD96 in 105 acute leukemia samples showing again significant variation of CD96 expression among AML samples and that patients with <10% expression of CD96 had higher rates of CR (Du et al. 2015). Similarly, Jiang et al. demonstrated that CD96 expression in $CD34^{+}CD38^{-}CD123^{+}$ LSCs was associated with shorter median survival (Jiang et al. 2017). Finally, Yabushita et al. showed that the combined expression of three LSC markers (CD25, CD123, and CD96) is associated with significantly decreased overall survival in patients with AML (Yabushita and Satake 2017). The don't eat me signal, CD47, was shown to be expressed on most primary AML specimens, bulk tumors, and LSCs compared with normal bone marrow HSCs, which expressed lower levels (Jaiswal et al. 2009; Majeti et al. 2009). Clinical trials targeting CD47 are just beginning.

CD25, also known as interleukin 2 receptor alpha (IL2Ra), is a type I transmembrane protein present on activated T and B cells and has been well described as a marker of regulatory T cells (Sakaguchi 2011). CD32, also known as FcγRII, is a surface receptor expressed in differentiated myeloid cells including monocytes, neutrophils, and dendritic cells and binds aggregated IgG (Warmerdam et al. 1992). Saito et al. showed that LSCs from patients with AML express either CD25 or CD32 or both, while normal HSCs maintained long-term multilineage hematopoietic capacity following depletion of those two surface antigens; thus, these markers appear to be possible candidates for targeting LSCs (Saito et al. 2010). Gonen et al. showed that CD25 positivity was correlated with adverse molecular findings such as FLT3-ITD and DNMT3A mutations (Gonen et al. 2012). CD25 expression was found to improve AML prognostication if added to the cytogenetic and mutational data, suggesting that it provides independent prognostic information for patients with AML (Gonen et al. 2012). Fujiwara et al. confirmed that CD25 is an independent marker of inferior rates of CR, worse PFS, and OS in AML patients older than 60 (Fujiwara et al. 2017) and Ikegawa et al. showed that CD25 expression on residual AML blasts after chemotherapy was associated with increased risk of relapse and worse OS for patients undergoing alloBMT (Ikegawa et al. 2016).

TIM-3 was initially identified as a molecule selectively expressed on IFN-γ-producing CD4+ Th1 and CD8+ T cytotoxic cells (Monney et al. 2002). Kikushige et al. demonstrated that TIM-3 is expressed in LSCs in most types of AML excluding APL, but not in normal HSCs (Kikushige et al. 2010). TIM-3 expression appears to distinguish LSCs from normal HSCs, but its expression is heterogeneous in

LSCs. Its expression by LSCs has also been associated with better outcomes and sensitivity to chemotherapy (Xu et al. 2017a). The latter finding is interesting and warrants further evaluation since the expression of most putative LSCs markers is associated with a worse overall prognosis.

## 9.5 Targeting LSCs in AML

The identification of markers which can distinguish LSCs from normal HSCs has led to the investigation of novel targeted therapies that can potentially eradicate the LSCs and at the same time spare normal HSCs in AML. Thus far, most of these targeted therapies have been primarily evaluated in the pre-clinical setting level. Moreover, as already discussed, the impact of targeting LSC antigens on the clinical outcomes of AML patients is probably going to be affected by the heterogeneity of LSCs, making it unlikely that one targeted therapy will be effective for all AML patients. Moreover, most clinical trials looking at the activity of new agents use response as an endpoint. Since response measures the bulk disease, it may either overestimate or underestimate the activity of the agent against LSCs. Prolonging leukemia-free survival should be the true clinical measure of LSC activity (Huff et al. 2006). Finally, the evaluation of these agents in the clinical setting will be critical for the identification of their side effects and off-target toxicity profiles.

Perhaps the best clinically studied putative LSC marker is CD33. Therapy targeting $CD33^+$ cells did show efficacy in both relapsed and elderly AML patients (Burnett and Mohite 2006; Majeti 2011; Tsimberidou et al. 2003). However, lack of an overall survival advantage, despite higher remission rates suggested that CD33 was probably expressed primarily by differentiated leukemia cells and not LSCs (Majeti 2011). Moreover, given the ubiquitous expression of CD33 on hematopoietic progenitors, it is probably not surprising that cytopenias were a common side effect. Interestingly, the one subgroup that appeared to show an overall survival improvement with CD33-targeting was the favorable cytogenetic AMLs (Giles et al. 2003; Taussig et al. 2005); these data may represent additional evidence that favorable AMLs arise from more differentiated, $CD33^+$ hematopoietic progenitors, whereas the LSCs from less favorable subtypes arise from $CD33^-$ progenitors.

Several groups have suggested that CD123 may be an ideal LSC target. Our own data demonstrate that LSCs regardless of the phenotype express CD123, while HSCs show little to no expression. Moreover, targeting CD123 in vitro eliminated LSCs, but showed no activity against HSCs (unpublished). Li et al. have recently presented pre-clinical data supporting the cytotoxic effect of SGN-CD123A, a potent CD123-directed antibody-drug conjugate in $CD123^+$ AML cell lines via induction of DNA damage, cell cycle alterations, and apoptosis (Li et al. 2018). The authors confirmed the activity of the conjugate in vivo in multiple xenograft models, including showing an additive effect with quizartinib, a novel FLT-3 inhibitor (Li et al. 2018). Similarly, Xie et al. demonstrated that CSL362, a monoclonal antibody binding to CD123 with high affinity, has significant antibody-dependent cell cyto-

toxicity (ADCC) against $CD34^{+}CD38^{-}CD123^{+}$ LSCs mediated by natural killer cells in vitro (Xie et al. 2017). Several groups have developed bispecific molecules attempting to direct T cells against AML blasts utilizing their CD123 expression. Particularly, CD3xCD123 is a dual-affinity retargeting (DART) antibody that showed dose-dependent T-cell mediated killing of AML cell lines and primary AML blasts in vitro and in vivo (Al-Hussaini et al. 2016; Chichili et al. 2015). More recently, Bonifant et al. generated T cells to secrete CD123/CD3-bispecific engager molecules and infused those into mice with AML xenografts demonstrating significant survival benefit associated with normalization of hematopoiesis (Bonifant et al. 2016). Finally, there are pre-clinical data supporting the efficacy of chimeric antigen receptor (CAR) T cells targeting CD123 in treating AML (Mardiros et al. 2013; Pizzitola et al. 2014). Currently, early phase clinical trials are ongoing, evaluating the clinical benefit of various CD123-targeting approaches.

Similar approaches have been introduced to target CLL-1 since this protein, as opposed to other LSC markers, is not expressed in normal hematopoietic cells, thus providing a great opportunity for targeted anti-leukemic effect and potential for good hematopoietic recovery. Particularly, a bispecific anti-CD3/anti-CLL-1 antibody was evaluated in in vitro and in vivo models of AML, demonstrating promising efficacy with regard to AML cell depletion (Leong et al. 2017). Laborda et al. designed anti-CLL-1 CAR T cells showing potent activity on AML cell lines and primary patient-derived AML blasts in vitro and on a disseminated CLL-1 positive mouse xenograft model, while sparing normal HSCs (Laborda et al. 2017). Similarly, Wang et al. developed CAR T cells expressing a CLL-1 receptor and demonstrated strong anti-leukemic activity in a xenograft model of disseminated AML, which was again associated with sparing of the normal HSCs and absence of significant myelosuppression (Wang et al. 2018). Finally, CLL-1 was recently used as a target for a novel anti-CLL-1 antibody-drug conjugate with a highly potent pyrrolobenzodiazepine dimer, showing high efficacy with regard to AML depletion in a xenograft model without any target independent toxicity (Zheng et al. 2018). Based on these data, targeting CLL-1 appears to be a very promising approach especially because of the absence of its expression in normal HSCs, but the design and development of early phase clinical trials is needed to confirm these results.

Targeting ALDH may be a promising approach for AML, but the high expression of ALDH in normal HSCs and the highly variable ALDH expression among LSCs make it challenging. Based on the role of ALDH in the metabolism of retinoic acid, our group has shown that all-trans retinoic acid (ATRA) can promote the differentiation of $ALDH^{int}$ LSCs in non-APL AML (Su et al. 2015), suggesting that ATRA could be an agent targeting specifically LSCs in AML which express intermediate levels of ALDH. Disulfiram, an ALDH inhibitor, has also been found to induce apoptosis selectively in LSCs via activation of ROS/JNK signaling and inhibition of NF-κB and Nrf2 (Conticello et al. 2012; Xu et al. 2017b). It was also recently found that disulfiram can overcome bortezomib and cytarabine resistance in $ALDH^{high}$LSCs from Down syndrome associated AML (Bista et al. 2017), further suggesting that disulfiram can be a targeted therapy for ALDH expressing LSCs. Finally, dimethyl ampal thiolester (DIMATE), another ALDH inhibitor, is active

specifically against LSCs but not against healthy HSCs (Chute et al. 2006; Venton et al. 2016). These results are intriguing since normal HSCs usually exhibit higher expression of ALDH and it would be expected that ALDH inhibitors should be more active against them. Potentially, signaling downstream of ALDH is different in LSCs and HSCs or LSCs are more dependent on ALDH activity due to increased DNA damage and sensitivity to ROS. Overall it would be critical to understand the impact of ALDH targeting in LSCs survival and its reflection in clinical outcomes.

Targeting of other antigens such as CD96, CD25, and CD32 has not been evaluated in such detail. However, anti-CD96 antibodies have been shown to promote antibody cell-mediated cytotoxicity which could be a promising approach against AML LSCs (Mohseni Nodehi et al. 2012). MicroRNAs such as miR-330-5p (Fooladinezhad et al. 2016) and miR-125a-3p (Emamdoost et al. 2017) can downregulate the expression of TIM-3 in AML cell lines, but there are no data with regard to the impact of this silencing on the survival of AML cells.

## 9.6 Conclusion and Future Directions

The failure of CRs to reliably translate into cures in AML can be explained by the LSC paradigm. Unfortunately, definitive clinical proof for the LSC concept, that targeting them improves outcomes, is currently lacking. The gold standard definition of LSCs focusing on immunocompromised mouse models of engraftment has led to potentially contradictory results that have proven difficult to translate into the clinical setting and across AML subtypes. Many studies have found that both phenotypic and genetic heterogeneity is less evident in MRD present during first CR than at diagnosis. Since first CR appears to be enriched for a "more homogeneous" population of LSCs, first CR may present an optimal time to target these cells with novel approaches. Moving forward, focusing on the most primitive cell phenotype present within a patient's AML cells may provide a broadly applicable means of studying clinically relevant LSCs and appropriate therapies to target these cells. Moreover, about a third of AML patients lack any usual molecular prognostic factors and, even when present, such prognostic factors may not be available for days or weeks. The most immature phenotype present within a patient's AML can be determined readily in essentially all patients by flow cytometry within hours of diagnosis. Rapid risk stratification may be particularly useful for patients harboring $CD34^{+}CD38^{-}ALDH^{high}$ leukemia cells, which identify high-risk patients who could be upfront candidates for novel LSC-targeted therapies.

In conclusion, targeting LSCs is a potentially effective strategy for patients with AML. However, the heterogeneity of LSCs and the different biological implications of potential LSC targets will probably affect the efficacy of the treatments as they move toward clinical trials. Finally, the identification of the appropriate context, such as maintenance therapy in first CR, is likely critical for optimizing their efficacy. The MRD state after allogeneic transplantation may be a particularly advantageous setting to study novel LSC-targeted therapies, in that they will be utilized at

lowest tumor burden as well as least tumor heterogeneity. Moreover, emerging data suggest that a new, non-tolerant, and non-exhausted transplanted immune system can augment the activity of many anticancer agents, small molecule as well as immunologic (Bouchlaka et al. 2010).

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# Chapter 10
# Contribution of Chronic Myeloid Leukaemia (CML) as a Disease Model to Define and Study Clonal Heterogeneity

**Marc G. Berger and Céline Bourgne**

**Abstract** Although tumour cell intra-clonal heterogeneity has been known for many years, its application in the oncology clinical practice (patient management, prognosis, etc.) remains limited. For this, chronic myeloid leukaemia (CML) is a remarkable model. Basic research studies revealed the heterogeneity of the initial clone, and led to the hypothesis of the existence of leukemic stem cells. Nevertheless, the indisputable evidence of the intra-clonal heterogeneity role in the therapeutic response came from the outcomes of the treatment with tyrosine kinase inhibitors (the first targeted therapy in medicine) combined with the early and rigorous clinical and molecular monitoring of these patients. CML management already takes this heterogeneity into account for personalized patient follow-up. The adventure continues with the objectives of better tailoring the treatment and of curing the disease in most of the patients.

**Keywords** Chronic myeloid leukaemia · Chronic phase · Leukaemia stem cell · Targeted therapy · Tyrosine kinase inhibitor · Intra-clonal heterogeneity · Precision medicine · Residual disease · Epigenetic · Single-cell transcriptome

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## 10.1 Introduction

Intra-clonal heterogeneity is a concept proposed by J. Dick's group in the 1990s, following the identification of a sub-population of cells that can initiate acute myeloid leukaemia after grafting in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) (Bonnet and Dick 1997; Lapidot et al. 1994). However, the transfer of this concept to the oncology clinical practice remains limited. In this sense, chronic myeloid leukaemia (CML) is a remarkable model. In most patients, CML is detected at the stage of chronic haematopathy with preservation of the differentiation capacity, and it always progresses to acute leukaemia after 4–6 years. This review focuses on the chronic phase of CML, before the progression to acute leukaemia, and summarizes the importance of the concept of intra-clonal heterogeneity in patient management in the era of targeted therapies and personalized treatments.

## 10.2 Chronic Myeloid Leukaemia

### *10.2.1 A Study Model in Oncology*

CML is a blood cancer model for studying the different aspects of tumour development/progression, and particularly the concept of leukemic clone. Indeed, the understanding of the pathophysiological mechanisms of this myeloproliferative syndrome, the development of tyrosine kinase inhibitors (TKI) and the study of their efficacy, and more recently the novel techniques of high through-put genetic analysis and single-cell approaches have allowed progressing from the concept of a cellular clone characterized by the presence of the Philadelphia chromosome (Ph1) in the 1980s to the detailed description of the remarkable complexity of the initial cellular clone. This synthetic mini-review summarizes the progress of knowledge in this field.

### *10.2.2 Philadelphia Chromosome: A Key Event in CML*

While the blood cell count modifications observed in patients with CML were considered initially as a benign proliferation that predisposed to the occurrence of acute leukaemia, the identification of a shorter chromosome 22 and then of the Philadelphia chromosome (Ph1) (Rowley 1973), which resulted from the translocation t(9;22)(q34;q11), asserted the clonal feature of the cells observed in the blood and in the bone marrow. Quite rapidly it was discovered that the translocation led to the fusion of the genes *BCR* and *ABL* that are normally distant in the genome (Groffen and Heisterkamp 1987; Heisterkamp et al. 1983). This chromosome translocation is still

considered as a key event in the development of the leukemic clone, particularly due to its capacity to induce a CML-type malignancy in mice transplanted with bone marrow infected with a retrovirus that encodes the $p210^{BCR\text{-}ABL}$ chimeric protein (Daley et al. 1990; Kelliher et al. 1990), or a transformed behaviour in normal cells (Zhao et al. 2001).

For long time, the Ph1 chromosome has been considered as the unique event leading to the leukemic transformation of immature haematopoietic cells; some recent results might question this paradigm (see below).

## 10.3 Elements in Favour of Intra-Clonal Heterogeneity Before the TKI Era

### *10.3.1 Cytological Features and Haematopoietic Cell Hierarchy*

The cytological features of the CML clone demonstrate the co-existence of different cell types within the clone. Although the most immature cells are rare, all the cells in the clone can be seen as a continuum from immature to differentiated cells, indicating that differentiation is globally maintained, like during normal haematopoiesis.

Very soon it was suspected that the Ph1 chromosome appeared in a primitive cell because (1) lymphoid B cells also harbour the Ph1 chromosome (Raskind and Fialkow 1987), although the clone includes mostly myeloid cells that can differentiate almost normally (Greaves et al. 1979; Tough et al. 1963; Whang et al. 1963); and (2) transformation into acute lymphoid leukaemia is observed in about 1/3 of patients with CML (Canellos et al. 1971).

The functional tests developed at the end of the 1980s, such as the culture of progenitor cells and the long-term culture-initiating cell (LTC-IC) assay (Sutherland et al. 1989), have allowed the identification within the CML clone of a more immature sub-population constituted of progenitors and of LTC-ICs that can support haematopoiesis in vitro (Udomsakdi et al. 1992). These cells express the antigen CD34, like the normal haematopoietic progenitors and stem cells. The *BCR-ABL* gene is expressed in this immature sub-population, strengthening the hypothesis that the Ph1 chromosome plays an essential role in all the cells of the clone (Maguer-Satta et al. 1996). The important clonogenic properties of this sub-population led to the idea that the strong proliferation capacity of CML cells gives them a competitive advantage compared to normal haematopoiesis.

This important proliferative potential justified the use of intensive chemotherapy regimens, but this approach allowed only a temporary improvement (Kantarjian et al. 1985). On the basis of this finding, Dr. T. Holyoake, at the time a post-doctoral fellow in Prof. C. Eaves' group at the Terry Fox Laboratory (Vancouver, Canada), hypothesized that a small sub-population of malignant cells could be quiescent and

resistant to anti-proliferative drugs. Indeed, she identified a sub-population of ($Ph1^+$/$BCR\text{-}ABL^+$) cells in the G0 phase of the cell cycle that have progenitor features and that can engraft in immunodeficient mice (Holyoake et al. 1999). This observation was a major element in the development of the concept of intra-clonal heterogeneity in this disease. Indeed, for the first time, it was demonstrated that chronic phase CML cells with the same membrane immature phenotype and the same cytogenetic abnormality could be subdivided into two distinct sub-populations in terms of cellular behaviour: one proliferative and the other quiescent.

In parallel, the molecular consequences of the t(9;22) translocation and of the constitutive tyrosine kinase activity of the chimeric protein $p210^{BCR\text{-}ABL}$ were described (Goldman and Melo 2001, 2003; Sawyers 1999). The permanent activation of different signalling pathways explained the features of CML cells, such as their aberrant proliferative potential, partly linked to their independence from some growth factors, such as IL-3 and G-CSF (Cilloni and Saglio 2012; Pluk et al. 2002; Skorski et al. 1995; Smith et al. 2003; Zhang et al. 2001; Zhao et al. 2002), and from the microenvironment, as well as their resistance to apoptosis.

Therefore, the quiescence of a small sub-population, despite the presence of the Ph1 chromosome and the detection of BCR-ABL transcripts, led to investigate the specific molecular mechanisms that explained the presence and the features of this sub-population from the early chronic phase of the disease.

## 10.4 Targeted Therapy Confirms the Intra-Clonal Heterogeneity of Chronic Phase CML

The findings concerning the effect of the chimeric protein BCR-ABL on the intracellular signalling pathways allowed the development of the first real targeted therapy in medicine: tyrosine kinase inhibitors (TKIs) to target the constitutive tyrosine kinase activity of the chimeric protein BCR-ABL. The outcomes (therapeutic response and tolerance) of treatment with imatinib, the first available TKI (Druker et al. 1996), were impressive (Deininger et al. 2005; Druker et al. 2006; Kantarjian et al. 2002).

Compared with the previous treatments (e.g., combination of interferon alpha and cytarabine) (Roy et al. 2006), the therapeutic response obtained with imatinib was so strong that the tools to detect the residual disease had to be rapidly optimized. Particularly, the qRT-PCR assay for the detection of the transcript of the chimeric protein BCR-ABL1 was standardized and recommendations were introduced for scoring the residual disease and the molecular response (Cross et al. 2015; Hughes et al. 2006).

It must be stressed that together, the remarkable follow-up of patients treated with this new therapeutic strategy, the optimization of the evaluation of the residual disease, and the study of CML cells resistant to TKIs have allowed the unparalleled progress in CML management and the acquisition of major knowledge on this blood malignancy that therefore remains a model in oncology.

### 10.4.1 Therapeutic Resistance and Intra-Clonal Heterogeneity

One would expect that by targeting the driver cytogenetic abnormality, TKIs should be effective in all patients. This is not the case (Fig. 10.1). It is estimated that <20% of patients are resistant to imatinib already in the chronic phase of the disease (Hochhaus et al. 2009; Nicolini et al. 2018). One of the best described mechanisms is the appearance of a point mutation in the gene encoding BCR-ABL (Gorre et al. 2001), attributed to a genetic instability that is probably multifactorial. In some cases, the presence of a BCR-ABL mutation may be responsible for the primary resistance at the beginning of the targeted treatment. Moreover, most patients seem to respond partially to the first-line treatment, before becoming resistant due to the emergence of a mutated clone (Soverini et al. 2013) (Fig. 10.1d). This mutated clone, which represents only a small fraction of malignant cells at the beginning of the disease, can expand during the treatment with TKIs that exert a selective therapeutic pressure. This confirms the presence of intra-clonal heterogeneity in some patients since the early phase of the disease.

Nevertheless, BCR-ABL point mutations represent a minority (<20%) of the cases of resistance during the early chronic phase (Soverini et al. 2006), and other still poorly known multifactorial mechanisms might lead to resistance to TKIs in the absence of mutations in the BCR-ABL transcript (see below).

### 10.4.2 Optimal Therapeutic Response: A Sub-Population of Malignant Cells Remains Difficult to Eliminate

Although TKI efficacy is remarkable because the survival of patients is similar to that of age- and sex-matched controls (Tauchi et al. 2011), it is instructive to analyse the dynamics and quality of the optimal therapeutic response (Fig. 10.1a).

In all patients, TKIs induce the apoptosis of most cells of the CML clone very rapidly because a 3 log reduction of the tumour mass is observed during the first year in the case of satisfactory response. However, a sub-population of CML cells, which can be detected by qRT-PCR, survives for many years (Baccarani et al. 2015). A threshold of 0.1% for the BCR-ABL/ABL ratio is considered to be protective against the progression to acute leukaemia, without a known explication. It is not clear whether this is linked to the features of this sub-population (genetically more stable), or whether a critical tumour mass predisposes to acute transformation, or whether the pool of more resistant cells is maintained small through an extrinsic control.

Over time, the residual sub-population progressively decreases, leading to BCR-ABL/ABL ratios <0.01% or even <0.001% that represents $10^8$ and $10^7$ malignant cells persisting in the organism, respectively.

In agreement, the therapy withdrawal trials for patients in whom residual disease is no longer detected by qRT-PCR (BCR-ABL/ABL ratio <0.001%) have reported

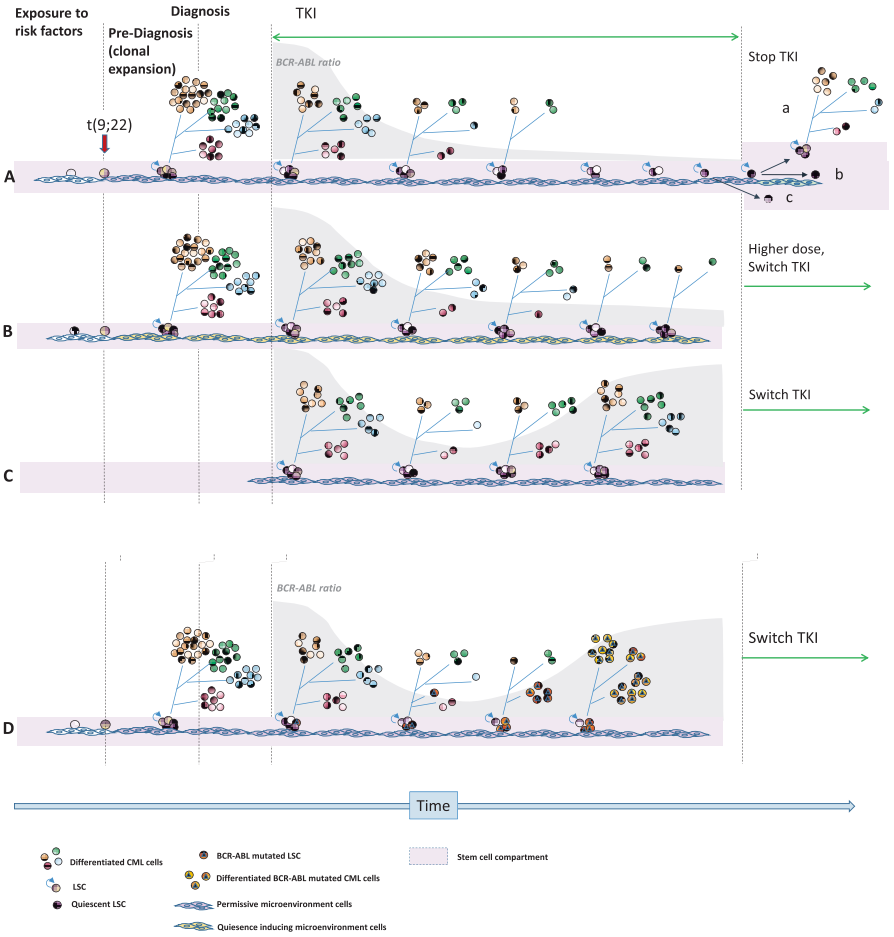


**Fig. 10.1** Influence of chronic phase CML intra-clonal heterogeneity on the response to targeted therapy. It is thought that CML is the consequence of the appearance of the driven *t*(9;22) translocation in a hematopoietic stem cell. This might be favoured by extrinsic, environmental, and/or intrinsic (epigenetic...) factors. The mutated cell is then clonally amplified with preservation of myeloid cell differentiation (schematized by a hierarchical tree in the figure). At diagnosis, the expansion phase has already led to invasion of the bone marrow cavity and the blood compartment. Within the clone, there is a subpopulation of leukemic stem cells (LSCs), among which some are quiescent. It is recognized that bone marrow microenvironment plays a role in the maintenance of LSCs, at least a permissive role. Upon treatment with a tyrosine kinase inhibitor (TKI), the following outcomes may be observed. (**A**) In the event of optimal response, the residual disease (monitored using the BCR-ABL/ABL ratio) decreases rapidly and then more slowly to very low or even undetectable values. After treatment withdrawal, half of the patients who showed optimal response will relapse due to the presence of LSCs in the bone marrow (a) that could be within a protective microenvironment. Other patients will maintain a very low level of residual disease without relapse (b), and other patients will not have any residual CML cell (c) and could be considered cured. (**B**) Some patients show a slower and partial therapeutic response called suboptimal response. A particular microenvironment favouring cell quiescence could contribute to this phenomenon. (**C**) While all patients show a partial response that reflects the heterogeneous TKI sensitivity of the cells within the clone, some patients show therapeutic resistance. Specifically, within a subpopulation of cells, particularly immature cells, including quiescent cells, some resistant cells (due to different, not yet fully elucidated mechanisms) will start expanding during treatment. In most cases, this leads to changing TKI molecule. (**D**) A possible resistance mechanism is the appearance of a point mutation in the BCR-ABL gene that impairs/prevents TKI binding/activity. As the TKI will eliminate only the cells harbouring wild-type BCR-ABL, the mutated clone will be selected

disease recurrence in about half of them (Mahon et al. 2010; Ross et al. 2013; Saussele et al. 2018). This indicates the persistence of a very small number, which cannot be detected by qRT-PCR, of leukemic stem cells (LSC) that are resistant to TKIs from the beginning of the treatment. This is the clinical demonstration of CML intra-clonal heterogeneity because a LSC sub-population resist to treatment. In agreement, CML LTC-ICs have been detected in the bone marrow of patients whose residual disease was undetectable with the routinely used techniques (Chomel et al. 2011; Chu et al. 2011). LSCs have some features in common with HSCs, such as their localization within the bone marrow microenvironment and a similar phenotype ($Lin^-$ $CD34^+CD38^-$) (Herrmann et al. 2012). Some specific features related to their stem cell status and the bone marrow microenvironment might favour LSC survival, even when the tumour mass is sufficiently reduced.

The CML research groups have rapidly focused on the resistance mechanisms of this small sub-population. Prof T. Holyoake found a link with the quiescent population that she previously described by showing that these cells are resistant to imatinib and are selected during treatment (Graham et al. 2002). Similar results were obtained with other TKIs, although the strongest compounds eliminate more cells (Copland et al. 2006; Jørgensen et al. 2007). Other studies have questioned the dogma of the main role of the BCR-ABL protein. Indeed, some LSCs survive despite BCR-ABL inhibition, suggesting resistance mechanisms that are independent of BCR-ABL tyrosine kinase activity (Corbin et al. 2011). Currently, one of the major challenges concerning CML is to understand these mechanisms for developing novel therapeutic strategies that, combined with TKIs, will allow eliminating this sub-population, and hopefully, curing a larger number of patients.

Finally, analysis of the treatment outcome results of the TKI stopping trials indicates that some patients are cured by TKIs because the disease remains undetectable for more than 5 years after treatment withdrawal. This suggests that TKIs can eradicate CML LSCs in some patients, possibly because these cells are more dependent on BCR-ABL activity. An interesting topic for future research is the determination of the intrinsic characteristics of LSCs in these patients.

Globally, these observations confirm the TKI sensitivity heterogeneity among the cells of the clone despite the fact that they all carry the molecular target. They also demonstrate that a small fraction of malignant cells, present already at diagnosis, can resist to TKI treatment in the long term, even in patients with an excellent response.

### *10.4.3 Intra-Clonal Heterogeneity and New Sequencing Techniques*

The new techniques of single-cell sequencing and bioinformatics analysis brought new information in cancer research. A recent remarkable study allowed obtaining some insights into the intra-clonal heterogeneity of the immature cell compartment

of the CML clone, by using approaches that associate single-cell sequencing and transcriptome analyses to characterize the $Lin^-$ $CD34^+CD38^-$ cell population in patients with chronic phase CML. This sub-population represents about 0.1% of the CML cells. This study showed that in CML, $CD34^+CD38^-$ cells have a specific transcriptome profile, with upregulation (e.g., *GAS2*) and downregulation (e.g., *CXR4*) of some known genes. It also highlighted the deregulation of the TGFβ and TNFα signalling pathways, associated with an increase of quiescent cells. Importantly, this technique allowed comparing BCR-$ABL^+$ and BCR-$ABL^-$ cells in each patient. The analysis of samples from patients at CML diagnosis and then during the treatment with TKIs identified a specific highly quiescent BCR-$ABL^+$ sub-population that is already present at diagnosis, and is selected in vivo by the TKIs (Giustacchini et al. 2017), an observation confirmed elsewhere (Warfvinge et al. 2017). The whole-transcriptome approach demonstrated that the transcriptomic profile of TKI-resistant LSCs is different from that of normal quiescent HSCs, particularly concerning the deregulation of some signalling pathways (TGFβ, TNFα, JAK-STAT, CTNNB1, and NFKB1A). In patients who displayed a sub-optimal therapeutic response to TKIs, TGFβ, TNFα, and IL-6 pathway were upregulated in CML cells and also in immature BCR-$ABL^-$ cells. This could be caused by a micro-environmental deregulation (Shah and Bhatia 2018), probably induced by the leukemic clone. These abnormalities reflect profound changes in the haematopoietic tissue that may contribute to the therapeutic resistance of a sub-population of quiescent immature cells within the CML clone.

The reasons why a sub-population of BCR-$ABL^+$ cells shows a very different profile (quiescent cells that can self-renew and amplify) are still poorly known. As all cells harbour the Ph1 chromosome, which is essential for CML physiopathology, epigenetic mechanisms could help explaining the intra-clonal heterogeneity. Indeed, it has been demonstrated that the gene encoding BCR-ABL must be transfected in immature ("stem") cells to develop the disease. This observation suggests that the stem status of cells might have a pathophysiological role. In HSCs, this status is controlled through a specific epigenetic regulation programme (Cabezas-Wallscheid et al. 2014; Hodges et al. 2011; Langstein et al. 2018; Lipka et al. 2014). Many observations support multiple deregulations of the epigenetic mechanisms that control genes in CML (Koschmieder and Vetrie 2018).

Our group recently reported that an epigenetic deregulation of the CML clone is present already at diagnosis and up to the chronic phase, and that an intra-clonal epigenetic heterogeneity can be detected already in $CD34^+$ cells, a subset that contains progenitors and LSCs and represents on average 1–2% of the chronic phase CML clone. In a global methylome analysis (Infinium Human Methylation 450 K BeadChip system) of $CD34^+CD15^-$ and $CD34^-CD15^+$ cells selected by flow cytometry, we could identify a specific DNA methylation profile of the CML clone relative to normal cells, and also a specific profile of $CD34^+CD15^-$ cells compared with all the other cells in the clone. Moreover, for some genes, we could establish a correlation between their DNA methylation status and their expression (e.g., *GAS2*, *PRAME*, etc.) (Maupetit-Mehouas et al. 2018). These results demonstrate that an epigenetic deregulation is present already in the early disease phases, although up to

now it was considered significant only during the transformation and blast phases. We are currently assessing the DNA methylation anomalies accumulated in TKI-resistant cells in vivo.

## 10.5 Future Challenges

Due to the intra-clonal heterogeneity present at the diagnosis of chronic phase CML, it is important and strategic to identify the LSC sub-population with tools that can be used also in the clinical practice. This is difficult because LSCs share strong phenotypic similarities with normal HSCs. Indeed, they are included in the $Lin^{-}CD34^{+}CD38^{-/low}$ cell fraction (Eisterer et al. 2005). Hermann et al. reported that expression of CD26 can be used to identify CML LSCs within the $CD34^{+}CD38^{-/low}$ fraction (Herrmann et al. 2014). The $CD26^{+}CD34^{+}CD38^{-/low}$ phenotype was then used to identify residual LSCs in patients with CML during treatment with TKIs and remission (Bocchia et al. 2018). However, these results need to be reproduced. Other markers, such as IL-1 RAP, could be interesting (Järås et al. 2010). Therefore, it is still difficult to distinguish the LSC population from the residual normal HSC population in the same patient before and during the TKI treatment (Warfvinge et al. 2017). This is an important challenge for assessing the initial LSC proportion within the tumour mass that seems to present a predictive value concerning the therapeutic outcome (Landberg et al. 2016; Mustjoki et al. 2013), and for monitoring the residual clonal population during the early follow-up.

The epigenetic specificities of immature CML cells and the anomalies observed in non-tumour cells of the same patient suggest a possible genetic background that might predispose to the appearance of the Philadelphia chromosome (Giustacchini et al. 2017). Indeed, after the discovery of a pre-leukemic mutation background that promotes the haematopoietic clonal potential (clonal haematopoiesis of indeterminate potential; CHIP) in healthy subjects without detectable disease (Genovese et al. 2014; Jaiswal et al. 2014; Xie et al. 2014) and before the appearance of acute myeloid leukaemia (Abelson et al. 2018; Desai et al. 2018), the question of a background that predisposes to the translocation (t9;22) remains a particularly interesting research topic. We know that the peak of CML appearance after irradiation occurs about 7 years after radiotherapy. The events occurring during this period of "incubation" remain unknown. Some mutations could contribute to this risk. However, few genetic mutations are detected at diagnosis of chronic phase CML, compared with other blood malignancies, and are more frequent in the case of progression. Moreover, they do not exactly concern the same genes as in CHIP (Branford et al. 2018; Kim et al. 2017; Togasaki et al. 2017). Other deregulations might be involved in the early phases of CML.

The influence of a microenvironmental alteration (Giustacchini et al. 2017) linked to genetic determinism (Janel et al. 2017) cannot be ruled out. Their identification is essential to envisage a predictive medicine, and to discover other therapeutic targets in addition to BCR-ABL.

## 10.6 Conclusion

The new knowledge accumulated in the last years on CML treated with TKIs highlight the importance of rigorously monitoring the therapeutic progress in precision medicine. Therefore, CML remains a unique model that brings important insights for the entire cancer field. All current strategies for curing an increasing proportion of patients are useful to better understand the mechanisms of intra-clonal heterogeneity and how to exploit them for therapeutic purposes.

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# Chapter 11
# The Heterogeneity of Osteosarcoma: The Role Played by Cancer Stem Cells


**Kristina Schiavone, Delphine Garnier, Marie-Francoise Heymann, and Dominique Heymann**

**Abstract** Osteosarcoma is the most common bone sarcoma and is one of the cancer entities characterized by the highest level of heterogeneity in humans. This heterogeneity takes place not only at the macroscopic and microscopic levels, with heterogeneous micro-environmental components, but also at the genomic, transcriptomic and epigenetic levels. Recent investigations have revealed the existence in osteosarcoma of cancer cells with stemness properties. Cancer stem cells are characterized by their specific phenotype and low cycling capacity, and are linked to drug resistance, tumour growth and the metastatic process. In addition, cancer stem cells contribute to the enrichment of tumour heterogeneity. The present manuscript will describe the main characteristic features of cancer stem cells in osteosarcoma and will discuss their impact on maintaining tumour heterogeneity. Their clinical implications will also be briefly addressed.

**Keywords** Tumour heterogeneity · Gene mutation drivers · Cancer stem-like cells · Circulating tumour cells · Monitoring · Clinical trials · Osteosarcoma · Cancer stem cell · Drug resistance · Cell dormancy

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## 11.1 Introduction

Osteosarcoma is part of the family of malignant bone sarcomas which originate from a common mesenchymal precursor located in the bone marrow, and known as mesenchymal stem cells (Brown et al. 2018a). Osteosarcoma is the main bone sarcoma in adolescents and young adults, with a peak of incidence at around 18 years old. Osteosarcomas are preferentially detected in the metaphysis of long bones and the tumour tissue is characterized by the presence of osteoid matrix produced by cancer cells (Fig. 11.1). Microscopic heterogeneity is the first marker for osteosarcoma with the presence of highly vascularized, necrotic, proliferating and osteoid foci. Depending on the morphological features of the cancer cells, osteosarcomas can be classified as osteoblastic, chondroblastic, fibroblastic or telangiectatic. Current treatment combines neo-adjuvant chemotherapy, surgery and adjuvant chemotherapy including at least three cytotoxic agents such as doxorubicin, methotrexate and ifosfamide. Unfortunately, prognosis remains poor and overall survival has stagnated in the last four decades (Heymann et al. 2016). Overall survival reaches 50–70% at 5 years depending on the series in the absence of detectable metastases, but drops to 30% when lung metastases are detected at the time of diagnosis.

Tumour heterogeneity can be directly related to both the natural history of the cancer cells and to their dialogue with the protagonists in the local microenvironment (Mutsaers and Walkley 2014; Tang et al. 2008; Mohseny et al. 2009;

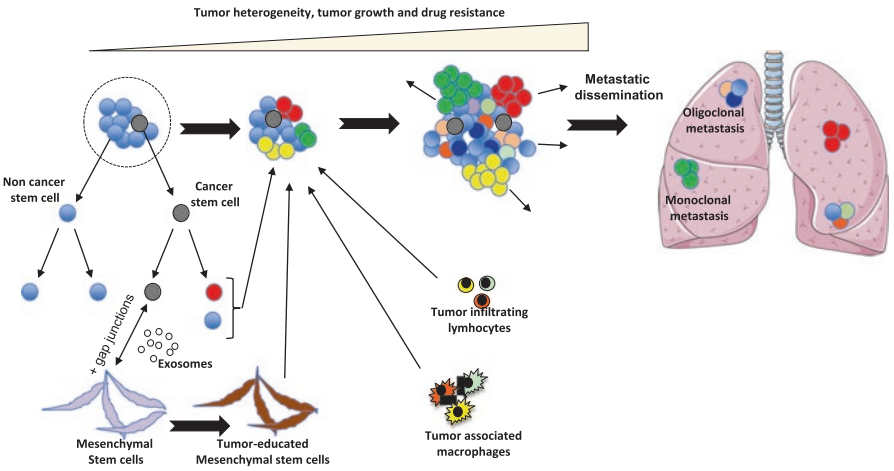


**Fig. 11.1** Clonal evolution of osteosarcoma cells and their role in tumour heterogeneity. Initially formed by mono- or oligoclonal subclones, dominant clones appear progressively, resulting in marked heterogeneity in the tumour mass. Of these cancer cells, a subpopulation exhibits stemness markers and educates mesenchymal stem cells to release exosomes, which in turn increase the stem cell phenotype and upmodulate tumour growth and the development of metastases. These two-way communications enrich tumour heterogeneity and increase the risk of drug resistance. Immune cells, with their diversity and induction of local immune tolerance, complete the heterogeneity of the tumour mass

Cortini et al. 2017; Alfranca et al. 2015). The local micro-environment is composed of numerous cell types, including immune (e.g. tumour-infiltrating lymphocytes and tumour-associated macrophages) (Heymann et al. 2017; Dumars et al. 2016; Théoleyre et al. 2005) and non-immune cells such as endothelial cells, fibroblasts and mesenchymal stem cells, which are spatially, temporally and functionally linked to cancer cells (Brown et al. 2018a). Cancer cells can control the behaviour of their neighbours, which in turn play a part in fuelling tumour growth and the metastatic process. Cancer cells are composed of numerous cell clones competing together to preserve the overall survival of their congeners through selective advantage. Some of these clones drive tumour initiation and are called cancer stem cells (Brown et al. 2017). Even if the term "stem cell" is not perfectly appropriate, it describes a subpopulation of cells capable of reconstituting the characteristics of all cancer cells detectable in the tumour mass. Consequently, cancer stem cells can generate a tumour mass after inoculation into an immunodeficient organism (Najafi et al. 2019).

The present review will discuss the main data available in the literature in favour of the existence of cancer stem-like cells in osteosarcoma, as well as their potential contribution to the enrichment of tumour heterogeneity. Their clinical impact in drug resistance will be also discussed.

## 11.2 Clonal Evolution of Cancer Cells in Osteosarcoma: A Combination of Oncogenic and Epigenetic Events

In parallel to histological heterogeneity, osteosarcoma is one of the most complex oncologic diseases in terms of genetic aberration. In 2014, Reimann et al. found wide genomic rearrangements in the tumour exome of a single case of osteosarcoma (Reimann et al. 2014). These authors detected 3,000 somatic single nucleotide variants, small indels and more than 2,000 copy number variants in diverse chromosomes. The osteosarcomas were thus characterized by a loss of heterozygosity. The complexity of the disease was confirmed by Bousquet et al. who studied a series of 44 osteosarcomas and observed recurrent somatic alterations to *TP53* and *RB1* and also detected 84 mutation points and 4 deletions related to 84 genes (Bousquet et al. 2016). Similarly, Smida et al. analysed 160 osteosarcoma samples by whole-genome sequencing in order to identify somatic copy number alterations. They found specific unstable genomic regions in which numerous tumour suppressor genes were included (e.g. *TP53, RB1, WWOX* and *DLG2*) (Smida et al. 2017). This very high number of alterations perfectly illustrates the genomic complexity of osteosarcomas. The development of cancer is sustained by two main theories: (1) the "linear model" theory, which is based on successive accumulations of oncogenic events in one cell leading to the development of a heterogeneous disease; (2) the branched evolution theory known as the "parallel model", characterized by the parallel evolution of subclones which accumulate DNA alterations and also lead to a polyclonal

tumour mass (Greaves and Maley 2012; Tellez-Gabriel et al. 2016). Of course, the nature and number of these oncogenic events drive tumour initiation as has been shown by Funes et al. who transformed mesenchymal stem cells using genetic alterations (Funes et al. 2007). They observed that four oncogenic hits made possible the formation of colonies in agar, although only five oncogenes were able to induce tumour development in immunodeficient mice. They also suggested that tumourigenesis of modified mesenchymal stem cells was dependent on the nature of the oncogene. For instance, disruption to the RB pathway was enough to induce anchorage-independent growth of mesenchymal stem cells (Funes et al. 2007). In addition, one oncogenic hit sensitized mesenchymal stem cells to carcinogenic agents such as pesticides and may have led to tumour development in immunocompromised mice (Hochane et al. 2017). In addition, the clonal evolution of cancers is tightly controlled by the selective pressure of the local micro-environment (e.g. immune infiltrate and hypoxia), and can be oriented under drug pressure toward resistant or tolerant cancer cells (Brown et al. 2018b; Vallette et al. 2018). Regardless of what the first oncogenic event is, a permissive local micro-environment is obligatory for protecting cancer-initiating cells against immune cells and fuelling these cells with adequate nutrients (de Groot et al. 2017).

Both types of clonal evolution have been described in osteosarcoma (Wang et al. 2019). Wang et al. analysed and compared 86 tumours in 10 osteosarcoma patients using whole exosome and genome sequencing. By analysing the architecture and relationships of the cancer subclones, they demonstrated a dynamic mutational process and, for the first time, two patterns of lung metastases—with a linear model in six patients and a branched model in four patients. Based on the low number of patients included, the co-existence of both models in a same patient can be excluded. The tumour evolution model has recently been enriched by a "plasticity" model identified in Ewing sarcoma (Franzetti et al. 2017). In Ewing sarcoma, the plasticity model is based on equilibrium between various cancer cell subclones differentially expressing the chimeric EWS1/FLI1 transcription factor, leading to major modifications in cell migration and invasion properties. The two populations create an ecosystem with dynamic fluctuation in cells differentially expressing the fusion protein depending on the stage of the disease.

Very recently, Gambera et al. established multicolour (RGB) $p53^{-/-}$ $Rb^{-/-}$ mouse mesenchymal stem cells (Gambera et al. 2018) that can form osteosarcomas when inoculated into bone micro-environment cells. They also deciphered the clonal evolution during tumour progression (Rubio et al. 2014). They identified two main steps in tumour progression. At an early stage of development (25 days), tumour growth is characterized by polyclonal expansion with no modification to the proportions of the coloured cells injected. At a late stage of tumour growth (50 days), Gambera et al. observed the emergence of dominant clones at the periphery of the tumour mass, corresponding to clonal evolution of the disease. Overall, these data provided evidence of marked clonal modifications in cancer cells from a polyclonal context to the formation of dominant clones which were oligoclonal and exhibited similar tumourigenesis properties (Gambera et al. 2018). In addition, the metastatic process to the lung was associated with an oligoclonal and monoclonal dynamic.

Although this model cannot be transposed to humans, there is some evidence of dominant clones in osteosarcoma. In 2015, Kovac et al. investigated the evolutionary landscape using exosome sequencing in 31 osteosarcoma samples (Kovac et al. 2015). They identified 14 genes associated with a BRCAness signature as the main drivers for tumour development not exclusively expressed in all subclones. *TP53* mutations were frequently observed in subclones. These authors hypothesized that osteosarcoma could be initiated by a mutation in *TP53* or *RB* in one specific subclone (monoclonal disease), leading to chromosal instability and chromatide breakages, and to new oncogenic events in various subclones (polyclonal disease). PARP inhibitors may then be a therapeutic option in osteosarcoma (Engert et al. 2017). The existence of dominant subclones was confirmed by Chen et al. by studying a case report of a chemoresistant osteosarcoma sample in which they identified a clone associated with a new *TP53-KPN3* translocation (Chen et al. 2016). The three models for cancer cell evolution are responsible for the considerable heterogeneity found in osteosarcoma and the emergence of dominant clones which evolve in a dynamic manner and in perfect symbiosis with their permissive ecosystem.

As shown in Ewing sarcoma, for which the heterogeneity of DNA methylation is a reflection of the spectrum of the disease (Surdez et al. 2017), epigenetic genetic alterations are observed in osteosarcomas and are associated with its pathogenesis (e.g. tumour growth and metastatic process) (Feng et al. 2018; Sarver and Subramanian 2016; Georges et al. 2018). Epigenetic modulations can regulate osteosarcoma cell differentiation and can concomitantly interfere with their microenvironment (Itoh et al. 2018; Lamoureux et al. 2014; Li et al. 2018). For instance, Lamoureux et al. demonstrated that selective inhibition of bromodomain epigenetic signalling induced an inhibitory effect in primary tumour growth and simultaneously in osteoblasts and osteoclasts, two cell types found in the local microenvironment (Lamoureux et al. 2014). More recently, Li et al. gave evidence of epigenetic downregulation in osteosarcoma cells of CXCL12 (SDF-1) via DNA methyltransferase-1, related to their ability to form lung metastases and, interestingly, to their impairment of cytotoxic T-cells homing in on the tumour mass (Li et al. 2018). They found a correlation between CXCL12 expression and the overall survival of osteosarcoma patients. Tumour heterogeneity and clonal evolution of osteosarcomas are thus regulated by epigenetic events.

## 11.3 Presence of Cancer Stem Cells in Osteosarcoma: Their Functional Impact

The conventional theories for clonal evolution described above can be completed by the "cell origin" theory. In this theory, the first oncogenic event may occur in a cancer stem cell or in a cell in the non-side population (López-Lázaro 2018), with cancer as the end result of successive cell divisions in stem cells with cumulative DNA replication errors (e.g. mutations and epigenetic mistakes) making possible

both the self-renewal of "differentiated" cancer cells and the maintenance of undifferentiated cells. The presence of a side population that excludes Hoechst 33342 dye has been demonstrated on osteosarcoma cell lines and in human primary osteosarcoma (Murase et al. 2009; Yang et al. 2011). These cells are able to regenerate both side- and non-side cells, show higher clonogenicity than non-side populations and sustained tumourigenicity. They have also shown increased multi-drug resistance and are phenotypically similar to stem cells thanks to the expression of Oct-4 and Nanog for instance. Through analogy with embryonic stem cells, this side population has been called cancer stem cells or stem-like cells. However, their immune-tolerant property, their low cycling characteristic and drug resistance have led to this population also being referred to as dormant, quiescent, tolerant and persister cells (Vallette et al. 2018). It has been suggested that cancer stem cells are unique subclones within a tumour, responsible for tumour progression, resistance to therapies and the initiation of metastases. This definition is supported by clinical cases showing metastases more than 20 years after complete remission (Halldorsson et al. 2009) or a local recurrent disease after inoculation of adipose tissue 13 years after complete remission (Perrot et al. 2010).

In the last few decades, numerous works have tried to identify specific markers and the properties of cancer stem cells in osteosarcoma (Table 11.1). Osteosarcoma cancer stem cells are supported by sox2, a stem cell transcription factor which inhibits the Hippo pathway (Basu-Roy et al. 2012, 2015). In addition to the expression of the stemness markers shared with embryonic stem cells, osteosarcoma cancer stem cells have been characterized by their ability to form cell spheroids in vitro, which are highly tumourigenic in vivo (Murase et al. 2009; Yang et al. 2011). Cancer stem-like cells expressed high levels of aldehyde deshydrogenase-1 (ALDH1) (Honoki et al. 2010; Greco et al. 2014). ALDH-1 expression was associated with resistance to chemotherapy (Honoki et al. 2010) and the metastatic potential of cancer cells (Greco et al. 2014). The receptors for stem cell growth factor (CD117) and stro-1 expressed by mesenchymal stem cells are expressed by osteosarcoma cancer stem cells and were associated with metastasis and drug resistance (Adhikari et al. 2010). CD133 was also linked to the stem cell phenotype in osteosarcoma (Tirino et al. 2008, 2011; He et al. 2012; Li et al. 2013; Fujiwara et al. 2014). CD133 or prominin-1 is a pentaspan transmembrane glycoprotein localized in cellular protrusions (Glumac and LeBeau 2018). Like $ALDH1^+$ cells, CD133-expressing osteosarcoma cells displayed high tumourigenicity in vivo (Tirino et al. 2011). Its high expression in patients predicted lung metastases and consequently correlated with poor prognosis (He et al. 2012; Li et al. 2013; Fujiwara et al. 2014). Tian et al. demonstrated the expression of CD271, a low-affinity nerve growth factor receptor, by osteosarcoma cancer stem cells (Tian et al. 2014) and defective autophagy led to the suppression of the stem-like properties of CD271+ (Zhang et al. 2016). Numerous other factors (CBX3, KLF4, SATB2, etc.) summarized in Table 11.1 controlled the biological properties/maintenance of stem cells. The biology of osteosarcoma cancer stem cells is also under the control of epigenetic networks. Several recently identified microRNAs regulate stem cell phenotype and their invasion and migration properties by targeting specific molecular pathways, such as PTEN, POU5F1

**Table 11.1** Main markers expressed by osteosarcoma cancer stem cells

| Markers | References |
|---|---|
| Oct4 (octamer-binding transcription factor 4), Nanog (Nanog Homeobox), transcription factors, stemness markers | Murase et al. (2009) and Yang et al. (2011) |
| Sox2 (SRY-related HMG-box-2) transcription factor, stemness markers | Basu-Roy et al. (2012, 2015) |
| ALDH1 (aldehyde desyhdrignease-1) | Honoki et al. (2010), Greco et al. (2014) and Wang et al. (2011) |
| CD24 (cell adhesion molecule) | Guth et al. (2014) |
| CD44 (receptor of hyaluronic acid) | He et al. (2015) |
| CD117 (receptor of stem cell growth factor) | Adhikari et al. (2010) |
| Stro-1 (marker of mesenchymal stem cells) | Adhikari et al. (2010) |
| CD133 (prominin-1) | Tirino et al. (2008, 2011), He et al. (2012), Li et al. (2013), Fujiwara et al. (2014) and Glumac and LeBeau (2018) |
| CD271 (low-affinity nerve growth factor receptor) | Tian et al. (2014) and Zhang et al. (2016) |
| CBX3 (Chromobox protein homolog 3) | Fujiwara et al. (2014) and Saini et al. (2012) |
| ABCA5 (ATP-binding cassette, sub-family A, member 5) | Saini et al. (2012) |
| KLF4 (Kruppel like factor 4) | Qi et al. (2018), Li et al. (2017) and Martins-Neves et al. (2016a) |
| SATB2 (special AT-rich sequence-binding protein 2) | Xu et al. (2017) |
| RAB39A (Rab small GTPase)-RXRB (retinoid X receptor Beta) | Chano et al. (2018) |
| TB1XR1 (transducin (beta)-like 1 × −linked receptor 1) | Xi et al. (2019) |
| SENP1 (Sentrin specific protease-1) | Liu et al. (2018) |
| hTERT (human telomerase reverse transcriptase) | Yu et al. (2013) |

Wnt or Jagged1 (Lu et al. 2017; Zhang et al. 2018a; Zou et al. 2017; Zhao et al. 2017; Guo et al. 2017; Di Fiore et al. 2016; La Noce et al. 2018).

Osteosarcoma cancer stem cells are resistant to chemotherapy and radiotherapy, and can drive cancer recurrence (Gibbs et al. 2005; Fujii et al. 2009). Consequently, conventional chemotherapy impacts cancer stem cells and enriches the tumour mass in stem cells (Martins-Neves et al. 2012). They play a significant role in tumour heterogeneity through permanent enrichment of new mutated cancer cells and dominant subclones, and by regulating their local micro-environment. Cancer cells dialogue permanently with locally based partners. These communications include direct exchanges of small mediators using channels of the gap junction type (Tellez-Gabriel et al. 2017), with some selectivity. For instance, endothelial cells use gap junctions to communicate with osteosarcoma cells, and cancer cells do not communicate using this mode of communication with undifferentiated mesenchymal stem cells, unlike mesenchymal stem cells, which initiate their differentiation toward the osteoblast lineage. The dialogue between both cell types is controlled by

acidosis. Acid-activated mesenchymal stem cells influence osteosarcoma cell behaviour such as their stemness properties (Avnet et al. 2017). Mesenchymal stem cells and cancer cells can also dialogue through the release of extracellular vesicles (Cortini et al. 2017; Baglio et al. 2017). Baglio et al. recently demonstrated the role of exosomes in osteosarcoma development and, more interestingly, that tumour cells educated mesenchymal stem cells by paracrine activity associating extracellular vesicles (Baglio et al. 2017). Tumour exosomes containing both IL-1 and TGFβ educated mesenchymal stem cells, which in turn promoted tumour growth and the development of lung metastases. In addition to identifying new therapeutic targets, these works show that osteosarcoma cells can regulate their microenvironment qualitatively and consequently enrich tumour heterogeneity. These data are reinforced by publications that underline the role of TGFβ in the stemness of osteosarcoma cells (Zhang et al. 2013; Lamora et al. 2016; Martins-Neves et al. 2016b). TGFβ1 is thus crucial for the differentiation of osteosarcoma cells for cancer toward cancer stem cells (Zhang et al. 2013). The second key molecular pathway for stemness in osteosarcoma is Wnt/beta catenin signalling, which supports stem cell formation (Lamora et al. 2016). Crosstalk between both pathways has been observed in both chronic inflammation and carcinogenesis (Martins-Neves et al. 2016b). Local immunity is also controlled by cell communications leading to an increase in tumour heterogeneity (Heymann et al. 2017). In parallel to the heterogeneity of cancer cells, immune heterogeneity with tumour-associated macrophages (Vallée and Lecarpentier 2018) and tumour-infiltrating lymphocytes (Sharma and Capobianco 2017) has been established and defines an immune-tolerant niche.

## 11.4 Tumour Heterogeneity, Cancer Stem Cells and New Therapeutic Options

Based on the data available in the literature, blocking agents have been developed as new therapeutic options for osteosarcoma patients to overcome drug resistance (Heymann et al. 2016; Dumars et al. 2016; Saraf et al. 2018; Makena et al. 2018). Thus, pimozide and resveratrol inhibit osteosarcoma cancer stem cells (Gonçalves et al. 2019; Peng and Jiang 2018). Pimozide blocks the epithelial-to-mesenchymal transition and both drugs interrupt the STAT-3 (IL-6 signalling pathways) and Wnt-β/catenin signalling modulated by TGBβ. Numerous therapeutic options target the TGFβ pathway. Miao et al. have recently developed single-walled carbon nanotubes to specifically inhibit TGFβ-induced osteosarcoma cell dedifferentiation and prevent the acquisition of the stem cell phenotype (Miao et al. 2017). Targeting the Wnt/beta catenin pathway may be a therapeutic alternative (Martins-Neves et al. 2018). Martins-Neves et al. used IWR-1, a tankyrase inhibitor to attenuate Wnt/beta catenin signalling in osteosarcoma cancer stem cells with promising results in preclinical mouse models (Martins-Neves et al. 2018). Blockading N-cadherin/NF-KB signalling also appears interesting with the administration of metformin to inhibiting the stem cell phenotype (Xu et al. 2017). Shang et al. revealed that metformin increased the sensitivity of stem cells to conventional chemotherapy and confirmed the advantages of this drug in the treatment of osteosarcoma (Shang et al. 2017).

Metformin should be considered as a metabolic modulator of osteosarcoma cancer stem cells (Paiva-Oliveira et al. 2018). Targeting EGFR and CD133 (Chen et al. 2018), Sox9 (Qu et al. 2018), TSSSC3 and Src/AKt pathways (Yan et al. 2017) FGFR2 (Zhang et al. 2018b) has also been assessed recently in osteosarcoma and exhibited high efficacy by repressing the self-renewal of stem cells, tumour growth and the metastatic process. Immunotherapies are interesting potential future options (Heymann et al. 2016; Mesiano et al. 2018), as shown recently by Mesiano et al. (2018) and D'Angelo et al. (2018). Mesiano et al. used cytokine-induced killer cells which are effective against cancer stem cells in sarcoma (Mesiano et al. 2018). D'Angelo et al. developed autologous T cells expressing NY-ESO-1$^{c259}$ expressed by synovial sarcomas (D'Angelo et al. 2018), and showed that patients with metastases treated with an affinity-enhanced T-cell receptor recognizing an HLA-A2-restricted NY-ESO-1/LAGE1a-derived peptide, increased the anti-tumour response by around 50%. In addition, circulating NY-ESO-1$^{c259}$ T cells were detectable in blood for at least 6 months in all responders, and most administered NY-ESO-1$^{c259}$ T cells exhibited an effector memory phenotype following ex vivo expansion (D'Angelo et al. 2018).

## 11.5 Conclusion

Cancer stem cells, which should be called cancer "stem-like" cells, are detectable in osteosarcoma. They contribute markedly to tumour heterogeneity and are responsible for drug resistance. Dialogue is established between cancer stem-like cells and their local micro-environment, and they are able to educate to facilitate their maintenance and development. This dialogue is a future potential target and there is drug development in combination with conventional chemotherapies (Fig. 11.1). Better characterization of cancer stem-like cells in osteosarcoma and their role in the clonal evolution of the disease is mandatory for improving the therapeutic response of poor responders, as well as for improving the overall survival of osteosarcoma patients which has changed little in the last four decades.

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# Chapter 12
# Ovarian Cancer Stem Cell Heterogeneity


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**Abstract** Ovarian carcinoma features pronounced clinical, histopathological, and molecular heterogeneity. There is good reason to believe that parts of this heterogeneity can be explained by differences in the respective cell of origin, with a self-renewing fallopian tube secretory cell being likely responsible for initiation of an overwhelming majority of high-grade serous ovarian carcinomas (i.e., type II tumors according to the recent dualistic classification), whereas there are several mutually non-exclusive possibilities for the initiation of type I tumors, including ovarian surface epithelium stem cells, endometrial cells, or even cells of extra-Müllerian origin. Interestingly, both fallopian tube self-renewing secretory cells and ovarian surface epithelium stem cells seem to be characterized by an overlapping array of stemness signaling pathways, especially Wnt/β-catenin. Apart from this variability in the respective cell of origin, the particular clinical behavior of ovarian carcinoma strongly suggests an underlying stem cell component with a crucial impact. This becomes especially evident in high-grade serous ovarian carcinomas treated with

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classical chemotherapy, which entails a gradual evolution of chemoresistant disease without any apparent selection of clones carrying obvious chemoresistance-associated mutations. Several cell surface markers (e.g., CD24, CD44, CD117, CD133, and ROR1) as well as functional approaches (ALDEFLUOR™ and side population assays) have been used to identify and characterize putative ovarian carcinoma stem cells. We have recently shown that side population cells exhibit marked heterogeneity on their own, which can hamper their straightforward therapeutic targeting. An alternative strategy for stemness-depleting interventions is to target the stem cell niche, i.e., the specific microanatomical structure that secures stem cell maintenance and survival through provision of a set of stem cell-promoting and differentiation-antagonizing factors. Besides identifying direct or indirect therapeutic targets, profiling of side population cells and other ovarian carcinoma stem cell subpopulations can reveal relevant prognostic markers, as exemplified by our recent discovery of the Vav3.1 transcript variant, which filters out a fraction of prognostically unfavorable ovarian carcinoma cases.

**Keywords** Ovarian cancer · Ovary · Fallopian tube · Tumor heterogeneity · Ovarian cancer cell of origin · Ovarian epithelium stem cell · Fallopian tube epithelium stem cell · Ovarian carcinoma stem cell · Stem cell heterogeneity

## 12.1 Ovarian Cancer: Clinical Features of a Highly Aggressive and Heterogeneous Tumor Type

Ovarian cancer is the most lethal malignancy among all gynecological tumors (Partridge and Barnes 1999; Cannistra 2004; Fleming et al. 2009). Despite significant progress in radical debulking surgery and the advent of several successive lines of chemotherapy with new cytotoxic drugs as well as maintenance therapy with targeted agents, the de facto cure rate from ovarian cancer has not changed visibly over the last five decades. This is because of very high rates of recurrence in excess of 85% even after optimal primary surgical debulking and adjuvant chemotherapeutic treatment. Indeed, ovarian cancer exhibits a high initial responsiveness to first-line standard platinum-based chemotherapy, which results in a high proportion of complete clinical remissions. Furthermore, an intriguing trait of ovarian cancer is that recurrent disease can be successfully re-challenged with platinum-based drugs, with the likelihood of a sustained remission continuously increasing with an expanding platinum-free interval. As a rule, however, the intervals between relapses become progressively shorter until platinum sensitivity is lost. This typical clinical course of ovarian cancer cannot be explained by simple, progressive, clonal selection occurring during the treatment of a primary multi-clonal disease (Zeimet et al. 2012). This has also been recently noted by Macintyre and co-workers, who analyzed copy number variations during the evolution of high-grade serous ovarian cancer (Macintyre et al. 2018). Specifically, the authors were unable to find

significant changes in the copy number signature of primary tumor specimens versus biopsies from the first or second relapse. Therefore, it is more likely that pre-existing cancer stem- or precursor cells known to exhibit constitutive resistance to cytotoxic/cytostatic agents increase in numbers as a result of continuous Darwinian selection under treatment, such that the overall mutational load of the tumor remains relatively constant. In summary, the typical clinical course of ovarian cancer suggests that stem cell-driven repopulation is a prominent phenomenon in this disease. Thus, ovarian cancer can be regarded as an ideal candidate to study the underlying role of cancer stem cells (CSCs) in the development and progression of solid malignant tumors.

Clinically, histologically, and molecularly, ovarian carcinoma presents as a highly heterogeneous disease. In recent years, it has become increasingly clear that ovarian carcinoma represents an umbrella term for a conglomerate of malignant diseases that all involve the ovary but need not to be necessarily inter-related. Still, even as a group of somewhat unrelated diseases, ovarian carcinoma shows several peculiarities, and principal clinical features seem to remarkably converge. Such a common denominator is especially the way and pattern of metastatic dissemination. Unlike other tumor types that ultimately involve distant tissues, metastasis of ovarian carcinoma is largely limited to the peritoneal cavity and its associated organs. There are two major routes for ovarian cancer peritoneal metastatic disease. Metastasizing tumors might involve the retroperitoneum in terms of lymphatic spread or other Müllerian organs through direct invasion and secondary site growth as so-called implants. Systemic peritoneal dissemination is achieved by detaching single tumor cells and/or small tumor cell aggregates that are transported and dispersed by peritoneal fluid, followed by adhesion and invasive growth. In contrast to most other solid tumor entities, hematogenous spread plays only a minor role in ovarian cancer metastasis (Fleming et al. 2009).

The four major histopathological subtypes of ovarian cancer are serous, mucinous, endometrioid, and clear cell, with the serous cancers being by far the most prevalent form (Table 12.1). Strikingly, the four subtypes can be molecularly distinguished based on the specific pattern of *HOX* gene activation. Specific members of the *HOXA* gene cluster (i.e., *HOXA9*, *HOXA10*, *HOXA11*, and *HOXA13*) play crucial roles in the development and differentiation of Müllerian organs, and are differentially expressed in the different types of ovarian carcinoma. Serous ovarian cancers show selective overexpression of HOXA9, whereas mucinous and endometrioid cancers selectively overexpress HOXA11 and HOXA10, respectively. Corroborating this clinical association, forced expression of such "fate-specifying" *HOX* genes in mice produces histological features that are reminiscent of the corresponding human disease (Bast et al. 2009).

The staging and grading system experienced quite a convoluted evolution. The current FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) staging system recognizes four principal stages, with rather complex sub-staging categories. Fundamentally, the stage I tumors include all lesions ranging from a single tumor limited to an ovary (stage IA) to a positive evidence for malignant cells in ascites or peritoneal washings (stage IC3). The stage II tumors show loco-

**Table 12.1.** The distinct nature and underlying biology of type I and type II ovarian cancers

| | Type I | Type II |
|---|---|---|
| FIGO stage at diagnosis | Early-advanced (I–II)↑ (III–IV)↓ | Advanced (III–IV) |
| Histopathological appearance (grading) | Well-differentiated | Poorly differentiated |
| Proliferation index and progression kinetics | Low and slow | High and fast |
| Manifestation of ascites | Uncommon | Common |
| Genomic instability | Rather not | Yes |
| p53 mutational inactivation | Possible but rare | >95% |
| Developmental origin | Variable—e.g., ovarian surface epithelium, endometrium, extra-Müllerian tissue | Fallopian tube |
| Long-term remission after primary treatment | Definitely possible | Unlikely due to recurrence |
| Prognosis | Favorable | Unfavorable |
| Chemosensitivity | Poor | High |

This table was modified from Kurman and Shih (2016)

regional involvement and extend to the uterus, the fallopian tube, or other pelvic intraperitoneal tissues (implant growth). The stage III tumors display extrapelvic peritoneal colonization, but otherwise remain confined to the retroperitoneal surface, lymph nodes, or the capsule of liver and/or spleen. The stage IV tumors finally manifest positive pleural effusion and/or growth within the liver and/or spleen parenchyma. The FIGO classification system also takes into account the differentiation status of the tumor, currently distinguishing three grades, namely low, intermediate, and high (Aust and Pils 2014).

It has been increasingly realized that high-grade serous (HGS) ovarian cancers behave rather differently from other types of ovarian carcinoma. Because of very fast tumor growth and a corresponding lack in early detection methods, HGS ovarian carcinomas are typically diagnosed at an advanced stage (i.e., FIGO stage III or IV), with limited therapeutic options. In fact, it is mainly because of the HGS subtype that ovarian cancer is sometimes referred to as "silent killer" or "whispering disease"(Aust and Pils 2014). The other subtypes of ovarian cancer, including the low-grade serous and mucinous forms, behave more predictably and seem to follow more a classical pathological progression pathway, starting from a clearly defined (benign) precursor lesion, subsequently passing through stages of atypical proliferation/hyperplasia ("borderline tumors"), and finally developing into carcinoma (invasive growth). HGS and non-HGS ovarian tumors also show important biological differences. HGS ovarian carcinomas consistently carry inactivating p53 mutations, with the remaining mutational load being highly variable and related to widespread genomic and chromosomal instability. Conversely, all other ovarian cancer subtypes exhibit largely stable genomes, with a much more confined mutational signature. In fact, most of the recurrent mutations in non-HGS ovarian cancers affect mitogenic signaling, of which many converge at the mitogene-activated

protein kinase (MAPK) pathway. This dichotomy of ovarian cancer led to the proposal of a new classification system taking into account these clinico-pathological and molecular features to distinguish between type I and type II tumors (Kurman and Shih 2016) (Table 12.1).

Due to its mutational burden imposed by unstable genomes and karyotypes, the type II class of tumors is per se highly heterogeneous, with further molecular subtypes to be definable. For instance, The Cancer Genome Atlas (TCGA) distinguishes four molecular subgroups of HGS ovarian cancer: (1) immune-reactive, (2) differentiated, (3) proliferative, and (4) mesenchymal. Along similar lines, the Australian Ovarian Cancer Study (AOCS) distinguishes five genomic rearrangement signatures and the OVCAD consortium (Ovarian Cancer—Diagnosis of a Silent Killer) defines two molecular subtypes (subtype 1 and 2), based on a specific gene panel consisting of 112 genes. Importantly, tumors of subtype 2 are characterized by peritoneal carcinomatosis at the time of diagnosis, resulting in insufficient surgical debulking and poor outcome (Pils et al. 2012).

It is important to note that the ovaries can also be a target site for metastasis from unrelated primary tumors, which may not be obvious at the time of the initial diagnosis; Krukenberg tumor, mostly gastric carcinoma metastasizing into ovary, is a pathologic classic for more than a century (reviewed in Agnes et al. 2017). Regarding the ovarian cancer histological subtypes, especially mucinous ovarian tumors with *pseudomyxoma peritonei* manifestations seem to be frequently of gastrointestinal (most often appendiceal) origin (Fleming et al. 2009).

In this chapter, we elaborate on the probable cell-of-origin in ovarian cancer and discuss the fallopian tube fimbrial epithelium as a candidate source for HGS ovarian tumor development. According to this concept, the ovary would act as a first site for metastatic deposition, which would represent a situation of quasi-obligatory, locoregional involvement of the ovary through an adjacent, female-specific tissue. Irrespective of the cell of origin, ovarian carcinoma as a disease and its stem cells component feature a pronounced heterogeneity, an issue that we have experimentally approached previously (Boesch et al. 2014).

## 12.2 Ovarian and Fallopian Tube Epithelial Stem Cells and the Origin(s) of Ovarian Cancer

The notion of ovarian epithelial stem cells is largely dominated by periodic regenerative postovulatory responses. The ovary is covered by a single-layered epithelium that morphologically oscillates between cuboidal and squamous cells, depending on the respective phase of sexual cycle. Interestingly, ovarian surface epithelial cells express both epithelial (e.g., keratins) and mesenchymal (e.g., vimentin) markers, suggesting their not entirely mature phenotype (this developmental dichotomy is somewhat reminiscent of mesothelial cells lining the peritoneum, which is the major site of ovarian carcinoma dissemination). A growing

follicle first leads to bulging of the ovarian surface epithelium (OSE) over the ovarian surface and finally to its rupture, with oocyte release and expulsion of follicular fluid. A secondary consequence is the degradation and release of ovarian surface epithelial cells together with the upper cell layers of the ovulating follicle which mainly include granulosa and theca cells. The resulting rupture stigma is rapidly repaired, with wound closure achieved latest after 3 days, at least in mice (Ng and Barker 2015). This periodic regenerative response that involves the replacement of cells lost during ovulation strongly suggests the existence of a long-term proliferative reserve within OSE cells, and an obvious explanation would be the existence of a self-renewing stem cell.

The mere existence of such stem cells, their signaling requirements (e.g., niche composition), possible heterogeneity, and anatomical location within OSE have been subject to some controversy in recent years. Not entirely surprisingly, marker-free approaches started the quest for potential OSE stem cells. One of the most frequently applied approaches is the identification of label-retaining cells (LRCs), which reveals a relatively quiescent subset, in turn believed to constitute a hallmark property of stem cells. Such slow-cycling cells are identified by first forcing intact tissues to accumulate DNA (BrdU or IdU) or chromatin (H2B-GFP) labels by a period of sustained exposition or expression ("pulse"), followed by a prolonged period of time without label supply, during which non-stem cells (especially transit-amplifying cells) dilute the mark due to their enhanced proliferation (chase) (Chang et al. 2008). Interestingly, such LRCs seem to be present at two anatomically distinct locations in mouse ovaries—at interfollicular clefts, i.e., those parts of the OSE that lie between follicles and flank postovulatory rupture wounds, and at a specific anatomical location, called the ovary hilum that constitutes a contact zone between three epithelial layers—OSE, oviduct fimbrial epithelium (particularly rich in LRCs), and ovarian ligament epithelium (Brenton and Stingl 2013; Ng and Barker 2015).

Another marker-free approach targets the self-protective capacity of stem cells, with two extensively used methodologies aimed at the identification and isolation of cells specifically expressing aldehyde dehydrogenase (ALDEFLUOR™ assay) and/or efflux pumps of the ABC drug transporter family (side population (SP) assay) (Hatina et al. 2013; Boesch et al. 2012, 2014, 2016a, b), respectively. Both these procedures have basically been adopted for FACS-based purification of putative stem cells. The ALDEFLUOR™ assay exploits a specific fluorescent substrate for the ALDHA1 isoenzyme, BODIPY-aminoacetaldehyde (BAAA), which is enzymatically converted in such a way that it becomes highly hydrophilic, thus unable to leave the cell, which yields a fluorescent signal selectively in stem cells. The SP assay uses from a certain point of view rather an opposed strategy to identify stem cells. ABC drug transporters, which normally protect the stem cells from environmental toxins such as xenobiotics, extrude several fluorescent dyes as well, including Hoechst 33342 (Goodell et al. 1996) and DyeCycle™ Violet (Boesch et al. 2012, 2014), which leads to distinctly lower accumulation in stem versus non-stem cells.

ALDEFLUOR™-bright cells have been purified from the ovary hilum and expression profiling ($ALDH^{bright}$ versus $ALDH^{dim}$ cells) was performed to further characterize these cells. Intriguingly, the canonical Wnt/β-catenin signaling pathway has been found strongly upregulated in the $ALDH^{bright}$ cell fraction, which also showed high expression of Lgr5, a specific marker for intestinal crypt, and bulge hair follicle stem cells (Ng et al. 2014; Ng and Barker 2015). Importantly, Lgr5 is not just a marker—it rather forms an integral part of the Wnt/β-catenin signaling pathway, thereby constituting an essential feed-forward loop in cells expressing it. As a known downstream target of β-catenin, Lgr5 amplifies transcriptional activation by serving as a receptor for a family of Wnt-unrelated ligands including R-spondins (together with Lgr4 and Lgr6). Strikingly, the Lgr family of proteins also includes receptors for pituitary gonadotropins such as FSH (Lgr-1) and LH (Lgr-2) (de Lau et al. 2014); it is especially the steep rise in FSH, which is believed to underlie the peri- and postmenopausal outbreak of ovarian carcinoma (see below). To support long-term stem cell maintenance, Wnt/β-catenin signaling must be carefully and tightly balanced, which is secured by another group of β-catenin downstream targets acting as negative regulators, including Axin2 and the TNFR family member Tnfrsf19 (Troy). Interestingly, cells co-expressing ALDHA1, Lgr5, Wnt4, Axin2, and Troy can be found in both anatomical locations specified above, namely the ovarian hilum and the interfollicular cleft (Ng and Barker 2015).

The fallopian tube epithelium (or oviduct epithelium in mouse), including the fimbrial epithelium, is clearly more mature than OSE, and two major epithelial cell types can be distinguished, (1) secretory cells (typical marker: PAX-8) and (2) ciliated cells (typical marker: acetylated tubulin beta 4 – TUBB4). Lineage tracing experiments to deduce ancestral relations and infer the developmental trajectories in vivo have recently been conducted (Ghosh et al. 2017). It turned out that both secretory and ciliated cells belonged to the same lineage, but the secretory cells represented an earlier phenotype and had the capacity to replenish the ciliated cells. Moreover, the study suggested that the putative oviductal epithelial stem cells corresponded to (a fraction of) secretory cells, which can both self-renew and give rise to differentiated progeny. The secretory fallopian tube cells, including the putative stem cells, are crucially dependent on Wnt/β-catenin signaling. It seems that estrogens, notorious mitogens for fallopian tube secretory cells responsible for their proliferation, exert their biological effect through modulation of the Wnt/β-catenin signaling pathway (Nagendra et al. 2016). However, the critical cellular source providing the Wnt ligands to OSE and fallopian tube stem cells still remains enigmatic. The respective stromal cells are an obvious candidate, but there might be another, more specific source—follicular fluid that is released upon ovulation. Indeed, a number of Wnt ligands (e.g., Wnt2, Wnt4, Wnt5A, Wnt11) as well as Wnt-regulating factors/decoy receptors (e.g., SFRP1 and SFRP4) have been detected in follicular fluid (Ng and Barker 2015). Intriguingly, some components of follicular fluid might induce genotoxic stress or otherwise harm the genome, as evidenced by sustained activation of the DNA damage response pathway in exposed mouse oviductal (King et al. 2011) and human fallopian tube epithelial cells (Huang et al. 2015). This might

be of relevance for the later discussion about the nature and identity of the probable cell of origin in ovarian cancer.

Why to deal with the fallopian tube epithelial cells in the context of ovarian CSCs? Traditionally, ovarian cancer has been automatically assumed to arise from the ovary, with OSE stem cells being the prime candidate for initial malignant transformation (Chang et al. 2008). The incessant ovulation hypothesis, formulated almost half a century ago (Fathalla 1971), tried to link inter-species variations in female cycle to the incidence and type of ovarian carcinogenesis. In support, epidemiological data demonstrated a significant rise in ovarian cancer incidence with age, as well as protective effects of pregnancy, lactation, and the use of oral contraceptives. Accordingly, ovulation would induce repeated cell cycle entry and proliferation in OSE cells, leading to gradual accumulation of genomic alterations and possibly, malignant transformation. Indeed, in vivo targeted mutagenesis of hilum-associated OSE cells in mice results in the formation of tumors that faithfully resemble human ovarian carcinoma (Flesken-Nikitin et al. 2013). However, this theory soon became challenged by pathologists, who were frequently unable to find pre-cancerous lesions in the ovary despite advanced-stage disease. This has been further corroborated in women undergoing prophylactic oophorectomy due to germline *BRCA1/2* mutations, whose ovaries frequently showed no signs of dysplasia or early hyperplasia. In contrast, pre-cancerous changes of the fallopian tube epithelium could frequently be observed in basic pathological examinations and molecular analyses. The healthy fallopian tube epithelium includes rather evenly distributed secretory and ciliated cells, while the *BRCA1/2*-mutant fallopian tube, especially in more distal regions, frequently presents with islets of pure secretory cells. Depending on the degree of hyperplasia, secretory cell expansions/outgrowths (SCE/SCOUT) and serous tubal intraepithelial neoplasia/carcinoma (STIN/STIC) are distinguished (Mehra et al. 2011). At the immunohistochemical level, such lesions present active p53 (called p53 signature) indicative of an ongoing process of mutagenesis and repair. Moreover, PAX-8, a typical marker for fallopian tube secretory cells, is also widely expressed in ovarian carcinoma (Adler et al. 2017; Ghannam-Shahbari et al. 2018). From an epidemiological point of view, it is interesting to note that prophylactic salpingo-oophorectomy, i.e., the combined removal of both fallopian tubes and ovaries, has a significantly higher impact on reducing the risk of ovarian cancer than oophorectomy alone (Falconer et al. 2015). Finally, the fact that secretory fallopian tube stem cells are governed by the same molecular circuits as OSE stem cells suggests overlapping niche requirements of stem cells from both sources. The integration of these various data led to another concept of ovarian cancer development in which the fallopian tube/fimbrial secretory cell acts as the cell of origin, especially in high-grade serous cancers (Perets and Drapkin 2016). However, it is important to note that this conceptual framework does not oppose the incessant ovulation theory, but rather complement it: firstly, the epidemiological evidence supporting the incessant ovulation theory still remains valid and, secondly, ovulation-related wounds or surface lesions could potentially represent an easy portal of entry for detached, migratory tubal cells with tumor-initiating capacity. This way of thinking can even be further

extended: while the fimbrial secretory cell might represent the cell of origin in serous ovarian carcinoma, one can speculate that endometriosis, an otherwise benign condition where cells of the endometrium detach and colonize the Müllerian tract, may similarly initiate ovarian cancer development, then biased towards other histological subtypes, such as endometrioid and clear cell (Fleming et al. 2009; Cardenas et al. 2016; Torng 2017). It is even conceivable that the roots of ovarian cancer can be traced back to entirely different tissues. For example, gene expression profiling of ovarian mucinous adenocarcinoma showed that its closest counterparts among solid tissues and cancers were the colonic epithelium and mucinous colorectal carcinoma, respectively (Heinzelmann-Schwarz et al. 2006). A surprising phenotypic convergence has also been found for ovarian cancer and renal clear cell carcinoma (Zorn et al. 2005). Finally, recall the probable appendiceal origin of mucinous ovarian tumors with *pseudomyxoma peritonei* mentioned above (Fleming et al. 2009). Apparently, ovary represents a fruitful "soil" (=niche) for dispersed cancer cells of variable origin and an ovarian metastasis could be clinically apparent before a primary tumor.

Importantly, the clinical, epidemiological, and molecular characteristics of ovarian carcinoma discussed above are neither comprehensive nor complete. In addition, the age-dependent rise in ovarian cancer incidence is not a linear function of the cumulative number of ovulations experienced; in fact, the peri- and postmenopausal periods seem to be especially critical. This led to another paradigm of ovarian carcinoma initiation, namely the gonadotropin theory (Cramer and Welch 1983). Here, the basic tenet is that with the cessation of periodic ovulations, the synthesis of ovarian steroids such as estrogens and progesterone ceases, which abolishes their regulatory function on the pituitary gonadotropins FSH and LH. In this context it is striking to note that a unique population of OSE stem cells, termed very small embryonic-like stem cells (VSELC), has been identified which feature overresponsiveness to FSH and nuclear expression of the pluripotency factor Oct-4 (Bhartiya and Singh 2015). Whereas these cells are currently regarded as possible candidates for neo-oogenesis, their potential contribution to ovarian cancer development remains to be seen.

The postmenopausal hormonal milieu is believed to substantially influence the composition and function of the ovarian niche (Cardenas et al. 2016). It is quite widely accepted that stem cells populating the inner part of ovary during postovulatory repair immediately enter quiescence and may rest in this state in the form of small inclusion cysts for decades. It is speculated that the tumor-preventive effects of the ovarian stromal cell niche are abolished, or at least reduced, in the aged, atrophic ovary, which then fosters tumor outgrowth from the cell cycle re-entering stem cells (OSE stem cells or fimbrial secretory stem cells). Data from a mouse model of heritable accelerated follicle depletion (Ww mouse, carrying a hypomorphic *c-Kit* mutation) indicated that active ovulation can indeed suppress the effects of *p53* loss-of-function, thus deferring the manifestation of the programmed phenotype to the onset of menopause (Wang et al. 2016).

In conclusion, it is quite apparent that ovarian cancer is a stem cell-driven tumor type, even though the exact cell of origin remains elusive to date. Variations in the

phenotypic identity and early niche of the cell of origin may contribute to ovarian cancer heterogeneity and form the basis for the broad clinical presentation characteristic of this tumor.

## 12.3 Ovarian Cancer Stem Cells: New Evidence for an Old Concept

Irrespective of the cell of origin, there is ample evidence that ovarian carcinoma features intrinsic heterogeneity. In contrast to the clonal evolution model where tumor characteristics and heterogeneity arise from stochastic mutations in any cell, the CSC model postulates that a mutated tumor-initiating cell with the stem cell characteristics of both self-renewal and differentiation produces progeny recapitulating, at least in part, the hierarchical differentiation pattern seen in normal tissue. Similar to tissue stem cells, these CSCs are equipped with increased capacity to resist harmful intrinsic and external events, such as a low proliferation rate, marked proficiency in DNA damage repair, and upregulation of detoxifying enzymes and efflux pumps, which results in reduced sensitivity to radiotherapy and cytotoxic as well as targeted drugs. With these assets and the ability to repopulate a complete tumor from a single cell, the CSCs take the center stage in tumor development, metastasis, and therapy resistance. Consequently, a lot of effort has been taken to define, isolate, and characterize these cells in ovarian cancer (summarized in Table 12.2). As CSCs are supposed to resemble their normal counterparts, initial studies have harnessed the same techniques as used for the definition of stem cells of the putative tissues of origin, notably fallopian tube and ovary (see above), such as the detoxifying enzymatic activity of ALDH1 (Deng et al. 2010; Landen et al. 2010; Kryczek et al. 2012; Kuroda et al. 2013). Other groups have used the side population assay to indicate the expression and activity of ABC drug transporters for the definition of cells with stem cell characteristics (Szotek et al. 2006; Moserle et al. 2008; Hu et al. 2010; Hosonuma et al. 2011). It has long been elusive, however, which MDR pump confers the SP phenotype. Some studies demonstrated that ABCG2 was the ABC transporter expressed in ovarian cancer SP cells (Dou et al. 2011; Zhang et al. 2015), but it was later found that both ABCG2 and ABCB1 can confer the SP phenotype to ovarian CSCs (Boesch et al. 2014). Another set of studies focused on surface proteins, which had previously been shown to be expressed on cells with stem cell properties in other tumor types, such as CD24, CD44, CD117, CD133, and lastly ROR1, to identify CSCs in ovarian cancer (Ferrandina et al. 2008; Zhang et al. 2008, 2014; Alvero et al. 2009; Gao et al. 2010). Finally, stimulated by the findings made during the development of induced pluripotent stem cells, several groups have investigated the expression of canonical stem cell transcription and reprogramming factors such as Lin28, Oct4, Nanog, Myc, and Sox2 (Peng et al. 2010; Meirelles et al. 2012; Siu et al. 2013; Di et al. 2013; Belotte et al. 2015; Yan et al. 2015; Wen et al. 2017). Although certainly important for the functional aspects and maintenance of stemness, these intracellular markers cannot

**Table 12.2** Stem cells markers used to identify and characterize ovarian carcinoma stem cells

| Marker | Biological function | References |
|---|---|---|
| CD24 | Cell surface sialoglycoprotein acting as adhesion molecule | Gao et al. (2010) |
| CD44 | Cell surface glycoprotein with widespread expression important in cell–cell interactions, cell adhesion, and migration; can interact with many ligands (HA, osteopontin, collagens, etc.) | Zhang et al. (2008) and Alvero et al. (2009) |
| CD117 (mast/stem cell growth factor receptor—SCFR; c-Kit proto-oncogene) | Receptor tyrosine kinase | Zhang et al. (2008) and Lai et al. (2009) |
| CD133 also as prominin-1 | Surface glycoprotein with five transmembrane domains localizing to membrane protrusions | Ferrandina et al. (2008), Curley et al. (2009) and Roy et al. 2018 |
| ROR1 | Tyrosine-protein kinase transmembrane orphan receptor | Zhang et al. (2014) and Henry et al. (2015) |
| CD326/EpCam | Transmembrane glycoprotein mediating $Ca^{2+}$-independent homotypic cell–cell adhesion | Tayama et al. (2017) |
| ALDH1 | Group of enzyme catalyzing the oxidation of intracellular aldehyde to carboxylic acid | Deng et al. (2010), Landen et al. (2010), Kryczek et al. (2012) and Kuroda et al. (2013) |
| CD338/ABCG2 | Protein transporting various molecules across extra- and intracellular membranes; can be involved in chemoresistance | Szotek et al. (2006), Moserle et al. (2008), Hu et al. (2010) and Hosonuma et al. (2011) |
| CD243/ABCB1/MDR1 | Protein transporting various molecules across extra- and intracellular membranes; can be involved in chemoresistance | Eyre et al. (2014) |
| NANOG | Transcription factor involved in self-renewal of undifferentiated embryonic stem cells | Siu et al. (2013) |
| SOX2 | Transcription factor involved in self-renewal of undifferentiated embryonic stem cells | Belotte et al. (2015) and Wen et al. (2017) |
| OCT4/POU5F1 | Transcription factor involved in self-renewal of undifferentiated embryonic stem cells | Peng et al. (2010) and Yan et al. (2014) |
| MYC | Oncogenic transcription factor; one of the reprogramming factors of induced pluripotent stem cells | Di et al. (2013) |
| CD184/CXCR4 (C-X-C chemokine receptor type 4)[a] | Alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1) overexpressed in $CD133^+$ NYO-1 ovarian carcinoma cells; it can constitute a possible therapeutic target | Mitsui et al. (2012) |

(continued)

**Table 12.2** (continued)

| Marker | Biological function | References |
|---|---|---|
| Endothelin receptor A[a] | G protein-coupled receptor overexpressed in $CD133^+$ cells of various ovarian carcinoma cell lines and primary tumor cells; it can constitute a possible therapeutic target | Coffman et al. (2013) |
| LIN28[a] | RNA-binding protein; one of the reprogramming factors of induced pluripotent stem cells, specifically overexpressed in $CD44^+CD24^+Epcam^+$ cells of various ovarian carcinoma cell lines | Peng et al. (2010) and Meirelles et al. (2012) |
| MISRs[a] | Müllerian inhibiting substance receptors overexpressed in $CD44^+CD24^+Epcam^+$ cells of various ovarian carcinoma cell lines and sensitive to MIS treatment; it can constitute a possible therapeutic target | Wei et al. (2010) |
| IL-17R[a] | Receptor for IL-17, a pro-inflammatory cytokine mainly produced by T-helper (Th17) cells and macrophages, specifically overexpressed in $CD133^+$ A2780 ovarian carcinoma cells; it can constitute a possible therapeutic target | Xiang et al. (2015) |

[a]Notice that C-X-C chemokine receptor type 4, endothelial receptor A, Müllerian inhibiting substance receptors, LIN28, and interleukin-17 receptor cannot be regarded as genuine ovarian carcinoma stem cell markers, as they have not been used to prospectively isolate cells exhibiting stem cell properties, but have been solely identified by virtue of their distinctly higher expression in cell subsets isolated by using different marker molecules

be used to isolate live cells for further in vitro or in vivo studies. Taken together, all these studies showed that cells isolated from patients based on these different types of markers displayed at least some characteristics of stem cells such as increased clonogenicity and tumor formation in xenograft models—or, as in the case of intracellular markers, were expressed at higher levels in such cells. In addition, high tissue expression of these different stem cell markers was associated with poor prognosis in most studies although few opposite associations with survival were also reported. One possible interpretation of all these findings with a large variety of markers is that the typical CSC encompasses all these characteristics and that the very same cell can be identified with any of these. Unfortunately, only few reports investigated more than one marker and if so, their expression did not completely overlap. Also, many of the studies showed that the difference in the stemness potential between putative CSCs and their non-CSC counterparts was not absolute. Lastly, there are also large technological differences between the studies with respect to the tissue of origin (ascites or primary tumor, or tumor cell lines), the way of isolation (direct marker-based sorting or selective culture under stem cell specific conditions), and the method to functionally define stemness (asymmetric division, spheroid formation, clonogenicity, and tumorigenicity). Thus, it is more likely that differences in phenotype and function rather reflect the heterogeneity between but

also within tumor specimens such as tissue of origin, histopathological subtype, or molecular subtype. However, such information has been rarely documented in the previous studies and in most instances, the number of cases would have been too small anyway for a definitive statement. In addition, even within a single patient, the composition of CSCs might vary between primary tumor, ascites, and metastases. This picture is further complicated by intratumoral heterogeneity at the single cell level (McPherson et al. 2016). Accordingly, it has recently been shown that two or more molecular subtypes are present in almost 30% of patients (Tan et al. 2018). Assuming that these subtypes represent progeny of different CSCs, it is highly probable that regional differences do exist also within the CSC compartment. Moreover, any additional mutation or change in the epigenetic pattern such as DNA methylation or histone modifications will further increase the variability of CSCs. Thus, a systematic study is needed to elucidate the complete heterogeneity of CSCs in ovarian cancer and to determine their relationship to histopathological subtypes, clinical parameters, and molecular aberrations. With the recent advances in single cell analysis at the genetic, transcriptomic, and proteomic level, it is clearly the right time for this endeavor.

## 12.4 Heterogeneity of Ovarian Cancer Stem Cells and Possibilities of Their Therapeutic Targeting

Although many markers have been reported to be specific for ovarian CSCs (see above), our own investigations based on a systematic screen suggested that the SP phenotype was the most robust and reliable in defining this minority population (Boesch et al. 2014, 2016b). Ovarian cancer SP cells exhibited bona fide stem cell characteristics including tumorigenicity in vivo, single cell clonogenicity and sphere formation in vitro, multi-drug resistance, and asymmetric cell division (Boesch et al. 2014). Continuative analyses of ovarian cancer SP cells conducted in our laboratory employed multi-color flow cytometry and were initially aimed at dissecting the phenotype of these stem-like cells to potentially reveal novel therapeutic targets. Unexpectedly, we found that many of the investigated markers showed biphasic expression patterns, such that the stem cell compartment was further subdivided. Overall, we found a degree of heterogeneity in SP cells that was comparable to that of the bulk of non-SP cells. As SP cells typically account for 2% or less of total cells, we considered this a remarkable finding and hypothesized that ovarian CSC heterogeneity might be clinically relevant (Boesch et al. 2014). Specifically, we reasoned that heterogeneity in stem cell compartments might add another layer of complexity to their therapeutic targeting by increasing the statistical odds for spontaneous escape variants which then persist under treatment and sustain the tumor as (minimal) residual disease. Cellular diversification of ovarian CSCs may therefore lead to a “disguise in heterogeneity” phenomenon (Boesch et al. 2016a) that counteracts the success of cancer therapies especially in long-term.

Using flow cytometric analysis, we found that ovarian cancer SP cells overexpressed the alpha chain of the platelet-derived growth factor receptor (PDGFRα). Overexpression of PDGFRα in the stem cell compartment might explain the association of PDGFR signaling with tumor progression in epithelial ovarian cancer (Henriksen et al. 1993) as well as the more recently disclosed role of the PDGF/PDGFR axis in marking and filtering out platinum-resistant cancers (Avril et al. 2017; Zhang et al. 2018). PDGFRα can be principally targeted using small molecule inhibitors (Cortes and Kantarjian 2004; Joglekar-Javadekar et al. 2017) or monoclonal antibodies (Tap et al. 2016) and PDGFRα-directed therapy has gained FDA approval for the treatment of advanced soft tissue sarcoma in late 2016, in combination with the anthracycline doxorubicin (Klug and Heinrich 2017). However, we ultimately refrained from targeting ovarian CSCs using PDGFRα inhibition since these cells exhibited a non-uniform expression pattern for PDGFRα, with approximately 5–10% of cells lacking expression of this potential therapeutic target (Fig. 12.1). Thus, treatment failure would have been likely—or even inevitable.

A main conclusion that we drew from the heterogeneity of ovarian CSCs is that their eradication using direct targeting approaches is difficult to accomplish. We thus currently resort to the concept of indirect targeting harnessing particular non-transformed cell types of the tumor microenvironment (Boesch et al. 2016a). For example, in breast cancer, we found that a distinct population of cancer-associated fibroblasts, molecularly characterized by activity of the interleukin-7 promoter, sustains breast cancer stemness through provision of the C-X-C motif chemokine CXCL12. Pharmacological antagonism of the cognate receptor (i.e., CXCR4) or cell type-specific ablation of Cxcl12 in interleukin-7-producing cells largely abrogates tumor-initiating potential and slows down tumor growth (Boesch et al. 2018a). Thus, interleukin-7-expressing cancer-associated fibroblasts bear "druggable" niche activity for breast CSCs. We envision a similar strategy to combat ovarian CSCs as well. Indeed, different research groups have already provided promising proof-of-concept data for this novel therapeutic concept (Ahmed et al. 2018). Although fibroblasts may be a promising target in ovarian cancer as well, the unique cellular and anatomical niches present in the peritoneal cavity and the omentum suggest that other microenvironmental cell types, most notably mesothelial cells (Zeimet et al. 2012; Boesch et al. 2014) and adipocytes (Nieman et al. 2011; Ladanyi et al. 2018) may also be harnessed for CSC-directed therapy in ovarian cancer. The stage is thus set to mechanistically investigate which of these cell populations provide(s) essential niche activity for ovarian CSCs, and by which paracrine or juxtacrine factors these effects are mediated. This could reveal novel microenvironmental stem cell targets and ultimately lead to a paradigm shift in the treatment of ovarian cancer. The hope is to leverage treatment concepts that specifically target the residual cancer cells in remission, hence to prevent recurrence and improve long-term survival.

Despite the ambiguous phenotypic identity of ovarian cancer SP cells, we used this stem cell marker as a valuable platform for ovarian cancer biomarker discovery (Reimer et al. 2018). Unbiased gene expression profiling based on microarray technology demonstrated a unique genetic signature of ovarian cancer SP cells, which overexpressed and downregulated specific sets of genes, respectively. One of the

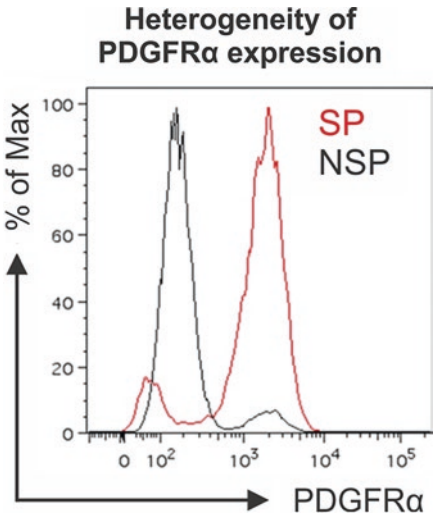


**Fig. 12.1** Expression pattern of PDGFRα in ovarian cancer stem cells. Flow cytometric analysis of PDGFRα (CD140a) expression in ovarian cancer SP and NSP cells from the cell line A2780. The stem-like SP cells show biphasic expression of PDGFRα, with roughly 10% and 90% of cells staining negative and positive for this marker, respectively. A similar, but inverse, distribution was detected in the bulk of NSP cells. Abbreviations used: NSP, non-side population; PDGFRα, platelet-derived growth factor receptor alpha chain; SP, side population

most highly expressed genes in the stem cell fraction happened to be a guanine nucleotide exchange factor that was specific for Rho/Rac family GTPases, Vav3 (Reimer et al. 2018). Vav3 serves specialized functions in cell motility/cytoskeletal rearrangements (Hornstein et al. 2004) and is further implicated in angiogenic responses within the tumor microenvironment (Brantley-Sieders et al. 2009). More importantly, we established that overexpression of Vav3 in the stem cell fraction was mainly due to a specific 5′-truncation variant, termed Vav3.1, which also showed high upregulation in primary ovarian cancer tissue (compared to the non-malignant ovary) (Boesch et al. 2018b; Reimer et al. 2018). Expression levels of Vav3.1 finally turned out to be of prognostic significance in ovarian cancer, with high-level expression correlating with disease progression and poor survival (Boesch et al. 2018b; Reimer et al. 2018). Vav3.1 message was also able to filter out those patients that never responded to platinum-based chemotherapy (Boesch et al. 2018b; Reimer et al. 2018), suggesting a particular role of Vav3.1 in mediating genuine (i.e., intrinsic, non-acquired) chemoresistance.

In sum, ovarian CSCs can be reliably identified using the SP phenotype. Notwithstanding, ovarian cancer stem cells are characterized by marked phenotypic and functional heterogeneity, which has implications for the design and conceptual realization of therapeutic targeting approaches. Heterogeneity of ovarian CSCs does not preclude meaningful (i.e., clinically relevant) biomarker inference from their transcriptomic landscape.

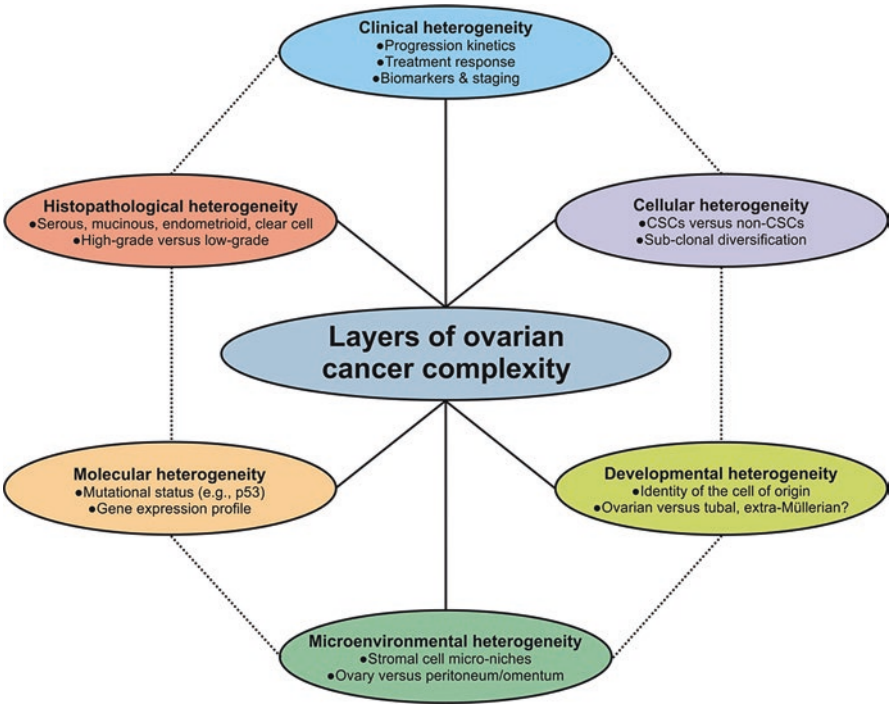


**Fig. 12.2** Factors implicated in ovarian carcinoma stem cell heterogeneity and complex relationships between them

The overall heterogeneity of ovarian cancer as seen in the clinic thus appears to be the final outcome of several distinct but interconnected mechanisms of diversification, several of which involve the subpopulation of CSCs either directly or indirectly (Fig. 12.2). These mechanisms establish several layers of tumor complexity that act along the entire path of the tumor evolution trajectory. It is likely that the clinical behavior of other solid tumor types is shaped at least in part by similar mechanisms of action.

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