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REVIEW

Lung cancer stem cells: a biological and clinical perspective

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Abstract
Introduction Lung cancer is the most lethal form of cancer in the world and despite significant therapeutic improvements that have been made, its survival rate still remains low. The latter is mainly due to the acquisition of resistance to systemic treatment regimens which, in turn, may be due to the presence of cancer stem cells (CSCs) within the primary tumors. CSCs constitute a subpopulation of cells that are highly tumorigenic and that exhibit biological properties similar to those of normal tissue stem cells, including an unlimited self-renewal capacity, an extensive proliferative capacity and a capacity to generate differentiated progeny. A better understanding of the signaling pathways that regulate lung CSC maintenance, proliferation, and tumorigenicity could thus lead to the design of improved approaches to lung cancer treatment.
Aim In this review we will discuss the current knowledge on lung CSCs, their biological properties and their putative clinical relevance. By employing currently available data, we will evaluate the prognostic value of several lung CSC markers. In addition, we will discuss the release of CSCs from tumor tissue into the blood circulation via epithelial-mesenchymal transition (EMT) as an important step towards acquiring a metastatic phenotype. Finally, we will provide an outlook into novel CSC-targeting approaches for achieving less invasive diagnostic procedures and improving long-term therapeutic options.
Conclusion Lung CSC research has gained considerable momentum to both basic and clinical applications, both aiming to identify a reliable panel of markers for lung CSCs and to clarify their function, with the final goal to develop a CSC-targeted therapy that will result in the complete elimination of CSCs for achieving significantly better long-time survival of lung cancer patients.

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1 Lung cancer characteristics

Lung cancer is the leading cause of cancer-related mortality in the world [1], which is due to its high incidence and recurrence rates. Despite advances in diagnostics and treatment achieved during the last two decades, the overall high mortality rate has remained [2]. Small cell lung cancer (SCLC) is a neuroendocrine tumor that represents about 20 % of all lung cancers, whereas the most common forms of non-small cell lung cancer (NSCLC) include adenocarcinoma (40 %), squamous cell carcinoma (25 %) and large cell carcinoma (10 %) [3]. Despite continuous efforts to improve therapeutic outcomes, including platinum doublets, maintenance chemotherapy and targeted therapy with epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors, the overall 5-year survival rate for lung tumors is still below 15 % and therefore, improvements in both diagnostics and treatment are urgently needed [1, 2].

2 Identification of lung tissue stem cells

Normal lung tissue comprises various cell types, including basal mucous secretory cells of the trachea and bronchi, Clara cells of the bronchioles, and Type I/II pneumocytes of the alveoli. All these mature cell types originate from lineage-restricted lung progenitor cells which, in turn, are derived from undifferentiated multi-potent lung tissue stem cells [4]. Like stem cells of other tissues, lung tissue stem cells are required for tissue maintenance and injury repair [5]. They are defined by their ability to self-renew, to produce differentiated progeny and to proliferate extensively [6]. A pulmonary stem cell population was first identified at the bronchioalveolar duct junction and, accordingly, these cells were termed bronchioalveolar stem cells (BASCs) [7]. Because cultured BASCs were found to

exhibit several stem cell properties, with their transformed counterparts even giving rise to adenocarcinomas, this work provided a first clue for the notion that pulmonary stem cells may represent a target population for lung cancer treatment.

3 A cancer stem cell model of tumorigenesis

The term cancer stem cells (CSCs) refers to a minute subset of exclusively tumorigenic cells with the ability to self renew and to generate the diverse cell types present within a given tumor [6, 8]. The exact origin of CSCs still remains to be resolved, but both the prerequisite of several mutations to occur in a cell to become malignant and the limited possibility for sufficient oncogenic mutations to accumulate in differentiated cells indicate that these mutations may preferentially arise in tissue stem cells [9]. It has also been revealed that normal tissue stem cells and CSCs share several stem cell-associated characteristics, such as the expression of primitive stem cell markers and the presence of telomere reverse transcriptase activity [10]. Also, both cell types exhibit resistance to therapeutic drugs, possibly through an elevated expression of multidrug resistance proteins [11, 12], the presence of efficient DNA repair mechanisms, and/or a slow cell cycle progression [13, 14]. Additionally, it has been reported that CSCs may arise from mutated differentiated cells that have regained self-renewal by de-differentiation to a progenitor-like state [15].

The similarities observed between normal tissue stem cells and CSCs has led to the development of a CSC model of tumorigenesis [6]. The hypothesis that tumors may originate from cancer-initiating cells with stem-like characteristics was first confirmed in acute myeloid leukemia [16, 17], and later in breast, brain, prostate, and other solid tumors, including lung tumors [18–22]. Since most tumors are clonal in origin, tumorigenic cancer stem cells must have the ability to give rise to a phenotypically diverse progeny [6] resulting, at e.g. the histological level, in heterogeneous tumor regions exhibiting different degrees of proliferation, differentiation, invasiveness, vascularity and inflammation [15, 23].

4 Identification of lung cancer stem cells

In the recent past, lung cancer stem cells have been identified as a subset of cells that exclude Hoechst 33342 dye, are drug-resistant, and are positive for CD133 (CD133+), aldehyde dehydrogenase ($ALDH^{high}$) or CD44 (CD44+) expression (Table 1). Ho et al. [29] were the first to isolate a subpopulation of cells (i.e., a side population, SP) by their ability to exclude Hoechst 33342 dye from six human lung cancer cell lines. These SP cells were found to exhibit tumor-initiating abilities, high invasive characteristics, and a resistance to chemotherapeutic drugs. In addition, they exhibited elevated membrane-bound transporter ABCG2 and telomerase expression levels when compared to non-SP cells. A consecutive study showed that SP cells from SCLC cell lines comprise less than 1 % of the bulk population, are highly tumorigenic and over-express genes associated with stemness and drug resistance features [30].

An inherent resistance to toxic compounds also allows for the selection of CSCs [6]. Following this approach, increased drug resistance to doxorubicin, cisplatin or etoposide was exploited for the isolation of chemoresistant lung CSCs from a drug treatment-enriched H460 lung cancer cell line. Isolated drug-resistant cells were found to be highly tumorigenic, to be able to self-renew and to generate differentiated progeny. Moreover, they predominated in SP cells expressing CD133 and several embryonic stem cell markers [31]. In support of this approach, a recent study confirmed that cisplatin treatment selects for multidrug-resistant CD133-positive cells in lung cancer H460 and H661 cells [32]. Similary, treatment with the tyrosine kinase inhibitor gefitinib was found to affect various stem cell properties, including sphere-forming capacity, increase in SP cells and ALDH1A1 over-expression, in a gefitinib-resistant lung cancer HCC827-derived cell line [33].

Next to drug resistance, the employment of stem cell markers remains the most widely used approach for identifying lung CSCs. Through the assessment of CD133 expression, Eramo et al. [20] managed to identify CD133-positive lung CSCs in 19 primary human NSCLC and SCLC specimens. These CD133-positive cells were shown to proliferate indefinitely as tumor spheres, to display resistance to chemotherapeutic drugs and to generate tumor xenografts in immunocompromised mice. Bertolini et al. [34] independently reported similar findings using CD133-positive/EpCAM-positive cells isolated from 60 primary human NSCLC samples, and another study suggested that CD133-positive progenitor cells may play a role in NSCLC tumor vasculogenesis [35]. In addition, the embryonic transcription factor Oct-4 was found to be crucial for maintaining the CSC phenotype in CD133-positive NSCLC cells [36].

ALDH enzyme activity was first successfully measured in lung cancer cell lines by Moreb et al. using an adapted Aldefluor assay [37]. Subsequently, the Aldefluor assay was used in conjunction with fluorescence-activated cell sorting (FACS) to isolate $ALDH^{high}$ cells from human lung cancer cell lines. These isolated cells showed both self-renewal and differentiation capacities, multidrug resistance and CD133 expression. After xenotransplantation into athymic nude mice, these cells generated tumors that recapitulated the heterogeniety of the parental lung tumors [38]. Two other groups independently reported similar results by utilizing both cell lines and primary tumor samples [39, 40]. Also, CD44-positive NSCLC cells were shown to be enriched for CSCs, as these cells were found to be tumorigenic both in vitro and in vivo, to express

Table 1 Lung CSCs may exhibit different biomarkers

CSC biomarker	Description	Reference
Hoechst SP	• Hoechst 33342 dye excluding cells, termed Side Population (SP) • Enriched with stem-like properties in a variety of tumor types • Activity in cells conferred by ABCG2	[24]
ABCG2	• Breast cancer–resistance protein • ATP-binding cassette superfamily G-member 2 protein, superfamily of ABC transporters • Displays affinity for numerous cytotoxic molecules	[25]
CD133/PROM1	• Cell-surface pentaspan glycoprotein • Expressed by stem cells and CSCs in various tissues • Role in organization of plasma membrane topology	[26]
ALDH1	• Belongs to the ubiquitous aldehyde dehydrogenase family • Cytosolic enzyme involved in cellular detoxification, differentiation, and drug resistance • Oxidizes cellular aldehydes (retinol to retinoic acid) and contributes to early stem cell differentiation	[27]
CD44	• Membrane-bound glycoprotein with multiple isoforms • Mediates tissue remodeling, cell migration, and cell-to-extracellular matrix adhesion • Major receptor of hyaluronan, carbohydrate polymers of the extracellular matrix.	[28]

embryonic stem cell markers and to be resistant to cisplatin treatment [41].

Taken together, it can be concluded that CD133-positive and $ALDH^{high}$ SP cells represent phenotypically distinct subpopulations, and that enrichment for CSCs using one marker set does not necessarily correlate with CSCs identified by another marker set [42]. It has even be reported that over 45 % of A549 and H446 lung cancer cells may serve as cancer-initiating cells, as determined by cloning and tumorigenicity assays, although many of them were neither CD133-positive nor otherwise specified as SP cells [43].

5 Prognostic significance of lung CSC markers

The therapy response rate has since long been considered a key factor in the outcome of advanced lung cancer treatment, where it is commonly reported as a secondary endpoint. However, especially in advanced disease, a discordance has been noted between the early response rate to therapy and long-term outcome. In the SCLC extensive disease, the response rate to standard platinum-based chemotherapy ranges from 70 % to 80 %, whereas the median survival time of these patients is only 9 to 10 months [44]. Therefore, only major differences in objective response rates will allow the prediction of a survival advantage [45]. Alternatively, the relationship between disease response and survival endpoints may depend on the percentage of CSCs present within a tumor. Chemotherapeutics primarily target the bulk of the tumor cells, allowing rare CSCs with increased resistance to survive and, ultimately, to dictate long-term disease outcome [46]. Moreover, standard drugs preferentially kill the rapidly proliferating cells within the tumor mass, thereby favoring an increase of quiescent CSCs within new emerging tumor niches [13]. According to this concept, the ultimate therapy goal would be a complete depletion of CSCs within a tumor. This does not necessarily have to be associated with an immediate reduction of the overall tumor mass.

The identification of new biomarkers for both diagnosis and treatment may provide new tools for improved lung cancer patient care [47, 48]. So far, several studies have been aimed at investigating the potential clinical impact of lung CSC markers. A summary of the results obtained so far is presented in Table 2. Although CD133 expression has been identified as an independent prognostic marker in hepatocellular carcinomas and gliomas [60, 61], in lung cancer only one study [50] out of several [10, 34, 35, 49–52, 55] has revealed an association between elevated CD133 protein expression and a shorter disease-free survival (DFS), with sustained significance also after a multivariate analysis [50]. Likewise, up-regulated CD133 mRNA expression was found to be associated with shorter DFS in another independent study [59]. Moreover, simultaneous CD133 and proliferating marker Ki-67 expression was found to be associated with a significantly higher crude recurrence rate (27.9 % vs. 4.5 %, $p<0.001$) and a worse DFS compared to non-expressers [50]. In addition, CD133 expression was found to correlate with the expression of several resistance-related proteins ($p=0.01$) [49], and co-expression of CD133 and ABCG2 was found to predict postoperative recurrence in Stage I NSCLC patients [51].

ALDH1 expression was found to be associated with a worse clinical outcome in patients with breast and prostate cancer [62, 63], whereas elevated ALDH1 expression has emerged as a favorable prognostic factor in ovarian carcinomas [64]. In four studies on lung cancer [38, 40, 55, 59], higher ALDH1 protein expression was found to be associated with a worse overall survival (OS) as assessed by univariate analysis [38, 40] and, in addition, to act as an independent prognostic marker for DFS and cancer-specific survival (CSS) [38].

Table 2 Prognostic value of CSC biomarkers in lung cancer tissues

Biomarker analyzed	Type	Tumor stage	Specimen type *(n)*	Method of analysis (cutoff value)	Therapy (% of patients)	DFS/PFS[a]/RFS[b]		OS/CSS[c]		First author (year, reference)
						UV	MV	UV	MV	
CD133	NSCLC	Local (Stage I–III)	Snap frozen (79)	IHC (0.7 %)	S (100 %)			NS		Hilbe (2004, [35])
CD133	NSCLC	Advanced (Stage IIIB/IV)	FFPE (42)	IHC (n.d.a.)	Cisplatin CT (100 %)	NS[a]				Bertolini (2009, [34])
CD133	NSCLC	Local (Stage I–III)	FFPE (88)	IHC (> 10 %)	S (69 %) S+CT (14 %) S+RT (17 %)			NS		Salnikov (2010, [49])
CD133	ADC	Local (Stage I)	FFPE (177)	IHC (> 17.5 %)	S (100 %)	p=0.004	p=0.01			Woo (2010, [50])
ALDH1	NSCLC	Local (Stage I)	FFPE (300)	IHC (>10 %)	n.d.a.		p=0.016	p=0.009 p=0.006[c]	p=0.016[c]	Jiang (2009, [38])
CD133 ALDH1A1	NSCLC	n.d.a.	FFPE (> 200)	IHC (n.d.a.) IHC (n.d.a.)	n.d.a.			NS p=0.025		Sullivan (2010, [40])
ALDH3A1				IHC (n.d.a.)				NS		
CD44	NSCLC	Advanced (Stage I–III)	FFPE AD (96) FFPE SCC (27)	IHC (moderate/ strong)	n.d.a.	NS[a] NS[a]		p=0.015 NS		Leung (2010, [41])
CD133 ABCG2	NSCLC	Local (Stage I)	FFPE (145)	IHC (> 1 %) IHC (> 10 %)	S (100 %)	NS[b] NS[b]				Li (2011, [51])
CD133+ABCG2							p=0.015[b]			
CD133 ABCG2	NSCLC	Local (Stage I/II)	FFPE (133)	IHC (> 0 %) IHC (> 0 %)	n.d.a.	NS NS	NS NS	NS NS	NS NS	Herpel (2011, [52])
C-kit				IHC (> 0 %)		NS	NS	NS	NS	
Bmi-1	NSCLC	Local (Stage I–III)	FFPE (179)	IHC (> 10 %)	S(62 %) S + adjuvant CT (12 %) S + neoadjuvant CT (26 %)	NS p=0.05 (Stage I/II); NS (Stage III) NS		NS NS NS		Vrzalikova (2008, [53])
Bmi-1 EZH-2	NSCLC	Local (Stage I–IV)	FFPE (157)	IHC (> 10 %) IHC (> 25 %)	S (100 %)			NS p=0.006 (Stage I)	p=0.048 (Stage I)	Kikuchi (2010, [54])
CD133 ALDH1	NSCLC	Locally advanced N2 or N3	FFPE (30)	IHC (> 1 %) IHC (> 10 %)	Induction chemoradiotherapy+ S	NS NS	NS NS	NS NS	NS NS	Shien (2012, [55])
ABCG2				IHC (> 10 %)		NS	NS	NS	NS	
Bmi-1				IHC (> 5 %)		NS	NS	NS	NS	
CD133/ALDH						p=0.05		p=0.042	p=0.047	
c-Myc	SCLC	All SCLC Local (minor RR) Advanced (minor RR)	FFPE (102)	IHC (> 10 %)	CT			NS NS p=0.0032		Micke (2003, [56])
Oct-4	ADC	Local (Stage IA–IIIB)	FFPE (107)	IHC (> 0.7)	S			p=0.019	p=0.015	Zhang (2010, [57])
Oct-4 Sox-2	NSCLC	Stage I–IV	FFPE (44)	IHC (>10 %) IHC (>10 %)	n.d.a.			NS	p=0.008	Li (2012, [58])

Table 2 (continued)

Biomarker analyzed	Type	Tumor stage	Specimen type (*n*)	Method of analysis (cutoff value)	Therapy (% of patients)	DFS/PFS[a]/RFS[b]		OS/CSS[c]		First author (year, reference)
						UV	MV	UV	MV	
CD133	ADC	Local (Stage I-III)	RNAlater (64)	qRT-PCR (n.d.)	S (100 %)	$p=0.033$				Cortes-Dericks (2012, [59])
ALDH						NS				
OCT4A						$p=0.047$				
BMI-1						n.d.a.				
SOX2						n.d.a				
µPAR						n.d.a.				

DFS disease-free survival; *PFS* progression-free survival; *RFS* recurrence-free survival; *CSS* cancer-specific survival; *UV* univariate analysis; *MV* multivariate analysis; *NSCLC* non–small cell lung cancer; *IHC* immunohistochemistry; *NS* non-significant; *n.d.a.* no data available; *FFPE* formalin fixed and paraffin embedded; *S* surgery; *CT* chemotherapy; *RT* radiotherapy; *CRT* chemoradiotherapy; *ADC* adenocarcinoma; *SCLC* small cell lung cancer; *qRT-PCR* quantitative reverse transcriptase-polymerase chain reaction

[a] Applies to PFS

[b] Applies to RFS

[c] Applies to CSS

Although lower CD44 expression was found to be associated with shorter survival rates in glioblastoma multiforme and breast carcinoma patients [65, 66], so far in only one study the prognostic value of CD44 expression in lung cancer has been evaluated. In this study, a negative or weak positive CD44 protein expression was found to be associated with a worse OS in the adenocarcinoma, but not in the squamous carcinoma patient group [41].

In addition, the expression of CSC-associated transcription factors could provide prognostic information on the clinical behavior of lung cancers. Among them, Bmi-1 has been shown to sustain stem cell properties in normal and cancerous lung tissues [67, 68]. Increased Bmi-1 expression has also been shown to correlate with a poor prognosis in hepatocellular and gastric carcinoma patients [69, 70], and with a favorable prognosis in breast cancer patients [71]. In lung cancer patients, however, Bmi-1 positivity was not found to be associated with OS in two studies encompassing in total 338 NSCLC [53, 54]. The embryonic stem cell markers Oct-4, Sox-2, c-Myc and Nanog, all expressed also by CSCs, are transcription factors known to be involved in early embryonic development and differentiated cell reprogramming [72, 73]. In extensive SCLC with a minor chemotherapy response rate, positive c-Myc expression was found to be associated with a decreased OS [56]. Similarly, Oct-4 expression was found to correlate significantly with a worse survival rate [57–59], even in multivariate analyses [57, 58], whereas a simultaneous increased expression of Oct4, Nanog and the transcriptional E-cadherin repressor Slug was found to be associated with a worse prognosis in lung adenocarcinoma patients [74].

Taken together, the picture emerges that the prognostic significance of the various lung CSC biomarkers tested so far remains, as yet, controversial. In case of CD133, only one study with 177 patients enrolled showed its independent prognostic value for a decreased PFS [50]. Similarly, ALDH1A1 expression was found to serve as an independent marker for decreased DFS and OS in only one study with 300 patients enrolled [38]. Both studies indicate that only validations in large cohorts of patients may reveal the true clinical significance of the currently recognized lung CSC markers. In addition, the identification of new CSC biomarkers will be crucial for a better understanding of the process of lung CSC-related pathogenesis and for its clinical translation into reliable prognostic and predictive therapy markers.

6 Targeting self-renewal signaling pathways in CSCs

Cancer has been denoted as a developmental disease in which normal developmental signaling pathways have been co-opted by oncogenic processes during pathogenesis [10]. Wnt, Notch, Hedgehog (Hh) and, more recently, Bmi-1 signaling pathways have been shown to regulate the behavior of normal stem cells, as well as their neoplastic transformation

when deregulated. Several reviews have addressed the roles of these genes and their concomitant pathways in the processes of stem cell renewal and tumorigenesis [6, 9, 75–78].

A possible therapeutic strategy for eliminating CSCs is to specifically target the signaling pathways and/or transcription factors that are involved in CSC maintenance and/or proliferation. The Hh signaling pathway is known to be active in SCLC tumors [79], and its blockade has been shown to reduce the growth of SCLC tumor xenografts in mice [80]. Likewise, impairment of the Wnt-2 signaling pathway, which is aberrantly activated in lung tumors, has been shown to induce apoptosis in human NSCLC cell lines [81], and suppression of elevated Notch signaling in $ALDH^{high}$ cells has been shown to result in a significantly decreased ALDH activity and a concomitant reduction in proliferative and clonogenic potential of lung CSCs [40]. Also, blocking the SCF-c-kit autocrine loop, or knocking down Oct-4 expression, has led to a complete elimination of lung CSCs [82, 83]. Moreover, Bmi-1 deficiency was shown to suppress de novo tumor development in a mouse lung cancer model by limiting the expansion potential of BASCs [67]. Nevertheless, because CSCs share several stemness characteristics with normal stem cells, targeted anti-CSC therapies should be designed to preserve normal stem cells and to only hit CSC-specific signaling pathways [84].

Although in preclinical models CSCs have amply been shown to be responsible for the behavior of cancers, clinical studies evaluating CSC-directed treatment strategies in lung cancer are still scarce and mostly in the very early phases of development. OMP-21M18 is a monoclonal antibody designed to specifically target CSCs. The combination of OMP-21M18 with carboplatin and pemetrexed is currently being investigated in a phase 1b trial as first line treatment for patients with NSCLC (ClinicalTrials.gov identifier: NCT01189968). In the phase 1b/2 »PINNACLE« trial, Anti-Notch 2/3 is being tested in combination with cisplatin and etoposide in first-line extensive-stage SCLC patients (ClinicalTrials.gov identifier: NCT01859741). Tariquidar, an ABC transporter inhibitor, in combination with docetaxel, has been explored for the treatment of recurrent metastatic solid tumors in a phase 2 trial (ClinicalTrials.gov identifier: NCT00072202). The results of this latter trial have pointed at a possible therapeutic efficacy of this drug, particularly in lung cancer patients [85]. Clearly, since most studies on the therapeutic efficacy of CSC-targeting drugs are still in their early phases, more information awaits to be gathered before any firm conclusions can be drawn on the clinical importance of these drugs.

7 Role of EMT in the acquisition of CSC traits and metastasis

Epithelial-mesenchymal transition (EMT) and reverse mesenchymal-epithelial transition (MET) represent highly conserved and fundamental processes that normally take place during embryonic development. Via EMT, certain polarized epithelial cells may undergo morphogenetic changes that lead to a loss of apical-basolateral polarity and a disintegration of cell-cell junctions. Consequently, this may result in the formation of migratory mesenchymal cells with a highly invasive potential. These cells can travel to distant locations of the developing embryo and participate at these locations in the formation of epithelial organs via reverse MET [86]. Loss of E-cadherin expression is considered to be a hallmark of EMT. This loss is followed by up-regulation of the expression of several mesenchymal markers, such as Vimentin and N-cadherin, and an increased activity of matrix metalloproteinases, which is associated with an increased invasive potential [87]. Recently, activation of the EMT/MET cascade has been implicated in the process of (lung) cancer metastasis [88, 89], in which disseminated cancer cells seem to acquire self-renewal capacities similar to those observed in tissue stem cells [90]. Ample studies have suggested that EMT may induce stemness properties in normal and malignant cells [91–93]. Also, activation of EMT has been associated with a decreased drug sensitivity and it has been found that it may even contribute to a decreased efficacy of therapy and resistance to tyrosine kinase inhibitors in EGFR-mutated NSCLCs [94–96], apparently through the acquisition of stem cell-like properties by the tumor cells [33]. Therefore, cancer cells undergoing EMT may indeed become metastatic drug-resistant cancer cell progenitors, or even metastatic CSCs.

8 Are CSCs present among circulating tumor cells?

The detection of circulating tumor cells (CTCs) in the peripheral blood of a patient with metastatic cancer was first described in 1869 [97]. CTCs were later defined as tumor cells originating from either a primary tumor or as metastatic cells that circulate freely in the peripheral blood of carcinoma patients [98]. According to the 'seed and soil' theory of metastasis development, tumor cells may enter the peripheral blood circulation after detaching from the primary tumor and circulate to reach distant organs, where they re-attach and give rise to metastases [99]. The presence of disseminated tumor cells (DTCs) in the bone marrow or CTCs in the peripheral blood of patients with primary lung cancer has been shown to correlate with shorter survival rates [100–104], or to predict disease recurrence [105] and chemotherapy response failure [101].

Current CTC detection methods mainly rely on the presence of epithelial markers, and thus tend to underestimate subpopulations of CTCs that lack epithelial marker expression but exhibit EMT and/or stem cell-like traits [106]. Breast cancer CTCs were shown to express mesenchymal markers as well [107], allowing the prediction of a worse

prognosis more accurately than the expression of cytokeratins alone [108]. Basically, each type of tumor cell can be 'circulating'. It is likely, however, that metastases develop from a restricted population of tumor cells that have undergone EMT and that have acquired the ability to migrate to distant sites via the blood circulation, to colonize other tissues, and to initiate de novo tumor growth (Fig. 1). It has been suggested that this process may be explained by the presence of cells expressing putative CSC markers among disseminated tumor cells in the bone marrow of e.g. early breast cancer patients [109]. Several studies have subsequently confirmed that a proportion of CTCs found in the peripheral blood of liver and breast cancer patients indeed exhibits CSC-associated markers [110, 111]. Accordingly, elevated CD133 mRNA expression levels in peripheral blood mononuclear fractions of progressive cancer patients with bone metastases were found to independently predict a shorter OS [112]. Circulating CSCs may evolve from stationary CSCs by combining two decisive features: stemness and the ability to undergo EMT [113]. In terms of clinical use, the number of circulating CSCs could reflect the degree of metastatic progression, and their decrease or absence could indicate a favorable response to systemic treatment. Phenotyping of CTCs using CSC markers may thus provide additional information on tumor aggressiveness.

9 Conclusions and future directions

Through the worldwide acceptance of the CSC paradigm, in recent years lung CSC research has gained considerable momentum for both basic and clinical applications. Both are aiming to (i) identify a reliable panel of markers for lung CSCs and (ii) clarify their function in order to be able to use them for more accurate disease outcome prediction and the development of novel therapies. The final goal is to develop a CSC-targeted therapy that will result in the complete elimination of CSCs. Presumably this could be achieved through the disruption of signaling pathways that control the self-renewal, proliferation and differentiation capacities of CSCs. Although scarce, such therapeutic approaches are already under development. However, in order to be able to only target CSC-specific pathways and, at the same time, preserve normal tissue (stem) cells, a better identification of drug targets unique for CSCs is a prerequisite. From a clinical perspective, a complete elimination of CSCs seems crucial for achieving a significantly better long-term survival of lung cancer patients. In addition, a precise and robust CSC quantification method would provide important information regarding tumor aggressiveness, treatment response and prognosis, and would help clinicians to design targeted personalized treatment regimen by selecting patient-tailored drugs. Because a single definite lung CSC marker has not been identified yet, a combination of markers may be required for a reliable quantification of CSCs. The acquisition of additional evidence that the elimination of CSC leads to improved patient survival will provide the most definitive proof of the clinical relevance of CSCs.

Finally, the identification of circulating CSCs in peripheral blood samples of (lung) cancer patients could represent a 'real-time biopsy' strategy for continuous monitoring of

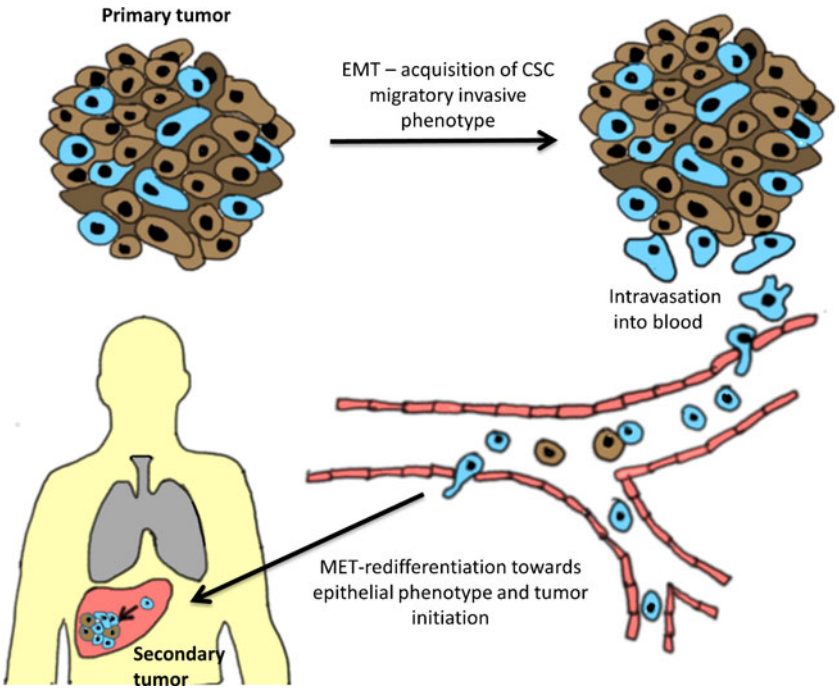

Fig. 1 Tumor tissue is heterogeneous because it consists of both bulk tumor cells (*brown*), composing the tumor mass, and a minute population of CSCs (*blue*). Tumor cells may acquire an invasive migratory CSC phenotype via EMT, which enables them to enter the blood circulation, via which they reach distant organs and eventually implant themselves via reverse MET and consequently give rise to metastases

treatment response and, thus, provide an early non-invasive diagnostic tool for detecting tumor progression. Accordingly, it is anticipated that the identification of circulating CSCs and their characterization will play an increasingly important role in the diagnosis and treatment of cancer patients.

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