Cancer Stem Cells in Lung Cancer: Roots of Drug Resistance and Targets for Novel Therapeutic Strategies

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Abstract Lung cancer represents the leading cause of cancer-related deaths worldwide due to its high incidence and the lack of effective therapies. Current pharmacological strategies for the treatment of advanced stage disease are in fact largely ineffective mostly due to the emergence of drug resistance. The cancer stem cell (CSC) hypothesis suggests that treatment failure and tumor relapse may be explained by the existence of a subset of self-renewing cancer cells endowed with tumor-initiating potential which are able to escape conventional and targeted therapies and to regenerate tumors.

In this chapter we will first focus on the description of studies which led to identification and characterization of CSCs in lung cancer according to their expression of specific markers and/or functional properties and will discuss the potential clinical value of CSC-related markers to predict patients' outcome and response to therapies. We will next review evidences supporting the proposed mechanisms of resistance of CSCs to chemotherapy and targeted therapies and in particular intrinsic CSCs' properties such as enhanced activation of the DNA damage repair machinery and anti-apoptotic signaling, increased expression of drug transporters, activation of self-renewal pathways, and quiescence status. The ability of tumor microenvironment (TME)-derived signals to modulate CSC phenotype, especially through the induction of epithelial mesenchymal transition, has also been demonstrated to contribute to drug resistance. Here we will discuss the interconnection among TME signals, modulation/generation of CSC, and development of resistance to both conventional and targeted therapy in lung cancer. Finally we will present novel strategies based on targeting of specific pathways activated in CSCs or able to impair the cross talk between TME and CSCs and aimed at eradication of the CSC subsets, which have been already tested or are currently under investigation in clinical trials in advanced lung cancer.

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C. Maccalli et al. (eds.), *Cancer Stem Cell Resistance to Targeted Therapy*,
Resistance to Targeted Anti-Cancer Therapeutics 19,
https://doi.org/10.1007/978-3-030-16624-3_3

Despite some still ongoing controversies regarding the best strategy/markers to define the stem cell population in lung cancer, several evidences support the resistance of lung CSC to conventional and targeted therapies providing a new perspective for the understanding of drug resistance mechanisms and indicating the path for development of innovative targeted therapies that may ultimately improve clinical outcome of lung cancer patients.

Keywords Lung cancer · Cancer stem cells · Chemoresistance · CD133 · ALDH Targeted therapy · Tumor microenvironment · EMT · ABC transporters · Notch Wnt

Abbreviations

ABC	ATP-binding cassette
ADC	Adenocarcinoma
ALCAM	Activated leukocyte cell adhesion molecule
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ATRA	All-trans retinoic acid
CAF	Cancer-associated fibroblast
CSC	Cancer stem cells
CXCR4	Chemokine receptor type 4
DDR	DNA damage response
DFS	Disease-free survival
Dhh	Desert hedgehog
DSBs	Double-strand breaks
Dvl	Disheveled proteins
EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor tyrosine kinase inhibitor
EMT	Epithelial to mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
Hh	Hedgehog
Ihh	Indian hedgehog
IL-6	Interleukin-6
LC	Large cell carcinoma
MIC	Metastasis initiating cell
MMP	Metalloproteinase
NSCLC	Non-small cell lung cancer
PDX	Patient-derived xenograft
PTCH	Patched

SCC	Squamous-cell carcinoma
SDF1	Stromal cell-derived factor 1
Shh	Sonic hedgehog
SP	Side population
TGF-β	Transforming growth factor beta
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
VEGFR	Vascular endothelial growth factor receptor
ZEB	Zinc finger E-box-binding

1 Lung Cancer Stem Cells (CSCs): Introduction

Lung cancer represents the leading cause of cancer-related mortality worldwide and is estimated to be responsible for more than 1.5 million deaths/year [1]. Despite recent advances in early detection strategies and development of novel pharmacological treatments, prognosis remains poor especially for advanced stage disease in which current strategies result in 5-year survival rates of less than 15% mainly due to inefficient control of relapsing disease and metastatic dissemination [2]. Inherent and acquired drug resistance represents therefore a significant clinical challenge in the treatment of lung cancer and, in particular for its most frequent type, non-small cell lung cancer (NSCLC) which accounts for 80–85% of all lung cancers. Drug resistance is a multifactorial phenomenon dependent on several characteristics of both cancer cells and surrounding microenvironment [3]: in this chapter we will review the role of cancer stem cells in this mechanism.

The cancer stem cell (CSC) model suggests that tumors are arranged in a hierarchical structure, at the apex of which a small subset of stem-like cells are responsible for tumor initiation and maintenance [4]. Mounting evidence suggests that CSCs play a critical role not only in tumor formation but also in metastasis and drug resistance [5]. Most current anticancer therapies in fact may fail to eradicate CSC clones due to their inherent drug resistance, resulting in their selection. CSCs spared by therapy may regenerate the original tumor (local relapse) or disseminate to distant organs driving tumor recurrence and metastasis. CSCs are characterized by a strong resistance to currently adopted therapies, such as chemotherapy and radiotherapy, due to their slowly proliferating nature, the intrinsic high levels of anti-apoptotic molecules, their relative resistance to DNA damage and the high activity of the detoxification machinery involved in drug extrusion [4, 6, 7]. Moreover CSCs can also resist to molecular targeted therapy due to the activation of specific pathways able to bypass drug activity [8].

The identification of CSC-specific markers and/or subsets, as the first step to devise novel therapeutic targets to specifically hit CSCs, is therefore becoming a compelling issue to overcome drug resistance and tumor recurrence to ultimately improve clinical outcomes of lung cancer patients.

1.1 Stem Cells in Lung Cancer

The cancer stem cell model proposes that tumors are organized into an aberrant "organ-like" hierarchy driven by a subset of cells endowed with the ability to self-renew and generate the heterogeneous cell population representing the tumor bulk [4]. The first experimental evidence supporting the existence of CSCs came from human acute myeloid leukemia (AML) with the demonstration that only rare malignant cells had the ability to reform the original disease over several transplantations, implying self-renewal and differentiation ability [9] [10]. Hierarchical organization in solid tumors was later experimentally demonstrated in breast cancer [11], and subsequently CSCs have been identified in other solid tumors including cancers of the brain [12, 13], colon [14–16], head and neck [17, 18], pancreas [19–21], melanoma [22–24], liver [25, 26], stomach [27], prostate [28, 29], ovary [30], and lung [31, 32].

Indeed the first experimental evidences for the existence of a stem-like clonogenic subpopulation in lung cancer were provided in the 1980s [33, 34]. These pioneering studies demonstrated that a very small proportion (<1.5%) of SCLC and lung adenocarcinoma cells from patient samples possessed the ability to generate colonies in soft agar that demonstrated tumor initiation potential when transplanted into nude mice. More recently the identification and isolation of lung CSCs have relied on the expression of specific surface markers [31, 32] or on their functional properties. Several markers have been proposed to identify lung CSCs, and up to now there is not a common consensus about the definition of the unique or combination of markers for identification of CSCs. Several studies reported similar CSC features for lung tumor cell subpopulation expressing different markers, and other works demonstrated that these cell subsets are not overlapping, presumably suggesting the existence of different lung CSC subpopulations [35].

The origin of CSCs remains a controversial issue: they may come from neoplastic transformation of normal stem cells in which the self-renewal machinery is already endogenously activated or from progenitor/differentiated cells that have reacquired properties of stem cells during the oncogenic transformation process [36]. A prerequisite for experimental investigation of CSCs is that these cells should be prospectively identified and isolated to test their functional properties: it follows that adequate cell-specific markers are needed and these can be sometimes inferred by properties of normal stem cells of the tissue of origin. Compared to other cancer types, however, less is known about the biology of lung cancer stem cells. This is in part due to the complexity of this disease in terms of phenotypic diversity and anatomically distinct sites of cancer origin in the pulmonary airways. The existence of distinct subsets of lung stem cells responsible for homeostasis of different anatomically defined regions of the respiratory tract which may represent the cells of origin of the different lung cancer histological subtype has been proposed to explain the diversity encountered in lung cancers. In support of this hypothesis, it has been demonstrated in murine models that sites of origin of the different histological subtypes of NSCLC (i.e., squamous-cell carcinoma (SCC),

adeno-/bronchoalveolar carcinomas (ADC), and large cell carcinomas (LC)) coincide with distinct airway stem cell niches [37]. Indeed exploiting transgenic mouse models in which lung cancer was induced by oncogene activation or tumor suppressor knockout under the control of lung epithelial cell-specific promoters, several studies have demonstrated that genetic modifications in the stem cell subsets specific for proximal airway (basal cells), mid-level airway (Clara cells), and distal airway (bronchoalveolar stem cells) give rise to histologically different lung tumors [38–40], thus supporting the concept that normal airway stem cells can act as cell of origin for lung cancers [41].

Until now, resident lung stem/progenitor cells of different anatomically defined regions of the airway epithelium had only been identified in the lungs of mice. However the evidence derived from murine model suffers from the constraint of genetic manipulation and cannot be easily translated to humans also because of species specificity of some of the markers adopted to identify murine stem cells (e.g., Sca-1) for which no human counterpart is known. Recently Kajstura et al. [42] presented the first evidence for the existence in adult human lungs of multipotent resident lung stem cells that could induce lung repair following injury. These cells, identified by the *c-kit* maker, are able in vivo to originate novel airway structures and vasculature successfully rebuilding the complete lung architecture. The existence of a multipotent lung stem cells in humans remains however controversial, and alternative evidence has been provided showing that c-kit(+) cells did not contribute to lung epithelium regeneration and homeostasis, but rather represented the progenitor endothelial cells able to reconstruct damaged lung vasculature [43].

Therefore until now no consensus has been reached on the definition of the human lung stem or progenitor cells specific for different regions of the respiratory tract. This lack of knowledge regarding normal lung stem cells also hampers the possibility to uniquely define lung cancer stem cells; indeed many controversies are still ongoing regarding the best strategy/markers to identify stem cell population in lung cancer.

An additional layer of complexity comes from recent evidence showing that differentiated tumor cells may also revert to CSCs' status under specific stimuli from tumor microenvironment [44–46]. The CSCs' compartment might even in itself be heterogeneous and comprise different subsets responsible for primary tumor initiation/maintenance, drug resistance, and metastatic dissemination [5, 20]. The intrinsic plasticity of tumor cells, which are capable of acquiring CSCs' properties under specific conditions, together with CSCs' heterogeneity makes therefore challenging the effort to design specific therapies able to efficiently target this evolving and dynamic population.

1.2 Lung CSC Markers

Identification of CSCs is mostly based on the expression of surface marker able to discriminate the stem-like subset from differentiated cells and allowing physical separation of different subpopulations using FACS sorting. Other strategies rely on

functional activities of CSCs exploiting their intrinsic elevated levels of drug transporters and enzymes deputed to detoxification. The ability to form clonal spheroids in vitro and the tumorigenic potential in mice represent the assays general use to test properties of isolated cells with the latter representing the gold standard to ascertain tumor-forming potential.

Side Population

The first isolation of lung CSCs was performed exploiting side population (SP) assay [47]. This assay was firstly described by Goodell et al. to identify hematopoietic stem cells [48] and relies on the ability of ABC transporters, highly expressed in stem cell populations, to drive efflux of the fluorescent dye Hoechst 33342. Cells able to exclude Hoechst 33342 dye are termed "side population" since they are identified in flow cytometry plots as a (generally) small fluorescence-negative subpopulation. Ho and colleagues demonstrated that the side population identified in lung cancer cell lines showed cancer stem-like characteristics such as tumor-initiating abilities, high invasiveness, chemoresistance, increased telomerase (hTERT) activity, and quiescence, compared to non-SP population. They also reported the existence of a small fraction of SP in primary lung cancer. Further evidence also confirmed the existence of the SP in NSCLC cell lines showing stem-like features including self-renewal ability and expression of embryonic stem cell transcription factors such as Oct4, Sox2, and Nanog [49].

Despite the fact that the side population assay is widely exploited to identify cells with CSCs' properties, there is some criticism regarding the use in this assay of a fluorescent DNA-intercalating dye: under certain conditions non-SP cells, unable to extrude the dye, may in fact suffer from cytotoxic effects due to the presence of this agent misleading the interpretation of functional assays comparing the behavior of SP vs. non-SP populations. Evidence in lung cancer and other tumor types supports the notion that the side population assay may identify cancer stem cells, but experimental variables such as incubation time, dye (and cell) concentration, and gating strategy may result in different frequencies of SP detection among experiments [50]. Therefore, a standardized and more stringent experimental procedure is needed to produce comparable and solid results and to determine the ability of this assay to accurately quantify and isolate CSCs.

ALDH

Another "function-based" method to isolate lung CSCs exploits their elevated activity of aldehyde dehydrogenase (ALDH) enzyme. ALDH family members are deputed to detoxification and are involved in chemoresistance process [51]. ALDH activity, that defines normal stem cells and CSCs, is generally measured by the Aldefluor assay (Stem Cell Technologies).

In NSCLC two aldehyde dehydrogenase isozymes, ALDH1A1 and ALDH3A1, were identified overexpressed in atypical pneumocytes possibly following malignant transformation after chronic carcinogen exposure [52]. Next, Sullivan et al. demonstrated for the first time the increased tumorigenic ability of $ALDH^+$ cells isolated from NSCLC cell lines compared to the negative counterpart. $ALDH^+$ cells showed an enhanced activation of the NOTCH pathway, and its targeting using

ɣ-secretase inhibitor resulted in a drastic decrease of ALDH$^+$ cells [53]. Following this seminal paper, other reports have substantiated the CSCs' properties of ALDH$^+$ cells. Akunuru et al. showed that ALDH^{high} cells isolated from NSCLC cell lines have an increased potential to generate spheroids in vitro and tumorigenic and metastatic activity in vivo [35]. Similarly Jiang et al. proved the self-renewal potential and high tumorigenic ability of NSCLC cells with high ALDH1 activity, as well as their resistance to chemotherapy [54].

CD133

The main method for identification and isolation of lung CSCs is based on FACS sorting of tumor cells expressing specific stem cells-related markers.

CD133 (Prom1) is a cell surface glycoprotein with five transmembrane domains and two large glycosylated extracellular loops [55] . The glycosylated epitope of CD133, AC133, has been shown to select normal human hematopoietic and neural stem cells and next to identify CSCs in several solid tumors such as glioblastoma and colon and pancreas carcinomas [55–57].

The first evidence for identification of lung CD133$^+$ CSCs in primary NSCLC tumors was provided by Eramo et al. who identified self-renewing and highly tumorigenic CD133$^+$ cells that could be cultured and expanded in vitro as floating spheroids. CD133$^+$ lung tumor spheroids were characterized by the expression of embryonic stem cells transcription factors (Oct4 and Nanog) and by their ability to generate tumor xenografts in immunocompromised mice with features resembling original patients' tumors. Spheroids induced to differentiate lost CD133 expression, stem-like features and tumorigenic ability. CD133$^+$ spheroids were additionally shown to resist in vitro to chemotherapy treatment [31]. We provided further evidence for the existence of CD133$^+$ lung CSCs using prospective isolation from freshly dissociated primary NSCLC samples or patients' derived xenografts (PDXs). CD133$^+$ cells were shown to possess high tumorigenic ability when injected at low dose in immunocompromised mice and to be able to give rise to tumors that recapitulate the complexity of primary tumors. Notably, we showed that both acute and chronic exposure of lung cancer cells to cisplatin resulted in the selection of chemoresistant CD133$^+$ cells and identified in this subpopulation frequent coexpression of the ABCG2 transporter and the CXCR4 chemokine receptor [32]. Recently we also reported that the subset of CD133$^+$CXCR4$^+$ lung CSCs possesses increased ability to disseminate and initiate metastasis, thus representing metastasis-initiating cells (MICs). Furthermore we demonstrated that this specific cellular subset shows mesenchymal features and can be directly modulated by tumor microenvironment signaling, providing support to the hypothesis of a tight interplay between microenvironment, stemness, and chemoresistance [46, 58].

Following another possible strategy, Levina and coworkers exploited chemotherapy to enrich for resistant CSCs in lung cancer cell lines. Tumor cells able to survive cisplatin, doxorubicin, and etoposide treatments were enriched for CD133$^+$ cells, lost expression of differentiation markers, and showed high tumorigenic and metastatic potential in vivo [59]. Several other papers also reported the existence of a CSC subset defined by CD133 expression. CD133$^+$ lung CSCs

identified in primary NSCLC tissue were shown to express high level of Oct-4 transcription factor. Oct-4 knockdown was able to prevent tumor sphere formation in vitro and inhibit CD133$^+$ cells' ability for tumor formation and also to chemosensitize CSCs thus increasing the efficacy of chemotherapy [60]. Similarly, Chiou et al. showed that Oct4 and Nanog transcription factors are key regulators of CD133$^+$ cell maintenance. Their ectopic expressions in lung ADC increased the percentage of CD133-expressing subpopulation and sphere formation, enhanced drug resistance, and promoted EMT. Double knockdown of Oct4 and Nanog suppressed the expression of Slug, reversed the EMT process and blocked the tumorigenic and metastatic ability [61].

Despite the wide use of CD133 marker, many controversies are still ongoing regarding its value as optimal marker for CSCs' isolation in different types of cancer, since several discordant evidences have been provided. One of the major issues to be considered is related to the glycosylation of CD133 antigen, since indeed only AC133 glycosylated epitope has been proven to select for CSCs and thus antibodies recognizing different CD133 isoforms and epitopes may be not properly distinguish between CSCs and differentiated tumor cells. In lung cancer it has been reported that also CD133$^-$ cells sorted from NSCLC cell lines maintained tumor-initiating potential and ability for self-renewal [62]. We however provided robust evidence indicating that even if CD133$^-$ cells could initially generate tumors in vivo, they failed to sustain tumor initiation in serial transplantation assays because of their limited tumorigenic potential, whereas CD133$^+$ cells endowed with sustained self-renewal ability can indefinitely propagate tumors [32].

CD44

CD44 is a cell surface glycoprotein that functions as a receptor for hyaluronic acid, an extracellular matrix-related glycosaminoglycan. It is expressed both in normal and in cancer stem cells, and it is involved in multiple cellular processes such as proliferation, differentiation, migration, and angiogenesis [63]. CD44 represents an important marker for definition of CSCs in breast, prostate, pancreatic, squamous head and neck, and more recently also lung cancer [64]. Leung et al. demonstrated that CD44$^+$ cells isolated from NSCLC cell lines possessed an enhanced self-renewal ability, were able to generate spheroids in vitro, expressed pluripotency genes (Oct-4, Nanog, and SOX2) and EMT makers (SNAI1, CDH2, and VIM), and showed an increased in vivo tumor-initiating ability compared to the subpopulation of CD44$^-$ cells. Tumors derived from CD44$^+$ cells recapitulated the same heterogeneity of the parental tumor indicating the ability of CD44$^+$ cells to give rise to all differentiated cells composing the tumor bulk. Moreover CD44$^+$ cells could resist cisplatin treatment [65]. Combination of the CD44 marker with ALDH activity also discriminated a subset of lung cancer cells with enhanced tumorigenic potential and drug resistance. The ALDH(hi)CD44(hi) subset sorted from NCSLC cell lines showed the highest invasion rate, pluripotency genes expression, tumor initiation ability with shortest latency and highest growth rates compared to ALDH(hi) CD44(lo), ALDH(lo)CD44(hi), ALDH(lo)CD44(lo) cells and unsorted controls. ALDH(hi)CD44(hi) were moreover able to efficiently survive chemotherapy and

targeted therapy, and in accordance, clinical lung cancer samples with high frequency of ALDH- and CD44-coexpressing cells were correlated with shorter recurrence-free survival [66].

CD166

Another surface marker described to select for the lung CSC population is represented by CD166, also known as activated leukocyte cell adhesion molecule (ALCAM). CD166 is a member of the immunoglobulin superfamily of cell adhesion molecules, and it is involved in angiogenesis, differentiation, homing, and maintenance of hematopoietic stem cells. It is known to be a marker for normal hematopoietic stem cells as well as for CSCs of colorectal and prostate cancer [67, 68].

More recently Zhang et al. identified CD166 as a novel marker for lung CSCs isolated from primary NSCLC tumors. $CD166^+$ $EpCAM^+$ cells were shown to be endowed with the ability to self-renew and to initiate primary and secondary xenograft tumors representing the phenocopies of parental patients' tumors when injected at low doses in immunocompromised mice. In vitro $CD166^+$ cells were able to form spheres, and as few as 1–5 single cells from dissociated lung spheres were capable to initiate tumors in vivo. Finally $CD166^+$ expression was also found to be a poor prognostic indicator for shorter overall survival in NSCLC patients [69].

2 Lung CSCs and Drug Resistance

2.1 Clinical Relevance of CSCs for Lung Cancer Treatment

The CSCs' paradigm has profound implications for cancer therapy but also represents a formidable challenge for clinical validation since our current understanding of tumor response during treatments mainly relies on imaging techniques that may not capture the complexity of the dynamics of small subpopulations. The clinical application of CSC-related concepts requires therefore evaluation of available evidences under a new perspective. In this chapter we will discuss potential implications of CSCs in light of the efficacy of current pharmacological treatments and the clinical value of CSC markers.

2.1.1 Lung Cancer Treatments, Drug Resistance, and CSCs

Surgery still represents the best option for long-term survival of NSCLC patients when the disease is detected at an early stage and results in 5-year survival rates of more than 70% in pathological stage Ia. The potential use of adjuvant platinum-based chemotherapy after surgery has also been widely investigated [70, 71], but its efficacy in stage I–II disease, the criteria for selection of patients that could benefit from this type of treatment, and the potential for novel drugs in this setting still

remain unclear [72]. Unfortunately, however, approximately 70% of patients are diagnosed with unresectable disease (locally advanced or metastatic). Combination chemotherapy, usually based on platinum doublets, is currently the first-line therapy of choice for advanced NSCLC with selective use of radiotherapy. The prognosis for chemo-/radio-treated patients remains disappointingly low with a 5-year survival rate less than 5%, largely due to the emergence of drug resistance (intrinsic or acquired) during treatments [73].

In recent years, new therapies directed against specific molecular targets (targeted therapy) have entered clinical trials for the treatment of lung cancer. Tyrosine kinase inhibitors (TKIs) against epidermal growth factor receptor (EGFR) or oncogenic fusion events (EML4-ALK) are currently used in clinical practice for specific patient subgroups as well as anti-angiogenic agents against vascular endothelial growth factor receptor (VEGFR) [74]. However, targeted therapies often result in short-term improvements of survival in responsive subsets and have a marginal impact on overall mortality since eventually most patients experience tumor recurrence [75]. More encouraging results have recently emerged from immunotherapeutic strategies based on the use of drugs targeting immune checkpoint inhibitors (anti-PD1/PDL-1) [76] which have been shown to induce relevant and long-lasting clinical responses especially; however more conclusive data on the real efficacy of immunotherapy in lung cancer are needed [77].

Resistance to therapy is one of the major hurdles in clinical management of lung cancer patients and contributes largely to disease progression, recurrence and mortality. Several mechanisms concur in mediating drug resistance including reduced drug uptake (or increased efflux) due to enhanced activity of drug transporters, the increased activity of detoxifying enzymes, the increased activity of the DNA damage repair machinery, and the enhanced resistance to apoptosis or altered cell-cycling properties [78]. The presence of specific subpopulations of cancer cells endowed with both increased tumor-forming potential and chemoresistance (all features of cancer stem cells) has also been suggested to be responsible for the observed tumor recurrence after therapy [79]. In particular in NSCLC patients, it was clinically demonstrated that induction chemotherapy induces a faster tumor regrowth in the waiting period between chemotherapy treatment and subsequent radiotherapy due to an accelerated regrowth of surviving tumor cells with deleterious implications for curative intervention [80]. This observation may support the concept that conventional therapies eliminate the bulk of tumor cells but may spare the subpopulation of CSCs able to survive treatment and to proliferate to reconstitute the tumor, thus explaining tumor recurrence and treatment failure following an apparently successful first line of therapy [81].

Several lines of evidence in experimental models have demonstrated that both conventional and targeted therapies may enrich for CSC subset through a positive selection of pre-existing and intrinsically resistant CSCs or through the induction of epithelial to mesenchymal transition (EMT) program linked to generation of cells with CSC-like features [82]. Different mechanisms have been proposed to confer CSCs' resistance to treatments that will be extensively discussed in Sects. 3.2.2 and 3.2.3, including the intrinsic high expression levels of ATP-binding cassette (ABC)

drug pumps or anti-apoptotic molecules, their relative resistance to DNA damage, and their quiescent/slowly proliferative nature [7].

In this context we postulate that only a deeper understanding of the mechanisms underlying CSCs' drug resistance and the development of novel combination treatments able to target both the tumor bulk and CSC subsets may lead to the eagerly awaited improvements in NSCLC patient outcome.

2.1.2 Prognostic Significance of Lung CSC Markers

Several studies have tried to correlate the expression of CSC-related makers with NSCLC patients' outcome and response to therapy. However, due to discordant results, the potential clinical impact of CSC markers is still controversial, and they have not yet entered the clinical practice. This is not surprising considering that these efforts are confronted by two great challenges: (i) the selection of validated CSC markers (discussed in Chap. 1) and (ii) the limitations of the techniques generally used to evaluate marker expression in clinical samples. Currently the most practical applications for prognostic markers in solid tumors rely on immunohistochemical (IHC) staining performed on archival tissues: this technique however may not adequately capture the CSCs' subpopulation (and its subsets), and we may have to wait for a paradigm shift and implementation of flow cytometry also in this setting (as in hematological malignancies) before CSC markers can usefully be applied in the clinic.

The prognostic/predictive value of CD133 expression has been extensively investigated in NSCLC. Woo et al. analyzed the expression of CD133 by IHC in 177 surgically resected stage I lung adenocarcinoma and found that CD133 is independent prognostic marker for shorter disease-free survival (DFS); moreover the combination of CD133 with proliferating marker Ki-67 could predict postoperative recurrence [83]. Similarly Li H et al. demonstrated in a case series of 145 stage I NSCLC patients that the coexpression of CD133 and ABCG2 is predictive of high risk of postoperative early relapse [84]. Mizugaki et al. reported, in a case series of 161 surgically resected NSCLCs, the correlation of CD133 expression with pathological advance stages and identified CD133 as an independent factor for poor prognosis [85]. Conversely, Salnikov et al. demonstrated in a retrospective series of 88 untreated NSCLC that CD133 does not represent a prognostic parameter for patient survival but is strongly correlated with the expression of chemoresistance-related proteins and therefore can potentially be useful to predict efficacy of anticancer therapies [86]. In NSCLC patients treated with platinum-containing regimens, we also observed a tendency toward shorter progression-free survival when $CD133^+$ cells were detected by IHC in pretreatment samples [32]. Interestingly using flow cytometry, we have been recently able to show that identification of the $CD133^+CXCR4^+EpACM^-$ lung CSC metastatic subset in primary tumors correlates with tumor recurrence and poor outcome [46].

Many other studies investigate the clinical and prognostic significance of CD133 in NSCLC reporting different results. This discrepancy may be due to

differences in clinical pathological features and size of patients cohort analyzed as well as to methodological differences such as the use of different antibodies to detect CD133 or different IHC scores used to evaluate CD133 positivity. A meta-analysis of 13 studies, with a total of 1004 NSCLC patients, proved that CD133 expression was associated with overall survival (OS) but not with disease-free survival (DFS) or any other clinicopathological parameters except tumor differentiation [87]. Another meta-analysis including 23 studies confirmed that CD133 level was significantly correlated with the overall survival of NSCLC patients but not with the disease-free survival; considering clinicopathological features, CD133 level was positively correlated with lymph node metastasis, but not with histological classification. Overall these meta-analyses support the possible use of CD133 as a biomarker for worse prognosis in NSCLCs [88] .

The ABCG2 drug transporter pump, one of the determinants of the "side population" phenotype, was demonstrated to be associated with a shorter survival in advanced NSCLC treated with platinum-based chemotherapy, although it did not predict response to chemotherapy [89]. A similar observation was reported in an independent study demonstrating that in NSCLC patients receiving cisplatin-based adjuvant chemotherapy, high ABCG2 expression as assessed by qPCR was correlated with short progression-free survival but not with response to treatment [90].

Different studies also investigated the prognostic potential of ALDH1 protein expression in NSCLC. Jinang and coworkers showed that high expression of ALDH1 was associated with poor prognosis in NSCLC patients and with a more aggressive and advanced pathological grade and stage [54]. Similarly, Sullivan et al. confirmed that tumors with higher numbers of $ALDH^+$ cells had a significantly poorer overall survival and this association was present also in patients with stage I and N0 disease [53]. Interestingly combined analysis of ALDH1A1 and CD133 revealed strong association with poor survival in resected early-stage NSCLC [91]. Furthermore, CD133 or ALDH1 positivity in NSCLC undergoing induction chemoradiotherapy was significantly correlated with worse overall survival and resulted as an independent prognostic factors for disease relapse [92].

Some evidence also demonstrated prognostic value of CSC-associated transcription factors. The increased expression of embryonic stem cells transcription factors Oct4 and Nanog together with Slug, an EMT-related marker, was found to be associated with worse prognosis in lung adenocarcinoma patients [61]. A retrospective analysis of 226 patients with lung adenocarcinoma showed that high Nanog expression was independently associated with a poor prognosis [93]. On the same lines, Vrzalikova et al. demonstrated that in NSCLC patients who had received adjuvant therapy, the expression of BMI-1, an oncogene belonging to the Polycomb group of ring finger transcription factors, was correlated with shorter DFS in stage I and II tumors [94].

Taken together these evidences sustain the prognostic and predictive significance of different lung CSC markers, even if some discordant results have been published, likely due to methodological variability and to selection criteria used in different studies. Moreover since no consensus has been reached regarding the use of optimal

markers to identify lung CSC, a combination of different markers possibly identifying distinct CSC subsets might improve the predictive/prognostic value of a potential CSC-based biomarker for clinical application.

2.2 *Molecular Pathways Sustaining Intrinsic Drug Resistance of Lung CSC*

The intrinsic drug resistance of CSC can be viewed as the consequence of several biological mechanisms that are constitutively activated in CSC including (i) enhanced activity of the DNA damage repair machinery and the ability to escape apoptosis; (ii) expression of specific transmembrane transporters with drug-extruding capability; (iii) activation of stemness pathways regulating and sustaining self-renewal; and (iv) quiescence status.

2.2.1 DNA Damage Response and Anti-apoptotic Pathways

Many chemotherapeutic drugs such as platinum-based agents as well as radiotherapy exert their anticancer activities by inducing lethal levels of DNA damage. Conversely, cancer cells can survive treatments by activating DNA damage response (DDR) pathways that allow DNA repairing. DDR mechanisms determine cell cycle arrest at specific checkpoints and recruitment of the DNA repair machinery leading to damage control: in-depth investigation of DDR pathways activity in cancer cells could therefore give information on basic principles of cancer development and also result in novel therapeutic strategies [95].

Enhanced DNA repair capacity has been demonstrated to contribute to increased resistance to therapy in the CSC population. The first evidence came from a pioneering study by Bao et al. showing that $CD133^+$ glioblastoma CSCs preferentially activate DNA damage checkpoint response and DNA repair mechanisms contributing to radioresistance and tumor regeneration. Accordingly, specific inhibitors of checkpoint-related kinases Chk1 and Chk2 could overcome CSCs' radioresistance [96]. In a seminal study, the CSC population in NSCLC was also found to strongly activate Chk1 kinase in response to chemotherapy compared to the counterpart of differentiated cells representing the tumor bulk. A combination of Chk1 inhibitors (AZD7762) with chemotherapy dramatically determined a reduction in CSCs' survival by inducing premature cell cycle progression and mitotic catastrophe. Furthermore in vivo combination treatment with Chk1 inhibitors and chemotherapy was able to abrogate the ability of CSCs to form tumor in immunocompromised mice [97]. Enhanced DNA repair ability was also reported in $CD133^+$ cells sorted from A549 NSCLC cell line due to the upregulation of DNA double-strand break (DSB) repair genes that caused an increase resistance to radiotherapy [98].

Overexpression of anti-apoptotic molecules represents another mechanism by which tumor cells can escape damage induced by therapy. Tumor cells can express high levels of anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-XL, and Mcl-1 that contribute to chemotherapy resistance [99]. In NSCLC primary tumors, the CSC subset was shown to express the anti-apoptotic Bcl-XL at particularly high levels. Treatment with a selective inhibitor of Bcl-XL, ABT-737, showed a preferential cytotoxic activity toward slowly proliferating CSCs in vitro and was able to impair tumor growth of CSC-derived xenografts and reduce CSCs' content in vivo, indicating its specificity in CSCs' targeting [100].

2.2.2 Proteins Involved in Drug Efflux and Detoxification

One of the most investigated mechanisms for anticancer treatment failure is the activity of specific transmembrane transporters mediating drug efflux. ATP-binding cassette transporter proteins (ABC transporters) are recognized as one of the main families of such transporters with the ability to drive the extrusion of a wide range of chemotherapeutic drugs such as doxorubicin, etoposide, paclitaxel and cisplatin using ATP hydrolysis as a source of energy to overcome chemical gradient [101].

The cancer resistance protein ABCG2, one of the members of ABC transporters family, is responsible for the efflux of Hoechst dye defining the "side population" (SP) enriched for CSCs and is one of the main transporters mediating CSCs' resistance to therapy in different cancers [102]. ABCG2 actively effluxes a wide variety of xenobiotic compounds from cells, and its overexpression in tumor cells confers multidrug resistance to several chemotherapeutic agents and targeted therapies [103]. Moreover in lung cancer patients, high expression of ABCG2 is also associated with lower response to carboplatin and cisplatin and poor overall survival [89, 104].

The first evidence proving that ABC transporters could confer chemoresistance properties to lung CSCs came from the study by Ho et al.; in this work SP cells, sorted for six lung cancer cell lines, showed stem-like features, an enhanced tumorigenic potential in vivo, and an increased resistance to various chemotherapeutic drugs such as cisplatin, gemcitabine, and vinorelbine, all of which are commonly used as first-line therapies for lung cancer, due to the high expression of ABC transporters [47]. In line with these observations, we also reported that $CD133^+$ lung CSCs expressed high level of ABC transporters compared to the $CD133^-$ counterpart. Coherently with this finding, we showed both in cell lines and in patient-derived xenografts (PDX) that cisplatin treatment resulted in a selection of $CD133^+$ CSCs that coexpressed the ABCG2 pump proving the contribution of this drug transporter in CSC-mediated chemoresistance [32].

ALDH are a group of $NAD(P)^+$-dependent enzymes that catalyze the oxidization of aldehydes into carboxylic acids, and their intrinsic detoxifying action can contribute significantly to the development of drug resistance [105]. ALDH11A and ALDH3A1 enzymes were demonstrated to identify CSC subpopulation in different

tumors, including lung cancer, and they can act as drug-detoxifying enzymes mediating CSCs' therapeutic resistance [105]. In particular in lung cancer, tumor cells with high ALDH1 activity isolated from cells line displayed CSC features and greater resistance to chemotherapeutic drugs commonly used as first-line therapy in clinical setting compared to ALDH1$^-$ cells [54]. Knockdown of ALDH1A1 and ALDH3A1 isozymes in NSCLC cell lines confirmed an increased sensitivity to cyclophosphamide and a decreased tumorigenic potential [106]. Treatment of H460 and H1299 lung cancer cell lines with paclitaxel resulted in the selection of resistant ALDH1$^+$ CSCs' population. Notably, in vivo treatment of xenografts with paclitaxel resulted in reduction of primary tumor growth but promoted the selection and priming of ALDH1-positive CSCs with a consequent increase in the number of metastatic nodules [107]. Resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) is a major issue in the treatment of EGFR-mutated lung cancer, and ALDH1 CSCs were proven to resist targeted therapy [108]. To mimic in vitro the acquisition of resistance to EGFR-TKI gefitinib, Shien and coworkers generated resistant sublines from four EGFR-mutated NSCLC cell lines, through stepwise escalation and high-concentration exposure to gefitinib. Resistant sublines exhibited an overexpression of ALDH1, increased EMT-associated markers, self-renewal potential, and higher tumor-initiating capability in vivo suggesting that acquired resistance to TKI may also rely on the expansion of drug-refractory CSC population. Moreover gefitinib-resistant sublines also displayed an enhanced resistance to the chemotherapeutic agents docetaxel and paclitaxel, an effect that may be mediated by the expansion of the ALDH1 CSC population [108]. Similar results were reported in another study showing that ALDH1-positive lung cancer cells isolated from EGFR-mutant PC-9 cell line displayed resistance to gefitinib and to conventional chemotherapeutic drugs such as cisplatin, etoposide, and fluorouracil, compared to ALDH1-negative cells. Remarkably, analysis of clinical sample confirmed a correlation between high expression of ALDH1 and resistance to both EGFR-TKI and chemotherapy drugs [109].

2.2.3 Self-Renewal Pathways

In normal stem cells' self-renewal, proliferation and differentiation processes are tightly controlled by several pathways including the embryonic Notch, Hedgehog, and Wnt/β-catenin signaling pathways. The same pathways are found aberrantly activated in cancer and may contribute to CSCs' generation and maintenance [110].

Notch Signaling Pathway

The Notch signaling pathway is crucial for cell fate determination [111]. Notch signaling is initiated by the binding of ligands of the Delta-like (DLL1/2/3) or Jagged-like (JLL1/2) families to the transmembrane receptors Notch1, Notch2, Notch3, and Notch4, which induce proteolytic cleavage of the receptor intracellular domains by enzymes of the γ-secretase complex. The intracellular domain is then

translocated into the nucleus where it induces the transcription of Notch target genes involved in cell fate determination [111].

Notch signaling plays an important role in embryogenesis, organogenesis and maintenance of adult lung homeostasis through fine regulation of the differentiation process of stem cells [112]. Suppression of Notch signaling during lung development determines premature tissue differentiation [113], whereas its overexpression results in accumulation of stem cells and arrest of differentiation [114]. Overexpression of Notch signaling has been frequently observed in lung cancer; however some controversies are still ongoing regarding the oncogenic or tumor-suppressive function of this pathway. Several evidences proved that blockade of Notch signaling pathway using γ-secretase inhibitor resulted in cancer cell growth arrest and increased apoptosis, supporting the role of Notch signaling as an oncogenic driver promoting tumor cell survival [115–117]. Conversely, other studies have demonstrated that overexpression of Notch in NSCLC cell lines determined cell death and reduction of tumor growth in vivo, suggesting that Notch may also act as a tumor suppressor [118].

Numerous evidences indicate the role of Notch pathway in maintenance of CSCs in different tumor type, including in lung cancer [119, 120]. Concerning the role of Notch in mediating CSC drug resistance, Liu et al. demonstrated that treatment of NSCLC cell lines with low-dose cisplatin was sufficient to enrich for chemoresistant $CD133^+$ CSC and that this selection was mediated by activation of the Notch pathway. Indeed pretreatment with a γ-secretase inhibitor or a Notch-targeted shRNA was able to reduce cisplatin-induced enrichment of $CD133^+$ cells and to enhance sensitivity of CSCs to chemotherapy. In vivo combination treatment with γ-secretase and cisplatin significantly reduced $CD133^+$ CSCs confirming that activation of Notch signaling is pivotal in mediating cisplatin-induced enrichment of resistant CSCs [121]. The pivotal role of Notch in maintenance of lung CSCs' properties was substantiated in a study by Hassan et al. showing that NSCLC cells with high Notch activity, identified using a Notch GFP reporter construct, displayed stem-like features, have enhanced in vivo tumorigenicity, and can survive cisplatin and docetaxel chemotherapy. Tumor xenografts treated in vivo with γ-secretase inhibitor and docetaxel failed to regenerate tumors in serial transplantation assays indicating exhaustion of the CSC subset [122]. Interestingly, Notch was also shown to mediate the resistance of CSCs to targeted therapy. Arasada et al. reported that treatment of EGFR-mutated lung cancer cell lines with erlotinib mediated selection and expansion of resistant ALDH-positive CSCs and that this enrichment was dependent on direct activation of Notch signaling [123].

Hedgehog Signaling Pathway

The Hedgehog (Hh) signaling pathway is involved in the regulation of cell differentiation and proliferation in embryonic development and in the maintenance of adult stem cells [124]. The Hh ligands (i.e., Sonic hedgehog, Shh; Indian hedgehog, Ihh; and Desert hedgehog, Dhh) bind to the Patched (PTCH) receptor triggering derepression of Smoothened (SMO) protein within the cell membrane and activation of GLI transcriptional regulators of Hh target genes [125].

The Hh pathway coordinates lung development during embryogenesis; indeed, knockout of Shh in transgenic mice determines aberrant lung development [126, 127]. According to some evidence, the Hh pathway remains active in adult lung stem cells as a mechanism for regeneration of tissue in response to airway epithelial injury [128]. Hedgehog pathway can be aberrantly activated in cancer, resulting in tumor growth, proliferation, and metastasis [129]. Activation of the Hh pathway has been shown in lung cancer, where GLI1 expression was found in a large percentage of primary NSCLC samples and in 85% of SCLC tumor samples, indicating constitutive activation [130, 131].

In particular in SCLC, Hh signaling pathway was demonstrated to play an important role in tumor initiation, and it may possibly represent a therapeutic target to prevent cisplatin resistance [132]. Constitutive activation of the Hedgehog signaling promoted the clonogenic ability of SCLC cells in vitro and the initiation and progression of SCLC in vivo. Conversely pharmacological blockade of Hh determined growth arrest of SCLC cells, also after chemotherapy treatments that are usually followed by quick recurrence and disease progression. These findings suggest a crucial role of Hedgehog signaling in the development and maintenance of SCLC and propose Hh inhibition as a therapeutic strategy to keep in check tumor progression and delay cancer recurrence [132]. In lung adenocarcinoma Hh inhibition was demonstrated to cause growth arrest and to significantly decrease the frequency of the side population endowed with tumor-initialing potential and chemoresistance. As a result, combination treatment with inhibitor of the Hh pathway and cisplatin resulted in an increased cytotoxic effect linked to depletion of the CSC population [133]. Additionally it has been shown that induction of EMT in NSCLC confers resistance to both EGFR-tyrosine kinase inhibitors and chemotherapy: interestingly, inhibition of the Hh pathway in NSCLC cell lines resistant to EGFR-TKI erlotinib resulted in attenuation of the EMT phenotype, decrease of CSC marker expression, and sensitization of cancer cells to erlotinib and cisplatin, thus further substantiating a connection between Hh signaling, CSC, and drug resistance [134].

Wnt/β-Catenin Signaling Pathway

Wnt signaling is essential both for the control of cell proliferation and cell fate determination during embryonic development and in the maintenance of adult stem cell [135]. Briefly, the binding of Wnt ligands to the Frizzled receptor results in recruitment of Disheveled proteins (Dvl) that in turn block Axin/APC/GSK-3β complexes thereby derepressing β-catenin. The accumulation and translocation of β-catenin into the nucleus promote transcription of Wnt target genes [136].

The Wnt pathway is well known to be deregulated in several tumor types, including lung cancer [137]. Some studies have demonstrated the overexpression of Wnt-1 and Wnt-2 in NSCLC cell lines and primary cancer tissues; moreover inhibition of Wnt signaling caused cell growth arrest and induced apoptosis in NSCLC cell lines [138, 139]. Giangreco et al. reported that membranous staining for β-catenin was observed in normal and metaplastic lung specimens, whereas carcinoma in situ and severely dysplastic lung tissues showed nuclear localization

of β-catenin, indicating activation of Wnt/β-catenin signaling during cancer progression [140].

Regarding activation of the Wnt pathway in lung CSC subsets, Levina et al. showed that lung cancer cells able to survive chemotherapy were enriched for CD133 CSC marker and expressed high nuclear level of β-catenin compared to their corresponding parental counterparts [59]. Teng et al. reported high activation of Wnt/β-catenin signaling in cisplatin-selected A549 lung cancer cells concomitantly with an increased expression of OCT-4 embryonic transcription factor. Knockdown of β-catenin expression using RNA interference in lung cancer cells resulted in downregulation of the Wnt target genes and in a reduction of OCT-4-expressing cells concomitantly with decreased proliferation and reduced clonogenic potential, migration, and drug resistance [141]. Taken together, these studies provide evidence for the involvement of Wnt signaling in maintenance of lung CSC and chemoresistance.

2.2.4 Intrinsic Quiescence

Quiescence is another mechanism contributing to the chemoresistance of tumor cells. Quiescent cells are arrested in the G0 phase of the cell cycle; this dormant state is reversible and can be modulated in response to the activation of signaling pathways induced by microenvironmental stimuli [142]. Quiescence is regulated by different signaling molecules including the well-characterized tumor suppressors p53 and RB and several cyclin-dependent kinase inhibitors, in particular p21, p27, and p57, all able to induce cell-cycle arrest [142].

Quiescence is a distinctive feature of normal stem cells, and it was proved to characterize specific subsets of CSCs [143]. Tumor cells endowed with stem-like features can disseminate to distant sites and survive in nonproliferative quiescent state for long time. This process occurs at early time of tumor progression or following therapeutic intervention and awakening of dormant cells may lead to tumor progression and relapse after very long periods from primary tumor removal or treatment [144]. The mechanisms leading to quiescent cell awakening are not well understood, but this process appears to be tightly regulated by microenvironment signals [145] as clearly demonstrated in breast cancer where two microenvironment-secreted factors, thrombospondin-1 and periostin, have been shown to play a crucial role in dictating cancer cells' quiescence and metastasis outgrowth [146, 147].

Quiescent state also allows CSCs to escape conventional chemotherapy that targets actively proliferating tumor cells [143]. Subsets of nonproliferative and drug-resistant CSCs could therefore "respond" to tumor shrinkage caused by treatments through reactivation and reconstitution of the tumor bulk. Three different strategies could be exploited to eradicate the quiescent CSCs' subpopulation. The first one paradoxically consists in promotion of cancer cells' proliferation to sensitize CSCs to conventional therapies; however this approach may also promote cancer progression due to CSCs' awakening and possible CSCs' dissemina-

tion. The second and more conservative approach proposes to maintain CSCs in a quiescent state avoiding their awakening with the final aim to treat tumor as a chronic disease. The last strategy consists in CSCs' eradication while these are still in a quiescent state: this is a fascinating approach, but at present a deeper understanding of molecular pathway governing CSCs' dormancy is still needed before such strategy could be implemented [144].

Long-term label retention is a widely used strategy for the identification of stem cells by exploiting their slow-cycling nature, whereas rapidly dividing progenitor cells dilute their labels [148]. The use of membrane-labeling dyes such as PKH67/PKH26 has been reported to track slow-cycling cells including both normal and cancer stem cells [149]. In lung cancer we demonstrated the existence of slow-cycling PKH^+ cells enriched for $CD133^+$ CSC; within this subset it was possible to distinguish a long-term quiescent PKH_{Bright} population, strongly enriched for $CD133^+$CXCR4-CSCs deputed to primary tumor maintenance, and a short-term quiescent PKH_{Dim} population enriched for $CD133^+CXCR4^+$ lung metastatic CSCs [150]. Both PKH^+ cell fractions were resistant to cisplatin treatment, suggesting that quiescent $PKH^+/CD133^+$ subpopulation overlaps with the already reported cisplatin-resistant $CD133^+$ CSCs [32]. Pretreatment with the differentiating agent all-trans retinoic acid (ATRA) counteracted cisplatin resistance, preferentially sensitizing PKH_{Dim} cells to chemotherapy suggesting an effect on metastatic CSC subset as proven by in vivo decrease of tumor dissemination. By exploiting the quiescent properties of CSCs, this study revealed therefore the heterogeneity of lung CSCs and suggested the potential use of retinoic acid in combination with standard chemotherapy to counteract lung cancer metastatic spread [150].

2.3 Tumor Microenvironment Signaling Promoting CSC Drug Resistance

It is becoming increasingly clear that cancer development and progression cannot be fully understood without considering the major role played by the surrounding tissue microenvironment which actively participates to tumor growth [151]. The tumor microenvironment (TME) is a complex environment composed by extracellular matrix and several different cell types, including immune cells, vascular endothelial cells and cancer-associated fibroblasts, all of which participate in different aspects of tumor formation [152]. In this context it is easily appreciated that drug resistance can both be driven by the intrinsic ability of tumor cells to survive pharmacological treatment (intrinsic resistance) and by indirect mechanisms involving TME signals able to protect cancer cells from the damage caused by different drugs (extrinsic resistance) [153].

Induction of epithelial-mesenchymal transition (EMT) in tumor cells by TME-related signals is currently seen as one of the most crucial processes responsible for extrinsic resistance [154]. EMT is a reversible process active during develop-

ment by which epithelial cells acquire mesenchymal traits losing their apical-basal polarity and cell-cell adhesion: the same process is crucial for cancer cells in acquiring invasiveness and metastatic features [155]. The concepts of EMT and stemness are closely interconnected as many of the signals inducing EMT have also been shown to regulate stemness properties of cancer cells [44, 156, 157]. In this chapter we will therefore review experimental and clinical evidences related to EMT and drug resistance together with studies highlighting the link between EMT and acquisition of CSC phenotype.

Activation of EMT is associated with increased expression of mesenchymal markers including vimentin, fibronectin, N-cadherin, enhanced activity of matrix metalloproteinases such as MMP-2, MMP-3 and MMP-9, and decrease of epithelial markers such as E-cadherin [158]. The modulation of mesenchymal and epithelial gene expression during EMT is regulated by specific transcription factors (TF) acting as master regulators and in particular by Snail, Twist, and zinc finger E-box-binding (ZEB) [159]. The Snail family of zinc finger transcription factors, consisting of Snail1 (Snail), Snail2 (Slug), and Snail3 (Smuc), was demonstrated to play a crucial role in promoting EMT in cancer cells through the transcriptional repression of E-cadherin [160]. A role for Slug in lung cancer progression has also been proposed [161]. Twist is an highly conserved basic helix-loop-helix transcriptional factor that drives lineage determination in healthy tissue and has been shown to actively regulate EMT and metastasis in breast cancer [162]. In lung cancer Twist appears to play a pivotal role in promoting EMT by repressing E-cadherin and promoting N-cadherin expression thus inducing acquisition of metastatic traits through upregulation of MMP and FAK activity [163]. The Zeb family which includes ZEB1 and ZEB2 transcription factors can promote EMT through the repression of epithelial genes such as E-, P-, and R-cadherins and components of tight and gap junctions and desmosomes [164–166]. Moreover Zeb family TF can activate mesenchymal genes such as vimentin and N-cadherin and induce metalloproteinases such as MMP1, MMP2, and MMP14 [167, 168]. A correlation between high expression of ZEB1 and aggressiveness of the disease, defined by metastasis and chemoresistance occurrence, has been reported in lung cancer [169].

Different signals from the tumor microenvironment are able to trigger EMT process in lung cancer cells. The most well-known and studied inductor of EMT is the transforming growth factor beta (TGF-β) that explicates its effects through the activation of SMAD transcription factor complexes and regulation of target genes [170, 171]. The SMAD complex transduces extracellular signals from TGF-β to the nucleus where it interacts with Snail, Twist, and Zeb transcription factor families to repress epithelial genes and induce mesenchymal traits [172–174]. Another potent inducer of EMT is represented by the pro-inflammatory interleukin-6 (IL-6). In particular IL-6 plays a crucial role in regulating EMT in lung cancer through aberrant activation of STAT3 phosphorylation particularly in the context of KRAS activation [175, 176]; the inhibition of this axis can prevent distant metastasis formation in lung cancer xenograft models and reverse IL-6-induced EMT [177, 178]. Notably, IL-6 has also been shown to correlate with a poor clinical outcome and shorter overall survival in NSCLC patients [179], and elevated serum levels of IL-6

have been detected in lung cancer patients and correlated to lung cancer risk [180]. In different experimental settings, however, the inhibition of IL-6 has also been shown to enhance tumor progression highlighting the complex interplay and timing of the interactions within the TME [181].

2.3.1 Epithelial to Mesenchymal Transition and Drug Resistance

It has been increasingly recognized that cancer drug resistance is frequently accompanied by EMT in different types of cancer [182]. Strong experimental evidence supporting this link comes from recent studies exploiting genetically engineered mice models of pancreatic and breast carcinomas proving the crucial role for EMT in inducing chemoresistance [183, 184]. Challenging commonly held beliefs, EMT impairment did not affect metastasis development; however, EMT cells were shown to survive chemotherapy due to reduced proliferation, apoptotic tolerance, and increased expression of resistance genes and significantly contributed to drug resistance and even to metastasis formation after chemotherapy [184]. In pancreatic cancer the suppression of EMT did not decrease tumor dissemination and metastasis formation but led to an increase in drug transporter expression that contributed to enhanced sensitivity to gemcitabine treatment [183]. Overall these studies indicate the potential use of an EMT inhibitor to enhance efficacy of conventional chemotherapies.

Other studies have reported that induction of EMT was associated with overexpression of ABC transporters and of DNA repair proteins increasing resistance to chemotherapy [185, 186]. In lung cancer, analysis of cisplatin-resistant cells displayed the acquisition of an EMT phenotype and an increased invasion and migration ability [187]. The mechanism through which chemotherapy enriched for EMT cells may rely on the eradication of epithelial cells with a consequent relative increase of mesenchymal cells or on the direct promotion of EMT in cancer cells. Notably, chemotherapy treatments can induce the release of both stroma and tumor cytokines able to trigger pro-survival pathways in surviving tumor cells as well as induction of EMT, paradoxically sustaining chemoresistant cells and conferring increased metastatic ability [188]. In this respect cisplatin treatment of NSCLC was proved to increase the pro-inflammatory cytokine IL-6 that contributes to both EMT induction and chemoresistance of cancer cells due to the upregulation of anti-apoptotic proteins and DNA repair machinery [189]. Moreover different studies have reported the role of tumor microenvironment, particularly of cancer-associated fibroblast, to contribute to EMT induction and chemoresistance of NSCLC cells through a paracrine loop based on IL-6 [190, 191]. In particular treatment of lung cancer cells with cisplatin increased the expression of TGF-β that determined fibroblast activation and increased their IL-6 production. IL-6 in turn activated EMT in cancer cells and caused resistance to chemotherapy [191].

Accumulating evidence indicates that EMT activation is also linked to the acquisition of targeted therapy resistance [192]. In particular in NSCLC, the resistance to epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) has been

associated with different mechanisms including the mesenchymal phenotype of tumor cells [193]. In detail, gene expression profiling of a panel of 42 NSCLC cell lines screened for erlotinib sensitivity demonstrated the correlation between a gene signature associated with epithelial to mesenchymal transition (EMT) and insensitivity to erlotinib. Notably, NSCLC patients that showed strong E-cadherin expression and thus an "epithelial" phenotype experienced a longer DSF and OS with erlotinib plus chemotherapy treatment versus chemotherapy alone [193]. Similar results were reported by Thomson and coworkers showing in vitro and in vivo that sensitivity of human NSCLC cell lines to EGFR-TKI treatment was dependent on the degree to which cells have undergone an epithelial to mesenchymal transition (EMT). NSCLC lines expressing high levels of E-cadherin showed greater sensitivity to EGFR inhibition compared to cell lines expressing vimentin and/or fibronectin that were insensitive to the growth-inhibitory effects of EGFR-TKI [194]. The same group also reported that induction of EMT in NSCLC line H358 by TGF-β treatment caused loss of EGF family ligand expression, increased EGFR-independent Mek-Erk pathway activation, and reduced sensitivity to EGFR inhibition [195]. Finally, it was demonstrated that restoration of E-cadherin expression was able to increase cancer cell sensitivity to EGFR-TKIs [196]. All these evidences support the role of EMT as potential determinant for insensitivity to EGFR inhibition in NSCLC patients highlighting a common mechanism of resistance to both conventional and targeted therapies. In an attempt to investigate the cause of EGFR-TKI resistance, Yao and colleagues uncovered the existence of a subpopulation of lung cancer cells intrinsically resistant to erlotinib that displayed EMT phenotypes [197]. This subpopulation presented autocrine activation of TGF-β signaling that determined its mesenchymal features and secretion of IL-6, enabling cells harboring mutant EGFR to overcome their EGFR dependency, resulting in decreased sensitivity to erlotinib treatment. These findings imply that resistance to molecular targeted therapy can be driven both by tumor cell-autonomous mechanisms and/or activation of the tumor microenvironment [197].

2.3.2 Epithelial to Mesenchymal Transition and Acquisition of CSC Properties

It has been reported that EMT endows tumor cells with stem-like features, and thus the frequency of CSCs may also be partially regulated as a result of EMT activation induced by microenvironment cues in differentiated tumor cells. This observation implies that selection and expansion of CSCs conferring drug resistance may be due to both selective pressure and survival advantage of pre-existing CSCs and/or their de novo generation through the EMT process induced by TME signals.

The first seminal paper proving the connection between EMT and CSCs was published in 2008 by Weinberg's group [44]. Overexpression of EMT-related transcriptional factors, Snail and Twist, or TGF-β treatment induced in breast can-

cer cells an increase of CD44 ^{high}/CD24^{low} cancer stem cells and enhanced the capability to form mammospheres in vitro and to initiate tumor in vivo, two hallmarks of functional cancer stem cells [44]. In fact there is remarkable overlap in signaling pathways able to maintain CSC properties and to activate EMT such as Wnt, Hedgehog, and Notch pathways. Therefore drug resistance related to the activation of EMT (discussed in Sect. 3.2.3.1) can be also mediated by CSCs' generation through self-renewal signaling activation. For this reason EMT, CSCs, and drug resistance have been described as "an emerging axis of evil" for cancer treatment [154].

In lung cancer activation of Hedgehog pathway was demonstrated to induce EMT providing tumor cells with metastatic potential and resistance to chemotherapy [198]. Hh pathway can also confer resistance to EGFR-TKIs by inducing EMT in lung cancer cells [199] and, importantly, inhibition of the Hh pathway can reverse the EMT phenotype with a concomitant reduction of CSC markers and sensitize cells to EGFR-TKIs [134]. Notch signaling activation was also demonstrated to promote EMT in lung cancer cells, linked to the acquisition of resistance to EGFR-TKI [200].

Several studies reported that treatment of NSCLC cells with TGF-β induces EMT associated with the acquisition of CSC phenotype, demonstrated by the expansion (or de novo generation) of CD133$^+$ cells, enhanced migratory potential and tumorigenicity [45, 46, 201]. Interestingly, we also observed that the ability of lung tumor cells to "sense" TGF-β stimuli and to generate CD133$^+$ cells through the EMT process was linked to their plasticity that could be measured as a ratio between epithelial (E-cadherin) and mesenchymal (SNAI2) gene expression. Cells showing an intermediate EMT state, thus expressing both markers, were the most prone to generate CSCs under microenvironment stimuli both in vitro and in vivo [45].

Besides TGF-β, other cues from the tumor microenvironment can induce EMT and generation of CSC subsets. Cancer-associated fibroblasts (CAFs) were demonstrated to facilitate the conversion of differentiated lung primary tumor cells into CSCs, through the paracrine activation of EMT program and WNT, Notch, and Hedgehog signaling [202]. CAFs are crucial for CSC maintenance and regulation through the overexpression of growth factors such as IGF-II, HGF, and SDF1 and concomitant induction of the expression of their corresponding receptors in CSCs [45, 46, 203, 204]. Interestingly, tumor cells co-cultured with CAF also showed an enhanced resistance to chemotherapeutic drugs that was linked to microenvironment-generated CSC subsets [204]. In particular we recently reported that microenvironment stimuli eliciting EMT, including signals from CAFs, are able to generate the subset of CD133$^+$CXCR4$^+$EpCAM cells that represent the metastatic and chemoresistant fraction of CSCs [46]. Stromal-derived SDF-1/CXCL12 cytokine, the ligand of the CXCR4 receptor, is able to trigger EMT in lung cancer cells, and inhibition of CXCR4 signaling can partially block the EMT program induced by CAF-conditioned medium and prevent metastatic dissemination induced by chemoresistant CSCs. This observation points at the SDF-1/

CXCR4 axis as one of the crucial mediators of tumor-stroma cross talk responsible for EMT induction and generation of chemoresistant CSCs [46].

3 Novel Therapeutic Strategies Targeting Lung CSCs

The therapeutic implications of the cancer stem cell concept encompass different areas ranging from the potential use of CSC markers as prognostic and/or predictive factors (discussed in Sect. 3.2.1.2.) to the rationale design of novel therapies targeting these "seeds" of drug resistance and tumor recurrence [205]. Building on information gathered in preclinical studies dissecting CSCs' biology, the main approaches that can be considered are (i) direct targeting of pathways implicated in CSCs' maintenance or specific CSCs' functional properties (i.e., high expression of drug transporters, detoxifying enzymes, and anti-apoptotic molecules) and (ii) interference with tumor microenvironment communication [58, 206].

3.1 Targeting Intrinsic CSC Drug Resistance

With the aim to eliminate CSCs and possibly overcome drug resistance, different compounds specifically targeting self-renewal pathways involved in CSCs' maintenance have been tested in preclinical models and clinical trials. In particular several inhibitors of the Notch signaling pathway have been developed and tested, including γ-secretase inhibitors (GSIs), monoclonal antibodies (mAb) against Notch receptors or ligands, blocking peptides, and natural compounds [207, 208]. To date, GSIs are the most extensively developed and investigated class of Notch inhibitors. In lung cancer, the γ-secretase inhibitor R04929097, previously evaluated in other solid tumors [209, 210], has been tested in a phase II clinical trial for treatment of patients with advanced NSCLC who had completed treatments with front-line chemotherapy (clinicaltrials.gov, NCT01193868). The same compound has been under evaluation in combination with the EGFR-TKI erlotinib in advanced NSCLC (NCT01193881). Although both trials were terminated as a result of discontinued production of the study drug, administration of Notch-targeting compounds in combination with other drugs was evaluated as safe and feasible indicating potential for development of novel molecules [211]. In addition to γ-secretase inhibitors, the monoclonal antibody demcizumab (OMP-21 M18, OncoMed) has been developed to target Notch ligand DLL4. This antibody has been evaluated in NSCLC cancer in combination treatment with carboplatin and pemetrexed (NCT01189968), and encouraging early clinical activity has been observed and reported at the 2016 Annual Meeting of the American Society of Clinical Oncology [212].

The Hedgehog (Hh) pathway has long been implicated in CSC maintenance, and many of its components have received considerable interest as targets for Hh signaling inhibition [213]. In particular pharmacological targeting of SMO has been

widely explored, and GDC-0449 (vismodegib, Genentech) has been the first SMO inhibitor to enter clinical trials and to show its antitumor efficacy in solid tumors, particularly in basal cell carcinoma [214]. GDC-0449 also demonstrated an effective antitumorigenic activity in lung adenocarcinoma and SCLC and was able to increase the cytotoxic effects of cisplatin by affecting the side population [133]. It is currently under evaluation in a phase II clinical trial in SCLC in combination with cisplatin and etoposide (ECOG-1508, NCT00887159).

Concerning the Wnt pathway, the evaluation of pharmacological activity of DKN-01, a neutralizing mAb targeting extracellular dickkopf-1 (Dkk-1) and inhibiting the canonical Wnt/β-catenin signaling pathway, has recently been completed in a phase I trial in patients with relapsed or refractory NSCLC, multiple myeloma and advanced solid tumors (NCT01457417). Results from the trial indicated a safe pharmacological profile and potential clinical activity suggesting potential for future development in combination with other agents [215]. The small molecule FJ9, an antagonist of Disheveled (Dvl) protein, has been demonstrated to significantly downregulate canonical Wnt signaling and to possess promising anticancer activity. Preclinical studies showed that treatment with FJ9 was able to induce apoptosis in several lung cancer cell lines and to inhibit tumor growth in murine xenograft models [216].

Targeting the "side population" may also represent another approach to overcome resistance to therapy by increasing drug retention within CSCs. Xia et al. developed an image-based high-content screening (HCS) to specifically identify and analyze the high drug-efflux cancer cells (HDECC) in lung cancer cells lines. They screened 1.280 pharmacologically active compounds and identified 12 effective HDECC inhibitors. In vitro testing demonstrated that these inhibitors were able to overcome multidrug resistance and sensitize HDECCs to chemotherapeutic drugs; in addition they were able to significantly decrease in vivo tumorigenic activity of tumor cells, possibly by affecting CSCs' content [217].

Inhibition of activity of ABC efflux transporters has long been investigated as a possible way to overcome multidrug resistance (MDR), but compounds developed so far have shown limited efficacy and generalized toxicity [101]. The possibility that selective inhibition of drug efflux could also help in overcoming CSC-mediated drug resistance might however open the way for investigation of new treatment schedules or novel compounds. In this respect the calcium-channel blocker verapamil is also known to inhibit ABC transporter P-glycoprotein (P-gp), one of the major determinants of the MDR phenotype [78]*:* clinical trials in NSCLC comparing verapamil plus chemotherapy vs. chemotherapy alone demonstrated an improved outcome with a median survival significantly improved in the verapamil arm ($p = 0.02$) [218]. Tariquidar, another inhibitor of P-gp, has been investigated in combination with docetaxel for the treatment of recurrent metastatic solid tumors in a phase II trial (NCT00072202), and the results have indicated some anticancer efficacy particularly in lung cancer patients [219]. Several other compounds, including cyclosporine A, biricodar, PK11195, and curcumin, have been found to inhibit the ABC transporter family and counteract multidrug resistance, but none of these has been exhaustively tested in clinical trials [220].

Targeting the ALDH family of enzymes, highly expressed in CSCs (see Sect. 3.1.2), represents another strategy to potentially overcome drug resistance induced by CSCs. Disulfiram (Antabuse), an FDA-approved pan-ALDH1 inhibitor originally used in the treatment of chronic alcoholism, has demonstrated its efficacy in targeting CSCs in several solid tumors including lung cancer [221]. In particular disulfiram was able to re-sensitize cancer cells to standard therapies or enhance the cytotoxic effects of chemotherapy [222]. In a small phase II clinical trial, disulfiram in combination with cisplatin and vinorelbine was well-tolerated and significantly prolonged overall survival in patients with metastatic NSCLC [223]. Salinomycin, traditionally used as an antibacterial drug, has also demonstrated anticancer activity by directly targeting $ALDH^+$ CSCs. In in vivo preclinical models of NSCLC, salinomycin in combination with paclitaxel was able to drastically decrease metastasis formation compared to chemotherapy alone by targeting $ALDH^+$ lung CSCs [107]. The natural compound silibinin, a bioactive flavonoid agent, was proven to target $ALDH1^+$ CSCs and to sensitize them to the EGFR-TKI erlotinib thus decreasing the ability of $ALDH^+$ cells to escape targeted therapy and to sustain tumor recurrence [224].

Other strategies have been reported to sensitize CSCs to standard chemotherapy in particular by acting on mechanisms sustaining CSCs' resistance to DNA damage or apoptosis (see Sect. 3.2.2.1). Combination therapy with an inhibitor of DNA damage checkpoint protein kinase-1 (Chk1), particularly activated in CSCs compared to differentiated cells counterpart, was able to drastically reduce tumor growth and CSC subset compared to chemotherapy alone [97]. Furthermore inhibition of the anti-apoptotic protein Bcl-XL (consistently expressed at high levels in lung CSCs) using the small molecule inhibitor ABT-737 showed a specific cytotoxic activity toward quiescent/slow-proliferating CSCs [100]. Finally a differentiation strategy using all-trans retinoic acid (ATRA) in combination with cisplatin was proven to sensitize the subset of chemoresistant and metastatic $CD133^+CXCR4^+$ CSCs to cisplatin treatment strongly reducing tumor dissemination [150].

It must be considered however that the intriguing possibility to target CSCs through inhibition of stemness-related signaling pathways or exploiting specific properties of CSCs such as high expression of ABC transporters or ALDH enzymes, ability to escape apoptosis, and relative cellular quiescence also raises serious concerns because similar pathways/functional activities are shared with normal stem cells: anti-CSCs' therapies should therefore potentially be designed to preserve normal stem cells and to specifically target only molecules uniquely expressed or functionally activated in CSCs.

3.2 Targeting Tumor Microenvironment Cross Talk

Strategies aimed at interfering with microenvironment stimuli able to regulate the stemness phenotype and/or CSCs functional activities could offer an innovative way to potentially bypass CSC-mediated chemoresistance.

As described in Sect. 3.2.3, EMT is a crucial process mediating chemoresistance also through the generation of the CSC subset; thus therapeutic strategies able to reverse or inhibit EMT could sensitize tumor cells to conventional drugs and impair CSCs' formation [154]. Metformin, one of the first-line medications for the treatment of type 2 diabetes, has been recently shown to possess anticancer activity and to inhibit EMT process [225, 226]. In lung cancer Li et al. demonstrated that metformin increases the sensitivity of TKI-resistant lung cancer cells to erlotinib or gefitinib by reversing EMT [227]. EMT inhibition was linked to decrease of IL-6 signaling activation in TKI-resistant cells induced by metformin treatment. Combinatorial therapy with TKI and metformin effectively inhibited tumor growth in xenografts derived from resistant cancer cells, which was associated with EMT reversal and decreased IL-6 signaling activation, thus potentially representing an effective treatment to overcome TKI resistance and prolong survival of EGFR-mutated NSCLC [227]. Similarly, another group showed in lung adenocarcinoma that metformin was able to inhibit EMT by blocking the IL-6/STAT3 axis. Enhanced IL-6 expression could promote EMT in lung cancer cells via STAT3 phosphorylation, and metformin was able to reverse such a mechanism by blocking STAT3 phosphorylation. Importantly, metformin inhibited tumor growth and distant metastases in xenograft-bearing mice due to inhibition of EMT [178]. Interestingly a recently identified inhibitor of the stemness phenotype, napabucasin (Boston Biochemicals), also acts through inhibition of STAT3 signaling [228], and preliminary clinical investigation of this compound in advanced NSCLC has provided promising results [229]. IL-6 is abundantly released by stroma cells in tumor microenvironments; thus, as suggested by these studies, metformin or other drugs interfering with stromal signals may effectively impair tumor-stroma cross talk preventing EMT activation in tumor cells and acquisition of drug resistance.

CXCR4/CXCL12 axis contributes to NSCLC progression, and targeting this axis has been considered a potential therapeutic approach for lung cancer treatment in particular to counteract metastatic disease [230]. CXCR4/CXCL12 pathway is able to guide tumor dissemination to distant site and also to activate pro-survival and self-renewal pathways in tumor cells [231]. In particular we have observed CXCR4 coexpression in a defined subset of $CD133^+$ CSCs was able to survive chemotherapy and endowed with high dissemination potential and ability to initiate metastasis [32, 46]. In several PDX models of lung cancer, we have observed that cisplatin treatment, although effective in reducing tumor size, induces a relative enrichment of chemoresistant $CD133^+CXCR4^+$ cells in the residual tumor and that this enrichment correlated to an increased metastasis formation. Combination treatment with CTCE-9908, a small molecule inhibitor of CXCR4, was able to counteract the relative increase of $CD133^+CXCR4^+$ cells induced by cisplatin and drastically reduce metastatic dissemination, suggesting that CXCR4 blockade could specifically impair dissemination of chemoresistant and metastatic CSCs [46]. Moreover since stromal CXCL12 was demonstrated to induce EMT and acquisition of stem-like properties in NSCLC cells, inhibition of CXCR4 can

also impair the microenvironment-derived modulation of CSCs and chemoresistance [46].

Altogether these evidences highlight the crucial role of tumor-stroma cross talk in mediating chemoresistance and tumor progression induced by CSCs and indicate the potential of novel strategies aimed at interfering with this interaction to sensitize CSCs to standard chemotherapy and impair their fostering by microenvironment stimuli.

4 Conclusions and Future Perspectives

The cancer stem cell hypothesis has provided a new perspective in the understanding of mechanisms subtending drug resistance and for the development of novel strategies that may increase the efficacy of current therapies for cancer [81]. In fact, despite increased knowledge of the molecular basis of cancer development, evaluation of novel early diagnosis methods and employment of targeted therapies, lung cancer remains the most lethal cancer worldwide with an overall 5-year survival rate of approximately 15% [2]. This clinical evidence strongly supports therefore the urgent need to identify novel strategies to overcome drug resistance and tumor progression. CSC research has been a field in great expansion in the last decade with the achievement of several milestones including the demonstration of their existence in solid tumors, their characterization, and the understanding of drug resistance properties that may allow the design of new anticancer strategies to potentially improve effectiveness of current treatments.

The first evidence of CSCs in primary lung cancers was independently provided by two groups that identified lung CSCs on the basis of their surface expression of the CD133 marker [31, 32]. However many other groups have reported the existence of different cellular subsets with stem-like properties and ability to initiate tumor that were identified using different markers. It must be stressed however that many studies used lung cancer cell lines that, even if informative, may not faithfully recapitulate the biology of primary tumors; therefore validation in clinical sample represents the best way to validate potential markers used for the selection of lung CSCs. Lack of consensus regarding optimal CSC markers and the possibility that indeed different lung CSC subsets may exist further complicate our understanding of such populations and consequently our ability to efficiently target these cells. These controversies also arise from the poor knowledge of normal stem cells counterpart in lung tissue: some evidence indicates the possibility of the existence of distinct stem/progenitor cell subsets deputed to the maintenance of anatomically defined regions of the respiratory tracts from which different lung cancer histological subtype may be generated with implications for the phenotype of corresponding CSCs. Although this notion is well proven in murine models, knowledge about lung stem cell biology in humans is still in its infancy.

Despite difficulties in optimal markers selection for lung CSC isolation, one of the common traits of these cell subsets is the ability to resist current therapy used for lung cancer treatment, both conventional chemotherapy and targeted therapy. While current treatments may shrink tumors by eradicating actively dividing and

differentiated tumor cells, CSCs can survive these insults due to their unique properties and lead to drug resistance and subsequent tumor relapse. Self-renewal and pro-survival pathways activated in CSC as well as expression of drug transporters concur in conferring drug resistance to CSCs and represent the ideal targets for development of novel treatment strategies to improve patient response and prolong their overall survival. Signaling pathways associated with stem cell properties, such as differentiation and self-renewal capacities, have all been found often hyper-activated in CSC, and specific inhibitors blocking signaling activation are under investigation in preclinical studies and clinical trials showing some promising results. Major concerns arise from the evidence that the same signaling pathways are shared by CSCs and normal stem cells; thus further studies are necessary to identify more precise therapies which can selectively target CSCs but avoid toxicity to normal stem cells.

Besides intrinsic properties of CSC, others extrinsic factor derived from the tumor microenvironment can mediate CSC-induced drug resistance. Several cytokines released from stromal cells may trigger the activation of EMT in cancer cells resulting in acquisition of stemness properties together with other capabilities such as increased invasion/dissemination, resistance to anoikis and resistance to apoptosis/chemotherapy. In lung cancer EMT is associated with metastatic progression, resistance to EGFR inhibitors, chemotherapy, and generation of CSCs. The documented plasticity of differentiated tumor cells able to convert to stem-like phenotype under microenvironmental signaling represents another layer of complexity in CSC targeting. Compounds able to impair tumor-stroma cross talk could prevent de novo generation of CSCs and acquisition of drug resistance through the induction of EMT thus possibly improving the effectiveness of current therapies.

A very significant aspect to be considered regarding CSC targeting is that current parameters used in clinical evaluation of treatment efficacy in particular in terms of local tumor shrinkage may not be appropriate for the evaluation of CSC subset depletion. Indeed, strategies that effectively target CSCs are not expected to have an immediate impact on tumor shrinkage but rather on long-term end points such as tumor recurrence or metastatic progression. For these reasons novel biomarkers are needed to evaluate the efficacy of innovative therapies in CSC targeting. Circulating tumor cells shed from primary tumor into blood stream represent a non-invasive liquid biopsy of tumors and may offer the unique possibility to monitor the modulation of CSC populations during treatment to ascertain therapy efficacy.

Compelling evidences have been provided in preclinical models for the existence of lung cancer stem cells and their drug resistance phenotypes although the inherent complexity of lung cancer and the difficulties related to establishment of primary cultures of lung-derived cells have restrained advancements in lung CSC characterization if compared to other solid tumors. Novel therapeutic strategies targeting CSCs have been tested in experimental models and already evaluated in clinical studies in advanced NSCLC, but further efforts are needed to translate current lung CSC knowledge into clinical practice and fulfill the expectation to provide innovative ways to overcome drug resistance in lung cancer.

Financial Support This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC) Special Program 12,162 (ED12162 to LR and GS) and IG16847 (to LR).

Conflicts of Interest No potential conflicts of interest were disclosed.

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