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Review

Are we Missing the Target? – Cancer Stem Cells and Drug Resistance in Non-small Cell Lung Cancer

SANDRA GOTTSCHLING[1], PHILIPP A. SCHNABEL[2], FELIX J.F. HERTH[3] and ESTHER HERPEL[2]

[1]*Department of Thoracic Oncology, Thoraxklinik, University of Heidelberg, Heidelberg, Germany;*
[2]*Institute of Pathology, University of Heidelberg, Heidelberg, Germany;*
[3]*Department of Pneumology, Thoraxklinik, University of Heidelberg, Heidelberg, Germany*

Abstract. *Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death in Western countries. Despite novel molecular therapies, the majority of patients with advanced or metastatic disease show rapid progression and a median survival time of not more than 18 months. In the last decade, there has been increasing evidence that cancer stem cells (CSC) play a pivotal role in drug resistance, tumour regeneration and metastasis of various cancer entities including lung cancer. In this review, we discuss the evidence for stem cells in NSCLC, their predictive and prognostic significance, their specific mechanisms of resistance and potential targets and strategies for eradication of these cells. Consideration of the specific properties of CSC in lung cancer therapy might substantially contribute to increased response and prolonged survival rates in this disease.*

Lung cancer is the leading cause of cancer-related death in Western countries (1). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases and is divided into the major histological subtypes of adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC). About 70% of all newly-diagnosed patients present with locally-advanced or metastatic disease and require systemic treatment (2). Despite novel molecular therapies, the prognosis of lung cancer is still poor and shows a median survival time of about 18 months in inoperable stages.

Correspondence to: Sandra Gottschling, MD, Department of Thoracic Oncology, Thoraxklinik, University of Heidelberg, Amalienstr. 5, 69126 Heidelberg, Germany. Tel: +49 62213968069, Fax: +49 62213961302, e-mail: sandra.gottschling@thoraxklinik-heidelberg.de

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Lung cancer is characterized by frequent intratumoural heterogeneity (3). Up to 63% of all NSCLC were reported to contain more than one histologic subtype. This led to the assumption that lung cancer derives from a multipotent stem-like cell which is able to generate these various histological components.

The major criteria of stem cells is their ability to self-renew and differentiate into various cellular lineages via asymmetric division. Moreover, stem cells are characterized by slow division kinetics, drug and irradiation resistance and an increased capacity of invasion, metastasis, tumour formation and proliferation (4-7). Two types of stem cells are distinguished: pluripotent embryonic stem cells (ESC) which are able to differentiate into cells of all three germ layers and multipotent adult stem cells which are able to differentiate into various cells of a specific tissue or organ. Since the knowledge on ESC in lung cancer is yet scant, this review focuses on the relevance of adult stem cells in this disease.

So far, serial transplantation experiments in rodent models represent the gold standard for the approval of stem cell characteristics. Moreover, several surface antigens such as CD24, CD34, CD44, CD117 and CD133 and the expression of *aldehyde dehydrogenase 1A1* (*ALDH1A1*) are used for the identification and prospective isolation of adult stem cells. In addition, the ability to extrude dyes such as Hoechst 33342 or Rhodamine 123 and the slow division kinetics of stem cells, which results in the retention of membrane or DNA dyes such as PKH, carboxyfluorescein succinimidyl ester (CFSE) or bromdesoxyuridine (BrDU), are used to identify these cells (Figure 1).

Two general concepts of stem cell-driven tumorigenesis exist (Figure 2): In the deterministic model, all tumour cells originate from a degenerated stem cell or a de-differentiated tumour cell which acquired stem cells characteristics. This cancer stem cell (CSC) simultaneously self-renews and generates committed tumour cells *via* asymmetric division (8, 9). The committed tumour cells are thought to follow a

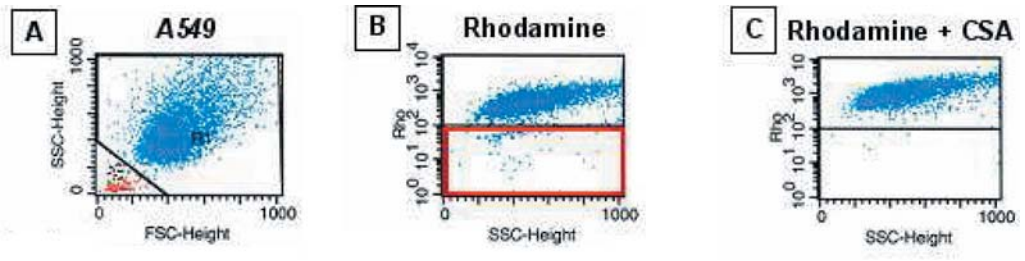

Figure 1. *Side population (SP) of the adenocarcinoma cell line A549. The adenocarcinoma cell line A549 was stained with Rhodamine 123 (Rho), a dye extruded by the ABC transporter multidrug resistance-1 (MDR1). After 2 h, a small subpopulation of cells had effluxed the dye and showed a decreased fluorescence intensity (red box; picture B). When the ABC transporter was blocked with ciclosporine A (CSA), the cells expressing this drug transporter were not able to extrude the dye and maintained their initial fluorescence intensity (picture C). Dead cells were excluded from analysis (picture A). Dot plots: FSC: foward scatter, SSC: side scatter, y-axis: fluorescence intensity (log), horizontal line within the dot plots: autofluorescence cut-off.*

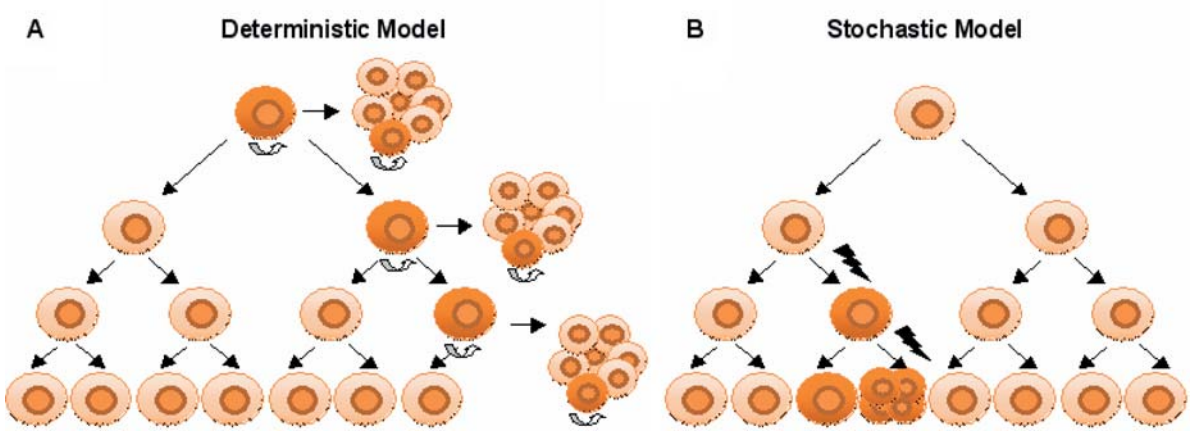

Figure 2. *Models of stem cell-driven tumorigenesis. A: Deterministic model. The tumor originates from a cancer stem cell (CSC; dark brown) which simultaneously generates committed tumour cells (light brown) and CSC by asymmetric cell division. B: Stochastic model. The tumour cells (light brown) acquire genetic alterations (flashes) and undergo clonal evolution resulting in the generation of dominant clones with CSC properties (dark brown). All cells undergo symmetric division. In reality both concepts might occur in parallel or in combination.*

pre-destinated and irreversible route of differentiation. The stochastic model, in contrast, assumes that one or more tumour cells randomly acquire genetic alterations which induce stem cell characteristics. Subsequent clonal selection results in the evolution of dominant clones which mainly sustain the tumour growth and proliferate *via* symmetric division. Differentiation of tumour cells occurs randomly and reversibly by events such as exposure to niche-specific factors or subsequent genetic changes. In reality both concepts might occur in parallel or in combination.

Irrespective of the way of stem cell-driven tumorigenesis, the presence of such cells in tumour tissue would require either elimination of this critical population or of all dominant clones for successful therapy. Since stem cells play a crucial role in tissue repair and homeostasis, they possess special mechanisms that protect them from lethal damage. These mechanisms represent an elementary obstacle for the successful therapy of cancer.

In this review, we will discuss the evidence for stem cells in normal lung tissue and lung cancer, their prognostic and predictive significance and novel therapeutic options for targeting these cells.

Evidence for Stem Cells in the Normal Lung Epithelium

The lung epithelium consists of several different cell types whose composition varies with the anatomical region (Figure 3): The epithelium of the trachea and the proximal airways consists of ciliated cells and Clara-like cells producing secretoglobins, and moreover, a small number of neuroendocrine cells and mucus-producing submucosal

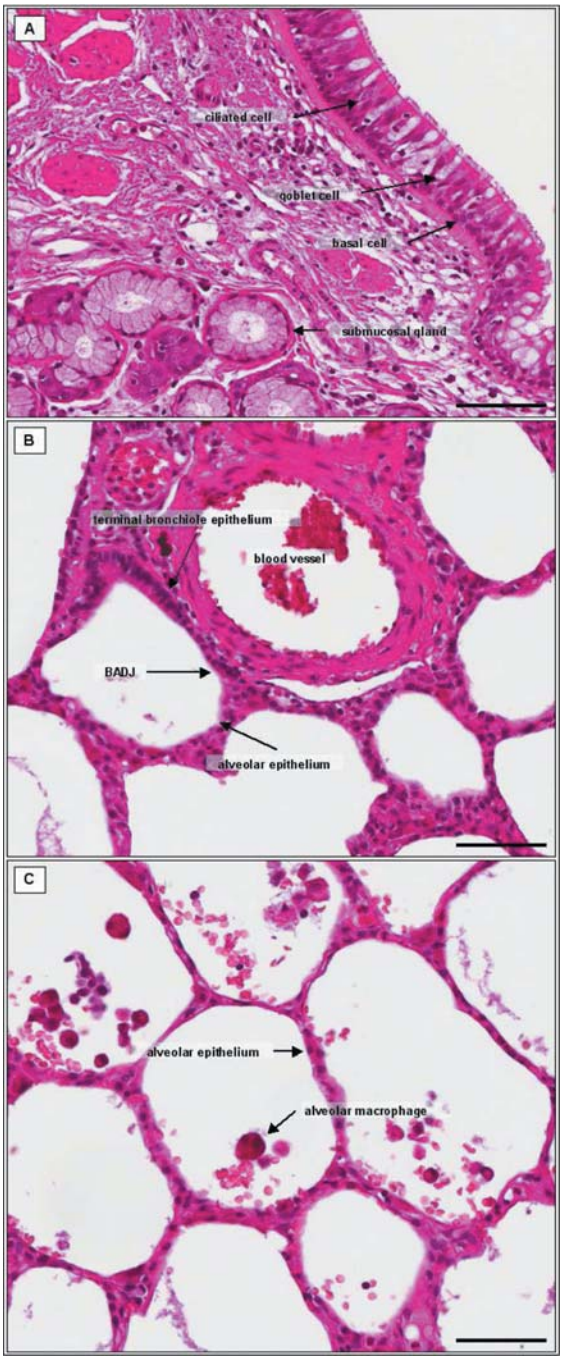

Figure 3. *The lung epithelium. A: Tracheobronchial epithelium. The columnar tracheobronchial epithelium contains ciliated cells, goblet cells, basal cells and submucosal glands. Clara cells and neuroendocrine cells are not shown. Basal cells and a variant of Clara cells are considered stem/progenitor cells B: Bronchioalveolar duct junction (BADJ). As shown in rodent models, the junction between the cuboidal terminal bronchiole epithelium and the alveolar epithelium contains bronchioalveolar stem cells (BASC). C: Alveolar epithelium. The alveolar epithelium consists of pneumocytes type I and II and alveolar macrophages. Pneumocytes type II are able to regenerate and differentiate into pneumocytes type I. Microphotographs: Stain: hematoxylin-eosin, magnification: ×200, scale bar: 50 μm.*

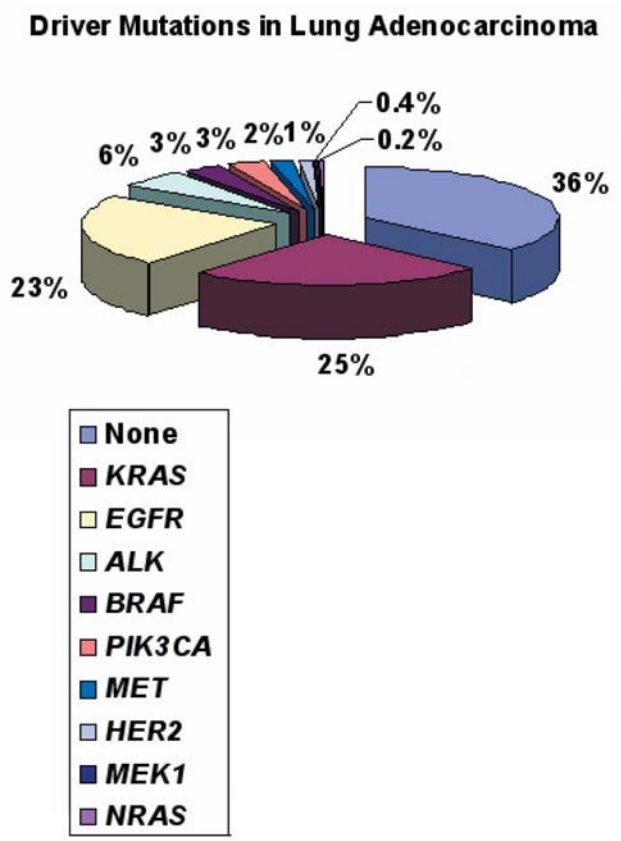

Figure 4. *Driver mutations in lung adenocarcinoma. Mutation analysis of 422 patients with lung adenocarcinoma revealed driver mutations in 60% of the cases. K-ras (Kirsten rat sarcoma) and EGFR (epidermal growth factor receptor-1) mutations represent the most prominent alterations. Other mutations, ALK (anaplastic lymphoma kinase) rearrangements and MET (Met proto-oncogene) amplifications are present in <10% of the tumours. BRAF: B-Raf proto-oncogene serine/threonine-protein kinase, PIK3CA: phosphatidylinositol-3-kinase, catalytic, alpha polypeptide, HER2: epidermal growth factor receptor-2, MEK1: dual specificity mitogen-activated protein kinase kinase-1, NRAS: neuroblastoma RAS viral oncogene homolog. Adapted from M.G. Kris et al., J Clin Oncol 2011 (May 20 Supplement).*

glands containing ciliated and basal cells (7). In the more distal airways, Clara cells predominate over ciliated cells and there are more neuroendocrine cells, but no basal cells. The alveolar epithelium consists of pneumocytes type I and pneumocytes type II, producing surfactant factor.

The first evidence for the presence of regenerating cells in the lung epithelium came from rodent injury models in the 1970s (7). Selective damage of specific cell types by inhaled oxidants such as NO_2 or ozone or intravenous administration of bleomycin revealed a regenerative potential of Clara cells and pneumocytes type II. In the nineties and 2000s, intraperitoneal injection of naphthalene showed that the

regenerative potential of Clara cells is restricted to a specific subpopulation, so called variant Clara cells, which are lacking expression of cytochrome *P450F2*. Additional genetic labeling studies showed that cytokeratin 14- positive basal cells also possess regenerative potential in the proximal airways and give rise to basal cells, Clara cells and ciliated cells (10).

The distal lung epithelium appears to be regenerated by variant Clara cells and *calcitonin gene-related peptide* (*CGRP*)-expressing pulmonary neuroendocrine cells, both located in the neuroendocrine bodies (11). Moreover, in 2005 Bender Kim *et al*. showed that the junction between the cuboidal epithelium of the terminal bronchiole and the alveolar epithelium contains a population of stem cell *antigen-1* (*Sca-1*)$^+$, CD31$^-$, CD34$^+$, CD-45$^-$, Clara cell antigen (CCA)$^+$ cells which maintain the terminal bronchiolar epithelium (12). These so-called bronchioalveolar stem cells (BASC) gave rise to Clara-like, pneumocyte type II-like and peumocyte type I-like cells *in vitro* and showed expansion and differentiation towards the alveolar lineage upon induction of a *Kirsten rat sarcoma* (*k-ras*) mutation *in vivo*.

So far, it is unclear whether these stem and progenitor cells are organized in a hierarchical structure, as known from the hematopoietic system, or represent different types of region-specific stem cells. An analysis of Qualter *et al*. in *epithelial cell adhesion molecule* (*EpCAM*)$^+$, Sca-1$^+$, CD24^{dim}, CD31$^-$, CD45$^-$, CD49$^+$, CD104$^+$ murine lung epithelial cells provided first evidence for a stem cell hierarchy in the lung (13). These cells were able to self-renew in cytokine-supplemented, stroma-free cultures and generated colonies of airway, alveolar and mixed lung epithelial lineages upon co-culture with pulmonary mesenchymal cells.

Although the use of different stem and progenitor cell markers in the various studies hamper an uniform view so far, there is strong evidence that the lung contains various stem and progenitor cells that regenerate specific regions of the pulmonary epithelium and might be organized in a hierarchical structure (14). The characteristics of these putative stem and progenitor cells are summarized in Table I.

Evidence for Stem Cells in NSCLC

First evidence for the presence of stem cells in NSCLC came from a mouse strain with inducible *k-ras G12D* mutation developed by Bender Kim *et al*. (12). In 2005 this group demonstrated an increased expansion and reduced differentiation of BASC upon activation of *k-ras* resulting in an atypical adenomatous hyperplasia. Combination of *k-ras* activation and naphthalene treatment led to an increase in tumour number and areas, strongly indicating that BASC might represent the progenitor cells of pulmonary adenocarcinoma. Subsequent analyses of Xu *et al*. demonstrated that not only BASC, but also pneumocytes type II and Clara cells in the terminal bronchioles proliferate in response to *k-ras* activation. In this study, only pneumocytes type II progressed to adenocarcinoma (15).

As for human lung cancer cell lines and primary cells derived from lung cancer samples, Ho *et al*. demonstrated the presence of a Hoechst dye 33342 extruding subpopulation, the so-called side population (SP), which accounted for 0.03%-6.1% of the tumour cells (16). In contrast to non-SP cells, this small subpopulation was able to regenerate SP and non-SP cells and showed an increased tumorigenicity in mice. In line with their resistance to multiple cytostatic drugs and enhanced proliferation capacity, these cells showed an elevated expression of various ATP-binding cassette transporters and *human telomerase reverse transcriptase* (*hTERT*).

Eramo *et al*. used the common stem cell antigen CD133 to prospectively isolate human lung cancer cells with stem cell properties. These cells were able to form tumour spheres, showed co-expression of the ATP-binding cassette transporter *breast cancer resistance protein-1* (*BCRP1*) and the ESC transcription factors *octamer binding protein* (*Oct*) *3/4* and *Nanog*. Moreover, these cells were highly tumorigenic in mice, able to regenerate the original tumour histology and resistant to various cytostatic drugs (17). Chen *et al*. reported very similar results for CD133-positive NSCLC cells and moreover showed that these cells lost their stem cell characteristics when the expression of *Oct4* was knocked down by siRNA (18).

Levina *et al*. who used a reverse approach to isolate lung cancer stem cells also reported corresponding findings (19). Exposure of the NSCLC cell line *H460* to cytostatic drugs enriched a Hoechst dye 33342-extruding SP. The antigen profile and functional properties of this SP was basically consistent with the stem cell population described by Eramo *et al*. and Chen *et al*. In addition to previous analyses, Levina *et al*. demonstrated that these cells had an activated *β-Catenin* pathway known to be involved in stem cell self-renewal, and moreover, that tumours originating from these cells show a high expression of angiogenic cytokines and growth factors.

Bertolini *et al*. confirmed an enrichment of CSC after cytostatic treatment *in vivo* (20). When mice carrying lung cancer xenografts were treated with cisplatin, the tumours showed an enrichment of epithelial cells expressing *CD133* and *BCRP1*. These cells shared several functional and molecular properties with the CSC described in previous studies including an extraordinary high tumorigenicity in mice and an increased expression of the transcription factors *Oct3/4* and *Nanog*, adhesion and motility genes and various ATP-binding cassette transporters.

As for human pulmonary CSC, CD133 and *BCRP1* appear to be the most appropriate markers so far. However, a recent study of Curtis *et al*. demonstrated that the phenotype of lung cancer propagating cells varies with the type of genetic alteration present in the tumour (21). Since recent analyses in

Table I. *Putative pulmonary stem and progenitor cells. The table shows the characteristics of putative stem/progenitor cells identified in the lung epithelium of rodent models. CK: Cytokeratin, CCSP: Clara cell secretory protein, CYP2F2: cytochrome P450, family 2, subfamily F, polypeptide 2, NEB: neuroendocrine body, PNEC: pulmonary neuroendocrine cells, CGRP: calcitonin gene-related peptide, SP-A, -C, -D: surfactant protein-A, -C, -D, AQP3: aquaporin 3, BASC: bronchioalveolar stem cell, Sca-1: stem cell antigen-1, BADJ: bronchioalveolar duct junction, y: yes, n: no.*

Putative stem/ progenitor cell	Marker	Niche	Descendant	Self-Renewal	Multi-lineage differentiation	References
Basal cell	$CK5^+$, $CK14^+$, $p63^+$	Conducting airway epithelium	Basal cells, Clara cells, ciliated cells	y	y	Hong *et al*., 2004
Variant Clara cell	$CCSP^+$, $CYP2F2^-$	NEB	Variant Clara cells, Clara cells, PNEC, ciliated cells	y	y	Hong *et al*., 2001
PNEC	$CGRP^+$	NEB	PNEC	y	n	
Type II pneumocyte	$SP\text{-}A^+$, $SP\text{-}C^+$, $SP\text{-}D^+$, $CD44v6^+$, $AQP3^+$	Alveoli	Type II pneumocytes, Type I pneumocytes	y	n	Evans *et al*., 1975
BASC	$CCSP^+$, $SP\text{-}C^+$, $CD34^+$, $Sca\text{-}1^+$, $CD45^-$, $CD31^-$	BADJ	Clara cells, type II pneumocytes	y	y	Bender Kim *et al*., 2005

lung adenocarcinoma revealed the presence of driver mutations in up to 87% of all cases, several phenotypes of pulmonary CSC might exist. This suggests that rather functional properties than surface antigens represent appropriate targets for specific therapies against CSC (22, 23).

Predictive and Prognostic Significance of CSC in Lung Cancer

In recent years, several immunohistochemical and a few microarray studies analyzed the prognostic and predictive significance of CSC antigens and gene signatures in NSCLC.

As for the most common human pulmonary CSC antigens CD133 and *BCRP1*, expression frequencies between 15%-have been 63% and 15%-51%, respectively, were reported (24-29). While our group and Salnikov *et al*. found no prognostic significance of CD133 and *BCRP1* expression in 133 stage I/II and 88 stage I-III patients who underwent complete pulmonary resection, two Asian studies in 145 stage I NSCLCs and 177 stage I AC patients found an up to 9.3-fold increased risk of postoperative disease recurrence in CD133/*BCRP1* and CD133-positive cases, respectively (25, 28-30).

In patients with locally-advanced or metastatic disease who received platinum-based therapy studies of Yoh *et al*. and Ota *et al*. demonstrated a predictive and prognostic significance of *BCRP1* (26, 27): while Yoh *et al*. reported a higher response rate (44% *vs*. 24%, $p=0.08$) of patients with *BCRP1* negative tumours and a 2.8-fold ($p=0.001$) and 1.8-fold ($p=0.08$) reduced risk of relapse and disease-related mortality, respectively, Ota *et al*. found no association between *BCRP1* expression and response to therapy or progression-free survival (PFS), but an association with a significantly reduced median survival time (7.8 *vs*. 14.9 months, $p=0.03$) in *BCRP1*-positive cases.

Also in neoadjuvant treatment, a recent study in 50 patients who received preoperative chemoradiotherapy, demonstrated that the 5-year overall survival (OS) was worse in patients with CD133 or *ALDH1* expression (45% *vs*. 90%, $p=0.042$) (31).

Authors who used other stem cell markers such as the basal cell antigen cytokeratin 14 or the ESC transcription factor *Oct4*, found a 1.6-fold increased risk of disease-related mortality in two large datasets of 399 ($p=0.003$) and 505 stage I-IV patients ($p=0.004$) and a 2.2-fold increased risk of disease-related mortality in a collective of 112 operated stage IB-IIIA AC patients ($p=0.015$), respectively (32, 33).

Onaitis *et al*. applied a 10-gene progenitor cell signature derived from a transgenic mouse model to a dataset of resected human AC patients and found a 3.5-fold increased risk of disease-related mortality ($p=0.0004$) when this gene signature was expressed (34).

All in all, these studies provide strong evidence that the presence of tumour cells expressing antigens or gene signatures associated with stem cells might play a prognostic and predictive role in NSCLC.

Significance of Drug Resistance in Current Lung Cancer Therapy

In recent years, increasing efforts were undertaken to identify therapy responders in advance. The presence of specific targets and the absence of resistance mechanisms represent the two pillars of tailored therapy.

Overexpression of key enzymes of the folate synthesis, particularly *thymidylate synthase* (*TS*), and components of the mitotic spindle apparatus such as *tubulin beta III* (*TUBB3*) show a high level of evidence for an association with response to folate antagonist such as pemetrexed and microtubule-inhibiting taxanes, respectively (35-37).

Particularly targeted therapies with tyrosine kinase inhibitors (TKI) such as erlotinib, gefitinib and crizotinib rendered impressive results when response predictors were considered. While conventional chemotherapy shows response rates of maximal 30% in the first-line treatment and about 10% in subsequent therapy lines, treatment with the *epidermal growth factor receptor* (*EGFR*)-TKI erlotinib or gefitinib or the *echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase* (*EML4-ALK*)-TKI crizotinib in patients with activating *EGFR* mutations or *EML4-ALK* rearrangements shows response rates of ≥70% and 57%, respectively (38, 39).

Also DNA repair proteins such as *excision repair cross-complementation group 1 (ERCC1)*, *breast cancer 1, early onset* (*BRCA1*) and *ribonucleotid reductase 1* (*RRM1*) gained growing significance for tailoring cisplatin or gemcitabine-containing therapy regimens. Operated patients who received cisplatin-based, adjuvant treatment showed a significantly longer survival, when their tumours were *ERCC1*-negative. Also in advanced disease, lower *ERCC1* mRNA levels were highly predictive for longer survival after cisplatin-based treatment (40-42). In metastatic disease, low *BRCA1* mRNA levels predicted a higher response rate, longer PFS and median OS towards platinum-containing second-line therapies (43). As for gemcitabine, studies of Bepler *et al*. demonstrated that patients with low *RRM1* mRNA levels experienced better response and longer PFS and OS to this antimetabolite (44, 45). However, none of these candidate biomarkers have been established to date since large, prospective trials are just getting under way.

So far, the association between these putative biomarkers and CSC is widely unknown. The findings of Ota *et al*. who demonstrated a correlation between protein expression of *ERCC1* and the stem cell antigen *BCRP1* (p=0.012) and the results of Salnikov *et al*. who demonstrated a correlation between protein expression of *TS* and the stem cell antigen CD133 (p<0.01), indicate that tumour cells with predominant expression of these enzymes might bear stem cell characteristics (27, 29).

Mechanisms of Drug Resistance in CSC

Hitherto, little heed has been taken of the specific drug resistance mechanisms of CSC in antineoplastic treatment. The ability of asymmetric cell division, the slow division kinetics or even quiescence and the expression of several drug efflux pumps and DNA repair proteins represent the major escape mechanisms of stem cells (46-48).

In 2010 Pine *et al*. demonstrated for the first time that a small subpopulation of lung cancer cells which were present in commercially available cell lines and primary tumour cell cultures was able to undergo asymmetric cell division: While the differentiation markers *prosurfactant protein C* and pan-cytokeratins were passed to one daughter cell, the stem cell marker CD133 was passed to the other and exclusively characterized cells with tumour repopulating capability (49). A subsequent study of Dey-Guha *et al*. in breast cancer cell lines showed that asymmetric division generated slowly cycling G_0-like cells which were enriched in tumour tissue after neoadjuvant therapy (50). The presence of such a quiescent and drug-resistant subpopulation has also been shown in lung cancer (51). Several pathways and molecules such as *v-akt murine thymoma viral oncogene homolog 1* (*AKT1*), *insulin-like growth factor receptor-1* (*IGF-1R*), *protein kinase C* (*PKC*), *aurora kinase A* (*AURAKA*), *Notch, phosphatidylinositol 3-kinase* (*PI3K*), *p53* or *phosphatase and tensin homolog deleted on chromosome 10* (*PTEN*) were found to be involved in the regulation of division symmetry and quiescence of stem cells (50-53). As most classical cytostatics require cell division for being effective, quiescence or slow division kinetics protects tumour cells against lethal DNA damage. Moreover, novel molecular drugs such as *AKT*-TKI might rather support than suppress CSC as they favor asymmetric cell division and the generation of slowly-cycling, drug-resistant tumour cells (50, 54).

Besides division symmetry and slow division kinetics, CSC show an increased expression of DNA repair proteins and drug efflux transporters. Salnikov *et al*. and Ota *et al*. demonstrated that overexpression of *TS* and *ERCC1* which might predict resistance against pemetrexed and cisplatin, respectively, correlated with the expression of the stem cell antigens *CD133* and *BCRP1* (27, 29). Moreover, several other proteins involved in detoxification and drug resistance such as *glutathione-S-transferase*, the drug transporter *ABCC2* (*multidrug resistance 1* [*MDR1*], *p-glycoprotein* [*p-gp*]), *metallothionein*, *catalase or O^6-methylguanine-DNA methyltransferase* showed an overexpression in CD133-positive cells. Ho *et al*. reported an increased expression of the drug transporters *ABCG2* (*BCRP1*), *ABCA2*, *MDR1* and *ABCC1* (*multidrug resistance protein-1* [*MRP1*]) in the SP of various NSCLC cell lines (16). In other tumour entities, *glutathione-S-transferase* and *MRP1* were shown to confer resistance to gemcitabine, while *MDR1* was associated with resistance to paclitaxel and *BCRP1* with resistance to the *EGFR*-TKI gefitinib (55-57).

All in all, CSC possess several mechanisms to overcome irreversible damage by cytostatic drugs. These mechanisms have neither been studied sufficiently nor have they been considered in cancer therapy so far.

Potential Targets and Approaches for CSC-directed Therapy

The ability of asymmetric cell division and the generation of slowly cycling or quiescent progenies represent important escape mechanim of stem cells. Thus, pivotal molecules and mechanisms involved in the regulation of division symmetry might provide one of the Achilles' heels to target these cells in NSCLC.

Table II. *Potential targets and therapeutic agents against cancer stem cells (CSC). The table shows a selection of substances which are currently tested in clinical trials including lung cancer (ClinicalTrials.gov). The listed target molecules and pathways are involved in the regulation of CSC. PARP1: poly(ADP-ribose)-polymerase 1, MDR1: multidrug-resistance 1, Akt: v-akt murine thymoma viral oncogene, PI3K: phosphatidylinositol 3-kinase, mTOR: mechanistic target of rapamycin (serine/threonine kinase), MAPK: mitogen-activated protein kinase, *: selection.*

Target	Function	Agent	Phase	ClinicalTrials.gov Identifier
PARP1	Single strand break damage repair	Iniparib (BSI-201)	III, II	NCT01082549, NCT01086254
Telomerase	Self-renewal	GV 1001 telomerase peptide,	II, III	NCT00509457, NCT01579188
		Imetelstat (GRN163L)	II	NCT01137968
MDR1	Drug resistance	Tariquidar (XR9576)	III, III	NCT00042315, NCT00042302
Wnt/β-Catenin	Self-renewal	PRI-724	I	NCT01302405
Hedgehog	Self-renewal	Vismodegib (GDC-0449)	I, I	NCT00968981, NCT00607724
		Saridegib (IPI-926)	I	NCT00761696
		LEQ506	I	NCT01106508
		BMS-833923	I, I	NCT00670189, NCT01413906
		PF-04449913	I	NCT01286467
Notch	Self-renewal	RO4929097	I, II,	NCT01193881, NCT01193868,
			I	NCT01131234
		LY2606368	I	NCT01115790
Akt	Self-renewal	MK-2206	I, II	NCT01147211, NCT01294306*
PI3K	Self-renewal	BKM-120	I/II, I,	NCT01487265, NCT01570296,
			II, II	NCT01297491, NCT01501604
mTOR	Self-renewal	Everolimus (RAD001)	I, I	NCT00457119, NCT00456833*
MAPK	Self-renewal	Selumetinib (AZD6244)	I, II	NCT01586624, NCT01229150*
		PD-0325901	I, II	NCT01347866, NCT00174369
		MSC1936369B	I, I	NCT01357330, NCT01390818*
		GSK1120212	I, II	NCT00955773, NCT01362296
		LY2228820	I	NCT1393990
		LY3007113	I	NCT01463631

The *Notch*, *Wnt* and *Hedgehog* pathway represent key mechanisms of stem cell regulation (58). In 2008 Tsao *et al*. demonstrated that disruption of *Notch* signaling by inhibition of gamma-secretase cleavage of *Notch* receptors dramatically expanded the population of distal pulmonary progenitors and prevented formation of proximal structures in murine embryonic lungs (59). Two years later, Sullivan *et al*. showed that the proliferation and clonogenicity of self-renewing *ALDH*-positive CSC in primary human NSCLC cell lines was significantly decreased by treatment with either a γ-secretase inhibitor or stable expression of shRNA against *Notch3* (60). A large study of Westhoff *et al*. in tumour tissue of more than 400 NSCLC patients demonstrated an activated *Notch* pathway in about one third of the cases and a significantly worse survival of these patients when concomitant *p53* mutations were lacking (61). Altogether, these studies indicate not only a pivotal pathogenetic role, but also a prognostic relevance of *Notch* signaling in NSCLC.

Wnt signaling represents another pathway that is critically involved in the regulation of CSC (62). Levina *et al*. demonstrated that NSCLC cells surviving cytostatic treatment show stem cell properties and an activated *Wnt* pathway (18). Similar findings were reported by Teng *et al*. in cisplatin-selected *A549* NSCLC cells: Activation of *Wnt* signaling led to a significantly enhanced proliferation, colony formation, migration and drug resistance (63). In a murine model, Zhang *et al*. demonstrated that *Gata6*-regulated Wnt signaling controls the balance between progenitor cell expansion and epithelial differentiation required for both, lung development and regeneration (64).

As for *Hedgehog* signaling, recent studies of Shi *et al*. and Tian *et al*. in a pulmonary LCC and an AC cell line showed that the proliferation of the SP was significantly impaired by inhibition of the Hedgehog pathway with Cyclopamine or Vismodegib (GDC-0449), respectively (65, 66).

Another molecule which has repeatedly been reported to be involved in the regulation of CSC in NSCLC is the tumour suppressor gene *PTEN*. In a murine model with constitutive loss of *PTEN*, epithelial cells residing in the bronchioalveolar duct junction underwent proliferation and formed uniform masses (67). Similar results were derived from a previous study analyzing a murine model with bronchioalveolar epithelium-specific null mutation of *PTEN*: The numbers of BASC, the putative progenitors of pulmonary adenocarcinoma, were likewise increased (68). In addition, *PTEN* is an inhibitor of the *PI3K/AKT/mTOR* (*mammalian target of rapamycin*) signal transduction axis

and loss of function leads to an activation of these molecules which have all been shown to be involved in the maintenance of CSC (50, 52, 69).

Currently, several phase I and II studies testing inhibitors of the *Wnt*, *Notch* and *Hedgehog* pathway as well as the *PI3K/AKT/mTOR* signal transduction axis are in progress (Table II). However, it remains to be elucidated whether targeting these pathways and molecules will lead to an increased therapeutic efficacy or even have an opposite effect in lung cancer (54).

As for drug efflux transporters, pharmacodynamic studies with the *MDR1* inhibitors CBT-1(R) and tariquidar showed an increased uptake of the radionuclid ^{99m}Tc-sestamibi, a substrate of *MDR1* (70-72). However, two phase III studies in stage IIIB/IV NSCLC combining tariquidar with first line single-agent vinorelbine or a carboplatin/paclitaxel doublet showed no advantage and had to be terminated prematurely (ClinicalTrials.gov ID NCT00042315, NCT00042302). Recent *in vitro* and *in vivo* studies analyzing the effect of TKI such as axitinib, gefitinib, vandetanib, pelitinib or crizotinib on the function of *ABCG2* (*BCRP1*) and *ABCB1* (*MDR1*) demonstrated an inhibition of these drug efflux transporters and an enhanced efficacy of cytostatic drugs in multidrug-resistant cell lines and murine xenograft models (51, 73, 74). These TKI might be worthwhile combination partners for specific cytostatics such as anthracyclines, *Vinca* alkaloids, taxanes or epipodophyllotoxins in subsequent clinical trials.

A recent analysis of Bartucci *et al*. in CSC derived from primary NSCLC cultures demonstrated a strong activation of the *DNA damage checkpoint protein kinase 1* (*Chk1*) upon exposure to cisplatin, gemcitabine or paclitaxel (75). In contrast, differentiated, chemosensitive NSCLC cells showed only a weak activation of *Chk1*. When the *Chk1* inhibitor AZD7762 and chemotherapy were co-administered tumour growth *in vivo* was suppressed, whereas chemotherapy alone was scarcely effective. Thus, AZD7761 might represent an interesting agent for future clinical trials directing CSC in NSCLC.

Poly [ADP-ribose] polymerase 1 (*PARP1*), *telomerase and mitogen-activated protein kinases* (*MAPK*) respresent other promising targets for the eradication of CSC. In patients with squamous NSCLC, low expression of the DNA repair protein *PARP1* showed a significant association with an increased chemotherapeutic efficacy (76). Although analyses on *PARP1* expression in pulmonary CSC are so far lacking, recent data on the pivotal significance of this enzyme in the regulation of pluripotency and differentiation of murine ESC suggest a general role in stem cells (77). As for human telomerase, Ho *et al*. demonstrated an increased expression of *hTERT* in the SP of various NSCLC cell lines and primary NSCLC cells corresponding to the enhanced proliferation capacity of this subpopulation (19, 78, 79). Also *MAPK* is involved in the regulation of pulmonary stem cell proliferation (80). However, the efficacy of *MAPK* inhibitors might depend on the type of *MAPK* that is blocked: While inhibition of *rat fibrosarcoma/mitogen-activated protein kinase kinase 1/2* (*Raf/ERK1/2*)-mediated downstream signaling might impair the self-renewal of NSCLC cells, blocking of the *p38 MAPKα* might have the opposite effect: In a murine model of Ventura *et al*., inhibition of *p38 MAPKα* generated an immature and hyperproliferative lung epithelium which was highly susceptible to *k-ras*-induced tumorigenesis (80-82).

Presently, several early phase studies analyzing the efficacy of *MAPK* inhibitors in lung cancer and various phase II and III trials with the *PARP1* inihibitor Iniparib and agents against telomerase are in progress (Table II). High throughput screening methods such as those developed by Gupta *et al*. for breast cancer stem cells, might represent an effective approach for the identification of novel compounds against CSC (83).

All in all, consideration of CSC and their specific mechanisms of drug resistance might pave novel ways for more effective treatment and long-lasting therapeutic success in lung cancer.

Future Perspectives

Although analyses in lung cancer and various other tumour entities provide strong evidence for a pivotal role of CSC in drug resistance and relapse, several questions remain to be answered.

So far, various markers and methods have been used to identify and isolate cancer cells with self-renewing and multilineage differentiation capability. However, comparative functional and molecular analyses of these differentially isolated cells are so far lacking. It is unclear, whether they represent the same cell population, a hierarchy of stem and progenitor cells or different cell types which share some common functional and molecular properties. Moreover, the origin and development of these cells is not yet understood: Do CSC derive from a neoplastic stem cell or a neoplastic tumour cell which acquired stem cell charcteristics? Does their development require specific mutations that occur in an orchtestrated manner or does it represent a clonal selection of randomly mutated cells by microenvironmental cues? The identification of specific markers and gene signatures of various differentiation stages might represent a first step towards better understanding of pulmonary stem cells. However, established differentiation assays are the prerequisite for such analyses and are yet lacking.

Moreover, several fundamental results on lung stem cells derive from murine models. So far, it is uncertain, whether these findings can be transferred onto human conditions. The common murine stem cell marker *Sca-1*, for instance, has no human homologue. In addition, the life expectancy of mice and men is very different and might affect the proliferation

capacities of murine and human stem cells. As for transplantation studies, the growth behaviour of human xenografts strongly varies under the murine background and thus, results from *in vivo* studies might in a large part reflect the experimental conditions and less the genuine behaviour of the human tumour cells.

The development of novel imaging techniques to identify and track stem cells in their natural niche might help to better understand their significance in drug resistance, tumour regeneration and metastasis (84-86). However, these methods are still in early stages of development.

Altogether, several *in vitro* and *in vivo* studies provide strong evidence for the presence of drug-resistant and highly reg-nerative tumour cells in NSCLC. Eradication of these cells might contribute to better response and prolonged survival in lung cancer.

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