Lung cancer stem cells
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Lung cancer remains the leading cause of cancer-related deaths despite recent breakthroughs in immunotherapy. The widely embraced cancer stem cell (CSC) theory has also been applied for lung cancer, postulating that an often small proportion of tumor cells with stem cell properties are responsible for tumor growth, therapeutic resistance and metastasis. The identification of these CSCs and underlying molecular maintenance mechanisms is considered to be absolutely necessary for developing therapies for their riddance, hence achieving remission. In this review, we will critically address the CSC concept in lung cancer and its advancement thus far. We will describe both normal lung stem cells and their malignant counterparts in order to identify common aspects with respect to their emergence and regulation. Subsequently, the importance of CSCs and their molecular features in lung cancers will be discussed in a preclinical and clinical context. We will highlight some examples on how lung CSCs attain stemness through different molecular modifications and cellular assistance from the tumor microenvironment. The exploitation of these mechanistic features for the development of pharmacological therapy will also be discussed. In summary, the validity of the CSC concept has been evidenced by various studies. Ongoing research to identify molecular mechanisms driving lung CSC have revealed potential new cell intrinsic as well as tumor microenvironment-derived therapeutic targets. Although successfully demonstrated in preclinical models, the clinical benefit of lung CSC targeted therapies has thus far not been demonstrated. Therefore, further research to validate the therapeutic value of CSC concept is required.

1. Introduction

Lung cancer is the most common cancer type worldwide with the highest mortality rate. In 2018, global estimates of incidence and death were at 2.1 million and 1.8 million respectively [1]. Lung cancer is histologically categorized into small cell (SCLC) and non-small cell lung carcinoma (NSCLC), constituting about 15% and 85% of the disease, respectively. Lung cancer is often diagnosed at late stages of disease, limiting the applicability of surgery and radiotherapy, making chemotherapeutic regimens the preferred treatment. Significant advancement has been achieved with NSCLC treatment, particularly in adenocarcinoma (ADC) and squamous cell carcinoma (SCC), indicated by the number of treatments approved by the FDA that include epidermal growth factor receptor (EGFR) and ALK targeted therapy and, more recent therapeutic targeting.
On the other hand, for SCLC, which is already metastatic at early stages of disease, no effective targeted therapy has been identified as yet. First line treatment chemotherapy in SCLC has not changed since the last decades, although recently promising results were obtained with immune checkpoint inhibitors that may lead to the long looked for improvement in survival of SCLC patients [3]. Despite these encouraging developments in subgroups of patients, therapeutic improvement for lung cancer patients as a whole are still needed.

In the last two decades, the cancer stem cell (CSC) hypothesis has attracted immense attention from both tumor conceptual as well as therapeutic perspective [4]. CSCs, representing a highly tumorigenic subpopulation, are deemed responsible for causing tumor growth, therapy resistance and recurrence, and metastasis possibly through epithelial-to-mesenchymal (EMT) reprogramming. A substantial amount of evidence points to the involvement of CSCs in tumorigenesis and progression of lung cancer. In this review, we will critically discuss lung tumorigenesis and therapeutic opportunities based on the CSC concept also taking into account possible cellular and molecular similarity with normal lung tissue homeostasis.

2. Adult lung epithelial stem cells and tissue repair

Adult stem cells are key cellular components of mature tissues or organs that participate in replenishing damaged and aged cells to maintain tissue homeostasis. These multipotent stem cells are able to self-renew and are restricted in their lineage to a specific subset of cells, only capable of giving rise to cellular offspring functional in that particular organ. Long-lived tissue-specific stem cells have been identified in various organs, such as the intestinal and skin stem cells, which are dedicated to differentiate into lineage-specific progenitor cells to secure proper tissue formation and organ functioning [5]. The progenitor cells of those two organs are required to cycle frequently to balance-off the shedding-prone environment.

The lungs have been recognized to possess remarkable tissue repair capability. This allows the lungs to recover efficiently from injury in response to inhaled particles/irritants and from bacterial and viral infections. At steady state, unlike in skin and intestine, the lung epithelia’s turn-over rate is extremely low with half-cycling time averaging at 17 months, referring to the terminally differentiated ciliated cells’ turnover using lineage-labeling system [6]. For proliferation, they rely on signals originating from surrounding mesenchyme to initiate cell cycling [7]. The tissue homeostasis and mild injury are regionally maintained and fixed by resident epithelial cells acting as lineage-committed progenitors in respective proximodistal locations (see Table 1). Basal cells provide maintenance functions in proximal regions (close to trachea), whereas club and alveolar epithelial type II (AEC II) cells are at distal sites. AEC II cells are restricted to the alveolar region of the lungs. In response to severe injury, different subsets of resident epithelia can dedifferentiate/differentiate and transdifferentiate into epithelial cells that aim to restore lung tissue homeostasis. In studies employing rodents lung injury models, lung resident epithelia such as basal cells, club cells, variant club cells and AEC II cells were found to serve as facultative stem cell reparative activity, which has been extensively reviewed recently (see [8]). In brief, basal cells maintain and repair large airway epithelium, whereas secretoglobin family 1A, member 1 (SCGB1A1)+ club cells are responsible for maintenance and repair of small airways under homeostatic conditions or conditions of mild injury [9,10]. In the alveolar space, AEC II cells can proliferate and differentiate into AEC I cells during steady state and mild injury [11]. Upon more severe injury, transformation related protein 63 (TRP63)+keratin 5 (KRT5)+KRT14+ basal cells were able to replenish naphthalene, SO2 and H1NI viral-induced damaged ciliated, club or even AEC I and AEC II cells. Recent studies demonstrated that mouse and human SRY-box 9 (SOX9)+ basal cells, presumably arising from SOX2+ progenitors, represent a rare subpopulation of basal cells that are able to regenerate recently, biological therapy using immune checkpoint inhibitors [2].

Table 1

<table>
<thead>
<tr>
<th>Human lung resident epithelia’s locations, functions and facultative stem activity</th>
<th>Proximodistal location</th>
<th>Function</th>
<th>Facultative stem activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal cells</td>
<td>Trachea, bronchi and bronchioles (absent in respiratory bronchioles)</td>
<td>Proximal</td>
<td>Structural support and stromal interactions</td>
</tr>
<tr>
<td>Ciliated cells</td>
<td>Trachea, bronchi and bronchioles</td>
<td>Proximal and distal</td>
<td>Clearance of irritants with cilia</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>Trachea, bronchi and bronchioles</td>
<td>Proximal</td>
<td>Clearance of irritants with mucus</td>
</tr>
<tr>
<td>Club cells</td>
<td>Bronchioles</td>
<td>Proximal and distal</td>
<td>Secretory for protection like secreting secretoglobin protein</td>
</tr>
<tr>
<td>Variant club cells</td>
<td>Neuroepithelial bodies and bronchoalveolar duct junctions</td>
<td>Proximal and distal</td>
<td>Not known, perhaps same as club cells</td>
</tr>
<tr>
<td>Neuroendocrine cells</td>
<td>Trachea, bronchi and bronchioles</td>
<td>Proximal and distal</td>
<td>Responders to neuronal signals by releasing hormone (receptosecretory)</td>
</tr>
<tr>
<td>Alveolar epithelial cell I (AEC I)</td>
<td>Alveoli</td>
<td>Distal</td>
<td>Facilitate gas exchange</td>
</tr>
<tr>
<td>Alveolar epithelial cell II (AEC II)</td>
<td>Alveoli</td>
<td>Distal</td>
<td>Provide secretory vesicles filled with surfactants and sheddings-prone environment</td>
</tr>
</tbody>
</table>
an important role in lung repair [15]. In general, EMT strips off epithelial phenotypes and switches cells to possess mesenchymal phenotypes termed alveolar epithelial progenitors expressing transmembrane 4 L six family member 1 (TM4SF1) and AXIN2 also function both during normal homeostasis and acute alveolar injury [14].

In addition to acquiring stem cell properties, EMT is known to play an important role in lung repair [15]. In general, EMT strips-off epithelial phenotypes and switches cells to possess mesenchymal phenotypes with migratory potential. This is modulated by transcription factors like snail family transcriptional repressors 1 (SNAI1), twist family bHLH transcription factor 1 (TWIST1) and zinc finger proteins like snail family transcriptional repressors 1 (SNAI1), twist family bHLH transcription factor 1 (TWIST1) and zinc finger E-Box binding homeobox 1 (ZEB1) by reducing expression of epithelial and increasing mesenchymal proteins [16]. Diverse signaling pathways are able to activate these transcription factors that initiate EMT, including Notch, Sonic Hedgehog (SHH), Wnt/β-catenin signaling, and tyrosine kinase receptors like EGFR, fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGF/MET), insulin-like growth factor receptor (IGF) and platelet-derived growth factor (PDGF) as well as phosphoinositide 3-kinase (PI3K)/AKT, and the most studied transforming growth factor β (TGFβ/SMAD pathway (see also Fig. 1) [16]. Lung repair involves migration of cells to the damaged region that, for example, was shown to involve Wnt/FGF10-dependent enhanced expression of SNAI1, vimentin (VIM) and alpha-smooth muscle actin (SMA, presently known as actin, alpha 2 (ACTA2)) in mouse models [17]. Overall, it appears that epithelial cell plasticity is regulated in a tightly controlled spatial and injury-dependent manner in the adult lung with the aim of maintaining airway homeostasis and repair (see also Fig. 2). This view of facultative stem cell-mediated lung repair is increasingly recognized and substantially different from the dedicated stem cell-mediated repair that is seen, for example, in hematopoiesis and small intestine’s regeneration [5].

3. CSCs in lung cancer

3.1. The origin of CSCs in the lung

The concept of attributing tumor-initiating and tumor expansion activity to a malignant stem cell compartment has been proposed almost two centuries ago [18]. This notion gave birth to the CSC theory that postulates the presence of cells possessing stem cell properties in tumors, which are crucial for tumorigenesis and progression. In analogy to normal stem cells, CSCs also undergo symmetric and asymmetric cell division to self-renew and to give rise to more differentiated offspring. Moreover, there is likely not only one specific CSC population, but multiple, each being generated from both overlapping and independent carcinogenic events. This concept is different to what is accepted in the conventional idea of potency in long-lived-normal stem cells. Nonetheless, CSCs should be defined by their functional capabilities, not by their supposed origin e.g., mutated normal stem cells [19]. Thus, heterogeneity represents not only vertical hierarchical relationship between parental cells and progenies, but also horizontal relationship among inter-clonal CSCs.

By considering the stem cell properties of lung CSCs and the likelihood of stem cell activities being triggered in lungs mostly through injury–repair responses, lung CSCs may have initially arisen from proliferative cells of the lung epithelia, i.e., the facultative stem cells. Different histological lung cancer subtypes are suggested to originate from different facultative stem cells. Wadden and colleagues provided an association between injury–repair responses of lung facultative stem cells and tumorigenesis where they demonstrated that TRP63 KRT5+ basal cells preferentially repaired DNA damage by the notoriously unreliable non-homologous end joining (NHEJ) repair mechanism, which may lead to genetic instability in basal cells and onset of oncogenic transformation [20]. Basal cells were suggested to be the origin of CSCs in SCC due to similarities of molecular markers and their spatial origin in proximal lung. In addition, the requirement of SOX2 to restrict division to self-renew and to give rise to more differentiated offspring.

In the distal lung of KRAS mutant mice models, AEC II cells have been exclusively shown to progress to ADC, whereas club cells became hyperplastic in bronchoalveolar duct junctions [24]. KRAS and EGFR’s involvement to AEC II cells self-renewal during injury has been proposed as triggers for continuous proliferation of ADC CSCs [25]. It was proposed that the normal involvement of EGFR and KRAS pathways in AEC II cells stemness is hijacked during oncogenesis leading to malignant stem cell activity. The likelihood of club cells to progress to ADC has not been demonstrated, which may be caused by elevated expression of SOX2 during injury [24]. Indeed, when NOTCH1 intracellular
domain (ICD) was overexpressed, SOX2 levels were reduced and club cells in bronchioles developed into ADC in a KrasG12D background, implicating SOX2 as a suppressor of tumor formation in club cells [26].

For SCLC, pulmonary neuroendocrine cells (PNECs) were shown to be the candidate of transformation upon inactivation of Ttp53, Rb transcriptional corepressor 1 (Rb1) and phosphatase and tensin homolog (Pten) in adult mouse lung [27]. The triple mutations in differentiated PNECs led to hyperplastic lesions, whereas mutations in proliferating PNECs resulted in tumor formation and invasion in mice. Taken together, current findings show that chronic injury is a tumor-initiating event in lungs and that the origin of lung CSCs is highly dependent on the type of injury inflicted and epithelial cell type affected (see also Fig. 2).

3.2. Identification of lung CSCs

Pioneering studies of CSCs in lung cancer have shown certain subpopulations displaying self-renewal, differentiating and tumor-regenerative ability. Those include cells expressing putative CSC cell surface markers, cells excluding Hoechst 33,342 dye (termed side population (SP)) and cells surviving chemotherapies (for recent review see [28]). Upregulation of several proteins, such as aldehyde dehydrogenase (ALDH), ATP binding cassette subfamily G member 2 (ABCG2), CD44, CD117/KIT, CD133/prominin 1 (PROM1), Nanog homeobox (NANOG) and POU class 5 homeobox 1 (POU5F1, formerly known as OCT3/4) have been extensively characterized to be associated with stem cell properties in lung cancer and have been used to identify CSC populations [28,29]. It should be noted that thus far no marker is deemed robust and reproducible enough to detect specific sets of CSCs in lungs. Regardless, the existence of lung CSCs according to both marker expression and functional properties has been clearly demonstrated.

Lung CSCs have some striking similarities with their normal counterparts, particularly reflecting their epithelial origin. The PNECs are similar to CSCs of SCLC that favor Notch inhibition to prevent differentiation upon injury and acquire a proliferative state [30]. SOX2 expression is needed for the emergence of proliferating basal cells upon injury as well as maintenance of CSCs in SCC [21,22]. Requirement of SOX9 to generate functional alveoli suggests that AEC II facultative stem cells in distal airways are akin to the SOX9-expressing CSCs giving rise to ADC [12,31]. Together this suggests that there are close resemblances between tumor and normal stem cells in terms of their pathways and transcription factor utilization.

On the other hand, variable stem cell marker expression may be dependent on the tissue and cellular context in which the CSCs develop. For example, progression of club cells or AEC II cells to ADC CSC might be expected when the right combinations of genetic alterations are given. However, in Pten⁻/⁻;Cdkn2ab⁻/⁻;Sox2 lung cancer mice models, Ferone and colleagues showed that SOX2 not only de-differentiate and acquire a proliferative state [30]. SOX2 expression is needed for the emergence of proliferating basal cells upon injury as well as maintenance of CSCs in SCC [21,22]. Requirement of SOX9 to generate functional alveoli suggests that AEC II facultative stem cells in distal airways are akin to the SOX9-expressing CSCs giving rise to ADC [12,31]. Together this suggests that there are close resemblances between tumor and normal stem cells in terms of their pathways and transcription factor utilization.

Fig. 2. Cellular components of lung tissue and lung cancer, and the microenvironment. Lung cancer, like normal tissue, consists of heterogeneous cellular components leading to complex cellular and molecular interactions. Across the proximodistal locations, three major histologically different tumor types can arise from respective regional facultative stem cells, except for the least known facultative stem potential of neuroendocrine cells. The figure depicts mirror image of normal lung epithelia and tumor epithelia and their respective microenvironments. The capability of those cells to undergo hyperplasia bridges their facultative stem potential with cancer stem cell (CSC) transformation. Cytokines secretions directed from stromal cells may also contribute to the stem cell phenotypes maintenance aside of their internal regulations (see text for more detail).

4. Lung CSC regulation, cellular plasticity and the tumor microenvironment

4.1. Signaling pathways in lung CSCs

Considering the cellular resemblance of lung CSCs with facultative stem cells, it is not surprising that lung CSCs also make use of signaling pathways regulating self-renewal and differentiation processes in normal tissue. In general, signaling pathways involved in CSCs’ perpetuation are Janus family tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT), nuclear factor kappa B (NFκB), Notch, PI3K/AKT serine/threonine kinase, SHH and Wnt/β-catenin pathways.
An overview is provided in Fig. 3. The general consensus on how these pathways work has been reviewed elsewhere [32]. In response to injury, SHH pathway is deactivated to promote proliferation of adjacent mesenchyme which encourages the proliferation of normal lung epithelium, possibly through paracrine signaling that enforces suppression of SHH signaling [7]. The Notch pathway has a role in promoting differentiation of PNECs, maintaining club cell identity, and driving proliferation and maturation of AEC II cells [30,33]. Wnt/β-catenin pathway is associated with epithelia hyperplasia and regeneration during injury, and more specifically, AEC II stem cell identity and proliferative potential [34,35]. The above mentioned embryonic signaling pathways were similarly found to be relevant in lung CSCs maintenance, likely being rooted from their respective cell of origins [36]. Activation of the JAK/STAT, PI3K/AKT and NFκB pathways are linked with extrinsic factors derived from chronic inflammatory conditions. This resembles the situation in chronic obstructive pulmonary disease (COPD) in which inflammation often leads to activation of these pathways in response to inflammatory cytokines [37]. Lung CSC maintenance and COPD are perhaps sustained by the same injury-dependent inflammatory processes.

4.2. Lung CSC plasticity

High cellular plasticity is a characteristic for lung malignancies. This is exemplified by Akunuru and colleagues who characterized different subpopulations of CSCs in human ADC expressing SP, CD133+, ALDHhigh and CD24highCD44high [38]. When comparing SP, CD133+ and ALDHhigh populations with marker negative populations, they noticed differential expression of self-renewal-related genes such as HES family bHLH transcription factor 1 (HES1), NANOG, NOTCH1, SHH and SOX2, and metastasis-related genes like C-X-C motif chemokine receptor 4 (CXCR4), tumor necrosis factor (TNF), homeobox B9 (HOXB9), TGFBI, vascular endothelial growth factor A (VEGFA), interleukin 1 beta (IL1B) and IL6. They also found that EMT reprogramming by TGFBI dynamically changed the proportion of the CSCs whereby SP cells decreased, and CD133+, ALDHhigh and CD24highCD44high populations increased. Concomitantly, the expression of self-renewal-related and metastasis-related genes was increased in primary ADC cells. In addition, stem cell marker negative populations could also give rise to CSC marker-positive population suggesting plasticity of the non-CSCs population. Finally, the observed inter-convertibility of lung CSCs may not be surprising considering high plasticity in lung epithelia as discussed earlier above. Together, this illustrates that cellular plasticity in normal lung has been replicated by lung cancers in which interactions with the tumor microenvironment play an important role.

4.3. Lung CSCs and the tumor microenvironment

Tumor microenvironment (TME) is a crucial immediate extrinsic
factor that influences how CSCs behave. Stromal cells located in the surroundings of tumor are often observed to assist CSCs to thrive by affecting their cellular machinery. As mentioned earlier, this is especially true when inflammation is sustained in the course of injury when stromal cells are recruited to facilitate the healing process. Those cells include cancer associated fibroblasts (CAFs) and infiltrating immune cells, particularly the tumor associated macrophages (TAMs). Such assistance can also be recruited from distant sites such as the mesenchymal stem cells (MSCs) from bone marrow. Much has been revealed on the role of TME, including hypoxia, and its players in lung cancer development [39,40]. Some evidence related to CAFs, TAMs and MSCs will be highlighted below.

Located in the connective tissue filled with extracellular matrix, fibroblasts are the closest stromal cells to epithelial cells. Under normal conditions, fibroblasts function to secrete matrices and soluble secreted factors to provide anchorage support and survival cues for epithelial cells. However in TME, fibroblasts are reprogrammed to support CSCs. In a report, CAFs have been identified to highly express ACTA2 and SNAI1 that are uniquely present if compared to normal fibroblasts [41]. When co-cultured with NSCLC cells, CAFs were able to stimulate a more migratory and invasive phenotype as well as EMT properties signified by elevation of VIM, TWIST1, SNAI1, ZEB1, matrix metallopeptidase 2 (MMP2) and MMP9 expressions through micro RNA (miR)-33b downregulation. MiRs are non-coding oligonucleotides that perform regulatory functions of gene expression and are known as dual players in both stimulating and suppressing tumor growth. An important role for miR-16 was demonstrated in regulating the FGFR1-dependent production of HGF by CAFs [42]. MiR-16-mediated inhibition of HGF production reduced the migration of adjacent lung cancer cells. It was proposed that low miR-16 levels in CAFs from smokers may be linked with tumor progression. In another study the production of IL6 by CAFs was shown to enhance NSCLC invasiveness via JAK2/STAT3 signaling [43]. Paracrine effects by CAFs was also shown to involve IGF2–IGF1R by maintaining stemness of CSCs characterized by high expression of NANOG and POU5F1 [44]. Recently, CAF heterogeneity was demonstrated by variable expression of the cell surface markers CD10 and G protein-coupled receptor 77 (GPR77). Both were found to promote tumor formation and chemoresistance [45]. GPR77-dependent IL6 and IL8 production were found to provide a CSC supportive niche and, moreover, targeting GRP77 with a neutralizing antibody restored chemosensitivity and reduced tumor growth in NSCLC models. During tissue regeneration and inflammation, macrophages with distinct phenotypes actively partake in all stages of inflammatory response and healing processes. Upon injury, resident and bone-marrow-derived macrophages arising from monocytes are recruited to mediate pro-inflammatory functions upon receiving appropriate stimuli like damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), cytokines, growth factors and other molecular mediators [46]. M1 and M2 macrophages alternatively mediate pro- and anti-inflammatory responses to facilitate tissue remodeling and repair. Tissue destructive M1 subset releases pro-inflammatory mediators like TNF, interferon gamma (IFNG) and IL12, whereas M2 subset releases anti-inflammatory mediators like IL4, IL10, TGFB1, HGF, IGFI, PDGF and VEGFA [46–48]. A recent study assessing the effect of different macrophages subsets revealed different consequences to NSCLC progression. The pro-inflammatory M1 subset suppressed tumorigenicity of AS49 ADC cells by increasing cisplatin sensitivity, inducing apoptosis and enhancing senescence [49]. M2a and M2c subsets promoted invasion and increased tumorigenicity. Therefore, M2 macrophages are associated with pro-tumorigenic behavior. Indeed, IL10-producer TAMS were detected at higher percentage in more advanced stages of NSCLC [50]. Moreover, high IL10 expression by TAMs was associated with poorer overall survival (OS). Cytokines released by TAMs like TGFB1, was characterized to contribute in EMT reprogramming through downregulation of miR-138 with consequent enrichment of CD44+/CD90− lung CSCs and a concurrent increase in colony-forming ability [51]. Mechanistically, TAMs may also modulate stemness of NSCLC through elevated expression of ubiquitin specific peptidase 17-like family member 9, pseudogene (USP17L9P, formerly known as USP17) [52]. This can be achieved by stimulation of wide array of cytokines, namely TNF, IL1B, IL4, IL6, IL8, IL10, CXCL12, C-C motif chemokine ligand 18 (CCL18) and CCL2 through the binding of hypoxia inducible factor 1 subunit alpha (HIF1A), STAT3, STAT6 and NFKB1 transcription factors. Elevated of USP17L9P expression consequence led to higher cancer intrinsic inflammation as indicated by the production of TNF, IL1B, IL6, IL8, IL12 and IL23, and increased stemness indicated by higher expression of stem cell markers like ABCG2, ALDH1, CD44, CD117, CD133, kruell like factor 4 (KLK4), MYC, NANOG, POU5F1 and SOX2.

Not much information has been obtained for the involvement of MSCs towards stemness maintenance of lung CSCs. One study showed that they modulated stemness of ADC CSCs through IL6/JAK2/STAT3 pathway [53]. Co-culture of MSC with lung ADC promoted sphere formation and increased expressions of NANOG, POU5F1 and SOX2.

Taken together, the role of CAFs, TAMs and MSCs within TME is to provide stimulatory molecules that directly or indirectly enhance stem cell properties of lung CSCs.

4.4. EMT cellular reprogramming

EMT has also been proposed to encourage stem cell maintenance. For example, Chang and colleagues has provided a clear link between mesenchymal reprogramming and stemness induction through Wnt signaling in NSCLC [54]. They showed that exposure of serially selected low and high motility A549 sphere-derived cell clones to EMT inducers alone is not sufficient to promote stemness. In fact, the cells have to be sufficiently mesenchymal at the beginning (low E-cadherin (ECAD) expression) in order to be susceptible to WNT3A-induced EMT. Increased stemness of high motility mesenchymal cell clones in response to WNT3A stimulation was demonstrated by elevated sphere-forming ability, higher number of SP and elevated expression of a number of mesenchymal, self-renewal, CSC-associated and ABC transporters genes. Chromatin immunoprecipitation (ChIP) experiments indicated that EMT helps switching in transcriptional activity involving catenin beta 1 (CTNNB1)/ECAD/SOX15 to CTNNB1/TWIST1/Transcription Factor 4 (TCF4) complex. The authors identified five gene signatures consisting of nuclear CTNNB1high/nuclear TWIST1high/ECADlow/SOX15low/CD133high to be associated with disease progression and metastasis of lung cancer.

A relationship between EMT and expression of CSC marker CD44 has been reported by Su and co-workers [55]. The authors demonstrated that CD133+ cancer cells co-expressing CD44 displayed higher migration and invasion capability when compared to CD44CD133- lung cancer cells. Only the double positive cell exhibited increased sphere formation and in vivo tumor regeneration. WNT3A was able to increase CTNNB1 and forkhead box M1 (FOXM1) expression in CD133+CD44+ population mimicking the phenotype of CD133+CD44+ population. This indicates that Wnt/β-catenin pathway is active in CD133+ CD44+ CSCs. Knock-down and overexpression of FOXM1 gene revealed that increased metastatic phenotype is mediated through TWIST in a FOXM1-dependent mechanism.

A different and striking way by which lung cancer cells can obtain mesenchymal properties has been reported to involve cell fusions between epithelial cancer cells and bone marrow-derived mesenchymal cells. Apparently, spontaneously formed hybrids between lung cancer cells and stromal MSCs was found to result in an EMT programmed cell death characterized by reduced ECAD and pan-cytokeratins expression, increased VIM, ACTA2 and fibronectin 1 (FN1) expression together with increased motility and invasiveness [56]. Moreover, the hybrid became more stem-like indicated by elevated expression of stem cell markers like ALDH1, BMI1, NANOG, NOTCH1, POU5F1 and SOX2, and increased sphere-forming ability. The hybrid also displayed enhanced in
vivo tumorigenic potential. The authors further speculated that the hybrid formation is stimulated during inflammation, thus providing a new link between inflammation and tumorigenesis. Furthermore, it appeared that the hybrids were rather instable and tend to revert back to epithelial phenotype, which would facilitate secondary colonization upon metastasis. Hybrid stability was found to be regulated by grainyhead like transcription factor 2 (GRHL2), miR-145 and Ovo like transcriptional repressor 1 (OVOL1) and regulation of the miR-200/ZEB1 balance that controls EMT [57].

5. Cell intrinsic regulation of lung CSCs

Aside from modulation of stem cell properties by external stimuli as described above, cell intrinsic genetic alterations are able to directly affect molecular mechanisms controlling stemness and boost tumor aggressiveness. Several examples of such molecular alterations covering genetic, epigenetic and other regulatory components in lung cancer are described below.

5.1. NSCLC

The accumulation of DNA damage is one of the main triggers of tumorigenesis leading to various genetic aberrations such as single nucleotide polymorphism (SNP), deletions, chromosomal translocations or breaks, aneuploidization and polyploidization. Commonly observed genetic aberrations in NSCLC include BRAF, EGFR, erb-b2 receptor tyrosine kinase 2 (ERBB2), KRAS, MET, ROS1 and ALK-echinoderm microtubule associated protein-like 4 (EML4) gene fusion [58]. For some of these genetic alterations, involvement in stem cell regulation has been suggested. As discussed earlier under 3.1., KRAS and EGFR have been implicated in self-renewal during lung injury and it has been proposed that their continuous activation may lead to ADC CSCs. In addition, constitutively activated EGFR mutant cells were shown to increase the expression of spalt like transcription factor 4 (SALL4), a zinc finger transcription factor that is known to interact with KLF4, POU5F1 and SOX2. This could lead to the maintenance of stem cell properties like spheroid formation and pluripotency genes expressions in lung ADC [59].

Recently, a novel fusion gene was identified in invasive mucinous subtype of lung ADC [60]. Being mutually exclusive with currently known mutations like KRAS, EGFR, BRAF and ERBB2, the CD74-NGR1 fusion was further characterized. CD74-NGR1 contains transmembrane domain of CD74 and EGF-like domain of neurogenin 1 (NGR1), which is a ligand for ERBB2/ERBB3. ERBB3 stimulation resulted in Nfib activation and enhanced secretion of IFG2 and spheroid growth in lung cancer cells [61]. Remarkably, lung cancer possessing this gene fusion is self-sustaining in vitro, independent of growth factors commonly supplied to enrich CSCs. This finding demonstrates yet another example that lung CSCs can develop a self-reliance mechanism to maintain stem cell phenotype through genetic alteration.

TM4SF4 expression, a protein within the same family as the marker of alveolar epithelial progenitor cells known to be involved in normal lung homeostasis as mentioned earlier (see section 2), was found elevated in ALDH1+ CSCs and in γ-irradiation resistant ADC cells and was associated with both enhanced stem cell properties and EMT phenotypes [62]. TM4SF4 stimulates the release of osteopontin, an extra-cellular matrix protein involved in the regulation of many processes in various tissues including bone formation, tissue repair and immune reaction. In A549 ADC cells, TM4SF4 via IGF1R-dependent glycosyn synthase kinase 3 beta (GSK3B)/CTNNB1 activation led to osteopontin production that in an autocrine loop, was able to stimulate migration, invasion, sphere formation and expression of EMT- and CSC-associated genes, including neuronal cadherin (NCAD or presently known as cadherin 2 (CDH2)), VIM, POU5F1 and SOX2. More in depth analyses revealed that the EMT and CSC phenotypes were regulated by osteopontin-dependent activation of CD44 and integrins, and subsequent activation of JAK2/STAT3 and focal adhesion kinase (FAK)/STAT3 pathways. In addition, osteopontin expression was shown to be increased in tumor specimens compared to normal lung tissue and correlated with poorer survival. This study highlights the intricate measures taken by CSCs to sustain their growth using feedback loop mechanism to stimulate common pathways like STAT3 for stemness maintenance.

MiRs also have been reported to regulate lung CSCs. Recent years of studies in lung cancer have revealed that miRs impact on multiple CSC components by post-transcriptionally regulating genes including stem cell genes, EMT machinery and epigenetic mechanisms [29,63,64]. For instance, overexpression of miR-708-5p was found to lead to DNA hypomethylation by promoting the downregulation of DNA methyltransferase 3 alpha (DNMT3A) in NSCLC cell lines [65]. This resulted in re-expression of ECAD, which consequently reduced the stem cell properties and tumor-generating capacity of CSCs in NSCLC through reduction of Wnt/β-catenin pathway activation. MiR-708-5p expression correlated with higher OS and relapse-free survival of NSCLC patients. Even though not discussed by the authors, it can be deduced that abolishing the mesenchymal phenotype due to restoring ECAD expression may lead to reduction of CSC properties. Moreover, this finding illustrates that the CSC phenotype is controlled at different levels involving a miR, epigenetic alterations via DNMT3A and EMT re-programming. Another miRNA, miR-150, was shown to promote cell invasion and metastasis in NSCLC [66]. This was achieved by inhibiting production of forkhead box O4 (FOXO4), a negative regulator of cell proliferation, resulting in increased anchorage-independent growth, migration and invasion that involved triggering of EMT through NFkB/SNAI1/YY1/Raf kinase inhibitor protein (RKIP) circuitry. Clinical data showed that miR-150 expression was associated with metastatic NSCLC. Although a more direct relation between miR-150 and CSC properties was not explored, its oncogenic and EMT activating properties are suggestive for such a role.

A novel class of non-coding RNAs called P-element induced wimpy tests (Piwi)-interacting RNAs (piRs) has recently gained attention for its role in tumorigenesis. PiRs are able to silence mobile elements like transposons, particularly in germ line cells, and the association between piRs and NSCLC has been examined by Peng and colleagues where they assessed tumor-suppressive function of piR-55490 [67]. PiR-55490 was shown to be downregulated in human lung carcinoma specimens that involved inhibition of mechanistic target of rapamycin (mTOR) at transcriptional level, rendering it incapable of supporting proliferation of lung cancer. In the same year, Li and co-workers investigated oncogenic piR-651’s effect on NSCLC [68]. First, they confirmed the clinical relevance of piR-651 by showing elevated expression in NSCLC tumor samples when compared to adjacent normal tissues. Although the CSC compartment was not directly studied, the authors identified significant correlation of cyclin D1 (CCND1) and cyclin dependent kinase 4 (CDK4) expressions with piR-651, indicative of either higher proliferative or self-renewal potential of cells in NSCLC upon piR-651 upregulation. Since the exploration of the role of piRs in CSCs maintenance is still in its infancy, further study is warranted.

5.2. SCLC

In SCLC, inactivating mutations in tumor protein 53 (TP53) and RB1 are major contributors to tumorigenesis, whereas tumor maintenance and progression are attributed to mutations in NOTCH and PTEF, amplification of proto-oncogene MYC family members, activation of FGFR pathway and hypermethylation activity such as by enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) [3,69]. Of note, the SCLC progression genes have all been implicated in regulating (cancer) stem cells. Different with NSCLC, SCLC does not seem to favor NFkB activation to develop, but instead blocking NFkB with dominant negative form of inhibitor of kappa B alpha (IκBα) accelerated tumor progression of SCLC in an inducible lentiviral SCLC mouse model [70].
SCLC appears to depend more on protein kinase A (PKA)-dependent activation of cyclic AMP response element-binding protein (CREB) pathway for mediating tumor growth and maintenance of a neuroendocrine phenotype.

As mentioned, amplification of MYC family members is commonly associated with SCLC tumors. Chen and co-workers recently identified a new target gene of MYCN and achaete-scute homolog 1 (ASCL1) transcriptional activity of a transcriptional repressor called INSM1 critical for neuroendocrine differentiation [71]. SHH signaling was found to act upstream of MYCN activation and SHH inhibitors reduced MYCN and INSM1 activation leading to inhibition of lung cancer growth. The two studies described above suggested that CSCs of SCLC heavily rely on signaling pathways that favor neuroendocrine phenotype maintenance. In addition, recently ASCL1 was identified as the primary activator of MYCL1, nuclear factor I B (NFIB), RET and SOX2, and for tumor formation in mouse models [72]. Different from the majority of SCLCs with high neuroendocrine features, recently a subtype with a low neuroendocrine phenotype was identified, characterized by EMT reprogramming, activated Hippo, Notch and TGFB pathway, MYC amplification and overexpression under influence of RE1 silencing transcription factor (REST), mechanisms known to contribute to stemness [73].

Taken together, CSCs in both NSCLC and SCLC are regulated through cell autonomous molecular mechanisms also including intrinsic genetic aberrations that have been linked with tumorigenesis and tumor progression in these malignancies.

5.3. Therapy resistance, relapse and lung CSCs

CSCs are deemed to be resistant for standard therapies according to the concept. Therapeutic resistance has been associated with expression of several CSC phenotypes which indirectly links CSCs involvements in resistance to therapies as also reviewed recently [74]. For example, Wang and colleagues showed the involvement of CSCs in therapeutic resistance frequently seen in the clinic in lung cancer cell lines [75]. In A549 and NCI-H460 cell lines, pre-treatment with cisplatin resulted in increased colony formation and sphere formation in the surviving fraction, while paclitaxel or doxorubicin pre-treatments did not. The increase in stemness correlated with upregulation of several pluripotency-related genes like NANOG, POU5F1 and SOX2, and several ALDH isoforms that are linked with multidrug resistance. aberrant activation of the CCAAT enhancer binding protein B (CEBPB)/Tribbles pseudokinase 1 (TRIB1)/histone deacetylase (HDAC)/TP53 axis was identified to induce cisplatin resistance. Knock-down of TRIB1 and chemical inhibition of HDAC restored sensitivity to cisplatin. In addition, TRIB1 was found to be clinically relevant as its expression is NSCLC-specific and elevated in cisplatin-treated NSCLC patients and correlated with poor treatment outcome and OS. With this finding, one wonders whether cisplatin usage as first line treatment is the best treatment option. This finding also emphasizes the importance of epigenetic mechanism such as histones acetylation in tumorigenic gatekeeping and specifically the targeting of lung CSCs.

The possible role of CSC in NSCLC in contributing to tyrosine kinase inhibitors (TKIs) resistance has been explored by various investigators. EGFR mutant NSCLC cells selected for gefitinib resistance demonstrated high expression levels of stem cell markers such as ALDH1A1, CD133, CXCR4, POU5F1 and SOX2 and, moreover, were highly tumorigenic in vivo suggesting that lung CSCs are refractory for this treatment [76]. Others pre-treated CSCs with different TKIs consisting of gefitinib, regorafenib and sorafenib and found an increase of stem-like phenotypes, which included colony formation, sphere formation, ALDH activity and the expression of POU5F1 and SOX2 [77]. The pre-treatments also evoked higher expression of mesenchymal markers like NCAD, VIM and MMP2 concomitantly with higher migratory potential. Further experiments employing transcriptional profiling identified the activation of AKT/FOXM1/stathmin 1 (STMN1) pathway in mediating resistance. Pharmacological inhibition of AKT restored TKI sensitivity. Furthermore, both STMN1 and FOXM1 appeared to be associated with unfavorable prognosis and survival outcome of NSCLC patients.

On the other hand, EMT that forms close association with stemness may also influence resistance phenotype. EMT induction was shown to mediate resistance towards EGFR inhibition in EGFR-mutant NSCLC through ZEB1 expression [78]. ZEB1 was found to directly cause transcriptional repression of BIM (presently known as BCL2 like 11), rendering insensitivity to EGFR inhibitor-induced apoptosis due to diminished levels of this pro-apoptotic protein. In other situation, embryonic signaling pathways that support CSC development like Notch may also confer resistance. High NOTCH1 expression in gefitinib-resistant lung cancer cell line was found to promote EMT phenotypes, whereas silencing it with small interfering RNA (siRNA) reversed the phenotypes in addition to restoring sensitivity to gefitinib [79]. Silenced NOTCH1 also reduced colony formation of the gefitinib-resistant lung cancer cell line, indicating reduced CSC potential due Notch abolishing.

The increase in CSC properties of therapy-resistant lung cancer cells will obviously favor relapse and progression of disease. However, direct evidence supporting therapeutic resistance and relapse due to increased tumor stemness, for example by comparing biopsies before and after relapse, has not been formally presented. Interestingly, a recent study in mice has linked the reactivation of latent/dormant tumors to altered activity of CD133 positive lung CSCs [80]. Li and co-workers showed that latent NSCLC xenografts in nude mice could be reactivated by co-implanting fresh NSCLC material, which was associated with a shift in asymmetric - to symmetric cell division in the CD133 positive lung CSC compartment. Further investigation revealed that this transition was initiated by IGF1 signaling involving the PI3K/AKT/β-catenin axis. The expansion of the lung CSC fraction subsequently stimulated angiogenesis further fueling tumor relapse. Although this study supports a proposed major role of lung CSCs in tumor relapse, more definitive proof in longitudinal biopsies from patients is currently missing. Use of liquid biopsies represented by circulating tumor cells (CTCs) will greatly facilitate the analyses of identity and levels of CSC markers during treatment and progression.

Overall these studies provide substantial evidence for the involvement of lung CSCs and EMT in drug resistance in NSCLC.

6. Pharmacological targeting of lung CSCs

Current knowledge of the molecular mechanisms that sustain lung CSCs, either being TME-derived or cell autonomous, has enabled studies to explore their value as therapeutic targets. Several examples are described below and summarized in Table 2.

6.1. Targeting the TME

As pointed out earlier the TME has an important contribution in CSC maintenance in lung cancer. Several studies have explored the possible therapeutic benefit of targeting specific components of the TME. A multikinase receptor inhibitor named nintedanib was found to have selective inhibitory effect on CAFs assisting ADC, but not CAFs assisting SSC [81]. Nintedanib was able to prevent TGFB-dependent activation of fibroblasts into CAFs and consequently suppressed tumor growth and invasion in vitro. This study revealed the importance of preventing activation of CAFs, hence eliminating the stromal support to cancer cells. Despite its promise in preclinical studies, nintedanib did not perform as well as expected in patients. Several clinical trials evaluating its efficacy in combination with chemotherapeutic drugs reported lack of efficacy or development of adverse events (NCT00806819, NCT00979576, NCT02231164 and NCT02300298). Nonetheless, new clinical trials have been initiated for evaluating combinatorial response with che- motherapies in SCLC patients even though limited activity was seen in recent phase II study in patients with relapsed SCLC (NCT01441297)
Examples of pharmacological targeting of lung cancer stem cells and their tumor microenvironment.

<table>
<thead>
<tr>
<th>Targeting drug</th>
<th>Target(s)</th>
<th>Clinical study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apatinib</td>
<td>VEGFR2 and HGF/MET</td>
<td>NCT02515435</td>
<td>[85,86]</td>
</tr>
<tr>
<td>CBP501</td>
<td>calmodulin</td>
<td>NCT00942825</td>
<td>[84]</td>
</tr>
<tr>
<td>Nintedanib</td>
<td>VEGFR, FGFR and PDGFR</td>
<td>NCT00860819, NCT00979576, NCT02231164, NCT02300298, NCT01441297</td>
<td>[81,82]</td>
</tr>
<tr>
<td>NSC12</td>
<td>FGF2</td>
<td></td>
<td>[83]</td>
</tr>
<tr>
<td>Targeting lung CSC directly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABV-399</td>
<td>MET</td>
<td>NCT02099058, NCT03574753, NCT03539536</td>
<td>[97,98]</td>
</tr>
<tr>
<td>Anlotinib</td>
<td>VEGFR1/2/3, FGFR1/2/3, PDGFR/A, BRAF, MET and CD117</td>
<td>NCT01924195, NCT02886919</td>
<td>[99]</td>
</tr>
<tr>
<td>AZD9150</td>
<td>STAT3</td>
<td>NCT02983578, NCT03421535</td>
<td></td>
</tr>
<tr>
<td>CUDC-101</td>
<td>HDAC, FGFR and HER2</td>
<td>NCT01171924, NCT01702285</td>
<td>[92]</td>
</tr>
<tr>
<td>Defactinib</td>
<td>FAK</td>
<td>NCT01943292, NCT01951690</td>
<td>[94]</td>
</tr>
<tr>
<td>DSF/Cu complex</td>
<td>ALDH1A1</td>
<td></td>
<td>[100]</td>
</tr>
<tr>
<td>MK-2206</td>
<td>Akt</td>
<td>NCT01294306</td>
<td>[96]</td>
</tr>
<tr>
<td>Napabucasin</td>
<td>STAT3</td>
<td>NCT01252441, NCT02826161</td>
<td>[95]</td>
</tr>
<tr>
<td>Omosomy</td>
<td>MYC</td>
<td>–</td>
<td>[90]</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>VEGFR1/2/3, PDGFR and CD117</td>
<td>NCT01262820, NCT01027598, NCT00871403</td>
<td>–</td>
</tr>
<tr>
<td>RO4929097</td>
<td>Gamma-secretase/Notch</td>
<td>NCT01193881, NCT01193868, NCT01217411</td>
<td>–</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>MYC</td>
<td>–</td>
<td>[89]</td>
</tr>
<tr>
<td>TMU-3535</td>
<td>HDAC</td>
<td>–</td>
<td>[93]</td>
</tr>
<tr>
<td>TTI-101</td>
<td>STAT3</td>
<td>NCT03195699</td>
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FGF ligands produced by both stromal and tumor cells contribute to tumor aggressiveness and stemness, providing interesting targets for therapy. In addition to FGFR receptor inhibitors, recently a FGF-ligand trap was developed based on the antagonistic effect of pentraxin 3 (PTX3) on FGF signaling leading to anti-tumor effects in NSCLC [83]. Using pharmacophore modeling analyses of the PTX3-FGF binding site allowed the development of an acetylated pentapeptide named NSC12 that sequesters FGFs and effectively inhibited angiogenesis and tumor growth in both murine and human lung cancer cell lines. Although not explored, eliminating FGF from external environment would potentially limit CSC growth in both NSCLC and SCLC.

Previously shown to increase the uptake of cisplatin to cancer cells, the calmodulin inhibitor CBP501 was also found to have anti-tumor effects by targeting the TME involving suppression of lung CSC activity [84]. CBP501 could suppress the lipopolysaccharide (LPS)-induced production of IL6, IL10 and TNF by macrophages and subsequently resulted in reduction of stem cell properties in Lewis lung carcinoma mouse model including decreased expression of the drug resistance molecule ABCG2, decreased spheroid formation and tumor initiation capacity, and reduced metastatic potential. Evaluation in clinical trial (NCT00942825), however, did not see additional benefit when CBP501 was employed along with cisplatin and pemetrexed as compared to cisplatin and pemetrexed regimen only. Progression free survival (PFS) was observed at 140 days vs. 165 days.

The VEGFR2 inhibitor apanitinib has recently been explored for its effect towards EMT [85]. It was reported that apanitin reduced macrophage-induced EMT reprogramming in NSCLC cell lines. Pre-treatment of apanitinib to polarized macrophages led to inability of macrophage's secretion to induce migration and invasion. In-depth investigation led to the finding that apanitin blocked HGF and MET expression that are probably utilized by NSCLCs for EMT reprogramming. The efficacy of this therapeutic modality proves that diminishing the secretory support within TME is important to attenuate EMT induction that may also affect stemness maintenance of lung CSCs, although not examined. Several clinical trials evaluating the efficacy of apanitinib have been initiated for NSCLC and SCLC. An active phase II trial (NCT02515435) in pre-treated advanced non-squamous NSCLC patients reported a clinical endpoint with median OS and PFS of 7.69 and 3.06 months, respectively [86].

Together these studies suggest that targeting of the fibroblast and macrophage components of the TME may have therapeutic value although likely more relevant targets in the TME need to be identified.

Whether these strategies also target lung CSCs remains to be demonstrated.

6.2. Targeting molecular features of lung CSCs

Targeting intricate mechanisms that maintain the undifferentiated stem cells state of lung CSCs is obviously an attractive therapeutic approach. Below several examples are discussed particularly those that have undergone clinical testing.

Based on the involvement of the Notch pathway in CSC maintenance, two clinical trials were initiated to explore its possible therapeutic value in NSCLC. RO4929097, a gamma-secretase inhibitor, was used in phase I dose-escalation study (NCT01193881) with erlotinib combination in stage IV or recurrent NSCLC patients and in a phase II efficacy study (NCT01193868) in chemotheraphy-treated advanced NSCLC patients. Both studies have since been terminated due to halt in production of RO4929097. The phase II trial, however, revealed poor drug efficacy indicated by observation of progressive disease in all five patients. The same drug was also evaluated in clinical trial (NCT01217411) in combination with radiotherapy on patients with extensive SCLC with brain metastasis. Low accrual and discontinued drug production led to premature termination of the study.

Inhibition of embryonic signaling pathways including Notch may sound attractive, but there is a risk of normal homeostasis disruption since those pathways are still operating in normal adult lung. Besides, it is important to realize that when such pathways are acting as morphogenetic gradients, inhibiting one might risk in promoting the other subtype of lung cancers along the proximodistal axis, e.g., NFKB and Notch pathways contradicting activation in SCLC and NSCLC. Indeed, progression of SCLC to SCLC has been observed during therapeutic resistance in the clinic, in which lung ADC in some patients transformed into SCLC displaying high grade neuroendocrine phenotypes after TKI treatment [87]. The opposite transformation was seen when chemotherapy was employed to treat SCLC patients; Notch pathway in this case was proposed to be the double-edge sword that facilitated the resistance [88].

MYC represents another attractive target for therapy although direct therapeutic targeting of transcription factors remains challenging. The phytochemical compound, sulforaphane, was found to induce apoptosis and suppress CSC properties in NSCLC [89]. Sulforaphane accomplishes this inhibition through the modulation of miR-214 and subsequent transcriptional repression of MYC. Interestingly, in addition to MYC, also CTNNB1, CCNE1, EZH2 and survivin are targets of miR-214
and reduced by sulforaphane treatment. Furthermore, chemotherapeutics like cisplatin and doxorubicin could induce MYC expression leading to higher sphere-forming ability and proportion of CD133+ and ALDH1+ cells, which could be reversed by sulforaphane and miR-214 mimic treatments. In addition, sulforaphane was able to sensitize NSCLC to chemotherapy both in vitro and in vivo. Its multi-targeting properties make sulforaphane a promising compound for further evaluation in clinical studies. A more targeted approach has been developed by overexpressing a dominant negative MYC, named Omomyc, and recently its therapeutic impact was tested in SCLC. Omomyc was determined to inhibit cell growth and induce cell death in SCLC cell lines with or without amplification of MYC, MYCL and MYCN, all with genetically silenced TP53 and RB1 [90]. Although effective in preclinical studies, application in the clinic is more difficult with respect to drug delivery issues. Regardless, targeting MYC family members that play an important role in the maintenance of lung CSCs provides a promising therapeutic option that remains to be further explored.

Epigenetic alterations have been connected with stemness in lung cancer. Preclinical studies with HDAC inhibitors, chemotherapy and targeted therapies have demonstrated efficacy in therapy-resistant NSCLCs [75,91]. Several phase I/II clinical trials have employed HDAC inhibitors as single or combined treatments both in SCLC and NSCLC. CUDC-101, a multi-targeting inhibitor for HDAC, EGF, and HER2 was evaluated in preclinical and clinical settings [92]. In vitro and in vivo studies showed anti-tumor activity in multiple tumor types, notably in erlotinib-resistant lung cancer models. A phase I clinical trial (NCT01171924) indicated the drug is well-tolerated when administered intravenously. Oral administration of the compound was also studied in phase I clinical trial (NCT01702285), however the study was recently terminated with unknown reason. A novel HDAC inhibitor named TMU-35435 has also been developed aiming to interfere with Wnt/β-catenin pathway [93]. TMU-35435 induces histone acetylations thus altering gene expression including negative regulators of Wnt/β-catenin pathway that were reactivated such as CDH1, secreted frizzled related protein 4 (SFRP4), beta-transducin repeat containing E3 ubiquitin protein ligase (BTGRC), AXIN2, retinoic acid receptor beta (RARB) and catenin beta interacting protein 1 (CTNNBIP1). Combined treatment with the demethylating agent 5-aza-dC synergized the anti-cancer effects both in vitro and in vivo. Usage of epigenetic modifying drugs leading to alterations in gene expression involving those that control lung CSCs may have use in clinical settings.

To counter the effect of FAK in a self-sufficient CSC feedback loop, FAK inhibitor, VS-6063 (defactinib) was examined for efficacy. A phase I trial (NCT01943292) in non-hematologic malignancies and phase II trial (NCT01951690) in KRAS mutant NSCLC have been conducted to assess safety, tolerability, OS and PFS. The phase I trial concluded well-tolerable treatment with reversible adverse events like fatigue and hyperbilirubinemia [94]. Results of the phase II trial have not yet been reported.

STAT3 is recognized as an attractive molecular target due to its frequent involvement in CSC maintenance. Napabucasin (BBI608), advocated as the first-in-class CSC inhibitor that blocks the transcriptional activity of STAT3 has been tested in combination with paclitaxel in previously treated patients with metastatic squamous or non-squamous NSCLC in a still-ongoing phase Ib/II clinical trial (NCT01325441) [95]. The study concluded with well-tolerable and manageable adverse events with encouraging signs of anti-cancer activity. Due to more favorable observation in patients with non-squamous NSCLC, it was quickly brought to phase III clinical trial (NCT02826161) to compare its combination with paclitaxel and paclitaxel alone in this patient group. However, the study was prematurely terminated due to change of treatment landscape and evolving standard of care. It is not clear if the study will be continued, but meanwhile, three clinical trials aiming at evaluating different STAT3 inhibitors (AZD9150 and TTI-101) are still recruiting patients (NCT02983578, NCT03195699 and NCT03421353).

Targeting of the PI3K/AKT pathway has also been explored. The pan-AKT inhibitor, MK-2206, has been evaluated in both preclinical and clinical studies. In an in vitro study involving various erlotinib-sensitive and -resistant NSCLC cell lines, MK-2206 sensitized erlotinib-resistant NSCLC cell line to erlotinib treatment and inhibited the growth by inhibiting MET/HGF-dependent resistance mechanism, rendering them incapable of sphere-forming [96]. The same concept was tested in the phase II clinical trial (NCT01294306) evaluating efficacy and tolerability in advanced NSCLC patients. No additional benefit was observed as patients with EGFR-mutated tumors had 10.6 months median OS compared to patients with EGFR wild-type tumors who had 11.1 months median OS.

In an effort to block aberrant signaling by MET receptor due to MET amplification and MET overexpression, ABBV-399, a MET antibody-drug conjugate was developed making use of the cytotoxic drug monomethylauristatin E (MMAE) [97]. High efficacy of this therapeutic relies on certain threshold overexpression of MET as examined in NSCLC cell lines and patient-derived xenografts (PDx). A recent ongoing phase I clinical trial (NCT02099058) revealed that monotherapy of the antibody–drug conjugate or combination with erlotinib was well-tolerated and exhibited anti-tumor activity with three MET+ treated patients of 16 displayed partial response [98]. Phase II clinical trial (NCT03574753) evaluating overall response in patients with MET+ stage IV or recurrent SCC is still ongoing and future phase II clinical trial (NCT03539536) to determine best-suited population for the therapy will be performed.

Pharmacological drugs targeting CD117/KIT aiming at inhibiting the receptor tyrosine kinase activity have been explored. Three multi-kinase inhibitors that also exert inhibitory effect towards CD117 have entered clinical evaluations, namely anlotinib, famitinib and pazopanib. Anlotinib (AL3818) targets VEGFR1/2/3, FGFR1/2/3, PDGFR/A/B, MET and CD117 and has been tested in phase II and III clinical trials (NCT01924195 and NCT02389919) addressing efficacy in patients with advance and previously treated NSCLC. Studies have been completed and reported superior performance of anlotinib over placebo group with median OS and PFS of 9.63 vs. 6.30 and 5.37 vs. 1.40 months, respectively [99]. The encouraging results have resulted in new clinical studies also exploring the possibility of its incorporation into first and second line therapies in both NSCLC and SCLC. Pazopanib, with similar targets as anlotinib, also has been tested in NSCLC and most encouraging results were obtained in clinical evaluation for stage IIB/IV NSCLC patients with progressive disease upon first line treatment of bevacizumab (NCT01268280). In the single intervention study, median PFS and OS were obtained at 10.9 and 24.1 weeks, respectively. Pazopanib has also been tested in combination with erlotinib (NCT01027598) and pemetrexed (NCT00871403) in both cases showing benefits for PFS but not OS.

The ALDH enzyme, often linked with CSCs, has also been evaluated for therapeutic value in preclinical studies. Liu and colleagues used disulfiram (DSF)/copper (Cu) complex for targeting ALDH1A1 positive lung CSCs [100]. The complex was able to reduce the proportion of ALDH1+ population in NSCLC cell lines and upon implantation as xenografts in nude mice. This was associated with treatment induced reduction of expression of NANOG, POU5F1 and SOX2, colony formation and invasion in vitro as well as decreased tumor size in vivo.

Lung CSCs are proposed to be less immunogenic and recent successes obtained with immune checkpoint inhibitors suggest that despite of this feature, lung CSC are likely eliminated or kept in check. For example, a 1-year survival rate of as high as 51% in patients treated with nivolumab was observed who were previously treated with doublet platinum-based chemotherapy [101]. Although not previously evaluated in lung cancer, some targeting methods may be potentially interesting. For example, bifunctional immune checkpoint-targeted antibody–ligand traps have been produced that aside from targeting immune checkpoints, also can sequester cytokines suppressing immune responses. Ravi and colleagues generated an antibody against cytotoxic T-lymphocyte associated protein 4 (CTLA4), programmed cell death 1
Conflict of interest statement

The authors declare that they have no conflict of interest.

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