Chapter 19
Lung Cancer Stem Cells, p53 Mutations and MDM2

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Abstract Over the past few decades, advances in cancer research have enabled us to understand the different mechanisms that contribute to the aberrant proliferation of normal cells into abnormal cells that result in tumors. In the pursuit to find cures, researchers have primarily focused on various molecular level changes that are unique to cancerous cells. In humans, about 50 % or more cancers have a mutated tumor suppressor p53 gene thereby resulting in accumulation of p53 protein and losing its function to activate the target genes that regulate cell cycle and apoptosis. Extensive research conducted in murine cancer models with activated p53, loss of p53, or p53 missense mutations have facilitated researchers to understand the role of this key protein. Despite the identification of numerous triggers that causes lung cancer specific cure still remain elusive. One of the primary reasons attributed to this is due to the fact that the tumor tissue is heterogeneous and contains numerous sub-populations of cells. Studies have shown that a specific sub-population of cells termed as cancer stem cells (CSCs) drive the recurrence of cancer in response to standard chemotherapy. These CSCs are mutated cells with core properties similar to those of adult stem cells. They reside in a microenvironment within the tumor tissue that supports their growth and make them less susceptible to drug treatment. These cells possess properties of symmetric self-renewal and migration thus driving tumor formation and metastasis. Therefore, research specifically targeting these

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cells has gained prominence towards developing new therapeutic agents against cancer. This chapter focuses on lung cancer stem cells, p53 mutations noted in these cells, and importance of MDM2 interactions. Further, research approaches for better understanding of molecular mechanisms that drive CSC function and developing appropriate therapies are discussed.

Keywords Cancer stem cells • Lung cancer • p53 mutations • MDM2

Lung Cancer

One of the leading causes of cancer-related deaths is cancer of the lung, of which 80–90 % being attributed to tobacco smoking [1, 2]. More than 50 different histological variants of lung cancer have been recognized by the World Health Organization (WHO) and classified based on their phenotype, their zone of origin in the lung, or by tumors arising from functionally diverse lung cells [3]. This diversity characterizes the neoplasms in lung as heterogeneous with different histological subtypes. Almost 98 % of lung cancers are carcinomas (tumors arising from epithelial cells) and based on the size of a cancer cell, categorized as small cell lung cancer (SCLSC) and non-small cell lung cancer (NSCLC) carcinomas. SCLSCs and NSCLCs constitute approximately 10–15 % and 85–90 % of lung cancers respectively [4, 5]. The SCLSCs are malignant small epithelial cells with scanty cytoplasm, while the NSCLCs are further classified based on their size and shape as large cell carcinoma, squamous carcinoma, and adenocarcinoma. NSCLCs are relatively larger in size and contain a high nucleus to cytoplasmic ratio [3, 4, 6]. Additionally, other rare subtypes of lung cancer include bronchioloalveolar carcinoma, carcinoid, glandular, and neuroendocrine tumors.

According to NCI PDQ®, of all the subtypes in lung cancer, the incidence of squamous and adenocarcinoma are considered to be the highest [7]. Studies comparing the major four subtypes of lung cancer recognized that the rate of development of adenocarcinoma is more common and constitute approximately 40 % of these lung cancer subtypes in humans, with the cause strongly associated with tobacco smoking [3, 7, 8]. Various other factors such as asbestos, arsenic, radon (radioactive gas formed as a result of breakdown of uranium in soil), and environmental air pollution also pose a risk of lung cancer [9]. Epidemiological studies and molecular biology studies have indicated a high risk of at least 20 carcinogens in tobacco smoke that can cause lung cancer [10]. Research has shown that tobacco carcinogens such as polycyclic aromatic hydrocarbons (benzo[a]pyren) target hot spots in codon regions of TP53 by forming DNA adduct, thus forming sites for mutations in cancer (reviewed and summarized in [11]). The systematic analysis based on different lung cancer research data uploaded at International Agency for Research on Cancer (IARC) indicates a moderate relationship between smoking exposure and mutation pattern in codon regions (157,158, 175,245, 248, 249 and

273) of TP53, suggesting that mutational pattern in cancers arising in smokers is not specific to a single codon. Studies also confirmed a high frequency in G: C to T: A transversions in TP53 coding region smokers (16 %) than in non-smokers (5.8 %). One key observation in their analysis is change of mutational spectrum based on gender with G:C to T:A transversions found to be higher in female smokers (36 %) than male smokers (27 %) [11, 12]. The advances at molecular level in understanding the cause of cancer and research studies targeting identification of cancer stem cells hold a promise for development of novel approaches in diagnosis and treatment of lung cancer.

Stem Cells in the Lung

The lung is considered to be a highly heterogeneous organ with a variety of cells located in distinct regions of the tissue. Functionally distinct putative stem cells were shown to reside in different anatomical regions of the respiratory system, which play a key role in repopulating the cells in their local area [13–17]. Studies have reviewed the role played by the local stem cells found in trachea (basal, mucous secretory), bronchus (basal, mucous secretory), bronchiole (Clara), and alveolus (type II pneumocyte) and have shown that they primarily contribute to regeneration of lost cells/tissues in response to injury [18]. Identification of resident multipotent lung stem cells that can regenerate any lung cell is still an area of active research. Two major types of stem cells, namely epithelial and mesenchymal stem cells have been reported in the lung so far [13, 16, 19]. These cells were isolated and characterized based on specific cell surface markers that are unique to certain cell lineages. In line with the heterogeneous nature of the lung, the cells that reside in different regions of the lung exhibit differential expression of various cell surface markers. A classic stem cell marker used in the identification of hematopoietic stem cells, Stem cell antigen (Sca-1), has also been found to be expressed in some cells of mesenchymal origin [20]. Studies have demonstrated that lung cells expressing Sca-1 were predominantly found in distal regions of lungs and were shown to possess a temporal emergence, indicated by enrichment of Sca-1 expressing cells in adult mouse lungs when compared to neonatal lungs [21]. Sca-1^{pos} cells have been shown to emerge in postnatal lung during the branching of the airways/lung vasculature and increase exponentially in adult lungs. Sca-1 [22]. Based on the expression of Sca-1 and other markers, various studies have identified unique sub-populations in lung tissue with stem cell characteristics. Thus Sca-1 emerged as a representative cells surface marker to identify the lung stem cells.

Bronchioalveolar stem cells (BASCs) isolated, from bronchioalveolar duct junction in adult mouse lungs, based on expression of Sca-1 and CD 34 (Epithelial and hematopoietic markers) were shown to exhibit self-renewal and multipotent capabilities. In *in vivo* studies, the BASCs were shown to participate in lung epithelial cell renewal and maintain bronchiolar, clara and alveolar cell populations in the distal lung [13]. Gene expression analysis on Sca-1^{neg}, $CD45^{neg}$, $CD31^{neg}$ lung populations and

corresponding Sca-1^{pos} cell lines were shown to possess epithelial and mesenchymal gene expression profiles respectively, signifying the presence of Sca-1pos cells with mesenchymal characteristics [16, 21]. Moreover, the Sca-1^{pos}, CD45^{neg}, CD31^{neg} were enriched with mesenchymal progenitor cells in culture as shown by their spindle shaped morphology and expression of mesenchymal markers (CD 104a, Vimentin). In contrast, Sca-1^{neg}, CD45^{neg}, CD31^{neg} cells were shown to possess cobblestone epithelial cell morphology and epithelial marker expression (E-cadherin, cytokeratins 5 and 14, and proSP-C) [21]. From a functional standpoint, the isolated and characterized stem cells in the lung are believed to play an important role in maintaining lung homeostasis. Bronchiolar stem cells have been functionally defined by their expression of clara cell secretory protein (CCSP), pro-surfactant protein C and they belong to airway epithelium [23, 24]. Tiesanu et al., in 2009 have identified bronchiolar stem cells as CD45^{neg}, CD31^{neg}, CD34^{neg}, Sca-l^{low} and AF^{low} as opposed to Sca-1^{pos}, CD45^{pos}, CD45^{neg}, CD31^{neg} reported by Kim et al. [13]. Their transgenic mice models study associated with stem cell expansion, ablation, and lineage tracing, demonstrated CD34^{pos} does not belong to air way epithelium and CCSP expressing cells are found in CD34^{neg}, Sca-l^{low} and AF^{low} [25]. It is useful to note that evidence of different subpopulations in lung cells with potential stem cell properties has been attributed to the method of isolation, culturing conditions, and choice of markers [22]. These studies indicate the complex nature of lung and presence of one or more putative stem cells in the pool Sca-1^{pos}CD 45^{neg}CD31^{neg}, details of isolation and characterization of these cells has been detailed in recent articles published by our group and others [16, 26].

In humans, a class of somatic lung stem cells with self-renewing, clonogenic, and multipotent (in vitro and in vivo) properties were shown to exist using c-kit as the stem cell marker [17]. The c-kit^{pos} cells were negative for hematopoietic and mesenchymal markers and interestingly demonstrated positive expression of key markers associated with pluripotency: *OCT4*, *NANOG*, *KLF4*, and *SOX2*. However, a key defining feature of somatic stem cells that differentiates them from pluripotent stem cells is that they undergo asymmetric division that results in the generation of a heterogeneous population of stem cells and progenitor cells. This study has been received with some skepticism putting forward several questions and need for independent studies to ascertain existence of somatic lung stem cells in human [27].

The fact that lung tissue is composed of a variety of cells with distinct phenotype and functions complicates our understanding of lung regeneration. This is evident from multiple research studies where lung cells characterized by different markers were shown to possess core stem cell properties of self-renewal, clonality, and multipotent characteristics [13, 16, 22]. While these research studies promise a step ahead in identifying putative lung stem cells, there are challenges that need to be addressed. One such challenge is to define a unique set of cells that play a crucial role in lung regeneration. This is expected to strengthen our attempts to develop focused therapeutic strategies in the context of wound healing but also in identifying and targeting putative cancer stem cells in the lung. Some of the studies carried out in identifying lung stem cells in mouse and their characteristic features described by experimental evidence are summarized in Table 19.1.

Table 19.1 Summary of putative lung stem cells sorted by cell surface markers

Sorted based on	Sca-1 expression anatomical location	Morphology	Comments	Reference
Sca-1^{pos}, CD 34^{pos}, CD 45^{neg}, CD31^{neg}	Bronchi alveolar duct junction	Epithelial	These cells were positive for Clara cell marker and Surfactant protein marker.	BASCcs [13]
Hoechst ^{low}, CD 45^{neg}, CD31^{neg}	Sca-1 expression is predominantly found in Distal Lung, restricted to Endothelial and Perivascular Cells	Mesenchymal	Similar to other mesenchymal progenitors, these cells express Sca-1, CD106, CD 140a (PDFGR-a) and CD44	[19]
Sca-1^{pos}, CD 45^{neg}, CD31^{neg}	–	Mesenchymal	Differentiated into endothelial and lung epithelial (alveolar type I, II, and Clara)	[16]
Sca-1^{low}, AF ^{low}CD34^{neg}, CD 45^{neg}, CD31^{neg}	Endothelium and proximal airway epithelium	Epithelial (Bronchiolar)	Sca-1^{low}, CD4^{5neg}, CD31^{neg}, CD34^{neg} include both clara cells & bronchiolar stem cells. Bronchiolar stem cells are distinguished from clara cells by low autofluorescence	[25]
Sca-1^{pos}, CD 45^{neg}, CD31^{neg}	Endothelium and distal parenchymal	Mesenchymal	Cells were also positive for CD 34 and Thy1. Capable of differentiating into lipofibroblastic, osteogenic and chondrogenic cell lineages	[21]

Cancer Stem Cells in the Lung

Numerous studies have demonstrated the presence of subpopulations of cells in tumors that play a critical role in initiating a tumor during post chemotherapy or radiation treatments [15, 28]. These cells are present as a small population within the tumor and appear to be more potent in initiating the tumor than other subpopulations and were classified as cancer stem cells (CSCs). These CSCs were characterized by independent research studies and were shown to sustain their malignant phenotype against drugs targeting cancer [28–30]. Interestingly, these CSC subpopulations were found to possess stem like properties of self-renewal and differentiation similar to those exhibited by somatic stem cells. Certain signaling pathways such as Hedgehog, Notch, and WNT that are important for maintenance of embryonic stem cells were also shown to have a role in putative CSCs found in the lung [14, 31–34]. Thus the discovery of CSCs has opened a new area of research in cancer that focuses on understanding and targeting the cells that drive the recurrence of tumor and metastasis.

CSCs share similarities with the resident somatic stem cells in their respective tissues of origin. Somatic stem cells are characterized by their oligopotent property, where they continuously renew themselves as well as differentiate into distinct descendants that are specific to a tissue. Somatic stem cells are found along with specialized cells of an adult tissue or organ as rare side populations. For prolonged periods of time they reside in quiescence (G-0/resting) phase of the cell cycle, a stage that is an actively controlled phase involving various epigenetic, transcriptional, and signaling pathways [35]. In response to injury or stimuli, these somatic stem cells enter mitosis and give rise to a stem cell and a progenitor cell by the process of asymmetric cell division. The stem cell resides back in quiescence stage until the next signal to re-enter the cell cycle, while the progenitor cells undergo a series of amplifications that give rise to post mitotic differentiated cells in respective tissues or organs of an animal (Fig. 19.1).

This characteristic asymmetric division not only plays a role in maintaining homeostasis in adult tissues by replacing the dead or aging cells, but also avoids repetitive entry of stem cells into the cell cycle, which may increase the chance of DNA damage. This similar kind of hierarchy is observed in CSCs, where a side population of cells forms the backbone to drive relapse of tumor and metastasis. However, unlike normal somatic stem cells these CSCs possess abnormal characteristics which are currently being explored in the context of understanding their role in specific cancers. A broad perspective and future directions in identifying cancer stem cells, *in vitro and in vivo* assays to characterize them, and developing drug screening strategies have been critically discussed [36].

Identification and isolation of these CSCs from the bulk of tumors have been reported based on presence of specific markers that differ from those from used to identify adult lung stem cells reviewed in [15]. The phenotypic characterization of CSCs include the activity of cytoplasmic enzyme aldehyde dehydrogenase (ALDH), expression of cell surface markers CD 133 and CD 44, or capacity of cells to efflux

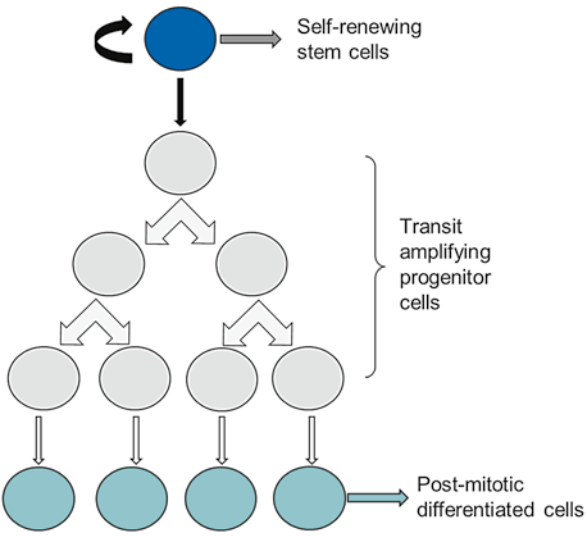

Fig. 19.1 Asymmetric division of somatic stem cells. Asymmetric self-renewal properties of somatic stem cells results in a stem cell and a progenitor cell. The progenitor cells divides repeatedly and give rise to post-mitotic terminally differentiated cells, thus maintaining homeostasis and stem cell pool of a tissue

membrane permeable dyes such as Hoechst 33342 dye and existing as a side population (SP) in bulk of tumor cells [15]. CSCs expressing CD133 cell surface markers were identified to be a putative marker for NSCLC and SCLC, while CD44 is found to be enriched only in NSCLC and not in SCLC. Similarly, NSCLC demonstrate positive activity for ALDH. Based on these studies, identifying a panel of universal markers to classify CSCs is an active area of research.

Signaling pathways such as Hedgehog (Hh), Notch, and WNT are important in the maintenance of stem cells and tissue homeostasis found in CSCs. It is believed that dysregulation of these pathways in CSCs could drive their tumorigenic activities with several reports focused on developing therapeutic strategies to target these pathways [15]. For example, inhibiting Hh signaling pathway in lung cancer cell lines resulted in loss of side population cells, while targeting Notch and Wnt signaling resulted in reduction of ALDH positive tumor cells or induction of apoptosis or growth inhibition in NSCLC [15]. These clinical trials provide some novel developments in treating lung cancer but further trials are needed to demonstrate efficacy. Despite these encouraging clinical results in treating cancer, other mechanisms that are being discovered in CSCs still need to be further researched to develop feasible therapies. One such key mechanism is the Epithelial-mesenchymal transition (EMT) in CSCs, first reported in breast cancer stem cells [37], where the CSCs were shown to exploit this EMT mechanism that is normally observed during developmental process of the mesoderm. It involves a process by which the epithelial cells lose their morphology and gain migratory and invasive properties to become mesenchymal cells. This EMT mechanism was found to be activated during cancer invasion and metastasis and results in the generation of mesenchymal cells that express the stem cell marker CD44 and form tumors effectively in mammary epithelial cancer cells [37] and believed to be associated with drug resistance and cancer progression. Our current understanding is limited on signaling pathways and transcriptional factors

that takes place in these CSCs. Expression of EMT associated genes is also being assessed in the context of lung cancer, but the role of EMT in progression of lung cancer is yet to be established [38].

P53 and MDM2 in Lung Cancer

In normal cells, p53 is expressed at low levels but as a result of stress or cellular damage, it activates a host of different proteins that are involved in cell cycle, apoptosis, and senescence, thereby prevents proliferation of cells that carry mutations or DNA damage. In unstressed cells, p53 function is regulated by its specific target murine double minute 2 (MDM2) by a process of ubiquitination. MDM2, an E3 ubiquitin-protein ligase, binds N-terminal transactivation domain of p53, thus mediating p53 degradation by nuclear and cytoplasmic proteasomes. This constant mono-ubiquitination by MDM2 regulates physiological levels and functions of p53 in normal cells [39].

Apart from known functions of p53, recent evidence suggests that p53 plays a crucial role in regulating stem cell homeostasis [40]. Studies involving re-programming of differentiated cells into induced pluripotent stem cells have noted that inhibition or loss of p53 increases the re-programming efficiency by 3–10 fold [41–43]. These studies indicate that p53 has a pivotal role in restricting the reprogramming process. Other studies involving adult mammary stem cells derived from p53−/− mice were shown to possess immortal behavior by increased self-renewal and symmetric division as opposed to limited self-renewal and asymmetric division observed in their wild type counterparts [44]. Similarly in haematopoietic stem cells (HSC), expression of p53 has been found to be critical for regulation of several aspects of HSC behavior. Deletion of p53 in mice was also shown to contribute to increased HSC self-renewal and as well as an increase in the HSC pool [45]. In HSCs, p53 was also found to regulate cellular response to oncogene expression in progenitor cells, where absence of p53 and expression of proto-oncogene KRAs was found to promote acute myeloid leukemia [46]. A recent study in hematopoietic stem cells and mammary stem cells has noted that DNA damage by irradiation induces up regulation of p21, a known p53 inhibitor [47]. These studies indicated that elevated levels of p21 prevent p53 activation and its basal activity, thus preventing stem cells from apoptosis, and allowing them to enter cell cycle. This study identified a unique mode of p21-dependent response to DNA damage in stem cells wherein p21 activates DNA repair, minimizing DNA damage accumulation, and exhausting the stem cells to divide symmetrically as opposed to less stressful asymmetric division [47]. In summary, these studies suggest that, apart from its normal functional role in tumor suppression and cell cycle regulation, p53 is able to restrain adult stem cell self-renewal, and impose asymmetric mode of cell division.

The loss of tumor suppressor function of p53 is either impaired by deletion of Tp53 gene or expression of mutated p53 protein. Alternatively in some human cancers even though wild type p53 is active, its function is diminished by its primary

cellular inhibitor, MDM2. These functional disparities are the most commonly observed causes of cancers in humans. MDM2 has a dual function towards p53, by acting as a positive regulator of p53 by interacting with p53 mRNA when the ATM (Ataxia telangiectasia mutated) pathway is active. As the ATM activity ceases, MDM2 acts as a negative regulator to suppress the p53 protein activity by mediating its degradation under normal conditions [48].

The incidence of p53 missense mutations (70 %) is highest in lung cancer compared to all cancer types. Approximately 90 % of these p53 mutations are missense mutations that result in accumulation of mutant p53 protein [49, 50]. These impact molecular activity of cells and cause novel tumors not commonly observed in p53−/− (p53 null) cancerous mice. These missense mutation effects have been explained based on two primary models: dominant negative (DN) activity or oncogenic gain-of-function (GOF) [51]. In the first model, it is proposed that the mutant protein forms a hetero tetramer with wild type p53 and exerts a dominant negative effect on wild type p53 function. In the second model, it is projected that the mutant allele confers oncogenic progression irrespective of the wild type p53 allele counterpart. Studies carried out to understand the GOF mutations by transforming p53 null mice with mutant p53 constructs have supported the effects based on the GOF model. Phenotypic characteristics ascribed to GOF activity of mutant p53 include increased tumorigenicity, growth rate, motility, metastasis, invasiveness and decreased sensitivity to chemotherapeutic drugs [51]. Understanding the changes in these cancer lines with GOF activities is basically proposed as an important area of research for drug targeting. Towards use in these studies, many researchers have generated p53 mutant mouse models [51] and human cell line models based on mutations reported by research studies complied in IARC TP53 database (http://www-p53.iarc.fr/). Recent studies have also highlighted the use of lentiviral approaches (endogenous expression) or transfections (transient expression) to express tumor-derived mutant p53 in cells [52]. Engineered lung cancer cell lines by lentiviral approaches, were shown to possess differential up-regulation of genes between the p53 mutants and differences in their GOF activities [53, 54]. It is observed that these mutant derived p53 show enhanced expression of NF-kappaB2 and receptor tyrosine kinase AXL [53, 55] that could be potential targets for therapies. However, mechanisms underlying these differential expression are unclear and yet to be defined.

Summary

Cancer stem cells are found to conserve many properties of normal somatic stem cells that relate to self-renewal and differentiation. However, they develop resistance to the action of drugs by activating new molecular mechanisms to protect themselves from apoptosis. Some of these mechanisms, specifically related to self-renewal and differentiation are defining features of somatic stem cells. Along with stem-cell like characteristics, CSCs also carry dysregulated activities in of p53, MDM2 and pathways

dependent on these proteins. It is thus important to understand pro-tumorigenic effects of missense mutations and their impact on cancer progression. Defining the mechanisms underlying the molecular level changes observed in cancer as it relates to CSCs will enable us to develop effective therapeutic strategies.

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