# Chemoresistance of Lung Cancer Cells: 2D and 3D *In Vitro* Models for Anticancer Drug Screening

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**Abstract**

Chemoresistance of lung cancer cells is a key factor that limits the treatment of lung cancer patients. Patients may initially respond to standard chemotherapy, but this is often followed by rapid development of drug resistance and disease progression. Tumor heterogeneity and the presence of putative cancer stem-like cells (CS-LCs) provide a viable explanation for the chemoresistance of several types of tumors. In this book chapter, we will first describe the current knowledge of the role of both tumor heterogeneity and CS-LCs in lung cancer chemoresistance, tumor progression and metastasis. Next, we will discuss ongoing strategies at the *in vitro* level to screen for more effective anticancer drugs. We will specifically focus in three-dimensional (3D) culture systems (Spheroids and tumorspheres) and their application in anticancer drug discovery for lung cancer.

**Keywords:** chemoresistance, tumor heterogeneity, cancer stem cells, spheroids tumorspheres, 3D systems

## 1. Introduction

Lung cancer is the leading cause of cancer death among men and the second leading cause of cancer death among women worldwide [1]. Despite important advances in our knowledge of cancer cell biology and anti-cancer therapies such as chemotherapy, radiotherapy and targeted therapies, the five-year survival rates remain poor (<15%). Lung tumors are broadly classified into small cell lung cancer (SCLC) and non-small lung cancer (NSCLC). SCLCs are defined by neuroendocrine differentiation and small cell morphology of the tumor cells and account for

15–20% of newly diagnosed lung malignancies. Interestingly, SCLC tumors tend to recur as chemoresistant variants and occasionally show progression to a NSCLC phenotype [2]. Regardless of the type, chemoresistance appears in most lung tumors, presenting a challenge to the development of new therapeutic regimes. The failure of the management of lung cancer is largely attributed to the inherent and/or acquired resistance that limits the efficacy of current therapies. Several characteristics of lung tumors have been identified for long time as key driving factors that lead to increased chemoresistance. Among them, mutations, amplifications and overexpression of multidrug-resistant proteins have been investigated *in vitro* using cell lines growing as adherent monolayers (2D systems). Intratumoral heterogeneity was also recognized long time ago as a key factor contributing to chemoresistance and soon tumor spheroids were developed with the aim to replicate *in vitro* “mini-tumor” with more complex and heterogeneous 3D architecture mimicking primary tumors. These tumor spheroids were routinely obtained by culturing cancer cells in serum-containing media under anchorage-independent conditions. The isolation of putative cancer stem cells from solid tumors was done by culturing cancer cells under anchorage-independent condition but in serum-free media (initially with few supplements). Under these conditions, cancer cells grow as “floating tumorspheres” and form complex 3D structures similar to spheroids. It is widely accepted that “floating tumorspheres” are enriched with cancer stem-like cells that are inherently chemoresistant. For clarity and consistency, we will call “spheroids” and “tumorspheres” to masses of cancer cells growing as floating spheres in serum-containing and serum-free media, respectively. The aim of this chapter is to (1) briefly describe the main factors—relevant to 3D *in vitro* models contributing to chemoresistance (intratumoral heterogeneity and the presence of CS-LCs) and (2) discuss the application of spheroids and tumorspheres as tools for screening anticancer drugs targeting chemoresistant cancer cells.

### 1.1. Intratumoral heterogeneity

Intratumoral heterogeneity is a term that refers to the presence of cells within a tumor with varying degrees of morphology, proliferation rate, ability to metastasize, sensitivity to drugs, dependence on growth signals and tumor initiation/repopulation capacity. It has long been recognized as a salient feature of most cancers and largely associated with tumor relapse. Both genotypic and phenotypic diversity exist within tumors that arise driven by genetic mutations, epigenetic alterations or microenvironmental influence. As a consequence, expansion of selected clones as well as establishment of differentiation hierarchies of cancer stem cells (CSCs) and non-CSCs creates a wide diversity of cells [3]. The basis for the genotypic heterogeneity is the inherent genetic instability of cancer cells and the clonal evolution theory that proposes that a tumor of monoclonal origin may become heterogeneous due to advantageous tumorigenic growth of clonal subpopulations. Over time, as the tumor progresses, cancer cells accumulate different mutations and different clones may compete or evolve in parallel generating a tumor composed of cells with varied genetic imprints. The latter is called branched evolution, and this process has been confirmed by genetic analysis in a variety of tumors [3]. Non-genetic heterogeneity (phenotypic heterogeneity) is the result of microenvironmental pressures due to, for instance, alterations in oxygen, pH and nutrient availability based on their regional location within the primary tumor largely influenced by their relative distance to blood vessels. Both genotypic and phenotypic heterogeneity are associated with chemoresistance that have a profound impact on the clinical outcome of lung cancer patients. For instance, at the clinical level, it has been recently suggested that increased metabolic heterogeneity should be considered as a high-risk subpopulation for early EGFR TKI failure [4].

### 1.2. Presence of cancer stem-like cells in lung tumors

The cancer stem cell hypothesis (CSCH) suggests that most cancers contain a rare subpopulation of cancer cells with properties such as indefinite self-renewal, slow replication and ability to give rise to differentiated progeny. These cells possess intrinsic resistance to chemotherapy and radiotherapy and are thought to be responsible for tumor initiation and growth and tumor relapse [5, 6]. The CSCH is a hierarchical model in which cancer stem cells (CSCs) can differentiate into non-cancer stem cells (non-CSCs) but not the other way around. According to this model, eliminating the CSC subpopulation would eventually lead to a cure. This concept has been recently challenged by several alternative models of cancer stem cell biology [7, 8] since experimental evidence demonstrated that cancer cells are extremely plastic [9, 10] and evidence of interconversion between CSCs and non-CSCs was found in a variety of cancer types including lung [11], breast [12] and colon [13] cancers. Contrary to the CSCH, all the alternative models propose that to cure cancer all cancer cells should be eliminated at once. At present, it is safe to assume that tumors may consist of a heterogeneous population of cancer cells with different "stemness" properties ranging from a pure non-CSC phenotype (typically sensitive to conventional anticancer drugs) to a pure CSC phenotype (usually highly resistant to conventional anticancer drugs). *In vitro*, CS-LCs are able to grow in the absence of serum as 3D spheres under anchorage-independent conditions as floating "tumorspheres" (FTs) and it is thought that the ability to form clonal spheres is a unique characteristic of CSCs [14, 15]. Because of its 3D architecture, and the notion that FTs consist of mostly CSCs with inherent chemoresistant properties, they have widely adopted as system models for drug screening (see Section 2.2.2.).

## 2. *In vitro* models for anticancer drug screening

Several *in vitro* models of lung cancer have been widely used for testing new anticancer drugs. Historically, 2D cultures were introduced first and chemoresistant cells lines were isolated and widely used to screen for more effective anticancer drugs. However, since 2D cultures typically consist of a more homogenous population of cells, 2D systems may not be able to account for intratumoral heterogeneity. As a result that 3D systems would recapitulate more faithfully, the heterogeneous nature of cancer cells existing *in vivo* were soon developed. Before describing these 3D systems, we will briefly discuss 2D systems.

### 2.1. 2D systems

Some cell lines were found to be inherently resistant to one or more drugs. In addition, several drug-resistant cancer cell lines have been generated in the laboratory to investigate either the underlying mechanism of resistance to a particular drug and/or to screen for alternative drug able to circumvent the acquired resistant. The next three sections will focus in the development and characterization of cancer-resistant cell lines and their application to identify mechanism of resistance and identification of new targets.

### 2.1.1. Development of drug-resistant cellular models of lung cancer

Developing models of chemoresistant cancer cells is a well-utilized approach to investigate mechanisms of acquired drug resistance, anticancer drug screening and developing novel drugs to resensitize resistant cancers to apoptotic stimuli. Developing a stable clinically relevant drug-resistant model involves using doses and exposure times consistent with the clinical setting [16]. In order to mimic clinical conditions, developing *in vitro* models of cancer resistance involves chronic exposure to lower concentration of drug with a pulsed treatment strategy that involves cycles of exposure to drug followed by recovery in drug-free medium [17]. Alternatively, continuous selection strategy with increasing doses or acute exposure to high concentration of gefitinib and erlotinib has been used to develop resistant models of NSCLC [18–20].

### 2.1.2. Characterization of drug-resistant models

Several assays have been used to characterize chemoresistance by comparing the resistant phenotype with the parental cell line. Measurement of drug sensitivity using viability assays such as the MTT assay, flow cytometry analysis to determine cell cycle arrest, clonogenic survival assay, characterization of stemness markers, determination of ALDH activity, expression of EMT markers, characterization of MDR modulators and quantification of cellular uptake of drug used to induce drug resistance are commonly used assays to characterize drug resistance. A dose and time-response curve of cell viability is used to calculate $IC_{50}$. The fold resistance is calculated by comparing the ratios of $IC_{50}$ of the resistant cells with parental cells [17]. A marked feature of drug-resistant cell lines is enrichment of cancer stem cells, a subpopulation of tumor cells with capacity for self-renewal and tumorigenicity potential. Aldehyde dehydrogenase (ALDH) is involved in differentiation of cancer stem cells and promoting resistance and survival mechanisms [21]. Aberration in regular cell cycle can circumvent or potentiate apoptosis, and drug-resistant cells undergo cell cycle arrest to prevent apoptotic cell death [22]. Epithelial mesenchymal transition has been associated with resistance to gefitinib and erlotinib in NSCLC [23, 24]. Quantifying decreased cellular drug accumulation in the resistant phenotype using inductively coupled plasma mass spectrometry (ICP-MS) due to overexpression of transporter proteins is another novel method of characterizing acquired drug resistance [25, 26].

### 2.1.3. Identifying mechanisms of resistance and novel drug targets for resistant lung cancer

Epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase (RTK), involved in cellular proliferation is overexpressed in NSCLC and SCLC. Targeting EGFR signaling using tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib was a reasonable clinical success, particularly in patients with EGFR mutations [27, 28]. Acquisition of resistance due to a secondary mutation of EGFR (T790M) is a major therapeutic problem necessitating discovery of novel drugs that can inhibit TKI-resistant NSCLC after developing T790 M mutation [29]. A panel of 12 NSCLC cell lines comprising wild-type EGFR (TKI-resistant), EGFR mutation with an additional TKI-resistance inducing mutation and EGFR mutation yet sensitive to TKI-inhibitor was used to screen 10 anticancer compounds. All 12 NSCLC cell lines showed inhibition of proliferation with 17-DMAG, an Hsp90 inhibitor and belinostat, a histone-deacetylase inhibitor (HDACi). 17-DMAG and belinostat inhibited EGFR and p-Akt expression in one cell line of each group. Combination of 17-DMAG and belinostat showed synergistic antiproliferative activity and inhibited the growth of TKI-resistant cell lines. These drugs were effective in mice xenografts and completely suppressed tumor growth with the combination being more effective than either drug alone. Immunoblotting of mice tumors showed decreased expression of p-EGFR, total EGFR and p-Akt substantiating a need to validate a combination therapy of 17-DMAG and belinostat in patients with EGFR-TKI-resistant NSCLC [30].

Some mechanisms shown to confer resistance to gefitinib and erlotinib in EGFR-mutated patients in addition to T790 M mutation, stemness and EMT have been shown to be MET amplification, FGFR1 overexpression and IGF1R overexpression [30–32]. These multiple resistance mechanisms can exist in parallel giving rise to heterogeneous resistant cell population. NSCLC grown resistant to erlotinib identified subclones that underwent MET amplification or induced EMT phenotype. MET subclones, while maintaining erlotinib resistance, showed increased sensitivity to MET inhibitors, crizotinib and capmatinib. EMT subclones overexpressed FGFR1 and showed increased sensitivity to FGFR1 inhibitor AZD4547. Inhibitors of MET and FGFR1 showed reduced sensitivity to mixed NSCLC cell line as compared to the individual subclones. This study highlights the coexistence of parallel resistance mechanisms giving rise to heterogeneous resistant population [33]. Anaplastic lymphoma kinase (ALK) gene rearrangements act as oncogenic drivers and are present in a small subset of NSCLC, which are responsive to ALK kinase inhibitors such as ceritinib (LDK378) [34]. Ceritinib-resistant cells were grown by chronic exposure to the drug and assayed for resistance using cell proliferation and viability assays. Ceritinib treatment upregulated Src, an oncogene involved in tumorigenesis and metastatic progression. Knockdown of Src with siRNA in resistant phenotype resensitized cells to ALK inhibition with ceritinib. Resistant ALK-positive cell lines showed sensitivity to Src inhibitor ADZ0530, which has promising therapeutic potential to be explored in patients with ALK-TKI-resistant tumors [35]. In another study, combinatorial treatment of ALK and IGF1R inhibitor was used to overcome crizotinib resistance in ALK-positive lung cancer, positing IGF1R to be an independent drug target in this subset of lung cancer [36]. Interleukin-8 is upregulated in gefitinib-resistant cells and is associated with shorter progression-free survival in EGFR-TKI-treated lung cancer patients. IL-8 expressing EGFR-mutant cell line showed increased phosphorylation of Akt and NF-κB translocation and decreased sensitivity to gefitinib-induced apoptosis. Knocking down IL-8 increased the apoptotic sensitivity to gefitinib. IL-8 expressing cells showed stemness as characterized by ALDH activity, increased expression of Nanog, Oct4 and Sox2 and forming more and larger colonies than controls. This acquisition of stemness was IL-8 dependent, as knocking down IL-8 reversed the stem cell-like properties in EGFR-TKI- resistant cells [37]. Gefitinib-resistant cells were developed to investigate the induction of EMT phenotype associated with EGFR-TKI resistance in NSCLC. While these resistant phenotypes were negative for T790 M mutation or MET-amplification, they had acquired the EMT phenotype with increased migratory and invasive phenotype. The acquisition of EMT phenotype was mediated via the activation of IGF1R/NF-κB signaling pathway. Inhibition of IGF1R/NF-κB signaling restored the sensitivity to gefitinib and suppressed the migration and invasion capability, suggesting that this pathway could be a novel target for gefitinib-resistant EGFR-mutant NSCLC therapy [38].

To summarize, despite the wide variety of cancer drugs available to treat lung cancer, acquired resistance to therapy is still a frequently encountered problem. It is important to identify the resistance conferring mechanisms, so that the resistance can be circumvented or reversed with novel anticancer drugs. Although drug-resistant cell lines provide robust preclinical models to interrogate underlying mechanisms of resistance and anticancer drug screening, they lack key features present *in vivo* in primary tumors that may provide additional mechanism associated with chemoresistance.

### 2.2. 3D systems

Although a plethora of 3D systems have been developed for anticancer drug screening at present, despite some technical traits, none of these systems showed a clear advantage over other regarding the ability to select successful clinically useful anticancer drugs. For space limitations, only floating spheroids and floating tumorspheres will be addressed in this chapter. **Figure 1** shows representative microscopy image of spheroids and tumorspheres.

#### *2.2.1. Floating spheroids*

The 3D multicellular aggregates formed from single cell suspensions in FBS containing media under anchorage-dependent or independent conditions are commonly known as "Spheroids." It is an excellent 3D model for drug screening as it can closely mimic tumor heterogeneity, tumor microenvironment niches (normoxic, hypoxic, pH gradient zones) and tumor structural as well as functional intricacies. Spheroids with constant size and consistent structural features are necessary to generate consistent and reproducible results.

Researchers have developed various techniques to develop spheroids to achieve these targets. The commonly used 3D cell culture methods can be divided into two main categories—(i) scaffold

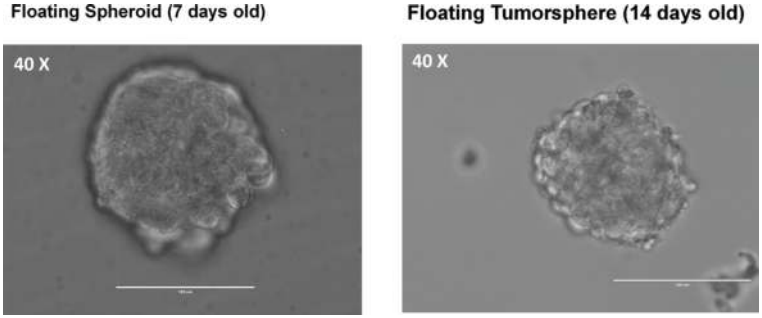

**Figure 1.** Representative images of H460 lung cancer cells growing as floating spheroids in serum-containing media (A) or as floating tumorspheres in serum-free media (B). Image in B was reprinted with permission from [39]. Bar = 100 μm.

based (hydrogel and inserts) and (ii) non-scaffold based. Scaffold-based methods involve collagen-, chitosan- and polycaprolactone-based biomaterials, which serve as extracellular matrix and provide a 3D architecture for the growth of cells in anchorage-dependent manner in 3D cellular entities. Non-scaffold-based approaches employ techniques such as forced floating, rotary devices, hanging drop arrays, microfluidics, etc. to generate spheroids. Forced floating is the simplest and most commonly used method for the spheroids in a laboratory setting. As the name suggests, forced floating of cells is achieved by culturing them in vessels that have been coated with poly-HEMA or agarose suspensions, which prevents attachment of cells to the substratum. This method has been successfully used to generate spheroids of various types of cancers. It is a simple, inexpensive and convenient method for generating spheroids; however, it produces spheroids of variable sizes and shapes. In a recent study, Ivascu et al. reported the use of centrifugation to generate spheroids of fixed size using forced suspension method [40]. They added cell suspensions containing a fixed number of cells in each well of poly-HEMA-coated 96-well plates and subsequently centrifuged the plates to colocalize them. They reported formation of uniform single spheroids per well as early as 24 hours. Rotary 3D culture techniques work on agitation principle, which prevents cells from attaching to the container and instead cells interact with each other and develop 3D spheroids. Rotary culture methods can be sub divided into two main categories: (i) spinner flask and (ii) rotational culture systems. In spinner flask method, cell suspensions are constantly agitated by magnetic stirrer, while in rotational culture system cell suspension media is moved by rotating the culture vessel itself. Both these methods can produce large amount of spheroids and can be used for long-term production of spheroids. As culture is constant, agitated cells receive constant supply of nutrients and oxygen. However, size and structure of the spheroids cannot be controlled in these 3D culture methods. As these methods rely on constant moving of culture media, it exerts extensive force on cells which can either damage or affect cellular physiology of the cells. Rotational culture system is a superior technique than spinner flask method as it exerts far less force on the cells [41]. Kelm et al. developed hanging drop method for the generation of multicellular spheroids [42]. This method employs gravity as a force to bring the cells together to generate spheroids. Small aliquots of cell suspension media (usually 20 μL) carrying fixed number of cells are placed in wells of microtiter plate. These plates are then inverted and suspension media forms a drop and cells migrate to the tip of the drop due to gravity. These cells are then allowed to proliferate and generate a single spheroid per well. This method is good for high throughput, and spheroids with consistent size can be generated with excellent reproducibility. One limitation of this method is the volume of the liquid (up to 50 μL) that can be used to generate drop as surface tension can keep only small volumes of media together. Since small volumes of media are used for drop generation, this method requires constant media replacement making maintenance of culture challenging [43]. Tan et al. used a microfluidic platform to generate spheres [44]. This platform consists of a main channel and an array of coated microwells. Media containing single cell suspension flows through the channel and the cells get trapped into the wells where they grow in close contact and generate a single spheroid per well. Size and shape of the spheroids can be controlled by this method. It is a very good platform for high throughput drug screening and compatible with multidimensional imaging. However, spheroids generated in microfluidic platform cannot be retrieved for further study and structural analysis. In addition, several coculture methods have been developed to mimic complex cancer cell and microenvironment interactions. Tumor microenvironment is a complex system that involves interactions between tumor cells and adjacent stroma cells (fibroblasts, endothelial and inflammatory cells) embedded in extracellular matrix (ECM). Spheroids generation using a coculture model by coculturing cancer cells and fibroblasts and/or immune cells has been reported and studied for their unique interaction and subsequent effect on carcinogenesis [45, 46]. Spheroids perfectly mimic an avascular tumor microenvironment and cellular heterogeneity. Similar to tumor a nutrient, oxygen and pH gradient exist in spheroids leading to three distinct cellular zones in the spheroids [47]. The central necrotic zone, which mostly constituted by dead cells, is devoid of oxygen and nutrients. This is followed by middle dormant (senescent) zone consisted of quiescent cells. The peripheral layer, which has sufficient supply of nutrients and oxygen, has proliferative cells. Microelectrodes and proton magnetic resonance with pH-sensitive indicators are commonly used techniques to study oxygen flow and pH inside the spheroids [48, 49]. **Figure 2** illustrates the complex 3 architecture of spheroids.

In general, 3D spheroids show less chemosensitivity towards various drugs compared to 2D *in vitro* cell culture models [50, 51]. This differential response can be attributed to structural and functional complexity of 3D model vs. a 2D model. Drug penetration is one of the reasons of this resistance as the structural and microenvironmental barriers prevent effective convection of drug in 3D spheroids. As a result, drug fails to achieve an effective concentration inside the tumor to deliver a strong anticancer response. Kerr et al. demonstrated that reduced penetration of anthracycline-based drugs was responsible for a mild cytotoxic effect of these drugs in lung spheroids and a more lipophilic analogue partitioned better hence can be a better therapeutic option against 3D spheroids [52]. A recent study by Gupta et al. showed improved efficacy of paclitaxel when coadministered with tumor penetrating peptide iRGD due to increased availability of the drug in the interior of A549 spheroids [53]. Similarly, tumor

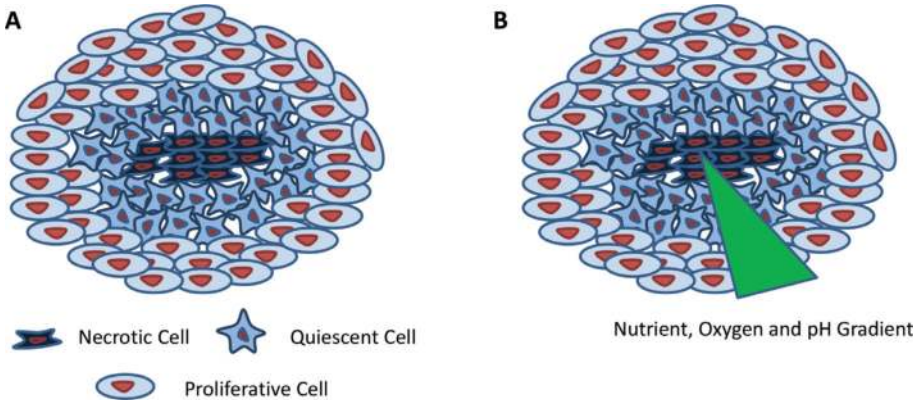

**Figure 2.** Structural organization of spheroids. (A) Cells are organized in three distinct zones based on viability and proliferative status of the contributing cells. The innermost necrotic layer is mostly composed of dead cells, which is followed by middle senescent layer consisting of slowly growing quiescent cells. The outermost layer is proliferative and contains rapid dividing cells. (B) A gradient of nutrients, oxygen and pH is responsible for this structural organization of cells in spheroids. Since the interior section of the spheroids receives least amount of nutrients and oxygen, the survival of cells in this part of the spheroid is compromised. As supply of nutrients and oxygen improve from moderate to high in the middle and outer section of spheroids, cells change from quiescent to highly proliferative in these zones.

heterogeneity arising from the distinct arrangement of cells in different proliferative zones in spheroids can promote resistance to certain drugs. For example, antiproliferative drugs such as paclitaxel, which confers most activity towards rapidly dividing cells, showed a reduced activity towards interior region of spheroid as it consists of quiescent cells [54]. Drugs like doxorubicin which induce anticancer effect by producing reactive oxygen species show limited response under hypoxic conditions [55]. However, drugs which get activated under hypoxic conditions can potentially treat hypoxic tumors. In 2012, Meng et al. observed that a hypoxia-selective drug TH-302 showed 650-fold greater activity in hypoxic H460 lung cancer cell spheroids than in monolayer cells [56]. Cancer cells switch their metabolism to glycolysis and produce lactic acid in the process. A lack of effective clearing of this excess lactic acid results in accumulation of lactic acid and subsequent reduction of pH in the interior of the tumor. A reduced pH of the tumor microenvironment can adversely affect the cytotoxicity of weak basic drugs such as doxorubicin, mitoxantrone, vincristine, etc. These drugs get protonated under acidic conditions resulting in decreased cellular uptake, hence lose their activity [57]. Activations of mechanisms related to drug efflux in spheroid can be responsible for increased resistance to the drugs. In 2015, Rodriguez et al. showed an increased expression of MDR-1 and P-glycoprotein in spheroids of INER-37 human NSCLC cell line compared to 2D culture likely responsible for increased drug resistance [58]. Several studies have indicated CSC enrichment in spheroids as possible explanation for drug resistance and metastasis [59, 60]. A 3D spheroid platform is a more relevant model for anti-cancer drug screening, and represents a much better approach to achieving therapeutic outcomes for cancer patients as compared to current therapeutic practices. There is need to develop drugs with better penetrating ability to improve its availability to the most interior sections of tumors. Improved drug delivery techniques employing vehicles, such as nanoparticles, liposomes, nanospheres, etc., can (i) improve drug delivery and (ii) protect drug decomposition under harsh tumor microenvironment. Tumor heterogeneity arising from cells of different metabolic, proliferative, chemosensitivity, metastatic and stemness profile calls for a more complex and versatile therapy approach for cancer treatment. For example—a cotherapy approach using multiple drugs which can target different populations of cancer cells will have better outcome for patients than a simple single therapy approach. Development of better drugs with single drugs targeting multiple pathways can remove the need to use multiple drugs simultaneously. So, an effective and improved drug development, drug delivery and drug testing programme are the need of hour to make significant inroads in a fight against cancer. It is important to mention that Steadman et al. raised concerns about the possibility that the chemoresistance mechanisms found in spheroids may differ from the resistance found in intractable solid tumors in patients [61]. If this is the case, drugs selected by this system will be ineffective in primary tumors. Future studies should be aimed to attempt to recapitulate *in vitro,* the complex architecture of lung tumors should take in consideration several factors known to simultaneously contribute to chemoresistance.

### *2.2.2. Floating tumorspheres*

Cancer is a complex disease with several preventive mechanisms put in place to escape elimination by immune response, drug efflux or expulsion mechanisms to mitigate drug activity and a microenvironment uniquely suitable for cancer cells. The matter is further complicated by tumor heterogeneity resulting in mixed population of cells with varying degree of chemoresistance, invasive and migratory potential. Several cancer models have been proposed to explain tumor heterogeneity and related tumor characteristics. Clonal evolution model (CEM) and cancer stem cell model (CSCM) are two of the most popular and widely accepted models. CEM postulates clonal evolution of tumor and suggests existence of several clones with varying genetic and epigenetic modifications as the contributor for tumor heterogeneity. Lately, CSCM has become most commonly used model for tumor biology. According to CSCM, a rare but fixed population of cells known as cancer stem cells (CSCs) with indefinite self-renewal potential and pluripotency are responsible for tumor origin, heterogeneity, metastasis, resistance and relapse. CSCs are rare cells and most of the times only constitute 1% of tumor volume. Over the period, various methods and techniques have been developed to enrich and study the CSC. Fluorescence-activated cell sorting (FACS) using surface markers, culture of cells in suspension for generating tumorspheres with increased stemness, sorting of cells based on the activity of intracellular enzymes such as aldehyde dehydrogenase (ALDH) and 26S proteosome and sorting of side population cells due to their ability to exclude Hoechst 33342 are some of the most commonly used methods for CSC sorting and subsequent enrichment. Generation of tumorspheres is most convenient and cost-effective way for enriching CSCs as it does not require previous knowledge of surface marker or enzyme expression and costly cell sorting FACS set up. For this chapter, we will focus on generation of floating tumorspheres and their use as a model for studying chemoresistance. In 1996, Reynolds et al. described the generation of normal neural stem cells as "neurospheres" upon culture of brain cells in floating conditions in serum-free medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF) [62]. Following the same culture protocol, Singh et al. generated tumorspheres from the brain tumor cells [63]. Soon after, the method was widely accepted and researcher all over the world used this protocol to generate tumorspheres from a wide variety of cancers [64–68]. Over the course of time, researchers have tried to improve upon the existing protocol of sphere formation in order to make it more efficient, consistent, reliable and physiologically relevant. Various types of scaffolds consisting of chitosan-alginate, collagen, alginate and agarose have been used as 3D matrices to substitute low attachment plates [69–72]. In 2014, Cao et al. generated tumorspheres from primary neuroblastoma cells driven from MYCN transgenic mice using a medium supplemented with fetal bovine serum (FBS) and β-mercaptoethanol. These spheres exhibited indefinite renewal as they could be passaged more than 20 times and also demonstrated enhanced metastatic potential [73]. More recently, in one of our studies, we described culture of lung tumorspheres (LTs) from H460 cells solely in serum-free media without supplementation of growth factors [39]. This method was further extended to mammospheres (MSs) generation in MCF-7 cells [74].

Over a period, several targeted therapies have been developed towards various oncogenic drivers (EGFR, ALK, ROS1, RET, etc.) in lung cancer [75]. However, most of these drugs have shown a transient effect on cancer as initial remission of disease is followed by outbreak of more aggressive and resistant cancer resulting in modest overall survival. Therapy resistance is a leading hurdle in cancer treatment and mostly responsible for poor outcome for the patients. Several researchers have identified acquired mutations during the prolonged treatment with a single drug as the leading cause for the therapy resistance. As discussed in more detail in the previous section (Section 2.1.3.), secondary mutations, bypass pathways such as MET, amplification of HER2, overexpression of AXL kinase, etc. have been shown to induce therapy resistance in lung cancer [75–78]. With the advent of concept of CSCs and subsequent discovery of CSCs in several types of cancers, researchers started to explore a possible link between therapy resistance and possible changes in the CSCs population. In 2008, Levina et al. were the first researchers to report the enrichment of cancer stem-like cells (CSLCs) in response to therapy treatment in lung tumor. They observed that the surviving cancer cell expressed stemness-related markers such as CD133, CD117, SSEA-3, TRA1-81, Oct4 and nuclear β-catenin. These cells retained a higher capability to form spheres, self-renewal, differentiation and showed high metastatic and tumorigenic potential [79]. Several studies have pointed towards enrichment of CSLCs as a contributing factor for acquired resistance in lung cancer in response to Cisplatin therapy [25, 80]. Similar findings have been reported for other commonly used anticancer therapies in lung cancer [81]. Normally, a 2D *in vitro* model is used for the screening of potential anticancer agents and more often they fail to translate *in vitro* antiproliferative efficacy of a drug in *in vivo* settings. This is due to an inherently flawed 2D model which does not replicate a real tumor at structural, physicochemical, mechanical and biochemical levels. A tumor is a 3D entity composed of cells exhibiting varying degree of resistance, proliferative and metastatic tendencies. Tumor microenvironmental conditions which play a critical role in determining tumor heterogeneity, resistance and metastasis cannot be replicated by an *in vitro* model, and hence it often fails to impress upon aforementioned attributes of tumor. Therefore, a more physiologically relevant drug screening tool is a real necessity to improve upon often failing *in vitro* cytotoxicity model. Tumorspheres with their 3D structure and often increased stemness can serve as more resistant and invasive model with closely relatable microenvironmental conditions as that of a real tumor. It can serve as a more realistic approach for drug screening with better chances of replication of drug efficacy in *in vivo* system. Tumorspheres assay can serve as a quick and more economical intermediary testing platform for *in vivo* tumor xenograft studies in a high-throughput setting while *in vivo* studies can be reserved for validating findings observed in the tumorsphere assays.

In recent years, researchers have successfully employed tumorsphere model for effective screening of potential anticancer drugs. A number of studies exploring anticancer efficacy of different classes of compounds such as inorganic [82], natural ingredients [83], antibiotics [84], Chinese medicine [85], cardiac glycosides [86], etc. against LTs have been published. *In vivo* studies have been performed to explore and extend observed drug efficacy against LTs in a more physiological setting [87, 88]. Several studies have tried combination therapy approaches in LTs setting to develop effective drug combinations to alleviate therapy resistance-related concerns and improve efficacy of existing cancer drugs [68, 89–92]. The promising anticancer drugs selected using tumorspheres described above need to pass the test of more relevant animal models and later on successful clinical trials to validate the applicability of this 3D system for anticancer drug screening.

### *2.2.3. 3D systems as models to test drug delivery and efficacy*

Drug delivery refers to approaches, formulations, technologies and systems for transporting and administering *in vivo* an active pharmaceutical ingredient (API) to achieve a therapeutic effect in the patient. In tumors, there are gradients of drug concentrations, oxygen and nutrients created by the distance from blood vessels. These similarities make 3D systems the more physiological models for drug delivery testing and therefore better predictors of chemosensitivity. Metha et al. [93] described in detail six characteristics of spheroids, which are absent in conventional culture formats, that mimic how drug delivery might occur *in vivo*: (1) spheroids model the 3D architecture of tissues, including multicellular arrangement and extracellular matrix deposition, found *in vivo*. (2) Spheroids have sizeable cell-cell interactions, including tight junctions that are known to influence response of cells to drugs, (3) spheroids have diffusional limits to mass transport of drugs, nutrients and other factors, (4) spheroids are formed with two or more cell types in varying ratios representing intercellular signaling and architecture that can help to understand how multiple cell types might impact drug delivery, (5) rare cells such as cancer stem cells or primary stem cells may be present or incorporated and maintained in spheroids which can facilitate targeting these specific cells with drugs and (6) larger spheroids develop central necrosis and regions of hypoxia present in many cancers. These specific microenvironments have been shown to contain cancer cells with increased chemoresistance. Tumorspheres share most of these characteristics with few differences: (1) they are considered to be enriched with CS-LCs that are inherently more resistant than non-CS-LCs and (2) tumorspheres can be generated in complete absence of external mitogenic stimulation that makes ideal system to study how specific factors (sometimes present in serum-containing media) may alter the response of cancer cells to anticancer drugs. For instance, lung tumorspheres grown in the absence of external mitogenic stimuli when exposed to exogenously added EGF demonstrated increased sensitivity to Erlotinib and Gefitinib [10]. In summary, despite their limitations, both spheroids and tumorspheres are useful and complementary systems for drug delivery testing.

## 3. Implications for translational oncology

The *in vitro* identification of effective anticancer is a crucial part of the anticancer drug screening program. Drug development is a long and expensive business, and billions of dollars are invested for a single successful drug release in market. It starts at identification of potential drug candidate with subsequent testing *in vitro* and *in vivo* setting followed by testing in various phases of clinical trials. Upon successful completion of clinical trial, drug is approved by FDA for marketing. Therefore, development of realistic models of drug screening is extremely important for vetting of drug candidates in earlier preclinical stages for them to have better chances to be successful in later clinical trials. Traditionally, 2D *in vitro* culture model is used for cancer drug screening. However, it completely fails to recapture finer intricacies of 3D tumor. It does not have any semblance with a 3D tumor at microenvironmental, biological and physiological levels and hence in most instances miserably fails in therapy translation from an *in vitro* to an *in vivo* setting. In order to address these issues, researchers have developed various drug screening 3D models, which align well, mimic essence of natural tumors and carry more significance as drug screening platforms. Despite these important technical advances, few drugs have been translated into clinical practice and the prognosis of lung cancer patients remains poor, suggesting that current *in vitro* 3D models are still not good models of primary tumors.

## 4. Conclusions

At present, current *in vitro* 3D models offer significant advantages over 2D systems in terms of recapitulating intratumoral heterogeneity and enrichment of cancer stem cells and have been extremely useful for understanding cancer cell biology. However, for anticancer drugs discovery, this success has not been translated into the clinic because the prognosis of lung cancer patients still remains poor. Future development in the field should concentrate on (i) efforts to better mimic *in vivo* conditions and (ii) identifying the underlying mechanism of chemoresistance of *in vitro* system in correlation with *in vivo* conditions. Otherwise, drugs selected with the current method will only target a subpopulation of chemoresistant cells or as suggested by Steadman et al. will be ineffective due to differences in the underlying mechanism of chemoresistance between *in vitro* 3D system and *in vivo* conditions.

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## Conflict of interest

The author declares no conflict of interest, financial or otherwise.

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