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Chapter

Recent Approaches Encompassing the Phenotypic Cell Heterogeneity for Anticancer Drug Efficacy Evaluation

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Abstract

Despite the advancements in biomarker-based personalized cancer therapy, the inadequacy of molecular and genetic profiling in identifying effective drug combinations was defined in most cases. Drug resistance remains a major limitation of the current predictive oncology. Emerging reports indicate that the success of anticancer therapy is usually limited by intratumoral heterogeneity which is not captured by the existing cancer cell biomarker-based approaches. Cell heterogeneity, not only genetic but also phenotypic, is considered to be the root cause of resistance to anticancer treatment, cancer progression, and the presence of cancer stem cells. Therefore, functional testing of live cells representing various cell types within the tumor exposed to potential therapies is needed for identification of effective drug combinations. Here we look at the different existing model systems, including ex vivo models of the patient's tumor cells, 2D/3D in vitro cultures/cocultures, patient-derived cellular organoids, single-cell models, ex vivo tumor platforms containing tumor microenvironment and extracellular matrix, etc., scoping at drug efficacy evaluation and solving the problem of cancer resistance.

Keywords: ex vivo model, cancer heterogeneity, tumor microenvironment, cancer stem cells, anticancer therapy, drug resistance, plasticity

1. Introduction

Fighting cancer is a major challenge for scientists and clinicians. As a result of human population aging, today cancer is the most common cause of death in the world. One of the most promising measures to reduce cancer deaths is believed to be prevention. However, only smaller part of cancers can be prevented by avoiding environmental risk factors. The recent results presented in Science suggest that only one-third of the cancer risk is attributable to environmental factors or inherited predispositions, and that unavoidable random errors or mutations (bad-luck mutations), occurring during DNA replication in normal stem cells, are a major contributing factor in cancer development [1]. Authors suggest that this stochastic factor is the major contributor to cancer overall. The results are consistent with

epidemiological estimates of the fraction of cancers that can be prevented by changes in the environment.

Therefore, random, replicative errors every time a cell divides can contribute to cancers all along tumorigenesis, and at the same time, environmental factors can play a role during all phases. Authors suggest two prevention strategies in cancer: primary and secondary [2].

The importance of spontaneous endogenously generated DNA damage in oncogenesis is supported by the other recent studies [3, 4]. This understanding is vital for designing strategies to reduce deaths from cancer, suggesting the essential role of intervention. From that point of view, choosing the most effective medication and the treatment strategy as well as the ability to predict the treatment outcome is of particular importance.

Nowadays, biomarker-driven personalized cancer therapy has emerged as a powerful concept and significantly improved the results of cancer therapy. However, although discoveries in tumor genomics have led to the identification of a number of new cellular targets and the development of novel targeted drugs, currently available FDA-approved targeted therapies fall over time due to the development of drug resistance after impressive initial response. Intrinsic and acquired resistances remain the major limitation of the current predictive oncology.

Data presented in Nature indicate that precision oncology strategy to target the specific mutations in a tumor (to link genetic testing of patients with the drugs that would work best for them) has not been shown to work well, and perhaps it never will [5–7]. Therefore, in addition to more targeted agents, there is a great need for new approaches and for more predictive model systems to substantially improve in vitro drug testing.

Emerging reports indicate that the success of anticancer therapy is usually limited by intratumoral heterogeneity which is not captured by the existing cancer cell biomarker-based approaches. Cell heterogeneity in the tumor is considered to be the root cause of resistance (both intrinsic and acquired) to anticancer treatment including conventional chemotherapy, radiation therapy, and molecularly targeted therapy. Traditionally, it is thought that it arises from distinct mutations in "driver" oncogenes; however, intratumoral heterogeneity depends not only on genetic modifications but also on nongenetic processes involving either stochastic events or epigenetic modifications [8].

The development of tumor cell heterogeneity is believed to be related to cancer stem cells (CSCs). Reversible transitions between CSCs and non-CSCs within the tumor contribute to intratumoral heterogeneity. The heterogeneity of cellular states in cancer has been linked to drug resistance, cancer progression, and the presence of cancer stem cells [9, 10]. Therefore, subpopulations of cancer cells can be morphologically and/or functionally distinct, and this phenotypic diversity of cancer cells may account for differences in drug resistance. These data propose a different strategy in treating cancer by exploiting the observed phenotypic heterogeneity in cellular types.

Although current therapies rarely take intratumoral heterogeneity into account when predicting clinical response, the future oncology will likely require designing new innovative approaches involving ex vivo platforms that consider intratumoral heterogeneity in the context of therapy resistance mechanisms [11]. Detailed analysis of the response of phenotypically different cancer cells to the applied drugs is required in each individual patient's case. This analysis could be performed by using ex vivo patient-derived cancer cell lines and functional testing of different tumor cells exposed to potential therapies. Patient-derived models are needed to identify effective drug combinations for cancer treatment. New research, combining large-scale molecular studies data from patients, laboratory cancer cell lines, and drug

sensitivity tests, showed that patient-derived cell lines did carry the same genetic alterations that drove cancer in patients, and thus are suitable to predict how tumor will respond to a drug [12]. This means that cell lines can be used to create tools for physicians to select the best available drugs for each patient individually. *A reliable patient-specific model system, which includes patient's ex vivo heterogeneous cancer cells as an experimental model, for the identification of effective drug combinations in individual cells in each individual cancer case should appear.*

In any case, before running complex and expensive trials of individualized cancer treatment (including clinical trials, genetic, epigenetic, cellular, and immunological research), studies involving the development of appropriate experimental cell culture models must be carried out. Only then the models can be used for the evaluation of cellular response of different tumor subpopulations to the chemotherapy/targeted therapy treatment in every individual case, as well as for the identification of the best cell-type-specific treatment along with a proposed molecular mechanism and characteristic markers of the case.

Currently, various models are used for tumor resistance studies. Most popular methods are these: in vitro acquired resistance models using commercial cancer cell lines, while the other is searching for presumptive biomarkers. However, the first strategy does not reflect the actual situation of the individual patient, whereas the second strategy, without live cancer cells, lacks functional component. Experimental models of mouse tumors, including genetically modified mice and human tumor xenografts, due to large requirement of space, duration, and relatively high price, are not very promising for use in the clinic. Also, these methods do not consider tumor cell heterogeneity. It is believed that cell-based functional studies and methods, once optimized, could bypass the need for whole animal cancer avatars [13].

Single-cell models (e.g., circulating tumor cells), being very promising for genomic, transcriptomic, and multiplex proteomic analyses, however, also have a disadvantage: functional studies in models of single cancer cells do not estimate the importance of extracellular interactions within a solid tumor, especially in response to the treatment [14]. Over the last few years, Science and other journals have published works on the use of patient-derived cell lines in the selection of targeted medicines in specific cases of resistant tumors, but cell subpopulations were not included in the studies [15, 16]. The most advanced model systems that contextually conserve the personalized tumor ecosystems, designed as a personalized ex vivo human cancer platform for the evaluation of the clinical response to the anticancer treatment—the CANScript and OncoCilAir—are a highly promising methodology that includes cell heterogenicity and tumor environment [17, 18]. The latter platform is new in vitro model of nonsmall cell lung cancer which combines a reconstituted human airway epithelium, human lung fibroblasts, and lung adenocarcinoma cell lines. These two are complex and expensive technologies and can emerge as a powerful platform for tailored cancer therapy. However, they do not evaluate the response of phenotypically different groups of cells to the treatment.

Until now, cellular in vitro technology has not been used in a clinic, but it is already in commercial use. According to BBC market research report, the global cell-based assays market was $ 15 billion in 2015 and is expected to reach over $ 28 billion by 2021. In the treatment of cancer, researchers return to individual, disease-determining cell research models ("Back-to-the-Future with Tumor Cell-Based Avatars"). There is no doubt that in the near future ex vivo models of tumor cells will be used in the clinic, and this would be an important step toward even more personalized cancer therapy. These approaches can be used to create tools for doctors to identify the best therapies for individual cancer patients.

Among these are the ex vivo models of the patient's tumor cells having a different phenotype. With the help of new models, we hope the importance of tumor cell subpopulations will be justified in predicting the response to the treatment for a particular patient and, in a specific case, to identify possible therapeutic efficacy.

Optimization of these tests, verification, and evaluation of the costs of the most effective therapeutic solutions are necessary.

In this review, we will present the current ex vivo tumor cell models and clinically relevant platforms to functionally test predicted drugs/drug combinations for individual patients.

2. Cancer genomics

Today, in the epoch of innovative technologies, a breakthrough in replacing conventional molecular diagnostics technologies with new ones such as next-generation sequencing (NGS), has led to higher content analyses of cancer disease with improved cost-effectiveness and speed, higher sensitivity, and specificity [19]. It has enabled cancer biologists and clinicians to obtain valuable data of complex molecular alterations from clinical cancer patient samples rapidly. Based on this, The Cancer Genome Atlas (TCGA) (a collaboration between the National Cancer Institute and the National Human Genome Research Institute) has characterized diverse genomic alterations underlying individual human cancers by using over 11,000 tumors from 33 cancer types. Multidimensional maps of key genomic changes in those tumors were generated, and systematic studies of these genetic changes were performed. Based on sequence and structure, 299 driver genes with >3400 putative missense driver mutations were identified with implications regarding their anatomical sites and cancer/cell types [20]. In addition, comprehensive characterization of 10 selected signaling pathways was performed across the 33 cancer types analyzed by TCGA. Alterations in those signaling pathways across 9125 samples from 33 cancer types were determined. RTK/RAS/MAP-kinase and PI3K/Akt signaling pathways, among others, were frequently found to be genetically altered in cancer [21]. Results from TCGA Pan Cancer Atlas project greatly deepened the current understanding in various fields of oncogenesis such as somatic driver mutations, germline pathogenic variants and their interactions in the tumor, the influence of the tumor genome and epigenome on transcriptome and proteome, and the relationship between tumor and the microenvironment, including implications of prevention and treatment [22]. Based on recent genomic cancer research, new data are currently employed in the development of novel targeted drugs focusing on genes that are mutated in a tumor, e.g., activated oncogenes and their downstream signaling components [23].

At present, in the post-genomic era of the cancer therapeutics, the shift away from the use of conventional cytotoxic drugs toward molecularly targeted agents has occurred. Conventional chemotherapy is mostly directed at the majority of proliferating cells in tumors. Cancer is a genetic disease with genotypic changes that include: mutations, insertions, gene amplifications, deletions, gene fusions, chromosomal rearrangements, transposition of the genetic elements, translocations, and microRNA alterations. Detection of genetic alterations in specific regulatory molecules that are responsible for oncogenic transformation has enabled the development of targeted therapies [24, 25]. According to Hanahan and Weinberg, six hallmarks of cancer can be highlighted: sustained proliferative signaling; evasion of growth-suppressive signaling, resistance to cell death; limitless replication;

induction of angiogenesis; invasion; and acquisition of metastatic capability. In a while, the same authors have emphasized additional cancer properties: deregulation of cellular energetics, avoidance of immune destruction, genome instability, and tumor-promoting inflammation [26]. The phenomenon of "oncogene addiction," describing a special cancer cell dependency on a particular "driver" alteration for their survival, was defined in 2002 by Weinstein. In the last decade, the strategy to eliminate acquired genetic dependencies on oncogene addiction drivers by blocking them in each patient and in each tumor cell specifically has been widely translated into the clinic. Accordingly, the major classes of current US Food and Drug Administration (FDA)-approved targeted therapies include drugs that were designed to inhibit specific oncogenic mutations and molecular alterations specific to cancer cells, or aimed to increase the antitumor immune response, or inhibiting neoangiogenesis. Additionally, beyond direct targeting of genomic defects, the process of differentiation, epigenetic alterations, and microenvironmental properties were taken into account in drug discovery. Novel treatments include advanced molecularly targeted therapies that may consist of cell-, hormone-, small molecule-, vaccine- or antibody-based therapies. They are tailored to inhibit signal transduction, regulate gene expression, induce apoptosis, diminish angiogenesis, and serve as an immunotherapeutic in specific cancers.

Individual genomic drivers are usually identified from the sequencing of the human genome. Molecular genotyping is now a customary practice applied to the cancer patients aiming to define their genetic profile. Consequently, genomic and other omics data are used to predict the drug sensitivity of an individual tumor [27, 28]. In parallel, a variety of molecularly targeted drugs are being studied in preclinical settings or clinical trials, and many targeted therapies have been approved by the FDA for treatment of different kinds of cancer. However, a range of drawbacks are monitored; for example, numerous drugs fail in early and late stages of clinical trials, partly due to insufficient drug efficacy. Before that, only 5–15% of investigational cancer drugs succeed in receiving clinical approval. Data suggest that new targeted drugs without proper stratification may reach only 10–20% efficacy. In other words, only for a small fraction of patients, the genomics-driven cancer therapy can be very effective and improve the clinical outcome. However, for the rest, such therapy will not work. It is considered that 20,000 proteins are implicated and play key roles in cancer biology; however, today, a majority of these structural and regulatory classes of proteins appear to be undruggable. Alternatively, some identified targets have no drugs developed [23, 29, 30].

Although prolonged progression-free survival is monitored after successful targeted therapy, patients with cancer usually develop resistance to the drugs within 6–12 months. As an example, in the case of lung adenocarcinoma, the initial clinical response to targeted drugs—protein kinase inhibitors—is almost always temporary due to acquired resistance [31]. In general, despite our increasing understanding of the molecular determinants of oncogenesis, many forms of the cancer remain incurable. Therefore, the complementary strategies to match their tumors to appropriate therapies are needed as genome profiling of tumors is insufficient to guide the therapy for most patients. Nowadays, molecularly guided therapy involves determination of DNA, RNA, proteins, metabolites, etc., as complementary biomarkers. Biomarker sets to detect individual and pathway drivers as molecular drug targets obtained from protein arrays, mouse avatars, and other models are also applied [24]. Among them, the application of proteomic technologies to study cell signaling networks, functionally important protein phosphorylation, and expression changes might play a key role in the discovery of both new biomarkers and new therapeutic targets [32].

Nevertheless, it should be stressed that regardless of the mechanisms of the therapies, occurring resistance is a near-universal phenomenon in patients with cancer.

It is considered that genome-based cancer therapeutic matching is limited by incomplete biological understanding of the relationship between phenotype and cancer genotype. Even more, resistance does not necessarily depend on the cancer cell genotype. Although cancer is primarily a genetic disease and genetic mutations are responsible for cancer initiation and progression, they are not necessarily responsible for the resistance to treatment and should not become the targets for therapy. In addition to more targeted agents, there is a great need for new approaches, more predictive model systems.

3. Cancer resistance

It is increasingly realized that cancer resistance occurs nearly in all patients regardless of the therapies used (targeted agents, chemotherapy or immunotherapy). Anticancer drug resistance is a complex process. The cause of tumor resistance could be both intrinsic and extrinsic and it develops through many different mechanisms.

Drug resistance can be categorized as primary (intrinsic) or secondary (acquired). Intrinsically resistant tumors do not respond to the therapy at all due to the resistance mutations which exist within cells prior to the treatment. Acquired resistance (*post-therapy resistance*) occurs due to selective propagation of pre-existing or de novo-induced molecular alterations after the anticancer therapy. Moreover, besides primary and acquired, authors name one more way for the resistance to arise. Adaptive resistance occurs due to cancer cell plasticity under the pressure of a drug, especially in the microenvironment which is throughout heterogenic. Further, acquired and adaptive resistance can also be subclassified as *on-target* or *off-target*.

Causative factors for cancer cell resistance are grouped into three categories: mutational events, non-mutational events, and changes in the surrounding microenvironment. Genetic and nongenetic resistance mechanisms can act alone or in concert, in order to confer resistance to the applied therapy [27, 33]. Genetically caused acquired resistance involves emergence of de novo mutations in cancer cells. These mutations allow escaping the oncogene-targeted therapies by many ways. *On-target* resistance (alteration of the driver oncogene) occurs when the primary target of the drug itself gets altered. In this case, resistance occurs via a second-site, or secondary, mutation in the drug target. For example, under first generation EGFR TKI treatment, appearance of EGFR-T790M mutation is known as a second-site mutation. *Off-target* resistance (activating mutations in other genes) occurs via activation of critical signaling molecules or pathways that are either parallel or downstream of the target. Upregulation of a distinct receptor tyrosine kinase is an example of such a bypass mechanism for TKI targeted therapy.

Downstream activation at the genetic level means that cancer cells can become resistant to targeted inhibitors by amplifying the target or acquiring activating mutations in signaling pathway genes downstream of the driver oncogene. In many cases, those are cell growth and survival-promoting pathways. An example of activation of downstream signaling is EGFR-driven tumors with resistant cells, which reactivate MAPK pathway at multiple downstream points by means of upregulated expression and/or enhanced activation of proteins in the EGFR/MAPK signaling pathway. Alternatively, activation of a new (parallel or surrogate) cell growth and survival-promoting signaling pathways beyond the oncogenic driver is another

escape mechanism for drug resistance. It can be exemplified that the inhibition of MAPK ERK signaling pathway molecules led to the activation of a parallel PI3K/AKT/mTOR signaling pathway in lung cancer [27]. In general, various and different types of drug resistances have been shown in cancer cells. They involve activation of pro-growth and/or pro-survival signaling, cell death inhibiting pathways, altering drug targets as well as drug metabolism, enhancing DNA repair. Additional mechanisms of resistance involve alteration in epigenetically regulated drug tolerance, gene expression, epithelial-to-mesenchymal transition (EMT), phenotypic transformation, etc. [28]. Interplay between different signaling pathways, e.g., to induce adaptive activation of bypass signaling, was shown during acquired resistance.

3.1 Tumor heterogeneity

Accumulating evidence suggests that intratumoral heterogeneity plays an important role in cancer resistance to applied therapy, both conventional and targeted [9, 10]. Tumors are composed of heterogeneous cancer cell populations, either phenotypically or genetically different, and are marked by spatial and temporal heterogeneity. Even after malignant transformation, cancer cell clones remain dynamic and continue accumulating mutations. This ongoing evolution ultimately generates distinct molecular signatures in different loci of a tumor. Intratumoral heterogeneity itself may be responsible for the evolution of cancers as well as for differential levels of sensitivity to treatment. Various drug-resistant clones with different properties appear within the tumor tissue both prior and after the cancer therapy. Importantly, cellular composition of a tumor can change substantially over the time [31, 33, 34]. In that respect, the combination of different approaches—one targeting pathways and alterations implicated in resistance of different tumor composing cells and the other directed to overcome heterogeneity—is a promising strategy in cancer treatment.

Causes of intratumoral heterogeneity may be intrinsic or extrinsic and genomics or phenotypic. Among intrinsic factors, genomic instability is critical for the development of intercellular genetic heterogeneity in cancer. Genomic instability along to cell-to-cell variation leads to acquired resistance. Extrinsic factors include pH, hypoxia, paracrine signaling, interactions with stromal and other tumor cells, surrounding matrix as well as other environmental influences. Genetic alterations might be caused by exogenous mutagenic factors (UV radiation, tobacco smoke) or endogenous defective processes such as errors during DNA replication, repair, etc. Meanwhile, phenotypic diversity can reflect differences in cell cycle stage, stochastic phenotypic alterations, or hierarchical position during development. Primary genotypic or phenotypic variations prior to therapy also describe intratumoral heterogeneity in many tumors. Therefore, temporal-spatial differences between distinctive subpopulations of tumor cells within the same tumor at both genetic and epigenetic levels cause tumor heterogeneity [34].

Various models have been proposed to explain intratumoral heterogeneity.

The clonal evolution model postulates that somatic mutations stochastically occur in various clones within the tumor at different sites and different disease stages, leading to differential growth patterns and resulting in spatial-temporal tumor heterogeneity. In clonal evolution model (linear or branched) genetic mutations gained by tumor cells contribute to their altered phenotype and differences in resistance. Those differences in the biological properties are inherited by individual cancer cells clones. In this way, genomic instability leads to cell-to-cell variation as well as acquired resistance.

In the model of stem cells, clonal expansion of slow cycling cells, displaying stem cell-like behavior (self-renewal and differentiation capability), leads to tumor

heterogeneity [34]. The role of cancer stem cells (CSC) in determining heterogeneity and resistance in cancer will be described below.

One more theory, the so-called phenotypic plasticity model, suggests that cancer cells are capable of switching between different transcriptional programs, and therefore, phenotypic and histological transformation occurs. In this model, unlike the clonal evolution and stem cell theories, reversible molecular changes result in tumor heterogeneity. Inconsistent drug responses between cancer cells can be a result of diversification of epigenomic, transcriptomic, proteomic, metabolic, and functional states driven by tumor cell microenvironment changes [8, 29, 35, 36]. One of the mechanisms of phenotypic change is via EMT, an evolutionarily conserved program of transdifferentiation. EMT is observed clinically in patients with acquired resistance.

Lastly, another mechanism of cancer cell evolution was described. Briefly, there are proofs that cancer cells fuse with fibroblastic or mesenchymal cells to produce genetic hybrids with enhanced malignant profile both in vivo and in vitro [37]. Taken together, all these models in concert explain all the attributes of tumor tissue heterogeneity as well as genetic cell-to-cell variation and acquired resistance. Indeed, the findings of multiple studies demonstrate that a combination of genetic, epigenetic, and functional mechanisms contributes to intratumoral heterogeneity with different levels of sensitivity to anticancer therapies. Moreover, heterogeneity is dynamically changing over time and in concert with changes in therapy [33]. Heterogeneity of cellular states in cancer has been linked to drug resistance, cancer progression, and the presence of cancer stem cells [9, 10].

3.2 Cancer stem cells

An important role in the development of cancer heterogeneity is attributed to CSCs. In this model, tumor heterogeneity is determined by CSCs existing at different levels of hierarchical tree and possessing different phenotypes. CSCs are present in almost all cancers as a small population of drug-resistant, tumor-initiating cells and make up less than 1% of the tumor cell population. Recent data show that during cancer progression, gradual loss of a differentiated phenotype and acquisition of progenitor and stem cell-like features are observed. Cells with dedifferentiated phenotype were mostly prominent in metastasis [38, 39].

Different factors are recognized as causes of CSC emergence. It can be cell fusion, horizontal gene transfer, and mutations in somatic or differentiated cancer cells, ultimately resulting in their dedifferentiation and reprogramming. Mutations leading to the aberrant regulation of a majority of stemness and proliferation pathways distinguish CSCs from non-CSCs residing within the tumor. In response to various factors such as wounding, stress, or hypoxia, a differentiated cancer cell can dedifferentiate into a CSC. Reversible transitions between CSCs and non-CSCs within the tumor contribute to intratumoral heterogeneity [40, 41].

Incidences of different oncogenic rearrangements in stem, progenitor, or mature cells can lead to the generation of CSCs too. Authors propose that breast cancer CSCs could have been generated from adult somatic cells, through acquired mutations responsible for uncontrolled reactivation of pluripotency-associated programs. Deregulated canonical and developmental signaling pathways are highly associated with CSC phenotype and regulation of CSC behavior. Alterations in proliferation, differentiation, and programmed cell death pathways have been demonstrated [42].

It is believed that CSCs are a quiescent, low-cycling cell population. Another specific property of CSCs is resistance to chemotherapeutic drugs. Self-renewal and cellular quiescence characterize both normal adult stem cells and CSCs; they also

can divide asymmetrically and are able to produce progenies of multilineage differentiation. CSCs are regulated by similar cell signaling pathways and express similar cell-surface and intracellular markers as normal stem cell. Growth factor TGF-β, signaling pathways FGFR/MAPK or Akt, Wnt, Notch, and Hh, and major transcription factors Oct3/4, SOX2, and Nanog are involved in maintaining self-renewal and pluripotency of CSCs as well as of embryonic stem cells.

Many markers have been associated with the CSC phenotype; however, no universal CSCs markers were found, suggesting the existence of heterogeneous CSC populations within a heterogeneous tumor [38]. Indeed, CSCs are both genetically and phenotypically heterogeneous. CSC plasticity is the major determinant of their intratumoral heterogeneity. Dysregulation of key effectors in the above-mentioned signaling pathways modulate CSC properties [34]. Recent research has emphasized extensive metabolic variability in CSCs—each distinct CSC population possessed a unique metabolic profile. Both intrinsic factors (genetic and epigenetic alterations) and extrinsic factors (mainly tumor microenvironment) were shown to be implicated in the regulation plasticity as well as in maintaining stemness. Signaling molecules of Wnt, Hh, and Notch pathways, along with metabolic regulators such as HIF1α, maintained CSC populations within tumors [34, 38]. Even more, increasing data show that cells with self-renewal potential can be generated from terminally differentiated somatic cells, thus reverting hierarchical developmental organization. For example, there is evidence that breast CSCs have originated from non-stem cancer cells [42]. Under the regulation of multiple factors, differentiated cancer cells could regain "stemness," in this way confirming bidirectional conversion between non-stem and stem cells [34]. In that case, transformation of "non-stem cell to stem cell" contributes to the development of the heterogeneity within a tumor.

Core signaling pathways that regulate the phenotype of normal stem cells are responsible for dedifferentiation and acquisition of stem cell features in a tumor when transcriptionally and epigenetically deregulated. There are data that CSCs are maintained in the undifferentiated state through extrinsically regulated epigenetic mechanisms [39]. Therefore, CSCs may be considered as a status rather than a fixed subset of cancer cells. Transformation between modes of non-stem cells to stem cells or vice versa contributes to the development of heterogeneity within tumor.

The studies of last decades have shown a link between cancer stem cell formation and the process of epithelial-mesenchymal transition. This connection might be responsible for tumor heterogeneity, progression, and acquired resistance to therapy, followed by disease recurrence [40, 41, 43]. Namely, epithelial cells undergoing EMT acquire CSC phenotype and chemoresistance. Moreover, these cells may go back and forth along this transition. As described in a breast cancer study, two distinct populations (epithelial-to-mesenchymal and mesenchymal-to-epithelial) of CSCs were found [44]. Current data indicate that CSCs are not fixed at full epithelial or full mesenchymal cell status, but maintain plasticity between EMT and MET states [42, 45]. Consequently, tumors are supposed to consist of continuous sequence of cell states along epithelial to mesenchymal positions [46]. Also, non-stem cancer cells were shown to regain stemness through an epithelial-to-mesenchymal transition-mediated process [38].

Processes of EMT are regulated in response to the environmental changes and involve canonical and developmental signaling pathways that induce the expression of transcription factors, referred as "master regulators" of EMT, as well as epigenetic and post-translational regulators. Those include members of the Snail and ZEB transcription factor families. Different signaling pathways, e.g., Wnt/β-catenin pathway or AKT/STAT3, contribute to EMT [47–49]. Although it is widely accepted to link stemness to the EMT, there is another view proposing that these processes

occur in parallel rather than through the same pathway [38]. Regardless, a wide spectrum of hybrid epithelial/mesenchymal (E/M) cellular states which combine epithelial and mesenchymal features are formed during epithelial-mesenchymal transition.

It is increasingly understood that cell plasticity in the tumor can affect treatment outcomes. EMT grants resistance to cell death induced by different agents, including conventional, targeted, and immunological, in cancer cells. Different phenotypes of cancer cells may confer changes to both sensitivity and intracellular regulation of cellular biology; different signaling may be acquired in response to one treatment. There are data about it in various cellular and preclinical models; however, more data from clinical cases are needed [50–54].

As discussed above, drug resistance is a specific property of a CSC. It involves various aspects of CSC functioning and is mediated by multiple mechanisms, such as: ATP-dependent ABC transporter-mediated drug efflux; inactivation of anticancer drugs; changing the targets of chemotherapeutic agents; slow cell cycle/quiescence; self-renewal property and stemness-regulating signaling; disrupted differentiation; metabolic modulation; antiapoptotic measures (upregulation of antiapoptotic and downregulation of proapoptotic genes); improved DNA damage response; epigenetic alterations (DNA methylation, histone alterations, microRNAs); immune escape mechanisms; etc. [34, 38].

Based on the abovenamed features and mechanisms of CSC resistance, different strategies to tackle CSC resistance were developed, mainly focusing on CSC elimination or induction of their differentiation. More than 60 CSC-targeted reagents have been registered for clinical trials (e.g., Reparixin, Demcizumab, Napabucasin, metformin, etc.) [55]. However, to our current knowledge, there are no drug discovery platforms that would include EMT reversal and overcoming its conferred resistance. Nevertheless, the results from TCGA project encourage stemness signatures were found, and certain inhibitors were proposed as potential drugs for various cancers [39].

3.3 Tumor microenvironment

Development of drug resistance in cancer, regardless of mechanism, is highly dependent on the tumor microenvironment [35]. Tumor microenvironment includes the cells composing tumor stroma (normal fibroblasts, cancer-associated fibroblasts (CAFs), vascular endothelial cells, infiltrating immune cells, etc.), soluble molecules, and the extracellular matrix (ECM). Other factors of tumor surroundings, such as availability of nutrients and oxygen, and biophysical properties of the extracellular matrix are also important factors in regulating tumor cell behavior. Cancer cell interaction with stroma cells, with other cancerous cells, and with ECM contribute to direct cell interaction-mediated drug resistance known as *tumor cell interaction-/adhesion-mediated resistance*. Probably the best-known example is the CAFs. Those specific cells are most abundant cells in the tumor stroma responsible for the buildup and remodeling of the tumor microenvironment. CAFs produce ECM components such as collagens (I, III, IV, and V), hyaluronic acid, fibronectins, and laminins; alternatively, they secrete matrix metalloproteinases. Cancer-associated fibroblasts possess high expression of α-SMA, CD44, HI-1α, MMP11, VEGF, CXCL12, TGF-β1, TGF-βRII, IL6, and TNFα biomarkers. In many studies, such cells were demonstrated to be involved in cancer progression and resistance to therapy [56–59]. As an example, it was demonstrated that functional and proliferating colon cancer stem cells that ensured tumor expansion predominantly resided at the tumor edge close to cancer-associated fibroblasts [60]. Furthermore, factors secreted by cancer cells and acting through autocrine mechanisms are involved in

protection of cancer cells against treatments; that is called *soluble factor-mediated drug resistance*. Various cancer cell-released molecules are known as contributors to chemoresistance; it can be growth factors, glycoproteins, inflammatory cytokines, enzymes, chaperones, tumor-derived exosomes, etc. [61]. As an example, the recent study highlighted that cancer-associated fibroblasts induced ovarian cancer cell chemoresistance in transmanner by secretion of CCL2/CCL5 and induction of IL-6 production [62]. Microenvironment-driven dynamic heterogeneity and phenotypic plasticity was recognized as mechanisms of therapy resistance in melanoma [35]. Hence, development of drug resistance in cancer, regardless of a mechanism, is highly dependent on the tumor microenvironment. Moreover, factors secreted by tumor cells themselves or by the cells of its microenvironment play a crucial role in the development of epithelial-mesenchymal transition at the invasive front of primary tumors. Paracrine and autocrine mechanisms of EMT induction in cancer are evidenced in literature and may include exosomes or free proteins and miRNAs [63]. Consequently, distinct subpopulations of cancer cells with distinctive biological features appear in the tumor and thus respond differently to the therapy.

The critical role of tumor microenvironment, which is composed of cellular and noncellular components in cancer development and progression, is evident. As EMT is considered to be one of the key factors in progression of tumors to malignancy [64, 65], the role of ECM in this process was demonstrated. ECM composition was partially responsible for insufficient therapy and appearance of heterogenic cell populations, which were characterized by different response/susceptibility to anticancer agent [66]. Dynamic nature of ECM regulates behavior of cancer cells by triggering signaling pathways through adhesion proteins like integrins [67]. Collagen I, which is the major component of extracellular matrix, has been shown to support phenotypic changes of cancer cells [68]. Studies conducted on 3D tumor-ECM in vitro cancer models demonstrated importance of ECM composition and stiffness on this transition regulation [69, 70]. On the other hand, noncellular components of ECM, e.g., proteins, glycoproteins, and proteoglycans, form a physical barrier for penetrating drug. Numerous cell-cell and cell-ECM contacts attenuate penetration of drug molecules into different layers of tumor [71, 72].

4. Models of cancer treatment prediction

Today, a lot of conferences addressed to advancing drug discovery, cancer pharmacology, and tumor modeling are held worldwide, e.g., Predict Tumor Models (London, UK, 2017; Boston, MA, 2019), 3D-Oncology (Boston, MA, 2019), EACR Goodbye Flat Biology (Berlin, DE, 2019), and SelectBio 3D-Culture and Organoids/Organ-on-a-Chip World Congress (San Diego, CA, 2019). Realization that new approaches in oncology are necessary to improve cancer treatment is increasing. The topics in such conferences include: How to satisfy the demand for clinically relevant models for understanding disease progression; Multicellular interactions and the immune microenvironment; Advancing 3D-based models for cancer biology and drug discovery; Influence of microenvironment on stemness; Microenvironment and metabolic regulation of cancer invasion; Highlighting the pitfalls of complex approaches with regards to cost, speed and accuracy; The true utility and translatability of 3D tumor models within drug research; etc. Likewise, big pharma companies and small start-ups are offering nonconventional, patient-specific and often patent-protected cancer models, desirably reflecting the complex nature of tumor microenvironment and cell heterogeneity. For example, Champions Personalized TumorGrafts™ have offered patients and physicians using

their in vivo xenograft mouse avatar-based diagnostic model at a cost of approx. $10,000 [73]. The demand for cancer models that reliably mimic the intrinsic ecosystem of a solid tumor in vivo is really high. However, to create and replicate a system consisting of malignant cells, normal and abnormal stroma, immune cells, as well as dynamic microenvironment containing gasses and nutrients, chemokines, cytokines, growth factors, together with specific extracellular matrix, is totally challenging [74, 75]. In addition, clinical application of such models may become reality only if the patient benefit justifies the cost of the methodology.

The use of reliable in vitro cell model, which precisely reflects the situation in vivo is critically important during preclinical drug studies. Due to the high mortality from cancer, the search of new and more effective anticancer agents still remains a highly challenging area of biomedical research. The principle of targeted cancer therapy is based on biochemical differences between cancer and healthy cells. In regard to low efficacy of drugs passed through Phase II and III of clinical studies and acquired resistance of tumor cells to therapy, more detailed studies of tumor structure, histology, and biology are required. These fields remain very important since a choice of inadequate in vitro drug screening system further results in low efficacy or high toxicity of anticancer agent during clinical studies [76, 77]. Advances made in cancer research in the last decades demonstrate critical role of tumor microenvironment in cancer development, progression, and response to therapy. According to different studies, potent anticancer drugs that demonstrated high efficacy in 2D cancer cell cultures had significantly reduced effectiveness during further studies in vivo [78]. In human body, tumor represents a highly complex architecture that is difficult to replicate in vitro. First of all, it is highly heterogenic multilayer structure, formed by different type of cells, e.g., cancer cells, cancer-associated fibroblasts, tumor infiltrated endothelial cells, and cells of immune system. Cellular components of tumor regulate different features of cancer cells such as proliferation, invasiveness, and susceptibility to drugs [79]. Tumor represents a mixture of genetically, epigenetically, and phenotypically different populations of transformed cells. Elimination of all viable cancer cells which are in different status makes it very challenging to target particular molecular drivers of cancer. It is known as well that cell heterogeneity is increasing with disease progression. Thus, even modern targeted therapy becomes insufficient in some cases of cancer [80–82].

Failure to advance in cancer therapy has been attributed to the complexity of the disease. A solid tumor is often called “an organ” but very heterogenic and of irregular structure with many niches and different conditions inside. Today, there is a gap between scientific understanding of tumor complexity and concrete measures to fight cancer in clinics. This gap is constantly being filled with preclinical cancer models, but up to date, no model, at the same time, is fast, accurate, affordable, and suitable for clinics.

One of the hypotheses to explain cancer resistance to therapy is cancer stem cells, or cancer-initiating cells. Although they are more recognized as a phenotypic status or mode rather than a fixed subset of cancer cells, various authors distinguish certain populations of cells within a tumor that propagate cancer [83–87]. The main trick regarding those cells is that when they are asleep, they evade chemotherapy; when they wake up, they cause cancer recurrence. Killing those cells selectively is a priority [9]. Other authors propose that cancer genome undergoes clonal evolution under the pressure of constant stress and after cancer therapy. One study compared the genomes of 100 early stage resected lung tumors and found driver mutations in EGFR, MET, BRAF, and TP53 are almost always clonal; moreover, cells from different parts of the same tumor had very heterogenic genomes [88]. Accordingly, every single case of cancer is very individual and may be genetically and in other

ways substantially different. Noncellular components of tumor environment such as secreted grow factors, RNA and DNA molecules, metabolites, and extracellular matrix proteins regulate various biological functions of cancer cells [71]. Therefore, commonly used purified cancer cell lines in vitro do not possess the heterogeneity, nor genetic nor phenotypic, and thus may represent only a small fraction of this multiplexed pathology [89]. Even when heterogenic and in an enriched medium, 2D cell cultures lack the architecture and complexity of a real tumor and thus are not suitable for cancer biology and immunology studies [90]. However, ordinary cancer cell cultures on flat plastic are successfully used in fundamental research.

Trying to describe cancer cells in molecular signature led to the emergence of "biomarkers"—certain molecules that are specific to cancer cells. Although this goal is not ultimately achieved yet, many cancer biomarkers are used in cell profiling, cancer research, and targeted drug development. For example, in a search for cancer stem cells in lung cancer, CD44, CD133, and CD90 had been considered as potential biomarkers, but CD44 showed the best specificity in one study [91], and CD133+CXCR4 in another study [92]. In pancreatic cancer, co-expression of CD24, CD44, EpCAM, and CD133 corresponded to more aggressive cell behavior [93]. As we have described above, an initiative called The Cancer Genome Atlas (TCGA) has generated comprehensive, multidimensional maps of key genomic changes in 33 types of cancer in order to investigate somatic driver mutations, germline pathogenic variants, and their interactions in the tumor, the influence of the tumor genome and epigenome on transcriptome and proteome, and the relationship between tumor and the microenvironment, including implications of prevention and treatment [22]. On its basis, other authors have performed analysis by using 26 computational tools and identified 299 driver genes [20] as well as alterations in signal transduction [21]. It is worth mentioning that DNA sequencing is becoming part of routine clinical care that may result in rapid and high-throughput analysis of complex germline and somatic alterations from clinical cancer patient samples [19].

Probably more complex and more ambitious project has been started in 2017—The Human Cell Atlas. Such atlas is believed not only to append new cell types of a human body to the known ones but also to help scientists to track cell lineage, dynamic states, and stemness of cells as well as communication between cells. This database may include not only genomic and transcriptomic data from a single cell, but also profiling the accessibility of the chromatin [94]. It is really important to investigate cells one by one for many reasons when talking about cancer. For example, conversion to stem-cell state in response to microenvironmental cues was regulated by balance between epithelial and mesenchymal regulators (described by expression of CDH1 and SNAI2, respectively) in lung cancer cells [95].

4.1 In vivo models

Human tumors can be grafted into immunodeficient mice to generate so-called patient-derived xenograft (PDX) mice. PDX is a powerful tool aiming to test multiple drugs or drug regimens. It is considered as a preferred option in cancer research as it mimics the native features of tumors more closely compared to conventional cell culture drug screening platforms. With its drawbacks as a system, it is still important, widely used, and remains a gold standard for preclinical development and individual drug discovery.

Different PDX repositories exist that represent models of various tumors. Conte and colleagues try to accumulate the data into one comprehensive open global catalog of PDX models and their associated datasets that would ease the identification of PDX models relevant to specific cancer research questions [96]. The standard was also developed to unify generation, quality assurance, and use of PDX models,

because the processes of creation and characterization of PDX models considerably differed among institutions [97].

Beyond PDX mouse model libraries, now patients can be offered individualized mice, which is a platform used for the measurement of efficacy of experimental treatments exploiting patient's own malignant cells. The aim is to have a model with experimental data as close as possible to human disease situation. Individual approach is vital here as cancer is the most genetically diverse and unique disease with different genetic alterations existing between individual patients, individual tumors, and even within a single tumor.

In PDX model, a patient tumor specimen is directly transplanted into immunocompromised mice. This allows representing critical molecular and biological properties of the original tumor, and immunodeficient mice prevent graft rejection. Several types of such mice are used for PDX generation. Currently, the most commonly used types of immunodeficient mice are nude, severe combined immunodeficiency (SCID), and nonobese diabetic (NOD) SCID γ (NSG). The latter is the most severely immunodeficient due to the lack of T, B, and NK cells [98]. NSG engrafting success rate is higher compared with that of in nude and NOD/SCID mice; therefore, NSG mice are considered the most suitable ones for the efficient engraftment of human tumor.

Tumor tissue fragments or single-cell suspension is used for PDX establishment, and both have their advantages and disadvantages. Tumor fragments preserve cell-cell interactions and tissue architecture of the tumor, and all this mirrors the tumor microenvironment. However, a fragment does not represent the whole tumor, unlike single-cell suspension that helps to avoid tumor subclones. One can distinguish an additional PDX model that also recapitulates the molecular heterogeneity of the corresponding tumor and can be used to study cancer biology—circulating tumor cells (CTCs). These are released from cancerous lesion into peripheral blood and may infiltrate distant tissues inducing metastasis. CTCs represent a method for liquid biopsy which is used to generate various CTC-derived preclinical models.

Tumor fragment can be implanted heterotopically, that is, into the area unrelated to the original tumor site (usually subcutaneously). A major disadvantage of heterotopic implantation is that microenvironment of the subcutaneous space differs greatly from that of the original organ. Thus, orthotopic transplant models have been developed in which tumor fragment is directly implanted into the corresponding anatomical organ. Orthotopic PDX is considered an exemplary one as unlike subcutaneous model it mimics primary tumor's natural environment that enables relevant tissue-related gene expression. In some cases, it reflects responses to therapy more closely than heterotopic PDX. Consequently, orthotopic models may be better predictors of therapeutic response [99]. Besides, subcutaneous model rarely produce metastasis [100], while orthotopic PDX is more likely to metastasize [101]. However, orthotopic PDX generation is technically demanding and tumor changes have to be monitored via expensive and laborious imaging process. Therefore, subcutaneous models are currently preferred for preclinical studies.

Tumor engraftment is a critical milestone in PDX generation. Time from tumor sample implantation to progressive growth may range greatly. Authors provide different timeframes for PDX generation or tumor graft latency ranging from 2 to 12 months [102–104]. The differences in time and engraftment rate are determined by tumor characteristics, like aggressiveness, histological type, and tumor cell percentage in the tissue [105]. Prasetyanti and colleagues showed that tumor cell proliferation was associated with successful PDX establishment and as one would expect this allowed to distinguish patients with poor clinical outcomes [106]. The others report the ability of NSCLC to grow as xenograft in mice as an accurate indicator of poor prognosis for patient survival [107, 108]. Similar data were received

with breast cancer [109] and head and neck squamous cell carcinoma (HNSCC) models [110]. Here, authors claimed that the clinical application of rapid engraftment as a biomarker for risk stratification could potentially improve the outcome of patients. Despite the wide array of PDXs generated for different tumors, some cancers, e.g., prostate, luminal ER+ breast, and neuroendocrine, remain underrepresented [111].

Tumor implant location is another important factor for successful engraftment. It was shown that tumors had higher success rates grafted in subrenal capsular sites [107, 112] due to better blood supply. The strain of immunodeficient mice used for engraftment also plays its role. Not surprisingly, engrafting success rate in NSG mice is increased compared to other strains.

After harvesting the tumor, it can be cryopreserved, characterized, or dissected again for reimplantation and propagation into other mice. This allows to expand PDX from one biopsy into multiple mice and to receive numerous data points from the same original tumor.

PDX retains tumor architecture and microenvironment with physiological conditions reflecting original tumor. Grafted tumor tissue maintains the genetic and epigenetic changes found in the patient, and also it retains patient's stroma [113, 114]. PDX mice models can preserve tumor heterogeneity at least in early passages. They retain heterogenous molecular pathways of drug resistance that exist in tumor or in the cells comprising tumor environment [115]. As these models retain the biological characteristics of the donor tumors, they become indispensable for drug safety and efficacy research in preclinical studies as well as in co-clinical trials. These models are also exploited to examine personalized treatment strategies—response to antitumor therapeutics and identification of the mechanisms of resistance that can be primary or acquired [116–118]. PDXs may represent intratumoral and intrametastatic heterogeneity and, therefore, more accurately predict mechanisms of resistance to clinical treatments [119].

Mice PDX models from various kinds of tumors have been established to study tumor biology, drug screening, and biomarker discovery. Authors report PDX generated for nonsmall cell lung carcinoma closely mimics the characteristics of patient primary tumors [120, 121], lung squamous cell carcinoma [122], and colorectal cancer [123]. Data are also available for renal cell carcinoma [124], melanoma [125], gastric [126], breast [127], and other cancers.

Data show that PDXs have a predictive power in clinical therapy outcomes since drug activity in PDXs phenomenally correlates with patient's clinical outcome. In 112 out of 129 therapeutic outcomes, a remarkable correlation was observed between drug responses in patients and the corresponding PDX models of various solid tumors. Data showed that PDXs reproduced patients' clinical outcomes, even in cases when patients underwent several additional cycles of therapy over the time. This indicates the potential of these models to provide the guidance on treatments [128]. More data with PDX models of breast cancer and lung adenocarcinoma origin confirmed high rate of consistency in patient and relevant PDXs [129, 130]. In addition to that, PDX models of colorectal cancers treated with EGFR inhibitor showed response rates like those of the patient [131]. Gu et al. evaluated the correlations between PDX model-based mouse trials and cancer patient-based clinical trials. Results disclosed a high correlation in the rates of RECIST criteria between mouse trials and Phase II and III studies [132]. Research with pancreatic cancer PDX revealed that metformin treatment did not inhibit the growth of pancreatic cancer; clinical trial echoed PDX data—there was no benefit of adding metformin to combination therapy [133]. Eventually, in renal cell cancer model, correlation was noticed between PDX and clinical results in responses to Sirolimus, Sunitinib, and Dovitinib, but not Erlotinib [134].

Correlation between PDX models and clinical trials also allowed discovery of biomarkers for drug susceptibility and resistance. Vemurafenib-resistant melanoma model was generated using PDX. Resistant tumors showed reliance on BRAF signaling due to the elevated BRAF (V600E) expression [135]. Accordingly, elevated BRAF expression could be a potential biomarker for Vemurafenib resistance. The other data showed relationship between Gemcitabine resistance and survival of pancreatic cells with deoxycytidine kinase activity [136].

Co-clinical trials are parallel studies with mouse PDX models and patients. PDX model is established from the patient participating in a study, and the same treatment is applied to patient and PDX [116]. On the other hand, PDX model can be treated not only with the same drug that is used in the donor patient, but also with other drugs or drug combinations. This concept integrates preclinical and clinical data and facilitates the selection of an individual treatment to the patient. In addition, screening for prognostic biomarkers can be conducted, and drug response as well as resistance mechanisms can be investigated [137]. Usually, with the progression of cancer, the drug becomes less effective partially due to appearance of resistance mechanisms. During a clinical study, it is not possible to reveal timing and mechanism of resistance. Here, PDXs are also of use allowing to predict both the development of resistance to the first-line therapy as well as the response to second-line therapy before this is observed in the donor patient, and to adjust further treatment accordingly [138]. Authors report correlation between clinical results and PDX models. Kim et al. conducted co-clinical trial to identify predictive biomarkers for the multikinase inhibitor Dovitinib in lung squamous cell carcinoma (LSCC) and revealed that FGFR gene expression signatures were predictors for the response to Dovitinib in LSCC [139]. Stebbing et al. provided data that supported the use of the personalized TumorGraft model as an investigational platform for therapeutic decision-making that could guide treatment for rare tumors such as sarcomas [140]. Others showed that PDXs reliably replicated clinical outcome in a Phase II co-clinical study of arsenic trioxide in relapsed small-cell lung cancer [141].

Currently, there are no much data, however co-clinical trials should find their place in the future as they give possibility to evaluate the efficacies of several drugs or drug combinations, save time, and reflect personalized medicine. However, while aforementioned advancements have been made in PDX applications, these models still retain some important limitations that should be noted.

Several practical challenges are related to PDX models. First of all, it is important to transplant the representative tissue, which is a matter of concern when it comes to larger tumors. Those tumors that have genetic heterogeneity cannot always be recapitulated in serial passages if the genetic heterogeneity is not all represented in the dissected tumor that is passaged [142]. Also, the best transplantation strategy should be chosen for specific tumor type. Orthotopic transplantation is considered more suitable; however, it is more complex and special surgical technique is necessary. Consequently, more time and labor are required for model establishment. Engraftment failures are also to be discussed as percentage is still low in some tumor types. It is known that clinically aggressive tumors with many proliferative cancer cells have the highest engraftment rate [143]. Studies in which PDX models had been created simultaneously from primary and metastatic tumors proposed that metastases had a higher engraftment rate [144].

The other limitation of PDX system is the substitution of human stroma with murine components that occurs over the time with repeated passages. Human stromal elements are maintained for only 2–3 passages [111]. Later, the interaction of human cells with the murine microenvironment changes cell functioning and characteristics due to interspecies incompatibility, and the heterogeneity of exact human tumor microenvironment might not be retained. Therefore, therapeutic

studies are more representative in low-passage models. To reduce the influence of murine stroma on the PDX model, co-engraftment of human mesenchymal stem cells or cancer-associated fibroblast cell lines in PDXs is explored. One promising approach is the isolation and co-engraftment of patient-derived fibroblasts [145].

How cancer emerges and develops depends on its interaction with the host immune system. In addition, cancer response to therapy is also determined by pre-existing immune phenotype; it also depends on immune system responses caused by drug introduction [146]. Lately, several cancer immunotherapies have been evaluated in preclinical and clinical studies. However, standard PDX models were inconvenient for the analysis of tumor immunology and immuno-oncology drugs (e.g., immune checkpoint inhibitors) as the immunodeficient mice had no cytotoxic T cells. To overcome this problem, humanized PDX models have been developed using injection of peripheral blood lymphocytes or tumor-infiltrating lymphocytes into NSG mice [138]. But this did not resolve the issue in general, as after several weeks severe graft-versus-host reaction appeared [147]. Therefore, such mice can be used only for short-term experiments. Other model system exploited transplantation of CD34-positive human hematopoietic stem cells into NSG mice and demonstrated the establishment of a humanized PDX model with hematopoietic restoration [148]. In addition, Morton and colleagues demonstrated that CD34-positive cells isolated from patient and injected into mouse blood successfully restored a functional immune system mimicking that of the patient [149]. Such PDX are invaluable for cancer immunology and immuno-oncology drug research. Nevertheless, the time required generating enough tumor material in sufficient number of mice, as well as patient survival, is of utmost importance for personalized medicine application. Time is the main obstacle impeding the extensive use of mouse PDX models, because right drug for the right patient must be given at a right time. Development of an individualized PDX takes anywhere from 3 to 6 or even 12 months, and in many instances, this time is too long, as in the case of metastases, patients may not even survive PDX creation time. Therefore, this method is restricted to patients with a less aggressive disease development.

In addition to that, long time needed to generate sufficient number of mice with the same tumor brings another problem—clonogenicity. Research showed that all PDX models studied experienced moderate drifts or even dramatic clonal selections even within the first mouse passage [150]. Due to selection of preexisting minor clones, authors observed rapid accumulation of copy number alterations (CNAs) during PDX passaging. Particular CNAs acquired during PDX passaging differed from those obtained during tumor development in patients. Some CNAs observed in primary tumors later disappeared in PDXs [151]. The results showed that PDXs were not static and underwent mouse-specific tumor development: PDX that originally had mirrored particular disease after some time changed into the form that did not anymore.

Financial aspects are also to be assessed. PDX models are costly as it takes much time to create PDX, while immunodeficient mice in addition are expensive. Furthermore, PDXs may have limited use in diagnostics due to their low-throughput character: they are restricted to test a few agents due to insufficient number of animal colonies with individual patient's tumor. However, it is still important for drug validation, biomarker development, and drug resistance analysis.

Other animal models exist; however, those may not meet the requirements for personalized medicine and will not be reviewed in detail here. Among others, cell line-derived xenografts did not reflect the complexity of tumor heterogeneity and displayed pronounce genomic differences compared to relevant tumor [152, 153]. Genetically engineered mouse models (GEMMs) carry mutations in genes of interest; however, generation of mutant mice carrying several genetic

alterations is time-consuming, and complex activation of genetic alterations reduces the attractiveness of the method [154]. Besides, GEMMs may not be able to mirror the personalized therapy as they have less heterogeneity due to only a few genes modified.

4.2 2D in vitro models

Many reviewers have argued that conventional cancer cell lines in a usual 2D culture were an oversimplified model to study cancer. Various studies have demonstrated that both molecular and functional parameters were different depending on cell culture conditions, including substrate dimensionality as well as the method by which it was created, leading to inconsistent data [155]. Alternatively, primary cell-derived 3D spheroids demonstrated different response to therapeutic drugs when compared to spheroids from long ago established breast cancer cell lines [156]. Growing body of evidence demonstrated that cancer cells growing in 3D or 2D environment possessed different susceptibility to anticancer treatment, showing either increased or decreased sensitivity to apoptosis-inducing agents [157, 158]. Therefore, the natural question which models for cancer research should be abandoned is very relevant [159]. Regarding cancer cell lines, in general, it is well known that they are unstable and possess genetic drift. Long-term cultivation causes other problems resulting in poor reproducibility of the results [160–162]. For example, a widely used glioblastoma cell line U87MG was found to be completely different from the original donor, as DNA profiling revealed [163], many other cell lines were misidentified or contaminated [164]. Due to such obstacles, cell line stability and bioproduction are the headache for pharmaceutical companies which try to comply with strict regulations of healthcare manufacturing.

Nevertheless, these cell lines proved to be useful in basic and fundamental molecular biology research, e.g., in studying molecular action mechanisms of anticancer drugs, as reviewed many times elsewhere. Easy handling, homogeneous character, limitless growth, and high-throughput made it the model of choice for many years. After a lot of criticism from more advanced cancer modeling experts, surprisingly, they suddenly appeared to be equally valuable for translational cancer research. For example, in a large-scale study summarizing data from the analysis of 1000 cancer cell lines, their response to 265 anticancer drugs, and 11,000 patient samples of 29 different tumor types, authors concluded that a majority of molecular abnormalities found in tumors including driver mutations were also found in cancer cell lines. Furthermore, the results suggested that cell lines could predict drug response of a tumor [12]. Earlier, by application of computational methods, cell line gene expression profiling and annealing with known pharmacological profiles of anticancer drugs successfully predicted unknown drug sensitivities [165]. Such methodology may be compared to target-based drug discovery approach. However, an alternative approach—agnostic phenotypic screening—has resulted in the discovery of majority of first-in-line drugs, and authors noticed the resurgence of sophisticated, high-content phenotypic screening. By testing the unlimited number of ex vivo cancer cell lines with large libraries of chemicals or biologicals, there is a chance of having a significant hit [166, 167]. The NCI-60 panel was the first widely used cancer cell line panel in drug discovery. By demand, their genomes have been sequenced in 2012 [168]. Today, there is a Human Glioblastoma Cell Culture (HGCC) resource that consists of a biobank of 48 cell lines representing all molecular subtypes of the disease, with an associated database containing high-resolution molecular data [169]. Similar work was done with 100 ovarian cancer cell lines to make an OCCP panel where the cells were described [170]. Cancer Cell Line Encyclopedia (CCLE) and the Cancer Genome Project (CGP) have served

well in identifying novel biomarkers in drug response. Recently, large collaboration efforts expanded the characterizations of CCLE to include genetic, RNA splicing, DNA methylation, histone H3 modification, microRNA expression, and reverse phase protein array data. These data were collected for 1072 cell lines of various lineages from individuals of different ethnicities to improve the understanding of the molecular features that contribute to cancer phenotypes, including drug responses. Computational integration of these data with functional characterization such as drug sensitivity, short hairpin RNA knockdown, and CRISPR-Cas9 knockout data revealed potential targets and associated biomarkers for further analysis. This information is publicly available [171]. Similarly, immortalized cell line models such as EBV-transformed lymphoblastoid cell lines (LCLs) from the International HapMap Project, Human Variation Panel, and 1000 Genomes Project with multiomics data became available for fundamental cancer research [172].

Various specific cell lines are created for scientific research. HAP1 is a semi-haploid human adherent cell line derived from the male chronic myelogenous leukemia cell line KBM-7, with a single copy of almost every human chromosome. These cells are easy to culture, and authors assert that such cell line is an invaluable tool for gene functional testing and drug discovery by facilitating gene-editing techniques [173]. The well-known resources of cell lines for research and commercial applications include ATCC, JCRB, and other cell culture biobanks with many species covered. The European Collection of Cell Cultures ECACC (UK) offers more than 1500 human iPSC lines, 450 monoclonal antibody-producing clones, and many normal tissue and cancer cell lines for disease modeling or bioproduction [174].

The first attempt to solve the disadvantage of cell line "purity" as well as personalization of the treatment approach was the introduction of primary ex vivo cancer cell lines into the cancer research, including drug testing, discovery and therapy response prediction, with improving methodologies and optimizations for cell culturing [175–178]. Heterogeneity found in cell cultures was suggested to be coded by biological mechanisms from the primary tumors, and it was acknowledged as an instrument for clinical implications. Some authors denied the global inadequacy of translating pharmacogenomic data from 2D to clinical settings suggesting that drug resistance was mainly intrinsic and did not depend on cell culturing conditions [179]. However, this question is controversial, as other authors demonstrated that DNA methylation profile rapidly changed when cells had been plated in vitro, namely there was a global loss of 5hmC modification in vitro [180]. In addition, there are also challenges at cellular level. In many reports, ex vivo cancer cell line generation often was not 100% effective, meaning that obtained cells did not proliferate sufficiently or at all. Too long time frame from biopsy to sufficient cell number (2–6 months in lung cancer) [7, 15] is not applicable in clinics as well as there is a risk to select only the fast-growing cells, the subclones that do not resemble the original tumor; moreover, in many cases, patient survival may not be that long. Nevertheless, authors suggested that drug screening may be done using fresh cells, or primary cultures, within days while still in the presence of stroma cells [73]. Additionally, the issue of cell line generation and expansion could be solved by advances in culture medium composition, cell isolation, passaging, and associated techniques, or by means of assisted cell expansion described in [181] which is favorable in the aspect that it also worked with normal cells from the same tissue. Briefly, they used human mammary and prostate epithelial cells from fresh and even frozen biospecimen using an irradiated feeder cell layer and a Rho kinase inhibitor Y-27632 in the culture medium and expanded the culture to 1 million cells in 7 days. In this way, both drug screening and toxicity studies can be carried out for the patient simultaneously in several weeks. Alternatively, recent advance in cell imaging (next-generation

phenotypic screening) may enable fast, miniaturized, and physiologically relevant analysis of fresh ex vivo cells in suspension within hours after sample acquisition (e.g., IntelliCyt iQue Screener). For adherent cells, various automated, high-throughput imaging platforms, such as Cellinsight, ImageXpress Micro, Celldicoverer, MACSima, IncuCyte, etc., are available.

Additional pro for ex vivo cell cultures is the evidence of cancer stem-like cells in such cultures [91, 182, 183] and similar molecular profiles to those of in vivo cancer cells [184]. The latter study suggested that only several existing breast cancer cell lines, namely BT483, T47D and MDAMB453, had satisfactory similarity to molecular pattern of tumors. Another report supplemented the study by stating that primary ovarian cancer cells also differed from the established cell lines in tumor-associated antigen expression (namely BIRC5, CA125, CEA, DDX43, EPCAM, FOLR1, Her-2/neu, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MUC-1, NY-ESO-1, PRAME, p53, TPBG, TRT, and WT1 mRNAs), but one cell line—OVCAR-3—was quite representative [185]. On the other hand, there is evidence that even in common commercial cancer cell lines established long time ago and being deposited in biobanks such as ATCC, putative cancer stem cells are present [186].

Up to this paragraph, the cell line model has been discussed. However, cells growing in monolayer lack typical interactions that are present in tumors. It was shown that cell cultivation in 2D and 3D cultures results in different cell morphology, intracellular signaling, proliferation rate, differentiation capacity, and thus different response to treatment. For example, PDGFR activity was significantly lower in three sarcoma cell lines grown in 2D than in spheroids; in addition, stem cell markers Nanog, Oct4, and Slug as well as EMT proteins Snail, Slug, and Zeb1 were significantly higher in spheroids [187]. Another study showed that different cytotoxic compounds retained their ranking both in 2D and 3D cultures; however, all the IC50 values were smaller in 3D spheroids [188]. As described by an example of anti-HER2 targeted drug trastuzumab in a beautiful review by Sokolova et al., HER2 formed heterodimers with HER3 in a monolayer culture, but in spheroids, HER2 homodimers prevailed; therefore, a greater antiproliferative effect of this antibody against spheroids in comparison to a monolayer was observed. Importantly, HER2/HER2 receptor led to activation of MAPK signal transduction pathway, whereas HER2/HER3 receptor activated alternative PI3K pathway. Another effect of dimensionality was changes in expression and the basal phosphorylation level of cell-surface receptors. It could be either higher or lower in 3D than in 2D [189]. Similarly, remarkable differences were observed in pancreatic cancer cell lines HPAF-II, HPAC and PL45 when cultivated on 2D or 3D [190]. 3D hepatocyte spheroids (named as human liver microtissues) have demonstrated better prediction of liver toxicity than two-dimensional primary human hepatocytes model [191]. Similarly, large-scale pharmacological profiling of PANC1 human pancreatic cancer and SN12C human kidney carcinoma cell lines in both 2D and 3D was performed, analyzing a collection of 1912 chemotherapeutic agents. Interestingly, comparison of pharmacological responses of cells cultured in traditional 2D monolayer conditions as well as responses obtained from cells forming spheres versus cells already in 3D spheres enabled the identification of those key signaling pathways and cellular processes that when modulated by drugs were able to reduce cancer cell viability in all growth conditions or, to our particular interest, selectively in the different cell growth modes [192]. Thus, 3D culture systems comparing to 2D cultures have more advantages for using them for drug investigation studies. Some of the disadvantages of 2D models were reduced by introducing 3D substrates, scaffolds, and extracellular matrix components, including well-known Matrigel. Such models may be placed between 2D and 3D, sometimes called

2.5D. Consequently, commercial cell lines or cells isolated from donor tissue can be used in cancer research.

Dimensionality alters cellular surface area for a drug to penetrate. On a porous scaffold, surface area of a cell may increase; however, in a compact spheroid, surface area may decrease when compared to that of a planar 2D adherent cell. One study described generation and properties of multicellular tumor spheroids from different pancreatic cancer cell lines. Briefly, large and dense spheroids represented type I, lose and of different sizes—type II, and floating monolayer films—type III. The three types displayed different expression of adhesion molecules E-cadherin, DSC, and DSG proteins; in addition, type I spheroids had higher resistance to doxorubicin and gemcitabine [155]. More importantly, cell-cell and cell-surface contacts generate intracellular signaling that has been reviewed elsewhere.

Despite the abovementioned in defense of cell cultures, other authors argue that in principle the approach is defective in one additional aspect—enzymatic digestion of a solid tissue/tumor. This procedure is almost absolutely necessary in generating cell cultures. However, the proteolytic enzymes (trypsin, collagenase, dispase, accutase, etc.) cleave both proteins of the extracellular matrix and cell-surface receptors. By doing that, digestion irreversibly changes cellular signaling [193]. However, comparison of different cell-dissociating agents for stem cell isolation from glioma tumorspheres revealed that there was a difference in CD133 antigen retention: the worst result was obtained for nonenzymatic dissociation solution NECDS, while the minimal impact was observed in Libertase-TL-treated cells [194]. On the other hand, disruption of cell-cell contacts during the initial steps of cell culture establishment favors the outgrowth of specific cells, the composition of which do not recapitulate the original heterogeneity of a tumor [177]. Therefore, organotypic tumor slices without application of proteolysis would be the best model for drug selection in the individualized cancer treatment, and it will be discussed here below. In addition, tissue slice generation time can be fast enough to receive the results in days.

4.3 3D in vitro models

Phenotypic screening-based drug discovery should rely on 3D models because monolayer culture cell concentration is strikingly low (10^5–10^6 in mL) when compared to in vivo tissue (40–50 million cells in a mL) [195]. However, this recommendation is not attributable to leukemia cell cultures where physiologically relevant concentration of the cells can be obtained in vitro for the research. Advances in cancer modeling were nicely reviewed in publications [25, 30, 89, 145, 177, 189, 196, 197]. In our previous publication [198], 3D drug testing models with end-point viability-measuring methods were presented, including scaffold-based 3D culture systems, tissue slices, cellular spheroids, organoids, and organs-on-chips. Having both models for normal tissues and a tumor from a single patient would improve the prediction of systemic toxicities to healthy stem cells. As covered by other authors, self-organized and stem cell-derived three-dimensional human organoids give promise to be applied in disease modeling, drug screening, regeneration therapy, and even for host-microbe interaction studies [199]. These models can be patient-specific and thus are suitable for personalized medicine. When generated from cancer patient cells, such organoids have the value in anticancer drug screening [200]. More frequently used 3D culture models of cancer include: tumor mono-cell spheroids, hetero-spheroids (tumoroids), tumor tissue explants, and tumor-on-a-chip. 3D printing, to our opinion, is more relevant to nongenetic pathologies where normal organ-like structures have to be replicated. Tumor, as known, is of

irregular, chaotic structure; thus, there is nothing to reconstruct with a precision of a 3D printer. One more three-dimensional approach is the application of decellularized cadaver organs. For example, rat lungs have been used to determine human adenocarcinoma cell lines H358, PC9, and SW1573 proliferation on the scaffold, cell viability over the culture period and in response to treatment with cisplatin or Erlotinib. A resazurin-based reagent was perfused through the scaffold to evaluate cell number [201].

Multicellular tumor spheroids represent aggregates of cells formed in conditions where cell-cell interactions predominate over cell-substrate interactions. Spheroids closely resemble avascular primary tumors and metastases with respect to their architecture, gradient of oxygen, nutrients and metabolites distribution, distinct proliferation rates as well as microenvironment and drug penetration [202, 203]. One-cell-type spheroids are cellular aggregate formed by one type of cells, in this case cancer cells. In principal, this type of spheroids can be used as a model system for primary avascular tumor or metastasis [204]. Enhanced expression of stem cell markers and drug resistance in sphere-forming nonsmall cell lung cancer cells were reported: tumor sphere-derived cells expressed stem cell markers CD44, CD133, Sox2, and Oct4. Moreover, expression of lung resistance-related protein (LRP), glutathione-S-transferase-π (GST-π), and multidrug resistance proteins-1 (MRP1) were all significantly enhanced in sphere-derived cells [205]. In addition to spheroid formed by one type of cells, more complex multicellular spheroids are used in drug studies. This type of spheroids contains not only cancer but also other tumor-constituting cells, such as fibroblasts, endothelial cell, and immune cell, and thus is closer to situation in vivo due to additional cancer-normal cell contacts.

Various techniques can be used to obtain spheroids: plastic culture dishes with ultra-low attachment surfaces, spontaneous aggregation under free-floating conditions, liquid overlay on agarose, hanging drop cultures, spinner-flask cultures, and micro-fabricated scaffolds [204]. Simplicity in production and use of spheroids has led to widespread use of these techniques. Cancer organoids may be created from CTCs, as nicely reviewed in [206], highlighting the techniques for CTC isolation from patient blood, cell culture enrichment, culture medium supplementation, as well as CTC-derived organoid application in disease modeling, genetic instability studies, drug discovery, and precision medicine. Interestingly, authors have demonstrated that replacing fetal bovine serum with human serum could enhance spheroid formation and increase the invasiveness of cancer cells in vitro [207]. However, not all techniques provide uniform-sized spheroids, resulting in poor reproducibility of experimental data. Cell biological functionality and sensitivity to drugs have a strong correlation with the size of spheroids [208, 209]. The methods of hanging drop and microfabricated microstructures enable researches to modulate spheroid dimensions but have a limitation for mass production potential and cause difficulties for effective harvesting of spheroids. Recently, a number of optimized spheroid production protocols were developed with various robust and effective platforms for high-throughput drug screening. Opera Phenix High Content Screening System with Synchrony Optics and automated image analysis were reported to be well suitable for microtissue spheroid analysis; ImageXpressMicro Confocal automatic imaging system with MetaXpress6 software was used for similar purpose.

In a strict sense, the term "organoid" means that the microtissue has originated from stem cells that had differentiated in tissue-specific manner and formed a mini-organ or organ-like structure. Such models of other than oncologic disease research have been nicely reviewed elsewhere. However, when microtissue is formed from primary cancer cells, some authors call it organoid, and some as spheroid. In either way, cancer cells propagated in three-dimensional (3D) culture systems exhibit physiologically relevant cell-cell and cell-matrix interactions, gene expression and

signaling pathway profiles, and heterogeneity and structural complexity that reflect in vivo tumors. In one study, three lines of ex vivo established colorectal tumor spheroids were analyzed. All three lines expressed EpCAM, CD133, CD44, CD24, ALDH1, and LGR5 biomarkers and maintained them stably for months in vitro. STR phenotyping and mutation profile for APC, KRAS, MLH1, serine-threonine kinase 11, and TP53 coincided with original tumors from which they had been derived [210].

Cancer modeling using organoids was nicely reviewed in [211, 212]; larger view on tumoroid application in cancer research was extensively discussed in [213]. Patient-derived tumor organoids were suggested as the best model enabling high-throughput drug testing in a clinically relevant time frame at the same time being cost-effective [23]. Although multicellular spheroids exhibit physiologically relevant structural complexity of tumor as well as cell-cell and cell-matrix interactions, smaller than 200 μm spheroids do not reflect the in vivo characteristics of tumor cell proliferation, metabolite, and oxygen gradient resulting in necrotic core formation. Authors developed an optimized protocol allowing production of large spheroids that mimic some of the complex tumor microenvironment, including hypoxia. However, in this model, spheroids were unable to develop tumor specific vascular architecture, which was achieved by transplanting of spheroids to immunosuppressed mouse .

Another study described a 3D cell culture system to study tumor-stroma interactions in nonsmall cell lung cancer cells by creating mono and co-culture spheroids of two NSCLC cell lines A549 and Colo699, and lung fibroblasts. The viability of tumor spheroids was determined after 5 and 10 days by using Annexin V/Propidium Iodide flow cytometry. Lower viability was observed in A549 monocultures compared to co-cultures, whereas Colo699 monocultures showed better viability compared to co-cultures. Additionally, tumor-fibroblast spheroid formation was characterized by scanning electron microscope, semi-thin sections, fluorescence, and immunohistochemistry of E-Cadherin, vimentin, Ki67, fibronectin, cytokeratin 7, and a-smooth muscle actin in addition to conventional histology [214].

It is rather difficult to consider which spheroid production method would be most suitable for potential drug studies. The main requirement for high-throughput analysis is reproducibility of uniform spheroids, as cell susceptibility to therapy depends on the spheroid size. It was demonstrated that the size of spheroid may depend on production method used, as growing of squamous carcinoma cells on ultralow attachment plates resulted in higher proliferation rates and increased spheroid size compared to Hanging drop production method. Despite numerous spheroid studies, it is not still clear which time point would be ideal to start treatments and evaluate drug response. Typical spheroid structure consisting of an outer layer of proliferating cells and inner layer of quiescent cells is detected in spheroids larger than 500 μm in diameter. Several agents have been found to be less effective in 3D models than in 2D cell cultures due to the drug concentration gradient in different layers of spheroid which is relative to drug delivery within solid tumor in vivo. Moreover, cell growing in 3D environment showed expression of different genes associated with resistance to therapy [205, 215].

Limited drug penetration to the tumor mass is one of the mechanisms which determines tumor resistance to applied therapy. This critical parameter is not reproduced in monolayer 2D cultures. Meanwhile, spheroids formed from cancer cells mimic both the heterogenic multilayer tumor structure and gradient drug penetration thus providing more physiologically relevant results as compared to monolayer cultures. Cells in the center of spheroid are exposed to nonoptimal drug concentrations; thus, increased drug doses or drug combinations are needed to provide antitumor effectiveness. Recent studies demonstrated the advantage of

spheroid model in evaluating activity of drug combinations and treatment schedules in order to promote drug delivery and accumulation inside a tumor. Therefore, spheroid models can be used to assess penetration of different drug formulations.

In drug development, potential agents are further progressed to antitumor activity screening in vivo where syngeneic mice model, genetically engineered mice models, or xenograft models are used. All these models have some significant limitations leading to inadequate results. First of all, tumors are often implanted subcutaneously—to the site which does not reflect their original in vivo location. Due to the compromised mouse immune system, xenografts are usually characterized by higher proliferation rates compared to primary human tumor [115, 216]. Moreover, transplanted tumor interacts with stromal components which are not of human origin, thus can behave in different manner. Strategy that could significantly increase the rate of success would be more complex, physiologically relevant cellular models used in early lead discovery. For solid tumors, this would include development of 3D in vitro tumor models which recapitulate human tumor architecture and biology, thus providing greater translational potential [217, 218].

4.4 Complex 3D models

Solid tumors consist of cancer and stromal cells surrounded by extracellular matrix. All these cellular and noncellular tumor components co-exist in highly interactive 3D environment. Gradient of nutrients, soluble factors, oxygen and metabolites occurring in different layers of tumor as well as different type of interactions such as cell-cell and cell-ECM regulate cell function and behavior. Cancer cells cultivated in 3D environment, if compared to cells growing as a monolayer, are exposed to different signals that modify their response to various stimuli [158, 219]. For example, cells growing in 3D cultures experience different mechanical forces compared to cells growing in 2D. It was demonstrated that mechanical properties can regulate cellular response to therapy and angiogenesis [220]. Additionally, various interactions occurring in multilayer tumor create physical barrier attenuating drug penetration. Recent studies demonstrated that 3D microenvironment had effect on numerous cellular functions including cell morphology, viability, proliferation, differentiation, and migration potential, as well as cell signaling, gene and protein expression following response to the applied therapy. Taken together, these results indicate that 3D models of cancer provide more physiologically relevant results and have greater translational potential to successful clinical studies.

There are several artificially engineered 3D cancer models that are close to natural tissues and thus partially recapitulate the environment into which a tumor cell may invade; for example, three-dimensional organotypic microfluidic model to study the phenotype and invasion of glioma stem cells [221]. Artificial skin was built of stratified terminally differentiated epidermal compartment of keratinocytes and melanocytes, a dermal compartment of fibroblasts embedded in collagen, and a basement membrane deposited by skin cells; such skin reconstruct was used for melanoma modeling [222]. Another example is a 3D bone marrow model derived from endothelial progenitor cells and multipotent mesenchymal stromal cells embedded in alginate and Matrigel. It was used to study immunotherapy on primary multiple myeloma [223]. More advanced bone marrow models are described in [224] and may involve various soluble factors, osteoblasts and the flow of nutrients. Similarly, a lung cancer model named OncoCilAir has almost everything that is available in vivo, except immune system of a patient. Briefly, OncoCilAir was developed by plating lung fibroblasts and bronchial epithelial cells on different sides of a porous membrane. Later, nonsmall cell lung cancer cells were

added to the fibroblastic side, and air-liquid interface was introduced to mimic the conditions in normal lungs [18]. In that way, OncoCilAir includes both healthy and cancerous cells; thus, it can be used to test both tumor-killing activity and the adverse effects of anticancer drugs. Moreover, the model could be maintained for up to 3 months, which theoretically enables studies of such long-term effects as air pollution toxicity, drug resistance, and tumor recurrence.

Organotypic 3D in vitro models are very close to realistic representation of a tumor. When a reasonably thin slice of a real tumor is carefully plated on a porous surface to ensure better diffusion of nutrients and metabolites, both cancerous and stroma cells are present in such a model, including the unchanged intratumoral tissue architecture with ECM and cellular contacts as well as immune cells with natural cytokines. Such a system is well suited to perform drug selection in personalized medicine as it captures the heterogeneity of a tumor, can be multiplexed for various biochemical applications, and also is transparent for optical microscopy. Probably one of the most advanced commercial systems available today is CANScript from Mitra Biotech (India). This platform is intended for prediction of clinical responses to anticancer treatment of a certain patient and is applicable in almost all types of cancer, including bladder, prostate, HCC, cervical, urogenital, NHL, and GBM. In contrast to other similar preclinical models, CANScript and its prediction algorithm has been tested in clinical settings for H&N, CRC, breast, pancreas, stomach, esophageal, lung, ovarian, AML, and CML cancers [ClinicalTrials.gov identifier (NCT number) NCT03253575]. The technology has been validated in mouse xenografts and extremely well correlated with the clinical outcome. Currently, the system is being adapted to become an automated platform available to hospitals. Briefly, the tumor sample from a patient is cut using a tissue chopper into 200–400 μm thick slices that are placed into 48-well cell culture plates with RPMI-1640 medium with supplements including patient blood serum (2%). Importantly, the cell culture plate itself is coated with tumor-grade-matched matrix. Tumor-stroma matrix proteins (TMPs) are analyzed from the same specimen by mass spectrometry after sample processing, selecting certain proteins to further constitute the protein cocktail for plate coating. It ensures the conservation of the tumor microenvironment in addition to the 3D structure of a tissue slice and native patient-specific ligands from their blood serum. It was demonstrated many times that the mentioned factors determine the fate of tumors in terms of integrity, survival, metastasis, and response to chemotherapy. In addition, the CANScript developers have demonstrated that explants in noncoated wells lost tumor architecture and exhibited decreased viability, proliferation, and activation of oncogenic pathways compared with the initial baseline [225]. The method utilizes both morphological and biochemical criteria, including measurement of cell viability, ATP utilization, proliferation status, changes in tumor area and nuclear fragmentation, activation and expression of various signaling proteins by reverse phase phosphoproteomic array, immunohistochemistry and qRT-PCR methods for molecular profiling, and quantification of biomarkers [17].

However, tissue slicing has some minor limitations. For example, when a tumor is larger than the well of a plate into which it has to be positioned, only a portion of a slice would be examined. In such a case, the unexamined part of a tumor and its response to the suggested treatment could be lost. There are data that drug sensitivity and biomarker expression vary considerably within individual samples [77, 226]; hence, multiple-site samples should be taken, making it difficult for the specialist to decide which sites to include in the sampling. Furthermore, cellular signaling and hence the drug response in slices may be altered due to suffered mechanical stress during the precision cutting and cellular necrosis signaling at the incision planes. In addition, it is characteristic to a solid tumor to maintain high interstitial fluid

pressure that limits the absorption, e.g., of a drug, and this pressure is not preserved in the current models. As noticed by PREDECT team, the ultimate goal—clinical application of this method—requires standardization which is impossible in obtaining the samples manually due to different intraoperative manipulation and pathology processing even with the same type of cancer [89]. Additionally, for CANScript technology, identification and individual plate coating with required TMPs appear to be the limiting (expensive and labor consuming) steps, as for these purposes, the biopsies firstly have to be analyzed by liquid chromatography-mass spectrometry (LCMS/MS), followed by Venn diagrams and heatmaps to select the proteins to be mixed in a cocktail for the plate coating.

Another and probably the most straight-forward approach in trying to understand the nature of cancer was the initiative The Cancer Genome Atlas (TCGA), as already described above in this review. Starting from 2006, 20,000 primary cancer and matched normal samples spanning 33 cancer types have been molecularly characterized, yielding over 2.5 petabytes of genomic, epigenomic, transcriptomic, and proteomic data that will remain publicly available at portal.gdc.cancer.gov for the research community to use [227]. Comparatively similar problem in data processing is already arising from the next step in cancer modeling that included real-time monitoring and gave the fourth dimension for in vitro cancer studies. Apparently, it is not applicable in vivo, but definitely it is the future of oncology.

4.5 Single-cell approach

When talking about cancer heterogeneity, we usually think about a complex organ-like tumor. That is correct, because human body is mainly solid, and almost all cell types form solid tissues. However, blood is liquid and it contains many types of different cells; thus, it is also heterogenic. When analyzing blood, we are familiar with flow cytometry and discrimination of cells by their physical parameters and CD antigens. The same is for blood cancer: flow cytometry can discriminate various subpopulations of abnormal blood cells. Additionally, mass cytometry has emerged as a method to evaluate cellular biomarkers on a single cell. Importantly, this method has enabled studying the antibody-based cancer therapies as well as metal containing chemotherapeutic agents at one-cell resolution [228]. Similarly, Western blot method has been miniaturized even to subcellular level [229]. As mentioned previously, blood cancer is quite well treated just because there is no need for a complex model to study the cell response or resistance to a certain drug or combinations. Cells in suspension and can be readily analyzed in vitro not only for the antigens, but also for cellular signaling. For example, phospho-flow cytometry was applied in the search for B-cell precursor acute lymphoblastic leukemia individualized therapy and enabled to screen for small molecule kinase inhibitors that induced cancer cell death [230]. Functional analysis of single-cell mass accumulation rate became available by means of microchannel resonance [231]. Recent technologies have enabled single-cell RNA sequencing thus providing the power to dissect intratumoral heterogeneity and to suggest combination therapies [232]. Today, a Phase II clinical trial (NCT01620216) started in 2012 is still recruiting leukemia patients for ex vivo drug response prediction. However, even here, the microenvironment plays a role. As reported previously, the responses of leukemia cells to chemotherapy in vivo, compared to in vitro, were partly related to the interactions of leukemic cells and the three-dimensional bone marrow/stromal microenvironment. To investigate this phenomenon, leukemic cell lines were co-cultured with human bone marrow mesenchymal stem cell (hu-BM-MSC) in 3D and compared to leukemic cells treated in suspension or grown on a hu-BM-MSC monolayer (2D conditions) [233]. The authors concluded that cultures in 3D were

more resistant to drug-induced apoptosis compared to cells cultured in 2D or in suspension.

Further, the modeling becomes complicated when it comes to circulating tumor cells (CTCs). During cancer progression, invasive cells evade the primary tumor and enter the vascular systems in search for better environment. Such CTCs are important in many aspects; the ability to target such cells and to eliminate them before the formation of metastasis is of high priority. CTCs are valuable for assessing the profile and heterogeneity of tumor-evading cancer cells. However, their direct use as a single-cell model in predicting tumor response to the treatment is disputable; therefore, researchers usually create CTC culture models as well as mouse xenografts [234]. However, to retrieve CTCs and later to expand their culture in vitro is challenging. One study compared three methods (RT-qPCR for cytokeratin 19, double immunofluorescence with A45-B/B3 and CD45 antibodies, and CellSearch system with CTC kit) to search for and count circulating tumor cells in breast cancer patient blood. Interestingly, there was virtually no agreement between the methods, including the cancer marker CK19 mRNA-positive cells that were also present in 26 healthy female donors [235]. This study demonstrated the limitations of biomarker-based cell characterization as well as weakness of technical standardization procedures. However, authors described that they have succeeded (16.5% success rate) to establish long-term cell cultures from breast cancer CTCs, growing them in serum free media supplemented with epidermal growth factor and basic fibroblast growth factor under nonadherent, hypoxic conditions [236]. Other reports demonstrated that in the case of colorectal cancer, CTC cell line generation was possible only from the patients with stage IV cancer and was not successful in earlier stages, and the cell cultures demonstrated all the attributes of cancer stem cells [182].

In general, pharmacologic response of every single cell from the heterogenic cancer tissue should be analyzed whenever possible. In the case of CTCs, single cell analysis is very helpful in defining their response to the drugs when cells have already escaped from their niche in the tumor tissue, i.e. when cancer cells are circulating in pre-metastatic fashion. However, their behavior does not necessarily correspond to the response of cells in solid tumor from which they have originated. Various microscopy imaging techniques that distinguish individual cells should be preferred over pan analysis methods both in vitro and in vivo [237, 238].

5. Upcoming technologies and perspectives

As of particular interest, there are several reports of drug response analysis in situ by microinjections into the intact solid tumor, as reviewed in "In situ functional diagnostics" [30]. Briefly, the drugs were delivered into the xenograft tumors either as a monotherapy compound or up to 16 slowly releasing drug reservoirs, and the response was evaluated by retrieving the whole tumor for analysis. Alternative approach was used to design acoustofluidic 3D tumor platform to investigate the localized release of temperature-sensitive liposomal doxorubicin in glioblastoma model [239]. Basically, focused ultrasound raises the temperature in the zone of the targeted several square millimeters. When directed at a tumor, such heating favors the disassembly of circulating cargo liposomes that contain toxic substances. This method shows promise in localized activation/release of chemotherapeutic drugs that are not well tolerated in organism and thus fail in dose-escalation trials. One more example is a nano device iNANIVID-assisted multiphoton microscopy of individual cells when physically inserted into a live solid tissue [240]. This short-term (up to 4 h) intravital mouse tumor monitoring was performed in order to validate

the nanodevice for drug release; in this particular case, for analyzing EGF-induced chemotaxis of metastatic mammary tumor cells. Although this technology seems to us not applicable to humans, at least for long-term drug response studies, such progress may indicate the evolution of 4D cancer modeling.

Summarizing the above stated, the best cancer model is fully natural tumor in a living human. Although it might sound wired, it is true to some extent when talking about child cancer in the United States. More than 80% of children who have cancer are cured successfully in United States so they are comparatively better served by available therapies, as compared to adults. Authors postulate that mainly it was a result of incredibly effective chemotherapy combinations that were established through highly empirical and incremental clinical trials [241]. However, more technical approaches may be emerging to advance noninvasive cancer response monitoring in a patient to guide the best treatment. For example, magnetic resonance methods are able to some extent visualize tumor characteristics and its metabolic phenotypes on an anatomical, microvascular, microstructural, microenvironmental, and metabolomics scale [242]. More specifically, magnetic resonance imaging of muscle-invasive bladder cancer revealed that quantification of apparent diffusion coefficient (ADC) of water could reliably predict patient response to the neoadjuvant chemotherapy, i.e., resistant tumors were more heterogeneous in their spatial distribution of ADC values [243]. Alternatively, positron emission tomography showed promising results in molecular cancer biomarker imaging in vivo by using newly developed imaging agents for precise molecular targets. This technique can be useful in clinics for measuring early treatment response to predict therapeutic efficacy and relating tumor response to survival [244].

The next best thing to the real tumor monitoring in vivo (so-called 4D cancer model) is sophisticated 3D in vitro and in vivo mouse models. For fundamental studies, light sheet fluorescent microscopy is a new technology for rapid, low phototoxicity 3D imaging with resolution similar to that of confocal microscopy; moreover, super-resolution variations of the method are becoming available. Probably the best model in both genetic and physiological relevance, more than a humanized mouse, is the putative "patient-on-a-chip." The model would be comprised of many organ-on-a-chip building blocks that presumably would be printed from patient-derived and differentiated iPSCs, later connecting those blocks in a physiological order by microfluidic channels [245]. For example, an array of 3D bioprinted tissues (skin, bone and cartilage), microfluidics-based kidney-on-a-chip, lumen forming intestine microtissues, and heart model as a multilayer of cardiac fibroblasts alternately layered with iPSC-differentiated myocytes on an elastic silicone membrane for efficient beating, are promising components of such a system [246]. However, this kind of model is considered only in a preclinical setting for drug research. Computational time-lapse movie data analysis also will require the creation of most intelligent software [247].

Photo-degradable gelatin may be used for very specific reasons in 3D gel cellular models. Using this delicate approach, heterogenous cell cultures may be cultivated in one dish until a cell or a colony of interest has to be extracted for analysis, leaving the remaining culture intact. This method was used to separate morphologically different subpopulations from a murine breast cancer cell line [248]. No digestion, no labeling, no flow cytometry is needed.

For 2D cell cultures, noninvasive, time-lapse compatible electrical impedance may be used for phenotypic screening, target identification, compound screening, lead selection, investigating the mechanism of action, and testing drug safety and toxicity [249]. The method also requires gold-plated electrode for cell adhesion.

For single-cell approach, advanced microfluidic techniques are being developed for multiomics data acquisition in nanodroplets. Briefly, high-throughput

single-cell isolation and nucleic acid barcoding methods are making it possible to measure the (epi)genomic, transcriptomic, or proteomic state of individual cells by elegant strategies and techniques described in [250]. Single-cell methods complemented with gene-editing technologies were recognized among the most perspective models for disease modeling [159]. Furthermore, a new technology directed at cellular spheroid generation using microwells that are much smaller than a well of a 1536-well plate may be paving its way. The method involves limiting dilution principle within traditional 2D cell culture plates coated with polydimethylsiloxane. The advantage of this method over traditional limiting dilution is that the small volume of a well limits diffusion of autocrine factors and improves extracellular survival signaling; at the same time, there is no danger of evaporation. Moreover, many clones (300 and more) and other types of cells from a heterogenous cancer tissue may be present in the same culture medium allowing paracrine signaling without complex microfluidic connections [251] or in a microchip format as developed earlier [252].

6. Concluding remarks

Regardless of the abundance of approaches and various models and methods developed, the question remains, which models are the best to assess cell heterogeneous response to anticancer treatment and to evaluate its clinical relevance? Recently, authors have performed SWOT (strengths, weaknesses, opportunities, and threats) analysis of 3D cell models and suggested that more transparent assessment of the value of new 3D models was needed. Currently, it looks like many popular 3D models have no standardized protocols for validation in clinics as well as they are not convenient enough, do not mimic cancer biology sufficiently, and thus are not suitable for use in clinic. As a result, early enthusiasm regarding 3D models is already followed by disillusionment and disappointing results. Development of long-term, low-throughput, inconsistent, and expensive models should be abandoned for clinical applications; however, such models may have merit in fundamental research [253].

We suggest that the best model would be simple, miniaturized, inexpensive and, at the same time, multifaceted, involving ex vivo analysis of individual cells in different states and in different conditions—suspension, 2D and 3D as well as co-cultures with stroma cells. Drug screening in such a model would require knowledge of which cellular states survive the specific treatment and, according to that, combinational therapy—not only by content but also organized in time and sequence—should be tested in the model system. The short-term cultivation in vitro should be complied for to preserve the patient-specific cellular heterogeneity, cell differentiation status, and molecular profile. For this particular reason, as well as for the ability to recheck the obtained results, patient's tumor specimen freezing protocols should be developed.

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