1 Introduction

In this review, we attempt to survey the current status of in vitro/ex vivo models of human cancer that try to better reproduce the complexity and heterogeneity of a human cancer in situ, with a perspective of their use in medicine. A historical background to the use of tumor cell lines in cancer drug discovery is followed by a survey of more recent attempts to mimic in vitro the three-dimensional characteristics of solid tumors, including those models which add elements of the important tumor stroma such as fibroblasts. Studies using bioreactors are reviewed; these not only permit changes in scalability but are permissive for dynamic, controlled flow-through conditions of cell culture. Although cell lines with or without stroma may be studied and used in three dimensions, they nevertheless constitute attempts to re-construct only some of the complexity of a complex and often heterogeneous tumor, and essentially remain reductionist models. The use of precision-cut tumor slices potentially permits all aspects of tumor complexity and heterogeneity to be captured in vitro, sometimes referred to as ex vivo, but in
reviewing progress with this technological platform, some important caveats are articulated. Finally, we address the rather vexed question of how any model may be “validated” sufficiently well so that it may be incorporated into mainstream activities not only relating to drug discovery but also to the study of tumor biology in vitro/ex vivo.

1.1 The current problems of pre-clinical models of cancer

In a recent and self-revelatory article addressing some of the current malaise in the pharmaceutical industry and the difficulties of novel drug discovery [1], it was suggested that ultimate project success in drug discovery depended very heavily on the confidence that teams had in studies of target validation. This critical first step in the pre-clinical cascade of cancer drug discovery depends almost entirely on the use of in vitro models, cell lines, that attempt to recapitulate pathology in a reductionist way (reviewed in [2]). These same models, expressing a drug target of interest, generally become incorporated into subsequent steps of the pre-clinical cascade of drug discovery, providing essential tools for compound screening and refinement, until candidate molecules are chosen for in vivo assays of efficacy; sometimes, the same long-established cell lines are grafted into mice for such a purpose. There has been a growing realization that the workhorse of robust reproducible cell lines, growing in suspension or in two-dimensions on plastic, amenable to high-throughput screening, is limited in its ability to capture the complexity and heterogeneity of a cancer. The compelling evidence for this lies in clinical trial data: the greatest source of failure in phase 2 is a lack of efficacy [1, 3–5] an efficacy that had presumably been robustly demonstrated in pre-clinical studies. Novel cancer therapeutics are the worst candidate drugs within all therapeutic areas which lack of efficacy in clinical trial [3] and thus, one could postulate that the pre-clinical models of cancer, including in vitro models, are among the least predictive.

1.2 Cell lines: Does genotype capture phenotype?

One defense of the use of single cell lines, growing in suspension culture or on plastic, as sufficient models of human cancers, largely rests on the data emerging from massive efforts to fully sequence their genomes and describe their transcriptomes [6, 7]. Representation of intra-tumor heterogeneity, within a pathology, has been argued to be satisfied by increasing the numbers of cell lines representing that pathology (e.g. colon cancer) (see [2]). Each cell line “representivity” of pathology has been claimed to be reflected in the fidelity by which they carry signature patterns of genetic alterations. In addition, the response of cell lines to pharmacological agents was claimed to reflect clinical sensitivity patterns [6, 7] although this is controversial [8]. What these studies do not address is whether the biochemical circuitry of these cells, such as signaling cascades initiated by growth factors (GFs), cytokines and heterogeneous cell contacts, is representative of a complex tumor in situ? Tumors have rightly been described as “organs” [9]. It is difficult to imagine how cell lines growing on plastic, lacking all the interplay with a dynamic and complex stroma, may be representative of a tumor. In addition, solid tumors grow in three dimensions, with homo- or hetero-intercellular contacts. However, in reviewing three-dimensional in vitro models of cancer, recently also addressed elsewhere [10, 11], and precision-cut slices, these same questions, about how representative their circuitry is, must also be posed. This challenge is addressed at the end of this review.

2 2D and 3D cell line models

2.1 Some history of 2D cancer models

In the 1950s, cell culture was introduced as a tool to screen compounds as potential drugs in vitro, using cells grown and passaged infinitely on glass in flasks in semi-synthetic medium ([12, 13] and references therein). However, the correlation between in vitro culture, mouse in vivo results, and clinical responses was very poor. This was not seen too negatively – rather it was suggested to compare the models and make use of their diversity: “At the present imperfect state of our knowledge, the most fruitful approach to the complexities of cancer chemotherapy may well be the imaginative exploitation of the discrepancies, which are bound to emerge from these comparisons” [13].

Perhaps the next major advance was the colony forming assay, developed by Hamburger and Salmon [14] in the 1970s, which allowed growing fresh human patient material embedded as single cell solution in soft agar. In an NCI-funded study, four laboratories evaluated the technology and its use in drug testing. Six tumor entities, breast, colorectal, kidney, lung, melanoma, and ovarian yielded cultures that passed the quality controls established by the laboratories, for 20–38% of patients tested at samples sizes of >160 patients each [15]. The protocol was then used to re-test 79 compounds, which had been inactive in the in vivo screening assay, on 15 tumors each. Fourteen compounds were found to be active in at least two tumors. Due to the low plating efficiency, with a concomitant bias of the assay toward tumors that grew in soft agar, and the requirement for a constant supply of fresh patient material, the assay was considered impractical for larger compound screens. The plating efficiency could be improved to 70–80%, at least for certain pathologies [16], but a review on clinical studies indicated rather limited success of in vitro soft agar assays to predict a patient’s
tumor’s sensitivity toward clinically approved drugs. Twelve prospective studies were investigated, including a total of 506 patients (33%) that had been treated with chemotherapy chosen via clonogenic assays. The mean response rate for these patients was 27% (range 10–100%; \(n = 12\) studies) compared to 18% (range, 0–100%; \(n = 7\) studies) for patients treated with empiric therapy. The effect on overall survival had not been properly addressed [17]. The absence of a functional tumor microenvironment in the assays – extracellular matrix, stromal cells – and selection for cells within the tumor that survive in soft agar may be explanations of poor predictivity.

As mentioned above, besides the unsatisfactory predictive power, the requirement for a constant supply of fresh patient material rendered the clonogenic assay unsuitable for larger scale compound screens. The availability of cell lines originally derived from patients, and adapted to grow on plastic, provided an ample, indefinite source of cell material (Fig. 1A). The NCI thus introduced its “NCI60” panel, originally starting from a smaller, lung focused panel, to cover a range of pathologies (leukemia, colon, lung, central nervous system [CNS], renal, melanoma, ovarian, breast, and prostate). An important reason for the expansion of the panel was the fact that “normal” fibroblasts or epithelial cell lines were not appropriate controls for normal tissue. Moreover, the differential sensitivity of the cell lines to cytotoxics allowed for their grouping into compound classes, and a rapid classification of novel compounds [18].

2.2 Genomically characterized cell lines growing in 2D on plastic

The recent decades have seen a surge of targeted molecules, some of them active against very small subsets of a given pathology. Thus sets of genomically characterized, diverse cellular models were required. The NCI panel was sequenced [19], but also more extensive cell line panels have been established, and with the help of next generation sequencing thoroughly characterized [6]. Large-scale testing of targeted compounds reported in the latter study indicated a good correlation between cell lines identified as sensitive, their genotype, and sensitivity markers confirmed in clinical studies (e.g. HER2 amplification and BRAF V600E mutation) [6]. Other investigators used sets of genetically defined tumor cell lines to explain why the BRAF inhibitor vemurafenib is only active in BRAF mutant melanomas [20, 21], or mTOR inhibition by rapalogs-induced AKT activation, potentially explaining their limited anti-tumor activity [22]. Both results lead to new drug discovery programs to tackle cell intrinsic resistance mechanisms.

However, these approaches completely neglected the role of the stromal compartment, commented on in Section 1, ignoring an important source of signals, potentially imparting tumor resistance. Indeed, in a study applying a matrix of stromal and tumor cells (Fig. 1A), Straussmann and coworkers showed that some combinations of stroma-derived signals may render sensitive tumor cell lines resistant to targeted drugs, e.g. hepatocyte growth factor (HGF) produced by stromal cells reverted vemurafenib sensitivity of V600E mutant cell lines [23]. In a complementary approach, Wilson and colleagues investigated the effect of six GFs (HGF, epidermal growth factor [EGF], FGF, platelet-derived growth factor [PDGF], neuregulin [NRG1], and insulin-like growth factor [IGF]) on the resistance of tumor cell lines toward a set of kinase inhibitors that they were originally sensitive to. Except for PDGF, all GFs provided rescue in some combinations [24]. Expression of HGF was detected in patients with melanoma, and indeed correlated with increased resistance to the BRAF inhibitor [23]. Thus, adding complexity to 2D models may increase their predictive power, particularly regarding signaling feed-back loops leading to resistance.
2.3 Moving into 3D cancer models

Even complex 2D models still ignore an important fact – solid tumors grow in three dimensions (3D). A number of techniques have been developed to cultivate cells as 3D cultures. These include aggregating cells at the bottom of a drop (“hanging drop”) [25], a liquid overlay technique, which prevents cells from attaching to the vessel surface using a non-adherent coating (agarose- or poly-HEMA treatment, ultra-low attachment plates with round bottom) [26–28], or growing cells in stirred culture systems, such as spinner flasks [29] (Fig. 1B), or stirred-tank bioreactors (Section 3). After initial aggregation, cells generally started to secrete extracellular matrix (ECM) components and upregulated proteins mediating cell–cell interactions, e.g. E-cadherin, and many, but not all, cell types formed tight cell–cell contacts [30]. The resulting 3D spheroids were compact, stable and showed decreasing gradients of proliferating cells, oxygen levels, and nutrition supplies from the periphery toward the spheroid’s center [27, 28, 31, 32]. These physiological characteristics of the 3D spheroid closely resembled avascular tumor nodules, micro-metastases, and inter-vascular regions of large solid tumors as presented by Sutherland [33].

Culturing cells in 3D tumor spheroids altered their sensitivity to cytotoxic agents such as 5-fluorouracil, cisplatin, or doxorubicin [34–38]. Several factors might confer this resistance to cytotoxic agents including – but not limited to – insufficient distribution of the drugs within the spheroid [39], non-proliferative and hypoxic cells in the core of the spheroid [40], induction of GF secretion (e.g. transforming growth factor β [41]), cell–cell interactions mediated by E-cadherin [42], and production of extracellular matrix proteins as well as expressing resistance markers like ABC1 multi-drug resistance pumps [37]. On the other hand, some targeted compounds appeared to be effective in 3D spheroids while having significantly reduced or no effect in 2D monocultures [38, 40, 43]. For example the PI-3 kinase inhibitor PX-866 suppressed the growth of U87 glioblastoma, T47D breast and HCT116 colon cancer cells in 3D spheroids but not when grown on plastic as monolayers [43]. PX-866 has demonstrated anti-tumor activity both as a single agent and in combination with other agents in a number of human tumor models [44, 45] and is currently being evaluated in clinical trials [46]. At least in some cases, 3D spheroids thus seem to predict in vivo and potentially clinical efficacy better than 2D models.

For the above reason, and since they are highly reproducible, robust, easy to use and suitable for high-throughput screening, 3D spheroids represent an important 3D platform in the biopharmaceutical drug discovery [11]. The size of cell aggregates as well as their proliferation, apoptosis, or drug responses may be measured based on fluorescence, luminescence, and absorbance by high-content imaging systems [40]. Also functional analyses based on phenotypic RNAi screening proved to be possible in high-throughput 3D spheroid systems [47].

2.4 3D and the stroma

A spheroid mono-culture system, however, still lacks the stromal compartment of a tumor. Investigators have addressed this issue by mixing tumor cells and fibroblasts suspensions, tumor spheroids with fibroblast/monocyte suspensions, tumor spheroids with embryonic bodies, or combining tumor spheroids with a fibroblast/endothelial cell monolayer. Paracrine interactions, and effects of stromal cells on tumor cell behavior and/or vice versa have been reported [48–53]. This heterotypic cell–cell crosstalk was shown to induce the formation of more compact 3D spheroids [54] and affect inter-cellular signaling and gene expression of the tumor cells, resulting in altered tumor cell proliferation and migration [49, 52, 55].

However, extensive characterization and validation of the 3D spheroid models for different cancer pathologies are still required in order to understand how closely they resemble the biological properties of in vivo tissue, including growth kinetics, gene expression, the architecture of signaling cascades, and drug treatment responses.

A first step toward a systematic comparison of pre-clinical models, including 3D spheroids, was the study performed by Lee et al. [27]. Histological and molecular features of 29 different ovarian cancer cell lines were compared between 3D spheroids, 2D monocultures, and xenografts, including a correlation with literature data available for the primary tumors. Comparison of 3D with 2D cultures suggested up-regulation of E-cadherin, down-regulation of vimentin, decreased expression of the proliferation marker MIB1, and increased expression of apoptotic marker caspase-3 in spheroids. While most of the cell lines formed poorly differentiated, high grade histology spheroids, the cell line OAW42 histologically resembled a well-differentiated (grade 1) serous ovarian tumor, and UWB1.289 and BRC1 resembled moderately well differentiated (grade 2) serous ovarian tumors. No differentiation was observed in 2D. The histology of the spheroids matched those of the original tumor in some cases, but there was a clear selection for the poorly differentiated high grade histology [27]. Adequate characterization of these models and their molecular signaling processes, as well as the addition and functional analysis of the stromal cells in co-culture with tumor cells, will allow for a more defined role of these models in the drug discovery process.

2.5 Incorporating the extracellular matrix (ECM)

Models based on spheroids growing in liquid medium still miss an essential component of tumor biology: the ECM. Pre-formed 3D spheroids embedded into, or sitting on top of, a gel matrix could be analyzed for cell invasion and migration upon treatment with compounds. Using this
assay, the HSP90 inhibitor 17-AAG and a phospholipase C (PLC) γ inhibitor could be shown to prevent dissemination on Matrigel™ (a laminin-rich basement membrane extract [BME] – see below) at concentrations below that to inhibit 50% of growth (GI50). The former was also shown to interfere with invasion of U87 MG glioblastoma spheroids into Matrigel, again at sub GI50 concentrations [28]. Cell lines like VCaP that do not form spheroids in ECM [56] can be pre-grown in medium before implantation into ECM (Fig. 1C). However, the ECM is not just an inert matrix that provides a 3D scaffold for tumor cells to grow and invade, but also plays an essential role in differentiation and maintenance of tissues, which may be missed in such hybrid systems. Moreover, the ECM has been shown to provide survival and drug resistance signals in cancer (for a review, see [57]). B1 integrin signaling was shown to protect small cell lung cancer (SCLC) cells in clonogenic soft agar assays with added fibronectin from DNA damaging agents [58] breast cancer cells plated on fibronectin or collagen I from paclitaxel [59] and HER2 amplified breast cancer cell lines in Matrigel from trastuzumab, pertuzumab, as well as lapatinib [60]. Activation of the MAPK-pathway by integrin αvβ3 also protected breast cancer cells from paclitaxel toxicity [61]. Multiple integrin-targeted small molecules and antibodies are currently in clinical development [62].

Assays including ECM components may be performed in 2D, by coating plates with the respective proteins, but due to the above outlined advantages of growing cells in three dimensions, and the impact of matrix stiffness on cell growth and differentiation, embedding of the cells in the matrix is preferred. Indeed, matrix stiffness was shown to affect stem cell differentiation [63, 64], tumor progression [65], invasion [66, 67], and drug sensitivity [68, 69]. 2D cultures have a stiffness of 1–2 GPa compared to normal breast tissue (160 Pa), and breast cancer tissue (4 kPa). Matrigel and collagen are around 200–400 Pa [57].

Commonly used matrices in 3D cell culture are a laminin-rich BME (also called Matrigel), purified from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, composed mainly of laminin-111, collagen IV, and heparan sulfate proteoglycan [70, 71], or collagen I. Mina Bissell's laboratory spearheaded much of the research on 3D extra-cellular matrix embedded models (Fig. 1C). Mouse mammary epithelial cells grown on floating collagen membrane, but not grown in 2D plastic, were shown to differentiate to form acini, and secrete casein [72, 73]. Embedding in Matrigel allowed them to form alveoli-like structures that secreted milk proteins into a hollow lumen [74]. Whereas normal mammary cells formed hollow acini formed by a single layer of epithelial cells, and stopped proliferating after 10–12 days in culture, mammary tumor cells continued to proliferate, generating large spheres [75]. Genes that were down-regulated in normal human mammary epithelial cells during differentiation in 3D cultures predicted for a poor outcome in breast cancer patients [76, 77]. Moreover, the morphology of the spheres formed by the 3D Matrigel-associated cultures allowed for a clustering into clinically relevant subgroups. Most luminal B subtype cell lines formed invasive stellar structures in Matrigel, whereas luminal A and basal subtypes formed round or grape-like structures. When correlated with gene expression analysis, cell lines forming stellar structures lacked E-cadherin and EGFR/ErbB2 expression, whereas grape-shaped colony forming cell lines mostly over-expressed ErbB2. Strikingly, when 3D gene expression was compared to 2D gene expression, the main differentiator was the culture system rather than the cell lines [78]. For other tumor entities, similar observations were made, e.g. prostate [56], colorectal cancer [79], and lung cancer [80]. A gene signature based on lung cancer cell lines growing in 3D as smooth versus branched colonies could classify lung cancer patients into good and bad prognosis groups, albeit on a limited patient set [80]. Moreover, not only primary normal and tumor cells from the colon may be grown in Matrigel, when supplied with a mix of GFs including R-spondin to stimulate WNT signaling, but potentially many other tumor types as well, thus allowing for the generation of large sets of patient-derived primary organoid cultures as potentially more predictive tumor models [81].

In an effort to compare the effect of cell culture methods on gene expression and drug responsiveness, JIMT1 cell cultures were grown in 2D, as spheroids and as a 3D model in Matrigel, as well as in a mouse xenograft model. Gene expression profiles revealed that JIMT1 cells cultured in Matrigel more closely resembled the xenograft model than the spheroid model. Pathway analysis of 54 genes down-regulated compared to 2D indicated that hormone (endoplasmic reticulum [ER]) and GF signaling (HGF and TGF) were most affected. One hundred and two drugs were tested against the models, and 63 showed an effect in at least one model. The 3D model in Matrigel was generally more sensitive against the selection of compounds tested [82].

This latter study is another important step toward a validation of 3D models for target validation and drug discovery. A recent Innovative Medicines Initiative (IMI) (www.imi.europa.eu) funded effort of a large consortium of Academic, SME and pharmaceutical partners, PREDECT (www.preject.eu), aims to provide better characterized in vitro 3D platforms for both target validation and subsequent pre-clinical studies with the goal to increase predictivity of drug efficacy in patients.

### 3 Bioreactors: Controlled culture systems for accurate recapitulation of tumor microenvironment

In order to increase the relevance of in vitro tumor modeling approaches, discussed above, recreation of cellular
architecture and the microenvironment is essential. This includes not only the recapitulation of 3D cellular architecture and the presence of different cellular players, ECM components, and nutrients, but also the maintenance at physiological levels of physicochemical environmental parameters known to influence drug response, such as temperature, pH, and oxygen [83–86], which could be achieved through utilization of bioreactors (Table 1).

Bioreactors are culture systems designed to provide efficient mass transfer; computer-controlled systems provide on-line monitoring and automated control of environmental culture variables (temperature, pH, and dissolved oxygen). Furthermore, the use of perfusion operation modes allows simulation in vitro of consequences of circulatory system connections, such as shear stress and pressure, nutrient and O₂ supply, and metabolite clearance [87], as well as supply of cytokines and GFs. Bioreactors have been extensively applied in biopharmaceutical industrial processing. The knowledge accumulated facilitated the translation to the production of advanced medical therapeutic products, such as stem cells for cell therapy [88] and tissue engineering applications [89], in which cells and their microenvironment are the product of interest. In recent years, bioreactor design and development have been toward improved accuracy in control of cellular microenvironment with reduced shear, reduced working volumes and parallelization.

Currently, the range of bioreactor types with potential application in cancer in vitro models is wide: from classical stirred culture vessels and rotary cell culture systems (RCCS) to last-generation microfluidic devices [88, 90] (Fig. 2), plus an array of custom-made designs [91–93], which have been applied for the generation of 3D cellular structures, as well as for their culture under dynamic and controlled conditions.

### 3.1 Stirred culture vessels

These include spinner vessels and computer-controlled stirred-tank bioreactors (Fig. 2), providing a dynamic stirred environment, overcoming mass transport and gas transfer limitations, as well as culture heterogeneity presented by other systems [88] (Fig. 2A, Table 1). Stirred vessels are scalable systems, with simple design and operation, extensively characterized hydrodynamically. Manipulation of parameters such as vessel and impeller design and stirring rate allow applicability to an array of cell types with distinct aggregative capabilities and sensitivities to shear stress, including a large panel of tumor cell lines [94–97].

Another important feature is the feasibility to perform non-destructive sampling along time, enabling continuous monitoring/characterization as well as retrieval of material for further applications [98, 99]. Importantly, stirred-tank bioreactors are highly flexible, and can accommodate different 3D culture strategies, from cell spheroids, to microcarrier/scaffold and microencapsulated mono and co-cultures [99–102], presenting widespread potential. Hirschhaeuser et al. [103, 104], evaluated the efficacy of catumaxomab (anti-epithelial cell adhesion molecule [EpCAM] × anti-CD3 bispecific antibody) in coculture of head and neck squamous cell carcinoma spheroids and human peripheral blood mononuclear cells in spinner vessels. A dose-dependency effect was observed, including reduced spheroid volume, increased immune-cell infiltration and cytokine secretion, decreased proportion of proliferating cells, and reduced ability of colony formation.

The main limitations of stirred-culture vessels are the hydrodynamic shear force-related cellular stress, promoted by stirring, and the high culture volumes associated with these systems (minimum of 50–80 mL). Microencapsulation and scaffold strategies can minimize cell expo-
sure to shear-stress [100] and improve microenvironment recapitulation by accumulation of secreted factors and ECM components [105]. Recent efforts have been made toward the development of smaller-scale systems, with commercially available solutions starting to emerge, such as the Ambr® system (TAP Biosystems, recently acquired by Sartorius), with working volumes of 10–15 mL.

The characteristics described make stirred-tank bioreactors particularly suitable for long term [106] and/or repeated-dose studies [99], as well for large-scale production for feeding of high-throughput and parallel testing in miniaturized devices, such as microfluidic-based bioreactors (see below). Nevertheless, these systems have not been used to their full potential in cancer research.

### 3.2 Rotary cell culture systems (RCCS)

These are based on the rotating wall vessel bioreactor (RWV) developed by National Aeronautics and Space Administration (NASA) for studying tissue generation and cell behavior under microgravity conditions (reviewed in [107]) and are commercialized by Synthecon (www.synthecon.com). RWV are horizontally rotating cylindrical culture vessels with no internal stirring mechanisms (Fig. 2B), providing low shear and turbulence. Cells remain suspended in near-free fall, simulating microgravity conditions, which promote cell–cell interactions and minimize mechanical cell damage [108, 109], in the absence or presence of scaffolds [110, 111]. Gas transfer is achieved by diffusion through a central silicon membrane and RCCS are compatible with perfusion operation modes. The main disadvantages are associated with mass transfer limitations throughout the vessel, due to restricted control of nutrient/gas concentrations, which may result in heterogeneous microenvironments [88]. RCCS have been applied to 3D cancer cell culture for almost 40 years, to study tumor formation and progression and tumor microenvironment [112]. Numerous co-culture models in which the stromal compartment is represented have been described, such as colon and prostate, using adenocarcinoma cell lines with human colonic fibroblasts [108] and human prostate carcinoma together with prostate or bone stromal cells [113]. These induced genetic, morphologic, and behavioral changes [114], namely structural organization similar to normal colon crypt development and maintenance of androgen responsiveness and prostate-specific antigen (PSA) production, respectively. Evidence cumulated through the years point to altered gene expression and cell behavior due to microgravity [112]; a recent study focused on characterization of poorly differentiated thyroid cancer cells cultured during the Shenzhou-8 space mission, under real microgravity conditions, identified mechanisms of growth inhibition by cytokine secretion and gene expression profiling [115]. Microgravity culture is an interesting and useful tool to study cancer mechanisms; nevertheless translation of findings under these conditions into human disease models may not be easy.

### 3.3 Microfluidic devices

Microfluidic devices, or micro-bioreactors, are efficient small-scale systems with precise control of cell microenvironment (Fig. 2C). Recent advances in microfluidic technology boosted the development of novel in vitro drug screening methods compatible with high-throughput applications [116] and the development of “organs-on-chips,” composed of biologically functional tissue mimetics, in which inter-tissue interactions can be reconstituted [117]. Microenvironment can be controlled by precise adjustment of fluid flows, allowing perfusion operation modes, resulting in shear stress and mechanical strain. The main limitations of these systems are associated with low scalability and high complexity.

An array of designs have been proposed in recent years, with highly diverse innovative approaches to tumor microenvironment recapitulation [116, 118–120]. Recently, a microfluidic model to analyze the specificity of human breast cancer metastases to bone has been described [121]. By recreating a vascularized osteo-cell conditioned microenvironment using bone marrow-derived mesenchymal stem cells and endothelial cells, the authors identified molecular pathways critical for extravasation of breast cancer cells, involving breast cancer cell surface receptor CXCR2 and bone-secreted chemokine CXCL5 [121]. Microfluidic systems for culture of patient-derived material have also been described, such as the “thick-tissue bioreactor” developed by McCawley and coworkers [122] for culture of biopsied portion of breast tumor tissue in parallel microchambers. As a proof-of-concept for drug testing by delivery, the chemotherapeutic agent docetaxel and protease inhibitor compounds were administered to breast tumor cell lines cultured in Matrigel [122]. Zhang et al. [123] described a microfluidic platform for culture of patient-derived myeloma cells by emulating the dynamic physiology of the bone marrow microenvironment. The system uses a three-dimensional ossified tissue to mimic the tumor niche and recapitulate interactions between bone marrow cells and osteoblasts [123]. Many of these technical advances should now be gradually incorporated into standard practice.

### 4 Tumor tissue slices: Culture system preserving the individual tumor microenvironment

In contrast to more simplistic models, tissue slices provide the opportunity to study tumor cells in the context of an intact microenvironment. This includes all other cell types of the particular tissue as well as the extracellular matrix, which results in the conservation of naturally
occuring interactions in slice culture (Table 1). Given the influence of the tumor environment on drug sensitivity and other aspects of tumor biology [124], fresh tumor samples would appear more suitable to study individual patient tumors than established immortalized cell lines. Furthermore, tissue slice culture has the potential to cover the whole clinical spectrum of solid tumors including all stages and grades from well-differentiated slow-growing to poorly differentiated fast-growing subtypes, whereas the vast majority of cell lines represent only high-stage and poorly differentiated tumors [125]. Consequently, short-term cultivated tumor tissue slices could mirror more closely the intra- and inter-tumoral variability of solid tumors typically observed in vivo including proliferative aspects as well as the response to drug treatment. The complexity of this model system comes along with challenges that have to be addressed. In the following section, we discuss these challenges together with the potential and limitations of tissue slice culture.

### 4.1 History of “organotypic tissue slices”

The idea of cultivating “organotypic tissue slices” is certainly not new. In the 1920s, decades before the first immortalized cell line was established, free-hand cut pieces of tissue from rat and human carcinomas have already been used to study cell metabolism [126, 127]. This was followed by a variety of studies with explants of human tumors cultivated in hanging drops [128], on plasma clots [129, 130] or in liquid media [131] many of them with the aim to use this model for prediction of individual drug sensitivity. However, after establishment of the HeLa cell line in 1952 [132], immortalized tumor cell lines became the most frequently used model system worldwide and still represent the backbone of basic cancer research (see Section 2). With the introduction of high-precision tissue slicers [133], which allowed preparation of slices with constant thickness and therefore improved reproducibility, tissue slice culture experienced a “renaissance”. Consequently, tissue slices became an attractive alternative to immortalized cell lines since at the time it was widely recognized that there is a need for more complex model systems. In the last decades, precision-cut slices from various patient tumors such as carcinomas from breast [134–139], lung [138, 140–142], brain [143, 144], ovary [145, 146], cervix [147], kidney [148], and prostate [149] were introduced and used in various fields of cancer research.

### 4.2 Tumor tissue slice cultivation and applications

Primarily, tumor tissue slices have been used to determine drug responses including responses to cytotoxic chemotherapeutics, small molecule inhibitors, and other drugs [135–138, 141, 142, 144, 146, 147, 150–154]. Cellular responses were assessed on different levels ranging from drug uptake, proliferation and induction of cell death to changes in protein levels, and gene expression. Most of these studies are restricted to the analysis of the tumor cells’ response to drug treatment, whereas some include the investigation of the role of cancer associated fibroblasts in the tumor’s response to cytotoxic substances [138, 142]. Furthermore, localization and migration of immune cells in tumor tissue was examined using tissue slice culture techniques [155]. Successful infections of cancer cells in tissue slices with different viruses have been described showing that this ex vivo model is also suitable for the validation of alternative anti-cancer approaches such as oncolytic viral infection or gene therapy [143, 156–160]. In addition, tissue slice culture has been used for validation purposes in studies utilizing immortalized cell lines from different tumor entities grown in 2D [139, 145, 148]. Meanwhile, drug testing services based on patient-derived tumor tissue slices are commercially available (http://indivumed.com).

Despite the prevalent use of tissue slices in cancer research, a systematic characterization and a comparison with the “original” tumor has only been performed at a fairly rudimentary level so far. If at all, such comparisons are restricted to viability [135, 140, 146], proliferation status [140, 142], apoptosis [135, 140], and marker expression [140]. All these studies come to the conclusion that tissues from different tumor entities can be maintained ex vivo for 3–7 days without significant loss of viability/proliferation or induction of apoptosis and the maintenance of overall tissue architecture. Whether their detailed biochemistry continued to reflect, with fidelity, that of the tumor from which they were derived is addressed below.

### 4.3 Factors that influence “tissue slice quality”, limitations and suggested solutions

In principle, the criteria for successful tumor slice cultivation were summarized previously in an excellent review on tissue slices from non-malignant organs by Fisher and Vickers [161]. However, due to different intraoperative manipulation and pathology processing it is challenging to meet such “minimum” standard criteria for tumor tissue slices. Even with minimized warm ischemia times, optimized preservation solution and rapid slicing procedures there will be a variability that is beyond the control of the researcher (Fig. 3). Furthermore, inter- and intra-tumoral heterogeneity [162] complicates slicing and the cultivation procedure itself. In different tumors from the same entity and even within an individual tumor one has to deal with variability in terms of consistency [135, 136, 140], but also regarding occurrence of necrosis, proliferation [140, 142] and cell composition [142]. Consequently, the question arises if a “standard operating” procedure of tumor tissue slice culture is feasible or if individually adapted procedures might be necessary. Even though an adequate characterization of the “original” tumor tissue
cannot be done before starting tissue slice culture, this should be a pre-requisite for the interpretation of any data obtained from cultivated tissue slices. Furthermore, every experiment should be performed in replicates with slices representing different sites of the tumor.

Besides availability of sufficient tissue material and ethical issues raised by research with patient tumors, the most relevant disadvantage of this cultivation technique is the difficulty to define adequate controls. This is simply based on the fact that ex vivo results from individual tumors cannot be reproduced in vivo. For this reason and in analogy to clinical studies, a considerable amount of patient samples are needed to achieve reliable results. Ultimately, to answer the question if tumor tissue slices will really be predictive of the patients’ therapy response, a systematic comparison with clinical trials using the same drugs in vivo and ex vivo will be required. Such comparisons have not been performed so far.

There are also important technical issues to be considered. Various mechanical stresses can influence tissue quality during surgery, pathology processing, and slicing. In addition, changes in temperature, oxygen levels and the availability of nutrients definitely occur both during the preparation of the slice and its maintenance (Fig. 3). Obviously, such non-reversible perturbations do result in a restriction of tissue slice culture to short-term experiments. These factors not only determine the tissue quality at the starting point of cultivation, and thereon, but might also influence both the drug sensitivity of cancer cells ex vivo and their use to investigate tumor biochemistry. It is therefore essential to optimize slicing procedures as well as culture conditions to minimize such artifactually induced changes (Fig. 3). Different slicing procedures with slicers such as Krumdieck [135, 138, 146, 151, 157, 163], manual choppers [153, 154], and vibratomes [140, 153, 154, 163] as well as different culture conditions including floating [135, 136, 138, 142, 152], filter-supported [140, 148, 153, 154], and rotating [149] systems have been published. However, to date no systematic analysis or comparison of the different preparation and culture conditions is available. Such “basic validation” studies could be performed with tumors from cell line derived xenografts, or even syngeneic models of mouse tumors, providing relatively infinite and homogenous source of tissue material. The syngeneic models would have the advantage of providing tumors with a complete stroma, particularly if genetically engineered mouse models were to be investigated as surrogates for human tumors.

What remains to be established, permitting tissue slices to be a valuable adjunct to a variety of 3D platforms in vitro, is with what fidelity the biochemical circuitry of a slice represents that of the in situ tumor from which it was derived. What is the impact of the slicing procedure itself on the activation of stress and inflammatory responses? Do these procedures “re-wire” the biochemical circuitry, both of the tumor cells and their stroma, sufficiently to influence canonical pathways that determine drug response, for example by modulating DNA repair pathways or signaling cascades? Then, on incubation, at what period can an optimal window of fidelity to the original tumor be described permitting investigation of either drug responses or the circuitry associated with a novel, potential drug target? Until these questions are addressed in some depth, tumor slices should be used with circumspection. Claims that fresh slices reflect the in situ tumor, permitting programs of personalized medicine ([http://indivumed.com, [139, 140, 153, 154]]) should be carefully scrutinized. The IMI program PREDECT (www.prepect.eu) has one of its goals the task of comprehensively characterizing the biochemistry of tumor tissue slices. It hopes to define the limits of this exciting technical platform, which should capture tumor complexity and heterogeneity in vitro.

5 Concluding remarks

In reviewing the considerable progress that has been made in attempts to create in vitro models that are more representative of tumor complexity, particularly with cells that are surviving and/or growing in three dimensions with stroma, a number of caveats were highlighted. These complex in vitro platforms are surely an advance, recognizing that existing models have limitations (Table 1). Several novel models have been shown to differ
in phenotypic and molecular characteristics from “standard” two-dimensional cultures. This is encouraging. Is this enough to move these advanced models alongside existing models or even replace them? The former, yes. The latter, no. These “new” models should be embraced by industry and the academic community so that they can be characterized, tested and refined further by the community. “Validation” that these novel models will be more predictive of clinical outcome will require a retrospective analysis of their utility based on clinical results.

What remains to be done? A two-pronged approach is required: first, there are many continuing technical questions to address. How to grow mixed stromal-tumor complexes in appropriate ratios reflecting pathology? What type of ECM to add – such as collagen – Matrigel mixes or how to move toward defined ECM components and what are the flow and physico-chemical environmental characteristics of a particular pathology/cancer stage? How to optimize tissue slice preparation and maintenance? The word “optimize” is key. Optimize toward what? This is where considerable effort is required. While cancer cell lines have been characterized for changes in their genomes and compared with human tumors, what has not been sufficiently characterized is how the biochemical circuitry of a novel in vitro platform (and even the 2D cell lines) compares with a tumor in situ. Demonstrating that a novel platform is “different” is insufficient. As it becomes clear that a single target approach to drug discovery is unsustainable, the ever increasing power of systems biology should allow hypotheses to be tested regarding the rational drugging of complimentary “nodes” in a network [164]. A systems approach is only viable if models of pathology and biology, used for validation, are representative of in vivo conditions. While very important strides have been made in modeling biology and pathology in animals, for example creating models to validate novel targets using genetically engineered mice [165], there is a continued need to develop and advance the use of appropriate in vitro models, such as those described here. However, the community should be prudent about the use of novel platforms which often remain as “black boxes” and should contribute to their further characterization.

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