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*Teaser This review highlights currently used 3D **in vitro** models of tumors expressing EGFR family receptors with special focus on factors affecting tumor cell biology and resistance to antitumor agents.*

3D *in vitro* models of tumors expressing EGFR family receptors: a potent tool for studying receptor biology and targeted drug development

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Carcinomas overexpressing EGFR family receptors are of high clinical importance, because the receptors have prognostic value and are used as molecular targets for anticancer therapy. Insufficient drug efficacy necessitates further in-depth research of the receptor biology and improvement in preclinical stages of drug evaluation. Here, we review the currently used advanced 3D *in vitro* models of tumors, including tumor spheroids, models in natural and synthetic matrices, tumor organoids and microfluidic-based models, as a potent tool for studying EGFR biology and targeted drug development. We are especially focused on factors that affect the biology of tumor cells, causing modification in the expression and basic phosphorylation of the receptors, crosstalk with other signaling pathways and switch between downstream cascades, resulting ultimately in the resistance to antitumor agents.

Introduction

In the modern conditions of high mortality from cancer, the search for new and more-effective antitumor drugs remains an extremely demanding area for biomedical research. The progress achieved in the study of the molecular basis of carcinogenesis and the detection of subtle biochemical differences between normal and tumor cells has led to the rapid development of targeted antitumor therapy. The development of targeted drugs relies on the use of distinctive features (targets) of tumor cells of a particular type to ensure the selectivity of the action of the therapeutic agent. Proteins of the human epidermal growth factor receptor (EGFR) family (HER1–4) are widely known as molecular targets of tumor cells. Normally, these receptors regulate proliferation, differentiation and apoptosis of cells, and their functioning disorders are characteristic of several carcinomas and are associated with poor disease prognosis [1]. To date, several drugs have been introduced into clinical practice for targeting HER-positive tumors (see below), but their inadequate efficacy and the acquired resistance of tumor cells remain a serious problem.

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In this regard, the study of the biology of the receptors of this family and the development of specific targeted drugs remain relevant.

Despite the rapid development in the field of cancer biology and development of therapeutic agents, the overall proportion of antitumor drugs that have successfully passed clinical trials and are approved for clinical use remains low [2]. At the same time, many agents being developed do not pass beyond Phase II and III clinical trials owing to inadequate efficacy [3]. The complex structure of solid tumors *in vivo* causes objective difficulties in studying the tumor biology and evaluating the therapeutic potential of the tested drugs *in vitro*. Despite the wide use of 2D monolayer culture of tumor cells, this approach does not reflect certain features of a real tumor, primarily its 3D organization. The 3D structure of the tumor suggests numerous cell–cell contacts, as well as significant gradients in the concentration of gases, nutrients and catabolites throughout the tumor mass, causing a specific microenvironment of cells in different layers. In turn, this leads to the heterogeneity of cellular populations in the tumor, resulting in the variation of the expression profiles of many genes and the metabolism of cells. Heterogeneity of the tumor largely determines the response of the neoplasm to the therapy. In addition, the presence of intercellular contacts and extracellular matrix (ECM) makes it difficult for large drug molecules to penetrate into the tumor, so their effectiveness depends strongly on their ability to diffuse through the tumor mass [4]. Thus, the improvement of model systems for testing potential antitumor drugs *in vitro* is currently important. In this regard, 3D tumor models *in vitro* are becoming increasingly widespread. The simplest 3D models are one-cell-type conglomerates of tumor cells (spheroids) and are mainly designed to take into account the presence of intratumoral gradients [5]. More-complex models include not only tumor cells but also cellular components of the tumor stroma (multicell-type 3D models), ECM (matrix-based 3D models) and its functional analogs (e.g., hydrogels), and/or liquid flow (microfluidics-based 3D models) [6,7]. These types of 3D models more realistically convey the interaction of the noted elements in the tissue structure. Structural proximity to a real tumor determines the greater relevance of 3D models and should ensure studying the biology of carcinogenesis and tumor growth more adequately. The practical aspect of the approach is in the more accurate prediction of the antitumor efficacy of the newly developed agents at the early stage of *in vitro* testing [8,9]. This review focuses on the currently used 3D *in vitro* tumor models with the expression of EGFR family proteins as promising targets for targeted antitumor therapy.

Receptors of the EGFR family as targets for drug delivery

The biology of the EGFR family proteins

The EGFR family is represented by four receptor tyrosine kinases: HER1 (EGFR), HER2, HER3 and HER4 (ErbB1–4). The EGFR family (or HER family) members activate a mitogenic signal transduction system, mediated by phosphatidylinositol (PI3K/Akt pathway) and G-protein Ras (Ras/Raf/MEK/MAPK pathway) metabolism. Normally, a complex signal transduction network triggered by HER controls cell proliferation and differentiation and regulates cell adhesion and apoptosis. At the same time, alterations at various levels of this network are at the heart of the malignant transformation of cells [1].

HER1 (EGFR) and HER4 proteins have a typical receptor tyrosine kinase structure, represented by three domains: the extracellular (receptor) N-terminal domain, the transmembrane domain and the intracellular (tyrosine kinase) C-terminal domain (Fig. 1). The extracellular domain, in turn, consists of four subdomains (I–IV) capable of changing the relative orientation in response to ligand binding. In the absence of a ligand, the extracellular domain is in a closed conformation supported by intramolecular disulfide bonds between cysteine-rich subdomains II and IV, which determines the monomer (inactive) state of the receptor. The ligand-binding site is formed by the subdomains I and III, so that interaction with the ligand induces their juxtaposition and the transition of the extracellular domain to the open conformation, thus breaking the connection between domains II and IV and changing the relative orientation of domains II and III. Such a rearrangement leads to the exposure of regions of domains II and IV that are necessary for dimerization with the receptor partner, which is also in an open conformation. In response to dimerization, the conformation of the kinase domain changes (activation) and autophosphorylation of the C-terminal tyrosine residues occurs. Phosphotyrosines of kinase domains of receptors serve as a starting point for signal transduction in the cell, being the binding sites of adapter proteins containing Src homology-2 (SH-2) domains or phosphotyrosine-binding domains (PBDs) [10,11].

Distinctive features of the HER2 protein concern the structure of the extracellular domain. For example, several amino acid substitutions have been found in the extracellular domain of HER2 in positions that provide interaction of subdomains II and IV of the HER1 and HER3, whereas the subdomains I and III, by contrast, stably contact each other. Thus, HER2 has a constantly open conformation that does not depend on the interaction with the ligand [i.e., the receptor is in the active (ready to dimerize) state] (Fig. 1). These structural features are in good agreement with the fact that the natural HER2 ligand has not been found. HER2 is considered as a co-receptor that normally forms functionally active heterodimers with other proteins of the HER family after their activation by the corresponding ligands: epidermal growth factor (EGF) and transforming growth factor (TGF)α, in the case of heterodimerization with HER1, or neuregulins, in case of heterodimerization with HER3 or HER4 [12]. The HER2 protein as a heterodimerization partner plays a very important part in signal transduction. It has been found, in particular, that heterodimers with HER2 are characterized by a greater ligand-binding affinity owing to a slower dissociation of the receptor–ligand complex. In addition, HER2 slows the internalization of the heterodimeric complex and/or accelerates its recycling to the cell surface, preventing lysosomal degradation [12–14]. The listed properties of HER2 cause its resistance to negative feedback regulation and were discovered a relatively long time ago [15,16]; however, their molecular mechanisms are still not completely clear. Thus, a mechanism of HER2 stabilization on the membrane is proposed owing to the association with proteins containing the PDZ domain (e.g., erbin) [17]. In addition, HER2 is characterized by a weak interaction with ubiquitin-ligase Cbl, which is necessary for effective internalization and degradation of the ligand-activated (autophosphorylated)

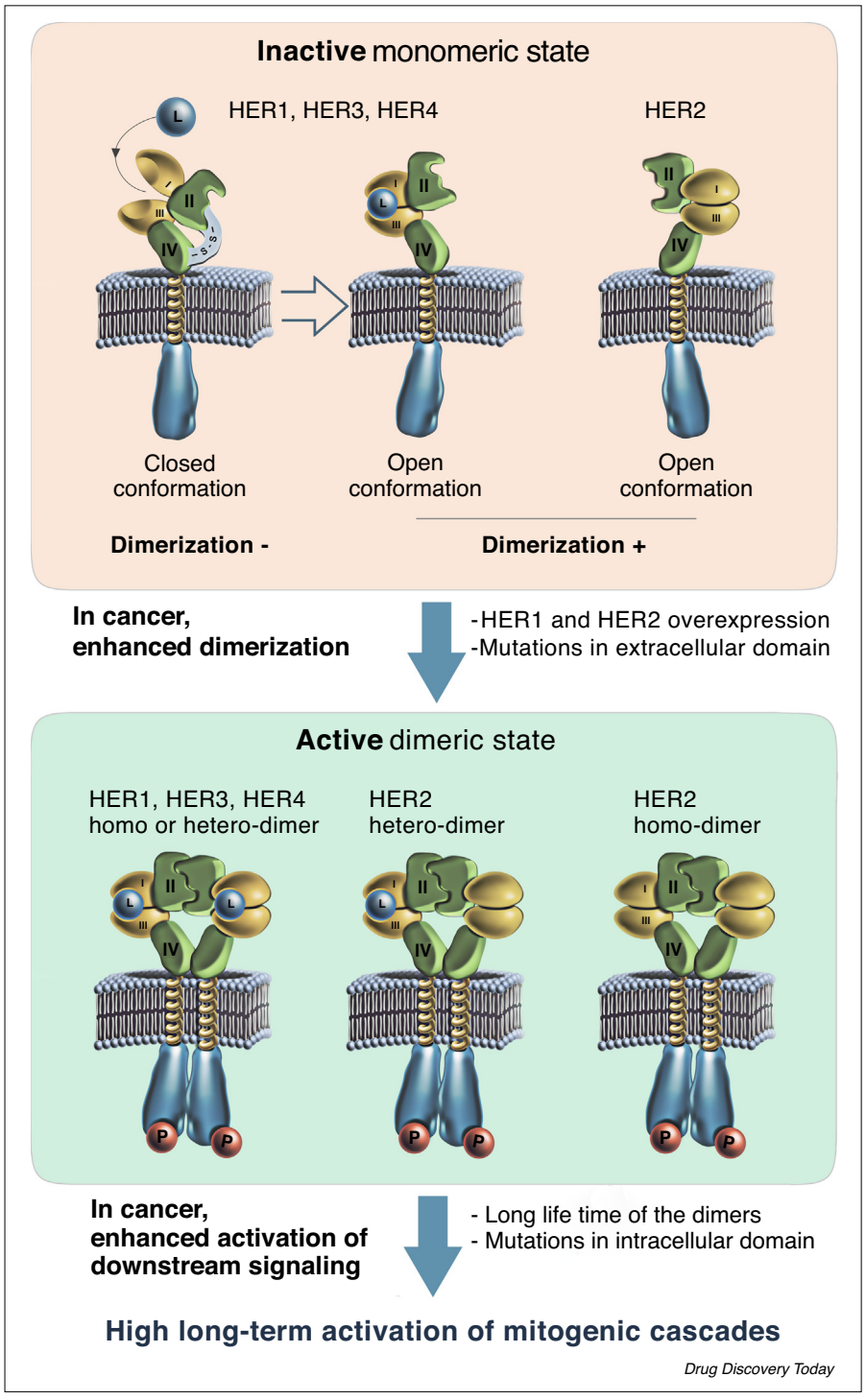

FIGURE 1

Structure and function of EGFR family receptors. The EGFR tyrosine kinase family includes EGFR (HER1), HER2, HER3 and HER4 proteins, which normally trigger a complex signal transduction network controlling cell proliferation, differentiation, adhesion and apoptosis. Receptor monomers exist in two conformations: closed (no ligand, no dimerization) and open (upon ligand binding, ready for dimerization), with HER2 being an exception that has a constantly open conformation but no known ligand. In cancer, HER1 and HER2 overexpression as well as mutations in their extracellular domains enhance receptor dimerization and activation. Activated homo- and hetero-dimers transduce proliferative signals into the cell. In cancer, longer life of dimers is observed along with mutations in the intracellular domain that strongly increase kinase activity and subsequently enhance mitogenic signal transduction.

HER1 [13]. Interaction with chaperone Hsp90 is also considered as an important criterion for HER2 stability; inhibition of Hsp90 with geldanamycin leads to the dissociation of its complex with the HER2 kinase domain, thus making recognition sites available to ubiquitin ligases, which is followed by internalization, ubiquitinylation and degradation of the receptor [18]. It is worth noting that the contribution of these various factors affecting the HER2 turnover in the cell and, accordingly, its functional activity,

could depend on the type of cells and the level of receptor expression. In general, these features provide a longer duration of signal transduction from HER2-containing heterodimers, which ultimately leads to more-efficient activation of mitogenic signaling cascades than in the case of homodimers of the rest of the family. Among the structural features of the HER3, the main ones are defects in the structure of the tyrosine kinase domain, which prevent the kinase activity of this receptor even when binding a specific ligand. Similarly to HER2, HER3 is not an autonomously functioning receptor tyrosine kinase, but is able to form functionally active heterodimers with other members of the family [19].

Role of the EGFR family in carcinogenesis

Alteration of the structure and functioning of the EGFR family accompanies the development of several tumors of epithelial origin. The main mechanisms for such alterations are somatic mutations and amplification of the gene, leading to the overexpression of the receptor (Fig. 1). Mutations in the form of small inserts or deletions can affect the kinase and extracellular domain of the receptor. Thus, small deletions and substitutions of single amino acids in the EGFR kinase domain are observed in ~17% of non-small-cell lung carcinomas (NSCLC) and ~10% of gliomas [20]. These mutations affect the ATP-binding pocket of the kinase domain and significantly enhance the catalytic function of the receptor by interfering with autoinhibition of the kinase [21]. Mutations in the kinase domain of HER2 occur in ~2–4% of lung adenocarcinomas [22], as well as in ≤5% of gastric, breast and colon carcinomas [23]. These mutations cause a conformation change in the kinase domain, increasing the affinity for ATP, which ultimately leads to an increase in ligand-independent kinase activity [24]. Deletion in the extracellular domain of EGFR (EGFRvIII variant), which causes the absence of subdomains I and II of the extracellular domain, is often found in glioblastomas. In the absence of subdomain II, the extracellular domain has a constitutively open (active) conformation [20]. In lung adenocarcinoma cells, the amino acid substitutions in subdomain II of the extracellular domain of HER2 were found to disrupt the formation of intramolecular disulfide bonds. Free cysteine residues form intermolecular disulfide bonds, causing constitutive homodimerization and kinase activation [25]. In breast carcinomas, mutant HER2 variants are often found with extracellular domain deletion, together referred to as p95HER2 [26]. The most active among them is a 100–115 kDa fragment. It was found that its small extracellular domain contains several cysteine residues, which form intermolecular disulfide bonds, thus causing constitutive homodimerization and kinase activity [26]. The expression of this truncated HER2 fragment has been shown to correlate with a high metastatic tumor potential [27]. Mutations of HER3 and HER4 and their role in the functioning of receptor dimers have been poorly understood. In particular, a number of HER3 mutants with amino acid substitutions in different receptor domains was found, but their effect on the growth of tumor cells was revealed only in the presence of the active HER2 protein [20].

Another alteration in the regulation of the EGFR family in tumors is their overexpression. In particular, in breast, pancreas, bladder and lung carcinomas (NSCLC) EGFR overexpression occurs [28–32], and in glioblastomas mutant variant EGFRvIII is overexpressed [32,33]. HER2 overexpression is observed in breast and ovarian (20–30%) [34,35], esophagus and gastric (15–20%) [36], and prostate (25%) [37] carcinomas and is associated with an increased risk of metastasis and resistance to chemotherapy [13]. In the case of HER2 overexpression along with ligand-dependent heterodimerization spontaneous homodimerization is possible, resulting in a constant ligand-independent activity of HER2 [38].

Targeting the EGFR family in cancer therapy

High levels of expression of the EGFR family receptors, characteristic for many types of carcinomas, causes significant differences in the number of receptor molecules on the surface of malignant and normal cells. Along with the involvement of these receptors in the pathogenesis of tumors, this opens the prospect of their use as a molecular target for targeted therapy. To date, several agents targeted to HER1 and/or HER2 have been approved for clinical use. Two strategies of the HER1/2-targeted therapy have been successfully applied in clinical practice: (i) intracellular kinase domain blocking by using low molecular weight inhibitors and (ii) affecting the extracellular domain of the receptor with specific monoclonal antibodies and their derivatives.

The mechanism of action of first-generation reversible low-molecular-weight inhibitors (e.g., gefitinib, erlotinib, lapatinib, vandetanib) is based on competition with ATP for the binding site of the receptor kinase domain [39–41]. The interaction of these inhibitors with receptors is characterized by a slow dissociation, which ensures a prolonged inhibition of autophosphorylation and further signal transduction. The advantages of next-generation low-molecular-weight inhibitors that covalently and irreversibly bind to the receptor kinase domain are their higher potency combined with a low level of secondary resistance [42,43]. For most FDA-approved (e.g., afatinib, osimertinib, olmutinib, neratinib) and clinically tested drugs, high activity is shown against mutant receptor forms.

Anti-HER therapeutic antibodies bind to the extracellular domain of the targeted receptor with high affinity, usually blocking the ligand-binding region. This principle of action underlies the therapeutic effect of the humanized anti-HER2 antibody trastuzumab (Herceptin®), the chimeric anti-HER1 antibody cetuximab (Erbitux®) and the fully human anti-HER1 antibodies panitutumab (Vectibix®) and necitumumab (Portrazza®) which are used in clinical practice. The presumed mechanism of action of these antibodies includes the activation of antibody-dependent cellular cytotoxicity, the inhibition of proteolytic cleavage of the extracellular domain of the receptor and the blocking of the receptor-mediated signaling pathways. The latter is possibly caused by the induction of endocytosis of the receptor with its subsequent degradation, as well as interruption of the interaction of the receptor with kinases (in particular, proto-oncogene tyrosine protein kinase Src), which govern the underlying signal transduction pathways [44–47]. The therapeutic effect of the humanized antibody perthuzumab (Perjeta®) is driven by another principle [48]. Pertuzumab binds to subdomain II of the HER2 extracellular domain and blocks its ligand-dependent heterodimerization with other receptors of the family, thereby disrupting signal transduction into the cell [49].

Despite the rather wide use of these HER-specific drugs, which is more successful with their combined action and combination with

chemotherapeutic agents, their insufficient effectiveness remains a serious problem. Several studies report the insufficient efficacy and discontinuation of clinical trials of anti-EGFR and anti-HER2 drugs based on monoclonal antibodies and tyrosine kinase inhibitors for the treatment of certain types of tumors (for detailed reviews, see Refs [50–52]). It is determined mostly by tumor resistance acquired in the course of treatment. The mechanisms of the resistance are very diverse and reflect, on the one hand, the branching of a complex system of HER-mediated signal transduction with the possibility of activating alternative signaling pathways and, on the other hand, high genetic diversity of tumor cells. Thus, resistance to monoclonal antibodies can be caused by mutations or altered transcriptional activity of genes of HER-mediated signaling pathway proteins. This leads to increased signal transduction (e.g., a decrease in the expression level of the PTEN phosphatase gene regulating PI3K/Akt signaling phosphorylation, as well as an activating mutation or overexpression of PI3K). Moreover, in response to receptor blockade by the antibody, bypass signaling pathways can be triggered by other receptor tyrosine kinases [i.e., insulin-like growth factor-1 receptor (IGF-1R), vascular endothelial growth factor receptor (VEGFR), hepatocyte growth factor receptor (c-MET, HGFR), etc.] [53,54]. The acquired resistance to low-molecular-weight inhibitors, in addition to triggering bypass signaling cascades, is often associated with mutations in the kinase domain of the receptors that increase its affinity to ATP and, in turn, reduce the efficiency of competitive binding with the inhibitor [55–57].

A promising strategy to increase the specific toxic effect on tumor cells is the creation of bifunctional structures containing a potent toxic component [58,59]. In such constructs, the antibody or its functional analog performs a targeting function, providing targeted delivery of the toxin to the tumor cells. Thus, the chemical conjugate of trastuzumab with the maytansinoid microtubule assembly inhibitor trastuzumab emtansine (Kadcyla®) has successfully passed clinical trials [60]. Similar conjugates can be obtained using effector components of a diverse nature and mechanism of action, allowing the implementation of various methods for treating tumors. Thus, agents for boron-neutron capture therapy in EGFR-positive brain tumors, which are conjugates of borated dendrimers and an EGFR-specific antibody (cetuximab) [61] or ligand (EGF) [62], have been created and tested in preclinical trials. It should, however, be noted that obtaining such antibody–drug conjugates (ADCs) is a time-consuming, technically complex and expensive process. An alternative approach is to obtain bifunctional recombinant targeted toxins. In this case, cytotoxic proteins of various origin are used as an effector substance. For example, bacterial and plant toxins, enzymes and cytotoxic factors of human origin (apoptosis-inducing factor AIF-1, granzymes, RNAse), as well as protein photosensitizers, among others. Several targeted toxins based on recombinant antibodies (immunotoxins) specific to the receptors of the EGFR family undergo preclinical [63–77] and clinical [78–80] trials. In addition, the use of alternative scaffold proteins, in particular designed ankyrin repeat proteins

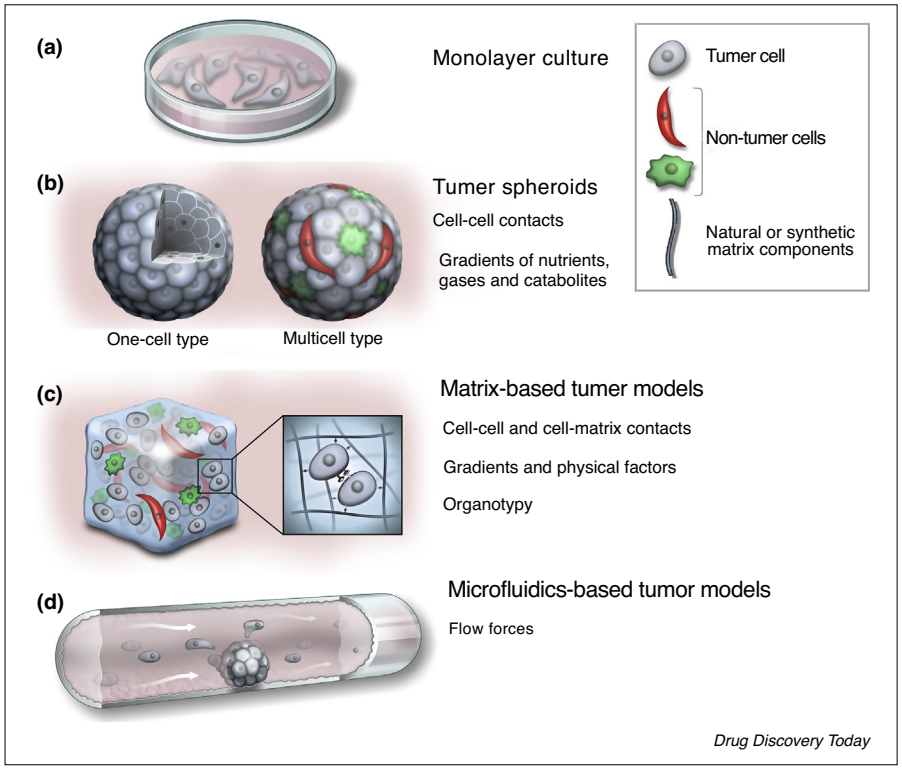

FIGURE 2

In vitro models of tumors expressing EGFR family receptors: 2D monolayer culture **(a)**, tumor speroids of one-cell and multi-cell types **(b)**, tumor models based on natural and synthetic matrices, including organotypic models **(c)**, and microfluidics-based models **(d)**. The more complex the model the greater the resemblance to the real tumor. Improvement of the *in vitro* tumor models is realized via introduction of stromal cells and/or matrix fibers to the tumor cell culture, thus providing morphological and functional features of the tumor *in vivo*. Physical factors, such as liquid flux force, can also be modeled using the principles of microfluidics.

(DARPins) and affibody as a targeting module for recombinant targeted toxins, has been proposed [81–83].

3D models of tumors expressing EGFRs

Tumor spheroids in a liquid-based environment

One-cell-type tumor spheroids

The most simple 3D model of a tumor is a compact spherical conglomerate of tumor cells: tumor spheroids (Fig. 2). Spheroids are formed by numerous contacts between tumor cells when they are cultured under free-floating conditions and reproduce the avascular stage of the tumor node development (i.e., a small primary tumor, an early metastasis or a tumor zone located far from the vessel). The most common methods to generate tumor spheroids are spontaneous aggregation, liquid overlay on agarose, hanging-drop cultures, spinner-flask cultures, rotary cell culture systems and ultra-low attachment plates [84]. Simplicity in the production and use of spheroids has led to their widespread use as models for studying cell biology in the context of the 3D tissue structure and developed intercellular contacts.

Using spheroids of tumor cells expressing EGFRs, several questions could be addressed concerning the biology of these receptors, as well as the influence of the 3D organization on the effectiveness of targeted antitumor agents. Thus, Pickl and Ries [85] compared signal transduction from HER2 in breast carcinoma cells (SKBR-3) and human ovarian carcinoma cells (SKOV-3) for the first time. It was found that, when the cells are aggregated in a spheroid, the HER2 protein is found in the lipid rafts of the plasmalemma. This leads to a change in its functioning – cells in the spheroid form the HER2 homodimers with subsequent activation of the MAPK-mediated signal transduction pathway. Cells in the monolayer culture are characterized by the formation of HER2/HER3 heterodimers and the initiation of signal transduction via PI3K. The HER2 localization on the membrane accounts for altered sensitivity of cells to the anti-HER2 antibody trastuzumab. Trastuzumab binds more efficiently to the HER2 homodimers, which leads to a greater antiproliferative effect of this antibody against spheroids, compared with the monolayer [85].

In addition to receptor localization, the 3D structure can influence the level of basal phosphorylation of receptors and its response to stimulation. A detailed comparison of EGFR biology in spheroids and monolayer culture of human lung carcinoma revealed that, under 3D conditions, the expression of this receptor is decreased but its basal phosphorylation increases, in comparison with the monolayer [86]. At the same time, the activation of phosphorylation in response to stimulation by specific ligands in spheroids is lower than in the monolayer. By contrast, for BT-474 human breast carcinoma cells, the level of EGFR and HER2 phosphorylation in spheroids was lower than in a monolayer [87], although the expression of both receptors was higher. It is interesting to note that the phosphorylation of EGFR and HER2 was observed only in cells from several outer layers of the spheroid. Reduced receptor activation in a 3D model, apparently, caused a greater sensitivity of spheroids to tyrosine kinase inhibitors gefitinib and lapatinib, compared with monolayer culture [87]. The principal difference in the activity of these receptors under different conditions of cell culture testifies to the importance of using a 3D model as more-approximate to the real tumor than the 2D monolayer system.

The greater structural proximity of 3D models to *in vivo* conditions suggests a greater similarity of the response to the therapeutic effect. Indeed, EGFR-positive human colon adenocarcinoma SW48 spheroids were shown to be more resistant to the therapeutic antibody cetuximab as compared with the monolayer of this line, coinciding with the effect in patients. The spheroid cell secretome, in contrast to the monolayer, was revealed to be sensitive to cetuximab therapy. The most sensitive component of the secretome was phosphorylated EGFR (pEGFR), the secretion of which increased under the cetuximab action on a cetuximab-sensitive tumor. Relying on these results, the authors suggested using pEGFR as a noninvasive marker of the response of EGFR-positive colorectal carcinoma (CRC) to cetuximab therapy, which is of great clinical importance [88].

Differences in the sensitivity of tumor spheroids and monolayer cell culture to HER2-specific recombinant immunotoxins based on *Pseudomonas* exotoxin A were shown [89]. Efficacy of photoimmunotherapy agents based on the HER2-specific antibody trastuzumab [90,91] or EGFR-specific antibodies cetuximab and panitumumab [92] also varied in 2D and 3D tumor models. It should be noted that, in the latter case, conjugates of photosensitizer and anti-EGFR antibody showed comparable activity against spheroids *in vitro* but different efficacy *in vivo* – the panitumumab-based conjugate was more effective owing to a longer circulation in the bloodstream.

Studies of the resistance mechanisms of head and neck squamous cell carcinoma (HNSCC) CAL27 to the anti-EGFR tyrosine kinase inhibitors [93] showed that resistant and sensitive tumor cells of the monolayer did not differ in growth parameters and metabolic profile, whereas in spheroids resistant cells showed more-aggressive growth, which was accompanied by an increase in the level of aspartate, alanine, glycerophosphocholine and creatine plus phosphocreatine. In the HNSCC xenograft model *in vivo*, in addition to the effects detected in the 3D model *in vitro*, the glucose transporter GLUT-1 was upregulated, indicating the Warburg effect. The appearance of the Warburg effect is probably related to the greater role of hypoxia and oxidative stress in a more complex microenvironment *in vivo*. These conditions stimulate the expression of hypoxia-inducible factor (HIF)-1, which, in turn, causes the switching of metabolic pathways.

Multicell-type tumor spheroids

In addition to the above examples of homotypic (one-cell-type) spheroids formed only by tumor cells, heterotypic (multicell-type) spheroids are also used (Fig. 2). Such spheroids represent a more complex 3D model including not only tumor cells but also the cellular components of the tumor stroma: fibroblasts, endotheliocytes, immune cells, among others, and allow assessment of the influence of non-tumor cells on tumor cell biology during carcinogenesis and tumor development. To obtain such models, several cell types are pre-mixed, and then the suspension is cultivated under nonadherent conditions [87,94], or cells are co-cultivated in a special adjacent co-culture system [95]. Thus, adjacent co-culturing of tumor cells with tumor-associated fibroblasts was used to create a model of epithelial–mesenchymal transition [95]. Co-cultivation has been shown to mutually affect both types of cells: fibroblasts underwent activation and proliferation and exhibited increased migration activity. In tumor cells (i) expression of fibronectin, alfa-smooth muscle actin α-SMA, EGFR and connective

tissue growth factor CTGF was increased, (ii) expression of laminin, type I collagen, membrane proteins β-catenin and E-cadherin was decreased and (iii) the content of active TGFβ1 in the culture medium was also increased. Changes in the expression profile of these proteins indicate a process of epithelial–mesenchymal transition and were observed only under 3D culture conditions, not in a monolayer culture.

3D tumor models in natural matrices

Along with cell–cell interactions, cell–matrix interactions can also be crucial for neovascularization, invasion and distant metastases formation, as well as induction of the immune response, among other things. High importance of the ECM in the development of tumors causes the widespread elaboration and use of appropriate model systems (Fig. 2). To model tumors that express EGFRs, 3D models are most widely used with collagen-1 gels and Matrigel® (laminin-rich ECM), derived from basal membrane proteins, as the natural matrix. When obtaining such models, the cells are cultivated either within or on top of the matrix.

Homotypic 3D tumor models in natural matrices

The simplest matrix-based models are those that include only tumor cells. HER2 signaling cascades and the response of cells to HER2-specific targeted drugs (i.e., trastuzumab, pertuzumab and lapatinib) were investigated in models of HER2-overexpressing human breast adenocarcinoma in Matrigel® [96]. All human breast adenocarcinoma cell lines studied (i.e., AU565, SKBR-3, HCC1569) exhibited a switch in HER2 signaling from the PI3K/AKT pathway to the RAS/MAPK pathway when grown in 3D lrECM. This resulted in a change in the response to therapeutic treatment. In particular, SKBR-3 cells in 3D lrECM were less sensitive to trastuzumab, compared with a monolayer of cultured cells. According to the study, inhibition of β1-integrin, a key membrane component of cells binding to ECM proteins, significantly increased the sensitivity of tumor cells to the therapeutic anti-HER2 antibodies trastuzumab and pertuzumab in the 3D model. In a similar model [97], inhibited phosphorylation of HER1–3 receptors in HER2-overexpressing human breast adenocarcinoma cells resistant to lapatinib or lapatinib plus trastuzumab combination was shown. At the same time, downstream kinases in the β1-integrin signaling pathway [i.e., focal adhesion kinase (FAK), Src] were, by contrast, upregulated (phosphorylated). Thus, it is assumed that, in the resistant cells, the bypass pathway for proliferative signal transduction occurs via β1-integrin. This is confirmed by the fact that the growth of these cells is suppressed when either β1-integrin is blocked with specific antibody or siRNA or FAK undergoes pharmacological blocking. Interestingly, in cells resistant only to trastuzumab, the phosphorylation of HER1–3 proteins persists and the anti-β1-integrin antibody has virtually no effect.

A matrix-induced shift in the activity of the signal transduction pathway from AKT to MAPK was also shown for the estrogen receptor/HER2-positive breast cancer model [98]. The authors also suggest that the decrease in the trastuzumab effectiveness against cells in 3D culture is caused by the activation of integrin-mediated signaling cascades in the laminin-rich matrix. This assumption is confirmed by the positive effect of blocking β1-integrin and mitogen-activated protein kinase kinase (MEK), which participate in the integrin-mediated signaling.

The effect of matrix components on EGFR downstream signaling pathways has also been revealed. In particular, on the 3D model of EGFR-positive CRC in type I collagen [99] it was shown that the resistance to cetuximab arising in such a model is caused by an increase in the phosphorylation of two other tyrosine kinases: MET and RON. This possibly testifies to switching EGFR-mediated signal transduction to the bypass pathways. So, inhibition of MET and RON tyrosine kinases with crizotinib restored the sensitivity of the cells to cetuximab.

In several studies, 3D tumor models in matrices were used to study the radiosensitivity of tumors [100–103]. In particular, it was demonstrated that the combined blocking of membrane proteins EGFR and FAK increased the radiosensitivity of HNSCC cell lines UTSCC15 and SAS more than separate inhibition of these proteins did [100]. This is considered to be caused by the non-overlapping of the downstream signaling pathways. In addition, blocking of EGFR signaling by the monoclonal antibody cetuximab in the 3D HNSCC model in matrices, the signal pathway through the kinase of the MAPK family, JNK2, is activated, thus reducing the effect of cetuximab and increasing radioresistance [103]. The results corresponded to the situation observed in a xenograft tumor *in vivo*, but not in monolayer cell culture. 3D tumor models in natural matrices were also used to evaluate the antitumor efficacy of the therapeutic agents being developed: HER2-specific gelonin-based immunotoxins against breast carcinoma [71]; recombinant EGFR-specific protein composed of single-domain antibody (sdAb) and tumor-penetrating peptide iRGD against gastric cancer [104]; gingerol against breast carcinoma [105].

3D tumor models in decellularized tissue matrices

A topical approach for obtaining 3D tumor structures is also the use of decellularized (DCL) tissue matrices. The technology of obtaining and subsequent recellularization of such matrices is widely used in regenerative medicine. Methods for obtaining the matrices of the liver, lungs, heart muscle, kidneys, pancreas, brain and spinal cord, skin, among others, have been developed [106]. The process is based on the 'dissolution' of cell membranes and the washing out of the cell debris with the maximum possible preservation of the ultrastructure of the tissue and the chemical composition of the matrix. The use of DCL matrices in oncology takes into account tissue-specific features of the chemical composition of the ECM and, accordingly, their influence on cellular invasion and development of primary tumors and metastases [107]. It was demonstrated on a DCL tissue-matrix-based model of human lung carcinoma [108] that the cells of the resulting 3D model proliferated more slowly than the monolayer culture and exhibited induction of invasion and epithelial–mesenchymal transition (EMT) in response to the TGFβ1 treatment, as well as a more pronounced response to the gefitinib.

Organotypic tumor models

There is also a so-called organotypic tumor model, which is a more sophisticated model that combines tumor cells with stromal cells. The development of organotypic models was pioneered by Bissell and co-workers [109]. Organotypic models allow even-more-accurate *in vitro* reconstitution of the morphological and functional properties of tumor tissue *in vivo* and enable study of the contribution of stromal components and matrix to a malignant transformation. Often, to create such a model, stromal cells are first cultured in the matrix and then tumor cells are seeded on such a

scaffold. Thus, collagen or Matrigel®-based organotypic models of EGFR-positive esophageal carcinoma were obtained, consisting of esophageal or skin fibroblasts and esophageal carcinoma cells [110]. Such models indicated malignant cell penetration to the ECM, with the concomitant MMP-9 activation, and a dependence of the tumor cell differentiation degree on the type of fibroblasts in their microenvironment. So, fetal esophageal fibroblasts contributed to the development of less differentiated invasive tumor cells, whereas fetal skin fibroblasts caused the development of a highly differentiated tumor. It is assumed that the differences shown are related to the activity of cytoplasmic AKT protein kinase in fibroblasts, because the level of AKT phosphorylation in stromal fibroblasts correlated with the degree of invasive potential of the tumor cells. The AKT protein kinase, being one of the key effectors in the signal transduction pathways from growth factors and cytokines, can be activated in response to TGFβ1, and is also regulated by β1-integrin [110]. It was further shown [111] that hepatocyte growth factor (HGF) secreted by cancer-associated fibroblasts (CAFs) enhances the ability of esophageal epithelial cells to invade the ECM. Thus, the organotypic models enable studying a complex network of interactions of tumor cells, stromal cells and matrix components.

It is known that the proinflammatory interleukin (IL)-6 is upregulated in certain tumors, including high-grade serous ovarian cancer, and has a pro-tumor effect. In clinical trials, anti-IL-6 antibodies are used, but their effectiveness is inadequate. To address this problem, organotypic models of ovarian carcinoma were obtained based on AOCS1 and IGROV-1 cell lines, recreating the microenvironment of the omentum as a common metastasis site for peritoneal tumors [112]. Models included fibroblasts, mesothelial cells and tumor cells cultured in a collagen matrix. Antibodies against IL-6 and transcription factor STAT3 have been shown to upregulate the EGFR signaling pathway in ovarian carcinomas, and the combined effect of anti-IL-6 antibody and anti-EGFR inhibitor gefitinib effectively inhibits tumor cell growth.

Organotypic models can also be used to study the initiation of carcinogenesis. Nash *et al*. [113] demonstrated an organotypic model of a normal mammary gland, consisting of three types of cells cultivated in type I collagen: normal luminal epithelial cells HB2, myoepithelial cells and fibroblasts. In this model, it was shown that HER2 overexpression causes a state close to ductal carcinoma *in situ* (DCIS). The same was demonstrated in another organotypic model of the mammary gland, consisting of populations of myoepithelial and luminal breast cells. It was shown that when these populations are co-cultivated in a collagen gel they form a physiologically relevant bilayer structure, and that the HER2 expression in the luminal compartment leads to the appearance of DCIS [114].

3D tumor models in hydrogels

To obtain 3D tumor models, not only matrices of animal origin but also other natural or synthetic polymers, collectively called hydrogels, can be used as an extracellular scaffold. Commonly, such matrices in their initial state are inert to the animal and human cells, which allows them to be used to create a 3D architecture for neoplasm and to provide physical factors for the tumor microenvironment, excluding biochemical aspects of interactions with cells. A serious advantage of using hydrogels is an opportunity to get reproducible results owing to their precise composition. One of the examples is the alginate hydrogel. An alginate-based model of hepatocellular carcinoma was used in a microtiter-scale in standard 96-well plates and a microarray platform in a chip format [115]. It was shown that, unlike the monolayer culture and the standard 3D model-on-a-plate, the model-on-a-chip enables circumvention of the dependence of the tumor cell proliferation on their density and associated resistance to chemotherapy. Moreover, a higher expression level of β-integrin, EGFR and VEGF was observed in the 3D model-on-a-chip unlike in the monolayer, which is considered as a feature of phenotype more approximate to the situation *in vivo*.

Depending on the research objectives, hydrogels can be modified by introducing components that react with cells or natural matrix of the tumor model. For example, a hydrogel system based on polyethylene glycol-norbornene (PEG8NB), crosslinked by a peptide linker sensitive to matrix metalloprotease (MMP-sensitive linker), was used to obtain a 3D model of pancreatic ductal adenocarcinoma [116]. By varying the PEG8NB concentration, hydrogels of different stiffness were obtained. This model showed that the cells proliferated better in the soft hydrogel than in the stiff one. Moreover, EGFR inhibition did not cause cell death in the soft hydrogel, whereas a high level of apoptosis was observed in the stiff one, which was caused by a decrease in the expression of EGFR signaling pathway proteins. Another interesting result is the observed effect of cell-mediated matrix remodeling on cell survival; when using a non-MMP-sensitive linker the proliferation and metabolic activity of the cells did not decrease under the EGFR inhibition at any hydrogel stiffness. Thus, the influence of the physical properties of the ECM and cell-mediated matrix remodeling on the efficacy of antitumor drugs was established.

Tumor organoids

One of the key features of a malignant tumor *in vivo* is its genetic, epigenetic and phenotypic heterogeneity as a result of the high instability and clonal diversity of tumor cells. These particular features determine the drug resistance of the tumor and its metastatic potential, resulting in the inefficiency of diagnosis, prognosis and treatment of the disease [117]. In this regard, the creation and use of experimental tumor models that reflect these features of the tumor have objective advantages. The creation of such models is based on the use of the patient's tumor tissue. For *in vivo* studies, the patient-derived tumor xenograft (PDTX) models are used, which involve the transplantation of a tumor fragment from an actual patient to an immunodeficient animal [118]. For *in vitro* research, the tumor organoids have become widespread during the past few years. A tumor organoid is a 3D organ-like model system derived from single cells or small clusters of cells including stem cells, after disaggregating a fragment of a patient's or experimental animal's tumor tissue, grown in an ECM (Matrigel®). Organoids of primary tissue cultures from a biopsy material make it possible, firstly, to study the activity of tumor stem cells and, secondly, to develop schemes for personalized medicine and evaluate their effectiveness [119,120]. The greater physiological nature of organoids, compared with other 3D tumor models *in vitro*, as well as their potential for assessing the sensitivity of a particular clinical tumor to therapeutic effects

and developing agents for personalized therapy are beyond question. Thus, using this technology, biobanks of tumor organoids are created, including the main molecular subtypes of tumors, in particular colon cancer [120]. It was shown that tumor organoids *in vitro* reproduce the molecular-genetic profile of real tumors *in vivo*. The colon cancer biobank was used to study a clinically relevant issue of a targeted therapy of colon cancer with a mutation in the RAS protein [121]. A correlation was shown between the presence of mutant RAS and resistance to the EGFR/MEK/ERK pathway inhibition. Thus, using tumor organoids it was confirmed that schemes of inhibition of the EGFR/MEK/ERK and PI–3K/AKT signaling pathways currently used in clinic are ineffective in the case of tumors mutated in RAS. By contrast, in the case of tumors with wild-type RAS, targeting the EGFR/MEK/ERK signaling pathway might be advanced compared with anti-EGFR monotherapy.

The correlation between RAS mutation and HER3 signaling was shown on colon cancer organoids [122]. It was revealed that the expression of mutant KRAS results in an increase in the expression of the HER3 ligands, heregulins (neuregulins), which increases autocrine signaling through HER3, and also leads to abnormal 3D morphogenesis (disturbance of the apical–basal epithelial polarity). In another study [123], human breast cancer organoids were used for quantitative optical imaging of the tumor's metabolic response to therapy. Optical metabolic imaging (OMI) is a promising method for assessing the metabolic status of cells, based on the visualization of the reduced and oxidized electron carriers NADH and FAD and quantifying their ratio (redox ratio) in terms of the intensity or lifetime of their fluorescence. Thus, a high redox ratio indicates the predominance of glycolysis over oxidative phosphorylation and is often observed in tumor cells even under normal oxygenation (the Warburg effect), associating with greater malignancy. The combined effect of trastuzumab and a new PI3K kinase inhibitor, XL147, on HER2-positive trastuzumab-resistant breast cancer organoids led to a significant reduction in the redox ratio, which corresponded to the inhibition of growth of the experimental tumors *in vivo*. It was also found that the value of the redox ratio in organoids depends on the molecular profile of tumor cells and, in particular, is maximal in HER2-positive cells, which corresponds to the known fact of greater aggressiveness of tumors of this type. Thus, tumor organoids are considered to be a relevant *in vitro* model for OMI, which makes it possible to correctly assess changes in the metabolism of tumor cells in response to therapy, predicting its efficacy *in vivo*.

To study the influence of mutation in several key genes on CRC development, migration and metastasis, a series of human colon organoids was created with mutated Wnt, EGFR, p53 and TGFβ, in various combinations [124]. Orthotopic transplantation of such organoids into mice revealed that all four mutations are necessary for the proliferation and metastasis of CRC, causing the acquisition of tumor cell independence from niche factors and the ability to grow in distant sites (metastasize). The unique features of the brain ECM deserve special attention in the modeling of brain tumors and the study of therapy effectiveness. In particular, overexpression of hyaluronic acid and other ECM components observed in glioblastoma is associated with tumor progression and resistance to therapy. To recreate the conditions of the microenvironment of tumor cells in glioblastoma, a 3D *in vitro* model was created based on a PEG-maleimide hydrogel platform comprising hyaluronic acid and ligands of integrin receptors (RDG-peptides) [125]. It was shown that resistance of glioblastoma cells to the EGFR-specific kinase inhibitor erlotinib resulted from the interaction of hyaluronic acid with CD44 and SD168 receptors, as well as from a ligand-bound state of integrins that probably activate EGFR or its mutant form EGFRvIII. It was also demonstrated that the level of expression of the ECM receptors as well as kinetics of the development of glioblastoma cell resistance to erlotinib in the created 3D model correspond to those in orthotopic xenografts *in vivo*, in contrast to the simpler ECM-free gliomasphere models. Thus, this model is promising for studying the effect of a complex organized brain matrix on tumor development and its resistance to therapy.

Microfluidics-based 3D tumor models

Modern experimental oncology is also mastering and actively developing lab-on-a-chip technology, based on microfluidics principles (Fig. 2) [126]. It has obvious advantages for testing drug effectiveness, because the physical characteristics of the laminar liquid flow, implemented in this approach, provide a high-precision drug dosage. Another promising application is the study of metastasis mechanisms. Rizvi *et al*. [127] used a flow system to study the influence of flux force on the metastasis of the ovarian tumor. It was found that, in flow conditions, the 3D ovarian cancer model exhibited an upregulation of EGFR and vimentin expression, as well as a downregulation of E-cadherin expression, and the appearance of other EMT features; that is, a phenotype of an aggressive metastatic tumor. In this case, the micronodules formed in the flow are smaller in size than in the absence of flow. An analogous model of human lung carcinoma was used to study the formation of invadopodia during metastasis [128]. The involvement of EGFR signaling pathways in this process was revealed; under the influence of EGF, the formation of the invadopodia was activated.

Concluding remarks

Beyond any doubt, 3D organization of tumor tissue significantly affects the biology of tumor cells and their resistance to the action of limiting factors. The spatial architecture of the tissue with the presence of various types of cells and matrix components causes the development of a significant number of cell–cell and cell–matrix contacts that perform a mechanical and signaling role. Stiffness of the matrix fibers and mechanical compression of cells within the tissue have a certain significance. Distance from the blood and lymphatic vessels gives rise to significant gradients of nutrients, gases and metabolites throughout the tissue. In solid tumors, such gradients reach a critical value owing to the time gap between the growth of the tumor mass and neovascularization.

Indicated factors condition the low relevance of the simplest 2D models of tumor growth, widely used in cell biology and experimental oncology. To some extent, these factors are taken into account when creating 3D *in vitro* models. Such models shed light on the changes in the biology of EGFR family receptors that are related to the complexity of structural and functional bonds in the 3D tumor structure and are responsible ultimately for the effectiveness of targeted drugs. Organization of the cells in 3D structure changes the content of EGFRs on the cell membranes and their

basal phosphorylation, as well as the expression level of downstream signaling proteins [86,87]. Notably, it has been shown in different models that the 3D structure can be characterized by up- and down-regulation of these parameters. For HER2, the ability to switch signal pathways from the PI3K/AKT to the RAS/MAPK pathway is registered [85,96,98]. Such switching is considered to be induced by the receptor localization in lipid rafts of plasmalemma or cross-interaction of signaling pathways from HER2 and β-integrin participating in cell–matrix contacts. β-integrin and HER1–3 signaling pathway crosstalk can be considered as one of the resistance mechanisms, confirmed in numerous studies on 3D models, so it requires close attention in the development of personalized treatment regimens [97,98]. Along with the direct impact on the localization and activity of EGFRs, the matrix can exert an effect through the overexpression of tumor genes, in particular MMPs involved in the cleavage of membrane receptors [129]. Metabolic changes associated with hypoxic conditions emerging far from blood vessels, which are shown to enhance the aggressive phenotype of tyrosine kinase inhibitor-resistant EGFR-positive cells, should also be taken into account [93,127]. Such an effect can be attributed to the activation of the oncogenic signaling network, including EGFR-related pathways, resulting from increased glucose uptake [130]. The epithelial–mesenchymal transition observed in 3D models of tumors with the expression of EGFRs is to be separately noted [95]. It is accompanied by a change in the expression of cell adhesion and cell–cell contact proteins and proteins of the ECM, cytoskeleton, growth factors and their receptors.

Summarizing, it should be emphasized that association in a 3D structure causes changes in the biology of tumor cells owing to the presence of multiple pathways by which various factors affect regulatory mechanisms in the cell. Along with limiting drug diffusion through the cellular mass, this, apparently, causes the observed diversity of the responses of 3D models to drug treatment. Most commonly the higher resistance to HER-targeted therapy is observed in 3D models; however, tumor cell sensitization has been also reported. The proximity of the spatial architecture, chemical and cell composition in 3D models led the community to consider them as potent tools that reproduce the behavior of tumors *in vivo* and have high prognostic value. It must be recognized that, despite the wide discussion, the scope of experimental evidence cannot be considered exhaustive. Focusing on EGFR-expressing tumors, it has been reported that, instead of monolayer cell culture, the 3D model of KRAS-mutated colon cancer recapitulates the cetuximab resistance that is characteristic for xenografts and, of particular importance, is observed in patients with the same cancer type [88]. Much more similarity in the metabolic signature was observed between xenografts of EGFR-expressing HNSCC and tumor spheroids compared with the monolayer [93]. It must be emphasized, however, that the similarity was not complete. Phosphorylation of EGFR and downstream proteins was similar in SCC xenografts and 3D but not 2D cell culture [103]. In line with the other mentioned reports, the physiology and kinetics of acquired drug resistance in brain-mimetic ECM-based 3D culture resembled that of orthotopic xenografts [125]. The great potential of the tumor organoids in producing models of high validity must be admitted. Using the patient-derived tumor tissue enables model clonal diversity of tumor cells as well as considering cell–cell and cell–matrix interactions.

Further systemic studies are required to elucidate the precise mechanisms of tumor cell biology regulation in the 3D environment, to model them in *in vitro* conditions and to evaluate the particular role of the individual factors. The latter is of practical importance, because the 3D models are more labor- and resource-consuming. Despite all the advantages of 3D models, it is necessary to note that 2D models will apparently find practical application for a long time, because of the ease of their creation and use. In particular, 2D models seem optimal in the case of HTS of potential antineoplastic agents. In addition, the simplest 2D (monolayer) models make it possible to precisely control the parameters of the medium and the external factors, facilitate monitoring experiments and simplify interpretation of the results when studying the intracellular mechanisms (Fig. 3). More-complex models taking into account such factors as intratumoral gradients, cell–cell and cell–matrix contacts, and me-

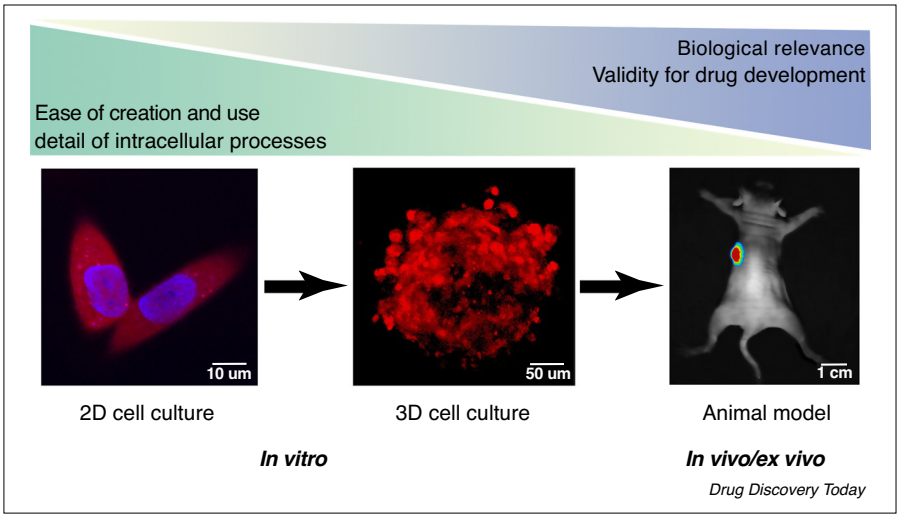

FIGURE 3

The balance when choosing the *in vitro* tumor model: ease and detail of analysis vs complexity and clinical relevance.

TABLE 1

Factors affecting tumor cells in various 3D models

Factors	Tumor models				
	One-cell-type spheriods	Multi-cell-type spheriods	Matrix-based models	Tumor organoids	Microfluidic-based models
Limiting the exchange area between the cell and the media	+	+	+	+	+
Gradients of nutriens, gases and catabolites	+	+	+	+	+
Interaction with non-tumor cells	_	+	+/−	+	+/−
Cell–matrix interaction	_	_	+	+	_
Mechanical pressure	_	_	+	+	_
Organotypicity	_	_	+/−	+	_
Tissue derived	_	_	_	+	_
Liquid flow	_	_	_	_	+

chanical forces are preferred for the next steps of drug discovery (Table 1). To predict the *in vivo* efficacy of the lead or drug candidate, tumor spheroids or matrix-based models should at least be employed for *in vitro* studies. A growing amount of experimental data proves their closer similarity to the drug sensitivity of real tumors. The advanced models in DCL matrices and organotypic models are indispensable tools for deciphering the complicated regulation network in carcinogenesis as well as in development of drug resistance. At the same time, their value for routine tasks (screening, efficiency evaluation) seems questionable. The tumor organoid technology has its particular niche; it was proposed to develop schemes for personalized medicine and the approach has made great progress in the past few years. To conclude, the creation of complex and multicomponent 3D tumor models with the expression of the EGFRs is expected to further develop our understanding of the biology of the receptors of this family, their role in the cell signaling network and the responsibility for cell malignancy. Today, it is already clear that 3D models are a potent tool that allows the search for effective HER-targeted drugs, developing personalized treatment regimens, establishing molecular mechanisms of drug action and identifying ways to overcome resistance to them.

Conflicts of interest

The authors state no conflicts of interest.

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