In vitro three-dimensional tumor microenvironment models for anticancer drug discovery

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Anticancer drug discovery has long been hampered by the poor predictivity of the preclinical models. There is a growing realization that the tumor microenvironment is a critical determinant of the response of cancer cells to therapeutic agents. The past 5 years have seen a great deal of progress in our understanding of how the three-dimensional microenvironment modulates the signaling behavior of tumor cells. The present review discusses how three-dimensional in vitro cell culture models can benefit cancer drug discovery through an accurate modeling of the tumor microenvironment, leading to more physiologically relevant experimental outcomes.

Keywords: 3D, angiogenesis, breast, cancer, esophaguscience, organotypic, skin, spheroid, therapy, tissue engineering


1. Introduction

Much of what is known about the activity of anticancer drugs has been derived from studies performed on adherent cultures of tumor cells. Under these conditions, the cells are grown flat, in two dimensions (2D) as monocultures on plastic tissue culture plates. This model has several important advantages in that the cells are easy to grow and maintain, the cultures are pure and free from contaminating cells and the methods of protein/RNA/DNA extraction are relatively simple. The adherent culture model ignores the fact that normal cells do not exist in isolation and are in fact orientated in three-dimensional (3D) space, attached to an extracellular matrix (ECM), in continuous dynamic interaction with the numerous cell types that come together to make up an organ. In a similar manner, tumors are not composed exclusively of malignant cells and the tumor organ is composed of a mixture of cancer cells, host fibroblasts, endothelial and immune cells working in concert to drive the tumor progression. We now know that the classical adherent-cell culture model of growing cancer cells on tissue culture dishes is completely inadequate for modeling the complexities of the tumor organ and that this is likely to give researchers an aberrant picture of how cancer cells respond to drugs. The obvious solution to these problems would be to perform the experiments in vivo using animal models, but this is also not without drawbacks. Animal models are highly complex and involve the interactions of many different cell types, often interacting in undefined ways, making it difficult to look at the effects of individual variables on the cellular phenotype under these conditions. 3D organotypic cultures represent a good compromise between the artificial environments encountered under adherent 2D cell culture conditions and the great complexity of the in vivo animal models. The goal of 3D organotypic cultures is to recreate as simply as possible the correct tissue-specific cell–cell and cell–matrix interactions so that the phenotypic and signaling behavior of the cells correlates more closely with that seen in vivo.
The present overview discusses how cellular phenotype and drug responses are modulated between 2D and 3D cancer cell culture systems and discusses new models that will hopefully allow a better candidate drug selection at an earlier stage of drug discovery.

2. Organotypic cell culture models of normal and malignant cells

Most of the pioneering work in 3D cell culture has emerged from the breast cancer and breast development fields. Unlike other organ systems, the mammary gland undergoes a well-characterized developmental cycle that can be easily studied and manipulated in vitro. Early studies showed that primary mammary epithelial cells grown in 2D adherent culture failed to form the phenotypically correct acini structures that are observed in breast tissue [1]. However, if the same epithelial cells were grown in recombinant basement membrane or were allowed to form their own matrix within an unconstrained collagen gel, they were able to form proper acini structures [1]. The formation of the acini structure also allowed the mammary epithelial cells to recapitulate their resistance to apoptotic stimuli that they were previously rendered sensitive to under 2D culture conditions [2]. Since this pioneering work, these methods have been adapted to a wide range of cells from other organ systems and are routinely used in cell biology studies that investigate the mechanics of cell polarity [3].

The major focus of the author’s laboratory for many years has been the melanoma and the adoption of organotypic cell culture models came about for the simple reason that mouse skin is not histologically similar to human skin. In the murine skin, the melanocytes are located in the hair follicle, where they are mostly responsible for the pigmentation of the mouse fur. In the human skin, the melanocytes are aligned along the basement membrane, in close contact with the epidermal keratinocytes. Here the melanocytes respond to ultraviolet radiation and generate the pigment melanin, which is then transferred to the surrounding keratinocytes, where it forms a protective cap over the nucleus, thus preventing the cells from ultraviolet-induced genetic damage. Hence, the mouse and human melanocytes inhabit very different microenvironments and serve different functions. There is a strong need for human melanocytes to be grown in organotypic cultures. In vitro monocultures of human melanocytes are characterized by aberrantly high proliferation rates, the expression of melanoma-specific antigens and a bi- or tripolar morphology [4]. The growth of the same melanocytes in the presence of human-skin keratinocytes brings the melanocytes under control so that they exhibit the same phenotype as observed in vivo [4]. For these reasons the authors decided not to pursue their melanoma studies using either mouse models or 2D in vitro cultures; instead they opted to recreate human skin in vivo from the constituent primary fibroblasts, keratinocytes and melanocytes.

Detailed protocols outlining the generation of human skin reconstructs have been published elsewhere and are only briefly described here [5]. The constituent skin cells are derived from newborn foreskins, with the stroma-supporting fibroblasts being isolated from the dermis and the stratified epithelium-forming keratinocytes and melanocytes being derived from the epidermis. The first procedure in reconstruct development is the establishment of a dermal or stromal layer (Figure 1), where a mixture of human-skin fibroblasts and collagen are mixed and seeded onto plastic reconstruct trays. After the fibroblasts have constricted the collagen (typically 4 – 7 days), a mixture of undifferentiated keratinocytes and melanocytes are added to the dermal layer in the ratio 1:3 – 1:20. After the culture becomes established, the tray is lifted and the top exposed to air, which facilitates the keratinocyte differentiation, inducing them to stack up and form the epidermal layer. After the reconstruct reaches maturity, the histology accurately represents that of normal human skin [5,6].

Melanoma cells can be introduced into the reconstructs by mixing them with keratinocytes (ratio 1:5) and adding them to the preformed dermal fibroblast/collagen layer. In this context, they exhibit tumor stage-specific behavior that is not seen under adherent 2D cell culture conditions. Thus, cells derived from the early radial growth phase of melanoma stay within the epidermal keratinocyte layer and do not breach the basement membrane [6,7]. In contrast, cells from the more progressed vertical growth phase of melanoma break through the basement layer and begin to invade the fibroblast-rich stromal layer. Cell lines from melanoma metastases spread rapidly into the dermal layer and quickly form nests of invasive tumor cells [6].

Melanoma cells grown in the organotypic human skin model typically exhibit different phenotypes to the same lines grown under adherent 2D culture conditions. The overexpression of β3-integrin, which is a melanoma progression marker, typically leads to an enhanced growth and invasion of early stage radial growth phase (RGP) melanoma cell lines in the organotypic skin culture model, but has little effect on either growth or motility when the same cells are grown under adherent 2D cell culture conditions [8]. Interestingly, the phenotypes observed in the organotypic skin reconstruct model are closely recreated when the same cells are grown in vivo, where the subcutaneous injection of these cells into nude mice leads to an enhanced tumor formation.

The strength of the organotypic culture model system is that it can be easily adapted to other organ systems by altering the source of the fibroblasts, epithelial cells and the growth factors in the media. This technique has been successfully used to generate reconstructs of human breast, human colon [9] and esophagus [10,11]. Of these, the esophagus model has been most extensively characterized.
Recent studies have shown that the esophageus reconstruct model accurately recapitulates the differentiated phenotype of human esophageal keratinocytes. Under 2D adherent cell culture conditions, the primary human esophageal keratinocyte line EPC2 does not express any of the claudin family of proteins, whereas immunostained sections of human esophagus show strong expression of claudin-4 and 7 in the differentiated keratinocyte layers [11]. However, when the same EPC2 esophageal keratinocytes were grown in the 3D organotypic esophagus culture the expression of claudin-4 and claudin-7 was regained and was expressed in a pattern that mirrored that of the normal human esophagus [11]. Esophageal keratinocytes also show differences in intracellular signaling between 2D and 3D organotypic culture. The increased Akt activity imposes growth arrest on the esophageal keratinocytes grown under 2D adherent cell culture conditions, but imposes an increase in both cell size and suppression of terminal differentiation when the cells are grown as an esophagus reconstruct [12].

A number of recent studies have begun to assess the effects of novel anticancer drugs within these 3D organotypic reconstruct models. A recent study from Meier and co-workers investigated the interaction between two signal transduction inhibitors in a simplified organotypic model of melanoma, where the epidermal keratinocyte layer was excluded [13]. The co-administration of the two inhibitors was associated with a synergistic induction of apoptosis in the 3D model that was not observed under adherent 2D culture conditions. Interestingly, within the same model there was very little apoptosis in the surrounding dermal fibroblasts, indicating some degree of tumor selectivity [13].

In another series of studies, the ability of the proteasome inhibitor bortezomib to inhibit the growth and invasion of esophageal squamous cell carcinoma cells was assessed in an organotypic culture system (esophageal reconstruct shown in Figure 2). In these experiments, increasing concentrations of bortezomib led to enhanced levels of apoptosis within the reconstruct as measured by TdT-mediated dUTp nick end labelling (TUNEL) staining. Numbers of TUNEL-positive nuclei were counted using the automated image processing software ImagePRO

Figure 1. Scheme showing the generation of human skin reconstructs. A. A layer of acellular collagen is allowed to polymerise in the organotypic culture tray inserts. B. A stromal layer of collagen mixed with fibroblasts is overlayed on top of the acellular collagen. C. The stromal layer is incubated for 4 – 7 days to allow the fibroblasts to constrict the collagen. D. The epithelial cells are layered on top of the fibroblast-containing stromal layer. E. The reconstruct is airlifted to promote differentiation and stratification of the epithelial layer.

3. Simplified collagen-implanted spheroid models for drug discovery

Although the organotypic reconstructs can be used successfully for investigating the efficacy and mechanisms of action of novel anticancer drugs, they are somewhat hampered by the prolonged culture times required (often > 21 days). The technique itself is quite technically demanding and requires constant monitoring by trained personnel. It is the authors’ opinion that these methods are poorly suited to higher throughput drug screening studies. To overcome the technical complexities and prolonged incubation times of the organotypic reconstructs, a simpler model has been developed, the so-called collagen-implanted spheroid model. This assay takes account of both cell–cell contact between adjacent tumor cells and the need for a 3D supporting matrix. For the initial spheroid studies using melanoma and esophageal squamous cell carcinoma (ESCC) lines, collagen...
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I was chosen as matrix, as this is the major structural ECM protein in most organs. Collagen also has the advantage of being easy to manipulate, so that the elastic modulus and tensional force of the gel can be easily altered [14]. It is worth noting that collagen is not the only matrix used in 3D organotypic culture studies. Some of the most popular substrates are the so-called recombinant basement membrane gels (Matrigel®) that are derived from the Engelbreth-Holm-Swarm mouse sarcoma. Although many cell types have been shown to exhibit tissue-specific architecture when grown in these gels, the basement membrane gels are somewhat poorly defined and consist of many ECM proteins and growth factors. Some caution should be applied when specific aspects of matrix biology are inferred from these studies. Other groups have also successfully used fibrin for both organotypic skin reconstructs and implanted spheroid studies [15].

Spheroids, or tumor cell aggregates, have had a long history of use and were first developed in the 1970s to assess the mechanism of action of chemotherapy and radiotherapy [16]. A variety of techniques exist for spheroid formation and the authors have mostly used the liquid overlay method. In this, a uniform cell suspension is plated on top of hard (1.5%) agar (sample scheme is shown in Figure 3). The presence of the agar prevents the tumor cells from adhering to the underlying tissue-culture plastic. After 48–72 h, the tumor cells form small aggregates – or spheroids. Most established protocols use the spheroids on top of agar for drug toxicity studies. The authors’ protocol differs in that the spheroids are manually harvested using a P1000 pipette and are gently mixed with a suspension of bovine collagen type I (Figure 3). The collagen concentration used in these studies is the same as that in the human skin reconstructs. New 24-well plates are first layered with acellular collagen to stop the spheroids settling onto the underlying plastic. The spheroid/collagen mixture is then gently pipetted onto the first acellular collagen layer and the matrix is allowed to polymerize in an incubator for 15 min. After this time the collagen layer is overlayed with fresh cell culture media. The potential of using these 3D aggregates was realized when it was found that the spheroid cultures accurately recapitulated the heterogeneity of tumor cell growth and drug resistance seen in vivo. In particular, it is known that in patient tumors, not all of the malignant cells are subject to the same microenvironment. Cells at the leading edge where the tumor and stroma interface, are known to be the most metabolically active and show the greatest invasive and proliferative capacities. In contrast, the cells that are located away from this leading edge, towards the tumor center are known to be more quiescent and less proliferative. In patient lesions, the cells close to the tumor core, which are normally poorly perfused and nutrient starved, may be necrotic. It is the authors’ practice to avoid growing over-large spheroids as these may be subject to necrosis, which could bias the results. These differing microenvironments within the tumor contribute towards the phenotype of the tumor organ in an important way and can contribute to altered gene expression through the generation of molecules such as hypoxia-inducible factor (HIF)-1α.

A detailed investigation has revealed that similar levels of tumor heterogeneity can be recreated within the tumor spheroids. Immunostained sections of tumor spheres have shown that there are a higher percentage of Ki67 positive cells at the exterior leading edge of the spheroid and that the cells toward the center of the structure tend to express the CDK inhibitor p27KIP-1, indicating that they are growth-arrested [17]. Likewise, as the tumor spheroids grow, their expression of cyclin dependent kinase (CDK) inhibitors (which arrest the cell cycle), such as p18, p21 and p27, also increases [18]. In this instance, the expression of these CDK inhibitors increases the most toward the center of the spheroid, leading to the complete downregulation of the CDK2 activity [18]. Further evidence for the spheroid model recreating the tumor microenvironment comes from studies on HIF-1, that showed that HIF-1 had little effects on the tumor cell growth in 2D adherent culture, but increased the tumor cell growth when grown as spheroids under hypoxic conditions [19].

There is also evidence that intracellular signaling pathways are regulated by the 3D microenvironment and that the different microenvironmental regions of the spheroid may have different signaling activities. In melanoma, there is hope that signal transduction inhibitors may be used as novel targeted therapies. The present favored molecular target for melanoma is the mitogen activated protein kinase (MAPK) pathway, whose activity can arise through the acquisition of activating mutations in BRAF, N-Ras, c-Kit and autocrine growth factors (reviewed in [20,21]).
Pathological examination of phospho-extracellular signal-regulated kinases (ERK) staining within melanoma lesions shows that much of the MAPK signaling activity is located in cells at the leading edge of the tumors [22]. This finding is intriguing when taken in the context of the relatively poor clinical activity of MAPK-targeting drugs as single agent therapies [23,24] and suggests that although the MAPK pathway is a major melanoma signaling pathway, its inhibition may only impact on a relatively small proportion of the malignant cells. In agreement with the pathological studies, the immunohistochemical staining of melanoma spheroid cultures reveals that high phospho-ERK levels are only found at the spheroid edge (Figure 4). Again this is interesting given that ERK was uniformly expressed throughout the tumor and all of the cells within the sphere are clonal – and all expressed the MAPK-activating BRAF V600E mutation (Figure 4). When the same melanoma cells are plated under adherent tissue culture conditions, a uniform phospho-ERK staining is seen in nearly every cell. It is likely that this regional and highly heterogeneous expression of active signaling molecules throughout solid tumors will have important implications for the successful clinical translation of the newer classes of molecularly targeted therapies and further study is definitely warranted.

In subconfluent 2D tumor cell culture, most of the cells are actively proliferating and progressing through the cell cycle. Most chemotherapeutic drugs work on actively proliferating tumor cells, by either crosslinking DNA or interfering with the formation of the mitotic spindle. These drugs are likely to have less effect on 3D tumor spheroids, as the major fraction of the cells are quiescent. In agreement with this idea, early studies have shown that EMT6 tumors in mice were drug-sensitive when grown in 2D adherent culture and then highly drug-resistant when grown as tumor spheroids [25]. One of the key determinants of drug resistance in 3D culture is the formation of polarized organoid structures and the receipt of survival signals from the surrounding ECM. This is best characterized in 3D breast carcinoma cultures where the upregulated expression and activation of β3-integrin drives the induction of polarity and hemidesmosome formation, leading to the suppression of apoptosis through enhanced NF-κB activity [2]. Other factors that also contribute to the enhanced drug resistance of tumor cells grown in 3D spheroid include the reduced diffusion of drugs through the multiple cell layers and the increased expression of drug transporters, such as P-glycoprotein, which are seen under 3D culture conditions [26]. There is also evidence that the change in metabolic activity seen in spheroids, where the tightly packed cells generate increased lactic acid, can also alter pharmacological responses through the reduction of drug uptake and permeability [16,27].

In initial studies, the authors compared the phenotypes of melanoma cells derived from the three stages of melanoma progression (RGP, vertical growth phase [VGP] and metastatic) when grown as collagen-implanted spheroids. Under 2D adherent cell culture conditions, cell lines derived from all three stages looked phenotypically alike and had similar motility characteristics. However, when grown as 3D collagen-implanted spheroids, the extent of collagen invasion was linked to the tumor stage: the cells from the early stage were poorly invasive, whereas the cell

**Figure 3. Scheme showing the generation of collagen-implanted spheroids.** A cell suspension 100 µl (25,000 cells/ml) is overlayed on top of hard (1.5%) agar. Plates are incubated for 48 – 72 h until spheroids have formed. The resulting spheroids are then harvested and transferred to a falcon tube and allowed to settle. The media is aspirated and replaced by a collagen suspension. 24-well plates are overlaid with 300 µl acellular collagen which is allowed to set. 500 µl of spheroid/collagen mixture is added to the acellular collagen layer and allowed to polymerize. Finally, media is added on top of the collagen.
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Figure 4. pERK is expressed largely at the leading edge of melanoma spheroids. A. Immunohistochemical staining of melanoma-cell spheroids (C8161) for phospho-ERK. Note the strong staining around the spheroid edge. B. Immunohistochemical staining for the total ERK in the same spheroids as shown in A. Note the staining of ERK is uniform throughout the entire spheroid.

ERK: Extracellular signal-regulated kinase.

line derived from the metastasis rapidly colonized the entire collagen gel [28]. Melanoma cells have a mesenchymal phenotype, lose E-cadherin expression and upregulate N-cadherin. The phenotype of the melanoma cells invading into the collagen is fibroblast-like, with the cells exhibiting an elongated morphology. In contrast, when spheroids from squamous cell carcinoma lines, which are epithelial in origin, are implanted into collagen, they invade as flat epithelial sheets [29]. Other sphere-like 3D models of melanoma have also been developed where the melanoma cells are coated onto microcarrier beads before implantation into a fibrin gel [15]. In this model, the melanoma cell lines are also found to invade in stage-specific manner. Interestingly, when fibroblasts are added to the fibrin matrix, the invasion of earlier-stage melanoma cells is curtailed, whereas the invasion of melanoma lines derived from metastases are not – a finding that mirrors the authors’ own experience [15].

The focus of the authors’ present research is to establish a rational basis for melanoma and ESCC therapy by targeting the signaling pathways known to be constitutively active. In 2D adherent culture, the proliferation of melanoma cells derived from RGP, VGP and metastatic stage lesions are blocked by inhibitors of PI3K, MAPK and Src [28,30,31]. However, when the same cells are grown as 3D-collagen implanted spheroids, only cells from the early stage RGP and VGP melanoma lesions are growth-inhibited and show reduced survival; the metastatic lines become completely resistant to these same inhibitors. The spheroids are scored for cell survival by removing the cell culture media, washing in PBS and then staining with calcein-AM and ethidium bromide (Invitrogen, LIVE/DEAD® assay kit). It is only when the two signaling pathways are inhibited simultaneously that any synergy is seen. This is in stark contrast to the results in 2D culture, where the simultaneous inhibition of PI3K and MAPK are additive at best [28]. The most likely explanation for this different response is that the dependence on these proliferative pathways becomes altered in 3D culture, the highly regional activity of phospho-ERK seen in both spheroids and in pathological melanoma lesions would seem to support this idea.

In studies on chemotherapy drugs, growing the tumor cells as 3D spheroids on top of the agar layer is sufficient to induce drug resistance [16]. With signal transduction inhibitors, such as MAPK and PI3K inhibitors, the situation is rather different. The high activity of MAPK seems to be critical for the maintenance of homotypic cell–cell adhesion and growing the spheroids on top of agar for prolonged periods of time in the presence of MAPK inhibitors leads to the disruption of the spheroid structure and cell death (unpublished observations). Indeed, the pharmacological profiles of the MEK inhibitor U0126 are identical between metastatic melanoma cells grown as spheroids on top of agar to those grown in 2D culture. The implantation into collagen seems to be critical for the resistance to signal transduction inhibitors in the melanoma spheroids. Culturing tumor cells on top of matrix proteins, such as fibronectin, laminin and collagen IV, is known to increase the drug resistance of tumor cells [32]. The mechanisms for this remain to be elucidated, but are likely to be a consequence of outside-in signaling via integrins. A recent study has shown that growing melanoma cells in 3D collagen leads to the inactivation of p53 via integrin αv signaling through the MAPK pathway [33].

Fibroblasts are not the only host cell type that has been successfully mixed with tumor spheroids under co-culture conditions. There is a considerable literature describing the invasion of host immune cells into tumor spheroids, often with the recapitulation of phenotypes that are not observed under 2D adherent cell culture conditions. In particular, a work has shown that the lactic acid derived from 3D tumor spheroids modulates the dendritic cell activation and antigen expression, leading to tumor-induced suppression of immune function [34].
reduce the tumor growth. Antiangiogenic drugs, such as the anti-VEGF antibody bevacizumab, have recently entered clinical development for colorectal carcinoma and now show great promise in a variety of other solid tumor systems. When administered as a single agent, the bevacizumab treatment is associated with minor responses; however, the co-administration of this antiangiogenic agent with chemotherapy can lead to dramatically improved rates of response in colorectal carcinoma patients [36]. Recent studies have also hinted that antiangiogenic therapies may have other, unintended, beneficial effects, with the co-administration of chemotherapy and bevacizumab leading to the depletion of the cancer stem cell compartment in a mouse glioblastoma model [37].

Data are emerging that suggest that even the therapy agents that are primarily targeted against the tumor cells may also have antiangiogenic effects that may contribute to their efficacy in vivo. The putative BRAF/multi-kinase inhibitor sorafenib was originally tested in melanoma as a BRAF inhibitor and can indeed inhibit the MAPK pathway within melanoma xenografts in vivo [38]. However, a recent study showed that this drug is more likely to work in vivo through blocking angiogenesis [38]. Clinical evaluation of these findings are at present in progress and there is some suggestion that sorafenib may reduce the tumor vascular permeability. In other tumor systems, the HER2 targeting antibody trastuzumab may also work in part through the suppression of angiogenesis and has been shown to inhibit the release of several antiangiogenic factors in vivo [39]. As more molecularly targeted therapy agents undergo evaluation, it is becoming increasingly important to assess whether these agents also possess off-target antiangiogenic activity. To address this in a simple, physiologically relevant way, a 3D model has been developed in which the co-culture of tumor cells and stromal fibroblasts interact to drive the vascular network formation in a 3D collagen gel. In this model, human microvascular endothelial cells (HMVECs) are cultured on a type I collagen-coated plate to 80% confluency (Figure 5). The HMVECs are then overlayed with acellular collagen, followed by a further layer of collagen containing a mixture of fibroblasts and tumor cells [40]. After 24 – 48 h, the fibroblasts start to contract the collagen, increasing the tensional force within the matrix. At the same time, the endothelial cells start to detach from the plate and grow up into the fibroblast-collagen layer. Ultrastructural analysis using electron microscopy has shown that in this model the endothelial cells link up to form self-junctions with a true lumen (10 – 300 µM), as well as close contacts between the surrounding fibroblasts [40]. The capillary networks first became apparent after 4 – 5 days and increase over an 11-day period [40]. Over time, the fibroblasts became concentrically arranged around the microvessels and helped to stabilize the lumen formation. Although the presence of fibroblasts alone can induce the vascular network formation, the co-culture of tumor cells

Figure 5. Scheme showing the generation of human angiogenic networks. A. HMVEC are plated on the bottom of 24-well plates, a layer of acellular collagen is plated on top of this. A mixture of cancer cells and fibroblasts in collagen is layered on top of the acellular collagen. B. After 4 – 7 days the collagen constricts and the endothelial cells detach from the plate and move upwards into the collagen layer. C. The endothelial cells form a mature vascular network.

HMVEC: Human microvascular endothelial cell.

4. Modeling tumor neo-angiogenesis in 3D culture

Much of the growth of solid tumors is dependent on the ready supply of nutrients and oxygen from a local blood supply. As tumors grow beyond a few millimeters in size they readily outstrip the host’s blood supply and begin to stimulate the formation of their own tumor vasculature. This is a complex process driven by the release of pro-angiogenic factors from the tumor cells and the surrounding stroma. Ultimately, this leads to the co-option of neighboring host vessels as well as the recruitment of circulating endothelial cell precursors leading to the establishment of new microvessels (reviewed in [35]). Compared with the normal vasculature, tumor blood vessels are very disorganized, leaky and tortuous. The flow of blood through the tumor-derived capillaries is often very slow and may be even stationary, there are also reports of blood flow patterns being reversed within the tumor vasculature. The tumor vasculature is recognized as an excellent therapeutic target as it is expected to cut off the nutrient supply and

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and fibroblasts appears to increase the maturity of the resulting microvessels, demonstrating the importance of the tumor/fibroblast interaction in driving this process. A preliminary analysis of a number of receptor tyrosine kinase inhibitors in this model have revealed that molecules targeted against the transforming growth factor receptor β1 (TGF-β1) and VEGF, as well as the proteasome inhibitor bortezomib, are able to completely block the neo-angiogenesis in the model when ESCCs are co-cultured with esophageal fibroblasts. Studies in melanoma using this model are ongoing.

5. Expert opinion

There can be no doubt that 3D in vitro models of tumor growth, progression and angiogenesis are becoming invaluable for the assessment of novel anticancer agents. The marked changes seen both in the activity and the spatial organization of intracellular signaling molecules between 2D and 3D cultures have very important implications for the development of molecularly targeted therapies. Understanding the nature of the rewired signaling that occurs in 3D culture and confirming this in solid tumor specimens is critical to understanding more about how targeted therapies might work as well as providing the rationale for future drug combination studies. The beauty of the 3D organotypic models lies in the wealth of the information they provide. There are few other simple biological assays that allow for a simultaneous readout of motility, invasive capacity, survival, growth and apoptosis. At present however, we lack the really sophisticated analysis tools that will allow us to get the most out of these extremely high-content assays. Cancer biologists will need to work closely with their cell imaging colleagues to optimize automated systems that allow the results from these models to be properly scored in an accurate and unbiased way.

The challenge for the future is how to turn the 3D assays into real screening tools. The main problem that hampers this transition is one of standardization. At present it is quite difficult to get thousands of spheroids or organotypic cultures with identical features. Spheroids have often uneven sizes, reconstructs often have areas where many tumor cells grow and other areas where there are few tumor cells. One solution to this could be to grow the tumor cells on the surface of microcarrier beads. Although this may allow for standardization and the possibility of huge scale-ups (microcarrier beads are suitable for use in bioreactors) there are disadvantages in that the cells are only one layer thick, so the potential for cell–cell contact is lost. Clearly organotypic culture requires a great deal of further work before real high-throughput screening can be performed using these 3D models.

One other major area of contention in 3D culture generation is the choice of matrix. As outlined earlier, the three most commonly used matrices are basement membrane type gels (Matrigel), collagen I and fibrin. The basement membrane gels are the most controversial as they consist of poorly defined mixtures of ECM molecules and growth factors; again, inter-batch variability is critical and different lots can lead to vastly different experimental outcomes. The best solution to this problem would be to allow the stromal cells to deposit their own ECM on an inert 3D scaffold before plating the tumor cells. Although progress is being made in these areas, they are largely the preserve of the tissue engineers, with little input from cancer biologists. Despite these challenges, the idea that 3D is best is now firmly entrenched in our thinking about cancer biology. We can only predict that there is an exciting future ahead for this technology which will hopefully lead toward the identification of better treatments to lessen the burden and suffering caused by cancer.

Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation of this manuscript.
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