Journal of Biotechnology 148 (2010) 3–15

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Multicellular tumor spheroids: An underestimated tool is catching up again

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ARTICLE INFO

Article history:
Received 2 October 2009
Accepted 6 January 2010

Keywords:
Multicellular spheroid
Mass culture
Micropatterning
Microfluidics
Spheroid coculture
Immune therapy
Cancer stem cells/tumor-initiating cells

ABSTRACT

The present article highlights the rationale, potential and flexibility of tumor spheroid mono- and cocultures for implementation into state of the art anti-cancer therapy test platforms. Unlike classical monolayer-based models, spheroids strikingly mirror the 3D cellular context and therapeutically relevant pathophysiological gradients of *in vivo* tumors. Some concepts for standardization and automation of spheroid culturing, monitoring and analysis are discussed, and the challenges to define the most convenient analytical endpoints for therapy testing are outlined. The potential of spheroids to contribute to either the elimination of poor drug candidates at the pre-animal and pre-clinical state or the identification of promising drugs that would fail in classical 2D cell assays is emphasised. Microtechnologies, in the form of micropatterning and microfluidics, are also discussed and offer the exciting prospect of standardized spheroid mass production to tackle high-throughput screening applications within the context of traditional laboratory settings. The extension towards more sophisticated spheroid coculture models which more closely reflect heterologous tumor tissues composed of tumor and various stromal cell types is also covered. Examples are given with particular emphasis on tumor-immune cell cocultures and their usefulness for testing novel immunotherapeutic treatment strategies. Finally, tumor cell heterogeneity and the extraordinary possibilities of putative cancer stem/tumor-initiating cell populations that can be maintained and expanded in sphere-forming assays are introduced. The relevance of the cancer stem cell hypothesis for cancer cure is highlighted, with the respective sphere cultures being envisioned as an integral tool for next generation drug development offensives.

1. Rationale for the use of spheroid monocultures in anti-tumor therapy testing

Recently, 3D culture gene expression profiles have been shown to more accurately reflect clinical expression profiles than those observed in 2D cultures. This has stimulated numerous offensives which adopt organotypic 3D culture systems into therapy test platforms. These culture models can be used to evaluate therapeutic methods and offer the promise of improving clinical efficacy predictions. Indeed, the potential of sophisticated, 3D culture systems to be incorporated into mainstream development processes for new anti-cancer therapeutic strategies is increasingly recognized; it is thought to improve the pre-animal and pre-clinical selection of both the most promising drug candidates and novel, future-oriented treatment modalities (Abbott, 2003; Friedrich et al., 2007a, 2009; Kunz-Schughart et al., 2004; Mueller-Klieser, 2000). Among the 3D *in vitro* culture systems which have been established to restore the histomorphological, functional and microenvironmental features of *in vivo* human tumor tissue, tumor spheroid or sphere cultures are a rather classical approach to obtain and maintain the functional phenotype of human tumor cells. The basic system was adapted to cancer research almost 40 years ago and has since then considerably contributed to our knowledge of cellular response to diverse therapeutic interventions. Initially, tumor spheroid cultures were applied in experimental radiotherapy followed by photodynamic treatment, hyperthermia, and chemotherapy including target-specific approaches as well as other contemporary lines of attack such as gene therapy or cell- and antibody-based immunotherapy (Desoize et al., 1998; Desoize and Jardillier, 2000; Dubessy et al., 2000; Mueller-Klieser, 1997, 2000; Olive and Durand, 1994; Santini et al., 1999). Some of these therapeutic strategies demand the establishment and/or utilization of advanced models, such as the coculturing of tumor spheroids with immune cell suspensions or endothelial cell culture systems as highlighted later in this synopsis. These advanced 3D spheroid models will receive greater interest as high-throughput manipula-

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doi:10.1016/j.jbiotec.2010.01.012

tion and analysis methods become available. However, great care is required to select the most appropriate model to match the needs of each therapeutic investigation. For example, monocultures of multicellular spheroids from human tumor cell lines have proven to be a prevailing tool in the study of the microenvironmental regulation of tumor cell physiology and therapeutic problems associated with metabolic and proliferative gradients in a 3D cellular context (Rodriguez-Enriquez et al., 2008). Spheroid monocultures have thus contributed to our understanding of the altered responsiveness of chronically hypoxic tumor cells and the importance of 3D cell–cell and cell–matrix interactions in radio- and chemoresistance (Shield et al., 2009). One continuing field of interest is the combination of various drugs and/or radiosensitizers with single and fractionated irradiation regimens. Here, the spheroid model is particularly relevant since transient G_2 delay, apoptosis induction and late onset of DNA strand breaks following radiosensitization are more pronounced in the 3D setting. The enormous body of literature on multicellular tumor spheroids used in combinatorial therapy-oriented studies is thus continuously increasing and has more or less frequently been reviewed by various researchers (Desoize and Jardillier, 2000; Dubessy et al., 2000; Durand and Olive, 2001; Friedrich et al., 2007b; Hirschberg et al., 2004; Kunz-Schughart et al., 1998; Mueller-Klieser, 1987, 1997, 2000; Santini et al., 1999).

According to the vast majority of literature reports, many treatments are expected to lose efficacy in the 3D pathophysiological environment, and spheroids are thus most frequently thought to be a tool for negative selection to reduce animal testing or to evaluate drug candidates with enhanced tissue distribution and efficacy. Indeed, most therapeutic approaches were found to be less effective in 3D than in 2D cultures. This, however, cannot be generalized, since some potential targets and signaling pathways especially or even exclusively play a role in the 3D environment or milieu (Barbone et al., 2008, Dardousis et al., 2007; Frankel et al., 2000; Howes et al., 2007; Jelic, 2005; Poland et al., 2002). Consequently, the spheroid model has also been increasingly recognized as a primary tool for positive selection in innovative drug development initiatives.

It is undisputed that other 3D culture models such as multilayer or gel-based assays are also of intermediate complexity relative to monolayer and *in vivo* models. These intermediate models reflect particular aspects of 3D cellular interactions and may even be preferable to resemble some specific tumor entities, carcinogenesis processes or conditions such as the ones used to remodel breast architecture, function, and neoplastic transformation (Nelson and Bissell, 2005; Weaver and Bissell, 1999; Weaver et al., 1996). It is also true that the continuous progress in tissue engineering, including the development of various 3D scaffolds and bioreactor systems has improved the diversity, fidelity and capacity of culture models that can be used in cancer research (Friedrich et al., 2007a; Kunz-Schughart et al., 2004). However, only a small number of these systems are sufficiently well characterized to resemble the tumor-like 3D cytoarchitecture as well as simulate the pathophysiological micromilieu and tumor cell responses of the *in vivo* tumor state. Hence, it seems important to once more underscore the flexibility and challenges of the classical tumor spheroid monoculture model in particular in the light of the latest methodological progress.

Some essential conceptual considerations for the successful establishment of an initial spheroid-based drug screening platform have been recently discussed (Friedrich et al., 2009). In this report, a basic set-up is described which could be integrated into a standard large scale drug test routine. The set-up requires a small number of spheroids and a limited amount of drug, and it implements only some basic analytical endpoints such as spheroid and

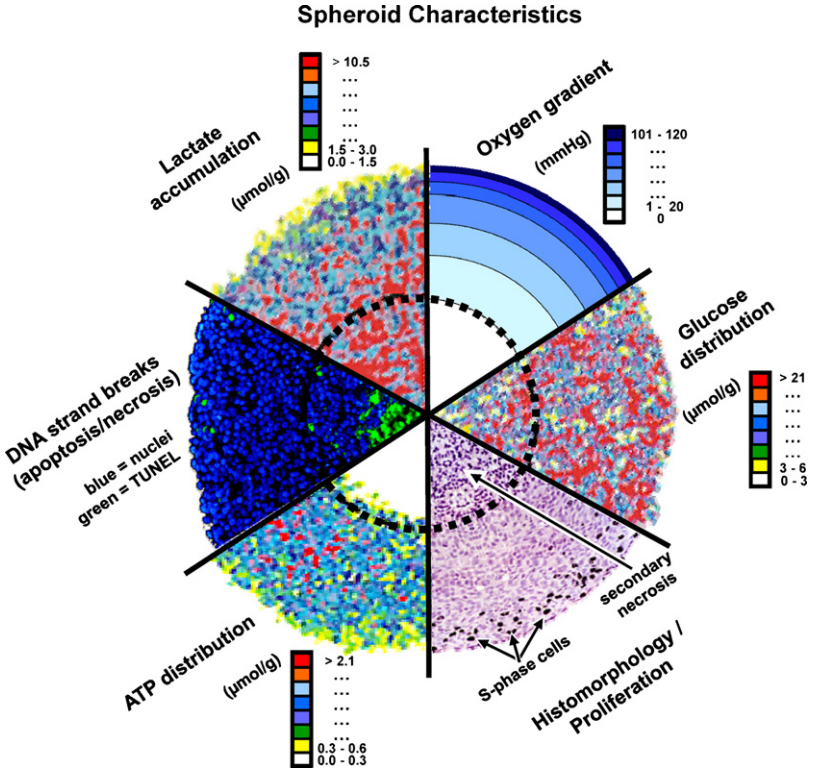

Fig. 1. Combination of analytical images of spheroid median sections studied with different technologies: autoradiography, the tunnel assay, bioluminescence imaging, and probing with oxygen microelectrodes. Together these measurements enable the concentric arrangement of cell proliferation, viability and the micromilieu in large spheroids to be understood.

cellular integrity after routine single treatment as well as spheroid regrowth and growth delay. Also, a list of spheroid-forming human carcinoma cell lines which do not require the addition of exogenous artificial or natural matrix materials is provided based in part on the 60-cell line screen of the Developmental Therapeutics Program of the National Cancer Institute (NCI-DTP). This list can be supplemented by the body of literature on human epithelial cancer cell line spheroids of different origin as reviewed for example by Friedrich et al. (2007a,b). Here, however, it is noted that the term spheroid and sphere, respectively, are not consistent throughout the literature, and yet this definition is critical to the rationale use of spheroid monocultures in anti-tumor therapy testing. The term aggregate is primarily but not always used to describe and eventually to discriminate loose packages of cells from compact spherical cultures (Ivascu and Kubbies, 2006). Unfortunately, some so-called spheres and even spheroids in the literature are no more than loose aggregates that easily detach, cannot be manipulated or transferred and may not only lack cell–cell and cell–matrix interactions, but also lack a true spherical geometry. Without these features the pathophysiological gradients, as depicted in Fig. 1, developed in genuine tumor spheroids will not be established. The clearly defined 3D structure and uniform geometry are mandatory requirements prior to scaling cultures for the efficient generation of a few up to thousands of individual, yet identical cultures for implementation of the model into primary drug testing routines.

It is worth mentioning that one has to precisely define the spheroid size at the onset of treatment as this clearly affects response to treatment. As an example, small spheroids with a size of <150 μm may exhibit 3D cell–cell and cell–matrix interactions and may also show an altered expression profile as compared to 2D cultures. Also, proliferative activity may differ to the respective 2D cultures but clear radial proliferative and pathophysiological inward and outward gradients may not yet be found. Tumor spheroid cultures develop chemical gradients (e.g. of oxygen, nutrients, and catabolites) at diameters between 200 and 500 μm with a central secondary necrosis typically established at sizes >500 μm. Here, cells located in the spheroid periphery reflect the *in vivo* situation of actively cycling tumor cells adjacent to capillaries while innermost cells become quiescent and eventually die via apoptosis or necrosis. The establishment of pathophysiological gradients and the concentric arrangement of heterogeneous cell populations with increasing spheroid size is cell-line dependent but in principle comparable to the situation in avascular tumor nodules, micrometastases or inter-capillary microregions of solid *in vivo* tumors as detailed earlier (Friedrich et al., 2007a; Kunz-Schughart et al., 2004; Mueller-Klieser, 1987, 1997, 2000; Sutherland, 1988). One of the most interesting features of the spheroid model is the metabolic adaptation of central cells to maintain intracellular homeostasis in response to the microenvironmental stresses created by diffusive gradients. The proliferative and functional features as well as micromilieu conditions combined with the extensive and complex 3D network of cell–cell and cell–matrix interactions affect cellular RNA and protein expression, the distribution and function of biomodulators and also the penetration, binding and bioactivity of therapeutic drugs and drug candidates. Overall response to treatment may be affected by a multitude of factors. These include potential penetration barriers, altered expression profiles and/or signaling pathways of particular targets, modulation of DNA damage and repair mechanisms, an *in vivo*-like distribution of biological response modifiers and survival signals, as well as the cell cycle distribution. By varying the size of spheroids for treatment, spheroid cultures are thus amenable to therapy-related studies with different emphasis, i.e. focusing on 3D cellular interactions and structure only or in combination with authentic pathophysiological milieu conditions.

2. Analytical endpoints – choices, considerations and clinical relevance

A broad range of basic and complex technologies have been used to monitor and analyze intact and viable, frozen or paraffin-embedded spheroid cultures (Friedrich et al., 2007a; Kunz-Schughart et al., 1998, 2004). All modern molecular methods can be applied to study cells from spheroids on the cellular, protein, RNA and DNA level. Thus, a number of new papers particularly describe genomic stability of cells in multicellular spheroids and their gene expression profiles and gene function. A recent genomic array hybridization study for example indicates that the genomic profile of human malignant glioma is preserved in spheroid but not in primary monolayer cultures (De Witt Hamer et al., 2009). Several comparative transcriptomic studies show that numerous genes associated with cell survival, proliferation, differentiation and resistance to therapy are differentially expressed in cells grown as multicellular spheroids versus 2D cultures. This was shown for example for cell lines originating from epithelial ovarian cancer (Cody et al., 2008; Zietarska et al., 2007), hepatocellular carcinoma (Chang and Hughes-Fulford, 2009; Shimada et al., 2007; Yamashita et al., 2004) or colon cancer (Gaedtke et al., 2007; Timmins et al., 2004). The expression profiles more closely resemble the profiles of the respective tumor tissue *in vivo* and are thus highly relevant for the establishment and testing of novel therapeutic interventions.

Global and selected gene expression responses to radio-/chemotherapy are currently under investigation as potential early markers for the onset of resistance mechanisms. Accordingly, microarray technology has also been considered to evaluate mechanisms of cytotoxin action and immediate adaptive responses to treatment in spheroids (L'Esperance et al., 2008). However, because of the lack of clear gene expression profiling data from clinical samples this approach cannot yet be used as a valid analytical endpoint to predict clinical efficacy and treatment success or failure. Accordingly, other defined, standard practice endpoints and easy to handle analytical tools are required with respect to drug and therapy testing. The determination of clonogenic survival via colony formation assays is an established endpoint to verify drug efficacy and radioresistance in monolayer cells. Such assays have also been performed with single cells isolated from spheroids following selective dissociation (Durand, 1990; Freyer and Schor, 1989; Kerr et al., 1987; Kunz-Schughart and Freyer, 1997; Wartenberg et al., 1998; Watanabe et al., 2007), but such experimental set-ups not only require large amounts of spheroids but artificial cell loss due to the dissociation process may become a particular problem after drug exposure. In fact, the cell preparation protocol is critical to the analytical power of this and all other endpoints that require single cell preparations including cell counting or flow cytometric analyses. Such analyses as well as histological and immunohistological processing of spheroid sections are of course basic tools for spheroid characterization, but cannot be used in real-world rapid drug testing routines. Also, some new technologies such as digital deconvolution, multiphoton confocal microscopy, single-plane illumination microscopy or optical projection tomography may be useful for advanced spheroid imaging but should at present be exclusively considered for sophisticated analyses (Lin and Chang, 2008).

In contrast, spheroid integrity can easily be visualized by (rapid) phase contrast imaging and is still the most popular technique to evaluate the effects of growth-promoting or growth-suppressing factors and drugs in spheroids. Spheroid growth delay, a classical analytical endpoint, is most frequently calculated as the difference between treated and untreated spheroids to reach a particular volume and has recently been discussed for standardized spheroid screening (Friedrich et al., 2009). Here, it is also pointed out that complex correlation analyses via mathematical modeling based on

the Gompertzian or equivalent functions which describe spheroid and tumor growth, respectively (Kunz-Schughart et al., 1996; Marusic et al., 1994; Tabatabai et al., 2005), are often not feasible for treated samples and also not necessary, if pronounced and not minor effects are to be assessed. With respect to clinical relevance, monitoring the proportion of spheroids which are capable of restarting growth and/or obtaining a specific volume should be emphasized. This approach allows the estimation of a 50% spheroid control concentration or dose (SCD_{50}) according to the tumor control dose (TCD_{50}) known from *in vivo* irradiation experiments which defines the dose that leads to tumor cure in 50% of the animals. Only combination treatments resulting in a left shift of the dose-response curves (i.e. lowering the TCD_{50}) are of particular relevance and expected to have a potential for enhancing the rate of tumor cure in humans.

Since loss of spheroid integrity may not result from total cell destruction and spheroid integrity does not necessarily reflect the presence of viable cells, an immediate measure for cell survival after treatment is desirable. Accordingly, a number of attempts to simplify the measurement of cell number and survival after treatment in tumor spheroids have been used in the past. These include the monitoring of respiratory activity via scanning electrochemical microscopy (SECM) (Torisawa et al., 2005) as well as the analysis of lactate dehydrogenase (LDH) activity in spheroid sections based on image cytometry (De Witt Hamer et al., 2005). Both approaches are not feasible for routine drug testing. Real-time, impedimetric monitoring of tumor spheroids either positioned in a capillary system or as a more recent development in a 3D multifunctional electrode-based microcavity array has great potential for evaluating cell characteristics and survival if spheroids maintain their spherical shape and cellular arrangement. However, one array type is not suitable for all spheroid size ranges as may occur when comparing treated and untreated spheroids (Bartholoma et al., 2005; Kloss et al., 2008). According to the wide range of commercially available cytotoxicity/viability assays based on absorption, luminescence or fluorescence that are routinely applied to monitor membrane integrity, intracellular activity or energetic status in monolayer cultures, numerous approaches are expected to be compatible with spheroid cultures. Indeed, some of them have already been used for small spheroids with a size of <200 μm (Kosaka et al., 1996; Mellor et al., 2005; Orlandi et al., 2005). Disappointingly, we found numerous approaches to be inappropriate since the assay signals did not correlate linearly with the number of cells per spheroid when using larger cultures. Instead the acid phosphatase assay, a simple, rapid and high-throughput compatible assay, can be used for monitoring membrane-intact cells in single spheroids without the need for spheroid dissociation (Friedrich et al., 2007b, 2009). Thymidine incorporation assays are useful to study proliferative activity but because of the handling of radioactive material they are not recommended for unrestricted drug testing routines.

The capacity for spheroid outgrowth in 3D matrices or on adherent, extracellular matrix (ECM) coated or non-coated surfaces, has previously been described as an interesting parameter to study the migratory behavior of tumor spheroid cells (Hirschberg et al., 2006; Lambert et al., 2006). However, this parameter can only be used with short incubation intervals of less than one cell cycle. Therefore, the approach is only useful for rapidly migrating cells, e.g. in glioma or glioblastoma spheroids (Hirschberg et al., 2006). At longer incubation intervals, the parameter does not allow clear discrimination of a drug's effects on cell adherence, migration or proliferation. In the past spheroid outgrowth was sometimes also used to evaluate spheroid control doses since single spheroid monitoring was not feasible and too time consuming at the time. Spheroids were thus transferred onto adherent surfaces and the potential of spheroid cells to form a monolayer after treatment was recorded. Today, because of the potential for long-term culturing in liquid overlay and semi-automated single spheroid monitoring, spheroid transfer can be avoided. It is unclear, however, whether SCD_{50} values determined by the various approaches would be consistent.

The application of spheroids to monitor local drug penetration capacity, tissue distribution and binding properties is also a challenge. Indeed, one advantage of the spheroid model is that drug candidates must penetrate in order to be highly effective. However, the analytical tools for such penetration studies, be they confocal laser scanning (Kostarelos et al., 2004; Martin et al., 2003; Mellor et al., 2005) or based on emerging optical imaging technologies as discussed earlier (Lin and Chang, 2008) still await intense validation as to their practical operating conditions. At present, they have limited potential to be incorporated into rapid, high-throughput approaches for spheroid analysis.

3. Emerging microtechnologies for the production of tumor spheroids

The semiconductor industry gave rise to microfabrication techniques involving the use of photolithography to define microscale material patterns. These fabrication techniques were later adopted for applications in the life sciences. They were further developed in the form of soft lithography for the 3D replication of elastomeric polydimethylsiloxane (PDMS) microstructures. These structures can be used for (bio)material microprinting and microfluidics (Whitesides et al., 2001). In the last few years, the collection of microtechnologies have begun to impact the life sciences with relevant highlights being cell patterning (Folch and Toner, 2000; Kane et al., 1999) with unprecedented geometric control (Singhvi et al., 1994; Thery et al., 2006) and microfluidics for the spatiotemporal manipulation of the cellular microenvironment (El-Ali et al., 2006; Meyvantsson et al., 2008). Although well suited for the challenge, the use of microtechnologies for the production of uniform tumor spheroids has only been explored in the most recent years. The vast majority of microtechnology research for spheroid production has focused on the use of hepatocytes for engineering the artificial extracorporeal 3D liver or stem cells as tissue progenitors. Besides these pursuits, microtechnologies are equally applicable to the production of spheroids from diverse tissue origins to model the varied cancer scenarios.

Tumor spheroids are conventionally produced by a variety of methods that share the common feature of promoting cell–cell coupling by resisting cell–surface interactions. Strategies either involve continuous agitation of the cell suspension (Friedrich et al., 2007a; Ingram et al., 1997) or cellular aggregation by sedimentation at concave and cell adhesion resistant surfaces, such as agarose (Friedrich et al., 2009), poly-hydroxyethyl methacrylate (poly-HEMA) (Ivascu and Kubbies, 2006) or a droplet's air–liquid interface (Kelm and Fussenegger, 2004; Timmins and Nielsen, 2007). Microtechnologies in the form of 3D microwells and planar micropatterns can provide the same cell aggregation condition, but unlike the macroscale approaches, these technologies offer precision feature replication across cellular and tissue length scales for the controlled development of uniform tumor spheroids. These approaches are inherently automated and can be parallelised for the mass production of tumor spheroids.

Material selection is vitally important for tissue engineering and microfabrication. Surfaces which resist cell adhesion are required for the assembly of spheroids, and there is an enormous repertoire of materials from which to choose. Among these, polyethylene glycol (PEG) has emerged as the preferred material that resists both protein and cell adhesion (Zhang et al., 1998) while also providing degrees of freedom for microfabrication. In recent years, PDMS has also been shown to resist cell adhesion (Frimat et al., 2009) and enable spheroid formation (Nakazawa et al., 2009), while also

affording access to the many benefits of soft lithography.

Arrays of microwells numbering in their thousands can be fabricated in a single cell culture substrate. Using such platforms, cells from suspension settle as discrete populations of equivalent number within the microwells, and during continued culture they assemble into spheroids with homogeneous characteristics. The principle is very similar to the hanging drop or the liquid overlay methods, but with the major advantage of eliminating the intensive task of dispensing individual cell populations, thereby making the automated and parallel production of tumor spheroids possible. Ungrin et al. (2008) have used an anisotropically etched silicon template to mould dense arrays of pyramidal PDMS microcavities for the high-throughput production of embryonic stem cell aggregates. Large arrays (~4000) of non-adherent 150 μm diameter microwells have also been prepared from photocrosslinkable PEG and used for the constrained formation of equal-sized embryoid bodies (Karp et al., 2007). Using 500 μm diameter PEG-coated microwells, HepG2 spheroids as large as 260 ± 8 μm can be produced (Sakai and Nakazawa, 2007). Alternatively, collagen adhesion sites have been microcontact printed onto the base of PEG-coated microwells to act as centres of attachment for the formation of uniformly sized hepatocyte spheroids (Fukuda et al., 2006).

Planar cell adhesion islands alone can serve as the foci for spheroid assembly. The physical walls of microwells are replaced by a biologically inert background that serves to guide the organization of the seeded cells onto the adhesion sites for unconstrained growth into spheroids. Again, the cell repellent properties of PEG surfaces have been exploited. In one example, Tamura et al. used 100 μm diameter collagen prints surrounded by a self-assembled PEG monolayer and arrayed with a 600 μm pitch for the well-defined formation of 267 ± 22 μm diameter HepG2 spheroids (Tamura et al., 2008). In a further two examples, patterned PEGylated surfaces were used to first generate a feeder layer of cells which then supported the growth of functional hepatocyte spheroids (Otsuka et al., 2004) or the differentiation of fetal mouse liver spheroids (Kojima et al., 2009). In place of printing cell adhesion proteins, these demonstrations instead relied on the differential adsorption of serum proteins, with bioactive adhesion proteins only presented in areas where the perforated PEG polymer exposed the underlying glass substrate. Building on similar principles, we have developed a novel thin film (~40 nm) PDMS printing method for the long-term patterned cultivation of a wide variety of cell lines on tissue culture substrates (Frimat et al., 2010). Shown in Fig. 2, we have used thin film PDMS printing for the automated mass production of uniformly sized and 3D human HT29 colon carcinoma spheroids. The platform is suitable for harvesting by gentle pipetting, with further analysis demonstrating that the spheroids are densely aggregated and truly spherical in character. Due to their suitability for easy harvesting, these microarrays can be used as mother dishes for spheroid maturation prior to transfer to standard microtiter plate formats for high-throughput screening applications.

Microfluidics is another microtechnology format that can be used to control the formation of tumor spheroids. But more than simply caging the cellular assembly process within a fluidic compartment, microfluidics can also be used for perfused culture to more accurately recapitulate the *in vivo* state of vascularized tumors. Perfusion can be achieved using a stacked bilayer of microfluidic channels interfaced with a semi-porous membrane (Hsiao et al., 2009; Powers et al., 2002; Torisawa et al., 2007), or by using rows of micropillars to interface a central culture compartment with flanking perfusion channels (Toh et al., 2007; Zhang et al., 2008). Microfluidic systems have also been developed for low volume drug testing. One approach removed the tubing, and associated volume requirements, from traditional microfluidic systems by instead using pipette dispensing for the delivery of a few microliters of reagent (Meyvantsson et al., 2008). Importantly, this system is configured for interfacing with industry standard robotic dispensing systems and microtiter plate reader platforms. As such, this is a critical step towards the cell biology community widely benefiting from the capabilities of the emerging microtechnologies.

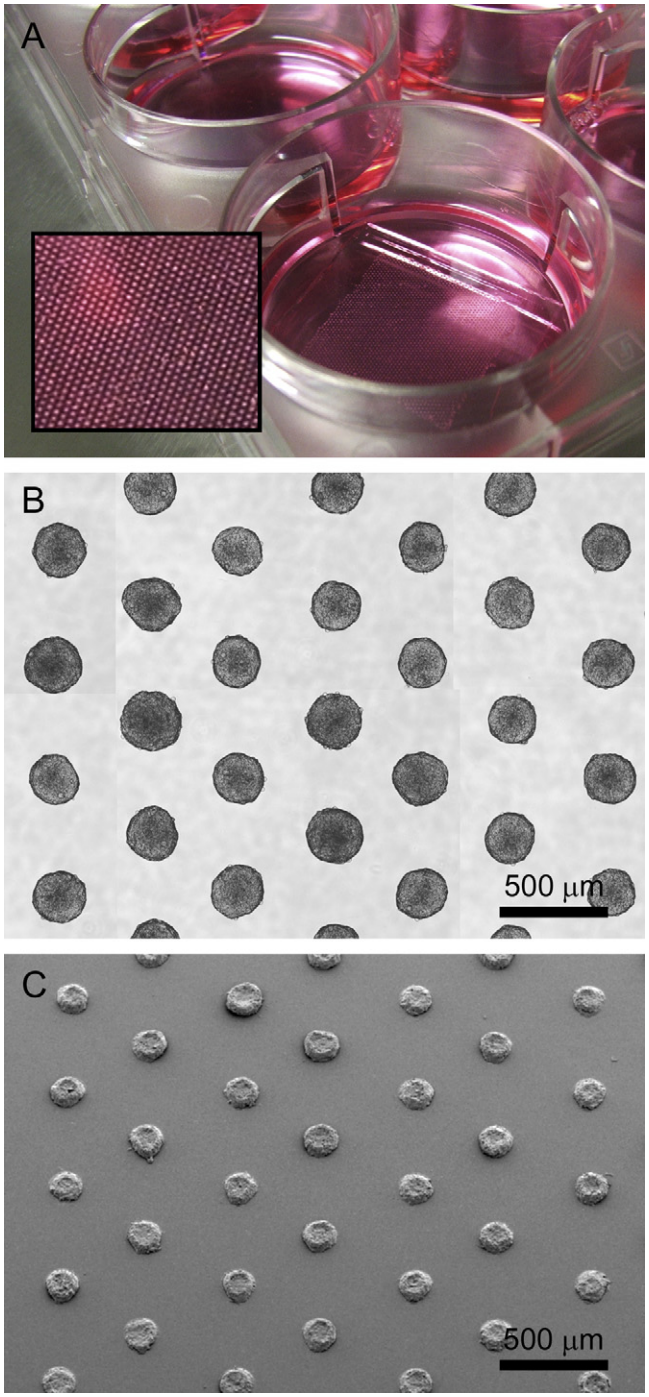

Fig. 2. An individual microarray can be accommodated within each of the wells of a standard 6-well tissue culture plate for the scalable production of over 10,000 spheroids (A). Microscopy image compilation of uniformly sized human HT29 colon carcinoma spheroids cultivated on the perforated thin film PDMS micropattern (B). The adhesion islands are 150 μm in diameter and pitched by 450 μm. Scanning electron microscopy image to illustrate the 3D nature of spheroids at an early stage of development (C).

Although only in their infancy, microtechnologies for spheroid cultivation have already demonstrated great potential. In the near future we can anticipate these systems and methods being tailored

for the development of larger spheroids to investigate hypoxia effects as well as being used to engineer more complex coculture and vascularized models. Beyond this there is tremendous scope to apply microtechnologies to advance both fundamental and applied spheroid research.

4. Spheroid cocultures – future-oriented models of increasing relevance

The tumor microenvironment plays a critical role in cell differentiation, and greatly impacts therapeutic efficiency. The multicellular tumor spheroid model has therefore emerged as a powerful tool to resemble tumor pathophysiology for anti-tumor research. For modeling the cellular heterogeneity in tumor tissues a variety of spheroid-based coculture systems have been developed. One of these is the so-called tumoroid model, which contains a mixture of different breast cancer cell types to mimic the cell fractions and their different *in vivo* responses to anticancer drugs (Karacali et al., 2007). The different cellular fractions within a spheroid critically affect behavior and response to therapy. Therefore, a method of digital morphological profiling using different histological stainings has been developed to characterise the models and strive towards standardization. Various spheroid coculture approaches have also been applied to analyze the interplay of tumor cells and stromal fibroblasts. Coculturing techniques are diverse and include mixed spheroids (Hoffmann et al., 2009), tumor spheroids cultured on fibroblast monolayers (Krueger et al., 2005), tumor spheroids cocultured with pre-established fibroblast spheroids (Kunz-Schughart et al., 2001; Seidl et al., 2002), fibroblast suspensions added to tumor spheroids and the incubation of fibroblast spheroids with tumor cell suspensions (Friedrich et al., 2007a). Lange, Djordjevic and co-workers (Djordjevic et al., 2006) have emphasized the importance of cell kinetics and clonogenicity in the evaluation of anti-cancer treatment modalities by using a hybrid spheroid (HS) assay which is based on the agglomeration of fibroblasts into spheroids under non-adhesive conditions. Using HeLa cells as model tumor cells, they mixed 1% HeLa cells and 99% fibroblasts to obtain 100 ± 7 μm HS containing 170 cells with a Poisson average multiplicity of 1.7 HeLa cells. By following the growth of the individual formed HS in 96-well plates after treatment with X-rays or drugs, they measured the HeLa cell multiplicity and single-cell survival curves (Djordjevic and Lange, 1991; Djordjevic et al., 1998). Using cervical cancer surgical samples, they obtained similar measurements for the putative cancer stem cells of individual patients – e.g., 0.5 ± 0.09% of the cancer cells were clonogenic in 88–105 μm HS and 0.76 ± 0.15% in 105–125 μm HS. Since no colonies were formed from 40,000 of the respective tumor cells in monolayer culture when 200–300 colonies would have been expected from the plating efficiency in HS, the fibroblasts in the HS seemed to provide a microenvironment or niche for the growth of a particular cancer cell subpopulation, supposedly the putative cancer stem cell fraction (Lange et al., 2009). The patented approach of Lange's group has promise for individualizing patient treatments.

Endothelial cell (EC) spheroids are increasingly used for evaluating the pro- and anti-angiogenic potential of drugs. As a recent example, vasohibin treatment of HUVEC (human umbilical vein endothelial cells) spheroids established in a 3D collagen gel resulted in sprouting inhibition (Kern et al., 2009). The relevance of this model is also supported by the successful development of an endothelial transplantation assay which is based on grafting *ex vivo* generated endothelial cell spheroids (Laib et al., 2009). With a similar approach it was found that subcutaneous implantation of HUVEC spheroids into SCID mice resulted in superior *in vivo* vascularization compared to the use of endothelial progenitor cells (Finkenzeller et al., 2009). Co-spheroids consisting of HUVEC and human fibroblasts have also been discussed for angiogenesis studies and tissue engineering applications (Kunz-Schughart et al., 2006, Wenger et al., 2005). In addition, tumor spheroids cocultured with EC demonstrated T-cadherin upregulation to potentiate intratumoral angiogenesis (Ghosh et al., 2007).

Further variations and combinations of different cell types are used in anti-cancer research. For example, cocultures of colon cancer spheroids with myofibroblasts, normal colon epithelium or with HUVEC monolayers have been developed for the analysis of TNF secretion and the expression of soluble TNF-receptors which play a role in colon cancer development and the formation of metastases (Paduch and Niedziela, 2009). Another group has applied prostate cancer spheroids in coculture with osteoblasts and/or endothelial cells to mimic the bone metastatic prostate cancer microenvironment and used this to evaluate growth behavior (Hsiao et al., 2009). In a different approach, tumor spheroids were studied in confrontation culture with embryoid bodies (EB). In this interesting system embryonic stem cells spontaneously express endothelial markers, followed two days later by differentiation into leukocytes. Leukocyte numbers increased and invaded breast tumor spheroids during coculture (Hannig et al., 2008). Unfortunately, the formation of EBs tends to result in heterogenously sized aggregates. To circumvent this problem, and as described in the previous section, microwell and microfluidic systems can be used to control the size of cultivated EBs (Karp et al., 2007; Torisawa et al., 2007).

Spheroids generated from tumor or tissue biopsies are of growing interest in the arena of individualized and patient-specific therapy. This approach extends the cocultures from cell lines and primary cells to so-called fragment spheroids. Recently, fragment spheroids from malignant tumor and benign mucosa of head and neck squamous cell carcinoma (HNSCC) were cocultured with monocytes. Increased cytokine levels from the culture supernatant correlated with tumor recurrence (Kross et al., 2008). The inter-individual variance observed in the study reflects the real heterogeneity of tumors. This presents a challenge for establishing a robust testing protocol and provides a rationale to pursue personalized therapeutic strategies. A further advantage of using biopsy material is that the cultivated spheres can contain tumor-initiating or tumor progenitor stem cells. As discussed in the last section of this review, these cell subpopulations are likely to provide valuable insights into the biology of tumor formation. A major problem with biopsy material is its availability for research purposes and the limitations of current preservation methods. To address this, a neurosphere cryopreservation technique using 90% serum and 10% dimethyl sulfoxide (DMSO) has been developed which seems to not alter genetic profiles or the cellular phenotype (Chong et al., 2009). This method could facilitate the establishment of a repository of tumor-initiating cells for a battery of standardized experiments.

Table 1 summarizes some relevant therapy-related work with spheroid coculture approaches. It also points to the intriguing use of tumor spheroids cocultured with various immune cell populations to address problems in tumor immunology and immunotherapy, which shall be further discussed herein.

5. Evaluating the efficacy of immunotherapy in 3D cultures

Multicellular tumor spheroids can be cocultured with different immune cells such as monocytes, macrophages, dendritic cells, T cells or natural killer (NK) cells. These cell types are predominantly added as cell suspensions to tumor spheroids followed by monitoring of migration, activation or maturation processes. In combination with therapeutic agents the cocultures are used to study cellular toxicity and therapeutic effects. In immunology research, the spheroid model was in common use in the 1990s and has re-entered the stage in the last few years to further impact the

Table 1
Summary of tumor spheroid coculture applications for therapy testing.

3D model	Cell types	Application/therapy	Reference
Mixed spheroids	HNSCC cell line + fibroblasts	Anti-EGFR monoclonal antibody	Hoffmann et al. (2009)
	Prostate cancer cell line + HUVEC + osteoblasts	Microfluidic system for growth analysis	Hsiao et al. (2009)
	Cervical cancer line + fibroblasts	Survival after irradiation (X-ray)	Djordjevic and Lange (2006)
	HeLa cell line + fibroblasts	Clonogenic fraction and survival curve – hybrid spheroid assay	Lange et al. (2009)
	Biopsy surgical cancer cells + fibroblasts	Clonogenic fraction and survival curves – hybrid spheroid assay	Lange et al. (2009)
	Mouse fibrosarcoma line (cisplatin sensitive and resistant)	Survival after irradiation (X-ray)	Frenzel et al. (1995)
	Rat brain tumor cells (sensitive and resistant to 1,3-bis(2-chloroethyl)-L-nitrosourea)	BCNU treatment	Tofilon et al. (1987)
(Transfectant) Mosaic spheroids	Glioma cell line transfected with noradrenaline transporter gene or GFP	β-emitting radio- pharmaceutical [^{131}I] MIBG to assess bystander effects	Boyd et al. (2002)
MCTS on monolayer	Colon carcinoma cells on monocytes/fibroblasts	Cathepsin B expression, invasiveness	Krueger et al. (2005)
	Melanoma cell lines on endothelial cell lines	Role of T-cadherine in tumor angiogenesis	Ghosh et al. (2007)
	Colon adenocarcinoma cell lines on HUVEC colon epithelium/myofibroblast	Role of TNF-α and TNF-receptors in colon cancer development	Paduch and Niedziela (2009)
Embryoid bodies	Embryonic stem cells + breast cancer spheroids	Leukocyte differentiation	Hannig et al. (2008)
Fragment spheroids	Malignant/benign HNSCC mucosa + monocytes	IL-6 secretion and prediction of prognosis	Kross et al. (2008)
MCTS + immune cell suspension	Benign/malignant urothelial cell line + monocytes/macrophages	Influence of cytokines on monocyte to macrophage differentiation	Konur et al. (1998)
	Benign/malignant urothelial cell line + BCG/lymphokine-activated killer cells	Immune cell infiltration studies	Durek et al. (1999)
	Breast cancer cell line + macrophages	VEGF expression and *de novo* vessel formation	Bingle et al. (2006)
	EC from benign/malignant pancreatic tissue endothelial + T cells/T_{reg}	T cell/T_{reg} infiltration studies	Nummer et al. (2007)
	Breast cancer cell line + monocytes	Monocytes as vehicles for gene therapy	Muthana et al. (2008)
	Bladder/renal carcinoma cell line + CTL/LAK	Assess number of T cells for adoptive therapy	Kawai et al. (2001)
	HNSCC cell line + PBMC	Therapeutic antibody EMD 72000	Hoffmann et al. (2009)
	HNSCC cell line + PBMC	Trifunctional bispecific antibody catumaxomab	Hirschhaeuser et al. (2009)
	Urothelial/prostate carcinoma, melanoma cell lines + monocytes	Differentiation of monocytes to DC	Gottfried et al. (2006)
	Diverse entities + CTLs	Cytotoxicity of CTLs	Fischer et al. (2007)
	Melanoma cell line + CTLs	TAA recognition by CTLs	Feder-Mengus et al. (2007)

development of new immunotherapies. This progression started with the development of different tumor spheroid and immune cell cocultures. Rather than an exhaustive coverage of this literature, we have instead opted to highlight key examples: Urothelial-bladder carcinoma cells have been cocultured with human monocytes or macrophages, and the released cytokines were compared to a coculture of the immune cells with a non-tumorigenic cell line. The tumor spheroids were found to induce the secretion of high levels of IL-1β and IL-6 and to influence the differentiation of monocytes into macrophages (Konur et al., 1998). The same benign and malignant bladder cell lines were cocultured with either Bacillus Calmette-Guérin (BCG) activated killer (BAK) cells or IL-2 (lymphokine) activated killer (LAK) cells and used to assess infiltration. In this setting BAK effector cells, in contrast to LAK cells, did not invade the benign urothelial tumor spheroids (Durek et al., 1999). With a similar approach, it was shown that the migration of monocytes into breast-tumor derived fibroblast spheroids was significantly higher than into normal fibroblast spheroids. This phenomenon relates to an IL-6 – MCP-1 (monocyte chemotactic protein-1 = CCL2) loop and supposedly contributes to the accumulation of tumor-associated macrophages in the desmoplastic stroma of breast tumors (Silzle et al., 2003, 2004). For the study of angiogenesis, T47D mammary carcinoma spheroids were cocultured with macrophages, and resulted in increased vascular endothelial growth factor levels. Transferring this to skin chamber experiments in nude mice, an increase in the number of *de novo* formed vessels was observed (Bingle et al., 2006). The role of EC in the infiltration of regulatory T cells (T_{reg}) has also been studied using a spheroid adhesion assay. T_{reg} cells, but not conventional T cells, showed a strongly enhanced capacity to bind to tumor-derived EC spheroids (Nummer et al., 2007).

Such coculture systems and experiments have been applied to several immunotherapy strategies. One of them is the use of cytokines to enhance natural immune defenses against tumor cells. For example, a superadditive growth inhibition effect was observed when culturing renal cell carcinoma spheroids with interferon-α in combination with retinoids (Rohde et al., 2004). As an alternative to cytokine therapy, cytokines can be targeted with antibodies to inhibit apoptotic protection mechanisms. In this experiment tumorigenic $CD133^+$ colon cancer stem cells were treated with anti-IL-4 to sequester IL-4, rendering the cells more susceptible to apoptosis (Todaro et al., 2008).

Another sophisticated strategy is the application of immune cells as vehicles for gene therapy. To enhance the natural migration of monocytes into tumor masses, they were pre-loaded with magnetic nanoparticles and a magnet was used to transport the cells into the spheroids (Muthana et al., 2008). In a different approach, an integrin binding motif was inserted into an adenovirus HI-loop to improve the gene transfer into organotypic glioma spheroids. Similarly, bispecific antibodies targeting endothelial growth factor receptor and the fiber knob of the adenovirus can also be used (Grill

et al., 2001). Beyond this strategy, great efforts are being made for the development of cell-based immunotherapies. Adoptive therapy with cytotoxic T lymphocytes (CTL) for example was simulated in a spheroid model to asses the number of required autologous T cells for efficient tumor cell killing (Kawai et al., 2001).

In immunotherapy monoclonal antibodies, with their diverse modes of action, are one of the most promising anti-cancer agents. They can be conjugated to radioisotopes, prodrugs, drugs, toxins and cytokines or they can be bispecific and additionally bind T cells. These antibody conjugates can be used to target tumor associated antigens (TAA), trigger antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), interact as receptor antagonists or inhibit vessel formation (Zafir-Lavie et al., 2007). For example, the antibody trastuzumab (Herceptin®) is an antagonist of the Her2/neu receptor which is frequently overexpressed in hormone-receptor negative breast tumors. Trastuzumab was assessed in 2D and 3D cultures. Interestingly, in monolayer cultures heterodimers of Her2 and Her3 were formed, whereas homodimers of Her2 were predominantly formed in spheroid cultures which are also found on breast tumor cells *in vivo*. This led to altered phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling. These findings indicate that spheroid cultures better reflect some *in vivo* aspects of Her2 signaling and offer a better understanding of trastuzumab's mode of action (Pickl and Ries, 2009). Monolayer and 3D (in a laminin-rich ECM gel) breast cancer cells have been treated with pertuzumab, another monoclonal antibody directed against Her2/neu, and also lapatinib, a small molecule tyrosin kinase inhibitor. The sensitivity of the cells to the agents differed between 2D and 3D settings and was even increased by the additional use of a β1-integrin inhibitor (Weigelt et al., 2009). An antibody targeting Her1, which is commonly known as epidermal growth factor receptor (EGFR), was used on a HNSCC spheroid and PBMC (peripheral blood monocytes) coculture model. Pre-treatment with the anti-EGFR monoclonal antibody EMD 72000 led to increased leukocyte infiltration into tumor spheroids overexpressing EGFR due to upregulated MCP-1/CCL2 chemokine secretion induced by anti-EGFR signaling (Hoffmann et al., 2009). Besides monoclonal antibodies targeting antigens or receptors with their two binding sites, the latest development is bispecific antibodies which can simultaneously bind to two different targets. We assessed the trifunctional bispecific antibody catumaxomab (anti-EpCAM × anti-CD3) in a spinner flask coculture of HNSCC spheroids and human PBMCs (Hirschhaeuser et al., 2009). Catumaxomab dose-dependently reduced spheroid volume, increased immune-cell infiltration and cytokine secretion, and decreased the proportion of proliferating cells, the ability of colony formation and the viability of disaggregated single cells.

Recent publications indicate that the metabolic milieu of the tumor cells and its effect on the surrounding immune cells should be considered for developing efficient therapies. It is well known that tumor cells gain most of their energy by glycolysis, producing pyruvate which is often converted to lactic acid. Another source for local lactate accumulation in tumor tissue is a truncated citrate cycle associated with enhanced glutaminolysis. It was shown that lactic acid modulates the differentiation of monocytes into dendritic cells and alters antigen expression (Gottfried et al., 2006). Furthermore, lactic acid disturbs the metabolism of T cells and reduces their cytotoxic activity by half (Fischer et al., 2007). Lactic acid produced in 3D melanoma models is further responsible for defective TAA recognition by CTLs (Feder-Mengus et al., 2007). The pathophysiological environment thus critically contributes to

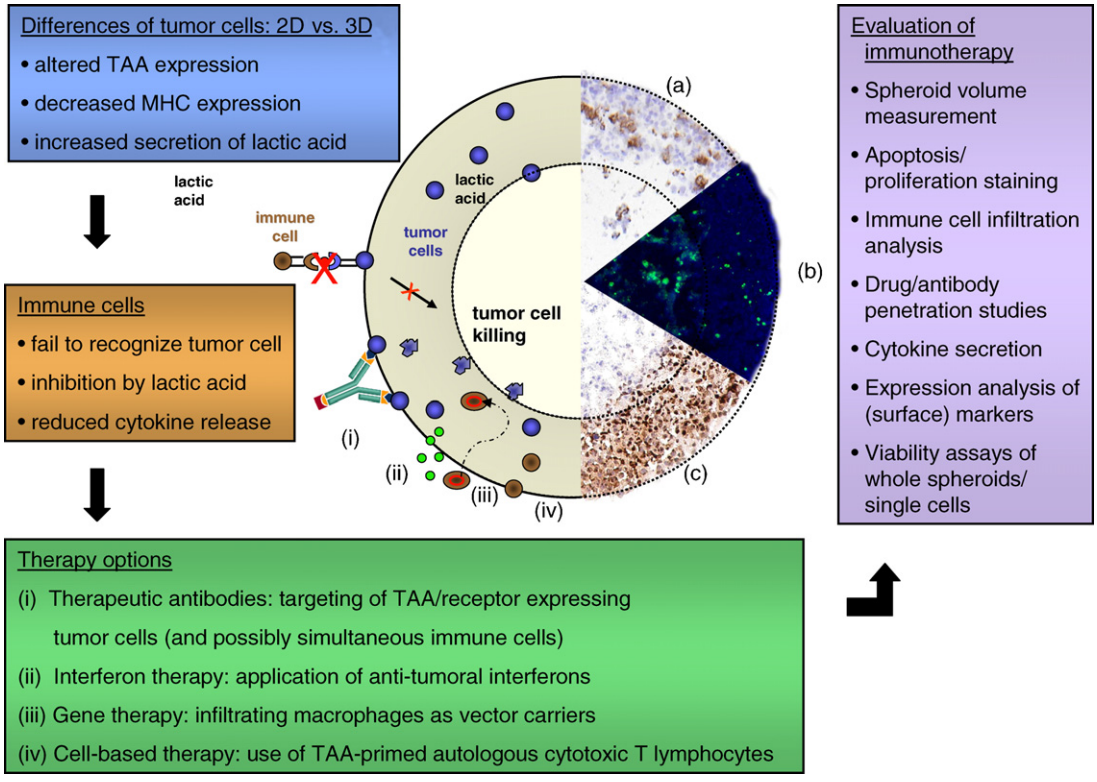

Fig. 3. Immunotherapy options and evaluation methods. Tumor cells cultured in 3D show many differences in biological functions compared to 2D cultures, including modulation of HLA class I molecules, decreased TAA expression and an increased production of lactic acid that leads to defective responsiveness of TAA-specific CTLs to tumor cells. Several therapeutic strategies were developed to increase anti-tumor responses. These include size measurements, penetrations studies, expression analyses, viability assays, secretion of cytokines and various immunohistological stainings. Examples of stained infiltrated $CD45^+$ immune cells (a), apoptosis (b) and $Ki67^+$ proliferating cells (c) on a FaDu spheroid section are shown. The section clearly demonstrates the typical architecture of a multicellular tumor spheroid with an outer viable rim and a necrotic core.

tumor-immune escape and modulation of the tumor metabolic milieu can therefore be used to enhance anti-tumor responses, and represents a striking opportunity for future immunotherapy investigations. Fig. 3 summarizes the potential of 3D cultures to be implemented in such studies.

6. Cancer stem cells – new potential of sphere culturing for therapy-testing?

A cancer 'developmental paradigm' emphasizes the hierarchy of heterogeneous cells present within the tumor mass (Clarke et al., 2006). Here, heterogeneity does not refer to infiltrating and activated tumor stromal cells as highlighted earlier but to differences in the phenotype and functional capacity of different cancer cell subpopulations within a single tumor. The theory of cancer stem cells (CSC) states that a small subset of cancer cells within the tumor mass has the exclusive capacity to both divide and expand the cancer stem cell pool and to differentiate into non-tumorigenic, more differentiated cancer cell lineages. Cancer stem cells are alternatively referred to as tumorigenic as well as tumor- or cancer-initiating cells (TIC or CIC) as these terms describe the human cancer cell's potential for tumor maintenance and propagation in immunodeficient mice in contrast to those lacking this capacity (Cho and Clarke, 2008; Clarke et al., 2006). Human cancers recently described as having such subpopulations of cells include not only cancers of the blood but also solid malignancies of brain, breast, pancreas, colon, and head and neck (Cho and Clarke, 2008; Dalerba et al., 2007; Lee et al., 2006; Ricci-Vitiani et al., 2009). It is beyond the scope of the present article to further discuss the cancer stem cell hypothesis with respect to any particular tumor entity and the ongoing controversy related to the identification and use of sufficient markers and *in vivo* models to define the cancer stem cell pool. However, it is noted that if this difference in tumorigenicity of cancer cells also occurs in patients, then the ability to enrich and maintain these cells *in vitro* lays the important groundwork for future mechanistic and therapy-oriented studies. The existence of small subpopulations of quiescent CSC/TIC which are responsible for tumor recurrence and metastasis, may particularly resist physiological/pathophysiological niches and potentially differ in therapy-response to the bulk tumor cells, has clear implications for the development of novel types of anti-tumor therapy. Only those treatment strategies that lead to a more efficient elimination of CSC/TIC will thus improve tumor cure (Baumann et al., 2008). Accordingly, there have been numerous attempts to isolate and propagate *in vitro* the cancer stem cell compartment from primary tumor material and to identify and verify the capacity of analogous subpopulations from tumor cell lines with similar characteristics. Interestingly, many recent reports state that such cancer stem cell populations are particularly and/or exclusively maintained in sphere culture. This intriguing aspect should no doubt receive thorough consideration in future drug screening initiatives. We will therefore give a few examples where researchers have applied sphere-culturing to isolate, enrich, maintain or expand potential CSC/TIC populations from various tumor sites.

The majority of literature in the field clearly relates to the study of human brain tumors and the culture of so-called neurospheres. In most of these studies serum-free neuronal stem cell or DMEM/F12 based media supplemented with various growth factors and nutrient mixtures (notably containing EGF, bFGF (basic fibroblast growth factor) and LIF (leucemia inhibitory factor)) were applied. With such a protocol, Yuan et al. isolated from patients a small subpopulation of glioblastoma multiforme (GBM) cells that showed self-renewal, could differentiate into multi-lineage progenies and were capable of forming spheres and subsequent subspheres. Only the spheroid-forming cells were capable of producing tumors in athymic nude mice (Yuan et al., 2004). Many others confirmed that neuronal stem cell properties were evident; e.g. a stem-like behavior and enhanced tumorigenic potential of primary glioma, glioblastoma and medulloblastoma cells grown in sphere-forming assays both when using the entire cellular material from tumor biopsies or some tumor cell subpopulations defined and enriched according to their surface expression pattern (Bao et al., 2006a,b; Galli et al., 2004; Lu et al., 2009; Singh et al., 2003, 2004; Wang et al., 2008; Zhang et al., 2006). In a convincing study, Gunther et al. (2008) recently expanded tumor cells from primary material from nine of nineteen glioblastoma patients and observed clearly distinct phenotypes of the cultures. Four cultures grew entirely spherically without cell attachment. These expressed neuro-developmental genes, displayed a full stem-like phenotype and showed 100% tumorigenicity in NMRI (nu/nu) mice. The other cultures grew mostly adherent or semiadherent and only occasionally formed spheres that tended to attach or remain attached to the culture plate. This set of cultures displayed an expression signature enriched for extracellular matrix-related genes, showed a restricted stem-like phenotype and a reduced or no tumorigenicity. A similar study was performed by Laks et al. who cultured neurospheres from 32 patients with glioma including 15 patients with primary glioblastoma. Neurosphere formation was renewable and the tumorigenic capacity of the neurospheres transplanted into the brain of immunosuppressed mice significantly correlated with clinical outcome. Their data imply neurosphere formation to be a robust and independent predictor of glioma tumor progression (Laks et al., 2009). By using neural crest stem cell conditions and sphere formation Hansford et al. (2007) were able for the first time to successfully expand tumor cells both from low-risk neuroblastomas and from the bone marrow metastases of high-risk tumors. The latter formed metastatic tumors in a murine xenograft model with as little as 10 cells and could also be serially passaged.

Spheroid-forming assays in serum-free media for stem cell culturing have also been successfully applied to generate cultures and maintain cancer cells with enhanced clonogenic, differentiation and tumorigenic potential from other solid tumors and their metastases. Colospheres from primary human colon cancer cells (Du et al., 2008; Ricci-Vitiani et al., 2007; Todaro et al., 2008; Vermeulen et al., 2008) and mammospheres of putative CSC/TIC from primary breast cancer patients (Grimshaw et al., 2008; Ponti et al., 2005; Wicha, 2008) are just two examples where tumor sphere culturing has considerably contributed to a better understanding of the behavior and heterogeneity of cancer cell subpopulations and supported the CSC/TIC hypothesis. One of those articles particularly states that CSC/TIC from colorectal cancers can only be maintained in 3D culture under serum-free conditions (Ricci-Vitiani et al., 2007). In their study transfer of the CSC/TIC into a serum-conditioned environment led to cell adherence, differentiation and loss of the tumorigenic potential. A similar observation by Lee et al. (2006) was described for human GBM cells, where the loss of self-renewal and differentiation capabilities of the cells was accompanied by alterations in the gene expression profile when using standard *in vitro* conditions and serum-containing media. Based on this, the use of established cell lines propagated as monolayers for lengthy periods in serum-supplemented media has to be questioned as a viable approach for the development of new therapies. On the other hand, most established cancer cell lines are tumorigenic in immunosuppressed mouse models and it may well be, that some established cancer cell lines by chance selected for the CSC/TIC population even under serum conditions.

Primary tumor biopsy materials which contain the CSC/TIC compartment may have major advantages for developing new therapeutic strategies but because of limited access it is unlikely to become the basis for large-scale test programs. Having said that, the expansion of the CSC/TIC population *in vitro* as well as the

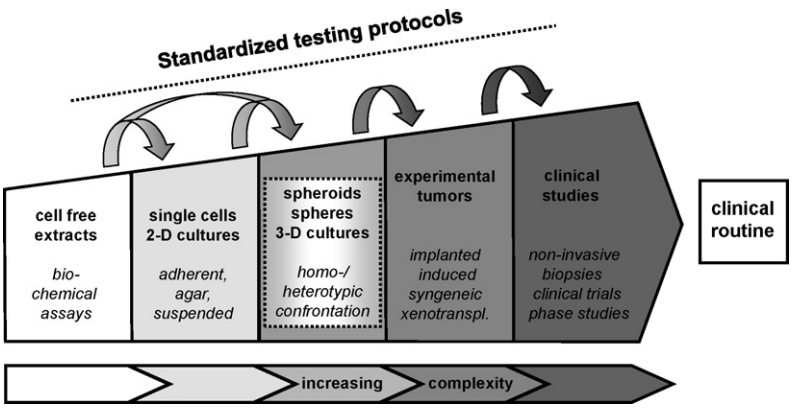

Fig. 4. Implementation of 3D culture assays as a tool for negative and positive selection in the drug development process.

evaluation of established cell lines for a CSC/TIC phenotype is a clear challenge for the coming years. Accordingly, several investigators have also applied serum-free sphere-culture protocols as an approach to (re)-establish or enrich putative CSC/TIC populations from various established human cancer cell lines including prostate cancer (Dubrovska et al., 2009), hepatocellular carcinoma (Kamohara et al., 2008), squamous cell carcinoma (Chiou et al., 2008), breast cancer (Phillips et al., 2007; Ponti et al., 2005; Zhou et al., 2009), pancreatic cancer (Hong et al., 2009); or brain/neuronal tumors (Mahller et al., 2009; Nakai et al., 2009; Qiang et al., 2009; Rappa et al., 2008). This interesting strategy, however, clearly requires further evidence and verification *in vivo* to become a meaningful tool for therapy testing. This is particularly relevant in light of recent, intriguing work by Pollard and co-workers who claim that the neurosphere culture paradigm, i.e. neurosphere culturing to be essential for brain tumor CSC/TIC maintenance and propagation, can be overcome by their strategy of using laminin-coated surfaces to produce adherent cultures (Pollard et al., 2009; Woolard and Fine, 2009). However, the potential of this alternative is not yet clear and further evaluation is required.

Viewing cancers from the developmental perspective and culturing primary cancer cell subpopulations *in vitro* in stem or progenitor cell conditions has clearly led to new insights into tumor biology that otherwise would not have been possible. Sphere-forming assays have become an integral yet compelling tool in these studies.

7. Conclusion and perspectives

Since the cost of drug development increases exponentially at the stage of *in vivo* tumor models and even more when entering clinical trial, the guiding principle in the pharmaceutical industry has been to select for the most promising candidates earlier rather than later. Cell-based assays have become a key element in drug discovery and therapy test programs. However, they have shown to be an expedient but still highly artificial representation of the living patient and its diseased tissue. It is expected that more sophisticated tissue-type and tissue-based assays bear the potential for an improved predictive power for clinical efficacy as they may contribute to both negative selection as well as identification of drugs that have just failed in classical 2D cell assays because of the poor reflection of *in vivo* cell and tissue characteristics (Fig. 4).

Our review has highlighted the unique potential and flexibility of tumor spheroids as a model system to test new treatment strategies in a 3D cellular context and a pathophysiological milieu. The aspect of standardisation and the validation of the most convenient and clinically relevant analytical endpoint(s) which may require long-term culturing are clear goals for the next years to accelerate the proposed implementation of the model into therapy test routines. This is expected to include conventional but also new analytical tools such as various imaging technologies that still have limited potential to be incorporated into rapid, high throughput approaches for spheroid analysis. One of the challenges is the establishment and validation of easy-handling, practical operating conditions for monitoring local drug penetration capacity, tissue distribution and binding properties of therapeutics in a standardized fashion. The extension towards more sophisticated spheroid coculture approaches such as those recommended for the testing of immune cell-based therapeutic strategies can be realized as clearly shown in experimental settings. Today, the reliable use of such models for therapy testing is still confined to experienced laboratories and personnel. Further progress towards standardization and (semi-)automation as well as the development and implementation of ready-to-use analytical tool-kits and instrumentation is required and should be promoted to transmit current experimental set-ups into routine work flows. Such initiatives will clearly be paralleled and may even be accelerated by further improvements in sufficient mass production of well-defined spheroids for screening purposes based on emerging microtechnologies such as micropatterning combined with microfluidics as particularly emphasized in the present article. This kind of work is extremely auspicious but still far from being completed and validated. The applicability of such technologies for the production of uniformly sized but more complex spheroid cocultures is clearly envisioned. Its further development into a culture platform or screening tool for cancer stem or tumor-initiating cells would be an ambitious venture requiring the expertise of various scientific disciplines. However, in light of recent literature which implies that sphere-formation is useful if not necessary to maintain and expand cells with tumor-initiating capability from various primary tumor entities, such devices could revolutionize anti-tumor screening operations and also provide a rationale for personalized therapeutic test strategies.

Acknowledgements

The authors gratefully acknowledge the assistance of Dr. Juergen Friedrich for image preparation and Prof. Christopher S. Lange from the State University of New York for fruitful discussions. The diverse aspects of work with spheroid cultures by the authors is supported by the German Federal Ministry of Education and Research (BMBF, grants 01ZZ0502 and 03NUK006 – LAKS), the Ministry of Innovation, Science, Research and Technology of the state of North Rhine-Westphalia (HM and JW), the German Research Foundation (DFG, grants KU971/7-1 to LAKS, MU576/15-1 to WMK and WE3737/3-1 to HM and JW), Fresenius Biotech GmbH and TRION Pharma GmbH (WMK). OncoRay is funded by the BMBF "Center for Innovation Competence" program.

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