Review

Spheroid culture as a tool for creating 3D complex tissues

Eelco Fennema[1], Nicolas Rivron[1,2], Jeroen Rouwkema[3], Clemens van Blitterswijk[1], and Jan de Boer[1]

[1] Department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands
[2] Laboratory of Pluripotent Stem Cells in Development and Disease, Hubrecht Institute, Utrecht, The Netherlands
[3] Department of Biomechanical Engineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands

3D cell culture methods confer a high degree of clinical and biological relevance to *in vitro* models. This is specifically the case with the spheroid culture, where a small aggregate of cells grows free of foreign materials. In spheroid cultures, cells secrete the extracellular matrix (ECM) in which they reside, and they can interact with cells from their original microenvironment. The value of spheroid cultures is increasing quickly due to novel microfabricated platforms amenable to high-throughput screening (HTS) and advances in cell culture. Here, we review new possibilities that combine the strengths of spheroid culture with new microenvironment fabrication methods that allow for the creation of large numbers of highly reproducible, complex tissues.

Spheroid culture compared to 2D culture

In the body, cells are typically surrounded by an ECM, or are in direct physical contact with cells from either the same or different lineages. However, most studies in cell biology are performed using the cells of interest cultured on cell culture-compatible polystyrene in a 2D monolayer. This approach results in a well-controlled and homogeneous cell environment, facilitates microscopic analysis and medium changes, and sustains cell proliferation for most cell types. However, this is generally not considered the natural microenvironment of the cells. Alternatively, in 3D culturing methods, cells are cultured as aggregates [1], are grown on 3D scaffold materials [2], or are embedded in gels [3]. Aggregates have many different names; ranging from mammosphere [4], micromass [5], and spheroids [6], to microfabricated tissues [7]. We prefer the term spheroid in this review to indicate aggregated cells (not necessarily constituting a perfect spherical shape), that do not adhere to any culture substrate (e.g., polystyrene).

Cells in a 3D environment behave fundamentally different from cells in monolayer culture. For instance, both primary articular chondrocytes as well as hepatocytes rapidly lose their normal phenotype once taken out of the body and put in 2D cell culture, but this loss can be attenuated or even reversed by 3D culturing methodology [8,9]. Additionally, multipotent mesenchymal stromal cell (MSC)-derived hepatocytes support key functions such as albumin and urea synthesis, as well as ammonia and drug clearance better in a 3D environment [10]. Many of the observed differences between 2D and spheroid culture (see Glossary) are due to differential cell–cell and cell–matrix interactions and hence play an important role in this review. For example, modifying cell–matrix adhesions in 3D through depletion of β_1-integrin decreases spheroid forming capability of PC3 prostate adenocarcinoma cells in suspension, whereas it does not hinder them from adhering to tissue culture plastic [11]. Moreover, the loss of articular chondrocyte phenotype is also accompanied by a change in the expression of cell adhesion molecules [12].

Several fabrication methods are used to create spheroids (see Figure I in Box 1). The earliest records describe the hanging drop method (see Figure Ia,b in Box 1), where cells spontaneously aggregate in the bottom of a drop after inverting a plate with drops of cell suspension [13]. This technique is often used to generate embryoid bodies from embryonic stem cells. Alternatively, spinner flasks enable spontaneous cell aggregation [14]. Another technique, the static liquid overlay technique (LOT), where suspended cells are cultured on a nonadherent substrate, causes cells

Glossary

Mature cells: are isolated from specific organs but have restricted proliferation capacity and large populations cannot be obtained.
Microcontact printing: uses these shapes as a die to transfer patterns or patterned substrates.
Microfabrication techniques: facilitate the formation of micrometer-sized shapes through the use of technology from computer chip design (e.g., silicon wafers).
Microfluidics: the handling of microliter volumes through micrometer-sized shapes (e.g., channels) to control the precise delivery of cells and/or substances.
Organoids: Mature cells, adult stem cells, and pluripotent stem cells are organized to recapitulate human physiology and diseases *in vitro*. They can be used as models for drug discovery, as tissue units for implantation, or to understand mechanisms of organ regeneration.
Organ-specific adult stem cells or progenitors: can form tissue structures closely recapitulating adult physiology. They are not tumorigenic but can only form part of an organ (e.g., epithelium).
Pluripotent stem cells: can form all tissues but also generate inadequate populations and tumors. Embryonic stem cells can form whole organs.
Spheroid culture: a non-substrate-adherent, aggregated, mutually adherent population of cells adopting a spherical (-like) shape.

Corresponding author: de Boer, J. (j.deboer@utwente.nl)
Keywords: spheroid; bioengineering; high throughput screening; biomimicry.

 0167-7799/$ – see front matter © 2013 Published by Elsevier Ltd. http://dx.doi.org/10.1016/j.tibtech.2012.12.003 Trends in Biotechnology, February 2013, Vol. 31, No. 2

to aggregate instead of adhere to a surface [15] (see Figure Ia in Box 1). Spheroids can also be formed by centrifugation [16]. More recently, cells have been seeded on nonadherent micropatterned surfaces in microfabricated devices [7] (see Figure Id in Box 1). These techniques open doors for new applications of spheroid cultures, such as high-throughput 3D tissue screening and the formation of complex, organotypical tissue grafts [17]. Here, we discuss spheroid culture models and how new fabrication methods combined with spheroid culture can lead to large numbers of highly reproducible, complex tissues.

Spheroids as a tool to investigate intercellular and cell–matrix interactions

As a result of the replication of a natural cellular niche, spheroids make excellent tools for tissue-based mechanistic assays, as well as for probing cell–cell and cell–matrix interactions. For instance, embryonic skeletogenesis starts with limb bud formation through condensation of MSCs. N-cadherin, an intercellular adhesion protein, is expressed in the condensed mesenchyme, and blocking it with a monoclonal antibody results both in reduced chondrogenesis in micromass culture and perturbed limb development *in vivo* [18]. Not only by direct blocking, but also indirectly through decreased Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling resulting in decreased N-cadherin expression, MSC condensation, and expression of chondrogenic marker genes. Increasing Rac1 signaling has the opposite effect [19]. Migration of cells relies on intercellular and cell–matrix interactions, and knockdown of another cadherin family member, cadherin-7, diminishes chondrogenic differentiation and reduces condensation through impaired migration [20].

Spheroids have also been used as a tool to investigate the role of adhesion molecules in tumor biology. Multicellular tumor spheroids have greater chemotherapeutic resistance than the same cells in monolayer culture. Expression of a dominant negative E-cadherin or wild type E-cadherin in a spheroid model of Ewing Sarcoma tumor cells decreases or increases the chemotherapeutic resistance, respectively [21]. In another model system, a role for the family of kallikrein (KLK)-related peptidases has been found in spheroid culture of serous epithelial ovarian carcinoma (EOC). Two different transcripts of KLK7, KLK7-253 and KLK7-181 are only expressed in EOC but not in healthy ovarian tissue. Increased expression of these transcripts causes increased spheroid formation through integrin-mediated adhesion, and spheroids expressing these transcripts show increased chemoresistance to paclitaxel [22].

Spheroid culture has also been pivotal in the analysis of a new immune-activation phenomenon, termed nemosis. It is described as programmed cell death in activated fibroblast spheroids without the expression of apoptotic markers and accompanied by production of cyclooxygenase (COX)2, proteinases [e.g., matrix metalloproteinase (MMP)1] and proinflammatory cyto- and chemokines [23]. Nemosis is initiated by integrin-fibronectin (FN) binding and regulates fibrocyte spheroid formation. Interfering with this interaction by either using fibronectin knockout cell lines or by blocking the integrin receptor with anti-α5, and -β1 integrin antibodies results in impaired spheroid formation for up to 12 h of treatment and increased COX2 induction [24].

Novel intercellular adhesion proteins, such as connexins and pannexins have also been investigated in spheroids. Pannexins (Panxs) are a class of gap junction proteins [25]. Panx1 induction in the C6 glioma cell line accelerates formation of multicellular aggregates and a more mature F-actin cytoskeleton, whereas interfering with Panx1 has the opposite effect. In a co-culture of Panx1 and Panx2 cells, the percentage of Panx1 cells determines the degree of compaction of the aggregates [26]. These examples illustrate that spheroids recapitulate more physiological cell–cell interactions and subsequent morphogenetic movements such as tissue contraction and condensation.

Engineering native tissues

Organs and tissues are not typically composed of a homogeneous cell population, but rather are complex structures with intricate relations between vessels, nerves, and stroma. This complex interplay of factors requires a 3D environment to account for the many complicated interactions. Classical examples of the use of spheroids for the

Box 1. Spheroid fabrication techniques

Classical methods of forming cellular aggregates include culturing cells in suspension, in round-bottom nonadherent plates and in hanging drops. New platforms based on microfabrication and microfluidics allow for the formation of higher numbers of smaller aggregates with a more homogeneous size. Such new methods are less laborious, more rapid, and amenable to HTS.

To be widely applied by the scientific community, such platforms must be easy and rapid to use, cost-effective, and compatible with machines (e.g., robotic liquid handling devices, and microscopes). Some platforms can be used directly by nontechnological laboratories, whereas others necessitate a technological capacity.

Liquid overlay/cell suspension culture. This method consists of forming cellular aggregates in suspension by stirring large volumes of culture medium. This is an attractive solution for mass production that mainly benefited from the development of bioreactors [69]. It currently does not allow for a precise control of the size of aggregates (Figure Ia).

(Microfabricated) hanging drop. This method consists of forming aggregates in drops hanging from a surface (Figure Ib). New platforms in 96- and 384-well plate formats allow formation of smaller aggregates, with a more controlled size and are less labor intensive [48,70]. These new platforms are commercially available (Figure Ic).

Microwell arrays. The original method consists of forming aggregates in round-bottom nonadherent 96-well plates. New platforms termed microwell arrays have a high number of wells in the micrometer scale [67,71,72]. Nontechnological laboratories might favor the use of stamps (e.g., elastomeric stamps made via soft lithography) to imprint routinely agarose microwell arrays (simple and cost-effective method [67,71]) (Figure Id). Technological laboratories can scale up this method to 96-well plates [73] or combine it with microfluidics [74]. A platform with wells of 400 μm is commercially available.

Microfluidic. Microfluidic channels are used to promote the formation of cellular aggregates. This method opens possibilities for the continuous production of highly controlled aggregates [75]. This method currently necessitates a technological capacity (Figure I e,f).

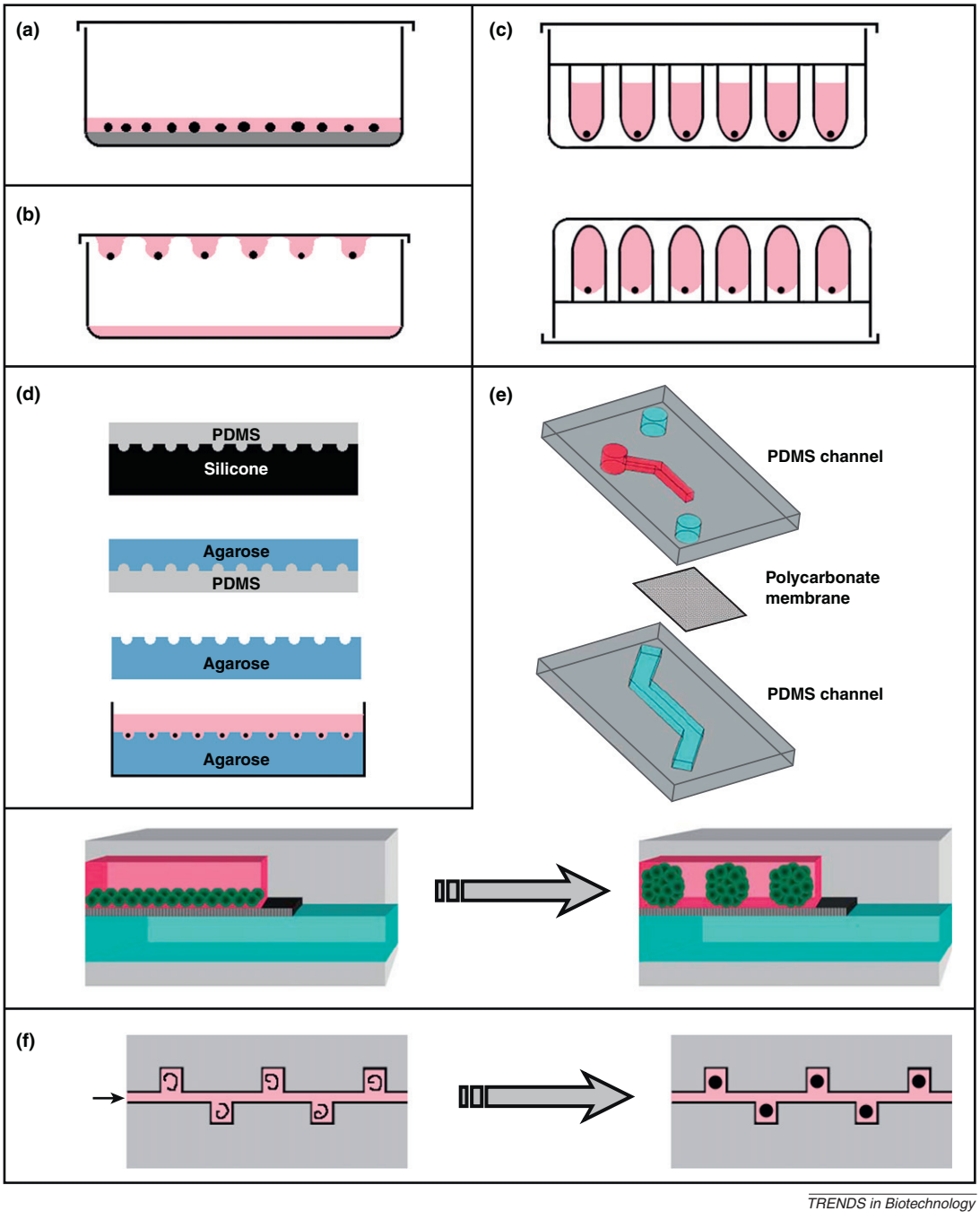

Figure I. Spheroid fabrication methods: **(a)** liquid overlay technique; **(b)** hanging drop technique; **(c)** microwell hanging drop technique; **(d)** microwell array from micropatterned agarose wells; **(e,f)** microfluidic spheroid formation. [(e) is adapted from [75]).

replication of a complex tissue environment are cartilage tissue [27], pancreatic tissue [28], and cardiac muscle [29]. More recent examples include the formation of organs such as the pituitary gland [30], optic cup [31], and layered cortical tissue [32]. We provide examples that are indicative of the possibilities for engineering complex tissues either for regeneration or basic biological research when using spheroid culture.

Tissue engineering constructs are limited in size because they generally lack a vascular network. This results in inadequate distribution of oxygen and nutrients to the implanted construct and limited integration with the host. A possible answer to this problem is the *in vitro* prevascularization of grafts [6,33,34] (Figure 1). Vascularization is a 3D process in which vascular structures develop within another tissue. This makes coculture of spheroids an interesting model system for vascularization, because the dense cellular environment combined with the presence of natural ECM and the possibility to include supporting cells such as mesenchymal precursors, offer a good resemblance of the natural environment in which vascularization takes place. Apart from tissues such as liver, muscle, and cardiac tissue [35], spheroids have been used to engineer prevascularized bone [6]. Blood vessels form in

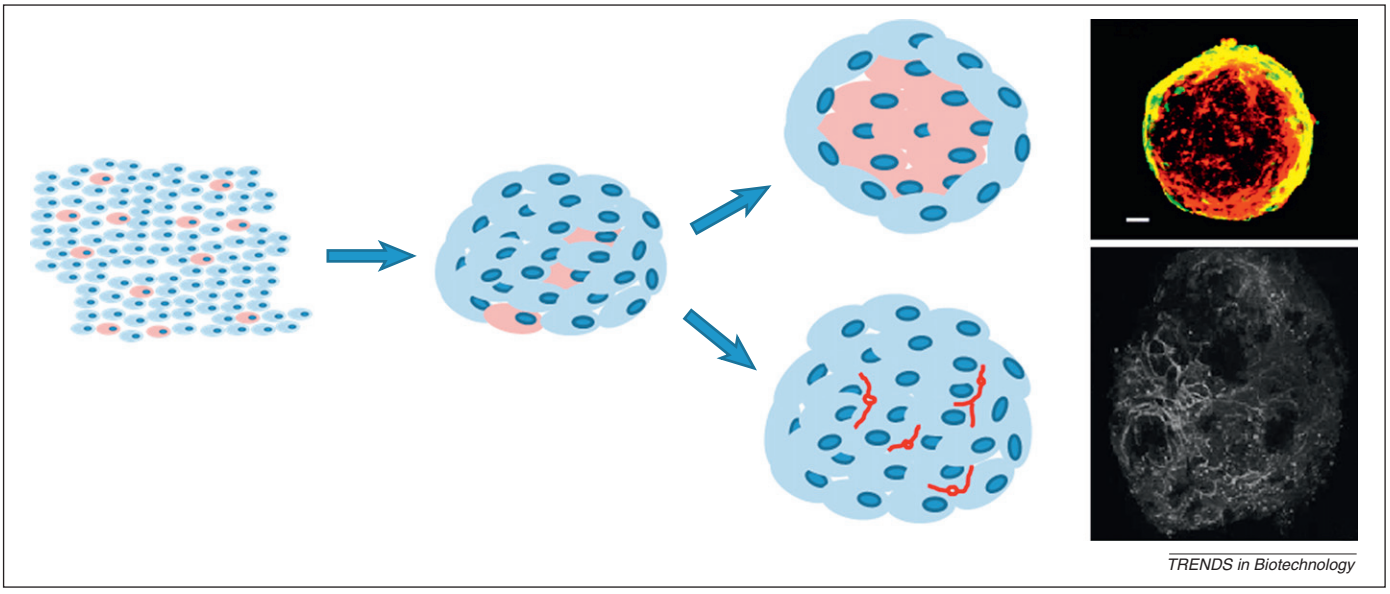

Figure 1. Schematic of two examples of a mixed population of two cell types incorporated in a spheroid resulting in either layer formation (upper path: mixed human aortic fibroblast cells (red) and human umbilical vein endothelial cell (HUVEC) (green) (ratio 10:1) or vascular structures [lower path: co-culture of human mesenchymal stem cells (hMSCs) and HUVECs; ratio unknown] (Scale bar, 20 μm). Adapted from [76] and [37], respectively.

embryoid bodies from endothelial cells that assembled into primitive vascular networks [36]. Spheroids formed of human MSCs and human umbilical vein endothelial cells (HUVECs) also develop primitive vascular structures [37] (Figure 1). Moreover, this heterotypic cell communication leads to increased osteogenic differentiation [37,38] and activation of the Wnt signaling pathway [38]. Such engineered vascular networks can connect with the vasculature of the host upon implantation and increase the survival of the implanted cells [39]. They must mature rapidly (e.g., undergo full tubulogenesis, and be covered with pericytes) and be rapidly perfused to be functional upon implantation [6,34]. The morphogen Sonic hedgehog (shh), which is critical for endothelial tube formation during embryonic development [40], has proven useful to regulate tubulogenesis in this coculture of endothelial and MSCs. Shh regulates the number of lumens and the hierarchy in the size of the lumens in a dose-dependent manner and leads to an increase in perfused lumens in a MSC/HUVEC coculture upon implantation in mice [6]. Functionally, these engineered vascular networks contribute to improve the survival of the implanted cells [39], the *de novo* formation of bone [6], and skeletal muscle tissues [34]. Thus, the formation of native features (e.g., blood and neural networks) in spheroids might be necessary to promote adequate tissue development *in vitro*.

Another complex tissue is the hematopoietic stem cell (HSC) niche. HSCs reside in a subendosteal niche in the bone marrow. They can exit the niche to enter the peripheral blood flow and eventually repopulate the niche in a process called homing. Several cell types have been proposed to create niches for HSCs. Osteoblasts, endothelial cells, and pericytes, at least, are of great importance in anchoring and modulating HSC fate [41]. Spheroids formed using different cell types model the interaction of HSCs with subendosteal cells [42]. Spheroids were formed with MSCs either predifferentiated toward the osteogenic lineage or not. When composed of differentiated cells and infiltrated by $CD34^+$ HSCs, spheroids form better and induce less proliferation of $CD34^+$ cells than when composed of undifferentiated cells. Spheroids formed with a mix of predifferentiated and undifferentiated MSCs organize in a double-layered structure at the interface at which $CD34^+$ cells preferentially reside. $CD34^+$ cells are able to move in and out of the spheroids while maintaining their stemness.

This model system can be used to investigate the dynamical mechanisms of HSC homing/anchorage that are difficult to assess *in vivo*. In the subendosteal niche, HSCs are close to sinusoidal blood capillaries [43]. The formation of engineered vascular networks in these spheroids could more closely mimic the endosteal niche *in vitro*.

Analytical challenges

Until recently, HTS has been predominantly performed with monolayer cultures. Static HTS technology has recently incorporated 3D culturing methods, and can be used for selecting clinically relevant drugs [44]. For toxicity screens however, HTS is still hampered by limitations in drug penetration, contact-dependent multidrug resistance, and oxygen deficiency. For instance, when screening for the therapeutic range of irinotecan (an anticancer drug) on HT29 colorectal cancer spheroids, basic parameters such as cell viability and spheroid volume can be automatically analyzed, but mechanistic assays that explain the difference between 2D and 3D cannot [44]. This means that mechanistic assays, showing for instance increased osteogenic properties of human MSC (hMSC)/HUVEC spheroid cocultures, are not readily available. Control of spheroid size can turn the oxygen deficiency of a large spheroid core from a disadvantage into an essential part of the assay, for instance, in anticancer drug screening [45]. Moreover, spheroids can be used to predict gradients of oxygen that determine cellular responses [46] or embryotoxicity [47].

Newer technologies facilitate long term HTS of spheroids, allowing 384 spheroids to be cultured in a hanging drop fashion, but permitting medium change and drug screening [48]. 3D culture can also be achieved with microfluidics to provide a constant flow of nutrients or substances to large arrays of spheroids [49,50] (see Figure Ie,f in Box 1).

2D cell-based HTS analytical protocols are well-established, as reviewed in [51], whereas 3D HTS methods pose new analytical challenges. When turning to 3D HTS analysis, many assays are rendered useless. For example, in the lactate dehydrogenase (LDH) cell viability assay, a crescent fluorescent signal lingers after the reaction is chemically stopped, making it difficult to assess cell viability, and only the acid phosphatase activity assay is reliable [52]. Alternatively, confocal microscopy can be used to image up to 320 μm depth in spheroids [53]. The necessity for such interventions hampers the applicability of confocal imaging to 3D HTS [54]. Moreover, most high-throughput analytical programs are designed to tailor the needs of 2D HTS by analyzing fluorescent or chemiluminescent signals [55], posing yet another obstacle for 3D HTS, where the layered structure might interfere with the signals. Apart from the analytical hurdles, HTS 3D imaging results in vast amounts of data that require a tailored infrastructure for processing and analysis [56]. 3D HTS screening has nevertheless been proven possible in specific cases. For example, spheroid volume, cell migration, and invasion into Matrigel can be analyzed in an automated fashion using bright field imaging upon treatment with three anticancer drugs [57]. To the best of our knowledge, high-throughput confocal structural analysis of 3D cultures has not yet been established. Other assays, such as gene and protein analysis using multiplex PCR and multiplex ELISAs, respectively, can be applied to spheroid lysate or conditioned medium.

Steering complex tissue architecture

Natural tissue has its own architecture in which cells receive mechanical cues that will steer their behavior. For instance, in bone tissue, matrix stiffness controls osteogenic gene expression [58], but cell migration also controls tissue architecture [59].

One of the theories that describes this architecture or organization of cell populations uses spheroids as a basis. In 1963, Steinberg postulated the 'differential adhesion hypothesis' (DAH), in which the sorting cell populations in development or tissue reconstruction are compared with mixing liquids, such that the intercellular adhesion forces that determine the final configuration of the populations minimize the total surface free energy [60,61]. Tissues with lower surface tension (*e.g.* liquid) envelop tissues with higher tissue tension (*e.g.* elastic-solid state) [62] and will take a spherical form, independent of its composition. Morphological evidence for the theory comes from embryology where tissues with a higher tissue surface tension (TST; e.g., heart) are enveloped by tissues with a lower TST (e.g., liver) [62]. For example, increasing α5β1 integrin levels by transfection leads to an increase in TST. Simultaneously increasing the available exogenous fibronectin leads to a transition of the spheroids from a liquid state to the elastic-solid state (increased TST). These data indicate that the behavior of cell populations can be tuned from

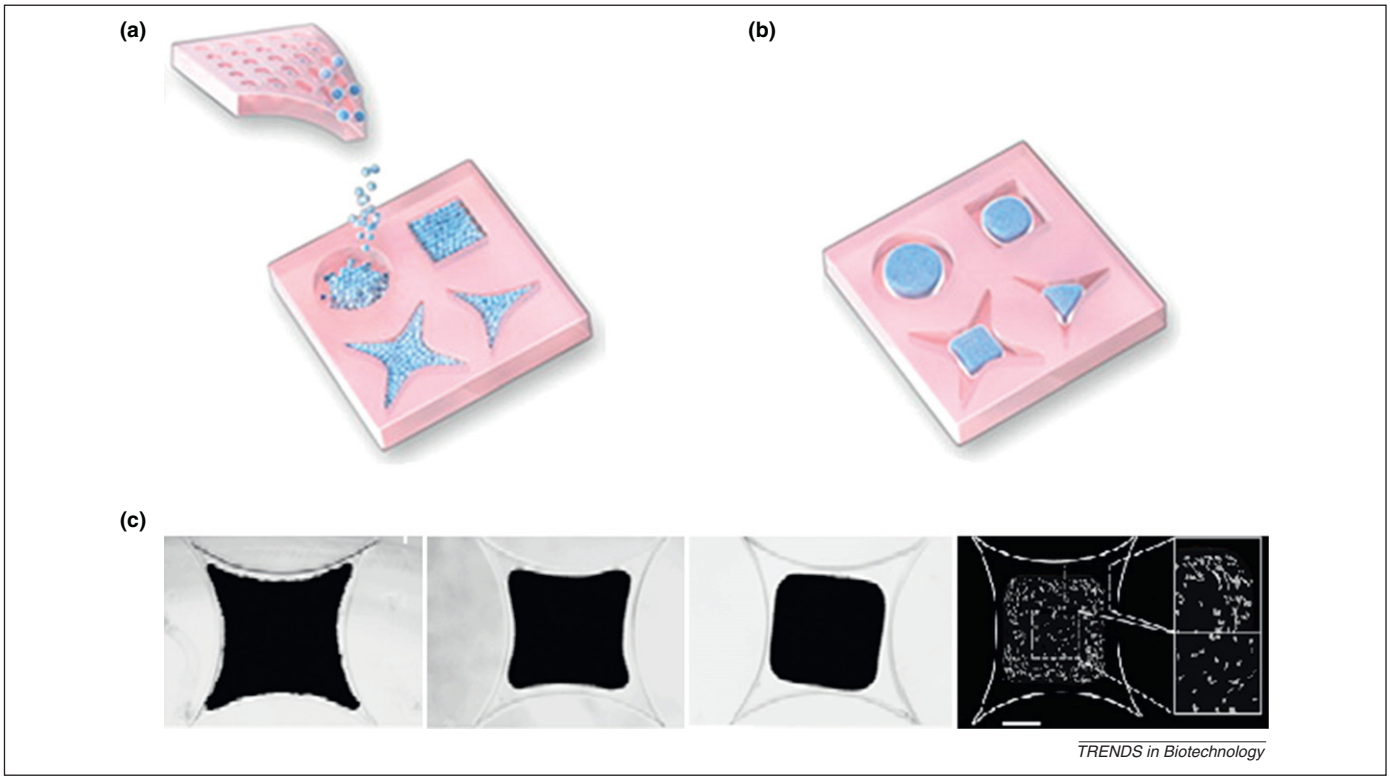

Figure 2. A cartoon schematic showing how spheroids are used to create a larger tissue. **(a)** Spheroids are seeded into shapes and **(b)** form a tissue. **(c)** Spheroids of human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) form complex tissues with a gradient of $PECAM^{+}$ cells in the areas of high deformation (inserts) (scale bar, 500 μm). Adapted from [67].

liquid to elastic by interfering with either receptor or ligand densities, leading to different mechanical properties [63], and can be seen as an engineering tool to manipulate tissue morphogenesis *in vitro*.

Another way of steering the spatial organization of cell populations is the use of micromolded thermoresponsive substrates, where cells are seeded inside a circular shape at 24 °C. Upon subsequent incubation at 37 °C, the substrates shrink, creating space around the first aggregated cells where a second population of cells can be cultured, thus determining the spatial organization of these cell types [64].

Tissue composition is typically repetitive in nature. The liver, for instance, is organized in lobules and composed of sinusoids that all have an identical architecture; the 'building blocks' of the liver. Microfabrication tools allow for high-precision fabrication of templates for microscale building blocks that can be combined to form complex tissues with diverse geometries. For instance, a patterned hyaluronic acid substrate can be created with a microfluidic mold. The nonpatterned surface is coated with fibronectin, where induced cardiomyocytes preferentially attach, elongate, and align to the pattern to eventually form millimeter-sized beating organoids [65]. Similar techniques make it possible to create shapes that force aggregated cells into a fixed geometry. Substrate geometry can change cell shape and fate of cells through actin–myosin tension mediated RhoA–ROCK signaling [66]. Microfabrication techniques have been applied to cocultures of aggregated cells to generate different tissue shapes (Figure 2). In star-shaped and triangular cocultures of HUVECs and hMSCs, PECAM-1$^+$ cells prefer areas of high deformation (corners) and correspond with areas of increased concentrations of vascular endothelial growth factor (VEGF)-A and VEGF receptor (VEGFR)-2 (Figure 2c). The effects of the external deformation can be attenuated through the inhibition of cytoskeletal tension by actin and myosin blocking agents [67].

Microcontact printing has made it possible to create 2D shapes that force cells into a fixed geometry. Microcontact printing has been extended to study multicellular behavior [68], or the patterned behavior of cells in structures similar to tissues. Microfabrication techniques have been further applied to assemble 3D, free-floating, geometric tissues using multicellular aggregates of endothelial cells and MSCs as building blocks. These 3D microfabricated tissues deform and change shape autonomously due to endogenous actin/myosin-generated forces. They form self-organized patterns of vascular structures in regions of high deformation, and correlate with the formation of a long-range gradient of VEGF in interstitial cells in these regions. The interstitial cells in the deformed regions overexpress VEGFR-2, and endothelial cells show an increased proliferation rate. Tissue contractility and deformation can induce the formation of gradients of angiogenic microenvironments that could contribute to the long-range patterning of the vascular system [67] (Figure 2c).

Summary and future perspectives

Spheroid culture is a versatile and powerful biomimicry tool in many areas of (regenerative) medicine, in basic science, and more application-oriented approaches. Technical improvements in the last decade have facilitated the fabrication of large numbers of highly reproducible spheroids. Complex tissues can be engineered by the use of spheroid cultures and microfabrication techniques. Although we see the aggregation of cells into spherical shapes in biological processes, these techniques should not be confined to the spherical shape *per se* because they might reveal mechanisms not present in spheroids (e.g., local deformation). We encourage studies that investigate the formation of complex tissues as biological models or engineered solutions, so that large patient populations could benefit from engineered cardiac or pancreatic tissue, for instance. Spheroid culture has also entered the realm of HTS, however, because there are no assays specifically developed for spheroid HTS, they must be tailor-made. Assay development and the analysis of large quantities of data are two important challenges to face for the future of spheroid HTS. When these challenges are met, HTS screening of complex tissue should be possible, which can yield a turnaround for many other fields of research, as it already did for cancer medicine with tumor spheroids on a small scale. To conclude, we believe that spheroid culture in concert with established techniques can provide new insights and solutions to challenging scientific questions.

Acknowledgements

The work of E.F. was sponsored by the *Perfectos* research grant from Agentschap NL. JR is supported by a VENI fellowship from the Dutch Technology Foundation STW, which is part of the Netherlands Organisation For Scientific Research (NWO).

References

1 Lin, R-Z. and Chang, H-Y. (2008) Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol. J.* 3, 1172–1184
2 Moroni, L. *et al.* (2008) Integrating novel technologies to fabricate smart scaffolds. *J. Biomater. Sci. Polym. Ed.* 19, 543–572
3 Slaughter, B.V. *et al.* (2009) Hydrogels in regenerative medicine. *Adv. Mater.* 21, 3307–3329
4 Klopp, A.H. *et al.* (2010) Mesenchymal stem cells promote mammosphere formation and decrease E-cadherin in normal and malignant breast cells. *PLoS ONE* 5, e12180
5 Greco, K.V. *et al.* (2011) High density micromass cultures of a human chondrocyte cell line: A reliable assay system to reveal the modulatory functions of pharmacological agents. *Biochem. Pharmacol.* 82, 1919–1929
6 Rivron, N.C. *et al.* (2012) Sonic Hedgehog-activated engineered blood vessels enhance bone tissue formation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4413–4418
7 Rivron, N.C. *et al.* (2009) Tissue assembly and organization: developmental mechanisms in microfabricated tissues. *Biomaterials* 30, 4851–4858
8 Bierwolf, J. *et al.* (2011) Primary rat hepatocyte culture on 3D nanofibrous polymer scaffolds for toxicology and pharmaceutical research. *Biotechnol. Bioeng.* 108, 141–150
9 von der Mark, K. *et al.* (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* 267, 531–532
10 Li, J. *et al.* (2010) 3D PLGA scaffolds improve differentiation and function of bone marrow mesenchymal stem cell-derived hepatocytes. *Stem Cells Dev.* 19, 1427–1436
11 Schooley, A.M. *et al.* (2012) β1 integrin is required for anchorage-independent growth and invasion of tumor cells in a context dependent manner. *Cancer Lett.* 316, 157–167
12 Mahmood, T.A. *et al.* (2004) Adhesion-mediated signal transduction in human articular chondrocytes: the influence of biomaterial chemistry and tenascin-C. *Exp. Cell Res.* 301, 179–188

13 Harrison, R.G. (1907) Observations on the living developing nerve fiber. *Proc. Soc. Exp. Biol. Med.* 4, 140–143
14 Sutherland, R.M. *et al.* (1971) Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J. Natl. Cancer Inst.* 46, 113–120
15 Yuhas, J.M. *et al.* (1977) A simplified method for production and growth of multicellular tumor spheroids. *Cancer Res.* 37, 3639–3643
16 Handschel, J.G.K. *et al.* (2007) Prospects of micromass culture technology in tissue engineering. *Head Face Med.* 3, 4
17 Moreira Teixeira, L.S. *et al.* (2012) High throughput generated micro-aggregates of chondrocytes stimulate cartilage formation in vitro and in vivo. *Eur. Cell Mater.* 23, 387–399
18 Oberlender, S.A. and Tuan, R.S. (1994) Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development* 120, 177–187
19 Woods, A. *et al.* (2007) Rac1 signaling stimulates N-cadherin expression, mesenchymal condensation, and chondrogenesis. *J. Biol. Chem.* 282, 23500–23508
20 Kim, Y. and Rajagopalan, P. (2010) 3D hepatic cultures simultaneously maintain primary hepatocyte and liver sinusoidal endothelial cell phenotypes. *PLoS ONE* 5, e15456
21 Kang, H.G. *et al.* (2007) E-Cadherin cell-cell adhesion in Ewing tumor cells mediates suppression of anoikis through activation of the ErbB4 tyrosine kinase. *Cancer Res.* 67, 3094–3105
22 Dong, Y. *et al.* (2010) Kallikrein-related peptidase 7 promotes multicellular aggregation via the α5β1 integrin pathway and paclitaxel chemoresistance in serous epithelial ovarian carcinoma. *Cancer Res.* 70, 2624–2633
23 Vaheri, A. *et al.* (2009) Nemosis, a novel way of fibroblast activation, in inflammation and cancer. *Exp. Cell Res.* 315, 1633–1638
24 Salmenperä, P. *et al.* (2008) Formation and activation of fibroblast spheroids depend on fibronectin–integrin interaction. *Exp. Cell Res.* 314, 3444–3452
25 Panchin, Y. *et al.* (2000) A ubiquitous family of putative gap junction molecules. *Curr. Biol.* 10, R473–R474
26 Bao, B.A. *et al.* (2012) Pannexin1 drives multicellular aggregate compaction via a signaling cascade that remodels the actin cytoskeleton. *J. Biol. Chem.* 287, 8407–8416
27 Jukes, J.M. *et al.* (2008) Endochondral bone tissue engineering using embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6840–6845
28 Lumelsky, N. *et al.* (2001) Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292, 1389–1394
29 Kehat, I. *et al.* (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* 108, 407–414
30 Suga, H. *et al.* (2011) Self-formation of functional adenohypophysis in three-dimensional culture. *Nature* 480, 57–62
31 Eiraku, M. *et al.* (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 472, 51–56
32 Eiraku, M. *et al.* (2008) Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell* 3, 519–532
33 Rouwkema, J. *et al.* (2008) Vascularization in tissue engineering. *Trends Biotechnol.* 26, 434–441
34 Koffler, J. *et al.* (2011) Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. *Proc. Natl. Acad. Sci. U.S.A.* 108, 14789–14794
35 Kelm, J.M. and Fussenegger, M. (2004) Microscale tissue engineering using gravity-enforced cell assembly. *Trends Biotechnol.* 22, 195–202
36 Gu, A. and Shively, J.E. (2011) Angiopoietins-1 and -2 play opposing roles in endothelial sprouting of embryoid bodies in 3D culture and their receptor Tie-2 associates with the cell–cell adhesion molecule PECAM1. *Exp. Cell Res.* 317, 2171–2182
37 Rouwkema, J. *et al.* (2006) Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct. *Tissue Eng.* 12, 2685–2693
38 Saleh, F.A. *et al.* (2011) Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional in vitro model. *Eur. Cell Mater.* 22, 242–257
39 Levenberg, S. *et al.* (2005) Engineering vascularized skeletal muscle tissue. *Nat. Biotechnol.* 23, 879–884
40 Vokes, S.A. (2004) Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development* 131, 4371–4380
41 Ding, L. *et al.* (2012) Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462
42 de Barros, A.P.D.N. *et al.* (2010) Osteoblasts and bone marrow mesenchymal stromal cells control hematopoietic stem cell migration and proliferation in 3D in vitro model. *PLoS ONE* 5, e9093
43 Bianco, P. (2011) Bone and the hematopoietic niche: a tale of two stem cells. *Blood* 117, 5281–5288
44 Friedrich, J. *et al.* (2009) Spheroid-based drug screen: considerations and practical approach. *Nat. Protoc.* 4, 309–324
45 Asthana, A. and Kisaalita, W.S. (2012) Microtissue size and hypoxia in HTS with 3D cultures. *Drug Discov. Today* 17, 810–817
46 Lee, S.Y. *et al.* (2010) Implication of necrosis-linked p53 aggregation in acquired apoptotic resistance to 5-FU in MCF-7 multicellular tumour spheroids. *Oncol. Rep.* 24, 73–79
47 Seiler, A.E. and Spielmann, H. (2011) The validated embryonic stem cell test to predict embryotoxicity in vitro. *Nat. Protoc.* 6, 961–978
48 Tung, Y.C. *et al.* (2011) High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *Analyst* 136, 473
49 Agastin, S. *et al.* (2011) Continuously perfused microbubble array for 3D tumor spheroid model. *Biomicrofluidics* 5, 024110
50 Montanez-Sauri, S.I. *et al.* (2011) Automation of three-dimensional cell culture in arrayed microfluidic devices. *J. Lab. Autom.* 16, 171–185
51 Michelini, E. *et al.* (2010) Cell-based assays: fuelling drug discovery. *Anal. Bioanal. Chem.* 398, 227–238
52 Friedrich, J. *et al.* (2007) A reliable tool to determine cell viability in complex 3-D culture: the acid phosphatase assay. *J. Biomol. Screen* 12, 925–937
53 le Roux, L. *et al.* (2008) Optimizing imaging of three-dimensional multicellular tumor spheroids with fluorescent reporter proteins using confocal microscopy. *Mol. Imaging* 7, 214–221
54 LaBarbera, D.V. *et al.* (2012) The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert Opin. Drug Discov.* 7, 819–830
55 Fan, F. and Wood, K.V. (2007) Bioluminescent assays for high-throughput screening. *Assay Drug Dev. Technol.* 5, 127–136
56 Kozak, K. *et al.* (2009) Data mining techniques in high content screening: a survey. *J. Comput. Sci. Syst. Biol.* 2, 219–239
57 Vinci, M. *et al.* (2012) Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol.* 10, 29
58 Shih, Y.V. *et al.* (2011) Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. *J. Bone Miner. Res.* 26, 730–738
59 Österholm, C. *et al.* (2012) Fibroblast EXT1-levels influence tumor cell proliferation and migration in composite spheroids. *PLoS ONE* 7, e41334
60 Steinberg, M.S. (1963) Reconstruction of tissues by dissociated cells. *Science* 141, 401–408
61 Steinberg, M.S. (1970) Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. *J. Exp. Zool.* 173, 395–433
62 Foty, R.A. *et al.* (1996) Surface tensions of embryonic tissues predict their mutual envelopment behavior. *Development* 122, 1611–1620
63 Caicedo-Carvajal, C.E. *et al.* (2010) α5β1 integrin-fibronectin interactions specify liquid to solid phase transition of 3D cellular aggregates. *PLoS ONE* 5, e11830
64 Tekin, H. *et al.* (2012) Controlling spatial organization of multiple cell types in defined 3D geometries. *Adv. Mater.* 24, 5543–5547
65 Khademhosseini, A. *et al.* (2007) Microfluidic patterning for fabrication of contractile cardiac organoids. *Biomed. Microdevices* 9, 149–157
66 McBeath, R. *et al.* (2004) Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev. Cell* 6, 483–495
67 Rivron, N.C. *et al.* (2012) Tissue deformation spatially modulates VEGF signaling and angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6886–6891

68 Nelson, C.M. *et al.* (2005) Emergent patterns of growth controlled by multicellular form and mechanics. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11594–11599
69 Want, A.J. *et al.* (2012) Large-scale expansion and exploitation of pluripotent stem cells for regenerative medicine purposes: beyond the T flask. *Regen. Med.* 7, 71–84
70 Hsiao, A. *et al.* (2012) Micro-ring structures stabilize microdroplets to enable long term spheroid culture in 384 hanging drop array plates. *Biomed. Microdevices* 14, 313–323
71 Napolitano, A.P. *et al.* (2007) Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Eng.* 13, 2087–2094
72 Truckenmüller, R. *et al.* (2011) Thermoforming of film-based biomedical microdevices. *Adv. Mater.* 23, 1311–1329
73 Markovitz-Bishitz, Y. *et al.* (2010) A polymer microstructure array for the formation, culturing, and high throughput drug screening of breast cancer spheroids. *Biomaterials* 31, 8436–8444
74 Chen, H. *et al.* (2011) Microwell perfusion array for high-throughput, long-term imaging of clonal growth. *Biomicrofluidics* 5, 44117–44117-13
75 Torisawa, Y.S. *et al.* (2007) Efficient formation of uniform-sized embryoid bodies using a compartmentalized microchannel device. *Lab Chip* 7, 770–776
76 Kelm, J.M. *et al.* (2005) VEGF profiling and angiogenesis in human microtissues. *J. Biotechnol.* 118, 213–229