Directed assembly and development of engineered tissues using microwell screening platforms

Chapter 7

Valorization of Research Findings

Valorization

The term “valorization” includes the use or application of an invention, object, process or activity so that it generates value. In the European Union’s perspective on academic research, this can be described by the dissemination and exploitation of the results obtained within a given project. Here, a few potentially interesting options are outlined for valorizing the research described in this thesis.

Microwell platforms

Two microwell screening platforms are described in this thesis, namely 1) the (wet) agarose hydrogel-based microwell screening platform which is incorporated into a standard transparent polystyrene 96-well culture plate, and 2) the thermoformed microwell platform which is based on a (dry) thermoformed COP film that is bonded to a bottomless standard 96-well plate. Both share a similar purpose but have their own characteristics. Above all, the hydrogel system is wet and thus requires additional conduct to preserve sterility. The benefit of a wet system is the absence of noticeable diffraction differences between the microwell material and the culture medium. Conversely, the advantages of using the thermoformed system are its accessible handling and compatibility with most (confocal) microscopy objectives, as the sample-objective distance is smaller.

The powerful aspect of microwell screening arrays is their versatility in assay application. First of all, many cell types allow their culture as unanchored aggregates, either as single cell type spheroids or multicellular aggregates that can organize and develop into structures that replicate tissues and organs, including diseased ones. Moreover, microwells can be modified to allow cells attaching onto a surface or to anchor cells within a substrate of ECM components. As of the hydrogel microwell screening platform, initial trials were performed of modifying the hydrogel material, in this case gelatin, as it is cheap and easy-to-handle. However, the system can expectedly be tailored with a range of hydrogel materials such as fibronectin, collagen or matrigel that could extent culture options to tissues and organoids necessitating their integration into such ECM-like microenvironments [1]. Similarly, thermoformed microwell plates permit the engraftment of ECM components such as a basal membrane-like substrates onto the polymer film. As such, functionalized surfaces can be introduced as a (patterned) interface to control cell adhesion, morphology and differentiation.

The competition

Recently, various companies have been founded that jump in on the trend of 3D cultures and their analysis. The company Microtissues sells the ‘3D Petri Dish’ which includes a polymeric stamp used for replica molding microwells into hydrogel, however not directly in multiwell plates but on a tissue culture surface such as a standard single dish. Alternatively, the company Stemcell licensed the product ‘AggreWell™’ which is based on standard multiwell plates, specifically 6-well plates. They offer a large array of polymeric microwells integrated into the multiwell dish. Similarly, the enterprise Elplasia offers a range of standard multiwell plate-based microwell platforms comprised of either square or round bottom microwells. As a 96-wellplate is also in their assortment, this

permits high-throughput screening (HTS) of aggregates with a decent biological replicate number per well/ condition.

Others also attempt to translate cell aggregate cultures to the realm om HTS. The company 3DBiomatrix sells the 'Perfecta3D® Hanging Drop Plate' in a 96 and 384 format that allows aggregation by the classical hanging drop methods and accessible imaging, although only one cellular structure is formed per "well". An identical approach is pursued by the company InSphero with their 'GravityTRAP™ ULA Plate'.

Then there is Alvetex, that does not focus on self-organizing tissues but offers cells a polymeric scaffold substrate in the form of inserts for existing standardized culture platforms. Others do not sell the platform but instead provide services for 3D high-content imaging and analysis of over 150 different 3D cellular models. The company Ocello generates these models in-house and employs them for phenotypic screening and compound profiling. As an extra service they perform in-depth 3D imaging and analysis on plates from customers.

Unique selling point

The valorization potential of the microwell screening platforms - as they are now – regards the massively parallelized culture of non-adherent and matrix-free 3D cellular aggregates. The unique selling point of both the thermoformed and hydrogel-based platform is this integration within a 96-wellplate format which renders their compatibility with HTS. Importantly, every well contains more than hundred microwells which provides statistical robustness in screening assays. Also, both platforms are compatible with liquid handling machines and high-content imaging systems. Comparing with competitor's platforms, the round-bottom plates from Elplasia display the highest similarity to our thermoformed and hydrogel microwell plates. However, the imaging possibilities of this plate are so far unknown as the information provided by Elplasia is limited.

The hydrogel microwell platform

The hydrogel microwell platform is already successfully applied in a variety of research projects. Examples include the controlled re-aggregation of primary human pancreatic islet cells [2] and the facilitation of changing identity of mature human beta-cells into glucagon-producing alpha-cells [3], which have potential impact on regenerative strategies in diabetes.

The polymeric mold is the most valuable part of this microwell replica-molding system. It contains pillars placed in an arrayed format for 96-well plates. Every pillar contains hundreds of micropillars that are copied into an agarose hydrogel by replica molding which is a sort of stamping. Two potential modifications to the system that may be considered for improvement:

- Speeding up the production; the stamp material may be substituted to something that is more stiff and easily releases from the agarose hydrogel without the need of a wet environment. For example, a thermoplastic polymer may be considered, such as polystyrene. However, polystyrene has a linear expansion coefficient of 0.7% per 100 degree, yielding in shrinkage. This in contrast to 0.03% for silicon. Alternatively, an anti-sticking coating may be applied such that the stamp can be released more easily after molding the hydrogel.

- The microwell geometry could be modified to one with a hemispherical or conical shaped bottom instead of a flat one. This would extend the rapid formation of aggregates to cells that are resilient to spontaneous aggregation.

The thermoformed microwell platform

Meanwhile, thermoformed microwell systems have been used for a plethora of cell biological studies in academic research. For example as a promising approach in confining Individual islets, thereby providing a protective environment to preserve islets during and after transplantation [4]. Another study showed augmented bone formation in 3D aggregate cultures from human osteoblasts under fluid flow-mediated biomechanical stimulation [5]. Such studies validate the thermoformed microwell system as a culture platform for 3D models that mimic native tissue.

Capitalization

Pioneers of microthermoforming technology have introduced a thermoformed microwell screening plate into the market through a newly founded company named 300Microns. Moreover, the focus is on providing tailored polymer film-based 3D cell culture systems. The technology for microthermoforming polymer film-based products is protected by intellectual property that is licensed from Karlsruhe Institute of Technology, where it was developed.

Scaffold-free tissues of clinically relevant size

The study that is described in Chapter 2 – The generation of scaffold-free tissues with defined shape and up to centimeter scale – is backed up by a patented invention that relates to the modular bottom-up method that was employed for producing these 3D tissues [6]. In particular, the invention describes the formation of tissues by combining living cells to form supracellular aggregates using geometrical confinements, followed by combining those aggregates and applying conditions that induce self-assembly and tissue morphogenesis. Chapter 2 described the formation of tissues with defined architecture by using intermediate tissue building blocks formed by human primary cells and stem cells. This opens the door of forming shaped tissues on demand from patient-derived cells. First, these cells can be multiplied in vitro, then aggregated into tissue building blocks, optionally guided using small molecules to differentiate into the appropriate tissue type, and then formed into a tissue of clinical relevant size with a defined geometry that fits to the site of implantation in the patient.

Using this method, cartilage tissue generation could be an attainable option to explore taking to the next level, for instance animal studies. Especially, recent developments in culture of articular-like cartilage from human primary chondrocytes or MSCs may contribute to this purpose [7, 8] and provide more authentic cartilage tissue that is adjusted in shape to fit into defect.

The blastoid model

Potential applications for the blastoid are modeling and studying embryonic development and basic biology, toxicology screenings and cloning.

Cloning

We envision that cherry picking the blastoids that show correctly aligned primitive endoderm, covering the ICM, could be an effective strategy to increase the chances of implantation. Obviously, this necessitates an non-invasive method to spatially resolve the formation of PrE. A fluorescent reporter for PrE can provide a solution, however, challenges await in forming blastoids using wildtype cells or cells which are custom gene-edited. A potential solution could be harnessing machine learning to find a relation between phenotypical data, obtained via bright-field microscopy, and correct specification of PrE.

Cloning livestock

In case blastoids show the competence of implanting and developing to term, cloning will be a very interesting and thought-provoking application. Particular impact would be in livestock cloning, preferably cattle [9]. For instance, the bovine breeding community showed increased interest for in vitro assisted development of bovine embryos (in vitro embryo production – IVP) in the last decades. Nowadays many bovine embryos are produced by in vitro fertilization using sex tested semen to secure sex and high genetic value. The significant next step would be reproductive cloning of adult cattle by nuclear transfer, which is technically feasible but expensive and inefficient. As such, switching to commercially interesting bovine blastoid development should be the priority.

Breeding genetically modified mice

Gene targeting has led to hundreds of different mouse models for human disorders and these genetically modified mice are used in 2/3 of all mice experiments done in the UK (Office 2014). Inactivating genes Knockout mice have revealed the roles of many genes important in embryonic development, healthy physiology and disease.

Generating knockout mice typically starts with targeting genes in ES cells which are then selected, proliferated and injected into mouse blastocysts where they mix with cells from the autologous ICM. The injected blastocysts first have to be implanted in a pseudo-pregnant mouse to grow to a full pup that hopefully contains the altered gene in its gametes. If so, Mendel's law applies to the mice that are bred allowing their altered genes to be inherited to offspring that contains this modification in all of their cells.

When blastoids are able to develop into full-grown mice, genetic modification can be easily introduced in these animals by targeting the embryonic stem cells that grow them. This would significantly speed up the process as it assures the genetic inheritance of modified genes and circumvents Mendel's law. This enables introducing multiple modifications at once to facilitate revealing functional redundancy between several genes that can mask gene function in specific knockdowns. Additionally, genetically identical mice are generated in contrast to conventional breeding methods. This would also include an advantage compared to novel in vitro methods of recapitulating complete meiosis from ES cell-derived germ cells leading to functional sperm-like cells that can lead to fertile offspring 10.

Toxicology and drug toxicity screenings

The blastoid can be generated in a massively parallel fashion which allows screening of drugs and (potential) hazardous or toxic compounds on a genetically identical model for preimplantation development. This can have important implications on the safety regulations for prescribing drugs, which are usually not tested on pregnant women or women in the process of becoming pregnant.

Basic biological studies and regenerative medicine

An in vitro model that accurately recaps preimplantation development would greatly contribute to the study of genetic, molecular and physical mechanisms governing cell fate specification and tissue and organ differentiation and morphogenesis. Presently, the state-of-the-art method for in vitro study of preimplantation development of the mouse includes flushing out embryos from the uterus or oviduct of a pregnant mouse and continue culture in chemically-defined medium. Mice are normally sacrificed in the process of harvesting embryos and natural embryos can only be obtained in relatively small numbers. Hence, a way to prevent sacrificing animals and increase to large-scale generation and screening on early embryonic development is to utilize artificial blastocysts. Clearly, the usefulness of such a model system critically depends on its resemblance to its natural equivalent. Interestingly, however, Intestinal organoids have already been used with success in identifying novel rare cell types [11] that would be difficult to discover directly in vivo.

References

1. Turner, D.A., Baillie-Johnson, P. & Martinez Arias, A. Organoids and the genetically encoded self-assembly of embryonic stem cells. *BioEssays : news and reviews in molecular, cellular and developmental biology* **38**, 181-191 (2016).
2. Hilderink, J. *et al.* Controlled aggregation of primary human pancreatic islet cells leads to glucose-responsive pseudoislets comparable to native islets. *Journal of cellular and molecular medicine* **19**, 1836-1846 (2015).
3. Spijker, H.S. *et al.* Conversion of mature human beta-cells into glucagon-producing alpha-cells. *Diabetes* **62**, 2471-2480 (2013).
4. Buitinga, M. *et al.* Microwell scaffolds for the extrahepatic transplantation of islets of Langerhans. *PloS one* **8**, e64772 (2013).
5. Altmann, B. *et al.* Differences in morphogenesis of 3D cultured primary human osteoblasts under static and microfluidic growth conditions. *Biomaterials* **35**, 3208-3219 (2014).
6. Nicolas C. Rivron, J.R., Severine Le Gac, Roman K. Truckenmüller, Clemens A. van Blitterswijk and Erik J. Vrij SELF-ASSEMBLING TISSUE MODULES. (2009).
7. Leijten, J. *et al.* Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 13954-13959 (2014).
8. Caron, M.M. *et al.* Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* **21**, 604-613 (2013).
9. Mapletoft, R.J. & Hasler, J.F. Assisted reproductive technologies in cattle: a review. *Revue scientifique et technique* **24**, 393-403 (2005).
10. Zhou, Q. *et al.* Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro. *Cell stem cell* **18**, 330-340 (2016).
11. Grun, D. *et al.* Single-cell messenger RNA sequencing reveals rare intestinal cell types. *Nature* **525**, 251-255 (2015).