Maastricht University

Engineering the bone marrow microenvironment

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Impact chapter

Introduction

Tissue engineering and regenerative medicine (TERM) are becoming more relevant in clinical practice. This multidisciplinary field, spanning from biology, materials science, engineering, medicine, chemistry, and many other sub fields, have amounted a total of $10.8 billion in sales worldwide in 2016 and are expected to reach $22 billion in 2025 [1]. Despite the obvious impact of this field on medicine, TERM therapies are still far for being widespread and match the research that has been developed in the past 26 years, since the launch of the Tissue Engineering Journal [2], when the first TERM applications were reported. The disparity between market and research is often attributed to a high number of failed clinical trials of tissue engineered medical products (TEMPs) (more than 86% when requesting Federal Drug Administration, USA, approval) [1] and a cost of manufacturing these products that exceeds the reimbursement, leading to a market that is not sustainable [3]. *Biomaterials-based* companies generated most of the sales for tissue engineering (TE) products, amounting roughly 99% of the total sales in 2017 in the US market. The remaining 1% of the sales came from *Cells and Biomaterials* companies, while *Stem Cells* companies generating less than 1% of the total sales [4]. This scenario reflects the gap between the vast number of pre-clinical TEMPs that have failed or are yet to reach the clinical stage and become a product for clinical use. However, it also illustrates the unexplored business opportunities that this field still has and the space for growth that still holds.

Three-dimensional cell expansion

Strategies developed during this thesis could contribute to wane the gap between the scientific output and the market. For instances, in **chapter 2** we have discussed the importance of optimizing an adequate system for culturing human mesenchymal stem cells (hMSCs) in a three-dimensional (3D) fashion. One of the biggest challenges that cell therapy products face is scarcity in the total cell number harvested from donors [5]. These cells have to be expanded, while keeping their regenerative potential intact for an overall better outcome of the developed cell therapy. Despite the recent efforts trying to standardize protocols for hMSCs expansion *ex vivo*, the field is yet to agree on a final standard protocol that ensures the aforementioned requisites for a successful therapy [6]. By comparing cell aggregates with alginate culture, we showed that alginate hydrogels are better at maintaining the cells viability for long-term cultures, while keeping a small, yet constant population of cells that underwent proliferation. This specific condition could then

be upscaled and applied to bioreactor culture systems, in order to produce enough hMSCs for a cell therapy. This sort of systematic research, comparing different well-established 3D culture conditions to single out the one with the best outcome, is perhaps one of the most fundamental and undervalued research in TE. With increasing material synthesis, with different chemistries that provide new complexity to 3D culture systems, it becomes imperative to compare materials and categorize them in what they do best, in order to provide better tools for TEMPs.

Back to fundamental biology

Systematic comparisons should also be followed by more comprehensive biological studies on the 3D materials that are being developed. Since most of the developed therapies are failing at the clinical trial stage [1], TERM scientists must focus on understanding how materials affect cells at the molecular level. Quite often, while developing new material formulations, scientists focus on viability, cell metabolism, proliferation and differentiation, while most of the intrinsic molecular regulations are not fully explored. In the recent years, mechanobiology has paved the way for the influence of materials on the molecular biology [7]. However, we are slowly decoding how each molecular pathway, which has been well described for two-dimensional (2D) standard *in vitro* culture systems, is actually different when cells are cultured in 3D. Not only cells "feel" the stiffness/mechanical properties of materials, but they also sense so many more biophysical properties that change when moving from 2D to 3D. Properties such as oxygen tension, cell adhesion, cell polarity, nutrients diffusion, and many others, will not produce the same results on cells that have been studied in 2D [8]. While these changes may look trivial when the goal is to expand or differentiate cells, the reality is that any cell behavior will ultimately be affected by the new dimension. Therefore, in **chapter 4** and **6**, we explored how 3D culture affects cells to change their molecular pathways. For instance, in **chapter 4**, when cultured in alginate hydrogels, hMSCs reduced their mammalian target of rapamycin complex 1 (mTORC1) expression, which ultimately led to the activation of forkhead box O3 (FoxO3) and cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27), which have been reported to induce cells towards quiescence. Instead, when cultured in 2D and deprived from fetal bovine serum (i.e. starved) hMSCs activated mTORC1, leading towards inhibition of FoxO3 and p27 expression, but kept their quiescence through retinoblastoma 1 (RB1) activation. Despite these differences between 2D and 3D, we only scratched the surface of how 3D may induce quiescence. We are still far from pinpointing the exact variable(s) responsible for these molecular pathway differences. In **chapter 6**, we also concluded that the secretion of stanniocalcin-1 (STC-1), a protein with anti-inflammatory properties, was

increased in hMSCs when these were cultured in soft materials such as alginate hydrogels and electrospun fibers, when comparing to 2D polystyrene surfaces [9]. We attributed this change in behavior to a shift in cell's mechanosensing. In fact, when stress fibers were abolished through molecular inhibitors, cells secreted STC-1 as if they were being cultured in 3D. This research clarifies even further the need to explore these differences between 2D and 3D for successful 3D cell therapy outcomes. If in the first study (**chapter 4**) we induced quiescence, therefore hampering the outcome of 3D cell expansion with hydrogels, in the second study (**chapter 6**) we showed that hMSCs anti-inflammatory power can be harnessed by using the very same material. More fundamental biology will, therefore, lead us to better therapy outcomes, while providing a real impact in cell biology and TEMPs industry.

The high-throughput solution

As already mentioned, in the past 30 years there has been great improvement in biomaterials. While the first materials to be developed and used in TERM therapies were inert, homogenous, artificial, and aimed to avoid the immune system, the new generation of materials are much more complex [10]. They have instead been developed with different functionalities, gradients, biophysical and biochemical cues, and designed to modulate the immune system. All these upgrades, and ease to chemically change the materials, had led to a vast number of biomaterials that can be tested for a variety of different applications. We now have the capacity to generate these many different materials, expose them to several different cell types and culture them with different cytokine cocktail formulations. However, we do not have the capacity, money, and time to study in details the combination of all these conditions. Hence, TE scientists have also been focusing on developing high-throughput techniques for both production and screening of tens of thousands of different conditions [11, 12]. These techniques, which have been applied to other fields and have been an essential part of the "omics research" (e.g. genomics, proteomics, transcriptomics) [13], are still in their infancy in biomaterials research [11]. In **chapter 5**, we have developed a high-throughput approach to study hMSCs behavioral changes when exposed to 3D artificial niches created by the multi-combinations of five different peptides found in the natural *in vivo* bone marrow stem cell niche. With this study, we were not only able to develop this stem cell niche library, but we were also successful on designing a platform for 3D hydrogel culture *in vitro* and *in vivo* that would allow us to high-throughput screen our conditions. Such initiatives will speed-up the identification of potential biomaterial formulations for specific applications, allowing the combination of material development with drug screening. By eliminating undesirable formulations and doing reverse

engineering at a faster pace, we will be more efficient in predicting the outcomes of our biomaterials, and therefore reduce the unforeseen results that might be responsible for clinical trial failures [11]. Investing in high-through techniques that span from production, to monitoring and screening is key for the next biomaterials revolution.

"Secretome factories": a stem cell tale

As previously mentioned, *Cells and Biomaterials* and *Stem Cells* companies have only accounted for 1% of the total sales in TE companies [4]. However, in the past year, we have been witnessing a revolution in cell manipulation. Fischbach *et al*, have even described cells as being part drug and part device, "having therapeutic capabilities that are distinct from those of small molecules and biologics" [14]. In fact, cells are often described as cytokine factories; changing their complex behavior according to the necessities of the environment where they are placed. If changing from 2D to 3D has a significant impact on cells behavior, one can wonder how many responses and changes a cell can uphold when facing with many other challenges. If these changes elicit a relevant outcome in terms of secretome, exploring them for therapeutically purposes might turn up the tides on cell therapies available in the market. Despite these progresses, TE strategies are still struggling to find ways to preserve *in vivo* implanted cells for as long as it is possible. Most of the experiments performed *in vivo* have shown that implanted cells do not last long in the host tissue leading to a poor cell integration on the newly formed strategies [15]. Instead, it seems that hMSCs participate in the regeneration of the wound tissue with secretion of pro-regenerative cytokines and other factors. If we are able to keep cells near the wounded site for longer times, while ensuring that their secretome is tuned to promote better regenerative outcomes, we may improve TE strategies with cells and biomaterials. In **chapter 7**, we followed this strategy by merging two different individually optimized materials for distinct purposes: a hydrogel that allows culturing hMSCs in 3D and an electrospun-hollowed cylinder opened at on end. By incorporating the hydrogel inside the electrospun and stitching the loose end, we have developed a new system for hMSCs secretome delivery, which we named *Hydrocup*. This elegant strategy showed promising preliminary results on harnessing the power of hMSCs secretome, while keeping hMSCs alive and in place *in vitro* and *in vivo*. Such device can potentially be used alone or in combination with other cell therapies to ensure that the implanted cells and host tissue constantly receive beneficial natural drugs for tissue regeneration. Diseases such as myocardial infarction may benefit from these strategies and patients might have their necrosis reduced [16]. Since there is still controversy on whether autologous or allogenic hMSCs transplants elicit the same responses on the host immune system, with recent

studies showing that under certain circumstances allogenic hMSCs can suffer immune rejection and keep a specific immunological memory [17], this cell delivery strategy could be beneficial on protecting cells from direct physical interaction with the host cells. Allogenic cell sources combined with the Hydrocup system, could then be used as an off-the-shelf product, without having to wait for cell expansion from the patients' own cells.

Conclusion

Overall, this thesis provides new insights and efforts that may lead TE strategies to more palpable clinical therapy outcomes. Going backwards and trying to decode the fundamentals of biology applied to the newly synthesized biomaterials seems now even more important if we were to be successful on this task. These new therapies will affect millions of people that today suffer from incurable diseases or live with permanent impairments for life. The ingenuity of our research in this field will ultimately lead us to find solutions to overcome some of the problems we have discussed here. We foresee that applying modern high-throughput techniques to screen interactions between cells and multiple biomaterials variations will be crucial to speed-up new relevant discoveries. Fine-tuning these communications between cells and biomaterials will also foster a deeper understanding and application of the cell's secretome, which could possibly replace cells altogether in the therapies to come. Finally, by bringing together scientists from different fields with different expertise, contributing to a multidisciplinary fertile environment, will be crucial to have a real impact on TERM therapies.

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