

TISSUE ENGINEERING APPLICATIONS FOR ADIPOGENESIS

**A Thesis Submitted to
the Graduate School of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in Bioengineering**

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**July 2025
İZMİR**

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ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest gratitude to my esteemed supervisor, Prof. Dr. Engin ÖZÇİVİCİ, for his invaluable guidance, unwavering support, understanding, and patience throughout my doctoral research. His trust in me, as well as the insights he shared regarding both scientific work and the functioning of academic life, have been truly invaluable.

I would also like to extend my sincere thanks to the members of my thesis monitoring committee, Prof. Dr. Özden YALÇIN-ÖZUYSAL and Assoc. Prof. Dr. Hüseyin Cumhur TEKİN, for their guidance, support and constructive feedback during my research process. I am equally grateful to the thesis jury members, Assoc. Prof. Dr. Sinan GÜVEN and Assoc. Prof. Dr. Gizem ÇALIBAŞI-KOÇAL, for their scientific contributions and thoughtful evaluations. Additionally, I would like to thank my reserve jury members, Prof. Dr. Zekiye Sultan ALTUN and Asst. Prof. Dr. Yavuz OKTAY, for their interest and for agreeing to contribute to my thesis defense process.

I am sincerely grateful to Prof. Dr. Gülistan MEŞE-ÖZÇİVİCİ, whom I consider a co-advisor, for her continuous support and generosity in sharing her laboratory and knowledge.

I am deeply thankful to my dear friend and senior lab colleague Müge Anıl-İnevi for her invaluable training, her generous sharing of scientific knowledge, and above all, for standing by my side and offering unwavering support through both experimental challenges and personal struggles. I would also like to thank our research group members and colleagues, Alara KARA, Ayşe ANAZ, Hüsniye BÜYÜKÇAKIR, İlayda ÖZKAN, Meltem GÜZELGÜLGEN, Melike ÇAĞAN, Ece İNAL-ERYEĞİT, Azra YILDIRIM, Şüheda YAŞARBAŞ, Ceylan ÇAKMAK, Yağmur Ceren ÜNAL, and our dedicated undergraduate students for their invaluable help and friendship during this journey.

Special thanks are due to my dear friends Alper ŞAHİN, Azra YILDIRIM, Ece İNAL-ERYEĞİT, Ekin KESTEVUR-DOĞRU, Eyüp BİLGİ, Gamze DOĞAN, Gülten KURU, Hilal Deniz YILMAZ-DAĞDEVİREN, Meltem GÜZELGÜLGEN, Müge ANIL-İNEVİ, Şüheda YAŞARBAŞ, Tolga SEVER, Tuğçe SAKALLI and many others whose names I may not be able to list here. Their presence, encouragement, and support have been invaluable to me, both academically and personally. I am truly fortunate to

have such wonderful people by my side throughout this journey. I am sincerely grateful for their kindness, advice, and camaraderie.

I am especially grateful to Ece İNAL-ERYEĞİT and Perge Bilgesu TOSUNOĞLU for their experimental contributions and warm encouragement throughout the research process. I would also like to thank Muhammet Semih BAŞLAR for his valuable help in the platform fabrication.

The financial support provided by the Scientific and Technological Research Council of Turkey (TÜBİTAK; projects 215S862, 22AG053, 22AG032, and the 2211-A scholarship) and the Council of Higher Education (100/2000 Ph.D. Scholarship Program) is gratefully acknowledged. This study was also supported by the Izmir Institute of Technology Research Fund (2024İYTE-1-0049 and 2020İYTE0069). Also, I would like to thank the Biotechnology and Bioengineering Application and Research Center (İYTE) for providing the resources and facilities needed for the RT-PCR experiments.

Lastly, I wish to express my heartfelt gratitude to my beloved mother and sister. Their unconditional love, unwavering support, and the unshakable faith they have always placed in me—often more than I had in myself—have been my greatest source of strength. For all that they are and all that they have given me, I am deeply and eternally grateful.

ABSTRACT

TISSUE ENGINEERING APPLICATIONS FOR ADIPOGENESIS

The engineering of physiologically relevant adipose tissue models is essential for advancing research in obesity, metabolic disorders, and soft-tissue reconstruction. This thesis therefore adopted scaffold-free biofabrication to build stable, functional 3D adipose constructs from mature adipocytes by integrating biological and physical approaches. Magnetic levitation (MagLev) was utilized as a label- and scaffold-free method for the assembly of adipocytes. However, the low density and lipid heterogeneity of differentiated adipocytes yielded uneven levitation and hindered cohesive tissue formation. To overcome this, various strategies were implemented to improve adipocyte culture, including modifications to the MagLev configuration and the culture environment. Also, hanging drop and liquid overlay methods were benchmarked against MagLev for spheroid assembly, compaction and viability.

In the study, multicellular models were developed by incorporating endothelial cells and macrophages into adipogenic 3D constructs. The impact of cellular composition and culture technique on the morphology and organization of spheroids was assessed, revealing that both the choice of cell type and physical platform significantly influence tissue architecture. Also, carrageenan was introduced as a macromolecular crowding agent to enhance extracellular matrix deposition and improve structural integrity in mature adipocyte cultures. Carrageenan promoted spheroid compaction and collagen accumulation without compromising cell viability or lipid content.

Collectively, the findings demonstrate that combining magnetic levitation, multicellular culture, and macromolecular crowding represents a promising strategy for generating biomimetic adipose tissue constructs. These models may serve as valuable platforms for metabolic studies, drug testing, and regenerative medicine applications.

ÖZET

ADİPOJENEZ İÇİN DOKU MÜHENDİSLİĞİ UYGULAMALARI

Fizyolojik olarak anlamlı adipoz doku modellerinin geliştirilmesi, obezite, metabolik hastalıklar ve yumuşak doku rekonstrüksiyonu alanlarında ilerleme sağlanması açısından büyük önem taşımaktadır. Bu tez kapsamında, olgun adipositlerin doğrudan kültürlenmesine dayanan, biyolojik ve fiziksel yaklaşımların entegre edildiği biyoüretim stratejileri kullanılarak stabil ve fonksiyonel üç boyutlu (3B) adipoz doku modelleri oluşturulmuştur. Manyetik levitasyon (MagLev) sistemi, etiketleme veya iskele içermeyen 3B yapı oluşturmak için uygulanmıştır. Ancak, farklılaşmış adipositlerin düşük yoğunluğu ve heterojen lipid içeriği nedeniyle hücreler levitasyon ortamında düzensiz bir şekilde konumlanmış, bu da sıkı ve bütünleşik yapılar oluşturulmasını zorlaştırmıştır. Bu sınırlamayı aşmak için MagLev konfigürasyonu üzerinde modifikasyonlar ve kültür ortamına bazı katkı maddeleri eklenmesi gibi çeşitli düzenlemeler yapılmıştır. Ayrıca, MagLev yöntemi, hanging drop ve liquid overlay teknikleri ile karşılaştırılmış; bu tekniklerin farklı kültür koşulları altında adiposit sferoidlerinin oluşumu, kompaktlığı ve canlılığını destekleme düzeyleri değerlendirilmiştir.

Çalışmada, endotel hücreleri ve makrofajlar kullanılarak adipositlerle birlikte çok hücreli modeller geliştirilmiştir. Hücresel kompozisyon ve kullanılan kültür tekniğinin sferoid morfolojisi ve organizasyonu üzerindeki etkileri analiz edilmiş; doku mimarisinin, hem hücre türüne hem de fiziksel platforma duyarlı olduğu gösterilmiştir. Ayrıca, olgun adiposit kültürlerinde hücre dışı matriks birikimini artırmak ve yapısal bütünlüğü güçlendirmek amacıyla makromoleküler kalabalıklaştırıcı ajan olarak karragenan kullanılmıştır. Karragenan, hücre canlılığını veya lipid içeriğini etkilemeden sferoidlerin sıkılaşmasını ve kollajen birikimini desteklemiştir.

Bu tez kapsamında elde edilen bulgular, manyetik levitasyon, çok hücreli kültür ve makromoleküler kalabalıklaştırma yaklaşımlarının bir araya getirilmesinin, biyomimetik adipoz doku yapılarının oluşturulması açısından etkili bir strateji sunduğunu göstermektedir. Geliştirilen bu modeller, metabolizma araştırmaları, ilaç taramaları ve rejeneratif tıp uygulamaları için değerli birer platform olma potansiyeli taşımaktadır.

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CHAPTER 1

INTRODUCTION

1.1. Adipose Tissue and Obesity

Obesity, characterized by excessive expansion of adipose tissue (AT), has become a major global health concern, with over 890 million adults classified as obese as of 2022.^{1,2} The main cause of obesity is the imbalance between energy intake and expenditure, leading to the accumulation of triglycerides in AT. This metabolic disorder is strongly associated with the development of type 2 diabetes, cardiovascular disease, hypertension, and certain cancers.^{2,3} AT remodeling in obesity leads to dysregulation of secreted factors, such as cytokines, chemokines, and hormones, and the elevated presence of inflammatory molecules within the tissue contributes to chronic low-grade inflammation (Figure 1.1).^{2,4}

Adipose tissue is a metabolically active, endocrine organ composed of mature adipocytes and stromal vascular fraction (SVF), including preadipocytes, endothelial cells (ECs), immune cells (e.g. monocytes, macrophages and leukocytes), and adipose-derived stem cells.⁵ This highly vascularized connective tissue is surrounded by extracellular matrix (ECM) mainly consisting of type IV collagen in basal lamina, as a major component, and also collagen type I, III, V, VI, fibronectin, laminin and elastin, forming a structural and signaling framework that regulates cell behavior.^{6,7}

The primary functions of AT include the regulation of energy balance and homeostasis, providing mechanical support for internal organs, and facilitating heat production through thermogenesis.⁸ However, after the discovery of leptin as an adipocyte-derived signaling molecule in the late 20th century, adipose tissue was considered an endocrine organ and research has increasingly focused on endocrine and paracrine functions of adipose tissue.⁹ AT secretes various chemokines, cytokines, and

hormones that play key roles in regulating processes such as glucose metabolism, immune responses, and angiogenesis.¹⁰⁻¹²

Adipose tissue expands via two mechanisms called hypertrophy (cell enlargement) and hyperplasia (formation of new adipocytes) to accommodate excess energy intake.^{8,13} Hyperplastic expansion is metabolically favorable, maintaining insulin sensitivity, while hypertrophy induces inflammation, fibrosis, and metabolic dysfunction.^{8,14,15} Childhood obesity is often characterized by a near doubling of adipocyte number compared to normal-weight peers, increasing the risk of persistent obesity into adulthood.¹⁶ In contrast, adult adipose tissue expansion predominantly occurs via hypertrophy, particularly in visceral fat depots. However, emerging evidence suggests that under chronic energy surplus, preadipocytes in adults can still be activated to differentiate, indicating that hyperplasia is not entirely absent. Understanding these mechanisms is crucial for creating targeted strategies to address metabolic disorders related to obesity.

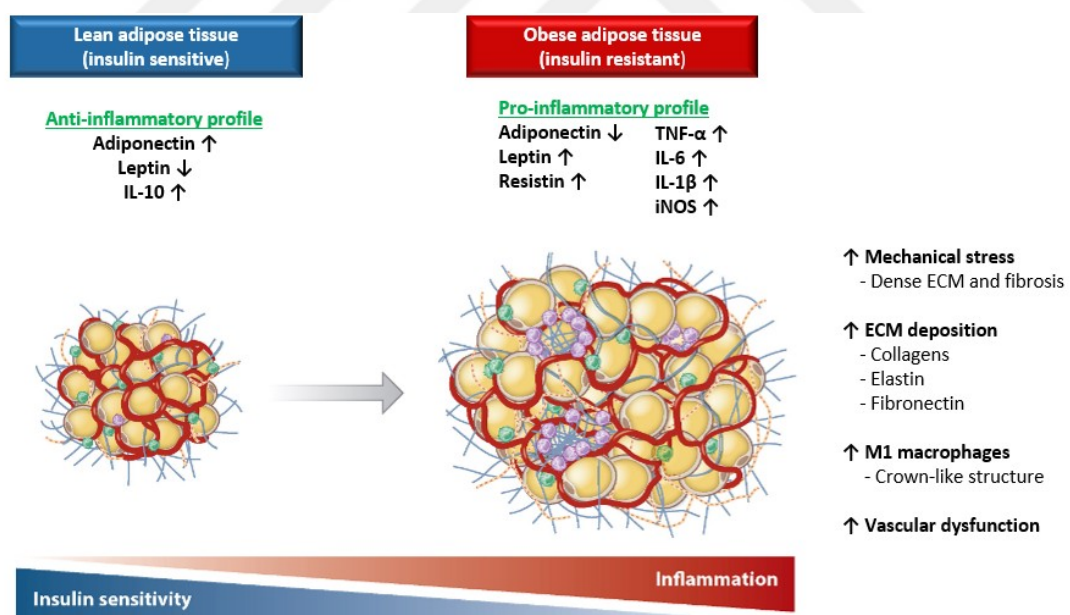


Figure 1.1. Schematic representation of lean and obese adipose tissue microenvironments and their metabolic-inflammatory profiles.^{17,18}

AT is a highly vascularized connective tissue, and in healthy AT, endothelial cells typically remain in a quiescent state.² However, ECs can be triggered to transition into a proliferative phase, leading to angiogenic sprouting and the development of new blood vessels, due to changes in metabolism and the expression of angiogenic factors.^{2,5} Blood vessels in AT are crucial not only for supplying nutrients and oxygen but also for transporting cytokines and hormones. The expansion of AT occurs with adipogenesis accompanied by angiogenesis. For angiogenesis to proceed correctly in AT, pro-angiogenic and anti-angiogenic factors must be balanced, including both adipokines and classical angiogenic molecules.^{2,19} For instance, leptin promotes angiogenesis by stimulating matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9, which play a role in the sprouting of new blood vessels, whereas adiponectin exerts an anti-angiogenic effect.^{19,20} Disruption of this balance can lead to endothelial dysfunction and abnormal angiogenesis, concomitant with conditions often associated with obesity.² Angiogenesis is initiated during adipose tissue expansion to meet the oxygen requirements of the growing tissue. However, when tissue expansion occurs too rapidly, the oxygen supply becomes insufficient, leading to hypoxia and triggering an inflammatory response.²

Macrophages, which constitute about 10% of the cells in AT, are also important regulators of AT function and remodeling.^{8,21} In lean AT, macrophages are involved in lipid buffering and tissue remodeling.^{21,22} However, during obesity, the number of macrophages infiltrating the tissue increases dramatically, making up as much as 40% of the total AT cell population.⁴ These macrophages form crown-like structures around dead adipocytes and secrete pro-inflammatory cytokines that contribute to chronic inflammation and insulin resistance. Some studies have suggested that adipose tissue macrophages (ATMs) may also influence angiogenesis.²¹ In obese AT, depletion of ATMs reduces endothelial cell tube formation, likely due to decreased expression of platelet-derived growth factor (PDGF), a key angiogenic molecule.^{8,23} This evidence supports the idea that ATMs produce angiogenic factors that promote new blood vessel growth and help alleviate tissue hypoxia.

1.2. 3D Adipose Tissue Engineering

Tissue engineering (TE) mainly aims to develop tissue substitutes to replace or repair diseased/damaged tissues to improve their function.²⁴ In addition to creating replacement tissues, TE also enables the production of three-dimensional (3D) cellular models that closely replicate native tissue architecture and microenvironment, allowing for more accurate studies of human physiology and pathophysiology. These advanced models have become invaluable tools in drug development and personalized medicine, offering deeper insights into disease mechanisms and potential therapeutic approaches.

Two-dimensional (2D) cell culture models have been widely used for *in vitro* studies, including cytotoxicity and migration assays, and they continue to be employed in the early stages of pharmacological research.²⁵ However, these systems have inherent limitations in replicating the complex *in vivo* environment, limited in their ability to replicate the *in vivo* environment, particularly in terms of cell-cell and cell-ECM interactions. To overcome these shortcomings, 3D culture models have been developed, offering more physiologically relevant environments that better mimic native tissue architecture and function (Figure 1.2).

3D cultures preserve the intricate spatial architecture, cell–cell and cell–matrix interactions that are fundamental to adipose tissue function. These features critically influence adipogenic processes, including preadipocyte differentiation, lipid accumulation, and the expression of key adipokines such as leptin and adiponectin. Adipocytes cultured in 3D environments exhibit more robust lipid droplet formation, enhanced insulin responsiveness, and gene expression profiles closer to native adipose tissue compared to their 2D counterparts.²⁶ Furthermore, 3D cultures better simulate the mechanical cues and diffusion gradients of oxygen and nutrients, which are known to regulate adipogenic maturation and metabolic activity.

Tissue engineering approaches to generate 3D adipose models are broadly classified into scaffold-based and scaffold-free strategies. Scaffold-based systems utilize natural (e.g., collagen, gelatin, silk) or synthetic (e.g., PLA, PLGA, PEG) biomaterials that support structural organization, adipogenic differentiation, and cell adhesion.^{24,27} Different scaffold materials or their combinations can exhibit diverse mechanical properties, supporting both the natural morphology of adipocytes and promoting

adipogenic differentiation.²⁸ However, scaffold-based techniques require careful consideration of several critical parameters, including well-defined porosity and pore size to facilitate cell interactions and nutrient transport, as well as biocompatibility and biodegradability to accurately mimic the physiological microenvironment and provide structural support for 3D *in vitro* models that closely mimic *in vivo* conditions.^{24,29} If the scaffold degrades too slowly or too quickly, it can lead to reduced tissue formation or structural collapse.³⁰ Although scaffold-based approaches allow control over ECM composition, the design and fabrication of suitable scaffolds remain complex processes.

Adipose tissue engineering (ATE) presents unique challenges for scaffold-based systems compared to other tissue types. Because of adipocyte expansion, which occurs through lipid accumulation, the design of scaffolds with optimal pore size and porosity is highly challenging.²⁸ Additionally, scaffold properties, such as mechanical stiffness and surface chemistry, significantly influence cell attachment, morphology, and the differentiation potential of precursor cells into mature adipocytes.^{31,32} Thereby, scaffold-free approaches for ATE have gained increasing attention as a promising alternative to overcome the inherent limitations of scaffold-based systems.

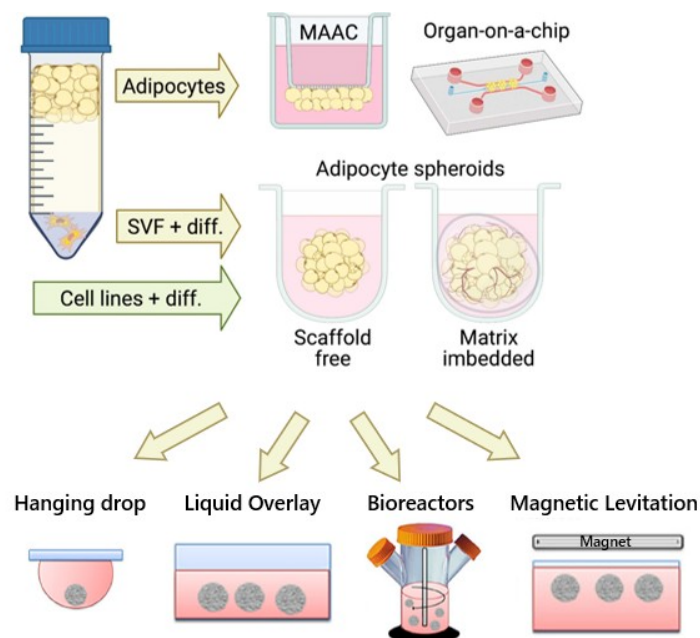


Figure 1.2. Overview of strategies for generating 3D adipose tissue models.^{33,34}

1.2.1. Scaffold-free Cell Culture Techniques

Cells can secrete and organize their own ECM components when cultured in conditions that promote aggregation.²⁸ This inherent capability reduces the need for external scaffolds and simplifies the culture process. A variety of techniques designed to encourage 3D spheroid formation have been extensively applied in AT culture, including hanging drop technique, low-adhesive surfaces, bioreactors, and microfluidic systems (Figure 1.2).³⁵⁻⁴¹

The hanging drop (HD) technique is particularly useful in producing spheroids with high uniformity in size and shape. By suspending cells in small droplets of culture medium, gravity causes them to aggregate into spherical structures.⁴² This method allows for excellent control over the cellular structure size, which is particularly important in experiments that require uniformity in cell density and morphology.⁴³ However, the hanging drop method has limitations, such as low working volumes and challenging medium exchange. During medium replenishment, fragile spheroids may break apart or undergo disruption, making reproducibility and long-term culture more difficult.³⁵ The liquid overlay technique (LOT) involves culturing cells on non-adhesive surfaces, such as agar- or agarose-coated plates, that prevent the cells from attaching to the substrate by encouraging the cells to form spheroids through cell-cell interactions.^{44,45} While this method is simple and cost-effective, it offers less precise control over spheroid size and uniformity compared to HD cultures, due to enabling the formation of several spheroids in culture plates. Bioreactor-based systems are designed to support long-term culture of 3D spheroids by providing an environment to support cell aggregation, growth, and differentiation.^{39,46} These systems are capable of maintaining high cell viability for extended culture periods and are suitable for large-scale culture. However, continuous rotation within bioreactors causes shear stress that can negatively affect cell viability and function, especially in sensitive cell types like adipocytes.⁴⁷ This becomes a significant challenge for ATE, as adipocytes are sensitive to mechanical stress, which can impair their metabolic activity and differentiation.

Another promising scaffold-free approach is cell sheet technology, where cells are cultured on materials that allow detachment without enzymatic digestion, enabling the formation of multilayered cell sheets to create tissue-like constructs.⁴⁸⁻⁵⁰ This method can

be applied for ATE as it allows for the creation of thick tissue constructs that closely resemble native adipose tissue. However, challenges include fabricating suitable polymer surfaces for attachment and detachment, as well as ensuring uniformity in the cell layers.⁵¹ Therefore, the optimization of the properties of the polymers is essential for successful application.

The Membrane Mature Adipocyte Aggregate Culture (MAAC) is also an innovative scaffold-free method designed for culturing isolated mature adipocytes *in vitro*. In MAAC, adipocytes are cultured beneath a permeable membrane, allowing them to assemble into high-density aggregates that preserve their unilocular lipid droplets and native phenotype.^{52,53} While MAAC effectively preserves adipocyte morphology and function over time, it has limitations, such as issues with scalability and standardization due to variability in aggregate size and cell density. Additionally, it does not easily facilitate the controlled integration of other cell types, like endothelial cells or macrophages, which restricts its use for studying complex adipose tissue interactions.

1.2.2. *In vitro* Adipose Tissue Models

Vascularized adipose tissue models are extensively investigated in the context of soft tissue reconstruction, particularly for breast tissue transplantation following mastectomy due to breast cancer. A major limitation in such applications is the volume loss frequently observed after implantation, which is primarily attributed to insufficient revascularization and the resulting hypoxic microenvironment, leading to tissue atrophy and eventual graft failure.^{27,54,55} Therefore, creating vascularized adipose tissue capable of maintaining both its structure and function post-transplantation requires careful attention to both adipogenesis and angiogenesis, which are also crucial for endocrine functions.⁵⁵

A number of strategies have been developed to engineer vascularized adipose tissue, with most of these focusing on the co-culture of preadipocytes (or stem cells) and endothelial cells. However, these co-culture systems may face several challenges. First, differentiation media used to stimulate adipogenesis can sometimes hinder cell proliferation or even block the formation of new vessels.⁵⁶⁻⁵⁸ Also, the use of stem cells

in these co-culture systems raises concerns about potential tumorigenicity, either through the transformation of stem cells into cancerous cells or through the promotion of endogenous tumor growth after implantation.⁵⁹

To address some of these challenges, the coculture of pre-differentiated adipocytes/mature adipocytes and endothelial cells has been explored.^{57,58} Scaffold-based co-culture systems have demonstrated enhanced adipogenic function and improved vessel formation.⁵⁷ However, as previously mentioned, they face significant challenges, including adipocyte loss, which is largely attributed to the fragile nature of mature adipocytes or their tendency to float during bioprinting.⁶⁰ For adipose tissue engineering, these techniques offer varying levels of success, and the choice of method depends on the specific application and the desired outcomes. However, adipose tissue is unique in that it is a relatively soft tissue that requires a careful balance between cell-cell interactions, ECM deposition, and adipocyte differentiation. Its nature makes it difficult to culture adipose tissue effectively, whether using scaffold-based or scaffold-free systems.

On the other hand, macrophages play a pivotal role in maintaining adipose tissue homeostasis, and thus, co-culture systems incorporating both adipocytes and macrophages provide models that more closely replicate the native adipose microenvironment, yielding physiologically relevant insights into metabolic and inflammatory processes. Recent studies have explored macrophage–adipocyte interactions using a variety of co-culture models to investigate obesity-related inflammation. However, most of these studies employed transwell systems or hydrogel scaffolds, which limit direct cell–cell contact or gradient-dependent signaling, thereby overlooking critical juxtacrine cues and phenomena such as crown-like macrophage structures typically observed in obese adipose tissue.⁶¹⁻⁶³ For example, Shen et al. demonstrated that co-culturing macrophages with adipocyte spheroids in a transwell setup led to a shift toward an M2 macrophage phenotype, yet the absence of direct contact constrained the study of contact-dependent signaling events.⁶³

One of the spheroid-based studies demonstrated that showed that co-culture of preadipocytes with macrophages in spheroid models induced lipid accumulation even without adipogenic induction, highlighting the strong influence of macrophage-derived signals.⁶⁴ Despite these advances, to our knowledge, no studies have yet explored direct spheroid co-culture systems involving mature adipocytes and macrophages, which could provide an even more faithful representation of cell–cell interactions in hypertrophic or inflamed adipose tissue.

1.3. Magnetic Levitation

Magnetic levitation (MagLev) is a technique enabling cell manipulation using magnetic force. It can be applied to cells based on the positive or negative magnetophoresis principle (Figure 1.3).⁶⁵ In positive magnetophoresis, cells labelled with magnetic nanoparticles are attracted toward regions of higher magnetic field strength. This allows for precise control of cell positioning, aggregation, and even tissue assembly within the 3D culture. Using positive magnetophoresis technique, previous studies showed potential mono- and co-cultures for aortic valve⁶⁶, bronchiole⁶⁷, adipose tissue⁶⁸ and tumor models⁶⁹. Although MagLev with positive magnetophoresis is a contact-free and straightforward method, it may suffer from challenges related to inconsistent cellular internalization of magnetic labels, time-consuming preparation steps, and cellular stress, affecting cell viability and behavior due to prolonged exposure to strong fields.⁶⁵

Alternatively, magnetic levitation conducted with negative magnetophoresis (MagLev) enables the levitation of diamagnetic objects, such as cells and organisms, which tend to move towards a lower magnetic field in a paramagnetic medium depending on their density.⁷⁰ The response of the cells repelled by magnetic force into the lower magnetic field region is amplified via paramagnetic agents, such as Gadavist (Gd), that alter the magnetic susceptibility of the medium.⁷¹ The sensitivity of the levitation system can be controlled by adjusting the positions of the magnets, thereby manipulating the net magnetic force acting on the target objects.⁷²

The MagLev has been previously applied with different shapes and configurations of magnets to improve accuracy and throughput in the detection of cell densities.^{65,73-75} The single-cell density-based principle of MagLev has enabled applications in diagnostics or drug testing for various healthcare problems such as sickle cell disease, cancer, and infectious diseases.⁷⁶⁻⁸² Also, cellular processes such as adipogenic differentiation and cell death can be detected by this system.^{80,83}

MagLev can also be applied for 3D self-assembly of both non-living objects and cells, offering the advantage of a contact- and label-free working principle, unlike positive magnetophoresis. Previous studies have demonstrated MagLev as an effective scaffold-free biofabrication method for creating cartilage and cancer cellular spheroids^{84,85} as well as monophasic or biphasic assemblies of stem cells, cancer cells, and fibroblasts^{86,87}. In

MagLev, varying configurations and shapes of permanent magnets can be used to create different 3D cellular patterns (Figure 1.3F-I).^{65,88} Additionally, the morphological parameters of 3D structures can be influenced by factors such as culture time, seeding density, and the concentration of paramagnetic agents.⁸⁹ In summary, this system enables the creation of 3D cellular constructs with specific patterns tailored to target tissues. Given its simplicity, ease of use, and tunability, MagLev holds significant promise for applications in tissue engineering and drug screening.

In our previous work, we demonstrated that adipogenic-differentiated cells exhibited a scattered vertical distribution within the levitation system due to their low buoyant density and varying intracellular lipid content.⁸³ This finding suggests that the intrinsic density heterogeneity of adipogenic cells can be exploited in MagLev systems to promote layer-by-layer organization. However, this same characteristic may pose a challenge for achieving uniform and cohesive 3D structures, as differences in buoyant density may cause cells to levitate at distinct vertical positions. To date, studies specifically utilizing mature or lipid-laden adipocytes in magnetic levitation systems remain limited. Notably, one study employing co-cultures of adipogenic cells with fibroblasts and cancer cells with distinct densities reported the successful formation of layered 3D structures for short-term culture periods using a ring magnet configuration⁹⁰. However, this system requires elevated concentrations of gadolinium, which may pose cytotoxic risks. These observations highlight both the potential and the limitations of using MagLev for adipose tissue engineering, emphasizing the need for further optimization to balance structural complexity with architectural stability.

1.4. Macromolecular Crowding to Improve Scaffold-free Culture

Cells in natural tissues reside in a densely packed, macromolecule-rich environment (e.g., adipose tissue with a concentration of 900 g/L), whereas cell culture systems have diluted media with low macromolecule concentrations (about 50 g/L), leading to slower biochemical and biological reactions.⁹¹ To replicate these natural, crowded conditions, macromolecular crowding agents, including polysaccharides (Ficoll, dextran, carrageenan), proteins (bovine serum albumin), and polymers (polyethylene

glycol (PEG)) have been employed.^{91,92} These macromolecules occupy a significant fraction of the volume, restricting molecular movement and thereby facilitating increased molecular interactions. This phenomenon, known as macromolecular crowding (MMC), has been utilized to investigate protein-protein interactions and the regulation of gene expression.⁹¹ MMC can stabilize gene expression by limiting the diffusion of transcription factors.⁹² Furthermore, crowding has been shown to stabilize nucleic acid interactions, such as DNA/DNA and DNA/RNA hybrids, by promoting hydrogen bonds.⁹²

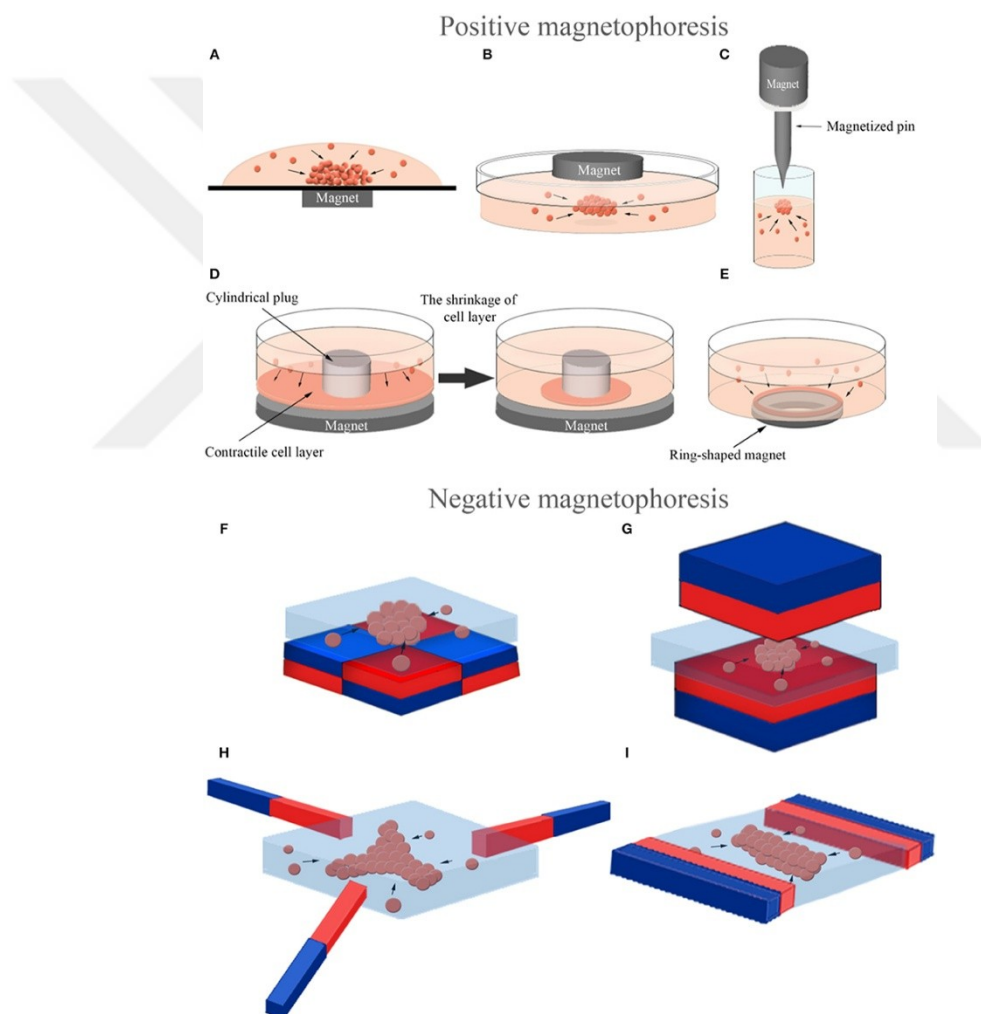


Figure 1.3. Magnetic-based biofabrication approaches using positive (A-E) and negative magnetophoresis (F-I).⁶⁵

Recently, the interest in the application of MMC in 3D cell culture systems has increased to utilize them as a tool to better mimic the complex, *in vivo* microenvironment. The application of MMC has been shown to significantly increase and accelerate ECM deposition *in vitro*, achieving up to an 80-fold increase within several days, depending on the crowding agent, cell type, and culture conditions (Figure 1.4).⁹³⁻⁹⁵ For instance, the use of carrageenan at specific concentrations has been demonstrated to significantly increase collagen type I, III and IV, fibronectin and laminin deposition in human umbilical cord-derived MSC cultures without adversely affecting cell viability or phenotype.⁹⁶ In addition, research suggests that the chemical properties of MMC agents can influence stem cell differentiation process.⁹⁷⁻⁹⁹ Specifically, nonsulfated polysaccharides, such as Ficoll, tend to promote adipogenic differentiation, while sulfated polysaccharides, including dextran sulfate and carrageenan, are more likely to drive osteogenic and chondrogenic differentiation.⁹¹ In this context, MMC shows promise for more accurate modeling of cellular behaviors, enhancing the relevance of *in vitro* studies and improving the understanding of cellular responses in 3D, tissue-like environments.

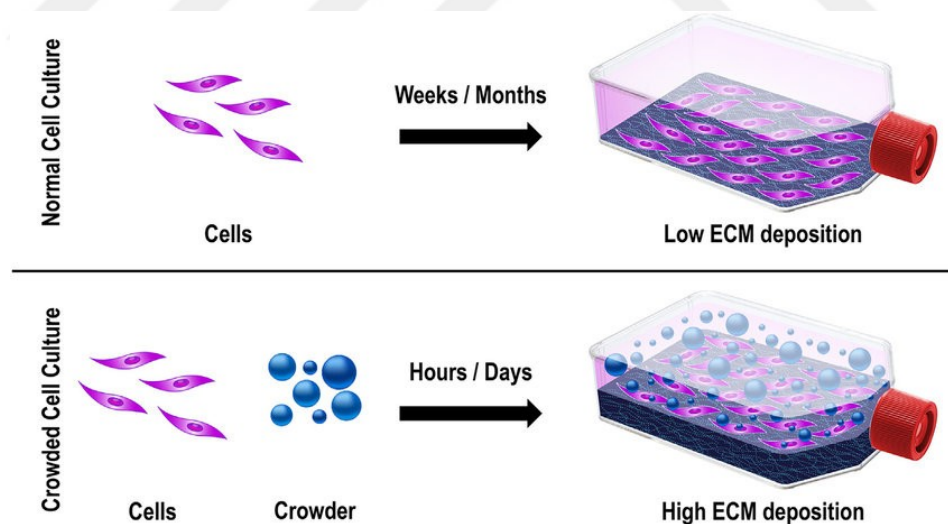


Figure 1.4. The effect of macromolecular crowding on extracellular matrix (ECM) deposition.⁹⁵

In this thesis, we aim to create adipose tissue models that can be utilized for investigating obesity and obesity-related diseases. The primary goal of the study was to

culture mature adipocytes directly, rather than relying on *in situ* differentiation of stem cells. To achieve this, we employed various configurations of the MagLev device to obtain uniform and stable adipogenic structures. Additionally, we leveraged the MMC phenomenon to enhance ECM deposition in culture and improve cell-cell interactions between adipocytes. Furthermore, we compared the MagLev system with traditional scaffold-free 3D cell culture techniques, including hanging drop and liquid overlay, to assess its advantages and effectiveness in creating more physiologically relevant tissue models.



CHAPTER 2

SCAFFOLD-FREE BIOFABRICATION OF ADIPOCYTE STRUCTURES WITH VERTICAL MAGNETIC LEVITATION SYSTEM

2.1. Background

Tissue engineering applications aim to repair the form and/or the function of impaired tissues.¹⁰⁰ Techniques related to tissue engineering, therefore rely heavily upon the optimization of cells, scaffolds, and proper chemical or mechanical signals to mimic the tissues of interest. Of these components, the scaffold serves as an extracellular matrix (ECM) biomimetic and can be tailored from materials that are natural or synthetic.^{101,102} ordered or amorphous¹⁰³⁻¹⁰⁶, bioactive or space-filling^{107,108} based on demand and specific to the tissue of interest. More specifically, utilization of scaffolds for tissue biofabrication requires careful consideration of crucial parameters such as porosity, biocompatibility, bioactivity, and biodegradability to recapitulate the physiological microenvironment and provide structural support for three-dimensional (3D) in vitro models to mimic in vivo conditions.^{29,109,110} However, these parameters are challenging to engineer. For example, an inadequate scaffold degradation rate may cause reduced tissue formation or tissue collapse.^{111,112} Furthermore, the designed scaffolds should possess proper mechanical properties to enable cell growth and to mitigate problems such as stress shielding.^{103,113,114} Other challenges related to scaffold design include durability of the material during sterilization and expensive and time-consuming fabrication processes.¹¹⁵⁻¹¹⁷ Scaffold-free biofabrication is emerging as an alternative approach to cope with the challenges inherent to the use of scaffolds.¹¹⁸⁻¹²² Most cells can create self-assembled 3D structures due to their tendency to form clusters. This natural tendency to aggregate enables cells to secrete and form ECM components per se, relieving the need to use a scaffold in the system.

Established tissue engineering protocols, such as the hanging drop method¹²³, low adhesive surfaces¹²⁴, bioreactors¹²⁵, and microfluidic systems¹²⁶ enable the formation of spheroids through this self-assembly behavior of cells. Some of these protocols, such as the hanging drop method, exert precise control over the size and shape of formed spheroids^{42,127}, however, the working volume is very low and replenishing the culture medium is challenging.³⁵ Utilization of bioreactors also presents a scaffold-free approach in tissue engineering for extended culture time and high throughput; however, continuous rotation in bioreactors causes prolonged shear stress on cells.¹²⁸ On the other hand, cell sheet technology is an alternative technique based on the detachment of the cells as sheets using thermo-, photo- or electro-sensitive surfaces for 3D construction of tissues.⁴⁸⁻⁵⁰ Cell sheet engineering produces layered cell constructs, and detached cell sheets with their own ECM can facilitate transplantation into host tissues.¹²⁹ Nevertheless, the fabrication of appropriate thicknesses of stimuli-responsive polymer surfaces for cell attachment and detachment is challenging.⁵¹ Furthermore, cell layer thickness can be a limiting factor for cell-dense tissue models due to poor nutrient diffusion and hypoxia.¹³⁰ It is also possible to encounter a folding problem with cell sheets.¹³¹ In these systems, the physical and chemical properties of polymers are important parameters for efficient cell manipulation.^{131,132} Another novel technique that enables scaffold-free biofabrication is the magnetic levitation and subsequent assembly of single cells or spheroids.^{86,133} Magnetic levitation can be applied to cells based on positive or negative magnetophoresis principle.⁶⁵ In positive magnetophoresis, cells labeled with magnetic nanoparticles (MNPs) can be levitated using an external magnetic force, and this manipulation can enable cells to form scaffold-free 3D structures. Previous studies showed potential applications using this technique for monocultures and co-cultures of aortic valve⁶⁶, bronchiole⁶⁷, adipose tissue⁶⁸, and tumor models⁶⁹. Although magnetic levitation with positive magnetophoresis is a contact-free and straightforward method, it requires additional labeling procedures and uniform distribution of MNP. Unfortunately, MNPs cannot be removed from the assembled biostructure and cause cytotoxicity and DNA damage once internalized.¹³⁴⁻¹³⁶ An alternative variant to accomplish magnetic levitation is to use negative magnetophoresis, which can levitate diamagnetic objects such as cells and organisms based on their tendency to avoid strong magnetic fields.^{76,137,138} Still contact-free, negative magnetophoresis has an additional advantage due to its label-free working principle. Magnetic levitation conducted with negative magnetophoresis can be applied for 3D self-assembly of both nonliving objects and cells.^{65,133,139,140} In previous

studies, magnetic levitation was used as an effective scaffold-free biofabrication method for cartilage and cancer cellular spheroids^{84,85} as well as monophasic or biphasic assembly of stem cells, cancer cells, and fibroblasts^{86,87}. Magnetic levitation based on negative magnetophoresis stratifies cells based on their inherent single cell density profile^{76,141}, and all of the previous studies showed biofabrication using cells of similar single cell density.^{86,142} However, some tissue types, such as adipose tissue, contain cells with highly variable single-cell density phenotypes.⁸³ Scaffold-free 3D biofabrication of adipose tissue was performed previously through magnetic levitation with positive magnetophoresis using iron oxide and gold nanoparticles.^{68,143} However, the label-free biofabrication of adipose tissues using negative magnetophoresis approach has never been shown. In this study, we demonstrate scaffold-free biofabrication of monophasic and biphasic structures consisting of normal and adipogenic cells of bone marrow origin. We believe this approach may constitute a viable alternative to meet the clinical demands of regenerative medicine and cell therapy for adipose tissue engineering.

2.2. Materials and Methods

2.2.1. Magnetic Levitation Experimental Setup

The magnetic levitation device will be composed of a microcapillary channel (1 mm × 1 mm cross-section, 50 mm length, and 0.2 mm wall thickness, Vitrocom) as a cell culture chamber between two N52-grade neodymium magnets (NdFeB, Supermagnete) (50 mm length, 5 mm height and 2 mm width) with same poles facing each other to create a magnetic field gradient and two parallel mirrors (Thorlabs) placed at 45° to visualize cell levitation in real-time. The components of the magnetic levitation device will be held together with photoreactive resin printed using 3D printer (Figure 2.1).

Cells suspended in a gadolinium (Gd, Gadavist)-containing paramagnetic medium are migrated from a region of high magnetic field to low magnetic field. This movement, that is caused by the magnetic susceptibility difference between cells and the surrounding medium ($\chi_{\text{cell}} - \chi_{\text{medium}}$), ends when magnetic force (F_{mag} , Eq. 1) is equal to

gravitational force (F_g , Eq. 2). In the magnetic field gradient created between the magnets, the point where the F_{mag} and F_g forces are equal is the equilibrium position of the cells for levitation.

$$\vec{F}_{mag} = \frac{V \cdot (\chi_{cell} - \chi_{medium})}{\mu_0} (\vec{B} \cdot \nabla) \vec{B} \quad (1)$$

$$\vec{F}_g = V \Delta \rho g \quad (2)$$

Here, V is the volume of cell, μ_0 is the magnetic permeability of free space ($1.2566 \times 10^{-6} \text{ kg} \cdot \text{m} \cdot \text{A}^{-2} \cdot \text{s}^{-2}$), B is the magnetic flux density, $\Delta \rho$ is the density difference between cell and the surrounding medium and g is the vector of gravity.

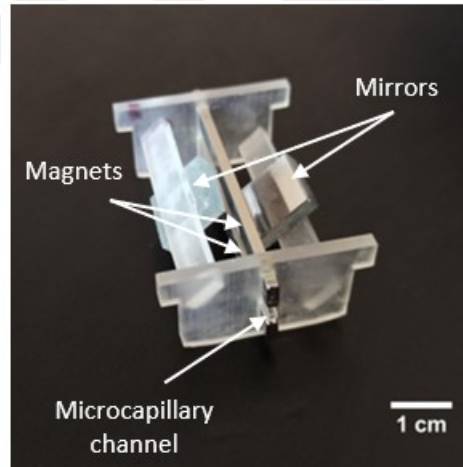


Figure 2.1. Vertical magnetic levitation device. Briefly, the device consists of (1) two N52-grade neodymium magnets with the same poles facing each other to create a magnetic force, (2) a microcapillary channel to load cell suspension, including Gd³⁺-based paramagnetic agent (Gadavist®, Gd), and (3) two mirrors placed at 45° to visualize levitation. The parts of the device were assembled using connecting pieces printed by a 3D printing system.

2.2.2. Infection of the Cells

7F2 cells were labeled with a red fluorescent protein (DsRed). For labeling, MSCV retrovirus expressing DsRed fluorescent protein and providing puromycin resistance for antibiotic selection was used. Retroviral vectors were produced in 293T cell lines. The NIH3T3 cell line was used for the determination of infectious retroviral vector concentration. After 48 h of infection, antibiotic selection was performed with 2 µg/ml puromycin and selection continued until all uninfected cells died. D1 ORL UVA cells were labeled with green fluorescent protein (GFP) as previously described.¹⁴⁴

2.2.3. Cell Culture

D1 ORL UVA^{eGFP} and 7F2^{DsRed} cells were cultured in growth medium supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco) at 5% CO₂ humidified atmosphere at 37°C. Dulbecco's modified Eagle's medium (DMEM high glucose; Gibco) and alpha Modified Essential Medium (αMEM; Biological Industries) were used as standard growth media for D1 ORL UVA and 7F2 cells, respectively. The medium was refreshed every 2–3 days. For differentiation of 7F2 cells into adipocytes, the cells were seeded in six-well plates at a concentration of 5000 cells/well. On the 2nd day of culture, the medium was replaced with adipogenic differentiation medium composed of 10 nM dexamethasone, 50 µM indomethacin, 5 µg/ml insulin and the cells were cultured for 5, 7, or 10 days. The adipogenic induction medium was replaced every 2–3 days. D1 ORL UVA cells were seeded under the same conditions and cultured in the growth medium only. Throughout the study, cells cultured in the adipogenic medium were called the “adipogenic” group, while non-induced cells that were cultured in the standard medium were called the “growth” group. In addition to murine cells, we also used human lung carcinoma cells (A549) and human fetal osteoblastic cells (hFOB 1.19) for scaffold-free biofabrication of human-derived cells. A549 cells, which are known to have the capacity for lipid accumulation^{145,146} were cultured in DMEM high glucose containing 10% FBS and 1% P/S at 5% CO₂ humidified

atmosphere at 37°C. hFOB cells were cultured in DMEM:F12 (Gibco) medium supplemented with 10% FBS and 1% P/S in a humidified incubator at 34°C. For adipogenic differentiation of A549 cells, the cells were seeded in 12-well plates at a concentration of 750 cells/well. Adipogenic differentiation was induced for 21 days by using the same inducers as in the 7F2 differentiation protocol. For growth group, the confluent cell cultures of A549 and hFOB cells were used. The cells were imaged at 10× under an inverted microscope (Olympus IX-83).

2.2.4. Cell Viability Assay

Cell viability was tested with a calcein-AM/propidium iodide (PI) double staining kit (Sigma-Aldrich). The 7F2 and D1 ORL UVA cells were seeded into 24-well plates with a concentration of 750 cells/well. For adipogenesis, 7F2 cells were induced with adipogenic inducers after 2 days of seeding and all groups were cultured for an additional 7 days. At day 7, the cells were treated with Gd concentrations of 15, 30, and 50mM during 24 h. Then, the cells were washed with phosphate-buffered saline (PBS) and the assay solution was added. The cells were incubated at 37°C for 15 min. The stained cells were imaged under the fluorescence microscope (Olympus IX-83).

2.2.5. Oil-Red-O Staining

To stain lipid droplets, A549 cells in the growth and adipogenic groups were washed with PBS, fixed with 10% Neutral Buffer Formalin (NBF) for 15 min at room temperature, and then rinsed with distilled water. Afterward, the cells were stained with Oil-Red-O (ORO) solution (Amresco) for 45 min at 37°C and rinsed with distilled water three times.

2.2.6. Fluorescent Staining of Human-Derived Cells

To label A549 cells with red tracer and hFOB cells with green tracer, the cells were trypsinized and centrifuged. The pellet was resuspended at a concentration of 1×10^6 cells/ml in serum-free medium. Then, the cell-labeling solution (Vybrant™) was added at a concentration of 5 μ M and incubated at 37°C for 20 min. The labeled cells were centrifuged at 1500 rpm for 5 min three times. Finally, the cells were suspended in complete medium and used for 3D levitation culture.

2.2.7. 3D Culture of the Cells in Vertical Magnetic Levitation System

Firstly, to show the single cell culture of adipogenic cells, 7F2 cells cultured in growth and adipogenic induction medium were trypsinized at Day 10. Then, they were centrifuged at 125g for 5 min and the pellet was resuspended to 10^5 cells/ml in the culture medium. Quiescent (growth) and adipogenic-differentiated cells were loaded into capillaries as 50 μ l samples (5000 cells/capillary) using concentrations of 15, 30 and 50mM gadolinium (Gd^{3+}) based solution (Gadavist®; Bayer). The cells were cultured in the levitation system for 24 h at 5% CO₂ humidified atmosphere at 37°C and then visualized under an inverted microscope at $\times 4$ magnification. Levitation images were subsequently analyzed by ImageJ Fiji software to determine morphological parameters [area, perimeter, thickness, length and elongation (1/circularity)] and levitation heights of 3D structures. All morphological indices of the self-assembled clusters were quantified as described previously.⁸⁶ Second, D1 ORL UVA^{eGFP} stem cells and adipogenic differentiated 7F2^{DsRed} cells were cocultured under magnetic levitation. The cells cultured for 5, 7, or 10 days were detached from culture plates and mixed at a 50:50 ratio with a final concentration of 10^5 cells/ml. Then, the mixed cells were loaded into capillaries using 50 mM Gd and cultured over 24 h at 37°C. Imaging and analysis of 3D structures were carried out as mentioned above. Three strategies were used to create different 3D cellular patterns. In the first strategy, two cell types were sequentially loaded into capillaries with different periods. For this, D1 ORL UVA^{eGFP} cells cultured for 7 days in

plates were loaded into capillaries with 10^5 cell/ml. After culture of the cells for 24 h in the levitation device, the second type of cells (7F2^{DsRed}) were loaded with the same concentration and cultured for an additional 4 h. 3D structures were monitored using both inverted and confocal microscopes. The vertical band thickness of overlapped 7F2^{DsRed} and D1 ORL UVA^{eGFP} cells was calculated with ImageJ Fiji. In the second strategy, one cell type was used to cover another cell type. For this, D1 ORL UVA^{eGFP} cells were cultured for 8 h in the levitation device, then 7F2^{DsRed} cells were loaded into capillaries containing D1 ORL UVA^{eGFP} clusters and capillaries were rotated 180° twice every 15 min. After additional culture for 8 h, the process was repeated by adding 7F2^{DsRed} cells. To measure the coating capacity of loaded 7F2^{DsRed} cells, fluorescence signals of the cells were quantified by ImageJ Fiji. The same loading strategy was performed by starting with culture of 7F2^{DsRed} cells and adding D1 ORL UVA^{eGFP} cells. A third strategy was used to form multilayered structures. The cells were loaded into capillaries at 8 h intervals in three steps by loading D1 ORL UVA^{eGFP}, 7F2^{DsRed}, and D1 ORL UVA^{eGFP} cells sequentially. During loading, the magnetic levitation device was horizontally rotated 180° and cells were cultured for 24 h in total. The same strategy was performed by starting with culture of 7F2^{DsRed} and adding, in turn, D1 ORL UVA^{eGFP} cells and 7F2^{DsRed}. All imaging processes for 3D cellular structures were performed using an inverted microscope (Olympus IX-83) at $\times 4$ and confocal microscope (Leica DMI8) at $\times 10$. To show the applicability of the system to 3D culture of human-derived cells, A549 and hFOB cells were cultured or cocultured in equal proportions during magnetic levitation and imaged under the inverted microscope.

2.2.8. Statistical Analysis

All the experiments were performed in triplicate, and data on levitation height and morphological parameters are presented as the mean and standard deviation (mean $\pm$ SD). Student's t test (two-tailed) was applied to determine statistical significance, and $p < 0.05$ was considered as statistically significant. Nonsignificant changes are also reported for completeness but marked with (p-NS).

2.3. Results

2.3.1. 3D Culture of Single Adipocytes in Magnetic Levitation Device

To evaluate whether the magnetic levitation setup that was previously developed^{83,86} is suitable for the scaffold-free biofabrication of adipocyte clusters, quiescent (growth) and adipogenic 7F2 cells were levitated and cultured for 24 h. The 3D structures that formed during magnetic levitation were evaluated based on their area, perimeter, thickness, length, elongation, and center of gravity height from the top surface of the bottom magnet (Figure 2.2A). At the beginning of the experiment, single cells that were cultured in growth medium for 10 days levitated to the heights of 622 ± 19.5 , 850 ± 6.3 , and 963 ± 4.4 μm in 15, 30, and 50mM Gd-containing solution, respectively (Figure 2.3A,B). The average single cell levitation height of adipogenic 7F2 cells was 34.1%, 13.6%, and 8.9% higher compared with the matching Gd concentration of growth cells (all $p < 0.0001$) (Figure 2.3). The clusters formed after 24 h in levitation culture by adipogenic cells at three different Gd concentrations (15, 30, and 50 mM) exhibited looser and more branched patterns compared with the packed structures in the growth group (Figure 2.2B). The levitation height of growth cell clusters was 442 ± 53 , 502 ± 83 , and 814 ± 54 μm in 15, 30, and 50mM Gd-containing solution, respectively (Figure 2.2C). Adipogenic cell clusters, on the other hand, exhibited 115% ($p = 0.006$), 119% ($p < 0.0001$), and 46% ($p < 0.0001$) higher levitation heights for matching Gd concentrations.

When the cells were cultured with levitation and 50mM Gd, the morphological indices of the clusters including area, perimeter, thickness, length and elongation of the whole structure were $117,196 \pm 74,871$, 2930 ± 2127 , 309 ± 83 , 907 ± 574 , and 6.3 ± 5.6 for the growth group, respectively (Figure 2.2D-H). Adipogenic clusters, on the other hand, showed 106% ($p = 0.02$), 36% ($p = 0.005$), and 292% ($p=0.01$) higher perimeter, thickness, and elongation values, respectively, compared with clusters formed by growth cells. However, no significant difference was detected for the area and length of clusters between growth and adipogenic groups. Furthermore, as criteria for loose packaging of cells, area/perimeter (Figure 2.2I), area/thickness (Figure 2.2J), and area/length (Figure 2.2K) values were also examined. These results showed that the observed differences

occurred not because of the length of the clusters that were measured but because of thickness and perimeter.

During levitation culture experiments, some clusters belonging to the growth group at 30mM Gd and to both the growth and adipogenic groups at 15mM Gd attached to the capillary bottom surface and those clusters were not analyzed for morphological parameters (Figure 2.4). At 30mM Gd, adipogenic 3D clusters had larger area (341%, $p = 0.002$), perimeter (463%, $p < 0.0001$), thickness (129%, $p < 0.0001$), length (176%, $p = 0.002$), and elongation (648%, $p = 0.001$) compared with growth controls (Figure 2.4D-H). At 15mM Gd concentrations, adipogenic clusters only differed from growth controls in thickness (241%, $p = 0.017$) and levitation height (115%, $p = 0.006$).

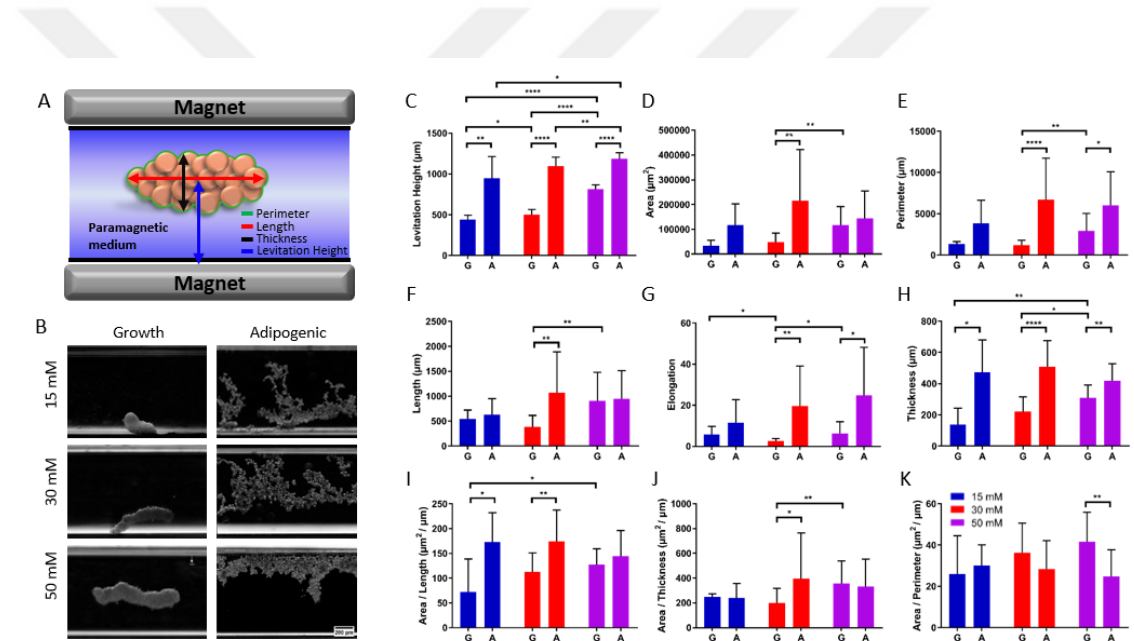


Figure 2.2. Magnetic levitation culture of 7F2 cells after adipogenic induction for 10 days.

(A) Illustration of levitation-based culture through scaffold-free technique (green line: perimeter of 3D cluster, red arrow: length, black arrow: thickness, blue arrow: levitation height). (B) Culture images of growth and adipogenic 7F2 cells at 15, 30, and 50mM Gd concentrations. Scale bar: 200 μm . Quantitative parameters of 3D cellular clusters; (C) levitation height, (D) area, (E) perimeter, (F) length, (G) elongation, (H) thickness, (I) area/length, (J) area/thickness, (K) area/perimeter. Data are represented as mean $\pm$ SD. Statistical significance was defined as * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

The effect of Gd concentration on the size of 3D structures formed during magnetic levitation culture was also examined for growth and adipogenic cells, excluding the samples that showed total attachment at lower concentrations. The 15mM Gd group showed differences in the growth group for elongation and thickness compared to the 30mM (118%, $p = 0.011$) and 50mM (55%, $p = 0.010$) Gd groups, respectively. No statistically significant difference was observed for the morphology of the 15mM Gd group compared with other concentrations in adipogenic clusters (Figure 2.2D-H). However, the clusters formed by cells at 50mM Gd were larger structures and the differences between their mean area, perimeter, thickness, length, and elongation were 58% ($p = 0.003$), 59% ($p = 0.003$), 28% ($p = 0.02$), 57% ($p = 0.002$), and 59% ($p = 0.013$) compared with the clusters formed at 30 mM Gd concentration. However, all size parameters were similar between adipogenic groups.

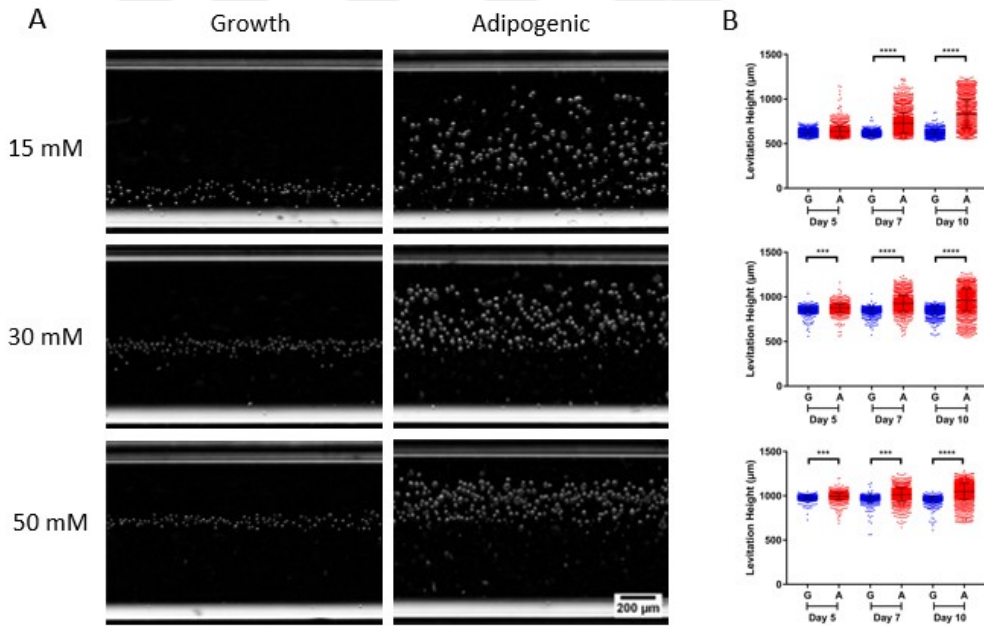


Figure 2.3. Single-cell levitation of growth and adipogenic 7F2 cells. (A) Levitation profile of growth and adipogenic cells by using 15, 30 and 50 mM Gd after 10 days of culture in 2D. Scale bar: 200 μm. (B) Scatter plots of levitation heights of growth and adipogenic cells with 15, 30 and 50 mM Gd at day 5, 7 and 10. Data are represented as scattered with mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

2.3.2. Co-culture Models of Adipocytes

To manipulate the self-assembly and single cell density phenotype of cells, adipocytes or growth 7F2^{DsRed} cells were mixed with D1 ORL UVA^{eGFP} (D1^{eGFP}) stem cells either pre- or post-levitation. Before mixing, D1^{eGFP} cells were levitation cultured in the microcapillary platform using similar time and Gd³⁺ concentration conditions (Figure 2.5A). Levitation heights of single D1^{eGFP} cells cultured for 7 days were 581 ± 19.7 , 831 ± 9.0 , 962 ± 7.5 μm at 15, 30, and 50mM Gd, respectively (Figure 2.6). On the other hand, 3D clusters formed by D1^{eGFP} cells after 24 h had levitation heights of 521 ± 54.2 , 801 ± 20.9 , and 914 ± 17.8 μm at 15, 30, and 50mM Gd, respectively (Figure 2.5B). D1^{eGFP} cell clusters did not show significant differences in area and thickness for all Gd concentrations, and exhibited minor differences for perimeter, length, and elongation.

The applied Gd concentrations (up to 50mM) were previously shown not to induce cytotoxicity on D1 ORL UVA cells⁸⁶. However, to ensure that 7F2 cells were also tolerant to Gd, live/dead staining for both growth and adipogenic 7F2 cells treated with Gd concentrations (15, 30, 50mM) for 24 h was assessed (Figure 2.7). Considering the viability and 3D culture results, the 50mM Gd concentration was deemed to be suitable for the following studies.

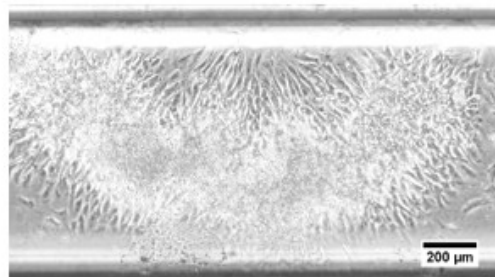


Figure 2.4. Representative image of microcapillary channel which results in cell attachment to the bottom of the capillary by using 15 mM Gd.

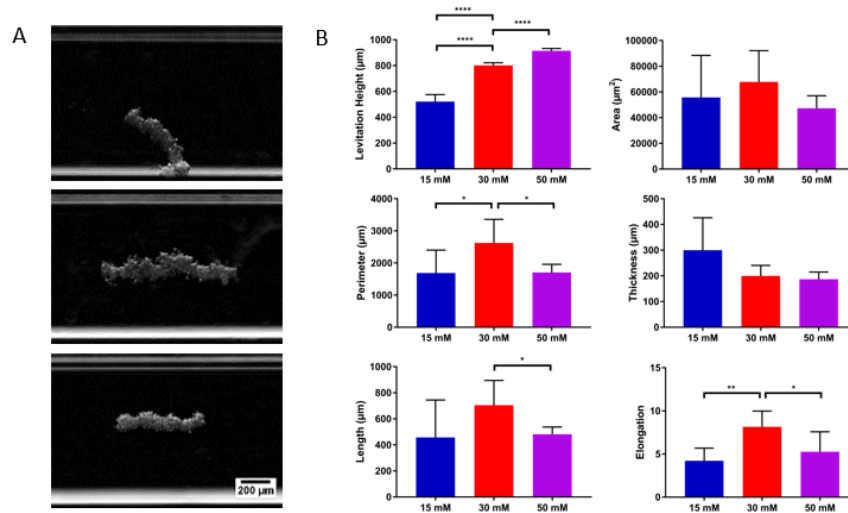


Figure 2.5. Magnetic levitation culture of D1 ORL UVA cells. (A) Culture images of D1 ORL UVA cells at 15, 30, and 50mM Gd concentrations. Scale bar: 200 μm . (B) Quantitative parameters of 3D cellular clusters were present as levitation height, area, perimeter, thickness, length, and elongation. Data are represented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

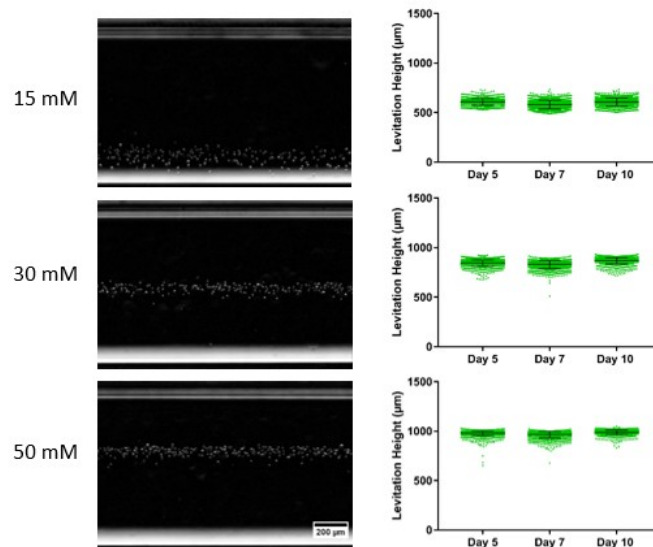


Figure 2.6. Single cell levitation profile of D1 ORL UVA cells levitated with 15, 30 and 50 mM Gd. Data are represented as mean $\pm$ standard deviation. Scale bar: 200 μm .

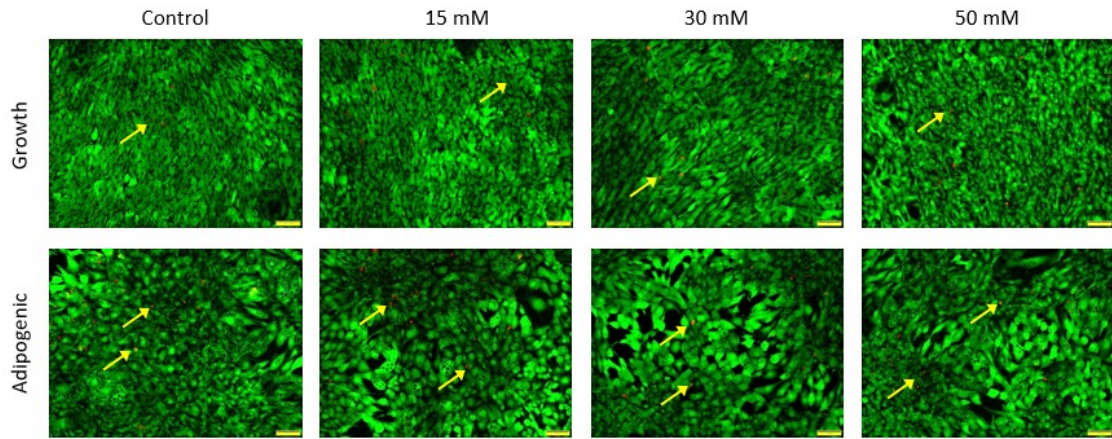


Figure 2.7. Live/dead assay conducted by calcein-AM (green-live)/PI (red-dead) staining for growth and adipogenic 7F2 cells that were treated with three Gd concentrations (15, 30, 50 mM). Similar ratios for dead cells were observed for all concentrations. Scale bar: 100 μm .

D1^{eGFP} cells that were cultured in growth media and 7F2^{DsRed} cells that were cultured in growth or adipogenic media were mixed in 1:1 ratio and levitation cultured for 24 h. The degree of adipogenesis in 7F2^{DsRed} cells was controlled by their exposure time to adipogenic induction for 5, 7, or 10 days; while D1^{eGFP} and growth 7F2^{DsRed} cells were cultured for the same time points in growth media (Figure 2.8). Single cell levitation of D1^{eGFP} and 7F2^{DsRed} cells showed homogeneous distribution for the growth group, while based on the adipogenic exposure time, a portion of 7F2^{DsRed} cells were observed to levitate above D1^{eGFP} cells (Figure 2.9). The clusters formed after 24 h of levitation culture appeared to have a homogeneous cell distribution for growth conditions; while with increased adipogenesis, 7F2^{DsRed} cells positioned themselves in the higher portion and D1^{eGFP} cells remained on the lower portion of clusters, resulting in multi-layered cell structures (Figure 2.10A, yellow arrows). The levitation height of clusters formed by coculturing adipogenic and stem cells was $1014.6 \pm 86.4 \mu\text{m}$ at Day 5 (Figure 2.10B). However, when the adipogenic induction time was increased to 7 and 10 days, 3D clusters of adipogenic group were located at 9% and 8% higher levels compared to 5 days. When the 3D clusters were analyzed for area (Figure 2.10B), at Day 5, cocultured adipogenic and stem cells had a size of $126,674 \pm 83,897 \mu\text{m}^2$. Compared with Day 5, the area of clusters increased two-fold ($p < 0.001$) at Day 10. A similar trend was observed for other

parameters such that average perimeter, thickness, length, and elongation increased 27.1%, 11.7%, 8.7%, and 44.2% at Day 7 (all p-NS) and 131.0% ($p < 0.001$), 19.2% (p-NS), 79.5% ($p = 0.001$), and 165.8% ($p = 0.005$) at Day 10 compared with Day 5 (Figure 2.10B). Also, comparison between Days 7 and 10 indicated 77.3% ($p = 0.005$), 81.8% ($p = 0.007$), 6.7% (p-NS), 65.1% ($p = 0.005$), and 83.6% (p-NS) increases in area, perimeter, thickness, length, and elongation, respectively. On the other hand, the sizes of 7F2^{DsRed} and D1^{cGFP} cells cultured in growth medium showed minor differences based on culture time. We continued further levitation coculture studies using 7 days of growth and adipogenic 7F2^{DsRed} cells and growth D1^{cGFP} cells for consistency.

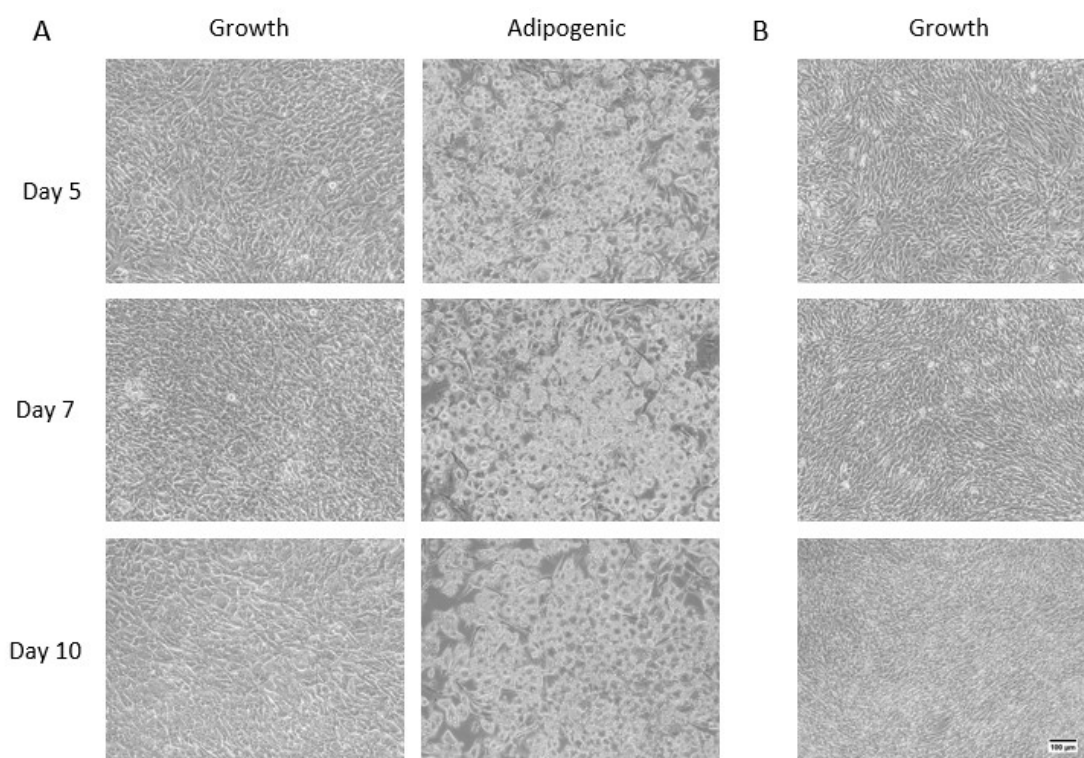


Figure 2.8. Cell culture images of the cells. (A) 7F2 cells cultured in growth medium and adipogenic differentiation medium for 5, 7 and 10 days. (B) D1 ORL UVA cells cultured in growth medium for 5, 7 and 10 days. Scale bar: 100 μm .

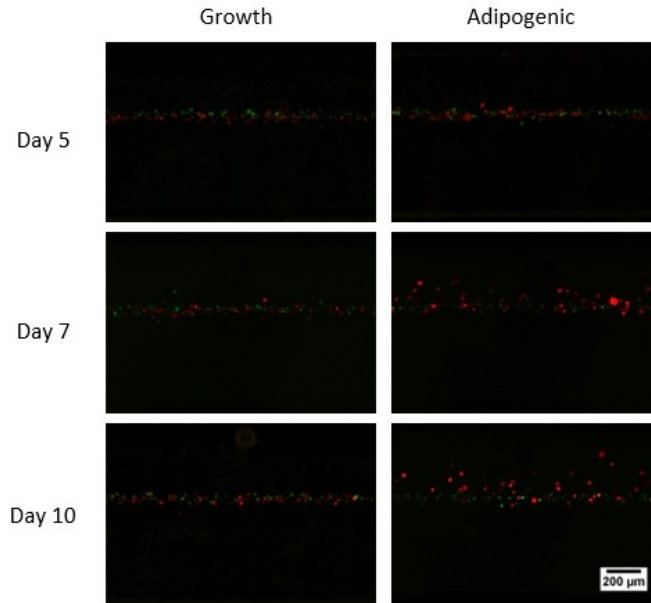


Figure 2.9. Single cell levitation of D1 ORL UVA cells (green) mixed with growth or adipogenic differentiated 7F2 cells (red) with a 1:1 ratio. Adipogenic differentiation was induced for 5, 7 or 10 days. Scale bar: 200 μm .

To demonstrate the applicability of the 3D culture method performed with the magnetic levitation system to human cells, human-derived hFOB osteoblast cells (Figure 2.11A) and A549 cells, which can accumulate lipid droplets (Figure 2.11B), were used. First, the single cell levitation profiles of A549 and hFOB cells were measured using 50mM Gd concentration as $988 \pm 64 \mu\text{m}$ for hFOB cells, $958 \pm 18 \mu\text{m}$ for growth A549 cells and $964 \pm 53 \mu\text{m}$ for adipogenesis-induced A549 cells (Figure 2.11C). In the adipogenic A549 group, several cells with relatively increased cell size, were observed at higher levitation heights (Figure 2.11C, yellow arrows). The cells were then grown for 24 h under levitation as single- or co-cultures and morphological parameters were analyzed (Figure 2.12A-C). Area, perimeter, thickness, length, and elongation of 3D clusters of single-cultured growth A549 cells were $207,331 \pm 55,586 \mu\text{m}^2$, $6474 \pm 1619 \mu\text{m}$, $212 \pm 37 \mu\text{m}$, $1822 \pm 406 \mu\text{m}$, and 16.5 ± 5.6 , respectively. For the adipogenic group, these parameters were mostly similar to the growth group. Only thickness exhibited a significant increase (36%, $p = 0.022$). On the other hand, when the cells were mixed with osteoblastic hFOB cells in the same ratio, the two cell types showed a homogeneous distribution in 3D structure (Figure 2.12C). In terms of morphological features and levitation profile, there

were no significant differences between growth and adipogenic groups in 3D cocultured cells (Figure 2.12B). However, it was observed that the coculture of hFOB cells with growth A549 cells resulted in decreases in perimeter (36%, $p = 0.016$), length (29%, $p = 0.04$), and elongation (56%, $p = 0.003$) and an increase in thickness (23%, $p = 0.037$) of the 3D cellular structures compared to single-cultured growth A549 cells. The comparison of adipogenic groups exhibited a similar trend with decreases in perimeter (40%, $p = 0.02$) and elongation (64%, $p = 0.003$).

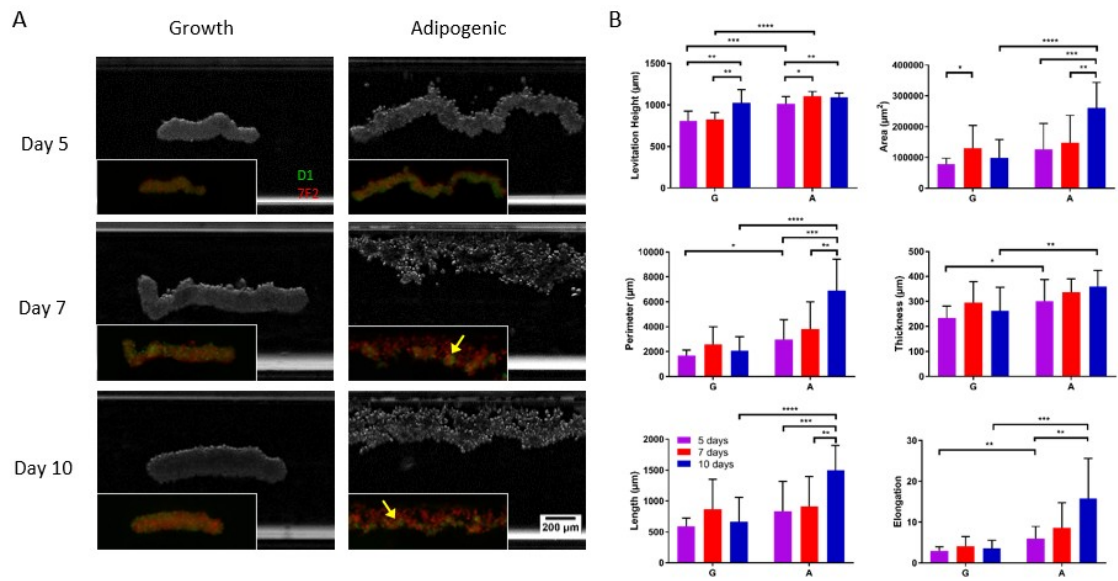


Figure 2.10. Homogeneous coculture of adipogenic cells and stem cells. (A) Phase contrast and fluorescence microscopy images of that 7F2 cells (red), which were cultured in growth and adipogenic medium during 5, 7, or 10 days, homogeneously mixed with D1 ORL UVA cells (green). Yellow arrows indicate D1 ORL UVA cells on the bottom layer. Scale bar: 200 μm . (B) Quantitative parameters of 3D cellular clusters formed by two cell types were present as levitation height, area, perimeter, length, elongation, and thickness. Data are represented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

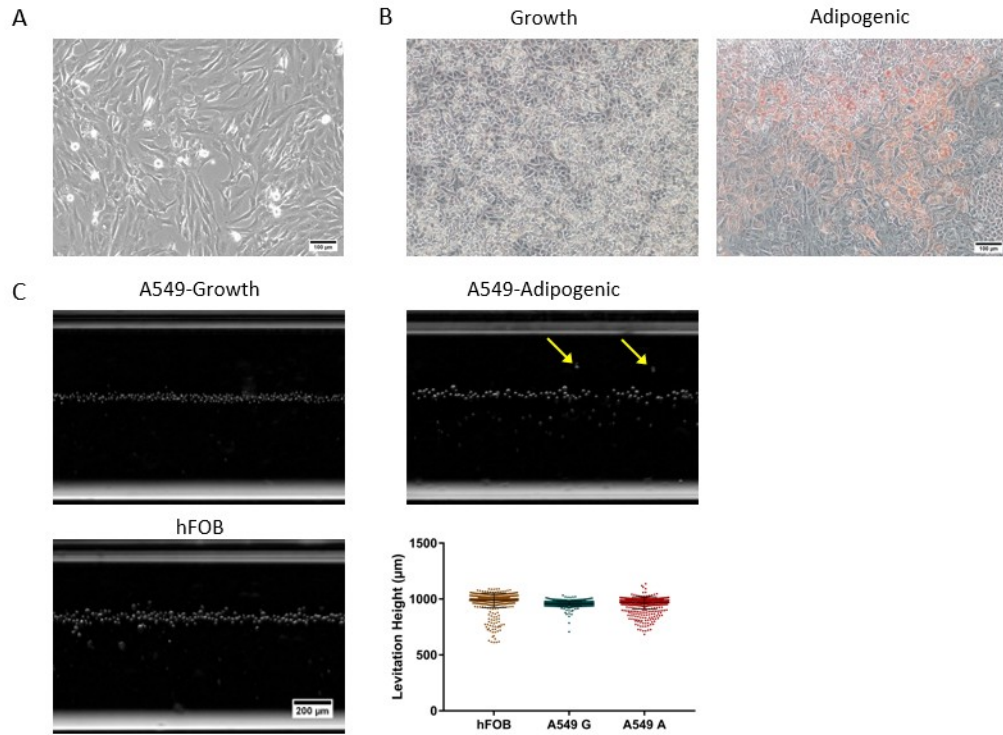


Figure 2.11. 2D culture and levitation of hFOB and A549 cells. (A) hFOB cells that were cultured in the growth medium. Scale bar: 100 μm. (B) Oil-red-O staining of A549 cells that were cultured in growth or adipogenic induction medium. Scale bar: 100 μm. (C) Single cell levitation of the hFOB, growth A549, and adipogenic A549 cells with 50 mM Gd. Yellow arrows indicate lipid accumulated A549 cells. Scale bar: 200 μm.

To form multilayered structures in the magnetic levitation system, mouse-derived cells were loaded into the system with various fabrication strategies (Figure 2.13A), resulting in distinct patterns (Figure 2.13B-F). By loading 7F2^{DsRed} cells after levitation culture of D1^{eGFP} cells for 24 h (1st strategy), pre-formed D1^{eGFP} aggregates were partially coated with 7F2^{DsRed} cells in both growth and adipogenic groups (Figure 2.13B). In the adipogenic group, 7F2^{DsRed} cells were more loosely integrated into this construct because of the disparity in the levitation height of these two cell types due to their different densities (Figure 2.9).

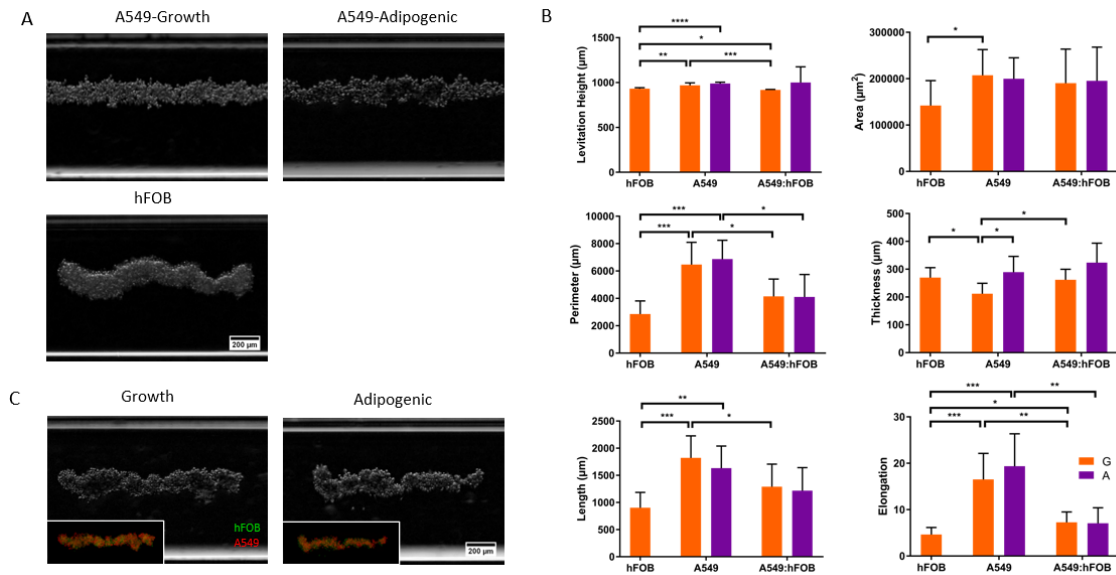


Figure 2.12. 3D magnetic levitation culture of growth/adipogenic A549 cells and growth hFOB cells (A) Single cell culture of the A549 and hFOB cells in the magnetic levitation system with 50 mM Gd. Scale bar: 200 μm. (B) Quantitative parameters of 3D cellular clusters; levitation height, area, perimeter, thickness, length, and elongation. Data are represented as mean ± SD. Statistical significance was defined as *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (C) Coculture of hFOB (green) and A549 (red) cells mixed at a 1:1 ratio in magnetic levitation with 50 mM Gd. Scale bar: 200 μm.

In contrast, for the growth group, separately assembled D1^{eGFP} clusters were mostly coated by 7F2^{DsRed} cells, forming a large aggregate. Analysis of the contact surface area of the two cell types showed that the vertical band of overlapped cells (Figure 2.13B, yellow bracket) was decreased threefold ($p = 0.015$) (Figure 2.14A). An alternative (2nd) strategy was performed to increase coating on the lower side of clusters in an effort to form multi-layered spheroid-like structures (Figure 2.13C,D). In this strategy, the second cell type was loaded in two consecutive runs to improve coverage of the pre-formed cluster; and 180° rotation of the microcapillary was performed between loadings.

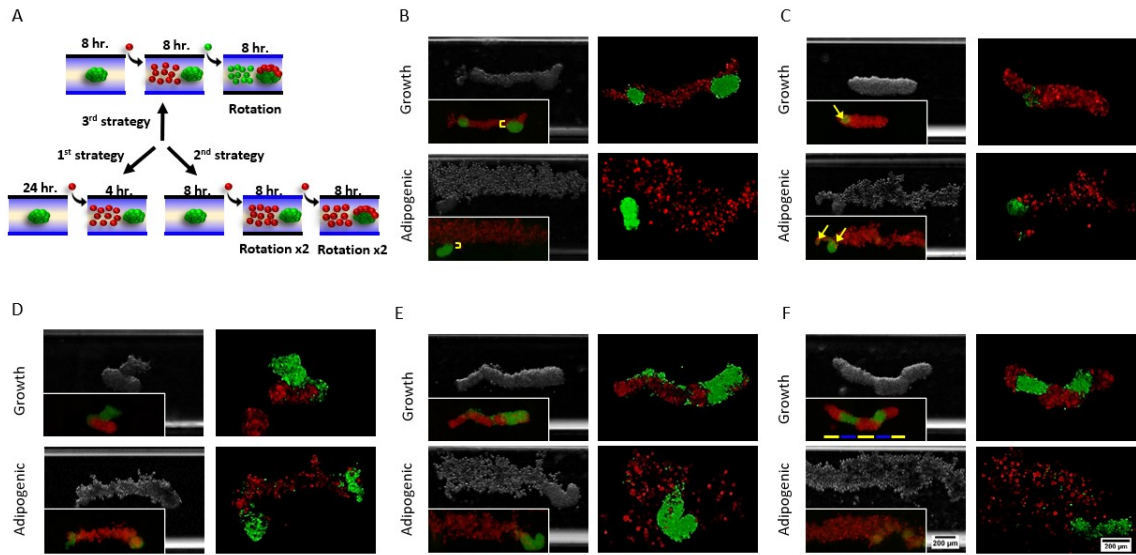


Figure 2.13. Magnetic levitation culture of adipocyte structures with various culture strategies. (A) Schematic illustration of cell loading strategies. First strategy: Adding 7F2 (red) cells after culture of D1 ORL UVA (green) cells for 24 h; second strategy: After culture of D1 for 8 h, adding 7F2 cells twice with intervals of 8 h and 180° rotation at last loading; third strategy: After culture of D1 for 8 h, adding 7F2 and D1 cells, respectively, with 180° rotation twice per 15 min during second and third cell loading. (B) After culturing D1 cells for 24 h, 7F2 cells were added and cultured for an additional 4 h. Yellow brackets indicate overlapped cells in the vertical direction. (C) After culturing D1 cells for 8 h, 7F2 cells were added by 180° rotation of the capillary twice per 15 min, and the addition of 7F2 was repeated after an additional 8 h. Yellow arrows show 7F2 cells positioned on D1 clusters. (D) The same strategy as with C was performed by starting with 7F2 cells and then adding D1 cells. (E) After culturing D1 cells for 8 h, 7F2 cells were added, and after an additional 8 h, D1 cells were added by 180° rotation of the capillary. (F) The same strategy as with E was performed by starting with 7F2 cells and adding D1 and 7F2 cells, respectively. Phase contrast (left), fluorescence (lower left), and confocal (right) microscopy images.

Based on this strategy, we first tried to coat D1^{eGFP} clusters with 7F2^{DsRed} cells and observed that small D1^{eGFP} clusters assembled with the 7F2^{DsRed} cells. Also, it was seen that 7F2^{DsRed} cells were located on the surface of D1^{eGFP} clusters in both groups without complete coating (Figure 2.13C, yellow arrows) and that the contact area of D1^{eGFP} clusters with 7F2^{DsRed} cells increased 7.6- and 17.3-fold for the growth ($p < 0.001$) and adipogenic ($p = 0.01$) groups, respectively, compared to the 1st strategy (Figure 2.14B). By using this strategy, although we did not observe effective coating, compared to the first loading strategy, the differences between the levitation heights of growth and adipogenic cells were minimized, leading to coded structures. We then tried to cover 7F2^{DsRed} clusters with D1^{eGFP} cells with the same strategy (Figure 2.13D) and, although we observed an increase in the contact area of growth cells, we failed to observe the same for adipogenic 7F2^{DsRed}, potentially due to the low density of adipogenic cells. Finally, we utilized a 3rd strategy for biofabrication of layered structures with an inoculation strategy similar to the 2nd approach, but we varied the cell type during inoculations. When cell loading started with D1^{eGFP} cells, we observed that D1^{eGFP} cells assembled with adipogenic cells and showed a similar pattern as that formed by the 2nd loading strategy (Figure 2.13E). When we started with 7F2^{DsRed} cells, we observed that adipogenic cells coated the D1^{eGFP} cells, while layered structures emerged in the growth group (Figure 2.13F, yellow and blue lines).

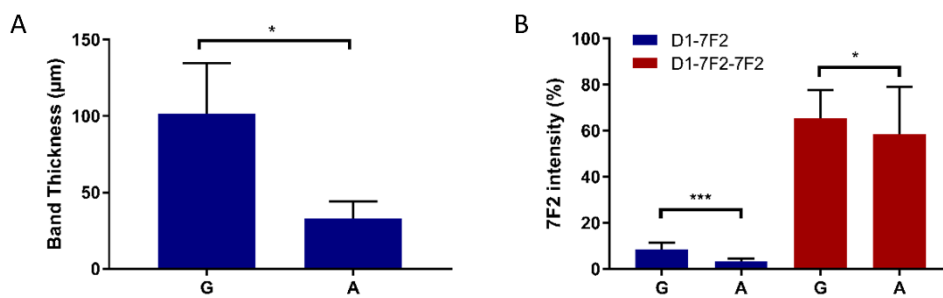


Figure 2.14. Coating of D1 ORL UVA^{eGFP} cells with 7F2^{DsRed} cells. (A) Band thickness of overlapped D1 and 7F2 cells in the vertical direction. (B) Fluorescence intensity of 7F2 cells on D1 clusters. 7F2 intensity was normalized to the D1 clusters in each sample. Data are represented as mean $\pm$ SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

2.4. Discussion

In this study, a magnetic levitation system was used as a biofabrication tool for only adipocytes or adipocytes with stem/osteoblastic cells. When adipocytes differentiated from mouse-derived cells were levitation-cultured under a magnetic gradient, it was observed that the cells created very loose 3D clusters compared to tightly-packed growth cell clusters. This difference in morphological phenotype may be due to altered intercellular interactions after adipogenic differentiation. On the other hand, when the adipocytes and stem cells were cocultured with different loading strategies, it was observed that denser cells tended to spontaneously self-assemble in the bottom part of the cell mixture. However, with different loading strategies, we obtained partially covered stem cells with adipocytes or asymmetrical attachments. Therefore, compared with some other spheroid-based scaffold-free culture techniques challenged with creating complex structures, it was shown that our platform can be used to form heterotypic and layered 3D cellular structures, and that the levitation system is also appropriate to form vertical cellular layers.

Adipose tissue defects caused by burns or tumor removal have prompted rising interest in adipose tissue engineering for clinical needs and adipose tissue engineering has started to be widely utilized in regenerative medicine and cell therapy.¹⁴⁷ Adipose tissue models are also important for the determination of the physiological and pathological processes, which are associated with obesity and obesity-related diseases. Several studies performed in static or dynamic conditions have been reported for adipose tissue engineering studying the relation between adipocytes and other cell types (e.g., cancer cells) in 3D environment.^{35,39,57,58,68,148-153} The complex nature of adipose tissue, which contains various cell types such as adipocytes, adipose-derived stem cells, and vascular smooth muscle cells, is the biggest obstacle for the fabrication of appropriate adipose tissue mimics.⁵ Studies on adipose tissue engineering mostly rely on using scaffolds to mimic the 3D architecture of adipose tissue because of the consensus that adipose tissue cells could not produce their own ECM and need supporting materials for maintenance of their 3D morphology during culture due to their fragile nature.^{35,153,154} In contrast, some studies showed that it is possible to achieve scaffold-free adipose tissue engineering, demonstrating that these cells have the ability to produce their own

ECM.^{35,155,156} Here, the microcapillary-based system provides an alternative scaffold- and contact-free method for biofabrication of a 3D adipose tissue. To recapitulate the 3D architecture of adipose tissue, we created self-assembled 3D structures of adipocytes and mesenchymal stem cells, which are used as model cells for the two components of adipose tissue.⁵ In this context, cell culture in the magnetic levitation system was performed with different Gadavist concentrations and loading strategies to assemble the cells which exhibit different intercellular interactions. As a result of the study, it was shown that the magnetic levitation system allowed monolayered or bilayered coculture of the adipose tissue cells using a scaffold-free culture approach. Although we only utilized cell culture techniques and immortal cell lines, we believe this approach can be adapted to use unilocular and multilocular adipocytes to better mimic various adipose tissues.

Magnetic levitation of cells can be performed with magnetic labeling of cells (positive magnetophoresis) or label-free (negative magnetophoresis).⁶⁵ Iron oxide and gold nanoparticles, which are used for cell manipulations in positive magnetophoresis, may be cytotoxic and they cannot be easily removed from assembled biostructures.^{67,134,136,157} Internalization of MNPs can cause increased production of intracellular reactive oxygen species (ROS) which is concomitant to DNA damage in a time- and concentration-dependent manner.¹³⁴⁻¹³⁶ Even when MNP concentrations are kept in the nontoxic range, their accumulation can cause cytotoxicity or carcinogenicity.¹⁵⁸ Increased ROS levels lead to structural and functional distortion of cellular components and, eventually, changes in cell metabolism that are remarkable drawbacks in tissue engineering applications.^{135,159} Although it is possible to reduce the cytotoxicity of MNPs by biotransformation¹⁶⁰⁻¹⁶², the efficacy of this process is dependent on the size, shape, and surface properties of nanoparticles.^{162,163} Furthermore, the use of MNPs requires additional time-consuming experimental steps for labeling.¹⁶⁴ Contrary to studies performed by positive magnetophoresis for 3D adipose culture^{68,143}, the label-free approach utilized in our study provides a simple technique to create 3D adipose models which have the possibility to be translated to fat grafting clinical applications, without involving magnetic particles that are likely to cause side effects in patients.

In recent studies, the label-free magnetic levitation technique has been used for detection and characterization of various cell types such as blood cells^{74,76,78,141}, cancer cells^{76,165}, adipocytes⁸³, and osteogenic cells¹⁶⁶. Also, it was shown that sickle cell disease which results in increased density of erythrocytes¹⁶⁷ and drug treatments that cause density changes in bacteria and yeast⁷⁶ can be tested with magnetic levitation systems and

portable platforms of the system enable applications for point of care testing.^{78,165,168} After the successful application of the magnetic levitation system to single-cell-based studies, it became a focus of interest as a biofabrication tool for tissue engineering.¹⁶⁹ Previously, the suitability of a long-term cell culture in a magnetic levitation platform was demonstrated by single culture and co-culture of cancer and stem cells.⁸⁶ In this study, we created 3D clusters of adipocytes with or without mesenchymal stem cells. The size of the structures containing adipogenic cells, which were formed with the same number of control cells, was significantly larger because the adipogenic cells showed loose packing and formed porous structures. This may be a result of our particular levitation system inducing single-cell density-based stratification of cells before self-assembly.⁸³ Because single cell density varies greatly in lipid-accumulating cells based on their heterogeneous differentiation and because cells with lower single cell density position themselves higher compared with others in the microcapillary system, the resulting structures may be looser compared with cells that form structures in a single plane. However, it is also possible that the loose packing of the cell constructs may be related to the reduced expression of cell adhesion molecules in adipocytes.^{170,171} In contrast to our method, magnetic label-based adipogenic cell levitation culture resulted in a tighter 3D structure of adipocytes, possibly due to the equal attractiveness of the magnetic force on the MNPs.^{68,143} Regardless of loading sequence and manipulations, it was seen that the system allows the formation of bilayer structures by using the density differences of the cells.

For better mimicking of the functions of complex native tissues, it is important to recapitulate heterotypic cell-to-cell interactions in layers. Previously, various studies have been performed to fabricate multilayered structures for different tissue/organ models such as blood-vessels¹⁷²⁻¹⁷⁴, skin^{175,176}, brain^{177,178}, bone^{179,180}, air-blood tissue barrier¹⁸¹, and cardiac muscle¹⁸². As in standard 3D culture studies, studies to produce layered cell structures are commonly based on the use of supporting materials such as collagen, hydrogel, and polymer composites, which facilitate the creation of layered cell constructs due to supporting cell-cell adhesion, with different technologies such as bioprinting and microfluidics. This strategy of using scaffold materials is not preferable because of limitations on fabrication and insufficient imitation of native tissues due to their incompatible physical and chemical properties¹¹¹⁻¹¹⁷. However, contact-free magnetic force-based 3D culture techniques are emerging as an alternative to mimic more accurate 3D constructs as sheets or spheroids in a scaffold-free manner. Whereas a number of studies on magnetic force-based culture methods including MNPs for cell manipulations

were performed to create homotypic or heterotypic 3D layered cell structures in previous studies^{67,183-186}, studies of label-free magnetic levitation used for the construction of layered structures are very few.⁸⁶

Scaffold-based tissue engineering strategies inherently present some drawbacks such as biocompatibility concerns, inhomogeneous distribution of cells, hindering self-assembly, and the disruption of the physiological circulation of signal molecules by non-physiological support materials.¹⁸⁷⁻¹⁸⁹ However, scaffolds also have inherent advantages for sustaining biomimetic properties for mechanical and chemical niches.¹⁹⁰⁻¹⁹² It is possible to combine magnetic guidance protocols with scaffold-based tissue engineering for materials, including methacrylated gelatin (GelMA) and laminin-coated polystyrene divinylbenzene beads.^{193,194} Although not applied for adipose tissue biofabrication, the combinatorial approach signifies the possibility that magnetic levitation can be utilized in the bottom-up engineering of other tissues.

In this study, our results showed that a low density of adipocytes caused attachment to the top surface of the capillary channels during levitation culture. It was seen that decreasing Gd concentration or coculture with denser cells could overcome this problem. Also, adipocyte-only cultures forming very loose structures tended to lose the integrity of these clusters under external forces. This suggested that the magnetic levitation system may enable the determination of weak homotypic cell-to-cell interactions. In this study, although we aimed to create multilayered cellular structures, the difference in single cell densities was so large between growth and adipogenic cells, it resulted in limited flexibility in imposing construct shape and layers. This observation warrants further studies on optimization of biofabrication, potentially by manipulating working volumes as well as the magnet array.

In conclusion, we demonstrated biofabrication of adipose tissue constructs using label-free magnetic levitation. To our knowledge, this is the first study using a magnetic levitation system to construct a 3D layered structure of adipose tissue. Compared with other scaffold-free culture techniques challenged with creating complex structures³⁸, magnetic levitation can enable the creation of the multilayered adipose tissue model with its complex nature by manipulation of system parameters or configurations. Although we used the system for the design of a 3D adipose tissue model, this novel technology offers the opportunity to mimic other tissues by providing a cost-effective and easy operation. As a result, it can be used in translational studies such as metabolism and drug testing.

CHAPTER 3

MAGNETIC LEVITATION-ASSISTED BIOFABRICATION AND 3D CULTURE OF ADIPOCYTES, ENDOTHELIAL CELLS, AND MACROPHAGES

3.1. Background

Obesity, characterized by excessive adipose tissue accumulation, has emerged as a significant global public health concern.² In 2022, approximately 1 in 8 people worldwide were living with obesity, with 890 million adults classified as obese and 37 million children under the age of 5 being overweight, highlighting a growing global health crisis.¹⁹⁵ This condition is associated with the increased risk of numerous chronic health issues, including type 2 diabetes, cardiovascular disease, hypertension, and certain types of cancer.²⁻⁴ The development of obesity is influenced by a complex interplay of genetic, epigenetic, and environmental factors.^{2,196} In biological terms, obesity triggers the remodeling of adipose tissue, leading to the dysregulation of secreted factors and a surge in inflammatory molecules, collectively contributing to chronic low-grade inflammation.^{4,196}

Adipose tissue is a complex and dynamic compartment that plays a crucial role in energy metabolism and overall health. It comprises a variety of cell types, including adipocytes, preadipocytes, endothelial cells and immune cells.⁵ These cells interact intricately to maintain the proper function of adipose tissue. The regulation of adipose tissue involves a diverse array of signaling molecules, whose relative abundance and activity can be influenced by factors such as diet, exercise, and inflammation.¹⁹⁷⁻¹⁹⁹ Adequate vascularization within adipose tissue is essential to ensure nutrient and oxygen supply as well as the transport of cytokines and hormones. Maintaining a balance between pro-angiogenic and anti-angiogenic factors is critical for normal angiogenesis, and

disruption of this balance can lead to endothelial cell dysfunction and abnormal angiogenesis, which are associated with obesity.^{2,3} Furthermore, the expansion of adipose tissue during obesity is accompanied by an increased number of macrophages, contributing to inflammation and insulin resistance.²⁰⁰ Therefore, understanding the roles of endothelial cells and macrophages in adipose tissue is important for describing the biology, pathophysiology, and the development of potential therapeutic interventions against obesity.

Interest in adipose tissue research has increased due to the recognition of its endocrine functions, in addition to its role as an energy source and insulation barrier.⁹ The traditional approaches to study adipose tissue in vitro involve the use of simple and cost-effective two-dimensional (2D) monolayer cell cultures. However, three-dimensional (3D) culture models offer a more accurate representation of the 3D microenvironment of adipose tissue, including cell-cell interactions, extracellular matrix (ECM) remodeling, and nutrient/oxygen diffusion. Consequently, 3D models enable the biofabrication of targeted tissue constructs that closely resemble native tissue compared to their 2D counterparts. To date, several 3D adipose tissue models have been developed using scaffold-based or scaffold-free techniques. Scaffold-based techniques utilize natural polymers such as collagen, gelatin, fibrin, silk, and hyaluronan, as well as synthetic polymers such as PLA, PLGA, and PEG, to support and mimic the extracellular matrix of adipose tissue in a 3D setting.^{27,201} However, scaffold-based methods face specific challenges when dealing with adipocytes, including issues related to the fragile nature of adipocytes, their tendency to float in media, and the determination of suitable scaffold pore sizes for cells as adipogenic differentiation involves lipid accumulation-dependent cell size increase, making the process more complex.^{60,202,203}

Alternatively, scaffold-free 3D adipose culture studies involve the generation of spherical aggregates or cell sheets consisting of adipose-derived stem cells, preadipocytes, or mature adipocytes, using various techniques such as hanging drop, low-adhesive surfaces, and microfluidics.^{35,44,155,204-206} These scaffold-free 3D adipose culture studies have been applied to various research areas, including adipogenesis, angiogenesis, inflammation, and drug screening. These studies have revealed transcriptomic and metabolic disparities between 2D and 3D models, however, they remain limited in their ability to fully mimic native adipose tissue due to its inherently heterogeneous cell population.

In recent years, magnetic levitation (MagLev) technology has emerged as a promising tool for the biofabrication of 3D cell culture models *in vitro*. This approach has been successfully employed to construct monolayered or bilayered cellular spheroids containing various cell types such as chondrocytes, fibroblasts, stem cells, and cancer cells.^{80,84-87,90} MagLev allows the utilization of different combinations and shapes of permanent magnets to generate varied patterns of 3D cellular structures.⁶⁵ Furthermore, it has been demonstrated that the morphological characteristics of these 3D structures can be controlled by adjusting culture time, cell seeding density, or the concentration of a paramagnetic agent.^{86,89} In a previous study, our team utilized the MagLev technique to design adipose tissue constructs formed by the self-assembly of adipogenic cells and stem cells.⁸⁹ However, the MagLev setup used in that research presented challenges in obtaining stable 3D adipogenic structures. To address these issues, our current aim is to develop a biofabrication method that allows for the creation of stable and uniform adipose tissue models incorporating adipocytes, macrophages, and endothelial cells, each of which plays a crucial role in adipose tissue. In this study, we developed a novel MagLev platform by optimizing the magnitude and the gradient of magnetic fields and generated viable and stable mono-, co- and tri-cultured adipose models. We further showed the extended viability of tissue constructs in 2D and 3D, and compared the efficacy of the biofabrication platform to the standard liquid overlay technique. Our results suggest that the proposed biofabrication platform can generate low-cost, rapid, versatile and stable 3D adipose tissues to be used in tissue culture and drug screening studies.

3.2. Materials and Methods

3.2.1. Design of Magnetic Levitation Platforms

Two different configurations of magnetic levitation devices were used in the study. First design was the vertical magnetic levitation system, used to determine the density of each cell type. This design was constructed by vertically placing two N52-grade neodymium magnets (50 mm length, 5 mm height and 2 mm width, Supermagnete) 2 mm

apart with the same poles facing each other to create a magnetic field gradient, a square micro-capillary channel ($1 \times 1 \times 50$ mm, Vitrocom) as a reservoir between the magnets and two mirrors ($12.7 \times 12.7 \times 3.2$ mm, Thorlabs) placed at 45° for visualization of the levitated cells. The components of the vertical levitation device were held together with photoreactive resin printed using a 3D printer (Formlabs Form 2). The second design was the horizontal magnetic levitation system, where two magnets were positioned horizontally at a distance of 1.5 mm from each other. Unlike the vertical setup, this novel platform did not require any reflective mirrors to image cellular structures. The parts of the horizontal levitation platform were assembled by holders printed by a 3D printing system. The magnetic field gradient formed in the magnetic platforms was simulated by the finite element method.

3.2.2. Cell Culture

7F2 (osteoblast) cells, known for their capacity to differentiate into adipocytes, bEnd.3 (endothelial cells), and Raw 264.7 (macrophages) cell lines were selected for the study to represent different cellular components commonly involved in adipose tissue. 7F2 cells were cultured in Alpha Modified Essential Medium (α MEM, Serox), while bEnd.3 and Raw 264.7 cells were maintained in Dulbecco's Modified Eagle Medium with high glucose (DMEM high glucose, Gibco™). All media were supplemented with 10% fetal bovine serum (FBS, Gibco™) and 1% penicillin/streptomycin (P/S, Gibco™), and the cells were incubated under standard conditions in a humidified atmosphere containing 5% CO₂ at 37°C. To induce adipogenic differentiation of 7F2 cells, they were seeded in 6-well plates at a concentration of 20,000 cells/well. On the second day of culture, the standard growth medium was replaced with adipogenic induction medium consisting of 10 nM dexamethasone (Sigma), 50 μ M indomethacin (Sigma), and 5 μ g/mL insulin (Sigma). The cells were then cultured under these conditions for a period of 7 days to promote differentiation. The adipogenic medium was refreshed every 2–3 days.

3.2.3. Fluorescently Tagged Cell Lines

7F2, bEnd.3 and Raw 264.7 cells were labeled with red, green and blue fluorescent tags. To obtain stable fluorescent cells of 7F2, bEnd.3 and Raw 264.7, infection of the cells was conducted using Retrovirus or Lentivirus vectors expressing related fluorescent proteins followed by puromycin selection. After 72 h of infection, the antibiotic selection was performed with 2 $\mu\text{g/mL}$ puromycin (Sigma) for 7F2 and bEnd.3, and 6 $\mu\text{g/mL}$ for Raw 264.7 cells. Also, uninfected 7F2 cells were labeled with blue fluorescent tracker dye according to the manufacturer's instructions for tri-culture experiments (CellTracker™).

3.2.4. Magnetic Levitation in Vertical Device and Density Determination of Cells

For the determination of single-cell density of 7F2 (growth and adipogenic-differentiated cells), bEnd.3 and Raw 264.7 cells, each cell type was suspended at a concentration of 10^5 cells/mL. Then, the cells were levitated using 25 mM gadolinium (Gd, Gadovist®, Bayer) for 10 min to reach equilibrium and visualized with an inverted microscope (Olympus IX-83). The calibration curve of the system was obtained by levitation of polymeric beads with densities of 1.00, 1.02, 1.05, 1.07 and 1.09 g/mL (Cospheric LLC., ABD).

3.2.5. 3D Culture of Cells in Horizontal Magnetic Levitation System

3D cellular structures were created with different cell combinations to mimic adipose tissue models with the magnetic levitation system. To form 3D structures composed of 1) adipogenic cells, 2) adipogenic and endothelial cells, 3) adipogenic cells and macrophages, and 4) adipogenic cells, endothelial cells, and macrophages, the cells

were cultured with total cell numbers of 1000, 2500, and 5000. For co-culture and tri-culture studies the cells were mixed at ratios of 1:1, 1:1:1, and 10:10:1. Subsequently, 50 μ l of the prepared cell suspensions were loaded into capillaries and cultured in the horizontal magnetic levitation system for 24 hours using 50 mM Gd. The resulting 3D structures were visualized with a fluorescent (Olympus IX-83) or a confocal (Leica DMI8) microscope. 3D structures were also viewed as a time-lapse movie for 8 hours under the fluorescent microscope.

To demonstrate the continued viability of the cells after levitation, microcapillaries containing 3D cellular structures were removed from the magnetic levitation system and placed in 6 cm plates. After the cells settled to the bottom surface and initial attachment occurred, the medium was added, and the cells were cultured for 96 hours. Viability on the standard culture plates was also evaluated by transferring the 3D structures to 96-well plates.

3.2.6. Culture of the Cells on the Low-attachment Surface Following Magnetic Levitation

To test the sustainability of 3D structures and to conduct long-term culture of the biofabricated adipose tissue models, 3D cellular structures formed by the magnetic levitation system were transferred on agar gel and cultured for 5 days. Briefly, a 2% (W/V) agar solution (Sigma-Aldrich) was prepared by dissolving powder agar in $1\times$ PBS, and the prepared solution was autoclaved. To prevent solidification of the autoclaved agar solution, the solution was kept in a water bath or on a magnetic hotplate stirrer set at 43°C. To obtain the final agar concentration (1%), 2% agar was mixed with the medium, and 50 μ l of 1% agar was used to cover the 96-well plate surface. After solidification at room temperature, pre-formed 3D structures were transferred onto agar and 100 μ l of the medium was added into wells. The cellular structures were cultured in a 37°C incubator for 5 days.

3.2.7. Spheroid Formation by Liquid Overlay Technique

The cells grown in a culture dish were detached using 0.25% trypsin-EDTA solution (Gibco™). Appropriate cell suspension concentrations were prepared, and 100 µl of these suspensions were transferred to wells in a culture plate coated with 50 µl of 1% agar (Sigma-Aldrich), with a total of 5000 cells per well. The cells were cultured for 5 days and the formed spheroids were visualized with an inverted microscope (Olympus IX-83).

3.2.8. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using the Monarch® Total RNA MiniPrep Kit (New England Biolabs, T2010S) according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometry (Nanodrop 2000, Thermo Scientific), and samples with A260/A280 ratios between 1.8 and 2.0 were used for downstream analysis. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) with 1 µg of total RNA in a 20 µL reaction volume. Quantitative RT-PCR was conducted using RealQ Plus 2x Master Mix Green (Ampliqon, APQ-A323402). Cycling conditions were: initial denaturation at 95°C for 15 min, followed by 45 cycles of 95°C for 30 sec (denaturation), 60°C for 30 sec (annealing) and 72°C for 30 sec (extension). A melting curve analysis was performed at the end of the amplification program, consisting of 95 °C for 10 sec, 65 °C for 60 sec, and a final step at 72 °C for 1 sec to confirm the specificity of the amplified products. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to GAPDH as the housekeeping gene. All reactions were performed in triplicate.

Table 3.1. Sequences of forward and reverse primers of mouse genes used in qRT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
PPARγ	GCCTTGCTGTGGGGATGTC	TCCTTGGCCCTCTGAGATGAG
C/EBPα	TGGACAAGAACAGCAACGAGTAC	GCAGTTGCCCATGGCCTTGAC
Adiponectin	CTGGCCACTTTCTCCTCATTTTC	GGCATGACTGGGCAGGATTA
FABP4	TCACCTGGAAGACAGCTCCT	AATCCCCATTACGCTGATG
Adipsin	GCTATCCCAGAATGCCTCGTT	CCACTTCTTTGTCCTCGTATTGC
Resistin	CAACTCCCTGTTTCCAAATGC	CTCAAGACTGCTGTGCCTTCT
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
IL-6	GTTCTCTGGGAAATCGTGGA	TTCTGCAAGTGCATCATCGT
GAPDH	GACATGCCGCCTCGAGAAAC	AGCCCAGGATGCCCTTTAGT

3.2.9. Statistical Analyses

All experiments were performed at least in triplicate, and data on density and morphological parameters (area, thickness, circularity, and length) are presented as the mean and standard deviation (mean $\pm$ SD). Student's t-test (two-tailed) or one-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significance. Also, linear regression analysis was performed to assess the relationship between the cell seeding density and morphological parameters. The results are presented as regression coefficients with corresponding 95% confidence intervals. The goodness of fit was evaluated using the R-squared value. Statistical significance was determined using p-values, where $p < 0.05$ was considered significant.

3.3. Results

To reduce differences in cell spatial positioning based on single-cell density differences of MagLev culture, we designed a novel magnetic levitation platform for the 3D adipose culture technique. The novel design implemented horizontal deployment of magnets around the microcapillary channel (Figure 3.1A), in contrast to the vertical deployment design that was used in our previous studies.^{83,169} This adjustment induced a local minimum for the magnetic gradient at the center of the microcapillary (Figure 3.1B, Figure 3.2A), similar to the vertical magnet setup. However, as intended, this adjustment reduced the distance resolution of cells based on the changes in single-cell density, effectively decreasing the levitation height differences by ~50% between adipocytes and other cells (Figure 3.1C,D).

Before starting the biofabrication of cells with the horizontal MagLev system, we analyzed the density profile of the cells critical for adipose tissue model, including adipocytes, endothelial cells, and macrophages. For this purpose, we utilized the vertical MagLev setup with 25 mM Gd, which allowed us to detect subtle variations in single-cell density with enhanced resolution (Figure 3.1D).⁸³ Using levitation positions of polymeric beads with known densities (Figure 3.2B), single-cell levitation height values were translated to density (Figure 3.3). The average single-cell densities for undifferentiated 7F2 cells (growth, 7F2 G), adipogenic-differentiated 7F2 cells (adipogenic, 7F2 A), bEnd.3 cells and Raw 264.7 cells were determined as 1.062 ± 0.005 g/mL, 1.042 ± 0.018 g/mL, 1.067 ± 0.004 g/mL, and 1.074 ± 0.004 g/mL, respectively. These results indicated that there was a significant difference in single-cell densities, with Raw 264.7 displaying high single-cell density values while adipogenic cells had the largest variability (Table 3.2).

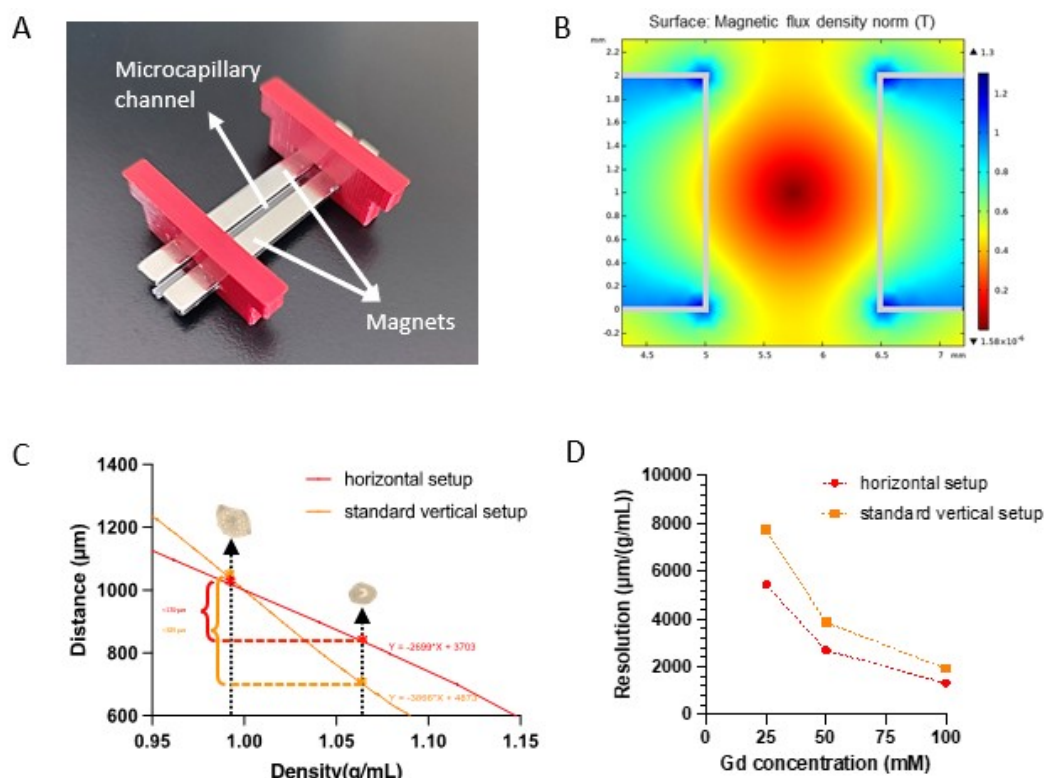


Figure 3.1. Horizontal magnetic levitation setup and theoretical foundation. A) Horizontal magnetic levitation device consisting of two neodymium magnets, fixed 1.5 mm apart with 3D-printed connecting components, with opposite poles facing each other, and a microcapillary channel (levitation chamber) placed between them. B) Simulation of magnetic flux density norm between two opposing magnets via Finite Element Methodology. C) Computationally modeled relationship between cell density and levitation heights in a 50 mM Gd^{3+} paramagnetic solution for horizontal and standard vertical magnetic levitation setups. The bottom surface of magnets is considered as $z = 0$. An adipocyte cell with lipid accumulation (approximately 0.99 g/mL density) and a quiescent 7F2 cell (approximately 1.06 g/mL density) are positioned closer along the z -axis in a horizontal magnetic levitation device compared to the standard configuration. D) Computational model of the density resolution for horizontal and standard vertical magnetic levitation setups as a function of Gd^{3+} concentrations.

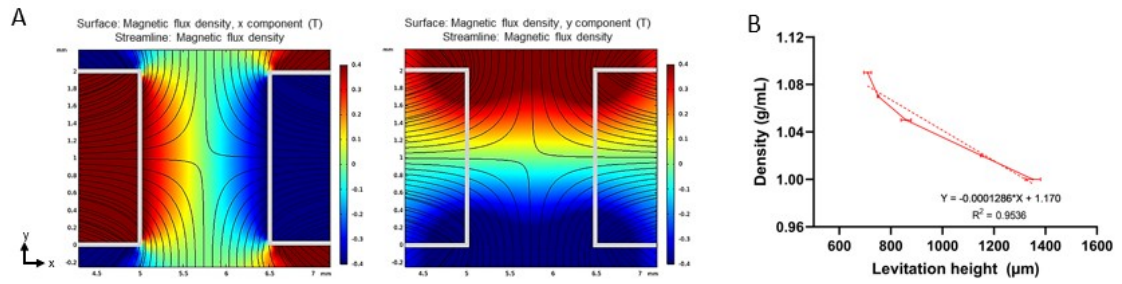


Figure 3.2. (A) Finite Element Methodology simulation of x component (B_x , left panel) and y component (B_y , right panel) of magnetic induction (B) between two opposing magnets. Streamlines on the images represent total magnetic induction ($B_x + B_y$). (B) Calibration curve of the vertical levitation system obtained by levitation of polymeric beads with known density using 25 mM Gd, to determine the single-cell density.

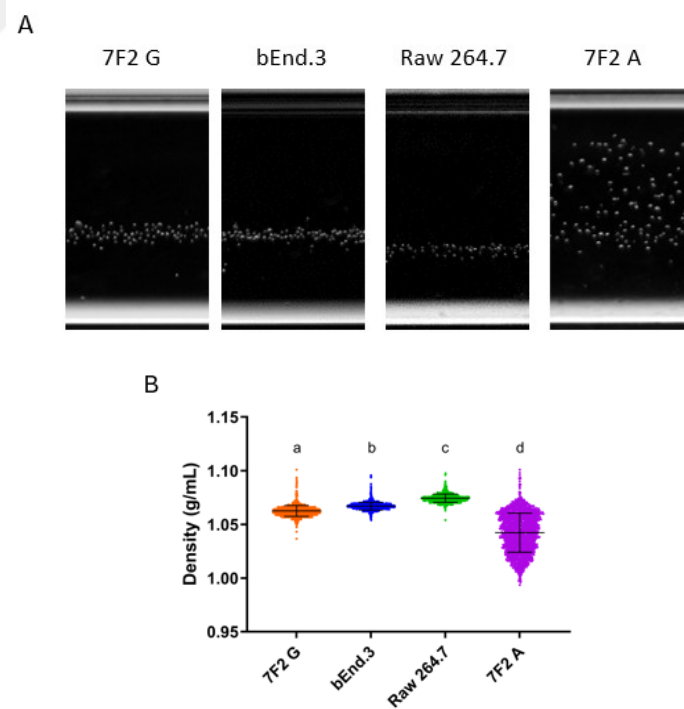


Figure 3.3. (A) Levitation images of growth and adipogenic-differentiated 7F2 cells, bEnd.3 and, Raw 264.7 cells and (B) single cell density of the cells levitated with 25 mM Gd. The letters (a, b, c, d) indicate different groups.

Table 3.2. Statistical measures for the density, including range, skewness, and coefficient of variance for each cell type.

CELL TYPE	RANGE	SKEW	COV(%)
7F2 Growth	0.064	1.308	0.48
bEnd.3	0.042	1.312	0.34
Raw 264.7	0.044	0.978	0.36
7F2 Adipogenic	0.107	-0.128	1.74

To evaluate the potential of the horizontal MagLev setup for biofabrication of adipose tissue models, we subsequently tested mono-, co-, and tri-culture of these cells in the horizontal MagLev platform using 50 mM Gd.^{89,207} Initially, we self-assembled growth and adipogenic 7F2 cells, bEnd.3 endothelial cells, and Raw 264.7 macrophages separately as mono levitation cultures on the setup for 24 hours. We examined the effect of the amount of cell loading on the formation of 3D structures using 1000, 2500, and 5000 cells per setup (Figure 3.4). The resulting 3D structures (Figure 3.4A,B) were assessed based on morphological indices, including area, thickness (area/length), circularity and length (Figure 3.5A, Table 3.2). Results indicated that the area of the 3D levitated cells at the lowest seeding cell density exhibited $0.026 \pm 0.006 \text{ mm}^2$ for 7F2 G; $0.018 \pm 0.011 \text{ mm}^2$ for bEnd.3 cells; $0.024 \pm 0.010 \text{ mm}^2$ for Raw 264.7 cells; and $0.086 \pm 0.039 \text{ mm}^2$ for 7F2 A. When the total number of cells in the capillary was increased to 2500, adipogenic constructs exhibited the largest area, measuring $0.186 \pm 0.071 \text{ mm}^2$, which was 131% ($p=0.003$), 231% ($p=0.0003$) and 263% ($p<0.0001$) greater than that of 7F2 G, bEnd.3, and Raw 264.7, respectively. This trend persisted at the highest cell loading (5000) as well. Linear regression analysis suggested a robust relationship between construct area and initial cell number for the MagLev platform as observed by R^2 values between 0.6 and 0.7 for all cell types (Figure 3.5B). In addition, all cell types exhibited similar increasing trends in construct area, except adipogenic cells which demonstrated a significantly higher rate of increase ($p<0.01$) compared to the other cell types. The influence of initial cell number and cell type on construct thickness was comparatively smaller than other morphological parameters. The thickness of the 3D levitated cells at the lowest cell loading was $0.089 \pm 0.016 \text{ mm}$ for 7F2 G, $0.073 \pm 0.014 \text{ mm}$ for bEnd.3,

0.077 $\pm$ 0.014 mm for Raw 264.7, and 0.092 $\pm$ 0.013 mm for 7F2 A on average. A slightly increasing trend in thickness of 7F2 G ($R^2=0.63$) and bEnd.3 ($R^2=0.63$) cells was observed with the increase in the amount of cell loading, while the thickness for adipogenic and Raw 264.7 constructs lacked trends. Notably, a significant difference in the thickness of Raw 264.7 cells (0.091 $\pm$ 0.019 mm, $p=0.001$) and 7F2 A cells (0.089 $\pm$ 0.015 mm, $p=0.002$) was observed compared to 7F2 G cells (0.128 $\pm$ 0.011 mm) only at 5000 cell loading. Except for the 7F2 G cells, a negative trend for circularity was observed, indicating that the structures increased elongation with higher cell loading. At the highest loading numbers, the circularity averaged 0.219 $\pm$ 0.054 for 7F2 G, 0.224 $\pm$ 0.079 for bEnd.3, 0.119 $\pm$ 0.040 for Raw 264.7, and 0.034 $\pm$ 0.010 for 7F2 A. Apart from 7F2 G and bEnd.3, all cell types exhibited significant differences from one another. Additionally, the length, another morphological parameter evaluated for the 3D structures, revealed a trend consistent with that observed in the area (Figure 3.6). At all seeding densities, the length of adipogenic cells was more than 150% greater compared to that of all other cell types. The results indicated that the increase in area is attributed to an increase in length (y-axis) rather than thickness (x-axis).

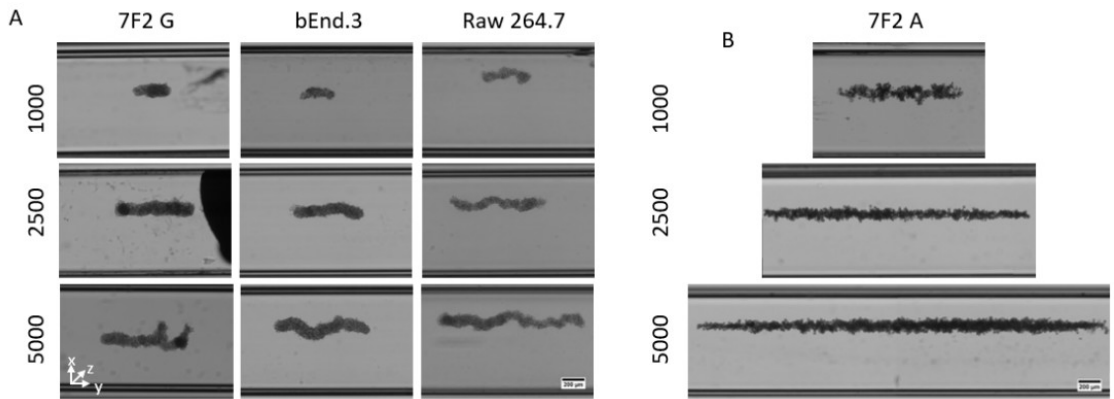


Figure 3.4. Monoculture of (A) undifferentiated 7F2 cells (7F2 G), bEnd.3 endothelial cells, and Raw 264.7 macrophages, and (B) adipogenic-differentiated 7F2 (7F2 A) cells in a horizontal magnetic levitation system for 24 hours using 50 mM Gd—scale bar: 200 μ m.

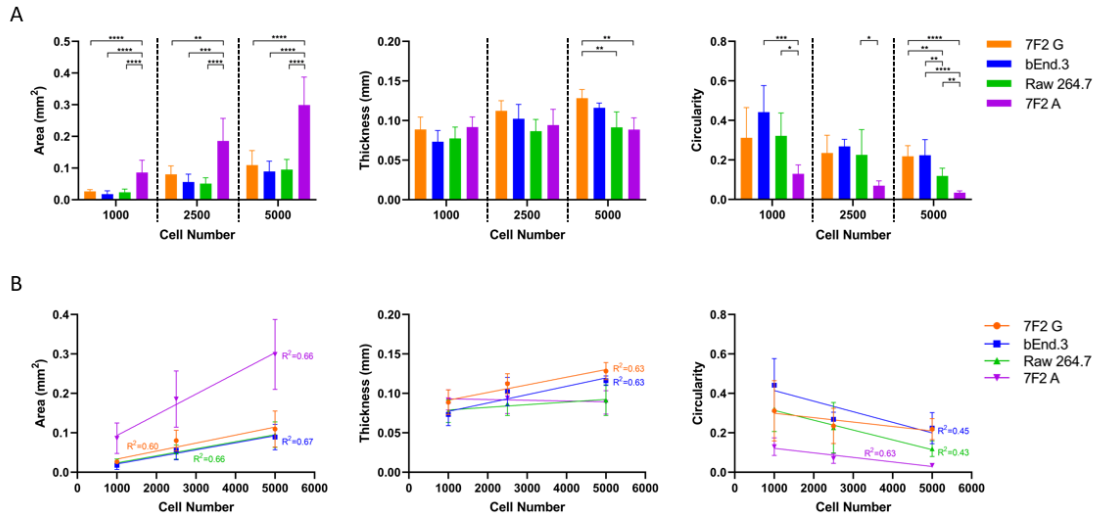


Figure 3.5. (A) Morphological analysis of 3D structures formed by initial seeding densities of 1000, 2500, and 5000 cells, evaluated for area, thickness and circularity. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Comparisons are shown only between related seeding densities for each cell type and not across different cell densities. (B) Linear regression analysis depicting the relationship between cell number and morphological parameters (area, thickness, and circularity). Different cell groups are represented by distinct colored lines, with corresponding R^2 values indicating significant goodness of fit for each group. Data are represented as mean $\pm$ SD.

Then, we proceeded to co-culture cells growth (Figure 3.7A) or adipogenic 7F2 (Figure 3.7B) with either bEnd.3 or Raw 264.7 for 24 hours. Resulting structures indicated that bEnd.3 cells distributed homogeneously in the growth 7F2 group but formed multiple clusters in adipogenic co-culture (Figure 3.7B, yellow arrows). In contrast, although Raw 264.7 cells were uniformly mixed within the structure in both groups, a distinct layer formed on the outer surface of 3D growth structures (Figure 3.7A, white arrows). Despite these observations, adding bEnd.3 or Raw 264.7 cells to growth or adipogenic 7F2 cells similarly affected the entire 3D structures with respect to morphological parameters. The primary difference was observed in the area ($p=0.041$), circularity ($p=0.013$), and length ($p=0.020$) between 7F2 G + bEnd.3 and 7F2 G + Raw

264.7 at 5000 cells, as well as in length ($p=0.030$) between 7F2 A + bEnd.3 and 7F2 A + Raw 264.7 (Figure 3.6B and 3.8A). When 7F2 A cells were co-cultured with bEnd.3 cells, there was a 27% increase in the area and significant decreases of 27% ($p<0.0001$) in thickness and 68% ($p<0.0001$) in circularity for the 5000-cell groups compared to growth cells. The differences were even more pronounced with Raw 264.7 cells, showing a 125% increase ($p<0.0001$) in area and reductions of 31% ($p<0.0001$) in thickness and 80% ($p<0.0001$) in circularity. Compared to monoculture conditions, co-culture induced changes in area, thickness and circularity indicated a potential for forming more compact structures, supported by a significant correlation between structure area and initial cell number for each group, with R^2 values of 0.53 for 7F2 G + bEnd.3, 0.85 for 7F2 A + bEnd.3, 0.61 for 7F2 G + Raw 264.7, and 0.87 for 7F2 A + Raw 264.7 (Figure. 3.8B).

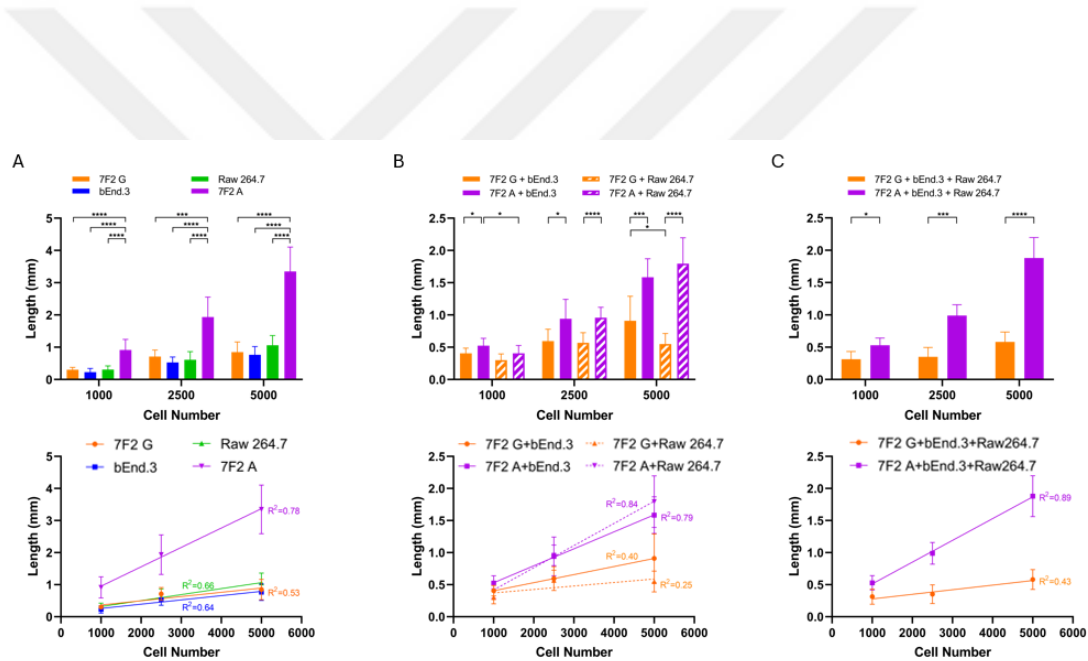


Figure 3.6. Analysis of the length of 3D structures formed by (A) mono-culture of growth and adipogenic-differentiated 7F2 cells, bEnd.3 endothelial cells and Raw 264.7 macrophages, (B) co-culture of growth and adipogenic-differentiated 7F2 cells with bEnd.3 and Raw 264.7 cells, and (C) tri-culture of growth and adipogenic-differentiated 7F2 cells with bEnd.3 and Raw 264.7 cells. Data are represented as mean $\pm$ SD. Comparisons are shown only between related seeding densities for each cell type and not across different cell densities.

Interestingly, while there was no correlation between thickness and cell number in the adipogenic mono-culture group (Figure 3.5B), the R^2 values for 7F2 A + bEnd.3 ($R^2 = 0.27$) and 7F2 A + Raw 264.7 ($R^2 = 0.37$) groups were significantly higher (Figure 3.8B). Additionally, unlike growth groups, both adipogenic co-cultured structures exhibited a strong negative correlation between initial cell number and circularity.

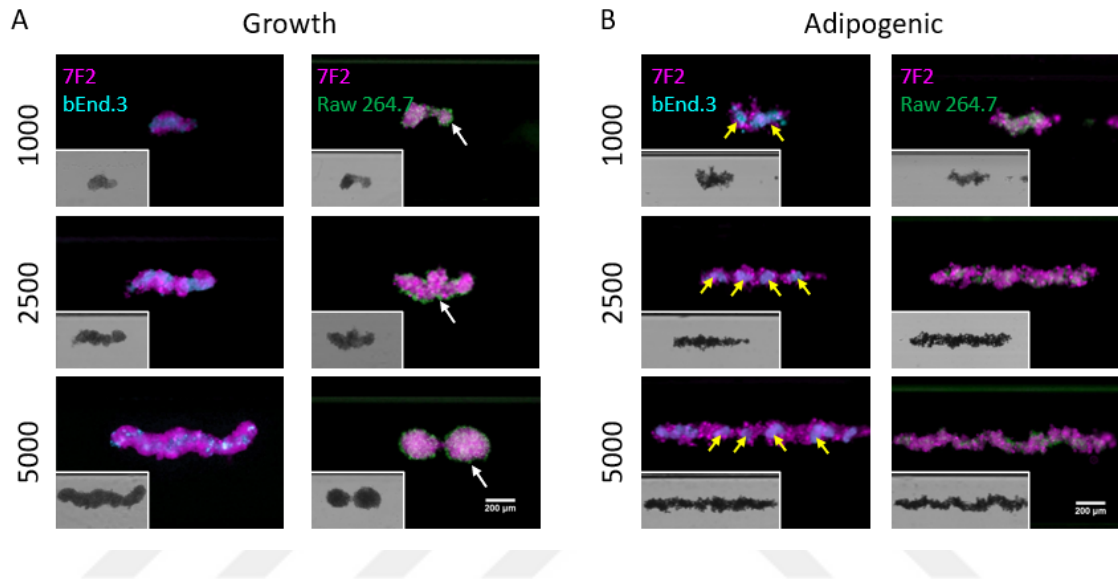


Figure 3.7. Co-culture of (A) undifferentiated 7F2 cells (7F2 G, magenta) and (B) adipogenic-differentiated 7F2 cells (7F2 A, magenta) with bEnd.3 (cyan) or Raw 264.7 (green) cells mixed in equal proportions in a horizontal magnetic levitation system for 24 hours using 50 mM Gd—scale bar: 200 μ m. White arrows indicate Raw 264.7 cells located at the outer surface of 3D structures, while, yellow arrows highlight clusters formed by bEnd.3 cells in adipogenic structures.

When three cell types were loaded together as either 7FG + bEnd.3 + Raw 264.7 controls or 7FA + bEnd.3 + Raw 264.7 adipogenic composition to novel MagLev culture setup, 3D constructs were observed to be similar in morphology to both mono- and co-culture systems at 24 hours (Figure 3.9). Using time-lapse imaging, we observed that Raw 264.7 cells were positioned peripherally in x-axis in the growth group for up to 2 hours, but their distribution along the axis became more homogeneous over time (Figure 3.10),

revealed at 24 hours that they populated the bottom surface (z-axis) of the structure due to their higher single-cell density. In the adipogenic groups, all cells exhibited a homogeneous distribution after 8 hours; notably, the formation of multiple clusters by bEnd.3 cells within the adipogenic structure was not observed during this period and in the end, formed structure appeared looser compared to the growth group.

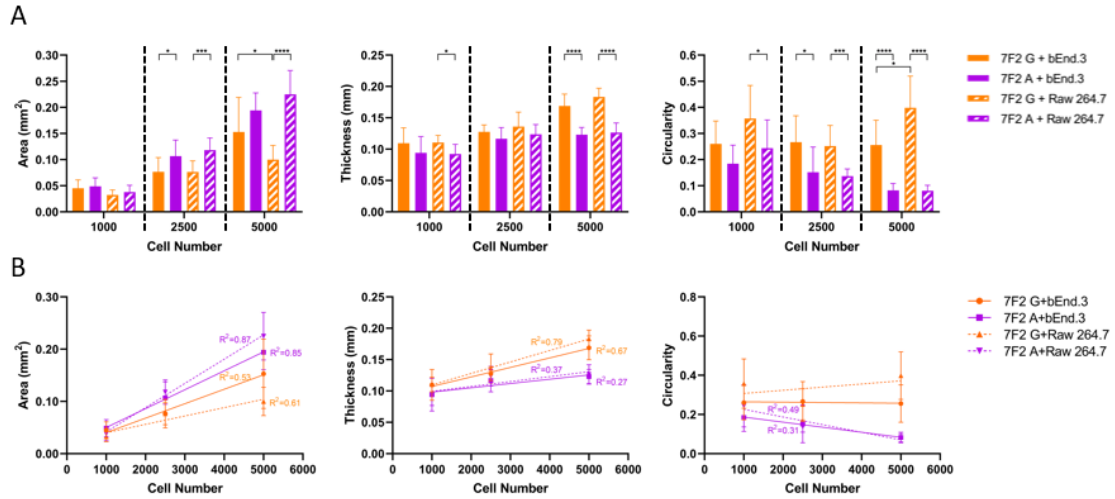


Figure 3.8. (A) Morphological analysis of 3D structures formed by initial seeding densities of 1000, 2500, and 5000 cells, evaluated for area, thickness, and circularity. Solid columns represent co-culture groups with bEnd.3 cells, whereas striped columns indicate co-culture with Raw 264.7 cells. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Comparisons are shown only between related seeding densities for each cell type and not across different cell densities. (B) Linear regression analysis depicting the relationship between cell number and morphological parameters (area, thickness, and circularity) for co-cultured cells. In all graphs, purple indicates adipogenic groups, while orange denotes growth groups cultured with bEnd.3 (solid lines) and Raw 264.7 (dashed lines). R² values indicate significant goodness of fit for each group. Data are represented as mean and $\pm$ SD.

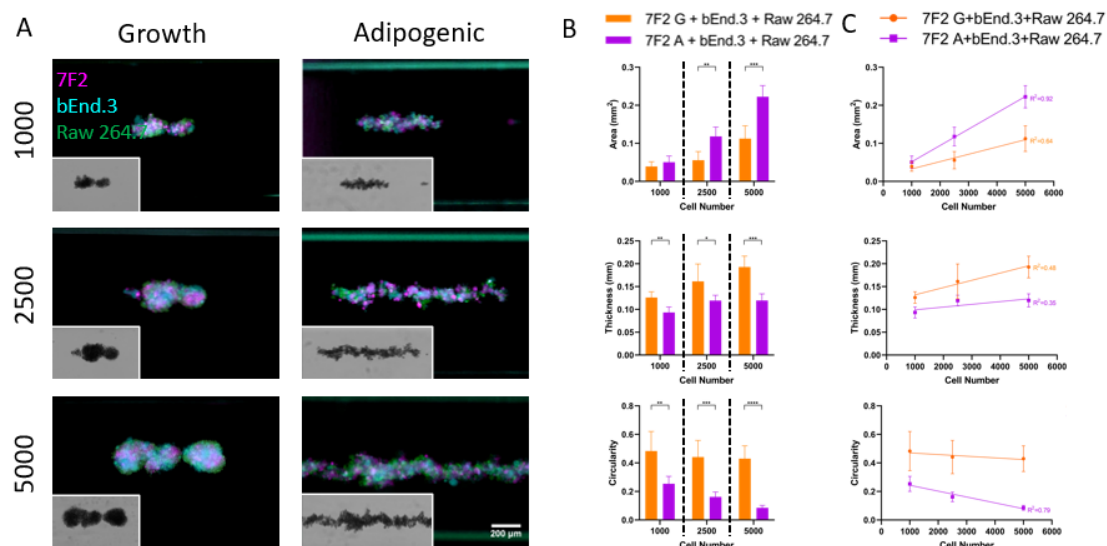


Figure 3.9. (A) Tri-culture of bEnd.3 (cyan) and Raw 264.7 (green) cells with undifferentiated 7F2 cells (7F2 G, magenta) and adipogenic-differentiated 7F2 cells (7F2 A, magenta) mixed in equal proportions in a horizontal magnetic levitation system for 24 hours using 50 mM Gd—scale bar: 200 μ m. (B) Morphological analysis of 3D structures formed by initial seeding densities of 1000, 2500, and 5000 cells mixed in equal proportions. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Comparisons are shown only between related seeding densities for each cell type and not across different cell densities. (C) Linear regression analysis depicting the relationship between cell number and morphological parameters (area, thickness, and circularity) for tri-cultured cells. R^2 values indicate significant goodness of fit for each group. Data are represented as mean $\pm$ SD. In all graphs, purple indicates adipogenic groups, while orange denotes growth groups.

Morphological analysis indicated that the adipogenic tri-culture composition had 113% ($p=0.003$), and 98% ($p=0.0006$) increase in area for 2500 and 5000 cell loading with no significant change at 1000 cells; 26% ($p=0.007$), 26% ($p=0.046$) and 38% ($p=0.0003$) decrease in thickness for 1000, 2500 and 5000 cell loading; and 48% ($p=0.007$), 63% ($p=0.001$) and 80% ($p<0.0001$) decrease in circularity compared to

growth groups (Figure 3.9B). Similar to mono- and co-culture results, cell loading was positively correlated to the area ($R^2=0.64$ for growth, $R^2=0.92$ for adipogenic), thickness ($R^2=0.48$ for growth, $R^2=0.35$ for adipogenic) and length ($R^2=0.89$ for growth, $R^2=0.43$ for adipogenic) but negatively correlated to circularity ($R^2=0.79$) only for adipogenic structures (Figure 3.6 and 3.9C).

To assess the sustained viability of 3D adipogenic constructs fabricated for 24 hours using the novel MagLev platform, we first ceased levitation by removing the microcapillary from the magnetic field and either allowed the settling of the cells on the glass bottom (Figure 3.11) or transferred 3D structures to the culture plates (Figure 3.12). Due to the excessive proliferation and dominance of Raw 264.7 on microcapillary (Figure 3.11A), we diluted Raw 264.7 to reach a 10:10:1 ratio of 7F2 A, bEnd.3, and Raw 264.7 cells (Figure 3.11B). For all conditions, the adhesion and spreading across the 2D surface of cells after levitation culture served as indicators of culture health. The majority of the cell population was comprised of bEnd.3 and Raw 264.7 cells, while adipocytes constituted a minor fraction of the overall population after 3 days.

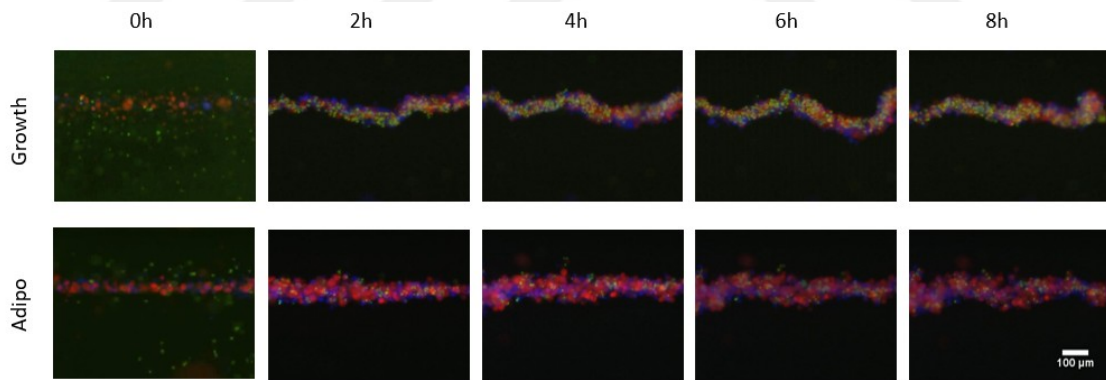


Figure 3.10. Time-lapse images of tri-cultured cells for 8h. Red: 7F2 G or 7F2 A, green: Raw 264.7, blue: bEnd.3. Scale bar: 100 μm .

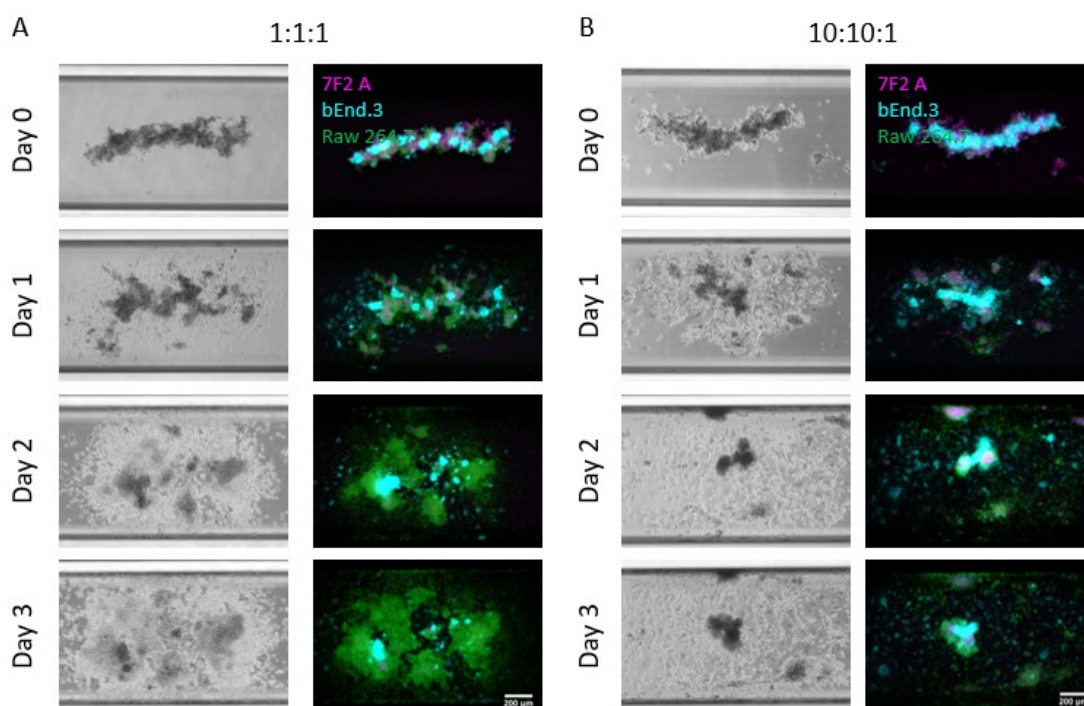


Figure 3.11. Culture of the pre-formed 3D structure of tri-cultured cells mixed in (A) equal proportions and (B) a 10-fold decreased ratio of Raw 264.7 cells on microcapillary surface for 3 days—scale bar: 200 μm . Magenta: 7F2 A, cyan: bEnd.3, and green: Raw 264.7.

After observing MagLev biofabricated 3D structures on 2D glass surfaces, we tested their sustained long-term 3D culture by transferring them onto agar using liquid overlay technique (LOT). We compared the culture of MagLev-assisted liquid overlay (ML+LOT), to 3D formation of single cells on simple liquid overlay (LOT). First, we tried monoculture adipogenic 7F2 cells. Due to the inherent properties of adipogenic cells, characterized by the formation of loose and fragile 3D structures, LOT required 5 days of culture for 3D adipogenic structures to form, yet they still appeared fragmented at the end of the protocol (Figure 3.13A). On the other hand, the ML+LOT condition induced a unified composition from day 1 and gradually continued forming a more compact structure. In wells employing LOT, the number of spheroids exceeded 180 on average, whereas with ML+ LOT, the number remained below 10 at day 1. By the end of the 5 days, the average number per well was 47 for LOT and 7 for ML+LOT (Figure 3.13B).

Notably, upon excluding dispersed single cells (Figure 3.13A, red arrows) in ML+LOT, the system facilitated the formation of a single, uniform 3D structure.

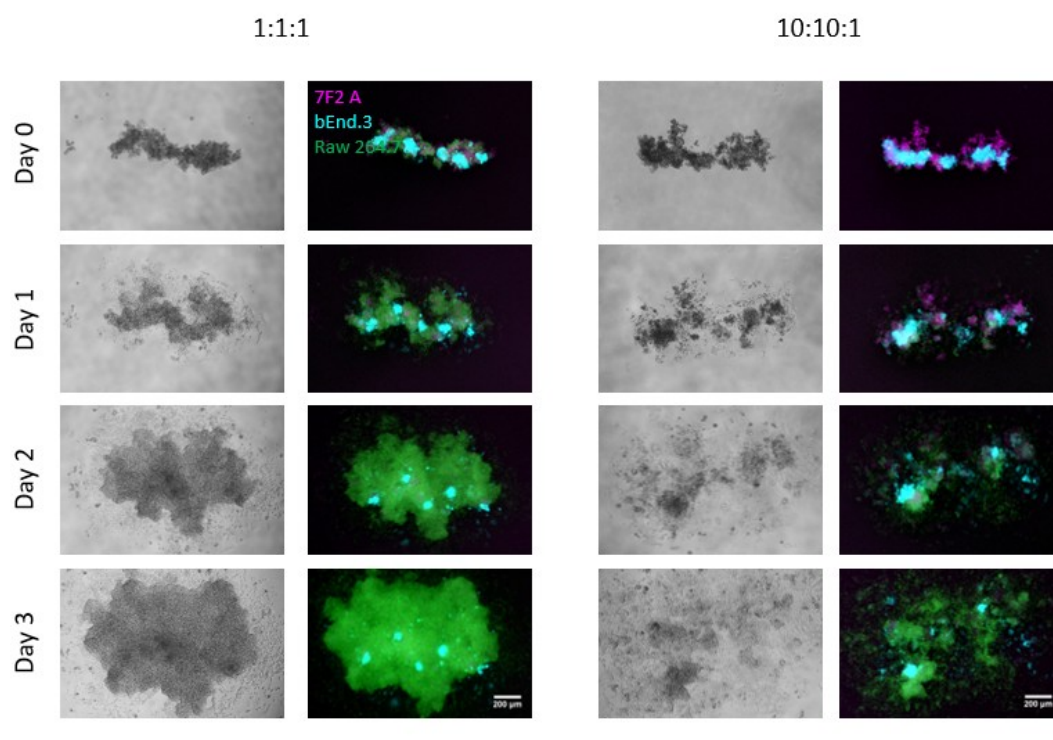


Figure 3.12. Culture of pre-formed 3D adipogenic structures on well-plates for 3 days. 3D structures are formed by adipogenic cells, bEnd.3 cells and Raw 264.7 cells mixed by the ratio of 1:1:1 (left panel) and 10:10:1 (right panel). Scale bar: 200 μ m.

When we extended the experiments with tri-culture conditions (Figure 3.14), LOT failed to aggregate cells, yielding an average of 32 clusters per well. However, with the assistance of MagLev, the ML+LOT group achieved the formation and sustained single spheroid-like structures. Thus, we have demonstrated that the developed technique is suitable for both mono-cultured and tri-cultured adipogenic cells, facilitating the creation of diverse adipose tissue models.

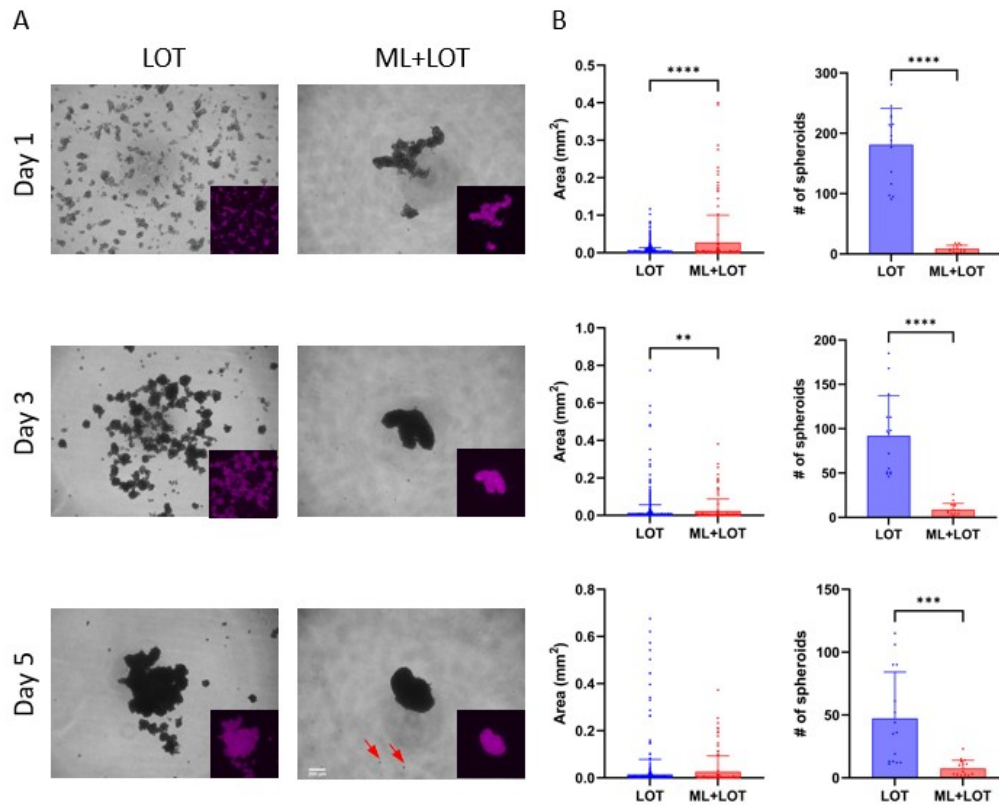


Figure 3.13. (A) Long-term culture of the adipogenic cells using the liquid overlay technique (LOT) or pre-formed 3D structure of adipogenic cells created by the magnetic levitation system (ML+LOT) on agar gel—scale bar: 200 μm . Red arrows indicate excluded cells dispersed on the agar gel (B) Area and number of 3D structures formed in culture wells at day 1, 3, and 5. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The expression levels of key adipogenic marker genes were also assessed to evaluate the adipogenic phenotype of 3D adipogenic structures produced by combined ML+LOT approach (Figure 3.15). As expected, all markers, including early regulators Cebpa and Pparg, as well as late markers Fabp4, Adipoq, Adipsin, and Resistin, were significantly upregulated in adipogenic cultures compared to undifferentiated 2D controls (2D G), with the exception of Cebpa in 3D culture. Notably, the 3D adipogenic cultures (3D A) displayed a distinctive expression profile: while Cebpa, Pparg and Adipoq were expressed at lower levels compared to 2D adipogenic culture (2D A) ($p < 0.0001$), the expression of Fabp4, Adipsin, and Resistin remained substantially elevated. In particular,

Fabp4 expression reached nearly 110-fold higher than in 2D G and was approximately six times higher than in 2D A ($p < 0.0001$). These findings suggest that under 3D conditions, there is a shift toward a more mature adipocyte phenotype for specific functional markers, despite reduced expression of early transcription factors.

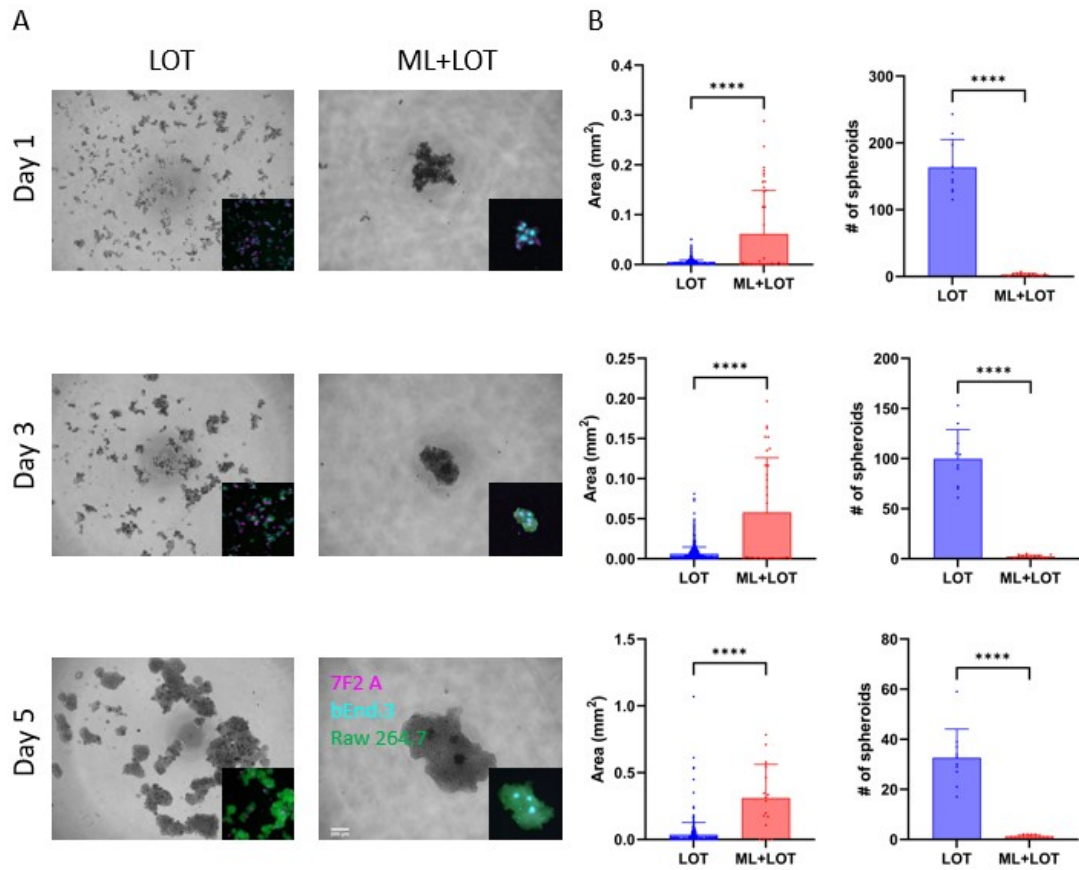


Figure 3.14. (A) Long-term culture of adipogenic-differentiated cells (magenta), endothelial cells (cyan) and macrophages (green) mixed with the ratio of 10:10:1 using the liquid overlay technique (LOT) or pre-formed 3D structures of them created by the magnetic levitation system (ML+LOT) on agar gel—scale bar: 200 μm . (B) Area and number of 3D structures formed in culture wells at day 1, 3, and 5. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The effects of adipocyte-conditioned media derived from 2D (2D A CM) and 3D (3D A CM) cultures on the inflammatory gene expression profile of Raw 264.7 macrophages were assessed and compared to both untreated controls and LPS-stimulated positive controls (Figure 3.16). As expected, TNF α , IL-6, and IL-10 mRNA levels were significantly elevated in LPS-treated macrophages ($p < 0.0001$ for all), confirming the robust inflammatory activation. Interestingly, macrophages cultured with 3D A CM exhibited a pronounced upregulation of IL-6 expression ($\sim 16,000$ -fold increase relative to control, $p=0.003$), which was significantly higher than that observed in the 2D A CM group. On the other hand, TNF α expression remained comparable to control levels in macrophages treated with 3D condition medium, while IL-10 expression was undetectable in cells exposed to 3D A CM. Meanwhile, treatment with 2D A CM did not induce substantial expression changes in any of the measured cytokines relative to the untreated control.

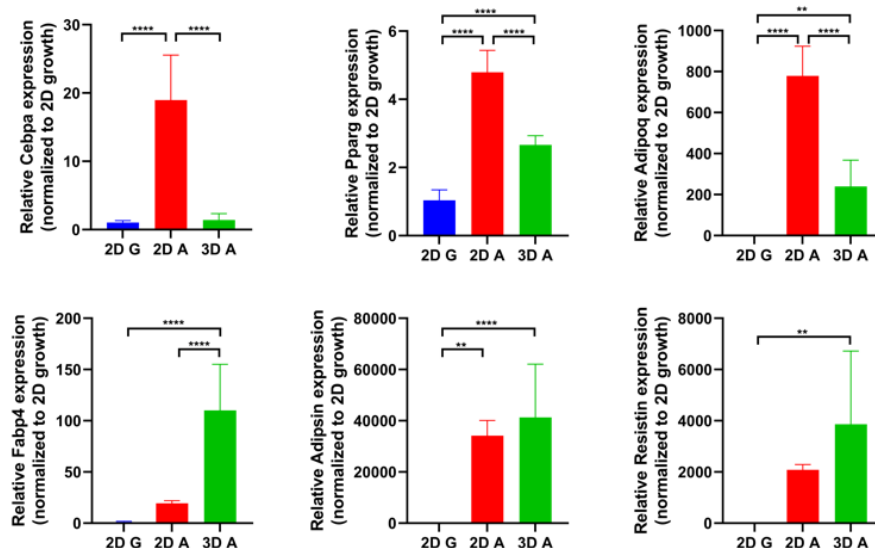


Figure 3.15. Adipogenic gene expression profiles in 2D and 3D cultures. Relative mRNA expression levels of key adipogenic markers, Cebpa, Pparg, Fabp4, Adipoq, Adipsin, and Resistin, were analyzed by qRT-PCR. Analysis of mRNA expression levels of adipogenic marker genes. Data are represented as mean $\pm$ SD. Statistical significance is by one-way ANOVA with post-hoc analysis (** $p < 0.01$, **** $p < 0.0001$).

3.4. Discussion

In this study, we evaluated the capacity of the MagLev system to form adipose tissue constructs including special cell types native to the adipose tissue. Specifically, we cultured adipogenic differentiated cells with endothelial cells and/or macrophages, which play crucial roles in the functions of adipose tissue and obesity. In the MagLev system, adipogenic groups formed thinner, string-like clusters compared to the growth control. To maintain the stability of our 3D cultures by promoting the formation of more robust structures and to enable long-term culture, we employed the liquid overlay technique following magnetic levitation. This approach was exclusively compared with the standalone application of the liquid overlay technique. Thus, we observed that the proposed method demonstrates its potential as an effective approach for the 3D culture of mature adipocytes with other resident cells in adipose tissue, enabling stable 3D cellular structures within a relatively short culture period, whereas traditional culture systems were not fully successful in achieving 3D adipose structure from mature adipocytes.

Previous to this study, we attempted the levitation culture of adipogenic cells, both alone and in combination with mesenchymal stem cells, utilizing a vertical magnetic levitation system.⁸⁹ Our observations revealed the emergence of 3D adipogenic structures exhibiting loose and branched morphologies, ascribed to the heterogeneous cellular density resulting from variable amounts of lipid accumulation.⁸⁹ Although higher gadolinium concentrations can yield tighter structures, it can reduce cell viability and lead to adhesion on the upper surface of the microcapillary channel because of the excessive levitation height.⁸⁶ In this study, we modified the magnet configuration to manipulate the magnetic gradient and resolution in the system, focusing cells with varying densities into a more compact space compared to our previous study.⁸⁹ Despite these improvements, adipogenic 3D structures in levitation monoculture still exhibited vulnerability to mechanical disturbances, potentially based on their weak adhesive properties.¹⁷⁰

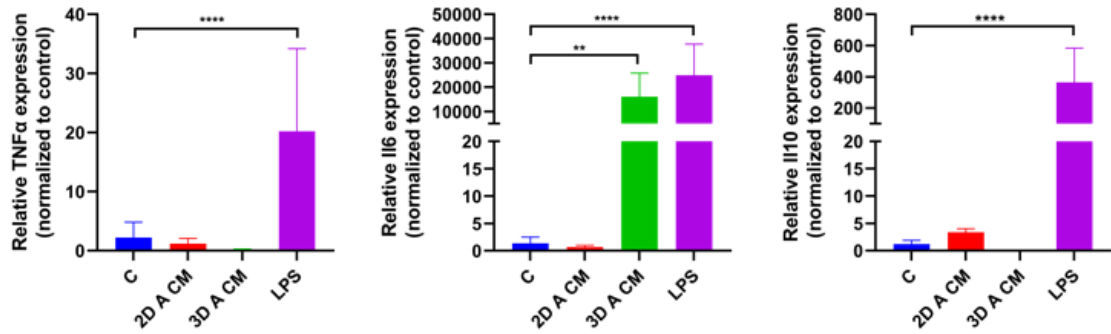


Figure 3.1. Effects of adipocyte-conditioned media on inflammatory gene expression in macrophages. Relative expression levels of TNF α , IL-6, and IL-10 mRNA in Raw 264.7 macrophages following 24-hour incubation with conditioned media from 2D (2D A CM) and 3D (3D A CM) adipogenic cultures, compared to untreated controls (C) and LPS-stimulated (500 ng/mL) positive controls. Data are represented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA (** $p < 0.01$, **** $p < 0.0001$).

In addition, we noted distinct phenomena in co-culture setups involving additional cell types: macrophages were uniformly dispersed in a 3D adipogenic structure, while endothelial cells displayed discrete multiple clusters within adipose constructs formed during levitation. This phenomenon likely contributed to the increased circularity and thickness of adipogenic clusters, indicative of the supportive role played by other cell types in promoting tighter structural integrity. The underlying mechanisms governing cellular self-assembly into spheroidal configurations involve intricate interactions between cells and the extracellular matrix (ECM), mediated by cell adhesion molecules such as integrins and cadherins.²⁰⁸ Although the literature concerning adipocyte-specific intercellular connections is sparse, previous studies have suggested a downregulation of cell surface adhesion proteins during adipogenic differentiation, potentially impeding the formation of cohesive 3D adipogenic clusters.^{170,209-211} However, our study revealed that co-culturing with additional cell types appears to enhance the suitability of adipogenic cells for scaffold-free culture techniques. Our observations also revealed the differential distribution of cells within the culture system. Macrophages, which have the highest density and smallest cell size compared to other cell types, likely contributed to the formation of the 3D structure later due to the longer levitation time required for smaller

objects to reach equilibrium in the MagLev system, as well as their positioning at lower levels in the microcapillary channel.²¹² Confocal imaging revealed that macrophages were predominantly localized at the base of growth clusters, which exhibited a more concave morphology, while they were more uniformly distributed in adipogenic clusters, which formed less cohesive structures compared to growth controls. The homogeneous distribution of macrophages, which have a higher density compared to other cells, within the adipogenic structure suggests that modifications to the MagLev system mitigate the formation of distinct layers based on density differences. In the growth group, the external displacement of macrophages may be attributed to strong intercellular connections formed by larger cells, which rapidly reach equilibrium and prevent macrophages from infiltrating the inner regions. These findings suggest that macrophages play a crucial role in shaping the morphology of growth clusters. In contrast, in the adipogenic group, adipogenic cells were primarily responsible for shaping the 3D structure.

In this study, we evaluated the effect of cell type and number on the 3D adipogenic structures formed by MagLev. Adipogenic cells with larger sizes predominated the overall dimensions of the 3D structure. However, results suggested that the thickness of the structure was less influenced by the number of cells, with a tendency for the cells to form elongated structures rather than increasing the aspect ratio of the structure. Consequently, we observed that adequate control over the thickness could not be achieved using the magnetic levitation system, which operates on the principle of escaping the lowest magnetic field region. The addition of other cell types resulted in a reduction in the area and length of adipogenic structures, while a slight increase was observed in their thickness and circularity. Although the rate of area reduction in 3D structures decreased as the number of cells increased, the highest increase rate of thickness and circularity was observed under conditions with the greatest total cell number. This suggests that as the number of non-adipogenic cells increases, these cells might facilitate a more compact formation of adipogenic structures through enhanced cell-cell and cell-ECM interactions.

Our findings demonstrated that while LOT alone was moderately effective for generating compact spheroids from mature adipocytes in monoculture, it was considerably less efficient compared to ML+LOT. Adipocytes cultured under LOT conditions often formed loosely aggregated or irregularly shaped structures, likely due to their low intrinsic adhesive capacity and buoyant phenotype, which limits spontaneous self-assembly on non-adhesive surfaces. In contrast, ML+LOT significantly improved spheroid cohesion and compaction, facilitating the formation of structurally stable

adipocyte aggregates by providing magnetic confinement and levitation-based spatial orientation. Interestingly, in triculture experiments involving adipocytes, endothelial cells, and macrophages, the limitations of LOT became more pronounced. The addition of heterogeneous cell types with distinct adhesive and migratory properties disrupted the already weak aggregation dynamics observed in monoculture LOT conditions, leading to poorly organized or dispersed cellular assemblies. This impaired aggregation may result from a combination of biophysical and biochemical incompatibilities between the cell types, including cadherin isoform mismatch, differences in cell buoyancy, uncoordinated ECM remodeling, and suboptimal medium conditions. Collectively, these factors hinder the formation of a cohesive and compact spheroid architecture. These observations suggest that, in the absence of additional physical forces, LOT alone may be inadequate to drive efficient aggregation and structural integration in complex multicellular systems.

Assessing the cell viability proved challenging due to the tri-culture system, where the different colors for tracking cells impeded live-dead staining techniques. Additionally, conventional viability and proliferation assays, such as MTT or LDH assays, which provide aggregate data on culture health, do not offer specific information on individual cell types within a tri-culture context.²¹³ Consequently, we assessed cell viability by evaluating culture adhesion, surface spreading, and cell number increase. Furthermore, the literature indicates that signals from fluorescent proteins produced by stably transfected cells can serve as indicators of cell viability and membrane integrity.^{214,215} Although adipogenic cells presented challenges in observation due to their lower proliferation activity and the tendency to become buoyant during maturity, it is evident that endothelial cells and macrophages maintained their viability based on these parameters. However, additional functional tests are necessary to confirm that the tri-culture system does not adversely affect cell viability and proliferation.

Adipose tissue engineering shows great potential for applications in regenerative medicine, cosmetic surgery, and disease modeling. However, conventional culture systems often fall short in providing the necessary cues for effective adipocyte differentiation, vascularization, and tissue integration.²¹⁶ Novel approaches are needed to replicate the complexity and functionality of native adipose tissue. Biofabrication techniques, such as 3D bioprinting and microfluidics, present potential solutions for generating intricate adipose tissue constructs with spatial organization and functional vasculature. However, operational conditions and shear stress associated with these methods can alter the potential for adipogenic differentiation and affect the expression of

adipocyte-specific markers.^{217,218} Furthermore, the buoyancy force due to the low density of adipocytes can lead to weak anchorage to scaffolds, leading to the cells floating after undergoing adipogenic differentiation.⁶⁰ For mature adipocytes, larger pore sizes in scaffolds are typically preferred, but this can restrict cell-cell interactions with other cell types in co-culture studies, potentially influencing cell signaling, paracrine communication, and tissue development. This study aims to introduce a technique for the direct culture of mature adipocytes, which differ significantly from adipogenic-differentiated stem cells or preadipocytes in morphology, metabolic activity, gene expression, and functional attributes.^{219,220} In addition, the application of adipogenic inducers after spheroid formation from precursor cells can negatively impact co-cultured cells, such as restriction of tube formation by endothelial cells.^{58,221} Furthermore, the heterogeneity of fat depots underscores the importance of considering the retention of depot-specific characteristics *in vitro*; which is a critical requirement for studying metabolism and developing targeted therapies for obesity and metabolic disorders.^{222,223} While 3D culture models better preserve depot-specific traits than conventional monolayer systems, handling mature adipocytes remains challenging due to their inherent fragility and buoyancy.^{224,225} Although some studies have utilized mature adipocytes, to our knowledge, there is a notable absence of applications using scaffold-free techniques.

Beyond the biofabrication aspects, we observed distinct transcriptional differences of adipocytes cultured in 3D compared to 2D culture. In 2D differentiated cultures, early adipogenic transcription factors C/EBP α and PPAR γ were highly expressed, suggesting adipogenic commitment under these conditions. In contrast, a pronounced reduction of C/EBP α and PPAR γ in the 3D aggregates may indicate a lower transcriptional activity of classical adipogenic regulators, consistent with the quiescent state seen in terminally differentiated adipocytes. Meanwhile, the elevated expression of Fabp4, Adipsin, and Resistin in 3D cultures may indicate advanced functional maturation and a shift toward a specialized endocrine phenotype. In addition, conditioned media collected from 3D adipogenic cultures exerted a distinctly different influence on macrophage inflammatory gene expression compared to that derived from 2D systems. Macrophages exposed to 3D adipocyte-conditioned media displayed markedly increased IL-6 expression alongside suppressed TNF α and undetectable IL-10 levels. These results indicate a unique inflammatory modulation that is distinct from the classical LPS-driven activation pattern; instead, they suggest that paracrine factors secreted by mature adipocytes can skew macrophages toward an IL-6–dominant phenotype with suppressed

IL-10 expression, closely resembling the chronic low-grade inflammatory milieu characteristic of obese adipose tissue.^{226,227} This may reflect the greater variety of adipose tissue macrophages (ATM) found in obesity. Notably, previous co-culture studies of adipocytes and macrophages have reported that TNF α expression remains unchanged or increased while IL-6 levels are elevated, causing impaired insulin signaling.^{228,229} Also, studies have identified different subgroups of ATMs in obese fat tissue, highlighting the diversity and adaptability of ATMs under metabolic stress.²³⁰ Overall, the combination of mature adipocytes displaying an endocrine profile marked by elevated expression of Fabp4, Adipsin, and Resistin but reduced levels of Adipoq, together with the pronounced IL-6-skewed transcriptional response of macrophages, highlights a transcriptional profile reminiscent of early obesity-associated inflammatory interactions.

In summary, we developed a method to enable the direct culture of adipocytes with other cell types in a scaffold-free manner, facilitating research on adipose tissue functions and pathophysiology. This approach primarily focused on the biofabrication aspects, providing insights into how mature adipocytes can be assembled with endothelial cells and macrophages to mimic certain features of the adipose microenvironment. However, some functional and phenotypic investigations, such as the formation of endothelial vascular networks or the emergence of crown-like macrophage structures typically observed in obese adipose tissue, were beyond the scope of this study. Future refinements of this technique could expand its utility to explore these complex interactions in greater depth, ultimately advancing our ability to model hypertrophic, fibrotic, and metabolically dysfunctional adipose tissue.

CHAPTER 4

ENHANCING SCAFFOLD-FREE 3D ADIPOSE TISSUE CONSTRUCTS WITH CARRAGEENAN-INDUCED MACROMOLECULAR CROWDING: COMPARATIVE EVALUATION OF 3D CULTURE TECHNIQUES

4.1. Background

To effectively mimic native tissue environments, strong cell–cell and cell–ECM interactions are essential. These interactions help maintain cellular functions, support proper tissue architecture, and regulate key biological processes such as proliferation, differentiation, and tissue remodeling. In their absence, both engineered constructs and 3D culture models may fail to replicate the complexity and functionality of natural tissues.

The fabrication of ECM-rich 3D tissue constructs *in vitro* is inherently time-consuming, as the deposition and organization of ECM components require extended culture periods. The required maturation time varies significantly depending on the tissue type and the structural complexity of the construct, ranging from a few weeks to several months.²³¹ However, such prolonged culture durations can give rise to issues such as cellular senescence and undesirable functional or phenotypical changes. Even though some approaches are used to increase ECM deposition in 3D cultures (such as using growth factors, adjusting the mechanical properties of scaffolds), they have still had limited success.²³¹ In recent years, using crowding agents in cellular environments has gained attention to increase ECM deposition.

The molecular phenomenon called macromolecular crowding (MMC) refers to the densely packed microenvironment with macromolecules such as proteins, nucleic acids, and polysaccharides.^{91,95} These macromolecules occupy a significant amount of

space and limit the volume available for other molecules to move and interact, thereby, it mimics the high-density conditions found within native tissues, and affects molecular interactions and biochemical processes.⁹⁵ Various macromolecules, such as dextran, Ficoll, methyl cellulose, and carrageenan, have been utilized as crowding agents to enhance ECM deposition, sustain cellular phenotype, and support the formation of biomimetic microenvironments.⁹² These effects are largely attributed to the ability of macromolecular crowding to promote supramolecular assembly of ECM proteins, facilitate crosslinking and stabilization of the matrix, and accelerate ECM remodeling processes, thereby enabling more rapid and organized matrix accumulation *in vitro*.^{232,233} While MMC agents have been extensively utilized in 3D culture systems involving fibroblasts, mesenchymal stem cells (MSCs), chondrocytes, endothelial cells, and cancer cell lines to promote ECM deposition and tissue-like architecture, their integration into adipocyte-based 3D models remains limited.^{94,96}

Adipogenic differentiation is a tightly regulated process that requires sustained exposure to adipogenic cues, including growth factors such as insulin, FGF-2, and IGF-1.²³⁴⁻²³⁶ However, in standard 2D or 3D cell culture systems, these bioactive molecules are often rapidly degraded, leading to a limited temporal availability and downstream signaling effects.²³⁷ In contrast, under MMC conditions, undifferentiated MSCs undergo extracellular matrix (ECM) remodeling, characterized by the enhanced deposition of key ECM components such as collagen types I and IV, fibronectin, and heparan sulfate proteoglycans (HSPGs), facilitating the local sequestration of growth factors.^{233,238} This localized enrichment of signaling molecules reinforces adipogenic maturation by linking ECM dynamics with sustained growth factor signaling.²³³

In 2D cultures, studies have shown that MMC enhances the adipogenic differentiation potential of MSCs during induction. In a study, the use of Ficoll to induce crowding has been shown to upregulate both early (e.g., C/EBP β) and late (e.g., PPAR γ , FABP4) adipogenic markers, leading to a more than 25% increase in lipid accumulation compared to conventional induction protocols.²³³ On the other hand, the application of carrageenan both in the pre-differentiation culture or during adipogenic induction significantly impaired adipogenic differentiation, as evidenced by reduced lipid accumulation and adipogenic marker expression.^{97,99} However, to our knowledge, the effects of carrageenan on mature adipocytes have not been thoroughly explored. It is possible that carrageenan-induced macromolecular crowding may enhance the deposition

of adipocyte-specific ECM components in mature adipocytes due to macromolecular crowding, which enables cell-specific ECM production.⁹²

Magnetic levitation (MagLev), based on the principle of negative magnetophoresis, has recently gained attention as a label- and scaffold-free approach for 3D cell culture, allowing multicellular structures by suspending cells within a magnetic field.^{86,87} Unlike traditional 3D spheroid culture techniques, such as the hanging drop and liquid overlay techniques, MagLev can enhance cell–cell and cell–ECM interactions by confining cells within a limited space through magnetic forces, thereby promoting more physiologically relevant tissue-like structures. Previously, we employed MagLev in various configurations to biofabricate adipose tissue models, including mono- and co-cultures of adipogenic cells with stem cells or with endothelial cells and macrophages. Here, we aim to further enhance adipose tissue biofabrication within the MagLev system by incorporating carrageenan as a crowding agent. Additionally, we will compare the performance of the MagLev system with traditional techniques, including hanging drop and liquid overlay, in terms of adipose tissue formation and differentiation.

4.2. Materials and Methods

4.2.1. Design of Magnetic Levitation Platform

In a horizontal magnetic levitation system, two N52-grade neodymium magnets with the same poles facing each other were positioned on the horizontal axis. A micro-capillary channel placed between the magnets was used as a cell culture chamber. The parts of the horizontal levitation platform were assembled by holders printed by a 3D printing system. During the culture, the levitation device was tilted about 3° using two pieces of slides.

4.2.2. Cell Culture

7F2 and bEnd.3 cells were selected as model cells in the study. α MEM for 7F2 cells and DMEM high glucose for bEnd.3 were used as standard growth media supplemented with 10% FBS and 1% P/S at 5% CO₂ humidified atmosphere at 37°C. For the differentiation of 7F2 cells into adipogenic cells, the cells were seeded in 6-well plates with a concentration of 40000 cells/well. On the 2nd day of culture, the medium was replaced with adipogenic differentiation medium composed of 10 nM dexamethasone, 50 μ M indomethacin, and 5 μ g/ml insulin and the cells were cultured for 7 days. The medium was replaced every 2–3 days. As a maintenance medium, insulin was added only to the culture medium.

4.2.3. 3D Culture of Cells in the Horizontal MagLev System

3D cellular structures were created with different cell combinations, including 3D structures composed of 1) adipogenic cells and 2) adipogenic and endothelial cells, to mimic adipose tissue models with the magnetic levitation system. To form 3D structures, the cells were cultured with total cell numbers of 25, 250 and/or 2500. For co-culture studies, the cells were mixed at ratios of 1:1. Subsequently, 50 μ l of the prepared cell suspensions were loaded into capillaries and cultured in the horizontal magnetic levitation system for 5-7 days using 50 mM Gd. The resulting 3D structures were visualized with a fluorescent microscope.

4.2.4. Spheroid Formation by Hanging Drop Technique

For hanging drop culture, the cells were trypsinized, and the cell suspension was prepared for seeding at densities of 25, 250, and 2500 cells per 20 μ l droplet on the inner side of a 60 mm petri dish lid. Then, the lid was inverted. To prevent evaporation, 3-4 mL

of PBS was added to culture dishes. The cells were mono- or co-cultured for 5-7 days. 3D structures were visualized with an inverted microscope.

4.2.5. Spheroid Formation by Liquid Overlay Technique

For gel coating of well plates, 2% (W/V) agarose solution was prepared by mixing agarose with dH₂O and used to cover the surface of the 96-well plate after autoclaving the solution. 50 µl of agarose solution was loaded into each well, and the agarose was solidified at room temperature. After solidification, the coated well plates were stored at 4 °C until use. The cells grown in a culture dish were detached using 0.25% trypsin-EDTA solution, and cell suspension concentrations were prepared for the spheroid culture of the cells, with 25, 250, and/or 2500 cells on the non-adhesive surface. 100 µl of the prepared cell suspensions were transferred to culture plates coated with 2% agarose, and the cells were cultured for 5-7 days. The formed spheroids were visualized under an inverted fluorescent microscope.

4.2.6. Culture of Cells with a Crowding Agent

To induce macromolecular crowding with the aim of enhancing ECM production and promoting more stable 3D adipose structures, carrageenan (Sigma) was sterilized under UV light for 60 minutes and subsequently dissolved in culture medium to prepare a stock solution at a final concentration of 5 mg/mL. Ascorbic acid was then added to the solution at a ratio of 1 µL per 1 mL of carrageenan solution, using a 0.1 M filtered stock to promote collagen synthesis. For the experimental groups, three different final concentrations of carrageenan were used—25 µg/mL, 50 µg/mL, and 100 µg/mL—by adjusting cell concentrations accordingly for the respective studies. Cell suspensions were also supplemented with a final concentration of 0.1 mM ascorbic acid. The control groups consisted of complete medium supplemented only with standard adipogenic differentiation agents.

4.2.7. MTT Assay

To assess the effect of carrageenan on the viability of adipogenic cells, 7F2 cells were seeded into a 96-well plate at a density of 2,000 cells per well. After 48 hours, the medium was replaced with adipogenic differentiation medium, and the cells were cultured for 7 days, with medium changes every 2-3 days. Following differentiation, the cells were treated with carrageenan at concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 $\mu\text{g/mL}$ for 72 hours. At 24, 48, and 72 hours post-treatment, the medium was removed, and MTT solution (10% in serum-free medium) was added. The cells were then incubated for 3 hours at 37°C. After incubation, the MTT solution was discarded, and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plate was shaken at 60 rpm for 10 minutes to ensure complete dissolution of the crystals, and absorbance was measured at 570 nm and 690 nm using a ScanIT spectrophotometer.

For bEnd.3 cells, 3,000 cells per well were seeded into a 96-well plate. After 24 hours, the cells were treated with carrageenan at the same concentrations used for the 7F2 cells (3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 $\mu\text{g/mL}$). Absorbance measurements were performed following the same MTT assay protocol described for the 7F2 cells.

4.2.8. Live/Dead Staining

After 7 days of adipogenic differentiation, cells were cultured with 50 $\mu\text{g/mL}$ carrageenan for an additional 5 days using three different spheroid culture techniques or on a conventional 2D culture plate. Following culture, cells were gently washed with PBS to remove residuals. A Calcein-AM/Propidium Iodide (PI) staining solution was then applied according to the manufacturer's instructions. Cells were incubated with the staining solution for 15 minutes at room temperature, protected from light. After incubation, live (Calcein-positive) and dead (PI-positive) cells were visualized and imaged using a fluorescence microscope.

4.2.9. Staining of Lipid Droplets

To assess lipid accumulation, Oil Red O (ORO) staining was performed. A 0.5% (W/V) ORO solution was prepared by dissolving 250 mg of ORO powder in 50 mL of isopropanol to create a stock solution. For staining, a working solution of 3:2 ORO:isopropanol was prepared freshly and filtered. The cells were first washed with PBS and then fixed with 10% neutral-buffered formalin (NBF) at room temperature for 15-30 minutes. After fixation, the cells were washed again, and the working solution was added. The cells were then incubated at 37°C for 45 minutes. Following incubation, excess dye was removed by washing, and the cells were imaged under a microscope. For quantitative analysis, to destain the ORO dye in the lipid droplets, isopropanol was added, and the cells were shaken at 60 rpm for 15 minutes. Subsequently, 100 µL of each sample was transferred to a 96-well plate, and absorbance was measured at 510 nm. The same protocol was applied for staining of 3D structures; however, the fixation time was doubled.

4.2.10. Sirius Red Staining

To evaluate the effect of carrageenan on ECM accumulation, collagen staining was performed using Sirius Red Staining (SRS). A 0.1% SRS solution was prepared by dissolving 50 mg of Sirius Red powder in 50 mL of 1% acetic acid, followed by filtration through a 0.45 µm filter. After adipogenic differentiation in 2D culture, the cells were cultured with carrageenan for 72 hours. For staining, the cells were washed with PBS and fixed in 10% NBF for 15-30 minutes. After fixation, the cells were washed with dH₂O and stained with the 0.1% Sirius Red solution for 1 hour at 37°C. After staining, excess dye was removed through several washes, and the cells were imaged under a microscope. For destaining, a NaOH:MeOH (1:1) solution was added, and the cells were shaken at 60 rpm for 30 minutes. Then, 100 µL samples from each well were transferred to a 96-well plate, and absorbance was measured at 540 nm. The same protocol was applied for

staining 3D structures, with the exception that the fixation time was increased to 30 minutes.

4.3. Results

4.3.1. Biofabrication of Monocultured and Co-cultured Adipogenic Models with Three Spheroid Culture Techniques

We evaluated the effects of cellular composition and various culture media, including those containing agents that maintain adipogenic conditions, on 3D structure formation using hanging drop, liquid overlay and magnetic levitation methods.

4.3.1.1. Optimization of Seeding Cell Density

We investigated the structural organization of adipogenic cells cultured under three different scaffold-free 3D culture techniques—hanging drop (Figure 4.1A), liquid overlay technique (LOT) (Figure 4.1B), and magnetic levitation (MagLev, ML) (Figure 4.1C)—at varying initial seeding numbers (25, 250, and 2500 cells) in maintenance medium and observed on Day 3 and Day 7. In the HD method (Figure 4.1A), low cell density (25 cells) led to the formation of small aggregates by Day 3, which appeared to disassemble by Day 7, indicating insufficient cell-cell adhesion or aggregate stability. At intermediate density (250 cells), compact clusters formed at Day 7. The highest density (2500 cells) resulted in a dense, multilayered cell mass, maintaining its structure over the culture period; however, due to the tendency of adipogenic cells to float, the clusters appeared less spherical and loose. In the LOT (Figure 4.1B), all three cell densities supported the formation of compact spheroids. The size and density of the aggregates positively correlated with seeding number, with the 2500-cell group producing large and well-defined spheroids. These structures remained stable over time, and even increased

slightly in compaction by Day 7, suggesting efficient self-assembly and cell-cell interaction in this technique. In the MagLev system (Figure 4.1C), the effect of magnetic field-assisted aggregation was apparent. At 25 and 250 cells, loose clusters formed initially and became more compact by Day 7. In the 2500-cell group, a dense and cohesive aggregate was already observable on Day 3, and it persisted through Day 7, indicating enhanced aggregation kinetics and structural integrity at higher cell numbers under MagLev conditions. Notably, adhesion of the cells to the capillary wall was observed in some samples. Based on the structural integrity and morphological consistency observed across the different seeding densities, a seeding density of 2500 cells was selected for subsequent experiments.

4.3.1.2. 3D Culture of Cells in Different Culture Medium Components for Adipogenic Conditions

To assess the effects of culture medium and cellular composition on the adipose tissue modeling, we cultured adipogenic cells with or without endothelial cells in either maintenance medium (medium + insulin) or differentiation medium (medium + insulin + dexamethasone + indomethacin). The cells (2500 cells in total) were cultured for 7 days, and morphological indices, including area, Feret diameter, and circularity, were analyzed.

When the adipogenic cells were cultured in maintenance medium (Figure 4.2A), the LOT and ML techniques yielded the most compact aggregates, with pronounced spheroid boundaries that remained consistent over time compared to the HD technique. Although the HD system allows aggregation, it may not provide sufficient mechanical or spatial constraints to maintain the cohesion of mature adipocytes over time, as compared to other techniques. In differentiation medium (Figure 4.2B), on the other hand, the adipogenic cells formed loosely connected clusters that remained relatively irregular during the culture period in both HD and LOT. However, ML cultures showed more robust aggregates with greater cohesion. In both conditions (maintenance and differentiation), the MagLev system initially formed string-like structures on day 1 due to the system's configuration. However, over time, the cells acquired a more spheroid-like morphology, indicating gradual aggregation.

In co-culture experiments performed in maintenance medium (Figure 4.3A), it was revealed that the presence of endothelial cells changed the cluster formation patterns, especially in LOT. Even though aggregates produced by HD and LOT exhibited intermediate compactness, with gradual structural refinement observed through day 7, they still formed a loose, spread-out structure that did not exhibit significant 3D compaction compared to ML. On the other hand, co-cultured cells did not aggregate closely, even by day 7, when cultured using the HD and LOT technique in differentiation medium (Figure 4.3B). However, in the ML system, similar 3D structures may have formed under all conditions due to the magnetic force facilitating initial aggregation.

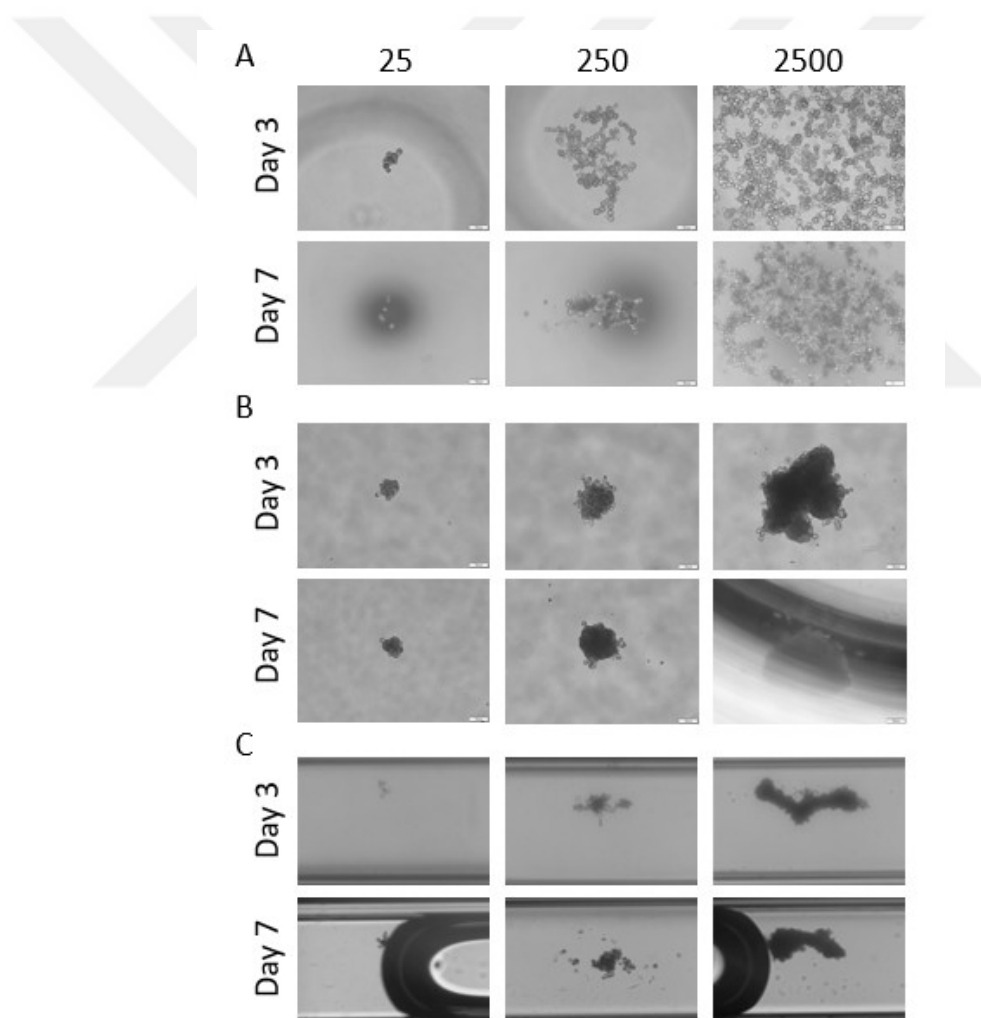


Figure 4.1. Culture of the adipogenic cells using the (A) hanging drop, (B) liquid overlay technique and (C) magnetic levitation in maintenance medium for 7 days—scale bar: 100 μm .

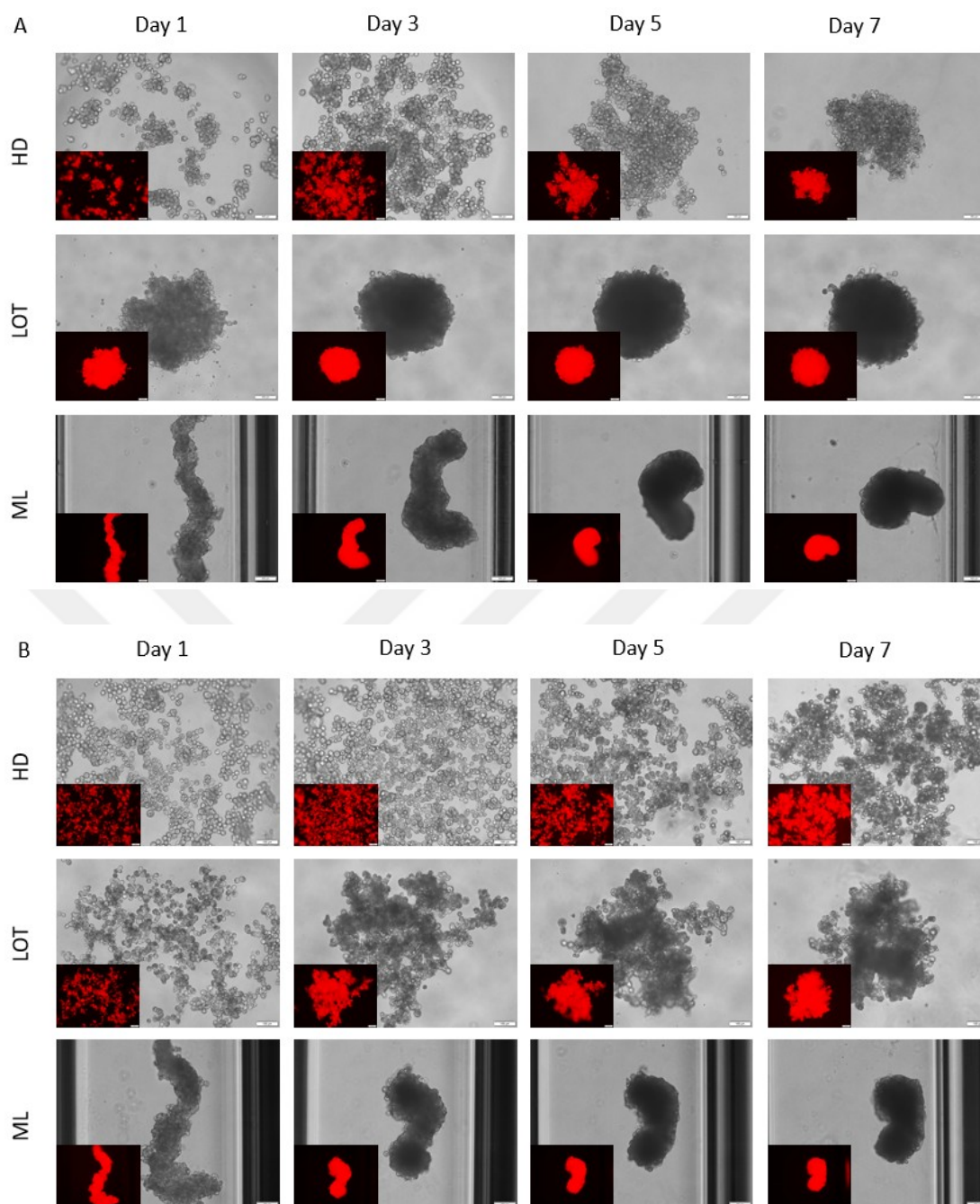


Figure 4.2. Mono-culture of the adipogenic cells using the hanging drop (HD), liquid overlay technique (LOT) and magnetic levitation (ML) in (A) maintenance or (B) differentiation medium for 7 days—scale bar: 100 μm .

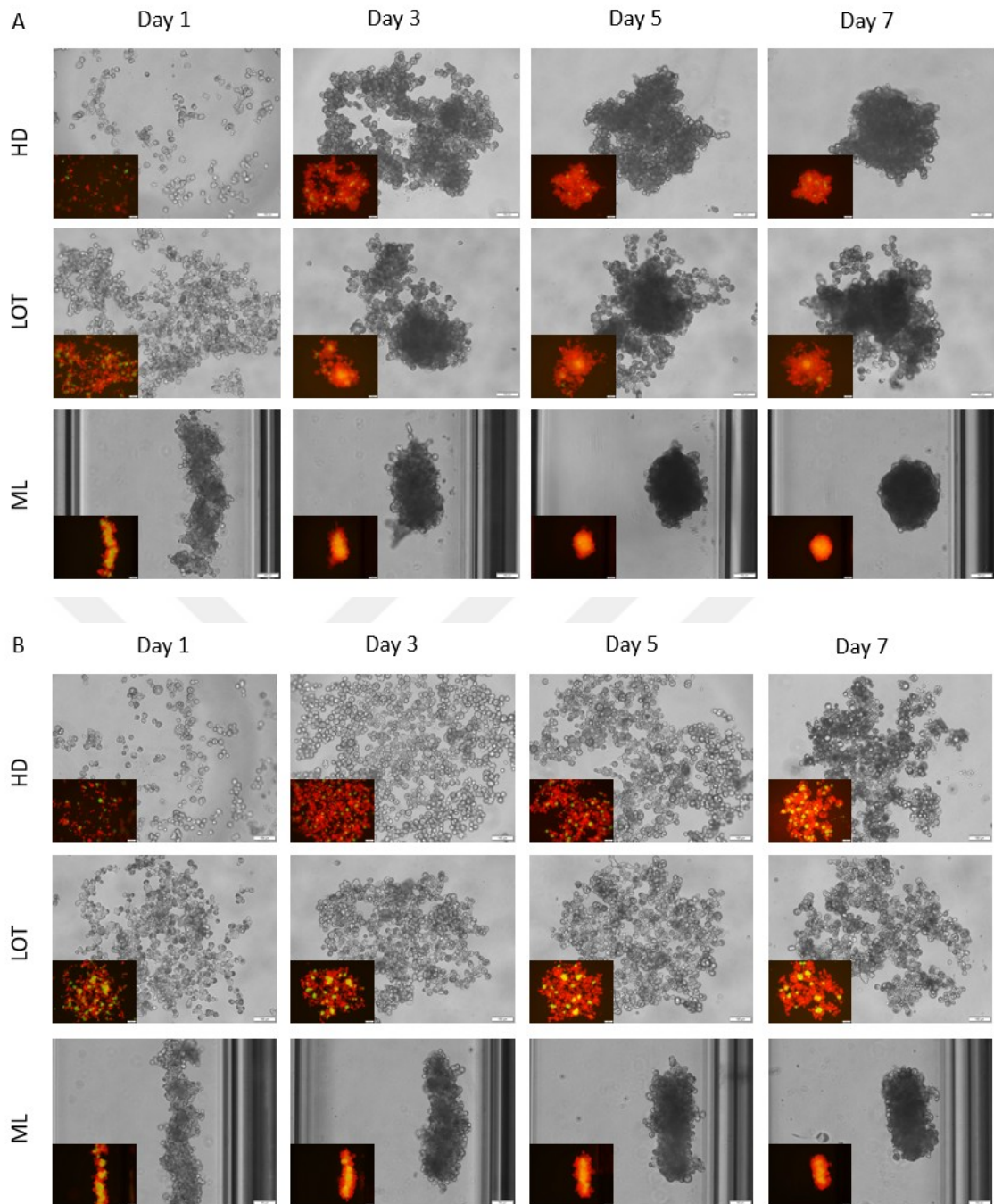


Figure 4.3. Co-culture of the adipogenic and endothelial cells using the hanging drop (HD), liquid overlay technique (LOT) and magnetic levitation (ML) in (A) maintenance or (B) differentiation medium for 7 days—scale bar: 100 μm.

To assess the effects of culture medium (maintenance vs. differentiation) and cellular composition (monoculture vs. co-culture) on the morphology of 3D structures, morphological indices were evaluated at four time points (Figure 4.4). Because HD

samples and several LOT samples had not yet formed stable aggregates on day 1, early morphology could not be quantified. The most of techniques showed a progressive compaction, reflected by decreasing area and Feret diameter and an increased circularity (Figure 4.4). In the HD technique, the area of the 3D structure in the maintenance medium decreased 67.9% ($p < 0.0001$) and 56.6% ($p < 0.0001$) for mono-cultured and co-cultured groups, respectively, between day 3 and day 7. Liquid-overlay (LOT) responses were more moderate; no significant area change was detected in mono-cultured cells in maintenance medium (2.3%, $p > 0.05$), while co-cultures expanded by 22.7% ($p = 0.003$). By contrast, differentiation medium drove a 32.0% area decrease in LOT mono-cultures ($p < 0.0001$) and had little effect on co-cultures (9.4% increase, $p > 0.05$). ML aggregates were the most stable, exhibiting only a 10–15 % non-significant decrease under maintenance conditions compared to day 3, and 30–40% compared to day 1 ($p = 0.0002$ for co-cultured group). On the other hand, a similar trend was observed in both mono- and co-cultured cells in differentiation medium under HD and ML conditions, with a significant decrease of 20–50% over time.

Diameter data mirrored the surface-area findings. In hanging-drop and magnetic-levitation cultures, the aggregates compacted continuously, losing ≈ 45 –55 % of their initial diameter in every ML condition ($p < 0.05$) and 27 % in HD cultures grown in differentiation medium ($p < 0.0001$). LOT behaved differently: a pattern in which the diameter increases at day 5 and then decreases at day 7 in all conditions was observed, the only exception was the differentiation-medium mono-culture. On the other hand, 3D structures became progressively rounder, showing a significant increase in circularity in all settings except co-cultures in maintenance medium under HD.

When we compare the effect of culture conditions at day 7, we observed that adding endothelial cells to the culture did not affect the area of the 3D structures formed by HD in maintenance medium, but decreased it in differentiation medium by 21.5% ($p < 0.0001$). 3D structures of co-cultured cells exhibited a larger area compared to mono-cultured cells in both maintenance (53.2%, $p = 0.015$) and differentiation medium in LOT (60.0%, $p = 0.020$). On the contrary, ML did not show any significant difference according to both culture medium and cell types ($p > 0.05$ for all).

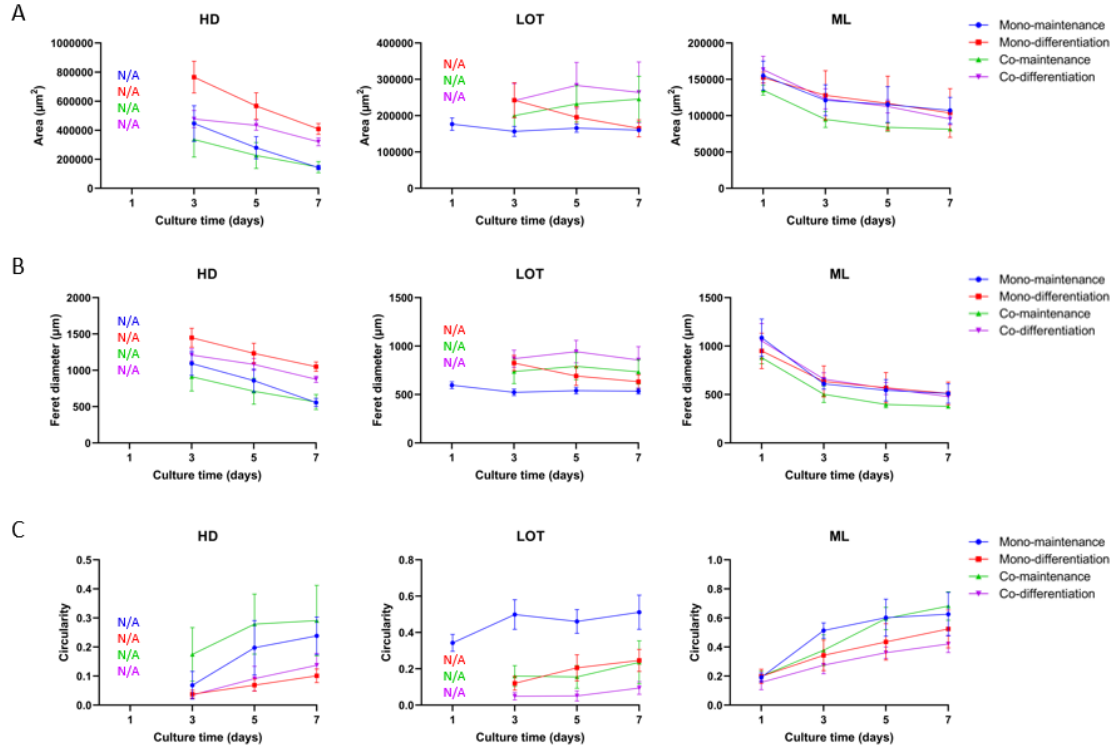


Figure 4.4. Analysis of morphological parameters of 3D structures of mono- or co-cultured cells grown in maintenance or differentiation medium for 7 days. N/A: not applicable, HD: hanging drop, LOT: Liquid overlay technique, ML: magnetic levitation.

The differences in diameter and circularity between culture conditions were more abundant in the LOT system. Aggregate diameter was significantly smaller in monocultures grown in maintenance medium than in those exposed to differentiation medium (-15.3%, $p = 0.0143$) and in co-cultures cultured with maintenance medium (-26.9%, $p = 0.0063$); likewise, monocultures in differentiation medium were smaller than their co-cultured counterparts (-26.1%, $p = 0.0012$), whereas the two co-culture media did not differ ($p > 0.05$). Monocultures with insulin were high round than those in differentiation medium (107.5%, $p = 0.022$) and than co-cultures with insulin (117.4%, $p = 0.0106$); aggregates formed in differentiation medium became markedly less spherical in co-culture compared to monoculture (-61.6%, $p < 0.0001$), and co-cultures were rounder in maintenance than in differentiation medium (148.7%, $p = 0.0308$). Besides, the circularity of 3D structures formed by HD was not affected by cell types in both

culture media; however, in differentiation medium, the diameter was smaller by 16.4% in co-cultured cells compared to monoculture ($p < 0.0001$). Also, culture medium affected the diameter in both mono and co-cultured groups ($p < 0.0001$). In contrast, circularity (61.8%, $p = 0.0067$) and feret diameter (-21.1%, $p = 0.0004$) of 3D structures from ML only differed in cocultured cells between maintenance and differentiation medium. In all cultures, the feret diameter of the 3D structures formed in differentiation medium was higher compared to maintenance medium. The smallest diameter was obtained by ML, ranging from 350-550 μm on average, which is suitable for the diffusion of nutrients and oxygen.

4.3.2. 3D Culture of Cells with Carrageenan

Carrageenan was utilized as a macromolecular crowding agent to improve culture conditions for adipogenic cells, which are characterized by low mass density and limited cell-cell interactions. To evaluate the cytotoxic effects of carrageenan on cultured cells, cell viability and metabolic activity were quantified by MTT assay (Figure 4.5). Initially, we assessed the growing profile of untreated cells over a 72-hour period (Figure 4.5A). Adipogenic cells displayed a gradual decline in metabolic activity, whereas bEnd.3 endothelial cells showed the expected time-dependent increase. The treatment with varying concentrations of carrageenan did not produce a strictly dose-dependent response. Nevertheless, at 72 h adipogenic cells cultured with 6.25, 25, or 400 $\mu\text{g/mL}$ carrageenan exhibited significantly higher MTT absorbance than untreated controls, indicating preserved and mildly enhanced metabolic activity (Figure 4.5B). bEnd.3 cells demonstrated an upward trend at increasing carrageenan concentrations, reaching statistical significance only at 400 $\mu\text{g/mL}$ (Figure 4.5C). Collectively, these data confirm that carrageenan is non-cytotoxic within the tested range and can be applied as a crowding additive without compromising cell viability.

Carrageenan was next assessed for its ability to modulate matrix deposition and lipid storage under conventional 2-D conditions. Adipogenic cultures were supplemented with 25, 50, or 100 $\mu\text{g/mL}$ carrageenan and analysed for collagen production (Figure 4.6) and lipid accumulation (Figure 4.7). Carrageenan significantly increased the collagen deposition in adipogenic cells on the first day at a 50 $\mu\text{g/mL}$ concentration (Figure 4.6B).

Over time, in all concentrations (25, 50, and 100 $\mu\text{g/mL}$), there was an increase in collagen deposition. By contrast, carrageenan had no measurable effect on lipid accumulation after five days (Figure 4.7B).

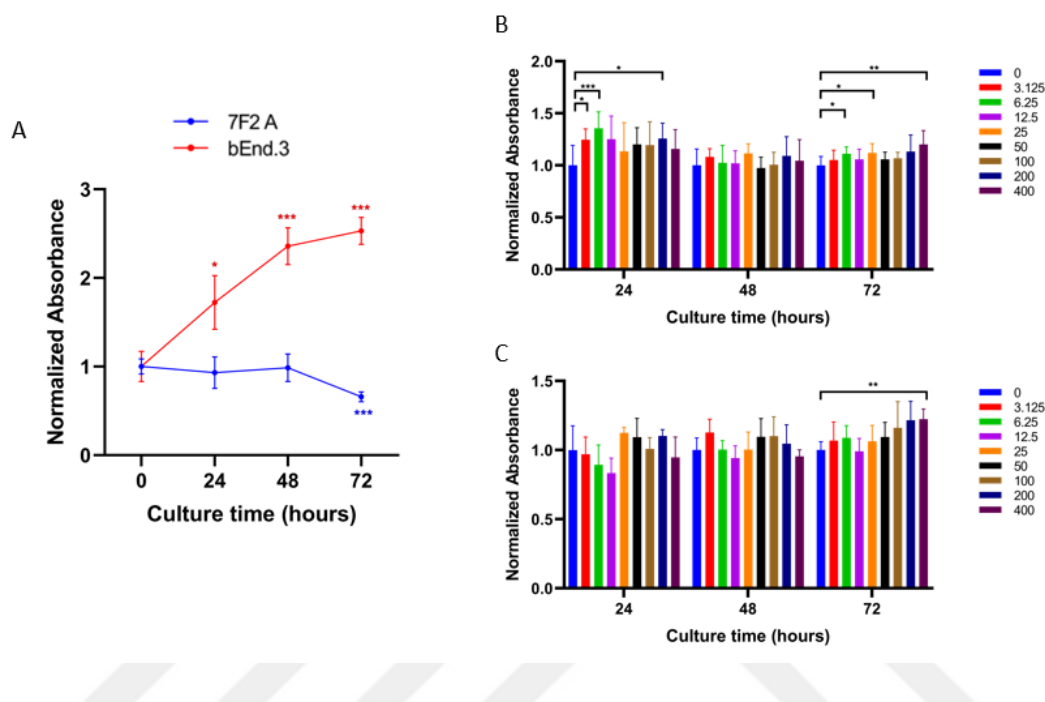


Figure 4.5. MTT analysis of adipogenic and endothelial cells cultured with different carrageenan concentrations. (A) Proliferation of the adipogenic and endothelial cells for 72h. Data are normalized to 0 hours. The effect of carrageenan on (B) adipogenic and (C) endothelial cell viability. Data are normalized to control for each day. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The impact of carrageenan on scaffold-free 3D morphogenesis was tracked for five days using HD, LOT and ML systems with the concentrations of 25, 50 and 100 $\mu\text{g/mL}$. In the HD system, cells initially formed dispersed and loosely aggregated clusters on Day 1 (Figure 4.8). However, carrageenan-treated samples produced compact spheroids that were smaller than untreated controls, and by Day 5 the area was reduced by 30–35 % across all concentrations ($p = 0.025$, 0.012 and 0.032 for 25, 50 and 100 $\mu\text{g/mL}$, respectively). This decrease in area was accompanied by increased circularity

(100-150%, $p < 0.0001$ for 25 and 50; $p = 0.0003$ for 100 $\mu\text{g/mL}$) and a falling Feret diameter (13-15%, $p > 0.05$), indicating pronounced compaction (Figure 4.11A-C).

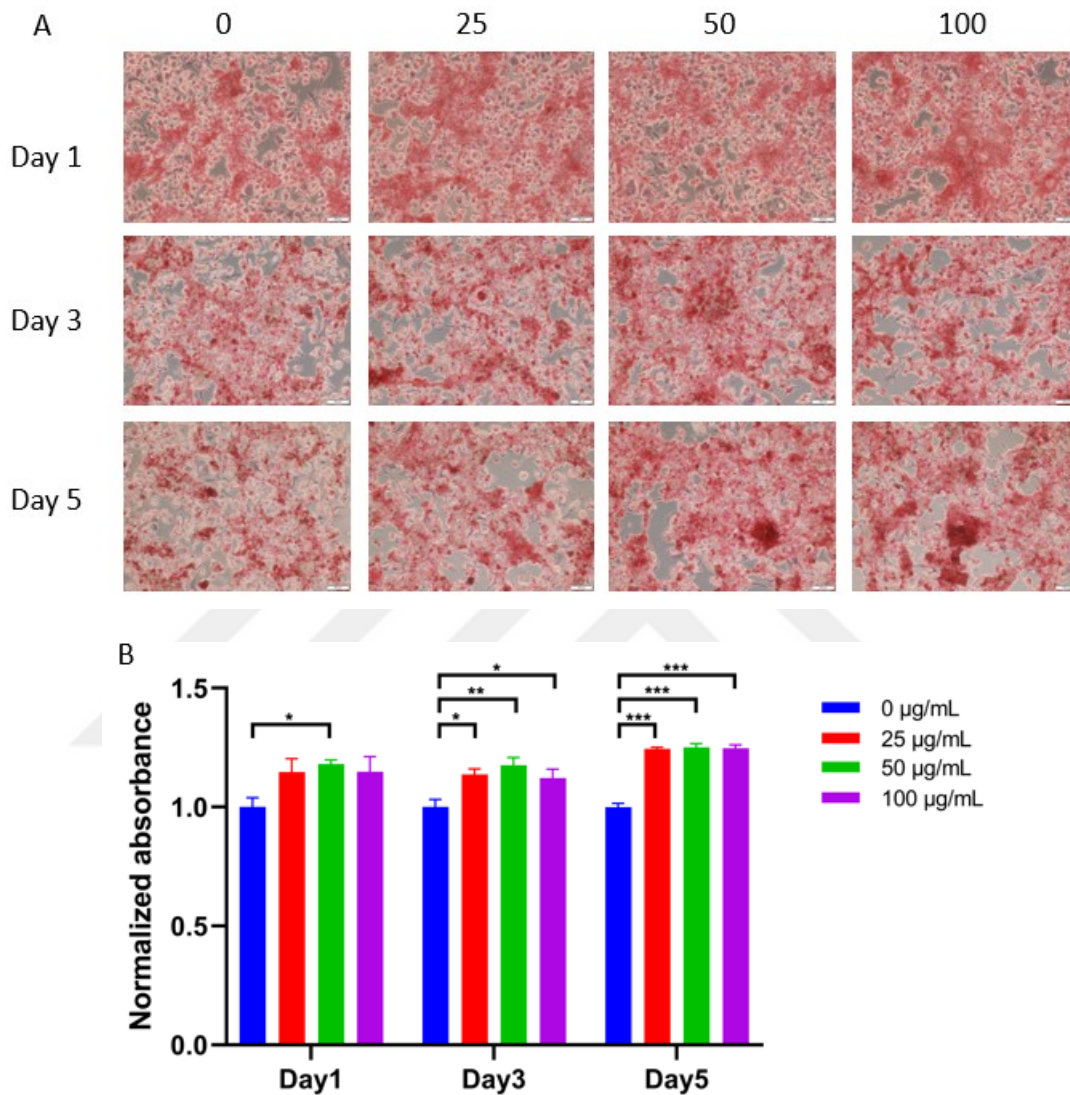


Figure 4.6. (A) Collagen deposition in adipogenic cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days.—scale bar: 100 μm . (B) Quantification of collagen deposition by absorbance measurement of eluted Sirius Red at 540 nm. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

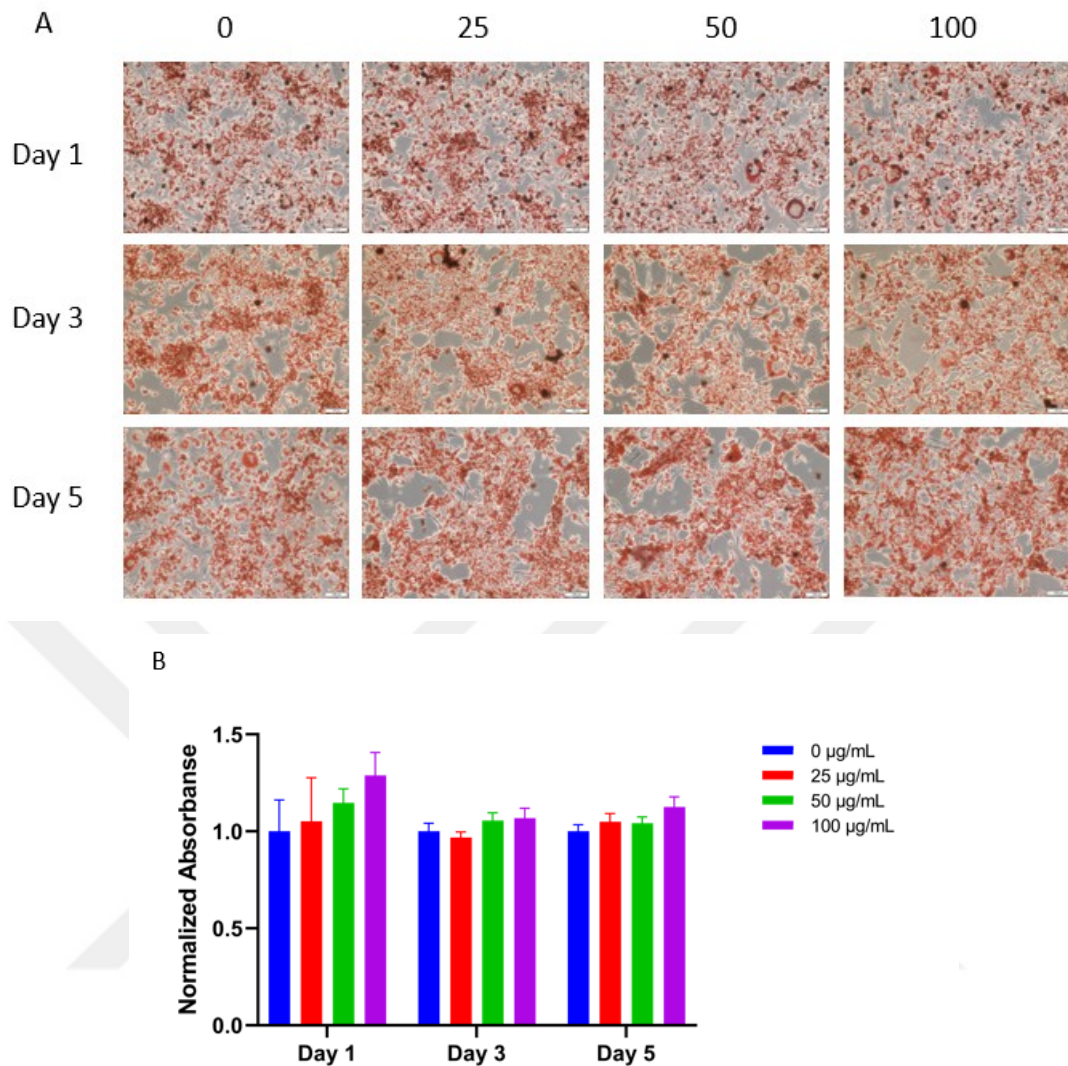


Figure 4.7. (A) Lipid accumulation in adipogenic cells treated with 25, 50 and 100 µg/ml of carrageenan for 5 days. Scale bar: 100 µm. (B) Quantification of neutral lipids by absorbance measurement of eluted Oil-Red-O at 510 nm. Data are represented as mean $\pm$ SD.

LOT facilitated faster radial compaction, with carrageenan-treated cultures condensing into well-defined spheroids within the first 24 hours, whereas the untreated control still displayed a loose, branched morphology (Figure 4.9). At day 5, both area and Feret diameter decreased in a concentration-dependent manner, in area: 41%, 63% and %66 for 25,50 and 100 µg/mL respectively and, in diameter: 21.6%, 40.1% and 44.4%, while circularity increased dramatically, %74.9%, 185.2% and 234.3%, compared to the control group (Figure 4.11A-C). Interestingly, control aggregates and the 25 µg/mL group

displayed an increase in size over time, whereas higher concentrations produced a > 20% reduction (Figure 4.11A,C).

Adipocytes formed characteristic elongated, cord-like assemblies across all concentrations due to the configuration of the MagLev device. During culture these cords thickened and shortened (Figure 4.10). Aggregate area declined steadily in all conditions, but changes were not statistically different from controls except at 25 $\mu\text{g/mL}$ on Day 3 ($p < 0.0001$) (Figure 4.11A). Also, circularity and Feret diameter in carrageenan-treated groups were mostly similar to those in the control group (Figure 4.11B,C).

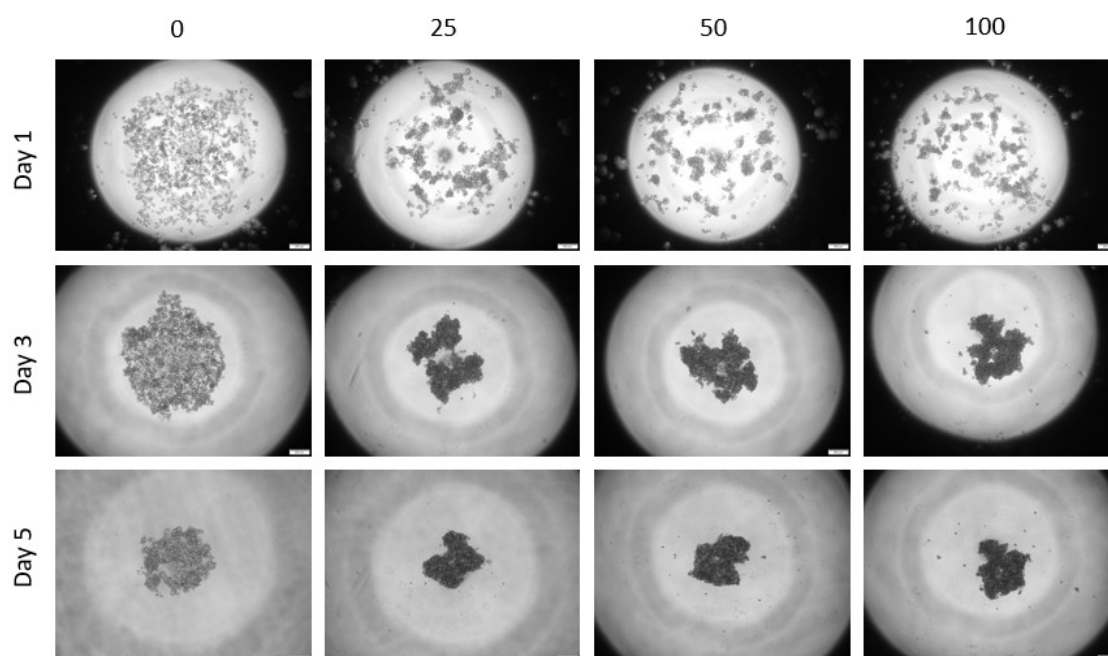


Figure 4.8. 3D culture of adipogenic cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using hanging drop technique. Scale bar: 200 μm .

A broadly comparable response emerged when carrageenan was introduced into adipocyte–bEnd.3 co-cultures (Figure 4.12) In LOT, the area of spheroids was already 20–25 % smaller than control by day 1 ($p = 0.026, 0.007$ and 0.019 for 25, 50 and 100 $\mu\text{g mL}^{-1}$, respectively), and the disparity widened over time; this widening, however, reflected a progressive expansion of the untreated aggregates rather than further shrinkage of the carrageenan-treated spheroids (Figure 4.13, 4.15A). In contrast, HD cultures

exhibited the greatest absolute compaction in the untreated control, yet these aggregates still remained the largest structures compared to carrageenan groups by day 5 (Figure 4.12, 4.15A,C). Besides, in ML, there was no difference between the groups at any time-point (Figure 4.14, 4.15A). By the end of culture, circularity and Feret diameter were comparable across carrageenan concentrations for each technique, although ML consistently yielded the most favorable overall morphology (Figure 4.15B,C).

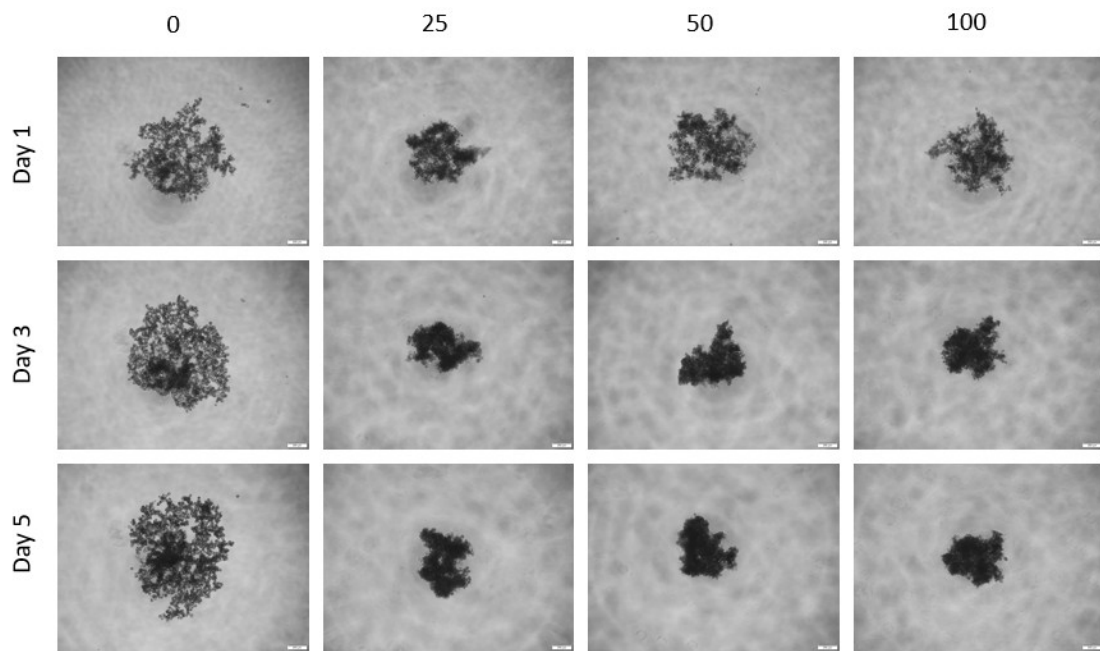


Figure 4.9. 3D culture of adipogenic cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using liquid overlay technique. Scale bar: 200 μm .

In addition, comparing the same carrageenan concentrations between mono- and co-cultures revealed that co-culture consistently produced larger structures (32–46% increase, $p=0.008$ for 50 and $p=0.003$ for 100 $\mu\text{g/mL}$) in HD, while it did not significantly differ from mono-cultured cells for LOT and ML (except for 50 $\mu\text{g/mL}$ in ML, $p=0.005$).

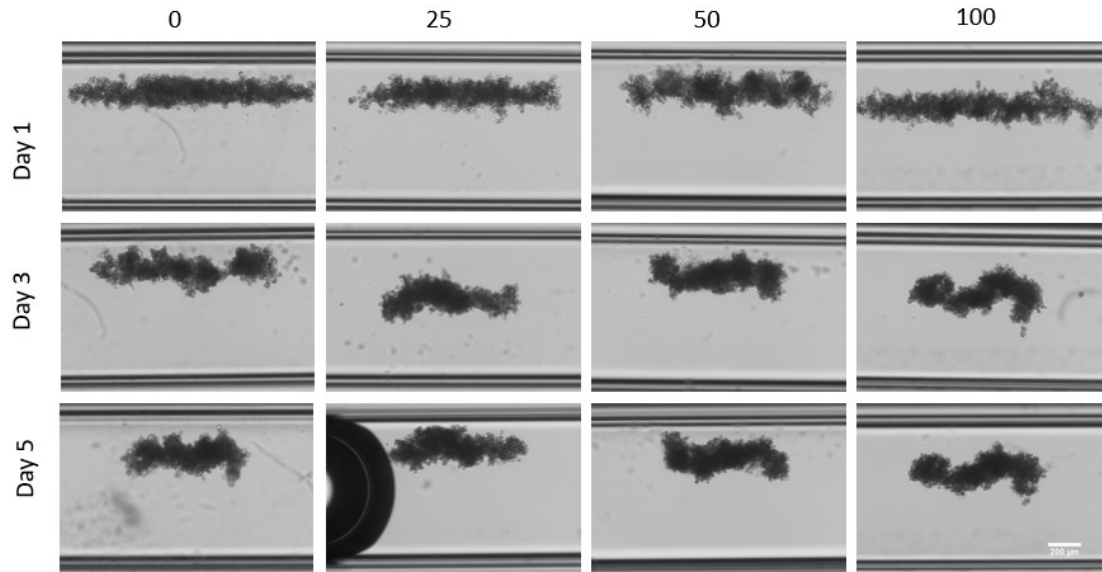


Figure 4.10. 3D culture of adipogenic cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using magnetic levitation. Scale bar: 200 μm .

The viability of 3D adipose constructs generated using three scaffold-free culture techniques was assessed via Calcein-AM/PI staining for both mono- (Figure 4.16A) and co-cultures (Figure 4.16B) with or without 50 $\mu\text{g/mL}$ carrageenan. In monocultures treated with carrageenan, cell viability remained high, measuring 97.4% for HD, 98% for LOT, and 85.7% for ML, indicating no detrimental effect of carrageenan on adipocyte survival (Figure 4.16C). Statistically, ML consistently displayed lower viability compared to the other techniques in monocultured cells ($p < 0.0001$). In cocultures, all groups maintained high viability across all platforms. However, HD showed slightly reduced viability compared to its corresponding control and LOT. Additionally, ML demonstrated lower viability than LOT within the carrageenan-treated group. Importantly, carrageenan did not significantly affect viability within each technique, supporting its compatibility with these culture systems.

In addition to viability assessment, matrix deposition was examined in mono-cultured 3D structures treated with 50 $\mu\text{g/mL}$ carrageenan by staining of collagen (Figure 4.17A). Because control samples were dispersed due to lower aggregation, quantitative analysis of collagen content was not feasible for control groups, and the assessment

remained qualitative. In all groups, positive collagen deposition was successfully detected, indicating ECM formation during scaffold-free culture.

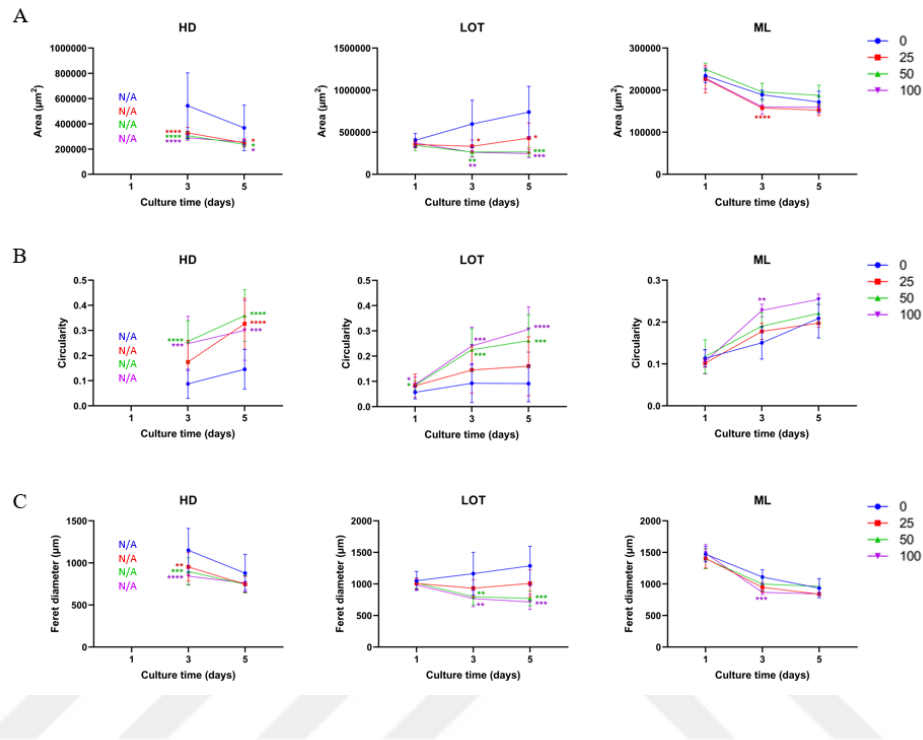


Figure 4.11. Analysis of morphological parameters of 3D structures formed by adipogenic cells treated with 25, 50 and 100 µg/mL of carrageenan for 5 days. N/A: not applicable, HD: hanging drop, LOT: Liquid overlay technique, ML: magnetic levitation. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Finally, Oil-Red-O staining of ML aggregates showed no measurable change in lipid accumulation in response to 50 µg/mL carrageenan (Figure 4.17B), suggesting that the crowding agent does not alter adipogenic lipid storage in the 3D context.

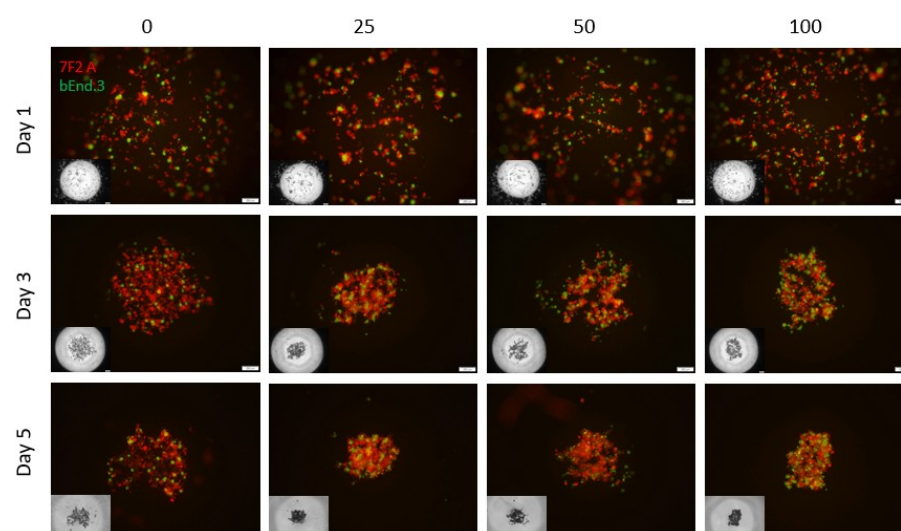


Figure 4.12. 3D co-culture of adipogenic and endothelial cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using hanging drop technique. Scale bar: 200 μm .

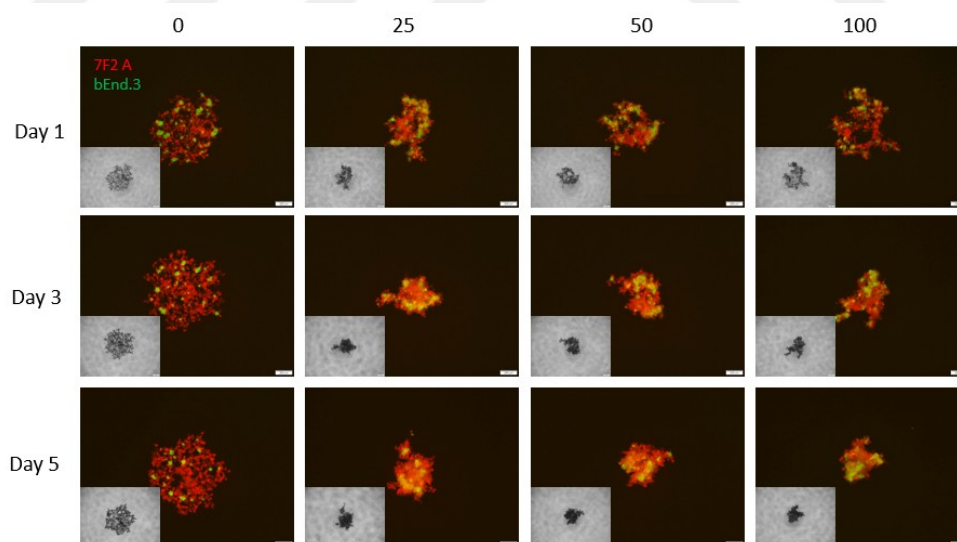


Figure 4.13. 3D co-culture of adipogenic and endothelial cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using liquid overlay technique. Scale bar: 200 μm .

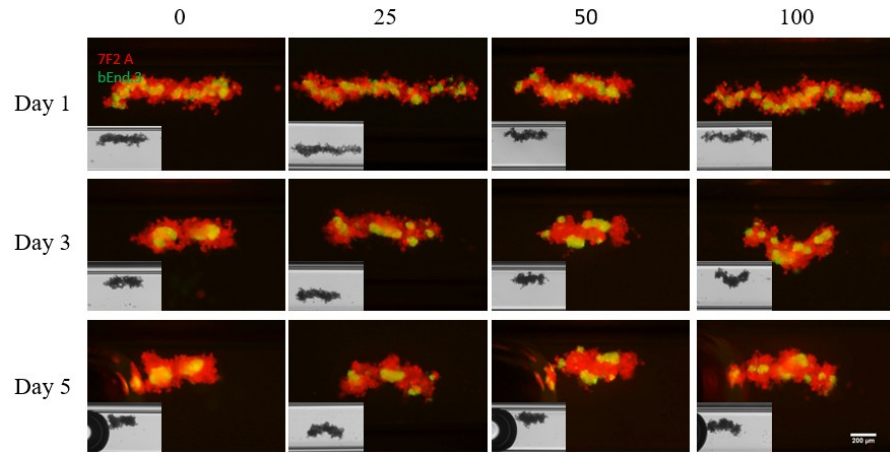


Figure 4.14. 3D co-culture of adipogenic and endothelial cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days using magnetic levitation. Scale bar: 200 μm .

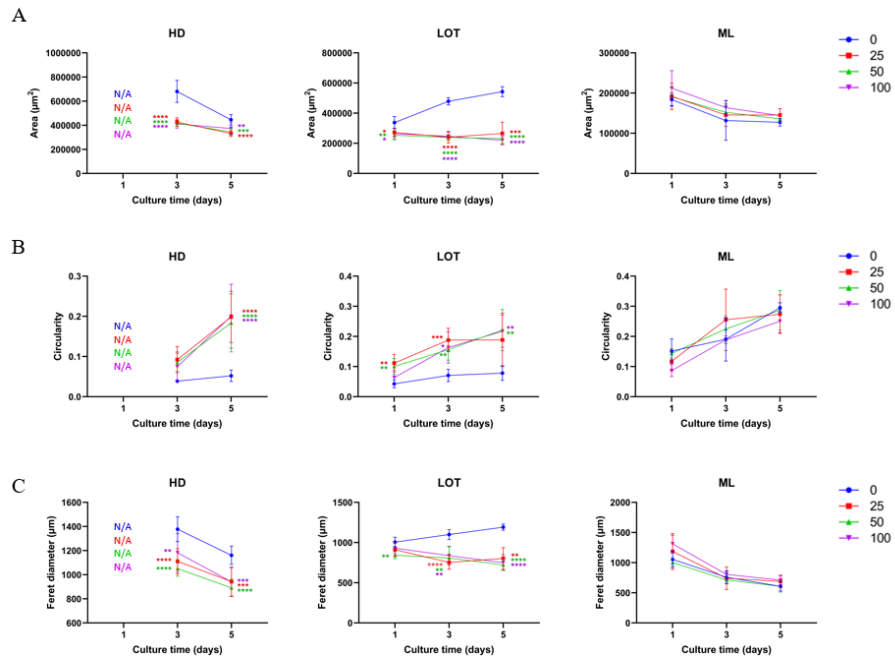


Figure 4.15. Analysis of morphological parameters of 3D structures formed by co-cultured adipogenic and endothelial cells treated with 25, 50 and 100 $\mu\text{g/mL}$ of carrageenan for 5 days. N/A: not applicable. Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

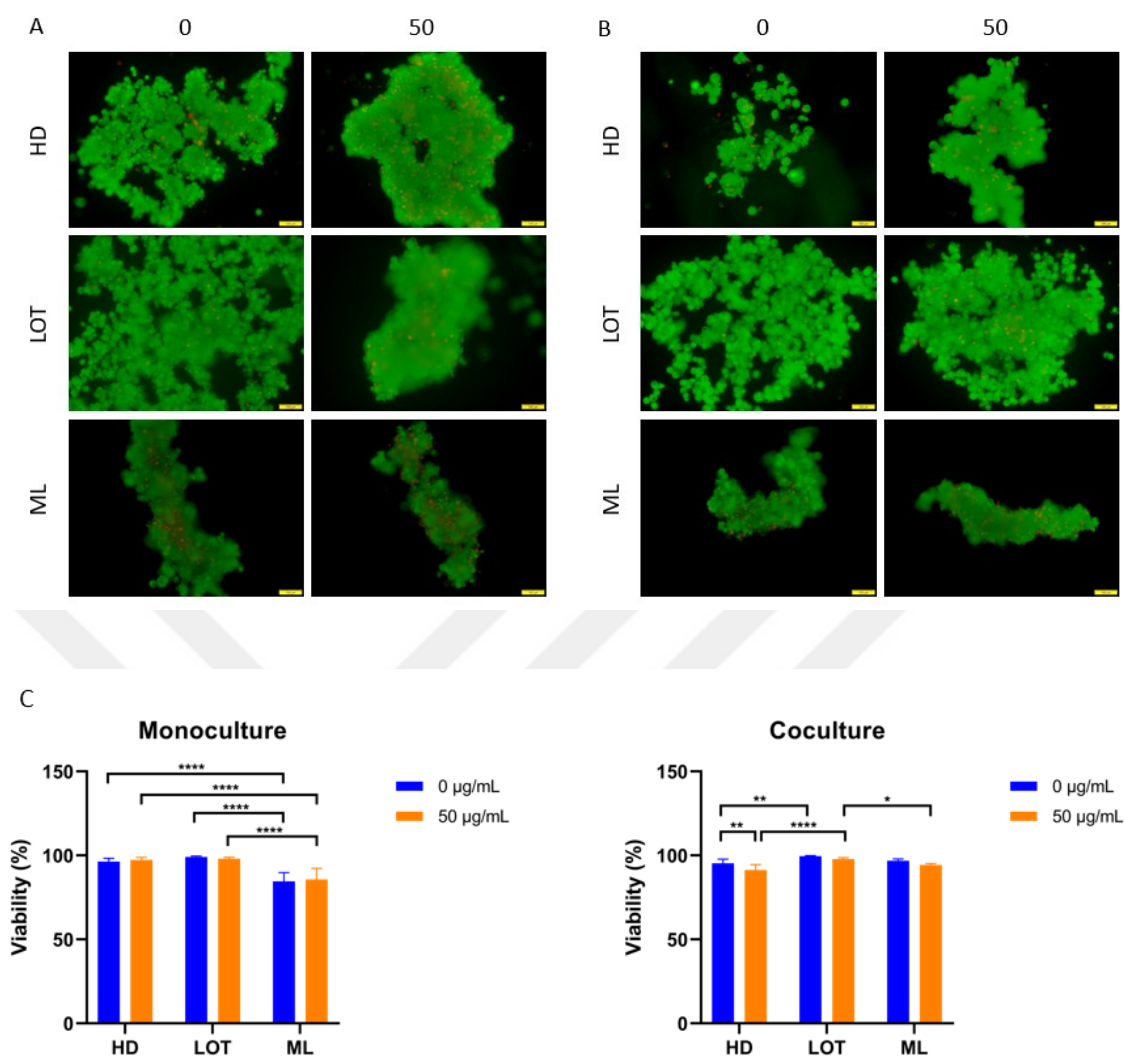


Figure 4.16. Live/dead assay conducted by calcein-AM (green-live)/PI (red-dead) staining for 3D (A) mono-culture or (B) co-culture of cells treated with 25, 50, and 100 $\mu\text{g/mL}$ of carrageenan for 5 days, cultured using hanging drop (HD), liquid overlay technique (LOT), and magnetic levitation (ML). Scale bar: 100 μm . (C) Quantitative analysis of cell viability Data are represented as mean $\pm$ SD. Statistical significance is defined as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

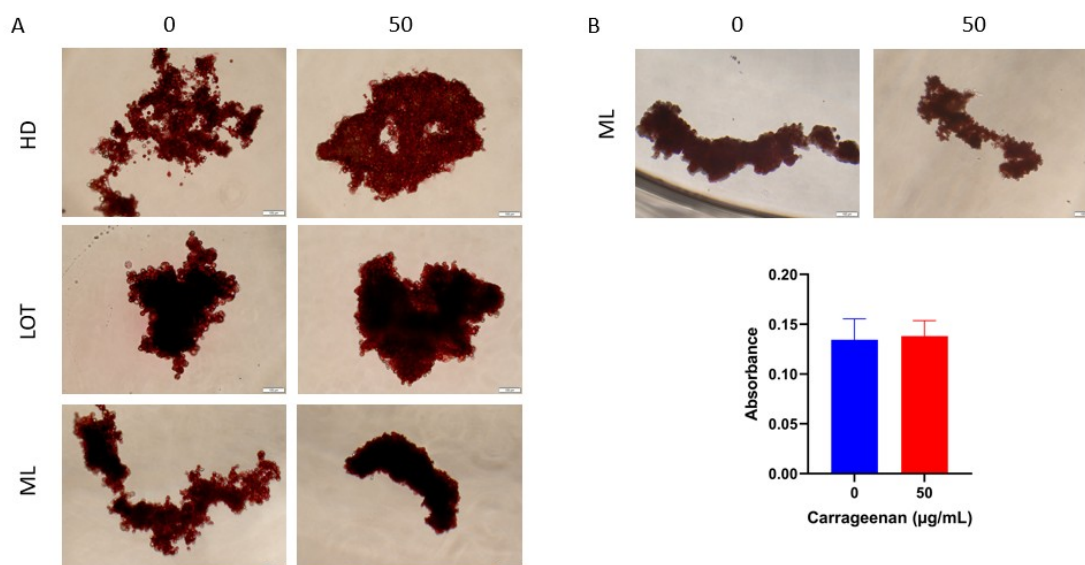


Figure 4.17. (A) Collagen deposition in adipogenic cells treated with 50 µg/mL of carrageenan for 5 days, cultured using hanging drop (HD), liquid overlay technique (LOT), and magnetic levitation (ML). Scale bar: 100 µm. (B) Oil-red-O staining indicating lipid droplets in adipocytes cultured with carrageenan in ML system. Scale bar: 100 µm. Quantification of neutral lipids was performed by absorbance measurement of eluted Oil-Red-O at 510 nm. Data are represented as mean ± SD.

4.4. Discussion

The present study set out to determine whether carrageenan can be utilized as a macromolecular crowding agent to improve the structural organization and compaction of scaffold-free, 3D adipose constructs generated from pre-differentiated adipocytes. Culture-derived “mature” adipocytes differ from native adipocytes in lipid-droplet architecture, cell size, endocrine and transcriptional profile, and mechanical environment.^{239,240} Moreover, their tendency to float and dedifferentiate limits the feasibility of using fully mature adipocytes in *in vitro* studies. These limitations can distort obesity research by exaggerating hyperplasia, underrepresenting hypertrophy-associated inflammation and insulin resistance, and misestimating drug responses related to the secretome or extracellular matrix (ECM) signaling. To address these issues, we employed

three widely used spheroid-assembly platforms, hanging drop (HD), liquid overlay technique (LOT), and magnetic levitation (MagLev). We used adipogenic-differentiated cells as a model for lipid-accumulated cells and cultured cell suspensions in the presence of 0 (control), 25, 50, or 100 $\mu\text{g/mL}$ carrageenan, thereby, we aimed to propose a suitable technique for the effective in vitro culture of mature adipocytes.

To investigate the effect of culture medium on structural organization before macromolecular crowding, adipocyte cultures were maintained either in maintenance or adipogenic differentiation media. Although cells cultured in insulin-containing maintenance medium formed more compact structures across all techniques, this condition may not accurately mimic the native adipose tissue environment. In fact, the observed reduction in the adipogenic cell population under this condition suggests limited maturation, highlighting its inadequacy for modeling mature or obese adipose tissue, which is characterized by high lipid content and fully differentiated adipocytes. Therefore, adipogenic differentiation medium can be proper to ensure the development of physiologically relevant adipose tissue constructs. Adipogenic differentiation medium provided better adipogenic morphology; however, this group exhibited more branched and loose structures. Additionally, during culture, we observed that lipid content varied among cells due to the progression of the differentiation process, which influenced their aggregation potential across replicates.

In this study, we also observed that co-culturing endothelial cells (ECs) with adipogenically differentiated cells influenced the ability of the cells to self-assemble into compact spheroids in a scaffold-free 3D culture system. While adipocytes alone formed tightly packed aggregates in the LOT, the addition of endothelial cells disrupted this organization, resulting in more dispersed structures. Interestingly, this disaggregation effect was not observed in the HD method. Several factors may underlie this behavior, including differences in cell-cell adhesion molecule expression, intrinsic cell motility, and interactions with the surrounding environment. In LOT, the lack of physical confinement may allow endothelial cells to migrate and interfere with the compaction process, whereas HD enforces close proximity and compensates for weak adhesive interactions.²⁴¹ These differences suggest that the success of spheroid formation depends not only on cell type but also on culture geometry and physical constraints. Notably, the MagLev system produced more consistent results across all culture conditions, including different culture media and cell types. Additionally, the circularity of the structures was superior in the MagLev group compared to the other culture techniques. These findings demonstrate that

the MagLev system is the most reliable and efficient technique for biofabrication of adipose tissue models, offering enhanced morphological consistency and cellular organization across different culture media and cell types.

Although ECs play a supportive role in adipose tissue development *in vivo* by promoting angiogenesis and facilitating adipogenic differentiation through paracrine signaling²³⁶, their co-culture with adipocytes in 3D *in vitro* systems may lead to unexpected outcomes. In a study, increasing the proportion of ECs in co-culture resulted in a marked reduction in lipid accumulation and the expression of key adipogenic markers such as PPAR γ and adiponectin.²⁴² This effect may arise from competition between cell types. Moreover, the differentiation medium may inhibit optimal EC functions, potentially compromising their pro-adipogenic signaling. These findings underscore the importance of cellular composition, culture conditions and biophysical context in the formation and stability of 3D adipose tissue models to faithfully recapitulate native tissue microenvironments.

Then, to enhance the structural integrity and uniformity of scaffold-free adipocyte spheroids, carrageenan was employed as an MMC agent to promote the formation of stable and cohesive 3D assemblies. Concentrations above 500 $\mu\text{g/mL}$ of carrageenan have been reported to significantly increase medium viscosity²⁴³, which may disrupt cell aggregation dynamics, limit the diffusion of nutrients and signaling molecules, and alter mechanotransduction pathways. This is particularly relevant for adipogenesis, which favors a compliant microenvironment with low stiffness to support proper differentiation and lipid accumulation. Therefore, we first tested the effect of carrageenan on cellular metabolism at concentrations up to 400 $\mu\text{g/mL}$. The MTT results demonstrate that carrageenan can be incorporated into adipogenic and endothelial cultures without eliciting obvious cytotoxicity. In endothelial cells, intermediate doses produced a modest reduction in proliferation, possibly reflecting transient engagement of stress-response programs and early adaptive remodeling. At the highest concentration (400 $\mu\text{g/mL}$), however, MTT absorbance increased, which may come from the emergence of stress tolerance or the activation of pro-survival signalling cascades. In contrast to the response observed in ECs, MTT analysis of adipogenically differentiated cells treated with varying concentrations of carrageenan for 72 hours revealed a dose-selective stimulatory effect. A significant increase in metabolic activity was observed compared to the untreated control group with at 6.25, 25, and 400 $\mu\text{g/mL}$ of carrageenan, suggesting enhanced cellular viability and/or mitochondrial activity at specific concentrations. The effect may be attributed to

carrageenan's ability to act as an MMC agent, which could increase ECM deposition and create a denser microenvironment that promotes adipocyte maintenance or function, possibly stabilizing cell membrane dynamics. However, since intermediate concentrations did not show significant changes, this suggests a non-linear relationship, indicating that the effect of carrageenan is not purely dose-dependent but may be influenced by dynamic changes in the local microenvironment, such as ECM remodeling or growth factor retention.

MMC is an increasingly employed strategy in tissue engineering to mimic the dense extracellular environment found *in vivo* and to enhance ECM deposition. However, the impact of MMC agents on adipogenesis is highly agent-specific. Among the commonly used agents, Ficoll (both Ficoll 70 and Ficoll 400) has consistently been shown to enhance adipogenic differentiation and facilitate brown adipocyte differentiation when applied during the early induction phase.^{233,244} Studies report that Ficoll promotes lipid accumulation and upregulates adipogenic markers such as PPAR γ , FABP4, and adiponectin, effects likely mediated by increased ECM density and enhanced local retention of pro-adipogenic growth factors.²³³ Similarly, Ficoll cocktail has demonstrated supportive roles in adipogenesis when used with serum-containing media although this effect is donor-dependent.²⁴⁵ In contrast, negatively charged and highly sulfated agents like fucoidan have been shown to inhibit adipogenic differentiation, potentially through disruption of mitogen-activated protein kinase (MAPK) signaling.²⁴⁶ In addition, fucoidan's high negative charge density and strong sulfation may contribute to its anti-adipogenic effect by disrupting key differentiation signaling pathways and altering the pericellular microenvironment required for adipogenic commitment. It has been also shown that higher concentrations of dextran sulfate inhibit lipid accumulation during adipogenic differentiation, whereas lower concentrations promote adipogenesis.²⁴⁷ Likewise, carrageenan, particularly when applied prior to or throughout the differentiation process, has been reported to impair adipogenesis, as evidenced by reduced lipid accumulation and downregulation of adipogenic gene expression.^{97,99} This can result from excessive ECM deposition or alterations in matrix stiffness that perturb the cytoskeletal dynamics and signaling pathways essential for adipocyte maturation. However, the effects of carrageenan applied after the completion of adipogenic differentiation remain largely unexplored. Mature adipocytes differ significantly from precursor cells in terms of morphology, gene expression, lipid metabolism, and matrix interaction. For example, as adipose tissue enlarges, its ECM is actively remodelled to

create the extra space and structural support required for adipocytes to develop and mature.²⁴⁸ Therefore, their response to macromolecular crowding agents such as carrageenan may also diverge. In contrast to reduced collagen deposition and lipid accumulation during adipogenic differentiation of stem cells treated with carrageenan, our findings extend the limited literature on post-differentiation crowding by showing that carrageenan not only augments collagen deposition in mature adipocytes without compromising neutral-lipid storage, but also markedly reorganises their 3D architecture. However, there is a lack of systematic investigation into how post-differentiation carrageenan exposure affects mature adipocyte function and the expression of adipogenic marker genes. Understanding these responses could offer novel insights into adipose tissue maintenance, modeling of hypertrophic obesity, or optimization of long-term adipose tissue culture systems.

In differentiation medium, adipocytes typically self-assemble into loosely connected, highly branched aggregates, however, when carrageenan was added after maturation, both the HD and LOT techniques produced compact, spheroidal constructs with higher circularity and pronounced radial compaction, indicating that macromolecular crowding can overcome the inherently poor cell–cell cohesion of lipid-laden adipocytes. Importantly, cellular composition modulated this response in a technique-dependent manner. In the HD system, higher carrageenan concentrations generated significantly larger aggregates in co-culture than in monoculture. By contrast, the same carrageenan levels in LOT led to comparable size and shape metrics in mono- and co-cultures, suggesting that the rapid meniscus-driven compaction characteristic of LOT dominates over cell-type–specific interactions. In MagLev, carrageenan exerted only minor effects on overall morphology, and a monoculture–co-culture divergence was detected solely at 50 µg/ mL, implying that the continuous magnetic force in ML masks subtle biomechanical cues.

Here, we evaluated the long-term culture of adipogenic structures using MagLev for the first time and compared it with other techniques. All three scaffold-free 3D culture techniques supported robust adipocyte and coculture survival, with viabilities generally exceeding 90%. LOT consistently outperformed HD and ML, yielding the highest viability in both mono- and coculture systems. MagLev consistently yielded slightly reduced viability than HD or LOT. This reduction could be attributed to prolonged exposure to paramagnetic medium (Gd). Although short-term studies have shown minimal Gd-related cytotoxicity, prolonged exposure could still impose metabolic

stress.⁸⁶ Nevertheless, MagLev drives extremely rapid radial compaction, enabling the formation of cohesive spheroidal cords within 1-2 days. By shortening the overall culture period, this accelerated aggregation may partly offset any viability loss associated with Gd. Collectively, these results indicate that while MagLev offers a high speed of 3D assembly, LOT provides a more permissive environment for sustained viability. Importantly, the addition of carrageenan as a macromolecular crowding agent did not compromise cell viability in any of the models. This suggests that carrageenan can be safely integrated into adipose tissue biofabrication workflows to enhance ECM deposition and structural integrity without inducing cytotoxic effects.

These findings suggest that carrageenan can serve as a tunable macromolecular crowding agent to modulate cell–cell interaction and matrix organization in mature adipocyte spheroids, and they provide a comparative framework for optimizing both concentration ranges and culture techniques when developing physiomimetic adipose tissue models for metabolic research and drug testing. However, given that several *in vivo* and *in vitro* studies have reported that carrageenan may trigger pro-inflammatory responses and increase the expression of cytokines such as TNF- α and IL-6, its potential to induce inflammation-related phenotypic alterations in adipocytes should be carefully considered when designing both monoculture and co-culture systems, particularly in the context of obesity research.

CHAPTER 5

CONCLUSION

In this thesis, a multifaceted approach was employed to address critical challenges in adipose tissue engineering. Basically, different magnetic levitation (MagLev) configurations and traditional scaffold-free 3D cell culture techniques and culture conditions were evaluated to understand how physical, chemical, and biological cues can be harnessed to improve the structural integrity, functional maturation, and biomimicry of adipose tissue constructs. Although adipose-derived stem cells and preadipocytes are widely used for in vitro adipogenesis, the resulting adipocytes do not fully mimic native mature adipocytes. They typically exhibit multilocular lipid droplets and produce markedly lower levels of key adipokines, such as leptin, compared to fully differentiated fat cells. Therefore, we focused on developing 3D culture models enabling the direct culture of mature adipocytes.

In Part 1 (Chapter 2), the vertical MagLev system was introduced as a scaffold-free biofabrication method to engineer adipogenic structures using negative magnetophoresis. This system enabled the assembly of both mono- and co-culture spheroids by exploiting cell density differences and eliminating the need for cytotoxic magnetic particles. While adipogenically differentiated cells formed looser, more branched clusters compared to their undifferentiated counterparts, density-driven aggregation permitted the construction of vertically layered biphasic structures. Despite the inherent fragility and heterogeneity of mature adipocytes, the MagLev system demonstrated the ability to generate viable 3D structures using various loading strategies. These results underscore the potential of MagLev for engineering tunable and heterocellular adipose tissue models without the drawbacks of scaffolds or labeling agents. However, in this system, we demonstrated that density difference and low cell-cell interactions limited the strong assembly of adipocytes.

In Part 2 (Chapter 3), the horizontal MagLev configuration was employed to enable compact adipose structures by eliminating cell density differences. Also, the

potential of collaboration of scaffold-free 3D culture techniques—specifically MagLev followed by liquid overlay technique (LOT)—was investigated to generate stable and physiomimetic adipose tissue constructs by incorporating multiple cell types. The study focused on modeling more complex cellular environments by combining adipogenic cells with endothelial cells or macrophages (co-culture) and with both endothelial cells and macrophages (tri-culture). We evaluated how these multicellular configurations affect the formation, stability, and morphology of 3D structures and cell functions. The results demonstrated that both ML+LOT enabled the formation of stable 3D adipose tissue-like structures across mono- and tri-culture setups. According to mRNA expression analysis, our data indicate that the scaffold-free 3D constructs developed using mature adipocytes maintain key functional properties, as evidenced by elevated expression of Fabp4, Adipsin, and Resistin, alongside a distinctive secretory profile that robustly induces IL-6 expression in macrophages. This suggests that these 3D aggregates may serve as an early-stage obesity-like *in vitro* model, capturing aspects of the low-grade chronic inflammatory crosstalk characteristic of hypertrophic adipose tissue, and providing a valuable platform for studying adipocyte–macrophage interactions in metabolic dysregulation.

In Part 3 (Chapter 4), the focus shifted to scaffold-free 3D culture techniques—namely hanging drop (HD), liquid overlay (LOT), and magnetic levitation (ML)—to assess their suitability for constructing mature adipocyte spheroids in combination with endothelial cells. In addition, the integration of medium additives, particularly carrageenan used as MMC agent, was assessed to modulate the microenvironment of mature adipocyte cultures. Comparative analyses revealed that HD and LOT were more sensitive to medium composition, carrageenan supplementation, and cell type inclusion. Interestingly, LOT promoted viability and circularity but was more susceptible to cell dispersion under suboptimal conditions. In the long-term culture studies conducted for the first time using the MagLev system, it was observed that MagLev produced consistent morphology across experimental variables, but viability remained lower, likely due to the prolonged exposure to gadolinium-based media. However, the rapid compaction dynamics under magnetic force may offset this limitation by reducing culture duration. Unlike traditional applications of MMC during adipogenic induction, this study uniquely evaluated carrageenan post-differentiation. It was shown that carrageenan enhanced collagen deposition without compromising cell viability or lipid content, indicating its suitability for promoting ECM-rich, stable 3D spheroids. Furthermore, morphological

analyses demonstrated that carrageenan improved spheroid compactness, while co-culture with endothelial cells differentially influenced these outcomes depending on the technique. Importantly, carrageenan concentrations above 500 $\mu\text{g/mL}$ were avoided due to adverse effects on medium viscosity, which could hinder nutrient diffusion and mechanotransduction—parameters critical for adipocyte function. Overall, this part highlighted the importance of selecting appropriate 3D assembly platforms based on the intended structural, functional, and biological readouts.

In summary, this thesis demonstrates that scaffold-free platforms, particularly magnetic levitation and crowding agent supplementation, offer versatile tools to develop physiomimetic adipose tissue models. By leveraging cell-intrinsic properties, minimizing artificial components, and strategically tuning culture conditions, these strategies present a promising avenue for constructing 3D adipose models applicable in metabolic research, drug testing, and regenerative medicine.

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SELECTED PUBLICATIONS

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