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Three-Dimensional Adipocyte Culture as a Model to Study Cachexia-Induced White Adipose Tissue Remodeling

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TITLE:

Three-Dimensional Adipocyte Culture as a Model to Study Cachexia-Induced White Adipose Tissue Remodeling

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KEYWORDS:

Cellular Biology, 3D culture, Magnetic levitation, Adipose Tissue, UCP-1^{+} Adipocytes, Cell Differentiation, Beige/Brite cells, Primary adipocytes, Cachexia, LLC, Conditioned medium.

SUMMARY:

This protocol describes a three-dimensional (3D) magnetic printing culture system that permits dissection of beige remodeling induced by a conditioned medium from cancer cells. Using a 3D culture system of UCP1+ adipocytes that express green fluorescent protein (GFP) allows the study of beige adipocytes contributing to adipose tissue remodeling.

ABSTRACT:

Cancer cachexia (CC) presents itself as a syndrome with multiple manifestations, causing a marked multi-organ metabolic imbalance. Recently, cachectic wasting has been proposed to be stimulated by several inflammatory mediators, which may disrupt the integrative physiology of adipose tissues and other tissues such as the brain and muscle. In this scenario, the tumor can survive at the host's expense. In recent clinical research, the intensity of depletion of the different fat deposits has been negatively correlated with the patient's survival outcome. Studies have also shown that various metabolic disorders can alter white adipose tissue (WAT) remodeling, especially in the early stages of cachexia development. WAT dysfunction resulting from tissue remodeling is a contributor to overall cachexia, with the main modifications in WAT consisting of morpho-functional changes, increased adipocyte lipolysis, accumulation of immune cells, reduction of adipogenesis, changes in progenitor cell population, and the increase of "niches" containing beige/brite cells.

To study the various facets of cachexia-induced WAT remodeling, particularly the changes in the population of progenitor cells and beige remodeling, two-dimensional (2D) culture has been the first option for in vitro studies. However, this approach does not adequately summarize WAT complexity. Improved assays for the reconstruction of functional AT ex vivo are helpful for the comprehension of physiological interactions between the distinct cell populations. Here, this protocol describes an efficient three-dimensional (3D) printing tissue culture system based on magnetic nanoparticles. The protocol is optimized for investigating WAT remodeling induced by cachexia induced factors (CIFs). The results show that a 3D culture is an appropriate tool for studying WAT modeling ex vivo and may be useful for functional screens to identify bioactive molecules for individual adipose cell populations applications and aid the discovery of WAT-based cell anticachectic therapy.

INTRODUCTION:

Living organisms are composed of cells that exist in 3D microenvironments with cell-cell and cell-matrix interplay and elaborate transport dynamics for nutrients and cells[1,2]. However, most of the fundamental knowledge gained in cell biology has been generated using monolayer cell culture (2D). Although 2D culture can answer some of the mechanistic questions, this approach inadequately recapitulates the natural environment within which cells reside and may be incompatible with predicting a complex drug response[1]. Moreover, cells sense their physical surroundings through mechanotransduction. Indeed, mechanical forces are translated to biochemical signals that ultimately influence gene expression patterns and the cell's fate. In the last few decades, 3D tissue culture has emerged as a new in vitro tool that can mimic the in vivo microenvironment with greater fidelity. This can avoid some mechanistic pitfalls generated by in vitro 2D approaches[3].

Cancer cachexia (CC) is defined as a syndrome with multiple manifestations, causing a marked multi-organ metabolic imbalance. During the development of cachexia, WAT undergoes multiple morphological changes resulting in an increased adipocyte lipolysis, accumulation of immune cells, reduction in adipogenesis, changes in progenitor cell population, and an increase in "niches" containing beige/brite cells (beige remodeling)[4]. However, recapitulating the mechanism by which cachexia effects WAT remodeling using in vitro models presents a significant technical challenge. Indeed, a few studies that attempted investigation of tumor/tissue communication have used monolayer in vitro cell culture (2D), circumventing the complexity of the 3D microenvironment of WAT.

Although there are several experimental approaches to generate 3D culture, three different assembly methods are preferred to produce adipospheroids: magnetic levitation or printing[5], hanging drop[6], and Matrigel-scaffold systems[7]. Despite being appropriate for adipospheroids, these systems have advantages and disadvantages and should be chosen according to the characteristics of each experimental design. Based on the limitations mentioned above, the magnetic printing method was used to generate 3D cell cultures[5]. This method uses a magnetic nanoparticle assembly consisting of gold nanoparticles and iron oxide, making the printing

method suitable for most cell types. Here, 3D cell cultures were used to induce adipogenesis, and CIFs were used to reproduce the environmental condition in CC.

PROTOCOL:

1. Incubation of 2D cells with magnetic nanoparticles

1.1. Grow adherent 2D cultures to ~ 70% confluence using standard cell culture procedures.

1.2. Prepare the magnetic nanoparticle assembly. Take it out of the refrigerator and let it warm to room temperature (20–25 °C) for about 15 min[1].

1.3. Mixed medium: Add the magnetic nanoparticles directly to 12 mL of medium in 100 mm cell culture plates. Suspend and resuspend the medium a few times to obtain a homogeneous distribution of the nanoparticles.

NOTE: The medium will appear dark because of the brown color of the iron oxide. A concentration of 2.5 μL/cm^2 of the culture area is recommended.

1.4. Wash the 100 mm 2D culture plate three times with phosphate-buffered saline (PBS).

1.5. Add 12 mL of mixed medium from step 1.3 to the 100 mm cell culture plates. Incubate the plates overnight in an incubator (37 °C, 5% CO_2) to allow attachment of the magnetic nanoparticles to the cells

2. Creating 3D cultures with spheroid assembly in 96-well plates

2.1. After overnight incubation, wash the cells to remove any residual medium and unattached magnetic nanoparticles by gently agitating the plates with 3 x 10 mL of PBS.

2.2. Aspirate the PBS from the Petri dish and detach the cells by incubation with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution for 2–5 min at 37 °C.

2.3. While waiting for the cells to detach, disinfect the magnetic drives with 70% ethanol[1].

2.4. After the cells have detached, add serum-containing medium at 4x the volume of the trypsin-EDTA solution to neutralize trypsin's effect, and then transfer the suspension into a conical tube.

2.5. Centrifuge the suspension at 500 × *g* for 10 min. Aspirate the supernatant, taking care not to touch the pellet.

NOTE: After centrifugation, the cells should appear brown, and cell suspensions in medium should appear darker than usual. Cells should appear peppered with the nanoparticles[1].

2.6. Count the cells in suspension, and calculate the volume of medium volumes needed to create 3D cultures. For adipospheroids, use 5,000 to 10,000 cells in 150 µL in 96-well plates.

2.7. Using a 96-well bioprinting kit, place a cell-repellent 96-well plate at the top of the Spheroid Drive.

2.8. Pipette 150 µL of cell suspension into each well of the 96-well plate, and close the plate to allow the cells to aggregate at the bottom in the shape of a magnet.

2.9. Leave the plate on the drive in the incubator for 1–2 h to yield a competent spheroid.

NOTE: These cultures should appear dense and brown and should be printed in the plate (**Figure S1**). **Figure S2** presents a summary workflow of the main steps of 3D magnetic printing of spheroid assembly in 96-well plates.

3. White adipogenesis induction

3.1 Prepare maintenance and induction media[8]; prepare induction medium before each use.

3.1.1. Prepare maintenance medium containing 5 µg/mL of insulin (10 mg/mL stock stored at 4 °C for one week) and 0.5 µM rosiglitazone (10 mM stock in dimethyl sulfoxide (DMSO)).

3.1.2. Prepare induction medium containing 125 µM indomethacin from a 0.125 M stock in ethanol, 2 µg/mL of dexamethasone from a 2 mg/mL stock in ethanol, 0.5 mM isobutyl-1-methylxanthine (IBMX) from a 0.25 M stock in DMSO, and 0.5 µM rosiglitazone from a 10 mM stock in DMSO.

NOTE: Heat indomethacin to 60 °C to dissolve.

3.2. After 24–48 h of printing spheroids, replace the regular complete medium with induction medium (day 0).

3.3. After 48 h (day 2), replace the induction medium with maintenance medium.

3.4. Change the medium every 3–5 days until the cells are fully differentiated.

NOTE: Generally, after 7–8 days of stimulation with the induction medium, cells differentiate into mature fat cells and are filled with oil droplets that can be viewed at the edges of the adipospheroids.

4. Production of Lewis lung carcinoma conditioned medium (LLC-CM)

4.1. Seed Lewis lung carcinoma (LL/2) cells in 100 mm cell culture plates in growth medium at a density of 6000 cells/cm^2.

NOTE: Growth medium contains Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin-streptomycin.

4.2. After 2 days, replace the medium in each plate with fresh growth medium.

NOTE: LL/2 cells contain a heterogeneous mix of adherent (higher number) and floating cells.

4.3. After 2 days (day 4), harvest the conditioned medium, and clear it of cells and debris by centrifugation (500 × *g*, 10 min).

4.4. Freeze aliquots of the conditioned medium in liquid nitrogen for later use.

NOTE: For treatment of spheroids with conditioned medium, use a combination of 75% fresh growth medium and 25% LLC-conditioned medium.

REPRESENTATIVE RESULTS:

Adipospheroids from 3D culture of stromal vascular fraction (SVF) cells

Both 3D and confluent 2D cultures were set up with the same numbers of SVF cells from the same mouse inguinal WAT preparation (**Figure 1A,B**) and subjected to the same experimental protocol to allow comparison of marker gene expression. Spheroids stimulated with induction medium expanded over time. **Figure 2B** shows an increase in the density of 2D multilocular cells, indicating the differentiation to mature adipocytes. Quantitation of adipospheroid volume showed expansion (~1-fold) after 10 days following the initiation of adipogenesis, whereas those in non-differentiating growth media (DMEM 2%) did not expand (**Figure 1B** (3D-WHITE), **Figure 1C**). Next, gene expression of adipospheroids was compared to that of 2D culture. The gene expression of mature adipocyte marker genes, such as *Adipoq* and *Fabp4*, extracellular matrix (ECM) *Fn1* and *Col4a1*, and thermogenic markers, *Ucp-1* and *Pgc1a,* were detected in both 2D and 3D cultures (**Figures 2A-C**). Adipospheroids expressed higher levels of mature adipocyte markers and ECM markers than non-differentiated 2D (DMEM 2%). Immunofluorescence analysis of paraffin sections of adipospheroids revealed robust expression of perilipin-1, a marker of mature lipid droplets, in the medium (**Figure 2C** (WHITE), **Figure S3**).

Induction of adiposheroid remodeling by LLC-CM

The induction of WAT remodeling in response to CC has been previously described[9-11]. In 2D culture, the impairment of adipogenesis induced by the addition of CM with secreted factors from LLC tumor cells has been previously described[12]. Co-culture of 3T3-L1 cells with LLC cells reduced white adipogenesis and adiponectin secretion and upregulated *IL-6* gene expression and protein synthesis[12]. In this respect, cell culture with LLC-CM, in both systems, showed a reduction in terminal differentiation. Adipospheroids treated with LLC-CM showed a lower increase (~58%) in their total area (**Figure 1C**) after 10 days following adipogenesis induction. This condition was

accompanied by a lower expression of mature adipocyte markers when compared to WHITE cells (**Figure 2A,B**) in both 2D and 3D cultures. However, gene expression of thermogenic markers was higher in LLC-CM (**Figure 2C**). An overall 2.6-fold increase in *UCP1* mRNA levels was observed in adipospheroids compared to those in 2D cultures; a potential, functional property of factors that regulate beige adipogenesis. Finally, this study examined whether printed spheroids can be used as an additional model of WAT remodeling. To address this question, SVF cells from Ucp1 $Cre^{+}/mTmG^{+}$ mice were used as expression of GFP is an indicator of *UCP1* transcription and beige adipogenesis[13]. Cells were magnetically printed (3D), and adipogenesis was induced with and without LLC-CM (**Figure 3**). Adipospheroids cultivated with LLC-CM showed an increase in the number of GFP-positive cells (*UCP-1* expression), which is absent in adipospheroids treated only with differentiation medium without LLC-CM.

FIGURE AND TABLE LEGENDS:

Figure 1: Adipocytes in 3D culture of primary WAT cells. Adipocyte morphology in different culture conditions. (**A**) Schematic of differentiated adipocytes as a monolayer (2D) on tissue-culture plastic (top) and using a 3D magnetic printing system (bottom). (**B**) The stromal vascular fraction of mouse inguinal WAT ($5\cdot\times 10^4$ cells) was used to start cultures. Phase-contrast images of differentiated adipocytes in the three different culture conditions: DMEM—Uninduced spheroids (left); adipogenesis-induced spheroids (center); and LLC-CM medium grown in the same conditions (right). (**C**) Increase in the volume of the sphere (πr^2) for DMEM—Uninduced spheroids. Abbreviations: LLC = Lewis Lung carcinoma; CM = conditioned medium; WAT = white adipose tissue. Scale bars are 100 µm.

Figure 2: Quantitative real-time PCR analysis of WAT remodeling and thermogenic genes induced by LLC-CM in 2D and 3D cultures. Expression of (**A**) mature adipocyte; *Fabp4* and *Adipoq*, (**B**) ECM, *Fn1* and *Col4a,* and (**C**) thermogenic, *Ucp-1*, *Pgc1a*, markers. After 10 days of uninduced (DMEM as growth medium), and adipogenesis-induced (induction medium WHITE) and WHITE + LLC-CM medium grown in the same conditions, total RNA was extracted from differentiated cells. Reverse transcription was performed using a cDNA reverse transcription kit, and quantitative reverse-transcription PCR was performed in duplicate with SYBR green fluorescent dye. Cyclophilin was used as the reference housekeeping gene. (**D**) After 10 days of adipogenesis induction, paraffin sections of spheroids were subjected to immunofluorescence analysis with perilipin antibodies (red), indicating lipid droplet maturation in adipospheroids upon culture in the adipogenesis induction medium containing LLC-CM. Abbreviations: CM = conditioned medium; DAPI = 4',6-diamidino-2-phenylindole (to stain DNA blue); WAT = white adipose tissue; ECM = extracellular matrix. Bars are mean ± standard error of the mean. Ordinary one-way analysis of variance was used to compare unstimulated (DMEM 2%) 2D versus 3D cultures from the same tissue source and media condition with *Sidak* correction for multiple comparisons, n = 3, *$p < 0.05$). Scale bars are 50 µm.

Figure 3: Visualization of adipospheroids formed by primary WAT cells from Ucp1 $Cre^{+}/mTmG^{+}$ mice. The stromal vascular fraction of mouse inguinal WAT ($5\cdot\times 10^4$ cells) was used to start 3D magnetic printing cultures. Adipogenesis induction was done in spheroids for 10 days in the

following experimental conditions: WHITE (CONTROL = induction medium) or WHITE+LLC-CM medium. After 10 days of adipogenesis induction, whole mounts were subjected to immunofluorescence. The presence of green (GFP-positive) cells in Ucp1 Cre^{+}/$mTmG^{+}$ images indicates transcription from the *UCP1* gene promoter (beige adipogenesis). Scale bars are 100 µm. Abbreviations: LLC = Lewis Lung carcinoma; CM = conditioned medium; DAPI = 4′,6-diamidino-2-phenylindole (to stain DNA blue); WAT, white adipose tissue.

Figure S1: Creating 3D cultures with spheroid printing in 96-well plates. Competent spheroid on the drive in the incubator after 1 day (24 h). Scale bar = 100 µm.

Figure S2: Workflow for 3D culture using a magnetic printing system. 1. Isolate cells from the SVF according to the standard protocol. When the cells are 70% confluent, add culture medium containing magnetic beads. 2. Incubate in an incubator (37 °C, 5% CO2) overnight. 3. After the incubation period, detach the cells, and add the desired number of cells in 96-well plates. Immediately, place a cell-repellent 96-well plate at the top of the spheroid drive, and place (plate plus Spheroid Drive) it in the incubator for 1–2 h. 4. Start the induction of differentiation (adipogenesis) after 24–48 h of printing the spheroids. Abbreviations: 3D = three-dimensional; SVF = stromal vascular fraction.

Figure S3: Perilipin staining in 3D culture. Paraffin sections of spheroids subjected to immunofluorescence analysis with perilipin antibodies (red), indicating lipid droplet maturation in adipocytes present in the spheroids upon culture in the adipogenesis induction medium containing Lewis Lung carcinoma-conditioned medium. Scale bar = 20 µm.

DISCUSSION:

This protocol sets up a 3D cell culture system to study the development of adipocytes in adipospheroids derived from primary WAT cells of mice. Compared to conventional 2D adherent culture, this 3D system facilitates the study of AT remodeling that more closely resembles in vivo conditions. In the last few years, studies have shown that culturing cells in 3D yields distinct cellular morphology and signaling compared to a 2D culture system[3]. Fibroblast morphology in 3D is different from that found in 2D[14]. In mammary epithelial cells, 3D culture can induce tissue-specific differentiation[15]. The investigation of multicellular drug resistance in mammary carcinoma cells is only efficient when analyzed in cells grown in 3D compared to evaluation performed with traditional 2D cultures[16].

CC is a very complex syndrome, and in vitro models for mechanistic studies are scarce. Lopes et al.[12] showed in a co-culture system that LLC compromised adipogenesis, as indicated by a decreased volume of lipid droplets in 3T3-L1 cells in vitro. This study showed that cells treated with LLC-CM demonstrated the same attenuation of adipogenesis in both 2D and 3D culture systems. However, despite the impairment of adipogenesis, an increase in the expression of major WAT remodeling and thermogenic markers was observed in adipospheroids. Currently, beige remodeling has been described as a prevalent phenotype of AT remodeling induced by CC. However, this phenotype is demonstrated only in vivo or ex vivo, and there is no description of beige adipogenesis in vitro. Hence, differentiation of cells from Ucp1 Cre^{+}/$mTmG^{+}$ mice was

induced in a 3D magnetic printing system in the presence of LLC-CM. An increase in GFP-positive cells, and hence, *Ucp-1* transcription was observed in response to LLC-CM, a fact that corroborates the browning description in response to cachexia (ex vivo).

This is the first study using a 3D adipocyte culture to induce WAT remodeling in CC. Magnetic bioprinting is an efficient tool as a non-scaffold 3D culture system. Moreover, the 3D system may provide a more physiologically relevant microenvironment than 2D culture. Additionally, adipospheroids can be used for large-scale studies with different tumor types and drug screening analyses. Another innovative approach was to use SVF from Ucp1 Cre^{+}/$mTmG^{+}$ mice and adapt them to 3D culture. Such a system can be extended to primary cells derived from other lineage-tracing animal models.

A limitation to be considered is that magnetic bioprinting could interfere with cellular functions, and this should be assessed on a case-by-case basis. Many other 3D spheroid-generating methods can be employed to construct adipospheroids, including non-scaffold 3D culture, such as hanging drop systems[6], and scaffold 3D culture[7]. Unlike these methods, which require specialized equipment or reagents, the procedure described here is fast and practical for manipulating adipospheroids for subsequent experiments. The simplicity of the method minimizes potential pitfalls. Whether the 3D culture system has advantages over functional assays, such as cachexia-induced lipolysis and/or lipogenesis, needs further analysis. Finally, the current method provides a robust and reliable experimental system to study WAT remodeling in vitro, leading to various applications such as investigating the dose-dependent effects of a particular drug in a specific cell type of interest to aid in the discovery of novel therapeutic interventions for CC.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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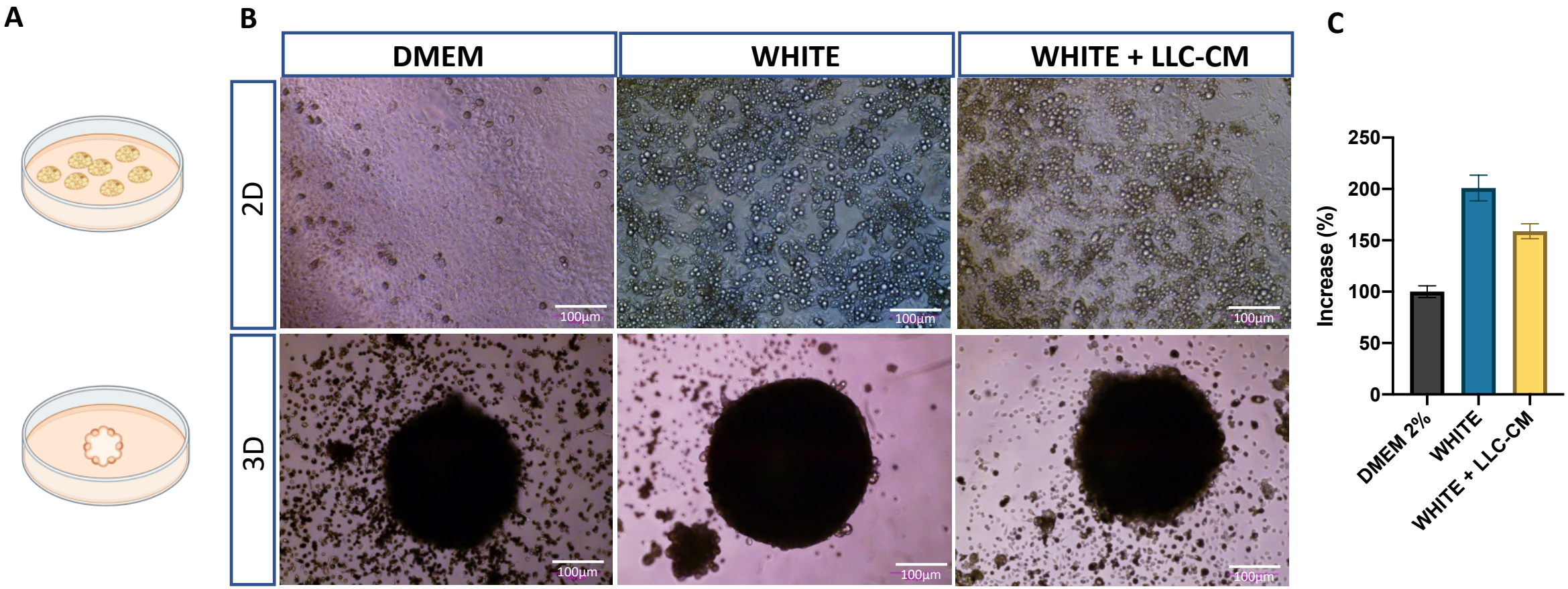
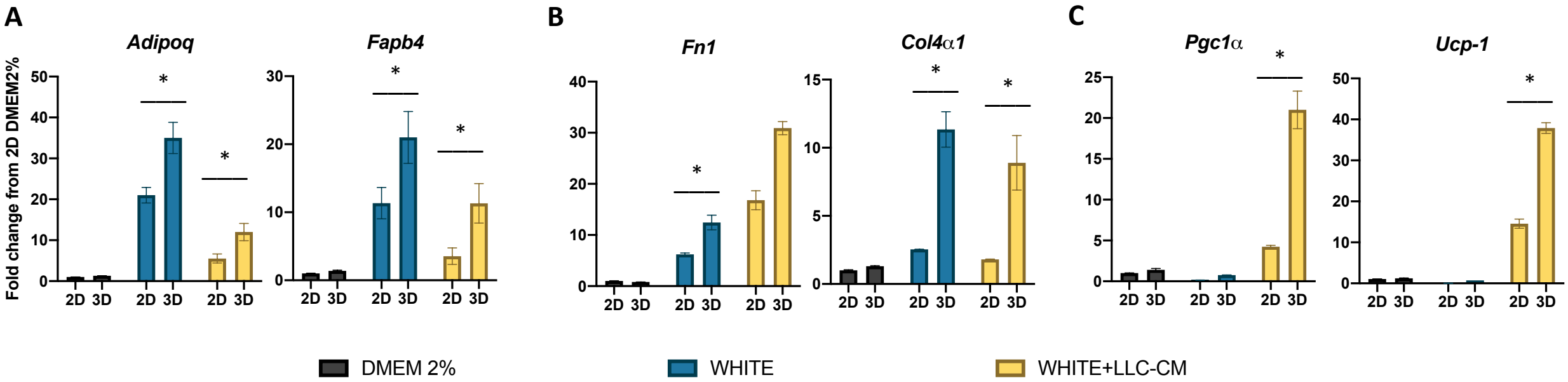


Figure 2



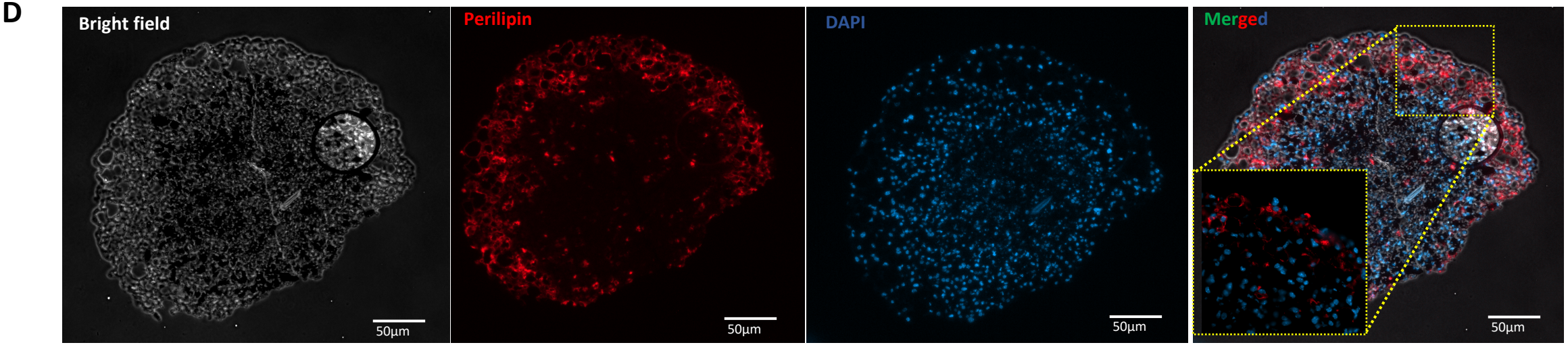


Figure 3

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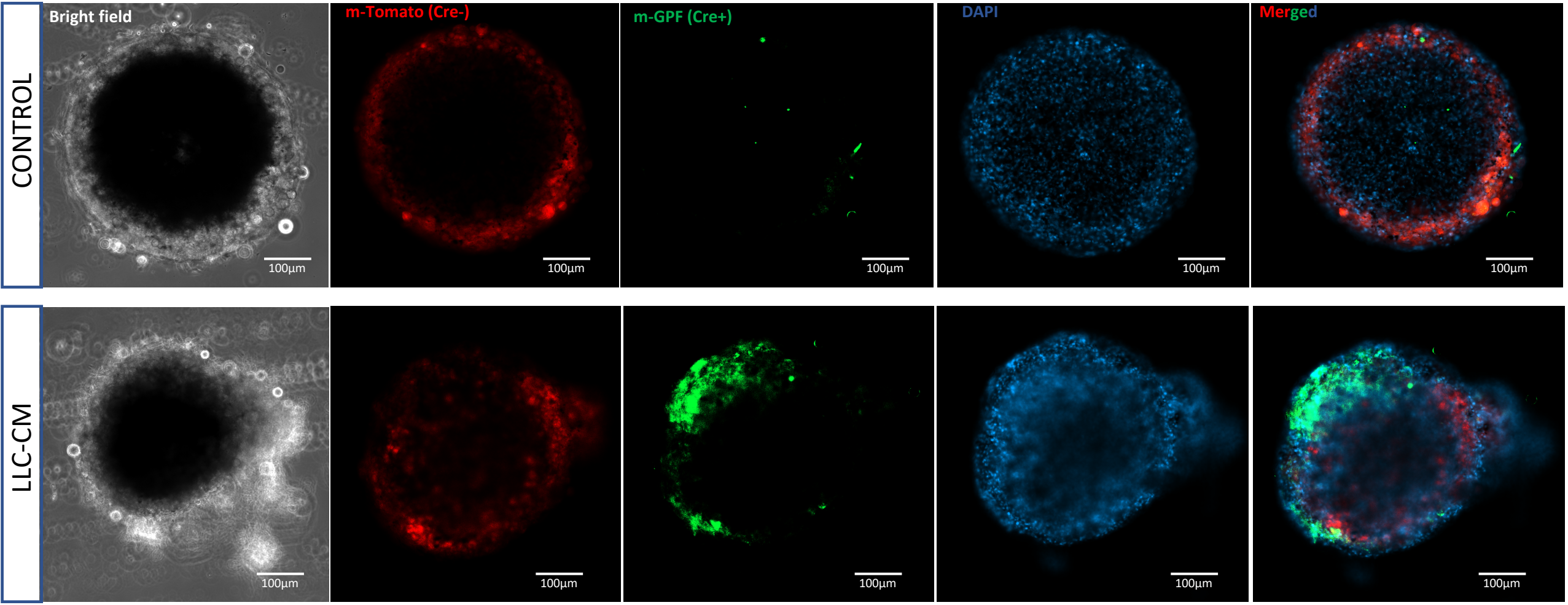

Name of Material/ Equipment

3-Isobutyl-1-methylxanthine
96-Well Bioprinting Kit, black
Alexa Fluor 647 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)
CELL CULTURE MICROPLATE, 96 WELL, PS, F-BOTTOM, µCLEAR, BLACK, CELLSTAR, CELL-REPELLENT SURFACE, LID, STERILE, 8 PCS./BA
Dexamethasone
DMEM
Fetal Bovine Serum (Tova)
Indomethacin
Insulin

LL/2 (LLC1) (ATCC CRL-1642)
NanoShuttle-PL
NucBlue Fixed Cell ReadyProbes Reagent (DAPI)
Pen strep

Perilipin-1 (D1D8) XP Rabbit mAb
Rosiglitazone
Trypsin-EDTA, 0.05%

Reverse-transcription PCR primers
Primer
Adipoq
Col4a1
Cyclophilin a
Fabp4
Fn1
Pgc1a
Ucp1

Mouse genotyping
Primer name

Cre F
Cre R
oIMR7318
oIMR7319
oIMR7320
WH336
WH337
WH338
WH339

Company	Catalog Number	Comments/Description
Sigma-Aldrich (St. Louis, MO, USA)	I-5879	Cell culture
Greiner (Monroe, NC, USA)	655841	Cell culture
Jackson ImmunoResearch	711-606-152	Immunofluorescence staining, secondary, 1:400 in TBS with 0.1% Twee
Greiner (Monroe, NC, USA)	655976	Cell culture
Sigma-Aldrich (St. Louis, MO, USA)	D-1756	Cell culture
Corning (Manassas, VA, USA)	10-017-CV	Cell culture
Gemini Bio (West Sacramento, CA)	100-500	Cell culture
Sigma-Aldrich (St. Louis, MO, USA)	I-7378	Cell culture
Sigma-Aldrich (St. Louis, MO, USA)	I0516	Cell culture
American Type Culture Collection (Manassas, VA, USA)	CRL-1642	Lewis Lung Carcinoma cell line
Greiner (Monroe, NC, USA)	657843	Cell culture
ThermoFisher (Waltham, MA, USA)	R37606	Immunofluorescence staining, following the manufacturer's instruction
Corning (Manassas, VA, USA)	30-002-CI	Cell culture
Cell Signaling Technology (Danvers, MA, USA)	9349	Immunofluorescence staining, primary, 1:1000 in TBS with 0.1% Tween
Sigma-Aldrich (St. Louis, MO, USA)	R-2408	Cell culture
Corning (Manassas, VA, USA)	25-052-CI	Cell culture

Forward	Reverse
GTTCCCAATGTACCCATTCGC	TGTTGCAGTAGAACTTGCCAG
TCCAAGGGCGAAGTGGGTTT	ACCCTTGCTCGCCTTTGACT
ATGGCACTGGCGGCAGGTCC	TTGCCATTCCTGGACCCAAA
TGGTGACAAGCTGGTGGTGGAATG	TCCAGGCCTCTTCCTTTGGCTCA
GCTTCCCCAACTGGTTACCCT	GGGTTGGTGATGAAGGGGGT
GAAAACAGGAACAGCAGCAGAG	GGGGTCAGAGGAAGAGATAAAG
TCCTAGGGACCATCACCACCC	AGCCGGCTGAGATCTTGTTTCC

Description	Sequence	Reference

Generic Cre forward	GCG GTC TGG CAG TAA AAA CTA TC	
Generic Cre reverse	GTG AAA CAG CAT TGC TGT CAC TT	
mT/mG forward	CTC TGC TGC CTC CTG GCT TCT	
mT/mG wild type reverse	CGA GGC GGA TCA CAA GCA ATA	
mT/mG mutant reverse	TCA ATG GGC GGG GGT CGT T	
UCP1 mutant forward	CAA TCT GGG CTT AAC GGG TCC TC	ncedirect.com/science/article/pii/S15504131
UCP1 mutant reverse	GTT GCA TCG ACC GGT TAA TGC AG	
UCP1 wild type forward	GGT CAG CCT AAT TAG CTC TGT	
UCP1 wild type reverse	GAT CTC CAG CTC CTC CTC TGT C	https://www.jax.org/strain/00767

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s

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October 19th, 2020

Dr. Nam Nguyen

JoVE

Dear Dr. Nguyen:

Thank you for forwarding the peer-review reports for our manuscript (VE61853) entitled **" ADIPOCYTE 3D CULTURE AS A MODEL TO STUDY CACHEXIA-INDUCED WHITE ADIPOSE TISSUE REMODELING "** by Batista Jr *et al*.

We would also like to thank the reviewers for their insightful comments and critiques of our manuscript. Their valuable comments and suggestions are much appreciated and have greatly helped us to improve the manuscript.

Considering the set of comments and suggestions, we chose to reorganize the manuscript and added some new analyzes. In this sense, we decided to highlight the advantages of the 3D vs. 2D model about the general parameters of WAT remodeling without emphasizing beige remodeling.

We hope you and the reviewers agree that our revised manuscript is now suitable for publication in Immunometabolism.

Sincerely,

Miguel Luiz Batista Júnior
Biochemistry Department
Boston University School of Medicine

Editorial comments:

Changes to be made by the Author(s):

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please use original language throughout the manuscript. Please revise the following lines: 38-41, 52-54, 63-64, 170-172,

Response: *Most of the overlaps are related to the description of the methods. Besides, they are all referenced appropriately. However, as far as possible, we changed words and substituted synonyms.*

3. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
Response: *In this new version, we added more details, and as suggested by reviewer 2, we added a Figure (S3) with the workflow for magnetic printing.*

4. 1.4: What are the incubation settings?
Response: *This information was updated.*

5. 2.2: How much trypsin-EDTA is used? Incubate at what temperature as well?
Response: *This information was updated.*

6. 4.3: What happens after centrifugation? Aspiration?
Response: *This information was updated.*

37. Please spell out all journal titles in the references.
Response: *This information was updated.*

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the manuscript "ADIPOCYTE 3D CULTURE AS A MODEL TO STUDY CACHEXIA-INDUCED BEIGE REMODELING OF WHITE ADIPOSE TISSUE " Batista et al. propose to improve methodology to study cachexia-induced AT remodeling. They describe a protocol for efficient three-dimensional (3D) printing tissue culture system based on magnetic nanoparticles. They optimized protocol for investigating white/beige AT remodeling. Their results suggest that 3D culture is an appropriate for studying AT remodeling ex vivo and may be useful for functional screens to identify molecules bioactive toward individual adipose cell populations applications and aid approaches to AT-based cell anticachectic therapy.

It is a potentially useful protocol and a well written manuscript. However, several conceptual and technical issues must be resolved.

Major Concerns:

First, while cancer-induced AT beiging and cachexia is observed in mice, there is no credible evidence that it happens in humans. Cancer clearly induces lipolysis in AT. In this manuscript, lipolysis is not mentioned. This must be fixed. In addition to discussing lipolysis, it's markers (lipase expression, glycerol / FA release) should be measured upon LLC CM exposure and compared for 2D vs 3D cultures.

Response: *We agree with this comment. Concerning cachexia-induced lipolysis, this item was added to the text. We also found the suggestion to compare some lipolysis markers between the two cultures exciting and timely. However, this manuscript has given priority to defining morphological changes at the expense of functional ones. It was highlighted in the discussion section as a limitation of the study.*

Line 189" "2D co-culture with LLC cells resulted in a reduction in white adipogenesis and an increase in beige adipogenesis (data not shown)". How was this measured? Evidence for beiging would be key for the paper with this title and conclusions. Data must be shown and be convincing. Beige adipocytes contain more mitochondria than white, which needs to be demonstrated for LLC CM culture. They also feature uncoupling, which could be measured by Seahorse. Without this evidence, the beiging conclusion does not stand.

Response: *Regarding the issue (Line 189), we removed this information since the presentation of these results is no longer part of this manuscript's scope. In this sense, we reformulated the manuscript to emphasize WAT remodeling. In this context, beige remodeling is treated as one of the changes that make up the entire remodeling process.*

Minor Concerns:
In Figure 3, only 3D data are presented. What is observed in 2D? If the same UCP1 induction is observed, what is the advantage of using this 3D model?

Response: *Indeed**,** Figure 3 shows only 3D culture data. The differential of this assay was to use the SVF of UCP1+ animals. In this new version, we present data on the gene expression related to; mature adipocytes (Adipoq and Fabp4), EMC components (Fn1 and Clo4a1), and beige adipocytes (Pgc1a and Ucp-1). For qPCR analysis, the fold-change was analyzed from 2D DMEM2% experimental condition. This further analysis shows that the 3D model's advantages are apparent, at least regarding the evaluated parameters.*

"LLC-CM induce an increase in SFV" is not a conclusion that can be made from the data and is inappropriate for section heading.
Response: *This comment was updated.*

Line 193: " we SFV cell": please fix the text.
Response: *It was corrected.*

It is not clear why the method used (Refs 7 and 4) is referred to as "magnetic printing". There is no printing involved in the published magnetic levitation approach to adipospheroids formation. Thus, reference call out should not discuss printing. Because the 96-Well Bioprinting Kit 655841 is used here, it should be better explained how the printing component changes the procedure from what had been published.
Response: *It was properly updated. We also add a Figure (S3) with the workflow for magnetic printing.*

Reviewer #2:
This paper describes a three-dimensional (3-D) preadipocyte culture, which turned out to be effective to study beige fat formation in white adipose tissues. The manuscript is well written, and the protocol and findings are presented very well. I have a few minor comments.

1. For readers who are not familiar with the method of magnetic 3-D bioprinting, please include a figure that explains the method.
Response*: We added Figure S3 detailing the workflow to magnetic 3-D bioprinting.*

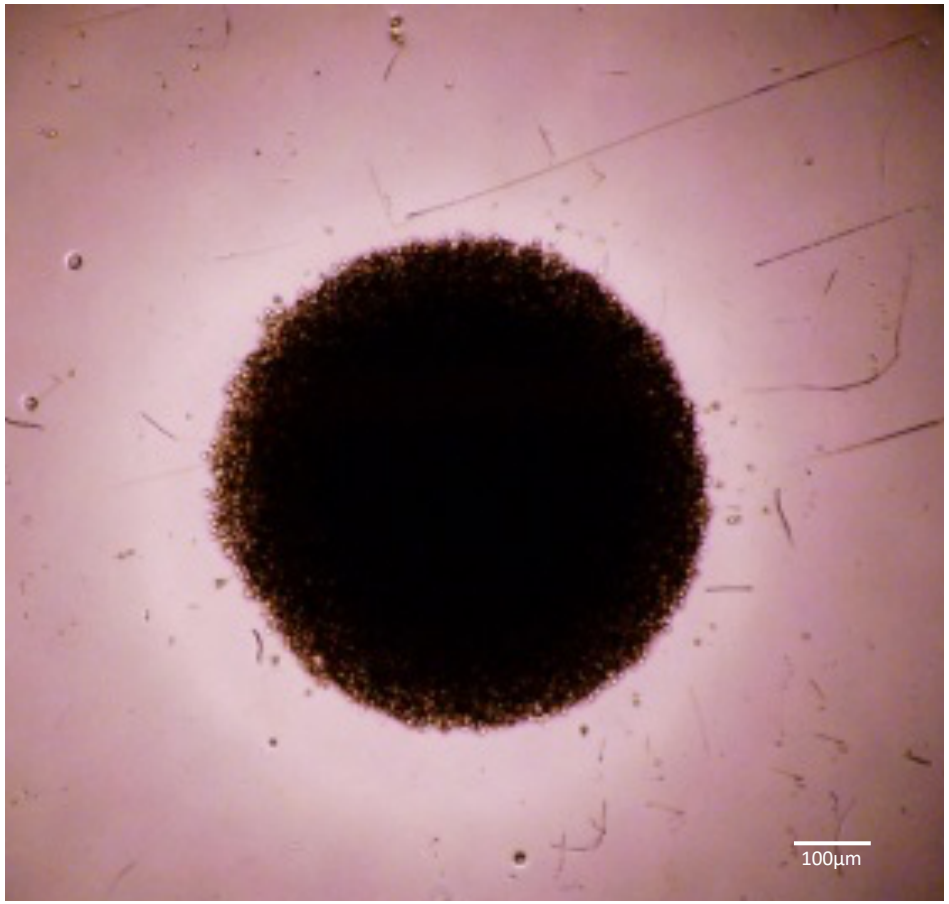
2. Do the magnetic beads remain in the spheroids? If yes, how many beads remain in the spheroid?
Response: *Yes, they remain in the spheroids. Considering the size of the nanobeads, I think this count is not so trivial. Nor is it a usual procedure described in studies using this technique. What we did was to optimize the concentrations of magnetic beads recommended by the supplier. In this sense, we reduced the need for these by more than 60%. That is, we use the minimum concentration necessary for the formation of the spheroids*

3. Please discuss the relationship between the diameter of spheroids and number of cells per spheroid.
Response: *In particular, in the 3D culture for adipocytes, during the cell differentiation process (adipogenesis), the most prevalent event is the increase in the accumulation of intracellular lipids at the expense of the rise in the number of cells, measured by quantifying total proteins (https://doi.org/10.1007/s10439-018-1993-y).*

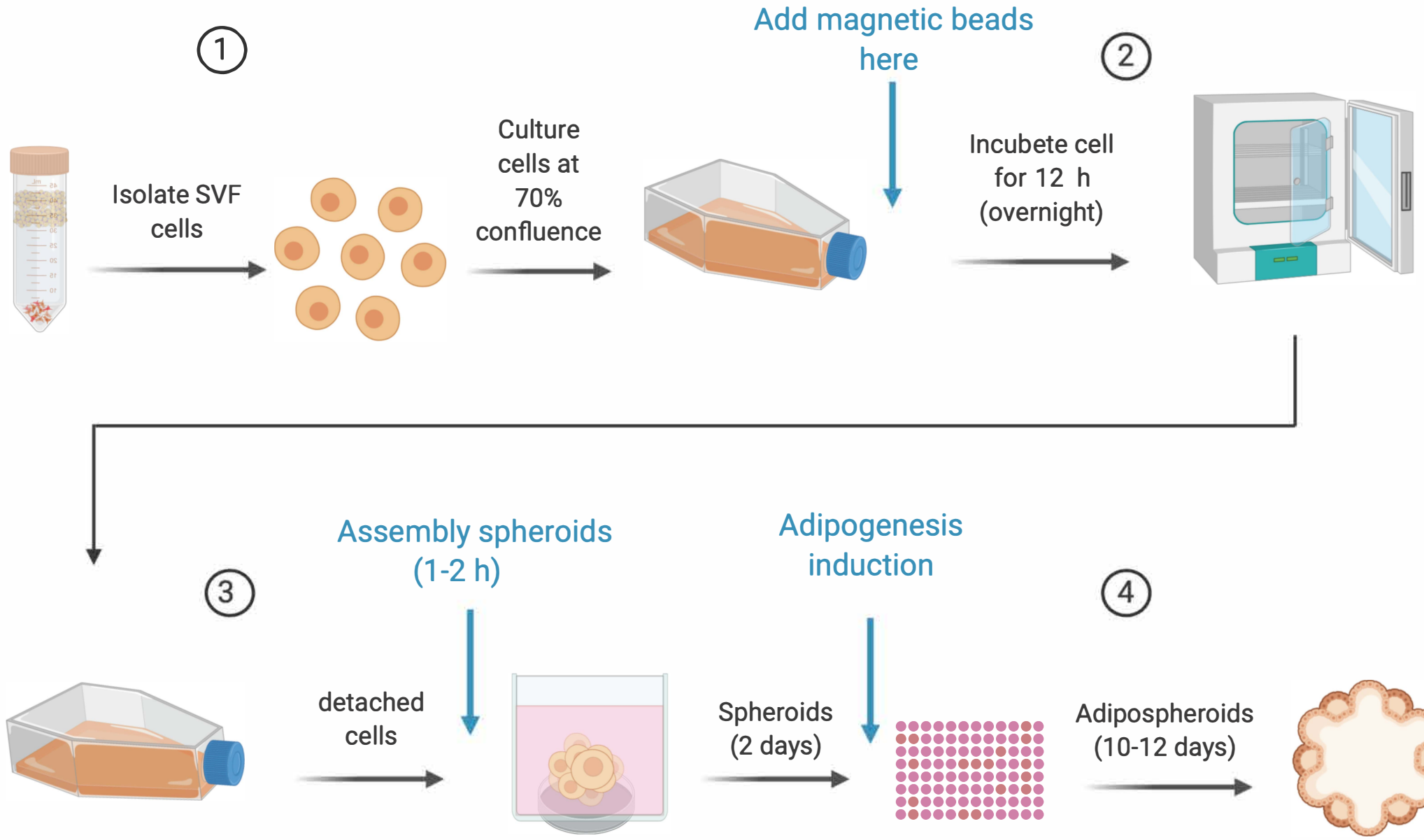
4. Did authors examine cell death and proliferation within the spheroids? It would be informative to know the degree of cell death in spheroids in determining the ideal number of cells per spheroid for individual experiment.
Response: *It is an interesting question. We didn't evaluate cell death and proliferation parameters. However, we use the concentration of cells and experimental conditions that have already been used in other studies to ensure the "health" of the spheroids (DOI:10.1038/s41598-017-19024-z). Still, in this aspect, Figure 2D shows an excellent density of positive nucleus marking, which suggests the absence of an adipospheroids necrotic center.*

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.pdf



Supplemental Figure 2



Supplemental Figure 3