Journal of Visualized Experiments

A human 3D extracellular matrix-adipocyte culture model for studying matrix-cell metabolic crosstalk

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60486R1
Full Title:	A human 3D extracellular matrix-adipocyte culture model for studying matrix-cell metabolic crosstalk
Section/Category:	JoVE Biology
Keywords:	adipocyte extracellular matrix adipose tissue glucose uptake insulin resistance cellular metabolism
Corresponding Author:	Robert O'Rourke University of Michigan Ann Arbor, MI UNITED STATES
Corresponding Author's Institution:	University of Michigan
Corresponding Author E-Mail:	rorourke@med.umich.edu;rorourke@umich.edu
Order of Authors:	Carmen G Flesher
	Nicki A Baker
	Clarissa Strieder-Barboza
	Dominic Polsinelli
	Phillip J Webster
	Oliver A Varban
	Carey N Lumeng
	Robert W O'Rourke
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ann Arbor, Michigan, United States

Cover Letter

University of Michigan Health System

Robert W. O'Rourke, MD
Professor of Surgery
Division of Minimally Invasive Surgery
Section of General Surgery

Department of Surgery
1500 East Medical Center Drive
2210 Taubman Center, SPC 5354
Ann Arbor, MI 48109-5354
(734) 936-7607

rorourke@umich.edu

August 19, 2019

Phillip Steindel, Ph.D.
Review Editor, JoVE

Dear Dr. Steindel and Editors and Reviewers of *J Vis Exp,*

Please accept this submission of our revised manuscript "A human 3D extracellular matrix-adipocyte culture model for studying matrix-cell metabolic crosstalk", by authors Flesher et al., for consideration for publication in *J Vis Exp*.

We appreciate the reviewers' thoughtful, thorough, and professional critiques. We have significantly revised the manuscript, added substantial additional data, and addressed each concern to the best of our ability in the revised manuscript and the point-by-point response below. We feel that these changes greatly strengthen the manuscript and we thank the reviewers for their suggestions.

We have included an email from Oxford University Press permitting republication of figure 4 of this revised manuscript, which was originally published in Baker et al., *J Clin Exp Metab* 2017.

The authors have no conflicts of interest. This work has not been previously published. We appreciate your attention to this manuscript and we look forward to your decision.

Sincerely,

Robert W. O'Rourke, MD

TITLE:
A Human 3D Extracellular Matrix-Adipocyte Culture Model for Studying Matrix-Cell Metabolic Crosstalk

AUTHORS & AFFILIATIONS:
Carmen G. Flesher[1], Nicki A. Baker[1], Clarissa Strieder-Barboza[1,2], Dominic Polsinelli[6], Phillip J. Webster[1,2], Oliver A. Varban[1], Carey N. Lumeng[2,3,4], Robert W. O'Rourke[1,7]

[1]Department of Surgery, University of Michigan Medical School, Ann Arbor, MI, USA
[2]Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI, USA
[3]Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, MI, USA
[4]Graduate Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI, USA
[6]Undergraduate Research Opportunity Program, University of Michigan, Ann Arbor, MI, USA
[7]Department of Surgery, Ann Arbor Veterans Affairs Healthcare System, Ann Arbor, MI, USA

Corresponding author:
Robert W. O'Rourke rorourke@med.umich.edu

E-mail addresses of co-authors:
Carmen Flesher (cflesher@med.umich.edu)
Nicki Baker (nickiba@med.umich.edu)
Clarissa Strieder-Barboza (cstriede@umich.edu)
Dominic Polsinelli (dompols@umich.edu)
Oliver Varban (ovarban@med.umich.edu)
Carey Lumeng (clumeng@med.umich.edu)
O'Rourke, Robert (rorourke@med.umich.edu)

KEY WORDS
adipocyte, adipose tissue, extracellular matrix, diabetes, glucose uptake, obesity

SUMMARY
We describe a 3D human extracellular matrix-adipocyte in vitro culture system that permits dissection of the roles of the matrix and adipocytes in contributing to adipose tissue metabolic phenotype.

ABSTRACT
The extracellular matrix (ECM) plays a central role in regulating tissue homeostasis, engaging in crosstalk with cells and regulating multiple aspects of cellular function. The ECM plays a particularly important role in adipose tissue function in obesity, and alterations in adipose tissue ECM deposition and composition are associated with metabolic disease in mice and humans. Tractable in vitro models that permit dissection of the roles of the ECM and cells in contributing to global tissue phenotype are sparse. We describe a novel 3D in vitro model of

human ECM-adipocyte culture that permits study of the specific roles of the ECM and adipocytes in regulating adipose tissue metabolic phenotype. Human adipose tissue is decellularized to isolate ECM, which is subsequently repopulated with preadipocytes that are then differentiated within the ECM into mature adipocytes. This method creates ECM-adipocyte constructs that are metabolically active and retain characteristics of the tissues and patients from which they are derived. We have used this system to demonstrate disease-specific ECM-adipocyte crosstalk in human adipose tissue. This culture model provides a tool for dissecting the roles of the ECM and adipocytes in contributing to global adipose tissue metabolic phenotype and permits study of the role of the ECM in regulating adipose tissue homeostasis.

INTRODUCTION

The extracellular matrix (ECM) not only provides a mechanical scaffold for tissues, but also engages in complex crosstalk with cells that reside within it, regulating diverse processes necessary for tissue homeostasis, including cell proliferation, differentiation, signaling, and metabolism[1]. While healthy ECM plays an essential role the maintenance of normal tissue function, dysfunctional ECM has been implicated in multiple diseases[2].

Adipose tissue plays an important role in the pathogenesis of metabolic disease. Obesity is associated with excessive adipocyte hypertrophy and cellular hypoxia, defects in adipocyte cellular metabolism, and adipose tissue endoplasmic reticulum and oxidative stress and inflammation. While poorly understood, these complex processes conspire to impair adipose tissue nutrient buffering capacity, leading to nutrient overflow from adipose tissue, toxicity in multiple tissues, and systemic metabolic disease[3-5].The sequence of events and specific mechanisms that underlie adipose tissue failure are poorly understood, but alterations in adipose tissue ECM have been implicated. The ECM composition is altered within adipose tissue in human and murine obesity, with increased deposition of ECM protein along with qualitative biochemical and structural differences in the adipose tissue ECM associated with human metabolic disease, including type 2 diabetes and hyperlipidemia[6-11].

Despite these observations, the role of adipose tissue ECM in mediating adipose tissue dysfunction is not well-defined. This is in part due to a lack of tractable experimental models that permit dissection of the specific roles of ECM and adipocytes in regulating ultimate adipose tissue function. ECM-adipocyte culture better simulates the in vivo environment of native adipose tissue in at least two respects. Firstly, ECM culture provides a molecular environment similar to native adipose tissue, including native collagens, elastins, and other matrix proteins absent in standard 2D culture. Secondly, culture on 2D plastic has been shown to alter adipocyte metabolism via mechanical effects due to decreased elasticity of plastic substrate[12], which ECM-culture eliminates.

Methods to engineer biological scaffolds by isolation of ECM from decellularized adipose and other tissues have been studied in the context of regenerative and reconstructive medicine and tissue engineering[13-18]. We have previously published methodology in which we adapted these methods to develop an in vitro 3D model of human ECM-adipocyte culture, using ECM and

adipocyte stem cells (preadipocytes) derived from human visceral adipose tissues[11]. In the present article, we describe these methods in detail. The decellularization procedure for human adipose tissue is a four-day process that involves mechanical and enzymatic treatments to remove cells and lipid, leaving a biological scaffold that maintains characteristics of the tissue from which it is derived. Decellularized ECM supports adipogenic differentiation of human preadipocytes, and when reconstituted with adipocytes, maintains microarchitecture and biochemical and disease-specific characteristics of intact adipose tissue and engages in metabolic functions characteristic of native adipose tissue. This matrix can be studied alone or reseeded with cells, permitting study of interactions and crosstalk between the cellular and extra-cellular components of adipose tissue.

PROTOCOL

Adipose tissues are procured from human subjects undergoing elective bariatric surgery under institutional review board approval.

1. Preadipocyte isolation and culture reagent preparation

1.1. Prepare 2% bovine serum albumin (BSA) in 1x phosphate buffered saline solution (PBS). Filter sterilize, and store at 4 °C.

1.2. Prepare Type II collagenase: 2 mg/mL in 2% BSA in 1x PBS. Prepare immediately before use.

1.3. Prepare Red Blood Cell (RBC) Lysing Solution: 1.5 M NH_4Cl, 100 mM $NaHCO_3$, 10 mM disodium EDTA in deionized water (DI/H_2O). Store at 4 °C. Prepare 1x RBC Lysing Solution from 10x stock solution in DI/H_2O immediately before use.

1.4. Prepare Growth Media: 15% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (ABAM) in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12). Filter sterilize, and store at 4 °C.

1.5. Prepare Preadipocyte Freezing Solution: 10% Dimethyl Sulfoxide, 15% FBS in DMEM/F12 media. Filter sterilize, and store at 4 °C.

1.6. Prepare Differentiation Media: 10 mg/L transferrin, 33 µM biotin**,** 0.5 µM human insulin solution, 17 µM D-pantothenic acid hemicalcium salt, 100 nM dexamethasone, 2 nM 3,3'-5,Triiodo,L-thyronine sodium salt (T3), 1 µM ciglitizone, 540 µM 3-Isobutyl-1-methylxanthine (IBMX), 1% ABAM in DMEM/F12. Filter sterilize, and store at 4 °C.

2. ECM reagent preparation

2.1. Prepare Freezing Buffer Solution: 10 mM Tris base, 5 mM EDTA, 1% ABAM, 1% phenylmethylsulfonyl fluoride (PMSF) in DI/H_2O. Stir solution to dissolve EDTA. Adjust pH to 8.0 with HCl or NaOH. Store at 4 °C for up to 3 months.

2.2. Prepare Enzymatic Solution #1: 1% ABAM in 0.25% trypsin-EDTA. Store at 4 °C for up to 3 months.

2.3. Prepare Rinsing Buffer Solution: 137 mM NaCl, 2.68 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1% ABAM, 1% PMSF in sterilized DI/H_2O. Stir to dissolve salts. Adjust pH to 8.0 with HCl or NaOH. Store at 4 °C for up to 3 months.

2.4. Prepare Enzymatic Solution #2: 55 mM Na_2HPO_4, 17 mM KH_2PO_4, 4.9 mM $MgSO_4 \cdot 7H_2O$, 1% ABAM, 1% PMSF in DI/H_2O. Store 4 °C for up to 3 months. Stir to dissolve salts. Immediately prior to use, add 80 U/mL lipase from porcine pancreas, type VI-S; 160 U/mL deoxyribonuclease I from bovine pancreas, type II-S; and 100 µg/mL ribonuclease A from bovine pancreas, type III-A.

2.5. Prepare Polar Solvent Extraction Solution: 1% ABAM, 1% PMSF in isopropanol.

CAUTION: Isopropanol is flammable; store in a flammable cabinet at 25 °C and dispose in flammable waste.

2.6. Prepare 70% ethanol, 1% ABAM, 1% PMSF in DI/H_2O. Add ABAM and PMSF just prior to use.

CAUTION: Ethanol is flammable; store in a flammable cabinet at 25 °C and dispose in flammable waste.

2.7. Prepare Storage Solution: 1% ABAM, 1% PMSF in 1x PBS. Store at 4 °C for up to 3 months.

3. Metabolic phenotyping reagent preparation

3.1. Glucose uptake

3.1.1. Prepare Serum Starvation Media: DMEM/F12, 1% ABAM. Filter sterilize, and store at 4 °C

3.1.2. Prepare 200 nM human insulin solution in 1x PBS immediately before use.

3.1.3. Prepare 200 nM human insulin, 0.1 mM 2-Deoxy-D-glucose, 1 µCi/well Deoxy-D-glucose, 2-[1,2-3H (N)]-, in 1x PBS. Prepare immediately before use.

3.2. Lipolysis

3.2.1. Prepare Isoproterenol diluted in PBS: 3 mM stock solution. Dilute to working concentration of 3 µM for assay.

3.3. Oil Red-O Staining

3.3.1. Prepare 4% Formalin in DI/H_2O. Store at room temperature.

3.3.2. Prepare Oil Red-O Working Solution. Dilute Oil Red-O Solution (ORO) with DI/H_2O in a 3:2 ratio (ORO:DI/H_2O). Prepare immediately before use. Filter through filter paper (**Table of Materials**).

4. Adipose tissue procurement

NOTE: Visceral adipose tissue (VAT) is collected from the greater omentum at the beginning of the operation by the surgeon and transported back to the laboratory on ice for immediate processing. Universal precautions should be used when handling all human tissues and caustic reagents, including performing all work in a laminar flow hood, using complete laboratory safety wear, and no recapping of needles.

4.1. Add 5–10 g of intact VAT to 15–25 mL of Freezing Buffer Solution in a 50 mL conical tube to immerse the tissue sample. Store samples at -80 °C until decellularization, for up to 1 month.

4.2. Use a separate fresh sample of VAT for preadipocyte isolation as described in section 5.

5. Preadipocyte isolation

5.1. Place 2 g of intact VAT in 20 mL of the collagenase, type II, solution in a 50 mL conical tube. Then mince thoroughly by inserting sterile scissors into the conical tube and mincing the tissue within the tube. Once fully minced to a fine slurry, incubate the tissue in the collagenase solution on an orbital shaker at 130 rpm and 37 °C for 60 min.

5.2. Filter the resultant digestate through a 100 µm nylon mesh into a fresh 50 mL conical tube by pouring the digestate from one conical tube through a piece of mesh folded over the top of a fresh conical tube. The digestate at this point should be a yellow-orange liquid with moderate viscosity, with small amounts of residual strands of undigested fibrous tissue. The mesh should capture larger pieces of undigested tissue, which are discarded.

5.3. Centrifuge the sample at 270 x *g* for 10 min. Remove the supernatant and resuspend the cell pellet in 2 mL of 1x RBC Lysing Solution with a pipette.

5.3.1. Incubate for 1 min at 25 °C and then add 10 mL of 15% FBS-DMEM/F12. Centrifuge at 270 x *g* for 10 min.

5.4. Remove the supernatant and resuspend the cell pellet in 10 mL of 15% FBS-DMEM/F12 with a pipette. Transfer the cell suspension to 100 mm Petri dish with a pipette and incubate at 37 °C and 5% CO_2, until cells reach 80–100% confluence, typically 2–6 days. Change the media every 2–3 days.

5.5. Detach and wash cells.

5.5.1. Remove media with a pipette and apply 4 mL of 0.25% trypsin-EDTA to adherent cells. Incubate at 37 °C for 10 min, periodically swirling the plate gently to detach the cells.

5.5.2. Add 20 mL of 15% FBS-DMEM/F12 and resuspend the detached cells in this media with a pipette. Then transfer to a fresh 50 mL conical tube and centrifuge 270 x *g* for 10 min.

5.5.3. Remove the supernatant and discard. Wash the cell pellet once in 1x PBS, and then resuspend the cell pellet in 20 mL of fresh 15% FBS-DMEM/F12 with a pipette. Transfer the cell suspension to a T-150 culture flask.

5.6. Culture cells at 37 °C and 5% CO_2. Split and expand cells every 2-3 days as they reach 80–100% confluence by applying 7 mL of 0.25% trypsin-EDTA, expanding from one flask to 8 flasks.

NOTE: This typically requires 3-4 passages, which permits appropriate expansion and retains adipogenic potential and patient- and depot-specific cellular metabolic phenotypes. Passaging preadipocytes in excess of 4-5 passages leads to loss of adipogenic potential.

5.7. Detach cells in 8 flasks with 7 mL of 0.25% trypsin-EDTA per flask as described above, and incubate at 37 °C for 10 min.

5.8. Add 8 mL of 15% FBS-DMEM/F12 per flask and resuspend the detached cells with a pipette. Transfer the entire cell suspension divided evenly into three 50 mL conical tubes and centrifuge at 270 x *g* for 10 min.

5.9. Resuspend the resultant cell pellets in 5 mL of 15% FBS-DMEM/F12 in a 15 mL conical tube and count cells using cell counter and Trypan blue.

5.10. Centrifuge the cell suspension at 270 x *g* for 10 min. Then resuspend the cell pellet in Preadipocyte Freezing Solution to a final cell concentration of 1 x 10^6/mL, and aliquot 1 mL of cell suspension per 1.5 mL cryovial tube.

5.11. Store cells in cryovials for 1 days at -80 °C. Then transfer cryovials to liquid nitrogen for long-term storage for 3–6 months.

5.12. When ready for use, thaw one cryovial in a 37 °C water bath for 3-5 min. Resuspend the cells in 20 mL of 15% FBS-DMEM/F12, and centrifuge at 270 x *g* for 10 min.

5.13. Resuspend the cell pellet in 20 mL of 15% FBS-DMEM/F12, pipette into a single T-150 flask, and then grow to 80% confluence over 2-3 days at 37 °C and 5% CO_2.

5.14. Detach cells with 7 mL of 0.25% trypsin-EDTA per flask as described above in step 5.6. Resuspend at 3 million cells per mL (i.e., 6 x 10^4 cells per 20 µL) in 15% FBS-DMEM/F12, and use as outlined below (section 7, step 7.4).

6. Adipose tissue ECM preparation

6.1. Day 1: Freeze-thaw and enzymatic digestion #1

6.1.1. Freeze-thaw previously frozen (step 4.2) VAT samples stored in Freezing Buffer Solution in 50 mL conical tubes from -80 °C to 37 °C in a preheated water bath, incubating 20 min with gentle periodic manual agitation. Once thawed, transfer back to -80 °C and incubate 20 min. Repeat freeze-thaw 3x, ending by thawing samples in a 37 °C water bath.

6.1.2. Using sterile forceps, transfer the VAT samples to fresh 50 mL conical tubes containing 15–25 mL of Enzymatic Solution #1, ensuring that the VAT samples are fully immersed. Then incubate overnight on an orbital shaker (130 rpm, 37 °C).

6.2. Day 2: Enzymatic digestion #2

6.2.1. Wash samples 3x with 15–25 mL of Rinsing Buffer Solution on an orbital shaker (130 rpm, 37 °C, 20 min each wash). Pour off Rinsing Buffer Solution after each wash.

6.2.2. Transfer samples to fresh 50 mL conical tubes containing 15–25 mL of Enzymatic Solution #2 and incubate on an orbital shaker (130 rpm, 37 °C, overnight).

6.3. Day 3: Delipidation

6.3.1. Wash samples 3x with 15–25 mL of Rinsing Buffer Solution on an orbital shaker (130 rpm, 37 °C, 20 min each wash). Pour off Rinsing Buffer Solution after each wash.

6.3.2. Transfer samples to fresh 50 mL conical tubes containing 15-25 mL of Polar Solvent Extraction Solution and incubate on an orbital shaker (130 rpm, 25 °C, overnight). After this step, a majority of the lipid should be removed, and the samples should be white or translucent in color.

CAUTION: The polar solvent extraction solution is flammable and should be stored and used at 25 °C.

6.4. Day 4: Wash and storage

6.4.1. Transfer samples to fresh 50 mL conical tubes containing 15–25 mL of Rinsing Buffer Solution. Wash samples 3x on an orbital shaker (130 rpm, 37 °C, 20 min each wash).

6.4.2. Wash samples 3x with 15–25 mL of 70% ethanol on an orbital shaker (130 rpm, 37 °C, 20 min each wash) pouring off the 70% ethanol solution after each wash.

6.4.3. Wash samples once with Storage Solution on an orbital shaker (130 rpm, 37 °C, 20 min each wash).

6.4.4. Using sterile forceps, transfer samples to fresh 50 mL conical tubes containing 15–25 mL of Storage Solution. Ensure enough Storage Solution is used to fully immerse samples. Store at 4 °C for up to 1 month.

7. ECM-adipocyte preparation

7.1. Transfer stored ECM fragments to individual wells of 24-well plate using sterile forceps. Add as many ECM fragments into as many wells as required for the planned downstream assay (e.g., glucose uptake or lipolysis, see below), including duplicates or triplicates. Wash with 500 µL of 70% ethanol 3x on an orbital shaker (130 rpm, 37 °C, 20 min each wash).

7.2. Rehydrate ECM by washing 3x in sterile 1x PBS on an orbital shaker (130 rpm, 37 °C, 20 min each wash).

7.3. Using sterile scissors, cut and weigh ECM into 100 mg fragments. Using sterile forceps, place one 100 mg fragment in each well of a 24-well plate. Incubate at 25 °C for 15 min to allow excess PBS to extrude from fragments. Carefully remove any excess PBS with a pipette.

7.4. Seed each 100 mg ECM fragment with 20 µL of preadipocyte cell suspension (3 million cells per mL, 6 x 10^4 cells per 20 µL, in 15% FBS-DMEM/F12, from step 5.10). Pipette the cells directly into the ECM by placing the tip of pipette in the ECM and gently expelling the cell suspension into center of the matrix, taking care that the cell suspension does not overflow and end up on the bottom of the well.

7.4.1. If the cell suspension is overflowing from the ECM where the pipette tip has been placed, remove the tip from that location and insert somewhere else in the ECM. Incubate seeded ECM for 40 min at 37 °C.

NOTE: For RNA extraction for qrtPCR, seed each 500 mg ECM fragments with 3 x 10^5 cells in 100 µL (3 million cells per mL, i.e., 3 x 10^5 cells per 100 µL, in 15% FBS-DMEM/F12).

7.5. Fill each well of the 24-well plate with 500 µL of growth media to cover the seeded ECM fragments. Culture at 37 °C and 5% CO_2 for 72 h.

7.6. After 72 h, carefully aspirate 15% FBS-DMEM/F12, tilting the plate slightly to allow media to pool below fragment, and placing the pipette tip just adjacent to the ECM fragment without disturbing it. After aspirating, add 500 µL of Differentiation Media, changing media every 2–3 days using a similar technique, for a total culture period of 14 days.

7.6.1. Check for differentiation using light microscopy: cells will accumulate lipids, turn brown-yellow in color and more spherical in shape.

NOTE: Seeded matrices can be used for metabolic testing (e.g., glucose uptake assay, lipolysis assay, ORO), histology or immunohistochemistry (IHC), or standard tissue imaging. For fixed tissue ORO staining and imaging, freeze ECM-adipocyte samples in liquid nitrogen.

8. Metabolic phenotyping

8.1. Scanning electron microscopy

8.1.1. Fix samples in 2.5% glutaraldehyde in Sorensen's phosphate buffer at 25 °C for 12 h. Postfix in 1% osmium tetroxide in Sorensen's phosphate buffer at 4 °C for 1 h.

8.1.2. Serially dehydrate samples in ethanol. Wash in hexamethyldisalizane, and air-dry. Then mount on a scanning electron microscopy stub with colloidal graphite. Dry, and sputter-coat with gold.

8.1.3. Capture images with a scanning electron microscope.

8.2. Oil Red-O Staining

8.2.1. Live Tissue: Oil Red-O Solution

8.2.1.1. Carefully aspirate media from wells with a pipette. Then wash samples once with 500 µL of 1x PBS per well.

8.2.1.2. Fix samples with 200 µL of 4% formalin in sterile deionized H_2O at 25 °C for 15 min. Aspirate formalin with a pipette, wash samples two times with 1x PBS (500 µL each wash).

8.2.1.3. Add 200 µL of 60% isopropanol samples at 25 °C for 5 min. Aspirate 60% isopropanol with a pipette.

8.2.1.4. Stain samples with Oil Red-O working solution at 25 °C for 5 min. Aspirate Oil Red-O with a pipette and then wash samples 3x with 1x PBS (500 µL each wash). Then image with an optical microscope.

8.2.2. Fixed Tissue: Oil Red-O Stain Kit

8.2.2.1. Flash-freeze ECM-adipocyte samples in optimal cutting temperature (OCT) compound and section (5 µm) on a cryostat.

8.2.2.2. Place the slide in 85% propylene glycol in DI/H_2O for 2 min. Place the slide in ORO stain at 60 °C for 6 min. Place steh lide in 85% propylene glycol in DI/H_2O for 1 min. Rinse the slide twice with DI/H_2O.

8.2.2.3. Place the slide in Modified Mayer's Hematoxylin from Oil Red-O staining kit for 1 min. Rinse the slide twice with tap water. Rinse slide twice with DI/H_2O.

8.2.2.4. Mount the coverslip using aqueous mounting medium and image on a microscope.

8.2.3. RNA extraction from ECM for qrtPCR

NOTE: To maximize RNA yield, use 500 mg ECM fragments seeded with 3 x 10^5 preadipocytes in 100 µL and differentiate as above in 6-well plates in 3 mL of Differentiation media per well.

8.2.3.1. Once differentiated, transfer each individual ECM-adipocyte sample into a 50 mL conical tube on ice using sterile forceps.

8.2.3.2. Wash empty well with 500 µL of Buffer RLT. Add Buffer RLT to 50 mL conical tube with matching ECM-adipocyte sample.

8.2.3.3. Using sterile scissors, finely mince each ECM-adipocyte sample within the 50 mL conical tube, while holding the tube on ice, inserting the scissors into the conical tube to mince the tissue.

8.2.3.4. Completely freeze and thaw the conical tubes from -80 °C to 37 °C 3x.

8.2.3.5. Centrifuge conical tubes at 500 x *g* and 4 °C for 10 min.

8.2.3.6. Carefully remove the supernatant with a pipette and use for RNA extraction with a Fibrous Tissue RNA extraction Kit (**Table of Materials**).

8.3. Glucose uptake assay

8.3.1. Differentiate 6 x 10^4 preadipocytes in 100 mg ECM fragments in 0.5 mL of differentiation medium in 24-well plates as described above (section 5).

8.3.2. After 14 days of differentiation, remove the medium and wash ECM-adipocytes once with 1x PBS. Add 0.5 mL/well Serum Starvation Medium and culture at 37 °C and 5% CO_2 for 12 h.

8.3.3. Remove medium and wash cells twice with 1x PBS. Add 0.5 mL/well 2% BSA in PBS and culture at 37 °C and 5% CO_2 for 2 h.

8.3.4. Wash cells once with 1x PBS, add 0.5 mL/well 1x PBS with or without 200 nM insulin, and incubate at 37 °C for 40 min.

8.3.5. Aspirate 1x PBS, add 0.5 mL/well 1X PBS with 0.1 mM 2-deoxy-D-glucose, 2 µCi/mL deoxy-D-glucose, 2- [1,2- ^{3}H(N)], with or without 200 nM insulin, and incubate at 37 °C and 5% CO_2 for 40 min. Use standard precautions for handling and disposal of all radioactive reagents and waste, as mandated by local institutional regulatory statutes.

8.3.6. Remove medium with a pipette and wash cells 3x with 1x PBS. Add 420 µL of 1% SDS solution in DI/H_2O, and lyse cells with vigorous pipetting. Incubate 25 °C for 10 min.

8.3.7. Collect 5 µL from each well for Bradford protein assay. Transfer 400 µL of remaining cell lysate into 2 mL of scintillation fluid in a scintillation vial. Count ^{3}H-2DG activity on scintillation counter. Analyze data as counts per minute normalized to protein, mg/mL.

8.4. Lipolysis assay

8.4.1. Differentiate 6 x 10^4 preadipocytes in 100 mg ECM fragments in 0.5 mL of human differentiation medium in 24-well plates as described above (section 5).

8.4.2. After 14 days of differentiation, remove medium and wash cells twice with warm 1x PBS. Add 0.5 mL of serum starvation medium (without insulin) with or without 3 µM isoproterenol, and culture adipocytes at 37 °C and 5% CO_2 for 72 h.

8.4.3. Collect culture supernatants, which may be stored at -80 °C until ready for assay. Collect the ECM in microcentrifuge tubes for DNA quantitation for data normalization.

8.4.4. Pipet 2 µL of each supernatant into a 96-well microplate. Reserve wells for blanks (distilled H_2O) and glycerol standard solution provided in Triglyceride Determination Kit.

8.4.5. Add 270 µL of free glycerol reagent from Triglyceride Determination Kit to each well, pipette to mix. Incubate plate at 37 °C for 5 min.

8.4.6. Measure absorbance at 540 nm on a microplate spectrophotometer.

8.4.7. Calculate the concentration of glycerol and normalize with DNA from ECM:

$$\text{Glycerol Concentration of Sample} = \left(\frac{\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}}{\text{Absorbance}_{\text{standard}} - \text{Absorbance}_{\text{blank}}}\right) \times \text{Concentration of Standard } \left(2.5 \frac{\text{mg}}{\text{mL}}\right)$$

REPRESENTATIVE RESULTS

Preparation of adipose tissue ECM, seeding with preadipocytes, and in vitro differentiation into mature adipocytes result in clear sequential morphologic changes in tissue that permits visual assessment of progress throughout the protocol (**Figure 1**). Preadipocytes used to seed the ECM are isolated using collagenase digestion from separate VAT samples (**Figure 2**). Scanning electron microscopy of ECM-adipocyte constructs at each stage of processing reveals decellularization of ECM and subsequent recapitulation of lipid-containing adipocytes upon reseeding and differentiation (**Figure 3**). Collagen 1-specific immunohistochemistry of decellularized ECM reveals maintenance of collagen microarchitecture, while Oil Red-O staining of live and fixed/sectioned 3D ECM-adipocyte constructs reveals lipid-containing adipocytes (**Figure 4A**). qrtPCR from adipocytes differentiated in ECM demonstrates marked upregulation of adipogenic genes relative to undifferentiated preadipocytes cultured in ECM (**Figure 4B**). Metabolic phenotyping of ECM-adipocyte constructs studying all combinations of ECM and adipocytes from diabetic (DM) and non-diabetic (NDM) subjects reveals impaired glucose uptake and lipolytic function in ECM-adipocyte constructs from DM relative to NDM tissues, recapitulating DM-specific metabolic defects we have previously reported in human adipocytes in standard 2D culture[11]. Importantly, NDM ECM rescues insulin-stimulated glucose uptake and lipolytic capacity in DM adipocytes, while DM ECM impairs lipolytic capacity in NDM adipocytes (**Figure 4C**)[11]. Together, these data demonstrate disease-specific regulation of adipocyte cellular metabolism by the ECM. Further details are reported in Baker et al.[11].

FIGURE LEGENDS:

Figure 1: Workflow for ECM-adipocyte preparation. Day 1: Whole visceral adipose tissue samples undergo three freeze-thaw cycles followed by overnight incubation with enzymatic digestion solution #1. Day 2: Following digestion with enzymatic solution #1, samples are digested overnight with enzymatic digestion solution #2. At this point, samples will be partially delipidated. Day 3: Following enzymatic digestion #2, samples undergo overnight incubation with polar solvent extraction solution, which will complete the delipidation. After Day 3, samples should be fully delipidated and white/translucent in color. Samples are washed thoroughly with rinsing buffer solution then 70% ethanol and stored in storage solution in 40 °C until ready for reseeding with preadipocytes. Day 4: Preadipocytes are seeded into decellularized VAT followed by adipogenic differentiation for 14 days.

Figure 2: Workflow for preadipocyte isolation. VAT is minced, digested with collagenase, filtered, centrifuged, and the resultant stromovascular cell pellet plated and cultured to expand preadipocytes. See Baker et al. 2017[21] for further details regarding human preadipocyte isolation and 2D adipocyte culture.

Figure 3: Scanning electron microscopy images. Intact adipose tissue, decellularized adipose tissue, and decellularized adipose tissue repopulated with preadipocytes and differentiated in adipogenic media for 14 days.

Figure 4: Characterization of adipocytes in ECM. (**A**) Decellularized adipose tissue ECM maintains microarchitecture and supports adipocyte differentiation: Top: Collagen 1 immunohistochemistry of whole human VAT before and after decellularization demonstrating

maintenance of microarchitecture; middle: 3D confocal photomicrographs of live human adipocytes within ECM; intact decellularized human VAT stained with Oil Red-O before reseeding with preadipocytes, 4 days after seeding, and 14 days after seeding with preadipocytes followed by adipogenic differentiation; blue: DAPI staining of cell nuclei; red: Oil Red-O staining of intracellular lipid; bottom: Formalin-fixed, paraffin-embedded, 5 µm sectioned, Oil Red-O-stained human VAT prior to decellularization, immediately after decellularization, and after decellularization, preadipocyte-seeding, and 14 days of adipogenic differentiation, demonstrating cytoplasmic lipid accumulation in adipocytes within ECM. (**B**) Adipocytes in ECM upregulate adipogenic gene expression: qrtPCR analysis comparing adipogenic gene transcript levels in RNA from human VAT adipocytes differentiated in VAT ECM for 14 days relative to undifferentiated preadipocytes in VAT ECM cultured for 72 h in nonadipogenic media. Data are mean ± SEM. *ACLY*: ATP citrate lyase, *ATGL*: adipose triglyceride lipase, *FASN*: fatty acid synthase, *PPARg*: peroxisome proliferative activated receptor gamma; ordinate: fold difference in transcript level in mature adipocytes relative to undifferentiated preadipocyte referent=1; all fold differences significant ($p<0.001$, paired t-test); ECM from 10 subjects, preadipocytes/adipocytes from n=11 subjects. (**C**) ECM regulates adipocyte metabolism in a DM-specific manner: 3D-ECM from DM or NDM subjects seeded with preadipocytes from DM or NDM subjects, differentiated into adipocytes, and studied with metabolic phenotyping. Data bars labeled with patient source (NDM, DM) of ECM and adipocytes (ECM/AD); e.g., NDM/NDM denotes both ECM and preadipocytes derived from NDM patients, while NDM/DM denotes ECM from NDM patients combined with preadipocytes from DM patients. Data are mean ± SEM. Ordinates: glucose uptake (cpm), normalized to ECM/cell lysate protein concentration(mg/mL); lipolysis: culture supernatant glycerol concentration(mg/mL) normalized to ECM/cell lysate DNA concentration(ng/mL); *$p<0.050$, **$p<0.100$ comparing indicated data-point to corresponding data-point (basal or insulin-stimulated for glucose uptake, basal or isoproterenol-stimulated for lipolysis) in DM/DM arm, using mixed model analysis adjusting for repeated measures; ECM from n= 9 NDM, 8 DM subjects, preadipocytes from 10 NDM, 9 DM subjects for glucose uptake; ECM from 6 NDM, 6 DM subjects, preadipocytes from 6 NDM, 6 DM subjects for lipolysis. This figure has been adapted from O'Rourke and Lumeng[11].

DISCUSSION

The ECM-adipocyte culture model provides a valuable tool for dissecting the individual roles of ECM and cells in dictating ultimate tissue phenotype. The ECM isolation protocol is quite reproducible, but variability in the decellularization process may be observed. The Day 3 delipidation step is a critical point in the protocol. At the completion of the overnight extraction, delipidation of the matrix should be evidenced by the Polar Solvent Solution turning yellow, while the matrix should transition from a yellow-orange color characteristic of intact adipose tissue to translucent/white (**Figure 1**). If these color changes do not occur, then delipidation is likely not complete. Inter-patient variability in delipidation efficiency is common, but to date we have not observed any correlation of efficiency of delipidation with subject DM status, age, BMI, or other clinical variables. Large samples (greater than 20 g) will not undergo efficient delipidation; processing samples <20 g may prevent this problem. If a sample does not appear completely delipidated after Day 3, divide the sample in half with sterile scissors, then

transfer samples to fresh 15-20 mL of Polar Solvent Extraction Solution and place on an orbital shaker for 3-5 h. Some samples may not fully decellularize after repeating the polar solvent extraction step. If a majority of the sample is decellularized, it is possible to cut out the sections containing lipid before using the sample in experiments.

Contamination may occur if sterility is not diligently maintained throughout the process. All work should be performed in a laminar flow hood using standard cell culture precautions. We typically store ECM for up to 3 months at 4 °C, and preadipocytes for up to 6 months in liquid nitrogen. Retention of biofunctionality and disease- and depot-specific properties beyond this time has not been tested.

Confocal microscopy permits visualization of mature adipocytes within the ECM, which may be enhanced with Oil Red-O staining. Scanning electron microscopy (SEM) also provides a non-quantitative method for visualizing adipocytes within ECM. Of note, SEM and Oil Red-O staining suggest unilocular adipocytes in the ECM-adipocyte constructs, a morphology that is similar to in vivo adipocytes and in contrast to the typical multilocular adipocytes observed when preadipocytes undergo adipogenic differentiation on 2D plastic (**Figure 3, Figure 4A**). This observation suggests that the ECM provides a more physiologic environment for adipogenic differentiation than standard tissue culture on plastic. Fixation and sectioning of ECM-adipocyte constructs may be difficult, as OCT-embedded constructs are fragile and do not section easily. Sectioning embedded tissues immediately after removal from storage from the -80 °C freezer and performing sectioning in a cold-room (4 °C) on a pre-chilled cryostat makes sectioning feasible.

ECM-adipocyte culture provides a physiologic environment that approximates the in vivo adipose tissue environment and provides a sustainable environment for preadipocyte stem cell growth and differentiation, as evidenced by upregulation of adipogenic genes in 3D-ECM-adipocyte culture. ECM-adipocyte cultures also engage in adipocyte metabolic functions, including glucose uptake and lipolysis[8]. We report glucose uptake as cpm normalized to either total protein extracted from ECM-adipocyte cultures measured with a Bradford assay, or to DNA content measured with a DNA assay kit, and have observed similar results with both methods. Similarly, we report lipolysis as mg/mL of glycerol release normalized to either protein or DNA, also with similar results obtained with either normalization method. Others suggest normalizing adipocyte metabolic data to lipid content, but to date our attempts to reliably extract lipid from ECM constructs has been unsuccessful. Reporting glucose uptake rate as moles of glucose per unit of time and lipolysis as moles of glycerol/free fatty acid per unit of time may permit more accurate comparisons between separate experiments. Isolation of RNA from ECM for qrtPCR analysis is characterized by lower yields compared with cells in 2D culture, but preparation of larger ECM fragments seeded with more cells overcomes this problem, as described in step 8.3.1. We have encountered difficulty in isolating adequate quantities of undegraded protein with phosphomoieties intact for Western blot analysis, which represents a limitation of the model.

Similar methodology for isolating ECM from adipose tissue and seeding with preadipocytes has been previously published, with evidence suggesting that ECM may promote adipogenic differentiation absent classic adipogenic mediators including cAMP agonists, insulin, and PPAR-γ agonists[12,14]. Our data is the first to demonstrate disease-specific regulation of cellular metabolism by ECM in adipose tissue, using methods in which adipocytes are stimulated with classic adipogenic differentiation factors[11]; similar studies in the absence of adipogenic stimuli are a target of future research. Others have demonstrated similar disease-specific ECM-cell crosstalk using ECM and cells isolated from lung tissue[19,20]. Alternative strategies have also been employed, including creation of matrices by mixing homogenized adipose tissue ECM preparations in peptide hydrogels[12].

While inter-patient variability in human adipocyte cellular metabolism is observed in both 2D-plastic and 3D-ECM culture, when analyzed in aggregate, these cells manifest disease-, depot- and sex-specific differences in cellular metabolism, as described by our group and others[11,21-24], validating the utility and generalizability of these culture models for the study of adipocyte cellular metabolism. We have demonstrated that adipocytes differentiated in disease-matched ECM (i.e., NDM adipocytes in NDM ECM, DM adipocytes in DM ECM) recapitulate metabolic phenotypes of isolated NDM and DM adipocytes in standard 2D culture, supporting ECM-adipocyte culture as a model for studying disease-specific alterations in adipocyte cellular metabolism in a more physiologic environment. We have not observed differences in the magnitude or direction of metabolic functions, including glucose uptake or lipolysis, when ECM and adipocytes from the same or different donors are studied. In either case, ECM-adipocyte constructs manifest DM-specific metabolic phenotype (e.g., decreased GU in DM/DM relative to NDM/NDM constructs), with no clear difference when comparing constructs comprised of ECM and adipocytes from the same or different donors.

ECM-adipocyte culture allows study of combinations of ECM and preadipocytes from distinct patient populations and tissue depots, permitting analysis of disease- and depot-specific contributions of ECM and adipocytes to ultimate adipose tissue metabolic phenotype. Demonstrating this strength of the ECM-adipocyte culture model, we have shown that ECM from NDM tissue has the capacity to rescue metabolic defects in DM adipocytes, while ECM from DM tissue impairs metabolic phenotype in NDM adipocytes (**Figure 4C**)[11]. Preliminary data in murine visceral and subcutaneous adipose tissues suggest that murine ECM regulates murine adipocyte metabolism similarly in a depot-specific manner (unpublished data, O'Rourke RW, 2019), suggesting that this model system may be used to study ECM-adipocyte crosstalk in both murine and human systems. Furthermore, this model permits study of isolated manipulation of ECM as a means to regulate adipose tissue phenotype. Preliminary study of ECM treated in conditions that induce glycation targeted specifically to the ECM, for example, suggest that these modifications regulate the cellular metabolic phenotype of cells subsequently seeded into treated ECM (unpublished data, O'Rourke RW, 2019), providing a model for study of the effects of targeted manipulation of the ECM on adipocyte phenotype.

ACKNOWLEDGEMENTS:

We thank Danielle Berger, Marilyn Woodruff, Simone Correa, and Retha Geiss for assistance with study coordination. SEM was performed by University of Michigan Microscopy & Image Analysis Laboratory Biomedical Research Core Facility. This project was supported by NIH grants R01DK097449 (RWO), R01DK115190 (RWO, CNL), R01DK090262 (CNL), Veterans Affairs Merit Grant I01CX001811 (RWO), Pilot and Feasibility Grant from the Michigan Diabetes Research Center (NIH Grant P30-DK020572) (RWO), Veterans Administration VISN 10 SPARK Pilot Grant (RWO). Scanning electron microscopy performed by the University of Michigan Microscopy & Image Analysis Laboratory Biomedical Research Core Facility. Figure 4 of this manuscript was originally published in Baker et al., *J Clin Endo Metab* 2017; Mar 1;102 (3), 1032-1043. doi: 10.1210/jc.2016-2915, and has been reproduced by permission of Oxford University Press [https://academic.oup.com/jcem/article/102/3/1032/2836329]. For permission to reuse this material, please visit http://global.oup.com/academic/rights.

DISCLOSURES:

The authors declare no conflicting interests.

REFERENCES

1. Frantz, C., Stewart, K.M., Weaver, V.M. The extracellular matrix at a glance. *Journal of Cell Science.* **123** (Pt 24), 4195-4200 (2010).

2. Berrier, A.L., Yamada, K.M. Cell-matrix adhesion. *Journal of Cell Physiology*. **213** (3), 565-573 (2007).

3. Trayhurn, P. Hypoxia and adipose tissue function and dysfunction in obesity. *Physiology Reviews*. **93**(1), 1-21 (2014).

4. O'Rourke, R.W., Lumeng, C.N. Obesity heats up adipose tissue lymphocytes. *Gastroenterology*. **145** (2), 282-285 (2013).

5. Engin, A. The Pathogenesis of Obesity-Associated Adipose Tissue Inflammation. *Advances in Experimental Medicine and Biology*. **960**, 221-245 (2017).

6. Dankel, S.N. et al. COL6A3 expression in adipocytes associates with insulin resistance and depends on PPARγ and adipocyte size. *Obesity (Silver Spring).* **22** (8), 1807-1813 (2014).

7. Divoux, A. et al. Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss. *Diabetes*. **59**, 2817-2825 (2010).

8. Lackey, D.E. et al. Contributions of adipose tissue architectural and tensile properties toward defining healthy and unhealthy obesity. *American Journal of Physiology, Endocrinology, and Metabolism.* **306** (3), E233-E246 (2014).

9. Muir, L.A. et al. Adipose tissue fibrosis, hypertrophy, and hyperplasia: correlations with diabetes in human obesity. *Obesity (Silver Spring)*. **24** (3), 597-605 (2016).

10. Spencer, M. et al. Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. *American Journal of Physiology, Endocrinology, and Metabolism*. **299** (6), E1016-E1027 (2010).

11. Baker, N.A. et al. Diabetes-specific regulation of adipocyte metabolism by the adipose tissue extracellular matrix. *Journal of Clinical Endocrinology and Metabolism*. **102** (3), 1-12 (2017).

12. Pellegrinelli, V. et al. Human adipocyte function is impacted by mechanical cues. *Journal of Pathology.* **233** (2), 183-195 (2014).

13. Flynn, L.E. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials*. **31** (17), 4715-4724 (2010).

14. Perea-Gil, I. et al. In vitro comparative study of two decellularization protocols in search of an optimal myocardial scaffold for recellularization. *American Journal Translational Research*. **7** (3), 558-573 (2015).

15. Porzionato, A. et al. Decellularized omentum as novel biologic scaffold for reconstructive surgery and regenerative medicine. *European Journal of Histochemistry*. **57** (1), e4 (2013).

16. Tebyanian, H. et al. A Comparative Study of Rat Lung Decellularization by Chemical Detergents for Lung Tissue Engineering. *Open Access Macedonian Journal of Medical Sciences*. **5** (7), 859-865 (2017).

17. Crapo, P.M., Gilbert, T.W., Badylak, S.F. An overview of tissue and whole organ decellularization processes. *Biomaterials*. **32** (12), 3233-3243 (2011).

18. Wang, L., Johnson, J.A., Zhang, Q., Beahm, E.K. Combining decellularized human adipose tissue extracellular matrix and adipose-derived stem cells for adipose tissue engineering. *Acta Biomaterials*. **9** (11), 8921-8931 (2013).

19. Booth, A.J. et al. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. *American Journal of Respiratory and Critical Care Medicine*. **186** (9), 866-876 (2012).

20. Parker, M.W. et al. Fibrotic extracellular matrix activates a profibrotic positive feedback loop. *Journal of Clinical Investigation*. **124** (4), 1622-1635 (2014).

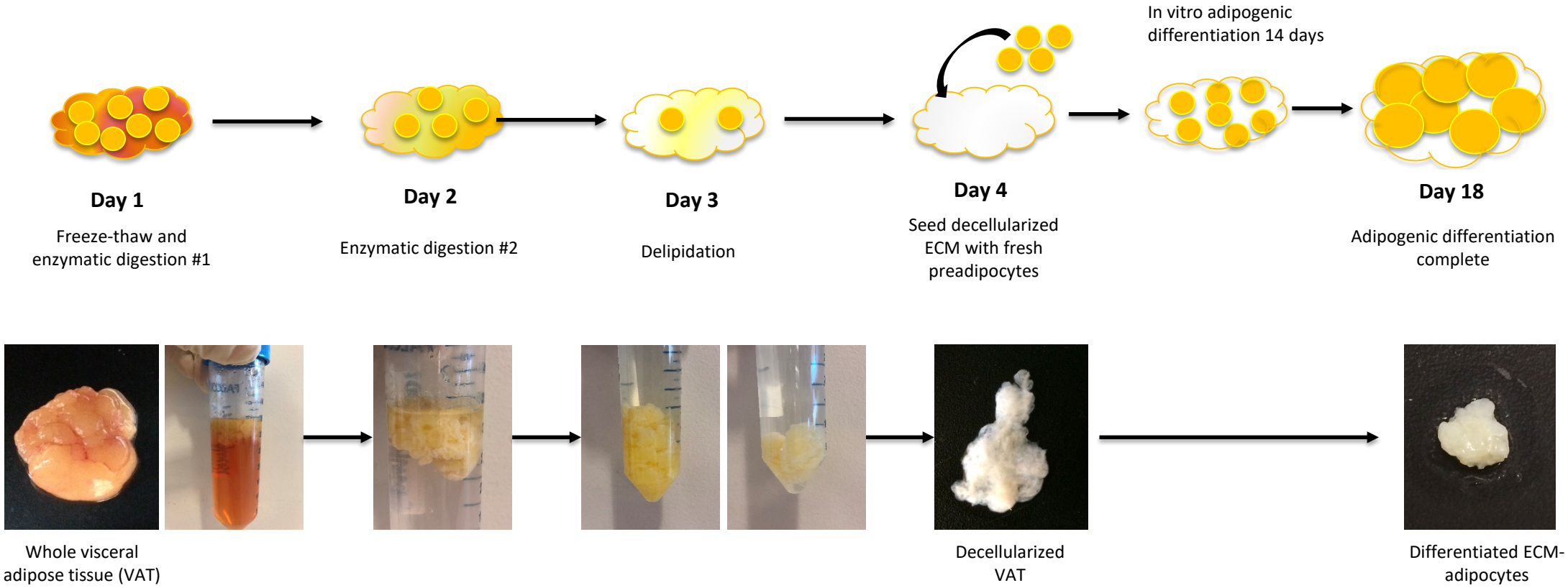
21. Baker, N.A., Muir, L.A., Lumeng, C.N., O'Rourke, R.W. Differentiation and Metabolic Interrogation of Human Adipocytes. *Methods in Molecular Biology*. **1566**, 61-76 (2017).

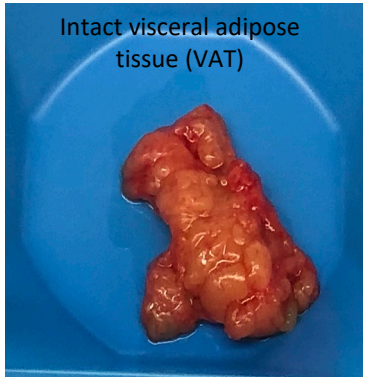
22. O'Rourke, R.W. et al. Hexosamine biosynthesis is a possible mechanism underlying hypoxia's effects on lipid metabolism in human adipocytes. *PLoS One*. **8** (8), e71165 (2013).

23. Tchkonia, T. et al. Fat depot-specific characteristics are retained in strains derived from single human preadipocytes. *Diabetes*. **55** (9), 2571-2578 (2006).

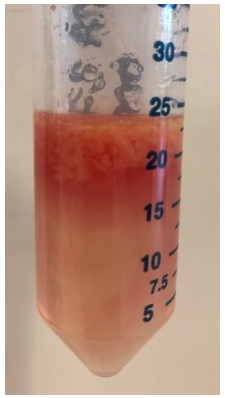
24. Tchoukalova, Y.D. et al. Sex- and depot-dependent differences in adipogenesis in normal-weight humans. *Obesity (Silver Spring)*. **18** (10), 1875-1880 (2010).

Figure1





Place VAT in collagenase solution

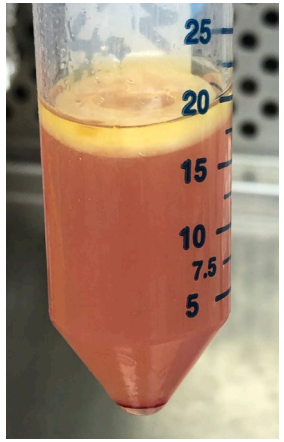


Mince tissue with sterile scissors

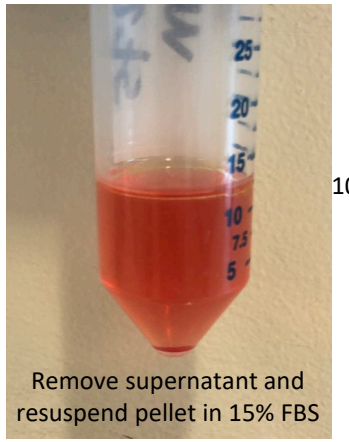
Incubate 37°C, 60 min, 130 rpm.

Centrifuge 270 rcf, 10 min

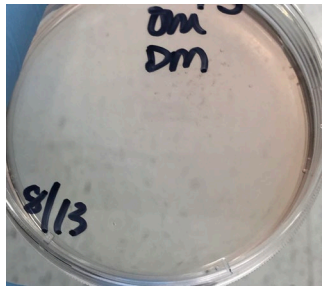
Filter digestate through 100 µm nylon mesh



Remove lipid layer and aqueous supernatant, resuspend cell pellet in RBC Lysing Solution, incubate, then centrifuge 270 rcf, 10 min

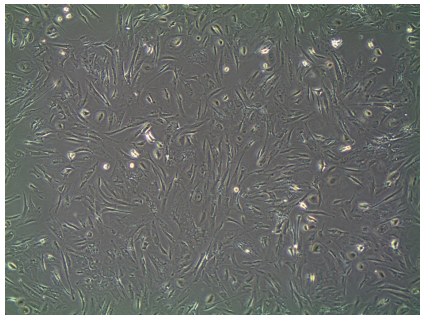


Transfer cell suspension to 100mm petri dish



Passage plastic-adhered cells 4 rounds to isolate/expand preadipocytes

Expanded preadipocytes



Intact adipose tissue

Decellularized adipose tissue

Repopulated decellularized adipose tissue

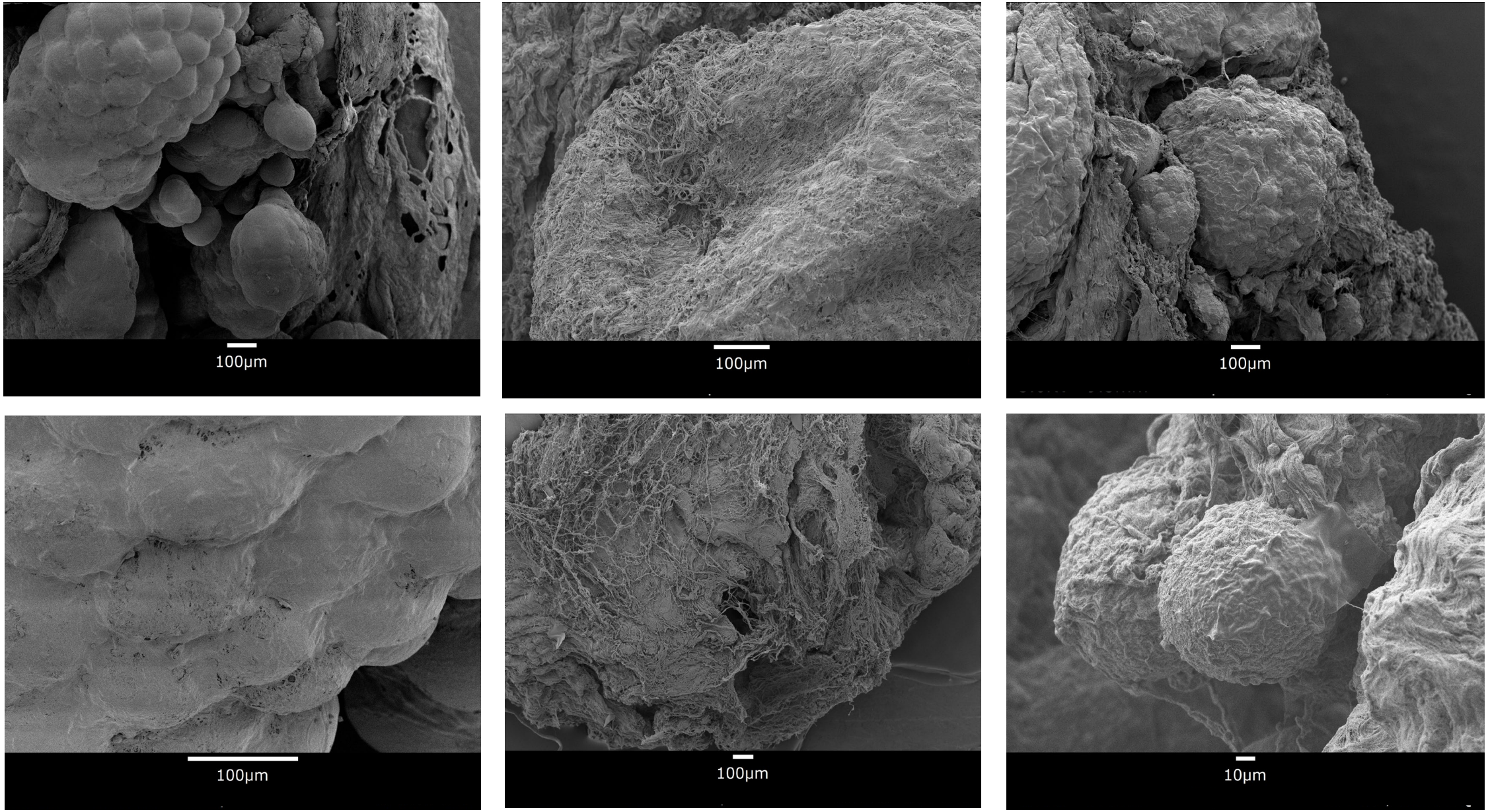
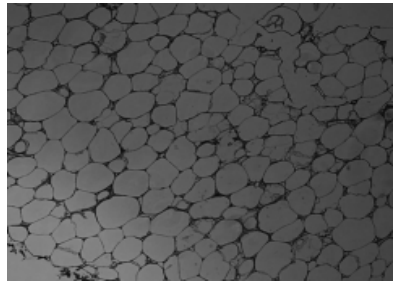


Figure4

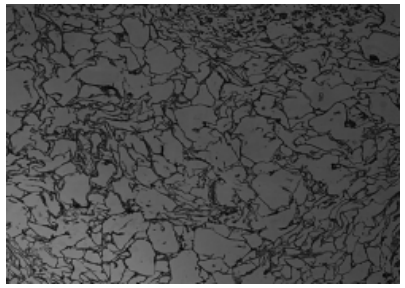
A

Collagen 1 Immunohistochemistry

Intact adipose tissue

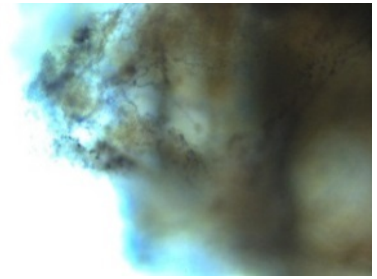


Decellularized ECM

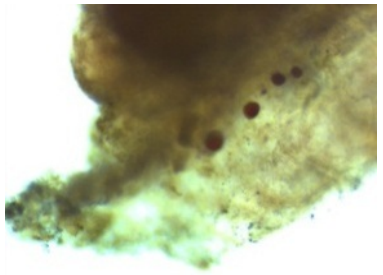


Oil Red-O stained live tissue

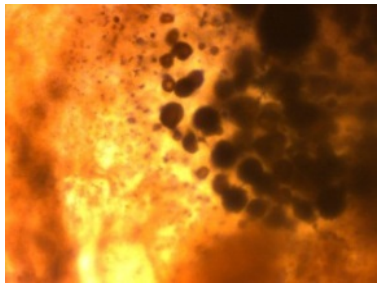
Decellularized ECM



ECM repopulated-
4 days diff

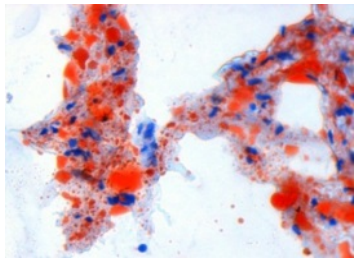


ECM repopulated-
14 days diff

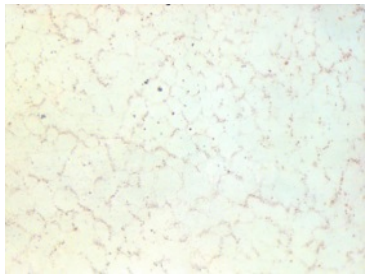


Oil Red-O stained fixed tissue

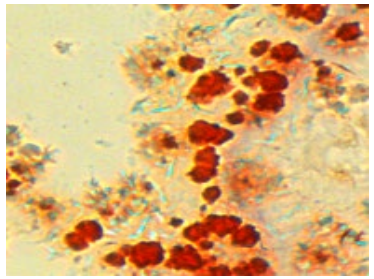
Intact adipose tissue



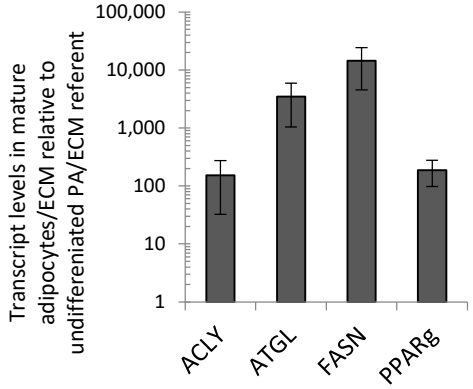
Decellularized ECM



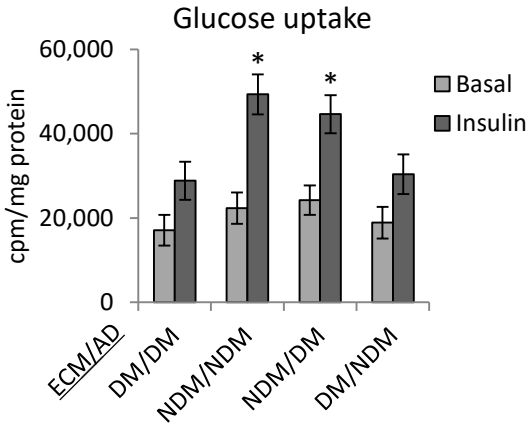
ECM repopulated-
14 days diff

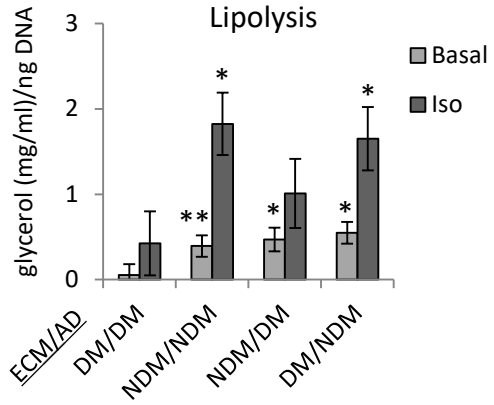


B



C





Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.25% trypsin-EDTA	Gibco, ThermoFisher Scientific Inc., Waltham, MA, USA	Cat#25200056	
1.5 mL cryovial tube	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#02-682-557	
10% Neutral Buffered Formalin	VWR International LLC., Radnor, PA, USA	Cat#89370-094	
100 µm nylon mesh filter	Corning Inc., Corning, NY, USA	Cat#352360	
2-Deoxy-D-glucose	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#D8375	
2 nM 3,3'-5,Triiodo,L-thyronine sodium salt (T3)	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#T6397	
24-well tissue culture plates	VWR International LLC., Radnor, PA, USA	Cat#10861-700	
3-Isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#I5879	
96-well tissue culture plates	VWR International LLC., Radnor, PA, USA	Cat#10861-666	

Antibiotic-Antimycotic Solution (ABAM)	Gibco, ThermoFisher Scientific Inc., Waltham, MA, USA	Cat#15240062	
Biotin	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#B4639	
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#A8806	
Buffer RLT	Qiagen, Hilden, Germany	Cat#79216	
Ciglitizone	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#C3974	
Deoxy-D-glucose, 2-[1,2-3H (N)]-	PerkinElmer Inc., Waltham, MA, USA	Cat#NET328A250UC	
Deoxyribonuclease I from bovine pancreas, type II-S	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#D4513	
Dexamethasone	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#D4902	
Dimethyl Sulfoxide	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#BP231	Flammable, caustic

Disodium EDTA	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#BP118	
D-pantothenic acid hemicalcium salt	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#21210	
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12	Gibco, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#11320033	
Ethanol	Decon Labs, Inc., King of Prussia, PA, USA	Cat#DSP-MD.43	Flammable
EVE Cell Counting Slides, NanoEnTek	VWR International LLC., Radnor, PA, USA	Cat#10027-446	
Fetal bovine serum (FBS)	Gibco, ThermoFisher Scientific Inc., Waltham, MA, USA	Cat#10437028	
Glutaraldehyde	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#G5882	Caustic
Hexamethyldisalizane	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#440191	Flammable, caustic

Human insulin solution	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#I9278	
Isopropanol	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#A415	Flammable
Isoproterenol	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#I5627	Flammable
KCl	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#S25484	
KH_2PO_4	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#P5655	
Lipase from porcine pancreas, type VI-S	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#L0382	
$MgSO_4*7H_2O$	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#230391	
Na_2HPO_4	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#S5136	
NaCl	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#S3014	

$NaHCO_3$	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#S233	
NH_4Cl	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#A661	
Optimal cutting temperature (OCT) compound	Agar Scientific, Ltd., Stansted, Essex, UK	Cat# AGR1180	
Oil Red-O Solution (ORO)	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#O1391	
Oil Red-O Stain Kit	American Master Tech Scientific Inc., Lodi, CA, USA	Cat#KTORO-G	
Osmium tetroxide	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#201030	Caustic
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#93482	Caustic
Phosphate Buffered Saline Solution (PBS)	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#SH3025601	

Ribonuclease A from bovine pancreas, type III-A	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#R5125	
RNAEasy Fibrous Tissue MiniKit	Qiagen, Hilden, Germany	Cat#74704	
Scintillation Fluid	Fisher Scientific, ThermoFisher Scientific Inc., Waltham, MA USA	Cat#SX18	
Scintillation Counter			
Scissors, forceps, sterile			
Sorensen's phosphate buffer	Thomas Scientific, Inc., Swedesboro, NJ	CAS #: 10049-21-5	
T-150 culture flask	VWR International LLC., Radnor, PA, USA	Cat#10062-864	
TaqMan Gene Expression Master Mix	ThermoFisher Scientific Inc., Waltham, MA USA	Cat#4369016	
Temperature-controlled orbital shaker			
Tissue Homogenizer, BeadBug Microtube Homogenizer	Benchmark Scientific	Cat#D1030	

Transferrin	Sigma-Aldrich, Inc. St Louis, MO, USA	Cat#T3309	
Triglyceride Determination Kit	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#TR0100	
Trypan blue stain, 0.4%	VWR International LLC., Radnor, PA, USA	Cat#10027-446	
Type II collagenase	Gibco, ThermoFisher Scientific Inc., Waltham, MA, USA	Cat#17101015	
Whatman Reeve Angel filter paper, Grade 201, 150mm	Sigma-Aldrich, Inc., St Louis, MO, USA	Cat#WHA5201150	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A human 3D extracellular matrix-adipocyte culture model for studying matrix-cell metabolic crosstalk
Author(s):	Carmen G. Flesher, Clarissa Strieder-Barboza, Nicki A. Baker, Dominic Polsinelli, Phillip J. Webster, Oliver A. Varban, Carey N. Lumeng, Robert W. O'Rourke

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Robert W. O'Rourke
Department:	Surgery
Institution:	University of Michigan
Title:	Professor
Signature:	
Date:	June 25, 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Dr. O'Rourke,
Your manuscript, JoVE60486 "A human 3D extracellular matrix-adipocyte culture model for studying matrix-cell metabolic crosstalk," has undergone editorial and peer review and your video has been reviewed by our production department. Note that editorial and production comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits. After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Your revision is due by Aug 19, 2019. To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article. Furthermore, please submit a high resolution version of your video (up to 2 GB) here: https://www.dropbox.com/request/YuLQrEYDZ3KHAqOS38B5
Sincerely, Phillip Steindel, Ph.D., Review Editor, JoVE, 617.674.1888

8/14/2019
Dear Dr Steindel and Editors and Reviewers of JoVE,
We appreciate the reviewers' thoughtful, thorough, and professional critiques. We have significantly revised the manuscript, added substantial additional data, and addressed each concern to the best of our ability in the revised manuscript and the point-by-point response below. We feel that these changes greatly strengthen the manuscript and we thank the reviewers for their suggestions.
Sincerely,

Robert W. O'Rourke, MD

_____

Editorial and production comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
3. JoVE format does not support a separate abbreviations list; instead, please ensure that all abbreviations are defined on first use.
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: EVE Cell Counting Slides, NanoEnTek, TaqMan, BeadBug, Solutions X-Stream, RNAEasy

Done

Protocol:
1. There is a 10-page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Details were added throughout the protocol as requested.

Specific Protocol steps:
1. 1-3: Protocol steps should largely be in the imperative; please ensure every substep has at least one instruction. Also, it is likely unnecessary to include kits and instruments in the protocol text; the Table of Materials should be sufficient.

Done

Results:
1. Please include a "Representative Results" section with at least one paragraph of text explaining the results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

A Representative Results section was added, along with additional data demonstrating applications of the ECM-adipocyte culture. The new Fig 4 is derived from our previously published work Baker et al., JCEM, 2017. This latter citation was referenced in the acknowledgment section as the source of the additional Figure 3 of the revised manuscript as well.

Figures:

1. Please upload each Figure individually to your Editorial Manager account. Please remove 'Figure 1' etc. as well as unnecessary whitespace.

Done

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]
2. Please do not abbreviate journal titles.

Done

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary: Clear and thorough; referenced appropriately.
Major Concerns: none -- protocols are clearly written; all supplies listed appropriately.
Minor Concerns:
If the authors wish to add a comment on the following it may be useful if answer is known:
It appears that the preadipocytes differentiated in decellularized matrix become unilocular. How consistent it this finding? all cells? Short of electron microscopy, is there a way to assess? Is it possible to digest the reseeded ECM to liberate adipocytes for metabolic assays or must one work with embedded cells?

A primary challenge with ECM-adipocyte culture is visualizing cells within the ECM. Scanning electron microscopy, while non-quantitative, is reliable and demonstrates unilocular adipocytes. We have had success with ORO staining of fixed ECM-adipocyte cultures, as described in this report, which also suggest unilocular adipocytes but is similarly non-quantitative. We have not yet perfected methods to liberate adipocytes from ECM once seeded but this is an excellent suggestion by the reviewer which we have considered. One of our hopes is that publication of this manuscript will prompt others to develop better methods for visualizing cells within the ECM and possibly liberate cells from the ECM. This important observation the reviewer raises regarding the possibility that the ECM induces unilocular lipid droplet formation adipocytes was added to the discussion section of the manuscript.

Also 2 little things:
-- section 5.4 states 10 mm Petri Dish. Typo for 100 mm?

Corrected-thank you.

-- protocol should mention safety precautions e.g. for PMSF and perhaps just briefly working with human tissues in general for less experienced workers - even though all procedures under the hood).

A sentence was added to 4.1 addressing human safety and reagent precautions

Reviewer #2:

Manuscript Summary: The authors provide a protocol isolating preadipocytes from adipose tissue, preparing ECM from adipose, and culturing/differentiating the preadipocytes in the ECM. Overall, the protocol is well-explained and should be useful for other researchers. Several concerns and edits should be addressed to improve the manuscript:

Minor Concerns:
1) The introduction is pretty simplistic with regard to how adipose tissue goes bad. It is certainly not just due to "nutrient overflow" as stated but is a complex process involving hypertrophy, inflammation, exposure to toxins, ECM interactions, etc. This could be expanded upon.
2) Also, in the Introduction, state a few examples as to how the ECM cultures better than other culture methods.

We have added text to the discussion to expand upon these two important points that the reviewer raises.

3) On protocol section 5.1, how do you mince the tissue when it is in a tube?

Details were added to 5.1 to answer this question.

4) On protocol section 5.4, should mention the thick layer of fat that will be at the top. It can cause problems when aspirating.

We do not see a significant fat layer at this point, but rather most of the fat resides in a liquid lipid layer. The reviewer nonetheless raises an important issue, namely variability in different tissue preparation methods from lab to lab leads to variability in the nature of the tissue materials at each step in the process. To address this and better describe what we see

in our lab, we added a new Figure 2 to the revised manuscript, that provides photographs of each step of the collagenase digestion process, so that readers may better understand this art of the procedure. Thank you for raising this issue.

5) On protocol section 5.5, there must be a PBS wash that is missing before trypsinizing the cells. Also, aren't the cells counted and a certain number plated? This seems important.

We added the PBS wash step-thank you for pointing out this omission. Cells are not counted until step 5.7, at which point in the protocol, this is specified. For step 5.5, a 100mm dish of confluent cells is transferred to a single T150 flask, as specified in the protocol.

6) On protocol section 5.6, how many passages would you expect to get?

We passage preadipocytes 3-4 times during the expansion process, as we have found that this number of passages permits appropriate expansion and retains adipogenic potential and patient- and depot-specific metabolic phenotypes. Passages beyond this reduce adipogenic potential and should be avoided. We added this information to section 5.6 of the revised manuscript. Of note, reviewer #2 asked a similar question.

7) On protocol section 7.1, it is unclear on the quantity of ECM fragments that you would use.

Details were added to 7.1 to answer this question.

8) On protocol section 7.6, it says to "carefully aspirate". It would be useful to provide some pointers on how to do this without sucking up the ECM.

Details were added to 7.6 to answer this question.

9) On protocol section 8.4.5 would be worthwhile to mention any safety/disposal precautions when using 3H.

Details were added to 8.4.5 to address this concern.

10) In the Discussion, it would be helpful to expand upon how to overcome the various challenges that are mentioned instead of just stating that something "is challenging".

The Discussion was edited to provide guidance on how to overcome the challenges mentioned.

11) What kinds of inter-patient variability do you see and how do you deal with it when interpreting experimental results?

We observe inter-patient variability with respect to efficiency of delipidation of adipose tissue during ECM preparation as mentioned in the discussion section, but we have not observed any correlation of delipidation efficiency with DM status, age, sex, BMI, or other patient clinical variables from whom tissue is derived. We do not use ECM that we cannot completely delipidate in metabolic experiments, and thus we exclude these samples from downstream assays and analysis. We discuss in the discussion section troubleshooting methods to address the problem of incomplete delipidation.

We also observe inter-patient variability in adipocyte cellular metabolism (glucose uptake, lipolytic function) in both 2D-plastic and 3D-ECM culture, although despite this variability, when analyzed in aggregate, cells manifest disease-, depot- and sex-specific differences in cellular metabolism, as described by our group and others in multiple publications (Baker et al., O'Rourke et al., Tchkonia et al., Tchoukalova et al.), validating the utility and generalizability of in vitro human adipocyte culture for the study of cell metabolism. We added a comment to the discussion section of the revised manuscript addressing this important issue.

12) Figures showing adipogenesis would be helpful for visualization.

To address this important concern, we added micrographs of Oil Red-O stained adipocytes within ECM, as well as qRTPCR data demonstrating upregulation of adipogenic genes in adipocytes within the ECM over the course of differentiation in the new Figure 4 added to the revised manuscript, to demonstrate maturation and adipogenesis of preadipocytes within the ECM.

Reviewer #3:

The ECM plays an important role in adipose tissue development and function, and is often overlooked when studying adipose tissue. Most studies investigate adipocyte function using either freshly isolated adipocytes or preadipocytes differentiated in vitro in 2D cultures. Here, Flesher et al. describe a 3D human extracellular matrix (ECM)-adipocyte in vitro culture system that allows the study of the role of the matrix-adipocyte interaction in contributing to adipose tissue function. The protocol described in the present manuscript outlines the steps necessary to decellularize human adipose tissue to isolate the ECM, and the process of differentiating human preadipocytes in the ECM scaffold to create 3D adipocyte-ECM constructs that are metabolically active. The manuscript is well prepared but will benefit from several recommendations:

1) The manuscript/protocol appears to be based on earlier research from the authors (Ref 8: J Clin Endocrinol Metab. 2017 Mar 1; 102(3): 1032-1043). However, this is not obvious when reading the current manuscript. Line 77-78, the authors write: "We adapted these methods to develop an in vitro 3D model of human ECM-adipocyte culture, using ECM and adipocyte stem cells (preadipocytes) derived from human visceral adipose tissues. 13,14". Refs 13 and 14 do not appear to be the authors' work and Ref 8 should be highlighted more prominently to refer the readers to the study and representative results corresponding to the current manuscript.

The Introduction was edited to clearly refer to and cite our prior publication describing the ECM-adipocyte culture system. We also added a new Figure 4 with data from this original manuscript, and we cite this manuscript and republished data in the acknowledgment section of the revised manuscript. Thank you for pointing out this omission.

2) The authors only provide images of the adipose decellularization and recellularization process. The manuscript would benefit from also showing representative glucose uptake and lipolysis data, and oil red O imaging to illustrate that the ECM-adipocyte constructs are functional after culture.

The requested functional data was added to the revised manuscript in the new Representative Results section and the new Figure 4. Some of these additional data were derived from our previously published work Baker et al., JCEM, 2017. This citation was referenced in the acknowledgment section as the source of these additional data.

3) Glucose uptake rate should be reported as moles of glucose per unit of time and not simply as radioactive cpm. Lipolysis as moles of glycerol/FFA per unit of time. This allows for easier comparisons across experiments and across studies.

The reviewer raises an important point. Unfortunately, we do not have free fatty acid data for the lipolysis experiment presented as we only measured glycerol release, nor do we have reagent information from the lot of ^{3}H-labeled glucose used for the specific glucose uptake experiment presented in Fig 3B to determine cpm/mole of glucose, so we are unable to revise the added figure 4B to accommodate this request. We do however acknowledge this limitation in the discussion of the revised manuscript and state that reporting data as the reviewer suggests provides for more accurate comparisons of data across experiments and across studies.

4) Normalization of glucose uptake/lipogenesis/lipolysis assays is often subject of debate (protein content vs lipid content vs adipocyte number). The authors recommend normalizing with protein content, but for experiments where preadipocytes are differentiated into adipocytes in vitro, results need to be normalized by lipid content in order to account for potential differences in differentiation capacity.

The reviewer raises an important point of ongoing debate in adipocyte biology, i.e. what is the best method for normalizing cell function per cell in adipocytes? Cell number, protein, DNA, and lipid have all been suggested as normalization factors. We typically normalize metabolic data to protein measured with Bradford assay, but we also normalize by DNA content, and have observed similar results with both of these methods in our model systems. We also seed each well for every experiment with the same number of preadipocytes, although admittedly this does not fully account for variability in differentiation. We have not developed methods for accurately quantifying lipid content, in part due to difficulty in fully releasing lipid from ECM constructs, and thus we do not have data regarding lipid content and have not tested concordance of lipid normalization with other methods. We added a sentence to the discussion addressing this issue and acknowledging this limitation.

5) Do the authors observe differences in ECM isolation and culture process between adipose tissue from lean/obese/diabetic patients? This should be discussed.

Reviewer #2 raised a similar question. We see inter-patient variability with respect to efficiency of delipidation of adipose tissue during the ECM preparation process as mentioned in the discussion section, but we have not observed any correlation of delipidation efficiency with DM status, age, sex, BMI, or other patient clinical variables from whom tissue is derived. We discuss in the revised discussion section troubleshooting methods to address the problem of incomplete delipidation and the lack of correlation between ECM delipidation and subject clinical characteristics. We do observe DM-specific difference in ECM-adipocyte constructs with respect to adipocyte metabolism, along with a rescue effect of DM adipocyte metabolic function by NDM ECM, which is discussed in detail in the revised manuscript's discussion section.

6) Is there an advantage to culture ECM and adipocytes from the same donor? How does culturing ECM from one donor with adipocytes from a different donor affect the results? This should be discussed

We have not observed differences in metabolic functions (glucose uptake, lipolysis) when ECM and adipocytes from the same or different donors are studied. In either case, ECM-adipocyte constructs manifest DM-specific metabolic phenotype (e.g. decreased glucose uptake in DM/DM relative to NDM/NDM constructs), with no clear difference in the magnitude of metabolic responses when comparing DM/DM or NDM/NDM constructs from the same or different donors. We speculate that in the absence of immune cells, which the ECM preparation eliminates, no alloresponse occurs, thus preserving adipocyte function despite donor mismatch between ECM and adipocyte. We added a brief paragraph to the discussion addressing this important point and we thank the reviewer for raising it.

Reviewer #4:

Manuscript Summary: I think this report describes quite clearly the procedures and the figures are complimentary to this. Thus, scientists will find this protocol useful in further exploring the importance of ECM.

Major Concerns:

1. It is stated that they are seeding cells in 10 mm Petri dishes but surely it is 100 mm dishes?

This was corrected-thank you for pointing out this error.

2. How many passages for cell amplification and what consequences of likely 7-8 passages? Has this been characterized?

We passage preadipocytes 3-4 times during the expansion process, as we have found that this number of passages permits appropriate expansion and retains adipogenic potential and patient- and depot-specific metabolic phenotypes. Passaging preadipocytes in excess of 4-5 passages leads to loss of adipogenic potential. We have added this information to section 5.6 of the revised manuscript. Of note, reviewer #2 asked a similar question.

3.The authors have performed gene analysis but do not comment on the usefulness/problems of the procedure for protein extraction. This is obviously an important issue.

The reviewer raises an important point. We have not yet developed a robust method for extracting un-degraded protein, especially with phospho-moieties intact, from the ECM. For this reason, to date we have relied on transcriptional analysis. We are currently refining such methods, but to date we have not successfully isolated protein that is non-degraded for Western blot from ECM. We added a brief discussion of this limitation to the revised discussion section. We hope that publication of this manuscript will prompt others to develop such methods.

4. It is surprising to this reviewer that preadipocyte differentiation is the same in T2D and ND cells. There are several reports with different data. Any comments on this?

We have not observed differences in adipogenic differentiation capacity between DM and NDM preadipocytes as measured by adipogenic gene expression or lipid droplet formation in either 2D or 3D culture. We have however observed significant differences in cellular metabolism between DM and NDM cells, with DM cells manifesting decreased glucose uptake and lipolytic capacity in both 2D and 3D systems, as reported in Baker et al. JCEM 2017. Of note, we also demonstrated that NDM ECM rescues these metabolic defects in DM adipocytes, also reported in Baker et al., and discussed in this manuscript and displayed in the new Figure 4. We discuss this latter effect as a useful application of ECM-adipocyte culture in detail in the revised manuscript.

From: JOURNALS PERMISSIONS
To: O"Rourke, Robert
Subject: RE: Copyright permission
Date: Monday, August 12, 2019 7:58:23 AM
Attachments: image001.png

External Email - Use Caution

Dear Robert W. O'Rourke,

RE. Figs 4B & 5A. Nicki A. Baker, Lindsey A. Muir, Alexandra, R. Washabaugh et al. Diabetes-Specific Regulation of Adipocyte Metabolism by the Adipose Tissue Extracellular Matrix. *The Journal of Clinical Endocrinology & Metabolism* (2017) 102 (3): 1032-1043, https://doi.org/10.1210/jc.2016-2915

Many thanks for your response. I can confirm that as this article is published under a Creative Commons CC-BY-NC license, this permits non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited. Therefore you don't require direct permission from us to reuse these figures.

Kind regards,
Katie

Katie Randall | Permissions Assistant | Rights Department
Academic and Journals Divisions | Global Business Development
Oxford University Press | Great Clarendon Street | Oxford | OX2 6DP

OXFORD
UNIVERSITY PRESS

From: O'Rourke, Robert [mailto:rorourke@med.umich.edu]
Sent: 12 August 2019 12:44
To: JOURNALS PERMISSIONS
Subject: Re: Copyright permission

Yes this would be non-commercial use- it's a methods paper we'd like to disseminate for scientific interest

Robert W. O'Rourke, MD
Professor, Department of Surgery, Michigan Medicine and University of Michigan Medical School
Chief, Division of General Surgery, Director, Bariatric Surgery Program, Ann Arbor Veterans Administration Hospital

Department of Surgery
Michigan Medicine
2210 Taubman Center-5343
1500 E. Medical Center Dr
Ann Arbor, MI, USA 48109-5343

Administrative Specialist Associate: April Schauer (734)936-5823 phone
(734)936-5830 fax

On Aug 12, 2019, at 7:40 AM, JOURNALS PERMISSIONS <Journals.Permissions@oup.com> wrote:

External Email - Use Caution

Dear Robert W. O'Rourke,

RE. Figs 4B & 5A. Nicki A. Baker, Lindsey A. Muir, Alexandra, R. Washabaugh et al. Diabetes-Specific Regulation of Adipocyte Metabolism by the Adipose Tissue Extracellular Matrix. *The Journal of Clinical Endocrinology & Metabolism* (2017) 102 (3): 1032-1043, https://doi.org/10.1210/jc.2016-2915

Thank you for your email. Please can you confirm whether this reuse within JOVE would be non-commercial?

Kind regards,
Katie

Katie Randall | Permissions Assistant | Rights Department
Academic and Journals Divisions | Global Business Development
Oxford University Press | Great Clarendon Street | Oxford | OX2 6DP

From: O'Rourke, Robert [mailto:rorourke@med.umich.edu]
Sent: 07 August 2019 16:55
To: JOURNALS PERMISSIONS; 'customercare@copyright.com'
Subject: RE: Copyright permission

Dear Oxford University Press,

I'd like to request permission to re-use two figures from a manuscript my laboratory published in *J Clin Endo Metab* in 2017 (Baker, Nicki A. et al., Diabetes-Specific Regulation of Adipocyte Metabolism by the Adipose Tissue Extracellular Matrix *J Clin Endo Metab* 2017; Mar 1;102(3):1032-1043. doi: 10.1210/jc.2016-2915.) for publication in a techniques manuscript in the Journal of Visualized Experiments (J Vis Exp, aka JOVE). Specifically, I'd like to republish figure 4B and figure 5A from Baker et al..

I attempted to get a permission vis the website:

https://s100.copyright.com/AppDispatchServlet?publisherName=OUP&publication=1945-7197&title=Diabetes-Specific%20Regulation%20of%20Adipocyte%20Metabolism%20by%20the%20Adipose%20Tissue%20Extracellular%20Matrix&publicationDate=2017-01-05&volumeNum=102&issueNum=3&author=Baker%2C%20Nicki%20A.%3B%20Muir%2C%20Lindsey%20A.&startPage=1032&endPage=1043&contentId=10.1210%2Fjc.2016-2915&oa=CC%20BY-NC&copyright=Oxford%20University%20Press&orderBeanReset=True

But was not able to register or logon. I emailed for help, and was referred to this website: https://global.oup.com/academic/rights/permissions/autperm/?lang=en&cc=gb, which states that:

After publication you may reuse the following portions of your content without obtaining formal permission for the activities expressly listed below:

- *one chapter or up to 10% of the total of your single author or co-authored book,*
- *a maximum of one chapter/article from your contribution to an edited book or collection (e.g. Oxford Handbooks),*
- *a maximum of one chapter/article of your contribution to an online only, or digital original publication, or*
- *three figures/illustrations/tables of your own original work*

OUP is pleased to grant this permission for the following uses:

- *posting on your own personal website or in an institutional or subject based repository after a **12 month** period for **Science and Medical** titles and a **24 month** period for **Academic, Trade and Reference** titles;*
- *inclusion in scholarly, not-for-profit derivative reuses, (these can include the extension of your contribution to a book-length work, or inclusion in an edited collection of your own work, or any work of which you are an author or editor);*
- *reproduction within coursepacks or e-coursepacks for your own teaching purposes, (with the proviso that the coursepacks are not sold for more than the cost of reproduction);*
- *inclusion within your thesis or dissertation.*

Permission for these reuses is granted on the following conditions:

- *that the material you wish to reuse is your own work and has already been published by OUP;*
- *that the intended reuse is for scholarly purposes, for publication by a not-for-profit publisher;*
- *that full acknowledgement is made of the original publication stating the specific material reused [pages, figure numbers, etc.], [Title] by/edited by [Author/editor], [year of publication], reproduced by permission of Oxford University Press [link to OUP catalogue if available, or OUP website];*
- *In the case of joint-authored works, it is the responsibility of the authors to obtain permission from co-authors for the work to be reuse/republished.*
- *that reuse on personal websites and institutional or subject based repositories includes a link to the work as published in an OUP online product (e.g. Oxford Scholarship Online), and/or or to the OUP online catalogue entry; and that the material is not distributed under any kind of Open Access style licences (e.g. Creative Commons) which may affect the Licence between yourself and OUP.*

Can I assume that my plans to republish figure 4B and figure 5A from Baker et al.. *J Clin Endo Metab* 2017; Mar 1;102(3):1032-1043 in a techniques manuscript in the Journal of Visualized Experiments falls under the stipulations above and is ok to proceed? Just wanted to confirm of course before moving forward.

Thanks

Robert W. O'Rourke, MD
Professor, Department of Surgery, Michigan Medicine and University of Michigan Medical School
Chief, Division of General Surgery, Director, Bariatric Surgery Program, Ann Arbor Veterans Administration Hospital

Department of Surgery
Michigan Medicine
2210 Taubman Center-5343
1500 E. Medical Center Dr
Ann Arbor, MI, USA 48109-5343

Administrative Specialist Associate: April Schauer (734)936-5823 phone; (734)936-5830 fax

From: JOURNALS PERMISSIONS <Journals.Permissions@oup.com>
Sent: Wednesday, August 7, 2019 11:46 AM
To: O'Rourke, Robert <rorourke@med.umich.edu>
Subject: Automatic reply: Copyright permission

External Email - Use Caution

All **Journal** permission requests should be directed to **Rightslink** either at article level on the Oxford Journals website via the 'Permissions' link or through the **CCC** website.

Any queries regarding this service should be addressed to: customercare@copyright.com

All **Academic** and **EDUK** permission requests are now handled through the **PLS Clear** system - details of how to request permission can be found here: https://global.oup.com/academic/rights/permissions/?lang=en&cc=gb

For information on how to navigate and/or use this online system please contact: plsclear@pls.org.uk

****
Electronic Mail is not secure, may not be read every day, and should not be used for urgent or sensitive issues

****
Electronic Mail is not secure, may not be read every day, and should not be used for urgent or sensitive issues

Figure 1

Figure 2

Click here to access/download

Supplemental Coding Files

Flesher-et-al.-J-Vis-Exp-2019-Figure-2.svg

Figure 3

Click here to access/download

Supplemental Coding Files

Flesher-et-al.-J-Vis-Exp-2019-Figure-3.svg

Figure 4

Click here to access/download

Supplemental Coding Files

Flesher-et-al.-J-Vis-Exp-2019-Figure-4.svg