ECM Hydrogel Derived from Decellularized Adipose Tissue for Adipose Derived Stem Cell Differentiation to Augment Breast Reconstruction

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Abstract

In 2020, over 130,000 women in the United States alone underwent breast reconstructive procedures.[1] These procedures include prosthetic implants or autologous tissue transfer, both supplemented with traditional fat grafting. However, due to low volume retention rates, poor supportive vasculature, and unsuitability for larger defect volumes, there is a clinical need to improve fat grafting.[2] As adipose tissue is the most abundant, consumable, and easily harvested tissue found in the human body, we proposed creating an extracellular matrix (ECM) hydrogel that is derived from human decellularized adipose tissue (hDAT) to support allogeneic adipose-derived stem cell (ADSCs) differentiation. Adipose tissue is rich in ECM and can secrete growth factors potentially supporting the proliferation and differentiation of ADSCs into adipocytes, thus allowing for controlled fat growth and high-volume retention rates. In this study, an hDAT hydrogel with a 15 mg/mL concentration was created that underwent gelation under physiological conditions. A DNA quantification assay confirmed an 89% reduction in DNA content, and microstructure analysis and rheology results suggest the potential for the hDAT hydrogel to create a hospitable environment for the differentiation of ADSCs. The hDAT hydrogel was embedded with ADSCs *in vitro* to assess differentiation and adipogenesis. Preliminary results suggest a significant difference between the lipid concentrations for the hDAT hydrogel embedded with ADSCs and the hDAT alone. These results, combined with future *in vivo* mouse models, could establish the hDAT hydrogel as a promising clinical alternative to traditional fat grafting used in breast reconstruction.

Keywords: human decellularized adipose tissue, adipose tissue, breast reconstruction, adipose-derived stem cells, fat grafting

Introduction

In 2022, over 200,000 women received a breast cancer diagnosis, with 72% of these women electing for a mastectomy and the other 28% choosing to have breast-conserving surgery.[1] Post-mastectomy reconstruction is typically done autologously from the patient's own body with varying muscle flaps known as autologous tissue transfer (ATT), or alloplastic with implants. In combination with either of these methods, autologous fat grafting (AFG) is used to provide contouring which addresses smaller deformities that remain after ATT or implantation methods. The choice of reconstruction method depends on the location of the resected tumor, the choice of the patient, or the need for follow-up treatment, such as radiation.[2]

The process of AFG includes taking a small amount of fat from another region of the body and reinjecting it in the site of the resected breast tumor. As only a small amount of fat can be removed from the body, AFG only serves

minor defects after tissue transfer or implantation and can be absorbed back into the body due to the poor vasculature at the implantation site. One study done on facial reconstruction AFGs found that only 33% of the initial volume inserted remains after a year.[3] Another complication associated with fat grafting includes weakness in certain areas following the removal of fat, thus making daily tasks harder for these patients.[4] Fat grafting is also limited if the patient does not have an excess of body fat available for harvesting. Thus, the existing complications within fat grafting create a clinical need for a more controlled and minimally invasive approach in post-mastectomy reconstructions.

Decellularized ECM hydrogels are a promising biomaterial for tissue engineering applications. These hydrogels have demonstrated potential as scaffolds to induce tissue repair and organ regeneration. As adipose tissue is the most abundant, consumable, and easily harvested tissue, it is an optimal choice for creating a hydrogel. Adipose provides an ample amount of ECM proteins and can secrete numerous growth factors, creating potential for an hDAT hydrogel to support stem cell viability and differentiation. Studies have investigated decellularized adipose ECM hydrogels. Pu et. al. produced a thermally sensitive hDAT-gel, quantified that the gel supports ADSC proliferation *in vitro*, and showed that an ADSC-embedded hDAT-gel accelerated wound healing of mouse skin.[5] Zhao et. al. found that hDAT-gel can induce adipogenesis and has better biocompatibility than many other biomaterials for soft tissue deformation when injected subcutaneously into mice.[6] By controlling the parameters of the hydrogel to improve volume retention rates and examining the nutrient and waste exchange of cells, this research shows potential use for an adipose-derived ECM hydrogel in breast reconstructive surgery post-mastectomy.

The overall objective of this Capstone project was to develop and characterize a human decellularized adipose tissue (hDAT) ECM hydrogel seeded with ADSCs that are able to effectively differentiate into mature adipocytes, providing controlled fat growth. The first aim was to fabricate and characterize the hDAT hydrogel by modifying established adipose decellularization protocols, verifying the decellularization through a DNA content assay, imaging the microstructure of the hydrogel through Wright staining, and quantifying the mechanical properties of the hydrogel through rheology testing. The second aim was to incorporate ADSCs into the ECM hydrogel *in vitro* and assess cell viability and adipogenesis through Oil Red O staining. With these results and further research, this ADSC-embedded hDAT ECM hydrogel could be established as a predictable and non-invasive soft tissue filler to improve or replace traditional fat grafting used in breast reconstruction.

Results

Fabrication of the hDAT Hydrogel

Fresh adipose tissue was harvested via a panniculectomy procedure performed at the UVA Health Center. All cells and cellular components were removed from the tissue through a series of chemical and enzymatic washes over the course of a week. The residual ECM was lyophilized until completely dehydrated, and this lyophilized ECM was ground into a fine powder using a sieve and grinding apparatus. The ECM powder was digested with pepsin in acidic solution into monomers to form a pre-gel. The pre-gel then self-assembled at physiologic temperature after 15-30 minutes to form a homogenous and polymerized gel (Figure 1).

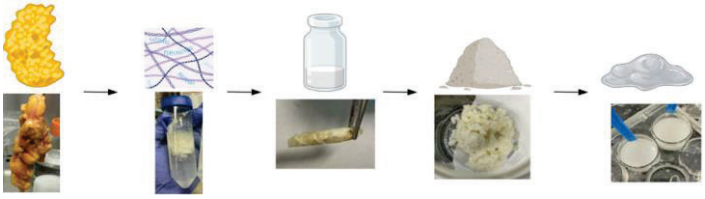

Figure 1. Decellularization of adipose tissue to create the hDAT ECM hydrogel.

Microstructure of Pre-Gel Creates a Hospitable Environment for Cells

The hydrogel underwent Wright staining in order to view the microstructure of the ECM hydrogel. Images were taken under 5x and 40x magnification to display a network of interconnecting fibers to create a variety of pore sizes throughout the gel. Utilizing ImageJ, analysis revealed that the pore sizes of the gel range from 10 to 15 μm in diameter (Figure 2).

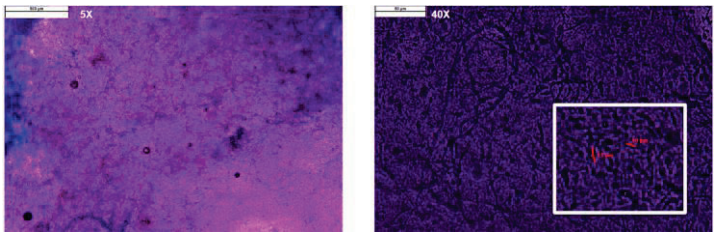

Figure 2. Wright staining, a combination of eosin and methylene blue dye, was performed to provide contrast to the pre-gel and allow visualization of the fibers and pores.

DNA Quantification Assay Verifies Decellularization

Decellularization of the hDAT hydrogel was verified by utilizing the Quant-iT PicoGreen dsDNA Assay Kit and Reagents, a fluorescent DNA quantification assay. Utilizing the created standard curve of known DNA concentrations, the concentration of DNA found within the hDAT hydrogel was 5.3 µg of DNA/mL (Figure 3). As adipose tissue contains 47.8 µg of DNA/mL, this demonstrates an 89% decrease in DNA content which can be attributed to the decellularization protocol.[7]

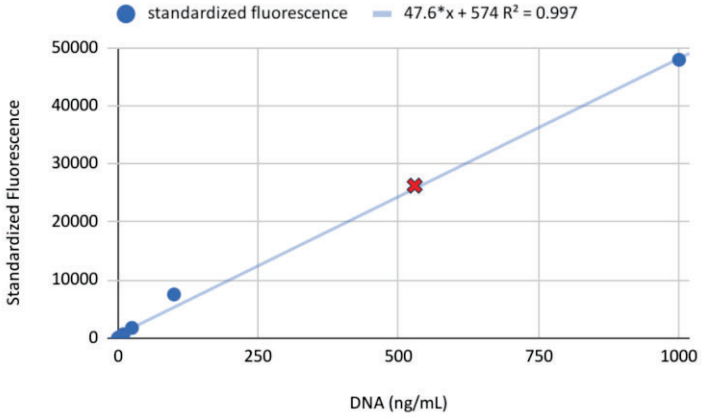

Figure 3. Standardized fluorescence values for known DNA concentrations are seen as blue points. The fluorescence of the hDAT hydrogel is denoted with the red X. Because the hydrogel was diluted 10-fold when tested, the quantified DNA concentration is multiplied by 10.

Soft Materials Favor Adipogenic Differentiation

The ECM powder was digested at different concentrations of 10, 15, and 20 mg/mL. Using a modular compact rheometer in the Innovations in Fabrication (IFAB) facility, the storage and loss modulus were measured for the varying concentrations over the course of 60 seconds (Figure 4). Three trials were performed for each concentration and average storage and loss modulus were calculated (Table 1). The similar storage and loss moduli of the 15 and 20 mg/mL concentrations could indicate that the pre-gel solution became saturated around 15 mg/mL.

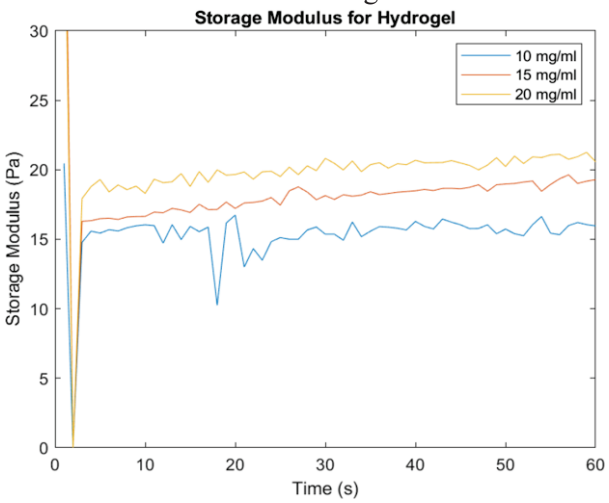

Figure 4. The storage moduli, a measure of stiffness, of the hDAT ECM hydrogel at concentrations of 10, 15, and 20 mg/mL measured with a rheometer at room temperature.

Table 1. Experimental average Storage and Loss Moduli of the hDAT ECM hydrogel at concentrations of 10, 15, and 20 mg/mL

Concentration (mg/mL)	Storage Modulus (Pa)	Loss Modulus (Pa)
10	15.3 ± 1.68	6.71 ± 1.46
15	18.2 ± 4.15	7.21 ± 0.91
20	20.1 ± 3.76	7.5 ± 1.49

hDAT ECM Hydrogel Promotes Adipogenesis

Passage 3 ADSCs were embedded into the ECM hydrogel and incubated alongside a positive and negative control, ADSCs plated on a glass well and the ECM hydrogel without cells, respectively. The cells were maintained in Adipogenic growth medium for 7 days. The ADSCs on day 7 were stained with Oil Red O to assess for lipid concentration, a marker for adipogenesis. Due to the opaqueness of the ECM hydrogel, it was difficult to visualize the cells embedded within (Figure 5a). Thus, the Oil Red O staining was quantified utilizing percent area staining in ImageJ, which resulted in a significant increase in percent area stained for the ECM hydrogel embedded

with ADSCs, 0.531 ± 0.079 %, then that of the ECM hydrogel alone, 0.243 ± 0.047 % (Figure 5b).

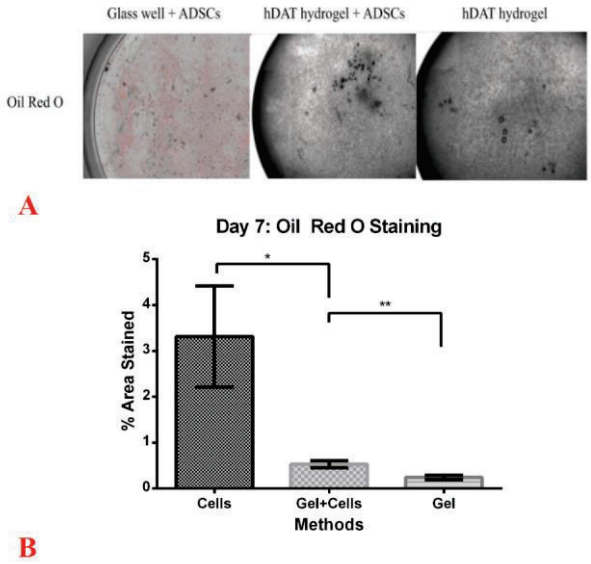

Figure 5. (**5a**: top) Images of Oil Red O staining of cell culture. (**5b:** bottom) Percentage area stained.

Discussion

The hDAT ECM hydrogel examined in this study has the potential to augment traditional fat grafting methods through utilizing adipose tissue that is often discarded as medical waste. By repurposing this tissue to an ECM hydrogel through a decellularization process, the hydrogel will improve volume retention rates and implementation into native tissue in breast reconstruction. Previous literature indicated the efficiency and optimization of the decellularization process detailed in *Methods in Molecular Biology*, which was thus implemented into this study.[8] The effectiveness of the decellularization process was analyzed through the quantification of DNA content within the pre-gel, resulting in an 89% reduction in DNA content from native adipose tissue. The DNA content of our hydrogel, 5.3 µg of DNA/mL, equates to 353 ng DNA/mg of ECM powder. Prior research has suggested that a DNA content of less than 50 ng DNA/mg of ECM powder is optimal for proper decellularization and a limited immune response.[9] Therefore, the extent of our decellularization could potentially be increased through longer incubation times with DNase, RNase, and lipase to reach this lower threshold.

Following the creation of the ECM powder and then digestion with pepsin, the ECM pre-gel was neutralized to reach a pH of 7 and inactivate pepsin. The ECM hydrogel underwent gelation under physiological conditions for 15-30 minutes, allowing for an increase in crosslinks and hydrophobic interactions within the ECM hydrogel. Physiological conditions for gelation are deemed preferable due to the clinical application of this study, and thus having this pre-gel undergo gelation when potentially implementing it within a patient is optimal.

Wright staining, a stain composed of eosin and methylene blue, is typically used to distinguish between blood cells. For the purpose of this project, however, Wright staining was utilized to emphasize and visualize the microstructure of the ECM hydrogel. The pore sizes within the structure were found to be in between 10 to 15 µm, and pre-adipocytes range in size from 10 to 20 µm in diameter.[10] These sizes are comparable, indicating that the pores of the ECM hydrogel could allow for proper pre-adipocyte support.[10] Because the hydrogel is a loosely crosslinked network of ECM proteins aggregated through guest-host, ionic, and hydrophobic interactions, the pre-adipocytes should be able to grow and migrate freely within the network. Furthermore, as the hydrogel is loosely connected, the ECM hydrogel should also grow and expand along with the differentiated mature adipocytes as they reach sizes of 50 to 200 µm.[11]

To assess the mechanical properties associated with the ECM hydrogel, the hydrogel was placed in a rheometer under room temperature to assess the storage and loss modulus. On average, the storage moduli were greater than the loss moduli for all concentrations, suggesting that the hydrogel is mainly elastic. Compared to other material types for the purpose of adipogenic differentiation such as a double-network PEG/collagen hydrogel and methacrylamide-modified gelatin (Gel-MOD), the hDAT hydrogel is relatively softer (Table 2). A softer hydrogel is ideal as pre-adipocytes tend to prefer softer surfaces for their differentiation and growth into mature adipocytes.[12,13]

Table 2. Average storage moduli of comparator materials.

Material Type	Storage Modulus (Pa)
Degradable PEG	87.7
Gel-MOD	538
Adipose Tissue[14]	2000

The hydrogel was then embedded with ADSCs to assess their *in vitro* survivability and differentiation into mature adipocytes. Although the differentiation process takes approximately 11 to 13 days, at the time of this report, data has only been collected up until day 7. The three groups assessed in this study were the gel embedded with ADSCs, a positive control, and a negative control. The positive control reflects the gold-standard in cell culture with the cells directly plated on 24-well plate and the negative control contains the ECM hydrogel on its own, without any cells. On day 7, the three samples were stained with Oil Red O to assess the progress in differentiation of the ADSCs into adipocytes. The results suggested a significant increase in the percent area dyed with Oil Red O staining in the gel embedded with cells compared to the gel on its own. The current results appear promising as there has been a significant increase in staining in the ECM hydrogel containing cells. However, a day 14 Oil Red O staining is required to assert adipocyte differentiation within the ECM hydrogel.

An ECM hydrogel derived from human decellularized adipose tissue was prepared, analyzed, and embedded with cells to potentially serve as a supplement in fat grafting post-mastectomy. The study demonstrates the hDAT ECM hydrogel creates a hospitable environment for the growth and migration of mature adipocytes within the hydrogel. Additionally, the study shows promising signs for the differentiation of adipose-derived stem cells into mature adipocytes, allowing for effective integration into native tissue in a clinical sense. Thus, the ECM hydrogel proves a promising biomaterial to further conduct *in vivo* studies on and potentially implement as a supplementary method for breast reconstruction.

Materials and Methods

Fabrication of Hydrogel

Fresh adipose tissue was harvested via a panniculectomy procedure performed at the UVA Health Center by Dr. Chris Campbell. This adipose tissue was decellularized according to an established protocol found in *Methods in Molecular Biology*. The protocol included three free-thawing cycles, followed by treating the tissue under agitation with: 0.25% trypsin for 16h, isopropanol for 16 h, three 30 min rinses of Sorensen's phosphate buffer solution (SPB), trypsin for 6h, and three 30 min rinses of SPB. The tissue shrank significantly after isopropanol washes, which aided in lipid removal. The tissue was then treated with Deoxyribonuclease, Ribonuclease, and Lipase for 16h, isopropanol for 8h, three 30 min rinses of SPB, three 30 min rinses of 70% ethanol, and three 30 min rinses of ddH2O. The tissue was transferred from all washes and treatments with a fine mesh strainer in order to prevent sample loss. The remaining ECM was partitioned into 1 mL aliquots and lyophilized until completely dehydrated by the Griffin Lab. The lyophilized ECM was then ground into a fine powder using a #40 sieve (426μm) and grinding apparatus.

This ECM powder was digested at a concentration of 15 mg/mL with pepsin at a concentration of 1 mg/mL in a 0.01 M HCl solution under constant agitation at room temperature for 72 hours. The pepsin was dissolved completely in the HCl, and then the ECM powder was added. For example, to make 5 mL of pre-gel, using a small beaker and a stir plate 5 mg of pepsin were dissolved into 5 mL of HCl. Once visually homogeneous, 75 mg of ECM powder was added, covered with parafilm, and left under agitation for 72 hours. The solution was checked periodically to ensure the ECM powder did not cling to the walls of the beaker. After 72 hours, the solution was base neutralized on ice and under agitation with 1/10 the volume of the solution with 0.1 M NaOH, then diluted with 1/9 the volume of the solution with 10x PBS. For example, to neutralize 5 mL of solution, 500 μL of 0.1 M NaOH were added. The pH was checked with pH strips to ensure a pH between 7-8. Then, 555 μL of 10x PBS was added. Then pre-gel could then be stored in a refrigerator for up to a week or used immediately. The pre-gel was viscous, pipettable,

and homogenous. When the pre-gel was placed at 37℃, a polymerized and soft gel formed after approximately 15-30 minutes.

Pre-gel Microstructure

To perform the Wright staining, 100 µL of pre-gel was pipetted onto a microscope slide and gently smeared to create a thin layer. The pre-gel was covered with undiluted Wright stain solution, about 50 µL, for 2-3 minutes (Figure 6). An equal amount of DI water was added to the slide and let sit for 5 minutes. The slide was then carefully flooded with DI water and then air dried for 10 minutes. The slide was then imaged with a microscope under 5x, 20x, and 40x magnification. ImageJ was used to measure the pore sizes.

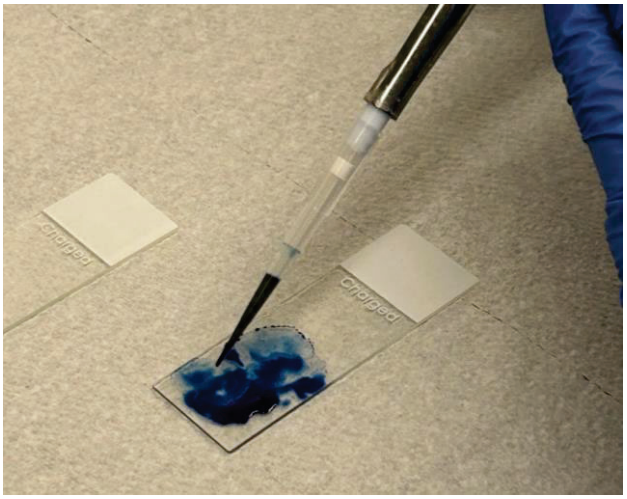

Figure 6. Wright stain solution is pipetted directly onto the pre-gel smear without overflowing the microscope slide.

Verification of Decellularization

The protocol outlined in the ThermoFisher Quant-iT PicoGreen dsDNA Reagent and Kit Manual was used to quantify the concentration of DNA in the hDAT hydrogel using a fluorescent nucleic acid stain (ThermoFisher #P7589).[15] A 1X TE buffer (Tris-HCl, EDTA) working solution was prepared by diluting the concentrated kit buffer 20-fold with nuclease-free water. Next, the DNA reagent working solution was prepared by diluting the concentrated kit DMSO solution 200-fold in 1X TE buffer. For a microplate assay of a total 200 µL assay volume per well, 100 µL of the DNA reagent was needed per well and was protected from light. To prepare the DNA standard curve, A 2 µg/mL stock solution of dsDNA in TE buffer was prepared by diluting the kit lambda DNA standard 50-fold in TE buffer. Next, this 2 µg/mL stock solution was diluted with TE buffer according to given manual volumes in Table 1 and Table 2 to final concentrations of 1 µg/mL, 100 ng/mL, 25 ng/mL, 10 ng/mL, 2.5 ng/mL, 250 pg/mL, and blanks (TE buffer) in a microplate for fluorescence-based assay (Cat. No. M33089) to a final volume of 100 µL. 3 replicates for each standard curve concentration were plated.

The experimental adipose ECM hydrogel was then prepared. The hydrogel was diluted 10-fold in TE buffer. 100 µL of the diluted hydrogel was plated in triplicate. Then, 100 µL of DNA reagent was added to each well, including the standard curve wells, and mixed thoroughly by pipetting up and down. The microplate was incubated for 5 minutes at room temperature, protected from light. The microplate fluorescence was then measured by a fluorescence microplate reader at 480 nm excitation and 520 nm emission.

To analyze the data, the raw fluorescence value of the reagent blanks (TE buffer) was subtracted from the fluorescence of each sample. This corrected data was used to generate a linear standard curve of fluorescence versus DNA concentration (Figure 3). The DNA concentration of the hydrogel was determined from the standard curve equation. Since the gel was diluted 10-fold, the given DNA concentration was multiplied by 10 to yield a final DNA concentration of 5.3 µg/mL for the hDAT adipose ECM hydrogel.

Rheological Characterization

To perform the rheology, a modular compact rheometer (Anton Paar, MCR 302) with a 25 mm cone plate was used. The storage and loss moduli values of the hydrogel were quantified under 1% shear strain and 0.25 Pa shear stress with a frequency of 1 Hz over 60 seconds, which were standard default settings using a rheometer. Due to the broken temperature sensor, the gelation process could not be quantified under physiological temperatures. 50 µL of pre-gel for each trial and concentrations were incubated at 37℃ for 15 minutes prior to the rheology to form the hydrogel.

Cell Culture

A 10 cm diameter cell culture dish with semi-confluent ADSCs at passage 3 was obtained from the Civelak Lab. Existing media was removed, and the cells were washed with room temperature PBS. 5 mL of 37°C trypsin-EDTA was added to the plate and then incubated for 4 minutes to detach the cells. The plate was removed from incubation and 12 mL of 37°C DMEM supplemented with FBS was added to neutralize the trypsin-EDTA. The cells were resuspended by gently pipetting up and down 3 times, and then gently transferred to a 50 mL sterile conical. The conical was centrifuged for 5 minutes at 1,000 rpm, and the supernatant was removed. The pellet was resuspended with 1 mL of 37°C DMEM supplemented with FBS. The cells were then counted (see Cell Counting).

498 µL of the cell suspension was combined with 786 µL of prepared adipose ECM hydrogel. 214 µL of this combination was then plated in triplicate into a 24-well plate to reach a total well volume of 1 mm for the experimental condition. 83 µL of the remaining cell suspension was plated in triplicate for the positive control, followed by 214 µL of the ECM hydrogel in triplicate for the negative control. This yielded $5x10^4$ cells for each positive control and experimental condition well. The final experimental set-up is shown in Figure 8. Two plates were prepared. The plates were incubated for 15 minutes until gelation occurred, and then 0.5 mL of adipogenic induction media (ThermoFisher #A1007001) was added into each well and changed every 3 days.

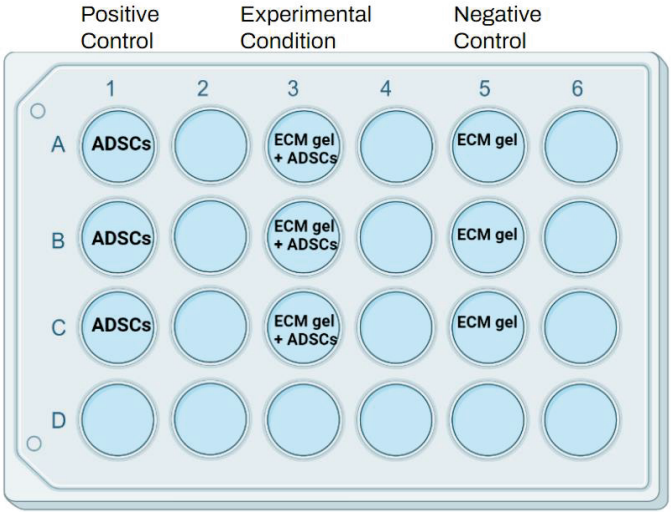

Figure 7. Cell culture experimental set-up.

Cell Counting

10 µL of cell suspension was added to 10 µL of trypan blue stain. This sample was then added to a hemocytometer prepared with a most coverslip. The live cells were counted under a light microscope, yielding a cell density of $6x10^5$ cells/mL (Figure 9).

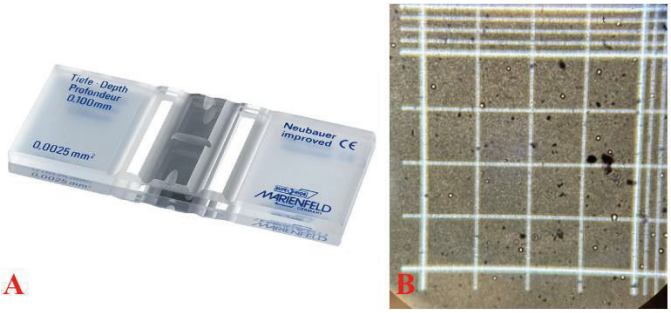

Figure 8. (**8a:** left) Glass hemocytometer.[16] (**8b:** right) One of four corners of the hemocytometer viewed under a light microscope. Live cells appear white and dead cells appear dark blue. The number of cells inside the 16 squares were counted, and then repeated for all four corners of the hemocytometer to yield a total cell count. This process was repeated for the opposite side of the hemocytometer. The cell counts were averaged and then multiplied by 10^4 to yield a final cell density of $6x10^5$ cells/mL.

Oil Red O Staining

Oil Red O (ORO) staining was performed after 7 days of culture. An ORO stock solution was prepared by combining 0.5 g ORO (Sigma #O-0625) with 100 mL of 98% isopropanol and stirred for 30 minutes. An ORO working solution was prepared by combining 24 mL of ORO stock solution with 16 mL of dH2O and stirred for 15 minutes. The working solution was then strained to collect any undissolved stain powder. One 24-well plate was removed from the incubator, and the wells were fixed with 125 µL of 4% PFA for 10 minutes. The wells were rinsed with PBS, then washed with 60% isopropanol and dried completely. 100 µL of ORO working solution was added to each well for 8 minutes. The ORO was then removed, and the wells were immediately washed in 100 µL of 60% isopropanol for 30 seconds. The wells were washed with dH2O and air dried, then imaged with a microscope. The percentage of ORO dye absorbed was quantified with ImageJ. ORO staining will also be performed on day 14 of culture with the second plate on 5/8/23.

End Matter

Author Contributions and Notes

A.C.K, O.G.M, and N.V.P.N contributed equally to research design, research performance, analysis, and writing.

P.C.D. contributed to research design, advising, and project funding.

The authors declare no conflict of interest.

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