Examination of ex-vivo viability of human adipose tissue slice culture

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Abstract

Obesity is associated with significantly higher mortality rates, and excess adipose tissue is involved in respective pathologies. Here we established a human adipose tissue slice cultures (HATSC) model ex vivo. HATSC match the in vivo cell composition of human adipose tissue with, among others, mature adipocytes, mesenchymal stem cells as well as stroma tissue and immune cells. This is a new method, optimized for live imaging, to study adipose tissue and cell-based mechanisms of obesity in particular. HATSC survival was tested by means of conventional and immunofluorescence histological techniques, functional analyses and live imaging. Surgery-derived tissue was cut with a tissue chopper in 500 μm sections and transferred onto membranes building an air-liquid interface. HATSC were cultured in six-well plates filled with Dulbecco’s Modified Eagle’s Medium (DMEM), insulin, transferrin, and selenium, both with and without serum. After 0, 1, 7 and 14 days in vitro, slices were fixed and analyzed by morphology and Perilipin A for tissue viability. Immunofluorescent staining against IBA1, CD68 and Ki67 was performed to determine macrophage survival and proliferation. These experiments showed preservation of adipose tissue as well as survival and proliferation of monocytes and stroma tissue for at least 14 days in vitro even in the absence of serum. The physiological capabilities of adipocytes were functionally tested by insulin stimulation and measurement of Phospho-Akt on day 7 and 14 in vitro. Viability was further confirmed by live imaging using Calcein-AM (viable cells) and propidium iodide (apoptosis/necrosis). In conclusion, HATSC have been successfully established by preserving the monovacuolar form of adipocytes and surrounding macrophages and connective tissue. This model allows further analysis of mature human adipose tissue biology ex vivo.

Introduction

The prevalence of overweight and obesity, commonly measured by a high body mass index (BMI), is notably increasing worldwide [1]. Meanwhile, current research has identified a high
BMI as an underlying risk factor for many severe chronic diseases [2]. Furthermore, epidemiologic studies provide clear evidence that, relative to normal weight, obesity is associated with significantly higher all-cause mortality [3]. In the USA alone, a morbid BMI is responsible for approximately 18% of all deaths in the age group of 40 to 85 year-olds [4]. Lastly, prolonged caloric excess leads to adipose tissue remodeling and thereby to chronic inflammation including type 2 diabetes [5,6], cardiovascular diseases [7,8], cancer [9,10], osteoporosis [11], chronic kidney disease [12] as well as depression [13], which altogether constitute the leading causes of disability and death in developed countries.

Although, in general, there are three different types of adipocytes differentiated (white, brown, beige) [2], white adipose tissue seems to occur most frequently [14]. Adipose tissue is an active metabolic and inflammatory organ [15,16]. It can emit adipokines as well as anti- and pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF) and variable interleukins [17,18]. Connected to the chronic inflammation, leukocytosis in adipose tissue has been observed, including macrophages [19,20], B cells [21], T cells [22], neutrophils [23], eosinophils [24], and mast cells [25].

Despite the apparent key role of adipose tissue in severe diseases, only few methodological approaches are available to study its biology *ex vivo*. Historically, fat cells could be isolated and first cell lines, mainly of rodent origin, were obtained [26–28]. However, these cell lines created differentiated adipocytes with multiple lipid droplets. Most frequently used are mice embryo-originated 3T3-L1, 3T3-F442A and C3H10T1/2 cells and DFAT-GFP cells derived from mature adipocytes of GFP transgenic mice [26,29–32].

A monovacuolar state was not reached until the invention of the ceiling cultures by Sugihara et al. in 1986. With this method adipocytes were incubated floating on top of completely filled culture flasks [33]. However, floating adipocytes cultures rapidly dedifferentiate into fibroblast-like cells [34–36]. Three-dimensional culture of isolated mature adipocytes and ceiling culture of adipose tissue fragments were also established by Sugihara [37,38]. These methods allow to investigate proliferation, differentiation and adipocyte functions of mature adipocytes and preadipocytes *in vitro* [39]. A combination of adipose tissue fragments derived from rats in three-dimensional collagen gel was described by Sonoda et al. 2008. They make it possible to observe regenerating preadipocytes and mesenchymal stem cells [40,41]. The first successful experiments on the cultivation of human adipose tissue explants were published by Smith in 1971, and recently Harms et al. published an advancement of the ceiling culture method with mature human adipocytes [42,43]. In recent years, first tissue engineering methods for human adipose tissue have been developed [44,45].

However, interspecies discrepancies impede possible translations of research findings. Amongst numerous obstacles, the significant negative correlations in gene regulation between mice and humans in caloric restriction make direct comparison prone to errors [46]. Further differences occur between sex and life stages. It could, for example, be found that the femoral adipose tissues of premenopausal females appear to have a greater capacity for adipose expansion via hyperplasia, hypertrophy, and insulin sensitivity compared to age-matched postmenopausal females [47].

In addition, the place of origin affects cell composition and the extracellular matrix [48]. Especially the non-cellular structure of the extracellular matrix seems to have a major impact on adipocyte metabolisms and is thus remodeled in diseases (e.g. in diabetes) pointing out the complexity of adipose tissue and the shortcomings of frequently used cell culture models [49,50].

Based on the previously established slice cultures of human tumor tissues, this study aims at investigating whether human adipose tissue can be kept in a human slice culture system model [51–54].
Materials and methods

Tissue samples

This study has been approved by the Ethical Committee at the Medical Faculty, Leipzig University (#290-13-07102013). All patients declared their informed consent in written form. Subcutaneous AT was obtained from the Department of Orthopedics, Trauma Surgery, and Plastic Surgery (University Hospital Leipzig, Germany). ATs were derived from abdomen, dorsum, mamma, pelvis and thigh (Table 1). The samples were transported in sterile Hanks’ Balanced Salt Solution (HBSS, Gibco, Life Technologies, Carlsbad, USA) or DMEM (Gibco) and were processed within one to six hours after dissection.

Preparation of slice culture

AT samples were dissected into 5 x 5 x 10 mm pieces and cut by a tissue chopper (Mc Ilwain, Redding, USA) in 350, 500, and 750 μm thick slices. Subsequently, slices were transferred onto cell culture inserts with a pore size of 0,4 μm (Millipore, Merck, Darmstadt, Germany) placed in six-well plates (Corning, New York, USA), and cultivated on a liquid-air-interface in a humidified incubator at 35 °C and 5% CO₂ (Fig 1). Each well contained 1 ml culture medium under the membrane inserts supplying the tissue via diffusion. The basic culture media consisted of DMEM, insulin-transferrin-selenium mixture (1:100, ITS, Sigma Aldrich, Saint Louis, USA), and Penicillin/Streptomycin (1:100, PenStrep, Gibco). Fetal bovine serum (1:10,

Table 1. Adipose tissue samples.

| Sample | Origin          | Indication of surgery | Age [years] | Sex   | BMI [kg/m²] | Secondary diagnoses          | Medium     | Max. Period [days] |
|--------|----------------|-----------------------|-------------|-------|-------------|-----------------------------|------------|-------------------|
| #001   | Abdomen        | Postbariatric         | 55          | male  | 29          | HT, HU, NIDDM, O             | I, II, III | 14                |
| #002   | Mamma           | Gynecomastia          | 21          | male  | 32          | -                           | I, II, III | 14                |
| #003   | Abdomen        | Postbariatric         | 70          | female| 34          | CAD, NIDDM                  | I, II, III | 14                |
| #004   | Abdomen        | Postbariatric         | 59          | male  | 40          | CAD, HT, HU, PHT, T2D       | I, III     | 14                |
| #005   | Mamma           | Gynecomastia          | 17          | male  | 27          | PHT                         | I, III     | 14                |
| #006   | Dorsum          | Postbariatric         | 34          | male  | 58          | HT                          | I, III     | 14                |
| #007   | Thigh           | Postbariatric         | 62          | female| 31          | HT                          | I, III     | 21                |
| #008   | Dorsum          | Postbariatric         | 42          | female| 30          | DL, HT, NIDDM               | I, III     | 14                |
| #009   | Mamma           | Macromastia           | 36          | female| 24          | -                           | I, III     | 14                |
| #010   | Dorsum          | Postbariatric         | 25          | male  | 36          | -                           | I, III     | 14                |
| #011   | Abdomen         | VRAM flap             | 57          | male  | 25          | CKD, PAD                    | I, III     | 14                |
| #012   | Abdomen         | Postbariatric         | 52          | female| 31          | PHT                         | I, III     | 14                |
| #013   | Abdomen         | Postbariatric         | 52          | male  | 29          | CAD, DL                     | I, III, IV, V, VI | 14        |
| #014   | Abdomen         | Postpartum            | 32          | female| 22          | -                           | I, III, IV, V, VI | 14        |
| #015   | Abdomen         | Postbariatric         | 41          | female| 29          | -                           | I, III, IV, V, VI | 14        |
| #016   | Abdomen         | Postbariatric         | 47          | female| 30          | NIDDM, PHT                  | I, III, IV | 14                |
| #017   | Thigh           | Postbariatric         | 46          | male  | 26          | -                           | I, III     | 14                |
| #018   | Abdomen         | Postbariatric         | 33          | male  | 35          | HT                          | I, III     | 14                |
| #019   | Pelvis           | Sarcoma               | 37          | male  | 23          | -                           | I, III     | 14                |
| #020   | Dorsum           | Sarcoma               | 77          | male  | 28          | COPD, HT, HU, PHT           | I, III, IV | 14                |
| #021   | Thigh           | Sarcoma               | 47          | female| 41          | MS, PHT                     | I, III, IV | 14                |
| #022   | Pelvis          | Exostosis             | 23          | male  | 22          | -                           | I, III, IV | 14                |

Coronary artery disease (CAD), chronic kidney disease (CKD), chronic obstructive pulmonary disease (COPD), dyslipidemia (DL), hypertension (HT), hyperuricemia (HU), multiple sclerosis (MS), non-insulin-dependent diabetes mellitus (NIDDM), osteoporosis (O), peripheral artery disease (PAD), primary hypothyroidism (PHT), insulin-dependent type 2 diabetes mellitus (T2D), vertical rectus abdominis myocutaneous (VRAM).

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FBS, Gibco) or human tumor necrosis factor alpha (50 ng/ml, TNFα, Pepro Tech, Rocky Hill, USA) were added (Table 2). The culture media were changed first after 24 hours, subsequently every 48 hours. Pictures were taken with an Olympus SZ61 Stereomicroscope (Olympus, Tokyo, Japan). Slices, which were fixed at the preparation day, were labeled as day 0. Each culture time point was compared with day 0 and is represented by an individual slice.

**Live imaging**

15 minutes prior to imaging the media were removed and replaced by basic culture media containing fluorescent dyes: Hoechst 33342 (Nuclei, 1:1000, Sigma Aldrich), propidium iodide (apoptosis/necrosis, 1:1000, PI, Calbiochem, Darmstadt, Germany) and Calcein-AM (unspecific metabolism 1:200, Life Technologies). Pictures for 3D reconstruction or videos were taken with an Olympus IX81 confocal microscope (FV1000, Olympus) equipped with a humidified incubator and a motorized stage. During the imaging procedure, inside temperature was adjusted to 35 °C, 5% CO₂, and 60% humidity.

Fig 1. Experimental setup. Tissue samples were derived from orthopedic, trauma and plastic surgeries. A—Tissue was transported in sterile culture medium into the lab. B—Samples were cut into 500 μm thick slices by a tissue chopper. C & D—The slices were incubated on top of filter membrane inserts on a liquid-air-interface in a humidified incubator. On defined points of time specimens were live imaged or fixed.

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Table 2. Culture media.

| Condition | Basic Medium | Serum | Other contents | Addition |
|-----------|--------------|-------|----------------|----------|
| I         | DMEM         | 0% FBS| ITS, PenStrep  |          |
| II        | DMEM         | 5% FBS| ITS, PenStrep  |          |
| III       | DMEM         | 10% FBS| ITS, PenStrep  |          |
| IV        | DMEM         | 0% FBS| ITS, PenStrep  | TNFα     |
| V         | DMEM         | 0% FBS| ITS, PenStrep  | K⁺       |
| VI        | DPBS         | 0% FBS| ITS, PenStrep  |          |

Dulbecco’s Modified Eagle’s Medium (DMEM), Dulbecco’s phosphate-buffered saline (DPBS), fetal bovine serum (FBS), insulin-transferrin-selenium mixture (ITS), Penicillin/Streptomycin (PenStrep), human tumor necrosis factor alpha (TNFα), potassium ion (K⁺).

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Tissue analysis
At 0, 1, 7 or 8, and 14 days in vitro (DIV), slices were fixed over-night in 4% paraformaldehyde (PFA) prior to paraffin embedding. Paraffin sections (10 μm) were cut, dewaxed in xylene, dehydrated in decreasing alcohol series, and stained with hematoxylin/eosin (H/E) for conventional histology. Pictures were taken using an optical microscope, Axioplan 2 (Carl Zeiss, Oberkochen, Germany). In order to establish immunofluorescence, sections were pretreated with citrate buffer (pH 6) in a microwave for 10 minutes and antibodies were incubated overnight at 4 °C with 0.5% bovine serum albumin (BSA, Sigma Aldrich) and 10% normal goat serum or normal donkey serum (NGS or NDS, Jackson Immuno Research, West Grove, USA). To observe proliferation, antibodies against Ki67 (1:400, rabbit, DCS, Hamburg, Germany) were used. Apoptosis was detected by staining for activated Caspase-3 (1:300, rabbit, Cell Signaling, Cambridge, United Kingdom). Macrophages were labeled using Anti-IBA1 (1:500, rabbit, Wako, Osaka, Japan; 1:500, guinea pig, Synaptic Systems, Göttingen, Germany) or Anti-CD68 (1:100, mouse, DAKO, Agilent, Santa Clara, United States). Viability of adipocytes was visualized using Perilipin A (1:500, rabbit; 1:250, goat, both Abcam, Cambridge, United Kingdom). The sections were washed and incubated at room temperature with secondary antibodies for one hour (1:500, goat-anti-rabbit Alexa 488/568; 1:500, goat-anti-guinea pig Alexa 488; 1:250, donkey-anti-rabbit Alexa 488; 1:250, donkey anti-goat Alexa 568, all Life Technologies). Nuclei were counterstained using Hoechst 33342 (1:10.000, Sigma-Aldrich). Pictures were taken using a fluorescence microscope BX40 (Olympus) or a LSM 710 (Carl Zeiss). In order to compare the cell size between conditions, the cross-section area of 20–60 adipocytes of each H/E stained section per condition of four experiments (#009, #010, #011 and #021) was measured manually using ImageJ (Version 1.8.0).

Western blot
48 hours prior to the experiment, adipocyte tissue cultures were serum starved overnight by changing medium to serum and insulin free medium. To determine Akt phosphorylation, adipocytes were stimulated with insulin (10 nM), diluted in pre-warmed serum free medium for 15 min. Separation of membranes and cytosol was performed by a protocol modified from Nishiumi and Ashida [55]. Briefly, adipocyte cultures were collected in buffer A (50 mmol/l Tris, 0.5 mmol/l dithiothreitol, adjusted to pH 8.0 and 1% phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium orthovanadate and 1% Sigma protease inhibitor cocktail freshly added) and stored at -80 °C until further analysis. Western blot analysis was performed as described earlier [56]. Blots were incubated with Phospho-Akt (1:1000, Cell Signaling) at 4 °C overnight. Immunoreactions were detected with the appropriate peroxidase-conjugated anti-
rabbit IgG secondary antibody (1:5000 for phospho-specific antibodies; Vector Laboratories, Peterborough, UK) at room temperature for 2 h. Peroxidase activity was visualized with an enhanced chemiluminescence kit (Amersham, Pharmacia, Freiburg, Germany). In addition, blots were stripped and incubated with pan-Akt antibody (1:3000, Cell Signaling) followed by a secondary antibody (1:10000, anti-rabbit IgG, Vector Laboratories). In addition, blots were stripped and incubated with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (diluted 1:100000, Research Diagnostics, Flanders, Netherlands) followed by the anti-mouse IgG secondary antibody (1:10000, Vector Laboratories). GAPDH antibodies were used as a loading control. Semiquantitative evaluation of arbitrary unit was performed with the ImageJ plugin for western blot analysis.

**Statistical analysis**

One-way-ANOVA with Bonferroni correction was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, USA). P < 0.05 was considered significant.

**Results**

Adipose tissues were derived from nine abdominal, four dorsal, three breast, three pelvic, and three limb surgeries (Table 1) and were kept in culture for up to 14 days. The tissue donors were 43.9 years (SD ±15.9) old on average, had a BMI of 31.0 kg/m² (SD ±8.0) on average, and 40.9% were female. In 14 cases, the patients had secondary diagnoses (Table 1). Tissue were transferred from surgery and cut on a tissue chopper between 1 and 6 hours after removal (Fig 1). Pioneering experiments investigating the optimal thickness for cultivation showed that 500 μm were ideal for tissue preparation and handling. 350 μm thick slices often collapsed during the preparation process while 750 μm slices proved difficult to embed into paraffin for further histological analysis. 500 μm thick slices maintained approximately five to seven cell layers and all layers were well preserved during cultivation.

Tissue integrity was macroscopically well preserved up to 14 DIV and adipocyte appearance did not change (Fig 2). Tissue slices cultivated with 10% FBS (Table 2, III) showed minor slice shrinkage (Fig 2B, 2E, 2H and 2K). To induce distinct tissue damage, TNFα was supplemented to serum-free media provoking cell death (Figs 2C, 2F, 2I, 2L and 4).

Histological analysis of H/E staining’s demonstrated the well-maintained characteristics of AT (Fig 3). The cross-section area of adipocytes increased under TNFα supplementation, while no measurable difference was observed between the two different culture media between 0 and 14 DIV (Fig 3). Slices cultivated without serum supplementation (I, Table 2) maintained their cellular composition up to 14 DIV. In medium supplemented with serum, stroma tissue appeared to expand, but no obvious discrepancy could be observed between conditions with a dose of 5% FBS (II, Table 2) as compared to 10% FBS (III, Table 2) (S1 Fig). Taking into consideration the high serum doses used in previous experiments (e.g. 15% FBS [57]), 10% FBS was used for our further experiments.

Viability of adipose cells was determined via immunofluorescence with antibodies against Perilipin A (Fig 4). No obvious differences between the endpoints of the standard conditions I and III (Table 2) could be observed. Homogeneous expression of the lipid droplet surface protein in both conditions proved the survival of adipocytes in slice cultures for 14 days. As a positive control for cell death, TNFα was added to the medium (IV, Table 2; Fig 4D). Thus, positive controls confirmed the predictive validity of Perilipin A. To further investigate function of adipocytes the phosphorylation of Akt, a key step in insulin signaling, was investigated after 15 min of insulin stimulation. After 7 and 14 DIV the phosphorylation of Akt remains low in the control condition, whereas the insulin condition shows an enhanced expression of
phosphorylated Akt (Fig 5A & 5B). Using antibodies against IBA1 and CD68 revealed viability of macrophages. Some CD68-positive macrophages were co-localized with Ki67, proving proliferation processes on 14th DIV (Fig 6, Circle).

In live imaging analyses medium with Calcein-AM was added 15 minutes prior to taking pictures. The non-fluorescent Calcein-AM diffused through cell membranes, intracellular...
Fig 3. Tissue analysis. A—No measurable difference in the cross-section area of adipocytes in medium with and without 10% FBS, but TNFα supplementation increased the cross-section area of adipocytes (y-axis in μm²).
Morphological analysis was performed via H/E staining and showed well-sustained AT. B—0th DIV. C & D—7th DIV. E & F—14th DIV. Left column—0% FBS. Right column—10% FBS.

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esterases hydrolyzed the acetoxy-methylester-group, and the fluorescent Calcein accumulated in cell plasma [58]. This activating process could be shown in vital adipocytes and stroma cells on day 7 and 14 in vitro (Fig 7, S1 Video). However, exposing the tissue to laser light provoked a positive control cellular death of both adipocytes and stroma cells after prolonged exposure (S2 Fig, S2 Video).

Discussion and conclusion

Organotypic slice cultures of human adipose tissue maintained their morphological characteristics and their metabolism for up to 14 days in culture. It can thereby be concluded that HATSC provides a platform to investigate human adipose tissue in a controlled ex vivo setting with little ethical constraints.

Morphological analysis of H/E staining’s cannot discriminate viable adipocytes from dead adipocytes; The distinction between living and dead adipocytes (lipid droplets with or without
cell nuclei) cannot be made on the basis of cell nuclear morphology. In histologic standard sections (approx. 10 μm) of normal-sized adipocytes (approx. 50–150 μm) their small nucleus is not necessarily part of the histological section. Therefore, the state of a given adipocyte cannot always be judged in the basis of its morphology (e.g. euchromatic, pyknotic, fragmented).

Studies in the literature on changes in the cell volume of adipocytes as a distinguishing feature of vitality or apoptosis/necrosis of adipocytes do not exist. Jo et al. did not find an alteration in cell volume during apoptosis, using mathematical models [59]. They could show that under prolonged weight-loss conditions large adipocytes shrink, but at the same time the smaller adipocytes die first [60]. A faster cell death of the small adipocytes could lead to an increase in the average cell volume, even if the large adipocytes themselves shrink. In a human study, Verboven et al. could show that people with obesity, i.e. those with an increased inflammation in fatty tissue, have more large and very large adipocytes, although they have an increased basal lipolysis [61]. They attribute this to a decrease in the number of small adipocytes. Both processes, early cell death of small adipocytes and the slow shrinkage of large adipocytes could explain the total increase in adipocyte surface area of the TNFα condition that was observed in the present study (see Fig 3A).

The survival of adipocytes was demonstrated by immunofluorescence staining and the viability of adipose tissue by live imaging and functional experiments. Perilipins are lipid droplet-associated proteins and their phosphorylation is essential in lipolysis. Perilipin A is a known marker for viability of adipocytes and has been used in immunological research [62–66]. Using our own experiments, we were able to confirm the sensitivity of Perilipin A as a viability marker of adipocytes (see Fig 4A and 4D).

Pathological remodeling of adipose tissue includes hypertrophy, accumulation of immune cells such as macrophages, decreased capillary density, and fibroblast activation [67]. There is some evidence that adipose tissue is able to control local regulation and proliferation of macrophages independently of the influx of blood precursors, but no evidence currently exists supporting the role of local myelopoiesis in adipose tissue [68–72]. The preservation of macrophages in HATSC was displayed with immunofluorescence staining with IBA1 and CD68. Both antibodies were selected for their wide distribution in the study of macrophages in adipose tissue [73,74]. Even the local proliferation in the absence of blood could be
demonstrated on DIV 14 in human adipose tissue, where CD68-positive macrophages were co-localized with Ki67 (Fig 6).

Live-imaging was used to further study the formation of crown-like structures consisting of proliferating macrophages around dying adipocytes in murine adipose tissue \cite{75,76}. The results show that such experiments could also be performed in human tissues. In a rodent study by Weisberg et al. TNF\(\alpha\) was shown to be distributed by macrophages and not by adipocytes as part of the stroma-vascular fraction during inflammation and diabetes \cite{19,77}. Contrary to this, human adipocytes have the potential to secrete TNF\(\alpha\), thus signaling to immune cells \cite{78–80}. Such potential species differences can now be worked out. Moreover, studying human adipose tissues from individuals with different BMI with and without type II diabetes might help to better understand how inflammation and oxidative stress drives insulin resistance, arteriosclerosis, angiogenesis, as well as cancer \cite{81–84}. This can be concluded since the open access of the system allows for studies of the accumulation of (secreted) molecules in the medium. The large number of standardized samples which can be prepared from small probes

Fig 6. Proliferation and inflammation. Proliferation and inflammation in abdominal tissue were determined via immunofluorescence with antibodies against Ki67 (green) and CD68 (red). Cell nuclei were counterstained (Hoechst 33342, blue). A & B—7th DIV. C & D—14th DIV. Left column—0% FBS. Right column—10% FBS. Circle triple positive cell - > Proliferating macrophage.

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further enables the investigation of therapeutics, targeting essential biochemical pathways, drug accumulation, and modern pharmaceutics for gene therapy [85].

In conclusion, slice cultures derived from human adipose tissue have been successfully established whereas the unique monovacuolar shape of the adipocytes as well as the complex organization of the tissue could be maintained. Hence, this method serves as a confirmation of the current findings about rodent adipose tissue while it allows to further dissect its biology in the human system.

Supporting information

S1 Fig. Analysis of different culture media. Analysis was performed via H/E staining. Top row—0% FBS, middle row—5% FBS and bottom row—10% FBS. A, C, E—1st DIV. B, D, F—8th DIV. The adipose tissue slice cultures maintain most of their morphologic properties, but high serum concentrations increased the fibrocyte fraction. (TIF)

S2 Fig. Live imaging of slice cultures on DIV 0. Viability and death of cells were determined, directly after preparation of slice cultures in basic media without serum, via Calcein-AM (cell metabolism, green) and propidium iodide (apoptosis/necrosis, red), e.g. arrows. A to C—60 min; D to F—180 min; H to J—300 min after laser exposure. (TIF)

S1 Video. Live imaging of slice cultures on 7th DIV (0% FBS). Viability and death of cells were determined via Calcein-AM (cell metabolism, green) and propidium iodide (apoptosis/necrosis, red). (AVI)

S2 Video. Live imaging of slice cultures on 0th DIV. Viability and death of cells were determined via Calcein-AM (cell metabolism, green) and propidium iodide (apoptosis/necrosis,
red), directly after preparation of slice cultures in basic media without serum. (AVI)

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References
1. Roberto CA, Swinburn B, Hawkes C, Huang TT-K, Costa SA, Ashe M, et al. Patchy progress on obesity prevention: emerging examples, entrenched barriers, and new thinking. Lancet. 2015 Mar; 385 (9985):2400–9. https://doi.org/10.1016/S0140-6736(14)61744-X PMID: 25703111
2. Heymsfield SB, Wadden TA. Mechanisms, Pathophysiology, and Management of Obesity. N Engl J Med. 2017 Jan; 376(3):254–66. https://doi.org/10.1056/NEJMra151409 PMID: 28099824
3. Flegal KM, Kit BK, Orpana H, Graubard BI. Association of All-Cause Mortality With Overweight and Obesity Using Standard Body Mass Index Categories: A Systematic Review and Meta-analysis. JAMA. 2013 Jan; 309(1):71–82. https://doi.org/10.1001/jama.2012.113905 PMID: 23280227
4. Masters RK, Reither EN, Powers DA, Yang YC, Burger AE, Link BG. The impact of obesity on US mortality levels: The importance of age and cohort factors in population estimates. Am J Public Health. 2013; 103(10):1895–901. https://doi.org/10.2105/AJPH.2013.301379 PMID: 23948004
5. Grant RW, Dixit VD. Adipose tissue as an immunological organ. Obesity (Silver Spring). 2015 Mar; 23 (3):512–8.
6. Blüher M. Adipose tissue inflammation: a cause or consequence of obesity-related insulin resistance? Clin Sci. 2016 Aug; 130(18):1603 LP– 1614.
7. Tirosch A, Shai I, Afek A, Dubnov-Raz G, Ayalon N, Gordon B, et al. Adolescent BMI Trajectory and Risk of Diabetes versus Coronary Disease. N Engl J Med. 2011 Apr; 364(14):1315–25. https://doi.org/10.1056/NEJMoa1006992 PMID: 21470009
8. Twig G, Yaniv G, Levine H, Leiba A, Goldberger N, Derazne E, et al. Body-mass index in 2.3 million adolescents and cardiovascular death in adulthood. N Engl J Med. 2016; 374(25):2430–40. https://doi.org/10.1056/NEJMoa1503840 PMID: 27074389
9. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2016 Feb; 371 (9612):569–78.
10. Louie SM, Roberts LS, Nomura DK. Mechanisms linking obesity and cancer. Biochim Biophys Acta—Mol Cell Biol Lipids. 2013; 1831(10):1499–508.
11. Palermo A, Tuccinardi D, De Feudis G, Watmabe M, Manfrini S. BMI and BMD : The Potential Interplay between Obesity and Bone Fragility. nt J Environ Res Public Heal. 2016;
22. Smitka K, Maresová D. Adipose Tissue as an Endocrine Organ: An Update on Pro-inflammatory and Anti-inflammatory Microenvironment. Prague Med Rep. 2015; 11(2):87–111.

23. Ellulu MS, Khaza’ai H, Rahma’t A, Patimah I, Abed Y. Obesity can predict and promote systemic inflammation in healthy adults. Int J Cardiol [Internet]. 2016; 215(2016):318–24. Available from: http://dx.doi.org/10.1016/j.ijcard.2016.04.089

24. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B Lymphocytes Promote Insulin Resistance through Modulation of T Lymphocytes and Production of Pathogenic IgG Antibody. Nat Med. 2011 May; 17(5):610–7. https://doi.org/10.1038/nm.2353 PMID: 21499269

25. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec; 112(12):1796–808. https://doi.org/10.1172/JCI19246 PMID: 14679176

26. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003 Dec; 112(12):1821–30. https://doi.org/10.1172/JCI19451 PMID: 14679177

27. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B Lymphocytes Promote Insulin Resistance through Modulation of T Lymphocytes and Production of Pathogenic IgG Antibody. Nat Med. 2011 May; 17(5):610–7. https://doi.org/10.1038/nm.2353 PMID: 21499269

28. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med. 2009 Jul; 15:914. https://doi.org/10.1038/nm.1964 PMID: 19633658

29. Talukdar S, Oh DY, Bandypadhyay G, Li D, Xu J, McNeilis J, et al. Neutrophils mediate insulin resistance in high fat diet fed mice via secreted elastase. Nat Med [Internet]. 2012; 18(9):1407–12. Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Neutrophils+mediate+insulin+resistance+in+high+fat+diet+fed+mice+via+secreted+elastase#0 PMID: 22863787

30. Wu D, Molofsky AB, Liang H-E, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. Science. 2011 Apr; 332(6026):243–7. https://doi.org/10.1126/science.1201475 PMID: 21436399

31. Liu J, Divoux A, Sun J, Zhang J, Clément K, Glickman JN, et al. Deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. Nat Med. 2009 Aug; 15(8):940–5. https://doi.org/10.1038/nm.1994 PMID: 19633655

32. Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. Cell. 1974; 3 (2):127–33. https://doi.org/10.1016/0092-8674(74)90116-0 PMID: 4426090

33. Rodbell M. Metabolism of Isolated Fat Cells: I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem. 1964 Feb; 239(2):375–80.

34. Wang QA, Scherer PE, Gupta RK. Improved methodologies for the study of adipose biology: Insights gained and opportunities ahead. J Lipid Res. 2014; 55(4):605–24. https://doi.org/10.1194/jlr.R046441 PMID: 24532650

35. Green H, Kehinde O. An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. Cell. 1975; 5(1):19–27. https://doi.org/10.1016/0092-8674(75)90087-2 PMID: 165899

36. Green H, Kehinde O. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell. 1976; 7(1):105–13. https://doi.org/10.1016/0092-8674(76)90260-9 PMID: 949738

37. Nobusue H, Endo T, Kano K. Establishment of a preadipocyte cell line derived from mature adipocytes of GFP transgenic mice and formation of adipose tissue. Cell Tissue Res. 2008; 332(3):435–46. https://doi.org/10.1002/ctn.2008-0593-9 PMID: 18386066

38. Ruiz-Ojeda JF, Rupérez IA, Gomez-Llorente C, Gil A, Aguilera MC. Cell Models and Their Application for Studying Adipogenic Differentiation in Relation to Obesity: A Review. Vol. 17, International Journal of Molecular Sciences. 2016.
33. Sugihara H, Yonemitsu N, Miyabara SYK. Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties. PubMed Commons. Differentiation. 1986;1986.

34. Asada S, Kuroda M, Aoyagi Y, Fukaya Y, Tanaka S, Konno S, et al. Ceiling culture-derived proliferative adipocytes retain high adipogenic potential suitable for use as a vehicle for gene transduction therapy. Am J Physiol Endocrinol Metab. 2011 Apr; 301(1):C181–5.

35. Lessard J, Pelletier M, Biertho L, Biron S, Marceau S, Hould F-S, et al. Characterization of Dedifferentiating Human Mature Adipocytes from the Visceral and Subcutaneous Fat Compartments: Fibroblast Activation Protein Alpha and Dipeptidyl Peptidase 4 as Major Components of Matrix Remodeling. PLoS One. 2015 Mar; 10(3):e0122065. https://doi.org/10.1371/journal.pone.0122065 PMID: 25816202

36. Wei S, Bergen WG, Hausman GJ, Zan L, Dodson MV. Cell culture purity issues and DFAT cells. Biochem Biophys Res Commun. 2013; 433(3):273–5. https://doi.org/10.1016/j.bbrc.2013.03.006 PMID: 23499844

37. Sugihara H, Yonemitsu N, Toda S, Miyabara S, Funatsumaru S, Matsumoto T. Unilocular fat cells in three-dimensional collagen gel matrix culture. J Lipid Res. 1988 May; 29(5):691–7. PMID: 3411243

38. Sugihara H, Funatsumaru S, Yonemitsu N, Miyabara S, Toda S, Hikichi Y. A simple culture method of fat cells from mature fat tissue fragments. J Lipid Res. 1989; 30(12):1987–95. PMID: 2559938

39. Zhang H, Kumar S, Barnett A, Eggo M. Ceiling culture of mature human adipocytes: use in studies of adipocyte functions. J Endocrinol. 2000; 164:119–28. https://doi.org/10.1677/joe.0.164119 PMID: 10657847

40. Sonoda E, Uchihashi K, Aoki S, Toda S, Soejima H, Izuhara K, et al. A New Organotypic Culture of Adipose Tissue Fragments Maintains Viable Mature Adipocytes for a Long Term, Together with Development of Immature Adipocytes and Mesenchymal Stem Cell-Like Cells. Endocrinology. 2008 Oct; 149(10):4794–8. https://doi.org/10.1210/en.2008-0525 PMID: 18535101

41. Toda S, Uchihashi K, Aoki S, Sonoda E, Yamasaki F, Piao M, et al. Adipose tissue-organotypic culture system as a promising model for studying adipose tissue biology and regeneration. Organogenesis. 2009 Apr; 5(2):50–6. https://doi.org/10.4161/org.5.2.8347 PMID: 19794899

42. Smith U. Morphologic studies of human subcutaneous adipose tissue in vitro. Anat Rec [Internet]. 1971 Jan 1; 169(1):97–104. Available from: https://doi.org/10.1002/ar.101690109 PMID: 4322670

43. Harms MJ, Li Q, Lee S, Zhang C, Kull B, Hallen S, et al. Mature Human White Adipocytes Cultured under Membranes Maintain Identity, Function, and Can Transdifferen tiate into Brown-like Adipocytes. Cell Rep. 2019; 27(1):213–225.e5. https://doi.org/10.1016/j.celrep.2019.03.026 PMID: 30943403

44. Louis F, Kitano S, Mano JF, Matsusaki M. 3D collagen microfibers stimulate the functionality of preadipocytes and maintain the phenotype of mature adipocytes for long term cultures. Acta Biomater [Internet]. 2019; 84:194–207. Available from: http://www.sciencedirect.com/science/article/pii/S1742706118307116 PMID: 30502481

45. Aubin K, Safoine M, Proulx M, Audet-Casgrain M-A, Côté J-F, Têtu F-A, et al. Characterization of In Vitro Engineered Human Adipose Tissues: Relevant Adipokine Secretion and Impact of TNF-α. PLoS One [Internet]. 2015 Sep 14; 10(9):e0137612. Available from: https://doi.org/10.1371/journal.pone.0137612 PMID: 26367137

46. Swindell WR, List EO, Berryman DE, Kopchick JJ. Transcriptional profiling identifies strain-specific effects of caloric restriction and opposite responses in human and mouse white adipose tissue. Aging (Albany NY). 2018; 10(4):701–46.

47. Karastergiou K, Fried SK. Cellular Mechanisms Driving Sex Differences in Adipose Tissue Biology and Body Shape in Humans and Mouse Models BT—Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes and Obesity. In: Mauvais-Jarvis F, editor. Cham: Springer International Publishing; 2017. p. 29–51.

48. Grandi G, Müller S, Moest H, Moser C, Wollscheid B, Wolfrum C. Depot specific differences in the adipogenic potential of precursors are mediated by collagenous extracellular matrix and Flotillin 2 dependent signaling. Mol Metab [Internet]. 2016; 5(10):937–47. Available from: http://dx.doi.org/10.1016/j.molmet.2016.07.008 PMID: 27889006

49. Baker NA, Muir LA, Washabaugh AR, Neeley CK, Chen SYP, Flesher CG, et al. Diabetes-specific regulation of adipocyte metabolism by the adipose tissue extracellular matrix. J Clin Endocrinol Metab. 2017; 102(3):1032–43. https://doi.org/10.1210/jc.2016-2915 PMID: 28399093

50. Bonnans C, Chou J, Verb Z. Remodelling the extracellular matrix in development and disease. Nat Rev Mol Cell Biol. 2014 Dec; 15(12):786–801. https://doi.org/10.1038/nrm3904 PMID: 25415508

51. Gerlach MM, Merz F, Wichmann G, Kubic K, Wittekind C, Lordick F, et al. Slice cultures from head and neck squamous cell carcinoma: a novel test system for drug susceptibility and mechanisms of resistance. Br J Cancer. 2014 Jan; 110(2):479–88. https://doi.org/10.1038/bjc.2013.700 PMID: 24263061
71. Pinho MB, Hurtado SP, El-Cheikh MC, Rossi MD, Dutra HS, Borojevic R. Myelopoiesis in the omentum of normal mice and during abdominal inflammatory processes. Cell Tissue Res [Internet]. 2002; 308(1):87–96. Available from: https://doi.org/10.1007/s00441-002-0550-y PMID: 12012208

72. Okabe Y, Medzhitov R. Tissue-Specific Signals Control Reversible Program of Localization and Functional Polarization of Macrophages. Cell [Internet]. 2014; 157(4):832–44. Available from: http://www.sciencedirect.com/science/article/pii/S0092867414004942 PMID: 24792964

73. Elewa YHA, Ichii O, Kon Y. Comparative analysis of mediastinal fat-associated lymphoid cluster development and lung cellular infiltration in murine autoimmune disease models and the corresponding normal control strains. Immunology [Internet]. 2016 Jan 1; 147(1):30–40. Available from: https://doi.org/10.1111/imm.12539 PMID: 26439309

74. Kang YE, Kim JM, Joung KH, Lee JH, You BR, Choi MJ, et al. The Roles of Adipokines, Proinflammatory Cytokines, and Adipose Tissue Macrophages in Obesity-Associated Insulin Resistance in Modest Obesity and Early Metabolic Dysfunction. PLoS One [Internet]. 2016 Apr 21; 11(4):e0154003. Available from: https://doi.org/10.1371/journal.pone.0154003 PMID: 27101398

75. Haase J, Weyer U, Immig K, Klötting N, Blüher M, Eilers J, et al. Local proliferation of macrophages in adipose tissue during obesity-induced inflammation. Diabetologia. 2014; 57(3):562–71. https://doi.org/10.1007/s00125-013-3139-y PMID: 24343232

76. Gericke M, Weyer U, Braune J, Bechmann I, Eilers J. A method for long-term live imaging of tissue macrophages in adipose tissue explants. Am J Physiol—Endocrinol Metab. 2015; 308(11):E1023–33. https://doi.org/10.1152/ajpendo.00075.2015 PMID: 25874903

77. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol—Endocrinol Metab. 2001 May; 280(5):E745–51. https://doi.org/10.1152/ajpendo.2001.280.5.E745 PMID: 11287357

78. Sewter CP, Digby JE, Blows F, Prins J, O’Rahilly S. Regulation of tumour necrosis factor-alpha release from human adipose tissue in vitro. J Endocrinol. 1999; 163(1):33–8. https://doi.org/10.1677/joe.0.1630033 PMID: 10495404

79. Hube F, Hauner H. The role of TNF-α in human adipose tissue: Prevention of weight gain at the expense of insulin resistance? Horm Metab Res. 1999; 31(12):626–31. https://doi.org/10.1055/s-2007-978810 PMID: 10688912

80. Rydéén M, Dicker A, Van Harmelen V, Hauner H, Brunnberg M, Perbeck L, et al. Mapping of early signaling events in tumor necrosis factor-α-mediated lipolysis in human fat cells. J Biol Chem. 2002; 277(2):1085–91. https://doi.org/10.1074/jbc.M109496200 PMID: 11694522

81. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010 Mar; 140(6):883–99. https://doi.org/10.1016/j.cell.2010.01.025 PMID: 20303878

82. Karin M, Greten FR. NF-κB: Linking inflammation to cancer development and progression. Nat Rev Immunol. 2005; 5(10):749–59. https://doi.org/10.1038/nri1703 PMID: 16175190

83. McNelis JC, Olefsky JM. Macrophages, Immunity, and Metabolic Disease. Immunity [Internet]. 2014; 41(1):36–48. Available from: http://dx.doi.org/10.1016/j.immuni.2014.05.010 PMID: 25035952

84. Mirza RE, Koh TJ. Contributions of Cell Subsets to Cytokine Production during Normal and Impaired Wound Healing. Cytokine. 2015 Feb; 71(2):409–12. https://doi.org/10.1016/j.cyto.2014.09.005 PMID: 25281359

85. Kallendrusch S, Schopow N, Stadler SC, Bünning H, Hacker UT. Adeno-Associated Viral Vectors Transduce Mature Human Adipocytes in Three-Dimensional Slice Cultures. Hum Gene Ther Methods. 2016 Sep; 27(5):171–3. https://doi.org/10.1089/hgltb.2016.137 PMID: 27650213