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Raman microspectroscopy reveals unsaturation heterogeneity at the lipid droplet level and validates an in vitro model of bone marrow adipocyte subtypes

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Author contribution statement

J.T. planned experiments, analyzed and interpreted the results. G.F. performed Raman acquisitions, interpreted and analyzed the results and compiled the corresponding figures. A.D. and N.B. performed HPLC analyses. J.T., C.B. and N.D.T. performed in vitro culture experiments. V.C. performed co-cultures. G.F. and L.D. performed statistical analyses for Raman data. J.T., G.F., A.D., C.C. and O.N. interpreted the Raman and HPLC data. O.N. and G.P. initiated the project, while C.C. and O.N. co-supervised the project. O.N. and J.T. wrote the manuscript, G.F. and C.C. significantly edited the manuscript.

Keywords

fatty acid, unsaturation (fatty acid), OP9 cell, lipid droplet diameter, Regulated, constitutive, Stroma, Bone marrow stromal cell (BMSC)

Abstract

Word count: 317

Bone marrow adipocytes (BMAd) constitute the most abundant stromal component of adult human BM. Two subtypes of BMAd have been described, the more labile regulated adipocytes (rBMAd) and the more stable constitutive adipocytes (cBMAd), which develop earlier and are more resilient to environmental and metabolic disruptions. In vivo, rBMAd are enriched in saturated fatty acids, contain smaller lipid droplets (LDs) and more readily provide hematopoietic support than their cBMAd counterparts. Mouse models have been used for BMAd research, but isolation of primary BMAd presents many challenges, and thus in vitro models remain the current standard to study nuances of adipocyte differentiation. No in vitro model has been described for the study of rBMAd/cBMAd.

Here, we present an in vitro model of BM adipogenesis with differential rBMAd and cBMAd-like characteristics. We used OP9 BM stromal cells derived from a (C57BL/6xC3H)F2-op/op mouse, which have been extensively characterized as feeder layer in hematopoiesis research. We observed similar canonical adipogenesis transcriptional signatures for spontaneously-differentiated (sOP9) and induced (iOP9) cultures, while fatty acid composition and desaturase expression differed at the population level. To resolve differences at the single adipocyte level we show that Raman microspectroscopy presents a high-resolution method for studying adipogenesis in vitro in a label-free manner, with resolution to individual LDs in this model. We find sOP9 adipocytes have lower unsaturation ratios, smaller LDs and higher hematopoietic support than iOP9 adipocytes, thus functionally resembling rBMAd and cBMAd, respectively. Validation in human samples confirms a higher unsaturation ratio for lipids extracted from stable cBMAd (femoral head upon hip-replacement surgery) versus labile rBMAd (iliac crest after chemotherapy). Moreover, the 16:1/16:0 fatty acid unsaturation ratio, already shown to discriminate cBMAd from rBMAd in rabbit and rat marrow, was validated in both the OP9 model in vitro system and in human samples. We expect our model will be useful for cBMAd and rBMAd studies, particularly where isolation of primary BMAd is a limiting step.

Contribution to the field

Taken together, we conclude that the spontaneous versus induced OP9 in vitro adipogenesis model presented here replicates key differences in lipid droplet size, FA unsaturation ratio and hematopoietic support respectively associated in vivo to regulated versus constitutive BMAd subtypes. We additionally validate the value of the 16:1/16:0 FA unsaturation ratio as a surrogate marker to segregate rBMAd-rich versus cBMAd-rich tissues both in vivo and for our in vitro model, and the feasibility of Raman microspectroscopy to reveal equivalent differences in the unsaturation ratio at the single adipocyte level.

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Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

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Inclusion of identifiable human data

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In review

Data availability statement

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In review

Raman microspectroscopy reveals unsaturation heterogeneity at the lipid droplet level and validates an *in vitro* model of bone marrow adipocyte subtypes

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18 **Keywords: fatty acid¹, unsaturation ratio², OP9 cells³, lipid droplet diameter⁴, regulated⁵,**
19 **constitutive⁶, stroma⁷, bone marrow stromal cell (BMSC)⁸. (Min.5-Max. 8)**

20

21 **Abstract**

22 Bone marrow adipocytes (BMAds) constitute the most abundant stromal component of adult human
23 bone marrow. Two subtypes of BMAds have been described, the more labile regulated adipocytes
24 (rBMAds) and the more stable constitutive adipocytes (cBMAds), which develop earlier and are
25 more resilient to environmental and metabolic disruptions. *In vivo*, rBMAds are enriched in saturated
26 fatty acids, contain smaller lipid droplets (LDs) and more readily provide hematopoietic support than
27 their cBMAd counterparts. Mouse models have been used for BMAds research, but isolation of
28 primary BMAds presents many challenges, and thus *in vitro* models remain the current standard to
29 study nuances of adipocyte differentiation. No *in vitro* model has yet been described for the study of
30 rBMAds/cBMAds.

31 Here, we present an *in vitro* model of BM adipogenesis with differential rBMAd and cBMAd-like
32 characteristics. We used OP9 BM stromal cells derived from a (C57BL/6xC3H)F2-op/op mouse,
33 which have been extensively characterized as feeder layer for hematopoiesis research. We observed
34 similar canonical adipogenesis transcriptional signatures for spontaneously-differentiated (sOP9) and

35 induced (iOP9) cultures, while fatty acid composition and desaturase expression of *Scd1* and *Fads2*
36 differed at the population level. To resolve differences at the single adipocyte level we tested Raman
37 microspectroscopy and show it constitutes a high-resolution method for studying adipogenesis *in*
38 *vitro* in a label-free manner, with resolution to individual LDs. We found sOP9 adipocytes have
39 lower unsaturation ratios, smaller LDs and higher hematopoietic support than iOP9 adipocytes, thus
40 functionally resembling rBMAds, while iOP9 more closely resembled cBMAds. Validation in human
41 primary samples confirmed a higher unsaturation ratio for lipids extracted from stable cBMAd-rich
42 sites (femoral head upon hip-replacement surgery) versus labile rBMAds (iliac crest after
43 chemotherapy). As a result, the 16:1/16:0 fatty acid unsaturation ratio, which was already shown to
44 discriminate BMAd subtypes in rabbit and rat marrow, was validated to discriminate cBMAds from
45 rBMAd in both the OP9 model *in vitro* system and in human samples. We expect our model will be
46 useful for cBMAd and rBMAd studies, particularly where isolation of primary BMAds is a limiting
47 step.

48 **1 Introduction**

49 Similar patterns of bone marrow adipocyte (BMAd) formation occur in vertebrate species (including
50 rodents, rabbits, and humans). Specifically, a decreasing rate of BMAd has been observed relative to
51 the decreasing size and lifespan of the animal, with larger skeletons containing more BM adipose tissue
52 (BMAT) which extends farther into the skeleton (1). The distal parts of the skeleton (ie. peripheral
53 bones in human, the distal tibia, paws, and caudal vertebrae in mice) predominantly contain adipocytic
54 marrow interspersed with some hematopoietic cells, comprising the more stable constitutive BMAds
55 (cBMAds) that appear just around birth. The proximal locations of the skeleton (ie. thoracic vertebrae
56 in human and mouse, as well as proximal tibia and other long bones in mouse) contain predominantly
57 hematopoietic marrow interspersed with the more labile regulated BMAds (rBMAds) (1,2, 36). The
58 rBMAds are smaller in size and respond readily to induction of bone marrow adipogenesis through
59 nutritional challenge (high fat diet or caloric restriction) or hematopoietic failure (irradiation or
60 chemotherapy), or BMAd mass reduction in the context of cold exposure or increased hematological
61 demand (e.g. phenylhydrazine). It is argued that cBMAds on the other hand, do not respond readily to
62 environmental demands (1,3–6). Stromal cells and BMAds are tightly linked to BM hematopoiesis.
63 Various stromal populations in mouse and human have shown to promote hematopoietic support while
64 more mature BMAds correlate with reduced hematopoietic proliferation ((7,8) and reviewed in (9,10)).
65 Meanwhile *Adiponectin* expressing cells within the BMAd lineage are beneficial to hematopoietic
66 regeneration in most bones but not the caudal vertebrae of mice (11,12). These studies indicate the
67 probability for population- and location-specific distinctions of BMAd maturation that specifically
68 affect hematopoiesis and respond to hematopoietic demand (reviewed in (13)).

69 Isolation of BM stroma for differentiation and analyses of BMAds *in vitro* to study these processes in
70 mouse models is challenging due to the limited amount of BMAd material obtained from each bone
71 or bone segment. Moreover, this is compounded by the fact that the defining surface markers that
72 would aid in purification of BMAd progenitor populations in homeostatic mice do not discriminate
73 between the labile and stable BMAds (7). With such constraints, a promising alternative to the use of
74 primary cells for *in vitro* studies are multipotent BM-derived stromal cell lines differentiated *in vitro*,

75 especially when studies are possible at the single cell level to address heterogeneity (14). For this
76 purpose, we developed a high-throughput image platform to monitor adipocytic differentiation
77 though LD accumulation quantified by Digital Holographic Microscopy in live cells, which requires
78 minimal handling to avoid perturbation of the culture (15). While not limited by end-point analysis or
79 the introduction of handling or preparation biases, this technique does not provide information on the
80 molecular composition of the cells. Methods capable of combining manipulation-free, label-free,
81 single-cell imaging with analysis of molecular composition are thus of great interest to the field of
82 adipogenesis at large. Raman microspectroscopy is a non-invasive and label-free method that does
83 not require specific sample preparation, with a sub-cellular resolution on the scale of $\sim 1\mu\text{m}$ that
84 provides the molecular composition with spatial data, where standard methods provide bulk
85 information (e.g. mass spectroscopic and chromatographic methods) (16). We thus hypothesized that
86 combining lipid profiling by Raman microspectroscopy with the inherently heterogeneous *in vitro*
87 stromal adipocytic differentiation model would serve as a powerful tool to aid in the understanding of
88 BM adipogenesis.

89 Specifically, LD formation (17) begins with fatty acids (FAs) that enter the cell via fatty acid
90 transport proteins or fatty acid translocase (18,19), or are synthesized through endogenous de novo
91 lipogenesis (DNL). FAs then enter a bioactive pool to form fatty acyl-CoA to be used by glycerolipid
92 synthesis enzymes in the endoplasmic reticulum (ER) to form neutral lipids such as triacylglycerols
93 (TAGs) and eventually coalesce and form LDs (20). If attached to the ER, LDs may grow in size
94 through diffusion of newly synthesized lipids to the LD, and if unattached, through local synthesis or
95 fusion of smaller LDs to larger ones. LDs increase in size during adipocytic differentiation and
96 perilipins (PLINs) bind to promote their stabilization (21). Lipases mobilize neutral lipids in LDs for
97 metabolic energy by fatty acid oxidation, which is triggered through nutritional, hormonal, or
98 inflammatory activation. Adipose triglyceride lipase (ATGL) catalyzes the initial step of intracellular
99 TAG hydrolysis followed by hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL)
100 into glycerol moieties (22,23). FAs are necessary for energy production and lipid synthesis for
101 cellular signaling and membrane formation. Despite their importance, increased concentrations of
102 non-esterified FAs can be detrimental contributing to lipotoxicity, and thus TAG hydrolysis and FA
103 cycling are carefully regulated (24,25). As illustrated in Figure S1, the primary product of DNL is
104 palmitic acid (16:0), in the family of saturated FAs that have cytotoxic effects and induce reactive
105 oxygen species (ROS) (26). The monounsaturated FA (MUFA) oleic acid (18:1) may counteract this
106 effect possibly by activating esterification of palmitic acid into TAGs and lipid droplet storage (27).
107 Palmitic acid accumulation is therefore prevented by increased desaturation to palmitoleic acid
108 (16:1n-7) or elongation to stearic acid (18:0) and further desaturation to oleic acid (28). Notably,
109 oleic acid inhibits palmitic-acid dependent osteoclastogenesis and palmitic acid is found to be
110 increased in BM serum of osteoporotic women (29). In fact, BMAT expansion throughout the red
111 marrow with age and disease is associated with bone loss and fracture risk, while cBMAd formation
112 is positively associated with bone accrual during early development through correlative and
113 descriptive studies (30).

114 While concrete definitions and BMAd classification markers are being established (30), the oldest
115 standard for classifying the two known types of BMAds is by the composition of their lipid content
116 (31, 32). The first reports by Mehdi Tavassoli showed that the adipocytic-rich yellow marrow -now
117 denominated cBMAd- from the os calcis of rabbits was higher in the proportion of unsaturated FAs
118 (palmitoleic -16:1n-7- and oleic -18:1n-9- acids), whereas fatty acid composition of BMAds from the
119 adipocyte-poor red marrow of thoracic vertebrae (rBMAds) was richer in saturated FAs (palmitic -
120 16:0- and stearic -18:0- acids) (33). In recent years, primary rat cBMAds from tail vertebrae and distal
121 tibia were likewise shown to contain a higher proportion of unsaturated fatty acids in caudal vertebrae
122 or distal tibia as compared to rat rBMAds isolated from either lumbar vertebrae or femur plus proximal
123 tibia combined (1). Congruently MRI, ¹H-MRS, and gas chromatographic data from human subjects
124 consistently showed that BM adipose tissue (BMAT) from sites of red marrow contained highly
125 saturated lipids, compared to the mostly unsaturated lipids detected at yellow marrow sites (1,33–35).
126 From murine samples, it is considerably more challenging to obtain sufficient BMAT for analysis due
127 to the size of the animal as well as the amount of BMAT present in the bones (32). To our knowledge,
128 the lipid composition of murine BMAds has not been described to date. However, between BMAT of
129 red and yellow marrow there is a conservation of the differences in both the 16:1/16:0 and 18:0/18:1
130 fatty acid unsaturation ratios relative to total lipid content across species (summarized and compiled
131 with our data in Table 1 and Figure S1).

132 In this study, we harness the inherent adipogenic potential of murine BM-derived OP9 stromal cells to
133 further dissect intrinsic properties of BMAds using standard techniques for lipid profiling at the cell
134 population-level, and Raman microspectroscopy for lipid profiling at the single-adipocyte level. We
135 subjected OP9 cells to basal culture conditions or to a standard adipogenic cocktail, allowing the OP9
136 cells to differentiate spontaneously through serum exposure and confluency (sOP9) or through specific
137 induction of the adipogenic differentiation program (iOP9). To test the hematopoietic supportive
138 capacity of OP9 cells from these conditions, we assessed their interaction with a hematopoietic
139 component in short-term co-culture assays with hematopoietic stem and progenitor cells (HSPCs). We
140 show that a full biochemical induction of the adipogenic differentiation program induces iOP9s to more
141 closely resemble cBMAds in their lipid content, lipid droplet size and hematopoietic support than the
142 sOP9 adipocytes that develop upon simple serum induction, which retain rBMAd-like properties.
143 Furthermore, we validated the significance of our unsaturation ratio-driven cBMAd versus rBMAd
144 definition. For this, we compared for the first time primary human samples from iliac crest samples
145 drawn after hematological aplasia, which represent the most extreme case of rBMAd remodeling (36),
146 to femoral specimens from hip replacement surgery, which represent one of the best characterized
147 cBMAd depots (37,38).

148 **2 Methods**

149 **2.1 Cell culture**

150 All stromal cells (MS5, C3H10T1/2, MC3T3-E1-4, MC3T3-E4-24 bone marrow stromal lines,
151 AFT024 and BFC012 stromal lines from fetal liver (39) or embryonic stem cell-derived fibroblasts as
152 in (40,41) were cultured in complete medium consisting of Minimum Essential Media alpha (MEM α)
153 with GlutaMax™ (Gibco, catalog no. 32561) and 1% Penicillin/Streptomycin (P/S, Gibco, catalog no.

154 15140) supplemented 10% fetal bovine serum (FBS, Gibco, catalog no. 10270-106) at 37°C and 5%
155 CO₂ with media changed every 2-3 days, and differentiated as described below (42). For subsequent
156 analysis OP9 cells were plated in 24-well plates (Falcon) with CaF₂ substrates (Crystran) for Raman
157 microspectroscopy and RT-qPCR, in 6-well plates (Falcon) for HPLC analysis, and in flat-bottom
158 tissue culture-treated 96-well plates (Falcon) for co-culture assays. OP9 cells were plated
159 subconfluently at a density of 5,000 cells per cm² (undifferentiated OP9 cells) or plated at confluency
160 of 20,000 cells per cm² in complete medium (spontaneous OP9 cells) for either 7 or 17 days, as
161 specified in figure legends. Both OP9 cells and OP9-EGFP cells (14) were authenticated by the ATCC
162 on June 24, 2021, by short tandem repeat (STR) analysis, indicating an exact match of the ATCC
163 reference OP9 cell line (CRL-2749). OP9 cells are available from the ATCC (CRL-2749), but this
164 source has not been tested for adipogenesis or hematopoietic support in our laboratory.

165 2.1.1 *In vitro* differentiation and quantification

166 All stromal lines were plated at 20,000 per cm² and cultured to confluency in 1% gelatin-coated plates
167 (prepared overnight at 4°C), then allowed to spontaneously differentiate over time by confluency or
168 induced toward adipocytic or osteogenic differentiation via a standard differentiation cocktail as in
169 (14,42). The adipogenic induction cocktail consisted of complete medium supplemented 1 μM
170 dexamethasone (Sigma, catalog no. D4902), 5 μg/ml insulin (Sigma, catalog no. I0516), and 0.5mM
171 isobutyl-methylxanthine (IBMX, Sigma, catalog no. I5879). After four days, the adipogenic induction
172 medium was changed to a maintenance medium consisting of complete medium with insulin and
173 dexamethasone only. Media was changed every 3-4 days with aliquots prepared fresh from stock
174 solutions (IBMX in DMSO, insulin in PBS, dexamethasone in ethanol) kept at -20°C in the dark. Short-
175 term differentiation experiments were performed at day seven of adipocytic differentiation with
176 minimal culture manipulation as in (15), and long-term experiments were carried out on day 17. Neutral
177 lipids were stained at day 17 of culture with Oil Red O for quantification of adipogenesis in 96-well
178 plates. First, cells were gently washed with phosphate buffered saline (PBS, Gibco, catalog no.
179 10010015) then fixed for 10min at room temperature with 4% paraformaldehyde (PFA) diluted in PBS
180 from 32% stock solution, and gently washed three times with PBS. 100 μl filtered Oil Red O solution
181 freshly prepared from stock solution (Sigma, catalog no. 01391-250ML) diluted 3:2 with distilled water
182 was added to the wells. Cells were incubated with Oil Red O at room temperature for 45min on a
183 shaker, then washed gently with PBS three times and light transmission micrographs were obtained.
184 100 μl isopropanol was then added to the wells and incubated at room temperature. After 10min, 70 μl
185 of the solution was transferred to a new 96 well plate and OD measurements read at 520nm with
186 isopropanol as background. Osteogenic differentiation was performed by addition of dexamethasone
187 1 μM, 2-phospho-L-ascorbic acid 50 μg/ml (1000x stock in PBS), glycerophosphate 10mM (100x stock
188 in PBS) and 1,25-hydroxyvitamine D3 0.01 μM (stock 1000x in ethanol) for 28 days. Every 3-4 days
189 differentiation media was changed. All media changes were made with fresh stock aliquots. Efficiency
190 of osteoblastic differentiation was determined with Alizarin Red stain (Sigma) in stromal cells fixed as
191 described above for O Red Oil stains. Alizarin Red staining solution (alizarin red 2% in distilled water,
192 filtered through 45-μm pore, pH adjusted to 4.0-4.3 with NH₄OH and filtered again) was then added
193 for 10-15 minutes until precipitates were visible. Alkaline Phosphatase development kits were
194 purchased from Promega (S3771), and OP9 stains were performed according to manufacturer's

195 instructions in lightly fixed cells (4%PFA for 1 minute at room temperature) by dilution of NBT in AP
196 staining buffer (100mM Tris HCl pH9.5, 50mM MgCl₂, 100mM NaCl and 0.1% Tween-20). Pictures
197 were taken immediately after staining.

198 2.2 Raman microspectroscopy

199 Fixed 24-well plates were kept in PBS at 4°C and shipped to the University of Lille. Raman acquisitions
200 were done on a LabRAM HR800 equipped with an immersion objective (Nikon, obj x100, numerical
201 aperture = 1, Japan) and a diode laser $\lambda=785$ nm. The laser power at the sample was 30mW. The lateral
202 resolution was 1-2 μ m. Spectral acquisition was made in the 400–1800cm⁻¹ range and spectral resolution
203 was 4cm⁻¹. The acquisition time was set at 60s per spectrum. Raman spectra were processed using
204 Labspec software (HORIBA, Jobin-Yvon, France). The water immersion objective focused the laser
205 on the center of individual lipid droplets where one spectrum corresponds to one adipocyte lipid
206 droplet. In total 2944 spectra were measured over 120 sOP9-adipocytes (on average 24 spectra per
207 adipocyte) and 2971 spectra over 138 iOP9-adipocytes (with an average of 21 spectra per adipocyte).
208 The number of spectra per well was between 60 to 110. The unsaturation ratio was calculated as ratio
209 of area under the curve of bands 1654cm⁻¹ / 1441cm⁻¹. The optical image of the adipocyte was saved for
210 each acquisition. The diameter of LDs was evaluated from the optical image by using the software FIJI
211 (43).

212 The instrument is also equipped with a XYZ motorized stage which allows the acquisition of Raman
213 images. Raman images were acquired using the point-by-point imaging mode (Pt-Img). The laser beam
214 was focused perpendicular to the sample surface. Acquisition time was set to 3sec (x2) for each
215 spectrum. The laser beam was stepped in two dimensions (x and y), and a spectrum was recorded at
216 each position (x,y). The step was set to 1 μ m between 2 positions. The Pt-Img mode generated xxy
217 spectra.

218 2.3 High-performance liquid chromatography

219 Cells were fixed with PFA as described for the adipocytic quantification above. After washing, 500 μ l
220 of PBS was left in the wells, plates were sealed with parafilm and stored at 4°C until processing for
221 HPLC analysis. Cells were detached from the plate with 1mL (2 x 0.5ml) of PBS and lipids extracted
222 with chloroform/methanol (2:1; v/v) under agitation for 30min at room temperature. The resulting lipid
223 extract was then subjected to a saponification, followed by a fatty acid derivatization into naphthacyl
224 esters as described previously (44). Fatty acid derivatives were applied into the HPLC Alliance system
225 (2695 Separations Module, Waters, Saint-Quentin-en-Yvelines, France) equipped of an autosampler
226 (200ml-loop sample), a photodiode array detector (model 2998), and the Empower software for data
227 analyses. Fatty acid derivatives were eluted on a reverse phase YMC PRO C18 column (3mm,
228 4.6x150mm, 120Å) by using two solvent systems: a) methanol/acetonitrile/water (64:24:12; v/v/v) and,
229 b) methanol/dichloromethane/water (65:28:7; v/v/v) under identical conditions reported previously
230 (44). Fatty acid derivatives were detected at 246 nm and quantified by an external standard curve
231 realized with the naphthacyl 19:0 derivative. Note that 19:0 was also used as internal standard to
232 evaluate FA recoveries. Lipidomics LC-HRMS standards and solvents are provided in Table S1.

233 2.4 Real-time quantitative PCR

234 At day seven or 17 of adipocytic differentiation, RNA was extracted with Trizol and cDNA was
235 synthesized using Taq DNA polymerase (catalog no. 10342020, Life Technologies) according to
236 manufacturer's instructions. Quantitative PCR was performed in technical triplicates using the Fast
237 SYBR Green qPCR Mastermix with 250 μ M of primer concentration (Applied Biosystems) on
238 QuantStudio 6 (Life Technologies). Primers were pre-validated for high efficiency and internally tested
239 not to vary upon OP9 differentiation. The geometric mean of housekeeping genes (*RPL13* and *YWHAZ*)
240 was used as reference to calculate the fold expression of each gene relative to the samples of
241 undifferentiated OP9 cells as described for the delta-delta normalization method (45,46). Primer
242 sequences obtained from Microsynth are listed in Supplementary Table S2.

243 2.5 Bone marrow extraction and HSPC sorting

244 Total bone marrow was extracted from eight-week-old B6 ACTb-EGFP females housed in 12-hour
245 day-night light cycles and provided ad-libitum sterile food and water as described previously, in
246 accordance to Swiss law and with the approval of cantonal authorities (Service Veterinaire de l'Etat de
247 Vaud) and complying with ARRIVE guidelines. Total bone marrow cells were extracted from femur,
248 tibia and pelvis by crushing using a mortar and pestle in ice-cold PBS supplemented 1mM
249 ethylenediaminetetraacetic acid (EDTA, catalog no. 15575020, Thermo Fisher Scientific). The
250 samples were dissociated and filtered through a 70 μ M cell strainer (catalog no. 352350, Falcon), lysed
251 for 30sec at room temperature in red blood cell lysis buffer (Biolegend, catalog no. 420301), washed
252 with ice-cold PBS-EDTA and centrifuged at 1300rpm for 10min at 4°C. For HSPC sorting prior to co-
253 culture with OP9 stroma, the cell pellet was stained with 50 μ l biotinylated 'lineage' antibody cocktail
254 (BD, catalog no. 558451) in 1ml PBS-EDTA per six bones for 15min on ice. The sample was washed
255 with PBS-EDTA and stained with 50 μ l magnetic beads of the same kit in PBS-EDTA for 10min on
256 ice. The samples were then washed and filtered through a 70 μ m cell strainer (catalog no. 352350,
257 Falcon) prior to lineage depletion using the AutoMACS Pro (Miltenyi Biotec, USA). After depletion
258 the negative fraction was resuspended in an antibody mix containing antibodies against Streptavidin-
259 TxRed (1:200), cKit-PECy7 (1:200), Sca1-APC (1:100) and PI (1:1000). Cell sorting was performed
260 on a FACSAria Fusion (Becton Dickinson, USA) cell sorter.

261 2.6 *In vitro* HSPC co-culture

262 OP9 cell seeding as detailed in Section 2.1, was followed by a short-term *in vitro* adipocyte
263 differentiation of OP9 cells, and then a gently wash with PBS and pre-warmed Iscove's Modified
264 Dulbecco's Medium (IMDM, catalog no. 12440053, Gibco) supplemented 10 % FBS and 1%
265 Pen/Strep (P/S) was added. The washing was done using the Caliper Sciclone ALH 3000 (Caliper Life
266 Sciences, USA) and consisted of four cycles of removing 140 μ l media and adding 140 μ l fresh IMDM
267 media, to dilute the previous media so as to keep mature adipocyte detachment to a minimum. The
268 wells were left with 100 μ l IMDM 10% FBS and 1% P/S. HSPCs extracted from B6 ACTb-EGFP mice
269 were plated at a ratio of 1:10 initial OP9 cells (in volume of 100 μ l IMDM, totaling 200 μ l volume per
270 well).

271 After seven days of co-culture, the 96-well plates were removed from the incubator and placed on ice.
272 To count non-adherent cells, the cells in suspension were retrieved through repeated washes and stained
273 with CD45-PacBlue at final concentration of 1:200, PI at 1:1000 and 5 μ l CountBright beads (catalog
274 no. C36950, Invitrogen) directly added to the media. Without any further manipulation, the plates were
275 analyzed using the High Throughput Sampler (HTS) module of a LSRII (Becton Dickinson, USA)
276 flow cytometer. For accuracy, the settings were programmed to mix the wells thoroughly before sample
277 uptake. To count adherent cells, a separate set of duplicate plates were taken from the incubator, the
278 media manually removed, and wells washed once with 200 μ l PBS. 40 μ l of Trypsin EDTA (0.5 %,
279 catalog no. 25300-054, Gibco) was added to each well and incubated for five minutes at 37°C. 160 μ l
280 of ice-cold PBS containing FBS (to neutralize the trypsin), CD45-APCCy7 (1:200), PI (1:1000) and
281 5 μ l CountBright beads were then added to the plates. The plates were stained on ice and directly
282 analyzed with flow cytomet.

283 **2.6.1 In vitro conditioned media preparation and culture**

284 Conditioned media was prepared for four different conditions as follows. For the undifferentiated
285 condition, undifferentiated OP9 cells were seeded in six-well plates at confluency (20,000 OP9
286 cells/cm²) in IMDM (catalog no. 12440053, Gibco) supplemented 10% FBS and 1% P/S (basal IMDM)
287 and conditioned medium was harvested after two days in culture and stored at -20°C. For the
288 spontaneous differentiation conditions, confluent OP9 cells were grown in MEM α as described for the
289 short-term differentiation assays except that at day five media was changed to IMDM following three
290 washes; conditioned medium was then harvested after two days and stored at -20°C. For the induced
291 adipocytic conditions, confluent OP9 cells underwent the short (5 day) or long (17-day) DMI
292 adipogenic induction protocols before changing media to basal IMDM for two additional days of
293 culture; conditioned medium was then harvested and stored at -20°C. Subsequently 2000 live-sorted
294 HSPCs were plated per well in 96-well round-bottom plates in the absence of stroma. Different ratios
295 of conditioned-to-basal IMDM media were added. After two-day culture the cells were stained with
296 Annexin V, Propidium Iodine (PI) and CD45 to select for live CD45⁺ hematopoietic cells via flow
297 cytometric analysis with an LSRII cytometer (Becton Dickinson, USA). CountBright beads were added
298 to count absolute cell numbers as described above.

299 **2.6.2 Cobblestone formation assay**

300 OP9 cells were plated at 20,000 cells/cm² in gelatin-pre-coated 6-well plates and cultured with α -MEM
301 media supplemented with 10%FBS and 1%P/S. The cells were induced to differentiate using the
302 standard differentiation cocktail DMI, which was changed two times per week. After seven days of
303 differentiation, the media was washed three times with PBS and changed to basal IMDM. On the same
304 day, 200 sorted KLS cells were added into each well. After 7 days of co-culture the hematopoietic
305 colonies were manually counted (47) and scored the following way: (i) shiny colonies (defined by
306 majority of round and bright cells, as they float above the OP9 stromal layer, with no more than 10
307 cobblestone cells visible per colony; (ii) cobblestone colonies (defined as darker 'cobblestone'-like
308 cells, because they are below the OP9 stromal layer) should not contain more than 20% of shiny cells;
309 and (iii) mixed colonies that contain both shiny and cobblestone cells(48–50).

310 **2.7 Clinical samples**

311 This study complied with the Declaration of Helsinki and the local ethical authorities (CER-VD). All
312 patients signed a specific consent for the reuse of biological samples in the context of our study. For
313 HPLC, iliac crest BM aspirates from female and male patients undergoing treatment for acute myeloid
314 leukemia (between ages 48 and 73, n=5) or surgical debris from patients undergoing hip replacement
315 surgery (between ages 56 and 80, n=4) were received for analysis from the Lausanne University
316 Hospital (Centre Hospitalier Universitaire Vaudois, CHUV). Floating adipose tissue, serum, and oil
317 fractions were isolated and processed for lipid extraction and processed as described for OP9
318 lipidomics.

319 **2.8 Statistical Analysis**

320 Values are shown as mean plus or minus the standard deviation or standard error of the mean as
321 indicated. Student's t-test was performed for all experiments when comparing two conditions only, or
322 a Two-Way ANOVA when comparing multiple conditions, with P-values indicated for statistical
323 significance.

324 Raman spectra were processed by using the PLS Toolbox and the MIA toolbox (v8.7 eigenvector
325 Research, Inc., USA). The spectra were baseline corrected, normalized and mean centered. Two
326 processes were done on Raman spectra. First, cluster analysis (CA) was performed using Ward's
327 method. CA clustered individual Raman spectra into 3 categories (saturated-rich lipids, unsaturated-
328 rich and mixture of saturated and unsaturated lipids). Second, principal component analysis (PCA) was
329 used which highlighted the difference in spectral features between the spontaneous and induced
330 conditions. All data are reported as mean \pm standard deviation. All statistical analyses were performed
331 using GraphPad Prism (version 8.0.0, GraphPad Software). Mean values were compared by Mann and
332 Whitney test, or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test.
333 Statistical significance was accepted for $P < 0.05$, and reported.

334

335 **3 Results**

336 **3.1 Differential adipocyte lipidation and similar transcriptional signature upon adipogenic** 337 **induction by confluency (spontaneous) versus DMI cocktail (induced).**

338

339 We chose murine BM-derived OP9 stromal cells as a model for this study because of their propensity
340 to differentiate into adipocytes while displaying both hematopoietic support and dual adipogenic and
341 osteogenic differentiation capacities as compared to other commonly used murine stromal lines
342 including BM-derived MS5 and C3H10T1/2 lines or MC3T3 subclones (Figure S2A-C and (42)). Note
343 retention of alkaline phosphatase activity in confluent and fully mature, lipidated OP9-derived O Red
344 Oil positive adipocytes (Figure S2D), reminiscent of properties assigned to hematopoietic supportive
345 AP+ reticular adventitial cells in the bone marrow (51) and possibly of AdipoCARs (52).

346 The non-clonal OP9 stromal cell line (53,54) was originally derived from the calvaria of newborn
347 osteopetrotic mice ((C57BL/6x3H)F₂-op/op) deficient in macrophage colony-stimulating factor (M-

348 CSF), and were shown optimal for studies of hematopoietic cell development and differentiation
349 sensitive to M-CSF (55,56). OP9 cells have been described as preadipocytes with multilineage
350 differentiation capacity which readily generate mature adipocytes, and express the transcriptional
351 activators CCAAT/enhancer binding proteins (C/EBP) α and β , together with the master regulator of
352 adipocyte differentiation peroxisome proliferator activated receptor- γ (PPAR γ), and perilipin (PLIN),
353 a phosphoprotein associated with the surface of lipid droplets (LDs) (21,54). Thanks to their
354 multipotency, hematopoietic support, and adipocytic differentiation capacities, OP9 cells are thus ideal
355 for *in vitro* studies on BM adipogenesis. Due to their non-clonal nature and inherent heterogeneity,
356 they serve as a BM model for studying BMA δ differentiation at the single cell level.

357 *In vitro*, the OP9 cell line spontaneously differentiates into adipocytes upon confluency in the presence
358 of serum (spontaneous OP9 adipocytes, sOP9), a phenomenon that can be more robustly promoted by
359 exposure to a classical adipocytic differentiation cocktail consisting of insulin, dexamethasone, and 3-
360 isobutyl-1-methylxanthine (induced OP9 adipocytes, iOP9) (21,53). After 17 days in culture, both
361 sOP9 and iOP9 culture conditions contained numerous differentiated OP9-adipocytes (Figure 1) with
362 a significantly greater prevalence of Oil Red O staining of lipids in the induced condition (iOP9)
363 ($p < 0.01$) as compared to control baseline undifferentiated OP9 cells plated at sub-confluency (uOP9)
364 (Figure 1G). Spontaneous OP9-adipocytes formed under the minimal confluency condition (sOP9) and
365 stained with Oil Red O at intermediate levels between undifferentiated uOP9 cells and induced iOP9
366 cultures (Figure 1G). No significant amount of mature adipocytes could be visualized in the uOP9
367 condition (Figure 1A,D). Adipocytes were defined as previously described, where cells containing at
368 least four identifiable lipid droplets were accounted as mature adipocytes (57). Morphologically, both
369 sOP9 and iOP9 adipocytes stained with Oil Red O at day 17, but iOP9 adipocytes constituted the
370 majority of the cultures and often contained large lipid droplets, while sOP9 adipocytes were sparser
371 and their lipid droplets seemed smaller.

372 Given the intermediate phenotype of sOP9 adipocytic cultures, containing a mixture of mature
373 adipocytes and non lipidated stromal cells likely including preadipocytes, we wished to determine
374 whether the transcriptional profile and lipid composition of these cultures more resembled the
375 undifferentiated uOP9 cultures or the highly lipidated iOP9 adipocytic cultures. We thus compared
376 transcriptional adipocyte differentiation markers by RT-qPCR and the global fatty acid (FA)
377 composition by high performance liquid chromatography (HPLC) for the three conditions.

378 RNA transcripts for the canonical adipocyte differentiation and maturation program (*Fabp4*, *Adipoq*,
379 *Cebpa*, *Pparg*, *Lpl*, *Pnpla2* -also known as *Atgl*-) in the sOP9 condition most resembled iOP9
380 adipocytic cultures, with average gene expression more than 10 times higher for the sOP9 and iOP9
381 conditions than for the control uOP9 condition (Figure 2A). **These results were further validated in the**
382 **OP9-EGFP cell line used in previous studies (Figure S3A-B, (14)).** Further analysis of the desaturation
383 and elongation pathway for fatty acid synthesis showed a similar pattern (Figure 2B), with overall
384 higher expression for the sOP9 and iOP9 adipocytic conditions as compared to the uOP9
385 undifferentiated condition. Of note RNA transcripts for *Sds1* and *Fads2* were however significantly
386 between sOP9 and iOP9 cultures. We thus conclude that, at the population level, expression of genes
387 related to the adipocyte differentiation and fatty acid desaturation programs is similar for both sOP9

388 and iOP9 adipocytic conditions after 7 days of culture, suggesting that the transcriptional program is
389 triggered in sOP9 cells prior to lipid droplet accumulation.

390 Conversely, total FA content and profiles were similar for control uOP9 cells and the sOP9 adipocytic
391 condition as measured by HPLC analysis. Classical adipogenic induction of iOP9 cells resulted in a
392 16-fold increase of total FA content as compared with sOP9 cells or uOP9 cells
393 ($2.4 \times 10^{\pm 2.2} \times 10^{\pm 2.2} \text{ pmol/cm}^2$ versus $1.5 \times 10^{\pm 7.0} \times 10^{\pm 7.0} \text{ pmol/cm}^2$ and $17.4 \times 10^{\pm 3.7} \times 10^{\pm 3.7} \text{ pmol/cm}^2$
394 respectively, Figure 2C). FA profiles revealed a preferential enrichment of MUFAs in iOP9 cells (47%
395 MUFAs versus 38% in uOP9s and 36% in sOP9 cells), mostly to the detriment of PUFAs (only 2% in
396 iOP9 cells versus 6% PUFAs in uOP9s and 10% in sOP9 cells, $p < 0.0001$) (Figure 2D, G). In terms of
397 individual FAs, the most striking finding was the marked high ratio of palmitoleic acid (16:1) in iOP9
398 cells, representing 29% of total FA (versus 9% in uOP9 or sOP9 cells, $p = 0.007$) (Figure 2D, E). This
399 result was associated to a reduction of 18 carbon FAs in iOP9 cells and a trend towards a consistent
400 reciprocal enrichment of 18 carbon FAs in sOP9 cells: stearic acid (18:0, sOP9 11% vs. iOP9 4%,
401 $p = 0.02$), oleic acid (18:1, sOP9 26% vs. iOP9 16%, $p = 0.07$) and linoleic acid (18:2 n-6, sOP9 4% vs.
402 iOP9 1%, $p = 0.007$). The most abundant PUFA species was arachidonic acid (20:4 n-6). In relative
403 terms, linoleic (18:2 n-6) and arachidonic acid (20:4 n-6) were significantly enriched in the sOP9-
404 adipocytes at 5% of total FA while making up only 1% of total FA in iOP9-adipocytes. Notably, the
405 ratio of palmitoleic acid (16:1) versus its saturated form (stearic acid, 16:0) in iOP9 adipocytes was
406 three times higher than in sOP9 adipocyte cultures or uOP9 control cells (Figure 2E). The ratio of oleic
407 acid (18:1) versus its saturated form (stearic acid, 18:0) showed a similar but non-significant trend
408 (Figure 2F). These results are very similar to those obtained on rats' tibiae when comparing lipid
409 profiles from constitutive adipocytes versus regulated adipocytes (1). Our data is also congruent with
410 the results shown for rabbit BMAdS when comparing FA composition of rabbit BMAdS from rBMAd-
411 rich versus cBMAd-rich sites ((33) and summarized in Table 1).

412 Overall, these results indicate that, at the population level, sOP9 adipocytic cultures share
413 transcriptional similarities in the adipocyte differentiation program with iOP9 cells, but are less
414 lipidated and present lower abundance of unsaturated FAs. This constellation would be consistent with
415 our hypothesis that sOP9 adipocytic cultures present similarities with regulated BMAdS, and thus an
416 enrichment in saturated FA, as compared to cBMAd-like iOP9 adipocytic cultures. Given the
417 heterogeneous lipidation of sOP9 adipocytic cultures and the inevitably bulk nature of our RT-PCR
418 and HPLC-based analysis, we set out to validate our hypothesis through a single cell analysis pipeline
419 that could directly compare sOP9 and iOP9 adipocytes on their relative FA acid composition at the
420 single cell level.

421

422 3.2 Raman microspectroscopy detects unsaturated spectra at the single lipid droplet level in 423 spontaneous and induced OP9-derived adipocytes.

424 Raman microspectroscopy represents a powerful yet non-invasive and label-free method to assess the
425 lipid composition of adipocytes *in vitro*. The unsaturation ratio is recognized as a measure of the

426 proportion of unsaturated FA obtained by the ratio of the peak area assigned to unsaturated bonds
 427 (C=C) divided by the peak area assigned to saturated bonds (CH₂) (Equation 1) (58–61).

428

$$429 \quad \text{Unsaturation ratio} = \frac{A(\nu_{C=C})}{A(\nu_{CH_2})} \quad (1)$$

430 **Equation 1:** Lipid unsaturation ratio measured by Raman microspectroscopy.

431 Our feasibility test with Raman microspectroscopy imaging showed that it was possible to acquire
 432 Raman spectra at single lipid droplet (LD) resolution in our model (Figure 3). After 17 days of culture,
 433 Raman spectra, representative of the molecular composition of individual LDs, were acquired for sOP9
 434 and iOP9 adipocytes through high resolution Raman imaging. No analysis was performed for the
 435 undifferentiated (uOP9) condition as it presented with very few, if any, LDs. Raman images of
 436 individual adipocytes were processed by PCA. The PCA shows that a mixture of spectra exists between
 437 the adipocytes but also within the LDs themselves, as illustrated by the Raman images (Figures 3B,
 438 3E). In Figure 3C, the PC1 scores (96.68%) show a representative spectrum rich in unsaturated lipids
 439 for the iOP9 adipocyte shown in Figure 3A-B. In Figure 3F, the PC1 scores (95.69%) show a spectrum
 440 representative of a saturated rich lipids for the sOP9 adipocyte shown in Figure 3D-E. Figure S4 shows
 441 the additional variation captured by PC2 and PC3 (<5%) for the same two adipocytes shown in Figure
 442 3. We conclude that LDs composed of predominantly unsaturated-rich or saturated-rich lipids can be
 443 identified through Raman microspectroscopy within OP9-derived adipocytes with single LD
 444 resolution.

445 **3.3 Raman microspectroscopy reveals differential predominance of unsaturated spectra in** 446 **large lipid droplets from induced adipocytes**

447 In order to determine if significant differences exist in the unsaturation ratio of FAs accumulated within
 448 sOP9 versus iOP9 adipocytes, we thus proceeded to the acquisition of Raman spectra from individual
 449 LD in both conditions and throughout 3 separate experimental campaigns (iOP9: n = 138 adipocytes
 450 and 2971 spectra; sOP9: n = 120 adipocytes and 2944 spectra; lateral resolution 1-2 μm), and combined
 451 all data for aggregated principal component (PC) analysis in both conditions. Figure S5 shows the
 452 classification of spectra using unsupervised hierarchical cluster analysis, which predicted three main
 453 categories: saturated-rich, unsaturated-rich and mixture. The representative spectrum of each category
 454 is shown in Figure 4A. PCA was then performed on the averaged spectra per adipocyte. The PC1
 455 loading plot contains characteristic spectra where the positive peaks correspond to saturated lipids
 456 (1061, 1129, and 1296 cm⁻¹, represented in red for Figures 4A-B) and the negative peaks correspond to
 457 unsaturated lipids (1080, 1267, and 1655 cm⁻¹, represented in blue for Figures 4A-B) (58). PC1 captures
 458 78.20% of the variability of the overall dataset, and separates the Raman spectra based on the saturated-
 459 rich and unsaturated-rich profiles (Figure 4B). On the PCA score plot, each point represents an
 460 averaged spectrum per adipocyte (Figure 4C). Most of the spectra from iOP9-adipocytes (81%) form
 461 a cluster in the negative score along PC1, while the spectra of sOP9-adipocytes are scattered along
 462 PC1. This indicates that the molecular composition of LDs in sOP9-adipocytes is heterogeneous
 463 compared to iOP9-adipocytes, and that iOP9-adipocytes are enriched in unsaturated-rich lipids. Score

464 plots in Figure 4C and 4D are equivalent, but the points in Figure 4C are colored according to PC1
465 scores, and the points in Figure 4D are colored according to the culture condition. Specifically, the
466 points in dark red and dark blue in Figure 4C correspond to Raman spectra of saturated-rich and
467 unsaturated-rich lipids, while intermediate colors point to Raman spectra of a rather balanced mixture
468 of saturated and unsaturated lipids. PCA analysis shows that LDs in iOP9-adipocytes are mainly rich
469 in unsaturated lipids, while LDs in sOP9-adipocytes are composed of a rather balanced mixture of
470 unsaturated and saturated lipids (Figure 4D). However, when comparing the averaged raw unsaturation
471 ratio (based on averaged spectra per adipocyte) we found no significant difference between iOP9
472 adipocytes and sOP9 adipocytes (Figure 4E). At the adipocyte level, both conditions have a similar
473 averaged unsaturation ratio. This result is explained by PCA analysis which shows that LDs of both
474 conditions are not solely composed of either uniformly saturated or unsaturated lipids, but rather by
475 different mixtures thereof. We thus took advantage of the 1-2 μ resolution of our setup and performed
476 the analysis at the single LD level.

477 First, we hypothesized that the smaller diameter of LDs in sOP9 adipocytes may be related to their
478 lower unsaturation ratio, owing to the higher mobilization rate of unsaturated FAs (62). We thus plotted
479 LD diameter versus unsaturation ratio (Figure 5A-B). The LD diameter did not correlate with the
480 unsaturation ratio. Specifically, correlation analysis for the LD diameter to unsaturation ratio shows
481 that the parameters are not linearly correlated in either condition (Figure 5A-B, $R^2_{\text{spot}}=0.0013$;
482 $R^2_{\text{min}}=0.0107$). Even when the spectra are separated according the 3 types of lipids (saturated-rich,
483 unsaturated-rich and mixture) the LD diameter and the unsaturation ratio are not linearly correlated.
484 All the R^2 are inferior to 0.05 (Figure S6). Since, no correlation was observed, the LD diameter and the
485 unsaturation ratio were explored separately.

486 The averaged LD diameter was significantly lower ($p<0.0001$) in the sOP9-adipocytes ($5.24\pm 1.85\mu\text{m}$)
487 than the iOP9-adipocytes ($7.87\pm 3.83\mu\text{m}$) (Figure 5C). When we further separated LDs by type of
488 lipids, we found that saturated-rich, unsaturated-rich or mixture LDs are not different in their average
489 composition when originating from iOP9 or sOP9 adipocytes, but unsaturated LDs are more frequent
490 in iOP9s while saturated LDs are more frequent in the sOP9 condition (Figure 5D-E, Figure S5B).
491 Unsaturated-rich LDs are overall bigger in size than saturated-rich LDs, with a predominance in iOP9
492 adipocytes, particularly above the diameter of $10\mu\text{m}$. In iOP9 adipocytes, the number of unsaturated-
493 rich LDs is higher compared to sOP9 adipocytes for LD diameter superior to $10\mu\text{m}$.

494 Then, the averaged unsaturation ratio per condition were obtained from individual spectra of LDs. The
495 unsaturation ratio of iOP9 condition (0.17 ± 0.05) is significantly higher than sOP9 condition
496 (0.14 ± 0.05 , $p=0.0014$). Also on average, LDs of iOP9 condition have more unsaturated-rich lipids
497 compared to the LDs in sOP9 condition. Since differences are observed between sOP9 and iOP9 around
498 a LD diameter of $10\mu\text{m}$ (Figure 5E), the unsaturation ratio of LDs was investigated as function of the
499 threshold of $10\mu\text{m}$ (Figure 5G). The larger LDs ($>10\mu\text{m}$) have the highest unsaturation ratio compared
500 to smaller LDs. Smaller LDs ($<10\mu\text{m}$) from iOP9 adipocytes have a higher unsaturation ratio compared
501 to sOP9-adipocyte. Then, we investigated the unsaturation ratio as a function of the 3 types of LDs,
502 namely saturated-rich, mixture or unsaturated-rich. Although we found consistent differences between
503 the three classes of LDs, there were no significant differences between LDs of the same class in sOP9

504 adipocytes versus iOP9-adipocytes (Figure 5H). Not surprisingly, mixture LDs showed the greatest
505 heterogeneity in their unsaturation ratio (Figure 5H). Overall, we conclude from this data that iOP9
506 adipocytes contain significantly bigger LDs and more frequently unsaturated LDs than sOP9
507 adipocytes. *In vivo*, both higher LD diameter and higher FA unsaturation are characteristics of
508 cBMAds, while smaller LD diameter and lower unsaturation ratio are characteristics of rBMAds. We
509 could not find a consistent relationship between LD diameter and unsaturation ratio.

510

511 **3.4 Hematopoietic support capacity of sOP9-adipocytes is superior to that of iOP9-adipocytes**

512 Another *in vivo* property of rBMAds as compared to cBMAds is their differential capacity to serve as
513 a supportive niche to hematopoietic progenitor cells. Indeed, cBMAds are associated to the
514 hematopoietic poor -yellow- regions of the marrow, while rBMAds are associated to the hematopoietic-
515 rich -red- regions of the marrow. To determine whether sOP9 versus iOP9 cultures behave different in
516 terms of hematopoietic support, we co-cultured murine hematopoietic stem and progenitor cells
517 (HSPCs) isolated as cKit⁺Lin⁻Sca1⁺ (KLS) cells for seven days with the differentiated OP9 cells at a
518 ratio of 1 HSPC to 10 OP9 cells. As in previous work (63), this high ratio of KLS to OP9 cells serves
519 to avoid dispersion due to HSPC heterogeneity and avoids downstream stochastic, clone effects on
520 analysis secondary to differences in the content of the most primitive and thus proliferative HSPCs
521 across wells, which although small, would be exponentially exaggerated after co-culture. At the end of
522 the seven-day co-culture period, flow cytometric analysis revealed that total hematopoietic expansion
523 of 2,000 seeded HSPCs was 4.5-fold lower ($p < 0.0001$) when iOP9 cultures were used as feeder as
524 compared to the sOP9 condition (Figure 6A). Of note, total hematopoietic cells in suspension were
525 significantly lower in the iOP9 versus sOP9 condition ($p < 0.05$) whereas the more immature progenitor
526 cells that are typically in close contact and adherent to the feeder cells were proportionally lower in the
527 sOP9 condition. This suggests that iOP9 cultures may preferentially favor support of the more primitive
528 hematopoietic progenitors, which is congruent with previous data from purified human BMAd as
529 compared to undifferentiated stromal cells (8). The more hematopoietic progenitor supportive function
530 of the sOP9 condition is also illustrated by the quantification of colonies formed by day 7 of co-culture
531 (Figure 6B). The total number of hematopoietic colonies per initial number of HSPCs seeded, was
532 significantly higher in the sOP9 coculture (0.13 colonies per initial KLS seeded) than in the iOP9
533 coculture (0.01 colonies per initial KLS seeded). All three colony categories, from the shiny consisting
534 of more mature hematopoietic cells to the mixed and cobblestone colonies which contain the most
535 immature hematopoietic progenitors were significantly increased ($P < 0.05$) in the sOP9 condition. To
536 understand to which extent secreted factors from either condition contributed to hematopoietic
537 expansion, HSPCs were cultured for two days in the absence of feeder cells but in the presence of
538 different concentrations of 48-hour conditioned media from either uOP9, sOP9 or iOP9 cultures. For
539 the iOP9 condition we tested both a short (5+2 days) and a long (17+2 days) DMI adipogenesis
540 induction protocol (Figure 7C). Hematopoietic expansion was greatest for HSPCs cultured in the
541 highest concentration of undifferentiated-conditioned medium. HSPCs cultured in conditioned
542 medium from iOP9-adipocytes did not show a significant expansion after two days in culture,
543 independently of the length of the iOP9 DMI induction protocol. However, the conditioned medium

544 from sOP9 cultures was substantially more supportive to HSPC maintenance than iOP9, although less
 545 than those cultured in conditioned media from undifferentiated OP9 cells. This effect increased with
 546 higher ratios of conditioned-to-basal medium. We therefore conclude that the *in vitro* hematopoietic
 547 progenitor support capacity of sOP9 cultures is superior to that of iOP9 cultures, aligning with the *in*
 548 *vivo* functionality of regulated versus constitutive BMAd.

549 **3.5 HPLC fatty acid quantification in primary human samples confirms a higher** 550 **unsaturation ratio in constitutive BMAd-rich marrow (femoral head) versus regulated** 551 **BMAd-rich marrow (iliac crest post-chemotherapy).**

552 In order to contextualize and validate the value of the FA unsaturation ratio used throughout this
 553 work as a surrogate marker for regulated versus constitutive BMAd, we set to test whether we could
 554 find differences in the unsaturation ratio of lipids extracted from one of the most extreme BM
 555 remodeling scenarios. Patients suffering from acute leukemias receive intensive aplastic
 556 chemotherapy, a regime that eliminates leukemic cells but also virtually all short-term hematopoietic
 557 progenitors in the BM, leading to a rapid adipocytic conversion of the marrow. BMAd formed in
 558 this context should represent a stereotypical example of rBMAd, and we thus set **out** to compare the
 559 unsaturation ratio of lipids obtained from iliac crest samples from these patients as compared to the
 560 lipid fraction obtained from surgical debris of adult patients undergoing elective hip replacement
 561 surgery, which is thought to represent a cBMAd-rich site. Analysis of these primary human BM lipid
 562 samples reflected a preferential enrichment of MUFAs in the post-chemotherapy iliac crest BM
 563 samples (regulated BMAd site) versus the femoral head BM samples (constitutive BMAd site) from
 564 hip replacement surgery (39.6% versus 51.2%, $p=0.02$) to the detriment of PUFAs (18.6% versus
 565 17.0% $p=0.80$) and SFAs (41.8% and 36.6%, $p=0.04$) (Figure 7D). While rapid conversion to
 566 adipocytic marrow is induced by chemotherapy, and thus iliac crest samples are interpreted as
 567 rBMAd-rich, the differences in fatty acid profiles displayed could also be an inherent function of the
 568 anatomical site. The largest difference between FA species in the regulated versus constitutive
 569 marrow sites was seen in the 18:1 to 18:0 unsaturation ratio (4.40 versus 7.91, $p=0.007$), and the 16:1
 570 versus 16:0 unsaturation ratio (0.11 versus 0.19, $p=0.031$) (Figure 7B-C). We thus conclude that
 571 human BM samples from a rBMAd-rich site in the context of BM remodeling driven by
 572 hematopoietic demand present a lower unsaturation ratio, as measured by 16:1/16:0 -and 18:1/18:0-
 573 FA content, than samples obtained from a cBMAd-rich site, which complements previously reported
 574 results for rabbit and rats marrow at steady-state (1,33–35) as summarized in Table 1.

575 **Table 1: Fatty acid unsaturation ratios in rat, rabbit, human, and OP9 cells from red and yellow**
 576 **BMAd.** The 16:1/16:0 and 18:1/18:0 unsaturation ratios were calculated from the proportion of total fatty
 577 acids. Rat BM data from Scheller et al. 2015 Supplementary Data 1 (regulated: femur and proximal tibia,
 578 constitutive: distal tibia). Rabbit BM data from Tavassoli 1977 Table 1 (regulated: vertebrae, constitutive: os
 579 calcis). Human original data from Figure 7 (regulated: iliac crest post-chemotherapy, constitutive: femoral head)
 580 and OP9 cell original data from Figure 6 (regulated: spontaneous, constitutive: induced) from HPLC analysis.
 581 Additional measurements in human BM have been made by MRI spectroscopy but cannot be compiled with
 582 these data due to the different methodology (35,64). c: constitutive, r: regulated.

| Origin | BMA _d type | 16:1/16:0 | c/r | 18:1/18:0 | c/r | Reference |
|------------------|------------------------|--------------|-----|--------------|-----|-----------------------|
| <i>Rat</i> | constitutive regulated | 0.25 0.09 | 2.8 | 1.18 0.81 | 1.5 | Scheller et al. 2015 |
| <i>Rabbit</i> | constitutive regulated | 0.53 0.16 | 3.3 | 7.52 2.68 | 2.8 | Tavassoli et al. 1977 |
| <i>Human</i> | constitutive regulated | 0.19 0.11 | 1.7 | 7.91 4.40 | 1.8 | original data |
| <i>OP9 cells</i> | induced spontaneous | 0.84 0.24 | 1.6 | 4.74 2.33 | 2.0 | original data |

583 Taken together, we conclude that the spontaneous versus induced OP9 *in vitro* adipogenesis model
584 presented here replicates key differences in lipid droplet size, FA unsaturation ratio and hematopoietic
585 support respectively associated *in vivo* to regulated versus constitutive BMA_d subtypes. We
586 additionally validate the value of the 16:1/16:0 FA unsaturation ratio as a surrogate marker to segregate
587 rBMA_d-rich versus cBMA_d-rich tissues both *in vivo* and for our *in vitro* model, and the feasibility of
588 Raman microspectroscopy to reveal equivalent differences in the unsaturation ratio at the single
589 adipocyte level.

590

591 4 Discussion

592 BMA_ds constitute a distinct fat depot with low responsiveness to cold and insulin, overall relative
593 resistance to lipolysis, and high expression of adiponectin as compared to subcutaneous fat depots
594 (37,65,66). Two BMA_d subtypes have been described based on their differential response to
595 physiological and metabolic stimuli: the more stable so-called constitutive (cBMA_d) depots,
596 preferentially located in the distal parts of the skeleton, and the more labile so-called regulated
597 (rBMA_d) adipocytes, which are interspersed within the hematopoietic marrow in more proximal
598 skeletal sites. Due to their fragility and location within the bone cavity, access to live BMA_d cells for
599 functional and cell trajectory analysis is not trivial, especially for murine BMA_d depots where BM
600 adipose tissue volume is very limiting. Although possibly limited on the extrapolation of specific
601 metabolic behaviors (37), *in vitro* models of adipogenesis from undifferentiated stromal progenitors
602 have been proposed as a complementary tool to study BMA_d at the cellular level, so as to facilitate
603 both gene function studies and cell behavior analysis (reviewed in (32,67)). To our knowledge, no *in*
604 *vitro* model has been described to recapitulate some or all of the differential characteristics of the *in*
605 *vivo* defined rBMA_d versus cBMA_d depots. Here we present a model of BM adipogenesis in the form
606 of a differential *in vitro* culture system for BM-derived OP9 stromal cells. When classically induced
607 by an adipogenic cocktail (iOP9s) OP9-derived adipocytes resemble cBMA_ds and, when allowed to

608 spontaneously differentiate by confluency in serum-containing conditions (sOP9s), they resemble
609 rBMAdS in lipid droplet size, overall lipid saturation, 16:1/16:0 FA unsaturation ratio and
610 hematopoietic supportive properties. Indeed, we validated the value of the 16:1/16:0 FA unsaturation
611 ratio, already described as a discriminating factor for cBMAd and rBMAd depots in rabbit and rat
612 homeostatic tissues, in both human BM and as a marker of cBMAd-like (iOP9) versus rBMAd-like
613 (sOP9) *in vitro* cultures. Furthermore, we have extended the reproducibility of the 16:1/16:0 FA
614 unsaturation ratio as a discriminating factor to highly remodeled rBMAd depots in the context of
615 patients undergoing intensive ablative chemotherapy. It is worth noting that the robustness of
616 differences in the 16:1/16:0 FA unsaturation ratio in our model (summarized in Table 1; (1,68), as
617 opposed to other proposed FA pairs (e.g. 18:1/18:0), is somehow surprising and may be associated to
618 the low mobilization rate for palmitate (16:0) and palmitoleate (16:1) (69,70). Further studies should
619 mechanistically address the relative contribution of *de novo* lipogenesis and FA re/uptake in lipid
620 composition differences underlying the 16:1/16:0 FA unsaturation ratio. Indeed, at the RNA level,
621 sOP9 adipocytes had significantly higher expression of *Lpl*, which mediates fatty acid uptake for
622 storage, while iOP9 adipocytes had increased expression of *Atgl*, the key enzyme that initiates
623 hydrolysis of TAGs.

624 After HPLC-based lipid composition analysis of OP9 cultures at the cell population level, and given
625 the relative heterogeneity of *in vitro* adipogenic cultures (67), we implemented a novel application of
626 Raman microspectroscopy to measure lipid unsaturation heterogeneity at the single cell level, with
627 individual LD resolution and in a label-free manner. This approach allowed us to focus lipid
628 composition analysis exclusively on mature adipocytes and not on the remaining unlipidated stromal
629 component. Previous studies have shown the application of Coherent anti-Stokes Raman
630 microspectroscopy to embryonic stem cells undergoing adipogenic differentiation, showing a measure
631 of chemical lipid saturation composition and an increase in LD accumulation with differentiation
632 (71,72). While, very interestingly, individual LDs within one adipocyte appear to have an
633 inhomogeneity in their chemical composition, this was not further analyzed. Raman microspectroscopy
634 has allowed for the characterization of LD lipid composition in animal and *in vitro* models. Lipid
635 droplets have been measured directly in the endothelial layer of murine aortic sections by, pointing to
636 smaller LDs induced by TNF α preferentially containing more unsaturated lipids whereas larger
637 LDs were more rich in saturated lipids (73). Another Raman-based study showed interesting
638 differences in the pattern of distribution between saturated FA species (palmitic acid -16:0- and stearic
639 acid -18:0), which were evenly distributed in broad areas of the cell, and unsaturated FA species (oleic
640 acid -18:1- and linoleic acid -18:2-), which were concentrated in LDs, after exposure and uptake in
641 macrophages (74). The effect of the high fat diet was evaluated on the lipidic composition of
642 perivascular adipocytes in a mouse model. The size of the LDs was increased as an adaptive response
643 to the excessive lipid income. In an *in-vitro* study, the effect of TNF- α on endothelial HMEC-1 cells
644 during 24h was monitored by Raman microspectroscopy. The composition and the distribution of
645 cellular lipids was modified upon exposure to TNF- α , and the increase in LD size was concomitant
646 with the formation LDs composed of unsaturated rich lipids (75,76). However, none of these studies
647 provide supporting statistical analysis for an univocal association between LDs size and molecular
648 composition. In all, we found Raman microspectroscopy to be robust for the measurement of the
649 unsaturation ratio at the lipid droplet level. Congruently to the HPLC-derived data, we could thus

650 confirm at the single adipocyte level the higher unsaturation ratio of iOP9 lipids as compared to the
651 sOP9 condition. It is worth noting that the overall differences in unsaturation ratio were more
652 pronounced when measured with HPLC analysis (1.02 ± 0.27 for iOP9-cultures versus 0.19 ± 0.11 sOP9-
653 cultures, $p=0.0004$, Figure 2E), than with Raman (0.17 ± 0.05 for iOP9-cultures versus 0.14 ± 0.05 sOP9-
654 cultures, $p=0.014$, Figure 5F). This difference could be related to the sample bias, as HPLC analysis
655 was done in lipid extracts from the bulk culture while Raman microspectroscopy acquired spectra from
656 mature adipocytes only, or to the method itself. For the HPLC analysis, the unsaturation ratio is
657 calculated from the contribution of isolated and purified lipids. On the other hand, Raman analysis is
658 done inside the LD where lipids are mixed. The unsaturation ratio is calculated from the contribution
659 of the mixture of lipids which hamper the signal. Indeed, the small differences we report in the
660 unsaturation ratio are in the range of previous Raman studies cited above, although differences are
661 more pronounced if only the more defined fractions are considered (unsaturation-rich versus saturated-
662 rich, Figure S5).

663 Diseases of lipodystrophy may provide insights into the formation of BMAd and the selective
664 accumulation of FAs that we are struggling to understand (1,77). Congenital generalized lipodystrophy
665 (CGL) is a family of diseases that include a complete or partial loss of adipose depots, which reveal
666 important insights into the consequences of genetic deficiencies involved in adipocytic maturation and
667 function (reviewed in (77)) and may point us in the direction of the underlying nature of BMAd
668 formation. One of the best understood CGL mouse models within the context of BMAd research are
669 the *Ptrf*^{-/-} deficient mice, which contain BMAT only in the tail vertebrae and very distal tibia with a loss
670 of BMAd at the tibia-fibula junction and proximal thereof (1,78–80). This is accompanied by a small
671 increase in cortical bone mineral content in adult male mice and a large increase in trabecular number
672 and cortical bone mineral content in females (1). The differential effect on BMAT upon *PTRF* loss of
673 function, points toward a differential regulation of LD formation in regulated versus constitutive
674 BMAd. Alternatively, differences in unsaturated lipid ratios could be involved in the specific
675 sensitivity of BMAd to such a mutation. More recently, diphtheria-toxin mediated ablation of Adipoq-
676 Cre expressing cells also revealed differences in BMAd plasticity throughout the long bones in "fat-
677 free" mice. In particular BMAd were increasingly present in the mid-diaphysis with age (81,82).
678 Furthermore, specific depletion of BMAd through a double conditional Osterix and Adipoq-
679 dependent strategy caused severe reduction in rBMAd with a milder reduction in the cBMAd mass,
680 while ATGL (*Pnpla2* gene) loss in BMAd through the same strategy caused massive rBMAd expansion
681 in the mid diaphysis (83,84). To what extent these discrepancies are due to divergent transcriptional
682 programs or part of a stabilization of the adipocytic differentiation program remains to be determined.
683 Moreover, seipin-deficient lymphoblastoid cell-lines derived from patients suffering congenital
684 generalized lipodystrophy type 2 were shown to have an increased proportion of SFAs with a decrease
685 in MUFAs, which are the principle products of *Scd1*, indicating decreased *Scd1* activity. LD number
686 and size were increased compared to control (85). Impaired $\Delta 9$ -desaturase activity in nematodes also
687 led to decreased TAG accumulation and LD size that could not be rescued completely by supplemented
688 dietary oleate (86). Therefore, it appears that LD size is at least partially driven by the ability of *Scd1*
689 to synthesize unsaturated FAs. Although our results did not show a strong correlation of LD size with
690 lipid unsaturation, we did observe that the largest LDs were always predominantly unsaturated. Taken
691 together, this points to tissue-specific regulation of lipid composition independently of nutrient and

692 metabolite availability, and possibly to sequential stages of LD (and adipocyte) maturation in the sOP9
693 versus the iOP9 conditions. Overall, it is tempting to suggest a continuum with smaller LD being
694 mostly saturated, an intermediate stage of LD maturation that are of largely mixed composition, and
695 the most mature LDs that are large and unsaturated. Follow-up studies should address this hypothesis
696 through Raman microspectroscopy in the context of inhibition versus activation of lipolysis and
697 reuptake with relation to lipid droplet and adipocyte size. Extrapolation to the *in vivo* scenario should
698 be done with caution, as the primary mechanism of lipid remodeling in primary human BMAd has
699 been shown to be independent of ATGL-mediated lipolysis (37).

700 Finally, we found that sOP9-adipocytes are more supportive to hematopoiesis than iOP9-adipocytes,
701 as seen by their capacity to form hematopoietic colonies and to support hematopoietic expansion. This
702 agrees with reports of adiponectin expressing BM pre/adipocytes -including so-called Adipo-CAR
703 cells- being necessary for hematopoietic expansion (12,87-88), whereas mature BMAds rather
704 inducing HSCs quiescence or reduced proliferation (7,8,11,89). Of note, depletion of recently
705 described marrow adipocyte lineage precursors (MALPs) did not affect hematopoiesis, at least in
706 homeostasis (90). The reduced proliferation of hematopoietic progenitors in iOP9 adipocytic
707 cocultures was replicated in a dose-dependent manner through condition media experiments (Figure
708 6C), pointing to availability of soluble factors as one of the implicated mechanisms. Indeed, previous
709 data revealed a higher expression of the hematopoietic supportive factors C-X-C Motif Chemokine
710 Ligand 12 (*Cxcl12*) and c-Kit Ligand (*KitL*) in undifferentiated OP9 cultures versus OP9-derived
711 adipocytes analogous to iOP9s (42), and the regulation of BM stromal cell differentiation precisely via
712 *Cxcl12* (91). Note however that the undifferentiated OP9 condition was shown even more supportive
713 of hematopoietic proliferation than sOP9s (Figure 6). In congruence with previous *in vivo* data (11),
714 this observation in the OP9 system suggests that hematopoiesis is favored when mature marrow
715 adipocytes are absent. We therefore suggest an optimum of maturation along the adipocytic
716 differentiation axis with regard to hematopoietic support, in particular through at least limited
717 expression of Angpt1, SCF, and *Cxcl12* that are attenuated in terminally differentiated adipocytes (92).
718 Taken together, our results indicate that, at the population level, sOP9 adipocytic cultures share
719 similarities in terms of hematopoietic support with the labile rBMAds of the red marrow, while iOP9
720 adipocytic cultures impair rapid hematopoietic progenitor proliferation as described for the stable
721 cBMAds of the yellow marrow.

722 The mechanisms for differential hematopoietic support *in vivo* are still poorly understood, but
723 extrapolation from the collective findings of the field suggests that cBMAd-dense regions of the distal
724 skeleton maintain HSCs, while rBMAd-containing red marrow would contribute to expansion of their
725 progenitors, possibly through increased FA transfer (83). Most HSCs have low mitochondrial potential
726 and rely mainly on anaerobic glycolysis, while downstream progenitors rely on high energy production
727 through mitochondrial oxidative phosphorylation (93–95). Previous studies have also revealed that
728 HSCs and leukemic cells can rely on PPAR-mediated FA oxidation (96), with activation of oxidative
729 metabolism being a predictive factor for leukemic stem cell behavior (97). Indeed, we have shown that
730 BM stromal cells with lower overall lipid content (sOP9 cultures) associate to increased hematopoietic
731 expansion capacity compared with more mature BMAds that are high in lipid content (iOP9 cultures).
732 Specific differences in FA of BMAds may contribute to the nuances of differential hematopoietic

733 support. For example, palmitic acid was shown to have a lipotoxic effect on hematopoietic-supportive
734 osteoblasts, and high levels are present in aged and osteoporotic bone associated with decreased
735 hematopoiesis (98,99). This may in part explain the overt myeloproliferation that is seen on the aged
736 skeleton. In the current study, the stromal OP9 cell line was selected for its adipocytic differentiation
737 potential. The inherent M-CSF deficiency may influence outcomes with regard to the hematopoietic
738 supportive function. Previous studies have shown lymphoid and myeloid support of bone marrow
739 adipocytes, although the extent of hematopoietic support as a function of the extent of adipocytic
740 differentiation has not been addressed. In future studies, a comparison to marrow stromal cell lines
741 without cytokine expression defects is warranted to provide a more representative understanding of the
742 marrow hematopoietic and adipocytic lineages.

743 Future studies to understand the functional metabolic differences of rBMAd-like sOP9 versus cBMAd-
744 like iOP9 adipocytes should focus on *in vitro* lipase- and desaturase- inhibition, complemented by the
745 study of their role in BMAd-deficient animal models (such as the Atgl-KO or Scd1-KO) to better
746 understand the role of rBMAd vs cBMAd FA mobilization on hematopoiesis. Altogether, our results
747 open avenues for future work on the OP9-adipocyte system to understand the mechanisms underlying
748 BMAd heterogeneity and its impact on both hematopoiesis and bone health.

749

750 **5 Conflict of Interest**

751 The authors declare no conflict of interest at this time.

752 *The authors declare that the research was conducted in the absence of any commercial or financial*
753 *relationships that could be construed as a potential conflict of interest.*

754 **6 Author Contributions**

755 J.T. planned experiments, analyzed and interpreted the results. G.F. performed Raman acquisitions,
756 interpreted and analyzed the results and compiled the corresponding figures. A.D. and N.B. performed
757 HPLC analyses. J.T., C.B. and N.D.T. performed *in vitro* culture experiments. V.C. performed co-
758 cultures. G.F. and L.D. performed statistical analyses for Raman data. J.T., G.F., A.D., C.C. and O.N.
759 interpreted the Raman and HPLC data. G.Q.D. and O.N. designed and optimized OP9 differentiation
760 protocols and associated hematopoietic support assays. O.N. and G.P. initiated the project, while C.C.
761 and O.N. co-supervised the project. O.N. and J.T. wrote the manuscript, G.F. and C.C. significantly
762 edited the manuscript.

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779 **9 Data Availability**

780 Original datasets are available in Mendeley Data publicly accessible repository:
 781 [<http://dx.doi.org/10.17632/wdm9gvz3bm.1>].

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1058

1059 **11 Supplementary Material**

1060 Supplementary Figures have been uploaded separately on submission. Please provide Frontiers link
1061 for access here.

1062

In review

1063 **Figure 1: Heterogeneity in the formation of lipidated adipocytes upon induction of adipogenesis**
 1064 **by confluency and serum exposure (spontaneous) versus DMI cocktail (induced).** OP9 cells were
 1065 (A, D) passaged subconfluently and remained undifferentiated on day 17 of culture, (B, E, H)
 1066 differentiated spontaneously upon confluency in the presence of serum for 17 days, or (E, F, I) induced
 1067 with a classical adipogenic differentiation cocktail in the same serum-containing conditions after
 1068 confluent plating and 17 days in culture. (G) Oil red O measurements show the highest adipogenic
 1069 differentiation in the induced condition at day 17 in culture. ** $P < 0.01$ by student's t-test. Error bars
 1070 represent mean \pm s.d. (n=3 biological replicates). DMI: 1 μ M dexamethasone, 0.5mM isobutyl-
 1071 methylxanthine, 5 μ g/ml insulin.

1072

1073 **Figure 2: Population level differences in lipid composition are more marked than differences in**
 1074 **the canonical adipocytic transcriptional signature for spontaneous versus induced cultures.** (A)
 1075 RT-qPCR markers of adipogenesis are increased in induced OP9-adipocytes. (B) Desaturase
 1076 expression is highest in day 17 induced OP9-adipocytes while one of the elongases in fatty acid *de*
 1077 *novo* synthesis is decreased. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by Bonferroni's multiple
 1078 comparisons test. Error bars represent mean \pm s.d. (n=3 independent experiments). (C-G) HPLC
 1079 analysis of spontaneously differentiated or induced OP9 adipocytes revealed (C) nuances of the most
 1080 abundant saturated, mono-, and polyunsaturated lipid species, with (D) overall total higher unsaturation
 1081 content in induced versus spontaneous conditions. (E) Percentage of individual fatty acid species
 1082 reveals (F) a greater 16:1/16:0 unsaturation ratio in the induced condition. (G) Unsaturation ratios
 1083 18:1/18:0 were not significantly different. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by
 1084 ANOVA test with Bonferroni's multiple comparisons adjustment. Error bars represent mean \pm s.d.
 1085 (n=3 independent experiments). HKGs: housekeeping genes.

1086

1087 **Figure 3: Raman microspectroscopy detects unsaturated spectra at the single lipid droplet level**
 1088 **in spontaneous and induced adipocytes.** (A) An optical image of an adipocyte in the induced
 1089 condition (iOP9) analyzed by Raman imaging. (B) Raman image of the scores of PC1 in induced
 1090 condition. Pixels in black represent a score of PC1 equal to zero, which corresponds to the absence of
 1091 PC1. Pixels in shades of blue correspond to the contribution of PC1. (C) Loading PC1 is characteristic
 1092 of a spectrum of unsaturated lipid-rich and captures more than 95% of the variation. (D) An optical
 1093 image of an adipocyte in spontaneous condition (sOP9) analyzed by Raman imaging. (E) Raman image
 1094 of the scores of PC1 in spontaneous condition. Pixels in black represent a score of PC1 equal to zero,
 1095 which corresponds to the absence of PC1. Pixels in shades of red correspond to the contribution of
 1096 PC1. (F) Loading PC1 is characteristic of a spectrum of saturated lipid-rich. The Raman images of the
 1097 scores and the loadings of PC2 and PC3 capture less than 2% and are presented in supplementary files
 1098 (Figure S4).

1099

1100 **Figure 4: Raman microspectroscopy reveals predominance of unsaturated spectra in induced**
 1101 **adipocytes, with heterogeneity in the unsaturation ratio for the spontaneous condition.** (A)
 1102 Representative Raman spectrum of saturated-rich, unsaturated-rich lipids and mixture identified by
 1103 unguided hierarchical cluster analysis. (B) Loading plot PC1 indicates the discriminant peaks for
 1104 saturated versus unsaturated designation, the color is associated to the attribution of specific Raman
 1105 bands: saturated in blue, unsaturated in red (n=3 independent experiments, pooled for PCA analysis).

1106 (C) PCA score plot of Raman spectra labelled according to spontaneous (orange) and induced (green)
 1107 OP9-adipocytes. (D) The same PCA score plot as (C) labelled according to the assignment of Raman
 1108 spectra as saturated (red) and unsaturated (blue) lipids based on the PC1 loading determined by B. For
 1109 panels C and D, each point is a single adipocyte with all its lipid droplet data points averaged. n
 1110 (induced) = 138 adipocytes, 2971 spectra; n (spontaneous) = 120 adipocytes, 2944 spectra. (E)
 1111 Unsaturation ratios of spontaneous and induced at adipocyte level. The unsaturation ratio of sOP9
 1112 adipocytes is not different from iOP9 adipocytes (P=0.0855 by Mann-Whitney test).

1113

1114 **Figure 5: Induced adipocytes are composed of larger lipid droplets than spontaneous adipocytes,**
 1115 **but there is no overall correlation between lipid droplet diameter and unsaturation ratio. (A-B)**
 1116 Unsaturation ratio as a function of the diameter of the LDs for sOP9 versus iOP9 adipocytes (n=3
 1117 independent experiments, pooled for LD analysis). The low R2 for both conditions indicates that the
 1118 diameter of the LDs is not correlated with the unsaturation ratio. Dashed lines represent the correlation
 1119 line. The Y axis is colored as function of the score of PC1. The circles in dark blue corresponds to LDs
 1120 identified as unsaturated rich. The circles in dark red correspond to LDs identified as saturated-rich.
 1121 The circles in yellow and light blue correspond to LDs identified as mixture. (C) Histogram for
 1122 frequency of LDs as a function of the diameter for the spontaneous and induced condition. Vertical
 1123 lines show the mean diameter of the LD for each condition. The mean diameter of LDs is 5.24 μ m
 1124 (\pm 1.85 μ m) and 7.87 μ m (\pm 3.83 μ m) for sOP9 adipocytes and iOP9 adipocytes, respectively. The LDs
 1125 mean diameter of sOP9-adipocytes is significantly lower compared to iOP9-adipocytes (P<0.0001,
 1126 Mann-Whitney test). (D, E) When separated according to LD type a trend toward larger unsaturated
 1127 lipid droplets in the induced condition is observed. (F) Unsaturation ratios of sOP9 versus iOP9 at LD
 1128 level. LDs from iOP9 adipocytes have a higher mean unsaturation ratio compared to LDs from sOP9
 1129 adipocytes. (P<0.0014, Mann-Whitney test). (G) At single droplet resolution, larger droplets (>10 μ m)
 1130 have a higher unsaturation ratio as compared to smaller lipid droplets, while smaller droplets (<10 μ m)
 1131 in induced OP9-adipocytes (n=1973) have a higher unsaturation ratio than those of spontaneous OP9-
 1132 adipocytes (n=2579). Highest unsaturation ratio overall is seen in large droplets from spontaneous
 1133 OP9-adipocytes (n=41). (F) Unsaturation ratio as a function of the condition (spontaneous and
 1134 induced) and the nature of the lipid (saturated-rich, unsaturated-rich and mixture). *P<0.05,
 1135 ****P<0.001 by Kruskal-Wallis test. The lipid droplet of spontaneous unsaturated-rich have the higher
 1136 unsaturation ratio compared to induced unsaturated-rich.

1137

1138 **Figure 6: Short-term co-culture of OP9-adipocytes with hematopoietic stem and progenitor cells**
 1139 **favours hematopoietic expansion in the spontaneous condition. (A)** Total hematopoietic cell
 1140 expansion of 2000 initial HSPCs after seven days in co-culture (including cells in adherent and cells
 1141 in suspension) is significantly higher (p<0.0001) when hematopoietic stem and progenitor cells,
 1142 HSPCs, (cKit⁺Lin⁻Sca1⁺, KLS) are co-seeded with spontaneous- compared to induced OP9 adipocytes.
 1143 (B) The colony forming capacity of HSPCs seeded with spontaneous OP9 adipocytes is significantly
 1144 higher (P<0.05) than when cultured with induced OP9 adipocytes. (C), Total hematopoietic expansion
 1145 (in the absence of stroma) after two-day co-culture of 2000 HSPCs in conditioned medium from OP9
 1146 cultures comprising increasing ratios of conditioned-to-fresh media. ****P<0.0001, **P<0.01,
 1147 *P<0.05 by Bonferroni's multiple comparisons test. Error bars represent mean \pm s.d. (n=3 biological
 1148 replicates).

1149

1150 **Figure 7: HPLC fatty acid quantification of primary human BM samples confirms a higher**
1151 **unsaturation ratio in constitutive BMAd-rich marrow (femoral head) versus regulated BMAd-**
1152 **rich marrow (ileac crest post-chemotherapy).** (A-C) HPLC analysis of lipids isolated from human
1153 iliac crest bone marrow aspirates after induction chemotherapy (regulated site, labile marrow) or from
1154 the bone marrow of femoral specimens collected upon hip replacement surgery (constitutive site, stable
1155 marrow). (A) Higher unsaturation content in stable versus labile marrow oil reveal nuances of
1156 individual saturated, mono-, and polyunsaturated lipid species. (B-C) Greater unsaturation ratios in the
1157 human marrow from the constitutive site versus the regulated site. (D) **P<0.01, *P<0.05 by
1158 student's t-test. Error bars represent mean \pm s.d. (n=4 experiments from independent donors).
1159 Regulated site (iliac crest): 3 females, 1 male (age = 55 ± 12 years). Constitutive site (hip replacement):
1160 3 males, 1 female (age = 68 ± 10 years).

1161

In review

1162 **Table 2:** List of general abbreviations

| Abbreviation | Definition |
|---------------------|--|
| ACTb | Actin beta |
| AdipoQ | Adiponectin |
| Angpt1 | Angiopoietin 1 |
| ANOVA | Analysis of variance |
| ATGL | Adipose triglyceride lipase |
| Ap2 | Activating protein 2 |
| BM | Bone marrow |
| BMAAd | Bone marrow adipocyte |
| BMAT | Bone marrow adipose tissue |
| BMSC | Bone marrow stromal cell |
| CA | Cluster of analysis |
| CAR cell | CXCL12 abundant reticular cell |
| cBMAAd | Constitutive bone marrow adipocyte |
| cDNA | Complementary deoxyribonucleic acid |
| CEBPa | CCAAT Enhancer Binding Protein Alpha |
| CFU | Colony-forming unit |
| CGL | Congenital generalized lipodystrophy |
| cKit | Kit proto-oncogene, receptor tyrosine kinase |
| CXCL12 | C-X-C motif chemokine ligand receptor 12 |
| DMI | Cocktail for Dexamethasone, IBMX and insulin |
| DNA | Deoxyribonucleic acid |
| DNL | De novo lipogenesis |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFP | Enhanced green fluorescent protein |
| ER | Endoplasmic reticulum |
| FA | Fatty acid |
| FABP4 | Fatty acid binding protein 4 |
| FACS | Fluorescence-activated cell sorting |
| FADS | Fatty acid desaturase |
| FBS | Fetal bovine serum |
| HKGs | Housekeeping genes |
| ¹ H-MRS | proton magnetic resonance spectroscopy |
| HPLC | High performance liquid chromatography |
| HSC | Hematopoietic stem cell |
| HSL | Hormone sensitive lipase |
| HSPC | Hematopoietic stem and progenitor cell |
| IBMX | isobutylmethylxanthin |
| IMDM | Iscove's Modified Dulbecco's Medium |
| iOP9 | induced OP9 cell |
| KitL | cKit ligand |
| KLS | cKit ⁺ Lineage ⁻ Sca1 ⁺ |

| | |
|---------------|--|
| LD | Lipid droplet |
| LPL | lipoprotein lipase |
| mRNA | Messenger ribonucleic acid |
| MRI | Magnetic resonance imaging |
| MUFA | Monounsaturated fatty acid |
| M-CSF | Macrophage colony-stimulating factor |
| MEM α | Minimum Essential Medium alpha |
| ORO | Oil red O |
| PC | Principle component |
| PCA | Principle component analysis |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PI | Propidium iodide |
| PLIN | Perilipin |
| PPAR γ | Peroxisome proliferator-activated receptor gamma |
| PTRF | Polymerase I and transcript-release factor |
| P/S | Penicillin/Streptomycin |
| PUFA | Polyunsaturated fatty acid |
| rBMAd | Regulated bone marrow adipocyte |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT-qPCR | Real-time quantitative polymerase chain reaction |
| Sca1 | Stem cell antigen 1 |
| Scd | Stearyl-CoenzymeA desaturase |
| SCF | Stem cell factor |
| SD | Standard deviation |
| SFA | Saturated fatty acid |
| sOP9 | spontaneous OP9 cell |
| STR | Short tandem repeats |
| TAG | Triacylglycerols |
| uOP9 | Undifferentiated OP9 cell |

1163

1164

1165 **Table 3:** List of fatty acid abbreviations

| Abbreviation | Definition | Formula | IUPAC Name |
|--------------|------------------|--|---|
| 14:1 | Myristoleic acid | C ₁₄ H ₂₆ O ₂ | (Z)-tetradec-9-enoic acid |
| 16:0 | Palmitic acid | C ₁₆ H ₃₂ O ₂ | hexadecanoic acid |
| 16:1n-7 | Palmitoleic acid | C ₁₆ H ₃₀ O ₂ | (Z)-hexadec-9-enoic acid |
| 16:1n-10 | Sapienic acid | C ₁₆ H ₃₀ O ₂ | (Z)-hexadec-6-enoic acid |
| 18:0 | Stearic acid | C ₁₈ H ₃₆ O ₂ | octadecanoic acid |
| 18:1n-9 | Oleic acid | C ₁₈ H ₃₄ O ₂ | (Z)-octadec-9-enoic acid |
| 18:2n-6 | Linoleic acid | C ₁₈ H ₃₂ O ₂ | (9Z,12Z)-octadeca-9,12-dienoic acid |
| 20:4n-6 | Arachidonic acid | C ₂₀ H ₃₂ O ₂ | (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid |

1166

In review

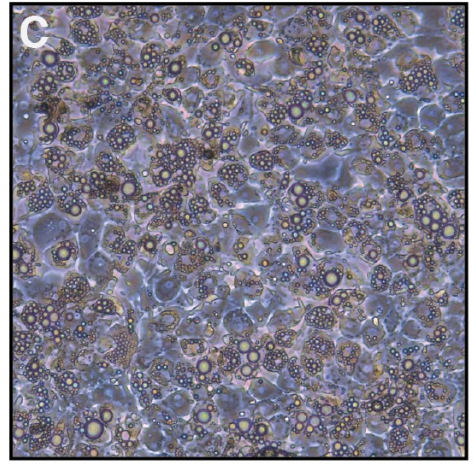
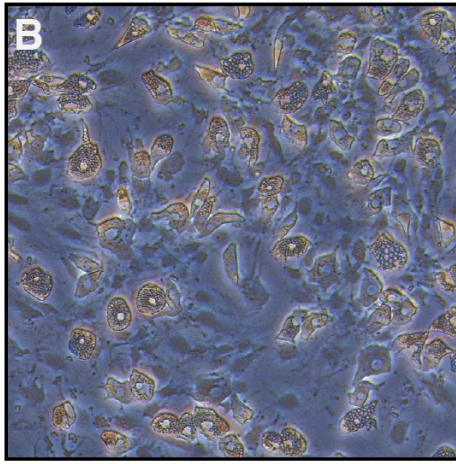
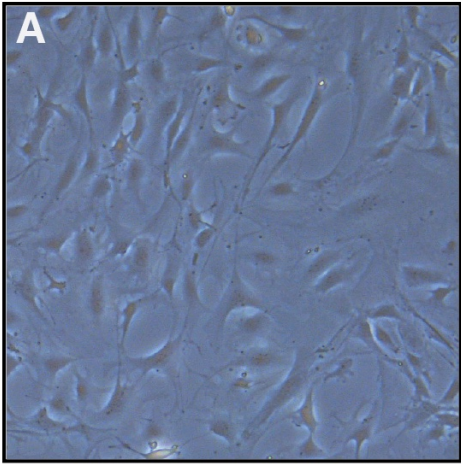
Figure 1.JPEG

Undifferentiated

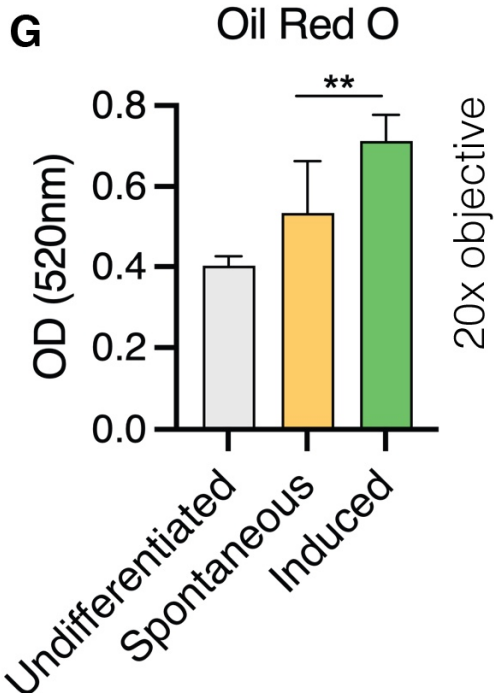
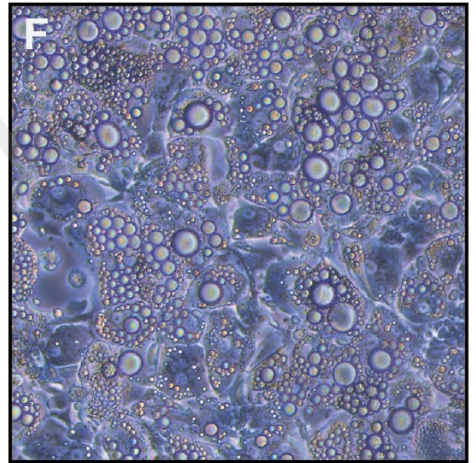
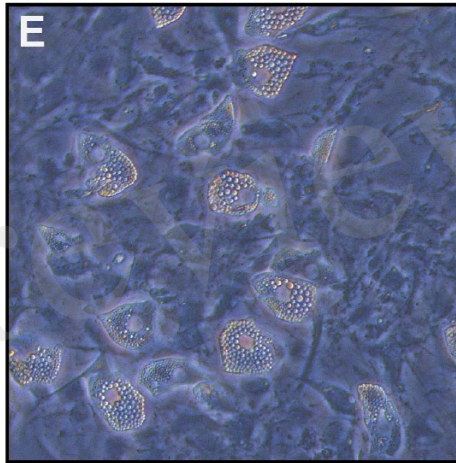
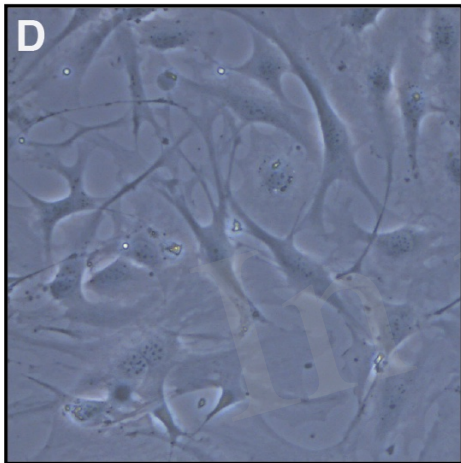
Spontaneous

Induced

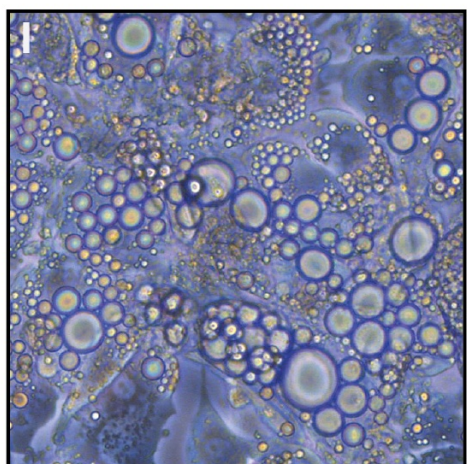
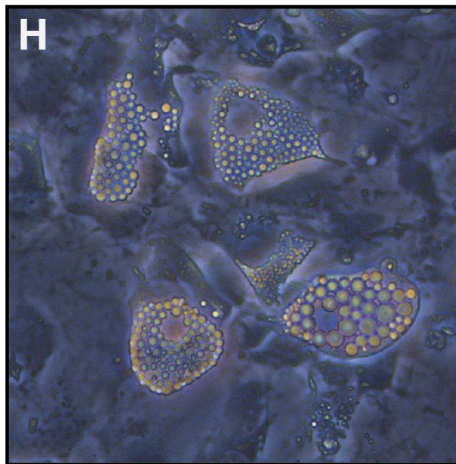
5x objective

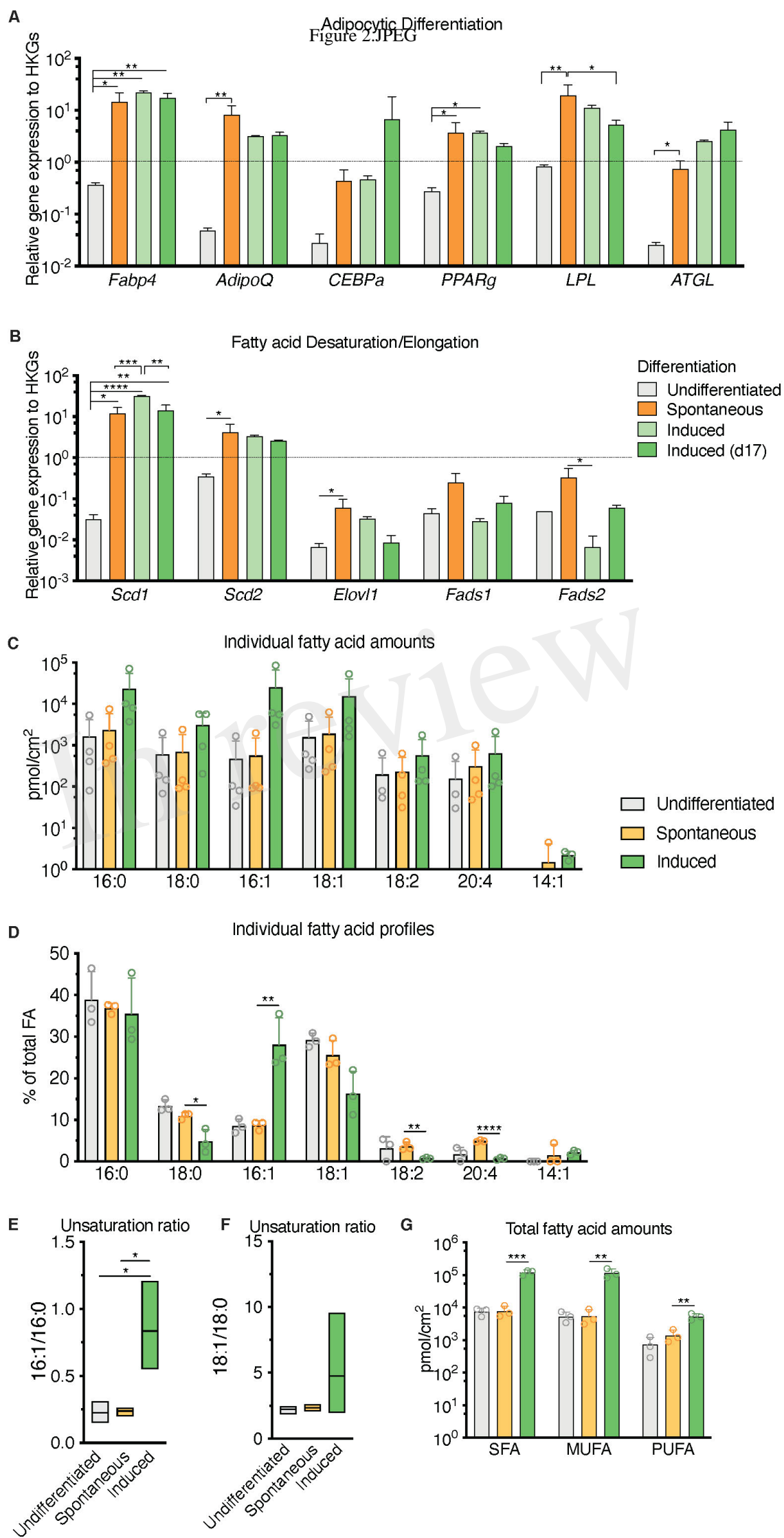


10x objective



20x objective





Induced condition

Optical image

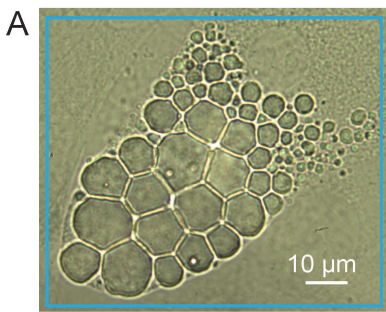
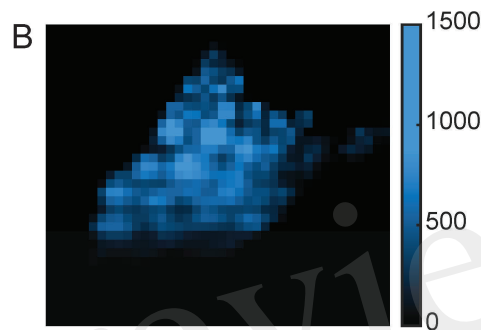
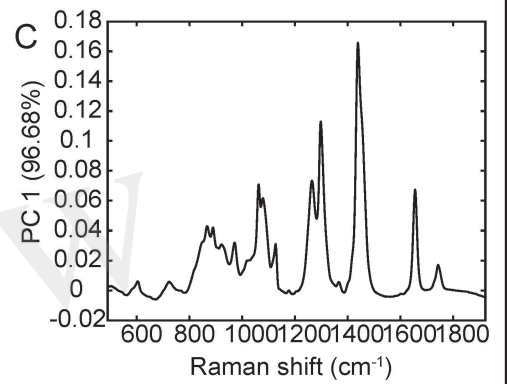


Image of scores on PC 1 (96.68%)



Loading PC 1



Spontaneous condition

Optical image

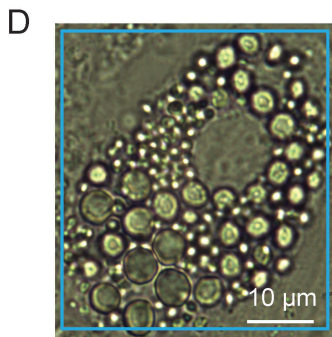
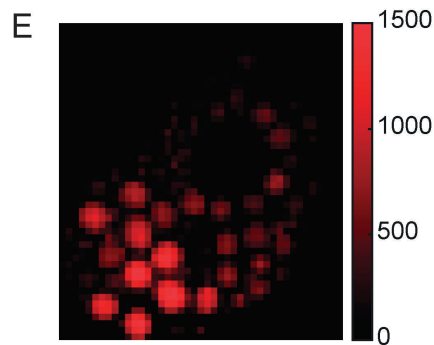


Image of scores on PC 1 (95.69%)



Loading PC 1

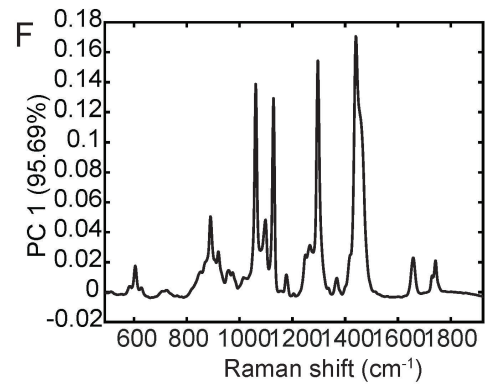


Figure 4.JPEG

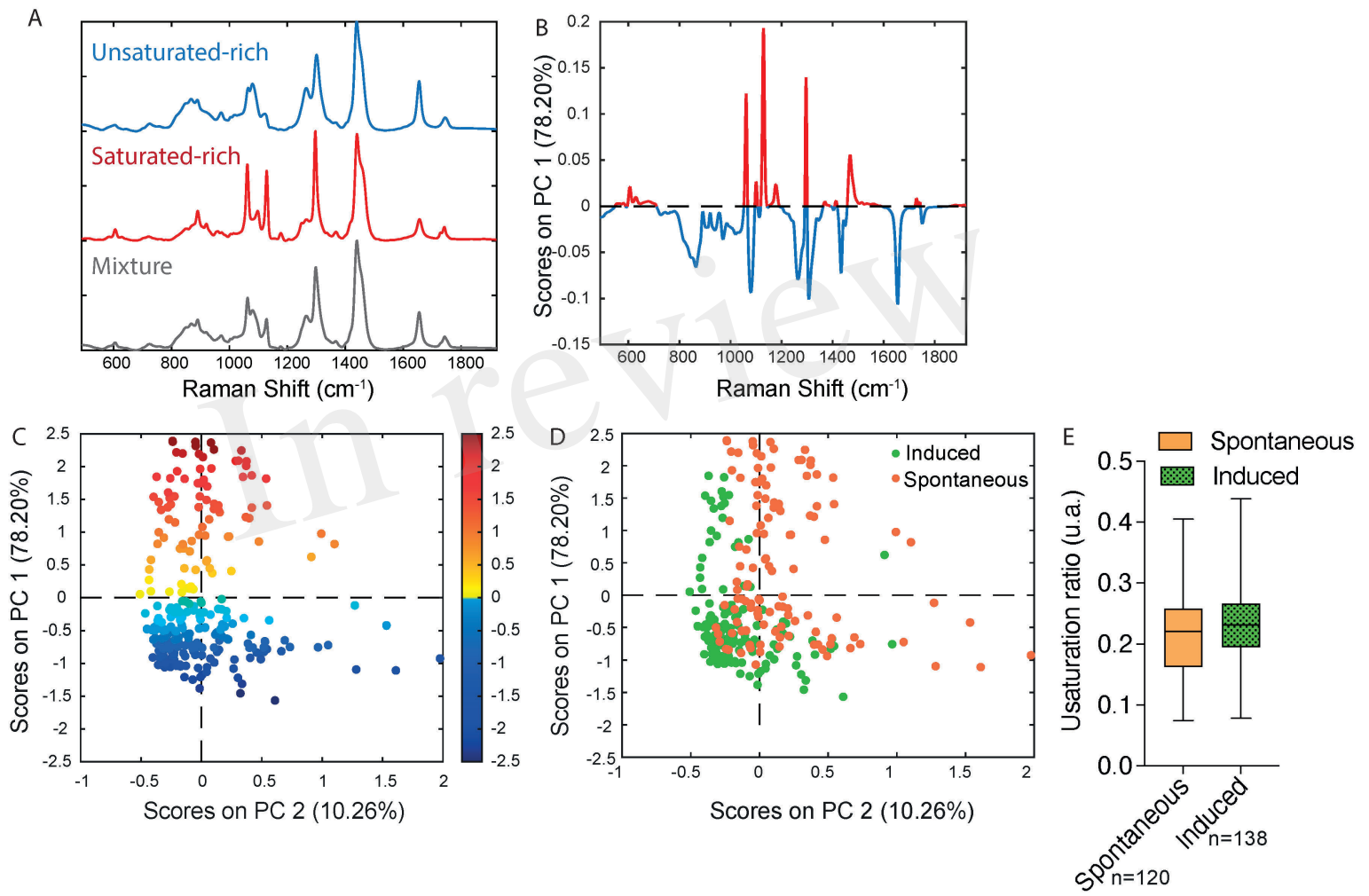


Figure 5.JPEG

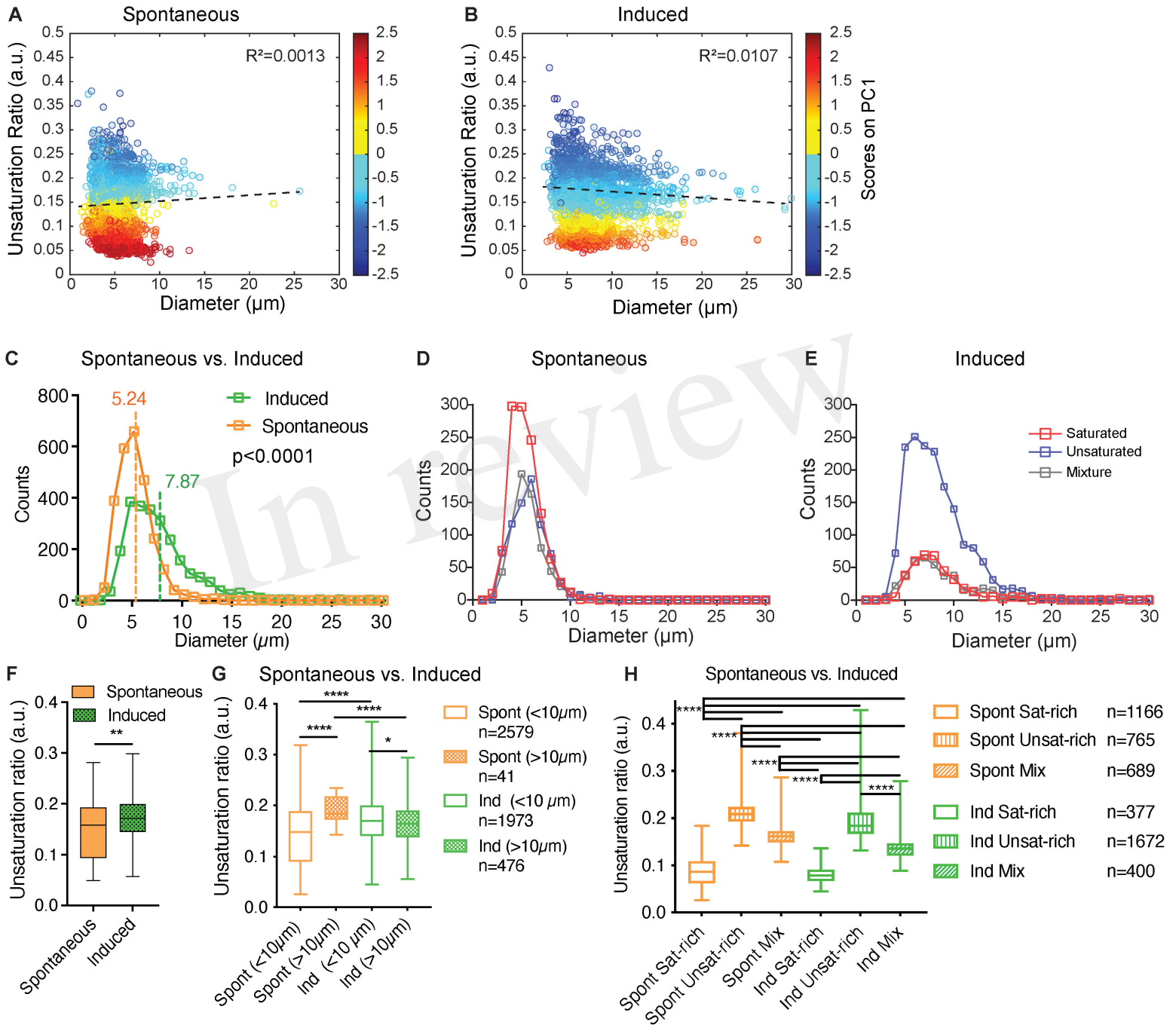
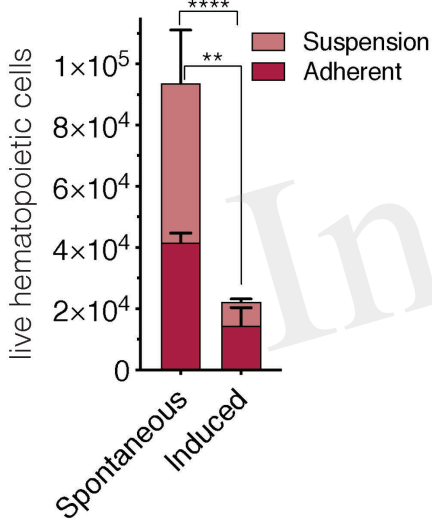
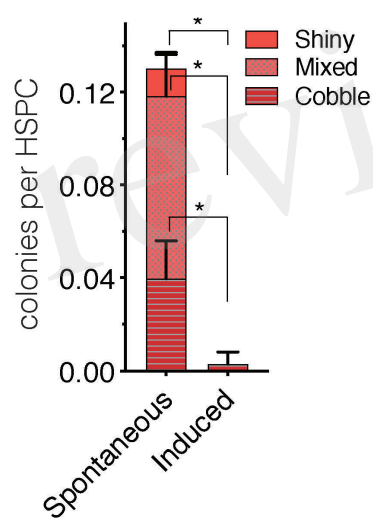


Figure 6.JPEG

A Hematopoietic expansion



B Colony Forming Cells



C Hematopoietic expansion

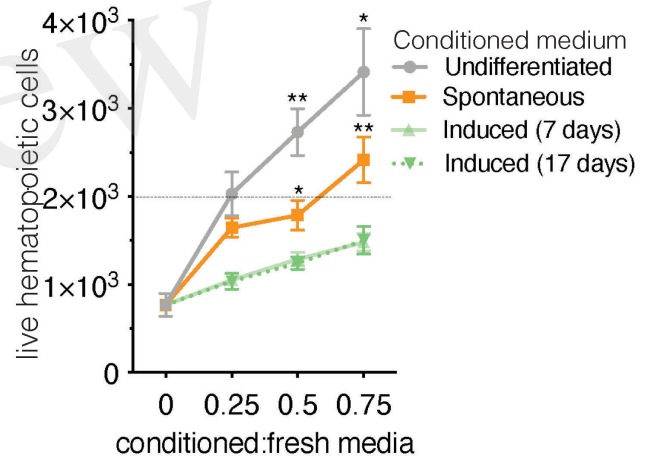


Figure 7.JPEG

