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► **To cite this version:**

Jérémy Dufau, Joanne X Shen, Morgane Couchet, Thais de Castro Barbosa, Niklas Mejhert, et al..
In vitro and ex vivo models of adipocytes. *American Journal of Physiology - Cell Physiology*, 2021,
320 (5), pp.C822 - C841. 10.1152/ajpcell.00519.2020 . hal-03866665

HAL Id: hal-03866665

<https://hal.science/hal-03866665>

Submitted on 23 Nov 2023

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1 Full Title: In vivo and ex vivo models of adipocytes

2 Running Title: Fat cell in a dish

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18

19 Keywords : adipocyte, fat cell, adipose tissue, cell culture, 2D and 3D

20

21

22 **Abstract**

23 Adipocytes are specialized cells with pleiotropic roles in physiology and pathology. Several types of fat cells
24 with distinct metabolic properties co-exist in various anatomically defined fat depots in mammals. White,
25 beige and brown adipocytes differ in their handling of lipids and thermogenic capacity promoting
26 differences in size and morphology. Moreover, adipocytes release lipids and proteins with paracrine and
27 endocrine functions. The intrinsic properties of adipocytes pose specific challenges in culture. Mature
28 adipocytes float in suspension culture due to high triacylglycerol content and are fragile. Moreover, a fully
29 differentiated state, notably acquirement of the unilocular lipid droplet of white adipocyte, has so far not
30 been reached in two-dimensional culture. Cultures of mouse and human differentiated preadipocyte cell
31 lines and primary cells have been established to mimic white, beige and brown adipocytes. Here, we survey
32 various models of differentiated preadipocyte cells and primary mature adipocyte survival describing main
33 characteristics, culture conditions, advantages and limitations. An important development is the advent of
34 three-dimensional culture, notably of adipose spheroids that recapitulate *in vivo* adipocyte function and
35 morphology in fat depots. Challenges for the future include isolation and culture of adipose-derived stem
36 cells from different anatomical location in animal models and humans differing in sex, age, fat mass and
37 pathophysiological conditions. Further understanding of fat cell physiology and dysfunction will be
38 achieved through genetic manipulation, notably CRISPR-mediated gene editing. Capturing adipocyte
39 heterogeneity at single cell level within a single fat depot will be key to understand diversities in
40 cardiometabolic parameters among lean and obese individuals.

41

42 **ABBREVIATIONS**

43 AT : adipose tissue

44 BAT : brown adipose tissue

45 C/EBP : CCAAT-enhancer-binding proteins

46 cAMP : cyclic adenosine monophosphate

47 CREB : cAMP-response element binding protein

48 CRISPR : clustered regularly interspaced short palindromic repeats

49 FOXO : forkhead box protein of class O

50 hADSC : human adipose derived stem cell

51 hMADS : human multipotent adipose derived stem cell

52 IBMX : 3-isobutyl-1-methylxanthine

53 LD : lipid droplet

54 MAAC : membrane mature adipocyte aggregate culture

55 MEF : mouse embryonic fibroblasts

56 PPAR : peroxisome proliferator-activated receptors

57 PRDM16 : PR domain containing 16

58 SGBS : Simpson-Golabi-Behmel syndrome

59 SVF : stromal vascular fraction

60 TNF- α : tumor necrosis factor alpha

61 UCP1 : uncoupling protein 1

62 WAT : white adipose tissue

63

64

65 INTRODUCTION

66 White adipose tissue (WAT) displays a remarkable capacity for undergoing changes in size and
67 morphology. The close relationship between WAT mass and common cardiometabolic disorders, such as
68 insulin resistance, type 2 diabetes and hypertension, have prompted intense research into the mechanisms
69 driving WAT plasticity and phenotype. In addition, WAT displays distinct cell heterogeneity. As an
70 example, the role of infiltrating immune cells in attenuating insulin sensitivity is well documented.
71 Nevertheless, adipocytes constitute almost 90% of the volume and up to 40% of the cells present in WAT
72 (146). Moreover, changes in adipocyte lipid storage (uptake and release) are believed to play a central role
73 in promoting ectopic lipid deposition in tissues like liver and skeletal muscle, which can ultimately lead to
74 lipotoxicity and insulin resistance in these tissues (24). Brown adipose tissue (BAT) is specialized in energy
75 dissipation as heat. Induction of BAT thermogenesis as well as transdifferentiation followed by activation of
76 white into brown-like (so-called, beige or brite for brown-in-white) adipocytes has raised interest as a
77 therapeutic strategy (146). Functional studies of adipocytes and their (dys)regulation under different
78 conditions are therefore of obvious interest for the understanding of systemic metabolic regulation.
79 However, studies of mature adipocytes are hampered by a number of technical hurdles, including high lipid
80 content of these cells that makes them float in suspension, their short life *ex vivo* span and the often harsh
81 methods that are required to isolate them, which may impact on their transcriptional and functional
82 fingerprint. In addition, understanding adipogenesis and the mechanisms that drive adipocyte differentiation
83 require a cell system that allows detailed temporal assessments and the ability to follow individual
84 cells/groups of cells (Figure 1). Finally, more mechanistic studies require approaches where gene expression
85 can be manipulated, which has proven very difficult for this cell type.

86 Consequently, many groups have aimed at developing renewable, cryopreservable cell sources of
87 adipocyte progenitors that would allow the study of relevant phenotypes of mature fat cells, i.e. uptake of
88 glucose/lipids, fatty acid esterification and oxidation, release of these fatty acids through lipolysis, effects of
89 insulin on glucose and lipid handling, and secretion of key adipokines such as leptin and adiponectin. This
90 review discusses the different models of adipocyte culture developed over the last decades. In addition to

91 widely used 2D-culture methods, we also discuss more recent advances in 3D-culture of adipocytes, which
92 can produce cells that are more similar to freshly isolated mature fat cells.

93

94 **Cell culture models for the study of white and brown adipocytes**

95 *Cell lines*

96 *Mouse cell lines.* Todaro and Green first reported cell isolation and culture from disaggregated 17 to
97 19 day-old Swiss mouse embryos in the early 1960's. They described several culture protocols leading to the
98 establishment of aneuploid fibroblast-like cell lines sensitive to contact inhibition (176). Different sublines
99 were generated, among which 3T3-L1 displayed an adipogenic potential (Table 1, Figure 2) (63). After an
100 intense proliferation phase leading to confluence, 10-50% of 3T3-L1 cells reached a resting state and started
101 accumulating lipids (Figure 1) (65). During subsequent years, investigations of the underlying mechanisms
102 of 3T3-L1 adipose conversion led to identification of highly pro-adipogenic compounds and their
103 transcription factor targets. An early observation was that adipogenesis could be stimulated by high insulin
104 concentrations (63). While this is mediated through pleiotropic mechanisms that are still not fully
105 understood, the adipogenic action of insulin is in part mediated by the transcription factor FOXO (60). It
106 was further shown that both 1-methyl-3-isobutylxanthine (IBMX), a phosphodiesterase inhibitor leading to
107 elevated intracellular cAMP levels (150), and dexamethasone, a glucocorticoid analogue (147), increase
108 adipose conversion of 3T3-L1 cells. Members of the C/EBP family were later characterized as adipogenic
109 transcription factors (23, 30) induced by cAMP-mediated CREB activation (215) and glucocorticoid
110 receptor stimulation (205). Upon induction of differentiation process, cells undergo about two rounds of
111 division. C/EBP β plays a crucial role during mitotic clonal expansion (168). The importance of mitotic
112 clonal expansion in human cell models remains debated. Thiazolidinedione effects on adipogenesis (91, 196,
113 213), along with the identification of PPAR γ as the predominant transcription factor of adipose conversion,
114 were also first described in 3T3-L1 cells (20). These breakthrough advances in the understanding of
115 adipogenesis were possible in 3T3-L1 cells, because they do not express the major adipogenic transcription
116 factors unless they are pharmacologically induced. In contrast, more committed 3T3 cell lines such as
117 F442A only require insulin for induction of adipogenesis (see below). Over the years, 3T3-L1 cells have

118 become the most commonly used preadipocyte model for the study of adipocyte development and biology
119 because of their ease of use, assay scalability and reproducibility in differentiation that can today reach
120 100% in optimal conditions (213). However, transfection of differentiated 3T3-L1 preadipocytes with
121 expression vectors often does not lead to robust expression of the corresponding proteins (149). Today, 3T3-
122 L1 cells are still extensively used to investigate the effects of molecules/compounds on adipogenesis (26),
123 inflammation (32) and metabolism (174). The function and importance of various proteins are studied
124 through gene silencing using siRNA (21, 106, 136). High-throughput approaches have been applied to 3T3-
125 L1 cells for screening of chemical compounds and siRNA (118, 170). Use of CRISPR-Cas9 mediated gene
126 editing has recently been reported (126). Differentiated 3T3-L1 preadipocytes express brown adipocyte
127 markers and increased mitochondrial respiration in response to catecholamines (117). While this has
128 prompted testing of the browning effect of various compounds (103, 125, 130), it should be kept in mind
129 that even upon β -adrenergic stimulation, the level of expression of the prototypical brown adipocyte marker
130 UCP1 is low (7). 3T3-L1 cells may not be considered as a bona fide brown adipocyte model.

131 Besides 3T3-L1, other sublines of 3T3 cells have been selected according to their capacity to
132 accumulate fat. Among them, Green and Kehinde described the 3T3-F442A clone obtained from a third
133 selection of clones as being more committed to the adipocyte lineage (64). The cells are differentiated
134 through mere addition of insulin into a serum-containing medium (40, 129). Despite a higher differentiation
135 potential, 3T3-F442A cells have been used to a much lesser extent than 3T3-L1 (149), primarily due to
136 initial lack of commercial availability. Nevertheless, 3T3-F442A cells helped characterizing the
137 transcriptional program of adipogenesis (151, 177), and are a useful tool when it comes to *in vitro* study of
138 pharmacological effects on adipocyte biology (25, 75, 137). The two 3T3 lines share common advantages
139 and limitations (Table 2).

140 The C3H/10T1/2 cell line was established during the early 1970's from C3H mouse embryos in a
141 similar way as 3T3 cells (139). Like 3T3 cells, C3H/10T1/2 cells are aneuploid and sensitive to post-
142 confluence arrest of proliferation. Using the demethylating agent 5-azacytidine after seeding, C3H/10T1/2
143 can differentiate into myocyte, adipocyte and chondrocyte cells which clones can be isolated (66, 95, 171).
144 Adipocyte differentiation protocols for C3H/10T1/2 cells have been refined over the years. Treatment with

145 bone morphogenic protein 4 during proliferation followed by treatment with the classical 3T3-L1 adipogenic
146 cocktail leads to more than 90% cells committed into the adipocyte lineage with high lipid accumulation and
147 adipose-specific gene expression (Table 1) (169). Of note, adipose conversion of C3H/10T1/2 cells is
148 strictly dependent on PPAR γ agonists (2). Besides study of adipogenesis, C3H/10T1/2 cells have been
149 useful for investigating brown adipocyte biology. Treatment of multipotent C3H/10T1/2 cells revealed the
150 importance of BMP7 in acquirement of a brown adipocyte phenotype (179). C3H/10T1/2 cells have also
151 been successfully used for siRNA-mediated gene silencing (29, 159).

152 Ob17 cells were derived through dedifferentiation of mature adipocytes (acquired through
153 collagenase digestion) obtained from the epididymal fat pad of leptin-deficient *ob/ob* mice (124). Upon
154 seeding, these cells gave rise to colonies, among which the Ob17 subclone was selected based on post-
155 confluence growth arrest and high adipogenic potential. Adipogenic conversion can be achieved without
156 transformation in both serum-containing or serum-free media supplemented with insulin after several
157 passages (53, 54, 67, 124). Ob17 cells have been used in the past to investigate the biology of adipocyte
158 lipoprotein lipase (4, 135, 191, 192). Similarly, the HGFu cell line has been isolated from epididymal fat pad
159 of lean wild type mice and these cells differentiate in a similar manner compared to Ob17 cells (22, 52).

160 BFC-1, a clonal aneuploid cell line established from the interscapular brown adipose tissue of the
161 C57 BL/6J mouse, was used to study β -adrenergic and insulin response (51). These cells differentiate into
162 adipocytes and do not express the uncoupling protein 1 UCP1 even upon β -adrenergic agonist treatment
163 (50). BFC-1 cells have been used for characterization of metabolic pathways in a few studies (133).

164 OP9 cells originate from mice deficient in macrophage colony stimulating factor (211). They were
165 derived from bone marrow cells isolated from new-born mouse calvaria and were cultured using 3T3
166 protocols, giving rise to different OP cell lines (Table 1). Among these, OP9 displayed the most pronounced
167 mesenchymal stem cell properties, including surface marker immunophenotype and multiple lineage
168 differentiation potential (57, 94). Following work on lymphohematopoietic differentiation (57, 121), the
169 adipogenic potential of OP9 cells was first described by the Wollins group (206). Unlike 3T3 and C3H cells,
170 induction of OP9 cells leading to expression of adipogenic genes and lipid accumulation is achieved in only
171 three days. A derived cell line from OP9, OP9-K, has been reported to be more robust in terms of

172 adipogenesis, easily imaged and useful for screening of molecules (99). Despite fast differentiation, OP9
173 cells keep adipogenic potential over time and passages even if propagated from confluent cells, unlike other
174 cell lines. The cells are easy to transfect with plasmids as well as with retroviruses and siRNAs (99, 206).
175 Using CRISPR-mediated genome editing, Bahrami-Nejad *et al.* recently generated single OP9 cell colonies
176 with tagged endogenous PPAR γ and C/EBP β proteins (10). While OP9 is one of the most recent
177 preadipocyte cell lines, it has been used over the past few years to investigate adipogenesis and crosstalks
178 between cell types in co-culture systems (16, 107, 208). Advantages and limitations are not yet fully
179 appreciated. Of note, OP9 cells need to be continuously cultured at high cell density to obtain optimal
180 differentiation. Moreover, differentiation protocols used for other preadipocyte cell lines lead to pronounced
181 fat accumulation, resulting in cell fragility that compromises cell manipulation, especially
182 immunocytochemistry (99, 149, 206).

183

184 *Human cell lines.* To our knowledge, PAZ6 is the first human preadipocyte cell line described in the
185 late 1990's (Figure 2) (220). PAZ6 are preadipocytes isolated from infant BAT transformed with a vector
186 containing the SV40 T and T-antigen coding genes. Once differentiated, PAZ6 adipocytes accumulate lipids
187 and express brown/beige markers such as UCP1 and β_3 -adrenergic receptors. PAZ6 cells have been used
188 both as white and brown preadipocyte models (83, 88, 221). Retrospectively, it appears that other models
189 better recapitulate white and brown adipocyte phenotypes (see below). Darimont *and coll.* described the
190 generation of the Chub-S7 cell line through transformation of human white subcutaneous preadipocytes with
191 papillomavirus E7 oncoprotein and human telomerase reverse transcriptase (34). An adipogenic capacity is
192 maintained across passages. Chub-S7 adipocytes display expression of adipogenic markers and classical
193 adipocyte metabolic pathway activities. Chub-S7 adipocytes have been used for studying cellular
194 metabolism (82), adipogenic differentiation and miRs regulation (1). Other human preadipocyte cell lines
195 were established through the transformation of human adipose derived stem cells (hADSC) from AT stromal
196 vascular fraction. In many attempts, immortalization was accompanied by a decrease in adipogenic
197 potential. Successful examples include the development of cell lines producing prolactin (81), retaining
198 depot-specific features (172), or the isolation of clones of brown and white preadipocytes allowing

199 characterization of molecular signatures and thermogenic potential (207). These immortalized human white
200 preadipocytes were recently genetically engineered through a CRISPR-derived system activating the UCP1
201 promoter. Transplantation of engineered precursor cells in mice led to improved metabolic phenotype (201).
202 Hansen *and coll.* isolated TERT-hBA and TERT-hWA cells upon immortalisation of brown and white
203 stromal-vascular cell from a single donor (111). TERT-hWA cells differentiate into adipocytes and are able
204 to convert into beige/brite adipocytes upon rosiglitazone treatment. These cells have not widely been used
205 yet.

206 Other cellular models of preadipocytes have been obtained from AT of patients with pathological
207 conditions without the need of transformation or immortalization. In the early 2000's, Wabitsch's group
208 described the development of two cell lines displaying adipogenic potential derived from patients with
209 cancer or rare genetic conditions. The first is the aneuploid LiSa-2 cell line, isolated from a poorly
210 differentiated pleomorphic liposarcoma. Following adipose conversion, LiSa-2 cells accumulate lipids and
211 exhibit adipose metabolic and gene expression features (200). While LiSa-2 cells have not been extensively
212 used as a preadipocyte model, it should be noted that transcriptomic analysis comparing isolated mature
213 adipocytes, LiSa-2 and PAZ6 cells revealed that LiSa-2 resemble primary white adipocytes more closely
214 than PAZ6 cells, but still display strong differences concerning lipid metabolism (186). An additional
215 preadipocyte line was derived from subcutaneous stromal-vascular fraction (SVF) of an infant with X-linked
216 Simpson-Golabi-Behmel syndrome (199). Interestingly, while the so-called SGBS cells do not display
217 karyotype aberrations even after several cell divisions and are not immortalized, they remain proliferative in
218 serum-containing medium for around 70 passages, likely due to point mutations and chromosomal
219 rearrangements in the *GPC3*, *GPC4* and *OFD1* genes, which encode proteins that control cell division and
220 growth (198, 210). Despite these chromosomal aberrations, SGBS have been widely used (49). Once
221 differentiated, SGBS adipocytes accumulate lipids and display classical adipocyte gene marker expression,
222 including *LPL*, *GPD1*, *SLC2A4* and *LEP*, and activities of metabolic pathways such as stimulated lipolysis
223 and glucose uptake (199). SGBS cells have been used in studies describing transcriptomic analysis of
224 adipogenesis (55), adipocyte metabolism (89) and inflammation (154). Recent work also highlights the
225 potential of SGBS cells for the study of adipocyte browning as the transcriptomic profile differ from that of

226 primary subcutaneous white adipocytes with UCP1 protein expression and metabolic signatures closer to
227 those of brown/beige adipocytes (210). Accentuated browning can be achieved through sustained PPAR γ
228 agonist or BMP7 treatment (93).

229 Recently, there has been a growing interest in the derivation of hADSCs that do not require cell
230 transformation. During the early 2000's, Rodriguez *et al.* described the isolation of human multipotent
231 adipose-derived stem (hMADS) cells obtained from the SVF of subcutaneous WAT from different infants
232 (144, 145). The different lines of hMADS cells display donor-specific differences. In the absence of
233 karyotype alterations, significant telomerase activity allows proliferation in serum-containing medium
234 supplemented with human fibroblast growth factor. It should however be noted that they will eventually
235 cease proliferating as they are not immortalized. hMADS cells can differentiate into adipocytes, osteoblasts
236 and chondrocytes. Adipose conversion leads to lipid accumulation in more than 90% of cells exhibiting key
237 adipocyte characteristics (144, 145). hMADS adipocytes have proven to be very useful tools for studying
238 adipocyte biology. Some examples are provided by studies of effects of miRNAs on adipogenesis (209), of
239 serum amyloid A on lipolysis and adipokine and inflammatory cytokine secretion (47), or the respective
240 importance of lipases in lipolysis through gene silencing, overexpression and pharmacological modulation
241 (15). Combining siRNA approaches with metabolic, lipidomic and molecular studies unveiled new pathways
242 in the control of human adipocyte insulin sensitivity (115). hMADS adipocytes acquire a beige adipocyte
243 phenotype upon sustained PPAR γ activation, resulting in strong UCP1 expression along with increased
244 expression of beige adipocyte markers and mitochondrial content (132). hMADS adipocytes therefore
245 facilitate studies of metabolic adaptations (11) and mitochondrial plasticity linked to beiging (131). hMADS
246 cells proliferate extensively without being transformed while maintaining a normal karyotype. As they
247 originate from healthy individuals, they may be considered as more physiologically relevant than SGBS
248 cells. Several groups have established equivalent hADSC models with similar characteristics and advantages
249 as hMADS cells (43). Importantly, cryopreserved hADSCs can be successfully established from adult
250 donors, although older donor age (>40-50 years of age) may negatively impact on the proliferative and
251 differentiation capacity of the cells. As further discussed below, it is a common experience that cell cultures

252 from subcutaneous WAT are relatively easily established, whereas cells from the visceral depot are much
253 less prone to differentiate in hADSC cultures.

254

255 *Primary cell culture*

256 *Mouse embryonic fibroblasts.* In addition to immortalized mouse embryonic fibroblast (MEF) lines,
257 such as the various 3T3 or C3H subclonal cell lines, primary MEF cells can also be used as preadipocyte
258 models (Table 1, Figure 2). These cells are generally subjected to the same differentiation protocols (167).
259 As MEFs represent a pool of diverse stem cells, only a small percentage of cells differentiate into adipocytes
260 (167). Moreover, earlier passages are used to ensure maximal adipose conversion. Primary MEFs isolated
261 from mice deficient for or overexpressing a gene of interest have been used to investigate the impact of
262 genetic modification on adipogenesis (48, 69). Modulation of gene expression can also be achieved *in vitro*
263 using siRNA or cDNA transfection (38, 112). Interestingly, similar to the cell types described above,
264 adipose conversion of MEFs leads to expression of classical brown adipocyte markers including PRDM16
265 and UCP1 (19). Functional aspects of adipocyte browning have not been thoroughly investigated in primary
266 MEFs.

267 *Rodent adipose tissue-derived progenitors.* In 1964, Rodbell first described the isolation of SVF
268 from rat AT (Table 1) (141). Once seeded, adipocyte conversion of progenitor cells present in the SVF can
269 be achieved in medium supplemented with fetal calf serum, glucose and insulin (190), and further enhanced
270 by addition of classical adipogenic compounds (17, 204). Of note, AT-derived SVF cells are able to
271 differentiate in serum-free media (35). Unlike preadipocyte cell lines, SVF cells proliferate not more than a
272 few passages and rapidly lose their adipogenic potential. However, primary preadipocyte cultures have seen
273 a renewed interest during the last few years as they better recapitulate *in vivo* adipocyte biology (72).
274 Preadipocyte adipogenic potential varies across depots being higher in subcutaneous than in perigonadal fat
275 in mice (110). Of interest, SVF progenitor cells can be obtained from various fat depots with preservation of
276 properties associated with anatomical origin (41, 73, 158). SVF can be prepared from rodents of different
277 ages (41, 87), fed different diets (157), and genetically (76, 160) or hormonally modified (98). Preadipocytes
278 can also be isolated and cultured from BAT. The cells differentiate into adipocytes displaying brown

279 adipocyte features when exposed to serum-containing media supplemented with insulin, IBMX,
280 dexamethasone, T3 and rosiglitazone (122, 123). Primary brown SVF cells have helped elucidate several
281 aspects of brown adipocyte development and biology (27, 86, 113, 153).

282 *Human adipose tissue-derived progenitors.* Similar to isolation from murine AT, adipocyte
283 progenitors can be routinely obtained from the SVF of human AT (Table 1). Pioneering work in the late
284 1980's characterized serum-free cell culture media that induced differentiation into adipocytes (71). These
285 protocols have subsequently been optimized by altering the composition, concentration and timing of pro-
286 adipogenic agents (188). Usually, SVF cells are plated in a medium containing fetal calf serum for the first
287 24 hrs. This enables the cells to rest and undergo one round of replication. The following day, cells are
288 washed and incubated in a chemically defined medium supplemented with a cocktail of pro-adipogenic
289 factors including insulin, T3, glucocorticoids, IBMX and PPAR γ agonists. This cell system has been used by
290 a large number of groups and has enabled detailed functional studies, for example the respective
291 contribution of PPAR α and PPAR γ to adipocyte lipid and glucose metabolism through pharmacological
292 modulation (140), as well as gene knock down on adipogenesis (105) or over expression on browning
293 capacity (175). As discussed below, the fact that serum can be omitted in the differentiation medium of
294 human adipocyte progenitors is a clear difference to cultures of murine preadipocytes. Nevertheless, similar
295 to other primary cell systems, an important caveat is the inter-individual differences between cells from
296 different donors. For example, it is commonly observed that cells from older donors (37, 152) or donors with
297 higher body mass index (189) display an attenuated differentiation capacity. Moreover, SVF from
298 subcutaneous and visceral (greater omentum) depots display qualitative differences in differentiation
299 capacity (152, 187). This implies that studies on *in vitro* differentiated primary adipocytes often require SVF
300 obtained from several donors in order to provide reproducible results. It should be noted that subcutaneous
301 WAT differentiated preadipocytes can be converted into brown-like adipocytes upon activation of PPAR γ
302 (97). Genome editing using CRISPR-Cas9 has been achieved in primary human preadipocytes leading to
303 robust downregulation of target genes (85).

304

305 *Common features and limitations of 2D adipocyte culture models*

306 A noticeable redundancy exists between all preadipocytes models concerning both advantages and
307 limitations. The Table 2 is an overview of such characteristics that may help in apprehending the interest and
308 limit of each model.

309 *Species differences in composition of differentiation medium.* As 3T3-L1 cells provided a framework
310 for the molecular mechanisms of adipogenesis, use of their specific pharmacological activators became the
311 gold standard of adipogenesis induction *in vitro*. As described in Table 1, the composition of pro-adipogenic
312 media is similar for most of the cell types. However, some peculiarities and species-specific features can be
313 observed. Briefly, the use of a cocktail containing insulin, dexamethasone and IBMX is broadly used in the
314 various cell models except for 3T3-F442A and LiSa-2 cells. While the requirement of PPAR γ agonists is
315 mandatory for optimal differentiation of human preadipocyte models, it is usually optional for rodent cells
316 with the exception of MEF and C3H/10T1/2 cells. This may be due to the ability of rodent cells to produce
317 endogenous PPAR γ ligands during adipose conversion in contrast to human cells (184). *In vivo*, PPAR γ
318 ligands have recently been suggested to be produced by endothelial cells of human WAT (61, 68). Another
319 major point is supplementation with serum. It was shown earlier that human primary cells and human cell
320 lines engage in adipose conversion using chemically-defined media (36, 71). On the other hand, mouse cells
321 require the use of serum, except for OP9 cell line differentiated in serum-free medium with high
322 concentrations of the hormonal cocktail compounds. Finally, the use of T3 is important for culturing human
323 cells, whereas it is often dispensable in mouse cell cultures as serum is present.

324 *Intrinsic cellular characteristics of 2D preadipocyte models.* *In vitro* differentiation of primary and
325 cell line-derived preadipocytes leads to lipid accumulation in small multilocular lipid droplets (LD),
326 reflecting the immaturity of such white adipocytes compared to the mature white adipocytes observed *in*
327 *vivo*, which contain a large unilocular LD. *In vitro* investigations of LD dynamics in 2D cultures remain
328 therefore limited and while recent advances have allowed better understanding of LD biogenesis (128), the
329 mechanisms controlling LD size remain elusive. It seems that components of the volume-regulated anion
330 current play an important role in the ability of adipocytes to expand, that is mainly linked to both insulin and
331 fibroblast growth factor signalling (202, 217, 218). The large variation in adipocyte unilocular LD size under
332 various pathophysiological conditions likely involves other molecular mechanisms.

333 While primary preadipocytes represent the most physiological model of adipose precursors, the cells
334 lose their adipogenic potential after a few rounds of division. Cell lines often display aneuploidy and
335 karyotype aberrations (Table 2). Specific consequences of aneuploidy on preadipocytes and adipocyte cell
336 biology are poorly documented. The general cellular consequences of aneuploidy have recently been
337 reviewed (31). Briefly, despite conferring a proliferative behaviour in embryonic mouse cells, aneuploidy is
338 associated with increased mRNA and protein expression due to increased chromosome copies that can result
339 in aberrant induction of signalling pathways, proteotoxic stress with increased autophagy and lysosomal
340 activity resulting from accumulation of misfolded proteins, and genomic instability that can further induce
341 DNA damage.

342 *Commercial availability and use.* The mouse preadipocyte cell lines described herein are
343 commercially available. Among them, 3T3-L1 is today the most widely used. The use of human cell lines is
344 much more limited and restricted to a few groups because they are not commercially available and usually
345 have to be provided under specific MTAs. As an example, Pubmed data from year 2015 to 2020 including
346 “3T3-L1” in title search gives around 3000 publications, while “3T3-F442A” and “SGBS” give 35 and 76,
347 respectively.

348

349 **Primary mature adipocyte survival**

350 To circumvent the limitations of 2D cultures, methods have been developed to directly investigate
351 mature adipocytes.

352

353 *Isolated mature adipocytes*

354 When Rodbell set up the method for mature adipocyte isolation through collagenase digestion, he
355 could describe novel aspects of fat cell glucose and lipid metabolism and their hormonal regulation (141-
356 143). Isolated adipocytes have since extensively been used to investigate fat cell metabolism. However,
357 isolated adipocytes are fragile and survive only a few hours (141), thus various attempts have been made to
358 develop protocols to maintain mature adipocytes in culture for longer periods.

359

360 *Scaffold-embedded adipocytes*

361 Because adipocyte survival is compromised *in vitro* due to their buoyancy in the liquid phase,
362 multiple methods embed mature adipocytes in a semi-solid matrix. Sugihara *et al.* were the first to describe
363 the maintenance of freshly isolated mature human and rat adipocytes in a collagen 3D-scaffold (164). In this
364 setup, the adipocytes could be maintained in culture for several days with preserved catecholamine-induced
365 lipolysis. However, cells rapidly showed signs of dedifferentiation, acquiring a fibroblast-like morphology
366 containing multilocular LD and re-entering the cell cycle. Moreover, collagen-derived matrices are semi-
367 liquid at 37°C, impairing proper cell embedding (78). A few years later, embedding of mature adipocytes in
368 Matrigel was reported to allow the maintenance of cells for at least 6 days, during which they retained a
369 unilocular LD as well as adipose marker protein expression and gene induction by insulin (74). More
370 recently, Hong *et al.* used a hyaluronan-based hydrogel to investigate the impact of hypoxia on murine and
371 human mature adipocytes. A small proportion of the adipocytes embedded in this hydrogel re-entered the
372 cell cycle and started to proliferate but most of them retained a unilocular shape for several days (78). The
373 proliferation observed might be due to dedifferentiation or to contaminating fibroblasts that remain attached
374 to adipocytes during cell isolation. Another major issue is the poor transduction of mature adipocytes with
375 lentiviral vectors (79). Altogether, only a few studies have been performed using adipocytes in 3D-matrix
376 cultures.

377

378 *Ceiling cultures*

379 For ceiling cultures, isolated mature adipocytes are placed in a classical plastic culture flask
380 completely filled in with culture medium supplemented with fetal calf serum to avoid cell-air interface
381 (Figure 3, (163)). Because of their buoyancy, floating mature adipocytes accumulate on the upper side of the
382 flask and eventually firmly adhere to it, allowing the flask to be turned upside down for inspection and
383 imaging of cell morphology. The adipocytes respond to insulin with increased lipogenic activity resulting in
384 increased LD size, or to norepinephrine with increased lipolysis-mediated glycerol release. Although
385 adipocytes survive in ceiling culture for several days, they display progressive changes towards fibroblastic
386 morphologies with multilocular LD due to dedifferentiation (163). In the early 2000's, Zhang *et al.* revisited

387 the ceiling culture system with small numbers of freshly isolated mature adipocytes cultured in six-well
388 plates covered with glass coverslips (Figure 3). Once adipocytes adhered to the coverslip, they survived for
389 10 to 14 days with preserved metabolic activity and no sign of dedifferentiation (214). Use of glass
390 coverslips facilitates imaging procedures such as confocal microscopy. However, mature adipocyte ceiling
391 cultures have not been extensively used. During the last few years, they have gained renewed interest in
392 research on cell therapy and tissue engineering, as mature adipocytes eventually de-differentiate into
393 precursors cells that retain the ability to undergo adipogenesis (6, 96) and display pluripotency markers
394 along with stem cell multiple lineage potential (84).

395

396 *Membrane mature adipocyte aggregate culture (MAAC)*

397 Recently, Harms *et al.* described a variant of the ceiling culture in which a small amount of human
398 mature adipocytes aggregate under permeable small pore transwell membranes (Figure 3, (70)). The authors
399 showed that cells retained mature adipocyte gene expression signature, functional response and fat depot-
400 specific difference. Dedifferentiation is moderate. Culturing under transwells facilitates the establishment of
401 co-culture systems. As a proof of concept, the authors co-cultured human MAAC and murine RAW264
402 macrophages with synergistic response to lipopolysaccharides. Similar to hADSC adipocytes and human
403 differentiated preadipocytes in primary culture, MAAC cells are efficiently transduced with adenoviruses.
404 Long-term treatments with rosiglitazone and insulin leads to the induction of a brown-like phenotype with
405 increased expression of UCPI. MAAC have also been established from mouse WAT.

406

407 *Adipose tissue explant cultures*

408 The first AT explant culture is attributed to Smith's work during the early 1970's (161, 162).
409 Explants are small pieces of WAT containing AT-resident cell types that can be maintained in culture for
410 several weeks between two coverslips. Whereas adipocyte spherical morphology in AT explant did not
411 change when maintained in the absence of serum, serum addition to the medium resulted in proliferation of
412 fibroblast-like cells and decreased adipocyte size, particularly of the most hypertrophic cells. Collagenase
413 digestion of AT explants yielded adipocytes containing large unilocular LD, but whose diameter was smaller

414 than freshly isolated adipocytes. Cell size directly measured on explants revealed that the decrease in cell
415 size was higher when cells were incubated with serum. Interestingly, even after 2 weeks of culture, AT
416 explants remain metabolically active (161, 162).

417 AT explants have been widely used to investigate the lipolytic response of AT under various conditions and
418 the impact of this lipolysis on stromal cells contained in the explant (8, 56). They also represent an
419 interesting model to investigate AT inflammation as well as angiogenesis (58), and have been used to
420 investigate adipokines and cytokine secretion in response to pharmacological treatments (116). While the
421 explant model of mature adipocyte maintenance seems to be a useful tool to investigate adipocyte biology,
422 explant culture is also associated with changes in adipocyte phenotype, such as increased expression of pro-
423 inflammatory factors, resulting in altered pattern of adipocyte marker gene expression and substantial
424 dedifferentiation (59). Recently, Huang *et al.* described mouse BAT explant culture for 24h to investigate
425 signalling pathways (80).

426

427 *Sandwiched WAT*

428 A new kind of AT explant method has recently been reported; a small human AT fragment being
429 enclosed between two monolayers of adipose stromal cells (100). The so called sandwiched WAT remains
430 viable for 7 weeks. Mature adipocytes can be released from the scaffold through collagenase digestion.
431 Sandwiched WAT has maintained secretory activity such as leptin release. Following decrease during the
432 first day of culture compared with native tissue, expression of adipose marker gene expression is maintained
433 thereafter for several weeks. Catecholamine and insulin stimulation lead to comparable lipolysis and
434 glycolysis as in freshly harvested WAT during at least 5 days. Sandwiched WAT can be re-implanted *in vivo*
435 in mice with viable adipocytes of normal size after 10 days. This model offers an interesting new tool to
436 investigate mid-to-long term mature adipocyte culture, as the sandwiched WAT adipocytes do not de-
437 differentiate into fibroblast-like cells for at least four weeks. The procedure is time consuming and not easy
438 to set up.

439

440 *Limitations and progress*

441 While various attempts have been made to allow middle and long-term maintenance of primary
442 mature adipocytes *in vitro*, each approach leads to changes in adipocyte characteristics, notably cell
443 morphology and gene expression pattern. Mature adipocyte buoyancy remains a constraint. Constant need
444 for fresh tissue is another important limitation, especially for human WAT. It must be kept in mind that
445 isolation of mature fat cells by collagenase digestion is *per se* responsible of phenotypic changes such as
446 decrease in adipocyte marker gene expression and induction of pro-inflammatory factors. The detrimental
447 effect of collagenase digestion on inflammatory response can however be mitigated through optimization of
448 digestion conditions (173).

449

450 **Advent of spheroid models**

451 Spheroid cultures have been developed for differentiation of progenitor cells in 3D as a mean to get closer
452 to mature cells in native tissues. Before describing the recent development in adipose spheroid culture, a
453 rapid survey of progress for other cell types provides essential features of this approach.

454 The context and microenvironment of a cell play essential roles in the control of cellular decision-
455 making. Cell-cell and cell-matrix interactions shape molecular phenotypes and regulate cell morphology (5),
456 migration (42), metabolism (165) and proliferation (108). In conventional 2D monolayer cultures, these
457 interactions are grossly perturbed. To more closely recapitulate the cellular microenvironment, 3D cell
458 cultures were developed, with the first seminal publications reporting carcinoma cell culture in spheroidal
459 aggregates published almost 50 years ago (166). Not long after, such spheroid cultures were also established
460 for the culture of primary cells. Specifically, the authors noted that 3D cultures of murine mammary
461 epithelium were phenotypically stable and more closely resembled their *in situ* tissue counterparts than
462 conventional 2D cultures (44). Despite these early successes, the use of 3D methods for the culture of
463 primary human cells has lagged considerably behind and only received widespread attention in the last
464 decade.

465 By now, a multitude of methodologically diverse 3D culture protocols have been developed that
466 support the maintenance of primary human cells, including spheroid cultures, hollow-fibre bioreactors and
467 microfluidic organ-on-a-chip systems (46, 101, 102, 138, 155, 185, 212, 219). Spheroids arguably constitute

468 the most extensively studied 3D culture method for mature human cells. Single-cell suspensions with
469 defined cell concentrations are seeded into ultra-low attachment plates or hanging drops, in the presence or
470 absence of extracellular scaffolds, such as matrigel, alginate or collagens. Over the course of hours to days,
471 depending on the cell type, the cells in suspension aggregate into spheroidal structures with highly
472 homogenous and well-defined sizes, typically in the range of 200-400 μm in diameter.

473 Spheroid cultures have been used extensively for the culture of human hepatocytes, cardiomyocytes
474 and neuronal cells. Spheroid cultures of primary human hepatocytes retained their transcriptomic (13, 127),
475 proteomic (14, 127) and metabolomic signatures (197) for multiple weeks, whereas 2D monolayer or
476 sandwich cultures from the same donors rapidly deteriorated. Spheroid cultures similarly extended the
477 functionality and lifespan of human stem cell-derived cardiomyocytes (12, 134) and of various neuronal cell
478 types (28, 39). Whereas early 3D cultures were mostly established from single cell types, more advanced
479 models have been developed that allow the co-culture of multiple tissue-resident cell types, including
480 immune cells, endothelial cells or pericytes.

481

482 **Spheroids of adipocyte progenitors**

483 Given the encouraging results on morphology, molecular phenotype and functional characteristics
484 observed with spheroid culture in various tissues, similar approaches have recently been established for the
485 differentiation of adipocyte progenitor cells in 3D. Adipose spheroid culture may address some of the
486 limitations observed with primary adipocyte survival.

487

488 *3T3-L1- and 3T3-F442A-based spheroid cultures*

489 Turner *and coll.* described one of the first 3T3-L1 spheroid culture generated by seeding cells on
490 plates coated with positively charged elastin-like polypeptide-polyethyleneimine conjugates (182).
491 However, attachment forces were mild, resulting in spheroids of heterogeneous sizes. Compared to 2D
492 cultures, these spheroids displayed increased triglyceride content and higher expression of *Pparg*, *Adipoq*,
493 *Cd36* and *Cd40* (180, 183). Spheroids also exhibited more robust effect of TNF- α on lipolysis compared
494 with monolayer cultures. Other spheroid cultures of adipocyte-like cell lines use a variety of different

495 scaffolds. Spheroid cultures of 3T3-L1 and 3T3-F442A cells in a cross-linked hyaluronic acid scaffold
496 featuring key adipose extracellular matrix components (collagens I and VI and the cell binding domain of
497 fibronectin) in microwell plates displayed greater lipid accumulation than those in 2D monolayer cultures
498 (109). Furthermore, stimulation with isoprenaline or caffeine resulted in an increase in lipolysis in 3D
499 differentiated 3T3-F442A cultures, with 3T3-F442A cells featuring more pronounced adipocyte features
500 than 3T3-L1 (148). Alternatively, 3T3-L1 spheroids can be generated using cellular bioprinting of cell-
501 hydrogel droplets in an oil mixture before gelation and subsequent transfer to low cell attachment microwell
502 plates (62). However, although 3D cultures in this setup exhibited higher expression of *Pparg*, *Fabp4*,
503 *Adipoq*, *Fasn* and *Plin1* compared to monolayer culture, spheroids displayed multilocular cells. Normalized
504 lipid levels, as well as leptin and adiponectin secretion, were higher in 2D cultures.

505 Interestingly, culture of 3T3-L1 cells suspended in agarose promoted differentiation without the need
506 for additional chemical adipogenic induction (9). In these cultures, a compact spheroid is not formed and
507 cells remain only loosely associated. The authors reported LD formation as early as after 24 hours of culture,
508 while prolonged culture with fetal bovine serum for 2 weeks resulted in the greatest lipid accumulation.
509 While this method allows for single cell observation and analysis, lipid accumulation remains limited.
510 Medium exchange and cell extraction complicate the approach.

511

512 *Human adipose-derived stem cell-based 3D cultures*

513 One of the first 3D reconstructions of AT included the encapsulation of hADSCs in poly(ethylene
514 glycol) diacrylate hydrogel (3, 193). These tissue constructs accumulated lipids, secreted adipokines and
515 were susceptible to β -adrenergic receptor-stimulated lipolysis. Verseijden *et al.* compared adipogenesis of
516 hADSCs in scaffold-free spheroids and fibrin-based constructs and found that fibrin-based constructs was
517 required for lipid accumulation and significant increase in *PPARG*, *PLIN1*, *FABP4* or *LEP* expression (194).
518 Notably, co-culture of 3D hADSC spheroids with human umbilical vein endothelial cells allowed for the
519 formation of vascular structures while adipogenic differentiation was induced (195). Implantation of the
520 spheroids in mice resulted in increased vasculogenesis within the constructs, suggesting interaction with the
521 host vascular system.

522 Other culture approaches used for the formation of hADSC-derived adipose spheroids include
523 hanging drops (120), ultra-low attachment plates (203), rotating bioreactors (120), bioprinting of
524 methacrylated gelatin ink-encapsulated spheroids (33), culture on elastin-like polypeptide-
525 polyethyleneimine surfaces (181) and hydrogels within well plates (178, 216). Spheroids can also be formed
526 from UET-13 cells which are bone marrow stromal cells with prolonged lifespan through retroviral
527 transduction using plates featuring microfabricated honeycomb arrangements (114). Furthermore, a recent
528 study generated hADSC spheroids using a liquid overlay technique by coating 96-well plates with agarose,
529 followed by cell culture in growth medium on an orbital shaker (77). While lipid accumulation was not
530 extensive, the authors reported the formation of an extracellular microenvironment containing collagens,
531 laminin and low fibronectin levels, which resembled native AT. The use of hADSCs may be beneficial for
532 vascularization and *in vivo* transplantation due to their ability to secrete angiogenic factors (194).

533

534 *Stromal vascular fraction-based 3D cultures*

535 To our knowledge, the first attempt of 3D culture of mouse adipose SVF was successfully achieved
536 in a collagen hydrogel (45). In this study, an increased adipogenic differentiation of subcutaneous AT-
537 derived progenitors compared with 2D cultures was observed, as indicated by higher expression levels of
538 adipocyte specific genes and unilocular LD formation. Even more interestingly, the study described similar
539 differentiation levels between subcutaneous and visceral progenitor in 3D culture, as shown by similar levels
540 of expression of *Adipoq*, *Fabp4* and *Pparg* and other metabolic genes, similar lipid accumulation and
541 unilocular LD formation. While both subcutaneous and visceral 3D cultures displayed inducible lipolysis
542 and thermogenesis when stimulated with the beta-adrenergic agonist isoproterenol, they retained depot-
543 specific features including distinct transcriptional profiles, but also higher thermogenic gene expression in
544 subcutaneous as well as higher cytokine expression and secretion in visceral AT-derived progenitor cultures.

545 Recently, human and mouse SVF-derived adipose spheroids were generated using scaffold-free
546 hanging drops before transfer to ultra-low attachment plates (92). A noticeable advantage of these primary
547 human SVF-derived spheroids is their scalability, large number of unilocular LD and relevant molecular
548 phenotype after 31 days of differentiation. Spheroids were also sensitive to environmental toxins, exhibiting

549 fewer and smaller LD, lower adiponectin secretion and higher IL-8 levels. However, expression of key
550 adipocyte markers in the spheroids were less than 2-fold higher compared to 2D cultures, suggesting the
551 need for further optimization of the protocols. A schematic overview of the main approaches to generate
552 adipose spheroids is given in Figure 4.

553 Co-culture of preadipocyte spheroids with the corresponding endothelial cells from the same SVF
554 allowed to recapitulate aspects of *in vivo* vascularization (119). Constructs were formed using ultra-low
555 attachment surfaces and stirring of cultures allowed for nutrient diffusion and sprouting before cells were
556 incorporated into Matrigel droplets. Subsequently, to maintain endothelial cells, differentiation was
557 performed using a medium omitting the typical pro-adipogenic differentiation medium components (IBMX
558 and rosiglitazone) and indomethacin, striking a balance between sufficient adipogenic induction and
559 endothelial cell preservation. Moreover, fatty acids in the differentiation medium facilitated lipid loading.
560 Consequently, endothelial cells self-assembled into vascular networks featuring tubular structures and
561 transplantation of the vascularized adipose constructs in mice resulted in vessels containing both human and
562 mouse endothelial cells. Although multilocular adipocytes were still present, several unilocular cells were
563 observable in the cultures, in which early and late adipocyte differentiation markers were upregulated after
564 10 days of differentiation.

565 Co-culture of adipocyte spheroids have also been generated from perinephric AT-derived SVF to
566 recapitulate the perinephric adipose microenvironment using liquid overlay-based culture (156). Ultimately,
567 co-culture with the adipocyte spheroids could shift unpolarized macrophages to acquire an alternatively
568 activated M2 phenotype. The heterogeneity of the SVF allows the possibility of incorporating different cell
569 types in addition to preadipocytes into the adipose model, more closely mimicking the tissue *in vivo*.
570 Another advantage of SVF is that it is more easily acquired than hADSCs (18).

571

572 *Main advantages and limitations*

573 Spheroid culture systems of adipose progenitor cells reveal striking advantages over conventional 2D
574 culture approaches. 3D cultured cells exhibit higher differentiation levels, as indicated by increased
575 adipocyte gene expression and also unilocular LD formation, which cannot be achieved in 2D cultures

576 (Figure 4). 3D cultures therefore recapitulate much more closely the adipocyte function and morphology
577 observed *in vivo*. Another interesting aspect is the ability to efficiently differentiate visceral adipose
578 progenitors, which is only poorly achieved in 2D culture. The preservation of depot-specific features is
579 promising but has so far only been shown for murine cells. Finally, the possibility of adding other cell types
580 like endothelial cells, which can provide vascular structures, enables advances beyond adipocyte
581 monocultures towards complex AT organoids. However, 3D culture is technically more challenging and
582 time consuming compared to 2D cultures, as the individual aggregates can be easily damaged and require
583 the use of expensive plates or scaffolds.

584

585 **Conclusions and perspectives: modelling dysfunction fat cell**

586 In mammals, white adipocytes ensure safe and efficient storage of triacylglycerols and their
587 mobilization via fatty acids. Lipid turnover in white adipocytes confers to AT an essential role in whole-
588 body metabolic homeostasis. Fat cells are also recognized as important producers of paracrine and endocrine
589 factors. The endocrine function of AT is considered as an essential link between increased fat mass and the
590 development of insulin resistance, and white adipocyte dysfunction constitutes a cardinal event in the
591 development of insulin resistance and associated disorders. Key to the recognition of these essential roles
592 has been the development of appropriate cellular models. Major methodological progress has been achieved
593 since the pioneering development of the 3T3-L1 mouse preadipocyte cell line. The main challenges today
594 include the establishment of better protocols to isolate proliferative and cryopreservable hADSCs from
595 donors of different sex, age, body mass index and pathophysiological conditions. Such efforts will help
596 defining the intrinsic and inter-individual diversity at the cellular level and how it links to clinical
597 phenotypes and may also reveal which properties are retained after multiple passages, e.g. through
598 epigenetic imprinting.

599 A major challenge remains to establish hADSCs from the visceral fat depot; these attempts have not
600 been fully satisfactory so far. Whether this is due to intrinsic differences of visceral compared to
601 subcutaneous hADSCs or to environmental cues (e.g. the influence of neighbouring immune and endothelial
602 cells, which are more abundant in visceral than subcutaneous WAT) remains unknown. Moreover,

603 functional investigation of adipocytes from smaller depots such as bone marrow, perivascular, mammary,
604 epicardial, joint, dermal, retro-orbital and plantar WAT are needed. ADSCs from other depots will help
605 understanding adipocyte heterogeneity in relation to anatomical and developmental origins and may possibly
606 uncover new disease mechanisms. Another layer of heterogeneity is provided by the recent recognition of
607 diversity among adipocytes within a fat depot. Several populations of white and beige adipocytes with
608 unique metabolic and endocrine properties and differential response to exogenous stimuli have been
609 identified within a fat depot (104). This challenge will be addressed by further developments in 2D and 3D
610 cultures of ADSCs and primary mature adipocytes possibly combined with novel single cell approaches.
611 Addressing the roles of proteins and cognate pathways will be achieved through the large number of novel
612 gene editing tools that can be used in vivo to produce mouse transgenic lines and derive ex vivo adipose
613 culture models and in vitro to generate genetically engineered hADSCs. A better understanding of adipocyte
614 biology may allow refined patient stratification and help identify individuals who could benefit the most
615 from existing therapeutic approaches. Moreover, it may enable the development of future adipocyte-targeted
616 strategies for the treatment of metabolic diseases.

617 **Acknowledgments**

618 This work was produced within the framework of the European Research Council (ERC) Synergy
619 Grant SPHERES (agreement No. 856404) under the European Union’s Horizon 2020 research and
620 innovation programme (erc-spheres.univ-tlse3.fr). D.L. is supported by Inserm, Paul Sabatier University,
621 Fondation pour la Recherche Médicale (DEQ20170336720), Agence Nationale de la Recherche (ANR-17-
622 CE14-0015Hepadialogue), Région Occitanie (DIALOGUE projects), FORCE/F-CRIN and AstraZeneca
623 France. D.L. is a member of Institut Universitaire de France.

624

625

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627

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224 **Table 1 Components frequently found in adipocyte culture media**

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	Cell model	Serum	Insulin	IBMX	Dexamethasone	PPAR γ agonist	Transferrin	T3	Fetuin	Cortisol
Mouse	3T3L1	+	+(63)	+(150)	+(147)	+/- (91, 196, 213)	-	-	-	-
	3T3F442A (40)	+	+	-	-	-	-	-	-	-
	C3H/10T1/2 (169)	+	+	+	+	+(2)	-	-	-	-
	OP9 (206)	+/-	+	+	+	-	-	-	-	-
	MEF (167)	+	+	+	+	+(2)	-	-	-	-
	SVF (90)	+	+	+	+	+	-	-	-	-
Human	PAZ6 (220)	-	+	+	+	+	-	+	-	-
	Chub-S7 (34)	-	+	+	+	+/-	+	+	+	-
	LiSa-2 (200)	-	+	-	-	-	-	+	-	+
	SGBS (199)	-	+	+	+	+	+(49)	+	-	+
	hMADS (144, 145)	-	+	+	+	+	+	+	-	-

227 IBMX, 3-isobutyl-1-methylxanthine; hMADS, human Multipotent Adipose-Derived Stem; hADSC, human
 228 adipose-derived stem cells; MEF, mouse embryonic fibroblasts; SGBS, Simpson-Golabi-Behmel syndrome;
 229 SVF, stromal-vascular fraction; T3, triiodothyronine;

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238 **Table 2 Advantages and limitations of adipocyte cell models**

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			Main advantages			Main limitations						
			Chromosomal status	Differentiation medium	General features	Chromosomal status	Differentiation medium	General features				
Mouse	Cell lines	3T3-L1			Precisely established protocol	Aneuploidy	Serum-supplemented media	Transfection issues				
		3T3-F442A		Insulin only	High differentiation potential							
		C3H/10T1/2			Browning potential							
		OP9	Unknown		Fast differentiation, CRISPR-Cas9 gene editing	Unknown		Limited hindsight				
	Primary cells	MEF	Euploidy	Fully-defined media	Large amount of cells	Use of knock-out mouse models, Recapitulate <i>in vivo</i> features (SVF)	Unknown	Immortalization procedure and its consequences				
		SVF										
Human	Cell lines	Immortalized cell lines	Unknown						Fully-defined media	Large amount of cells	Unknown	AT availability, numerous models with poor cross-comparison
		Cells derived from pathological conditions										
		Derived hADSC	Euploidy									
	Primary cells	hADSC							Better recapitulate <i>in vivo</i> features	Chromosomal rearrangement and mutations		

241 AT, adipose tissue; MEF, mouse embryonic fibroblasts; hADSC, human adipose-derived stem cells; SVF,

242

243

244 **Figure legends**

245

246

247 **Figure 1 : Schematic overview of adipogenesis.**

248 White adipose tissue is composed of mature adipocytes and cells from the stroma vascular fraction including
249 immune, endothelial and neural cells, as well as adipocyte precursors named adipose-derived stem cells. De
250 novo formation of adipocytes through adipogenesis occurs mainly during adipose tissue development. It
251 may take place during adulthood allowing adipocyte turnover and adipose tissue expansion in response to
252 overfeeding. Upon adipogenic stimuli, that partly remain elusive *in vivo*, adipose stem cells are committed to
253 preadipocytes. Once committed, preadipocyte progressively acquire an adipocyte specific gene expression
254 pattern along with lipid droplet (LD) formation. Eventually, multilocular LD fuse to form a large and unique
255 LD that characterize mature white adipocytes.

256

257 **Figure 2 : Origin of preadipocyte models for cell culture.**

258 **A :** Mouse 3T3 immortalization scheme. The vast majority of rodent preadipocyte cell lines has been
259 obtained from mouse embryos or pups. Disaggregation of mouse embryos allowed the obtention of mouse
260 embryonic fibroblasts that are used as preadipocyte models in primary culture or were immortalized to
261 obtain 3T3 subclones (Swiss mouse strain) or a variant for C3H subclones (C3H mouse strain). The OP9 cell
262 line has been obtained submitting bone marrow stroma cell isolated from new born calvaria deficient in
263 macrophage colony stimulating factor to the same immortalization scheme as 3T3 cells.

264 **B :** Mouse primary culture. Following collagenase digestion of adipose tissue, mature adipocytes may be
265 directly investigated and stem cells from the stroma vascular fraction may be differentiated in primary
266 cultures. The Ob17 cell line model of preadipocytes was obtained through dedifferentiation of mature
267 adipocytes.

268 **C :** Human adipocyte models. Following collagenase digestion, stem cells from the stroma vascular fraction
269 may be differentiated in primary cultures (not shown). Immortalization of human preadipocytes is achieved
270 through cell transformation or extensive cell passaging. LiSa-2 and SGBS cell lines have been obtained

271 through collagenase digestion of adipose tissues from patients with rare poorly differentiated pleomorphic
272 liposarcoma, and X-linked Simpson-Golabi-Behmel syndrome, respectively.

273

274 **Figure 3 : Ceiling culture techniques for mature adipocyte survival.**

275 **A** : Conventional ceiling culture. Conventional ceiling can be achieved culturing isolated mature adipocytes
276 in a flask fully filled with culture media. Due to buoyancy, adipocytes adhere the top of the flask and avoid
277 direct contact with air. Culture under a glass coverslip in multi-well plate improves adipocyte survival,
278 facilitates microscopy and reduces dedifferentiation.

279 **B** : Membrane mature adipocyte aggregate culture (MAAC). A transwell membrane is used as support. The
280 setting allows maintenance of mature adipocytes without dedifferentiation for several days and co-cultures
281 with other cell types.

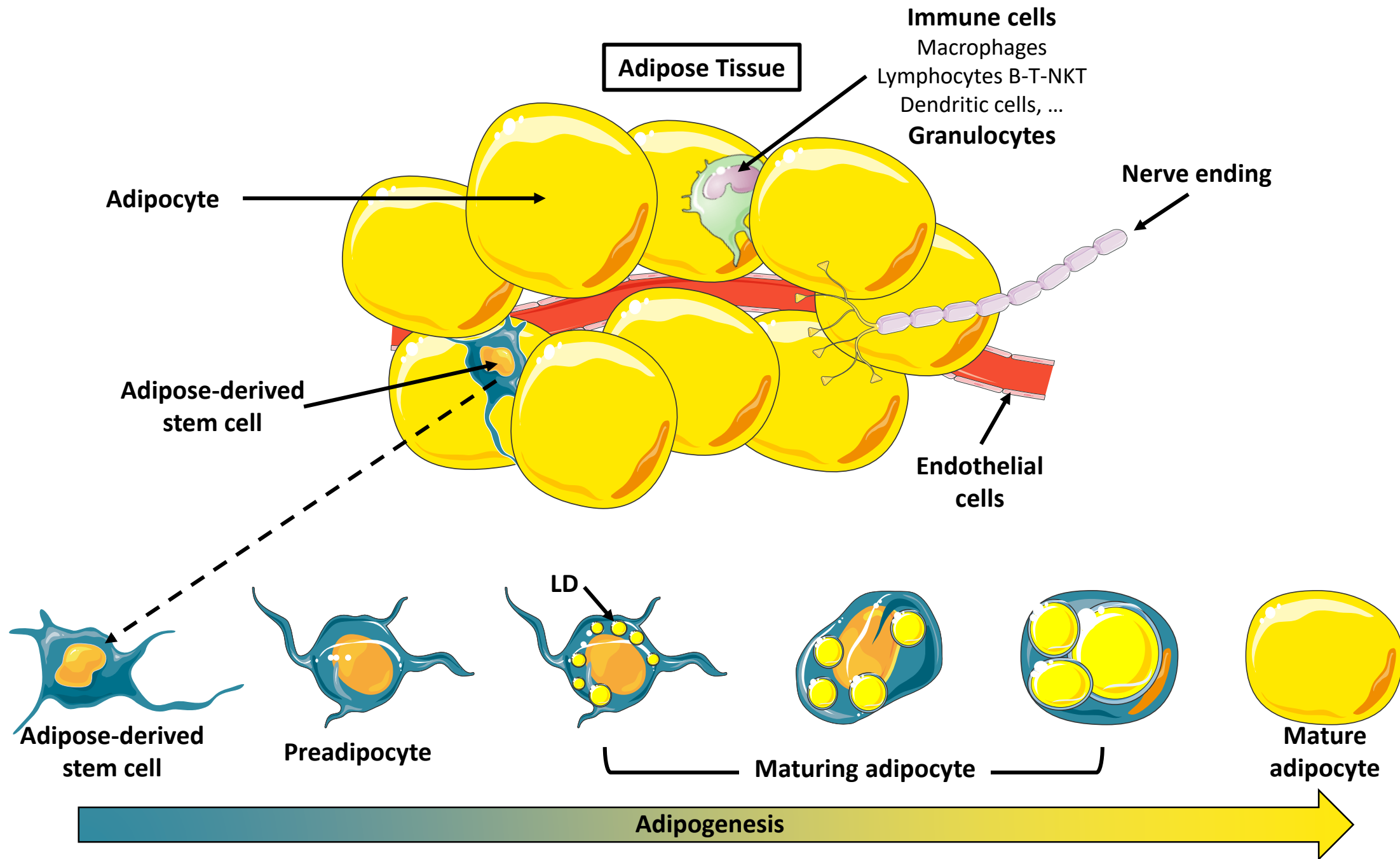
282

283 **Figure 4 : Schematic overview of preadipocyte spheroid culture.**

284 **A** : Spheroid formation. Isolated primary adipocytes spontaneously gather in a spheroid shape when placed
285 in ultra low attachment plate or in hanging drop. Once formed, the spheroid are kept in ultra low
286 attachment wells to be differentiated scaffold-free or following matrix inclusion.

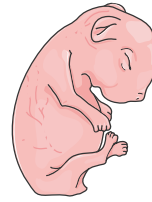
287 **B** : Improved differentiation in adipose spheroids. Compared to conventional 2D culture, primary
288 preadipocytes differentiated into spheroid structures display increased adipose marker gene expression,
289 adipokine secretion and lipid droplet size. The formation of unilocular fat cell can be achieved in such
290 condition which is not the case in 2D culture.

291

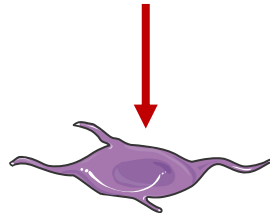




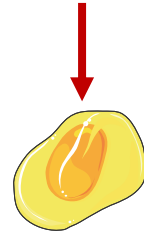
Mouse embryo



New born calvaria



Mouse embryonic fibroblast



Bone marrow stroma cells

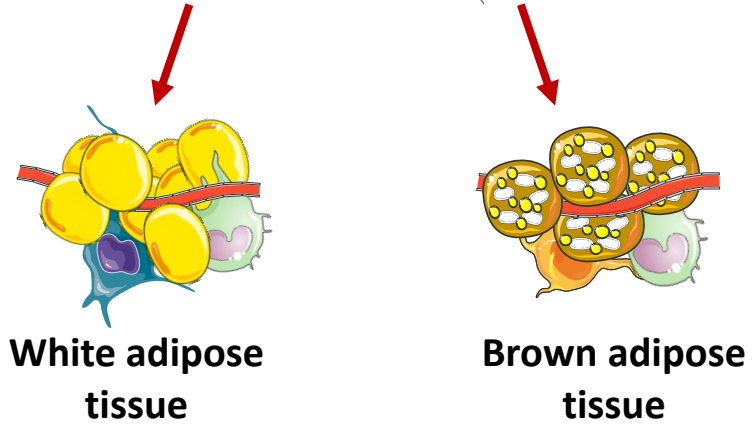
3T3 immortalization scheme



**3T3 subclones
C3H/10T1/2**



OP9



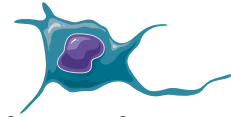
White adipose tissue

Brown adipose tissue

Collagenase digestion



Mature adipocyte



White adipose-derived stem cell

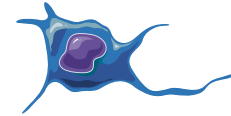


Mature adipocyte

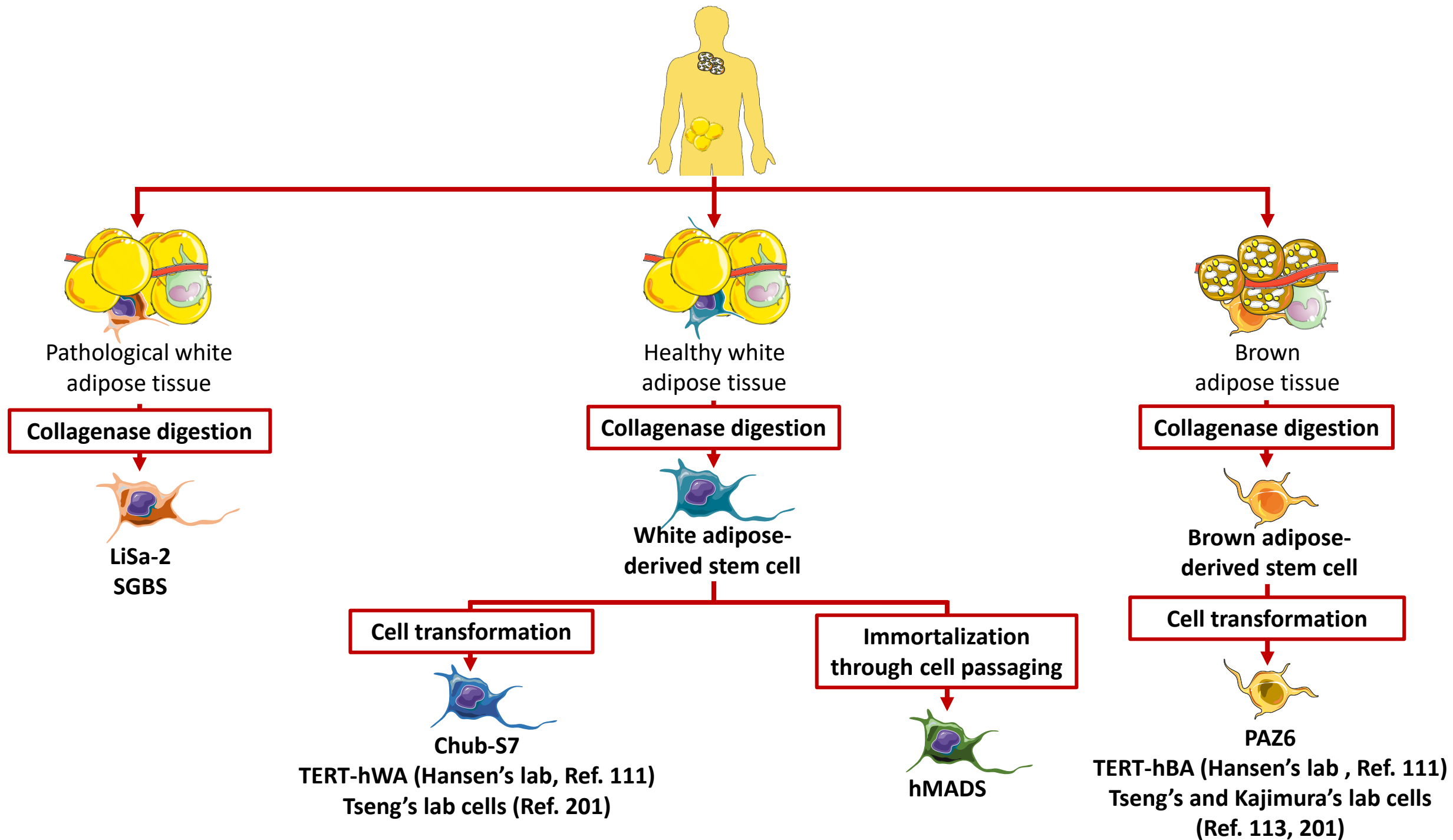


Brown adipose-derived stem cell

Dedifferentiation

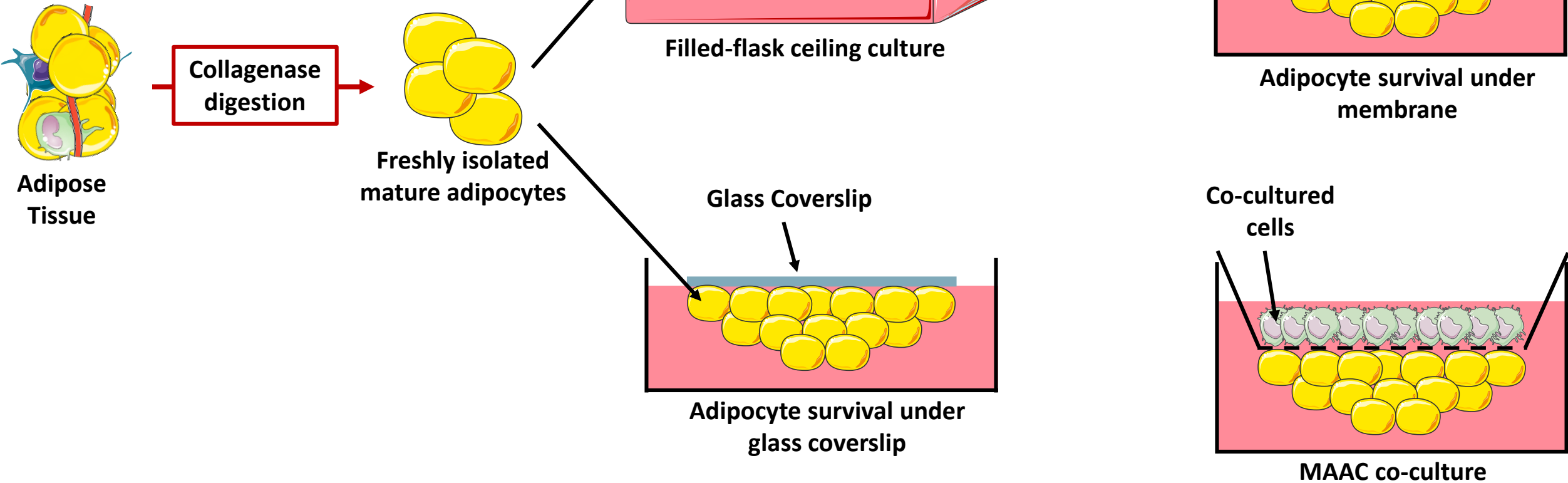


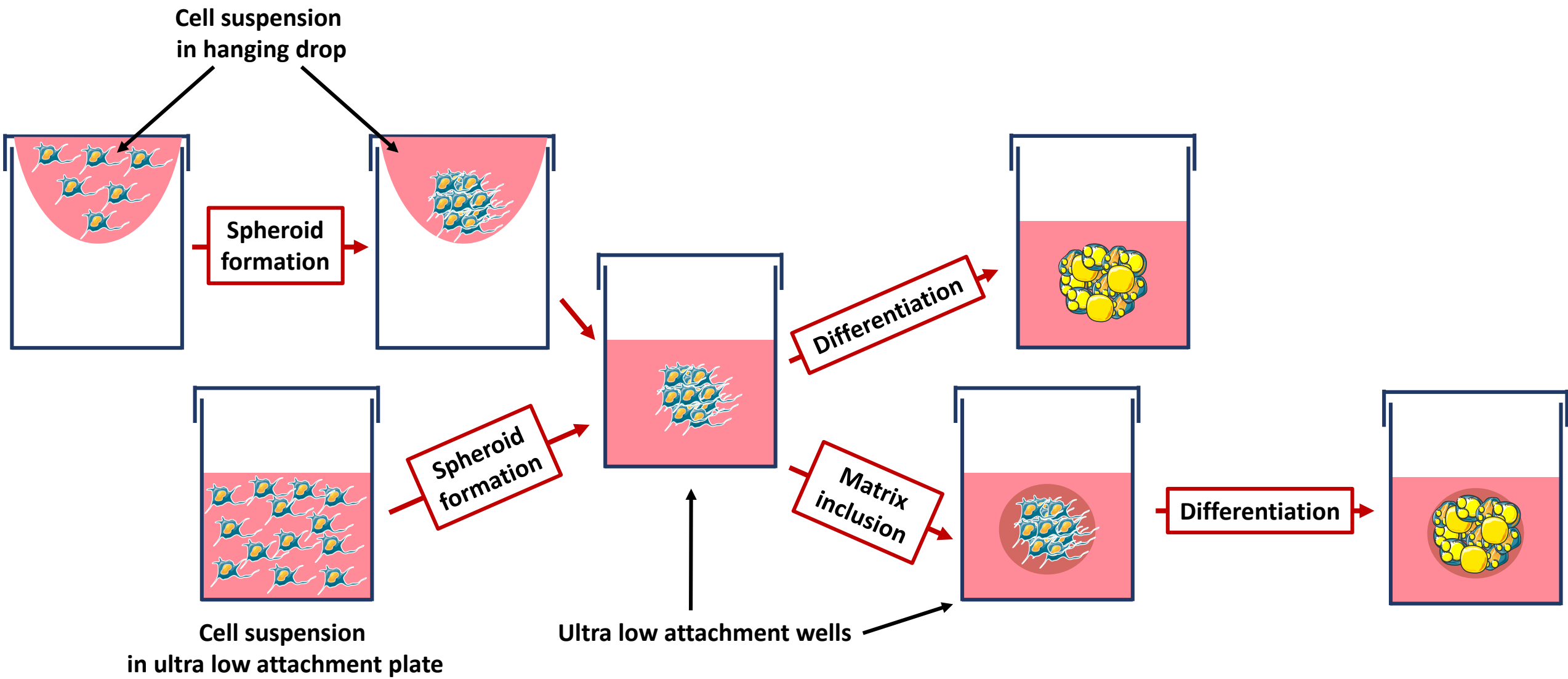
Ob17



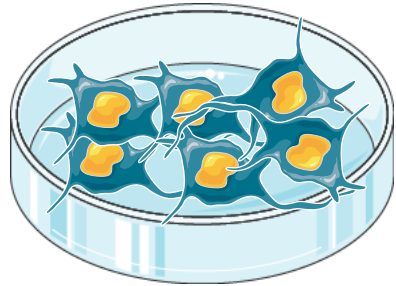
B : Membrane mature adipocytes aggregate culture (MAAC)

A : Conventional ceiling culture

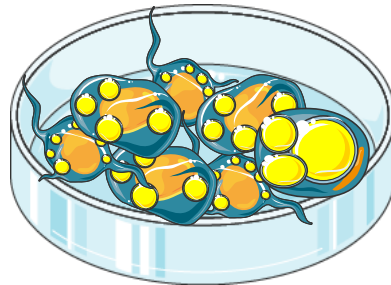




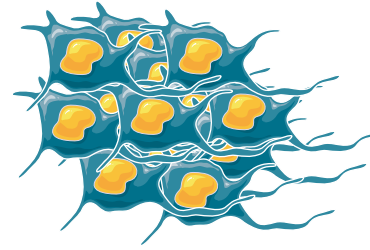
**Conventional
2D culture**



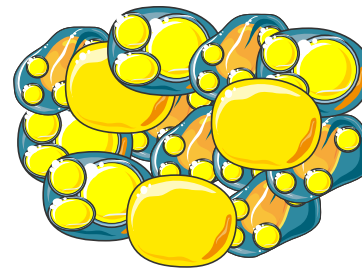
Differentiation



**Spheroid
3D culture**



Differentiation



Expected improvements in spheroids vs 2D culture :

- ↗ Adipose marker gene expression
- ↗ Adipokine secretion
- ↗ Lipid droplet size

Unilocular adipocyte formation