

1 **Mammalian adipogenesis regulators (Aregs) exhibit robust non- and anti-**
2 **adipogenic properties that arise with age and involve retinoic acid signalling**

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22 **Abstract**

23 Adipose stem and precursor cells (ASPCs) give rise to adipocytes and determine the
24 composition and plasticity of adipose tissue. Recently, several studies have demonstrated that
25 ASPCs partition into at least three distinct cell subpopulations: *Dpp4*⁺ stem-like cells, *Aoc3*⁺
26 pre-adipocyte-like cells, and the enigmatic *CD142*⁺ cells. A great challenge now is to
27 functionally characterize these distinct ASPC populations. Here, we focus on *CD142*⁺ ASPCs
28 since discrepant properties have been assigned to this subpopulation, from adipogenic to non-
29 and even anti-adipogenic. To address these inconsistencies, we comprehensively
30 characterized mammalian subcutaneous *CD142*⁺ ASPCs across various sampling conditions.
31 Our findings demonstrate that *CD142*⁺ ASPCs exhibit high molecular and phenotypic
32 robustness, firmly supporting their non- and anti-adipogenic properties. However, these
33 properties emerge in an age-dependent manner, revealing surprising temporal *CD142*⁺ ASPC
34 behavioural alterations. Finally, using multi-omic and functional assays, we show that the
35 inhibitory nature of these adipogenesis-regulatory *CD142*⁺ ASPCs (Aregs) is driven by
36 specifically expressed secretory factors that cooperate with the retinoic acid signalling
37 pathway to transform the adipogenic state of *CD142*⁻ ASPCs into a non-adipogenic, Areg-like
38 one.

39 Introduction

40 Although adipogenesis is one of the best-studied cell differentiation paradigms (Rosen and
41 Spiegelman, 2014), we still have limited knowledge of the *in vivo* origin and composition of
42 adipose stem and precursor cells (ASPCs, (Ferrero, Rainer and Deplancke, 2020)). This is
43 partially due to the highly heterogeneous and unstructured nature of adipose tissue depots,
44 which are present in multiple anatomical locations (including subcutaneous and visceral white
45 adipose tissue) and consist of a mixture of different cell types (Cristancho and Lazar, 2011),
46 whose origin and identity differ between distinct fat depots (Cleal, Aldea and Chau, 2017).
47 Driven by the resolving power of single-cell RNA-sequencing (scRNA-seq), several studies
48 have recently investigated and confirmed mammalian ASPC heterogeneity (Burl *et al.*, 2018;
49 Hepler *et al.*, 2018; Schwalie *et al.*, 2018; Cho, Lee and Doles, 2019; Gu *et al.*, 2019; Merrick
50 *et al.*, 2019; Spallanzani *et al.*, 2019; Sárvári *et al.*, 2021). Our own integrative analysis of
51 publicly available scRNA-seq data has thereby allowed the compiling of an ASPC
52 subpopulation consensus based on the fact that the three main identified subpopulations
53 exhibit a remarkable molecular consistency throughout the analysed datasets (Ferrero, Rainer
54 and Deplancke, 2020). These subpopulations include adipose stem-like cells with high
55 expression of *Cd55* and *Dpp4*, pre-adipocyte-like cells with high *Aoc3* and *lcam1* expression,
56 and a rather enigmatic, third population characterized by high *F3* (coding for CD142) and
57 *Clec11a* expression. A hierarchy of these ASPCs has also been proposed with the highly
58 proliferative DPP4+ stem-like cells giving rise to the two other subpopulations: ICAM1+ and
59 CD142+ ASPCs (Merrick *et al.*, 2019) which themselves may be able to interconvert (Merrick
60 *et al.*, 2019; Sárvári *et al.*, 2021). These distinct ASPC subpopulations have been shown to
61 be established as early as post-natal day 12 (P12) in mouse, based on scRNA-seq data
62 (Merrick *et al.*, 2019). At an even earlier developmental stage (P2), immunofluorescence-
63 based *in situ* analyses revealed the presence of anatomically partitioned DPP4+ adipose
64 stem-like and ICAM1+ pre-adipocyte-like cells (Merrick *et al.*, 2019), however, the presence
65 of CD142+ ASPCs in such young mice has not yet been demonstrated.

66
67 A great challenge in the field now is to explore whether these molecularly distinct ASPC
68 subpopulations also have different functional properties. In this study, we decided to focus on
69 CD142+ ASPCs. This is because previous work has defined these cells as being not only non-
70 adipogenic but also anti-adipogenic, which is why they were termed “adipogenesis regulators”
71 (Aregs) (Schwalie *et al.*, 2018). More recent, independent findings supported the notion that
72 adipose tissue may harbour a negatively regulatory cell type (Lee *et al.*, 2019), yet both its
73 identity as well as the underlying molecular mechanisms have so far remained ill-defined
74 (Shamsi, Tseng and Kahn, 2021). The presence of an anti-adipogenic cell population could
75 have tremendous implications with regard to how adipose tissue development and
76 homeostasis is regulated in health and disease. This is why further studies are warranted that
77 explore its existence as well as its molecular and functional properties, especially in light of
78 recent, divergent findings showing that CD142+ ASPCs are in fact adipogenic (Merrick *et al.*,
79 2019). The reasons for these functional discrepancies between studies have remained unclear
80 but are hypothesized to reflect differences in cell isolation and sorting criteria, antibodies,
81 culturing conditions, sex or age (Merrick *et al.*, 2019; Ferrero, Rainer and Deplancke, 2020;
82 Corvera, 2021).

83
84 Given the importance of resolving the molecular and functional heterogeneity of ASPCs, here,
85 we set out to systematically address these inconsistencies. Our findings validate the molecular

86 and phenotypic robustness of murine CD142+ ASPCs, authenticating these cells as non-
87 adipogenic inhibitors of adipogenesis. Interestingly however, we demonstrate that these
88 functional properties are age-dependent. Specifically, we show that the molecular identity of
89 CD142+ cells is already established before post-natal day 16 (P16), while their non-adipogenic
90 properties become apparent only four weeks after birth. Importantly, we also confirm the anti-
91 adipogenic properties of adult CD142+ ASPCs and, using a diverse range of multi-omic- and
92 functional assays, provide insights into the molecular mechanisms that control their activity.
93 Particularly, we show that the inhibitory nature of CD142+ ASPCs appears to be driven by a
94 set of specifically expressed secretory factors, involving Tissue factor (CD142) itself as well
95 as Matrix Gla protein (MGP), whose actions may possibly converge onto the retinoic acid (RA)
96 signalling pathway. These factors/pathways seem to function to render CD142+ ASPCs
97 refractory to adipogenesis, while exerting their anti-adipogenic activity by transforming the
98 adipogenic state of CD142- ASPCs into a non-adipogenic, CD142+-like state.

99 **Results**

100 **CD142+ ASPCs are defined by a specific transcriptomic signature and a robust non-** 101 **adipogenic phenotype**

102 In recent years, numerous studies have dissected white adipose tissue (WAT) composition at
103 the single-cell level (subcutaneous WAT: (Burl *et al.*, 2018; Schwalie *et al.*, 2018; Cho, Lee
104 and Doles, 2019; Merrick *et al.*, 2019); visceral WAT: (Hepler *et al.*, 2018; Spallanzani *et al.*,
105 2019; Sárvári *et al.*, 2021); perivascular WAT: (Gu *et al.*, 2019)). Together, these studies
106 provide an opportunity to acquire high-resolution insights into ASPC heterogeneity through
107 data integration, essentially allowing to validate and possibly expand or revise our initial
108 scRNA-seq-based observations (Schwalie *et al.*, 2018). To do so, we integrated data from our
109 own and relevant publicly available mouse subcutaneous adipose tissue scRNA-seq studies
110 (Burl *et al.*, 2018; Schwalie *et al.*, 2018; Merrick *et al.*, 2019) revealing that ASPCs robustly
111 partition among three principal subpopulations in line with earlier observations (**Fig. 1A**,
112 **Suppl. Fig. 1**, Ferrero *et al.*, 2020). One of these three main ASPC clusters, characterized by
113 high and specific *F3* (coding for CD142) gene expression, proved to be highly robust and
114 stable across increasing clustering resolution (**Fig. 1B**), indicating that these cells are
115 characterized by a clearly delineated and specific transcriptomic signature.

116
117 Given the particular interest in the *F3*+ cluster, previously identified to represent cells having
118 inhibitory properties toward adipogenesis (Schwalie *et al.*, 2018), we set out to better
119 understand the specific molecular markers of this population. Using the integrative analysis,
120 we identified a set of robust markers differentially expressed in each individual scRNA-seq
121 dataset that are specific to the *F3*+ cluster. Combined with the top 20 markers that were
122 detected by bulk RNA-seq as being differentially expressed in CD142+ *versus* CD142- freshly
123 isolated ASPCs in our previous study (Schwalie *et al.*, 2018), this resulted in a list of 100
124 markers that are representative of CD142+ ASPCs (top CD142+ markers, **Suppl. Table 1**,
125 **Methods**). To assess the relevance of this list of gene expression markers, we carried out an
126 in-depth quantitative transcriptomic (BRB-seq, Alpern *et al.*, 2019) and proteomic
127 characterization of FACS-sorted CD142+ *versus* CD142- ASPCs (defined as: SVF Lin-
128 (CD31- CD45- TER119-) SCA-1+ CD142+ and SVF Lin- SCA-1+ CD142- respectively,
129 **Suppl. Fig. 2, Methods**). The identified top CD142+ markers revealed to be specific to
130 CD142+ cells both at the transcriptomic and proteomic level (**Fig. 1C-D, Suppl. Fig. 3A-B**,
131 **Suppl. Table 1-2**). In addition, we found that overall gene and protein expression levels
132 correlated well between all scRNA-seq, bulk RNA-seq, and proteomic datasets (**Fig. 1D**,
133 **Methods**), indicating that the observed transcriptomic signature of CD142+ ASPCs is a
134 reasonable proxy of their protein/functional characteristics. To assess to which extent this
135 signature was affected by culturing or differentiation conditions, we performed bulk RNA-seq
136 of CD142- and CD142+ ASPCs post-expansion and post-differentiation (i.e. after exposure
137 to adipogenic medium) as well as a proteomic analysis of expanded, respective populations
138 (**Suppl. Table 2**). Interestingly, we observed that, under these conditions, the expression of
139 many top CD142+ markers including *Gdf10*, *Cpe*, *Rbp1*, *F3*, *Bgn*, *Clec11a*, *Mgp* and *Aldh1a2*
140 is maintained both at the transcriptomic and/or proteomic level in CD142+ ASPCs compared
141 to their CD142- counterparts (**Suppl. Fig. 3C-D, Suppl. Fig. 4**).

142
143 To examine the functional properties of these cells, we set out to test the phenotype of CD142+
144 ASPCs across a wide range of experimental conditions and functional assays, aiming to
145 possibly reconcile discrepant findings of CD142+ ASPC behaviour. First, we recapitulated our

146 earlier findings (Schwalie *et al.*, 2018), showing that the top 5-7% most positive CD142+
147 ASPCs, isolated using the previously employed anti-CD142 antibody, have very low to no
148 adipogenic capacity as compared to CD142- ASPCs when stimulated with a standard white
149 adipogenic differentiation cocktail (**Fig. 1E-F, Methods**). However, since the nature of this
150 antibody may be one of the possible reasons underlying discrepant CD142+ cell behaviour
151 read-outs, we tested three more antibodies. While the flow cytometry profiles of the four
152 assessed antibodies differ to a certain extent, the isolated cellular fractions yielded consistent
153 non-adipogenic phenotypic results (**Fig. 1E-F, Suppl. Fig. 5A, Methods**). Indeed, when
154 freshly isolated with different antibodies, the respective CD142+ ASPC samples revealed a
155 consistent transcriptional signature exhibiting a significantly higher expression score based on
156 the top CD142+ markers (here named the “CD142+ score”, **Suppl. Table 1, Methods**)
157 compared to the other tested cellular fractions (total and CD142- ASPCs) (**Fig. 1C and G**).
158 Finally, the observed non-adipogenic properties of post-differentiation CD142+ cells (i.e. post-
159 exposure to a standard, adipogenic cocktail) were consistent with the expression profiles of
160 adipogenesis-relevant genes. Specifically, genes involved in “white fat cell differentiation”
161 (GO:0050872) or “negative regulation of fat cell differentiation” (GO:0045599) were
162 significantly lower or higher expressed in CD142+ ASPCs compared to total or CD142-
163 ASPCs, respectively (**Fig. 1H-I**). Moreover, fat and lipid-related terms that were detected as
164 significant by gene set enrichment analysis (GSEA) were negatively enriched in CD142+
165 versus CD142- ASPCs (**Suppl. Fig. 5B**).

166
167 Finally, since CD142 surface expression shows a continuum across ASPCs (**Suppl. Fig. 5A**),
168 it is possible that the stringency of the cell isolation procedure (so far typically 5-7%) has an
169 impact on downstream cell behaviour. To investigate this, we isolated CD142+ ASPCs using
170 a less stringent gating (~20% **Suppl. Fig. 7A**), yet we did not observe a notable difference in
171 overall differentiation potential compared to the more stringently isolated cells (**Fig. 1J-K**,
172 **Suppl. Fig. 7B-C**).

173
174 Next, we examined the influence of the differentiation medium as it is widely established that
175 adipogenic potential varies as a function of the utilized differentiation cocktail. To do so, we
176 used the standard “complete DMEM” differentiation medium (with insulin, IBMX and
177 dexamethasone) as well as three additional ones (1, “Min DMEM”, with insulin only; 2,
178 “Complete + T3 + Indo DMEM/F12”, with insulin, IBMX, dexamethasone, T3 and
179 indomethacin; 3, “Min DMEM/F12”, with insulin only, **Methods**, Schwalie *et al.*, 2018; Merrick
180 *et al.*, 2019). However, we did not observe notable CD142+ ASPC differentiation differences
181 across these distinct culturing conditions (**Fig. 1J-K**).

182
183 Another source for discrepant cell behaviour could be the sex of the animals. To test this, we
184 isolated CD142+ and CD142- ASPCs from both male and female mice, revealing that, upon
185 exposure to an adipogenic cocktail, the adipogenic propensity of total and CD142- ASPCs
186 was significantly higher (adjusted p-value (p-adj) < 0.001 and < 0.05, respectively) in males
187 compared to females (**Fig. 1L-M, Suppl. Fig. 8**). However, both male and female CD142+
188 cells were completely refractory to adipogenic differentiation (**Fig. 1L-M, Suppl. Fig. 8**). This
189 is in line with transcriptomic results, as our scRNA-seq analysis of male and female cells
190 (**Suppl. Fig. 9A**) (Schwalie *et al.*, 2018) revealed the existence of a clearly delineated
191 F3(CD142)+ cluster in both sexes with a highly consistent overlap of specific markers (**Suppl.**
192 **Fig. 9B-C**). In addition, the integration of the different publicly available scRNA-seq datasets

193 included cells from mice of different sexes and clearly showed the existence of a robust *F3*+

194 cluster (Burl *et al.*, 2018; Schwalie *et al.*, 2018; Merrick *et al.*, 2019) (**Fig. 1B, Suppl. Fig. 1**).

195

196 Together, these in-depth computational and experimental analyses validate the previously

197 observed molecular identity of CD142+ ASPCs and demonstrate the robustness of their non-

198 adipogenic phenotype across a wide range of conditions and experiments.

199

200 **Age-dependent molecular and phenotypic emergence of *bona fide* CD142+ ASPCs**

201 The findings reported above indicate that CD142+ ASPCs constitute a distinct cell population

202 with a well-defined molecular identity and a clear non-adipogenic character. However, all of

203 these analyses were performed on cells derived from adult mice, prompting the question

204 whether the observed CD142+ cell properties could perhaps be age-dependent. Analysis of

205 publicly available scRNA-seq data of post-natal day 12 (P12) mice revealed a CD142+ cluster

206 that shares many of the adult top CD142+ markers (Merrick *et al.*, 2019, **Suppl. Fig. 10A-B**)

207 and overlaps with adult CD142+ ASPCs upon data integration (**Suppl. Fig. 10C-D**). Nevertheless,

208 we uncovered that the identity of these P12 *F3*(CD142)+ cells, as defined by

209 their “CD142+ score” (**Suppl. Table 1**), is significantly less pronounced compared to their

210 adult counterpart (p-value < 0.001, **Fig. 2A, Suppl. Fig. 10E**). To investigate whether this

211 more subtle molecular identity would also manifest itself at the phenotypic level, we assessed

212 the proportion and adipogenic propensity of distinct ASPC subpopulations at distinct

213 developmental time points including newborn (P0), 12-17-day-old (P12-17) mice as well as

214 juvenile (4-week-old (4wo)) and adult (7wo and 11wo) animals with these two groups being

215 separated by weaning at post-natal day 21 (P21). Given the overlap between P12 and adult

216 CD142+ ASPCs in the scRNA-seq analysis and the specificity of *F3*(CD142) in both age

217 groups (**Suppl. Fig. 10B**) (Merrick *et al.*, 2019), we used the CD142 marker to enrich for our

218 cellular fractions of interest across ages. We observed significant differences in the

219 proportions of cellular fractions assessed by flow cytometry, with the Lineage negative (SVF

220 Lin-) portion being significantly higher (p-adj < 0.0001) and the ASPC portion (SVF Lin-

221 SCA-1+) significantly lower (p-adj < 0.05) in pre-weaning (P0 and P12-17) compared to post-

222 weaning mice (4wo, 7wo and 11wo) (**Fig. 2B-C, Suppl. Fig. 11A**). In addition, ASPCs from

223 pre-weaning mice exhibited significantly lower (p-adj < 0.01) CD142 cell surface expression

224 compared to post-weaning animals (**Fig. 2B-C**). Indeed, we found that at the gating stringency

225 corresponding to 5% of CD142+ cells in adult (11wo) mice, only 2.5% of newborn (P0)

226 CD142+ ASPCs were captured (**Fig. 2B-C**), consistent with the observed gradual decrease

227 of the CD142- ASPC fraction as mice mature (**Suppl. Fig. 11A-B**).

228

229 Next, we assessed the adipogenic capacity of the different ASPC fractions as a function of

230 age. Using the standard differentiation cocktail (**Methods**), we unexpectedly observed that all

231 cellular fractions (total, CD142- and CD142+ ASPCs) derived from pre-weaning mice

232 exhibited a remarkable adipogenic propensity with virtually all cells displaying lipid

233 accumulation. However, based on visual inspection, the overall size of the pre-weaning cells

234 and the size of the accumulated lipid droplets tended to be smaller compared to post-weaning

235 cells (**Fig. 2D**). Perhaps most interestingly, pre-weaning-derived CD142+ ASPCs gave rise to

236 *in vitro* adipocytes, which is in stark contrast to the marked non-adipogenic properties of their

237 post-weaning counterparts (**Fig. 2D-E, Suppl. Fig. 11C-E**). Indeed, we observed that, despite

238 non-negligible variability between independent replicates, the adipogenic propensity of total

239 and CD142- ASPCs gradually decreases with age, whereas CD142+ ASPCs exhibit a very

240 sharp drop (p-adj < 0.0001) in their ability to give rise to *in vitro* adipocytes between the 16th
241 and 28th (4wo) day of post-natal development (**Fig. 2D-E, Suppl. Fig. 11C-E**).

242
243 To further explore this age-dependent functional change of CD142+ ASPCs, we performed
244 bulk RNA-seq on freshly isolated ASPC cellular fractions at different time points after birth
245 (P0, P16, 4wo, 7wo and 11wo, **Suppl. Table 2**). Analysis of the resulting data revealed that
246 age is the variable that explains the largest variation across samples as they are ordered by
247 this feature along the first principal component (PC1) (**Fig. 2F**). GSEA revealed that terms
248 such as “cell fate commitment” (GO:0045165), “mesenchymal cell differentiation/proliferation
249 and development” (GO:0048762, GO:0010463, GO:0014031), “stem cell differentiation”
250 (GO:0048863), “stem cell population maintenance” (GO:0019827) and cell cycle-related terms
251 are negatively enriched in the genes driving PC1 (**Suppl. Fig. 12A**). This suggests that genes
252 linked to these terms are up-regulated in freshly isolated young ASPCs, likely reflecting their
253 more naïve “stem” condition. Indeed, all ASPC fractions that were freshly isolated from the
254 newborn bourgeoning fat pads expressed substantially higher levels of *Ccnd1* (coding for
255 CyclinD1) and *Dlk1/Pref1*, and lower levels of markers such as *Pdgfrb*, *Cd34* and *Ly6a* (coding
256 for SCA-1) compared to ASPCs from older animals (**Suppl. Fig. 12B-F**). Such a signature
257 was proposed to select for a primitive and naïve precursor population (Wang *et al.*, 2003;
258 Atanassova, Rancic and Georgieva, 2012; Hepler and Gupta, 2017). Interestingly, it has also
259 been shown that foetal and early post-natal ASPCs from murine subcutaneous depots
260 surprisingly express perilipin (*Plin1*) and adiponectin (*Adipoq*) and exhibit highly proliferative
261 and differentiation properties (Hong *et al.*, 2015; Hepler and Gupta, 2017), consistent with our
262 observations for newborn-derived ASPCs (**Suppl. Fig. 12G-H, Supp. Fig. 11C-E**). In contrast,
263 lipid/fat-related terms were enriched in freshly isolated adult ASPCs, implying a molecular
264 state which is less naïve and more committed towards adipogenesis (**Suppl. Fig. 12A**). We
265 further found that the “retinol/retinoid metabolic process” (GO:0042572/GO:0001523) gene
266 expression signature is much more prominent in adult-derived ASPCs compared to young
267 ASPCs, which correlates with the decreased adipogenic capacity of adult *versus* young
268 ASPCs. (**Suppl. Fig. 13A-B**). In addition to this general increase with age, this same RA-
269 related expression signature was even more pronounced in 4wo and adult CD142+ compared
270 to CD142- ASPCs (**Suppl. Fig. 13C**). Interestingly, the “CD142+ score” (**Suppl. Table 1**)
271 increased in all the cellular fractions of ASPCs (CD142-, CD142+ and total ASPCs) in an age-
272 dependent manner (**Fig. 2G, Suppl. Fig. 14A**). We thereby observed that this set of top
273 markers becomes significantly enriched in CD142+ ASPCs (compared to CD142- ASPCs) at
274 P16 and is particularly prominent in adult cells (**Suppl. Fig. 14B-E**). Moreover, when we
275 removed the age-driven source of variation from the analysed samples, CD142+ ASPCs from
276 all developmental time points, except P0, were marked by a relative and substantial increase
277 of the “CD142+ score” compared to the other assessed fractions (all ages, total and CD142-
278 ASPCs), while being once again more pronounced in adults, reflecting the results from our
279 scRNA-seq analysis of P12- and adult-derived ASPCs (**Fig. 2A**) (Merrick *et al.*, 2019). This
280 increase was accompanied by a gradual decrease of the “CD142+ score” in all CD142-
281 ASPCs with age (**Fig. 2H**).
282

283 Together, these findings suggest that ASPCs and their considered subpopulations are
284 molecularly and phenotypically naïve at birth, after which they gradually acquire their
285 respective properties throughout the early post-natal developmental stages. Furthermore, the
286 definite “CD142+-specific expression signature” appears to emerge in CD142+ ASPCs before

287 P16, followed by the manifestation of their completely non-adipogenic phenotype by post-natal
288 week 4.

289

290 **CD142+ ASPC-dependent adipogenic inhibition and mediating factors**

291 The findings above validate the previously described molecular identity of CD142+ ASPCs,
292 provide additional evidence as to the robustness of their non-adipogenic character, and add
293 the interesting dimension of this phenotypic property emerging with age. Importantly, our
294 results also corroborate the notion that adult CD142+ ASPCs are not only non-adipogenic, but
295 also anti-adipogenic. This is because adult CD142- ASPCs reproducibly exhibit a greater
296 adipogenic propensity than total ASPCs (**Fig. 1E-F, Fig. 2D-E**), suggesting that the presence
297 of CD142+ ASPCs dampens the adipogenic capacity of their CD142- counterparts (as a
298 progenitor population devoid of CD142+ ASPCs and featuring a high adipogenic potential).
299 To better understand underlying mechanisms governing the inhibitory function of CD142+
300 ASPCs, we first re-explored the anti-adipogenic nature of these cells using a transwell set-up
301 allowing specific ASPC subpopulations to be co-cultured but without cell-to-cell contact
(Methods). These experiments revealed that CD142- ASPCs co-cultured with CD142+
303 ASPCs show a significantly decreased capacity to generate adipocytes (p-value < 0.01)
304 compared to CD142- ASPCs cultured on their own (**Fig. 3A-B**). To molecularly support these
305 results, we performed bulk RNA-seq of differentiated CD142- ASPCs and differentiated
306 CD142- ASPCs co-cultured with CD142+ ASPCs (**Suppl. Table 2**). Consistent with our
307 phenotypic observations, CD142- ASPCs cultured alone showed a significantly higher
308 expression of genes related to “white fat cell differentiation” (GO:0050872) (**Fig. 3C**), while
309 key genes linked to the “negative regulation of fat cell differentiation” (GO:0045599) were up-
310 regulated in CD142- ASPCs co-cultured with CD142+ cells (**Fig. 3D**). These results
311 recapitulate CD142+ ASPCs’ previously reported capacity to inhibit adipogenesis through
312 paracrine signalling (Schwalie *et al.*, 2018). Together with a recent, independent report
313 showing a comparable capacity of CD142+ stromal cells in muscle (Camps *et al.*, 2020), we
314 therefore decided to reintroduce the original “Adipogenesis regulators” (Aregs) nomenclature
315 to conceptually define CD142+ ASPCs.

316

317 Given that Aregs can exert their activity *via* paracrine signalling, we explicitly mined the
318 transcriptome and proteome data to identify Areg-specific secreted factors. This resulted in a
319 stringent set of highly Areg-specific candidates including *F3* (coding CD142) itself, *Mgp*,
320 *Gdf10*, *Clec11a*, *Cpe* and *Bgn* (**Fig. 3E, Suppl. Table 1, 2 and Methods**). To assess the
321 inhibitory potential of these factors, we treated CD142- ASPCs with various concentrations of
322 recombinant candidate proteins, aiming to mimic the physiological presence of Aregs. Similar
323 to the above-described experiments, the extent of adipogenesis was inherently variable
324 across distinct assays and cell populations. Nevertheless, both recombinant CD142 (p-adj <
325 0.001) and GDF10 (p-adj < 0.001) significantly inhibited adult-derived CD142- ASPC
326 adipogenesis at a concentration of 100 ng/ml, while recombinant MGP inhibited adipogenesis
327 at 1 µg/ml (p-adj < 0.001) (**Fig. 3F-G, Suppl. Fig. 15 and 16, Methods**). Interestingly, P0-
328 derived CD142- ASPCs appeared to a large extent refractory to such an inhibition, illustrated
329 by a much less striking decrease in the extent of adipogenesis upon treatment with
330 recombinant EGF, a well-established adipogenesis inhibitor (Harrington, Pond-Tor and
331 Boney, 2007) that was used as a positive control (**Suppl. Fig. 17, Methods**). Nevertheless,
332 we still observed a significant decrease in the extent of adipogenesis when newborn CD142-
333 ASPCs were exposed to recombinant CD142, GDF10, MGP and BGN (p-adj < 0.01, < 0.01,

334 < 0.001 and < 0.05 respectively), with MGP now exerting the most pronounced inhibitory effect
335 on differentiating newborn-derived CD142- ASPCs (**Suppl. Fig. 17**).

336

337 Amongst other Areg candidates, we also identified a few retinoic acid (RA)-related genes
338 including *Aldh1a2*, *Epha3*, *Osr1* and *Rbp1* (**Fig. 3H, Suppl. Fig. 18, Suppl. Table 1**,
339 **Methods**). Their specificity to CD142+ ASPCs raises the possibility that this pathway could
340 also be implicated in the anti-adipogenic character of Arebs. This is further supported by the
341 significant enrichment of the GO term “retinol metabolic process” (GO:0042572) (with retinol
342 being a precursor of RA) in transcriptomic and proteomic data from freshly isolated CD142+
343 compared to CD142- ASPCs (**Fig. 3I, Suppl. Fig. 19A-B**). We further uncovered that
344 transcriptomic data from cultured CD142+ ASPCs treated with standard white adipogenic
345 cocktail is enriched for the “cellular response to RA” (GO:0071300) and “RA receptor signalling
346 pathway” (GO:0048384) terms compared to CD142- ASPCs post-differentiation (**Suppl. Fig.**
347 **19C-D, Suppl. Fig. 20**). Given that RA has been previously shown to inhibit adipogenesis of
348 3T3 cells (Murray and Russell, 1980), these findings suggest that Arebs might auto-suppress
349 their own adipogenic differentiation capacity by actively responding to RA. To test the
350 functional implication of RA in Arebs’ inhibitory properties, we treated CD142- ASPCs with
351 RA (**Methods**) and observed a significant decrease (p-adj < 0.001) in the extent of
352 adipogenesis (from 0.1 μ M RA, **Fig. 3J-K, Suppl. Fig. 21**). Together, these findings suggest
353 that the inhibitory character of Arebs might be mediated *via* RA signalling in concert with other
354 factors such as CD142, GDF10 and MGP.

355

356 **Molecular (auto-)regulation of Areg-mediated inhibitory activity**

357 To validate the involvement of factors shown implicated in the inhibitory nature of CD142+
358 ASPCs on adipogenesis, i.e. *F3* (CD142), *Mgp*, *Gdf10* and *Aldh1a2*, we first knocked the
359 respective genes down in adult CD142+ and total ASPCs in an siRNA-dependent manner and
360 examined its impact on adipogenesis. We observed that for each of these four genes, their
361 respective knockdown (KD) lead to a variable but consistent increase of adipogenic propensity
362 both in CD142+ and total ASPCs, with more pronounced effects in the total ASPC population
363 (**Suppl. Fig. 22, Methods**). Indeed, we found that siRNA-mediated changes in lipid
364 accumulation in CD142+ ASPCs were small, suggesting an inherent inability of these cells to
365 give rise to adipocytes, at least in the imposed culturing conditions. Given the Areg-specific
366 (among ASPCs) expression of *F3*, *Mgp*, *Gdf10* and *Aldh1a2* genes, we interpret the increase
367 of total ASPC adipogenesis upon knockdown of these respective genes as a consequence of
368 specifically inactivating Areg function. To support this interpretation in a more rigorous way,
369 we performed transwell assays, allowing CD142- ASPCs to be exposed to the secretome of
370 CD142+ ASPCs in which the respective candidate factors were knocked down. We observed
371 that inactivating all four genes caused an increased adipogenesis of co-cultured CD142-
372 ASPCs compared to control (scr siRNA), suggesting that they are indeed involved in the
373 inhibitory activity of Arebs. However, while we observed that *F3* and *Mgp* inactivation caused
374 a dramatic increase in overall differentiation of CD142- ASPCs (p-adj < 0.01 and < 0.01
375 respectively, **Fig. 4A-B, Suppl. Fig. 22H-I**), *Gdf10* and *Aldh1a2* KD had a lower effect (p-adj
376 = 0.052 for *Gdf10*, p-adj = 0.029 for *Aldh1a2*).

377

378 To further characterise the molecular mechanism(s) underlying the observed Areg-mediated
379 inhibitory signalling, we profiled the transcriptomes of CD142- ASPCs responding to CD142+
380 ASPCs, whose activity was modulated *via* individual knockdowns (**Suppl. Table 2**). We
381 observed that the respective gene expression profiles reflected the image-based

382 differentiation results to a great extent, with adipogenic propensity differences driving the first
383 principal component (PC1) and correlating with the overall transcriptomics-based “white fat
384 cell differentiation score” (GO:0050872) (**Fig. 4C, Suppl. Fig. 23A-B**). Interestingly, distinct
385 CD142- ASPC samples grouped as a function of how effectively they were impacted by
386 CD142+ ASPC signalling, forming two clusters corresponding to “active Areg” signalling
387 (CD142- ASPCs co-cultured with si*Gdf10*, si*Aldh1a2*, as well as WT and scr Aregs) and what
388 we interpret as “dysfunctional Areg” signalling (co-cultured with si*F3*, si*Mgp* Aregs and with an
389 empty transwell, **Fig. 4C-E**). Furthermore, we found that in CD142- ASPCs that responded to
390 “active Aregs”, the expression of most top CD142+ markers (i.e. Areg-specific markers)
391 including *F3*, *Bgn*, *Rbp1*, *Osr1*, *Cpe*, *Mgp* and *Gdf10* is higher compared to the other CD142-
392 ASPC samples, suggesting that the molecular state of CD142- ASPCs that were exposed to
393 “active Aregs” became itself more Areg-like (**Fig. 4D, Suppl. Fig. 23C**). To test this hypothesis,
394 we integrated transcriptomic profiles of CD142- and CD142+ ASPCs that were on their own
395 exposed to an adipogenic cocktail for 6-8 days (**Suppl. Table 2**) into the analysis of bulk RNA-
396 seq data derived from CD142- ASPCs that were co-cultured with distinct KD CD142+ ASPCs.
397 Remarkably, post-differentiation CD142+ and CD142- ASPCs fell into the two distinct clusters
398 corresponding to “active” and “dysfunctional Aregs” respectively, indicating that the CD142+
399 ASPCs were transcriptionally similar to CD142- ASPCs that were exposed to “active Aregs”
400 (**Fig. 4E-D, Suppl. Fig. 23D**). Furthermore, we observed a strong anti-correlation between the
401 Areg *versus* “white fat cell differentiation” signatures inferred from all the considered samples
402 (**Fig. 4F, Suppl. Fig. 23A and C, Suppl. Table 1**). Specifically, we found that the expression
403 of most top Areg markers, and particularly those of all tested candidates, strongly anti-
404 correlated with the “white fat cell differentiation score” (**Fig. 4G, Suppl. Fig. 23E, Methods**).
405 Our findings therefore point to a strong association between Areg marker (**Suppl. Table 1**)
406 expression (especially of the tested candidates) and the inability of ASPCs to undergo
407 adipogenic differentiation.

408
409 Finally, we aimed to provide additional evidence that the effect of functional Aregs on
410 differentiating CD142- ASPCs is at least in part regulated by RA signalling-related genes.
411 Remarkably, the “cellular response to RA” (GO:0071300) and “RA receptor signalling
412 pathway” (GO:0048384) terms were enriched in CD142- ASPCs subjected to “active Areg”
413 signalling (**Suppl. Fig. 23F-G**), and the expression of the genes involved in these two terms
414 were anti-correlated with “white fat cell differentiation score” (**Fig. 4G-I, Suppl. Fig. 23A, F**
415 and **G**). To further demonstrate the involvement of RA signalling in this Areg-mediated
416 inhibitory effect, we performed bulk RNA-seq of CD142- ASPCs treated with RA or EGF in
417 order to compare their gene expression profiles to those of CD142- ASPCs that were co-
418 cultured with “active Aregs”. This analysis revealed that CD142- ASPCs that were exposed
419 to “active Aregs” were transcriptionally more similar to CD142- ASPCs treated with RA than
420 those treated with EGF (**Suppl. Fig. 24**). In addition, we observed that the receiving CD142-
421 ASPCs exhibited remarkably coherent transcriptional dynamics of a number of genes and
422 pathways that were previously reported to be involved in the RA-mediated inhibition of
423 adipogenesis, a pattern of expression supported by the analysis of CD142- ASPCs treated
424 with RA. Specifically, Wnt signalling pathway-related terms, RA receptors (*Rarb* and *Rarg*) as
425 well as Catenin beta-1 (*Ctnnb*) were enriched upon the Areg-mediated inhibition of
426 differentiating CD142- ASPCs (**Suppl. Fig. 25**), consistent with previously reported findings
427 on the effect of RA on preadipocytes (Goldstein, Scalia and Ma, 2009; Kim *et al.*, 2013).
428 Moreover, CD142- ASPCs exposed to the secretome of CD142+ ASPCs during adipogenic
429 differentiation showed a decreased expression (compared to CD142- ASPCs being exposed

430 to “dysfunctional” or no Aregs) of a substantial collection of genes reported to be
431 downregulated in RA-mediated adipogenesis suppression, namely *Rxra*, (Sagara *et al.*, 2013),
432 *Pparg*, *Cebpa* (Schwartz *et al.*, 1996), *Mapk14* (Lee *et al.*, 2011), *Zfp423* (Wang *et al.*, 2017)
433 and *Asct2* (Takahashi *et al.*, 2015) (**Suppl. Fig. 25**). Conversely, the expression of genes
434 involved in the EGF-mediated inhibition of adipogenesis, *Erk1* (MAPK3), *Erk2* (MAPK1) and
435 *Prkaca* (PKA C alpha) (Boney, Smith and Gruppuso, 1998; MacDougald and Mandrup, 2002;
436 Harrington, Pond-Tor and Boney, 2007) did not show substantial changes in CD142- ASPCs
437 that were exposed to “active Aregs” compared to CD142- ASPCs exposed to “dysfunctional
438 Aregs” (**Suppl. Fig. 25**). Finally, we found that RA-treated CD142- ASPCs, next to an
439 impaired adipogenic capacity (**Fig. 3J-K**), exhibit also a strikingly consistent transcriptomic
440 signature, with a number of highly specific Areg markers being regulated by RA in a
441 concentration-dependent manner. Indeed, we observed that the expression of *F3* and *Mgp*
442 but also of *Cpe*, *Gdf10*, *Bgn* and *Clec11a* and other Areg-specific genes is gradually
443 upregulated by increasing concentrations of RA (**Fig. 4J, Suppl. Fig. 26**). However, for a
444 number of genes (*F3*, *Mgp*, *Bgn*, *Clec11a*), RA administered at concentrations higher than 10
445 μ M tended to reverse this up-regulation, potentially pointing to a specific RA concentration
446 range (0.01-1 μ M) that may be physiologically relevant for the Areg-dependent inhibition of
447 adipogenesis. Together, these findings strongly suggest that the RA signalling pathway plays
448 an important role in mediating the inhibitory activity of Aregs through a synchronised regulation
449 of Areg-specific genes.

450 Discussion

451 In this study, we systematically examined the molecular and phenotypic properties of murine
452 subcutaneous CD142+ ASPCs, motivated by the reported discrepancy regarding their
453 behaviour in the context of adipogenesis (Schwalie *et al.*, 2018; Hwang and Kim, 2019; Merrick
454 *et al.*, 2019; Corvera, 2021). Using numerous functional and multi-omic assays across distinct
455 experimental settings and sampling conditions, we unambiguously validated the previously
456 proposed non-adipogenic and anti-adipogenic nature of these cells, which is why we suggest
457 to retain their initially proposed name: “Aregs” for adipogenesis regulators (**Fig. 1** and **Suppl.**
458 **Fig. 1-9**).

459

460 These analyses also led to unexpected findings regarding in particular the age-dependent
461 nature of CD142+ ASPCs’ functional properties. It has been shown that the developmental
462 timing of adipose tissue formation varies largely between species (Carberry, Colditz and
463 Lingwood, 2010; Louveau *et al.*, 2016) and different anatomical fat depots (Birsoy *et al.*, 2011;
464 Han *et al.*, 2011; Rosen and Spiegelman, 2014; Hong *et al.*, 2015). Subcutaneous stromal
465 vascular fraction (SVF) cells were shown to be capable of differentiating into lipid-filled
466 adipocytes *in vitro* under adipogenic differentiation medium from embryonic day E16.5 (Birsoy
467 *et al.*, 2011). However, the dynamics of post-natal ASPC differentiation, as well as the
468 emergence of their cellular heterogeneity, are still poorly understood. Having experimentally
469 investigated diverse murine post-natal developmental stages, we found that all fractions of
470 newborn (P0) ASPCs displayed a molecular identity and behaviour (high proliferative and
471 adipogenic propensity) that resemble those of “naïve preadipocytes” (**Suppl. Fig. 12**) (Wang
472 *et al.*, 2003; Atanassova, Rancic and Georgieva, 2012; Hong *et al.*, 2015; Hepler and Gupta,
473 2017). Importantly, P0-derived CD142+ ASPCs did not show a higher “Areg/CD142+ score”
474 (**Suppl. Table 1**, **Fig. 2G-H**, **Suppl. Fig. 14**) nor higher expression of “retinol metabolic
475 process”-related genes (**Suppl. Fig. 13**) compared to the other tested ASPC fractions. This is
476 consistent with the notion that all P0 ASPCs are likely still naïve and indicates that the
477 mesenchymal cellular landscape that is observed in adults has not yet been established in
478 newborns. Further inquiry revealed an age-dependent evolution of both the molecular
479 signature as well as the diverse adipogenic phenotypes of ASPCs. Indeed, we found that the
480 Areg-specific molecular characteristics emerge in CD142+ ASPCs before P16, consistent with
481 their detection in P12 scRNA-seq data (Merrick *et al.*, 2019) and become most prominent in
482 adulthood (**Fig. 2A**, **Suppl. Fig. 10**). Interestingly however, the non- and anti-adipogenic
483 phenotype of CD142+ ASPCs only emerged between post-natal day 16 and 28 (4wo), thus
484 after the establishment of their molecular identity, with, intriguingly, the weaning of the litters
485 occurring during this time period (**Fig. 2D-E**). We cannot formally point to weaning as the
486 primary causal factor for the observed timing offset between Aregs’ molecular and functional
487 appearance. Yet, the fact that weaning entails a dramatic nutritional alteration makes it an
488 intriguing candidate for further investigation.

489

490 While the observed adipogenic phenotype of pre-weaning CD142+ ASPCs is rather striking,
491 it still does not fully resolve the reported functional discrepancy for CD142+ ASPCs given that
492 an adipogenic propensity has also been reported for adult CD142+ APSCs (Merrick *et al.*,
493 2019), which contrasts with our results. Amongst remaining possible reasons for this
494 discrepancy is genetic background. Indeed, Merrick and colleagues used CD1 as opposed to
495 C57BL/6J mice used in other studies, including our own (Burl *et al.*, 2018; Hepler *et al.*, 2018;
496 Schwalie *et al.*, 2018; The Tabula Muris Consortium *et al.*, 2018; Cho, Lee and Doles, 2019;

497 Zhang *et al.*, 2019; Sárvári *et al.*, 2021). Metabolic variation as a function of genetic
498 background is widely recognized in the field (Fontaine and Davis, 2016). Yet, how ASPC
499 heterogeneity and function may vary across individuals / strains has not yet been investigated
500 and will thus constitute an exciting downstream research avenue.

501
502 Given the demonstrated non-adipogenic and inhibitory properties of Aregs, understanding
503 how these functional properties are molecularly regulated is highly relevant. Since our findings
504 demonstrated that Aregs exert their inhibitory properties *via* paracrine signalling, we focused
505 on genes coding for secreted factors. We identified six candidates that were specific to Aregs
506 across multi-omic datasets: *F3* (coding for CD142) itself, *Mgp*, *Gdf10*, *Clec11a*, *Cpe* and *Bgn*,
507 with CD142 and MGP the most interesting functionally, based on recombinant protein as well
508 as knockdown assays (**Fig. 3F-G, Suppl. Fig. 16, Fig. 4A-B**). The involvement of CD142 in
509 Aregs' inhibitory activity is surprising given the reported physiological role of CD142 as a
510 coagulation factor (Chu, 2011). CD142, also known as Tissue factor, is the primary initiator in
511 the extrinsic coagulation pathway (Petersen, Valentin and Hedner, 1995) and has not been
512 explicitly shown to be involved in adipogenesis-related processes. MGP (Matrix Gla protein,
513 a member of a family of vitamin-K2 dependent, Gla-containing proteins) has been
514 demonstrated to act as an inhibitor of calcification in cartilage and vasculature (Bäck *et al.*,
515 2019), implying its possible specificity to mesenchymal cells with multilineage potential.
516 Finally, while the inhibitory effect of GDF10 has been previously demonstrated in the context
517 of adipogenesis in Areg-like/CD142+ muscle-resident stromal cells (Camps *et al.*, 2020) as
518 well as in differentiating 3T3-L1 cells (Hino *et al.*, 2012), we found that inactivating *Gdf10* in
519 Aregs did not majorly interfere with their inhibitory activity towards other ASPCs.

520
521 Next to these secretory proteins, we uncovered the RA-signalling pathway as another likely
522 actor that is involved in the Areg-mediated inhibition of adipogenesis. Retinoic acid has long
523 been linked to adipogenic inhibition (Murray and Russell, 1980; Kuri-Harcuch, 1982; Salazar-
524 Olivo *et al.*, 1994; Schwartz *et al.*, 1996; Lee *et al.*, 2011; Sagara *et al.*, 2013; Wang *et al.*,
525 2017) and demonstrated to be protective against diet-induced obesity (Berry and Noy, 2009;
526 Bonet, Ribot and Palou, 2012). Throughout our analyses, we identified a substantial number
527 of genes related to RA signalling to be specific to Aregs and some of them, including *Aldh1a2*,
528 *Epha3*, *Osr1* and *Rbp1* are *bona fide* Areg markers (**Fig. 3H, Suppl. Fig. 18**). Furthermore,
529 transcriptomic profiling of freshly isolated Aregs suggests that they produce retinol (**Fig. 4I**,
530 **Suppl. Fig. 19A-B**), a precursor of RA. In addition, RA-related terms, particularly "cellular
531 response to RA" and "RA receptor signalling pathway" were enriched in CD142- ASPCs that
532 were subjected to active Areg signalling (**Fig. 4I, Suppl. Fig. 4A, Suppl. Fig. 19C-D**).
533 Together, these results suggest that Aregs might exert their anti-adipogenic activity *via* RA
534 itself, as further supported by the remarkably coherent transcriptional dynamics of a set of
535 genes involved in RA-mediated inhibition in CD142- ASPCs exposed to active Aregs. For
536 example, we found that Areg-treated CD142- ASPCs were transcriptionally more similar to
537 CD142- ASPCs treated with RA than those treated with EGF, another well-established
538 adipogenesis inhibitor (**Fig. 4J, Suppl. Figs. 24-25**) (Harrington, Pond-Tor and Boney, 2007).
539 This transcriptional similarity reflects a more general, intriguing phenomenon in that Areg-
540 treated CD142- ASPCs exhibited a significantly increased expression of many Areg-specific
541 genes, a number of which were also upregulated in RA-treated CD142- ASPCs (**Fig. 4D-G**,
542 **Suppl. Fig. 26**). These results point to a potential conversion of CD142- ASPCs, when
543 subjected to inhibitory signals, into Areg-like cells, a phenomenon of interconversion of various

544 ASPC subpopulations that has been recently proposed to occur within the subcutaneous
545 adipogenic stem cell niche (Merrick *et al.*, 2019). It is thereby worth noting that the
546 transcriptome of Aregs exposed to adipogenic cocktail also showed a response to RA (**Suppl.**
547 **Fig. 19C-D**), suggesting that they exert an auto-inhibitory effect that may impair their own
548 ability of generating *in vitro* adipocytes.

549

550 In conclusion, our findings systematically authenticated Aregs as a molecularly, phenotypically
551 and functionally robust inhibitory subpopulation of murine subcutaneous ASPCs. This
552 subpopulation emerges within the ASPCs during early stages of post-natal development. The
553 establishment of the molecular signature of Aregs precedes the manifestation of their
554 phenotypic (non-adipogenic) and functional (anti-adipogenic) properties, with those two
555 events being separated by weaning. We finally uncovered that the murine Areg-mediated
556 inhibition of adipogenesis involves the secreted factors CD142 (encoded by *F3*) and Matrix
557 Gla protein (MGP), which act together with the RA signalling pathway. This set of factors
558 seems at first glance unrelated, but intriguingly, the expression of *F3* and *Mgp* (as well as
559 *Aldh1a2* and *Bgn*), has already been shown to be regulated by retinoic acid (Balmer and
560 Blomhoff, 2002; Takeda *et al.*, 2016) as we also demonstrated for these and other Areg-
561 specific markers (**Fig. 4J, Suppl. Fig. 26**). Furthermore, these genes were reported to be
562 higher expressed in visceral compared to subcutaneous ASPCs and have as such been
563 associated with the impaired capacity of visceral ASPCs to give rise to *in vitro* adipocytes
564 (Reichert *et al.*, 2011; Meissburger *et al.*, 2016; Takeda *et al.*, 2016; Li *et al.*, 2020). Further
565 studies will now be required to investigate how this seemingly diverse collection of molecules
566 may cooperate within the retinoic acid signalling pathway to steer the developmental,
567 phenotypic and functional properties of Aregs.

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729

730 **Author contributions**

731 B.D., M.Z. and P.Y.R. designed the study and wrote the manuscript. M.Z. and P.Y.R.
732 conducted the experiments and analyses: M.Z. performed all the experimental assays
733 including murine SVF extraction, FACS-based isolation, cell culture experiments, imaging,
734 image analyses and quantification. P.Y.R. performed all single-cell-, bulk RNA-sequencing-
735 and mass-spectrometry-related analyses. J.R. and D.A. performed bulk RNA sequencing and
736 data pre-treatment, H.H., R.F. and M.L. assisted with experimental procedures and analyses.
737 All authors read and approved the final manuscript.

738

739 **Conflict of interest**

740 The authors declare that they have no competing interests.

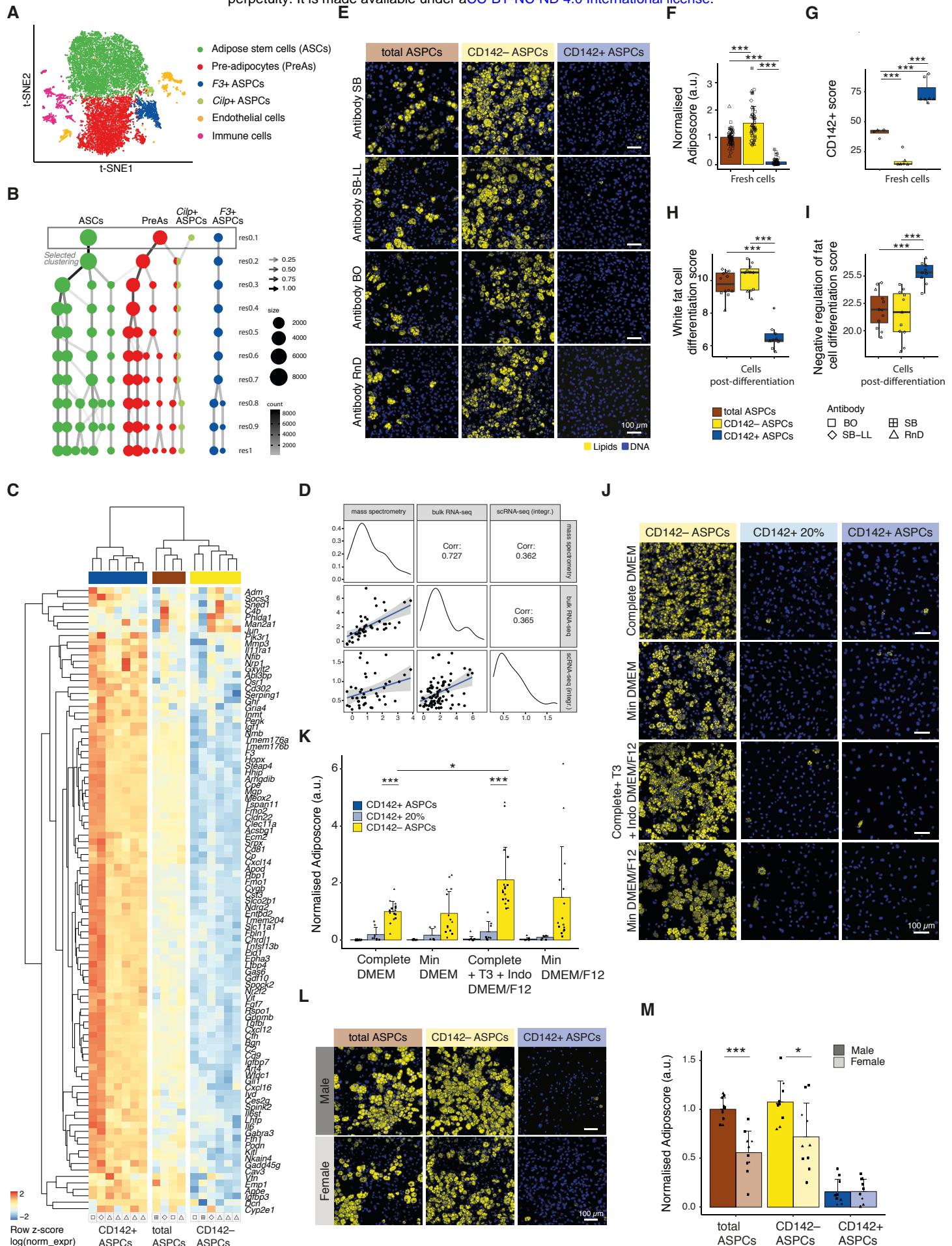


Figure 1 | See next page for caption

Figure 1. CD142+ ASPCs constitute a robust ASPC subpopulation defined by a stable non-adipogenic phenotype and a highly specific transcriptomic signature

(A) t-SNE cell map of integrated scRNA-seq datasets (see **Methods**) visualizing the main identified subpopulations of murine subcutaneous ASPCs: adipose stem cells (ASCs) in green, pre-adipocytes (PreAs) in red, *F3*(CD142)+ ASPCs in blue, as well as *Cilp*+ ASPCs, endothelial and immune cells (see also **Suppl. Fig. 1**);

(B) Clustering tree of the Seurat-based clustering result of the integrated analysis described in **A**, visualizing the relationships between clustering at different resolutions of the three main ASPC subpopulations as well as *Cilp*+ ASPCs, demonstrating a high stability of the *F3*(CD142)+ ASPC cluster;

(C) Gene expression heatmap of the top CD142+ markers (**Suppl. Table 1**) across bulk RNA-seq samples of freshly isolated total, CD142– and CD142+ ASPCs; log normalized expression scaled by row;

(D) Correlation of the logFC of top CD142+ markers (**Suppl. Table 1**) across scRNA-seq, bulk RNA-seq and mass spectrometry data; logFC was defined as the \log_2 FC of freshly isolated CD142+ over CD142– ASPCs for bulk RNA-seq and mass spectrometry, and as the average logFC of *F3*(CD142)+ over the remaining cells in scRNA-seq across integrated datasets described in **A** (**Methods**);

(E) Representative fluorescence microscopy images of total, CD142– and CD142+ ASPCs isolated with the use of the respective anti-CD142 antibodies: SinoBiological (SB), SinoBiological-LL (SB-LL), BiOrbyt (BO) and R&D Systems (RnD) (**Suppl. Fig. 2B**, **Suppl. Fig. 5A**, **Methods**), after *in vitro* adipogenic differentiation;

(F) Fraction of differentiated cells per ASPC type shown in **E**, as quantified by the “adiposcore”; marker shapes correspond to different anti-CD142 antibodies used for isolation as indicated, n=9-15, 3-4 biological replicates, 3-5 independent wells for each;

(G) Boxplot showing the distribution of the “CD142+ score” based on the expression of the top CD142+ ASPC markers (**Suppl. Table 1**, **Methods**);

(H) Boxplot showing the distribution of the “white fat cell differentiation score” based on the expression of the genes linked to the GO term “white fat cell differentiation” (GO:0050872) (**Methods**);

(I) Boxplot showing the distribution of the “negative regulation of fat cell differentiation score” based on the expression of the genes linked to the GO term “negative regulation of fat cell differentiation” (GO:0045599) (**Methods**);

(J) Representative fluorescence microscopy images of CD142–, 20% CD142+ and (5-7%) CD142+ ASPCs isolated following the sorting strategy shown in **Suppl. Fig. 7A**, after *in vitro* adipogenic differentiation with the indicated white adipogenic differentiation cocktails (**Methods**);

(K) Fraction of differentiated cells per ASPC type and differentiation cocktail shown in **J**, as quantified by the “adiposcore”; marker shapes correspond to different biological replicates, n=8-17, 3-5 biological replicates, 2-5 independent wells for each;

(L) Representative fluorescence microscopy images of male- and female-derived total, CD142– and CD142+ ASPCs, isolated following the sorting strategy shown in **Suppl. Fig. 8A** after *in vitro* adipogenic differentiation;

(M) Fraction of differentiated cells per ASPC type and per sex shown in **L**, as quantified by the “adiposcore”; bar colour shading corresponds to male- and female-derived cells as indicated; marker shapes correspond to different biological replicates, n=9-10, 4 biological replicates, 2-3 independent wells for each;

In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow); scale bars, 100 μ m; bar colours: total ASPCs — brown, CD142– ASPCs — yellow, CD142+ ASPCs — blue, 20% CD142+ ASPCs — turquoise; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, pairwise two-sided *t*-test (**K**, **M**) or one-way ANOVA and Tukey HSD *post hoc* test (**F-I**), for statistical details see **Methods**.

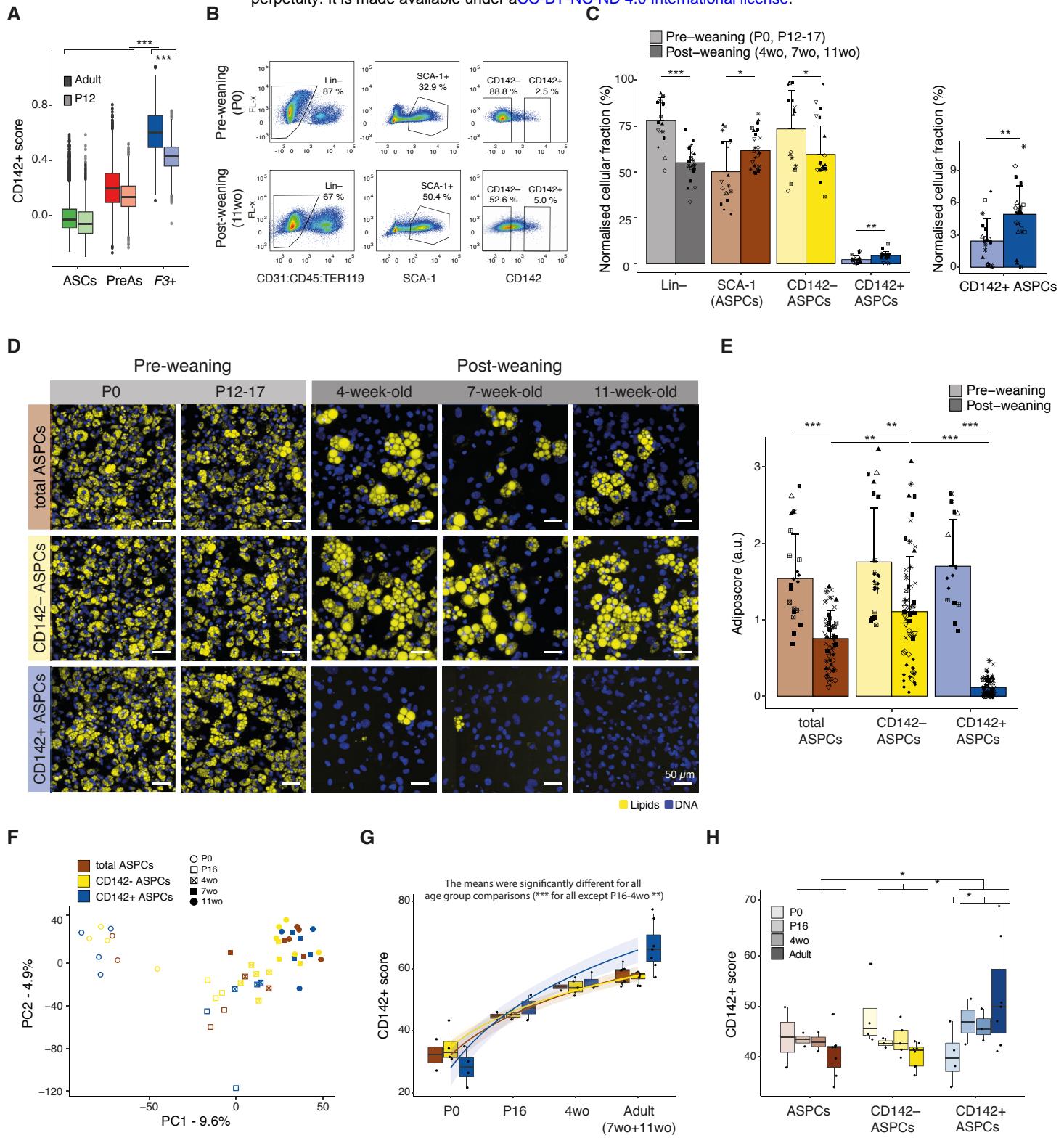


Figure 2 | See next page for caption

Figure 2. Age-dependent molecular and phenotypic emergence of *bona fide* CD142+ ASPCs

(A) Boxplot showing the distribution of the “CD142+ score” (**Suppl. Table 1**) in adult (darker colours) and P12 (lighter colours) ASPCs across the three main ASPC subpopulations (adipose stem cells (ASCs) in green, pre-adipocytes (PreAs) in red and *F3*(CD142)+ ASPCs in blue);

(B) FACS-based gating strategy of pre-weaning (new-borns (P0))- and post-weaning (11-week-old (wo))-derived Lin- (defined as CD31– CD45– TER119–), SCA-1+ (total ASPC), CD142– and CD142+ ASPC cellular fractions within the subcutaneous adipose SVF; at least 8 biological replicates were performed, shown here is one representative biological replicate;

(C) Bar plots showing the normalised parental percentage of pre- and post-weaning indicated cellular fractions; the graph on the right represents the fractions of CD142+ ASPCs plotted separately; marker shapes correspond to different biological replicates, n=8-10;

(D) Representative fluorescence microscopy images of P0-, P12-17-, 4wo-, 7wo- and 11wo-derived total, CD142– and CD142+ APSCs after *in vitro* adipogenic differentiation;

(E) Fraction of differentiated cells per ASPC type shown in D, as quantified by the “adiposcore”; bar colour shading corresponds to pre- and post-weaning-derived cells as indicated; marker shapes correspond to different biological replicates, n=14-65, 12-13 biological replicates, 2-6 independent wells for each;

(F) PCA based on the bulk RNA-seq data of freshly isolated P0-, P16-, 4wo-, 7wo- and 11wo-mice-derived total, CD142– and CD142+ ASPCs;

(G) Boxplot showing the distribution of the “CD142+ score” (**Suppl. Table 1**) across ages and tested cellular fractions (freshly isolated total, CD142– and CD142+ ASPCs) showing the age-dependent emergence of the CD142+ signature; see colour legend in F;

(H) Boxplot showing the distribution of the “CD142+ score” (**Suppl. Table 1**) across ages and tested cellular fractions (freshly isolated total, CD142– and CD142+ ASPCs) using the log normalized expression corrected for age-driven source of variation; see colour legend in F;

In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow); scale bars, 50 μ m; bar colours: total ASPCs — brown, CD142– ASPCs — yellow, CD142+ ASPCs — blue; pre-weaning data are represented in lighter shades; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, pairwise two-sided *t*-test (A, C, E, H) or one-way ANOVA and Tukey HSD *post hoc* test (G, null hypothesis: no difference in means across age), for statistical details see **Methods**.

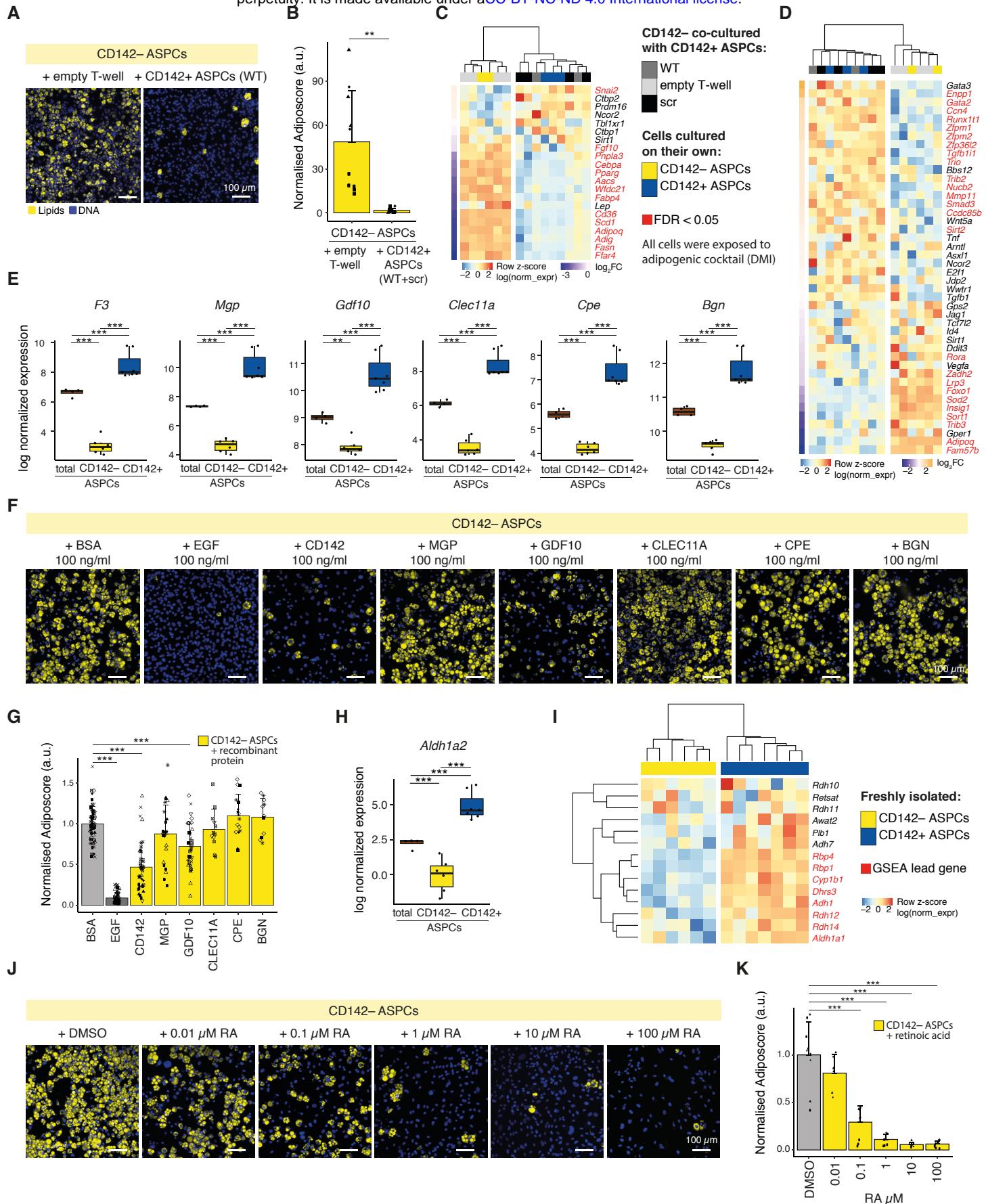


Figure 3 | See next page for caption

Figure 3. CD142+ ASPC (Areg)-specific candidates and their involvement in adipogenic inhibition

(A) Representative fluorescence microscopy images of CD142- ASPCs co-cultured with an empty transwell (T-well) or with wild-type (WT) CD142+ ASPCs or CD142+ ASPCs carrying control siRNA (scr) after *in vitro* adipogenic differentiation;

(B) Fraction of differentiated CD142- ASPCs indicated in **A**, as quantified by the “adiposcore”; marker shapes correspond to different biological replicates, n=10, 4 biological replicates, 2-3 independent wells for each;

(C) Expression heatmap listing genes linked to “white fat cell differentiation” (GO:0050872) across bulk RNA-seq samples of CD142+ and CD142- ASPCs after adipogenic differentiation (i.e. after exposure to an adipogenic cocktail, **Methods**); the genes are ordered from top to bottom by the log₂FC of CD142+ over CD142- ASPCs after adipogenic differentiation; significantly differentially expressed genes (FDR < 0.05) are coloured in red; log normalized expression scaled by row;

(D) Expression heatmap listing genes linked to “negative regulation of fat cell differentiation” (GO:0045599) across bulk RNA-seq samples of CD142+ and CD142- ASPCs after adipogenic differentiation; the genes are ordered from top to bottom by the log₂FC of CD142+ over CD142- ASPCs after adipogenic differentiation; significantly differentially expressed genes (FDR < 0.05) are coloured in red; log normalized expression scaled by row;

(E) Bulk RNA-seq-derived expression plots of CD142+ ASPC (Areg) markers coding for secreted proteins that were selected for downstream validation: *F3* (coding for CD142), *Mgp* (coding for Matrix Gla protein, MGP), *Gdf10* (GDF10), *Clec11a* (CLEC11A), *Cpe* (Carboxypeptidase E, CPE) and *Bgn* (Biglycan, BGN);

(F) Representative fluorescence microscopy images of adult-derived CD142- ASPCs after *in vitro* adipogenic differentiation; the induction cocktail was supplemented with recombinant proteins corresponding to the selected Areg-specific candidates: CD142, MGP, GDF10, CLEC11A, CPE and BGN at 100 ng/ml (**Methods**);

(G) Fraction of differentiated adult-derived CD142- ASPCs, as quantified by the “adiposcore”, treated with the indicated recombinant proteins shown in **F**; marker shapes correspond to different biological replicates, n=11-59, 2-10 biological replicates, 3-9 independent wells for each;

(H) Bulk RNA-seq-derived expression plot of *Aldh1a2* (coding for Retinal dehydrogenase, RALDH2);

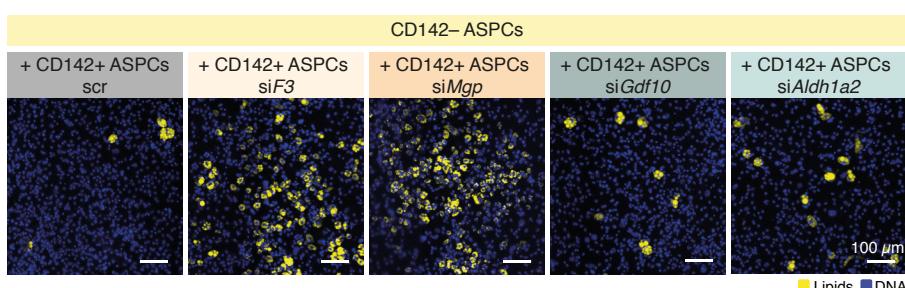
(I) Expression heatmap listing genes linked to “retinol metabolic process” (GO:0042572) across bulk RNA-seq samples of freshly isolated CD142- and CD142+ ASPCs; genes identified as lead by GSEA (**Methods**) are coloured in red; log normalized expression scaled by row;

(J) Representative fluorescence microscopy images of adult-derived CD142- ASPCs after *in vitro* adipogenic differentiation with the differentiation cocktail supplemented with DMSO (RA carrier) and RA at the indicated concentrations;

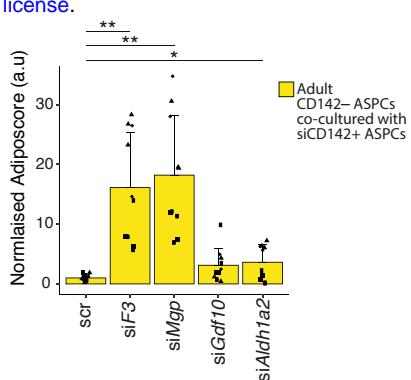
(K) Fraction of differentiated CD142- ASPCs, as quantified by the “adiposcore”, treated with the indicated recombinant proteins shown in **J**; marker shapes correspond to different biological replicates, n=9, 3 biological replicates, 3 independent wells for each;

In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow); scale bars, 100 µm, bar colours: total ASPCs — brown, CD142- ASPCs — yellow, CD142+ ASPCs (Aregs) — blue, recombinant BSA or DMSO treatment (negative controls) — light grey, recombinant EGF treatment (positive control) — dark grey; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, pairwise two-sided *t*-test (**B**, **G**, **K**) or one-way ANOVA and Tukey HSD *post hoc* test (**E**, **H**), for statistical details see **Methods**.

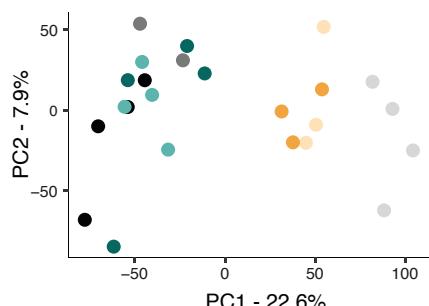
A



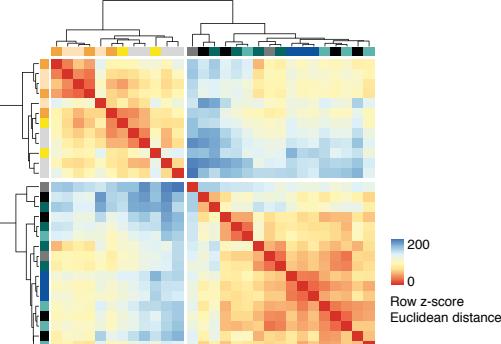
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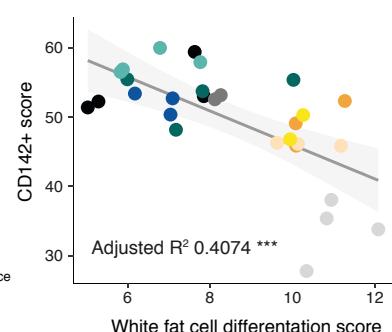
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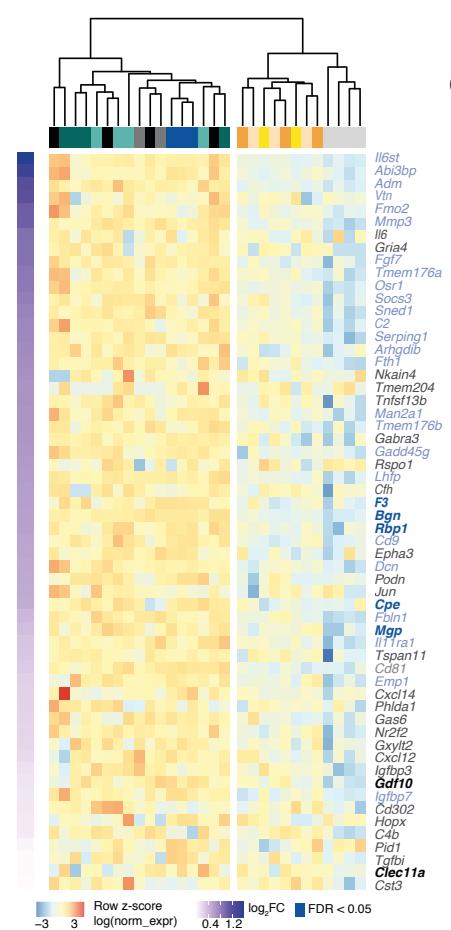
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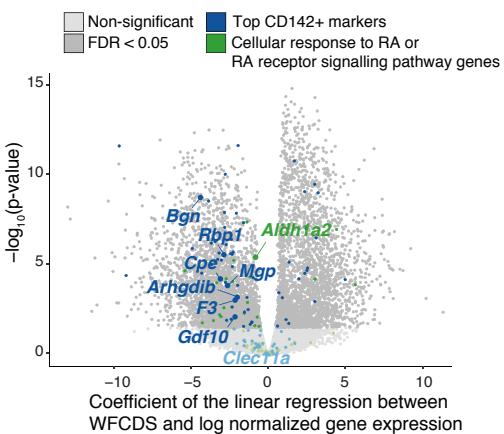
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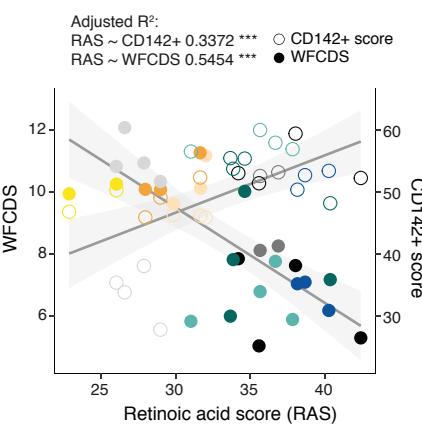
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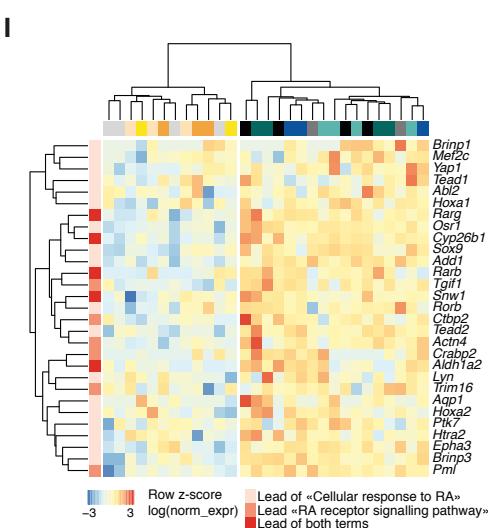
CD142- co-cultured with CD142+ ASPCs:

WT	scr	siF3
empty T-well		siMgp
		CD142- ASPCs
		CD142+ ASPCs
siGdf10		
siAldh1a2		

Cells cultured on their own:

CD142- ASPCs

All samples were exposed to an adipogenic cocktail (DMI)



I

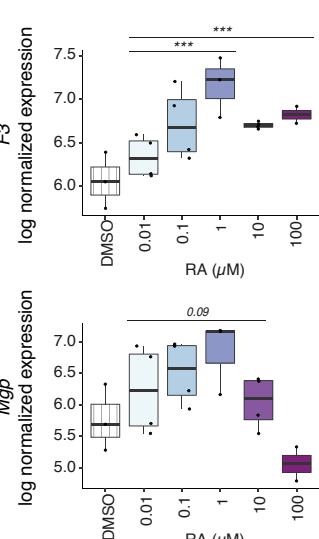


Figure 4 | See next page for caption

Figure 4. Molecular (auto-)regulation of CD142+ ASPC (Areg)'s inhibitory activity *via* RA signalling

(A) Representative fluorescence microscopy images of CD142- ASPCs, co-cultured with CD142+ ASPCs (Aregs) carrying control (scr) or siRNA-mediated knockdowns of selected CD142+ ASPC (Areg)-specific candidate genes: *F3*, *Mgp*, *Gdf10* and *Aldh1a2* after adipogenic differentiation (i.e. after exposure to an adipogenic cocktail, **Methods**);

(B) Fraction of differentiated CD142- ASPCs, as quantified by the “adiposcore”, co-cultured with CD142+ ASPCs (Aregs) carrying knockdowns indicated in **A**; marker shapes correspond to different biological replicates, n=10, 4 biological replicates, 2-3 independent wells for each;

(C) PCA based on bulk RNA-seq data of CD142- ASPCs co-cultured with CD142+ ASPCs (Aregs) carrying knockdowns as indicated in **A**; see colour legend at the bottom of the figure;

(D) Expression heatmap of top CD142+ markers (i.e. Areg markers, **Suppl. Table 1**) with a positive \log_2 FC when performing differential expression analysis between CD142- ASPCs that were exposed to “active Aregs” (WT, scr, si*Gdf10*, si*Aldh1a2*) *versus* CD142- ASPCs that were exposed to “dysfunctional Aregs” (si*F3*, si*Mgp*) across the same bulk RNA-seq samples; genes are ordered from the highest (top) to the lowest (bottom) \log_2 FC; significantly differentially expressed genes (FDR < 0.05) are coloured in blue; Areg candidates are highlighted in bold; log normalized expression scaled by row; see colour legend at the bottom of the figure;

(E) Heatmap showing the Euclidian distance of the transcriptomic data of CD142- ASPCs co-cultured (*via* transwell) with distinct CD142+ ASPC knockdown types or controls as well as CD142+ or CD142- ASPCs post-differentiation, calculated on the five first principal components of the PCA shown in **Suppl. Fig. 23D**;

(F) Correlation of “white fat cell differentiation score” *versus* “CD142+ score”; see colour legend at the bottom of the figure;

(G) Volcano plot showing the coefficient of the linear regression performed between “white fat cell differentiation score” (WFCDS) and log normalized gene expression (WFCDS \sim log(normalized expression)) (x axis) *versus* the $-\log_{10}$ (p-value) (y axis); top CD142+ markers (i.e. Areg markers, **Suppl. Table 1**) are highlighted in blue and genes linked to “cellular response to RA” (GO:0071300) and “RA receptor signalling pathway” (GO:0048384) terms in green;

(H) Correlation plot of “RA score” (RAS) (based on the expression of genes linked to “cellular response to RA” (GO:0071300) and “RA receptor signalling pathway” (GO:0048384)) *versus* “CD142+ score” (**Suppl. Table 1**) or “white fat cell differentiation score” (WFCDS) (GO:0050872);

(I) Expression heatmap of the lead genes identified by GSEA of the significantly enriched terms “cellular response to RA” (GO:0071300) and “RA receptor signalling pathway” (GO:0048384); GSEA identified these two terms as enriched when performed on the genes driving PC1 shown in **C**; see colour legend at the bottom of the figure;

(J) Boxplots showing the distribution of the log normalized expression of *F3* (**top**) or *Mgp* (**bottom**) across transcriptomic data of CD142- ASPCs treated with an adipogenic cocktail supplemented with DMSO or different concentrations of RA (0.01 to 100 μ M); the indicated significance is based on the result of differential expression analysis between all RA treated samples or samples treated with RA of a concentration between 0.01 and 1 μ M *versus* controls (treatment with DMSO with or without DMSO);

In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow); scale bars, 100 μ m; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, pairwise two-sided *t*-test (**B**), for statistical details see **Methods**.