

1 **Title: Toxicity impacts on human adipose MSCs acutely exposed to Aroclor and non-  
2 Aroclor mixtures of PCBs.**

3 **Authors**

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8 **ABSTRACT**

9 PCBs accumulate in adipose where they may impact the growth and function of cells within the  
10 tissue. This is particularly concerning during adolescence when adipocytes expand rapidly.  
11 Herein we sought to understand how exposure to PCB mixtures found in U.S. schools affects  
12 human adipose mesenchymal stem/stromal cell (MSC) health and function. We investigated how  
13 exposure to Aroclor 1016 and Aroclor 1254, as well as a newly characterized non-Aroclor  
14 mixture that resembles the PCB profile found in cabinets, Cabinet Mixture, affects adipose MSC  
15 growth, viability, and function in vitro. We found that exposure to all three mixtures resulted in  
16 two distinct types of toxicity. At PCB concentrations >20  $\mu$ M, the majority of MSCs die, while  
17 at 1-10  $\mu$ M MSCs remained viable but display numerous alterations to their phenotype. At these  
18 sublethal concentrations, MSC rate of expansion slowed, and morphology changed. Further  
19 assessment revealed PCB-exposed MSCs had impaired adipogenesis and a modest decrease in  
20 immunosuppressive capabilities. Thus, exposure to PCB mixtures found in schools negatively  
21 impacts the health and function of adipose MSCs. This work has implications for human health  
22 due to MSCs' role in supporting the growth and maintenance of adipose tissue.

23 **SYNOPSIS**

24 PCB mixtures found in schools are toxic to human adipose mesenchymal stem/stromal cells,  
25 stunting their growth and altering their function in ways that could contribute to metabolic  
26 diseases.

27 **Key Words (words not in title):** Mesenchymal stem cell, persistent organic pollutants,  
28 endocrine disrupting chemical, EDC

29 **INTRODUCTION (1.5 pages total)**

30 There is significant need to understand how exposure to polychlorinated biphenyl (PCB)  
31 mixtures found both in old and new schools contributes to the dysfunction of adipose tissue.  
32 PCBs are a group of environmental toxins containing 209 distinct congeners that were heavily  
33 produced globally from the late 1920s until being banned in 1979.<sup>1</sup> Despite being banned,  
34 mixtures of different PCB congeners can still be found in capacitors (Aroclor 1016),  
35 transformers (Aroclor 1254), caulk (Aroclor 1254), and many other building materials making  
36 them ubiquitous in public spaces across the United States.<sup>1-4</sup> Furthermore, evidence of Aroclor  
37 1016 and 1254 has been found in schools as recent as 2021.<sup>5</sup>

38 While intentional production of PCBs is banned, PCBs continue to be produced as contaminants  
39 in products such as pigments and varnishes used as finishes.<sup>6-8</sup> One study found finished  
40 cabinetry to be a novel, non-Aroclor source of PCB mixtures leading to elevated levels of PCBs

47 in residential houses and apartments.<sup>9</sup> It was also shown that factory workers exposed to these  
48 materials accumulate significant levels of cabinet mixture PCBs (PCB 47, PCB 51, and PCB 68)  
49 in their plasma and urine.<sup>10</sup>

50  
51 Occupational exposure is not the only way humans are exposed to PCBs. While exposure to  
52 PCBs was once thought to be primarily through ingestion of contaminated food, it is now clear  
53 that inhalation is a major route of human exposure, specifically for the semi-volatile, lower-  
54 chlorinated PCBs.<sup>11</sup> These semi-volatile PCBs do not remain fixed in place, but become  
55 volatilized over time, making them a persistent source of PCB exposure to those in the  
56 environment.<sup>10</sup> Not only have non-Aroclor sources been found on small scales in residential  
57 homes, but these sources now have widespread distribution in the air of places such as Chicago  
58 despite not being manufactured in high levels prior to the PCB ban.<sup>12</sup> Due to the wide variety of  
59 old and new products containing PCBs and the lack of natural degradation pathways for many  
60 PCBs, there continues to be widespread contamination from both Aroclor and non-Aroclor  
61 sources in buildings made with PCB-containing materials.

62  
63 A particularly vulnerable population to PCB exposure is school-aged children, as many schools  
64 still in use today were built prior to the PCB-ban and newer buildings are likely to contain  
65 pigments and cabinetry finishes that contain non-Aroclor mixtures of PCBs. Because PCBs are  
66 semi-volatile, adolescents are exposed through inhalation of PCBs while at school. Studies of  
67 school air have found significantly elevated levels of PCBs in the air of many schools.<sup>13,14</sup> One  
68 study found concentrations inside schools were 10-100 times higher than outdoors.<sup>15</sup> While  
69 attending these schools, children accumulate PCBs. One study found that lower-chlorinated  
70 PCBs were detected in 95% of pupils attending a contaminated school compared to only 27% of  
71 the students at a non-contaminated school.<sup>14</sup> Further rat studies using PCB mixtures similar to  
72 those found in schools (a combination of Aroclor 1254 and 1221) have reported PCB  
73 accumulation in tissues such as the liver and adipose tissue.<sup>16,17</sup> Since children continue to be  
74 exposed to these PCB sources in their day to day lives, there is a dire need to understand how  
75 exposure to PCB mixtures affects lipid rich tissues, such as adipose.

76  
77 While often thought of as just a storage depot for fat, adipose communicates with multiple other  
78 organ systems through endocrine signaling and plays a critical role in regulating whole-body  
79 energy metabolism. For example, adipose tissue stores excess lipids not only for caloric reserve,  
80 but also to avoid ectopic fat deposition in other organs such as the liver, muscle, and heart which  
81 would promote systemic complications such as non-alcoholic fatty liver disease, diabetes, and  
82 heart disease.<sup>18</sup> Adipose tissue also releases adipokines, like adiponectin, which enhances insulin  
83 sensitivity and suppresses production of inflammatory cytokines such as TNF $\alpha$ .<sup>19</sup> Thus,  
84 disruptions to adipose tissue metabolism and/or endocrine signaling can result in metabolic  
85 syndromes such as obesity, diabetes, and hyperlipidemia.<sup>20</sup> During adolescence adipose tissue  
86 undergoes massive expansion via the proliferation and differentiation of adipose progenitors,  
87 also called adipose mesenchymal stem/stromal cells (MSC). Although adipose MSCs are  
88 responsible for maintaining healthy turnover rates of adipocytes throughout a person's lifespan,  
89 these progenitor cells are particularly important during adolescence, when the number of adipose  
90 cells more than quadruples before becoming relatively constant in adulthood.<sup>21</sup> Additionally,  
91 these adipose MSCs are active contributors to regulating local inflammation, particularly in the  
92 early stages of obesity.<sup>22,23</sup> Early in the development of metabolic syndromes, adipose MSCs will

93 produce high levels of MCP-1 leading to increased immune cell infiltration and inflammation.<sup>24</sup>  
94 Other studies have shown stimulation of adipose MSCs is critical to regulating adipose  
95 inflammation.<sup>25</sup> Since proper tissue expansion and immune function is imperative for adipose  
96 health, disruption of these adipose MSCs by environmental toxins would lead to disruption of  
97 metabolic health as the adipose becomes less able to replace adipocytes or control adipose  
98 inflammation.<sup>20,26,27</sup> Therefore, it is imperative to understand if PCBs directly impact adipose  
99 MSCs. While many studies have been performed on adipocytes or pre-adipocytes exposed to  
100 PCBs, to date, the effects of PCB exposure on primary human adipose MSC has not been  
101 previously evaluated.<sup>28</sup>

102  
103 Herein we systematically analyze how three different PCB mixtures impact human adipose  
104 MSCs. Two of the mixtures are found in legacy sources, Aroclor 1016 and Aroclor 1254, while  
105 the third mixture has been derived to mimic the PCB congeners recently found to be emitted  
106 from new cabinetry, Cabinet Mixture.<sup>9,29</sup> All three mixtures were recently identified in a study  
107 looking at room-to-room variations in PCBs. PCB 47, the primary congener found in Cabinet  
108 Mixture, was identified in rooms built after 2012. Whereas rooms built before 1970 had evidence  
109 of Aroclor 1016 and 1254 likely from the use of fluorescent light fixtures and caulking  
110 respectively.<sup>5,15</sup> Not only are these mixtures relevant to modern human exposure, but they also  
111 represent a wide range of congeners. Aroclor 1016 is comprised of primarily lower-chlorinated,  
112 non-dioxin-like PCBs (Dioxin TEQ: 0.09)<sup>30</sup>; Aroclor 1254 is comprised of higher-chlorinated  
113 PCBs including several dioxin-like congeners (Dioxin TEQ: 21)<sup>30</sup>; Cabinet Mixture is three non-  
114 dioxin-like congeners.<sup>9,31</sup> A range of concentrations of each mixture are used to assess how  
115 short-term exposure impacts human adipose MSC growth, viability, and functional phenotype.  
116

## 117 **METHODS**

118

### 119 **Materials**

#### 120 *Sources of PCBs*

121 Aroclor 1016 and Aroclor 1254 (lot number KC 12-638) in the original containers from  
122 Monsanto (St. Louis, MO) were provided by the Synthesis Core of the Iowa Superfund Research  
123 Program (ISRP). The PCB congener profiles of both Aroclors have been reported previously.<sup>32,33</sup>  
124 The cabinet PCB mixture was prepared by mixing 2,2',4,4'-tetrachlorobiphenyl (PCB 47),  
125 2,2',4,6'-tetrachlorobiphenyl (PCB 51), and 2,3',4,5'-tetrachlorobiphenyl (PCB 68) from  
126 AccuStandard (New Haven, CT, USA) in a weight ratio of 75:17:8. The original data and  
127 characterization of cabinet mixture are openly available through the Iowa Research Online  
128 repository at <https://doi.org/10.25820/data.006184>.  
129

130

#### 131 *Cell culture media*

132 Unless otherwise specified, cells were cultured in MEM-alpha (Thermo Fisher, Cat#: 12561049)  
133 supplemented with 1% (v/v) penicillin/streptomycin (Life Technologies), 1% (v/v) L-glutamine  
134 (Life Technologies), and 0.5% or 15% fetal bovine serum (VWR) depending on the experiment.  
135 For differentiation of adipose MSCs to adipocytes, two additional media formulations were used.  
136 Initiation of differentiation was done with Preadipocyte Differentiation Media (PDM-2) (Lonza,  
137 Cat: #PT-8002). Maintenance of differentiation was done with DMEM supplemented with 1.9  
138 ng/mL Insulin (Sigma-Aldrich, Cat: #91077C) and 10% FBS.

139 **Isolation and characterization of adipose-derived MSC**

140 MSCs were isolated from the stromal vascular fraction of human adipose. Briefly, adipose tissue  
141 from three breast reduction surgeries, donors 20-40 years of age, were obtained from the  
142 University of Iowa Tissue Procurement Core. The core collects tissue specimens from surgeries  
143 performed at the University of Iowa Hospitals and Clinics after obtaining informed consent  
144 according to an approved IRB held by the core. The core then removes any identifying  
145 information and provides the de-identified tissue to researchers. Once the tissue was obtained,  
146 adipose was dissected out, minced into small pieces, and incubated overnight in collagenase. The  
147 next day, the tissue was further disrupted via serial pipetting and centrifuged to separate the  
148 stromal vascular fraction from the lipid-rich layer. The SVF was collected, washed 3 times, and  
149 plated in polystyrene flasks with MEM-alpha growth media supplemented with 15% FBS. 4  
150 hours after plating, any unattached cells were discarded, and the remaining cells were cultured  
151 until 70% confluent. The cells were then passaged 1:3 and expanded into a P1 generation for  
152 cryobanking and analysis of surface markers and differentiation potential.

153

154 To determine if the isolated cells were indeed MSCs, they were tested for conformance to the  
155 MSC minimal criteria.<sup>34</sup> Cells between passage 1 and 2 were stained for CD90, CD73, CD105  
156 CD34, CD45, CD11b, CD19, and HLA-DR surface expressions. Positive surface marker  
157 expression staining was carried out using PE-CD90 antibody (BD Biosciences, A15794), PE.  
158 Cy7-CD73 antibody (BD Biosciences, Cat #561258), and FITC-CD105 antibody (BD  
159 Biosciences, Cat #561443) with their corresponding isotype controls: PE-CD90 Mouse IgG1  
160 (Invitrogen, Cat #GM4993), PE. Cy7 Mouse IgG1k (BD Biosciences, Cat #557872), and FITC  
161 Mouse IgG1k (BD Biosciences, Cat #56649) respectively. CD34, CD45, CD11b, CD19, and  
162 HLA-DR were assessed using a PE-conjugated hMSC Negative Cocktail (BD Biosciences, Cat  
163 #562530). After staining, cells from each donor were analyzed on a Cytek Northern Lights  
164 Spectral Cytometer (**Supplemental Figure 1**).

165

166 **Metabolic Function Assay**

167 To determine the toxicity of the PCB mixtures on MSC metabolic function, we used an XTT  
168 assay (Biotium, Cat #30007). Using either 15% FBS or 0.5% FBS media, 2,000 MSCs were  
169 plated in 96-well plates containing 167 µL of media with a DMSO control (1 µL/mL) or media  
170 with 5 or 25 µM of PCBs dissolved in DMSO. After 48 hours, the culture media was removed  
171 and replaced with 100 µL of 15% FBS or 0.5% FBS media, depending on the original media  
172 composition. Then, 50 µL of XTT solution was added to the 100 µL of media in each well  
173 followed by incubation at 37 °C for 2 hours. After incubation, absorbance was read at 490 nm  
174 and 650 nm. Wells without MSCs and MSCs that had been permeabilized with 0.2% TritonX-  
175 100 were used as internal controls for each experiment. To show the change in XTT signal as a  
176 percent of the vehicle treated controls, all samples divided by the average of the vehicle treated  
177 controls.

178

179 **Cell Proliferation and Morphology**

180 Cell proliferation was determined by counting nuclei stained with Hoechst 33342 (Thermo  
181 Fisher Scientific, Cat # H3570) and morphology was evaluated using Hoechst 33342 and  
182 ActinGreen 488 (Thermo Fisher Scientific, Cat # R37110) after 48 hours of PCB exposure.  
183 MSCs were seeded on 24-well plates with a seeding density of 12,000 cells/well. One mL of

184 0.5% FBS media with a DMSO control (1  $\mu$ L/mL) or 0.5% FBS media with 1, 5, 10, 20, 25  $\mu$ M  
185 of PCBs dissolved in DMSO was added to each well at the same time as seeding. After 48 hours  
186 of incubation, media was removed from each well. The MSCs were then fixed for 5 minutes  
187 using 10% formalin followed by permeabilization with 0.05% Triton X-100 in PBS. A staining  
188 solution was made by diluting Hoechst 33342 and ActinGreen 488 with PBS to concentrations of  
189 5  $\mu$ L/mL and 2 drops/mL, respectively. After washing the cells with PBS, the staining solution  
190 was added to each well, and cells were incubated for 30 minutes at room temperature. The  
191 staining solution was removed and replaced with PBS. Imaging was performed at 10x  
192 magnification on an inverted fluorescent microscope (Leica DMI6000). To prevent bias in field  
193 selection, 5x5 tile scans were performed around the center of the well (25 images per well). The  
194 number of nuclei was counted using ImageJ (NIH) with an automated cell counting macro that  
195 ran a Gaussian blur, threshold, convert to mask, and watershed before analyzing particles to  
196 obtain the number and size of nuclei.

197

### 198 **Cell Viability**

199 Cell death was assessed using propidium iodide staining and the LDH-Glo Cytotoxicity Assay  
200 (Promega, Cat #J2380). For the propidium iodide staining, MSCs were seeded at 61,000  
201 cells/well in a 6-well plate. The MSCs were cultured for 48 hours in 5 mL of 0.5% FBS media  
202 with a DMSO control (1  $\mu$ L/mL) or 0.5% FBS media with 1, 5, 10, 20, 25  $\mu$ M of PCB mixtures  
203 dissolved in DMSO. The cells were then lifted and stained with propidium iodide (Sigma-  
204 Aldrich, Cat # P4864) to a final concentration of 0.01  $\mu$ M. Controls included unstained MSCs as  
205 well as dead control cells which had been permeabilized with 0.2% TritonX-100 for 10 minutes.  
206 The cells were then incubated in the staining solution for 10 minutes before being analyzed via  
207 flow cytometry using a Cytek Northern Lights spectral cytometer. For the LDH assay, MSCs  
208 were plated in a 24-well plate as described in the “Cell Proliferation and Morphology” section.  
209 After 48 hours of incubation, 2  $\mu$ L of media was collected from each well and each sample was  
210 diluted with 48  $\mu$ L of LDH storage buffer. LDH Detection Reagent was prepared and added to  
211 each sample as directed by the manufacturer’s protocol. After incubation for 1 hour at room  
212 temperature, luminescence was recorded and normalized by the positive control to obtain LDH  
213 release as a % of the dead cell control.

214

### 215 **MSC-PBMC Direct Contact Co-culture**

216 Peripheral blood mononuclear cells (PBMCs) were isolated from a leukapheresis reduction cone  
217 from a de-identified donor via the DeGowin Blood Center at the University of Iowa Hospital and  
218 Clinics. PBMCs were cryopreserved in a solution of 40% FBS, 50% RPMI, and 10% DMSO  
219 until use. Immunosuppressive capabilities of MSC were investigated utilizing a co-culture  
220 method as previously described.<sup>35</sup> MSCs in a T75 flask were exposed to 1  $\mu$ L/mL DMSO  
221 (“Vehicle Control”), 5, or 10  $\mu$ M of PCB mixtures in 0.5% FBS containing media. Cells were  
222 then cultured within these conditions for another 48 hours. After pre-exposure to PCB mixtures,  
223 MSCs were harvested, counted, and plated for co-culture. PBMCs were stained with CFSE Cell  
224 Division Tracker dye (BioLegend; Cat: #423801) for 15-minutes. Any unbound CFSE dye was  
225 quenched with RPMI (15% FBS). Two-hundred fifty thousand cells were then added to each  
226 well to establish a 1:3 ratio of MSC to PBMCs. To activate PBMCs, 250,000 CD3/CD28  
227 Dynabeads (Thermo Fisher Scientific, Cat: #11132D) were added to each well. A stimulated  
228 control with Dynabeads but no MSCs and an unstimulated control without MSCs or Dynabeads  
229 were performed in parallel and used for gating and statistical comparison. After 4-days of co-

230 culture, PBMCs were collected and analyzed by flow cytometry. Gates were first set on FSC-A  
231 vs SSC-A for excluding any Dynabeads, MSCs, and debris within the samples. The resultant  
232 cells were then analyzed for percent proliferation as measured by CFSE intensity using the  
233 unstimulated control to set a gate.

234

### 235 **Adipogenic Differentiation Assay**

236 To investigate the PCB mixtures' potential disruption on adipogenesis through pre-exposure,  
237 cells were plated at a confluent density and cultured with 0.5% FBS MEM-alpha with 1 and  
238 10 $\mu$ M of Aroclor 1016, 1254, or Cabinet Mixture for two days to achieve confluence. As a  
239 vehicle control, cells were cultured in 0.5% MEM-alpha containing 1 $\mu$ M of DMSO. After the  
240 pre-exposure period, all wells were washed with 1x PBS to remove remaining PCBs, and media  
241 was switched to 10% FBS Preadipocyte Differentiation Media (PDM-2) for 7 days, with media  
242 changes every 3-4 days. After 7 days the media was switched to 10% FBS DMEM + 1.9 ng/mL  
243 Insulin for another 7 days, with media changeouts every 3-4 days. As a negative control, cells  
244 received complete 0.5% FBS DMEM for all 14 days. At the end of the 14 days, media was  
245 collected for adiponectin analysis and cells were either stained with AdipoRed and imaged or  
246 harvested for RT-qPCR analysis.

247

### 248 *Adiponectin ELISA*

249 Media was collected after 14-days of differentiation and stored at -20 C until analysis.  
250 Quantification of adiponectin production was performed using an ELISA kit (BioLegend; Cat  
251 #442304) with no dilutions of samples to provide absorbance values within the linear range of  
252 the standard curve. Four biological replicates were used for each condition.

253

### 254 *RT-qPCR Analysis*

255 In preparation for RNA isolation, all samples were lifted with Accutase, spun down at 500g for 5  
256 minutes, and washed with PBS. Total RNA was isolated via RNeasy kit (Qiagen; Cat #74104) as  
257 per the manufacturer's protocols and eluted in 50  $\mu$ L nuclease-free water. Each RNA elution was  
258 run on nano-drop for nucleotide quantification and ensuring protein/organic solvent purification.  
259 cDNA was synthesized utilizing a high-capacity cDNA reverse transcriptase kit (Applied  
260 Biosystems; Cat #4375575). ABI QuantStudio (model 7 Flex) was used for quantitative PCR  
261 reactions with SYBR green master mix (Applied Biosystems; Cat #4367659). The catalog of  
262 chosen primers can be found in supplementary data (**Supplementary Table 1**). GAPDH was  
263 chosen for normalizing gene expression, and fold changes were compared to fully differentiated  
264 vehicle controls via  $2^{-\Delta\Delta Ct}$  method.

265

266

### 267 **Statistical Analysis**

268 GraphPad Prism 9 was used for graphing and performing statistical analyses on quantitative data.  
269 One-way ANOVA with Dunnett post-hoc analysis was used for statistical comparisons. A  
270 p<0.05 was considered statistically significant for post-hoc analyses. IC50 values were  
271 determined using Nonlinear Regression (least squares fit) for the [Inhibitor] vs. response (three  
272 parameters) model. Bottom constraint was set to 0 and top constraint was set to 152, the average  
273 number of cells/frame for the vehicle control condition. Further statistical details are provided  
274 within each figure caption.

275

276 **RESULTS AND DISCUSSION**

277 Toxicity of PCB mixtures is heavily influenced by serum

278 To investigate the effect of the PCB mixtures on MSC health, we decided to first determine if  
279 PCBs are toxic to MSCs. For assessing toxicity, MTT and XTT assays are often a first choice as  
280 they can detect a broad range of cellular responses. Since these assays measure NADH  
281 production, changes in the viability, metabolic function, or proliferative capacity of the cells will  
282 all lead to a change in signal. During our first XTT experiment, we performed a 48-hour  
283 exposure of MSCs to PCB mixtures dissolved in 15% FBS media. We found the PCB mixtures  
284 had little to no effect on the MSCs at both 5 and 25  $\mu$ M concentrations (**Figure 1A**). These data  
285 were surprising since previous work has shown various PCBs negatively impact many cell types  
286 such as human preadipocytes, neural stem cells, and astrocytes.<sup>36-38</sup> Therefore, we were  
287 expecting to see a similar negative impact of the PCB mixtures on MSCs.

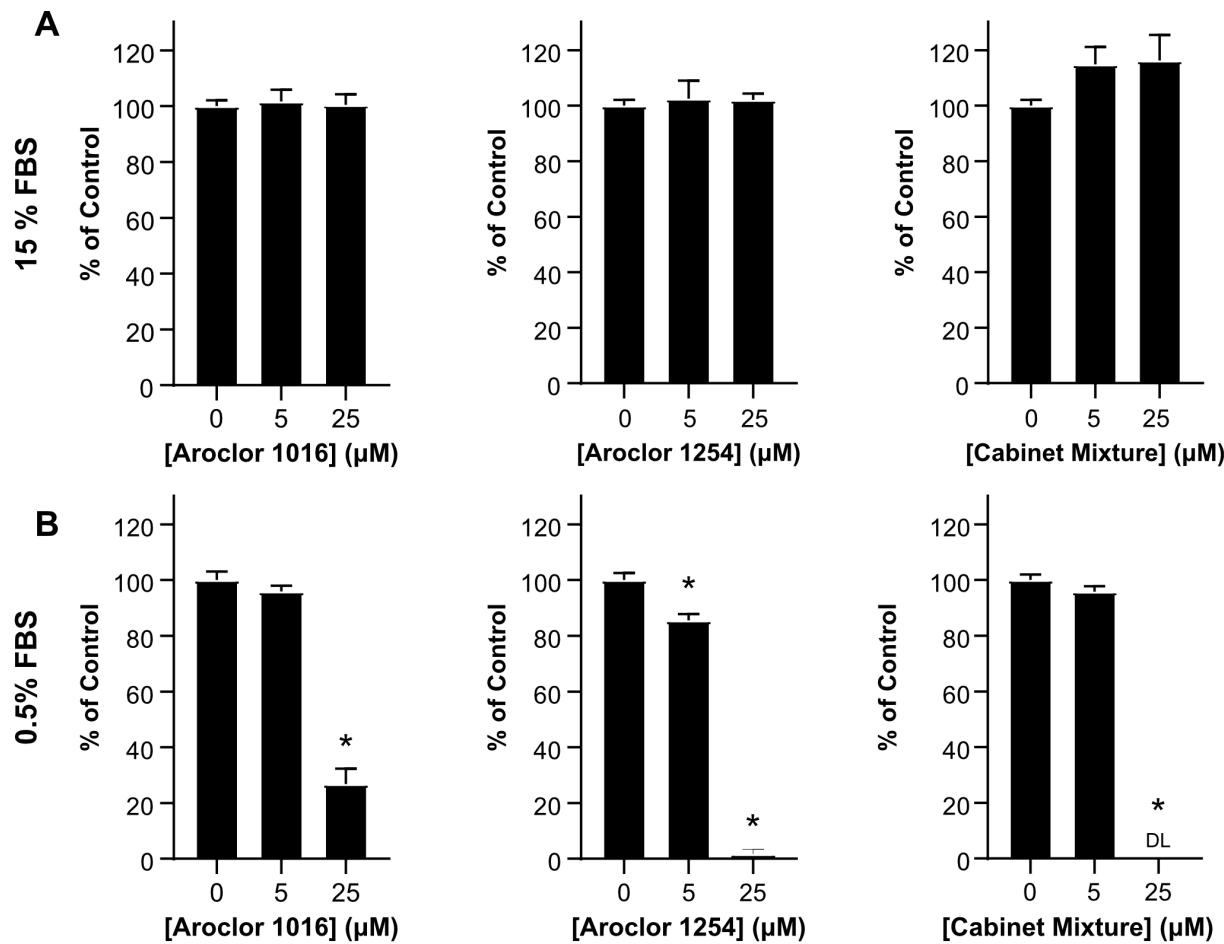
288

289 We were curious if there was a component of our cell culture system preventing PCBs from  
290 exerting an effect on MSCs. Previous work shows the drug binding sites of albumin strongly  
291 bind PCB congeners.<sup>39-41</sup> For our first experiment, 15% of the media was FBS, of which almost  
292 half of the proteins were albumin.<sup>42</sup> We hypothesized that the albumin in our media was  
293 significantly decreasing the amount of free PCB available to MSCs, thus masking any toxic  
294 effects of PCB exposure. To test this hypothesis, we repeated our first experiment, exposing the  
295 MSCs to the PCB mixtures for 48 hours, however, we replaced the 15% FBS media with a 0.5%  
296 FBS media.

297

298 After 48 hours of PCB exposure in this 0.5% FBS media, we found striking differences between  
299 the vehicle control and 5 and 25  $\mu$ M conditions. Aroclor 1254 had close to a 20% reduction in  
300 NADH production at 5  $\mu$ M, and all three PCB mixtures had 75% or greater reductions in NADH  
301 production at 25  $\mu$ M exposures (**Figure 1B**). Compared to the 15% FBS media, the 0.5% FBS  
302 media allowed for much greater insight into the effects of PCBs on MSCs and revealed PCB  
303 exposure disrupts adipose-derived MSCs cellular processes. Thus, all subsequent cell  
304 experiments were performed using MEM-alpha with 0.5% FBS unless otherwise stated.

305



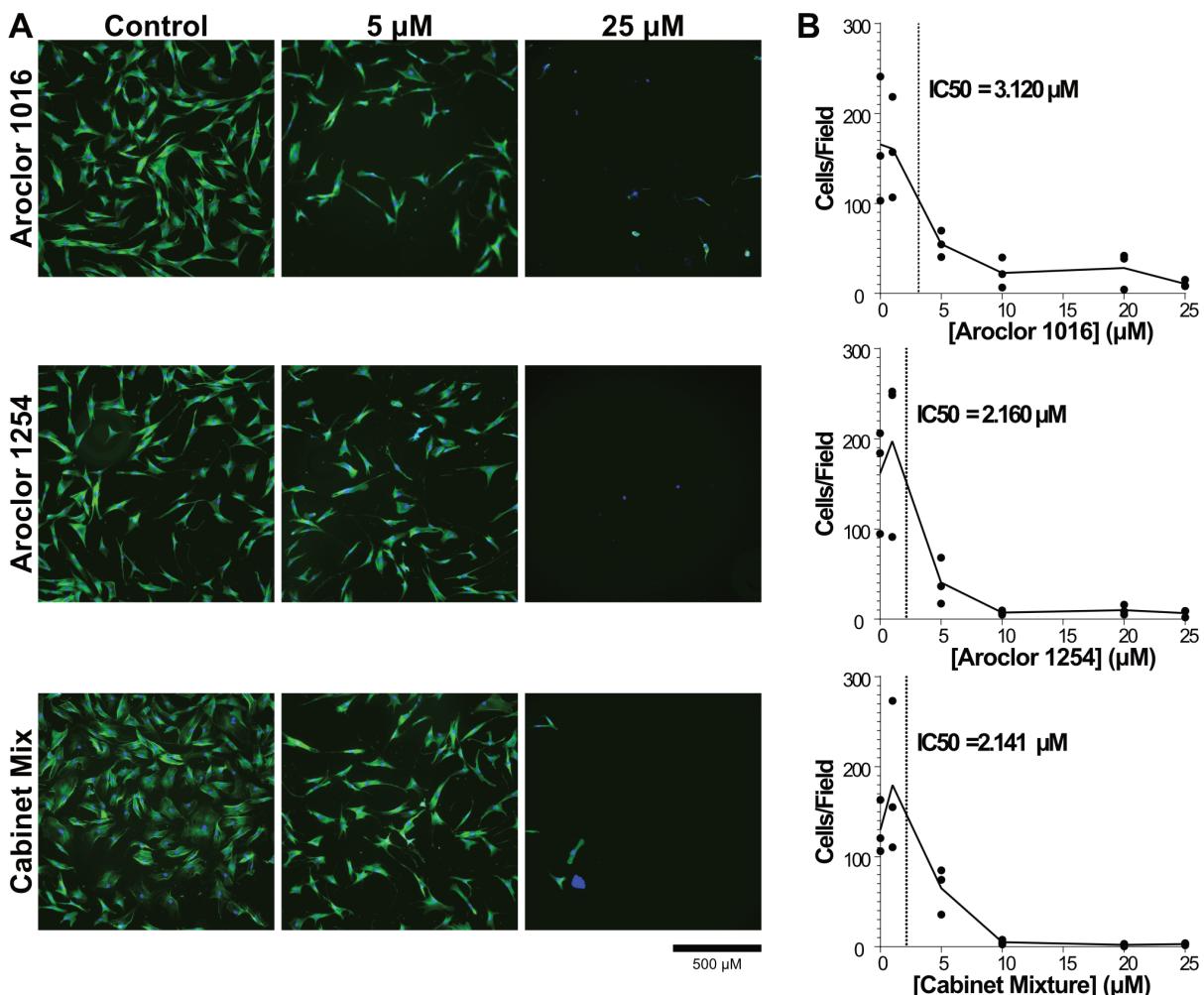
306  
307 **Figure 1: The effect of PCBs on MSC metabolic activity is highly dependent on serum**  
308 **concentration.** The metabolic activity of MSCs was determined using XTT after 48 hours of  
309 exposure to 5 or 10 μM concentrations of Aroclor 1016, Aroclor 1254, or Cabinet Mixture  
310 dissolved in (A) 15% FBS media or (B) 0.5% FBS media. Bars represent mean and error bars are  
311 SEM. Ordinary one-way ANOVA, \* designates significant difference ( $p < 0.05$ ) between the  
312 indicated group and vehicle control (0 μM) treated MSCs after Dunnnett multiple comparison  
313 corrections. n=8 biological replicates with adipose MSC donor 2334 (representative of 4  
314 experiments).

315 Short term exposure to PCB mixtures reduces adipose derived MSC expansion

316 While the XTT assay showed there was decreased NADH as the amount of PCB increased the  
317 decrease could have been due to direct PCB cytotoxicity, decreased proliferation, or decreased  
318 cellular metabolism. We next wanted to determine which of these potential mechanisms of  
319 cellular disruption were at work. Throughout the course of the XTT assay, we observed that the  
320 number of MSCs visible in the wells under a microscope at the end of the experiment was  
321 decreased in the PCB exposed conditions compared to the vehicle control. These observations of  
322 led us to hypothesize that increasing MSC exposure to PCBs would lead to decreased cell  
323 numbers. To determine the effect of PCBs on MSC expansion, we exposed MSCs from three  
324 donors to the three PCB mixtures at concentrations ranging from 1-25 μM. After 48 hours of  
325 incubation, we stained the cells with Hoechst and Actin Green and imaged them to assess cell  
326 counts and morphology.

328

329 We found that increasing concentrations of PCB mixtures led to a significant decrease in the  
330 number of cells in each well. All three MSC donors had significant decreases in cell counts with  
331 exposure to increasing concentrations of all three PCB mixtures (**Figure 2B**). In fact, all samples  
332 exposed to 20  $\mu$ M of PCBs had at least a 90% reduction in the number of MSCs at 48 hours.  
333 Combining all donors together, IC<sub>50</sub> values for each PCB mixture were calculated to be in the 2-  
334 5  $\mu$ M range showing that even low concentrations of Aroclor 1016, Aroclor 1254, and Cabinet  
335 Mixture can significantly impact MSC health. In addition, there were also significant changes to  
336 the morphology of the MSCs with increasing concentrations of PCB mixtures. The cells that  
337 remained in the wells at the high concentrations (20, 25  $\mu$ M) had what looked to be only  
338 fragments of cytoskeleton left and the portion that remained took on a spindle-like appearance  
339 and smaller overall footprint compared to cells in the vehicle control (**Figure 2A**). In addition,  
340 the size of cell nuclei generally became larger and the variability of nuclear size increased  
341 (**Supplemental Figure 2**). It should be noted that due to wash steps in the staining procedure,  
342 any small nuclei of detached dead cells would have been washed out before imaging. Of the cells  
343 that remained attached, the increased frequency of large nuclei at higher concentrations could be  
344 an indication that the cells have entered senescence, which is characterized by large, flattened  
345 nuclei.<sup>43</sup>

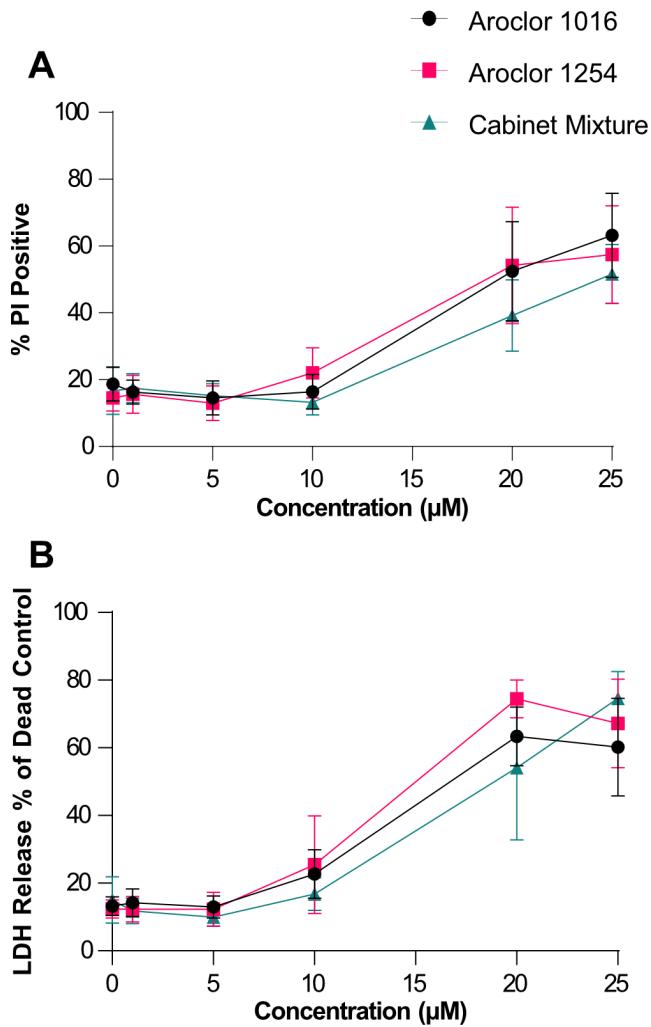


346

347 **Figure 2: Cell count decreases with increasing exposure to PCB mixtures. (A)**  
348 Representative images of adipose MSCs exposed to 0, 5, or 25  $\mu$ M concentrations of Aroclor  
349 1016, Aroclor 1254, and Cabinet Mixture. (B) The number of cells was determined by using  
350 ImageJ software to count the number of nuclei per image field. Quantification was performed on  
351 25 images per condition for three independent adipose MSC donors. Data are represented via a  
352 point for the mean of each donor (n=25 image fields/point) with a line connecting the mean value  
353 of all donors (n=3 donors). IC50 values calculated using Prism for Aroclor 1016, Aroclor 1254,  
354 and Cabinet Mixture are 3.120  $\mu$ M, 2.160  $\mu$ M, and 2.141  $\mu$ M, respectively.  
355

356 PCB mixtures increase cell death at high concentrations  
357 After the XTT and imaging studies, it was clear that exposure to PCB mixtures was causing  
358 cytotoxicity, but it was not yet clear if this was due primarily to suppression of cell proliferation  
359 or increases in cell death. To assess cell death directly, we used two complimentary assays, a  
360 propidium iodide (PI) stain to measure membrane permeability and lactate dehydrogenase  
361 (LDH) assay to measure release of LDH from dead cells. We again exposed MSCs to three PCB  
362 mixtures ranging from 1-25  $\mu$ M and compared them to DMSO treated control cells. After 48  
363 hours, the cells were stained with PI for analysis by flow cytometry and the media was assessed  
364 for LDH activity.  
365

366 Based on the prior imaging experiments (**Figure 2**), we expected to see increases in cell death  
367 starting at 5  $\mu$ M, however, with both the PI staining (**Figure 3A**) and the LDH assay (**Figure**  
368 **3B**), we observed minimal cell death after exposure to 1, 5, or even 10  $\mu$ M of the PCB mixtures.  
369 It was only at high exposure levels, 20 and 25  $\mu$ M, that we observed significant levels of cell  
370 death. Thus, at higher concentrations  $>20$   $\mu$ M, the decrease in cell numbers is due to lethal  
371 cytotoxicity of the PCBs on MSCs. While the decreased cell counts we saw with 5 and 10  $\mu$ M  
372 exposure but without increased levels of cell death suggests that low concentrations of PCB  
373 mixtures cause a cytostatic rather than a cytotoxic effect on adipose MSCs. These results indicate  
374 that PCB mixtures at lower concentrations are disrupting cellular processes involved in cell  
375 proliferation and raise the possibility that exposure to low concentrations of PCB mixtures alter  
376 other aspects of adipose MSC phenotype.  
377



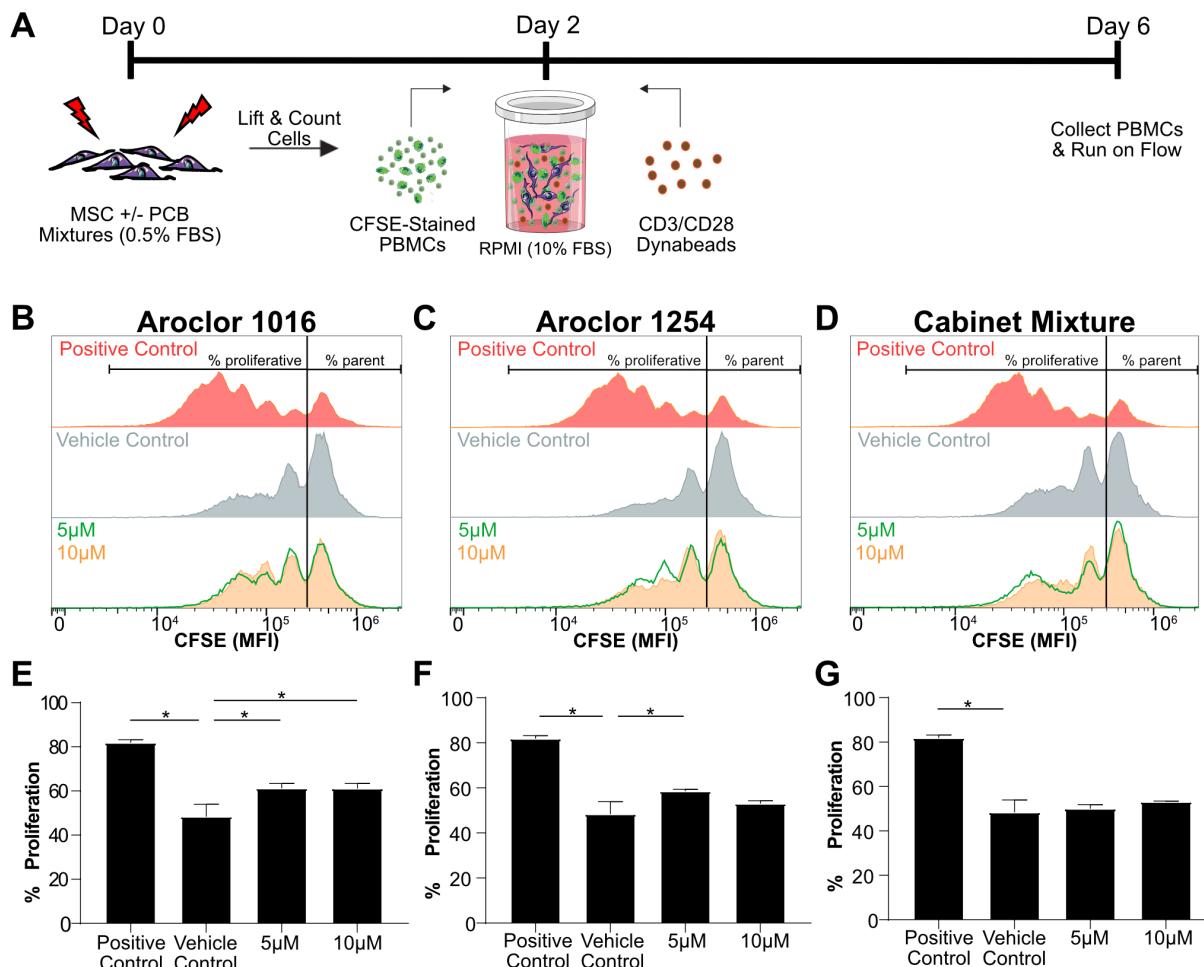
378  
379 **Figure 3: High concentrations of PCB mixtures kill the majority of adipose MSCs.** Cell  
380 death was assessed using (A) propidium iodide staining and flow cytometry or (B) LDH assay  
381 after 48 hours of exposure to media formulated to contain 0, 1, 5, 10, 20, or 25  $\mu\text{M}$  of Aroclor  
382 1016, Aroclor 1254, and Cabinet Mixture. Each data point represents mean of 3 donors and error  
383 bars are SEM.

384  
385 Exposure to PCB mixtures only modestly impacts adipose MSC's immunomodulatory properties  
386 After determining the PCB mixtures' cytotoxic effects on MSCs at higher concentrations, we  
387 wanted to investigate if exposure to non-lethal concentrations alters functional characteristics  
388 critical for adipose MSCs. An important property of adipose MSCs is their immunosuppressive  
389 capability. When in an inflammatory environment, MSCs tend to drive the surrounding immune  
390 cells towards a more immune-resolving phenotype, and as such serve as a key regulator of  
391 adipose inflammation.<sup>25</sup> To determine how MSC exposure to non-lethal concentrations of PCB  
392 mixtures impacts their immunosuppressive properties, we pre-exposed the MSCs to each mixture  
393 at 5 or 10  $\mu\text{M}$  for 48 hours within 0.5% FBS supplemented media. The cells were then washed to  
394 remove any dead cells and residual PCBs, counted, and seeded at a ratio of 1 MSC to 3  
395 Dynabead-stimulated PBMCs (**Figure 4A**).

396

397 Since the PBMCs are stained with the cell proliferation dye, CFSE, prior to stimulation, each  
398 division will lead to a cytosolic partitioning of the fluorescent dye and leftward shift in CFSE  
399 intensity. As seen in the “Positive Control” panels of (Figure 4B-D), the absence of MSCs  
400 allows the PBMCs to freely proliferate, leading to increased peaks at lower fluorescent  
401 intensities, each peak signifying a new generation of inflammatory cells. Upon adding vehicle  
402 treated MSCs, the divisions are substantially reduced. To summarize the degree of inflammatory  
403 suppression, we use a gate to separate the un-proliferated parent peak from cells that have  
404 undergone cell division and calculate the “% Proliferated”. The presence of vehicle control  
405 MSCs decreases the % of proliferated cells from ~84% to ~48% (Figure 4E-G). Pre-exposure of  
406 MSCs to Aroclor 1016 at 5 and 10  $\mu$ M both lead to a small but statistically significant increase in  
407 % proliferation (Figure 4E). This was also observed for Aroclor 1254 (Figure 4F) at 5  $\mu$ M, but  
408 the difference at 10  $\mu$ M was not large enough to reach statistical significance. Interestingly,  
409 while both Aroclor 1016 and 1254 had modest effects on MSC suppression of PBMCs, Cabinet  
410 Mixture had no measurable effect (Figure 4G). Based on prior cytotoxicity assays (Figure 2)  
411 that showed a change in cell behavior at these same concentrations, namely a dramatic reduction  
412 in proliferation, we expected to see a much larger impact on adipose MSCs immunosuppressive  
413 potency. This result paints a more complex portrait of adipose MSC response to PCB exposure  
414 and suggests the surviving adipose MSCs retain some functionality.

415



416

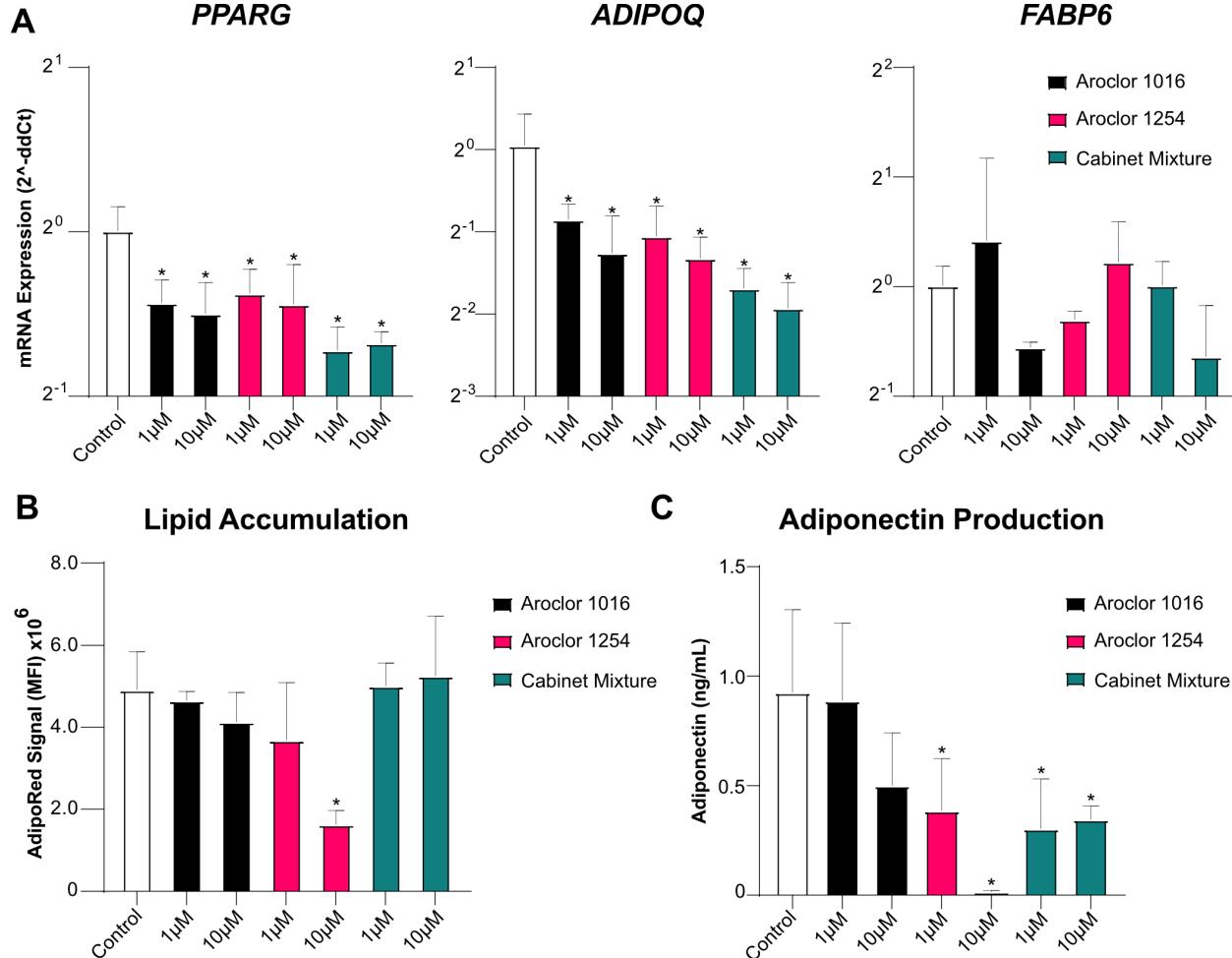
417 **Figure 4: MSC immunosuppressive capabilities are only slightly reduced following**  
418 **exposure to PCB mixtures.** A)Timeline of the PBMC-MSC coculture. Immunosuppressive  
419 capabilities of MSCs was assessed by measuring the dilution of CFSE dye in PBMCs after  
420 coculture with MSCs that had been pre-exposed for 48-hours to B) Aroclor 1016, C) Aroclor  
421 1254 or D) Cabinet Mixture. The percent of PBMCs that proliferated was quantified to assess  
422 MSCs immunosuppressive potency after exposure to E) Aroclor 1016, F) Aroclor 1254 or G)  
423 Cabinet Mixture. Bars are mean +/- SD of n=3 independent adipose donors. Ordinary one-way  
424 ANOVA, \* designates significant difference (p<0.05) between the indicated group and vehicle  
425 control (DMSO) pre-exposed MSCs after Dunnett multiple comparison corrections. Cell figures  
426 were adapted from <https://smart.servier.com/> and licensed under CC-BY 3.0.  
427

428 Pre-Exposure to PCB Mixtures Disrupts MSCs' Adipogenic Potential  
429 With recent studies correlating persistent organic pollutants, such as PCBs, with the development  
430 of metabolic syndromes, we next wanted to investigate the influence of PCB mixtures on adipose  
431 MSCs adipogenic potential.<sup>44</sup> To assess this, we pre-exposed MSCs to sublethal concentrations  
432 of PCB mixtures for 48 hours, and then induced adipogenic differentiation for 14-days in the  
433 absence of PCB exposure. After 14 days, we analyzed the transcript levels of key genes involved  
434 or indicative of adipocyte differentiation, namely, peroxisome proliferator activated receptor  
435 gamma (*PPARG*) a gene that plays a vital role as a master switch for the adipogenic  
436 differentiation pathway, contributing to protein transcription that influences lipid accumulation  
437 and insulin sensitivity, fatty acid binding protein (*FABP6*) a gene involved with fatty acid uptake  
438 and metabolism, which is a precursor to lipid production, and adiponectin (*ADIPOQ*) which is  
439 exclusively expressed by mature adipocytes for encoding adiponectin, a major protein involved  
440 in regulating whole-body metabolism.<sup>45,46</sup> We found exposure to any PCB mixture led to a  
441 significant reduction in the transcript levels of both *PPARG* and *ADIPOQ* (**Figure 5A**). While  
442 significant, the magnitude of reduction of *PPARG* was fairly modest, with exposure leading to a  
443 1.35-1.65-fold reduction compared to vehicle-treated controls. The reduction of *ADIPOQ* was  
444 more pronounced with 10  $\mu$ M cabinet mixture exposure leading to a nearly 4-fold reduction in  
445 expression. Interestingly, no significant changes were observed for *FABP6*. Thus, exposure of  
446 adipose MSCs for just 48 hours has a long-term impact on gene expression, even after 14 days of  
447 differentiation in PCB-free media.  
448

449 To determine if alterations in gene expression led to changes in adipocyte phenotype, we  
450 repeated the experiment and analyzed lipid accumulation and adiponectin production directly.  
451 MSCs exposed to Aroclor 1016 or 1254 showed a dose-dependent decrease in lipid accumulation  
452 while MSCs exposed to Cabinet Mixture showed no decline in lipid accumulation (**Figure 5B**).  
453 Interestingly, the only exposure that resulted in a statistically significant decline in lipid  
454 accumulation was 10  $\mu$ M Aroclor 1254. Analysis of adiponectin secreted by the adipocytes after  
455 14 days of differentiation revealed a stark decrease in production after pre-exposure to all three  
456 of the PCB mixtures (**Figure 5C**). Specifically, pre-exposure to Aroclor 1254 at 5  $\mu$ M or Cabinet  
457 Mixture at both concentrations reduced adiponectin output in half. Increasing the Aroclor 1254  
458 exposure to 10  $\mu$ M nearly completely blocked adiponectin production. Overall, the observed  
459 changes in adiponectin secretion were consistent with the *ADIPOQ* transcript levels (**Figure**  
460 **5A**). This is important, as adiponectin production assists in maintaining insulin-sensitivity and  
461 attenuating chronic inflammation, while decreased serum levels have been associated with obesity  
462 and type-2 diabetes.<sup>47,48</sup> Taken collectively, these data demonstrate that even a short window of

463 exposure to PCB mixtures disrupts the quality of adipogenesis which alters the properties of the  
464 resultant mature adipocytes.

465  
466



467  
468 **Figure 5: Adipogenic differentiation of MSCs are slightly diminished after pre-exposure to**  
469 **PCB mixtures.** A) Fold-change of the expression of prominent genes in the adipogenesis  
470 signaling pathway, *PPARG*, *ADIPOQ*, and *FABP6*. Delta-CT was calculated using GAPDH and  
471 then compared to vehicle treated controls using the Delta-Delta-CT method. n=3 experiments  
472 with Adipose MSC donor 2334 B) Lipid accumulation as measured by AdipoRed staining  
473 measured using a 96-well plate reader. C) adiponectin present in culture media measured using  
474 ELISA. Bars are mean +/- SD of n=4 experiments with adipose MSC donor 2334. Ordinary one-  
475 way ANOVA with Dunnett post-hoc test, \* indicates p<0.05 compared vehicle controls.  
476

477

### IMPLICATIONS FOR HUMAN HEALTH

478 Our work shows that exposure to Aroclor 1016, Aroclor 1254, and the Cabinet Mixture all result  
479 in adverse effects on adipose MSCs, a cell type that is critical for the maintenance and function  
480 of adipose tissue and overall health. We examined PCB concentrations that are similar to tissue  
481 concentrations measured in adipose. In vivo studies usually report PCBs in terms of ng/g of lipid  
482 and have found adipose tissue levels ranging from 700-9000 ng/g of lipid depending on the  
483 severity of exposure<sup>49,50</sup>. Considering the molecular weight of PCBs, the density of lipids, and

484 that adipose is ~60% lipids<sup>51</sup>, these tissue levels correspond to an adipose tissue concentration  
485 range of 1.5-18  $\mu$ M of total PCB. While we examined mixtures rather than single congeners in  
486 this study, mixtures are more physiologically relevant as people are exposed to mixtures and not  
487 single congeners. Furthermore, studying mixtures made our assays sensitive to possible  
488 interaction effects between congeners which have been reported for other cell types<sup>52</sup>. Our data  
489 show that exposure to these mixtures at adipose tissue relevant concentrations results in  
490 significant toxicity and functional disruption of adipose MSCs, an important stem/mesenchymal  
491 cell population. In reality, PCB profiles found within current U.S. school air show contributions  
492 from all three of these investigated sources, but only specific congeners are detectable within the  
493 serum of students.<sup>53</sup> Regardless, these findings have potential implications for the health of  
494 school-aged children, especially those attending schools that were built during Aroclor  
495 production. Adipose MSCs are particularly important during adolescence, as the number of  
496 adipocytes goes through massive expansion before plateauing and maintaining numbers  
497 throughout adulthood. This expansion of adipocytes relies on the MSC niche found within  
498 adipose tissue. We have shown here that exposure to non-lethal concentrations of PCB mixtures  
499 disrupts both adipose MSC proliferation and impairs their ability to differentiate into mature  
500 functional adipocytes. These effects of PCBs could have profound implications on the number  
501 and quality of adipocytes that are generated during expansion. Mature adipocytes with altered  
502 adiponectin signaling could have significant physiological effects such as decreased insulin  
503 sensitivity in peripheral tissues, disrupted androgen signaling, and increased chronic  
504 inflammation: all of which are aspects of metabolic syndrome.  
505

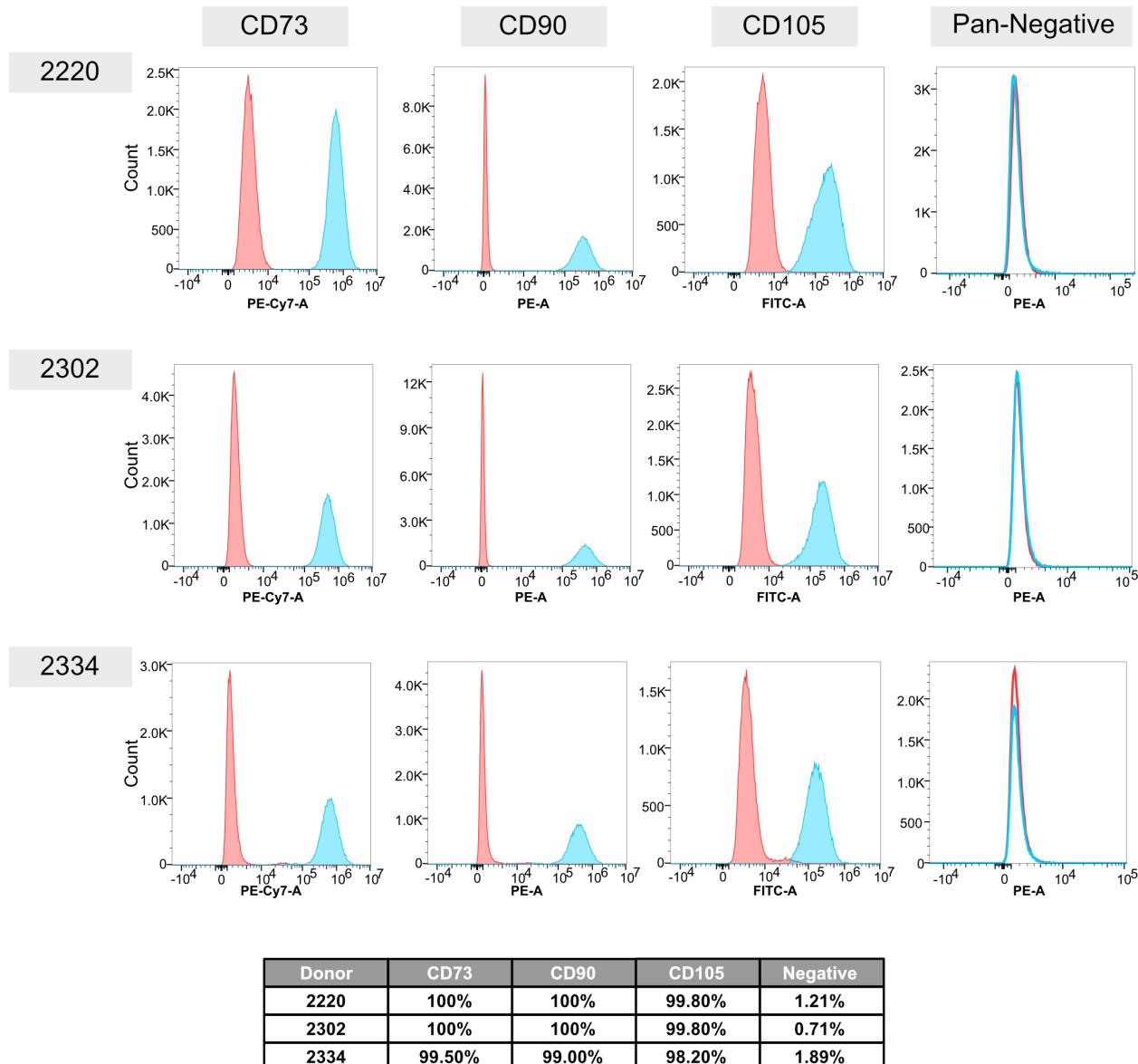
506 While, in adults, MSCs and preadipocytes do not proliferate at the same rate as they do in  
507 adolescents, about 10% of all adipocytes are still replaced annually, and a healthy MSC niche is  
508 needed to support this replacement.<sup>21</sup> Since PCBs are known to accumulate in adipose tissue,  
509 adipose MSCs within adults are susceptible to the negative effects of PCB exposure.<sup>54,55</sup>  
510 Disruption of adipose MSCs would lead to a lower rate of adipocyte replacement which has been  
511 linked to hypertrophic obesity.<sup>56</sup> Additionally, recent work suggests that proper expansion of  
512 adipose tissue is fundamental to the prevention of metabolic disease.<sup>20,27</sup>  
513

514 While the effects of PCB mixtures on adipose MSCs were fairly consistent between different  
515 mixtures, there were also distinct differences between the groups. Exposure of MSCs to Aroclor  
516 1254 leads to much lower levels of adiponectin and lipid accumulation compared to Aroclor  
517 1016 or Cabinet Mixture. One likely explanation for this difference is the congeners which  
518 compose the mixtures. Aroclor 1254 contains both higher chlorinated and dioxin-like PCB  
519 congeners, while Aroclor 1016 contains lower chlorinated non-dioxin-like congeners.<sup>32,33</sup>  
520 Moreover, each congener has its own partitioning coefficient and effective free concentration.  
521 Due to the differences in congener profile and relative abundance, it is likely that multiple  
522 distinct congeners, or congener subsets, are responsible for the biological effects we have  
523 observed here on adipose MSCs  
524

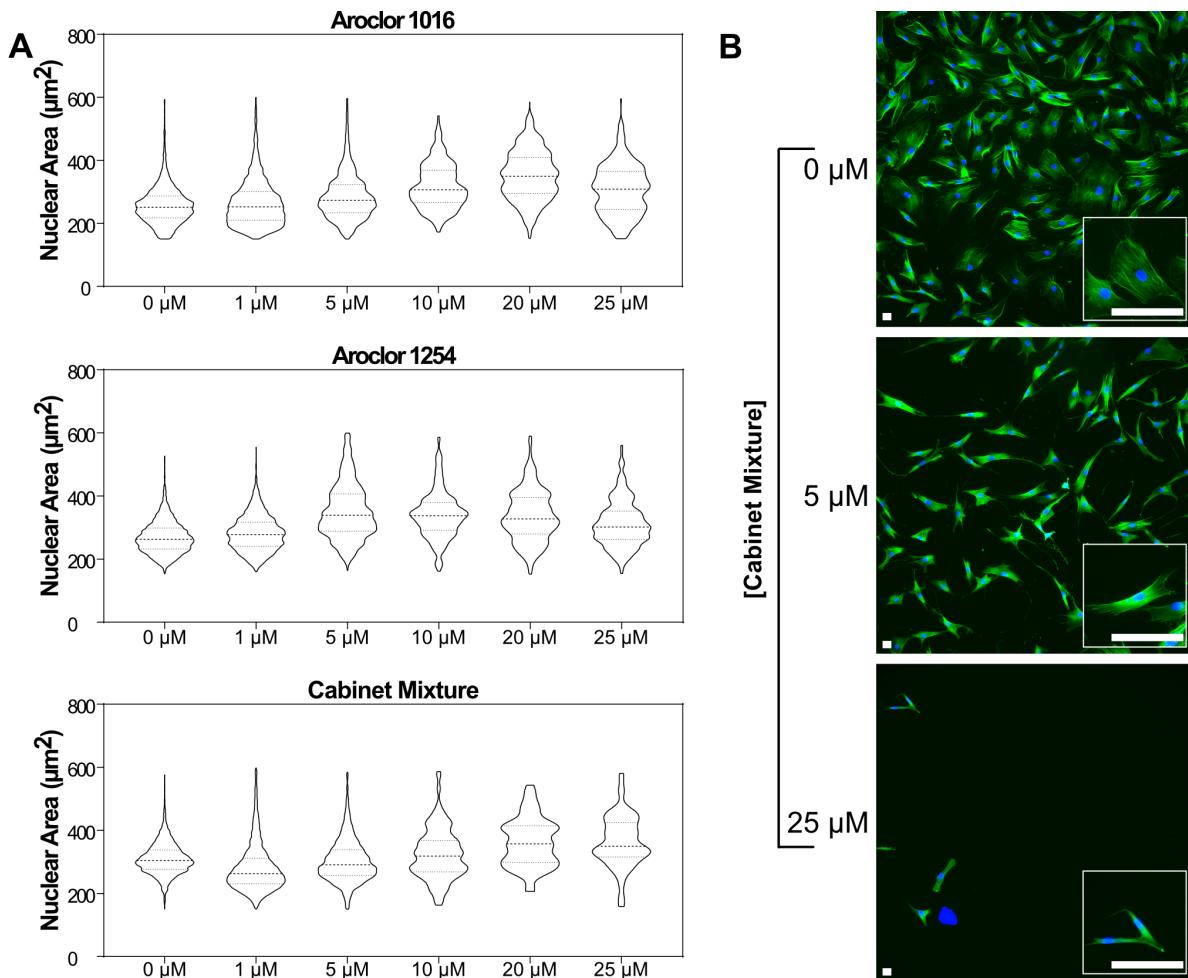
525 Another potential reason for the differences between mixtures observed in the adipogenic  
526 differentiation assay is the different mechanisms of action of different PCBs. Previous work has  
527 focused primarily on elucidating the effect of dioxin-like PCBs on adipocytes. PCB 126, a  
528 dioxin-like PCB, activates the aryl hydrocarbon receptor (AhR) which suppresses PPARG  
529 transcription and, subsequently, adipogenesis.<sup>36</sup> Further, transcriptome sequencing of adipocytes

530 after exposure to PCB 126 showed marked activation of AhR genes but also activation of  
531 proinflammatory pathways and the AGE-RAGE pathways which is known to be associated with  
532 the development of obesity and insulin resistance.<sup>57</sup> In our experiments, Aroclor 1254 is the only  
533 mixture that contains significant amounts of dioxin-like PCBs (Aroclor 1016 contains trace  
534 amounts of DL congeners but has a Dioxin TEQ of 0.09 compared to a TEQ of 21 for Aroclor  
535 1254)<sup>30</sup>. Aroclor 1254 has also been shown to lead to higher levels of DNA damage,  
536 tumorigenesis, and disruption of central nervous system neurochemical function than Aroclor  
537 1016.<sup>3,58,59</sup> This disruption may be due to a dose-dependent inhibition of creatine kinase activity  
538 as seen in L6 myoblasts.<sup>60</sup> Creatine kinase-B activity is decreased in adipocytes during obesity,  
539 thus opening the potential for a mechanistic tie between Aroclor 1254 exposure and adipocyte  
540 dysfunction.<sup>61</sup> The other two mixtures are comprised of non-dioxin-like congeners, thus the  
541 previous proposed mechanisms are unlikely to explain the effects of Aroclor 1016 and Cabinet  
542 Mixture. Non-dioxin-like PCBs, such as those found in Aroclor 1016 and Cabinet Mixture, have  
543 been shown to induce neuronal apoptosis via a p53-independent mechanism.<sup>62</sup> They also bind to  
544 ryanodine receptors (RyR) and increase calcium release from the endoplasmic reticulum of  
545 neurons. Increased RyR activity is associated with neuronal apoptosis and dendritic growth.<sup>62</sup> In  
546 adipocytes, RyR3 activity is inversely related to adiponectin mRNA expression, thus  
547 overactivation of RyR by non-dioxin-like PCBs is one potential mechanism of disrupted adipose  
548 function.<sup>63</sup> While further studies will be required to understand which congeners or congener  
549 interactions are responsible for toxicity and their mechanisms of action, this work provides direct  
550 evidence that short-term exposure to environmentally relevant mixtures of PCBs disrupt the  
551 health and function of adipose MSCs.

552  
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555  
556 **Supplementary Figure 1: Adipose MSCs meet the ISCT Minimal Criteria.** Adipose MSCs  
557 were analyzed for expression of markers according to the ISCT MSC Minimal Criteria. Flow  
558 cytometry histogram plots are shown for CD73, CD90, CD105 and a Negative Cocktail (CD34,  
559 CD45, CD11b, CD19, and HLA-DR). Each plot contains the isotype control (red peak) and on  
560 target sample (blue peak). Table displays the percent of each on-target population considered  
561 positive based on gates set using the isotype control.



562  
563 **Supplemental Figure 2: Exposure to PCB mixtures leads to dose dependent changes to**  
564 **MSC morphology.** Morphology of nuclei and cytoskeleton after 48 hours of exposure to PCB  
565 mixtures. (A) Violin plot of nuclear area after exposure to 0, 1, 5, 10, 20, or 25  $\mu\text{M}$   
566 concentrations of Aroclor 1016, Aroclor 1254, and Cabinet Mixture. (B) Representative images  
567 of cells after exposure to 0, 5, or 25  $\mu\text{M}$  concentrations of Cabinet Mixture. All scale bars  
568 represent 50  $\mu\text{m}$ .  
569

570 **Supplemental Table 1. qRT-PCR Primers**

Primer Name; Exon Location	IDT Catalog #
GAPDH; 3 – 4	39a.22214836
ADIPOQ; 1 – 3	58.39189358
CCL2; 1 – 2	58.45467977
FABP6; 5 – 6	58.1261702
PPARG; 7 – 8	58.25464465
SCL2A4; 1 – 2	58.2557238

571

572

### 573 **ACKNOWLEDGEMENTS**

574 This study was supported by NIH [P42 ES013661](#) (JAA, AJK, HJL) and a pilot grant from the  
575 Environmental Health Research Center [P30 ES005605](#) (AJK, JAA). RB is supported by the

576 University of Iowa MSTP Grant, NIH T32 GM139776. We also acknowledge the University of  
577 Iowa Tissue Procurement Core which manages the University of Iowa Biobank (UIBB –  
578 IRB#201103721) for services provided related to acquisition of study specimens. The TPC is  
579 supported by an award from NIH (NCI award number P30CA086862) and the University of  
580 Iowa Carver College of Medicine.

581

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584 Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. Jesse Liszewski:  
585 Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing –  
586 Original Draft, Writing – Review & Editing, Visualization. Michael Schrodt: Methodology,  
587 Investigation, Writing – Original Draft. Bhavya Vats: Investigation. Xueshu Li: Resources, Data  
588 Curation, Writing – Original Draft. Hans-Joachim Lehmler: Resources, Data Curation, Writing –  
589 Original Draft, Funding Acquisition. Aloysius J. Klingelhutz: Conceptualization, Resources,  
590 Writing – Review & Editing, Funding Acquisition. James Ankrum: Conceptualization,  
591 Methodology, Formal analysis, Resources, Data Curation, Writing – Review & Editing,  
592 Visualization, Supervision, Funding Acquisition.

593

594

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