Bisphenol S moderately decreases the expression of syncytiotrophoblast marker genes and induces apoptosis in human trophoblast lineages

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Abstract

Bisphenol S (BPS) is currently used in the manufacturing of several household equipment such as water pipes and food containers. Hence, its entrance into the human body is almost inevitable. The presence of BPS in body fluids has been reported. However, its potential toxicity, especially on human placenta development and pregnancy progression, has not been explored. In this study, we assessed the impacts of BPS on self-renewal and differentiation potentials of trophoblast stem cells (TSCs) by exposing them to three different BPS concentrations during both self-renewal and differentiation of TSCs into syncytiotrophoblast (ST), extravillous trophoblast (EVT), and trophoblast organoids. Interestingly, BPS treatment did not affect the proliferation, cell cycle, and stemness of the TSCs but it led to induction of apoptosis in each trophoblast lineage. BPS altered the expression of several fusion-related genes. However, this alteration did not translate into significant morphological defects in the STs and organoids. Moreover, BPS did not impair the differentiation of TSCs into EVTs. These findings suggest that the presence of BPS at the feto-maternal interface may exaggerate trophoblast apoptosis and moderately inhibit the trophoblast fusion pathway to affect placenta development and pregnancy. Our study offers valuable insights into the toxicity of BPS on human placenta development, emphasizing the need for further research on the relationship between maternal serum levels of BPS and pregnancy complications.

Keywords: Bisphenol S, Placenta, Trophoblast, Proliferation, Differentiation, Apoptosis

1. Introduction

The human placenta is a transient organ which is indispensable for pregnancy progression and maternal health. The major cell type in the placenta is the trophoblast, which has three main lineages: cytotrophoblasts (CTs), syncytiotrophoblasts (STs) and extravillous trophoblasts (EVTs). During placenta development, the CTs, which are stem cells, proliferate and differentiate into the STs and EVTs, with a continuous occurrence of strictly controlled apoptosis to remove superfluous and dysfunctional cells (Adu-Gyamfi et al., 2021; Knöfler et al., 2019). While the EVTs invade the uterus to establish the uteroplacental circulation and the histotrophic nutrition, the STs serve as a barrier that controls the exchange of substances between the fetus and the mother (Adu-Gyamfi et al., 2021; Knöfler et al., 2019). It has been found that abnormal proliferation, differentiation, and apoptosis of the trophoblasts lead to defective placenta and pregnancy complications such as miscarriage, preeclampsia, and fetal growth restriction (Adu-Gyamfi et al., 2020b; Adu-Gyamfi et al., 2019; Erel et al., 2001; Farah et al., 2020; He et al., 2013; Sharp et al., 2010; Zhang et al., 2020). It is proposed that the accumulation of xenobiotics at the feto-maternal interface could be one of the contributing factors to this anomaly.

Some xenobiotics can compromise or exaggerate the functionality of several cells, tissues, and organs. One group of these chemicals is the bisphenols, among which are bisphenol A (BPA) and bisphenol S (BPS). BPA is used in the production of food and beverage containers, water pipes, medical equipment, coatings, adhesives, high-performance composites, and automotive and aircraft parts (Adu-Gyamfi et al., 2022). Ever since it was shown that BPA significantly affects several developmental processes, such as impairing decidualization (Nelson et al., 2020), inducing abnormal placentation, and causing pregnancy complications (Adu-Gyamfi et al., 2022), its use has been banned in some geographical settings. BPS has now been recommended as an alternative substitute for BPA. Hence, this chemical is currently used in the production of several equipment which were previously produced with BPA. Due to this, human exposure to this chemical is now on the rise. Contemporary evidence suggests that BPS is detectable in the follicular fluid (Žalmanová et al., 2023), blood, and urine (Jin et al., 2018; Smarr et al., 2018), raising concerns about its potential toxic effects. Therefore, it is imperative to conduct a comprehensive examination of the potential toxic effects of this chemical to ascertain whether it is safe to the human body and pregnancy. It has been demonstrated that BPS affects EGF signaling in human trophoblast cells (Ticiani et al., 2022), impairs endocrine functions in the sheep placenta (Gingrich et al., 2018), and induces morphological defects in the junctional zone of the mouse placenta (Mao et al., 2020). However, the impacts of BPS on human placenta development remains to be clarified.

93 Previous research on the effects of xenobiotics on placenta development primarily relied on animal models
94 and immortalized or choriocarcinoma trophoblast cell lines such as JEG3, Bewo and HTR8/SVneo cells.
95 These models exhibit varying gene expression signatures and do not closely mirror human placenta
96 development. Therefore, experimental findings from them are not directly applicable to humans (Abbas et
97 al., 2020; Hemberger et al., 2020). Since it is unethical to administer xenobiotics to pregnant women, it
98 would be ideal to use an in vitro model that closely mirrors the human placental micro-environment for
99 studying the toxic effects of xenobiotics on placenta development. The use of human trophoblast stem cells
100 (TSCs) has been recommended in that regard (Adu-Gyamfi et al., 2022). The TSCs are multipotent and
101 have the ability to differentiate into trophoblast lineages that exhibit transcriptomic signatures which closely
102 resemble those of the early human placenta (Okae et al., 2018). Therefore, they serve as a better model for
103 studying the mechanisms of early human placenta development.
104
105 In this study, we aimed to determine the impacts of BPS on trophoblast cell cycle, proliferation, stemness,
106 differentiation, and apoptosis. We differentiated human TSCs (which are the in vitro counterparts of the
107 CTs) into STs, EVTs, and organoids in the presence or absence of BPS. Our findings would contribute to
108 policy formulation regarding BPS use.
109
110 **2, Materials and methods**
111
112 **2.1 TSC culture**
113 Human TSCs were grown in TSC culture medium [Dulbecco's modified Eagle's medium (DMEM)/F12
114 (Gibco)] supplemented with 0.2% fetal bovine serum (FBS; GeminiBio), 1% insulin-transferrin-selenium-
115 ethanolamine (ITS-X; Gibco), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 0.3% bovine serum albumin
116 (BSA; Sigma-Aldrich), 0.5% penicillin–streptomycin (Gibco), 0.5 μM A83-01 (Wako Pure Chemical
117 Corporation), 0.5 μM CHIR99021 (Selleck Chemicals), 0.5 μM SB431542 (Stemcell Technologies), 0.8
118 mM valproic acid (VPA; Wako Pure Chemical Corporation), 5 μM Y27632 (ROCK inhibitor, Selleck
119 Chemicals), 1.5 μg/ml L-ascorbic acid (Sigma-Aldrich) and 50 ng/ml epidermal growth factor (EGF;
120 PeproTech) in accordance with a previous protocol (Okae et al., 2018). Cell culture plates that had been
121 coated with 5 μg/ml of Collagen IV (Corning) were used for the cell culture. On reaching 70–80%
122 confluency, the cells were detached with TrypLE (Gibco) for 8 min at 37°C and then passaged at a 1:3 split
123 ratio. TSCs were cultured in a 37°C and 5% CO_2 incubator. The medium was replaced every day with a
124 fresh medium.
125
126 **2.2 Differentiation of TSCs to STs**

127 Previously cultured TSCs at ~80% confluence were trypsinized. 3×10^5 of the TSCs were seeded in 6 cm
128 Petri dishes and cultured in 3 mL of ST medium [DMEM/F12 supplemented with 2 μM forskolin, 0.3%
129 BSA, 1% ITS-X supplement, 0.1 mM 2-mercaptoethanol, 4% KSR, 50 ng/ml EGF, 0.5% Penicillin-
130 Streptomycin, and 2.5 μM Y27632] in accordance with a previous protocol (Okae et al., 2018). On the
131 second and fourth days, the medium was replaced with a fresh medium. On the 5th day, the STs were
132 collected, and washed to remove dead cells after which RNA was extracted from them.
133
134 **2.3 Differentiation of TSCs to EVTs**
135 Trypsinized TSCs were seeded in 6-well plates pre-coated with 1 μg/ml of Collagen IV at a density of $1 \times$
136 10^5 cells per well and grown in 2 mL of EVT medium [DMEM/F12 supplemented with 0.3% BSA, 0.1 mM
137 2-mercaptoethanol, 4% KSR, 0.5% Penicillin-Streptomycin, 1% ITS-X supplement, 2.5 μM Y27632,
138 100 ng/ml NRG1, and 7.5 μM A83-01] in accordance with a previous protocol (Okae et al., 2018). Shortly
139 after suspending the cells in the medium, Matrigel was added to a final concentration of 2%. On day 3, the
140 medium was replaced with EVT medium without NRG1, and Matrigel was added to a final concentration
141 of 0.5%. On day 6, the medium was replaced with EVT medium without NRG1 and KSR. Matrigel was
142 then added to a final concentration of 0.5%. The cells were harvested on day 8 for further experiments.
143
144 **2.4 Differentiation of TSCs to organoids**
145 TSCs at ~80% confluence were trysinized. 5 x 10^3 TSCs were suspended in 50 uL of 72% Matrigel droplet
146 in Advanced DMEM/F12 medium. 5 droplets of the cell-matrigel mixture were introduced into the middle
147 of 6 cm Petri dishes. It was ensured that the droplets were separated from one another and never adhered to
148 the walls of the Petri dishes. The droplets were polymerized by putting the Petri dishes in a humidified 5%
149 CO_2 incubator at $37^{O}C$ for 30mins. The Petri dishes were then removed from the incubator, and then 2ml
150 of trophoblast organoid medium was added to the droplets and the Petri dishes put back in the incubator.
151 The medium was replaced every 2 days. The culture was continuously monitored for the appearance of
152 small organoid clusters. On day 8, the organoids were harvested, and total RNA was extracted from them.
153 The trophoblast organoid medium comprised Advanced DMEM/F12 supplemented with N2, B27, 2 mM
154 L-glutamine, 100ng/mL Recombinant human FGF-2, 500nM A83-01, 1.5μM CHIR99021, 50 ng/mL
155 Recombinant human EGF, 1.25 mM N-Acetyl-L-cysteine, 50 ng/mL Recombinant human HGF, 100
156 μg/mL Primocin, 80 ng/mL Recombinant human R-spondin-1, 2.5 μM Prostaglandin E2, and 2 μM Y-
157 27632, same as that used in a previous study in which organoids were established from human primary
158 trophoblast cells (Turco et al., 2018).
159
160 **2.5 Treatment of the TSCs, STs, EVTs, and organoids with BPS**

161 BPS (Sigma-Aldrich) was dissolved in absolute ethanol, and then transferred into cell culture media to yield
162 final concentrations of 0.01, 0.1 and 1μM, which are concentrations that fall within the reported urine and
163 blood levels of BPS in humans (Hehn, 2016; Jin et al., 2018; Smarr et al., 2018). Since TSCs, STs, EVTs,
164 and organoids grow and develop in special media, specific BPS-containing media and their negative
165 controls (NCs) were prepared for each model. The TSCs were treated with the various BPS concentrations
166 for 2 days and were then harvested for cell cycle analysis, apoptosis assessment, and total RNA extraction.
167 Differentiation of the TSCs into STs, EVTs, and organoids was done as already described above, with the
168 only modification being the addition of BPS and ethanol to the media. On day 5, the STs were harvested,
169 and on day 8, the EVTs and organoids were harvested for subsequent experiments.
170

171 **2.6 Cell proliferation assay**

172 The CCK8 kit (Abcam, Massachusetts, UK) was used to assess the proliferative rate of the TSCs. 3×10^3
173 cells were seeded in 96-well plates in triplicates. After treating the TSCs with 0.01, 0.1 and 1μM of BPS
174 and the NCs for 1 day, 2 days or 3 days in 100ul of media per well, we added 10 μl of the CCK8 solution
175 to each well, and then incubated the cells for 1 h at 37 ◦C. We measured the optical density at 450 nm with
176 a microplate reader (Thermo Fisher Scientific, MA, USA).
177

178 **2.7 Flow cytometric analysis of the cell cycle, and apoptosis assay**

179 Only TSCs were used for cell cycle analysis while both TSCs and EVTs were used for the apoptosis assay.
180 The cells were trypsinized and washed. For cell cycle analysis, about $1x10^5$ TSCs were fixed with 70%
181 ethanol and incubated for, at least, 3 hours at -20ºC. After removing the ethanol, 150ul of Muse Cell Cycle
182 reagent (Millipore, MCH100106) was used to incubate the cells for 30 minutes at room temperature in the
183 dark. The cells were analyzed by using the Muse flow cytometer. For apoptosis assay, about $1x10^5$ TSCs
184 or EVTs were incubated with 150ul of Muse Annexin V & Dead Cell reagent (Millipore, MCH100105) for
185 20 minutes at room temperature in the dark. The cells were then analyzed with the Muse flow cytometer
186 (Luminex).
187

188 **2.8 Total RNA extraction and RT-qPCR**

189 Total RNA was extracted from the TSCs, STs, EVTs and the organoids by using the single cell RNA
190 purification kit (Norgen Biotek Corp, 51800). The quality of the RNA was evaluated with 1% agarose gel
191 electrophoresis and UV spectrophotometry. Complementary DNAs (cDNAs) were generated from 300 ng
192 of total RNA using the SuperScript™ III first strand synthesis SuperMix kit (Thermo Fisher Scientific,
193 18080400) and then diluted 20 times with DNase-free water. RT-qPCR was carried out by using the
194 PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, A46110). Primers were designed to

amplify the junction between two exons using a web-based primer design program, Primer 3 (http://bioinfo.ut.ee/primer3/). The primer sequences are listed in Supplementary Table 1. Cycle threshold (Ct) values of the samples and the controls were normalized against GAPDH while the relative gene expression was calculated as fold enrichment by using the $2^{-\Delta\Delta CT}$ method. The relative gene expression was calculated from an average of two replicates. All primers used in qPCR are listed in Supplementary Table 1.

2.9 Statistical analyses

The data were analyzed using the GraphPad Prism 9 software, and are presented as mean ± SD of, at least, two independent experiments. Significant differences between groups were assessed with t-test. *, **, and *** denote significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results

3.1. Successful establishment of the ST, EVT, and organoid models

We established in vitro models of villous and extravillous trophoblasts of the placenta by differentiating TSCs into STs, EVTs, and organoids (**Fig. 1A**). Each trophoblast lineage displayed gene expression patterns that are comparable to those of the placenta. In the ST model, we verified the upregulation of ST markers, including fusogenic genes (CGA, CGB3, CSH1, ERVW1, ERVFRD, GCM1, PSG1, SDC1 and CYP19A1) (**Figs. 1B** and 1**C)** as well as downregulation of TEAD4 (a stem cell marker) and CDH1 (an epithelial marker) (**Fig. 1D**) compared to TSCs. In the EVT model, we confirmed the upregulation of EVT markers (HLA-G, MMP2, ASCL2, ITGA5 and FN1) (**Fig. 1E**), accompanied by a decrease in TEAD4 expression (**Fig. 1F**) compared to TSCs. In the organoid model, we validated an increase in the expressions of CGA, CGB3, CSH1, ERVW1, ERVFRD, GCM1, PSG1, SDC1 and CYP19A1 (**Figs. 1G** and **1H**) and a decrease in the expression of CDH1 (Figure 1J) compared to TSCs. Interestingly, expressions of proliferation markers (Ki67 and PCNA) (**Fig. 1I**) and stem cell markers (TEAD4, ELF5, GATA3, and TP63) (**Fig. 1J**) displayed no significant changes in organoids, likely due to the organoids comprising a mixture of TSCs and STs. In summary, we successfully established in vitro models representing both villous and extravillous trophoblasts of the human placenta.

3.2. BPS exhibit no significant effect on stemness, proliferation, and cell cycle of TSCs.

We exposed TSCs to three different BPS concentrations (0.01, 0.1, and 1μM) for 2 days, as illustrated in **Fig. 2A**. We observed that BPS treatment did not affect the expressions of the stem cell marker genes (TEAD4, ELF5, GATA3 and TP63) (**Fig. 2B**). Consistently, proliferation assay showed no difference in growth rate between the BPS-treated and the NC TSCs (**Fig. 2C**), along with no significant expression

229 change in the proliferation marker genes Ki67 and PCNA upon BPS treatment (**Fig. 2D**). Flow cytometric
230 analysis further indicated that the cell cycle did not differ between the BPS-treated and the NC TSCs (**Fig.**
231 **2E** and **Supplementary Fig. 1**). Moreover, we assessed the impact of each BPS concentration on the
232 proliferation and stemness of trophoblast organoids that consist of both TSCs and STs. On day 8 of TSC
233 differentiation into the trophoblast organoids, we found that, just like the TSCs, BPS treatment did not
234 affect the expressions of the stem cell markers (TEAD4, ELF5, GATA3 and TP63) as well as the cell
235 proliferation markers (Ki67 and PCNA) (**Supplementary Figs. 2** and **3**). Collectively, these results indicate
236 that BPS has no significant effect on self-renewal of TSCs as well as trophoblast organoids.
237

238 **3.3. BPS moderately decreases the expression of ST marker genes in STs and organoids, while**
239 **inducing no discernable effect on EVT formation**

240 To explore the influence of BPS on the multipotency of TSCs, we treated the TSCs with three different
241 BPS concentrations (0.01, 0.1, and 1μM) during differentiation of TSCs into STs, EVTs, and trophoblast
242 organoids (**Fig. 2A**). On day 5 of ST formation, we observed no significant morphological differences
243 between the BPS-treated STs and their corresponding NC STs (**Fig. 3A**). However, BPS treatment
244 moderately reduced the expressions of several ST marker genes. For instance, in the STs treated with
245 0.01μM of BPS, CGB3, CSH1, GCM1, and PSG1 were downregulated, while CDH1 and TEAD4 were
246 upregulated. However, the expressions of CGA, ERVW1, ERVFRD and CYP19A1 remained unchanged
247 compared to the NCs (**Fig. 3B**). Importantly, in the STs treated with 0.1μM and 1μM of BPS, we observed
248 that 8 out of 9 ST marker genes (CGA, CGB3, CSH1, ERVW1, GCM1, PSG1, SDC1 and CYP19A1) were
249 downregulated while CDH1 and TEAD4 were upregulated compared to the NCs (**Fig. 3B**), suggesting that
250 higher concentrations of BPS have a broader impact on the downregulation of ST marker genes. Consistent
251 with STs, the organoids treated with BPS exhibited no significant difference in morphology (**Fig. 3C**) but
252 a reduction in multiple ST marker genes compared to the NCs, particularly with the treatment of 1μM of
253 BPS. These genes include CGA, CGB3, CSH1, GCM1, ERVFRD, SDC1, and CYP19A (**Fig. 3D**). In
254 contrast with ST formation, EVTs on day 8 of differentiation did not show significant morphological
255 differences or changes in the expression of EVT marker genes between the BPS-treated EVTs and their
256 corresponding NCs (**Figs. 4A** and **4B**). Taken together, these findings suggest that BPS moderately inhibits
257 the induction of ST marker genes during ST formation, while eliciting no effect on EVT formation.
258

259 **3.4. BPS induces apoptosis in trophoblast lineages.**

260 We determined the impacts of 0.01, 0.1 and 1μM of BPS on the apoptosis of TSCs. Additionally, during
261 the differentiation of TSCs into the STs, EVTs, and organoids, we investigated the influence of these three
262 different BPS concentrations on apoptosis in each trophoblast model. In the TSCs, we found that each BPS

concentration increased the proportion of early and total apoptotic cells compared to the NC TSCs (**Fig. 5A** and **Supplementary Fig. 4A**). In agreement with this observation, TSCs treated with each concentration of BPS displayed an increase in the expressions of the pro-apoptotic genes BAX, TP53, and CDKN1B (**Fig. 5B**). Notably, higher concentrations of BPS significantly intensified apoptosis (**Fig. 5A)**. With induction of pro-apoptotic genes, TSCs treated with BPS concentrations of 0.1μM and 1μM also showed diminished expression of the anti-apoptotic genes. Particularly, treatment with 1μM of BPS significantly lowered the levels of BCL2, SIRT1, MCL1, and PTMA compared to the NC TSCs (**Fig. 5B**). In the EVTs, we found that only 1μM of BPS induced apoptosis (**Fig. 5C** and **Supplementary Fig. 4B**), leading to an increase in the proportion of late and total apoptotic cells compared to the NC EVTs. Further assessment showed that BAX, TP53, and CDKN1B were upregulated while BCL2 was downregulated in the EVTs treated with 1μM of BPS (**Fig. 5D**). However, unlike TSCs, the EVTs treated with 1μM of BPS did not show any change in the expressions of SIRT1, MCL1 and PTMA compared to the NC EVTs (**Fig. 5D**). In the ST model, 0.01μM of BPS did not affect the expressions of the apoptosis markers. However, 1μM of BPS was able to reduce the expressions of anti-apoptotic genes (BCL2, SIRT1 and MCL1) but had no effect on the expressions of pro-apoptotic genes (**Fig. 5E**). In the organoid model, only the 1μM of BPS was able to downregulate BCL2, SIRT1, and MCL1, while upregulating TP53 and CDKN1B. However, it did not affect the expressions of PTMA and BAX (**Fig. 5F**).

4. Discussion

In this study, we have successfully established in vitro placental models. In these models, the TSCs organized into either extravillous trophoblast or villous-like structures. This establishment was done in accordance with published protocols (Okae et al., 2018; Turco et al., 2018). Human placenta development mainly encompasses trophoblast proliferation, differentiation, and apoptosis which are critically regulated by a myriad of molecules, including transcription factors (Papuchova and Latos, 2022), hormones (Adu-Gyamfi et al., 2020c), and cytokines (Adu-Gyamfi et al., 2020a; Appiah Adu-Gyamfi et al., 2020). During differentiation, the CTs either transform into STs or EVTs. Formation of the STs, a process known as syncytialization, involves the fusion of the cell membranes of adjacent CTs, leading to protoplasmic merging (Kar et al., 2007). This occurrence is mediated by molecular processes such as upregulation of the syncytins (ERVW1, ERVFRD) (Roberts et al., 2021) and the hormonal genes (CGA, CGB3) (Okae et al., 2018) as well as the downregulation of epithelial markers such as CDH1 (E-cadherin) (Adu-Gyamfi et al., 2021). We confirmed these patterns of gene expression upon differentiation of TSCs into STs and organoids, providing evidence that we successfully established ST and trophoblast organoid models. Formation of the EVTs, a process known as epithelial-mesenchymal transition, is characterized by the CTs acquiring a spindle-shaped morphology. This cellular process is mediated by the upregulation of HLA-G

297 and mesenchymal markers such as ASCL2, MMP2 and ITGA5 (Okae et al., 2018; Varberg et al., 2021).
298 As expected, we observed this expression pattern of the molecular markers in the EVT lineage we
299 established. By using these models, we have demonstrated that BPS may moderately interfere with placenta
300 development.
301
302 Proliferation of the CTs is the first stage in human placenta development. Both hypo- and hyper-
303 proliferative rates of trophoblast cells are detrimental to the formation of a functional placenta. For instance,
304 when CT proliferation is reduced, few CTs become available to undergo differentiation, leading to defective
305 ST and EVT formation. Conversely, when proliferation is excessively stimulated, CT differentiation
306 becomes inhibited, resulting in impairment of ST and EVT formation. A previous study demonstrated that
307 BPS does not affect the proliferation of HTR8/SVneo cells (Ticiani et al., 2022). This cell line is
308 immortalized and has characteristics of the non-proliferative extravillous trophoblast cells which are
309 completely different from the CTs or TSCs. Therefore, we assessed the impacts of BPS on the proliferation
310 of TSCs and organoids. We observed that BPS did not affect the proliferation, cell cycle, and stemness of
311 TSCs. Hence, BPS may not be detrimental to the proliferation and renewal of the trophoblast progenitor
312 cells within the placenta.
313
314 At the feto-maternal interface, physiologic regulation of trophoblast differentiation is vital to the
315 establishment of a normal placental architecture while dysregulation of this process, either due to the
316 abnormal expression of some genes or the presence of a xenobiotic, induces placental abnormalities to
317 affect pregnancy (Gong et al., 2021; Mohamad et al., 2020; Rosenfeld, 2021; Wu et al., 2023). In this study,
318 we found that BPS did not induce noticeable defects in the expression of the EVT marker genes and EVT
319 morphology. We also found that BPS did not significantly affect the morphologies of the STs and
320 organoids. However, BPS led to a moderate reduction in the transcript levels of several ST marker genes
321 that promote trophoblast fusion. Additionally, it elevated the transcript levels of the epithelial marker,
322 CDH1, and the stem cell marker, TEAD4 (Saha et al., 2020) in STs. Notably, BPS elicited these effects in
323 a dose-dependent manner, with the highest concentration causing the most marked effect. Consistent with
324 our observation, it was found in sheep that BPS impaired the placental cell fusion pathway, but it did not
325 affect the morphology of the placenta (Gingrich et al., 2018). In a previous in vitro study, ~0.8 μM of BPS
326 did not affect the spontaneous fusion of primary CTs (Ticiani et al., 2021). Collectively, these findings
327 suggest that BPS causes a potential molecular-level defect in CT syncytialization but has no significant
328 effect on CT differentiation into EVTs.
329

Trophoblast apoptosis is an integral process in placenta development. The interplay between pro- and anti-apoptotic regulators at the feto-maternal interface tightly regulates the rate of trophoblast apoptosis. During normal pregnancy, trophoblast apoptosis increases with advancing gestation. This serves to eliminate unwanted, superfluous, and dysfunctional cells, promoting placental growth and functionality. However, excessive apoptosis impairs placenta development, potentially leading to pregnancy pathologies (Sharp et al., 2010; Straszewski-Chavez et al., 2005; Tomas et al., 2011). We have shown that BPS triggers apoptosis in each trophoblast lineage. This was evidenced by an increase in the proportion of total apoptotic TSCs and EVTs, an increase in the expression of pro-apoptotic genes and a reduction in the expression of anti-apoptotic genes in the TSCs, STs, EVTs and the organoid model. While all the concentrations of BPS triggered apoptosis in the TSCs, only the higher BPS concentrations were able to induce apoptosis in the STs and EVTs. This suggests that CTs may be more susceptible to the apoptotic effects of BPS than STs and EVTs. Due to this apoptotic effect, the presence of BPS within the placental micro-environment may have a synergistic effect with physiological apoptosis; thereby, amplifying trophoblast apoptosis to the detriment of placenta formation and functioning.

5. Conclusions

This study is the first to use human TSCs and trophoblast differentiation models to assess the impacts of BPS on human placenta development. Our findings suggest that the presence of BPS at the feto-maternal interface may exaggerate trophoblast apoptosis while moderately inhibiting trophoblast fusion to impair placenta development. Since BPS and its products are in use, the global consumption of BPS is bound to rise exponentially in the coming decades. Hence, epidemiological studies should monitor BPS levels in the blood and placentas of pregnant women and assess the relationship between maternal serum levels of BPS and pregnancy complications. The findings will assist in policy formulations concerning the use of BPS and its products in daily life.

Data Availability

The original data can be obtained from the corresponding author via an email.

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Conflict of Interest

364 The authors have declared that they have no conflict of interest.
365
366 **Figure legends**
367
368 **Figure 1: The established placental models and their characteristics.** (**A**) Images showing the
369 differentiation of TSCs into EVT, ST, and organoid models. (**B and C**) Expression fold changes of ST
370 marker genes in STs relative to TSCs. (**D**) Transcript levels of epithelial (CDH1) and stem cell (TEAD4)
371 markers in STs relative to TSCs. (**E**) Expression fold changes of EVT marker genes in EVTs relative to
372 TSCs. (**F**) Transcript level of a stem cell marker, TEAD4, in EVTs relative to TSCs. (**G and H**) Expression
373 fold changes of ST marker genes in trophoblast organoids relative to TSCs. (**I and J**) Transcript levels of
374 proliferation markers (I) and stem cell markers (J) in organoids relative to TSCs.
375
376 **Figure 2: BPS does not affect TSC marker gene expression, cell cycle, and proliferation.** (**A**) A
377 schematic diagram illustrating the experimental design we used to investigate the impacts of BPS on the
378 self-renewal of TSCs and their differentiation potentials. We treated TSCs with BPS for 2 days. Also, during
379 the differentiation of TSCs into STs, EVTs, and organoids, we treated them with BPS (0.01, 0.1, or 1 μM).
380 (**B**) Transcript levels of stem cell markers in the BPS-treated TSCs relative to NCs. (**C**) Proliferation rates
381 in the BPS-treated and NC TSCs. (**D**) Transcript levels of proliferation markers in the BPS-treated TSCs
382 relative to NCs. (**E**) Cell population distribution in different cell cycle phases in BPS-treated and NC TSCs.
383
384 **Figure 3: BPS leads to a moderate downregulation of trophoblast fusion marker genes in the STs and**
385 **organoids.** (**A**) Representative images showing BPS-treated and NC STs after 5 days of TSC differentiation
386 into STs. (**B**) Transcript levels of trophoblast fusion, stem cell, and epithelial markers in the BPS-treated
387 STs relative to NC STs. (**C**) Representative images showing BPS-treated and NC organoids after 8 days of
388 TSC differentiation into organoids. (**D**) Transcript levels of trophoblast fusion, stem cell, and epithelial
389 markers in the BPS-treated organoids relative to NC organoids.
390
391 **Figure 4: BPS does not affect EVT morphology and the expressions of EVT marker genes.** (**A**)
392 Representative images showing BPS-treated and NC EVTs after 8 days of TSC differentiation into EVTs.
393 (**B**) Transcript levels of EVT markers in the BPS-treated EVTs relative to NC EVTs.
394
395 **Figure 5: BPS induces apoptosis in TSCs, STs, EVTs, and organoids.** (**A**) The distribution of cell
396 population in different apoptotic phases (EAC: Early apoptotic cells, LAC: late apoptotic cells, TAC: Total
397 apoptotic cells, and DC: Dead cells) of BPS-treated and NC TSCs. (**B**) Transcript levels of apoptosis

398 markers in the BPS-treated TSCs relative to NC TSCs. (**C**) The distribution of cell population in different
399 apoptotic phases of BPS-treated and NC EVTs. (**D**) Transcript levels of apoptosis markers in the 1uM BPS-
400 treated and NC EVTs. (**E**) Transcript levels of apoptosis markers in the BPS-treated STs relative to NC
401 STs. (**F**) Transcript levels of apoptosis markers in the BPS-treated organoids relative to NC organoids.
402

403 **References**

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Figure 1

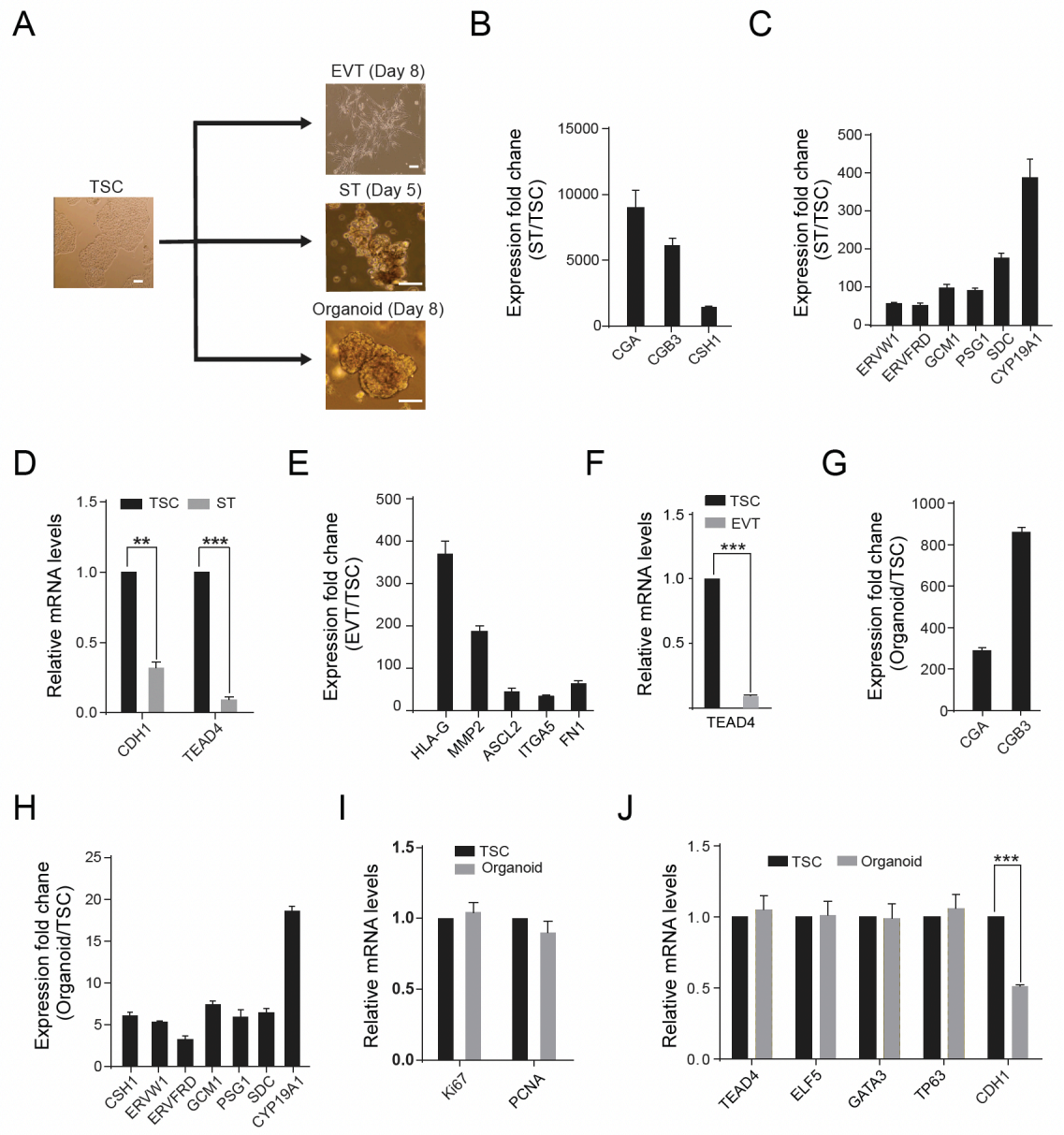

A
TSC
EVT (Day 8)
ST (Day 5)
Organoid (Day 8)
B
Expression fold chane (ST/TSC)
15000
10000
5000
0
CGA
CGB3
CSH1
C
Expression fold chane (ST/TSC)
500
400
300
200
100
0
ERVW1
ERVFRD
GCM1
PSG1
SDC
CYP19A1
D
Relative mRNA levels
TSC
ST
**
***
1.5
1.0
0.5
0.0
CDH1
TEAD4
E
Expression fold chane (EVT/TSC)
500
400
300
200
100
0
HLA-G
MMP2
ASCL2
ITGA5
FN1
F
Relative mRNA levels
TSC
EVT
***
1.5
1.0
0.5
TEAD4
G
Expression fold chane (Organoid/TSC)
1000
800
600
400
200
0
CGA
CGB3
H
Expression fold chane (Organoid/TSC)
25
20
15
10
5
0
CSH1
ERVW1
ERVFRD
GCM1
PSG1
SDC
CYP19A1
I
Relative mRNA levels
TSC
Organoid
1.5
1.0
0.5
0.0
Ki67
PCNA
J
Relative mRNA levels
TSC
Organoid
***
1.5
1.0
0.5
0.0
TEAD4
ELF5
GATA3
TP63
CDH1

Figure 2

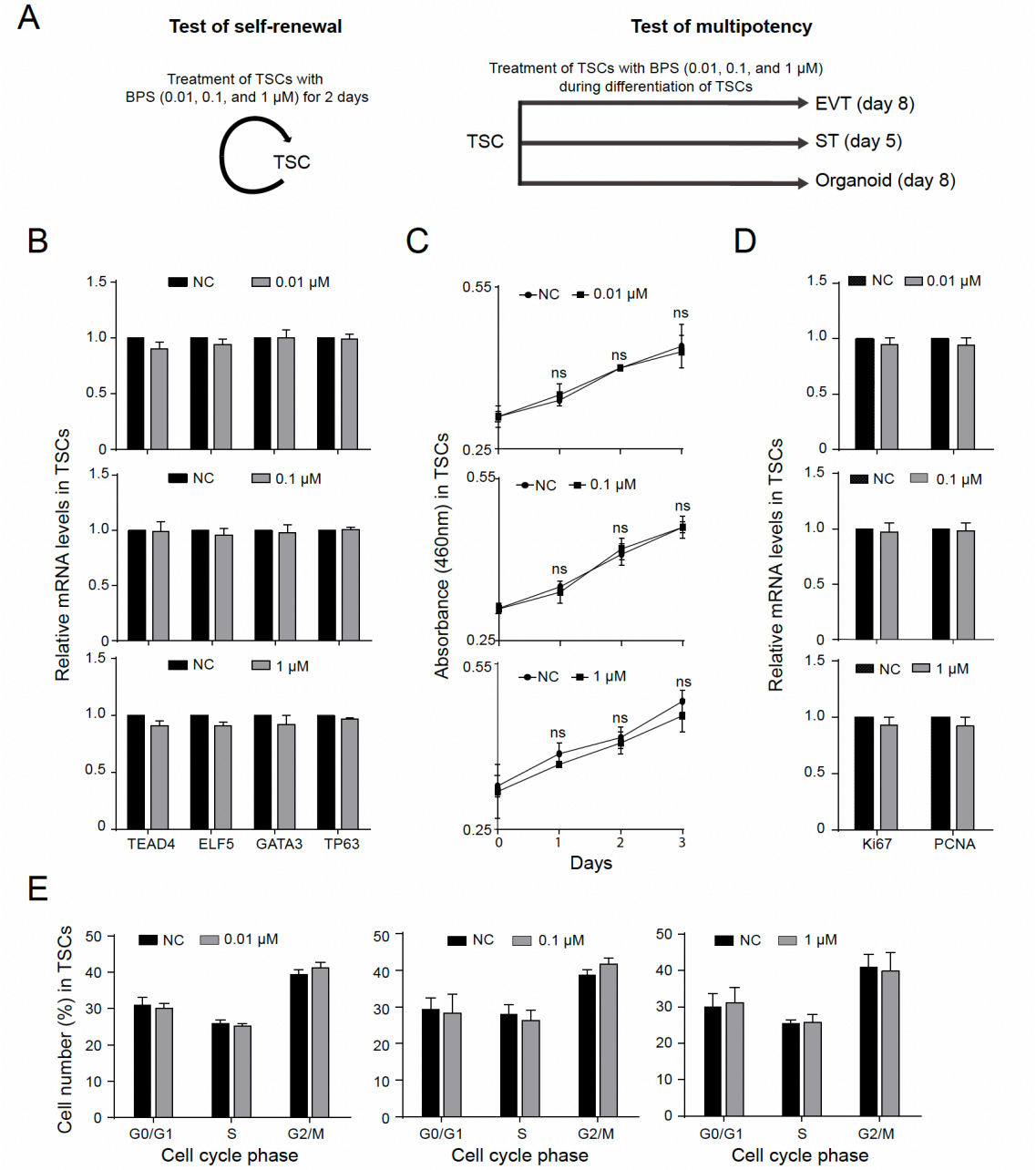

A
Test of self-renewal
Treatment of TSCs with
BPS (0.01, 0.1, and 1 μM) for 2 days
TSC
Test of multipotency
Treatment of TSCs with BPS (0.01, 0.1, and 1 μM)
during differentiation of TSCs
TSC
EVT (day 8)
ST (day 5)
Organoid (day 8)
B
Relative mRNA levels in TSCs
NC
0.01 μM
0.1 μM
1 μM
TEAD4
ELF5
GATA3
TP63
C
Absorbance (460nm) in TSCs
ns
Days
D
Relative mRNA levels in TSCs
Ki67
PCNA
E
Cell number (%) in TSCs
G0/G1
S
G2/M
Cell cycle phase

Figure 3

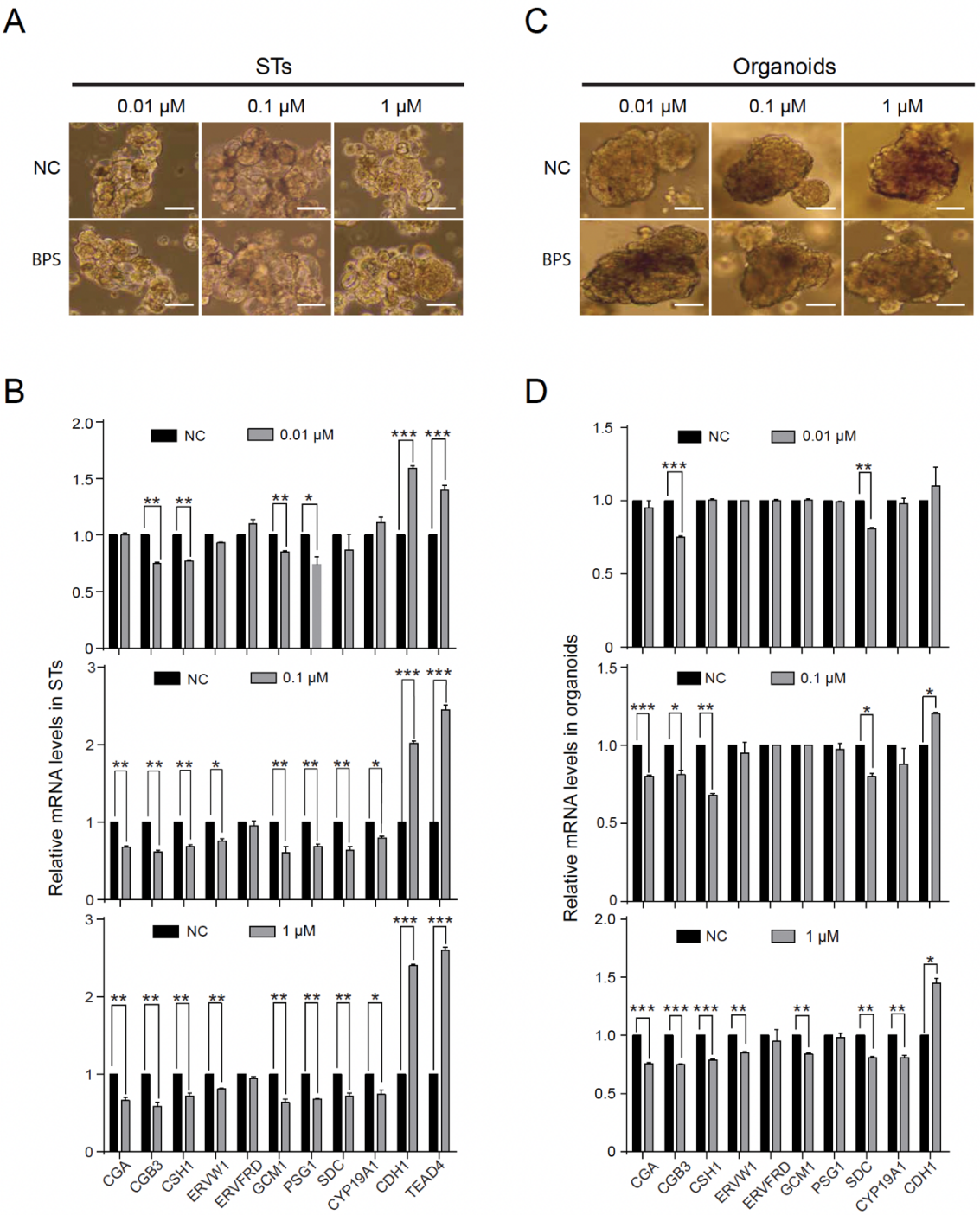
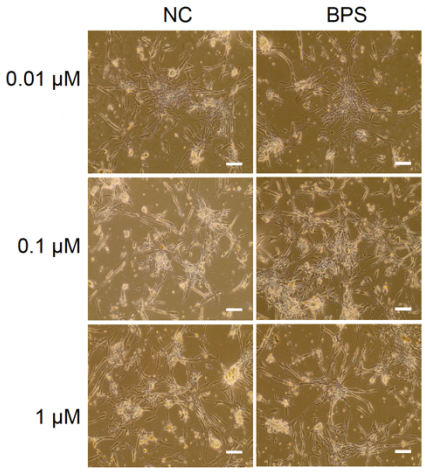

A
STs
0.01 μM
0.1 μM
1 μM
NC
BPS
C
Organoids
0.01 μM
0.1 μM
1 μM
NC
BPS
B
D
Relative mRNA levels in STs
Relative mRNA levels in organoids
NC
0.01 μM
0.1 μM
1 μM
CGA
CGB3
CSH1
ERVW1
ERVFRD
GCM1
PSG1
SDC
CYP19A1
CDH1
TEAD4

Figure 4

A


NC
BPS
0.01 μM
0.1 μM
1 μM

B

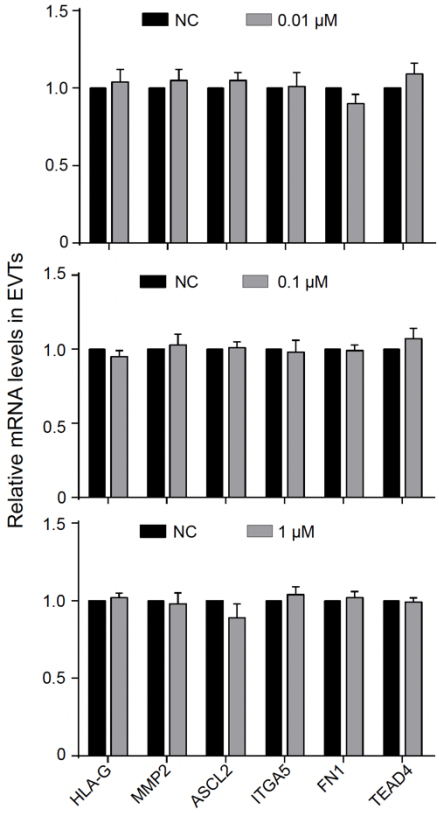
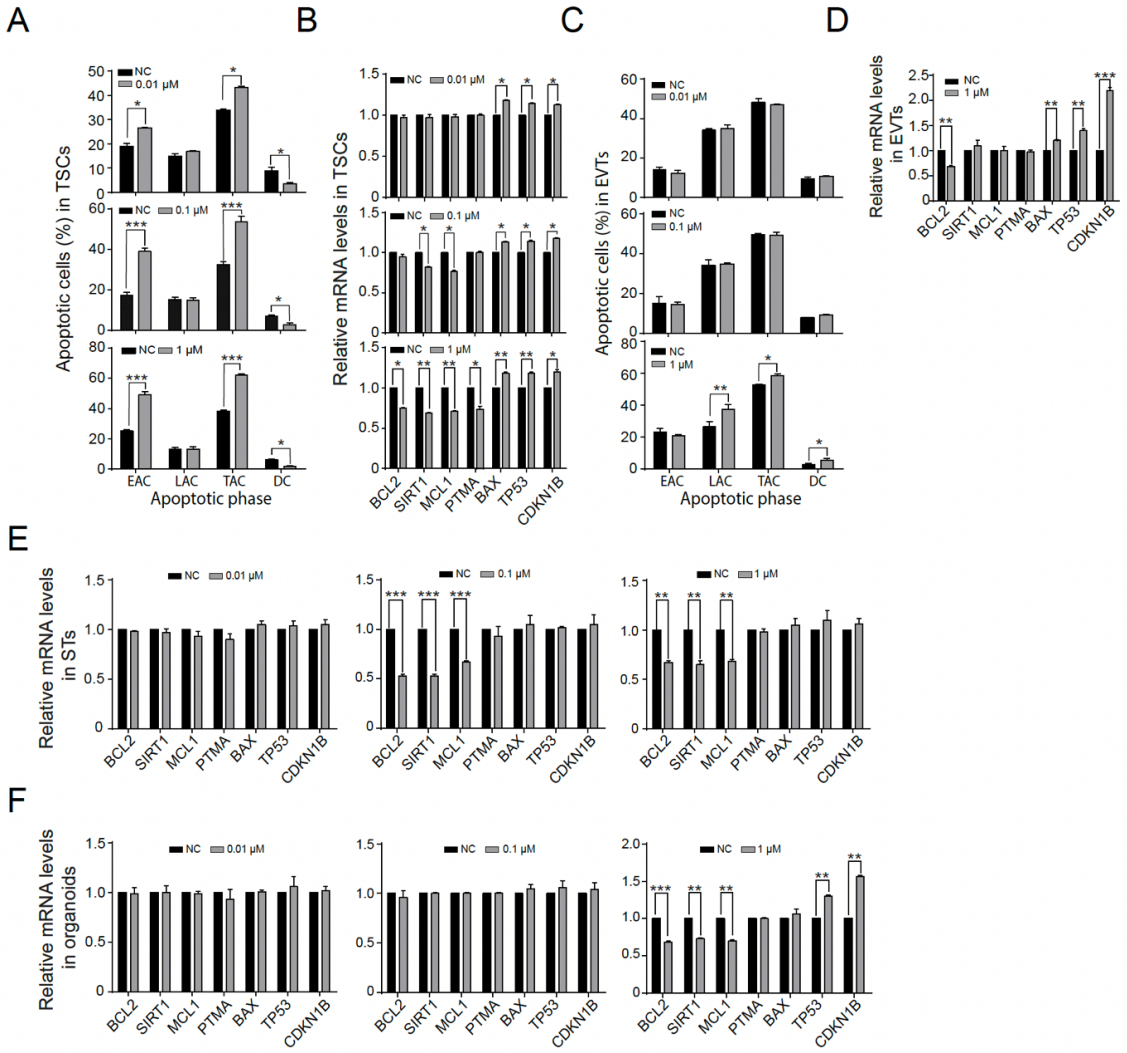
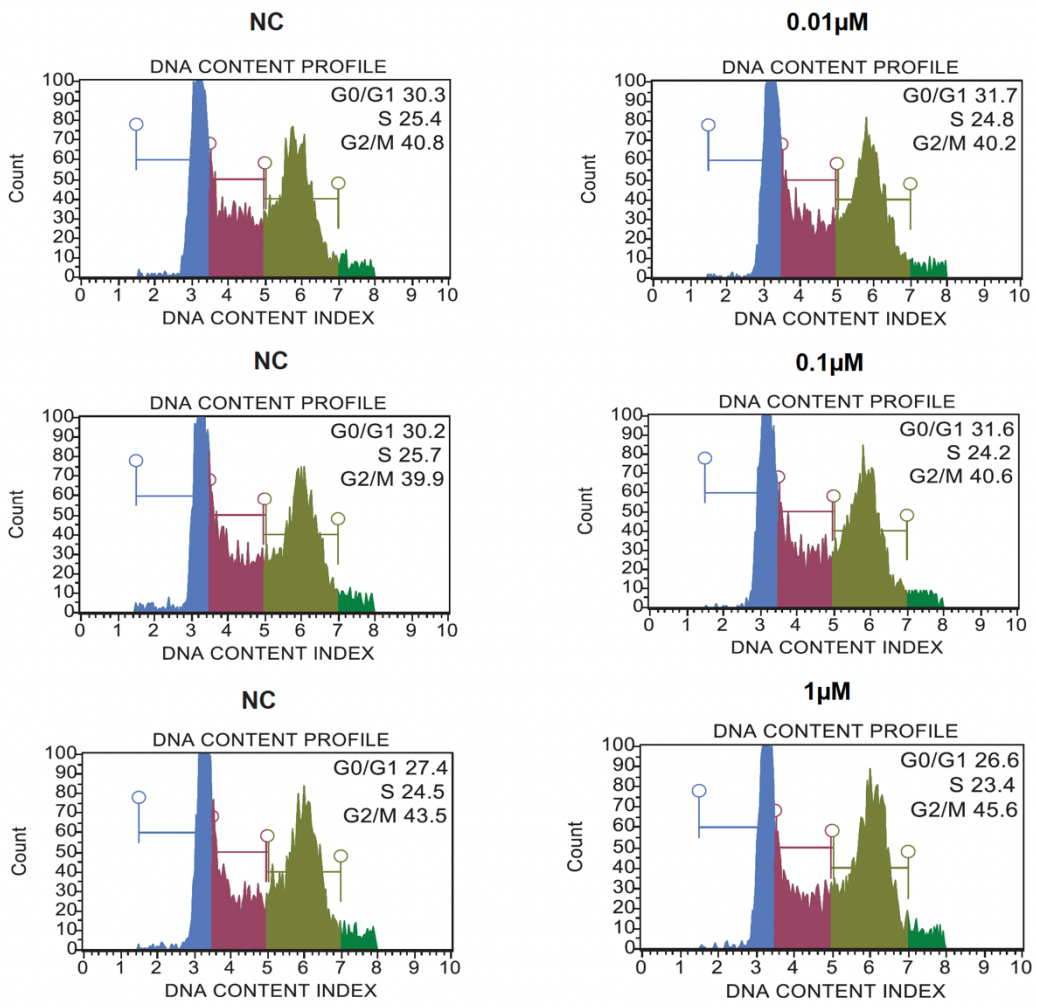

Relative mRNA levels in EVTs
NC
0.01 μM
0.1 μM
1 μM
1.5
1.0
0.5
0
HLA-G
MMP2
ASCL2
ITGA5
FN1
TEAD4

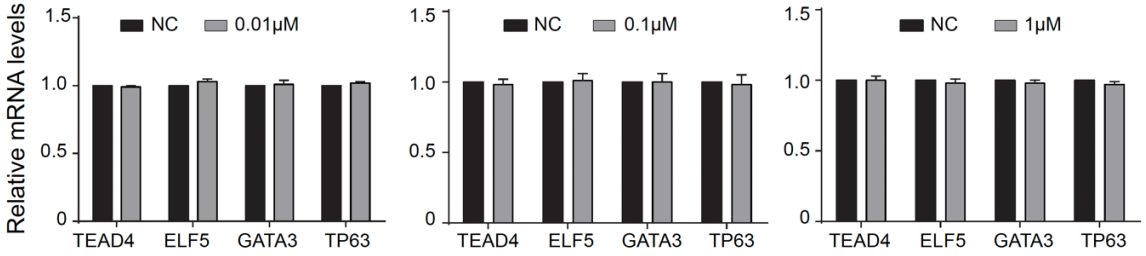
Figure 5


A
B
C
D
E
F
Apoptotic cells (%) in TSCs
Relative mRNA levels in TSCs
Apoptotic cells (%) in EVTs
Relative mRNA levels in EVTs
Relative mRNA levels in STs
Relative mRNA levels in organoids
NC
0.01 μM
0.1 μM
1 μM
EAC
LAC
TAC
DC
Apoptotic phase
BCL2
SIRT1
MCL1
PTMA
BAX
TP53
CDKN1B

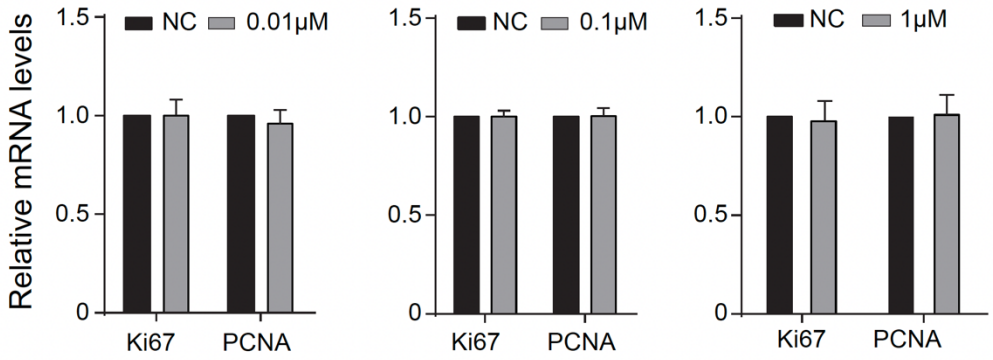
Supplementary data



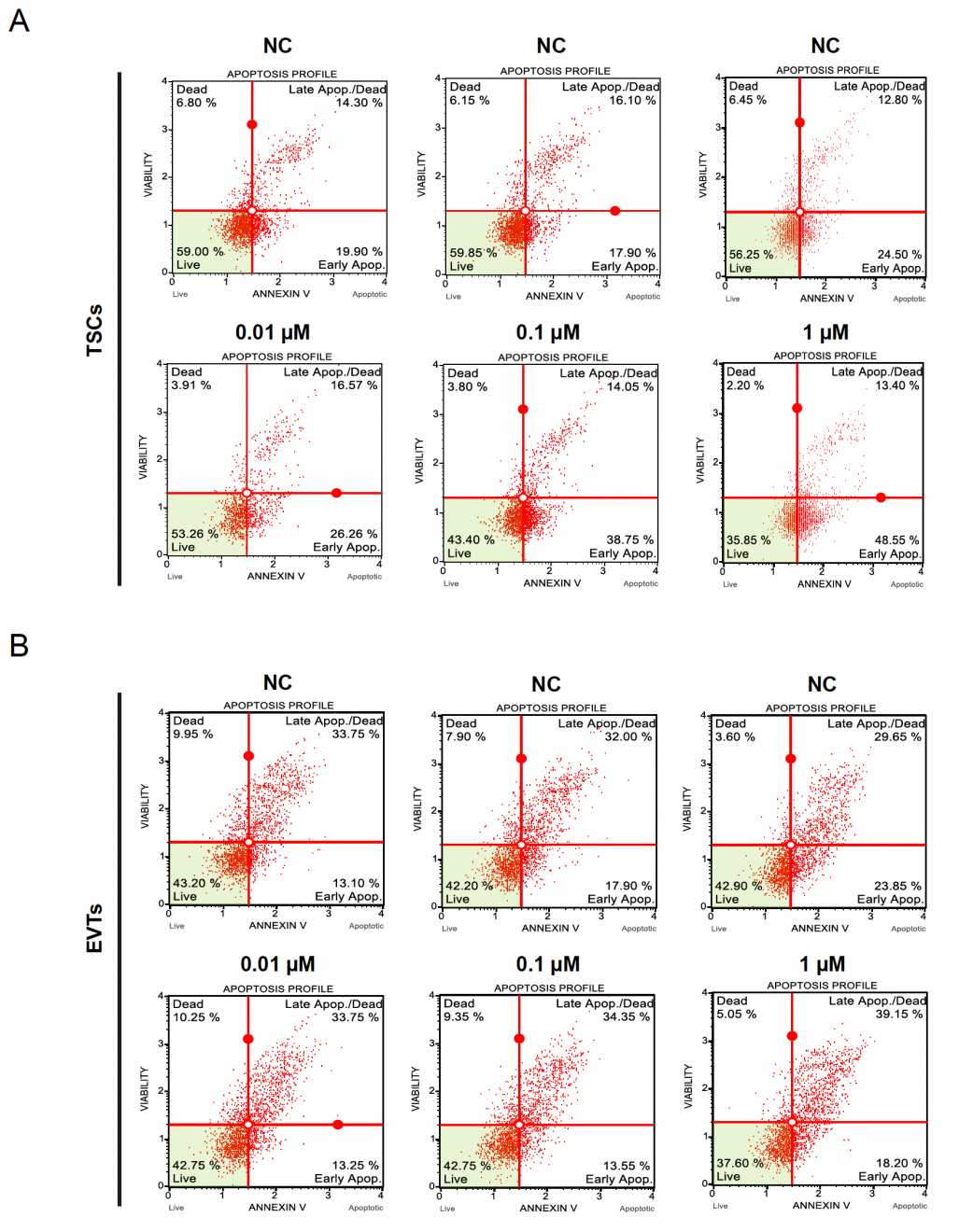
Supplementary Fig.1. Representative histogram from cell cycle analysis presenting the proportion of cells in each phase of the cell cycle.



Supplementary Fig.2. Transcript levels of stem cell markers in the BPS-treated organoids relative to NC organoids.



Supplementary Fig.3. Transcript levels of proliferation markers in the BPS-treated organoids relative to NC organoids.



Supplementary Fig.4. (A and B) Representative graphs showing the proportion of apoptotic cells among the BPS-treated TSCs and NC TSCs (A), and among the BPS-treated EVTs and NC EVTs (B).

Supplementary Table 1. A list of primers ued for quantitative PCR	
GAPDH _L	TGACTTCAACAGCGACACCC
GAPDH_R	TTCGTTGTCATACCAGGAAATGAG
CGA _L	GTCCAAAAGAACGTCACCTCA
CGA _R	CGTGTGGTTCTCCACTTTGAA
CGB3_L	GTCAACACCACCATCTGTGC
CGB3_R	GGTAGTTGCACACCACCTGAG
CSH1_L	CAGACCTCCTTCTGCTTCTCA
CSH1_R	GATGCGGAGCAGCTCTAGATT
ERVW-1_L	CAACTGCTATCACTCTGCCAC
ERVW-1_R	TCCAACAGACAGTGACTCCAA
ERVFRD-1_L	GCTGGTTCTCATTCTCACGC
ERVFRD-1_R	GGGGATCCTGTACTTTGGAGC
GCM1_L	GCAAGAAGAGCCATGAAGAAA
GCM1_R	TCCCCTGACTTTGTGTTTCAC
PSG1_L	GGGAGATGATGGGACTAGAGG
PSG1_R	CTGGGATTTAAGTTGCTGCTG
SDC1_L	TGAAGATCAAGATGGCTCTGG
SDC1_R	GGTCTGCTGTGACAAGGTGAT
CYP19A1_L	GACTCGAGTTTTTCCCCAAAC
CYP19A1_R	CGATGTACTTTCCTGCACAGC
CDH-1_L	GAACTAACACACGGGGCGAG
CDH-1_R	TTTGTAGTCACCCACCTCTAAG
TEAD4_L	CTCCACGAAGGTCTGCTCTTT
TEAD4_R	GGATGCGGTAAGAGTAGTGTCC
HLA-G_L	TCCACAGATACCTGGAGAACG
HLA-G_R	AGGGTGGCCTCATAGTCAAAG
MMP2_L	AGCTCCCGGAAAAGATTGAT
MMP2_R	GTGCTGGCTGAGTAGATCCAG
ASCL2 _L	CGTGAAGCTGGTGAACTTGGG
ASCL2 _R	CTCCACCTTGCTCAGCTTCTT
ITGA5_L	CCTGCTGTCCACCATGTCTAT
ITGA5_R	GAGCTGCTGACCTTCCAGAG
FN1_L	TAAGGTTCGGGAAGAGGTTGT
FN1_R	AACTGTGTAGGGGTCAAAGCA
PCNA_F	AGCTCTTCCCTTACGCAAGTCT
PCNA_R	AGGAAAGTCTAGCTGGTTTCGG
Ki67_F	ACAGCAACGACATGAAAACCA
Ki67_R	GCCTCAGTCTTATTTTGGCGT
ELF5 _L	CGGGTGGTTAAATCGGAAGC
ELF5_R	ACCCGCTCCAAAATTCCTGT
GATA3_L	GGGCTCTACTACAAGCTTCAC
GATA3_R	CACTTTTTGGATTTGCTAGAC
TP63_L	AGAAACGAAGATCCCCAGATGA
TP63_R	CTGTTGCTGTTGCCTGTACGTT
BCL2_F	ATGACTGAGTACCTGAACCGG
BCL2_R	GAAATCAAACAGAGGCCGCAT
SIRT1_F	CCTAATAGAGTGGCAAAGGAGC
SIRT1_R	ATATACCTCAGCGCCATGGAA
MCL1_F	GGATGGGTTTGTGGAGTTCTT
MCL1_R	AGATATGCCAAACCAGCTCCT
PTMA_F	AATGAGGAAAATGGGGAGCAG
PTMA_R	CCATCCTCTTCCTCACCATCA
BAX_F	TGTCGCCCTTTTCTACTTTGC
BAX_R	CCAATGTCCAGCCCATGATG
TP53_F	CGAGATGTTCCGAGAGCTGAA
TP53_R	TTTTGGACTTCAGGTGGCTG
CDKN1B_F	AATGCGCAGGAATAAGGAAGC
CDKN1B_R	GAACCGGCATTTGGGGAAC