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1 **Title:** Single-cell analysis of prostaglandin E₂-induced decidual cell differentiation: does extracellular
2 8-Br-cAMP cause artifacts?

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30 **Running Title:** PGE₂ as the natural decidual ligand

31
32 **Summary Sentence:** In vitro decidualization of human endometrial stromal fibroblasts with
33 prostaglandin E₂ and progestin induces a markedly different, and potentially more physiological,
34 decidual cell state through PTGER2-dependent PKA activation, compared to the conventional cyclic
35 AMP/MPA protocol.

36
37 **Keywords:** prostaglandin E₂, natural ligand, decidua, decidual stromal cell, endometrial stromal
38 fibroblast, PTGER2

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41 **Abstract**

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43 Development of the uterine decidua, the transient maternal tissue contacting the fetus during
 44 extended gestation, is the hallmark of reproduction in many placental mammals. Differentiation of
 45 decidual stromal cells is known to be induced by stimuli that activate the nuclear progesterone receptor
 46 and the cyclic AMP/protein kinase A (cAMP/PKA) pathways. The nature of the stimulus upstream of
 47 PKA has not been clearly defined, although a number of candidates have been proposed. To bypass this
 48 uncertainty for in vitro experiments, direct addition of membrane-permeable cAMP along with
 49 progesterone has been the prevailing method. Phylogenetic inference suggests that the inflammatory
 50 eicosanoid prostaglandin E₂ (PGE₂) was the stimulus that ancestrally induced decidualization.
 51 Accordingly, we developed a protocol to decidualize human endometrial stromal fibroblasts using
 52 progesterone and PGE₂ and analyzed the response in comparison with a cAMP-based protocol.
 53 Transcriptomic comparison reveals a common activation of core decidual cell genes between both
 54 treatments, and a set of senescence-related genes exaggerated under cAMP treatment. Single-cell
 55 transcriptomic analysis of PGE₂-mediated decidualization revealed a major transcriptomic transition
 56 between an early activated cell state and a differentiated decidual state, but notably did not identify a
 57 developmental trajectory representing a distinct senescent decidual state as reported in recent literature.
 58 Furthermore, investigation of the signal transduction process underlying PGE₂-mediated
 59 decidualization showed that it depends upon progesterone-dependent induction of PGE₂ receptor 2
 60 (PTGER2 aka EP2) and PKA, the kinase activated by PTGER2. This progesterone-dependent induction
 61 of PTGER2 is absent in the opossum, a species incapable of decidualization. Together, these findings
 62 suggest that the origin of the decidual cell type involved the evolution of progesterone-dependent

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63 activation of the PGE₂/EP2/PKA axis. We propose the use of PGE₂ for in vitro decidualization studies
64 as a potentially more physiological model than 8-Br-cAMP.

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66 **Introduction**

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68 The uterine decidua, the transient maternal tissue that forms the maternal side of the fetal-
69 maternal interface, plays an essential role in the reproduction in mammals with invasive placentation
70 (Gellersen and Brosens, 2014). The decidua is a novel tissue which evolved in the stem lineage of
71 placental (eutherian) mammals (Mess and Carter, 2006), and was a key innovation for the evolution of
72 the elaborated placenta of eutherians (Chavan et al. 2017; Stadtmauer and Wagner 2020). Decidual
73 development entails the differentiation of the endometrial stromal fibroblasts (ESF) into mature
74 decidual stromal cells (DSC). The ability of endometrial stromal fibroblasts to differentiate into DSC is
75 an evolutionarily derived trait (Park et al., 2016; Erkenbrack et al., 2019), and the stromal cells that are
76 able to form DSC are called “neo-ESF” (Kin *et al.*, 2016). Neo-ESF and DSC evolved from an
77 ancestral endometrial stromal cell type unable to decidualize called “paleo-ESF.” Paleo-ESF are still
78 found in the opossum, *Monodelphis domestica* (Kin *et al.*, 2016), and likely exist in other marsupials. It
79 follows that comparison of the response of paleo-ESF and neo-ESF to decidualogenic stimuli is a way to
80 identify the molecular key innovations of neo-ESF to enable differentiation into a DSC. Here we
81 investigate which signal, in addition to progesterone (P4), was likely the ancestral trigger for decidual
82 differentiation and come to the conclusion that PGE₂ likely is the “natural” ligand for decidualization.
83 We have previously described the response of paleo-ESF to this ligand (Erkenbrack et al., 2018), and
84 here we describe the response of the human ESF to PGE₂ and progestin. We propose that one of the

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85 unique functions of neo-ESF is the specialized progesterone-dependent response to PGE₂ identified in
86 this study.

87 A large amount of molecular biology has been done on the mechanisms underlying
88 decidualization (Gellersen and Brosens, 2014). A broadly accepted model suggests that decidualization
89 results from the convergence of two signaling pathways, progesterone signaling via isoform A of the
90 nuclear progesterone receptor, PR-A, and the cAMP/PKA pathway (Brosens et al., 1999; Christian et
91 al., 2001). While many other factors have been shown to influence or are associated with
92 decidualization, the main evidence for the two signaling pathway model is that a membrane-permeable
93 form of cAMP (8-Bromo-cAMP) and progesterone are sufficient to decidualize human endometrial
94 stromal fibroblasts in vitro. It is less clear what the ligand is that activates the cAMP/PKA pathway in
95 vivo. Candidates include: gonadotropins such as follicle stimulating hormone (FSH), luteinizing
96 hormone (LH), or human chorionic gonadotropin (hCG) (Tang and Gurpide, 1993), relaxin (RLN)
97 (Telgmann et al., 1997), corticotropin releasing hormone (CRH) (Ferrari et al., 1995) and PGE₂ (Frank
98 et al., 1994) (see review in Gellersen and Brosens, 2014). However, the receptors of gonadotropins,
99 RLN, and CRH are either not or only marginally (*RXFP1*) expressed in cultured human ESF, both
100 immortalized as well as primary cells (Wagner, unpublished), and are thus unlikely to be critical for
101 decidualization. Also, other candidates have evolved more recently, for instance hCG only exists in
102 anthropoid apes (Maston and Ruvolo, 2002) and therefore cannot be the ancestral stimulus for
103 decidualization. In contrast, PGE₂ receptors are broadly expressed across mammalian ESF, from
104 opossum to human, with the exception of species that secondarily lost invasive placentation, e.g. the
105 cow (Kin et al., 2016). In addition, PGE₂ is present at the implantation site after embryo attachment in a
106 wide range of eutherian mammals (Table S1), and even in the opossum (Griffith et al., 2017;
107 Erkenbrack et al., 2018). This means that PGE₂ was a signal present in the uterus at the time of embryo

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108 attachment even before the evolution of the DSC, and the evolution of decidualization would have
 109 entailed evolution of a new response to PGE₂, rather than a new decidualizing signal. This follows from
 110 the finding that decidual cells evolved after the most recent common ancestor of opossum and placental
 111 mammals (Kin et al., 2014), and that embryo attachment in the opossum is also associated with PGE₂
 112 production. Hence, we investigated whether PGE₂, in addition with P4, is sufficient for in vitro
 113 decidualization of human ESF. PGE₂ has been implicated in decidualization before (Frank et al., 1994;
 114 Brar et al., 1997), and was then interpreted as a modulating factor enhancing decidualization.

115 The widely-used standard protocol uses extracellular cAMP to activate the PKA pathway could
 116 lead to artifacts because the cell is unable to internally regulate the level of cAMP. In fact, here we
 117 show that the cAMP-based protocol causes the expression of a large set of genes, including markers of
 118 a recently reported senescent decidual cell state (snDSC) (Lucas et al., 2020), which are much more
 119 highly expressed compared to our PGE₂-based protocol. To investigate this interpretation we perform
 120 single-cell transcriptome analysis on PGE₂/MPA-stimulated human ESF and find that in fact during in
 121 vitro decidualization ESF are producing two kinds of cells, an activated ESF population and mature
 122 DSC, where the former has some characteristics of senescent cells but does not seem to represent a
 123 distinct population of senescent cells.

124

125 **Materials and Methods**

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127 ***Cell Culture and in Vitro Decidualization***

128 Immortalized human endometrial stromal fibroblasts (equivalent to ATCC line CRL-4003) were
 129 cultured in growth media containing the following: 1x antibiotic-antimycotic (15240, Gibco; contains
 130 penicillin, streptomycin, and Amphotericin B), 15.56 g/L DMEM/F-12 without phenol red (D2906,

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131 Sigma Aldrich), 1.2 g/L sodium bicarbonate, 10 mL/L sodium pyruvate (11360, Thermo Fisher), and 1
132 mL/L ITS supplement (354350, VWR) in 10% charcoal-stripped fetal bovine serum (100-199,
133 Gemini). Media was replaced every 3-4 days during cell growth.

134 For in vitro decidualization, cells were grown in a base medium containing antibiotic-
135 antimycotic (15240, Gibco), 15.56 g/L DMEM/F-12 (D8900, Sigma Aldrich), and 1.2 g/L sodium
136 bicarbonate in 2% fetal bovine serum (100-106, Gemini). Additives included medroxyprogesterone
137 acetate at a concentration of 1 μ M, prostaglandin E₂ at a concentration of 1 μ M, or 0.5 mM 8-
138 bromoadenosine 3'-5'-cyclic monophosphate. Unless otherwise noted, media was changed daily during
139 the decidualization process. Decidualization was begun when cells had reached approximately 80%
140 confluency in 6-well (9 cm²) culture plates.

141 For inhibitor experiments, in vitro decidualization was conducted with the addition of either 1
142 μ M PTGER2 inhibitor (PF-04418948, Sigma), 1 μ M PTGER4 inhibitor (ER-819762, Sellek), or 5 μ M
143 of the PKA inhibitor H-89 (B1427, Sigma). Concentrations were determined based upon published
144 literature (PF-04418948: Birrel et al., 2012; ER-819762: Chen et al., 2010; H-89: Yoshino et al., 2003;
145 Matsuoka et al., 2010) and preliminary experiments.

146

147 ***Bulk RNA Sequencing***

148 RNA was isolated from cells lysed using Qiagen Buffer RLT and processed using the RNeasy
149 Mini Kit (74104, Qiagen) according to the manufacturer's specifications. Transcriptome libraries were
150 generated from isolated RNA by the Yale Center for Genomic Analysis using poly-A selection and
151 sequenced using an Illumina NovaSeq.

152 Sequencing reads were mapped to the Ensembl human transcriptome GRCh38 version 96 using
153 the pseudoalignment software kallisto (version 0.42.5; Bray et al., 2016). Transcript read counts were

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154 normalized using the transcripts per million (TPM) metric (Wagner et al., 2012) for comparison
155 between samples. The Pvcust software package (Suzuki et al., 2006) was used to ascertain the
156 uncertainty in hierarchical clustering of samples.

157 Principal component analysis was conducted on a subset of transcript per million
158 transcriptomes, only taking into consideration genes with an average expression of between 3 and 1000
159 TPM across all conditions. Genes were subset to only those with false discovery rate $< 10^{-6}$, fold change > 1.5
160 (treatment/control expression > 1.5 for upregulated or control/treatment > 1.5 for downregulated), and excluding
161 genes designated “off” (TPM < 3) in both conditions.

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163 Differential expression analysis was conducted using edgeR (version 3.28.0; Robinson et al.,
164 2010). The resulting differentially expressed genes were filtered to only those expressed at greater than
165 2 transcripts per million, an operational threshold for being considered “on” (Wagner et al., 2013).
166 Furthermore, only genes with a false discovery rate lower than 10^{-6} and an average fold change of
167 greater than 1.5 between treatment and control (treatment/control expression > 1.5 for upregulated, or
168 control/treatment > 1.5 for downregulated) were considered. For analyses that only took protein-coding
169 genes into consideration, filtering using the Ensembl BioMart “protein_coding” gene type annotation
170 reduced the total number of genes from 40275 to 22688, and transcripts per million were recalculated
171 accordingly. The number of genes that met all of the above criteria was 2668.

172 K-means clustering of genes by square root-transformed TPM values averaged for each
173 treatment condition was conducted to generate a number of clusters ranging from 4 to 10. Following
174 manual comparison, $k = 5$ clusters appeared to capture the most prominent patterns from our heatmap
175 including all genes, so more extensive analysis was conducted on these five clusters. Pathway analysis

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176 and gene ontology annotation were performed using Enrichr software (version 2.1; Kuleshov et al.,
177 2016).

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179 *Single-cell RNA Sequencing*

180 Single-cell RNA sequencing was conducted on endometrial stromal fibroblasts decidualized in
181 vitro for 2 or 6 days by daily administration of media containing 1 μ M PGE₂ and 1 μ M MPA. Single-
182 cell suspensions were prepared by a protocol modified from the 10X Genomics Sample Preparation
183 Demonstrated Protocol for Cultured Cells (CG00054 Rev. B). Briefly, cells were rinsed with TrypLE
184 express (12604, Thermo Fisher) and treated with TrypLE express for 5-10 minutes at 37°C. 10 mL
185 growth medium was added and the cells were pelleted by centrifugation for 5 minutes at 400 g, then
186 resuspended in 5 mL growth medium by pipetting up and down 10-20 times. Next, an 18-gauge needle
187 was used to transfer the cells onto a 40- μ m strainer, and the flowthrough was centrifuged and
188 resuspended in PBS containing 0.04% bovine serum albumen (A9647, Sigma). The resulting
189 suspension was passed through a FlowMi 30- μ m cell strainer and used for single-cell library
190 preparation using the 10X Chromium platform (3' chemistry, version 3) and sequenced using an
191 Illumina NovaSeq apparatus. In total, 10073 unique capture bead barcodes (each roughly
192 corresponding to a unique cell) were sequenced, composed of 4706 at 2 days and 5367 at 6 days with
193 an average of 34364 reads per cell.

194 Initial alignment of raw sequencing reads to the human genome (GRCh38 3.0.0 preparation by
195 10x Genomics), filtering, barcode and unique molecular indicator counting was conducted using 10X
196 Genomics cellranger software version 3.1.0. Resulting feature-barcode matrices were processed using
197 scprep software version 1.0.5 (github.com/KrishnaswamyLab/scprep). In attempt to remove data points
198 originating from beads that had captured ambient RNA (low library size), multiple cells (high library

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199 size), or dying cells having lost membrane integrity (disproportionately high mitochondrial genes), data
200 were further thresholded based on library size (between the 16th and 90th percentile of features/library
201 for 2-day treated samples, and 9th and 90th percentile of features/library for 6-day treated samples), and
202 below the 93rd percentile of mitochondrial gene content of the library after examination of distributions
203 (Figure S3). Genes expressed in fewer than 5 cells from either sample were excluded from the analysis.
204 The result was a total of 7279 cells and 16496 genes considered in downstream analyses.

205 Read counts were normalized to counts per million and square root transformed. Low-
206 dimensional embedding was performed using PHATE (version 1.0.5; Moon et al., 2019) For the
207 purpose of visualizing individual gene expression, denoising was performed using MAGIC (version
208 2.0; van Dijk et al., 2018). The statistical dependency between genes or between genes and
209 pseudotemporal scores was quantified using k-nearest neighbor conditional density resampled estimate
210 of mutual information (knn-DREMI) and rescaled for visualization using DREVI where noted
211 (Krishnaswamy et al., 2014; van Dijk et al., 2018).

212 Cells were partitioned using k-means clustering with increasing k parameters (Figure S4) and
213 the lowest number of clusters was chosen for which each prominent region of the embedding was
214 partitioned into a unique cluster, but before a precipitous drop in silhouette score. Marker genes for
215 each cluster were identified using scprep's differential expression function which performed Welch's *t*-
216 test between cells within and without each cluster.

217 Unbiased annotation of single cells with their most similar bulk RNA-seq treatment condition
218 (base media, PGE₂+MPA, or cAMP+MPA) was performed using SingleR (v1.0.6; Aran et al., 2019).
219 Trajectory inference was performed individually on 2- and 6-day treated samples using Slingshot
220 (Street et al., 2018). A starting cluster of most ESF-like cells, as determined by SingleR and
221 visualization of ESF marker genes, was used as a constraint on the Slingshot algorithm.

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223 ***Senescence-Associated β -Galactosidase***

224 Human endometrial stromal fibroblasts were cultured in base differentiation media with one of
225 the following sets of additives: none (control), 1 μ M palbociclib (PD0332991, Sigma) plus 1 μ M PGE₂
226 and 1 μ M MPA, 1 μ M PGE₂ plus 1 μ M MPA, 0.5 mM cAMP plus 1 μ M MPA, or 1 μ M MPA. Media
227 was replenished daily over the course of six days. At the end of the time course, senescence-associated
228 β -galactosidase activity was ascertained using a staining kit (9860S, Cell Signaling) following the
229 manufacturer's instructions. In this protocol, cells were rinsed in PBS and treated with fixative solution
230 for 10-15 minutes, rinsed with PBS twice, and incubated overnight at 37°C before imaging.

231

232 **Results**

233

234 ***Progestin + Prostaglandin E₂ Treatment Activates Classical Hallmarks of Decidualization***

235 Immortalized human endometrial stromal fibroblasts were treated with either progestin (MPA,
236 metoxyprogesterone acetate) and prostaglandin E₂, progestin and cyclic AMP, or base culture media
237 alone. PGE₂+MPA treatment induced a morphological change from an elongate fusiform morphology
238 to a more quasi-epithelial, laterally expanded shape (Figure 1a). They did not become globular to the
239 same extent observed in response to treatment with progestin and cyclic AMP (Figure 1a).
240 Nevertheless, the expression of key marker genes *prolactin (PRL)* and *insulin-like growth factor*
241 *binding protein 1 (IGFBP1)* were upregulated to a similar extent in response to both treatments as
242 determined by quantitative real-time PCR (qPCR) (Figure 1b).

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244 ***PGE₂+MPA and cAMP+MPA Treatments Modulate an Overlapping Set of Genes***

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245 Bulk transcriptome sequencing was conducted on cells exposed to both decidualization regimes,
 246 as well as to a base media control. Hierarchical clustering analysis found with high confidence that
 247 PGE₂+MPA-treated cells clustered with cAMP+MPA-treated cells, to the exclusion of the untreated
 248 control group (Figure S1). Principal component analysis (Figure 2a) was used to visualize the
 249 collective similarity of the three gene expression states. Transcriptomes were found to separate along
 250 the first two principal component axes primarily by treatment condition. The first axis, explaining
 251 47.3% of the variance, separated the two decidualization treatments from the control treatment most
 252 notably. The second principal component axis, explaining 26.6% of the variance, primarily separated
 253 PGE₂+MPA treatment from the cAMP+MPA samples. This pattern suggests that cells from both
 254 treatments differ in similar ways from ESF and also display expression differences specific to their
 255 treatment.

256 Differential gene expression analysis was conducted on all pairwise combinations of the three
 257 treatment regimes: PGE₂+MPA, cAMP+MPA and control. There was substantial overlap of 652 genes
 258 found to be upregulated under cAMP+MPA treatment (56% of 1158 genes) as well as under
 259 PGE₂+MPA treatment (79% of 824 genes) (Figure 2b). The overlap of downregulated genes (520 of
 260 1166, 45%, under cAMP+MPA, 520 of 599, 87%, under PGE₂+MPA) was similarly large. In both cases
 261 the set of genes differentially expressed in response to cAMP+MPA treatment was larger than the
 262 number of genes uniquely responsive to PGE₂+MPA (506 versus 172 genes uniquely upregulated, 646
 263 versus 79 genes uniquely downregulated). This pattern suggests that cAMP is inducing expression of
 264 genes that are not affected by PGE₂. The question, addressed below, is whether that means that PGE₂ is
 265 only inducing a subset of decidualization-related genes, or whether cAMP is causing artifactual gene
 266 expression.

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268 ***Both PGE₂ and cAMP Activate the Decidual Cell Core Regulatory Network***

269 Several prominent patterns emerged in the gene expression of cells under both decidualizing
 270 treatments. If expression scores are divided into “low/off” “medium” and “high” expression categories
 271 (Table S2), there are 27 possible patterns among the three treatments, 24 of which are differential
 272 (excluding identical categories in all 3 groups). Heatmap clustering of differentially expressed genes
 273 (Figure 2c) revealed, however, that some of these conceivable combinations were much more
 274 prominent in the data than others. To investigate this further, k-means clustering of genes was
 275 conducted using a range of hyperparameters from k = 3 to k = 10 clusters, and it was concluded that the
 276 data were best represented by five major patterns of differential gene expression between the three
 277 treatment conditions (Figure 2d). The full output from this analysis is provided as Supplemental Data.

278 Two of the resulting gene clusters represented genes lowly expressed in the control treated
 279 group, and three represented genes highly expressed in ESF but downregulated in response to either
 280 cAMP+MPA or PGE₂+MPA treatment. Of the former two, Cluster 4 included 566 genes that generally
 281 were moderately to highly upregulated in response to both decidualizing regimes. This cluster included
 282 almost all of the genes that have been identified as belonging to the decidual cell type’s core gene
 283 regulatory network (Erkenbrack *et al.*, 2018; Liang, 2018), including transcription factors c-Fos *FOS*,
 284 nuclear receptor related 1 protein *NR4A2*, forkhead box protein O1 *FOXO1*, odd-skipped-related 2
 285 *OSR2*, hypoxia-inducible factor 1-alpha *HIF1A*, zinc finger and BTB domain-containing protein 16
 286 *ZBTB16*, heart- and neural crest derivatives-expressed protein 2 *HAND2*, Krueppel-like factor 9 *KLF9*,
 287 zinc finger protein 331 *ZNF331*, and zinc fingers and homeoboxes protein 2 *ZHX2*. This cluster also
 288 included phenotypic effector genes encoding signaling peptides associated with decidual cells such as
 289 *PRL*, *IGFBP1*, left-right determination factor 2 *LEFTY2*, bone morphogenetic protein 2 *BMP2*, and

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290 somatostatin *SST* (Table S3). This consistent pattern suggested that a true differentiation event to the
291 decidual cell type had taken place in both treatment regimes.

292 The latter three gene clusters consisted of genes highly expressed in ESF and more lowly
293 expressed under either treatment. Of these, Cluster 2 included 733 genes that were similarly
294 downregulated by both decidualization regimes. Pathway analysis of this cluster returned terms
295 primarily associated with cytoskeletally-mediated fibroblast functions, such as “focal adhesion”
296 (KEGG, $p = 8.0 \times 10^{-12}$), “tight junction” (KEGG, $p = 2.2 \times 10^{-4}$), and “regulation of actin cytoskeleton”
297 (KEGG, $p = 2.0 \times 10^{-6}$). These represented genes mediating cellular interactions including
298 thrombospondin-2 *THBS2*, tubulins *TUBB6*, *TUBB4B*, *TUBA1C*, and *TUBA1B*, ezrin *EZR*, and the
299 claudin *TMEM47*. Furthermore, genes related to cell proliferation, including terms related to “cell
300 cycle” (KEGG, $p = 4.3 \times 10^{-5}$), including mitosis (GO:0000070; GO:0044772; 0000086; 0044839) and
301 cytokinesis (GO:0000281; GO:0061640) were included in this cluster, indicative of the cell cycle exit
302 expected to accompany a differentiation event (Takano et al., 2007).

303 There were two patterns of genes highly expressed in endometrial stromal fibroblasts and
304 downregulated in one or the other decidualization regime. One of these, Cluster 5, consisted of 216
305 genes for which the PGE₂+MPA treatment led to stronger downregulation than cAMP+MPA. This
306 included growth factors such as pleiotropin *PTN* and neuregulin 1 *NRG1*, and the GDNF receptor
307 *GFRA1* (Table S5). This pattern also applied to genes involved in extracellular matrix production
308 associated with decidualization such as osteopontin *SPP1* and hyaluronic acid synthase *HAS2* (Table
309 S5).

310 Lastly, Cluster 1 comprised 270 genes highly expressed in ESF that were more strongly
311 downregulated by cAMP+MPA treatment than PGE₂+MPA treatment. Some of the genes most
312 specifically affected by cAMP+MPA (i.e. non-significant difference between PGE₂+MPA and base

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313 treatments) were genes having to do with translation such as the aminoacyl-tRNA synthetases *WARS*,
 314 *YARS*, *MARS*, *GARS*, and *SARS*. This cluster of also included the matrix proteoglycans testican
 315 *SPOCK1* and versican *VCAN*, as well as the mesenchymal regulator SLUG *SNAI2*. This cluster also
 316 contained genes belonging to the KEGG pathway “PI3K-Akt signaling pathway” ($p = 9.3 \times 10^{-4}$),
 317 including the GTPase *HRAS*, PH domain and leucine-rich repeat protein phosphatase *PHLPP1*, and
 318 fibronectin *FN1*.

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320 Genes Specifically Induced by cAMP+MPA

321 Genes whose expression was specifically induced by cAMP+MPA (Figure 2b) fell into Cluster
 322 3 under k-means clustering (Figure 2d). Notably included in this cluster of 883 genes were almost all of
 323 the markers identified in a previous study (Lucas et al., 2020), and were proposed to represent a
 324 senescent subpopulation of decidual cells. These included ABI family member 3 binding protein
 325 *ABI3BP*, A disintegrin and metalloproteinase with thrombospondin motifs 5 *ADAMTS5*, cell migration-
 326 inducing and hyaluronan-binding protein *CEMIP* (*KIAA1199*), clusterin *CLU*, type II iodothyronine
 327 deiodinase *DIO2*, insulin-like growth factor 2 *IGF2*, lumican *LUM*, epididymal secretory protein E1
 328 *NPC2*, and the SRY-related HMG-box transcription factor *SOX4* (Table S4). Lucas and colleagues
 329 (2020) have proposed that decidualization leads to two fates, decidualization and senescence, with both
 330 playing important biological roles in implantation. The preferential elevation of this signature in
 331 cAMP+MPA decidualization prompted further investigation into whether such a senescent cell
 332 population was absent in PGE₂+MPA-decidualized cells.

333 As a first test for cellular senescence, chromogenic staining for senescence-associated β -
 334 galactosidase (Sa β G) activity was conducted on endometrial stromal fibroblasts decidualized for 3 or 6
 335 days by cAMP+MPA or PGE₂+MPA regimes (Figure S2). Highest β -galactosidase activity was

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336 observed in the cells treated for 6 days with decidual stimulus, regardless of whether it was PGE₂+MPA
337 or cAMP+MPA. On the other hand, cells treated with MPA alone for an equivalent duration showed
338 fewer SAβG⁺ cells. Cells decidualized with PGE₂+MPA in the presence of palbociclib, an inhibitor of
339 CDK4/CDK6 previously used to increase susceptibility of cells to senescence (Brighton et al., 2017),
340 showed no noticeable effect in our semi-quantitative test. We interpret this result as indicating that
341 under both treatment regimes the cells experience cellular stress, probably caused by the energy
342 demand associated with transcriptional reprogramming.

343

344 *Single-Cell Transcriptomic Analysis of Prostaglandin E₂-Induced Decidualization*

345 Since gene expression associated with senescent DSC was largely limited to cAMP+MPA
346 treatment in our bulk RNA-seq experiments but simple SAβG tests were inconclusive with respect to
347 the presence of senescent cells, we investigated the question of whether PGE₂+MPA treatment leads to
348 the formation of a senescent cell population. We approached this question by single-cell RNA
349 sequencing.

350 Cell cultures were subjected to single-cell transcriptomic analysis after 2 or 6 days of
351 PGE₂+MPA treatment. This design was chosen because according to Lucas et al. (2020), the senescent
352 cells arise after four days of decidualization treatment. Single-cell transcriptomes were embedded in
353 two dimensions, and kernel density estimation, a method to visualize data point density when normally
354 points would overlap in the ordination plot, was used as a first-pass attempt to judge the number of
355 potential cell types by looking for hotspots of cell density (Moon et al., 2018). The prediction was that
356 this number would increase by one after 6 days indicating the emergence of a senescent population.
357 However, this visualization instead revealed two centers of data density at 2 days, connected by a

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358 continuous line, and a single center in the 6-day treated samples (Figure 3a). This is more indicative of
359 a convergence than a bifurcation.

360 The second preliminary assessment was exploration of intrinsic dimensionality of the data, in
361 order to identify potential branch points: the prediction was that cells in transition between cell types
362 would have more complex transcriptomes (higher intrinsic dimensionality), whereas metastable,
363 differentiated states would have lower intrinsic dimensionality (Moon et al., 2019). This analysis
364 identified a single locus of cells ranked highest in intrinsic dimensionality between the two cell density
365 hotspots at 2 days, and slightly below the major hotspot identified at 6 days (Figure 3a). This indicated
366 that the major transcriptomic transition was occurring in this region.

367 The next step was the identification of the cell states observed with stages of ESF
368 differentiation. Visualization of cell transcriptomes revealed that the majority of cells lie on or around
369 the identified density hotspots, but that at both time points a sparse string of cells extended out to form
370 a curved tail continuous with the line between the two hotspots. The curve formed by this sparse tail
371 leading into the denser cell area was hypothesized to represent progression along the decidualization
372 pathway. This was tested by assignment of each cell to its transcriptionally most similar group from the
373 bulk RNA-seq experiment, which revealed that the less densely populated end of the curve was indeed
374 most ESF-like (Figure 3b). The more densely-populated end of the ordination plot was most similar to
375 PGE₂+MPA-decidualized bulk transcriptomes, with some cAMP+MPA interspersed but not
376 characterizing a distinct region.

377 Cells were partitioned into transcriptomically similar clusters using k-means clustering (Figure
378 3c). The optimal number of clusters was determined from a range of 2 to 7 (Figure S4). The first
379 partition recognized was between cells above and below the previously identified point of highest
380 intrinsic dimensionality, which presumably represented the transition point between these two distinct

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381 cell states or even cell types. Next, at both time points, a second division occurred at $k > 3$ clusters
 382 between the sparse ESF-like tail and the rest of the accumulation of cells below the transition point.
 383 Further divisions sharply reduced clustering silhouette scores, so a total of 3 clusters seems to be the
 384 most meaningful classification of cells for both, the 2- and 6-day time points.

385 Gene expression analysis was used to identify marker genes for each cluster (Supplemental
 386 Data) and explore expression of known markers of various DSC and ESF cell states (Figure 4). At the
 387 sparsely-populated ESF-like end of the curve, ESF markers such as *VIM*, *EZR* and *TUBB6* (Figure 4a)
 388 were most highly expressed at both 2 and 6 days. Furthermore, these cells also had a distinct cell cycle
 389 signature (e.g. GO:1901990, “regulation of mitotic cell cycle phase transition”, 2d $p = 5.84 \times 10^{-15}$, 6d p
 390 $= 3.79 \times 10^{-13}$). Indeed, cell cycle scoring identified this tail as cells in S and G2/M phase, whereas the
 391 bulk of cells were G1 (Figure S5a). This pattern included the cytokinesis gene anillin *ANLN* (Figure
 392 4a), which was previously identified as a marker of proliferative ESF (Lucas et al., 2018), indicating a
 393 distinction between proliferation (highly proliferative ESF; hpESF) and differentiation. Nevertheless,
 394 regression out of cell cycle-associated genes (Tirosh et al., 2016), a step sometimes included in scRNA-
 395 seq analyses although not always advisable due to its potential to mask biological signal (Luecken and
 396 Theis, 2019), failed to remove this tail of ESF-like cells (Figure S5b). This confirmed that the cluster
 397 was not a cell-cycle artifact but had a broader and persistent ESF-like gene expression signature.

398 On the other end of the putative decidualization curve, the cluster of cells above the transition
 399 point at 6 days showed expression of decidual genes. This cluster was characterized by the gene set
 400 “BMP2-WNT4-FOXO1 Pathway in Human Primary Endometrial Stromal Cell Differentiation”
 401 (WikiPathways WP3876, 6d $p = 8.03 \times 10^{-6}$), indicating that these were indeed decidual stromal cells.
 402 Decidual cell signaling peptides such as *PRL*, *IGFBP1*, and *LEFTY2* were expressed in this region, and
 403 decidual transcription factors appearing earlier such as *FOXO1* and *HAND2* (Supplemental Data) or

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404 later such as *KLF9* (Figure 4b). Decidual markers were lowly expressed but detectable at 2 days, and at
405 a much greater magnitude at 6 days. At 2 days, this cluster was predominately consisted of ribosomal
406 genes (e.g. GO:0006412 “translation”, $p = 5.85 \times 10^{-73}$), which was interpreted as a signal of cells still
407 undergoing the reprogramming associated with differentiation. This end of the decidualization curve
408 was also characterized by expression of the prostaglandin E₂ receptor 2 *PTGER2*, which was
409 unexpressed in the more ESF-like cells.

410 The dense cluster of cells located below the point of highest dimensionality, more densely
411 populated at 2 days, were also ESF-like, expressing some ESF markers including tropomyosins *TPM1*
412 and *TPM2*, as well as alpha smooth muscle actin *ACTA2* and transgelin *TAGLN* (Figure 4c) but not the
413 decidual markers *PRL* or *IGFBP1* (Figure 4b). At both 2 and 6 days, this cluster was enriched in gene
414 sets such as “regulation of cell migration” (GO:0030334, 2d $p = 1.22 \times 10^{-5}$, 6d $p = 2.19 \times 10^{-7}$),
415 “homotypic cell-cell adhesion” (GO:0034109, 2d and 6d $p = 3.87 \times 10^{-6}$), and “muscle contraction”
416 (GO:0006936, 2d $p = 9.64 \times 10^{-11}$, 6d $p = 1.33 \times 10^{-7}$). This was interpreted as a pre-decidual ESF state, as
417 it lies between the most ESF-like cells and the point of highest intrinsic dimensionality believed to
418 resemble the transition to later-stage decidualization. To differentiate these early-stage decidualizing
419 ESF from proliferative ESF, we term them activated ESF (acESF).

420 Finally, marker genes of the senescent/progesterone-resistant decidual cell state identified by
421 Lucas et al. (2020) were examined to see whether their expression characterized a subset of cells
422 arising from our experiments with PGE₂. These genes, including *SOX4*, *ABI3BP*, *ADAMTS5*, *CLU* and
423 *DIO2* (see Table S4) were expressed in some cells, and found on day 6 to have a stronger correlated
424 expression with each other than on day 2. Particularly at 2 days, expression of most of these genes
425 spread across the putative branch point rather than being exclusive to one region (Figure 4d), but at 6
426 days expression of several of these genes was concentrated towards one corner of the acESF cells.

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427 Strongest expression of the fibroblast markers *TPM2*, *ACTA2*, and *TAGLN* was, by contrast, more
 428 biased towards the opposite side of this cluster, and *THBS1* was more broadly expressed. Sub-
 429 partitioning of the acESF cluster in two divided the cells generally between these two patterns (Figure
 430 3d), and revealed the *SOX4^{high}* cells expressed gene sets enriched for “p53 signaling pathway” (KEGG p
 431 = 1.80×10^{-5}) and “PI3K-Akt signaling pathway” (KEGG p = 6.98×10^{-3}). On the other hand, *ACTA2^{high}*
 432 *TAGLN^{high}* cells were the source of the highest signal for “muscle contraction” (GO:0006936, p =
 433 5.01×10^{-8}), “homotypic cell-cell adhesion” (GO:0034109, p = 1.48×10^{-12}) and “wound healing,
 434 spreading of cells” (GO:0044319, p = 1.46×10^{-3}). Due to the large decline in clustering optimality
 435 (silhouette score) when calculating k = 4 rather than 3 clusters at 6 days, the distinction between the
 436 *SOX4^{high}* and *ACTA2^{high}* *TAGLN^{high}* activated ESF was interpreted as gradation within a cluster rather
 437 than discrete clusters.

438 Finally, having identified three major gene expression patterns by marker gene exploration and
 439 comparison to bulk RNA-seq, developmental trajectories were inferred between the three shapes and
 440 determine whether a new subpopulation was arising the later time point. Trajectory inference was
 441 conducted at both time points, with the constraint that all trajectories must begin at the most ESF-like
 442 cell cluster. This resulted in only one principle curve (putative trajectory) in the 2-day PGE₂+MPA
 443 decidualization sample, and one in the 6-day PGE₂+MPA decidualization sample (Figure 3c), i.e. no
 444 bifurcation event was predicted using this method. The identified trajectory extended to the most DSC
 445 cell cluster and represented the axis of decidualization. Partitioning of the 6-day sample into 4 clusters
 446 rather than 3 (one representing snESF) did not affect this outcome.

447 Our single-cell analysis does not support the model that PGE₂+MPA treatment leads to the
 448 emergence of a separate decidual cell population after more than 4 days of treatment. If such a
 449 population exists, it should be seen in our six day treatment. However, we did pick up a weak signal of

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450 ESF, not DSC, expressing the snDSC-associated genes identified by Lucas and colleagues (2020), and
451 enriched for genes in the PI3K/AKT pathway. This raises the possibility that the prominent presence of
452 senescent decidual cells after cAMP+MPA treatment may be an artifact of the use of extracellular
453 cAMP rather than a natural ligand to induce decidualization.

454

455 *PGE₂-Mediated Decidualization Depends Upon PGE₂ Receptor 2 and Protein Kinase A*

456 A series of experiments was conducted to determine the signal transduction pathway by which
457 prostaglandin E₂ signaling led to decidualization. Of the two PGE₂ receptors known to be expressed in
458 vitro in ESF and DSC, *PTGER2* (a.k.a. EP2) was found to increase over pseudotemporal progression
459 along the axis of decidualization whereas *PTGER4* (a.k.a. EP4) decreased (Figure 5a). Likewise,
460 *PTGER2* expression was positively correlated with *PRL* expression as a proxy for decidualization,
461 whereas *PTGER4* was low in almost all *PRL*⁺ cells (Figure 5a). These findings suggested that *PTGER2*
462 activation, and possibly *PTGER4* deactivation, has roles in PGE₂-mediated decidualization.

463 To investigate this further, in vitro PGE₂+MPA decidualization was conducted in the presence of
464 small molecule prostaglandin E₂ receptor antagonists, the PTGER2 antagonist PF-04418948 and the
465 PTGER4 antagonist ER-819762. H-89, an inhibitor of protein kinase A, downstream of PTGER2
466 signaling, was also administered. Treatment with PTGER2 inhibitor significantly reduced decidual
467 marker genes after 6 days: *PRL* was reduced approximately 50%, and *IGFBP1* approximately 78%
468 relative to uninhibited PGE₂+MPA controls (Figure 5b). This effect was marginally improved by
469 treatment with both PTGER2 and PTGER4 inhibitors (57% and 80% reduction, respectively). In
470 contrast, treatment with PTGER4 inhibitor alone resulted in a non-significant 9% reduction in *PRL*
471 expression, and a 31% reduction in *IGFBP1* expression. Administration of the PKA inhibitor H-89

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472 significantly reduced *PRL* expression by 40%, and *IGFBP1* expression by 44%. From these results we
473 conclude that the decidualizing effect of PGE₂ is mediated through the PTGER2-PKA axis.

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475 ***PGE₂ Receptor 2 Is Under Progestin Regulatory Control***

476 Despite the demonstrated importance of PTGER2 to PGE₂-mediated decidualization, bulk
477 transcriptomic quantification revealed *PTGER2* to be below or marginally equal to functional levels of
478 expression in endometrial stromal fibroblasts (Figure 5c). However, the gene was clearly turned on
479 after progestin treatment. Expression of *PTGER2* was approximately quadrupled after treatment with
480 MPA plus either PGE₂ or cAMP, but not after treatment with either PGE₂ or cAMP alone (Figure 5c).
481 This was confirmed in bulk RNA sequencing, which showed an approximate quadrupling of *PTGER2*
482 from ~3 TPM after control treatment to ~12 TPM after treatment with MPA and either PGE₂ or cAMP.
483 That the effect was indeed due to MPA was confirmed by treatment of ESF for 6 days with MPA alone,
484 after which the expression of *PTGER2* increased 7.14-fold, passing from “off” to “on” (Figure S6).

485 This effect of progestin on *PTGER2* appears to not be shared by the paleo-ESF cell type.
486 Analysis of previously published transcriptomes from opossum paleo-ESF treated with MPA alone,
487 MPA+PGE₂, or MPA+cAMP (Erkenbrack et al., 2018) revealed that *PTGER2* expression was
488 unaffected by the presence of progestin, and in all cases remained below the operational threshold for
489 being considered “on” (Figure 5d).

490

491 **Discussion**

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493 In this study we have investigated the biology of PGE₂+MPA-induced in vitro decidualization
494 and compared the results with the effects of cAMP+MPA treatment. We found that in vitro PGE₂+MPA

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495 treatment alone is sufficient to cause the hallmarks of decidualization. Mechanistically, the results
496 suggest that PGE₂ mediated decidualization depends on the induction of PTGER2 by progesterone, and
497 proceeds via the EP2-cAMP-PKA axis, while the dominant PGE₂ receptor in ESF, EP4, is
498 downregulated.

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500 ***Prostaglandin E₂ as a Natural Ligand for Decidualization***

501 Whereas previous studies of PGE₂-mediated decidualization only performed marker gene and
502 morphological assessment (Frank et al., 1994; Brar et al., 1997), the whole-transcriptome approach
503 paints a more complete picture. The transcriptomic response of endometrial stromal fibroblasts to
504 PGE₂+MPA comprised of the widespread activation of genes in the core regulatory network of decidual
505 stromal cells (Table S3). This suggests that the response to PGE₂ was a true transformation into the
506 DSC cell type. This conclusion is also supported by the results of our single-cell transcriptomic
507 analysis, which identified a hotspot of cells with high-complexity transcriptomes separating apparently
508 mature DSC from more fibroblast like cells (Figure 3a).

509 The sufficiency of this molecule, plus the cross-species conservation of prostaglandin signaling
510 in reproduction (Table S1), suggest that both, progesterone and PGE₂, can be considered “natural
511 signals” for decidualization. We consider a natural ligand for the differentiation of a cell type to be a
512 shared derived signal that was present and effective at the evolutionary origin of that cell type, and
513 which continues to have an active physiological role in derived species. In decidualization, cAMP
514 mediates a key signal determining decidual cell differentiation but is not the natural ligand itself,
515 whereas PGE₂ and progesterone fit the definition of a natural ligand. It has yet to be seen whether other
516 signals, such as those from the local tissue microenvironment including the uterine glands or lumen,

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517 also fit this definition. Given its simplicity and the conservation of PGE₂ expression in pregnancy
518 among placental mammals, it should be easily applicable to other species.

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520 ***PGE₂ Signaling through PTGER2 versus PTGER4***

521 Secreted prostaglandin E₂ is bound by G-protein coupled receptors in the membrane of the
522 receiving cell. Among the eight members of the prostanoid receptor subfamily, four paralogous
523 receptors are specific for PGE₂: PTGER1, PTGER2, PTGER3, and PTGER4 (Breyer et al., 2001). In a
524 resting endometrial stromal fibroblast in culture, only one of these, *PTGER4*, is expressed above the
525 operational threshold for a gene to be considered actively transcribed (Rytkönen et al., 2018). We find
526 that another prostaglandin E₂ receptor, *PTGER2*, becomes expressed in human ESF after exposure to
527 progesterin.

528 PTGER2 appears to be functionally adapted to receive sustained PGE₂ stimulus, whereas the
529 PTGER4 response is transient and self-limiting (Desai et al., 2000). PTGER4 undergoes more rapid
530 desensitization to ligand binding (Nishigaki et al., 1996), on the order of minutes, due to the action of
531 an additional C-terminal protein domain absent in PTGER2 (Bastepe and Ashby, 1997). Therefore, it is
532 likely that the sequence of events is as follows (Figure 6): At first, PTGER4 is expressed and PTGER2
533 is absent. Progesterone produced by the corpus luteum after ovulation induces PTGER2. Now, in
534 response to PGE₂ stimulus at the time of implantation, PTGER4 becomes quickly desensitized, and
535 signaling through PTGER2 becomes dominant. Indeed, in our bulk RNA-seq data *PTGER4* expression
536 was reduced in response to PGE₂+MPA or cAMP+MPA treatment from an average of 5 TPM to 2 or 1
537 TPM, respectively. Single-cell data also supported this sequence of events, where *PTGER2* was
538 expressed in the mature decidual end of the curve and by contrast *PTGER4* was unexpressed in *PRL*⁺
539 cells.

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540 Progestin-dependent expression of *PTGER2* has previously been reported in human cells (Brar
541 et al., 1997). Dependence of decidualization upon progesterone has been found to be the result of
542 cAMP-activating PGE₂ receptor activation (i.e. *PTGER2*) in the rabbit (Fortier et al., 1987; Stadtmauer
543 and Wagner, unpublished) and dog (Graubner et al., 2020). Both *PTGER2* and *PTGER4* are G-protein
544 coupled receptors that signal through the G_{os} subunit which activates adenylyl cyclase to produce
545 cAMP. However, *PTGER4* activates two signal transduction pathways, protein kinase A (PKA), which
546 promotes decidualization, and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), which opposes
547 it (Rundhaug et al., 2011; Gellersen and Brosens, 2014). Of the two kinases, *PTGER4* favors AKT
548 (Fujino et al., 2005), whereas *PTGER2* is specific for PKA (Fujino et al., 2002). Decidualization is
549 therefore unlikely to be inducible by *PTGER4* signaling alone, given the demonstrated necessity of
550 PKA signaling. Instead, it is likely that upon induction of *PTGER2* and desensitization of *PTGER4*,
551 preferential signaling through *PTGER2* favors a PKA-dominated intracellular state, and thus promotes
552 decidualization.

553 *PTGER2* has been characterized as the least expressed of the four paralogous PGE₂ receptors
554 (Ricciotti and Fitzgerald, 2011), suggesting that its physiological role is more specialized. In
555 pulmonary fibroblasts, prostaglandin E₂ signaling through *PTGER2* inhibits PI3K/AKT signaling, the
556 myofibroblast phenotype and fibrosis (Mukherjee et al., 2019). Through PGE₂-*PTGER2* signaling, the
557 same antagonistic mechanism may channel decidualizing endometrial stromal cells away from a more
558 myofibroblast-like ESF state. In our single-cell expression data, *PTGER2* was associated with the
559 mature decidual end of the decidualization curve, and had little to no intersection with markers of the
560 acESF state (Figure 4). Phosphorylated (activated) AKT has been shown to decrease in cells
561 decidualizing under the influence of progesterone, but phospho-AKT is elevated in the absence of
562 progesterone or in the presence of progesterone plus a PKA inhibitor (Yoshino et al., 2003). This is

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563 consistent with a progesterone-dependent mechanism for shifting the balance from AKT-dominated to
564 PKA-dominated state such as the one proposed here.

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566 ***Cellular Heterogeneity and Prostaglandin E₂-Driven Decidualization***

567 Decidual cell heterogeneity is the hypothesis that decidualization gives rise to multiple cell
568 types, or multiple states of the same cell type. Different cell populations may coexist at the same time
569 and fulfill distinct biological functions, in contrast to (but compatible with) the notion of temporal
570 phases, including an early pro-inflammatory and later mature decidual state (Salker et al., 2012;
571 Gellersen and Brosens, 2014; Rytönen et al., 2019).

572 Single-cell RNA sequencing studies of the peri-implantation fetal-maternal interface (Vento-
573 Tormo et al., 2018; Suryawanshi et al., 2018) support the model of decidual cell heterogeneity, with
574 each partitioning DSC-like cells into three distinct transcriptional states. In the analysis by Vento-
575 Tormo and colleagues (2018), these included a cluster lacking *PRL* and *IGFBP1* expression but
576 expressing *ACTA2* and *TAGLN*, markers of the acESF cells identified in our study particularly prevalent
577 at 2 days, as well as two mature decidual cell population characterized by expression of standard
578 markers *PRL* and *IGFBP1*, although to a higher degree in one cluster with the other expressing markers
579 such as *LEFTY2* and *IL15*. Notably, *LEFTY2* was one of the genes we found more highly elevated in
580 PGE₂+MPA treatment than cAMP+MPA, and the majority of the DSC cluster identified at 6 days was
581 *LEFTY2*⁺ and *IL15*⁺, with an offshoot more highly expressing *PRL* and *SST*. The authors implied a
582 possible identification of the *LEFTY2*⁺ cluster as snDSC although their justification was unclear
583 (Vento-Tormo et al., 2018). Our *LEFTY2*⁺ cells from in vitro PGE₂+MPA treatment lack expression of
584 the putative senescence markers, so it is unlikely that the reason we find a single cluster of DSC is
585 because *all* DSC are snDSC. The difference between these two DSC populations was not deemed great

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586 enough to warrant unique cluster assignment (appearing at $k \geq 5$ clusters, Figure S4), but nevertheless
 587 this intriguing if weak pattern will need to be pursued by extended sampling. In the analysis by
 588 Suryawanshi and colleagues, the three subtypes of DSC were analyzed explicitly as a developmental
 589 trajectory, with one being an intermediate more ESF-like starting point, an *ACTA2*⁺ and *TAGLN*⁺ state,
 590 and a canonical decidual state expressing *PRL* and *IGFBP1*. Overall, comparison between the present
 591 study and the two in vivo analyses suggests that the most robust pattern is the identification of
 592 canonical *PRL*⁺ *IGFBP1*⁺ DSC and an *ACTA2*⁺ *TAGLN*⁺ cell we deem activated ESF.

593 The leading form of decidual cell heterogeneity discovered in vitro is what has been labeled as
 594 the senescent decidual cell state, proposed to have a physiological role in decidual development
 595 (Brighton et al., 2017) as well as a pathological role in recurrent pregnancy loss (Lucas et al., 2020).
 596 According to this theory, development of decidual stromal cells along a senescence pathway promotes
 597 tissue turnover and makes room for the implantation of the blastocyst, and is aided by the active
 598 phagocytic and cytolytic activities of uterine natural killer cells. Single-cell transcriptomic studies of in
 599 vitro decidualized human endometrial stromal fibroblasts revealed a trajectory splitting event at 4 days
 600 of decidualization after which “mature” and “senescent” decidual cells diverge (Lucas et al., 2020), but
 601 used the cAMP+MPA decidualization regime.

602 Our PGE₂-mediated decidualization experiment focussed on 2- and 6-day time points to bracket
 603 this splitting event at day 4 reported by Lucas and collaborators (2020). Nevertheless, we did not
 604 identify a unique cluster cells co-expressing decidual and senescent markers after six days of
 605 PGE₂+MPA treatment, although some of the cells we deem activated ESF did express a suite of genes
 606 associated with so-called snDSC in the absence of decidual markers. The lack of discretely identifiable
 607 snDSC after PGE₂+MPA treatment could be interpreted as indicating that their induction under
 608 cAMP+MPA is an artifact of the stimulation with extracellular cAMP. Alternatively, the lack of

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609 senescent cells could be an artifact of the PGE₂+MPA treatment. However, since our experiments show
610 that decidualization by PGE₂+MPA treatment is acting through the PTGER2 → cAMP → PKA
611 pathway, we are inclined to believe that treatment with extracellular cAMP may overload the cell with
612 cAMP, leading to cell senescence.

613 The senescence DSC model of Lucas et al. makes several assertions with which we agree. First,
614 a group of genes including *ABI3BP*, *ADAMTS5*, *DIO2*, *CLU*, and *CEMIP* show correlated expression
615 with *SOX4* or *STAT1*. Our data support this pattern, which is stronger at 6 days relative to 2 days.
616 Second, with respect to the finding that cells with this gene expression pattern increase after 4 days, our
617 PGE₂+MPA data also show expression of several of these genes at 2 and 6 days, although to a lesser
618 degree than seen after cAMP+MPA treatment and not co-expressed with decidual markers. As a
619 consequence, the finding that these cells are the product of a branching event or new endpoint that
620 emerges after 4 days was not supported in our study.

621 The senescent and mature decidual cells can also be framed as a dichotomy between
622 progesterone-resistant and progesterone-susceptible cells (Lucas et al., 2020), due to snDSC
623 transcription factors being negatively regulated by progesterone (e.g. *SOX4*: Cloke et al., 2008). Since
624 the upstream components of the decidualization pathway (such as PTGER2) are demonstrated to be
625 regulated by progesterone signaling, the production of cells entering this state under the cAMP+MPA
626 method could be increased as a consequence of exogenous cAMP bypassing this layer of regulation.

627 The findings from our study are compatible with the following model (Figure 7): Endometrial
628 stromal fibroblasts decidualizing in response to PGE₂ go through a pro-inflammatory phase in which
629 they may assume an activated state similar to the myofibroblast-like transcriptomic profile identified in
630 vivo. At 2 days, most of the cells are either in this state or in transition to DSC, whereas by 6 days
631 many have adopted a more mature, canonical DSC state although some remain in the activated ESF

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632 state. Activated ESF are enriched for genes in the protein kinase B pathway and cell adhesion, whereas
 633 cells at or approaching the DSC state express *PTGER2*, the receptor through which PGE₂ activates
 634 cAMP and protein kinase A signaling, even by day 2. At 6 days, activated ESF exist along a continuum
 635 where some have taken on a state where they express the putative senescence markers, PI3K/AKT-
 636 associated genes, and genes related to control of cell survival and adhesion (snESF). If 8-Br-cAMP is
 637 used as the decidualogenic stimulus, and if the snESF phenotype is indeed indicative of progesterone
 638 resistance, the production of snDSC under 8-Br-cAMP treatment but not PGE₂+MPA can be explained
 639 by the bypassing of the surface receptor level of signal transduction upstream of cAMP/PKA. As PGE₂
 640 receptor expression, such as the EP2 switch we identify, seem to be influenced by progesterone, direct
 641 cAMP addition allows for the expression of the normally progesterone-dependent decidual program
 642 with the senescence-like program, resulting in snDSC.

643 Our results are consistent with the notion of temporal phases to decidualization, where decidual
 644 cells must progress from a proliferative fibroblast-like state to a pro-inflammatory and ultimately an
 645 anti-inflammatory state. Such a transition may require a distinct cellular program of resolution (Serhan
 646 and Savill, 2005). Notably, resolution of inflammation involves cell turnover through auto-opsoniaztion
 647 with cell surface molecules such as thrombospondin 1 (Serhan and Savill, 2005) which promotes
 648 immune cell recognition and phagocytosis of inflammatory cells. Indeed, *THBS1* was a top marker of
 649 the activated ESF cell state, along with the thrombospondin motif-containing *ADAMTS5* enriched in
 650 snESF. Since this phenomenon can occur in many cell types, its evolution predates the decidual stromal
 651 cell and thus it may be expected that development towards an anti-inflammatory state would activate
 652 programs for, if not direct cell death, then immune-assisted cell death, a cornerstone of the senescence
 653 model (Brighton et al., 2017). Virtually none of the recognized products of the acute senescence-
 654 associated secretory phenotype (Coppé et al., 2010) are expressed in our data.

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655 In vitro decidualization is inevitably removed from in vivo biology. As it has become evident
 656 that the decidual stromal cell type takes several forms depending upon developmental time and
 657 situation (e.g. blastocyst rejection or acceptance), when choosing an in vitro model system it is
 658 important to note which of these states it produces. Our PGE₂+MPA in vitro decidualization appears to
 659 generate mature decidual stromal cells as well as a subset of activated ESF similar to the
 660 myofibroblast-like activated ESF identified in in vivo studies (Suryawanshi et al., 2018; Vento-Tormo
 661 et al., 2018). On the other hand, PGE₂+MPA decidualization appears to less readily generate senescent
 662 DSC. Should senescent DSC be a biologically relevant entity, the identification and use of natural
 663 ligands for this specific cell state could be an improvement to their generation in response to exogenous
 664 cAMP. Method choice would then depend upon the endometrial state one intends to simulate in vitro.
 665 Importantly, our failure to reliably generate snDSC using PGE₂+MPA does not contradict the
 666 possibility that stromal cell senescence takes place in vivo and can contribute to recurring early
 667 pregnancy loss (Lucas et al., 2020).

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669 ***The Evolution of Decidual Cell Differentiation***

670 According to the sister cell type model (Arendt, 2008), novel cell types come into existence
 671 through an evolutionary bifurcation event which results in the production of two distinct cell types to
 672 take the place of the ancestral cell type – hence, sister cell types. Within this framework, the neo-ESF
 673 cell of placental mammals is the sister cell type to the decidual stromal cell (Kin *et al.*, 2015). In this
 674 case one sister cell type, the neo-ESF, also gives rise to the other, the DSC, during ontogeny. This
 675 developmental linkage is itself a derived state: exposure of opossum paleo-ESF to deciduogenic
 676 conditions – either cAMP+MPA or PGE₂+MPA – results in cellular stress rather than decidualization
 677 (Erkenbrack *et al.*, 2018). Most notably, the same study also provides data that shows that MPA does

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678 not induce the expression of PTGER2 in the opossum ESF, suggesting that the progesterone
679 responsiveness of the *PTGER2* gene is a key innovation in the evolution of the neo-ESF/DSC sister cell
680 type pair. These findings suggest that it was only upon the evolution of progesterone-dependent
681 regulation of PTGER2 that the two natural ligands for decidualization, PGE₂ and progesterone, become
682 functionally linked. This linkage may be a way in which the progesterone channels the inflammation
683 induced PGE₂ expression into a signal for decidual cell type differentiation, rather than stress and
684 senescence.

685

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687

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698 Tables

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Species	PGE ₂ at attachment	PGE ₂ at parturition	Deciduate?	References
Quail	N/A	Yes	No	Hertelendy, 1974
Opossum	Yes	Yes	No	Griffith et al., 2017
Armadillo	Yes	?	Yes	Chavan and Wagner, 2016 Chavan et al., 2018
Dog	Yes	Yes	Yes	Gram et al., 2014 Kautz et al., 2014
Pig	Yes	?	2° Modified	Wilson et al., 2002 Waclawik et al., 2017
Horse	Yes	Yes	2° Modified	Watson and Sertich, 1989 Bauersachs and Wolf, 2012
Guinea Pig	No	Yes	Yes	Poyser, 1984 Rajabi and Cybulsky, 1995
Mouse	Yes	Yes	Yes	Holmes and Gordashko, 1980 Kimura et al., 1999
Human	Yes	Yes	Yes, Spontaneous	Holmes et al., 1990 Sangha et al., 1994

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703 **Table S1.** Distribution of prostaglandin E₂ in pregnancy (attachment and parturition) in selected
704 amniotes, with phylogenetic relationships sketched on the left. Cursory ancestral state inference
705 suggests that PGE₂ involvement in attachment predated the evolution of the decidua, consistent with
706 PGE₂ being a natural ligand for decidualization.

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Cluster	Base	cAMP+MPA	PGE ₂ +MPA
1	+0.85 (H)	-1.06 (L)	+0.21 (M)
2	+1.12 (H)	-0.75 (L)	-0.37 (L)
3	-0.87 (L)	+1.05 (H)	-0.17 (M)
4	-1.09 (L)	+0.49 (H)	0.60 (H)
5	+1.01 (L)	-0.10 (M)	-0.90 (L)

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711 **Table S2.** Centers of standardized distributions of expression value scores (Z-score derived from
712 square root TPM) for each gene cluster as determined by k-means clustering. For the purposes of
713 interpretation, these values values can be binned into “low/off” ($z < -0.33$), “medium” ($-0.33 < z <$
714 $+0.33$), and “high” ($z > +0.33$) levels of expression.

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Gene Name	TPM (Base)	TPM (cAMP)	TPM (PGE ₂)	FDR (Base vs. PGE ₂)	FDR (Base vs. cAMP)	FDR (PGE ₂ vs. cAMP)
<i>FOS</i>	4.1	6.5	7.1	1.2E-10	8.4E-04	5.3E-03
<i>FOXO1</i>	1.9	77.2	43.4	0.0E+00	0.0E+00	6.0E-26
<i>HAND2</i>	33.4	157.3	114.4	4.2E-181	1.2E-252	1.2E-06
<i>HIF1A</i>	174.6	365.1	364.0	2.8E-61	2.5E-48	1.1E-01
<i>KLF9</i>	5.6	19.0	23.7	1.2E-160	3.1E-94	1.1E-10
<i>NR4A2</i>	0.7	13.0	9.5	1.5E-59	1.4E-65	5.1E-01
<i>OSR2</i>	18.8	44.7	38.9	1.9E-27	3.6E-31	6.5E-01
<i>ZBTB16</i>	0.1	4.4	4.8	1.4E-36	9.5E-33	5.9E-01
<i>ZHX2</i>	4.4	10.5	8.9	4.6E-19	2.2E-18	8.8E-01
<i>ZNF331</i>	7.0	14.5	12.0	2.2E-22	1.9E-23	9.4E-01
<i>BMP2</i>	0.5	6.5	4.5	8.2E-34	2.2E-43	1.4E-01
<i>IGFBP1</i>	0.0	4.8	7.5	3.3E-33	2.9E-25	5.2E-02
<i>LEFTY2</i>	0.0	14.5	40.2	4.7E-85	1.4E-56	1.4E-08
<i>PRL</i>	0.2	13.3	9.7	1.5E-26	2.9E-31	4.7E-01
<i>SST</i>	0.1	8.3	221.1	3.9E-195	2.4E-20	1.6E-125

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718 **Table S3.** Expression levels of select genes functionally related to decidual cell identity or phenotype,
719 all falling into Cluster 4 in k-means clustering analysis.

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Gene Name	TPM (Base)	TPM (cAMP)	TPM (PGE ₂)	FDR (Base vs. PGE ₂)	FDR (Base vs. cAMP)	FDR (PGE ₂ vs. cAMP)
<i>ABI3BP</i>	37.6	469.7	211.3	6.4E-140	2.1E-248	1.1E-22
<i>ADAMTS5</i>	140.3	216.8	122.2	5.9E-02	3.8E-34	5.6E-46
<i>CEMIP</i>	6.9	40.7	21.0	4.2E-08	2.5E-18	2.3E-03
<i>CLU</i>	154.8	436.1	136.8	6.1E-01	8.4E-28	2.0E-30
<i>DIO2</i>	11.6	25.8	20.0	8.2E-04	9.4E-08	1.3E-01
<i>IGF2</i>	14.0	306.9	89.0	2.5E-71	1.4E-159	4.4E-28
<i>LUM</i>	81.0	377.6	250.4	8.7E-94	5.9E-144	1.3E-06
<i>NPC2</i>	692.7	1962.9	1369.2	3.6E-35	4.9E-63	5.2E-05
<i>SOX4</i>	111.8	176.3	99.0	1.8E-01	7.9E-22	1.6E-28

Table S4. Expression levels of select genes proposed to be markers of the senescent decidual cell state (Lucas et al., 2020), all falling into Cluster 3 in k-means clustering analysis.

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Gene Name	TPM (Base)	TPM (cAMP)	TPM (PGE ₂)	FDR (Base vs. PGE ₂)	FDR (Base vs. cAMP)	FDR (PGE ₂ vs. cAMP)
<i>NRG1</i>	48.7	62.4	20.9	5.0E-47	4.8E-19	5.3E-122
<i>GFRA1</i>	33.3	25.0	13.2	1.3E-117	2.6E-13	3.3E-53
<i>PTN</i>	189.4	162.4	63.9	5.4E-76	4.1E-04	1.9E-48
<i>TLE4</i>	223.2	135.3	71.6	5.2E-135	2.1E-30	3.8E-39
<i>HAS2</i>	8.9	11.3	4.0	3.0E-20	1.3E-02	5.6E-32
<i>MEX3A</i>	12.4	13.8	6.4	1.4E-17	3.4E-02	5.9E-27
<i>PCDH9</i>	14.7	10.3	5.1	1.2E-79	3.6E-16	4.3E-25
<i>GBE1</i>	78.3	74.6	44.2	1.1E-35	1.4E-02	5.2E-22
<i>PAG1</i>	13.6	11.4	8.2	4.4E-30	1.1E-01	3.9E-21
<i>LRRC17</i>	52.7	21.0	7.6	6.2E-92	2.3E-30	4.4E-21
<i>AC055839.2</i>	84.6	65.4	33.0	7.1E-49	3.3E-07	3.2E-20
<i>TRIM5</i>	25.6	31.6	16.4	6.9E-09	2.3E-03	1.6E-19
<i>ZNF804A</i>	16.8	9.5	4.5	7.6E-84	7.0E-26	5.7E-19
<i>DCHS1</i>	11.5	7.5	3.5	3.9E-58	4.8E-12	9.2E-19
<i>SPP1</i>	21.4	27.1	7.7	4.6E-12	1.7E-01	7.1E-17

Table S5. Expression levels of genes falling into Cluster 5, with reduced expression after PGE₂+MPA treatment compared to base or cAMP+MPA treatment.

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Figure 1. In vitro decidualization of human ESF using PGE₂+MPA. **a.** Cells treated with culture media for 6 days (left) retained spindle morphology, whereas cells treated with MPA+PGE₂ for 6 days became wider (right). Scale bar = 20 μ m. **b.** qPCR quantification of decidual markers PRL and IGFBP1 in response to various decidualization regimes, measured as linearized expression relative to control gene TBP. Experimental n = 2 for cAMP+MPA and PGE₂+MPA treatments, experimental n = 1 for all other treatments, and technical n = 3 for all conditions.

Figure 2. Bulk RNA sequencing analysis. **a.** Principal component analysis conducted on protein-coding genes. Transcriptomes cluster by treatment, with PC1 representing a putative decidual differentiation axis. **b.** Venn diagrams of genes differentially upregulated or downregulated in response to cAMP+MPA or PGE₂+MPA treatments with respect to control treatment. **c.** Heatmap of differentially expressed protein-coding genes ($n = 2$ per condition). **d.** K-means clustered ($k = 5$) heatmap of differentially expressed protein-coding genes showing 5 major patterns of comparative gene expression among the 3 treatment conditions ($n = 2$).

Figure 3. Single-cell RNA sequencing analysis. **a.** Kernel density estimation shows the concentration of most cell density in a region to the lower left of 2-dimensional PHATE embedding, with either two centers of density in the 2-day treated cells (top), or one in the 6-day treated cells (bottom). **b.** All cells from 2-day (left) or 6-day (right) treatments were plotted in a 2-dimensional space and color-coded by best match to bulk RNA-seq treatment conditions (this study). This revealed a curve representing decidual progression from ESF-like (base treatment) to DSC-like (mature) **c.** PHATE visualization for 2- and 6-day PGE₂+MPA decidualized cells, partitioned into clusters and overlaid with inferred developmental trajectories. **d.** Subdivision of activated ESF cluster into two partitions, SOX4⁺ snESF and ACTA2⁺ myoESF.

Figure 4. Expression of select genes (MAGIC-denoised square root counts per million) plotted over reduced-dimensionality single-cell data for either 6-day or 2-day time points. **a.** Proliferative ESF marker genes: *VIM*, *EZR*, *TUBB6*, *ANLN*. **b.** Canonical DSC markers: *FOXO1*, *KLF9*, *PRL*, *IGFBP1*, *LEFTY2*. **c.** Pre-decidual ESF marker genes: *TPM2*, *ACTA2*, *TAGLN*, *THBS1*. **d.** Markers of proposed progesterone-resistant/senescent/*SOX4*^{high} DSC: *SOX4*, *ABI3BP*, *ADAMTS5*, *CLU*, *DIO2*.

Figure 5. *PTGER2* expression is upregulated by progestin and contributes to PGE₂-induced decidualization. **a.** Expression of *PTGER2* (left) and *PTGER4* (right) relative to pseudotemporal progression towards DSC or to *PRL* expression in single-cell data. Conditional probability is a density-resampled estimate of mutual information of *PTGER2*/*PTGER4* and the x-axis value. **b.** qPCR quantification of decidual markers in response to PGE₂+MPA decidualization as well as inhibitors of *PTGER2* (EP2i) or *PTGER4* (EP4i), or protein kinase A (PKAi), measured as fold change relative to control gene *TBP* relative to PGE₂+MPA group. Experimental n = 2, technical n = 2 for all. **c.** qPCR quantification of *PTGER2* showed upregulation only in the presence of MPA. Experimental n = 2 for both treatments containing MPA, n = 1 otherwise; technical n = 3. **d.** Expression of PGE₂ receptors 2 and 4 in *Monodelphis domestica* paleo-ESF (data from Erkenbrack et al., 2018).

Figure 6. Schematic diagram of induction of decidual cell differentiation. Self-limitation of PTGER4, plus P4-induced transcriptional upregulation of PTGER2, results in a transition to a PKA-dominated, rather than AKT-dominated, signaling state.

Figure 7. Conceptual diagram of cell states and types produced during in vitro decidualization with the PGE₂+MPA (left) or 8-Br-cAMP+MPA (right) protocols. Three categorical cell states of (proliferative) ESF, activated ESF (acESF), and decidual stromal cells (DSC). Activated ESF exist along a continuum, one end of which (snESF) expresses a “senescence” gene program associated with stress-susceptibility/progesterone-resistance; under 8-Br-cAMP+MPA treatment these can become senescent DSC (snDSC) whereas under the PGE₂+MPA model the single inferred endpoint is the canonical DSC.

Figure S1. Bulk RNA sequencing analysis. **a.** Clustering dendrogram of all genes for 3 treatment conditions ran in duplicate. **b.** Covariance matrix between protein-coding genes of the 6 bulk transcriptomes generated.

Figure S2. Senescence-associated β -galactosidase staining. **a.** Representative images from cells treated for 3 days (1 of 2 replicates ran). **b.** Representative images from cells treated for 6 days. Scale bar = 20 μ m.

Figure S3. Quality control metrics and filtering cutoffs for single-cell RNA sequencing analysis. **a.** Library size cutoffs were chosen for each sample (2-day, left: 16%, 90%; 6-day, right: 9%, 90%) by a visual attempt to isolate a single symmetrical distribution by removal of a low-size peak and removal of a high library size tail. **b.** Mitochondrial gene expression was cut off at the 93rd percentile of both samples in combination. **c.** Genes expressed in fewer than 5 cells in total from both samples were excluded.

Figure S4. K-means clustering of transcriptomes at 2 days **(a)** and 6 days **(b)** was performed with k parameters from 2 to 7 clusters. Silhouette = silhouette score.

a **b**

Figure S5. Cell cycle inference. **a.** Scoring of most likely cell cycle phase, based upon marker genes identified by Tirosh and colleagues (2016). **b.** Regression out of cell cycle-associated genes did not remove the sparse tail of ESF-like cells, so it was not performed in the primary analysis.

Figure S6. Additional qPCR quantification of *PTGER2* and *PTGER4* endometrial stromal fibroblasts after 6 days of daily treatment with media $\pm$ MPA. *PTGER2* increased 7.14-fold in response to MPA, passing from “off” to “on”, whereas *PTGER2* was reduced to 48% of its original value. Red line (y-intercept = 0.327) shows approximate threshold for being actively transcribed, based on an average TPM value of 9.17 for *TBP* (this study) and cutoff of 3 TPM. Experimental n = 2 for all conditions and technical n = 3.