

23 **Abstract**

24 Decidual remodelling of midluteal endometrium leads to a short implantation window after
25 which the uterine mucosa either breaks down or is transformed into a robust matrix that
26 accommodates the placenta throughout pregnancy. To gain insights into the underlying
27 mechanisms, we established and characterised endometrial assembloids, consisting of
28 gland organoids and primary stromal cells. Single-cell transcriptomics revealed that
29 decidualized assembloids closely resemble midluteal endometrium, harbouring differentiated
30 and senescent subpopulations in both glands and stroma. We show that acute senescence
31 in glandular epithelium drives secretion of multiple canonical implantation factors, whereas in
32 the stroma it calibrates the emergence of anti-inflammatory decidual cells and pro-
33 inflammatory senescent decidual cells. Pharmacological inhibition of stress responses in
34 pre-decidual cells accelerated decidualization by inhibiting senescence and mesenchymal-
35 epithelial transition, processes involved in endometrial breakdown and regeneration,
36 respectively. Accelerated decidualization resulted in entrapment of co-cultured human
37 blastocysts in a largely static decidual matrix. By contrast, the presence of senescent
38 decidual cells created a dynamic implantation environment, enabling embryo expansion and
39 attachment, although their persistence led to gradual disintegration of assembloids. Our
40 findings demonstrate that senescence controls endometrial fate decisions at implantation
41 and highlight how endometrial assembloids may accelerate the discovery of new treatments
42 to prevent reproductive failure.

43 **Introduction**

44 Upon embryo implantation, the cycling human endometrium transforms into the decidua of
45 pregnancy to accommodate the placenta (Gellersen and Brosens, 2014). Transition between
46 these physiological endometrial states requires intensive tissue remodelling, a process
47 termed decidualization. Notwithstanding that decidualization in early pregnancy cannot be
48 studied directly, a spectrum of prevalent reproductive disorders is attributed to perturbations
49 in this process, including recurrent implantation failure and recurrent pregnancy loss
50 (Dimitriadis et al., 2020, Macklon, 2017, Zhou et al., 2019). By contrast, the sequence of
51 events that renders the endometrium receptive to embryo implantation has been
52 investigated extensively, starting with obligatory estrogen-dependent tissue growth following
53 menstrual repair. As a consequence of rapid proliferation of stromal fibroblasts and glandular
54 epithelial cells, which peaks in the upper third of the functional layer (Ferenczy et al., 1979),
55 endometrial volume and thickness increases multi-fold prior to ovulation (Raine-Fenning et
56 al., 2004, Dallenbach-Hellweg, 1981). After the postovulatory rise in progesterone levels,
57 proliferation of epithelial cells (EpC) first decreases and then ceases altogether in concert
58 with the onset of apocrine glandular secretions, heralding the start of the midluteal window of
59 implantation (Dallenbach-Hellweg, 1981). Concurrently, uterine natural killer (uNK) cells
60 accumulate and endometrial stromal cells (EnSC) start decidualizing in a process that can
61 be described as ‘inflammatory programming’ (Brighton et al., 2017, Chavan et al., 2020,
62 Erkenbrack et al., 2018, Salker et al., 2012). Phenotypic decidual cells emerge upon closure
63 of the 4-day implantation window, meaning that the endometrium has become refractory to
64 embryo implantation (Gellersen and Brosens, 2014). In pregnancy, decidual cells form a
65 robust, tolerogenic matrix in which invading trophoblast cells cooperate with local immune
66 cells to form a haemochorial placenta (Aplin et al., 2020, Vento-Tormo et al., 2018). In non-
67 conception cycles, however, falling progesterone levels and influx of neutrophils leads to
68 breakdown of the superficial endometrial layer and menstrual shedding (Jabbour et al.,
69 2006).

70 Recently, we highlighted the importance of cellular senescence in endometrial remodelling
71 during the midluteal implantation window (Brighton et al., 2017, Lucas et al., 2020, Kong et
72 al., In press). Senescence denotes a cellular stress response triggered by replicative
73 exhaustion or other stressors that cause macromolecular damage (Munoz-Espin and
74 Serrano, 2014). Activation of tumour suppressor pathways and upregulation of cyclin-
75 dependent kinase inhibitors p16^{INK4a} (encoded by *CDKN2A*) and p21^{CIP1} (*CDKN1A*) lead to
76 permanent cell cycle arrest, induction of survival genes, and production of a bioactive
77 secretome, referred to as the senescence-associated secretory phenotype (SASP). The
78 composition of the SASP is tissue-specific but typically includes proinflammatory and
79 immuno-modulatory cytokines, chemokines, growth factors, and extracellular matrix (ECM)
80 proteins and proteases (Birch and Gil, 2020). Acute senescence, characterised by transient
81 SASP production and rapid immune-mediated clearance of senescent cells, is widely
82 implicated in processes involving physiological tissue remodelling, including during embryo
83 development, placenta formation and wound healing (Munoz-Espin and Serrano, 2014, van
84 Deursen, 2014). By contrast, persisting senescent cells cause chronic inflammation or
85 ‘inflammaging’ (Birch and Gil, 2020), a pathological state that underpins many age-related
86 disorders. We demonstrated that inflammatory reprogramming of EnSC burdened by
87 replication stress leads to the emergence of acute senescent cells during the implantation
88 window (Brighton et al., 2017, Lucas et al., 2020, Kong et al., In press). Upon successful
89 implantation and continuous progesterone signalling, decidual cells co-opt uNK cells to
90 eliminate their senescent counterparts through granule exocytosis (Brighton et al., 2017,
91 Lucas et al., 2020, Kong et al., In press). Turnover of senescent decidual cells likely
92 promotes recruitment of bone marrow-derived decidual precursor cells, which confer tissue
93 plasticity for rapid decidual expansion in early pregnancy (Diniz-da-Costa et al., In press).
94 Importantly, lack of clonogenic decidual precursor cells and a pro-senescent decidual
95 response are linked to recurrent pregnancy loss (Lucas et al., 2016, Lucas et al., 2020,
96 Tewary et al., 2020).

97 Based on these insights, we hypothesized that acute senescence is integral to successful
98 implantation, by creating conditions for anchorage of the conceptus in an otherwise tightly
99 adherent decidual matrix. To test this hypothesis, we developed an ‘assembloid’ model,
100 consisting of endometrial gland organoids and primary EnSC, which recapitulates the
101 complexity in cell states and gene expression of the midluteal implantation window. We used
102 this model to establish co-cultures with human blastocysts and demonstrate that different
103 pathological states associated with implantation failure and miscarriage can be recapitulated
104 in endometrial assembloids by modulating decidual senescence.

105 **Results**

106 *Establishment of simple endometrial assembloids*

107 Organoids consisting of gland-like structures are established by culturing endometrial
108 epithelial progenitor cells seeded in Matrigel in a chemically defined medium containing
109 growth factors and signal transduction pathway modulators (Supplementary Table 1) (Turco
110 et al., 2017, Boretto et al., 2017). Gland organoids grown in this medium, termed expansion
111 medium, are genetically stable, easily passaged, and can be maintained in long-term
112 cultures (Boretto et al., 2017, Turco et al., 2017). Estradiol (E2) promotes proliferation of
113 gland organoids and cooperates with NOTCH signalling to activate ciliogenesis in a
114 subpopulation of EpC (Haider et al., 2019). Further, treatment with a progestin (e.g.
115 medroxyprogesterone acetate, MPA) and a cyclic AMP analogue (e.g. 8-bromo-cAMP)
116 induces secretory transformation of gland organoids in parallel with expression of luteal-
117 phase marker genes (Turco et al., 2017).

118 We modified the gland organoid model to incorporate EnSC. To this end, midluteal
119 endometrial biopsies (Supplementary Table 2) were digested and gland organoids
120 established from isolated EpC (Figure 1A). In parallel, purified EnSC were propagated in
121 standard monolayer cultures. At passage 2, single cell suspensions of EnSC were combined
122 with organoid EpC in a 1:1 ratio (v/v), seeded in hydrogel, and cultured in expansion medium

123 supplemented with E2 (Figure 1A). The hydrogel matrix comprised 97% type I and 3% type
124 III collagens, which are both present in midluteal endometrium (Oefner et al., 2015, Aplin et
125 al., 1988, Aplin and Jones, 1989, Iwahashi et al., 1996), and has a predicted in-use elastic
126 modulus (Pa) of comparable magnitude to non-pregnant endometrium (Abbas et al., 2019,
127 Bagley, 2019). As shown in Figure 1B, gland formation was unperturbed by the presence of
128 EnSC and assembloids resembled the architecture of native endometrium more closely than
129 organoids. Further, decidualization of assembloids with 8-bromo-cAMP and MPA for 4 days
130 (Figure 1C) resulted in robust secretion of decidual prolactin (PRL) and C-X-C motif
131 chemokine ligand 14 (CXCL14) (Figure 1D). Immunofluorescent microscopy provided further
132 evidence that decidualizing assembloids mimic luteal phase endometrium, exemplified by
133 laminin deposition by decidualizing EnSC, induction of osteopontin (*SPP1*) and
134 accumulation of glycodefin (encoded by *PAEP*) in the lumen of secretory glands, and
135 downregulation of the progesterone receptor (*PGR*) in both stromal and glandular
136 compartments (Figure 1E).

137 We reasoned that once established assembloids may no longer require exogenous growth
138 factors and pathway modulators for differentiation because of the presence of EnSC. To test
139 this hypothesis, parallel gland organoids and assembloids were established from 3
140 endometrial biopsies and decidualized with E2, 8-bromo-cAMP and MPA for 4 days in either
141 expansion medium, base medium (Supplementary Table 1), or base medium with each
142 exogeneous factor added back individually. Induction of *PAEP* and *SPP1* was used to
143 monitor the glandular differentiation response. As shown in Figure 2, differentiation of gland
144 organoids in base medium markedly blunted the induction of *PAEP* and *SPP1* when
145 compared to expansion medium. Further, add-back of individual factors did not restore the
146 glandular response. By contrast, addition of N-acetyl-L-cysteine (NAC), a potent antioxidant,
147 to the base medium was sufficient to induce a robust glandular response in assembloids.
148 Thus, in subsequent experiments simple assembloids were grown in expansion medium

149 supplemented with E2 and then decidualized in minimal differentiation medium (MDM),
150 consisting of base medium containing NAC, E2, 8-bromo-cAMP and MPA.

151 *Cellular complexity of decidualizing assembloids mimics midluteal endometrium*

152 We hypothesized that, depending on the level of replicative stress, individual EpC and EnSC
153 adopt distinct cellular states upon decidualization of endometrial assembloids. Based on
154 previous time-course experiments in 2D cultures, we further speculated that divergence of
155 cells into distinct subpopulations would be apparent by day 4 of differentiation (Brosens et
156 al., 1999, Lucas et al., 2020). To test this hypothesis, we performed single-cell RNA
157 sequencing (scRNA-seq) on undifferentiated assembloids grown for 4 days in expansion
158 medium and assembloids decidualized in MDM for 4 additional days (Figure 3A). Eleven
159 distinct cell clusters were identified by Shared Nearest Neighbour (SNN) and Uniform
160 Manifold Approximation and Projection (UMAP) analysis, segregating broadly into epithelial
161 and stromal populations within the UMAP-1 dimension and into undifferentiated and
162 differentiated subpopulations within the UMAP-2 dimension (Figure 3B). Each cell cluster
163 was annotated based on expression of curated marker genes, which were cross-referenced
164 with a publicly available data set (GEO: GSE4888) to determine their relative expression
165 across the menstrual cycle *in vivo* (Talbi et al., 2006)

166 We identified five unambiguous EpC subsets. The glandular component of undifferentiated
167 assembloids harboured actively dividing EpC (EpS1; n=198) as well as EpC expressing
168 marker genes of E2-responsive proliferative endometrium (EpS2; n=692), including *PGR*
169 and *CPM* (Figure 3C). EpS3 (n=29) consisted of ciliated EpC, expressing an abundance of
170 genes involved in cilium assembly and organization, including *DNAI1* and *TUBA4B* (Figure
171 3C). Ciliated cells are the only glandular subpopulation present in both undifferentiated and
172 decidualized assembloids. *In vivo*, EpS3 marker genes transiently peak during the early-
173 luteal phase (Figure 3C). Decidualization of endometrial assembloids led to the emergence
174 of two distinct EpC subsets, EpS4 (n=434) and EpS5 (n=208). Both clusters expressed
175 canonical endometrial 'receptivity genes' (Figure 3C), i.e. genes used in a clinical test to aid

176 the timing of embryo transfer to the window of implantation in IVF patients (Diaz-Gimeno et
177 al., 2011). In agreement, induction of EpS4 and EpS5 marker genes *in vivo* coincides with
178 the transition from early- to mid-luteal phase. However, while expression of EpS4 marker
179 genes, including *SOD2*, *MAOA* and *PTGS1*, generally peaks during the midluteal window of
180 implantation, EpS5 genes tend to persist or peak during the late-luteal phase (Figure 3C).
181 Additional mining of the data revealed that transition from EpS4 to EpS5 coincides with
182 induction of p16^{INK4a} and p21^{CIP1} in parallel with upregulation of 56 genes encoding secretory
183 factors (Figure 3-figure supplement 1). Notably, several canonical implantation factors
184 secreted by this subpopulation are also well-characterised SASP components, including
185 dipeptidyl peptidase 4 (*DPP4*) (Kim et al., 2017), growth differentiation factor 15 (*GDF15*)
186 (Basisty et al., 2020), and insulin-like growth factor binding protein 3 (*IGFBP3*) (Elzi et al.,
187 2012). Thus, EpS5 consists of senescent EpC producing an implantation-specific SASP.

188 Decidualized endometrial assembloids also harboured a sizable population of ambiguous
189 cells expressing both epithelial and stromal genes (Figure 3C and Figure 3-figure
190 supplement 2). A hallmark of this subset, termed 'transitional population' (TP; n=472), is the
191 induction of long non-coding RNAs involved in mesenchymal-epithelial and epithelial-
192 mesenchymal transition (MET/EMT), such as *NEAT1* (nuclear paraspeckle assembly
193 transcript 1) and *KCNQ1OT1* (KCNQ1 opposite strand/antisense transcript 1) (Bian et al.,
194 2019, Chen et al., 2021). GO analysis showed that both EpS5 and the transitional population
195 are comprised of secretory cells involved in ECM organization (Figure 3D). However, while
196 EpS5 genes are implicated in neutrophil activation (a hallmark of premenstrual
197 endometrium), genes expressed by the transitional population are uniquely enriched in GO
198 terms such as 'wound healing', 'regulation of stem cell proliferation', 'blood coagulation' and
199 'blood vessel development' (Figure 3D), which points towards a putative role in tissue repair
200 and regeneration.

201 The stromal fraction of undifferentiated assembloids consisted of actively dividing EnSC
202 (stromal subpopulation 1 [SS1]; n=434) and E2-responsive EnSC (SS2; n=874) expressing

203 proliferative phase marker genes, such as *PGR*, *MMP11*, and *CRABP2* (Figure 3E). As
204 anticipated, decidualization of assembloids for 4 days led to a preponderance of pre-
205 decidual cells (SS3; n=495) as well as emerging decidual cells (SS4; n=87) and senescent
206 decidual cells (SS5; n=118) (Figure 3E). Each of these subpopulations expressed marker
207 genes identified previously by scRNA-seq reconstruction of the decidual pathway in
208 standard primary EnSC cultures (Lucas et al., 2020). Pre-decidual cells in SS3 express
209 *HAND2*, a key decidual transcription factor (Marinic et al., 2021), as well as previously
210 identified genes encoding secreted factors, including *VEGFA* (vascular endothelial growth
211 factor A), *CRISPLD2* (a progesterone-dependent anti-inflammatory response gene coding
212 cysteine rich secretory protein LCCL domain containing 2), *IL15* (interleukin 15) and *TIMP3*
213 (TIMP metalloproteinase inhibitor 3) (Lucas et al., 2020). Novel pre-decidual genes were also
214 identified, such as *DDIT4* (DNA damage inducible transcript 4), encoding a stress response
215 protein intimately involved in autophagy, stemness and antioxidative defences (Ho et al.,
216 2020, Miller et al., 2020). Decidual cells (SS4) and senescent decidual cells (SS5) express
217 *SCARA5* and *DIO2*, respectively (Figure 3E), two stroma-specific marker genes identified by
218 scRNA-seq analysis of mid- and late-luteal endometrial biopsies (Lucas et al., 2020). SS3
219 and SS4 genes mapped to the early- and mid-luteal phase of the cycle whereas SS5 genes
220 peak in the late-luteal phase, i.e. prior to menstrual breakdown. Notably, the transcriptomic
221 profiles of SS3 and SS5 are enriched in GO terms such as 'Wound healing', 'Response to
222 hypoxia', and 'Inflammatory response', suggesting that both clusters comprise stressed cells
223 (Figure 3F). However, the nature of the cellular stress response differs between these
224 populations with only senescent decidual cells (SS5) expressing genes enriched in
225 categories such as 'Embryo implantation' 'Cellular senescence', 'Aging', and 'Leukocyte
226 activation'. By contrast, few notable categories were selectively enriched in decidual cells
227 (e.g. 'Mesenchymal cell differentiation'), rendering the lack of GO terms that pertain to
228 stress, inflammation, or wound healing perhaps the most striking observation. In keeping
229 with the GO analysis, senescent decidual cells (SS5) express a multitude of SASP-related
230 genes (Figure 3-figure supplement 3), including matrix metalloproteinases (e.g. *MMP3*, 7, 9,

231 10, 11 and 14), insulin-like growth factor binding proteins (e.g. *IGFBP1*, 3, 6 and 7), growth
232 factors (e.g. *AREG*, *FGF2*, *FGF7*, *HGF*, *PDGFRA*, *PDGFRB*, and *VEGFA*), cytokines (e.g.
233 *LIF*, *IL6*, *IL1A*, and *IL11*), chemokines (e.g. *CXCL8* and *CXCL1*), and members of the TGF- β
234 superfamily of proteins (e.g. *GDF15*, *INHBA*, and *BMP2*). By contrast, decidual cells are
235 characterised by expression of an unique network of secretory genes, some encoding ECM
236 proteins (e.g. *COL1A1*, *COL3A1*, and *LAMA4*) and others known decidual markers (e.g.
237 *PRL*, *PROK1* and *WNT4*) as well as factors involved in uNK cell chemotaxis and activation
238 (e.g. *CCL2*, *CXCL14*, and *IL15*) (Figure 3-figure supplement 3).

239 Taken together, single-cell analysis of undifferentiated and decidualized assembloids
240 revealed a surprising level of cellular complexity. Each epithelial and stromal subpopulation
241 appears functionally distinct and maps to a specific phase of the menstrual cycle. Transition
242 between cellular states is predicated on changes in cell cycle status, ranging from actively
243 dividing cells in proliferating assembloids to the emergence upon differentiation of highly
244 secretory senescent epithelial and decidual subpopulations, resembling premenstrual
245 endometrium. However, the dominant subpopulations on day 4 of decidualization are EpS4
246 and SS3, which map to the midluteal implantation window *in vivo*.

247 *Receptor-ligand interactions in decidualizing assembloids*

248 We used CellPhoneDB to explore putative interactions between subpopulations in
249 decidualizing assembloids. This computational tool takes into account the subunit
250 architecture of both ligands and receptors in heteromeric complexes (Efremova et al., 2020,
251 Vento-Tormo et al., 2018). The number of predicted interactions is depicted in Figure 4A,
252 showing a conspicuous lack of crosstalk between the transitional population and any other
253 populations. Conversely, the most abundant interactions centre around the secretory
254 subpopulations, EpC5 and SS5.

255 A total of 270 significantly enriched (non-integrin) receptor-ligand interactions (FDR-
256 corrected $P < 0.05$) were identified between epithelial and stromal subsets in decidualizing

257 assembloids (Figure 4-Source Data 1), a representative selection of which are shown in
258 Figures 4B. Within the multitude of predicted complex interactions, three broad categories
259 can be discerned. First, there are non-selective interactions involving ligands produced by all
260 subpopulations in one compartment acting on receptors expressed by all subsets in the
261 other compartment. Second, there are semi-selective stromal-epithelial interactions involving
262 3 or 4 subpopulations across both compartments. For example, binding of WNT5A secreted
263 by all decidual stromal subsets to FZD3 (frizzled class receptor 3) expressed on all epithelial
264 cells represents a non-selective receptor-ligand interaction whereas binding of WNT5A or
265 WNT4 to FZD6 is a predicted semi-selective interaction, involving all stromal subsets (SS3-
266 5) and EpS4 but not EpS5 (Figure 4B). While FZD3 activates the canonical β -catenin
267 pathway, FZD6 functions as a negative regulator of this signalling cascade (Corda and Sala,
268 2017). Finally, we identified only three highly-selective receptor-ligand interactions (Figure
269 4B), two of which involved secretion of decidual ligands, prolactin (PRL) and C-X-C motif
270 chemokine ligand 12 (CXCL12), acting on their cognate receptors expressed on receptive
271 EpC (EpC4). CXCL12-dependent activation of C-X-C motif chemokine receptor 4 (CXCR4)
272 has been shown to promote motility of EpC (Zheng et al., 2020); whereas PRL is a
273 lactogenic hormone that stimulates glandular secretion in early pregnancy (Burton et al.,
274 2020).

275 In contrast to stromal-epithelial communication, non-selective interactions are predicted to
276 be rare between decidual subsets. Instead, communication appears governed largely by a
277 combinatorial network of receptor-ligand interactions (Figure 4C). For example, colony
278 stimulating factor 3 (CSF3) and vascular endothelial growth factor A (VEGFA) produced by
279 senescent decidual cells (SS5) is predicted to impact selectively on pre-decidual cells (SS3)
280 whereas secretion of inhibin A (INHBA) may engage both pre-decidual and decidual cells
281 (SS4). Other interactions are predicted to govern crosstalk between SS3 and SS4, such as
282 modulation of the WNT pathway in response to binding of R-spondin 3 (RSPO3) to leucine-
283 rich repeat-containing G protein-coupled receptor 4 (LGR4). A striking observation is the

284 overrepresentation of receptor tyrosine kinases implicated in SS3 and SS5 signal
285 transduction as well as the involvement of receptors that signal through downstream
286 cytoplasmic tyrosine kinases, including CSF3 receptor (CSF3R) and CD44 (Figure 4C)
287 (Corey et al., 1998, van der Voort et al., 1999).

288 *Tyrosine kinase-dependent stress responses determines the fate of decidual cells*

289 The CellPhoneDB analysis inferred that epithelial-stromal crosstalk in assembloids is robust,
290 buffered by numerous non-selective interactions, whereas decidual subsets are reliant on
291 selective receptor-ligand interactions and activation of distinct signal transduction pathways.
292 For example, the predicted tyrosine kinase-dependency of pre-decidual (SS3) and
293 senescent decidual cells (SS5) raised the possibility that these subpopulations can be
294 targeted by tyrosine kinase inhibitors, such as dasatinib (Brighton et al., 2017, Zhu et al.,
295 2015). To test this supposition, we generated single-cell transcriptomic profiles of
296 assembloids decidualized for 4 days in the presence of dasatinib (Figure 5A). We found that
297 tyrosine kinase inhibition upon decidualization had a dramatic impact on stromal
298 subpopulations, virtually eliminating senescent decidual cells (SS5, n=7) and increasing the
299 abundance of decidual cells 9-fold (SS4, n=882) (Figure 5B). Apart from a modest reduction
300 in pre-decidual cells (SS3), dasatinib also impacted markedly on transitional cells, reducing
301 their numbers by 76%. By contrast, the effect on epithelial populations was confined to a
302 modest reduction in senescent EpC (EpS5) (Figure 5B). Further, relatively few genes were
303 perturbed significantly (FDR-corrected $P < 0.05$) upon dasatinib treatment in epithelial
304 populations (Figure 5C). In the stroma, dasatinib triggered a conspicuous transcriptional
305 response in pre-decidual (SS3) and transitional cells, whereas gene expression in decidual
306 cells (SS4) and the few remaining senescent decidual cells (SS5) was largely unaffected
307 (Figure 5C). In transitional cells, dasatinib simultaneously upregulated genes encoding
308 canonical mesenchymal markers (e.g. *SNAI2*, *TWIST2*, *ZEB1*, *COL1A1*, and *FBN1*)
309 (Owusu-Akyaw et al., 2019) and decidual factors (e.g. *SCARA5*, *FOXO1*, *GADD45A*, *IL15*,
310 *CXCL14*, and *SGK1*) (Gellersen and Brosens, 2014), revealing that MET accounts for the

311 emergence of this population upon decidualization (Figure 5-Source Data 1). In pre-decidual
312 cells, dasatinib inhibited the expression of a network of genes enriched in GO categories
313 such as 'Response to wounding' (FDR-corrected $P = 3.5 \times 10^{-5}$), 'Response to stress' (FDR-
314 corrected $P = 3.8 \times 10^{-5}$), and 'Response to oxidative stress' (FDR-corrected $P = 1.3 \times 10^{-4}$),
315 indicative of a blunted stress response. To substantiate this finding, we measured the
316 secreted levels of CXCL8 (IL-8), a potent inflammatory mediator implicated in
317 autocrine/paracrine propagation of cellular senescence (Acosta et al., 2008, Kuilman et al.,
318 2008), in assembloids decidualized with or without dasatinib. CXCL14, IL-15, and TIMP3
319 levels were also measured to monitor the decidual response. As shown in Figure 5D,
320 dasatinib completely abrogated the release of CXCL8 by pre-decidual cells while markedly
321 enhancing subsequent secretion of CXCL14, IL-15, and TIMP3, which are involved in
322 effecting immune clearance of senescent decidual cells (Brighton et al., 2017, Lucas et al.,
323 2020, Kong et al., In press). Together, these observations not only support the CellPhoneDB
324 predictions but also indicate that the amplitude of the cellular stress response during the pre-
325 decidual phase determines the subsequent decidual trajectory, with low levels accelerating
326 differentiation and high levels promoting cellular senescence and MET.

327 *Modelling the impact of decidual subpopulations on human embryos.*

328 We postulated that decidual invasion by human embryos that have breached the luminal
329 endometrial epithelium depends on an acute cellular senescence and transient SASP
330 production, rich in growth factors and proteases. Conversely, we reasoned that lack of
331 senescent decidual cells or unconstrained SASP should simulate pathological implantation
332 environments associated with implantation failure and early pregnancy loss, respectively. To
333 test this hypothesis, we constructed a simple implantation model by embedding human
334 embryos in endometrial assembloids. To this end, assembloids were first decidualized for 96
335 hours in the presence or absence of dasatinib, washed and cultured in embryo medium,
336 consisting of MDM with added supplements (Figure 6A and Supplementary Table 1). Day 5
337 human blastocysts were placed into small pockets created in the decidualized assembloids

338 (Figure 6B) and individual embryo-assembloid co-cultures imaged using time-lapse
339 microscopy over 72 hours. Co-cultured blastocysts (n=4) expanded markedly when placed in
340 decidualized assembloids that were not pre-treated with dasatinib (Figure 6C and D). Time-
341 lapse microscopy revealed intense cellular movement in the stromal compartment as well as
342 evidence that interaction between migratory decidual cells and polar trophectoderm
343 promotes adherence and early invasion of the embryo (Video 1). Upon retrieval and
344 processing, we were able to identify proliferating polar trophectoderm in one firmly attached
345 embryo as well as expression of OCT4 and GATA6 in the epiblast and hypoblast,
346 respectively (Figure 6E). A major limitation of this implantation model is that persistence of
347 senescent decidual cells also causes gradual disintegration of the assembloids (Figure 6–
348 figure supplement 1). By contrast, pre-treatment with dasatinib, which accelerates
349 decidualization and all but eliminates decidual senescence, resulted in much more robust
350 assembloids. However, all embedded blastocysts (n=4) failed to expand in this model
351 (Figure 6C and D). Further, movement of the decidual matrix was greatly reduced and
352 directed migration or attachment of decidual cells to the blastocyst was not observed (Video
353 2). Secreted levels of human chorionic gonadotropin (hCG) did not differ between co-
354 cultures (Figure 6E), indicating that all embryos remained viable over the 72-hour
355 observation period. Thus, while our experimental design precluded modelling of
356 physiological embryo implantation, different pathological endometrial states underlying
357 reproductive failure were recapitulated in simple assembloids.

358 **Discussion**

359 There is ample clinical evidence that insufficient endometrial growth decreases the likelihood
360 of successful embryo implantation (Kasius et al., 2014), although the underlying reasons are
361 less clear. Following menstruation, E2-dependent proliferation of both glandular EpC and
362 EnSC accelerates with increasing distance from the endometrial-myometrial interface
363 (Ferenczy et al., 1979), a response linked to presence of an IFN- γ gradient generated by
364 lymphoid aggregates residing in the basal endometrial layer (Christian et al., 2001,

365 Tabibzadeh, 1991, Tabibzadeh et al., 1993). Stromal remodelling and secretory
366 transformation of the glands during the luteal phase are also confined to the superficial
367 endometrial layer, indicating that rapid proliferation is intricately linked to the post-ovulatory
368 differentiation responses, cumulating in a midluteal implantation window. Our single-cell
369 analysis of simple endometrial assembloids provides compelling evidence that the sequence
370 of events leading up to the implantation window, and beyond, requires divergence of both
371 glandular EpC and EnSC into differentiated and senescent subpopulations, a process likely
372 determined by the level of replication stress incurred by individual cells in the preceding
373 proliferative phase (Brighton et al., 2017). Importantly, we demonstrate that acute
374 senescence in glandular EpC (EpS5) underpins production of an implantation-specific
375 SASP, comprising canonical implantation factors and growth factors, such as amphiregulin
376 (*AREG*) and epiregulin (*EREG*), implicated in transforming cytotrophoblasts into extravillous
377 trophoblasts (Cui et al., 2020, Yu et al., 2019). On the other hand, the transcriptome profile
378 of differentiated EpC (EpS4) revealed a pivotal role for this subpopulation in prostaglandin
379 and glycodelin synthesis. Prostaglandins, and specifically PGE2, are indispensable for
380 implantation (Ruan et al., 2012), whereas glycodelin is an abundantly secreted, multifaceted
381 glycoprotein involved in blastocyst attachment, trophoblast differentiation, and immune
382 modulation in early pregnancy (Lee et al., 2016). Further, differentiated EpC highly express
383 *SLC2A1*, encoding the major glucose transporter GLUT1. Glucose is required for glycogen
384 synthesis, an essential component of glandular secretions that nourishes the conceptus prior
385 to the onset of placental perfusion around 10 weeks of pregnancy (Burton et al., 2020). The
386 fate and function of senescent EpC in pregnancy is unknown. Arguably, localised secretion
387 of proteinases by senescent EpC may promote breakdown of the surrounding basement
388 membrane, thereby facilitating endoglandular trophoblast invasion and access to
389 histotrophic nutrition in early gestation (Huppertz, 2019, Moser et al., 2010). In non-
390 conception cycles, the abundance of p16^{INK4}-positive glandular EpC rises markedly during
391 the late-luteal phase (Brighton et al., 2017), indicating that senescent EpC are progesterone-

392 independent and likely responsible for glandular breakdown in the superficial endometrial
393 layer at menstruation.

394 Decidual transformation of EnSC in assembloids unfolded largely as anticipated from
395 previous studies, i.e. starting with an acute pre-decidual stress response and leading to the
396 emergence of both decidual and senescent decidual subpopulations (Brighton et al., 2017,
397 Lucas et al., 2020, Kong et al., In press). Like their epithelial counterparts, senescent
398 decidual cells have a conspicuous secretory phenotype. We identified 56 and 72 genes
399 encoding secreted factors upregulated in senescent epithelial and decidual subpopulations,
400 respectively. However, only 15 genes were shared, indicating that the SASP generated in
401 both cellular compartments is distinct. As glandular secretions drain into the uterine cavity,
402 the embryonic microenvironment is therefore predicted to change abruptly upon breaching of
403 the luminal epithelium. Based on computational predictions of ligand-receptor interactions,
404 we demonstrated that the decidual response in the stroma can be targeted
405 pharmacologically with only modest impact on glandular function and, by extension, the
406 preimplantation embryo milieu. Specifically, dasatinib, a tyrosine kinase inhibitor, was highly
407 effective in blunting the pre-decidual stress response, leading to a dramatic expansion of
408 anti-inflammatory decidual cells and near-total elimination of senescent decidual cells.
409 Dasatinib also inhibited MET and shifted the transcriptional profile of the remaining
410 transitional cells towards a decidual phenotype. An analogous population of ambiguous cells
411 expressing both epithelial and mesenchymal marker genes was recently identified in
412 midluteal endometrium by scRNA-seq analysis (Lucas et al., 2020). Further, based on
413 CellPhoneDB and GO analyses, decidualization-associated EpC are predicted to be highly
414 autonomous and involved in tissue regeneration, in line with experimental evidence that
415 MET drives re-epithelization of the endometrium following menstruation and parturition
416 (Owusu-Akyaw et al., 2019, Patterson et al., 2013). Thus, the level of endogenous cellular
417 stress generated by the endometrium during the window of implantation calibrates the
418 subsequent decidual trajectory, either promoting the formation of a robust decidual matrix or

419 facilitating tissue breakdown and repair. Further, an in-built feature of both trajectories is
420 self-enforcement, as decidual cells recruit and activate uNK cells to eliminate their
421 senescent counterparts (Brighton et al., 2017, Lucas et al., 2020, Kong et al., In press),
422 whereas senescent decidual cells induce secondary senescence in neighbouring decidual
423 (Brighton et al., 2017, Ozaki et al., 2017) and, plausibly, uNK cells (Rajagopalan and Long,
424 2012) .

425 Clinically, recurrent pregnancy loss, defined as multiple miscarriages, is associated with loss
426 of endometrial clonogenicity (Lucas et al., 2016, Diniz-da-Costa et al., In press), uNK cell
427 deficiency and excessive decidual senescence (Lucas et al., 2020, Tewary et al., 2020), and
428 rapid conceptions (also referred to as 'superfertility') (Dimitriadis et al., 2020, Ticconi et al.,
429 2020). Conversely, lack of a proliferative gene signature in midluteal endometrium and
430 premature expression of decidual PRL have been linked to recurrent implantation failure
431 (Berkhout et al., 2020b, Koler et al., 2009, Koot et al., 2016), a pathological condition defined
432 by a failure to achieve a pregnancy following transfer of one or more high-quality embryos in
433 multiple IVF cycles (Polanski et al., 2014). We reasoned that these aberrant implantation
434 environments can be recapitulated in assembloids by manipulating the level of decidual
435 senescence. In line with these predictions, the presence of senescent decidual cells created
436 a permissive environment in which migratory decidual cells interacted with expanding
437 blastocysts, although continuous SASP production also promoted breakdown of the
438 assembloids. Conversely, in the absence of senescent decidual cells, non-expanding
439 embryos became entrapped in a robust but stagnant decidual matrix. These observations
440 are in keeping with previous studies demonstrating that implantation of human embryos
441 depends critically on the invasive and migratory capacities of decidual cells (Berkhout et al.,
442 2020a, Gellersen et al., 2010, Grewal et al., 2008, Weimar et al., 2012). Our co-culture
443 experiments also highlighted the shortcomings of simple assembloids as an implantation
444 model, including the lack of a surface epithelium to create distinct pre- and post-implantation

445 microenvironments and the absence of key cellular constituents, such as innate immune
446 cells.

447 In summary, parsing the mechanisms that control implantation has been hampered by the
448 overwhelming complexity of factors involved in endometrial receptivity. Our single-cell
449 analysis of decidualizing assembloids suggests that this complexity reflects the reliance of
450 the human endometrium on rapid E2-dependent proliferation and replicative exhaustion to
451 generate both differentiated and senescent epithelial and stromal subpopulations in
452 response to the postovulatory rise in progesterone. We demonstrate that senescent cells in
453 both cellular compartments produce distinct bioactive secretomes, which plausibly prime
454 pre-implantation embryos for interaction with the luminal epithelium and then stimulate
455 encapsulation by underlying decidual stromal cells. Based on our co-culture observations,
456 we predict that a blunted pre-decidual stress response causes implantation failure because
457 of a lack of senescence-induced tissue remodelling and accelerated decidualization.
458 Conversely, a heightened stress response leading to excessive decidual senescence may
459 render embryo implantation effortless, albeit in a decidual matrix destined for breakdown and
460 repair. Finally, we demonstrated that pre-decidual stress responses can be modulated
461 pharmacologically, highlighting the potential of endometrial assembloids as a versatile
462 system to evaluate new or repurposed drugs aimed at preventing reproductive failure.

463 **Materials and Methods**

464 *Ethical approvals, endometrial samples and human blastocysts*

465 Endometrial biopsies were obtained from women attending the Implantation Research Clinic,
466 University Hospitals Coventry and Warwickshire National Health Service Trust. Written
467 informed consent was obtained in accordance with the Declaration of Helsinki 2000. The
468 study was approved by the NHS National Research Ethics Committee of Hammersmith and
469 Queen Charlotte's Hospital NHS Trust (1997/5065) and Tommy's Reproductive Health
470 Biobank (Project TSR19-002E, REC Reference: 18/WA/0356). Timed endometrial biopsies
471 were obtained 6 to 11 days after the post-ovulatory LH surge using a Wallach Endocell™
472 Endometrial Cell Sampler. Patient demographics for the samples used in each experiment
473 are detailed in Supplementary Table 2.

474 The use of vitrified human blastocysts was carried out under a Human Fertilisation and
475 Embryology Authority research licence (HFEA: R0155) with local National Health Service
476 Research Ethics Committee approval (04/Q2802/26). Spare blastocysts were donated to
477 research following informed consent by couples who had completed their fertility treatment
478 at the Centre for Reproductive Medicine, University Hospitals Coventry and Warwickshire
479 National Health Service Trust. Briefly, women underwent ovarian stimulation and oocytes
480 were collected by transvaginal ultrasound-guided aspiration and inseminated with prepared
481 sperm (day 0). All oocytes examined 16-18 hours after insemination and classified as
482 normally fertilised were incubated under oil in 20-25 µL drops of culture media (ORIGIO
483 Sequential Cleav and Blast media, CooperSurgical, Denmark) at 5 % O₂, 6 % CO₂, 89% N₂
484 at 37°C. Following culture to day 5 of development, the embryo(s) with the highest quality
485 was selected for transfer, whereas surplus embryos considered top-quality blastocysts were
486 cryopreserved on day 5 or 6 by vitrification using Kitazato vitrification media (Dibimed,
487 Spain) and stored in liquid nitrogen. Prior to their use in the co-culture, vitrified blastocysts
488 were warmed using the Kitazato vitrification warming media (Dibimed, Spain) and underwent
489 zona pellucida removal using a Saturn 5 Laser (CooperSurgical, Denmark). Blastocysts

490 were then incubated for 1 hour under oil in 20 μ L drops of culture media (ORIGIO Sequential
491 Blast media, CooperSurgical, Denmark) at 5 % O₂, 6 % CO₂, 89% N₂ at 37°C and allowed to
492 re-expand.

493 *Processing of endometrial biopsies and primary EnSC cultures.*

494 Unless otherwise stated, reagents were obtained from Life Technologies (Paisley, UK). Cell
495 cultures were incubated at 37°C, 5% CO₂ in a humidified incubator. Centrifugation and
496 incubation steps were performed at room temperature unless stated otherwise. Fresh
497 endometrial biopsies were processed as described previously (Barros et al., 2016). Briefly,
498 tissue was finely minced for 5 minutes using a scalpel blade. Minced tissue was then
499 digested enzymatically with 0.5 mg/ml collagenase I (Sigma Aldrich, Gillingham, UK) and 0.1
500 mg/ml deoxyribonuclease (DNase) type I (Lorne Laboratories, Reading, UK) in 5 ml phenol
501 red-free Dulbecco's Modified Eagle Medium (DMEM)/F12 for 1 hour at 37°C, with regular
502 vigorous shaking. Dissociated cells were washed with growth medium [DMEM/F12
503 containing 10% dextran-coated charcoal stripped FBS (DCC-FBS), 1% penicillin-
504 streptomycin, 2 mM L-glutamine, 1 nM E2 (Sigma Aldrich) and 2 mg/ml insulin (Sigma
505 Aldrich)]. Samples were passed through a 40- μ m cell sieve. EnSC were collected from the
506 flow-through, while epithelial clumps remained in the sieve and were collected by
507 backwashing into a 50 ml Falcon tube. Samples were resuspended in growth medium and
508 centrifuged at 400 \times g for 5 minutes. EnSC pellets were resuspended in 10 ml growth
509 medium and plated in tissue culture flasks. To isolate EnSC from other (non-adherent) cells
510 collected in the flowthrough, medium was refreshed after 24 hours. Thereafter, medium was
511 refreshed every 48 hours. Sub-confluent monolayers were passaged using 0.25% Trypsin-
512 EDTA and split at a ratio of 1:3.

513 *Endometrial gland organoid culture*

514 Endometrial gland organoids were established as described previously (Turco et al., 2017),
515 with adaptations. Freshly isolated endometrial gland fragments were resuspended in 500 μ l

516 phenol red-free DMEM/F12 medium in a microcentrifuge tube and centrifuged at $600 \times g$ for
517 5 minutes. The medium was aspirated and ice-cold, growth factor-reduced Matrigel®
518 (Corning Life Sciences B.V., Amsterdam, Netherlands) was added at a ratio of 1:20 (cell
519 pellet: Matrigel®). Samples mixed in Matrigel® were kept on ice until plating at which point
520 the suspension was aliquoted in 20 μ l volumes to a 48 well plate, one drop per well, and
521 allowed to cure for 15 minutes. Expansion medium supplemented with E2 (Supplementary
522 Table 1) (Turco et al., 2017) was then added and samples cultured for up to 7 days. For
523 passaging, Matrigel® droplets containing gland organoids were collected into
524 microcentrifuge tubes and centrifuged at $600 \times g$ for 6 minutes at 4°C. Samples were
525 resuspended in ice-cold, phenol red-free DMEM/F12 and subjected to manual pipetting to
526 disrupt the organoids. Suspensions were centrifuged again, resuspended in ice cold additive
527 free DMEM/F12 and then subjected to further manual pipetting. Suspensions were
528 centrifuged again and either resuspended in Matrigel® and plated as described above for
529 continued expansion or used to establish assembloid cultures.

530 *Establishment of assembloid cultures*

531 At passage 2, EnSC and gland organoid pellets were mixed at a ratio of 1:1 (v/v) and ice-
532 cold PureCol® EZ Gel (Sigma Aldrich) added at a ratio of 1:20 (cell pellet: hydrogel).
533 Samples were kept on ice until plating. The suspension was aliquoted in 20 μ l volumes using
534 ice-cold pipette tips into a 48-well plate, one droplet per well, and allowed to cure in the cell
535 culture incubator for 45 minutes. Expansion medium supplemented with 10 nM E2 was
536 overlaid and the medium was refreshed every 48 hours. For decidualization experiments,
537 assembloid cultures were grown in expansion medium supplemented with E2 for 4 days to
538 allow for growth and expansion. Assembloids were then either harvested or decidualized
539 using different media as tabulated in Supplementary Table1 for a further 4 days. Again, the
540 medium was refreshed every 48 hours and spent medium stored for further analysis. For
541 tyrosine kinase inhibition, MDM was supplemented with 250 nM dasatinib (Cell Signaling
542 Technology, Leiden, NL).

543 *Fluorescence microscopy*

544 For fluorescent microscopy, assembloids were removed from culture wells and transferred
545 into tubes for fixation. Samples were washed in PBS and fixed in 10% neutral buffered
546 formalin in the tube for 15 minutes, then washed three times with PBS and stored for use.
547 Samples were dehydrated in increasing concentrations of ethanol (70% then 90% for 1 hour
548 each, followed by 100% for 90 minutes), then incubated in xylene for 1 hour. After paraffin
549 wax embedding, 5 μm sections were cut and mounted, then incubated overnight at 60 °C.
550 Slides were then stored at 4°C until further processing. De-paraffinization and rehydration
551 were performed through xylene, 100% isopropanol, 70% isopropanol and distilled water
552 incubations. Following antigen retrieval, permeabilization was performed where appropriate
553 by incubation with 0.1% Triton X-100 for 30 minutes. Slides were then washed, blocked, and
554 incubated in primary antibodies overnight at 4°C. Antibody details are presented in
555 Supplementary Table 3. After washing three times, slides were incubated with secondary
556 antibodies for 2 hours, then washed as before and mounted in ProLong Gold® Antifade
557 Reagent with DAPI (Cell Signaling Technology). Slides were visualised using the EVOS
558 Auto system, with imaging parameters maintained throughout image acquisition. Images
559 were merged in ImageJ and any adjustments to brightness or contrast were applied equally
560 within comparisons.

561 *Real-time quantitative polymerase chain reaction*

562 After removal of spent medium, gland organoid cultures were washed in PBS and harvested
563 in 200 μl Cell Recovery Solution (Corning). Gel droplets were transferred to nuclease-free
564 microcentrifuge tubes and placed at 4°C for 30 minutes. Samples were then washed in PBS,
565 centrifuged at 600 $\times g$ for 6 minutes twice and snap frozen as cell pellets. Assembloid
566 cultures were washed with PBS and then recovered by directly scraping the samples into
567 nuclease-free microcentrifuge tubes. Samples were centrifuged at 600 $\times g$ for 6 minutes.
568 The cellular pellet was resuspended in 500 μl of 500 $\mu\text{g/ml}$ collagenase I diluted in additive-

569 free DMEM/F12 and incubated at 37°C for 10 minutes with regular manual shaking.
570 Samples were washed twice in PBS, with centrifugation at 600 × *g* for 6 minutes, then cell
571 pellets were snap frozen. RNA extraction was performed using the RNeasy Micro Kit
572 (QIAGEN, Manchester, UK) according to manufacturer's instructions. RNA concentration
573 and purity were determined using a NanoDrop ND-1000. All RNA samples were stored at -
574 80°C until use. Reverse transcription was performed using the QuantiTect Reverse
575 Transcription (RT) Kit according to manufacturer's protocol (QIAGEN). Input RNA was
576 determined by the sample with lowest concentration within each experiment. Genes of
577 interest were amplified using PrecisionPlus SYBR Green Mastermix (PrimerDesign,
578 Southampton, UK). Amplification was performed in 10 µl reactions containing 5µl
579 PrecisionPlus 2× master mix, 300nM each of forward and reverse primer, nuclease free
580 water and 1µl of cDNA or water control. Amplification was performed for 40 cycles on an
581 Applied Biosystems QuantStudio 5 Real-Time PCR System (qPCR). Data were analysed
582 using the Pfaffl method (Pfaffl, 2001) and *L19* was used as a reference gene. Primer
583 sequences were as follows: *L19* forward: 5'-GCG GAA GGG TAC AGC CAA T-3', *L19*
584 reverse: 5'-GCA GCC GGC GCA AA-3', *PAEP* forward: 5'-GAG CAT GAT GTG CCA GTA
585 CC-3', *PAEP* reverse: 5'-CCT GAA AGC CCT GAT GAA TCC-3', *SPP1* forward: 5'-TGC
586 AGC CTT CTC AGC CAA A-3', *SPP1* reverse: 5'-GGA GGC AAA AGC AAA TCA CTG-3'.

587 *Enzyme-linked immunosorbent assay*

588 Spent medium was collected every two days during a 4-day decidual time course, with or
589 without dasatinib treatment. DuoSet solid phase sandwich enzyme-linked immunosorbent
590 assay (ELISA) kits (Bio-Techne, Abingdon, UK) were used for the detection of PRL (DY682),
591 TIMP3 (DY973), IL-8 (DY208), IL-15 (DY247), CXCL14 (DY866) and HCG (DY9034).
592 Assays were performed according to manufacturer's instructions. Absorbance at 450 nm
593 was measured on a PheraStar microplate reader (BMG LABTECH Ltd, Aylesbury, UK), with
594 background subtraction from absorbance measured at 540 nm. Protein concentration was
595 obtained using a 4-parameter logistic regression analysis and interpolation from the curve.

596 *Single-cell capture, library preparation and sequencing*

597 Assembloids were dissociated to single cells by incubation of gel droplets with 0.5 mg/ml
598 Collagenase I for 10 minutes in a 37°C water bath for 10 minutes with regular vigorous
599 shaking. Samples were washed with additive free DMEM/F12 phenol free medium and
600 incubated with 5×TrypLE Select diluted in additive free DMEM/F12 phenol free medium for 5
601 minutes in a 37°C water bath. Cell clumps were disrupted by manual pipetting, then
602 suspended in 0.1% BSA in PBS and passed through a 35 µm cell sieve. Droplet generation
603 was performed using a Nadia Instrument (Dolomite Bio, Cambridge, UK) according to the
604 manufacturer's guidelines and using reagents as described by Macosko et al., (2015) and
605 the scRNAseq v1.8 protocol (Dolomite Bio). Pooled beads were processed as described
606 previously (Lucas et al., 2020) and sequenced using a NextSeq 500 with high output 75
607 cycle cartridge (Illumina, Cambridge, UK) by the University of Warwick Genomics Facility.

608 *Bioinformatics analysis*

609 Initial single cell RNAseq data processing was performed using Drop-Seq_tools-2.3.0
610 (DropseqAlignmentCookbook_v2Sept2018, <http://mccarrolllab.com/dropseq>) and as
611 described previously (Lucas et al., 2020). To select high-quality data for analysis, cells were
612 included when at least 200 genes were detected, while genes were included if they were
613 detected in at least 3 cells. Cells with more than 5000 genes were excluded from the
614 analysis as were cells with more than 5 % mitochondrial gene transcripts, to minimize
615 doublets and low quality (broken or damaged) cells, respectively. The Seurat v3 standard
616 workflow (Stuart et al., 2019) was used to integrate datasets from biological replicates.
617 Clustering and nearest neighbour analysis was performed on the full integrated dataset
618 using principle components 1:15 and a resolution of 0.6. The 'subset' function was applied
619 for interrogation of specific experimental conditions and timepoints. Gene ontology (GO)
620 analysis was performed on differentially expressed genes from specified 'FindMarkers'
621 comparisons in Seurat v3 using the Gene Ontology Consortium database (Ashburner et al.,
622 2000, The Gene Ontology, 2019, Mi et al., 2013). Dot plots of significantly enriched GO

623 terms (FDR adjusted $P < 0.05$) were generated in RStudio (version 1.2.5042). CellPhoneDB
624 was used to predict enriched receptor-ligand interactions between subpopulations in
625 decidualizing assembloids (Efremova et al., 2020, Vento-Tormo et al., 2018). Significance
626 was set at $P < 0.05$. Annotated tyrosine kinase interactions were curated manually.

627 *Co-culture of human blastocyst and endometrial assembloids*

628 Prior to co-culture, decidualised assembloids were washed in PBS and medium was
629 replaced with embryo medium (Supplementary Table 1). Assembloids were lightly punctured
630 with a needle to create a small pocket, to enable one re-expanded day 5 human blastocyst
631 to be co-cultured per assembloid. The plate was transferred to a pre-warmed and gassed
632 (humidified 5% CO₂ in air) environment chamber placed on an automated X-Y stage
633 (EVOS® FL Auto Imaging System with onstage incubator) for time-lapse imaging. Brightfield
634 images were captured every 60 minutes over 72 hours. Captured images were converted
635 into videos using ImageJ.

636 For fixation, assembloid co-cultures were removed from culture wells and transferred into
637 tubes. Samples were washed in PBS and fixed in 10% neutral buffered formalin in the tube
638 for 15 minutes, then washed three times with PBS. Assembloids were permeabilised for 30
639 minutes in PBS containing 0.3% Triton X-100 and 0.1 M glycine for 30 minutes at room
640 temperature. Samples were incubated overnight at 4°C in primary antibodies diluted in PBS
641 containing 10% FBS, 2% bovine serum albumin (BSA), and 0.1% Tween-20. Samples were
642 then washed in PBS (0.1% Tween-20) and incubated for 2 hours at room temperature
643 protected from light in fluorescently conjugated Alexa Fluor secondary antibodies 1:500
644 (ThermoFisher Scientific) and DAPI (D3571, ThermoFisher Scientific, dilution 1/500), diluted
645 in PBS containing 10% FBS, 2% BSA, and 0.1% Tween-20. Samples were imaged on a
646 Leica SP8 confocal microscope using a ×25 water objective, with a 0.6 μm z-step and 2×
647 line averaging.

648 *Statistical analysis*

649 Data were analysed using GraphPad Prism. For paired, non-parametric significance testing
650 between multiple groups, the Friedman test and Dunn's multiple comparisons *post hoc* test
651 were performed. Only values of $P < 0.05$ were considered statistically significant.

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661 References

- 662 ABBAS, Y., CARNICER-LOMBARTE, A., GARDNER, L., THOMAS, J., BROSENS, J. J., MOFFETT,
663 A., SHARKEY, A. M., FRANZE, K., BURTON, G. J. & OYEN, M. L. 2019. Tissue stiffness at
664 the human maternal-fetal interface. *Hum Reprod*, 34, 1999-2008.
- 665 ACOSTA, J. C., O'LOGHLEN, A., BANITO, A., GUIJARRO, M. V., AUGERT, A., RAGUZ, S.,
666 FUMAGALLI, M., DA COSTA, M., BROWN, C., POPOV, N., TAKATSU, Y., MELAMED, J.,
667 D'ADDA DI FAGAGNA, F., BERNARD, D., HERNANDO, E. & GIL, J. 2008. Chemokine
668 signaling via the CXCR2 receptor reinforces senescence. *Cell*, 133, 1006-18.
- 669 APLIN, J. D., CHARLTON, A. K. & AYAD, S. 1988. An immunohistochemical study of human
670 endometrial extracellular matrix during the menstrual cycle and first trimester of
671 pregnancy. *Cell and Tissue Research*, 253, 231-240.
- 672 APLIN, J. D. & JONES, C. J. P. 1989. Extracellular Matrix in Endometrium and Decidua. In:
673 GENBAČEV, O., KLOPPER, A. & BEACONSFIELD, R. (eds.) *Placenta as a Model and a*
674 *Source*. Boston, MA: Springer US.
- 675 APLIN, J. D., MYERS, J. E., TIMMS, K. & WESTWOOD, M. 2020. Tracking placental
676 development in health and disease. *Nat Rev Endocrinol*, 16, 479-494.
- 677 ASHBURNER, M., BALL, C. A., BLAKE, J. A., BOTSTEIN, D., BUTLER, H., CHERRY, J. M., DAVIS,
678 A. P., DOLINSKI, K., DWIGHT, S. S., EPPIG, J. T., HARRIS, M. A., HILL, D. P., ISSEL-
679 TARVER, L., KASARSKIS, A., LEWIS, S., MATESE, J. C., RICHARDSON, J. E., RINGWALD,
680 M., RUBIN, G. M. & SHERLOCK, G. 2000. Gene ontology: tool for the unification of
681 biology. The Gene Ontology Consortium. *Nat Genet*, 25, 25-9.
- 682 BAGLEY, B. 2019. *Collagen Gelation Kinetics and Shear Modulus [White paper]*. [Online].
683 Advanced Biomatrix. Available: [https://advancedbiomatrix.com/3d-collagen-](https://advancedbiomatrix.com/3d-collagen-hydrogel-stiffness.html)
684 [hydrogel-stiffness.html](https://advancedbiomatrix.com/3d-collagen-hydrogel-stiffness.html) [Accessed 01/10/2020].
- 685 BARROS, F. S., BROSENS, J. J. & BRIGHTON, P. J. 2016. Isolation and Primary Culture of
686 Various Cell Types from Whole Human Endometrial Biopsies. *Bio-protocol*, 6, e2028.
- 687 BASISTY, N., KALE, A., JEON, O. H., KUEHNEMANN, C., PAYNE, T., RAO, C., HOLTZ, A., SHAH,
688 S., SHARMA, V., FERRUCCI, L., CAMPISI, J. & SCHILLING, B. 2020. A proteomic atlas of
689 senescence-associated secretomes for aging biomarker development. *PLoS Biol*, 18,
690 e3000599.
- 691 BERKHOUT, R. P., KEIJSER, R., REPPING, S., LAMBALK, C. B., AFINK, G. B., MASTENBROEK, S.
692 & HAMER, G. 2020a. High-quality human preimplantation embryos stimulate
693 endometrial stromal cell migration via secretion of microRNA hsa-miR-320a. *Hum*
694 *Reprod*, 35, 1797-1807.
- 695 BERKHOUT, R. P., LAMBALK, C. B., REPPING, S., HAMER, G. & MASTENBROEK, S. 2020b.
696 Premature expression of the decidualization marker prolactin is associated with
697 repeated implantation failure. *Gynecol Endocrinol*, 36, 360-364.
- 698 BIAN, Y., GAO, G., ZHANG, Q., QIAN, H., YU, L., YAO, N., QIAN, J., LIU, B. & QIAN, X. 2019.
699 KCNQ1OT1/miR-217/ZEB1 feedback loop facilitates cell migration and epithelial-
700 mesenchymal transition in colorectal cancer. *Cancer Biology & Therapy*, 20, 886-896.
- 701 BIRCH, J. & GIL, J. 2020. Senescence and the SASP: many therapeutic avenues. *Genes Dev*,
702 34, 1565-1576.
- 703 BORETTO, M., COX, B., NOBEN, M., HENDRIKS, N., FASSBENDER, A., ROOSE, H., AMANT, F.,
704 TIMMERMAN, D., TOMASSETTI, C., VANHIE, A., MEULEMAN, C., FERRANTE, M. &
705 VANKELECOM, H. 2017. Development of organoids from mouse and human

- 706 endometrium showing endometrial epithelium physiology and long-term
707 expandability. *Development*, 144, 1775-1786.
- 708 BRIGHTON, P. J., MARUYAMA, Y., FISHWICK, K., VRLJICAK, P., TEWARY, S., FUJIHARA, R.,
709 MUTER, J., LUCAS, E. S., YAMADA, T., WOODS, L., LUCCIOLA, R., HOU LEE, Y.,
710 TAKEDA, S., OTT, S., HEMBERGER, M., QUENBY, S. & BROSENS, J. J. 2017. Clearance
711 of senescent decidual cells by uterine natural killer cells in cycling human
712 endometrium. *Elife*, 6.
- 713 BROSENS, J. J., HAYASHI, N. & WHITE, J. O. 1999. Progesterone receptor regulates decidual
714 prolactin expression in differentiating human endometrial stromal cells.
715 *Endocrinology*, 140, 4809-20.
- 716 BURTON, G. J., CINDROVA-DAVIES, T. & TURCO, M. Y. 2020. Review: Histotrophic nutrition
717 and the placental-endometrial dialogue during human early pregnancy. *Placenta*,
718 102, 21-26.
- 719 CHAVAN, A. R., GRIFFITH, O. W., STADTMAUER, D., MAZIARZ, J., PAVLICEV, M., FISHMAN, R.,
720 KOREN, L., ROMERO, R. & WAGNER, G. P. 2020. Evolution of embryo implantation
721 was enabled by the origin of decidual stromal cells in eutherian mammals. *Mol Biol*
722 *Evol.*
- 723 CHEN, Y., LI, J., XIAO, J.-K., XIAO, L., XU, B.-W. & LI, C. 2021. The lncRNA NEAT1 promotes the
724 epithelial-mesenchymal transition and metastasis of osteosarcoma cells by sponging
725 miR-483 to upregulate STAT3 expression. *Cancer Cell International*, 21, 90.
- 726 CHRISTIAN, M., MARANGOS, P., MAK, I., MCVEY, J., BARKER, F., WHITE, J. & BROSENS, J. J.
727 2001. Interferon-gamma modulates prolactin and tissue factor expression in
728 differentiating human endometrial stromal cells. *Endocrinology*, 142, 3142-51.
- 729 CORDA, G. & SALA, A. 2017. Non-canonical WNT/PCP signalling in cancer: Fzd6 takes centre
730 stage. *Oncogenesis*, 6, e364.
- 731 COREY, S. J., DOMBROSKY-FERLAN, P. M., ZUO, S., KROHN, E., DONNENBERG, A. D., ZORICH,
732 P., ROMERO, G., TAKATA, M. & KUROSAKI, T. 1998. Requirement of Src kinase Lyn for
733 induction of DNA synthesis by granulocyte colony-stimulating factor. *J Biol Chem*,
734 273, 3230-5.
- 735 CUI, X., WANG, H., LI, Y., CHEN, T., LIU, S. & YAN, Q. 2020. Epiregulin promotes trophoblast
736 epithelial-mesenchymal transition through poFUT1 and O-fucosylation by poFUT1 on
737 uPA. *Cell Prolif*, 53, e12745.
- 738 DALLENBACH-HELLWEG, G. 1981. The Normal Histology of the Endometrium.
739 *Histopathology of the Endometrium*. Berlin, Heidelberg.: Springer.
- 740 DIAZ-GIMENO, P., HORCAJADAS, J. A., MARTINEZ-CONEJERO, J. A., ESTEBAN, F. J., ALAMA,
741 P., PELLICER, A. & SIMON, C. 2011. A genomic diagnostic tool for human endometrial
742 receptivity based on the transcriptomic signature. *Fertil Steril*, 95, 50-60, 60 e1-15.
- 743 DIMITRIADIS, E., MENKHORST, E., SAITO, S., KUTTEH, W. H. & BROSENS, J. J. 2020. Recurrent
744 pregnancy loss. *Nat Rev Dis Primers*, 6, 98.
- 745 DINIZ-DA-COSTA, M., KONG, C.-S., FISHWICK, K. J., RAWLINGS, T., BRIGHTON, P. J., HAWKES,
746 A., ODENDAAL, J., QUENBY, S., OTT, S., LUCAS, E. S., VRLJICAK, P. & BROSENS, J. J. In
747 press. Characterization of highly proliferative decidual precursor cells during the
748 window of implantation in human endometrium. *Stem Cells*.
- 749 EFREMOVA, M., VENTO-TORMO, M., TEICHMANN, S. A. & VENTO-TORMO, R. 2020.
750 CellPhoneDB: inferring cell-cell communication from combined expression of multi-
751 subunit ligand-receptor complexes. *Nat Protoc*, 15, 1484-1506.

- 752 ELZI, D. J., LAI, Y., SONG, M., HAKALA, K., WEINTRAUB, S. T. & SHIIO, Y. 2012. Plasminogen
753 activator inhibitor 1--insulin-like growth factor binding protein 3 cascade regulates
754 stress-induced senescence. *Proc Natl Acad Sci U S A*, 109, 12052-7.
- 755 ERKENBRACK, E. M., MAZIARZ, J. D., GRIFFITH, O. W., LIANG, C., CHAVAN, A. R., NNAMANI,
756 M. C. & WAGNER, G. P. 2018. The mammalian decidual cell evolved from a cellular
757 stress response. *PLoS Biol*, 16, e2005594.
- 758 FERENCZY, A., BERTRAND, G. & GELFAND, M. M. 1979. Proliferation kinetics of human
759 endometrium during the normal menstrual cycle. *Am J Obstet Gynecol*, 133, 859-67.
- 760 GELLERSEN, B. & BROSENS, J. J. 2014. Cyclic decidualization of the human endometrium in
761 reproductive health and failure. *Endocr Rev*, 35, 851-905.
- 762 GELLERSEN, B., REIMANN, K., SAMALECOS, A., AUPERS, S. & BAMBERGER, A. M. 2010.
763 Invasiveness of human endometrial stromal cells is promoted by decidualization and
764 by trophoblast-derived signals. *Hum Reprod*, 25, 862-73.
- 765 GREWAL, S., CARVER, J. G., RIDLEY, A. J. & MARDON, H. J. 2008. Implantation of the human
766 embryo requires Rac1-dependent endometrial stromal cell migration. *Proc Natl Acad
767 Sci U S A*, 105, 16189-94.
- 768 HAIDER, S., GAMPERL, M., BURKARD, T. R., KUNIHS, V., KAINDL, U., JUNTILLA, S., FIALA, C.,
769 SCHMIDT, K., MENDJAN, S., KNOFLER, M. & LATOS, P. A. 2019. Estrogen Signaling
770 Drives Ciliogenesis in Human Endometrial Organoids. *Endocrinology*, 160, 2282-
771 2297.
- 772 HO, K. H., CHEN, P. H., CHOU, C. M., SHIH, C. M., LEE, Y. T., CHENG, C. H. & CHEN, K. C. 2020.
773 A Key Role of DNA Damage-Inducible Transcript 4 (DDIT4) Connects Autophagy and
774 GLUT3-Mediated Stemness To Desensitize Temozolomide Efficacy in Glioblastomas.
775 *Neurotherapeutics*, 17, 1212-1227.
- 776 HUPPERTZ, B. 2019. Traditional and New Routes of Trophoblast Invasion and Their
777 Implications for Pregnancy Diseases. *Int J Mol Sci*, 21.
- 778 IWAHASHI, M., MURAGAKI, Y., OOSHIMA, A., YAMOTO, M. & NAKANO, R. 1996. Alterations
779 in distribution and composition of the extracellular matrix during decidualization of
780 the human endometrium. *J Reprod Fertil*, 108, 147-55.
- 781 JABBOUR, H. N., KELLY, R. W., FRASER, H. M. & CRITCHLEY, H. O. 2006. Endocrine regulation
782 of menstruation. *Endocr Rev*, 27, 17-46.
- 783 KASIUS, A., SMIT, J. G., TORRANCE, H. L., EIJKEMANS, M. J., MOL, B. W., OPMEER, B. C. &
784 BROEKMANS, F. J. 2014. Endometrial thickness and pregnancy rates after IVF: a
785 systematic review and meta-analysis. *Hum Reprod Update*, 20, 530-41.
- 786 KIM, K. M., NOH, J. H., BODOGAI, M., MARTINDALE, J. L., YANG, X., INDIG, F. E., BASU, S. K.,
787 OHNUMA, K., MORIMOTO, C., JOHNSON, P. F., BIRAGYN, A., ABDELMOHSEN, K. &
788 GOROSPE, M. 2017. Identification of senescent cell surface targetable protein DPP4.
789 *Genes Dev*, 31, 1529-1534.
- 790 KOLER, M., ACHACHE, H., TSAFRIR, A., SMITH, Y., REVEL, A. & REICH, R. 2009. Disrupted gene
791 pattern in patients with repeated in vitro fertilization (IVF) failure. *Hum Reprod*, 24,
792 2541-8.
- 793 KONG, C.-S., ORDOÑEZ, A. A., TURNER, S., TREMAINE, T., MUTER, J., LUCAS, E. S.,
794 SALISBURY, E., VASSENA, R., FOULADI-NASHTA, A. A., TISCORNIA, G., HARTSHORNE,
795 G., BROSENS, J. J. & BRIGHTON, P. J. In press. Involvement of uterine natural killer
796 cells in the natural selection of human embryos at implantation. *FASEB J*.
- 797 KOOT, Y. E., VAN HOOFF, S. R., BOOMSMA, C. M., VAN LEENEN, D., GROOT KOERKAMP, M.
798 J., GODDIJN, M., EIJKEMANS, M. J., FAUSER, B. C., HOLSTEGE, F. C. & MACKLON, N. S.

- 799 2016. An endometrial gene expression signature accurately predicts recurrent
800 implantation failure after IVF. *Sci Rep*, 6, 19411.
- 801 KUILMAN, T., MICHALOGLU, C., VREDEVELD, L. C., DOUMA, S., VAN DOORN, R., DESMET,
802 C. J., AARDEN, L. A., MOOI, W. J. & PEEPER, D. S. 2008. Oncogene-induced
803 senescence relayed by an interleukin-dependent inflammatory network. *Cell*, 133,
804 1019-31.
- 805 LEE, C. L., LAM, K. K., VIJAYAN, M., KOISTINEN, H., SEPPALA, M., NG, E. H., YEUNG, W. S. &
806 CHIU, P. C. 2016. The Pleiotropic Effect of Glycodelin-A in Early Pregnancy. *Am J*
807 *Reprod Immunol*, 75, 290-7.
- 808 LUCAS, E. S., DYER, N. P., MURAKAMI, K., LEE, Y. H., CHAN, Y. W., GRIMALDI, G., MUTER, J.,
809 BRIGHTON, P. J., MOORE, J. D., PATEL, G., CHAN, J. K., TAKEDA, S., LAM, E. W.,
810 QUENBY, S., OTT, S. & BROSENS, J. J. 2016. Loss of Endometrial Plasticity in Recurrent
811 Pregnancy Loss. *Stem Cells*, 34, 346-56.
- 812 LUCAS, E. S., VRLJICAK, P., MUTER, J., DINIZ-DA-COSTA, M. M., BRIGHTON, P. J., KONG, C. S.,
813 LIPECKI, J., FISHWICK, K. J., ODENDAAL, J., EWINGTON, L. J., QUENBY, S., OTT, S. &
814 BROSENS, J. J. 2020. Recurrent pregnancy loss is associated with a pro-senescent
815 decidual response during the peri-implantation window. *Commun Biol*, 3, 37.
- 816 MACKLON, N. 2017. Recurrent implantation failure is a pathology with a specific
817 transcriptomic signature. *Fertil Steril*, 108, 9-14.
- 818 MACOSKO, E. Z., BASU, A., SATIJA, R., NEMESH, J., SHEKHAR, K., GOLDMAN, M., TIROSH, I.,
819 BIALAS, A. R., KAMITAKI, N., MARTERSTECK, E. M., TROMBETTA, J. J., WEITZ, D. A.,
820 SANES, J. R., SHALEK, A. K., REGEV, A. & MCCARROLL, S. A. 2015. Highly Parallel
821 Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*,
822 161, 1202-1214.
- 823 MARINIC, M., MIKA, K., CHIGURUPATI, S. & LYNCH, V. J. 2021. Evolutionary transcriptomics
824 implicates HAND2 in the origins of implantation and regulation of gestation length.
825 *Elife*, 10.
- 826 MI, H., MURUGANUJAN, A., CASAGRANDE, J. T. & THOMAS, P. D. 2013. Large-scale gene
827 function analysis with the PANTHER classification system. *Nat Protoc*, 8, 1551-66.
- 828 MILLER, W. P., SUNILKUMAR, S., GIORDANO, J. F., TORO, A. L., BARBER, A. J. & DENNIS, M.
829 D. 2020. The stress response protein REDD1 promotes diabetes-induced oxidative
830 stress in the retina by Keap1-independent Nrf2 degradation. *J Biol Chem*, 295, 7350-
831 7361.
- 832 MOSER, G., GAUSTER, M., ORENDI, K., GLASNER, A., THEUERKAUF, R. & HUPPERTZ, B. 2010.
833 Endoglandular trophoblast, an alternative route of trophoblast invasion? Analysis
834 with novel confrontation co-culture models. *Hum Reprod*, 25, 1127-36.
- 835 MUNOZ-ESPIN, D. & SERRANO, M. 2014. Cellular senescence: from physiology to pathology.
836 *Nat Rev Mol Cell Biol*, 15, 482-96.
- 837 OEFNER, C. M., SHARKEY, A., GARDNER, L., CRITCHLEY, H., OYEN, M. & MOFFETT, A. 2015.
838 Collagen type IV at the fetal-maternal interface. *Placenta*, 36, 59-68.
- 839 OWUSU-AKYAW, A., KRISHNAMOORTHY, K., GOLDSMITH, L. T. & MORELLI, S. S. 2019. The
840 role of mesenchymal-epithelial transition in endometrial function. *Hum Reprod*
841 *Update*, 25, 114-133.
- 842 OZAKI, R., KURODA, K., IKEMOTO, Y., OCHIAI, A., MATSUMOTO, A., KUMAKIRI, J., KITADE,
843 M., ITAKURA, A., MUTER, J., BROSENS, J. J. & TAKEDA, S. 2017. Reprogramming of
844 the retinoic acid pathway in decidualizing human endometrial stromal cells. *PLoS*
845 *One*, 12, e0173035.

- 846 PATTERSON, A. L., ZHANG, L., ARANGO, N. A., TEIXEIRA, J. & PRU, J. K. 2013. Mesenchymal-
847 to-epithelial transition contributes to endometrial regeneration following natural
848 and artificial decidualization. *Stem Cells Dev*, 22, 964-74.
- 849 PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-
850 PCR. *Nucleic Acids Res*, 29, e45.
- 851 POLANSKI, L. T., BAUMGARTEN, M. N., QUENBY, S., BROSENS, J., CAMPBELL, B. K. & RAINE-
852 FENNING, N. J. 2014. What exactly do we mean by 'recurrent implantation failure'? A
853 systematic review and opinion. *Reprod Biomed Online*, 28, 409-23.
- 854 RAINE-FENNING, N. J., CAMPBELL, B. K., CLEWES, J. S., KENDALL, N. R. & JOHNSON, I. R.
855 2004. Defining endometrial growth during the menstrual cycle with three-
856 dimensional ultrasound. *BJOG*, 111, 944-9.
- 857 RAJAGOPALAN, S. & LONG, E. O. 2012. Cellular senescence induced by CD158d reprograms
858 natural killer cells to promote vascular remodeling. *Proc Natl Acad Sci U S A*, 109,
859 20596-601.
- 860 RUAN, Y. C., GUO, J. H., LIU, X., ZHANG, R., TSANG, L. L., DONG, J. D., CHEN, H., YU, M. K.,
861 JIANG, X., ZHANG, X. H., FOK, K. L., CHUNG, Y. W., HUANG, H., ZHOU, W. L. & CHAN,
862 H. C. 2012. Activation of the epithelial Na⁺ channel triggers prostaglandin E(2)
863 release and production required for embryo implantation. *Nat Med*, 18, 1112-7.
- 864 SALKER, M. S., NAUTIYAL, J., STEEL, J. H., WEBSTER, Z., SUCUROVIC, S., NICOU, M., SINGH, Y.,
865 LUCAS, E. S., MURAKAMI, K., CHAN, Y. W., JAMES, S., ABDALLAH, Y., CHRISTIAN, M.,
866 CROY, B. A., MULAC-JERICEVIC, B., QUENBY, S. & BROSENS, J. J. 2012. Disordered IL-
867 33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in
868 women with recurrent pregnancy loss. *PLoS One*, 7, e52252.
- 869 STUART, T., BUTLER, A., HOFFMAN, P., HAFEMEISTER, C., PAPALEXI, E., MAUCK, W. M., 3RD,
870 HAO, Y., STOECKIUS, M., SMIBERT, P. & SATIJA, R. 2019. Comprehensive Integration
871 of Single-Cell Data. *Cell*, 177, 1888-1902 e21.
- 872 TABIBZADEH, S. 1991. Induction of HLA-DR expression in endometrial epithelial cells by
873 endometrial T-cells: potential regulatory role of endometrial T-cells in vivo. *J Clin*
874 *Endocrinol Metab*, 73, 1352-9.
- 875 TABIBZADEH, S., SUN, X. Z., KONG, Q. F., KASNIC, G., MILLER, J. & SATYASWAROOP, P. G.
876 1993. Induction of a polarized micro-environment by human T cells and interferon-
877 gamma in three-dimensional spheroid cultures of human endometrial epithelial
878 cells. *Hum Reprod*, 8, 182-92.
- 879 TEWARY, S., LUCAS, E. S., FUJIHARA, R., KIMANI, P. K., POLANCO, A., BRIGHTON, P. J.,
880 MUTER, J., FISHWICK, K. J., DA COSTA, M., EWINGTON, L. J., LACEY, L., TAKEDA, S.,
881 BROSENS, J. J. & QUENBY, S. 2020. Impact of sitagliptin on endometrial
882 mesenchymal stem-like progenitor cells: A randomised, double-blind placebo-
883 controlled feasibility trial. *EBioMedicine*, 51, 102597.
- 884 THE GENE ONTOLOGY, C. 2019. The Gene Ontology Resource: 20 years and still GOing
885 strong. *Nucleic Acids Res*, 47, D330-D338.
- 886 TICCONI, C., PIETROPOLLI, A., D'IPPOLITO, S., CHIARAMONTE, C., PICCIONE, E., SCAMBIA, G.
887 & DI SIMONE, N. 2020. Time-to-Pregnancy in Women with Unexplained Recurrent
888 Pregnancy Loss: A Controlled Study. *Reprod Sci*, 27, 1121-1128.
- 889 TURCO, M. Y., GARDNER, L., HUGHES, J., CINDROVA-DAVIES, T., GOMEZ, M. J., FARRELL, L.,
890 HOLLINSHEAD, M., MARSH, S. G. E., BROSENS, J. J., CRITCHLEY, H. O., SIMONS, B. D.,
891 HEMBERGER, M., KOO, B. K., MOFFETT, A. & BURTON, G. J. 2017. Long-term,

892 hormone-responsive organoid cultures of human endometrium in a chemically
893 defined medium. *Nat Cell Biol*, 19, 568-577.

894 VAN DER VOORT, R., TAHER, T. E., WIELENGA, V. J., SPAARGAREN, M., PREVO, R., SMIT, L.,
895 DAVID, G., HARTMANN, G., GHERARDI, E. & PALS, S. T. 1999. Heparan sulfate-
896 modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal
897 transduction through the receptor tyrosine kinase c-Met. *J Biol Chem*, 274, 6499-
898 506.

899 VAN DEURSEN, J. M. 2014. The role of senescent cells in ageing. *Nature*, 509, 439-46.

900 VENTO-TORMO, R., EFREMOVA, M., BOTTING, R. A., TURCO, M. Y., VENTO-TORMO, M.,
901 MEYER, K. B., PARK, J. E., STEPHENSON, E., POLANSKI, K., GONCALVES, A., GARDNER,
902 L., HOLMQVIST, S., HENRIKSSON, J., ZOU, A., SHARKEY, A. M., MILLAR, B., INNES, B.,
903 WOOD, L., WILBREY-CLARK, A., PAYNE, R. P., IVARSSON, M. A., LISGO, S., FILBY, A.,
904 ROWITCH, D. H., BULMER, J. N., WRIGHT, G. J., STUBBINGTON, M. J. T., HANIFFA, M.,
905 MOFFETT, A. & TEICHMANN, S. A. 2018. Single-cell reconstruction of the early
906 maternal-fetal interface in humans. *Nature*, 563, 347-353.

907 WEIMAR, C. H., KAVELAARS, A., BROSENS, J. J., GELLERSEN, B., DE VREEDEN-ELBERTSE, J. M.,
908 HEIJNEN, C. J. & MACKLON, N. S. 2012. Endometrial stromal cells of women with
909 recurrent miscarriage fail to discriminate between high- and low-quality human
910 embryos. *PLoS One*, 7, e41424.

911 YU, Y., FANG, L., WANG, S., LI, Y., GUO, Y. & SUN, Y. P. 2019. Amphiregulin promotes
912 trophoblast invasion and increases MMP9/TIMP1 ratio through ERK1/2 and Akt
913 signal pathways. *Life Sci*, 236, 116899.

914 ZHENG, J., QU, D., WANG, C., DING, L. & ZHOU, W. 2020. Involvement of CXCL12/CXCR4 in
915 the motility of human first-trimester endometrial epithelial cells through an
916 autocrine mechanism by activating PI3K/AKT signaling. *BMC Pregnancy Childbirth*,
917 20, 87.

918 ZHOU, Q., YAN, G., DING, L., LIU, J., YU, X., KONG, S., ZHANG, M., WANG, Z., LIU, Y., JIANG,
919 Y., KONG, N., SUN, J. & SUN, H. 2019. EHD1 impairs decidualization by regulating the
920 Wnt4/beta-catenin signaling pathway in recurrent implantation failure.
921 *EBioMedicine*, 50, 343-354.

922 ZHU, Y., TCHKONIA, T., PIRTSKHALAVA, T., GOWER, A. C., DING, H., GIORGADZE, N., PALMER,
923 A. K., IKENO, Y., HUBBARD, G. B., LENBURG, M., O'HARA, S. P., LARUSSO, N. F.,
924 MILLER, J. D., ROOS, C. M., VERZOSA, G. C., LEBRASSEUR, N. K., WREN, J. D., FARR, J.
925 N., KHOSLA, S., STOUT, M. B., MCGOWAN, S. J., FUHRMANN-STROISSNIGG, H.,
926 GURKAR, A. U., ZHAO, J., COLANGELO, D., DORRONSORO, A., LING, Y. Y.,
927 BARGHOOUTHY, A. S., NAVARRO, D. C., SANO, T., ROBBINS, P. D., NIEDERNHOFER, L. J.
928 & KIRKLAND, J. L. 2015. The Achilles' heel of senescent cells: from transcriptome to
929 senolytic drugs. *Aging Cell*, 14, 644-58.

930

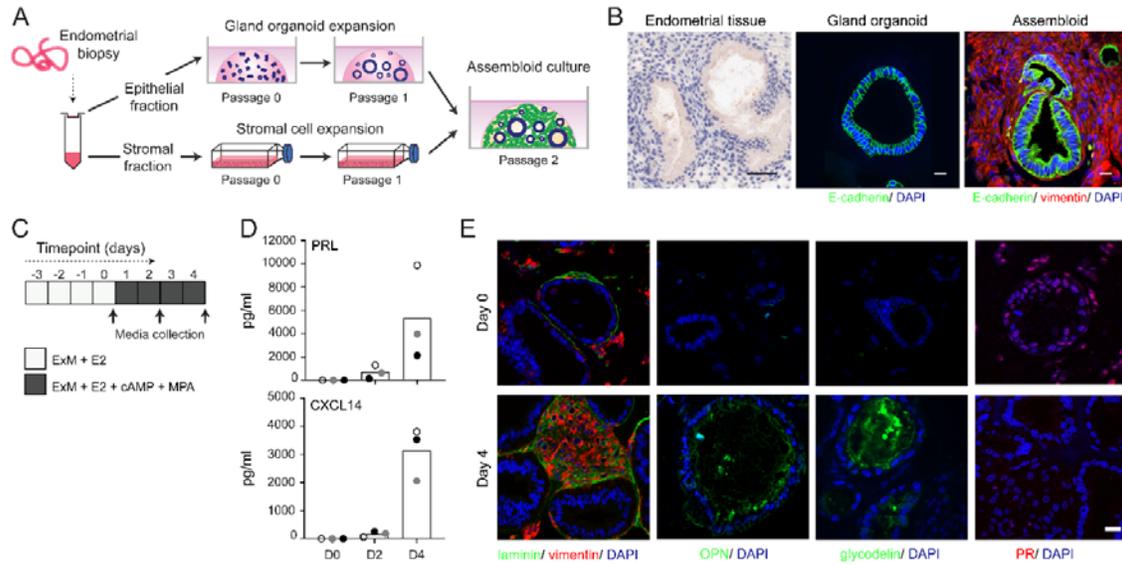


Figure 1: Establishment of endometrial assembloids.

(A) Schematic for establishing endometrial assembloids. (B) Structural appearance of hematoxylin and eosin stained secretory endometrium, E-cadherin labelled gland organoids, and E-cadherin and vimentin stained endometrial assembloids. Scale bar = 50 μ m. (C) Schematic summary of experimental design. (D) Secreted levels of PRL and CXCL14 were measured by ELISA in spent medium at the indicated timepoints. Data points are coloured to indicate secretion in assembloids established from different endometrial biopsies (n=3). (E) Representative immunofluorescence labelling of laminin and vimentin, progesterone receptor (PR), glycodeilin and osteopontin (OPN) in undifferentiated (Day 0, top panels) and decidualised (Day 4; bottom panels) assembloids. Nuclei were counterstained with DAPI. Scale bar = 50 μ m. ELISA data in Panel B are available in Figure 1-Source Data 1.

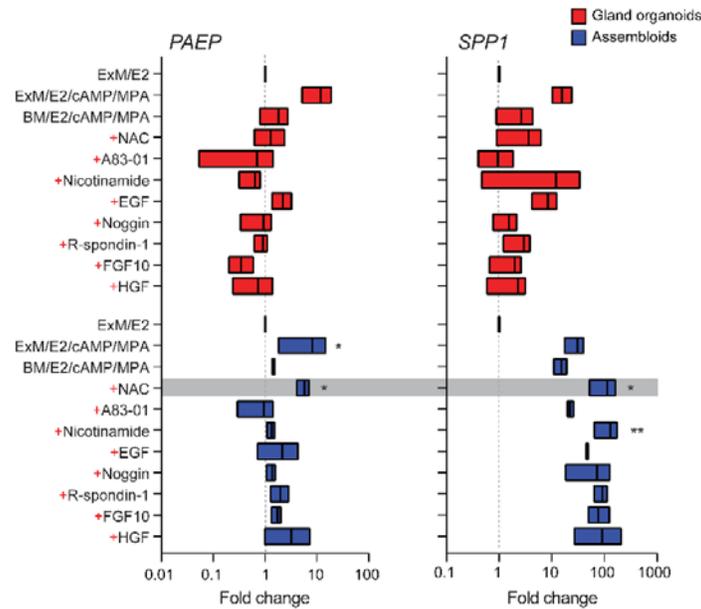


Figure 2: Characterisation of a minimal differentiation medium for endometrial assembloids. Parallel gland organoids (blue) and assembloids (red) were established from 3 endometrial biopsies and decidualized with 8-bromo-cAMP and MPA for 4 days in either expansion medium (ExM), base medium (BM) or BM with each exogenous factor added back individually (+). Induction of *PAEP* and *SPP1* was used to monitor the glandular differentiation. The grey bar indicates the composition of the minimal differentiation medium selected for further use (BM supplemented with NAC, E2, cAMP, and MPA). Data are presented as fold-change relative to expression levels in undifferentiated organoids or assembloids cultured in ExM+E2. Bars present minimal, maximal, and median fold-change. * and ** indicate $P < 0.05$ and $P < 0.01$ obtained by Friedman's test for matched samples. Relative expression values for biological replicates are available in Figure 2-Source Data 2.

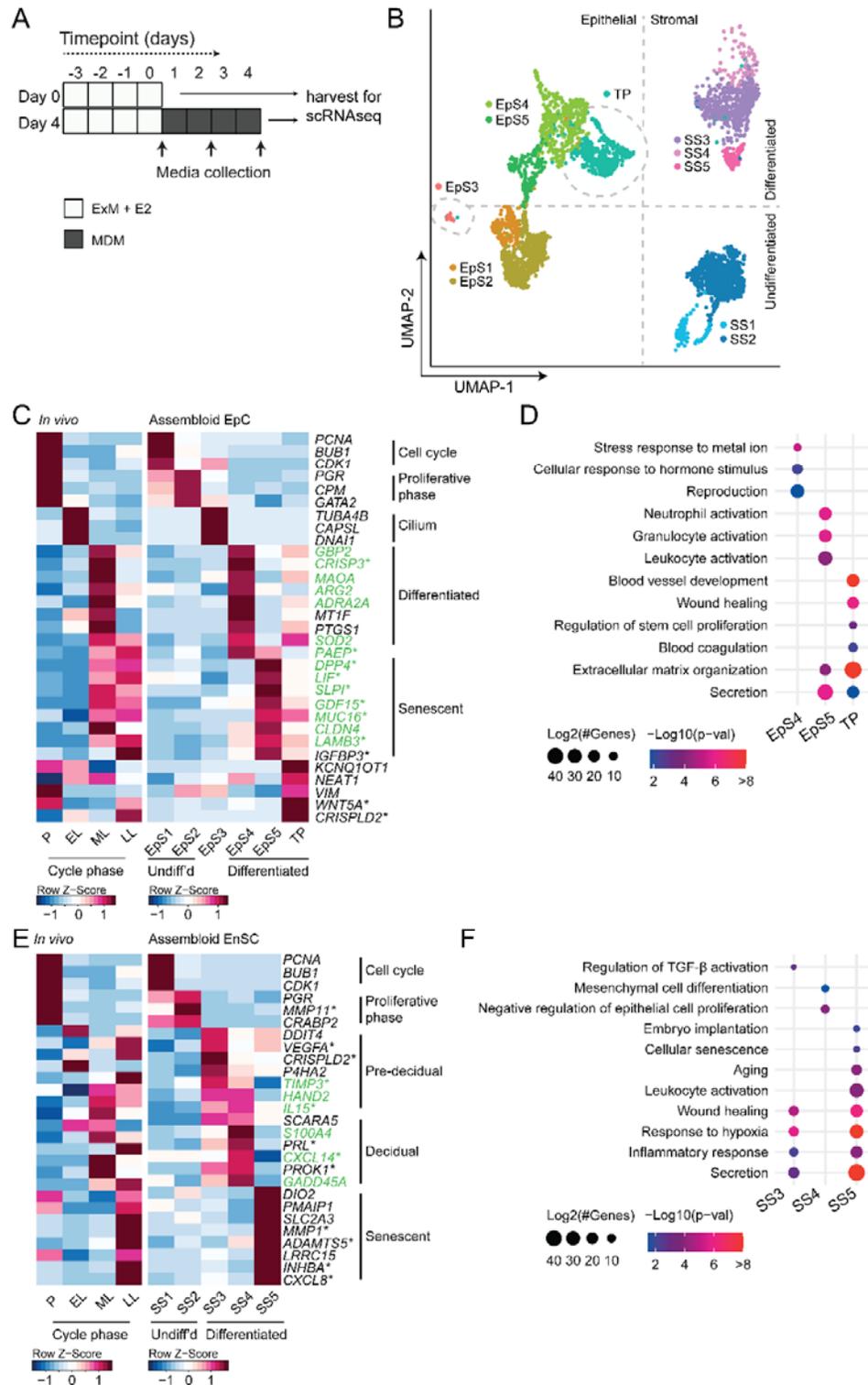


Figure 3: Decidualizing assembloids mimic midluteal endometrium

(A) Schematic overview of experimental design. ExM, expansion medium; MDM, minimal differentiation medium. (B) UMAP visualizing epithelial and stromal subsets (EpS and SS,

respectively) identified by single-cell transcriptomic analysis of undifferentiated and decidualized assembloids. A transitional population (TP) consisting of cells expressing epithelial and stromal markers is also shown. Dotted lines indicate the separation of EpS and SS in UMAP_1 and of undifferentiated and differentiated subpopulations in UMAP_2. (C) Composite heatmaps showing relative expression (Z-scores) of epithelial marker genes across the menstrual cycle *in vivo* and in undifferentiated and decidualized assembloids. Highlighted in green are genes that mark the midluteal window of implantation whereas genes encoding secreted proteins are indicated by *. See also Figure 3-figure supplement 1. (D) Dot plots showing GO terms related to biological processes enriched in different epithelial populations in decidualizing assembloids. The dot size represents the number of genes in each GO term and the colour indicates FDR-corrected *P*-value. (E) Composite heatmaps showing relative expression (Z-scores) of stromal marker genes across the menstrual cycle *in vivo* and in undifferentiated and decidualized assembloids. Highlighted in green are genes that mark the midluteal window of implantation whereas genes encoding secreted proteins are indicated by *. (F) Dot plots showing GO terms related to biological processes enriched in different stromal subpopulations in decidualizing assembloids. See also Figure 3-figure supplements 1, 2 and 3. Complete epithelial subpopulation marker lists can be found in Figure 3-Source Data 1. GO analysis outputs can be found in Figure 3-Source Data 2. Complete stromal subpopulation marker lists can be found in Figure 3-Source Data 3.

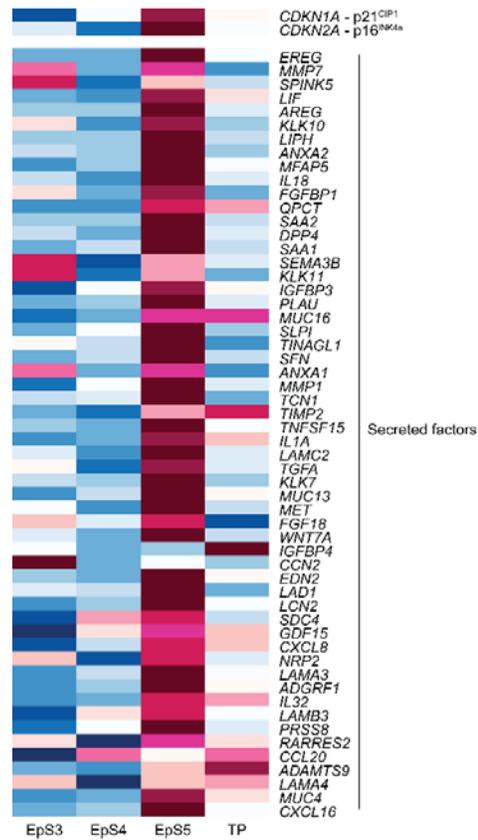


Figure 3–figure supplement 1:

Heatmap showing relative expression (Z-scores) of genes encoding the cyclin-dependent kinase inhibitors p16^{INK4a} and p21^{CIP1} as well as SASP-related genes in epithelial and transitional subpopulations in decidualizing assembloids. Supports Figure 3C.

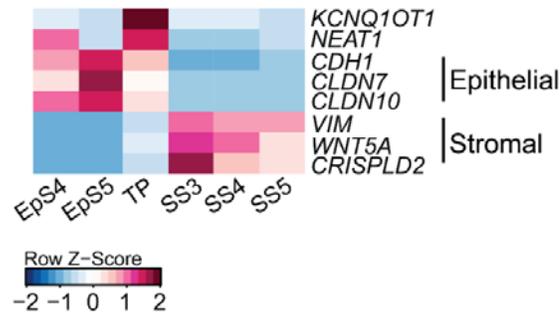


Figure 3—figure supplement 2:

Heatmap showing relative expression (Z-scores) of EMT/MET, epithelial and mesenchymal marker genes in the transitional population (TP), epithelial (EpS4-5) and stromal (SS3-5) subpopulations in decidualizing assembloids. Supports Figure 3C.

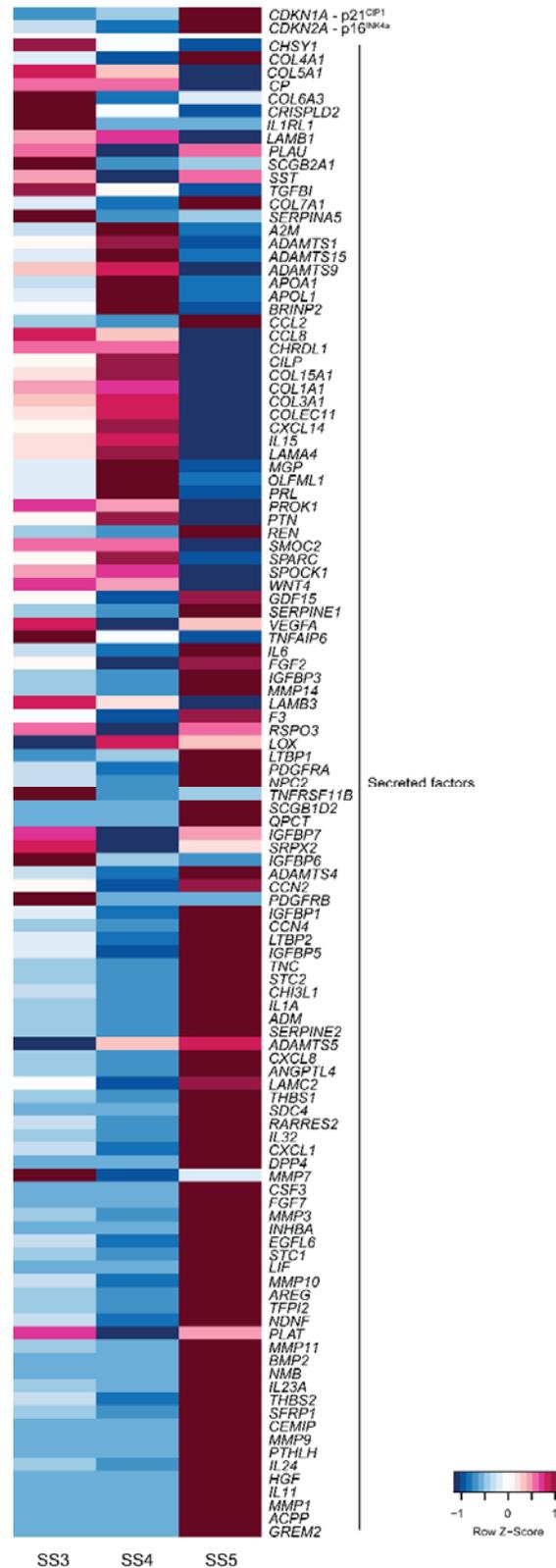


Figure 3–figure supplement 3:

Heatmap showing relative expression (Z-scores) of genes encoding the cyclin-dependent kinase inhibitors p16^{INK4a} and p21^{CIP1} as well as secretory and SASP-related genes in stromal subpopulations (SS3-5) in decidualizing assembloids. Supports Figure 3D.

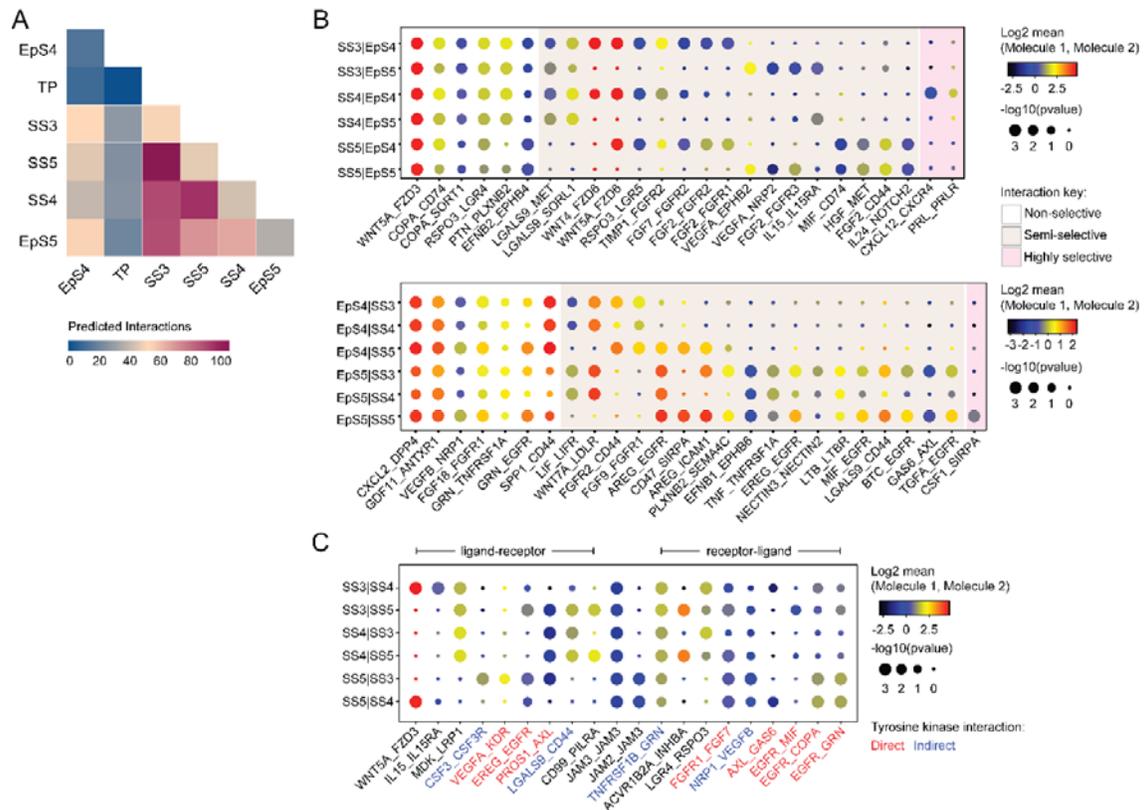


Figure 4: Putative receptor-ligand interactions in decidualizing assembloids

(A) Heatmap showing the total number of cell-cell interactions predicted by CellPhoneDB between different subpopulations in decidualizing assembloids. (B) Dot plots of representative ligand-receptor interactions between SS and EpS (upper panel) and EpS and SS (lower panel) in decidualizing assembloids. Circle size and colour indicate P -value and the means of the average expression value of the interacting molecules, respectively. Shaded boxes were used to group putative interactions by level of selectivity. (C) Dot plot of representative ligand-receptor and receptor-ligand interactions between stromal subpopulations in decidualizing assembloids. Direct and indirect tyrosine kinase interactions are indicated by red and blue labels, respectively. Complete tables of predicted ligand-receptor interactions can be found in Figure 4-Source Data 1.

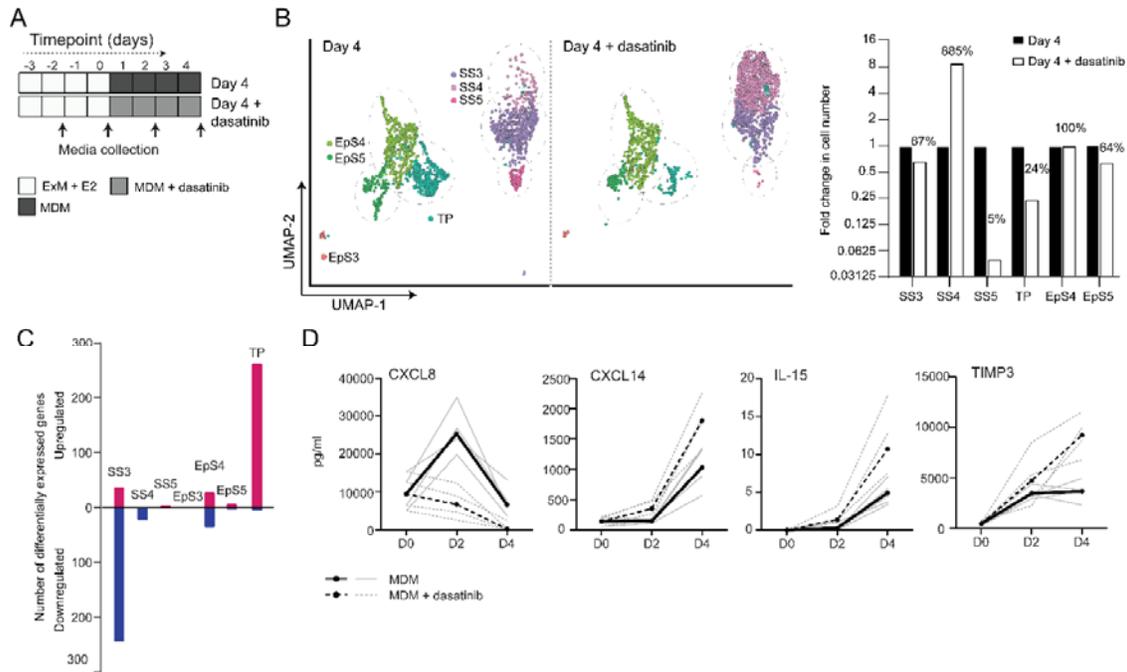


Figure 5: Tyrosine kinase-dependent stress responses determines the fate of decidual cells

(A) Schematic overview of experimental design. ExM, expansion medium; MDM, minimal differentiation medium. (B) UMAP visualisation (left panel) and relative proportions (right panel) of subpopulations in endometrial assembloid decidualized in the presence or absence of dasatinib. (C) Number of differentially expressed genes (DEG) in each subpopulation in response to dasatinib pretreatment. (D) Secreted levels of CXCL8 and decidual cell factors in spent medium from assembloids treated with or without dasatinib. Secreted levels in individual assembloids established from 4 different endometrial assembloids decidualized with or without dasatinib are shown by dotted and solid lines, respectively. Full lists of differentially expressed genes and associated gene ontology analysis can be found in Figure 5-Source Data 1 and 2, respectively. Data used in Panel D are available in Figure 5-Source Data 3

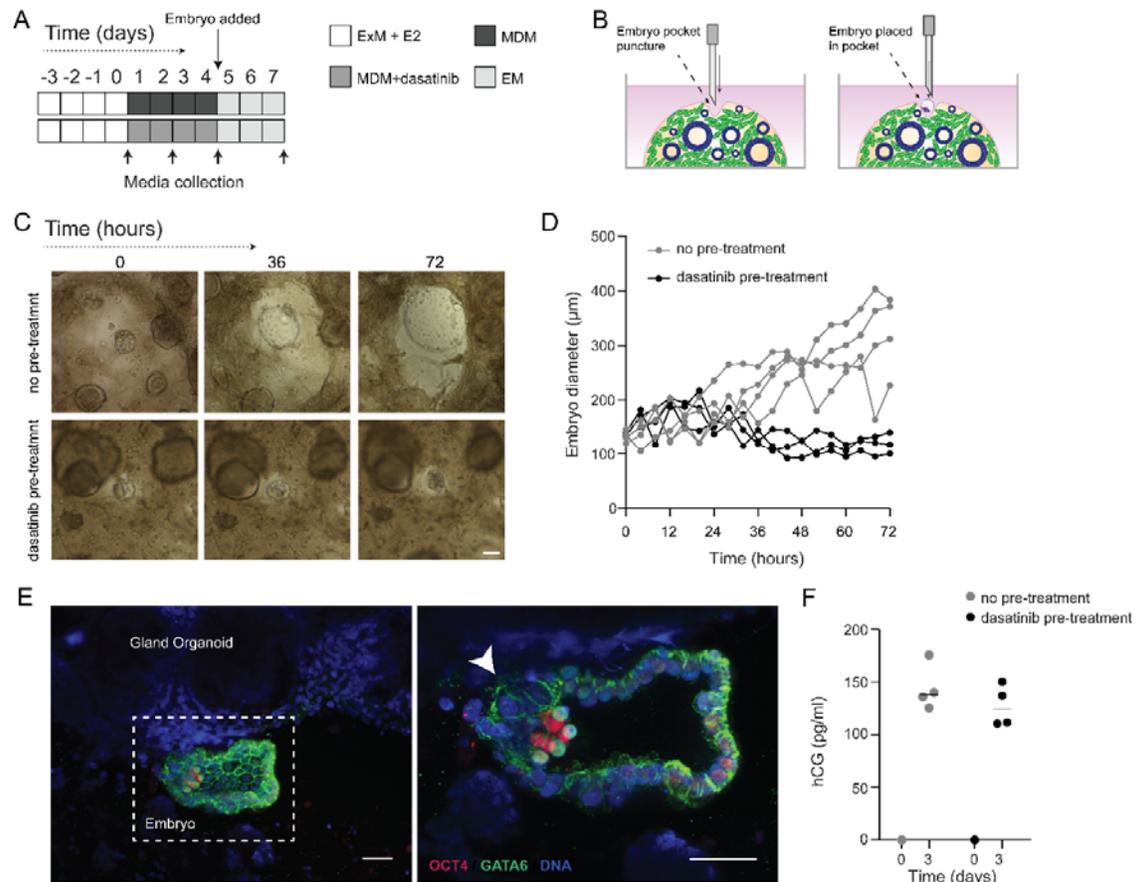


Figure 6: Impact of decidual senescence in assembloids on co-cultured human blastocysts

(A) Diagram showing experimental design. ExM, expansion medium; MDM, minimal differentiation medium; EM, embryo medium. (B) Schematic drawing of co-culture method. (C) Representative time-lapse images of blastocysts embedded in assembloids following decidualization for 96 hours in the absence (upper panels) or presence (lower panels) of dasatinib. See also Figure 6–figure supplement 1. (D) Expansion of embryos over 72 hours when embedded in decidualizing assembloids pre-treated with or without dasatinib. Scale bar = 100 μm . (E) OCT4 and GATA6 immunofluorescence marking the epiblast and hypoblast, respectively, in a blastocyst attached by proliferating polar trophoblast (arrowhead) to decidual assembloids. Scale bar = 50 μm . (F) Secreted levels of hCG in blastocyst-endometrial assembloid co-cultures. Individual embryo diameter measurements for biological replicates in Panel D are available in Figure 6-Source Data 1. Individual ELISA data used in Panel F are available in Figure 6-Source Data 2.

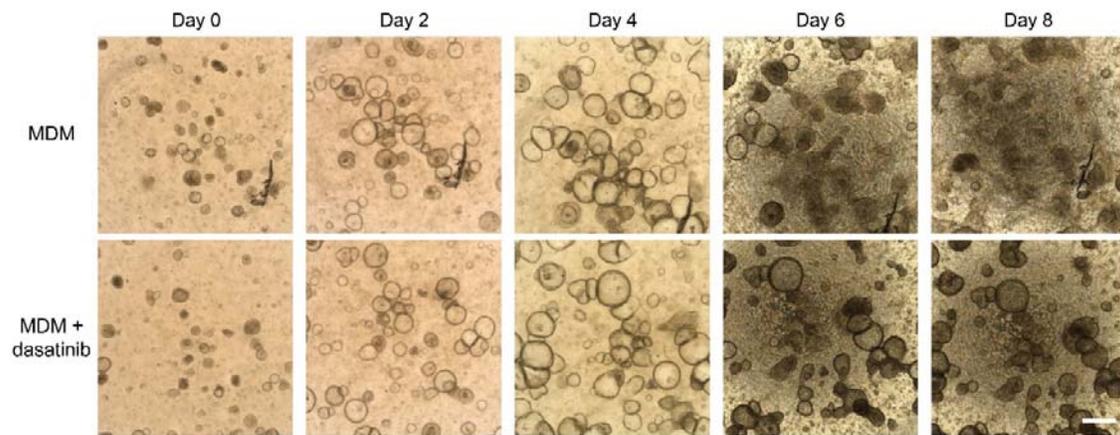


Figure 6—figure supplement 1: dasatinib prevents disintegration of decidualizing assembloids

Time-course images of endometrial assembloids in minimal differentiation medium (MDM) supplemented or not with dasatinib. Scale bar = 50 μm .