

1 **Clearance of senescent decidual cells by uterine natural killer cells drives endometrial**  
2 **remodeling during the window of implantation**

3  
4 Paul J Brighton<sup>1\*</sup>, Yojiro Maruyama<sup>1,2\*</sup>, Katherine Fishwick<sup>1</sup>, Pavle Vrljicak<sup>1,3</sup>, Shreeya  
5 Tewary<sup>1,3</sup>, Risa Fujihara<sup>1,2</sup>, Joanne Muter<sup>1</sup>, Emma S Lucas<sup>1,3</sup>, Taihei Yamada<sup>2</sup>, Laura Woods<sup>4,5</sup>,  
6 Raffaella Lucciola<sup>1</sup>, Yie Hou Lee<sup>6,7</sup>, Satoru Takeda<sup>2</sup>, Sascha Ott<sup>3</sup>, Myriam Hemberger<sup>4,5</sup>,  
7 Siobhan Quenby<sup>1,2</sup>, and Jan J Brosens<sup>1,3,8</sup>

8  
9 <sup>1</sup>Division of Biomedical Sciences, Clinical Science Research Laboratories, Warwick Medical  
10 School, University of Warwick, Coventry CV2 2DX, UK.

11 <sup>2</sup>Department of Obstetrics and Gynecology, Juntendo University Faculty of Medicine, Tokyo,  
12 113-8421, Japan.

13 <sup>3</sup>Tommy's National Centre for Miscarriage Research, University Hospitals Coventry &  
14 Warwickshire, Coventry CV2 2DX, UK

15 <sup>4</sup>Centre for Trophoblast Research, University of Cambridge, Downing Street, Cambridge CB2  
16 3EG, UK

17 <sup>5</sup>Epigenetics Programme, The Babraham Institute, Babraham Research Campus, Cambridge  
18 CB22 3AT, UK

19 <sup>6</sup>Obstetrics & Gynaecology -Academic Clinical Program, Duke-NUS Medical School,  
20 Singapore.

21 <sup>7</sup>KK Research Centre, KK Women's and Children's Hospital, Singapore.

22 <sup>8</sup>Corresponding Author and Lead Contact: Jan Brosens M.D., Ph.D. Clinical Science Research  
23 Laboratories, Warwick Medical School, University of Warwick, Coventry CV2 2DX, UK. Tel:  
24 +44 2476968704; FAX: +44 2476968653; Email: [J.J.Brosens@warwick.ac.uk](mailto:J.J.Brosens@warwick.ac.uk)

25 \*These authors contributed equally.

26 **Summary**

27 In cycling human endometrium, menstruation is followed by rapid estrogen-dependent growth.  
28 Upon ovulation, progesterone and rising cellular cAMP levels activate the transcription factor  
29 Forkhead box O1 (FOXO1) in endometrial stromal cells (EnSCs), leading to cell cycle exit and  
30 differentiation into decidual cells that control embryo implantation. Here we show that FOXO1  
31 also causes acute senescence of a subpopulation of decidualizing EnSCs in an IL-8 dependent  
32 manner. Selective depletion or enrichment of this subpopulation revealed that decidual  
33 senescence drives the transient inflammatory response associated with endometrial receptivity.  
34 Further, senescent cells prevent differentiation of endometrial mesenchymal stem cells in  
35 decidualizing cultures. As the cycle progresses, IL-15 activated uterine natural killer (uNK)  
36 cells selectively target and clear senescent decidual cells through granule exocytosis. Our  
37 findings reveal that acute decidual senescence governs endometrial rejuvenation and  
38 remodeling at embryo implantation, and suggest a critical role for uNK cells in maintaining  
39 homeostasis in cycling endometrium.

40

41 **Key words:** endometrium, decidualization, senescence, uterine natural killer cells,  
42 homeostasis, implantation, senescence-associated secretory phenotype, IL-8, FOXO, granule  
43 exocytosis, senolytics.

## 44 **Introduction**

45 Different mammalian species employ divergent strategies to ensure successful embryo  
46 implantation. In mice, synchronized implantation of multiple embryos (average 6-8) is  
47 dependent on a transient rise in circulating estradiol (E2) that not only renders the progesterone-  
48 primed endometrium receptive, but also activates dormant blastocysts for implantation (Paria  
49 et al., 1998). Upon breaching of the uterine luminal epithelium, implanting murine embryos  
50 trigger extensive remodeling of the endometrial stromal compartment. This process, termed  
51 decidualization, is characterized by local edema, influx of uNK cells and differentiation of  
52 stromal fibroblasts into specialized decidual cells that coordinate trophoblast invasion and  
53 placenta formation (Gellersen and Brosens, 2014; Zhang et al., 2013). Likewise, the human  
54 endometrium transiently expresses a receptive phenotype, lasting 2-4 days, during the mid-  
55 luteal phase of the cycle. However, this implantation window is not controlled by a nidatory  
56 E2 surge (de Ziegler et al., 1992; Groll et al., 2009), perhaps reflecting that synchronized  
57 implantation of multiple human embryos is neither required nor desirable. Further,  
58 decidualization of the stromal compartment is not dependent on an implanting embryo but  
59 initiated during the mid-luteal phase of each cycle in response to elevated circulating  
60 progesterone levels and increased intracellular cAMP production (Gellersen and Brosens,  
61 2014). In parallel, CD56<sup>bright</sup> CD16<sup>-</sup> uNK cells accumulate in luteal phase endometrium. In  
62 pregnancy, uNK cells exert an evolutionarily conserved role in orchestrating vascular  
63 adaptation and trophoblast invasion (Hanna et al., 2006; Xiong et al., 2013), but their function  
64 in cycling human endometrium is unclear.

65        Differentiation of human endometrial stromal cells (EnSCs) into decidual cells is a  
66 multistep process (Gellersen and Brosens, 2014). Following cell cycle exit at G0/G1,  
67 decidualizing EnSCs first mount a transient pro-inflammatory response, characterized by a  
68 burst of free radical production and secretion of various chemokines and other inflammatory

69 mediators (Al-Sabbagh et al., 2011; Lucas et al., 2016b; Salker et al., 2012). Exposure of the  
70 mouse uterus to this inflammatory secretome activates multiple receptivity genes, suggesting  
71 that the nidatory E2 surge in mice is supplanted by an endogenous inflammatory signal in the  
72 human uterus. Feedback loops purportedly limit the inflammatory decidual response to 2-4  
73 days (Salker et al., 2012). The next decidual phase coincides with embedding of the implanted  
74 embryo into the stroma. At this stage, fully differentiated decidual cells, which are now tightly  
75 adherent and possess gap junctions (Laws et al., 2008), form an immune privileged matrix  
76 around the semi-allogenic conceptus (Erlebacher, 2013). In the absence of implantation, falling  
77 progesterone levels trigger a second inflammatory decidual response which, upon recruitment  
78 and activation of leukocytes, leads to tissue breakdown, focal bleeding and menstrual shedding  
79 of the superficial endometrial layer. Scar-free tissue repair involves activation of mesenchymal  
80 stem-like cells (MSCs) and epithelial progenitor cells that reside in the basal layer (Evans et  
81 al., 2016). Following menstruation, rising follicular E2 levels drive rapid tissue growth, which  
82 over ~10 days increases the thickness of the endometrium several-fold. Clinically, suboptimal  
83 endometrial growth is strongly associated with reproductive failure (Yuan et al., 2016); but  
84 how MSC activation followed by intense proliferation is linked to the decidual process is  
85 unclear.

86 FOXO1 is a core decidual transcription factor that controls cell cycle exit of EnSCs in  
87 response to differentiation signals and activates expression of decidual marker genes, such as  
88 *PRL* and *IGFBP1* (Park et al., 2016; Takano et al., 2007). Here we demonstrate that FOXO1  
89 also induces acute senescence in a subpopulation of EnSCs. We show that the senescence-  
90 associated secretory phenotype (SASP) drives the initial auto-inflammatory decidual response  
91 linked to endometrial receptivity and provide evidence that uNK cells target and eliminate  
92 senescent decidual cells as the cycle progresses. Our findings reveal a hitherto unrecognized  
93 role for acute cellular senescence in endometrial remodeling at the time of embryo

94 implantation; and suggest a major role for uNK cells in maintaining tissue homeostasis from  
95 cycle to cycle.

96

## 97 **Results**

### 98 **Decidualization induces acute senescence in a subpopulation of EnSCs**

99 To determine if cycling human endometrium harbor dynamic populations of senescent cells,  
100 we first stained primary EnSC cultures for senescence-associated  $\beta$ -galactosidase (SA $\beta$ G)  
101 activity. At passage 1 (P1), SA $\beta$ G<sup>+</sup> cells were detectable in variable numbers in different  
102 cultures (Figure 1A). Strikingly, the number of SA $\beta$ G<sup>+</sup> cells increased markedly upon  
103 decidualization with 8-bromo-cAMP and medroxyprogesterone acetate (MPA, a progestin).  
104 Typically, SA $\beta$ G<sup>+</sup> cells formed islets surrounded by SA $\beta$ G<sup>-</sup> EnSCs in differentiating cultures  
105 (Figure 1A). Quantitative analysis confirmed a time-dependent increase in SA $\beta$ G activity upon  
106 decidualization (Figure 1B). The abundance of SA $\beta$ G<sup>+</sup> cells in undifferentiated cultures  
107 declined upon passaging of cells (Figure S1A). Initially, this was paralleled by a reduction in  
108 SA $\beta$ G activity, which was reversed at later passages (P6) (Figure S1B), presumably reflecting  
109 emerging replicative exhaustion of EnSCs (Figure S1C). However, even after ~60 days in  
110 continuous culture, exposure of EnSCs to a decidualogenic stimulus enhanced SA $\beta$ G activity  
111 and triggered the appearance of SA $\beta$ G<sup>+</sup> cells (Figure S1A and S1B).

112 Although SA $\beta$ G activity is a commonly used biomarker for senescent cells, it lacks  
113 specificity (Matjusaitis et al., 2016). Hence, we examined the expression of other putative  
114 senescence markers in undifferentiated and decidualizing EnSCs. Many senescence signals  
115 converge onto the tumor suppressor protein p53 (p53) and induce the expression of cyclin-  
116 dependent kinase (CDK) inhibitors, leading to proliferative arrest and cell cycle exit (Munoz-  
117 Espin and Serrano, 2014; van Deursen, 2014). We reported previously that downregulation of  
118 MDM2 proto-oncogene, an E3 ubiquitin ligase, stabilizes p53 in differentiating EnSCs

119 (Pohnke et al., 2004). Western blot analysis showed that p53 stabilization upon decidualization  
120 is paralleled by upregulation of p16<sup>Ink4a</sup> (p16; Figure 1C), a CDK inhibitor presumed specific  
121 for senescence. Notably, confocal microscopy revealed that induction of p16 upon  
122 decidualization is confined to a subpopulation of EnSCs (Figure 1D). Loss of lamin B1  
123 (LMNB1) and high mobility group box 2 (HMGB2) drives many of the chromatin and  
124 epigenetic changes that underpin cellular senescence (Aird et al., 2016; Sadaie et al., 2013).  
125 Decidualization resulted in downregulation of both effector proteins and a reciprocal increase  
126 in the histone H2A variant macroH2A (mH2A) and trimethylated lysine 9 on histone H3  
127 (H3K9Me3) (Figures 1C and S1E). This histone variant and modification are involved in  
128 senescence-associated heterochromatin formation (SAHF). Unexpectedly, the nucleosome  
129 linker histone H1 (H.H1), which purportedly is lost in senescent cells (Funayama et al., 2006),  
130 was upregulated upon decidualization (Figures 1C and S1E). These observations were  
131 confirmed by immunofluorescence confocal microscopy (Figure 1E, left panel), which also  
132 revealed that the nuclei of EnSC increase in size (~ 40%) upon decidualization (Figure 1E,  
133 right panel). Next, we examined if the transient inflammatory decidual response also  
134 encompasses secreted factors typical of the canonical senescence associated secretory  
135 phenotype (SASP). As shown in Figure 1F, secretion of IL-8 (CXCL8), GRO $\alpha$  (CXCL1), and  
136 IL-6 peaked transiently on day 2 of decidualization and returned to baseline by day 4. By day  
137 8, the level of secretion of GRO $\alpha$  and IL-6 was lower than that observed in undifferentiated  
138 cultures.

139 Taken together, the data reveal striking similarities between cellular senescence and  
140 differentiation of EnSCs into decidual cells. However, only a subpopulation of EnSCs became  
141 strongly SA $\beta$ G<sup>+</sup> or expressed p16 upon decidualization. Further, while SASP is often a  
142 sustained response, decidual inflammation is temporally restricted to 2-4 days.

143

144 **Temporal regulation of senescent cell populations in cycling endometrium**

145 To extrapolate these observations to the *in vivo* situation, protein lysates from whole  
146 endometrial biopsies were subjected to Western blot analysis. As the cycle progresses from the  
147 proliferative to the secretory phase, the abundance of p53, p16, LMNB1, HMBG2, mH2A and  
148 H3K9me3 in the endometrium mimicked the changes observed in decidualizing EnSC cultures  
149 (Figure 2A). Further, analysis of snap-frozen biopsies showed a sharp increase in SA $\beta$ G  
150 activity upon transition from proliferative to early-secretory endometrium with levels peaking  
151 in the late-luteal phase (Figure 2B). Disintegration of the stromal compartment upon  
152 cryosectioning of frozen tissue samples precluded a meaningful analysis of SA $\beta$ G<sup>+</sup> cells.  
153 Instead, we used immunohistochemistry to examine the abundance and tissue distribution of  
154 p16<sup>+</sup> cells in 308 formalin-fixed endometrial biopsies obtained during the peri-implantation  
155 window, i.e. 6 to 12 days after the luteinizing hormone (LH) surge (Figure 2C). The statistical  
156 distribution of p16<sup>+</sup> cells in the glandular epithelium, luminal epithelium and stromal  
157 compartment is presented as a centile graph (Figure 2D). Interesting, p16<sup>+</sup> cells were most  
158 prevalent in both the glandular and luminal epithelium at LH+10 and +11, which coincides  
159 with the onset of the late-luteal phase of the cycle. The relative abundance of p16<sup>+</sup> cells was  
160 ~10-fold higher in the luminal compared to the glandular compartment. Typically, stretches of  
161 p16<sup>+</sup> cells were interspersed by p16<sup>-</sup> cells in the luminal epithelium (Figure 2C). By contrast,  
162 p16<sup>+</sup> cells gradually increased in the stromal compartment during the mid-luteal phase and this  
163 was accelerated in late-luteal endometrium. Occasionally, swirls of p16<sup>+</sup> cells were observed  
164 in the stroma, seemingly connecting the deeper regions of the endometrium to p16<sup>+</sup> luminal  
165 epithelial cells (Figure 2C). Collectively, the data indicate that the endometrium harbors  
166 dynamic and probably spatially organized populations of senescent cells during the luteal phase  
167 of the cycle.

168

## 169 **FOXO1 drives EnSC differentiation and senescence**

170 To gain insight into the mechanism that drives decidual senescence, SA $\beta$ G activity was  
171 measured in cultured EnSCs treated for 8 days with either 8-bromo-cAMP, MPA or a  
172 combination. Both 8-bromo-cAMP and MPA were required for significant induction of SA $\beta$ G  
173 activity ( $P < 0.05$ ) (Figure 3A). In differentiating EnSCs, cAMP and progesterone signaling  
174 converge on FOXO1, a core decidual transcription factor responsible for cell cycle arrest and  
175 induction of decidual marker genes, such as *PRL* and *IGFBP1* (Takano et al., 2007).  
176 Interestingly, FOXO1 was also shown to induce cellular senescence of ovarian cancer cells  
177 treated with progesterone (Diep et al., 2013). siRNA-mediated knockdown of FOXO1 in  
178 EnSCs not only abolished the induction of *PRL* and *IGFBP1* (Figure S2A) but also the surge  
179 in IL-8, GRO $\alpha$ , and IL-6 secretion upon treatment of cultures with 8-bromo-cAMP and MPA  
180 (Figure 3B). After 8 days of decidualization, FOXO1 knockdown was less efficient but  
181 nevertheless sufficient to significantly blunt SA $\beta$ G activity ( $P < 0.05$ ) (Figure 3C). Several  
182 components of the SASP have been implicated in autocrine/paracrine propagation of  
183 senescence, including IL-8 acting on CXCR2 (IL-8 receptor type B) (Acosta et al., 2008). In  
184 agreement, decidualization of EnSCs in the presence of SB265610, a potent CXCR2 inhibitor,  
185 attenuated SA $\beta$ G activity in a dose-dependent manner (Figure 3D). Conversely, recombinant  
186 IL-8 upregulated SA $\beta$ G activity in undifferentiated EnSCs in a concentration-dependent  
187 manner (Figure 3D), although spatial organization of SA $\beta$ G<sup>+</sup> cells into ‘islets’ was not  
188 observed (Figure S2B). Further, siRNA-mediated *CXCL8* (coding IL-8) knockdown in  
189 undifferentiated EnSCs not only blunted the surge in IL-8 secretion upon decidualization  
190 (Figure S2B), but also abolished the increase in SA $\beta$ G activity (Figure 3E). Unexpectedly, IL-  
191 8 knockdown compromised the induction of *PRL* and *IGFBP1* in cultures treated with 8-  
192 bromo-cAMP and MPA (Figure 3F), indicating that autocrine/paracrine signals involved in  
193 EnSC differentiation also drive decidual senescence.



194 In an attempt to block decidual senescence selectively, EnSCs were differentiated in  
195 the presence or absence of the mTOR inhibitor rapamycin, a pharmacological repressor of  
196 replicative senescence (Demidenko et al., 2009). Rapamycin prevented expansion of SA $\beta$ G<sup>+</sup>  
197 cells upon decidualization but expression of *PRL* and *IGFBP1* was again compromised  
198 (Figures S2C and S2D). By contrast, withdrawal of 8-bromo-cAMP and MPA from cultures  
199 first decidualized for 8 days reversed the induction of decidual marker genes (Figure 3G), albeit  
200 without impacting on either SA $\beta$ G activity or expression of p53, p16, LMNB1 and HMGB1  
201 (Figure 3H). Taken together, the data demonstrate that FOXO1 drives both differentiation and  
202 senescence of distinct EnSC subpopulations in an IL-8 dependent manner; however, while  
203 expression of differentiation markers requires continuous cAMP and progestin signaling, the  
204 senescent phenotype does not.

205

### 206 **Pleiotropic functions of senescent decidual cells**

207 The abundance of SA $\beta$ G<sup>+</sup> cells in undifferentiated cultures correlated closely with the number  
208 of SA $\beta$ G<sup>+</sup> cells upon decidualization (Pearson's  $r = 0.97$ ,  $P < 0.0001$ ) (Figure S3A). A  
209 congruent correlation was apparent upon measuring SA $\beta$ G activity in paired undifferentiated  
210 and decidualizing cultures (Pearson's  $r = 0.91$ ,  $P < 0.0001$ ) (Figure 4A); inferring that senescent  
211 decidual cells arise from stressed (presenescent) EnSCs. Hence, we tested if decidualization-  
212 associated senescence could be blocked by pretreating undifferentiated cultures with senolytic  
213 drugs. Exposure of primary EnSCs for 72 h to increasing concentrations of ABT-263  
214 (Navitoclax), a BCL-X<sub>L</sub> inhibitor (Zhu et al., 2016), had no discernible impact on the induction  
215 of SA $\beta$ G activity upon decidualization (Figure S3B). By contrast, pretreatment of primary  
216 cultures with dasatinib, a broad-spectrum tyrosine kinase inhibitor (Childs et al., 2017; Zhu et  
217 al., 2015), inhibited SA $\beta$ G activity upon decidualization in a dose-dependent manner (Figure  
218 S3C). Conversely, to increase the abundance SA $\beta$ G<sup>+</sup> cells, undifferentiated EnSCs were treated

219 with the CDK4/CDK6 inhibitor palbociclib (PD0332991), a functional p16 mimetic (Mosteiro  
220 et al., 2016). Dose-response analyses showed that treatment with palbociclib for 4 days was  
221 sufficient to increase SA $\beta$ G activity in undifferentiated EnSCs to the level observed in cells  
222 decidualized with 8-bromo-cAMP and MPA for 8 days (Figure S3C). Notably, neither  
223 dasatinib nor palbociclib pretreatment impacted on the induction of *PRL* or *IGFBP1* upon  
224 decidualization (Figure 4C). However, dasatinib pretreatment markedly blunted the surge in  
225 IL-8, IL-6, and GRO $\alpha$  secretion that characterizes the initial decidual phase. By contrast, this  
226 auto-inflammatory decidual response was amplified in response to palbociclib pretreatment  
227 (Figure 4D).

228 In other cell systems, transient - but not prolonged - exposure to SASP has been shown  
229 to promote tissue rejuvenation by reprogramming committed cells into stem-like cells  
230 (Ritschka et al., 2017). We reasoned that SASP-dependent tissue rejuvenation during the  
231 window of implantation may be relevant for the transition of the cycling endometrium into the  
232 decidua of pregnancy. To test this hypothesis, we examined the clonogenic capacity of paired  
233 undifferentiated cells and cells decidualized for 8 days. Analysis of 12 primary cultures  
234 demonstrated decidualization is associated with a modest but significant increase in colony-  
235 forming cells ( $P < 0.05$ ), although the response varied between primary cultures (Figure 4E).  
236 However, pretreatment of undifferentiated cultures with dasatinib or palbociclib consistently  
237 increased and decreased the clonogenic capacity of decidualizing cultures, respectively (Figure  
238 4F). Likewise, rapamycin also depleted decidualizing EnSC cultures of clonogenic cells  
239 (Figure S3D). Taken together, the data suggest that senescent decidual cells produce a transient  
240 inflammatory environment that not only renders the endometrium receptive but also increases  
241 tissue plasticity prior to pregnancy.

242

243

## 244 **Immune clearance of senescent decidual cells**

245 Recognition and elimination of senescent cells by immune cells, especially NK cells, play a  
246 pivotal role in tissue repair and homeostasis (Iannello and Raulet, 2013; Krizhanovsky et al.,  
247 2008). During the luteal phase, uNK cells, characterized by their CD56<sup>bright</sup> cell surface  
248 phenotype (Figure S4A), are by far the dominant endometrial leukocyte population. Analysis  
249 of a large number of LH-timed endometrial biopsies (n = 1,997) demonstrated that the  
250 abundance of CD56<sup>+</sup> uNK cells in the endometrial stromal compartment increases on average  
251 3-fold between LH+5 and +12; although inter-patient variability was marked (Figure 5A, left  
252 panel). The heatmap in Figure 5A (right panel) depicts the uNK cell centiles across the peri-  
253 implantation window. Notably, uNK cells often appear to amass in edematous areas that are  
254 relatively depleted of stromal cells, especially during the transition from the mid- to late-luteal  
255 phase of the cycle (Figure 5B, left panel). Quantitative analysis of 20 biopsies obtained between  
256 LH+9 and LH+11 confirmed the inverse correlation between the density of uNK cells and  
257 endometrial stromal cells (Figure 5B, right panel). In co-culture, uNK cells isolated from  
258 secretory endometrium had no impact on proliferation or viability of undifferentiated EnSCs  
259 (Figure S4B, left panel). By contrast, co-culture of uNK cells with EnSCs first decidualized for  
260 8 days resulted in loss of cell viability. Visually, uNK cells transformed the monolayer of  
261 decidual cells into a honeycomb pattern with cell-free islets (Figure 5C).

262         These observations suggested that uNK cells actively eliminate senescent EnSCs but  
263 only upon decidualization. In agreement, co-culture of EnSCs with uNK eliminated the  
264 induction of SA $\beta$ G activity upon decidualization without affecting basal activity in  
265 undifferentiated cells (Figure 5D). Two independent mechanisms underpin NK cell-mediated  
266 clearance of stressed cells (Sagiv et al., 2013). First, binding of the NK cell surface ligands  
267 TRAIL and FAS ligand (FasL) to the corresponding receptors on target cells can lead to caspase  
268 activation and cell death. However, incubation of primary EnSCs with increasing

269 concentrations of FasL or TRAIL had no impact on SA $\beta$ G activity in either undifferentiated or  
270 decidualizing cells (Figure S4C), inferring that death receptor activation in uNK cells is not  
271 required for senolysis. The second mechanism involves secretion by activated NK cells of  
272 granules containing perforin and granzyme (A, B). Perforin forms pores in the plasma  
273 membrane of target cells and triggers apoptosis upon release of granzyme into the cytoplasm  
274 (Chowdhury and Lieberman, 2008). As shown in Figures 5D and S4B, both the pan-caspase  
275 inhibitor Z-VAD-FMK and the granzyme B inhibitor 3,4-Dichloroisocoumarin (3,4-DCI)  
276 negated the impact of uNK cells on SA $\beta$ G activity and cell viability in decidualizing cultures.  
277 To explore why uNK cell-mediated clearance of SA $\beta$ G<sup>+</sup> EnSCs is restricted to decidualizing  
278 cultures, we focused on IL-15, a pivotal cytokine that regulates NK cell proliferation and  
279 activation (Marcais et al., 2014). IL-15 secretion was below the level of detection in  
280 undifferentiated cells but, after a lag-period of 2 days, rose markedly upon decidualization of  
281 EnSCs in a time-dependent manner (Figure 5E). Notably, pretreatment of cultures with  
282 dasatinib or palbociclib had no impact on impact on IL-15 secretion, suggesting that decidual  
283 cells orchestrate the uNK-mediated clearance of their senescent counterparts (Figure 5E).  
284 Incubation of co-cultures with an IL-15 blocking antibody antagonized, at least partly, uNK  
285 cell-mediated clearance of senescent decidual cells (Figure 5F).

286

## 287 **Tissue homeostasis**

288 Our findings indicate that endometrial homeostasis during the luteal phase is dependent on  
289 balancing induction and clearance of senescent decidual cells. We speculated that this process  
290 is *a priori* dynamic, which should be reflected in varying numbers of uNK cells in different  
291 cycles. As proof of concept, we quantified uNK cells in biopsies from 3 patients obtained  
292 around the same time in the mid-luteal phase ( $\pm$  1 day) in 3 different cycles. As shown in Figure  
293 6A, the abundance of uNK cells in the subluminal endometrial stroma can vary profoundly

294 between cycles. As levels both rose and fell, the observed inter-cycle changes in uNK cell  
295 density are unlikely triggered by the tissue injury caused by the biopsy, although an impact on  
296 the magnitude of change cannot be excluded. Additional examples of uNK cell fluctuations in  
297 two consecutive cycles are presented in Figure S5A.

298 Cyclic surveillance and elimination of senescent cells should protect the endometrium  
299 against chronological ageing. To substantiate this hypothesis, we performed RNA-sequencing  
300 on LH-timed endometrium biopsies obtained from 10 women aged  $\leq 30$  years and 10 women  
301 aged  $\geq 40$  years (Gene Expression Omnibus accession no. GSE102131). The samples were  
302 matched for body mass index, parity and day of biopsy but were separated by approximately  
303  $\sim 170$  menstrual cycles (Table S1). A total of 84 genes were identified as differentially  
304 expressed between the two groups (Figure S5B). However, 7 biopsies in the older age group  
305 expressed a receptive phenotype compared to 4 samples from younger women (Figure 6B).  
306 Thus, differential gene expression was accounted for by the state of receptivity of individual  
307 biopsies but not age. Taken together, the data suggest that cyclic endometrial senescence and  
308 rejuvenation may lead to short-term fluctuations in endometrial homeostasis during the  
309 window of implantation but long-term functional stability.

310

## 311 **Discussion**

312 In contrast to chronic senescence associated with organismal ageing, acute senescence is a  
313 tightly orchestrated biological process implicated in embryo development, wound healing and  
314 tissue repair. Typically, acute senescent cells produce a context-specific SASP with defined  
315 paracrine functions and self-organize their elimination by various immune cells (van Deursen,  
316 2014). Here we provide evidence that acute decidual senescence is a pivotal process that  
317 coordinates acquisition of a receptive phenotype with endometrial remodeling and rejuvenation  
318 during the implantation process. We reported previously that decidual transformation of

319 primary EnSCs is a stepwise process that starts with a NOX4-dependent burst of free radicals  
320 and release of multiple inflammatory mediators (Al-Sabbagh et al., 2011; Lucas et al., 2016b;  
321 Salker et al., 2012). Exposure of the mouse uterus to this inflammatory secretome induced  
322 expression of multiple implantation genes and enabled efficient implantation of *in vitro*  
323 cultured mouse embryos (Salker et al., 2012). We now demonstrate that this nidatory decidual  
324 signal is driven foremost by acute senescence of a subpopulation of EnSCs. The close  
325 correlation between SA $\beta$ G activity before and after decidualization suggests that polarization  
326 of EnSCs upon cell cycle exit into differentiating and senescent cells is not stochastic but  
327 determined by the level of replicative stress incurred by individual EnSCs during the preceding  
328 proliferative phase. Acute senescence rejuvenates the receptive endometrium through two  
329 distinct mechanisms. First, decidual SASP not only ‘locks in’ endometrial MSCs upon  
330 decidualization but, dependent on the amplitude of the inflammatory response, also de-  
331 differentiates more committed cells into clonogenic MSCs. Arguably, an adequate MSC  
332 population may be essential for expansion of the decidua in pregnancy. Second, clearance of  
333 senescent decidual cells upon uNK cell activation ensures that the embryo embeds in a  
334 preponderance of mature decidual cells. In co-culture, uNK cell mediated clearance of SA $\beta$ G<sup>+</sup>  
335 cells transformed the decidual cell monolayer into a honeycomb pattern. If recapitulated *in*  
336 *vivo*, this observation suggests a role for uNK cells in creating ingresses in the tightly adherent  
337 decidual cell matrix to facilitate trophoblast invasion and anchoring of the conceptus.  
338 Compared to undifferentiated EnSCs, decidual cells are highly resistant to various stress  
339 signals, convert inactive cortisone into cortisol through the induction of 11 $\beta$ -hydroxysteroid  
340 dehydrogenase type 1, and protect the embryo-maternal interface from influx of T-cells by  
341 silencing genes coding for key chemokines (Erlebacher, 2013; Gellersen and Brosens, 2014).  
342 Taken together, these observations suggest that senescent decidual cells trigger a dynamic  
343 tissue reaction that ultimately results in enclosure of the conceptus into an immune-privileged

344 decidual matrix. In pregnancy, uNK cells express senescence markers and are proangiogenic  
345 rather than cytotoxic (Rajagopalan and Long, 2012). Whether prior exposure to senescent  
346 decidual cells contributes to this gestational phenotype of uNK cells is an intriguing but as yet  
347 untested possibility.

348 Notably, p16<sup>+</sup> epithelial cells were present throughout the peri-implantation window,  
349 although relatively much more so in the luminal compared to glandular epithelium. It is  
350 conceivable that p16<sup>+</sup> luminal epithelial cells play a role in directing the embryo to preferential  
351 sites of implantation. However, the abundance of p16<sup>+</sup> cells in both epithelial compartments  
352 peaked on the transition of mid- to late-luteal phase, which in turn points towards a potential  
353 role for cellular senescence in rendering the endometrium refractory to further implantation.

354 Clinically, recurrent pregnancy loss (RPL) is a distressing disorders that often remain  
355 unexplained despite extensive investigations (Lucas et al., 2016a). Embryonic chromosome  
356 instability accounts for a majority of sporadic failures. However, the likelihood of an  
357 underlying endometrial defect compromising the development of a euploid embryo increases  
358 with each additional failure. Nevertheless, the cumulative live birth rate following multiple  
359 miscarriages or IVF failures is high (Lucas et al., 2016a; Smith et al., 2015), which suggests  
360 that embryo-endometrial interactions are intrinsically dynamic. Our findings point towards a  
361 new paradigm that accounts for the observation that RPL does not preclude a successful  
362 pregnancy. If the level of replicative stress during the follicular phase is efficiently  
363 counterbalanced by uNK cell mediated clearance of senescence decidual cells during the luteal  
364 phase, implantation competence of the endometrium is assured and, in the absence of other  
365 pathology, reproductive fitness should be maximal. If not, the frequency of aberrant cycles,  
366 and thus the likelihood of reproductive failure, is predicted to increase in line with the degree  
367 of endometrial dyshomeostasis. For example, the endometrium in RPL patients is characterized  
368 by MSC deficiency, heightened cellular senescence and a prolonged and disordered decidual

369 inflammatory response (Lucas et al., 2016b; Salker et al., 2012). Our model predicts that  
370 excessive decidual senescence can be counterbalanced by increased uNK cell proliferation and  
371 activation, thus tending towards homeostasis and leading to intermittent normal cycles.  
372 Importantly, the degree of endometrial MSC deficiency correlates with the number of previous  
373 miscarriages and, by extension, the likelihood of further failure (Lucas et al., 2016b). This  
374 observation provides credence to our assertion that the chance of a successful pregnancy  
375 correlates inversely with the severity of endometrial dyshomeostasis. The corollary of an  
376 intrinsic ability to balance induction and clearance of senescent cells from cycle to cycle is that  
377 the human endometrium seems refractory to ageing and maintains its function throughout the  
378 reproductive years.

379           In summary, acute senescence of a subpopulation of stromal cells upon decidualization  
380 triggers a multi-step process that transforms the cycling endometrium into a gestational tissue.  
381 Endometrial remodeling at the time of embryo implantation is controlled spatiotemporally by  
382 the level of decidual senescence and the efficacy of immune clearance.



## 383 **Experimental Procedures**

384

### 385 **Patient recruitment and sample collection**

386 This study was approved by NHS National Research Ethics Committee (1997/5065).  
387 Participants provided written informed consent in accordance with the Declaration of Helsinki,  
388 2000. A total of 2,131 biopsies were used in this study, including 109 samples processed for  
389 primary EnSC cultures. Patient demographics are summarized in Table S2. See Supplemental  
390 Experimental Procedures for details.

391

### 392 **Decidualization of EnSCs and uNK cell isolation**

393 Primary EnSC cultures were decidualized with 0.5 mM 8-bromo-cAMP and 1  $\mu$ M  
394 medroxyprogesterone acetate (MPA). For co-culture experiments, the supernatant from freshly  
395 isolated EnSCs was collected 6-18 h post-seeding and uNK cells isolated using magnetic  
396 activated cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). See  
397 Extended Experimental Procedures for details. In co-culture, the ratio of EnSCs to uNK cells  
398 was 2:1. See Supplemental Experimental Procedures for details.

399

### 400 **siRNA transfection**

401 Confluent EnSCs in 24-well plates were transfected using jetPRIME Polyplus transfection  
402 reagent (VWR International, Lutterworth, UK) according to the manufacturer's instructions.  
403 Culture medium was refreshed 18 h post-transfection. See Supplemental Experimental  
404 Procedures for details.

405

406

407

408 **Colony-forming unit (CFU) assay**

409 CFU assays were performed as described (Lucas et al., 2016b). Briefly, 500 EnSCs per well  
410 were seeded into 10 $\mu$ g/ml fibronectin-coated 6-well plates and cultured in 10% DMEM/F12  
411 containing 10 ng/ml basic fibroblast growth factor for 12 days. Cells were stained with  
412 hematoxylin and colonies of more than 50 cells were counted. Cloning efficiency (%) was  
413 calculated as the number of colonies formed / number of cells seeded  $\times$  100.

414

415 **Real-time Quantitative (RTq)-PCR**

416 Total RNA was isolated using STAT-60 (AMS Biotechnology, Oxford, UK), reverse  
417 transcribed and subjected to real-time PCR using Power SYBR Green Master Mix (Fisher  
418 Scientific, Loughborough, UK), according to manufacturers' instructions. See Supplemental  
419 Experimental Procedures for details.

420

421 **Enzyme-linked immunosorbent assay**

422 Detection of individual secreted factors was achieved by commercially available DuoSet  
423 ELISA kits (BioTechne, MN, USA) according to the manufacturer's instructions. See  
424 Supplemental Experimental Procedures.

425

426 **Senescence-Associated- $\beta$ -Galactosidase (SA $\beta$ G)**

427 SA $\beta$ G staining was performed on confluent EnSC in 24-well plates using Senescence  $\beta$ -  
428 Galactosidase Staining Kit (Cell Signalling Technology, MA, USA) according to the  
429 manufacturer's instruction. SA $\beta$ G activity in cell and tissue lysates was quantified using the  
430 96-Well Cellular Senescence Activity Assay kit (Cell Biolabs Inc; CA, USA). Activity was  
431 normalized to protein content, as determined by Bradford assay (Sigma Aldrich, UK). See  
432 Supplemental Experimental Procedures for details.

433 **Western blot analysis**

434 Protein lysates (25 µg per lane) were separated in 12% poly-acrylamide gels by standard SDS-  
435 PAGE electrophoresis. Proteins were transferred onto nitrocellulose (GE Healthcare,  
436 Amersham, UK) and probed with antibodies targeting Lamin B1 (Abcam, Cambridge, UK;  
437 1:1000), HMGB2 (Abcam; 1:500), p16<sup>INK4</sup> (Abcam; 1:1500) and p53 (Agilent Technologies,  
438 Santa Clara; 1:3000), Histone H1 (Abcam; 1:2000), MacroH2A (Abcam; 1:5000) H3k9me3  
439 (Abcam; 1:1000), FOXO1 (Cell Signaling Technologies, Denvers, M.A, USA; 1:1000) and β-  
440 actin (Sigma, Poole; UK), 1:100000). See Supplemental Experimental Procedures for details.

441

442 **Immunocytochemistry**

443 Cytospin preparations from 100,000 uNK cells were fixed in 10% formalin and probed with  
444 anti-CD56 antibody (Agilent Technologies) (1:250, overnight, 4°C). CD56<sup>+</sup> cells were  
445 identified using the Novolink<sup>TM</sup> polymer detection system exactly as per manufacturer's  
446 instructions (Leica Biosystems). For immunofluorescence analysis, EnSCs seeded in 35mm  
447 glass-bottomed culture dishes were treated and then fixed in 4% paraformaldehyde and  
448 permeabilized in 0.5% Triton X-100. See Supplemental Experimental Procedures.

449

450 **Immunohistochemistry**

451 Formalin fixed paraffin embedded endometrial sections were stained for CD56 (NCL-L-CD56-  
452 504, Novocastra, Leica BioSystems; 1:200) or p16<sup>INK4</sup> (CINtec<sup>®</sup> clone E6H4, Roche, Basel,  
453 Switzerland; 1: 5). See Supplemental Experimental Procedures for details.

454

455 **RNA-sequencing:** Total RNA isolated from snap frozen LH-timed endometrium biopsies from  
456 10 women aged ≤ 30 years and 10 women aged ≥ 40 years was processed for RNA-sequencing.

457 See Supplemental Experimental Procedures.

458 **Statistical analysis**

459 GraphPad Prism v6 (GraphPad Software Inc.) was used for statistical analyses. Data were  
460 checked for normal distribution using Kolmogorov-Smirnov test. Unpaired or paired *t*-test was  
461 performed as appropriate to determine statistical significance between two groups. For larger  
462 data sets, significance was determined using one-way ANOVA and Tukey's post-hoc test for  
463 multiple comparisons.  $P < 0.05$  was considered significant.

464 **Author Contributions**

465 Conceptualization, J.J.B.; Methodology, P.J.B., P.V., E.S.L, S.O and M.H.; Investigation,  
466 Y.M., P.J.B., K.F., R.F., J.M., T.Y., L.W., R.L., Y.H.L. and M.H.; Writing – Original Draft,  
467 J.J.B., P.J.B and E.S.L.; Funding Acquisition, S.Q., S.T and J.J.B.; Resources, K.F., Sh.T.,  
468 M.H., S.Q. and J.J.B.; Supervision, M.H., S.Q and J.J.B.

469

470 **Acknowledgement**

471 We are grateful to all the women who participated in this research. We thank Gnyaneshwari  
472 Patel, Anatoly Shmygol, and Jesús Gil for advice and technical assistance. This work was  
473 supported by funds from the Tommy’s National Miscarriage Research Centre and the  
474 Biomedical Research Unit in Reproductive Health.

## 475 References

- 476 Acosta, J.C., O'Loughlen, A., Banito, A., Guijarro, M.V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M.,  
477 Brown, C., Popov, N., *et al.* (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*  
478 *133*, 1006-1018.
- 479 Aird, K.M., Iwasaki, O., Kossenkov, A.V., Tanizawa, H., Fatkhutdinov, N., Bitler, B.G., Le, L., Alicea, G., Yang,  
480 T.L., Johnson, F.B., *et al.* (2016). HMGB2 orchestrates the chromatin landscape of senescence-associated  
481 secretory phenotype gene loci. *J Cell Biol* *215*, 325-334.
- 482 Al-Sabbagh, M., Fusi, L., Higham, J., Lee, Y., Lei, K., Hanyaloglu, A.C., Lam, E.W., Christian, M., and Brosens,  
483 J.J. (2011). NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial  
484 stromal cells in response to cyclic AMP signaling. *Endocrinology* *152*, 730-740.
- 485 Childs, B.G., Gluscevic, M., Baker, D.J., Laberge, R.M., Marquess, D., Dananberg, J., and van Deursen, J.M.  
486 (2017). Senescent cells: an emerging target for diseases of ageing. *Nat Rev Drug Discov*.
- 487 Chowdhury, D., and Lieberman, J. (2008). Death by a thousand cuts: granzyme pathways of programmed cell  
488 death. *Annu Rev Immunol* *26*, 389-420.
- 489 de Ziegler, D., Bergeron, C., Cornel, C., Medalie, D.A., Massai, M.R., Milgrom, E., Frydman, R., and Bouchard,  
490 P. (1992). Effects of luteal estradiol on the secretory transformation of human endometrium and plasma  
491 gonadotropins. *J Clin Endocrinol Metab* *74*, 322-331.
- 492 Demidenko, Z.N., Zubova, S.G., Bukreeva, E.I., Pospelov, V.A., Pospelova, T.V., and Blagosklonny, M.V.  
493 (2009). Rapamycin decelerates cellular senescence. *Cell Cycle* *8*, 1888-1895.
- 494 Diep, C.H., Charles, N.J., Gilks, C.B., Kalloger, S.E., Argenta, P.A., and Lange, C.A. (2013). Progesterone  
495 receptors induce FOXO1-dependent senescence in ovarian cancer cells. *Cell Cycle* *12*, 1433-1449.
- 496 Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annu Rev Immunol* *31*, 387-411.
- 497 Evans, J., Salamonsen, L.A., Winship, A., Menkhorst, E., Nie, G., Gargett, C.E., and Dimitriadis, E. (2016).  
498 Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol* *12*,  
499 654-667.
- 500 Funayama, R., Saito, M., Tanobe, H., and Ishikawa, F. (2006). Loss of linker histone H1 in cellular senescence. *J*  
501 *Cell Biol* *175*, 869-880.
- 502 Gellersen, B., and Brosens, J.J. (2014). Cyclic decidualization of the human endometrium in reproductive health  
503 and failure. *Endocr Rev* *35*, 851-905.
- 504 Groll, J.M., Usadi, R.S., Lessey, B.A., Lininger, R., Young, S.L., and Fritz, M.A. (2009). Effects of variations in  
505 serum estradiol concentrations on secretory endometrial development and function in experimentally induced  
506 cycles in normal women. *Fertil Steril* *92*, 2058-2061.
- 507 Hanna, J., Goldman-Wohl, D., Hamani, Y., Avraham, I., Greenfield, C., Natanson-Yaron, S., Prus, D., Cohen-  
508 Daniel, L., Arnon, T.I., Manaster, I., *et al.* (2006). Decidual NK cells regulate key developmental processes at  
509 the human fetal-maternal interface. *Nat Med* *12*, 1065-1074.
- 510 Iannello, A., and Raulet, D.H. (2013). Immune surveillance of unhealthy cells by natural killer cells. *Cold Spring*  
511 *Harb Symp Quant Biol* *78*, 249-257.
- 512 Krizhanovskiy, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe,  
513 S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. *Cell* *134*, 657-667.
- 514 Laws, M.J., Taylor, R.N., Sidell, N., DeMayo, F.J., Lydon, J.P., Gutstein, D.E., Bagchi, M.K., and Bagchi, I.C.  
515 (2008). Gap junction communication between uterine stromal cells plays a critical role in pregnancy-  
516 associated neovascularization and embryo survival. *Development* *135*, 2659-2668.
- 517 Lucas, E.S., Dyer, N.P., Fishwick, K., Ott, S., and Brosens, J.J. (2016a). Success after failure: the role of  
518 endometrial stem cells in recurrent miscarriage. *Reproduction* *152*, R159-166.
- 519 Lucas, E.S., Dyer, N.P., Murakami, K., Lee, Y.H., Chan, Y.W., Grimaldi, G., Muter, J., Brighton, P.J., Moore,  
520 J.D., Patel, G., *et al.* (2016b). Loss of Endometrial Plasticity in Recurrent Pregnancy Loss. *Stem Cells* *34*,  
521 346-356.
- 522 Marçais, A., Cherfils-Vicini, J., Viant, C., Degouve, S., Viel, S., Fenis, A., Rabilloud, J., Mayol, K., Tavares, A.,  
523 Biennu, J., *et al.* (2014). The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the  
524 development and activation of NK cells. *Nat Immunol* *15*, 749-757.
- 525 Matjusaitis, M., Chin, G., Sarnoski, E.A., and Stolzing, A. (2016). Biomarkers to identify and isolate senescent  
526 cells. *Ageing Res Rev* *29*, 1-12.
- 527 Mosteiro, L., Pantoja, C., Alcazar, N., Marion, R.M., Chondronasiou, D., Rovira, M., Fernandez-Marcos, P.J.,  
528 Munoz-Martin, M., Blanco-Aparicio, C., Pastor, J., *et al.* (2016). Tissue damage and senescence provide  
529 critical signals for cellular reprogramming in vivo. *Science* *354*.
- 530 Munoz-Espin, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell*  
531 *Biol* *15*, 482-496.

- 532 Paria, B.C., Lim, H., Wang, X.N., Liehr, J., Das, S.K., and Dey, S.K. (1998). Coordination of differential effects  
533 of primary estrogen and catecholesterogen on two distinct targets mediates embryo implantation in the mouse.  
534 *Endocrinology* 139, 5235-5246.
- 535 Park, Y., Nnamani, M.C., Maziarz, J., and Wagner, G.P. (2016). Cis-Regulatory Evolution of Forkhead Box O1  
536 (FOXO1), a Terminal Selector Gene for Decidual Stromal Cell Identity. *Mol Biol Evol* 33, 3161-3169.
- 537 Pohnke, Y., Schneider-Merck, T., Fahnenstich, J., Kempf, R., Christian, M., Milde-Langosch, K., Brosens, J.J.,  
538 and Gellersen, B. (2004). Wild-type p53 protein is up-regulated upon cyclic adenosine monophosphate-  
539 induced differentiation of human endometrial stromal cells. *J Clin Endocrinol Metab* 89, 5233-5244.
- 540 Rajagopalan, S., and Long, E.O. (2012). Cellular senescence induced by CD158d reprograms natural killer cells  
541 to promote vascular remodeling. *Proc Natl Acad Sci U S A* 109, 20596-20601.
- 542 Ritschka, B., Storer, M., Mas, A., Heinzmann, F., Ortells, M.C., Morton, J.P., Sansom, O.J., Zender, L., and  
543 Keyes, W.M. (2017). The senescence-associated secretory phenotype induces cellular plasticity and tissue  
544 regeneration. *Genes Dev* 31, 172-183.
- 545 Sadaie, M., Salama, R., Carroll, T., Tomimatsu, K., Chandra, T., Young, A.R., Narita, M., Perez-Mancera, P.A.,  
546 Bennett, D.C., Chong, H., *et al.* (2013). Redistribution of the Lamin B1 genomic binding profile affects  
547 rearrangement of heterochromatic domains and SAHF formation during senescence. *Genes Dev* 27, 1800-  
548 1808.
- 549 Sagiv, A., Biran, A., Yon, M., Simon, J., Lowe, S.W., and Krizhanovsky, V. (2013). Granule exocytosis mediates  
550 immune surveillance of senescent cells. *Oncogene* 32, 1971-1977.
- 551 Salker, M.S., Nautiyal, J., Steel, J.H., Webster, Z., Sucurovic, S., Nicou, M., Singh, Y., Lucas, E.S., Murakami,  
552 K., Chan, Y.W., *et al.* (2012). Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine  
553 receptivity in women with recurrent pregnancy loss. *PLoS One* 7, e52252.
- 554 Smith, A., Tilling, K., Nelson, S.M., and Lawlor, D.A. (2015). Live-Birth Rate Associated With Repeat In Vitro  
555 Fertilization Treatment Cycles. *JAMA* 314, 2654-2662.
- 556 Takano, M., Lu, Z., Goto, T., Fusi, L., Higham, J., Francis, J., Withey, A., Hardt, J., Cloke, B., Stavropoulou,  
557 A.V., *et al.* (2007). Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and  
558 the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal  
559 cells. *Mol Endocrinol* 21, 2334-2349.
- 560 van Deursen, J.M. (2014). The role of senescent cells in ageing. *Nature* 509, 439-446.
- 561 Xiong, S., Sharkey, A.M., Kennedy, P.R., Gardner, L., Farrell, L.E., Chazara, O., Bauer, J., Hiby, S.E., Colucci,  
562 F., and Moffett, A. (2013). Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *J*  
563 *Clin Invest* 123, 4264-4272.
- 564 Yuan, X., Saravelos, S.H., Wang, Q., Xu, Y., Li, T.C., and Zhou, C. (2016). Endometrial thickness as a predictor  
565 of pregnancy outcomes in 10787 fresh IVF-ICSI cycles. *Reprod Biomed Online* 33, 197-205.
- 566 Zhang, S., Lin, H., Kong, S., Wang, S., Wang, H., Wang, H., and Armant, D.R. (2013). Physiological and  
567 molecular determinants of embryo implantation. *Mol Aspects Med* 34, 939-980.
- 568 Zhu, Y., Tchkonja, T., Fuhrmann-Stroissnigg, H., Dai, H.M., Ling, Y.Y., Stout, M.B., Pirtskhalava, T., Giorgadze,  
569 N., Johnson, K.O., Giles, C.B., *et al.* (2016). Identification of a novel senolytic agent, navitoclax, targeting the  
570 Bcl-2 family of anti-apoptotic factors. *Aging Cell* 15, 428-435.
- 571 Zhu, Y., Tchkonja, T., Pirtskhalava, T., Gower, A.C., Ding, H., Giorgadze, N., Palmer, A.K., Ikeno, Y., Hubbard,  
572 G.B., Lenburg, M., *et al.* (2015). The Achilles' heel of senescent cells: from transcriptome to senolytic drugs.  
573 *Aging Cell* 14, 644-658.
- 574

575 **Figure legends**

576

577 **Figure 1. Decidualization induces acute senescence in a subpopulation of EnSCs.**

578 (A) Representative SA $\beta$ G staining in undifferentiated EnSCs (Day 0) or cells decidualized for  
579 the indicated time points with 8-bromo-cAMP and MPA. Scale bar = 100  $\mu$ m.

580 (B) SA $\beta$ G activity, expressed in fluorescence intensity units (FIU), in undifferentiated EnSCs  
581 (day 0) or cells decidualized for the indicated time points.

582 (C) Representative Western blot analysis of p53, p16, LMNB1, HMGB2, mH2A, H3K9me3  
583 and H.H1 levels in undifferentiated EnSCs and cells decidualized for the indicated time points.  
584  $\beta$ -actin served as a loading control.

585 (D) Left panel: representative immunofluorescence staining for p16 expression in  
586 undifferentiated cells and cells decidualized for 8 days. Nuclei were counterstained with DAPI.  
587 Scale bar = 50  $\mu$ m. Right panel: percentage of p16<sup>+</sup> cells.

588 (E) Left panel: representative confocal microscopy images of undifferentiated (Day 0) or  
589 decidualized (Day 8) EnSCs immune-probed for LMNB1, mH2A, H3K9me3 and H.H1. Scale  
590 bar = 10  $\mu$ m. Right panel: nuclear size of undifferentiated EnSCs (n = 48) and of cells first  
591 decidualized for 8 days with 8-br-cAMP and MPA (C+M) (n = 48) was measured in 3 primary  
592 cultures.

593 (F) Secretion of IL-8, GRO $\alpha$ , and IL-6 was measured in the supernatant of primary EnSCs  
594 collected every 48 h over an 8-day decidualization time-course.

595 Data are mean  $\pm$  SEM of 3 biological replicates unless stated otherwise. \*\*  $P < 0.01$ , \*\*\*  $P <$   
596 0.001. Different letters above the error bars indicate that those groups are significantly different  
597 from each other at  $P < 0.05$ .

598 See also Figure S1.

599



600 **Figure 2. Senescent cells in cycling human endometrium**

601 (A) Left panel: representative Western blot analysis of p53, p16, LMNB1, HMGB2, mH2A,  
602 H3K9me3 and H.H1 levels in whole tissue biopsies from proliferative endometrium (PE) and  
603 secretory endometrium (SE).  $\beta$ -actin served as a loading control. Right panel: protein levels  
604 quantified relative to  $\beta$ -actin by densitometry and expressed as arbitrary units (a.u.).

605 (B) SA $\beta$ G activity, expressed in fluorescence intensity units (FIU) / mg protein, was measured  
606 in biopsies from proliferative endometrium (PE; n = 7), early-secretory (ES; n = 9), mid-  
607 secretory (MS; n = 38) and late-secretory (LS; n = 19) endometrium.

608 (C) Immunohistochemistry demonstrating distribution of p16<sup>+</sup> cells in the stromal  
609 compartment and luminal epithelium. Scale bars = 200  $\mu$ m

610 (D) The abundance of p16<sup>+</sup> cells during the luteal phase in glandular epithelium, luminal  
611 epithelium and stroma compartment was analyzed by color deconvolution using ImageJ  
612 software in 308 LH-timed endometrial biopsies (average 48 samples per time point; range: 22  
613 to 69). The centile graphs depict the distribution of p16<sup>+</sup> cells across the peri-implantation  
614 window in each cellular compartment. Color key is on the right.

615 Data are mean  $\pm$  SEM of 3 biological replicates unless stated otherwise. \*\*  $P < 0.01$ , \*\*\*  $P <$   
616 0.001. Different letters above the error bars indicate that those groups are significantly different  
617 from each other at  $P < 0.05$ .

618

619 **Figure 3: A FOXO1 / IL-8 axis drives EnSC differentiation and senescence.**

620 (A) SA $\beta$ G activity in EnSCs either undifferentiated, or decidualized for 8 days with 8-bromo-  
621 cAMP, MPA, or a combination.

622 (B) Top left panel: *FOXO1* mRNA levels in undifferentiated EnSCs and cells treated with 8-  
623 br-cAMP and MPA (C+M) following transfection with non-targeting (NT) or FOXO1 siRNA.

624 Other panels: Secretion of IL-8, IL-6 and GRO $\alpha$  was measured following FOXO1 knockdown  
625 in the supernatant of primary EnSCs every 48 h over an 8-day decidualization time-course.

626 (C) SA $\beta$ G activity in EnSCs following transfection with NT or FOXO1 siRNA. The cultures  
627 either remain untreated or decidualized for 8 days.

628 (D) SA $\beta$ G activity in undifferentiated EnSCs treated for 8 days with increasing concentrations  
629 of recombinant IL-8 and in cells decidualized for 8 days in the presence of increasing  
630 concentrations of the CXCR2 antagonist, SB265610.

631 (E) SA $\beta$ G activity in EnSCs following transfection with IL-8 siRNA. The cultures either  
632 remain untreated or decidualized for 8 days.

633 (F) *PRL* and *IGFBP1* transcript levels in EnSCs following transfection with IL-8 siRNA. The  
634 cultures either remain untreated or decidualized for 8 days.

635 (G) *PRL* and *IGFBP1* expression in undifferentiated EnSCs, cells decidualized for 8 days, and  
636 upon withdrawal of 8-br-cAMP and MPA (C+M) for the indicated days.

637 (H) Left panel: SA $\beta$ G activity in undifferentiated EnSCs, cells decidualized for 8 days, and  
638 following withdrawal of C+M for the indicated days. Right panel: representative Western blot  
639 analysis of p53, p16, LMNB1 and HMGB2 levels in undifferentiated EnSCs, cells decidualized  
640 for 8 days, and following withdrawal of C+M for the indicated days.  $\beta$ -actin served as a loading  
641 control.

642 Data are mean  $\pm$  SEM of 3 biological replicates unless stated otherwise. \*  $P < 0.05$ , \*\*  $P <$   
643 0.01 and \*\*\*  $P < 0.005$ . Different letters above the error bars indicate that those groups are  
644 significantly different from each other at  $P < 0.05$ .

645 See also Figure S2.

646

647

648

649 **Figure 4: functions of senescent decidual cells.**

650 (A) Pearson's correlation analysis of SA $\beta$ G activity in 75 matched undifferentiated primary  
651 cultures and cultures decidualized for 8 days.

652 (B) Representative SA $\beta$ G staining in undifferentiated (Day 0) and decidualizing EnSCs (Day  
653 8) following 4 days of pretreatment with vehicle, dasatinib (250 nM) or palbociclib (1  $\mu$ M).  
654 Scale bar = 100  $\mu$ m.

655 (C) *PRL* and *IGFBP1* mRNA expression in response to pretreatment with vehicle, dasatinib or  
656 palbociclib. The cultures then remained undifferentiated or were decidualized for 8 days.

657 (D) IL-8, IL-6 and GRO $\alpha$  secretion was measured every 48 h in the supernatant of primary  
658 EnSCs decidualized for the indicated time-points following pretreatment with vehicle,  
659 dasatinib or palbociclib.

660 (E) Colony forming unit (CFU) activity in paired EnSC cultures that either remain  
661 undifferentiated (Day 0) or were decidualized for 8 days (n = 10).

662 (F) Left panel: representative clonogenic assays established from EnSC cultures first pretreated  
663 with vehicle, dasatinib or palbociclib and then decidualized for 8 days. Right panel: CFU  
664 activity in EnSC cultures first pretreated with vehicle, dasatinib or palbociclib and then  
665 decidualized for 8 days.

666 Data are mean  $\pm$  SEM of 3 biological replicates unless stated otherwise. \*  $P < 0.05$ , \*\*  $P <$   
667 0.01 and \*\*\*  $P < 0.001$ . Different letters above the error bars indicate that those groups are  
668 significantly different from each other at  $P < 0.05$ .

669 See also Figure S3.

670

671

672

673

674 **Figure 5: uNK cell mediated immune surveillance and clearance of senescent cells.**

675 (A) Left panel: uNK cell density in the subluminal stroma was quantified using a standardized  
676 immunohistochemistry protocol in LH timed endometrial biopsies (n = 1,997). Right panel:  
677 corresponding centile graph. Color code on the left.

678 (B) Left panel: example of the tissue distribution of CD56<sup>+</sup> uNK cells (brown staining) at  
679 LH+10. Scale bar = 250  $\mu$ m. Right panel: Pearson's correlation analysis of stromal cell and  
680 uNK cell densities. A total of 80 randomly selected images from 20 biopsies were analyzed.

681 (C) Representative images of an eosin stained primary culture decidualized for 8 days  
682 incubated for 18 h with or without uNK cells isolated from luteal phase endometrium. Scale  
683 bar = 100  $\mu$ m.

684 (D) SA $\beta$ G activity in undifferentiated or day 8 decidualized EnSCs co-cultured with or without  
685 uNK cells in the presence or absence of the apoptosis inhibitor Z-VAD-FMK (Z-VAD, 10  $\mu$ M)  
686 or the granzyme activity inhibitor 3,4-DCI (25  $\mu$ M).

687 (E) Secretion of IL-15 secretion was measured every 48 h in the supernatant of primary EnSCs  
688 decidualized for the indicated time-points following pretreatment with vehicle, dasatinib (250  
689 nM) or palbociclib (1  $\mu$ M).

690 (F) SA $\beta$ G activity in undifferentiated or day 8 decidualized EnSCs co-cultured with or without  
691 uNK cells in the presence or absence of an IL-15 blocking antibody (1 $\mu$ g/ml).

692 Data are mean  $\pm$  SEM of 3 biological replicates unless stated otherwise. Different letters above  
693 the error bars indicate that those groups are significantly different from each other at  $P < 0.05$ .

694 See also Figure S4.

695

696 **Figure 6:**

697 (A) CD56 immunohistochemistry of LH-timed endometrial biopsies obtained in 3 different  
698 cycles in 3 subjects. The day of the biopsy and the percentage of CD56<sup>+</sup> uNK cells versus

699 stromal cells are indicated. The color of the box indicates the percentile range of uNK when  
700 adjusted for the day of biopsy. Scale = 200  $\mu$ m.

701 (B). Heatmap showing that the 84 differentially expressed genes identified by intensity  
702 difference analysis ( $P < 0.05$ ) following RNA-sequencing of endometrial biopsies of women  
703 aged  $\leq 30$  years or  $\geq 40$  years are accounted for by the receptive status of the biopsy. Note that  
704 more biopsies from the older group expressed a receptive phenotype when compared to  
705 samples from younger women.

706 See also Figure S5.













