

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

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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^{bright} CD16⁻ 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 β -galactosidase (SA β G)
101 activity. At passage 1 (P1), SA β G⁺ cells were detectable in variable numbers in different
102 cultures (Figure 1A). Strikingly, the number of SA β G⁺ cells increased markedly upon
103 decidualization with 8-bromo-cAMP and medroxyprogesterone acetate (MPA, a progestin).
104 Typically, SA β G⁺ cells formed islets surrounded by SA β G⁻ EnSCs in differentiating cultures
105 (Figure 1A). Quantitative analysis confirmed a time-dependent increase in SA β G activity upon
106 decidualization (Figure 1B). The abundance of SA β G⁺ cells in undifferentiated cultures
107 declined upon passaging of cells (Figure S1A). Initially, this was paralleled by a reduction in
108 SA β 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 β G activity
111 and triggered the appearance of SA β G⁺ cells (Figure S1A and S1B).

112 Although SA β 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^{Ink4a} (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 α (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 α 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 β G⁺ 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 β 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 β G⁺ cells.
153 Instead, we used immunohistochemistry to examine the abundance and tissue distribution of
154 p16⁺ 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⁺ cells in the glandular epithelium, luminal epithelium and stromal
157 compartment is presented as a centile graph (Figure 2D). Interesting, p16⁺ 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⁺ cells was
160 ~10-fold higher in the luminal compared to the glandular compartment. Typically, stretches of
161 p16⁺ cells were interspersed by p16⁻ cells in the luminal epithelium (Figure 2C). By contrast,
162 p16⁺ 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⁺ cells were observed
164 in the stroma, seemingly connecting the deeper regions of the endometrium to p16⁺ 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 β 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 β 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 α , 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 β 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 β G activity in a dose-dependent manner (Figure 3D). Conversely, recombinant
186 IL-8 upregulated SA β G activity in undifferentiated EnSCs in a concentration-dependent
187 manner (Figure 3D), although spatial organization of SA β G⁺ 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 β 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 β G⁺
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 β 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 β G⁺ cells in undifferentiated cultures correlated closely with the number
208 of SA β G⁺ cells upon decidualization (Pearson's $r = 0.97$, $P < 0.0001$) (Figure S3A). A
209 congruent correlation was apparent upon measuring SA β 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_L inhibitor (Zhu et al., 2016), had no discernible impact on the induction
215 of SA β 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 β G activity upon decidualization in a dose-dependent manner (Figure
218 S3C). Conversely, to increase the abundance SA β G⁺ 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 β 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 α 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^{bright} 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⁺ 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 β 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 β 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 β G activity and cell viability in decidualizing cultures.
277 To explore why uNK cell-mediated clearance of SA β G⁺ 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 ≤ 30 years and 10 women
301 aged ≥ 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 ~ 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 β 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 β G⁺
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 β -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⁺ 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⁺ luminal epithelial cells play a role in directing the embryo to preferential
351 sites of implantation. However, the abundance of p16⁺ 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 μ 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 μ 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- β -Galactosidase (SA β G)**

427 SA β G staining was performed on confluent EnSC in 24-well plates using Senescence β -
428 Galactosidase Staining Kit (Cell Signalling Technology, MA, USA) according to the
429 manufacturer's instruction. SA β 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^{INK4} (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⁺ cells were
445 identified using the NovolinkTM 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^{INK4} (CINtec[®] 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

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- 574

575 **Figure legends**

576

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

578 (A) Representative SA β 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 μ m.

580 (B) SA β 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 β -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 μ m. Right panel: percentage of p16⁺ 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 μ 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 α , 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). β -actin served as a loading control. Right panel: protein levels
604 quantified relative to β -actin by densitometry and expressed as arbitrary units (a.u.).

605 (B) SA β 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⁺ cells in the stromal
609 compartment and luminal epithelium. Scale bars = 200 μ m

610 (D) The abundance of p16⁺ 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⁺ 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 β 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 α 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 β 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 β 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 β 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 β 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. β -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 β G activity in 75 matched undifferentiated primary
651 cultures and cultures decidualized for 8 days.

652 (B) Representative SA β 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 μ M).
654 Scale bar = 100 μ 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 α 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

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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⁺ uNK cells (brown staining) at
679 LH+10. Scale bar = 250 μ 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 μ m.

684 (D) SA β 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 μ M)
686 or the granzyme activity inhibitor 3,4-DCI (25 μ 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 μ M).

690 (F) SA β 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 μ 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

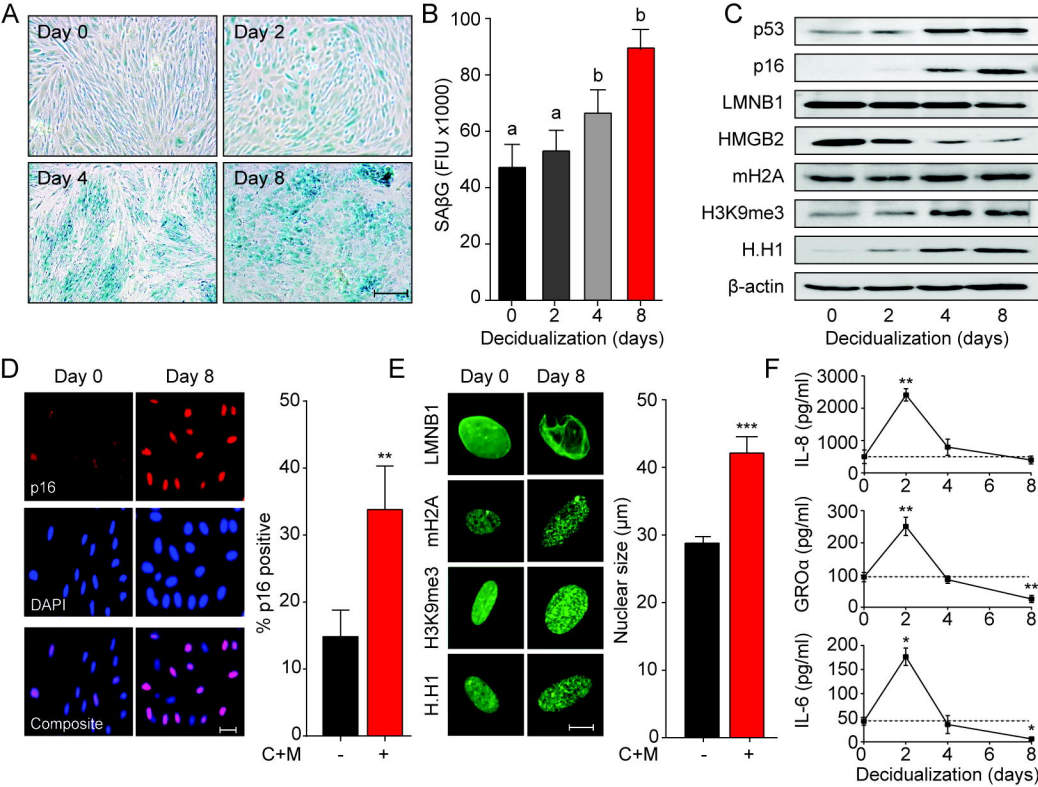
696 **Figure 6:**

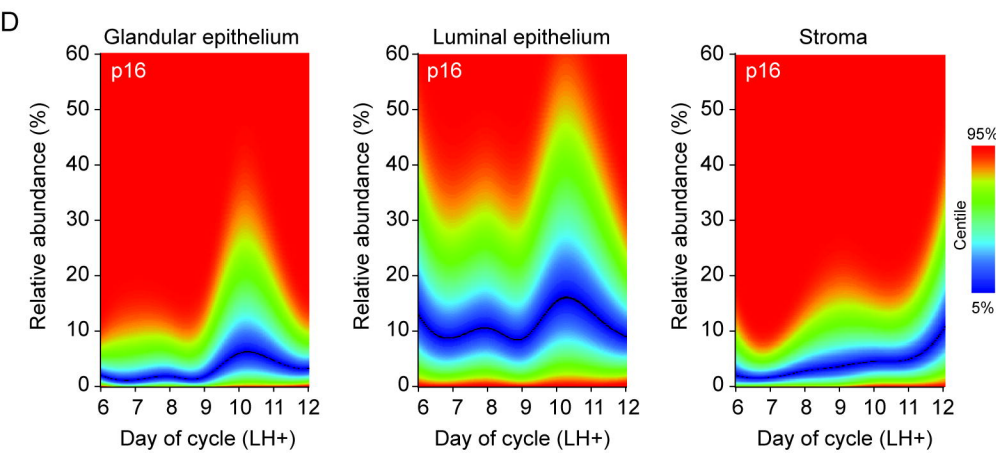
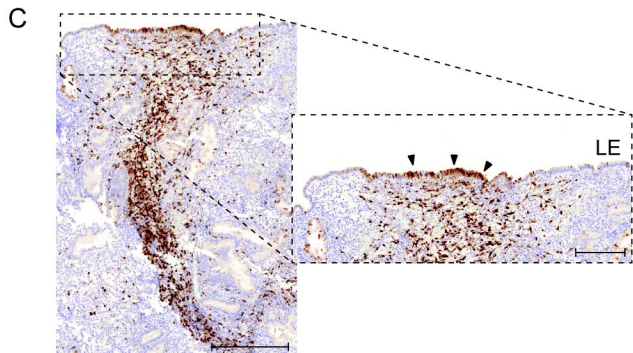
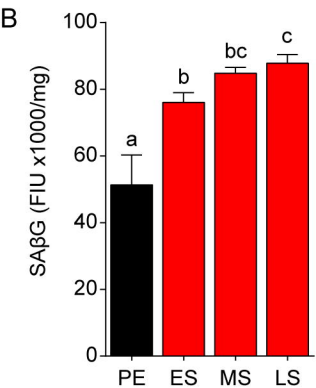
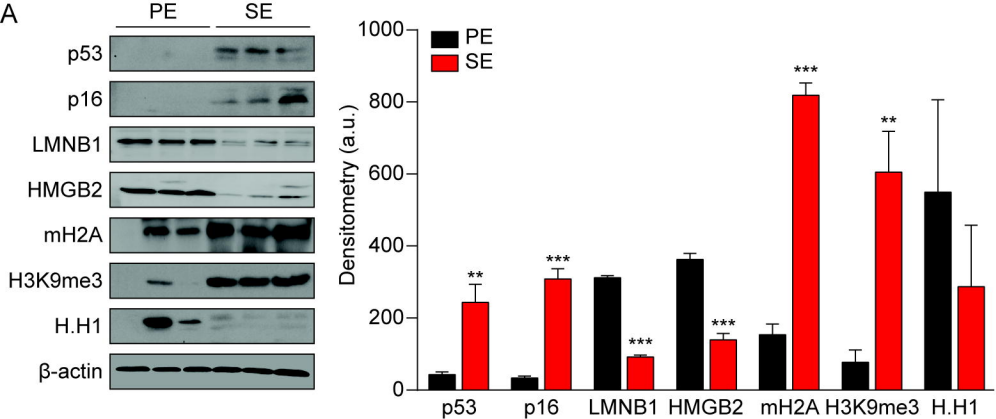
697 (A) CD56 immunohistochemistry of LH-timed endometrial biopsies obtained in 3 different
698 cycles in 3 subjects. The day of the biopsy and the percentage of CD56⁺ uNK cells versus

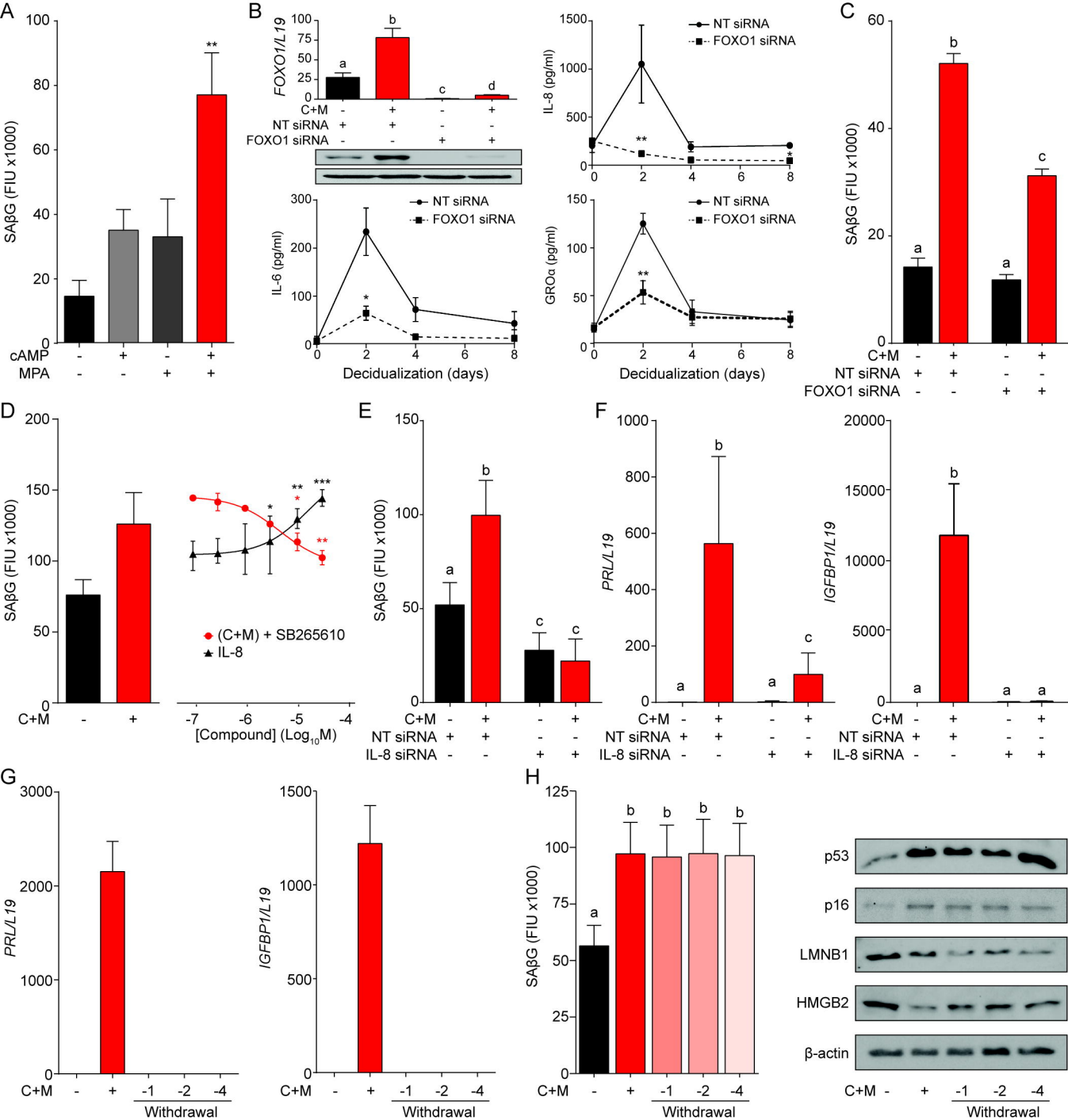
699 stromal cells are indicated. The color of the box indicates the percentile range of uNK when
700 adjusted for the day of biopsy. Scale = 200 μ m.

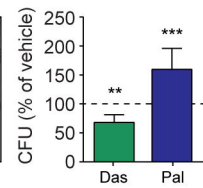
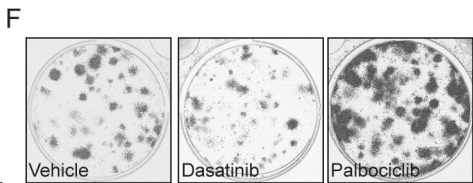
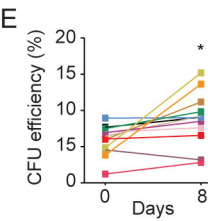
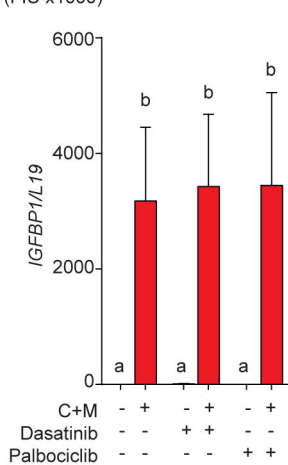
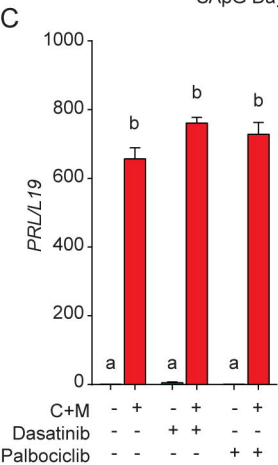
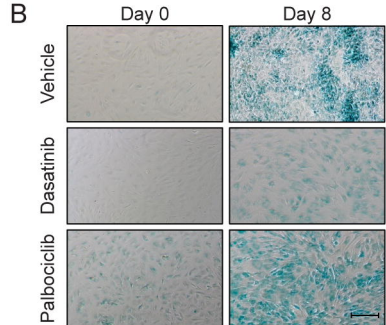
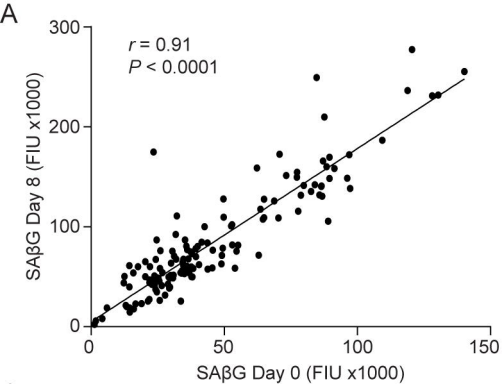
701 (B). Heatmap showing that the 84 differentially expressed genes identified by intensity
702 difference analysis ($P < 0.05$) following RNA-sequencing of endometrial biopsies of women
703 aged ≤ 30 years or ≥ 40 years are accounted for by the receptive status of the biopsy. Note that
704 more biopsies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

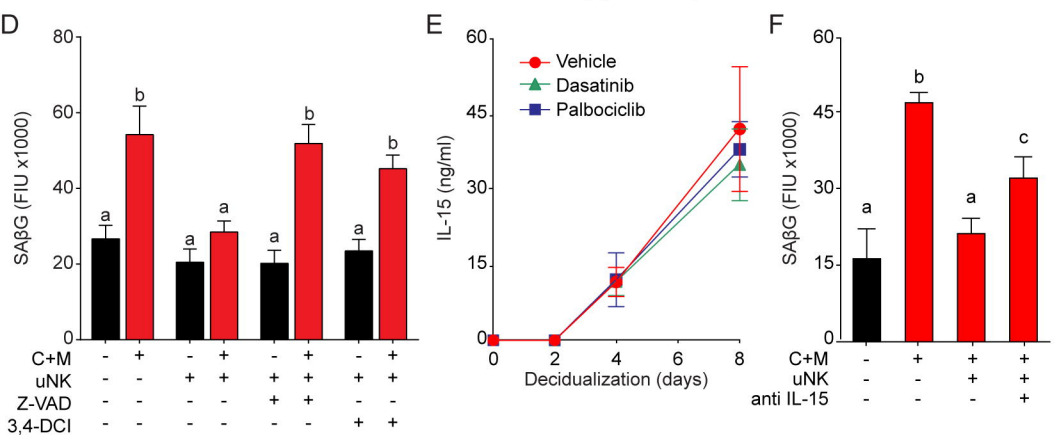
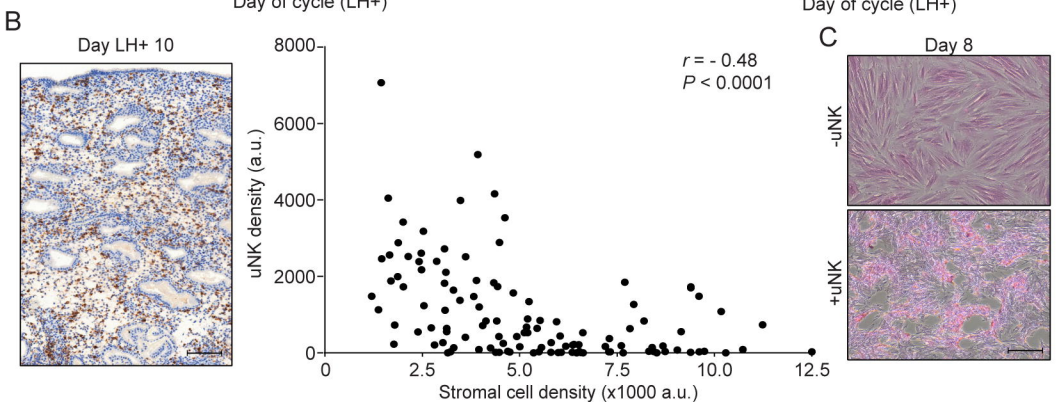
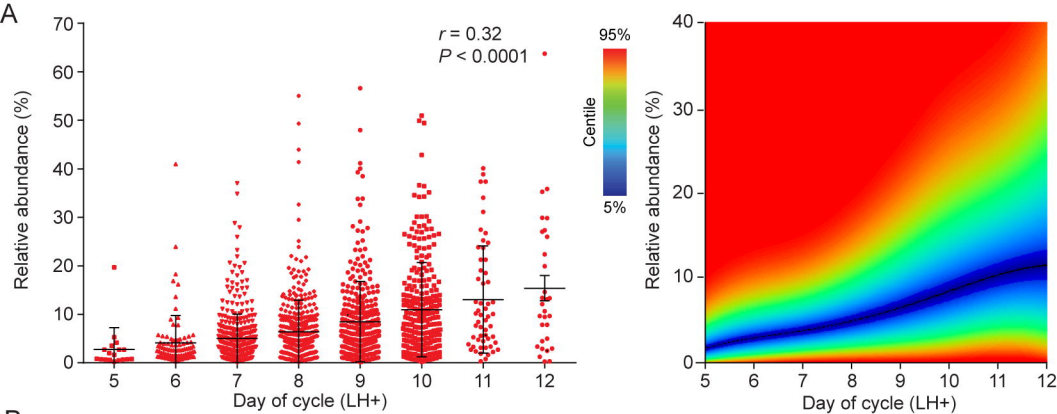
706 See also Figure S5.

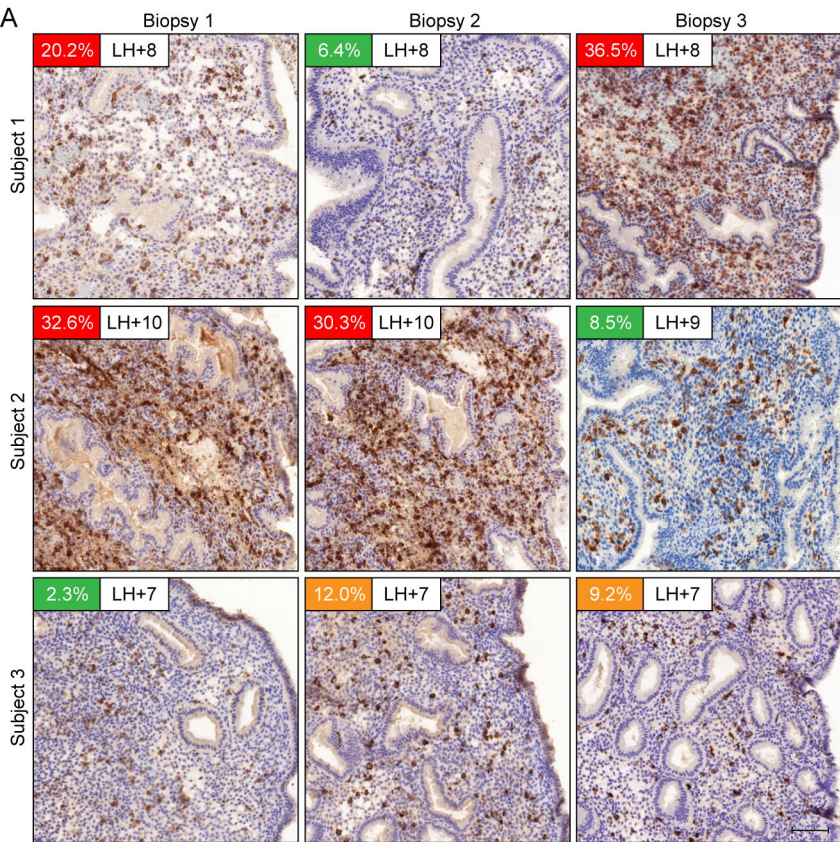












■ <75th percentile
 ■ 75th-94th percentile
 ■ >95th percentile

