

1 **Single cell RNAseq provides a molecular and cellular cartography of** 2 **changes to the human endometrium through the menstrual cycle**

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21 22 **Summary**

23 In a human menstrual cycle, the endometrium undergoes remodeling, shedding, and
24 regeneration, all of which are driven by substantial gene expression changes in the
25 underlying cellular hierarchy. Despite its importance in human fertility and
26 regenerative biology, mechanistic understanding of this unique type of tissue
27 homeostasis remains rudimentary. We characterized the transcriptomic
28 transformation of human endometrium at single cell resolution, dissecting the
29 multidimensional cellular heterogeneity of this tissue across the entire natural
30 menstrual cycle. We profiled the behavior of 6 endometrial cell types, including a
31 previously uncharacterized ciliated epithelial cell type, during four major phases of
32 endometrial transformation, and found characteristic signatures for each cell type
33 and phase. We discovered that human window of implantation opens with an abrupt
34 and discontinuous transcriptomic activation in the epithelia, accompanied with
35 widespread decidualized feature in the stromal fibroblasts. These data reveal
36 signatures in the luminal and glandular epithelia during epithelial gland
37 reconstruction, and suggest a mechanism for adult gland formation.

38 39 **Introduction**

40 The human menstrual cycle – with its monthly remodeling, shedding, and
41 regeneration of the endometrium – is not shared with many other species. Similar
42 cycles have only been consistently observed in human, apes, and old world monkeys
43 (Emera et al., 2012; Martin, 2007) and not in any of the model organisms which
44 undergo sexual reproduction such as mouse, zebrafish, or fly. This cyclic
45 transformation is executed through dynamic changes in states and interactions of

46 multiple cell types, including luminal and glandular epithelial cells, stromal
47 fibroblasts, vascular endothelial cells, and infiltrating immune cells. Although
48 different categorization schemes exist, the transformation can be primarily divided
49 into two major phases by the event of ovulation: the proliferative (pre-ovulatory) and
50 secretory (post-ovulatory) phase (Noyes et al., 1950). During the secretory phase, the
51 endometrium enters a narrow window of receptive state that is both structurally and
52 biochemically ideal for embryo to implant (Croxatto et al., 1978; Wilcox et al., 1999),
53 i.e. the mid-secretory phase or the window of implantation (WOI). To prepare for this
54 state, the tissue undergoes considerable reconstruction in the proliferative phase,
55 during which one of the most essential elements is the formation of epithelial glands
56 (Filant and Spencer, 2014).

57
58 Given the broad relevance in human fertility and regenerative biology, a systematic
59 characterization of endometrial transformation across the natural menstrual cycle
60 has been long pursued. Histological characterizations established the morphological
61 definition of menstrual, proliferative, early-, mid-, and late- secretory phases (Noyes
62 et al., 1950). Bulk level transcriptomic profiling advanced the characterization to a
63 molecular and quantitative level (Riesewijk et al., 2003; Ruiz-Alonso et al., 2012) and
64 demonstrated the feasibility of translating the definition into clinical diagnosis of the
65 WOI (Díaz-Gimeno et al., 2011). However, it has been a challenge to derive unbiased
66 or mechanism-linked characterization from bulk readouts due to the uniquely
67 heterogeneous and dynamic nature of endometrium.

68
69 The complexity of endometrium is unlike any other tissue: it consists of multiple cell
70 types which vary dramatically in state through a monthly cycle as they enter and exit
71 the cell cycle, remodel, and undergo various forms of differentiation with relatively
72 rapid rates. The notable variance in menstrual cycle lengths within and between
73 individuals (Guo et al., 2006) adds an additional variable to the system. Thus,
74 transcriptomic characterization of endometrial transformation at our current stage
75 of understanding requires that cell types and states be defined with a minimum of
76 bias. High precision characterization and mechanistic understanding of hallmark
77 events, such as the WOI, requires that we study both the static and dynamic aspects
78 of the tissue. Single cell RNAseq provides an ideal platform for these purposes. We
79 therefore performed a systematic transcriptomic delineation of human endometrium
80 across the natural menstrual cycle at single cell resolution.

81

82 **Results**

83 To characterize endometrial transformation across the natural human menstrual
84 cycle, we collected endometrial biopsies from 19 healthy and fertile women, 4-27
85 days after the onset of menstrual bleeding (**Figure S1**). All women were on regular
86 menstrual cycles, with no influence from exogenous hormone or gynecologic
87 pathology. Single cells were captured and cDNA was generated using Fluidigm C1
88 medium chips. The fraction of reads mapped to the spike-in controls developed by
89 the External RNA Controls Consortium (ERCC) was used as a metric for quality
90 filtering (Method).

91

92 **Human endometrium consists of six cell types across the menstrual cycle**
93 Dimensional reduction via t-distributed stochastic neighbor embedding (tSNE)
94 (Maaten and Hinton, 2008) on the top over-dispersed genes (Method) revealed clear
95 segregation of cells into distinct groups (**Figure 1A**). We defined cell types as
96 segregations that were not time-associated, i.e. groups encompassing cells sampled
97 across the menstrual cycle. Six cell types were thus identified; canonical markers and
98 highly differentially expressed genes enabled straightforward identification of four of
99 these: stromal fibroblast, endothelium, macrophage, and lymphocyte (**Figure 1B**).
100 The two remaining cell types both express epithelium-associated markers; one of
101 these cell types is characterized by an extensive list of uniquely expressed genes.
102 Functional analysis (Ashburner et al., 2000; Mi et al., 2017; The Gene Ontology
103 Consortium, 2017) revealed that 56% of genes in this list are annotated with a cilium-
104 associated cellular component or biological process (**Figure 1C, Figure S2**), thereby
105 identifying this cell type as “ciliated epithelium”, specifically with motile cilia
106 (Mitchison and Valente, 2017; Zhou and Roy, 2015). We defined the other epithelial
107 cell type as “unciliated epithelium”.

108
109 Using RNA and antibody co-staining (Method), we validated previously unannotated
110 discriminatory markers, epithelial lineage identity, and visualized the spatial
111 distribution of ciliated epithelium *in situ*. Four genes were selected for RNA staining:
112 they were identified as highly discriminatory for the cell type (**Figure 1B**) but either
113 have no previous functional annotation (C11orf88, C20orf85, FAM183A) or are
114 annotated with non-cilia-associated functionality (CDHR3) (**Table S1**). We found
115 consistent co-expression of all four genes with FOXJ1 (canonical master regulator for
116 motile cilia with epithelial lineage identity) antibody staining in both glandular
117 (**Figure 2A, C**) and luminal (**Figure 2B, D**) epithelia on day 17 (**Figure 2A, B**) and day
118 25 (**Figure 2C, D**) of the menstrual cycle. The results validated these ciliated cells as
119 an epithelial subpopulation of both luminal and glandular epithelia in healthy human
120 endometrium across the menstrual cycle. This data also demonstrates the consistent
121 discriminatory power of the new markers we identified (**Figure 2E**) across the cycle.
122 Lastly, the co-expression of these unannotated markers in ciliated cells helps confirm
123 a likely cilia-associated functionality for them and for other unannotated markers we
124 found, which constituted 44% of all markers identified for this cell type (**Figure S2,**
125 **Table S1**).

126
127 **Human endometrial transformation consists of four major phases across the**
128 **menstrual cycle**

129 Samples were taken throughout the menstrual cycle and annotated by the day of
130 menstrual cycle (the number of days after the onset of last menstrual bleeding). While
131 the time variable serves as an informative proxy for assigning endometrial states, it
132 is susceptible to bias due to variances in menstrual cycle lengths between and within
133 women (Guo et al., 2006), and limited in resolution due to variance of cells within an
134 individual. To study transcriptomes of endometrial transformation in an unbiased
135 manner, we performed within-cell type dimension reduction (tSNE) using whole
136 transcriptome data from unciliated epithelia and stromal fibroblasts, respectively.
137 The results revealed four major phases for both cell types, which we refer to as phases

138 1-4 (**Figure S3A insets**). The four phases were clearly time-associated (**Figure S3A**),
139 confirming the overall validity of the time annotation. Examples where the orders
140 between two women in their phase assignments and time annotation were reversed
141 and cases where cells with the same time annotation were assigned into different
142 phases demonstrate the bias and imitated resolution if we were to use time directly
143 for characterizations (**Figure S3A**).

144

145 **Constructing single cell resolution trajectories of menstrual cycle using mutual** 146 **information based approach**

147 Endometrial transformation over the menstrual cycle is at least in part a continuous
148 process. A model that not only retains phase-wise characteristics but also allows
149 delineation of continuous features between and within phases will enable higher
150 precision characterizations. To build such a model, we used a mutual information
151 (MI) (Tkačik and Walczak, 2011) based approach, such that we exploited the
152 information provided by the time annotation, minimized its limitation noted in the
153 previous section, and accounted for potential continuity between and within phases.
154 Briefly, we enriched for genes that were changing across the menstrual cycle based
155 on the MI between gene expression and time annotation regardless of underlying
156 model of dynamics (Method). In total we obtained 3,198 and 1,156 “time-associated”
157 genes for unciliated epithelia and stromal fibroblasts, respectively (FDR<0.05)
158 (**Figure S3B**). For both cell types, dimensional reduction (tSNE) using time-
159 associated genes revealed the same four major phases that we obtained using
160 unsupervised approach (**Figure S3C, insets**), demonstrating that the MI-based
161 approach reduced the bias of the time annotation to the same extent as unsupervised
162 approach. Meanwhile, the MI-based approach enabled identification of a clear
163 trajectory that connected the phases and was time-associated within phases. We
164 defined the trajectories using the principal curve (Hastie and Stuetzle, 1989) (**Figure**
165 **3A**), and assigned each cell an order along the trajectory based on its projection on
166 the curve (Ji and Ji, 2016; Kim et al., 2016; Marco et al., 2014; Petropoulos et al., 2016),
167 which we refer to as pseudotime (**Figure 3A**). We observed high correlations
168 between time and pseudotime for both unciliated epithelia and stromal fibroblasts
169 (**Figure 3B**). The high correlation between pseudotimes of the two cell types from
170 the same woman (**Figure 3C**) further supports the validity of the trajectories.

171

172 **The WOI opens with an abrupt and discontinuous transcriptomic activation in** 173 **unciliated epithelium**

174 Interestingly, we observed a notable discontinuity in the trajectory of unciliated
175 epithelia between phase 4 and the preceding phases (**Figure 3A, left**). This
176 discontinuity was consistently observed regardless of the method we used for
177 dimension reduction (**Figure S4A, S4B**) or feature enrichment (**Figure S4C**). It is
178 unlikely to be an artifact of sampling density given that the involved biopsies were
179 taken with a maximum interval of one day (**Figure S1**) and that a similar
180 discontinuity was not observed in the stromal fibroblast counterpart (**Figure 3A,**
181 **right**). To understand the nature of this discontinuity, we explored the genes and their
182 dynamics that contributed to it. Briefly, we identified genes that were dynamically
183 changing along the single-cell trajectories of endometrial transformation by

184 calculating the MI between gene expression and pseudotime, obtaining 1,382 and 527
185 genes for unciliated epithelia and stromal fibroblasts, respectively (FDR<1E-05,
186 **Figure S5A**). Ordering these genes based on the pseudotime at which their global
187 maximum was estimated to occur (pseudotime_{max}, Method) revealed the global
188 features of transcriptomic dynamics across the menstrual cycle (**Figure S5B**). In
189 unciliated epithelia, the dynamics demonstrated an overall continuous feature across
190 phase 1-3, until an abrupt and uniform activation of a gene module marked the
191 entrance into phase 4 (**Figure 4A, S5B left**). Genes in this module included *PAEP*,
192 *GPX3*, and *CXCL14* (**Figure 4A**), among others which were relatively consistently
193 reported by bulk transcriptomic profilings as overexpressed in the WOI despite
194 notable discrepancies among bulk profiling results (Díaz-Gimeno et al., 2011; Talbi et
195 al., 2006; reviewed by Ruiz-Alonso et al., 2012). Thus, entrance into phase 4 can be
196 identified with the opening of the WOI. Our analysis shows that this transition into
197 the receptive phase of the tissue occurs with an abrupt and discontinuous
198 transcriptomic activation that is uniform among all cells and activated genes in the
199 unciliated epithelia.

200

201 **The WOI is characterized by widespread decidualized features in stromal** 202 **fibroblasts**

203 Unlike their epithelial counterparts, transcriptomic dynamics in stromal fibroblasts
204 demonstrate more stage-wise characteristics, where genes are up-regulated in a
205 modular form, revealing boundaries between phases (**Figure 4B, S5B right**). In phase
206 4 stromal fibroblasts, the up-regulated gene module includes *DKK1* and *CRYAB*,
207 among a few others that were recapitulated by consensus among bulk analysis and
208 further confirm the identity of WOI (Díaz-Gimeno et al., 2011; Talbi et al., 2006;
209 reviewed by Ruiz-Alonso et al., 2012), although the transition was not as abrupt as in
210 their epithelial counterparts (**Figure 4A**). In the same module we noticed the
211 decidualization initiating transcriptional factor FOXO1 (Park et al., 2016) and
212 decidualized stromal marker IL15 (Okada et al., 2014). Importantly, while their
213 upregulation in phase 4 was obvious, their expression was already noticeable in
214 phase 3 in a lower percentage of cells and with lower expression level. Decidualization
215 is the transformation of stromal fibroblasts where they change from
216 elongated fibroblast-like cells into enlarged round cells with specific cytoskeleton
217 modifications, playing essential roles for embryo invasion and for pregnancy
218 development (for review see Ramathal. et al., 2010). Our data suggested that this
219 process is initiated before the opening of WOI in a small percentage of stromal
220 fibroblasts, and that at the receptive state of tissue decidualized features are
221 widespread in stromal fibroblasts.

222

223 **The WOI closes with continuous transcriptomic transitions**

224 While the WOI opened up with an abrupt transcriptomic transition in unciliated
225 epithelia, it closed with more continuous transition dynamics (**Figure 4A, S5B left**).
226 Genes expressed in phase 4 unciliated epithelium are featured by three major groups
227 with distinct dynamic characteristics. Group 1 genes (e.g. *PAEP*, *GPX3*) have sustained
228 expression throughout the entire phase 4, and their expression remains noticeable
229 until phase 1 of a new cycle. Group 2 genes (e.g. *CXCL14*, *MAOA*, *DPP4*, and the

230 metallothioneins (*MT1G*, *MT1E*, *MT1F*, *MT1X*), on the other hand, gradually decrease
231 to zero towards the later part of phase 4, whereas group 3 genes (e.g. *THBS1*, *MMP7*)
232 are upregulated at later part of the phase and their expression is sustained in phase
233 1 of a new cycle. These characteristics indicate a continuous and gradual transition
234 from mid-secretory to late-secretory phase (Talbi et al., 2006; reviewed by Ruiz-
235 Alonso et al., 2012), and hence the closure of the WOI.

236

237 The parallel transition in stromal fibroblasts is also characterized with three similar
238 groups of genes (**Figure 4B, S5B right**) and continuous dynamics. Specifically, we
239 observed a transition towards the later part of phase 4: gradual down-regulation of
240 decidualization-associated genes (e.g. *FOXO1* and *IL15*) and up-regulation of a
241 separate module of genes (e.g. *LMCD1*, *FGF7*). These transitions reveal the final phase
242 of decidualization at the transcriptomic level, which, differing from that during
243 pregnancy, ultimately leads to the shedding of the endometrium in a natural
244 menstrual cycle.

245

246 **WOI associated transcriptional regulators are featured with characteristic**
247 **regulatory roles at the opening and closure of WOI**

248 Cell type identity and cell state are primarily driven by small groups of transcriptional
249 regulators. We therefore sought to identify WOI-associated transcriptional factors
250 (TF) to understand what drives the opening and closure of WOI. We first
251 characterized all TFs that are dynamic across the menstrual cycle (Method) and found
252 for both unciliated epithelia and stromal fibroblasts, these TFs can be primarily
253 assigned to two main categories (**Figure S6A, B, Table S2**), i.e. with 1 or 2 peak(s)
254 of expression detected within one menstrual cycle. Similar to what we observed at
255 whole transcriptome level, the global TF dynamics of the two cell types are notably
256 distinct at the opening of WOI, where in unciliated epithelia a single major
257 discontinuity occurred (**Figure S6A**), whereas in stromal fibroblasts no comparable
258 discontinuity was observed (**Figure S6B**). These, at the level of transcriptional
259 regulators, validated the WOI-associated transcriptomic dynamics described in
260 previous sections.

261

262 We next define WOI-associated TFs as those with a peak expression detected after
263 the opening of WOI (**Figure S6C, D**), i.e. the boundary between phase 3 and 4. We
264 further divided these TFs into those 1) that peaked during, and 2) that peaked at the
265 end of phase 4, with the hypothesis that the former are more likely related to the
266 opening of the WOI and the latter the closure. Interestingly, we found that these two
267 groups of TFs are enriched with notably different functional roles. For unciliated
268 epithelia, group 1) TFs are dominated by regulators of early developmental process,
269 especially in differentiation (*IRX3*, *PAX8*, *MITF*, *ZBTB20*); whereas group 2) TFs
270 include those associated with ER stress (*DDIT3*) and immediate early genes (*FOS*,
271 *FOSB*, *JUN*). For stromal fibroblasts, group 1) TFs are primarily consisted of
272 regulators of chondrocyte differentiation via cAMP pathways (*BHLHE40*, *ATF3*),
273 hence are likely drivers for decidualization, and *HIVEP2* - binder to the enhancer of
274 MHC class I genes (discussed more in later sections on immune cells); group 2) TFs
275 include those with roles in ER stress (*YBX3*, *ZBTB16*) as well as in regulation of

276 inflammatory (XEBPD) and apoptosis (STAT3). Of note, we highlight the concurrent
277 upregulation of MTF1, which activates the promoter of metallothionein I (**Figure**
278 **S6C**), with metallothionein I genes (MT1F, 1X, 1E, 1G, **Figure 4A**) in unciliated
279 epithelia, revealing these heavy metal binding proteins as a key regulatory module
280 associated with WOI.

281

282 In summary, our analysis enabled the identification of key drivers for the opening and
283 closure of the human WOI as well as transitions between other major cycle phases
284 (**Figure S6C, D**, top panels). We also highlight the dynamics of nuclear receptors for
285 major classes of steroid hormones (**Figure S6E**), as a special group of TFs mediating
286 the communication between endometrium and other female reproductive organs.
287 Lastly, we performed similar analyses on genes encoding secretory proteins (**Figure**
288 **S7, Table S3**) and report those associated with the WOI (**Figure S7C, D**).

289

290 **The relationship between endometrial phases identified at the transcriptome**
291 **level is consistent with canonically defined endometrial phases**

292 Since its formalization in 1950 (Noyes et al., 1950), a histological definition of
293 endometrial phases, i.e. the proliferative, early-, mid-, and late-secretory phases, has
294 been used as the gold standard in determining endometrial state. It also usually
295 serves as the ground truth in bulk-based profiling studies in categorizing endometrial
296 phases. Given that there were clear differences between our phase definition and the
297 canonical definition, we investigated the relationship between the two.

298

299 Cell mitosis is one of the most distinct features of the pre-ovulatory (proliferative)
300 endometrium, hence the naming of proliferative phase. Thus, to identify the boundary
301 between proliferative and secretory phases, we first explored cell cycle activities
302 across the menstrual cycle. Specifically, we defined endometrial cell cycle associated
303 genes (**Figure S8A, B**, Method) and assigned cells into G1/S, G2/M, or non-cycling
304 states (**Figure S8C, D**). For both unciliated epithelia and stromal fibroblasts, cell
305 cycling was observed in only a small fraction of cells across the menstrual cycle
306 (**Figure S8C, D**, left). This fraction demonstrated phase-associated dynamics, where
307 it was most elevated in phase 1, slightly decreased in phase 2, and almost completely
308 ceased in later phases (**Figure S8C, D**, right), indicating that the transition from phase
309 2 to 3 is between pre-ovulatory to post-ovulatory phases.

310

311 To further validate this assignment, we defined characteristic signatures for phase 1-
312 4 (**Table S4**, Method) and identified major hierarchies of biological processes that
313 were enriched by the signatures (**Table S5**, Method). While phase 1 was
314 characterized with processes such as tissue regeneration, e.g. Wnt signaling pathways
315 (unciliated epithelia: epi), tissue morphogenesis (epi), wound healing (stromal
316 fibroblasts: str), and angiogenesis (str) and phase 2 by cell proliferation (epi), phase
317 3 was dominated by negative regulation of growth (epi) and response to ions (epi)
318 and phase 4 by secretion (epi) and implantation (epi). The transition from a positive
319 to a negative regulation in growth from phase 2 to 3 further confirmed a pre-
320 ovulatory to post-ovulatory transition (Talbi et al., 2006).

321

322 Lastly, we used previous bulk tissue analyses to help differentiate the pre-ovulatory
323 and post-ovulatory phases. We reasoned that although bulk data is confounded by the
324 varying proportion of the major cell types, i.e. stromal fibroblasts and unciliated
325 epithelia, bulk and single cell data taken together should have high level of consensus
326 on genes that 1) are in synchrony between the two cell types or 2) have negligible
327 expression in one cell type but significant phase-specific dynamics in another. We
328 therefore identified genes with these characteristics using our single cell data (**Figure**
329 **4**). As expected, among these genes we identified are those that have been
330 consistently reported by bulk studies to be characteristic of canonical endometrial
331 phases, confirming the validity of using them to identify the WOI. Particularly, the
332 upregulation of the metallothioneins (MT1F, X, E, G) from phase 2 and 3 was
333 characteristic of proliferative to early-secretory transition based on bulk reports
334 (Ruiz-Alonso et al., 2012; Talbi et al., 2006). Therefore, considering all of the evidence
335 above, phases 1 and 2 can be identified as pre-ovulatory (proliferative) phases, and
336 phases 3 and 4 as post-ovulatory (secretory) phases. With the anchor provided by the
337 WOI, phase 3 can thus be identified as the early secretory phase.

338
339 In phase 1, we observed sub-phases in both unciliated epithelia and stromal
340 fibroblasts that are primarily characterized with genes that are gradually decreasing
341 or increasing towards later part of the phases (**Figure 4A, S5B**). In the unciliated
342 epithelia, the gradually decreasing genes included phase 4 genes (e.g. *PAEP*, *GPX3*), as
343 well as *PLAU*, which activates the degradation of blood plasma proteins. The down-
344 regulation of these genes suggested the end of menstruation, and hence the transition
345 from menstrual to proliferative phase in the canonical definition. Phase 2 can
346 therefore be identified as a second proliferative phase at the transcriptome level. At
347 histological level, transformation in the proliferative endometrium was reported to
348 be featured with morphological changes so gradual that they do not permit the
349 recognition of distinct sub-phases (Noyes et al., 1950). We however have discovered
350 that at the transcriptomic level, proliferative endometrium can be divided into two
351 subphases in both unciliated epithelia and stromal fibroblasts that can be
352 quantitatively identified by transcriptomic signatures (**Figure S9**).

353
354 Lastly, we explored interactions between unciliated epithelia and stromal fibroblasts
355 by identifying ligand-receptor pairs that were expressed by the two cell types across
356 the major phases/subphases of the cycle (**Table S6**, Method). We note one major
357 feature within the identified ligand-receptor pairs: they are dominated by a diverse
358 repertoire of extracellular matrix (ECM) proteins paired with integrin receptors,
359 suggesting that ECM-integrin interaction is a major route of communication between
360 the two cell types. We were also able to identify key interactions at the WOI such as
361 between LIF and IL6ST, with LIF being a key gene implicated in endometrial
362 receptivity (Evans et al., 2009, 2016; White et al., 2007).

363
364 **Transcriptome signatures in deviating glandular and luminal epithelium**
365 **supports a mechanism for adult epithelial gland formation**

366 In unciliated epithelia, we noticed further segregation of cells (**Figure 5A**) in the
367 direction perpendicular to the overall trajectory of the menstrual cycle. To validate

368 this segregation, we independently performed dimension reduction (tSNE) on cells
369 from each of the major phases (**Figure S10A**), excluding genes associated with cell
370 cycles (**Figure S8**). The results confirm the observed segregations when tSNE was
371 done on all unciliated epithelia (**Figure 5A**).

372
373 To identify the nature of this segregation, we performed differential expression
374 analysis and found genes that consistently differentiated the subpopulations across
375 multiple phases (**Figure 5B**). We examined immunohistochemistry staining of these
376 genes in the Human Protein Atlas (Uhlen et al., 2015) and found that genes
377 upregulated in one population stained intensely in epithelial glands, whereas genes
378 upregulated in the other demonstrated had no to low staining. Moreover, among
379 these genes we found a few that were associated with luminal and glandular epithelia.
380 *ITGA1*, which was reported to be consistently upregulated in glandular epithelia than
381 in luminal epithelia (Lessey et al., 1996), started to differentially express between the
382 two populations at phase 2 and the differential expression persisted for the rest of
383 cycle. *WNT7A*, reported to be exclusively expressed in the luminal epithelium of both
384 humans (Tulac et al., 2003) and mice (Yin and Ma, 2005), overexpressed in the other
385 population in all proliferative phases (**Figure 5C**); *SVIL*, differentially expressed in
386 the same population in all but phase 4, encodes supervillin, which was associated with
387 microvilli structure responsible for plasma membrane transformation on luminal
388 epithelium (Khurana and George, 2008). Taking the above evidence together, the
389 deviating subpopulations can be identified as the glandular and luminal epithelia.

390
391 Within the differentially expressed genes, we also noticed some that were previously
392 reported to be critical for endometrial remodeling and embryo implantation (**Figure**
393 **5C**), and discovered that they were characterized with unique dynamic features. For
394 example, the metallothioneins (*MT1E*, *MT1G*, *MT2A*, *MT1F*) were upregulated in the
395 luminal and glandular cells with a consistent lag in one phase. *LIF*, which was
396 implicated in endometrial receptivity (Evans et al., 2009, 2016; White et al., 2007),
397 was down-regulated in glandular epithelium throughout phase 2, 3, and early phase
398 4. *MMP26*, a metalloproteinase reported to be up-regulated in proliferative
399 endometrium (Ruiz-Alonso et al., 2012), was differentially expressed in glandular
400 epithelia until phase 4. Of note, we observed no such differential expression in phase-
401 defining genes presented in the earlier sections or housekeeping genes (**Figure**
402 **S10B**).

403
404 Compared to the consistent distinction between the ciliated and unciliated epithelia,
405 the deviation between luminal and glandular epithelia at transcriptome level was
406 subtler and more dynamic: it became noticeable at late phase 1 and was most
407 pronounced in phase 2 (**Figure 5A** and **Figure S10A**). This observation is further
408 supported by the dynamics of differentially expressing genes such as *HPGD*, *SULT1E1*,
409 *LGR5*, *VTCN1*, and *ITGA1* (**Figure 5C, D**), among many others (**Figure S10C**), in that
410 the maximum deviation of their expression in luminal and glandular cells was
411 reached in phase 2 (the latest phase before ovulation).

412

413 Functional enrichment analysis of genes overexpressed in the luminal epithelia
414 proliferative phase revealed extensive enrichments in morphogenesis and
415 tubulogenesis which lead to the development of anatomic structures, as well as
416 morphogenesis at the cellular level which lead to differentiation (**Figure 5E**). The
417 Wnt signaling pathway, associated with gland formation during the development of
418 the human fetal uterus, was also enriched in this gene group, along with growth, ion
419 transport, and angiogenesis. On the other hand, the most pronounced feature of the
420 glandular subpopulation in the same phase was a consistently higher fraction of
421 cycling cells compared to their luminal counterparts (**Figure S8C**, left). The co-
422 occurrence of the ceasing of cell cycle activity and maximized deviation between the
423 two subpopulations in phase 2 also suggests that the important role proliferation
424 plays in the process.

425
426 In addition, we identified a third cell group in the first three biopsies on the
427 pseudotime trajectory (ordered by the median of pseudotime of all cells from a
428 woman) (**Figure 5A, S10A, S12**). This cell group is transcriptomically in between
429 luminal and glandular epithelia (**Figure S10D**), expressing markers from both,
430 suggesting either an intermediate state undergoing transition between two
431 populations or a bipotential progenitor state giving rise to both populations. To
432 explore whether our data supports one state over the other, we examined genes that
433 are overexpressed in this cell group over both luminal and glandular epithelia
434 (**Figure S10E**), where we found genes that are of mesenchymal origin, including *CD90*
435 (*THY1*) and fibrillar collagens (*COL1A1*, *COL3A1*) as well as transcriptional factors
436 that are associated with transitions between mesenchymal and epithelial states,
437 including *TWIST1*, slug (*SNAI2*) (reviewed by Zeisberg and Neilson, 2009), and *WT1*
438 (reviewed by Miller-Hodges and Hohenstein, 2011). The downregulation of these
439 genes from the ambiguous cell group to unciliated epithelia later in the pseudotime
440 trajectory suggested that it is a bipotential mesenchymal progenitor population that
441 develops into luminal and epithelia through mesenchymal to epithelial transition
442 (MET). In fact, we observed the transition between epithelial and mesenchymal states
443 in cells both at the earliest and the latest timepoints on the pseudotime trajectory
444 (**Figure 5A**), indicating that the transition peaked both immediately before and after
445 menstruation. This characteristic dynamic is further evidenced by the temporal
446 expression of vimentin (*VIM*), a canonical mesenchymal marker, in unciliated
447 epithelia (**Figure S10F**), where its expression is sustained in phase 1 and 2
448 (menstrual and proliferative phases), repressed in phase 3 and early phase 4 (early-
449 and mid-secretory phases) and rises again in late phase 4 (late-secretory phase).
450 Surprisingly, several previously proposed markers for endometrial cells with
451 clonogenic and mesenchymal characteristics (reviewed by Evans et al., 2016)
452 including *MCAM* (*CD146*) and *PDGFRB* (Schwab and Gargett, 2007) as well as *SUSD2*
453 (Miyazaki et al., 2012) were not significantly upregulated in the ambiguous cell group.

454
455 Adult human endometrial gland formation in menstrual cycles have been proposed
456 to originate from the clonogenic epithelial, or mesenchymal progenitors, or both, in
457 the unshed layer of the uterus (basalis) (Nguyen et al., 2017; W.C. et al., 1997). Our
458 data indicates that endometrial re-epithelization is through MET from mesenchymal

459 progenitors, a process that has been demonstrated in transgenic mouse models
460 (Cousins et al., 2014; Huang et al., 2012; Patterson et al., 2013) but had yet to be
461 observed in human. Our data also shows that following re-epitheliation, endometrial
462 gland reconstruction in adult human endometrium is driven by tubulogenesis in
463 luminal epithelium, which involves the formation of either linear or branched tube
464 structures from a simple epithelial sheet (Hogan and Kolodziej, 2002; Iruela-Arispe
465 and Beitel, 2013)- a mechanism that also contributes to gland formation during the
466 development of human fetal uterus (for review, see Cunha et al., 2017; Robboy et al.,
467 2017). This process is also characterized by proliferation activities that are locally
468 concentrated at glandular epithelium.

469

470 **Relative abundance of other endometrial cell types demonstrates phase-** 471 **associated dynamics**

472 Using the phase definition of unciliated epithelia and stromal fibroblasts, we assigned
473 other endometrial cell types from the same woman into their respective phases and
474 quantified their relative abundance across the cycle (**Figure S11A**). We observed an
475 overall increase in ciliated epithelial cells across proliferative phases and a
476 subsequent decrease in secretory phases as well as a notable rise in lymphocyte
477 abundance from late-proliferative to secretory phases. The change in macrophages
478 was in contrary to previous histological reports (Bonatz et al., 1991; Kamat and
479 Isaacson, 1987); factors such as sampling size for a low abundance cell type and
480 sampling bias in choice of spatial locations in microscopic observations of the tissue
481 may have caused the discrepancy and should be taken into account for future studies.

482

483 **Decidualization in natural human menstrual cycle is characterized by direct** 484 **interplay between lymphocytes and stromal fibroblasts**

485 Infiltrating lymphocytes were reported to play essential roles in decidualization
486 during pregnancy, where they were primarily involved in decidual angiogenesis
487 and regulating trophoblastic invasion (Hanna et al., 2006). Their functions in
488 decidualization during the natural human menstrual cycle, however, remain to be
489 defined. The dramatic increase in lymphocytes abundance in the early secretory
490 phase in our data strongly suggests their involvement in decidualization (**Figure**
491 **S11A**). We therefore characterized their transcriptomic dynamics across the
492 menstrual cycle to explore their roles and their interactions with other endometrial
493 cell types during decidualization.

494

495 Compared to their counterparts in non-decidualized endometrium (i.e. secretory
496 (phase 3) and proliferative phases), lymphocytes in decidualized endometrium
497 (phase 4) in natural menstrual cycle have increased expression of markers that are
498 characteristic of uterine NK cells during pregnancy (*CD69*, *ITGA1*, *NCAM1/CD56*)
499 (**Figure S11B**). More interestingly, they express a more diverse repertoire of both
500 activating and inhibitory NK receptors (NKR) responsible for recognizing major
501 histocompatibility complex (MHC) class I molecules (**Figure 6A**). We observed
502 lymphocytes expressing both NK and T cell markers and those expressing only NK
503 markers (**Figure S11B**), and therefore classified them as “*CD3+*” and “*CD3-*” cells
504 based on their expression of markers characteristic of T cells (**Figure S11B**).

505 Particularly, for both “*CD3+*” and “*CD3-*” cells, a noticeable rise in the fraction of cells
506 expressing *CD56*, the canonical NK marker during pregnancy, occurs as early as the
507 tissue transitioned from proliferative to secretory phase (**Figure S11C**), suggesting
508 again that decidualization was initiated before the opening of the WOI.

509
510 We next identified genes that are dynamically changing in the immune cells across
511 the menstrual cycle and characterized those that are associated with immune
512 functionality (**Figure 6B**). In “*CD3-*” cells, we observed a significant rise in cytotoxic
513 granule genes in decidualized endometrium (phase 4), with the exception of *GNLY*. In
514 “*CD3+*” cells, this rise in cytotoxic potential is manifested by an increase in *CD8*, while
515 the elevation in cytotoxic granule genes is only moderate. For both “*CD3+*” and “*CD3-*”
516 cells, the increase in IL2 receptors expression is noticeable in phase 4. Equally
517 notable are genes involved in IL2 elicited cell activation. As for the
518 cytokine/chemokine repertoire, “*CD3-*” cells in decidualized endometrium express a
519 high level of chemokines. Their “*CD3+*” counterparts, although expressing a more
520 diverse cytokine repertoire, demonstrate much lower chemokine expression. Lastly,
521 both “*CD3+*” and “*CD3-*” cells in decidualized endometrium have negligible expression
522 in angiogenesis associated genes (**Figure 6B**), contrary to their counterparts during
523 pregnancy.

524
525 Intriguingly, decidualized stromal fibroblasts upregulate immune-related genes that
526 reciprocated those upregulated in phase 4 immune cells. With the diversification of
527 NKR observed in immune cells in the decidualized endometrium (**Figure 6A**), we
528 observed an overall elevation in MHC class I genes in decidualized stromal fibroblasts
529 (**Figure 6C**), including *HLA-A* and *HLA-B*, which are recognized by activating NKR, as
530 well as *HLA-G*, recognized by inhibitory NKR. Worth noting was concurrent
531 upregulation of *HIVEP2* (**Figure S6D**), a TF responsible for MHC class I genes
532 upregulation. With the IL2-elicited activation observed in immune cells in the
533 decidualized endometrium (**Figure 6B**), we noticed not only the elevation of *IL15*
534 (plays similar roles as *IL2*) in decidualized stromal fibroblasts, as well as IL15-
535 involved pathways that regulate lymphocyte activation and proliferation (**Figure 6C**,
536 function annotation #4). Lastly, an angiogenesis associated pathway is elevated in
537 decidualized stromal fibroblasts, complementing the lack of this functionality
538 observed NK cells in the same phase.

539
540 Using immunofluorescence, we compared the spatial proximity between the
541 identified immune subsets and stromal fibroblasts before (**Figure 6D, E**) and during
542 (**Figure 6F, G**) decidualization. We observed notable increase in the number of both
543 *CD3+* (**Figure 6D, F**) and *CD56+* (**Figure 6E, G**) subsets that are in close proximity
544 with stromal fibroblasts during decidualization compared to pre-decidualization,
545 validating the direct interplays between these immune and stromal subsets during
546 decidualization.

547

548 Discussion

549 In this work, we studied both static and dynamic characteristics of the human
550 endometrium across the menstrual cycle with single cell resolution. At the

551 transcriptomic level, we used an unbiased approach to identify 6 major endometrial
552 cell types, including a ciliated epithelial cell type, and four major phases of
553 endometrial transformation. For the unciliated epithelia and stromal fibroblasts, we
554 used high-resolution trajectories to track their remodeling through the menstrual
555 cycle with minimum bias. Based on these fundamental units and structures, we
556 identified and characterized the receptive state of the tissue with high precision and
557 studied the dynamic cellular and molecular transformations that lead to the receptive
558 state.

559
560 The use of single cell RNAseq to characterize human endometrium is at an early stage.
561 Using endometrial biopsies, a previous study was only limited to the most abundant
562 stromal fibroblasts (late-secretory phase, Krjutskov et al., 2016). Coincident with our
563 work, the feasibility of generating data from other endometrial cell types was also
564 demonstrated by a group using full-thickness uterus (secretory phase, Wu et al.,
565 2018), but cell types were only analyzed at a single time point on a single patient
566 diagnosed with uterine leiomyoma (a gynecological pathology known to cause
567 menstrual abnormalities). Another coincident study modeled decidualization using
568 in vitro cultures of human endometrial stromal fibroblasts and compared the result
569 to the transition of stromal fibroblasts from mid- to late-secretory phase biopsies
570 (Lucas et al., 2018). In our study, biopsies were sampled from 19 healthy women
571 across the entire menstrual cycle. Each of the reported biological phenotypes was
572 supported by multiple biological replicates (i.e. women, **Figure S12**), such that none
573 of the biological results we reported in the study were due to “individual-specific”
574 results, undersampling, or confounded by pathological conditions.

575
576 An important result of our work is the molecular characterization of the ciliated
577 epithelium as a transcriptomically distinct endometrial cell type; these cells are
578 consistently present but dynamically changing in abundance across the menstrual
579 cycle (**Figure S11A**). Although the existence of ciliated cells in the human
580 endometrium has been speculated upon based on microscope studies since the
581 1890’s (Benda, 1894), researchers have been hesitant to include them as an
582 endometrial cell type due to two persisting controversies: 1) whether they exist solely
583 due to pathological conditions (Novak and Rutledge, 1948) and 2) whether they
584 persist across the entire menstrual cycle. The controversies have not been
585 satisfyingly resolved by studies in the 1970’s or recently, due to the confounding
586 gynecological conditions of the examined tissue (Ferenczy et al., 1972; Masterton et
587 al., 1975; Wu et al., 2018) and undersampling (Bartosch et al., 2011). In addition, no
588 standardizable features or signatures were available to identify or isolate this cell
589 type from endometrium. In addition to providing strong evidence that this cell type
590 exists in healthy endometrium throughout the menstrual cycle, we have provided a
591 comprehensive transcriptomic signature along with functional annotations which
592 can serve as molecular anchors for future studies.

593
594 In general, ciliary motility facilitates the material transport (e.g. fluid or particles).
595 The notable increase of ciliated epithelia in second proliferative phase (**Figure S11A**)
596 suggests that they may play a role in sperm transport towards fallopian tubes through

597 the uterine cavity. Moreover, their epithelial lineage identity and their consistent
598 presence in glandular epithelia, as shown by our *in situ* results (**Figure 2**), suggest
599 they may function as the mucociliary transport apparatus, similar to those in the
600 respiratory tract, to transport the secretions and provide proper biochemical milieu.
601 Further elucidation of this role may facilitate more accurate diagnosis of infertility. In
602 addition, we highlight the notably high fraction of genes (~25%) in the derived
603 signature with no functional annotations (**Figure S2, Table S1**). Co-expression of
604 these genes (**Figure 1C, 2**), with known cilium-associated genes and their exclusive
605 activation in ciliated epithelium provides evidence for their cilium-associated
606 functionality, e.g. in signal sensing and transduction (Bisgrove and Yost, 2006, PNAS
607 Mao et al), whose dysfunction can lead to both organ-specific diseases and multi-
608 system syndromes (Bisgrove and Yost, 2006; Fliegauf et al., 2007). Thus, functional
609 studies that link the roles of these un-annotated genes with cilia functionality will also
610 facilitate the understanding of this organelle.

611
612 We identified the opening of the WOI and discovered unique transcriptomic dynamics
613 accompanying both the entrance and the closure of the WOI. It was previously
614 postulated that a continuous dynamics would better describe the entrance of WOI
615 since human embryo implantation doesn't seem to be controlled by a single hormonal
616 factor as in mice (Hoversland et al., 1982; Paria et al., 1993), while discontinuous
617 characteristics were also speculated based on morphological observation of plasma
618 membrane transformation (Murphy, 2004). Our data suggest that the WOI opens with
619 an abrupt and discontinuous transcriptomic transition in unciliated epithelia,
620 accompanied by a more continuous transition in stromal fibroblasts. The abruptness
621 of the transition also suggests that it should be possible to diagnose the opening of
622 the WOI with high precision in clinical practices of *in vitro* fertilization and embryo
623 transfer.

624
625 It is intriguing that the mid- and late- secretory phases fall into the same major phase
626 at the transcriptomic level, especially since the physiological differences between
627 mid- (high progesterone level, embryo implantation) and late-secretory phase
628 (progesterone withdraw, preparing for tissue desquamation) seem to be as large as
629 that between early- to mid-secretory phase, if not larger. In fact, the characteristic
630 transition at the closure of the WOI is largely contributed by the same group of genes
631 that contributed to the abrupt opening of the WOI, except that while at the opening
632 their upregulation is rapid and uniform across all cells, at the closure the
633 downregulation was executed less uniformly and across a longer period of time. From
634 a dynamic perspective, the difference suggests that the transition between mid-to-
635 late secretory phases, although in magnitude may be similar to that between early-
636 to-mid secretory phases, is slower in rate, perhaps reflective of the rate of
637 progesterone withdrawal. From a molecular perspective, the less uniform
638 downregulation of genes suggests that the closure of the WOI may be mediated
639 through paracrine factors and cell-cell communications.

640
641 The abrupt opening of the WOI also allowed us to elucidate the relationship between
642 the WOI and decidualization. As noted earlier, decidualization is the transformation

643 of stromal fibroblasts that is necessary for pregnancy in both human and mouse, and
644 supports developments of implanted embryo. However, contrary to the mouse
645 where decidualization is triggered by implanting embryo(s) (Cha et al., 2012) and
646 thus occurs exclusively during pregnancy, in human, decidualization occurs
647 spontaneously during natural human menstrual cycles independent of the
648 presence of an embryo (Evans et al., 2016). Thus, the relative timing between the
649 WOI and the initiation of decidualization in human is unclear. While histological
650 observation suggests that decidualization starts around mid-secretory phase, our
651 data indicates that decidualization is initiated before the opening of the WOI, and that
652 at the opening of the WOI, decidualized features are widespread in stromal fibroblasts
653 at the transcriptomic level. This lag of morphological signals relative to
654 transcriptomic signals could result from the delay of phenotypic manifestation after
655 transcription either due to inherent delay between transcription and translation or
656 through post-transcriptional modifications.

657
658 We identified transcriptomic signatures in the luminal and glandular epithelia during
659 epithelial gland formation. The original definition of luminal and glandular epithelia
660 was established based on the distinct morphology and physical location between the
661 two. Their distinction at the transcriptome level had not been previously established,
662 and we found markers that differentiate the two across multiple phases of the
663 menstrual cycle. Moreover, we discovered signatures that are differentially up-
664 regulated in glandular and luminal epithelium during the formation of epithelial
665 glands. Epithelial glands create a proper biochemical milieu for embryo implantation
666 and subsequent development of pregnancy. In humans, the mechanism for their
667 reconstruction during proliferative phases, however, is unclear. Previous studies
668 through clonogenic assays reported that the cyclic regeneration of both glandular
669 and luminal epithelia was executed by progenitors with stemness characteristics
670 in the unshed layer of the uterus (basalis) (Huang et al., 2012; Nguyen et al., 2017;
671 W.C. et al., 1997). Our analysis suggests a mechanism that involves MET for re-
672 epithelization, followed by tubulogenesis in the luminal epithelium as well as
673 proliferation activities that were locally concentrated at glandular epithelium for
674 reformation of epithelial glands. Our data however cannot rule out the possibility that
675 cells that re-epitheliate the endometrium are the progeny of previously reported
676 candidates with stemness characteristics.

677
678 Lastly, we provide evidence for the direct interplay between stromal fibroblasts and
679 lymphocytes during decidualization in menstrual cycle. Our analysis suggests that
680 during decidualization in the cycling endometrium, stromal fibroblasts are directly
681 responsible for the activation of lymphocytes through IL2-elicited pathways. The
682 diversification of activating and inhibitory NKR in immune cells and the overall up-
683 regulation of MHC class I molecules in stromal fibroblasts is particularly interesting.
684 During pregnancy, cytotoxic NK cells are tolerant towards the semi-allogeneic fetus
685 (Schmitt et al., 2008). This paradoxical phenomenon was hypothesized to be
686 mediated by 1) the upregulation of non-classical MHC class I molecule (HLA-G) (Apps
687 et al., 2007), the ligand to NK inhibitory receptor, and 2) the downregulation of
688 classical MHC class I molecules (HLA-A, HLA-B) (Moffett-King, 2002; Sivori et al.,

689 2000) that engage with NK activating receptors. Our results demonstrate that similar
690 suppression in NK cells with high cytotoxic potential occurs during natural menstrual
691 cycle, however exerted by decidualized stromal fibroblasts.

692

693 **Conclusion**

694 In summary, we systematically characterized the human endometrium across the
695 menstrual cycle from both a static and a dynamic perspective. The high resolution
696 of the data and our analytical framework allowed us to answer previously
697 unresolved questions that are centered on the tissue's receptivity to embryo
698 implantation. We envision that our findings and the molecular signatures we
699 discovered will provide conceptual foundations and practical molecular anchors
700 for future studies.

701

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711

712 **Author Contributions**

713 WW, WP, FV, CS, and SRQ conceived and designed the study. WW, FV, and IM
714 performed experiments. WW performed single cell experiments, RNAscope
715 experiments and imaging. FV optimized the tissue dissociation protocol. IM
716 performed tissue dissociation and immunofluorescence experiments. PA collected
717 endometrial biopsies. WW and SRQ analyzed the single cell RNAseq data. MM and
718 WW analyzed RNAscope data. WW, FV, CS, and SRQ interpreted the results. WW, FV,
719 CS, and SRQ wrote the manuscript.

720

721 **Declaration of Interests**

722 A patent disclosure has been filed for the study under the inventors SRQ, CS, WW, and
723 FV.

724

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937

938 **Figure title and legends**

939 **Figure 1. Human endometrium consists of six cell types across the menstrual cycle**

940 (A) Dimension reduction (tSNE) on 2149 single cells from 19 healthy human endometria
941 across the menstrual cycle using top 1000 over-dispersed genes across all cells. Top right
942 inset: tSNE on immune cells using top 1000 over-dispersed genes across immune cells only.
943 Boundaries of cell types were defined by DBSCAN on the 2d-tSNEs.

944 (B) Top discriminatory genes for each identified cell type. Shown are differentially expressed
945 genes ($-\log_{10}(p_{\text{adj}} \text{ of a Wilcoxon's rank sum test}) > 50$, $\log_2(\text{FC}) > 2$) that are expressed in
946 $> 85\%$ cells in the given cell type. For each cell type, genes are ordered, from top to bottom,
947 by the ratio of (% cells within the cell type expressing a gene) and (% cells from other cell
948 types expressing the same gene). In red are canonical markers for the cell type.

949 (C) Cellular components and biological processes enriched in top discriminatory genes for
950 ciliated epithelium.

951 (FC: foldchange, FDR: false discovery rate, p_{adj} : adjusted p-value)

952 See also Figures S1 and S2

953

954 **Figure 2. Validation of markers, epithelial lineage, and spatial visualization for** 955 **endometrial ciliated cells using RNA and antibody co-staining**

956 (A-D) Representative images of human endometrial glands (A, C) and lumen (B, D) on day 17
957 (A, B) and day 25 (C, D) of the menstrual cycle.

958 (Single CDHR3 and C11orf88 RNA molecules appear as dots in cyan and magenta,
959 respectively. FOXJ1 antibody staining is in green and nuclei in gray. Scale bar: 50 μm . Zoomed-
960 in areas contain triple-expressing cells in the white dashed box in the corresponding panel)

961 (E) Integrated intensity of FOXJ1 antibody for double RNA positive (++) and negative (- -)
962 cells from all images on day 17 (left) and day 25 (right) of the menstrual cycle.

963 (+: cells expressing ≥ 4 RNA molecules of both markers. Horizontal line: median. ****: p-value
964 of a Wilcoxon's rank sum test < 0.0001)

965 See also Figures S1 and S2

966

967 **Figure 3. Constructing single cell resolution trajectory of endometrial transformation**
968 **across the human menstrual cycle**

969 (A) Pseudotime assignment of unciliated epithelia (epi) and stromal fibroblasts (str) across
970 the trajectory of a menstrual cycle. For both cell types, the trajectory was constructed as a
971 principal curve on the 2d-tSNE obtained using “time-associated” genes (see text and Method
972 for details). Pseudotime was assigned as a cell’s order along the trajectory based on its
973 projection on the curve. 1-4: the four major phases consistently identified using either whole
974 transcriptome (Figure S3A) or time-associated genes (Figure S3C). Start: pseudotime=0,
975 assigned based on the clinical definition of the start of a cycle.

976 (B) Correlation of pseudotime and time (day) for epi and str.

977 (C) Correlation of pseudotimes of epi and str from the same woman.

978 (In (B-C), dot and error bar are the median and the median absolute deviation of all epi or str
979 from a woman, respectively. Day: the day of menstrual cycle, i.e. the number of days after the
980 onset of last menstrual bleeding)

981 See also Figures S3-S5

982

983 **Figure 4. Temporal transcriptome dynamics of endometrial transformation across the**
984 **human menstrual cycle**

985 Exemplary phase and sub-phase defining genes, and the relationship between
986 transcriptomically defined and histologically defined (canonical) endometrial phases for

987 (A) unciliated epithelia (epi) and

988 (B) stromal fibroblasts (str) across

989 (C) a human menstrual cycle.

990 Shown are genes that differentially expressed ($-\log_{10}(p_{\text{adj}} \text{ of a Wilcoxon's rank sum test}) > 10$, $\log_2(\text{FC}) > 1$) in a phase or sub-phase, and are not differentially expressed between
991 luminal and glandular cells in the phase where the gene peaked. Genes were further filtered
992 for their potential to be deconvoluted between unciliated epithelia and stromal fibroblasts
993 in bulk data to obtain those that are temporally in synchrony between the two cell types or
994 those with negligible expression in one cell type across the cycle but significant phase-specific
995 dynamics in another.

996 (Cells (column) were ordered by pseudotime. Dashed line: continuous transition. WOI:
997 window of implantation. pro: proliferative. sec: secretory)

998 See also Figures S6-S9

1000

1001 **Figure 5. Deviating subpopulations of unciliated epithelia across the human menstrual**
1002 **cycle**

1003 (A) Subpopulations of unciliated epithelia. The color-coded classification was based on
1004 dimension reduction independently performed for each phase or subphase shown in Figure
1005 S10A (gray: cells that are transcriptomically in between the two subpopulations).

1006 (B-D) Dynamics of genes

1007 (B) that differentially expressed ($-\log_{10}(p_{\text{adj}} \text{ of a Wilcoxon's rank sum test}) > 0.05$,
1008 $\log_2(\text{FC}) > 2$) between the two subpopulations across multiple phases,

1009 (C) that were previously reported to be implicated in endometrial remodeling or embryo
1010 implantation, and

1011 (D) that exemplified those that reached maximum differential expression in phase 2.

1012 (In (B-D), cells are ordered by pseudotime. Dashed lines: boundaries between the 4 phases)

1013 (E) Gene ontology enrichment (FDR < 0.05) in genes overexpressed in luminal epithelia
1014 during proliferative phases. Shown are the enriched GO hierarchies. For each hierarchy,
1015 shown are the term with the highest specificity (indented) and the term with the highest
1016 significance value (un-indented).

1017 See also Figure S10

1018

1019 **Figure 6. Endometrial lymphocytes across the human menstrual cycle and their**
1020 **interactions with stromal fibroblasts during decidualization**

1021 (A) Expression of inhibitory and activating NK receptors (NKR) in endometrial lymphocytes.
1022 Cells (columns) were sorted based on percent NKR expressed.

1023 (B) Dynamics of genes related to lymphocyte functionality (shown are the medians). “CD3+”
1024 and “CD3-” cells are classified based on the expression of markers characteristic of T
1025 lymphocytes shown in Figure S11B.

1026 (C) Functional annotation (left) and expression (right) of genes that were overexpressed in
1027 decidualized stromal fibroblasts (phase 4) that are implicated in immune responses.

1028 (D-G) Spatial distribution of CD3 (D, F, open arrow) and CD56 (E, G, arrow) positive immune
1029 cells and stromal fibroblasts (arrowhead) before (D, E, day 17) and during (F, G, day 24)
1030 decidualization.

1031 See also Figure S11

1032

1033 **STAR Methods**

1034 **CONTACT FOR REAGENT AND RESOURCE SHARING**

1035 Further information and requests for resources and reagents should be directed to
1036 and will be fulfilled by the Lead Contact, SRQ (quake@stanford.edu).

1037

1038 **SUBJECT DETAILS**

1039 All procedures involving human endometrium were conducted in accordance with
1040 the Institutional Review Board (IRB) guidelines for Stanford University under the IRB
1041 code IRB-35448 and IVI Valencia, Spain under the IRB code 1603-IGX-016-CS.
1042 Collection of endometrial biopsies was approved by the IRB code 1603-IGX-016-CS.
1043 There were no medical reasons to obtain the endometrial biopsies. Healthy ovum
1044 donors were recruited in the context of the research project approved by the IRB.
1045 Informed written consent was obtained from each donor in her natural menstrual
1046 cycle (no hormone stimulation) before an endometrial biopsy was performed. De-
1047 identified human endometrium was obtained from women aged 18-34, with regular
1048 menstrual cycle (3-4 days every 28-30 days), BMI ranging 19-29 kg/m² (inclusive),
1049 negative serological tests for HIV, HBV, HCV, RPR and normal karyotype. Women with
1050 the following conditions were excluded from tissue collection: recent contraception
1051 (IUD in past 3 months; hormonal contraceptives in past 2 months), uterine pathology
1052 (endometriosis, leiomyoma, or adenomyosis; bacterial, fungal, or viral infection), and
1053 polycystic ovary syndrome.

1054

1055 **METHOD DETAILS**

1056 **Endometrium tissue dissociation and population enrichment**

1057 A two-stage dissociation protocol was used to dissociate endometrium tissue and
1058 separate it into stromal fibroblast and epithelium enriched single cell suspensions.
1059 Prior to the dissociation, the tissue was rinsed with DMEM (Sigma) on a petri dish to
1060 remove blood and mucus. Excess DMEM was removed after the rinsing. The tissue
1061 was then minced into pieces as small as possible and dissociated in collagenase A1
1062 (Sigma) overnight at 4 °C in a 50 mL falcon tube at horizontal position. This primary
1063 enzymatic step dissociates stromal fibroblasts into single cells while leaving epithelial

1064 glands and lumen mostly undigested. The resulting tissue suspension was then briefly
1065 homogenized and left un-agitated for 10 mins in a 50 mL Falcon tube at vertical
1066 position, during which epithelial glands and lumen sedimented as a pellet and stromal
1067 fibroblasts stayed suspended in the supernatant. The supernatant was therefore
1068 collected as the stromal fibroblast-enriched suspension. The pellet was washed twice
1069 in 50 mL DMEM to further remove residual stromal fibroblasts. The washed pellet
1070 was then dissociated in 400 μ L TrypLE Select (Life technology) for 20 mins at 37°C,
1071 during which homogenization was performed via intermittent pipetting. DNaseI (100
1072 μ L) was then added to the solution to digest extracellular genomic DNA. The digestion
1073 was quenched with 1.5 mL DMEM after 5 min incubation. The resulting cell
1074 suspension was then pipetted, filtered through a 50 μ m cell strainer, and centrifuged
1075 at 1000 rpm for 5 min. The pellet was re-suspended as the epithelium-enriched
1076 suspension.

1077

1078 **Single cell capture, imaging, and cDNA generation**

1079 For cell suspension of both portions, live cells were enriched via MACS dead cell
1080 removal kit (Miltenyi Biotec) following the manufacturer's protocol. The resulting cell
1081 suspension was diluted in DMEM into a final concentration of 300-400 cells/ μ L before
1082 being loaded onto a medium C1 chip for mRNA Seq (Fluidigm). Live dead cell stain
1083 (Life Technology) was added directly into the cell suspension. Single cell capture,
1084 mRNA reverse-transcription, and cDNA amplification were performed on the
1085 Fluidigm C1 system using default scripts for mRNA Seq. All capture site images were
1086 recorded using an in-house built microscopic system at 20x magnification through
1087 phase, GFP, and Y3 channels. 1 μ L pre-diluted ERCC (Ambion) was added into the lysis
1088 mix, resulting in a final dilution factor of 1:80,000 in the mix.

1089

1090 **Single cell RNAseq library generation**

1091 Single-cell cDNA concentration and size distribution were analyzed on a capillary
1092 electrophoresis-based automated fragment analyzer (Advanced Analytical).
1093 Tagmented and barcoded cDNA libraries were prepared only for cells imaged as
1094 singlet or empty at the capture site and with > 0.06 ng/ μ L cDNA generated. Library
1095 preparation was performed using Nextera XT DNA Sample Preparation kit (Illumina)
1096 on a Mosquito HTS liquid handler (TTP Labtech) following Fluidigm's single cell
1097 library preparation protocol with a 4x scale-down of all reagents. Dual-indexed
1098 single-cell libraries were pooled and sequenced in pair-end reads on Nextseq
1099 (Illumina) to a depth of 1-2 $\times 10^6$ reads per cell. bcl2fastq v2.17.1.14 was used to
1100 separate out the data for each single cell by using unique barcode combinations from
1101 the Nextera XT preparation and to generate *.fastq files.

1102

1103 **Tissue preparation for *in situ* hybridizations**

1104 Endometrial tissues were fixed for 24-48 h in 4% paraformaldehyde (PFA) at room
1105 temperature, trimmed, embedded in paraffin, and sectioned into 3 μ m in thickness
1106 onto APES-coated slides.

1107

1108 **Immunofluorescence**

1109 Tissue sections were baked at 60 °C for 1h, deparaffined with Histoclear and

1110 rehydrated with ethanol series. Antigen retrieval was performed by boiling tissue
1111 sections in 10 mM sodium citrate buffer (pH 6.0) for 20 min, followed by immediate
1112 cool down in cold water for 10 min. Tissue permeabilization was done with 0.25%
1113 Triton X 100 in PBS for 5 min, followed by wash in 0.05% Triton X100 in PBS for 5
1114 min twice. Non-specific binding was blocked with 5% BSA-0.05% Triton X100-4%
1115 goat serum in PBS for 1h at room temperature. Tissue sections were then incubated
1116 with primary antibodies over night at 4 °C and secondary antibodies for 1h at room
1117 temperature. Primary antibodies used and dilution ratios are Vimentin (2 µg/mL,
1118 ab8978, Abcam), Prolactin (1:10, PA5-26006, Thermo Fischer Scientific), CD3 (1:100,
1119 ab5690, Abcam), CD56 (1:50, ab133345, Abcam). Secondary antibodies used and
1120 dilution ratios are: Goat anti-mouse IgG (H+L) Superclonal™ Alexa Fluor 488 (1:200,
1121 A27034, Thermo Fischer Scientific) and Goat anti-rabbit IgG (H+L) Superclonal™
1122 Alexa Fluor 555 (1:200, A27039, Thermo Fisher Scientific). All sections were
1123 counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) (Thermo Fisher
1124 Scientific) and mounted with Aquatex® (Merck-Millipore). Images were captured
1125 with a confocal microscope (FV1000, Olympus) at 20X and 60X magnification with oil
1126 immersion and analyzed using Imaris (Bitplane).

1127

1128 **RNAscope for ciliated cells**

1129 Combined RNA and antibody *in situ* hybridizations were performed according to the
1130 manufacturer's technical note "RNAscope Multiplex Fluorescent v2 Assay combined
1131 with Immunofluorescence" for FFPE samples (Advanced Cell Diagnostics). 15 min
1132 and 30 min incubation were used for target retrieval and Protease Plus treatment,
1133 respectively. RNA probes (Advanced Cell Diagnostics) with the following channel
1134 assignment (C), fluorophore, and dilution in TSA buffer were used: CDHR3 (C1,
1135 cyanine 3, 1:1500), C11orf88 (C2, cyanine 5, 1:750); C20orf85 (C1, cyanine 3,
1136 1:1500), FAM183A (C2, cyanine 5, 1:1500). Tissue sections were blocked with
1137 SuperBlock (PBS) blocking buffer (Fisher Scientific) for 30 min at room temperature,
1138 incubated in anti-human FOXJ1 (1:500, eBioscience) over night at 4 °C and goat anti-
1139 mouse IgG secondary antibody (1:500, Life Technologies) for 2 h at room
1140 temperature. All sections were mounted with Prolong Diamond Antifade Mountant
1141 (Thermo Fisher Scientific). Imaging was carried out on an Axio-plan epifluorescence
1142 microscope equipped with an AxioCam 506 mono camera (Zeiss) using a 20x/0.8
1143 Plan-Apochromat objective (Zeiss). For each sample, 8-10 fields of view were
1144 captured with 10-15 z-stacks.

1145

1146 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1147 **Single cell RNAseq data analysis**

1148 Raw reads in the *.fastq files were trimmed to 75bp using fastq, aligned to Ensembl
1149 human reference genome GRCh38.87 (dna.primary_assembly) using STAR (Dobin et
1150 al., 2013) with default parameters, duplicate-removed using picard MarkDuplicates.
1151 Aligned reads were converted to counts using HTSeq (Anders et al., 2015) and
1152 Ensembl GTF for GRCh38.87 under the setting -m intersection-strict \-s no.
1153 Downstream data analysis was performed in R and Java. For each cell, counts were
1154 normalized to log transformed reads per million (log2(rpm+1)) by the equation

1155 $\log_2(rpm + 1) = \log_2(1 + \frac{1e06 * ct_{ij}}{\sum ct_i})$ where i is for cell i and j for gene j .

1156

1157 **Quality filtering of single cells**

1158 For quality filtering, fraction of reads mapped to ERCC (f_{ERCC}) was used as the quality
1159 metric and empirical cumulative distribution of f_{ERCC} in empty capture sites recorded
1160 on the C1 chip was calculated and used as the null model ($ecdf_{null}$). Single cells
1161 retained for downstream analysis were those with ($ecdf_{null}(f_{ERCC})$) < 0.05. 2149
1162 cells were retained for downstream analysis.

1163

1164 **Cell heterogeneity analysis**

1165 Over-dispersion of genes was calculated as $\frac{CV_i^2}{CV_e^2}$, where CV_i^2 is the squared variation of
1166 coefficient of gene i across cells of interest and CV_e^2 is the expected squared variation
1167 of coefficient given mean, fitted using non-ERCC counts. All pairwise distances
1168 between cells were calculated as (1-Pearson's correlation). Dimensional reduction
1169 was performed using the R implementation of tSNE (Rtsne).

1170

1171 **Differential expression analysis**

1172 To obtain differentially expressed genes for a cell type or state, for each gene,
1173 Wilcoxon's rank sum test (Mann and Whitney, 1947) was performed and fold change
1174 (FC, dummy variable = 1E-02) was calculated between cells within a cell type / state
1175 and the cells from other cell types / states. P-values obtained from the Wilcoxon's
1176 rank sum test were adjusted for multiple comparisons by Benjamini-Hochberg's
1177 procedure (Benjamini and Hochberg, 1995) to obtain p.adj. To evaluate the
1178 "sensitivity" and "specificity" of a gene in identifying a cell type / state, we also
1179 calculated the percent of cells within the cell type/state of interest that are expressing
1180 the gene (pct_{in}) and the percent of cells from other cell types / states expressing the
1181 gene (pct_{out}), as well as the ratio between the pct_{in} and pct_{out}.

1182

1183 **Gene ontology functional enrichment**

1184 Functional enrichment analysis was performed using Gene Ontology Enrichment
1185 Analysis (<http://www.geneontology.org>) and each enriched ontology hierarchy
1186 (FDR<0.05) was reported with two terms in the hierarchy: 1) the term with the
1187 highest significance value and 2) the term with the highest specificity.

1188

1189 **Enrichment of "time-associated" genes via mutual information (MI) based approach**

1191 The "time-associatedness" of a gene was calculated as the MI between the expression
1192 of a gene and time (or pseudotime) using the Java implementation of ARACNe-AP
1193 (Lachmann et al., 2016). For each gene, $MI_i = MI((e_{1i}, e_{2i}, \dots, e_{ni}), (t_1, t_2, \dots, t_n))$, where i is
1194 for gene i , e_{ni} is for expression of gene i in cell n , and t_n is the time (or pseudotime)
1195 annotation of cell n . The statistical significance of the MI_i was evaluated using the null
1196 model where the time (or pseudotime) annotation was permuted for 1000 times
1197 with respect to cells, based on which an empirical cumulative distribution function
1198 ($ecdf_{null,i}$) between the expression of gene i and the permuted time (or pseudotime)

1199 was constructed using R function `ecdf`. The p-value for MI_i was calculated as (1-
1200 $ecdf_{null,i}(MI_i)$). The p-values were then adjusted for multiple comparisons by
1201 Benjamini-Hochberg's procedure (Benjamini and Hochberg, 1995) to obtain FDR for
1202 each gene.

1203

1204 **Smoothing of "time-associated" genes and assignment into characteristic** 1205 **phases**

1206 To estimate the pseudotime at which a gene reached maximum expression
1207 ($pseudotime_{max}$), smoothing of gene expression was performed with respect to
1208 pseudotime using the R function `smooth.spline` (`spar=1`) and the pseudotime(s) at
1209 which a smoothed curve reached local maximum was estimated using the R function
1210 `peaks` and inflection point estimated using a custom R script.

1211

1212 Characteristic signatures for phase 1-4 (**Table S4**) were identified by assigning each
1213 pseudotime-associated gene we identified (**Figure S5A, B**) to the phase where its
1214 peak expression occurred (i.e. $pseudotime_{max}$)

1215

1216 **Characterization of dynamics of transcriptional factors (TF) and genes** 1217 **encoding secretory proteins (sec genes) across the menstrual cycle**

1218 We define a dynamic TF/sec gene (**Figure S6**) as a "time-associated" gene (**Figure**
1219 **S5B**) that is annotated as a transcriptional regulator/encoding a secretory protein by
1220 the Human Protein Atlas (Uhlen et al., 2015). Dynamic TFs/sec genes were first
1221 categorized into major groups using hierarchical clustering on smoothed and [0,1]
1222 normalized curves. In each group, TFs/sec genes were ordered by the pseudotime
1223 where a peak or a major peak (for curves with two peaks) occurred, and ties were
1224 broken by the pseudotime where the inflection point occurred.

1225

1226 **Cell cycle analysis**

1227 We took a two-step approach in identifying cycling cells and defining endometrium-
1228 specific cell cycle signatures. We first used a published gene set encompassing 43
1229 G1/S and 55 G2/M genes (Tirosh et al., 2016), representing the intersection of four
1230 previous gene sets (Kowalczyk et al., 2015; Macosko et al., 2015; Whitfield, 2002), and
1231 calculated a G1/S and a G2/M score for all single cells in unciliated epithelia and
1232 stromal fibroblasts, respectively, following the scoring scheme in (Tirosh et al., 2016).
1233 Briefly, cells with at least 2x average expression of either G1/S or G2/M genes than
1234 the average of all cells in the respective cell type was assigned as putative cycling cells.
1235 We next performed Wilcoxon's rank sum test (Mann and Whitney, 1947) between the
1236 putative cycling cells and the rest of cells in the cell type to enrich for cell-cycle
1237 associated transcriptome signatures that were specific to endometrium (**Figure**
1238 **S8A**). To assign cells into G1/S or G2/M phases, we performed dimension reduction
1239 on putative cycling cells using the identified signature, which revealed two major
1240 populations enriched with known G1/S or G2/M signatures. We assigned genes as
1241 either G1/S or G2/M associated by estimating the population at which peak
1242 expression of the gene occurred. We then recalculated the G1/S and G2/M scores for
1243 each cell using the signature customized for endometrium and finalized the
1244 assignment of G1/S and G2/M cells with at least 2x average G1/S or G2/M expression

1245 with respect to all cells in that cell type.

1246

1247 **Identification of putative ligand-receptor interactions between unciliated** 1248 **epithelia and stromal fibroblasts**

1249 For each identified phase and subphase, the expression of a known ligand or receptor
1250 was evaluated as the percent of unciliated epithelia or stromal fibroblasts expressing
1251 the genes to obtain $p_{(epi, j)}$ and $p_{(str, j)}$, where j is for phase j . A ligand or receptor is only
1252 considered expressed by a cell type in a phase if p is greater than 25%. The interaction
1253 between a ligand-receptor pair is established if when a ligand is expressed in one cell
1254 type and its known receptor is expressed in the other. The ligand- receptor pairing
1255 information was based on the database provided by (Ramilowski et al., 2015). In
1256 **Table S6**, ligand-receptor pairs were sorted, from top to bottom, by the level of
1257 interaction, quantified as the total number of interactions normalized by the total
1258 number of possible interactions between the two cell types within a phase.

1259

1260 **Analysis of RNAscope images**

1261 Z-stacks were projected (maximum intensity projection, MIP) using ImageJ. The
1262 resulting MIP images were analyzed using CellProfiler 3.0.0 as follows: 1) Correct
1263 background by subtracting the lower quartile of the intensity measured from the
1264 whole image. 2) Detect cell nuclei using the DAPI channel and cell boundaries using
1265 Voronoi distance (25 pixels) from the nuclei. 3) Enhance RNA signals using a tophat
1266 filter (5 pixels) and detect signals by intensity threshold (0.004 and 0.002 for Cy3 and
1267 Cy5, respectively). 4) Measure antibody intensity for each detected cell. All images
1268 were analyzed in the same way, with no image excluded.

1269

1270 **DATA AND SOFTWARE AVAILABILITY**

1271 The datasets generated and analyzed in the study are available in the NCBI Gene
1272 Expression Omnibus (GEO) and Sequence Read Archive (SRA) and can be accessed
1273 upon request. All custom scripts can be accessed upon request to the Lead Contact.

1274

1275 **Supplemental Figure titles and legends**

1276 **Figure S1. Number of single cells sampled across the human menstrual cycle**

1277 (Day: the day of menstrual cycle, i.e. the number of days after the onset of last
1278 menstrual bleeding)

1279 (Related to Figure 1)

1280 **Figure S2. Classes of functional annotation and their distribution for uniquely** 1281 **expressed genes in ciliated epithelium**

1282 (Related to Figure 1, 2)

1283 **Figure S3. Constructing single cell resolution trajectories of the human menstrual cycle** 1284 **using mutual information (MI) based approach**

1285 (A) Unbiased definition of four major phases of endometrial transformation across the
1286 human menstrual cycle via tSNE on all genes detected for unciliated epithelia (epi)
1287 and stromal fibroblasts (str) (Inset: phase assignment using Ward's hierarchical
1288 agglomerative clustering)

1289 (B) MI between gene expression and time (red) or permuted time (black) (Genes are
1290 ranked by MI)

1291 (C) tSNE using time-associated genes and trajectories of endometrial transformation defined

1292 by principal curves (Inset: phase assignment using Ward's hierarchical agglomerative
1293 clustering)
1294 (Related to Figure 3)
1295 **Figure S4. Discontinuity between phase 3 and 4 unciliated epithelia is supported by**
1296 **different analysis methods**
1297 Dimension reduction of unciliated epithelia (epi, left) and stromal fibroblasts (str, right) via
1298 (A) principal component analysis (linear) and
1299 (B) multidimensional scaling (non-linear) using whole transcriptome information
1300 (C) tSNE on top 50 principal components obtained via principal component analysis on whole
1301 transcriptome information
1302 (Phase 1-4 assignment followed Figure S3C)
1303 (Related to Figure 3)
1304 **Figure S5. Global temporal transcriptome dynamics across the human menstrual cycle**
1305 (A) MI between expressions of pseudotime-associated genes (FDR<1E-05) and pseudotime
1306 (red) or permuted pseudotime (black) for unciliated epithelia (epi) and stromal
1307 fibroblasts (str)
1308 (B) Dynamics of pseudotime associated genes across the menstrual cycle
1309 (Related to Figure 4)
1310 **Figure S6. Dynamic transcriptional factors (TF) across the menstrual cycle**
1311 (A, B) All pseudotime associated TFs for unciliated epithelia (epi, A) and stromal fibroblasts
1312 (str, B) (genes bracketed by red bars are zoomed in C, D)
1313 (C, D) TFs that are associated with the entrance/exit of WOI (bottom) or phase-defining (top)
1314 in epi (C) and str (D)
1315 (E) Expression of TFs that are nuclear hormone receptors for estrogen (ESR1), progesterone
1316 (PGR), glucocorticoid (NR3C1), and androgen (AR)
1317 (For heatmap, TFs were ordered first by the pseudotime of the major peak and then by the
1318 pseudotime of the peak's inflection point)
1319 (Related to Figure 3, 4)
1320 **Figure S7. Dynamic genes for secretory proteins (sec genes) across the menstrual cycle**
1321 (A, B) All pseudotime associated sec genes for unciliated epithelia (epi, A) and stromal
1322 fibroblasts (str, B) (genes bracketed by blue bars are zoomed in C, D)
1323 (C, D) Sec genes that are associated with the entrance/exit of WOI (bottom) in epi (C) and str
1324 (D)
1325 (For heatmap, sec genes were ordered following the same strategy as in Figure S6)
1326 (Related to Figure 4)
1327 **Figure S8. Endometrial cell cycle activities across the menstrual cycle**
1328 (A, B) Endometrial G1/S and G2/M signatures for unciliated epithelia (epi, A) and
1329 stromal fibroblasts (str, B)
1330 (C, D) Distribution (left) and factional dynamics (right) of cycling cells across major phases of
1331 the menstrual cycle
1332 (Related to Figure 4)
1333 **Figure S9. Top phase-defining genes for the two proliferative phases for unciliated epithelia**
1334 (epi) and stromal fibroblasts (str)
1335 (Related to Figure 4)
1336 **Figure S9. Top phase-defining genes for the two proliferative phases for unciliated**
1337 **epithelia (epi) and stromal fibroblasts (str)**
1338 (Related to Figure 4)
1339 **Figure S10. Deviating subpopulations of unciliated epithelia and their transcriptomic**
1340 **signature across the human menstrual cycle**
1341 (A) Dimension reduction (tSNE) on unciliated epithelia in major identified phases/sub-

- 1342 phases across the menstrual cycle
- 1343 (B) Dynamics of phase-defining and housekeeping genes in unciliated epithelial
- 1344 subpopulations across the menstrual cycle (dashed lines: boundaries between the 4
- 1345 phases)
- 1346 (C) Dynamics of differentially expressed genes between the two sub-populations in phase 2
- 1347 (D) Relationship between the ambiguous cell population with luminal and glandular cells in
- 1348 early phase 1. Shown are differentially expressed genes ($-\log_{10}(p_{\text{adj}})$ of a Wilcoxon's
- 1349 rank sum test) >0.05 , $\log_2(\text{FC})>2$) between luminal and glandular epithelia in early
- 1350 phase 1. Cells (column) are ordered by the ratio of (average expression of genes
- 1351 upregulated in the luminal) and (average expression of genes upregulated in the
- 1352 glandular)
- 1353 (E) Genes over-expressed and under-expressed in the ambiguous cell population relative to
- 1354 in luminal and glandular epithelia in early phase 1. Cells (column) are ordered by the
- 1355 ratio of (average expression of genes under-expressed) and (average expression of
- 1356 genes over-expressed)
- 1357 (F) Expression of vimentin (VIM) in unciliated epithelia
- 1358 (Related to Figure 5)
- 1359 **Figure S11. Changes in other endometrial cell types across the human menstrual cycle**
- 1360 (A) Normalized abundance of other endometrial cell types demonstrated phase-associated
- 1361 dynamics. Normalization was done against the total number of unciliated epithelia
- 1362 (for ciliated epithelium) or stromal fibroblasts (for lymphocyte, endothelium,
- 1363 macrophage) captured for each biopsy
- 1364 (B) Expression of major lymphoid lineage markers. Cells (columns) were sorted based on
- 1365 percent NK receptors expressed (as in Figure 6A)
- 1366 (C) Percent CD56+ cells in all "CD3+" and "CD3-" lymphocytes across major phases of the
- 1367 cycle
- 1368 (Related to Figure 6)
- 1369 **Figure S12. Data summary**
- 1370 For each woman:
- 1371 (A) Relationship between the day of menstrual cycle and her assignment to one of the four
- 1372 major phases (Figure 3) based on unbiased single cell analysis
- 1373 (B) Total number of single cells analyzed
- 1374 (C) Distribution of the six cell types
- 1375 (D) Distribution of glandular and luminal epithelia (Gray: cells in the ambiguous cell
- 1376 population in Figure 5A)
- 1377 (Each dot (A, B) or each bar (C, D) represents a woman. From left to right, women were
- 1378 ordered, based on the median pseudotime of her stromal fibroblasts and unciliated
- 1379 epithelia)
- 1380
- 1381 **Table S1. Classification of unannotated markers for ciliated epithelial cells**
- 1382 **Table S2. Dynamic transcriptional factors across the menstrual cycle ordered as in**
- 1383 **Figure S6**
- 1384 **Table S3. Dynamic genes encoding secretory genes across the menstrual cycle**
- 1385 **ordered as in Figure S7**
- 1386 **Table S4. Characteristic pseudotime-associated genes for each major phase identified**
- 1387 **Table S5. Hierarchies of biological processes enriched in characteristic pseudotime-**
- 1388 **associated genes for each major phase**
- 1389 **Table S6. Ligand receptor pairs between unciliated epithelia and stromal fibroblasts**
- 1390 **identified for each phase and subphase identified**

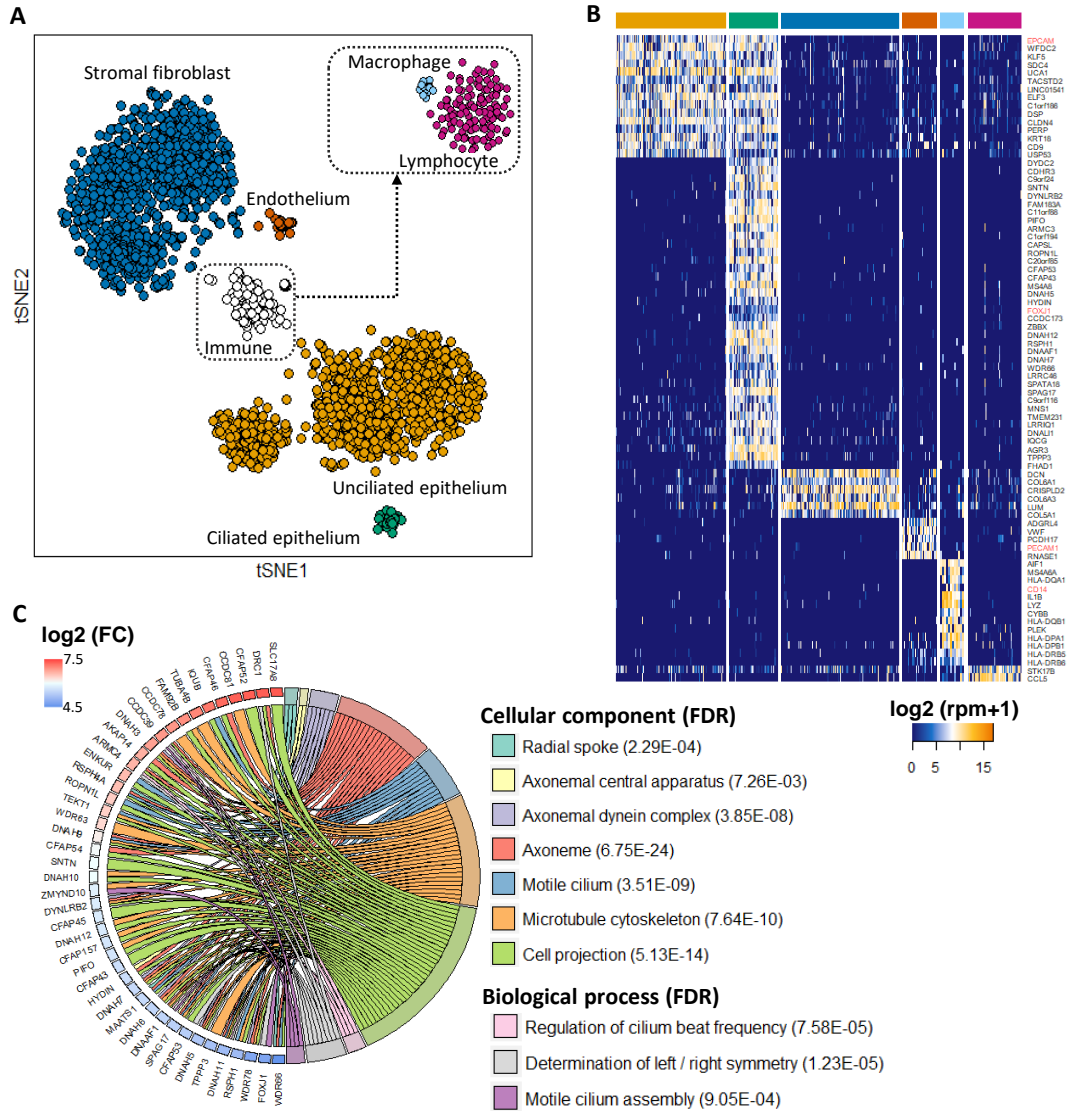


Figure 1. Human endometrium consists of six cell types across the menstrual cycle
 (A) Dimension reduction (tSNE) on 2149 single cells from 19 healthy human endometria across the menstrual cycle using top 1000 over-dispersed genes across all cells. Top right inset: tSNE on immune cells using top 1000 over-dispersed genes across immune cells only. Boundaries of cell types were defined by DBSCAN on the 2d-tSNEs.

(B) Top discriminatory genes for each identified cell type. Shown are differentially expressed genes ($-\log_{10}(p.\text{adj})$ of a Wilcoxon's rank sum test) >50 , $\log_2(\text{FC})>2$ that are expressed in $>85\%$ cells in the given cell type. For each cell type, genes are ordered, from top to bottom, by the ratio of (% cells within the cell type expressing a gene) and (% cells from other cell types expressing the same gene). In red are canonical markers for the cell type.

(C) Cellular components and biological processes enriched in top discriminatory genes for ciliated epithelium.

(FC: foldchange, FDR: false discovery rate, p.adj: adjusted p-value)

See also Figures S1 and S2

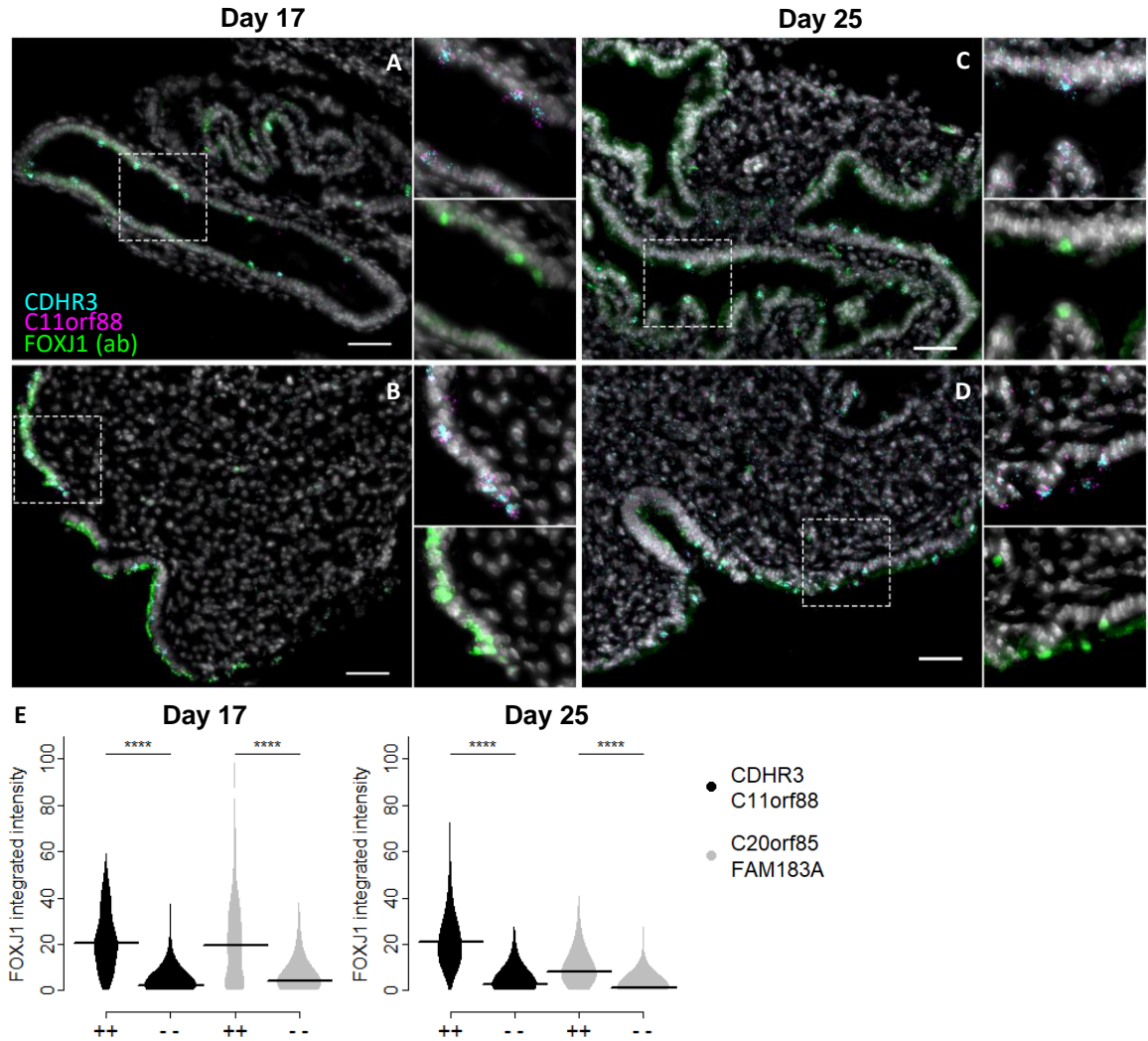


Figure 2. Validation of markers, epithelial lineage, and spatial visualization for endometrial ciliated cells using RNA and antibody co-staining

(A-D) Representative images of human endometrial glands (A, C) and lumen (B, D) on day 17 (A, B) and day 25 (C, D) of the menstrual cycle.

(Single CDHR3 and C11orf88 RNA molecules appear as dots in cyan and magenta, respectively. FOXJ1 antibody staining is in green and nuclei in gray. Scale bar: 50 μ m. Zoomed-in areas contain triple-expressing cells in the white dashed box in the corresponding panel)

(E) Integrated intensity of FOXJ1 antibody for double RNA positive (++) and negative (- -) cells from all images on day 17 (left) and day 25 (right) of the menstrual cycle.

(++: cells expressing ≥ 4 RNA molecules of both markers. Horizontal line: median. ****: p-value of a Wilcoxon's rank sum test < 0.0001)

See also Figures S1 and S2

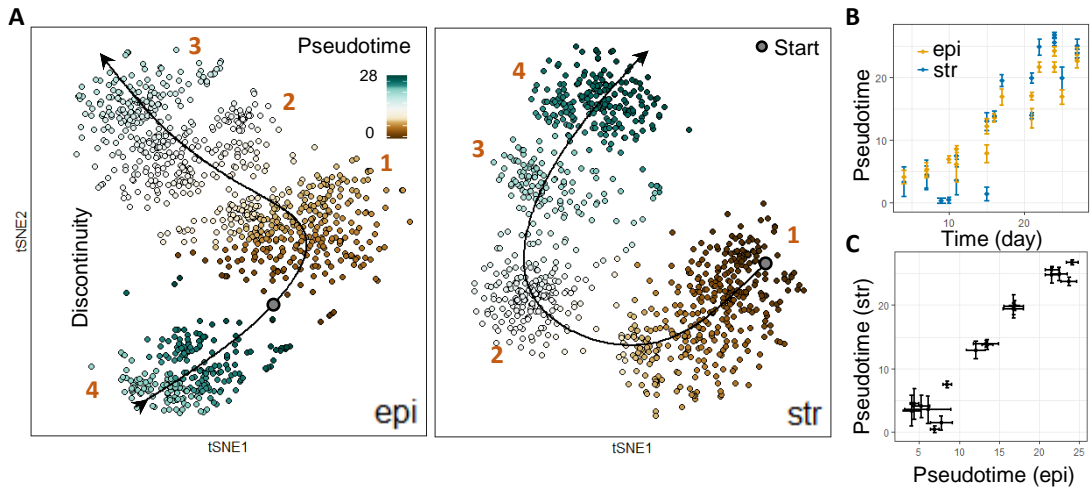


Figure 3. Constructing single cell resolution trajectory of endometrial transformation across the human menstrual cycle

(A) Pseudotime assignment of unciliated epithelia (epi) and stromal fibroblasts (str) across the trajectory of a menstrual cycle. For both cell types, the trajectory was constructed as a principal curve on the 2d-tSNE obtained using “time-associated” genes (see text and Method for details). Pseudotime was assigned as a cell’s order along the trajectory based on its projection on the curve. 1-4: the four major phases consistently identified using either whole transcriptome (Figure S3A) or time-associated genes (Figure S3C). Start: pseudotime=0, assigned based on the clinical definition of the start of a cycle.

(B) Correlation of pseudotime and time (day) for epi and str.

(C) Correlation of pseudotimes of epi and str from the same woman.

(In (B-C), dot and error bar are the median and the median absolute deviation of all epi or str from a woman, respectively. Day: the day of menstrual cycle, i.e. the number of days after the onset of last menstrual bleeding)

See also Figures S3-S5

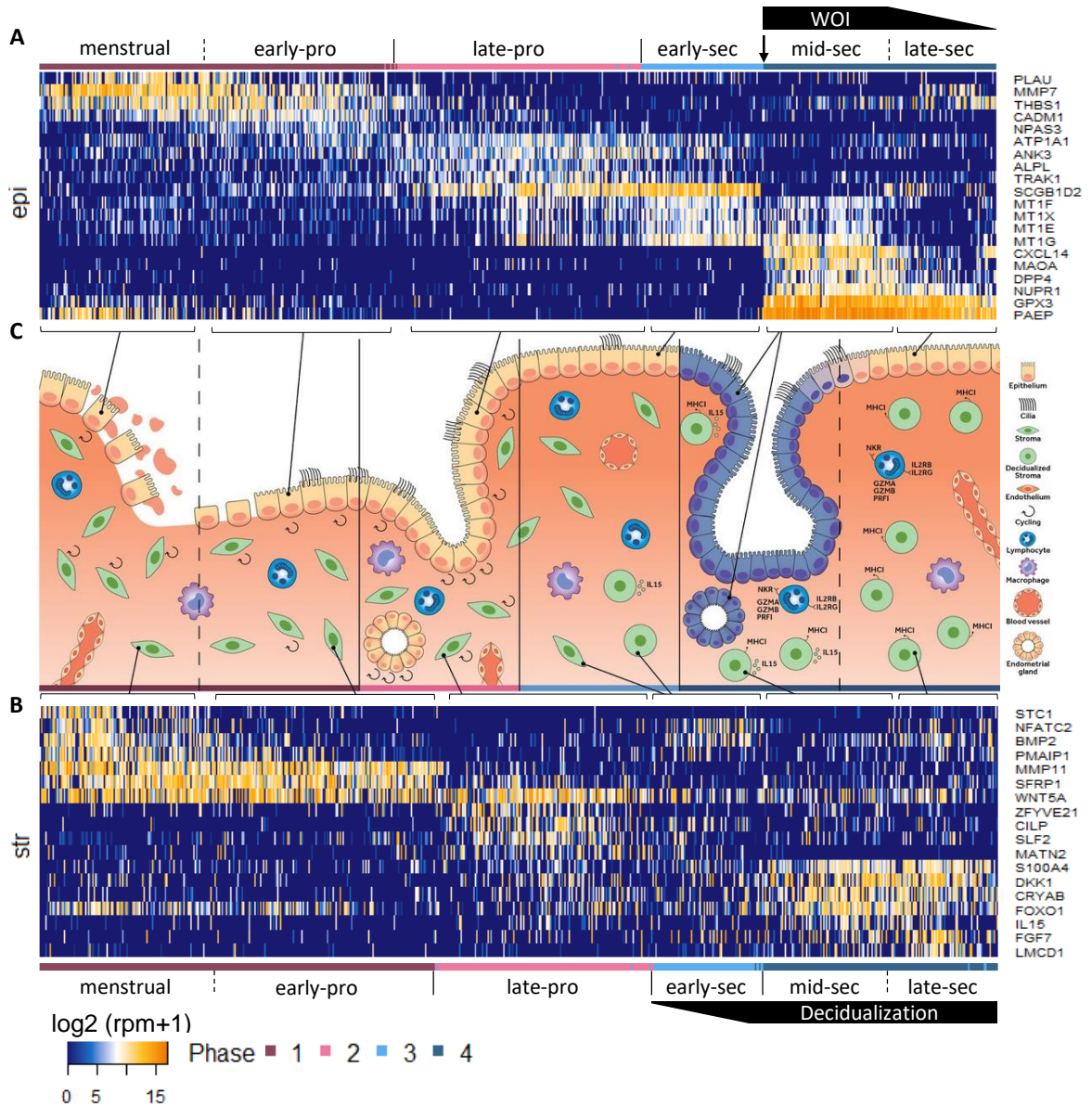


Figure 4. Temporal transcriptome dynamics of endometrial transformation across the human menstrual cycle

Exemplary phase and sub-phase defining genes, and the relationship between transcriptomically defined and histologically defined (canonical) endometrial phases for

- (A) unciliated epithelia (epi) and
(B) stromal fibroblasts (str) across
(C) a human menstrual cycle.

Shown are genes that differentially expressed ($-\log_{10}(p.\text{adj}) > 10$, $\log_2(\text{FC}) > 1$) in a phase or sub-phase, and are not differentially expressed between luminal and glandular cells in the phase where the gene peaked. Genes were further filtered for their potential to be deconvoluted between unciliated epithelia and stromal fibroblasts in bulk data to obtain those that are temporally in synchrony between the two cell types or those with negligible expression in one cell type across the cycle but significant phase-specific dynamics in another.

(Cells (column) were ordered by pseudotime. Dashed line: continuous transition. WOI: window of implantation. pro: proliferative. sec: secretory)

See also Figures S6-S9

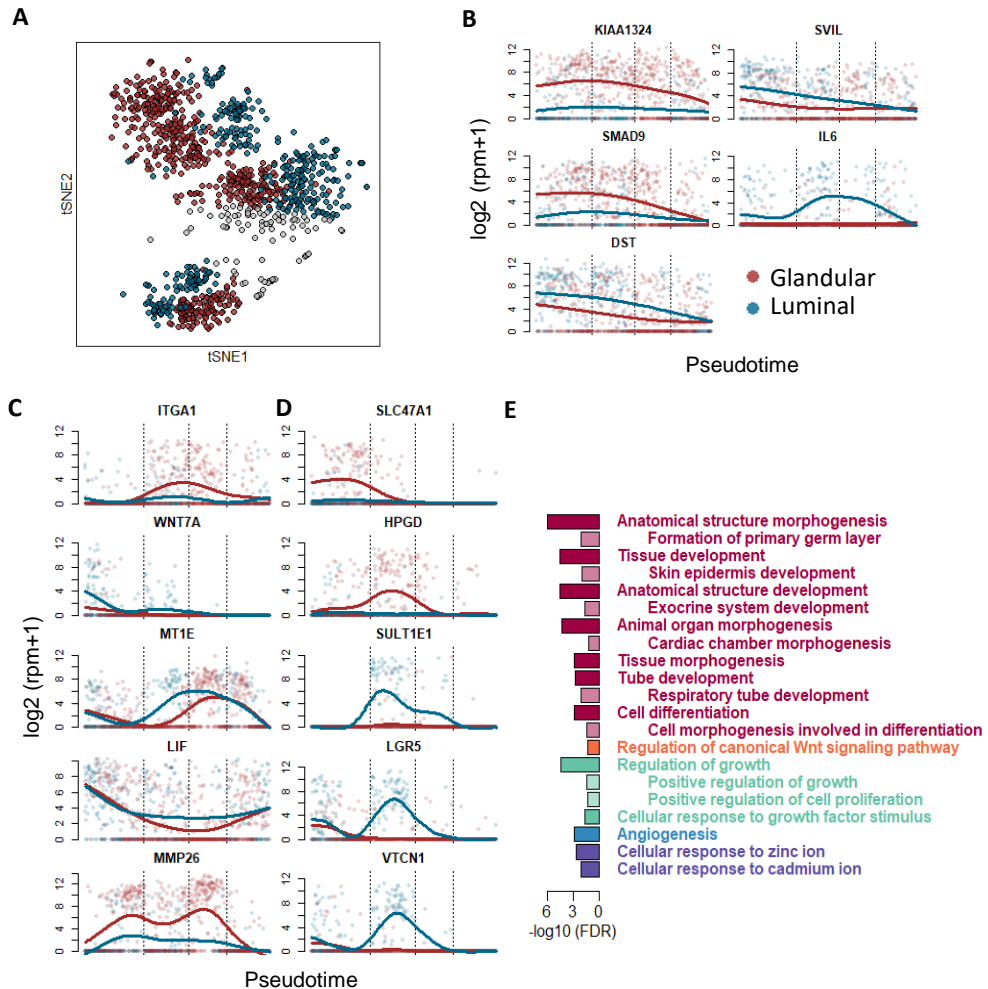


Figure 5. Deviating subpopulations of unciliated epithelia across the human menstrual cycle

(A) Subpopulations of unciliated epithelia. The color-coded classification was based on dimension reduction independently performed for each phase or subphase shown in Figure S10A (gray: cells that are transcriptomically in between the two subpopulations).

(B-D) Dynamics of genes

(B) that differentially expressed ($-\log_{10}(\text{p.adj})$ of a Wilcoxon's rank sum test) >0.05 , $\log_2(\text{FC})>2$ between the two subpopulations across multiple phases,

(C) that were previously reported to be implicated in endometrial remodeling or embryo implantation, and

(D) that exemplified those that reached maximum differential expression in phase 2.

(In (B-D), cells are ordered by pseudotime. Dashed lines: boundaries between the 4 phases)

(E) Gene ontology enrichmented ($\text{FDR}<0.05$) in genes overexpressed in luminal epithelia during proliferative phases. Shown are the enriched GO hierarchies. For each hierarchy, shown are the term with the highest specificity (indented) and the term with the highest significance value (un-indented).

See also Figure S10

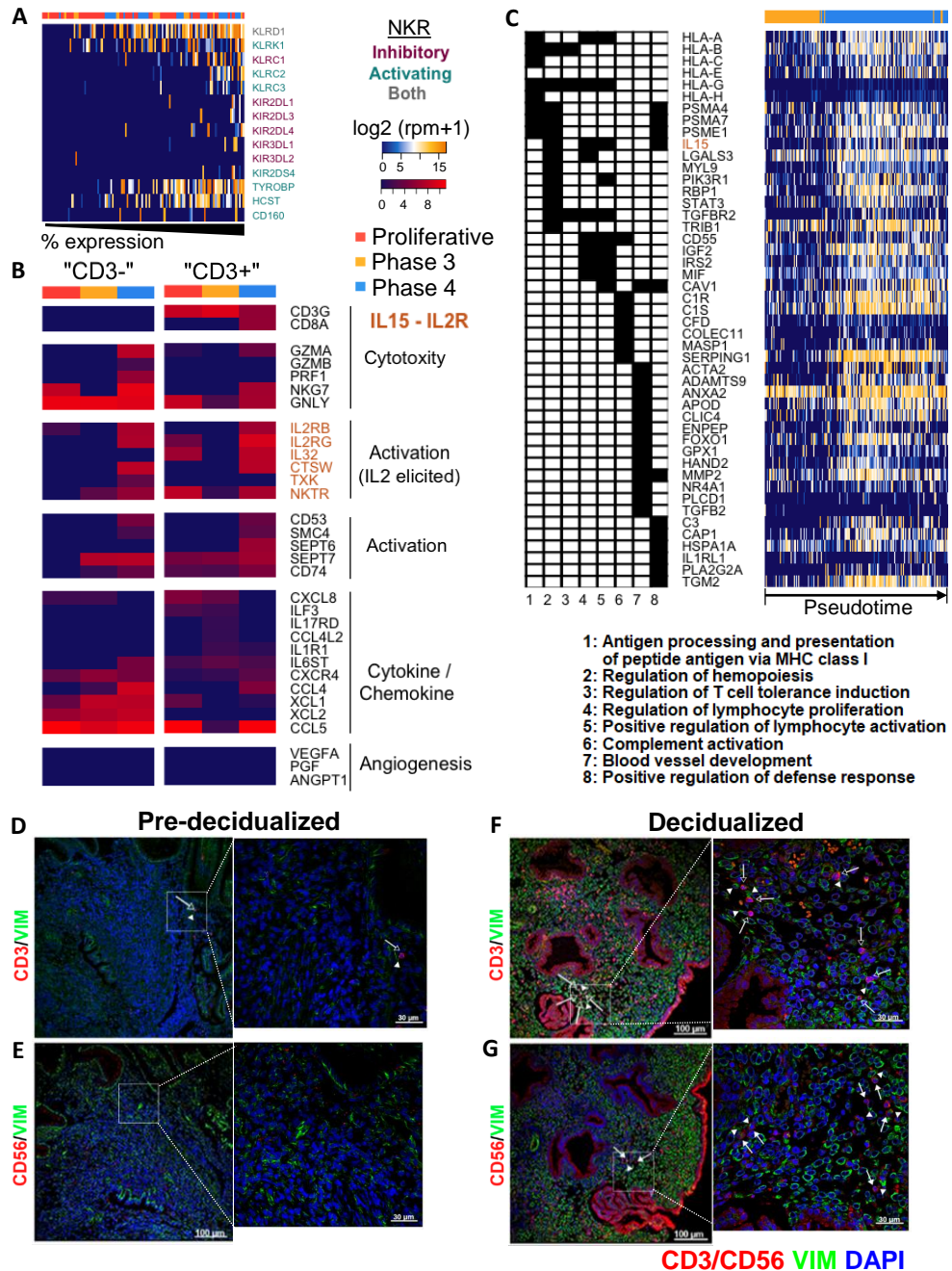


Figure 6. Endometrial lymphocytes across the human menstrual cycle and their interactions with stromal fibroblasts during decidualization

(A) Expression of inhibitory and activating NK receptors (NKR) in endometrial lymphocytes. Cells (columns) were sorted based on percent NKR expressed.

(B) Dynamics of genes related to lymphocyte functionality (shown are the medians). "CD3+" and "CD3-" cells are classified based on the expression of markers characteristic of T lymphocytes shown in Figure S11B.

(C) Functional annotation (left) and expression (right) of genes that were overexpressed in decidualized stromal fibroblasts (phase 4) that are implicated in immune responses.

(D-G) Spatial distribution of CD3 (D, F, open arrow) and CD56 (E, G, arrow) positive immune cells and stromal fibroblasts (arrowhead) before (D, E, day 17) and during (F, G, day 24) decidualization.

See also Figure S11