Decidual cell differentiation is evolutionarily derived from fibroblast activation

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Abstract

32   What the molecular mechanisms underlying the evolutionary origin of novel cell types are is a
33   major unresolved question in biology. The uterine decidual cell is a novel cell type of placental
34   mammals which serves as the interface between maternal and fetal tissues during pregnancy. In
35   this paper, we investigate two models for the nature of the differentiation of decidual cells: first,
36   that it represents a mesenchymal-epithelial transition (MET), and second, that it evolved from
37   wound-induced fibroblast activation (WIFA). Immunocytochemistry and RNA-seq analysis of
38   decidualizing human endometrial fibroblasts cast doubt on the MET hypothesis and instead
39   demonstrate a similarity between decidualization and fibroblast activation, including a central role
40   for TGFB1. Through single-cell RNA-seq, we found a transient myofibroblast-like cell
41   population in the *in vitro* differentiation trajectory of human decidual cells and found that these
42   cells represent a pre-decidual state approaching the inferred transcriptomic transition to decidual
43   cells. We propose an evolutionary developmental model wherein the decidual cell is a novel cell
44   type not equivalent to the myofibroblast, but the process of decidual differentiation itself evolved
45   as an endometrial-specific modification to fibroblast activation in response to the wound caused
46   by embryo implantation.

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Introduction

49   The origin of novel cell types is a major mode for the origin of evolutionary novelties and for the
50   evolution of organismal complexity (Valentine et al., 1994; Arendt, 2008; Wagner et al., 2014;
51   Arendt et al., 2016). The uterine decidual cell (DSC) is a cell type unique to eutherian (Placental)
52   mammals and is critical for the establishment and maintenance of pregnancy in these species
53   (Mess and Carter, 2006; McGowen et al., 2014). DSCs form in response to embryo implantation,
54   or spontaneously during the menstrual cycle in species with menstruation (Gellersen and Brosens,
55   2014), a derived feature of a minority of eutherian species (Critchley et al., 2020). The
56   evolutionary origin of the DSC along the stem lineage of eutherian mammals was an important
57   step to establish extended pregnancy in eutherian mammals (Wagner et al., 2014; Chavan et al.,
58   2016; Chavan et al., 2020). Decidualization, the process through which endometrial stromal
59   fibroblasts (ESFs) differentiate into DSCs, is a novel cellular response to embryo attachment that
60   does not exist in non-eutherian mammals such as opossum (Kin et al., 2014; Erkenbrack et al.,
61   2018). In this paper we investigate the evolutionary roots of the decidualization process and
62   provide evidence that this process is homologous to, i.e. derived from, the process of WIFA,
63   Wound-Induced Fibroblast Activation.

64 In previous work, our lab has provided evidence suggesting that the decidualization pathway is
65 derived from an ancestral cellular stress response: ESFs of the opossum, a marsupial whose
66 reproduction represents the inferred ancestral state for viviparous mammals, do not decidualize
67 (Kin et al., 2014), but respond to decidualization signals by activating a cellular stress response
68 (Erkenbrack et al., 2018). Here we address the question of what the biological nature and context
69 of that stress response was.

70 Resident fibroblasts of mucosal tissues become activated in response to injury, and convert to an
71 activated state called the myofibroblast (Hinz et al., 2007). Myofibroblasts function in wound
72 closure and are marked by the upregulation of proteins such as vimentin (VIM) and alpha-smooth
73 muscle actin (a-SMA) (Darby et al., 2014). Embryo implantation at the beginning of pregnancy
74 causes a local lesion of tissue integrity, i.e. a wound, as the fetus destroys the uterine epithelium
75 and burrows into the stroma (Carson et al., 2000). This traumatic process is the ancestral state for
76 eutherian mammals, as their most recent common ancestor has been inferred to have had
77 hemochorial (invasive) placentation, while non-invasive forms of pregnancy, as observed in
78 hoofed animals and their relatives, evolved later (Wildman et al., 2006; Mess and Carter, 2006;
79 Elliot and Crespi, 2009). Because fibroblasts in all mucosal tissues are activated under similar
80 conditions, it follows that early in the evolution of embryo attachment and implantation, resident
81 fibroblasts of the endometrium would have become activated by the wound caused by embryo
82 attachment. We hypothesize that this routinely-expected stress from tissue injury is the ancestral
83 cell state from which the DSC evolved. We argue that the original function of the DSC was to
84 counteract the inflammation caused by embryo attachment and implantation (Griffith et al., 2017;
85 Chavan et al., 2017; Chavan et al., 2020), an example of stress-induced evolutionary innovation
86 (Wagner et al. 2019).

87 In this study, we present evidence that decidualization involves induction of some of the
88 characteristic WIFA genes. In addition, we report that transforming growth factor β1 (TGFB1)
89 signaling is involved in decidualization: This is a significant observation because TGFB1 is
90 secreted by epithelial cells upon tissue injury and is a major inducer of fibroblast activation
91 (McCartney-Francis and Wahl, 1994; O'Kane and Ferguson, 1997; Singer and Clark, 1999; Wang
92 et al., 2006). Finally, a single-cell analysis of *in vitro* decidualized cells reveals a transient
93 population of myofibroblast-like cells. These results support the conclusion that WIFA is the
94 developmental as well as evolutionary precursor of the decidualization process. The WIFA
95 derivation model predicts evolutionary links and mechanistic parallels between decidualization,
96 wound healing and cancer malignancy (Kshitiz et al., 2019).

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Results and Discussion

Decidualization Is Not a Mesenchymal-Epithelial Transition

Our hypothesis that decidualization is derived from WIFA contradicts an alternative hypothesis, namely that decidualization is a mesenchymal-epithelial transition (MET) (Zhang et al., 2013; Pan-Castillo et al., 2018). This idea was inspired by the cell shape changes during decidualization: ESF have fusiform (spindle-shaped) cell bodies typical of mesenchymal cells whereas DSCs are more rounded or "epithelioid", and express epithelial markers like collagen IV and other basal membrane components (Wewer et al., 1985). Experimental reports of downregulation of genes such as *SNAIL* and *VIM* during decidualization (Zhang et al., 2013; Pan-Castillo et al. 2018) have purported to show that decidualization is a MET-like process. Transcription factors *TWIST1* and *SNAIL* are among the most important regulators of MET and its inverse, EMT, where they shift the balance towards the mesenchymal state (De Herreros et al., 2010; Taube et al., 2010).

To re-test the MET model of decidualization, we analyzed transcriptomes from immortalized human ESF cells decidualized with medroxyprogesterone acetate (MPA, a stable progestin) and 8-Bromo-cAMP (a cell membrane-permeable derivative of cyclic AMP) for 3 and 8 days (Rytkönen et al., 2019). These data show that *TWIST1* and *SNAIL* mRNA expression levels were upregulated (Figure 1A; see also Schroeder et al., 2011), rather than downregulated as expected in a MET. These results were further supported by transcriptomic data from our lab on two primary human ESF cell lines (Figure 1B) (Pavličev et al., 2017), and are consistent with the report of *SNAIL* mRNA being upregulated in the decidual zone of the mouse (Ma et al., 2006). VIM is also an important marker for mesenchymal identity (Bindels et al., 2006; Grande and López-Novoa, 2009). However, contrary to the expectation under the MET model that this mesenchymal marker would decrease, immunocytochemistry showed that VIM protein expression was strongly increased after decidualization (Figure 1C). Consistent with this observation, VIM protein has been reported to be highly expressed *in vivo* in decidual cells of the rat (Glasser and Julian, 1986; Korgun et al., 2007) and human (Can et al., 1995). We note that the *VIM* mRNA level did decrease over decidualization, although at all times exceeded 1000 transcripts per million. We conclude that, during decidualization, the mesenchymal character of endometrial stromal cells is not lost, but enhanced, as documented by the increased VIM protein expression and the upregulation of *TWIST1* and *SNAIL*.

Decidualization has Affinities to Fibroblast Activation

Next, we tested whether decidualization is related to WIFA. The WIFA derivation model predicts a transcriptomic signature of fibroblast activation during decidualization. Signaling and regulatory genes of WIFA include the platelet-derived growth factor receptors A and B (PDGFRA and

133   PDGFRB), cell surface receptors essential for wound healing-related fibroblast activation (Floege
134   et al., 2008; Chen et al., 2011; Miyazaki et al., 2019), fibroblast activation protein (FAP)
135   (Tillmanns et al., 2015), and TGF-β receptor II (TGFBR2), a component of TGF-β signaling
136   pathways (Inamoto et al., 2010). Cultured human ESFs increased mRNA expression of all of
137   these genes in response to deciduogenic stimuli (Figure 1D-H), consistent with the WIFA model.

138   Phenotypic markers for activated fibroblasts also include *ACTA2* (a-SMA), *DES* (desmin) and
139   *FN1* (fibronectin). In accordance with previous findings (Kim et al., 1998; Schwenke et al.,
140   2013), we found a greatly reduced mRNA level during decidualization for all three markers
141   (Figure 1I-K). At the protein level, *in vitro* decidualized cells (8-Br-cAMP+MPA, 6 days) show
142   low levels of a-SMA (Figure 1L). In contrast, control cultures express high levels of a-SMA after
143   6 days of culture in the medium without MPA or 8-Br-cAMP, but not after 2 days (Figure 1L).
144   This pattern may be explained if the fibroblast activation-like stage of decidualization is transient,
145   as indicated by single-cell analysis of *in vitro* decidualization (see below). The high levels of a-
146   SMA in 6 days of no MPA or 8-Br-cAMP culture compared to 2 days could indicate fibroblast
147   activation by prolonged culture in medium containing only 2% fetal bovine serum, crowding, or
148   by potential active TGFB1 in the medium, which is suppressed under deciduogenic conditions.
149   Next, we turn to a signaling pathway that is critical for wound-induced fibroblast activation,
150   TGFB1.

151

TGFB1 Is Involved in Regulating Decidualization

153   TGFB1 is a cytokine controlling fibroblast activation during wound healing (Brunner and
154   Blakytny, 2004; Oberringer et al., 2008). We reasoned that, if decidualization is derived from
155   fibroblast activation, TGFB1 should also play a role in decidualization, consistent with its role in
156   embryo implantation in various eutherian mammalian species (Singh et al., 2011, Monsivais et
157   al., 2017). We investigated the effects of TGFB1 and pharmacological inhibitors of its receptor on
158   differentiation of human decidual cells *in vitro*. Under prostaglandin E2 (PGE2) and MPA-
159   induced decidualization, TGFB1 protein addition increased mRNA expression of the classic
160   decidual marker *PRL* (Figure 2A), consistent with previous findings (Kim et al., 2005; Chang et
161   al., 2008). In addition, LY2109761, a dual inhibitor of type I and II TGF-β receptors (Melisi et al.,
162   2008) significantly reduced *PRL* mRNA levels (Figure 2B) and reduced the characteristic
163   polygonal morphology of decidual cells (Wynn, 1974) (Figure 2C, D). Hence, TGF-β receptor
164   inhibition weakened decidualization. Taken together, these results support a facilitating role for
165   TGFB1 in human decidualization. The involvement of the TGFB1 signaling pathway in
166   decidualization is consistent with the hypothesis of evolutionary derivation from WIFA.

167   Given that decidualization increases the expression of *TGFB1* mRNA (Figure 2E) and TGF-β
168   receptor inhibition decreases the expression of decidual markers, we hypothesized that TGFB1

169 expression itself may be enhanced via an autocrine feedback loop. This seemed plausible given
170 that a positive autocrine feedback loop for TGFB1 has been reported in activated skin fibroblasts
171 during wound healing (Messadi et al., 1994; Moulin, 1995; Blaauboer et al., 2011). However,
172 *TGFB1* mRNA expression in human decidual cells did not show a significant change when
173 treated with the TGF-β receptor inhibitor (Figure 2F), inconsistent with the autocrine feedback of
174 TGBF1 on its own expression.

175 Next, we asked whether this result is a general feature of the decidualization processes, or
176 whether it is an evolutionarily derived state in humans or other primates. Unlike the vast majority
177 of eutherian mammals, in which decidualization requires the stimulus of an embryo or uterine
178 injury, in humans and a limited number of other menstruating mammals such as some primates,
179 decidualization is independent of the presence of an embryo, or "spontaneous" (Emera et al.,
180 2011). Thus, in humans the increasing expression of TGFB1 in decidualization could be
181 overwhelmingly regulated by hormones or hormone-related pathways over TGFB1 itself.

182 In order to approach this question, we turned to the ESF cells of the rabbit, a species lacking
183 spontaneous decidualization. We treated primary ESF cells isolated from the rabbit endometrium
184 with PGE2 and MPA. We found that *TGFB1* mRNA expression did increase in rabbit ESFs
185 during treatment with PGE2 and MPA like in human ESFs (Figure 2G). In addition, TGF-β
186 receptor inhibitor was able to block the upregulation of *TGFB1* expression under these conditions
187 (Figure 2H). These results suggest that in the rabbit ESF, increasing TGFB1 expression seems to
188 involve an autocrine feedback loop during PGE2 and MPA treatment, similar to the situation in
189 activated fibroblasts.

190 These findings, together with the other evidence for an evolutionary relationship between WIFA
191 and decidualization, suggest that the situation in the rabbit ESF could be the ancestral response of
192 mucosal stromal fibroblasts, while the lack of positive feedback in humans could be derived.
193 Clearly, evidence from more species is necessary to resolve the question whether the presence of
194 positive autostimulation is derived or ancestral, but the fact that activated skin fibroblasts have a
195 positive feedback loop of TGFB expression suggests that it is ancestral. On the other hand, in
196 species with spontaneous decidualization, the positive feedback loop may not be necessary:
197 decidualization already happens before implantation rather than in response to embryo attachment
198 and invasion.

199

Single-Cell Sequencing Reveals a Myofibroblast-like Cell Population

201 To further dissect the process of decidualization, we re-analyzed single-cell data from our lab of
202 human ESF decidualized *in vitro* for 2 and 6 days (Stadtmauer and Wagner, 2020). Dimensional
203 reduction and clustering divide the cells into three major clusters: highly proliferative ESF
204 (hpESF), activated ESF (acESF), and DSC, and trajectory analysis infers a single developmental

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205 trajectory from ESF to DSC (Figure 3A, B bottom plane). At this point, a population of cells
206 express genes falling into gene ontology (GO) categories such as muscle contraction and actin
207 filament organization (Figure 3C). This expression pattern includes high levels of *ACTA2*,
208 tropomyosin 2 (*TPM2*) and the actin cross-linking protein transgelin (*TAGLN*). This observation
209 suggests that there are myofibroblast-like cells present during the decidualization process; we
210 refer to them as "activated ESFs."

211 This activated ESF population observed *in vitro* corroborates two single-cell sequencing studies
212 of the first-trimester human fetal-maternal interface which also reported myofibroblast-like cells
213 (Suryawanshi et al., 2018; Vento-Tormo et al., 2018). These myofibroblast-like cells are
214 characterized by genes such as *ACTA2* and *TAGLN*. The presence of such cells *in vivo* suggests
215 that our identification of a myofibroblast-like ESF cell population is not an *in vitro* artifact; on the
216 contrary, our *in vitro* finding suggests that the *in vivo* finding of myofibroblast-like cells may
217 reflect part of the decidualization process rather than activated tissue fibroblasts from other parts
218 of the uterus.

219 As these studies of the first-trimester human fetal-maternal interface were *in vivo* observational
220 data rather than controlled differentiation studies, the developmental trajectories between these
221 myofibroblast-like ESFs and DSCs could not be clearly ascertained. To assess whether
222 myofibroblast-like ESFs are a distinct population or transitory state, we took a closer look at our
223 single cell experimental decidualization data. Intrinsic dimension (Kerstin, 2016) of a cell
224 transcriptome is a measure of transcriptomic complexity reflecting the number of genes
225 simultaneously expressed. A high level of transcriptomic complexity has been related to cells
226 undergoing transcriptomic reprogramming to assume a new cell type identity (Brunskill et al.,
227 2014), as they will transiently co-express two transcriptional programs. Based on this insight, and
228 previous work using intrinsic dimensionality to identify branch points (Moon et al., 2019), we
229 visualized single-cell transcriptomes of decidualizing human ESFs as a landscape — a two-
230 dimensional PHATE embedding (Moon et al., 2019) and in the third dimension a measure of
231 intrinsic dimensionality (Kerstin, 2016; Moon et al., 2019). The resulting landscape (Figure 3A,
232 B) contained a single point of high transcriptomic complexity (indicated by arrowheads),
233 corresponding to the ESF-DSC developmental transition (Stadtmauer and Wagner, 2020). The
234 myofibroblast-like cells fell squarely into the ESF side of this transition point, as visualized by the
235 marker *ACTA2* (highlighted in red) characterizing the ascent of the complexity hill but not its
236 descent towards DSC (Figure 3A, B). The location of these cells suggests that myofibroblast-like
237 ESFs represent an activated ESF state transitional to DSC, rather than an alternative cell type fate.
238 We interpret this as consistent with our model that the process of decidualization is an
239 evolutionary modification of WIFA.

240 The single-cell transcriptomic data suggest that decidualization is a two-step process. First, ESFs
241 assume an activated pre-decidual state, characterized by a myofibroblast-like gene expression
242 profile, which is followed by differentiation into the actual DSC. The latter process departs from

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243 this activated, myofibroblast-like state, rather than from the quiescent ESF. Such a scenario would
244 also explain the partial affinity between the DSC and WIFA, which are similar at the signaling
245 and transcription factor level but divergent in their functional effector phenotype. Whether this
246 two-phase process corresponds to the idea that decidualization is a biphasic process with an early
247 inflammatory and a later anti-inflammatory phenotype (Salker et al., 2012, Rytkönen et al., 2019)
248 will require more detailed investigation. The fact that myofibroblast-like cells have been
249 identified in the *in vivo* implantation site (Suryawanshi et al., 2018; Vento-Tormo et al., 2018)
250 suggests that the second phase of the process (transition from the activated pre-decidual state to
251 DSC) could be reversible: a dynamical equilibrium like a chemical reaction.

252 Generation of a myofibroblast-like ESF cell state *in vitro*, distinct from DSC, has also been
253 achieved through exposure of ESFs to platelets, proposed to act through and dependent upon
254 TGFB1: excess production of these myofibroblast-like ESF was proposed to drive a constitutive
255 wound healing phenotype in endometriotic lesions (Zhang et al., 2016). It is possible, therefore,
256 that normal progression from the fibroblast activation-like stage to decidualization and prevention
257 of its persistence (Nancy et al., 2018) is dependent upon proper receipt of uterine-specific
258 regulatory factors. The physiological role of myofibroblast-like pre-decidual cells warrants further
259 study.

260

A Model for the Evolution and Nature of the Decidualization Process

262 We present our evolutionary/developmental model in Figure 4. Based on this model, we suggest
263 that the developmental process of decidualization likely derived from WIFA caused by the
264 implanting embryo. Our single-cell analysis suggests that the decidualization process includes at
265 least two steps: one where the cells assume a myofibroblast-like state, probably homologous to
266 the generic WIFA, and from that cell state the actual differentiation of the mature decidual cell
267 proceeds. Hence, we have the following cell state changes:

268   ESF → **pre-decidual cells** → DSC,

269 where the first step corresponds to

270   Quiescent FB → **activated Myo-FB**

271 during the wounding response. This model suggests that the pre-decidual cell is homologous to
272 the activated fibroblast, and the DSC is a novel cell type arising on the foundation of activated
273 fibroblast. Based on the phylogenetic distribution of placental types (Wildman et al., 2006; Mess
274 and Carter, 2006; Elliot and Crespi, 2009) we infer that this evolutionary event must have
275 occurred in the stem lineage of eutherian mammals. While some similarities between
276 myofibroblasts and mature DSC have been previously noted (Oliver et al., 1999), this model does

277   not require identifying myofibroblasts as a sister cell type to DSC, something which a previous
278   cell type phylogenetic analysis did not support (Kin et al., 2015). In contrast to generic fibroblast
279   activation, decidualization occurs after estrogen priming and under sustained progesterone
280   stimulation (Gellersen and Brosens, 2014). The latter signal indicates to the endometrium that
281   ovulation has happened and eventual wounding of the uterine epithelium might be caused by an
282   embryo. Indeed, wounding in the uterus in the presence of high progesterone levels is sufficient to
283   cause decidualization, as demonstrated in the so-called deciduoma model of rodent
284   decidualization (Krehbiel, 1937; Finn, 1986). Our model explains at the one hand the numerous
285   mechanistic similarities between decidualization and wound healing, because of their homology,
286   but on the other hand also accommodates the fact that mature decidual cells display a distinct
287   phenotype at maturity adapted to their role in embryo implantation. Among them are the active
288   suppression of genes dedicated to the actual wound healing process (Nancy et al., 2018). Given
289   the possible linkage of wound healing to both decidualization and to cancer malignancy (Dvorak,
290   1986, 2015), we note that suppression of wound healing may be relevant for cancer progression.

291   This model of novel cell type evolution is distinctive from other models by emphasizing
292   intermediate processes: transient cell states which could be homologous to pre-existing cellular
293   processes. Since cellular processes are responses to stimuli (an environment under which novel
294   cell types could thrive), we hypothesize that novel cell types may often build upon the foundation
295   of transient cell states. Both activation, as in WIFA, and differentiation are types of cell state
296   transitions differing primarily in the stability of the assumed state (Pope and Medzhitov, 2018),
297   and the origin of differentiation processes from activation processes may be a repeated trend in
298   evolution. For instance, there is growing evidence that cellular stress responses can be the seed for
299   the evolutionary origin of novel cell types (e.g. Wagner et al., 2019; König and Nedelcu, 2020).
300   Furthermore, under this model, it seems that the transient cell state does not necessarily need to be
301   stress-induced: any cellular process induced by internal or external signaling may do.

302   Dynamic transient cell states like this could have been missed in studies based on bulk cell
303   populations such as western blots, qPCR and bulk RNA-seq analysis. The present study highlights
304   the important insights that become possible from employing single-cell analysis to capture these
305   transient states. In sum, a single-cell level of analysis may open up a new direction to understand
306   the evolutionary origin of novel cell types.

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316   Figure 1

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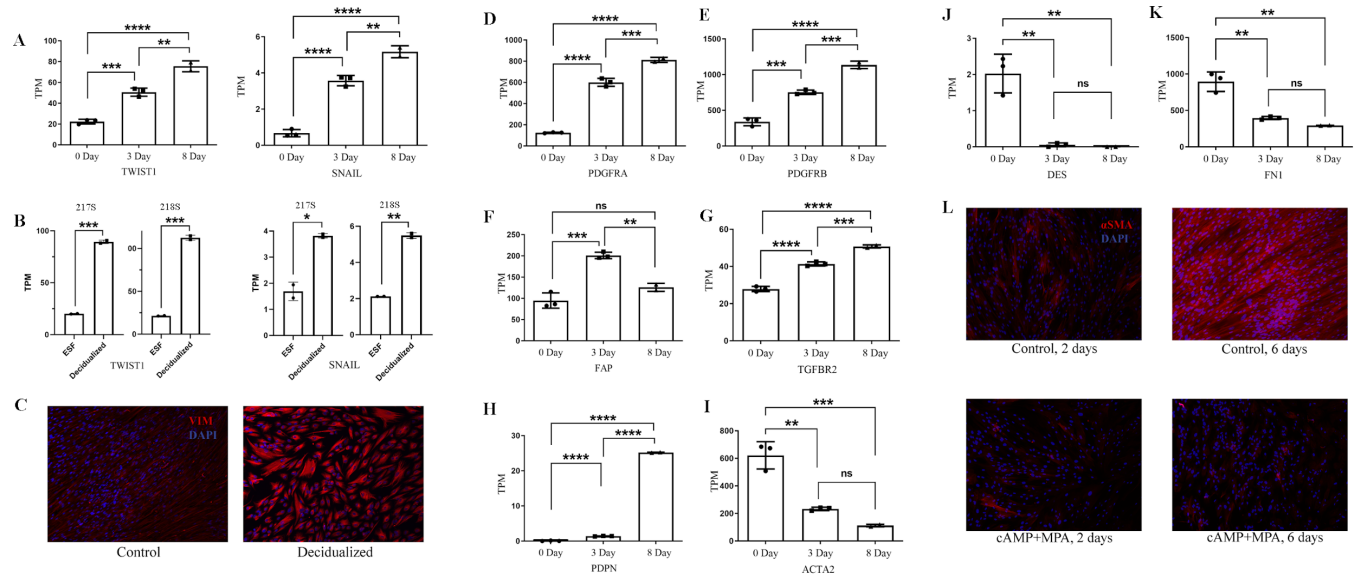
320 **Figure 1. Expression of MET and WIFA pathway components in decidualization.**

321 **A-C: MET hallmark genes in decidualization. A:** *TWIST1* and *SNAIL*, transcription factors and
322 markers for fibroblast activation and EMT, are upregulated during *in vitro* decidualization (8-Br-
323 cAMP+MPA, 0, 3, 8 days: Rytkönen et al., 2019). **B:** *TWIST1* and *SNAIL* are upregulated in human
324 primary decidual cell line 217s and 218s during *in vitro* decidualization (Pavličev et al., 2017). **C:**
325 Fibroblast activation and epithelial mesenchymal transition (EMT) marker VIM protein is strongly
326 upregulated during *in vitro* decidualization (8-Br-cAMP+MPA, 8 days).

327 **D-L: Fibroblast activation hallmark genes in decidualization. D-H:** Fibroblast activation
328 regulatory genes *PDGFRA*, *PDGFRB*, *FAP*, *TGFBR2* and *PDPN*, are upregulated during *in vitro*
329 decidualization (8-Br-cAMP+MPA, 0, 3, 8 days: Rytkönen et al., 2019). **I-K:** Phenotypic markers
330 of activated fibroblast cells, *ACTA2*, *DES* and *FN1* show decreased mRNA expression at later time
331 points of *in vitro* decidualization (8-Br-cAMP+MPA, 0, 3, 8 days: Rytkönen et al., 2019). **L:**
332 Decidual cells (8-Br-cAMP+MPA, 6 days) show low levels of myofibroblast maker a-SMA protein
333 while control cells (6 days) show high levels of a-SMA protein. Early decidual cells (8-Br-
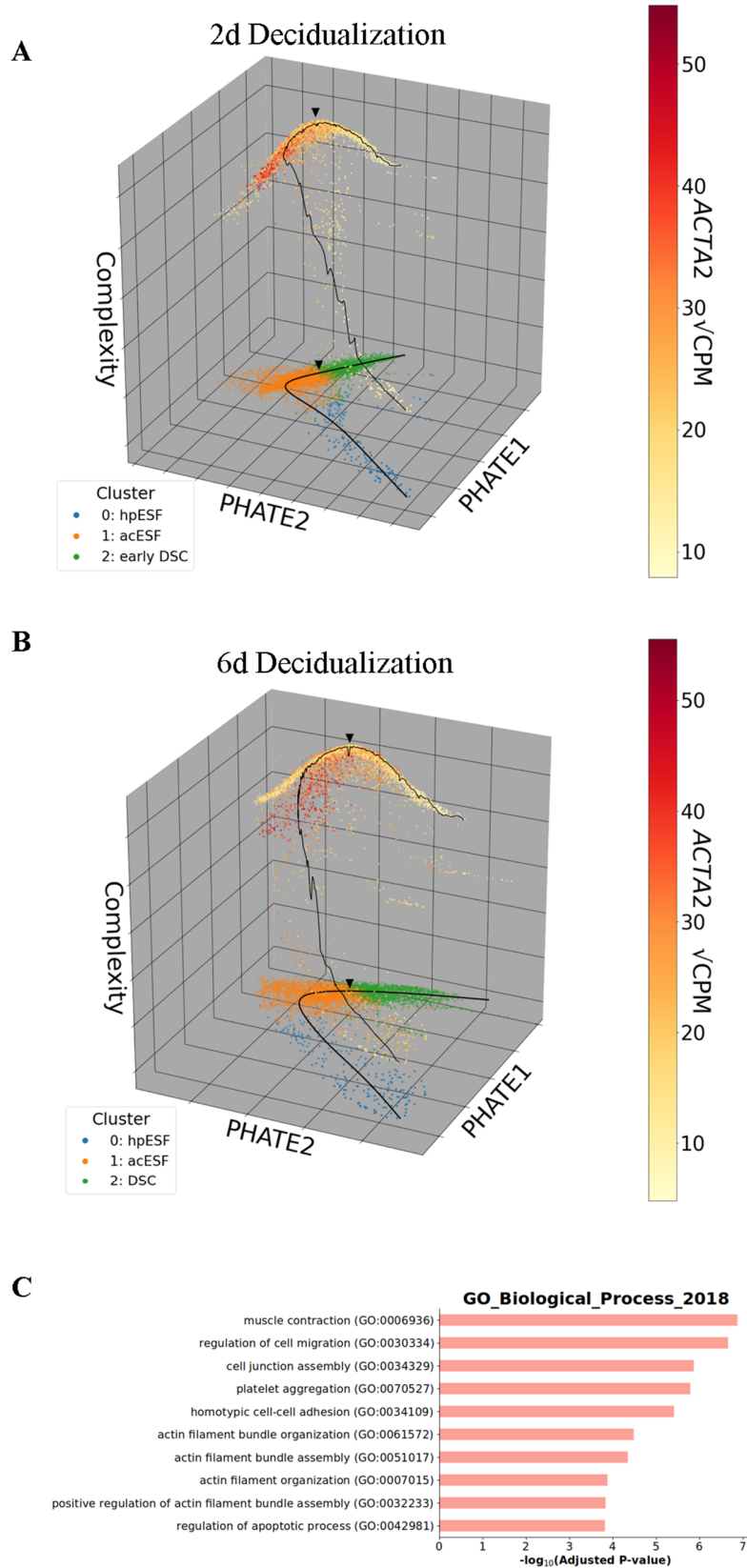334 cAMP+MPA, 2 days) and control cells (2 days) show low levels of a-SMA protein.

335 Statistical analysis was performed by unpaired t test (C-F) and one-way ANOVA (others). *:
336 $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$; ns: not significant.
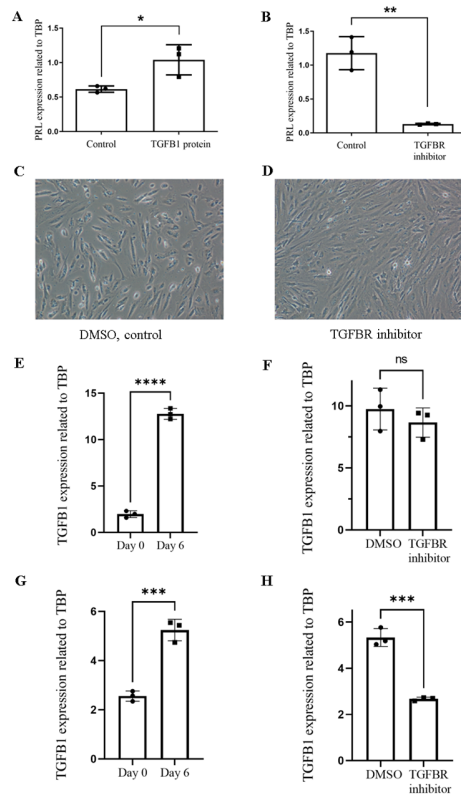
Figure 2: TGFB1 signalling in decidualization.

A-D: TGFB1 facilitates decidualization. **A)** TGFB1 protein (0.5 ng/mL) increases mRNA expression of decidualization maker *PRL* compared to control (PGE2+MPA, 6 days). **B)** TGF-β receptor inhibitor LY2109761 (5 uM) strongly suppresses mRNA expression of *PRL* (PGE2+MPA, 6 days). **C, D)** Decidual cell polygonal morphology is largely impaired by TGF-β receptor inhibitor (8-Br-cAMP+MPA, 6 days). **E)** In Human ESFs, TGFB1 mRNA expression increased after decidualization (PGE2+MPA). **F)** In Human ESF, TGFB1 expression was not affected by TGF-β receptor inhibitor (PGE2+MPA, 6 days). **G)** In Rabbit ESFs, TGFB1 mRNA expression increased after decidualization (PGE2+MPA). **H)** In Rabbit ESF, TGFB1 mRNA expression was downregulated significantly with TGF-β receptor inhibitor treatment (PGE2+MPA, 6 days). Statistical analysis was performed by unpaired t test, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant.

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A 2d Decidualization

Cluster
- 0: hpESF
- 1: acESF
- 2: early DSC

B 6d Decidualization

Cluster
- 0: hpESF
- 1: acESF
- 2: DSC

C GO_Biological_Process_2018

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Figure 3. Single-cell RNA sequencing reveals a myofibroblast-like ESF population during Human *in vitro* decidualization. **A, B)** Reduced-dimensionality embeddings for cells 2 days **(A)** and 6 days **(B)** after PGE$_2$+MPA decidualization treatment. Cells are plotted in the lower PHATE1-PHATE2 plane, colored by k-means cluster (k = 3), and overlaid with an inferred developmental trajectory. Above, each point is plotted again at a height corresponding to the cell's intrinsic transcriptomic dimensionality and colored according to expression of the myofibroblast marker *ACTA2* (MAGIC-denoised square-root counts per million). Thus, each cell is represented by a pair of points directly above and below each other. A region of myofibroblast-like *ACTA2+* (red) cells is identified on the ascending side of a complexity peak. The point of highest transcriptomic complexity and its corresponding point in the lower PHATE1-PHATE2 plane are indicated by arrowheads. **C)** Output from gene ontology analysis of the myofibroblast-like cell cluster from the 6-day sample, ranked by Enrichr P-value adjusted for multiple comparisons.

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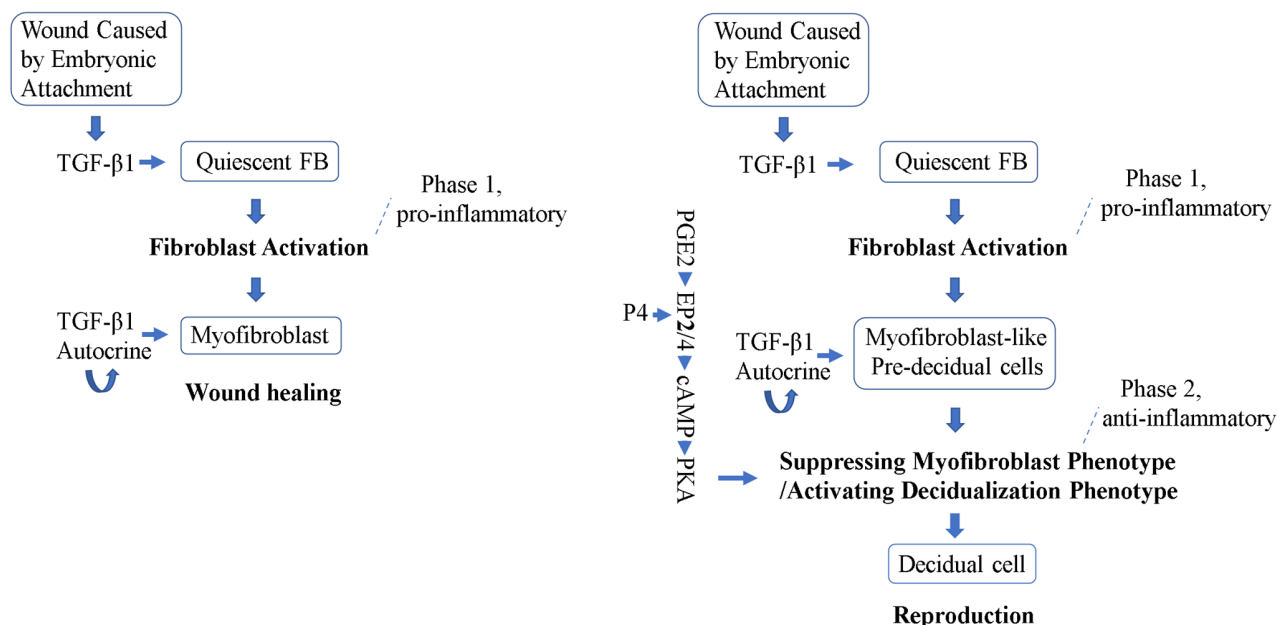
Figure 4. A developmental and evolutionary model for decidualization. Two stages of the evolution of the embryonic attachment reaction are shown. In the first **(left)** the attachment leads to a generic fibroblast activation and wound healing, in the second **(right)** the attachment reaction is transformed into decidualization. Hormone regulations required for the second phase. P4: progesterone, PGE2: Prostaglandin E_2, E2: Estradiol, EP2/4: Prostaglandin E2 receptors 2/4.

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Materials and Methods

Cell culture

Immortalized human ESFs (ATCC CRL-4003) were cultured in growth media containing 1x antibiotic-antimycotic (15240, Gibco; contains penicillin, streptomycin, and Amphotericin B), 15.56 g/L DMEM/F-12 without phenol red (D2906, Sigma-Aldrich), 1.2 g/L sodium bicarbonate, 10 mL/L sodium pyruvate (11360, Thermo Fisher), and 1 mL/L ITS supplement (354350, VWR) in 10% charcoal-stripped fetal bovine serum (100-119, Gemini).

ESFs from rabbit (*Oryctolagus cuniculus*) were isolated from uterine tissues by enzymatic digestion. The uterus was cut in half longitudinally and then cut into 2-3 mm fragments. These were digested with 0.25% trypsin-EDTA for 35 min at 37°C, followed by dissociation buffer (1 mg/mL collagenase, 1 mg/mL Dispase, 400 mg/mL DNaseI) for 45 minutes at 37°C. Cells were homogenized by passing through a 22-gauge needle and syringe followed by a 40 mm nylon mesh filter to remove remaining clumps. A single-cell suspension was obtained from the lysate, transferred to fresh growth medium, and cultured in T25 flasks. To facilitate enrichment of fibroblasts over epithelial cells, media was exchanged after fifteen minutes in order to remove less adherent cells (differential attachment). Cells were grown to confluency and sub-passaged by scraping the cells off of the surface and splitting into 2 T25 flasks, and one additional round of differential attachment was performed. All endometrial stromal fibroblasts were cultured in growth media (as above), with no antibiotic-antimycotic. To validate our fibroblast cell line, we used immunocytochemistry to detect proteins that mark mesenchymal (vimentin+) rather than epithelial (cytokertain+) cells cells. Primary antibody solutions used as follows: 1:200 rabbit anti-cytokeratin (ab9377, Abcam); 1:200 mouse anti-vimentin (sc-6260, Santa Cruz).

Decidualization treatment

Decidualization was effected with medium containing antibiotic-antimycotic (15240, Gibco), 15.56 g/L DMEM/F-12 (D8900, Sigma-Aldrich), and 1.2 g/L sodium bicarbonate in 2% fetal bovine serum (100-106, Gemini). Control wells were grown in medium alone while experimental wells additionally received 0.5 mM 8-Br-cAMP (B7880, Sigma-Aldrich) and 1 µM MPA (M1629, Sigma-Aldrich), or 1 µM PGE2 (HY-101952, MedChemExpress) and 1µM MPA. Media was replenished every two days.

Immunocytochemistry

Human ESFs were grown in an 8-well chamber slide (12-565-18, Fisher) to 70-80% confluency before treatment and decidualized for the duration indicated. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 mins at room temperature after treatment. Cells were then washed three times with PBS. Permeabilization was performed with 0.25% Triton X-100 in PBS for 10 mins at room temperature, followed by three more washes

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417 with PBS. For blocking, cells were incubated with 1% BSA in PBST (PBS with 0.1% Tween 20)
418 for 30 mins at room temperature. Primary antibody incubation (1:100 mouse-anti-Vim: sc6260,
419 Santa Cruz or 1:100 mouse-anti-aSMA: ab7817, Abcam) was performed with the aforementioned
420 blocking solution at 4°C overnight. Cells were then washed three times with PBS. Secondary
421 antibody incubation (1:100 Alexa Fluor 555 goat anti-mouse: A21422, Thermo Fisher) was
422 performed with blocking solution at room temperature for 1.5 hours. Cells were washed with PBS
423 three times before staining with DAPI (10236276001, Roche) for 1 min. Cells were then washed
424 with PBS three times and mounted with 50% glycerol. Staining results were imaged on a Nikon
425 Eclipse E600 microscope equipped with a Spot Insight camera.

426 *TGFB1 Treatment*

427 TGFB1 experiments were performed in media containing 1 μM PGE2, 1 μM MPA, 1x antibiotic-
428 antimycotic, 15.56 g/L DMEM/F-12 (D2906) without phenol red, 1.2 g/L sodium bicarbonate, 10
429 mL/L sodium pyruvate, 1 mL/L ITS supplement, and with no fetal bovine serum to exclude the
430 effects of potential active TGFB1 in fetal bovine serum. Recombinant human TGFB1 protein
431 (ab50036, Abcam) was added to the media of experimental wells at the beginning of the treatment
432 (0.5 ng/mL). Control wells received the same media but no TGFB1 protein. For the TGF-β
433 receptor inhibition experiment, LY2109761 (S2704, Selleckchem) was added into the media at
434 the beginning of the treatment (5 μM). 0.5 μL DMSO was added in the control group. Decidual
435 cell morphology experiment was performed under decidualization treatment.

436 *Quantitative PCR*

437 Human ESFs were cultured to 70-80% confluency before treatment. After treatment, media was
438 removed and cells were washed two times with PBS and then lysed with Buffer RLT Plus plus
439 1% beta-mercaptoethanol. RNA was extracted using an RNeasy Plus Micro Kit (74034, Qiagen),
440 and cDNA was constructed with a High-Capacity cDNA Reverse Transcription kit (4368814,
441 Applied Biosystems). qPCR was carried out in triplicate using ThermoFisher TaqMan reagent on
442 StepOne Plus Real Time PCR System 482 (Applied Biosystems). Gene TBP was used as the
443 internal reference gene to normalize the expression of target genes. qPCR primers used are as
444 follow: Human *TBP*: Hs99999910_m1, Human *PRL*: Hs00168730_m1, Human *TGFB1*:
445 Hs99999918_m1, Rabbit *TBP*: Oc05211505_m1, Rabbit *TGFB1*: Oc04176122_u1.

446 *Single cell transcriptomic analysis*

447 Published sequence data (NCBI PRJNA679324) of human ESF treated with PGE2+MPA daily
448 for 2 or 6 days were aligned and pre-processed using scprep software version 1.0.5
449 (github.com/KrishnaswamyLab/scprep) following the original study to filter irregularly-sized
450 libraries resulting from dead cells or doublets (Stadtmauer and Wagner 2020). Briefly,
451 thresholding between the 16th and 90th percentile of features per library for 2-day treated samples,
452 between the 9th and 90th percentile of features per library for 6-day treated samples, and below the

453   93rd percentile of mitochondrial gene content of all libraries resulted in 4077 remaining cells from
454   the 6-day group, and 3202 cells in the 2-day group, from a combined total of 10073. Thresholding
455   to genes only expressed in 5 or more cells left 16496 genes in consideration. Read counts were
456   normalized to square root counts per million.

457   A two-dimensional embedding was calculated using PHATE (v1.0.5, Moon et al., 2019). K-
458   means clustering was performed with a hyperparameter of 3, and differential gene expression
459   between clusters was calculated using scprep's differential expression function which performed
460   Welch's t-test between cells within and without each cluster. Gene set enrichment analysis on the
461   markers of the cluster identified as activated ESF was conducted by querying Enrichr (Kuleshov
462   et al., 2016). Intrinsic dimensionality for landscape plotting was calculated following Moon et al.
463   (2019), using a method based on the intrinsicDimension R Package and Kerstin (2016). A
464   developmental trajectory between clusters was drawn using Slingshot (Street et al., 2018) with the
465   starting cluster set to that with lowest dimensionality, and interpolation was used to draw the
466   trajectory in the intrinsic dimensionality axis as well to aid visualization. Expression of *ACTA2*
467   was denoised using MAGIC (v2.0; van Dijk et al., 2018) for clarity of visualization.

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