**SOX4 facilitates PGR protein stability and FOXO1 expression conducive
for human endometrial decidualization**

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Abstract

The establishment of pregnancy in human necessitates appropriate decidualization of stromal cells, which involves steroids regulated periodic transformation of endometrial stromal cells during menstrual cycle. However, the potential molecular regulatory mechanism underlying the initiation and maintenance of decidualization in humans is yet to be fully elucidated. In this investigation, we document that SOX4 is a key regulator of human endometrial stromal cells (HESCs) decidualization by directly regulating FOXO1 expression as revealed by whole genomic binding of SOX4 assay and RNA-Seq. Besides, our immunoprecipitation and mass spectrometry results unravel that SOX4 modulates progesterone receptor (PGR) stability through repressing E3 ubiquitin ligase HERC4 mediated degradation. More importantly, we provide evidence that dysregulated SOX4-HERC4-PGR axis is a potential cause of defective decidualization and recurrent implantation failure (RIF) in IVF patients. In summary, this study evidences that SOX4 is a new and critical regulator for human endometrial decidualization, and provides insightful information for the pathology of decidualization-related infertility and will pave the way for pregnancy improvement.

Introduction

Adequate crosstalk between implantation-competent embryo and receptive endometrium is prerequisite for successful pregnancy(Cha, Sun et al. 2012). The receptive endometrium in human requires remodeling of stromal cells under the regulation of rising progesterone and intracellular cyclic AMP to initiate the decidualization, which will undergo more extensive transformation after embryo implantation (Gellersen and Brosens 2014). The human endometrial stromal compartment can decidualize during the progesterone-dominant early secretory phase of a non-conception cycle(Wang, Vilella et al. 2020). During the pregnancy, the decidual process is poised to transit through distinct phenotypic phases that underpin endometrial receptivity, embryo selection, and ultimately resolution of pregnancy (Gellersen and Brosens 2014). The decidual reaction plays a central role in the establishment of a pregnancy and continues throughout the pregnancy. Insufficient decidualization in endometrium is related to failed embryo implantation(Peter Durairaj, Aberkane et al. 2017), unexplained infertility, recurrent spontaneous abortion(Coulam 2016), intrauterine growth retardation(Lefevre, Palin et al. 2011), and preeclampsia(Garrido-Gomez, Dominguez et al. 2017). However, the underlying molecular mechanism governing the endometrial decidualization remains enigmatic(Okada, Tsuzuki et al. 2018).

A wide range of transcription factors crucial for stromal cell decidualization have been successively identified(Gellersen and Brosens 2014). FOXO1, a member of FOXO fork-head transcription factors subfamily, is one of the earliest identified transcriptional factors in human endometrial stromal cells (HESCs) responding to decidualization stimulation (progesterone and cAMP)(Labied, Kajihara et al. 2006). Accumulative evidence has demonstrated that FOXO1 regulates transcription of PRL and IGFBP1 through direct binding to their promoters(Christian, Zhang et al. 2002, Kim, Buzzio et al. 2005). Progesterone receptor (PGR), which is a critical master factors for the

77 endometrial stromal cells (ESCs) decidualization, imparts endometrium
78 receptivity through binding with P4 ligand and its nuclear translocation(Keller,
79 Wiest et al. 1979, Mulac-Jericevic, Mullinax et al. 2000). An array of PGR
80 direct target genes had been unraveled by PGR ChIP-Seq in both human and
81 mice(Rubel, Lanz et al. 2012, Mazur, Vasquez et al. 2015, Chi, Wang et al.
82 2020). Abnormal PGR expression is closely relevant with unexplained
83 infertility(Keller, Wiest et al. 1979) and endometriosis (EMS) (Zhou, Fu et al.
84 2016, Pei, Liu et al. 2018). Although many transcription factors and
85 downstream events in decidualization of both mice and human have been
86 unraveled(DeMayo and Lydon 2020), the precise mechanism orchestrating
87 transcriptional regulatory network underpinning endometrial decidualization
88 remained not fully explored.

89 SOX4 is a highly conserved transcription factor belonging to the SOX
90 (SRY-box) family. Studies have shown that SOX4 is vital to a variety of
91 biological processes, including embryogenesis, neural development, and
92 differentiation(Moreno 2020). Further, it has been noticed that SOX4 knockout
93 mice die of cardiac malformation on the fourteenth day of pregnancy,
94 suggesting the key role of SOX4 in embryonic development(Ya, Schilham et al.
95 1998). In addition, an increasing number of reports indicate that SOX4 is
96 related to tumor cell proliferation, metastasis, and epithelial-mesenchymal
97 transformation(Li, Liu et al. 2020). Report also shows that SOX4 is highly
98 expressed in breast cancer under the regulation of progesterone(Graham,
99 Hunt et al. 1999). Additionally, SOX17, another member of the SOX family,
100 has also been observed to be a direct target of PGR in epithelium regulating
101 IHH expression(Wang, Li et al. 2018). While the significance and regulation of
102 SOX4 in female pregnancy remain intangible.

103 Here, we provide evidence that SOX4, under the regulation of P4-PGR,
104 guides human endometrium stromal cells (HESCs) decidualization by
105 regulating FOXO1 expression as revealed by ChIP-Seq and RNA-Seq.

106 Mechanism studies also unravel the importance of SOX4 in maintaining the
107 protein stability of PGR by repressing ubiquitin E3 ligase HERC4. Moreover,
108 both SOX4 and PGR have been demonstrated to be aberrantly downregulated
109 in the endometrium of EMS patients suffering from implantation failure.

110

111 **Results**

112 **SOX4 is dynamically expressed in human endometrial stroma cells** 113 **regulated by P4-PGR signaling**

114 To investigate the expression of transcription factors in HESCs, RNA
115 sequencing (RNA-Seq) was performed in normal endometrium stromal cells.
116 We noted that among the transcription factors expressed in endometrium
117 stromal cells, SOX4 was the 17th highest expressed (Fig. 1A), and as the most
118 abundant member in SOX family (Fig. 1B). Meanwhile, previous RNA-Seq
119 data in isolated mouse stromal and epithelial cells revealed that SOX4
120 expression was the most abundant SOX family in stromal cells (Fig. Figure
121 1-figure supplement 1A), while the expression of SOX17 was restricted to the
122 epithelium(Deng, Yuan et al. 2019). Thus, we speculated that the conserved
123 expression of SOX4 in stroma was linked to decidualization. To explore
124 whether SOX4 was under the regulation of dynamic change of estrogen and
125 progesterone during the menstrual cycle, we first analyzed the expression of
126 SOX4 in endometrial biopsy samples obtained from healthy,
127 reproductive-aged volunteers with regular menstrual cycles. Albeit SOX4 was
128 detected at a low level in the E2-dominant proliferative phase, its expression
129 was progressively increased in the nucleus of stromal cells in P4-dominant
130 early, middle, late secretory phases and this high expression in stroma cell
131 sustained in the decidual cells in early pregnancy (Fig. 1C).

132 This upregulation of SOX4 in differentiated stromal cell in secretory phase
133 was also substantiated in *in vitro* decidualized stromal cells induced by E2,
134 MPA and cAMP (EPC) cocktail, displaying gradually cumulated mRNA and

135 protein levels in immortalized HESCs (Fig. 1D, E) as well as in primary stromal
136 cells (Figure 1-figure supplement 2A, B). Immunofluorescence also manifested
137 that SOX4 was mainly localized in the nucleus of HESCs after EPC treatment
138 (Fig. 1F).

139 The intense expression of SOX4 in the stroma of secretory phase
140 accompanied with rising P4 and induced by EPC in in vitro stromal cells,
141 suggesting that progesterone may be involved in the regulation of SOX4
142 expression. This assumption was supported by the observation that SOX4
143 expression was induced by 2 days treatment of MPA, a progesterone analogue
144 commonly used in decidualization induction, and abrogated in the presence of
145 PGR antagonist RU486 (Fig. 2A). Progesterone induced SOX4 mRNA and
146 protein levels were significantly attenuated with PGR knockdown in
147 immortalized HESCs (Fig. 2B-D). Thus, the P4-PGR signaling was critical for
148 SOX4 expression in HESCs. Based on previous ATAC-Seq data from
149 undecidualized and decidualized stromal cells and PGR ChIP-Seq from
150 proliferative and secretory endometrium (Chi, Wang et al. 2020), we found that
151 there was intensive potential PGR binding at SOX4 promoter (Fig. 2E). This
152 speculation was validated by PGR ChIP-qPCR at SOX4 promoter in
153 decidualized stromal cells (Fig. 2F). Furthermore, the binding of PGR on SOX4
154 promoter was also confirmed by luciferase reporter assay that overexpression
155 of PGR increased the reporter activities in the presence of MPA in 293T cells
156 (Fig. 2G). These results indicated that PGR guided SOX4 expression via direct
157 transcriptional regulation.

158

159 **SOX4 is required for human endometrial stromal cell decidualization**

160 To depict the significance of SOX4 during decidualization, SOX4 was
161 knockout by CRISPR/Cas9 which was ascertained by Sanger-sequencing in
162 both alleles (Figure 3-figure supplement 1A) and further verified by
163 western-blot and immunofluorescence (Figure 3-figure supplement 1B, C).

164 The expression of *PRL* and *IGFBP1* were dramatically decreased in *SOX4*
165 knockout HESCs after decidualization for 2, 4, 6 days compared with *SOX4*
166 intact cells (Fig. 3A-C). Similar results were obtained in primary HESC after
167 *SOX4* knockdown by shRNA prior to EPC administration (Figure 3-figure
168 supplement 2 A-D). On the other side, overexpressing *SOX4* in HESCs
169 significantly augmented the expression of *PRL* and *IGFBP1* in both
170 immortalized (Fig. 3D-F) and primary HESC (Figure 3-figure supplement 2 A-C)
171 decidualized for four days.

172 Moreover, RNA-Seq was utilized in *SOX4* intact or depleted immortalized
173 HESCs treated with EPC for two days. The genes significantly downregulated
174 after *SOX4* knockdown included aforementioned *IGFBP1* and *PRL* as well as
175 other genes critical for decidualization, such as *FOXO1*, *LEFTY2*, *WNT5A*,
176 *WNT4*, *FOSL2*, *STAT3*, *LEFTY2*, *IL-11*, *BMP2* (Fig. 3G, H). Kyoto
177 Encyclopedia of Genes and Genomes (KEGG) analysis showed that the
178 differentially expressed genes were enriched in the FOXO signaling pathway,
179 TGF-beta signaling pathway, and MAPK signaling pathway (Fig. 3I), consistent
180 with the observed defective decidualization in the absence of *SOX4*. The Gene
181 Ontology (GO) annotation analysis showed that the differentially expressed
182 genes were related to autophagy-associated pathway, insulin-like growth
183 factor binding, collagen-containing extracellular matrix, cell-substrate adhesion
184 (Fig. 3J). Together, these results revealed the critical role of *SOX4* in HESCs
185 decidualization.

186

187 **Genome binding of *SOX4* in decidualized stromal cells**

188 To interrogate genes directly regulated by *SOX4*, we detected the
189 chromatin-wide binding of *SOX4* by ChIP-Seq. Our results showed that there
190 were total of 23,709 *SOX4* binding peaks with most of them enriched in
191 promoters, distal intergenic and intron (Fig. 4A). Peak enrichment indicated
192 that *SOX4* binding was primary closed to TSS as well as evidenced by

193 heatmap of peak distribution (Fig. 4B, C). We also noticed that SOX4 mainly
194 binds to those genes with higher expression (RPKM ≥ 1) accompanied with
195 few SOX4 binding on those less to no expression genes (RPKM < 1) (Fig. 4B).
196 Motif analysis results revealed that the most significantly enriched motif was
197 SOX4 consensus binding sites, further supported the reliability of this SOX4
198 ChIP-Seq. We also noticed that FRA2 and FOSL2, two critical members of
199 AP1, were also highly enriched at SOX4 binding sites, indicating the potential
200 cooperation between SOX4 and AP1 (Fig. 4D). Considering the correlation
201 between SOX4 binding and gene expression, we overlapped SOX4 binding
202 genes with those down-regulated genes after SOX4 knockdown and found that
203 389 genes with SOX4 binding decreased after SOX4 ablation, indicating the
204 direct regulation of SOX4 on these genes (Fig. 4E).

205 It was gratifying that there were several latent SOX4 binding sites on the
206 STAT3, FOXO1 and FOSL2 in both stromal cell line and primary cultured
207 stromal cells (Fig. 4F). ChIP-qPCR also confirmed the binding of SOX4 on
208 these genes (Fig. 4G-J). Those down-regulated genes (900) after SOX4
209 knockdown possess more SOX4 binding reads compared with upregulated
210 genes (1,022) (Fig. 4K), indicating the transcriptional activation effect of SOX4.
211 Importantly, those SOX4 direct target genes also showed specific enrichment
212 of the FOXO signaling pathway (Fig. 4L). To further assess the regulation of
213 SOX4 on FOXO1, SOX4 was knockout and the expression of FOXO1 was
214 overtly decreased accompanied with compromised decidualization as marked
215 by lessened IGFBP1 during decidualization from days 2 to 6 (Fig. 4M, N). On
216 the other hand, FOXO1 mRNA and protein were upregulated in SOX4
217 over-expression stromal cell decidualized for 4 days, as well as IGFBP1
218 protein (Fig. 4O, and Figure 4-figure supplement 1B). Moreover, the effects of
219 SOX4 over-expression on decidualization was blocked when the FOXO1 was
220 knockdown, suggested the FOXO1 as a crucial effector downstream of SOX4
221 (Figure 4-figure supplement 1 A, B). Besides the down-regulated genes, there

222 were also 438 genes with SOX4 bind sites were upregulated and GO analysis
223 revealed that cell cycle regulators were mostly enriched (Figure 4-figure
224 supplement 2 A, B). During the decidual induction, the stroma cell would stop
225 proliferation and initiate the differentiation program, the aberrant cell mitotic
226 division in the absence of SOX4 was consistent with the defective
227 decidualization. In summary, these results suggested that SOX4 directly
228 regulated transcription of transcription factor FOXO1, as well as other
229 molecules conducive for human endometrial decidualization.

230

231 **SOX4 stabilizes PGR through repressing ubiquitin-proteasome pathway**

232 Previous studies showed that P4-PGR signaling was critical for both
233 initiation and maintenance of decidualization (Gellersen and Brosens 2014),
234 which incited us to assess PGR expression in the absence of SOX4 during the
235 decidualization. We were attracted by the finding that PGR protein levels,
236 including both the PRA and PRB isoforms, were dramatically decreased in the
237 absence of SOX4 on days 0, 2, 3 and 6 during decidualization without overt
238 transcription reduction (Fig. 5A, 5B). Similar results were also observed in
239 primary stromal cells, implying that SOX4 stabled PGR protein at
240 post-transcriptional level (Figure 5-figure supplement 1A, B). Furthermore,
241 treatment of cells with the proteasome inhibitor MG-132 for 6 hours efficiently
242 attenuated the rapid degradation of PGR protein in SOX4 deficient cells (Fig.
243 5C), but the autophagy inhibitor 3-MA cannot rescue or attenuate the
244 degradation of PGR protein (Figure 5-figure supplement 1C), suggested the
245 PGR protein was mainly degraded through the proteasome in the absence of
246 SOX4. Likewise, PGR protein degeneration was faster in SOX4 depleted
247 HESCs two days after EPC treatment in the presence of the protein synthesis
248 inhibitor cycloheximide (CHX) for 0, 3, 6, and 9 h (Fig. 5D, E). Thus, these
249 results revealed that SOX4 knockdown led to shortened the half-life of PGR
250 protein.

251 To further explore the regulatory apparatus of PGR protein stability, the
252 ubiquitination status of PGR was determined. Immunoprecipitated PGR was
253 blotted against ubiquitin antibody in decidualized immortalized HESCs

254 pretreated with MG-132 for 6 h to postpone protein degradation. Notably, PGR
255 ubiquitination was increased after SOX4 depletion (Fig. 5F). These results
256 suggested that SOX4 knockdown reduced PGR protein stability through the
257 polyubiquitin-mediated proteasome degradation pathway.

258

259 **SOX4 inhibits PGR degradation by regulating E3 ligase HERC4**

260 Since ubiquitin E3 ligases were mainly responsible for protein
261 ubiquitination(Rape 2018), we next intended to identify the potential ubiquitin
262 E3 ligase for PGR protein. Mass spectrometry (MS) was employed for PGR
263 immunoprecipitated (IP) proteins in decidualized immortalized HESCs to
264 estimate PGR associated proteins (Figure 6-figure supplement 1A).
265 Reassuringly, several E3 ubiquitination ligases were identified, including
266 HERC4, RNF213 and UBR3 whose expressions were also upregulated
267 according to RNA-Seq in SOX4-silenced HESCs (Fig 6A). Real-time PCR
268 confirmed the abnormal upregulation of these E3 ligases when SOX4 was
269 downregulated (Fig. 6B, and Figure 6-figure supplement 1B, C). The protein
270 level of HERC4 was consistently upregulated upon the ablation of SOX4 (Fig.
271 6C). As HERC4 showed the most significant change after SOX4 knockdown
272 and its interaction with PGR as a potential E3 ubiquitin ligase was also
273 supported by UbiBrowser database (<http://ubibrowser.ncpsb.org>), we mainly
274 focused on this E3 ligase in our following experiments.

275 To verify whether HERC4 was a potential E3 ligase for PGR protein, we
276 first detected the protein interaction between HERC4 and PGR. The
277 co-immunoprecipitation result demonstrated a conserved physical interaction
278 between endogenous HERC4 and PGR in decidualized stromal cells as well
279 as exogenous expressed in 293T cells (Fig. 6D, E). Immunofluorescence
280 analysis showed the colocalization of HERC4 and PGR in decidualized
281 HESCs (Fig. 6F). To figure out whether HERC4 could induce endogenous
282 ubiquitination of PGR, HERC4 was overexpressed in immortalized HESCs
283 followed by decidualization for two days. The ubiquitination of PGR was higher
284 in the presence of HERC4 than in control, as shown in Figure 6G, consistent
285 with a reduced level of PGR protein in immortalized HESCs cells (Figure
286 6-figure supplement 1D). Conversely, HERC4 abolished by siRNA significantly

287 reduced PGR ubiquitination in 293T cells (Fig. 6H). The above studies
288 demonstrated that HERC4 was a critical E3 ligase for PGR ubiquitination.
289 Moreover, overexpression of HERC4 rescued the decreasing PGR
290 ubiquitination in the presence of SOX4 in 293T cells (Fig. 6I). Hence, SOX4
291 affected PGR ubiquitination through E3 ubiquitin ligase HERC4.

292 Since HERC4 was a ubiquitin ligase of PGR and the increased
293 ubiquitination of PGR was observed in the presence of HERC4, we next
294 interrogated whether HERC4 mediated PGR degradation. This posit was
295 underpinned by decreased PGR protein level with HERC4 overexpression in
296 both 293T cells (Fig. 6J) and immortalized HESCs (Fig. 6K). PGR protein was
297 also rescued by siRNA-mediated knockdown of HERC4 (Figure 6-figure
298 supplement 1E) in SOX4 abolished decidualized immortalized HESCs (Fig.
299 6L), and consistently, the IGFBP1 level was also rescued (Figure 6-figure
300 supplement 1F). Likewise, PGR half-life increased in both normal and SOX4
301 abolished immortalized HESCs after HERC4 repression (Fig. 6M and 6N). In a
302 word, these studies demonstrated that SOX4 mediated PGR degradation by
303 modulating E3 ligase HERC4.

304 Since there were 41 lysine residues in the PGR protein, UbiBrowser
305 (<http://ubibrowser.ncpsb.org>) was applied to predict the potential domain
306 recognized by E3 ligase HERC4 (Figure 7-figure supplement 1A). As the latent
307 recognition domains were located in DBD and LBD domains of PGR, PGR was
308 divided into four fragments (F1, F2, F3, and F4) accordingly (Fig. 7A). Only F3
309 (DBD domains of PGR) exhibited protein degradation after co-transfected with
310 HERC4 in 293T cells (Fig. 7B). Point mutation of all Lysine(K) to arginine(R) in
311 DBD region (Fig. 7C) showed that K588, K613, K617, K638 were critical for
312 PGR degradation (Fig. 7D), which was further sustained by incapable PGR
313 ubiquitination mediated by HERC4 in these mutant forms when compared with
314 WT or K565R in 293T cells (Fig. 7E). Collective, these results suggested that
315 K588, K613, K617 and K638 were vital for PGR ubiquitination by HERC4.

316

317 **Aberrantly decreased endometrial SOX4 expression is associated with**

recurrent implantation failure undergoing IVF treatment

There was considerable evidence indicating that HESCs decidualization was severely impaired in patients with the EMS, both in eutopic and ectopic lesions(Klemmt, Carver et al. 2006). **Recurrent implantation failure** (RIF) has also been reported to be associated with compromised decidualization(Zhou, Yan et al. 2019). We next analyzed the expression levels of SOX4, PGR, FOXO1, HERC4, IGFBP1, and PRL in the mid-secretory endometrium of healthy women (control, n = 12) versus women who suffered RIF due to endometriosis (EMS-RIF, n = 12). The expressions of *SOX4*, *FOXO1*, *IGFBP1*, and *PRL* were significantly decreased in EMS-RIF group with increased HERC4 compared with the control, but the mRNA level of *PGR* was comparable in both groups (Fig. 8A–F). At protein level, a large portion of endometrial samples from women with EMS-RIF showed reduced expression of SOX4, FOXO1, IGFBP1. Although the *PGR* mRNA levels were comparable between healthy and RIF samples, PGR protein was significantly decreased accompanied by increased HERC4 in EMS-RIF group (Fig. 8G, H). Immunostaining analysis further revealed significantly reduced PGR, SOX4, IGFBP1 and FOXO1 expression and increased HERC4 in stromal cells in women with EMS-RIF (Fig. 8I).

Additionally, primary human endometrial stromal cells (HESCs) were obtained from the proliferative phase endometrium of three healthy and three EMS-RIF patients (Figure 8-figure supplement 1A). The expression levels SOX4, FOXO1, PGR, IGFBP1 were lower in the primary HESCs of EMS-RIF cultured with EPC for 4 days compared to the control (Fig. 8J). Moreover, overexpression of SOX4 and/or PGR at least partially restored FOXO1 and IGFBP1 expression in EMS-RIF stromal cells (Fig. 8K). Collectively, our in vivo and in vitro evidence strongly suggested the crucial role of SOX4 in decidualization and female fertility.

347 **Discussion**

348 Here, we find that SOX4 is the most abundant SOX family member in
349 HESCs and plays a vital role in decidualization. Previous studies have
350 demonstrated that the SOX family controls cell fate and differentiation in
351 various developmental processes (She and Yang 2015, Angelozzi and
352 Lefebvre 2019). A previous study has confirmed that epithelial SOX17, one of
353 SOX transcription factors, is indispensable for uterine receptivity and embryo
354 implantation by regulating IHH expression (Wang, Li et al. 2018). The embryo
355 adhesion of implantation process occurred between the embryo and epithelia,
356 and the proper differentiation of epithelia is required for this event (Ye 2020).
357 Moreover, for the endometrial receptivity establishment, the stromal
358 compartment also needs to differentiate in response to the endocrine hormone
359 progesterone and estrogen that is known as decidualization, and the cross-talk
360 between the stroma and epithelia has been widely approved to participate to
361 the establishment of endometrial receptivity (Li, Kannan et al. 2011).

362 In the present study, we uncover the critical role of SOX4 in stromal cell
363 decidualization by RNA-Seq and ChIP-Seq. Several previously unappreciated
364 genes of SOX4 are unveiled through RNA-seq. FOXO1 is indispensable in the
365 process of decidualization account for the widely overlapping of binding peaks
366 with PGR (Vasquez, Mazur et al. 2015). In this investigation, FOXO1 is
367 regulated by SOX4 at transcriptional level, indicating the essential role of
368 SOX4 in decidualization. We are also very surprised to notice that the direct
369 regulation of SOX4 on FOSL2, an important part of AP1 complex involving
370 inflammation (Renoux, Stellato et al. 2020), expression directly. Moreover, we
371 also find that FOSL2 and FRA2 motif are significantly enriched in SOX4
372 binding sites, suggesting the complex interplay between these factors. The
373 regulation of SOX4 on FOSL2 implicates an alternative role of SOX4
374 regulating decidualization, which deserves further investigation.

375 PGR is a master regulator of the decidualization process since
376 decidualization is mainly influenced by the progesterone-PGR signaling. After
377 dimerization and nuclear translocation, PGR protein interacts with the following
378 proteins to synergistically direct the differentiation program: FOXO1, C/EBP β ,
379 STAT3, STAT5, HOXA10, and HOXA11(Gellersen and Brosens 2014). An
380 array of modulators regulates the functional plasticity of PGR, including
381 subcellular distribution, protein modification, and interaction with
382 co-regulators(Wu, Li et al. 2018). In this study, we were intrigued by the finding
383 that SOX4 affects PGR protein stability rather than its transcription.

384 During the menstrual cycle, estrogen-ER signaling induced endometrial
385 PGR mRNA in the proliferative phase, recapitulating the regulation manner of
386 PGR in mouse uteri(Tan, Paria et al. 1999, McKinnon, Mueller et al. 2018).
387 Here we provided evidence that PGR protein is further stabilized by increased
388 SOX4 at post-transcriptional regulation in the later secretory phase. Previous
389 studies have validated that SOX4 regulates P53 stability through E3 ligase
390 Mdm2(Pan, Zhao et al. 2009). In breast cancer cells, E3 ligase BRCA1(Calvo
391 and Beato 2011) and CUEDC2(Zhang, Zhao et al. 2007) have been shown to
392 regulate PGR protein stability. Multiple evidence in our research have verified
393 that the E3 ligase HERC4 functions as a ubiquitin-modified enzyme for the
394 PGR protein. Further exploration revealed that lysine residual in the PGR
395 DNA-binding domain possess modified sites for ubiquitination. In the
396 endometrial cells, there are very limited reports regarding PGR protein
397 modification. Our previous study proved that Bmi1 facilitates the PGR
398 ubiquitination through E3 ligase E6AP, which promotes PGR transcriptional
399 activity instead of protein degradation(Xin, Kong et al. 2018). Besides,
400 SUMOlation of PGR has been reported to occur at the K388 site and
401 fine-tunes the transcriptional activity of PGR(Jones, Fusi et al. 2006) . The
402 Lys-388 is a key site not only for PGR-B SUMOlation, but also a key site for
403 CUEDC2-mediated ubiquitination in proteasome in breast cancer(Zhang, Zhao

et al. 2007). Here, we characterized that K588, K613, K617, and K638 were critical sites for E3 ligase HERC4, which mediated PGR ubiquitination and degradation. There were other E3 ligases interacting with PGR as revealed by our mass spectrum, whether these E3 ligases also mediated PGR ubiquitination remains largely unknown. Multiple specific E3 ligases may exist for PGR ubiquitination rely on individual lysine residues.

Here, we report a previously unrecognized PGR ubiquitination and degradation modified by the ubiquitin E3 ligase HERC4 regulated by SOX4 in endometrial stromal cells. There are several potential postulations concerning the underlying mechanism by which SOX4 inhibits HERC4 expression in stromal cells. SOX4 has been reported to interact with repressive histone modifiers, such as H3K27 trimethylation enzyme EZH2, to directly repress the target gene transcription(Koumangoye, Andl et al. 2015). On the other hand, SOX4 indirectly repress the target gene expression through regulating EZH2 expression(Tiwari, Tiwari et al. 2013). Since no direct SOX4 binding on HERC4 is observed, the detailed mechanism by which SOX4 regulates HERC4 transcription in endometrial stroma cells requires further exploration.

Considering the critical role of progesterone during the pregnancy establishment and maintenances, both natural and synthetic progestogens have been widely used to improve endometrial function in women with a history of RIF and unexplained recurrent pregnancy loss. However, some of these patients still suffered from RIF and recurrent miscarriages due to progesterone resistance. The causes of progesterone resistance may be related to defects in the PGR signaling and its molecular chaperones as well as decreasing PGR transcriptional activity(McKinnon, Mueller et al. 2018). PGR functional deficiency is related to abnormal PGR mRNA levels, post-transcriptional modifications, post-translational modifications and protein stability(Xin, Qiu et al. 2016, McKinnon, Mueller et al. 2018). However, the

432 specific causes of PGR defects are, at present, poorly explored and
433 understood.

434 Endometriosis is frequently accompanied by progesterone resistance and
435 infertility. Several transcription factors, including the factors in GATA and HOX
436 family can regulate the progesterone signaling, and the epigenetic differential
437 methylation of PR-B, HOX, and GATA family transcription-factor can lead to
438 their decreased expression, leading to the disturbed progesterone signaling in
439 the endometriosis (Zondervan, Becker et al. 2020). It is interesting that lower
440 SOX4 expression is strongly relevant with reduced PGR protein expression in
441 endometrial stromal cells of EMS who experience RIF, and the level of PGR is
442 restored to some extent when SOX4 is overexpressed in these stromal cells. It
443 is conceivable that PGR defects caused by insufficient SOX4 may potentially
444 result in implantation failures, even with high doses of progestogens
445 supplementation during IVF. In our previous study using human endometrial
446 samples from different patients with recurrent spontaneous abortion cohort, we
447 also observed progesterone resistance as revealed by defective
448 PGR-signaling, with normal PGR protein level but diminished transcriptional
449 PGR activity(Xin, Kong et al. 2018). This implies that any disturbances in the
450 transduction of P4-PGR signaling pathway will severely influence the
451 endometrial cell responsiveness to progesterone and contribute to infertility.

452 Collectively, our investigation provides compelling evidence that SOX4
453 plays a key role in HESCs decidualization through the transcriptional
454 regulation of critical factors related to decidualization. Meanwhile, SOX4
455 endows human stromal cells appropriate progesterone responsiveness by
456 fine-tuning PGR protein stability. Aberrant SOX4 expression is strongly
457 associated with decreased PGR and FOXO1 expression in the endometrium
458 of women who have experienced RIF with EMS, implying the high clinic
459 relevance of SOX4 in female fertility and pregnancy maintenance. A better

460 understanding of the regulatory network of SOX4 will facilitate the
461 development of therapeutic strategy for the clinical treatment of RIF in EMS.

462 **Materials and Methods**

463

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
cell line (H. sapiens)	T HESCs	ATCC	CRL-4003 RRID: CVCL_C464	
Antibody	Anti-SOX4 (Rabbit polyclonal)	Abcam	Cat # ab80261 RRID:AB_1658989	WB (1:600) ICC (1:1000) IF (1:300)
Antibody	Anti-SOX4 (Rabbit polyclonal)	Diagenode	Cat # C15310129	IP (1:100)
Antibody	Anti-PGR (Rabbit monoclonal)	CST	Cat # 8757 RRID:AB_2797144	WB (1:500) ICC (1:3000) IF (1:200) IP (1:50) ChIP (1:50)
Antibody	Anti-HERC4 (Rabbit polyclonal)	Protein tech	Cat # 13691-1-AP RRID:AB_10596480	WB (1:1000) ICC (1:1000) IF (1:300)
Antibody	Anti-FOXO1 (Rabbit polyclonal)	Abcam	Cat # ab39670 RRID:AB_732421	WB (1:1000) ICC (1:3000)
Antibody	Anti-IGFBP1 (Rabbit polyclonal)	Abcam	Cat # ab228741	WB (1:1000) IHC (1:500)
Antibody	Anti-Ub (Mouse	CST	Cat # 3936 RRID:AB_3	WB (1:1000)

	monoclonal)		31292	
Antibody	Anti-HA (Rabbit monoclonal)	CST	Cat # C29F4	WB (1:1000) IP (1:100) ChIP (1:100)
Antibody	Anti-Myc (Mouse monoclonal)	CST	Cat # 2276 RRID:AB_3 31783	WB (1:1000)
Antibody	Anti-Flag (Mouse monoclonal)	Sigma	Cat # F9291 RRID:AB_4 39698	WB (1:1000)
Antibody	Anti-GAPDH (Rabbit polyclonal)	Abmart	Cat # P30008	WB (1:2000)
chemical compound, drug	E2	Sigma	E8875	Final concentration: 10 nM
chemical compound, drug	MPA	Sigma	M1629	Final concentration: 1 μM
chemical compound, drug	cAMP	MCE	HY-B0764	Final concentration: 0.5 mM
chemical compound, drug	MG132	Selleck	S2619	Final concentration: 20 μM
chemical compound, drug	3-MA	Selleck	S2767	Final concentration: 20 mM
chemical compound, drug	CHX	MedChem Express	HY-12320	Final concentration: 20 μg/ml
chemical compound, drug	insulin-transferrin -selenium	Thermo Fisher	51500-056	Final concentration: 1%

chemical compound, drug	puromycin	MedChem Express	HY-15695	Final concentration: 500 ng/ml
chemical compound, drug	RNAi MAX	Thermo Scientific	13778030	7.5 µl/6well
chemical compound, drug	Lipofectamine 2000	Thermo Scientific	11668030	5µl/6well/2500ng DNA
commercial assay or kit	Dual-Luciferase® Reporter Assay System	Promega	E1910	
commercial assay or kit	ChIP-IT high Sensitivity	Active Motif	53040	
commercial assay or kit	KAPA DNA HyperPrep Kit	Roche	KK8502	

Sample of clinical cases

Healthy women who had already gave birth and infertile women with RIF due to endometriosis were recruited from Liuzhou Maternity and Child Health Care Hospital in China from March 2018 to December 2020. The study had been approved by hospital ethics committee and all participants signed informed consent. The age of participates are between 20 and 38 years of age with Body Mass Index (BMI) between 18 and 23. The thickness of the endometrium on ovulation days was between 8 and 16 mm with menstrual cycle between 28 ± 7 days and no steroid treatment or other medication for at least 2–3 months before biopsy. Detailed information of patients was listed in Supplementary file 1. Patients with polycystic ovary syndrome, endometrial polyps, chronic endometritis, and hydrosalpinges were excluded. Only endometrial tissue with no apparent pathology assessed by a pathologist was kept for further experiments. Decidual tissue in early pregnancy were collected

479 from the women who underwent legal termination of an apparently normal
480 early pregnancy (about 8 weeks). Recurrent implantation failure (RIF) referred
481 to the inability to achieve a clinical pregnancy after transferring at least four
482 good-quality embryos in a minimum of three fresh or frozen cycles in a woman
483 under 40 years old(Coughlan, Ledger et al. 2014).

484

485 **Isolation and culturing of primary endometrial stromal cells**

486 Primary HESCs were obtained from healthy, reproductive-aged volunteers
487 with regular menstrual cycles or Endometriosis stage IV patients with infertility.
488 An endometrial biopsy was performed during the proliferative phase of the
489 menstrual cycle. These participants were recruited from The First Affiliated
490 Hospital of Xiamen University in China from July 2019 to October 2020.
491 Staging of endometriosis was according to rAFS (1985). Detailed information
492 of patients was listed in Supplementary file 1, and pathology of pelvic
493 endometriosis was showed Figure 8-figure supplement 1A. The study had
494 been approved by hospital ethics committee and all participants signed
495 informed consent. Participants were documented not under hormone
496 treatments for at least 3 months before surgery. Only endometrial tissue with
497 no apparent pathology assessed by a pathologist was kept for further
498 experiments. Primary human endometrial stromal cells (HESCs) of
499 endometriosis women were isolated by laparoscopy, and the isolation and
500 culturing of primary endometrial stromal cells were performed as follows. The
501 endometrial tissues were first cut into pieces as small as possible and
502 subjected to type IV collagenase (2% concentration) digestion for 1h. Two
503 hours after cell seeding, culture medium was changed to remove the floating
504 cells.

505

506 **Establishment of SOX4 knockout cell lines**

507 Immortalized HESCs cell lines were purchased from ATCC Corporation
508 (American Type Culture Collection, ATCC® cri-4003TM). SOX4 knockout was
509 generated by CRISPR/Cas9 approach as previously described(Zhang, Meng
510 et al. 2019). (1) sgRNAs targeting SOX4 gene (NM_003107.3) were designed
511 on the website (<https://zlab.bio/guide-design-resources>) and subcloned into
512 the pL-CRISPR.EFS.GFP vector (Addgene plasmid #57818). (2) Cas9
513 plasmid was packaged with lentivirus. After infecting stromal cells, Cas9
514 positive cells were sorted and were plated into a 96-well plate with one cell per
515 well. (3) knockout efficiency was verified by DNA sequencing and Western blot
516 after single cell derived clone expansion.

517

518 **In vitro culture of HESCs and decidualization**

519 Immortalized HESCs were maintained in DMEM/F12 without phenolic red
520 (Gibco) in the presence of 10% charcoal stripped fetal bovine serum (CS-FBS,
521 Biological Industries), glucose (3.1 g/L), sodium pyruvate (1 mM, Sigma);
522 sodium bicarbonate (1.5 g/L, Sigma), Penicillin-Streptomycin (50 mg/ml,
523 Solarbio); insulin-transferrin-selenium (ITS, 1%, Thermo Fisher) and
524 puromycin (500 ng/ml). The primary cultured cells were maintained in
525 DMEM/F12 (Gibco) without phenolic red and 10% CS-FBS. For
526 decidualization, HESCs were cultured in medium at the present of estrogen
527 (E2, 10nM, Sigma), medroxyprogesterone acetate (MPA, 1 mM, Sigma), and
528 dibutyl cyclophosphinoside (db-cAMP, 0.5 mM, MCE) in 2% CS-FBS with
529 different days. All the cells were cultured in 5% CO₂, 95% air, 100% humidity at
530 37 °C and culture medium was replaced every 48 h. The endometrial stromal
531 cell line was purchased from ATCC and authenticated using STR profiling by
532 ATCC, and
533 tested to be free from mycoplasma contamination.

534

535 **Plasmid construction and siRNA transfection**

536 The overexpressed plasmids of SOX4 (pLVML-FLAG-SOX4-IRES-puro,
537 and pLVX-HA-IRES-ZSgrena-SOX4), HERC4
538 (pLVX-FLAG-HERC4-IRES-Zsgreen, pENTER-FLAG-HERC4, and
539 HA-PCMV-HERC4), PGR (pLVX-MYC-PGR-IRES-Zsgreen and
540 HA-pCMV-PGR), and ubiquitin (pRK5-HA-Ubiquitin-WT and
541 MYC-Ubiquitin-WT) were purchased from Wu Han Miao Ling Company or
542 home-made. The list for plasmid used in this study was showed
543 Supplementary file 3. HESCs were transfected with these plasmids by
544 Lipofectamine 2000 transfection reagent (Invitrogen) or infected with the
545 lentivirus, and 48 hours later, HESCs were decidualized with EPC treatment.
546 ShSOX4 lentivirus was purchased from Shanghai Ji Man Company. The
547 ShSOX4 lentivirus and the control lentivirus were placed in the HESCs
548 medium, 48h after infection, HESCs were decidualized with EPC. siRNAs
549 targeting SOX4, HERC4, PGR and FOXO1 were purchased from Guangzhou
550 RiboBio Biological Company (see the specific sequence for details in
551 Supplementary file 2). RNA interference was carried out according to the
552 manufacturer's instructions. Briefly, 10 mM siRNA was transfected into HESC
553 with Lipofectamine RNAi MAX (Invitrogen, Carlsbad, USA). For the knockdown
554 and overexpression experiment, the silencing and overexpression was first
555 performed without differentiation stimulus, and 24 hours later hESCs were
556 decidualization for indicated time.

557

558 **ChIP-seq, ChIP-QPCR**

559 ChIP-seq was performed according to manual of ChIP-IT high Sensitivity
560 (Active motif, catalog NO. 53040). Briefly, both immortalized HESCs and
561 primary cultured endometrial stroma cells (treated with EPC to induced
562 decidualization for two days) with exogenous expressed HA-SOX4 were
563 cross-linked and immunoprecipitated with HA (CST, c29F4) and PGR (CST,
564 8757) antibodies. Immunoprecipitated and input DNA were quantified using

565 Qubit 4.0 fluorometer. Libraries were prepared using the KAPA
566 DNA HyperPrep Kit (KK8502) and sequenced with an Illumina Nova-PE150.
567 ChIP-QPCR was performed as ChIP-seq with antibodies HA (CST, c29F4) and
568 PGR (CST,8757) and the immunoprecipitated DNA was detected by QPCR. All
569 the PCR primers used in ChIP-qPCR were listed in Supplementary file 2.

570

571 **Immunoprecipitation**

572 For protein interaction, IP experiments were performed as previously
573 described(Xin, Kong et al. 2018). Anti-PGR (CST, 8757), Anti-HA (CST, c29F4),
574 SOX4 (Diagenode, C15310129), and mouse IgG or rabbit IgG (Mouse
575 Anti-Rabbit IgG (Conformation Specific, L27A9 mAb) were used for
576 immunoprecipitation. The immunoprecipitants were washed four times in lysis
577 buffer, resolved with SDS-PAGE, and immunoblotted with corresponding
578 antibodies.

579

580 **RNA-Seq**

581 Scramble or siSOX4 RNA were transfected into HESCs at 50%
582 confluence with RNAi MAX (Invitrogen). Immortalized HESCs were
583 decidualized for 2 days after transfection and RNAs were collected for
584 RNA-Seq of BGISEQ (China, BGI). Purified RNA was prepared and subjected
585 to 50-bp single-end RNA sequencing.

586

587 **IP mass spectrometry**

588 Immortalized HESCs were used to prepare IP samples after treated with
589 EPC 2 days. After Co-IP with PGR-conjugated agarose beads, the
590 immunoprecipitants were resolved with SDS-PAGE and visualized using
591 Coomassie brilliant blue stain. The discrete bands between PGR and IgG
592 control were isolated, digested, purified, and subjected to Liquid
593 Chromatograph Mass Spectrometer (LC-MS) in School of Life Sciences,

594 Xiamen University.

595

596 **Dual-luciferase reporter Assay**

597 Construction of luciferase reporter was performed as previously
598 described(Jiang, Liao et al. 2015). The promoter regions of SOX4 were
599 amplified from genomic DNA and subcloned into pGL3 plasmid. All constructs
600 were transiently transfected into 293T cells using Lipofectamine 2000. Total
601 cell lysates were prepared 36 h after transfection, and luciferase activity was
602 measured using the Dual-Luciferase Reporter Assay System (Promega
603 Corporation). Firefly luciferase activity was normalized by Renilla luciferase
604 activity.

605

606 **PGR ubiquitination assay**

607 The ubiquitination assays were performed as previously described(Xin,
608 Kong et al. 2018). MG-132 (Sigma M8699, 20 μ M) was added to the cultured
609 medium 6 h before cells collection. Cell lysis was immunoprecipitated with
610 antibodies against PGR, and HA, respectively. Proteins were released from
611 the beads by boiling in SDS-PAGE sample buffer, and ubiquitination was
612 analyzed by immunoblotting with different antibodies.

613

614 **Protein stability experiment and degradation assay**

615 To determine the protein stability of PGR protein, SOX4 knockdown and
616 control immortalized HESCs decidualized for 2 days followed by treatment with
617 cycloheximide (CHX, MedChemExpress, 20 μ g/ml), a protein synthesis
618 inhibitor, for 0, 3, 6, 9 h. or 0, 4, 8, 12 h. The protein degradation assay of PGR,
619 the immortalized HESCs with 50% confluency were induced decidualization for
620 2 days, followed by treatment with the proteasome inhibitor MG132 (Selleck,
621 20 μ M, 6 hours), and the autophagy inhibitor 3-methyladenine (3-MA, Selleck,
622 20 mM, 6 hours). A group treated with DMSO served as the negative control

623 group.

624

625 **Immunostaining**

626 For healthy endometrial tissue, proliferative phase samples were timed
627 based on the patient's cycle day, and luteal phase samples were timed using
628 the subject's urinary luteinizing hormone (LH) surge. All the samples from the
629 biopsy were fixed in formalin and embedded in paraffin for section. After
630 deparaffinization and hydration, formalin-fixed paraffin embedded endometrial
631 sections (5 μ m) were subjected to antigen retrieval by autoclaving in 10 mM
632 sodium citrate solution (pH=6.0) for 10 min. A diaminobenzidine (DAB, Sigma)
633 solution was used to visualize antigens. Sections were counter-stained with
634 hematoxylin. In immunofluorescence studies, formalin-fixed HESCs cells were
635 blocked with 5% BSA in PBS and immune-stained by antibodies for SOX4
636 (Abcam, ab80261), PGR (CST, 8757), FOXO1 (Abcam, ab39670),
637 IGFBP1(Abcam, ab228741) and HERC4(Proteintech,13691-1-AP). Signals
638 were visualized by secondary antibody conjugated with Cy2 or Cy3
639 fluorophore (Jackson ImmunoResearch). Sections were counter-stained with
640 Hoechst 33342 (2 μ g/ml, Life technology).

641

642 **Real-time PCR**

643 Quantitative real-time PCR was performed as described previously(Jiang,
644 Liao et al. 2015). Total RNA was extracted from HESCs or endometrium using
645 TRIzol reagent (Invitrogen) following the manufacturer's protocol. The quality
646 of RNA was determined by electrophoresis and concentration of RNA was
647 measured by Nanodrop. A total of 1 μ g RNA was used to synthesize cDNA.
648 Quantitative real-time PCR was performed with SYBR Green (Takara) on an
649 ABIQ5 system. All expression values were normalized against GAPDH. All
650 PCR primers are listed in Supplementary file 2.

651

652 **Western-blot and**

653 Western blot analysis was performed as described previously(Zhang,
654 Meng et al. 2019). Antibodies against SOX4, IGFBP1, FOXO1, PGR, HERC4,
655 FOSL2, HA, Myc, Flag, and ubiquitin were used. GAPDH served as a loading
656 control. The value for relative protein level represents the quantification of
657 band intensity of indicated protein with the loading control GAPDH using
658 Image J.

659

660 **Bioinformatic analysis**

661 The softwares for RNA-Seq and ChIP-Seq analysis including STAR
662 (2.7.3a) for alignment, MACS2 (2.2.7.1) for peakcall of ChIP-Seq, ggplot2
663 (3.3.5) for visualization, ngs.plot.r (2.61) for peak heatmap of ChIP-Seq and
664 the packages of edgeR (3.9), Complexheatmap (2.4.3), ChIPseeker (1.24.0) in
665 R (4.1). For ChIP-Seq, after filter the raw data to remove adapter from read by
666 Trimgalore, the clean reads were aligned to the human genome (Hg38) by
667 HISAT2. Only uniquely aligned reads were kept for downstream analysis.
668 MACS2 was applied for peak call using default parameters. The enrichment of
669 SOX4 binding on specific gene was visualized in Integrative Genomics Viewer
670 (IGV). RNA-seq raw data were initially filtered to obtain clean data after quality
671 control by Trimgalore. Clean data were aligned to the human genome (Hg38)
672 by HISAT2. RPKM value of each gene was calculated by EdgeR package in R.
673

674 **Statistics**

675 Statistical analysis was performed with Graphpad Prism. The data were
676 shown as the mean $\pm$ SEM. Statistical analyses were performed using 2-tailed
677 Student's *t*-test or ANOVA. All experiments were repeat at least three times
678 and *p*-values less than 0.05 were considered statistically significant.

679

680 **Data availability**

681 The sequencing data generated in this study have been deposited in the

682 Gene Expression Omnibus database under accession code GSE146280
683 (RNA-Seq), GSE174602 (ChIP-Seq in stromal cell line) and GSE116096
684 (ChIP-Seq in primary cultured stromal cell).

685

686 **Competing interests**

687 The authors declare that they have no competing interests.

688

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696

697 **Ethics**

698 The studies for collecting the endometrial tissue was approved by The Ethical
699 Committee of the Faculty of the Liuzhou Maternity and Child Health Hospital
700 (2020-074). The studies for isolating the primary endometrial cells was
701 approved by The Ethical Committee of The First Affiliated Hospital of Xiamen
702 University (XMY-2021KYSB044). All participants signed informed consent.

Figure Legends:

Fig. 1. SOX4 is dynamically expressed in human ESCs.

(A, B) Expression of all transcription factors (A) and SOX family genes (B) in human non-decidualized ESCs by RNA-Seq. The value in Y axis indicated the RPKM (Reads Per Kilobase per Million mapped reads) in RNA-Seq data. (C) Immunohistochemical analysis of endometrial SOX4 protein expression in proliferative, secretory phases (early, middle, and late) of the menstrual cycle and early pregnancy (about 8 weeks). GE: Gland epithelium; S: stromal. Scale bar: 100 μ m. (D) Expression of SOX4 mRNA levels in decidualized stromal cells at different time points after the E2, MPA and cAMP treatment. Results are presented as means $\pm$ SEM; n = 3; ** indicates P<0.005; *** indicates P<0.0001. (E) Expression of SOX4 protein levels in decidualized stromal cells at different time points after the E2, MPA and cAMP treatment. (F) Immunofluorescent detection of SOX4 protein localization in the undecidualized (D0) and decidualized (D4) HESCs. Scale bar: 100 μ m.

Fig. 2. SOX4 is regulated by P4-PGR signaling in human ESCs.

(A) Expression of SOX4 mRNA in the presence of MPA or MPA+RU486 for 2 days in immortalized HESCs. Results are presented as means $\pm$ SEM; n = 3; *** indicates P<0.0001. (B) RNA level of PGR after siRNA mediated knockdown. Results are presented as means $\pm$ SEM; n = 3; *** indicates P<0.0001. (C) Expression of SOX4 mRNA in the presence of MPA for 2 days with PGR knockdown. Results were presented as means $\pm$ SEM; n = 3; *** indicates P<0.0001. (D) Protein level of SOX4 and PGR after PGR knockdown in the presence of MPA for 2 days. Band quantification of PGR and SOX4 protein, relative to loading control GAPDH. *indicates P<0.05. (n=3). (E) Visualization of PGR binding and chromatin accessibility on SOX4 locus. The chromatin accessibility is depicted in undecidualized and decidualized stromal cells and genome-wide PGR binding is generated from proliferated and middle secretory endometrium as revealed from previous reports. Undec: undecidualized HESCs; Dec: decidualized HESCs; Prolif: proliferative

735 endometrium; Middle: middle phase of secretory endometrium. (F) ChIP assay
 736 of potential PGR binding on SOX4 as indicated from (E) in decidualized
 737 immortalized HESCs for 2 days. Data are plotted as mean $\pm$ SEM; n = 3; ***
 738 indicates P<0.0001. (G) Luciferase activity assay of SOX4 promotor in the
 739 presence of MPA and PGR in 293T cells. Results are presented as means $\pm$
 740 SEM. n = 3; * indicates P<0.05; ** indicates P<0.005; *** indicates P<0.0001.

741

742 **Fig. 3. SOX4 regulated genes in decidualized HESCs.**

743 (A, B) mRNA levels of *PRL* (A) and *IGFBP1* (B) in undecidualized and
 744 decidualized immortalized HESCs after SOX4 knockout at indicated time
 745 points after the E2, MPA and cAMP treatment. Results are presented as
 746 means $\pm$ SEM; n = 3; * indicates P<0.05; *** indicates P<0.0001. (C) Protein
 747 levels of IGFBP1 in undecidualized and decidualized HESCs after SOX4
 748 knockout at indicated time points. (D, E) mRNA levels of *PRL* (C) and *IGFBP1*
 749 (D) after SOX4 overexpression in undecidualized or decidualized HESCs for 4
 750 days. n = 3; * indicates P<0.05. (F) Protein levels of SOX4 and IGFBP1 in
 751 decidualized HESCs for 4 days with control or SOX4 overexpression. Band
 752 quantification of SOX4 and IGFBP1 protein, relative to GAPDH. (G) Differential
 753 expressed genes detected by RNA-Seq in immortalized HESCs decidualized
 754 for 2 days with control or SOX4 knockdown as visualized by volcano plot. (H)
 755 Heatmap of top differential expressed genes from RNA-Seq after SOX4
 756 knockdown in decidualized HESCs. (I and J) KEGG (J) and GO (K) analysis of
 757 the differentially expressed genes in RNA-Seq. Results were presented as
 758 means $\pm$ SEM. * indicates P<0.05; ** indicates P<0.005; *** indicates
 759 P<0.0001. The above experiments were repeated three times.

760

761 **Fig. 4. Genome wide binding of SOX4 in decidualized stromal cells.**

762 (A) Distribution of SOX4 binding peaks as revealed from SOX4 ChIP-seq in
 763 SOX4 overexpressed (HA-SOX4) immortalized HESCs decidualized for 2
 764 days.
 765 (B) Distribution SOX4 binding in genebody. (C) Heatmap of SOX4 binding sites
 766 distribution. (D) Motif analysis of SOX4 binding sites. (E) Venn diagram of
 767 SOX4 directly binding peaks and SOX4 regulated genes after SOX4

knockdown. (F) SOX4 binding site in the STAT3, FOXO1, FOSL2 with PGR binding and chromatin accessibility in both primary stroma cell and stromal cell line. The chromatin accessibility is depicted in undecidualized and decidualized stromal cells and genome-wide PGR binding is generated from proliferated and middle secretory endometrium as revealed from previous reports. Undec: undecidualized HESCs; Dec: decidualized HESCs. (G-J) ChIP-qPCR assay of SOX4 binding on PRL, STAT3, FOXO1 and FOSL2 in HA-SOX4 overexpressed decidualized HESCs. Results were presented as means $\pm$ SEM; n=3; * indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.001$. (K) Read number of SOX4 binding in SOX4 regulated genes after SOX4 knockdown. (L) KEGG analysis of overlapped genes of SOX4 directly binding and SOX4 downregulated genes as well as non-overlapped genes. (M) mRNA levels of *FOXO1* in SOX4 knockout undecidualized and decidualized HESCs at indicated time points. Results were presented as means $\pm$ SEM; n=3; **indicates $P<0.005$. (N) Protein levels of SOX4, FOXO1 and IGFBP1 in SOX4 knockout undecidualized and decidualized HESCs at indicated time points. Band quantification of indicated proteins, relative to loading control GAPDH. *indicates $P<0.05$, ***indicates $P<0.001$. n=3. (O) mRNA levels of *FOXO1* after SOX4 overexpression in undecidualized or decidualized HESCs for 4 days. Results were presented as means $\pm$ SEM; n =3; **indicates $P<0.005$.

The above experiments were repeated three times.

Fig. 5. SOX4 stabilizes PGR through repressing the ubiquitin-proteasome pathway.

(A) mRNA levels of *PGR* after SOX4 knockout in decidualized immortalized HESCs at indicated time points after the E2, MPA and cAMP treatment. Results were presented as means $\pm$ SEM; n=3. (B) Protein levels of PGR and SOX4 after SOX4 knockout in decidualized HESCs at indicated time points after the E2, MPA and cAMP treatment. (C) Protein levels of PGR in the presence of shSOX4 or MG132. MG-132 is added 6 hours before collecting the immortalized cells. (D, E) Protein levels of PGR in the presence of protein synthesis inhibitor cycloheximide (CHX) in SOX4 knockdown cells. (F) PGR ubiquitination after immunoprecipitation with PGR antibody and blotted with

ubiquitin antibody in SOX4 knockdown immortalized HESCs decidualized for 2 days. Cells were treated with MG-132 before cell lysis. The value for relative protein level is band quantification of indicted protein, relative to GAPDH.

804

Fig. 6. SOX4 inhibits PGR degradation by regulating E3 ligase HERC4.

(A) Venn-diagram of overlapping genes between SOX4 up-regulated genes in SOX4 knockdown from RNA-Seq and PGR associated protein from IP-MS. (B and C) mRNA and protein levels of HERC4 in the absence of SOX4 by qPCR (B) and Western-blot (C). Results were presented as means $\pm$ SEM; n =3. (D) Protein interaction between PGR and HERC4 after overexpression of HA-PGR and Flag-HERC4 in 293T cells. (E) Protein interaction between PGR and HERC4 after overexpression of HA-PGR and Flag-HERC4 in decidualized immortalized HESCs for two days, D2: two days. (F) Localization of PGR and HERC4 after over-expression of PGR and HERC4 in decidualized HESCs for two days, scale bar: 100 μ m. (G) PGR ubiquitination at the present of MG-132 after over-expression of HERC4 in decidualized HESCs for two days. (H) PGR ubiquitination at the present of MG-132 and ubiquitin with HERC4 over-expression or knockdown in 293T cells. (I) PGR ubiquitination at the present of MG-132, ubiquitin and SOX4 with or without HERC4 in 293T cells. (J) Degeneration of PGR after HERC4 overexpression in 293T cells. (K) PGR protein levels at the present of HERC4 overexpression and in HERC4 knockdown decidualized immortalized HESCs for 2 days. (L) Protein levels of PGR, HERC4 and SOX4 after SOX4 and/or HERC4 knockdown in decidualized immortalized HESCs for 2 days. (M, N) The protein half-life of PGR after HERC4 knockdown at the present of protein synthesis inhibitor cycloheximide (CHX) in 2 days decidualized immortalized HESCs. (O, P) The protein half-life of PGR after SOX4 and/or HERC4 knockdown at the present of protein synthesis inhibitor cycloheximide (CHX) in 2 days decidualized immortalized HESCs. The value for relative protein level is band quantification of indicted protein, relative to GAPDH.

The above experiments were repeated three times.

832

Fig.7. HERC4 mediates PGR ubiquitination at K588, K613, K617 and

K638.

(A) Schematic diagram of PGR structure. F1 to F4 represents four different functional domains, respectively. (B) Protein levels of different fragments of PGR (Myc tagged F1-F4) at the present of HERC4 in 293T cells. (C) Lysine (K) sites in PGR DBD domain. (D) Protein levels of PGR after Lysine mutant to Arginine in DBD with HERC4 over-expression in 293T cells. The value for relative protein level is band quantification of indicted protein, relative to GAPDH. (E) PGR ubiquitination after Lysine mutant to Arginine in DBD at the present of HERC4, ubiquitin and MG-132 in 293T cells.

Fig. 8. Endometrial SOX4 expression in RIF of women with EMS undergoing IVF treatment.

(A, B, C, D, E, and F) Expression of *SOX4*, *FOXO1*, *IGFBP1*, *PRL*, *PGR*, and *HERC4* in mid-secretory endometrium from control (n=12) and EMS-RIF (n = 12). Results were presented as means $\pm$ SEM. * indicates $P < 0.05$. (G and H) Protein levels of *SOX4*, *FOXO1*, *IGFBP1*, *PGR*, and *HERC4* in mid-secretory endometrium from control (n=12) and EMS-RIF (n = 12). C1-C12 and R1-R12 represent tissues from different patient. (I) Localization of *SOX4*, *FOXO1*, *IGFBP1*, *PGR*, and *HERC4* in control (n = 3) and EMS-RIF groups as detected by immunostaining. C4, C7, C11, R4, R5 and R12 represent tissues from different patient. GE: Gland epithelium; S: Stromal. Scale bar: 100 μ m. (J) Protein levels of *SOX4*, *IGFBP1*, *FOXO1*, and *PGR* in primary HESCs of control and EMS by Western-blot. (K) Protein levels of *IGFBP1*, *FOXO1*, *PGR* and *SOX4* in decidualized primary endometrial stromal cells from EMS-3 after overexpression of *SOX4* and/or *PGR*.

860 **Figure 1-figure supplement 1.**

861 **Sox family expression in mouse uterine stromal and epithelial cells.**

862 (A) Heatmap of Sox family members in mouse uterine epithelial and stromal
863 cells by RNA-Seq, n = 3.

864

865 **Figure 1-figure supplement 2.**

866 **SOX4 expression in the primary stromal cell during the decidualization.**

867 (A and B) Expression of SOX4 mRNA and protein in decidualized primary
868 HESCs from day 0 to day 6. Results were presented as means $\pm$ SEM; n =3;
869 *** indicates $P < 0.001$.

870

871 **Figure 3-figure supplement 1.**

872 **SOX4 knockout in the stroma cell by CRISPR-Cas9.**

873 (A Schematic diagram of SOX4 knockout by CRISPR/Cas9.

874 (B, C) SOX4 knockout confirmation by immunoblot and immunofluorescence in
875 HESCs. Scale bar: 50 μ m.

876

877 **Figure 3-figure supplement 2.**

878 **SOX4 knockdown by the shRNA derailed the decidualization in primary**
879 **stromal cell.**

880 (A) Knockdown efficiency of SOX4 by shRNA. Results were presented as
881 means $\pm$ SEM; n =3; *** indicates $P < 0.001$. (B, C) Expression of of *PRL* and
882 *IGFBP1* in the SOX4-knockdown primary decidualized HESCs from days 0 to
883 6. Results were presented as means $\pm$ SEM. n =3; * indicates $P < 0.05$, **
884 indicates $P < 0.005$. (D) Protein levels of SOX4 and IGFBP1 in the
885 SOX4-knockdown primary decidualized HESCs from days 0 to 6.

886

887

888 **Figure 3-figure supplement 3.**

889 **SOX4 over-expression upregulated the expression of decidualization**
890 **marker genes.**

891 (A, B) Expression of *PRL* and *IGFBP1* in primary decidualized HESCs for 4
892 days after SOX4 over-expression. Results were presented as means $\pm$ SEM; n
893 =3; * indicates $P<0.05$, ** indicates $P<0.005$. (C) SOX4 expression after SOX4
894 overexpression in the primary decidualized HESCs for four days.

895

896 **Figure 4-figure supplement 1.**

897 **FOXO1 knockdown abolished the effect of SOX4 over-expression on**
898 **decidualization**

899 (A) FOXO1 expression after FOXO1 knockdown in the primary decidualized
900 HESCs. (B) Protein levels of FOXO1, IGFBP1 and SOX4 after overexpression
901 of SOX4 in the presence of FOXO1 knockdown or not in decidualized hESCs
902 for four days.

903

904 **Figure 4-figure supplement 2.**

905 **The upregulated genes after SOX4 knockdown with SOX4 binding site in**
906 **their regulatory regions.**

907 (A) Venn diagram of SOX4 directly binding peaks and upregulated genes after
908 SOX4 knockdown. (B) GO analysis of the overlapped gene list in A.

909

910 **Figure 5-figure supplement 1.**

911 **Regulation of SOX4 on PGR in primary decidualized stroma cells.**

912 (A) Expression of *PGR* mRNA after SOX4 knockdown in primary decidualized
913 HESCs cultured with EPC from 0–6 days. Results were presented as means
914 $\pm$ SEM; n =3. (B) Protein levels of PGR after SOX4 knockdown in primary
915 decidualized HESCs cultured with EPC from 0–6 days. (C) Protein levels of
916 PGR and SOX4 in the presence of proteasome inhibitor MG132 or autophagy
917 inhibitor 3MA. The inhibitors were added 6 hours before collecting the
918 immortalized cells.

919 **Figure 6-figure supplement 1.**

920 **The SOX4 regulated ubiquitin E3 ligase expression during the**
921 **endometrial stroma decidualization.**

922 (A) Coomassie blue staining of PAGE-Gel with PGR immunoprecipitated
923 complex. (B and C) Expression of *RNF213* and *UBR3* in SOX4 knockdown
924 HESCs. Results were presented as means $\pm$ SEM; n =3; * indicates $P < 0.05$. (D)
925 PGR expression after HERC4 over-expression in HESCs as detected by
926 immunofluorescence, scale bar: 100 μ m. (E) Knockdown efficiency of HERC4
927 by siHERC4. The above experiments were repeated three times. (F) Protein
928 levels of IGFBP1 in the indicted treatment.

929

930 **Figure 7-figure supplement 1.**

931 **The predicted lysine sites for the ubiquitin modification in PGR protein**
932 **by the E3 ligase HERC4.**

933 (A) UbiBrowser (<http://ubibrowser.ncpsb.org>) was applied to predict the
934 potential site recognized by E3 ligase HERC4.

935

936 **Figure 8-figure supplement 1.**

937 **Histology of pelvic endometriosis tissue.** (A) HE staining of paraffin section
938 from the pelvic endometriosis.

939

940 **Supplementary files**

941 Supplementary file 1. Clinical information of all patients and controls.

942 Supplementary file 2. All primers used in this study.

943 Supplementary file 3. The plasmids used in this study

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