

1 **Early-life environment programs reproductive strategies through epigenetic regulation of *SRD5A1***

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13

14 **Abstract**

15 Reproductive function and duration of the reproductive life span are phenotypically plastic and
16 programmed in response to the early-life environment. Such adaptive responses are described and
17 rationalized in life history theory in the context of resource availability, but the molecular mechanisms
18 responsible have remained enigmatic. In this study, we hypothesized that epigenetic modifications
19 underlie adaptive reproductive strategies, and found distinct methylation patterns in buccal DNA of
20 Bangladeshi women who grew up in Bangladesh or the UK. The later pubertal onset and lower ovarian
21 reserve associated with Bangladeshi childhood was seen to correlate with more numerous childhood
22 infections, so we adopted a mouse model of pre-pubertal colitis to mimic these conditions. These mice
23 have a similarly-altered reproductive phenotype, which enabled us to determine its mechanistic basis.
24 Several genes encoding proteins with known functions in follicle recruitment were differentially
25 expressed in the mice ovaries, and were also differentially methylated in the women's buccal DNA. One
26 of these, *SRD5A1* which encodes the steroidogenic enzyme 5 α reductase-1, was down-regulated in
27 the mice ovaries and hyper methylated at the same putative transcriptional enhancer as in the women's
28 DNA; the levels of methylation correlating with gene expression levels. *Srd5a1* expression was down-
29 regulated also in the hypothalamus where 5 α reductase-1 catalyzes production of neurosteroids that
30 regulate gonadotropin releasing hormone (GnRH). Chemical inhibition of this enzyme affected both
31 GnRH synthesis and release, and resulted in delayed pubertal onset *in vivo*. The activity of 5 α reductase-
32 1 in hypothalamus and ovary and the sensitivity of *SRD5A1* to epigenetic regulation attest to its role in
33 directing long-term physiological strategies in response to environmental conditions. In the reproductive
34 axis, this includes timing of pubertal onset, adult reproductive function and duration of the reproductive
35 lifespan.

36
37 **Keywords:** reproduction; epigenetics; endocrine; ovary; hypothalamus; GnRH; migrants; women; mice;
38 *SRD5A1*; 5 α reductase-1; neurosteroids

39

40

41 **Introduction**

42 Reproductive function is plastic, responding and adapting to environmental signals with changes in age
43 of sexual maturation, hormone levels, rates of ovulation and fertility, as well as length of a woman's
44 reproductive lifespan^{1,2}. The adult reproductive phenotype is particularly sensitive to the early life
45 environment and is largely programmed by mid-childhood. This was evident in studies on Bangladeshi
46 migrants, in which we found that women who spent their childhood in Bangladesh, at least until aged 8
47 years, experienced later pubertal onset, earlier menopause and had a lower ovarian reserve than
48 Bangladeshi women who grew up in the UK³⁻⁶. Children who migrated at a younger age, or second-
49 generation Bangladeshis in the UK, had similar reproductive phenotypes to their European ethnic
50 neighbors, while women who were adults at migration maintained the "Bangladeshi childhood"
51 reproductive phenotype even after many years in the UK. Such adaptive strategies might enhance the
52 likelihood of reproductive success over the life course, and often involve trade-offs between growth and
53 reproduction, as described and rationalized in life history theory⁷⁻¹⁰. Although this theory explains
54 phenotypic diversity and plasticity in the context of resource availability¹¹⁻¹³, a mechanistic
55 understanding at the molecular level, detailing how an altered reproductive strategy can be
56 implemented and maintained throughout the life course, is completely lacking.

57 Epigenetic modification provides a means of sensing the environment and translating diverse signals
58 into altered patterns of gene expression, which can have a profound and long-term effect on the
59 phenotype. The epigenome undergoes considerable modification during various stages of development
60 and, as is becoming clear, plays a role in the maturation of the reproductive axis at puberty as well as
61 adult reproductive function¹⁴⁻¹⁶. We therefore hypothesized that the early-life environment programs
62 reproductive strategies through epigenetic-driven mechanisms.

63 Multiple hurdles exist in studying the epigenetic and mechanistic bases to human reproductive function
64 and plasticity, not least of which is the inaccessibility of hypothalamic-pituitary-gonadal (HPG) tissues in
65 healthy subjects¹. In this study, we first examined buccal tissues from Bangladeshi women to look for
66 changes in DNA methylation associated with their childhood environment. In order to address the

67 functional relevance of these findings, we then employed a mouse model of early-life challenges
68 comparable to those experienced in Bangladesh. The women's distinct reproductive phenotype is
69 associated specifically with higher disease load in Bangladesh^{3,4}, where individuals are exposed to
70 recurrent immune challenge in a country prone to seasonal floods, outbreaks of disease and relatively
71 poor healthcare¹⁷. The Bangladeshi women in our studies are, however, well-nourished, rarely perform
72 manual work and are relatively affluent. To match these conditions, we adopted a mouse model of pre-
73 pubertal (equivalent of human age ~6.5-9 y) mild colitis to expose the mice to immunological challenges.
74 The treatment leads to a similarly altered reproductive phenotype and permits access to the functional
75 reproductive tissues for gene expression and epigenetic analysis. This approach, combining all the
76 advantages of an experimental mouse model with observations and proxy tissue DNA analysis from
77 distinct groups of women, has revealed a pivotal role for the steroidogenic enzyme, 5 α reductase-1 and
78 its epigenetic regulation in programming adult reproductive strategies, affecting the timing of pubertal
79 onset, adult reproductive function and duration of the reproductive lifespan.

80

81 **Results**

82 *Differential methylation patterns in Bangladeshi women who grew up in Bangladesh or in the UK*

83 Methylation analysis of buccal DNA revealed that adult Bangladeshi women (aged 28.1 ± 5.0 y) living in
84 London had distinct methylation signatures, depending on whether they had experienced childhood in
85 Bangladesh (n=15) or the UK (n=13). Illumina-Methylation Epic array data revealed 17,004 CpG sites
86 with a mean methylation difference >20%, most of which (14,509) mapped to "open sea" regions of the
87 genome; a smaller number (2,423) were associated with "shores" and "shelves" and 72 mapped within
88 CpG islands. Methylation of CpG islands harboring promoters is a strong indicator of suppressed gene
89 expression, and we first investigated genes with known functions in fertility. We identified, and
90 confirmed by targeted bisulfite sequencing (Fig S1A), elevated methylation levels in CpG islands
91 associated with *FZD1* and *RUNX3*, both of which encode proteins that regulate ovarian

92 folliculogenesis^{18,19}, and also *RASAL3*, which controls a magnitude of inflammatory responses²⁰ and has
93 been linked specifically to inflammatory bowel disease²¹. Pathway analysis revealed that genes with
94 differentially methylated CpGs were significantly enriched in the Hippo (FDR 6.99E-12) and PI3K-Akt
95 (FDR 2.83E-07) signaling pathways (Fig S1B-D), both of which play central roles in regulating follicle
96 recruitment and growth^{22,23}.

97

98 *The altered reproductive phenotype of a mouse model of early life colitis is similar to that of the women*
99 *who experienced childhood in Bangladesh*

100 In order to determine whether these epigenetic modifications occur also in the reproductive tissues and
101 play a functional role in the altered phenotype, we set up an appropriate animal model. We induced
102 temporal colitis in newly-weaned female mice (22-23 d old: approximately equivalent to 6-6.5 y in
103 human age²⁴), by administration of dextran sodium sulfate (DSS) in the drinking water for 7 days, in
104 order to mimic early-life immune challenges experienced by girls in Bangladesh^{3,4}. The mice stopped
105 gaining weight during the latter part of the treatment and blood was evident in the feces, but they
106 quickly recovered (Fig 1A). Notably, however, the DSS-treated mice had delayed onset of puberty by an
107 average of 6 days (Fig 1B), corresponding to just over 1.5 y in human lifespan²⁴. This was evident only in
108 the treated mice and, as in the women, was not inherited transgenerationally³, with puberty in the
109 female off-spring occurring at a similar age to that in off-spring of littermate controls (Fig 1C). Levels of
110 circulating anti-Müllerian hormone (AMH) comprise a clinical marker for size of the ovarian reserve and,
111 as in women who had experienced Bangladeshi childhood⁵, were significantly lower in the DSS-treated
112 mice than the controls (Fig 1D). Thus, the reproductive phenotype of the mouse model mirrors that of
113 the women who were exposed to the early-life immune challenges of childhood in Bangladesh, enabling
114 us to study the underlying mechanisms of this adaptive response.

115

116 *The mouse model ovaries have altered follicle numbers and gene expression in pathways regulating*
117 *follicle growth and recruitment*

118 Given that these findings pointed to altered ovarian function, we examined histological sections of the
119 mouse ovaries. Follicle counts showed fewer primary and antral follicles in the DSS-treated mice than in
120 the controls, and there were significantly more atretic follicles (Fig 2A-E). This concurs with the lower
121 AMH levels, and reveals an altered reproductive trajectory involving increased rates of oocyte depletion
122 from the limited ovarian pool.

123 In order to determine the pathways responsible for the altered ovarian activity, we carried out RNA-seq
124 transcriptome analysis of ovaries from the treated mice and their litter-mate controls. Both coding and
125 non-coding RNAs were found to be differentially expressed: 92 were upregulated, while 13 were down-
126 regulated ($P_{adj} < 0.05$; Fig 2F). Pathway analysis (Fig S2) of the differentially-expressed genes ($P < 0.05$)
127 revealed enrichment specifically for oocyte-meiosis (FDR 1.9-E02) and, as in the women's differentially
128 methylated DNA, also for the Hippo signaling pathway (FDR 4.3-E01). Also similar to the women's
129 distinct methylation patterns, genes in the PI3K-AKT signaling pathway that stimulates the recruitment
130 of ovarian follicles²³ were enriched, with up-regulation of several activators of the pathway, while the
131 expression of PTEN which represses this pathway was reduced (Fig 2G).

132

133 *SRD5A1 is hypermethylated in women and mice following early-life immune challenge*

134 Among the most significantly differentially expressed genes in the mouse ovaries, three were associated
135 specifically with differentially methylated regions in the women's DNA. *PKIB* and *GIGYF2* (both encode
136 proteins that activate AKT; Fig 2G) were less methylated in the women who experienced childhood in
137 Bangladesh and their expression was up-regulated in the ovaries from the DSS-treated mice, while
138 *SRD5A1* was more methylated in these women and its expression down-regulated in the mouse model
139 (Fig 3A-C).

140 *SRD5A1* encodes the steroidogenic enzyme, 5 α reductase-1, which converts testosterone to
141 dihydrotestosterone (DHT). DHT inhibits follicle activation through decreasing cyclin D2 expression and
142 inducing cell cycle arrest²⁵, and via activation of PTEN which represses PI3/AKT signaling²⁶. DHT also
143 activates progesterone production²⁷. Thus the drop in 5 α reductase1 levels would not only facilitate
144 oocyte exit from the primordial follicle pool, in accordance with the ovarian histology, but would also
145 lower progesterone production, as seen in the women who spent their childhood in Bangladesh³.

146 We therefore examined whether methylation of *Srd5a1* was also affected in our mouse model. The
147 *Srd5a1* promoter was reported to be hypermethylated in the prefrontal cortex of mice in response to
148 psychological stress²⁸, but it was completely unmethylated in ovaries of both DSS and control groups of
149 mice (Fig S3A). Only ~570 bp separate the start of the mouse *Srd5a1* gene and its neighboring gene,
150 *Nsun2*, while in the human genome, these divergent genes are just ~50 bp apart, which points to
151 additional key gene-specific *cis* regulatory regions beyond the proximal promoter. We therefore
152 examined the mouse genomic region, homologous to that differentially methylated in the human
153 samples, which is located in both genomes at the start of the first intron. DNA bisulfite conversion and
154 deep-sequencing revealed that this region was significantly more methylated in the ovaries of the DSS-
155 treated group than in the controls (Fig 3D). This intronic region carries all of the marks of a
156 transcriptional enhancer²⁹ (Fig S3B), supporting the impact of this methylation on gene expression
157 levels, which was observed also in the strong correlation between levels of mRNA expression and CpG
158 methylation at the locus (Fig 3E). Furthermore, this putative enhancer region contains a SNP, rs494958,
159 which was reported to be associated with age at natural menopause, as well as two other significant
160 SNPs in high linkage disequilibrium with this trait³⁰.

161

162 *The up-regulation of Srd5a1 by estradiol is blunted by anti-inflammatory cytokines*

163 To determine the pathways leading to a reduction in *Srd5a1* expression as a result of the early-life
164 adversity, we first examined how its levels normally change with sexual maturation. Comparison of
165 *Srd5a1* mRNA levels in ovaries from sexually immature and mature mice showed that they increased

176 around 6-fold over this time (Fig 4A). To determine the mechanisms involved, we exposed ovarian KK-1
177 mouse granulosa cells to various steroids, which revealed a stimulatory effect of estradiol (E2), while
178 neither DHT, progesterone nor dexamethasone (synthetic cortisol) had any notable effects (Fig 4B, S4).
179 This suggests that the rise in *Srd5a1* expression at the time of puberty is due to the increase in E2 levels.
180 We then verified whether *Srd5a1* expression is affected adversely by the anti-inflammatory cytokines,
181 IL4, IL-10 and IL-13, which are elevated in the general stress response. Although these cytokines alone
182 did not reduce basal *Srd5a1* levels (Fig 4C), when given together with E2, both IL-10 and IL-13 blocked
183 the E2-stimulatory effect on this gene without affecting its up-regulation of *Cyp19a1* (Fig 4D). Thus, early
184 life adversity leading to an increase in IL-10 and/or IL-13, can cause a reduction in 5 α reductase-1 levels
185 by dampening the stimulatory effect of E2, explaining the particularly significant impact of this response
186 at early stages of pubertal development.

177

178 *5 α reductase-1 regulates the central control of reproduction and pubertal timing*

179 5 α reductase-1 is widely expressed and we considered that its altered expression in non-ovarian tissues
180 might play additional roles in mediating the distinct reproductive phenotype. We therefore measured
181 *Srd5a1* expression in the hypothalamus and the prefrontal cortex of the brain, and in the pituitary of the
182 mouse model. *Srd5a1* mRNA levels were reduced in the hypothalamus of the treated mice, but not in
183 the prefrontal cortex or the pituitary (Fig 5A). Using additional mice, separation of the hypothalamus
184 into distinct regions confirmed the reduced expression of *Srd5a1* specifically in the pre-optic area (Fig
185 5B), which contains most of the neurons that control reproduction, suggesting a possible role in the
186 timing of pubertal onset. This connection was supported by the fact that in second generation mice,
187 both age of pubertal onset (Fig 1C) and levels of *Srd5a1* mRNA in the ovaries and hypothalami (Fig 5C)
188 were similar to those of controls.

189 The drop in *Srd5a1* expression in the hypothalamus led us to examine the expression levels of *Gnrh* and
190 other genes that encode factors regulating the reproductive axis. The mRNA levels of *Gnrh* were

191 significantly lower in the DSS-treated mice than in their litter-mate controls, as were those of *Kiss1*;
192 notably *Tac2* mRNA levels were elevated, while those of *Kiss1r* and *Esr1* were unaltered (Fig 5D). The
193 expression of *Fkbp5*, which is highly sensitive to glucocorticoids, was not different in these mice (Fig 5D),
194 indicating that the mice were not suffering chronic stress.

195 To establish whether 5 α reductase-1 activity regulates expression of these genes in GnRH neurons, we
196 exposed the GT1-7 GnRH neuronal cell line to the inhibitor, dutasteride. As in the DSS-treated mice,
197 both *Gnrh* and *Kiss1* mRNA levels were repressed by the drop in 5 α reductase-1 activity, while the mRNA
198 levels of *Kiss1r* and *Fkbp5* were unaffected (Fig 5E). Given previous reports that some neurosteroids
199 catalysed by this enzyme activate the stimulatory GABA-A receptor and can augment GABA effects on
200 GnRH release at puberty³¹⁻³³, we also examined the effects of 5 α reductase-1 inhibition on GnRH
201 release. Dutasteride repressed GnRH release from these cells and completely blocked the stimulatory
202 effects of a GABA-A agonist, muscimol; however, levels were restored to those of controls by addition of
203 the neurosteroid, allopregnanolone (Fig 7F).

204 Having established the impact of early-life adversity on hypothalamic expression of *Srd5a1*, and the
205 effects of 5 α reductase-1 on regulation of GnRH synthesis and secretion, we went on to demonstrate
206 the role of this enzyme in determining pubertal onset *in vivo*. Young female mice were treated with
207 dutasteride daily starting soon after weaning. The treatment delayed their first vaginal opening (FVO) by
208 3-4 days compared to the sham-treated litter-mate controls (Fig 7G), which corresponds to an estimated
209 ~1 y in human lifetime²⁴. Thus the reduced expression of this enzyme due to down-regulation of *Srd5a1*
210 expression following early-life adversity appears to play a role in the delay in pubertal onset.

211

212 **Discussion**

213 Our study demonstrates that down-regulation of *SRD5A1* plays a pivotal role in shaping adult
214 reproductive function in response to experiences during pre-pubertal development. Having found that
215 this gene was differentially-methylated in buccal DNA of Bangladeshi women according to their

216 childhood environment, we have been able to confirm, in an appropriate mouse model, its altered
217 expression in the functional reproductive tissues and its function in the distinct reproductive phenotype
218 that follows this early-life adversity. Through manipulations *in vitro* and *in vivo*, we have demonstrated
219 the role of 5 α reductase-1 in determining pubertal timing through regulation of GnRH synthesis and
220 secretion. Moreover, the modified expression in the mouse model of *Srd5a1* and other genes in the
221 pathways to ovarian follicle recruitment, explains the differing rates of oocyte depletion and disparate
222 ages at menopause in the Bangladeshi women. We have thus uncovered a key role for this enzyme in
223 determining the reproductive trajectory throughout the adult lifespan, from puberty to menopause,
224 which is established by epigenetic programming during childhood development.

225 Given that *Srd5a1* mRNA levels increase across puberty and in response to E2, the immediate pre-
226 pubertal period evidently comprises a particularly sensitive time to any signals that diminish this up-
227 regulation. Such signals include the anti-inflammatory cytokines that we examined, and likely
228 encompass additional signals arising from other forms of physiological or psychological stress, given that
229 activity of this enzyme was seen to be decreased following various stressors and this was most
230 pronounced when the stress was experienced at a young age (e.g.^{34–38}). These reports support a role for
231 epigenetic modification of *SRD5A1* during childhood in determining additional aspects of the adult
232 phenotype, particularly relating to the stress axis.

233 Apart from its role in pubertal timing, reduced expression of 5 α reductase-1 in the hypothalamus
234 undoubtedly has consequences for other endocrine axes, especially those controlling growth and the
235 stress response which are also regulated by neurosteroids catalysed by this enzyme^{38–40}. In the face of
236 adversity and limited resources, altered epigenetic regulation of *SRD5A1* in the hypothalamic control
237 center, would allow differential regulation of these major endocrine axes to mediate the adaptive
238 response to changing environmental conditions which underlie the trade-offs between growth,
239 reproduction and homeostasis described through life history theory.

240 Such reprogramming of these axes might well be beneficial for the individual, but the altered
241 reproductive phenotype presents health consequences, given that timing and duration of the

242 reproductive lifespan dictate risks for steroid- and age-related disease. The epigenetic basis of plasticity
243 that we describe here explains some of the diversity in reproductive characteristics and how they are
244 shaped by early childhood environment, while also opening up the possibility that these characteristics
245 might be susceptible to manipulation to mitigate health issues across the lifespan.

246

247 **Methods**

248 ***Human methylation analysis***

249 British-Bangladeshi women (20-35 y), were recruited in London through community contacts using
250 snowballing techniques. The first group comprised women who were born in Bangladesh and moved to
251 the UK when aged over 16 y. The second group comprised women who were second-generation British-
252 Bangladeshis, born in the UK to Bangladeshi migrant parents. Protocols for human data collection were
253 approved by the Ethical Committee of the Department of Anthropology, Durham University. Women
254 gave informed consent to participate in the study, and the data were anonymized at source.

255 Buccal swabs were collected with iSWAB (Mawi DNA Technologies) and genomic DNA isolated using the
256 DNeasy Blood & Tissue Kit (Qiagen). Genome-wide DNA methylation data acquisition was carried out on
257 the Infinium MethylationEPIC BeadChip platform (Illumina) and performed by Tepnel Pharma Services,
258 UK using 'Bangladeshi childhood' (n=16) and 'UK childhood' (n=13) DNA samples, which passed the
259 quality control checks. Multidimensional scaling (MDS) plots indicated that no significant batch effects
260 were skewing our MethylationEPIC BeadChip data sets. The data were processed with the
261 Bioconductor/minfi package. CpG probes associated with known SNPs were removed, as were those
262 with a detection probability of <0.01. Probes on both X and Y chromosomes were retained. Methylation
263 beta values (0-1) were normalized by SWAN. Dmpfinder/minfi was applied to determine probes with
264 significantly differentially methylation levels between the 'Bangladeshi-childhood' and the 'UK-
265 childhood' groups. FDR was set at <0.05. The pathway analysis utilized NIPA, a tool that performs
266 enrichment tests by hypergeometric statistics (<https://github.com/ADAC-UoN/NIPA/>).

267 For validation of the array data, targeted bisulfite sequencing was conducted on a subset of the samples
268 used to generate the MethylationEPIC BeadChip data (chosen on the basis of their DNA quality and
269 concentration) via amplicon sequencing of 10 CpG sites using the MiSeq system (Barts and the London
270 School of Medicine and Dentistry, GenomeCentre). The interrogated CpGs were cg25470148
271 (chr1:25257931) lower-strand, *RUNX3*; cg16696646 (chr17:19861616), lower-strand *AKAP10*;
272 cg26916966 (chr17:40274524), upper-strand, *KAT2A*; cg07357279 (chr17:43318735), upper-strand,
273 *FMNL1*; cg01062942 (chr19:15568935), upper-strand, *RASAL3*; cg08470875 (chr2:26401718), upper-
274 strand, *FAM59B*; cg08700190 (chr5:6636046), upper-strand, *SRD5A1*; cg08198075 (chr6:123033536),
275 upper-strand, *PKIB*; cg12914114 (chr6:170687002), lower-strand, *FAM120B*; cg01480180
276 (chr7:90896329), lower-strand, *FZD1*. For each interrogated genomic region, >100 sequencing reads
277 were obtained. Primers are given in Table S1.

278 **Mice**

279 All animals were held and handled humanely, after protocol approval, and in accordance with IACUC
280 guidelines. For DSS-treatments, upon weaning, female mice from each litter were divided randomly into
281 two groups to provide littermate controls for all experiments. After ~2 d recovery, the mice were ear-
282 marked and weighed, and one group received 3 % dextran sodium sulfate (DSS: 35-50 kDa, MP
283 Biochemicals) in the drinking water for 7 d. The DSS water was changed every 2-3 d and the mice were
284 weighed each day for at least 16 d. All mice were observed daily for signs of pubertal onset, as
285 determined by FVO. In order to assess the impact of this early life exposure on the second generation, a
286 single male was housed with the DSS-treated and the littermate control female mice.

287 For harvest of brain tissue, the brains were removed and whole hypothalamus or prefrontal cortex
288 isolated into 1 ml TRIzol for RNA extraction. For isolation of specific regions (preoptic area, arcuate
289 nucleus or cerebellum), brains were transferred into a brain matrix (RWD-800-00149-00) for coronal
290 sectioning following isolation of each region from the relevant section, as determined using Allen Brain
291 Atlas. All tissues were collected from females in estrous, verified by cytological smears. Blood was
292 collected by cardiac puncture at the time of sacrifice, and circulating AMH levels were measured by

293 ELISA (Ansh labs, Webster, Texas) according to the manufacturer's protocol, after dilution of all samples
294 x20.

295 For the administration of dutasteride *in vivo*, after weaning, female mice from each litter were marked,
296 weighed and divided to two groups. A transparent plastic separator (kindly given by Madaf Plazit
297 Packaging) was inserted into each cage, with one mouse in each half. Dutasteride (SML1221, Sigma) was
298 dissolved in oleic acid (O1383, Sigma) at 15 mg/ml, and added to a ~60 mg piece of enriched diet pellet
299 (D12451i, Research Diets). Immediately after separation, each mouse received the dutasteride-treated
300 diet (~13 µg/g BW) or a similar amount of vehicle-treated diet for controls. The pellet was consumed
301 fully within a few minutes, and the separator was then removed. The treatment was repeated daily, and
302 mice were weighed and observed daily for signs of FVO.

303 ***Histology and follicle counts***

304 Ovaries were harvested from the mice at ~60 d and were fixed with 4 % paraformaldehyde for 4 h
305 before transferral to 70 % ethanol. Paraffin embedding, sectioning (4 µm), and hematoxylin and eosin
306 (H&E) staining were carried out at the Biomedical Core Facilities at the Rappaport Faculty of Medicine,
307 Technion-Israel Institute of Technology. Identification of follicle stage (using CaseViewer software) and
308 counting were performed (as in^{41,42}), while blind to the treatment group. In short, every fifth section per
309 ovary was analyzed, and the follicular stage was determined by size and morphological characteristics:
310 primary follicles containing a single layer of cuboidal granulosa cells; secondary follicles showing more
311 than one layer of granulosa cells but no antrum, and antral follicles containing an antral space. Atretic
312 follicles were identified based on the presence of *zona pellucida* remnants, stained bright pink.
313 Secondary and antral follicles were counted only if a nucleus was present, and the atretic follicles, which
314 vary considerably in size, were counted every 8th stained section, to avoid counting the same follicle
315 twice. The "follicle counts" presented comprise the number of follicles at each of these stages counted
316 in each ovary using this approach.

317 ***Quantitative PCR, transcriptome and methylation analysis***

318 RNA was isolated using TRIzol, DNase I-digested and cleaned using R1014 RNA Clean & Concentrator-5
319 kit (Zymo Research), cDNA synthesized using the qScript Flex cDNA kit (95049 Quanta) using oligo dT,
320 and real-time quantitative PCR (qPCR) was carried out using the PerfeCTa SYBR Green FastMix (Quanta),
321 both as previously reported⁴³, using primers listed in Table S2. Amplicon levels were quantified using
322 standard curves and normalized to levels of *Rplp0*.

323 For transcriptome analysis, RNA was extracted from the mice ovaries and purified as above, and
324 sequenced by CEL-seq, using Illumina HiSeq 2500 at the Technion Genome Center (as in⁴⁴). FastQC was
325 used for quality control and the reads were mapped by TopHat algorithm to mm10 genome assembly.
326 HTSeq-count was used to count the reads, and the normalization of raw counts and differential
327 expression were calculated using DESeq2 in R platform, with *Padj* using Benjamini and Hochberg
328 correction for false discovery. Pathway analysis was performed using the Database for Annotation,
329 Visualization and Integrated Discovery (DAVID).

330 DNA was extracted from the mouse tissues using TRIZOL, and the genomic DNA cleaned with the Quick-
331 DNA Miniprep Plus Kit (D4068; Zymo), before bisulfite conversion using the EZ-DNA Methylation-Gold
332 Kit (D5005 Zymo), and two rounds of PCR-amplification (nested, with outer and inner primers: Table S2)
333 using Red Load Taq Master (Larova). After purification of the amplicons (PCR purification kit; Qiagen)
334 and cloning into pGEM-T-easy, inserts from 7-8 randomly selected clones were sequenced and analyzed
335 as previously⁴⁵. Subsequently, the region in the first intron homologous to that differentially methylated
336 in the human samples, was amplified and cleaned as above. Additional rounds of PCR were then
337 performed using KAPA HiFi HotStart Ready mix (Roche), initially with primers containing the adaptors
338 (Universal adaptors; Illumina) and subsequently another 8-12 PCR cycles with the specific primers
339 (Illumina Nextera XT index kit); samples were cleaned with PCR purification kit (Qiagen) between each
340 stage. These libraries, after addition of 50% Phi-X, were then deep-sequenced by 150 bp paired-end
341 sequencing on Mi-seq (Illumina), at the Technion Genome Center.

342 ***Cell culture***

343 The KK-1 granulosa cell line was cultured as reported⁴⁶, maintained at 37 °C with 5 % CO₂ at 30-80 %
344 confluency, passaging 2-3 times a week. The cells were exposed either to 1-100 nM of the steroids
345 (Sigma) for 24 h, or to anti-inflammatory cytokines: IL-4, IL-10 or IL-13 (1-100 ng/ml for 24h), alone or
346 before addition of E2. Alternatively, the GT1-7 mouse hypothalamic GnRH neuronal cell line was
347 cultured with high glucose DMEM containing 10 % FBS, 1 % penicillin-streptomycin, sodium pyruvate
348 and sodium bicarbonate, maintained at 37 °C with 5 % CO₂ at 50-90 % confluency, passaging 1-2 times a
349 week. For mRNA measurements, the cells were cultured in charcoal-stripped FBS medium for 24 h
350 before some were exposed to dutasteride (5 µM) for 24 h. Cells were then harvested for RNA extraction
351 and qPCR analysis as before.

352 For analysis of GnRH release, cells (in 6-well plates) were washed twice in medium without FBS,
353 incubated in the same medium, and some exposed to dutasteride (10 µM). After 30 min, P4 (2 µM) was
354 added for 5 h, and then muscimol (100 µM) or/and AP were added for 1 h. The medium was collected
355 into 1.5 ml tubes, centrifuged for 2 min at 3000 g and kept at -80 °C for measurement of GnRH in the
356 supernatant by ELISA (Phoenix Pharmaceuticals, catalog # EK-040-02CE), according to the
357 manufacturer's protocol.

358 ***Statistical analysis***

359 All data are from multiple biological repeats (n-value) which were assayed individually. Results are
360 shown as box plots (whiskers show minimum and maximum values, boxes represent 25–75% data
361 ranges, horizontal lines within boxes indicate the median values), individual values, or as mean ± SEM
362 values. Statistical analysis for parametric data was using a Student's *t*-test (two-tailed), and differences
363 considered significant at $p \leq 0.05$, or alternatively One-way analysis of variance (ANOVA), followed by
364 the Tukey-Kramer or Bonferroni *t*-test for multiple comparisons. Methylation analysis (% methylation)
365 utilized Mann-Whitney non-parametric *t*-test.

366 ***Data Availability*** Data are available at NCBI's Gene Expression Omnibus (GEO), GSE133355 (human DNA
367 methylation data) and GSE133633 (mouse RNA-seq data).

368

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372

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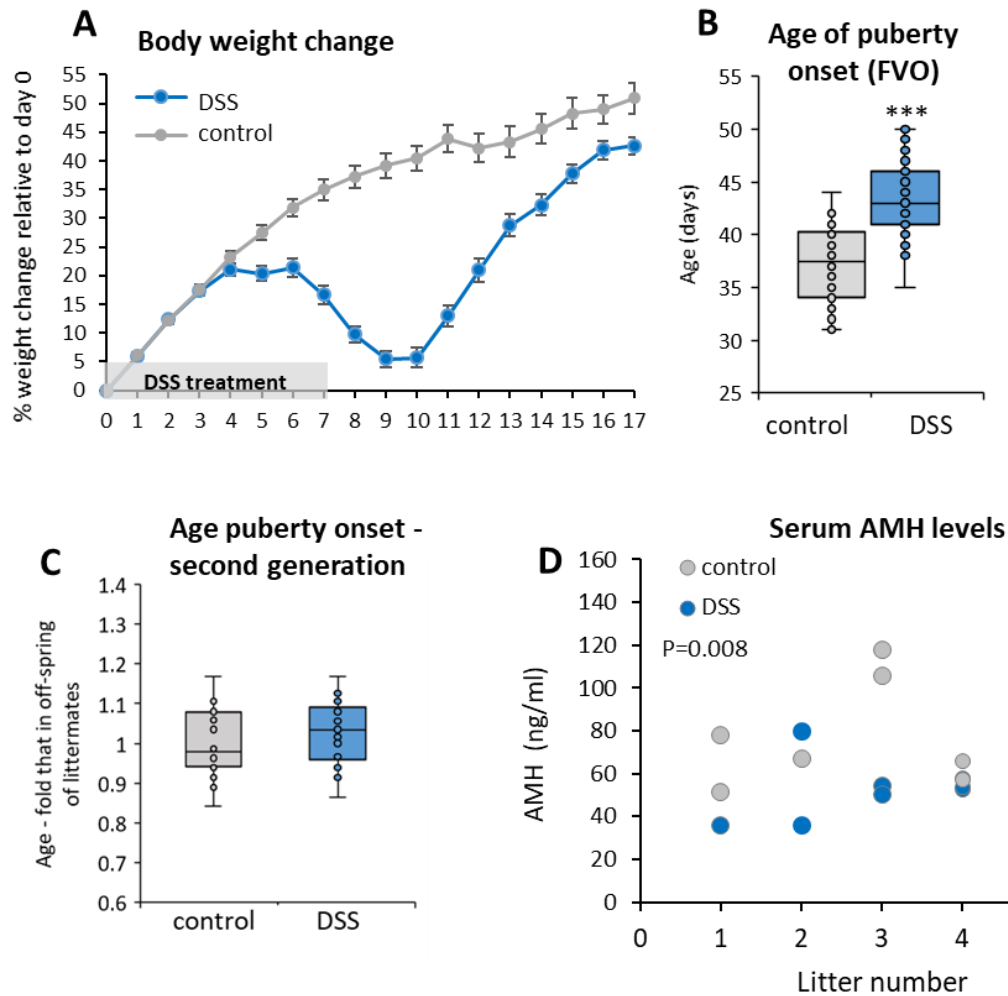
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481 **Figures**

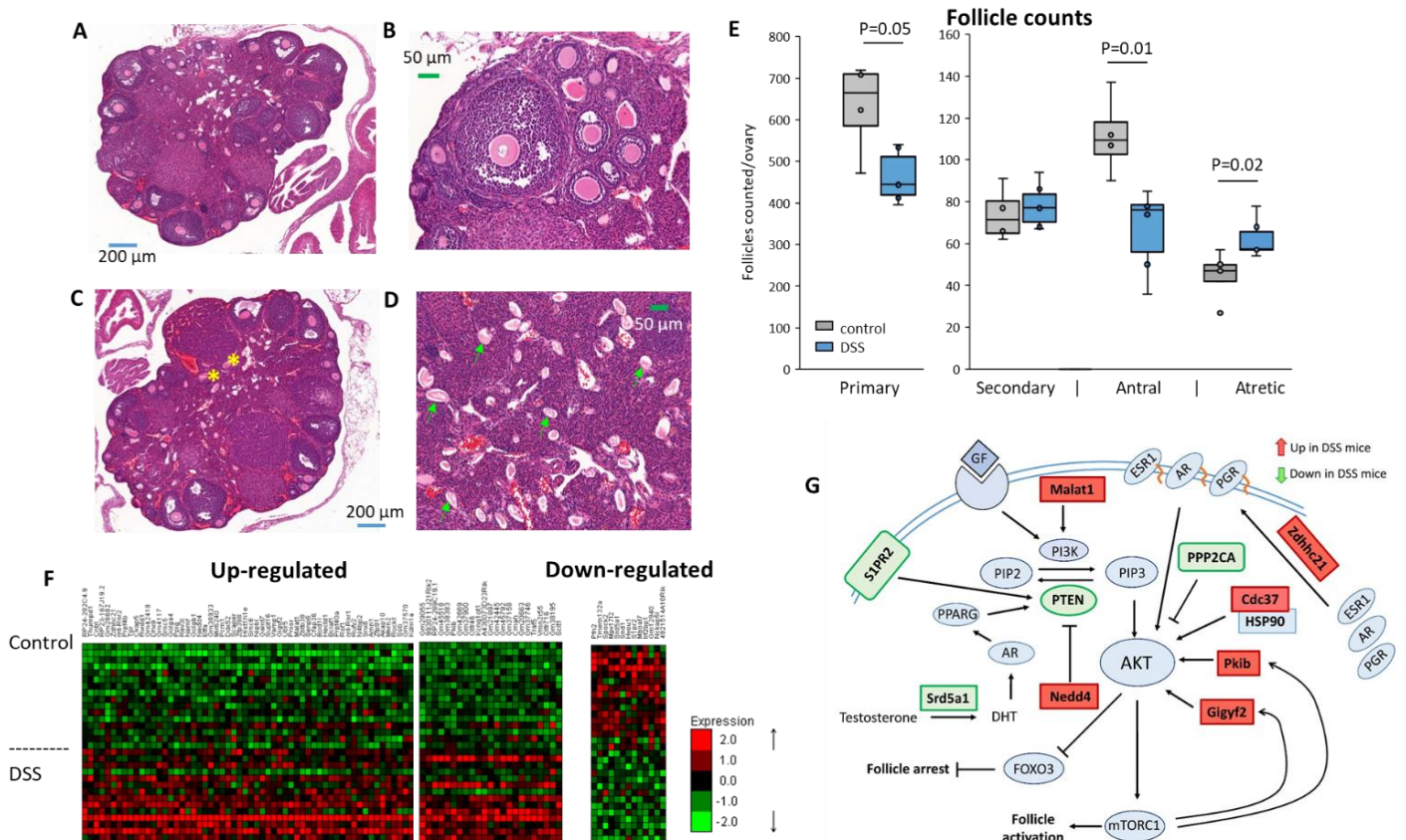
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483 **Fig 1:** Reproductive phenotype of the mouse model of early-life colitis is similar to that of the women who
 484 experienced childhood immunological challenge. (A) Changes in body weight in control (n=48) and DSS-
 485 treated (n=45) mice, relative to their initial weight. Mean±SEM. (B) Age of first vaginal opening (FVO),
 486 indicating onset of puberty. ***P<0.001; n=44, 48. (C) Age of FVO in second generation, relative to that
 487 in off-spring of parent littermate controls. P>0.05; n=20, 30. (D) Circulating AMH (ng/ml) in control and
 488 DSS-treated mice, shown separately for each of six litters. Mean between groups (control: 88.07 ng/ml
 489 [n=11] and DSS: 57.06 ng/ml [n=14]: P=0.008).

490

491



492

493 **Fig 2: The mouse model ovaries exhibit altered follicle numbers and pathways of gene expression**

494 *regulating follicle activation.* (A-D) H&E stained ovarian histological sections from (A,B) control and (C,D)

495 DSS-treated groups. Some atretic follicles (yellow asterisks) and *zona pellucida* remnants (green arrows)

496 are marked. (E) Follicle counts from sections of mice ovaries (n=4, n= 6), compared by t-test. (F) Heat

497 map of differentially expressed ovarian genes (DEGs) at $P_{adj} < 0.05$ from RNA-seq analysis. Each column

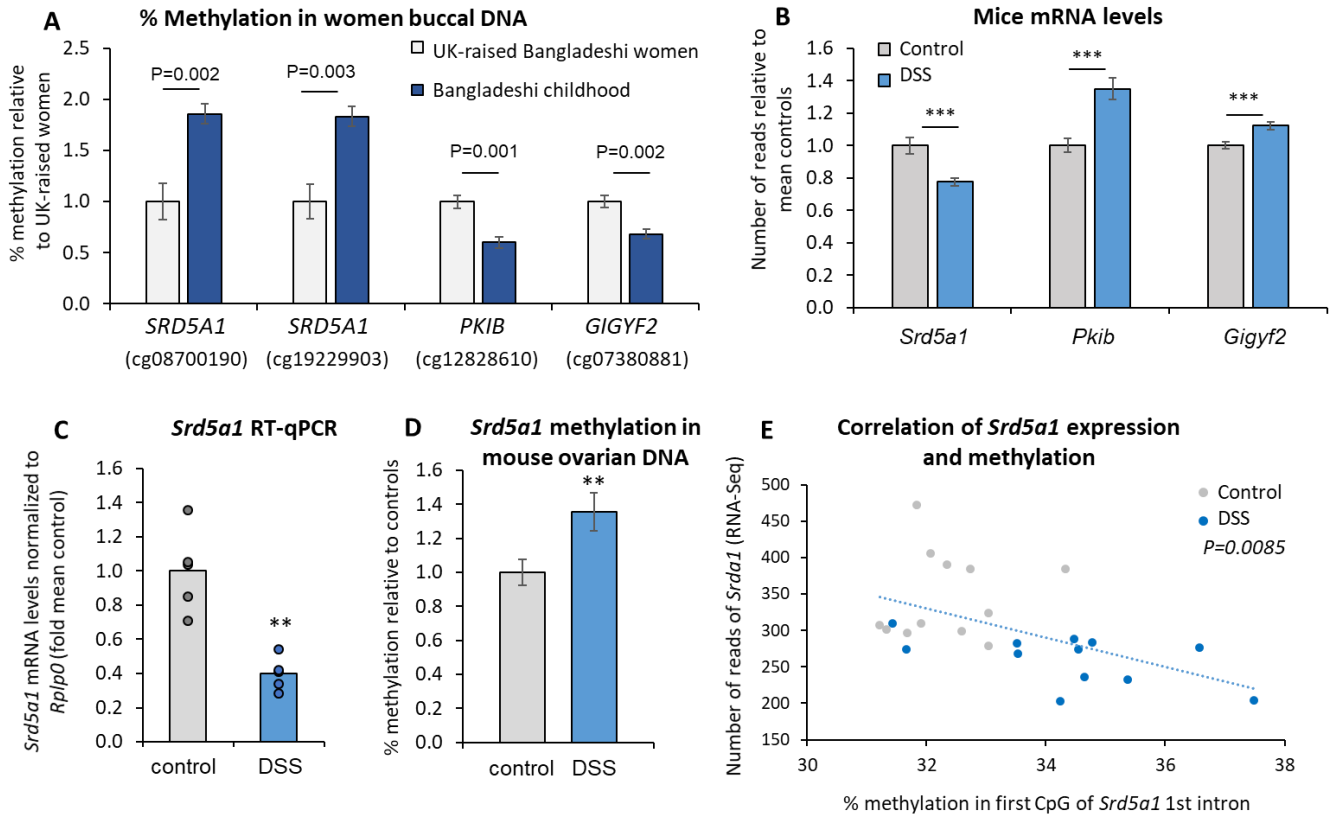
498 represents a gene and each row represents the expression level in one ovary. (G) Signaling pathway to

499 follicle activation, showing some of the DEGs ($P < 0.05$). Red boxes signify up-regulated genes; green

500 boxes signify down-regulated genes.

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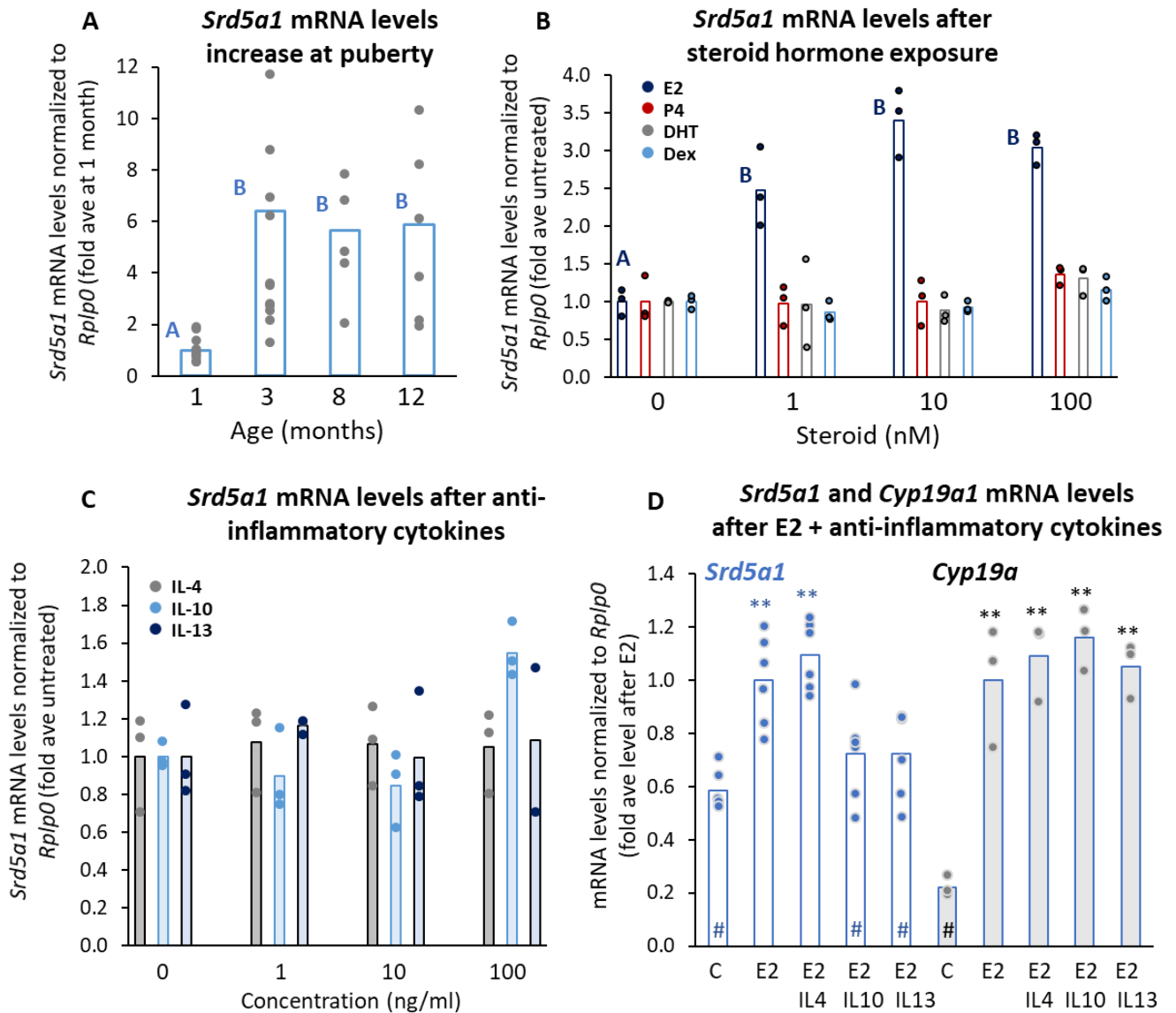
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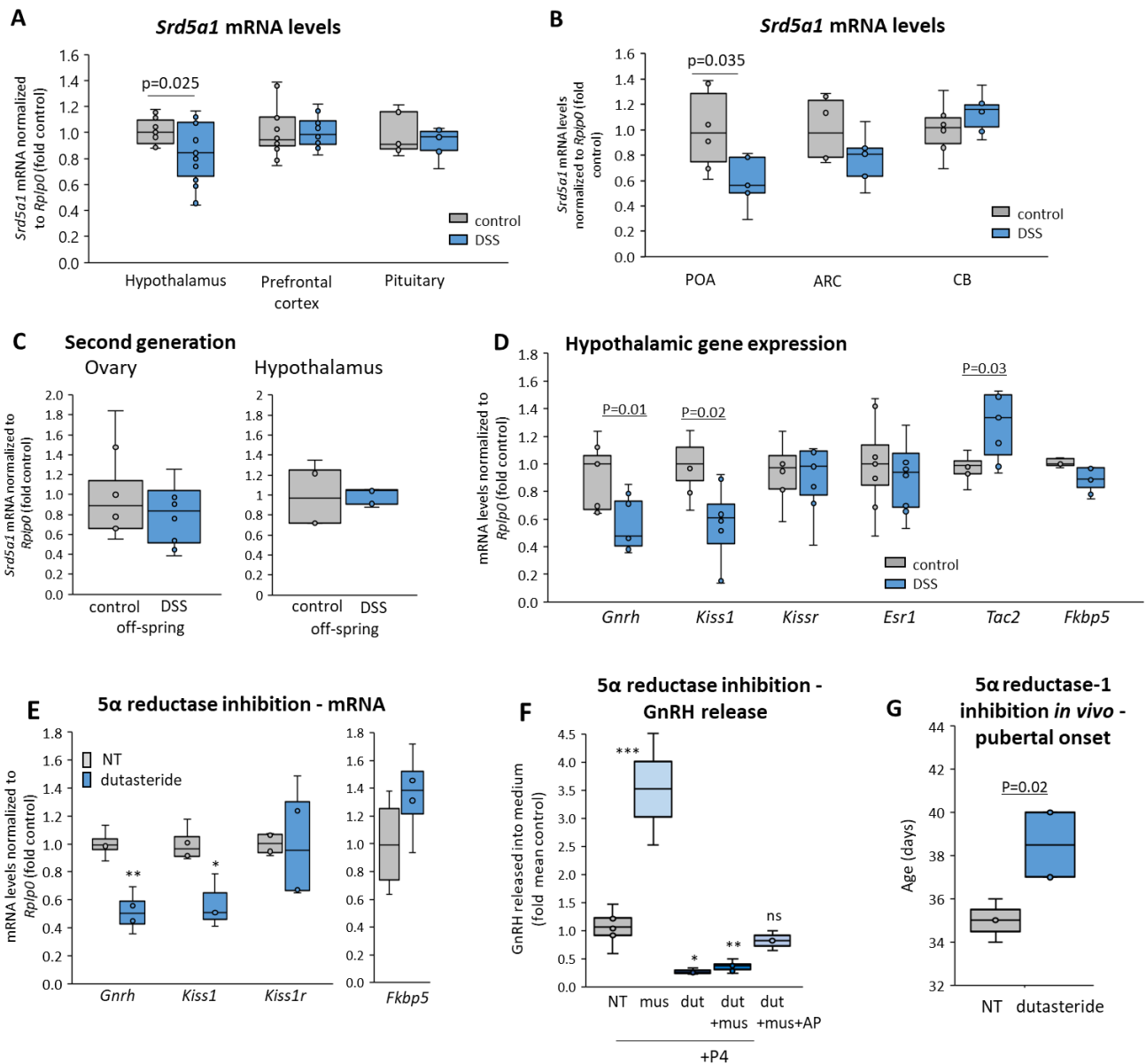
504 **Fig 3:** *Srd5a1* is hypermethylated in women and mice following early-life immune challenge. (A) Three
 505 genes (Illumina EpicMethylation sites) associated with differentially methylated regions in buccal DNA of
 506 Bangladeshi women who grew up in Bangladesh (n=16) or UK (n=13); mean±SEM. (B) The mRNA levels
 507 of these genes in control (n=16) and DSS-treated (n=14) mice ovaries from the RNA-seq analysis; ***:
 508 $P_{adj}<0.001$; mean±SEM. (C) qPCR analysis of the *Srd5a1* mRNA levels (n=5), **: $P=0.007$; showing means
 509 with individual data points. (D) Levels of CpG methylation in the 5' region of the *Srd5a1* first intron
 510 (corresponds with first site in Fig 3A: see S3), in control (n=24) and DSS-treated (n=29) mice ovaries,
 511 mean±SEM shown relative to controls; **: $P=0.015$ (Mann-Whitney t-test). (E) Correlation between the
 512 levels of *Srd5a1* mRNA (from RNA-seq analysis) and methylation measured in the same samples;
 513 $P=0.0085$.

514



515 **Fig 4:** The up-regulation of *Srd5a1* by estradiol is blunted by anti-inflammatory cytokines. (A) *Srd5a1*
 516 mRNA levels in ovaries of mice of various ages (n=12, 14, 5 or 6); for groups sharing same letter: p>0.05
 517 (ANOVA, Tukey-Kramer t-test). (B) *Srd5a1* mRNA levels in KK-1 cells (n=3) after exposure to estradiol
 518 (E2), progesterone (P4), dihydrotestosterone (DHT) or dexamethasone (Dex). For E2, ANOVA is as in Fig
 519 4A; otherwise P>0.05 (C) *Srd5a1* mRNA levels after cytokine exposure (n=3). (D) *Srd5a1* (n=6) and
 520 *Cyp19a* (n=3) mRNA levels after E2 alone (10 nM) or with cytokine (100 ng/ml); **: p<0.02 vs control; #:
 521 p<0.02 vs E2; where not marked p>0.05. All graphs show mean with individual data points.

522



523 **Fig 5:** 5 α reductase-1 regulates the central control of reproduction and pubertal timing. *Srd5a1* mRNA
 524 levels were measured in (A) hypothalamus (n=14), prefrontal cortex (n=15,14) and pituitary (n=7) of
 525 control and DSS-treated mice; or (B) preoptic area (POA: n=6, 5) and arcuate nucleus (ARC: n=6, 5) of the
 526 hypothalamus, and the cerebellum (CB: n= 6). (C) *Srd5a1* mRNA levels in the ovary (n=8) and
 527 hypothalamus (n=4,5) of female off-spring of DSS-treated mice and their littermate controls. (D) The
 528 mRNA levels of genes encoding reproductive regulatory factors were measured in the hypothalamus of
 529 the DSS-treated and control mice, with *Fkbp5* as an indicator for stress. (E,F) The effect of 5 α reductase
 530 inhibitor, dutasteride in GT1-7 GnRH neuronal cells on (E) gene expression (n=8, 4) and (F) GnRH release,
 531 in which some cells were also exposed to the GABA $_A$ agonist, muscimol, alone (n=2), with dutasteride
 532 (n=6) or together with AP (n=3), all in the presence of AP precursor, P4. Significant differences ($p<0.05$)
 533 are shown for comparisons with control mice or untreated cells, otherwise $P>0.05$. (G) Age of FVO
 534 following dutasteride: female mice and their control littermates were given dutasteride (or vehicle) in
 535 the diet each day after weaning, and checked daily for FVO (n=3, 4).