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1 **Title:**

2 **Single-cell exploration of gonadal somatic cell lineage specification during** 3 **human sex determination**

4
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27 **Summary**

28 Gonad development is an exciting model to study cell fate commitment. A better understanding of sex
29 determination requires the identification of all involved cell types and their dynamic expression
30 programs. Here we present an atlas of 128,000 single cells from human gonads between 5 and 12
31 postconceptional weeks. A focused analysis of somatic cells uncovered a population of bipotential
32 progenitors derived from the coelomic epithelium of both testes and ovaries, which may have the
33 capacity to commit to either a steroidogenic or a supporting fate. Moreover, our analyses suggest that
34 early supporting cells, prior to differentiation into Sertoli or pre-granulosa cells, also give rise to the
35 *rete testis/ovarii* and that the ovary retains the capacity to feed the supporting cell pool for an extended
36 period of time, directly from the surface epithelium. Finally, the potential involvement of the GnRH
37 signaling pathway in regulating testis differentiation was assessed ex vivo.

39 **Keywords**

40 Human development; fetal gonad; ovary; testis; single-cell RNA-seq; cell differentiation; GnRH

42 **Introduction**

43 Gonad development is a unique differentiation process that starts from a common bipotential
44 primordium and leads to the formation of two functionally distinct but complementary reproductive
45 organs, the testis and the ovary. In therian mammals this process is genetically controlled: the Y
46 chromosome of male individuals carries the sex determining region Y (*SRY*) gene which encodes for

47 the so-called testis-determining factor^{1,2}. Genital ridges first arise from the thickening and
48 differentiation of the coelomic epithelium at the surface of the mesonephroi at around the fourth
49 postconceptional week (PCW) in humans, a time when primordial germ cells also colonize the nascent
50 gonads³⁻⁵. At this stage, gonads contain multipotent progenitors of supporting and steroidogenic
51 lineages and are considered bipotential until 5.5 PCW, when the expression of SRY begins⁶. Upon
52 expression in supporting cell precursors, SRY induces SOX9 which triggers the differentiation of
53 Sertoli cells, the formation of testis cords and the development of Leydig cells⁷⁻¹⁰. In the absence of
54 SRY, the coordination of the RSPO1/WNT4/ β -catenin pathway and FOXL2 promotes ovarian
55 development, including the differentiation of supporting precursors into pre-granulosa cells and the
56 subsequent commitment of germ cells into meiosis¹¹⁻¹⁸. It is also well established that male and female
57 molecular expression programs antagonize each other, thereby enabling to reinforce the initiated fate
58 and repressing the alternative one¹⁹⁻²⁶. Altogether, the decisive commitment to a male or female fate
59 during the gonadal sex determination process directs the differentiation of several cell lineages towards
60 one of these two developmental pathways (for review, see²⁷).

61 While progress has been made in the identification of factors involved in gonad formation and
62 differentiation, our understanding of sex determination remains incomplete. Accordingly, the etiology
63 of disorders of sex development remains unexplained in more than half of the cases (for review, see²⁸).
64 One of the primary reasons for this is a lack of knowledge regarding the different cells that actually
65 make up the gonads. Notably, the lack of specific markers for progenitor cell populations and the
66 inability to perform lineage tracing experiments have prevented the study of gonadal cell specification
67 in humans. Ignoring the existence of certain cell types or subtypes may oversimplify or, even worse,
68 distort our view of gonadal sex determination. The deconvolution capability of single-cell RNA-
69 sequencing (scRNA-seq) has been used in several studies to characterize the large number of cell
70 populations that differentiate within human gonads²⁹⁻³⁴. In addition to the identification of marker genes
71 for distinct cell lineages, such approaches notably allowed to highlight candidate pathways involved in
72 gonadal cell-cell communication networks, to establish the molecular profiles of fetal *rete testis* and
73 *ovarii* cells, or to rule out the mechanisms of X-chromosome dosage in female germ cells. However,
74 these studies included few, if any, samples younger than 7 PCW, and in most cases cells from XX and
75 XY gonads were analyzed separately. In contrast to mouse gonad development, which has been
76 thoroughly characterized at the single-cell level³⁵⁻⁴⁰ (for review, see⁴¹), the early events of gonad
77 formation and the cell fate decisions made in male versus female somatic lineages remain to be
78 elucidated in humans.

79 Here, we present an in-depth characterization of the transcriptome of human first-trimester gonads from
80 the 5th PCW onwards at the single-cell level. Taking advantage of the large number of early gonads
81 and of the time series design of these scRNA-seq data, we investigated how distinct gonadal cell
82 populations could emerge and differentiate before, during and after sex determination. This single-cell
83 transcriptomic atlas allowed us to identify marker genes for each cell population, gain insight into the
84 specification of supporting and steroidogenic cell lineages, suggest different contributions of coelomic-
85 epithelial cells in male and female gonads and characterize the expression programs underlying these
86 processes. We also investigated the potential intra-gonadal role of gonadotropin releasing hormone
87 receptor (GnRHR) and found that this signaling pathway could regulate gene expression in human pre-
88 Sertoli cells, providing insights into the molecular mechanisms underlying early gonadal differentiation.
89 Our single-cell atlas is available via the ReproGenomics Viewer (<https://rgv.genouest.org>)^{42,43}.

90 **Results**

91 **A single-cell expression atlas composed of 128,000 cells from developing gonads**

92 To characterize the cell composition and evolution of human embryonic and fetal gonads as they
93 differentiate in a sex-specific manner, we collected and dissociated 17 testes and 16 ovaries from 5 to
94 12 PCW (Fig. 1, panel A). Half of the samples (17 out of 33) originated from early embryonic samples
95 aged from 5 to 7 PCW, to decipher early cell differentiation events during and right after sex
96 determination (Table S1). The present single-cell atlas is composed of 127,903 cells closely distributed
97 between females (67,427 cells, 53%) and males (60,476 cells, 47%). A principal component analysis of
98 the “pseudobulk” expression matrix showed that the first two components clearly distinguished the
99 samples by sex (35% of the variance explained by PC1) and by developmental stage (26% by PC2)
100 (Fig. 1, panel B). Samples at 5 PCW, which included emerging gonads together with the underlying
101 mesonephros, consistently segregated from 6-12 PCW gonads that were dissected free of mesonephros.
102 The proximity of male and female samples at this earliest stage also suggested that the expression
103 programs that drive sex-specific differentiation of gonadal cells were barely initiated.

104

105 In accordance with the “pseudobulk” analysis, gonadal cells from individuals of the same
106 developmental stage and sex distributed similarly within the UMAP space (Fig. 1, panel C). Most cells
107 from early developmental stages (5-6 PCW) overlapped in the central part of the UMAP, illustrating
108 again the initial transcriptional similarity of XY and XX gonadal cells. Conversely, male and female
109 cells at latter developmental stages occupied outermost and opposite regions of the UMAP, and those
110 from intermediate stages positioned midway between 5 PCW and 12 PCW cells.

111

112 **Single-cell transcriptomes distinguish differentiating gonadal cell populations**

113 We next partitioned the 127,903 gonadal cells into 41 clusters (named c1-c41) associated with 20 broad
114 cell types (Fig. 1, panel D) and corresponding to gonadal specific cell lineages as well as common cell
115 types that can be found in most tissues. The expression of known gene markers as well as the percentage
116 of cells belonging to specific sex and/or developmental stage (Fig. 1, panels E-G; Table S2) allowed us
117 to identify : primitive (cluster c32; *HBE1*,+) and definitive (c39; *HBB*+) erythrocytes ; endothelial cells
118 (c35; *CD34*+, *ECSCR*+) ; immune cells (c26; *PTPRC*+, *TYROBP*+) ; perivascular cells (c41; *ACTA2*+,
119 *TAGLN*+, *NR2F1*+) ; primordial (c5, c33; *DMRT1*+, *POU5F1*+, *NANOG*+, *DAZL*+) and female meiotic
120 (c38; *DMRT1*+, *SYCP3*+, *DAZL*+) germ cells; coelomic epithelial cells (c9, c18, c29, c27; *KRT19*+,
121 *UPK3B*+, *LHX9*+) ; ovarian surface epithelial cells (c10, c22; *LHX2*+, *IRX3*+, *KRT19*+, *UPK3B*+,
122 *LHX9*+) ; somatic progenitor cells (c3; *IL17B*+, *LHX9*+) ; interstitial cells (c1, c13, c15, c16, c21, c23,
123 c25; *PDGFRA*+, *TCF21*+, *LHX9*+) ; fetal/pre-Leydig cells (c27, c34; *INSL3*+, *CYP17A1*+) ; distinct
124 supporting subpopulations (*WNT6*+) including undifferentiated supporting cells (c2, c24; *DMRT1*+,
125 *TAC1*+, *TSPAN8*+) , pre-Sertoli cells (c12; *DMRT1*+, *SRY*+) , Sertoli cells (c14, c20, c31; *DMRT1*+,
126 *SOX9*+, *AMH*+) , pre-granulosa cells (c6, c7, c8, c11; *FOXL2*+, *IRX3*+) ; *rete testis/ovarii* cells (c17;
127 *PAX8*+, *TBX1*+) . The spatial distribution of the main gonadal somatic cell lineages was also illustrated
128 thanks to associated markers, i.e. *KRT19* for coelomic epithelial cells, *PDGFRA* for interstitial cells,
129 *FOXL2* and *SOX9* for pre-granulosa and Sertoli cells, respectively (Fig. 1; panel G). Additionally,
130 several extragonadal cell types (*GATA4*-, *LHX9*-) that originate mostly from 5 PCW samples (i.e.
131 gonads dissociated together with the mesonephroi) were captured: non-gonadal coelomic epithelial cells
132 (c28; *UPK3B*+, *KRT19*+, *GATA2*+) ; mesonephric mesenchymal cells (c19; *PDGFRA*+, *TCF21*+,
133 *NR2F1*+) ; genital duct cells (c37; *LHX1*+, *PAX8*+, *GATA2*+) ; neuroendocrine cells (c40; *ASCL1*+,
134 *PHOX2B*+, *GATA2*+) ; and adrenocortical cells (c36; *NR5A1*+, *CPLX1*+, *NR2F1*+) . A small cluster of
135 residual low-quality cells (c30) was also identified.

136

137 Several observations are worth mentioning: first, the exact nature from cluster c3 was initially intriguing
138 as these cells expressed neither supporting nor interstitial markers (Fig. 1, panel E). Considering these
139 cells were almost exclusively observed in early gonads at 5-6 PCW (~94%), expressed *NR5A1*, *GATA4*
140 and *LHX9* but not *UPK3B* or *KRT19*, together with their central position within the UMAP in-between
141 early interstitial and supporting cells (Fig. 1, panels D-F), we speculated they could represent
142 progenitors of gonadal somatic cell lineages. Second, we identified two clusters related to Leydig cells:
143 cluster c27 and cluster c34, which express higher levels of *INSL3* and of *CYP17A1* respectively, and
144 may represent two functionally distinct subpopulations or two successive steps during their
145 differentiation process (Fig. 1, panel E). As expected, female interstitial cells did not yet express
146 canonical steroidogenic markers (for review, see⁴⁴). Finally, two populations of *PAX8*⁺ cells could be
147 distinguished: a relatively small cluster of genital duct cells (c37; *LHX1*⁺, *KRT19*⁺, *GATA4*⁻), that were
148 mostly recovered from extragonadal tissue at 5 PCW, and a rather important number of *rete* cells (c17;
149 *TBX1*⁺, *NR5A1*⁺, *GATA4*⁺), captured mostly from gonads dissected free of mesonephros from 6 PCW
150 onwards (Fig. 1 panels D-F). 3D imaging on cleared whole-gonads using light-sheet fluorescence
151 microscopy indeed confirmed that *PAX8* was strongly expressed in ducts and glomeruli in the
152 mesonephros as well as in intra-gonadal cells located in the dorsal region of both testes and ovaries,
153 nearby the mesonephros (Fig. 2, panels A and B; Movies S1 and S2). Co-staining with *GATA4* also
154 confirmed the gonadal origin of these *PAX8*⁺ cells (Fig. 2, panel C).

155

156 **Disentangling the origin and differentiation of gonadal somatic cells**

157 To tease out the heterogeneity of supporting and steroidogenic cell lineages over developmental stages,
158 we subsetted 111,855 *GATA4*⁺ gonadal somatic cells and their putative progenitors. Notably, all
159 selected cell populations (i.e. 28 clusters from Fig. 1) were also *LHX9*⁺ and/or *NR5A1*⁺. They were
160 partitioned into 42 cell clusters (named s1-s42) (Fig. 3, panels A-D and Fig. S1, panel A). Among them,
161 13 clusters consisted of both male and female cells, mostly from 5 to 7 PCW, while the remaining ones
162 were almost composed of testicular and ovarian cells only (Fig. 3, Panel B). We observed marked
163 changes in terms of cell composition early during male gonad development, especially between 5 and
164 8 PCW (Fig. 3, Panel C), while such changes appeared more progressive in female gonads.

165

166 We next investigated the sex-specific developmental trajectories that somatic subpopulations follow as
167 human gonads differentiate. We first used known marker genes to annotate the 42 somatic cell clusters
168 (Fig. 3, panels D and E, Fig. S1, panel B and Table S3). We then performed pseudotemporal ordering
169 of XX and XY somatic cells separately using Monocle3. Since gonads are expected to originate from
170 the coelomic epithelium, at least based on knowledge gathered from animal models, we used coelomic
171 epithelial cells (cluster s30) at 5 PCW from each sex as starting points, yielding eight major trajectories
172 (Fig. 3, panels F and G). Strikingly, the trajectories were highly consistent with developmental stages,
173 both male and female cells being ordered with increasing pseudotime from 5 to 12 PCW. Initial
174 trajectories for male and female cells were highly overlapping (Fig. 3, panel F): disregarding their sex,
175 early coelomic epithelial cells (cluster s30) first evolved through cluster s34 and cluster s2, prior to
176 bifurcating towards either the interstitial lineage or the supporting lineage. The specification of these
177 two lineages from coelomic epithelium-derived cells in both males and females suggests that clusters
178 s2 and s34 may correspond to the multipotent somatic progenitors of the bipotential gonad. Of note is
179 that most cells from cluster s2 (88%) belonged to cluster c3 in the global analysis (Fig. S1, panel A),
180 which we hypothesized to correspond to gonadal progenitors. The initial commitment of XX and XY
181 putative somatic progenitors to the interstitial fate involved similar trajectories through cluster s8 and
182 s18, prior to acquiring sex-specific paths. These included clusters s10, s22, s1 and s38 up to the fetal
183 Leydig cell (s40) for testicular cells, and clusters s37 and s17 up to s15 for ovarian cells (Fig. 3, panel
184 F). Similarly, initial trajectories that reflect the specification of supporting lineages overlapped up to

185 cluster s19 but quickly diverged towards two sexually-dimorphic supporting subpopulations: cluster
186 s24 in testes and cluster s5 in ovaries, which differentiate further into Sertoli cells up to clusters s31 and
187 s16 and pre-granulosa cells up to clusters s39 and s3, respectively (Fig. 3, panel F). Overall, these
188 trajectories recapitulate the widely accepted model in which coelomic epithelium-derived progenitors
189 give rise to both supporting and interstitial lineages that rapidly commit to one or the other of their sex-
190 specific fates (Fig. 3, panels G and H). Importantly, very similar paths were obtained when using
191 SlingShot as an alternative single-cell trajectory inference method (Fig. S1, panel C).

192

193 In addition to this canonical model, our analysis also uncovered branching points along the supporting
194 lineage of both male (cluster s24) and female (cluster s5) gonads. The two resulting secondary
195 trajectories lead to the *rete testis* and *rete ovarii* cells (cluster s13; Fig. 3, panels D, F, G and H),
196 suggestive of a supporting origin of these cells as recently evidenced in the mouse^{39,45}. Furthermore, for
197 XX gonads we observed a second trajectory that originated from ovarian surface epithelial cells
198 (clusters s21 and s6) and led to pre-granulosa cells (clusters s39 and s3). Importantly these two
199 trajectories converged at a pseudotime composed of cells from cluster s20 at 9 PCW, therefore making
200 a loop towards a common pathway for the differentiation of most advanced pre-granulosa cells at 12
201 PCW (Fig. 3, panels D, F, G and F). It is thus tempting to speculate that this alternative pathway could
202 correspond to the specification of cortical granulosa cells directly from the ovarian surface epithelium,
203 as previously identified in the mouse⁴⁶. In contrast, coelomic epithelial cells in developing testes only
204 evolved towards a slightly different state (s33).

205

206 Noteworthy, this revisiting of early human gonadal sex differentiation could only be achieved thanks
207 to the inclusion of an important number of early embryonic samples, at 5 and 6 PCW. We indeed
208 reanalysed first trimester samples from several previously published datasets^{29,30,32,34} and retrieved all
209 major ovarian and testicular cell types (Fig. S2 and Fig. S3). However, when gonadal somatic cells were
210 selected and mapped onto our dataset, many gaps were highlighted along the reconstructed lineages
211 (Fig. S2, panels D, H and L and Fig. S3, panels D, H and L). Indeed, except for ovarian cells which
212 differentiation was well covered in the study by Garcia-Alonso and collaborators³², all other reanalysed
213 datasets lacked initial steps of gonadal cell specification, especially for the testis^{29,30,32,34}.

214

215 **Cell trajectory inference deciphers dynamic gene expression during differentiation of supporting 216 and interstitial cell lineages**

217 To highlight potential key regulators driving somatic cell specification and differentiation in human
218 gonads, we focused on 4,387 differentially-expressed genes (DEGs) with dynamic expression over the
219 eight major cell trajectories. Among these, 138 genes are known to be important or involved in sex
220 determination (GO:0007530), gonad development (GO:0008406) and/or steroid biosynthetic process
221 (GO:0006694) and 219 additional genes encode for transcription factors (GO:0003700) that could be
222 required for the establishment or maintenance of gonadal somatic cell lineages (Table S4). We further
223 classified the DEGs into 24 co-expression modules (termed m1-m24) according to their overall
224 expression patterns in distinct somatic cell lineages (Fig. 4, panel A and Table S4). The modules
225 revealed waves of expression along each lineage, starting with the coelomic epithelial cells at early
226 pseudotime and developmental stages, followed by the female and male supporting cells, the *rete*
227 *ovarii/testis* and the interstitial cells (Fig. 4, panels A and B; Fig. 3, panel H). Four modules
228 corresponded to genes that were already expressed in early coelomic epithelial cells (m1-m3 and m17).
229 These genes were subsequently downregulated in interstitial cell lineages (m1; *GATA4*, *NROB1*,
230 *RSPO1*, *WT1*), supporting cell lineages (m17; *SFRP1*, *LHX9*, *WNT5A*), all somatic cell lineages (m2;
231 *HMGAI*, *WNT2B*) or were further upregulated in late coelomic epithelial cells (m3; *BNCI*, *KRT19*,
232 *UPK3B*). A single module consisted of genes showing peak expression in gonadal somatic progenitor

233 cells (m4; *KDR*, *CCL21*, *GEM*, *IL17B*), prior to the differentiation of somatic cell lineages, with the
234 exception of the second wave of pre-granulosa cells. Regarding genes with preferential expression
235 within supporting cell lineages, eleven modules could distinguish those induced in both female and
236 male supporting cells (m5-m8), specifically in pre-granulosa cells (m9-12) or in (pre-)Sertoli cells
237 (m13-m15). More specifically, some genes showed a prolonged (m5; *GADD45G*, *WNT6*) or transient
238 (m6; *DMRT1*, *GFRA1*, *REG3G*, *TAC1*, *TSPAN8*) expression in all supporting cell lineages except
239 during the second wave of pre-granulosa cells, while others were also activated during the second wave
240 of pre-granulosa cells, again in a prolonged (m7; *TAF4*, *NRIP1*) or transient (m8; *CYP26B1*, *RUNX1*)
241 manner. Concerning genes expressed more specifically in pre-granulosa cells, four modules were
242 identified and included genes induced early (m9; *ESR1*, *KITLG*, *IRX3*, *TOX3*) or late (m10; *FOXL2*,
243 *YBX3*) during the two differentiation waves, only during the second wave (m11; *LHX2*, *POU6F2*) or
244 only during the first wave (m12; *CYP19A1*, *GHRL*). On the other hand, for male supporting cells, three
245 modules of genes that showed early (m13; *SRY*), intermediate (m14; *DHH*, *FATE1*, *INHBB*, *SOX10*,
246 *SOX9*) or late (m15; *AMH*, *CITED1*, *NKX3-1*) induction were identified. Module m16 corresponded to
247 genes with peak expression in *rete ovarii/testis* cells (m16; *FBXO32*, *PAX8*, *TBX1*). In addition to
248 module m17 (see above), several modules were associated with distinct expression patterns within the
249 interstitial cell lineages (m18-m22). These included genes expressed throughout the entire interstitial
250 lineage of both male and female gonads (m18; *ARX*, *HIC1*, *PDGFRA*, *TCF21*) or more sequentially and
251 transiently, with early (m19; *RXFP2*), intermediate (m20; *CYP11B1*, *NUPR1*), late and male-biased
252 (m21; *INHBA*, *MSC*, *OSR2*, *PTCH1*, *PTCH2*), or with peak expression at the end of the male interstitial
253 cell lineage, in Leydig cells (m22; *CYP17A1*, *INSL3*, *FOXO4*, *LHCGR*, *NR5A1*). Finally, two modules
254 included genes from the Y chromosome that were expressed only in male lineages (m23) and genes
255 involved in cell cycle (m24), which were expressed in coelomic epithelial cells and transiently within
256 the interstitial cell lineages.

257
258 Next, we investigated further the spatial distribution of supporting cells thanks to newly identified
259 markers: *WNT6* (module m5) for all supporting cells, *TAC1* (m6) for early supporting cells, and *LHX2*
260 (m11) for pre-granulosa cells derived from the coelomic epithelium (Fig. 4, panel A, Table S3 and Table
261 S4). We first validated *WNT6* as a supporting cell marker for both male and female gonads, as evidenced
262 by its coexpression with *SOX9* and *FOXL2*, respectively, on top of *WT1* (Fig. S4, panels A and B).
263 Consistent with its early expression in undifferentiated supporting cells, we detected *WNT6* in a similar
264 pattern to that of *SRY* prior to cord formation in the testis at 5 PCW and before the onset of *FOXL2*
265 expression in the ovary at 6 PCW (Fig. 5). On the other hand, *TAC1* was detected mostly in outer male
266 supporting cells at 5 PCW, in a complementary manner to *SOX9* that was found to be preferentially
267 expressed in inner supporting cells (Fig. 5). While *TAC1* expression sharply decreased in testes from 6
268 PCW onwards (Fig. S4, panels C and D), it was detected at high levels in inner supporting cells of
269 ovaries, at least up to 8 PCW (Fig. 5 and Fig. S5). Strikingly, *FOXL2* also showed a gradient expression
270 with decreasing levels from center to periphery, albeit with a broader expression pattern than *TAC1*
271 (Fig. 5 and Fig. S5, panels A-C). Conversely, *LHX2* was strongly detected in female coelomic epithelial
272 cells and in outer pre-granulosa cells, with decreasing expression levels from periphery to center of
273 ovaries (Fig. 5 and Fig. S5).

274 275 **The GnRHR signaling pathway regulates gene expression in Human pre-Sertoli cells**

276 Among the factors identified in our analyses, we chose to further investigate the potential role of the
277 GnRH receptor. *GNRHR* showed a transient expression pattern early during supporting lineage
278 differentiation, with a higher expression in pre-Sertoli cells (Fig. 6, panel A). This male-biased
279 expression was confirmed by qPCR on several human embryonic tissues (Fig. 6, panel B). A high
280 expression was observed in testes at 6 PCW, followed by a sharp decrease at 7 PCW. A similar

281 expression profile was observed in ovaries, albeit at a much lower level (Fig. 6, panel B). In addition to
282 the gonads, the adrenal was the only assessed tissue in which *GNRHR* expression was detected (Fig. 6,
283 panel B and Fig. S6, panel A). Interestingly, the expression of *GNRHR* in testes at 6 and 7 PCW was
284 correlated to that of *SRY* (Fig. 6, panel C and Fig. S6, panels B and C), suggesting *GNRHR* may be a
285 direct target of *SRY* or regulated by the same factors. To evaluate the functional role of GnRHR in pre-
286 Sertoli cells, we performed hanging drop cultures of human embryonic testes at 5-6 PCW (N=10
287 individuals), with or without Leuprolide⁴⁷, one of its well-characterized agonists (Fig. 6, panel D). After
288 24 hours, testis explants were recovered and changes in gene expression were investigated using BRB-
289 seq⁴⁸. A total of 18 DEGs were identified when comparing Leuprolide-treated explants to DMSO
290 controls, out of which 17 were also interrogated in our scRNA-seq dataset (Fig. 6, panel E; Table 1;
291 Fig. S6, panel D). Importantly, no significant change in expression could be observed for typical
292 markers of gonadal cell types, demonstrating unaltered cell proportions in testis explants upon GnRHR
293 activation for a relatively short duration. On the other hand, almost all DEGs were enriched in *GNRHR*+
294 pre-Sertoli cells (cluster s14 from Fig. 3), consistent with their regulation by the GnRH signaling
295 pathway *in vivo* too. Among DEGs, the expression pattern of *HSD3B2* was intriguing as it showed a
296 preferential expression in pre-Sertoli cells at 5-6 PCW, which was later restricted to Leydig cells from
297 7 PCW onwards (Fig. 6, panels E and F). This unexpected profile prompted a more systematic
298 investigation and comparison of steroidogenic factor expression during testis development (Fig. 6, panel
299 G; see Fig. S6, panel E for the backdoor pathway). The reanalysis of a previously published mouse
300 dataset³⁹ allowed us to identify all relevant cell types for this comparison (Fig. 6, panel G; Fig. S6,
301 panels F-H). As expected, most enzymes involved in the classical pathway for androgen biosynthesis
302 were preferentially expressed in both human and mouse Leydig cells. The main intra-testicular
303 regulators of steroidogenesis, i.e. *LHCGR* and *Lhcgr*, were also specifically expressed in these cells.
304 Noteworthy is that several of these genes (e.g. *STAR*, *CYP11A1*, *CYB5A*) expected to be specifically
305 expressed in Leydig cells were also found in early supporting and/or (pre-)Sertoli cells, albeit at lower
306 levels. These expression profiles are consistent with the dynamics of *NR5A1* that we found
307 preferentially expressed in Sertoli cells at 6-7 PCW, prior to being enriched in Leydig cells from 8 PCW
308 onwards (Fig. S7). The cellular distribution of *HSD17B3/Hsd17b3* was clearly different from other
309 factors, with a specific expression in Sertoli cells in both species (Fig. 6, panel G). This indicates that
310 the functional interplay between Sertoli and Leydig cells for the synthesis of androgens during fetal
311 life⁴⁹ might also apply to humans. The only but striking species difference was for *HSD3B1/Hsdb3b1*
312 and *HSD3B2/Hsd3b2* (Fig. 6, panel G). As described above, *HSD3B2* was expressed in pre-Sertoli cells
313 and Leydig cells, while *HSD3B1* was not expressed at all. Instead in the mouse, *Hsd3b1* was expressed
314 in Leydig cells, and *Hsd3b2* was barely detected in any cell type, as was *Gnrhr*. It is therefore tempting
315 to speculate that the specific expression of *HSD3B2* in human pre-Sertoli cells may be under the direct
316 control of GnRHR.

317

318 **Discussion**

319 **An atlas of gonadal sex determination at the single-cell level in humans**

320 For this study we captured and sequenced individual cells from 33 male and female gonads that covered
321 a developmental window of seven weeks from sex determination onwards. While a strong focus was
322 made to densely sample and finely characterize cell composition of undifferentiated gonads, our
323 experimental design also allowed us to recover more advanced cell differentiation states during gonad
324 development. Our dataset contains most, if not all, expected cell types of the gonad, including red cells,
325 immune cells, endothelial cells and perivascular cells, on top of germ cells, supporting cells and
326 interstitial cells at distinct differentiation stages, as well as their presumptive progenitors. We
327 characterized gene expression in each of these populations and identified additional markers, including

328 human-specific ones. Several studies also investigated human gonad development at the single-cell
329 level. These were however focused on gonads from one sex or the other, included only few samples at
330 early stages and/or no biological replicates^{29–31,33,34}. Noteworthy, one study provided an exhaustive
331 single-cell transcriptomic analysis of 38 human gonads during the first and second trimesters of
332 development³². Again, very few samples younger than 8 PCW were included, and most samples
333 included extragonadal tissue, making the use of spatial transcriptomics needed to eventually reattribute
334 all captured cells to their tissue of origin. By reanalysing the data from four of these benchmark studies,
335 we demonstrated that most of them did not include early steps of gonadal cell differentiation, making
336 the link between all somatic lineages impossible. This was especially true for the testis where
337 undifferentiated supporting and pre-Sertoli cells were fully missing. Overall, the dataset described here
338 represents to our knowledge the most comprehensive single-cell transcriptomics resource of early
339 human gonad development.

340

341 **Origin and specification of gonadal somatic cell lineages**

342 Our main objective was to explore the differentiation pathways of somatic cells in early human
343 embryonic gonads. In the absence of an adequate *in vitro* system and given the practical impossibility
344 of performing lineage tracing experiments, we conducted cell trajectory analyses, followed by a
345 comprehensive evaluation of differential gene expression across all identified somatic lineages. Since
346 such approaches rely solely on RNA expression dynamics, the conclusions that can be drawn regarding
347 reconstructed lineages should be interpreted with caution. Nevertheless, these analyses suggested that
348 the specification of human gonadal somatic cell lineages may follow a general scheme similar to that
349 observed in the mouse. Notably, putative gonadal progenitors derived from the coelomic epithelium
350 appeared to give rise to both the interstitial and supporting cell lineages, which subsequently commit to
351 sex-specific fates. It is also important to note that the outcome and interpretation of these trajectories
352 are closely tied to the arbitrarily chosen starting point. Even though we believe this is the most likely
353 scenario, slightly different paths could have been obtained if using cell populations other than the
354 coelomic epithelium as starting points. For instance, the use of potential progenitors could bring
355 plausible results since this population would still give rise to supporting and steroidogenic lineages.
356 However, they would also give rise to coelomic epithelial cells, meaning these cells would not
357 contribute to the gonad, with the exception of the second wave of pre-granulosa cells. Starting from
358 more differentiated cells already committed to a sex-specific path would however make no sense.
359 Besides, we found that the expression programs that underlie the specification of these lineages are not
360 necessarily conserved in Humans. This is notably striking for early supporting cells that specifically
361 express strong levels of e.g. *TAC1* or *TSPAN8*, which orthologs are not expressed in the mouse
362 gonads^{32,37}. Importantly, we also characterized the molecular phenotype of potential human gonadal
363 progenitors, whose cell population is almost exclusively present in gonads at 5 and 6 PCW. These cells
364 lose the coelomic epithelial cell markers *UPK3B* and *KRT19*, they maintain the expression of the
365 gonadal somatic cell markers *NR5A1* and *GATA4*, and they co-express markers characteristics of the
366 interstitial and the supporting lineages such as *TCF21*, *PDGFRA*, *LHX9*, *DLK1*, *NPY* and *SPRR2F*.
367 Additionally, they display a preferential expression for *GEM*, *IL17B*, *CCL21* and *KDR*. Together with
368 coelomic epithelial cells (44-58% of all gonadal somatic cells), they constitute the most abundant
369 somatic cell population (37-44%) within 5 PCW gonads. At 6 PCW, their proportion drops to 17% and
370 7% in female and male gonads, respectively. At this stage, early supporting cells represent 21 to 54%
371 of somatic cells, and already include *SOX9*⁺ Sertoli cells in the testis, demonstrating that sex
372 differentiation is initiated earlier than previously described^{31,50}. These results also suggest that sex
373 differentiation in humans, especially when compared to the mouse, is a relatively slow and
374 asynchronous process, since supporting and interstitial cells at distinct differentiation stages could be
375 found in the same gonad together with progenitor cells.

376

377 **Distinct spatio-temporal pathways for the differentiation of supporting cells**

378 The inclusion of an important number of early gonads allowed us to finely characterize the initial steps
379 of supporting cell differentiation. We notably identified strong, human-specific markers for
380 undifferentiated supporting cells, including *TAC1*, *TSPAN8* or *REG3G*. All of them are expressed
381 transiently, right after the potential somatic progenitor step and before the onset of the canonical
382 markers *SOX9* and *FOXL2*. A recent study proposed *TAC1*+ cells to be exclusively found in ovaries
383 and to represent pre-granulosa cell progenitors³¹. In our dataset, we clearly detected *TAC1* in all testes
384 at 6 PCW, but not at 5 PCW as these undifferentiated gonads did not yet include early supporting cells.
385 Moreover, we found *TAC1* to be strongly expressed in *SRY*+/*SOX9*- pre-Sertoli cells at the periphery of
386 the testis at 5 PCW, while it was expressed at lower levels in *SRY*+/*SOX9*+ Sertoli cells which were
387 located deeper. This clearly indicates that *TAC1*+ cells represent an early step during supporting cell
388 specification rather than an “ovarian-specific” progenitor population. This spatial distribution further
389 suggests that the differentiation of male supporting cells proceeds from the center to the periphery of
390 the testis, with first-specified pre-Sertoli cells being surrounded by newer ones. Such gene expression
391 gradients were also reported for *SOX9*, *DMRT1* and *GSTA2* in chicken fetal testes⁴¹. In ovaries, we
392 detected *TAC1* in *WNT6*+/*FOXL2*- pre-granulosa cells at 6 PCW as well as in *WNT6*+/*FOXL2*+ pre-
393 granulosa cells at least up to 11 PCW. Nevertheless, unlike in the testis, *TAC1* was restricted to the
394 innermost, medullary pre-granulosa cells and was absent in the outermost, cortical pre-granulosa cells,
395 which expressed *LHX2* instead. The specific locations of these distinct female supporting cell
396 populations is consistent with our lineage analysis that uncovered two waves for the differentiation of
397 pre-granulosa cells: a first one that may initiate from the coelomic epithelium-derived somatic
398 progenitors, which also gives rise to pre-Sertoli cells in male gonads, and a second one that would derive
399 directly from late female coelomic epithelial cells. This potential developmental scheme appears similar
400 to the mouse model where *Foxl2*+ medullary pre-granulosa cells were described to emerge from *Wnt6*+
401 bipotential progenitors while *Lgr5*+ cortical pre-granulosa cells were shown to derive from *Krt19*+
402 epithelial progenitors^{37,51}. However, in contrast to the mouse, we found that *LGR5* was expressed in
403 early supporting cells derived from somatic progenitors in both male and female gonads, and not in
404 cortical pre-granulosa cells derived from coelomic epithelial cells (Fig. S5, panels A-C).

405

406 **Evolution of the gonadal sex differentiation scheme**

407 While they are known at anatomical and histological levels, the *rete* cells have remained poorly
408 explored and somehow excluded from the sex differentiation process for decades. A few years ago,
409 these cells were shown to harbor supporting-like features and to express *PAX8* in the mouse^{45,52}. More
410 recently, scRNA-seq analysis of fetal mouse gonads identified the *Pax8*-expressing cell population as
411 being the first lineage to emerge from the coelomic epithelium, before the specification of supporting
412 cells. Lineage tracing experiments further suggested they contribute to the pool of male and female
413 supporting cells³⁹. We and others^{32,34} also characterized this not so new cell population in the human
414 developing gonad, with *PAX8* representing an evolutionary-conserved marker for this *rete* lineage.
415 Unlike in the mouse, our data suggest that human *rete* cells would differentiate from early supporting
416 cells rather than directly from the coelomic epithelial cells. Whether *PAX8*-expressing cells could also
417 have the capacity to give rise to supporting cells in humans remains to investigate. Our analyses also
418 highlighted two distinct populations of Leydig cells that differentially express *INSL3* and steroidogenic
419 genes. The coexistence of these two populations from around 8 PCW raises several questions regarding
420 their origins, differentiation mechanisms and fates. It could be important to investigate whether these
421 populations arise from two distinct progenitor lineages as described in the mouse⁵³. We believe that this
422 is not the case, as these two populations express *GATA4* and *NR5A1*, but rather that they represent two
423 sequential states during Leydig cell differentiation, as suggested by trajectory analyses. It will also be

424 interesting to evaluate whether signaling mechanisms known to regulate the specification of mouse
425 Leydig cells such as the PDGFRA⁵⁴ or the DHH⁵⁵ pathways may also trigger the differentiation of one
426 and/or the other of these two populations in humans. One might also hypothesize that one of these
427 populations could represent progenitors of adult Leydig cells, whose origin is still heavily debated (for
428 review, see⁵⁶), while the other population would represent fetal Leydig cells intended to involute later
429 during development. Further investigations are therefore needed to answer these questions, within the
430 limits of available experimental procedures for human tissues.

431

432 **Potential involvement of the GnRH signaling pathway during early testicular development**

433 GnRH is well known for its central role in regulating reproduction. Upon secretion by the hypothalamus,
434 GnRH binds to its receptor, triggering the release of LH and FSH from the anterior pituitary. These
435 gonadotrophic hormones subsequently regulate the growth and functions of the gonads, via their
436 respective receptors, LHCGR and FSHR (for review, see⁵⁷). Patients that exhibit defects in this
437 signaling pathway, either due to inappropriate migration of GnRH neurons, secretion of GnRH or
438 responsiveness to GnRH, are therefore characterized by idiopathic hypogonadotropic hypogonadism
439 (for reviews, see^{58,59}). Some of the phenotypes observed in such patients may also stem from roles for
440 GnRH outside the hypothalamic-pituitary-gonadal axis. Indeed, multiple forms of GnRH and its
441 receptors have been reported in various systems, reproductive or not, including in humans. The potential
442 for GnRH or GnRH-like molecules to directly regulate testicular and ovarian steroidogenesis was
443 extensively investigated, particularly in rodents (for reviews, see⁶⁰⁻⁶²). Here we show that a functional
444 GnRHR is transiently expressed in Human pre-Sertoli cells, where it may regulate specific gene
445 expression. The ontogenesis of GnRH neurons could be consistent with the activation of this testicular
446 GnRHR by the hypothalamic GnRH since these cells appear as early 5.5 PCW⁶³. Such a long-range
447 action is however unlikely, especially when considering dilution effects together with the short half-life
448 of GnRH (2-4 minutes), and similar limitations apply to placental GnRH⁶⁴. To identify a potential local
449 source of GnRH, we investigated the expression of *GNRH1* and *GNRH2* in various embryonic tissues.
450 We observed low but consistent levels of *GNRH1* in several organs (Fig. S6, panel B). Notably, *GNRH1*
451 expression increased in testes and decreased in ovaries between 6 and 7 PCW. While we barely detected
452 *GNRH1* in our scRNA-seq dataset, we actually found that *GNRH1* was preferentially expressed in (pre-
453)Sertoli cells (Fig. S6, panel C) using FACS-sorted testicular cell populations⁶⁵. These results suggest
454 that the GnRH-GnRHR system may represent an active autocrine/paracrine regulatory mechanism
455 during a critical period of human testicular differentiation. Given the central role of Sertoli cells in this
456 process, any alterations of the GnRH signaling pathway could impact male sex differentiation. In
457 addition to genetic causes, maternal exposure to environmental factors capable of agonizing or
458 antagonizing GnRHR, as recently identified⁶⁶, may also directly alter normal differentiation of pre-
459 Sertoli cells.

460

461 **Limitations of the Study**

462 To gain insights into gonadal somatic cell specification and differentiation, we performed single-cell
463 RNA sequencing on freshly collected embryonic and fetal gonads. However, the cell dissociation
464 procedure may bias against fragile cell types, which could be partly overcome by single-nucleus RNA
465 sequencing. Also, the technology we used relies on the 3' end sequencing of polyadenylated RNA,
466 which does not discriminate between transcript isoforms. Future work focusing on isoform-specific
467 expression will therefore be important prior to elucidating their potential roles in each cell type during
468 sex determination. Furthermore, lineage and trajectory inferences were based solely on computational
469 analyses that would require experimental validation using lineage-tracing approaches, despite the
470 challenges of applying such methods to human tissues. Finally, the in vivo relevance of our findings

471 regarding the functional role of the GnRH signaling pathway remains to be confirmed, here again within
472 the inherent limitations of such a biological model.

473

474 **Resource availability**

475 **Lead contact**

476 Further information and requests for resources and reagents should be directed to and will be fulfilled
477 by the lead contact, Antoine Dominique Rolland (antoine.rolland@univ-rennes.fr).

478

479 **Materials Availability**

480 This study did not generate new unique reagents.

481

482 **Data and Code Availability**

483 Single-cell RNA sequencing data have been deposited at the European Genome-phenome Archive
484 (EGA) as EGAS00001006568 and are publicly available as of the date of publication. BRB sequencing
485 data have been deposited at GEO as GSE288161 and are publicly available as of the date of publication.
486 This paper analyzes existing, publicly available data, accessible at GEO (GSE143381, GSE143356,
487 GSE181558, GSE184708) and ArrayExpress (E-MTAB-10551).

488

489 This paper does not report original code.

490

491 Any additional information required to reanalyze the data reported in this paper is available from the
492 lead contact upon request.

493

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509

510 **Author contributions**

511 A.D.R. and F.C. supervised the research. A.L., A.D.R., F.C., and S.M.G. designed the study. A.L., F.C.
512 and A.D.R. wrote the manuscript. A.L., A.D.R. and F.C. analyzed and interpreted sequencing data. S.L.,
513 O.C., L.N., C.D., P.R. and A.B. contributed to the analysis. A.L., A.D.R., S.M.G., B.E., M.T., L.L., I.C.
514 and V.L. prepared the biological samples. A.S. performed ex vivo organotypic culture experiments.
515 A.S., J.B. and S.M.G. validated expression data. C.L.M. and A.C. performed tissue clearing and light-
516 sheet imaging. S.M.G., S.N. and A.C. contributed to the manuscript. All authors approved the final
517 version of the manuscript.

518

519 **Declaration of interests**

520 The authors declare no competing interests.

521

522 **Main figure titles and legends**

523

524 **Figure 1. Profiling human embryonic and fetal gonads at single-cell resolution.** (A) Biological
525 sample collection for the generation of single-cell RNA-seq data from XX and XY human embryonic
526 and fetal gonads across eight developmental stages (5 to 12 PCW). At 5 PCW (scale bar = 1 mm),
527 gonads (circled in yellow) were collected together with the underlying mesonephros. From 6 PCW
528 onwards (scale bar = 3 mm), gonads (yellow arrowhead) were dissociated free of mesonephros (white
529 arrowhead). (B) 2D PCA plot of “pseudobulk” RNA-seq profiles showing diverging transcriptomes as
530 XX and XY gonads differentiate from 5 PCW onwards according to the color code. (C) UMAP
531 representation showing all gonadal cells coloured by sex and developmental stage (left). Deconvolution
532 of the global UMAP plot (right) per developmental stage (from 5 to 12 PCW) and sex (XX and XY
533 samples, top and bottom, respectively). In each UMAP plot, all cells from the dataset are in gray while
534 those from a given sex at a specific developmental stage are coloured accordingly. (D) UMAP
535 representation of all gonadal cells coloured by cluster (c1-c41) and annotated based on the expression
536 of well-established lineage or cell type markers. (E) Dot plot showing the expression of lineage or cell
537 type markers in each cluster (c1-c41). CE = Coelomic epithelial; SE = Surface epithelial; Meso mesench
538 = mesonephric mesenchymal. (F) Stacked bar plots illustrating the distribution of the sex and
539 developmental stage (left) and of the cell cycle phase assigned to the cells (G1, S or G2/M; right) for
540 each cluster (c1-c41). The number of cells in each cluster is indicated in brackets. (G) RNAscope *in situ*
541 hybridization for gonadal somatic lineage markers, i.e. *KRT19* for coelomic epithelial cells (green),
542 *PDGFRA* for interstitial cells (red), and *SOX9* or *FOXL2* for supporting cells (white), on sections from
543 XY and XX gonads at 7 and 11 PCW, respectively. When applicable, “Meso.” indicates the
544 mesonephros located dorsally to the gonad. Scale bars represent 100 or 20 μm , as indicated. See also
545 Figure S1, Table S1 and Table S2.

546

547 **Figure 2. Three-dimensional (3D) and two-dimensional (2D) imaging of PAX8+ cells in human**
548 **embryonic gonads.** (A) 3D visualisation (top panel) of PAX8+ (cyan) and SOX9+ (magenta) cells in
549 an XY gonad at 6 PCW. Two-dimensional (2D) digital slices (bottom panels) through the gonad and
550 mesonephros highlight intragonadal PAX8+ cells and the continuity with SOX9+ cells within the
551 gonad. (B) 3D visualisation (top panel) of PAX8+ (cyan) and OCT4+ (magenta) cells in an XX gonad
552 at 6 PCW. 2D digital slices (bottom panels) through the XX gonad also reveal a continuity of PAX8+
553 cells from the mesonephros to the dorsal region of the gonad. A, P, D and V in the top panels indicate
554 the anterior, posterior, dorsal and ventral poles, respectively. White asterisks and double daggers in the
555 bottom left panels indicate genital ducts and glorumeli, respectively, while yellow arrowheads indicate
556 intra-gonadal PAX8+ cells. “Meso.” indicates the mesonephros located dorsally to the gonads. (C) Co-
557 immunolabeling for GATA4 (red), PAX8 (green) and PAX2 (magenta) on a 2D section of a testis at 7
558 PCW. Intragonadal GATA4+/PAX8+ cells appear in yellow in the overlay image, while
559 PAX2+/PAX8+ genital ducts within the mesonephros appear in white. “Meso.” indicates the
560 mesonephros located dorsally to the testis. Scale bars represent 100, 200 or 300 μm , as indicated. See
561 also Movie S1 and Movie S2.

562

563 **Figure 3. Differentiation of somatic cell lineages during human gonad development.** (A) UMAP
564 representation of gonadal somatic cells coloured by sex and developmental stage. (B) Stacked bar plots

565 of the sex and developmental stage in each cluster (s1-s42), with the number of cells for each cluster
566 indicated in brackets. (C) Stacked bar plots of the cell cluster in each of the 33 samples. (D) UMAP
567 visualization of the scaled expression of gene markers in gonadal somatic cells. (E) UMAP
568 representation of gonadal somatic cells coloured by cluster (s1-s42) and annotated by cell population
569 based on the expression of established lineage or cell type markers. (F) Sex-specific trajectories of
570 gonadal somatic lineages. Starting points of each trajectory are indicated as circles located in cluster
571 s30. Pink and blue lines correspond to female and male trajectories, respectively, while cells are
572 coloured from light to dark gray according to increasing pseudotime. (G) Visualization of female and
573 male gonadal somatic cell lineages (top and bottom, respectively). Cell density along pseudotime (x-
574 axis) within each branch of the lineages is presented using violin plots that are coloured either by sex
575 and developmental stage (left) or by cell cluster (right), with cell fate decisions visualized as branch
576 points. CE = Coelomic epithelial. (H) A proposed model for the specification and differentiation of
577 somatic cell lineages in first trimester human gonads. The cell lineages derived from the gonadal
578 coelomic epithelium are presented together with associated gene markers. See also Table S3, Figure S1,
579 Figure S2 and Figure S3.

580

581 **Figure 4. Expression dynamics during gonadal somatic cell differentiation.** (A) Heatmap
582 illustrating 24 modules (m1-m24) of coexpressed genes along the pseudotime in female (left) and in
583 male (right) lineages. For each lineage, horizontal bars illustrate the pseudotime and the developmental
584 stage according to the color codes. Differentially-expressed genes (DEGs) between somatic clusters
585 were identified using the one-tailed Wilcoxon rank sum test as implemented in Seurat package (v4.0.1,
586 p-values adjusted for multiple testing using the Bonferroni correction) prior to being subjected to the
587 tradeSeq package (v1.22.0) onto the eight major pseudotemporal cell trajectories. The number of DEGs
588 is indicated in brackets on the right of the module identifier and some associated markers are indicated
589 on the right of the heatmap. A color scale for scaled expression data is given. CE = Coelomic epithelial.
590 (B) Gene expression profiles smoothed over pseudotime and coloured by cell lineage. See also Table
591 S4.

592

593 **Figure 5. Spatial localization of supporting cells in human embryonic and fetal gonads.** (A) UMAP
594 visualization of the expression of *WNT6* (Supporting cells), *TAC1* (Early supporting cells), *SRY* (Pre-
595 Sertoli cells), *SOX9* (Sertoli cells), *FOXL2* (Pre-granulosa cells) and *LHX2* (Ovarian surface epithelial
596 cells and pre-granulosa cells). (B) RNAScope *in situ* hybridizations for *SRY* or *TAC1* (red), *SOX9* or
597 *FOXL2* (white), *WNT6* or *LHX2* (green) on sections from a XY gonad at 5 PCW and XX gonads at 6
598 and 8 PCW; the short side of the inset (bottom panel) is 100 μm . When applicable, "Meso." indicates
599 the mesonephros located dorsally to the gonad. Scale bars represent 100 or 200 μm , as indicated. See
600 also Figure S4 and Figure S5.

601

602 **Figure 6. Involvement of GnRHR during early testis differentiation.** (A) *GNRHR* expression profile
603 smoothed over pseudotime and coloured by cell lineage. (B) Relative expression of *GNRHR* in human
604 embryonic organs as determined by qPCR. All samples were collected from individuals aged 6 PCW,
605 except gonads that were collected at 6 and 7 PCW. Data are presented as means \pm SEM. Meso =
606 mesonephros. (C) Expression correlation of *SRY* and *GNRHR* as determined by qPCR in testes at 6 and
607 7 PCW. (D) Experimental design for the functional investigation of GnRHR. Testes (yellow line) at 5-
608 6 PCW from N=10 individuals were collected, dissected free of mesonephros and cut into two explants
609 each prior to hanging drop cultures for 24 hours. For each individual, one testis was used as a control
610 (DMSO only) and the other one was supplemented with Leuprolide at 20 $\text{ng}\cdot\text{mL}^{-1}$. Changes in
611 expression were then assessed by bulk RNA-seq. (E) Expression profiles of differentially-expressed
612 genes (expression value higher than the background cutoff in at least one experimental condition, fold-

613 change cutoff equal or higher than 1.5 and a Wilcoxon signed-rank with an adjusted p-value with the
614 Benjamini and Hochberg method ≤ 0.05) in BRB-seq data (left panel) and scRNA-seq data (right panel).
615 Histograms represent the median expression ratio (Leuprolide over DMSO) \pm SEM. Dots represent the
616 percentage of XY cells in each cluster that express a given gene and are coloured according to scaled
617 expression. s24 and s14 refer to clusters from Fig. 4. Gonadal cell markers, *GNRHR*, upregulated genes
618 and downregulated genes are coloured in grey, blue, green and red, respectively. (F) RNAScope in situ
619 hybridizations for *GNRHR* or *HSD3B2* (red), *NR5A1* or *PDGFRA* (green), *SOX9* or *DHH* (white) on
620 sections from testes at 6 and 10 PCW. When applicable, “Meso.” indicates the mesonephros located
621 dorsally to the gonad. Scale bars represent 200 μ m, as indicated. (G) Cross-species comparison of
622 steroidogenic factors in human (top) and mouse (bottom) testicular cells. Dots represent the percentage
623 of XY cells in each cluster that express a given gene and are coloured according to scaled expression.
624 Gonadal cell markers, *GNRHR*, genes with conserved or nonconserved expression are coloured in black,
625 blue, green and red, respectively. Undiff. supporting = Undifferentiated supporting. See also Figure S6
626 and Figure S7.

627

628 Main tables and legends

Gene Name	Description	Expression profile	Fold change	p-value*
CCL2	C-C motif chemokine ligand 2	Upregulated	2,836	0,002
SERPINA3	serpin family A member 3	Upregulated	2,447	0,0059
TM4SF1	transmembrane 4 L six family member 1	Upregulated	2,022	0,0039
MIA	MIA SH3 domain containing	Upregulated	1,954	0,002
TAGLN	transgelin	Upregulated	1,862	0,0195
MIA-RAB4B	MIA-RAB4B readthrough (NMD candidate)	Upregulated	1,856	0,002
IL32	interleukin 32	Upregulated	1,850	0,0039
PDLIM1	PDZ and LIM domain 1	Upregulated	1,743	0,002
HMG5	high mobility group nucleosome binding domain 5	Upregulated	1,601	0,0273
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	Upregulated	1,552	0,0137
TUBA4A	tubulin alpha 4a	Upregulated	1,536	0,0059
PNLIP	pancreatic lipase	Upregulated	1,529	0,002
GADD45G	growth arrest and DNA damage inducible gamma	Upregulated	1,522	0,0273
BIRC3	baculoviral IAP repeat containing 3	Upregulated	1,514	0,0039

MTRNR2L2	MT-RNR2 like 2 (pseudogene)	Upregulated	1,511	0,0273
RAB8B	RAB8B, member RAS oncogene family	Upregulated	1,505	0,0059
SLC35F6	solute carrier family 35 member F6	Downregulated	-1,510	0,0195
CCDC117	coiled-coil domain containing 117	Downregulated	-1,543	0,0273

629 **Table 1. Differentially expressed genes (DEGs) identified upon GnRHR activation, related to**
630 **Figure 6.**

631 For each DEG, gene name and description, expression profile (Upregulated or Downregulated), median
632 paired expression fold change (Leuprolide over DMSO) and p-value (2-tailed Wilcoxon signed-rank
633 test) are provided. See also Figure S6.

634

635 **STAR Methods**

636

637 **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

638

639 **Human samples**

640 First trimester human embryos and fetuses (5-12 PCW) were obtained from legally induced
641 terminations of pregnancy performed in Rennes University Hospital. No termination of pregnancy was
642 due to fetal abnormality. Tissues were collected following women's written consent, in accordance with
643 the legal procedure agreed by the National agency for biomedical research (authorization #PFS09-011;
644 Agence de la Biomédecine) and the approval of the Local ethics committee of Rennes Hospital (advice
645 # 11-48).

646

647 The terminations of pregnancy were induced using a standard combined Mifegyne® (mifepristone) and
648 Cytotec® (misoprostol) protocol, followed by aspiration. A subset of women received either 400 or 800
649 mg of ibuprofen for preventive analgesia or none. Gestational age was determined by ultrasound, and
650 further confirmed by measurement of foot length^{68,69}. The samples were recovered from the aspiration
651 products using a binocular microscope (Olympus SZX7, Lille, France) and were immediately placed in
652 ice-cold phosphate-buffered saline (PBS). For 5 PCW samples, gonads and the underlying mesonephros
653 were collected together while from 6 PCW onwards gonads were dissected free of mesonephros. The
654 sex of individuals was determined by morphological evaluation of the gonads, except for embryos
655 younger than 7 PCW, for which a qPCR was performed on genomic DNA using primers specific for
656 *SRY* (Forward: ACAGTAAAGGCAACGTCCAG; Reverse: ATCTGCGGGAAGCAAAGTGC)⁷⁰ and
657 for a genomic upstream of the *GAPDH* gene (Forward: CCACAGTCCAGTCTGGGAACC; Reverse:
658 GAGCTACGTGCGCCCGTAAAA)⁷¹.

659

660 **METHOD DETAILS**

661

662 **Tissue dissociation**

663 Single-cell RNA-sequencing was performed on human embryonic and fetal gonads from 33 individuals
664 (16 females and 17 males) ranging from 5 to 12 PCW. The 5 PCW samples included n=1 female and
665 n=2 males. Then, for each sex, samples included n=4 at 6 PCW, n=3 at 7 PCW, n=2 at 8 PCW, n=2 at
666 9 PCW, n=2 at 10 PCW and n=2 at 11-12 PCW (Table S1). Single cell suspensions were obtained by a
667 standard enzymatic and mechanical digestion procedure. Briefly, gonads were cut into small pieces and

668 digested in PBS containing 0.25% Trypsin-0.02% EDTA (#T4049, Sigma-Aldrich) and 0.05 mg/ml
669 DNase (#DN25, Sigma-Aldrich) for 10 min at 37 °C, followed by gentle up and down pipetting. Trypsin
670 digestion was stopped by adding 10% fetal bovine serum in M199 medium and centrifugation at 350 g
671 for 5 min at 37°C. Dissociated cells were resuspended and washed twice in PBS containing 0.04%
672 weight/volume BSA. The final cell suspensions were filtered on 30 µm strainers (Miltenyi Biotec) and
673 counted on a Malassez hemocytometer after Trypan blue staining.

674

675 **Single-cell capture, library preparation and sequencing**

676 All steps for the construction of single-cell libraries (Chromium™ Single Cell 3' Library & Gel Bead
677 Kit v2) were carried out according to the manufacturer's instructions (10x Genomics). In brief, cell
678 concentration was systematically adjusted to 400 cells/µL in PBS-BSA prior to loading on the
679 Chromium™ Single Cell A Chip. For each sample, ~7,000 cells were loaded on a single reaction well
680 in order to target ~4,000 recovered cells. Following construction, the 33 single-cell libraries were
681 quality-controlled using a bioanalyzer (Agilent Technologies), pooled and sequenced on a single lane
682 of an Illumina HiSeq 4000 instrument using a 2x100 paired-end sequencing protocol (Illumina, San
683 Diego, CA) to validate quality and to adjust library pooling according to actual cell capture rates.
684 Subsequently, the 33 pooled libraries were sequenced on 33 lanes of an Illumina HiSeq 4000 instrument
685 to achieve an average sequencing depth of at least 350 million paired-reads per sample.

686

687 **scRNA-seq data pre-processing, quality controls and normalization**

688 Demultiplexed raw sequencing reads were processed, mapped to the GRCh38 human reference genome
689 (official Cell Ranger reference, v3.0.0) and quantified based on unique molecular identifiers (UMIs)
690 with the Cell Ranger pipeline (v7.0.0, 10x Genomics). The cell-calling algorithm from Cell Ranger,
691 which relies on the EmptyDrops method⁷², was also used to establish a first set of potential valid cells
692 and to merge the resulting raw count matrices from the 33 individual samples without normalization.
693 After library preparation and sequencing, our initial dataset consisted of a total of 158,663 cells with an
694 average of 77,769 reads per cell and a median number of 3,762 detected genes per cell.

695 Subsequently, cells with fewer than 500 detected genes; fewer than 2000 counts or higher than 100,000
696 counts; more than 30% of mitochondrial genes; or detected as doublets by the DoubletFinder (v3.0) R
697 package⁷³ were removed from the aggregated matrix. After quality control filtering, our final dataset
698 described the expression of 29,323 genes in 127,903 single cells, with an average of 3,876 cells per
699 sample and of 3,935 detected genes per cell (Table S1). Cell cycle phases were assigned using the Seurat
700 (v4.0.1) R package⁷⁴. Finally, the data were normalized and scaled using the SCTransform function
701 with cell-cycle score and mitochondrial-encoded gene transcript levels as covariates.

702 “Pseudobulk” samples (log₂-transformed and normalized count matrix from the aggregated counts from
703 all the analyzed cells of each sample) were also projected on a 2D PCA-based space using the
704 FactoMineR package⁷⁵ (v2.11) to graphically evaluate their distribution (Fig. 1, panel B).

705 Regarding the focused analysis of gonadal somatic cells, we first selected relevant clusters identified
706 from the primary analysis (i.e. the 28 *GATA4*⁺ cell clusters, which are also *LHX9*⁺ and/or *NR5A1*⁺)
707 and further reanalyzed the 111,855 subsetted cells with a similar strategy.

708

709 **Reduction of dimensionality and clustering**

710 Variable genes were detected using the SCTransform function from Seurat (v4.0.1) and used to perform
711 a PCA reduction of dimensionality. The first 50 principal components were then used in the Louvain
712 graph-based clustering using the FindNeighbors function followed by the FindClusters functions with
713 a resolution of 1.2 and 1.6 for the initial analysis and for the somatic cell analysis, respectively. Finally,
714 we used the uniform manifold approximation and projection (UMAP) method implemented in the
715 Seurat package to project single cells in a reduced 2D space.

716

717 **Cluster annotation and differential gene expression analysis**

718 Cell clusters and subpopulations were annotated using known marker genes as well as computed
719 differentially expressed genes using the Wilcox rank sum test implemented in Seurat package (v4.0.1)
720 (one-tailed Wilcoxon rank sum test, p-values adjusted for multiple testing using the Bonferroni
721 correction).

722

723 **Pseudotemporal cell trajectory inference**

724 To order gonadal somatic cells in pseudotime and infer male or female trajectories over developmental
725 stages, we used the learnGraph and orderCells functions using default parameters (except for
726 learnGraph: close_loop = loop) implemented in the Monocle3 R package (version 1.0.0)⁷⁶. To specify
727 the starts of the male and female lineage trajectories and to smooth expression values over pseudotime,
728 we used the medoids of XY and XX coelomic epithelial cells (cluster s30) at the earliest developmental
729 stage (5 PCW), respectively. The position of the root node of each trajectory was then determined by
730 extracting the closest neighboring nodes of the selected cells.

731 Differentially expressed genes over each of the eight major pseudotemporal cell trajectories were
732 determined with the tradeSeq package⁷⁷ (v1.22.0) with a number of knots (nknots) = 6. Briefly a list of
733 cells for each trajectory and their related pseudotime values were extracted from Monocle3 and used as
734 input for tradeSeq. The 4,387 genes identified were further clustered into 24 modules (m1-m24) of co-
735 expression along the eight major cell trajectories using the find_gene_modules function from the
736 Monocle3 R package.

737 Slingshot⁷⁸ (v1.8.0) was used as an additional approach to infer somatic cell trajectories in both female
738 and male gonads and therefore validate hypotheses made from Monocle3. The analysis was performed
739 in a PCA-reduced dimensional space. Slingshot then estimated pseudotime values along the inferred
740 differentiation trajectories, enabling the identification of putative lineage progression patterns.

741

742 **Analysis and comparison with external human scRNA-seq datasets**

743 To assess the complementarity between publicly available human gonadal scRNA-seq datasets and the
744 present one, we processed and analysed each dataset independently, restricting our analysis to first-
745 trimester samples. Three ovarian datasets (Fig. S2) were analysed, including i) three samples collected
746 at 7 (n=1), 9 (n=1) and 10 (n=1) PCW²⁹, ii) four samples collected at 6 (n=1), 9 (n=2) and 10 (n=1)
747 PCW³⁴, and iii) 23 samples collected from 17 individuals at 6 (n=2), 7 (n=1), 8 (n=7), 9 (n=3), 10 (n=1),
748 11 (n=2) and 12 (n=1) PCW³². Three testicular datasets (Fig. S3) were also analysed : i) four samples
749 collected at 6 (n=1), 7 (n=1), 8 (n=1) and 12 (n=1) PCW³⁰, ii) four samples collected at 7 (n=2), 9 (=1)
750 and 12 (n=1) PCW³⁴, and iii) nine samples collected at 7 (n=2), 8 (n=1), 9 (n=1), 11 (n=2), and 12 (n=3)
751 PCW³². Of note, one sample collected from a male individual at 6 PCW from Garci-Alonso and
752 collaborators³² was not included as it had been subjected to depletion of CD45+ cells and contained 44
753 cells only. Also, all datasets needed to be corrected for batch effects (Fig. S2 and Fig. S3).

754 For every dataset the same strategy as described above was used, from data pre-processing to cell
755 clustering and annotation. Additionally, inter-donor variability was corrected using Harmony⁷⁹ (v0.1)
756 prior to computing UMAP embedding. Once each dataset was annotated, *GATA4+* gonadal cell
757 populations were selected and mapped onto our somatic cell atlas using the FindTransferAnchors and
758 MapQuery functions implemented in the Seurat package, with default parameters.

759

760 **Mouse scRNA-seq data analysis**

761 The raw count matrix from 20 mouse gonads covering five developmental stages and captured using
762 the Chromium™ Single Cell 3' Library & Gel Bead Kit v2 (10x Genomics) was downloaded from the
763 GEO repository as provided by the authors (GSE184708)³⁹. Following quality controls on cells and

764 genes, a total of 53,410 cells from mouse genital ridges and testes aged 10.5 to 16.5 dpc. The data were
765 analysed as described above for human data, except that batch effects were corrected using the RPCA
766 method as implemented in Seurat v4.0.1. The resulting cell clusters were annotated based on the sex,
767 the developmental stage as well as the expression of known gene markers (Fig. S6, panels F-H).

768

769 **Hanging drop culture experiments**

770 Pairs of testes from N=10 individuals aged 5-6 PCW were collected, dissected free of mesonephros and
771 cut into two explants each. For each experimental condition, the two explants from one testis were used
772 and cultured under a petri dish lid in agreement with a previously established hanging drop culture
773 model of human fetal tissue explants⁸⁰, with minor modifications. Briefly, each testicular explant was
774 put in a 35 μ L a drop of M199 medium, no phenol red (Invitrogen Life Technologies), containing 50
775 μ g/mL gentamycin and 2.5 μ g/mL amphotericin B (Fungizone, Sigma Aldrich Chemicals). Exposures
776 consisted in the addition of 0.04 % v/v dimethyl sulfoxide (DMSO) for controls, or of Leuprolide 20
777 ng/mL (L0399-1MG, Sigma-Aldrich Chemicals) together with 0.04 % v/v DMSO for treated samples.
778 Explants were cultured in a humidified petri dish for 24 hours at 37°C, 5% CO₂. At the end of the
779 cultures, explants were collected, rinsed in PBS and stored at -80°C.

780

781 **RNA extraction and real-time quantitative PCR**

782 Total RNAs were extracted from cultured explants or uncultured tissues using the RNeasy Mini Kit
783 (Qiagen), quantified using a NanoDropTM 8000 spectrophotometer (Thermo Fisher Scientific) and
784 reverse transcribed (200 ng) with the iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative
785 PCR were performed with the iTaq[©] Universal SYBR Green Supermix (Bio-Rad Laboratories) using
786 a Cfx384 OneTouch Real-Time PCR system (Bio-Rad Laboratories). *RPLP0* and *RPS20* were used as
787 controls for normalization purposes and the relative expression of each gene in each sample was
788 determined using the 2 ^{Δ CT} method. Primer specificity was checked using primer-BLAST on human
789 RefSeq transcripts and their efficiency was evaluated using serial dilutions of amplicons.

790

791 **BRB-seq experiment and data analysis**

792 The 3' Bulk RNA Barcoding and sequencing (BRB-seq)⁴⁸ was performed as previously described⁸¹.
793 Briefly, total RNAs (10 ng per sample) were distributed in a 96-well plate, reverse transcribed and
794 barcoded using sample-specific indexes and UMIs. The resulting cDNAs were pooled, converted into
795 double strand cDNAs and tagmented using the Illumina Nextera XT Kit (Illumina, #FC-131-1024). The
796 tagmented library was then PCR-amplified, size-selected and sequenced in a paired manner (2x100 bp)
797 on a NovaSeq sequencer following Illumina's instructions. The first read contains the sample-specific
798 barcode (6 bp) and the UMI (10 bp) while the second read is mapped to the human reference
799 transcriptome (downloaded from the UCSC genome browser, release hg38) using BWA⁸² (v0.7.4.4;
800 with the parameter "-l 24"). The UMIs associated with each gene for each sample are counted, resulting
801 in a gene expression matrix that is normalized thanks to the rlog transformation from the DESeq2
802 package⁸³ (v1.40.2). Differentially expressed genes (DEGs) between controls (DMSO) and treated
803 samples (Leuprolide) were finally identified using a three-step filtration procedure: i) an expression
804 value higher than the background cutoff (median value of all genes in all samples) in at least one
805 experimental condition, ii) a fold-change cutoff equal or higher than 1.5, and iii) a Wilcoxon signed-
806 rank with a p-value adjusted with the Benjamini and Hochberg method⁸⁴ as a significance cutoff ($p \leq$
807 0.05).

808

809 **RNAScope *in situ* hybridization**

810 The spatial distribution of cell subpopulations was investigated by *in situ* hybridization using the
811 RNAScope[®] technology. Freshly collected human embryonic and fetal gonads were fixed in 4%

812 paraformaldehyde at 4°C for 24 hours, embedded in paraffin, and sectioned to a thickness of 5 µm. *In*
813 *situ* hybridization was next performed using the RNAscope® Multiplex Fluorescent v2 assay. Briefly,
814 sections were pretreated with H₂O₂ for 10 min at room temperature, boiled in 1X Target Retrieval buffer
815 for 15 min and submitted to a Protease Plus treatment for 30 min at 40°C. The following steps, i.e.
816 hybridization of probes, amplification and signal development, were performed according to the
817 manufacturer's instructions. Signals were detected using three different fluorophores, Opal 520 (1:500),
818 Opal 620 (1:750) and Opal 690 (1:750) (Akoya Biosciences) diluted in TSA buffer. Finally, slides were
819 covered with ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Scientific), images were
820 acquired on a ZEISS LSM 880 with Airyscan confocal laser microscope (Zeiss) and processed using
821 the ZEN 2.3 lite software (Zeiss). Initial settings were made with the "Best Fit" option which adjusts
822 the display characteristic curve (that defines the range of gray value histogram to be displayed) so that
823 the 0.1% of the darkest pixels contained in the image are black and the 0.1% of the brightest pixels are
824 white in the display. Individual channels were then independently adjusted (left/right limits of the
825 display characteristic curve) for each section, without modifying the so-called gamma value (curvature
826 of the curve). Indeed, depending on the tissue quality, the developmental stage, the sex and the opal
827 dye, the signal intensity for a given targeted gene could be very different. Furthermore, the degrees of
828 background signal and auto-fluorescence could significantly differ from one section to another. As a
829 result, for a given multiplex combination, the experiment was repeated on several sections with dye-
830 swap design to reach the best rendering. The image processing was therefore necessarily specific to
831 each section. Of note, the use of slightly different settings should not be problematic since the signal
832 intensities were never compared between sections. Furthermore, most targeted gene markers were
833 specific enough to a single cell population so that contrast enhancements only allowed to provide a
834 better rendering.

835

836 **Tissue clearing and light-sheet imaging**

837 After collection, samples were fixed overnight in 4% paraformaldehyde at 4°C, prior to being rinsed
838 and stored in 1X PBS at 4°C. Samples were then dehydrated at room temperature in a graded series of
839 MeOH concentrations in 1X PBS (20%, 40%, 60%, 80% and 2x 100%) with agitation before bleaching
840 by overnight incubation in 6% H₂O₂ in 100% MeOH at 4°C shielded from light. Samples were washed
841 3 times in 100% MeOH before being rehydrated in a decreasing concentration of MeOH in 1X PBS
842 (80%, 60%, 40%, 20%) with agitation before blocking and permeabilization in PBSGT (1X PBS with
843 0.2% gelatin and 0.5% Triton X-100) overnight. Following blocking, samples were incubated with
844 primary antibodies against PAX8 (Proteintech, 10336-1-AP) and SOX9 (R+D Systems, AF3075) or
845 OCT4 (R+D Systems, AF1759) at 1:500 dilution in PBSGT with Saponin (10mg/mL) with agitation
846 for 7 days. Samples were then washed 6 times in PBSGT before incubation with secondary antibodies
847 (ab150063 and ab175745 from Abcam, at 1:500 and 1:250 dilution, respectively) diluted in PBSGT
848 with Saponin at 37°C with agitation for 2 days. After washing 6 times in PBSGT, samples were
849 embedded in 1.5% Agarose in 1X TAE buffer to facilitate mounting. Embedded samples were then
850 dehydrated in a graded series of MeOH concentrations in 1X PBS (20%, 40%, 60%, 80% and 2 times
851 100%) with rotation before overnight incubation in 2/3 Dichloromethane (DCM) followed by a 30-
852 minute incubation in 100% DCM before immersion in Benzyl ether (DBE) and stored at RT in the dark
853 until imaging. Light-sheet imaging was performed on an Ultramicroscope Blaze light-sheet (Miltényi
854 Biotech) equipped with a 12x objective (MI Plan 12x/0.53) and tile scans of the whole samples were
855 performed using a z-step size of 2µm. Images were processed using the Oxford's Imaris software suite.

856

857 **Immunolabelling on cryosections**

858 Multiplex immunofluorescence experiments were performed as previously described⁸⁵. Briefly, freshly
859 collected samples were fixed overnight in 4% paraformaldehyde at 4°C, rinsed in 1X PBS and

860 cryoprotected by immersion in 15% sucrose overnight at 4°C. They were then embedded (mixture of
861 7.5% gelatin and 10% sucrose in 0.12 M phosphate buffer) prior to being frozen in isopentane at -55°C
862 and stored at -80°C. Samples were next cut into 20µm sections using a cryostat (Leica CM3050).
863 Cryosections were blocked in PBS-GT (0.2% gelatin, 0.25% Triton-X100 in 1X PBS) for 1 hour and
864 incubated overnight at room temperature with goat anti-PAX2 (R and D Systems, AF3364; 1:250),
865 mouse anti-GATA4 (Santa Cruz Biotechnology, sc-25310; 1:250) and rabbit anti-PAX8 (Cell Signaling
866 Technology, 59019; 1:200) primary antibodies diluted in PBS-GT. After 3 washes in 1X PBS, Alexa-
867 conjugated secondary antibodies were incubated (1:500 in PBS-GT) for 1hr at room temperature. After
868 3 washes in 1X PBS, sections were counterstained with DAPI and mounted with Mowiol. Imaging was
869 conducted using an epifluorescence microscope (Leica DM6000).

870

871 **QUANTIFICATION AND STATISTICAL ANALYSIS**

872 **Statistics**

873 All statistical analyses were performed using R.

874

875 For scRNA-seq data analysis, differentially-expressed genes between clusters were computed using the
876 Wilcoxon rank sum test implemented in Seurat package (v4.0.1), which relies on the one-tailed Wilcoxon
877 rank sum test, with p-values adjusted for multiple testing using the Bonferroni correction, as indicated
878 in Figure legends and Supplemental table legends. For pseudotemporal cell trajectories, differentially
879 expressed genes identified using the Wilcoxon rank sum test were further subjected to the tradeSeq
880 package⁷⁷ (v1.22.0), as indicated in Figure legends and Supplemental table legends

881

882 BRB-seq was performed on N=10 individuals aged 5-6 PCW, as indicated in the legends. For each
883 individual, one testis was cultured in DMSO (control sample) and the second was cultured in Leuprolide
884 (treated sample). Differentially expressed genes between controls (DMSO) and treated samples
885 (Leuprolide) were identified using a three-step filtration procedure: i) an expression value higher than
886 the background cutoff (median value of all genes in all samples) in at least one experimental condition,
887 ii) a median fold-change cutoff equal or higher than 1.5, and iii) a Wilcoxon signed-rank with a p-value
888 adjusted with the Benjamini and Hochberg method⁸⁴ as a significance cutoff ($p \leq 0.05$). Data are
889 presented as median expression (Leuprolide over DMSO) \pm standard error of the mean (SEM), as
890 indicated in the legends.

891

892 qPCR data on tissues and FACS-sorted cell populations are presented as mean \pm standard error of the
893 mean (SEM), as indicated in the Figure and Supplemental figure legends. For the validation of BRB-
894 seq data on cultured testis explants, data are presented as mean expression ratios (Leuprolide over
895 DMSO) \pm standard error of the mean (SEM), as indicated in Supplemental figure legends.

896

897 **Supplemental information**

898 **Document S1.** Figure S1-S7 and supplemental references

899

900 **Movie S1.** Light-sheet fluorescence microscopy video of ~6.4 PCW XY gonad stained with PAX8
901 (cyan) and SOX9 (magenta), related to Figure 2.

902

903 **Movie S2.** Light-sheet fluorescence microscopy video of ~6.4 PCW XX gonad stained with PAX8
904 (cyan) and OCT4 (magenta), related to Figure 2.

905

906 **Table S1.** Description of single-cell RNA-seq samples, related to Figure 1.

907 Development stage in PCW, sex and sequencing data are presented for each sample.

908

909 **Table S2. Differentially expressed genes (DEGs) between the 41 clusters (c1-c41) in the total**
910 **dataset, related to Figure 1.**

911 DEGs were identified using the Wilcox rank sum test implemented in Seurat package (v4.0.1) (one-
912 tailed Wilcoxon rank sum test, p-values adjusted for multiple testing using the Bonferroni correction).

913

914 **Table S3. Differentially expressed genes (DEGs) between the 42 somatic cell clusters (s1-s42) in**
915 **the somatic cells dataset, related to Figure 3.**

916 DEGs were identified using the Wilcox rank sum test implemented in Seurat package (v4.0.1) (one-
917 tailed Wilcoxon rank sum test, p-values adjusted for multiple testing using the Bonferroni correction).

918

919 **Table S4. Differentially expressed genes (DEGs) over the eight major cell trajectories, related to**
920 **Figure 4.**

921 DEGs over the eight major pseudotemporal cell trajectories were determined with the tradeSeq package
922 (v1.22.0).

923

924 **Table S5. Primer sequences used for qPCR, related STAR methods.**

925 For each target gene, the sequences of the forward and reverse primers used for quantitative PCR
926 experiments are provided.

927

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-PAX8	Proteintech	Cat# 10336-1-AP; RRID:AB_2236705
Goat anti-SOX9	R&D Systems	Cat# AF3075; RRID:AB_2194160
Goat anti-Oct3/4	R&D Systems	Cat# AF1759; RRID:AB_354975
Goat anti-PAX2	R&D Systems	Cat# AF3364; RRID:AB_10889828
Mouse anti-GATA4	Santa Cruz Biotechnology	Cat# sc-25310; RRID:AB_627667
Rabbit anti-PAX8	Cell Signaling Technology	Cat# 59019; RRID:AB_2799553
Donkey anti-rabbit IgG H&L, Alexa Fluor® 647	Abcam	Cat# ab150063; RRID:AB_2687541
Donkey anti-goat IgG H&L, Alexa Fluor® 750	Abcam	Cat# ab175745; RRID:AB_2924800
Donkey Anti-Mouse IgG H&L, Alexa Fluor® 647	Abcam	Cat# ab150107; RRID:AB_2890037
Donkey Anti-Goat IgG H&L, Alexa Fluor® 555	Abcam	Cat# ab150130; RRID:AB_2927775
Donkey Anti-Rabbit IgG H&L, Alexa Fluor® 488	Abcam	Cat# ab150073; RRID:AB_2636877
Biological samples		
First trimester human gonads or genital tracts	CHU DE RENNES - Hôpital Sud, Rennes, France	See Table S1
Critical commercial assays		
Chromium™ Single Cell 3' Library & Gel Bead Kit v2, 16 rxns	10x Genomics	Cat# PN-120237
Chromium™ Single Cell A Chip Kit, 48 rxns	10x Genomics	Cat# PN-120236
Chromium™ i7 Multiplex Kit, 96 rxns	10x Genomics	Cat# PN-120262
RNAscope™ Multiplex Fluorescent Detection Kit v2	ACD	Cat# 323110
RNAscope™ Probe Hs-FOXL2-C3	ACD	Cat# 586451-C3
RNAscope™ Probe Hs-KRT19	ACD	Cat# 310221
RNAscope™ Probe Hs-KRT19-C3	ACD	Cat# 310221-C3
RNAscope™ Probe Hs-LGR5-C2	ACD	Cat# 311021-C2
RNAscope™ Probe Hs-LHX2	ACD	Cat# 401651
RNAscope™ Probe Hs-NPY-C2	ACD	Cat# 416671-C2
RNAscope™ Probe Hs-NR5A1-C2	ACD	Cat# 553151-C2
RNAscope™ Probe Hs-PDGFRA	ACD	Cat# 604481
RNAscope™ Probe Hs-PDGFRA-C2	ACD	Cat# 604481-C2
RNAscope™ Probe Hs-SOX9-O2-C3	ACD	Cat# 518661-C3
RNAscope™ Probe Hs-SRY-C2	ACD	Cat# 476861-C2
RNAscope™ Probe Hs- TAC1-C2	ACD	Cat# 310711-C2
RNAscope™ Probe Hs-WNT6	ACD	Cat# 429451
RNAscope™ Probe Hs-WT1-C2	ACD	Cat# 415581-C2

RNAscope™ Probe Hs-GNRHR-3UTR	ACD	Cat# 411401
RNAscope™ Probe Hs-HSD3B2	ACD	Cat# 467681
Illumina Nextera XT Kit	Illumina	Cat# FC-131-1024
Deposited data		
scRNA-seq data, raw fastq files	This paper	EGA: EGAS00001006568
BRB-seq data, raw count and normalised gene expression matrix	This paper	GEO: GSE288161
scRNA-seq data, raw count matrix	Chitashvili et al. ²⁹	GEO: GSE143381
scRNA-seq data, raw count matrix	Guo et al. ³⁰	GEO: GSE143356
scRNA-seq data, raw count matrix	Garcia-Alonso et al. ³²	ArrayExpress: E-MTAB-10551
scRNA-seq data, raw count matrix	Taelman et al. ³⁴	GEO: GSE181558
scRNA-seq data, raw count matrix	Mayère et al. ³⁹	GEO: GSE184708
Oligonucleotides		
Primers for qPCR experiments	This paper	See Table S5
Software and algorithms		
Cell Ranger (v7.0.0)	10x Genomics	https://www.10xgenomics.com/support/software/cell-ranger/downloads
DoubletFinder (v3.0)	McGinnis et al. ⁷³	https://github.com/chrismcginnis-ucsf/DoubletFinder
Seurat (v4.0.1)	Satija et al. ⁷⁴	https://satijalab.org/seurat/
FactoMineR (v2.11)	Lê et al. ⁷⁵	http://factominer.free.fr/index.html
Monocle3 (v1.0.0)	Cao et al. ⁷⁶	https://cole-trapnell-lab.github.io/monocle3/
tradeSeq (v1.22.0)	den Berge et al. ⁷⁷	https://github.com/statOmics/tradeSeq
Slingshot (v1.8.0)	Street et al. ⁷⁸	https://github.com/kstreet13/slingshot
Harmony (v0.1)	Korsunsky et al. ⁷⁹	https://portals.broadinstitute.org/harmony/
BWA (v0.7.4.4)	Li and Durbin ⁸²	https://bio-bwa.sourceforge.net/
DESeq2 (v1.40.2)	Love et al. ⁸³	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
ZEN 2.3 lite software	Zeiss	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen-lite.html

Imaris software	Oxford Instruments	https://imaris.oxinst.com/
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eTOC Blurb

To investigate gonadal somatic cell lineage specification in Humans, Lardenois and Suglia et al. combine single-cell transcriptomics, trajectory analyses and organotypic cultures. They identify coelomic epithelium-derived progenitors, capable of adopting either a steroidogenic or supporting fate, and report expression changes upon pharmacological activation of GnRHR in embryonic testes ex vivo..

Highlights

Gonadal somatic cell lineages stem from coelomic epithelium-derived progenitors

Supporting cells commit to sex-specific fates by postconceptional week 6.

PAX8+ rete cells arise from ovarian and testicular undifferentiated supporting cells.

A second wave of pre-granulosa cells derives from the ovarian surface epithelium.