Title: Contribution of the Wolffian duct mesenchyme to the formation of the female reproductive tract

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26 **Abstract:**

27 The female reproductive tract develops from its embryonic precursor, the Müllerian duct.
 28 In close proximity to the Müllerian duct lies the precursor for the male reproductive tract, the
 29 Wolffian duct, which is eliminated in the female embryo during sexual differentiation. We
 30 discovered that a component of the Wolffian duct, its mesenchyme, is not eliminated after sexual
 31 differentiation. Instead, the Wolffian duct mesenchyme underwent changes in transcriptome and
 32 chromatin accessibility from male tract to female tract identity, and became a unique
 33 mesenchymal population in the female reproductive tract with localization and transcriptome
 34 distinct from the mesenchyme derived from the Müllerian duct. Partial ablation of the Wolffian
 35 duct mesenchyme stunted the growth of the fetal female reproductive tract. These findings reveal
 36 a new fetal origin of mesenchymal tissues for female reproductive tract formation and reshape our
 37 understanding of sexual differentiation of reproductive tracts.

39 **Significance statement:**

40 A female embryo initially possesses both the primitive female and male reproductive
 41 tracts, also known as the Müllerian and Wolffian duct. During sexual differentiation, the female
 42 eliminates the Wolffian duct and maintains the Müllerian duct that eventually differentiates into
 43 the female reproductive tract organs. However, in this paper, we show the female embryo retains
 44 mesenchymal cells surrounding the Wolffian duct for its reproductive tract formation. When
 45 incorporated into the female reproductive tract organs, the Wolffian duct mesenchyme shows
 46 unique anatomical localization and transcriptome and plays critical roles in female reproductive
 47 tract growth. This discovery provides new insights into female reproductive tract development
 48 and advances our understanding of sexual differentiation of reproductive tracts.

50 **Introduction:**

Sexually dimorphic differentiation of reproductive tracts in the mammalian embryo

ensures the establishment of a sex-specific reproductive tract in adulthood (1-3). Prior to sexual differentiation, male and female embryos possess both primitive male and female reproductive tracts, known as the Wolffian and Müllerian ducts, respectively (4). During sexual differentiation, in the female embryo, the Wolffian duct regresses in the absence of androgens; whereas the Müllerian duct is maintained and eventually gives rise to the oviduct, uterus, cervix and upper vagina (1). In the male embryo, on the other hand, fetal testes produce two hormones to reverse the fates of the two primitive reproductive tracts: anti-Müllerian hormone (AMH) induces regression of the Müllerian duct while androgens maintain the Wolffian duct, which eventually differentiates into the epididymis, vas deference and seminal vesicle (5).

Prior to sexual differentiation (i.e. before embryonic day or E13.5 in mice), the Wolffian duct provides critical guidance for the formation of the Müllerian duct (4, 6-9). The Wolffian duct derives from the intermediate mesoderm and extends caudally till its distal part fuses with the urogenital sinus (4, 8). Shortly after the Wolffian duct formation, coelomic epithelium in the cranial part of the mesonephros is specified to become Müllerian duct precursor cells, which then invaginate caudally towards the Wolffian duct and form a canalized tube (7). Once in contact with the Wolffian duct, the Müllerian duct begins to elongate in the craniocaudal direction under the guidance of the pre-established Wolffian duct and eventually fuses at the urogenital sinus by E13.5 in mice (6, 10). The elongation of the Müllerian duct requires the activation of PI3K/AKT signaling pathway in rat Müllerian duct epithelia(9). It was thought that specification and invagination of specified cells in the Müllerian duct are independent of the Wolffian duct (4). However, a study in chicken embryos found that surgical removal of the cranial portion of the Wolffian duct before the onset of Müllerian duct formation abolished the expression of *Lhx1* (also known as *Lim1*) (11), the essential gene for the specification of coelomic epithelium to become Müllerian duct precursor cells (12). Therefore, the role of the Wolffian duct in specification and

76 invagination during Müllerian duct formation may require further investigations. Nevertheless,
 77 substantial evidence has demonstrated that the subsequent step of Müllerian duct formation, its
 78 elongation, is dependent upon structural supports and regulatory signaling of the Wolffian duct.
 79 The tip cells of the elongating Müllerian duct contact closely with the basal membrane of the
 80 Wolffian duct, which serves as a physical guide for the Müllerian duct elongation (13). Physical
 81 interruption of the Wolffian duct in chicken embryos and genetic ablation of the caudal portion of
 82 the Wolffian duct in murine embryos both led to the arrested elongation of the Müllerian duct at
 83 the point of the interruption (6, 14). In addition, the Wolffian duct specifically expresses and
 84 secretes *Wnt9b*, a critical paracrine factor, without which the extension of the Müllerian duct is
 85 arrested at the cranial-most portion (15). Despite of the guiding roles of the Wolffian duct on
 86 Müllerian duct formation, the Wolffian duct epithelium itself does not contribute to or transform
 87 into the Mullerian duct. When the Wolffian duct epithelium was permanently labeled with a
 88 reporter *LacZ* that is induced by the Wolffian duct epithelium specific Cre (*Hox7b-Cre*), the entire
 89 Müllerian duct remained *LacZ* negative (6).

90 Despite its involvement in initial formation of the female reproductive tract before the
 91 onset of sexual differentiation, it is not known whether the Wolffian duct plays any contributing
 92 roles to the development of the female reproductive tract after sexual differentiation. When the
 93 Müllerian duct is formed under the guidance of the Wolffian duct, the two ducts are closely
 94 adjacent to each other and are surrounded by their respective mesenchymes, which are distinct in
 95 terms of microscopic appearance, gene expression and hormonal responsiveness. In the female
 96 mouse embryo on E13.5, the Müllerian duct mesenchyme lacks any recognized pattern in
 97 arrangement while mesenchymal cells surrounding the Wolffian duct appear elongated and
 98 radially disposed around the Wolffian duct (16). In terms of gene expression, the Wolffian duct
 99 mesenchyme but not the Müllerian duct mesenchyme expresses androgen receptor (*Ar*) (17, 18)
 100 and *Gli1*, a readout gene of the morphogen sonic hedgehog (SHH) that is specifically secreted

from the Wolffian duct epithelium (19-21). On the other hand, the Müllerian duct mesenchyme expresses *Amhr2*, the specific receptor for AMH (22), whereas the Wolffian duct mesenchyme does not (17, 18). As a result, in the female embryo, the Müllerian duct mesenchyme can respond to anti-Müllerian hormone (23) while the Wolffian duct mesenchyme possesses the capability of responding to exogenous androgens (24-26).

During sexual differentiation, the epithelium of the Wolffian duct is eliminated in the female embryo. However, the fate of the remaining mesenchyme surrounding the Wolffian duct in the female embryo after sexual differentiation is unclear. Extensive studies have demonstrated the essential roles of the mesenchyme in the growth and differentiation of female reproductive tract organs after its early formation (4, 5, 27, 28). Given the juxtaposition of the Wolffian duct to the Müllerian duct, we set up to investigate whether the Wolffian duct mesenchyme is a source of functional mesenchymal tissues in the female reproductive tract .

Results:

The Wolffian duct mesenchyme contributes to mesenchymal tissues in the female reproductive tract.

Wolffian duct mesenchyme and Müllerian duct mesenchyme in the mesonephros can be distinguished by their specific expression of *Gli1* and *Amhr2*, respectively, during fetal development (19, 22). We confirmed this expression pattern by RNA-scope (Fig. S1A) and *Amhr2-Cre; Rosa-tdTomato; Gli1-lacZ* double reporter mouse line (Fig. S1B). To investigate what the Wolffian duct mesenchyme becomes in XX embryos, we developed the *Gli1-CreER; Rosa-tdTomato* tamoxifen-inducible lineage tracing model, where the *Gli1*-positive Wolffian duct mesenchymal cells (*Gli1*⁺) were permanently labeled with red fluorescent protein tdTomato only at the time of tamoxifen injection (Fig. 1). Tamoxifen injection was performed on E13.5 and E14.5 before Wolffian duct regression in XX embryos. One day after the injection, a

few mesenchymal cells surrounding the Wolffian duct in the cranial (future oviduct) and caudal (future uterus) portion of the mesonephros were positive for *tdTomato* (**Fig. 1A & F**). On E16.5 when the epithelium of the Wolffian duct disintegrated, the labeled *Gli1*⁺ Wolffian duct mesenchyme remained present in the female reproductive tract (**Fig. 1B, 1C, 1G & 1H**). On postnatal day 0 (PND0 or birth), PND21 when the basic structures of the oviduct and uterus is established (29), and PND56 when the females are sexually mature for supporting pregnancy, the presence of *Gli1*⁺ Wolffian duct mesenchyme persisted in the oviduct and uterus (**Fig. 1D, E, I & J**). These *Gli1*⁺ Wolffian duct mesenchyme-derived cells expressed the markers for fibroblasts (Vimentin) (30) and smooth muscle cells (smooth muscle α -actin, α SMA) (31) (**Fig. S2**), indicating that they differentiated into typical cell types in the mesenchymal compartment of the female reproductive tract. These results demonstrate that the Wolffian duct mesenchyme in the XX embryo contributes to mesenchymal tissues in the female reproductive tract.

Transcriptomic and epigenetic changes underlying sexually dimorphic differentiation of the Wolffian duct mesenchyme

The Wolffian duct mesenchyme is masculinized to become components of the male reproductive tract in normal XY embryos or XX embryos exposed to exogenous androgens (25, 32, 33). Our discovery of the contribution of Wolffian duct mesenchyme to the female reproductive tract further indicates that the Wolffian duct mesenchyme is bipotential with sexually dimorphic differentiation capacity (**Fig. 2A**). We then set out to understand molecular changes and potential transcriptional regulation of the Wolffian duct mesenchyme when it follows either male or female fate. We performed RNA-seq and ATAC-seq to profile the transcriptome and chromatin accessibility landscape, respectively, on isolated *Gli1*⁺ Wolffian duct mesenchymal cells from XX and XY mesonephroi on E14.5 and E16.5, the window of sexual differentiation of reproductive tracts. We plotted principal component analyses (PCA) to illustrate the degrees of

151 differences among these four groups in terms of transcriptome and chromatin accessibility (**Fig.**
 152 **2B & 2C**). From E14.5 to E16.5, the distances between XX and XY groups in both PCA plots
 153 were increased, suggesting that transcriptome and chromatin accessibility landscape of the
 154 Wolffian duct mesenchyme became more sexually divergent. These results were consistent the
 155 higher number of differential expressed genes (DEG) between XX and XY from E14.5 to E16.5
 156 (**Fig. 2D & 2G**) and the increased number of differential ATAC-seq peaks (**Supplementary table**
 157 **1**).

158 To deduce biological events underlying male and female differentiation of the *Gli1*⁺
 159 Wolffian duct mesenchyme, we performed gene enrichment analysis of upregulated genes in XY
 160 and XX when they are compared (**Fig. 2D & 2G**). In these upregulated genes in XY *Gli1*⁺
 161 Wolffian duct mesenchymal cells (280 genes from E14.5 XY > E14.5 XX and 950 genes from
 162 E16.5 XY > E16.5 XX with 175 genes in common between the two stages), myogenesis,
 163 epithelial-mesenchymal transition and apical junction and KRAS signaling were significantly
 164 enriched with myogenesis or smooth muscle cell differentiation being the most significant
 165 enriched (**Fig. 2E**). This observation was consistent with the notion that Wolffian duct
 166 masculinization was associated with smooth muscle differentiation (34). On the other hand, when
 167 analyzing upregulated genes in XX *Gli1*⁺ Wolffian duct mesenchymal cells (158 genes from
 168 E14.5 XX > E14.5 XY or 762 genes from E16.5 XX > E16.5 XY) (**Fig. 2G**), we found that the
 169 statistically significant pathways were Hedgehog signaling (a morphogen signaling), EMT
 170 (features extracellular matrix remodeling), KRAS signaling, IL-6/JAK/STAT3 signaling and
 171 allograft rejection signaling (**Fig. 2H**), which suggested remodeling in cellular morphology,
 172 extracellular matrix and immune responses during the female fate differentiation of the Wolffian
 173 duct mesenchyme.

174 At mRNA transcriptional level, actions of specific transcriptional factors (TFs) and
 175 accessible chromatin landscape for transcriptional factor bindings govern cellular state and

176 differentiation (35). To identify transcriptional factors that potentially involve in male and female
 177 fate differentiation of the *Gli1*⁺ Wolffian duct mesenchyme, we determined the upregulated TFs
 178 in XY or XX *Gli1*⁺ Wolffian duct mesenchymal cells by overlapping the lists of upregulated
 179 genes in either of them with the mouse transcriptional factors database (36). We also examined
 180 TF binding motifs enriched in regions with increased chromatin accessibility in XY *Gli1*⁺
 181 Wolffian duct mesenchymal cells (E16.5 XY > E16.5 XX or E14.5 XY > E14.5 XX) and XX
 182 (E16.5 XX > E16.5 XY or E14.5 XX > E14.5 XY) with the focus on those distal from
 183 transcription start sites, which contain a majority of differential peaks (85.7%, -95.5%,
 184 **Supplementary table 1**) and are expected to harbor TF-binding motifs critical for cellular
 185 differentiation (37, 38). The top 20 motifs enriched in regions of increased chromatin
 186 accessibility in XY and XX *Gli1*⁺ Wolffian duct mesenchymal cells were shown in
 187 **Supplementary tables 2-5**. Finally, we overlapped the top motifs enriched at the chromatin
 188 regions with increased accessibility with the significantly upregulated TFs (TFs with the
 189 expression level TPM<10 were removed) in XY or XX *Gli1*⁺ Wolffian duct mesenchymal cells.

190 For the male differentiation, this overlapping analysis yielded five transcriptional factors
 191 and their motifs, *Tcf21*, *Hoxc9*, *Mef2c*, *Foxl2*, and *Bach2* (**Fig. 2F**). Of note, *Ar* was not in the
 192 list because *Ar* mRNA level in our RNA-seq datasets was not significantly different between
 193 XX and XY *Gli1*⁺ Wolffian duct cells on either E14.5 or E16.5, which is consistent with
 194 previous findings (17, 39). However, androgen responsive elements (ARE) or AR-associated
 195 nuclear receptor motifs were the top motifs in regions with increased chromatin accessibility in
 196 XY *Gli1*⁺ Wolffian duct mesenchymal cells. This observation demonstrates the benefits of
 197 including chromatin accessibility data for our analysis in identifying the predominant actions
 198 of AR. Among these five transcriptional factors, it has been shown that TCF21 modulates AR
 199 transcriptional regulation in vitro cancer cell lines (40); *Hoxc9* involves in regionalization of the
 200 Wolffian duct (41); and *Mef2c* (Myocyte-specific enhancer factor 2C) is a target of AR during

androgen enhanced myogenesis (42). The roles of *Fox2* and *Bach2* in Wolffian duct differentiation has not been reported in the literature. These results demonstrate that the male fate differentiation of the *Gli1*⁺ Wolffian duct mesenchyme is associated with multiple TFs that are potential critical components in AR regulatory network.

We adopted the same strategy to infer potential TFs in the female fate differentiation of the *Gli1*⁺ Wolffian duct mesenchyme (**Fig. 2I**). The upregulated genes in the XX *Gli1*⁺ Wolffian duct mesenchymal cells included dozens of TFs, among which *Esr1*, *Wt1*, *Hand2*, *Etv4*, *Tcf7* and *Mafb* had their binding motifs enriched in regions with increased chromatin accessibility in XX (E16.5 XX > E16.5 XY or E14.5 XX > E14.5 XY) (**Supplementary tables 4 & 5**). The roles of *Wt1* and *Hand2* in fetal female reproductive tract development have not been reported. *Esr1* mediates estrogen actions and is critical for female reproductive tract function (43). However, the absence of *Esr1* does not affect female reproductive development (44). In the absence of either *Etv4* or *Tcf7*, female mice are fertile, suggesting that they play dispensable roles in the female reproductive tract development (45, 46). These observations demonstrate that the repurposing of the *Gli1*⁺ Wolffian duct mesenchyme in XX embryos is associated actions of multiple transcriptional factors, which are more likely to be the outcome of the female fate differentiation.

Similarities and differences in gene expression between *Gli1*⁺ Wolffian duct mesenchyme and *Amhr2*⁺ Müllerian tract mesenchyme in the neonatal uterus

Once becoming a part of the female reproductive tract, the *Gli1*⁺ Wolffian duct mesenchyme-derived cells exhibited unique distribution along the tract: they were localized more in the uterus than the oviduct (**Fig. 1E and 1F**). Additionally in the uterus, *Gli1*⁺ Wolffian duct mesenchyme was clustered at the mesometrial side, where the uterine horn is connected to blood vessels and body cavity (**Fig. 1H & 1J**). Conversely, when we tracked the fate of the Müllerian duct-derived mesenchyme with the *Amhr2-Cre; Rosa-tdTomato* reporter model, we found that

226 *Amhr2*⁺ Müllerian tract mesenchyme contributed to the majority of mesenchymal cells in the
 227 oviduct and their localization in the uterus was exclusive to the anti-mesometrial side (**Fig. S3**).
 228 These results demonstrate that *Gli1*⁺ Wolffian duct mesenchyme gives rise to a mesenchymal
 229 population distinct from *Amhr2*⁺ Müllerian tract mesenchyme in the female reproductive tract.

230 To understand molecular similarities and differences between these two mesenchymal
 231 populations with their contrast localizations in the uterus (mesometrial vs anti-mesometrial side),
 232 we isolated these two cell types from the respective reporter models using FACS and compared
 233 their transcriptomic differences by RNA-seq at birth, when the Wolffian duct mesenchyme has
 234 been incorporated into the uterus. Both mesenchymal populations had high expression of typical
 235 mesenchymal genes, such as vimentin (*Vim*) and collagens (*Colla1*, *Colla2* and *Col3a1*).
 236 Nevertheless, their transcriptomes differed in 1,705 genes with 1049 genes expressed significantly
 237 higher in the *Gli1*⁺ Wolffian duct mesenchyme and 656 genes higher in the *Amhr2*⁺ Müllerian
 238 tract mesenchyme, respectively (**Fig. 3A**). To identify what signaling pathways have associations
 239 with these genes, we performed IPA upstream regulator analysis on the genes upregulated in
 240 each of the two mesenchymal cells. Interestingly, the analyses of higher expressed genes in these
 241 two populations showed enrichment of angiotensin (i.e. angiotensinogen and VEGF) and estrogen
 242 (i.e. beta-estradiol and diethylstilbestrol) signaling in common. Nevertheless, in the *Gli1*⁺
 243 Wolffian duct mesenchyme, the transforming growth factor beta one (TGFβ1) pathway was
 244 predicted to be one of the top signaling pathways that lie upstream of the 809 higher expressed
 245 genes. On the other hand, in the *Amhr2*⁺ Müllerian tract mesenchyme, the WNT/beta-catenin or
 246 CTNNB1 pathway was enriched (**Fig. 3B**). Consistent with this analysis, a downstream target of
 247 TGFβ pathway *Akap12* (also known as SSeCKS) (47) was upregulated in the *Gli1*⁺ Wolffian
 248 duct-derived mesenchymal cells and its protein was specifically localized to the Wolffian duct
 249 mesenchyme and the mesometrial side of the uterus on E16.5 and 18.5 (**Fig. 3C**). On the other
 250 hand, *Hand2*, a downstream target of WNT/beta-catenin signaling (48), was expressed higher in

251 *Amhr2*⁺ Müllerian tract mesenchyme and its protein was detected on the anti-mesometrial side of
 252 the E14.5-18.5 uterus (**Fig. 3D**). The enrichment of WNT/beta-catenin signaling pathway in
 253 *Amhr2*⁺ Müllerian tract mesenchyme is consistent with the observation of higher *Wnt* signaling in
 254 uterine anti-mesometrial side (49). Taken together, our results unequivocally demonstrate the
 255 *Gli1*⁺ Wolffian duct mesenchyme contributes a unique mesenchymal population different from
 256 the *Amhr2*⁺ Müllerian duct mesenchymal cells in the female reproductive tract.

257

258 **Partial ablation of the Wolffian duct mesenchyme stunts the growth of female reproductive** 259 **tracts ex vivo**

260 Next, we investigated the functional significance of the *Gli1*⁺ Wolffian duct mesenchyme
 261 in fetal female reproductive tract development by using the tamoxifen-inducible *Gli1-CreER*;
 262 *Rosa-tdTomato*; *Rosa-DTA* genetic cellular ablation model. In this model, the tamoxifen
 263 treatment not only induces *tdTomato* expression to label *Gli1*⁺ cells, but also turns on the
 264 expression of diphtheria toxin or *DTA* that induces cellular apoptosis specifically in DTA-
 265 expressing cells (50). Because *Gli1* is expressed in mesenchymal compartments of other critical
 266 organs (51), *in vivo* cellular ablation of *Gli1*⁺ cells led to multiple organ failures and embryonic
 267 lethality. To circumvent this problem, we performed the ablation experiment in organ culture,
 268 which is an established technique to study sexual differentiation of reproductive tracts ex vivo
 269 (52). Mesonephroi with *Gli1-CreER*; *Rosa-TdTomato* (control group) or *Gli1-CreER*; *Rosa-*
 270 *tdTomato*; *Rosa-DTA* (ablation group) from E14.5 XX embryos were cultured for two days with
 271 tamoxifen to induce *Cre* activities and tdTomato/DTA expression in the *Gli1*⁺ Wolffian duct
 272 mesenchyme before Wolffian duct regression. Upon the tamoxifen treatment, *tdTomato* was still
 273 specifically expressed in the *Gli1*⁺ Wolffian duct mesenchyme in both groups (**Fig. S4**). However,
 274 in the ablation group, we observed statistically significant increase in the number of apoptotic
 275 cells positive for the apoptotic marker, cleaved PARP1(53) in the mesenchyme (**Fig. S4**). In

addition, the total fluorescence signal of *tdTomato* in the mesonephroi after the culture was significantly reduced in the ablation group to 60% of that in the control group under the identical imaging setting (**Fig. 4A & C**), indicating a partial ablation of the *Gli1*⁺ Wolffian duct mesenchyme in the ablation group. Despite that the ablation was partial, we observed a noticeable phenotype that the lumen area of the Müllerian duct in the ablation group was significantly smaller than those in the control (**Fig. 4A & D**).

Next, we investigated how the partial loss of the *Gli1*⁺ Wolffian duct mesenchyme led to the reduced lumen areas. It is well established that the paracrine growth factors from the mesenchyme regulates the growth and differentiation of the epithelium (54). Therefore, we searched for known growth factors (55) whose expression was significantly higher in the *Gli1*⁺ Wolffian duct mesenchyme than in the *Amhr2*⁺ Müllerian duct mesenchyme in our RNA-seq data (**Fig. 3A**). We then examined the expression pattern of the potential growth factors in fetal female reproductive tract and mouse knockout phenotypes in the Müllerian duct development through the online gene expression and mouse genome informatics databases (56). As a result, we uncovered that growth factors (*Angpt1*, *Angpt2*, *Bdnf*, *Fgf7*, *Fgf12*, *Igf1*, *Pdgfa*, and *Vegfd*) were enriched in the *Gli1*⁺ Wolffian duct mesenchyme compared to the *Amhr2*⁺ mesenchymal cells. Among these factors, the mesenchyme-derived growth factor *Igf1* appeared to be a putative candidate with its receptor *Igfr1* expressed in the Müllerian duct epithelium (57, 58). Absence of *Igf1* or its receptor *Igfr1* led to hypoplastic reproductive tract organs in the XX embryo (58, 59), indicating the critical roles of *Igf1* signaling in the Müllerian duct growth. When we examined spatiotemporal expression of *Igf1* in the normal XX embryo, we found its specific and transient expression in the Wolffian duct mesenchyme at the mesometrial side of the caudal mesonephros (future uterus) on E16.5 although it was also expressed in the anti-mesometrial side of the cranial mesonephros (future oviduct) (**Fig. 4B**). These observations prompted us to investigate whether IGF1 was able to rescue the phenotype of the stunted growth of the Müllerian duct in the ablation

group, where the ablation of *Gli1*⁺ mesenchymal cells could lead to the decrease in mesenchymal IGF1 production. We supplemented exogenous IGF1 in the media of the ablation group and found that IGF1 treatment did not affect the ablation efficacy in the ablation groups (**Fig. 4A & 4C**). However, the phenotype of the decreased lumen areas was partially rescued in the ablation group with IGF1 treatment (**Fig. 4A & 4D**). Taken together, these results indicate the *Gli1*⁺ Wolffian duct mesenchyme is critical for the proper growth of the fetal female reproductive tract, probably through the paracrine growth factor IGF1.

Discussion

We provide the molecular and genetic evidence for the dual origins of mesenchymal tissues in the female reproductive tract. We used *in vivo* genetic cell tracking approach to reveal that the *Gli1*⁺ Wolffian duct mesenchyme continues to exist in the developing female reproductive tract, corroborating with another study with slightly different approach (60). We also revealed transcriptome and chromatin accessibility changes in sexual differentiation of the *Gli1*⁺ Wolffian duct mesenchyme, transcriptomic similarities and differences between the *Gli1*⁺ Wolffian duct mesenchyme and the *Amhr2*⁺ Müllerian duct mesenchyme, and the functional significance of the *Gli1*⁺ Wolffian duct mesenchyme in the fetal growth of the female reproductive tract (**Fig. 5**).

The Wolffian duct mesenchyme gives rise to a distinct mesenchymal population for uterine formation after sexual differentiation of reproductive tracts.

Despite of the critical roles of mesenchyme in female reproductive tract development, our understanding about the embryonic origin of its mesenchymal tissues has not been complete. When using *Amhr2-cre; Rosa-LacZ* mouse line to track the Müllerian duct mesenchyme that expresses *Amhr2* in the female mouse embryo, all the LacZ positive mesenchymal cells are

located at the anti-mesometrial side of the uterus and never observed in the mesometrial side (61). Our results from the *Amhr2-Cre; Rosa-Tomato* model also confirm this observation. This absence of *Amhr2*⁺ cells in the mesometrial side cannot be due to inefficient Cre activities because the Cre is under the control of *Amhr2* promoter and endogenous *Amhr2* expression is also absent at the mesometrial side (22). These results demonstrate that the entire *Amhr2*⁺ Müllerian duct mesenchyme contributes to mesenchymal tissues solely at the anti-mesometrial side of the uterus. As a result, when using *Amhr2-Cre* to ablate critical genes for female reproductive tract development and function, the ablation and phenotype only occurs in the anti-mesometrial side (62-64). For example, when β -catenin, the intracellular signaling transducer of the canonical WNT pathway, was inactivated in the *Amhr2-Cre; Ctnnb1-flox* conditional knockout mouse, only at the anti-mesometrial side did mesenchymal cells become deficient in smooth muscle differentiation. Those at the mesometrial side remained intact and differentiated normally into myometrium (62, 63). These observations imply the presence of a distinct mesenchymal populations in the mesometrial side of the uterus.

Using in vivo genetic lineage tracing, we reveal that the Wolffian duct mesenchyme gives rise to these mesenchymal cells at the mesometrial side of the uterus. Transcriptomic analysis and specific gene expression examination reveal gene expression similarities and differences between mesenchymal populations at the mesometrial and anti-mesometrial side of the uterus at birth. Both mesenchymal populations express typical mesenchymal markers (*Vim*, *Colla1*, *Colla2* and *Col3a1*) and show enrichment of angiotensin and estrogen signaling in higher expressed genes in them. However, compared to the *Amhr2*⁺ Müllerian duct mesenchyme, the *Gli1*⁺ Wolffian duct mesenchyme seems to be conditioned with smooth muscle differentiation signaling. For example, *Tgfb1* signaling, one major driver for smooth muscle differentiation (65), and its downstream gene AKAP12 is enriched only in the *Gli1*⁺ Wolffian duct mesenchyme. On the other hand, the

Müllerian duct mesenchyme specific gene *Hand2* is exclusively expressed in the stroma and remains absent in the smooth muscle layer in the postnatal uterus (66).

We also found that, after birth, the localization of Wolffian duct mesenchyme-derived cells remain at the mesometrial side of the uterus. It is well-noted that the mesometrial and anti-mesometrial mesenchyme in the uterus exhibit differential expression in postnatal development and function. In postnatal development, the *Amhr2*⁺ Müllerian duct mesenchyme (giving rise to the anti-mesometrial mesenchyme) had higher WNT signaling activity, which is essential for limiting gland formation to the anti-mesometrial side of the uterus (49). During pregnancy, estrogen and progesterone receptors coordinate actions of estrogen and progesterone in the preparation of the uterus for implantation and placentation, which occur at the anti-mesometrial and mesometrial side, respectively (67, 68). Interestingly, dynamic and differential expression of estrogen receptor α (ESR1) and progesterone receptor (PGR) are observed between mesenchymal cells at the mesometrial and anti-mesometrial poles during pregnancy (69, 70). Multiple single cell RNA transcriptomic studies have revealed mesenchymal heterogeneity in the uterus and have identified several stromal populations in the uteri at the age of postnatal 6 (2 mesenchymal populations, inner and outer stromal populations) (71), postnatal 12 (4 mesenchymal populations) (72) and in the adult uterus on the estrus stage (3 populations of fibroblasts and 2 population of perivascular cells) (73). However, the spatial expression of marker genes for these mesenchymal populations along the mesometrial-anti-mesometrial axis were not examined in these studies. Our discovery of the differential embryonic origins of mesometrial and anti-mesometrial mesenchyme in the uterus has potential implications in the differential gene expression along the mesometrial-anti-mesometrial pole for gland formation and uterine functions.

The transcriptome and chromatin accessibility analyses during sexual differentiation highlight how hormonal environment alters the fate of the Wolffian duct mesenchyme.

The contribution of Wolffian duct mesenchyme to the female reproductive tract

demonstrates that the Wolffian duct mesenchyme is bipotential with sexually dimorphic differentiation capacity. Androgen is known to be the predominant hormone for masculinizing Wolffian ducts and promoting the differentiation of the Wolffian duct mesenchyme into smooth muscle cells in the male embryo (18, 34). Our observations of myogenesis as the top signaling pathway in the upregulated genes in the Wolffian duct-derived mesenchymal cells and increased chromatin accessibilities in genomic regions harboring the top motif ARE corroborate with this notion. On the other hand, in the female embryo where androgens are absent, the differentiation of the Wolffian duct mesenchyme into smooth muscle cells does not occur. Although these mesenchymal cells remain undifferentiated in the female embryo, their chromatin accessibility increases in genomic regions containing multiple transcriptional factor motifs with estrogen receptor α (ESR1) as the top one. These observations suggest the estrogen action may play a role in modulating epigenetic landscape in the Wolffian duct mesenchyme in the female embryo. The existence of the ESR1 action in the fetal reproductive tract tissues in the female embryos has been supported by the expression of *Esr1* (74) and ESR1 (75) in the mesonephric mesenchyme and detection of widespread ER transcriptional activities in the ERE-luciferase reporter mouse (76). Consistent with these results on the ESR1 action, we observed increased expression of *Igf1*, a classic ESR1 transcriptional target gene in the mesometrial mesenchyme of the uterus (77). Despite the action of ESR1 in the female embryo, the *Esr1* knockout female mouse appears to have normal morphology and histology of the female reproductive tract (44). However, a recent study using the aromatase knockout mouse (the critical enzyme for estrogen production) demonstrates that the lack of peri- and postpubertal estrogen diminished uterine responses to estrogen later in life (78). Therefore, these observations raise the possibility that the fetal estrogen/ESR1 signaling plays a role in establishing epigenetic landscape for optimizing the

399 Wolffian duct mesenchyme's responsiveness to estrogen when it is incorporated into the female
400 reproductive tract.

401

402 **The Wolffian duct mesenchyme provides critical paracrine growth factors for Müllerian**
403 **duct development after sexual differentiation of reproductive tracts.**

404 Extensive studies have focused on the functional significances of the Wolffian duct in
405 early formation of the Müllerian duct. However, our results indicate that in the female embryo,
406 the remaining mesenchyme of the Wolffian duct still plays a role after Wolffian duct regression
407 and provides critical paracrine growth factors for the Müllerian duct growth. Although we
408 focused on *Igf1* that is expressed higher in mesometrial side where the *Gli1*⁺ Wolffian duct
409 mesenchyme are localized, IGF1 supplementation did not completely rescue the phenotype of
410 decreased lumen expansion in the ablation group. These results indicate other paracrine growth
411 factors from the Wolffian duct mesenchyme may also contribute to the fetal growth of the
412 Müllerian duct. A complementary experiment that ablates the *Amhr2*⁺ Müllerian duct
413 mesenchyme in the female reproductive tract was performed by ectopic postnatal AMH
414 administration (PND1-6) to the female rats (71). The AMH treatment inhibited *Amhr2*⁺
415 subluminal mesenchyme expansion and led to stromal hypoplasia and dysregulated mesenchymal
416 paracrine signals. Consequently, these treated female rats failed to develop uterine gland
417 formation and were infertile at the adulthood. It would be intriguing to investigate the impact of
418 the loss of the Wolffian duct mesenchyme on postnatal development and function of the female
419 reproductive tract using our *Gli1*⁺ cell ablation model. However, the expression of *Gli1* in other
420 tissues at the fetal stage (51) and its ubiquitous expression in the mesenchyme of the female
421 reproductive tract after birth (79) preclude the use of our *Gli1-CreER*; *Rosa-DTA* model from
422 specifically ablating the Wolffian duct mesenchyme or its derived cell to determine its postnatal
423 functions.

To put our discovery in the context of human diseases, the most common gynecological benign tumor in women of reproductive ages is uterine fibroids also known as leiomyomas, which derive from genetically altered mesenchymal cells in the female reproductive tract (80, 81). Interestingly, in two uterine fibroid mouse models where gain-of-function mutant forms of *Med12* (82) or *Ctnnb1* (83) were induced in the *Amhr2*⁺ Müllerian duct mesenchyme (giving rise to anti-mesometrial mesenchyme), large leiomyoma nodules grew at the mesometrial side of the uterus. These observations suggest the potential involvement or susceptibility of the Wolffian duct mesenchyme-derived cells or their microenvironments for facilitating the formation/growth of leiomyomas. Therefore, our discovery of the contribution of the Wolffian duct mesenchyme to the female reproductive tract not only provide new perspectives on sexual differentiation of reproductive tracts but also potentially promote our understanding of mesenchyme-associated gynecological complications in the female reproductive tract.

Methods and Materials

Animals

Gli1-CreER knock-in mice (stock# 007913) on mixed genetic backgrounds (Swiss Webster and C57BL/6J), *Rosa-tdTomato* (stock# 007909) on the C57BL/6J genetic background, *Rosa-DTA* (stock# 006331) on mixed genetic backgrounds (C57BL/6J and CD1), *Gli1-LacZ* (#008211) on mixed genetic backgrounds (Swiss Webster and 129S1/SvImJ) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Amhr2-Cre* were derived from previously-described colony (84) and maintained on C57BL/6J genetic background. CD-1 mice were from in-house CD-1 colony. Timed mating was produced by housing two or three females with a male. Vaginal plugs were checked daily and the day when the vaginal plug was found was designated as embryonic day E0.5. All animal procedures were approved by the National Institute of Environmental Health Sciences (NIEHS) and the University of Wisconsin-Madison (UW-

Madison) Animal Care and Use Committees and are in compliance with our NIEHS and UW-Madison approved animal study proposals and public laws. Genotyping was determined by Transnetyx or PCR based on genotyping protocols provided by the Jackson Laboratory. All experiments were performed on at least three animals for each genotype.

Tamoxifen treatment

CreER activity was induced by IP injection of tamoxifen (T-5648, Sigma-Aldrich) per mouse in corn oil, receptively. The dose and timing of tamoxifen treatment were described in the results section. The specificity of *Gli1-CreET*; *Rosa-Tomato* genetic lineage tracing were confirmed in our previous study (85).

C-section and pup fostering

Tamoxifen injection during pregnancy results in dystocia. To avoid the loss of pups due to dystocia, C-section was performed on the expected day of delivery (E18.5 or E19.5) following NIEHS SOP on Caesarian Section Rederivation (Terminal). Briefly, forceps and blunt scissors were used to incise the uterus, rupture amniotic sacs, and clamp the cord of the pups. Once all pups were free of the uterus, fluid from the nose and mouth of each pup were cleared using sterile cotton swabs. Pups were stimulated to breathe by rolling swabs length wise along the pup from mouth to anus. When all pups were breathing regularly without stimulation, and pup color was pink, the rederived pups were fostered to the CD-1 mom whose pups were removed. To ensure the success of fostering, CD-1 foster moms who delivered less than 3 days ago were used. The fostered pups were monitored closely for any signs of abandonment or cannibalism.

LacZ staining

The LacZ staining solution was made by dissolving X-gal (Invitrogen) into dimethylformamide to make 40 mg/ml stock solution. The working solution was further prepared by diluting the stock solution to 1 mg/ml in pre-warmed tissue stain base solution (Chemicon). Fresh tissues harboring *Amhr2-Cre*; *Rosa-Tomato*; *Gli1-LacZ* were fixed in 4% paraformaldehyde in 1xPBS at 4 °C for 1 h and then stained in the LacZ staining solution at 37 °C for 1–2 h followed by further fixation in 4% paraformaldehyde/PBS at 4 °C overnight.

Immunofluorescence

Tissues were fixed in 4% paraformaldehyde at 4°C overnight. The tissues and sections were processed for immunostaining as previously described (52). Tissues were dehydrated, embedded, and cyrosectioned at 10 µm. The sections were treated for antigen retrieval using commercial antigen unmasking solution (H-3300, VECTOR) and underwent immunostaining procedures. The following primary antibodies were used: rabbit anti-PAX2 (1:200, PRB-276P, Covance), rabbit anti-alpha-smooth muscle (1:200, ab5694, Abcam), mouse anti-vimentin (1:300, Abcam, ab8978), rabbit anti-AKAP12 (86) (1:500, a gift from Irwin H. Gelman, Roswell Park Cancer Institute), rabbit anti-Cleaved PARP1(1:300, Abcam, ab32064) . The secondary antibodies conjugated with different fluorescent dyes were used (1:200): Alexa Fluor@ 647 donkey anti-mouse IgG, Alexa Fluor@ 488, 568 or 647 donkey anti-rabbit IgG (Invitrogen). All the sections were imaged under a Leica confocal microscope.

For quantifying the number of cleaved PARP1+ cells (the apoptotic cells), at least 8 serial sections with 75 µm apart between each section from each cultured tissue were pictured.

Immunohistochemistry

Frozen sections were treated for antigen retrieval using commercial antigen unmasking solution (H-3300, VECTOR). Endogenous peroxidase was inactivated with 3% H₂O₂ (H325,

498 Fisher Scientific). Sections were incubated with blocking reagent, with primary antibodies goat
 499 polyclonal anti-HAND2 (Santa Cruz, sc-9409, a gift from Francesco DeMayo's lab at NIEHS) at
 500 4 °C overnight. Sections was washed three times, and then incubated with biotinylated anti-rabbit
 501 secondary antibody (#94583, Jackson ImmunoResearch) for 30 min at room temperature.
 502 Sections were then incubated with ABCComplex/HRP (Vectastain ABC kit, VECTOR), and DAB
 503 substrate (SK4100, VECTOR), counterstained with hematoxylin (HHS16, Sigma), and mounted
 504 for imaging.

505

506 **RNAscope**

507 RNAscope was performed in formalin fixed paraffin embedded tissues using
 508 RNAscope® 2.5 HD Detection Reagent (for the detection of *Igfl*) and RNAscope Duplex
 509 Detection Kit (for dual detections of *Amhr2* and *Glii*) according to the manufacturer's
 510 instructions (Advanced Cell Diagnostics, Newark, CA). Tissues were fixed in fresh 10%
 511 formalin for 24h at the room temperature. After fixation, tissues were processed, embedded in
 512 paraffin, sectioned at 5 µm, deparaffinized, and rehydrated. Then sections were treated with
 513 antigen retrieval buffer and proteinase. Sections were exposed to *Igfl* (443901) or *Amhr2*
 514 (489821) + *Glii* (311001-C2) probes and incubated at 40°C in a hybridization oven for 2 h.
 515 Following a series of rinsing, for the single detection of *Igfl*, the signaling is amplified and
 516 stained with a red substrate; for the dual detection, *Amhr2* and *Glii* signaling were amplified
 517 using amplifier conjugated horseradish peroxidase and alkaline phosphatase, respectively.
 518 Then, sections were incubated sequentially with a green and then a red substrate solution to
 519 generate chromogenic colors for *Amhr2* and *Glii* expression, respectively. After the staining,
 520 all the sections were counter-stained with Gill's hematoxylin I, air-dried and mounted. All
 521 sections are imaged under a Zeiss compound microscope.

522

523 **Ex vivo organ culture**

524 E14.5 mesonephroi with ovaries attached were cultured at 37°C with 5% CO₂/95% air on
 525 MilliCELL-CM culture plate insert 0.4 µm filters (Millipore) in Dulbecco's Minimal Eagle
 526 Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal calf serum
 527 (Hyclone), and 100 U/ml Penicillin-Streptomycin in the presence of and 500 nM 4-
 528 hydroxytamoxifen (H7904, Sigma) and 200ng/ml SHH (464-SH-025, R&D system). The
 529 hydroxytamoxifen is the active metabolite of tamoxifen in vivo for inducing the nuclear
 530 translocation of CreER to elicit gene recombination (87). The supplementation of SHH enhanced
 531 CreER expression and thus the CreER mediated ablation efficiency. After 48h of culture in the
 532 presence of SHH and 4-hydroxytamoxifen, tissues were cultured for another 48h with or without
 533 IGF1 (291-G1-200, R&D system). After culture, the whole tissues were imaged under a
 534 fluorescence stereo microscope for *tdTomato* expression and bright-light images. Image J was
 535 used to quantify the corrected total fluorescence (88) and lumen areas of the whole mount tissues.

537 **Fluorescence-activated cell sorting**

538 After tamoxifen treatment at the dose of 1 mg per dam on E12.5 and E13.5, tomato+
 539 cells were isolated from E14.5 and E16.5 *Gli1-CreER*⁺; *Rosa-tdTomato*⁺ mesonephroi, E19.5
 540 *Gli1-CreER*⁺; *Rosa-tdTomato*⁺ uterus or E19.5 *Amhr2-Cre*⁺; *Rosa-tdTomato*⁺ uterus using
 541 the following protocol for tissue dissociation. Tissues were dissected in cold 1xPBS and
 542 enzymatically dissociated in TrypLE Express (Gibco, 12604-013 - no phenol red) at 37 °C for
 543 20–30 minutes with 1000 rpm shaking in a VorTemp™ 56 Shaking Incubator (Labnet). Cells
 544 were further dissociated mechanically using a P200 pippette. After quenching enzyme
 545 activities by addition 2 volume of sorting buffer (1% FBS in 1xPBS), cell were pelleted for 10
 546 min at 500 g at 4 °C and suspended in sorting buffer, which was added through the cell strainer
 547 in the cap (remove tissue clogs) to 5 ml polystyrene round-bottom tube (#352235, Falcon).

Cell sorting was performed on a BD FACS Aria II in the NIEHS Flow Cytometry Center and tdTomato⁺ cells were collected in 5 ml polystyrene round bottom tube that had been coated in sorting buffer overnight.

RNA extraction and RNA-seq

Each group in RNA-seq experiments had three biological replicates with each pooled from cells in two or three times cell sortings. RNA extractions were performed using PicoPure RNA Isolation kit (Life technologies, USA) according to the manufacturer's protocol. The quality of RNA was measured in a 2100 Bioanalyzer Instrument (Agilent) with bioanalyzer high-sensitivity RNA kits according to the manufacturer's protocol. The RNA integrity number (RIN) of RNAs were all above 9.7. The RNA concentration was measured in a Qubit Fluorometer (ThermoFisher Scientific). For sorted Gli1⁺ mesenchymal cells, 250 ng RNA were used to generate libraries using Truseq RNA non-stranded kit (Illumina), which were sequenced in NextSeq 500 platform at NIEHS Epigenomics Core with the sequencing parameter, single end 75 nt reads. For sorted Gli1⁺ and Amhr2⁺ cell from E19.5 uterus, TruSeq Stranded mRNA kit was used to generate libraries that were sequenced by ActiveMotif on the Illumina platform with the sequencing parameter, paired end 75 nt reads.

ATAC-seq

ATAC-seq was performed with Illumina Nextera DNA preparation kit (Illumina, FC-121-1030) according to the previous protocol (89). Two replicates were included in each group. 20,000 sorted tdTomato⁺ cells from Gli1-CreER⁺;Rosa-tdTomato⁺ E14.5 and E16.5 mesonephroi were collected, and permeabilized with ice cold lysis buffer. The transposition reactions were carried out at 37 °C for 30 min in 50 µl volume containing (25 µl 2x TD buffer, 2.5 µl transposase (100 nM final), 16.5 µl PBS, 0.5 µl 1% digitonin, 0.5 µl 10% Tween-20, 5 µl

H₂O). Digested DNA was purified with Zymo DNA Clean and Concentrator-5 Kit (cat# D4014). Library pre-amplification was done with KAPA HiFi HotStart ReadyMixPCR Kit. Thermocycler conditions were 72°C for 5 min, 98°C for 30 sec, followed by 5 cycles of [98°C for 10 sec, 63°C for 30 sec, 72°C for 1 min] then hold at 4°C. The qPCR amplification using 5 ul of pre-amplified mixture was performed to determine additional cycles, which were between 5-6. Final amplification was performed with the remainder of the pre-amplified DNA and final PCR products were purified with Zymo DNA Clean and Concentrator-5 Kit and eluted in 20 µl H₂O. KAPA Pure beads were used to size select 150-450 bp DNA fragments (x0.6 and x1.5 cut). The size selected library was measured in a Cubic II for concentration and in a Bioanalyzer for size distribution, and sequenced in NextSeq 500 platform at NIEHS Epigenomics Core. Sequencing parameter was paired end 2x35 nt reads.

Bioinformatic analyses of next generation sequencing data

For analysis of the RNA-seq datasets, raw sequences were filtered to remove all reads with mean base quality score < 20; for paired-end data, both reads were required to pass this filter. Filtered reads were mapped against the mm10 reference assembly by STAR v2.5 (90) with parameters "--outSAMattrIHstart 0 --outFilterType BySJout --alignSJoverhangMin 8 --limitBAMsortRAM 55000000000 --outSAMstrandField intronMotif --outFilterIntronMotifs RemoveNoncanonical". Counts per gene were determined via featureCounts (Subread v1.5.0-p1) (91) with parameters "-s0" for the unstranded single-end data or "-s2 -Sfr -p" for the stranded paired-end data. Evaluated gene models are GENCODE VM18 annotations, as defined in the wgEncodeGencodeBasicVM18 table downloaded from the UCSC Table Browser (<https://genome.ucsc.edu/cgi-bin/hgTables>) on December 17, 2018. Entries were associated with Entrez gene identifiers when possible. Differential expression analysis was performed with DESeq2 v1.14.1 (92) in R v3.3.2. Differentially expressed gene thresholds were set at FDR 0.05,

fold change 1.5, and minimum average TPM 1. Pathway analysis was performed in Enrichr (93) by job submission at <https://maayanlab.cloud/Enrichr/>. Presented results are from MSigDB Hallmark 2020, with significant pathways identified at adjusted p-value < 0.05. For the E19.5 Gli1+ and Amhr2+ mesenchyme dataset, upstream regulatory analysis was performed according to the (Ingenuity Pathway Analysis) IPA guidelines (94).

For analysis of the ATAC-seq dataset, raw sequences were filtered to remove all pairs with mean base quality score < 20 for either read. Filtered read pairs were mapped against the mm10 reference assembly by Bowtie v1.2 (95) with parameters "-m 1 -X 2000 --chunkmbs 1024". Hits to chrM were discarded via samtools v1.3.1 (96). Duplicate mapped read pairs were removed by Picard tools MarkDuplicates.jar (v1.110) (<http://broadinstitute.github.io/picard>). Downstream analysis considered only the 9bp at the 5' end of each read. Peak calls per sample were made by MACS2 v2.1.1 (97) with parameters "callpeak -g mm -q 0.0001 --keep-dup=all --nomodel --extsize 9", followed by combining nearby peaks via BEDTools v2.24.0 (98) "merge -d 200". From the initial peak sets, a single set of unified peaks was generated to facilitate comparisons across samples. The unified peaks were defined by collapsing peaks from all samples and collecting regions that overlapped a called peak from 2 or more samples. Nearby unified peaks were combined via BEDTools v2.24.0 "merge -d 200", then filtered to require a minimum length of 50bp. ATAC-seq signal per peak was determined with BEDTools v2.24.0 coverage with the "-counts" option. EdgeR v3.16.5 (99) in R v3.3.2 was used to identify differential ATAC-seq signal between sample groups at FDR 0.01. Differential peaks were stratified into TSS proximal or TSS distal subsets based on a distance cutoff of 1Kb. Enriched motif analysis was performed by HOMER v4.10.3 (100) findMotifsGenome.pl with "-size given" after expanding differential peaks to a minimum width of 200bp; results presented here are for queries limited to peaks more than 1Kb from the nearest annotated TSS (distance measured after expansion to minimum 200bp size).

PCA plots were generated with the plotPCA function in DESeq2 v1.14.1, using the default setting of 500 most variant entries (either genes or ATAC-seq peak regions).

Statistical analyses

A minimum of three biological replicates were used in each examined point in lineage tracing, immunofluorescence, immunohistochemistry, and RNA-scope. Quantitative data is presented as mean±SEM and the sample sizes are indicated in figure legends. One way ANOVA test was used for evaluating significant differences among three groups in Figure 4C&4D. Two-tail Student's t test was used for evaluating significant differences between control and ablation groups in supplementary figure 5E. The significance level was set at $p < 0.05$.

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Author Contributions:

FZ performed most of the experiments; SJ performed staining and quantifications of lumen areas and fluorescent signaling in Figure 4 and Supplementary figure 5; FZ and HHCY designed the study, analyzed the data, and wrote the paper; SAG analyzed the next-generation sequencing data and edited the paper.

Competing interests:

Authors declare that they have no competing interests.

Data and materials availability:

All data are available in the main text or the supplementary materials upon reasonable request. RNA-seq and ATAC-seq data have been deposited in the GEO database under the accession code GSE179876.

References:

1. A. Kobayashi, R. R. Behringer, Developmental genetics of the female reproductive tract in mammals. *Nat Rev Genet* **4**, 969-980 (2003).
2. G. Shaw, M. B. Renfree, Wolffian duct development. *Sex Dev* **8**, 273-280 (2014).
3. S. E. Hannema, I. A. Hughes, Regulation of Wolffian duct development. *Horm Res* **67**, 142-151 (2007).
4. R. D. Mullen, R. R. Behringer, Molecular genetics of Mullerian duct formation, regression and differentiation. *Sex Dev* **8**, 281-296 (2014).
5. F. Zhao, H. H. Yao, A tale of two tracts: history, current advances, and future directions of research on sexual differentiation of reproductive tracts. *Biol Reprod* **101**, 602-616 (2019).
6. G. D. Orvis, R. R. Behringer, Cellular mechanisms of Mullerian duct formation in the mouse. *Dev Biol* **306**, 493-504 (2007).
7. Z. Y. Roly, B. Backhouse, A. Cutting, T. Y. Tan, A. H. Sinclair, K. L. Ayers, A. T. Major, C. A. Smith, The cell biology and molecular genetics of Mullerian duct development. *Wiley Interdiscip Rev Dev Biol* **7**, e310 (2018).
8. A. Murashima, B. Xu, B. T. Hinton, Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice. *Asian J Androl* **17**, 749-755 (2015).

- 681 9. A. Fujino, N. A. Arango, Y. Zhan, T. F. Manganaro, X. Li, D. T. MacLaughlin, P. K. Donahoe,
682 Cell migration and activated PI3K/AKT-directed elongation in the developing rat
683 Mullerian duct. *Dev Biol* **325**, 351-362 (2009).
- 684 10. F. Zhao, J. Zhou, R. Li, E. A. Dudley, X. Ye, Novel function of LHFPL2 in female and male
685 distal reproductive tract development. *Sci Rep* **6**, 23037 (2016).
- 686 11. Y. Atsuta, Y. Takahashi, Early formation of the Mullerian duct is regulated by sequential
687 actions of BMP/Pax2 and FGF/Lim1 signaling. *Development* **143**, 3549-3559 (2016).
- 688 12. A. Kobayashi, W. Shawlot, A. Kania, R. R. Behringer, Requirement of Lim1 for female
689 reproductive tract development. *Development* **131**, 539-549 (2004).
- 690 13. M. Jacob, K. Konrad, H. J. Jacob, Early development of the mullerian duct in avian
691 embryos with reference to the human. An ultrastructural and immunohistochemical
692 study. *Cells Tissues Organs* **164**, 63-81 (1999).
- 693 14. M. Chiga, T. Ohmori, T. Ohba, H. Katabuchi, R. Nishinakamura, Preformed Wolffian duct
694 regulates Mullerian duct elongation independently of canonical Wnt signaling or Lhx1
695 expression. *Int J Dev Biol* **58**, 663-668 (2014).
- 696 15. T. J. Carroll, J. S. Park, S. Hayashi, A. Majumdar, A. P. McMahon, Wnt9b plays a central
697 role in the regulation of mesenchymal to epithelial transitions underlying organogenesis
698 of the mammalian urogenital system. *Dev Cell* **9**, 283-292 (2005).
- 699 16. W. J. Dyche, A comparative study of the differentiation and involution of the Mullerian
700 duct and Wolffian duct in the male and female fetal mouse. *J Morphol* **162**, 175-209
701 (1979).
- 702 17. A. Crocoll, C. C. Zhu, A. C. Cato, M. Blum, Expression of androgen receptor mRNA during
703 mouse embryogenesis. *Mech Dev* **72**, 175-178 (1998).
- 704 18. A. Murashima, S. Miyagawa, Y. Ogino, H. Nishida-Fukuda, K. Araki, T. Matsumoto, T.
705 Kaneko, K. Yoshinaga, K. Yamamura, T. Kurita, S. Kato, A. M. Moon, G. Yamada, Essential
706 roles of androgen signaling in Wolffian duct stabilization and epididymal cell
707 differentiation. *Endocrinology* **152**, 1640-1651 (2011).
- 708 19. I. Barsoum, H. H. Yao, Redundant and differential roles of transcription factors Gli1 and
709 Gli2 in the development of mouse fetal Leydig cells. *Biol Reprod* **84**, 894-899 (2011).
- 710 20. H. L. Franco, H. H. Yao, Sex and hedgehog: roles of genes in the hedgehog signaling
711 pathway in mammalian sexual differentiation. *Chromosome Res* **20**, 247-258 (2012).
- 712 21. M. H. Little, J. Brennan, K. Georgas, J. A. Davies, D. R. Davidson, R. A. Baldock, A.
713 Beverdam, J. F. Bertram, B. Capel, H. S. Chiu, D. Clements, L. Cullen-McEwen, J. Fleming,
714 T. Gilbert, D. Herzlinger, D. Houghton, M. H. Kaufman, E. Kleymenova, P. A. Koopman, A.
715 G. Lewis, A. P. McMahon, C. L. Mendelsohn, E. K. Mitchell, B. A. Rumballe, D. E. Sweeney,
716 M. T. Valerius, G. Yamada, Y. Yang, J. Yu, A high-resolution anatomical ontology of the
717 developing murine genitourinary tract. *Gene Expr Patterns* **7**, 680-699 (2007).
- 718 22. N. A. Arango, A. Kobayashi, Y. Wang, S. P. Jamin, H. H. Lee, G. D. Orvis, R. R. Behringer, A
719 mesenchymal perspective of Mullerian duct differentiation and regression in Amhr2-lacZ
720 mice. *Mol Reprod Dev* **75**, 1154-1162 (2008).
- 721 23. R. R. Behringer, R. L. Cate, G. J. Froelick, R. D. Palmiter, R. L. Brinster, Abnormal sexual
722 development in transgenic mice chronically expressing mullerian inhibiting substance.
723 *Nature* **345**, 167-170 (1990).
- 724 24. A. Dean, L. B. Smith, S. Macpherson, R. M. Sharpe, The effect of dihydrotestosterone
725 exposure during or prior to the masculinization programming window on reproductive
726 development in male and female rats. *Int J Androl* **35**, 330-339 (2012).

25. M. B. Renfree, J. Fenelon, G. Wijayanti, J. D. Wilson, G. Shaw, Wolffian duct differentiation by physiological concentrations of androgen delivered systemically. *Dev Biol* **334**, 429-436 (2009).

26. F. M. Schultz, J. D. Wilson, Virilization of the Wolffian duct in the rat fetus by various androgens. *Endocrinology* **94**, 979-986 (1974).

27. L. Santana Gonzalez, I. A. Rota, M. Artibani, M. Morotti, Z. Hu, N. Wietek, A. Alsaadi, A. Albukhari, T. Sauka-Spengler, A. A. Ahmed, Mechanistic Drivers of Mullerian Duct Development and Differentiation Into the Oviduct. *Front Cell Dev Biol* **9**, 605301 (2021).

28. A. M. Kelleher, F. J. DeMayo, T. E. Spencer, Uterine Glands: Developmental Biology and Functional Roles in Pregnancy. *Endocr Rev* **40**, 1424-1445 (2019).

29. P. S. Cooke, T. E. Spencer, F. F. Bartol, K. Hayashi, Uterine glands: development, function and experimental model systems. *Mol Hum Reprod* **19**, 547-558 (2013).

30. K. De Clercq, A. Hennes, J. Vriens, Isolation of Mouse Endometrial Epithelial and Stromal Cells for In Vitro Decidualization. *J Vis Exp*, (2017).

31. Y. Gao, K. J. Bayless, Q. Li, TGFB1 is required for mouse myometrial development. *Mol Endocrinol* **28**, 380-394 (2014).

32. A. Jost, *Recherches Sur La Differentiation Sexuelle De L'embryon De Lapin .1. Introduction Et Embryologie Genitale Normale. *Arch Anat Microsc Mo* **36**, 151-200 (1947).

33. A. Jost, Problems of Fetal Endocrinology - the Gonadal and Hypophyseal Hormones. *Recent Prog Horm Res* **8**, 379-418 (1953).

34. M. Welsh, P. T. Saunders, R. M. Sharpe, The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology* **148**, 3185-3195 (2007).

35. S. L. Klemm, Z. Shipony, W. J. Greenleaf, Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet* **20**, 207-220 (2019).

36. M. Kanamori, H. Konno, N. Osato, J. Kawai, Y. Hayashizaki, H. Suzuki, A genome-wide and nonredundant mouse transcription factor database. *Biochem Biophys Res Commun* **322**, 787-793 (2004).

37. S. Maezawa, M. Yukawa, K. G. Alavattam, A. Barski, S. H. Namekawa, Dynamic reorganization of open chromatin underlies diverse transcriptomes during spermatogenesis. *Nucleic Acids Res* **46**, 593-608 (2018).

38. J. Wu, B. Huang, H. Chen, Q. Yin, Y. Liu, Y. Xiang, B. Zhang, B. Liu, Q. Wang, W. Xia, W. Li, Y. Li, J. Ma, X. Peng, H. Zheng, J. Ming, W. Zhang, J. Zhang, G. Tian, F. Xu, Z. Chang, J. Na, X. Yang, W. Xie, The landscape of accessible chromatin in mammalian preimplantation embryos. *Nature* **534**, 652-657 (2016).

39. F. M. Bentvelsen, A. O. Brinkmann, P. van der Schoot, J. E. van der Linden, T. H. van der Kwast, W. J. Boersma, F. H. Schroder, J. M. Nijman, Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol* **113**, 245-253 (1995).

40. C. Y. Hong, E. Y. Gong, K. Kim, J. H. Suh, H. M. Ko, H. J. Lee, H. S. Choi, K. Lee, Modulation of the expression and transactivation of androgen receptor by the basic helix-loop-helix transcription factor Pod-1 through recruitment of histone deacetylase 1. *Mol Endocrinol* **19**, 2245-2257 (2005).

41. E. M. Snyder, C. L. Small, D. Bomgardner, B. Xu, R. Evanoff, M. D. Griswold, B. T. Hinton, Gene expression in the efferent ducts, epididymis, and vas deferens during embryonic development of the mouse. *Dev Dyn* **239**, 2479-2491 (2010).

- 774 42. A. S. Al Madhoun, A. Voronova, T. Ryan, A. Zakariyah, C. McIntire, L. Gibson, M. Shelton,
775 M. Ruel, I. S. Skerjanc, Testosterone enhances cardiomyogenesis in stem cells and
776 recruits the androgen receptor to the MEF2C and HCN4 genes. *J Mol Cell Cardiol* **60**, 164-
777 171 (2013).
- 778 43. J. F. Couse, K. S. Korach, Estrogen receptor null mice: what have we learned and where
779 will they lead us? *Endocr Rev* **20**, 358-417 (1999).
- 780 44. D. B. Lubahn, J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach, O. Smithies, Alteration of
781 reproductive function but not prenatal sexual development after insertional disruption of
782 the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* **90**, 11162-11166 (1993).
- 783 45. M. A. Laing, S. Coonrod, B. T. Hinton, J. W. Downie, R. Tozer, M. A. Rudnicki, J. A. Hassell,
784 Male sexual dysfunction in mice bearing targeted mutant alleles of the PEA3 ets gene.
785 *Mol Cell Biol* **20**, 9337-9345 (2000).
- 786 46. S. Verbeek, D. Izon, F. Hofhuis, E. Robanus-Maandag, H. te Riele, M. van de Wetering, M.
787 Oosterwegel, A. Wilson, H. R. MacDonald, H. Clevers, An HMG-box-containing T-cell
788 factor required for thymocyte differentiation. *Nature* **374**, 70-74 (1995).
- 789 47. L. Levy, C. S. Hill, Smad4 dependency defines two classes of transforming growth factor
790 {beta} (TGF- β) target genes and distinguishes TGF- β -induced epithelial-
791 mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol*
792 **25**, 8108-8125 (2005).
- 793 48. M. Abe, I. Michikami, T. Fukushi, A. Abe, Y. Maeda, T. Ooshima, S. Wakisaka, Hand2
794 regulates chondrogenesis in vitro and in vivo. *Bone* **46**, 1359-1368 (2010).
- 795 49. J. Goad, Y. A. Ko, M. Kumar, S. M. Syed, P. S. Tanwar, Differential Wnt signaling activity
796 limits epithelial gland development to the anti-mesometrial side of the mouse uterus.
797 *Dev Biol* **423**, 138-151 (2017).
- 798 50. A. Ivanova, M. Signore, N. Caro, N. D. Greene, A. J. Copp, J. P. Martinez-Barbera, In vivo
799 genetic ablation by Cre-mediated expression of diphtheria toxin fragment A. *Genesis* **43**,
800 129-135 (2005).
- 801 51. C. C. Hui, D. Slusarski, K. A. Platt, R. Holmgren, A. L. Joyner, Expression of three mouse
802 homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-
803 3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during
804 postimplantation development. *Dev Biol* **162**, 402-413 (1994).
- 805 52. F. Zhao, H. L. Franco, K. F. Rodriguez, P. R. Brown, M. J. Tsai, S. Y. Tsai, H. H. Yao,
806 Elimination of the male reproductive tract in the female embryo is promoted by COUP-
807 TFII in mice. *Science* **357**, 717-720 (2017).
- 808 53. D. D'Amours, F. R. Sallmann, V. M. Dixit, G. G. Poirier, Gain-of-function of poly(ADP-
809 ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. *J*
810 *Cell Sci* **114**, 3771-3778 (2001).
- 811 54. G. R. Cunha, Mesenchymal-epithelial interactions: past, present, and future.
812 *Differentiation* **76**, 578-586 (2008).
- 813 55. D. W. Kufe, J. F. Holland, E. Frei, American Cancer Society., *Cancer medicine* 6 (BC Decker,
814 Hamilton, Ont. ; Lewiston, NY, ed. 6th, 2003).
- 815 56. C. J. Bult, J. A. Blake, C. L. Smith, J. A. Kadin, J. E. Richardson, G. Mouse Genome
816 Database, Mouse Genome Database (MGD) 2019. *Nucleic Acids Res* **47**, D801-D806
817 (2019).
- 818 57. D. M. Klotz, S. C. Hewitt, P. Ciana, M. Raviscioni, J. K. Lindzey, J. Foley, A. Maggi, R. P.
819 DiAugustine, K. S. Korach, Requirement of estrogen receptor-alpha in insulin-like growth

factor-1 (IGF-1)-induced uterine responses and in vivo evidence for IGF-1/estrogen receptor cross-talk. *J Biol Chem* **277**, 8531-8537 (2002).

58. J. Baker, M. P. Hardy, J. Zhou, C. Bondy, F. Lupu, A. R. Bellve, A. Efstratiadis, Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol* **10**, 903-918 (1996).

59. J. P. Liu, J. Baker, A. S. Perkins, E. J. Robertson, A. Efstratiadis, Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75**, 59-72 (1993).

60. R. Haraguchi, G. Yamada, A. Murashima, D. Matsumaru, R. Kitazawa, S. Kitazawa, New Insights into Development of Female Reproductive Tract-Hedgehog-Signal Response in Wolffian Tissues Directly Contributes to Uterus Development. *Int J Mol Sci* **22**, (2021).

61. C. C. Huang, G. D. Orvis, Y. Wang, R. R. Behringer, Stromal-to-epithelial transition during postpartum endometrial regeneration. *PLoS One* **7**, e44285 (2012).

62. E. Deutscher, H. Hung-Chang Yao, Essential roles of mesenchyme-derived beta-catenin in mouse Mullerian duct morphogenesis. *Dev Biol* **307**, 227-236 (2007).

63. N. A. Arango, P. P. Szotek, T. F. Manganaro, E. Oliva, P. K. Donahoe, J. Teixeira, Conditional deletion of beta-catenin in the mesenchyme of the developing mouse uterus results in a switch to adipogenesis in the myometrium. *Dev Biol* **288**, 276-283 (2005).

64. W. Winuthayanon, S. L. Lierz, K. C. Delarosa, S. R. Sampels, L. J. Donoghue, S. C. Hewitt, K. S. Korach, Juxtacrine Activity of Estrogen Receptor alpha in Uterine Stromal Cells is Necessary for Estrogen-Induced Epithelial Cell Proliferation. *Sci Rep* **7**, 8377 (2017).

65. X. Guo, S. Y. Chen, Transforming growth factor-beta and smooth muscle differentiation. *World J Biol Chem* **3**, 41-52 (2012).

66. P. S. Cooke, G. C. Ekman, J. Kaur, J. Davila, I. C. Bagchi, S. G. Clark, P. J. Dziuk, K. Hayashi, F. F. Bartol, Brief exposure to progesterone during a critical neonatal window prevents uterine gland formation in mice. *Biol Reprod* **86**, 63 (2012).

67. X. Wang, S. P. Wu, F. J. DeMayo, Hormone dependent uterine epithelial-stromal communication for pregnancy support. *Placenta* **60 Suppl 1**, S20-S26 (2017).

68. H. Wang, S. K. Dey, Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* **7**, 185-199 (2006).

69. J. Tan, B. C. Paria, S. K. Dey, S. K. Das, Differential uterine expression of estrogen and progesterone receptors correlates with uterine preparation for implantation and decidualization in the mouse. *Endocrinology* **140**, 5310-5321 (1999).

70. C. A. Rubel, H. L. Franco, J. W. Jeong, J. P. Lydon, F. J. DeMayo, GATA2 is expressed at critical times in the mouse uterus during pregnancy. *Gene Expr Patterns* **12**, 196-203 (2012).

71. H. D. Saatcioglu, M. Kano, H. Horn, L. Zhang, W. Samore, N. Nagykerly, M. C. Meinsohn, M. Hyun, R. Suliman, J. Poulo, J. Hsu, C. Sacha, D. Wang, G. Gao, K. Lage, E. Oliva, M. E. Morris Sabatini, P. K. Donahoe, D. Pepin, Single-cell sequencing of neonatal uterus reveals an Misr2+ endometrial progenitor indispensable for fertility. *Elife* **8**, (2019).

72. M. L. Mucenski, R. Mahoney, M. Adam, A. S. Potter, S. S. Potter, Single cell RNA-seq study of wild type and Hox9,10,11 mutant developing uterus. *Sci Rep* **9**, 4557 (2019).

73. P. M. Kirkwood, D. A. Gibson, J. R. Smith, J. R. Wilson-Kanamori, O. Kelepouri, A. Esnal-Zufiaurre, R. Dobie, N. C. Henderson, P. T. K. Saunders, Single-cell RNA sequencing redefines the mesenchymal cell landscape of mouse endometrium. *FASEB J* **35**, e21285 (2021).

74. J. G. Lemmen, J. L. Broekhof, G. G. Kuiper, J. A. Gustafsson, P. T. van der Saag, B. van der Burg, Expression of estrogen receptor alpha and beta during mouse embryogenesis. *Mech Dev* **81**, 163-167 (1999).

75. T. L. Greco, J. D. Furlow, T. M. Duello, J. Gorski, Immunodetection of estrogen receptors in fetal and neonatal female mouse reproductive tracts. *Endocrinology* **129**, 1326-1332 (1991).

76. S. Della Torre, G. Rando, C. Meda, P. Ciana, L. Ottobriani, A. Maggi, Transcriptional activity of oestrogen receptors in the course of embryo development. *J Endocrinol* **238**, 165-176 (2018).

77. S. C. Hewitt, Y. Li, L. Li, K. S. Korach, Estrogen-mediated regulation of Igf1 transcription and uterine growth involves direct binding of estrogen receptor alpha to estrogen-responsive elements. *J Biol Chem* **285**, 2676-2685 (2010).

78. S. C. Hewitt, M. Carmona, K. G. Foley, L. J. Donoghue, S. L. Lierz, W. Winuthayanon, K. S. Korach, Peri- and Postpubertal Estrogen Exposures of Female Mice Optimize Uterine Responses Later in Life. *Endocrinology* **161**, (2020).

79. H. Matsumoto, X. Zhao, S. K. Das, B. L. Hogan, S. K. Dey, Indian hedgehog as a progesterone-responsive factor mediating epithelial-mesenchymal interactions in the mouse uterus. *Dev Biol* **245**, 280-290 (2002).

80. V. Medikare, L. R. Kandukuri, V. Ananthapur, M. Deenadayal, P. Nallari, The genetic bases of uterine fibroids; a review. *J Reprod Infertil* **12**, 181-191 (2011).

81. X. Santamaria, A. Mas, I. Cervello, H. Taylor, C. Simon, Uterine stem cells: from basic research to advanced cell therapies. *Hum Reprod Update* **24**, 673-693 (2018).

82. P. Mittal, Y. H. Shin, S. A. Yatsenko, C. A. Castro, U. Surti, A. Rajkovic, Med12 gain-of-function mutation causes leiomyomas and genomic instability. *J Clin Invest* **125**, 3280-3284 (2015).

83. P. S. Tanwar, H. J. Lee, L. Zhang, L. R. Zukerberg, M. M. Taketo, B. R. Rueda, J. M. Teixeira, Constitutive activation of Beta-catenin in uterine stroma and smooth muscle leads to the development of mesenchymal tumors in mice. *Biol Reprod* **81**, 545-552 (2009).

84. S. P. Jamin, N. A. Arango, Y. Mishina, M. C. Hanks, R. R. Behringer, Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nat Genet* **32**, 408-410 (2002).

85. C. Liu, J. Peng, M. M. Matzuk, H. H. Yao, Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat Commun* **6**, 6934 (2015).

86. X. Lin, E. Tomblar, P. J. Nelson, M. Ross, I. H. Gelman, A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. *J Biol Chem* **271**, 28430-28438 (1996).

87. T. D. Littlewood, D. C. Hancock, P. S. Danielian, M. G. Parker, G. I. Evan, A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**, 1686-1690 (1995).

88. O. Gavet, J. Pines, Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. *Dev Cell* **18**, 533-543 (2010).

89. M. R. Corces, A. E. Trevino, E. G. Hamilton, P. G. Greenside, N. A. Sinnott-Armstrong, S. Vesuna, A. T. Satpathy, A. J. Rubin, K. S. Montine, B. Wu, A. Kathiria, S. W. Cho, M. R. Mumbach, A. C. Carter, M. Kasowski, L. A. Orloff, V. I. Risca, A. Kundaje, P. A. Khavari, T. J. Montine, W. J. Greenleaf, H. Y. Chang, An improved ATAC-seq protocol reduces

background and enables interrogation of frozen tissues. *Nat Methods* **14**, 959-962 (2017).

90. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T. R. Gingeras, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).

91. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930 (2014).

92. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).

93. M. V. Kuleshov, M. R. Jones, A. D. Rouillard, N. F. Fernandez, Q. Duan, Z. Wang, S. Koplev, S. L. Jenkins, K. M. Jagodnik, A. Lachmann, M. G. McDermott, C. D. Monteiro, G. W. Gundersen, A. Ma'ayan, Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* **44**, W90-97 (2016).

94. A. Kramer, J. Green, J. Pollard, Jr., S. Tugendreich, Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523-530 (2014).

95. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**, R25 (2009).

96. H. Li, B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, S. Genome Project Data Processing, The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

97. Y. Zhang, T. Liu, C. A. Meyer, J. Eeckhoutte, D. S. Johnson, B. E. Bernstein, C. Nusbaum, R. M. Myers, M. Brown, W. Li, X. S. Liu, Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**, R137 (2008).

98. A. R. Quinlan, I. M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841-842 (2010).

99. M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140 (2010).

100. S. Heinz, C. Benner, N. Spann, E. Bertolino, Y. C. Lin, P. Laslo, J. X. Cheng, C. Murre, H. Singh, C. K. Glass, Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* **38**, 576-589 (2010).

Tamoxifen was injected on E13.5 and E14.5 to permanently label *Gli1*⁺ cells with tdTomato in *Gli1-CreER; Rosa-tdTomato* XX embryos. (A-E) cranial and (F-J) caudal sections of the female reproductive tract from tamoxifen treated females on E14.5 (A & F), E16.5 (B & G), PND0 (C & H), PND21 (D & I) and PND56 (E & J) to visualize *tdTomato*-labeled *Gli1*⁺ cells (Red). All images are oriented with the mesometrial side of the tissues up and the anti-mesometrial side down. Epithelial cells are stained with PAX2 (Cyan). Pink and blue dashed lines circle Müllerian and Wolffian ducts, respectively. Scale bars in sections: 50 µm. N=3 in each time point examined.

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Figure 2. Profiling transcriptome and chromatin accessibility and deducing associated transcription factors in sexual differentiation of the *Gli1*⁺ Wolffian duct mesenchyme. (A) The sexually dimorphic fate of the Wolffian duct mesenchyme in the presence (XY embryos) and absence (XX embryos) of androgens. (B) Principal component analysis (PCA) of the 500 most variant genes in the RNA-seq dataset including E14.5 XX (lighter red), E14.5 XY (lighter blue), E16.5 XX (bright red) and E16.5 XY (bright blue) *Gli1*⁺ cells. N=3 in each group. (C)

962 PCA of the 500 peak regions with the most variant signal in our ATAC-seq dataset. N=2 in
963 each group. **(D & G)** Venn diagrams comparing significantly upregulated genes in XY **(D)** or
964 XX **(G)** on E14.5 and E16.5. **(E & G)** Gene enrichment analyses of the upregulated genes in
965 XY **(E)** (E14.5 XY>E14.5 XX or E16.5 XY>E16.5 XX) and in XX **(G)** (E14.5 XX > E14.5 XY
966 or E16.5 XX > E16.5 XY). **(F & I)** Transcription factors (TF) which are in the list of
967 upregulated genes in XY **(D)** and XX **(G)** and whose motifs are enriched in regions with
968 increased chromatin accessibility in XY **(F)** and XX **(I)** in their comparisons on either E14.5 or
969 E16.5.
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Figure 3. Gene expression similarities and differences between the *Amhr2*⁺ Müllerian tract mesenchyme and *Gli1*⁺ Wolffian duct mesenchyme in the neonatal uterus. (A) Volcano plot displaying differentially expressed genes between *Amhr2*⁺ and *Gli1*⁺ cells in neonatal uterus. N=3 in each group. A maximum value of 100 for $-\log_{10} (p\text{-value})$ is assigned so that all genes are shown in the plot. Differentially expressed gene thresholds for significance and fold change are shown as solid lines. **(B)** The top five upstream regulators enriched in the higher

978 expressed genes in *Amhr2*⁺ and *Gli1*⁺ cells, respectively. (C) Immunofluorescent staining of
 979 AKAP12 in the uterus on E14.5, E16.5 and E18.5; DAPI was used to stain nuclei (Grey). (D)
 980 Immunohistochemical staining of HAND2 in the uterus on E14.5, E16.5 and E18.5; Nuclei (blue)
 981 were stained with hematoxylin. All images in (C & D) are oriented with the mesometrial side
 982 of the tissues up and the anti-mesometrial side down. Pink and blue dashed lines circle
 983 Müllerian and Wolffian ducts, respectively. Scale bars in all sections: 50 µm. N=3 in each time
 984 point examined.

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987 **Figure 4. Partial ablation of the *Gli1*⁺ Wolffian duct mesenchyme stunts the growth of**
988 **female reproductive tracts ex vivo. (A)** Whole mount *tdTomato* and bright-field images of
989 the female reproductive tract with ovaries attached in the control (*Gli1-CreER*⁺; *Rosa-*
990 *tdTomato*⁺), ablation (*Gli1-CreER*⁺; *Rosa-tdTomato*⁺; *Rosa-DTA*) and ablation+IGF1 (*Gli1-*
991 *CreER*⁺; *Rosa-tdTomato*⁺; *Rosa-DTA* with IGF1 treatment) groups after 4-day culture. Scale
992 bars: 1.4 mm. White and black arrows indicate the mesometrial side and Müllerian tract lumen,
993 respectively. **(B)** Detection of *Igf1* in the cranial and caudal sections of the fetal female
994 reproductive tract on E14.5, E16.5 and E18.5 by RNAscope. Pink and blue dashed lines circle
995 Müllerian and Wolffian ducts, respectively. Scale bars: 100 μ m. **(C)** Corrected total
996 fluorescence of cultured female reproductive tracts in control, ablation, and ablation+IGF1
997 groups. NS: No significantly difference. *: P<0.05. **(D)** Lumen areas (mm²) of the fetal female
998 reproductive tracts in the control, ablation, and ablation+IGF1 groups. N=4 for the control and
999 ablation+IGF1 groups; N=5 for the ablation group. *: P<0.05; **: P<0.01; ***: P<0.001.

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Figure 5. The model summarizing the major results on the fates and roles of the *Gli1*⁺ Wolffian duct mesenchyme in reproductive tract development. At the onset of sexual differentiation of reproductive tracts (E14.5 in the mouse), the XX embryo possesses both the Wolffian duct (WD) and the Müllerian duct (MD), which are surrounded by their own mesenchymes, the Wolffian duct mesenchyme (WDM) and Müllerian duct mesenchyme (MDM). The WDM and MDM are localized in the mesometrial and anti-mesometrial side of the mesonephros, respectively. On E16.5, the WD epithelium in the XX embryo degenerates in the absence of androgen while the WD mesenchyme (WDM) is maintained and differentiates into a part of the mesenchymal compartment of the oviduct and uterus on E18.5 (Birth). During the female fate differentiation from E14.5 and E16.5 in the XX embryo, the WDM undergoes cellular remodeling and increases chromatin accessibility in regions enriched with

1013 estrogen response element (ERE+). The WDM also produces IGF1 transiently to facilitate the
 1014 growth of the MD. In the E18.5 uterus, the WDM derived cells reside predominately in the
 1015 mesometrial side with specific expression of AKAP12 and higher TGFB1 signaling; on the
 1016 other hand, the MDM-derived cells are exclusively localized in the anti-mesometrial side with
 1017 specific expression of HAND2 and higher WNT signaling. In the XY embryo, the WDM
 1018 differentiates into smooth muscle (SM) cells from E14.5 to E16.5 under the action of
 1019 androgens. During the male fate differentiation, the WDM in the XY embryo increases
 1020 chromatin accessibility in regions enriched with androgen response element (ARE+) and
 1021 express higher smooth muscle differentiation related transcriptional factors (TFs).