1    **Fadrozole-mediated sex reversal in the embryonic chicken gonad involves a PAX2 positive**

2    **undifferentiated supporting cell state**

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6

7      **Abstract**:

8      Gonadal sex differentiation among vertebrates involves divergent fates of a common groups of

9    progenitor cells present in both presumptive ovaries and testes. The first cell type to differentiate gives

10   rise to pre-Sertoli cells in the testis, and pre-follicular cells in the ovary. These cells derive form a

11   common lineage of so-called "supporting cells". In birds and other egg-laying vertebrates, locally

12   synthesised estrogen has a central role in ovarian development and influence the fate of these supporting

13   cells. Manipulation of estrogen levels during embryonic development induces gonadal sex reversal,

14   providing an experimental setting to evaluate the process of gonadal sex differentiation. Recently, we

15   identified PAX2 as a novel marker of the undifferentiated supporting cell lineage in the chicken embryo,

16   expressed in both sexes prior to overt gonadal sex differentiation. *PAX2* expression is downregulated

17   at the onset of gonadal sex differentiation in both males and females. The analysis of this

18   undifferentiated supporting cell marker, together with Sertoli (male) and pre-granulosa (female) will

19   enhance our understanding of supporting cell differentiation. Here we characterized the supporting cells

20   differentiation process and identified undifferentiated supporting cells in estrogen-mediated sex

21   reversal experiments. Female embryos treated with the aromatase inhibitor fadrozole developed

22   ovotestis, containing pre-granulosa cells, Sertoli cells and PAX2 positive undifferentiated supporting

23   cells. In contrast, male embryos treated with 17β-estradiol showed no $PAX2^+$ undifferentiated gonadal

24   supporting cells. Fadrozole time-course as well as multiple dose analysis suggests that supporting cell

25   transdifferentiation involves a dedifferentiation event into a $PAX2^+$ undifferentiated supporting cell

26   state, followed by a redifferentiation towards the opposite sex lineage.

27

28   **Key Words**: Fadrozole, PAX2, gonad, sex-reversal, estrogen, chicken

## Introduction

29

30    Gonadal sex differentiation describes the process of ovary or testis formation among vertebrate

31    embryos. In most species, the gonads differentiate during embryonic or larval life, or shortly thereafter.

32    Among humans, gonadal and external genital differentiation are sometimes atypical. Differences of

33    Sex Development (DSDs) in humans have an incidence of around 1% of all live births. DSDs occurs

34    when the chromosomal, gonadal or anatomical sex are discordant or ambiguous (1, 2). Gonads exhibit

35    a wide range of phenotypes, from total to partial sex reversal, ovotestis or gonadal dysgenesis (3). In

36    some cases, gonads develop normally, and the external genitalia are ambiguous, as in Androgen

37    Insensitivity (AIS) or CAH (Congenital adrenal hypoplasia) (4, 5). Despite the advances genetic

38    diagnosis, many DSD cases lack a definitive molecular diagnosis (6). All current DSDs diagnostical

39    methods do not provide functional information. This is crucial for understanding the etiology and risks

40    associated with specific DSDs mutations, and for clinical management (7). In recent years, animal

41    models have been very instructive in elucidating the molecular genetics of typical and atypical gonadal

42    sex differentiation (8-12).

43    Comparative analysis of chicken and mouse embryos has demonstrated significant genetic and

44    morphological conservation of gonadal sex differentiation (13, 14). As in human embryos, both chicken

45    and mouse exhibit the same groups of progenitor cell types in the gonad. These are the supporting cells

46    (presumptive Sertoli cells in the testis and granulosa cells in the ovary), steroidogenic cells (presumptive

47    Leydig and theca cells), germ cells (sperm or ova) and other interstitial cells such as vascular

48    progenitors (15-17). This conservation largely extends to the genetic level. *DMRT1*, *AMH* and *SOX9*

49    are expressed and play decisive roles in testicular morphogenesis, while *WNT4*, *RSPO1*, *FOXL2* and

50    *CYP19A1* (aromatase) are important in the ovary (18-21). Therefore, the chicken provides a very

51    tractable model for studying embryonic gonad development (22, 23). In birds and other egg-laying

52    vertebrates, locally synthesised estrogen is required for ovarian development (24-28). The estrogen

53    synthesising enzyme, aromatase, is only expressed in female gonads at the onset of ovarian

54    differentiation (24, 29). Experimental manipulation of estrogen levels during embryonic development

55    has been associated with gonadal sex reversal (30, 31). Inhibition of aromatase enzyme leads to testis

56   or ovotestis formation in genetic females (29, 32-34), while over-expression in genetic males can induce

57   transient ovary formation (35). This model provides an experimental setting to evaluate the process of

58   gonadal sex reversal and gonadal sex diff more broadly.

59      Recently, we identified PAX2 as a novel marker of the undifferentiated supporting cell population

60   in the embryonic chicken gonad. Furthermore, we found that PAX2 is also expressed in the

61   undifferentiated gonads of all major avian lineages (36). In chicken, PAX2 expression is sharply

62   downregulated at the onset of gonadal sex determination in both males and females (15, 36). The

63   identification of this undifferentiated supporting cell marker, together with Sertoli (male) and pre-

64   granulosa (female) markers now allows for a more complete understanding of the timing and nature of

65   supporting cell differentiation.

66      Here we characterize the supporting cells differentiation process, and the prevalence of

67   undifferentiated supporting cells in estrogen-mediated sex reversal experiments. Female embryos

68   treated with the aromatase inhibitor, fadrozole, developed ovotestis, containing pre-granulosa cells,

69   Sertoli cells and PAX2 positive undifferentiated supporting cells. In contrast, male embryos treated

70   with 17β-estradiol showed no PAX2$^+$ undifferentiated gonadal supporting cells. Fadrozole time-course

71   as well as multiple dose analysis suggests that supporting cell transdifferentiation involves a

72   dedifferentiation phase into a PAX2$^+$ undifferentiated supporting cell state, followed by a

73   redifferentiation towards the male (Sertoli) lineage.

74

## Materials and Methods

### Chicken eggs

77      Fertilized *Gallus gallus domesticus* HyLine Brown eggs were obtained from Research Poultry

78   Farm (Victoria, Australia) and incubated at 37.5°C under humid conditions. Embryos were staged

79   according to according to Hamburger and Hamilton (37). Experiments were performed in accordance

80   with our institutional animal ethics requirements (Approved Monash University AEC #17643).

81

### Fadrozole treatment

83    Eggs were injected at E3.5 (Hamburger and Hamilton stage HH19) (37) with 1mg of fadrozole

84    (Novartis) in 100 µL of PBS or vehicle, as described previously (38). Embryonic urogenital systems

85    were collected at E6.5 (HH30), E9.5 (HH35) or E12.5 (HH38). For double injection experiments, eggs

86    were injected at E3.5 and at E6.5 with 1 mg of fadrozole in 100 µl of PBS or vehicle. Tissues were

87    collected at E9.5.

88

89    **17β-estradiol treatment**

90    Eggs were injected at E3.5 (HH19) with 0.1 mg of 17β-estradiol in 10% ethanol in sesame oil or

91    vehicle, as described previously (38). Tissues were collected at E9.5 (HH35).

92

93    **Sexing PCR**

94    Genetic sexing was performed by PCR, as described previously (39). Genetic females

95    (chromosomally ZW) were identified by the presence of a female-specific (W-linked) XhoI repeat

96    sequence in addition to a 18S ribosomal gene internal control. Genetic males (chromosomally ZZ)

97    showed the 18S band only (39).

98

99    **qRT-PCR**

100    Quantitative RT-PCR (qRT-PCR) was performed as reported before (36). Briefly, E9.5 (HH35)

101    gonadal pairs were collected and snap frozen in dry ice. After PCR sexing, 3 same-sex gonadal pairs

102    from the same treatment were pooled for each sample, homogenized and RNA extracted using 1mL

103    TRIzol as per the manufacturer's instructions, (TRIzol, ThermoFisher). Genomic DNA was removed

104    using DNA-free™ DNA Removal Kit (Invitrogen) and 1 µg of total RNA was reversed transcribed into

105    cDNA using Promega Reverse Transcription System (A3500). RT-qPCR was performed using the

106    QuantiNova SYBR® Green PCR Kit. *PAX2* expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method

107    using β-actin as internal control. PCR primers were: *PAX2* Fw: GGCGAGAAGAGGAAACGTGA,

108    *PAX2* Rv: GAAGGTGCTTCCGCAAACTG, *β-actin* Fw: CTCTGACTGACCGCGTTACT and *β-*

109    *actin* Rv: TACCAACCATCACACCCTGAT. Data was analysed using two-way ANOVA.

110    Significance was determined using Tukey posttest.

111

112 **Immunofluorescence**

113     Immunofluorescence of frozen sections was performed as reported previously (15). Briefly,

114 gonadal samples were fixed in 4% paraformaldehyde/PBS for 15 minutes, cryoprotected in 30% sucrose

115 in PBS overnight, embedded in OCT embedding compound and snap frozen at -80°C. 10µm gonadal

116 cryosections were permeabilized with 1% Triton X-100 in 1X PBS for 10 minutes, blocked in 2% BSA

117 in 1X PBS for 1 hour, incubated overnight at 4°C with the primary antibody in 1% BSA in 1X PBS.

118 Primary antibodies used: rabbit anti-PAX2 (Biolegend 901001, 1;500), rabbit anti-DMRT1 (in house

119 antibody; 1:2000), rabbit anti-SOX9 (Millipore AB5535, 1:4000), rabbit anti-Aromatase (in house

120 antibody; 1:4000) and rabbit anti-AMH (Abexa ABX132175; 1:1000). Samples were washed and

121 incubated with the secondary antibody Alexa Fluor 488 donkey anti-rabbit (1:1000) in 1% BSA in 1X

122 PBS. Samples were counterstained with DAPI and mounted in Fluorsave (Millipore). Images were

123 collected on a Zeiss Axio Imager A1 microscope using a Zeiss Axiocam MRc5 camera, using the same

124 exposure time between treatments for expression comparisons. For PAX2 immunostaining, antigen

125 retrieval was performed using the Dako PT Link automated system.

126

127 # Results

128 *PAX2 expression is induced upon fadrozole mediated masculinization*

129     In the chicken embryos, a typical genetic female develops a large ovary, characterised by thickened

130 outer cortex and vacuolated underlying medulla (32, 33). Treatment with a single dose of the aromatase

131 inhibitor Fadrozole results in gonads lacking a cortex and developing testicular cords rather that lacuna

132 in the medulla (33). Here, embryonic day 3.5 (E3.5) chicken eggs were injected with the Fadrozole or

133 vehicle solution (Fig. 1A). Embryos were collected at E9.5 (Fig. 1A), genotypically sexed and *PAX2*

134 gonadal mRNA expression was measured by qRT-PCR. *PAX2* mRNA levels were significantly higher

135 in females treated with fadrozole (FAD), compared with the vehicle control (Fig. 1B).

136     In males, *PAX2* expression levels remained unchanged upon treatment (Fig. 1B). To validate the

137 qRT-PCR results, immunofluorescence against PAX2, aromatase (female marker) and male markers

138   SOX9 and DMRT1 was performed in E9.5 female (ZW) gonads treated with fadrozole (FAD) or vehicle

139   (Control). As expected, control left ovaries were larger, expressing aromatase in the medulla but no

140   PAX2, SOX9 or DMRT1 (Fig. 1C). In contrast, fadrozole-treated female left gonads lacked a cortical

141   compartment, and both male (SOX9) and female (aromatase) positive supporting cells co-existed in the

142   same gonad, but in separated defined regions (Fig. 1C). Aromatase positive cells were located in the

143   apical region of the gonad whereas SOX9 positive cells were detected more basally, adjacent to the to

144   the mesonephric kidney. Testis-associate DMRT1 was up-regulated throughout the medulla. PAX2$^+$

145   cells were identified in the gonadal medulla, between SOX9 and aromatase positive supporting cells

146   (Fig. 1C). Taking all together, these results indicate that fadrozole mediated masculinization results in

147   an increase in gonadal PAX2$^+$ expression among cells of the female gonad. Based on our previous data,

148   showing PAX2 to be a marker of prior to differentiation, these cells are interpreted to be undifferentiated

149   supporting cells.

150

151   *PAX2 expression is not induced upon estrogen-induced feminization*

152      To evaluate if PAX2 positive undifferentiated cells were also present in estradiol-mediated male

153   to female sex reversed gonads, E3.5 chicken eggs were injected with 17β-estradiol (E2) or vehicle

154   solution (Fig. 2A). Embryos were collected at E9.5 (Fig. 2A), genotypically sexed and *PAX2* expression

155   was measured by qRT-PCR. No significant differences were found in *PAX2* mRNA expression levels,

156   between treated (E2) and control gonads in both sexes (Fig. 2B). PAX2 Immunofluorescence was

157   performed on E9.5 male (ZZ) gonads treated with 17β-estradiol or vehicle, but no PAX2 positive cells

158   were detected in the gonadal medulla (Fig. 2C), consistent with the qRT-PCR results. To evaluate if, in

159   fact, the gonads were sex reversed, aromatase, SOX9 and DMRT1 protein expression were evaluated

160   by immunofluorescence. As expected, control male gonads showed no aromatase expression and

161   medullary expression of SOX9 and DMRT1 (Fig. 2C). In contrast, E2 treated gonads showed higher

162   ectopic activation of aromatase and lower levels of the male markers, SOX9 and DMRT1 in the medulla

163   (Fig. 2C). Additionally, DMRT1 positive germ cells were detected in the cortical region of the gonad,

164   as is seen in typical left ovaries. These results indicate that 17β-estradiol mediated sex reversal did not

165   induce PAX2 positive undifferentiated supporting cells at E9.5.

166    *PAX2 expression is not altered in E6.5 and E12.5 in fadrozole mediated masculinization*

167    To evaluate if fadrozole treatment in ZW embryos resulted in an increase of undifferentiated

168    supporting cells throughout gonadal development, fadrozole was injected in E3.5 eggs and gonads were

169    collected at E6.5 and E12.5 (Fig. 3A). Samples were genetically sexed and ZW gonads were

170    immunostained for male (SOX9 and DMRT1), female (aromatase) and undifferentiated (PAX2)

171    supporting cell markers. At E6.5, PAX2 was detected in both control and FAD-treated embryos. Some

172    numbers of Aromatase$^+$ cells were also detected, indicating the onset of ovarian differentiation.

173    However, the testis determining factor DMRT1, showed clear upregulation in the female gonad treated

174    with fadrozole aromatase inhibitor. By E12.5, PAX2 expression had been extinguished suggesting that

175    by this developmental stage, all the supporting cells committed to a pre-granulosa or Sertoli cell fate

176    (Fig. 3C). Control gonads showed a typical ovarian structure, with aromatase$^+$ vacuolated medulla and

177    an enlarged cortex, containing DMRT1 positive germ cells, and an aromatase positive medulla (Fig.

178    3C). As expected, E12.5 fadrozole treated gonads lacked a properly defined cortex (Fig. 3C). Aromatase

179    positive pre-granulosa cells located extensively throughout the gonad, whereas SOX9$^+$ DMRT1$^+$,

180    presumably Sertoli cells, are in the basal region of the gonad (Fig. 3C (Fig. 1C). This data suggests that

181    by E12.5, fadrozole treated gonads at E3.5 reverted to an ovarian phenotype.

182

183    *PAX2 undifferentiated supporting cells are lost upon booster injection of fadrozole*

184    As fadrozole treatment at E3.5 only results in changes in PAX2 expression pattern at E9.5, but not

185    in E6.5 or E12.5, we wanted to know if those PAX2 undifferentiated cells were a consequence of

186    fadrozole decay over time. To address this question, a double dose experiment was performed, where

187    eggs were injected at E3.5 and E6.5 with vehicle solution (control) or fadrozole, generating 4 different

188    conditions (Fig. 4A). Embryos were collected at E9.5 and immunofluorescence for AMH, SOX9,

189    DMRT1, PAX2 and aromatase was performed (Fig. 4B). As expected, control ovaries expressed

190    aromatase but no PAX2, SOX9, DMRT1 or AMH. Similar to the results from figure 1C, a single dose

191    of fadrozole at E3.5 resulted in gonads expressing male supporting markers (DMRT1, SOX9, AMH),

192    female markers (aromatase) and undifferentiated PAX2 positive cells (Fig. 4B). In contrast, single dose

193    of fadrozole at E6.5 resulted in no PAX2 expression in the gonads, but both Sertoli and pre-granulosa

194    cells coexisting in the gonadal medulla (Fig. 4B). These results indicate that PAX2 upregulation is not

195    a direct result of fadrozole treatment, and that the timing of fad administration is important. Moreover,

196    a similar phenotype was seen in gonads treated with double doses of fadrozole at E3.5 and E6.5 (Fig.

197    4B). Despite both male and female markers coexisted in the gonadal mesenchyme, no PAX2 expression

198    was detected (Fig. 4B). This suggests that despite one dose at E3.5 results in PAX2 expression, a booster

199    dose at E6.5 inhibits PAX2 expression at E9.5. Taking all together, these results suggests that the

200    undifferentiated supporting cells present at E9.5 are not a direct result of fadrozole injection. Instead,

201    this PAX2 undifferentiated population could be a result of the decay or metabolism of fadrozole at E9.5.

202    The lack of PAX2 positive cells in the gonadal medulla when a booster dose of fadrozole is injected

203    E6.5 supports with this idea. The lack of functional fadrozole at E9.5 results in disinhibition of

204    aromatase and the production of estrogen. Estrogen then could induce the differentiation into pre-

205    granulosa cells while inhibiting Sertoli differentiation. The upregulation of PAX2 suggests that Sertoli

206    to pre-granulosa trans-differentiation involves a dedifferentiation into undifferentiated PAX2$^+$

207    supporting cells, followed by a redifferentiation towards pre-granulosa cells.

208

## Discussion

210    Gonadal sex reversal occurs when there is a discordance between the genetic/chromosomic and

211    gonadal sex (40). Despite the genetic sex is determined at fertilization, the gonads are sexually

212    determined later during embryonic development (6, 41). Initially, both male and female gonads develop

213    similarly (42). These undifferentiated gonads have the potential of becoming a testis or an ovary,

214    depending on the genetic or environmental signals they receive (43). Among the genetic signals, sex

215    chromosome linked genes like *SRY* in humans or *DMRT1* in birds are sufficient and necessary for

216    testicular development (44-46). Misexpression of those genes in chromosomal females results in

217    testicular development (19, 47). Additionally, *SRY* translocation from the Y to the X chromosome

218    during male meiosis is the cause of the majority of the 46,XX DSD cases (48).

219    Among the estrogen plays a strong role in ovarian differentiation. Modulation of estrogen levels

220    resulted in gonadal feminization in birds, reptiles, and eutherian mammals (25, 29, 31, 49-51). In

221 placental mammals such as mice, estrogen seems to have a role in gonadal maintenance, as estrogen

222 receptor α and β double knock out result in postnatal gonadal sex reversal (52). In birds, exogenous

223 modulation of estrogen levels can influence the embryonic gonadal fate, independent of the genetic sex.

224 Injection of fadrozole, an estrogen synthesis inhibitor, in female embryos results in testicular

225 development, upregulating *DMRT1* and *AMH* and downregulating *FOXL2* and *aromatase* (29, 34, 38,

226 53). In some cases, these gonads reverted to ovotestis, upregulating aromatase at around day 7 to 9 of

227 development (32, 38, 54). Our results agree with these reports, showing both aromatase and SOX9

228 expression in the same gonads at E9.5 and E12.5, upon fadrozole treatment (Fig. 1C and 3C).

229 Surprisingly, aromatase-expressing embryonic pre-granulosa cells were confined to the apical region

230 of the gonad whereas the SOX9 positive Sertoli cells were present at the base of the gonad, suggesting

231 that some cell populations are more susceptible to redifferentiation. Two main Sertoli cell types were

232 identified in chicken embryonic testis (15). One population located in the most apical region of the

233 gonad expressing low levels of Sertoli markers *SOX9* and *DMRT1*, and the other located more basally,

234 expressing higher levels of *SOX9* and *DMRT1* (15). *DMRT1* is required to maintain the Sertoli cell

235 identity in testis (46, 55, 56). Lower *DMRT1* expression in the apical region could explain the higher

236 susceptibility of these supporting cells to transdifferentiate. On the other hand, a higher sensitivity to

237 estrogens could be also responsible to this phenomenon, although this remains unexplored. Further

238 research is required to evaluate why the apical supporting cell population is more sensitive to

239 transdifferentiation than the basal one.

240 The process of supporting cell re-differentiation coincides with the upregulation of PAX2 in the

241 gonads, a marker only expressed in undifferentiated supporting cells (15, 36). This suggests that Sertoli

242 cells should revert into a more undifferentiated or bipotential state before they re-differentiate into pre-

243 granulosa cells. These PAX2$^+$ "undifferentiated" supporting cells are located in between the SOX9$^+$

244 Sertoli and the aromatase positive pre-granulosa cells. In transdifferentiating adult murine gonads, cells

245 expressing both FOXL2 and SOX9 are detected, suggesting the presence of intermediate double

246 positive cell states (55). Further research is required to confirm that if in fact these are three independent

247 populations or if double or triple positive cells are detected. PAX2, SOX9 and aromatase or FOXL2

248 immunolabelling in the same gonadal sections will be crucial to address these inquiries.

249    It is curious that PAX2 was upregulated only during fadrozole mediated but not in estrogen

250    mediated sex reversal. This could suggest that female to male sex reversal occurs differently than male

251    to female sex reversal, perhaps due to the early elevated expression of DMRT1 is males that prevents

252    such de-differentiation. Another possibility is that we missed the PAX2 expression / undifferentiated

253    window in our time series. In fadrozole mediated sex reversal, PAX2 was differentially expressed at

254    E9.5 but not at E6.5 or E12.5. This suggest that this process occurs only at a short period of time. Only

255    E9.5 gonads were evaluated in 17β-estradiol mediated sex reversal. Further research should expand our

256    analysis to other embryonic stages before and after E9.5 to elucidate if pre-granulosa to Sertoli re-

257    differentiation also involves an undifferentiated PAX2$^+$ state. Similarly, intermediate states between

258    E6.5 and E12.5 in fadrozole mediated sex reversal are required to precisely define the timeframe of this

259    dedifferentiation and redifferentiation process. This would expand our knowledge in this poorly studied

260    mechanism.

261    The chicken model is an ideal system to study genetic, environmental, and hormonal factors that

262    control normal or abnormal gonadal differentiation. Estrogenic mediated sex reversal provides a

263    valuable tool to model and study DSDs, especially partial sex reversion and ovotestis development.

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415  secondary sex characteristics. Proc Natl Acad Sci U S A. 2021;118(10).
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417

418 **Figure legends**

419    **Fig 1. PAX2 expression is induced upon fadrozole mediated masculinization**. (A) Schematic

420    figure of the experimental plan. Fadrozole or vehicle solution was injected in chicken eggs at E3.5.

421    Samples were collected at E9.5. (B) *PAX2* qRT-PCR was performed in E9.5 gonadal samples of

422    fadrozole (FAD) or vehicle (Control) treated embryos. Expression level is relative to β-actin and

423    normalized to the male control. Bars represent mean±s.e.m., n=6. * adjusted P<0.05. 2-way ANOVA

424    and Tukey's post-test. (C) Immunofluorescence against PAX2, aromatase, SOX9 and DMRT1 in E9.5

425    female (ZW) gonads treated with fadrozole (FAD) or vehicle solution (Control). White arrows indicate

426    positive cells.

427

428    **Fig 2. PAX2 expression is not induced upon estrogen-induced feminization**. (A) Schematic

429    figure of the experimental plan. 17β-estradiol or vehicle solution was injected in chicken eggs at E3.5.

430    Samples were collected at E9.5. (B) *PAX2* qRT-PCR was performed in E9.5 gonadal samples of 17β-

431    estradiol (E2) or vehicle (Control) treated embryos. Expression level is relative to β-actin and

432    normalized to the male control. Bars represent mean±s.e.m., n=6. 2-way ANOVA and Tukey's post-

433    test. (C) Immunofluorescence against PAX2, aromatase, SOX9 and DMRT1 in E9.5 male (ZZ) gonads

434    treated with 17β-estradiol (E2) or vehicle solution (Control). White arrows indicate positive cells.

435

436    **Fig 3. PAX2 expression is not altered in E6.5 and E12.5 in fadrozole mediated**

437    **masculinization**. (A) Schematic figure of the experimental plan. Fadrozole or vehicle solution was

438    injected in chicken eggs at E3.5. Samples were collected at E6.5 or E12.5. (B) Immunofluorescence

439    against PAX2, aromatase, SOX9 and DMRT1 in E6.5 female (ZW) gonads treated with fadrozole

440    (FAD) or vehicle solution (Control). (C) Immunofluorescence against PAX2, aromatase, SOX9 and

441    DMRT1 in E12.5 female (ZW) gonads treated with fadrozole (FAD) or vehicle solution (Control).
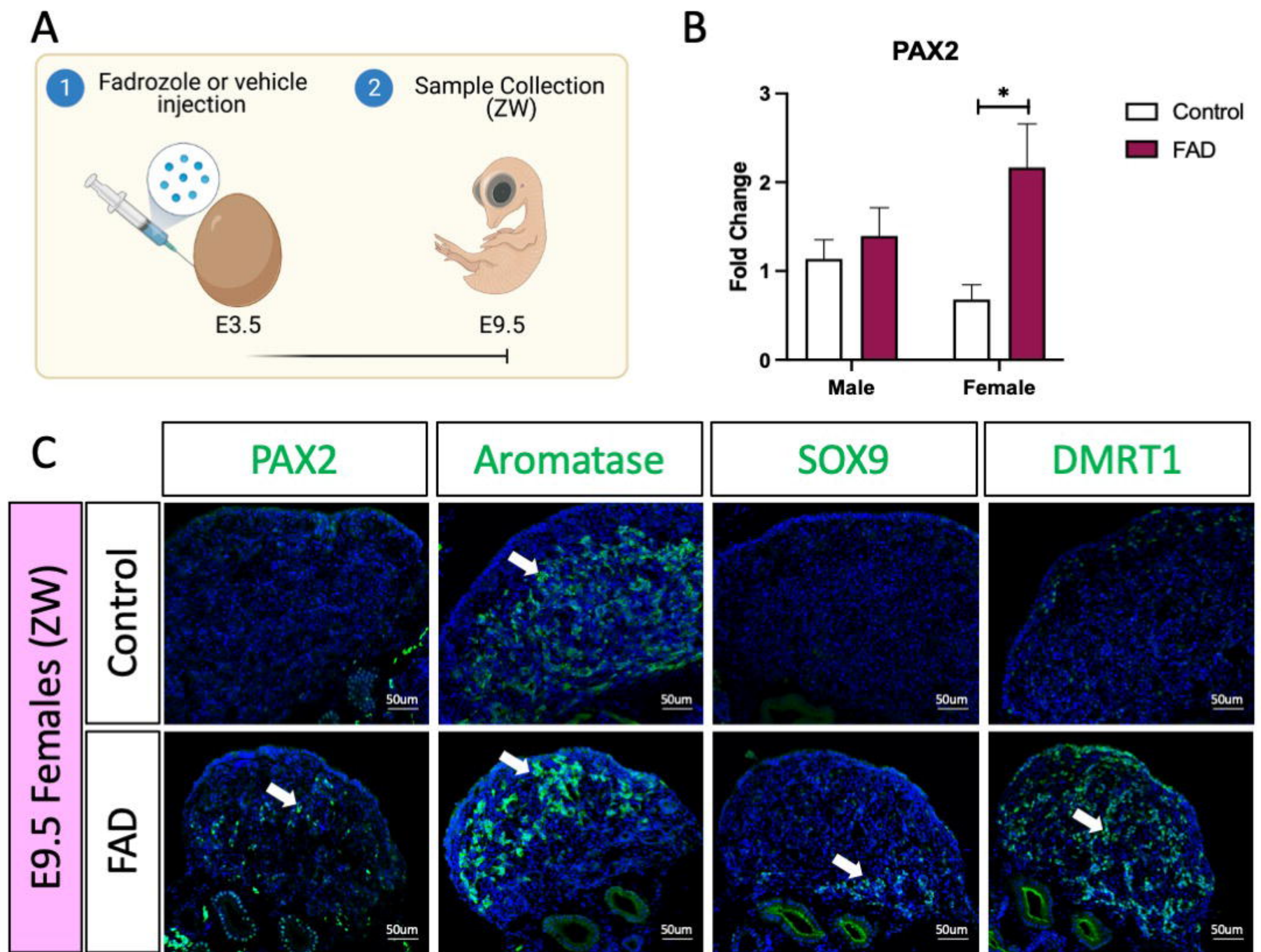
442    White arrows indicate positive cells.

443

444        **Fig 4. PAX2 undifferentiated supporting cells are lost upon booster injection of fadrozole**.

445     (A) Schematic figure of the experimental plan. Fadrozole or vehicle solution was injected in chicken

446     eggs at E3.5 and E6.5. Samples were collected at E9.5. (B) Immunofluorescence against PAX2,

447     aromatase, SOX9 and DMRT1 and AMH in E9.5 female (ZW) gonads treated with fadrozole or vehicle

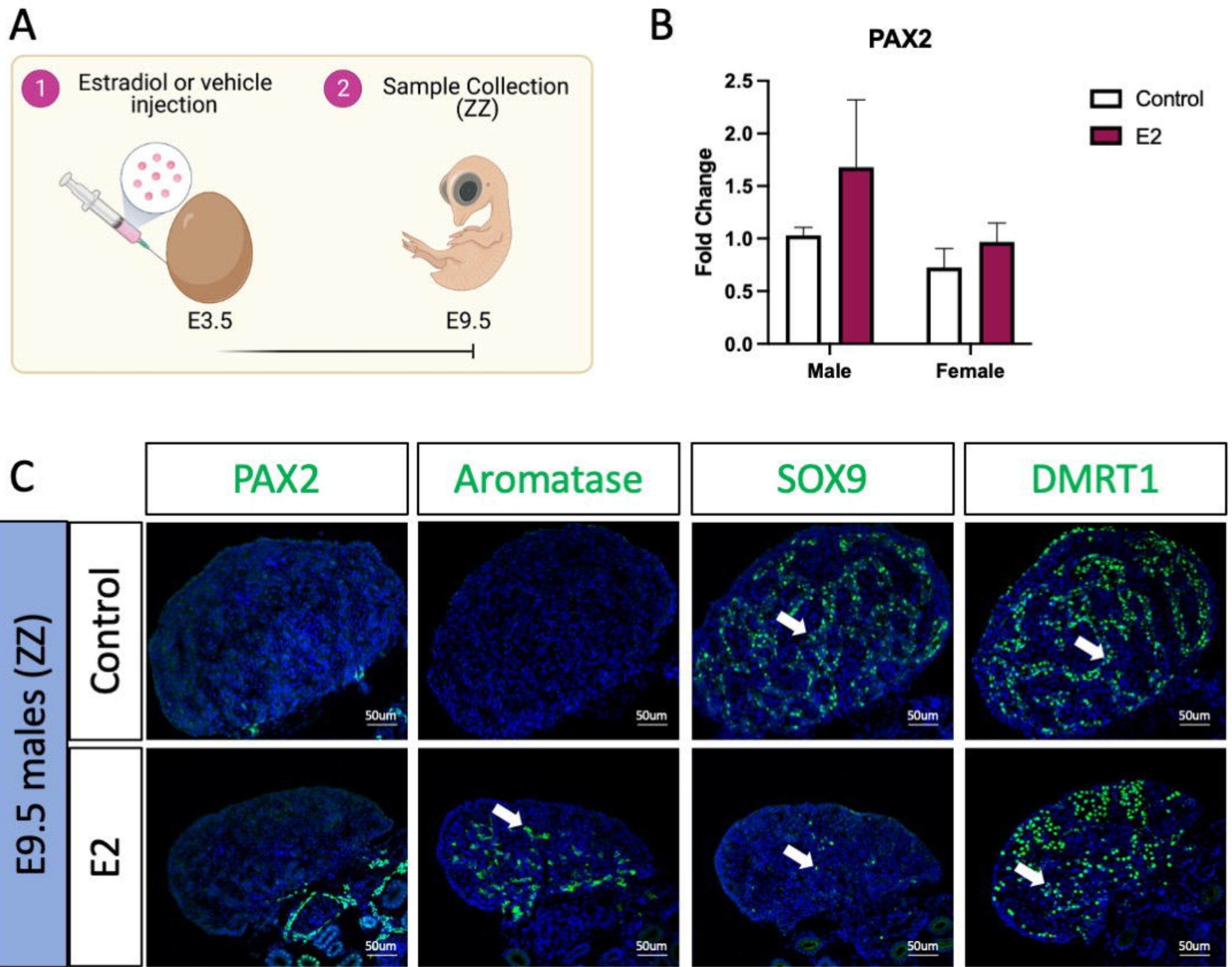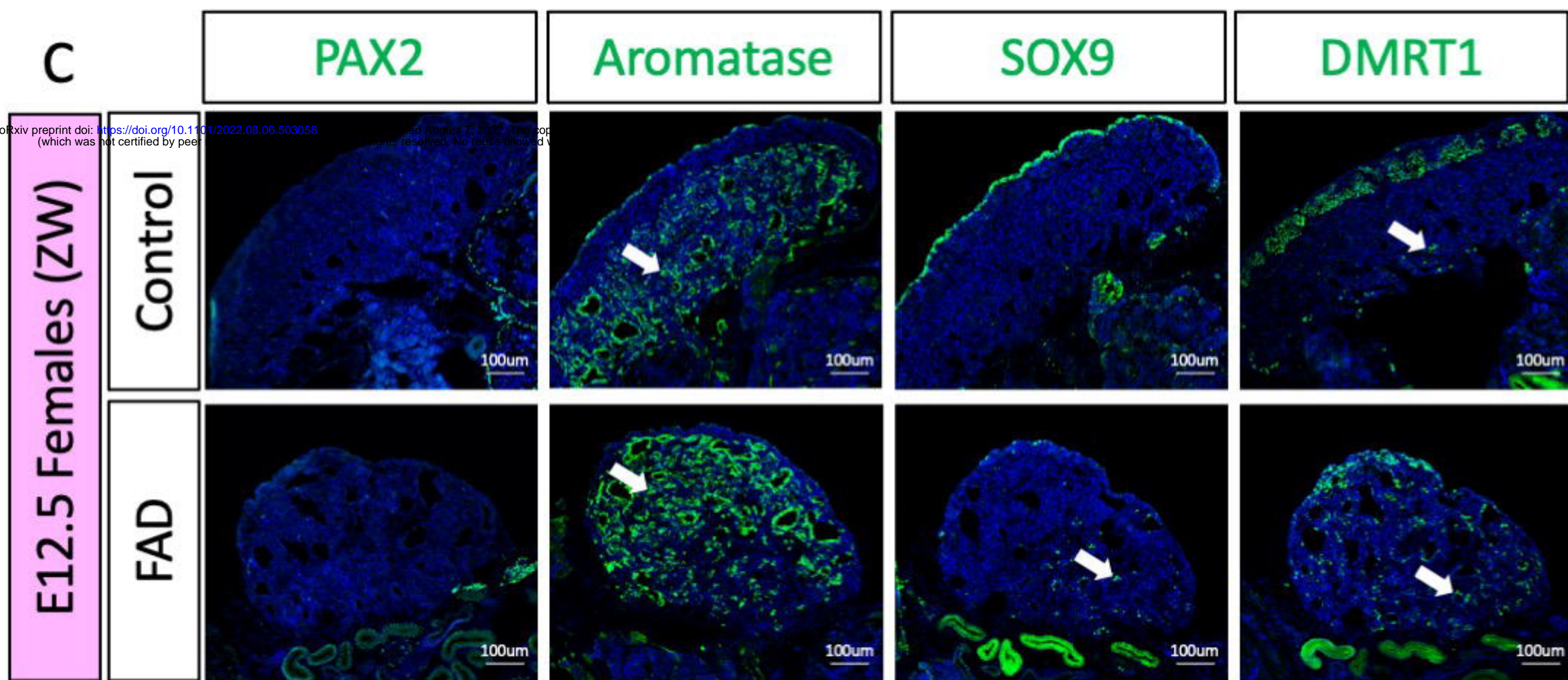448     solution (Control). White arrows indicate positive cells.

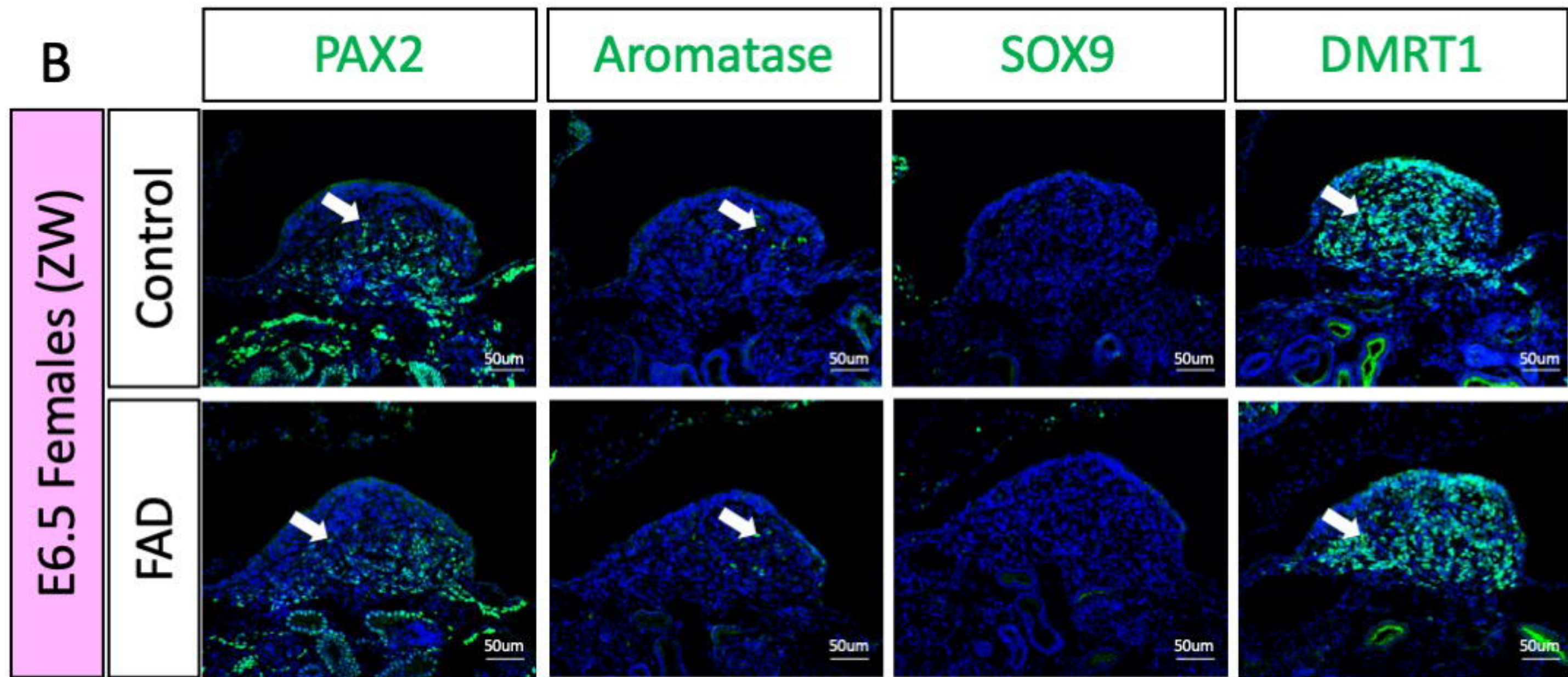Fig 1

**Fig 2**

**A**
1. Estradiol or vehicle injection
2. Sample Collection (ZZ)
E3.5 — E9.5

**B** PAX2

**C**

E9.5 males (ZZ)

| | PAX2 | Aromatase | SOX9 | DMRT1 |
| Control | | | | |
| E2 | | | | |

Fig 3

Fig 4

A

1. Fadrozole or vehicle injection — E3.5
2. Fadrozole or vehicle injection — E6.5
3. Sample Collection (ZW) — E9.5

B

E9.5 Females (ZW)

| E3.5 / E6.5 | Control Control | Fadrozole Control | Control Fadrozole | Fadrozole Fadrozole |
|---|---|---|---|---|
| PAX2 | | | | |
| Aromatase | | | | |
| SOX9 | | | | |
| DMRT1 | | | | |
| AMH | | | | |

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