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In the chick embryo, estrogen can induce chromosomally male ZZ left gonad epithelial cells to form an ovarian cortex, which supports oogenesis

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Abstract

In chickens, the embryonic ovary differentiates into two distinct domains before meiosis: a steroidogenic core, the female medulla, overlain by the germ cell niche, the cortex. The differentiation of the medulla is a cell autonomous process based on chromosome sex identity (CASI). In order to address the extent to which cortex differentiation depends on intrinsic or extrinsic factors, we generated models of gonadal intersex by mixing ZW (female) and ZZ (male) cells in gonadal chimeras, or by altering estrogen levels of ZW and ZZ embryos. We found that CASI does not apply to the embryonic cortex. Both ZW and ZZ cells can form the cortex and this can happen independently of the phenotypic sex of the medulla as long as estrogen is provided. We also show that the cortex promoting activity of estrogen signalling is mediated via Estrogen Receptor alpha within the left gonad epithelium. However, the presence of a medulla with an “intersex” or male phenotype may compromise germ cells progression into meiosis, causing cortical germ cells to remain in an immature state in the embryo.

Introduction

The gonads provide a paradigm in which to investigate organogenesis given that there are two possible outcomes, ovaries and testes, which arise from a common anlagen and cell lineages.

In the chick, gonadal sex specific morphological differences become grossly apparent from around day (D) 8 (HH34) (Hamburger and Hamilton, 1951) with the localisation of the germ cells to the appropriate domain. In chromosomally male (ZZ) embryos the germ cells become embedded within somatic cells to form the sex cords of the gonadal core or medulla, while in chromosomally female (ZW) embryos the germ cells aggregate at the periphery of the gonad and intermingle with somatic cells to form the cortex (Carlson and Stahl, 1985). In the embryonic phase of ovary differentiation, the germ cells organise in nests. It is only after hatching, when the cortex undergoes a major restructuring, in a process known as folliculogenesis, that the nests are broken and each germ cell becomes enveloped by layer(s) of granulosa cells. The embryonic cortex is therefore transient, but it is a common feature of most amniotes, including many mammals. It precedes the entry of germ cells into meiosis. Mouse and rat are exceptions to this rule, because their germ cells remain distributed in the whole gonad long after meiotic entry (Byskov, 1986; DeFalco and Capel, 2009).

The formation of the embryonic cortex is side dependent in the chick. This is due to the interaction of the canonical left-right asymmetry pathway with the gonadal development pathway via the transcription factor *Pitx2*, expressed asymmetrically in the left gonadal epithelium (Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008). During sex specific differentiation of the gonads, this asymmetry is ignored or overwritten in males, while it is exacerbated in females. Indeed, at sex determination, the medulla of both left and right embryonic ZW gonads differentiate into steroidogenic domains, but only the left gonad develops a cortex and proceeds to differentiate further into a functional ovary, while the right ovary remains just as a steroidogenic organ and later atrophies. Moreover all the germ cells in the right ovary are scattered in the medulla, do not properly enter meiosis and are lost post hatching, a fate shared with the germ cells of the left ovary which remain in the medulla (Guioli et al., 2014). This asymmetry points to the gonadal epithelium as pivotal in the formation of the ovarian cortex and establishes the importance of the embryonic cortex for survival and maturation of the germ cells.

Recent chimera studies have demonstrated that somatic cells of the chick gonad medulla differentiate into either testicular cells or ovarian steroidogenic cells in a cell autonomous manner, i.e. dependent on their sex chromosome constitution (CASI: Cell Autonomous Sex Identity) (Zhao et al., 2010), however the importance of intrinsic sex chromosome identity in the formation of the cortical germ cell niche has yet to be addressed.

Like lower vertebrates and many mammals, although not the mouse, the embryonic ovaries of birds produce estrogen from the start of female specific differentiation. For chicken and some other vertebrate species, including some mammals, it has been shown that ectopic manipulation of estrogen levels at the time of sex determination can override the effects of chromosomal sex. Notably, blocking the synthesis of estrogen in ZW embryos before sex differentiation, results in female-to-male sex reversal. In these birds, both the left and right medulla start to express male markers, such as *SOX9*, in differentiating cords and the left epithelium fails to make a cortex. Conversely, injecting estrogen before sex differentiation in ZZ embryos results in male-to-female sex reversal. Although this phenotype is reported to revert to normal in the adult, in the embryo both medullas express *FOXL2* and *CYP19A1* and a cortex develops on the left side (Akazome and Mori, 1999; Bruggeman et al., 2002; Vaillant et al., 2003; Yang et al., 2008).

During chick sex determination, *Estrogen Receptor alpha* (*ER α*) is expressed in both the left and right medulla, but asymmetrically in the epithelium of the left gonad, (Andrews et al., 1997; Guioli and Lovell-Badge, 2007). This makes it a good candidate for the estrogen transducer, with the hypothesis that estrogen affects the differentiation of both medulla and cortex by acting on different cell types and different pathways. Furthermore, it suggests once again the pivotal role of the epithelium in the formation of the cortex.

In order to understand the process of embryonic cortex morphogenesis we investigated the importance of estrogen signalling in cortex differentiation in relation to the chromosomal sex of gonadal cells. By following the fate of mixed sex gonadal chimeras and of gonads derived from embryos with manipulated estrogen levels, we show that cortex formation is not a CASI process and that estrogen is the only signal necessary for induction. However, the progression of cortical germ cells to meiosis is compromised in gonadal intersex phenotypes. Finally, we show that downregulating epithelial *ER α* is sufficient to severely affect cortex differentiation, indicating epithelial *ER α* as the relevant signal transducer.

Results

Modifying estrogen levels after the point of sex determination affects cortex formation without affecting the sex identity of the medulla

In order to understand the role of estrogen in cortex differentiation and the relationship between sex specific differentiation of cortex and medulla we altered estrogen levels beyond the time when sex reversal can be achieved (Bruggeman et al., 2002). To block/reduce estrogen levels we injected D7-7.5 (HH31) ZW embryos with the aromatase inhibitor fadrozole and repeated the treatment every two days (ZW-Fa embryos) (Fig. 1). Gonads recovered at D10 (HH36) showed a female medulla as expected, with no sign of masculinisation, as no male markers such as SOX9 were identified by immunostaining, similar to the ZW wildtype. However, the cortical domain of the left ovaries appeared reduced compared to controls and contained fewer germ cells (Fig. 1A-C). ZW left ovaries collected at D17 (HH43) were morphologically much smaller compared to ZW controls (suppl. Fig. 1), but still had a cortical domain. However, this was generally limited to the central part of the ovary (Fig. 1E-G).

To upregulate estrogen in ZZ embryos after sex determination, we injected β -estradiol *in ovo* at D7-7.5 (HH31) (ZZ-E2 embryos) (Fig. 1). The resulting ZZ left gonads collected at D10 (HH36) comprised a male medulla containing cords made of SOX9-positive somatic cells and germ cells, overlain by a narrow cortex-like domain (Fig. 1A-B, D). Left and right gonads recovered at D17 (HH43) showed a more striking phenotype, with a well-developed male medulla on both sides and a quite thick cortical domain on the left side, containing germ cell nests (Fig. 1E-F, H). Similar results were obtained when β -estradiol was injected much later (i.e. at D9 (HH35), suppl. Fig. 2).

These results show that the early differentiation of the cortex can be independent of the sex of the medulla and points to estrogen as the key inducer of this process.

ZW and ZZ cells can contribute to the cortical domain in mixed sex gonadal chimeras

It has previously been shown that, in the chick, the medullary fate is determined by CASI (Zhao et al., 2010). As the differentiating embryonic ovary is composed of two distinct sex specific functional domains: a steroidogenic medulla and a cortical domain essential for germ cells maturation, it remains to be assessed how important cell autonomous sex

identity is for the formation and differentiation of a proper ovarian germ cell niche. The estrogen manipulation experiments have demonstrated that a cortex can form in a ZZ embryo, however it is not clear that this is possible under normal physiological conditions. To address this issue, we generated gonadal chimeras comprised of male and female cells by transplanting D2 (HH10) lateral plate/intermediate mesoderm from the left side of donor GFP embryos into matching age and same side recipient embryos of the same or opposite sex, *in ovo*. The manipulated embryos were re-incubated and the gonads were collected at D18 (HH44) (Fig. 2A) and the cortex cellular composition was examined.

The resulting manipulated embryos contain a host derived right gonad and a left gonad containing somatic cells of host and donor origin. The germ cells, however, are always host derived on both sides, as they segregate to the germinal crescent of the embryo at stage HH5 and later migrate via the bloodstream to reach the intermediate mesoderm at HH15 (Ginsburg and Eyal-Giladi, 1987; Karagenc et al., 1996; Nakamura et al., 1988; Nakamura et al., 2007).

The chimeric gonads derived from left-to-left transplants between ZW embryos (ZW/ZW, same-sex chimeras) were ovaries composed of a medulla and a cortex similar to those in a normal left ovary. In the donor enriched area, the cortical epithelial and sub-epithelial somatic cells were mainly contributed by GFP donor cells (Fig. 2B(a-e), 2C(a-b) and suppl. Fig. 3A).

Fig. 2B(f-j) and suppl. Fig. 3B shows a typical chimeric left ovary from a ZW embryo containing donor cells derived from ZZ left mesoderm (ZZ/ZW mixed-sex chimera). This ovary displays a cortex along the entire length. As expected from previous studies (Zhao et al., 2010), the area enriched in donor tissue was composed of a medulla containing host (ZW), P450aromatase positive steroidogenic cells, surrounded by host (ZW, GFP negative) and donor (ZZ, GFP positive) derived interstitial cells. Surprisingly, in the GFP enriched area, the medulla was overlain by a cortex formed by GFP somatic donor (ZZ) cells. These areas were continuous with areas poor in donor cells, whose medulla and cortex were mainly host derived. At the junction between the two areas, intermingling of host and donor cells of the cortex was evident (Fig. 2C(c-d)). Transplants of ZW donor tissue into ZZ hosts (ZW-ZZ mixed-sex chimeras) often produce chimeric ovotestis. Fig. 2B(k-o), 2C(e-f) and suppl. Fig. 3C show one such example. The ovarian portion of the

chimera comprised a female medulla containing P450aromatase positive steroidogenic cells derived from the donor (ZW, GFP positive cells) as expected, overlain by a cortex made of both host (ZZ) and donor (ZW) somatic cells. This ovarian domain was contiguous with a testicular domain (Fig. 2B(k-o) and suppl. Fig. 3C).

We concluded that both ZZ and ZW cells can normally contribute the somatic component of the embryonic cortex without a sex bias and therefore independently of their chromosomal sex. Therefore it is clear that while medullary fate is determined by CASI (Zhao et al., 2010), this concept does not apply to the gonadal cortex.

Meiotic entry of cortical germ cells is severely compromised in gonads with a masculinised medulla

The mitotic to meiotic switch is quite asynchronous in the chicken ovarian cortex, with many germ cells entering meiosis by D12 (HH38) and the majority initiating the synaptic process by D16 (HH42) (de Melo Bernardo et al., 2015).

We followed germ cell meiotic progression in D17 (HH43) ovaries from ZW embryos subject to late fadrozole treatment (ZW-Fa) and from ZZ embryos subject to late estradiol treatment (ZZ-E2), by analysing the expression of the double strand breaks (DSB) marker γ H2AX and the synapsis marker SYCP3 (Guioli et al., 2012; Smith et al., 2008). At this stage, both markers are normally expressed in most germ cells of the wildtype ovary (Fig. 3A-B). Similarly, many germ cells expressed both markers in the estradiol treated ZW left gonads (ZW-E2). In fadrozole treated ZW left gonads (ZW-Fa) the cortex contained less germ cells, but positive for γ H2AX and SYCP3. Meiosis was most affected in estradiol treated ZZ left gonads (ZZ-E2), composed of a medulla organised in testicular cords (suppl. Fig. 4), overlain by a cortical domain made of ZZ somatic cells and ZZ germ cells: some cortical germ cells expressed γ H2AX but none were positive for SYCP3 as assessed by immunostaining (Fig. 3B).

To investigate the importance of germ cell chromosomal sex for the correct initiation of meiosis in the cortex, we generated a series of testis-to-ovary reversed ZZ embryos and a series of ovary-to-testis reversed ZW embryos, by *in ovo* injection of estradiol or fadrozole, respectively, at D4 (HH23) (ZZ-E2(sr) and ZW-Fa(sr) embryos). From both experiments, we obtained D17 embryos with incomplete gonadal sex reversal which typically showed an “intersex” phenotype. In the left gonad, the inner part of the medulla

contained SOX9-positive cells, organised in cords like structures, while the outer medulla contained more FOXL2 positive cells (suppl. Fig. 4). The medulla was generally overlain by a cortex like structure (Fig. 3A). Importantly, in ZZ-E2(sr) embryos the primordial germ cells (PGCs) are male (ZZ), while in ZW-Fa(sr) embryos they are female (ZW). Synapsis was compromised in both these models, because in both cases some cortical germ cells expressed γ H2AX but none or very few expressed SYCP3 by immunostaining (Fig. 3A-B).

STRA8 is a major factor involved in meiotic initiation and should be expressed in germ cells of the left cortex in a wave starting from D12 (HH38) (Smith et al., 2008). We analysed the *in situ* expression pattern of *STRA8* in the cortex of ZZ embryos exposed to late estradiol treatment (ZZ-E2) (Fig. 4A). These were negative for *STRA8* at D14 (HH40) and showed only a few very faint foci at D17 (HH43), compared to the cortex of normal ZW controls, estradiol treated ZW embryos (ZW-E2) and ZW embryos exposed to late fadrozole treatment (ZW-Fa) (Fig. 4A).

We also checked the expression of *DMRT1*, a known key factor involved in the mitotic-meiotic switch in mouse (Matson et al., 2010; Zarkower, 2013). In chick, as in the mouse, *DMRT1* is normally expressed in the proliferating germ cells in both differentiating ovary and testis and it is downregulated soon after meiosis starts (Guioli et al., 2014; Omotehara et al., 2014). As shown in Fig. 4B(a-c), *DMRT1* is downregulated by D17 (HH43) in most germ cells of the wildtype ovary. *DMRT1*-positive cells were only observed at the poles of the control ovary where the maturation of germ cells is delayed (de Melo Bernardo et al., 2015). In the experimental samples ZZ-E2, ZZ-E2(sr) and ZW-Fa(sr), that were severely compromised for SYCP3 expression, numerous germ cells throughout the cortex were strongly positive for *DMRT1* (Fig. 4B(d-l)). Altogether, these data suggest that it is the presence of testicular tissue that blocks or delays the start of meiosis of the cortical germ cells.

A cortical domain contributed by ZZ male somatic cells provides a proper niche for ZW and ZZ germ cell meiotic entry

In order to assess if a cortical domain made of ZZ somatic cells can sustain the progression of the germ cells into meiosis in physiological conditions, we analysed the expression of the SYCP3 meiotic marker in D18 (HH44) gonadal chimeras (Fig. 5).

In the same sex female left chimeric ovary (ZW/ZW) most germ cells localised along the entire cortex were positive for SYCP3 as expected (Fig. 5A, D). In ZZ/ZW mixed-sex left chimeric ovary, where the germ cells are female (ZW), the pattern of cortical PGCs was similar to the ZW/ZW controls (Fig. 5B, D). This means that SYCP3 was expressed in most PGCs throughout the entire cortex, both in areas mainly contributed by host female (ZW) somatic cells and in areas mostly contributed by donor male (ZZ) somatic cells, demonstrating that the latter are competent to form a proper cortical germ cell (oocyte) niche. However, in ZW/ZZ left chimeric ovotestis the results were more complex. Within the ovarian portion the cortical germ cells showed a variable pattern depending on localisation. In some cortical areas, many germ cells were found positive for SYCP3, while in other areas few or no germ cells were positive for these markers (Fig. 5C, D). The fact that at least some ZZ germ cells enter meiosis in this ZW/ZZ chimera, indicates that both female and male germ cell chromosomal sex identity is compatible with starting the female meiotic program in the embryo. However the SYCP3 patchy pattern suggests that the ovotestis condition of the chimera (see suppl. Fig. 3 for the chimera structure) may generate a “confused” sex environment which blocks or severely delays meiotic entry, similarly to what observed in the ZZ-E2, ZZ-E2(sr) and ZW-Fa(sr) models.

ER α downregulation in the ZW left gonad epithelium disrupts cortex differentiation

At sex determination, ER α is asymmetrically expressed in both ZW and ZZ embryos, being restricted to the epithelium of the left gonad although present in both medullas (Andrews et al., 1997; Guioli et al., 2014). This pattern makes it a candidate estrogen signal transducer in promoting cortical differentiation. In this study, we showed that the ZZ left gonad is sensitive to estrogen beyond the initiation of male specific differentiation, implying that an estrogen transducer should be expressed asymmetrically in the differentiating testis. However, this had not been specifically addressed in previous studies. To address this issue we performed a timecourse analysis of ER α during testis differentiation by immunostaining. Its expression was clearly detected in the epithelium of the left ZZ gonad at D7 (HH31). Although weaker than in the differentiating ovary, the staining was maintained in the epithelium of the left testis until at least D12 (suppl. Fig. 5). This indicates that the left epithelium of the ZZ developing testis retains the

potential to quickly respond to estrogen, even though it would not normally be exposed to it at any significant level.

In order to knock down the activity of ER α in the epithelial cells of the left ovary, a dominant negative form of ER α (cER α 524) (see suppl. Fig. 6A for assessment of dnER α activity) cloned into a Tet-ON plasmid for conditional gene expression, was transfected into the left gonadal epithelium at D2.5 (HH15-17) by *in ovo* electroporation of dorsal coelomic epithelium on the left side (Guioli et al., 2007). The DNA was co-electroporated with other plasmids of the Tet-ON system, including T2TP for expression of the Tol2 transposase and pT2K-CAG-rTA-M2, the doxycyclin-inducible activator (Watanabe et al., 2007) (Fig. 6A(a) and M&M). Expression of cER α 524 was induced at D4.5 (HH25) or D5.5 (HH27) and the screening was carried out at D10 (HH36) based on the EGFP reporter expression, as a measure of the quality of transfection. Electroporation typically results in mosaic transfection which varies from sample to sample. ZW embryos displaying high levels of EGFP (e.g. Fig. 6A (b-c)) were processed for immunostaining with markers specific to different cell types. The most severe phenotype was observed in ovaries exposed to doxycycline at the earliest time point. An example is shown in Fig. 6A (b,e,h,k). In the posterior part of the ovary, most of the medulla highlighted by P450aromatase was overlain by a simple epithelium, with the exception of the central part, which displayed some stratification and contained a small aggregate of germ cells (Fig. 6A (e,h)) (see suppl. Fig. 7 for better visualisation of the GFP pattern). A milder phenotype is shown in Fig. 6A (c,f,i,l) where the cortex appears markedly thin in areas where stretches of the epithelium were still enriched in EGFP compared to the non targeted portion. In these areas few germ cells were present (Fig. 6A(i)). Although by D10 (HH36) many EGFP epithelial derived cells were found in the medulla, the medulla maintained an ovarian identity, being positive for P450 (Fig. 6A (e-f)) and negative for SOX9 (data not shown). No other obvious phenotype was observed in the medulla. All samples still expressed *Pitx2* within the left epithelium as seen in the normal controls (Fig. 6A (j-l)).

To make sure that the data observed were due to specific knock down of ER α activity we performed an alternative set of experiments aiming to suppress ER α via RNAi. Fig. 6B shows a left ovary screened at D9 (HH35-36) and analysed for germ cell localisation and cortical structure. It is an example of a left gonad very well targeted in large areas along the anterior/posterior axis. As with cER α 524 most severe phenotype, the ER α shRNA

targeted left gonad showed a female medulla similar to the ZW wildtype control, but a severely compromised cortex in correspondence of well targeted areas of the epithelium. The medulla, marked by P450aromatase was found to be extended up to the base of the epithelium in the middle portion of the ovary, where most epithelial cells were EGFP positive, indicating lack of cortical development in this region (Fig. 6B(d)). The absence of a stratified cortex at D9-10 was never observed in ZW wild type gonads (see controls in Fig. 6A-B) and in gonads electroporated with various control vectors expressing an EGFP reporter, even in areas very well transfected (Suppl. Fig. 8).

Discussion

In recent years great advances have been made uncovering key sex regulators that control the commitment to the male and female programmes, mainly based on work carried out in mammals, primarily in the mouse.

However ovarian differentiation is still poorly understood. One of the main reason is that mouse ovarian morphogenetic changes, including the formation of a cortex, are much delayed to the perinatal period, making the discovery of female sex promoting factors more challenging. Moreover, estrogen is dispensable for mouse embryonic ovary differentiation, maybe reflecting the evolution of a different hierarchy of early regulators, compared even to other mammals. One example of this is *Foxl2* which is critical for ovary differentiation early in gonadal development in the goat, but not until after birth in the mouse (Boulanger et al., 2014; Schmidt et al., 2004; Uda et al., 2004; Uhlenhaut et al., 2009).

The chicken ZW gonad produces estrogen and differentiates into an ovary with a clear cortex and medulla soon after the female molecular pathway initiates, making it a good model system to address early ovarian differentiation. In this study, we focused on cortical morphogenesis with the aim of understanding the differentiation of the female (i.e. oogenic) germ cell niche.

Estrogen signalling is an important factor in chicken female sex determination, as it can override the genetic sex if perturbed at the time of sex determination (Yang et al., 2008). This holds true in lower vertebrates and some mammals including marsupials (Coveney et al., 2001; Guioli et al., 2014). However, its potential role as anti-testis and ovarian promoting factor are still ambiguous. To explore its action in promoting cortex formation

and to understand if this process can be dissociated from female specification of the medulla, we observed the effect of manipulating estrogen levels after D7 (HH31), when sex reversal is no longer achievable and medulla differentiation is consistent with genetic sex. Moreover, as the fate of the medulla, in chicken, is determined by a cell autonomous process strictly based on sex chromosome identity, we also generated a series of male:female gonadal chimeras to assess if CASI holds true for the cortical domain.

The analysis of same-sex and mixed-sex left gonadal chimeras showed that ZZ and ZW cells can become part of a cortex which can provide a proper oogenic niche like the female counterpart. In both cases, after an initial phase of proliferation, the germ cells entered meiosis at the appropriate time, excluding the involvement of W or Z specific genes in the control of early cortical development. Therefore, the cortex does not form based on the CASI process that determines the medullary fate (Zhao et al., 2010). It is known that the Z gene *Dmrt1*, a current candidate as a male primary sex determinant in chicken (Guioli et al., 2014; Lambeth et al., 2014; Smith et al., 2009), is expressed symmetrically in the medulla, but also asymmetrically in the gonad epithelium of ZZ and ZW embryos at the time of sex determination (Guioli and Lovell-Badge, 2007; Omotehara et al., 2014). As *Dmrt1* is not subject to dosage compensation (McQueen and Clinton, 2009), our chimeras show that any differences in *Dmrt1* expression levels due to gene dosage between female and male somatic cells does not impact cortex formation.

The estrogen injection in D7 ZZ embryos generated an “intersex” gonad composed of a male medulla overlain by a female cortex, while downregulation of estrogen in ZW embryos perturbed proper cortex formation, maintaining a female medulla.

These results show that the cortex domain can, at least initially, differentiate independently from the sex of the medulla if estrogen is provided. This makes estrogen the major promoting signal for cortex development and the left epithelium a naïve tissue capable of responding to any sex environment hormonal influence.

Therefore, early cortex development depends on a cell non autonomous process triggered by sex promoting signals, notably estrogen, normally produced in the ovarian medulla. Consistent with our results a previous study inhibiting estrogen synthesis in *T.scripta* at female producing temperature has shown that cortical expansion does not require a feminised medulla in *T.scripta*, supporting the idea that the differentiation of medulla and cortex may be unlinked, at least initially (Barske and Capel, 2010).

However, we did observe that in the left gonad of ZZ embryos subject to late estradiol treatment and in ovotestes commonly obtained from ZW/ZZ chimeras, cortical germ cells progression into meiosis was compromised, as SYCP3, a marker of the synaptic process was absent or expressed in very few cells at D17-18 of embryogenesis. Although in these severely compromised models the PGCs were genotypically male, there is evidence that this failure is not a result of the germ cell genotype. For example, although meiosis was compromised in ZW/ZZ chimeras, patches of SYCP3-positive ZZ germ cells were found, suggesting that the ZZ germ cells are capable of initiating meiosis at the right time in some areas of the cortical domain. Moreover, left ovotestes, generated by manipulation of estrogen levels in either D4 ZZ or ZW embryos, both contained cortical germ cells with compromised SYCP3 expression. This therefore occurs independently of the germ cell chromosomal sex.

In chicken, as in the mouse, *STRA8* is a key factor for germ cell entry into meiosis (Bowles et al., 2016; Koubova et al., 2014; Smith et al., 2008; Yu et al., 2013). In our SYCP3 compromised models *STRA8* was almost undetectable throughout development, while DMRT1, which should be downregulated at meiosis, was still present in many D17 (HH43) germ cells. This shows that at D17 (HH43) these cortical domains contained germ cells that have not progressed properly into meiosis, suggesting meiotic arrest or at least a severe delay.

The mitotic-meiotic switch is a very complex process, including many germ cell intrinsic and extrinsic factors which may act as promoters or suppressors of meiosis that are not yet completely elucidated. Our results suggest that signalling originating outside the cortex must be involved. It has been shown that FGF9 secreted by the Sertoli cells antagonises meiotic entry via activation of Nodal-Smad signaling in mouse spermatogonia (Tassinari et al., 2015) and in chicken ovary bFGF was found to act as a suppressor of meiosis and as a mitogenic factor (He et al., 2012). The male medulla in the ovotestes may antagonise meiotic entry via FGF signalling or other male driven signalling. Alternatively, meiosis may be impaired by the lack or deficiency of some female inducer signals from the medulla, or both.

In humans, disorders of sexual development (DSD) are associated with an increased risk of type II germ cell cancer which are derived from two types of *in situ* neoplastic lesions: carcinoma *in situ* (CIS) or gonadoblastoma, depending on the supporting cells being

Sertoli cells or granulosa cells, respectively (Hersmus et al., 2017). The observation of mitotic and meiotic signals within CIS cells, together with the inability of these cells to ever enter meiosis, points to a dysfunctional mitotic:meiotic switch that could provide the genetic instability initiating the tumour (Cools et al., 2006; Jorgensen et al., 2013). The long-standing hypothesis is that this process is initiated in fetal life, where a gonad with an intersex phenotype provides a sexually confused niche which can result in the delay or arrest of germ cell development. If these cells are not eliminated they may retain the embryonic phenotype and later transform (Jorgensen et al., 2013).

These human studies support the idea that the meiotic phenotype in our intersex chick models is due to the sexually confused environment and predict that if these germ cells are not later eliminated, they could become transformed into tumour cells. The chicken is already used as a model of ovarian cancer as it develops spontaneous ovarian cancer at a rate similar to human (Bernardo et al., 2015). The estrogen manipulated chick models might provide an important system in which to study the origin and dynamics of neoplastic transformation of germ cells.

We have previously proposed that estrogen signalling could be transduced via ER α on the basis of the RNA expression pattern at sex determination (Andrews et al., 1997; Guioli and Lovell-Badge, 2007). The symmetric expression in left and right medulla and the asymmetric expression in the gonad left epithelium of both female and male embryos suggested that estrogen, via ER α , may have multiple roles in promoting ovary differentiation which affects both the differentiation of the medulla and the cortex. However the protein pattern in the male has always remained elusive, making it difficult to explain how the radiolabelled estradiol injected in the embryo found to be binding both female and male gonad at D5-10 may do so via ER α (Gasc, 1980). In the present study we were able to show that some levels of ER α protein are present in the male gonad at sex determination in a pattern which reflects the RNA expression, indicating that both ZW and ZZ gonad could quickly respond via ER α to estrogen stimulation. Moreover, the maintenance of expression within the left epithelium in the differentiating testis shows that the epithelium remains exquisitely sensitive to estrogen and able to rapidly respond to the hormone even several days into the male differentiation pathway.

In agreement with predictions, the knock down of ER α activity in the epithelial cells of the left ZW gonad has resulted in the impairment of cortical development, the most severe

cases displaying areas devoid of a cortex and overlain by a simple epithelium, similar to the phenotype observed in the D10 embryos treated with fadrozole from D7-7.5 (HH31). The downregulation only affected the cortical domain without affecting the sex identity of the medulla. Moreover, the cortical phenotype was not linked to variation in *PITX2* expression within the left epithelium. All together these data indicate ER α as the main transducer of estrogen signalling in cortex formation and that this process strictly depends on its activity within the left epithelial cells of the gonad.

ER is a complex molecule. It has been shown in different systems that upon estrogen activation, it can have non-genomic and genomic activities. It can rapidly respond at the plasma membrane by triggering signalling pathways and it can mediate transcriptional regulation within the nucleus (Hamilton et al., 2017). Our future work will be addressing the mechanism of action of ER α in promoting cortical development upon estrogen stimulation, through the identification of the downstream targets of its action via transcriptomics approaches.

In summary, our data suggest a model whereby the gonads, at the time of somatic sex specific differentiation, are formed of two distinct domains: the epithelium and the medulla. Left and right medulla differentiates along the male or female pathway strictly based on CASI, depending on whether the cells comprising it are ZW or ZZ. CASI does not apply to the formation of the cortex. The epithelium of the left gonad is naïve with respect to chromosome sex identity. If exposed to estrogen will develop a cortex, via activation of ER α . The finding that this activation can occur above a male medulla, shows that estrogen is sufficient for this step and suggests that the initial formation of the cortex relies on positive inductive signals more than the lack of antagonising signals from the medulla.

Later progression of the cortical germ cells into meiosis requires a medulla of the correct phenotypic sex, suggesting that meiotic entry is a checkpoint of a well-coordinated ovarian cortex/medulla differentiation pathway.

Our results provide new insights into the process of chicken ovarian differentiation and provide a model that could be extended to other systems, including many mammals. Moreover, our data suggest that the chick intersex models may be a potential valuable system for investigating the aetiology of germ cell tumours.

Material and Methods

Animals

Most experiments were performed using Dekalb white chicken eggs obtained from Henry Stewart, Louth, UK, except for the generation of chimeras, done using GFP transgenic chickens (Roslin greens) and ISA brown chickens held at the National Avian Research Facility (NARF) at Roslin Institute, Edinburgh, UK. All experiments carried out on chicken were performed in accordance with regulations of the Francis Crick and Roslin Institutes's Animal Welfare and Ethical Review Body (AWERB), under the UK Animal (scientific procedures) Act.

Plasmids and *in ovo* electroporation

The Tol2 integration plasmid system was a kind gift of dr. Yoshiko Takahashi (Kyoto, Japan) and includes: the transposase expression plasmid pCAGGS-T2TP, the expression plasmid pT2K-CAGGS-EGFP and the conditional expression plasmid PT2K-B1-TRE-EGFP (both carrying an EGFP reporter), the Tet-ON activator plasmid pT2K-CAG-rTAM2 which responds to doxycycline (Sato et al., 2007; Watanabe et al., 2007). A truncated isoform of *ER α* lacking the last 196bp of the open reading frame (last 65aminoacids of the ligand binding domain) was generated by PCR using primers F-5'ACTAGTTCTAGAGCCATTAGCAATGACCATGAC and R-5'GTCGACTCTAGATTAACACTTCATATTGTACAGGTG, cloned in pCRII-TOPO (Invitrogen) and then moved into PT2K-B1-TRE-EGFP (using BamHI and EcoRV sites) to make *cER α 524*. This isoform corresponds to a human *ER α* isoform shown to have a powerful dominant negative activity (Ince et al., 1993).

Three *ER α* shRNA molecules were cloned downstream of a mouse U6 promoter into pCRIITOPO using a PCR based approach aiming to amplify the U6 promoter along with the shRNA, similar to the approach described in (Harper et al., 2005). Common forward primer: U6-Forward, 5'TCTAGATCGACGCCCATCTCTAG; U6-reverse-shRNA specific primers:

(ER1)CTCGAGAAAAAAGGAACACCCAGGAAAGCTTTCCCATCTGTGGCTTTA
CAGAAAGCTTTCCTGGGTGTTCCAAACAAGGCTTTTCTCCAAGGG;

(ER2)CTCGAGAAAAAAGCCACTAACCAGTGTACTATCCCATCTGTGGCTTTA
CAGATAGTACTGTTAGTGGCAAACAAGGCTTTTCTCCAAGGG,

(ER3)CTCGAGAAAAAAGGTACCCTACTACCTTGAAACCCATCTGTGGCTTTA
CAGTTTCAAGGTAGTAGGGTACCAAACAAGGCTTTTCTCCIAAGGG.

The sequences include restriction enzymes for cloning. Dominant negative activity of *cER α 524* was confirmed by luciferase assays *in vitro*, following transfection in HEK293 cells (ATCC-CRL-3216) together with full length *cER α* (cloned into pSG1) and a 3XERE-TATA-Luc luciferase reporter (Lemmen et al., 2004), kind gift of dr. Paul T. van der Saag, Ubrecht Institute, Utrecht, Netherlands; the three shER RNA molecules were also tested by luciferase assay using the same *in vitro* system; three biological replicates were performed in triplicate (see suppl. Fig. 6 for details and result). U6-shER ER1, displaying the best silencing activity was cloned into pSLAX13, previously modified to carry IRES-EGFP. U6-shER-IRES-EGFP (ER1) was then moved to a RCAS(A) retroviral vector to generate RCAS-U6-shER-IRES-EGFP (RCAS-shER1).

In ovo electroporation of plasmid DNA into the left gonadal epithelium was achieved by DNA injection into the left coelomic cavity of HH15-17 embryos using a glass capillary needle and an Inject+matic pico pump, followed by electroporation using a NEPA21 electroporator (Sonidel) (five 50ms pulses at 26V, transferring pulse only). Detailed procedure as previously described (Guioli et al., 2007). Experiments were repeated more than three times using at least 40 eggs per experiment. The surviving rate at collection was around 30%. The dissected gonads were screened on the basis of the EGFP expression pattern in the gonads. A minimum of four of the best electroporated gonads, per type, and wildtype gonads collected at D10 were analysed. See suppl. Fig. 8 for details of electroporation of other control vectors.

***In ovo* drug treatment**

For manipulation of estrogen levels after sex determination (late treatment), eggs were incubated at 37.7°C pointed end down. At D7-7.5 (HH31) or D9 (HH35) a first injection in the air chamber was performed through a hole made at the rounded end. To downregulate estrogen, the P450aromatase inhibitor Fadrozole (Sigma F3806) was injected at 0.5mg/egg in 50ul of PBS and then re-injected at 0.3mg/egg every other day. To increase estrogen, β -Estradiol (Sigma E2758) was injected at 120 μ g/egg in 25 μ l of 95% ETOH; embryos collected at D17 (HH43) were injected once more at D13 (HH39). For manipulation of estrogen levels before sex determination (early treatment) eggs were injected once at D4 (HH23) with 0.5mg or 1mg/egg of Fadrozole, or 120 μ g/egg of β -

estradiol. Three or more gonadal pairs were analysed from each type of experimental treatment, per developmental stage. A minimum of two stages were assessed (D10 and D17).

Generation of gonadal chimera

D2 (HH10/12) (13–15 somites) GFP transgenic embryos (Roslin greens) and ISA brown embryos were used as donor and host, respectively. Details of the procedure are described in (Zhao et al., 2010). The manipulated host embryos were then incubated until D18 (HH44). Two or three well contributed same sex or mixed sex gonadal chimeras per type were analysed further at D18.

Antibodies, Immunohistochemistry and in situ hybridisation

The following antibodies were used: rat anti-VASA (1:1000) (Aramaki et al., 2009), rat anti ER α (1:200, Fitzgerald 10R-E124AX), rabbit anti-DMRT1 (1:1500) (Guioli and Lovell-Badge, 2007), goat anti-SOX9 (1:500, R&D AF3075), goat anti-FOXL2 (1:400, Abcam ab5096), mouse anti-P450aromatase (1:200, Serotec MCA2077S), mouse anti- γ H2AX (1:200, Merk 05-636), rabbit anti-SYCP3 (1:500, Novus NB300-232), mouse anti-Fibronectin (1:100, Developmental Studies Hybridoma Bank B3/D6), goat anti-LHX9 (1:200, Santacruz sc-19350). The *STRA8* probe template for RNA *in situ* was generated by PCR cloning into PCRITOPPO (Invitrogen), using primers F-5'TACCCAGACACCTCATCCCC and R5' TCAAAGGTCTCCGTGCACCG. The *PITX2* probe was previously described in (Logan et al., 1998). Urogenital ridges were fixed in 4% paraformaldehyde at 4°C overnight, rinsed in PBS at Room Temperature (RT), then transferred to 30% sucrose overnight and finally embedded in OCT and stored at -80°C. Cryosections for immunofluorescence were rinsed 3X five minutes in PBS and transferred for 1 hour to a blocking solution (PBS/0.1% triton, 2% donkey serum) before adding the primary antibody ON at 4°C (or 37°C for ER α). After 3X ten minute washes in PBS/0.1% tween, the sections were incubated with secondary antibodies (Invitrogen Alexa fluor-conjugated donkey antibodies 1:400 in PBS/0.1% tween). The results from the *in situ* hybridization on cryosections were imaged using a Leica DM-RA2 upright microscope equipped with a Retiga 200R camera and Q-capture Pro7 software (Q imaging). Fluorescence images were collected on a Olympus VS120 slide scanner

equipped with a XM10 monochrome camera and a VS-ASW-L100 software (Olympus), or on a Leica upright SPE confocal system.

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Author contributions

Conceptualisation: SG, RLB, MC; Methodology: SG, DZ; Validation: SG, DZ; Formal analysis: SG, DZ; Investigation: SG, DZ, SN; Visualisation: SG; Writing- original draft: SG; Writing- review and editing: SG, RLB, MC; Project administration: RLB, MC; Funding acquisition: RLB, MC.

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Figures

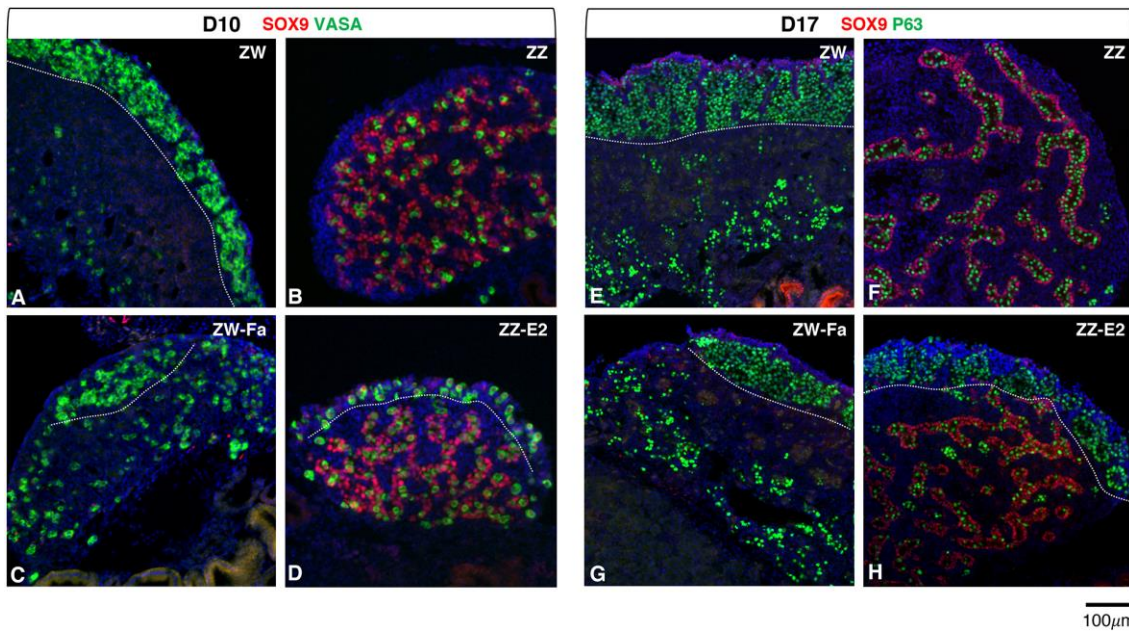


Fig. 1 Perturbing estrogen levels at embryonic D7-7.5 (HH31) affects cortex formation in ZW and ZZ embryos. Sections from left gonads at D10 (HH36) (A-D) or D17 (HH43) (E-H) double-stained for the Sertoli marker SOX9 (red) and a germ cell marker: VASA or P63 (green). (A, E) ZW controls; (B, F) ZZ controls; (C, G) ZW gonads treated with Fadrozole (ZW-Fa); (D, H) ZZ gonads treated with β -estradiol (ZZ-E2). Decreasing estrogen in ZW embryos after sex determination compromises the differentiation of the ovarian cortex; adding β -estradiol in ZZ embryos after sex determination induces the formation of a cortex on top of a male medulla. White dotted lines highlight the cortex-medulla border.

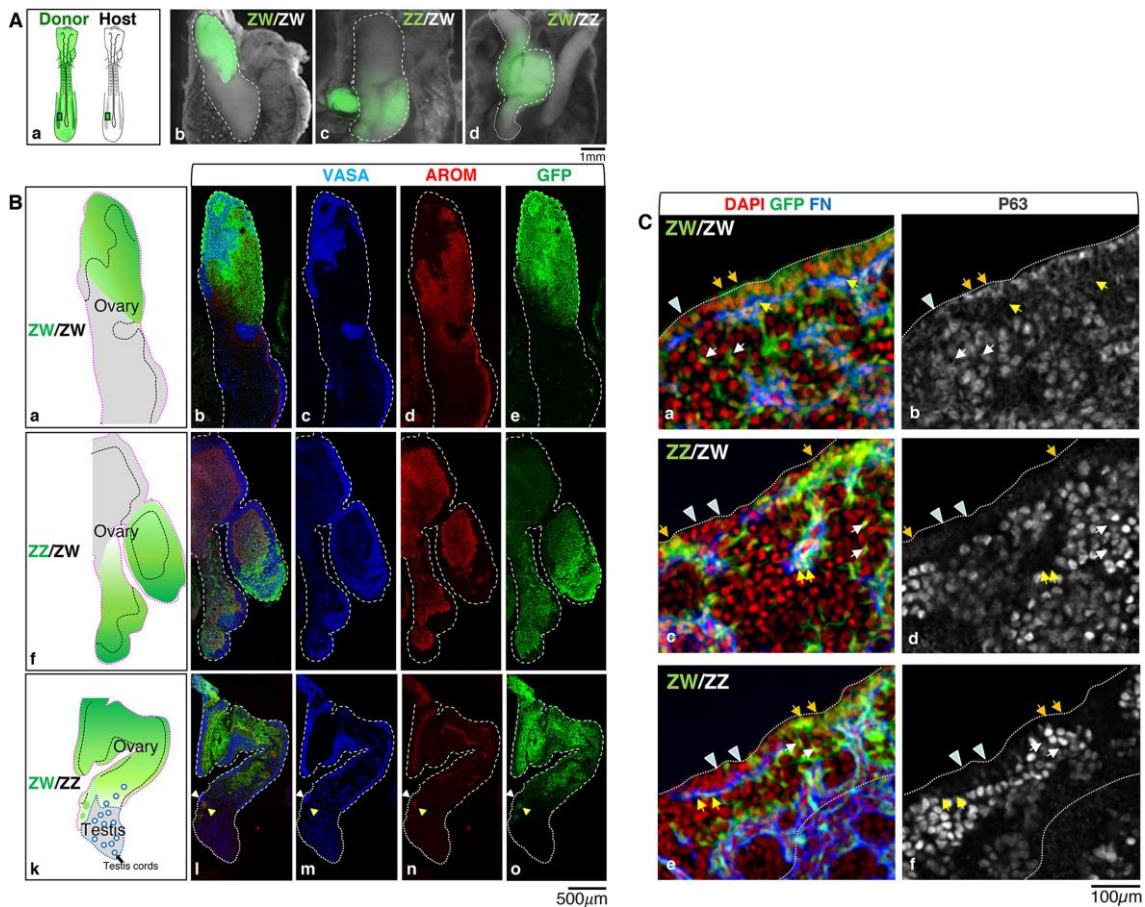


Fig. 2 ZW and ZZ cells can both contribute to the somatic component of the cortex domain. D18 (HH44) gonadal chimeras generated by transplanting posterior lateral plate/intermediate mesoderm from the left side of donor GFP embryos (green) into the left side of recipient wildtype embryos at D1.5-2 (HH10). (A) (a) Schematic of the transplant; (b, c, d) whole-mount images of the resulting gonads dissected at D18 (HH44) showing the chimeric left gonad enriched in donor GFP (green) cells; (b) ZW donor into ZW host (ZW/ZW), (c) ZZ donor into ZW host (ZZ/ZW) and (d) ZW donor into ZZ host (ZW/ZZ). White dashed or dotted lines highlight the chimeric left gonad (ovarian and testis domain, respectively). (B) immunofluorescent images of sections from the left chimeric gonads showing the germ cells using the marker VASA (blue), the female specific steroidogenic cells using the marker P450aromatase (AROM) (red) and the GFP positive donor cells (green); (a-e) ZW/ZW control chimera, (f-j) ZZ/ZW chimera, (k-o) ZW/ZZ chimera.

The schematic in (a, f, k) summarises the immunostaining results (see also suppl. Fig. 3), showing the ovarian domain (pink dotted line border), the cortical germ cells nests (superficial areas delimited by black dashed lines), the testis domain (blue dotted line)

and the GFP enriched area (green). ZW/ZW (a-e) and ZZ/ZW (f-j) chimeras are ovaries, and the ZW/ZZ (k-o) is an ovotestis. White arrowhead in (l-o) indicate a small cortical germ cell nest overlaying a patch of AROM donor cells (yellow arrowhead) in the transition area between ovary and testis. (C) High resolution images of cortical areas from ZW/ZW, ZZ/ZW and ZW/ZZ chimera sections immunostained for the extracellular matrix protein Fibronectin (FN) (blue) and the germ cell marker P63 (grey), nuclei are counterstained with DAPI (red). GFP (green) can be found in the cortex epithelium (orange arrows) and in somatic cells close to the germ cells nests, either positive (yellow arrows) or negative (white arrows) for FN. The ZZ donor cells in the ZZ/ZW chimera (c, d) and the ZW donor cells in the ZW/ZZ chimera (e, f) can both contribute the somatic components of the cortical domain. Epithelial donor cells (orange arrows) and host cells (arrowheads) may intermingle.

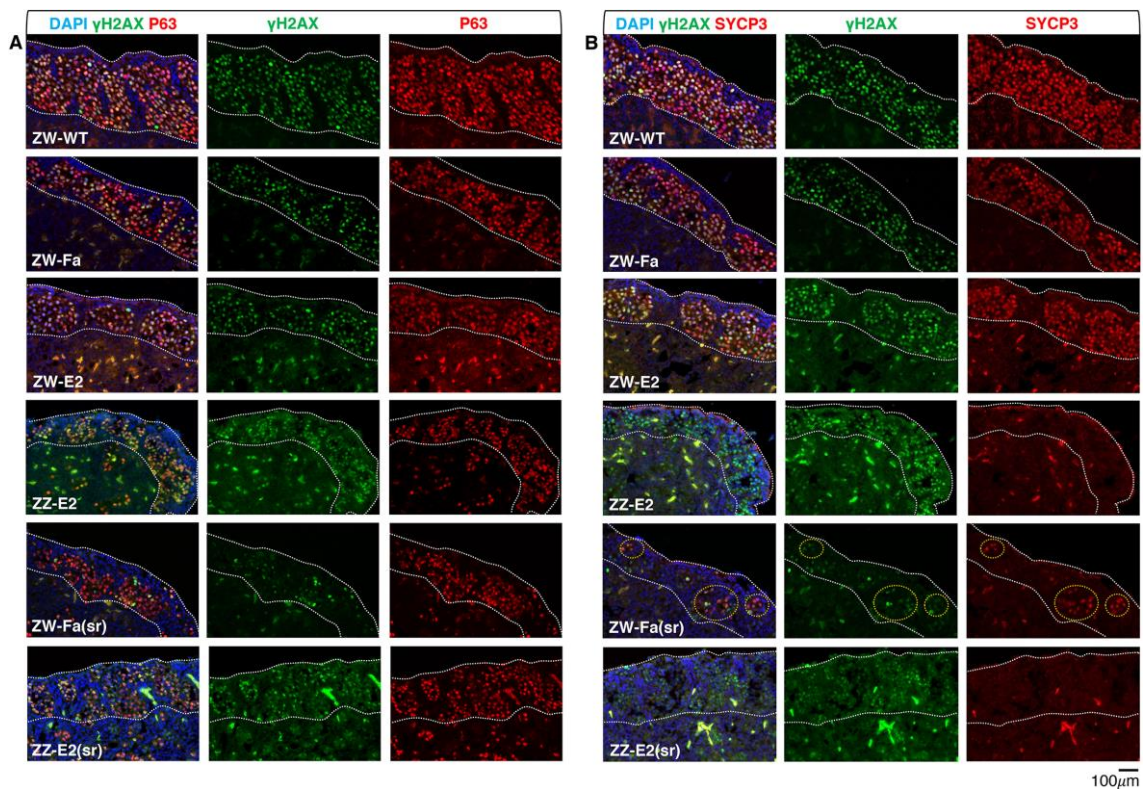


Fig. 3 Induction of meiosis is compromised in the cortical germ cells of D17 embryos subject to estrogen level alterations. (A) D17 (HH43) left gonad sections immunostained for γ H2AX (green) and P63 (red); nuclei are counterstained with DAPI (blue). (B) Sections from the D17 (HH43) left gonad shown in (A) immunostained for γ H2AX (green) and SYCP3 (red). ZW-WT: ZW wildtype; ZW-Fa: ZW treated with fadrozole from D7-7.5 (HH31); ZW-E2: ZW treated with β -estradiol at D7-7.5; ZZ-E2: ZZ treated with β -estradiol at D7-7.5; ZW-Fa(sr): ZW, partially sex reversed gonad, treated with Fadrozole at D4; ZZ-E2(sr): ZZ, partially sex reversed gonad, treated with β -estradiol at D4. All gonadal models have a cortical domain containing germ cells. In ZW-Fa ovary and ZW-E2 ovary most cortical germ cells express SYCP3 like the ZW-WT control. In ZW-Fa(sr) ovotestis very few germ cells express γ H2AX and SYCP3 (orange dotted circled areas). In ZZ-E2(sr) ovotestis and ZZ-E2 testis overlain by a cortex some cortical germ cells express γ H2AX but none express SYCP3. White dotted line highlights the cortical domain borders. See suppl. Fig. 4 for the medullary structure of the ZZ-E2, ZW-Fa(sr) and ZZ-E2(sr) models.

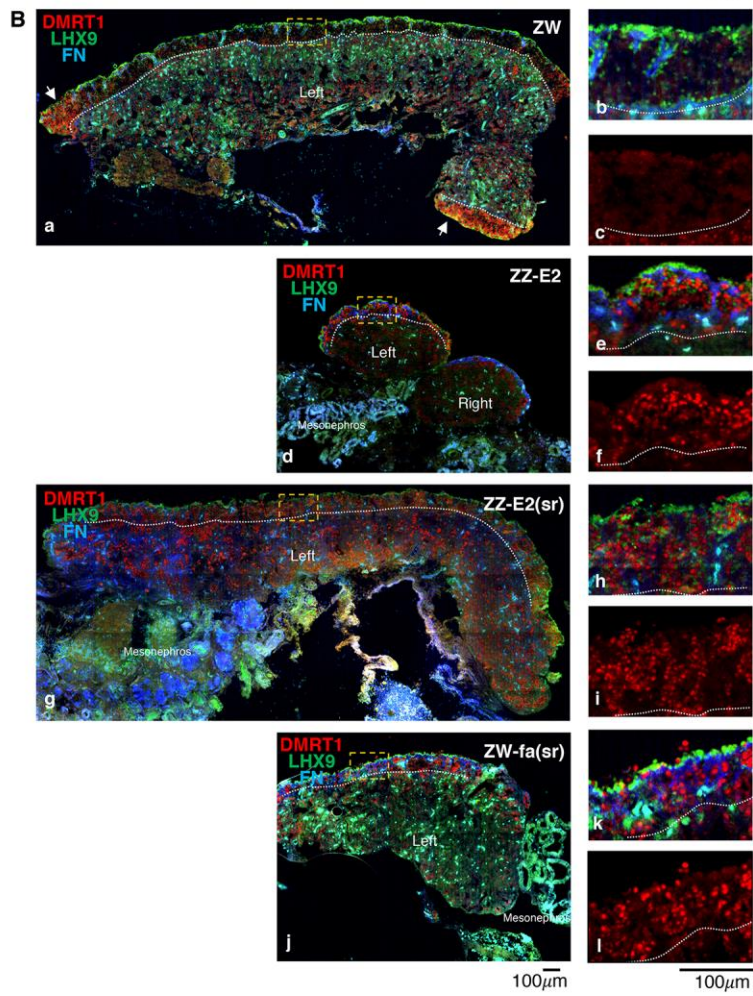
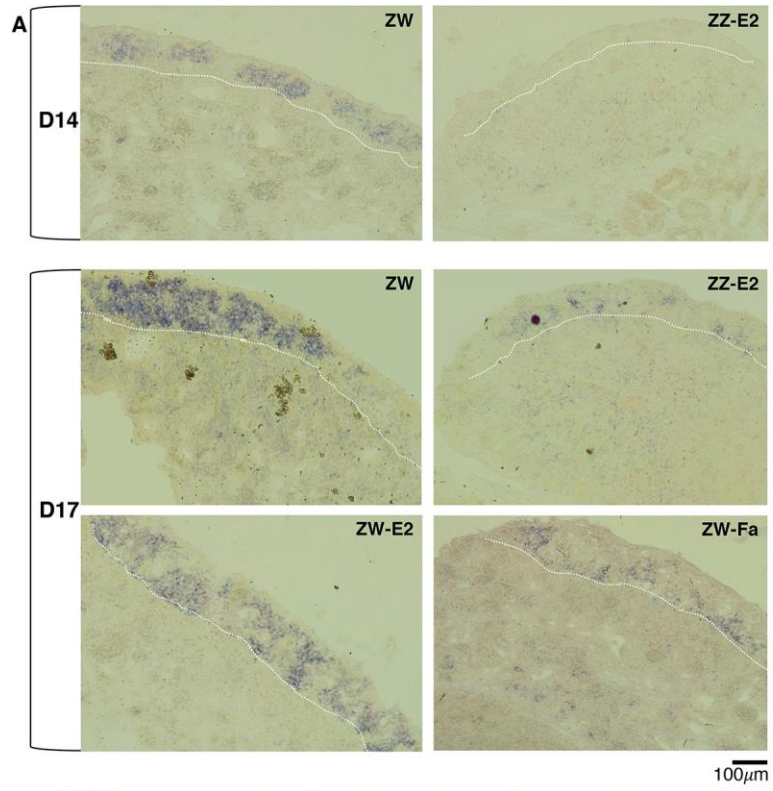


Fig. 4 The expression pattern of mitotic-meiotic switch markers is affected in the cortical germ cells of embryos subject to estrogen levels alteration. (A) RNA *in situ* analysis of *STRA8* expression on sections from the left gonad of D14 (HH40) and D17 (HH43) embryos. ZW: female wildtype control; ZZ-E2: ZZ, β -estradiol treated at D7-7.5 (HH31); ZW-E2: ZW, β -estradiol treated at D7-7.5 (HH31); ZW-Fa: ZW, Fadrozole treated at D7-7.5 (HH31). *STRA8* expression is severely compromised in the gonadal cortical germ cells from ZZ embryos exposed to β -estradiol.

(B) Immunofluorescent detection of DMRT1 (red), LHX9 (green), and fibronectin (FN) (blue) on gonadal cryostat sections from D17 (HH43) embryos. In the left wildtype ovary LHX9 marks cortical somatic cells, FN highlights the cortex-medulla border cells, respectively (Guioli and Lovell-Badge, 2007) and DMRT1 marks cortical germ cells, not somatic cortical cells (LHX9 positive). (a) ZW: female wildtype control; (d) ZZ-E2: ZZ, treated with β -estradiol at D7-7.5 (HH31); (g) ZZ-E2(sr): ZZ, treated with β -estradiol at D4 (HH23) (partially sex reversed); (j) ZW-Fa(sr): ZW, treated with fadrozole at D4 (HH23) (partially sex reversed). (b-c), (e-f), (h-i), (k-l) high magnification images of cortical areas (orange dashed box) from (a) (d) (g) (j) respectively; (b) (e) (h) (k) LHX9+FN+DMRT1, (c) (f) (i) (l) DMRT1 only. All samples are cut sagittally (left gonad shown) apart from ZZ-E2, transversally (left and right gonads shown). White dotted lines mark the cortex-medulla border. In the control ZW female only the cortical germ cells at the gonad poles are DMRT1 positive (white arrows). In ZZ-E2, ZZ-E2(sr) and ZW-Fa(sr) DMRT1 is expressed in many germ cells across the cortex.

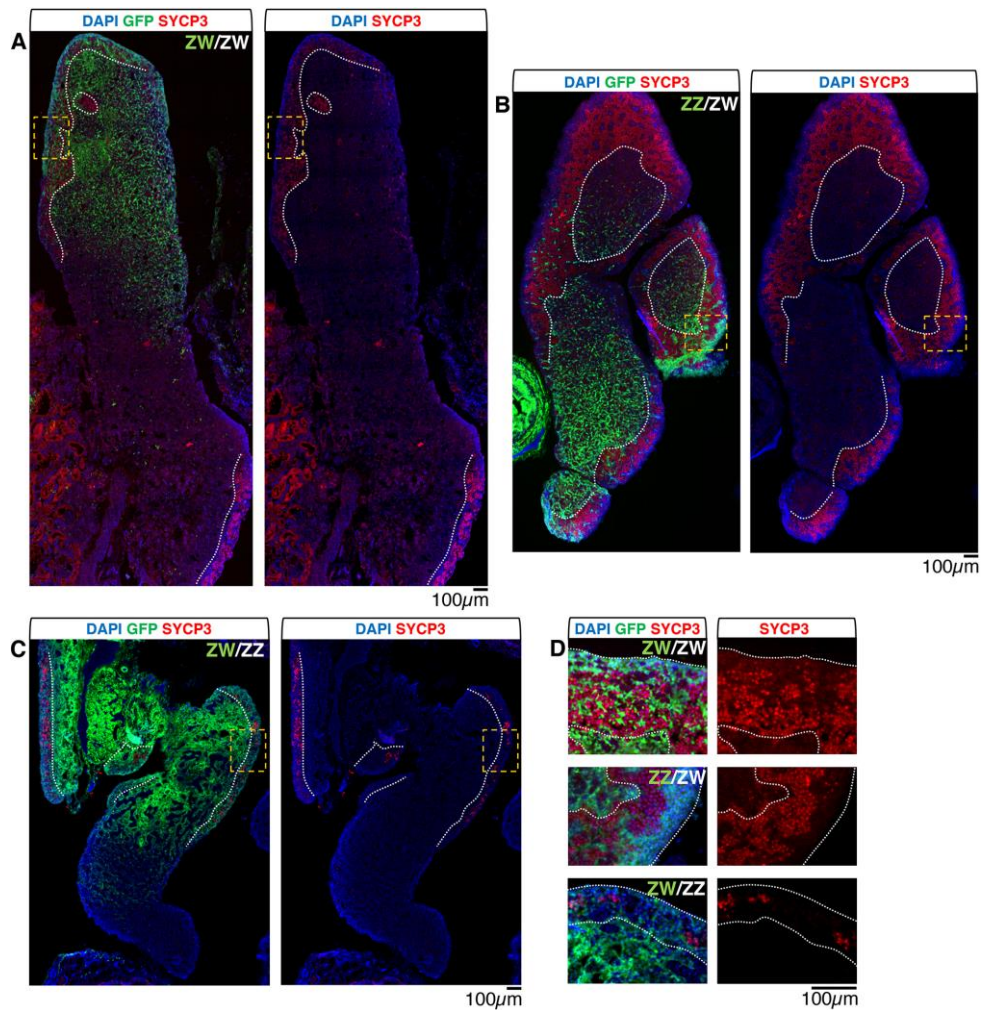


Fig. 5 In mixed sex gonadal chimeras ZZ somatic cells provide an adequate niche for progression of cortical germ cell into meiosis. Sections from chimeric left gonads at D18 (HH44) immunostained for SYCP3 (red); donor cells are GFP positive (green); nuclei are counterstained with DAPI (blue). (A) ZW donor into ZW host (ZW/ZW) control chimeric ovary; (B) ZZ donor into ZW host (ZZ/ZW); (C) ZW donor into ZZ host (ZW/ZZ); (D) high magnification images of cortical areas (orange dashed box) from (A, B, C). In ZW/ZW and in ZZ/ZW chimeric left ovaries most cortical germ cells express SYCP3. In the ZW/ZZ chimeric left ovotestis, SYCP3 is expressed only in some cortical germ cells (see suppl. Fig. 3 for visualising the distribution of all the germ cells). The ZZ donor cortical somatic cells in the ZZ/ZW chimera provide an adequate niche for progression of germ cells into meiosis. Both ZW and ZZ cortical germ cells can progress into meiosis. However in the ZW/ZZ ovotestis meiosis is compromised. White dotted line shows the cortex-medulla border.

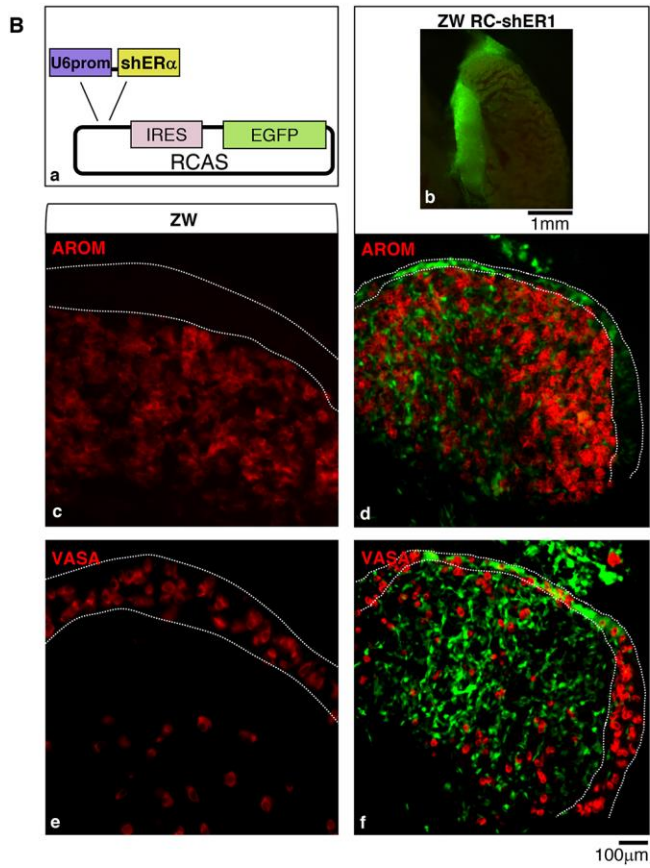
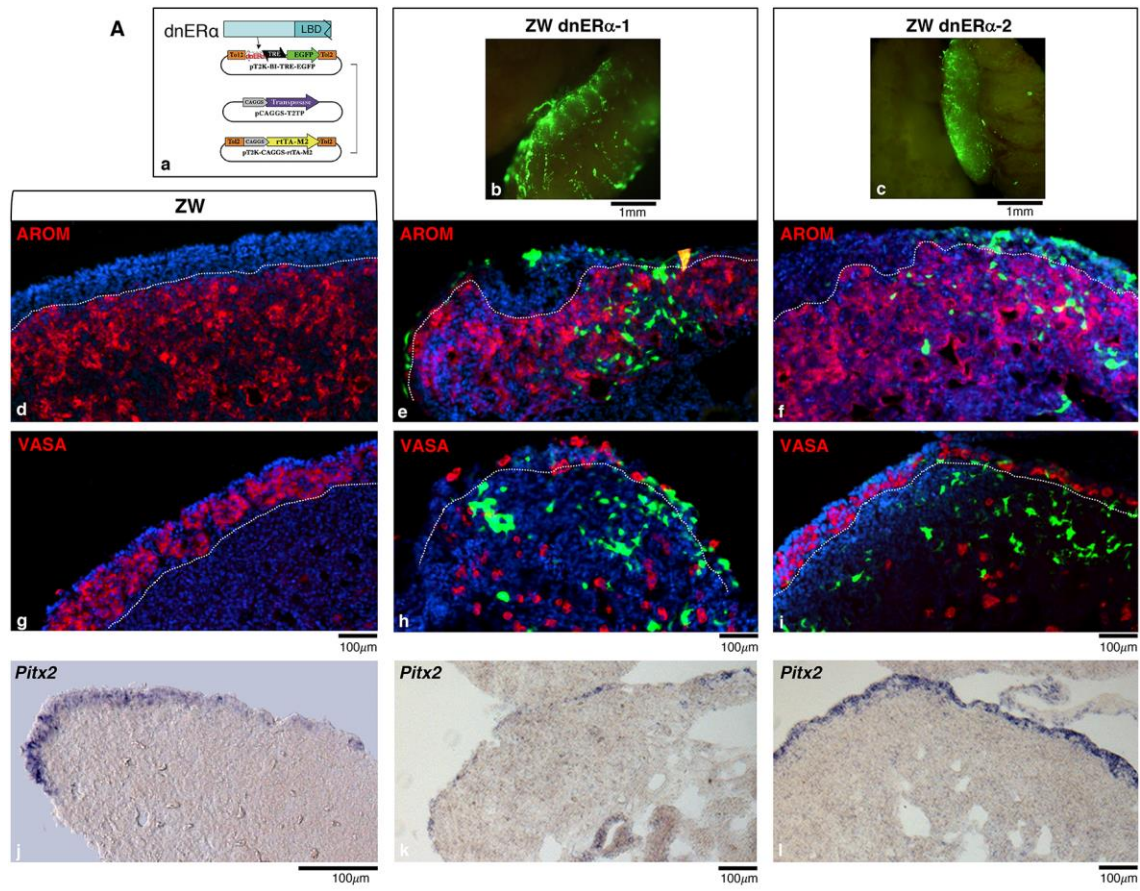


Fig. 6 *In ovo* suppression of epithelial ER α activity disrupts cortex differentiation.

(A) Electroporation of a dominant/negative isoform of ER α (dnER α) to the gonadal epithelium; (a) schematic of the inducible TET-ON plasmid system used to express dnER α (see Material and Methods for details), LBD= Ligand Binding Domain; (b, c) whole mount images of two ZW left gonads electroporated at D2.5 (HH15-17) and screened at D10 (HH36); targeted cells are highlighted by expression of an EGFP reporter (green); (d-i) fluorescence images of D10 (HH36) gonad sections immunostained for P450aromatase (AROM) (red), or the germ cell marker VASA (red) and GFP (green); (see suppl. Fig. 7 for the EGFP pattern alone); (d, g) ZW control, (e, f) and (h, i) ZW electroporated gonads. (j-l) *Pitx2* RNA in situ expression pattern: (j) ZW control, (k, l) ZW electroporated gonads. White dotted lines show the cortex/medulla border. (B) Electroporation of RCAS retroviral DNA expressing ER α shRNA (RCAS-shER1) to the gonadal epithelium; (a) schematic of the construct: RCAS-shER α expressing ER α specific short hairpin RNA molecules in tandem with an EGFP reporter; (b) whole mount image of a ZW left gonad electroporated with RC-shER1 at D2.5 (HH15-17) and screened at embryonic D9 (HH35-36) based on the EGFP reporter expression; (c-f) fluorescence images of D9 (HH35-36) gonad sections immunostained for P450aromatase (AROM) (red) or the germ cell marker VASA (red), (see suppl. Fig. 7 for the EGFP pattern alone); (c,e) ZW control, (d,f) ZW-RCAS-shER1 gonad.

The cortex is compromised by downregulation of ER α activity in both model systems compared to controls. Four out of eight samples from the two experiments showed the severe cortical phenotype in well electroporated regions. See suppl. Fig. 8 for immunofluorescence results on sections from electroporation of various control vectors expressing an EGFP reporter (minimum of three replicates per control vector analysed).

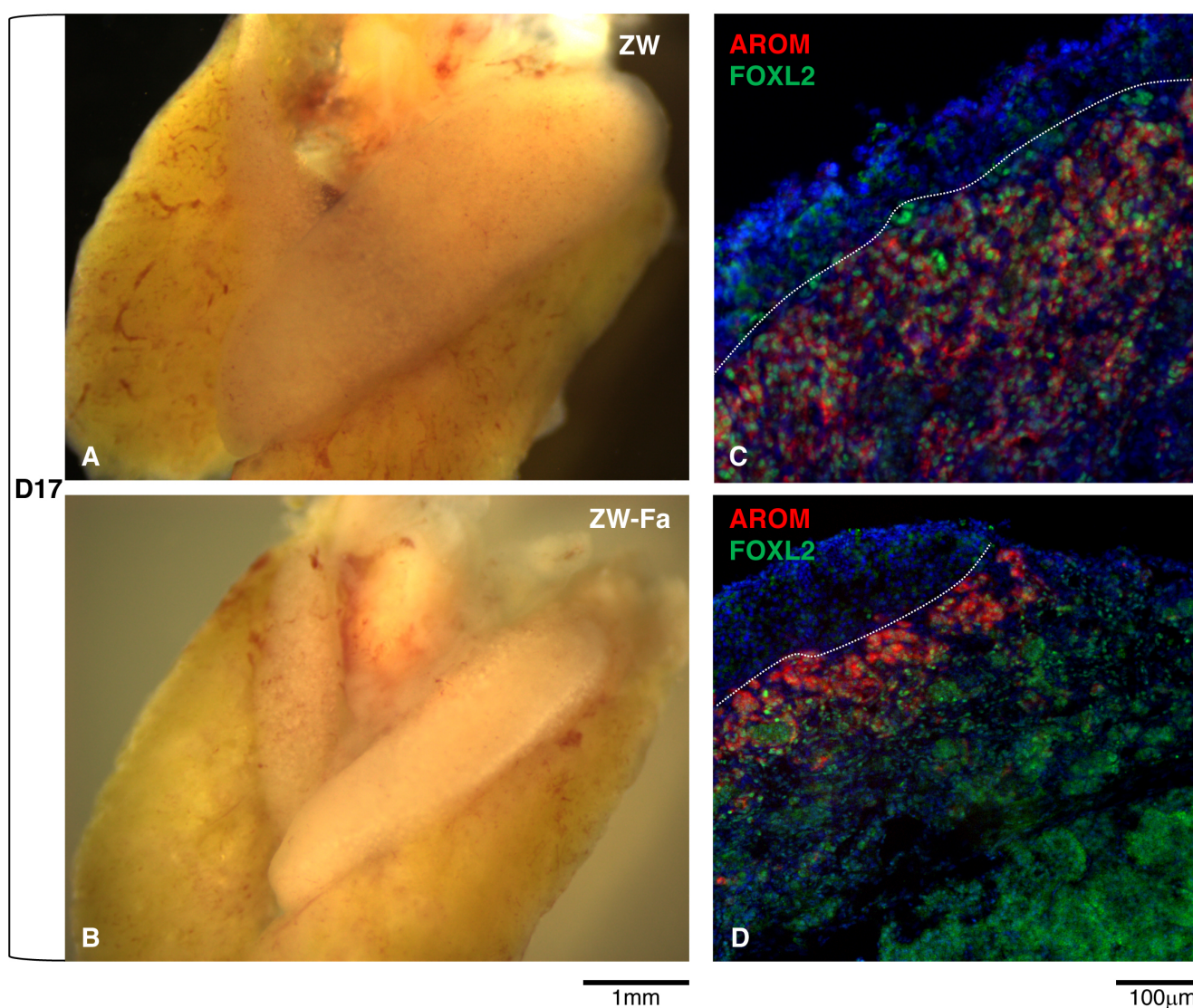


Fig. S1 In ZW embryos treated with Fadrozole at D7-7.5 (HH31) the growth of the left ovary is affected. (A-B) D17 (HH43) ZW gonads, (A) ZW control (B) ZW treated with Fadrozole from D7-7.5 (ZW-Fa); (C, D) fluorescence images of cryostat sections from the left gonad in (A) and (B) respectively, stained for the female markers P450aromatase (AROM) (red) and FOXL2 (green); nuclei counterstained with DAPI (blue). White dotted lines highlight the cortex-medulla border.

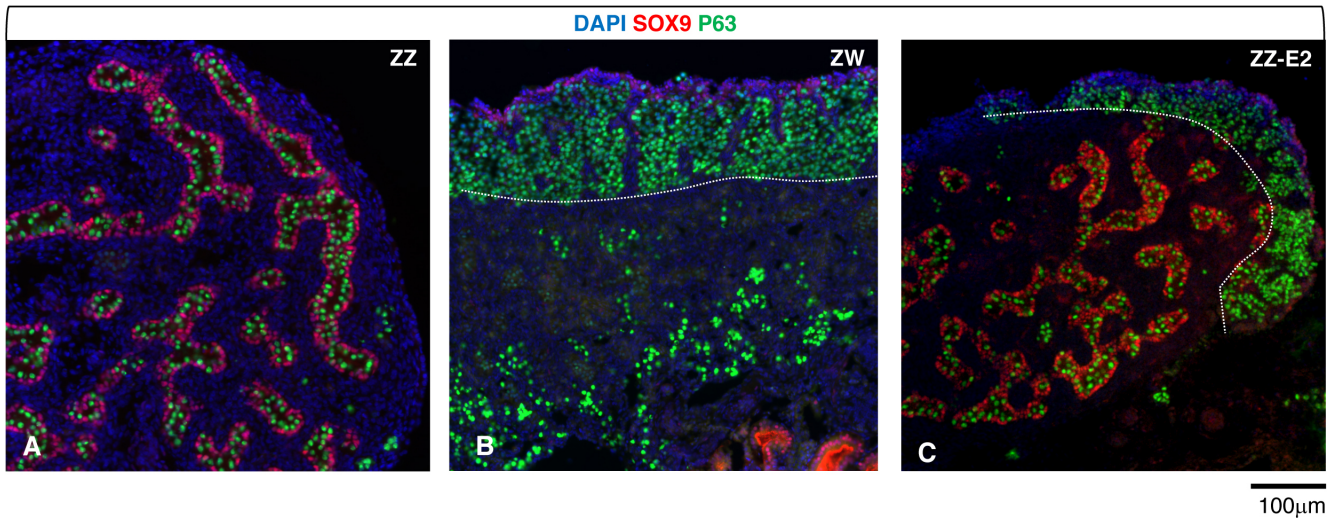


Fig. S2 The left testis of ZZ embryos exposed to β -estradiol at D9 (HH35) develops a cortical domain. Sections from the left gonad of D17 (HH43) embryos, immunostained for the Sertoli cell marker SOX9 (red) and the germ cell marker P63 (green); nuclei counterstained with DAPI (blue). (A) ZZ control, (B) ZW control, (C) ZZ treated with β -estradiol (ZZ-E2) at D9 (HH35). White dotted lines highlight the cortex-medulla border.

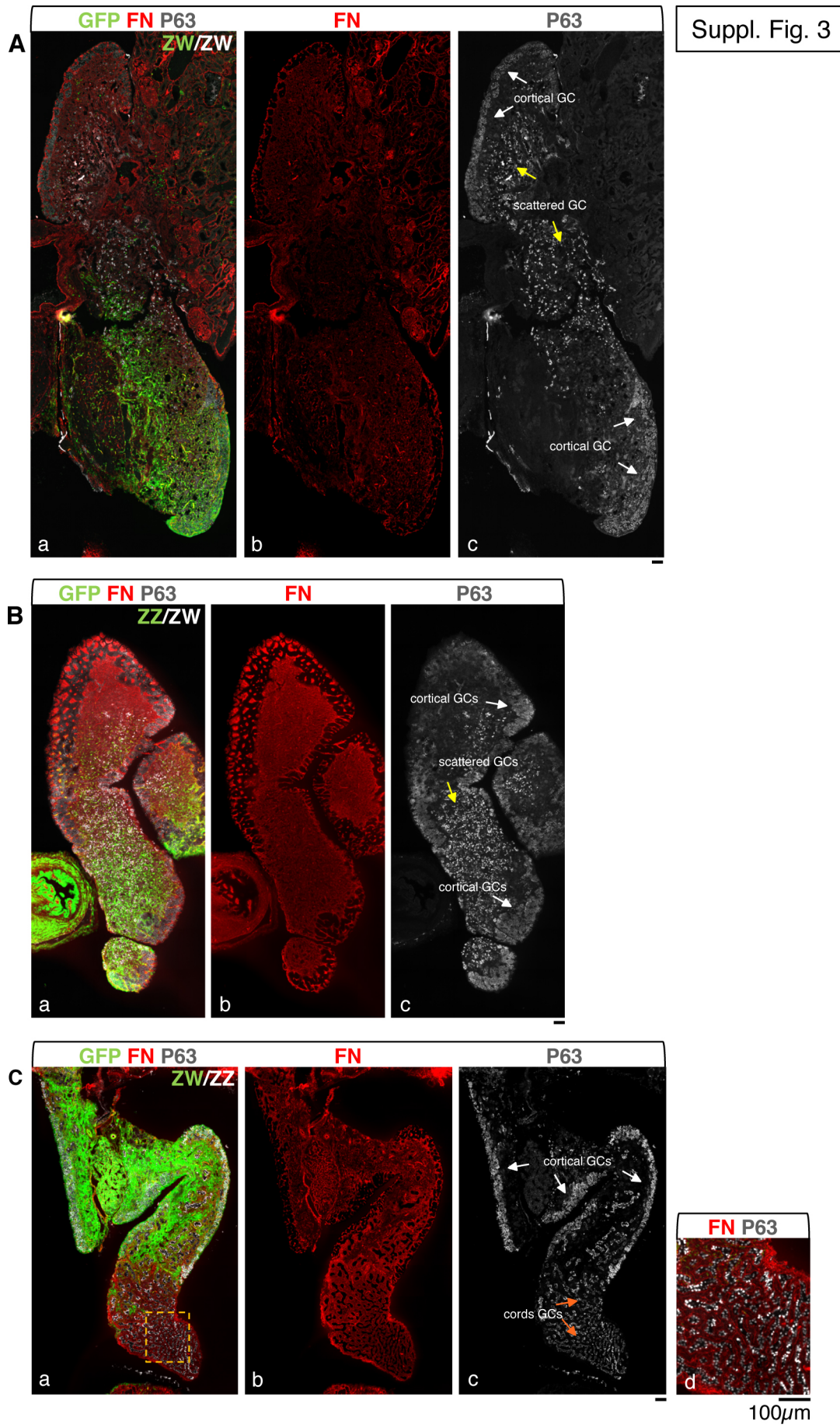


Fig. S3 ZW and ZZ cells can both contribute to the somatic component of the cortex.

Immunofluorescent images of sections from the D18 left chimeric gonads shown in Fig. 2, displaying germ cells localisation using the marker P63 (grey), the structure of the gonad using the extracellular matrix (ECM) marker Fibronectin (FN) (red) and the GFP positive donor cells (green). (A) ZW donor into ZW host (ZW/ZW); (B) ZZ donor into ZW host (ZZ/ZW); (C) ZW donor into ZZ host (ZW/ZZ). FN and P63 combined (Aa), (Ba), (Ca) and separate (Ab) (Bb) (Cb) and (Ac) (Bc) (Cc) stainings, are shown. Cortical germ cells (GCs) and medullary scattered GCs are indicated with white and yellow arrows respectively. GCs within testicular cords of the ovotestis are indicated by orange arrows. Details of ZW//ZZ chimeric ovotestis cords is shown in panel (Cd).

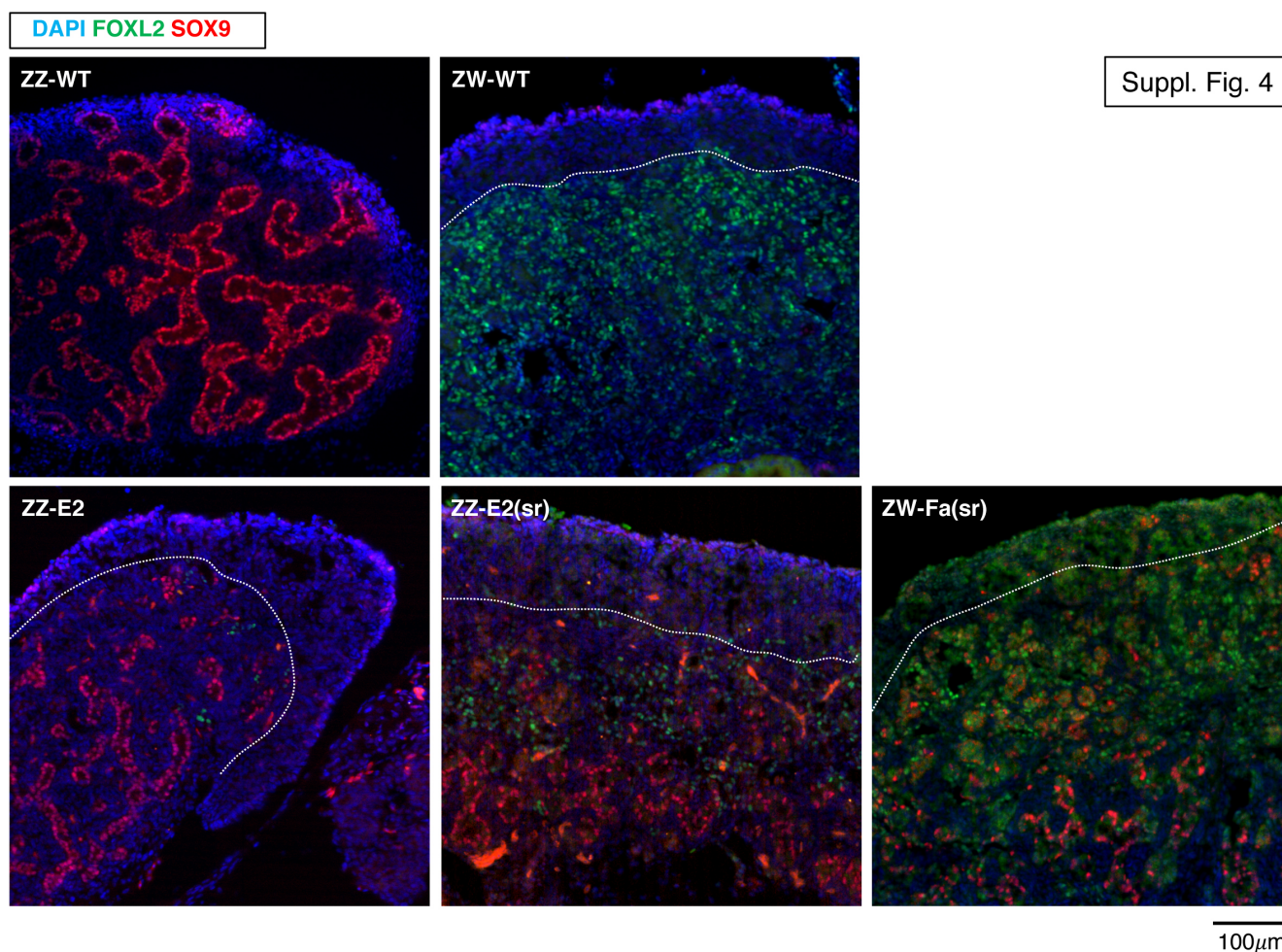


Fig. S4 Phenotypic sex of the gonadal medulla in D17 embryos subject to estrogen levels alteration. D17 (HH43) left gonad sections immunostained for the medulla female specific marker FOXL2 (green) and male specific markers SOX9 (red); nuclei are counterstained with DAPI (blue). ZZ-WT: ZZ wildtype; ZW-WT: ZW wildtype; ZZ-E2: ZZ treated with β -estradiol at D7-7.5; ZW-Fa(sr): ZW, partially sex reversed gonad, from treatment with Fadrozole at D4; ZZ-E2(sr): ZZ, partially sex reversed gonad, from treatment with β -estradiol at D4. White dotted line highlights the cortical domain borders.

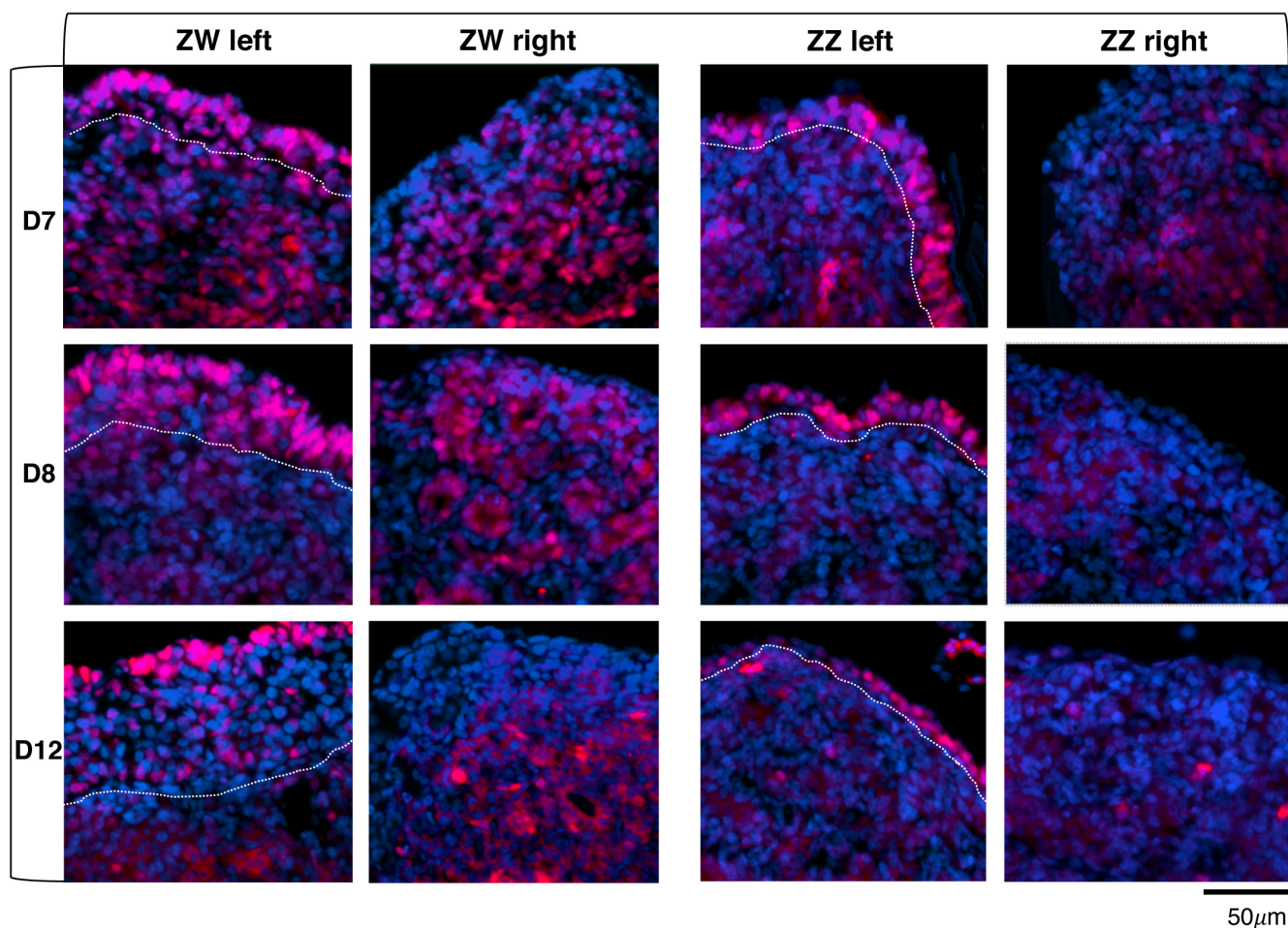


Fig. S5 ER α protein is produced in the epithelium of the left ZZ gonad during differentiation. Sections from ZW and ZZ left and right wild type gonads at embryonic D7 (HH31), D8 (HH34) and D12 (HH38), immunostained for ER α (red). White dotted lines in left gonad panels highlight the cortex-medulla border.

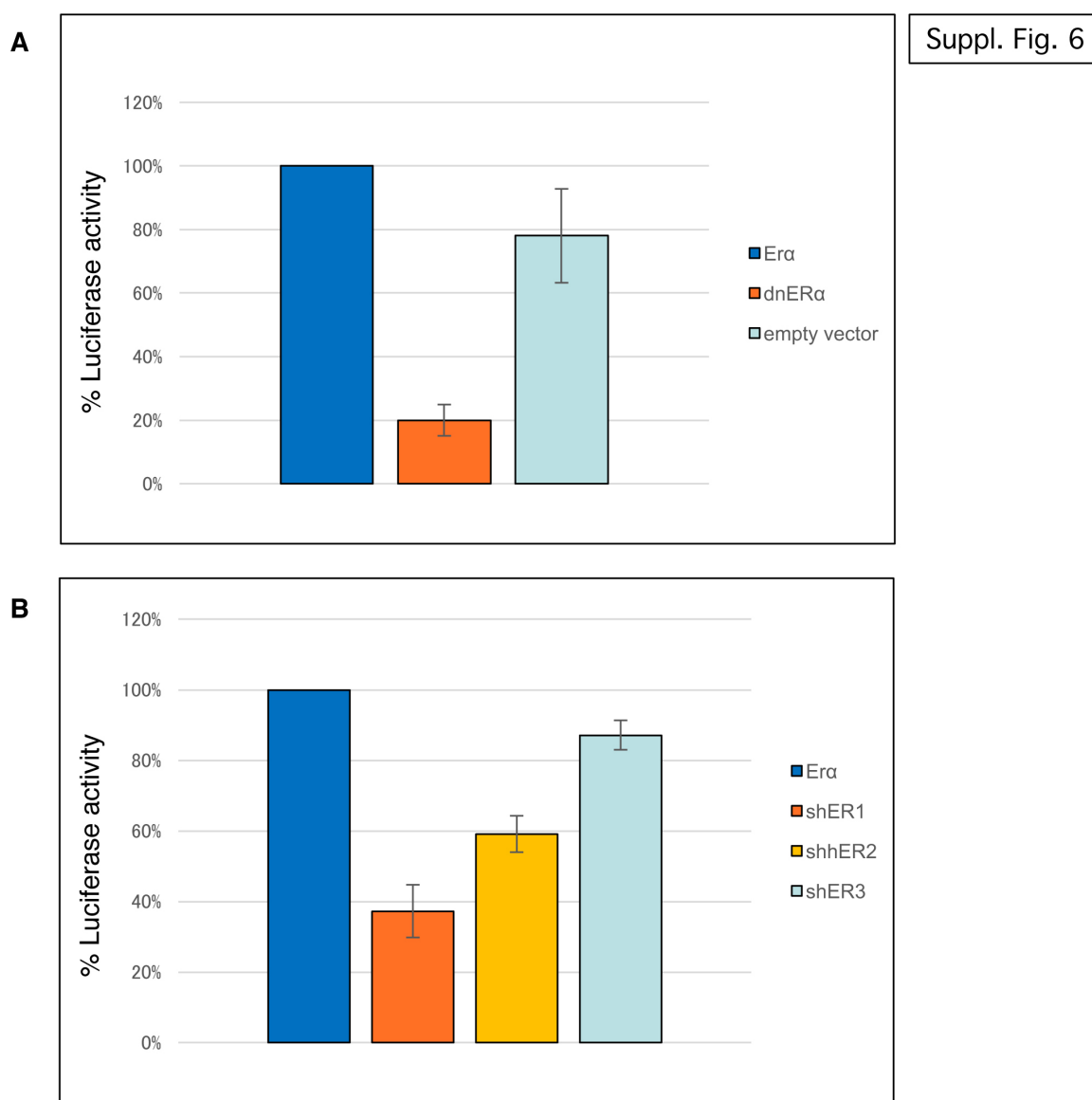


Fig. S6 Luciferase assay. (A) The diagram shows the activity of a Luciferase reporter under the control of a ERE enhancer in HEK293T cells co-transfected with ER α and the activator plasmid pT2K-CAG-rTA-M2 only (column 1), or in combination with the dominant negative form of ER α (dnER α) (column 2), or with the empty vector PT2K-B1-TRE-EGFP (column 3) (see material and methods for plasmids reference). The luciferase bioluminescence was normalised to β -galactosidase activity using ONPG. The activity of ER α alone (column 1) is taken as reference and set at 100%. Three biological replicates were performed in triplicate. The fold change is expressed as a percentage relative to the reference, with error bars showing the standard deviation (STD). (B) The diagram shows the activity of a Luciferase reporter under the control of a ERE enhancer in HEK293T cells co-transfected with ER α in combination with psilencer2-U6 empty vector (column 1), or with shER1 (column 2), or shER2 (column 3), or shER3 (column 4) cloned in psilencer. The luciferase bioluminescence was normalised to Renilla activity. The activity of ER α in column 1 was taken as reference and set at 100%. The fold change is expressed as a percentage relative to the reference. Error bars show the STD of three biological replicates performed in triplicate.

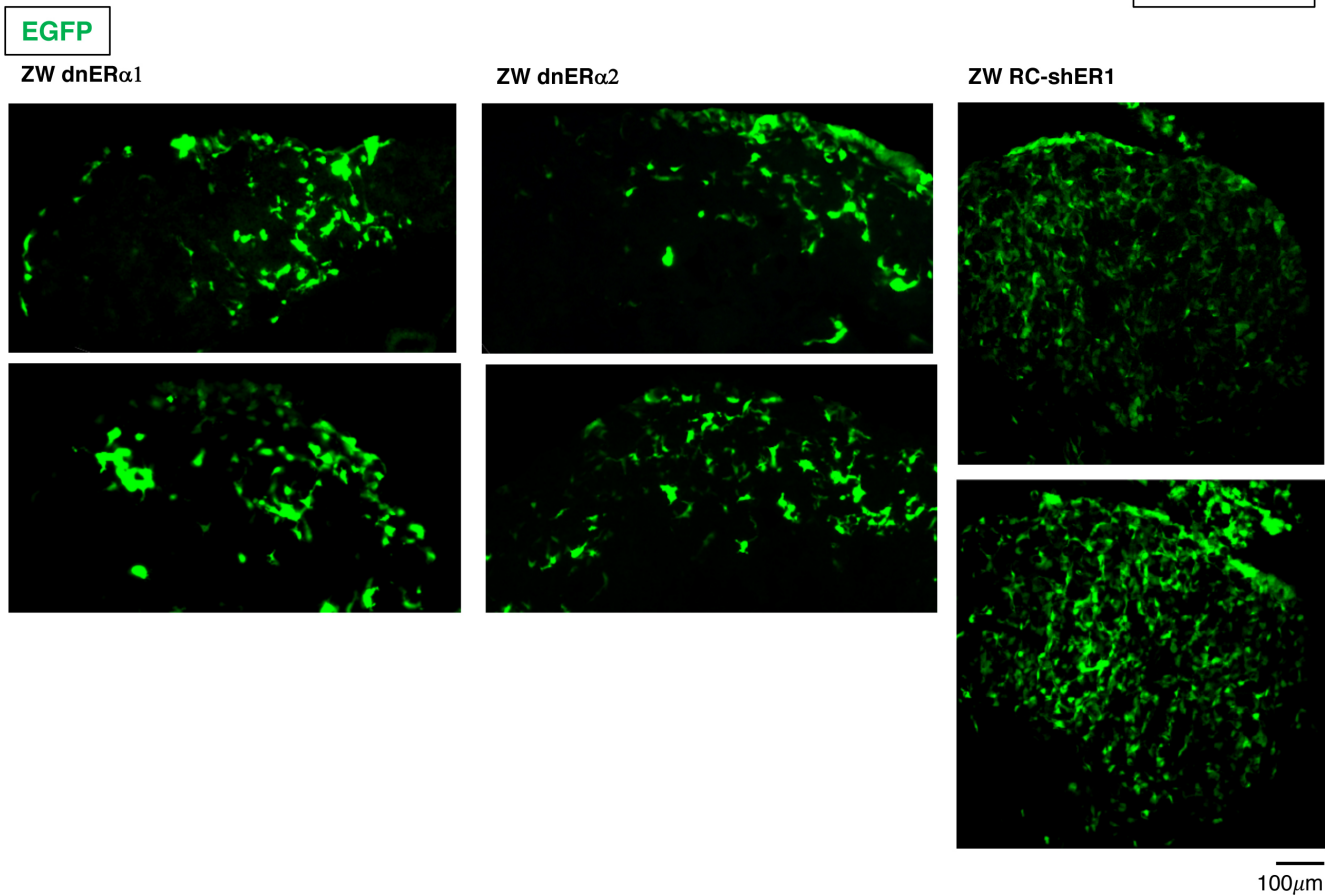


Fig. S7 EGFP reporter expression pattern in the sections from electroporated gonads shown in Fig. 6A-B.

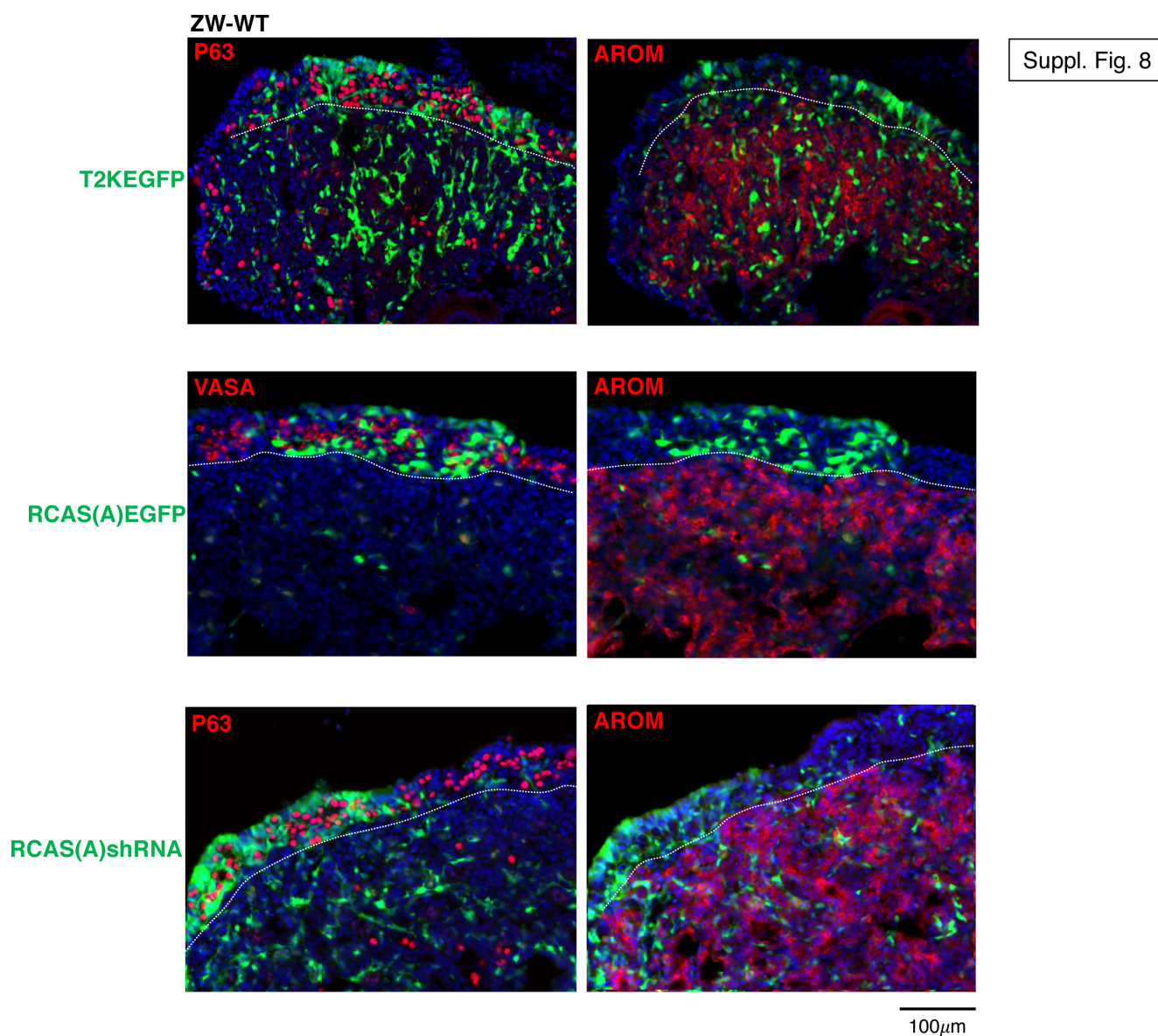


Fig. S8 The electroporation of gonadal epithelium does not affect cortex formation.

Immunofluorescent sections from D10 ZW embryonic ovaries electroporated at D2.5 (HH15-17) with Tol2 control expression vectors T2K-EGFP (see Material and Methods), the retroviral vector RCAS(A) expressing EGFP (RCAS(A)EGFP), or RCAS(A) expressing EGFP and a shRNA targeting the gene Foxl2 (RCAS(A)shRNA) (Oligo sequence: 5'-GGATCTACCAGTACATCAT-3'), which is not normally expressed in the epithelial cells. In red the germ cell marker (P63 or VASA) and P450aromatase (AROM); in green EGFP. White dotted lines show the cortex/medulla border. A minimum of 3 well electroporated embryos were analysed per construct.