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Retinoic acid signaling is dispensable for somatic development and function in the mammalian ovary

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ABSTRACT

Retinoic acid (RA) is a potent inducer of cell differentiation and plays an essential role in sex-specific germ cell development in the mammalian gonad. RA is essential for male gametogenesis and hence fertility. However, RA can also disrupt sexual cell fate in somatic cells of the testis, promoting transdifferentiation of male Sertoli cells to female granulosa-like cells when the male sexual regulator *Dmrt1* is absent. The feminizing ability of RA in the *Dmrt1* mutant somatic testis suggests that RA might normally play a role in somatic cell differentiation or cell fate maintenance in the ovary. To test for this possibility we disrupted RA signaling in somatic cells of the early fetal ovary using three genetic strategies and one pharmaceutical approach. We found that deleting all three RA receptors (RARs) in the XX somatic gonad at the time of sex determination did not significantly affect ovarian differentiation, follicle development, or female fertility. Transcriptome analysis of adult triple mutant ovaries revealed remarkably little effect on gene expression in the absence of somatic RAR function. Likewise, deletion of three RA synthesis enzymes (*Aldh1a1-3*) at the time of sex determination did not masculinize the ovary. A dominant-negative RAR transgene altered granulosa cell proliferation, likely due to interference with a non-RA signaling pathway, but did not prevent granulosa cell specification and oogenesis or abolish fertility. Finally, culture of fetal XX gonads with an RAR antagonist blocked germ cell meiotic initiation but did not disrupt sex-biased gene expression. We conclude that RA signaling, although crucial in the ovary for meiotic initiation, is not required for granulosa cell specification, differentiation, or reproductive function.

1. Introduction

In mammals, sex is initially determined in bipotential somatic progenitor cells of the genital ridge (the gonadal primordium). These cells eventually differentiate to form Sertoli cells in males or granulosa cells in females. This pivotal cell fate decision in a single precursor cell type triggers sexual differentiation in other gonadal cell types, ultimately establishing male or female sex throughout the body. After gonadal sex is determined, Sertoli and granulosa cells function to support male or female gametogenesis throughout reproductive life. In mice, male sexual development is triggered around embryonic day 10.5 (E10.5) by expression of the Y-linked *Sry* gene in the bipotential somatic progenitor cells. SRY activates the related gene *Sox9* and initiates a cascade of Sertoli-specific expression events that lead to testis differentiation (reviewed by Lin and Capel (2015)). In the absence of *Sry*, WNT4 and RSPO1 stabilize β-catenin/CTNNB1 and promote granulosa cell fate, leading to ovarian differentiation (Chassot et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999).

Once fetal Sertoli and granulosa cells are determined, germ cells in the ovary begin to undergo meiosis while germ cells in the testis enter mitotic arrest. This sex-specific divergence in germ cell fate involves FGF9 and WNT signaling (Bowles et al., 2010; Chassot et al., 2011; DiNapoli et al., 2006), as well as retinoic acid (RA), a diffusible derivative of vitamin A produced in the fetal ovary and in the mesonephroi of both sexes (Bowles et al., 2016, 2006; Koubova et al., 2006; Niederreither et al., 2002). RA also triggers meiosis in males but it does so with different timing due to somatic cell control of RA levels. Germ cells in the fetal ovary are exposed to RA during fetal life and therefore initiate meiosis before birth; by contrast, germ cells in the fetal testis are protected from RA by the RA-degrading cytochrome P450 enzyme CYP26B1 expressed in Sertoli cells (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). As a

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consequence, spermatogenesis is delayed until shortly after birth when CYP26B1 is down-regulated and new RA, produced by Sertoli cells, triggers the onset of spermatogonial differentiation and meiosis (Raverdeau et al., 2012).

Even after sex determination and sexual differentiation are completed, Sertoli and granulosa cell fates are actively maintained throughout postnatal life. Loss of the male transcriptional regulator *Dmrt1*, even in the adult gonad, causes postnatal transdifferentiation of Sertoli cells into granulosa-like cells, while loss of the female transcriptional regulator *Foxl2* causes a reciprocal cell-fate reprogramming in the ovary (Matson et al., 2011; Uhlenhaut et al., 2009). We found recently that RA can modulate somatic sexual cell fate. In *Dmrt1* mutant XY gonads, retinoid treatment enhances male-to-female trans-differentiation while vitamin A depletion, inhibition of RA synthesis, or deletion of the RA receptor *Rara* all strongly suppress the process (Minkina et al., 2014). Thus a crucial function of DMRT1 in Sertoli cells is to allow the use of RA to control male gametogenesis by sheltering the Sertoli cells from the feminizing action of RA, most likely by blocking the ability of RAR to activate or repress inappropriate target genes.

Given that RA is essential for mammalian spermatogenesis and hence for male reproduction, it seems paradoxical that RA can have such devastating consequences for Sertoli cells when DMRT1 is absent. The ability of DMRT1 to prevent inappropriate RA signaling activity allows males to use RA to control gametogenesis, but the evolutionary persistence of RA feminizing activity suggests that this function of RA might be advantageous in other settings. The most likely settings would be during fetal ovarian differentiation, where RA might promote establishment of the granulosa cell fate, or postnatally, when RA might help to maintain somatic cell fates or support reproductive function in the somatic ovary. The XX fetal somatic gonad is exposed to RA during differentiation: RA is produced in the adjacent mesonephros (Niederreither et al., 2002); RA synthesis genes *Aldh1a1* and *Aldh1a2* are expressed in the developing ovary by E12.5 (Bowles et al., 2016; Sutton et al., 2011; Teletin et al., 2017); and RA also becomes detectable in the XX gonad by E13.5 (Bowles et al., 2016). RAR-dependent signaling in XX germ cells commences by about E12.5 to E13.5, when a wave of meiosis sweeps the ovary and activates RA target genes in XX germ cells (Bowles et al., 2016). RAR-dependent signaling in XX somatic gonad cells may begin later, as cell type-specific microarray analysis indicates that *Rar* mRNA levels remain low in supporting cells between E11.5 and E13.5 (Jameson et al., 2012). Postnatal granulosa cells, like Sertoli cells, express components of the RA signaling pathway and thus also are candidates to respond to RA (Bagavandoss and Midgley, 1988; Kawai et al., 2016; Kipp et al., 2011; Minegishi et al., 2000a, 2000b).

We have investigated whether RA signaling in granulosa cells is important for sex determination, sex differentiation, or sex maintenance. We employed four distinct approaches to disrupt RA signaling in somatic cells of the genital ridge: 1) selectively deleting all three RA receptors by conditional genetics; 2) disrupting RA synthesis by selectively deleting three enzymes required for conversion of retinoid precursors to RA 3) cell-type specifically activating a dominant-negative RA receptor; and 4) culturing fetal gonads with an inhibitor of RA signaling. All four approaches indicated that RA signaling is not required for granulosa cell determination, differentiation or function: indeed XX animals lacking all three RA receptors in the somatic ovary are fertile females. We therefore conclude that RA is unlikely to be instructive for female somatic sex determination or for granulosa cell fate and function.

2. Results

2.1. Conditional deletion of Rar genes

To test the role of RA in granulosa cell differentiation, we first genetically disrupted RA signaling in somatic cells of the early fetal gonad. RA influences gene expression via RA receptors (RARs), which function as DNA binding transcription factors (reviewed by Rochette-Egly and Germain (2009)). Vertebrates have three genes encoding isoforms of RAR: *Rara*, *Rarb*, and *Rarg*. These genes express structurally similar proteins that are capable of functional redundancy (Mark et al., 2006). Although the three RAR proteins and their binding partners exhibit tissue and stage-specific expression, cross-regulation can cause loss of one *Rar* to induce aberrant expression of another (de The et al., 1990; Sucov et al., 1990). Therefore, to disrupt RA signaling as completely as possible we conditionally deleted all three receptors in granulosa cells. We combined "floxed" alleles of the three RARs ($Rara^{fl}$, $Rarb^{fl}$, $Rarg^{fl}$) (Chapellier et al., 2002a, 2002b, 2002c) with an *Sf1-cre* transgene that is active in the somatic gonad of both sexes starting around E10.5-E11, approximately when sex is determined (Bingham et al., 2006). In the conditional *Rar* alleles, *loxP* sequences flank exons harboring either the DNA-binding domain (in RARA and RARG) or the ligand-binding domain (in RARB), and Cre-mediated deletion removes sequences encoding these essential domains and also introduces a frameshift, producing null mutations (Chapellier et al., 2002a, 2002b, 2002c).

Rara deletion in Sertoli cells of the testis has previously been reported to disrupt spermatogenesis and also to interfere with RA-dependent cyclical gene expression in Sertoli cells (Vernet et al., 2006). Since *Sf1-Cre* is active in the genital ridges of both sexes (Bingham et al., 2006) including in pre-Sertoli cells, we were able to examine triple-mutant testes as a control for successful disruption of RAR-dependent signaling. Cyclical gene expression in Sertoli cells occurs as they promote successive stages of spermatogenesis, moving in waves down each testis tubule; a cross-section of the testis therefore reveals tubules at different stages of spermatogenesis, differing in Sertoli cell gene expression tubule-by-tubule (Fig. 1A, top). As expected, by two months mutant Sertoli cells had lost cycle-dependent expression of GATA1, instead exhibiting uniform expression in all tubules (Fig. 1A, bottom). In addition we observed the previously described vacuolization of the seminiferous epithelium and progressive germ cell depletion (Vernet et al., 2006), which was severe by six months (Fig. 1A, bottom). These results confirmed efficient disruption of *Rara*-dependent signaling by *Sf1-cre*. However it is important to note that because the other two RARs are not required in Sertoli cells (Vernet et al., 2006), the effectiveness of their deletion was not addressed by this particular experiment.

We directly confirmed efficient excision of floxed *Rar* exons in the ovary using RNA-seq data from adult ovaries (Fig. 1B). Reads mapping to the floxed exons of *Rara* and *Rarg* were specifically reduced in ovaries of mice carrying the *Sf1-Cre* transgene by twenty-one-fold and eleven-fold, respectively. *Rarb* mRNA was not expressed significantly above background levels in control ovaries lacking the *Sf1-Cre* transgene, making the efficiency of *Rarb* deletion difficult to quantify, though the floxed *Rarb* exons did appear reduced in expression in mutants (Fig. 1B). The apparent efficiency of *Rar* gene deletion measured by RNA-seq was unexpectedly high, as we had anticipated a background of unaffected mRNA expressed in germ cells, in which *Sf1-Cre* is not active. Cell-type specific RAR expression in ovaries has historically been difficult to assess due to lack of specific antibody reagents, and our data suggest that *Rar* genes are not highly expressed in adult germ cells. Together, therefore, these data strongly suggest that somatic cells of *Sf1-Cre*-positive *Rar* triple mutant ovaries had little or no functional RAR expression.

Next we examined the phenotype of *Rar* triple-mutant ovaries. Surprisingly, loss of functional RARs in fetal somatic cells and their descendants had no apparent effect on ovarian development or ability to support germ cells, either prenatally or postnatally. Triple-mutant females were fertile with normal litter sizes (seven dams produced an average of 6.7 pups in sixteen litters), confirming the ability of mutant granulosa cells to support oogenesis, meiosis and ovulation. At 6 weeks after birth, granulosa cells depleted of all three receptors appropriately