Global Deletion of ALDH1A1 and ALDH1A2 Genes Does Not Affect Viability but Blocks Spermatogenesis

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The transition of undifferentiated A spermatogonia to differentiated spermatogonia requires the action of retinoic acid (RA). The synthesis of retinoic acid from retinal in the seminiferous epithelium is a result of the action of aldehyde dehydrogenases termed ALDH1A1, ALDH1A2, and ALDH1A3. We used a mouse with a global deletion of the Aldh1a1 gene that is phenotypically normal and the CRE-loxP approach to eliminate Aldh1a2 genes globally and from Sertoli cells and germ cells. The results show that global elimination of Aldh1a1 and Aldh1a2 genes blocks spermatogenesis but does not appear to affect viability. The cell specific elimination of Aldh1a2 gene showed that retinoic acid synthesis by Sertoli cells is required for the initial round of spermatogonial differentiation but that there is no requirement for retinoic acid synthesis by germ cells. In both the global gene deletion and the cell specific gene deletions the maintenance of Aldh1a3 activity could not compensate.

Keywords: ALDH1A1, ALDH1A2, retinoic, spermatogenesis, viability

INTRODUCTION

The active form of vitamin A is retinoic acid that is synthesized in precise cellular locations by a two-step mechanism. First, retinol which is the circulating form of the vitamin is oxidized in a reversible reaction to retinyl dehydrogenase (Rdh10). Retinal is then oxidized to retinoic acid in a nonreversible reaction by one of 3 retinal dehydrogenases known as ALDH1A1, ALDH1A2, and ALDH1A3 (1).

ALDH1A2 and ALDH1A3 are required during fetal development. ALDH1A2−/− mice die during embryonic development and ALDH1A3−/− mice die shortly after birth (2, 3). However, ALDH1A1−/− mice develop normally (4). In humans, ALDH1A1 mRNAs is found in the liver, kidney, testis, brain, lung, red blood cells, and lens of the eye while ALDH1A2 mRNA is found in the testis, uterus, and skeletal muscle, and ALDH1A3 mRNA is localized in the prostate, trachea, intestine, and testis (5). Clearly all three ALDH1A enzymes contribute to RA synthesis during postnatal life. All together these studies underscore the tissue-specific central roles that ALDH1A enzymes play in animal physiology, and the vital significance of obtaining information concerning the expression and essential nature of the activity of these enzymes in human tissues.

In the mouse testis, retinoic acid is essential for the progression of undifferentiated spermatogonia A to become differentiating spermatogonia A1 and enter into spermatogenesis (6). In the absence of retinoic acid, undifferentiated spermatogonia never begin this progression (7). We have previously shown that deletion of the Rdh10 gene in Sertoli cells alone will inhibit the

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progression of undifferentiated spermatogonia and also the deletion in both germ cells and Sertoli cells blocks this progression (8). Enzyme inhibitors have been used to eliminate the activity of all 3 aldehyde dehydrogenases and using the CRElox P approach all 3 Aldh1a genes have been deleted only in germ cells, only in Sertoli cells and in both cell types (9, 10). Both of these approaches have shown that the retinal dehydrogenases are essential for spermatogenesis and both enzymes are present in germ cells and Sertoli cells. Aldh1a1 is most highly expressed in the Sertoli cells and Aldh1a2 and Aldh1a3 are expressed primarily in the germ cells but all 3 enzymes appear to be expressed at some level in both cell types (11).

It has been known that global deletion of the Aldh1a1 gene in mice has little effect and does not significantly alter fertility (4). Recent studies have shown that the knockout of Aldh1a2 alone or the simultaneous knockout of Aldh1a1-3 in germ cells has little effect on successful spermatogenesis and fertility (9, 12). However, the simultaneous knockout of Aldh1a1-3 in Sertoli cells does not allow the undifferentiated A spermatogonia to progress to differentiating A1 spermatogonia (9). In this study we have broadened these previous observations by examining the effect on spermatogenesis of leaving the Aldh1a3 gene intact. We started with a mouse mutant with a global deletion in Aldh1a1.

From that genotype we used the CRE-loxP system to remove the Aldh1a2 gene in germ cells and/or Sertoli cells and globally in all cells. In addition, and we have included sperm counts and fertility studies.

**MATERIALS AND METHODS**

**Animal Care, Breeding and Genotyping**

All procedures involving mice were approved by the Washington State University Committee on the Use and Care of Animals. The mouse colonies were maintained in a temperature-controlled environment with access to food and water ad libitum. Mice were euthanized by CO2 asphyxiation following cervical dislocation. Four mouse lines were generated for this study, each expressing Cre recombinases to inactivate the Aldh1a2 gene in Sertoli cells or globally. The Aldh1a2flfl, ERT-Cre line was created by breeding Aldh1a2flfl, ERT-Cre (a gift from John Amory and Jisun Paik at the University of Washington with permission from Jackson labs, JAX stock #012247). The offspring who were heterozygous for all 3 alleles were paired with females, generated above, who were Aldh1a1flfl, Aldh1a2flfl, Amh-Crefl. Male offspring from this pairing who were Aldh1a1+/−, Aldh1a2−/−, Amh-Cre− were paired with females, Aldh1a1+/−, Aldh1a2−/−, Amh-Cre− to generate experimental and control mice for both lines.

To determine the genotypes of the mice, PCR reactions were performed on template generated from a tail clip from each mouse. The primer sets for Amh-Cre and ALDH1A1 are as follows: Amh-Cre forward primer GCGGTCTGGCAGTAAACTATC and reverse primer GTGAAACAGCATTGTGCTC; ALDH1A1 forward primer CAACCCCTGAAATCCTC and reverse primer for the knockout TGGATTGGAATGTGTGAG AG and reverse primer for wild-type GACAGATTGAGAGC TGTTTACC. All others have been reported elsewhere (12).

**Fertility and Sperm Counts**

Males with confirmed KO in germ cells or Sertoli cells or both germ and Sertoli cells or ERT-Cre, tamoxifen treated males and controls were aged to 7 weeks and then were paired with a female of known fertility for 2 months to assess fertility. At the end of the 2 months the males were euthanized for study and the females left for 3 more weeks to continue to monitor for litters. The number of offspring and number of litters for each male was recorded. Following this timeline, each male in this study was euthanized at approximately 4 months. The body was fixed testes were embedded in paraffin, sectioned and incubated at 37°C for 15 minutes. Three µl of the sperm suspension was applied to a Cell Topping and Griswold Retinoic Acid Synthesis in Testis

**Histology**

Bouin’s fixative for immunohistochemistry and one was detunicated, snap frozen and weighed. Both cauda epididymides were placed in DMEM at room temperature and processed for counting sperm. The cauda epididymides were cut into approximately 1mm³ pieces and incubated at 37°C for 15 minutes. Three µl of the sperm suspension was diluted 4 fold with DMEM before counting the sperm.

**Tamoxifen Preparation and Administration**

Tamoxifen (Sigma T5648) was dissolved in 10% ethanol and 90% sesame oil at a concentration of 10 or 20 mg/ml, and the solution...
was wrapped in aluminum foil to protect from light. Mice were injected intraperitoneally with 40 mg/kg tamoxifen once per day from postnatal day 8 to 10 and/or with 80 mg/kg tamoxifen for 5 days starting at day 21 postpartum. Alternatively, at day of birth and postnatal day 1, mice were injected intraperitoneally with 100 mg/kg tamoxifen dissolved in sesame oil at only a concentration of 5 mg/ml. Tamoxifen was stored for a maximum of one week at 4°C and warmed to room temperature before injections. To confirm that the action of tamoxifen on the ERT-Cre resulted in excision of the ALDH1A2 gene, Aldh1a2<sup>fl/fl</sup> genotyping was performed on tail clips collected after euthanasia.

### Retinoic Acid Injections

Retinoic acid (Sigma R2625) was made fresh each day in DMSO. For the mice expressing the Stra8-cre and/or AMH-cre, 10 µl of 20 mg/ml was intraperitoneally injected once at day 21. For the males expressing the ERT-cre, RA at a concentration of 10 mg/ml was injected intraperitoneally at a dose of 12.5 µg/g body weight once at day 21. Males were euthanized after one round of spermatogenesis, 42 days later. As a control the same volume of DMSO was injected at day 21.

### RESULTS

Using the Aldh1a1<sup>−/−</sup>, Aldh1a2<sup>−/−</sup> mice as our starting point we first wanted to see whether the presence of Aldh1a3 altered the results from the previous studies of Teletin et al. (9). Their data showed that the deletion of all 3 Aldh1a genes in Sertoli cells blocked spermatogenesis at the conversion of A spermatogonia to A1 spermatogonia in mice. However, if these mice were injected with retinoic acid once, the block was removed, and spermatogenesis proceeded normally and continuously. They also showed that spermatogenesis was normal with the deletion of all 3 Aldh1a genes in germ cells alone. They concluded that RA from Sertoli cells was necessary for the initial A to A1 conversion of spermatogonia but that RA from germ cells could maintain the process. If RA synthesis was normal in Sertoli cells but the mice were routinely aged to 4 months and some were left for over 6 months and showed normal body weight and no obvious pathologies.

### DISCUSSION

The action of retinoic acid (RA) is required for normal spermatogenesis in rodents and possibly all mammals (15). We have previously shown that RA is synthesized locally in pulses along the seminiferous tubules (16). These pulses are required for the transition of undifferentiated A spermatogonia into A1 spermatogonia and into the differentiation pathway (7). The location of these pulses corresponds to the onset of spermatogenesis and the initiation of the cycle of the seminiferous epithelium. In the absence of RA there is no cycle, and no germ cells advance beyond undifferentiated spermatogonia. It has been
established that the pulse of retinoic acid is a result of the localized synthesis of retinal by retinol dehydrogenase 10 (RDH10) and the conversion of retinal to retinoic acid by 3 aldehyde dehydrogenases designated ALDH1A1, ALDH1A2 and ALDH1A3 (8, 9, 17, 18). Both the Sertoli cells and the germ cells have the capacity to synthesize RA (9).

Deletion of either the Aldh1a1 gene or Aldh1a2 gene alone has no major consequences to spermatogenesis or the mice. Teletin et al. (9) used a Cre-Lox P approach to eliminate all 3 Aldh1a genes from Sertoli cells or from germ cells or from both cell types. From these experiments they determined that RA from Sertoli cells was essential to begin the first wave of germ cell development. Deletion of all 3 genes from germ cells had no effect on spermatogenesis. However, in the Sertoli cell specific triple gene deletion, if RA was present during the first wave in the form of a single injection, spermatogenesis proceeded normally and was continuous suggesting that the germ cell RA was sufficient to maintain spermatogenesis once it had been initiated. We addressed these studies using a different genetic approach where we left the Aldh1a3 gene intact. While there are only low levels of ALDH1A3 in the testis we wanted to determine if it was sufficient to maintain spermatogenesis.

Our cell specific deletions of only Aldh1a1 and Aldh1a2 recapitulated the results from Teletin et al. (9) who deleted all

**TABLE 1** | The Aldh1a1<sup>-/-</sup>, Aldh1a2<sup>+/+</sup> mice were crossed with the designated Cre to delete gene in Sertoli cells or germ cells or both.

| Experiment                        | N   | Testis wt. | Sperm/cauda |
|----------------------------------|-----|------------|-------------|
| control                          | 10  | 0.129+/-.013 | 92+/-.27    |
| Stra8 Cre                        | 7   | 0.118+/-.013 | 79.6+/-.37  |
| AMH Cre                          | 6   | 0.018+/-.002 | zero        |
| AMH Cre + RA                     | 9   | 0.077+/-.018 | 84+/-.21    |
| Stra8 CRE and AMH CRE            | 8   | 0.025+/-.005 | zero        |
| Stra8 CRE and AMH CRE plus RA   | 7   | 0.029+/-.005 | zero        |

In some experiments (4 and 6) mice were treated with RA and analyzed 4 weeks later to determine if spermatogenesis could recover. N is number of individual mice. Values plus standard deviation are shown.
it has been proposed that inhibition of the synthesis or the action of RA may be initiated. From germ cells can only maintain spermatogenesis after it has been initiated. For ALDH1A enzyme studies it is possible that ALDH1A3 alone is not sufficient to maintain spermatogenesis. Nonetheless, inhibitors targeting ALDH1A1 and ALDH1A2 would certainly act as effective contraceptive compounds while not affecting gross viability. While we did not examine the physiopathology of potentially affected systems such as the immune system these results are significant in attesting to the feasibility of a RA focused contraceptive approach.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The animal study was reviewed and approved by WSU IACUC.

**AUTHOR CONTRIBUTIONS**

Experimental protocols were done by TT and the experiments were planned by TT and MG.

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