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The Role of Dosage Sensitive Sex Reversal Adrenal Hyperplasia Congenita 1 (DAX-1) in Mouse Oogenesis

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in Biology with Honors
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ABSTRACT

Although critically important to fertility and reproduction, the mechanisms controlling development of mammalian primordial germ cells into functional oocytes are poorly understood. Understanding the mechanisms that regulate oocyte development will ultimately reveal significant insights into female infertility problems in mammals, including humans. In order to decipher what mediates the formation of a functional oocyte, we are studying oogenesis in the mammalian model *Mus musculus*. During mouse embryonic development the oocytes develop as interconnected clusters of cells called germline cysts. Evidence suggests that in humans, oocytes are also organized into cysts. Eventually the cysts undergo programmed cell breakdown, each giving rise to at least one oocyte. In the mouse, a subset of cells in each cyst dies, with only a third of the original number of oocytes surviving. DAX-1 encodes a transcription factor that has been implicated in sex determination and gonad differentiation. Adult female mice lacking the Dax-1 gene exhibit an abnormal multiple oocyte follicle phenotype. These multiple oocyte follicles may be cysts that never completed the cyst breakdown process. To determine if Dax-1 plays a role in the process of cyst breakdown, ovaries from animals lacking Dax-1 were examined at postnatal day (PND) 7 which is just after cyst breakdown is completed. PND7 mutants had reduced cyst breakdown and reduced oocyte survival. In addition, expression of Dax-1 was examined in normal mice and was found to be present during the cyst breakdown period.
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As I begin the writing of my honors thesis, I realize that simultaneously my researching days in BRL are ending. These last two years in the Pepling Lab have impacted me more than all my biology courses combined.

I began my research the summer before my junior year, after consulting with Dr. Erdman. He had taught me that spring and encouraged me to pursue research. As a premedical student looking to beef up the future medical school applications and a strong recommendation from a professor I trusted, I decided to enroll in the Honors Thesis Program.

Next came finding a lab, which can sometimes be an impossible task, due to a high volume of students desiring research opportunities. I was not exactly picky as to where I ended up, I just wanted to do research. Chances had it that a new professor by the name of Dr. Pepling had no undergraduates in her lab. I believe there was a reason for this, that being the guaranteed busy schedule of a university tenure candidate. Luckily, as I came to see over the next two years Melissa is not exactly the best at saying no. If you are reading this now and looking for a lab to be placed in, I cannot recommend highly enough to speak with her about available space. She is extremely nice, patient (which was definitely needed with me), and has a genuine interest in the learning experience of her students. Perhaps the best part about working with Melissa was that although she is serious about the lab, she also knows how to make the experience a fun one. With many experiments gone wrong under my belt, I know that if it was not for the genuine fun and relaxed learning atmosphere that she provides, I may not have become as
dedicated to my thesis project over the years. No matter how many experiments went wrong she always found a possible reason explanation that didn’t involve it being my fault, and encouraged me to explore the possibilities of what could optimize my results. For that, I am grateful. I would have to say the first time I really knew that I was truly invested in my thesis work was junior year. It was a cold winter’s night in Syracuse around 4 am and I found myself hustling to BRL because I was convinced that the first PCR reactions that I ever set up alone had caught on fire in the thermocycler and the lab was burning down. Now, hundreds of PCR reactions later, I can laugh at myself for even thinking that was possible, but more importantly I can be proud of the level of dedication I had to my research. Any student at SU I believe would agree that it takes something pretty important to get you out of bed before dawn on a snowy Syracuse winter’s night. I consider myself lucky to have been able to do research at Syracuse these last two years. Without a doubt I am a better biology major and scientist than I would have ever been if I had not signed up to complete an honors thesis.
INTRODUCTION

Oogenesis is the process that leads to the formation of a healthy egg cell from a primordial germ cell. The word was created from the prefix "oo-" (Greek oon, egg) and "genesis" (the coming into being of something). Together these two mean the coming into being of an egg. The differentiation of primordial germ cells into oocytes is essential for fertility. In the mouse, at approximately 10.5 days post coitum (dpc) germ cells migrate to the developing gonad (Monk and McLaren 1981) (Figure 1). After arrival at the genital ridge, germ cells arrange into clusters or germ cell cysts. Cysts grow progressively by mitosis from 10.5 dpc to about 13.5 dpc (Pepling and Spradling, 1998). After this mitotic stage, the cells of the cyst then proceed to enter meiosis and then become arrested in the diplotene stage of prophase I. The cytokinesis is incomplete.

Figure 1. Timeline of Germ Cell Development in the Mouse (adapted from Pepling and Spradling, 2001). Green staining is for vasa (an oocyte specific marker) to show oocyte cytoplasm and red staining is propidium iodide to show nuclei of all cells, both somatic cells and oocytes.
which results in interconnected cells joined by bridges (Pepling and Spradling, 1998).

After this mitotic stage, the cysts then proceed to enter meiosis and then become arrested in the diplotene stage of prophase I.

Cysts have been well studied in *Drosophila*. During *Drosophila* oogenesis a sixteen cell cyst is formed as a product of four mitotic divisions of a cystoblast (Figure 2).

Following formation of the cyst, organelles and specific mRNAs are transported into one cell. This is the step that underlies the determination of one cystocyte as an oocyte, while the others become nurse cells. Although mouse oogenesis is not identical to that of *Drosophila*, both appear to operate through a very similar underlying mechanism.

![Figure 2: Cyst formation in the Drosophila ovary results in a 16 cell cyst. One cell will become the oocyte while the others act as nurse cells (shown in gray) by providing the oocyte (blue) with organelles, mRNA’s and proteins.](image)

In the mouse shortly after birth (PND 1), there are two major processes occurring simultaneously in the ovary affecting germ cell cysts. These two processes are germ cell death and cyst breakdown (Figure 1). Once a cyst breaks down, the single oocytes become encased by granulosa cells. A single oocyte surrounded by a layer of granulosa cells is considered a primordial follicle. Currently the factors controlling cyst breakdown, oocyte survival and primordial follicle assembly are unknown.

Studies show that several mammalian species lose one half to two thirds of their oocyte population early in development before individual follicles are formed (Baker,
In the human fetus approximately 7 million oocytes will develop, but after birth only about 2 million will remain. This germ cell loss also occurs in the mouse (Borum, 1961, Ratts et al., 1995). From 13.5 dpc to birth germ cell numbers remain rather consistent in the mouse. However, between PND 2 and PND 4 (shortly after birth) there is a drastic drop in the number of oocytes (Pepling and Spradling, 2001). The germ cells die off via programmed cell death, or apoptosis (Figure 3). The result of this process is that one third of the initial germ cell population remains (Pepling and Spradling, 2001). The function of germ cell loss is not understood, but one hypothesis is that it is a method of defective germ cell elimination (Baker, 1972).

Cyst breakdown is the second process occurring leading to the formation of the primordial follicle. By two days after birth, mouse germ cell cysts have broken down into single oocytes (Pepling and Spradling, 2001). Figure 1 shows this occurring from PND 2 to PND 4. The current model is that one cell of a cyst dies resulting in two smaller
cysts (Figure 4). This process repeats until a few individual oocytes remain. Only a subset of the oocytes survives suggesting that there are two types of germ cells that make up a cyst: (1) cells that will survive and become functional oocytes and (2) cells that support the oocytes and then die. The dramatic germ cell loss seen in the mouse occurs at the same time as cysts are breaking down (Figure 5).

This suggests that germ cell loss and cyst breakdown are regulated processes involved in the

Figure 4: Model of Cyst Breakdown (Pepling and Spradling, 2001). Shown is the progressive breakdown of an 8 cell cyst. Note that gaps represent intercellular bridges. Dying cells (green) cause breakage into smaller cysts, yielding three oocytes (right) that will become surrounded by granulosa cells to become primordial follicles.

Figure 5: Germ cell death and cyst breakdown coincide (from Pepling and Spradling, 2001).
formation of oocytes. However, the genes regulating these processes are unknown.

Dax-1 encodes a transcription factor that has been implicated in both sex determination and gonad differentiation (Yu et al, 1998). It belongs to the nuclear hormone receptor family. Steroid hormone receptors are found in both the cytosol and the nucleus of cells. When these receptors bind their respective ligands they undergo a conformational change that activates them so that they may recognize and bind to specific nucleotide sequences. These specific nucleotide sequences in the DNA are known as hormone response elements (HREs). When ligand-receptor complexes interact with DNA they alter the transcriptional activity of the associated gene. It is through this mechanism that steroid hormones turn genes on. The steroid family of receptors all have three distinct domains: a ligand-binding domain, a DNA-binding domain and a transcriptional regulatory domain. The idea that steroid hormone receptors may play a role in the process of cyst breakdown is based on the observation of multiple oocyte follicles in mice lacking one steroid hormone receptor called Dax.

The Dax-1 protein is 470 amino acids long and contains two distinct protein domains. Dax-1 is classified as an orphan nuclear hormone receptor, because currently there is no known ligand. Like other steroid hormone receptors, Dax-1 contains the ligand binding domain in the C-terminus. However, Dax-1 lacks the DNA binding

Figure 6. Structure of Dax-1 protein. The ligand binding domain in the C-terminus classifies Dax-1 as a hormone receptor despite lacking a ligand. Note the N-terminus has a 3.5 repetitive region of amino acids instead of the traditional DNA binding domain.
domain that is usually present in the N-terminus. The N-terminus of Dax-1 instead contains a three repeat leucine rich domain that may function as a protein interaction domain and a RNA binding domain (Zhang et al., 2000). Dax-1 has been shown to interact with both estrogen receptor alpha and estrogen receptor beta through coimmunoprecipitation and using the yeast two hybrid system (Zhang et al, 2000). The structure of Dax-1 can be seen in Figure 6.

The expression of Dax-1 is localized strictly to tissues involved in steroid hormone production and reproductive function: adrenal cortex, testis, Leydig and Sertoli cells, ovarian theca and granulosa cells, and the pituitary gonadotropes (Lalli and Sassone-Corsi, 2003). Traditionally, Dax-1 was considered an “anti testis” gene because duplication of Dax-1 resulted in male (XY), to female sex reversal, better known as dosage sensitive sex reversal (DSS). Male humans with DSS have no testis cord, while mice with DSS have disorganized testis cord formation (Swain et al. 1998). Deletions and other mutations of Dax-1 result in adrenal hyperplasia congenita (AHC). This disease is characterized by a lack of adrenal cortex. Hypergonadotropic hypogonadism (HH ) patients fail to release GnH and have a pituitary defect in gonadotropin production which results in small gonads.

In a normal mouse ovary, a follicle consists of a single oocyte surrounded by several layers of granulosa cells (Figure 7A). Follicles that contain more than one oocyte so-called multiple oocyte follicles occur but are extremely rare (less than 1 percent; Kent, 1960). Dax-1 deletion female ovaries exhibit abnormal multiple oocyte follicles (Figure 7B). These abnormal follicles may be cysts that did not complete the cyst breakdown process. With this observation, we propose that Dax-1 is important in the
cyst breakdown process. In situ hybridization shows the first expression of Dax-1 in the somatic cells of the female gonad at 11.5 dpc (Swain et al, 1999). Dax-1 expression continues to be expressed until 15.5 dpc and has been reported in the stromal, theca, and granulosa cells of the adult ovary (Swain et al, 1996). To determine if Dax-1 plays a role in the process of cyst breakdown, the expression of Dax-1 protein in the ovary was examined during cyst breakdown and ovaries from PND 7 females were examined for differences in cyst breakdown and oocyte survival.
MATERIALS AND METHODS

Mouse strains

Two strains of *Mus musculus* were maintained for this project. The CD-1 strain was obtained from Charles River Labs. The Dax-1 knockout strain was obtained from Dr. Larry Jameson at Northwestern University and was on a C57BL6 background. Dax-1 transgenic mice were based on a Cre/Lox-P genetics construct. The gene cre, short for cyclization recombination, encodes a site-specific DNA recombinase named cre. The protein product of the cre gene has the ability to recombine DNA at specific sites. These sites are known as Lox-P (locus of X-over P1) sequences. They work in pairs and are used to target DNA. Each sequence is 34 base pairs long and interacts specifically with Cre via recombination to alter gene expression. For this specific research project the Cre-Lox-P system was used to delete the DNA that codes for Dax-1. Mice that expressed Dax-1 flanked by Lox-P sites were bred with mice that expressed Cre driven by a constitutive promoter. Offspring expressing Lox-P sites flanking Dax-1 DNA and the Cre gene resulted in deletion of Dax-1. This was achieved through a recombination mechanism that involved Cre cutting each Lox-P site in half. All DNA between the two sites is removed and the remaining halves of the Lox-P sites are ligated together again. The excised DNA is degraded. The Dax-1 transgenic strain was housed in a controlled sterile environment on the 4th floor of BRL to prevent infection.
Dissection

Neonatal ovaries were dissected in lower case phosphate buffered saline (PBS). Ovaries were harvested using a Nikon dissecting microscope and collected for evaluation. Tails snips were also collected from Dax-1 mutants so that they could be used for genotyping.

Isolation of DNA from Mouse Tails

A DNAeasy kit was obtained from QIAGEN to isolate DNA from mouse tails. Each mouse tail was treated with the enzyme proteinase K and placed in a 55°C water bath overnight. Samples were next treated with a 1:1 concentration mixture of Buffer ATL and 100 percent ethanol and then vortexed. The vortexed sample was placed in a spin column and centrifuged for 1 minute at 8000 rotations per minute (rpm). Flow through was discarded and then each spin column was placed in a new collection tube and treated with 500 µl AW1 buffer plus ethanol. Samples were then centrifuged for 1 minute at 8,000 rpm. Flows through tubes were discarded and spin columns were placed in fresh eppendorf tubes and treated with 100 µl AE buffer. Samples were treated with 500 µl AW2 buffer plus ethanol and centrifuged for 3 min at 14,000 rpm. Flow through was discarded and spin columns were placed in fresh eppendorf tubes and treated with AE buffer. Samples were then centrifuged 1 min at 8,000 rpm. Flow through was collected and stored at -20°C.

DNA Amplification by Polymerase Chain Reaction

PCR reactions were a mixture (per sample for PCR) of 4 µl of dNTP mixture (containing
1.25 mM of dATP, dCTP, dGTP, and dTTP), 2.5 µl Buffer A, 1 µl of each primer, 0.5 µl of Taq polymerase and distilled water to a volume of 20 µl. Then, 5 µl of DNA was added to each tube. Reactions were placed in a thermocycler for amplification. There were 3 Dax-1 primers. The primers for the Dax-1 gene were:

Primer 1 (Mdxio2-FOR) is CCT TAG AAG TGT TGC TTC TG
Primer 2 (Mdxio1-rev1) is ACA GCT CAC CAC AGG ATC TT
Primer 3 (Mdx5ec3100rev1) is GCA CAT TGT TCT GAG TGG CT

Primers 1 and 2 amplify a 180 bp fragment from the wt allele. Primers 1 and 2 amplify a 200 bp fragment from the lx allele (the loxP site is about 20 bp). Primers 1 and 3 amplify a 300 bp fragment from the del allele (but do not amplify a fragment from the lx allele or the wt allele because the primers are too far apart).

There were two primers for the Cre gene:

5’Cre GGA CAT GTT CAG GGA TCG CCA GGC G
3’Cre GCA TAA CCA GTG AAA CAG CAT TGC TG

PCR cycles were run at the following temperatures:

5 min 94°C
1 min 94°C
1 min 54°C
1 min 30 sec 72°C repeat 38X

8 min 72°C

**Verification of PCR product by Gel Electrophoresis**

Two percent agarose gels were used for verification of PCR product by gel electrophoresis. 100 µl of 10X TBE buffer was diluted with 900 µl dH2O to reach 1X TBE concentration. 100 ml of the 1X TBE buffer mix was added to 2 grams agarose and microwaved for 2 minutes. 5 µl of ethidium bromide was added to the heated gel and then the gel was poured onto a gel tray and allowed to cool until solidified. 10 ul of each PCR reaction was then added to 2 µl of 6x loading dye. 12 µl of each sample was loaded on a gel and electrophoresed for 1 hr at 100-110 volts in a bath of 1X TBE sample buffer. A 100 bp marker was also electrophoresed as a reference for band size. An Image Station equipped with UV light was used to visualize bands on all agarose gels.

**Whole Mount Antibody Staining (Indirect)**

Indirect immunoflorescence was used for staining neonatal ovaries. Alexa fluor 488 conjugated to an anti rabbit antibody was used as the secondary antibody. It was preabsorbed over night with embryo powder and incubated overnight on a nutator at 4°C. Ovaries were dissected in PBS and stored in eppendorf tubes. In order to fix the tissues ovaries were incubated in 600 µl of 5% formaldehyde solution overnight at 4°C. Ovaries were then rinsed 2x in 1 ml PT quickly and then 1 ml PT for at least 30 minutes. They were then incubated in 1 ml PT + 5%BSA overnight 4°C. Next, ovaries were incubated with a primary antibody diluted in 500 µl PT + 5% BSA overnight at 4°C. Dax-1
antibody was used at 1:500 (K-17 from Santa Cruz Biotechnology), Stat3 (C20 from Santa Cruz Biotechnology) at 1:500 and EMA at 1:1. Following incubation with a primary antibody, ovaries were washed 1x in 1 ml PT + 1% BSA for 30 minutes at room temperature. Next ovaries were treated with 10 µl of 10mg/ml RNase A diluted in 1 ml PT + 1% BSA for 30 minutes. Eppendorf tubes containing the ovaries were wrapped in foil to block light exposure and then 10 µl of 0.5mg/ml propidium iodide in 1 ml PT + 1% BSA was applied to the ovaries for 20 minutes to stain the nuclei. Ovaries were then rinsed for 30 minutes with PT + 1% BSA. Ovaries were incubated in 500 µl of preabsorbed secondary antibody diluted 1:200 in PT + 5% BSA overnight at 4°C. Next the ovaries were rinsed with 1 ml of PT + 1% BSA 3x for 30 minutes. The last wash was with 1 ml PBS for 5 minutes. A few drops of vectashield were added to the ovaries. Ovaries were mounted on a slide and stored at -20°C.

**Confocal Microscopy**

All indirect immunofluorescence was observed through a confocal microscope. Ovaries were collected and stained with a nuclear and germ cell marker. Confocal analysis was performed. Eight sections of each ovary were selected. A single section picture was taken as well as a 10 stack picture that was centered at the single section picture. For each ovary total oocyte count number and single oocyte number were counted.
Western Blotting

Western blotting was used to detect the Dax-1 protein during cyst breakdown as well as in developing male gonads. Tissue extracts were prepared from 10 dissected ovaries or testis. These samples were stored in 1X PBS on ice. They were then homogenized in sample buffer on ice. Samples were then boiled for 5 minutes and centrifuged for 3 minutes at 6000 rpm. 2-mercaptoethanol was added to each sample (2 µl per 20 µl). The samples were then boiled for 3 minutes, centrifuged for 1 minute at 6000 rpm and placed on ice. Gel electrophoresis was run on a 4-20% polyacrylamide gel. 10 µl of Precision Plus Standard Protein marker was used as a reference for gel band size. Each gel well was cleaned using a syringe. 20 µl of extract was then loaded into each well. Any empty wells were loaded with 2X sample buffer. Western protein gels were run at 150 volts for one hour. The gel was transferred to a polyvinylidene fluoride (PVDF) nitrocellulose membrane through the following steps. First, the gel was soaked in transfer buffer for 5 minutes before transfer. The transfer buffer consisted of 3.0 grams of Tris base, 14.4 grams of Glycine and 200 ml of methanol diluted with dH2O up to 1 liter. The membrane was soaked in methanol for 1 minute and then washed in dH2O for 5 minutes on shaker. The membrane was then soaked in transfer buffer for 5 minutes. The gel and the membrane were placed in a transfer cassette along with filter paper and fiber pads. Transfer cassette was placed in transfer unit and the transfer unit was filled with transfer buffer. The transfer ran at 100 volts for one hour at 4°C. After transfer, the membrane was blocked overnight with blocking buffer at 4°C. The gel was stained with Comassie blue for 30 minutes and then treated several times with destain solution overnight. The membrane was incubated in 1° Ab diluted in Blocking buffer for 1 hr
(1:1000, usually) on nutator. It was then washed in Blocking buffer 3X for 10 minutes on shaker. Afterwards, it was incubated with 2° Ab-HRP diluted in Blocking buffer (1:10,000) for 1 hr on nutator. Again, the membrane was washed in Blocking buffer 2X for 10 minutes on shaker. It was then washed in wash buffer got 10 minutes on shaker. The membrane was then rinsed in PBS. Next, it was incubated in detection buffer + enhancer for 5 minutes, 5 ml per filter on shaker. This step was followed by rinsing the membrane in dH₂O. the membrane was covered in clear plastic, mounted to cardboard and exposed to film for 1 minute or 5 minutes for visualization of bands.
RESULTS

Dax-1 is expressed during the cyst breakdown period

Dax-1 had previously been found to be present in the developing female ovary from ages 11.5 dpc to 15.5 dpc (Lalli and Sassone-Corsi, 2003), but little information was available about Dax-1 during the cyst breakdown period. We first wanted to determine if Dax-1 was present during the cyst breakdown period. Ovaries and testes were analyzed via western blotting to detect the Dax-1 protein (Figure 8). The F9 cell extract was used as a positive control to confirm that the Dax-1 antibody was functional. The expected size band of 60 kd corresponding to the Dax-1 protein was detected in the F9 control.

![Figure 8. Western blot for Dax-1 protein using both ovary and testis extracts. F9 cells served as the control.](image)

Extracts were prepared from PND 1 and PND 4 ovaries, and 13.5 dpc, PND 1 and PND 4 testes. In the female, Dax-1 was present at PND 4. Dax-1 was also present in all male extracts tested. Unexpectedly, Dax-1 was not present in the PND 1 female extract.

Whole mount immunocytochemistry of 11.5 dpc ovaries was done to confirm that our Dax-1 antibody could function on whole tissue. EMA served as an embryonic germ cell marker, and TOTO-3 was used to label nucleic acid. Results confirmed Dax-1 activity at 11.5 dpc, and a functional antibody. Positive staining for Dax-1 was seen in the urogenital ridge as expected. Staining was in both the somatic cells and in the
oogonia that were forming cysts (Figure 9). In order to determine if Dax-1 was expressed during the cyst breakdown period, CD-1 (wild type) ovaries were processed by immunocytochemistry from ages PND 1 to PND 4. Examination using confocal microscopy gave positive results for Dax-1 protein during the cyst breakdown period for all ages examined. Figure 10 shows an example of Dax-1 expression in PND 1 ovaries and Figure 11 in PND 4 ovaries.

At PND 1 Dax-1 is ubiquitously expressed in the neonatal ovary (Figure 10). Within a day there is noticeable change in Dax-1 localization. At PND 2, Dax-1 is more
strongly expressed in oocytes and the surrounding granulosa cells (data not shown). Staining at PND 4 clearly shows strict localization of Dax-1 to the oocyte cytoplasm and nucleus as well as the surrounding granulosa cells (Figure 11).

![Figure 11. Dax-1 expression at PND 4 in the mouse ovary. Shown in the left panel is Dax-1 expression (green). The center panel shows DNA (propidium iodide, red) and the right panel shows overlay. Dax-1 is now localized to oocytes and granulosa cells.](image)

**Dax-1 gene affects oocyte endowment**

Dax-1 mutant ovaries were collected at PND 7 which is after cyst breakdown to determine if Dax plays a role in oocyte endowment. Mice heterozygous (+/-) for Dax-1 deletion were used as a control, while mice homozygous for Dax-1 deletion (-/-) were used to evaluate changes in oocyte endowment. After being processed for immunocytochemistry with Stat-3, a germ cell marker, ovaries were analyzed for total number of oocytes per section. Dax-1 heterozygous (del/lx) mice at PND 7 had an average of 58 ± 1.6 oocytes per section. The Dax-1 homozygous (del/del) mice were also analyzed and had an average of 46 ± 2.4 oocytes per section (Figure 12)
Dax-1 gene affects cyst breakdown

Dax-1 mutant ovaries were collected at PND 7 to determine if Dax is important for cyst breakdown during oogenesis. Heterozygous Dax-1 mice (del/lx) were used as the control, while mice homozygous for Dax-1 (del/del) were used to evaluate any problems with cyst breakdown. Immunohistocytochemistry revealed differences in the amount of cyst breakdown occurring in the homozygotes in comparison to the control. Ovaries were analyzed for total number of oocytes that were in cysts versus the total number oocytes that were single, or not in cysts. In the control Dax-1 heterozygotes, 40% ± 3 of the oocytes were single. In the Dax-1 homozygotes, only 25% ± 3.5 percent of the oocytes were single (Figure 13).
Fig 13. Comparison of cyst breakdown (% single oocytes) at PND7 in wildtype and mutant mice. Fewer oocytes are single in the mutants indicating a problem in cyst breakdown.
DISCUSSION

The experiments discussed in this paper were all geared towards obtaining a better understanding of the role of Dax-1 in mouse oogenesis. Currently, little is known about the early stages of oogenesis during which cyst breakdown and germ cell death occur and primordial follicles form. In order to better understand Dax-1 three main goals were set: 1) to characterize the ovarian cell types that contain Dax-1 during cyst breakdown; 2) to determine the effects of loss of Dax-1 on oocyte endowment; and 3) to determine the effects of loss of Dax-1 on cyst breakdown.

Dax-1 is known to be present during male gametogenesis, and therefore testes extracts were analyzed in addition to the ovary extracts by Western blotting. We found the expected 60 kd band in testes at 13.5 dpc, PND 1, and PND 4 and in ovaries at PND 4. These results confirmed that the Dax-1 antibody was functional and present in neonatal gonads. More importantly, the detection of a band at PND 4 confirmed that Dax-1 protein was present during the cyst breakdown process. There was no band detected for Dax-1 at PND 1 in the female. Immunocytochemistry experiments with Dax-1 antibody were performed to localize the protein to specific cell types. At PND 1 the staining revealed that Dax-1 is ubiquitously expressed in the neonatal mouse ovary. This contrasted with the Western blot which indicated that there was no Dax-1 present in the PND 1 ovary. A possible reason for the absence of a PND 1 band in the Western is that the sample could have been lost or degraded. The localization of Dax-1 drastically changed at PND 4, just a few days later. At PND 4, Dax-1 was shown to be present strictly in the oocyte and the granulosa cells. This change in localization suggests that
Dax-1 is present in cells that are specifically active in follicle assembly during the cyst breakdown time period.

The PND 7 data showed that homozygous (−/−) Dax-1 mutants had fewer oocytes than the heterozygous (+/−) mutants. This enhanced germ cell loss suggests Dax-1 may have a role in germ cell death rates or the apoptosis pathway. Ovaries were also analyzed for cyst breakdown defects. Quantitative analysis revealed that in comparison to the heterozygous Dax-1 mutant, the homozygous Dax-1 mutant contained more cysts. Mice lacking Dax-1 have multiple oocyte follicles that are possibly a product of cysts that did not complete the cyst breakdown process. The presence of more cysts in the homozygotes supports the idea that cyst breakdown is disrupted in female mice lacking the Dax-1 gene. A lower level of cyst breakdown suggests that as follicle assembly progresses, multiple oocyte follicles would be more prevalent.

Future work that could investigate the role of Dax-1 during cyst breakdown is evaluation of oocyte endowment and cyst breakdown defects at birth and at adulthood in the homozygous versus the heterozygous mice. These two checkpoints would give an idea of what the overall changes were between the two genotypes from birth, when oocyte number is high and oocytes are in cysts, until adulthood when cysts oocyte number is lower and oocytes are no longer in cysts. The role of Dax-1 in oogenesis may also be better understood by studying other conditions under which increased numbers of multiple oocyte follicles are observed. For example, treatment of neonatal ovaries with genistein, a soy bean estrogen, is known to induce the multiple oocyte follicle phenotype (Jefferson et al., 2002). It has been shown that when Estrogen Receptor (ER)-β knockout mice are treated with genistein that multiple oocyte follicles are no longer observed.
However, when ER-α knockout mice are treated with genistein the multiple oocyte follicles persist. Together this data suggests that the effect of genistein is mediated specifically through ER-β. The similar phenotype seen in genistein treated animals and Dax-1 mutant mice suggests that Dax could potentially interact with the estrogen signaling pathway during oogenesis.
REFERENCES


