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Role for endogenous estrogen in prepubertal Sertoli cell maturation

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ABSTRACT

Reducing prepubertal endogenous estrogens led to increased numbers of Sertoli cells and the associated increased testicular size and testicular sperm production capacity in boars. The increased number of Sertoli cells might be due to a longer time for proliferation; delayed differentiation of Sertoli cells during suppressed endogenous estrogens would be consistent with this hypothesized, prolonged proliferation interval. This study used immunohistochemical detection of anti-Müllerian hormone (AMH), a marker of immature Sertoli cells, and of CDKN1B, a cell cycle inhibitor associated with more mature Sertoli cells, to determine if suppressing endogenous estrogens detectably delayed "differentiation" of porcine Sertoli cells. Testes were from littermate pairs of boars previously treated with Letrozole, an aromatase inhibitor, or vehicle, from the first week of age until tissue collection at 2, 3, 4, 5 or 6 months of age. Four animals were examined at each age following Letrozole treatment and their corresponding littermates evaluated following treatment with vehicle. Amount of AMH protein in Sertoli cells decreased with age of boar and could not be detected at 6 months of age. The AMH labeling was greater in the Letrozole-treated boars compared with littermate vehicle controls at 4 months of age ($P = 0.03$). The percentage of CDKN1B-labeled Sertoli cells apparently increased with age through 5 months of age. At 4 and 5 months of age, the mean percentage of CDKN1B-labeled Sertoli cells was less in the Letrozole-treated animals than in the vehicle control animals ($P = 0.03$ and 0.04, respectively). These results are consistent with the hypothesis that continual inhibition of aromatase (and concomitatant reduced estrogen synthesis) causes a delay in Sertoli cell maturation in boars.

1. Introduction

Sertoli cells are supportive cells, which aid in nurturing developing male germ cells. Sertoli cells accomplish this function by providing structure to the seminiferous tubules, secreting paracrine substances such as desert hedgehog (Makela et al., 2011), and forming a blood–testis barrier to prevent the germ cells from being recognized by the immune system. Each Sertoli cell has the ability to support development of a limited number of germ cells at any point in time, hence Sertoli cell numbers are a limiting factor in determining testicular sperm production capacity.

The proliferation of Sertoli cells is a major determinant of germ cell number, ultimate testis size and sperm production (Berndtson et al., 1987; Okwun et al., 1996; Sharpe et al., 1999; McCoard et al., 2001; Sharpe et al., 2003). However, there is not a full understanding of determinants of proliferation and subsequent differentiation (Bar-Shira Maymon et al., 2000, 2004; Tarulli et al., 2006; Guo et al., 2007; Wood et al., 2011) Differentiation/maturation of Sertoli cells may be chronic arrested proliferation rather than terminal differentiation. The timing and duration of Sertoli cell proliferation varies among different species (Wang et al., 1989; Franca et al., 2000; Sharpe et al., 2003); Sertoli

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cell proliferation is thought to occur in two phases. In boars, the first phase of postnatal Sertoi cell proliferation occurs immediately after birth, and the second phase occurs just before puberty between 3 and 4 months of age (Franca et al., 2000). Sertoli cells are thought to differentiate after proliferation ceases (Walker, 2003). When undergoing differentiation, Sertoli cells change morphologically (including increased cytoplasmic volume, further elongation of cell shape, increased smooth endoplasmic reticulum, development of indented nuclear outline), secrete proteins required by germ cells, and form tight junctions to establish the blood–testis barrier (Chevalier, 1978; Tran et al., 1981; Gondos and Berndston, 1993). Whether the entire Sertoli cell population has ceased proliferation before some cells "differentiate" is currently unknown. After Sertoli cells differentiate completely, cell division essentially ceases, and cells are not thought to be able to return to a proliferative phase (Walker, 2003; Sridharan et al., 2007b). Thus, Sertoli cell number present at puberty sets the upper limit for potential sperm production in adulthood.

Aromatase is the enzyme that converts the carbonyl group into the hydroxyl group and eliminates the methyl group on androgens in the biosynthesis of estrogens. Letrozole is a non-steroidal aromatase inhibitor and effectively inhibited aromatase activity in the porcine testis by reversible competition (Bhatnagar et al., 1990; At-Taras et al., 2006b; Bhatnagar, 2007; Jeong et al., 2009). Furthermore, aromatase inhibition by Letrozole does not cause changes in concentrations of gonadotropins or testosterone in boar serum, strongly suggesting the increased number of Sertoli cells observed following Letrozole treatment (At-Taras et al., 2006b) was due to local effects rather than changes in systemic hormones such as FSH. Our initial experiments also suggested that the increased number of Sertoli cells was due to a prolonged interval of proliferation and concomitant delay in differentiation as an approximate 1 month delay in testicular maturation (assessed by appearance of lumens in seminiferous tubules) was observed (At-Taras et al., 2006a). Treated boars did reach full maturity with larger testes and the accompanying increase in sperm production measured by number of detergent resistant spermatids per testis.

The current study was designed to test the hypothesis that suppressing endogenous estrogens was accompanied by delayed maturity of Sertoli cells consistent with a prolonged window of Sertoli cell proliferation. The slight changes in steroid receptor amounts (ESR1 and AR) previously observed (Ramesh et al., 2007) conform to this hypothesis. However, non-steroidal markers of immaturity or maturity in Sertoli cells would more clearly evaluate the hypothesis as observations would have less potential to be directly influenced by aromatase inhibition. Anti-Müllerian Hormone (AMH) is generally recognized as a marker for immature Sertoli cells and is not detectable in Sertoli cells from mature boars (Ford and Wise, 2009). Cyclin-dependent kinase inhibitor 1B (CDKN1B) appears when cells have completed mitotic divisions, is not detectable in Sertoli cells from neonatal animals, and has been used as a marker for mature Sertoli cells. Hence, these two proteins were chosen as markers of immature and mature Sertoli cells respectively, for this study.

2. Materials and methods

2.1. Animal treatment

One member of each littermate pair of boars was orally treated with Letrozole (Ciba-Geigy, Basal, Switzerland, 0.1 mg/kg body weight) and the littermate was treated with the corn oil vehicle. Oral treatment was weekly beginning at 1 week of age and continued until tissue retrieval. Forty boars (20 littermate pairs) were divided into five groups based on the timing of tissue retrieval (2, 3, 4, 5, and 6 months of age) with each group containing four littermate pairs. Animals were housed with littermates until they reached about 110 kg and housed with single littermate boar thereafter. Breeding stock (PIC Lines 65 and C24) for these animals was donated by PIC USA (Franklin, KY) to the University of California, Davis swine facility; boars were crossbred with at least 50% PIC ancestry with some litters containing 50% Hampshire or 50% Yorkshire ancestry. Testes from four additional untreated animals (four litters) were obtained at 6 weeks of age and testes from 15 individuals (10 litters) were obtained at 6½ weeks of age. All animal use and experimentation was approved by the Institutional Animal Care and Use Committee. Previous studies documented that this Letrozole treatment decreased endogenous estrogen levels by 90% (Josso et al., 2001; At-Taras et al., 2006b). Treatment at weekly intervals effectively maintained inhibition of testicular aromatase, reduced circulating estrogens and increased number of Sertoli cells in these specific 2–6 month old boars (At-Taras et al., 2006a,b).

2.2. Immunohistochemistry

Testis samples were obtained at the estimated midpoint (equator) of the testis oriented on its longitudinal axis. The entire cross section was obtained from younger animals and a trapezoid with the narrow end extending toward the mediastinum was obtained from the older animals; much of the tunica albuginea was trimmed away and tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin as previously described (At-Taras et al., 2006a). Sections were cut at 5 μm thickness and mounted on glass slides. Paraffin was removed by two sequential, 4 min soaks in Citrosolv Hybrid (Fisher Scientific, Pittsburgh, PA, USA). Sections were rehydrated during 2 min transfers through two containers each of 100% ethanol, 95% ethanol, and 70% ethanol following by 5 min in running tap water. Antigen retrieval was performed by heating sections to 93 °C in diluted Antigen Unmasking Solution (Vector Laboratories Inc., Cat no. H3300). After 5 min, sections were allowed to cool to room temperature, rinsed in Tris-buffered saline (TBS; 50 mM Tris buffer with 1.5% NaCl, pH 7.6) and incubated for 30 min in 0.3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were then blocked in 1.5% normal serum in TBS for 20 min at room temperature and incubated overnight at 4 °C with either polyclonal

goat anti-human AMH IgG (sc-6886, 1:1000 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA) to observe AMH presence within the Sertoli cells or polyclonal rabbit anti-human CDKN1B IgG (sc-528, 1:150 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) to observe CDKN1B in paraffin sections of testes obtained from 2–6 month old boars. A second paraffin section from each testis was incubated with normal goat serum or normal rabbit serum instead of the anti-AMH or anti-CDKN1B, respectively, but otherwise subjected to the same procedures to verify specificity of immunohistochemistry in each assay. Apoptosis was evaluated in paraffin sections from 6 to 6½ week untreated boars by immunohistochemical analysis of cleaved caspase-3 (CASP3, 9661, Cell Signaling Technology, Inc., Danvers, MA, USA) (Gown and Willingham, 2002). The primary antibody was diluted 1:500 and additional testis sections were incubated with normal rabbit serum to verify specificity. In addition, each immunohistochemical assay contained sections from porcine ovary with aged luteal tissue or porcine mammary tissue as a positive control since CASP3 labeling of tissues from young testes was so low. Sections were washed with TBS twice for 4 min each and incubated with biotinylated second antibody (Vectastain Elite ABC kit PK6105 for AMH or PK6101 for CDKN1B or CASP3) at room temperature for 40 min. Sections were washed again two times in TBS prior to incubation with an avidin–biotin–peroxidase complex (ABC reagent) for 40 min. Incubation with NovaRed (Vector Laboratories) for 6 min was used to visualize bound AMH or CDKN1B antibody and incubation with 3-amino-9-ethylcarbazole (AEC, Vector Laboratories) was used to visualize bound CASP3 antibody. Sections were lightly counterstained with hematoxylin (American Master Tech, Lodi, CA, USA) and mounted in Faramount aqueous mounting medium (DAKO, Carpenteria, CA, USA). One section from each boar was evaluated for labeling by the primary antibody and one section was evaluated for nonspecific labeling by the respective normal serum substitution for the primary antibody.

2.3. Quantification of immunochemistry

2.3.1. Anti-Müllerian Hormone

Immunolabeling intensity was evaluated by one observer blind to treatment. Sertoli cells in random fields spread across the tissue section were evaluated and an average score was assigned to each boar. In a three-point scale, zero represented no labeling of Sertoli cells, one represented faint to moderate labeling, and two represented strong labeling. Since the anti-AMH antibody labeled the cytoplasm of Sertoli cells, the percentage of labeled cells could not be determined.

2.3.2. Cyclin-Dependent Kinase Inhibitor 1B

Sertoli cell nuclei were labeled by the anti-CDKN1B antibody; the percentage of immunolabeled Sertoli cell nuclei was used to quantify immunolabeling. Each tissue section was divided into five parts (upper, lower, left, right and central). Three fields were chosen randomly in each part and examined at a magnification of 400×. An observer blind to treatment classified at least 500 Sertoli cells as positive or negative based on staining intensity (red) of each nucleus. The percentage of labeled cells was determined.

Labeling of apoptotic cells was detected in the pig ovarian or mammary tissue positive control in each immunohistochemical assay indicating the assay was functional for each analysis. All the Sertoli cells in randomly chosen fields were evaluated with a minimum of 299 Sertoli cells per individual examined.

2.4. Seminiferous tubule diameter

The horizontal and vertical diameters of a single round tubule were measured in a microscope field in the region defined as the sub-tunica (Ford and Wise, 2009). If these two measurements differed by 10% or more, the tubule was considered to be other than round, not representing a cross-section of a tubule and measurements not recorded. The smaller diameter of acceptable measurements was used and the mean diameter for ten tubules, each in a different field, was used to represent seminiferous tubule diameter. Calculated intra-assay coefficient of variation for tubule diameter was 3% when means were based upon 9 tubules in 3 and 4 month old boars.

2.5. Gel electrophoresis and western blots

Specificity and cross-reactivity for the antibodies was confirmed by gel electrophoresis and western blot analysis. Testis and liver tissues were homogenized in 0.1 M potassium phosphate buffer containing 20% glycerol, 5 mM mercaptoethanol and 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), pH 7.4. Proteins (10 μg) were separated by SDS-PAGE (11% acrylamide gels) and transferred to Immobilon P (Millipore, Billerica, MA, USA). After blocking, blots were probed for 1 h with a 1:200 dilution of the AMH antibody or a 1:100 dilution of the CDKN1B antibody in PBS containing 0.5% Tween-20 (PBST) and 5% normal donkey serum (control lanes were probed with diluted goat or rabbit serum, respectively to provide equivalent goat or rabbit IgG concentrations). Bound antibody was visualized with 1:20,000 dilution of peroxidase-labeled donkey anti-goat IgG in PBST or donkey anti-rabbit IgG in Tris-buffered saline containing 0.5% Tween-20 followed by chemiluminescent detection (Western Lightning, PerkinElmer, Waltham, MA, USA).

2.6. Statistical analysis

Data (AMH, CDKN1B, and seminiferous tubule diameter) were subjected to analysis of variance (ANOVA) using SAS statistical programs (Proc GLM, SAS Institute, Cary, NC, USA). Treatment was a fixed factor; residuals were analyzed after adsorbing litter effects. Because some data grouped across ages did not meet normality criteria; data for each age were analyzed separately for all parameters. Previously reported body weight, testis weight, and number of Sertoli cells for these boars (At-Taras et al., 2006a) were subjected to the same analysis (Table 1). The relationship between seminiferous tubule diameter and CDKN1B was evaluated in 4 month and in 5 month samples with

Table 1
Prepubertal development of boars under the continuous influence of aromatase inhibion compared with their vehicle-treated littermate controls[a].

Age	Body weight (kg)			Testis weight (g)			Seminiferous tubule diameter (μm)			Number of Sertoli cells (billions)		
	Vehicle	Letrozole	SEM	Vehicle	Letrozole	SEM	Vehicle	Letrozole	SEM	Vehicle	Letrozole	SEM
2 months	21.4	18.9	1.9	8.2	8.4	0.6	49	47	1	2.83[b]	3.74[c]	0.50
3 months	35.2	37.1	2.1	19.1	17.7	1.5	61	51	7	6.54[b]	8.68[c]	0.80
4 months	69	72	1.2	70.7[c]	37.1[d]	16.6	111	83	15	24.96[c]	19.01[d]	2.81
5 months	96	93	3.5	193.5[c]	125.1[d]	51.2	168[b]	131[c]	9	19.35	27.53	6.14

[a]Data other than seminiferous tubule diameter was presented or discussed in a previous paper (At-Taras et al., 2006a).
[b,c]Vehicle and Letrozole treatment means differ, $P < 0.01$.
[c,d]Vehicle and Letrozole treatment means differ, $P < 0.05$.

treatment as a fixed factor and CDKN1B expression as a continuous variable.

3. Results

3.1. Anti-Müllerian hormone labeling

Anti-Müllerian hormone expression was immunohistochemically detected in Sertoli cells from 2 month old boars and had completely disappeared from Sertoli cells from 6 month old boars, regardless of treatment. Labeling intensity of Sertoli cells was statistically similar at 3 months of age although the mean score for Sertoli cells from Letrozole-treated boars (2) appeared higher than the mean score for Sertoli cells in control boars (1.25; Figs. 1 and 2). The AMH labeling intensity was greater in the Letrozole-treated boars at 4 months of age compared with the littermate vehicle controls ($P = 0.03$, 1.25 ± 0.35 compared with 0.25 ± 0.35). This is consistent with delayed Sertoli cell maturity in Letrozole-treated boars. The AMH labeling was low in all boars at 5 months of age (no labeling in most boars) and did not differ significantly with treatment. The 6 month old boars exhibited no detectable immunolabeling with the AMH antibody. Western blotting confirmed the AMH antibody recognized a protein matching AMH size in porcine testicular extracts (Fig. 3).

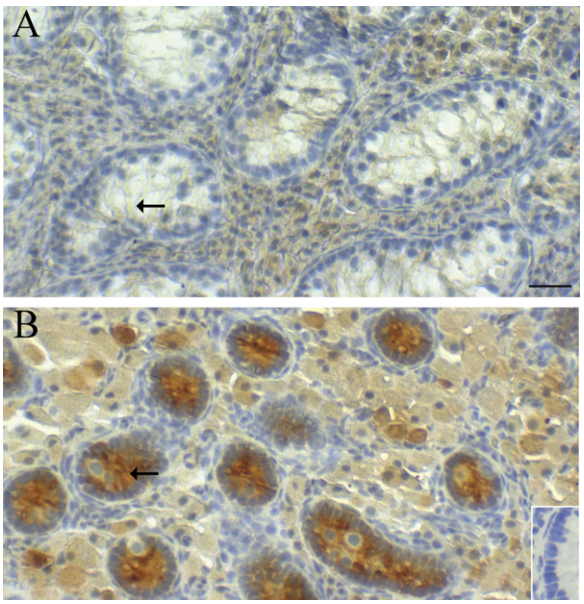

Fig. 1. Immunohistochemical labeling of AMH in porcine testis. (A) Testis section from 3 month old boar treated weekly with vehicle and (B) testis section from 3 month old littermate treated weekly with Letrozole. Arrows point to Sertoli cell cytoplasm within seminiferous tubules. The AMH labeling is indicated by reddish brown color. Inset represents testis section incubated with normal rabbit serum in place of anti-AMH; bar represents 30 μm.

3.2. Cyclin-dependent kinase inhibitor 1B labeling

No Sertoli cell labeling was detectable by immunohistochemistry with the CDKN1B antibody in testes at 2 months of age. Very few Sertoli cells were labeled by the anti-CDKN1B antibody in testes from 3 month old

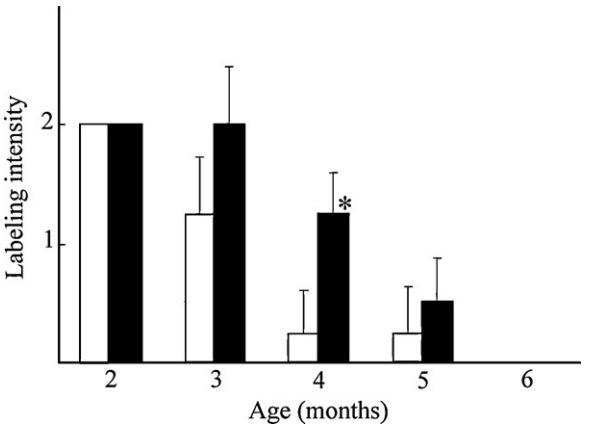

Fig. 2. Labeling intensity of Sertoli cells by AMH antibody during development. Each bar represents the mean of four boars; the vehicle-treated boars are represented by the open bars and their Letrozole-treated littermates by the closed bars. Lines above bars are SEM from analyses; * indicates values differ, $P < 0.05$.

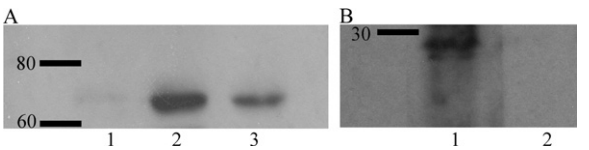

Fig. 3. Western blots of porcine testis. (A) The AMH antibody recognizes a protein of approximately 70 kDa in testis homogenates from 6.5 (lane 2) and 11 week (lane 3) boars but not detected in liver homogenates (lane 1). (B) The CDKN1B antibody recognizes a protein of approximately 27 kDa in testis homogenate (lane 1) that does not cross-react with normal serum (lane 2).

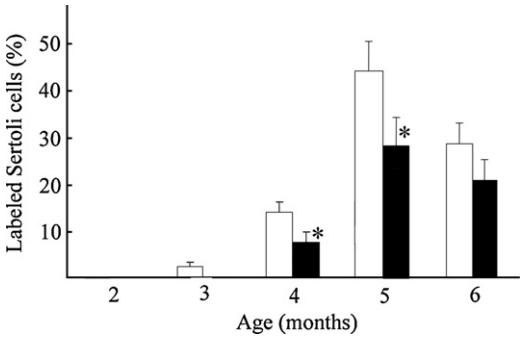

Fig. 4. Percentage of labeled Sertoli cell nuclei by CDKN1B antibody during development. Each bar represents the mean of four boars; the vehicle-treated boars are represented by the open bars and their Letrozole-treated littermates by the closed bars. Lines above bars are SEM from analyses; * indicates values differ, $P < 0.05$.

boars and treatment was not significant ($P = 0.1$; Fig. 4). The percentage of Sertoli cells expressing CDKN1B was greater in vehicle-treated boars at 4 months of age than in their Letrozole-treated littermates (14% compared with 8%, SEM = 2; $P = 0.03$; Fig. 5). This was also observed at 5 months of age (44% compared with 29%, SEM = 6; $P = 0.04$; Fig. 4). Immunolabeling of Sertoli cells by the CDKN1B antibody no longer differed between vehicle controls and Letrozole-treated boars at 6 months of age ($P = 0.095$). Western blotting confirmed the CDKN1B antibody recognized a protein matching CDKN1B size in porcine testicular extracts (Fig. 3).

3.3. Labeling of apoptotic cells

No CASP3 labeling was detected in the 2131 Sertoli cells examined in the testes from 6 week old untreated boars. Similarly, only 1 Sertoli cell in the 8226 Sertoli cells evaluated in testes from 6½ week old untreated boars demonstrated CASP3 labeling indicative of apoptosis.

3.4. Seminiferous tubule diameter

The diameter of the seminiferous tubules was greater in the 5 month old vehicle control boars than in their Letrozole-treated littermates (Table 1, $P < 0.01$). However, the arithmetic differences in tubule diameter between vehicle controls and their Letrozole-treated littermates at the younger ages were not statistically different. Within treatment, mean seminiferous tubule diameter was not correlated with CDKN1B labeling at 5 months of age or at 4 months of age although there was a tendency for a positive correlation at 4 months ($P < 0.10$).

4. Discussion

The increased number of Sertoli cells previously observed following weekly Letrozole treatment postnatally could result from increased rate of proliferation, an increased duration of proliferation, or a reduced rate of cell death (At-Taras et al., 2006a). Young growing animals are expected to have extremely low rates of cell death such that further reduction would not be sufficient to cause the approximate 25% increase in postpuberal Sertoli cell numbers observed. We recently reported that the Letrozole-induced increase in Sertoli cell numbers is present at 6½ weeks of age (Berger et al., 2012). Immunohistochemical analysis of apoptosis confirmed an almost undetectable rate of apoptosis at and prior to this time indicating the increased number of Sertoli cells resulted from an increased rate of proliferation or a prolonged duration of proliferation or a combination of both these factors. Previously, we reported that testicular development was delayed in animals treated weekly with Letrozole (At-Taras et al., 2006a). This was apparent in each littermate pair examined at 5 months of age and in two of the four littermate pairs evaluated at 4 months of age; the remaining two 4 months old littermate pairs were more immature and histological examination did not detect differences. Such delayed development is consistent with delayed maturation of Sertoli cells and delayed Sertoli cell maturity might be assumed from such observations. However, interactions of peritubular myoid cells with other

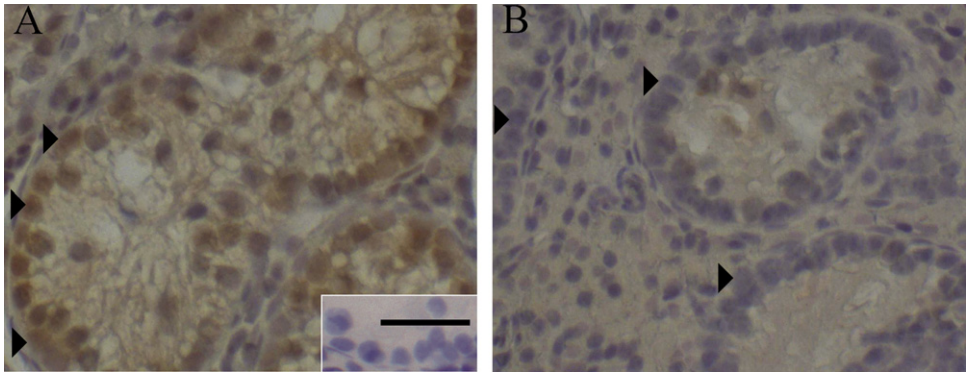

Fig. 5. Immunohistochemical labeling of CDKN1B in porcine testis. (A) testis section from 4 month old boar treated weekly with vehicle and (B) testis section from 4 month old littermate treated weekly with Letrozole. Arrowheads point to Sertoli cells. Labeled nuclei are brown and unlabeled nuclei are stained blue by the hematoxylin counterstain. Inset represents testis section incubated with normal rabbit serum in place of anti-AMH; bar represents 30 μm.

tubular components are an uncharacterized possibility. The present study directly evaluated Sertoli cells using two markers for transitions in this maturation process.

AMH is widely recognized as a marker for immature Sertoli cells (Sharpe et al., 2003) and the decreased intensity of labeling observed with increasing age in vehicle-treated boars is consistent with that use and with a previous study in boars (Ford and Wise, 2009). The decreased intensity of labeling in Sertoli cells in the 4 month old vehicle-treated boars compared with their Letrozole-treated littermates, is consistent with delayed maturation of Sertoli cells following Letrozole treatment. Although not statistically significant in 3 month old boars, a similar trend was observed. Cyclin-dependent kinase inhibitor 1B is recognized as a marker for more mature Sertoli cells, arrested in the G1 phase of the cell cycle (Holsberger et al., 2005) and the increased percentage of positive Sertoli cell nuclei with increasing age is again consistent. These observations are also consistent with a previous study of Sertoli cell differentiation in boars although the percentage of labeled Sertoli cells was analyzed in the current study and the percentage of tubules with at least one labeled Sertoli cell reported in the previous study (Ford and Wise, 2009). A significantly greater proportion of Sertoli cells from the 4- and 5-month vehicle-treated boars were labeled by antibody to CDKN1B compared with Sertoli cells from Letrozole-treated littermates, again consistent with delayed maturation of Sertoli cells in the Letrozole-treated boars, at least at 4 and 5 months of age. Sertoli cells in Letrozole-treated animals had reached a similar amount of CDKN1B labeling as those in vehicle-control littermates at 6 months; the arithmetic decrease in vehicle controls at 6 months may be due to different genetic background compared with 5 months. These two markers suggest a delay in maturation of Sertoli cells detectable one month earlier than demonstrable by histological analysis of spermatogenic development in the seminiferous tubules or seminiferous tubule diameter. The arithmetic values at 3 months of age are consistent with treatment delay in maturity as well, at least in litters in which vehicle controls appeared to demonstrate some advancement in maturity. Variation among litters in timing of observable changes may obscure treatment effect; the variation in labeling of testes from 3 month old boars by these two markers within a testicular region was apparent in previous research (Ford and Wise, 2009). Previous research has not suggested differences in development along the longitudinal axis of the testis (McCoard et al., 2003) but a development gradient may exist from the mediastinum to the tunica albuginea (Ford and Wise, 2009). Tubular diameters were similar in all three regions in a second study (Avelar et al., 2010) although tubules in the tunica albuginea region were arithmetically the smallest at 90 and 120 days. In this second report, Sertoli cell proliferation was greater in the mediastinum region than in the tunica albuginea region at 120 days, a surprising observation if the mediastinum was the more mature region. Regardless of whether there is or is not a detectable gradient in development, most (if not all) of the fields evaluated for AMH and CDKN1B for each boar in the present study would have been located in the large subtunica region. Because the variation among boars appeared to be greater than the variation among regions at 90 days of age in the previous study (Ford and Wise, 2009), regional variation would not have made a large contribution to the present results.

In previous research, we noted that the Letrozole-treated boars had more Sertoli cells than their littermate vehicle controls except at 4 months of age, when the Letrozole-treated boars had only 78% as many Sertoli cells as their control littermates. This 4 month age corresponds to the estimated time for completion of the second wave of Sertoli cell proliferation, at least in control boars (Franca et al., 2000). Collectively, these data suggest the first wave of Sertoli cell proliferation is prolonged following continuous Letrozole treatment, leading to more Sertoli cells. In addition or as a result, initiation of the second wave of proliferation is delayed, leading to delayed maturity of Sertoli cells.

The increased number of Sertoli cells following aromatase inhibition in prepuberal boars contrasts with observations in rats suggesting increased Sertoli cell numbers concurrent with increased aromatase activity and estradiol (McDonald et al., 2006) although very small doses of diethylstilbestrol were proposed to directly inhibit rat Sertoli cell proliferation (Atanassova et al., 2005). Estradiol inhibited apoptotic pathways in *in vitro* cultured rat Sertoli cells obtained at the end of the normal proliferative period (Royer et al., 2012; Lucas et al., 2011). We observed no effect of aromatase inhibition during the second wave of Sertoli cell proliferation on subsequent Sertoli cell numbers in the pig (Berger et al., 2012), so this may be another difference between the pig and rat Sertoli cell responses.

Cytokeratin 18 has been used as a marker for fetal and prepuberal Sertoli cells in humans and rodents as well as dedifferentiated Sertoli cells in humans, monkeys and rodents. Because cytokeratin 18 labeled Sertoli cells following transplants of neonatal boar testicular tissue to nude mice (Caires et al., 2008), we considered it as a potential marker for immature porcine Sertoli cells. Although we used the antibody source reported for these neonatal tissues and readily detected cytokeratin 18 in porcine liver tissues by immunohistochemistry and by western blot analysis, we were unable to detect cytokeratin 18 in 1 or 2 week old boar testicular tissue by either immunohistochemistry or western blots. The difference may be that the transplanted tissue had further dedifferentiated as reported for some of the human testicular dysgenesis syndrome tissues and following thermal injury (Bar-Shira Maymon et al., 2000, 2004; Guo et al., 2007). Additional markers for Sertoli cell maturity include proteins associated with junction protein complexes such as connexin-43, JAM-1, claudin and vimentin (Franke et al., 2004; Sridharan et al., 2007a). However, the immunohistochemical labeling of junctional complexes is not readily evaluated for quantitative comparisons.

Less CDKN1B and more AMH were observed in Letrozole-treated animals at 4 months of age compared with the littermate vehicle controls. This is consistent with a prolonged first wave of Sertoli cell proliferation, which delays the initiation of the second wave and the ultimate differentiation phase until 5 months of age, events which are presumably initiated in control animals at 3–4 months

of age. The rate and length of the second wave of proliferation is not necessarily compromised by the delayed initiation. In addition, the final status of differentiation was unaffected by the inhibition of estrogen production since no significant difference in the maturational marker labeling was noted between Letrozole-treated and control groups at the oldest age evaluated, and these testes from treated boars still functioned normally.

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