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Germ Cell Development in Meishan and White Composite Gilts

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Abstract

This study compared dynamics of the germ cell population in two swine breeds that differ in prolifacy, White Composite (WC) and Meishan (MS), during fetal and neonatal life and in mature sows. Germ cell populations developed in a similar pattern in these two diverse breeds during fetal life. Maximal germ cell number was observed at 90 days postcoitum (dpc) in both WC and MS gilts, and substantial oogonial apoptosis was evident thereafter with approximately 30% of maximal numbers present at 25 days postpartum (dpp). Neither gilt nor sow germ cell number was correlated with maternal ovulation rate. Postnatal MS gilts had larger pools of primordial follicles and consistently greater proportions and numbers of primary and secondary follicles compared to postnatal WC gilts, indicative of enhanced follicular recruitment and primordial follicle activation. Occasional antral follicles were present in MS ovaries by 25 dpp and numerous surface follicles were observed at 56 dpp in MS but not WC ovaries, indicative of more rapid ovarian maturation and early onset of puberty. Total germ cell number is unlikely to influence or to predict subsequent ovulation rate. These observations highlight important developmental events during late fetal and early postnatal life that prepare the ovarian environment for early onset of puberty and subsequent ovulation in MS gilts.
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Keywords: Ovary; Fetus; Pig; Germ cells; Morphology

1. Introduction

Prolificacy in pigs is generally defined as the number of viable piglets born per litter and is an important limiting factor affecting sow productivity. Some Chinese breeds exhibit exceptional prolificacy (Zhang et al., 1983; Sellier and Legault, 1986; Yun, 1988). Notably, Chinese Meishan (MS) pigs farrow on average three–five more pigs per litter, possess more teats and reach sexual maturity 60–90 days sooner than their European counterparts (Bolet et al., 1986; Haley and Lee, 1990; Rothschild and Bidanel, 1998). These substantial breed differences offer a unique model to study regulatory mechanisms that lead to high prolificacy in MS pigs that may improve reproductive efficiency in other pig breeds.

Several studies indicate that increased prolificacy of MS sows is primarily due to low levels of embryonic and fetal mortality (Bidanel and Legault, 1986; Bazer et al., 1988; Haley et al., 1990; Haley and Lee, 1993) possibly resulting from slower rates of preimplantation embryonic development (Anderson, 1978; Anderson et al., 1993; Pope et al., 1986, 1990; Bazer et al., 1988; Youngs et al., 1993). Whilst differential embryogenesis in European breeds is associated with heterogeneity of preovulatory follicle populations (Foxcroft and Hunter, 1985; Grant et al., 1989; Hunter et al., 1989; Hunter and Wiesak, 1990; Pope et al., 1990; Xie et al., 1990a,b,c), follicular heterogeneity is similar between MS and White Composite (WC) sows (Biggs et al., 1993). Increased ovulation rate is also implicated in enhanced litter size (Ashworth et al., 1992; Christenson, 1993; Faillace et al., 1994; Hunter et al., 1994). Consistent with this hypothesis, MS sows have high ovulation rate and, during the follicular phase, maintain greater numbers of follicles (Miller et al., 1998) at a more advanced stage of development (Faillace and Hunter, 1994) compared to WC gilts.

In European pig breeds, primordial germ cells colonize the gonad by 26 days postcoitum (dpc; Takagi et al., 1997) and ovarian differentiation has begun by 33 dpc characterized by enclosure of germ cells in egg cell nests (Allen, 1904; Pelliniemi and Lauteala, 1981; Byskov, 1986; McCoard et al., 2001a). At 60 dpc, all germ cells (now termed oogonia) are contained within egg cell nests (Mauléon, 1964; Oxender et al., 1979) corresponding with peak mitosis and maximal germ cell number (Black and Erickson, 1968). Germ cell maturation is initiated thereafter by termination of mitosis and progressive entry of a finite number of germ cells (now termed oocytes) into the growing pool. Once germ cells reach the diplotene phase of meiosis, they undergo folliculogenesis whereby somatic follicular cells (presumptive granulosa cells) surround diplotene oocytes and basal lamina is laid down forming a follicle (Byskov and Hoyer, 1994). In this way, primordial follicles appear by 70 dpc and primary and secondary follicles are present by birth (Oxender et al., 1979). Coincident with follicle formation and growth, atresia destroys many oogonia and oocytes (henceforth termed "germ cells") before entry into the growing pool or long before they are fully grown (Beaumont and Mandl, 1962). In pigs, marked germ cell degeneration during late fetal life is apparent with less than 50% of the peak number of germ cells present at birth and a further 70% loss by 130 days postpartum (dpp) (Black and Erickson, 1968). Initiation of these key events of germ cell mitosis, follicle formation, growth and atresia during fetal life, highlights the importance of this early period for ovarian development.

Despite studies characterizing follicular dynamics during the estrous cycle in MS and WC sows, little is known about initiation of folliculogenesis and subsequent development of follicle populations in these two diverse breeds, and potential contribution of these early

developmental events to increased prolificacy in MS sows. Therefore, the aim of this study was to compare the dynamics of germ cell and follicle population during fetal, neonatal and prepubertal life in MS and WC gilts.

2. Materials and methods

2.1. Animals and sample collection

Purebred MS (304–383 days of age; $n = 26$) and WC (Yorkshire × Landrace) females (324–894 days of age; $n = 29$) were bred to MS and WC boars, respectively. These females were housed indoors segregated by breed and body weight within breeds in groups of 10 or less females per pen. During the breeding seasons, females were fed to appetite. The day after estrus and during the first 85 days of gestation, diet was provided at 1.81 kg per day, fed once daily. During the last month of gestation, diet was provided at 2.3 kg per day, fed once daily. During lactation, sows were housed individually and fed to appetite. Piglets were weaned at a mean age of 17 days. Ovaries from each sow sacrificed during gestation were collected, weighed and corpora lutea (CL) dissected from stromal tissue and remaining ovarian tissue weighed. Corpora lutea number was recorded as a measure of ovulation rate. Ovaries were collected at 60, 75, 90 and 105 dpc from fetal gilts and from their dams, and at 1, 7, 14, 25 and 56 dpp from newborn gilts by either postmortem dissection or surgical ovariectomy. Fetal ovaries were collected from two gilts per litter with three–five litters represented per breed at each age. Postnatal ovaries were collected from 1 gilt from 10 different litters per breed on day 1. One sibling to each of these gilts was sampled at each subsequent age through 25 dpc. Gilts sampled on 56 dpp were contemporaries but from different litters. Procedure for handling all animals in this study complied with those specified in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

2.2. Enzymatic digestion

Right ovaries from each fetal/neonatal gilt ($n = 230$) and sow ($n = 31$) were trimmed of excess oviduct tissue and weighed. Each ovary was subjected to collagenase digestion to dissociate somatic and germ cells (oogonia and oocytes) in order to estimate total germ cell number per ovary using a modification of the procedure described by Greenwald and Moor (1989). Intact ovaries from gilts were chilled on ice, placed in 5 ml of collagenase digestion buffer (0.025% collagenase type I, 240 U/mg; Worthington Biochemical Corporation, NJ; 0.5% bovine serum albumin and 400 IU/ml DNase in Hank's balanced salt solution pH 7.6) and incubated overnight at 37 °C in a shaking water bath. Ovaries of sows minus their CL were dissected in approximately 1 g portions, minced and incubated overnight in collagenase digestion buffer (approximately 5 ml/g). The following morning, complete disaggregation of cellular material was achieved by pipetting through successively smaller tips. Cellular suspensions were not filtered. Oocytes were distinguished from somatic cells primarily due to their large size and spherical shape. A limitation of this technique is that large cumulus–oocytes complexes found in large antral follicles (100–300 μm in diameter)

are excluded from the hemacytometer. Atretic, and possibly some damaged oocytes were identified by a ruptured cell membrane, shrunken nucleus and absence of a spherical shape. Thus, only healthy oocytes were enumerated. Total germ cell number was estimated from duplicate subsamples using a hemacytometer (Bright-Line, AO Scientific Instruments, Buffalo, NY) and differential interference microscopy. One operator conducted all determinations. The correlation between counting subsets was 0.93 and 0.90 for gilt and sow ovaries, respectively. The overall coefficient of variation was 20.3 and 26% for gilt and sow ovaries, respectively.

2.3. Immunohistochemistry and histological evaluation

Left ovaries from each gilt were trimmed of excess oviduct tissue and weighed. Tissues were fixed intact overnight at 4 °C in 4% paraformaldehyde in PBS with gentle agitation. Each sample was washed in PBS (2× 1 h), dehydrated through graded ethanol (50, 70, 80, 95, 100%; 2× 1 h each), cleared in xylene (2× 1 h; Sigma), infiltrated with paraffin (60 °C; 4× 1 h), and embedded in paraffin wax. Serial cross-sections (5 μm) were made along the longitudinal axis from the middle of each ovary. The oviduct was used as a landmark and only sections containing oviduct tissue and distinct medulla and cortex compartments were used. Cross-sections (CS) were dried overnight onto glass slides at 37 °C and stored at room temperature until staining. Sections were stained immunohistochemically with GATA4 as described previously (McCoard et al., 2001a,b). GATA4 was used as a marker for follicular cells enabling rapid identification and classification of oogonia in egg cell nests and follicles in each developmental phase.

2.4. Follicle classification

Total germ cell number refers to all germ cells within the CS irrespective of their stage of development. Germ cells clustered into nests that were surrounded by a layer of squamous GATA4-positive follicular cells (Fig. 1A) are termed oogonia. Germ cells in early meiotic prophase (leptotene, zygotene, pachytene) located within egg cell nests, could not be reliably distinguished from mitotic oogonia based on morphological characteristics. Therefore, all germ cells located within egg cell nests, including both mitotically active oogonia and early meiotic prophase oocytes, are termed "oogonia." Primordial follicle classification (Fig. 1A) was based on presence of a single layer of GATA4-positive flattened follicular cells surrounding an individual oocyte (diplotene stage of meiosis or quiescent). An oocyte surrounded by a single layer of cuboidal follicular cells was classified as a primary follicle (Fig. 1B). Secondary follicles had more than one distinct layer of cuboidal GATA4-positive granulosa cells (Fig. 1B), and presence of a fluid filled cavity within follicles characterized antral follicles. In addition to healthy germ cells, large numbers of oogonia in egg nests and a small number of atretic follicles were observed. In addition, spaces in the ovarian stroma, resulting from complete oocyte degeneration, were present. Due to the large variation in stages of degeneration, it was not possible to accurately determine total numbers of atretic follicles. Only follicles containing a distinct spherical oocyte nucleus (i.e. presumably healthy) were counted. Total numbers of oogonia and follicles in each growth phase were expressed as a proportion of total germ cell number present within each section.
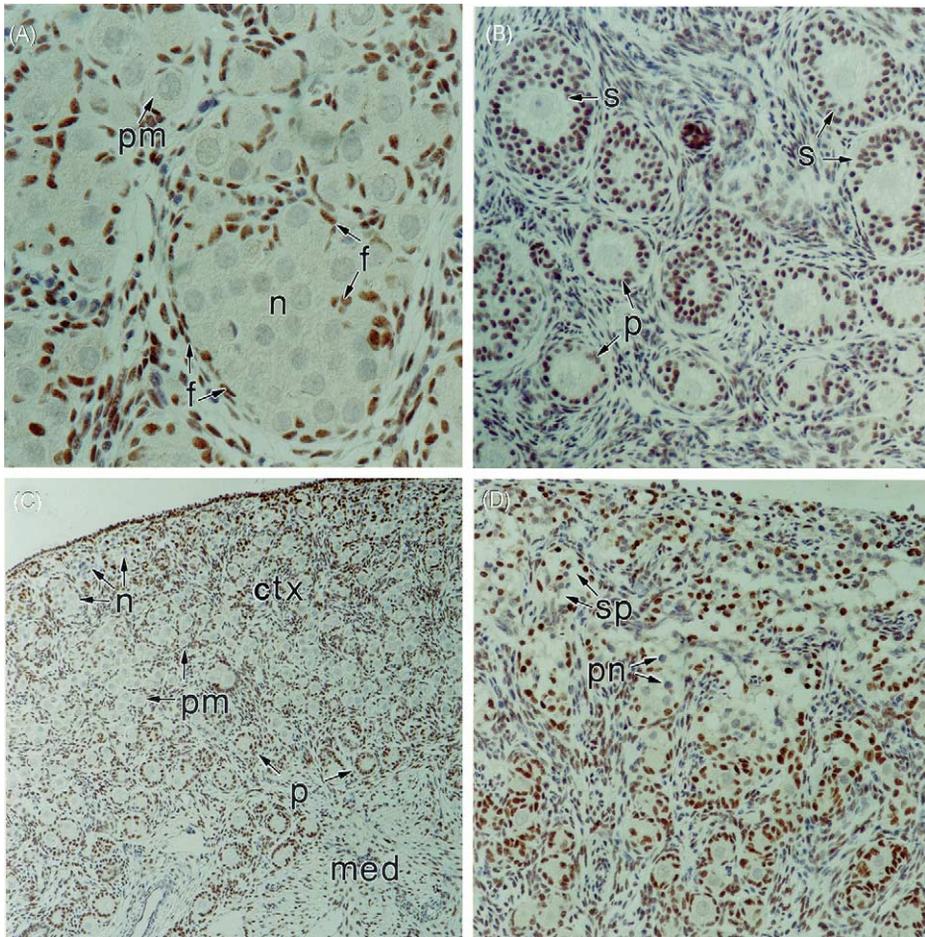
Fig. 1. GATA4 immunostaining of follicular/granulosa cells (depicted by red color) of the fetal porcine ovary. (A) Germ cells clustered into egg cell nests (n) and primordial follicles (pm) surrounded by a layer of flattened (f) GATA4-positive follicular cells at 90 days postcoitum. (B) Primary follicles (p) surrounded by a layer of GATA4-positive cuboidal granulosa cells and secondary follicles (s) surrounded by two or more layers of GATA4-positive cuboidal granulosa cells at 105 days postcoitum. (C) Transition of follicle development from the peripheral cortex (ctx) region of the ovary dominated by egg cell nests (n) and primordial follicles (pm), to primary follicles (p) at the border between the cortex and medulla (med). (D) Pyknotic nuclei (pn) and spaces (sp) within egg cell nests resulting from atresia and subsequent disappearance of germ cells at 105 days postcoitum.

2.5. Stereological evaluation of ovarian composition and follicle classification

Previous studies in pigs have evaluated ovarian follicular characteristics from small randomly selected areas from one or two sections per ovary (Oxender et al., 1979; Black and Erickson, 1968; Colenbrander et al., 1983). Careful examination of ovarian sections in this study revealed considerable variation in rates of development and atresia within various

regions of the cortical compartment. Due to this considerable variation in a single CS, every germ cell within a given CS was evaluated in this study to reduce potential error likely to be encountered using small randomly-selected areas. Five micrometer sections immunostained for GATA4 were examined under brightfield microscopy and used to evaluate ovarian composition. All measurements were made using computerized morphometric planimetry (Bioquant Nova Advanced Image Analysis, R&M Biometrics Inc., Nashville, TN). Proportions of each ovary composed of either medullary or cortex (Fig. 1C) tissue was determined at a magnification of $50\times$. Each entire ovary section was projected onto a computer screen and medulla and cortex regions of ovaries were measured by tracing outlines of each compartment using a computer mouse connected to a high-resolution digital pad. Total germ cell number, individually classified as either oogonia, primordial, primary, secondary or antral follicles, were counted in an entire ovarian CS from each ovary using Bioquant topography and morphometry software at $200\times$. Due to substantial maturation of MS ovaries at 56 dpp, histological evaluation of ovarian compartments (cortex and medulla) and proportions of primordial, primary, secondary and antral follicles were not compared between breeds at this age.

2.6. Follicle size

Average size of individual primordial, primary, secondary and antral follicles was determined by tracing each follicle at $400\times$. For each follicular phase, outlines (perimeter) of each follicle were traced and mean CS area calculated using Bioquant software. Only follicles containing a distinct nucleus were measured. At least 30–60 primordial follicles were measured per ovary and all primary, secondary and antral follicles present in each cross-section were measured per ovary at either $400\times$ (primordial) or $200\times$ (primary, secondary and antral follicles).

2.7. Statistical analysis

Differences between breeds in all components estimated were evaluated statistically using a mixed model procedure (SAS, 1999). For data from sows, the model included fixed effects of breed, day of gestation and the interaction of breed by day of gestation; age of sow was used as a covariate. For data from fetal gilts, the model included fixed effects of breed, age, breed $\times$ age interaction and sow age as a covariate; the random effect was litter nested within breed. For data from postnatal gilts, 1–25 dpp, the model included fixed effects of breed, age, breed $\times$ age interaction and sow age on the day of farrowing as a covariate; the random effect was litter. Age and parity were confounded in the animals used in the current study, but age was used as a covariate because age has a greater effect on litter size than parity (French et al., 1979). Data from 56 dpp gilts were evaluated separately. Paired comparisons were conducted using the Tukey–Kramer procedure. For statistical analysis, data for fetal and postnatal body weight were transformed to logarithms to adjust for heterogeneity of variances. Correlation coefficients were estimated by linear regression (SAS, 1999). All data are presented as least square means and standard errors. The influence of sow age was not significant ($P > 0.10$) except for the three traits described.

Table 1
Ovarian and reproductive performance characteristics (L.S.M. ± S.E.) of Meishan and White Composite sows[a]

	White Composite	Meishan
N	15	16
Mean age (days)	415	332
Age (range in days)	324 to 517	304 to 365
Parity[b]	0.44 ± 0.10	0 ± 0
Ovarian weight (g)	15.9 ± 0.8	20.4 ± 0.7***
Ovarian weight minus CL (g)[c]	6.5 ± 0.4	8.2 ± 0.4*
CL weight, (g):		
Total	9.4 ± 0.6	12.2 ± 0.6**
Individual	0.6 ± 0.03	0.7 ± 0.03
CL number	15.3 ± 0.8	17.9 ± 0.7*
Live fetuses[d]	9.7 ± 0.9	12.4 ± 0.9
Live fetuses/number of CL	63.7 ± 5.4	69.7 ± 5.0
Germ cell number ($\times 10^5$, right ovary)[e]	11.6 ± 1.6	8.7 ± 1.5

Meishan vs. White Composite; CL, corpora lutea.

[a] Ovaries were obtained on days 60, 75, 90 and 105 days of gestation. Breed by day of gestation interactions were not significant ($P > 0.05$).

[b] 0, first pregnancy; 1, second pregnancy.

[c] Residual ovarian tissue was less on day 105 than on days 60 and 75 ($P < 0.05$); day 90 was intermediate.

[d] Number of live fetuses increased with age of sow ($P < 0.05$).

[e] Germ cell number decreased with age of sow ($P < 0.01$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

3. Results

3.1. Sow traits

Meishan sows had heavier intact paired ovarian weights than WC sows ($P < 0.001$; Table 1). Greater number of CL ($P < 0.05$), greater total CL weight ($P < 0.01$) and greater weight of residual tissue after dissection of CL ($P < 0.05$), all contributed to the heavier ovaries in MS than in WC sows. Total germ cell number per ovary (right) was not different between breeds and was not significantly correlated to ovulation rate ($r = 0.2$). Age of sow affected total germ cell number in the right ovary ($P < 0.01$), due to the negative correlation among these two variables ($r = -0.37$), and affected number of live fetuses ($P < 0.05$), but the correlation was weak ($r = 0.2$).

3.2. Gilt body weight and ovary characteristics

Throughout both fetal and early postnatal life, body weight followed an exponential growth curve in both breeds. Body weight favored WC gilts from 60 to 25 dpp; however, this difference was not evident in the gilts sampled at 56 dpp (Fig. 2A). Both breeds exhibited similar profiles for ovarian weight, characterized by an increase in ovarian weight from 60
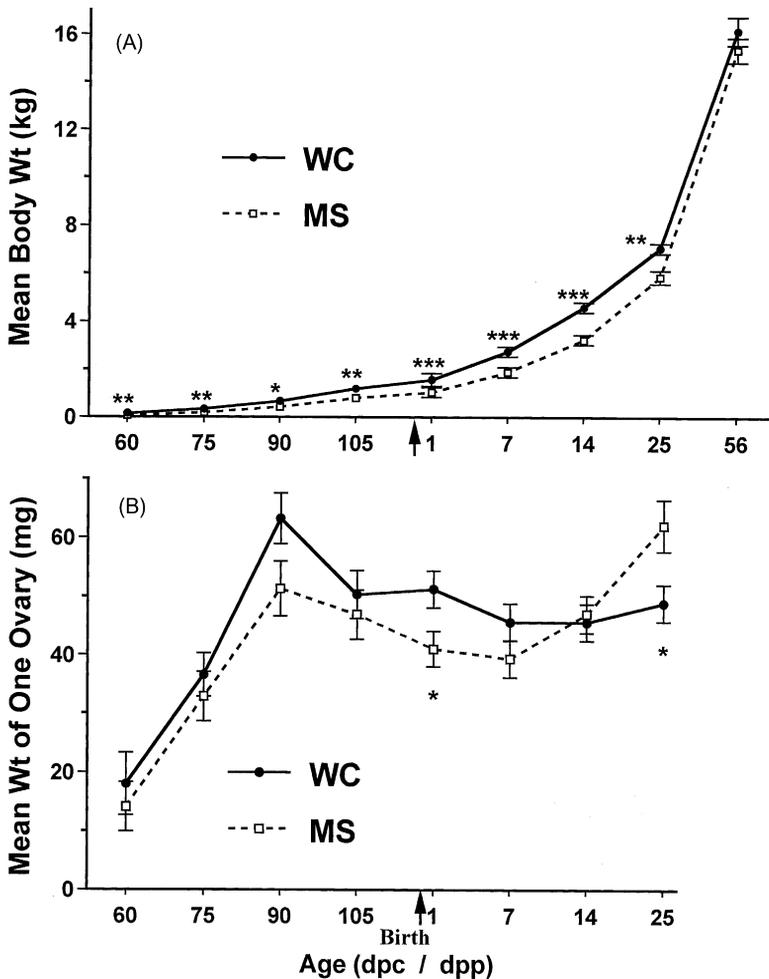
Fig. 2. Body weight (A) and ovarian weight (B) of Meishan (MS) and White Composite (WC) gilts during fetal ($n = 176$) and early postnatal life ($n = 86$; L.S.M. ± S.E.). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for MS vs. WC at specific ages; dpc, days postcoitum; dpp, days postpartum. Overall significance values: (A) body weight: fetal: age, $P < 0.0001$; breed, $P < 0.0001$; breed × age, NS. Postnatal: age, $P < 0.0001$; breed, $P < 0.001$; breed × age, NS. (B) Ovarian weight: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS. Postnatal: age, $P < 0.01$; breed, NS: breed × age, $P < 0.01$.

to 90 dpc, after which ovarian weight remained relatively constant (Fig. 2B). By 56 dpp, MS gilts had considerably heavier ovaries than WC gilts ($861.1 ± 53.1$ g versus $86.9 ± 59.5$ g; $P < 0.001$) due to substantial development of antral follicles on ovaries of MS gilts.

Proportions of ovaries comprised of cortical tissue were similar in MS and WC ovaries during fetal life ($58.6 ± 1.7\%$ versus $54.9 ± 1.8\%$) and from 1 to 25 dpp ($56.5 ± 2.1\%$ versus $53.1 ± 2.0\%$). From 75 dpc to birth, ovaries of both breeds had larger proportions of cortical than medullary tissue ($P < 0.05$). However, after 7 dpp, proportions of cortical

tissue declined and similar proportions of cortical and medullary tissue were present at 25 dpp.

3.3. Total germ cell number—enzymatic digestion

In both breeds, total germ cell number increased rapidly from 60 to 90 dpc (Fig. 3). In both breeds, germ cell number declined ($\sim$50%) from 90 to 105 dpc ($P < 0.01$) but germ cell number remained relatively constant thereafter reaching $\sim$35% of maximal numbers by 25 dpp (Fig. 2). From 60 to 90 dpc, WC gilts had greater number of total germ cells than MS gilts ($P < 0.05$). In prenatal gilts, total number of germ cells decreased with increasing age of the sows ($P < 0.01$; $r = -0.19$); this weak association of germ cell number and sow age was not apparent in early postnatal gilts ($P > 0.90$). Germ cell number was not correlated with maternal ovulation rate (data not shown). One, 1 dpp MS gilt had 10-fold greater number of germ cells than the group mean for this age group. Germ cell number for this gilt was greater than 2 S.D. of the mean for other gilts in this age group and, thus, was not included in the analysis.

3.4. Follicle maturation—cross-sectional morphometry

3.4.1. Oogonia

Irrespective of breed, ovaries had distinct cortical and medullary regions by 60 dpc, and oogonia were exclusively located within the cortex. In both breeds, the vast majority (99.7%) of germ cells were oogonia enclosed in egg cell nests located at the ovarian periphery (Fig. 1A and C). Egg nests were easily identified by GATA4-positive follicular cells
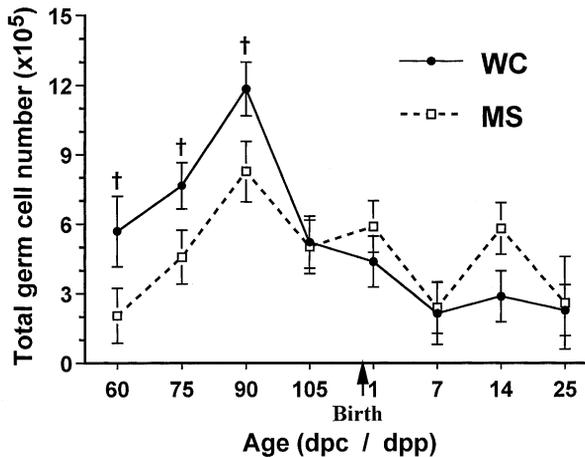
Fig. 3. Total oocyte number (L.S.M. $\pm$ S.E. $\times 10^5$) in the left ovary of fetal ($n = 160$) and early postnatal ($n = 70$) Meishan (MS) and White Composite (WC) gilts estimated by enzymatic digestion; dpc, days postcoitum; dpp, days postpartum. $^\dagger P < 0.10$ for MS vs. WC at specific ages. Overall significance values: fetal: age, $P < 0.001$; breed, ($P < 0.05$); breed $\times$ age, NS, sow age, ($P < 0.01$). Postnatal: age, $P = 0.06$; breed, NS; breed $\times$ age, NS; sow age, NS.

Fig. 4. Percentage of oogonia and primordial, primary and secondary follicles per ovary (L.S.M. ± S.E.) in Meishan (MS) and White Composite (WC) gilts during fetal ($n = 65$) and early postnatal ($n = 80$) development. $^{*} P < 0.05$; $^{**} P < 0.01$; $^{***} P < 0.001$; dpc, days postcoitum; dpp, days postpartum. Overall significance valu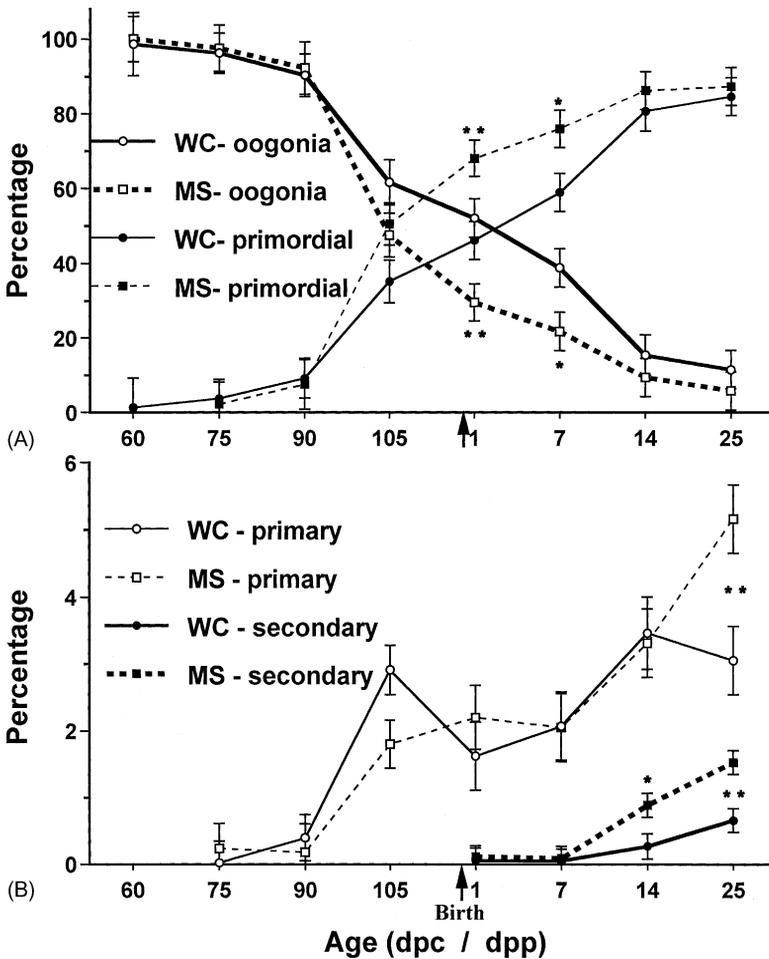es for (A) percentage of oogonia: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS; postnatal: age, $P < 0.0001$; breed, $P < 0.05$; breed × age, NS. Percentage of primordial follicles: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS.; postnatal: age, $P < 0.001$; breed, $P < 0.05$; breed × age, $P < 0.09$. (B) Percentage of primary follicles: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS; postnatal: age, $P < 0.0001$; breed, NS; breed × age, $P = 0.10$. Percentage of secondary follicles: postnatal: age, $P < 0.0001$; breed, $P < 0.06$; breed × age, $P < 0.05$.

surrounding clustered oogonia (Fig. 1A). From 60 to 90 dpc, the ovarian cortex was dominated by oogonia with similar proportions in each breed (Fig. 4A). However, by 105 dpc, numbers of oogonia decreased dramatically in both breeds representing only ∼50% of total germ cells present. Proportions of oogonia declined at a faster rate in MS than WC ovaries from 105 to 7 dpp resulting in a greater proportion of oogonia in WC than MS ovaries at birth (52% versus 30%; $P < 0.01$) and 7 dpp (39% versus 22%; $P < 0.05$), but breed
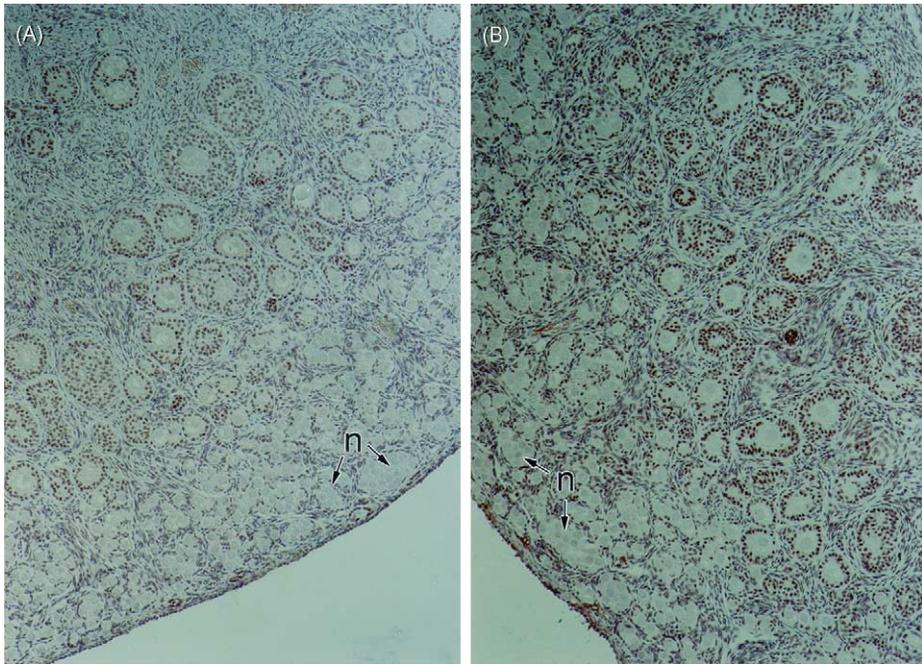
Fig. 5. (A) Ovary from a Meishan gilt at 25 days postpartum illustrating the presence of egg cell nests (n). (B) Ovary from a White Composite gilt at 56 days postpartum illustrating the presence of egg cell nests.

differences were absent at 14 or 25 dpp (Fig. 4A). Approximately 10% of the total germ cell pool was present as oogonia in both breeds at 25 dpp (Fig. 5A). However, whilst oogonia represented 3% of total germ cells at 56 dpp in WC ovaries (Fig. 5B), oogonia were absent from MS ovaries at this age (data not shown). Numbers of oogonia per CS exhibited a similar ontogenic profile as total germ cell number per CS (Fig. 6A and B). Postnatally, WC ovaries contained more oogonia compared to MS ovaries (Fig. 6B).

3.4.2. Primordial follicles

A small number of primordial follicles (2%) were observed at 60 dpc, located at the cortical–medullary border. GATA4 immunoreactive follicular cells interspersed with the oogonia in some interior egg nests indicated that primordial follicles form by invasion of follicular cells into egg nests resulting in dispersion of maturing oogonia (Fig. 1A). In this way, primordial follicles appeared in clusters reminiscent of egg nests.

Proportions of primordial follicles increased slowly between 60 and 90 dpc but increased sharply ($P < 0.01$) from 90 to 105 dpc in both breeds (Fig. 4A). Ovaries from MS gilts had a greater proportion of primordial follicles than WC ovaries at 1 and 7 dpp (Fig. 4A). Although proportions of primordial follicles continued to increase until 25 dpp in both breeds, there were no differences between breeds at either 14 or 25 dpp. Number of primordial follicles per CS diverged at 105 dpc and remained greater in MS than WC ovaries through 25 dpp (Fig. 6C).
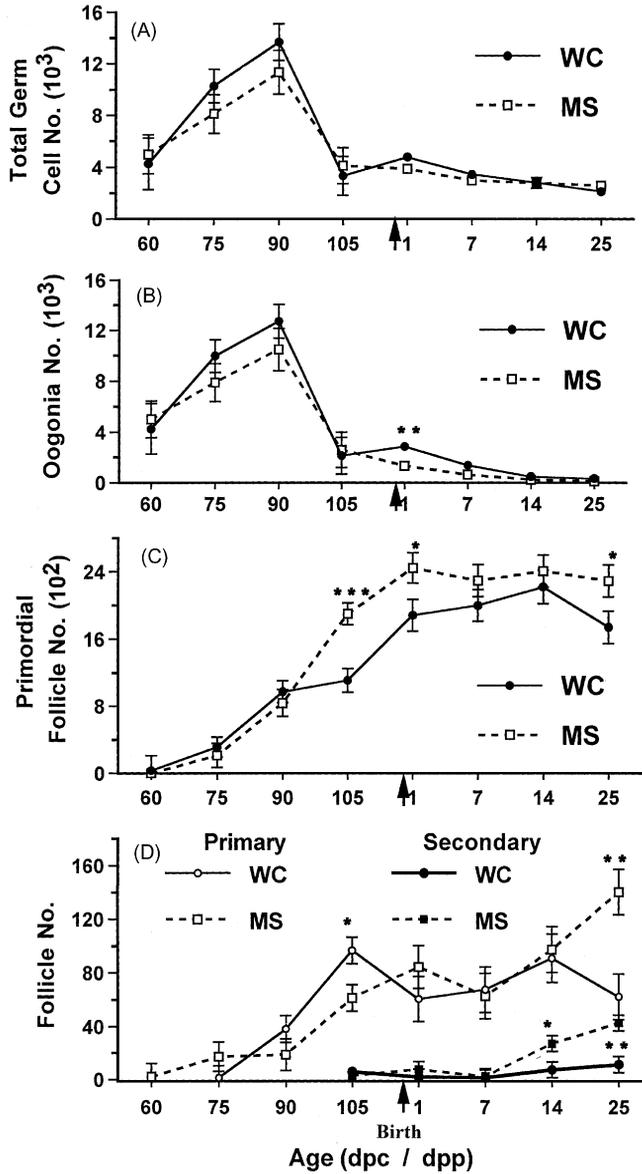
Fig. 6. Total germ cell number (A), oogonia number (B), primordial follicle number (C), primary and secondary follicle number (D) per ovarian cross-section in White Composite (WC) and Meishan (MS) gilts during fetal ($n = 65$) and early postnatal development ($n = 80$). $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ for MS vs. WC at specific ages; dpc, days postcoitum; dpp, days postpartum. Overall significance values: (A) total germ cell number: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS; postnatal: age, $P < 0.05$; breed, NS; breed × age, NS. (B) Oogonia number: fetal: age, $P < 0.0001$; breed, NS; breed × age, NS; postnatal: age, $P < 0.0001$; breed, $P < 0.05$; breed × age, $P < 0.08$. (C) Primordial follicle number: fetal: age, $P < 0.0001$; breed, NS; breed × age, $P < 0.01$; postnatal: age, NS; breed, $P < 0.05$; breed × age, NS. (D) Primary follicle number: fetal: age, $P < 0.0001$; breed, NS; breed × age, $P < 0.08$; postnatal: age, NS; breed, $P < 0.07$; breed × age, $P = 0.08$. Secondary follicle number: fetal: age, $P < 0.01$; breed, NS; breed × age, NS; postnatal: age, $P < 0.0001$; breed, $P < 0.06$; breed × age, $P < 0.01$.

3.4.3. Primary follicles

Primary follicles were first observed in both breeds at 75 dpc representing a small fraction of the total germ cell pool. Appearance of primary follicles clearly illustrated directional transition in follicle maturation from immature oogonia in egg nests dominating peripheral regions of the ovary, through to primary follicles at the innermost cortical–medullary border (Fig. 1C). Proportions of primary follicles did not increase from 75 to 90 dpc and were similar between breeds. Proportions of primary follicles increased dramatically from 90 to 105 dpc in both breeds ($P < 0.001$; Fig. 4B). Proportions of primary follicles remained constant through 7 dpp and breed differences were absent at 1, 7 and 14 dpp (Fig. 4B). However, by 25 dpp proportions of primary follicles diverged in favor of MS ovaries (Fig. 4B). Primary follicle number per CS was also greater in MS than WC ovaries at 25 dpp (Fig. 6D).

3.4.4. Secondary follicles

Secondary follicles were occasionally observed at 90 and 105 dpc but were more frequently encountered from 1 dpp onward. Consistent with directional transition in follicle maturation, secondary follicles were always located at the cortical–medullary border forming a layer inside the primary follicles. Proportions of secondary follicles increased ($P < 0.001$) during the postnatal period with a greater proportion of secondary follicles in MS than WC ovaries at both 14 and 25 dpp (Fig. 4B). Numbers of secondary follicles per CS was similar between breeds from 105 to 7 dpp, but secondary follicles increased more rapidly with age in MS than in WC ovaries from 7 to 25 dpp (Fig. 6D).

3.4.5. Antral follicles

Antral follicles were occasionally observed at 25 dpp in MS but not WC ovaries. By 56 dpp, MS ovaries had undergone a dramatic metamorphosis that accounted for their dramatic increase in weight. Large (>2 mm) fluid filled surface follicles ($n = 2$–24 per ovary) were observed in 90% of MS ovaries. Due to development of large antral follicles, and their migration from their central location to the periphery of the ovary, distinction between medulla and cortex was not possible at 56 dpp. In this way, MS ovaries at 56 dpp, histologically and visibly resembled adult ovaries. Antral follicles, but not surface follicles, were only observed in one WC gilt at 56 dpp, located at the cortical–medullary border and had not initiated migration.

3.5. Follicle size

Primordial follicles decreased in size from 75 to 1 dpp ($P < 0.001$) but remained constant thereafter in both breeds (Table 2). Primordial follicles were larger in WC than MS ovaries at 7 and 56 dpp. Primary follicles did not differ in size between breeds during fetal life, but were larger in MS than WC ovaries at 1, 25 and 56 dpp (Table 2). Secondary follicles increased in size following birth ($P < 0.001$) and MS ovaries had larger secondary follicles than WC ovaries at 25 and 56 dpp (Table 2). Antral follicles were rarely observed in WC ovaries and, thus, were not measured. However, large antral follicles were seen in MS ovaries from 25 dpp and surface follicles (>2 mm diameter) were frequently seen at 56 dpp.

Table 2

Cross-sectional area (μm^2 ($\times 10^2$); L.S.M. $\pm$ S.E.) of primordial, primary and secondary follicles in Meishan (MS) and White Composite (WC) gilts during fetal and early postnatal development

Age[a]	Primordial[b,c]		Primary[c,d]		Secondary[c,d]	
	WC	MS	WC	MS	WC	MS
75 dpc	11.8 ± 0.4	10.8 ± 0.5	19.7 ± 2.1	20.8 ± 2.6	–	–
90 dpc	10.2 ± 0.4	9.9 ± 0.5	20.7 ± 1.4	18.0 ± 2.0	–	–
105 dpc	9.4 ± 0.4	8.1 ± 0.4	13.2 ± 1.6	13.6 ± 1.6	–	–
1 dpp	6.7 ± 0.3	6.6 ± 0.3	12.3 ± 1.0	14.8 ± 1.0[†]	30.6 ± 8.3	36.6 ± 9.4
7 dpp	7.7 ± 0.3	6.0 ± 0.3***	20.8 ± 1.0	18.2 ± 1.0[†]	41.1 ± 7.9	37.2 ± 8.3
14 dpp	6.6 ± 0.3	5.8 ± 0.3[†]	18.0 ± 1.0	18.6 ± 1.0	40.0 ± 7.9	54.0 ± 7.9
25 dpp	7.0 ± 0.3	6.7 ± 0.3	15.9 ± 1.0	20.0 ± 1.0**	51.8 ± 7.6	88.0 ± 7.9**
56 dpp	6.8 ± 0.5	4.6 ± 0.5***	15.8 ± 1.5	21.3 ± 1.3**	125.8 ± 18.1	225.4 ± 16.1***

MS vs. WC within day and follicle classification.

[a] dpc, days postcoitum; dpp, days postpartum.

[b] $P < 0.001$, dpc vs. dpp.

[c] $P < 0.01$, effect of age; dpc in primordial follicles; dpc and dpp in primary follicles and dpp in secondary follicles.

[d] Breed $\times$ age interaction, dpp in primary ($P < 0.001$) and secondary ($P < 0.07$) follicles.

[†] $P < 0.10$.

** $P < 0.01$.

*** $P < 0.001$.

3.6. Oogonia/oocyte apoptosis

At 75 dpc, empty spaces were observed in egg cell nests, formed in place of degenerated and disappearing oogonia (Fig. 1D). Apoptotic oogonia were frequently observed in egg cell nests from 90 to 105 dpc, characterized by shrunken condensed nuclei (Fig. 1D). As observed for oogonia, atresia of follicles during these early stages of development appeared to result from death of germ cells followed by follicular/granulosa cells. Numbers of apoptotic oogonia or follicles were not determined directly in this study as those cells in early stages of degeneration were indistinguishable from healthy oogonia. In addition, it was not possible to determine numbers of oogonia that had degenerated and disappeared leaving large spaces in the nests. However, from 90 to 105 dpc there was a 39% ($P < 0.05$) and 56% ($P < 0.01$) reduction in total germ cell numbers (Fig. 3) in MS and WC ovaries, respectively, illustrating the rapid rates of germ cell apoptosis during this stage of development. Further, by 25 dpp, MS and WC ovaries contained 24 and 32% of their maximal germ cell complement (at 90 dpc), respectively (Fig. 3).

4. Discussion

This is the first study to describe fetal and neonatal ovarian development in MS gilts as compared to European breeds. Despite differential growth of MS and WC gilts, likely resulting from inherent breed differences (Rivera et al., 1994; Ford, 1997), ovarian growth followed a similar pattern in both breeds during fetal and neonatal life and was consistent with previous studies (Oxender et al., 1979; Colenbrander et al., 1983). However by 56 dpp,

MS ovaries visibly resembled adult ovaries with several large surface follicles that were absent from WC ovaries, indicative of early and substantial ovarian maturation in this breed. Gilt ovarian weight was not correlated to germ cell number and thus could not be used to predict germ cell population size.

Sow reproductive characteristics observed in this study are consistent with previous reports. Despite a lower mean age (332 days versus 415 days), MS sows exhibited a greater ovulation rate than WC sows. Since total germ cell number in adult ovaries did not differ between breeds, and was not correlated to ovulation rate or litter size, it is unlikely that total germ cell number is related to increased reproductive performance of MS sows.

In general, patterns of germ cell development were similar between breeds during fetal life. Rates of proliferation and atresia were not measured directly in this study; however, observations of changes in germ cell population size with advancing age, provide an indirect measure of these phenomena. Rapid increases in germ cell number from 60 to 90 dpc presumably correspond to rapid rates of germ cell proliferation, contrasting observations of Black and Erickson (1968), the only other known study with fetal pigs. Maximal germ cell numbers were observed at 50 dpc by Black and Erickson (1968), 40 days earlier than maximal germ cell number observed in this study. Germ cell number at 50 dpc was low in gilts evaluated independent of the animals used in the present study (WC: $(23 \pm 7) \times 10^4$; $n = 6$; unpublished observations), further emphasizing discrepancies between these two studies. Although methodologies used to determine germ cell number differ greatly, numerical estimates of total germ cell number are similar during late fetal and neonatal life, and similar numbers of animals were used. Ontogenic changes in total germ cell number observed in this study are supported by histological estimates of germ cell number in a representative CS (Fig. 4). Thus, estimates of germ cell numbers in this study, likely reflect true changes in the germ cell complement. There is no clear explanation for observed differences between these two studies; however, inherent breed differences cannot be discounted.

A marked decline in total germ cell numbers from 90 to 105 dpc indicates substantial apoptosis. Histological evaluations confirmed presence of large numbers of germ cells with condensed nuclei and shrunken cytoplasm characteristic of apoptosis. Large spaces formed by degeneration and subsequent disappearance of germ cells were found predominantly within egg cell nests. Marked transformation from oogonia to primordial follicles during this time is consistent with proposed increased germ cell apoptosis coincident with entry into meiosis (Hirshfield, 1991). Precipitous decline in germ cell numbers after 90 dpc is consistent with observations by Black and Erickson (1968) who attributed this decline to a preponderance of purebred animals (Duroc or Hampshire) with low germ cell numbers, sampled from 90 dpc onwards, compared to largely crossbred gilts (Yorkshire × Hampshire) with high germ cells numbers evaluated prior to 90 dpc. We also observed differences in germ cell number between two independent composite lines at 105 dpc: WCs (present study) versus a composite of 1/4 Yorkshire, 1/4 Landrace, 1/4 Large White, 1/4 Chester White established 15 years before the WC line ($(43 \pm 7) \times 10^4$ versus $(20 \pm 3) \times 10^4$; $P < 0.01$; unpublished observations). The genetically diverse breeds evaluated in the current study exhibited similar patterns of germ cell development. Declining germ cell numbers between 90 and 105 dpc were attributed to hybrid vigor by Black and Erickson (1968), but extensive apoptosis during late gestation seems a more likely cause of this rapid decline. An appropriately designed study is required to address these differences in interpretation.

Factors creating this volatile environment for fetal germ cells remains elusive; however, these observations highlight a critical period for germ cell survival. Substantial oogonial apoptosis was observed after 90 dpc but only occasional atretic follicles were observed. Notably, apoptosis was prevalent in groups of adjacent oogonia (nests) illustrating that oogonia exhibit a coordinated fate not only demonstrated by synchronous mitosis (Bielanska-Osuchowska, 1974; Guthrie and Garrett, 2001), but also synchronous cell death. Thus, oogonia that fail to reach a "threshold" stage of development seem to lose the race for survival and consequently degenerate. Alternatively, ovaries may possess a predetermined "quota" for number of germ cells that can be accommodated. Rapid transformation of oogonia to primordial follicles and appearance of primary follicles, coupled with a constant cortex area, suggest that limitations in physical space may set the upper limit for germ cell number. Thus, once the quota has been filled, larger and developmentally advanced primordial and primary follicles dominate the environment to the detriment of smaller less well-developed oogonia. Subsequent germ cell loss during early postnatal life, and gradual but progressive disappearance of germ cells with advancing age, is consistent with observations in other species (human: Block, 1951; Baker, 1963; mouse: Jones and Krohn, 1961; monkeys: Miller et al., 1999). Collectively, these observations illustrate the hazardous environment germ cells are exposed to during early development, but despite all odds, a sufficient number survive to provide the stockpile for subsequent ovulation.

Although few differences were observed in total germ cell pool size between breeds, striking differences were apparent in follicular maturation. While initiation of follicle maturation occurred at a similar time, the period from 105 dpc to birth appeared to represent a transition phase of development in MS ovaries. This phase was characterized by rapid transition from oogonia to primordial follicles in MS ovaries resulting in a larger primordial follicle pool in MS than WC ovaries by birth. Full complements were established by birth in MS gilts and primordial follicle number remained constant during the first 25 days of life presumably due to recruitment of oogonia resulting in a depletion of oogonia by 56 dpp. In contrast, in WC ovaries the primordial pool was not established until 14 dpp followed by a decline in number indicative of atresia, and 3% of the germ cell pool were still oogonia at 56 dpp. Similarly, despite primary and secondary follicle formation at a similar age in both breeds, MS ovaries exhibited enhanced primary and primordial recruitment indicative of increased primordial follicle activation. Antral follicles were also present in MS ovaries by 25 dpp and transformation of ovarian tissue reminiscent of adult ovaries (characterized by numerous surface follicles) at 56 dpp, further emphasizes advanced follicular maturation in MS compared to WC gilts.

Gonadotrophins have been implicated in germ cell survival in monkeys (Gulyas et al., 1977), rabbits (Jost et al., 1973) and humans (Ch'in, 1938; Baker and Scrimgeour, 1980). Fetal pig pituitaries differentiate after 70 dpc (Liwska, 1975; Ma et al., 1996), after which circulating gonadotrophins are detectable (Colenbrander et al., 1977, 1982). However, exogenous gonadotrophins are unable to stimulate primary follicle granulosa cell proliferation in 1–3-day-old pigs in vitro (Morbeck et al., 1993), and fail to modify ovarian development in 1-month-old pigs (Oxender et al., 1979). FSH can stimulate proliferation of granulosa cells from secondary follicles collected from 50- to 60-day-old pigs in vitro (Morbeck et al., 1993), and appearance of antral follicles represents a stage when follicles become sensitive to gonadotrophins (Mauléon, 1964; Oxender et al., 1979). Thus, pigs appear insensitive

to gonadotrophins during early postnatal development; however, follicular development is slightly retarded at birth in decapitated gilts (Colenbrander et al., 1983) suggesting that some pituitary hormone or combination of hormones influences fetal follicular maturation.

Several other factors have been implicated in initial recruitment of follicles into the growing pooling of developing follicles. Androgens increase primary follicle number and insulin-like growth factor-1 receptor expression in mice (Vendola et al., 1999a,b), and may regulate FSH receptor mRNA levels in ovarian follicles thereby influencing ovulation rate in gilts (Cardenas et al., 2002). Vascular endothelial growth factor controls follicular phase follicle recruitment in non-human primates (Zimmermann et al., 2002) and diet can influence follicle recruitment in heifers (Gong et al., 2002). Stem cell factor or kit ligand (Parrott and Skinner, 1999), growth-differentiation factor-9 (Vitt et al., 2000), basic fibroblast growth factor (Nilsson et al., 2001) and nerve growth factor (Dissen et al., 2001) promote initiation of primordial follicle growth. Müllerian inhibiting substance, a member of the transforming growth factor-β superfamily of growth and differentiation factors (Cate et al., 1986), is also implicated in primordial follicle recruitment (Durlinger et al., 1999) inhibiting initiation of primordial follicle growth (Durlinger et al., 2002). The role of these factors in recruitment of porcine follicles remains to be investigated.

Increased ovulation rate in adult MS sows has been attributed to a greater number of small developmentally advanced follicles recruited into the growing pool (Miller et al., 1998; Hunter et al., 1994; Biggs et al., 1993; Faillace and Hunter, 1994) compared to European breeds. Whilst differential follicle size was not observed in this study, increased recruitment of growing follicles in MS ovaries is consistent with this hypothesis. Moreover, the size of primordial follicle pool regulates numbers of follicles entering the growth phase (mouse: Krarup et al., 1969; human: Gougeon and Lefèvre, 1984), and a direct relationship between follicle numbers in the growth phase and ovulation rate has been reported in ewes (Cahill et al., 1979). Establishment of a larger primordial follicle pool in MS compared to WC ovaries during development could therefore provide a mechanism to explain differential ovulation rate between these two breeds. Differential recruitment of growing follicles may also be related to factors that prepare the ovarian environment for early onset of puberty in MS gilts (Bolet et al., 1986; Rothschild and Bidanel, 1998; Haley and Lee, 1990; Youngs et al., 1993). However, enhanced recruitment of primary and secondary follicles in MS gilt ovaries is also consistent with increased follicular recruitment during the estrous cycle associated with increased ovulation rate (Miller et al., 1998), indicating mechanisms that increase growing follicle recruitment are initiated early in development. Collectively, observations from this study indicate an association between a larger primordial follicle pool coupled with increased follicular recruitment during early development and subsequent ovulation rate in MS sows compared to European breeds. Irrespective of physiological roles of these developmental differences, this study highlights a window of opportunity to study factors/mechanisms that influence/regulate ovarian maturation in pigs.

5. Conclusion

This study highlights key developmental events that occur during early ovarian development in pigs. Although total germ cell pools may contribute little to reproductive efficiency,

numbers of germ cells that survive to enter primordial and developing follicle pools, may influence future reproductive performance. Certainly, substantial breed differences in rates of follicle maturation offer a unique opportunity to study factors or mechanisms that initiate and coordinate follicle development which have the ultimate potential to influence age at puberty and ovulation rate in sows.

Acknowledgements

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