Temporal changes in histomorphology and gene expression in goat testes during postnatal development1,2


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ABSTRACT: Testicular cell proliferation and differentiation is critical for development of normal testicular function and male reproductive maturity. The objective of the current study was to evaluate histological architecture and expression of genes marking specific cells and important functions as well as testosterone production of the developing goat testes. Testes were harvested from Alpine bucks at 0, 2, 4, 6, and 8 mo of age (n = 5/age group). Paired testes weight increased from 2 to 4 (P < 0.001) and 4 to 6 mo (P < 0.01). The greatest increases in seminiferous tubule and lumen diameters and height of the seminiferous epithelium occurred between 2 and 4 mo (P < 0.001). Genes expressed in haploid germ cells (Protamine1 [PRM1], Outer Dense Fiber protein 2 [ODF2], and Stimulated by Retinoic Acid gene 8 [STRA8]) increased dramatically at the same time (P < 0.001). Expression of other genes decreased (P < 0.05) during testicular maturation. These genes included P450 side chain cleavage (CYP11A1), Sex determining region Y-box 9 (SOX9), Insulin-like Growth Factor 1 Receptor (IGF1R), and Heat Shock Protein A8 (HSPA8). The Glutathione S-Transferase A3 (GSTA3) gene, whose product was recently recognized as a primary enzyme involved in isomerization of androstenedione in man and livestock species including goats, sheep, cattle, pigs, and horses, uniquely peaked in expression at 2 mo (P < 0.05). Follicle-Stimulating Hormone Receptor (FSHR) mRNA abundance tended to steadily decrease with age (P = 0.1), while Luteinizing Hormone Receptor (LHCGR) mRNA abundance in testes was not significantly different across the ages. Testosterone content per gram of testicular tissue varied among individuals. However, testosterone content per testis tended to increase at 6 mo (P = 0.06). In conclusion, major changes in cellular structure and gene expression in goat testes were observed at 4 mo of age, when spermatogenesis was initiated. Male goats mature rapidly and represent a good model species for the study of agents that enhance or impair development of testicular functions.

Key words: buck goat, spermatogenesis, steroidogenesis, testis development, testosterone biosynthesis


INTRODUCTION

Testicular development is a complex process that requires the coordinated proliferation, differentiation, and maturation of both somatic and germ cells. In mammals, postnatal development of the testis includes growth of seminiferous tubules containing the Sertoli and germ cells and expansion of the population of steroidogenic Leydig cells (Carmon and Green, 1952; Ren et al., 2009). Leydig cells grow in the interstitial spaces surrounding the seminiferous tubules and produce testosterone, which supports spermatogenesis (Roberts and Zirkin, 1991; Baker and O’Shaughnessy, 2001). In male sheep and goats, spermatocytes and other mature sperm cells first
appear between 3 and 4 mo of age, which corresponds with the onset of puberty (Carmon and Green, 1952; Nishimura et al., 2000).

The goat is an economically important species worldwide that provides meat, milk, and fiber, yet our understanding of the testicular physiology of the male goat (buck) is relatively limited. The available literature reports aspects of seminiferous tubule cycle (Bilaspuri and Guraya, 1984; França et al., 1999), changes in sperm nuclear proteins (Courten and Loir, 1981), testis development and sexual behavior (Nishimura et al., 2000), and high spermatogenic efficiency (Johnson et al., 2000; Leal et al., 2004). However, little is known about temporal changes in the pattern of gene expression during testicular development in the buck. In this study, selected genes known to be involved in spermatogenesis and steroidogenesis were investigated during postnatal development and reproductive maturation in the buck testis in relation to histomorphological changes and testosterone synthesis. The objective of this study was to characterize the developmental and maturational process of the goat testis as a function of temporal changes in the expression of genes critical for testicular function.

**MATERIALS AND METHODS**

**Animal and Sample Preparations**

All animal procedures were approved by the Prairie View A&M University Animal Care and Use Committee. Twenty-five Alpine buck kids from the International Goat Research Center (Prairie View A&M University, Prairie View, TX) born in March were castrated at 0, 2, 4, 6, and 8 mo of age (n = 5 each age). Testes were separated from the epididymis and tunic vaginalis, weighed, and sliced midsagittally. Parenchyma from the right testis was minced, snap-frozen in liquid nitrogen, and stored at −80°C, and a 1-cm³ cube was placed in a 50 mL of 4% paraformaldehyde fixative for 24 h before washing and storage in 70% ethanol until paraffin embedding.

**Testosterone Content of Testis Tissues**

Serum samples were not available. Therefore, testosterone content of testes tissues was measured. Right testis tissue samples (1 g) from bucks aged 2, 4, 6, and 8 mo were minced in a weigh boat, transferred to 10-mL Corex tubes (Corning, Tewksbury, MA), and homogenized in 2 mL of PBS with a Teflon-coated pestle (PRO250 model; PRO Scientific, Inc., Monroe, CT). There was not enough tissue available to extract samples for the 0-mo group of goats. The homogenates were mixed by vortexing and double extracted with 5 mL of diethyl ether. By use of a liquid nitrogen bath, the aqueous layer was frozen and the organic supernatant layer was transferred into 20-mL glass scintillation vials to evaporate the ether solvent. The dried extract was reconstituted with 1 mL of Tris buffered saline to use in the testosterone enzyme immunoassay (catalog number ADI-900-0065; ENZO Lifesciences, Farmingdale, NY). Aliquots of the reconstituted testis extracts were diluted 1:10 with Tris buffered saline and duplicate 100-μL aliquots of the diluted unknowns were assayed to determine the concentration of testosterone per gram of testis tissue. The cross-reactivity of the mouse monoclonal antibody to testosterone is 100, 7.2, 0.7, and 0.001% for the androgens testosterone, androstenedione, dehydroepiandrosterone, and dihydrotestosterone, respectively. Cross-reactivity of the testosterone antibody was 0.4% with estradiol and <0.001% for progesterone and pregnenolone. All unknown samples were processed in 1 single assay. The testosterone standard curve ranged from 7.8 to 2,000 pg/mL. The testosterone ELISA was conducted per the manufacturer’s suggested incubation times and temperatures. The optical density of each standard and unknown was determined at 405 nm with a correction between 570 and 590 nm by use of a BIO-RAD Model 680 Microplate Reader (BIO-RAD Laboratories, Hercules, CA). The concentration of testosterone for each goat testis extract was then calculated by use of AssayZap software (Biosoft, Cambridge, UK).

**Histomorphology and Tubule Measurements**

Paraformaldehyde fixed, paraffin-embedded tissue blocks of the right testis were cut in 20-μm sections using a microtome. Tissue sections were mounted on glass slides and processed for hematoxylin and eosin staining. Histological sections were observed in bright field on a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY). Images of stained sections were captured using a DS-Qi1Mc digital camera (Nikon Instruments, Inc.). Images of 30 round cross-sections of tubules were used to determine the mean diameter of the seminiferous tubule, the mean height of the seminiferous tubule, and the mean diameter of the tubule lumen for each goat. Image analysis was performed using the NIS-Elements BR 3.2 software (Nikon Instruments, Inc.).

**Ribonucleic Acid Isolation**

Total RNA was isolated from right testis samples collected from bucks at 0, 2, 4, 6, and 8 mo of age using TRIzol (Life Technologies, Grand Island, NY) according to manufacturer’s instructions. Concentrations of RNA samples were determined on a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Integrity of RNA was determined on a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA).
Cloning of PRM1, STRA8, and SOX9 cDNA from Goat Testes

To make probes for detecting PRM1, STRA8, and SOX9 mRNA on northern blots, 2 µg of mRNA from 2 bucks (ages 0 and 8 mo) were reverse transcribed with Superscript II (Invitrogen, San Diego, CA) and random octamer primers. Two sets of primers (Table 1), the second nested inside the first, were designed from Bos taurus cDNA sequences for PRM1, STRA8, and SOX9 (GenBank accession numbers BC108207, NM_182489, and AF278703, respectively). The T7 promoter sequence (TA-ATACGACTCACTATAGGG) was added to the 5′ end of the second antisense primer. Initial PCR used reverse-transcribed cDNA and the first set of primers, while secondary PCR used one-tenth of the initial PCR as template along with the second set of primers. The PCR-generated cDNA were cloned using the TA Cloning Kit protocol (Life Technologies). Plasmids were purified using Qiagen Plasmid Purification kit (Qiagen, Valencia, CA). The goat PRM1, STRA8, and SOX9 cDNA were sequenced, and sequences were submitted to GenBank (accession numbers HM773246, HM773245, and HM773244, respectively).

In Situ Hybridization Localization of Selected mRNA to Specific Testis Cells

The mRNA for PRM1, STRA8, SOX9, HSPA8, and ODF2 were localized on serial cross-sections from the right testis of goat buck kids (ages 0, 2, 4, 6, and 8 mo) by in situ hybridization. Tissue sections (7 µm) were hybridized with radiolabeled antisense complementary RNA (cRNA) probes produced by in vitro transcription with [α-35S] uridine triphosphate (UTP; 1,250 Ci/mmol; New England Nuclear, Boston, MA). The cloned cDNA for PRM1, STRA8, and SOX9 in plasmids linearized with EcoRI were used as templates. In addition, rat ODF2 cDNA in pBluescriptII vector was linearized with NcoI and transcribed with T7 RNA polymerase. Also, a PstI fragment of bovine HSPA8 cDNA in pBS-HSP70kdp8 (GenBank accession number NM_174381) was linearized with StyI and transcribed with T3 RNA polymerase. After hybridization, washing, and ribonuclease A digestion, slides were coated with photographic emulsion (Eastman Kodak, Rochester, NY). After 1.5 to 6 wk at 4°C, silver grains were developed with Kodak D-19. Then, sections were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO), dehydrated, and cover slipped. Digital photomicrographs of in situ hybridization in bright field and dark field were captured using the Nikon microscope, camera, and software described above.

Northern Blot Analyses of Gene Expression

Total testes RNA samples (8 µg) were denatured, subjected to electrophoresis on a 1.2% agarose gels alongside RNA Millennium Markers (Ambion, Austin, TX), and transferred to nitrocellulose membranes. The blots were hybridized with radiolabeled antisense cRNA probes produced by in vitro transcription with [32P]-UTP (3,000 Ci/mmol; New England Nuclear). The templates for in vitro transcription were as described above. After washing, hybridization signals were detected by exposing the blots to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Piscataway, NJ).

Quantitative PCR Analyses of Gene Expression

Testicular RNA samples (100 ng) were reverse transcribed with oligo-dT and random octamer primers and Superscript II (Life Technologies), according to manufacturer’s instructions. Primers for CYP11A1, FSHR, GAPDH, GSTA3, IGF1R, and LHCGR PCR (Table 2) were designed to bovine or goat cDNA sequences (GenBank accession numbers NM_001244612.1, D50058.1, NM_174061.1, NM_174381.1, and XM_001252511.3, respectively) in regions conserved with human sequences using Primer Express software (Life Technologies). Duplicate 10-µL reactions were performed with Power SYBR Green Master Mix (Life Technologies), PCR grade H2O (Ambion), 300 nM of forward and reverse primers, and 0.5 µL cDNA in a 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The efficiencies of PCR amplification were measured for each primer pair, with concentrations of two cDNA samples varied in concentration over 5 logs. The efficiencies (Table 2) averaged 96% for the target mRNA, very close to that of the normalizer GAPDH mRNA (98%). Threshold cycles were normalized to GAPDH values and

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Table 1. Primer sequences used in cloning cDNA from buck testes
Structure and gene changes in developing testes

Their central location in the tubules was consistent with 

were greater than at all other ages, even 8 mo (Fig. 2B;  

mRNA in testes from 4-, 6-, and 8-mo-old bucks. Concentration of testosterone per gram of testis did not 

mRNA had weak hybridization signals in the 2-mo-old testis despite the 6-wk exposure to photographic emul- 

In situ hybridization for SOX9, HSPA8, STRA8, PRM1, and ODF2 mRNA was performed on cross-sections of goat testes to localize gene expression to specific cells. Hybridization signals for both SOX9 and HSPA8 mRNA appeared strongest in the 0- and 2-mo-old animals due to the high concentrations of Sertoli cells and spermatogonia in seminiferous tubules (Fig. 4A through 4D). SOX9 mRNA had weak hybridization signals in the 2-mo-old testis despite the 6-wk exposure to photographic emulsion. In contrast, there were strong signals for STRA8, PRM1, and ODF2 mRNA in testes from 4-, 6-, and 8-mo-old bucks, indicating expression of these genes in the matured germ cells (Fig. 4E through 4J). At these ages, hybridization signals for STRA8 mRNA were intense in type B spermatogonia in the basal compartment and moderate in the adluminal compartment of the seminiferous tubules (Fig. 4E and 4F). Round and elongated spermatids and spermatozoa had intense hybridization signals for PRM1 and ODF1 mRNA (Fig. 4G through 4J).

Temporal Changes in Gene Expression in the Developing Testes

Northern blot analyses were selected for characterization of the sizes of the initially selected mRNA targets because such information is lacking, especially for live-stock testes. The abundance of SOX9 (3.9 kb in length) and HSPA8 (2.3 kb) mRNA appeared to be greater at early ages, while those of STRA8 (1.8 kb), PRM1 (580 bp), and ODF2 (2.5 kb) mRNA increased to high levels between 2 and 4 mo (Fig. 5). Changes in gene expression during testes development were quantitated on northern blots (Fig. 6A through 6E; for ODF2, PRM1, STRA8, HSPA8, and SOX9) or by quantitative PCR (Fig. 6F through 6J; for CYP11A1, GSTA3, LHCGR, IGF1R, and FSHR) for all 25 testes samples. The expression of ODF2, PRM1, and STRA8, unique to sper-
Figure 1. The weight of combined left and right testes of bucks from 0 to 8 mo of age is presented as means (±SEM). Analysis of variance indicated significant differences between age groups ($P < 0.001$). Means without a common superscript differ ($^{a,b}P < 0.0001$; $^{b,c}P < 0.01$).

Figure 2. Concentrations of testosterone per gram of testis (panel A) and concentrations adjusted per total right testis weight (panel B) of bucks at 2, 4, 6, and 8 mo of age (means ±SEM are graphed). Analysis of variance indicated a tendency for a difference in testosterone concentration per total right testis weight at 6 mo of age compared to the 2-mo-old group ($^{a,b}P = 0.06$). Means without a common superscript differ.

Figure 3. Histomorphological analysis of developing buck testes. Representative photomicrographs of hematoxylin- and eosin-stained sections of testes of bucks at 0 (A), 2 (B), 4 (C), 6 (D), and 8 (E) mo of age (scale bar = 100 μm). Higher magnification images of tubules indicated by arrows are shown in insets a through e (scale bar = 25 μm). In the 0-mo-old testis, degenerating cells were present in the centers of many seminiferous tubules (asterisk in inset a). Means (±SEM) are graphed for the diameter of the seminiferous tubule (F), the diameter of the lumen of the seminiferous tubule (G), and the height of the seminiferous epithelium (Semin. Epith. in panel H) in bucks at 0, 2, 4, 6, and 8 mo of age. Analysis of variance indicated significant increases between the 2 early time points (0 and 2 mo of age) and the older age groups ($^{a,b}P < 0.001$). Means without a common superscript differ ($P < 0.05$).
matogenic cells, increased in testes between 2 and 4 mo (Fig. 6A through 6C). The increases in ODF2 and PRM1 expression were 177- and 330-fold \((P < 0.001)\) while that of the STRA8 was 17-fold \((P < 0.05)\). In contrast, the expression of the SOX9, exclusive to Sertoli cells, decreased by 65% in testes between 2 and 4 mo (Fig. 6E; \(P < 0.001\)). The decrease in HSPA8 mRNA levels was more gradual and occurred between 4 and 8 mo (Fig. 6D; \(P < 0.01\)). Quantitative PCR identified distinct patterns of expression for Leydig cell–specific genes CYP11A1, GSTA3, and LHCGR. Expression of the CYP11A1 decreased from 0 to 8 mo in testes (Fig. 6F; \(P < 0.05\)). Expression of the GSTA3 appeared to increase between 0 and 2 mo and then it dropped precipitously between 2 and 4 mo (Fig. 6; \(P < 0.05\)). LHCGR expression varied in testes across ages (Fig. 6H; \(P = 0.3\)). Relative expression of the IGF1R decreased by 70% between 0 and 4 mo (Fig. 6I; \(P < 0.05\)). FSHR mRNA abundance tended to decrease with increasing age (Fig. 6J; \(P < 0.1\)).

**DISCUSSION**

The goat is a livestock species that grows and matures rapidly. In the buck, testes reach mature size and start producing sperm at 4 mo, similar to Blackbelly rams but earlier than bulls (Schanbacher, 1979; Bagu et al., 2006; Herrera-Alarcón et al., 2007). The decrease in testis size detected between 6 and 8 mo of age (sampled in September and November, respectively) is due to seasonality, driven by changes in photoperiod. In the northern hemisphere, the size of buck testes peaks in September, with a nadir in January (Ahmad and Noakes, 1995). In addition, this study demonstrates steroidogenic activity in the buck testis at young ages including 2 mo. Testosterone is critical for testicular development and spermatogenesis, although the mechanisms by which it exerts its support are still unclear (Roberts and Zirkin, 1991; Hall, 1994). To our knowledge, this is the first report of degenerating cells in the center of seminiferous tubules in 0-mo-old bucks, which are probably germ cells because of their location (Curtis and Amann, 1981). However, this is consistent with reports in other mammalian species of extensive degeneration of male germ cells in early life (Vergouwen et al., 1991; Dunkel et al., 1997). The observation that the seminiferous tubule cross-sectional dimension increases during testicular development in the goat adds to previous studies that demonstrated increases in tubule lengths and expansion of the var-

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**Figure 4.** Representative bright field and dark field images (left and right panels, respectively) are shown for in situ hybridization of selected mRNA in buck testes. Heat Shock Protein A8 (HSPA8) and Sry-like HMG box gene 9 (SOX9) mRNA images are from 2-mo-old buck testes while Outer Dense Fiber 2 (ODF2), Protamine1 (PRM1), and Stimulated by Retinoic Acid gene 8 (STRA8) mRNA data are from a 6-mo-old testis. Hybridization signals are silver grains (white on dark field images) appear over the cells containing the mRNA of interest.

**Figure 5.** Representative northern blot data depict expression of SOX9, HSPA8, STRA8, PRM1, and ODF2 genes in buck testes at the ages in months shown. Radioactive bands (black) identify the specific mRNA, as indicated. Sizes of SOX9, HSPA8, STRA8, PRM1, and ODF2 mRNA are 3.9 kb, 2.3 kb, 1.18 kb, 580 bp, and 2.5 kb, respectively. The lanes labeled “M” contained RNA markers, which do not hybridize to the mRNA probes.
ious testis cell populations (germ, Sertoli, and Leydig cells) during postnatal development (Baker and O’Shaughnessy, 2001; Bagu et al., 2006; Herrera-Alarcón et al., 2007).

To begin addressing the molecular pathways involved in testis development, expression levels of selected genes were measured in testis tissues and related to morphometric changes. Overall, the biggest differences were observed between 2 and 4 mo of age. This was true for the increases in testes weight, seminiferous tubule and lumen diameters, and the expression of STRA8, PRM1, and ODF2 genes in germ cells that have committed to undergoing meiosis and spermiogenesis (Hoyer-Fender et al., 1998; Horowitz et

Figure 6. Relative expression of selected genes (means ± SEM) in buck testes during postnatal development. Relative levels of mRNA are represented with 0 mo of age adjusted to 100%. Exceptions were panels A through C, where 6 mo levels were set at 100%. Panels A through E represent data obtained by northern blot analyses and panels F through J represent data obtained by quantitative PCR. Analysis of variance indicated increasing concentrations of ODF2, PRM1, and STRA8 mRNA between 2 and 4 mo (panels A to C; \( P < 0.001 \), \( P < 0.001 \), and \( P < 0.05 \), respectively). Concentrations of PRM1 mRNA decreased from 6 to 8 mo (panel B; \( P < 0.001 \)). HSPA8 mRNA concentrations decreased from 4 to 8 mo (panel D; \( P < 0.01 \)). SOX9 mRNA concentrations increased from 0 to 2 mo (panel E; \( P < 0.01 \)) and then decreased from 2 to 4 mo (\( P < 0.001 \)). CYP11A1 mRNA levels decreased from 0 to 8 mo (panel F; \( P < 0.05 \)). Concentrations of GSTA3 mRNA decreased from 2 to 4 mo (panel G; \( P < 0.05 \)). IGF1R mRNA concentrations decreased from 0 to 4 mo (panel I; \( P < 0.05 \)). Concentrations of FSHR mRNA tended to decrease over the ages (panel J; \( P = 0.1 \)) but there was no difference in LHCGR mRNA concentrations (panel H; \( P = 0.3 \)). Means without a common superscript differ (\( P < 0.05 \)). Rel. = Relative.
Structure and gene changes in developing testes

An intriguing finding of this study is that the patterns of expression of the Leydig cell–specific genes CYP11A1, GSTA3, and LHCGR were distinct. Expression of the CYP11A1 gene decreased between 0 and 8 mo of age, while that of the GSTA3 gene dropped rapidly between 2 and 4 mo. In contrast, expression of the LHCGR gene was variable in testes across the ages investigated. CYP11A1 and GSTA3 mRNA both encode enzymes that are directly involved in testosterone biosynthesis. The human GSTA3 enzyme has recently been discovered to have the greatest activity in isomerizing Δ^5-androstenedione to Δ^4-androstenedione, the immediate precursor of testosterone (Johansson and Mannervik, 2001). No GSTA3 gene homologue is expressed in rodent testes, however, because those species use the Δ^4-steroidogenic pathway (Conley and Bird, 1997). Our results appear to contrast with one study, in which expression of Cyp11a1 and Lhcgr genes increased in Leydig cells isolated from developing testes of mice through postnatal d 25 (O’Shaughnessy et al., 2002). The difference is likely due to decreasing numbers of Leydig cells relative to the expanding number of haploid germ cells in the maturing goat testes in our study. Nevertheless, the high levels of expression of the CYP11A1 and GSTA3 genes in goat testes at 0 and 2 mo of age may be critical for steroidogenesis and the initiation of spermatogenesis at 4 mo of age.

In conclusion, numerous qualitative and quantitative changes were observed in goat testes over the course of postnatal development. The seminiferous tubules attained mature cross-sectional dimensions by 4 mo of age and acquired a predominance of maturing germ cells. The unique patterns of expression of Sertoli- and Leydig-specific genes indicate finely tuned regulation. Future investigations are needed to fully understand the development of optimal testes functions: steroidogenesis and spermatogenesis.

LITERATURE CITED


