Histol Histopathol (2008) 23: 151-156

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Effects of hypothyroidism on anti-mullerian hormone expression in the prepubertal rat testis

S.M.L. Chamindrani Mendis-Handagama and H.B. Siril Ariyaratne*

Department of Comparative Medicine, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, USA

*Present address: Department of Veterinary Basic Sciences, The University of Peradeniya, Sri Lanka

Summary. Differentiation of adult Leydig cells (ALC) in the prepubertal rat testis is stimulated by thyroid hormone (Thy) and inhibited by the Anti-Mullerian Hormone (AMH) produced by the immature Sertoli cell (SC). As Thy induces SC maturation in the prepubertal rat testis, we hypothesized that Thy stimulation of ALC differentiation is mediated via inhibition of AMH production by the SC with their maturation. If this hypothesis is true, AMH production by the prepubertal Sertoli cells in hypothyroid rats should not decline immediately after birth as in euthyroid rats, but should be maintained throughout the hypothyroid period at a similar or higher level to that of day 1 rats. This concept was tested using control rats of postnatal days (pd) 1, 7 and 14 and hypothyroid (fed 0.1% propyl thiouracil/PTU to lactating mothers) rats of pd7 and pd14. Presence of AMH in SC was examined by immunocytochemistry for AMH. Results demonstrated that testes of pd1 rats had intense AMH positive labeling exclusively in cytoplasm of SC. In testes of pd7 and pd14 control and PTU rats, a positive but weak labeling was also observed in cytoplasm of some SC; Germ cells and testicular interstitial cells were negative for AMH at all tested ages in both experimental groups. These findings suggest that AMH production by the prepubertal SC is independent of Sertoli cell maturation and not regulated by Thy. Therefore, Thy regulation of ALC differentiation in the prepubertal rat testis is unlikely to be mediated via inhibition of AMH produced by the SC with their maturation.

Key words: Anti-Mullerian Hormone, Sertoli Cells, Testis, Hypothyroidism, Leydig cell differentiation

Offprint requests to: Dr. C. Mendis-Handagama, Department of Comparative Medicine, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN 37996, USA. e-mail: mendisc@utk.edu.

Introduction

Establishment of the adult Leydig cell population in the postnatal testis is critical to the male mammal for his general health and reproduction. Stem cells for the adult Leydig cells in the postnatal testis are shown to be the mesenchymal cells by many previous investigators (Roosen-Runge and Anderson, 1959; Lording and de Kretser, 1972; Mendis-Handagama et al., 1987) and studies from our laboratory have confirmed this speculation for prepubertal (Ariyaratne et al., 2000a-c) as well as the adult rats (Ariyaratne et al., 2000d). One unresolved issue in the process of adult Leydig cell differentiation is the mechanism(s) of the regulation of the onset of this process, where a non steroidogenica mesenchymal precursor cell differentiates into a steroidogenic Leydig progenitor cell. The importance of thyroid hormone in this triggering process is being repeatedly seen in several studies; hypothyroidism inhibits (Mendis-Handagama et al., 1998; Teerds et al., 1998; Ariyaratne et al., 2000b-d) and hyperthyroidism accelerates (Teerds et al., 1998; Ariyaratne et al., 2000b,c) this initial step in the Leydig cell differentiation process in prepubertal rats, as well as in adult rats following ethane dimethane sulphonate treatment (EDS, Ariyaratne et al., 2000d); EDS is an unique toxin for Leydig cells in rats and kills them within 48 hours of exposure (reviewed by Morris, 1996).

Anti-Mullerian hormone (AMH) is a 140 kDa protein produced by the immature Sertoli cells. It causes the degeneration of the female genital ducts (i.e. Mullerian/ paramesonephric ducts) in the male fetus. AMH is present in Seroli cells at birth in rats, however, rapidly disappears during the neonatal period (Tran et al., 1987; Kuroda et al., 1991). Therefore, it may be possible that AMH content in the neonatal-prepubertal Sertoli cells is inversely correlated with Sertoli cell maturation during the neonatal-prepubertal period. As AMH has been reported as a possible negative regulator

of the process of Leydig cell differentiation (Racine et al., 1998; Teixeira et al., 1999; Flynn-Thompson et al., 2003; Salva et al., 2004) and thyroid hormone causes maturation of Sertoli cells in the neonatal-prepubertal rat testis (Van Haaster et al., 1993), it is logical to hypothesize that the stimulatory effects of thyroid hormone on Leydig precursor cell differentiation may possibly be exerted via its effect on Sertoli cell maturation and thereby, inhibiting the production of AMH. This hypothesis is supported by the study of Arambepola et al. (1998), which reported that triiodothyronine (T3) inhibits AMH/Mullerian inhibiting substance mRNA production by the neonatal rat Sertoli cells in culture. Based on this observation these investigators (Arambepola et al., 1998) suggested that AMH/MIS production by the neonatal rat Sertoli is regulated by T3. However, studies performed with fetal rat Sertoli cells, it has also been shown that AMH/MIS mRNA levels cannot be taken as a reliable index of AMH/MIS protein expression (Kuroda et al., 1991).

Based on the findings of Arambepola et al. (1998), it is possible to hypothesize that withdrawal of the negative effect of AMH produced by the Sertoli cells on Leydig cell differentiation should trigger the onset of mesenchymal cell differentiation into the Leydig progenitor cells. If this concept is true, AMH production by the neonatal-prepubertal Sertoli cells in the rat testis could be maintained under a hypothyroid status where Sertoli cells fail to undergo maturation (van Haaster et al., 1993). In the present investigation we designed the experiment to prevent Sertoli cell maturation in the neonatal rats by subjecting these rats to a hypothyroid status. We tested whether AMH protein content in neonatal-prepubertal Sertoli cells could be maintained under a hypothyroid condition to add support to the hypothesis that the arrest in Leydig stem cell differentiation in the neonatal rat testis under a hypothyroid status results from continued AMH production by the immature Sertoli cells. The results revealed that irrespective of the thyroid hormone status in the neonatal rats, AMH in Sertoli cells gradually decline with age advancement, similar to the age-matched euthyroid rats.

Materials and methods

Female Sprague Dawley rats were purchased from Harlan Industries (Madison, WI) at mid pregnancy. They were maintained under conditions of controlled lighting (14 hours light: 10 hours darkness) and temperature (25°C), and were housed one to a cage. Agway Prolab rat formula (Syracuse, NY) and water *ad libitum* were fed to the pregnant rats until the birth of pups. Only the male pups were retained with the mothers and were then divided into two groups. First group of mothers, was continued to be fed with normal rat chow and water *ad libitum*; their pups were used as controls. The other group of rat mothers was also fed with normal rat chow but the reversible goitrogen 6-n-propyl-2-thiouracil (PTU, Sigma, St. Louis, MO, 0.1%, w/v) was added to their drinking water beginning immediately after parturition in order to induce a hypothyroid status in the offspring rats (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c). Control rat pups were sacrificed on postnatal days 1, 7, and 14 and hypothyroid rat pups were sacrificed on postnatal days 7 and 14 to perform the studies as described below. The approved animal protocol number for this study was #731.

Testicular tissue preparation for immunocytochemistry

Rats were euthanized by overdose of Isoflurane (Abbots Laboratories, Deer Park, MI) inhalation and their testicles were removed immediately after and fixed in Bouin's fixative and processed for immunocytochemistry as published previously (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a-d).

Immunocytochemistry for AMH

Tissue processing for immunocytochemistry and the protocol for the immunolocalization is identical to what we have published previously for other immunocytochemical studies (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a-d) except AMH antibody was used. In brief, testis tissue sections were de-waxed with xylene and rehydrated with decreasing concentrations of ethanol and brought to deionized water. This step was followed by washing the tissue sections in phosphate buffered saline (PBS, pH 7.3) for 5 minutes and incubating in 3% hydrogen peroxide for 20 minutes. After incubation, sections were washed again with PBS, and normal goat serum was added to tissues overnight (4°C) to bind nonspecific proteins. Rabbit polyclonal anti-AMH (gifted by Dr. R. Rey, Centro de Investigaciones Endocrinologicas, Buenos Aires, Argentina). This anti-AMH has previously been used for AMH immunolocalization in testes of rats and many other species (Tran et al., 1987). The final concentration of the antibody for immunolocalization was 1:1000 and was determined after preliminary studies performed with a range of 1:200-1:3000). Control incubations were carried out with preimmune serum. These incubations were carried out at 4°C overnight. AMH was detected using a commercially available biotin-streptavidin kit (BioGenex, San Ramon, CA) according with the manufacturer's instructions. Sections were counterstained with Harris' hematoxylon, dehydrated with increasing concentrations of ethanol, brought to xylene and cover-slipped using Permount. These studies were performed three times to confirm the repeatability of the outcome.

Results

AMH Immunocytochemistry

Figures 1 and 2 show the results of immunolabeling

for AMH in prepubertal testes of rats at low and high power, respectively in control and hypothyroid rats. Even at low power (2.5x objective lens), AMH immunolabeling was clearly evident in testes of postnatal day 1 rats (Fig. 1A). All Sertoli cells in every seminiferous cord showed positive labeling for AMH at postnatal day 1 (Fig. 1A). However, at this low magnification positive labeling for AMH was difficult to see in both control and hypothyroid rats of days 7 and 14 (Fig. 1C-F).

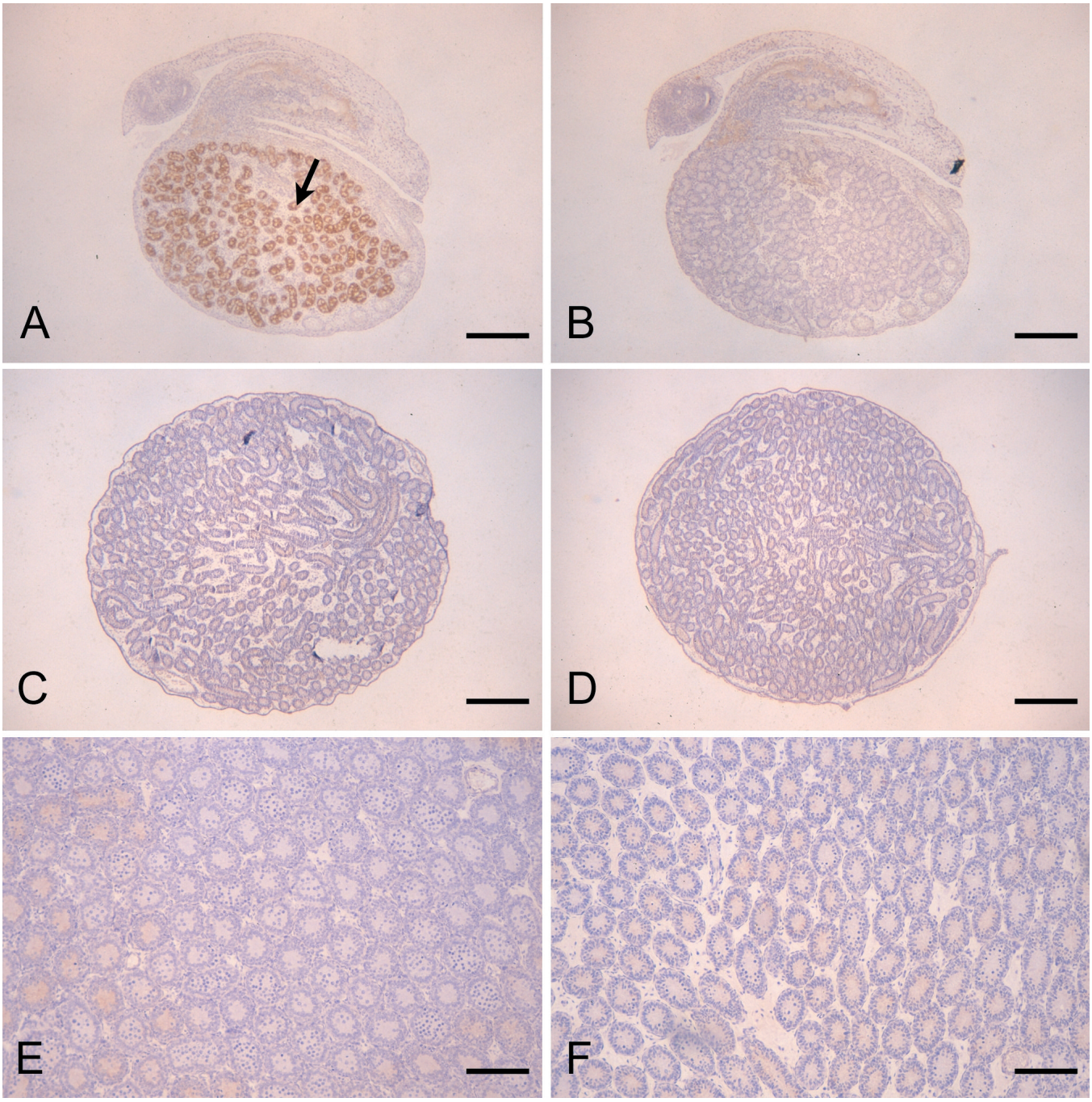

Fig. 1. A. Representative low power micrographs to show AMH immunolabeling (arrow) in rat testes. Postnatal day 1. **B.** Control incubation (no primary antibody) -day 1 testis. **C.** Postnatal day 7 control. **D.** Postnatal day 7 hypothyroid. **E.** Postnatal day 14 control. **F.** Postnatal day 14 hypothyroid. Bar: 35 μm.

Detailed studies performed at high power (100x objective lens) on AMH immunolabeling in testicular tissue revealed the following. At postnatal day 1, AMH label was detected exclusively in the seminiferous cords, but only in the Sertoli cell cytoplasm; gonocytes/germ cells, the nuclei of Sertoli cells and the cells in the testis interstitium were negative for AMH (Fig. 2A); control incubations did not show positive labeling for AMH

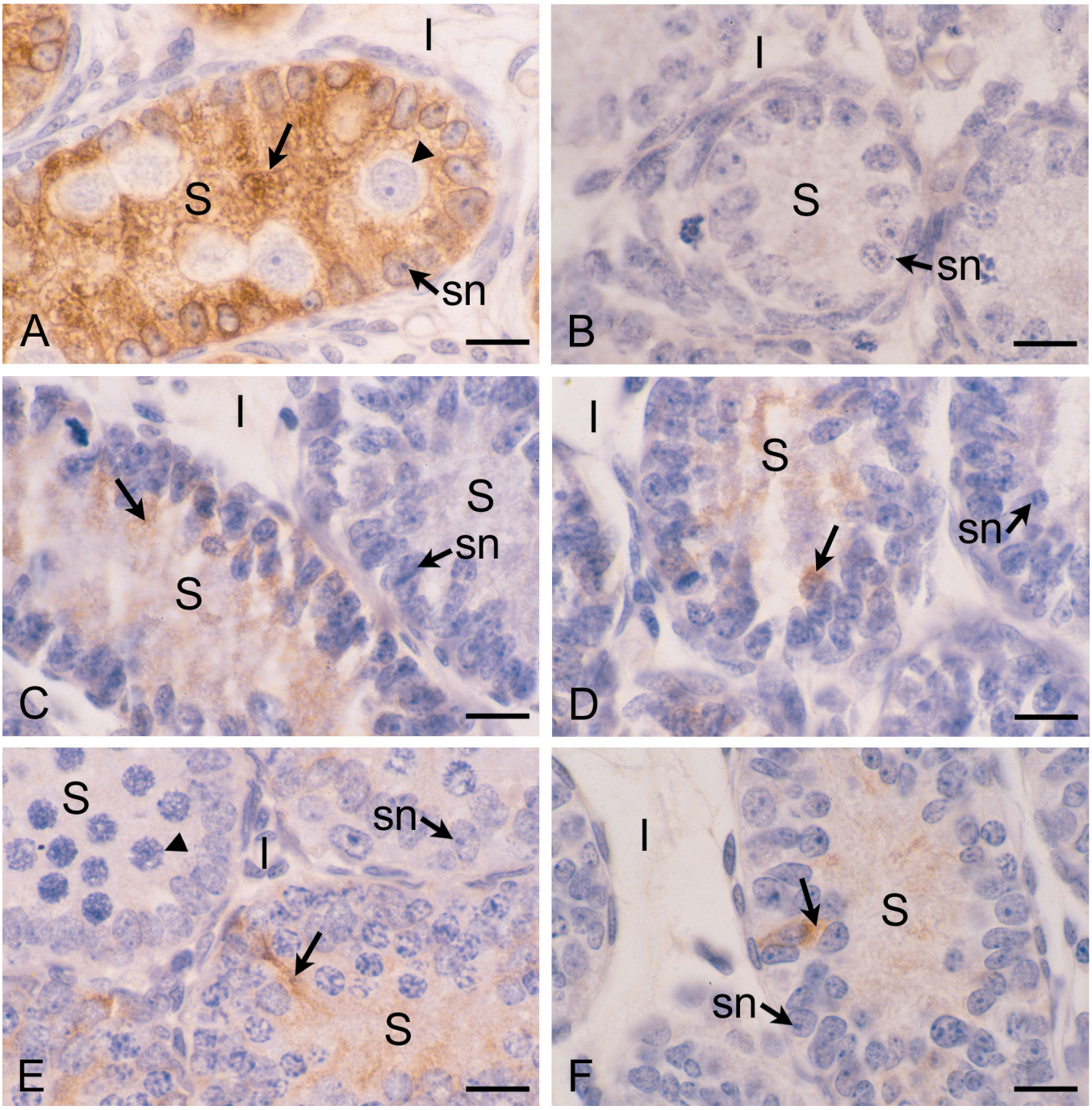

Fig. 2. Representative high power micrographs to show AMH immunolabeling (arrows) in rat testes. **A.** Postnatal day 1. **B.** Control incubation (no primary antibody)-postnatal day 1 testis. **C.** Postnatal day 7 control. **D.** Postnatal day 7 hypothyroid. **E.** Postnatal day 14 control. **F.** Postnatal day 14 hypothyroid. Germ cells (arrow heads), nuclei of Sertoli cells (sn), and cells in the testis interstitium **(I)** show negative labeling for AMH. S: seminiferous tubule Bar: 35 μm.

(Fig. 2B). In each seminiferous cord, cytoplasm of all Sertoli cells (i.e. 100%) showed the AMH label at postnatal day 1 (Fig. 2A). In contrast, in rats of postnatal days 7 and 14, in both control and hypothyroid groups, positive immunolabeling for AMH was seen in cytoplasm of few Sertoli cells, (Fig. 2C-F); germ cells, Sertoli cell nuclei and the cells in the testis interstitium were negative for AMH.

Discussion

AMH is well known for its role during Müllerian duct regression during fetal life. However, AMH actions in the postnatal testis is still to be identified. Immunocytochemical localization of AMH/MIS in Sertoli cells of the postnatal testes in many mammalian species have been documented. These include human (Tran et al., 1987; Meyts et al., 1999; Rey et al., 2000; Franke et al., 2004), mouse (Al-Attar et al., 1997), rat (Tran et al., 1987), pig (Tran et al., 1987; McCoard et al., 2003), sheep (Tran et al., 1987), goat (Tran et al., 1987) and cattle (Tran et al., 1987). To our knowledge, the present study is the first that demonstrates and compares the immunoexpression of AMH protein in Sertoli cells of prepubertal rat testis under euthyroid and hypothyroid conditions.

One function of AMH in the postnatal testis is on Leydig cell steroidogenesis. Several *in vivo* and *in vitro* studies have shown that AMH negatively regulates Leydig cell steroidogenic function (Teixiera et al., 1999; Flynn-Thompson et al., 2003). Findings from our laboratory recently showed that the receptors for AMH, i.e. AMHRII are first detected in the postnatally differentiated Leydig cells (i.e. adult Leydig cells/ALC) in the rat testis on postnatal day 13 (Mendis-Handagama et al., 2006). As ALC are first detected in the rat testis is on postnatal day 10 (Mendis-Handagama et al., 1987; Ariyaratne et al., 2000a) it was clear that these newly differentiated ALC do not gain AMHRII immediately upon their differentiation, but three days later. This observation suggested that the negative regulatory role of AMH on the steroidogenic function of the ALC in the rat testis is established on postnatal day 13 and continues to be present throughout adult age as positive labeling for AMHRII was clearly evident through 90 days of age (Mendis-Handagama et al., 2006).

Another role that AMH has on the postnatal testis is on the differentiation of the ALC population. Establishment of the ALC population in the postnatal testis is essential for the attainment of puberty and regulation of testicular functions in the adult male mammal. This ALC population differentiates primarily, if not exclusively, from the peritubular mesenchymal cells of the postnatal testis interstitium (Ariyaratne et al., 2000a-c). Among the factors that regulate the postnatal differentiation of ALC, it is reported that AMH is a negative regulator of this process (Racine et al., 1998; Teixeira et al., 1999; Flynn-Thompson et al., 2003; Salva et al., 2004). This suggestion was based on the findings of Leydig cell hyperplasia and absence of postnatally differentiated Leydig cells in testes of AMH deficient and over expressing mice, respectively (Racine et al., 1998). Later, it was reported that 21 day-old rat Leydig cells respond to AMH by decreasing DNA synthesis (Salva et al., 2004) and that AMH inhibits regeneration of rat Leydig cells after EDS treatment (Salva et al., 2004)

In the present study, we focused on the negative role of AMH on the onset of Leydig stem cell differentiation. As ALC differentiation is arrested in prepubertal hypothyroid rats (Mendis-Handagama et al., 1998; Teerds et al., 1998) we hypothesized that this arrest is due to continued production of AMH by the Sertoli cells as they maintain their immature status because of thyroid hormone deficiency. This concept was tested in the present study and the results revealed that the observations of Sertoli cell AMH in hypothyroid rats are similar to those of controls, i.e., AMH in Sertoli cells markedly reduce after birth in control and hypothyroid prepubertal rats, irrespective of their thyroid hormone status. This information was confirmed by immunoblot analyses (unpublished data). Therefore, findings of the present study were not in agreement with the concept that hypothyroidism could maintain Sertoli cell AMH protein content in prepubertal rats. It appears that Sertoli cell maturation in response to thyroid hormone stimulation (Van Haaster et al., 1993; Arambepola et al., 1998; McCord et al., 2003) is independent of the function of AMH production by the Sertoli cells. Based on these observations, it is possible to suggest that stimulation of the onset of Leydig stem cell differentiation in the postnatal testis is unlikely to be mediated via an arrest in AMH production by the Sertoli cells due to their maturation in response to thyroid hormones.

Acknowledgements. We thank Dr. Rodolfo Rey (Centro de Investigaciones Endocrinologicas, Buenos Aires, Argentina) for the AMH antibody and the assistance of Tommy Jordan (Instructional Resources, The University of Tennessee College of Veterinary Medicine) in preparing the color photographic plates. Supported in part by the University of Tennessee Professional Developmental Award Program and the World Health Organization.

References

Al-Attar L., Noel K., Deserter M., Bellville C., Forest M.G., Burgoyne P.S., Joss N. and Rey R. (1997). Hormonal and cellular regulation of Sertoli cell anti-Mullerian hormone production in the postnatal mouse. J. Clint. Invest. 100, 1335-1343.

Arambepola N.K., Buick D. and Cooke P.S. (1998). Thyroid hormone and follicle stimulating hormone regulate Mullerian-inhibiting substance messenger ribonucleic acid expression in cultured neonatal rat Sertoli cells. Endocrinology 139, 4489-4495.

Ariyaratne H.B.S., Mendis-Handagama S.M.L.C., Hales D.B. and Mason J.I. (2000a). Studies on the onset of Leydig precursor cell differentiation in the prepubertal rat testis. Biol. Rep. 63, 165-171.

Ariyaratne H.B.S., Mason J.I. and Mendis-Handagama S.M.L.C. (2000b). Effects of triiodothyronine on testicular interstitial cells and androgen secretory capacity of the prepubertal rat. Biol. Reprod. 63, 493-502.

Ariyaratne H.B.S., Mason J.I. and Mendis-Handagama S.M.L.C. (2000c). Effects of thyroid and luteinizing hormones on the onset of precursor cell differentiation into Leydig cells in the prepubertal rat testis. Biol. Reprod. 63, 898-904.

Ariyaratne H.B.S., Mills N., Mason J.I. and Mendis-Handagama S.M.L.C. (2000d). Thyroid hormone and Leydig cell regeneration in the adult rat following ethane dimethane sulphonate treatment. Biol. Reprod. 63, 1115-1123.

Flynn-Thompson E., Cheng H. and Teixeira J. (2003). Inhibition of steroidogenesis in Leydig cells by Mullerian inhibiting substance. Mol.Cell. Endocrinol. 211, 99-104.

Franke F.E., Paula K., Rey R., Marks A., Bergmann M. and Steger M. (2004). Differentiation markers of Sertoli cells and germ cells in fetal and early postnatal human testis. Anat Embryol (Berl) 209, 168-177.

Kuroda T., Lee M.M., Ragin R.C., Hirobe S. and Donohoe P.K. (1991). Mullerian inhibiting substance production and cleavage is modulated by gonadotropins and steroids. Endocrinology 129, 2985-2992.

Lording D.W. and de Kretser D.M. (1972). Comparative ultrastructural and histochemical studies of the interstitial cells of the rat testis during fetal and postnatal development. J. Reprod. Fert. 29, 261-269.

McCoard S.A., Wise T.H. and Ford J.J. (2003). Endocrine and molecular influences on testicular development in Meishan and White composite boars. J. Endocrinol 178, 405-416.

Mendis-Handagama S.M.L.C., Risbridger G.P. and de Kretser D.M. (1987). Morphometric analysis of the components of the neonatal and the adult rat testis interstitium. Int. J. Androl. 10, 525-534.

Mendis-Handagama S.M.L.C., Ariyaratne H.B.S., Teunissen van Manan K.R. and Haupt R.L. (1998). Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism. Biol. Reprod. 59, 344-350.

Mendis-Handagama S.M.L.C., Ariyaratne H.B.S., Di Clemente N. and Mrkonjich L. (2006). Detection of Anti-Mullerian hormone receptor type II in cells of the rat testis interstitium from birth to sexual maturity. Histol. Histopathol. 21, 125-130

Meyts E.R., Jorgensen N., Graem N., Muller J., Cate R.L. and Skakkebaek N.S. (1999). Expression of anti-Mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulose cells. J. Clin. Endocrinol. Metab. 84, 3836-3844.

Morris I.D. (1996). Leydig cell toxicology. In: The Leydig cell. Payne A.H., Hardy M.P. and Russell L.D. (eds). Cache River Press, Vienna, IL, USA. pp 573-596

Racine C., Rey R., Forrest M., Louis F., Ferre A., Huhtaniemi I., Josso N. and di Climente N. (1998). Receptors for anti-Mullerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. Proc. Natl. Acad. Sci. USA 95, 594-599.

Rey R., Sabourin J.C., Venara M., Long W.Q., Jaubert F., Zeller W.P., Duvillard P., Chemes H. and Bidard J.M. (2000). Anti-Mullerian hormone s a specific marker of Sertoli – and granulosa- cell origin in gonadal tumors. Human Pathol. 31, 1202-1208.

Roosen-Runge E.C. and Anderson D. (1959). The development of the interstitial cells in the testis of albino rat. Acta Anat. 37, 125-137.

Salva A., Hardy M.P., Wu X., Sottas C.M., MacLaughlin D.T., Donohoe P.K. and Lee M.M. (2004). Mullerian-inhibiting substance inhibits rat Leydig cell regeneration after ethylene dimethanesulphonate ablation. Biol. Reprod. 70, 600-607.

Teerds K.J., Rooij D.G., de Jong F.H. and van Haaster L.H. (1998). The development of the adult-type Leydig cell population in the rat is affected by neonatal thyroid hormone levels. Biol. Reprod. 59, 344-350.

Teixeira J., Fynn-Thompson E., Payne A.H. and Donohoe P. (1999). Mullerian-inhibiting substance regulates androgen synthesis at the transcriptional level. Endocrinology 140, 4732-4738.

Tran D., Picard J.Y., Jacqueline C. and Josso N. (1987). Immunocytochemical detection of anti-Mullerian Hormone in Sertoli cells of varioua mammalian species. Endocrinology 35, 733-743.

Van Haaster L.H., de Jong F.H., Doctor R. and de Rooij D. (1993). High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. Endocrinology 133, 755-760.

Accepted July 27, 2007