

Comparison of testis structure, function and thyroid hormone levels in control C57BL/6 mice and anti-mullerian hormone over expressing mice

S.M.L. Chamindrani Mendis-Handagama¹, H.B. Siril Ariyaratne^{1*},
Kellie A. Fecteau¹, Judith M. Grizzle² and Nilanthi K. Jayasundera¹

¹Department of Comparative Medicine, The College of Veterinary Medicine and ²Department of Animal Science, The University of Tennessee, College of Veterinary Medicine, Knoxville, USA

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

Summary. Anti-Mullerian hormone (AMH) is considered as a negative regulator of postnatal Leydig cell (LC) differentiation, because AMH over expressing mice (Mt-hAMH mice) testes are deficient in LC. Therefore, in the present study Mt-hAMH mice was used as a model to examine the process of postnatal LC differentiation. Testis structure-function studies were performed in age-matching Mt-hAMH and C57BL/6 (controls) mice; testicular components were quantified and circulating testosterone and thyroid hormone levels (thyroxine/T4 and triiodothyronine/T3; necessary for postnatal LC differentiation) were determined. Results revealed that Mt-hAMH mice were heavier and their testis weights were smaller compared to controls. Mast cells were present in Mt-AMH testis interstitium, but absent in controls. The absolute volumes of seminiferous tubules (ST), testis interstitium, LC and blood vessels per testis were lower and lymphatic space was higher in Mt-hAMH mice than in controls ($p < 0.05$). The average cell LC volume and their number per testis, ST length, plasma testosterone, luteinizing hormone-stimulated testosterone secretion per testis and per LC *in vitro*, plasma T4 and T3 were significantly lower in Mt-hAMH mice compared to controls ($p < 0.05$). Increased body weight in Mt-hAMH mice could be attributed to the reduced T4 and T3. Reduced testis weight in Mt-AMH mice is explained by the reduced ST volume in them. Reduced plasma testosterone, testicular and LC testosterone secretion *in vitro* in Mt-hAMH mice can be explained by the reduced number, size and steroidogenic potential of LC in Mt-hAMH mice. Study revealed several structure-function deficiencies in Mt-AMH

mouse compared to controls, which were not documented in previous investigations. As hypothyroidism causes arrest in postnatal LC differentiation, it is suggested that the reduced LC number in Mt-hAMH testes could be at least in part due to their reduced thyroid hormone levels. However, latter concept needs to be further tested in future investigations.

Key words: AMH, Leydig Cells, Hypothyroidism, Testosterone

Introduction

Testosterone, primarily secreted by the Leydig cells in the testis, is important for general health and reproduction of the adult mammalian male. Therefore, postnatal differentiation of Leydig cells in the developing testis is an important process to the adult male mammal to establish his adult Leydig cell population in the testis. Stem cells for the adult population of Leydig cells in the postnatal testis are shown to be the peritubular 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 fact for prepubertal (Ariyaratne et al., 2000a-c) as well as the adult rat (Ariyaratne et al., 2000d) using immunocytochemical labeling studies for 3 β -hydroxy steroid dehydrogenase enzyme.

In the process of postnatal differentiation of Leydig cells, five cell stages have been identified (Mendis-Handagama and Ariyaratne, 2001). The mesenchymal cells surrounding the seminiferous tubules/peritubular mesenchymal cells are the stem cells; they are non-

steroidogenic, and upon stimulation, they differentiate into Leydig progenitor cells which have limited steroidogenic potential (Mendis-Handagama and Ariyaratne, 2001). This step is followed by differentiation of Leydig progenitor cells into newly formed adult Leydig cells, immature Leydig cells and finally the mature adult Leydig cells as reviewed previously (Mendis-Handagama and Ariyaratne, 2001).

One of the unresolved issues in the process of postnatal Leydig cell differentiation is the mechanism(s) of the regulation of the onset of this process. We and others have shown that thyroid hormones play a critical role in triggering this process; 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 process.

Anti-Mullerian hormone (AMH) is a glycoprotein and a member of the transforming growth factor-beta (TGFB) family. It is well established that AMH, which is a 140 kDa protein, is produced by the immature Sertoli cells (Josso et al., 2001) and is an important factor in male sexual differentiation, because in the male fetus, AMH causes the degeneration of the female genital ducts (i.e. Mullerian/paramesonephric ducts). It is also shown that AMH represses aromatase activity of fetal Sertoli cells and inhibits testosterone synthesis by fetal Leydig cells (Rouiller-Fabre et al., 1998).

The role of AMH in the postnatal testis is not clearly recognized at present, however, the following observations suggest that AMH is a negative regulator of postnatal Leydig cell differentiation. AMH deficient male mice show marked Leydig cell hyperplasia (Racine et al., 1998), and male transgenic mice expressing very high levels of human AMH (hAMH) under the control of the mouse metallothionein-1 promoter (MT-hAMH) are incompletely masculinized externally and rapidly become infertile (Behringer et al., 1990). It is also shown that these male Mt-hAMH mice have a reduced number of postnatally differentiated Leydig cells or adult Leydig cells (Racine et al., 1998). Based on this observation, the authors (Racine et al., 1998) suggested that AMH has a negative regulatory role in the process of postnatal LC differentiation. However, it is logical to ask the question whether the reduced number of Leydig cells in Mt-AMH mice is solely due to the high AMH levels in them or are there any other factors which are not yet detected, contributing to this situation.

Therefore, the present study was designed to perform structure-function studies in Mt-hAMH male mice, as well as determining their circulating levels of thyroid hormones; latter hormone levels were measured because it has been shown previously that thyroid hormones are essential for postnatal Leydig cell differentiation (Mendis-Handagama et al., 1998; Teerds et al., 1998; Ariyaratne et al., 2000a-c); to date, this information is not available for Mt-AMH mice. Our aim was to detect the differences between the two experimental groups, which may possibly add new insight to further understand the regulation of postnatal

Leydig cell differentiation in the mammalian testis.

Materials and methods

Animals

The present study was approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Tennessee, Knoxville (Protocol #1519). Five month old C57BL/6 and Mt-hAMH male mice were used. Male and female C57BL/6 mice were obtained from Harlan Industries (Harlan, WI). Mt-hAMH male mice for breeding were provided by Dr. R. Behringer (M.D. Anderson Cancer Center, TX).

Establishment of Mt-hAMH transgenic mouse colony

Heterozygous Mt-hAMH males (chronically over-express human AMH under the control of mouse metallothionein-1 promoter) were bred to C57BL/6 females. Pups were either transgenic or normal, and distinguished by genotyping. In brief, DNA was isolated from tail clips of male mice using commercially available kits (Qiagen, Valencia, CA) and using the protocol provided by the manufacturer. The AMH transgene was amplified by PCR and identified by Southern blotting (Fig. 1).

Harvesting blood and preparation of plasma for radioimmunoassays

Heparin (Heparin solution for injection, USP, 10,000 units/ml, Steris Laboratory Inc. AZ; 10 U/kg. body weight) was injected 20 minutes before euthanasia of mice. Overdose of inhalation of Isoflurane (Abbott Laboratories, Deerpark, IL) was used to euthanize mice. Blood was collected by cardiocentesis, plasma was prepared and stored at -80°C until radioimmunoassays were performed for testosterone, thyroxin (T4) and triiodothyronine (T3).

Tissue harvesting

One testis was removed from each euthanized mouse (n=8/group), freed from the epididymis and weighed to obtain the fresh testis weight; this measurement is required to determine the fresh testis volume for the stereological analyses. This testis was used to determine the luteinizing hormone (LH, 100 ng/ml)-stimulated testicular testosterone secretory capacity *in vitro* as described previously (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c,d). The other testis of each mouse in each experimental group was fixed *in-situ* by whole body perfusion with 5% glutaraldehyde solution in 0.1M cacodylate buffer (pH 7.4; Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c,d). Fixed testes were weighed, cut into approximately 2-3 mm³ cubes, post fixed in a 1:1 mixture of 2% aqueous osmium tetroxide and 3% potassium ferrocyanide, dehydrated in graded ethanols and embedded in epon araldite (Mendis-

C57BL/6 vs Mt-hAMH male mice

Handagama et al., 1988, 1998; Ariyaratne et al., 2000a,c,d). Shrinkage of testis tissue was determined as described previously by Mendis-Handagama and Ewing (1990).

Light microscopy and stereology

From the polymerized blocks of testis tissue, 1 μ m thick sections were cut using a LKB ultramicrotome V and glass knives. They were mounted on pre-cleaned glass slides, stained with Methylene Blue Azure II stain and cover-slipped with Permount (Fisher Scientific company, Fair Lawn, NJ).

Volume density of testicular components

Volume density of a testicular component is defined as the volume of the component per unit volume of testis, and expressed as a percentage. This was determined using the point counting method (Weibel, 1969) as described (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c,d) using an Olympus BH-2 light microscope (Tokyo, Japan), fitted with a x40 objective, x10 ocular; a test grid containing 121 test points was fitted to the ocular lens of the microscope for counting (20 fields/block, 10 blocks/animal, 8 animals/group) was used. The components tested were seminiferous tubules (total, cellular area, lumen), testis interstitium, Leydig cells, blood vessels (total, wall, lumen) and lymphatic space.

The absolute volumes of testicular components

The absolute volumes of (mm³) of testicular components per testis were calculated by multiplying the volume density of each component by the fresh testis volume (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c,d). As the specific gravity of the testis is approximately 1, the values of testis weight in grams were taken as values for testis volumes in cm³.

Leydig cell number per testis

The numerical density (N_V) of Leydig cells (defined as the number of Leydig cells per unit volume of the testis tissue) was determined by the Disector method (Sterio, 1984) as described by Mendis-Handagama and Ewing (1990) with modifications as published previously (Ariyaratne and Mendis-Handagama, 2000). Twenty to 30 areas per block and 8 blocks per mouse were scored. The total number of Leydig cells per testis was calculated by multiplying the numerical density of Leydig cells by the fresh testis volume (Mendis-Handagama et al., 1988, 1998; Ariyaratne et al., 2000a,c,d).

Length of seminiferous tubules

Tissue sections used for stereology (as described

above) were used. Diameters of seminiferous tubules were measured using a stage and an ocular micrometers, and radius (r) was calculated under x40 objective and x10 ocular lenses (8-10 tubules/section, 4 sections/mouse, 8 mice/experimental group). Using the formula $\pi r^2 h$ (volume of a cylinder where $\pi = 22/7$ and h = length of seminiferous tubules/testis) and the value of absolute volume of seminiferous tubules (VST), h was calculated as shown below.

$$h = V_{ST} / \pi r^2$$

LH-Stimulated testicular testosterone production in vitro

Each fresh testis removed from each mouse was weighed, decapsulated and incubated in 2 ml of Krebs-Ringer bicarbonate solution (pH-7.4) supplemented with glucose (0.004 g/ml) and LH (100 ng/ml; Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a,c,d). These incubations were performed in 20 ml scintillation vials at 34°C in an oscillating water bath (90 oscillations/minute). At the end of 3 hours, the incubation medium was collected, centrifuged at 3000 g for 10 minutes, and the supernatant was separated and stored at -80°C until further analysis by radioimmunoassay.

Radioimmunoassay of hormones

Hormonal assays were performed using commercially available kits, validated for use in mice, and performed three times for each hormone to assure reproducibility and to assess inter-assay variability. Each sample was run in duplicate to estimate intra-assay variability. Testosterone in testis incubation media and plasma T4 and T3 levels were determined by Coat-A-Count RIA kits (DPC, Los Angeles, CA). The inter-assay coefficients of variation for testosterone, T4 and T3 were 8%, 8% and 9%, respectively. The intra-assay coefficients of variation ranged from 5-7% for all hormones tested.

Statistics

PC SAS was used for statistical analysis. Differences between the means in the two experimental groups were determined using unpaired t-test. P values of 0.05 or less was considered to be significant.

Results

Figure 1 shows the Southern blot used for genotyping mice, to identify Mt-hAMH positive mice and controls (Mt-hAMH negative). Table 1 shows body weight, testis weight, and volume densities of many testicular components in C57BL/6 and Mt-AMH mice. As shown, body weight was significantly greater and testis weight was significantly smaller in Mt-AMH mice compared to their age-matching controls. Volume densities (Vv%) of seminiferous tubules (total, cellular

C57BL/6 vs Mt-hAMH male mice

area, lumen), and blood vessels (total, wall, lumen) were not significantly different between the two groups. However, Vv% of testis interstitium and Leydig cells were significantly lower and the lymphatic space was significantly higher in Mt-hAMH mice compared to control C57BL/6 mice. Because Vv% is a relative measure, these results will not be discussed further.

Figure 2 shows representative light micrographs of testes of control C57BL/6 and Mt-hAMH mice. In control mice, seminiferous tubules demonstrated ongoing spermatogenesis in contrast to those in Mt-hAMH mice; some seminiferous tubules in them showed loss of germ cells and vacuolation of Sertoli cells (Figure 2, arrow with asterisk). Abundant Leydig cells were present in the testis interstitium of control mice. By contrast, few Leydig cells were present in the testis interstitium of Mt-hAMH mice. Additionally, testis interstitium of Mt-hAMH mice showed a considerable

number of mast cells, which were not observed in the testis interstitium of control mice.

Table 2 shows the absolute volumes of components per testis in C57BL/6 and Mt-AMH mice. All components tested, except for the lymphatic space were significantly greater in C57BL/6 mice compared to Mt-AMH mice. The average volume of a Leydig cell and Leydig cell number per testis were greater in C57BL/6

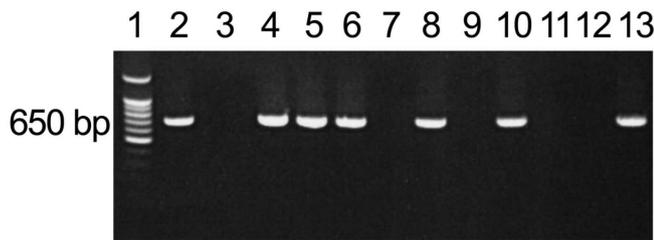


Fig. 1. Identification of Mt-AMH Mice by Genotyping. Lane 1: standards, Lane 2: transgene band from the positive control (DNA isolated from a tail of a Mt-hAMH mouse provided by Dr R. Behringer), Lane 3: negative control (DNA isolated from a normal C57BL6 mouse), Lanes 4, 5, 6, 8, 10, 13: positive banding with DNA from 6 mice identified as transgenics (used for breeding), Lanes 7, 9, 11, 12: absence of signal (normal males).

Table 1. Body weight, testis weight and volume density of testicular components in control C57BL/6 and Mt-hAMH mice.

Parameter	Normal C57BL6 Mice	Mt-AMH mice
Body weight (g)	33±3	48±2*
Testis weight (mg)	102±2*	77±1
Vv% of Seminiferous tubules (ST)	90.67±0.3	92.07±0.79
Vv% of ST cellular area	86.82±0.29	89.55±0.84
Vv% of ST lumen	3.85±0.3	2.52±0.5
Vv% Testis Interstitium	9.33±0.34*	7.93±0.45
Vv% of Leydig cells	5.10±0.04*	1.1±0.01
Vv% Blood Vessels (BV)	1.40±0.27	1.44±0.11
Vv% of BV wall	0.8±0.04	0.73±0.04
Vv% of BV lumen	0.6±0.2	0.61±0.06
Vv% of Lymphatic space	0.41±0.02	1.37±0.01*

Mean±SE. n=8 mice per group. Asterisks (*) show statistical significance at P<0.05.

Table 2. Absolute volume of testicular components in control C57BL/6 and Mt-hAMH mice.

Testicular Component (mm ³)	Normal C57BL/6 Mice	Mt-AMH mice
Seminiferous tubules (ST)	92.48±0.4*	70.89±0.92
ST cellular area	88.56±0.3*	68.95±0.48
ST lumen	3.92±0.2*	1.94±0.2
Testis Interstitium	9.51±0.02*	7.93±0.03
Leydig cells	5.190±0.05*	0.85±0.04
Blood Vessels (BV)	1.43±0.02*	1.12±0.01
BV wall	0.82±0.02*	0.55±0.01
BV lumen	0.61±0.02*	0.47±0.01
Lymphatic space	0.42±0.01	1.06±0.01*

Mean±SE. n=8 mice per group. Asterisks (*) show significantly greater value at P<0.05.

Table 3. Average volume of a Leydig cell, Leydig cell number per testis, seminiferous tubule diameter and length in control C57BL/6 and Mt-hAMH mice.

Leydig Cell Parameter	Normal C57BL/6 Mice	Mt-AMH mice
Average volume of a Leydig cell μm ³	3707±102*	2511±89
Leydig cell number per testis (x10 ⁶)	1.41±0.01*	0.34±0.02
ST diameter (μm)	176±1.1	180±1.3
ST length (μm)	5795±22*	4278±23

Mean±SE. n=8 mice per group. Asterisks (*) show significantly higher value at P<0.05.

Table 4. Hormones in control C57BL6 and Mt-hAMH mice.

Hormone	Normal C57BL/6 Mice	Mt-AMH mice
Plasma testosterone (ng/ml)	2.8±0.2*	0.9±0.03
LH-stimulated testicular testosterone secretion (10 ² ng/testis)	26±0.04*	2±0.01
Testosterone Secretion per Leydig cell (pg)	1.84±0.02*	0.59±0.01
Plasma T4 (ng/ml)	43±1.5*	18±0.02
Plasma T3 (ng/ml)	0.50±0.001*	0.24±0.001

Mean±SE. n=8 mice per group. Asterisks (*) show significantly greater value at P<0.05.

C57BL/6 vs Mt-hAMH male mice

mice than Mt-AMH mice (Table 3). The seminiferous tubular diameter was not significantly different in the two groups (Table 3), however, the Mt-AMH mice showed a lower value for the length of seminiferous tubules per testis (Table 3) and a reduced volume of germ cells per testis (Table 2).

Hormonal parameters of the two experimental groups are shown in Table 4. Mt-AMH mice demonstrated lower values for plasma testosterone levels, LH-stimulated testosterone secretion per testis

and per Leydig cell, and plasma T4 and T3 levels compared to controls.

Discussion

Our primary aim of the present study was to detect the differences in structure-function parameters and thyroid hormone levels between Mt-hAMH and C57BL/6 mice which may add new insight to further understand the regulation of postnatal Leydig cell

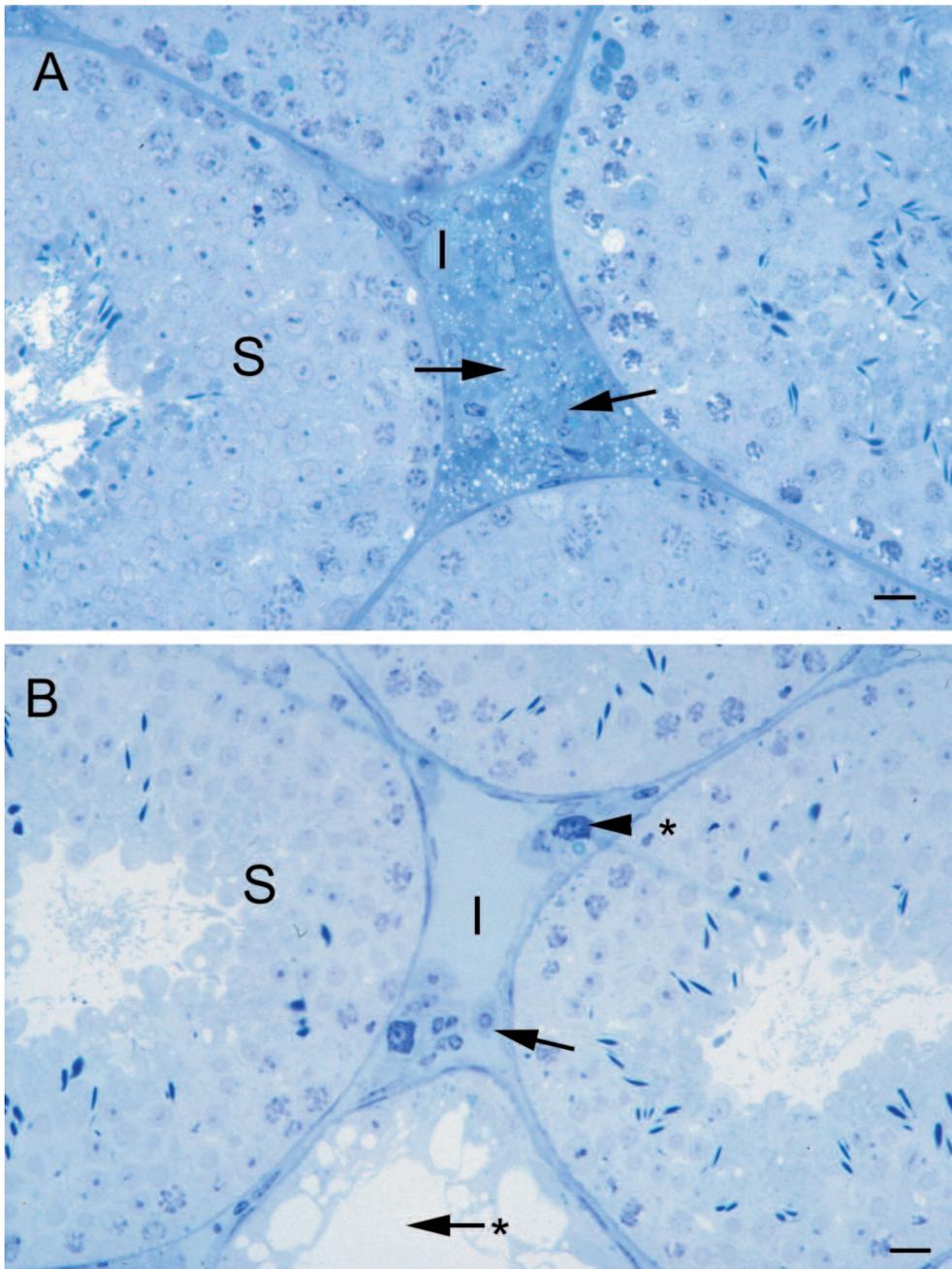


Fig. 2. Representative light micrographs of testis tissue in control and Mt-AMH mice. **A.** Control mouse. **B.** Mt-AMH mouse. Leydig cells (arrows) are abundant in the testis interstitium (I) in the control mouse and very few Leydig cells are seen in the Mt-AMH mouse. Arrow heads depict mast cells, which are present in the testis interstitium of Mt-AMH mouse but not in control mouse. Seminiferous tubules (S) in the control mouse do not show any damage. A seminiferous tubule with germ cell loss and vacuolation of Sertoli cells in the Mt-AMH mouse is shown by an arrow + asterisk. Bar: 20 μ m.

differentiation in the mammalian testis. Results showed that Mt-hAMH mice have reduced number of Leydig cells in their testes and reduced plasma testosterone levels compared to C57BL/6 mice. These observations agree with previous findings of Racine et al. (1998), however, the present study is the first to demonstrate quantitative information on these Leydig cell parameters, i.e. volume and number per testis, average size of a Leydig cell and testosterone secretion per Leydig cell *in vitro* in Mt-AMH mice. The reduced LH-stimulated testicular testosterone secretory capacity *in vitro* in Mt-hAMH mice also agrees with these observations on Leydig cell parameters in Mt-hAMH mice. It is also interesting and important to document that the values of plasma testosterone levels (Mendis-Handagama et al., 1990a; Alvarez et al., 2008; Monsefi et al., 2009; Mandal and Das, 2009), Leydig cell number per testis and average volume of a Leydig cell (Mendis-Handagama et al., 1990b) and testosterone secretion per testis (Mendis-Handagama et al., 1990b; Lin et al., 2009) in control mice of the present study compare favorably with those published previously.

The present study demonstrated several other differences between C57BL/6 and Mt-hAMH mice which were not reported previously. Mt-hAMH mice of five months of age had significantly greater body weights than their age-matched controls. Based on the published reports on obesity and hypothyroidism (Fox et al., 2008; Weaver, 2008), we suggest that this increased body weights of five month old Mt-hAMH mice are possibly due to the reduced thyroid hormone levels in them. Mt-hAMH had lower values of testis weights than age-matching C57BL/6 mice and this observation also agrees with the previous study of Racine et al. (1998). However, latter study (Racine et al., 1998) did not provide any other information on why the testes of Mt-hAMH have reduced testis weights. In the present study we show that Mt-hAMH mice have reduced seminiferous tubular length per testis, which resulted in reduced volume of seminiferous tubules per testis; this is in addition to the germ cell loss evident in the seminiferous tubules, supported by the reduced volume of germ cells per testis observed in Mt-AMH mice. These findings are further strengthened by the following observations. Litter size of normal (C57BL/6) dams paired to Mt-hAMH males was smaller (3-5 pups/litter) in contrast to 10-14 pups per litter when a normal male was paired to a normal female. Additionally, Mt-hAMH males became infertile after about 3 rounds of consecutive pairing. Loss of germ cells in Mt-hAMH mice can be suggested to be associated with reduced levels of testosterone in Mt-hAMH mice, because testosterone is one of the major factors essential for the process of spermatogenesis.

We also report for the first time the presence of considerable number of mast cells in the testis interstitium of Mt-hAMH mice compared to controls. Increased numbers of mast cells in testes have been associated with testicular dysfunction (Roaiiah et al.,

2007; Allam et al., 2009). Furthermore, it has been reported that mast cells affect sperm function as mast cell derived tryptase may reduce sperm motility (Allam et al., 2009). Interestingly, the fertility of Mt-hAMH male mice used in our study did not last long as in the control mice, and the absolute volume of germ cells per testis was significantly lower in Mt-hAMH mice compared to controls; these findings also support the above concept.

The present study showed that Mt-hAMH mice testes have reduced volumes of blood vessels (whole blood vessel volume, the blood vessel wall and the lumen) and an increased volume of lymphatic space, which are not reported previously. Increase in lymphatic space is in agreement with the fact that Mt-hAMH mice had reduced volume and number of Leydig cells in their testis interstitium, which provided a larger space in the testis interstitium for the lymphatics; this was also evident in viewing the tissue sections (see Fig. 2).

Mt-hAMH mice had reduced levels of plasma T4 and T3 levels, which are not reported previously. This finding is intriguing, because we (Mendis-Handagama et al., 1998; Ariyaratne et al., 2000a-c) and others (Teerds et al., 1998) have previously shown that thyroid hormone deficiency results in arrest in postnatal differentiation of Leydig cells. In previous studies, it has been shown that cells in Leydig cell lineage (including the stem cells) contain thyroid hormone receptor mRNA (Hardy et al., 1996). Therefore, it is logical to question whether the reduced number of Leydig cells in Mt-hAMH mice is at least in part due to the arrest in postnatal Leydig cell differentiation in their testis as a result of reduced thyroid hormone levels.

Mt-hAMH mice have high levels of AMH, which is suggested as a factor that exerts an inhibitory effect on the process of postnatal Leydig cell differentiation (Racine et al., 1998). AMH is present in high concentration in Sertoli cells at birth (Tran et al., 1987; Kuroda et al., 1991; Mendis-Handagama and Ariyaratne 2008); however, it disappears rapidly during the neonatal period (Tran et al., 1987; Kuroda et al., 1991; Mendis-Handagama and Ariyaratne, 2008). Because AMH and thyroid hormones have negative and positive effects respectively, on postnatal Leydig cell differentiation, in a previous study (Mendis-Handagama and Ariyaratne, 2008) we hypothesized that under a hypothyroid status where Leydig cell differentiation is arrested and Sertoli cells are maintained under an immature state, AMH in Sertoli cells will not disappear. However, results showed otherwise, i.e., disappearance of AMH protein in Sertoli cells of the neonatal testis occurs similarly under both euthyroid and hypothyroid status. This result suggested that AMH and thyroid hormones acts independently to regulate the process of postnatal Leydig cell differentiation.

It is also important to document that although AMH is considered as a negative regulator of postnatal Leydig cell differentiation, receptors for AMH, i.e. AMHR-II were not detected in any spindle-shaped cell type in the

C57BL/6 vs Mt-hAMH male mice

testis interstitium (rat model), at any postnatal age, i.e. birth to adulthood; these include the peritubular mesenchymal cells which are the stem cells for Leydig cells, (Mendis-Handagama et al., 2006). These findings suggested that the negative regulatory role of AMH on postnatal Leydig cell differentiation is unlikely to be a direct effect on the stem cells/peritubular mesenchymal cells. However, it should be mentioned that other cell stages in the Leydig lineage, namely, the progenitor cells, newly formed adult Leydig cells, immature Leydig cells and mature Leydig cells showed positive labeling for AMHR-II (Mendis-Handagama et al., 2005), and agree with the findings of Lee et al. (1999).

In the present study we have demonstrated that Mt-hAMH mice have reduced numbers of Leydig cells in their testes and they also have low levels of circulating T4 and T3. These findings suggest, but not prove, that low numbers of Leydig cells in Mt-hAMH mice may not be exclusively due to their high levels AMH, but at least in part due to the low circulating levels of thyroid hormones that exert an inhibitory effect on their differentiation. Therefore, we consider that the Mt-AMH mouse provides us an excellent model to investigate the regulatory roles of AMH and thyroid hormones on the process of postnatal Leydig cell differentiation. Whether exogenous supplementation of thyroid hormones to Mt-hAMH mice could stimulate the process of postnatal Leydig cell differentiation by reducing the AMH levels or in the presence of unaltered high AMH levels are important questions to be addressed in future studies.

Acknowledgements. We thank Dr. R. Berhinger for his gift of two Mt-AMH male mice to establish our Mt-AMH mouse colony, and Dr. Mary Ann Handel (University of Tennessee, Knoxville and The Jackson Laboratory, Bar harbor, ME) for advice and training on genotype determination. Thanks are also due to Ms. L. Mrkonjich for technical assistance and Greg Hirshoren and Phil Snow for their assistance in preparation of the figures. The financial support provided by the UT Center of Excellence is also gratefully acknowledged.

References

- Allam J.P., Langer M., Fathy A., Oltermann I., Bieber T., Novak N. and Haidl G. (2009). Mast cells in the seminal plasma of infertile men as detected by flow cytometry. *Andrologia* 41, 1-6.
- Alvarez J.D., Hansen A., Ord T., Bebas P., Chappell P.E., Giebultowicz J.M., Williams C. Moss, S. and Sehgal A. (2008). The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J. Biol. Rhythms* 23, 26-36.
- Ariyaratne H.B.S., Mason J.I. and Mendis-Handagama S.M.L.C. (2000a). Effects of tri-iodothyronine 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. (2000b). 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., Mendis-Handagama S.M.L.C., Hales D.B. and Mason J.I. (2000c). Studies on the onset of Leydig precursor cell differentiation in the prepubertal rat testis. *Biol. Reprod.* 63, 165-171.
- 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.
- Behringer R.R., Cate R.L., Froelick G.J., Palmiter R.D. and Brinster R.L. (1990). Abnormal sexual development in transgenic mice chronically expressing Mullerian inhibiting substance. *Nature* 345, 167-170.
- Fox C.S., Pencina M.J., D'Agostino R.B., Murabito J.M, Seely E.W., Pearce E.N. and Vasan R.S. (2008). Relations of Thyroid Function to Body Weight. Cross-sectional and Longitudinal Observations in a Community-Based Sample. *Arch Intern Med.* 168, 587-592.
- Hardy M.P., Sharma R.S., Arambepola, N.K., Sottas C.M., Russell L.D., Bunick D., Hess R.A. and Cooke P.S. (1996). Increased proliferation of Leydig cells induced by neonatal hypothyroidism in the rat. *J. Androl.* 17, 231-238.
- Josso N., di Clementi N. and Gouedard L. (2001). Anti-Mullerian hormone and its receptors. *Mol. Cell. Endocrinol.* 179, 25-32.
- 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.
- Lee M.M., Seah C.C., Masiakos P.T., Sottas C.M., Preffer F.I., Donohoe P.K., Maclaughlin D.I. and Hardy M.P. (1999). Mullerian-inhibiting substance type II receptor expression and function in purified rat Leydig cells. *Endocrinology* 140, 2819-2827.
- Lin H., Hu G-X., Dong L., Dong Q., Mukai M., Chen B-B., Holsberger D., Sottas S., Cooke P.S., Lian Q-Q., Li X-K. and Ge R-S. (2009). Increased proliferation but decreased steroidogenic capacity in Leydig cells from mice lacking cyclin-dependent kinase inhibitor 1B. *Biol. Rep.* 80, 1232-1238.
- 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 Fertil.* 29, 261-269.
- Mandal T.P. and Das N.L. (2009). Effect of δ -9-tetrahydro-cannabinol on altered antioxidative enzyme defense mechanisms and lipid peroxidation in mice testes. *Eur. J. Pharmacol.* 607, 178-187.
- Mendis-Handagama S.M.L.C. and Ewing L.L. (1990). Sources of error in the estimation of Leydig cell numbers in control and atrophied mammalian testes. *J. Microsc. (Oxford)* 159, 73-82.
- Mendis-Handagama S.M.L.C. and Ariyaratne H.B.S. (2001). Differentiation of the adult Leydig cell population in the postnatal testis. *Biol. Reprod.* 65, 660-671.
- Mendis-Handagama S.M.L.C. and Ariyaratne H.B.S. (2008). Effects of hypothyroidism on anti-mullerian hormone expression in the prepubertal rat testis. *Histol. Histopathol.* 23, 151-156.
- Mendis-Handagama S.M.L.C., Risbridger G.P. and de Kretser D.M. (1987). Morphometric analysis of the components of the neonatal and adult rat testis interstitium. *Int. J. Androl.* 10, 525-534.
- Mendis-Handagama S.M.L.C., Zirkin B.R. and Ewing L.L. (1988). Comparison of components of the testis interstitium with testosterone secretion in hamster, rat and guinea pig testes perfused *in vitro*. *Am. J. Anat.* 181, 12-22.
- Mendis-Handagama S.M.L.C., Kerr J.B. and de Kretser D.M. (1990a). Experimental cryptorchidism in the adult mouse I: qualitative and quantitative light microscopic morphology. *J. Androl.* 11, 539-547.
- Mendis-Handagama S.M.L.C., Kerr J.B. and de Kretser D.M. (1990b). Experimental cryptorchidism in the adult mouse II: a hormonal study.

- J. Androl. 11, 548-554.
- Mendis-Handagama S.M.L.C, Ariyaratne H.B.S., Teunissen van Manen K.R. and Haupt R.L. (1998). Differentiation of adult Leydig cells in the neonatal rat testis is arrested by hypothyroidism. *Biol. Reprod.* 59, 351-357.
- Mendis-Handagama S.M.L.C., Di Clementi N., Ariyaratne H.B.S. and Mrkonjich L. (2006). Detection of anti-Mullerian hormone receptor-II protein in the postnatal rat testis from birth to sexual maturity. *Histol. Histopathol.* 21, 125-130.
- Sterio D.C. (1984). The unbiased estimation of numbered and sizes of arbitrary particles using the disector. *J. Microsc.* 134, 127-136
- 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.
- Tran D., Picard J., Jacqueline C. and Josso N. (1987). Immunocytochemical detection of anti-Mullerian Hormone in Sertoli cells of various mammalian species. *Endocrinology* 35, 733-743.
- Racine C., Rey R., Forest M.G., Louis F., Ferre A., Huhtaniemi I., Josso N. and di Clementi N. (1998). Receptors for anti-Mullerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. *Pro. Natl. Acad. Sci. USA* 9, 594-599.
- Roaiah M.M., Khatab H. and Mostafa T. (2007). Mast cells in testicular biopsies of azoospermic men. *Andrologia* 39,185-189.
- 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.
- Rouiller-Fabre V., Carmona S., Abou Merhi R., Cate R., Habert R. and Vigier B. (1998). Effect of anti-Mullerian hormone on Sertoli and Leydig cell function in fetal and immature rats. *Endocrinology* 139, 1213-1220.
- Weaver J.U. (2008). Classical endocrine diseases causing obesity. *Front. Horm. Res.* 36, 212-228.
- Weibel E.R. (1969). Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* 26, 235-301.

Accepted February 1, 2010