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THYROID AND ANTI-MULLERIAN HORMONES ON LEYDIG STEM CELL DIFFERENTIATION

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SUMMARY

Androgens are essential to the male mammal for the general health and reproductive functions; Leydig cells in the testis interstitium are their main source. Therefore, the establishment of the adult Leydig cell population in the postnatal testis is an important event in the mammalian male, where the process begins during the prepubertal life. It is established that the adult population of Leydig cells is differentiated primarily from the peritubular mesenchymal cells in the testis. Five distinct cell stages, namely the mesenchymal cells (*i.e.* stem cells), progenitor cells, newly formed adult Leydig cells, immature Leydig cells and mature Leydig cells are identified in the Leydig cell lineage. At the onset of Leydig cell differentiation in the postnatal testis, a mesenchymal cell which is non-steroidogenic, differentiates into a progenitor cell which is still similar in shape to a mesenchymal cell but contains some steroidogenic enzymes. The trigger mechanism of this process is yet to be determined. Recent research has shown that this differentiation process is arrested under hypothyroid conditions and accelerated with hyperthyroid conditions. These findings suggest that thyroid hormones have a positive regulatory role in this process. Nevertheless, anti-Mullerian hormone (AMH) secreted by the immature Sertoli cells is considered to be a negative regulator of Leydig cell differentiation. Thyroid hormones and AMH could act directly on the mesenchymal precursor cells to trigger and inhibit, respectively, the process of Leydig cell differentiation. Additionally, thyroid hormones could act on Sertoli cells to induce maturation and inhibit the AMH production; this withdrawal of the inhibitory effect of AMH could possibly trigger the onset of mesenchymal cell differentiation. These concepts were addressed in this paper using evidence from experiments conducted in rats.

Key words : Anti-Mullerian hormone; Leydig cells; Stem cell differentiation; Thyroid hormone; Testis.

INTRODUCTION

Leydig cells

Franz Leydig, a scientist from Germany, first described the Leydig cells in the testis interstitium in 1850 (1) of various mammalian species (primates, carnivores, rodents, etc.). Pol Bouin (1870-1962) and Paul Ancel (1873-1961) were the first to strongly emphasize a possible endocrine role for Leydig cells, *i.e.* that internal secretion of Leydig cells controls male secondary characteristics (2). It is established now that the Leydig cells are the main source of androgens in the

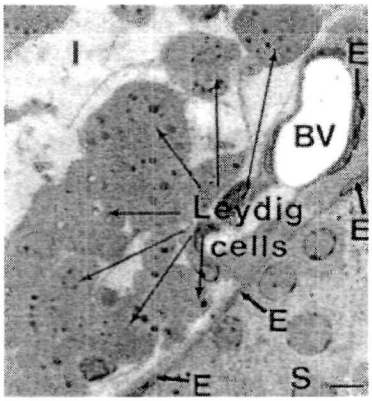

Figure 1. A light micrograph of a guinea pig testis interstitium (I) to show Leydig cells, which appear as large polygonal-shaped structures. BV = blood vessels, E = elongated spindle-shaped cells in the testis interstitium; those present at the periphery of the seminiferous tubules (S) are commonly known as peritubular mesenchymal cells and are the stem/precursor cells to Leydig cells. Bar= 20 μm

mammalian male and that the population of Leydig cells in the adult testis, identified as the adult Leydig cells, are differentiated postnatally during the neonatal pre-pubertal period (3-7). The postnatally differentiated Leydig cells of the adult population, hereafter referred to as Leydig cells in many places in the text, reside in the testis interstitium as large polyhedral cells (Figure 1). Variations are seen among species for Leydig cell number, size, morphological characteristics and their relationship to blood vessels and other surrounding structures; these characteristics are somewhat unique to each species (8), however, this aspect will not be discussed in this review. Luteinizing hormone (LH) produced by the gonadotrophs of the anterior pituitary gland, is considered as the primary regulator of Leydig cell structure and function in the postnatal testis.

Thyroid hormones (TH)

In 1912, Gudernatsch (9) provided the first evidence for TH and their role in cellular differentiation. It is now established that thyroxine (T_4) and triiodothyronine (T_3) are produced by the thyroid gland and T_3 is at least five times more potent than T_4. The most characteristic effect of TH is their ability to stimulate oxidative metabolism in many tissues in the body, however, in this sense, testis is not considered as a target organ for these hormones. TH secretion is regulated by thyroid hormone releasing hormone (TRH) and thyroid stimulating hormone (TSH) from the hypothalamus and the anterior pituitary, respectively. Recently, studies from our laboratory using the rat model demonstrated that thyroid hormone is critical for postnatal Leydig cell differentiation in the prepubertal (10, 11, 13) as well as in the adult rat -testis (12).

Leydig cell lineage

Figure 2 shows a schematic representation of the Leydig cell lineage. Mesenchymal cells of the testis interstitium are the precursors/stem cells to Leydig cells (4-8) and they embryologically originate either from the mesonephric tubules or loose connective tissue of the developing gonad derived from the embryonic mesoderm (14). In the postnatal testis, they are either found at the peritubular region or central interstitium (randomly scattered); those in the peritubular region have been identified as the precursor cell type for Leydig cells (7, 15-17) and several recent studies have confirmed this observation in the pre-pubertal rat (11, 13, 18) and adult rat testes following ethane-dimethane sulfonate (EDS)-treatment. EDS kills Leydig cells within 48 hrs (12) and therefore, EDS-treated adult rat testis is a good model to study Leydig cell differentiation using the universally accepted marker, 3ß-hydroxy steroid dehydrogenase (3ß-HSD) for all steroid secreting cells.

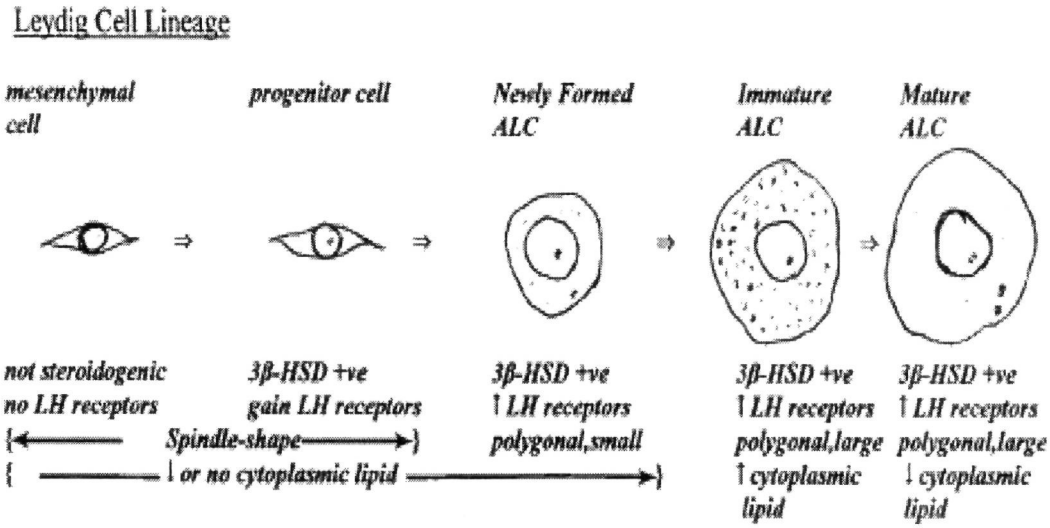

Figure 2. Schematic diagram of Leydig cell lineage, (used with permission from the publisher, *Mendis-Handagama and Ariyaratne, 2001, Biol.Reprod. 65, 660-671)*. The mesenchymal cells in the testis intersitium are the stem cells for Leydig cells; these are spindle-shaped and non-steroidogenic. They first differentiate into progenitor cells, which are also spindle-shaped, but contain some steroidogenic enzymes (*e.g.*3β-HSD) and LH receptors. Thyroid hormone is essential to stimulate the mesenchymal cell differentiation into the Leydig progenitor cells (the first step in Leydig cell differentiation) to begin this process. Leydig progenitor cells differentiate into mature adult Leydig cells through stages of newly formed adult Leydig cells and immature adult Leydig cells, respectively.

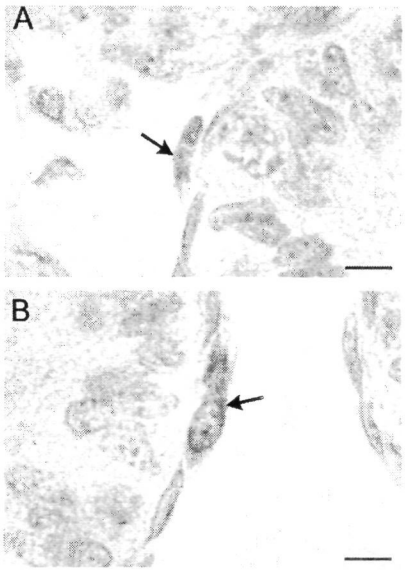

Figure 3. Representative light micrographs from a rat testis at postnatal day 10 immunolabeled for 3ß-HSD (shown in brown color) to demonstrate the early steps in Leydig cell differentiation. With thyroid hormone stimulation, mesenchymal cells in the periphery of the seminiferous tubules (S) differentiate into progenitor cells (arrows in Figures A and B); these are still spindle-shaped in configuration. With the progression of their differentiation towards the newly formed adult Leydig cells, they become rounder in and move gradually away from the peritubular region towards the central part of the testis interstitium. (used with permission from the publisher, *Ariyaratne et al., 2000, Biol. Reprod. 65, 660-671)*. Bar=20 μm

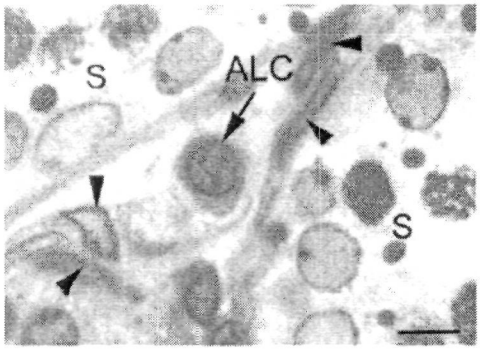

Figure 4. A representative light micrograph of a newly formed adult Leydig cell (arrow) at postnatal day 21 in a rat testis. The newly formed adult Leydig cells are much smaller than the mature adult Leydig cells, and have relatively little cytoplasm in contrast to the adult Leydig cells in a sexually mature testis. S = seminiferous tubules, I = testis interstitium, Bar = 8 μm (used with permission from the publisher, *Mendis-Handagama et al., 1998, Biol. Reprod.* 59,351-357).

At the onset of the process of Leydig cell differentiation, a mesenchymal cell which is a non-steroidogenic cell is differentiated into the second cell type in the lineage, the progenitor cell, which is steroidogenic (19) (Figure 3). Following differentiation, these progenitor cells proliferate and also differentiate into the next cell stage, the newly formed adult Leydig cells; progression of these events accompany the movement of these cells away from the peritubular location towards the central interstitium (19). With advancement of age and the progression of the Leydig cell differentiation process, the newly formed adult Leydig cells (Figure 4) transform into immature Leydig cells and finally attain the status of mature adult Leydig cells found in the sexually mature testis (Figure 1).

Thyroid hormones in postnatal Leydig cell differentiation

Until recent years, little was known about the effects of TH on the differentiation of Leydig cells in the postnatal testis. The observation that the adult rats subjected to transient neonatal hypothyroidism contain twice the number of Leydig cells per testis compared to the age-matched untreated controls (20, 21) raised the question of the mechanism of this Leydig cell hyperplasia following transient neonatal hypothyroidism. It was shown later that Leydig cell differentiation in the neonatal-prepubertal testis is arrested with hypothyroidism (10,21) and increased numbers of precursor cells/mesenchymal cells are generated during this period (10). Although it is reported elsewhere (22) that increased proliferation of postnatally differentiated Leydig cells from day 8 through 50 postpartum is the principal mechanism responsible for this Leydig cell hyperplasia in adult rats subjected to transient neonatal hypothyroidism, evidence is available to discard this conclusion. First, the postnatally differentiated Leydig cells are not present at day eight in the prepubertal rat testis (19, 23) and the only Leydig cell type present at this time (*i.e.* at postnatal day 8) is the fetal Leydig cell (19, 23). Second, it is a proven fact that Leydig cell differentiation in the prepubertal testis (10,11) and in the adult testes following EDS treatment (12) is arrested with hypothyroidism. As revealed by immunocytochemistry for 3ß-HSD, the universal marker for all steroidogenic cells, not only Leydig cells, but all other cell types in the Leydig cell lineage (*i.e.* progenitors, newly formed Leydig cells and immature Leydig cells) were absent in testes of these hypothyroid rats. These observations clearly showed that postnatal Leydig cell differentiation does not take place under hypothyroid conditions, *i.e.* TH is critical for the onset of mesenchymal cell differentiation into Leydig cell progenitors to begin the process.

Continuous exposure of lactating mothers to polychlorinated biphenyls (PCB) causes significant effects on Leydig cell structure and function; hypotrophy and reduced capacity to produce testosterone *in vitro* in response to LH stimulation (24). Additionally, it is reported that PCBs disrupts the thyroid gland function in humans (25-27) as well as in many other mammalian species such as the rat (28-34) and the grey seal (35). Based on the observations of Cooke *et al.* (31) and Kim *et al.* (24) it appears that PCB exposure during the neonatal period has subjected these rats to undergo a transient hypothyroid status, which has caused an interference in the normal process of Leydig cell differentiation during prepuberty and produce a defect in the steroidogenic function of the Leydig cells in the adult testis.

It is also being demonstrated that hyperthyroidism causes accelerated Leydig cell differentiation (11, 13, 21). Additionally, it is reported that greater numbers of mesenchymal cells are recruited into Leydig cells with TH treatment in prepubertal (13) and EDS treated (12) adult rats. These findings indicate that thyroid hormone causes proliferation of mesenchymal precursor cells and acceleration of their differentiation into Leydig progenitors; this is in addition to its effects of enhanced proliferation of progenitors and newly formed Leydig cells in the prepubertal testis (11, 13). A yet unresolved question on the effect of TH on the initiation of mesenchymal precursor cell differentiation to begin the process of Leydig cell differentiation is whether this effect is direct or indirect. Based on the demonstration of the presence of thyroid receptor mRNA in mesenchymal precursor cells (22), it is possible to suggest that TH act directly on the mesenchymal precursors to trigger this process. However, it still needs to be demonstrated that this mRNA expression is followed by protein synthesis to confirm this fact. Irrespective of this possible direct action of thyroid hormone on mesenchymal cells, it is also possible to predict that thyroid hormones may have an indirect effect on mesenchymal cell differentiation into progenitors in the postnatal testis. A logical hypothesis could be built on the known facts on TH action of Sertoli cell maturation and anti-Mullerian hormone (AMH) production by the Sertoli cells in the neonatal-prepubertal testis. This is because AMH is suggested as a negative regulator of Leydig cell differentiation (36).

Anti-Mullerian hormone and thyroid hormone on Leydig precursor cell differentiation

Anti-Mullerian hormone (AMH), which is also called the Mullerian-inhibiting factor (MIF) or Mullerian inhibiting substance (MIS) is a member of the transforming growth factor (TGF) (family of cytokines that includes TGFs, activins, inhibins and the bone morphogenetic proteins). Anti-Mullerian hormone produced by the Sertoli cells in the developing male fetal testis causes regression of the Mullerian ducts (37, 38). AMH production by the rat Sertoli cells decreases gradually and dramatically after the 3rd and 5th postnatal days, respectively and present at a very low level on the 20th postnatal day (39). Although it has been reported that the measurement of AMH mRNA is not an accurate index of AMH production (40), the fact that a dose-dependent decrease occur in AMH mRNA production by the

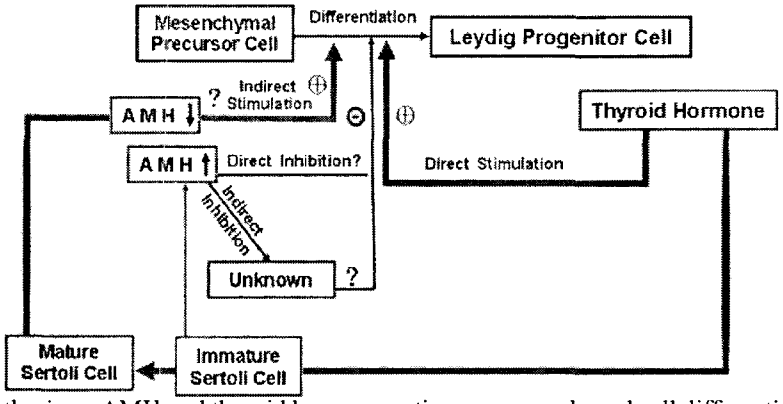

Figure 5. Hypothesis on AMH and thyroid hormone action on mesenchymal cell differentiation into Leydig progenitor cell to begin the process of Leydig cell differentiation. Thyroid hormone could act directly on the mesenchymal cells to trigger the onset (*i.e.* + ve regulation). AMH produced by the immature Sertoli cells inhibits, (– ve regulation) this differentiation. As thyroid hormones act on immature Sertoli cells to cause cell maturation, it is a possibility that this action inhibits the production of AMH, and thereby inhibits the inhibitory action of AMH on mesenchymal cell differentiation, which triggers the onset of mesenchymal cells differentiation (+ve regulation).

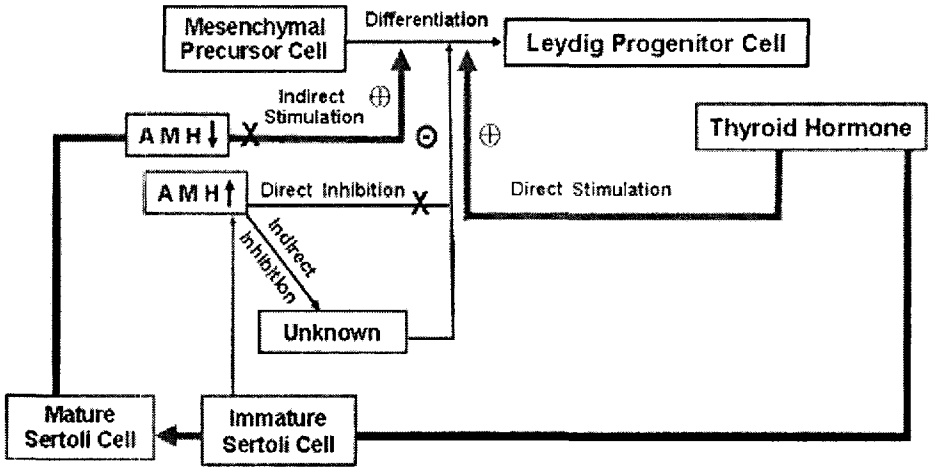

Figure 6. Summary of the findings on AMH and thyroid hormone regulation of the onset of Leydig stem cell differentiation.

immature Sertoli cells with triiodothyronine (T_3) is an interesting observation with respect to the hypothesis on the indirect role of TH on Leydig cell differentiation. This is because AMH has been suggested as a negative regulator for postnatal differentiation of Leydig cells (36). A schematic diagram (Figure 5) of this hypothesis on the mechanism of action of TH and AMH on regulation of precursor cell differentiation into Leydig progenitors in the postnatal testis is shown. According to this hypothesis, both hormones, *i.e.* TH and AMH could act directly on mesenchymal precursor cells to stimulate and inhibit, respectively, the onset of postnatal Leydig cell differentiation. Additionally, TH could indirectly act on this process; TH causes immature Sertoli cells to mature and thereby inhibit the AMH production, and the removal of the inhibitory action of AMH would cause the triggering stimulus for the mesenchymal cells to differentiate into Leydig progenitors.

Recent studies

In the process of solving this mystery on what triggers the onset of Leydig cell differentiation in the postnatal testis, we performed two experiments to add new insight to this area in male reproductive tract development.

Experiment I: Expression of AMH in Sertoli cells euthyroid, and hypothyroid neonatal-prepubertal rat

Hypothesis 1: The positive regulatory role of thyroid hormone on the onset of postnatal Leydig cell differentiation is induced by causing Sertoli cell maturation and inhibition of Sertoli cell AMH production, *i.e.* inhibition of the inhibitory action of AMH on Leydig cell differentiation.

Rationale/Objectives 1: Leydig cell differentiation is accelerated in prepubertal rats with T_3 treatment (13) and pronounced in AMH knockout mice (36), arrested in hypothyroid prepubertal rats (10,12) and in mice over-expressing AMH (36). AMH mRNA expression in the immature Sertoli cells is regulated at least in part by T_3 (41). AMH in Sertoli cells in the postnatal testis is shown to be abundant on day one, however, a sharp decline is reported thereafter, with the postnatal maturation of the Sertoli cells. Therefore, we asked the question whether the action of the thyroid hormone on postnatal Leydig cell differentiation is via the Sertoli cells, *i.e.* causing Sertoli cell maturation and thereby inhibiting the production of AMH, which is a negative regulator of the process. Thus, the objective of this study is to monitor the expression of AMH in Sertoli cells of control and hypothyroid rats to determine whether Sertoli cell AMH production could be maintained by inhibiting the maturation of Sertoli cells.

Design and Method 1 : Two groups of male Sprague Dawley rat pups were used. The first group of rats were controls and their mothers were fed with normal rat chow and water *ad libitum.* These pups were euthanized according to our approved protocol (#731) on day 1,7, and 14 (n=4 rats per group) and their testes were harvested. One testis was fixed in Bouins solution and prepared for immunocytochemistry and the other testis was snap frozen using liquid nitrogen and stored at -80° C until used for Western blot analyses for AMH. Mothers of rat pups in group 2 were also fed with normal rat chow, however, 0.1% propyl thiouracil (PTU) was added to their drinking water from the day of parturition so that hypothyroidism could be induced in the rat pup as previously reported (10, 20). Pups were euthanized and testes were harvested as similar to the control pups on postnatal days 7 and 14 to perform immunocytochemistry (Avidin-Biotin Method, 10-13) and Western blot analysis for AMH. Immunocytochemistry was performed for AMH (polyclonal antibody was provided by Dr. R. Rey, Argentina) using testis tissue of both control (days 1,7 and 14) and PTU-treated rats. Pre-immune serum was used instead of the primary antibody in all studies as control incubations. Our positive and negative controls for AMH were Sertoli cells and fetal Leydig cells in one day old rat testes, respectively.

Results 1: In control rats, all Sertoli cells in each seminiferous cord showed abundance of AMH labeling (100%) at postnatal day one. At days seven and 14, very little labeling was observed in some Sertoli cells in both control and PTU-treated rats demonstrating a sharp decline in AMH content in Sertoli cells after the postnatal day 1; Western blot analyses showed a similar pattern ;

(results not shown). The sharp decline in the immunolabeling for AMH in Sertoli cells of control rats on days 7 and 14 agrees with previously published reports (39). However, we were surprised to observe that PTU-treatment/hypothyroidism was unable to maintain Sertoli cell AMH production.

Conclusion 1: These findings suggested that Sertoli cell AMH production and maturation are two independent processes and therefore, the stimulatory effect of thyroid hormone on Leydig cell differentiation is unlikely to be mediated via causing Sertoli cell maturation and thereby inhibition of AMH production by the Sertoli cells.

Experiment 2: Expression of AMH type II receptors in cells of Leydig Cell Lineage in the rat testis from birth to sexual maturity.

Hypothesis 2: The negative regulatory role of AMH on postnatal Leydig cell differentiation is a direct action, *i.e.* inhibiting the differentiation of mesenchymal cells to Leydig progenitor cells

Rationale/Objectives 2: If AMH exert it's negative regulatory role on postnatal Leydig cell differentiation directly on mesenchymal cells to inhibit the onset of Leydig cell differentiation, mesenchymal cells should possess AMH type II receptors (AMHR-II). Therefore, the objective of this experiment is to investigate whether mesenchymal cells in the testis interstitium contain AMHR-II in rats from birth to sexual maturity.

Design and method 1 : Sprague Dawley rats, of postnatal day 1, 7-21 (every age), 28, 40, 60 and 90 were used (n=4 rats per group). They were euthanized according to our approved protocol (#731), their testes harvested, fixed in Bouins solution and processed for immunocytochemistry (10-13). The antibody used was a polyclonal raised in rabbits against the extra cellular part of the human AMHR-II (42). Immnuolocalization of AMHR-II in cells of Leydig cell lineage was performed using Avidin-Biotin method as published previously (10-13).

Results 2: We observed that none of the elongated spindle-shaped cells in the testis interstitium, which includes the mesenchymal precursor cells and the progenitor cells of the Leydig cell lineage (the first two cell types in the adult Leydig cell lineage) were positive for AMHR-II. The newly formed adult Leydig cells are the first cell type of the Leydig cell lineage to express AMHR-II and the first detection in these cells was on postnatal day 13, although these cells were present in the testis from the postnatal day 10. Thereafter, immunolabeling for AMHR type II was seen in Leydig cells at all ages studied (results not shown).

Conclusions 2: The negative immunolabeling in mesenchymal cells in the testis interstitium in rats from birth to sexual maturity suggested that it is unlikely that the negative action of AMH on postnatal Leydig cell differentiation is direct. The time lag between the first detection of the newly formed Leydig cells in the testis interstitium and the first detection of AMHR-II activity in them suggests that the establishment of the role of AMH on these Leydig cells, *i.e.* the negative action of AMH on Leydig cell steroidogenesis (43) does not take place immediately with their differentiation but few days after ; no change in cell size was observed during this period.

SUMMARY AND CONCLUSIONS

There are five recognizable stages of cells in the Leydig cell lineage in the postnatal testis as described and schematically diagrammed in Figure 1. At the onset of the process of Leydig cell differentiation, the mesenchymal cells or the stem cells differentiate into Leydig progenitor cells. Previous studies have shown that thyroid hormone is a positive regulator (10-13) and AMH is a negative regulator (36) of postnatal differentiation of Leydig cells. Recent studies in our laboratory using rats attempted to further investigate the effects of AMH and thyroid hormone on postnatal Leydig cell differentiation. We investigated the possibility of Sertoli cell maturation caused by the thyroid hormone is associated with inhibition of Sertoli cell AMH production to trigger the onset of mesenchymal cell differentiation into Leydig progenitor cells . This was to understand whether thyroid hormone has an indirect action on mesenchymal precursor/stem cell differentiation in addition to its direct action. The findings suggested that Sertoli cell maturation and AMH production by the Sertoli cells are two independent processes. We also tested whether negative action of AMH on postnatal Leydig cell differentiation is direct by investigating whether the mesenchymal precursor/ stem cells possess AMHR-II to respond to AMH. The findings revealed that mesenchymal precursor/ stem cells in the testis interstitium of postnatal testes were negative for AMHR-II from birth to sexual maturity. Therefore, it appears that the negative regulatory action of AMH on the process of postnatal Leydig cell differentiation is indirect. Figure 6 summarizes these findings using a schematic diagram.

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