Changes in expression and distribution of attractin in the testes of rats at different developmental stages

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Abstract. Attractin (Atrn), an autosomal recessive gene, is widely expressed in the body and displays multiple physiological and pathological functions in different types of tissues. The objective of this study was to localize Atrn protein and mRNA in the testis and epididymis of rats at different stages of maturation. Testis and epididymidis samples were obtained from the following 5 groups of Sprague Dawley (SD) rats in different developmental stages: newborn (8 h after birth), prepubertal (5 days), pubertal (20 days), postpubertal (50 days) and mature (70 days). Tissues were fixed and prepared for indirect immunofluorescence, immunohistochemistry, *in situ* hybridization, confocal laser scanning microscopy and western blot assays. A polyclonal antiserum against mouse Atrn and oligonucleotide riboprobes were used in the above assays. At the different stages of maturation, Atrn protein and mRNA were both widely expressed in the rat testis, including Leydig cells, primitive spermatogonia, primary spermatocytes, spermatids, Sertoli and peritubular myoid cells. Staining of the Atrn protein was mainly located on the cell membrane and in the cell cytoplasm while Atrn mRNA was distributed in both the nucleus and cytoplasm. No immunopositive staining was detected in spermatozoa and epididymides. In the epididymis, comprised of the caput, corpus and cauda, there was no definitive immunopositive staining within the efferent ductules or epididymal ducts. Taken together, Atrn protein and mRNA are both expressed widely in the rat testis at different stages of maturation, which suggests that Atrn protein is involved and plays an important role in the development of the reproductive system. In addition, the rat testis has the ability to synthesize Atrn protein throughout sexual development.

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Introduction

Attractin (Atrn), a dipeptidyl peptidase IV/CD26-like enzyme rapidly expressed and released on activated T cells, has attracted much attention in recent years (1). Scientists used positional cloning to identify the candidate gene, mahogany (Mgca). The predicted protein encoded by Mgca is a 1,428-amino acid, single-transmembrane-domain protein expressed in many tissues, including pigment cells and the hypothalamus (2-4). The extracellular domain of the Mgca protein is the orthologue of human attractin, a circulating molecule produced by activated T cells that has been implicated in immune-cell interactions. Atrn mRNA was found to be widely distributed throughout the central nervous system by *in situ* hybridization (ISH). In the hypothalamus, Atrn mRNA is observed in paraventricular and supraoptic nuclei, suggesting a potential role in the regulation of posterior pituitary gland function (5). Investigations have focused on the functional activity of attractin in every allelic line, in addition to its physiological properties, subcellular location, unifying mechanism and possible therapeutic interventions. Research has shown that attractin is widely distributed in most organisms and is involved in a number of physiological and pathological functions, including immune system regulation, body weight control, myelinization and tumor susceptibility. Particularly, attractin plays a role in the switch of pigment synthesis of hair color and in degenerative diseases of the central nervous system. Regarding pigmentation, it has been reported that mice with a mutation at the Atrn locus are darkly pigmented as the agouti-induced yellow band on the hair is suppressed and the mutation also suppresses the yellow fur caused by the overexpression of agouti in mutant animals (6).

Paz *et al* (7) found that the age-dependent progressive neurodegeneration, such as neuronal cell death, hypomyelination and vacuolation, is closely correlated with loss-of-function of attractin. This indicates that Atrn may have a potential therapeutic effect on neurodegenerative diseases. It

is known that the function of the male reproductive system is brought into play by the hypothalamus-pituitary-gonadal axis. However, there are few reports of attractin expression in the male reproductive system. To determine whether Atrn is expressed in the reproductive system of the male rat and to determine its localization, immunohistochemistry (IHC), indirect immunofluorescence (IIF), ISH and western blotting were conducted on testicular and epididymal tissue from male Sprague Dawley (SD) rats of different ages.

Materials and methods

Tissue collection. SD male rats used in all of the experiments were purchased from the animal center of Huazhong University of Science and Technology, so as to exclude any effects of immunologic interference. The animals were maintained at the animal facilities of Tongji Medical College, Huazhong University of Science and Technology. The experiments were conducted in accordance with the guidelines approved by the China Association of Laboratory Animal Care.

Testes and epididymides were obtained from 5 groups of SD rats: newborn (8 h after birth), prepubertal (5 days), pubertal (20 days), postpubertal (50 days) and mature (70 days). Rats of the same age were born on the same day, and each group included 8 animals.

Tissue preparation and staining

Preparation of paraffin tissue sections. Following castration, testes and epididymides from all animals of each age group (n=8) were transported in cold Hanks' Balanced Salt Solution to the laboratory. Sections (~1 cm^3) of the testicular parenchyma were removed and placed in 4% paraformaldehyde at 4˚C for 24 h. Tissues were then transferred to 0.1 M phosphate-buffered saline (PBS) solution at 4˚C for 24 h, dehydrated with 70, 80, 95 and 95% of ethanol successively at room temperature for 15 min for each treatment and then treated with 100% ethanol at room temperature for 10 min. The sections were cleaned twice with xylene at room temperature for 20 min and were embedded in paraffin. Tissue sections (4 μm) of each testis and epididymis were cut, mounted on poly-L-lysine-coated slides (Sigma Chemical Co., St. Louis, MO, USA), dried and stored at 4˚C.

Preparation of frozen tissue sections. Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.). The chest of the rat was opened by midline incision, and then perfused transcardially with 300 ml of 0.9% NaCl containing 2% sodium nitrite and 600 ml of 4% paraformaldehyde successively at 4˚C. The testes and epididymides were removed and immersed in a post-perfusion fixative of 4% fresh paraformaldehyde solution for 4 h at 4˚C, and then in 25% sucrose distilled water solution at 4˚C for 4-6 days until they sank to the bottom of the sucrose solution. Fresh sucrose solution was replaced daily. Testes and epididymides were carefully embedded at optimal cutting temperature (OCT) and cut into 20-μm sections and stored in cryoprotectant.

Direct immunofluorescence. Frozen tissue sections were washed 3 times with PBS for 5 min each time, and then incubated in normal rabbit serum (New Step Reagent Co., Fujian, China) for 10 min at 37˚C and washed 3 times with PBS for 5 min each time. Afterwards, tissues were incubated in the primary antibody (rabbit anti-mouse attractin antibody) (provided by Dr Shiliang Shen, Department of Genetics and HHMI Stanford University School of Medicine) diluted (1:400) in Tris-buffered saline (TBS) containing 10% rabbit serum for 2 h at 37˚C and then in goat anti-rabbit IgG labeled with fluorescein isothiocyanate (FITC) for 10 min at 37˚C in a dark humidified chamber. Slides were rinsed 3 times in PBS and then 3 times in distilled water. Each rinse lasted for 5 min. The tissue sections were mounted in glycerin buffer onto slides and covered by coverslips. Images of the tissues were captured immediately under fluorescence microscopy. Controls for the specificity of the antisera consisted of incubating tissue in antisera that had been pre-absorbed with an antigen which blocked all staining.

Immunohistochemistry. Paraffin tissue sections were dewaxed in xylene, rehydrated and washed in distilled water, and then immersed in 0.01 M citrate buffer (pH 6.0), and finally heated at 98˚C for 15 min in a microwave oven. After the tissue sections were cooled down to room temperature, the slides were washed 3 times with distilled water and 3 times with PBS successively. The slides were blocked for endogenous peroxidase by incubation with 0.3% H_2O_2 in PBS for 10 min at 37˚C, and then blocked with 10% goat serum (New Step Reagent Co., Fujian, China) for 20 min at 37˚C to prevent nonspecific binding of the antibodies. Subsequently, the slides were incubated at 4˚C overnight with the primary antibody diluted (1:200) in TBS containing 10% goat serum. After washing in PBS, the sections were incubated for 20 min at 37˚C with biotin-conjugated goat anti-rabbit IgG (New Step Reagent Co., Fujian, China). The sections were then rinsed in PBS and incubated for 20 min with enyzme-conjugated horseradish peroxidase (HRP)-streptavidin (New Step Reagent Co., Fujian, China), then rinsed in PBS. The attractin antibody-peroxidase complex was stained with a solution containing nickel sulfate (0.250 g), 3,3'-diaminobenzidine (0.002 g), and H_2O_2 (8.3 ml of 3%) in 10 ml of 0.175 M sodium acetate buffer for 2-3 min. Some sections were counterstained with Mayer's hematoxylin (New Step Reagent Co., Fujian, China), dehydrated step-wise with an ethanol series, cleaned and coverslipped. Controls for the specificity of the antisera consisted of incubating tissue in antisera that had been preabsorbed with a stain-blocking antigen.

Confocal laser scanning microscopy. Paraffin tissue sections were dewaxed in xylene, rehydrated and washed in PBS. Tissues were then incubated in normal rabbit serum for 10 min at 37˚C, washed, and incubated again in the primary antibody which was diluted with TBS containing 10% rabbit serum at 1:400 for 2 h at 37˚C. Goat anti-rabbit IgG was labeled with FITC for 30 min at 37˚C in a darkened humidified chamber. Slides were rinsed 3 times with PBS buffer, and then three times with distilled water for 5 min each rinsing. Sections were coverslipped in 1,4-diasabicyclo [2.2.2]octane (Dabco). The tissue sections were examined and photographed under confocal laser scanning microscopy immediately. Controls for the specificity of the antisera consisted of incubating tissue in antisera that had been preabsorbed with stain-blocking antigen.

In situ hybridization. Paraffin tissue sections were dewaxed in xylene, and rehydrated and washed in PBS. Endogenous peroxidase was blocked by incubating the sections in 0.3% H_2O_2 in PBS for 5 min at 37˚C to reduce the non-specific background staining. The sections were subsequently rinsed in distilled water, treated with pepsin diluted in 3% fresh citric acid, and fixed in 4% paraformaldehyde for 10 min at room temperature. Prehybridization was performed for ~2 h at 40˚C in prehybridization buffer [50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 10% dextran sulfate, and 1X Denhardt's solution, and 10 mM NaH_2PO_4]. Hybridization was performed for ~18 h at 40˚C in hybridization buffer containind digoxigenin (DIG)-labeled oligonucleotide riboprobes. The *in situ* hybridization kit was synthesized by Wuhan Boshide Biological Co. Ltd. The 5'-labeled attractin digoxigenin oligonucleotide probe was synthesized using multi-phase oligonucleotide probes and a highly sensitive labeling technique. The mRNA sequences of attractin target genes for the rat were, 5'-CCGCTTCAGACTAACTGGAT CTTCTGGATTTGTAA-3', 5'-ATATGTCTCCATTCACA AATAGTTTGCTGCAGTGG-3' and 5'-TATAAAGACTGT TCCTAAGCCCATTGCCCTGGAGC-3'.

After hybridization, the coverslips were removed in 5X SSC (standard saline citrate). Sections were washed in 2X SSC at 37˚C for 10 min, in 0.5X SSC at 37˚C for 15 min, and then in 0.2X SSC for 15 min successively. The hybridized DIG-labeled probes were detected with anti-DIG monoclonal antibodies. Sections were incubated with biotin-conjugated mouse anti-DIG (Boehringer, Mannheim) for 1 h at 37˚C, and then with 10% horse serum in TBS [0.1 M Tris (pH 7.6) and 0.15 M NaCl] for 30 min at 37˚C. After washing, the sections were incubated in the streptavidin-biotin-peroxidase complex (SABC) (Biological Co., Ltd., Wuhan Boshide, China) and then in enyzme-conjugated HRP-streptavidin at 37˚C for 20 min for each incubation. To visualize the complex, sections were covered with 0.5 mg/ml of DAB (Dako Corp., Carpintera, CA, USA) in 0.05 M Tris-HCl (pH 7.6) containing 0.01% H_2O_2. Counterstaining and other steps were performed as described above. Controls for the specificity of the oligonucleotide riboprobe consisted of incubating the tissue in oligonucleotide riboprobe hybridization buffer that had been preabsorbed with prehybridization buffer which blocked all staining.

Western blotting. Fresh testis tissues were isolated and put in 3 volumes of extract buffer. The sample was centrifuged at 10,000 rpm for 10 min at 4˚C and the supernatant was maintained at -70˚C. Then, 50 μl of the sample was added to an equal volume of 2X SDS gel-loading buffer. The sample in the loading buffer was heated at 100˚C for 3-5 min and then loaded for electrophoresis. The applied voltage was 8 V/cm (6 x 8 = 48 V). When the dye (bromophenol blue) front entered the separating gel, the voltage was increased to 15 V/cm (6 x 15 = 90 V). The power was turned off when the bromophenol blue reached the bottom of the separation gel. The gel was prepared for transfer in transfer buffer: 0.65 mA/cm^2 (~100 V) for 1.5-2 h, or 30 V overnight, on ice, and the filter was blocked with blocking buffer for 1-2 h at room temperature (0. ml blocking solution/cm^2 filter), with gentle agitation on a platform shaker. After blocking solution was discarded, the filter was immediately incubated with the attractin antibody. The attactin antibody (0.005 ml) (1:2,000) was added to the blocking solution and incubated at 4˚C for 2 h or overnight with gentle agitation on a platform shaker. The blocking solution was discarded, and the filter was washed 3 times (10 min each time) with 250 ml of PBS. The filter was incubated in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) (phosphate-free, azide-free blocking solution) 3 times for 10 min each time. The filter was then immediately incubated with the secondary antibody. Ten milliliters of phosphate-free, azide-free solution [150 mM NaCl, 50 mM Tris-HCl, 5% nonfat dry milk (pH 7.5)] was added. Secondary antibody solution (0.005 ml) (1:2,000) was added, and incubated for 1-2 h at room temperature with gentle agitation. The secondary antibody solution was discarded, and the filter was then washed with 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) (phosphate-free, azide-free solution) 3 times for 10 min each time. Five milliliters of the substrate 5-brono-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma) was added. Blue color on the filter (~20 min) was observed. BCIP/NBT solution was discarded when the bands were clear (~20 min). The enzymatic reaction was halted immediately by adding water. The filter was finally covered with a plastic membrane and the filter was stored.

Results

In the rat, maturation of the testis starts early after birth. Before puberty, the lumen of the seminiferous tubule has not yet formed. The diameter of this tubule is small and has no basement membrane. It contains gonocytes and undifferentiated cells that will later be transformed into Sertoli cells. Comparatively, in the interstitial tissue surrounding the seminiferous tubules, there are only undifferentiated interstitial cells which later give rise to Leydig cells.

Immunofluorescence and confocal laser scanning microscopy. In the testis of the male mature rat, there was distinct immunopositive staining of attractin on the cell membrane and in the cytoplasm of Leydig cells, primitive spermatogonia, primary spermatocytes, spermatids, Sertoli cells, and peritubular myoid cells. In the epididymis, there was no definitive immunopositive staining within the efferent ductules or the epididymal ducts (Figs. 1-4).

Immunohistochemistry. The attractin protein was positively stained with DAB in undifferentiated interstitial cells, in the newborn (1 day) gonocytes, in prepubertal (5 days) spermatogonia, in pubertal (20 days) primary spermatocytes, in postpubertal (50 days) spermatids as well as in Sertoli cells, peritubular constrictive cells and Leydig cells. The expression of Atrn in interstitial tissue was stronger than that in the seminiferous tubule. Attractin protein was also positively stained in Leydig and germ cells of the mature rat, exhibiting a strong membrane and cytoplasmic presence. No immunopositive staining was noted in spermatozoa or in the epididymides (Figs. 5-10).

In situ hybridization. Expression of attractin mRNA by ISH was consistent with the results of attractin protein staining

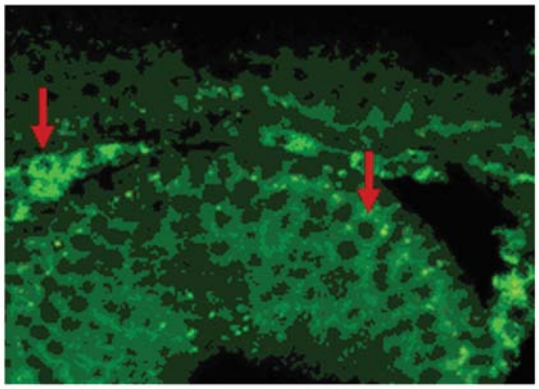

Figure 1. Positively stained Leydig cells and seminiferous tubules of the mature rat (x400).

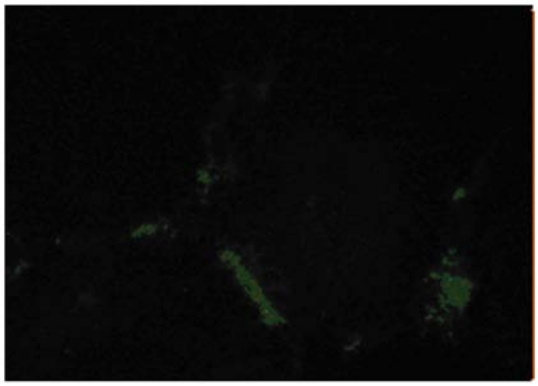

Figure 2. No definitive immunopositive staining was noted within Leydig cells and seminiferous tubules (x200).

within Leydig cells, primitive spermatogonia, primary spermatocyte, spermatid, Sertoli cells and peritubular myoid cells. Attractin mRNA was distributed in the nucleus and the cytoplasm of these cells. No immunopositive staining was noted in spermatozoa or in the epididymides (Figs. 11-16).

Western blot analysis. Color development was according to the protein Marker Mix molecular weight between 215 and 120K. The Atrn protein band was noted (Fig. 17). Lanes 1-3 show the 200-kDa Atrn protein band, respectively; lane 4 shows the 215-kDa protein marker.

Discussion

The mouse mahogany gene mutation was first recognized as a modifier of the Agouti phenotype in the late 1950s. It was only in the last few years however, that a clear image of mahogany gene structure and function emerged. In 1999, Gunn *et al* (2) and Nagle *et al* (3) discovered that it encodes a 1428-residue single transmembrane-spanning protein with a large extracellular domain that contains two epidermal growth factor (EGF) repeats, a complement C1r/C1s, Uegf, Bmp1 (CUB) domain and two laminin-type EGF repeats by positional cloning. Concurrently, Duke-Cohan *et al* (8,9) cloned human attractin, a human serum glycoprotein secreted from activated T cells. The extracellular domain of the mouse protein was found to be 93% identical

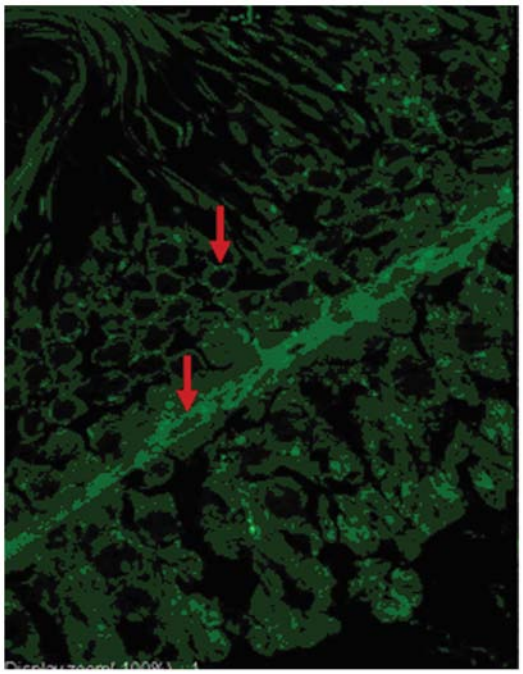

Figure 3. Positively stained Leydig cells and seminiferous tubules of the mature rat (x60).

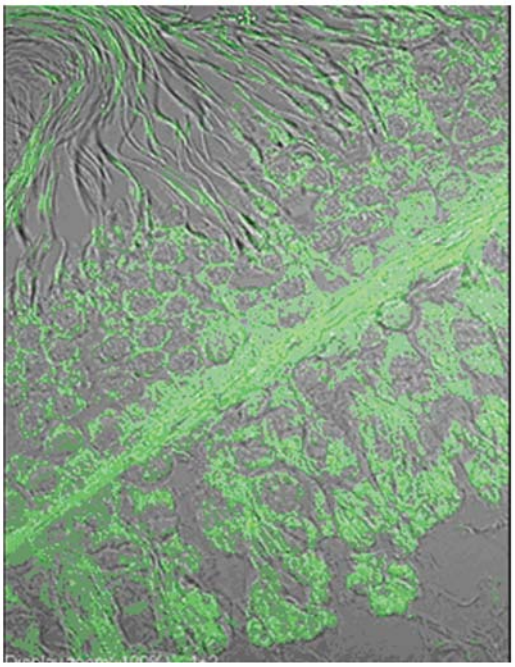

Figure 4. Positively stained Leydig cells and seminiferous tubules of the mature rat (x60).

to attractin. Due to this homology between the mouse mahogany protein and human attractin, the mouse gene was renamed the attractin gene. Further analysis demonstrated that humans produce two major isoforms of the Atrn protein: a secreted isoform that is truncated just short of the transmembrane domain, and a membrane-spanning isoform that is homologous to mouse Atrn. The secreted isoform is present in humans and rats but not mice, and is expressed

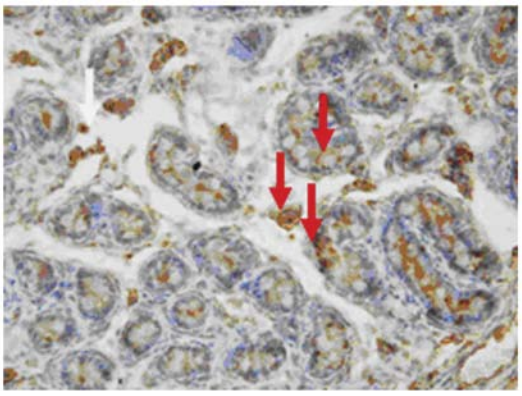

Figure 5. Positive staining of attractin protein with DAB in undifferentiated interstitial cells, gonocytes and Sertoli cells. Strong membrane and cytoplasmic staining is noted in the testis of the newborn (1 day) rat (x400).

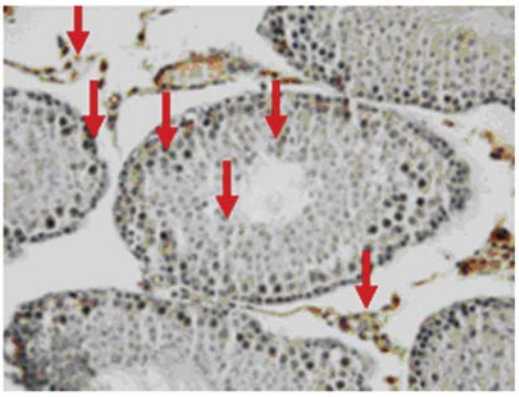

Figure 8. Positive staining of attractin protein with DAB in Leydig cells, spermatogonia, primary spermatocytes, spermatids, Sertoli cells, peritubular myoid cells. Strong membrane and cytoplasmic staining is noted in testis of the postpubertal (50 days) rat (x400).

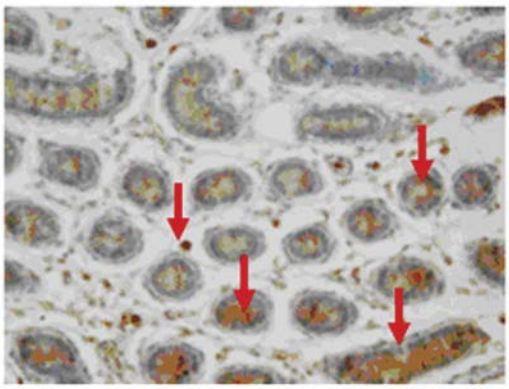

Figure 6. Positive staining of attractin protein with DAB in undifferentiated interstitial cells, spermatogonia, Sertoli cells, peritubular myoid cells. Strong membrane and cytoplasmic staining in testis of the prepubertal (5 days) rat (x400).

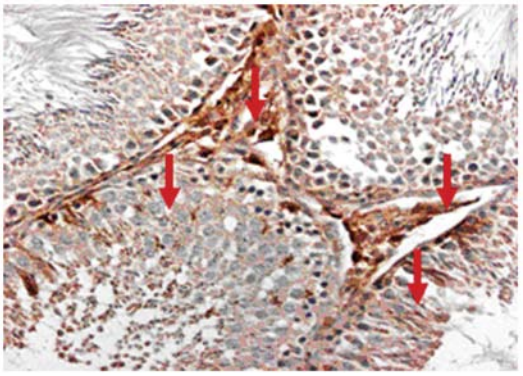

Figure 9. Distinct immunopositive staining within Leydig cells, peritubular myoid cells, Sertoli cells and germ cells (x400).

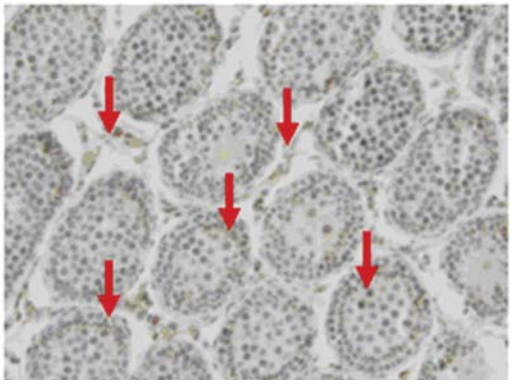

Figure 7. Positive staining of attractin protein with DAB in Leydig cells, spermatogonia, Sertoli cells, primary spermatocytes, peritubular myoid cells. Strong membrane and cytoplasmic staining is noted in testis of the pubertal (20 days) rat (x400).

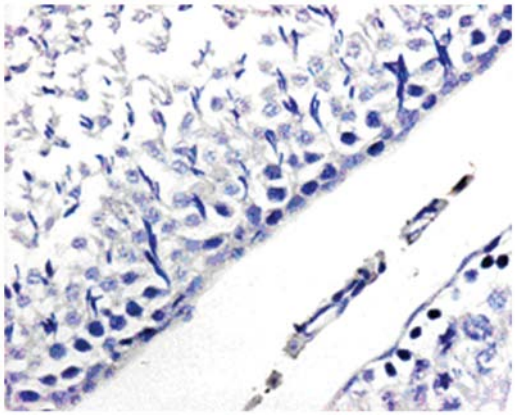

Figure 10. No definitive immunopositive staining within the testis tissue (x400).

in nearly every tissue. The membrane-spanning isoform has a wide (10) but not a ubiquitous distribution and is found at high levels in melanocytes and other components of the skin, as well as in the brain, the heart, the kidney, the liver, and the lung, but excluding the uterus, muscles or the spleen.

In recent years, extensive research has been conducted in regards to attractin in its involvement in melanogenetic shifts of coat color, in diseases of the central nervous system and in the development of obesity-regulating drugs, with the development of molecular biology techniques (11). Recently, involvement of attractin in the aspects of mammalian egg-sperm interactions has been suggested (10). However, there

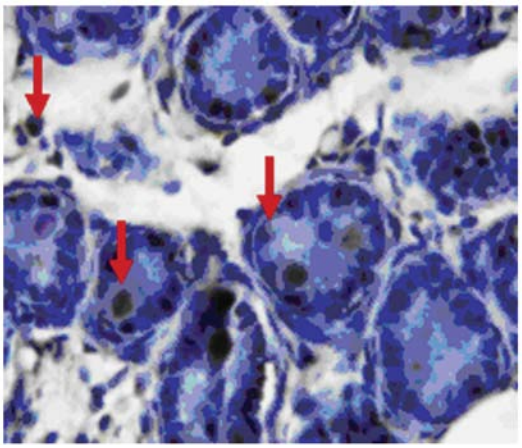

Figure 11. Positive staining of attractin mRNA with DAB in undifferentiated Leydig cells, gonocytes and Sertoli cells. Strong nucleic and cytoplasmic staining is noted in testis of the newborn (1 day) rat. Strong staining is mainly in gonocytes (x1,000).

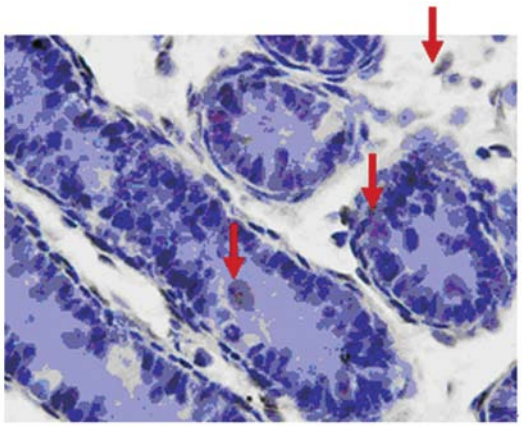

Figure 12. Positive staining of attractin mRNA with DAB in Leydig cells, spermatogonia and Sertoli cells. Strong nucleic and cytoplasmic staining is noted in testis of the prepubertal (5 days) rat. The expression of attractin in seminiferous tubules is stronger than that in the interstitial tissue (x400).

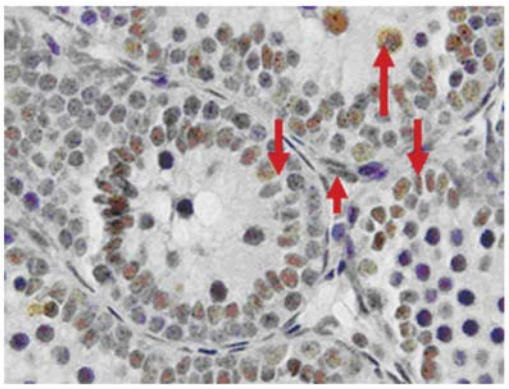

Figure 13. Positive staining of attractin mRNA with DAB in Leydig cells, spermatogonia, Sertoli cells, primary spermatocytes, peritubular myoid cells. Strong nucleic and cytoplasmic staining is noted in testis of the pubertal (20 days) rat (x400).

are no studies concerning the role of attractin in the male reproductive system.

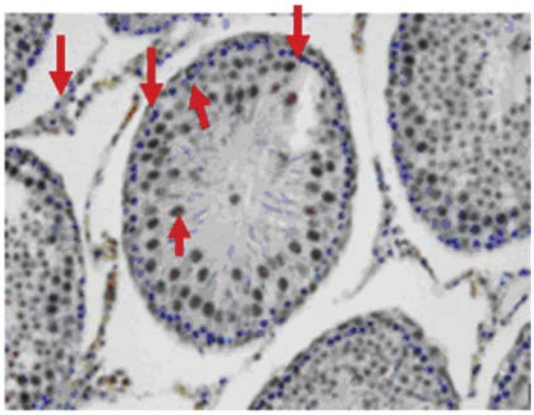

Figure 14. Positive staining of attractin mRNA with DAB in Leydig cells, spermatogonia, primary spermatocytes, spermatids, Sertoli cells, peritubular myoid cells. Strong nucleic and cytoplasmic staining in testis of the postpubertal (50 days) rat (x400).

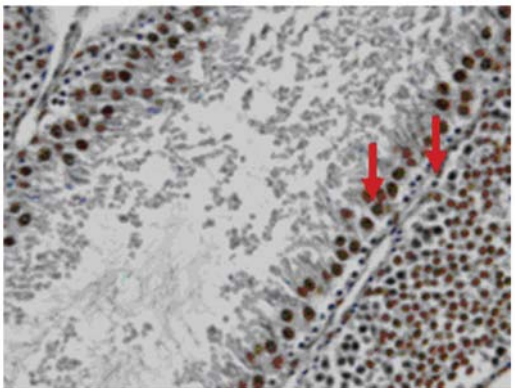

Figure 15. Positive staining of attractin mRNA with DAB in the seminiferous tubule and interstitial tissue. Strong nucleic and cytoplasmic staining is noted in testis of the mature (70 days) rat (x400).

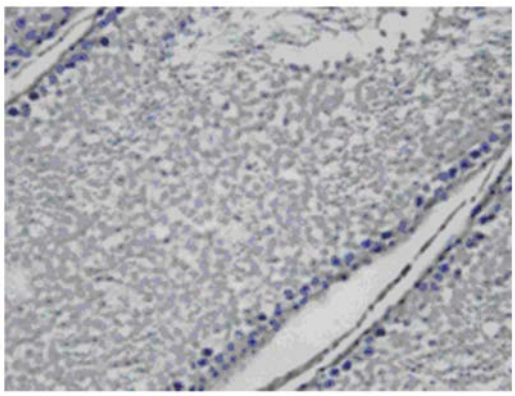

Figure 16. Staining with DAB was not observed in the negative control testis tissue of the mature (70 days) rat (x400).

This study provides evidence based on IIF, IHC, confocal laser microscopy techniques and ISH that Atrn protein and mRNA may be localized within both interstitial tissues and the seminiferous tubules in an age-dependent manner. In spermatozoa and epididymides, including the efferent duct-

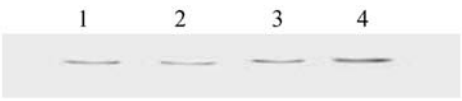

Figure 17. Lanes 1-3 show the 200-kDa Atrn protein band, respectively; lane 4 shows the 215-kDa protein marker.

ules and the epididymal ducts, no definitive immunopositive staining was noted. This suggests that rat testis has the ability to synthesize attractin protein throughout sexual development, and Atrn has likely little importance on maturation and deposition of spermatozoa. In the testis of the male rat, there was distinct immunopositive staining as detected by IHC on many cell membranes and also in the cytoplasm. This is the result of antigen dispersement, since Atrn is a transmembrane protein.

IIF, IHC, confocal laser microscopy data suggest that the expression of Atrn protein in Leydig cells gradually becomes stronger when compared with germ cells with the increasing age of the rat. This phenomenon may be due to the fact that seminiferous tubules contain only spermatocytes and Sertoli cells during the prepubertal stages of the male rat. From puberty, by the action of pituitary gonadotropin, Leydig cells gradually mature and testosterone secretion starts to increase. Upon stimulation of testosterone, the transcription of the Atrn gene is accelerated and protein secretion is increased. There are two isoforms of rat Atrn protein: the secreted isoform and the membrane-spanning isoform. The secreted isoform of Atrn protein may act on Leydig cells in an autocrine and paracrine manner by mechanisms which promote the expression of Atrn protein and secretion of testosterone.

However, contrary to the above mentioned results from IIF, IHC, confocal laser microscopy, ISH data indicate that the expression of Atrn mRNA within germ cells was stronger than that in Leydig cells, and increased with the age of the rats. This may be due to the interaction of Atrn protein and testosterone which results in increased secretion of testosterone, accelerated transcription of Atrn gene in germ cells, and facilitation of the development and maturation of germ cells. In addition, androgens are the most important hormones in the regulation of spermatogenesis, and testicular Sertoli cells and peritubular myoid cells are the target cells of androgens. The expression of Atrn protein and mRNA in Sertoli cells and peritubular myoid cells gradually increased along with the maturation of the rat. Spermatogenesis is the result of programmed differentiation of germ cells with the help and regulation of Sertoli cells. It is logical and foreseeable that the quantity of mRNA in a cell would be representative of the protein expression. But in fact, this is not entirely true. There are three levels of regulation from DNA and RNA to protein: the transcription level, translation level and post-translation level. From the mRNA point of view, it is only affected by the regulation of the transcription level and cannot represent the level of protein expression as the end result. Recent experimentation has confirmed that there is a poor correlation between the level of mRNA and the amount of protein in tissues, particularly proteins with low expression levels or contents (12). More importantly, based on the abundance of mRNA, it is almost impossible to estimate the complex modification afer protein translation, the subcellular localization or migration of the protein, and protein-protein interactions.

In the present study, Atrn protein in the testis of the mature male rat using dot blotting and western blot techniques was analyzed and the results showed that the protein content of Atrn was lower at this age.

In prepubertal stages, Leydig cells in the interstitial tissue of the testis have no ability to synthesize or secrete androgens. However, ISH analysis showed that attractin mRNA was positively stained in the gonocytes of newborn (1 day) and prepubertal (5 days) rats, which implies that Atrn plays a key role in gonocyte development and maturation (and its shift to spermatogonia independently from the effects of androgens secreted by mature Leydig cells). Prior to puberty, spermatogonia can still continuously differentiate and proliferate in this manner.

We conclude that the expression of Atrn protein and mRNA is related to the functional status of the cell. For example, seminiferous tubules have no lumens in the newborn (1 day) rat and are mainly composed of gonocytes and Sertoli cells. Strong mRNA staining was present mainly in gonocytes at this age. Spermatogonia emerged in the seminiferous tubules of the prepubertal (5 days) rat, and the expression of Atrn in spermatogonia became the strongest when compare to the other cells. This phenomenon was the same in the spermatocytes that emerged in the seminiferous tubules of the pubertal (20 days) rat and in spermatides that emerged in the seminiferous tubules of the postpubertal (50 days) rat.

Models by Huckins and Oakberg (13) and Huckins (14) of spermatogonial stem cells indicate that type A spermatogonia can be characterized into a reserve type of spermatogonial stem cell (AS), renewing spermatogonial stem cell (Apr-Aal-Aal), and differentiating spermatogonial stem cell (A1-A4, type B). This experiment did not sort type A spermatogonial stem cells into detailed types, but results showed that from puberty, the expression of Atrn was stronger in type A spermatogonia than type B, which suggests that Atrn may play a distinct role in the differentiation and proliferation of spermatogonia. Once type A spermatogonia had differentiated into type B spermatogonia, cell mitosis stopped, and the expression of Atrn was reduced or even disappeared. The expression of Atrn protein and mRNA was found to be strongly expressed in pachytene spermatocytes, which was demonstrated by the experimental phenomenon of significantly increased brown particles when compared to other cells. This suggests that, upon the action of pituitary gonadotropin, spermatogenic cells continuously proliferated and differentiated. Atrn may promote the meiosis of spermatocytes which leads to the differentiation of spermatocytes into spermatids and ultimately into spermatozoa.

The present study confirmed that Atrn protein and mRNA is widely distributed in the testes of rats at different ages of maturation and suggests that Atrn may take part in the physiological function and differentiation of germ cells. Thus, investigation of the function of Atrn in the male reproductive system in future preclinical and clinical studies is warranted.

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