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Demonstration of Follicle-Stimulating Hormone Receptor in Cauda Epididymis of Rat[1]

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ABSTRACT

FSH receptor has been shown to be specifically expressed only in the Sertoli cells in males. In one of our studies that consisted of deprival of endogenous FSH in immature rats and adult bonnet monkeys, atrophy of the epididymis was observed, cauda region being the most affected. Although epididymis is an androgen-dependent tissue, the changes in histology of the cauda region were observed without any associated change in the levels of testosterone in FSH-deprived animals. Considering this, it was of interest to evaluate the possibility of epididymis being a direct target for FSH action. In the present study, we have examined the expression of FSH receptor in the epididymis of rat and monkey. In the cauda region of rat epididymis, FSH receptor expression was demonstrated by RT-PCR and Northern and Western blot analyses. FSH receptor was found to be functional as observed by its ability to bind ^{125}IoFSH, by an increase in cAMP production, and by BrdU incorporation following addition of FSH under in vitro conditions. These results suggest the possibility of a role for FSH in regulating the growth of the epididymis.

epididymis, follicle-stimulating hormone, follicle-stimulating hormone receptor, male reproductive tract

INTRODUCTION

FSH plays an important role in regulation of mammalian reproduction by interacting with its specific receptor [1, 2]. FSH receptor (FSHR) belongs to the superfamiliy of G-protein-coupled receptors (GPCRs). GPCRs have a large ligand binding extracellular domain (ECD), seven transmembrane helices, and a short intracellular domain for activation of the effectors [3]. FSHR activation initiates a cascade of intracellular events leading to modification of cellular response and receptor desensitization [4]. It has been well established that FSHR, like several other GPCRs, act via the cAMP pathway. However, recent studies have shown that action of FSH on its receptor also activates the Ca^{2+} pathway [5]. Studies using Sertoli cells and granulosa cells have revealed the existence of alternatively spliced variants of *Fshr* having varied roles [6, 7].

Although it was believed that the expression of FSHR is restricted to only Sertoli cells in males [8, 9], the presence of FSHR in metastatic and prostate cancer cell line PC3 and DU145 has been reported. As in the case of Sertoli cells, in which FSH has an important role in proliferation, FSH triggered a proliferative response in prostate cancer cells [10–14]. It is to be noted that FSH is important for the proliferation of Sertoli cells during the fetal and the early neonatal period [15], which is until Day 18 in the case of rats [16]. However, during the adult stages in the rat, FSH regulates only the functional parameters in Sertoli cells for providing the microenvironment for developing sperms. In response to FSH, Sertoli cells synthesize and secrete a variety of proteins that are essential for spermatogenesis and maintenance of normal sperm production in the adult [4]. This critical function of the Sertoli cells of providing support for the differentiation of germ cells [17] is regulated by FSH as well as testosterone, which is produced by the Leydig cells in response to LH.

In a study that involved elucidating the mechanism of differential action of FSH on Sertoli cells by employing specific antiserum to neutralize endogenous FSH in rats, we observed significant changes in the histology of different regions of epididymis (unpublished observations). Although epididymis is an androgen-dependent tissue [18–21], the possibility of the observed effects in epididymis being due to deprival of testosterone was ruled out by the fact that there was no change in the level of serum testosterone. This suggested the possibility that epididymis could be a direct target for FSH action. In the present study, we demonstrate the presence of receptor for FSH in the cauda region of the epididymis and evaluate its significance.

MATERIALS AND METHODS

Animals and Treatment

Seven-day-old male Wistar rats were obtained from the Central Animal Facility, Indian Institute of Science, Bangalore, India, and maintained under standard conditions (12L:12D schedule, with water and pelleted food ad libitum). The animal procedures employed in the study have been approved by the Institutional Ethical Committee.

Antiserum to highly purified ovine FSH (oFSH) free of LH contamination was raised in adult male bonnet monkey (*Maccaca radiata*). The absence of contaminating antibodies to LH was established by the lack of binding to ^{125}IhCG and the inability of the antiserum to inhibit the LH-stimulated testosterone production by the rat Leydig cells. The effect of antiserum to neutralize endogenous FSH was established in immature male rats. Normal monkey serum (NMS)-administered rats served as controls [22].

Semiquantitative RT-PCR

Total RNA was extracted from cells or tissues from either rat or monkey using TRI reagent (Sigma) [23]. Following DNase (Amersham Pharmacia Biotech) treatment, 3 μg of total RNA were subjected to reverse transcription using random hexamers (Roche Molecular Biochemicals) and MMLV-RT (Amersham) in a 20-μl reaction mixture. PCR amplification was carried out using a 2-μl aliquot of cDNA and specific primers in a 25-μl reaction volume. A control reaction without reverse transcriptase was included for every sample

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TABLE 1. RT-PCR details for primers used in this study.

Primer[a]	Primer sequence (5′- 3′)	Annealing temp. (°C)	Product size (bp)	Reference no.	Species
Fshr ECD1	FP-134-GGCGGATCCCTGTGTCATTGCTCTAATAGG RP-1079-GGCGGATCCCTGTGTCATTGCTCTAATAGG	55	945	[48]	Rat
Fshr ECD2[b]	FP-134-GGCGGATCCCTGTGTCATTGCTCTAATAGG RP-497-GGCGAATTCTTACTGGATCTTGTGAACAGCTGG	55	363	[48]	Rat
Fshr ECD3[b]	FP-720-GGCGGATCCTCTGGGCCAGTCMTTTAGAT RP-1079-GGCGGATCCCTGTGTCATTGCTCTAATAGG	54	359	[48]	Rat
Fshr TM-ICD	FP-1841-GCCATCTCTGCCTCCCTCAAGGTG RP-2077-GCTCTTTCGGGCATGGAAGTTGTG	62	236	Novel	Rat/ mouse/ monkey/ human
Androgen receptor	FP-618-GAGGTAATATCCGAAGGCAGCA RP-1067-CTTATACAGAGACAGTGAGGAC	58	449	Novel	Rat
Cyclophilin	FP-263-GTGGCAAGTCCATCTACG RP-644-CAGTGAGAGCAGAGATTACA	55	381	[22]	Rat

[a] Primers were synthesized by Microsynth GmbH, Switzerland.
[b] *Fshr* ECD2 & 3 are the reamplified products of the first set of *Fshr* ECD1 primers.

of RNA isolated to verify absence of genomic DNA contamination. A 20-μl aliquot of the PCR product was electrophoresed on a 1.5% agarose (Amersham) gel containing ethidium bromide (Sigma). Cyclophillin was used as an internal control. The sequences of the primers used in the present study are given in Table 1.

The authenticity of all the RT-PCR products described in this study was confirmed by sequencing (data not presented) using the ABI Prism 377 automated DNA sequencer, following the purification of amplicon from agarose (Amersham) gels using a gel purification kit (Qiagen).

Northern Blot Analysis

Poly-A RNA was purified using an mRNA purification kit (Qiagen) from total RNA isolated from Sertoli cells and the cauda epididymis. Northern blot analysis was carried out by resolving 30 μg (cauda) and 10 μg (Sertoli cells) of poly-A RNA on 1% MOPS-formaldehyde gel followed by transferring the RNA on the nylon membrane. Expression of the *Fshr* gene was analyzed using a probe corresponding to the ECD region of *Fshr*, which was labeled with α^{32}P-dCTP (NEN Life Science Products, Inc.; specific activity of label used: 3000 Ci/mmol) using DNA labeling kit (Amersham). Hybridization was carried out at 60°C in church buffer (1% BSA, 0.5 M $[Na]_2$ HPO_4, 7% SDS, 1 mM EDTA, pH 8.0) for 18–20 h. The blot was washed at 65°C twice in 2× SCC for 10 min followed by two washes in 0.2× SCC and 0.2% SDS for 15 min each. The blot was exposed to a PhosphorImager screen for detection of the signal. Labeled tags were used to mark the 18 S and 28 S rRNA.

Preparation of Protein Lysates and Western Blot Analysis

For Western blot analysis, all tissue samples were homogenized in ice-cold PBS (pH 7.4) containing Complete Protease Inhibitors (Roche Molecular Biochemicals; diluted according to the manufacturer's instructions) and according to previously described procedures [6]. Western blot analysis was carried out using standard protocol with an antibody raised in monkey corresponding to the three overlapping decapeptides of the ECD region of FSHR corresponding to 18–27, 25–34, and 29–38 a.a. expressed in filamentous phage or the antibody against the phage coat [24] at a dilution of 1:100. Bound antibody was visualized using an enhanced chemiluminescence ECL kit (Amersham) according to the manufacturer's instructions using ECL films (Amersham). Analysis was carried out using the Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120). Western blot analysis to determine the proliferative status of the cells was carried out using antibodies to proliferating cell nuclear antigen (PCNA) (Santa Cruz Inc.), and α-tubulin (Calbiochem) was used as internal control.

Isolation of Sertoli Cell from 10-Day-Old Rats

Sertoli cells were isolated by previously described protocol [22, 25]. Briefly, seminiferous tubules were digested in 0.125% trypsin (Sigma) and 10 μg/ml of DNase (Worthington) in 1× HBSS for 20 min. After three washes in 1× HBSS, the tubules were subjected to two enzymatic digestions with 1 mg/ml collagenase (Worthington) and DNase followed by 1 mg/ml collagenase, 1 mg/ml hyaluronidase (Sigma), and DNase for 30 min each. The cells were washed three times with 1× HBSS and pelleted down by centrifugation. The cells were resuspended in 1 ml of DMEM Ham-F12 (Sigma) and counted in a hemacytometer.

Isolation of Cells from Cauda and Culture

Cauda was excised from 40-day-old rats and minced in sterile medium. The minces were subjected to collagenase digestion (1 mg/ml) in medium containing 10 μg/ml DNase for 30 min. The enzyme activity was arrested by diluting with ice-cold medium. Following extensive washes, the cellular clumps were subjected to a second collagenase digestion (1 mg/ml along with 10 μg/ml DNase) for 30 min. The cells were centrifuged and washed in medium. The cells were incubated in DMEM Ham-F12 at 34°C in the presence of 5% CO_2 for 24 h. The epithelial cells were purified from the fibroblast cells, and the cells were characterized by alkaline phosphatase staining, a specific marker of fibroblast cells. The two cell types from the cauda epididymis were used for determining the expression of *Fshr*.

^{125}IoFSH Binding Assay

For binding assay, iodination of 5 μg of oFSH was carried out with 50 μCi of ^{125}I-Na by the iodogen method. Cauda tissue from 10- and 80-day-old rat was minced in cold RRA buffer (50 mM Tris-Cl and 10 mM $MgCl_2$). Seminiferous tubules and brain were taken from 5- and 10-day-old rats, respectively. The samples were weighed and subjected to homogenization in the presence of 1× complete protease inhibitors in a dounce homogenizer using 10 strokes with dounce A and six strokes with dounce B. Different quantities of tissue samples were incubated with 100 000 cpm of ^{125}I-oFSH (specific activity 83.9 μCi/μg oFSH). The binding assay was carried out as per previously published protocol [6].

cAMP Production Assay

Production of cAMP was monitored using the cAMP Enzyme Immunoassay Biotrak (EIA) system as per the manufacturer's instructions (Amersham). Samples were initially incubated with 100 mM isobutyl-methyl xanthene (Sigma) for 30 min to inhibit the phosphodiesterase activity and then stimulated either with vehicle or with different concentrations of purified oFSH in medium for another 20 min.

Immunohistochemistry

For immunohistochemical analysis, the epididymides from immature (5-day-old) and adult (80-day-old) rats were fixed in Bouin fluid overnight and processed for paraffin embedding. The sections on the glass slides were dehydrated in gradients of alcohol into water. The slides were pretreated in PBS containing 0.1% Triton X-100 (PBST). Nonspecific binding was blocked by incubating the slides in PBST containing 10% normal goat serum and 2% BSA (blocking buffer) for 1 h. The incubation with the primary antibody or serum against phage coat was carried out in blocking buffer containing FSHR antibody at a dilution of 1:50. The FSHR antibody was raised in monkey against the three overlapping decapeptides of the ECD region of FSHR corresponding to 18–27, 25–34, and 29–38 a.a. expressed in filamentous phage. Following overnight incubation with the primary antibody in a humidified chamber, the slides were washed in PBST three times for 10 min each. Incubation with the secondary antibody was carried out at a concentration of 1:400 in blocking buffer for 1 h. The slides were washed three times in PBST to remove nonspecific binding. Detection of the antibody was carried out using the DAB Substrate Kit (Pierce Biotechnology) as per the manufacturer's protocol. Reaction was arrested by adding PBS.

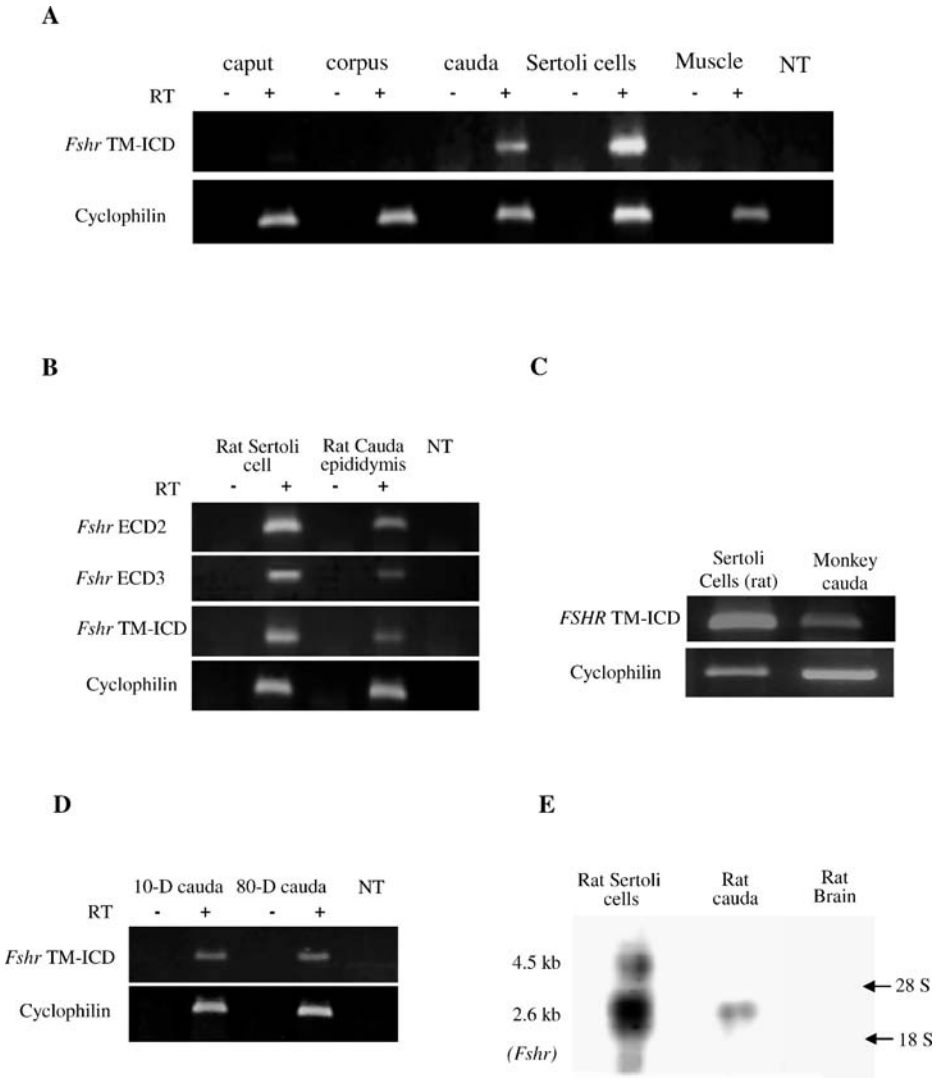

FIG. 1. Expression of *Fshr* in cauda. **A**) RT-PCR analysis for *Fshr* using RNA isolated from the caput, corpus, and cauda region of rat epididymis. Amplification was observed only in the RNA from the cauda region. Sertoli cells and muscle tissue were included as positive and negative controls, respectively. Absence of nonspecific amplification was confirmed by including –RT (no RT enzyme) and NT (no template) controls. **B**) Three micrograms of total RNA isolated from epididymis and Sertoli cells of rat were subjected to RT-PCR using specific primers corresponding to the extracellular domain (ECD), transmembrane domain (TM), and intracellular domain (ICD) region of rat *Fshr*. A positive signal was observed in cauda, corresponding to that seen in the rat Sertoli cells. **C**) RT-PCR analysis for *FSHR* expression in monkey cauda epididymis. Total RNA isolated from cauda of monkey gave amplification when subjected to RT-PCR using specific primers for *FSHR*. **D**) Analysis for the expression of *Fshr* FSH-R in the cauda of different-age rats. RT-PCR analysis carried out using RNA isolated from the 10-day (10-D) and 80-day (80-D)-old rat cauda epididymis. The expression of FSH-R mRNA was observed to be the same during the two stages studied. **E**) Northern blot analysis. Thirty micrograms of poly-A RNA from rat cauda and 10 μg of poly-A RNA from rat Sertoli cells were subjected to Northern blot analysis using a probe corresponding to the ECD region of rat Sertoli cell *Fshr*. Two transcripts of 2.6 and 4.5 kb were observed in RNA from rat Sertoli cells. Only one transcript corresponding to the 2.6-kb transcript was observed in RNA from rat cauda. The figures are representative of at least three independent experiments.

Proliferation Assay

The incorporation of BrdU (5-bromodeoxyuridine) by the cauda cells or Sertoli cells was used as a measure of proliferative activity. Sertoli cells were isolated from 10-day-old rats. Cauda cells were isolated from 10- and 80-day-old rats. Fifty thousand cells were cultured per well in quadruplets in a 96-well culture plate. Each group was treated with different concentrations of either oFSH, hCG (oFSH and hCG were kind gifts from Dr. M.R. Sairam, IRCM), testosterone (Sigma), or vehicle. The cells were cultured in serum-free medium (DMEM Ham-F12) containing 10 μg/ml transferrin (Sigma) and 5 μg/ml insulin (Sigma) for 16 h at 34°C in 5% CO_2. The cells were then incubated in the presence of 10 μM of BrdU for 4 h. The BrdU incorporation was assessed using Cell Proliferation ELISA Biotrak system as per the manufacturer's protocol (Amersham).

Statistical Analysis

Data are represented as mean ± SEM of at least three separate experiments performed with the same treatment protocol. For statistical comparison among groups involving only two columns, significance was evaluated using the unpaired two-tailed *t*-test. For comparison of data making up three or more columns, statistical significance was evaluated using the two-way ANOVA test. In both instances, a *P*-value # 0.05 was considered statistically significant.

RESULTS

RT-PCR Analysis for the Expression of Fshr *in Epididymis*

RT-PCR analysis for *Fshr* was carried out using specific primer sets corresponding to the different regions of *Fshr* (Table 1). RT-PCR analysis gave an amplification product of expected size from the cauda region of rat epididymis that corresponded to the amplification product seen in the Sertoli cells (Fig. 1A). Muscle tissue, which was used as negative control, did not give any amplification for *Fshr*. RT-PCR analysis using various primer sets corresponding to different regions of *Fshr* gave amplification both in the Sertoli cells and in the cauda of rat (Fig. 1B). RT-PCR analysis using cDNA from monkey cauda also revealed an amplification product corresponding to *FSHR* (Fig. 1C). The identity of the amplified product as *Fshr*/*FSHR* was ascertained by sequencing. The expression of *Fshr*/*FSHR* was very low in the cauda as compared to that in Sertoli cells from both rat and monkey. The ontological analysis of *Fshr* revealed that there was no change in the expression as analyzed in 10- and 80-day-old rat cauda epididymis (Fig. 1D).

Northern Blot Analysis for the Expression of Fshr *in Epididymis*

Northern blot analysis revealed two transcripts of *Fshr* in immature rat Sertoli cells corresponding to 2.6 and 4.5 kb and was in agreement with the sizes reported previously [26]. The mRNA from cauda gave a signal corresponding to the 2.6-kb transcript of *Fshr*, and no signal was observed with an equal quantity of brain mRNA used as negative control (Fig. 1E).

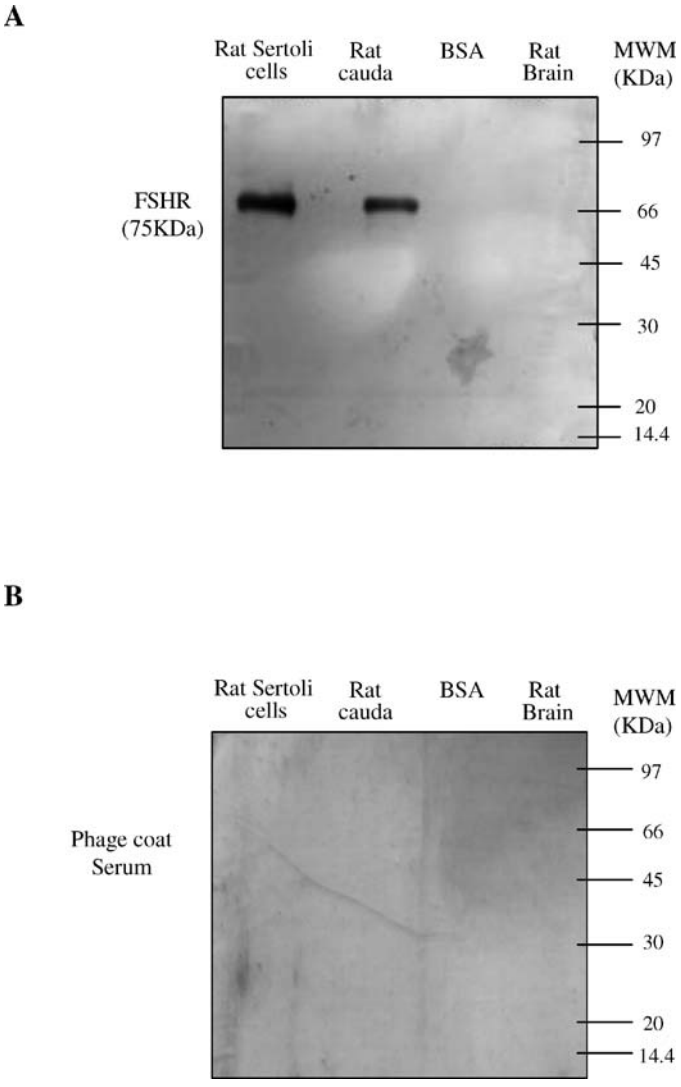

FIG. 2. Western blot analysis. **A**) Solubilized membrane preparation of rat Sertoli cells (100 μg), rat cauda (200 μg), and rat brain (200 μg) were subjected to Western blot analysis using antibody raised in monkey against the three overlapping decapeptides of the ECD region of FSHR corresponding to 18–27, 25–34, and 29–38 a.a. expressed in filamentous phage. BSA (10 μg) was included to determine the nonspecific binding. A band corresponding to 75 kDa was observed in the cauda and Sertoli cells from rat. (Abbreviations are provided in text.) **B**) Western blot analysis using solubilized membrane preparation of rat Sertoli cells, cauda, and brain, and BSA using the antibody against the phage coat raised in adult monkey did not reveal any signal. The figures are representative of at least three independent experiments.

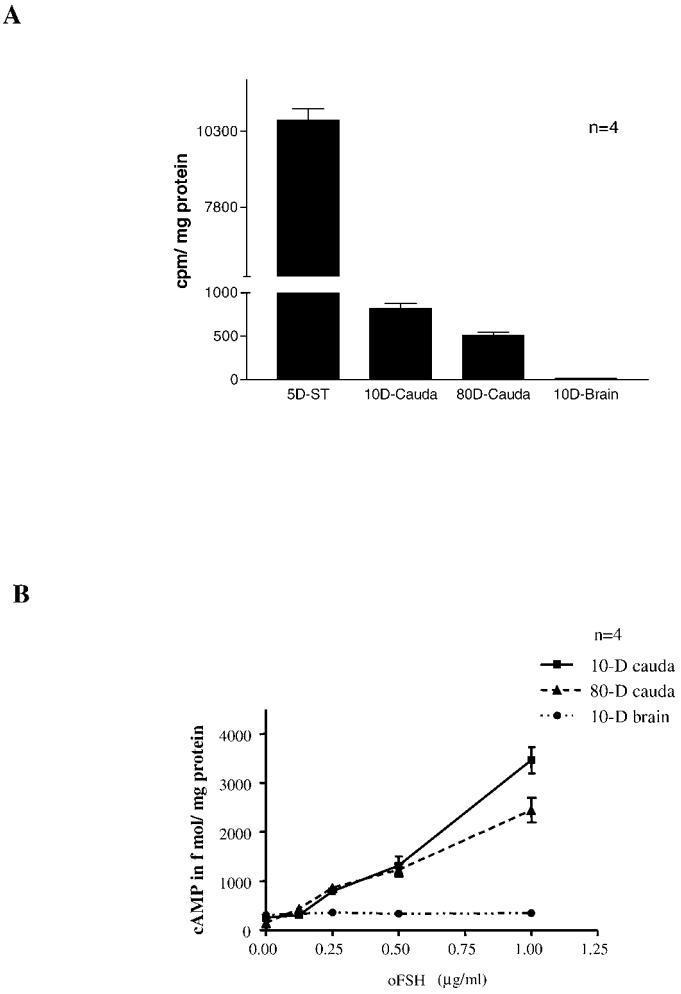

FIG. 3. Functional significance of FSHR. **A**) Receptor binding assay. Seminiferous tubules (ST) from 5-day (5-D)-old rat, cauda from 10-day (10-D) and 80-day (80-D)-old rat, and brain from 10-day-old rat were homogenized in dounce homogenizer and incubated with ^{125}I-oFSH (100 000 cpm). The specific binding was calculated by subtracting the nonspecific binding in the presence of an excess of unlabeled oFSH. Specific binding was observed with seminiferous tubules and 10- and 80-day-old rat cauda extracts. No binding was observed even with as much as 100 mg of brain protein. **B**) Stimulation in cAMP production. Cauda extract from 10-day (10-D) and 80-day (80-D)-old rat when stimulated with 0.125, 0.25, 0.5, and 1 μg/ml of oFSH revealed a concentration-dependent increase in the stimulation of cAMP production above the controls. Production of cAMP was more from 10-day-old rat cauda compared to the 80-day-old cauda at the concentration of 1 μg/ml of oFSH. There was no increase in cAMP production from 10-day-old rat brain extract stimulated with as much as 1 μg/ml of oFSH. All samples were preincubated with IBMX before stimulation with oFSH. Values represent mean ± SEM from four independent experiments.

The level of expression of *Fshr* in the cauda epididymis was very much lower than in the Sertoli cells (Fig. 1E).

Western Blot Analysis for the Expression of FSHR in Epididymis

Western blot analysis using the solubilized membrane preparation from cauda and Sertoli cells revealed a band of 75 kDa [2], and no signal was seen with brain and BSA (Fig. 2A). Western blot analysis carried out with phage coat serum did not give any signal with the membrane preparations of either Sertoli cells or cauda (Fig. 2B), thus establishing the specificity of the antiserum employed.

Binding Analysis of Cauda Epididymis to ^{125}I-oFSH

Specific binding of ^{125}I-oFSH by the cauda extract from 10- and 80-day-old rat was observed, and no binding was seen with brain extracts (Fig. 3A).

cAMP Production Assay

The action of FSH is known to be mediated by cAMP, which acts as its second messenger in the Sertoli cells [27] and granulosa cells. Following addition of oFSH, an increase in cAMP levels in cauda from both immature and adult rats (Fig.

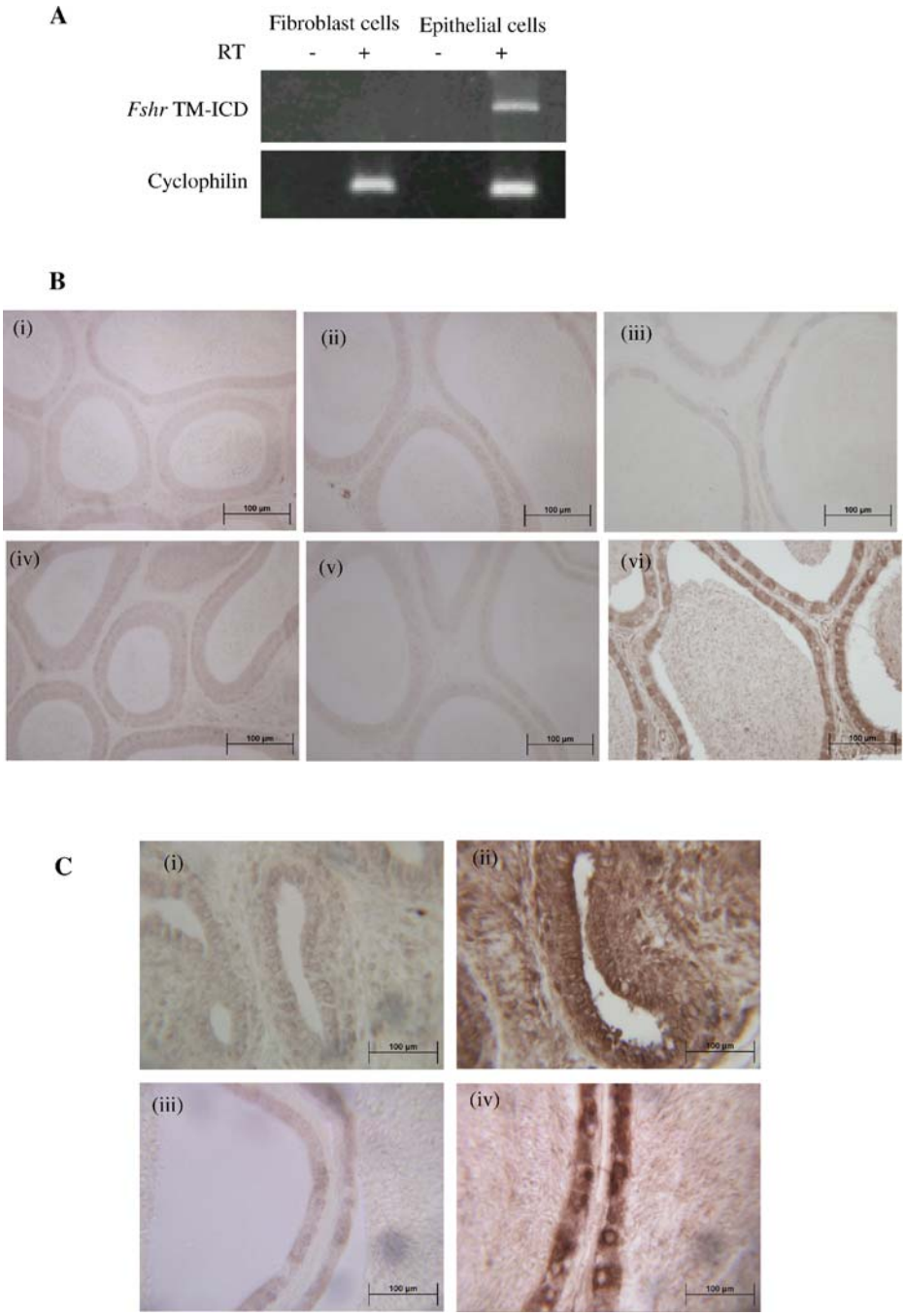

FIG. 4. Localization of FSHR expression in rat cauda. **A**) RT-PCR analysis for *Fshr* in fibroblast cells and epithelial cells isolated from rat cauda revealed the presence of signal for *Fshr* only in the epithelial cells. Absence of nonspecific amplification was confirmed by including –RT (no RT enzyme) and NT (no template) controls. **B**) Immunohistochemical localization of FSHR in the adult rat (80-day-old) epididymis using antibody raised against the three overlapping peptides from the ECD region of the oFSHR, which was expressed in filamentous phage in adult bonnet monkey. Images (i), (ii), and (iii) represent the sections of caput, corpus, and cauda, respectively, probed with serum of the monkeys immunized only with the phage coat. Images (iv), (v), and (vi) represent the sections from caput, corpus, and cauda, respectively, probed with FSHR antibody. The images were taken at ×40 magnification. **C**) Immunolocalization of FSHR in the immature (5-day) and adult (80-day) rats revealed intense staining on the membrane of the epithelial cells of cauda of immature rats. The images were taken at ×100 magnification. The figures are representative of at least three independent experiments.

3B) was observed, although the fold stimulation was much lower than seen with Sertoli cells, which were included as positive controls.

Localization of FSHR in Rat Epididymis

In order to determine the cells in which *Fshr* is expressed in cauda, RNA was isolated from the epithelial cells and the fibroblast cells of cauda, and RT-PCR analysis was carried out using specific primers for *Fshr*. It was observed that only RNA from the epithelial cells of rat cauda gave amplification of *Fshr* (Fig. 4A), and no signal for *Fshr* was seen with RNA from the fibroblast cells.

The localization of FSHR in rat epididymis was also demonstrated by immunohistochemistry using antibody raised in monkey against the three overlapping peptides of FSHR expressed in the filamentous phage. Sections obtained from the caput, corpus, and cauda of rat epididymis were probed either with phage coat serum of monkey or with FSHR antibody. Intense staining was observed with the cauda probed with FSHR antibody, whereas no staining was observed with the cauda probed with normal serum, suggesting that the staining is specific (Fig. 4B). The specificity of the FSHR staining in cauda was also validated by the absence of staining in caput and corpus when probed either with FSHR antibody or with normal serum (Fig. 4B). These results validate our observation that FSHR is expressed only in the cauda region of epididymis. Furthermore, the localization of FSHR on the membrane of the epithelial cells was more intense in the cauda from immature rats compared to the adult rats (Fig. 4C).

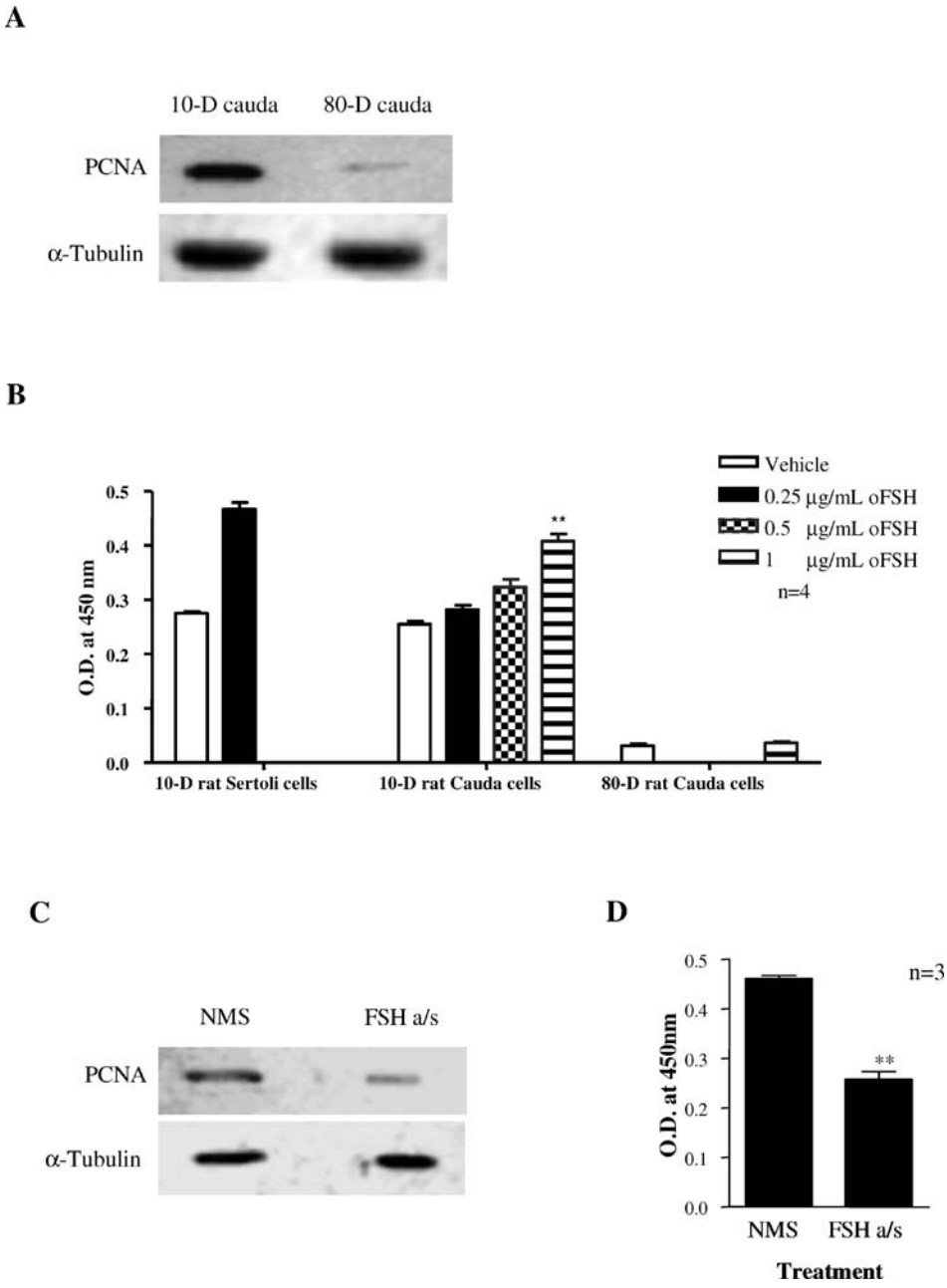

FIG. 5. Role of FSH in proliferation of cauda cells. **A**) Analysis for the maximum proliferative stage of cauda. Western blot analysis was carried out for PCNA, a cell proliferation marker, in the 10-day (10-D) and 80-day (80-D)-old rat cauda. The expression for PCNA was observed to be high in 10-day-old rat cauda. **B**) BrdU incorporation assay. Fifty thousand cauda and Sertoli cells were cultured in quadruplets in 96-well culture plates. A concentration-dependent increase in the proliferation of 10-day (10-D)-old rat cauda cells was observed in the presence of oFSH. However, no stimulation in proliferation of 80-day (80-D)-old rat cauda cells was observed even in the presence of 1 μg/ml of oFSH. The stimulation in proliferation of 10-day (10-D)-old rat Sertoli cells was included as a positive control. **C**) Effect of FSH deprival on proliferation of cauda. Western blot analysis was carried out using protein samples from cauda of immature rats treated with FSH a/s and NMS. Expression of PCNA was downregulated following deprival of FSH. **D**) BrDU incorporation assay in the FSH a/s- and NMS-treated immature rat cauda cells revealed that the proliferation of the cauda cells was decreased following neutralization of endogenous FSH. The figures are representative of at least three independent experiments. Values represent mean ± SEM of at least three independent experiments.

Proliferation of Cauda Cells by FSH and Testosterone

Since it is known that FSH stimulates proliferation of Sertoli cells [15], the possibility of such an effect by FSH in cauda cells was also examined. Initially, the maximum proliferative stage of cauda was determined by Western blot analysis for PCNA. It was observed that the level of expression of PCNA was maximal on Day 10 and decreased by Days 20 and 40. The expression of PCNA was very low in 80-day-old rat cauda (Fig. 5A), indicating that cauda cells are highly proliferative during the early neonatal period. BrdU incorporation analysis revealed that proliferation was high in the 10-day-old rat cauda cells compared to the 80-day-old rat cauda cells (Fig. 5B). Following addition of FSH, a concentration-dependent increase in the proliferation of 10-day-old rat cauda cells was observed. However, no stimulation in proliferation of 80-day-old rat cauda cells was observed even with 1 μg/ml of oFSH (Fig. 5B).

The role of FSH in the regulation of proliferation of immature rat cauda was validated by in vivo studies. FSH was neutralized in the immature rats from Day 7 until Day 13 of age, and, as expected, deprival of FSH resulted in a decrease in the expression of PCNA in the cauda epididymis (Fig. 5C). It was observed that following neutralization of endogenous FSH, there was a decrease in the incorporation of BrdU by immature rat cauda cells (Fig. 5D). This agrees with the decrease in PCNA, and these results establish that proliferation was decreased in cauda following neutralization of FSH.

As it is well known that epididymis is an androgen-regulated tissue [18, 28], it was of interest to evaluate the involvement of androgens in the proliferation of immature rat cauda cells. Analysis for expression of mRNA for androgen receptor in the immature and adult rat cauda cells by RT-PCR revealed that the expression was quite low in the 10-day-old rat cauda cells compared to the 80-day-old rat cauda cells (Fig. 6A). It was also observed that neither hCG nor testosterone was

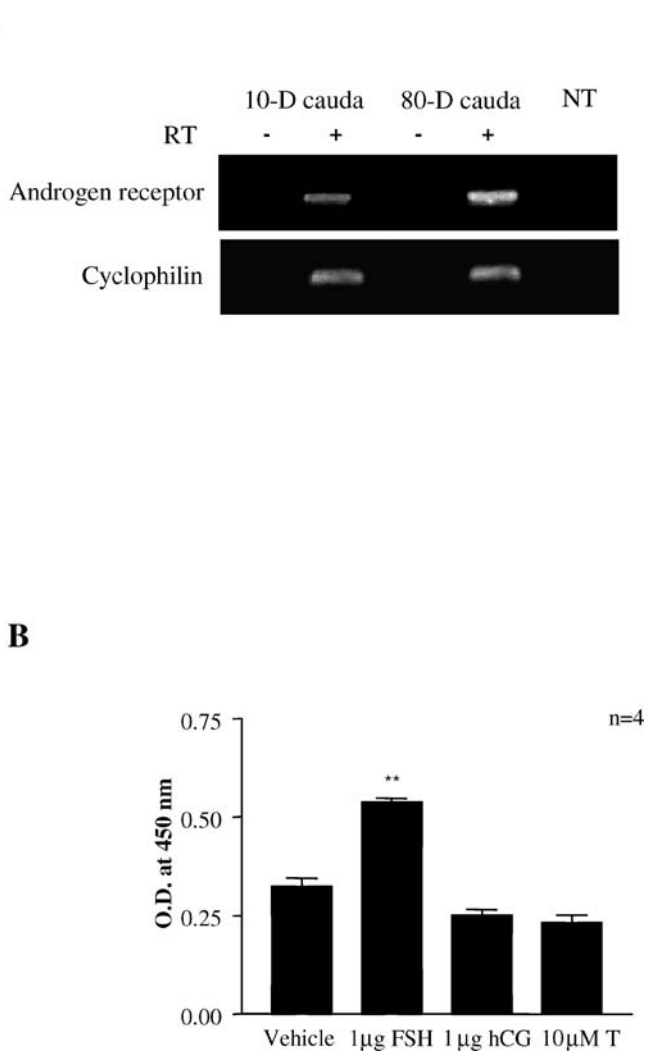

FIG. 6. **A**) RT-PCR analysis for the expression of androgen receptor in the 10-day (10-D) and 80-day (80-D)-old rat cauda epididymis. The expression was quite low during the immature stage, while the adult cauda expressed high levels of androgen receptors. Absence of nonspecific amplification was confirmed by including –RT (no RT enzyme) and NT (no template) controls. **B**) Fifty thousand cauda cells were cultured in quadruplets in 96-well culture plates. The proliferation in the cauda was determined by stimulating the cells with 1 µg/ml oFSH or 1 µg/ml hCG or 10 µM testosterone (T) or vehicle. Testosterone and hCG did not stimulate the proliferation of cauda cells in vitro. Values represent mean ± SEM from four independent experiments.

able to stimulate the proliferation of cauda cells from 10-day-old rats (Fig. 6B).

DISCUSSION

Our observations that there was a decrease in weight along with a change in the histology of rat epididymis following neutralization of endogenous FSH suggested the possibility that epididymis could be a target for FSH action. The conclusion is supported by the fact that the change in epididymis histology was seen without any change in the concentration of serum testosterone (data not presented), which is one of the important hormones required for the growth and function of epididymis [29]. As a first step toward this, we have been able to establish the expression of *Fshr* in the cauda region of epididymis of both rat and monkey by RT-PCR. The caput and corpus did not express *Fshr* as analyzed by RT-PCR (Fig. 1A). The presence of *Fshr* in the cauda region in the rat was confirmed by Northern and Western blot analyses. Although two transcripts of mRNA to *Fshr* of 4.5 and 2.6 kb are reported in the Sertoli cells, we could demonstrate the presence of a smaller transcript in cauda. The failure to detect the 4.5-kb transcript may be due to the extremely low levels of expression, as double the quantity of poly-A^+ RNA from cauda had to be used for Northern analysis. In all the studies reported so far, an appropriate size signal for 2.6 and 4.5 kb could be detected by Northern blot only with poly-A^+ RNA from Sertoli cells [26, 30], which suggests that the level of expression is quite low, and this also decreases with age. In this respect, it should be noted that the level of the 4.5-kb transcript is very low even in Sertoli cells compared to the 2.6-kb transcript. Invariably, the level of expression of Fshr/FSHR is quite low both at the mRNA and at the protein level compared to the Sertoli cells, and this is also reflected in the extent of response to FSH by way of cAMP production or BrdU incorporation. Justifiably, the subdued response to FSH raises doubts about its physiological significance. However, the fact that it is highly specific in that neither hCG nor testosterone was able to stimulate proliferation (Fig. 6B) provides support to the conclusion that FSH has a role in the regulation of proliferation of cauda cells from immature rats. In this respect, it should be noted that the level of expression of receptor for androgen, which is known to be important for regulation of the growth and function of epididymis, is also quite low in the immature cauda compared to adult stages (Fig. 6A) and that the addition of testosterone did not stimulate BrdU incorporation (Fig. 6B).

The low level of FSHR expression in cauda and the subdued response suggest the possibility that the receptor may be of low affinity. However, there are several examples where the receptors are functional in spite of the fact that these are of low affinity. Thus, in the case of human placenta, although the receptors for GnRH are of low affinity compared to pituitary receptors, it is known that GnRH stimulates chorionic gonadotropin synthesis and secretion in both humans [31] and nonhuman primates. Studies have also demonstrated that the self-peptides that bind to diabetes-associated I-Ag7 with low affinity can induce tolerance in nonobese diabetic mice [32]. Low-affinity cholecystokinin receptors are able to inhibit agonist-induced (Ca^{2+}) oscillations in pancreatic acinar cells [33]. These studies indicate that even low-affinity receptors are capable of eliciting physiological responses.

The level of expression of *Fshr* varies with age in rat Sertoli cell; only in immature rat does FSH stimulate proliferation, whereas in adult rat Sertoli cells, FSH stimulates functional parameters. Although there is no discernible change in level of expression of message for *Fshr* with age in cauda (Fig. 1D), it is possible that FSH stimulates proliferation in caudal cell only from immature rat, indicating that in the adult rat, cauda FSH may have a different function. The proliferation status of cauda cells in the two age-groups, as determined by the level of expression of the PCNA, was quite low in adult compared to the immature cauda cells (Fig. 5A). Neutralization of endogenous FSH resulted in the decreased level of PCNA protein in the immature cauda (Fig. 5C), thus further validating the importance of FSH in the proliferation of cauda cells. It is also pertinent to note in this respect that we have demonstrated that LH exerts a differential role on Leydig cells, depending on the stage of differentiation of Leydig cells. Thus, in the case of Leydig cells from 18-day-old rats, LH stimulates proliferation, while in the case of Leydig cells from adult rats, only an increase in testosterone production can be observed without any effect on proliferation [34]. Furthermore, results of our preliminary study also revealed that the expression of FSHR in

cauda cells is subject to regulation by FSH, suggesting its functional significance (data not presented).

It should be noted that the cauda is the region where the storage and nourishment of sperm takes place [29, 35, 36]. Transit of sperms through the epididymis is an obligatory requirement under normal conditions for the acquisition of forward motility and fertilizing capacity. Recently, reversible infertility was induced in adult male bonnet monkey by active immunization against eppin [37], an epididymal sperm protein that may be involved in acquisition of motility. Although this is the first report on the presence of FSHR in the epididymis, change in the histology of epididymis from the FSHR knockout (FORKO) mice has been reported. In this respect, it is important to note that testosterone levels did not decrease until Day 49 postpartum in FORKO mice [38], indicating that the observed changes are not due to a decrease in the testosterone levels. In addition, studies have revealed that testosterone replacement failed to completely reverse the effects of castration on the epididymis weight of adult rats [39–41], indicating the involvement of factors other than testosterone in the regulation of epididymal function. Studies have revealed that although androgens are very important, other regulatory molecules are also involved in controlling the epididymal function [42–47]. Considering these facts, the present observation of expression of functional receptors for FSH in the rat cauda epididymis may be of significance.

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