

## Seasonal Changes in Immunoreactivity of Inhibin/Activin Subunits in the Epididymis of Wild Ground Squirrels (*Citellus dauricus* Brandt)

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**Abstract.** The inhibin/activin subunits ( $\alpha$ ,  $\beta_A$  and  $\beta_B$ ) have been found in epididymal tissue of many mammals, but there have been no data available for wild seasonal breeders so far. The aim of this study was to investigate the immunoreactivities of inhibin/activin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits in the epididymis of wild ground squirrels during the breeding and nonbreeding seasons. Immunohistochemistry and Western blotting were performed to detect the epididymal immunolocalizations and immunoreactivities of the three subunits. Strong immunostaining of  $\alpha$  subunit was present in the interstitial part of the caput epididymis and epithelial parts of the corpus epididymis and cauda epididymis during the breeding season, whereas no  $\alpha$  subunit was found in the nonbreeding season.  $\beta_A$  and  $\beta_B$  subunits were expressed in all cell types of the epithelium throughout the whole seasonal cycle, and immunostaining in the breeding season was likely stronger compared with that of the nonbreeding season. These results suggested that the epididymis might be a potential source of inhibin and activin in the wild male ground squirrel, and the secretion of epididymal inhibin and activin showed distinct seasonal changes. Furthermore, inhibin and activin might function as paracrine and/or autocrine factors that have an effect on the epididymis.

**Key words:** Epididymis, Immunoreactivity, Inhibin/activin subunits, Wild ground squirrel

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The mammalian epididymis is derived from the anterior Wolffian (or mesonephric) duct and serves a critical function in sperm maturation and acquisition of the capacity to fertilize [1, 2]. At birth, the epididymis consist mainly of the epididymal duct and interstitial tissue, and it must then undergo elongation, expansion, coiling and segmentation to morph from a straight tube to an elaborately convoluted organ [3]. As a main male accessory reproductive organ, the development of a fully differentiated epididymis is dependent not only on androgens but also requires a mesenchyme-epithelium interaction to determine the regional specialization of the epithelium [4, 5]. Thus some mesenchyme-derived factors involved in the mesenchyme-epithelium interaction were thought to have a functional role in this process.

A number of paracrine factors involved in the control of proliferation and differentiation of various types of cells have been identified, including members of the transforming growth factor  $\beta$  (TGF- $\beta$ ) family, which are essential for the development and functional control of the male reproductive system [6–9]. Activins and inhibins are members of the TGF- $\beta$  family, initially characterized by their ability to regulate FSH secretion from the pituitary [10, 11]. They are dimeric glycoproteins formed by two of three different subunits ( $\alpha$ ,  $\beta_A$  and  $\beta_B$ ). Inhibins consist of either of the  $\beta$ -subunits dimerized

with a common  $\alpha$ -subunit ( $\alpha$ - $\beta_A$  and  $\alpha$ - $\beta_B$ ; inhibin A and inhibin B, respectively). Activins are dimers of  $\beta$ -subunits ( $\beta_A$ - $\beta_A$ ,  $\beta_A$ - $\beta_B$  and  $\beta_B$ - $\beta_B$ ; activin A, activin AB and activin B, respectively). There is also evidence that inhibin and activin are not only expressed in the testis but also in other tissues of the male reproductive tract, e.g., the prostate, seminal vesicles and epididymis [12]. In addition, previous studies have shown the expression of mRNA and protein of inhibin/activin subunits in the epididymis of the mouse, monkey, ovine and human [5, 13–18]. Inhibin B was suggested to have a possible role in epididymal function or an effect on the epididymis parallel to that of testosterone [19]. Furthermore,  $\beta_A$  subunits were proven to be mesenchyme-specific factors that act collectively with testosterone to facilitate epididymal coiling by stimulating epithelial proliferation in the mouse [5].

The wild ground squirrel (*Citellus dauricus* Brandt) is a typical seasonal breeder with a short sexually active period in April and May that is followed by a long period of sexual dormancy from June to March [20]. The testis and epididymis of this species exhibits distinct seasonal morphology changes from the breeding season to the nonbreeding season [21–24]. Our published results have indicated that seasonal changes in the distribution of inhibin/activin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits and activin signaling proteins were accompanied by changes in testicular activity in male ground squirrels and other mammals [21, 22, 25–29]. However, little is known about the role of inhibin/activin subunits in the epididymal tissue of this wild species. Thus, the aim of the present study was to investigate the localization of inhibin/activin subunits in the epididymis during the breeding and nonbreeding seasons, and to elucidate the relationship between the

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immunoreactivity of inhibin/activin subunits and the epididymal function in the wild male ground squirrels.

## Materials and Methods

### Animals

Forty-six wild male ground squirrels (twenty six in the breeding season and twenty in the nonbreeding season) that were thought to be adults based on their body weights (general standard range of body weight for adult squirrel: 242–412 g) were captured in April to September (breeding season, April and May; nonbreeding season, June to September) of 2007 in Hebei Province, China [22, 23]. After anesthesia, all animals were euthanized by decapitation. The obtained epididymal tissues were fixed in Bouin's solution for histological and immunohistochemical observation or rapidly subdivided into three separate regions (caput, corpus and cauda), weighed and immediately stored at  $-80^{\circ}\text{C}$  until protein extraction. All procedures on animals were carried out in accordance with the Policy on the Care and Use of Animals of the Ethics Committee of Beijing Forestry University and approved by the Department of Agriculture of Hebei Province, China (JNZF11/2007).

### Histology

Epididymal samples were dehydrated in ethanol series and embedded in paraffin wax. Serial sections ( $4\ \mu\text{m}$ ) were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA). Some sections were stained with hematoxylin-eosin (HE) for observation of general histology. All sections in this study were assessed using an Olympus microscope (BX51, Olympus, Tokyo, Japan), digital camera (DS126181, Canon, Tokyo, Japan) and the Image-Pro Plus 6.0 image-analyzing system (Media Cybernetics, Rockville, MD, USA).

### Immunohistochemistry

The serial sections of epididymis were incubated with 10% normal goat serum to reduce nonspecific binding of primary antibodies and background staining caused by the secondary antibody. The sections were then incubated with primary antibody (1:2000) raised against porcine inhibin  $\alpha$  chain (1-30)- $\text{NH}_2$  conjugated to rabbit serum albumin, porcine inhibin/activin  $\beta_A$  (81-113)- $\text{NH}_2$  (#305-24D) and cyclic acetyl human inhibin/activin  $\beta_B$  (81-113)- $\text{NH}_2$  (#305-25D) [30] for 12 h at room temperature. The inhibin  $\alpha$  subunit peptide was kindly provided by Dr N Ling (Neuroendocrine, San Diego, CA, USA), and the antibodies of inhibin/activin ( $\beta_A$  and  $\beta_B$ ) were kindly provided by Dr W Vale (Salk Institute for Biological Studies, La Jolla, CA, USA). The specificity of these three antibodies in the wild ground squirrel has already been confirmed in our previous reports on testicular and ovarian tissues in this wild rodent species [21, 31]. The sections were then incubated with a secondary antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin<sup>TM</sup> staining kit (Sigma, St. Louis, MO, USA.), followed by visualization with 30 mg 3,3'-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 ml of 0.05 mol Tris-HCl  $1^{-1}$  buffer, pH 7.6, plus 30  $\mu\text{l}$   $\text{H}_2\text{O}_2$ . Finally, the reacted sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal goat serum (Sigma) instead of the primary antisera.

### Western blotting

Frozen epididymal tissues from both the breeding and the nonbreeding seasons were removed from storage at  $-80^{\circ}\text{C}$  and then used in the subsequent procedures together. Epididymal tissue was diced into small pieces using a clean razor blade. Tissue was homogenized in a homogenizer containing 300  $\mu\text{l}$  of 10 mg/ml phenylmethylsulfonyl fluoride (PMSF) stock and incubated on ice for 30 min, maintaining the temperature at  $4^{\circ}\text{C}$  throughout all procedures. Homogenates were centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Protein extracts were mixed with an equal volume of  $2 \times$  Laemmli's sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH=6.8). Equal amounts of each sample were loaded and run on a 12% SDS-PAGE gel at 18 V/cm and transferred to nitrocellulose membranes using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membranes were blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membranes was carried out using the same primary antibodies as described in the immunohistochemistry (dilution 1:1000 for all antibodies) for 60 min. Secondary incubation of the membrane was then carried out for 60 min using a 1:1000 dilution of goat anti-rabbit IgG tagged with horseradish peroxidase. Finally, the membrane was colored with 25 mg 3,3'-diaminobenzidine (Wako) solution in 25 ml TBS-T buffer (0.02 M Tris, 0.137 M NaCl, and 0.1% Tween-20, pH 7.6) plus 3  $\mu\text{l}$   $\text{H}_2\text{O}_2$ .  $\beta$ -actin was used as a constitutively expressed protein product for comparison of inhibin/activin subunit protein abundance between samples. The negative control was treated with normal goat serum (Sigma) instead of the primary antisera.

### Statistical analysis

Mean values ( $\pm$  SD) were calculated and analyzed using one-way ANOVA. Tukey's test was used for detection of significant differences using the SPSS computer package.

## Results

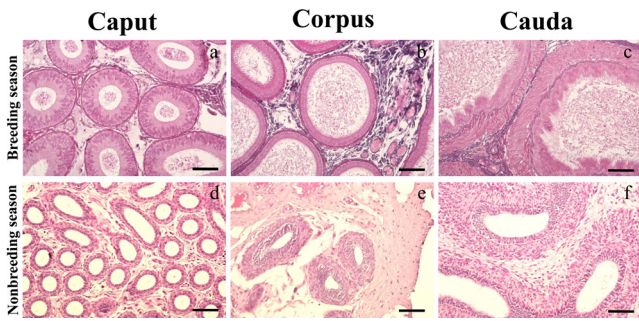
### Histology

Seasonal morphological changes in the epididymis of the wild male ground squirrel are shown in Fig. 1. Distinct morphological transformation was observed between the epididymis of the breeding season and that of the nonbreeding season. During the breeding season, there were larger epithelial cells and a wide epididymal lumen with abundant sperm. On the other hand, during the nonbreeding season, only small epithelial cells and a cramped epididymal lumen could be found; meanwhile, no sperm was observed in the epididymis. This result was consistent with those of our previous studies, which described in detail the seasonal changes in epididymal morphology [24]. The epithelial heights of the caput, corpus and cauda epididymis in the breeding and nonbreeding seasons are shown in Fig. 2. The mean height of the caput epididymis changed from 22.24  $\mu\text{m}$  (May; breeding season) to 5.46  $\mu\text{m}$  (July; nonbreeding season), and those of the corpus and cauda epididymis changed from 22.44  $\mu\text{m}$  to 6.18  $\mu\text{m}$  and 11.22  $\mu\text{m}$  to 8.12  $\mu\text{m}$ , respectively.

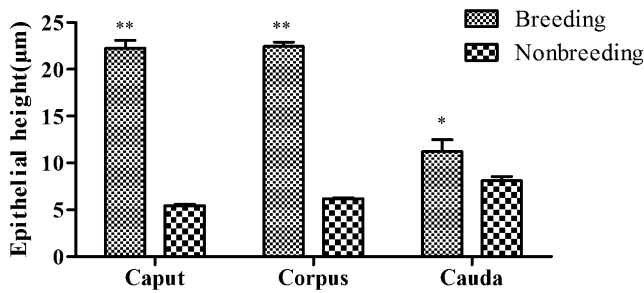
### Immunocytochemistry

Immunohistochemistry for inhibin/activin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits was performed in the epididymis of the breeding and nonbreeding



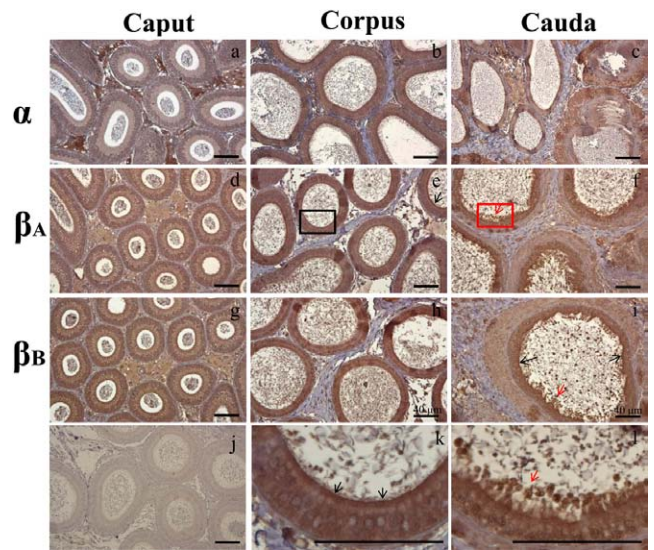


**Fig. 1.** Histology of the epididymis in the wild male ground squirrels in the breeding season (a, caput; b, corpus; c, cauda; May) and nonbreeding season (d, caput; e, corpus; f, cauda; July). Bar: 40  $\mu$ m.

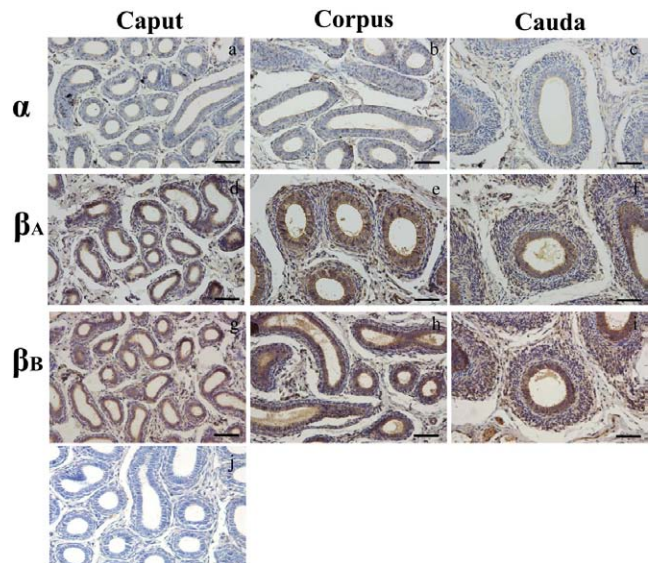


**Fig. 2.** The figure shows the epithelial heights of the caput, corpus and cauda epididymis in the breeding and nonbreeding seasons. Distinct changes were shown between the breeding and nonbreeding seasons. \* Significant ( $P < 0.05$ ). \*\* Very significant ( $P < 0.01$ ).

seasons, respectively. Most immunohistochemical signals were observed in the cytoplasm in both the breeding and nonbreeding seasons. Marked seasonal changes in immunolocalization of these proteins were observed between the breeding and nonbreeding seasons (Fig. 3 and Fig. 4). During the breeding season,  $\alpha$  subunit was immunolocalized intensely in interstitial cells of the caput epididymis (Fig. 3a). It was also detected in epithelial cells and myoid cells of the corpus and cauda epididymis (Fig. 3b and c). During the nonbreeding season, no immunostaining of  $\alpha$  subunit was observed in all segments of the epididymis (Fig. 4a–c). The  $\beta_A$  and  $\beta_B$  subunits had similar immunolocalization patterns during the breeding season. The most extensive immunostaining of  $\beta_A$  and  $\beta_B$  was present in epithelial cells throughout the whole epididymis. For the caput epididymis, the staining was mainly localized in the basal part of the epithelial cells, while stainings in the corpus and cauda epididymis were mainly detected in the apical part (Fig. 3k). In addition, the interstitial cells of the caput epididymis also showed a positive signal (Fig. 3d–i). Meanwhile, epididymosome-like structures of the cauda epididymis were immunostained for both subunits during the breeding season (Fig. 3f, i and l). During the nonbreeding season, the immunoreactivities of  $\beta_A$  and  $\beta_B$  subunits remained positive in the interstitial part and epithelial part. However, the intensity decreased, and the area of the positive signal was smaller



**Fig. 3.** Immunolocalization of the  $\alpha$  (a, b, c),  $\beta_A$  (d, e, f) and  $\beta_B$  (g, h, i) subunits in the epididymis of the wild male ground squirrel in the breeding season (a, d, g, caput; b, e, h, corpus; c, f, i, cauda). j: Negative control. k: Magnified view of the framed part in e. l: Magnified view of the framed part in f. The  $\alpha$  subunit was immunolocalized in interstitial cells of the caput epididymis and in epithelial cells and myoid cells of the corpus and cauda epididymis. The  $\beta_A$  and  $\beta_B$  subunits were present in epithelial cells throughout the epididymis, especially in the basal part of the caput epididymis and apical parts of the corpus and cauda epididymis (e, l, k, black arrows). Moreover, positive immunostaining was also found in epididymosome-like structures of the cauda epididymis (f, i and l, red arrows). Bar: 40  $\mu$ m.



**Fig. 4.** Immunolocalization of the  $\alpha$  (a, b, c),  $\beta_A$  (d, e, f) and  $\beta_B$  (g, h, i) subunits in the epididymis of the wild male ground squirrel in the nonbreeding season (a, d, g, caput; b, e, h, corpus; c, f, i, cauda). j: Negative control. No immunostaining of the  $\alpha$  subunit was observed in the epididymis. Positive signals of  $\beta_A$  and  $\beta_B$  were present in epithelial cells throughout the epididymis, but due to shrinkage of the cytoplasm, the signals are not as strong as those in the breeding season. Bar: 40  $\mu$ m.

when compared with the breeding season. (Fig. 4g–i). In the negative control, no signal was detected in epididymal tissues during the breeding and nonbreeding seasons (Figs. 3j and 4j).

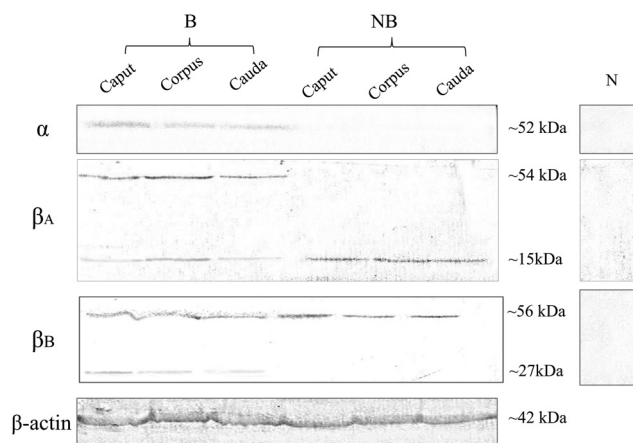
#### Western blotting

The results of Western blotting analysis for inhibin/activin subunits in the epididymis of the breeding and nonbreeding seasons are shown in Fig. 5. A strong positive signal of  $\alpha$  subunit was detected in protein extracted from the caput, corpus and cauda epididymis in the breeding season, while no signal was detected in the nonbreeding season. For the inhibin  $\alpha$  subunit antibody, a band was detected in the epididymis that migrated to a position of about 52 kDa, which was in accordance with our finding in the ovarian tissue [31]. Two bands of  $\beta_A$  subunit (15 kDa mature  $\beta_A$  subunit and 54 kDa free  $\beta_A$  subunit) were detected with strong positive signals throughout the whole epididymis in the breeding season. Only a weak signal was observed in the nonbreeding season. On the other hand, two strong positive signals of  $\beta_B$  subunit (27 kDa mature  $\beta_B$  subunit and 56 kDa free  $\beta_B$  subunit) were found in protein extracted from the caput to cauda epididymis in the breeding season, and only the immature form was found in the July sample. No signal was found in the negative control (lane N).

#### Discussion

The data from the present study extended our understanding of the role of inhibin/activin system in the epididymal recrudescence and regression process of the wild ground squirrel. The results presented here proved that the epididymal tissue was a potential cellular source of inhibin and activin in the wild ground squirrel, and the seasonal changes in the epididymal distribution of inhibin/activin subunits  $\alpha$ ,  $\beta_A$  and  $\beta_B$  were accompanied by changes in epididymal morphology.

This was the first study to identify the presence of inhibin/activin subunits in the epididymis of the wild ground squirrel. Although the inhibin/activin subunits have been localized mainly to the testis, activin subunits have also been found in a variety of locations, mainly the pituitary, brain, liver, kidney, pancreas and ovary [32]. In the present study, two activin subunits,  $\beta_A$  and  $\beta_B$ , were detected in the epididymis of wild ground squirrels during the breeding and nonbreeding seasons, while the inhibin  $\alpha$  subunit was only detected in the breeding season. These results showed that epididymal tissue might be able to assemble and secrete dimeric and bioactive activin and inhibin in the breeding season but only activin in the nonbreeding season. These findings are not unique in the wild ground squirrel, as the mRNA and protein of inhibin/activin subunits have also been identified in the epididymis of other species previously, such as the monkey [14], ovine [18], mouse [5] and human [13, 15–17]. In the monkey, inhibin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  mRNAs were detected in the epididymis by using *in situ* hybridization, with  $\beta_A$  mRNA being more strongly expressed in the caput than in other regions of the epididymis [14]. In the mouse, the *Inhba*, inhibin  $\beta_A$  gene, was expressed specifically in the mesenchyme of the anterior Wolffian duct at embryonic day 12.5 before the production of androgens [5]. In the human, different results have been reported by different laboratories. Phadke *et al.* showed the presence of inhibin-like molecules in different regions of the epididymis by using polyclonal antibodies generated against



**Fig. 5.** Western blotting results for the three inhibin/activin subunits ( $\alpha$ ,  $\beta_A$  and  $\beta_B$ ) and  $\beta$ -actin in the epididymis of the wild ground squirrel, respectively. On the right is the negative control (lane N). Total protein was extracted from different epididymal segments (caput, corpus, cauda) of the breeding season (B; May) and nonbreeding season (NB; July).

a 13 kDa human testicular inhibin [17]. On the other hand, the findings of Anderson *et al.* and Bahathiq *et al.* demonstrated that human fetal and adult epididymides were immunonegative for the inhibin  $\alpha$  subunit and immunopositive for both the activin  $\beta_A$  and  $\beta_B$  subunits [15, 16]. On the other hand, Roberts *et al.* showed that only a  $\beta_A$  subunit mRNA signal was present in the epididymis [13]. In addition, Nistal *et al.* recently reported that the epididymis was not responsible for the production of inhibin in men [33]. Although the presence of inhibin in the human epididymis is still debated, the expression of activin was confirmed. Taken together, the expression pattern of inhibin/activin subunits in epididymal tissue is species dependent. Considering our findings in the wild ground squirrel, the expression of inhibin/activin subunits also exhibits a region- and time-dependent manner, suggesting their active involvement in the seasonal changes of epididymal morphology and function.

In males, Sertoli cells were thought to be the major source of inhibin [34]. In addition to the feedback regulation of FSH, inhibin has many other functional roles in cellular growth, differentiation, steroidogenesis and neoplastic proliferation [33–37]. Besides, some extragonadal tissues have been shown to express inhibin, including the pituitary gland, placenta and bone marrow [17, 35]. However, the function of inhibin in the epididymis is still controversial. Some studies in humans and rats have shown that the epididymis was not the source of inhibin and that the proximal epididymis would reabsorb the inhibin produced by the testis [33, 38]. But other studies indicated that inhibin was detected in the epididymis and could have a role in acquisition of sperm fertilizing capabilities [17, 18, 39]. In the present study, the inhibin  $\alpha$  subunit was detected in the epididymis of the wild ground squirrels with a time- and region-specific pattern in which the interstitial part of the caput epididymis and epithelial part of the corpus and cauda epididymis were only expressed in the breeding season. Compared with studies of other species, the results here suggested that the epididymis was a potential source of inhibin in male wild ground squirrels and that inhibin may be involved



in the mesenchyme–epithelium interaction in the epididymis and serve as a paracrine and/or autocrine regulator(s) for epididymal functions (such as sperm maturation) during the breeding season. Moreover, the most distinctive seasonal changes in immunoreactivity of inhibin/activin subunits were found in the interstitial part of the caput epididymis where most epithelial coiling occurs. This might suggest a potential role for inhibin or activin [5].

In contrast to inhibin, gonadal activin was not the major source of activin in the male reproductive tract, and there have been several reports of activin subunit mRNA and protein expression in the prostate, seminal vesicles and epididymis [13, 35, 40–42]. In the present study, both the  $\beta_A$  and  $\beta_B$  subunits were detected in the epididymis of the wild ground squirrel, showing the possible secretion of dimeric and bioactive activin in epididymal tissues of this species. Different from the variable immunolocalization of the  $\alpha$  subunit, the  $\beta_A$  and  $\beta_B$  subunits were constantly expressed in epithelial cells, and the majority of immunoreactions were observed at apical parts of the cytoplasm in the corpus and cauda epididymis, which suggested that activin might be secreted into the tubal lumen from the epithelial cells. This finding was similar to that in the human epididymis, which was found to express the  $\beta_A$  subunit in the apical part of epithelial cells [15]. Previously, there have been some studies on the role of activin in the epididymis. Activin receptor IIB and phospho-SMAD2/3 were detected in the Wolffian (precursor of the epididymis) epithelium, suggesting that the activin pathway is activated and targeted to this tissue [5, 43]. Moreover, follistatin, the high-affinity binding protein for activin, was found in the epididymis, suggesting that the activin dimer probably acts in a paracrine or autocrine role rather than being secreted into the circulation, because follistatin would be highly bound to any free activin before entry into the plasma [15, 44]. Further study using the *Inhba*<sup>-/-</sup> mouse proved that the  $\beta_A$  subunit or its protein product activin A predisposed the anterior Wolffian duct epithelium to obtain the coiling phenotype and was responsible for the upregulation of the androgen receptor (AR) in the epididymal epithelium [5]. Here, our results showed strong immunoreactivity of the  $\beta_A$  and  $\beta_B$  subunits during the breeding season, as it decreased during the nonbreeding season. Meanwhile, these changes also accompanied marked seasonal changes in epididymal morphology. Therefore, it is reasonable to postulate that the  $\beta_A$  and  $\beta_B$  subunits or their protein product activin might exert local effects on changes in morphology and functional control of the epididymis in paracrine or autocrine manners. Besides, there is also evidence showing that activin might secrete into the tubal lumen and affect sperm maturation through action on sperm mitochondria in the breeding season [45].

In conclusion, this was the first study concerning the inhibin/activin  $\alpha$ ,  $\beta_A$  and  $\beta_B$  subunits in the epididymis of a seasonal breeding rodent. We showed that the inhibin and activin subunits were localized and synthesized by the epididymis, and the immunoreactivities of the inhibin and activin subunits changed from the breeding season to the nonbreeding season. Both inhibin and activin might exert local effects on epididymal morphology and function in a paracrine and/or autocrine manner(s). Further studies are needed to investigate the abundance and cellular localization of activin signaling components in the epididymis of the wild ground squirrel during the breeding and nonbreeding seasons to complete the basic foundation for understanding the significance of inhibin/activin signaling activity

in the epididymides of wild ground squirrels.

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