Mammal Study 35: 57–64 (2010)
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Immunohistochemical localization of the cytoskeletal proteins in the testes of the lesser mouse deer (*Tragulus javanicus*)

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Abstract. This study demonstrated the immunohistochemical localization of cytoskeletal proteins, cytokeratin, desmin, vimentin, α-tubulin and α-smooth muscle actin (α-SMA) in the testes of adult and immature lesser mouse deer (*Tragulus javanicus*). Desmin was found in the sub-epithelial myoid cells of epididymal ducts. The immunoreactivity for vimentin was shown in the peritubular myoid cells and around the nuclei of the Sertoli cells of which the positions indicated various arrangements. In the Sertoli cells, vimentin filaments between the basement membrane and the nucleus were elongated with the migration of the nucleus toward the upper part. Furthermore, vimentin was detected in the Leydig cells and the sub-epithelial myoid cells of epididymal ducts. Alpha-tubulin was apparently extended to the tip of each Sertoli cell cytoplasm, and α-SMA was found in the peritubular myoid cells and the sub-epithelial myoid cells of epididymal ducts. On the other hand, cytokeratin and desmin were not detected in the Sertoli cells, Leydig cells, spermatogenic cells and peritubular myoid cells. In the mouse deer testes, vimentin filaments of the Sertoli cells may not be involved in the migration of the elongated spermatids and spermiation like as rat Sertoli cells because the supranuclear extension of vimentin was scanty.

Key words: cytoskeletal proteins, lesser mouse deer, Sertoli cell, testis, vimentin.

The lesser mouse deer (*Tragulus javanicus*) belonging to the family Tragulidae (order Artiodactyla, suborder Ruminantia), are the smallest and the most primitive ungulates within existing ruminants (Dubost 1984; Chikuni et al. 1995; Nowak 1999; Breukelman et al. 2001; Meijaard and Groves 2004; Beck et al. 2006; Marcot 2007) and inhabit Southeast Asia including Thailand, Laos, Cambodia, Myanmar, Vietnam, Peninsular Malaysia and several islands such as Sumatra, Java and Borneo (Dubost 1984; Lekagul and McNeely 1988; Nowak 1999).

The mouse deer have an unpaired intermandibular gland under the chin, well-developed in male and used for an insistence of their territories or their sexual communications (Ralls et al. 1975; Davison 1980; Dubost 1984; Nowak 1999). It has been reported that the lesser mouse deer reach the sexual and physical maturity at about 5 months and have the gestation periods of 140–177 days (Tubb 1966; Dubost 1984; Lekagul and McNeely 1988; Nowak 1999; Francis 2001).

With regard to the genital organ of the mouse deer, several studies have been reported. Kimura et al. (2004) demonstrated histologically that the placenta of the mouse deer is a transitional type between diffuse epitheliochorial and polycotyledonary synepitheliochorial categories. In the mouse deer testes, the different patterns of lectin-bindings have been reported (Agungpriyono et al. 2009). Moreover, a multivesicular nuclear body

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(MNB) was observed in the nucleus of the mouse deer Sertoli cells same as other ruminants (Andriana et al. 2003a, 2005). In the Leydig cells, bundles of two types of filaments (actin and intermediate filaments) were ultrastracturally detected (Andriana et al. 2003b, 2005).

In the testes, the immunohistochemical localization of cytoskeletal proteins was investigated in many mammals (e.g. van Vorstenbosch et al. 1984; Steger and Wrobel 1994; Zhu et al. 1997; Komatsu et al. 1998; Sasaki et al. 1998; Rodríguez et al. 1999; Cruzana et al. 2006; Devkota et al. 2006). These studies revealed that the testicular localization of each cytoskeletal protein was different, and the distributional pattern was changed with testicular development and among species. However, any studies on the cytoskeletal proteins have not been reported about lesser mouse deer testes. In this study, therefore, we examined immunohistochemically the distribution of cytoskeletal proteins in the testes of the lesser mouse deer.

Materials and methods

Six testes with epididymides were obtained from male lesser mouse deer bred in a mouse deer farm in Kota Kinabalu, Malaysia (5°56'N, 116°3'E) (Table 1). The deer were kept under the natural environmental conditions (6–8 hours for monthly average sunshine duration; 30–32°C and 23–24°C for monthly maximum and minimum average temperature, respectively). Animals were sacrificed by an intraperitoneal overdose injection of ketamine hydrochloride. The testes were immediately fixed in Bouin's fluid or 10% formalin. After 24 h, the samples were transferred to 70% ethanol, then dehydrated in graded series of ethanol, cleared in xylene, and embedded in paraffin (Paraplast Plus, Kendall, MA, USA). Tissue samples were cut serially at 4 μm of thickness and placed on aminopropyl-trietoxy-silan-coated slides (S8226, Matsunami Glass Int. Inc., Osaka, Japan). Deparaffinized sections were used for hematoxylin and eosin (HE) and immunohistochemically stained using the avidin-biotin peroxidase complex (ABC) method (Hsu et al. 1981). The sections were treated by microwave in distilled water for 20 min to retrieve antigens, immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to block the endogenous peroxidase activity, and then incubated with normal goat serum (1:50, S-1000, Vector Laboratories Inc., CA, USA) for 30 min at RT to prevent the nonspecific staining. Then the sections were incubated overnight with each primary antibody at 4°C in a moisture chamber. The primary antibodies used in this study were the following: monoclonal anti-human lung tumor cell cytokeratin in mouse (1:100, Clone Lu-5, Biogenesis Ltd., Poole, UK), monoclonal anti-porcine stomach desmin in mouse (1:50, code M724, DAKO, Glostrup, Denmark), polyclonal anti-calf lens vimentin in rabbit (1:100, code VIP, MEDAC, Hamburg, Germany), polyclonal anti-human vimentin in rabbit (1:200, code ab15248, abcam Ltd., Cambridge, UK), monoclonal anti-human α-smooth muscle actin (α-SMA) in mouse (1:1000, Clone 1A4, Sigma Chemical Co., St. Louis, USA), and monoclonal anti-chick brain α-tubulin in mouse (1:200, Clone DM1A, Neomarkers, CA, USA). For secondary antibodies, biotinylated anti rabbit IgG in goat (1:200, BA-1000, Vector Laboratories Inc.,) or biotinylated anti mouse IgG in goat (1:200, BA-9200, Vector Laboratories Inc.) were applied for 30 min, and then the sections were incubated with ABC reagent for 30 min (1:2, PK-6100, Vectastain *Elite* ABC kit, Vector Laboratories). The binding sites were visualized with Tris-HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.006% H_2O_2. After incubation, the sections were washed with 0.01 M phosphate buffered saline (PBS, pH 7.4), dehydrated in graded series of ethanol, cleared in

Table 1. Individual profiles of the lesser mouse deer used in this study

Sample No.	Sex	BW (kg)	HBL (mm)	TL (mm)	HFL (mm)	EL (mm)	Spermiogenesis	Maturation
14	Male	2.30	498	85	126	42	C	M
15	Male	1.35	413	71	124	39	N	I
19	Male	1.65	440	72	125	36	C	M*
23	Male	1.75	438	70	135	41	C	M
25	Male	1.55	465	55	125	49	C	M
29	Male	2.00	462	57	133	39	C	M

BW, body weight; HBL, head and body length; TL, tail length; HFL, hind foot length; EL, ear length; C, completed; N, not completeted; M, mature; I, immature.

*: a few spermatozoa in the seminiferous tubules and none in the epididymal ducts.

xylene, coverslipped and observed under conventional light microscope. The negative control sections were treated with normal goat or normal horse antisera instead of each primary antibody.

Results

In five lesser mouse deer, the spermiogenesis was recognized in the testes and the epididymal ducts were filled with many spermatozoa except one animal (Fig. 1A, C, Table 1). The testis of one lesser mouse deer did not complete the spermatogenesis, producing no spermatozoa although the spermatogonia and spermatocytes were found in the seminiferous tubules (Fig. 1B). Therefore, this individual was regarded as immature (Table 1). In all samples, the immunoreactivity for cytokeratin was not shown in the testes and the epididymides. Desmin was detected in the walls of blood vessels (Fig. 2A) and the sub-epithelial myoid cells of epididymal ducts (Fig. 2B). The immunoreactivity for vimentin was shown in the myoid cells of the seminiferous tubules (peritubular myoid cells) and around the nuclei of the Sertoli cells of which the positions were arranged in different height (Fig. 3A). Vimentin filaments were extended from the basement membrane to the nucleus of the Sertoli cells when the nucleus kept some distance from the basement membrane. The filaments extended slightly into the supranuclear part when the nucleus was close to the base of the Sertoli cells (Fig. 3A). The strong immunoreactivity was found in the blood vessel walls, the modified Sertoli cells of the terminal segment of the convoluted seminiferous tubules and the epithelia of the straight seminiferous tubules and rete testis (Fig. 3B). Furthermore, vimentin immunoreactivity was shown in the sub-epithelial myoid cells of epididymal ducts and the stromal cells among the ducts (Fig. 3C). In the Leydig cells, the immunoreactivity for vimentin was detected (Fig. 3A).

The strong immunoreactivity for α-tubulin was detected in the Sertoli cells (Fig. 4A) and the modified Sertoli cells of the terminal segment (Fig. 4B). Tubulin filaments were obviously extended to the tip of each Sertoli cell cytoplasm. Positive immunoreaction with α-SMA was found in the walls of blood vessels and the peritubular myoid cells (Fig. 5A). The sub-epithelial myoid cells of rete testis and epididymal ducts also showed the immunoreactivity for α-SMA (Fig. 5B). Distribution pattern of each cytoskeletal protein was not different between mature and immature testes.

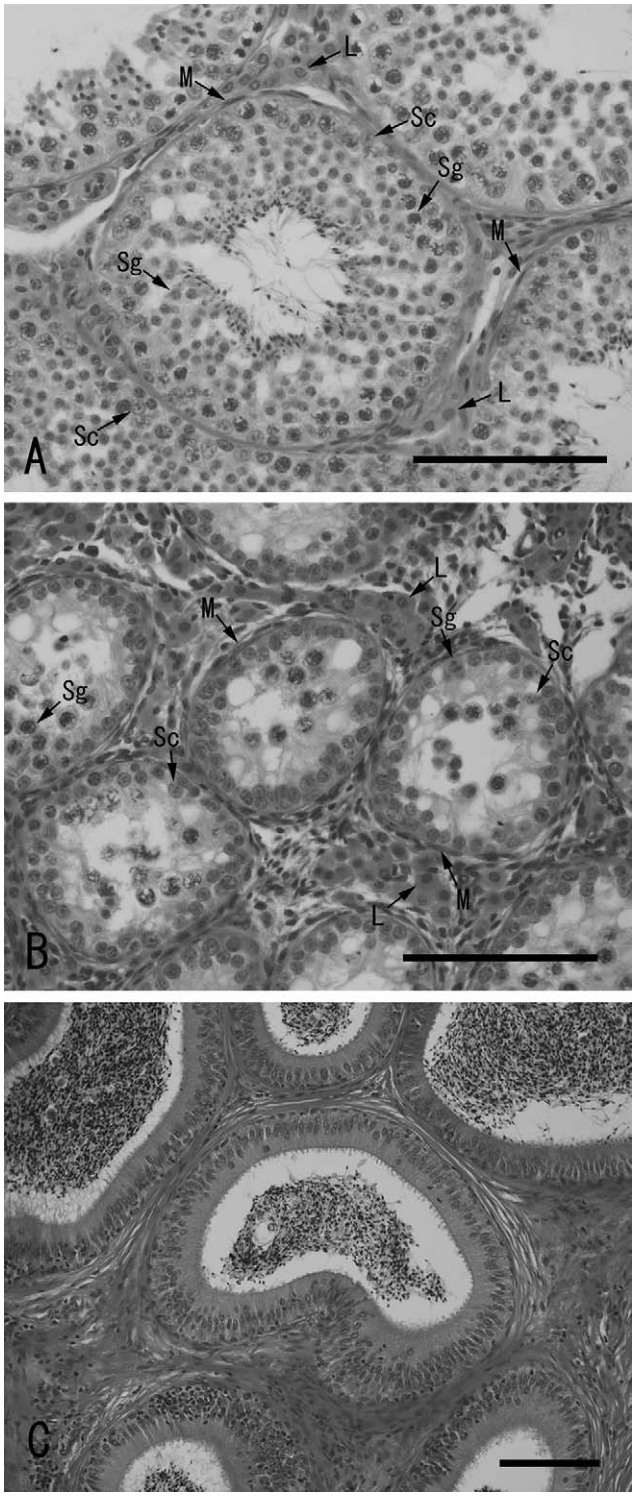

Fig. 1. General structure of the testis and epididymis of the lesser mouse deer. A: In mature testis, the spermiogenesis was confirmed, producing spermatozoa. B: There were no spermatozoa in immature testis, although spermatogenesis was already started. C: Epididymal ducts were filled with many spermatozoa in mature epididymis. Sc, Sertoli cells; L, Leydig cells; M, peritubular myoid cells; Sg, spermatogenic cells. HE staining. Bar: 100 μm.

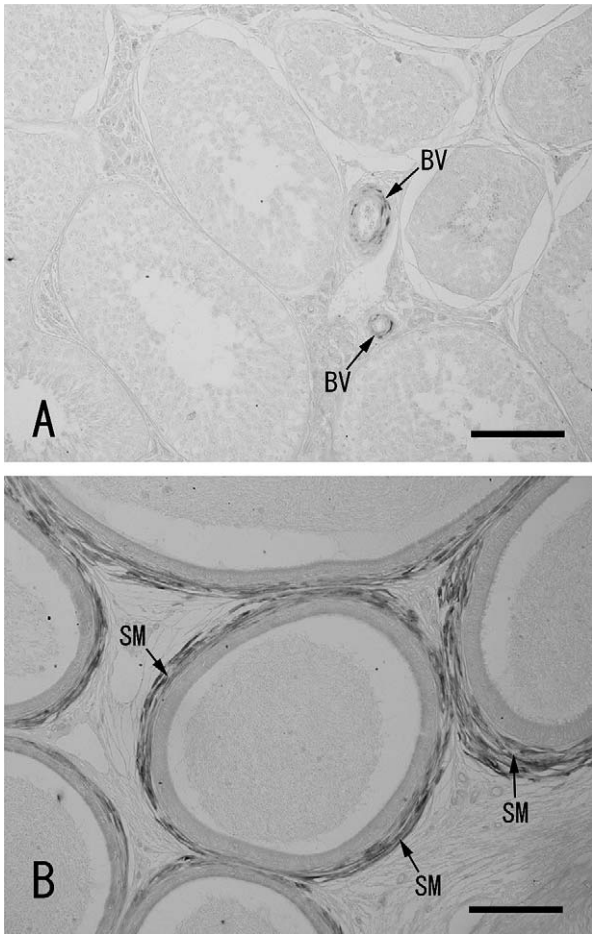

Fig. 2. Immunolocalization of desmin in the testis and epididymis of the lesser mouse deer. A: In the testis, desmin was found only in the walls of blood vessels (BV). B: Immunoreactivity for desmin was detected in the sub-epithelial myoid cells (SM) of epididymal ducts. Bar: 100 μm.

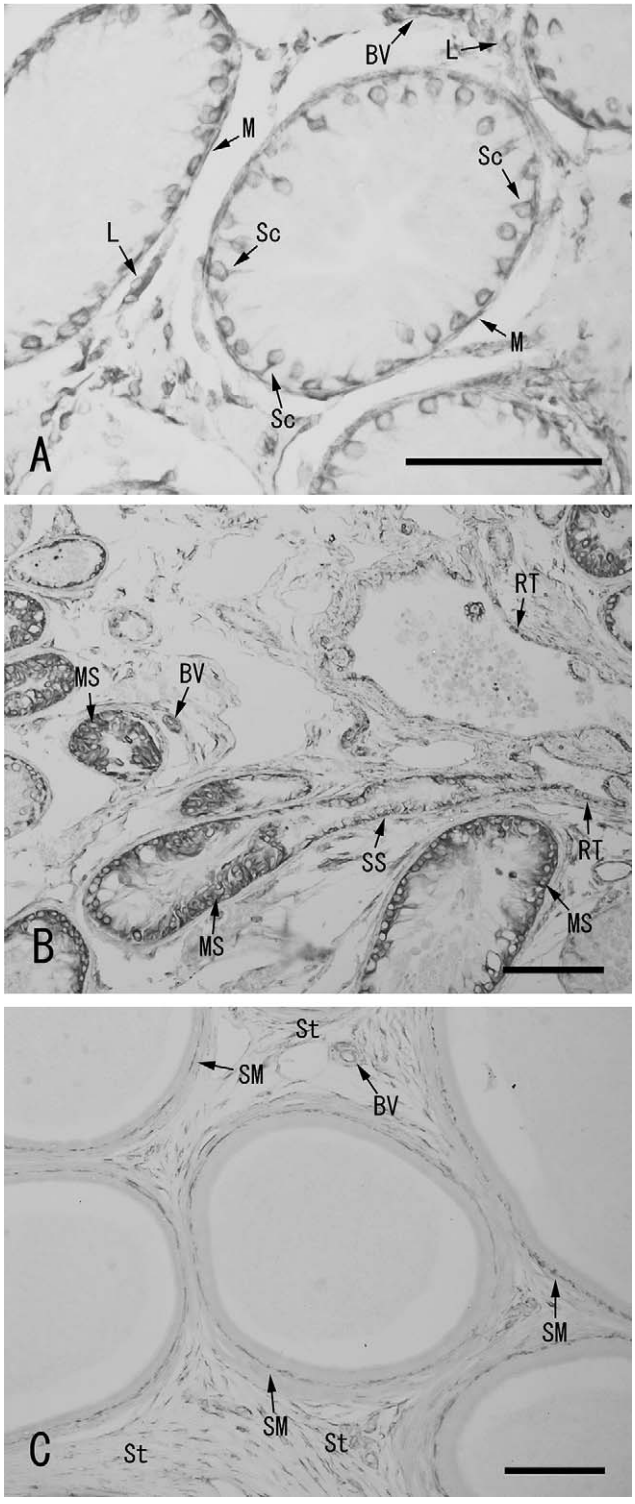

Fig. 3. Immunolocalization of vimentin in the testis and epididymis of the lesser mouse deer. A: Vimentin was found in the Sertoli cells (Sc), Leydig cells (L), peritubular myoid cells (M), and the walls of blood vessels (BV). B: Immunoreactivity for vimentin was also detected in the modified Sertoli cells (MS) of the terminal segment of the convoluted seminiferous tubules, and the epithelia of the straight seminiferous tubules (SS) and rete testis (RT). C: Vimentin was expressed in the sub-epithelial myoid cells (SM) of epididymal ducts and the stromal cells (St) among the ducts. Bar: 100 μm.

Discussion

In this study, the different distribution of each cytoskeletal protein was demonstrated in the testis of the lesser mouse deer. Intermediate filaments are subdivided into cytokeratin, desmin, vimentin, neurofilament proteins and glial fibrillary acidic protein (GFAP). It has been reported in various researches that cytokeratin, desmin and vimentin compose the cytoskeleton of the testes and are related to their structural support and functions (Amlani and Vogl 1988; Fridmacher et al. 1992; Allard et al. 1993; Zhu et al. 1997; Sasaki et al. 1998; Vogl et al. 2000; He et al. 2007).

In this study, cytokeratin and desmin were not expressed in the testicular cells, such as the Sertoli cells, spermatogenic cells, Leydig cells, peritubular myoid

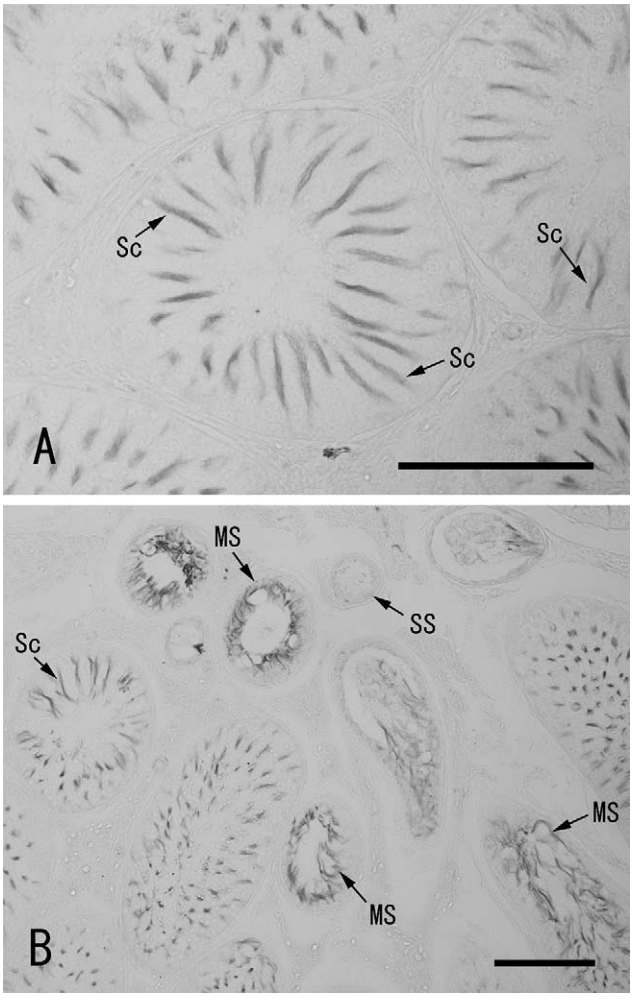

Fig. 4. Immunolocalization of α-tubulin in the testis of the lesser mouse deer. A: Alpha-tubulin was extended from basal to tip of each Sertoli cell (Sc). B: Immunoreactivity for α-tubulin was detected in the modified Sertoli cells (MS) of the terminal segment, but not in the epithelia of the straight seminiferous tubules (SS) and rete testis. Bar: 100 μm.

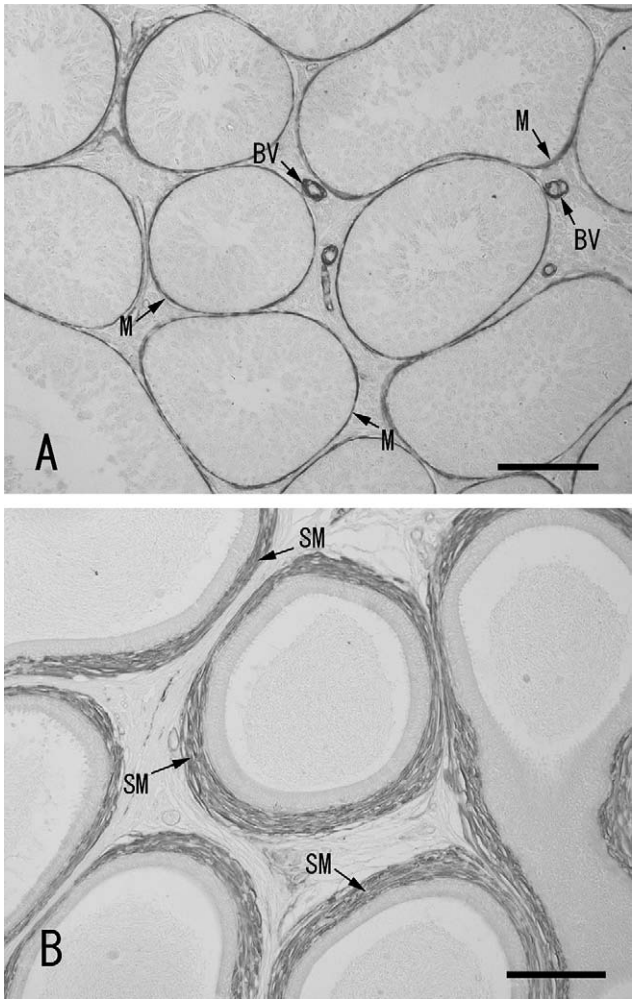

Fig. 5. Immunolocalization of α-smooth muscle actin (α-SMA) in the testis of the lesser mouse deer. A: In the testis, α-SMA was found in the peritubular myoid cells (M) and the walls of blood vessels (BV). B: Immunoreactivity for α-SMA was detected in the sub-epithelial myoid cells (SM) of epididymal ducts. Bar: 100 μm.

cells, modified Sertoli cells, and epithelial cells of the straight seminiferous tubules and rete testis. In fetal and neonatal testes, cytokeratin was present in the Sertoli cells (Paranko et al. 1986; Fridmacher et al. 1992; Rogatsch et al. 1996; Sasaki et al. 1998), but completely disappeared by adulthood (Paranko et al. 1986; Rogatsch et al. 1996). In pathological and culture conditions, however, cytokeratin was revealed in adult testes (Miettinen et al. 1985; Guillou et al. 1990; Rogatsch et al. 1996; de Miguel et al. 1997). In the mouse deer, it may be assumed that cytokeratin is expressed in the Sertoli cells of the fetal and more immature testes. It has been demonstrated that desmin is also expressed in the Sertoli cells of the fetal and immature testes (Fröjdman et al. 1992; Cruzana et al. 2006). In several mammals, moreover, desmin was found in the peritubular myoid cells (Virtanen et al. 1986; Palombi and Di Carlo 1988; Fröjdman et al. 1992; Steger and Wrobel 1994; Komatsu et al. 1998; Cruzana et al. 2006). In the ovine testes, desmin was shown in early prepubertal period, but vanished completely with its arrival to age of puberty (Steger and Wrobel 1994). In this study of the mouse deer, the immunoreactivity for desmin was detected only in the walls of blood vessels and the sub-epithelial myoid cells of epididymal ducts. In the mouse deer, therefore, it is possible that desmin expression of the Sertoli cells and the peritubular myoid cells might be disappeared with postnatal testicular development.

In the mouse deer testes, the immunoreactivity for vimentin was detected in the Sertoli cells, peritubular

myoid cells, Leydig cells, modified Sertoli cells, and epithelial cells of the straight seminiferous tubules and rete testis. In immature bovine testes, the Sertoli cells experienced the elevation of nucleus with the elongation of vimentin filaments connecting between nucleus and basement membrane from 5 to 8 months of age. In the mature testes, however, the nuclei were settled close to the basement membrane with short supranuclear flame of vimentin (Devkota et al. 2006). In the rat testes, the temporary elevation of the Sertoli cell nuclei was shown at 14 days of postnatal age (Zhu et al. 1997). In adult rat Sertoli cells, moreover, the nuclei were basally positioned and the extension of vimentin in the supranuclear part was remarkably changed during the seminiferous epitherial cycle (Allard et al. 1993; Zhu et al. 1997). In adult ovine (Steger and Wrobel 1994) and bovine testes (Steger et al. 1994; Devkota et al. 2006), however, any changes of vimentin distribution were not observed during the seminiferous cycle. In the adult and immature mouse deer, the Sertoli cell nuclei with different height were intermingled unlike the previously reported animals (Steger et al. 1994; Zhu et al. 1997; Komatsu et al. 1998; Rodríguez et al. 1999; Cruzana et al. 2006; Devkota et al. 2006). Although the Sertoli cells of the adult mouse deer were larger in size than those of the immature deer, the localization and intensity of vimentin positive reactions were not different between mature and immature Sertoli cells. Furthermore, the Sertoli cells showed no remarkable changes of vimentin distribution during the seminiferous epitherial cycle. It has been reported that vimentin filaments play an important role to anchor the spermatogenic cells to the Sertoli cells, and may be involved in the traverse of the elongated spermatids and spermiation (Amlani and Vogl 1988; Zhu et al. 1997; Vogl et al. 2000; He et al. 2007). In the mouse deer Sertoli cells, it is suggested that vimentin may not be related to the migration of the elongated spermatids with controlling its apical cytoplasmic distribution. Also in the Leydig cells of the mouse deer testes, the immunoreactivity for vimentin was detected. In the previous study, the bundles of intermediate filaments were noticed in the Leydig cell cytoplasm of the lesser mouse deer testes by ultrastructual observation (Andriana et al. 2003b, 2005). It is reasonable to suppose that the intermediate filament bundles of the Leydig cells are vimentin filaments.

Microtubules are composed of α- and β-tubulin subunits. In the Sertoli cells, microtubules are concerned with the structural support for developing spermatogenic cells, migration of spermatids, shaping of the spermatid head and acrosome, and preservation of the cell shape (Vogl et al. 1983; Redenbach and Vogl 1991; Allard et al. 1993; Steger et al. 1994; Vogl et al. 2000). In this study, α-tubulin was detected and extended toward the apical part of the Sertoli cell cytoplasm. In the mouse deer, therefore, microtubules may be main cytoskeletal proteins for the Sertoli cells and actively function for the supporting of spermatogenic cells and migration of spermatids. Microfilaments (actin filaments) are the thinnest in the cytoskeletal filaments. Alpha-SMA is localized in the smooth muscles, but never in the skeletal and cardiac muscle. It has previously been reported that α-SMA is expressed in the peritubular myoid cells of the testes and its circular formation is gradually completed with the testicular development (Tung and Fritz 1990; Palombi et al. 1992; Devkota et al. 2006).

It is obvious that the peritubular myoid cells play an important role in contractions of the seminiferous tubules to transport the spermatozoa and testicular fluid (Miyake et al. 1986; Maekawa et al. 1996). This study showed that the peritubular myoid cells contain α-SMA and vimentin in the mouse deer testes. Therefore, both α-SMA and vimentin may be related to the myoid cell contractions. On the other hand, the sub-epithelial myoid cells of epididymal ducts expressed not only α-SMA and vimentin but also desmin. Accordingly these three cytoskeletal filaments may function for the contractions of the epididymal ducts.

Acknowledgments: This study was financially supported in part by a Grant-in-Aid for Scientific Research (No. 13575027, No. 15405037, No. 18405036) and a Grant-in-Aid for Scientific Research (The 21st Century Center-of-Excellence Program) (E-1) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received 26 May 2009. Accepted 21 July 2009.