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Disrupted expression of intermediate filaments in the testis of rhesus monkey after experimental cryptorchidism

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Summary

Cytoskeletons in Sertoli cell play an important role in process of spermatogenesis. The expression and distribution of the intermediate filaments, vimentin, keratin and desmin, were studied in the Sertoli cells of the cryptorchid testis of rhesus monkey. Vimentin was localized in the perinuclear region of Sertoli cells of the normal testis. An intense increase in vimentin immunoreactivity was observed with appearance of disorganized staining in the Sertoli cells of the cryptorchid testes. Cytokeratin 18, a marker of immature Sertoli cells, re-expressed in the cells of the adult cryptorchid testes. Desmin was also observed in the Sertoli cells in addition to the peritubular myoid cells on 30 days after the cryptorchid operation. These data suggest that Sertoli cells in primate can be affected by the heat stress. The altered changes in intermediate filaments could be possible to induce the Sertoli cell functional changes that would partially contribute to the germ cell apoptosis leading to azoospermia or oligozoospermia.

Keywords: cryptorchidism, cytokeratin, desmin, Sertoli cell, vimentin

Introduction

Cryptorchidism, failure of testes to descend into scrotum at birth, constitutes the most common genital abnormality in boys. The subsequent infertility associated with cryptorchidism is attributed to testicular suprascrotal temperature. It is well known that the elevated temperature may be responsible for cellular, histological, and hormonal changes in the testis, which impair spermatogenesis and fertility (Hezmail & Lipshulz, 1982; Jegou *et al.*, 1984). Several reports have suggested that the elevation of testicular temperature induced by cryptorchidism not only causes germ cell loss, but also affects the morphology and function of Sertoli cells (Hagenas & Ritzen, 1976; Kerr *et al.*, 1979).

Sertoli cells are the primary supportive cells of seminiferous epithelium and play a key role in triggering and regulating the process of spermatogenesis (Russell, 1993; De Kretser *et al.*, 1998). Sertoli cell processes a highly organized and very active cytoskeleton, which probably is important in spermatogenesis. An important component of Sertoli cell cytoskeleton is intermediate filaments, which, in normal adult Sertoli cells, are mainly of the vimentin type (Franke *et al.*, 1979). Vimentin filaments are abundant around the nucleus, resulting in a clear zone free of organelles surrounding the nucleus (Aumüller *et al.*, 1988; Aumüller *et al.*, 1992). Collapse of vimentin filaments by chemicals was correlated with the loss of structural integrity of seminiferous epithelium along with germ cell apoptosis. Increase in Sertoli cell intermediate filaments and vimentin immunoreactivity was observed in men with severe germ cell depletion (Chemes *et al.*, 1977; Richburg & Boekelheide, 1996). Cytokeratin and vimentin are typically co-expressed in the Sertoli cell cytoplasm of foetus and childhood (Stosiek *et al.*, 1990; Aumüller *et al.*, 1992). Expression of cytokeratin in adult seminiferous epithelium

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has been regarded as a sign of either maintaining or regaining undifferentiated immature feature (Miettinen *et al.*, 1985; Stosiek *et al.*, 1990; Aumüller *et al.*, 1992; Bergmann & Kliesch, 1994; Steger *et al.*, 1996). Presence of desmin has been reported in Sertoli cell of foetal rats, which might shed some light on their ontogeny (Fröjdman *et al.*, 1992).

Changes in Sertoli cell intermediate filaments have been reported in the pathological and ageing human testes (Rogatsch *et al.*, 1996; Miguel *et al.*, 1997; Steger *et al.*, 1999) and in rat cryptorchid testis (Wang *et al.*, 2002). The following experiments have been designed to analyse the changes of the intermediate filament in monkey Sertoli cells during cryptorchidism-induced azoospermia. Our findings that rhesus monkey Sertoli cell can express all the three intermediate filaments under certain conditions indicate that this cell type reacts to abdominal temperature with complex changes of its cytoskeletal protein.

Materials and methods

Animals and tissue preparation

Adult (5–7 years old) and pre-pubertal male rhesus monkeys were raised in the Kunming Institute Primate Center, Kunming, Chinese Academy of Sciences (CAS). The experiments were approved by the Animal Ethical Committees of the Institute of Zoology and the Kunming Institute Primate Research Center, CAS. To induce unilateral cryptorchidism, adult monkeys were anaesthetized and a midline incision was made in the abdomen. The gubernaculums was cut on the right side to displace the testis into the abdomen. Suturing the inguinal canal on the right side prevented the testis decent. The testes of three monkeys for each group were removed on day 5, 10, 15 and 30 after surgery. Some of tissue slices were snap frozen in liquid nitrogen, and stored at −70 °C for RNA and protein analysis later and the others were fixed in Bouin's solution, embedded in paraffin and cut into 5-μm sections for immunohistochemistry.

Immunohistochemistry

The testicular tissues were dehydrated and embedded in paraffin. Sections at 5 μm thick were processed with the avidin–biotin–peroxidase complex (ABC) method. Following deparaffinization, the sections were hydrated and incubated for 20 min in 0.3% H_2O_2 in distilled water to quench endogenous peroxidase activity. The sections were then digested with 0.4% pepsin in 0.1 mM HCl for 10 min at 37 °C and incubated overnight at 4 °C with the primary antibody diluted in phosphate-buffered saline (PBS) containing 10% normal goat serum (NGS). The primary antibodies used for this study were anti-human monoclonal antibodies (Zymed, San Francisco, CA, USA) and the dilutions found to be optimal were as follows: vimentin 1 : 40, cytokeratin 18 (CK-18) 1 : 50, and desmin 1 : 40. After washing twice in PBS the sections were incubated with horse anti-mouse biotinylated immunoglobulin (Vector, Burlingame, CA, USA) for 30 min at 1 : 200 dilution in 2% NGS. Afterwards, the sections were washed twice in PBS and then incubated with the ABC (Vector) for 30 min at room temperature and developed with diaminobenzidine. The sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted for microscopic examination. Negative control means that normal immunoglobulin G was used instead of the primary antibody.

Western blot

The testes were homogenized in dounce homogenizer. The supernatants were obtained by centrifugation at 10 000 ***g*** for 30 min. After boiling for 3 min at 100 °C, aliquots equivalent to 50 μg of protein were separated in SDS-polyacrylamide (12%) slab mini gels. Separated proteins were transferred for 3 h at 75 V to nitrocellulose membrane, and the membranes were then soaked in blocking solution (5% non-fat milk in PBS, pH 7.6) for 1 h at room temperature and then incubated with the primary antibodies, at the dilutions of 1 : 1000 for vimentin, 1 : 500 for CK-18 or 1 : 300 for desmin overnight in blocking solution at 4 °C. After extensive washing with PBS the membranes were incubated with a peroxidase-conjugated second antibody (goat anti-mouse immunoglobulin; Jackson Immuno Research Lab., West Grove, PA, USA), at 1 : 2000 in blocking solution. The filters were developed with an enhanced chemiluminescence western blotting analysis, following the procedure described by the manufacturers (Pierce, Rockford, IL, USA).

Statistical analysis

Testes from three individual monkeys for each group were analysed. Experiments were repeated at least three times, and one representative from at least three similar results was presented. The quantitative data represent mean±SD of three results at least from three animals. Statistical analysis was conducted using the paired or unpaired Student's test. p-Values of <0.05 were considered statistically significant.

Result

The specificity of antibodies

Western blot analysis showed a single band, corresponding to the respective vimentin, CK18 and desmin antigen molecular weight, for each antibody (Fig. 1).

Intermediate filaments staining

The seminiferous epithelium was uniformly immunostained with the vimentin antibody. The Sertoli cells, Leydig cells and interstitial cells were positive. In the control testis, vimentin filaments were mainly immunolocalized in the perinuclear region of the basal and lateral cytoplasm in Sertoli cells, occasionally in the apical

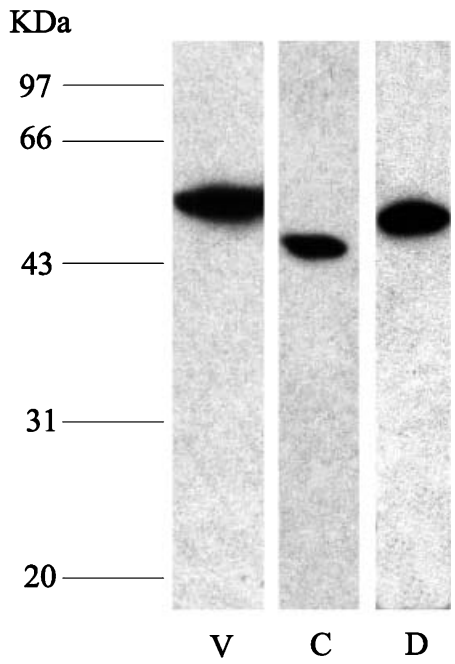

Figure 1. Western blotting analysis for testicular extracts from cryptorchid testis on day 15 after surgery. V, western blot stained with anti-vimentin antibodies. C, western blot stained with anti-cytokeratin 18 antibodies. D, western blot stained with anti-desmin antibodies.

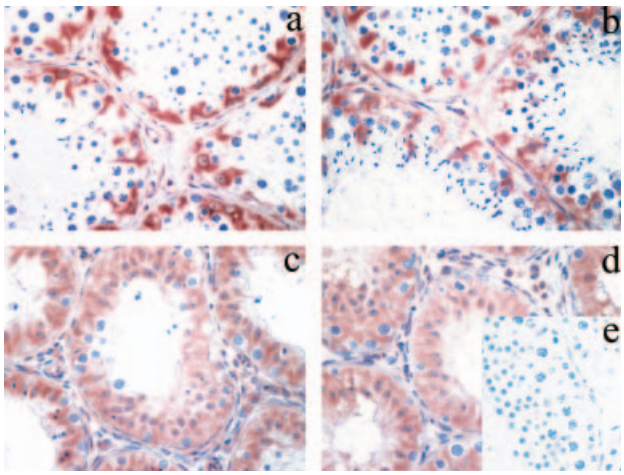

Figure 2. Immunohistochemical staining for vimentin. (a) normal seminiferous tubules exhibiting concentrated vimentin staining mainly in the perinuclear region of Sertoli cell. (b)–(d) Cryptorchidism exhibiting the appearance of disorganized vimentin staining on day 5 (b), day 10 (c) and day 15 (d). (e) Negative control (original magnification × 400).

cytoplasmic extensions in Sertoli cells (Fig. 2a). Appearance of disorganized vimentin staining was observed in the cryptorchid testes on 5 days (Fig. 2b). Immunohistochemical reaction was extended in the similar proportions throughout the entire cytoplasm of Sertoli cells on 10 and 15 days (Fig. 2c,d) after operation.

No CK-18 immunoreactivity was observed in the adult normal testis, but a strong CK-18 immunoreaction was detected in the Sertoli cells in the normal testis of prepubertal monkeys. A weak expression of this filament was detected in the Sertoli cells of the cryptorchid testis on day 5. On day 10 there was a remarkable increase in the number of the Sertoli cells with the cytokeratin immunoreactivity. On day 15 the most of the seminiferous tubules were positive with cytokeratin immunoreaction (Fig. 3).

An immunohistochemical reaction to desmin was observed in the peritubular myoid and the interstitial cells in the normal testis (Fig. 4a) and the cryporchid testes (Fig. 4b,c). However, a few desmin-positive staining in the intratubular Sertoli cells was detected in some tubules only on day 30 after operation (Fig. 4c).

Western blot analysis of changes in vimentin expression

The content of vimentin filaments in the cryptorchid testes was analysed by western blotting. Figure 5 revealed that the levels of vimentin antigen were significantly increased in the cryptorchid testes on days 10 and 15 after surgery.

Discussion

Cryptorchidism exposes the testis to an elevated temperature, which causes an increased incidence of germ cell apoptosis (Socher *et al.*, 1997). It has been reported that Sertoli cell function was severely disturbed by an elevated temperature (Hagenas & Ritzen, 1976; Hagenas *et al.*, 1978). Hagenas *et al.* (1982) and Karpe *et al.* (1982) have demonstrated that the secretion of androgen-binding protein, a functional marker of Sertoli cells, was impaired by the heat stress. Our data also suggest that abnormal cytoskeletal changes in Sertoli cells were induced in the cryptorchid testes of rhesus monkeys. These findings indicate that not only germ cells but also Sertoli cells were affected by heat-treatment.

Sertoli cells are responsible for orchestrating differentiation of germ cells and providing their structural and nutritional support in testis. All nutrients or hormonal stimuli to germ cells must pass through Sertoli cell cytoplasm (Ritzen *et al.*, 1989). It has been known that the paracine factors produced by Sertoli cells are involved in the regulation of spermatogenesis (Parvinen & Ventela, 1999). Vimentin filaments are known to be involved in maintaining the integrity of Sertoli cells to adjacent spermatogenetic cell contacts (Amlani & Vogl, 1988). It has been suggested that vimentin filament acts as mediators of cell signal transduction between plasma membrane and nucleus (Ritzen *et al.*, 1989). It has been reported that expression of vimentin filaments in the Sertoli cells increased in the ageing human testes (Miguel *et al.*, 1997) and its distribution in cryptrochid rats altered (Wang *et al.*, 2002). Our experiment further demonstrated that cryptorchidism in monkey caused disorganized distribution of vimentin in the Sertoli cells and lost its extension

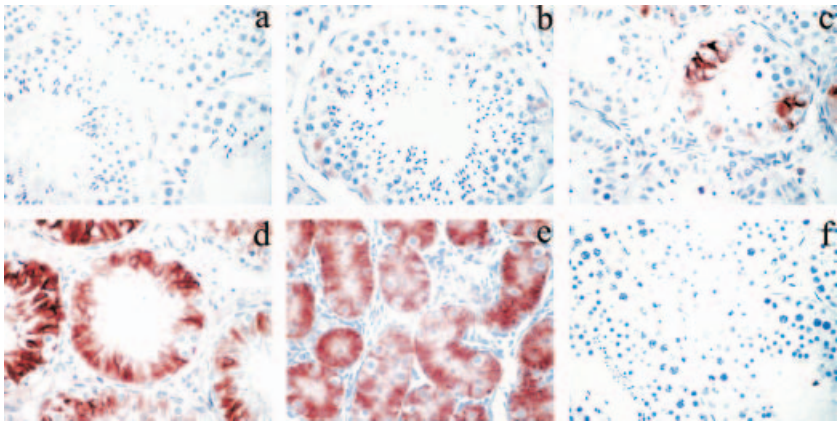

Figure 3. Immunohistochemical staining for cytokeratin-18 (CK-18). (a) Normal seminiferous tubule not exhibiting the CK-18 staining. (b)–(d) Cryptorchidism exhibiting the re-appearance of CK-18 staining on day 5 (b), day 10(c) and day 15 (d). (e) The distribution of CK-18 in Sertoli cell of pre-pubertal testes. (f) Negative control (original magnification × 400).

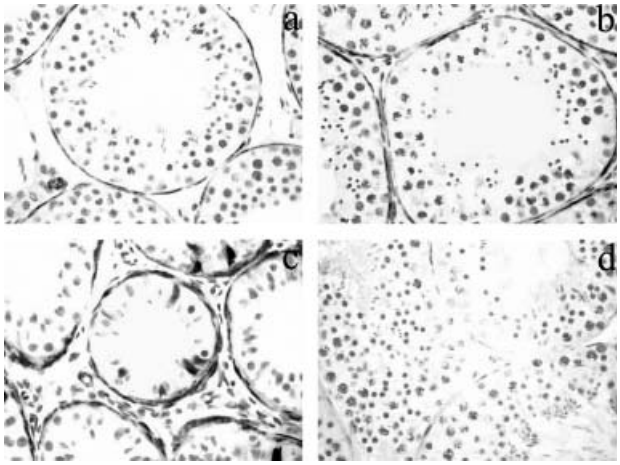

Figure 4. Immunohistochemical staining for desmin. (a)–(b) Desmin immunoreactivity in peritubular myoid cells in normal testis (a) and cryptorchid testis on day 5 (b). (c) A few desmin-positive Sertoli cells in cryptorchid testes on 30 days after surgery. (d) Negative control (original magnification × 400).

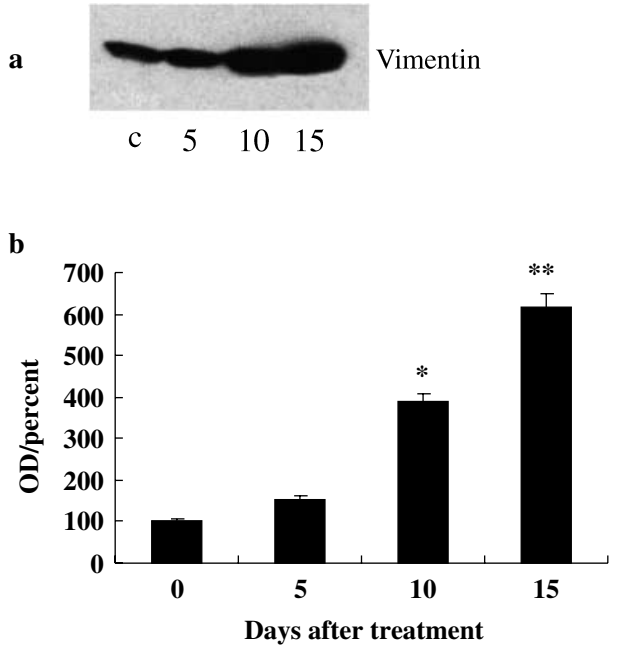

Figure 5. Temporal changes in vimentin expression in the cryptorchid testes. (A) Western blot analysis was performed using testis samples obtained from control monkeys and from monkeys 5, 10 and 15 days after surgery. (B) Quantitative analysis of changes in vimentin expression. Each data point represents the mean ± SEM (n = 3). OD, optical density (*$p < 0.05$, **$p < 0.01$ vs. control group).

in the perinuclear area. Vimentin immunolabelling increased in the Sertoli cells of the cryptorchidism testis with severe germ cell depletion. The quantitative study also revealed a significant increase in vimentin level. The possible cause and significance of such increase in vimentin expression in the cryptorchid testis are uncertain. It might be related to an alteration in the cell morphology (Georgatos, 1993). In the most cases with testicular disorders a vimentin increase in the Sertoli cells was accompanied with a higher content of follicle-stimulating hormone and a lower testosterone level in the blood (Nistal & Paniagua, 1997). The vimentin increase in the Sertoli cells may be related to certain local factors regulated by androgen and associated with the completion of spermatogenesis through a paracrine or autocrine response (Spiteri-Grech & Nieschlag, 1993) rather than a change in the endocrine function. Among these factors inhibin and plasminogen activators secreted by the Sertoli cells and the factors secreted by the germ cells might cause the functional changes in Sertoli cells (Vihko *et al.*, 1984; Saez *et al.*, 1991; Gérard & Jégou, 1993; Liu *et al.*, 1995; Anderson & Sharpe, 2000; Mu *et al.*, 2000; Zhou *et al.*, 2002).

Several studies in azoospermic men have revealed that the expression of cytokeratin may represent the status of differentiation of the Sertoli cells in different spermatogenic impairments (Maymon *et al.*, 2000, 2002). We have demonstrated in the present study that cytokeratin was strongly expressed in the Sertoli cells of the monkey cryptorchid testicular tubules with spermatogenetic arrest. Western blot showed that the molecular weight of the single cytokeratin band coincided with that of cytokeratin no. 18 (45 kDa);

Cytokeratin no. 18, together with cytokeratin no. 8, are the only keratins reported in Sertoli cells. According to the previous study, CK-18 expression can be regarded as a marker of immature or undifferentiated Sertoli cells in the seminiferous epithelium (Miettinen *et al.*, 1985; Stosiek *et al.*, 1990; Bergmann & Kliesch, 1994; Steger *et al.*, 1996). These data suggest that Sertoli cells in cryptorchid testis undergo a process of dedifferentiation, as indicated by the re-expression of cytokeratin intermediate filaments, and may subsequently result in a loss of Sertoli cell function and lead to a cession of spermatogenic activity. Our observation of an abnormal expression of desmin within the Sertoli cells in the cryptorchid testes represents the more abnormal changes of Sertoli cells induced by abdominal temperature.

Desmin, another member of intermediate filaments, was also present in few Sertoli cells of the cryptorchid testes observed in the present study. The biological significance of desmin is uncertain. Rogatsch *et al.* (1996) suggested that the expression of desmin may be related to a progressing reacquisition of a more undifferentiated phenotype, which should be further cleared.

In summary, an altered expression of the three intermediate filaments was present in the Sertoli cells of the cryptorchid testis in rhesus monkey. The molecular basis of these complex changes of Sertoli cells is still not clear. An impaired interaction of the Sertoli cells with abnormal germ cells or a complete lack of spermatogenesis might also induce the observed alteration of Sertoli cell intermediate filaments expression. The molecular mechanisms still remain to be further investigated.

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References

Amlani, S. & Vogl, A. W. (1988) Changes in the distribution of microtubules and intermediate filaments in mammalian Sertoli cells during spermatogenesis. *Anatomical Record* **220**, 143–160.

Anderson, R. A. & Sharpe, R. M. (2000) Regulation of inhibin production in the human male and its clinical applications. *International Journal of Andrology* **23**, 136–144.

Aumüller, G., Steinbrück, M., Krause, W. & Wagner, H. J. (1988) Distribution of vimentin-type intermediate filaments in Sertoli cells of the human testis, normal and pathologic. *Anatomy and Embryology* **178**, 129–136.

Aumüller, G., Schulze, C. & Viebahn, C. (1992) Intermediate filaments in Sertoli cells. *Microscopy Research Technique* **20**, 50–72.

Bergmann, M. & Kliesch, S. (1994) The distribution pattern of cytokeratin and vimentin immunoreactivity in testicular biopsies of infertile men. *Anatomy and Embryology* **190**, 515–520.

Chemes, H. E., Dym, M., Fawcett, D. W., Javadpour, N. & Sherins, R. J. (1977) Pathophysiological observations of Sertoli cells in patients with germinal aplasia or severe germ cell depletion: ultrastructure findings and hormone levels. *Biology of Reproduction* **17**, 108–123.

De Kretser, D. M., Loveland, K. L., Meinhardt, A., Simorangkir, D. & Wreford, N. (1998) Spermatogenesis. *Human Reproduction* **13**, 1–8.

Franke, W. W., Grund, C. & Schmid, E. (1979) Intermediate-sized filaments present in Sertoli cells are of the vimentin type. *European Journal of Cell Biology* **19**, 269–275.

Fröjdman, K., Paranko, J., Virtanen, I. & Pelliniemi, L. J. (1992) Intermediate filaments and epithelial differentiation of male rat embryonic gonad. *Differentiation* **50**, 113–123.

Georgatos, S. D. (1993) Dynamics of intermediate filaments. Recent progress and unanswered questions. *FEBS Letter* **318**, 101–107.

Gérard, N. & Jégou, B. (1993) In-vitro influence of germ cells on Sertoli cell-secreted proteins: a two-dimensional gel electrophoresis analysis. *International Journal of Andrology* **16**, 285–291.

Hagenas, L. & Ritzen, E. M. (1976) Impaired Sertoli cell function in experimental cryptorchidism in the rat. *Molecular and Cellular Endocrinology* **4**, 25–34.

Hagenas, L., Ritzen, E. M., Svensson, J., Hansson, V. & Purvis, K. (1982) Temperature dependence of Sertoli cell function. *International Journal of Andrology* **2**(Suppl.), 449–453.

Hezmail, H. P. & Lipshulz, L. I. (1982) Cryptorchidism and infertility. *Urologic Clinics of North America* **9**, 361–369.

Jegou, B., Peake, R. A., Irby, D. C. & de Kretser, D. M. (1984) Effects of the induction of experimental cryptorchidism and subsequent orchidopexy on testicular function in immature rats. *Biology of Reproduction* **30**, 179–187.

Karpe, B., Hagenas, L., Ploen, L. & Ritzen, E. M. (1982) Studies on the scrotal testis in unilateral experimental cryptorchidism in rat and guinea pig. *International Journal of Andrology* **5**, 59–73.

Kerr, J. B., Rich, K. A. & de Kretser, D. M. (1979) Effects of experimental cryptorchidism on the ultrastructure and function of the Sertoli cell and peritubular tissue of the rat testis. *Biology of Reproduction* **21**, 823–838.

Liu, Y. X., Liu, K., Zhou, H. M., Du, Q., Hu, Z. Y. & Zou, R. J. (1995) Hormonal regulation of tissue-type plasminogen activator and plasminogen activator inhibitor type-1 in cultured monkey Sertoli cells. *Human Reproduction* **10**, 719–727.

Maymon, B. B., Paz, G., Elliott, D. J., Hammel, I., Kleiman, S. E., Yogev, L., Hauser, R., Botchan, A. & Yavetz, H. (2000) Maturation phenotype of Sertoli cells in testicular biopsies of azoospermic men. *Human reproduction* **15**, 1537–1542.

Maymon, B. B., Yogev, L., Paz, G., Kleiman, S. E., Schreiber, L., Botchan, A., Hauser, R. & Yavetz, H. (2002) Sertoli cell maturation in men with azoospermia of different etiologies. *Fertility and Sertility* **77**, 904–909.

Miettinen, M., Virtanen, I. & Talerman, A. (1985) Intermediate filament proteins in human testis and testicular germ-cell tumors. *American Journal of Pathology* **120**, 402–410.

Miguel, M. P., Bethencourt, F. R., Arenas, M. I., Fraile, B. & Paniagua, R. (1997) Intermediate filaments in the Sertoli cells of the ageing human testis. *Virchows Arch* **431**, 131–138.

Mu, X. M., Liu, Y. X., Collins, L. L., Kim, E. & Chang, C. X. (2000) The p53/retinoblastoma-mediated repression of testicular orphan receptor-2 in the rhesus monkey with cryptorchidism. *Journal of Biological Chemistry* **275**, 23877–23883.

Nistal, M. & Paniagua, R. (1997) Non-neoplastic diseases of the testis. In: *Urologic surgical pathology* (eds Bostwick, D. G. & Elbe, J. N.), pp. 457–565. Mosby, St Louis.

Parvinen, M. & Ventela, S. (1999) Local regulation of spermatogenesis: a living cell approach. *Human Fertility (camb)* **2**, 138–142.

Richburg, J. H. & Boekelheide, K. (1996) Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. *Toxicology and Applied Pharmacology* **137**, 42–50.

Ritzen, E. M., Hansson, V. & French, F. S. (1989) The Sertoli cell. In: *The Testis*, 2nd edn (eds H. Burger & D. D. Kretser), pp. 269. Raven Press, New York.

Rogatsch, H., Jezek, D., Hittmair, A., Mikuz, G. & Feichtinger, H. (1996) Expression of vimentin, cytokeratin and desmin in Sertoli cells of human fetal, cryptorchid, and tumour-adjacent testicular tissue. *Virchows Arch* **427**, 497–502.

Russell, L. D. (1993) Morphological and functional evidence for Sertoli-germ cells relationships. In: *The Sertoli Cell* (eds L. D. Russell & M. D. Griswold), pp. 365–390. Cache River Press, Clearwater, FL, USA.

Saez, J. M., Avallet, O., Lejeune, H. & Chatelain, P. G. (1991) Cell–cell communication in the testes. *Hormone Research* **36**, 104–115.

Socher, S. A., Yin, Y., Dewolf, W. C. & Morgentaler, A. (1997) Temperature-mediated germ cell loss in the testis is associated with altered expression of the cell-cycle regulator p53. *Journal of Urology* **157**, 1986–1989.

Spiteri-Grech, J. & Nieschlag, E. (1993) Paracrine factors relevant to the regulation of spermatogenesis – a review. *Journal of Reproduction and Fertility* **98**, 1–14.

Steger, K., Rey, R., Kliesch, S., Louis, F., Schleicher, G. & Bergmann, M. (1996) Immunohistochemical detection of immature Sertoli cell markers in testicular tissue of infertile adult men: a preliminary study. *International Journal of Andrology* **19**, 122–128.

Steger, K., Rey, R., Louis, F., Kliesch, S., Behre, H. M., Nieschlag, E., Hoepffner, W., Bailey, D., Marks, A. & Bergmann, M. (1999) Reversion of the differentiated phenotype and maturation block in Sertoli cells in pathological human testis. *Human Reproduction* **14**, 136–143.

Stosiek, P., Kasper, M. & Karsten, U. (1990) Expression of cytokeratins 8 and 18 in human Sertoli cells of immature and atrophic seminiferous tubules. *Differentiation* **43**, 66–70.

Vihko, K. K., Suominen, J. J. O. & Parvinen, M. (1984) Cellular regulation of plasminogen activator secretion during spermatogenesis. *Biology of Reproduction* **31**, 383–389.

Wang, Z. Q., Watanabe, Y., Toki, A. & Itano, T. (2002) Altered distribution of Sertoli cell vimentin and increased apoptosis in cryptorchid rats. *Journal of Pediatric Surgery* **37**, 648–652.

Zhou, X. C., Wei, P., Han, X. B., Zou, R. J. & Liu, Y. X. (2002) Expression of $P16^{INK4a}$ in testis of rhesus monkey during heat stress and testosterone undecanoate induced azoospermia or oligozoospermia. *Contraception* **25**, 251–255.

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