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·Original Article·

Activation of extracellular signal-related kinases 1 and 2 in Sertoli cells in experimentally cryptorchid rhesus monkeys

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Abstract

Aim: To assess the spatiotemporal changes in the expression of extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPK) in response to heat stress in the cryptorchid testis, and to investigate a possible relation to Sertoli cell dedifferentiation. **Methods:** Immunohistochemistry and western blot were used to examine the expression and activation of ERK1/2, p38 and JNK in the cryptorchid testis at various stages after experimental cryptorchidism. **Results:** The abdominal temperature did not obviously change the total ERK1/2 expression but significantly activated phospho-ERK1/2 in the Sertoli cells of the cryptorchid testis. Heat stress increased total JNK expression in the Sertoli cells of the cryptorchid testis but did not activate phospho-JNK. Neither total p38 nor phospho-p38 was induced by heat stress in the Sertoli cells of the cryptorchid testis. Changes in the spatiotemporal expression of cytokeratin 18 (CK18), a marker of immature or undifferentiated Sertoli cells, were induced in the cryptorchid testis in a pattern similar to the activation of ERK1/2. **Conclusion:** The activation of ERK1/2 in the testis may be related to dedifferentiation of Sertoli cells under heat stress induced by experimental cryptorchidism. *(Asian J Androl 2006 May; 8: 265–272)*

Keywords: rhesus monkey; cryptorchidism; Sertoli cell; dedifferentiation; extracellular signal-regulated kinases 1 and 2

1 Introduction

Infertility associated with cryptorchidism is attributed to testicular suprascrotal temperature [1]. 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 [2]. We have found that abnormal expression and distribution of the intermediate filaments, such as vimentin, cytokeratin 18 (CK18) and desmin, in Sertoli cells are induced in the experimental cryptorchid testes of rhesus monkeys [2]. CK18 expression can be regarded as a marker of immature or undifferentiated Sertoli cells in the seminiferous epithelium [3]. These data suggest that Sertoli cells in the cryptorchid testes of rhesus monkeys undergo a process of dedifferentiation in response to heat stress. However, the underlying molecular mechanism has not been elucidated.

Mitogen-activated protein kinases (MAPK) is a fa-

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mily of evolutionary conserved protein kinases that play a critical role in transducing extracellular chemical and physical signals into intracellular responses. They play important roles in many signal transduction pathways involved in regulation of the cellular cycle, differentiation, proliferation and survival [4]. The MAPK family is composed of three subfamilies, namely, the extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 [5]. ERK 1 and 2 (ERK1/2) are activated by various ligands, such as vascular endothelial growth factor and transforming growth factor-β. They execute their functions by phosphorylating several downstream cytoplasmic kinases and nuclear transcription factors in their signal transduction pathways to promote cell survival and differentiation [6]. ERK3 appears to represent a distinct evolutionary branch of ERK MAPK and can be controlled by other cell signaling pathways. A number of observations suggest that the kinase may regulate cell differentiation [7]. JNK and p38 MAPK show similarity in organization but are distinct from the ERK MAPK cascade. These kinase pathways are implicated in signaling apoptosis, and cell differentiation and transformation [6].

In the present study, we examined the spatiotemporal expression and activation of ERK1/2, JNK and p38 MAPK in Sertoli cells as a first step to understand their possible roles in the process of Sertoli cell dedifferentiation in response to heat stress in the cryptorchid testis.

2 Materials and methods

2.1 Animals

Adult rhesus monkeys (5–7 years old) were raised in the Kunming Institute of Zoology, Chinese Academy of Sciences (CAS). The experiments were approved by the Animal Ethical Committees of both the Institute of Zoology and the Kunming Institute Primate Research Center, CAS. To induce unilateral experimental cryptorchidism, the animals were anesthetized by injection of pentobarbital sodium. A small incision was then made in the abdomen, and the gubernaculum was cut on the right side to displace the testes into the abdomen. Descent of the testes was prevented by closure of the inguinal canal on the right side by suturing. The testes in abdomen of three monkeys for each testicular castration time-point were removed on days 5, 10, 15 and 30 (D5, D10, D15 and D30) after surgery. Sham-operated scrotal testes were used as controls. The testes were decapsulated and divided into slices. Some of the tissue slices were fixed in Bouin's solution and embedded in paraffin prior to sectioning for immunohistochemistry. The others were snap-frozen in liquid nitrogen and stored at –70ºC for protein analysis.

2.2 Reagents

Polyclonal anti-phospho-ERK1/2 (No. 9101), anti-ERK1/2 (No. 9102), anti-phospho-p38 (No. 9211), anti-p38 (No. 9212), anti-phospho-JNK (No. 9251) and anti-JNK (No. 9252) antibodies were all purchased from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-CK18 antibody (No. 0073) was obtained from Zymed Laboratories (San Francisco, CA, USA).

2.3 Immunohistochemistry

Bouin-fixed, paraffin-embedded testicular sections were deparaffinized, hydrated and then incubated in 3% H_2O_2 to quench endogenous peroxidases. The sections were subsequently washed in phosphate-buffered saline (PBS) followed by antigen retrieval in 10 mmol/L ethylenediaminetetraacetic acid solution, pH 8.0. After two washes with PBS, the sections were blocked with 10% normal goat serum (NGS) for 30 min and then incubated at 4°C overnight with the primary antibodies (1:150 for each) diluted in PBS containing 10% NGS. Immunoreactivity was detected using the biotinylated goat antirabbit or the horse antimouse immunoglobulin G (IgG) secon dary antibodies (1: 200; Santa Cruz Biotechnology, CA, USA) followed by sequential incubation with avidin-biotinylated horseradish peroxidase complex and diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. The control sections were similarly processed, except that the primary antibodies were replaced with the normal rabbit or mouse IgG.

2.4 Western blot analysis

The frozen testes (1 g) were homogenized in 3 mL ice-cold preparation of modifed radioimmunoprecipitation (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 1% sodium dodecylsulfate [SDS] in PBS with protease inhibitors). The protein concentration of the extracts was determined by Bradford assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA). Between 30 μg and 50 μg protein per lane was resolved on a 12% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membranes at 100 V for 1 h. After being blocked

in 5% non-fat milk in PBST (PBS containing 0.05% Tween-20), the membranes were incubated with the primary antibodies at the dilution of 1: 500 each for anti-CK18, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK and anti-JNK overnight at 4ºC. Following 3 × 10 min washes in PBST, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies at a 1: 4000 dilution. The reactive bands were visualized by the SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and the membranes were then subjected to X-ray autoradiography. Band intensities were determined by Quantity one software (Bio-Rad, Hercules, CA, USA).

2.5 Statistical analysis

Samples from three individual animals at each tissue collection time point were analyzed. Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS, Chicago, IL, USA). Statistical significance was determined by one-way ANOVA. Post-hoc comparisons between means of treatment group were made using Fisher's protected least-significant-difference test. Differences were considered significant if $P < 0.05$. Values shown in all the figures were given as mean ± SEM. Data are representative of one of at least three separate experiments.

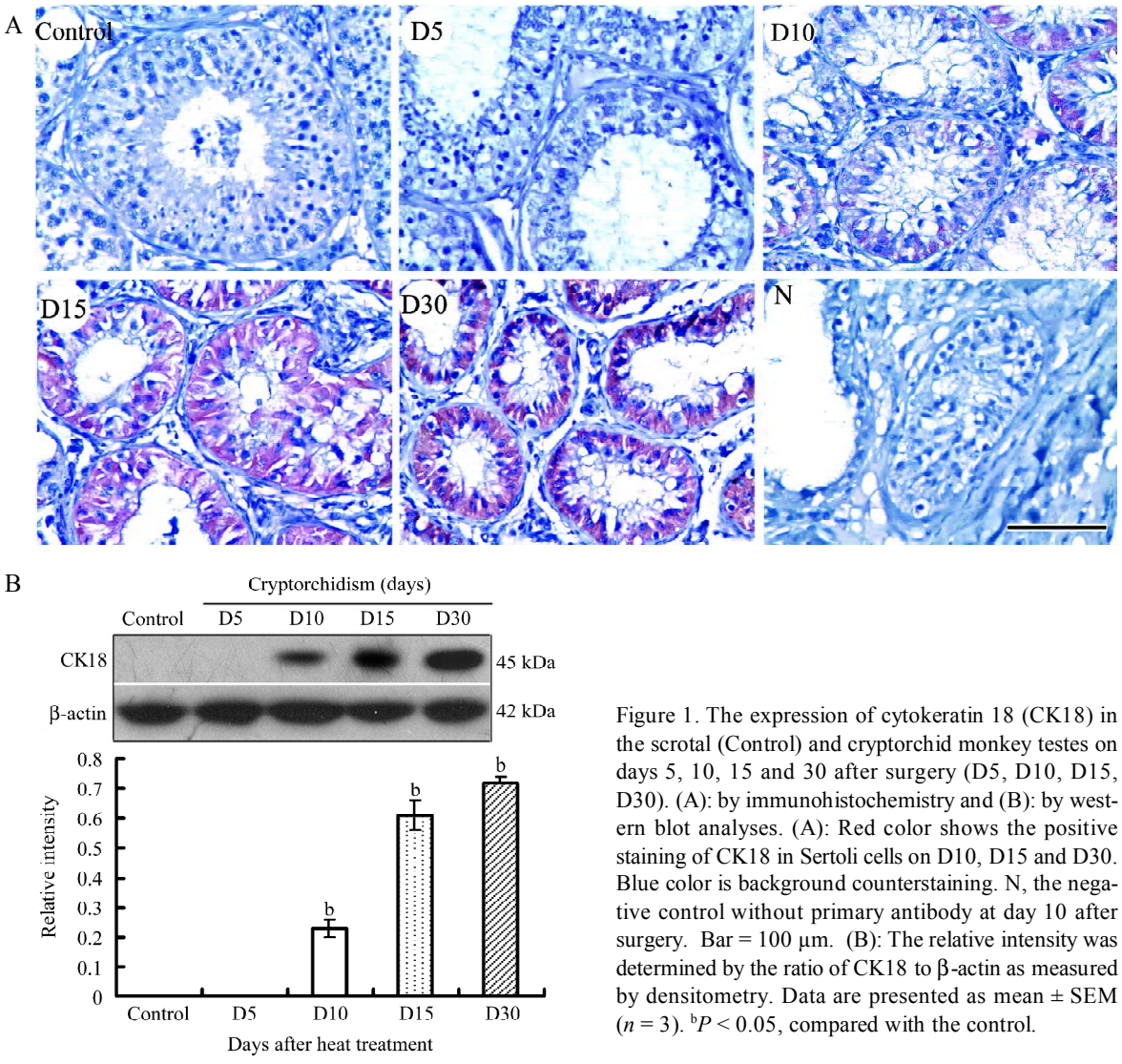

Figure 1. The expression of cytokeratin 18 (CK18) in the scrotal (Control) and cryptorchid monkey testes on days 5, 10, 15 and 30 after surgery (D5, D10, D15, D30). (A): by immunohistochemistry and (B): by western blot analyses. (A): Red color shows the positive staining of CK18 in Sertoli cells on D10, D15 and D30. Blue color is background counterstaining. N, the negative control without primary antibody at day 10 after surgery. Bar = 100 μm. (B): The relative intensity was determined by the ratio of CK18 to β-actin as measured by densitometry. Data are presented as mean ± SEM ($n = 3$). $^bP < 0.05$, compared with the control.

3 Results

3.1 Heat-induced cytokeratin 18 (CK18) re-expression in Sertoli cells of experimentally cryptorchid adult rhesus monkeys

No obvious immunoreactivity for CK18 was observed in the scrotal testis (Figure 1A, control) or on day 5 after experimental cryptorchidism (Figure 1A, D5). Weak expression of this molecule was detected in the Sertoli cells of the cryptorchid testis on day 10 after surgery (Figure 1A, D10). On days 15 and 30, a remarkable increase in the CK18 immunoreactivity in Sertoli cells was observed (Figure 1A, D15, D30). From day 10 to day 30, spermatogenesis was arrested, and only Sertoli cells and a few spermatogonia remained. The re-expression of CK18 was also confirmed by western blot analysis. As shown in Figure 1B, CK18 was not detected in the scrotal testis or on day 5 after surgery, but significantly increased on days 10, 15 and 30 ($P < 0.05$) in the experimental cryptorchid testis.

3.2 Temporal changes of ERK 1/2, JNK and p38 MAPKs in the testis of the experimentally cryptorchid adult rhesus monkeys

To detect the expression and phosphorylation of ERK1/2, JNK and p38 MAPKs in the experimental cryptorchid monkey testis, their proteins were studied in the scrotal and the cryptorchid testes on days 5, 10, 15 and 30 after surgery. As shown in Figure 2, phospho-ERK1/2 was not detected in the scrotal testis or on day 5 after surgery, but obviously increased on days 10, 15 and 30 ($P < 0.01$). No obvious difference in ERK1/2 expression was observed between the scrotal and cryptorchid testes. Interestingly, phospho-JNK was not detected in either the scrotal testis or the cryptorchid testis, whereas the total JNK expression level significantly increased on day 5 ($P < 0.05$) and days 10, 15 and 30 ($P < 0.01$) after surgery. However, there was neither an activation of phospho-p38 MAPK nor a variation in the total expression of p38 MAPK induced by heat stress.

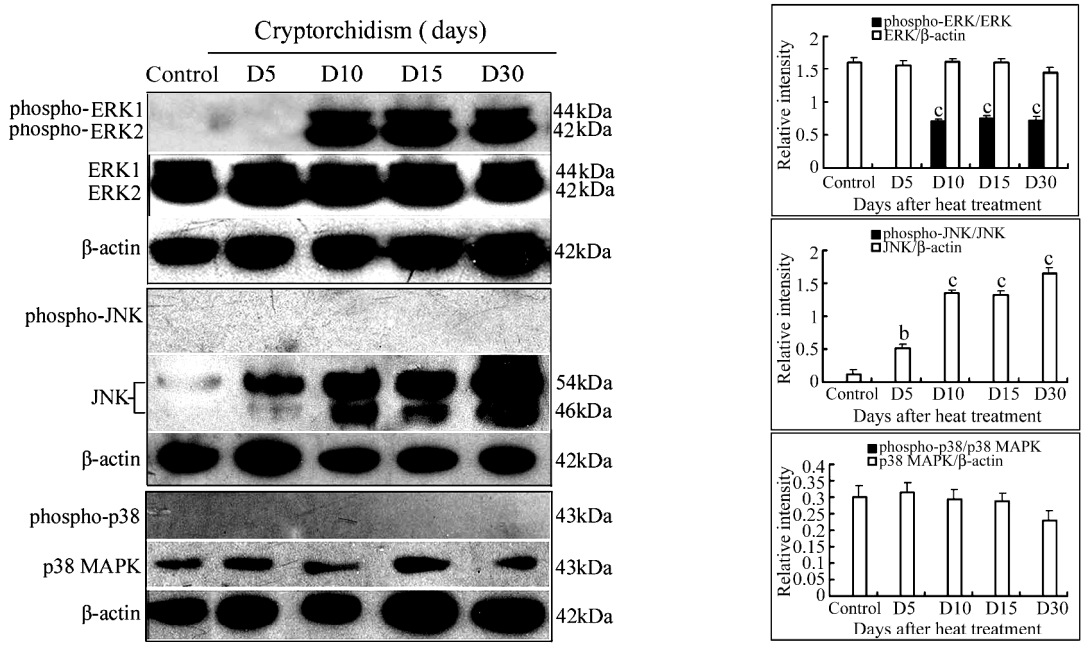

Figure 2. Western blot analysis of phospho-extracellular signal-regulated kinases 1 and 2 (ERK1/2), ERK1/2, phospho-c-Jun N-terminal kinases (JNK), JNK, phospho-p38 mitogen-activated protein kinases (MAPK), and p38 MAPK in the scrotal (control) and cryptorchid monkey testes on days 5, 10, 15 and 30 after surgery (D5, D10, D15, D30). β-actin was used as an internal control. The relative intensity was determined by the ratio of phospho-ERK1/2 to ERK1/2, phospho-JNK to JNK, phospho-p38 MAPK to p38 MAPK, ERK1/2 to β-actin, JNK to β-actin, and p38 MAPK to β-actin, respectively, as measured by densitometry. Data are presented as mean ± SEM ($n = 3$). $^{b}P < 0.05$; $^{c}P < 0.01$, compared with the control.

3.3 Immunohistochemical localization of phospho-ERK1/2, EK1/2 and JNK in the testis of the experimentally cryptorchid adult rhesus monkey

Immunohistochemical staining was used to detect the expression and phosphorylation of ERK1/2 in the scrotal and the cryptorchid testes. Heat stress induced a marked phosphorylation of ERK1/2 in the Sertoli cells on days 10, 15 and 30 after surgery (Figure 3A, D10, D15, D30). ERK1/2 immunostaining was predominantly distributed to the cytoplasm of Sertoli cells (Figure 3B). No obvious difference in ERK1/2 expression was observed between the scrotal and cryptorchid testes. Ex-

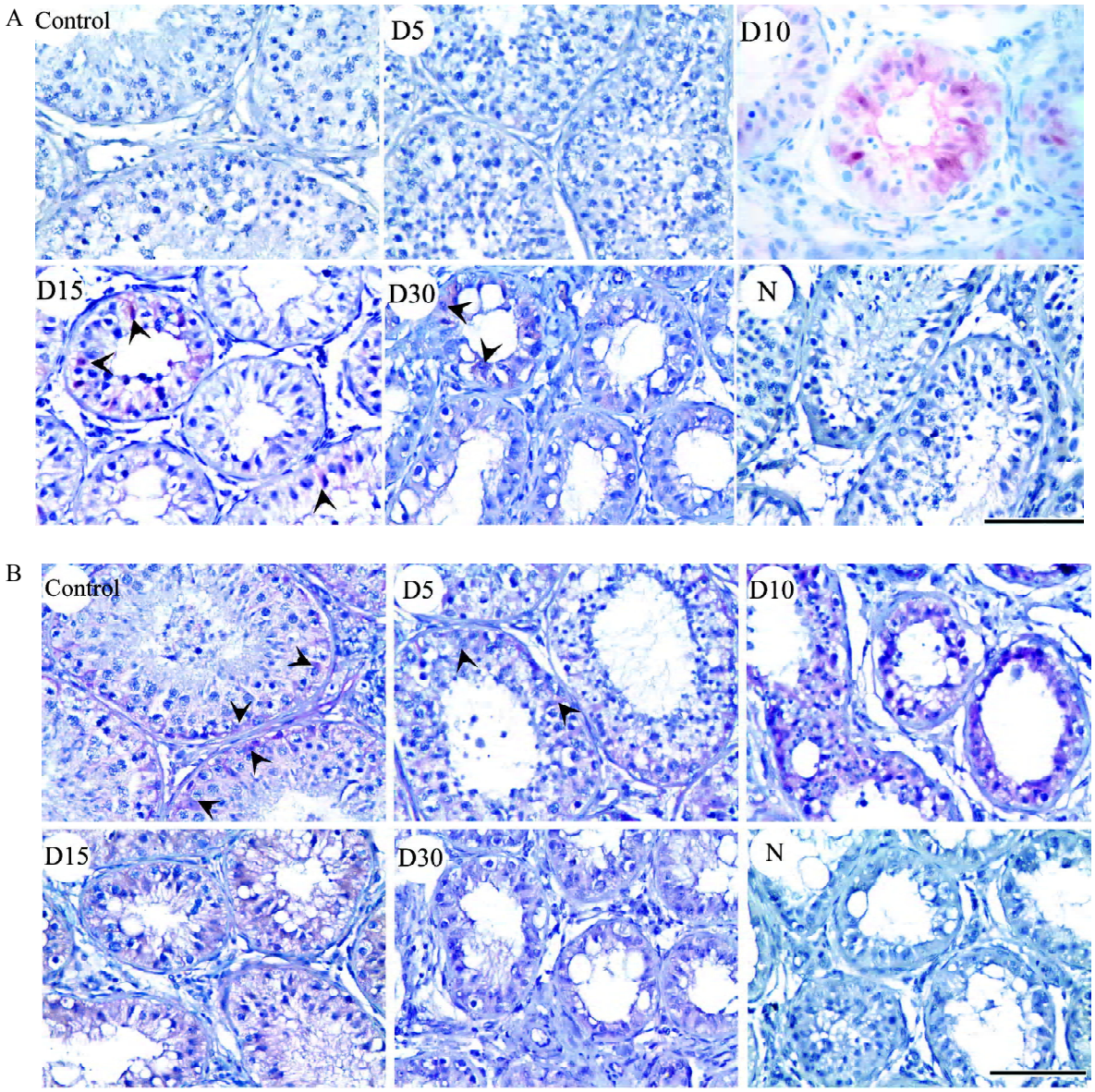

Figure 3. The expression of (A): phospho-extracellular signal-regulated kinases 1 and 2 (ERK1/2) and (B): ERK1/2 in the scrotal (control, Con) and cryptorchid monkey testes on days 5, 10, 15 and 30 after surgery (D5, D10, D15, D30) by immunohistochemical analysis. (A): Red color shows the positive staining of phospho-ERK1/2 in Sertoli cells on D10, D15 and D30 (arrow heads). (B): Red color shows the positive staining of ERK1/2 in Sertoli cells on Control and D5, D10, D15 and D30. Blue color is background counterstaining. N, the negative control without primary antibody at day 10 after surgery. Bar = 100 μm.

pression of the phosphorylated ERK1/2 was also seen in the interstitial and Leydig cells on day 10 after surgery (Figure 3A, D10). Expression of JNK was not detected in the scrotal testis, but was dominant in Sertoli cells on days 5, 10, 15 and 30 after surgery (Figure 4). And there were only few positive JNK signals in the nuclei of spermatids on day 5. However, we did not detect any expression of phospho-JNK (data not shown). Neither the phospho-p38 nor the total p38 MAPK was detected by immunohistochemistry in the scrotal and experimental cryptorchid testes (data not shown).

4 Discussion

It is known that exposure of the testis to abdominal temperature causes germ-cell loss, but the effect of abdominal testis temperature on Sertoli cells remains to be studied in detail. Sertoli cells provide an essential physical and trophic support for the developing spermatogenic cells in the seminiferous tubules [8]. In the present study, changes in the expression of CK18, a marker of immature or undifferentiated Sertoli cells [3], in the Sertoli cells of the cryptorchid testis indicates that Sertoli cells undergo dedifferentiation and return to an immature state in response to heat stress. The molecular mechanisms that underlie dedifferentiation of Sertoli cells in response to heat stress in the cryptorchid testis remain largely unknown. In the present study, we investigated the pattern of expression and activation of ERK1/2, JNK and p38 MAPKs in the rhesus monkey testis in response to heat treatment in order to shed some light on the possible mechanism of heat stress-induced dedifferentiation of Sertoli cells.

The switch of Sertoli cells from proliferation to differentiation is related to the activation state change of ERK [9]. While transient activation is most commonly associated with cellular proliferation, and sustained activation may be required for differentiation [10, 11]. Heat shock has been well documented as a stress that initiates long-term changes within cells. It is now clear that ERK1/2 MAPK can also be activated by various stress stimuli including heat shock [12]. It has also been demonstrated that sustained signaling through the ERK MAPK kinase pathway is capable of driving dedifferentiation of Schwann cells [13], while blocking the ERK MAPK kinase pathway could induce undifferentiated tumor cells to undergo a process of differentiation. Perhaps such a situation might also exist in Sertoli cells after heat treatment. We have demonstrated that ERK 1/2 are present in the testis of the rhesus monkey, and cryptorchidism induces

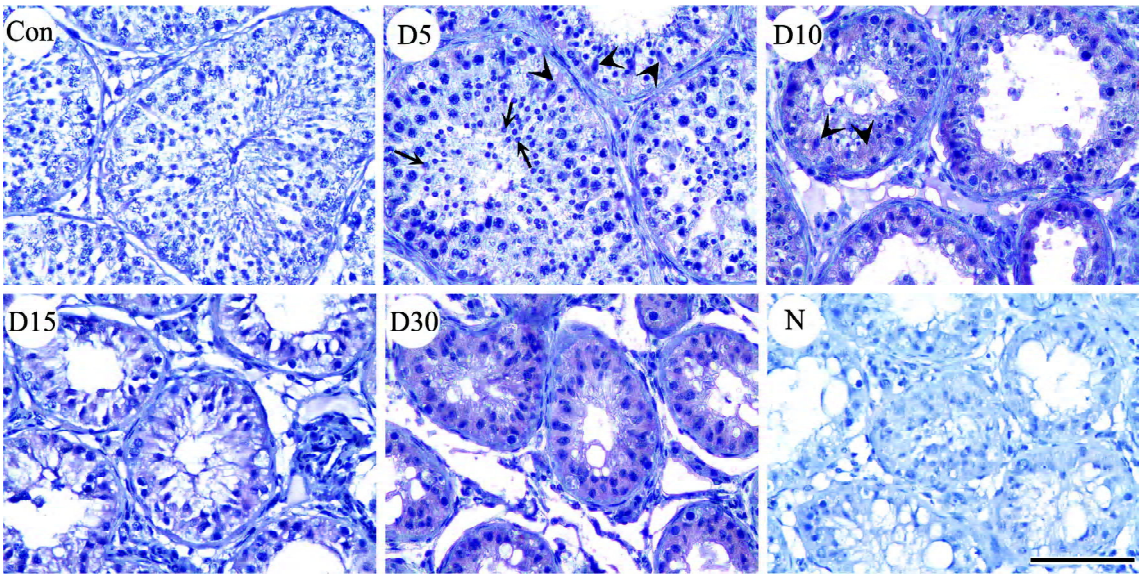

Figure 4. The expression of c-Jun N-terminal kinases (JNK) in the scrotal (control, Con) and cryptorchid monkey testes on days 5, 10, 15 and 30 after surgery (D5, D10, D15, D30) by immunohistochemical analysis. Red color shows the positive staining of JNK in Sertoli cells on D5, D10, D15 and D30 (arrow heads). Arrows show the positive staining of spermatids on day 5. Blue color is background counterstaining. N, the negative control without primary antibody at day 10 after surgery. Bar = 100 μm.

their activation by phosphorylation in the Sertoli cells 10 days after experimental cryptorchid surgery. The spatial and temporal expression and activation of ERK1/2 correlate well with the re-expression of CK18, suggesting that heat stress may induce Sertoli cell dedifferentiation through the activation of ERK signal transduction cascades.

A single stimulus can activate two or more MAPK cascades to varying degrees and the regulation of a cellular response could therefore be the result of several signaling cascades working in concert [14, 15]. For example, heat shock stimulated the phosphorylation and activation of ERK and "stress-activated" JNK MAPK in an interleukin 3-dependent cell line, BaF3, but p38 was not phosphorylated and activated. In other cell types, heat shock fails to activate ERK MAPK but does activate the stress-activated MAPK, such as JNK [16, 17]. Obviously, these responses are cell type-dependent. In our experimentally cryptorchid monkey model, we found that heat stress activated neither p38 MAPK nor JNK MAPK in Sertoli cells. However, heat stress significantly upregulated the expression levels of JNK in Sertoli cells 5 days after experimental cryptorchidism surgery. The increased expression level of JNK lasted until day 30, when the expression level of CK18 was still high, suggesting that JNK might also modulate the dedifferentiation process of Sertoli cells after heat treatment. Similarly, in mild testicular hyperthermia monkey models (local heating of the testis in a 43°C water bath), we found that although both CK18 and ERK1/2 activation occurred shortly after heat shock, the activation of CK18 lasted longer than that of ERK1/2 [18]. This means that in addition to the ERK MAPK signal pathway, other signaling pathways might exist that mediate the dedifferentiation of Sertoli cells marked by re-expression of CK18 after heat treatment. JNK might be one of the important signaling molecules in these pathways.

It has been reported that spermatogonia, spermatocytes and Leydig cells are also capable of expressing the kinases mentioned above. Using whole testes rather than isolated Sertoli cells in the present study, the observed changes in the kinases might partly represent changes in germ cells and Leydig cells. However, our immunohistochemical studies clearly show that the studied kinases were predominantly expressed in the Sertoli cells, indicating that the western blot result mainly represents kinases expressed in Sertoli cells.

In summary, the data from the present study demonstrate for the first time that ERK1/2 are activated in the cryptorchid testis of the rhesus monkey in response to heat stress, which may be related to dedifferentiation of Sertoli cells.

Acknowledgment

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References

1 Frankenhuis MT, Wensing CJ. Induction of spermatogenesis in the naturally cryptorchid pig. Fertil Steril 1979; 31:428–33.
2 Zhang ZH, Hu ZY, Song XX, Xiao LJ, Zou RJ, Han CS, *et al.* Disrupted expression of intermediate filaments in the testis of rhesus monkey after experimental cryptorchidism. Int J Androl 2004; 27: 234–9.
3 Steger K, Rey R, Kliesch S, Louis F, Schleicher G, Bergmann M. Immunohistochemical detection of immature Sertoli cell markers in testicular tissue of infertile adult men: a preliminary study. Int J Androl 1996; 19:122–8.
4 Kyriakis JM, Avruch J. Mammalian mitogen activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001; 81: 807–69.
5 Cobb MH. MAP kinase pathways. Prog Biophys Mol Biol 1999; 71: 479–500.
6 Matsuzawa A, Ichijo H. Molecular mechanisms of the decision between life and death: regulation of apoptosis by apoptosis signal-regulating kinase 1. J Biochem 2001; 130: 1–8.
7 Turgeon B, Lang BF, Meloche S. The protein kinase ERK3 is encoded by a single functional gene: genomic analysis of the ERK3 gene family. Genomics 2002; 80: 673–80.
8 Huleihel M, Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. Asian J Androl 2004; 6: 259–68.
9 Crepieux P, Marion S, Martinat N, Fafeur V, Vern YL, Kerboeuf D, *et al.* The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene 2001; 20: 4696–709.
10 Traverse S, Gomez N, Paterson H, Marshall C, Cohen P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. Biochem J 1992; 288: 351–5.
11 Bokemeyer D, Sorokin A, Dumm MJ. Multiple intracellular MAP kinase signaling cascades. Kidney Int 1996; 49: 1187–98.
12 Ng DC, Bogoyevitch MA. The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the

interleukin 3-dependent ProB cell line BaF3. J Biol Chem 2000; 275: 40856–66.
13 Harrisingh MC, Perez-Nadales E, Parkinson DB, Malcolm DS, Mudge AW, Lloyd AC. The Ras/Raf/ERK signalling pathway drives Schwann cell dedifferentiation. EMBO J 2004; 23: 3061–71.
14 Canman CE, Kastan MB. Signal transduction. Three paths to stress relief. Nature 1996; 384: 213–4.
15 Rausch O, Marshall CJ. Cooperation of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways during granulocyte colony-stimulating factor-induced hemopoietic cell proliferation. J Biol Chem 1999; 274: 4096–105.
16 Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, *et al*. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 1994; 369: 156–60.
17 Elbirt KK, Whitmarsh AJ, Davis RJ, Bonkovsky HL. Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. J Biol Chem 1998; 273: 8922–31.
18 Zhang XS, Zhang ZH, Jin X, Wei P, Hu XQ, Chen M, *et al*. Dedifferentiation of adult monkey Sertoli cells through activation of ERK1/2 kinase induced by heat treatment. Endocrinology 2005; [Epub ahead of print].