

## Expression of Orphan Receptors TR2, TR3, TR4, and p53 in Heat-Treated Testis of Cynomolgus Monkeys (*Macaca fascicularis*)

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**ABSTRACT:** To investigate the possible role of testicular orphan receptors (TR) TR2, TR3, and TR4 in the process of germ cell apoptosis in the heat-treated testis of monkey, we have examined the spatiotemporal expression of the 3 TR mRNAs in relation to p53 mRNA levels in the monkey testis by in situ hybridization and reverse transcription polymerase chain reaction techniques. The results showed that TR2 mRNA was confined to spermatocytes; TR4 and TR3 mRNAs were expressed in both spermatocytes and spermatids. The heat treatment did not change TR2 mRNA level but significantly reduced TR4 mRNA expression in spermatocytes on days 3 and 8 after the heat treatment. TR3 mRNA expression was affected by the heat treatment in a time-dependent manner, with the lowest level on day 30 after the heat shock. Low to moderate signal for p53 mRNA

was detected in spermatocytes before treatment, which increased dramatically on days 3, 8, and 30 after the heat shock. The coincident expression of the testicular TR3 and p53 mRNA, spatially and time dependently, implied that the decrease in TR3 expression in the heat-treated testis might be closely related to the p53 signal pathway, whereas the temporal decrease in TR4 production in the testis at the early stage indicated that this orphan receptor might be also involved in germ cell apoptosis. The data suggest that TR3, TR4, and p53 could be important regulators of germ cell apoptosis induced by the heat treatment, whereas TR2 might not be a key regulator in this process.

Key words: Heat shock, spermatogenesis, primate.

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In most mammals, the testicular temperature is maintained constantly lower than the core body temperature to allow normal spermatogenesis (Kormano, 1967; Sailer et al, 1997). Well-defined evidence indicates that a single exposure of rat or monkey testis to heat (43°C for 30 minutes) results in a selective and reversible germ cell apoptosis, leading to oligozoospermia or azoospermia (Mieusset and Bujan, 1995; Setchell, 1998; Lue et al, 2002; Zhang et al, 2005). Using the terminal uridine nick end labeling (TUNEL) assay, we have found that heat-induced germ cell apoptosis predominantly occurs in spermatocytes and round spermatids (Lue et al, 1999; Zhang et al, 2005).

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However, the exact cellular and molecular mechanisms underlying germ cell apoptosis are unclear.

Orphan receptors belong to a category of the steroid/thyroid hormone receptor superfamily in which cognate ligands have not been identified. Hence, these receptors are designated as orphan nuclear receptors (O'Malley and Conneely, 1992; Lee et al, 1995).

Testicular orphan receptor (TR)2 (also named NR2C1) has been shown previously to be specifically expressed in adult mouse testis and is confined to advanced germ cells (Lee et al, 1996). We have provided data to show that the TR2 mRNAs mainly localize in the spermatocytes and the elongated spermatids in the testes of both rat and rhesus monkey (Mu and Liu, 1999; Guo et al, 2000). The data from TR2 knockout mice suggest that TR2 might not play an essential role in normal testicular development and spermatogenesis (Shyr et al, 2002). Most likely, the function of TR2 could be replaced by other close members of the nuclear receptor superfamily, such as TR4 (also named TAK1 and TR2C2), which shares many similar functions with TR2 (Shyr et al, 2002). Sequence analysis shows that TR4 has high sequence homology with TR2, suggesting

that these 2 orphan receptors constitute a unique subfamily within the steroid receptor superfamily and that they might have similar biochemical and physiological functions (Chang and Kokontis, 1988; Chang et al, 1989, 1994). In the normal mouse testis, TR4 was dramatically increased in the spermatocytes at meiotic prophase, reaching the highest level at this phase during the first wave of spermatogenesis. Results from TR4 knockout mice indicate that TR4 might play an important role in the late meiotic prophase and subsequent meiotic divisions, suggesting that TR4 is essential for normal spermatogenesis (Mu et al, 2004).

TR3 (also called Nur77 or NGFI-B) mainly acts as transcriptional factors to positively or negatively regulate gene expression (Chang and Kokontis, 1988; Hazel et al, 1988; Milbrandt, 1988; Zhang and Pfahl, 1993; Kastner et al, 1995; Mangelsdorf and Evans, 1995). As a product of an immediate-early response gene, TR3 expression could be induced rapidly by various mitogenic inducers, including serum growth factor, epidermal growth factor, and fibroblast growth factor (Chang and Kokontis, 1988; Fahrner et al, 1990; Williams and Lau, 1993; Crawford et al, 1995; Lim et al, 1995). It is reported that TR3 expression is directly associated with cell growth (Kolluri et al, 2003). In contrast to this, reports show an association of TR3 with cell apoptosis (Liu et al, 1994; Woronicz et al, 1994). It has been suggested that TR3 acts in the nucleus to confer its mitogenic effect, but initiates apoptosis when it migrates to the mitochondria (Kolluri et al, 2003).

In our previous report (Zhang et al, 2005), we have demonstrated that a single exposure of monkey testis to 43°C water for 30 minutes once daily for 2 consecutive days resulted in a selective and reversible decrease in spermatogenesis because of an increase in germ cell apoptosis. To investigate the possible involvement of orphan receptors in the process of the heat-induced germ cell apoptosis, we have analyzed changes in expression of the orphan receptors TR2, TR3, and TR4 in relation to p53 mRNA levels in the monkey testis exposed to a higher (43°C) temperature.

## Materials and Methods

### Animals and Experimental Protocol

Sixteen male adult (7–10 years old), healthy, and fertile cynomolgus monkeys (*Macaca fascicularis*) were obtained and housed at the Guangxi Hongfeng Primate Research Center, Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS). These monkeys were only used for mating and did not receive any previous treatment. Animal handling and experimentation were in accordance with the recommendation

of the American Veterinary Medical Association and were approved by the Chinese academic committees of IOZ, CAS. These monkeys were housed in a animal facility under controlled temperature (22°C) and photoperiod (12 23 hours light dark) with free access to water and monkey chow. Eight monkeys were randomly assigned to receive no heat-treatment (control group), and a group of 8 monkeys were given daily testicular exposure to heat (43°C for 30 minutes) for 2 consecutive days (days 1 and 2). Heating of the scrota of the 8 adult monkeys was performed as described previously by Lue et al (1999). Briefly, under light sedation, testicular hyperthermia was induced by immersing the monkey scrota containing the testes into a thermostatically controlled water bath at 43°C for 30 minutes once daily for 2 consecutive days. After heat treatment, the scrotal area was dried and examined for any redness or injury, then the monkeys were returned to their cages and allowed to recover from the effect of the anesthesia. Inspection of the scrota after heat exposure showed no evidence of thermal injury to the scrotal skin after this short duration of modest increase in temperature. In each group, 3 monkeys were used only for semen and blood sample collection, and the other 5 were used for testicular tissue collections. To guarantee that we could get portions of testicular tissue from the 3 monkeys at each time point, testicular biopsies or unilateral castrations were performed in one testis of the animals on the day before (Pre, control) and on days 3, 8, 30, 84, and 144 (D3, D8, D30, D84, D144) after heat exposure.

### Testicular Tissue Collection

Testis biopsy or unilateral castration was performed under general anesthesia with ketamine (10 mg/kg) and atropine (0.05 mg/kg). Postoperatively, the animals were treated with oxymorphone (0.1 mg/kg) for analgesia. The operation was performed under aseptic conditions, and only 1 testis from each monkey was used for tissue collection. One portion of the tissue was immersion-fixed in Bouin's solution, embedded in paraffin, and subsequently sectioned for in situ hybridization, and the other portion was snap-frozen in liquid nitrogen for RNA isolation.

### In Situ Hybridization Analysis

The digoxigenin (DIG)-labeled antisense and sense cRNA probes of each gene were synthesized according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Deparaffinized and rehydrated tissue sections (5 µm) were pretreated with 10 µg/mL proteinase K at 37°C for 15 minutes and then subjected to fixation in 4% paraformaldehyde to preserve mRNA and terminate proteinase K digestion. Before hybridization, the sections were dehydrated through a graded ethanol series and allowed to air dry. After prehybridization at 50°C for 3 hours in prehybridization buffer containing 50% deionized formamide and 2× saline sodium citrate (SSC) without probe, hybridization was carried out at 55°C overnight with DIG-labeled antisense or sense probe in hybridization buffer (10 mM Tris-Cl, pH 7.5, 2× SSC, 50% deionized formamide, 1× Denhardt, 2.5 mM dithiothreitol, 5% dextran sulfate, 250 µg/mL yeast tRNA,

and 0.5% sodium dodecyl sulfate). After hybridization, the sections were washed thoroughly in  $2\times$ ,  $1\times$ , and  $0.1\times$  SSC twice for 15 minutes each at  $42^{\circ}\text{C}$ , rinsed in DIG buffer I (0.1 M maleic acid, 150 mM NaCl, pH 7.5) for 5 minutes, blocked with DIG buffer II (1% blocking reagent in DIG buffer I) at room temperature for 1 hour, incubated with alkaline phosphatase-conjugated anti-DIG antibody at  $4^{\circ}\text{C}$  overnight (1:5000), and washed in DIG buffer I, 3 times. The hybridization signals were visualized with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt in alkaline phosphatase buffer. The slides were rinsed in 95% ethanol for 30 minutes and mounted. Sense probe hybridizations were performed as in the negative controls.

#### Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from the testis tissue with Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions. The first strand of complementary DNA was synthesized by reverse transcription (RT) of 2  $\mu\text{g}$  of total RNA with oligo-dT primer and Superscript II reverse transcriptase (Gibco-BRL). After the RT reaction, 1  $\mu\text{L}$  of the incubation mixture was used as the template for the subsequent polymerase chain reaction (PCR) amplification. The sense strand (S) and antisense strand (AS) PCR primer sequences are: TR2(S), 5'-TCCAAAGACTGCGTTATCA-3'; TR2(AS), 5'-GCTGGCTCTGTTTTATTTC-3'; TR3(S), 5'-GACGGCTACACAGGAGAG-3'; TR3(AS), 5'-AACTTGAAGGAGGCAGAGG-3'; TR4(S), 5'-CAGCAGTTCA TCCTGACCAGCCC-3'; TR4(AS), 5'-CTGCTCCGGCAGC TGTAGGTC-3'; p53(S), 5'-GTTTCCGTCTGGGCTTCT-3'; p53(AS), 5'-ACCTCAGGCGGCTCATAG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH(S)), 5'-ACCA CAGTCCATGCCATCAC-3'; GAPDH(AS), 5'-TCCACC ACCCTGTTGCTGTA-3'. The PCR products were collected between 23 and 38 cycles, and the exponential increase in PCR products was confirmed. PCR reactions of TR2, TR3, TR4, p53, and GAPDH were performed for 28, 28, 26, 28, and 26 cycles at an annealing temperature of  $50^{\circ}\text{C}$ ,  $55^{\circ}\text{C}$ ,  $55^{\circ}\text{C}$ ,  $50^{\circ}\text{C}$ , and  $55^{\circ}\text{C}$ , respectively. Amplification of GAPDH gene transcripts was used to confirm RNA integrity and efficiency. The PCR products of 3 separate PCR reactions were run on 1% agarose gel. Intensities of autoradiographic bands were estimated by densitometric scanning with the BioImage scanner (Cheshire, United Kingdom).

#### Data Analysis

Samples from 3 individual animals at each tissue collection time point were analyzed. Statistical analysis was performed with Statistical Package for Social Science (SPSS for Windows package release 10.0, SPSS Inc, Chicago, Ill). Statistical significance was determined by 1-way ANOVA. Post hoc comparisons between treatment group means were made with Fisher's protected least significance difference test. Differences were considered significant at  $P < .05$ . Values shown in all the figures were given as the mean  $\pm$  SEM.

## Results

### Expression of TR2, TR4 mRNAs in the Heat-Treated Monkey Testes

To detect the spatiotemporal expression and variation of TR2 and TR4 mRNAs in the heat-treated testes of the cynomolgus monkeys, their mRNA levels were analyzed before (Pre) and after heat stress on days 3, 8, 30, 84, and 144 by in situ hybridization and RT-PCR. As shown in Figure 1, TR2 mRNA was mainly localized in the spermatocytes within the seminiferous epithelium before the heat treatment (Figure 1A, Pre), with no obvious changes in the mRNA levels on days 3, 8, 30, 84, and 144 after the heat treatment. The in situ hybridization result was confirmed by the RT-PCR data, shown in Figure 1B. The densitometry analysis showed no significant difference of the expression levels of TR2 mRNA before and after the heat stress ( $P > .05$ ). As another member of the TR2/TR4 heterodimer, the TR4 mRNA was especially prominent in the spermatocytes and the spermatids (Figure 2A, Pre). The positive signal of TR4 mRNA in the spermatids significantly decreased ( $P < .05$ ) on days 3 and 8 (Figure 2A [D3, D8] and B) associated with loss of germ cells, and then the signal was gradually increased to reach baseline, untreated levels ( $P > .05$ ) on days 30, 84, and 144 (Figure 2A [D3, D30, D84, D144] and B).

### Expression of TR3 mRNAs in the Heat-Treated Monkey Testes

The positive signals of TR3 mRNA were most abundant in the spermatocytes and the spermatids. The TR3 expression levels dropped significantly on day 3, reaching the lowest level on day 30 after the heat shock. Consistent with spermatogenic recovery, TR3 mRNA expression returned to pretreatment levels on days 84 and 144 (Figure 3A). The in situ changes in TR3 mRNA expression were further confirmed by the RT-PCR data, as shown in Figure 3B. The mRNA expression levels decreased significantly on days 3, 8, and 30 ( $P < .05$ ), and then returned to basal levels on days 84 and 144.

### Localization of p53 mRNA and Its Temporal Changes in Monkey Testes After Heat Shock

As shown in Figure 4A, low to moderate p53 mRNA expression was mainly detected in the primary spermatocytes at baseline before the heat shock (Pre), which dramatically increased from day 3 to day 30 after the heat treatment. On day 84, the positive germ cells hybridized with p53 cRNA probe seemed to be less than was observed on day 30 and then dropped back to the pretreatment level on day 144. Semiquantitative analysis of p53 mRNA expression by RT-PCR also confirmed

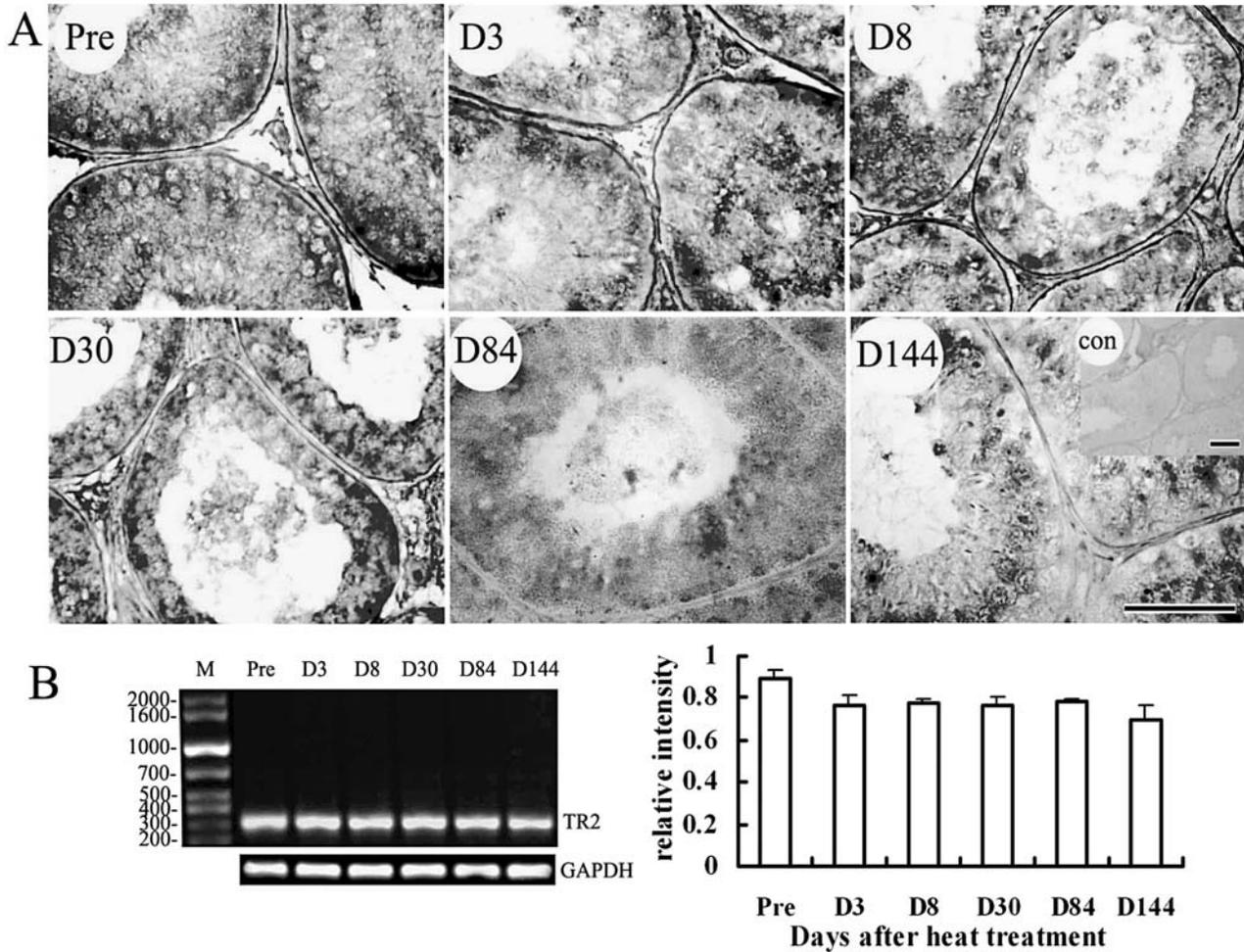


Figure 1. Expression of testicular orphan receptors (TR)2 mRNA in the heat-treated monkey testis. **(A)** In situ hybridization analysis of TR2 mRNA expression in testis of monkey before (Pre) and after heat stress (day [D]3, D8, D30, D84, D144). Dark blue color shows the positive signal. Con indicates negative control with sense probe on Pre sections. Scale bar = 100  $\mu$ m. **(B)** Reverse transcription polymerase chain reaction analysis of TR2 mRNA expression in the heat-treated monkey testis. mRNA levels were determined by densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative intensity was the ratio of TR2 mRNA to GAPDH. Data are presented as mean  $\pm$  SEM (n = 3). M indicates 1 kb plus DNA marker (bp).

the observed results by in situ hybridization. The RT-PCR data showed that the content of p53 mRNA significantly increased on day 3 ( $P < .05$ ); a fourfold increase was detected on day 8 ( $P < .01$ ), reaching a maximum level on day 30 ( $P < .01$ ), and then dropped to the control level on day 144 (Figure 4B).

**Discussion**

In our previous study, we used nonhuman primates as a model to test the effect of heat on spermatogenesis by heating the scrota at 43°C for 30 minutes once daily for 6 consecutive days, and we found that the treatment could induce oligozoospermia (Lue et al, 2002). The higher the temperature and the longer the duration of

heat exposure, the more severe the germinal epithelium damage. Then we found that a mild testicular hyperthermia (43°C for 30 minutes once daily for 2 consecutive days) induced reversible oligozoospermia or azoospermia (Zhang et al, 2005). We also found no differences in serum testosterone, follicle-stimulating hormone, or luteinizing hormone levels after the testicular heat treatment (Lue et al, 2005). We demonstrated that a Bcl-2 family member was involved in regulation of spermatogenesis in the cryptorchid monkey testis (Guo et al, 2001; Zhou et al, 2001; Zhang et al, 2003, 2005), and the Fas signaling system might be dispensable for heat-induced germ cell apoptosis in the testis (Hikim et al, 2003). In this study, we further demonstrated the involvement of TR3, TR4, and the tumor suppressor protein p53 in the regulation of

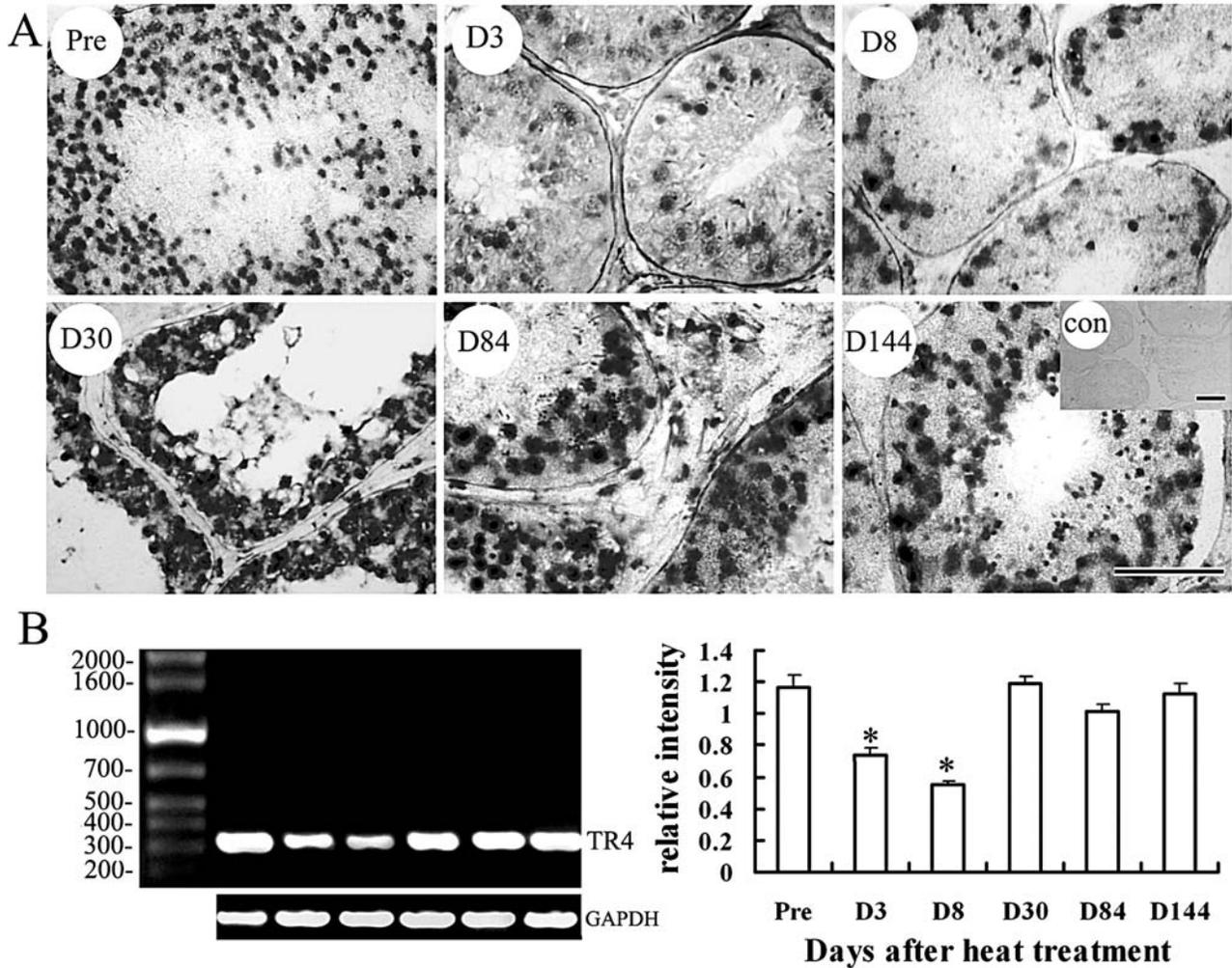


Figure 2. Expression of testicular orphan receptors (TR)4 mRNA in the heat-treated monkey testis. (A) In situ hybridization analysis of TR4 mRNA expression in testis of monkey before (Pre) and after heat stress (day [D]3, D8, D30, D84, D144). Dark blue color shows the positive signal. Con indicates negative control with sense probe on Pre sections. Bar = 100  $\mu$ m. (B) Reverse transcription polymerase chain reaction analysis of TR4 mRNA expression in the heat-treated monkey testis. mRNA levels were determined by densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative intensity was the ratio of TR4 mRNA to GAPDH. Data are presented as mean  $\pm$  SEM (n = 3). \* Significantly different at  $P < .05$ . M indicates 1 kb plus DNA marker (bp).

spermatogenesis through germ cell apoptosis in the heat-treated monkey testis.

As a subfamily of the nuclear receptors and transcriptional factors, orphan receptors regulate specific gene expression during the process of cell growth, development, and differentiation (Evans, 1988; Mangelsdorf et al, 1995). In this study, we have demonstrated that TR2 might not be the major factor involved in germ cell apoptosis. This observation is consistent with the finding of Shyr et al (2002). Evidence from TR4 knock-out male mice showing a reduced sperm production because of disruption of spermatogenesis at stages X–XII indicated the specific essential role of TR4 in regulation of spermatogenesis at the late phases of meiotic division (Mu et al, 2004). In this study, no obvious change in TR4 expression in the testis was observed at later time points,

implying the specific functional role of TR4 in regulation of spermatogenesis in the heat-treated monkey testis. Interestingly, heat-induced germ cell apoptosis in rats predominantly occurred in pachytene spermatocytes at stages I–IV and IX–XII, diplotene and dividing spermatocytes at stages XIII and XIV, and early (steps 1–4) spermatids, which were most susceptible to heat (Lue et al, 1999). Therefore, TR4 could be one of the putatively important molecules regulating germ cell apoptosis induced by heat treatment.

The TR3 mRNA was expressed mainly in the spermatocytes; dramatically decreased on days 3, 8, and 30 when germ cell apoptosis took place mostly in the heat-treated testis (Zhang et al, 2005); and recovered to the control level on days 84 and 144 after the heat treatment. TR3 is known to be a transcription factor

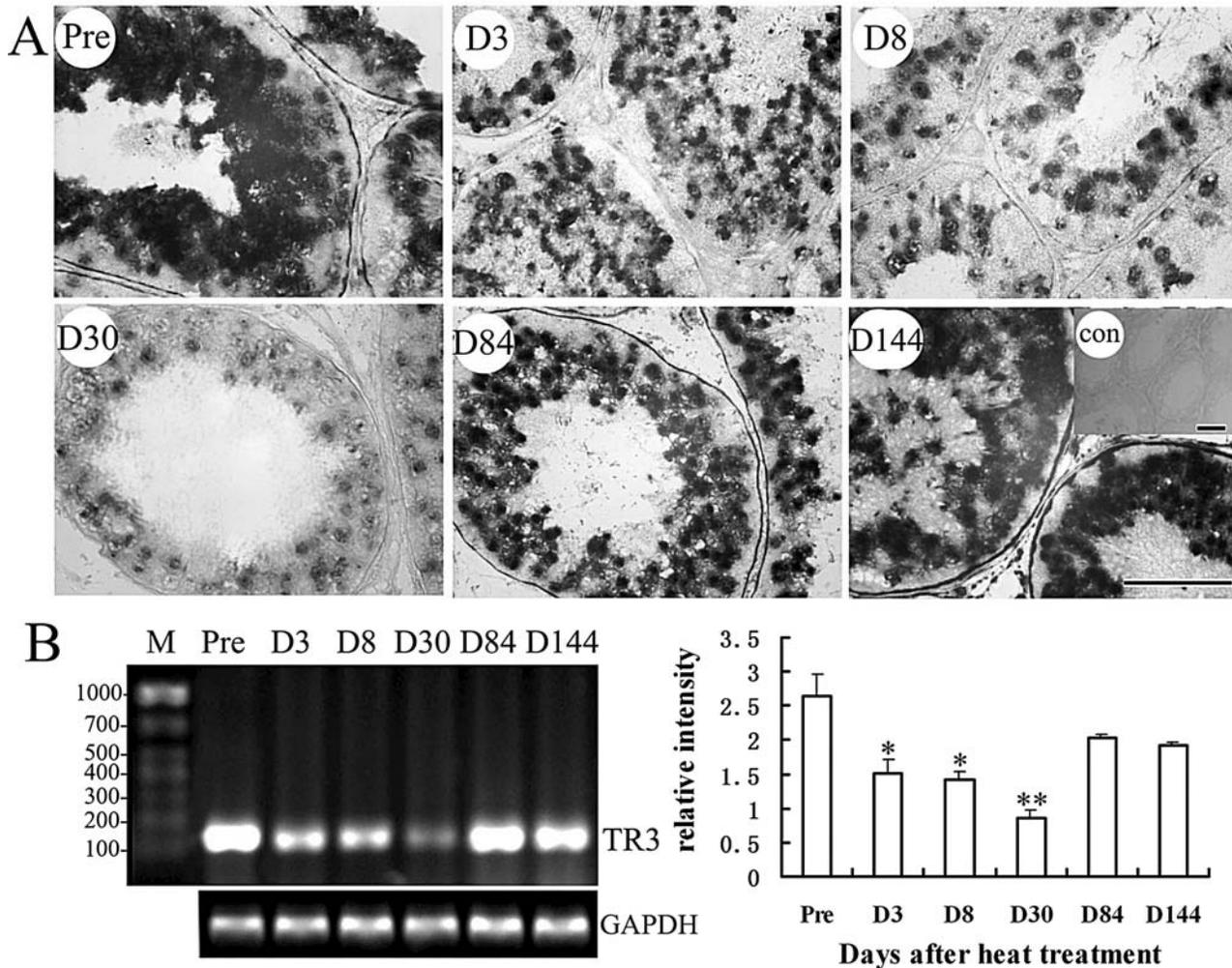


Figure 3. Expression of testicular orphan receptors (TR3) mRNA in the heat-treated monkey testis. **(A)** In situ hybridization analysis of TR3 mRNA expression in testis of monkey before (Pre) and after heat stress (day [D]3, D8, D30, D84, D144). Dark blue color shows the positive signal. Con indicates negative control with sense probe on Pre sections. Bar = 100  $\mu$ m. **(B)** Reverse transcription polymerase chain reaction analysis of TR3 mRNA expression in the heat-treated monkey testis. mRNA levels were determined by densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative intensity was the ratio of TR3 mRNA to GAPDH. Data are presented as mean  $\pm$  SEM ( $n = 3$ ). \*  $P < .05$ ; \*\*  $P < .01$ . M indicates 1 kb plus DNA marker (bp).

involved in modulation of gene expression linked to cell proliferation and apoptosis (Weih et al, 1996; Wu et al, 2002). We have reported that both TR3 mRNA and its protein are expressed in a significant amount in germ cells and suggested that the molecule might play an essential role in regulating germ cell development at an early stage in mice (Mu and Liu, 1998). It is known that p53 is capable of inhibiting cell cycle progression or inducing cell apoptosis in response to "stress" or DNA damage (Levine, 1997; Giaccia and Kastan, 1998; Koumenis et al, 2001; Boekelheide, 2005). Higher testicular temperature induces p53-mediated germ cell apoptosis (Yin et al, 1998). The p53 expression in the testis is abundant and confined to the pachytene spermatocytes (Schwartz et al, 1993). A p53-mediated

cell cycle checkpoint eliminates heat-induced DNA damages by apoptosis in the cryptorchid testis (Mu et al, 2000; Yin et al, 2002). We demonstrated in this study that p53 mRNA was restricted to the primary spermatocytes and was increased significantly by heat treatment in monkeys. On the basis of the same localization of TR3 with p53 in the germ cells, we presume that the heat-induced TR3 repression could be closely related to the p53 signal pathway; however, further investigation is necessary to test this hypothesis. These 2 transcription factors might be key regulators of spermatogenesis in the heat-treated monkey testis. In addition, TR3 is able to translocate from the nucleus to mitochondria and interact with Bcl-2 to induce apoptosis (Lin et al, 2004). Our present investigation, taken together, has identified

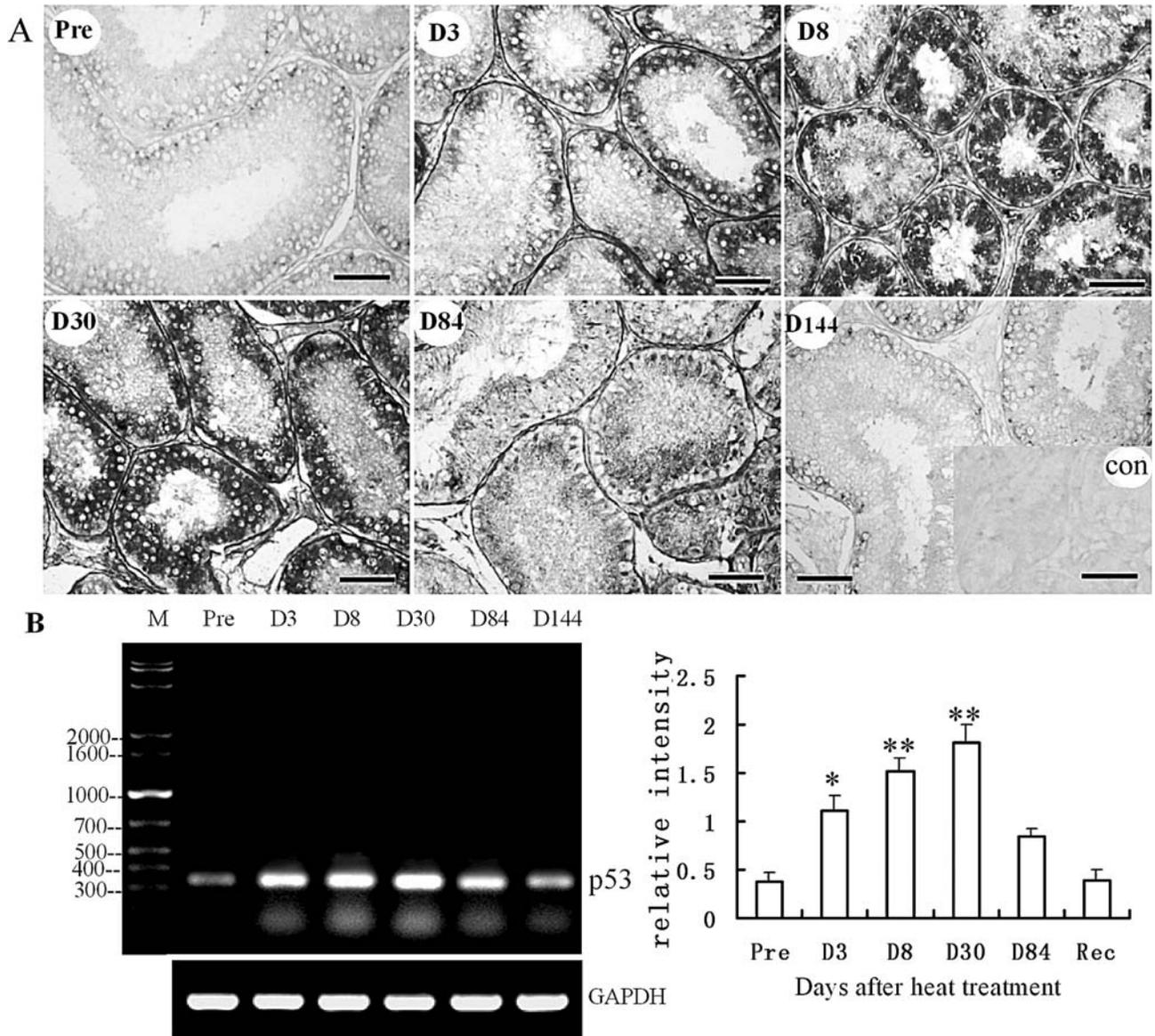


Figure 4. Expression of p53 mRNA in the heat-treated monkey testis. (A) In situ hybridization analysis of p53 mRNA expression in testis of monkey before (Pre) and after heat stress (day [D]3, D8, D30, D84, D144). Dark blue color shows the positive signal. Con indicates negative control with sense probe on D8 sections. Bar = 100  $\mu$ m. (B) Reverse transcription polymerase chain reaction analysis of p53 mRNA expression in the heat-treated monkey testis. mRNA levels were determined by densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative intensity was the ratio of p53 mRNA to GAPDH. Data are presented as mean  $\pm$  SEM (n = 3). \*  $P < .05$ ; \*\*  $P < .01$ . M indicates 1 kb plus DNA marker (bp).

the expression patterns of TR2, TR3, TR4, and p53 mRNAs in the heat-treated monkey testis. Our results suggest that TR3, TR4, and p53 might be involved in the process of germ cell apoptosis induced by the heat treatment. The 2 orphan receptors, however, might act on the testis via different signal pathways; TR3 expression is closely related to the p53 signal pathway; and the molecular mechanism for TR4 action on germ cell loss (occurring at an earlier time after testicular warming) is

not clear. TR2 expression was not significantly changed in the testis, thus suggesting that it might not be a key regulator of spermatogenesis in the heat-treated testis.

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