计划生育生殖生物学国家重点实验室简况及论文集

State Key Laboratory of Reproductive Biology Brief Introduction and Papers

2000

中国科学院动物研究所
Institute of Zoology, Chinese Academy of Sciences
计划生育生殖生物学国家重点实验室
State Key Laboratory of Reproductive Biology

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研究方向：

1995 年经学术委员会讨论，计划生育生殖生物学国家重点实验室的研究方向已集中为三大方面：(1)配子发生、成熟、排放以及黄体形成、萎缩的分子机理；(2)受精的分子机理和生殖过程；(3)胚胎着床的分子机理和抗着床作用。目的是从形态学、生理学、生物化学、细胞生物学、分子生物学等不同角度，在细胞和分子水平上探讨生殖调控的基本规律，同时为发展有效、安全、经济、实用的避孕方法奠定基础。

课题申请指南：

所在课题申请将围绕计划生育生殖生物学国家重点实验室的三大研究方向进行。

1. 生殖内分泌学研究
2. 生殖腺的细胞和分子生物学研究
3. 生殖细胞发育、成熟和排放机理的研究
4. 受精机理和生殖工程研究
5. 胚泡着床分子机理的研究
6. 妊娠早期人胎盘的细胞和分子生物学研究
7. 与生殖相关的生物活性物质（蛋白质、细胞因子、激素）的基因调控与基因工程研究
8. 有关避孕药物作用机制、开发应用与产业化的研究

2000 年资助课题一览表

（2000.05—2001.04）

序号	课　题　名　称	申请人	资助额（万元）
1999 年延续课题:			
1	实验动物体细胞克隆的若干影响因素的研究	王敏康（孙青原）	2.0
2	蛋白激酶在卵细胞周期调控中的作用 cAMP 和 PKC 对 MAPK 活性的影响	孙青原	2.0
3	胚胎滋养层 MHCII 类抗原表达调控机理的研究	彭景楩	2.0
4	卵巢的甾素——血管紧张素与卵泡发育和闭锁	吴尔若（王　红）	2.0
5	恒河猴胚胎植入过程中母胎界面细胞外基质和整合素的协同表达	李维智（朴允尚）	1.4
6	青春期 SF-1 对睾酮的调控	沙家豪（朴允尚）	1.3
7	Nesterone 对垂体促性腺细胞作用的分子机理	冷　颖（朴允尚）	1.3
2000 年新申请课题:			
1	牛体细胞克隆继代再克隆的研究	章孝荣（陈大元）	2.0
2	人胚泡着床调控机制的研究	陈士岭（刘以训）	2.0
3	血管内皮生长因子在小鼠胚胎着床中的作用及其与整合素关系的研究	郑　行（段恩奎）	2.0
4	新生血管新标记基因在植入期小鼠子宫内的表达和功能	阎锡蕴（刘以训）	2.0

序号	课 题 名 称	申请人	资助额（万元）
5	小鼠胚泡植入早期细胞外基质、整合素和基质金属蛋白酶之间的作用关系	赵兴绪（段恩奎）	2.0
室内课题资助:			
1	细胞因子对胚胎着床的作用机理及其应用	彭景楩	3.0
2	子宫内膜血管易变性调节机制的探讨	王 红	3.0

论文与专著目录

（一） 研究论文

性腺功能及配子发生

受精机理

胚胎着床机理

其它内容

（二）综述及科普文章

注：标*为 SCI 收录论文

（三）专 著

专著

陈大元主编 (2000)《受精生物学》
科学出版社 (ISBN 7-03-008504-3/Q 974)

专著中的章节

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 31, Issue of August 4, pp. 23877–23883, 2000
Printed in U.S.A.

The p53/Retinoblastoma-mediated Repression of Testicular Orphan Receptor-2 in the Rhesus Monkey with Cryptorchidism*

Received for publication, December 16, 1999, and in revised form, May 1, 2000
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M910158199

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Whereas the linkage of infertility to cryptorchidism, the failure of the testis to descend into the scrotum at birth, has been well documented, the detailed molecular mechanism remains unclear. Here we report that the testicular orphan receptor-2 (TR2) expression, which modulates many signal pathways, was completely repressed in the surgery-induced cryptorchidism of the rhesus monkey. Further studies link TR2 repression to the induction of p53 and results suggest that induced p53 could repress TR2 expression via the p53→p21→CDK→Rb→E2F signal pathway. In return, TR2 could also control the expression of p53 and Rb through the regulation of human papillomavirus 16 E6/E7 genes. Together, our data suggest a feedback control mechanism between TR2 and p53/Rb tumor suppressors, which might play important roles in male infertility associated with cryptorchidism.

With the exception of elephants and whales, most male mammals have a scrotum with the scrotal temperature always lower than that of the abdomen (1). This decrease of a few degrees in the scrotum is believed to contribute to an optimal environment for testes function. Cryptorchidism, the failure of the testes to descend into the scrotum at birth, affects 1% of newborn boys in the United States (2) and reports suggest that the worldwide incidence is rising (3). The subsequent infertility associated with cryptorchidism is attributed to testicular suprascrotal temperature, because *in situ* cooling of abdominal testes in dogs and pigs results in normal spermatogenesis (4, 5). In mice, spermatogenesis ceased when the testis was displaced surgically into the abdominal cavity and then was restored when the testis was surgically returned back into the scrotum (6). Other clinical conditions that raise scrotal temperature, such as varicocele and fever (7) or even high ambient temperature (8), can also reduce sperm production. Early reports suggested that cryptorchidism could induce apoptosis in testes (9). However, the detailed molecular mechanism of infertility associated with cryptorchidism remains unclear.

Nuclear receptors constitute a superfamily of transcription factors that regulate gene expression in a wide variety of biological processes, such as growth, differentiation, and development (10, 11). The orphan receptors belong to the nuclear receptor superfamily, although their biological significance has been debated because of the lack of known ligands for these receptors (12). The testicular orphan receptor-2 (TR2)[1] was isolated from testes and prostate cDNA libraries and its cDNA encodes a protein of 603 amino acids with a calculated molecular mass of 67 kilodaltons (13, 14). The expression of TR2 has been detected in testes, ventral prostate, seminal vesicles, and many other tissues. Among these tissues and organs, the TR2 is most highly expressed in testes. Immunohistochemical staining has shown that the TR2 was localized specifically in advanced germ cells in mice (15).

The p53 protein is a tumor suppressor that arrests the cell cycle in response to DNA damage. The p53 expression in testis is high and thought to be confined to the tetraploid (4N) pachytene spermatocytes (16). Primary spermatocytes may be particularly sensitive to DNA damage because of the active DNA rearrangement events that occur with meiosis (17). The p53 plays a role in normal differentiation and development, and this role is strongly supported by the observation that p53 expression at midgestation is confined to the differentiation region (18). Furthermore, *in situ* hybridization analyses of testes sections of the p53 promoter-CAT mice, with either a chloramphenicol acetyltransferase (CAT) or p53 probe, demonstrated a predominant CAT activity, indicating a cyclical pattern of p53 expression in the testes of adult mice (19).

The retinoblastoma gene product (Rb) is a phosphoprotein, which can both regulate cell cycle progression and inhibit apoptosis (20–22). Rb can be regulated through phosphorylation by cyclin-dependent kinase (CDK) and when hyperphosphorylated Rb loses its ability to block cell-cycle progression. Upon dephosphorylation, Rb is activated and induces growth arrest at the G_1 phase of the cycle. Interestingly, the Rb activity can be regulated by p53 through the induction of p21 (23), which is a p53 target gene and a CDK inhibitor. Increased levels of p21 result in an active, hypophosphorylated Rb that can mediate G_1 arrest. Overexpression of p21 can inhibit apoptosis (24–26), presumably through blocking Rb phosphorylation. Hence, the functional status of Rb has some potential correlation to the cellular outcome of p53-mediated events (27). Interestingly, like the p53, Rb is also highly expressed in tetraploid pachytene spermatocytes (28).

Although cryptorchidism is known to cause infertility in man because of disruption of spermatogenesis, the exact cellular and molecular mechanism is unclear. Because the biological mechanisms of reproduction of the rhesus monkey are very similar to those of the human, surgically induced cryptorchidism is a reproducible model in which to study spermatogenesis.

* This work was supported by National Institutes of Health Grant DK47258, the National Natural Sciences Foundation of China Grant 39870368, and the Chinese Fundamental Basis Research Program (973). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TR2, TR2 testicular orphan receptor; CAT, chloramphenicol acetyltransferase; Rb, retinoblastoma; CDK, cyclin-dependent kinase; p21, $p21^{waf1/cip1}$; HPV-16, human papillomavirus type 16.

To investigate the molecular mechanism of male infertility caused by cryptorchidism, a model of unilateral surgical cryptorchidism in the rhesus monkey was employed. In this model, one testis was restored to its predescended position in the abdomen, whereas the other testis remained in the scrotum as an euthermic model. We report here, for the first time, that the high temperature created by the cryptorchidism repressed the TR2 and that this repressive effect may proceed through a p53→p21→CDK→E2F signal pathway.

MATERIALS AND METHODS

Plasmids and Probes—p2709TR2CAT, which links the CAT reporter gene to the TR2 5′-promoter encompassing −2709 to +22 nucleotides of

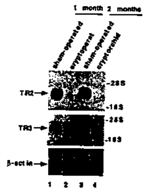

FIG. 1. **Repression of orphan receptor TR2 by surgery-induced cryptorchidism in the rhesus monkey.** Both the cryptorchid and sham-operated contralateral scrotal testes were removed from the body 1 and 2 months after surgery. Total RNA was isolated, and 30 μg was then separated by 1% formaldehyde agarose gel electrophoresis and then transferred onto nylon membrane. The membrane was probed with α-^{32}P-labeled human TR2-11 and TR3 5′-cDNA fragments. The same hybridization membrane was stripped and rehybridized with a β-actin gene cDNA probe to serve as an internal control for the normalization of RNA. Molecular weight markers shown on gel are 18 and 28 S rRNAs. This figure is representative of results from three experiments.

the 5′-flanking region of the TR2 gene, was previously reported (29). The wild type p53 expression plasmid pC53-SN3 and the mutant p53 expression plasmid pC53-SCX-3, gifts of Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) were described previously (30). The plasmids full-length pGEX-Rb, pGEX-Rb large A/B pocket, and pGEX-Rb C-pocket were gifts of Dr. John Ludow (University of Rochester, Medical Center, Rochester, NY). The plasmids pBluescript-p21 and pDC-E2F1 were generous gifts from Dr. Xiao-Fan Wang (Duke University Medical Center, Durham, NC) and Dr. David M. Livingston (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA), respectively. The plasmids full-length pCDNA-Rb, pCDNA-Rb large A/B pocket, and pCDNA-Rb C pocket were generated by digesting with *Bam*HI and *Eco*RI, and the *Bam*HI-*Eco*RI fragments of each plasmid were then cloned separately into the *Bam*HI-*Eco*RI site of the vector pCDNA3. All plasmids were verified by restriction enzyme analysis and DNA sequencing.

The TR2 probe was a 423-base pair fragment upstream of the *Aat*II site in the N-terminal of the human TR2 cDNA. The TR3 probe was a 485-base pair *Xcm*I fragment in the N-terminal of the human TR3 cDNA. The p53 probe was a 557-base pair fragment upstream of *Pvu*II site in the N-terminal of the human p53 cDNA. The p21 probe was a 218-base pair *Pst*I fragment in the N-terminal of human p21 cDNA. Each probe was labeled with [α-^{32}P]dCTP using the Rediprime™ Random Primer Labeling System (RPN 1633, Amersham Pharmacia Biotech) and purified using Probe Quant™ G-50 Micro Columns (27-5335-01, Amersham Pharmacia Biotech).

Animal and Tissue Preparation—Adult male rhesus monkeys were raised in the Kunming Primate Center, Kunming, China. To induce unilateral cryptorchidism, monkeys were anesthetized and a small incision was made in the abdomen. The gubernaculum was cut on the right side to displace the testis into the abdomen. Suturing the inguinal canal on right side prevented the testis decent. The left testis was manipulated into the abdomen and then placed back into the scrotum to serve as an euthermic control. Both the surgically induced cryptorchid and contral-lateral sham-operated scrotal tests were removed either 1, 1.5, or 2 months after surgery. The testes were decapsulated, snap-frozen in liquid nitrogen, and stored at −70 °C for later RNA and protein analysis.

Northern Blot Analysis—Total RNA was extracted using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol and then purified by guanidine-isothiocyanate CsCl gradient ultracentrifugation as described by Davis *et al.* (31). Each RNA sample (30 μg) was size-fractionated on 1% agarose, 30% formaldehyde gels and transferred to nylon membrane (Nytran, Schleicher & Schuell,

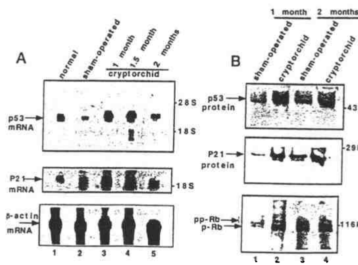

FIG. 2. **Induction of p53 and p21 and hypophosphorylation of Rb in the context of surgery-induced cryptorchidism in the rhesus monkey.** Testis total RNA was prepared as described in Fig. 1. *A*, the total RNA samples obtained from the indicated rhesus monkey testes were subjected to Northern blot analysis using α-^{32}P-labeled human p53 or p21 cDNA fragments. The same hybridization membrane was stripped and rehybridized with a β-actin gene cDNA probe to serve as an internal control for the normalization of RNA. Molecular weight markers shown on the gel are 18 and 28 S rRNAs. *B*, protein extracts obtained from the indicated rhesus monkey testes were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membrane. The membrane represented in the *upper panel* was first incubated with blocking buffer and then incubated with an anti-human p53 polyclonal antibody followed by an alkaline phosphatase-conjugated secondary antibody. The protein extracts were also immunoprecipitated with either 5 μl of anti-human p21 (*middle panel*) monoclonal antibody or 5 μl of anti-human Rb (*lower panel*) monoclonal antibody. Immunoprecipitates were separated and transferred to membranes, and Western blot analysis was performed using the same method described for p53. *pp-Rb*, hyperphosphorylated (inactive) form of Rb. *p-Rb*, hypophosphorylated (active) form of Rb.

Inc.). Hybridization was carried out for 24 h at 42 °C in 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA (pH 7.4)), 5× Denhardt's solution (1× Denhardt's solution is 0.02% ficoll, 0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 20 μg/ml of salmon sperm DNA, and the appropriate ^{32}P-labeled probes at 2 × 10^6 cpm/ml. The filters were then washed sequentially with 0.1% SDS 1× SSC, then 0.1% SDS 0.5× SSC both at room temperature, and then 0.1% SDS 0.25× SSC at 55 °C (1× SSC is 0.15 M NaCl plus 0.3 M sodium citrate), followed by exposure to PhosphorImager screen (Molecular Dynamics).

Immunoprecipitation and Western Blot Analysis—For the immunoprecipitation of p21 and Rb, testes samples were homogenized with polytron (SPT 10–35, Kinematica, Switzerland), lysed in cold homogenization buffer (20 mM HEPES (pH 7.9), 20 mM KCl, 1% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing proteinase inhibitor (0.4 mg/ml pefabloc^{SC}, 10 μg/ml leupeptin, 10 μg/ml pepstain, (Roche, 1206893) and then centrifuged at 16,000 × *g* at 4 °C for 15 min. The protein concentration of the supernatant was evaluated with the Bio-Rad reagent kit. The supernatant containing 500 μg of protein was incubated with primary antibody, either 65951A (Pharmingin) for p21 or 14001A (Pharmingin) for Rb, at 4 °C for 2 h before the addition of protein A/G-Sepharose beads (Santa Cruz). The beads were collected after another 2-h incubation at 4 °C. After being washed four times with lysis buffer, the bound antigens were resuspended in Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.003% bromphenol blue), separated via SDS-polyacrylamide gel electrophoresis, transferred to Immunobilon-P membrane (Millipore), and detected by Western blotting.

For the Western blot analysis, 100 μg of protein obtained from each indicated monkey testis tissue was separated by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane to be probed with antibody sc-6243 (Santa Cruz) to detect the p53 probe. The membranes containing protein from the p21 and Rb immunoprecipitation procedures were also subjected to Western blot analysis and probed with antibodies to either p21 or Rb as appropriate. The membranes were blocked in TBST (20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.1% Tween 20 containing 5% nonfat dry milk) for 2 h at room temperature or 4 °C overnight. Primary antibodies in TBST were added for binding at room temperature for 2 h, and then the appropriate alkaline phosphatase-conjugated secondary antibodies (Santa Cruz) in TBST were added and incubated for 2 h at room temperature. Again, the membranes were washed three times in TBST (10–15 min at room temperature), and immunoblots were developed with an alkaline phosphatase substrate kit (Bio-Rad, 170–6342) according to the manufacturer's recommendation.

In Situ Hybridization Analysis—Testis samples were fixed in Bouins's solution and embedded in paraffin. Six-micrometer sections were then mounted on gelatin-coated slides. The sections were deparaffinized, rehydrated, treated with 0.2 M HCl for 25 min, 0.3% Triton for 15 min, and pretreated with proteinase K (10 μg/ml) for 30 min at 37 °C. Sections were postfixed in 4% paraformaldehyde in phosphate-buffered saline for 5 min and acetylated for 10 min in 0.1 M triethanolamine containing 0.25% acetic anhydride. Subsequently, the sections were prehybridized at 42 °C for 2 h in prehybridization buffer (4× SSC, 50% formamide, 10 mM Tris-HCl (pH 7.5), 1 mg/ml yeast tRNA, 1 mg/ml salmon sperm DNA, 2× Denhardt's, 10% dextran sulfate). Then the sections were incubated with antisense and sense Dig-cRNA probes in prehybridization buffer at 48 °C for 18 h and then washed sequentially with 2× SSC, 1× SSC, 0.1× SSC (2× 15 min in each concentration). The detection of Dig-labeled probes was carried out using alkaline-phosphatase-conjugated anti-Dig Fab (Roche, 1093274), as well as a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, Roche) according to the manufacturer's instructions.

Cell Culture, Transfection, and CAT Assay—Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml) (Sigma) at 37 °C in 5% CO_2, for 24 h before transfection. Cells were transfected with a total of 11-μg plasmid DNA (plasmid types and amounts are described in the figure legends) using the calcium phosphate method (32). β-Galactosidase expression plasmids (1 μg) were co-transfected as an internal control by which to normalize the transfection efficiency. After 24 h of transfection, the medium was changed once, and cells were cultured for another 24 h before harvesting. Cells were then lysed in 250 mM Tris-HCl (pH 7.8) by subjection to three freeze-thaw cycles, and the resulting supernatants were assayed for CAT activity. The reaction product was extracted with ethyl acetate (Mallinckrodt Specialty Chemicals Co.), then applied to a thin-layer chromatography plate (TLC, Sigma), and developed in a 95% chloroform, 5% methanol solvent. The CAT activity was quantitated by ImageQuant software (Molecular Dynamics Inc.).

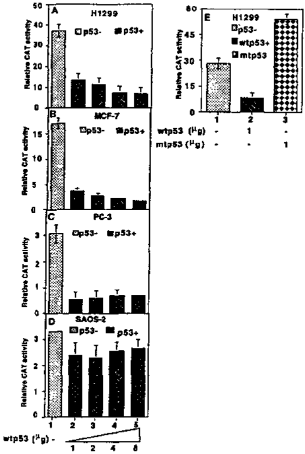

FIG. 3. Wild type p53 specifically represses TR2 expression in H1299 (*A*) and MCF-7 (*B*) cells, while mutant p53 (*E*) induces TR2 expression. The repression effect of wild type p53 on TR2 expression in the Rb-positive cell line PC3 (*C*) is dramatic, whereas the repression effect in Rb defective SAOS-2 cells is marginal (*D*). *A—D*, dose-response effects of wild and mutant p53 levels on the activation of p2709TR2CAT. p2709TR2CAT was transiently transfected into p53 null H1299 cells, MCF-7 cells, Rb-positive PC-3 cells, and Rb defective SAOS-2 cells. The wild-type p53 expression plasmid pC53-SN3 was co-transfected at various concentrations: 1, 2, 4, and 8 μg (*lanes 2, 3, 4,* and *5*, respectively). No p53 expression plasmid was transfected in *lane 1* of each panel. *E*, p2709TR2CAT was transiently transfected into H1299 cells without (*lane 1*) or with wild type p53 expression plasmid pC53-SN3 (*lane 2*) or mutant type p53 expression plasmid pC53-SCX3 (*lane 3*) was transiently transfected into H1299 cells. Cell extracts were prepared, and CAT activity was assayed as described under "Material and Methods." Results were quantitated with a PhosphorImager and displayed in terms of relative CAT activity. The values are the mean ± S.D. from at least three independent experiments.

RESULTS

The Repression of TR2 and Induction of p53 in Surgically Induced Cryptorchid Testis Tissue from the Rhesus Monkey—To study the potential physiological roles of the TR2 in its major target, the testis, the total RNA from the surgery-induced cryptorchid testis *versus* sham-operated contralateral scrotal testis, within the same rhesus monkey, was isolated and assayed for TR2 expression. Northern blot analysis showed unexpected results with the TR2 highly expressed in sham-operated contralateral scrotal testis but completely repressed in the undescended testis (Fig. 1, *upper panel*). In contrast, the expression of another testicular orphan receptor, TR3, isolated from the prostate and testes (33–35) was not significantly dif-

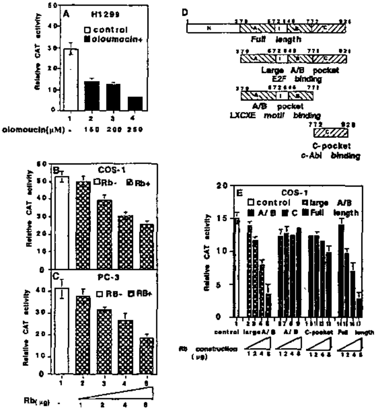

FIG. 4. The CDK inhibitor olomoucin and Rb inhibits TR2 expression, and the repression of TR2 by Rb requires E2F binding. *A*, CDK inhibitor olomoucin represses TR2 expression. p2709TR2CAT was transfected without (*lane 1*) and with increasing concentrations of CDK inhibitor olomoucin treatment in H1299 cells (*lanes 2–4*). *B* and *C*, Rb inhibits TR2 expression. p2709TR2CAT without (*lane 1*) and with increasing concentrations of PSG5-Rb (*lane 2–5*) were transfected into COS-1 (*B*) and PC-3 cells (*C*). *D*, diagrammatic representation of the full-length human Rb and the distinct binding domains of the Rb large A/B pocket, A/B pocket, and C pocket fragments. Numbers above the schematic diagram designate amino acid location. *E*, effect of the Rb large A/B pocket, A/B pocket, and C pocket fragments on TR2 expression. The repression of TR2 by Rb requires E2F binding. p2709TR2CAT was transiently transfected into COS-1 cells, without (*lane 1*) or with increasing concentrations of pCDNA3 Rb large A/B pocket (*lanes 2–5*), pCDNA3 Rb A/B pocket (*lanes 6–9*), pCDNA3 Rb C pocket (*lanes 10–13*), or pCDNA3 Rb full-length (*lanes 14–17*). Cell extracts were prepared, and CAT activity was assayed as described under "Material and Methods." Results were quantitated with a PhosphorImager and displayed as relative CAT activity. The values are the mean ± S.D. from at least three independent experiments.

ferent between the cryptorchid and sham-operated contralateral scrotal testis (Fig. 1, *middle panel*). The contrasting expression patterns between TR2 and TR3 suggest that the suppression of TR2 in cryptorchidism is specific to this orphan receptor. We also examined the expression of androgen receptor and orphan receptor TR4. The expression level of androgen receptor did not change significantly or slightly increased, and the expression level of TR4 decreased but was not completely inhibited in cryptorchid testis compared with contralateral sham-operated testis (data not shown). No change or a slight increase of androgen receptor expression level in cryptorchid testis rules out the possibility of any androgen inactivation in the model. Because TR4 is closely related to TR2, the decrease of TR4 expression level is expectant. We then analyzed the p53 expression in cryptorchid testis. Northern blot and Western blot analyses showed that the expression of p53 was induced significantly, at both the mRNA (Fig. 2*A*, *upper panel*) and protein levels (Fig. 2*B*, *upper panel*) in cryptorchid testis, as compared with the sham-operated contralateral scrotal testis.

Suppression of TR2 via p53→p21→CDK→Rb Signal Pathway—One possible mechanism to explain repression of TR2 following p53 induction in cryptorchid testis is that p53 can repress TR2 expression. To test this hypothesis, a plasmid with the TR2 promoter linked to a CAT reporter, p2709TR2CAT, was co-transfected with wild-type p53 expression plasmid pC53SN3 in various cell lines. The results showed that coexpression of wild-type p53 can repress TR2 expression in both of the p53 null cell lines, H1299 (Fig. 3*A*) and MCF-7 (Fig. 3*B*), as well as in the Rb-positive prostate cancer PC-3 cells (Fig. 3*C*). In contrast, transfection of mutant p53 expression plasmid (pC53-SCX3) enhances the p2709TR2CAT activity (Fig. 3*E*). Coexpression of wild-type p53 in the Rb-defective SAOS-2 cells, however, causes only marginal repression of TR2 (Fig. 3*D*), suggesting the p53 may repress TR2 expression via the Rb signal pathway. As there is a lack of p53 response elements in the TR2 promoter (p2709TR2CAT) preventing direct p53 binding (36), indirect suppression of TR2 via a p53-related signaling pathway is likely.

We then tested the potential linkage of TR2 repression in cryptorchid testis to the p53→p21→CDK→Rb signaling pathway. The same Northern blot membrane that showed the p53 induction and repression of TR2 in cryptorchid testis was reprobed with a p21 cDNA fragment. As shown in Fig. 2*A*, *middle panel*, the p21 mRNA was induced in cryptorchid testis as compared with the sham-operated contralateral scrotal testis. Western blot analysis further confirmed that the p21 protein expression was also induced in cryptorchid testis as compared with the sham-operated contralateral scrotal testis (Fig. 2*B*, *middle panel*). The immunoprecipitation and Western blot analysis showed that the active hypophosphorylated Rb form was significantly increased in cryptorchid testis as compared with the sham-operated contralateral scrotal testis (Fig. 2*B*, *lower panel*). Together, data in Fig. 2 are consistent with the hypothesis that p53 may proceed through the p53→p21→CDK→Rb signaling pathway to repress TR2 expression in cryptorchidism.

We then used the CDK inhibitor, olomoucin, to support our hypothesis that the TR2 repression in cryptorchid testis may involve the p53→p21→CDK→Rb signaling pathway. As shown in Fig. 4*A*, the expression of TR2 in p53 null H1299 cells was repressed by the addition of 150–250 µM olomoucin in a dose-dependent manner, suggesting that CDK activity, and thus Rb phosphorylation (37), is necessary for derepression of TR2.

Repression of TR2 Expression by Rb—To further strengthen our hypothesis that the repression of TR2 expression may function through the p53→p21→CDK→Rb signaling pathway, we co-transfected Rb and p2709TR2CAT into COS-1 cells. As shown in Fig. 4*B*, Rb repressed TR2 expression in a dose-dependent manner. Similar results also occurred when we replaced COS-1 cells with PC-3 cells (Fig. 4*C*).

It has been well documented that Rb contains several distinct domains that are able to bind to different proteins for various functions. For example, 1) the A/B pocket binds to other proteins with a *LXCXE* motif, 2) the C pocket binds to the C-Abl tyrosine kinase, and 3) the large A/B pocket binds to the E2F family of transcription factors (for detailed map, see Fig. 4*D*). These three domains, as well as the full-length Rb, were ligated into pCDNA3 expression vectors and then separately co-transfected with p2709TR2CAT into COS-1 cells. As shown in Fig. 4*E*, both the full-length Rb and Rb with large A/B domain can repress the TR2 expression in a dose-dependent manner. In contrast, the A/B pocket and C pocket of Rb showed only marginal effects on the repression of TR2 expression, suggesting that the large A/B domain, which binds to the E2F transcriptional factor, is required for Rb to repress TR2 expression.

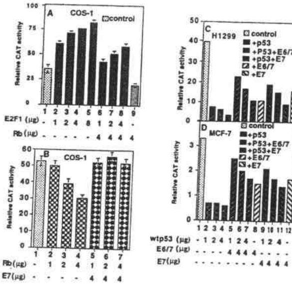

FIG. 5. E2F specifically induces TR2 expression and the repression of TR2 by p53 and Rb can be reversed by HPV-16 E6 and E7. *A*, E2F1 induces TR2 expression, yet the induction effect can be reduced by Rb. p2709TR2CAT was transiently transfected into COS-1 cells, without (*lane 1*) or with E2F1 expression plasmid pDC-E2F1, 1 (*lanes 2* and *6*), 2 (*lanes 3* and *7*), 4 (*lanes 4* and *8*), or 8 µg (*lane 5*). Four µg of PSG5-Rb were also transfected in *lanes 6–9*. *B*, HPV-16 E7 can reverse the repression of TR2 expression by Rb. p2709TR2CAT was transiently transfected into COS-1 cells, without (*lane 1*) or with varying concentrations of PSG5-Rb (*lanes 2–7*); 4 µg of HPV-16 E7 expression plasmid p1302 were co-transfected in *lanes 5–7*. *C* and *D*, HPV-16 E6/7 or E7 alone can partially reverse the repression of TR2 expression by p53. p2709TR2CAT was transiently transfected into H1299 cells (*C*) and MCF-7 cells (*D*), without (*lanes 1, 8,* and *12*) or with varying concentrations of wild-type p53 expression plasmid pC53-SN3 (*lanes 2–7*, and *9–11*); E6 and E7 expression plasmid p1321 was co-transfected in *lanes 5–8*, and E7 expression plasmid p1434 was co-transfected in *lanes 9–12*. Cell extracts were prepared and CAT activity was assayed as described under "Material and Methods." Results were quantitated with a PhosphorImager and displayed as relative CAT activity. The values are the mean ± S.D. from at least three independent experiments.

Induction of TR2 Expression by E2F1—Co-transfection of E2F1 expression plasmid pDC-E2F1 with the p2709TR2CAT into COS-1 cells demonstrated that E2F1 could induce TR2 expression in a dose-dependent manner (Fig. 5*A*). The addition of Rb repressed this dose-dependent induction, suggesting that the interaction of E2F and Rb is involved in the regulation of TR2 expression. It is possible that free E2F can induce TR2 expression, but the presence of Rb may titrate out this free E2F and therefore repress the induction of E2F-mediated TR2 expression.

Feedback Control of TR2 Expression by E6/E7—It is known that the human papillomavirus type 16 (HPV-16) gene products E6 and E7 can alter the cell cycle, and the TR2 has been shown to induce the HPV-16 expression via binding to a TR2 response element (HPV-direct repeat 4) in the long control region of HPV-16.[2] We also demonstrated that the HPV-16 gene products E6/E7 could regulate TR2 expression presumably through modulation of p53 and Rb expression or function. E6 functions to bind and degrade p53, whereas E7 can bind and inactivate Rb (39). To determine if p53- and Rb-mediated repression of TR2 expression could be reversed by the addition of E6 or E7, three E6/E7 expression plasmids were utilized. The plasmid p1304 effectively expresses E7 (40); the plasmid p1321 effectively expresses E6 and E7. The plasmid p1434 is identical to p1321 except that it contains a translation termination linker in the middle of E6 and therefore only effectively expresses E7 (41). As shown in Fig. 5*B*, co-transfection of p1304 can completely reverse the Rb repression effect on TR2 expression in COS-1 cells. In H1299 cells and MCF-7 cells, we demonstrated that both p1321 and p1434 repress TR2 expression (Fig. 5, *C* and *D*, *lanes 8* and *12*, respectively). When these

[2] C. Chang, D. L. Lin, L. Collins, and X.-M. Mu, manuscript in preparation.

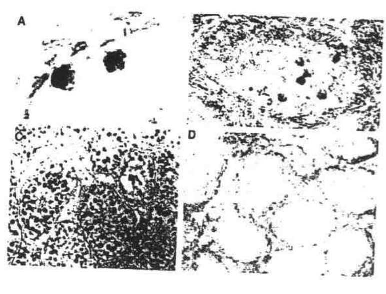

FIG. 6. Localization of TR2 mRNA in the rhesus monkey testis by *in situ* hybridization. Testis sections from normal rhesus monkey were subjected to *in situ* hybridization. The hybridization was performed employing TR2 Dig-labeled antisense (*A–C*) or sense (*D*) cRNA probes specific for the TR2 gene as described under "Material and Methods." *A*, two pachytene primary spermatocytes with positive signals. *B*, pachytene primary spermatocytes with positive signals in one of the seminiferous tubules. *C*, primary spermatocytes with positive signals. *D*, no signal can be detected with sense TR2 cRNA probe. Magnification: *A*, ×1000; *B*, ×400; and *C* and *D*, ×200.

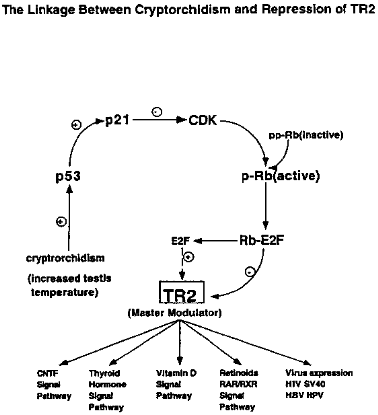

FIG. 7. **Our model linking cryptorchidism and the repression of TR2 involves the p53-Rb signaling pathway.** Due to increased testis temperature via cryptorchidism, expression of p53 is increased. This increase in p53 then up-regulates p21, which in turn reduces CDK activity. Rb, existing in either its hyperphosphorylated (inactive) or hypophosphorylated (active) form, is pushed toward an increase in activity due to the decrease in CDK function. Active Rb protein then binds transcription factor E2F, effectively reducing expression of TR2. It has been demonstrated that TR2 has the ability to regulate expression of several target genes, thereby modulating other signaling pathways. Thus, the repression of TR2 through up-regulation of p53 caused by cryptorchidism may have widespread effects. *pp-Rb*, hyperphosphorylated (inactive) form of Rb; *RAR*, retinoic acid receptor; *RXR*, retinoid receptor X; *HBV*, human hepatitis B virus.

E6/E7 expression plasmids were co-transfected with wild type p53 expression plasmid pC53-SCN3, E6 and E7 together (Fig. 5, *C* and *D*, *lanes 5–7 versus lanes 2–4*) or E7 alone (Fig. 5, *C* and *D*, *lanes 9–11 versus lanes 2–4*) could significantly reverse the p53 repression effect on TR2 expression. Therefore, our data demonstrate a feedback control mechanism that can further strengthen our hypothesis that the repression of TR2 expression is influenced by actions of p53 and Rb (Fig. 7).

In Situ Localization of TR2 in Rhesus Monkey Testis—As p53 expression was confined to the tetraploid (4N) spermatocytes at the pachytene phase of meiosis (16), the TR2 localization in the same area would provide further evidence that the TR2 repression in cryptorchid testis could be mediated through the p53 signal pathway. Using the Dig-labeled antisense TR2 cRNA probe, our *in situ* hybridization data showed that TR2 mRNA is significantly expressed in primary spermatocytes (Fig. 6*C*), the cell type of testes cells most sensitive to high temperature (17). Within the primary spermatocytes, the pachytene primary spermatocytes showed the highest TR2 mRNA expression (Fig. 6, *A* and *B*). Similar TR2 and p53 expression patterns in the pachytene primary spermatocytes therefore provide indirect but strong evidence supporting our hypothesis that p53 could repress TR2 expression in cryptorchid testis.

DISCUSSION

Spermatogenesis is a complicated process of germ cell differentiation, involving programmatic expression of developmental stage-specific genes in diverse cell types (15, 17, 42). It is possible that disruption of spermatogenesis in cryptorchidism results from the repression of some essential genes in specific cell types or differentiation stages (43). The TR2 is highly expressed in testis and specifically localized in germ cells (15). Earlier reports also demonstrated that the TR2 is a master regulator that controls many signaling pathways, such as the retinoids RAR/RXR (44–46), thyroid receptor (47), vitamin D receptor (48), ciliary neurotophic factor receptor (49), histamine H1 receptor (50), and human erythropoietin expression (51), as well as the expression of many viruses, such as human hepatitis B virus (52) and SV40 (53). It is likely that the TR2 subfamily may play very important roles via control of these signal pathways in the process of spermatogenesis. We demonstrated here that the TR2 was repressed by the higher temperature of the testis in the cryptorchid state, which may represent the first molecular linkage between cryptorchidism and spermatogenesis.

Higher testis temperature created by cryptorchidism could induce p53-mediated apoptosis in testis (54). Early reports suggested that p53-mediated apoptosis in testis could be a result of unrepairable DNA damage induced by high temperatures, which provides a protective mechanism in the human and in other species for the avoidance of propagation of damaged DNA. The direct linkage between p53-mediated apoptosis in cryptorchid testis and male infertility and the mechanisms behind reversal of cryptorchidism-related infertility via lowering of the testis temperature remain unclear (4, 5). Perhaps p53 could mediate other nonapoptotic signal pathways that play essential roles in spermatogenesis.

Both p53 and Rb play important roles in controlling cell-cycle progression, differentiation, development, and apoptosis. In testes, the p53 expression level is unusually high compared with other tissues, and mice with reduced levels of the p53 protein exhibit the testicular giant cell degenerative syndrome (55). Mice deficient in p53 are susceptible to spontaneous tumors (56). Cells lacking the p53 fail to arrest in response to a wide variety of DNA-damaging agents (57, 58). Mouse embryos without functional Rb fail to survive past embryonic stages, dying by gestation day 14.5 with defects in erythroid and neuronal development (59–61). Moreover, Rb has been shown to be involved in the differentiation of several cell types, including myoblasts, monocytes, and adipocytes (62–64). The feedback control between p53/Rb and TR2 in cryptorchidism may further strengthen the findings that p53/Rb and TR2 play important roles in the germ cell development and differentiation.

Combining our *in situ* hybridization data and earlier reports, we conclude that the TR2, p53, and Rb are all expressed in pachytene primary spermatocytes. Pachytene primary spermatocytes are of the cell type undergoing meiosis, which is an important step in the spermatogenesis. Furthermore, as primary spermatocytes and round spermatids are the germ cell stages most sensitive to heat injury (17) and the most frequently observed apoptotic cells in the experimentally induced cryptorchid mouse testis (38), it may be reasonable to hypothesize that primary spermatocytes need to maintain high levels of p53 for the control of DNA quality during the premeiotic period. Higher temperatures caused by cryptorchidism may therefore increase p53 expression and repress TR2 expression. Male infertility may be the consequence of these changes, in an attempt to avoid damaged DNA replication transmission.

By combining the data from the rhesus monkey model with that from multiple cell lines, we demonstrated that higher testis temperature created by cryptorchidism represses TR2 expression. We also determined that this TR2 repression was mediated by the activities of p53 and Rb and that the mechanism was primarily through the p53→p21→CDK→Rb→ E2F signaling pathway (Fig. 7). Although we do not rule out the possibility that other pathways or components within the p53→p21→Rb→CDK→E2F pathway may also play roles in modulating the expression of TR2, our data strongly indicate that p53 and Rb play a significant and central role in the repression of TR2 in the context of cryptorchidism.

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