

## Stage dependent and androgen inductive expression of orphan receptor TR4 in rat testis <sup>☆</sup>

Xiaomin Mu <sup>b</sup>, Lin Yang <sup>a</sup>, Chawnshang Chang <sup>a,\*</sup>

<sup>a</sup> George Whipple Laboratory for Cancer Research, Department of Pathology, University of Rochester, Rochester, NY 14620, USA

<sup>b</sup> Contraceptive Research and Develop (CONRAD) Program, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA 23507, USA

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### Abstract

In this study, we investigated the expression of TR4 in different stages of seminiferous tubules and the relationship between TR4 and androgen in rat testis. We found that TR4 was stage-dependently expressed in rat seminiferous tubules, T withdrawal induced by high doses of testosterone undecanoate and ethane dimethane sulfonate inhibit TR4 expression in rat testis, and testosterone induced TR4 expression in co-cultured primary germ/Sertoli cells. Furthermore, we demonstrated that androgen receptor could enhance TR4-mediated transactivation activity in testis cells in the presence of testosterone. Together, these data indicate that the expression of TR4 in rat testis is stage dependent and androgen inductive, and suggest the important role of orphan receptor TR4 in spermatogenesis.

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**Keywords:** Orphan receptor TR4; Stage dependent; Testosterone; Seminiferous tubules

Spermatogenesis is a complex process in which germ cells, supported by Sertoli cells, undergo mitotic and meiotic divisions and differentiation to produce spermatozoa. Spermatogenesis is highly organized with germ cells at different differential phases associated together into distinct stages, and 14 stages of spermatogenesis in rat are well characterized [1]. Several key regulators of spermatogenesis, like androgen receptor (AR), have been shown to be expressed in a stage-dependent manner [2]. Testosterone (T), secreted by the Leydig cells under the influence of pituitary luteinizing hormone (LH), is a major regulator of the spermatogenic process [3]. T regulates spermatogenesis through the AR in Sertoli cells [4].

Nuclear receptors constitute a superfamily of transcription factors that regulate gene expression in a wide variety of biological processes [5,6]. The orphan receptors belong to this superfamily, although their biological significance has been debated due to the lack of identified ligands [7]. We have cloned the human testicular orphan receptor-4 (TR4) from human testis and prostate cDNA libraries, and we have detected the high level of expression of TR4 in testis [8]. Recently, we demonstrated that targeted inactivation of TR4 delayed and disrupted late meiotic prophase and subsequent meiotic divisions of spermatogenesis in mice [9]. However, the expression of TR4 in different stages of seminiferous tubules and relationship between TR4 and androgen in spermatogenesis are still not clear.

In this study, we investigate the expression of TR4 in different stages of seminiferous tubules in rat and the relationship between TR4 and androgen in rat testis and testis cells. We report here that TR4 expression in rat testis is stage dependent and androgen inductive, and that T/AR

<sup>☆</sup> Abbreviations: T, testosterone; TU, testosterone undecanoate; AR, androgen receptor; EDS, ethane dimethane sulfonate; FSH, follicle stimulating hormone; LH, pituitary luteinizing hormone.

\* Corresponding author. Fax: +1 585 756 4133.

E-mail address: [chang@urmc.rochester.edu](mailto:chang@urmc.rochester.edu) (C. Chang).

signal could enhance TR4-mediated transactivation activity in testis cells.

## Materials and methods

**Animals and treatments.** Adult male Sprague–Dawley rats aged 70–100 days were maintained under standard animal house conditions. Twenty rats were injected with 50 mg/kg body weight TU or placebo every other day for 30 days. Twenty rats were injected with a single dose of ethane dimethane sulfonate (EDS) (75 mg/kg body weight). EDS was synthesized as previously described [10] and was dissolved in dimethyl sulfoxide:water (1:3). Control rats received injection of vehicle. Rats were killed one week after EDS or vehicle injection.

**Rat tubule micro-dissection.** Testes of adult Sprague–Dawley rats were excised and subjected to transillumination-assisted micro-dissection [11]. Segments of different stages of seminiferous tubules (from Stage V to X) were collected.

**Northern blot analysis.** Total RNAs were extracted from the micro-dissected seminiferous tubule segments, testes, and cultured testes cells. Northern blot analyses were performed by methods described previously [12,13]. The TR4 probe used for Northern blot analyses was the 598 bp *AatII* site fragment located in the 5' portion of the human TR4 cDNA.

**Quantitative real-time RT-PCR.** Quantitative real-time RT-PCR was performed as described previously [9,13]. Each PCR was triplicated, repeated two times, and normalized with  $\beta$ -actin.

**Cell culture, transfection, and luciferase assay.** Testes cell lines TM3, TM4, GC-1spg, and GC-2spd were purchased from ATCC. TM3 and TM4 were routinely maintained in 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F12 with 10% fetal bovine serum (FBS). GC-1spg and GC-2spd were maintained in DMEM medium containing 10% FBS and 1.5 g/L sodium bicarbonate. Cells were set in 60 mm Petri dishes, after 24 h the media were changed to DMEM or DMEM/F12 with 10% charcoal/dextran treated FBS, and transfected with a total of 2  $\mu$ g plasmid DNA by using superfect according to manufacturer's procedure (Qiagen, Chatsworth, CA) [13]. Plasmid pRLSV40-Luc or phRL-TK (1  $\mu$ g) was co-transfected as internal controls to normalize the transfection efficiency. After 16–24 h transfection, the medium was changed again for hormone treatments and then cultured for another 24 h before harvesting. The cell lysates were extracted and luciferase assays were performed as described previously [13,14].

**Primary germ/Sertoli cell co-culture.** Mixed cultures of Sertoli and germ cells were prepared from testes of adult rat. Tissues were incubated in 0.25% trypsin containing 1  $\mu$ g/ml DNase I (Sigma DN25) and then 0.1% type IV collagenase (Sigma, 9891) for 15 min at 33 °C with occasional gentle agitation and washed three times with Hanks' balanced salt solution after each incubation. The precipitated tubule tissue was resuspended in culture medium and set into 100 mm culture dish (10 ml per dish) or two-well glass slide culture chamber (2 ml per well) at a cell concentration of  $4 \times 10^5$ /ml. The cells were cultured in a humidified atmosphere, 5% CO<sub>2</sub> and 95% air at 33 °C with DMEM/F12 (1:1) containing 10% FBS, 4.0 mM glutamine, 1 mM pyruvate, and 1% insulin–transferrin–selenium (Gibco, 51500-056) and 10 nM T. The T and FSH for treatment of cells were purchased from Sigma (F2293).

**Statistical analysis.** Statistical difference between control and TU treatment, and control and EDS treatment was evaluated by student's *t* test. A value of  $P < 0.05$  was considered significant.

## Results

### Stage-dependent expression of TR4 in micro-dissected rat seminiferous tubules

Since rat seminiferous tubule stage micro-dissection technique is well established [1,11] and the characterization of stages VI–IX is more obvious, after a long term of

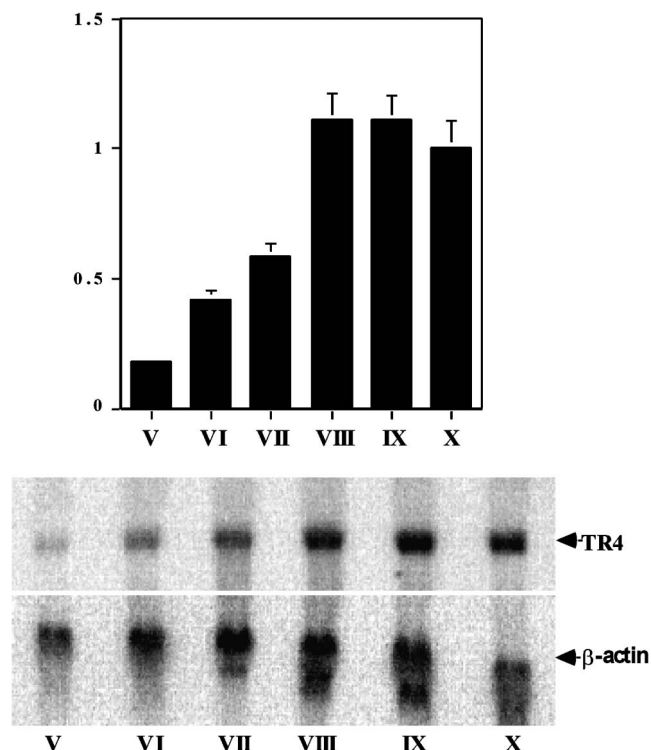


Fig. 1. Stage-dependent expression of TR4 in normal rat testis. Stage V–X tubules were micro-dissected, RNAs were extracted, and Northern blot analyses were performed. Lower panel: A representative Northern blot. Upper panel: Quantitative data from three Northern blots.

training, we could precisely micro-dissect stages V–X of tubules from normal adult rat. We pooled the same stage of tubules from more than ten micro-dissections, extracted RNAs, and Northern blot analyses were performed. As shown in Fig. 1, TR4 expression level is low at stage V, gradually increases from stage VI and stage VII, and reaches the highest level at stage VIII and remains at a relatively high level in stage IX and X. The data from Fig. 1 clearly indicate the stage-dependent expression of TR4 in rat seminiferous tubules.

### T withdrawal induced by high doses of TU and EDS injection suppresses TR4 expression in stage VIII tubule and testis in rat

High doses of T administration is well-established model to induce intratesticular T withdrawal in rat [15,16], and we have used high doses of TU injected rat model for several different types of studies in our laboratory. We found that the transillumination characteristics of different stages especially stage VII and VIII were still typical after 30 days TU injection. So we also micro-dissected stage VIII tubules from both placebo injected and high dose TU injected rats, extracted RNAs, and performed Northern blots. As shown in Fig. 2A, TR4 expression was significantly suppressed in stage VIII tubules from high dose TU injected rat compared with that from placebo injected rat (Lane 2 vs 1).

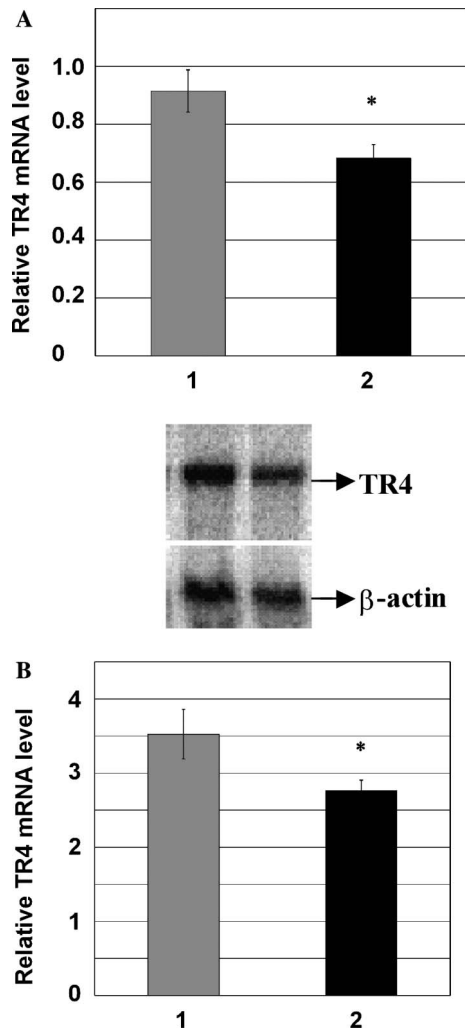


Fig. 2. T withdrawal induced by high doses of TU and EDS injection suppresses TR4 expression in stage VIII tubule and testis in rat. (A) Suppression of TR4 expression by high dose of TU in stage VIII tubule. Stage VIII tubules from both control placebo and TU injected rat were microdissected, RNAs were extracted, and Northern blots were performed. Lower panel: A representative Northern blot. Upper panel: Quantitative data from three Northern blots. Values are means  $\pm$  SD. \* $P < 0.05$ . (B) Suppression of TR4 expression by EDS injection. RNAs from both control placebo (lane 1) and EDS injected rat testes (lane 2) were extracted, and quantitative real-time RT-PCRs were performed. Values are means  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

To confirm that intratesticular T decrease can suppress TR4 expression, we also used the EDS treated rat model. EDS can selectively destroy Leydig cells and thus induce intratesticular T withdrawal, and the EDS treated rat model has been widely used to study hormonal regulation of spermatogenesis [10,17]. As shown in Fig. 2B, TR4 expression was suppressed in testis from EDS treated rats compared with that from placebo treated rats.

*T induces TR4 expression in primary germ/Sertoli cell co-culture*

To determine whether T could regulate TR4 expression, we established a Sertoli/germ cell co-culture system supple-

mented with T as described in Materials and methods. In our Sertoli/germ cell co-culture system, over 90% of the cells are Sertoli cells and germ cells, with very few contaminating Leydig cells and peritubular cells, as judged by microscope examination. Sertoli cells grew to confluence about 6 days after plating, and different germ cell types could be seen on the top of Sertoli cells about 4 days after plating. We treated primary germ/Sertoli co-cultured cells with different T concentrations. As shown in Fig. 3, we detected TR4 expression with 10 nM T, and the higher concentrations of 100 and 1000 nM, which is roughly the physiological range of intratesticular T, significantly induced TR4 expression (Fig. 3A, lanes 2–3 vs 1). As a control, follicle-stimulating hormone (FSH) did not induce but suppressed TR4 expression in the co-cultured primary germ/Sertoli cells (Fig. 3B, lane 2 vs 1).

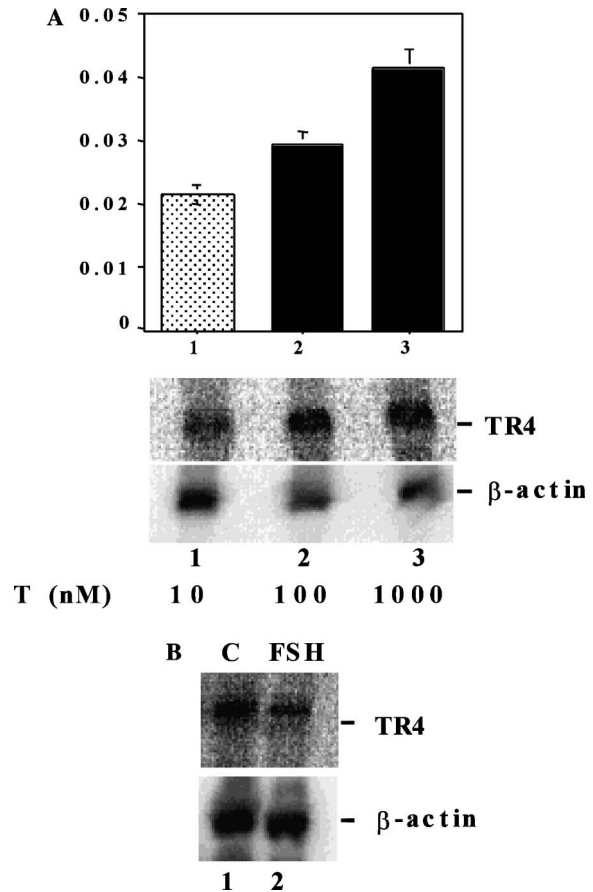


Fig. 3. Induction of TR4 expression by testosterone in co-cultured primary germ/Sertoli cells. Co-cultured primary germ/Sertoli cells were treated with indicated concentrations of T (A) and 0.5 U/ml FSH (B). After 16 h total RNAs were extracted and Northern blot analyses were performed with <sup>32</sup>P labeled TR4 and  $\beta$ -actin probe. The same amounts of total RNA (30  $\mu$ g) were loaded in all the Northern blots and three independent experiments were performed. (A) Lower panel: A representative Northern blot. Upper panel: Quantitative data from three Northern blots. (B) A representative Northern blot with RNAs from control C and FSH treated co-cultured primary germ/Sertoli cells.

*Induction of TR4-mediated transactivation by androgen receptor in the presence of testosterone in testis cells*

TR4 itself can also function as a transcriptional factor controlling many important signal pathways via

modulation of the expression of several important key genes [18–20]. We therefore also examined the potential T and AR effects on the TR4-mediated transactivation. As shown in Fig. 4, in Sertoli TM4 cells, Leydig TM3 cells, spermatogonia GC-1spg, and spermatocyte GC-2spd cells,

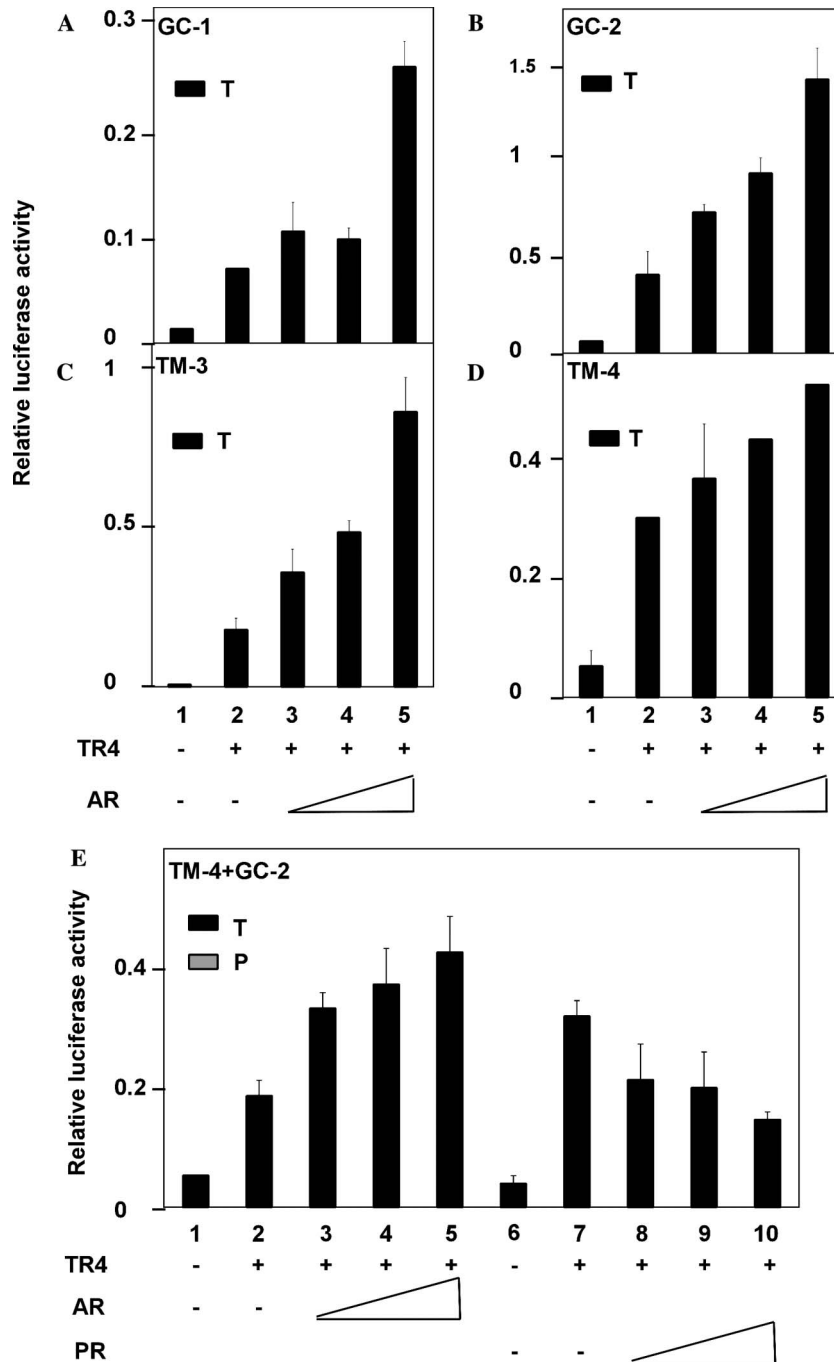


Fig. 4. AR enhances TR4 transactivation in Sertoli cell TM4, spermatogonia GC-1spg, Leydig Cell TM-3, spermatocyte cell GC-2spd, and Sertoli cell TM4 and co-cultured TM4 and spermatocyte GC-2spd cells in the presence of T. TR4 reporter gene pGL-TK-HCR-1-Luc (0.2 µg) was transiently transfected into Sertoli cell TM4, Leydig cell TM3, spermatogonia cell GC-1spg, spermatocyte and spermatid cell GC-2spd, and co-cultured TM4 and GC-2spd cells, co-transfected without (A–E, lanes 1, E, lane 6) or with 1 µg pCMV-TR4 expression plasmid (A–E, lanes 2–5, and E, lanes 7–10), pSG5-AR expression plasmid at various concentrations, 0.2, 0.4, and 0.8 µg (A–E, lanes 3–5, respectively), and pSG5-PR expression plasmid at various concentrations, 0.2, 0.4, and 0.8 µg (E, lanes 8–10, respectively). After 16–24 h transfection, 10 nM T (A–E, lanes 1–5) or 10 nM progesterone (E, lanes 6–10) was added. Cell extracts were prepared and luciferase activity was assayed, results were normalized by either pRL-TK (A) or pRLSV40-Luc (B–E), and displayed in terms of relative luciferase activity. The values are means ± SD from at least three independent experiments.

TR4 induced luciferase reporter linked with TR4RE-DR1 [21]. Addition of AR will then further enhance the TR4 transactivation in the presence of 10 nM of T (Figs. 4A–D, lane 2 vs 3–5). Interestingly, in co-cultured Sertoli TM4 cell and spermatocyte GC-2spd cells, AR can also significantly enhance TR4 transactivation in the presence of T (Fig. 4E, lane 2 vs 3–5). In contrast, addition of progesterone receptor shows suppression effects in the presence of 10 nM progesterone (Fig. 4E, lane 7 vs 8–10). The transactivation data from Fig. 4 indicated that T and AR could enhance TR4 transactivation activity, and this effect is specific in Sertoli TM4 cells and TM4/spermatocyte GC-2spd co-cultured cells.

## Discussion

In this study, we found stage-dependent TR4 expression in rat testis. So far, very few genes have been detected in a stage-dependent manner in seminiferous tubules [2]. Our findings suggest that TR4 plays an important role in spermatogenesis. Previous reports showed that AR was also stage-dependently expressed in the rat V–VIII seminiferous tubules with the highest expression in stage VI–VIII [22], suggesting the co-regulation between TR4 and AR. The stage dependence of TR4 in testis suggests the important role of orphan receptor TR4 in spermatogenesis.

Hirose et al. [23] investigated the TR4 mRNA in vitro expression in various isolated testicular cells, and detected strong TR4 signals in primary spermatocytes and weak TR4 signals in round spermatids. We previously also detected the biphasic expression of TR4 in testis by in situ hybridization [9]. TR4 is highly expressed in primary spermatocytes. TR4 is also expressed in round spermatids, but at a much lower level than that in primary spermatocytes. We believe that TR4 expression is stage dependent and androgen inductive in only certain type of germ cells. This could explain why TR4 expression level is only partially suppressed in the stage VIII tubules from higher doses of TU injected rat (Fig. 2A) and in the EDS injected rat testis (Fig. 2B). Interestingly, AR also shows biphasic roles in spermatogenesis. Spermatogenesis was arrested in premeiotic phase in testicular feminized male (Tfm) [24]. We recently developed AR knockout (ARKO) mice and Sertoli cell AR-specific knockout mice, and we found spermatogenesis also arrested in premeiotic phase [25,26]. Recently, Holdcraft et al. [27] showed that AR function is required for the differentiation of haploid spermatids by conditional inactivation of AR in Sertoli cells. All these findings strongly support the linkage between TR4 and androgen/AR signals.

We also used all available testis cells to study the effect of T/AR on TR4-mediated transactivation activity. Interestingly, AR could enhance TR4-mediated transactivation in the presence of T in all testis cells we tested. We previously reported that AR could suppress TR4 transactivation through heterodimerization with TR4 in H1299 and

HepaG2 cells [28]. There are complicated communications between different types of testis cells [29]. Testis is the only organ where meiosis takes place in male and intratesticular T concentration (around 250 nM) is much higher than in the other organs (1–10 nM), it is therefore likely many distinct biochemical events occur only in testis and induction of TR4 by testosterone in testis represents an unique regulation mechanism.

GC-2spd cell was generated from the preleptotene spermatocytes transfected with SV40 large tumor antigen gene and the gene coding for a temperature sensitive mutant of p53. GC-2spd cell can undergo meiosis and generate cells with haploid DNA content and morphological and biochemical feature of round spermatids including the appearance of an acrosomic granule [30]. It was later found that the several germ cell markers could not be detected in GC-2spd cells [31], but it was still confirmed that GC-2spd cells could be a model for mitochondrial differentiation during meiosis [32]. When we co-cultured GC-2spd cells with TM4 cells, we found some typical characteristics of testicular cell growth. We believe GC-2spd cell still keeps the basic characteristics of germ cells, especially when co-cultured with Sertoli TM4 cells.

## Acknowledgment

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