Am J Transl Res 2022;14(9):6067-6081
www.ajtr.org /ISSN:1943-8141/AJTR0133158

Original Article

Discoidin domain receptor 2 expression increases phagocytotic capacity in sertoli cells of sertoli cell-only syndrome testes

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Received March 15, 2021; Accepted July 25, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: Discoidin domain receptor 2 (DDR2) belongs to the receptor tyrosine kinase (RTK) family, other RTKs have been reported to regulate phagocytic function of Sertoli cells (SCs), yet little is known about the function of DDR2 in Sertoli cells. In the present study, we aim to explore the function and mechanism of ectopic discoidin domain receptor 2 (DDR2) expression in Sertoli cells of Sertoli cell-only syndrome (SCOS) testes. We found that discoidin domain receptor 2 (DDR2) was absent in Sertoli cells of normal testis but was expressed in Sertoli cells of SCOS testes. This Sertoli cell DDR2 expression was induced by impaired androgen receptor (AR) signaling, but was inhibited by increased AR signaling from testosterone administration. The Sertoli cell DDR2 expression led to an increase in phagocytosis through up-regulation of Scavenger receptor class B member 1 (SR-BI) levels. However, loss of DDR2 by knock-out or knock-down weakened the phagocytotic capacity of Sertoli cells. Furthermore, the expression of DDR2 in Sertoli cells activated matrix metallopeptidase 9 (MMP-9) to consume abnormal collagen increase in seminiferous tubules which was responsible for the block of testosterone transportation and AR loss and to compensate for the impaired blood-testis-barrier (BTB). Our data suggest that the AR/DDR2 cascade may serve as a negative feedback mechanism to help compensate for the homeostasis of seminiferous epithelium in SCOS testis.

Keywords: Sertoli cell-only syndrome (SCOS), sertoli cell, discoidin domain receptor 2 (DDR2), androgen receptor (AR), phagocytosis

Introduction

Mammalian spermatogenesis consists of a complex series of events including proliferation and differentiation of spermatogonia stem cells, meiosis of differentiating spermatogenic cells, and maturation of spermatids [1]. During this process, more than half of the differentiating spermatogenic cells undergo apoptosis before maturing into spermatozoa [2]. While apoptosis in spermatogenic cells has been widely reported throughout various stages of differentiation, only a few apoptotic spermatogenic cells can be detected at any given time [2-6]. This rare occurrence is likely due to the rapid elimination of apoptotic cells by phagocytic Sertoli cells (SCs) [7, 8]. This clearance of apoptotic substrates by phagocytosis is vital for the proper maintenance of seminiferous tubules, and without it can lead to impaired spermatogenesis [9, 10]. Androgen regulation through Sertoli cells was considered essential in spermatogenesis since mice with selective androgen receptor (AR) knockout in SCs showed a sterile phenotype of uncompleted spermatogenesis associated with increased germ cell apoptosis even when the serum testosterone concentration is normal [11]. However, whether the phagocytosis was impaired due to AR loss and whether impaired phagocytosis was involved remain largely unknown.

Azoospermia due to spermatogenic failure, or nonobstructive azoospermia (NOA), affects approximately 1% of the general population [12-14]. In patients presenting with azoospermia, Sertoli cell only syndrome (SCOS) is the common cause. This syndrome occurs in patients with disorders of sexual development (DSDs) who have genetic mutations, but the mutation has remained unrevealed [15]. It has been reported that mast cell infiltration as well as

extracellular matrix proteins are increased in SCOS testis [16-19]. Although many studies have focused on the role of SCs in SCOS testes and found low androgen receptor (AR) levels, inhibin B, anti-muller hormone and lactate production [20], whether their phagocytic function is affected is still unknown.

The discoidin domain receptor 2 (DDR2), as well as its family member DDR1, belongs to the receptor tyrosine kinase (RTK) family [21]. As opposed to other RTKs, DDRs respond to collagens rather than soluble peptide growth factors, and display a relatively slow onset of phosphorylation, occurring in hours rather than minutes [22, 23]. Since other RTKs have been reported to regulate phagocytic function of SCs [24, 25], and DDR1 is associated with idiopathic non-obstructive azoospermia [26], we hypothesized that DDR2 may also be involved in SCOS through the mis-regulation of phagocytosis in SCs.

In the present study, we found that ectopic DDR2 expression in Sertoli cells of SCOS testis was induced by a reduction in androgen receptor (AR) signaling, leading to an increase in phagocytosis through the up-regulation of Scavenger receptor class B member 1 (SR-BI) levels. Furthermore, the expression of DDR2 in Sertoli cells activated matrix metallopeptidase 9 (MMP-9) to consume abnormal collagen increase in seminiferous tubules which was responsible for block of testosterone transportation and AR loss and compensated for the impaired blood-testis-barrier (BTB). Our data suggest that the AR/DDR2 cascade may serve as a negative feedback mechanism to help compensate in the homeostasis of seminiferous epithelium in SCOS testis.

Materials and method

Human tissues

All patients gave informed written consent to the procedures and to further studies with their redundant testicular tissue material. To be specific, testicular biopsies from patients with Sertoli cell only syndrome (n=11) were analyzed. In addition, testicular tissues from normozoospermia patients who underwent testicular biopsy during genital surgery procedures for varicocele or epididymal cysts (n=8) were also included as normal controls after identification of normal spermatogenesis status in accordance with the criteria described by Suarez-Quian et al. The use of the human tissue in this study was approved by the Human Research Committee of The PLA General Hospital of Central Command Theater for Approval of Research Involving Human Subjects ([2017]006-1).

Animal models

For the EDS treated rat model, Sprague-Dawley male rats (90 days old) (Fourth Military Medical University, Xi'an, China) were injected intraperitoneally with either a single dose of EDS (75 mg/kg for rat) or with vehicle as described previously [27]. Animals were euthanized at 6 h, 12 h, 24 h, 48 h, or 72 h after treatment. Testes were collected for histologic examination. The Ethics Committee for Animal Experiments of the PLA General Hospital of Central Command Theater approved all animal work and experimental protocols (2018015).

For the mouse vasectomy model, the experimental vasectomy operation was performed under sodium pentobarbital (50 mg/kg body weight, i.p) anesthesia via a lower midabdominal incision as described previously [28].

H&E and immunohistochemistry staining

For H&E staining, paraffin sections of the testes were stained with hematoxylin for 3 min, followed by 1 min incubation with Clarifier I and Bluing Reagent, and then stained with eosin-Y for another 30 s.

For immunohistochemistry, the sections were blocked with 10% normal donkey or goat serum for 1 h and probed with anti-DDR2 antibody or anti-Collagen I antibody at 4°C overnight. Then they were incubated with biotinylated donkey anti-goat or goat anti-rabbit IgG at room temperature for 2 h and incubated with Extr Avidin Peroxidase for 1 h. Positive staining was detected with 0.05% DAB/0.01% H_2O_2 in 0.05 mol/l Tris-HCl buffer. Next, testicular sections of were counterstained with hematoxylin for 2 min. In the negative control group, the primary antibodies were omitted and replaced with normal IgG. All photographs were taken under a light microscope.

Immunofluorescence

The immunofluorescence staining of tissue sections or 4% paraformaldehyde-fixed cells

was carried out according to our previous work [27]. Briefly, samples were blocked with 2% goat and donkey serum in PBS for 30 min at room temperature in order to reduce nonspecific background. The sections were then incubated with the blocking solution containing the primary antibody, at 4°C overnight in a moist box. Slides were washed three times in PBS for 10 min prior to addition of rhodamine-labeled antibodies (Jackson Immune Research Laboratories, West Grove, PA, USA). Nuclei were visualized by 10-min staining of 40,6-diamidino-2-phenylindole (DAPI; dilution 1:2000; Sigma). The sections were mounted in 80% glycerol and examined with an inverted microscope (Axio Imager M1 microscope; Zeiss).

Oil Red O (ORO) staining

We prepared 10-μm-thick frozen sections. After air drying, the sections were fixed in 4% PFA for 15 min and rinsed with 60% (vol/vol) isopropanol. The sections were stained with ORO (Sigma) solution (ORO saturated solution in isopropanol: water, 3:2) for 15 min. The sections were then washed with 70% alcohol for 5 s to remove background staining. After rinsing with tap water, the sections were counterstained with Harris hematoxylin and mounted in glycerol-PBS (9:1) for further analysis.

Masson's trichrome staining

Testicular sections were deparaffinized, rehydrated and then washed in distilled water for 5 minutes. After being stained in Weigert's iron hematoxylin working solution for 10 minutes, sections were stained in Biebrich scarlet-acid fuchsin solution for another 10 minutes. Subsequently, sections were differentiated in phosphomolybdic-phosphotungstic acid solution for 10 minutes until collagen did not display red. After that, sections were transferred directly (without rinse) to aniline blue solution, stained for 5 minutes. Next, sections were rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2 minutes. Finally, sections were dehydrated very quickly, and were cleared in xylene and mounted with resinous mounting medium.

Real-time PCR

Total RNA was extracted from frozen mouse testis using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized with Superscript III (Rnase H-Reverse Transcriptase; Invitrogen), exactly according to the manufacturer's instructions and PCR was set up according to Promega's reverse transcription system protocol. The primers used in this study were shown in the supplemental figures (Table S1). Amplification of Gapdh mRNAs served as internal controls. All PCR reactions for all samples were repeated at least three times. PCR products were then quantified by SYBR green intercalation using the MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gapdh was used to obtain the $^{\Delta\Delta}$Ct values for the calculation of fold increases.

Western blotting

TM4 SCs were homogenized in ice-cold RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 1% vol/vol, sodium deoxycholate 1% wt/vol, and SDS 0.1% wt/vol pH 7.5) supplemented with complete proteinase-inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany). For western blot analysis, 30 μg lysate was separated on 8-10% SDS/PAGE and transferred to nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were then incubated with primary antibodies including anti-DDR2 (R&D Systerms, dilution 1:1000), anti-β-actin (Santa Cruz biotechnology, dilution 1:2000) and anti-AR (Abcam, dilution 1:1000) in blocking solution overnight at 4°C. After washing with PBS containing 0.05% Tween-20 (PBST), the membranes were incubated with peroxidase conjugated donkey anti-goat IgG or goat anti-rabbit IgG (diluted at 1:10000) for 60 min. The bands were finally detected by using an ECL kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

Laser capture microdissection (LCM) and RNA extraction

The LCM was performed at the core facilities of the Department of Pathology in Xi Jing hospital, the Fourth Military Medical University. Microdissection and capture of Sertoli cells were done followed the manufacturer's instruction (MDS Analytical Technologies, Sunnyvale, CA). Sertoli cells in approximately 80 tubules were collected from SCOS testis. Total RNA

from the captured SCs was extracted with the Pico Pure RNA Purification kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed as above described.

Phagocytosis assay

RBs were isolated from testes of 70-day-old mice and were then rodamin labeled as described elsewhere [29]. Briefly, seminiferous tubules were incubated in 25 ml of 0.01 M PBS (pH 7.2) containing 0.1% glucose, 3 mM lactate, 0.25 mg/ml trypsin for 20 min at 33°C, and then cells were dispersed by gentle pipetting. The suspension was filtered through sterile surgical gauze. Cells were pelleted by centrifugation at 800× g for 10 min and resuspended in 40 ml of 0.01 M PBS (pH 7.2) containing 0.1% glucose, 3 mM lactate, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. Supernatants were collected gently by pipetting and were pelleted twice at 200× g for 3 min. The resulting suspension containing RBs and GCs was allowed to sediment overnight at 4°C, and then was centrifuged at 800× g for 10 min. The supernatant was centrifuged again at 800× g for 30 min. The quality of RBs preparations were monitored by microscopic observation. RBs were transferred into labeling buffer (10 mM Na borate, pH 8.8, 150 mM NaCl), followed by incubation in biotin labeling solution (50 μg/ml in DMSO) at 20°C for 15 min. The labeling was ended by adding 10 mM NH_4Cl. To measure RB phagocytosis activity, SCs seeded in eight-well Labtek chambers were incubated with 1.2×10^7 biotinylated RBs for 2, 4, or 6 hours at 34°C in a humidified atmosphere of 5% CO_2. Unbound RBs were washed away with DMEM, and cells were fixed for 5 min with 4% paraformaldehyde/PBS (pH 7.4). Subsequently, RBs were labeled with avidin-rodamin for 1 h at 25°C. The ingested RBs were observed using an inverted microscope (Axio Imager M1 microscope, Zeiss) and phagocytic index was calculated as the intensity of rodaminin each well.

Statistical analysis

Experiments were repeated at least three times, and one representative from at least three similar results was presented. Ddr2 or COL1 immunoreactive content was determined based on Image J, with the aid of SPSS 15.0 software. Quantitative data are presented as mean ± SEM. Results were analyzed for statistically significant differences using analysis of variance, followed by Tukey's test. Differences were considered significant at $P < 0.05$.

Results

DDR2 is expressed in SCs of SCOS testes

To examine the histology of normal human testes and SCOS testes, we used hematoxylin and eosin staining and confirmed that most seminiferous tubules in SCOS testes contained only SCs compared with tubules of normal testes contain SCs and germ cells. In addition, most (9/11) SCOS testis of patients showed a significant increase in basal membrane thickness around the seminiferous tubules, indicating a fibrosis like phenotype (**Figure 1A** and **1B**). This was further confirmed with collagen I (COL1) staining, showing that COL1 levels in the basal membrane of SCOS tubules increased dramatically (**Figure 1C** and **1D**). Since COL1 was the known ligand for the DDR2 receptor, we sought to determine if DDR2 expression was affected in SCOS testes. In normal mammalian testes, DDR2 is exclusively expressed in the Leydig cells [30], yet SCOS testes show expression in both Leydig cells and SCs (**Figure 1E** and **1F**). This indicates that the spatial expression of DDR2 changes under pathological conditions.

COL1 does not cause DDR2 expression in SCs of SCOS testes

The increase of COL1 levels in seminiferous tubules of SCOS testes and the fact that DDR2 is a collagen receptor, led us to ask whether the abnormal expression of DDR2 was induced by the increase of collagen. To answer this question we used a mouse vasectomy model, which causes seminiferous tubule fibrosis similar to SCOS testes [28], to detect whether the increase of COL1 in the basal membrane can induce DDR2 expression in SCs. While COL1 levels were elevated significantly along the basal membrane of 5 months old mice who hadundergone a vasectomy as detected my Masson staining (Figure S1A), no DDR2 expression was seen in SCs indicating that DDR2 expression in SCs was not induced by COL1 up-regulation in SCOS testes (Figure S1B).

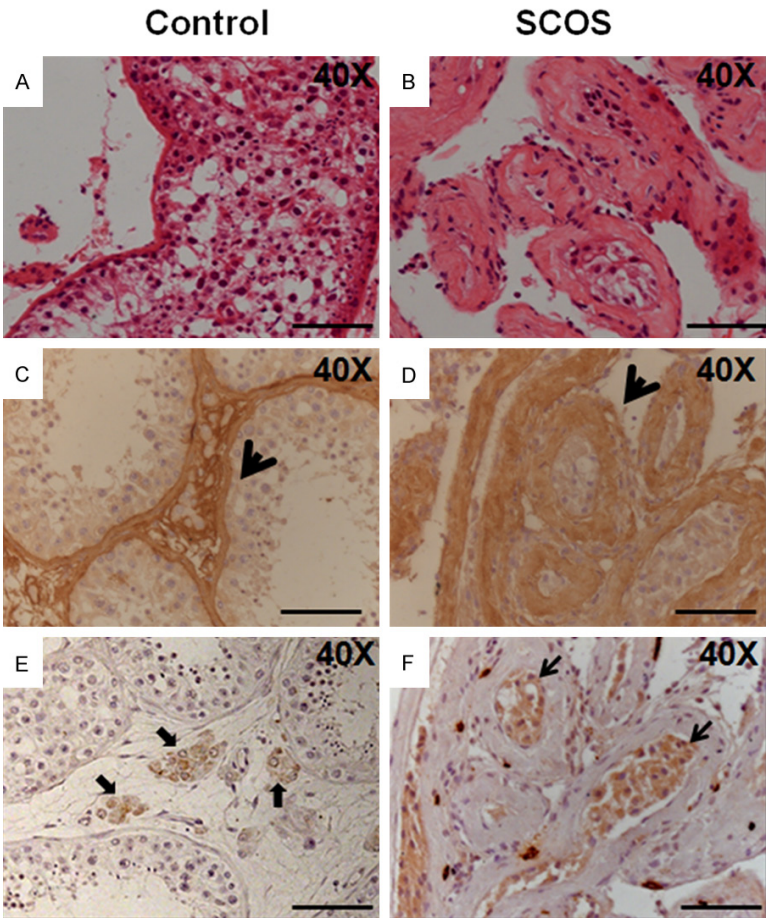


Figure 1. Histology and immunohistochemistry of Sertoli cell only syndrome testis (40X). H&E staining of control testis (A) and Sertoli cell only syndrome (SCOS) testis (B). COL1 expression in control testis (C) and SCOS testis (D). COL1 immunoactivity was found in basal membrane of control testis and Sertoli cell only syndrome testis, the COL1 immunoactivity in SCOS testis was stronger than control testis. Arrow head indicates basal membrane. (E, F) DDR2 immunohistochemical staining in control human testis and SCOS patient testis. DDR2 was exclusively expressed in Leydig cells in control testis but was both expressed in Leydig cells and Sertoli cells in SCOS testis. Arrow indicates Leydig cell and arrow head represent Sertoli cell. Bar =50 μm.

DDR2 expression in SCs is induced by decreaseof AR signaling in SCOS testes

Given that the androgen receptor (AR) is specifically expressed in SCs, and only a few patients with SCOS have an androgen deficiency [31], we wanted to know if SCOS testes had a defect in AR expression. Interestingly, DDR2 up-regulation in SCOS testes corresponded with a loss of AR expression compared with AR nuclear localization seen in SCs of control testes (**Figure 2**).

This led us to ask whether the abnormal expression of DDR2 in SCOS testes may be caused by a decrease in AR signaling. To test this hypothesis, we treated rats with EDS to kill their Leydig cells causing an androgen deficiency and subsequently a decrease in SCs AR expression [32]. Although DDR2 was not detected in SCs of control rat testes, strong DDR2 expression was seen in SCs after 12-24 hour of EDS treatment (**Figure 3A-F**). However, this expression was not found in each tubule and the strength of expression varied among tubules, suggesting that DDR2 expression in SCs might be stage specific. Further analysis of each tubule indicated that DDR2 levels in SCs reached their peak between stages VII-VIII then began to decline, becoming absent after stages XII-XIII (Figure S2). These findings suggest that a decrease in AR expression regulates DDR2 expression in SCs in a stage specific manner.

To demonstrate that lack of AR signaling is sufficient to regulate DDR2 expression, we used cultured mouse TM4 Sertoli cell lines and administered different concentrations of testosterone (T). Unlike SCs *in vivo*, control SCs cultured *in vitro* showed slight DDR2 expression due to a decrease in androgen signaling. Adding increasing concentrations of T corresponded to a gradual increase in AR levels, leading to a decline in DDR expression (**Figure 3G**). Pre-treatment of these cells with flutamide, the androgen receptor inhibitor, led to a decrease in AR expression and an increase in DDR2 expression even in the presence of T (**Figure 3H**). This further demonstrated that activation of AR signaling in SCs is necessary to prevent ectopic DDR2 expression.

DDR2 regulates phagocytosis in mouse SCs

Since tyrosine receptor kinases have been reported to regulate phagocytosis in Sertoli cells [24], we wanted to ask if ectopic DDR2

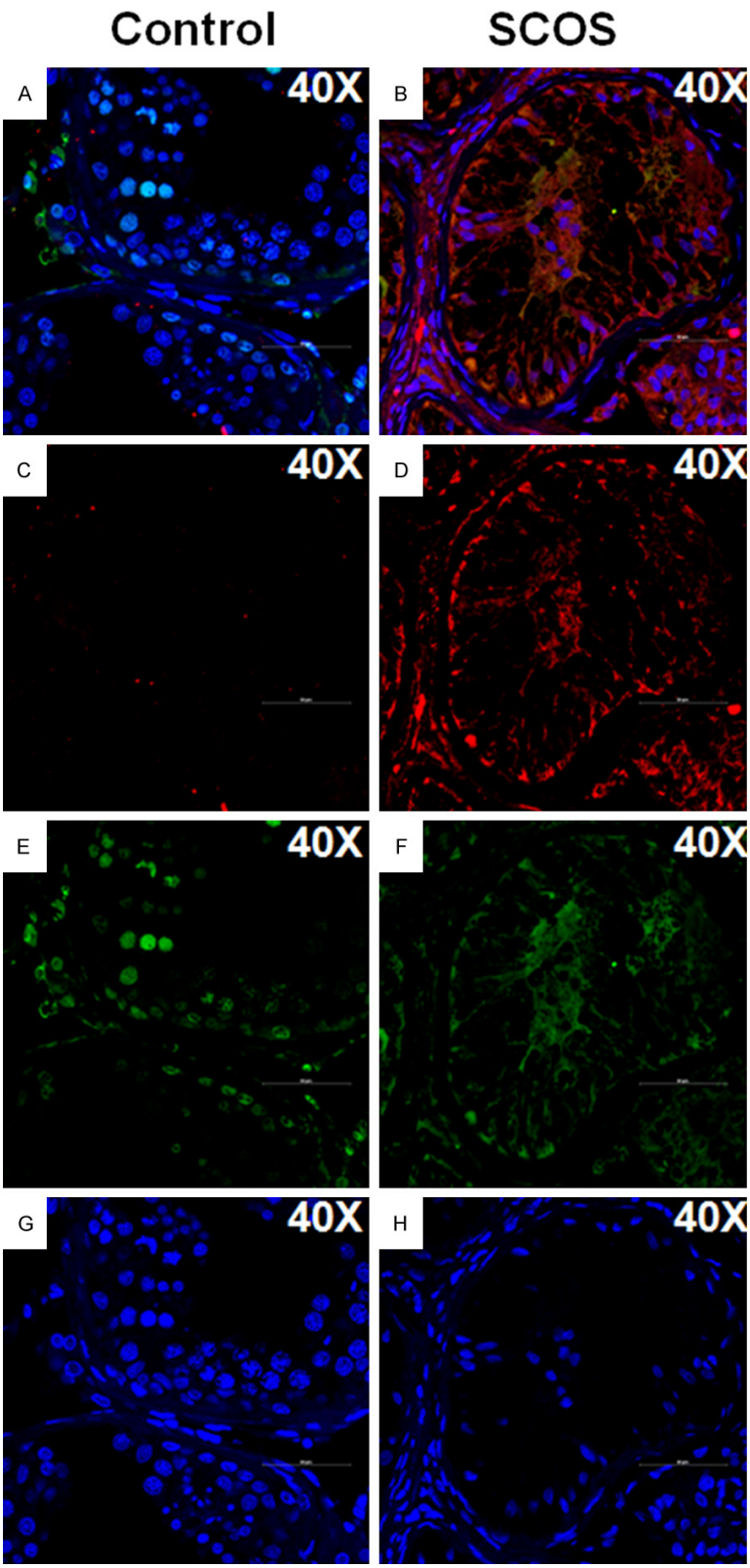


Figure 2. AR and DDR2 expression in human control testis and SCOS testis (40X). AR and DDR2 co-localization in human control testis (A, C, E, G). No DDR2 (C) immunoreactivity was found in seminiferous tubules of control testis while AR (E) was found located in Sertoli cells. AR and DDR2 co-localization in SCOS testis (B, D, F, H). Strong DDR2 (D) expression was found in Sertoli cell of SCOS tubules while AR (F) expression was lost in SCOS seminiferous tubules. AR was stained with green, while DDR2 was stained with red. DAPI was stained with blue indicating nuclear. Arrow heads indicate Sertoli cells. Bar =50 μm.

had a similar role in SCOS testes. Using adenovirus packaged plasmid, we over-expressed DDR2 in TM4 mouse Sertoli cell lines and saw an increase in DDR2 mRNA expression compared to GFP controls (**Figure 4B**). We next tested whether these cells had enhanced phagocytic function by culturing them for 2, 4, or 6 hours with isolated rhodamine tagged residual bodies and quantifying how many were taken up by the cells. Sertoli cells over-expressing DDR2 showed a significant increase in the amount of residual bodies that were phagocytosed compared to their control counterparts (**Figure 4A** and **4C**). Furthermore, these cells showed about a 2-fold increase in class B scavenger receptor type I (SR-BI) mRNA levels (**Figure 4D**), a receptor expressed in the Sertoli cells that is necessary for the uptake of apoptotic cells [33, 34]. In contrast, knockdown of DDR2 in cultured Sertoli cells using siRNA showed the opposite effect. Knockdown efficiency was measured using RT-PCR (**Figure 4F**) and those cells with reduced levels of DDR2 showed a statistically significant decrease in phagocytosis using the same assay as described above (**Figure 4E** and **4G**). Additionally, SR-BI mRNA levels were also reduced when DDR2 was

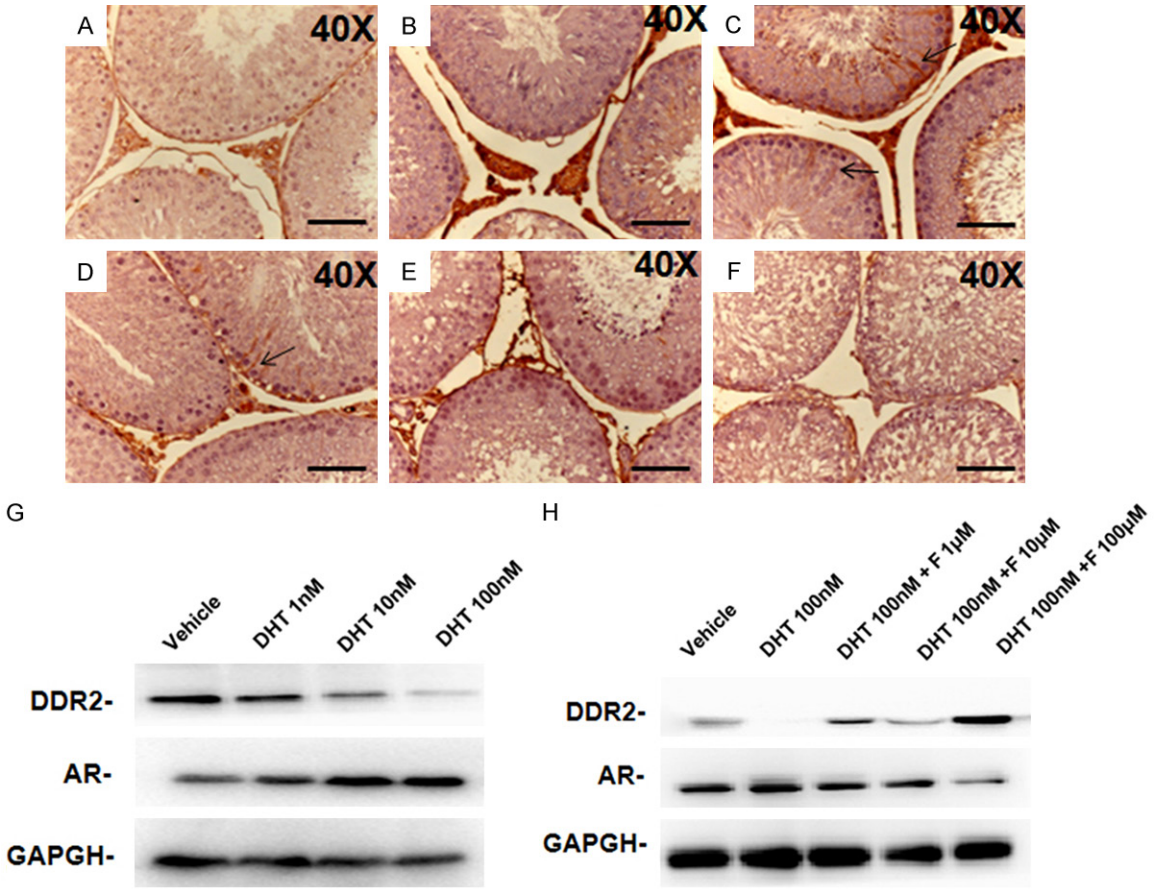


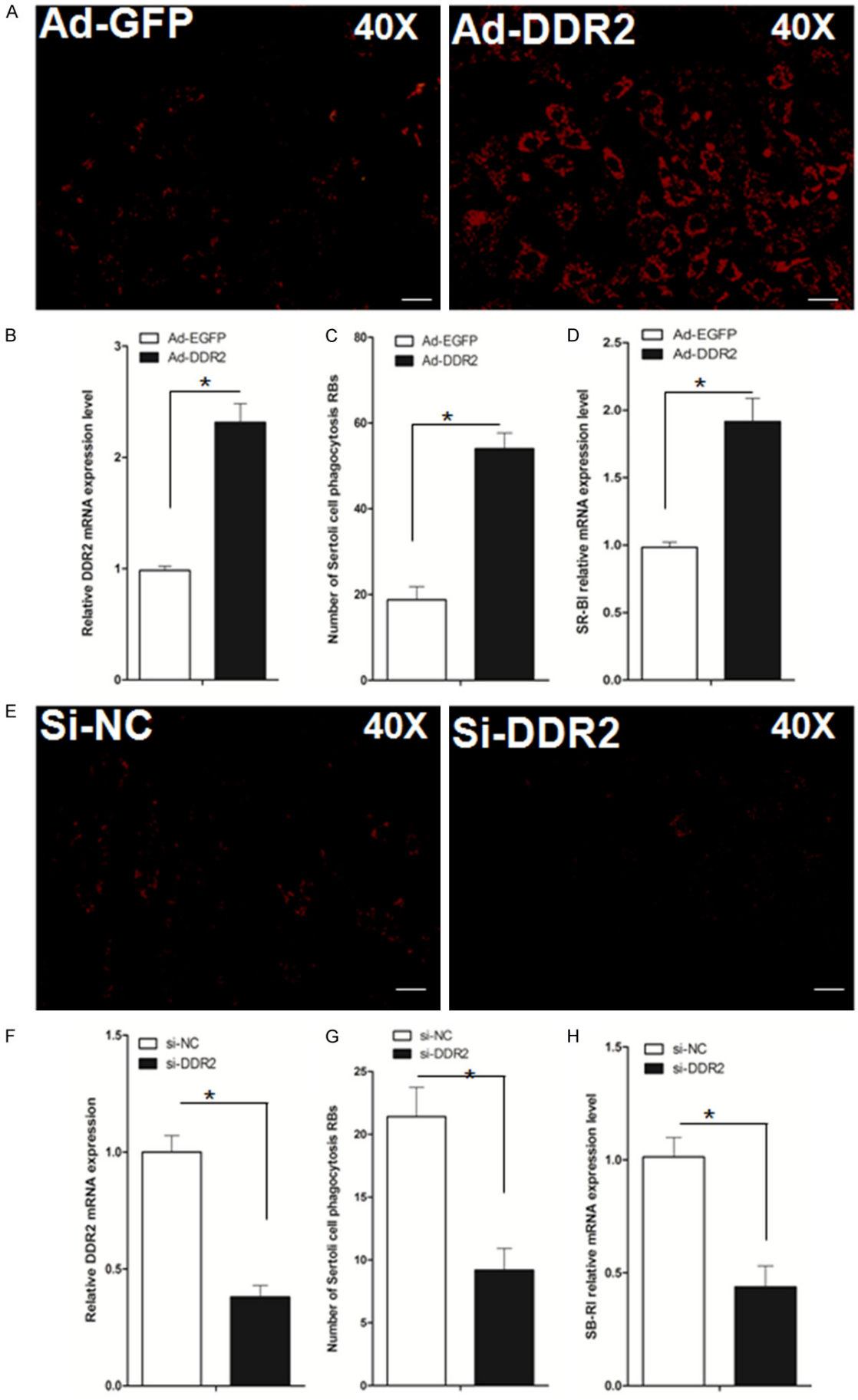
Figure 3. DDR2 expression was induced in Sertoli cell by decrease of AR signaling (A-F, 40X). DDR2 expression in Sertoli cell of seminiferous tubules after EDS 0 h (A), 6 h (B), 12 h (C), 24 h (D), 48 h (E) and 72 h (F). DDR2 expression was negative in EDS 0h testis (A) but became positive in EDS treated 12 h (C) and 24 h (D) testis. Arrow head indicates DDR2 positive Sertoli cell. Bar =50 μm. (G) Typical western blot results of DDR2 and AR expression in TM4 Sertoli cells with different concentration T treatment. AR levels in TM4 cells increased with elevated testosterone levels. DDR2 expression was found in TM4 cells but was inhibited by 100 nmol/L testosterone treatment. (H) Flutamide pre-treatment reverses the testosterone inhibition to DDR2.

knocked-down (**Figure 4H**). Altogether this indicates that DDR2 can cell autonomously regulate phagocytosis via SR-BI expression.

DDR2 expression in 70-day old wild type mice as well as DDR2$^{slie/slie}$ mice was detected by immunohistochemical staining, and no positive signaling was found in SCs (**Figure 5A** and **5B**). Periodic Acid-Schiff Stain (PAS) staining revealed that the remaining residual body number in seminiferous tubules of DDR2$^{slie/slie}$ testis increased compared with wild type testes (**Figure 5C**, **5D** and **5G**), indicating that the phagocytosis in DDR2$^{slie/slie}$ testicular SCs decreased. To confirm this, Oil red O (ORO) staining was performed and the results showed that lipid droplets to nuclei of SCs in DDR2$^{slie/slie}$ testis also increased significantly compared with wild type testes (**Figure 5E**, **5F** and **5H**). We also examined the SR-BI expression which is a marker for phagocytosis as previous study, and a significantly decrease of SR-BI mRNA level in the DDR2$^{slie/slie}$ testis was observed compared with wide type testis (**Figure 5I**).

DDR2 expression in SCs of SCOS activated the phagocytosis and MMPs signaling

To explore the role of SCs DDR2 expression in SCOS, DNA was extracted from the laser capture dissected SCs from the SCOS testis (**Figure 6C** and **6D**) and control testis (**Figure 6A** and **6B**). We found the SR-BI mRNA level in SCOS SCs increased significantly compared with control group, which indicated an increased


A
Ad-GFP
40X
Ad-DDR2
40X
B
Ad-EGFP
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Relative DDR2 mRNA expression level
C
Ad-EGFP
Ad-DDR2
Number of Sertoli cell phagocytosis RBs
D
Ad-EGFP
Ad-DDR2
SR-BI relative mRNA expression level
E
Si-NC
40X
Si-DDR2
40X
F
si-NC
si-DDR2
Relative DDR2 mRNA expression
G
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Number of Sertoli cell phagocytosis RBs
H
si-NC
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SB-RI relative mRNA expression level

Figure 4. Phagocytotic capacity increased in DDR2 over-expressed Sertoli cell but decreased in DDR2 knock-down Sertoli cell. (A) Staining of rodamin labeled RBs phagocytosis in control and DDR2 over-expressed Sertoli cells. Bar =50 μm. The magnification of (A) was 40X. (B) DDR2 mRNA level in control and DDR2 over-expressed Sertoli cells. The mRNA level of DDR2 in over-expressed group was 2.6 folds higher than control group. (C) Number of phagocytotic RBs in control and DDR2 over-expressed Sertoli cells. The number of phagocytotic RBs in DDR2 over-expressed Sertoli cells was 3 folds higher than control group. (D) SR-BI mRNA level in control and DDR2 over-expressed Sertoli cells. The SR-BI mRNA level in DDR2 over-expressed Sertoli cells was 2 folds higher than control group. (E) Staining of rodamin labeled RBs phagocytosis in negative control Sertoli cells and DDR2 knock-down Sertoli cells. Bar =50 μm. The magnification of (E) was 40X. (F) DDR2 mRNA level in negative control and DDR2 knock-down Sertoli cells. The DDR2 mRNA level in knock-down Sertoli cells was 40% of control group. (G) Number of phagocytotic RBs in negative control and DDR2 knock-down Sertoli cells. The number of phagocytotic RBs in DDR2 knock-down Sertoli cells was less than 50% of control group. (H) SR-BI mRNA level in negative control and DDR2 knock-down Sertoli cells. The SR-BI mRNA level in DDR2 knock-down Sertoli cells was less than 50% of control group.

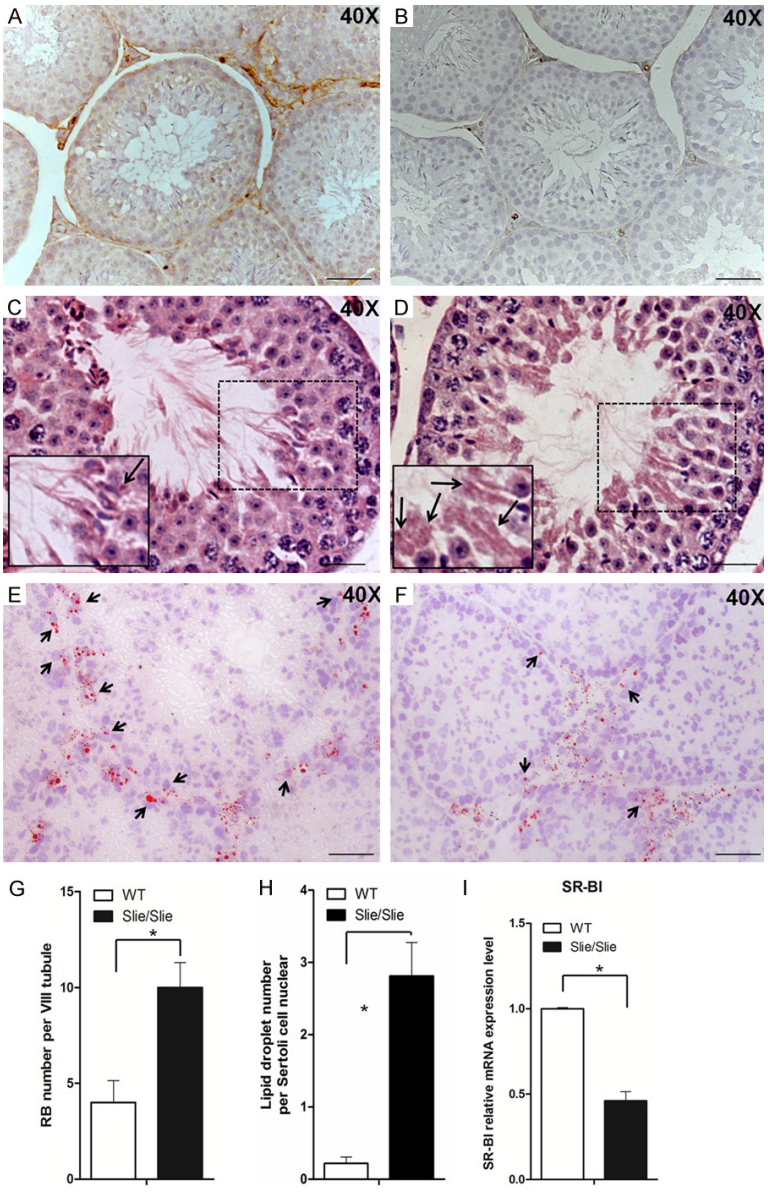

Figure 5. Phagocytosis capacity decreased in Sertoli cell of DDR2 deficiency mouse testis. (A, B) DDR2 immunostaining in wild type (A) and DDR2$^{slie/slie}$ (B) mouse testis. Bar =50 μm. (C, D) PAS staining of wild type (C) and DDR2$^{slie/slie}$ (D) testicular section. Arrow head indicates the residual body. Bar =50 μm. (E, F) Oil red O staining of wild type (E) and DDR2$^{slie/slie}$ (F) testicular section. Arrow heads represents the lipid drop in Sertoli cell. Bar =50 μm. (G) RBs number in wild type and DDR2$^{slie/slie}$ mouse testis. The average RBs number in tubule at stage VIII of DDR2$^{slie/slie}$ mouse testis was 2 folds higher than control group. The magnification was 40X. (H) Number of lipid drop in per Sertoli cell of wild type and DDR2$^{slie/slie}$ mouse testis. The number of lipid drop in per Sertoli cell of DDR2$^{slie/slie}$ mouse testis was 2.6 folds higher than control testis. (I) SR-BI mRNA level in wild type and DDR2$^{slie/slie}$ testis. The SR-BI mRNA levels in DDR2$^{slie/slie}$ testis were less than 50% of control testis.

phagocytosis capacity and was consistent with the results DDR2 over-expressed TM4 cells exerted an elevated phagocytosis (**Figure 6E**). Additionally, the level of adhered molecules that participated in blood-testis-barrier (BTB) formation such as N-cadherin and Occludin were also elevated in SCOS SCs, and represented BTB was impaired in SCOS testis (**Figure 6F** and **6G**).

Previous study had shown a feedback loop that COL1 activated DDR2 then led to increased MMPs signaling to consume the collagen [35]. We wonder whether the induced DDR2 by AR decrease can trigger MMPs expression to consume the collagen. The

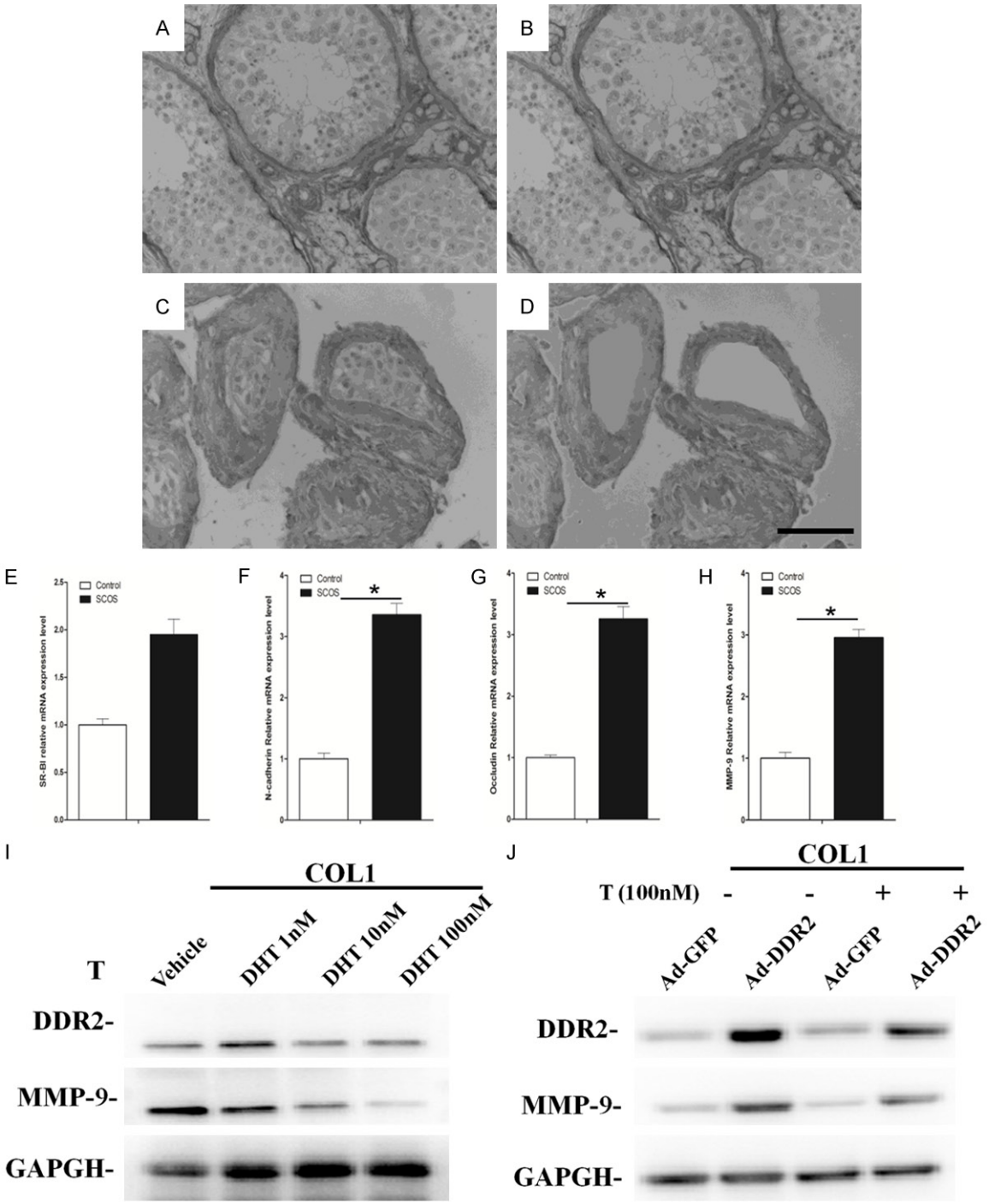

Figure 6. Up-regulated DDR2 expression in SCs of SCOS testis activated MMP-9 to consume the abnormal increased collagen and to compensate the impaired blood-testis-barrier. (A-D) Laser captured dissection of Sertoli cell from control testis (A and B) and SCOS testis (C and D). Bar =50 μm. The magnification of was 40X. (E) SR-BI mRNA level in control and SCOS testicular Sertoli cell. The SR-BI mRNA level in SCOS testicular Sertoli cell was 1.8 folds higher than control group. (F) N-cadherin mRNA level in control and SCOS testicular Sertoli cell. The N-cadherin mRNA level in SCOS testicular Sertoli cell was 3.1 folds higher than control group. (G) Occludin mRNA level in control and SCOS testicular Sertoli cell. The occludin mRNA level in SCOS testicular Sertoli cell was 3.3 folds higher than control group. (H) MMP-9 mRNA level in control and SCOS testicular Sertoli cell. The MMP-9 mRNA level in SCOS testicular Sertoli cell was 3 folds higher than control group. (I) Immunoblot of DDR2 and MMP-9 expression in TM4 Sertoli cells with different dose T administration plus collagen I pre-treatment. (J) Immunoblot of DDR2 and MMP-9 expression in DDR2 over-expressed TM4 Sertoli cells with 100 nM T administration plus collagen I pre-treatment.

qRT-PCR results showed the MMP-9 level in SCOS SCs significantly increased compared with control SCs, which suggested DDR2 expression up-regulated the MMP-9 level (**Figure 6H**). To demonstrate DDR2 expression activating MMP-9 in Sertoli cell, we detected DDR2 and MMP-9 expression in TM4 cells with COL1 and/or testosterone stimulus. The results showed DDR2 expression induced by COL1 activated MMP-9 and low dose testosterone (1 nM) increased the activation, whereas high dose testosterone (100 nM) inhibited the activation (**Figure 6I**). Moreover, over-expression of DDR2 increased MMP-9 activation and alleviated the inhibition induced by high dose testosterone (**Figure 6J**). All above data confirmed the up-regulated DDR2 in SCs activated MMP-9 to compensate for the abnormal increase of COL1 in SCOS testis.

Discussion

SCs only syndrome is a very common type of azoospermia, the discoid domain receptor family member DDR1 related with azoospermia as well as the DDR2 deficiency mouse had infertile phenotype, which triggered us to consider the role of DDR2 in infertility [26, 30]. In the present study, we demonstrated ectopic DDR2 expression induced by decreased AR signaling which led to an increase of phagocytosis and activation of MMP-9 to compensate for the impaired BTB caused by excessive collagen in SCOS testis.

Intra-testicular androgen concentration is essential for homeostasis in seminiferous tubules [36, 37]. AR is mainly expressed on SCs in human seminiferous tubules. Our hypothesis that the ectopic DDR2 expression in SCs is induced by decrease of AR is supported by the absence of AR in SCOS seminiferous tubules, by the stage specific DDR2 expression in EDS treated rat testis, and by the inhibition of T stimulation to DDR2 expression as well as flutamide treatment reversing the inhibition. The previous study that EDS induced stage specific loss of AR in Sertoli cell in rat testis also supported our hypothesis [32]. Germ cells exist in an environment surround by SCs; therefore, paracrine signaling between these intimately associated cells must regulate the process of germ cell death. In turn, SCs functions are also critically influenced by the status of adjacent germ cells [38]. In the EDS treated rat testis, germ cells undergo apoptosis due to the impaired SCs function, so there is a question whether the DDR2 expression is directly induced by decrease of AR or indirectly induced by the germ cells apoptosis caused by AR decrease in SCs. To answer this, we detected the DDR2 immunoreactivity in SCs of busulfan treated mouse testis, a classic model to induce germ cell apoptosis and resulted in only Sertoli cell left for a period [39], but we found no DDR2 expression in Sertoli cells (data not shown), indicating ectopic DDR2 expression in Sertoli cell is not induced by apoptotic germ cells but directly by decrease of AR.

Previous studies revealed a stage-dependent formation of SC phagocytosis where the highest phagocytic activity was shown in stage VIII and IX within the seminiferous epithelium [40]. Interestingly, the stage-specific expression of DDR2 throughout the seminiferous epithelial cycle after EDS treatment showed a similar pattern with the phagocytic activity. Emerging evidence points to a key role of the receptor tyrosine kinases (RTKs) family in phagocytic activity. For example, the Tyro 3 RTKs subfamily (Tyro 3, Axl, and Mer) regulate the phagocytic function of SCs [24, 41]. Considering DDR2 belongs to RTKs family, the patterns of DDR2 expression that occur during the highest phagocytosis of the spermatogenic cycle do suggest a potential involvement for DDR2 in SCs phagocytosis. This contention is supported by our present data that the DDR2 over-expressed SCs displayed an elevated phagocytic capacity whereas SCs in DDR2 deficiency mice as well as the DDR2 knock-down SCs showed a reduced phagocytic activity. It's interesting that even DDR2 is not expressed in SCs of wild type mouse testis, phagocytosis in Sertoli cells of $DDR2^{slie/slie}$ mouse testis still decreased. The $DDR2^{slie/slie}$ mouse was sterile, and the ectopic DDR2 expression was only observed in SCs of SCOS testis as well as EDS treated rat testis which both showed impaired spermatogenesis. Thus, our explanation is that DDR2 involved in phagocytosis is non autonomous and the effect of DDR2 on phagocytosis can only be activated in pathological condition. Another interesting finding is that SCs still exert increased phagocytotic capacity though no germ cells exist in SCOS testis. The possible reason is that those that remained were rela-

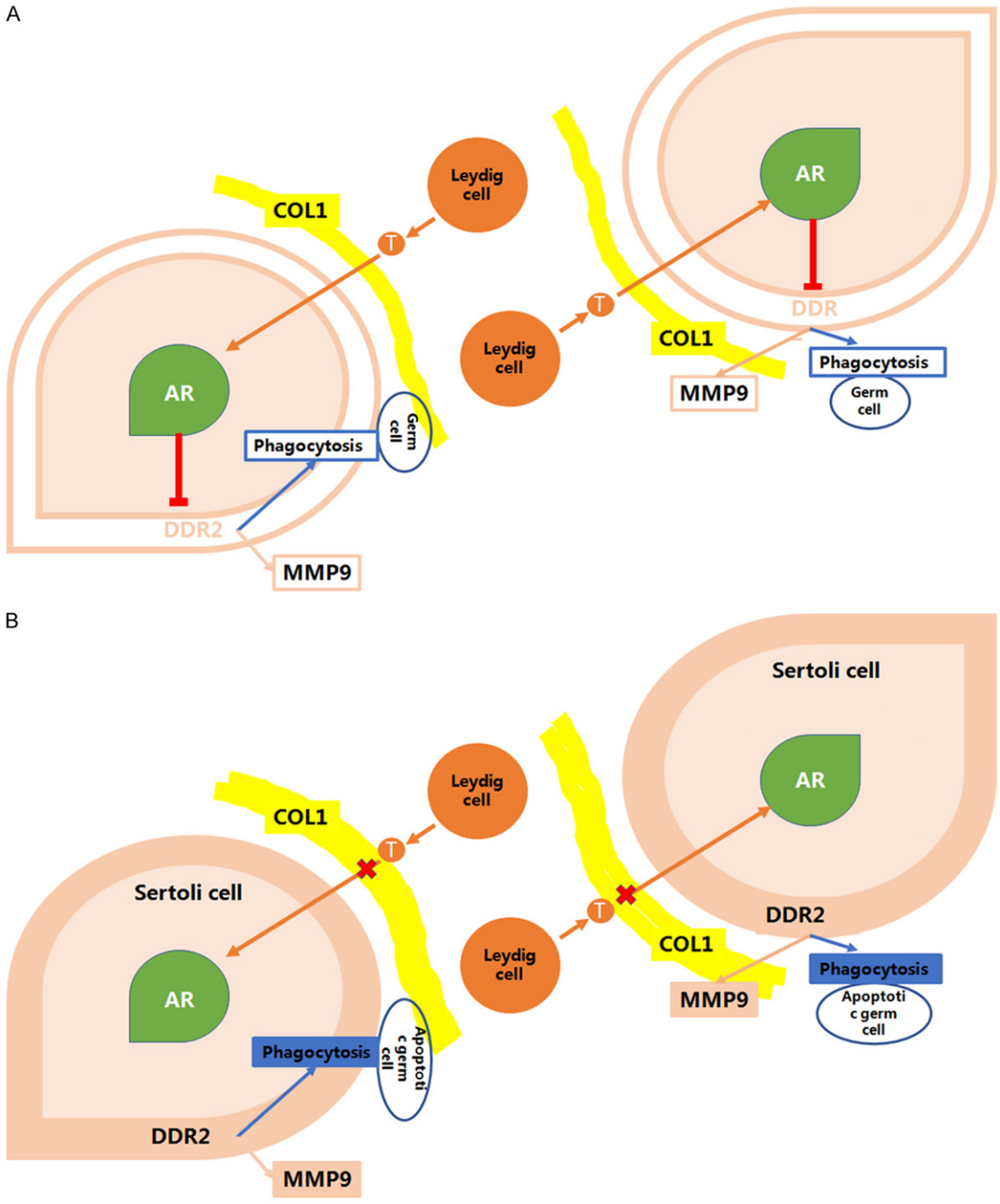

Figure 7. Scheme about the signaling and the role of up-regulated DDR2 in control human and SCOS testis. A. In control testis, the Sertoli cell DDR2 expression was inhibited by androgen receptor (AR) activated by Leydig cell produced testosterone. B. In SCOS testis, the increased collagen in basal membrane impaired the testosterone transportation and led to AR inactivation. The AR inactivation induced DDR2 expression, which promoting phagocytosis and activating MMP-9.

tive healthy SCs that phagocytize the apoptotic SCs since previous studies found some SCs that exerted apoptosis in SCOS tubules [27, 42].

Class B scavenger receptor type I (SR-BI), known as a receptor for high-density lipoprotein (HDL), was shown to bind to apoptotic germ cells through recognizing membrane phospho-

lipid phosphatidylserine (PS) in apoptotic cells by other investigators [33, 34, 43]. Our hypothesis that DDR2 expression may be involved in the phagocytosis through regulating SR-BI activity which is demonstrated by the finding that over-expressed DDR2 increased the SR-BI expression while knock-down DDR2 decreased it, and by DDR2 positive SCs showing increased SR-BI mRNA level in SCOS tubules.

Since most SCOS patients have normal serum testosterone concentration as well as normal steroidogenesis capacity [44], we wondered why the Sertoli cell AR level was decreased in SCOS seminiferous epithelium [31, 45]. Our present study showed an increased thickness and COL1 level in basal the membrane as well as BTB adherence molecules in SCs which is consistent with previous study showed a fibrosis phenotype in SCOS seminiferous tubules [17, 46], indicating a seriously impaired BTB. Thus, we consider the decrease of AR as a result of T transportation block caused by the stop periodic BTB opening due to the seminiferous tubules fibrosis in SCOS testis. MMP-9 expression at the BTB was found from stage VIII to stage X and was strongest in stage IX, which is similar with, but a little later than DDR2 expression in EDS treated testis [47]. Considering the finding that MMP-9 levels increased in Sertoli cell of SCOS testis and DDR2 over-expression reversed the inhibition of high dose testosterone to MMP-9 activation, the up-regulated DDR2 in SCs may act as a compensatory effect of blocked T transportation and thus consume collagen to alleviate BTB impairment through MMP-9 activation (**Figure 7**). This assumption is also supported by previous study: the steady-state level of MMP-9 in SCs was up-regulated following MMP-9 inhibitor treatment due to a physiological response of the SCs in the presence of MMP-9 inhibitor, in an attempt to maintain BTB homeostatic functions [47, 48]. However, how the increased collagen in seminiferous tubules blocks testosterone transportation still requires further study.

Conclusion

In summary, our current data substantiate DDR2 expression is induced by AR decrease in SCs of SCOS testis. The up-regulated DDR2 expression promotes phagocytosis by regulating SR-BI levels and compensates to alleviate BTB impairment by activating MMP-9. Our finding may shed light on the pathogenesis of SCOS.

Acknowledgements

This work was supported by the Natural Science Foundation of China (NO. 81801524), Natural Science Foundation of Hubei Province (2018CFB336).

Disclosure of conflict of interest

None.

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Table S1. Primer used in the present study

Gene name	Forward Primer (5'-3')	Reverse primer (5'-3')
Mouse SR-BI	TTTGGAGTGGTAGTAAAAAGGGC	TGACATCAGGGACTCAGAGTAG
Mouse DDR2	GCAGAAGCCGACATAGTGAA	CAAAGTTGAGAGACGAAAGG
Mouse β-actin	TGGAGAAGAGCTATGAGCTGCCTG	GTGCCACCAGACAGCACTGTGTTG
Human SR-BI	TGATGATGGAGAATAAGCCCAT	TGACCGGGTGGATGTCCAGGAAC
Human N-cadherin	CGAGCCGCCTGCGCTGCCAC	CGCTGCTCTCCGCTCCCCGC
Human Occuldin	TGCATGTTCGACCAATGC	AAGCCACTTCCTCCATAAGG
Human MMP-9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCCTTAT
Human GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

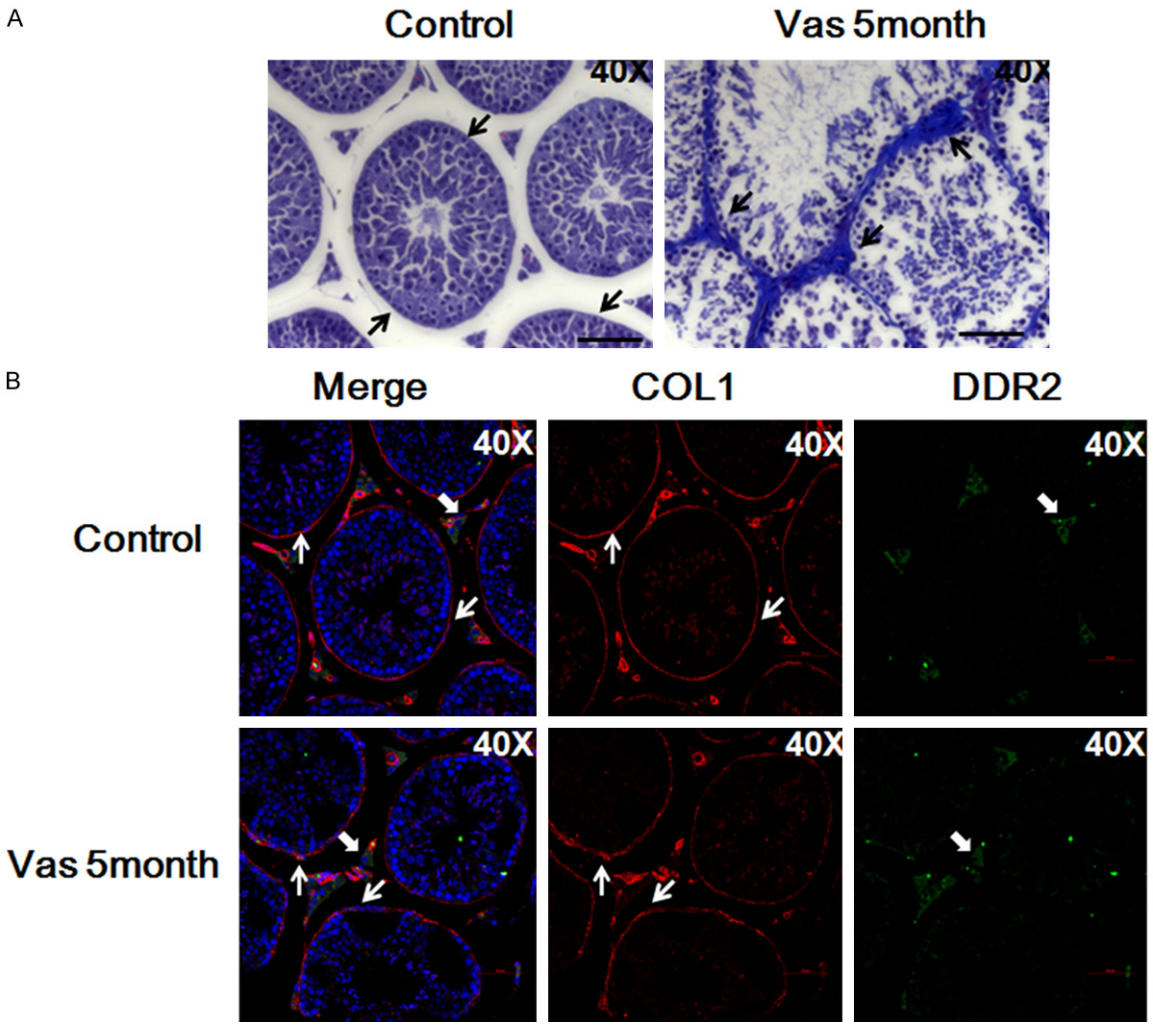

Figure S1. Increased collagen content in basal membrane couldn't induce Sertoli cell DDR2 expression in 5 month mouse vasectomy testis (40X). A. Masson staining of mouse control testis and vasectomy 5 month testis. Collagen level in basal membrane of vasectomy 5 month testis significantly increased than control testis. Arrow head represents basal membrane of seminiferous tubule and blue indicates collagen. Bar =50 μm. B. DDR2 and COL1 immunofluroscence staining in mouse control testis and vasectomy 5 month testis. COL1 expression increased in basal membrane of vasectomy testis and no DDR2 expression was found in seminiferous tubules. Bar =50 μm.

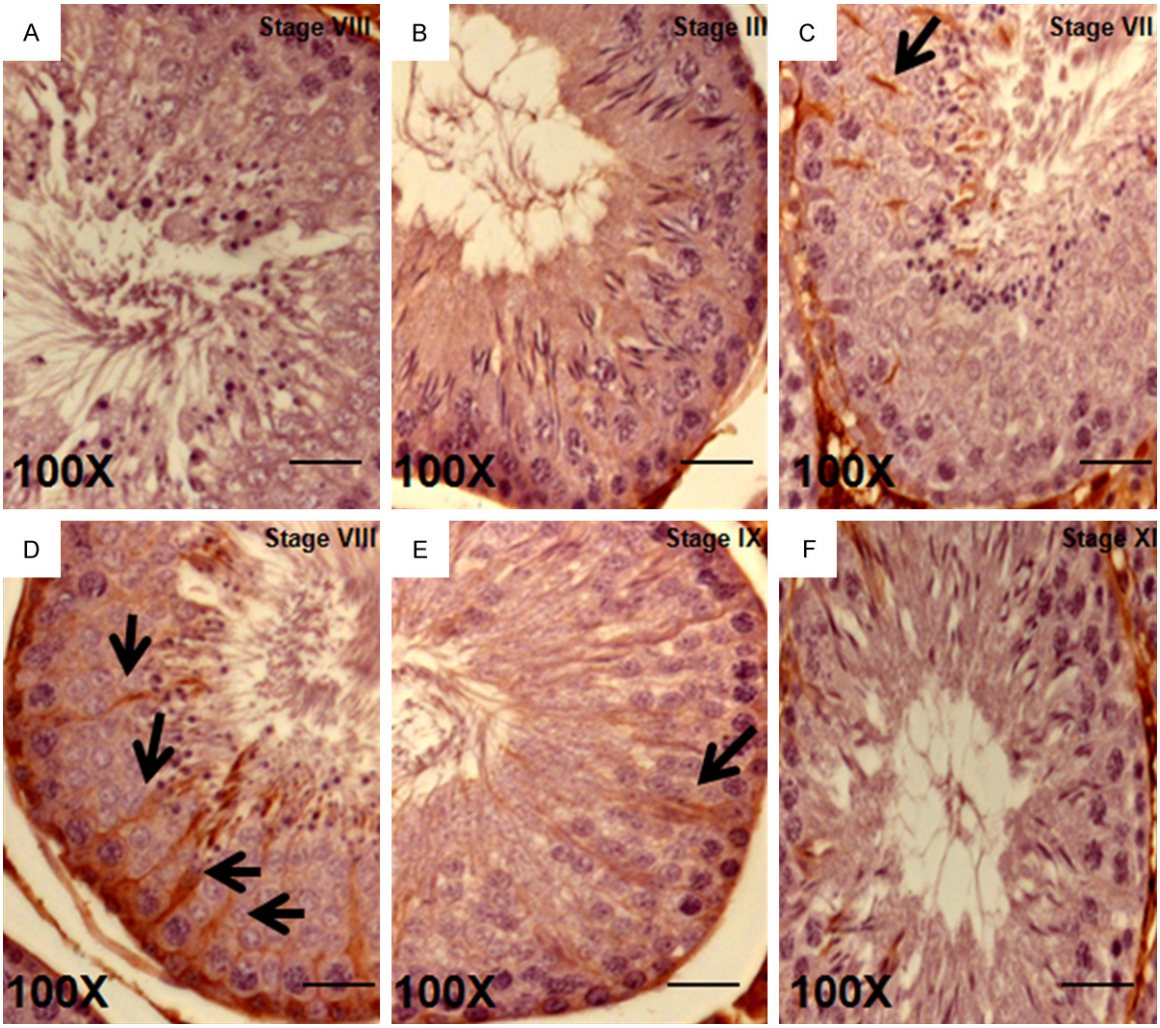

Figure S2. DDR2 expression at different stages of the epithelial cycle in sections of testicular seminiferous tubules from EDS treated 24 h rat testis (100X). DDR2 expression was found in stage VII-IX but was absent from stage IX-VI. Bar =100 μm.