BIOLOGY OF REPRODUCTION (2012) **87**(3):61, 1–8
Published online before print 13 June 2012.
DOI 10.1095/biolreprod.112.099978

Localization and Regulation of Murine *Esco2* During Male and Female Meiosis[1]

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ABSTRACT

Meiosis is essential for generation of healthy gametes in both sexes and involves recombination and segregation of homologous chromosomes to produce haploid gametes. The initiation of meiosis in both sexes relies upon retinoic acid (RA) (Griswold MD, Hogarth CA, Bowles J, Koopman P. Initiating Meiosis: The Case for Retinoic Acid. Biol Reprod 2012; 86(35):1–7). Previous studies have demonstrated that the stimulated by retinoic acid gene 8 (*Stra8*) was required for meiotic progression in both the mouse ovary and postnatal testis. To identify additional candidates that may play a role during meiosis, we used microarray databases to generate lists of transcripts with expression profiles similar to that of *Stra8* in the embryonic ovary and postnatal testis. One such gene, establishment of cohesion 1 homolog 2 (*Saccharomyces cerevisiae*) (*Esco2*), has been described as a regulator of sister chromatid cohesion during mitosis. This study describes the first in-depth analysis of ESCO2 localization and regulation during meiosis in both males and females. ESCO2 colocalized with the gamma H2A histone family member X (H2AFX) in pachytene spermatocytes, indicating that ESCO2 is a component of the XY body. In pachytene cells of the embryonic ovary, ESCO2 colocalized with H2AFX, which is consistent with the presence of ESCO2 in areas of double-stranded breaks. In addition, the expression of *Esco2* was found to be regulated by RA in the postnatal testis. These data indicate that ESCO2 may play a vital role in meiosis in both males and females.

germ cells, meiosis, ovary, testis

INTRODUCTION

Meiosis is a sex-specific event where germ cells undergo cellular differentiation to form oocytes and spermatozoa. Abnormal gene expression during meiosis can lead to aberrant gamete formation and is often a major cause of infertility in both males and females. However, there is still much to be learned about how meiotic entry and progression is regulated in both sexes. The progression of meiosis varies significantly between females and males. In the female mouse, meiosis is initiated around Embryonic Day 13.5 (E13.5) and arrests in the dictyate stage of meiosis I by E18.5 [1]. The meiotic cell cycle is reinitiated after birth and can continue for weeks or months, with oocytes arresting at metaphase of meiosis II and then not completing meiosis until after fertilization [2]. In contrast, gonocytes do not enter into meiosis in the male embryonic gonad but instead arrest in mitosis beginning at E14.5 [3, 4]. Gonocytes resume proliferation and differentiation shortly after birth and then initiate meiosis at approximately 8 days postpartum (dpp) [3]. Meiosis is completed in approximately 10 days with the production of four haploid spermatids that undergo a series of morphological changes before being released from the testis as immature spermatozoa.

One of the major differences between meiotic progression in males and that in females is how male germ cells handle carrying an X and a Y chromosome. During meiosis in males, the X and Y chromosomes for a pair and can undergo recombination at the pseudoautosomal region. In addition, the sex chromosomes are transcriptionally silenced either just before or during the first meiotic prophase [5–7], known as meiotic sex chromosome inactivation (MSCI) [8], and this results in the formation of the XY body. In the absence of MSCI, spermatocytes undergo apoptosis during pachynema, ultimately leading to infertility (reviewed in ref. [9]). Silencing will occur in females carrying unsynapsed X chromosomes as well as in unsynaptic autosomes in both spermatocytes and oocytes [10]. In other cell types carrying X and Y chromosomes, such as sex-reversed XY females, MSCI will not occur, and an XY body does not form [11]. This leads to the conclusion that MSCI and XY body formation are not intrinsic to an XY pair [6]. Proteins that localize to the XY body fall into several categories including modified histone variants, DNA damage repair proteins, and proteins that are involved with heterochromatin modifications and/or transcriptional repression [6]. Inactivating mutations in genes that associate with the XY body result in a meiotic block and subsequently male infertility [12, 13]. Some of the proteins that are associated with MSCI and the XY body are the gamma H2A histone family, member X (H2AFX), ataxia telangiectasia mutated homolog (ATM), mediator of DNA damage checkpoint 1 (MDC1), ubiquitin-conjugating enzyme E2B, RAD6 homology (*Saccharomyces cerevisiae*) (UBE2B), and breast cancer 1 (BRCA1). H2AFX plays a key role in chromatin remodeling, XY body formation, and possibly MSCI. Mice deficient in H2AFX have asynaptic X and Y chromosomes [13, 14]. ATM is involved in DNA repair; MDC1 is critical for establishing chromosome-wide silencing; and UBE2B may be involved in histone modifications. Knockouts of ATM, MDC1, or UBE2B result in infertility [12, 15–17]. BRCA1 is thought to recruit the kinase ataxia telangiectasia and Rad3-related (ATR) protein to the X and Y chromosomes, resulting in phosphorylation of H2AX and subsequently MSCI and XY body formation [18]. However, what other factors may contribute to the development of MSCI and the XY body are unknown.

Retinoic acid (RA), a metabolite of vitamin A, is required for normal meiotic progression in both males and females [19, 20]. Male animals that are vitamin A deficient (VAD) develop a block during spermatogonial differentiation [21, 22]. In

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Received: 16 March 2012.
First decision: 17 April 2012.
Accepted: 4 June 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

addition, germ cells of the embryonic VAD ovary display a block in meiotic prophase I [23]. Previous studies have also demonstrated the RA-responsive gene stimulated by the retinoic acid gene 8 (*Stra8*) gene is required for meiotic progression in both the murine ovary and testis [24–26], and ablation of this gene halts germ cell development early in meiotic prophase I in both sexes.

In order to investigate additional genes that might be involved in meiotic processes, a list of transcripts with expression profiles similar to that of *Stra8* was generated [27]. This study identified establishment of cohesion 1 homolog 2 (*S. cerevisiae*) (*Esco2*) as a candidate regulator of meiosis [28]. ESCO2, an ortholog of ECO1 in yeast, is a regulator of sister chromatid cohesion [28]. Mutations in ESCO2 have been shown to cause Roberts syndrome in humans, leading to a deficiency in chromatid cohesion around the centromeres of chromosomes [28]. Roberts syndrome is characterized phenotypically by symmetrical limb reduction, craniofacial anomalies, growth retardation, mental retardation, and cardiac and renal abnormalities. In addition, spontaneous abortions have been reported in females [29]; however, there are no published reports detailing whether male fertility is affected.

In this study, we examined the localization of ESCO2 throughout meiotic prophase and also its regulation by RA in both males and females to expand on our previously published data [27]. We found that ESCO2 localizes to the XY body in pachytene spermatocytes and is confined to double-stranded breaks in pachytene oocytes. In addition, we determined that *Esco2* expression can be driven by RA in the postnatal testis. Our findings suggest that ESCO2 may be involved in the meiotic process in both males and females.

MATERIALS AND METHODS

Animals and Tissues

All animal experiments were approved by Washington State University Animal Care and Use Committees and were conducted in accordance with the guiding principles for the care and use of research animals of the National Institutes of Health. The BL/6-129 mouse colony was maintained in a temperature- and humidity-controlled environment with food and water provided ad libitum. The animals were euthanized, and their testes and ovaries were dissected. Fetal gonad tissues were collected from embryos staged by fore and hind limb morphology [30]. Tissue samples for RNA preparation and protein isolation were snap frozen immediately after collection and stored at −80°C until used. Tissues used for immunofluorescence were placed in 4% paraformaldehyde for either 6 h (fetal gonads and 5–10 dpp testis), 8 h (10–30 dpp testis), or overnight (adult testis) directly after collection and then dehydrated through a series of ethanol washes and embedded in paraffin [27]. Four-micrometer sections were placed on sample adhesion microscope slides (Superfrost Plus; Menzel-Glaser, Braunschweig, Germany).

Germ Cell Isolation

Spermatocyte and oocyte meiotic prophase spreads were prepared using a previously published protocol [31]. Testes were detunicated, and the tubules were separated using forceps. Tubules or embryonic ovaries were placed in a hypotonic extraction buffer and macerated in a sucrose solution to form a cell suspension. The cell suspension was spread across a slide, using a 1% paraformaldehyde-Triton X-100 solution. Samples were incubated overnight in a humid chamber at 37°C and allowed to dry the next day.

Western Blotting

Mouse tissue was homogenized on ice in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) in the presence of protease inhibitors (Protease Inhibitor Cocktail Set; Roche) as described by Hogarth et al. [32]. Protein concentration was determined using the DC protein assay (Bio-Rad), and 30 μg of total protein was loaded per lane of a 12% SDS polyacrylamide gel with protein size standards (Page Ruler prestained protein ladder; Fermentas). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond XL; Amersham Biosciences). The membrane was blocked and incubated overnight at 4°C with rabbit anti-ESCO2 (product no. A301-689A; Bethyl Laboratories). The secondary horseradish-peroxidase-coupled antibody (1:5000 dilution; Zymed) was then applied, and membranes were incubated for 1 h at room temperature. Antibody binding was detected using the Western lighting chemiluminesence reagent (Perkin Elmer) and visualized using a luminescent image analyzer (model LAS-4000; Fujifilm).

Immunofluorescence

Immunofluorescence staining of sectioned tissues with rabbit anti-ESCO2 was performed as previously described [27]. Antigen retrieval was performed in a 0.01 M citrate buffer (pH 6; >90°C for 5 min), and the primary antibody was diluted (1:100) in a solution of 0.1% bovine serum albumin-PBS-10% goat serum and applied to the tissue. Slides were incubated in primary antibody overnight at 4°C. The following steps were performed at room temperature with PBS washes between incubations. Primary antibody was detected using AlexaFluor 488 goat anti-rabbit secondary antibody (product code A-11008; Invitrogen) at a 1:500 dilution in 0.1% bovine serum albumin-PBS-10% goat serum for 1 h. Following mounting under coverslips, using mounting medium (Vectashield; Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI), localization of ESCO2 was visualized and photographed using a Zeiss LSM 510 Meta model confocal microscope located at the Washington State University Franceschi Microscopy and Imaging Center. For all experiments, tissues from at least three embryos or animals were analyzed for protein localization. Control sections were incubated only in secondary antibody.

Immunofluorescence detection on meiotic prophase spreads was performed essentially as described by Koehler et al. [33]. Slides were placed in antibody dilution buffer (0.3% bovine serum albumin-0.01% normal donkey serum-PBS-Triton X-100). Primary antibodies (anti-ESCO2 [1:100 dilution], 200 μg/ml antisynaptonemal complex protein 3 [SYCP3; 1:50 dilution; product code SC-33874; Santa Cruz Biotechnology], and 1 mg/ml antiphospho-histone H2A.X [1:200 dilution; product code 05–636; Millipore) were diluted in antibody dilution buffer and incubated for periods ranging from 45 min to overnight at 37°C. Primary antibody binding was detected using AlexaFluor 488 donkey anti-rabbit secondary antibody (1:50 dilution; product code A21206; Invitrogen) for ESCO2, AlexaFluor 568 donkey anti-goat secondary antibody (1:200 dilution; product code A11057; Invitrogen) for SYCP3, and biotinylated horse anti-mouse antibody (1:1000 dilution; product code BA-2000; Vector Laboratories) for Histone H2A.X. Slides were placed in a 4 μM solution of DAPI for 30 min. Coverslips were mounted on slides using mounting medium (Vetashield, product no. H-1400; Vector Laboratories), and cells were viewed using a Zeiss confocal microscope or a Zeiss fluorescence microscope.

Grooved Agar Mold Cultures

Whole embryonic gonads or 2- or 6-dpp mouse testes cut into four pieces were cultured on 1.5% agar blocks [34, 35] placed in a 24-well plate with 300 μl of Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and cultured at 37°C with 5% CO_2. Prior to organ culture, the agar block was equilibrated in medium plus treatment or vehicle control (dimethyl sulfoxide [DMSO]) for 24 h. The medium containing treatment was replaced after 24 h, and the tissue was placed inside the groove. All tissue used for *Esco2* expression analysis was cultured in 0.35 or 0.7 μM RA [26, 36] or in 0.7 μM R115866 [26]. Dose-curve analysis was performed to determine the optimal RA concentration for the 6-dpp animals (data not shown). Neonatal testes were cultured for 24 h, and embryonic gonads were cultured for 48 h. During the 48-h culture, medium containing treatment was replaced after 24 h. After culture, tissue was flash-frozen on dry ice and stored at −80°C until RNA isolation. Each experiment was performed in triplicate.

Animal Injections

Six-day postpartum BL6/129 neonatal animals received an intraperitoneal injection of either vehicle control (DMSO) or 150 μg of RA. Testes were collected 24 h after injection and flash-frozen for RNA isolation.

RNA Isolation and Real-Time RT-PCR

RNA was extracted using an RNA extraction kit (PicoPure; Molecular Devices). The extracted RNA was used as a template to produce cDNA, using the iScript kit (Bio-Rad). Quantitative RT-PCR analysis was performed with a 7500 Fast real-time PCR system (Applied Biosystems) using Fast SYBR Green Mastermix (Applied Biosystems). Each RNA sample was analyzed in triplicate

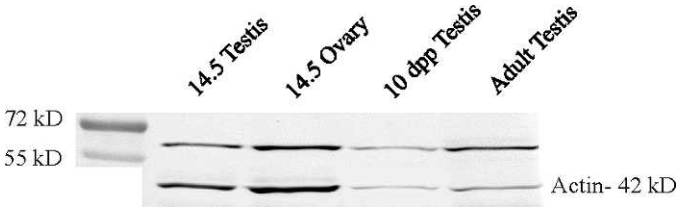


FIG. 1. ESCO2 expression in the embryonic gonad and postnatal testis. Western blot shows the expression of ESCO2 in the embryonic testis and ovary and postnatal testis. Total protein lysates from E14.5 ovary, E14.5 testis and 10-dpp and adult testis were incubated with antibody that recognizes ESCO2 protein.

with *Stra8* primers that amplified a 151-bp product (primers: 5′-GTTTCCTGCGTGTTCCACAAG-3′ and 5′-CACCCGAGGCTCAAGCTTC-3′) and *Esco2* primers that amplified a 125-bp product (5′-AAAGACCA GCTCGTCATTGACG-3′ and 5′-TCGGTGGTGATGTTGGAGATG-3′). Each RNA sample was analyzed in triplicate, using the delta-delta-C_T method, and statistical significance between vehicle control and treatment was determined using the Student *t*-test.

Analysis of Esco2 *Expression in VAD Testes*

Adult female mice (129/SvJ) were maintained on a VAD diet (Harlan Teklad) for at least 2 wk before mating and throughout pregnancy. Their male offspring were fed a VAD diet for 13–14 wk. At deficiency, one testis was removed from each animal and cut into two pieces; one piece was fixed in Bouin solution for histological assessment of spermatogenesis, and the other was placed in TRIzol (Invitrogen) reagent for RNA extraction and served as a preinjection control in RT-PCR analysis. Incisions were sutured, and animals were allowed to recover for 24 h. Three animals with similarly deficient spermatogenesis (as judged by preinjection testicular histology) were injected subcutaneously with 100 μl of 7.5 mg/ml all-*trans* RA (Sigma) in a solution of 10% ethanol-90% sesame oil. The remaining testis was harvested 8 or 24 h after the injection and placed in TRIzol for RNA extraction. Quantitative RT-PCR analysis was performed as described above.

RESULTS

ESCO2 Protein Is Present in Embryonic Gonad, Juvenile Testis, and Adult Testis

Previously published data indicate that ESCO2 is expressed in the postnatal testis [27]. Therefore, to determine if ESCO2 was present in the embryonic gonads, we performed Western blotting using a commercially available antibody. A 65-kDa band was detected in E14.5 testis and ovaries, 10-dpp testis, and adult testis (Fig. 1). This is similar to the theoretical relative molecular weight (M_r) of 67 kDa.

ESCO2 Localizes to the XY Body in the Postnatal Testis

Previous studies in our laboratory have demonstrated that ESCO2 localized to the nuclei of spermatocytes [27]. More

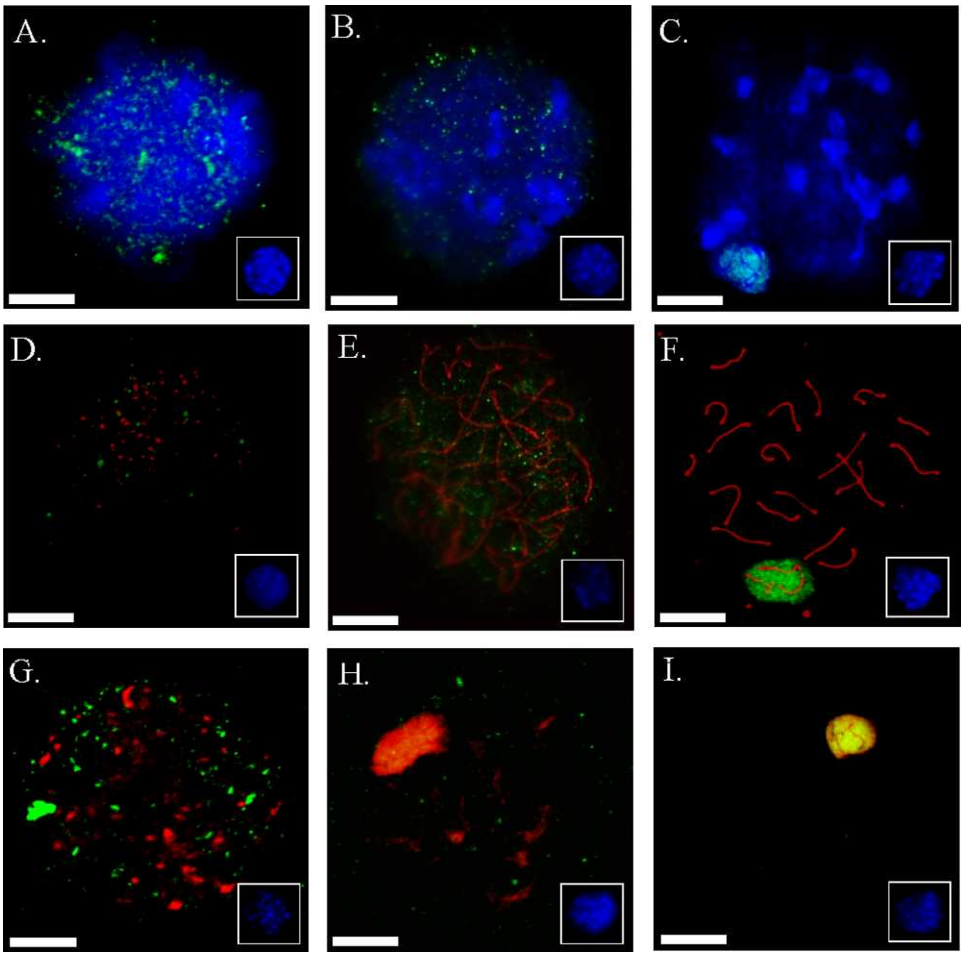


FIG. 2. ESCO2 localization in spermatocyte meiotic spreads. **A**–**C**) Immunofluorescence detection of ESCO2 in 10-, 15-, and 20-dpp spermatocytes. No antibody control is shown in the insets (**A**–**C**). **D**–**F**) Immunofluorescence detection of ESCO2 (green) and SYCP3 (red) in 10-, 15-, and 20-dpp mouse testes. **G**–**I**) Immunofluorescence detection of ESCO2 (green) and H2AFX (red) in 10-, 15-, and 20-dpp mouse testes. DAPI staining is shown in the insets of panels **D**–**I**. Bars = 10 μm.

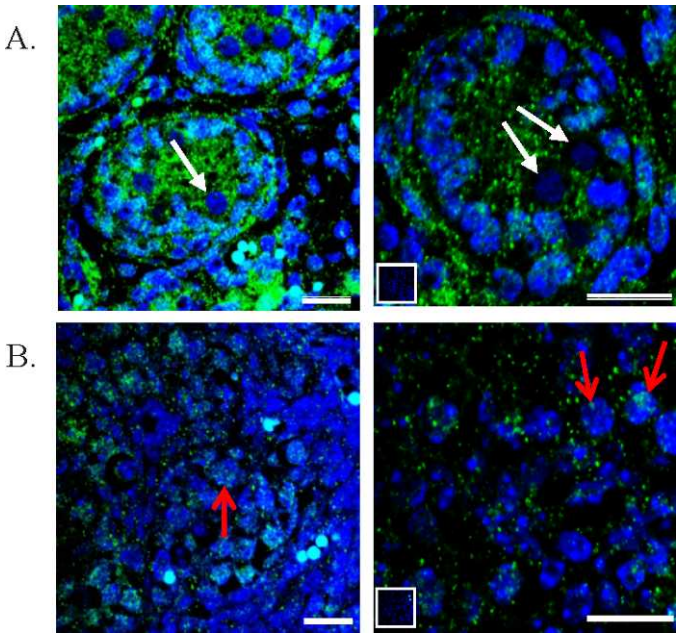


FIG. 3. ESCO2 localization in the embryonic gonads. **A**) Immunofluorescence detecting ESCO2 in cross-sections of E18.5 mouse testis. White arrows represent gonocytes. **B**) Immunofluorescence detection of ESCO2 in cross-sections of E18.5 mouse ovary. Red arrows represent oocytes. Bars = 20 μm. No antibody control is shown in the insets of **A** and **B**.

specifically, ESCO2 displayed a diffuse localization pattern in preleptotene and leptotene spermatocytes that became concentrated to a discreet domain in pachytene cells. Therefore, to closely examine ESCO2 localization specifically in the nuclei of spermatocytes, meiotic prophase spreads were stained with ESCO2 and SYCP3 to label the synaptonemal complexes. ESCO2 localized diffusely throughout the nuclei of leptotene (Fig. 2, A, D, and G) and zygotene cells (Fig. 2B). In pachytene cells, ESCO2 appeared to be confined to the XY body (Fig. 2, C and F). Co-immunofluorescence with ESCO2 and a known XY body marker, H2AFX, confirmed that ESCO2 localized to the XY body within early and late pachytene spermatocytes (Fig. 2, H and I).

ESCO2 Colocalizes with H2AFX, Which Is Consistent with the Presence of ESCO2 in Areas of Double-Stranded Breaks in the Embryonic Gonad

Previous work has shown that *Esco2* transcript is present in oogonia in the embryonic ovary and gonocytes in the embryonic testis [27]. Nevertheless, there are no published studies showing localization of the ESCO2 protein in the embryonic gonad. As a result, immunofluorescence detection on E14.5–E18.5 testis and ovary cross-sections was performed to determine ESCO2 localization in the embryonic gonad. At each age analyzed in the embryonic testis, ESCO2 was localized to Sertoli cell nuclei and cytoplasm (Fig. 3A, only E18.5 is shown). Notably, ESCO2 was absent from the gonocytes (Fig. 3A, arrows). In contrast, ESCO2 was detected in pregranulosa cells as well as in oocytes in all ages of the embryonic ovary examined (Fig. 3B, only E18.5 is shown). To further analyze ESCO2 localization in meiotic cells of the embryonic ovary, we performed co-immunofluorescence with SYCP3 within meiotic prophase spreads. ESCO2 was diffusely localized throughout the nucleus of leptotene and zygotene cells (Fig. 4, A and B). Interestingly, in pachytene cells, ESCO2 was concentrated in clumps that resembled loop-like structures extending off of the synaptonemal complexes (Fig. 4C), and these clumps persisted, although they were not as prominent, in diplotene cells. Colocalization of H2AFX and ESCO2 in pachytene oocytes is consistent with the formation of these loop-like clumps in areas of double-stranded breaks (Fig. 5).

Esco2 *Expression Is Driven by RA in Postnatal Testis*

Esco2 was found to have an expression profile similar to that of *Stra8* [27]. Since *Stra8* expression is known to be regulated by RA, it was of interest to determine if *Esco2* expression was also RA-regulated. Adult VAD mice were treated with RA, and *Esco2* transcript levels were measured and compared by real-time RT-PCR, pre- and post-RA injection. *Esco2* mRNA levels were 2.5-fold increased at 8 h postinjection, and a 9.1-fold increase was detected 24 h postinjection (Fig. 6). To further evaluate *Esco2* regulation by exogenous RA, we cultured neonatal testes in either RA or vehicle control for 24 h, using an agar culture system as described by Hogarth et al. [35]. Specifically, regulation of *Esco2* in gonocyte and spermatogonia populations was determined using 2- and 6-dpp testis cultures, respectively. *Esco2* expression was 2.1-fold increased in 2-dpp testes cultured with RA and significantly 2.5-fold increased in 6-dpp testis cultures (Fig. 7A). To determine if these results could be replicated in vivo, 6-dpp male animals were injected with 150 μg of RA, and testes were harvested 24 h postinjection. A significant 1.7-fold increase in *Esco2* expression was detected in these testes (Fig. 7B).

RA Does Not Regulate ESCO2 in the Embryonic Gonad

RA is essential for meiosis in the embryonic ovary, but it has not been determined whether RA can regulate *Esco2* expression in the embryonic gonad. Cytochrome p450, one of the 26-member family of enzymes (CYP26), is known to degrade RA in mammalian cells [37–40]. Previous studies have shown that an inhibitor of the CYP26 enzymes, R115866, can be used to raise endogenous levels of RA and examine the resulting effects on gene expression [26, 41]. Therefore, to determine whether endogenous RA regulates *Esco2* expression in the embryonic testis, E12.5 gonads where dissected and cultured in R115866 or control for 48 h, as described by Koubova et al. [26]. Expression levels of *Stra8*, a gene known to be RA-responsive in the embryonic gonad, and *Esco2* were measured using real-time RT-PCR. In embryonic testis, *Stra8* expression was significantly 5.4-fold increased compared to that in control, whereas in the embryonic ovary, *Stra8* expression was unchanged (Fig. 8A), consistent with published results [26]. However, *Esco2* expression remained unchanged in the presence of R115866 in both the embryonic testis and ovary (Fig. 8A). To further examine RA regulation of *Esco2* in the embryonic gonad, E11.5 urogenital ridges were collected and cultured in exogenous RA for 48 h, as described by Hogarth et al. [35]. In the presence of RA, *Esco2* expression was unchanged in either cultured embryonic testes or ovaries, compared to control (Fig. 8B).

DISCUSSION

This study represents the first detailed investigation of ESCO2 localization and regulation during meiosis in both males and females. We discovered that ESCO2 localized to the XY body in spermatocytes and was concentrated to chromatin regions containing double-stranded breaks in oocytes. Further-

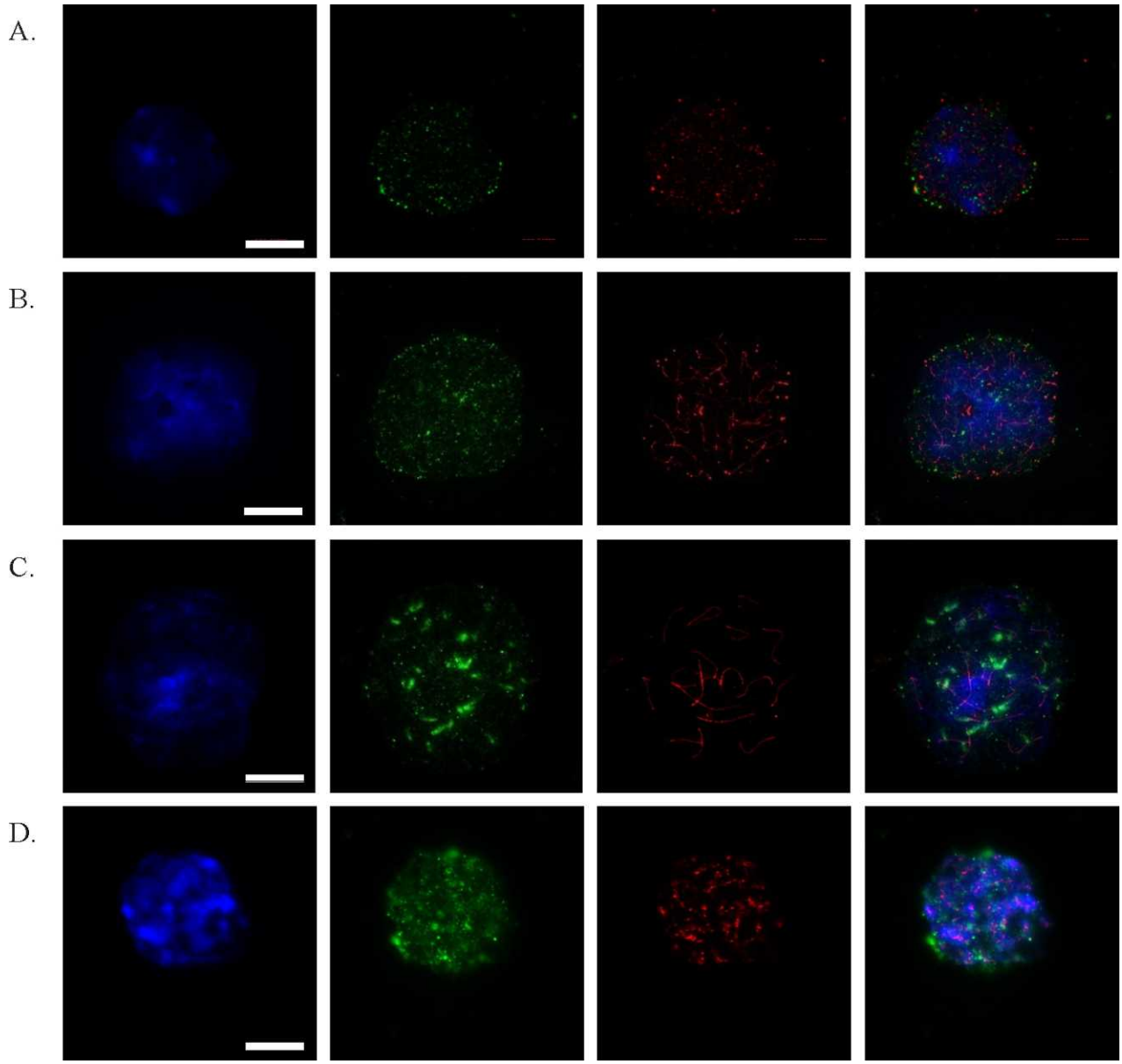


FIG. 4. ESCO2 localization in oocyte meiotic spreads. **A**–**D**) Immunofluorescence detection of ESCO2 (green), SYCP3 (red), DAPI (blue), and a merged image in E18.5 oocytes at leptonema, zygonema, pachynema, and diplonema, respectively. Bars = 10 μm.

more, we determined that RA can stimulate the expression of *Esco2* in the postnatal testis. These results imply that ESCO2 may be involved in meiosis and that RA could possible regulate XY body formation. Additionally, these results suggest that the fertility problems seen in female patients with Roberts syndrome could in part be due to meiotic errors in the oocyte.

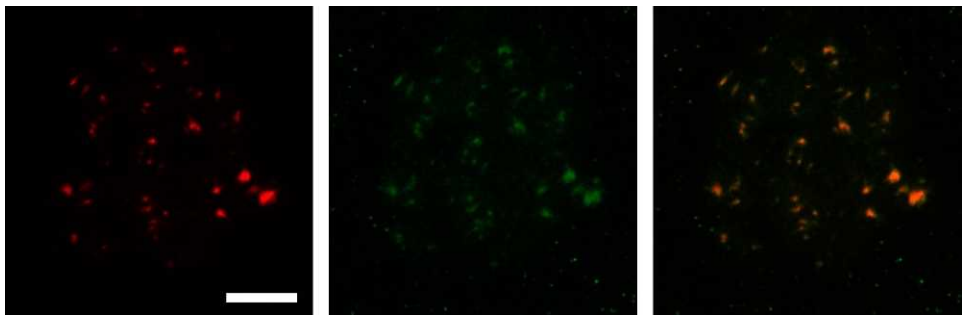


FIG. 5. Immunolocalization of ESCO2 and H2AFX in an E18.5 pachytene oocyte. Immunofluorescence detection of H2AFX (red), ESCO2 (green), and a merged image in a pachytene oocyte. Bars = 10 μm.

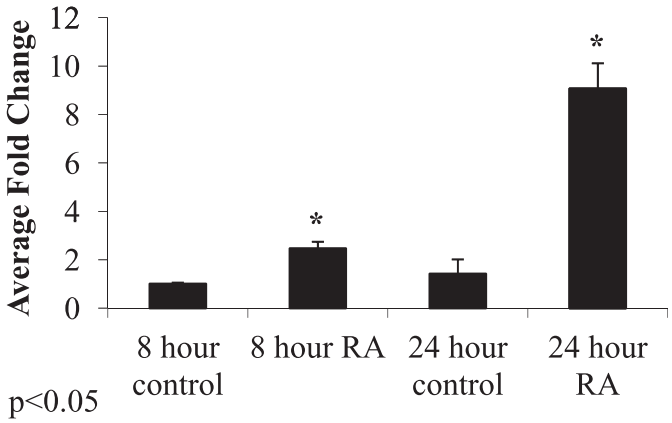


FIG. 6. *Esco2* expression in VAD animals. Quantitative real-time RT-PCR analysis of *Esco2* expression in VAD mice treated with control or RA at either 8 or 24 h. Error bars represent standard error of the mean. Asterisks indicate significance at specified level determined using Student *t*-test.

Mutations in ESCO2 have been shown to cause Roberts syndrome, a disease which can lead to severe birth defects in both sexes, and most patients do not live to reproductive age. However, normal life spans are reached by people with milder forms of Roberts syndrome, and fertility problems, specifically spontaneous pregnancy loss, have been reported in female patients, but to date, it is unknown whether male patients are infertile [28, 42, 43]. Roberts syndrome is characterized by loss of sister chromatid cohesion during mitosis. Still, the role of ESCO2 in meiosis in these patients has remained unexplored. It is possible that a loss of ESCO2 during meiosis may cause double-stranded breaks not to be repaired efficiently and consequently results in spontaneous pregnancy loss. Thus, a basic understanding of how ESCO2 functions during meiosis may help Roberts syndrome patients conceive children in the future.

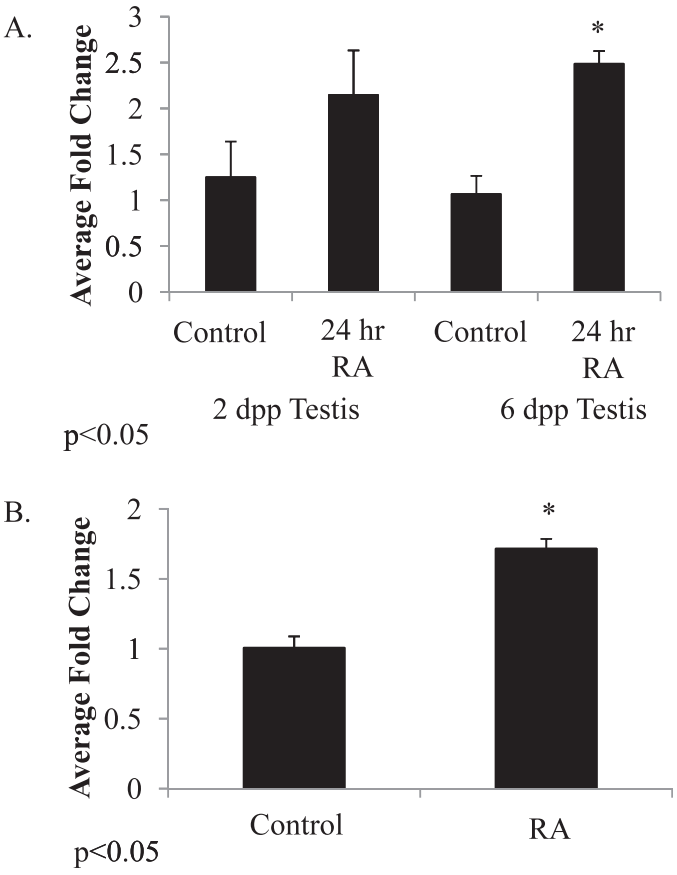
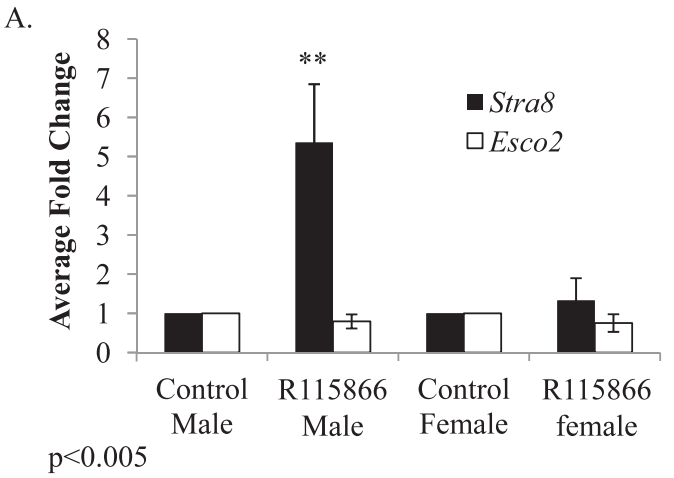


FIG. 7. *Esco2* expression in neonatal mice treated with RA for 24 h. **A**) Quantitative real-time RT-PCR analysis of *Esco2* expression in cultured neonatal testis at 2 dpp (n = 6) and 6 dpp (n = 8). **B**) *Esco2* expression in 6-dpp animals injected with 150 μg of RA and taken down 24 h post-injection. Error bars represent standard errors of the mean. Asterisks indicate significance at specified level using Student *t*-test.



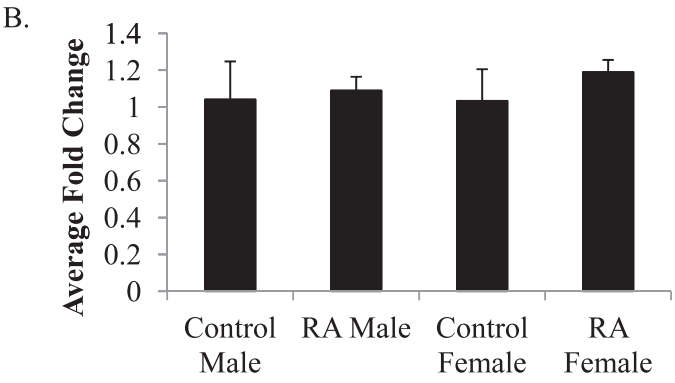


FIG. 8. *Esco2* expression in cultured embryonic gonads. **A**) Quantitative real-time RT-PCR analysis of *Stra8* and *Esco2* expression levels in E12.5 embryonic testis and ovary cultured with 0.7 μM R115866 for 48 h (n = 4). **B**) Quantitative real-time RT-PCR analysis of *Esco2* expression in E11.5 testis and ovary cultured with 0.7 μM RA for 48 h (n = 3). Error bars represent standard errors of the mean. Asterisks indicate significance at specified levels, using Student *t*-test.

In the embryonic gonad, ESCO2 localized to Sertoli cell nuclei and cytoplasm in the testis and was detected in pregranulosa cells of the ovary. These expression results agree with other previous data suggesting that ESCO2 is involved in the mitotic cell cycle [42, 44]. A previous study in our laboratory showed that *Esco2* transcript was present in E14.5 gonocytes [27]. However, our study did not detect ESCO2 protein in E14.5–E18.5 gonocytes. One possible reason is that a higher level of transcriptional regulation may be occurring to allow gonocytes to stockpile transcript in order for protein to be available during meiosis. One potential way in which this can occur is through P bodies, which are found in gonocytes and have been shown to sequester transcripts in a quiescent state [45, 46]. Furthermore, we have determined that ESCO2 expression differs between the embryonic and postnatal testis. Hogarth et al. [27] reported *Esco2* transcript in germ cells of

the postnatal testis from 5 dpp onward and protein present at 10 dpp, whereas the present study identified ESCO2 protein in the somatic cells of fetal testis from E14.5 to E18.5. The combination of these studies implies that ESCO2 may aid the Sertoli cell in governing mitotic quiescence of the gonocytes in the fetal gonad while only being required in the germ cells at the onset of meiosis. Further investigation of whether ESCO2 has a role outside of cell cycle control and whether its function differs between germ and somatic cells will be required.

In the embryonic ovary, ESCO2 displayed punctate nuclear localization in leptotene and zygotene cells and was concentrated into clump-like foci along the synaptonemal complexes in pachytene cells. It is interesting to note that the clumps seemed to form in areas containing double-stranded breaks, suggesting that ESCO2 may play a role in double-stranded break repair. ECO1, an ortholog of ESCO2 in yeast, has been shown to be an important cohesion factor during double-stranded break repair [47]. It is possible that if double-stranded breaks are not fully resolved by the end of pachytene within oocytes, then ESCO2 may act as a cohesion factor to assist in the resolution of those breaks. However, further studies need to be performed to investigate this hypothesis.

In the postnatal testis, ESCO2 displayed punctate localization in leptotene and zygotene cells and localized to the XY body in pachytene cells. ESCO2 is a known sister chromatid cohesion factor [28], and a recent study by Whelan et al. [44] demonstrated that ESCO2 deficiency caused mitotic chromosome cohesion loss in mouse embryos. In addition, cohesin complex proteins such as REC8 and RAD21L are concentrated in the XY body in late pachytene spermatocytes [48]. Therefore, it is plausible that ESCO2 may be necessary for cohesion between X and Y chromosomes, triggering XY body formation, and that loss of this gene may cause a meiotic block. Previous studies have shown that MSCI is initiated by DNA repair proteins such as H2AFX and ATR [9]. Because ECO1 is known to be an important cohesion factor during double-stranded break repair, ESCO2 may also play a role in MSCI, specifically in repairing double-stranded breaks. Nevertheless, future loss-of-function studies in meiotic cells will need to performed in order to fully investigate the function of ESCO2 during meiosis.

There is compelling evidence to suggest that RA is a regulator of germ cell differentiation, and meiosis in both males and females [19] and our study imply that RA may play a indirect role in driving formation of the XY body. Our data indicate that *Esco2* expression can be increased by RA in the postnatal testis. It is interesting to note that other proteins localizing to the XY body, such as REC8 and RAD51, are also RA-responsive. Additionally, the ATM and suppressor of variegation 3–9 homolog 2 (*Drosophila* spp) (SUV39H2) proteins have expression profiles similar to that of *Stra8* and also localize to the XY body [27]. Therefore, it is possible that RA can drive the expression of proteins important for XY body formation and maintenance, thereby indirectly regulating these processes. Still, future studies need to be conducted to verify the exact function of ESCO2 and RA during XY body formation and maintenance.

Esco2 exhibits an expression profile similar to that of the RA-regulated *Stra8* gene [27]; however, RA cannot drive *Esco2* expression in the embryonic ovary and testis. While *Esco2* does not contain an RA response element (RARE) in its promoter region, it does contain an SP1 binding site [49], and RA receptor-alpha has previously been shown to interact with SP1 to drive transcription in the absence of RARE [49, 50]. In the postnatal testis, it is possible that *Esco2* expression is regulated via a secondary RA activation response. The lack of an RA response in the embryonic gonad may be caused by the absence of other important transcription factors necessary for SP1-mediated transcriptional activation. However, further studies need to be conducted to determine the exact mechanism of RA regulation in the postnatal testis and to determine what causes the lack of RA regulation in the embryonic gonad.

Data presented here represent the first in-depth study to determine the localization and regulation of ESCO2 in the gonad and suggest that ESCO2 may play a role during meiosis in both males and females. Specifically, ESCO2 may be important in repairing double-stranded breaks in both males and females. Additionally, these results suggest that RA may be an important regulator of XY body formation during spermatogenesis. Future loss-of-function studies will determine whether ESCO2 is essential for germ cell development in both sexes and enhance our understanding of the underlying cause of infertility in female Roberts syndrome patients.

ACKNOWLEDGMENT

We would like to acknowledge M.A. Handel for help and expertise in XY body formation. We would also like to acknowledge T. Hassold and P. Hunt for their technical assistance.

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