Chapter 3

Studying Meiosis-Specific Cohesins in Mouse Embryonic Oocytes

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Abstract

Distinct meiotic cohesin complexes play fundamental roles in various meiosis-specific chromosomal events in spatiotemporally different manners during mammalian meiotic prophase. Immunostaining is one of the essential methods to study meiotic cohesin dynamics. For the study of cohesins in the meiotic prophase of oocytes, ovaries should be taken from the embryos during a very limited period before birth. Here we focus on some technical tips concerning the preparation of oocyte chromosome spreads for immunostaining. Further, we describe a method for chromosome fluorescence in situ hybridization (FISH) against immunostained oocytes.

Key words: Fetal oocyte, Embryonic ovary, Meiosis, Cohesin, Prophase, Axial element, Homolog synapsis, Synaptonemal complex, Chromosome spread, FISH

1. Introduction

Cohesin is essential for faithful chromosome segregation to establish cohesion between sister chromatids (1). The meiotic cohesin complex differs from that of mitosis since the mitotic RAD21/SCC1 subunit of the cohesin complex is largely replaced by meiotic counterparts, REC8 and RAD21L (2–5) in mammals. Also other meiosis-specific cohesin subunits, SA3 and SMC1β, are known to be expressed (6, 7). During meiotic prophase I, sister chromatids are organized into proteinaceous structures of axial elements (AEs) on which the synaptonemal complex (SC) is assembled to promote interhomolog recombination, a process yielding chiasmata between homologues (8, 9). The meiotic cohesin complexes, which interact with the SC components and localize along AEs, might act as a basis for SC assembly (10–14). Thus the cohesin complex is crucial

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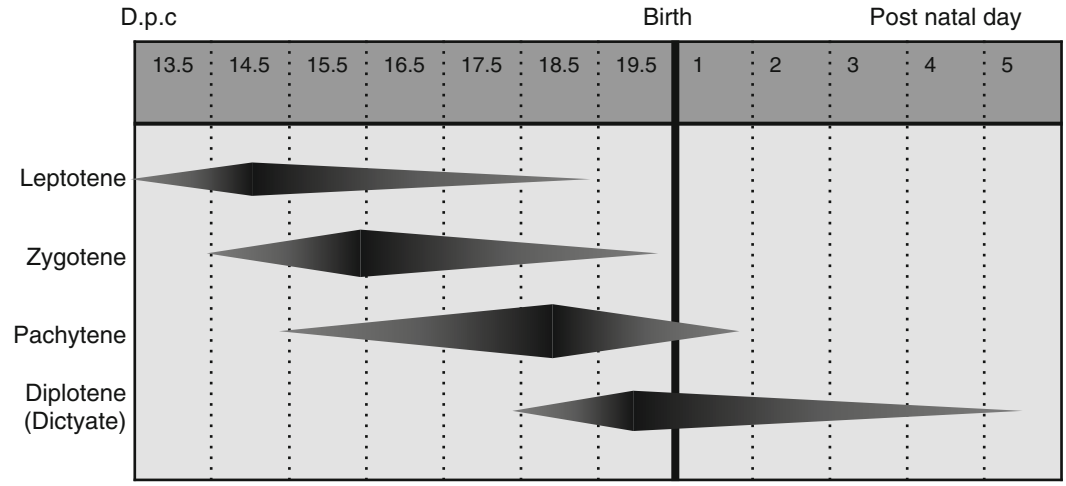

Fig. 1. Progression of meiotic prophase during female embryonic development. Shown is the embryonic age in days post corresponding with each specific meiotic prophase stage.

not only for sister chromatid cohesion but also for various meiosis-specific chromosomal events in meiotic prophase.

In contrast to male germ cells, which enter meiosis continuously and go straight into diakinesis after birth, female germ cells enter meiosis during fetal development and finally reach dictyate arrest that persists until puberty (Fig. 1) (15–17). Here we describe our technical knowledge concerning the study of cohesin in mouse fetal oocytes. Well-scheduled collection of embryos is required for oocyte preparation because meiotic prophase progression is chronologically coupled with the stage of embryogenesis. Unlike spermatocyte sampling from testis, special handling is required under a microscope due to the small size of the fetal ovary. Rapid preparation of single oocytes is achieved by physically crushing the fetal ovary followed by collagenase digestion with less physiological damage. The chromosome spread of oocytes based on a dry down technique (18) is made by hypotonic treatment and/or cytocentrifugation followed by fixation together with pre-extraction of free proteins providing a well-preserved chromosome axis structure. Fluorescence in situ hybridization (FISH) against immunostained chromosomes (19, 20) provides pairing/synapsis status of a pair of given homologues during meiotic prophase. These techniques are applicable not only to wild type but also to any strains of meiotic prophase deficient mouse.

2. Materials

2.1. Collection of Embryonic Ovaries

1. Fine-tipped tweezers.
2. Phosphate buffered saline (PBS): 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl.

3. M2 medium: containing 10% (v/v) fetal bovine serum, 1/100 (v/v) 200 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin. Store at 4°C.
4. Stereo microscope.
5. 1 mg/ml type I Collagenase in PBS. Store aliquots at –20°C.
6. 1 ml syringe with 18 G needle.
7. 0.05% trypsin
8. DMEM containing 10% (v/v) FBS
9. Hypotonic buffer: 17 mM trisodium citrate dihydrate, 50 mM sucrose, 5 mM EDTA, 0.5 mM DTT, 30 mM Tris–HCl, pH 8.2. Store aliquots at –20°C.

2.2. Processing of the Embryonic Oocyte Chromosome Spread by Dry Down

1. 2% (w/v) Paraformaldehyde (PFA), 0.2% (v/v) Triton X-100 in PBS, pH 9.2.
2. MAS-coated slide glass (MATSUNAMI).
3. PAP (hydrophobic barrier) pen (Vector laboratories, H-4000).

2.3. Embryonic Oocyte Spreading by Cytocentrifugation

1. 1% (w/v) PFA, 0.15% (v/v) Triton X-100 in PBS, pH 9.2.
2. Slide glass for cytocentrifuge (Thermo).
3. PAP (hydrophobic barrier) pen.
4. Disposable sample chamber (Thermo).
5. Cytocentrifuge machine (Thermo, Shandon cytospin 4).

2.4. Immuno-fluorescence

1. PBS.
2. 0.1% (v/v) Triton X-100 in PBS, pH 7.4.
3. Blocking solution: 5% (w/v) BSA in PBS. Store at 4°C.
4. Vectashield mounting medium containing 0.1 mg/ml of DAPI (Vector laboratories).
5. Coverslips.
6. Slide chamber.
7. Nail polish.
8. Anti-mouse-Alexa 555 (Cy3).
9. Anti-rabbit-Alexa 488 (FITC).

2.5. Chromosome Fluorescence In Situ Hybridization

1. 70, 80, 90, 100% ethanol.
2. 70% formamide/6× SSC.
3. 4% (w/v) PFA in PBS, pH 7.4.
4. 2× SSC.
5. 0.4× SSC/0.3% Tween-20.
6. PBS/0.1% Tween-20.

7. Mouse Chromosome FITC-labeled point probe (e.g., ID labs).
8. STARFISH hybridization buffer (ID labs) or 50% formamide/2× SSC.
9. Mouse Cot-1 DNA (Invitrogen).
10. Anti-rabbit-Alexa 488 (FITC).
11. Slide chamber.
12. 22×22 mm cover glass.
13. Nail polish.
14. Coplin jar.
15. Thermal cycler tube.

3. Methods

3.1. Collection of Embryonic Ovaries

1. Sacrifice the pregnant female at day 14.5–19.5 d.p.c or postnatal day 1–5 (see Note 1) by cervical dislocation.
2. Dissect out the uterine horns; remove embryos from the placenta; and transfer into a petri dish containing PBS.
3. Wash embryo with fresh PBS, cut away head, incise abdomen, and cut out side of legs (see Note 2) (Fig. 2).

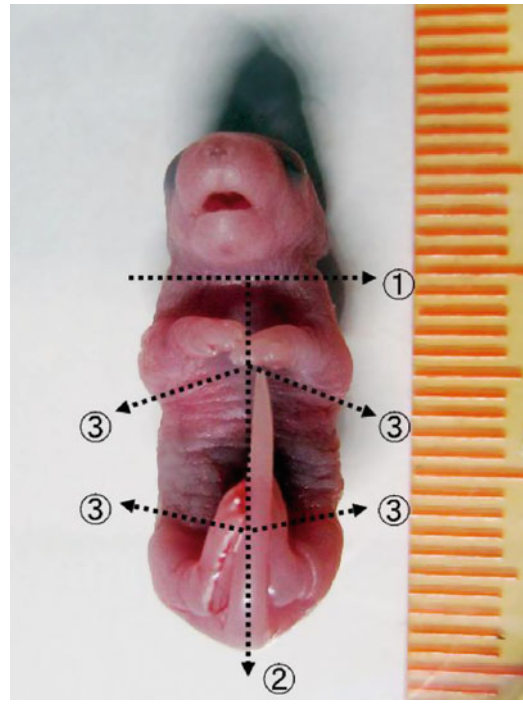

Fig. 2. Positioning of the d.p.c. 18.5 embryo for dissection. Dissect embryo by making incisions as indicated by the *dotted lines.*

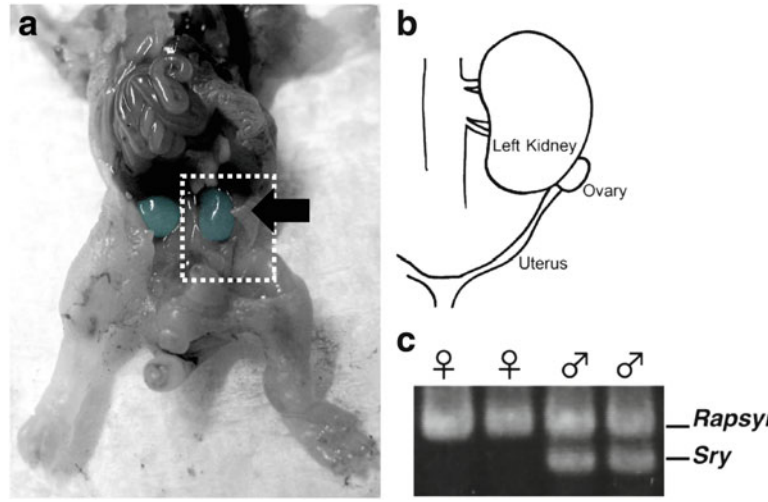

Fig. 3. (**a**) Exposed abdominal contents of a female embryo. The kidney is pseudocolored in *blue* and highlighted by a *black arrow*. (**b**) Shown is a schematic representation of some of the main structures located within the region demarcated by the *dashed box* in (**a**) illustrating that the ovary lies in close proximity to the infero-lateral surface of the kidney. (**c**) Sex is determined by PCR with male-specific *Sry* and autosome *Rapsyn* primers.

4. Open abdomen, displace loops of bowel upwards thereby exposing the ovaries located immediately inferior to the kidneys in the paravertebral gutters at the posterior wall of the peritoneal cavity (Fig. 3a, b) (see Note 3).
5. Remove both ovaries from each female fetus with fine-tipped tweezers under a stereo microscope and place in a 1.5 ml centrifuge tube containing 0.7 ml of M2 medium (see Note 4).
6. Crush ovaries into small pieces using an 18 G needle attached to a 1 ml syringe (see Note 5).
7. Spin down for 3 min at 400 × *g*.
8. Discard the supernatant (see Note 6) and add 1 ml of PBS.
9. Spin down for 3 min at 400 × *g*.
10. Discard the supernatant and add 0.5 ml of 1 mg/ml collagenase in PBS.
11. Incubate ovarian fragments with collagenase at 37°C for 15–30 min.
12. Gently pipette until large fragments of ovarian tissue are no longer visible.
13. Spin down for 3 min at 400 × *g*.
14. Discard the supernatant and add 0.5 ml of 0.05% trypsin (see Note 7).
15. Gently pipette and incubate at 37°C for 1 min.

16. Add 0.5 ml of DMEM containing 10% (v/v) FBS.
17. Gently pipette and spin down for 3 min at 400 × *g*.
18. Discard the supernatant and add 1 ml of PBS (see Note 8).
19. Resuspend cells by pipetting and spin down for 3 min at 400 × *g*.
20. Discard the supernatant and add hypotonic buffer (see Note 9).
21. Resuspend cells by pipetting and keep for 10 min or more at room temperature (see Note 10).

3.2. Chromosome Spread by Dry Down

1. Mark a circle approximately 1.0–1.5 cm in diameter on the slide with a hydrophobic barrier pen.
2. Put 10 μl of 2% (w/v) PFA containing 0.2% (v/v) Triton X-100 into each circle (see Note 11).
3. Add 10 μl of cell suspension in hypotonic buffer (prepared as in Subheading 3.1) to the PFA/Triton X-100 solution in each circle.
4. Slowly dry down in a humidity slide chamber for at least 2 h or overnight.
5. After drying, store the slides at −80°C.

3.3. Chromosome Spread by Cytocentrifuge

1. Mark a circle of approximately 1 cm in diameter on the slide using a hydrophobic barrier pen around the area where the cells will become attached following cytocentrifugation.
2. Install the slide equipped with the disposable sample chamber in the metal holder.
3. Add 100 μl of cell suspension in hypotonic buffer (prepared as in Subheading 3.1) to each chamber (see Note 12).
4. Install the holder equipped with slide and disposable sample chamber into the slot in the cytocentrifuge machine.
5. Cytocentrifuge for 5 min at 600–900 rpm.
6. Remove the disposable sample chamber from its slide.
7. Fix the attached cells on the slide in the marked area by adding 10–20 μl of 1% PFA containing 0.15% (v/v) Triton X-100.
8. Slowly dry down in a humidity slide chamber for a minimum of 2 h as in step 4 of Subheading 3.2.
9. After drying, store the slides at −80°C.

3.4. Immunostaining

1. Incubate frozen dry down or cytocentrifuge slides with 0.1% Triton X-100 in PBS for 10 min at room temperature.
2. Wash slides in PBS three times for 5 min.
3. Incubate slides with 100 μl of 5% BSA in PBS for 30 min to block nonspecific binding of the antibodies.

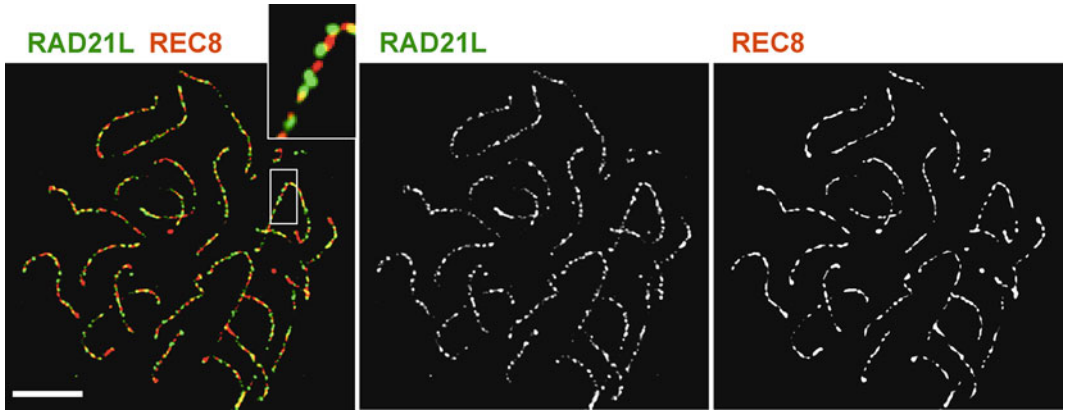

Fig. 4. Chromosome spread of pachytene oocyte obtained using the cytocentrifuge method immunostained for REC8 (*red*) and RAD21L (*green*). Shown inset is a magnified image of the *boxed* region. Scale bar, 5 μm.

4. Incubate slides with 50–100 μl of appropriately diluted mouse anti-REC8 and rabbit anti-RAD21L antibodies in PBS containing 5% BSA in a humidified chamber for 1 h at room temperature or overnight at 4°C.
5. Discard the solution and wash the slides three times in PBS, 5 min each.
6. Incubate slides with 50–100 μl of anti-mouse-Alexa 555 (Cy3) and anti-rabbit-Alexa 488 (FITC) secondary antibodies in PBS containing 5% BSA for 1 h at room temperature in the dark.
7. Discard the secondary antibody solution and wash three times with PBS for 5 min each in the dark.
8. Mount cover glass with a drop of Vectashield mounting medium containing DAPI and seal the edges of the cover glass using nail polish. Using this approach, both REC8 and RAD21L can be simultaneously imaged with fluorescence microscopy (Fig. 4).

3.5. Chromosome Fluorescence In Situ Hybridization and Immuostaining

1. Make chromosome spreads of oocytes as described in Subheading 3.2.
2. Stain the chromosome spreads with mouse anti-Sycp3 and rabbit anti-Sycp1 as described for REC8 and RAD21L in Subheading 3.4 (see Note 13).
3. Stain slides with the secondary antibodies anti-mouse-Alexa 555 (Cy3) and anti-rabbit-Alexa 647 (Cy5) as described in Subheading 3.4.
4. Wash with PBS and mount in Vectashield (see steps 7 and 8 in Subheading 3.4). Observe the slide under a microscope for confirmation of immunostaining.
5. Wash out mounting medium using PBS/0.1% Tween-20.

6. Fix antibodies on the slide glass with 4% PFA for 8 min at room temperature (see Note 14).
7. Wash three times with PBS for 5 min at room temperature.
8. Dehydrate immunostained samples by sequentially immersing in 70–80–90–100% ethanol for 5 min each at room temperature and air dry.
9. Denature double-stranded DNA by immersing immunostained slide samples in 70% formamide/6× SSC at 72°C for 10 min.
10. Dehydrate the samples by sequentially immersing in ice-cold 50–70–90–100% ethanol for 5 min each and air dry.
11. Mix 3 μl FITC-labeled chromosome point probe with 7 μl STARFISH hybridization buffer and 1 μl of Cot-1 DNA (final 0.1 μg/μl) in a thermal cycler tube.
12. Incubate 11 μl of the FISH probe/Cot-1 DNA/hybridization buffer mixture at 75°C for 5 min then chill immediately on ice.
13. Apply 11 μl of the probe mixture to the slide, cover with a 22 × 22 mm cover glass, and seal the cover with nail polish (see Note 15).
14. Place the slide in a humid chamber.
15. Hybridization: Incubate the chamber at 37°C for 12–16 h.
16. Carefully remove the nail polish from the slides and submerse the slides in a Coplin jar with 2× SSC at room temperature until the cover slips slide off (see Note 16).
17. Place the slides in Coplin jars with pre-warmed 0.4× SSC/0.3% Tween-20 solution at 73°C for 2 min (see Note 17).
18. Wash the slide in 2× SSC at room temperature for 1 min.
19. Mounting using Vectashield/DAPI (see step 8 in Subheading 3.4). Using this approach, chromosome-specific signals can be obtained whilst simultaneously labeling components of the SC (Fig. 5).

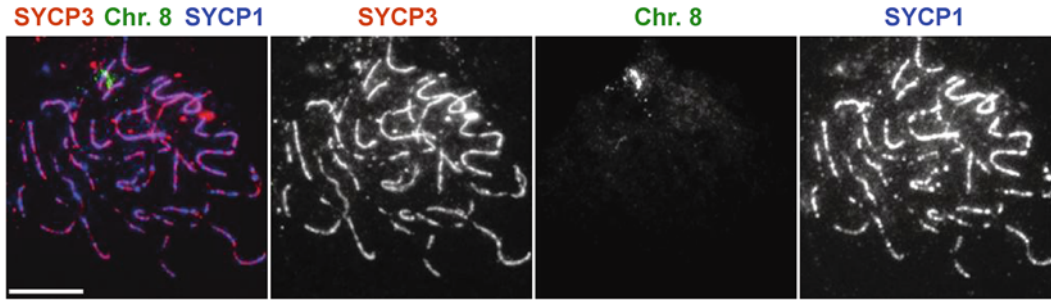

Fig. 5. FISH with Chromosome 8 A2 probe (*green*) against spread chromosomes immunostained with SYCP1 (*blue*) and SYCP3 (*red*). Scale bar, 5 μm.

4. Notes

1. The progression of meiosis in both sexes differs dramatically. In females, cells enter meiotic prophase during embryogenesis and arrest until the mouse matures after birth. After entering into meiosis in the embryonic ovary, oocytes pass through leptotene, zygotene, and pachytene stages before arresting in diplotene or dictyate stage, the last stage of meiotic prophase I. At leptotene, chromatin loops extend and condense, forming a filamentous meshwork called axial elements (AEs), which start undergoing synapsis or pairing at the zygotene stage. At the pachytene stage, the chromosome cores are completely synapsed and cells have 20 centromeres. The synaptonemal complex (SC) begins to disassemble at the diplotene stage, and homologous chromosomes move apart except at the chiasmata.
2. Making cuts deeply in the side of the legs is useful to fix the mouse firmly and to pick up the very small ovaries in the paravertebral gutters.
3. The early embryonic gonads are indistinguishable between the sexes; however, ovaries and testes become discernible from d.p.c 14.5 in terms of both morphology and location. The testes translocate to their final location at the anterior abdominal wall from d.p.c. 15.5.
4. If the sex of a given embryo is obscure, keep any part of the embryo body for genomic DNA extraction. The sex of each embryo from which gonads (fetal ovary) have been removed can be confirmed by PCR using primer sets for Y-chromosome-specific *Sry* and autosome control *Rapsyn* (Fig. 3c).

   Sry-F: 5′-CAGCCCTACAGCCACATGAT-3′

   Sry-R: 5′-GAGTACAGGTGTGCAGCTCTA-3′

   Rapsyn-F: 5′-AGGACTGGGTGGCTTCCAACTCCCAGACAC-3′

   Rapsyn-R: 5′-AGCTTCTCATTGCTGCGCGCCAGGTTCAGG-3′
5. Using this approach, ovarian tissue can become retained and inadvertently lost within the syringe and needle. An alternative approach which minimizes such loss involves repeatedly pipetting ovaries through P1000 tips.
6. Supernatants should be discarded carefully using a pipetman because of the very small volume of the cell pellets.
7. Trypsin treatment is not mandatory. However, its use will improve the number of single cells.

8. Usually, a total of approximately 10^5 cells will be obtained per ovary after collagenase treatment. Meiotic prophase cells comprise approximately 5–10% of the total cells.
9. The volume of hypotonic buffer is adjusted in the next step of the method.
10. Longer hypotonic treatment gives a better chromosome spread that preserves intact axis associated proteins such as cohesins or SC components. For some other proteins, however, localization signals are decreased or even eliminated by prolonged hypotonic treatment.
11. Triton X-100 works for pre-extraction of free cytoplasmic/chromatin-unbound proteins during PFA fixation. The pre-extraction and fixation are simultaneously done until the nuclei are attached to the slide glass.
12. Cytocentrifugation aids in the rapid preparation of well-spread chromosomes. Cytocentrifugation induces cell nuclei to become physically attached to the slide glass prior to extraction of free proteins during the fixation stage. However, chromosome spreading is greatly affected by the input cell concentration. There is tendency for cytocentrifugation at a low cell concentration to give more widely spread chromosomes; however, nuclei with broken chromosome morphology are occasionally observed. Also significant numbers of cells can be lost through flow out from the slide glass. The cell concentration should be empirically determined for the next application.
13. The SYCP3 staining shows axial/lateral elements while SYCP1 staining shows central elements, both comprising the SC.
14. Fixation of antibodies following immunostaining is mandatory in order to prevent their dissociation from the chromosome during the subsequent heat denaturing step in 70% formamide/6× SSC. Poor fixation results in massive aggregation of dissociated antibodies, which results in high background noise. In canonical chromosome FISH, pepsin digestion of the spread chromosomes would usually be applied to enhance hybridization. However such protein digestion should be avoided when immunostaining is combined with FISH.
15. Rubber cement can be used for sealing the cover glass instead of nail polish.
16. Carefully remove the nail polish along the edge of the cover glass using a knife or forceps.
17. The appropriate conditions for the post-hybridization washing step should be determined empirically for each probe. Poor washing results in high background with a low signal:noise ratio, whereas extensive washing will reduce hybridization signals.

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