

Chapter 1

Isolation of Fetal Gonads from Embryos of Timed-Pregnant Mice for Morphological and Molecular Studies

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Abstract

Gonadal sex differentiation is an important developmental process, in which a bipotential primordial gonad undergoes two distinct pathways, i.e., testicular and ovarian differentiation, dependent on its genetic sex. Techniques of isolating fetal gonads at various developmental stages are valuable for studies on the molecular events involved in cell-fate determination, sex-specific somatic and germ-cell differentiation and structural organization. Here we describe various procedures for isolation of embryonic gonads at different developmental stages from embryos of timed-pregnant mice. The isolated fetal gonads can be used for a variety of studies, such as organ culture, gene and protein expression. As examples of applications, we describe the immunofluorescence detection of SOX9 expression in gonadal tissue sections and microRNAs profiling/expression in fetal gonads at a critical stage for sex determination.

Key words: Timed-pregnant mice, Fetal gonad, Mesonephros, Testis cords, Immunofluorescence, microRNAs profiling

1. Introduction

Gonad development is a complex organogenesis involving the sexual dimorphic differentiation of both somatic and germ cells (1, 2). The laboratory mouse has been a key model for research in gonadal differentiation, in which precise morphological and cellular changes during the developmental process(es) have been well documented and characterized. The mouse gonad emerges at the coelomic surface of mesonephros at 9.5 days post coitum (dpc) in a bipotential manner, being morphologically identical in XX and XY embryos. At 10.5–11.5 dpc, *Sry* begins to be expressed in the XY gonad (3) and initiates the differentiation of a subset of somatic cells into Sertoli cells by directly upregulating *Sox9* (4) and other immediate downstream

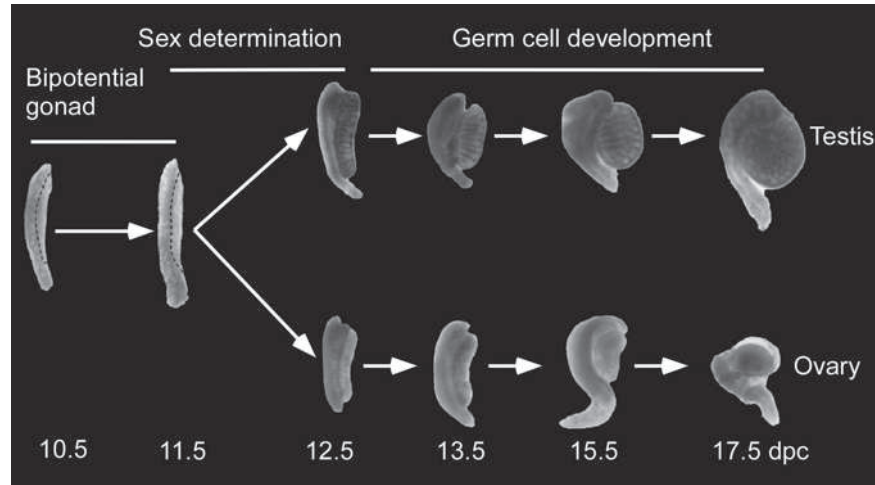


Fig. 1. Morphology of gonads isolated at different developmental stages. The testis cords are seen in the male gonads at 12.5 dpc and later but not at earlier stages.

testis differentiating genes. Upon Sertoli cell differentiation, the testis cords are formed, distinguishing morphologically the male gonads from female gonads (see Fig. 1). Ectopic expression of *Sry* in XX gonads induces testis development (5). Therefore, ovary development used to be thought as a default pathway. However, recent studies suggest that ovarian determining genes, such as *Rspo1* (6), *Wnt4* (7), and *FoxL2* (8) play important roles in ovary determination and differentiation in mammals.

Gonadogenesis also involves differentiation of primordial germ cells (PGCs). In the developing mouse embryo, PGCs migrate from the extraembryonic mesoderm into the urogenital ridge and populate the gonads between 10.0 and 11.5 dpc. Once inside the gonad, the germ cells continue to proliferate and go through several rounds of mitotic divisions in both sexes. Then the germ cells lose their motility and start to colonize in the gonad. Depending on the sex of gonadal somatic cells, germ cells in male and female gonads undergo two distinct developmental pathways. Male germ cells are arrested as prospermatogonia at G1/G0 from 13.5 dpc to a few days after birth (9). On the other hand, germ cells in the ovary enter the prophase of the first meiotic division and are arrested at the diplotene stage at birth.

Isolation of fetal gonads provides opportunity to study the sex differentiation of gonads and germ cells at morphological and molecular levels (3, 10, 11). Recombinant organ culture of gonads from wild-type mice (CD1) and mesonephroi from the transgenic mice ubiquitously expressing β -galactosidase (ROSA26) has shown the importance of the migration of mesonephric cells into the XY but not XX gonad for testis cord formation (12). Microarray analysis of

Sf1-GFP-positive somatic cells isolated from gonads has made it possible to identify many upregulated and downregulated genes associated with sex differentiation (13, 14). In this chapter, we describe a protocol for the preparation of timed-pregnant mice, isolation of urogenital complexes from embryos, separation of the gonad from the mesonephros, and the method to sex the embryos younger than 12.5 dpc. Immunofluorescence of histological sections and miRNA (miRNAs) profiling of male and female gonads at 12.5 dpc will be illustrated as the examples of morphological and molecular studies on dissected fetal mouse gonads.

2. Materials

1. 70% ethanol.
2. Regular surgical forceps and scissors.
3. Dumont #5 Forceps, or any dissection forceps with straight fine tips.
4. Dumont #5/45 Forceps, or dissection forceps with fine tips angled at 45 degrees.
5. Micro Dissecting Scissors (ROBOZ RS-5880).
6. Micro Dissecting Knife-Needle, 4 1/2", size 1 (Biomedical Research Instruments, Inc.).
7. Stereomicroscope.
8. Tissue culture dish (10-cm).
9. MIRASCORB Sponge.
10. 6–8 week old CD1 male and female mice (Charles River Laboratory).
11. Dulbecco's modified Eagle's medium (DMEM).
12. Fetal bovine serum (FBS).
13. Dissection medium: DMEM containing 10% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin, sterilized through 0.2-µm filter.
14. Phosphate-buffered saline (PBS).
15. 1.5-ml microcentrifuge tube.
16. 0.2-ml 8-strip PCR tubes.
17. DirectPCR lysis reagent (Viagen Biotech, Inc.).
18. Proteinase K.
19. RNAlater tissue stabilization solution (Ambion).
20. GoTaq DNA polymerase (Promega).
21. GeneAmp® PCR system 9700 (Applied Biosystems).

3. Methods

3.1. Preparation of Timed-Pregnant Females

Timed-pregnant mice can be purchased from commercial vendors, but since the time point is not always accurate, we prefer preparing timed-pregnant mice in our own laboratory.

1. Obtain 6–8 week old CD1 male and female mice from Charles River Laboratory, or other vendors.
2. Pair up female mice with male mice in cages just prior to the end of the daily light cycle.
3. Check the females for vaginal plugs early next morning. When a plug is found, the female is considered as pregnant at 0.5 dpc.
4. Transfer the plugged females into a new cage marked with the date on which a plug was detected and house them until dissection.

3.2. Isolation of Gonads from Embryos at 12.5 dpc or Later

Procedure for the isolation of gonads from 12.5 dpc embryos is shown as an example. This procedure can be used to isolate gonads from other gestational ages of embryos from timed-pregnant mice (Fig. 1). Dissection is usually carried out around noon on each day.

1. Sacrifice the timed-pregnant CD1 mice by cervical dislocation or other approved methods of the host Institutional Animal Care and Use Committee (IACUC).
2. Place the mouse on its back on absorbent paper and spray the mouse with 70% Ethanol thoroughly.
3. Lift the skin over the belly with forceps, make a lateral incision with regular surgical scissors, and pull the skin toward the head and the tail until the abdomen is completely exposed.
4. Open the abdominal cavity by lifting the abdominal muscle with forceps and cutting with scissors.
5. Grab the uterus with one set of forceps just above the cervix and cut the uterus cross the cervix with scissors. Then pull the uterus upward and cut beneath the oviduct.
6. Transfer the uterus into a 10-cm culture dish containing PBS.
7. Hold one end of the uterus with regular surgical forceps, insert one tip of a pair of Micro Dissecting Scissors into the antimesometrial wall of the uterus near the holding forceps, and cut the muscle by carefully pointing and sliding the scissors along the uterus. The embryos in amnionic membranes attached to the placenta will be exposed.
8. Separate each embryo from its placenta and surrounding membranes by carefully holding the placenta with forceps and cutting with Micro Dissecting Scissors (see Note 1).
9. Transfer the embryos to a new 10-cm dish and wash embryos with fresh PBS to remove the blood.

10. Place one layer of sponge in a 10-cm culture dish, add small amount of dissection medium to the dish (just enough to cover the bottom), and transfer the embryos onto the wet sponge (see Note 2).
11. Place the dish under a dissecting stereomicroscope, rotate the embryo on its side, hold the embryo with Dumont #5 forceps and cut off the anterior half of the embryo just below the forelimbs with Dumont #5/45 forceps (Fig. 2a, step 1). Then cut along the ventral midline of the posterior half of the embryo with Dumont #5/45 forceps, remove the liver and intestine (Fig. 2a, step 2), and cut off the hindlimbs and tail (Fig. 2a, step 3) (see Note 3).
12. Turn the embryo, and carefully remove the remaining intestines and liver. The urogenital complex must be visible on the dorsal wall of the embryo.
13. Hold the embryo with Dumont #5 forceps, cut out the urogenital complex with Dumont #5/45 forceps, and place it in a new 10-cm dish containing dissection medium.

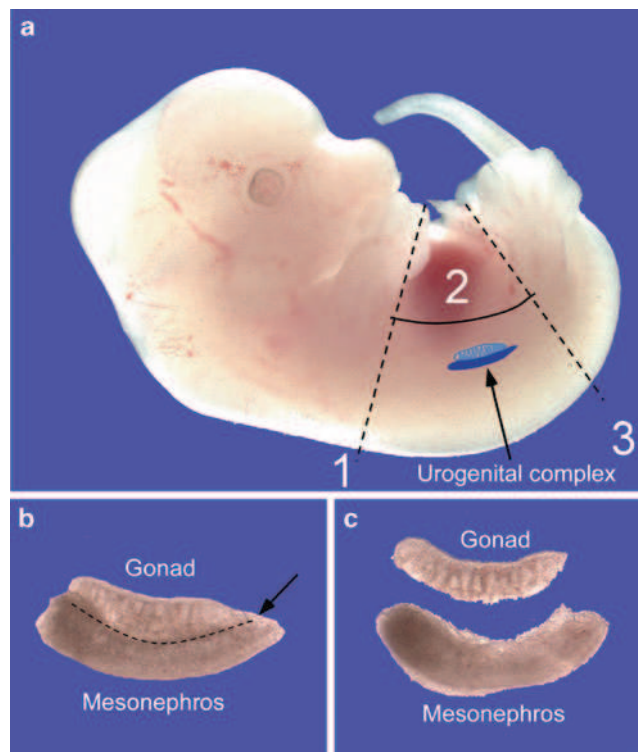


Fig. 2. Illustration of the procedure for the isolation of gonads from embryos at 12.5 dpc. (a) 12.5 dpc embryo, 1, 2, and 3 indicate the step of cutting, the position of urogenital complex is drawn on the embryo. (b and c) show the separation of gonad from mesonephros. The *dash line* in b shows the position for cutting.

14. Remove the connective tissues from the urogenital complex by holding with Dumont #5 forceps and cutting with Dumont #5/45 forceps.
15. Check the presence of testis cords in the dissected gonads under the dissecting stereomicroscope. At 12.5 dpc or later, the sex of the gonads can be morphologically distinguished by the presence of testicular cords in the testis.
16. Place the male and female urogenital complexes in separate 10-cm dishes containing dissection medium.
17. For immunohistochemistry, transfer the male and female urogenital complexes into the tubes containing a fixative (see Note 4).
18. For analyses of mRNA and protein, the gonads must be separated from the adjacent mesonephroi. Hold the mesonephros with Dumont #5 forceps and gently cut the connective tissue between them with Micro Dissecting Knife-Needle (Fig. 2b, c).
19. Pool the testes and ovaries in the 1.5-ml tubes containing 500 μ l RNAlater solution. Keep the tubes overnight at 4°C (see Note 5).
20. Add an equal volume pre-cooled PBS, and mix well.
21. Spin down at 5,000 $\times g$ for 2 min and discard the supernatant. These gonads can be used for the isolation of RNA immediately or stored at -80°C until use.

3.3. Isolation of Gonads from Embryos Younger than 12.5 dpc

The major steps for dissecting gonads from embryos younger than 12.5 dpc are the same as Section 3.2. However, at these developmental stages, the urogenital ridges are morphologically indistinguishable between XX and XY embryos. If the sex of each gonad needs to be identified, an extra step is required for determining the sex of the embryos. Staining of Bar body in amnions is a quick method for identifying the XX embryo by the presence of condensed chromatin body (Bar body) and is particularly advantageous when the sex of gonad needs to be known before further procedures (10). Otherwise, a piece of embryonic body (e.g., tail) can be saved during dissection and used for genotyping later. PCR detection of Y-chromosome-specific sequences is accurate and convenient particularly when a large number of gonads must be collected. We describe here the modifications of the Section 3.2 for this purpose.

1. Follow Step 1 to 9 as described under Section 3.2
2. Place embryos in order, so that they can be identified.
3. Keep the tails of embryos in individual tubes marked with their identifications. Store them at -20°C until PCR genotyping.
4. Instead of pooling the gonads, isolate urogenital complexes or gonads from each embryo one by one and store them individually

in proper containers with either fixative or RNAlater solution (see Note 6).

3.4. Sexing the Embryos by PCR Amplification of the Sry Sequence

1. Add 50 μ l DirectPCR Lysis Reagent containing freshly prepared 0.2 mg/ml Proteinase K to the 5 ml tube containing the tail of the embryo.
2. Incubate at 55°C for 5–6 h or overnight until no tissue clumps are seen.
3. Briefly spin down the tubes and add 50 μ l H₂O to each tube.
4. Incubate the tubes at 95°C for 10 min to inactivate Proteinase K.
5. Use 1 μ l of the lysate for total 25 μ l PCR reaction containing a pair of *Sry* primers (*Sry*-BA: 5'-GTCAAGCGCCCCAT-GAATGC-3'; *Sry*-EA 5'-TAGTTTGGGTATTCTCTCT-3').
6. PCR conditions: 95°C for 5 min, 30 cycles of 1 min at 95°C, 1 min annealing at 55°C, 1 min extension at 72°C, and a final extension at 72°C for 10 min.
7. Run a 1.5% agarose gel to analyze the PCR products.
8. If a 203-bp fragment is amplified by PCR, the embryo is considered to be XY male; the absence of such a DNA fragment, the embryo is considered to be XX females.

3.5. Examples of Studies

1. Immunofluorescence detection of SOX9 expression in histological sections:

Testicular differentiation is morphologically distinct by the formation of testis cords in the XY gonad by 12.5 dpc. The involvement of various molecules in this process can be studied by immunofluorescence detection of proteins in histological sections of the gonads. The mesonephros is usually left attached to the gonad so that the orientation of gonad can be identified. We show here an example of double staining of SOX9 and mouse Vasa homolog (MVH). *Sox9* is a direct target of the primary testis-determining gene *Sry* (4) and represents Sertoli cell differentiation. MVH is a marker for the germ cells of both sexes (15).

Methods: Isolated urogenital complexes at 12.5 and 14.5 dpc are fixed in 2% paraformaldehyde in microtubule stabilizing buffer (3) for 1 h and embedded in paraffin. Slides from the mid part of gonads are de-paraffinized and subjected to antigen retrieval procedures as previously described (3). The slides are then incubated with the mouse IgG blocking reagent provided in the MOM kit (Vector Laboratories, Burlingame, CA) for 60 min, followed by an anti-SOX9 mouse monoclonal antibody (Abnova Laboratories, Taiwan) and a rabbit anti-MVH antibody (kind gift from Dr. T. Noce) both at 1:1,000 dilutions, in PBS containing the protein concentrate (provided in the MOM kit) at 4°C overnight. On the next day, all slides are washed with

PBS and incubated with a horse anti-mouse IgG antibody conjugated with biotin (provided in the MOM kit) and a goat anti-rabbit IgG antibody conjugated with Rhodamine (Pierce Endogen, Rochford, IL), both at 1:1,000 dilutions, in PBS containing the protein concentrate for 30 min. All slides are washed with PBS, followed by distilled water, and mounted in the Prolong Antifade mounting medium (Molecular Probe, Eugene, OR) containing 0.4 µg/ml 4',6-diamidin-2'-phenylindole dihydrochloride (DAPI) (Roche Diagnostics, Mannheim, Germany). Fluorescent signals are examined under an epifluorescence microscope (Zeiss Axiophot, Germany). All images are captured with a digital camera (Retiga 1300, QImaging, Burnaby, BC) and processed with Northern Eclipse digital imaging software, version 6.0 (Empix Imaging, Mississauga, ON).

Results: SOX9 immunofluorescence staining can be clearly seen in the nuclei of numerous cells in the testes but not ovaries at either 12.5 or 14.5 dpc (Fig. 3). In the testis at 12.5 dpc, SOX9 staining is clearly seen in the nucleus. SOX9-positive cells are mainly localized within the testis cords but also occasionally seen in the interstitium, and less organized in the caudal pole. MVH staining is seen in the cytoplasm of germ cells. They are enclosed in SOX9-positive cells in the testis cords. By 14.5 dpc, both the volume of testis and number of testis cords increase considerably. SOX9-positive cells are predominantly localized within the testis cords, forming palisade-like alignments. In the ovary at 12.5 dpc, no SOX9 staining is seen. MVH-positive germ cells are scattered over the ovary. The number of MVH-positive cells is visibly increased in the ovary from 12.5 to 14.5 dpc.

2. MicroRNA profiling of 12.5 dpc fetal gonads

MicroRNA (miRNA)s are a class of short (~22 nt), naturally occurring, noncoding RNAs. They regulate protein expression by targeting the mRNAs of protein coding genes, resulting in either translation repression or transcriptional regulation (16). It has been shown that miRNAs are important for the proliferation of PGCs and spermatogonia. In addition, Dicer, the key enzyme of miRNAs biogenesis, is required for Sertoli cell functions and survival (17). Since miRNAs are important for organogenesis and cellular differentiation, it is reasonable to hypothesize that miRNAs also play some important roles in regulating fetal testis and ovary differentiation. Indeed, sexually dimorphic miRNA expression was observed during chicken fetal gonadal development (18). Accordingly, we used the isolated male and female gonads at 12.5 dpc for profiling the differentially expressed miRNAs.

Methods: Male and female gonads at 12.5 dpc from six CD1 mice are collected and pooled separately in 1.5-ml tubes containing RNAlater solution. Total RNA is isolated with RNeasy®-

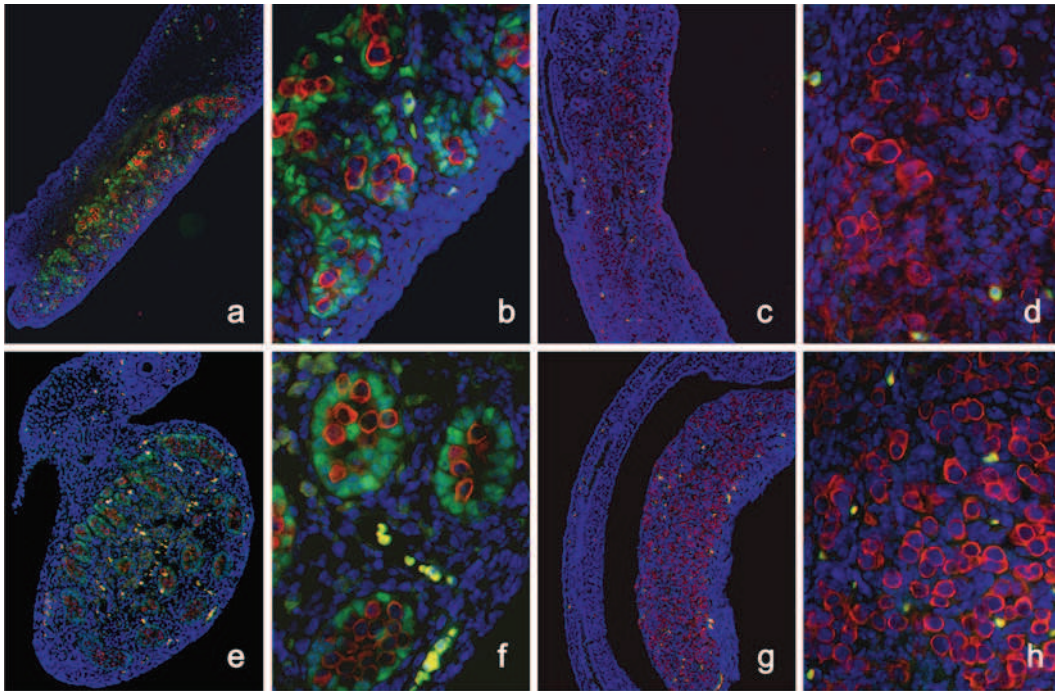


Fig. 3. Immunofluorescence detection of SOX9 (green) and MVH (red) with DAPI counterstaining (blue). The mesonephros is placed on the *left* and the gonad on the *right*. Bar indicates 200 μm in **a**, **c**, **e**, and **g**, or 40 μm in **b**, **d**, **f**, and **h**. SOX9 staining is predominantly seen in the nucleus of Sertoli cells and their precursors, whereas MVH staining is in the cytoplasm of germ cells. Yellow staining shows blood cells. (**a** and **b**) Male urogenital complex at 12.5 dpc. (**c** and **d**) Female urogenital complex at 12.5 dpc. (**e** and **f**) Male urogenital complex at 14.5 dpc. (**g** and **h**) Female urogenital complex at 14.5 dpc.

Micro Kit (Ambion) according to the manufacturer's instruction. RNA is quantified by using the NanoDrop ND-1000 Spectrophotometer. Mouse mmu-miRNome MicroRNA Profiling Kit (System Biosciences), which detects the expression of 709 miRNAs in mouse, is used to profile the miRNAs in male or female gonads. 1 μg of total RNA is tagged with poly(A) to its 3' end by poly A polymerase, and reverse-transcribed with oligo-dT adaptors by QuantiMir RT technology. Expression levels of the miRNAs are measured by quantitative PCR using SYBR green reagent and 7900HT Fast Real-Time PCR System (Applied Biosystems). All miRNAs can be measured with miRNAs specific forward primers and a universal reverse primer (SBI). Expression levels of the miRNAs can be normalized to U6 snRNA, RNU43 snoRNA, and U1 snRNA.

Results: Of the 709 miRNAs analyzed, 52 miRNAs were upregulated threefolds or higher in male gonads (Fig. 4a) and 69 miRNAs were upregulated at least threefolds in female gonads (Fig. 4b). The differential expression of miRNAs between male and female gonad at 12.5 dpc suggests that miRNAs may play important roles in sex differentiation. For the male gonads, the top ten miRNAs are miR-34b-5p,

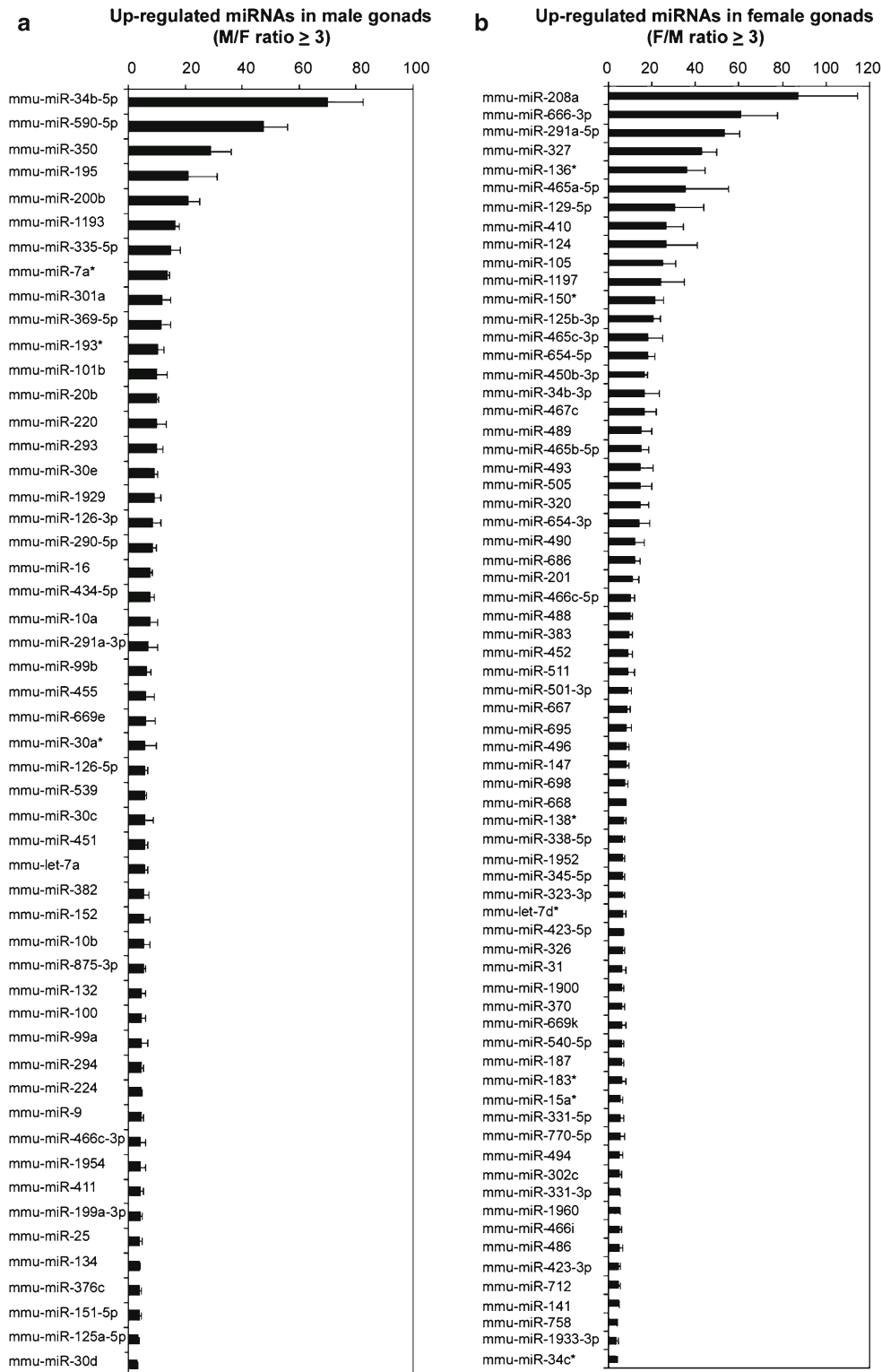


Fig. 4. Differential expression of miRNAs in mouse fetal gonads at 12.5 dpc. Total RNAs extracted from male and female gonads were used to measure the levels of miRNAs expression, and the ratio of male to female gonads was calculated. (a) miRNAs upregulated in male gonads as M/F ratios. (b) miRNAs upregulated in female gonads as F/M ratios. Data are derived from three independent experiments. Those showing $\geq$ threefold changes are presented here.

miR-590-5p, miR-350, miR-195, miR-200b, miR-1193, miR-335-5p, miR-7a*, miR-301a, and miR-369-5p, while for the female gonads, the top 15 miRNAs are miR-208a, miR-666-3p, miR-291a-5p, miR-327, miR-136*, miR-465a-5p, miR-129-5p, miR-410, miR-124, miR-105, miR-1197, miR150*, miR125b-3p, miR-465c-5p, and miR-654-5p. Since the isolated gonads at 12.5-dpc stage contain various somatic cell types in addition to germ cells, the cellular origins and exact functions of these differentially expressed miRNAs in embryonic testis and ovary are uncertain. Nevertheless, other studies have suggested some possible roles of these miRNAs in regulation of gene expression and biological processes. Among the miRNAs upregulated in the embryonic testis, miR-34, miR-200, miR-195, and miR-7 are putative tumor suppressors (19, 20). miR-34b-5p has been demonstrated to mediate the expression of an alternatively polyadenylated variant of the mouse β -actin gene (21), which might be important for testis cord formation. The miR-200b, miR-20b, and miR-369-5p are involved in mesenchymal stem cell phenotype (22) and epithelial-mesenchymal transition (23). Further, miR-200b is associated with regulation of growth and function of stem cells, in which its upregulation is associated with differentiation while its inhibition is related to maintenance of stemness (24). miR-7a is highly expressed in testis-derived male germ stem cells (25) and could be derived from the male germ cells in the embryonic testis.

Among the miRNAs upregulated in the embryonic ovary, miR-208a represents one of the well-characterized miRNAs. It is encoded within an intron of α -cardiac muscle myosin heavy chain gene and is a regulator of cardiac hypertrophy and conduction (26, 27) via its regulation of a number of target genes, including GATA4 (26), correct dosage of which is needed for proper sex determination (28). miR-124 has been demonstrated to play critical role in neurogenesis (29, 30), particularly in its regulation of SOX9 expression in the developing nervous tissues (31). Hence, SOX9 could be a target for inhibition by miR-124 in ovarian cells, in which SOX9 expression is preferentially repressed, as compared to the testicular cells of the same stage. Interestingly, miR-105 has been shown to control human ovarian cell proliferation (32), its elevated expression in the fetal ovary suggests a potentially similar role in the mouse. Further detailed studies on the expression of miRNAs and identification of their corresponding targets at various stages of gonadal development in both male and female should shed critical insights on how these miRNAs regulate the sex-specific cell fate determination and organogenesis in this interesting developmental system.

4. Notes

1. Since embryos are very soft and fragile, gentle handling is required to avoid breaking the embryos, especially when you need to count the tail somites.
2. Do not add too much dissection medium to the dish during dissection. Otherwise, embryos will move around and be difficult for the dissection manipulations. It is much easier to position embryos on a sponge with a minimal less volume of medium.
3. In step 2, cut just below the liver. If cut too deep, the urogenital complex may be removed at this step.
4. We recommend 2% paraformaldehyde in microstabilizing buffer (3). Since this fixative is powerful, antigen retrieval is necessary prior to immunofluorescence procedures.
5. For RNA isolation from gonads of 12.5 dpc or older embryos, the gonads can also be snap-frozen in individual microcentrifuge tubes in liquid nitrogen at this step.
6. For RNA isolation from gonads of younger than 12.5 dpc, if the sex of the gonads is needed, it is convenient to place the gonads in 8-tube PCR strips containing RNAlater solution, which preserves the integrity of the RNAs for RNA purification procedures at a later time (per supplier of this reagent kit, Ambion Inc.) instead of being snap-frozen in liquid nitrogen.

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