Video Article

Organ Culture and Whole Mount Immunofluorescence Staining of Mouse Wolffian Ducts

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Abstract

Tubal morphogenesis is a fundamental requirement for the development of most mammalian organs, including the male reproductive system. The epididymis, an integral part of the male reproductive tract, is responsible for sperm storage, maturation, and transport. The adult epididymis is a highly coiled tube that develops from a simple and straight embryonic precursor known as Wolffian duct (WD). Proper coiling of the epididymis is essential for male fertility, as sperm in the testis are unable to fertilize an oocyte. However, the mechanism responsible for epididymal development and coiling remains unclear, partially due to the lack of whole organ culture and imaging methods. In this study, we describe an *in vitro* culture system and whole mount immunofluorescence protocol to better visualize the process of WD coiling and development, which may also be applied to study other tubular organs.

Video Link

The video component of this article can be found at https://www.jove.com/video/55134/

Introduction

The male reproductive system primarily consists of testis, the site for germ cell development and differentiation, and a complex ductal system that is required for the maturation, transport, and storage of sperm. Epididymis is a tubular organ that connects testis with vas deferens and mainly involved in post-testicular development and maturation of germ cells [1]. The highly coiled adult mouse epididymis develops from a simple and straight precursor tube, Wolffian duct (WD) [1]. The complex and coiled structure of epididymis is essential for sperm to acquire the capacity to fertilize female germ cells [2]. How such an essential organ for male fertility develops and gets into shape is not well understood. Various signaling pathways have been shown to be involved in the development of WD such as the Wnt signaling pathway [3,4] and the androgen signaling pathway [5]. Our recent work has established the requirement of balanced Wnt signaling for WD coiling during prenatal development [4]. However, further studies are required to understand the mechanisms involved in postnatal epididymal coiling, cellular differentiation, and cell-to-cell communication within the epididymal epithelium and with interstitial cells.

Genetically modified mouse models have been proven to be a powerful tool for identification/validation of molecular mechanisms underlying development and disease of various organ systems [6]. Despite its extensive use, there are significant limitations of genetically modified mouse models, including the unpredicted phenotype of targeted mouse mutants with regards to presumed gene function, and no phenotype in null mutants in some models, influence of genetic and environmental factors, labor intensive and time-consuming process of developing mouse models. Therefore, the interpretation of the significance of the findings only from genetically modified mouse models is not always straightforward [6]. These limitations can be overcome by using *in vitro* organ culture system which gives us the flexibility to manipulate multiple signaling pathways in real time in controlled culture conditions. Organ culture system is instrumental in understanding the physiology and pathology of whole organs [7]. Moreover, imaging of whole organs stained with fluorophore labeled antibodies enables us to visualize the markers of interest in a three-dimensional context, as it exists *in vivo,* thereby providing a better understanding of the organ's shape, structure, and function [8].

Here, we have described the methods for isolation of mouse embryonic gonadal ridges, *in vitro* culture and whole mount immunofluorescence imaging of WDs, that can be applied to answer a variety of questions concerning the morphogenesis of tubular organs such as WDs. In this protocol, we isolated mouse embryonic urogenital ridges from 15.5 days post coitum (dpc) pregnant dams and cultured them for 3 d followed by immunostaining for an epithelial cell marker (cytokeratin 8, CK8), a cell proliferation marker (phospho-Histone 3, PH3) and active βcatenin.

Protocol

Animal care and experimental procedures were conducted in accordance with the guidelines of the Animal Care and Ethics Committee of the University of Newcastle and confirmed to the New South Wales Animal Research Act, New South Wales Animal Research Regulation, and the

Australian code for the care and use of animals for scientific purposes. All the procedures undertaken on mice were approved by the Animal Care and Ethics Committee of the University of Newcastle.

1. Time Mating

1. Pair 6 - 8 weeks old male and female mice just prior to the end of daylight cycle.
2. Check the females for the presence of vaginal plugs early morning (on or before 8.00 AM), every day. Day of plug is considered as 0.5 dpc.
3. Transfer the female with vaginal plug into a new cage (without male) and label the date of plug.

2. Isolation of Mouse Embryonic Gonadal Ridges

1. Isolate mouse embryonic gonadal ridges from 15.5 dpc pregnant females as described in [9], with a few modifications as described below.
2. Carry out dissection before noon as the time pregnant females are considered to enter into the next day of pregnancy by afternoon.
3. Sacrifice 15.5 dpc pregnant females using cervical dislocation (or as per the approved protocol by the institutional Animal Ethics Committee).
4. Keep the mouse on its back on absorbent tissue paper and spray the ventral side of the abdomen with 70% ethanol.
5. Lift the lower abdominal skin (ventral side) with forceps and make a lateral incision using surgical scissors, extending from urogenital opening to the end to rib cage. Pull the skin towards the head of the animal to expose the abdomen.
6. Cut the abdominal muscles with surgical scissors to expose peritoneal cavity. Grab the gravid uterus with forceps just above the cervix and make a cut. Now lift the uterus by holding on to the urinary bladder and cut the uterine broad ligament followed by cuts at the uterotubal junctions to detach uterus from the peritoneal attachment.
7. Transfer the uterus into a 50 mL tube containing ice-cold HBSS (Hank's Balanced Salt Solution). Wash the gravid uterus gently with ice-cold HBSS to remove the excess blood.
8. Keep the uterus in a Petri dish containing ice-cold HBSS and keep on ice. Cut the uterine wall and take out the embryos. Keep the embryos in a fresh Petri dish with HBSS on ice.
9. Take a clean sterile tissue paper and put it on a new Petri dish. Spray 70% ethanol on the tissue paper. Keep embryo on this tissue paper on the lateral side and cut it from lower abdomen using a sterile blade in the direction as shown by dotted line in **Figure 1**.
10. Fix the embryo on a sterile sponge base by pinning the vertebral column.
11. Place it under the dissecting stereomicroscope and carefully cut along the ventral midline. Remove the liver and intestines. The mouse urogenital system (kidney, ureter, testis, WD, and vas deferens) should now be visible.
12. Cut out the testis and WD, and keep in a fresh Petri dish with HBSS, on ice. To take out the WD and testis, cut the vas deferens close to its attachment to urethra and cut the attachment of lower portion of WD from gubernaculum.
13. Pool the testes and WDs from the embryos of the same group (this may vary as per the experimental plan).

3. Culture of Embryonic Gonadal Ridges (Figure 2)

1. Prepare medium for culturing the embryonic gonadal ridges by supplementing DMEM/F12 with 10% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin and 1% L-glutamine. Warm the media to 37 °C in a water bath.
2. Add 300 µL of media per well of a 24-well cell culture plate.
3. Take a fresh Petri plate and put a small drop of sterile HBSS on it. Put a polycarbonate track etch membrane (0.8 µm) on this drop of HBSS with its shining surface facing the HBSS.
4. Using clean forceps put two WDs and gonads on the membrane (on its rough surface, facing upward) and remove the maximum amount of HBSS using sterile wipes/absorbent paper. Do not touch the tissue with absorbent paper; otherwise, it will damage the tissue.
5. Ensure that neither the WDs nor the gonads are touching each other. Otherwise, they will stick together during subsequent incubation.
6. Transfer the membrane with the tissues in the well of a 24-well plate containing 300 µL culture medium and culture them at air-medium interface. Too much culture medium on membranes results in a cystic growth of WDs.
7. Incubate the plate at 37 °C incubator, in the presence of 5% CO_2.
8. Change the culture medium every day. To change the medium with the pipette, remove the medium from the well first and then add 300 µL of the prewarmed fresh medium.
   Note: For studying the effect of different signaling pathways on WD morphogenesis, chemical activators and/or inhibitors at the required concentrations can be added to the culture medium.
9. Culture the tissues for 3 d. Within 3 d, uncoiled WDs collected from a 15.5 dpc embryo transform into highly convoluted tubes (**Figure 3B**). The addition of the Wnt inhibitor, IWR1, results in inhibition of WD coiling (**Figure 3C**).
10. For harvesting these tissues take a Petri dish with ice-cold PBS. Transfer the membrane with cultured gonad and WD into the Petri plate. The membrane will float on the PBS. Press the membrane to sink it in PBS and take out the gonad and WD.
11. Fix the tissues with 4% paraformaldehyde (PFA) O/N at 4 °C or for 1 h at RT. If needed, take bright field images of the WDs before fixation.

4. Whole Mount Immunofluorescence

1. Wash the fixed tissues 3x with PBS-T (PBS + 1% Triton X-100) with slow rocking, 10 min each, at RT.
2. Dehydrate the tissues in a graded series of ethanol (25%, 50%, 75% and 100%), 10 min each, at 4 °C, with slow rocking (~30 rpm). At this step, the tissues can be stored at 4 °C in 75% ethanol.
    1. To change the ethanol, keep the tissues undisturbed for 2 min and let the tissues settle down. Take out as much supernatant as possible and then add the next higher concentration of ethanol. To avoid any damage to the tissues, the pipette tip should not touch the tissues at any stage.
3. Following dehydration, rehydrate the tissues in a graded series of ethanol (100%, 75%, 50% and 25%), 10 min each, at 4 °C, with slow rocking.

4. Wash the tissues with PBS-T (PBS + 0.1% Triton X-100), 4x, 20 min each, at RT, with gentle rocking (~30 rpm).
5. Block the tissues for 1 h at RT with blocking buffer (PBS + 1% BSA + 0.2% non-fat dry milk powder + 0.3% Triton X-100).
6. After blocking, transfer the tissues to primary antibody solution (diluted in blocking buffer) and incubate O/N at 4 °C with gentle rocking (~30 rpm). Dilutions of the primary antibodies used are described in the **Materials Table**.
7. The next day, wash tissues with PBS-MT (PBS + 2% non-fat dry milk powder + 0.5% Tween-20), 4x, 30 min each, at RT, with gentle rocking (~30 rpm).
8. Following this washing step, add secondary antibody at a dilution of 1:250 (this step needs optimization for individual antibodies) in blocking buffer and incubate for 1 h at RT, with gentle rocking.
9. From now onwards keep the tissues covered with aluminum foil to prevent exposure to light as secondary antibodies are labeled with light sensitive fluorophores.
10. Wash the tissues 3x with PBS-T (PBS + 0.1% Tween-20), 30 min each, with gentle rocking.
11. Prepare slides for mounting the stained tissues.
    1. For this, cut the glass cover slips into thin strips using a diamond pen and stick two strips one over the other on the glass slide using nail enamel. Make two boundaries and place tissues in between these two boundaries.
    2. Add DAPI containing mounting medium and put on a cover slip. Apply nail enamel at the corners of the cover slip to fix it to the slide. Note: Tissues are now ready for imaging. Store these slides at -20 °C.

Representative Results

During development, the WD undergoes significant changes wherein a simple and straight tube is transformed into a highly complex and coiled duct. Using methods described above, WDs undergo a similar transformation in culture conditions. Here we have shown the results from WDs cultured for 3 d (**Figure 3**). To dissect molecular mechanisms involved in WD morphogenesis, the culture medium can be supplemented with inhibitors and activators targeting different signaling pathways. **Figure 3C** shows uncoiled WD after 3 d of culture in the presence of the Wnt signaling specific inhibitor, IWR1 [10]. Arrows mark coiled (**Figure 3B**) and uncoiled WDs (**Figures 3A** and **C**) indicating that suppression of Wnt signaling results in inhibition of WD coiling (**Figure 3C**). Thus, this culture system can be used to dissect the molecular mechanisms involved during the development of WDs (or other organs).

To label different cell types, we performed whole mount immunostaining on WDs (**Figure 4**). Here we present data validating the use of this protocol for labeling cytoskeletal, cytoplasmic, and nuclear proteins. **Figure 4A** represents cytokeratin 8 (CK8, a marker of epithelial cells) immunostaining of WDs (marked by an arrow) cultured for three days. We have also applied the same protocol to assess cell proliferation by immunostaining for PH3 (phospho-Histone 3, a cell proliferation marker). **Figure 4B** shows a representative image of PH3 whole mount immunostaining. Green dots marked by an arrow represent PH3 positive hence proliferating cells. **Figure 4C** shows immunostaining for active β-catenin on WDs freshly isolated from 18.5 dpc mouse embryos. The negative control (IgG control) in **Figure 4D** shows no staining. These results highlight the robustness of this whole mount immunostaining protocol, which can also be used for many different antibodies.

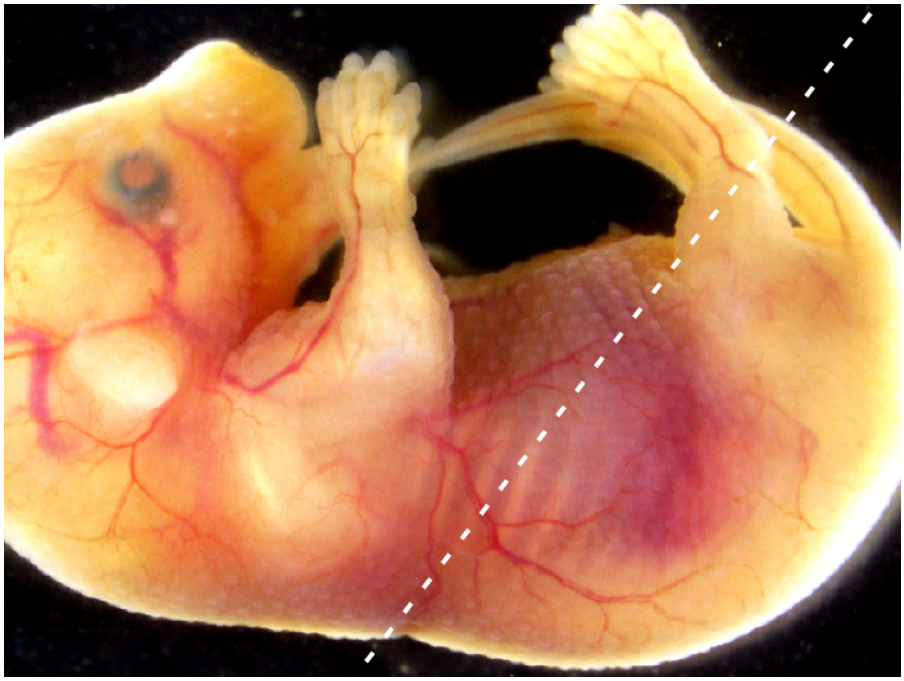

Figure 1. Illustration of the site of incision for isolation of embryonic gonads from 15.5 dpc embryo. The white dotted line marks the site for making an incision in a 15.5 dpc embryo to isolate urogenital ridges. Please click here to view a larger version of this figure.

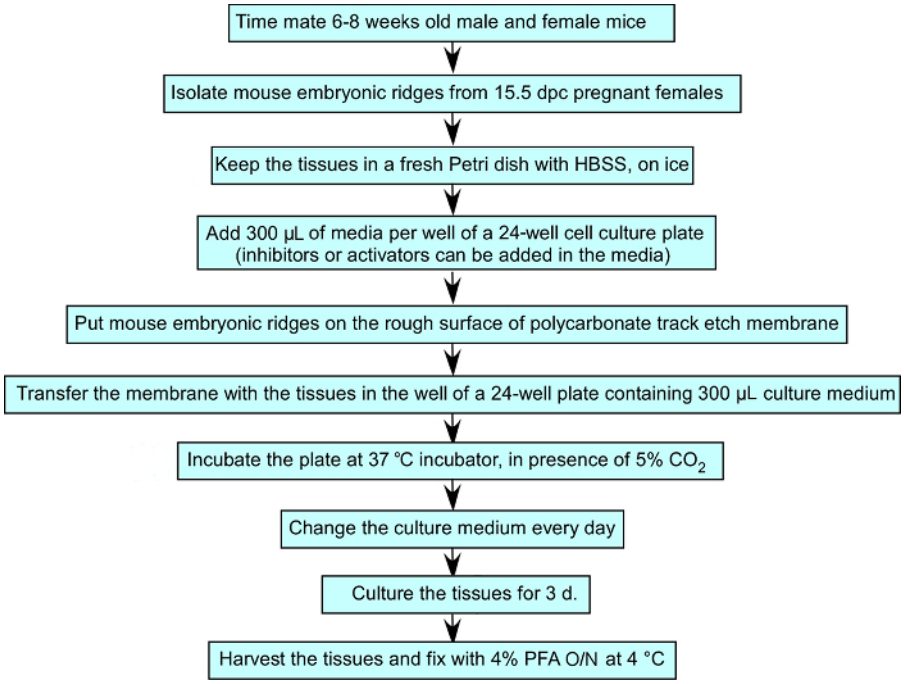

Figure 2. Flow diagram describing stepwise procedure for organ culture. Please click here to view a larger version of this figure.

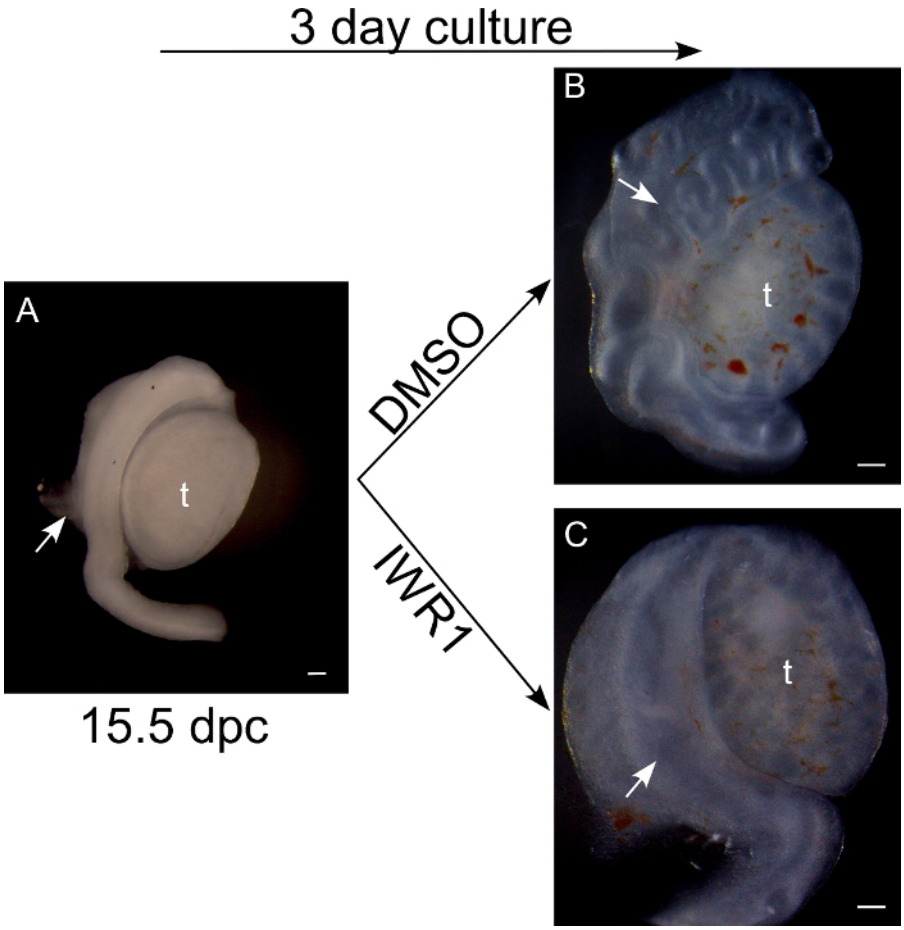

Figure 3. Normal WD morphogenesis in culture conditions. (**A**) Testis and WD isolated from 15.5 dpc embryo. (**B**) Coiled WD after 3 d culture in the presence of DMSO (control). (**C**) No WD coiling was observed with IWR1 treatment (a Wnt inhibitor). Arrow marks WD; t, marks testis. Bars equal 100 µm. Please click here to view a larger version of this figure.

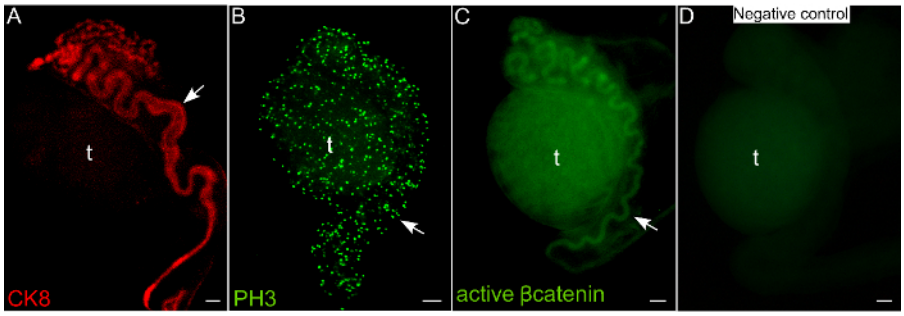

Figure 4. Whole mount immunolabeling of WDs. (**A - B**) Whole mount immunostaining of WDs isolated from 15.5 dpc embryos and cultured for 3 d for CK8 (**A**) and PH3 (**B**). (**C**) Active βcatenin whole mount immunofluorescence on WD isolated from 18.5 dpc embryo. (**D**) Negative control (IgG control) for active βcatenin on 18.5 dpc WD showing no staining. Arrow marks coiled WD; t, marks testis. Bars equal 100 µm. Please click here to view a larger version of this figure.

Discussion

The organ culture system has many advantages over the traditional cell culture system. This system retains the original structural relationships and interactions between different cell types. It mimics *in vivo* systems and gives more accurate information than cell culture studies where the niche factor is absent. The use of organ culture systems even have advantages over using the whole animal. These include the higher cost of using the whole animal, easy care and maintenance of organ culture system, *etc.* Moreover, various pharmacological agents or drugs can be tested on isolated/cultured organs and direct response of the organ and not the whole body can be studied.

There are several critical steps that need to be considered for successful culture of embryonic WDs. Tissues must be collected carefully without damaging their integrity, as damaged tissues do not grow well and become cystic. Excess of media on the top of filter also leads to a cystic growth of WDs. We performed tissue isolation and culture outside the tissue culture hood without any contamination. Culture medium was prepared and added to 24-well plates inside the tissue culture hood. Therefore, using proper technique, working quickly and taking necessary precautions, WDs collected outside tissue culture hood can be cultured without any contamination. While harvesting the cultured tissues, care should be taken as they are very fragile and should not be grabbed directly with forceps. Tissues should instead be picked up in a thin film of PBS that forms between the two arms of forceps.

We have also described a detailed whole mount immunofluorescence procedure in this manuscript. Z-stack imaging of tissues after whole mount staining using confocal microscopy or stereoscopy gives an advantage to section through the whole tissue without serial sectioning and staining multiple slides. Thus, it gives a better idea of the location of expression of target proteins. Antibody penetration is difficult in tissues with high fiber/extracellular matrix content resulting in a lower signal. Longer permeabilization and increased duration of incubation with primary antibodies may be useful in such cases. The other disadvantage of whole mount staining is that the resolution at a single cell level is hard to achieve and may need special objectives/microscopes. We performed both 1 h and O/N fixation in 4% PFA and found that these methods worked well for the majority of antibodies tested in our laboratory including CK8, PH3, and active βcatenin antibodies. We have also successfully colocalized CK8 with PH3 or active βcatenin. For colocalization, primary antibodies for both the markers were added simultaneously. These antibodies must be raised in different species. During the whole mount immunofluorescence procedure, utmost care should be taken when adding or removing solutions as the tissues can be easily drawn up into the pipette tip/transfer pipette and get stuck to the plastic. To avoid this, change solutions with a 200 µL pipette tip under a stereoscope or dissection microscope and leave a little bit of solution at the bottom of tubes. We recommend collecting the waste solutions in a clean transparent glass beaker. After each solution change, count the number of tissues in the tubes under a stereoscope. In case of accidental loss of tissues, they can be recovered from the glass beaker. Pipette tips used for transferring solutions should never touch the tissues as this will damage the tissues and leave them unsuitable for imaging.

For mounting the stained WDs, we used cavity slides as putting coverslips directly on the top of WDs can compress them and distort their morphology. Depending upon the thickness of tissues, the depth of the cavity can be easily manipulated by varying the number of coverslip strips used. This method of slide preparation is economical as it requires only cover slips and a diamond pen. Imaging can also be done before coverslipping as it is easy to orient and move the stained WDs before they are fixed under coverslips. With the whole mount immunostaining, Z-stack imaging gives better images and provides more information. For taking images from different treatment groups, the exposure should be kept same.

Disclosures

The authors have nothing to disclose.

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