Video Article

Using *Ex Vivo* Upright Droplet Cultures of Whole Fetal Organs to Study Developmental Processes during Mouse Organogenesis

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Abstract

Investigating organogenesis *in utero* is a technically challenging process in placental mammals due to inaccessibility of reagents to embryos that develop within the uterus. A newly developed *ex vivo* upright droplet culture method provides an attractive alternative to studies performed *in utero*. The *ex vivo* droplet culture provides the ability to examine and manipulate cellular interactions and diverse signaling pathways through use of various blocking and activating compounds; additionally, the effects of various pharmacological reagents on the development of specific organs can be studied without unwanted side effects of systemic drug delivery *in utero*. As compared to other *in vitro* systems, the droplet culture not only allows for the ability to study three-dimensional morphogenesis and cell-cell interactions, which cannot be reproduced in mammalian cell lines, but also requires significantly less reagents than other *ex vivo* and *in vitro* protocols. This paper demonstrates proper mouse fetal organ dissection and upright droplet culture techniques, followed by whole organ immunofluorescence to demonstrate the effectiveness of the method. The *ex vivo* droplet culture method allows the formation of organ architecture comparable to what is observed *in vivo* and can be utilized to study otherwise difficult-to-study processes due to embryonic lethality in *in vivo* models. As a model application system, a small-molecule inhibitor will be utilized to probe the role of vascularization in testicular morphogenesis. This *ex vivo* droplet culture method is expandable to other fetal organ systems, such as lung and potentially others, although each organ must be extensively studied to determine any organ-specific modifications to the protocol. This organ culture system provides flexibility in experimentation with fetal organs, and results obtained using this technique will help researchers gain insights into fetal development.

Video Link

The video component of this article can be found at https://www.jove.com/video/53262/

Introduction

Organ regeneration *in vivo* in humans is very limited; therefore, tissue engineering, the development of tissues and organs from individual cells donated by a host, is becoming an attractive potential therapy for organ replacement. However, for this therapeutic strategy to be successful, factors and cellular interactions involved in morphogenesis of the organ must be thoroughly studied and well-understood. Due to the inability to study development of specific organs with traditional approaches, researchers have turned to alternative whole embryo or whole organ cultures. Kalaskar *et al.*[1] have shown that *ex vivo* whole embryogenesis culture yields comparable results (in 58% of cultured embryos) to *in utero* development, suggesting that *ex vivo* culture methods are a feasible alternative for organogenesis studies.

An individualized organ culture system, such as this *ex vivo* droplet culture system, allows for whole organ analysis independent of systemic effects, while permitting manipulation of a specific signaling pathway or cellular interactions via addition of pharmacological reagents or antibodies. Traditionally, the study of fetal organ development has been limited to transgenic and knockout mouse technologies, in addition to pharmacological reagents delivered maternally. However, there are technical issues involving these techniques and treatments *in vivo*; most concerns revolve around the effects of influencing various organs simultaneously which often results in embryonic lethality. An additional concern of studies manipulating fetal development pharmacologically is the maternal effect of drugs on embryonic development *in utero* (*e.g.,* maternal metabolism of the drug before it reaches the embryo) and if such reagents can pass through the placental barrier.

The whole organ culture technique described here was adapted from a protocol first described by Maatouk *et al.*[2], in which whole fetal gonads are incubated in *ex vivo* upright droplet cultures. One significant advantage of culturing fetal gonads is that small-molecule inhibitors can readily access the whole organ by simple diffusion. DeFalco *et al.* have shown that utilizing this *ex vivo* droplet culture method in conjunction with small-molecule inhibitors can be used to study signaling processes and interactions occurring during gonad development[3]; these processes would be difficult to examine *in vivo* due to technical challenges (*e.g.*, passage of drugs through the placenta or lethality of affecting multiple organs using genetic or pharmacological approaches).

The droplet culture is not only an improvement in certain aspects over *in utero* experimentation, but also it is an improvement over *in vitro* and *ex vivo* systems as well. The use of cell lines to study morphogenesis is extremely difficult because they lack the diverse cell types, lack critical

extracellular matrix (ECM) components that permit the formation of organ architecture, and can exhibit artifacts in signaling cascades. Although tissue engineering has made significant improvements in creating scaffolds simulating ECM, the lack of knowledge with regard to which signals are required by each cell type during organogenesis makes it challenging to build an organ system *in vitro*. Other *ex vivo* systems have been previously established to study organogenesis, or more specifically morphogenesis, and have been very successful for live imaging of fetal organs in agar[4], transwells[5], filters[6], and other scaffold matrices[7,8]. The advantage of the droplet culture system is that it allows the study of morphogenesis by providing the ability to utilize less reagents, which are often expensive, but also giving the organ surface tension, which is important for growth and signaling capabilities[9].

In the mouse, initial testis morphogenesis takes place between embryonic (E) stages E11.5 and E13.5; these stages comprise the optimal time window for examining factors that influence sex-specific differentiation. Among the critical processes that occur during testis formation are the generation of testis cord architecture and the formation of a testis-specific vascular network. Utilizing this *ex vivo* whole organ droplet culture system, one is able to alter male-specific vascularization and inhibit testis morphogenesis through the use of a small-molecule inhibitor that blocks the activity of the receptors for vascular endothelial growth factor (VEGF); VEGF-mediated vascular remodeling is critical for testis development[10-12]. This technique can successfully be applied to other organs and can target specific time windows of development. Whole-mount organ imaging allows the visualization of vital structures as well as structural and cellular changes resulting from the administration of various inhibitors. Importantly, this system is advantageous in that the researcher can bypass potential confounding effects from maternal drug administration or systemic disruption during *in vivo* targeted gene strategies. Thus, this whole organ *ex vivo* droplet culture system can significantly improve the ability to understand the interactions and signaling which occur specifically within particular organs during fetal development.

Protocol

All mice used in these studies were CD-1 mice obtained from Charles River Laboratories. Previous culture experiments have also been performed on other strains, such as C57BL/6J (data not shown), but any strain can be used. Pregnant adult females were approximately 2-3 months old and were euthanized via CO_2 inhalation followed by cervical dislocation and bilateral thoracotomy prior to embryo removal. Mice were housed in accordance with NIH guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

1. Preparation of Instruments, Culture Media, and Dishes

1. Prepare a 70% ethanol solution. Autoclave deionized water (for making humidified chambers and for making/diluting solutions). Sterilize dissection tools (forceps, scissors, and 27 G needle syringes) by spraying down with 70% ethanol.
2. Prepare 10X phosphate-buffered saline (PBS) containing calcium and magnesium (80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, 6.75 ml of 1 M $CaCl_2$, 3.75 ml of 1 M $MgCl_2$). Stir to dissolve in 1 L sterile autoclaved water and then filter with filter paper. Dilute 10-fold with water to make 1X PBS.
3. Heat inactivate fetal bovine serum (FBS) at 55 °C for 30 min and filter sterilize with a 0.22 µm syringe filter.
4. Prepare 38 ml 1X complete culture medium (called complete DMEM, cDMEM), consisting of Dulbecco's Modified Eagle Medium (DMEM), 10% filtered heat-inactivated FBS and 1/100 dilution of penicillin-streptomycin stock. This usually should be used fresh, but can be stored at 4 °C for 1 month.
5. Reconstitute the VEGFR Tyrosine Kinase Inhibitor II (TKI II) in DMSO for a stock concentration of 5 mg/ml, and dilute stock with cDMEM to make a working solution. The final concentration for this study is 1.875 µg/ml in cDMEM. According to the company's recommendations, stock solutions are stable for up to 6 months at -20 °C. As for working solutions, make fresh and use on the same day.
   Note: The concentration of this drug in the working solution should be two-fold higher than the desired final concentration (since it will be diluted two-fold in the droplet). At the same time, prepare the same volume of cDMEM containing the same volume of DMSO for the "control" treatment.
6. Prepare the tail digestion reagents (50 mM NaOH and 1 M Tris-HCl [pH 8.0]) separately in distilled water.
7. Make a 25 µM stock of the XY PCR primers (XY Forward 5'-TGA AGC TTT TGG CTT TGA G-3' and XY Reverse 5'-CCG CTG CCA AAT TCT TTG G-3') together in nuclease-free water.
8. Prepare 50X TAE buffer (242 g Trizma Base, 57.1 ml Glacial Acetic Acid, 100 ml 0.5 M EDTA, pH 8.5, and add water to 1 L final volume). Prepare 1X TAE Running buffer (20 ml 50X TAE diluted with water to 1 L total volume).
9. To create a 2.5% agarose solution (0.625 g agarose, 0.5 ml 50X TAE Buffer, 24.5 ml water), heat agarose solution in microwave until boiling, then let cool until about 55-65 °C (able to touch). Once cool, add 2 µl of 1% ethidium bromide solution, and pour gel.
   CAUTION! Ethidium bromide is a mutagen and teratogen; please use nitrile gloves and proper safety protocol when handling. Alternatively, other potentially less toxic gel resolving agents or dyes can be used.
10. Prepare Blocking Solution (PBS with 0.1% Triton X-100, 10% fetal bovine serum [FBS], and 3% bovine serum albumin [BSA]) for immunofluorescence.
11. Prepare Washing Solution (PBS with 0.1% Triton X-100, 1% FBS, and 3% BSA) for immunofluorescence.
12. Prepare PBTx (PBS with 0.1% Triton X-100) for immunofluorescence.
13. Prepare the incubation chambers for culture. Fill large (100 mm diameter) petri dishes with 35 ml autoclaved water. Place near where dissection and cultures will be performed.

2. Isolation of Fetal Testes from *Mus musculus*

1. Check the female mouse for vaginal plugs each morning. Noon on the day at which the vaginal plug is detected is considered E0.5. Euthanize the pregnant female mouse at E11.5, in a manner consistent with approved animal protocols.
2. Spray down mouse abdomen with 70% ethanol.
3. Open the belly skin and peritoneum with a V-shaped incision using fine scissors and pull back flap of tissue to expose interior organs.

4. Locate the ovaries at both ends of the uterine horn. Using scissors, cut at the ovary and the connective tissue to separate the uterus containing the embryos from the mother's body.
5. Open the uterine wall by gently cutting the side opposite the placentae, exposing the yolk sacs. Be careful not to puncture yolk sac to avoid damaging or losing embryos.
6. Cut near the placentae to remove the embryo-containing yolk sacs and place them into a dish containing PBS with calcium and magnesium. The presence of calcium and magnesium ions will help support cell adhesion molecules to maintain tissue architecture and prevent the tissue from sticking to the dissection tools.
7. With forceps, remove the yolk sac and amnion from the embryo by carefully puncturing the yolk sac to create a hole in which the embryo can slide out. Once the embryo is outside of the yolk sac, cut the umbilical cord.
8. From this point forward, use a microscope located in a sterile tissue culture hood. Remove the head of embryo and discard by pinching with forceps on either side of neck.
9. Remove the tail and place into new microcentrifuge tube for XY PCR analysis to determine sex of embryo.
   Note: While it is optimal to culture gonads as soon as possible after harvest, it is also possible to remove tail DNA and perform XX/XY genotyping prior to culture, so that the sex of each embryo is known and only the desired sex needs to be cultured. While the genotyping is being done, embryos can be kept separated (so that they can be tracked) at 4 °C or on ice until ready for culture. One caveat is that this period outside *in utero* or culture conditions may potentially affect viability for culture or subsequent development during culture.
10. Secure the embryo in a supine position against the bottom of the culture dish by pinning the armpits of embryo with one pair of forceps (usually the forceps held in the weak hand). Maintain these forceps in place to hold the embryo still during the entire dissection process.
11. With other pair of forceps, remove skin covering abdomen and gently remove liver, intestines, and other organs to expose back body wall.
12. As the gonads will be located on the back body wall on either side of the dorsal aorta, a large blood vessel running along the midline of the body, scoop underneath the urogenital ridge with closed forceps and remove the urogenital ridge by opening the forceps and lifting up. As the gonads will be loosely attached to the wall, be careful not to pull up too fast without removing these connections or the gonads may stretch, causing damage to the organs.
13. Move the urogenital ridge into cDMEM in a dish to acclimate tissue to media.
14. Separate the gonad-mesonephros complex from the rest of the urogenital ridge using 27 G needles. Use one needle to cut by pressing down, and use the other to guide the tissue correctly to allow optimal separation. Avoid using a sawing motion against the dish bottom, as sharp needles will create shards of plastic that can stick to the tissue. Make sure to keep the mesonephros completely attached to the gonad.

3. Culturing of Gonad with a Small-molecule Inhibitor

1. Set two 20 µl pipettes to 15 µl each. Cut about 1-2 mm off one of the barrier pipette tips using a clean, sterilized razor blade for transfer of the gonads and addition of control cDMEM, while the other pipette will be used solely for drug-treated cDMEM.
2. Line up gonads with their long axis parallel to the pipette tip so they can easily be pipetted using the cut-off tip. Be careful of gonads sticking to the inside of the pipette tip.
3. Label one side as the control droplet and the other as the drug-containing droplet. Only two droplets can fit on a 35 mm dish lid. Make sure that labels on lids match the labels on tail-tissue-containing microcentrifuge tubes.
4. Pipette 15 µl of cDMEM containing a single gonad into a droplet in the lid of a small (35 mm) culture dish (use only the lids, as the bottoms are too tall to fit within a humidified petri dish chamber). Place two separate gonad-containing droplets on either side of the dish, well-separated from one another. Check under the microscope to ensure that the gonads have been transferred to the droplets.
5. To the droplet designated as the control, add an additional 15 µl of cDMEM containing DMSO (made in step 1.5) to make a droplet with a 30 µl total volume. Use only the "control" pipette that will not contact the drug.
6. To the other droplet (drug-treated sample), add 15 µl of TKI-II-containing cDMEM to make a droplet with a 30 µl total volume. Use only the pipette designated for drug-treated samples.
7. Using the pipette, spread out the droplets in an expanding circular pattern until they are about 15-18 mm in diameter and the gonad is located roughly in the middle. Orient the gonad so that it lies on its side and the gonad and mesonephros are easily distinguishable. Orient the lung so that the two lobes lay flat.
   1. Although droplet diameter may vary slightly, ensure that gonads are not floating and are held in place by surface tension. Make sure that droplets do not touch each other or the side of the lid. Without the surface tension, the organs will grow onto the lid during the culture and flatten, thus distorting organ morphology.
8. Carefully place the culture dish lid containing the droplets upright onto the surface of the water in the large (100 mm) humidifier dish. Ensure there are no bubbles trapped underneath the bottom of the lid and that it is lying flat on the surface of the water. Do not invert the lid and be careful not to let any water touch the top of the lid where the droplets are located.
9. To create a small, humidified chamber, immediately cover with the lid of the larger humidifier dish to enclose the gonad cultures. Act as quickly as possible to minimize any evaporation of media. Ensure that the smaller dish has room between it and the lid of the larger dish to move around freely; otherwise, condensation may make a seal and block air exchange to the tissue.
10. Once two small lids are placed into the chamber, immediately place the chamber into the incubator. Incubate the droplet cultures for 48 hr in a humidified CO_2 incubator at 37 °C.

4. Polymerase Chain Reaction for Determining the Sex of Embryos

1. Add the embryonic tails to bottom of a 1.5 ml microcentrifuge tube.
2. Add to each tube 200 µl of 50 mM NaOH.
3. Place at 95 °C for 15 min or until tissue is completely dissolved.
4. Add 50 µl of 1M Tris-HCl and vortex lightly and briefly.
5. In a 1.5 ml microcentrifuge tube, make a fresh PCR master mix by multiplying each volume by the total number of samples: 0.5 µl 25 µM Primer solution, 2.5 µl 10X Taq Buffer, 0.5 µl 10 mM dNTPs (nucleotides), 19.3 µl nuclease-free water, 0.2 µl DNA Taq polymerase. Mix well.
6. Add 23 µl of PCR master mix to PCR tubes containing 3 µl lysate of digested tails.
7. Flick tubes to mix them, then perform a short spin to bring the samples to the bottom of the tube.

8. Run XY (Short) PCR program (**Table 1**).
9. Load the samples (with 5x dye) and ladder in a 2.5% agarose gel in 1X TAE buffer.
10. Run the agarose gel electrophoresis until XX and XY bands can be resolved.
11. Image the gel with UV light and capture image.
12. Analyze the X-chromosome-specific vs Y chromosome-specific bands (X chromosome=331 base pairs [bp] and XY=302 bp)[13]; XY (male) samples will have two bands of 331 and 302 bp, whereas XX (female) samples will appear as a single 331 bp band.

5. Whole Mount Organ Immunofluorescence

Day 1:

1. Remove the gonads from the culture using a 1,000 µl pipette with a cut-off tip. If desired, they may be placed in a dish of PBS to wash off media and reagents. Place into PBS in a 0.5 ml microcentrifuge tube. The smaller tube size will conserve reagents and make it easier to keep track of samples in the tube.
    1. Be sure to keep control and treated samples, as well as XX and XY samples, in separate tubes and remove them from culture with separate sets of pipettes to avoid potential drug contamination.
2. Wash the gonads twice with PBS. From this point on, this protocol can use 1X PBS lacking calcium and magnesium. To wash them, allow the gonads to sink to the bottom of the tube (by gravity) and then remove the liquid above. Be careful not lose gonads by pipetting too close to them.
3. Remove as much PBS as possible from the tube. Add 250 µl of 4% paraformaldehyde in PBS containing 0.1% Triton X-100, and let incubate O/N at 4 °C on a rocker. Alternatively, this fixation can be done for 2 hr at 4 °C on a rocker. CAUTION! Paraformaldehyde is a toxic substance, so follow safety protocol when handling.

Day 2:

4. Rinse the gonads twice in 250 µl PBTx at RT.
5. Wash the gonads 3 times with 250 µl PBTx for 10 min each at RT on a rocker.
6. Block the gonads at least 1 hr in 250 µl Blocking solution at RT on a rocker.
7. Stain the gonads O/N at 4 °C on a rocker in 250 µl primary antibody diluted in Blocking Solution.

Day 3:

8. Rinse the gonads twice in 250 µl Washing Solution.
9. Wash the gonads 3 times with 250 µl Washing Solution for 10 min each at RT on a rocker.
10. Block the gonads 1 hr in 250 µl Blocking Solution at RT on a rocker.
11. Cover the microcentrifuge tube with aluminum foil to protect fluorescent secondary antibodies from light.
12. Incubate the gonads 2-4 hr at RT on a rocker in 250 µl secondary antibody diluted in Blocking Solution containing Hoechst 33342. Alternatively, perform secondary antibody and Hoechst 33342 incubation O/N at 4 °C on rocker.
13. Rinse the gonads twice in 250 µl PBTx.
14. Wash the gonads 3 times in 250 µl PBTx for 10 min each at RT on a rocker.
15. Using a cut-off pipette tip, transfer gonads onto a slide with minimal liquid. Remove excess liquid with pipette, but make sure that tissue does not dry out.
16. Orient the gonads in desired fashion with forceps, and quickly add a drop of mounting media to mount the gonads on the slide. Make sure the mounting media coats all samples completely; it is critical to avoid letting samples dry out.
17. Use coverslips (number 1.5 style) of appropriate size to gently cover samples. Let mounting media spread to contact the entire surface of coverslip. If necessary, very lightly press on the corners of the coverslip so that there are no large air bubbles.
18. Seal the slides with clear nail polish around the edges to reduce evaporation of the mounting media. Store mounted slides in the dark at 4 °C.

Representative Results

The *ex vivo* droplet culture allows one to manipulate whole organs, such as the gonad, to study cellular interactions and dynamics. **Figure 1** demonstrates in a step-wise fashion how to prepare an E11.5 gonadal droplet culture. The first steps in the culture protocol include the initial removal of the embryo-containing uterus from the mother mouse (**Figure 1A and 1B**). After removal of the uterus from the mother, the uterine wall is cut and the embryos are liberated from the yolk sac into PBS for further dissection (**Figure 1C-E**). After the removal of visceral organs, the urogenital ridge is clearly visible along the back body wall of the embryo (**Figure 1F**) and is isolated (**Figure 1G**). The gonad-mesonephros complex is then dissected away from the urogenital ridge (**Figure 1H**), and is placed into droplet culture with small-molecule inhibitor (**Figure 1I,** left: “T” for treated) and without small-molecule inhibitor (**Figure 1I**, right: “C” for control). The small dishes containing the droplets are enclosed in a make-shift humidified chamber (**Figure 1J**) and incubated at 37 °C and 5% CO_2 for 48 hr.

After 48-hr incubation the cultured organs are removed, washed with PBS, and are subjected to a whole mount immunofluorescence protocol to assess the effectiveness of the culture and the drug treatment; alternatively, they can be processed for RNA extraction for gene expression analyses. Fetal whole gonad-mesonephros complexes (E11.5 and older) have a high survival rate when transferred to culture media immediately after a clean dissection and cultured under normal conditions (37 °C and 5% CO_2) for 48 hr (but they can be potentially cultured for longer if necessary). Comparisons of E11.5 gonads under brightfield microscopy at initial incubation versus after 24 or 48 hr of culture reveal a dramatic change in gonad shape and the appearance of stripe-like cord structures in the control XY gonad (**Figure 2)**. Therefore, after culturing for 48 hr, the development is comparable to that of E13.5 *in utero* gonads (**Figure 2**). Additionally, the E11.5 fetal lung grows and displays increased branching that normally occurs during this phase in development (**Figure 2**). The culture process generally results in smaller organs as

compared to *in utero*, most likely due to fact that the culture conditions are not as optimal for growth relative to the *in utero* environment (see Discussion).

Although the size of the organs resulting from the 48 hr culture differs from that of *in utero*-developed organs, *ex vivo* cultured organs show similar tissue architecture and can serve as reasonable surrogates (**Figures 3 and 4**). To characterize organ architecture and morphogenesis, markers that specifically label critical organ cell types were used, such as SOX9 for testis Sertoli cells and lung branching cells, E-cadherin for lung epithelial cells, PECAM1 for germ cells and vasculature, and cleaved Caspase 3 for apoptotic cells (**Figures 3 and 4**). *Sox9*, encoding a transcription factor, plays an important role in fetal organ proliferation, differentiation, and extracellular matrix formation. Therefore, in both organs, SOX9 is utilized as a common architectural marker that labels Sertoli cells located within the testis cords[14-16] and branching structures within the lungs[17].

Gonad cultures in particular recreate *in utero* morphogenesis effectively. The mouse gonad is specified at embryonic (E) stage E10.0 and is initially morphologically identical in XY (male) and XX (female) embryos. The expression of the *Sry (Sex determining region of Y chromosome*) gene in XY gonads starting at E10.5 drives major molecular and morphological changes that occur rapidly between E11.5 and E13.5 in the fetal testis[19], including: the specification of Sertoli cells, the supporting cell lineage of the testis; the formation of testis cords, which are comprised of Sertoli and germ cells and are the fetal precursors to adult seminiferous tubules; and major vascular remodeling. In male-specific vascular remodeling, endothelial cells released from a vascular plexus in the neighboring mesonephros migrate into the gonad to form a testis-specific arterial system[4,20]. Immunofluorescent analyses reveal well-developed testis cord structures and vasculature in E13.5 *in utero* testes relative to E11.5 testes (**Figure 3**). As the images in **Figure 3** show, the droplet culture system can recreate testis differentiation events *ex vivo*, as it is possible to visualize SOX9-positive Sertoli cells in XY gonads forming into tubule-like cords and vasculature forming throughout the organ. With respect to lungs, 48-hr culture results in increased branching of SOX9/E-cadherin double-positive epithelial branches over the course of 2 days (**Figure 4**). Furthermore, we see similar levels of apoptosis in control cultured and *in utero* gonads, while there is some increase in apoptotic cells in lungs in the same culture conditions (**Figures 3 and 4**), suggesting that the gonad is particularly amenable to the culture conditions.

Small-molecule inhibitors can be used to study organ development by affecting cellular localization, proliferation, and cell cycle status, as well as organ architecture and various signaling cascades. The *ex vivo* whole organ droplet method allows the researcher to administer pharmacological reagents easily to fetal organs in a very small volume of culture media. To examine the effects of vascularization and vascular remodeling on testis differentiation and morphogenesis, we used the small-molecule inhibitor TKI II, a reagent that disrupts testis vascular development[3] by blocking the activity of VEGF receptors; the formation of fetal testis architecture occurs in a vascular-dependent manner, acting through vascular endothelial growth factor A (VEGFA)[11,12]. While vascularization of the testis is critical for the export of testosterone that drives virilization of the embryo, it is also a major driver of testis cord morphogenesis: previous work has shown that when VEGFA signaling is blocked at or prior to E11.5, Sertoli cells fail to partition out from surrounding interstitial cells and no cord structures form[3,12]. The results shown here demonstrate that disruption of vascular remodeling in the fetal testis is effective in the droplet culture system, and subsequent defects in testis morphogenesis (*i.e.*, abnormal testis cord formation) can be visualized (**Figure 3**). It should be noted that PECAM1, a marker for endothelial cells, is also expressed by germ cells (**Figure 3**); this germ cell staining is an internal control that shows that lack of vascular staining in treated gonads is not due to technical reasons, and also demonstrates that other cell types such as germ cells are not affected by the drug treatment. Given that most initial testis morphogenesis takes place between E11.5 and E13.5, these stages are the optimal window for determining factors which influence sex-specific differentiation, in particular the role of VEGF and vascular remodeling in the gonad.

The use of TKI II during lung development between E11.5 and E13.5 shows that small-molecule inhibition of vasculature can be reproduced in another organ (**Figure 4**). These result shows the efficacy of the *ex vivo* fetal organ droplet culture model system and the ability to use small-molecule inhibitors to alter signaling pathways within organs. Given the ease, flexibility, and efficacy of this protocol, the droplet culture provides a suitable alternative for experimental questions regarding organ development that cannot be addressed *in vivo*.

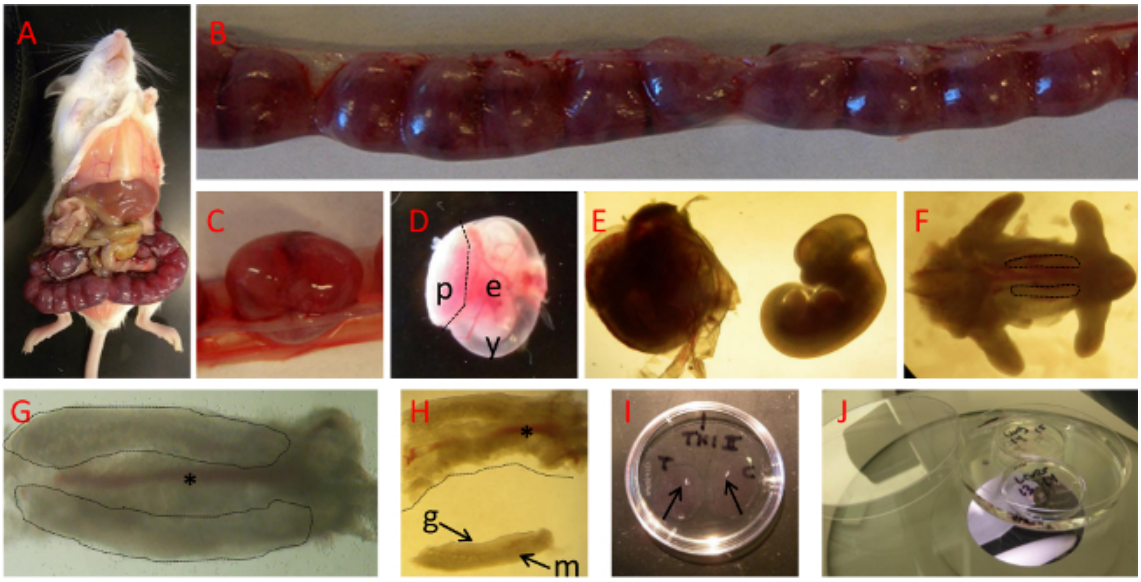

Figure 1. Steps in the *in vitro* whole organ droplet culture protocol. (A) Euthanized pregnant mouse with opened peritoneal cavity. **(B)** Uterine horn with embryos enclosed. **(C)** Close-up view of opened uterine wall with exposed embryo-containing yolk sac. **(D)** A single embryo (e) is attached to the placenta (p) enclosed within the yolk sac (y). **(E)** Embryo separated from placenta and yolk sac. **(F)** Dissected embryo with visceral organs removed and urogenital ridge exposed (gonads within urogenital ridge outlined in black). **(G)** Close-up of isolated of urogenital ridge (gonad-mesonephros complexes outlined in black). Asterisk denotes dorsal aorta in G and H. **(H)** Separation of gonad-mesonephros complex from urogenital ridge (dissection depicted by black dashed line). g, gonad; m, mesonephros. **(I)** Set-up of droplet cultures within 35-mm culture dish lid. Black arrows point to gonads within the droplets. T, treated; C, control. **(J)** Two droplet culture dish lids placed within an open humidified chamber. Please click here to view a larger version of this figure.

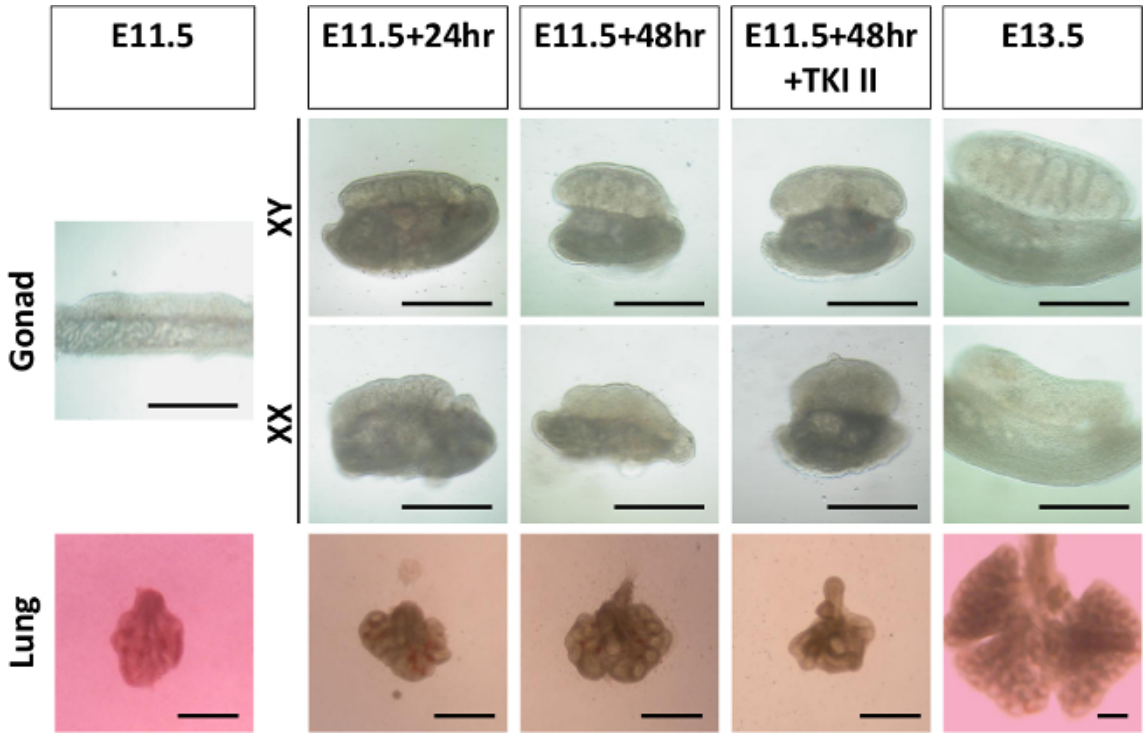

Figure 2. Development of *ex vivo* droplet cultured organs relative to *in utero* organs. Brightfield images of: E11.5 *in-utero*-developed organs (gonads and lungs) (first column); E11.5 organs after 24 hr (second column) and 48 hr droplet control culture (third column); E11.5 organs cultured for 48 hr with TKI II VEGFR inhibitor (fourth column); and E13.5 *in-utero*-developed organs (fifth column). Gonads are oriented with gonad (clear structure) above the mesonephros (opaque structure). E11.5 gonads are bipotential and appear morphologically identical in XY (male) and XX (female) samples. Scale bar, 500 µm. Please click here to view a larger version of this figure.

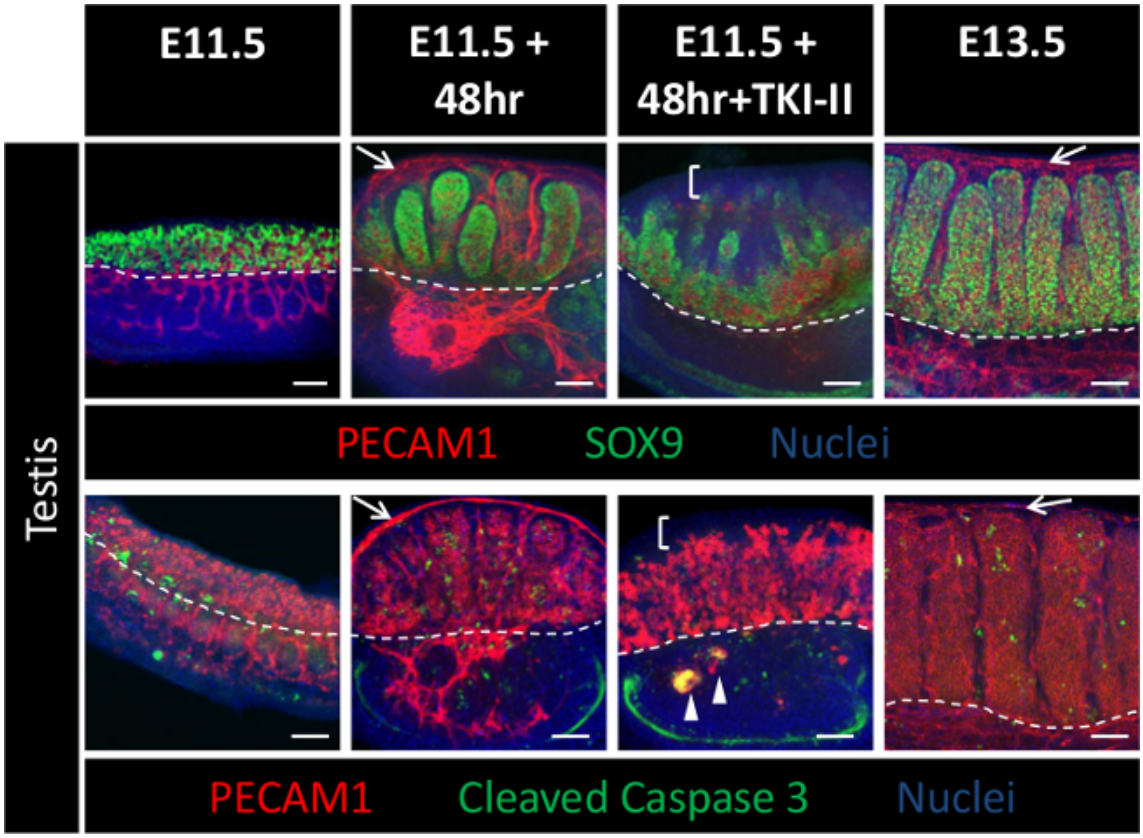

Figure 3. *Ex vivo* droplet culture testes are comparable in tissue architecture and cell death to *in-utero*-developed*counterparts.* Immunofluorescent images of: E11.5 *in-utero*-developed fetal testes (first column); control E11.5 testes after 48 hr of control *ex vivo* droplet culture (second column); E11.5 testes after 48 hr of *ex vivo* droplet culture with TKI II VEGFR inhibitor (third column); and E13.5 *in-utero*-developed testes (fourth column). Dashed lines indicate gonad-mesonephros border (gonad is oriented on top). SOX9 is a marker of Sertoli cells and is used to visualize testis cord architecture; PECAM1 is expressed in germ and vascular endothelial cells (therefore, remaining PECAM1 staining in treated gonads is almost exclusively germ cells); and cleaved Caspase 3 is a marker of apoptotic cells. Cultured control gonads showed similar testis cord formation, testis-specific vascularization (arrows throughout figure), and levels of apoptosis relative to *in-utero*-developed counterparts, while TKI-II-cultured gonads exhibited disrupted vasculature and abnormal testis cord morphogenesis. Brackets indicate surface domain of gonad where vasculature normally is present but is not detected in treated samples. Arrowheads point to dying vascular cell clumps in TKI-II-treated testes. Scale bar, 100 µm. Please click here to view a larger version of this figure.

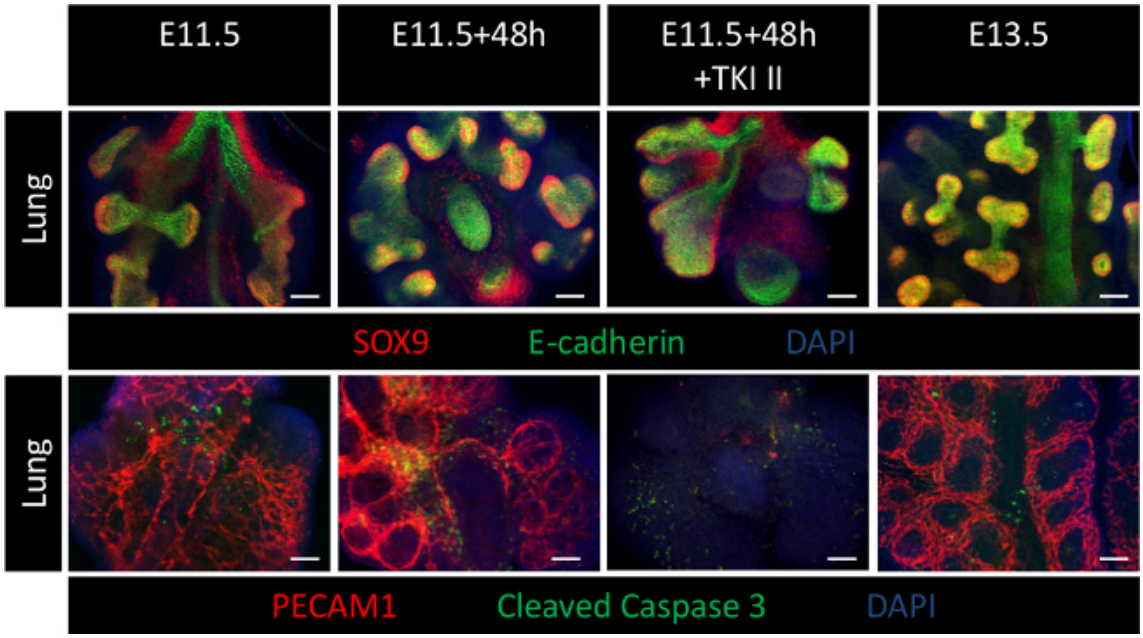

Figure 4. Other fetal organs (lung) cultured *ex vivo* in droplets are comparable to *in- utero*-developed organs. Immunofluorescent images of: E11.5 *in-utero*-developed lungs (first column); E11.5 lungs after 48 hr of control *ex vivo* droplet culture (second column); E11.5 lungs after 48 hr of *ex vivo* droplet culture with TKI II VEGFR inhibitor (third column); and E13.5 *in-utero*-developed lungs (fourth column). SOX9 labels branching cells; E-cadherin marks epithelial tubular structures; PECAM1 labels vascular endothelium; and cleaved Caspase 3 marks apoptotic cells. *Ex vivo* control cultured organs show similar architecture and expression of SOX9, E-cadherin, and PECAM1 in cultured organs compared to *in-utero*-developed organs, but varying amounts of cell death. Upon treatment with TKI II, vascular development is significantly reduced in lungs (as revealed by PECAM1 staining). Scale bar, 100 µm. Please click here to view a larger version of this figure.

Step	Temperature	Time	Number of Cycles
Starting	94 °C	2 min	1 cycle
Denaturing	94 °C	15 sec	35 cycles
Annealing	57 °C	30 sec	
Elongation	72 °C	30 sec	
End	72 °C	5 min	1 cycle

Table 1: XY PCR Analysis Program. Cycle parameters for “XY (Short)” PCR program used for XX/XY genotyping of embryos.

Discussion

This study demonstrates an *ex vivo* whole organ droplet method that has many potential applications for studying fetal development. This technique can be used for multiple organs, and allows the researcher to address biological questions that are difficult to examine using *in vivo* approaches due to inaccessibility of embryos and potential embryonic lethality. This culture method has additional benefits over other *in vitro* approaches such as mammalian cell lines: whole organs can be used, therefore maintaining critical intercellular interactions that are present *in utero*; and the culture volume is very small (~30 µl), thus using very small amounts of rare or expensive pharmaceutical reagents.

Critical aspects of the droplet culture protocol include: a) clean dissection of the organ. Clean dissection allows tissue architecture to be maintained and minimizes the number of exposed cut or damaged surfaces, which may be attracted to the culture dish surface and may disturb the culture; b) proper orientation of the organ within the droplet, in order to allow organ growth and morphogenesis; c) making the droplet the appropriate size, so that the droplet maintains the organ in place by surface tension but also does not dry out; d) using the correct droplet volume for the organ. Droplet volume should be determined empirically and according to organ size. However, 30 µl should be a reasonable starting point. e) choosing a relevant stage of development to address the biological question of interest; and f) maintaining a sterile culture environment, to avoid contamination which can damage the tissue within the droplet.

This paper mainly focuses on the E11.5-E13.5 gonad (*i.e.*, during initial sexual differentiation), but also shows that this protocol can be applied to other organs. Potential modifications of this protocol for other organs include: a) altering the droplet volume for a particular organ and/or stage of development. This culture method is applicable for all fetal stages for the gonad, but the volume should be adjusted for later fetal stages of larger organs. There is likely a limit to which fetal stages can be used for larger organs, given that simple diffusion will not allow nutrients or reagents to penetrate large tissues very effectively at later stages. It is therefore recommended to use this culture for fetal organs at the earliest stage possible for the experiment. b) adding exogenous growth factors, hormones, or other factors to the culture media. Certain organs may require a given protein or hormone to undergo normal morphogenesis; therefore, this protocol can be modified by the addition of such reagents to the culture media. c) adjusting the length of time in culture. While initial testis development can occur in as little as 24 hr, other organs may require longer periods of time in culture. If the droplets are kept sterile, the tissue may be amenable to up to 4 or even 5 days in culture. For longer

periods of culture (more than 48 hr), to remove ammonia or other built-up waste byproducts it may be necessary to refresh the media daily (with associated reagents) in the droplets by removing a set volume of the droplet and replacing that same volume with fresh media; for treated droplets, fresh media should contain the same concentration of drug/reagent as in the initial treatment. d) altering composition of media and/or associated reagents. Some tissues may require more or less of a given pharmaceutical reagent to elicit a desired effect. It is recommended to try a serial dilution of concentrations and assessing cell death (via cleaved Caspase 3 staining) to find an optimum concentration that will block/activate the desired pathway but not induce significant cell death in the cultured organs. e) the use of internal controls. Since organs such as the gonad come in pairs, one gonad can serve as an ideal internal control for the contralateral treated gonad. Other organs such as liver do not come in pairs; if the mouse strain used is rare and samples are limited, it is possible to dissect the organ in half and use each half for control and treated samples. One must keep in mind that organs, even those that come in pairs, may show asymmetry (such as different number of lobes for each side of the lung), so one must take note of which side of the organ is being used for control and treatment samples.

While droplet cultures can potentially recreate virtually all aspects of *in utero* development, there are some limitations to this technique. One is that droplet cultures, and *ex vivo* culture techniques in general, result in consistently slower growth rates relative to *in utero* development[1,5]; this is likely due to a number of factors, such as lower concentrations of required growth factors in culture media (and their access to the tissue) and limited neovascularization that occurs *ex vivo*. In addition to size of the organ, there is a concern that, if the explant is not properly oriented or the droplet is the incorrect size, tissue morphology may become flattened or distorted with a lack of a support structure that is provided by an agar bed in other protocols. However, this problem can be avoided with optimization of the protocol parameters. Another potential limiting factor is how well media and reagents can diffuse throughout the entire organ; while these cultured tissues have blood vessels, there is no perfusion of nutrients and oxygen through these vessels *ex vivo* due to lack of blood flow, so instead diffusion becomes a factor. This limitation is especially evident in postnatal testis cultures, in which *ex vivo* spermatogenesis is well-sustained in the periphery of the explant but the center-most portion of the tissue (*i.e.*, farthest from the media) degenerates[21-23]. Given those observations, it appears that size is a general limiting factor for whole organ culture or large-size explant protocols. However, general morphogenetic programs, such as branching morphogenesis in the fetal lung and *de novo* testis cord formation in the fetal gonad, still occur in droplet cultures. Therefore, these basic processes can still be studied using this technique.

This whole organ protocol was first described by Maatouk *et al.*[2], who adapted it from the previous agar-based culture method by Coveney *et al.*[4] The benefit of culturing organs *ex vivo* via droplets rather than in agar wells is that it uses at least a 10-fold reduced culture volume, thus conserving reagents; additionally, the droplet method does not involve pre-incubating the agar wells in reagent-containing media, which saves time and bypasses any concerns about the efficiency of reagents being delivered to the tissue through agar. While agar-based methods are generally thought to preserve more faithfully three-dimensional architecture of the tissue, proper orientation of the explant and optimization of culture conditions (see above) will ensure normal morphology of droplet-cultured organs.

These results demonstrate that *ex vivo* droplet cultured fetal gonads exhibit growth and morphogenesis comparable to that of *in-utero*-developed organs. This culture technique is not restricted to the gonad, but also can be applied to other fetal organs such as the lung. This *ex vivo* organ droplet method will be useful for studies of whole organ signaling and organ-specific cellular interactions, helped in part by the ability to image whole organs after culture and drug treatment. The study described here utilized a small-molecule inhibitor to investigate the influence of vascularization on morphogenesis, but a plethora of commercially available pharmacological reagents is available to study a multitude of signaling pathways and biological processes. Therefore, there are many potential future uses for this droplet culture method that will allow researchers to probe interesting and significant questions in developmental biology.

Disclosures

The authors declare that they have no competing financial interests.

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