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TITLE:

Generation of Porcine Testicular Organoids with Testis Specific Architecture using Microwell Culture

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KEYWORDS:

Testes, organoid, microwell, germ cells, Sertoli cells, organotypic

SUMMARY:

Here, we present a protocol for the reproducible generation of porcine testicular organoids with testis specific tissue architecture using the commercially available microwell culture system.

ABSTRACT:

Organoids are three dimensional structures composed of multiple cell types that are capable of recapitulating tissue architecture and functions of organs in vivo. Formation of organoids has opened up different avenues of basic and translational research. In recent years, testicular organoids have garnered interest in the field of male reproductive biology. Testicular organoids allow for the study of cell-cell interactions, tissue development, and the germ cell niche microenvironment and facilitate high throughput drug and toxicity screening. A method is needed to reliably and reproducibly generate testicular organoids with testis specific tissue architecture. The microwell culture system contains a dense array of pyramid-shaped microwells. Testicular cells derived from pre-pubertal testes are centrifuged into these microwells and cultured to generate testicular organoids with testis-specific tissue architecture and cell associations. Thousands of homogeneous organoids can be generated via this process. The protocol reported here will be of broad interest to researchers studying male reproduction.

INTRODUCTION:

In recent years, there has been a resurgence of interest in three-dimensional (3D) organoids. Different organs such as intestine[1], stomach[2], pancreas[3,4], liver[5], and brain[6] have been successfully derived into 3D organoid systems. These organoids have architectural and functional similarities to the organs in vivo and are more biologically relevant for study of tissue microenvironment than monolayer culture systems[7]. As a result, testicular organoids have started to garner interest as well[8-12]. The majority of methods reported so far are complex, non-high throughput[10] and require the addition of ECM proteins[8,10]. This complexity also leads to issues with reproducibility. A simple and reproducible method is needed that allows for the generation of testicular organoids with cell-associations that are like testis in vivo.

We have recently reported a system to address these requirements[12]. Using the pig as a model, we employed a centrifugal forced aggregation approach in the microwell system. In the microwell system, each well contains a large number of identical smaller microwells[13]. This allows for the generation of numerous spheroids of uniform size. The microwell system enabled generation of large numbers of uniform organoids with a testis-specific architecture. The system is simple and does not require addition of ECM proteins.

PROTOCOL:

NOTE: Testes from 1-week-old piglets were obtained from a commercial pig farm as by-product from castration of commercial pigs. Sourcing of testes was approved by the Animal Care Committee at the University of Calgary.

1. Preparation of enzyme solutions for tissue digestion

NOTE: Three different enzymatic solutions are needed, which include two different collagenase IV solutions (solution A, B) and a deoxyribonuclease I (DNase I) solution.

1.1. To prepare solution A, dissolve 20 mg of collagenase IV S (**Table of Materials**) and 40 mg of collagenase IV W (**Table of Materials**) in 25 mL of high glucose Dulbecco's Modified Eagle's Medium (DMEM). Then filter sterilize with a 0.22 µm filter. Add 0.4 mL of fetal bovine serum (FBS) to solution A to inhibit trypsin activity.

1.2. To prepare solution B, dissolve 80 mg of collagenase IV W (**Table of Materials**) in 40 mL of DMEM. Then filter sterilize with a 0.22 µm filter.

1.3. To prepare DNase I solution (7 mg/mL), dissolve 70 mg of DNase I in 10 mL of DMEM. Then filter sterilize with a 0.22 µm filter.

NOTE: The enzyme concentration for collagenase IV solutions outlined here, varies from the concentrations (2 mg/mL) stated in the original article[14]. The current protocol yields cells with slightly higher viability. However, cells isolated by both protocols produce organoids with identical tissue architecture.

2. Testis tissue enzymatic digestion

2.1. Collect the testes into a sterile beaker and wash with phosphate buffered saline (PBS) containing 1% penicillin/streptomycin (P/S). After washing, transfer the testes to a 100 mm tissue culture dish with PBS containing 1% P/S and remove the tunica vaginalis and epididymis using autoclaved scissors and forceps. Transfer the isolated testes to a new 100 mm dish and wash thoroughly with PBS containing 1% P/S.

2.2. To maintain sterility, use another set of sterile scissors and forceps to peel the testicular parenchyma out of the tunica albuginea. Cut the testes along the longitudinal axis directly under the tunica. Then peel the testes out of the tunica using two forceps and place into a new 100 mm dish containing of 1 mL DMEM with 1% P/S.

2.3. Mince the peeled testes with sterile scissors into 1-2 mm tissue pieces. After chopping, use sterile forceps to remove white fragments of connective tissue.

2.4. Transfer the minced tissue pieces into solution A and top it up to 50 mL with DMEM to obtain a concentration of 0.4 mg/mL for collagenase IV S (**Table of Materials**) and a concentration of 0.8 mg/mL for collagenase IV W (**Table of Materials**). Place solution A containing the tissue pieces into a 37 °C water bath for 30 min, gently invert the tubes every 5 min and check visually for the release of DNA.

2.4.1. Add 500 µL of DNase I if free floating DNA is observed. After 30 min, centrifuge the tube at 90 x *g* with brakes at 25 °C for 1.5 min and discard the supernatant.

NOTE: DNA would appear as a cloudy substance. When the tubes are shaken, the tissue pieces settle down whereas the DNA would stay afloat.

2.5. Add solution B to the tube and top up to 50 mL with DMEM to obtain a concentration of 1.2 mg/mL of collagenase IV W (**Table of Materials**). Place the tube into 37 °C water bath for 30 min and gently invert the tube every 5 min. Add 500 µL of DNase I if free floating DNA is observed.

2.6. After 30 min, centrifuge the tube at 90 *x g* with brakes at 25 °C for 1.5 min. After that discard the supernatant and wash once with PBS with 1% P/S.

NOTE: The discarded supernatants from both solution A and B will primarily contain interstitial cells.

2.7. Top up the tube with PBS up to 50 mL. Carefully collect the tubules from the top and place into a new 50 mL tube.

NOTE: The big undigested tissue fragments quickly settle at the bottom, while digested seminiferous tubules will remain in suspension. No big tissue fragments should be collected. This procedure can be repeated several times until the solution in the original tube is almost clear and

just undigested tissue fragments are remaining, which can be disposed.

2.8. Centrifuge the tubules at 90 *x g* with brakes at 25 °C for 1.5 min and discard the supernatant. Add fresh PBS and centrifuge again. Repeat this wash step twice.

2.9. After the last PBS wash, remove the supernatant and resuspend the seminiferous tubules in 5 mL of PBS. Then add 15 mL of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) to the tubules. Place the tube in 37 °C water bath and gently invert every 2 min. If a lot of free-floating DNA is observed, add 500 µL of DNase I with 5 mL of DMEM.

2.10. Evaluate the enzymatic digestion of tubules to single cells under the microscope (first after 5 min, then every 2 min). If mostly single cells can be detected, stop the reaction with 5 mL of FBS and filter through a 70 µm mesh and then through a 40 µm mesh.

NOTE: Care should be taken that the caps on the 50 mL tubes used for digestions (Solution A, B and 0.25% trypsin-EDTA) are tightened properly. If contamination in the water bath is a concern, paraffin film may be wrapped around the cap to ensure sterility.

2.11. Centrifuge the single cells at 500 *x g* with brakes at 25 °C for 5 min, resuspend in the enrichment medium (Dulbecco Modified Eagle Medium F/12 (DMEM/F12) containing 5% FBS and 1% P/S) and count the number of viable cells. This is the starting cell population. Fix 100 x 10^3 cells and perform immunocytochemistry for germ cell marker UCHL1 (Ubiquitin C-Terminal Hydrolase L1) as described[12] and determine the percentage of germ cells in this starting cell population.

NOTE: Germ cell marker Promyelocytic leukemia zinc finger protein (PLZF)[15] could also be used as a suitable alternative for UCHL1. In the starting cell population, the expected cell yield is around 700-800 x 10^9/g of tissue. The percentage of germ cells in this cell population should be 4-5%.

2.12. Place around 20 x 10^6 of this starting cell population in two ultra low attachment 100 mm Petri dishes (10 x 10^6 cells suspended in 10 mL of DMEM/F12 containing 1% P/S in each dish) for 2 days in a tissue culture incubator (37 °C, 5% CO_2, 21% oxygen). Perform the germ cell enrichment with the remaining cells.

NOTE: To ensure optimum viability and cell quality, while the germ cell enrichment and subsequent quantification takes place, the starting cell population is placed in culture in ultra low attachment tissue culture dishes for 2 days.

3. Germ cell enrichment

NOTE: The procedure described above yields primarily Sertoli cells and germ cells. Different adhesion properties allow for the separation of Sertoli cells and germ cells via differential plating.

3.1. Seed 20-25 x 10^6 cells of the starting cell population per 100 mm tissue culture dish in a total volume of 8 mL of enrichment medium (DMEM/F12 with 5% FBS and 1% P/S) for differential plating. Place the cells into an incubator (37 °C, 5% CO_2, 21% O_2) and after 1.5 h, ensure that the majority of Sertoli cells attach to the plate.

3.2. Collect and combine the supernatant (mainly containing germ cells) of 2 plates to one new 100 mm plate and place it back into the incubator. After 1 h, again combine the supernatant of 2 plates to a new 100 mm plate. Place the plates back into the incubator for overnight.

NOTE: The supernatants combined will primarily contain germ cells. The adhered cells that are discarded along with the plates are primarily testicular somatic cells such as Sertoli and peritubular myoid cells.

3.3. Collect the enriched germ cells in two fractions as follows.

NOTE: Enriched germ cells adhere differently, non adherent fraction and slightly adhered fraction. Both these fractions together form the enriched germ cell population

3.3.1. Non adherent fraction: collect the supernatant.

3.3.2. Slightly adherent fraction: wash the plates gently with PBS, treat with 2 mL of 1:20 dilution of 0.25% trypsin-EDTA for 5 min at room temperature, stop the reaction with 2 mL of enrichment medium and collect in the same tube as non adherent fraction.

NOTE: 0.25% Trypsin-EDTA needs to be diluted with PBS to produce a 1:20 trypsin solution.

3.4. Count the total number of cells using a cell counter, fix 100 x 10^3 cells and perform immunocytochemistry for germ cell marker UCHL1 as described[12] and determine the percentage of germ cells in this enriched cell population.

NOTE: The percentage of germ cells in this cell population should be at least 60-70%. Since the immunocytochemistry for quantification would require 1 day, the enriched cells should be placed in an ultra low attachment 100 mm Petri dish with DMEM/F12 supplemented with 1% P/S for the duration to ensure optimum cell quality and viability.

4. Preparation of cells for seeding

4.1. To harvest the starting cell preparation, collect the supernatant in a 50 mL tube from the ultra low attachment 100 mm dishes. Wash the plates vigorously with PBS to collect the adhered cells. No enzymatic digestion is needed.

NOTE: Some of the testicular cells, primarily Sertoli cells, can slightly adhere to the ultra low attachment plates.

4.2. Combine the starting cell preparation and enriched germ cells to obtain a working cell preparation containing 25% germ cells. Centrifuge at 500 *x g* with brakes at 25 °C for 5 min, discard the supernatant and resuspend the cells in organoid formation medium (DMEM/F12 supplemented with insulin 10 µg/mL, transferrin 5.5 µg/mL, selenium 6.7 ng/mL, 20 ng/mL epidermal growth factor, 1% P/S). Adjust the cell density to 2.4 x 10^6 per mL.

NOTE: Each well in the microwell plates used[13] in this protocol contained 1,200 microwells.

4.3. Use the formula below to calculate the number of cells per well as described below.

Number of cells per well = number of microwells x number of cells to be clustered for each organoid.

4.4. To generate organoids from 1, 000 cells each, seed each with (1,200 x 1,000 cells each=) 1.2 x 10^6 cells. Adjust the cell density in the cell suspension in a way that the cells are seeded in a volume of 0.5 mL of medium. For organoids formed from 1,000 cells each, use a density of 2.4 x 10^6 cells per mL (1.2 x 10^6 cells in 0.5 mL).

5. Preparation of microwells to receive cells

NOTE: To ensure that the cells do not adhere to the microwell surface, treat with a surfactant rinsing solution that is available for purchase by the manufacturer.

5.1. Add 0.5 mL of rinsing solution to each well. Ensure that no air bubbles are trapped in the well. To remove air bubbles, if any, centrifuge the plate at 2,000 x *g* with brakes at 25 °C for 2 min.

5.2. Observe the plate under a low-magnification inverted microscope, to verify that the bubbles have been removed from the microwells. If trapped bubbles are observed, centrifuge again at 2,000 x *g* with brakes at 25 °C for 2 min to remove any remaining bubbles.

5.3. Cover the plate with the lid and incubate for 30 min at room temperature. After the treatment is complete, remove the rinsing solution and immediately wash the plate with sterile water or PBS. Ensure that the plate do not dry out after the treatment with the rinsing solution.

6. Generation of testicular organoids

6.1. Add 0.5 mL of organoid formation medium (without any cells) to each well and centrifuge at 2,000 x *g* with brakes at 25 °C for 2 min to remove any trapped air bubbles. Observe the well under a low-magnification inverted microscope to verify that the air bubbles have been removed. If trapped bubbles are observed, centrifuge again at 2,000 x *g* with brakes at 25 °C for 2 min to remove any remaining bubbles.

6.2. Add 0.5 mL of the working cell suspension and gently mix by pipetting up and down.

Centrifuge at 500 x *g* with brakes at 25 °C for 5 min. Use an inverted microscope to verify that the cells have clustered within the microwells.

6.3. Transfer the plate into a cell culture incubator and culture for 5 days. Change half of the medium every other day.

6.3.1. To change medium without losing any organoids, touch the pipette tip to the wall of the well and lower gently to touch the meniscus and slowly draw the medium. Make sure to follow the meniscus as it drops down. To add fresh medium, touch the pipette tip to the wall of the well on the same side as the medium withdrawal and add fresh medium gently against the well wall, allowing it to slowly flow down the wall.

6.4. To recover the organoids, gently pipette the medium up and down using a wide mouth pipette. This would allow the organoids to come out of the microwells.

6.5. Collect the organoids, wash with PBS, fix and perform immunocytochemistry for germ cell marker (UCH-L1), Sertoli cell marker (GATA Binding Protein 4-GATA4), peritubular myoid cell marker (α-Smooth Muscle Actin-αSMA) and Leydig cell marker (Cytochrome P450-CYP450) as described[12] and visualize under a confocal microscope.

REPRESENTATIVE RESULTS:

Isolated cells from 1-week old porcine testes that were cultured in the microwells self-organized into spheroids (**Figure 1A, Figure 2**), with delineated and distinct exterior (seminiferous epithelium) and interior compartments (interstitium) (**Figure 1B, Figure 2**). The two compartments were separated by a collagen IV^{+ve} basement membrane. UCH-L1^{+ve} germ cells and GATA4^{+ve} Sertoli cells were in the exterior compartment on the basement membrane (**Figure 1B, Figure 2**). α-SMA^{+ve} peritubular myoid cells were localized along the inside of the basement membrane while Cytochrome P450^{+ve}Leydig cells were in the center of the interstitium (**Figure 1B, Figure 2**). This structure (**Figure 2**) is similar to in situ conditions (**Figure 1C**), where Leydig cells, peritubular myoid cells are located in the interstitium in the interstitial compartment; and germ cells, Sertoli cells are located at the seminiferous epithelium.

FIGURE AND TABLE LEGENDS:

Figure 1: Microwell-derived testicular organoids exhibit testis-specific tissue architecture with an inverted topography. (**A**) Porcine testicular cells at 0, 3, and 5 days of microwell culture. (**B**) Immunofluorescence images of testicular organoids identifying specific cell types: Sertoli cells (GATA4), basement membrane (Collagen IV); germ cells (UCH-L1); peritubular myoid cells (α-SMA); Leydig cells (CYP450). (**C**) Histological appearance (H&E) and schematic representation of 1-week-old pig testis. Specific cell types are indicated with corresponding arrows. Scale bars = 50 µm. This figure has been modified from Sakib et al.[12].

Figure 2: Schematic representation of organoid formation. Isolated single testicular cells are seeded and cultured in the microwells for 5 days to produce testicular organoids. This figure has been modified from Sakib et al.[12].

DISCUSSION:

We have established a simple method that allows the consistent, repeatable generation of large numbers of testicular organoids with tissue architecture that is similar to testis in vivo[12]. While the approach was developed using porcine testis cells, it is more broadly applicable also to mouse, non-human primate and human testis[12]. A number of different methods have been reported for producing testicular organoids[8-11]. Baert et al. generated human testicular organoids from adult and pre-pubertal samples by culturing testicular cells with extra cellular matrix (ECM) obtained by decellularization of adult human testes[11]. Although this model did not have distinct testicular morphology, organoids could secrete testosterone and cytokines[11]. Another human organoid model was reported by Pendergraft et al. using a hanging drop culture method that utilized solubilized testicular ECM proteins and organoids could produce testosterone[8]. Alves-Lopes et al. used a unique three-layer basement membrane matrix (e.g., Matrigel) gradient system to generate testicular organoids from rats[10]. The cells in this system generated tubular structures that had a blood testes barrier. The germ cells in these tubule-like structures were also responsive to retinoic acid stimulation[10]. All these methods are somewhat complex and challenging to use for high throughput assays. In contrast, the microwell system is simple, reproducible, can generate organotypic testicular organoids and can be used for high throughput drug and toxicity assays.

Although in the method outlined here we have used a cell density 1,000 cells/microwell (1,000 cells/organoid), this method can be used to generate organoids with as little as 125 cells/microwell[12]. This can be of particular use when experimenting with limited samples.

If the plate is not correctly balanced during centrifugation, uneven distribution of the cells may cause generation of organoids with variable size and shape. Care should be taken to balance the microwell plate properly. Once the cells have been seeded, attention should be paid to handling the plate during media changes. Shaking the plate too much when taking it out of the incubator or creating turbulence during media changes can cause some of the organoids to come out of the microwells and fuse with others[13].

The mammalian germ cell niche is complex and multicellular. The different cells in the testis such as Sertoli cells, peritubular myoid cells, Leydig cells all contribute to germ cell maintenance and fate[16,17]. Our organoid system can be used to manipulate different signaling pathways in specific cell types. A gene of interest can be up or downregulated in germ cells or other testicular somatic cells such as peritubular myoid cells, Sertoli cells. These modified cells can be then be combined with other testicular cells to generate modified testicular organoids, which can then be used to study the effects of the editing on ECM deposition, morphogenesis, cell-cell signaling, and spermatogenesis. Such modifications can also be performed to generate specific disease phenotypes for drug screenings. Compared to other methods for generation of spheroidal organoids such as culture in hanging drops or ultra low attachment U-bottom plates[8], using the microwell system has allowed for a testicular organoid model that is more accessible and allows for modifications. For example, germ cells may be genetically modified or treated with experimental factors and placed on a premade wild type organoid by simple centrifugation and

observed for downstream effects.

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DISCLOSURES:

Dr. Ungrin has a financial interest in the AggreWell technology as an inventor.

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Figure 1

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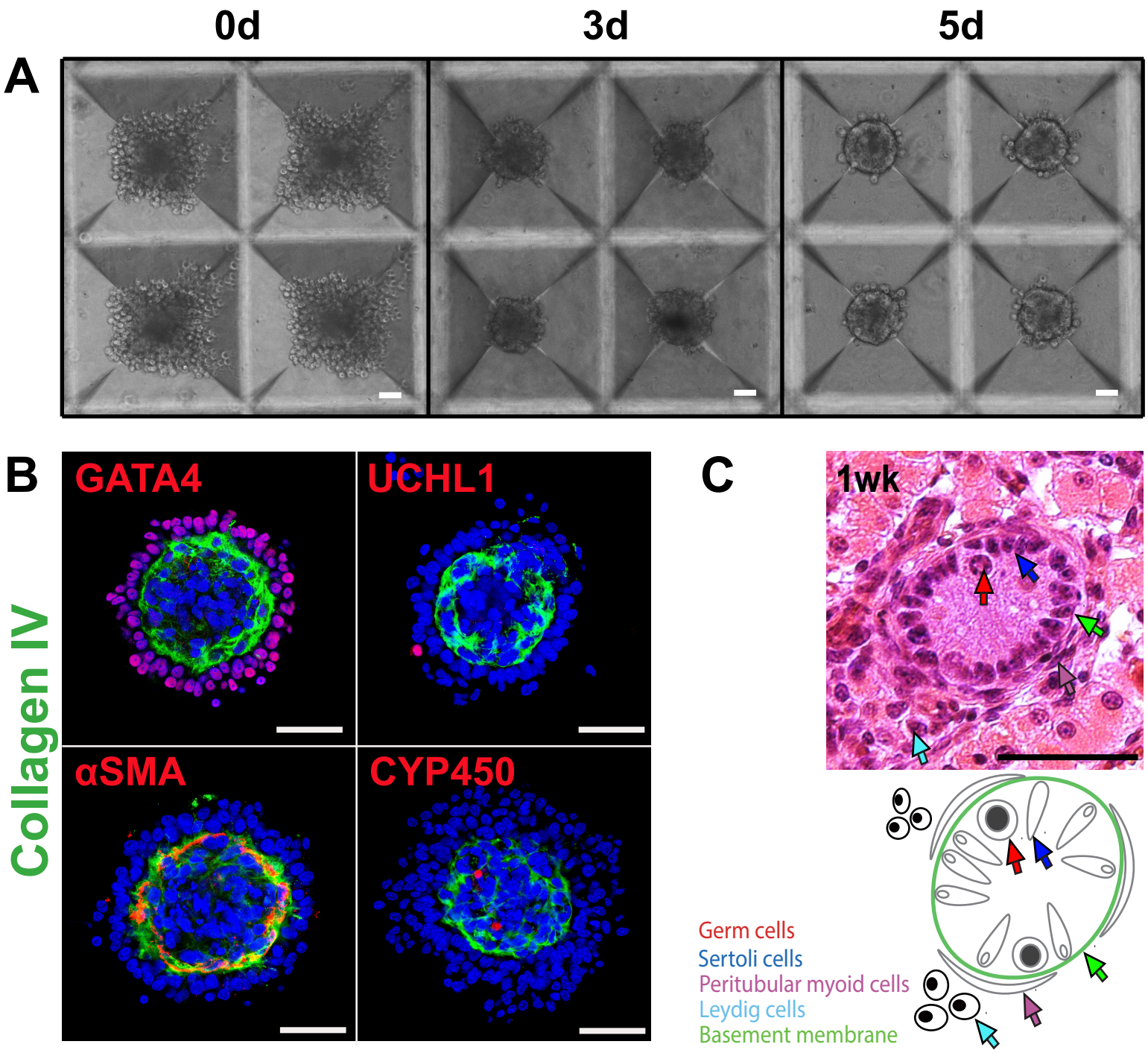

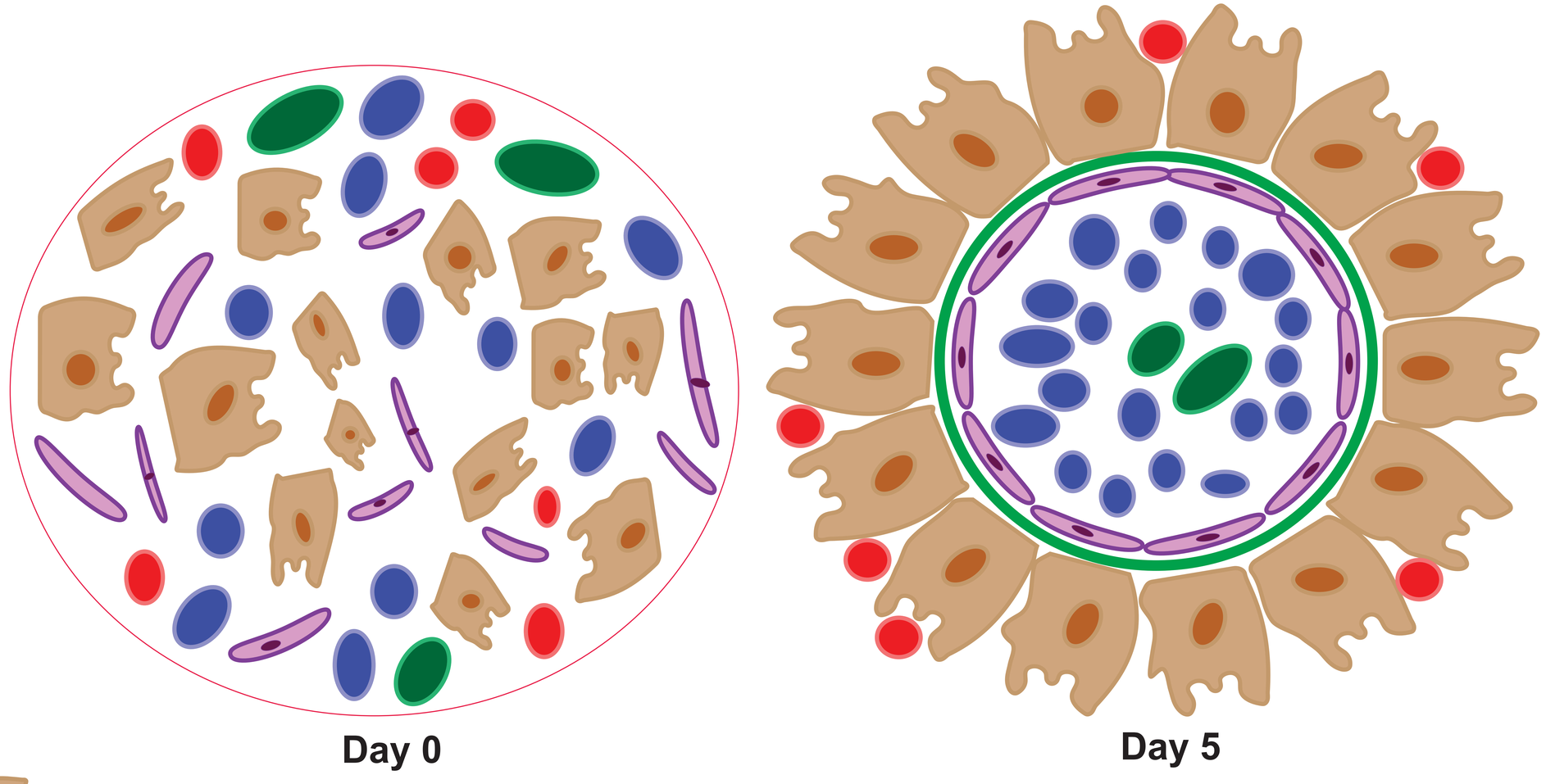

Name of Material/Equipment	Company	Catalog Number
100 mm ultra low attachment tissue culture dish	Corning	#CLS3262
100 mm tissue culture dish	Corning	#353803
Aggrwell 400	Stemcell Technologies	#34411
Anti-Adherence Rinsing Solution	Stemcell Technologies	#07010
Collagenase type IV from Clostridium histolyticum	Sigma-Aldrich	#C5138
Collagenase type IV Worthington	Worthington-Biochem	#LS004189
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	#DN25
Dulbecco's Modified Eagle's Medium/F12	Gibco	#11330-032
Dulbecco's Modified Eagle's Medium - high glucose	Sigma-Aldrich	#D6429
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	#D8537
Epidermal Growth Factor	R&D Systems	#236-EG
Falcon Cell Strainers 70 µm	FisherScientific	#352350
Falcon Cell Strainers 40 µm	FisherScientific	#352340
Fetal Bovine Serum	ThermoFisher Scientific	#12483-020
Insulin-Transferrin-Selenium	Gibco	#41400-045
Penicillin-Streptomycin	Sigma-Aldrich	#P4333
Porcine testicular tissue	Sunterra Farms Ltd (Alberta, Canada)	
Steriflip-GP Sterile Centrifuge Tube Top Filter Unit	Millipore	#SCGP00525
Trypsin-EDTA	Sigma	#T4049

Comments/Description
referred as Collagenase IV S
referred as Collagenase IV W

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