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Title: Scaffold-based and scaffold-free testicular organoids from primary human testicular cells

Running head: Human testicular organoids

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Abstract

Organoid systems take advantage of the self-organizing capabilities of cells to create diverse multi-cellular tissue surrogates that constitute a powerful novel class of biological models. Clearly, the formation of a testicular organoid (TO) in which human spermatogenesis can proceed from a single-cell suspension would exert a tremendous impact on research and development, clinical treatment of infertility, and screening of potential drugs and toxic agents. Recently, we showed that primary adult and pubertal human testicular cells auto-assembled in TOs either with or without the support of a natural testis scaffold. These mini-tissues harboured both the spermatogonial stem cells and their important niche cells, which retained certain specific functions during long-term culture. As such, human TOs might advance the development of a system allowing human *in vitro* spermatogenesis. Here we describe the methodology to make scaffold-based and scaffold-free TOs.

Keywords

Testis / organoid / primary cells / extracellular matrix / scaffold / *in vitro* spermatogenesis

1 Introduction

Organoid systems leverage the self-organization ability of cells to form tissue-specific multi-cellular structures. They are capable of recapitulating many important properties of a stem cell niche, thereby offering a promising new class of biological models. As organoids provide a more advanced *in vitro* tool which is amendable to extended cultivation and manipulation, they enable physiologically relevant experiments that cannot be conducted in animals or humans. *De novo* formation of functional testicular tissue from isolated mammalian testicular somatic and germ cells has been demonstrated *in vivo* and *in vitro* (1).

On the one hand, when immature rodent and pig testicular cells, obtained by enzymatic digestion, were transplanted under the dorsal skin of mice, the cells were able to rearrange into a functional endocrine and spermatogenic unit, supporting complete maturation and development of functional haploid male gametes (2, 3). Other reports confirmed this morphogenic ability of isolated testicular cells *in vivo* in ovine and bovine models (4, 5). On the other hand, various approaches aimed at *in vitro* sperm production using reassembled testicular somatic cells and germ cells either with or without scaffold support. In a mouse study, single cell suspensions were turned into aggregates in suspension culture and afterwards cultured at the gas-liquid interphase. In this scaffold-free approach, and thus only by relying on the auto-assembly properties of the testicular cell suspension, the seminiferous tubular structure was reorganized. However, spermatogenesis was arrested at the meiotic phase (6). More promising results were obtained when artificial 3D scaffolds were employed. Cultivation in a 3D collagen gel matrix system succeeded in generating spermatids from recombined immature rat testicular cells (7). Taking it further, the combination of a primary pre-meiotic testicular cell suspension and a 3D agarose or methylcellulose matrix resulted in morphologically normal mouse spermatozoa (8).

Recently, we published the first human *in vitro* study and reported the generation of scaffold-based and scaffold-free testicular organoids (TOs) with biomimetic activities using primary testicular cells from either adult or pubertal donors (Fig. 1). Although, the testicular cells were not able to reorganize into the typical testis cytoarchitecture during long-term culture, the niche cells maintained testis-specific functions, including *de novo* matrix production by elongating peritubular myoid cells, testosterone production by Leydig cells, inhibin b secretion and tight junction formation by Sertoli cells, and germ

cell renewal. Cytokine secretion profiling confirmed recapitulation of testicular processes *in vitro* (9). The necessity of the scaffold in TO formation is debatable, given that the spatial-temporal behaviour and hormone and cytokine secretion profiles of testicular cells in scaffold-free TOs were comparable. Nevertheless, the testis scaffold might still be of value for future experiments as tissue-derived scaffolds generally contain important regulatory cues (10, 11).

Human TOs take the development of a human *in vitro* spermatogenesis culture system a step forward. Such a system would be of great value in basic studies revealing the detailed mechanisms behind spermatogenesis and its disorders, and has an enormous clinical value in preventing and curing male infertility (12). Also, it has potential to become an industrial tool to find male contraceptives and to screen for reprotoxic compounds (13, 14). The methodology to prepare scaffold-based and scaffold-free TOs is summarized in Fig. 2 and described below.

2 Materials

2.1 Digestion of testicular tissue

1. Shaking water-bath
2. Petri dishes with a diameter of 100 mm
3. Sterile surgical tweezer and scissor or scalpel
4. Human testis biopsy
5. 50 ml conical tubes
6. KnockOut Dulbecco's Modified Eagle Medium (KnockOut DMEM)
7. Serological pipettes
8. Cell strainer with a mesh size of 100 µm
9. Cell strainer with a mesh size of 40 µm
10. Cell counter
11. Parafilm
12. Analytical balance

13. Centrifuge
14. Sterile enzyme mix 1: DMEM-F12 containing collagenase IA (1 mg/ml)
15. Sterile enzyme mix 2: DMEM-F12 containing collagenase (1 mg/ml), DNAse type I (0.5 mg/ml) and hyaluronidase type I-S (0.5 mg/ml)

2.2 Generation of organoids

1. Sterile 0.7% agarose in PBS (m/v)
2. Testicular scaffold discs (15)
3. Microwave
4. 24-well plate
5. Hanging culture inserts for 24-well plate
6. Culture medium: 10% (v/v) CTS KnockOut SR XenoFree medium, 1x GlutaMAX and 1% (v/v) penicillin-streptomycin diluted in KnockOut DMEM (9)
7. Incubator at 35°C with 5% CO_2 and humidified atmosphere

3 Methods

3.1 Digestion of testicular tissue

1. Preheat the water bath at 37°C.
2. Weigh the testicular tissue and prepare sterile enzyme mix 1 and 2 according to the following rule: 10 ml enzyme mix/1 g of tissue.
3. Add 10 ml of enzyme mix 1 to a petri-dish.
4. Place the testicular tissue piece into the enzyme mix and cut the tissue into fragments of $2mm^3$ with a sterile surgical scissor or scalpel.
5. Transfer the tissue pieces and the enzyme mix 1 with a serological pipette to a 50ml conical tube containing the remaining volume of enzyme mix 1 (*see* **Note 1**). Aspirate several times.

6. Seal the tube with parafilm and place in the pre-heated water bath (37°C) for 10 minutes at 120 RPM.

7. Take the tube out of the water bath and let the remaining fragments (tubules) sediment for 10 minutes.

8. Collect the supernatant (interstitial cells) in a new 50 ml Falcon tube and centrifuge at 300 *g* for 10 minutes. Resuspend the pellet in KnockOut DMEM with DNAse type I (0.5 mg/ml) and store until step 13 is reached.

9. Add enzyme mix 2 to the sedimented tissue pieces and aspirate several times (*see* **Note 1**).

10. Seal the 50 ml tube with parafilm. Place it in the shaking water bath (37°C) for 10 minutes at 120 RPM.

11. Take the tube out of the water bath and aspirate several times to mechanically help the digestion.

12. After re-sealing the tubes, shake for another 10 minutes in the water bath. Aspirate several times. If tubular fragments are still visible, extend the incubation time.

13. Run the cell suspensions from step 8 and 12 through a 100 µm-mesh sized cell strainer to remove large non-digested tubular fragments.

14. Run the obtained cell suspension over a cell strainer with a mesh size of 40 µm to obtain a homogenous single-cell suspension.

15. Centrifuge the tube at 300 *g* for 10 min and resuspend the cell pellet in KnockOut DMEM.

16. Count the cells.

3.2 Generation of organoids

1. Heat the 0.7% agarose in PBS (m/v) in the microwave for 1 minute (500W) until it is completely liquid.

2. Dilute the liquid agarose 1/2 with culture medium to obtain a 0.35% agarose solution.

3. Place the trans-well inserts in a 24-well plate.

4. Pipette 75µl of the 0.35% agarose mix in each trans-well insert (*see* **Note 2**).

5. Let the agarose solution jellify for 5 minutes at room temperature.

6. Place a testicular scaffold disc in each trans-well insert if scaffold-based TOs are needed (*See* **Note 3**). Skip this step to generate scaffold-free TOs (Fig. 2).

7. Spin the cells down at 300 *g* for 10 minutes and adjust the cell concentration to 10^6 cells/10µl culture medium.

8. Seed 10µl of cell suspension on top of the agarose gel (scaffold-free) or on top of the thin scaffold disc (scaffold-based) in each trans-well insert (Fig. 2).

9. Pipette 600 µl of culture medium in the wells of the 24-well plate by pipetting next to the inserts (*see* **Note 4**).

10. Place the 24-well plate with the inserts in the incubator at 35°C with 5% CO_2 and a humidified atmosphere.

11. Refresh the medium every 7 days.

4 Notes

1. It is recommended to not completely fill the tubes with enzyme mix. Leave the solution with tissue pieces some room for shaking.

2. A height of approximately 2 mm is advised for the agarose support. The presence of the agarose support is crucial in the formation of TOs for two reasons. On the hand, it prevents cell attachment to the membrane of the trans-well insert. On the other hand, the surface of the agarose hydrogel is non-adhesive and, consequently, promotes cell-cell attachment and 3D growth.

3. The protocol to produce thin testicular scaffold discs was described before (15).

4. Culture medium may be adapted according to the experimental set-up. Check regularly whether the level of culture medium reaches the cells in the trans-well insert to avoid dehydration.

5 Acknowledgements

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Figure Captions

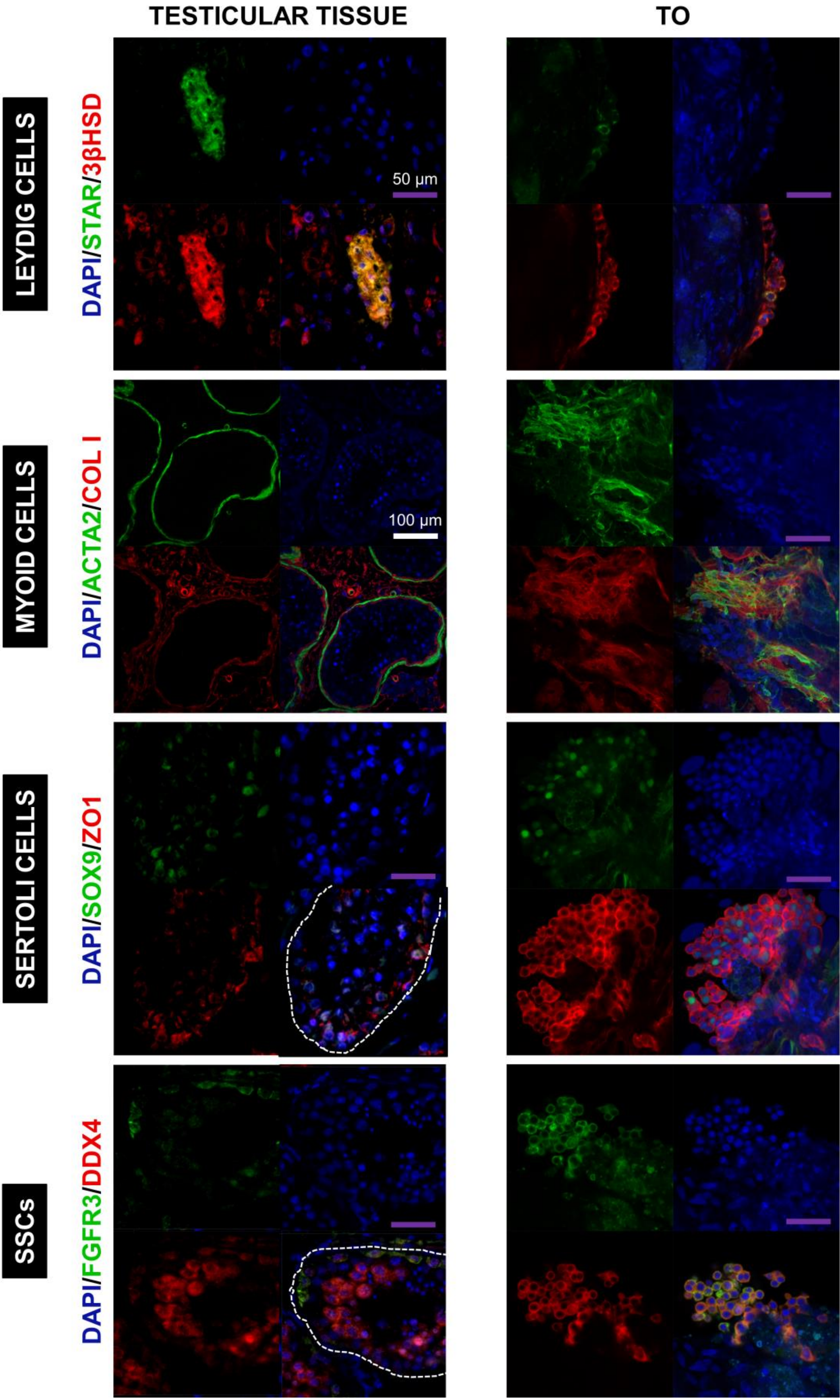

Fig. 1 Long-term cultured TOs contain spermatogonial stem cells (SSCs) and their niche cells. STAR$^+$/3β-HSD$^+$ cells represent steroidogenic Leydig cells (first row), SOX9$^+$/ZO-1$^+$ indicates tight junction-forming Sertoli cells (second row) and ACTA2$^+$/ COL I$^+$ cells are ECM-producing peritubular myoid cells (third row). Spermatogonial stem cells (SSCs) are stained by combining the spermatogonial marker FGFR3$^+$ with the germ cell lineage marker DDX4$^+$ (fourth row). The dotted lines delineate seminiferous tubules. Purple scale bars: 50 µm; white scale bars: 100 µm.

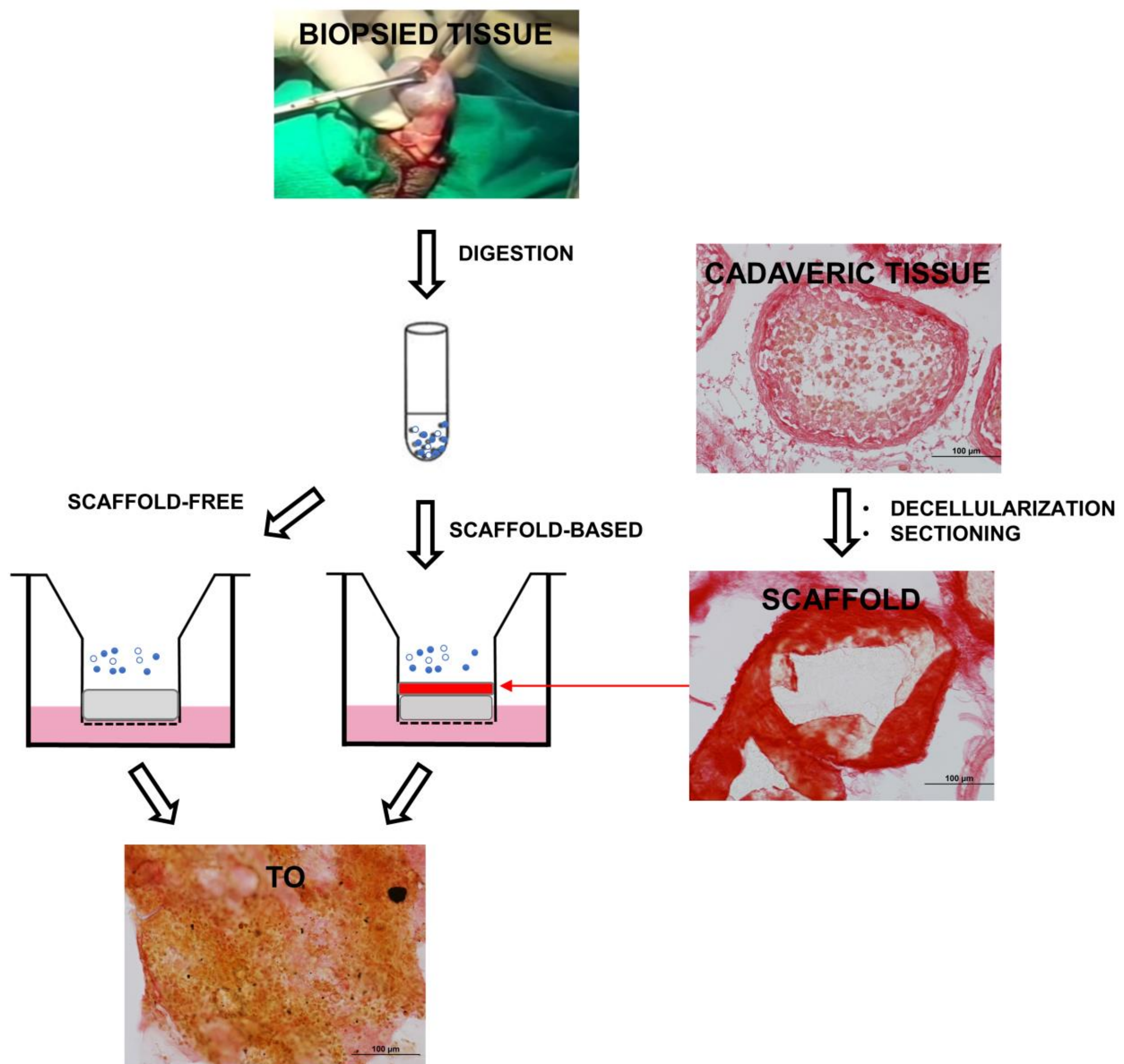

Fig. 2 Generation of scaffold-based and scaffold-free TOs. Testicular cells can be isolated from a testicular biopsy by enzymatic digestion. To form scaffold-based TOs, the cells are seeded into a trans-

well insert containing a testicular scaffold (red arrow) supported by an agarose hydrogel (grey rectangle). The scaffold is prepared as described before (15). Alternatively, addition of the scaffold to the trans-well insert can be circumvented to generate scaffold-free TOs. Black scale bars: 100 µm.