

TITLE: Creation of a bioengineered ovary: isolation of female germline stem cells for the repopulation of a decellularized ovarian bio-scaffold.

Running Title: Generation of a bioengineered ovary

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Abstract

Ovarian failure is the most common cause of infertility and affects about 1% of young women. One innovative strategy to restore ovarian function may be represented by the development of a bioprosthesis ovary, obtained through the combination of tissue engineering and regenerative medicine.

We here describe the two main steps required for bioengineering the ovary and for its ex vivo functional re-assembling. The first step aims at producing a 3D bio-scaffold, which mimics the natural ovarian milieu in vitro. This is obtained with a whole organ decellularization technique that allows the maintenance of microarchitecture and biological signals of the original tissue. The second step involves the use of magnetic activated cell sorting (MACS) to isolate purified female germline stem cells (FGSCs). These cells are able to differentiate in ovarian adult mature cells, when subjected to specific stimuli, and can be used them to repopulate ovarian decellularized bio-scaffolds. The combination of the two techniques represents a powerful tool for in vitro re-creation of a bioengineered ovary that may constitute a promising solution for hormone and fertility function restoring. In addition, the procedures here described allow for the creation of a suitable 3D platform with useful applications both in toxicological and transplantation studies.

Key Words: 3D bio-scaffold, Bioprosthesis ovary, Extracellular matrix, Female germline stem cells, Ovary bioengineering and repopulation, Whole- ovary decellularization.

1. Introduction

Infertility is a growing issue in modern society. According to the World Health Organization, it represents the fifth highest serious global disability, with an alarming incidence of one out of 1000 women, under the age of 30, rising to 1.0-1.5% in women younger than 40 years (*1, 2*). Ovary dysfunction and premature ovarian insufficiency (POI) represent the main causes of infertility and can occur as a result of an inherited condition, de novo mutation or from insults to the ovarian tissue, including viral infections and environmental factors (*2–4*). Furthermore, therapy-induced ovarian failure due to chemotherapy and radiation treatments, can cause oocyte and/or surrounding support cell apoptosis in cancer survivors (*5–9*). To date, several approaches have been developed and used in clinics to restore ovarian functions, including oocyte, embryo and ovarian tissue cryopreservation (*10–18*). However, since these procedures are largely devoted to cancer patients, the high risk of malignant cell re-introduction pose a severe limit to their use in clinical practices (*19, 20*). Development of a bioengineered ovary may provide a safe option in fertility restoration for all patients, including cancer survivors.

We here describe the two main steps required for the ex vivo creation of a bioprosthesis ovary and its functional assembling. The first step is based on a decellularization technique that produces an extracellular matrix (ECM)-based 3D-scaffold. The second one allows the repopulation of the decellularized bio-scaffold in order to create a functional in vitro bioengineered ovary. To date, many reports in the literature describe the regeneration in vitro of different organs using decellularized scaffolds (*21–30*). However, limited studies have been performed in the reproductive system, and, more specifically, in the ovarian tissue (*31, 32*). Indeed, the majority of decellularization protocols was specifically developed for ovarian tissue fragments and cortical slides (*33–35*), while the use of an entire ovary was limited to the bovine (*32*), the mouse (*36, 37*) and the porcine (*38*). The decellularization protocol here described leads to the creation of a whole-ovary 3D bio-scaffold preserves intact microarchitecture as well as ECM structures and components (*38*) (Figure 1). It combines the use of physical and chemical methods to remove cellular components and generate 3D

ovarian bio-scaffold that recreates in vitro the complex in vivo ovarian milieu, facilitating the necessary interactions between cells and their surroundings and ensuring a correct cell growth, differentiation and function (39).

The obtained whole-ovary bio-scaffold can be repopulated with a single cell type or with different ovarian cell populations, including fibroblasts, stromal and granulosa cells, or follicles. Among the several cell types present in the ovary, female germline stem cells (FGSCs) can be a promising candidate. Indeed, when subjected to specific stimuli, these cells are able to differentiate in ovarian adult mature cells and generate fully functional oocytes (40, 41). We here describe the isolation of FGSCs by using magnetic activated cell sorting (MACS). Obtained cells can be stably maintained in culture, undergo mitotic division and steadily express germline and pluripotency-related genes (Figure 2). Moreover, when used for the recellularization of decellularized ovary, FGSCs are able to rapidly migrate into the bio-scaffold, adhering and colonizing the ECM within 24 hours, and, during the subsequent days of culture, they increase in number and form cluster-like structures (Figure 3). Overall, the method here reported is simple, fast and highly efficient and paves the way for a possible in vitro ovarian tissue re-construction that may result advantageous for a general improvement of reproductive technologies and, possible future application to organ transplantation for hormone and fertility function restoring.

2. Materials

Prepare all solutions immediately before use (unless indicated otherwise).

2.1 Ovary collection

1. Porcine ovaries collected from a local slaughterhouse.
2. 500 mL plastic bottle.
3. Ice container.
4. Surgical scissor.

5. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na₂HPO₄ (8 mM) and 240 mg of KH₂PO₄ (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
6. Antibiotic/Antimycotic Solution.

2.2 Whole -ovary decellularization

1. 50 mL centrifuge polypropylene tubes.
2. Water bath.
3. Orbital shaker.
4. 500 mL plastic or glass bottle.
5. Deionized water (DI-H₂O).
6. 0.5% sodium dodecyl sulfate (SDS): dissolve 2.5 gr of SDS in 500 mL of DI-H₂O.
7. 1% Triton X-100: add 5 mL in 495 mL of DI-H₂O.
8. 2% deoxycholate: dissolve 10 gr of deoxycholate in 500 mL of DI-H₂O.

2.3 FGSC isolation

1. 4-well dish.
2. 100 mm petri dish.
3. Surgical scalpels.
4. 15 mL centrifuge polystyrene tube.
5. 50 mL centrifuge polypropylene tubes.
6. Centrifuge.
7. CO₂ incubator.
8. MACS cell separator.
9. 30-µm nylon mesh cell strainer.

10. Cell counting chamber.
11. Anti-SSEA-4 MicroBeads.
12. LS Column.
13. Inverted microscope.
14. 0.5% porcine gelatin: dissolve 0.5 gr of porcine gelatin in 100 mL of distilled water. Sterilize solution with autoclave.
15. Dulbecco's phosphate-buffered saline (PBS): dissolve 8 gr of NaCl (137 mM), 200 mg of KCl (2.7 mM), 1.44 gr of Na₂HPO₄ (8 mM) and 240 mg of KH₂PO₄ (2 mM) in 800 mL of distilled water. Adjust pH to 7.4. Add distilled water until volume is 1 L. Sterilize solution with autoclave and store at +4°C.
16. Hank's Balanced Salt Solution (HBSS) with phenol red.
17. 1 mg/ml collagenase (type IV): dissolve 5 mg of collagenase in 5 mL of HBSS with phenol red. Sterilize solution with 0.22 µm filter.
18. Trypsin-EDTA solution: dissolve 0.5 gr of porcine trypsin and 0.2 gr of EDTA 4Na in 1 L of HBSS with phenol red.
19. Fetal bovine serum (FBS).
20. FGSC culture medium: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), 40 ng/ml Human Stem Cell Factor (SCF) Recombinant Protein, 1% B27, 1 mM MEM Non-Essential Amino Acids, 0.1 mM b-mercaptoethanol, 10% Knock-out serum replacement (KO serum), 2 mM L-glutamine, 1% Antibiotic Antimycotic Solution (see Note 1).

3. Methods

All the procedures described below must be performed under sterile conditions. Instruments touching or in connection to the ovary have to be sterilized. Cell isolation must be carried out under laminar a flow hood and cell cultures have to be maintained at 37 °C during their handling using thermostatically controlled stages.

3.1 Ovary collection

1. Collect ovaries from gilts weighing approximately 120 kg.
2. Separate ovaries from fallopian tubes by cutting them with surgical scissor.
3. Transfer ovaries in cold sterile PBS containing antibiotic/antimycotic solution (5mL/500 mL) and transport them to the laboratory using ice container.

3.2 Whole-ovary decellularization

1. Wash ovary in fresh PBS, completely remove the PBS, place ovary in 50 mL tube and store organ at -80°C for at least 24 hours (see Note 2, Figure 4).
2. Thaw whole-ovary at 37°C in a water bath for 30 min.
3. Transfer whole-ovary in a bottle containing 500 mL of 0.5% SDS. Place the bottle onto an orbital shaker at 200 rpm and incubate for 3 hours at room temperature.
4. Remove SDS solution from the bottle containing the whole-ovary and add 500 mL of 1% Triton X-100. Incubate whole-ovary over-night at room temperature in 1% Triton X-100, using an orbital shaker at 200 rpm.
5. Remove Triton X-10 solution from the bottle containing the whole-ovary and wash ovary with 500 mL of DI-H₂O twice. Add for a third time 500 mL of DI-H₂O and extensively wash whole - ovary for 9 hours at room temperature, using an orbital shaker at 200 rpm.
6. Remove DI-H₂O from the bottle and add 500 mL of 2% deoxycholate for 12 hours at room temperature, using an orbital shaker at 200 rpm.
7. Remove deoxycholate and wash whole-ovary in DI-H₂O for 6 hours at room temperature, using an orbital shaker at 200 rpm. Changes DI-H₂O every 2 hours (see Note 3).

3.3 FGSC isolation

1. Add 500 μ L of sterile 0.5% porcine gelatin to 4-well dishes. Wait 2 hours to polymerize, maintaining them at room temperature.
2. Wash ovary twice in 50 mL of fresh sterile PBS at room temperature.
3. Place ovary into a 100 mm sterile petri dish and cut ovarian cortex into small pieces (approximately cubes of 1-2 mm²) using a surgical scalpel.
4. Wash fragments 4 times in sterile PBS at room temperature.
5. Enzymatically digest 20-30 fragments by 30 min incubation with 5 mL of 1 mg/ml collagenase (type IV) in 15 mL tube, with gentle shaking every 5 min.
6. Centrifuge digested tissue at 300 g for 5 min. Remove supernatant, add 5 ml of HBSS and resuspend digested tissue.
7. Centrifuge at 300 g for 5 min. Remove supernatant, add 5 ml of trypsin-EDTA solution and resuspend digested tissue. Incubate for 15 min at 37°C.
8. Remove the 0.5% porcine gelatin excess for the 4-well dish and let the dish open under laminar flow hood to dry (see Sect. 3.3, step 1).
9. Neutralize trypsin by adding 500 μ l FBS. Disperse digested tissues into single cells by gentle pipetting and centrifuge at 300 g for 5 min. Remove supernatant and resuspend pellet in FGSC culture medium.
10. Dissociate to single-cell suspension by pipetting up and down using a 10 mL serological pipette.
 1. Pass cell suspension through a 30- μ m nylon mesh filter to remove cell clumps which may clog the column (see Note 4).
11. Count cells using a counting chamber under an optical microscope at room temperature. Calculate the volume of medium needed to re-suspend cells in order to obtain 10⁷ cells in 80 μ L (see Note 5).
12. Centrifuge cell suspension at 300 g for 5 min. Aspirate supernatant completely and resuspend cell pellet in 80 μ L of pre-cooled FGSC culture medium per 10⁷ total cells (see Note 6).

13. Add 20 μL of Anti-SSEA-4 MicroBeads per 10^7 total cells (see Note 7). Mix well and incubate for 15 min at $+4^\circ\text{C}$ (see Note 8).
14. Wash cells by adding 2 mL of FGSC culture medium and centrifuge at $300\times g$ for 5 min. Aspirate supernatant and resuspend in 500 μL of fresh pre-cooled FGSC culture medium.
15. Place column in the magnetic field of a MACS Separator and prepare column by rinsing 3 mL of pre-cooled FGSC culture medium.
16. Apply cell suspension onto the column and collect flow-through containing unlabeled cells (Note 9). Wash column three times with 3 mL of FGSC culture medium (see Note 10).
17. Remove column from the separator and place it on a 50 mL collection tube. Pipette 5 mL of FGSC culture medium onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column (see Note 11).
18. Centrifuge cell suspension at 300 g for 5 minutes. Aspirate supernatant completely and resuspend cell pellet in FGSC culture medium and plate cells in gelatin pre-coated 4-well dish (see Sect. 3.3, steps 1 and 8) and culture the incubator under aseptic conditions with 5% CO_2 at 37°C .

4. Notes

1. FGSC culture medium can be stored at $+4^\circ\text{C}$ a maximum of 10 days.
2. Intact ovaries can be stored at -80°C for long time periods without causing matrix alteration.
3. The obtained bio-scaffold can be either directly used for histological analysis or sterilized for cell repopulation. Its sterilization procedure can be performed using 70% Ethanol and 2% Antibiotic in sterile H_2O for 30 min at room temperature. Before cell repopulation, wash bio-scaffold extensively with PBS and 4% Antibiotic at room temperature using an orbital shaker at 200 rpm.
4. Moisten filter with culture medium before use.
5. The formula to be used depends on the specific type of chamber. $\text{Cells}/\mu\text{L} = \text{Average number of cells per small grid} \times \text{chamber multiplication factor} \times \text{dilution}$.

6. Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labelling.
7. The microbead volume here reported is necessary for up to 10^7 cells. When working with fewer cells, use the same quantity. When working with higher cell numbers, scale up the volumes accordingly.
8. The incubation temperature and period are fundamental for specific cell labeling. Higher temperatures and/or longer incubation times may lead to nonspecific cell labelling.
9. FGCS are labelled cells. The unlabeled ones, collected in this step, can be discarded or cultured for negative control.
10. Add medium only when the column reservoir is empty.
11. To increase the purity of SSEA-4+ cells, the eluted fraction can be enriched with a new second column, repeating the magnetic separation procedure.

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References

1. Hewlett M and Mahalingaiah S (2015) Update on primary ovarian insufficiency. *Curr Opin Endocrinol Diabetes Obes* 22:483–9
2. Qin Y, Jiao X, Simpson JL, et al (2015) Genetics of primary ovarian insufficiency: new developments and opportunities. *Hum Reprod Update* 21:787–808
3. Yalcinkaya TM, Sittadjody S, and Opara EC (2014) Scientific principles of regenerative medicine and their application in the female reproductive system. *Maturitas* 77:12–19
4. Sadri-Ardekani H and Atala A (2015) Regenerative medicine for the treatment of

reproductive system disorders: Current and potential options. *Adv Drug Deliv Rev* 82–83:145–152

5. Siegel RL, Miller KD, and Jemal A (2019) Cancer statistics, 2019. *CA Cancer J Clin* 69:7–34
6. WH W, AB T, F S, et al (2005) Predicting Age of Ovarian Failure After Radiation to a Field That Includes the Ovaries. *Int J Radiat Oncol Biol Phys* 62
7. Wallace WHB, Critchley HOD, and Anderson RA (2012) Optimizing reproductive outcome in children and young people with cancer. *J Clin Oncol* 30:3–5
8. Duffy C and Allen S (2009) Medical and psychosocial aspects of fertility after cancer. *Cancer J* 15:27–33
9. Jadoul P, Dolmans M-M, and Donnez J (2010) Fertility preservation in girls during childhood: is it feasible, efficient and safe and to whom should it be proposed? *Hum Reprod Update* 16:617–30
10. Vajta G and Kuwayama M (2006) Improving cryopreservation systems. *Theriogenology* 65:236–244
11. Wong KM, Mastenbroek S, and Repping S (2014) Cryopreservation of human embryos and its contribution to in vitro fertilization success rates. *Fertil Steril* 102:19–26
12. Sparks A (2015) Human Embryo Cryopreservation—Methods, Timing, and other Considerations for Optimizing an Embryo Cryopreservation Program. *Semin Reprod Med* 33:128–144
13. Chen C (1986) Pregnancy after human oocyte cryopreservation. *Lancet* 327:884–886
14. Westphal LM, Massie JAM, and Lentscher JA (2019) Embryo and Oocyte Banking, In: *Textbook of Oncofertility Research and Practice*, pp. 71–79 Springer International Publishing, Cham
15. Porcu E (2001) Oocyte freezing. *Semin Reprod Med* 19:221–230
16. Davis VJ (2006) Female gamete preservation. *Cancer* 107:1690–1694

17. Gook DA and Edgar DH (2019) Cryopreservation of female reproductive potential. *Best Pract Res Clin Obstet Gynaecol* 55:23–36
18. Laronda MM (2020) Engineering a bioprosthetic ovary for fertility and hormone restoration. *Theriogenology*
19. Meirow D, Hardan I, Dor J, et al (2008) Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Hum Reprod* 23:1007–1013
20. Dolmans M-MM, Marinescu C, Saussoy P, et al (2010) Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* 116:2908–2914
21. Rajabi-Zeleti S, Jalili-Firoozinezhad S, Azarnia M, et al (2014) The behavior of cardiac progenitor cells on macroporous pericardium-derived scaffolds. *Biomaterials* 35:970–982
22. Lecht S, Stabler CT, Rylander AL, et al (2014) Enhanced reseeded of decellularized rodent lungs with mouse embryonic stem cells. *Biomaterials* 35:3252–62
23. Lee H, Han W, Kim H, et al (2017) Development of Liver Decellularized Extracellular Matrix Bioink for Three-Dimensional Cell Printing-Based Liver Tissue Engineering. *Biomacromolecules* 18:1229–1237
24. Yu YL, Shao YK, Ding YQ, et al (2014) Decellularized kidney scaffold-mediated renal regeneration. *Biomaterials* 35:6822–6828
25. Aulino P, Costa A, Chiaravalloti E, et al (2015) Muscle extracellular matrix scaffold is a multipotent environment. *Int J Med Sci* 12:336–40
26. Baiguera S, Gaudio C Del, Kuevda E, et al (2014) Dynamic decellularization and cross-linking of rat tracheal matrix. *Biomaterials* 35:6344–6350
27. Sjöqvist S, Jungebluth P, Lim ML, et al (2014) Experimental orthotopic transplantation of a tissue-engineered oesophagus in rats. *Nat Commun* 5:3562
28. Singh A, Bivalacqua TJ, and Sopko N (2018) Urinary Tissue Engineering: Challenges and

Opportunities. *Sex Med Rev* 6:35–44

29. Kajbafzadeh A-M, Khorramirouz R, Kameli SM, et al (2017) Decellularization of Human Internal Mammary Artery: Biomechanical Properties and Histopathological Evaluation. *Biores Open Access* 6:74–84
30. Zhang J-K, Du R-X, Zhang L, et al (2017) A new material for tissue engineered vagina reconstruction: Acellular porcine vagina matrix. *J Biomed Mater Res Part A* 105:1949–1959
31. Laronda MM, Rutz AL, Xiao S, et al (2017) A bioprosthesis ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nat Commun* 8:15261
32. Laronda MM, Jakus AE, Whelan KA, et al (2015) Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials* 50:20–9
33. Pors SE, Ramløse M, Nikiforov D, et al (2019) Initial steps in reconstruction of the human ovary: survival of pre-antral stage follicles in a decellularized human ovarian scaffold. *Hum Reprod* 34:1523–1535
34. Liu W-Y, Lin S-G, Zhuo R-Y, et al (2017) Xenogeneic Decellularized Scaffold: A Novel Platform for Ovary Regeneration. *Tissue Eng Part C Methods* 23:61–71
35. Hassanpour A, Talaei-Khozani T, Kargar-Abarghouei E, et al (2018) Decellularized human ovarian scaffold based on a sodium lauryl ester sulfate (SLES)-treated protocol, as a natural three-dimensional scaffold for construction of bioengineered ovaries. *Stem Cell Res Ther* 9:252
36. Eivazkhani F, Abtahi NS, Taviana S, et al (2019) Evaluating two ovarian decellularization methods in three species. *Mater Sci Eng C* 102:670–682
37. Alshaikh AB, Padma AM, Dehlin M, et al (2019) Decellularization of the mouse ovary: comparison of different scaffold generation protocols for future ovarian bioengineering. *J Ovarian Res* 12:58
38. Pennarossa G, Ghiringhelli M, Gandolfi F, et al (2020) Whole-ovary decellularization generates an effective 3D bioscaffold for ovarian bioengineering. *J Assist Reprod Genet* 1–

39. Gilpin A and Yang Y (2017) Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications. *Biomed Res Int* 2017:9831534
40. Telfer EE and Anderson RA (2019) The existence and potential of germline stem cells in the adult mammalian ovary. *Climacteric* 22:22–26
41. Clarkson YL, McLaughlin M, Waterfall M, et al (2018) Initial characterisation of adult human ovarian cell populations isolated by DDX4 expression and aldehyde dehydrogenase activity. *Sci Rep* 8:6953

Figure legends

Figure 1. (A) Whole-ovary decellularized bio-scaffold preserves an intact macrostructure, maintaining the original shape and tissue homogeneity. (B) Decellularized ovary displays three-dimensional microarchitecture and ECM integrity with complex and well -organized fiber network. (C) Masson's trichrome (left panel), Gomori's aldehyde-fuchsin (middle panel) and Alcian Blue (right panel) staining show the persistence of collagen fibers (dark blue), elastic fibers (magenta) and the retention of glycosaminoglycans (GAG, light blue) after decellularization process.

Figure 2. (A) FGSCs isolated using MACS in vitro cultured. (B) FGSCs could be expanded in vitro with an estimated cell doubling time of 48-72 h. (C) FGSCs express pluripotency-related genes (OCT4, NANOG, REX1, and SOX2) and (D) germline specific markers (DDX4/VASA, FRAGILIS, BLIMP1, and DAZL).

Figure 3. (A) Re-seeded FGSCs rapidly migrate into the bio-scaffolds, adhering and colonizing the ECM within 24 hours (left panel) and, during the subsequent days of culture, cell form of cluster-like structures (right panel). (B) H&E staining demonstrates the presence of cells into the bio-scaffolds after recellularization. (C) DAPI staining confirms the positivity for nuclei.

Figure 4. Schematic representation of decellularization protocol and chronological macroscopic images illustrating changes in ovary colour, turning from red to white, while maintaining original shape.