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Initiation of puberty in mice following decellularized ovary transplant

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Abstract

Clinical interventions to preserve fertility and restore hormone levels in female patients with therapy-induced ovarian failure are insufficient, particularly for pediatric cancer patients. Laproscopic isolation of cortical ovarian tissue followed by cryopreservation with subsequent autotransplantation has temporarily restored fertility in at least 27 women who survived cancer, and aided in pubertal transition for one pediatric patient. However, reintroducing cancer cells through ovarian transplantation has been a major concern. Decellularization is a process of removing cellular material, while maintaining the organ skeleton of extracellular matrices (ECM). The ECM that remains could be stripped of cancer cells and reseeded with healthy ovarian cells. We tested whether a decellularized ovarian scaffold could be created, recellularized and transplanted to initiate puberty in mice. Bovine and human ovaries were decellularized, and the ovarian skeleton microstructures were characterized. Primary ovarian cells seeded onto decellularized scaffolds produced estradiol in vitro. Moreover, the recellularized grafts initiated

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puberty in mice that had been ovariectomized, providing data that could be used to drive future human transplants and have broader implications on the bioengineering of other organs with endocrine function.

Keywords

Bioactivity; Decellularization; Endocrine function; ECM (extracellular matrix); SEM (scanning electron microscopy); Xenotransplantation

Introduction

Eighty percent of children with cancer in the United States will survive for 5 or more years[1], and by 2015, 1 in 250 adults will have survived childhood cancer[2,3]. Although survival rates are increasing due to a variety of improving diagnostics and therapeutics, childhood cancer survivors have a variety of chronic health conditions. For example, adult survivors of childhood cancer are significantly more likely to be infertile or have difficulty becoming pregnant than their siblings[4]. This is due in part to the gonadotoxic effects of many chemotherapeutic and radiation therapies used to treat cancer[5–7]. In addition to the iatrogenic effects of radiation and chemotherapy, girls and young women can lose ovarian function due to a genetic predisposition to premature ovarian failure (e.g. fragile X syndrome and Turner's syndrome) or early onset familial cancer (BRCA1/2)[8]; or to treatments for rheumatologic disease[9], chronic kidney disease[10,11] and HIV[12,13]. The ovary is the endocrine organ of the female reproductive system producing steroid and peptide hormones necessary for the onset and progression through puberty and entrance into the normal, recurrent menstrual cycle. Ovarian hormones also significantly influence bone, skin, breast, vessels and additional endocrine tissues[14–18]. Ovarian hormones are produced by the somatic cells, granulosa and theca, that surround the female gamete, which combined make the ovarian follicle unit. Chemo- and radiation treatment destroy the follicles with their enclosed oocyte, reducing fertility and causing premature menopause[19]. The ability to develop a tissue-engineered ovarian system to sense, replace and titrate sex hormone production would more fully restore systemic functions of the ovarian endocrine system than conventional exogenous hormone replacement therapy.

There are limited hormone and fertility restoration options for patients with hormone responsive cancers and pre-pubertal patients. A method that is still considered experimental involves removal of an ovary or ovarian biopsy prior to cancer treatment, and storage of that tissue for later use. Cryobanked ovarian cortical tissue transplants have resulted in 27 reported live births, including a live birth obtained from egg retrieval following xenotransplant to a peritoneal pocket after bilateral oophorectomy[20,21]. Additionally, a pre-pubertal girl, sterilized by her cancer treatment, underwent transplant surgery to initiate puberty with ovarian tissue preserved prior to treatment[22]. However, there is a limited window during which these transplants allow recipients to maintain normal hormonal regulation with regular menstrual cycles and produce offspring[23]. There is also a risk of reintroducing cancer cells into the patient from the transplanted tissue, especially when the primary disease is disseminated, is a blood cancer[23–25], or is a soft tissue malignancy

such as Ewing's Sarcoma[26]. Because of the increase in young female cancer survivors and inherent risk associated with direct transplantation of cryobanked tissues, there is an urgent need for safe and more sustainable alternatives.

One such alternative would be to implement an engineered ovary that provides hormones and an appropriate follicle niche while eliminating the risk of reintroducing cancerous cells. In this study, we focus on recapitulating the endocrine function of the ovary in a tissue-engineering mimic. We describe steps taken toward this goal by establishing an effective decellularization technique for human ovaries, and utilizing model organisms to provide demonstrable support for a transplantable endocrine organ. Within the fields of tissue engineering and regenerative medicine, decellularized organs and tissues have shown significant promise for restoring structure and function in such systems as lung, liver, kidney and heart[27–29]. Building upon previous successes of regenerating transplants from decellularized organs and tissues, we created human and bovine decellularized ovarian scaffolds as a proof-of-concept to show that ovarian cells retain viability and endocrine function in a natural three-dimensional scaffold, to induce a pubertal transition in ovariectomized mice after transplant.

Materials and Methods

Human ovarian tissue acquisition and transport

Human ovarian tissue was obtained from participants following informed consent under Institutional Review Board (IRB)-approved protocols. These participants were undergoing ovarian tissue removal and cryopreservation for fertility preservation at National Physicians Cooperative (NPC) sites that are part of the Oncofertility Consortium (oncofertility.northwestern.edu). As part of this investigational protocol, 80% of participant ovarian tissue was cryopreserved for their future clinical use and up to 20% of the tissue was designated for research that would support future use of the cryopreserved portion. Most tissue can be carefully divided and utilized for several experiments. The representative native images (Figure 1B, 2A–H) are from archival blocks for the same participant and are established from every sample received under this protocol. All participants were enrolled in 2013 and ranged in age from 6 to 34 years old. All participants had a cancer diagnosis and 4 out of 5 had a previous history of therapy (radiation, chemotherapy, immunosuppression) prior to ovarian tissue removal (Fig. 1A). The research tissue was transported to the laboratory in SAGE OTC Holding Media (Copper Surgical, Trumbull, CT) at 4°C for 14–24 hours. A portion of the tissue was fixed in 10% neutral buffered formalin. The remaining tissue was allowed to equilibrate to room temperature upon arrival and then processed for decellularization as described below.

Bovine ovarian tissue acquisition and decellularization

Bovine ovaries were collected from Aurora Packing Company (Aurora, IL) from young cows and transported to the lab in BoviPro Oocyte Holding Medium (1182/1210). Upon arrival the ovaries are rinsed in fresh medium and the excess fat is removed. Ovaries were butterflied along the hilus and cut into manageable pieces. Ovarian tissue was processed into 500 μm -thick sections of cortex and medulla using a Thomas Stadie-Riggs Tissue Slicer.

The first slice was considered cortical tissue. The next superficial slice was discarded, while the slices at the remaining depths were considered medulla. These pieces were placed in excess 0.1% SDS solutions and rotated at room temperature for 24 hours. Slices from human participant tissues were treated the same way, with additional analysis under a dissecting scope used to verify the cortical region of the ovary.

An approximately 5–10 mm³ piece of tissue was isolated for DNA quantification prior to decellularization. A similar sized piece was removed post-decellularization, washed several times in deionized water and both pieces were stored at –80°C until used. Individual native and decellularized bovine ovarian tissue samples were weighed following lyophilization overnight. Each sample was sonicated in individual 2 ml tubes containing 1 ml of 0.02 wt.% triton-X 100 (Bio-Rad) solution in ultrapure water for 45 minutes at room temperature. The resulting 1 ml liquid lysates were collected and stored at –80°C until analyzed. Double stranded DNA content (n g/ml lysate) in each sample was quantified in triplicate using Quanti-iT™ Picogreen® dsDNA Assay Kit (Invitrogen, #P7589) according to the manufacturer's instructions. These values were then normalized to the individual original dry tissue masses to obtain ng/ml DNA per mg dry tissue for both native and decellularized samples. Percent DNA remaining within decellularized tissue samples was obtained by calculating the ratio of (ng/ml DNA)/(mg dry tissue) between native and decellularized tissues.

Tissue fixation and histological analysis

Native and decellularized tissue pieces, and scaffolds were fixed in modified Davidson's Fixative (Electron Microscopy Sciences) overnight at room temperature. All tissue processing and hematoxylin and eosin (H&E) staining was performed by the Northwestern University Center for Reproductive Sciences Histology Core. Fixed tissue was processed using an automated tissue processor (Leica) and embedded in paraffin. Serial sections were cut 5 µm thick and selected slides were stained with H&E using a Leica Autostainer XL (Leica Microsystems). Immunohistochemistry was performed on 3–5 sections per sample and at least 3 samples per group. Each experiment was performed 2 or more times and included no-primary controls. Sections stained on the same day were imaged within the same block of time and with the same set of exposures. Sections were imaged on a Nikon E600 Fluorescent microscope (Nikon Instruments) with a Retiga Exi Fast 1394 camera (QImaging). Antibodies against SALL4 (1:1000; Abcam; ab29112), COL4 (1:500; Abcam; ab6586), Laminin (1:500; Sigma; 041M4799), Fibronectin (1:500; Santa Cruz Biotechnology; sc-6953), FOXL2 (1:50; Abcam; ab5096), CYP17a (1:500; gifted from Alan J. Conley [30]), anti-inhibin alpha (1:400; gifted #379-223-ET) were used and visualizing with AlexaFluor secondaries (1:1000; Life Technologies) or DAB (Vector Labs). Mounting medium with DAPI counterstain (Vector) was used to visualize nuclear material. Pathology of renal grafts was performed with antibodies against CD45 and F4/80 in the Mouse Histology and Phenotyping Laboratory at NU. The individual who analyzed the slide images was blinded to the tissue treatment.

Scanning Electron Microscopy (SEM)

Decellularized tissue samples were prepared for SEM by first rinsing in deionized water to remove detergent residues that might remain from the decellularization process. Samples were then flash frozen at -80°C and lyophilized overnight (VirTis advantage Plus; VirTis, USA) to remove all moisture. Samples were then cut with a scalpel to expose regions of interest, mounted with carbon tape, and coated with 15 nm osmium metal via plasma (Osmium Coater, SPI Supplies, USA). Samples containing cells were first fixed for 25 minutes in 2 wt.% glutaraldehyde (Sigma) plus 3 wt.% sucrose (Sigma) aqueous solution at room temperature. This was followed by a quick rinse in 70% ethanol and storage in 70% ethanol at 4°C until ready for further SEM preparation. Cellularized samples were sectioned to expose regions of interest and gradually dehydrated through an elevated series of ethanol washes: 80, 90, 100% for 15 minutes each. Dehydrated samples were CO_2 critically point dried (Critical Point Dryer, Tousimis Samdri). Samples were mounted on carbon tape and coated with 15 nm of osmium metal. All samples were imaged using a LEO Gemini 1525 SEM at 3kV accelerating voltage.

Isolation of primary ovarian cells and seeding on bovine scaffold

CD1 mice (Harlan) were treated in accordance with the Northwestern University's Animal Care and Use Committee policies. Primary ovarian cells were isolated from 3–4 week old mice as performed previously in rats[31–33]. Briefly, cells are digested with collagenase and DNase, followed by a mechanical shredding of tissue pieces with increasingly smaller pipettes to an approximately 0.3 mm inner diameter. Cells were cultured in plating medium of DMEM:F12 (Gibco; 11320-033) supplemented with 1x Insulin-Transferrin-Selenium (Sigma Aldrich), 12.75mM HEPES (pH 7.4; Sigma Aldrich, H6147) 1x Penicillin/Streptomycin (Cellgro, 30-002-CI), 10% FBS (Gibco, 16140) and 40 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma Aldrich, H0888) at 37°C and 5% CO_2 in air. Differential plating of the cell preparation occurred on fibronectin-coated plates (BD Biosciences, 354403) overnight and dead cells, debris, red blood cells and any non-adherent follicles or oocytes were washed away with PBS.

Scaffolds were created from decellularized bovine medulla sections of 0.5 mm thickness and 3 mm in diameter. Each scaffold was sterilized with ethanol and washed in PBS prior to seeding. Primary ovarian cells isolated the previous day were collected using 0.025% Trypsin-EDTA (Life Technologies, 25300-054, diluted 1:1) and 2×10^6 of these cells were seeded onto each scaffold and cultured on a transwell (Millicell, PICM01250) in a 24-well plate (Corning, 3470) with 400 μl of plating medium. The seeded scaffolds were cultured in a 37°C incubator with 5% CO_2 for 2 days.

Functional analysis of ovarian grafts

A power analysis determined that 3 animals per group was sufficient to efficiently detect (80% chance) a minimal difference of $50\% \pm 10\%$ in serum estradiol levels. These surgeries were performed 2 separate times. Postnatal day 18 CD1 mice were ovariectomized. Mice were anesthetized with ketamine/xylazine. A single incision was made through skin in the back and separate incisions over the location of the ovaries were made through the subcutaneous layers. The fat pad attached to the ovary was pulled out of the body cavity and

removed by cauterizing through the top of the uterine horn. Each incision in the body wall was sutured with non-absorbable sutures (Ethicon Vicryl). Meloxicam was administered near the skin incision and it was closed with 1–2 staples. Lidocaine ointment was applied around the staple. Two weeks following ovariectomy surgery, the vaginal opening of the ovariectomized mice was examined to determine if the surgery was successful in removing the ovaries. Based on this criterion, no animals were removed from this study. The ovariectomized mice were randomly assigned to receive a graft with or without cells. The mice were reopened and a 3mm graft, described above, was applied under each kidney capsule. Briefly, an incision was made through the subcutaneous layers above the location of the kidney. The kidney was massaged out of the body cavity and the exteriorized kidney was allowed to rest on the body wall. A 4 mm incision in the capsule was made and a pocket between the capsule and kidney parenchyma was made using a glass rod. The grafts inserted into the pocket under the capsule. The kidney was placed back inside and the same technique was repeated with the other kidney. Each incision in the body wall was sutured with non-absorbable sutures (Ethicon Vicryl). Meloxicam was administered near the skin incision and it was closed with 1–2 staples. Lidocaine ointment was applied around the staple. 3–4 ovariectomized mice contained grafts with cells, 3–4 ovariectomized mice contained grafts without cells and 1–2 ovariectomized mice that did not have grafts were used in each experiment. Age-matched control, or litter-mate CD1 females that did not have any surgeries were used as cycling control animals. Animals with like treatment were housed in the same cage and were not mixed with animals with different treatments to avoid undesirable estradiol exposure between groups. These experiments were performed 2 separate times.

Terminal blood draws and renal graft removal was performed 2–4 weeks after the surgery. Grafts with a small piece of kidney were put through histology as described above. Serum estradiol (Elisa; Calbiotech, ES180S) and inhibin A (Ansh, AL-123) were tested and analyzed using GraphPads Prism software. The individual who interpolated the raw data was blinded to the animal treatment. The control female mice were collected while cycling and not at a defined point in their cycle. Therefore, the serum hormone levels are not expected to be within a statistically normal distribution. The estimated variances between the compared groups were not significantly different.

Results

SALL4-positive cells in ovarian cortex biopsies from ALL patients

In order to assess the risk of reintroducing cancer cells through transplant of cryopreserved ovarian tissue, we analyzed a group of human ovarian cortical tissues that were cryopreserved for research purposes from women undergoing fertility preservation interventions and that were predicted to have disseminated disease. We evaluated 4 individuals with an acute lymphoblastic leukemia (ALL) diagnosis and 1 participant with a non-hematopoietic cancer for the presence of malignant cells in ovarian tissue (Fig. 1a). All human ovarian cortical tissue was obtained through the Oncofertility Consortium National Physicians Cooperative (NPC) following institutional approval and informed consent. We examined the tissue for the presence of Spalt-like transcription factor 4 (SALL4), an

oncogene expressed in metastatic leukemia cells and not expressed in normal ovarian tissue[34,35]. Ovarian tissue from 3 of 4 ALL-diagnosed participants (Fig. 1a, A–D) contained clear regions of SALL4-positive cells, while tissue from participant D who was diagnosed with pancreatic cancer did not (Fig. 1b–c). These results confirm that circulating cancer cells can be present within ovarian tissue obtained as a fertility preservation option, and highlights the inherent risk of transplanting this tissue to restore ovarian function.

Decellularization of human and bovine ovarian tissue

We next investigated if cells could be effectively removed from human ovarian cortical sections, while maintaining the integrity and microstructure of the extracellular matrix (ECM), a process known as decellularization. We tested a variety of reagents used in the decellularization literature[36] (data not shown) and found that the anionic detergent SDS was highly effective for human and bovine ovarian tissues. Ovarian tissues from participants with ALL were treated with SDS and removal of cellular material was analyzed. The starting tissue samples (~1–2 x 0.5 mm) contained primordial follicles and stroma of varying cell density (Fig. 2a,c,e,g). Incubation in 0.1% SDS for 24 hours eliminated all cells, leaving only the ECM in all cases analyzed (Fig. 2b,d,f,h). Indeed, the decellularized tissue was also free of SALL4 antigen staining.

Because human ovarian tissue is a limited resource, we utilized bovine tissue obtained from an abattoir for all remaining experiments and processed it into tissue strips (~1–3 x 0.5 mm) identical to the processing technique used for human patient samples. Bovine were chosen as our model because they are large mono-ovulatory mammals that have ovarian compartments that closely resemble those of humans. We first examined the follicular compartments in bovine tissue pieces. Tissue from the outer cortex contained primordial follicles (Fig. 2i), while tissue from the inner medulla had fewer, but larger follicles in advanced stages of folliculogenesis (Fig. 2k). We then treated the tissue sections with SDS and confirmed by hematoxylin and eosin (H&E) histological staining that the cortical and medullary tissues were completely decellularized (Fig. 2j,l).

To assess whether or not there was substantial remaining nuclear material within the decellularized pieces, we stained sections from each participant and representative cortical and medulla bovine pieces with DAPI. We found no visible nuclear material within the decellularized sections (Fig. S1). To further confirm that our decellularization technique removed most DNA within the samples, we quantified the double-stranded DNA content of native bovine samples and compared it to adjacent samples that had been decellularized. The decellularized samples contained 15.01% (SEM $\pm$ 5.29%, n = 7 pairs) of the DNA contained in the matched native samples.

Decellularization of an entire bovine ovary and characterization of the tissue skeleton

The future goal of this project is to create a fully functioning engineered ovary. Therefore, we asked whether or not the entire organ could be decellularized while maintaining the overall structure and compartmentalization. Bovine ovaries were incubated in 0.1% SDS for approximately 3 weeks at 4°C, with frequent solution changes. The organ maintained its macrostructure, based on visual inspection and handling. The ovary was lyophilized to

remove all water and bisected. Cavities once occupied by large maturing follicles could be observed by eye (Fig. 2m).

In order to better understand the native physical structure of the ovary, we examined the ECM skeleton using scanning electron microscopy (SEM; Fig. 3 and Fig. S2). A low magnification (gross) SEM survey across the length of the ovary shows regions once occupied by follicles, stromal cells and blood vessels (Fig S2). Higher magnification SEM inspection of the cortex begins with the intact ovarian surface where the ovarian surface epithelial (OSE) cells once resided (green arrow, Fig. 3a). Just interior to the OSE, a primordial follicle pore that once enclosed a 33.6 μm follicle is visible (Fig. 3b). This primordial follicle pore is of the same size as those shown in the histology images from the human cortical tissue (Fig. 2a, arrows, 35.8 μm and 35.0 μm). The interior of the decellularized cortex is composed of monodisperse pores, previously occupied by cells, with solid ECM walls (Fig. 3c,g,h). Large follicle pores are visible within the decellularized medulla in addition to pores that are similar in size to cortical follicles (Fig. 3d,e). Lumens from spiral arteries that are recruited by the large follicles are evident in the decellularized medulla (red arrows, Fig. 3d).

Pore walls within the cortex are primarily composed of radially-aligned collagen fibers (black arrows, Fig. 3h). In contrast, the medulla is characterized by a hierarchical pore architecture (pores within pores) with anisotropically oriented collagen fibers (black arrow, Fig. 3j) and additional flexible, structural proteins such as fibronectin (white arrow, Fig. 3j). The decellularized structure of human ovarian tissue was also examined through SEM (Fig. 3k,l). The tissue clearly contains collagen bundles similar to what is found in the bovine cortex (black arrows Fig. 3l). However, these bundles appear thicker and more uniform than the bovine tissue. Of note, all ovarian tissue collected are from diseased patients and may not represent a “normal” population.

ECM of decellularized bovine cortex and medulla were examined through immunohistochemistry, and collagen IV was present throughout the decellularized cortex, while it was limited in medullary regions (Fig. 4a–d). Laminin and fibronectin are both expressed by granulosa cells, but have different expression patterns within the decellularized tissues, likely due to the changes in granulosa cells across folliculogenesis. Laminin, is present in the cortex (Fig. 4c), but is not as prominent in the medulla ECM (inset, Fig. 4c). However, fibronectin is present in the decellularized medulla (Fig. 4d), but not the cortex region (inset, Fig. 4d). These data largely confirm the SEM analysis of the ovarian skeleton.

Limited human cortex material was used to assess the ECM by immunohistochemistry. Four out of the five human ovarian cortical tissues stained positive for collagen I (Fig. 4e), while all five tissues contained collagen IV, which was concentrated around follicle pores (Fig. 4f–g). No decellularized cortex samples stained for laminin. However, a single tissue stained positive for fibronectin (participant A, Fig. 4h), which is consistent with the stromal heterogeneity found in human ovary samples[37]. Human medulla was not available for this analysis.

Follicle-like structures form from primary ovarian cells seeded onto decellularized ovary scaffold

We next asked whether primary murine ovarian cells (selected because of their relative ease of isolation, abundance and species match to recipient animals) could grow on the bovine ECM scaffolds and produce hormones by utilizing a digestion technique that isolates and supports predominantly granulosa cells[32]. Mouse ovarian cells were isolated, and cultured overnight on fibronectin plates before seeding. The cellular preparation contains most ovarian cells types (Fig. S3 a–b). Red blood cells, tissue remnants and non-adherent follicles and oocytes are washed off, while adherent follicles, granulosa cells and theca cells remain (Fig S3 c–e). The cells were then trypsinized (another process that breaks up follicles) and seeded onto bovine ovarian ECM scaffolds, which consisted of 3 mm-diameter sections of 0.5 mm-thick decellularized tissue. After 48 hours, scaffolds were processed and sectioned through the transverse plane to examine cellular organization across the circular punch. Primary ovarian somatic cells formed a confluent sheet on the surface of the scaffold (Fig. 5c). While most primary ovarian cells remained on the surface or along the periphery of the scaffold, some cells did migrate through the decellularized medulla scaffold within 48 hours (Fig. 5a–b). To determine the types of cells present on the scaffold, we stained samples with FOXL2 and alpha-Inhibin, granulosa cell markers, and CYP17, a theca cell marker (Fig. 5ab, Fig S3 f). As expected, both major ovarian cell types were present on the scaffold and several cells grew within the scaffold (below dotted line Fig. 5a–b). Some granulosa cells organized into a circular, follicle-like pattern on the scaffolds (Fig. 5d). The primary ovarian cells also produce steroid blebs when seeded on decellularized scaffolds (Fig. 5e), similar to what was observed by others using SEM of mural granulosa cells after ovulation[38].

Initiation of puberty in mice after decellularized ovary scaffold transplant

To determine if the recellularized scaffolds could restore endocrine function in vivo, we ovariectomized mice prior to puberty and maintained them for 2 weeks. Our two primary endpoints were vaginal opening and serum hormone levels. Therefore, vaginal orifices were verified to be closed in ovariectomized animals prior to xenograft surgeries. Decellularized ECM scaffolds were seeded with mouse ovarian cells, as before, and transplanted under the kidney capsule of the ovariectomized mice to represent ‘ovary replacement grafts’. Sham grafts were treated the same except media without cells was added to the scaffolds. Serum estradiol levels of normal cycling control mice ranged from 5.8–10.6 pg/ml (Fig. 6a) consistent with animals sacrificed on different days of the estrous cycle. Ovariectomized mice with or without sham grafts had basal serum estradiol (5.1–6.7 pg/ml), while 6 out of 7 ovariectomized mice with ‘ovary replacement grafts’ had normal or elevated circulating estradiol levels 2–4 weeks after surgery. A one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test and 2-tailed independent t-tests were performed. The serum estradiol levels of ovariectomized animals with grafts were significantly different than the ovariectomized with sham group (5.90 ± 0.24 pg/ml, $N=6$; ANOVA $p<0.05$; t-test, $p=0.042$), and was not different from control levels (mean $\pm$ SEM: 9.17 ± 1.30 pg/ml, $N=7$; ANOVA not significant (ns); t-test, $p=0.07$). We also measured the peptide hormone inhibin A in the normal cycling, ovariectomized and ovary replacement graft transplanted animals. Inhibin A levels showed cycle-dependent scatter in the control mice and were largely undetectable in the ovariectomized mice with or without sham grafts (Fig. 6b). The

ovariectomized mice with ovary replacement grafts had cycle-range levels of serum inhibin A in all but one case (0.96 ± 0.84 pg/ml, N=5; ANOVA ns; t-test compared to control $p=0.040$). Since the graft is unlikely to be cycling, the inhibin A levels are consistent with continuous production of this ovarian hormone.

To verify ovary replacement functionality and puberty initiation in mice ovariectomized prior to puberty, we examined the vaginal orifices of mice with grafts. The vaginal orifices of mice ovariectomized prior to puberty are imperforate and maintained a visible hymen, while the orifice of ovariectomized mice with ovary replacement scaffold grafts opened within 4 weeks of surgery, similar to the cycling littermate control mouse (Fig. S4).

It is important to note that these grafts were transplanted into normal CD1 strain mice and not an immunodeficient strain. Therefore, to evaluate the general health of the grafts we examined their gross histology within the kidney capsule 2–4 weeks post-surgery (Fig. 6c). There appeared to be no gross rejection of the graft on the kidney (i.e. no fibrous capsule formation). Each graft was sectioned sagittally and further analyzed for T-cell and macrophage infiltration with CD45 and F4/80 antibodies respectively. Both sham and recellularized grafts contained immune cells. However, the number of immune cells was not enough to elicit a pathologic response (Fig. S5 a–d). The grafts were additionally stained with H&E. Granulosa cells formed follicle-like structures of 2–5 cells within the transplanted decellularized scaffold (Fig. 6f). Several follicle-like structures consisting of FOXL2-positive cells were identified (Fig. S5 e, arrow and inset). Alpha-Inhibin and CYP17-positive cells were also present within the grafts and visible at necropsy (Fig. S5 e–g). Additionally, the primary ovarian cells seeded onto the decellularized scaffold provided a niche for the few remaining primordial follicles or denuded oocytes to grow into large antral follicles. One graft contained 2 antral follicles, with 1 exceeding 600 μ m (Fig. 6d–e).

Discussion

There is a significant need for safe artificial ovaries that initiate puberty, provide endocrine support and restore fertility. We have described a new approach toward this goal by studying proof-of-concept transplants in model organisms, and identifying techniques that effectively decellularized human ovarian tissue pieces to create a tissue-specific ECM scaffold. In line with previous reports of decellularized liver and kidney, we demonstrated that a detergent removes cells, cellular debris and nuclear material from a whole ovary or ovarian slices[27,28,38]. Here, bovine and human ovaries were decellularized while maintaining the microstructure of the monodisperse pores of the stroma, the follicle pockets and the ovarian vessel architecture. We utilized bovine decellularized ovaries as a model for human ovaries that are neither diseased nor treated with chemo- or radiation therapy.

The physical environment contributes to the selection of quiescent follicles to begin growing and producing hormones[31,33]. The SEM micrographs confirm the similarities and differences of the two ovarian compartments, which provide defined niches for follicles at varying stages of folliculogenesis. Protein composition and alignment are important aspects of the physical environment that can impact the function of the organ. The image of the whole decellularized ovary in Fig. 2m underscores the immense amount of ECM that

contributes to the ovarian skeleton and, in turn dictates aspects of folliculogenesis. Likewise, the murine ovarian cells, comprised primarily of granulosa and theca cells, formed follicle-like pores, produced androgenic blebs on the decellularized bovine ECM and created a supportive niche for follicular growth.

Both the composition and architecture of the ECM regulates access to cytokines and nutrients and induces mechanotransduction cues that contribute to tissue formation. For example, human endothelial cells attach to decellularized porcine small intestinal submucosa better than to defined ECM-coated plastic dishes[39] and human adrenocortical cells attach, proliferate and produce more cortisol when seeded onto decellularized adrenal porcine ECM[40]. Rodent liver, lung, kidney and heart have been decellularized and reseeded to restore a measured degree of functionality such as metabolic activity and oxygen exchange when implanted into rodent recipients[27–29,41,42]. This manuscript now adds a novel approach to restoring ovarian endocrine function to the decellularized regenerative medicine literature and has broader implications to the bioengineering of other endocrine organs. In contrast to decellularized whole organs, we employ an immersion technique to organ decellularization. As such, our scaffolds lack a vascular bed, but cells grown within the scaffold survive from the vascularity of the adjacent surroundings as do tissue engineered tracheal grafts[43].

The ovary transplants of primary mouse ovarian cells cultured on a bovine scaffold restored serum estradiol levels, produced circulating inhibin A and induced a secondary sex characteristic – vaginal opening – in pre-pubertal ovariectomized CD1 mice. The serum estradiol levels of 6 out of 7 animals were equivalent to the range found in cycling controls. We speculate that the 1 graft that did not produce estradiol at necropsy equivalent to the others, ceased after a few weeks, since the vaginal orifice opened within 2 weeks following surgery. Furthermore, the transplant recipients were not immunodeficient and produced minimal reaction to the recellularized and sham grafts. Additionally, the decellularized scaffold and somatic cell niche supported the growth of small follicles or denuded oocytes into large antral follicles.

The future of a safe artificial ovary for human use would rely on personalized medicine techniques. Patient-derived induced pluripotent stem (iPS) cells cultured on patient or donor-derived ovarian decellularized scaffolds could be the key to personalized ovarian transplants that are free from metastatic disease. In fact, stem cells grown on tissue-specific decellularized scaffolds differentiate into the desired cells types. For example, human iPS cell-derived precursor alveolar epithelial cells seeded onto the human and rat lung decellularized scaffolds matured to a more advanced stage to express markers of mature pneumocytes. Therefore, the future clinical implication of our work would reduce the transmission of residual cancer cells by utilizing iPS-derived ovarian cells (e.g. from recipient dermal fibroblasts) with decellularized ovarian tissue from a xenogeneic source (e.g. bovine) or from human cadaveric organ donors. The groundwork for much of this future clinical model has been established as good manufacturing practice guidelines have already been formulated to aide in the preparation of patient-derived cells using integration-free techniques [44,45] and primordial germ cells and granulosa-like stem cells have been derived from human stem cells [46,47]. Building upon previous successes of regenerating

transplants from decellularized organs and tissues, we created human and bovine decellularized ovarian scaffolds as a proof-of-concept to show that ovarian cells retain viability and endocrine function in a natural three-dimensional scaffold, to induce a pubertal transition in ovariectomized mice after transplant.

Conclusions

While hurdles remain before this technology can be introduced as a regenerative therapy, we describe three main accomplishments: 1) Bovine and human ovaries are decellularized with 0.1% SDS, while maintaining their microstructure as visualized through SEM; 2) Compartmentalization of ECM that once occupied ovarian surface epithelium, immature and maturing follicles and vasculature are preserved within the ovarian skeleton after decellularization; 3) An ovarian transplant made of primary ovarian cells on a decellularized matrix provides a niche for steroid and peptide hormone production, which initiates puberty in ovariectomized mice. These data provide the first steps toward creating an artificial ovary that functions as an endocrine organ and supports viable gametes. Biomimetics of a larger scale and testing with human tissue needs to occur in order for this technique to be translated beyond proof-of-principle to provide long-term endocrine support for patients. Nonetheless, this report provides the first evidence that such an approach may be viable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mariotto AB, Rowland JH, Yabroff KR, Scoppa S, Hachey M, Ries L, et al. Long-Term Survivors of Childhood Cancers in the United States. *Cancer Epidemiology Biomarkers & Prevention*. 2009 Mar 31; 18(4):1033–40.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA A Cancer Journal for Clinicians*. 2012 Jan 4; 62(1):10–29. [PubMed: 22237781]
3. Woodruff TK. Fertility in female survivors of childhood cancer. *Nat Rev Endocrinol Nature Publishing Group*. 2013 Sep 3; 9(10):571–2.
4. Barton SE, Najita JS, Ginsburg ES, Leisenring WM, Stovall M, Weathers RE, et al. Infertility, infertility treatment, and achievement of pregnancy in female survivors of childhood cancer: a report from the Childhood Cancer Survivor Study cohort. *Lancet Oncol Elsevier Ltd*. 2013 Jul 22; 14(9):873–81.

5. Blumenfeld Z. Best Practice & Research Clinical Obstetrics and Gynaecology. Best Practice & Research Clinical Obstetrics & Gynaecology Elsevier Ltd. 2012 Jun 1; 26(3):379–90.
6. Morgan S, Anderson RA, Gourley C, Wallace WH, Spears N. How do chemotherapeutic agents damage the ovary? Hum Reprod Update. 2012 Jul 27; 18(5):525–35. [PubMed: 22647504]
7. Torino F, Barnabei A, De Vecchis L, Sini V, Schittulli F, Marchetti P, et al. Chemotherapy-induced ovarian toxicity in patients affected by endocrine-responsive early breast cancer. Critical Reviews in Oncology / Hematology Elsevier Ireland Ltd. 2014 Jan 1; 89(1):27–42.
8. Oktay K, Kim JY, Barad D, Babayev SN. Association of BRCA1 Mutations With Occult Primary Ovarian Insufficiency: A Possible Explanation for the Link Between Infertility and Breast/Ovarian Cancer Risks. Journal of Clinical Oncology. 2010 Jan 7; 28(2):240–4. [PubMed: 19996028]
9. Le thi Huong, Du; Amoura, Z.; Duhaut, P.; Sbai, A.; Costedoat, N.; Wechsler, B., et al. Risk of ovarian failure and fertility after intravenous cyclophosphamide. A study in 84 patients. The Journal of Rheumatology [Internet]. 2002; 29(12):2571–6. Available from: <http://www.jrheum.org/content/29/12/2571>.
10. Lim VS. Reproductive function in patients with renal insufficiency. Am J Kidney Dis. 1987 Apr; 9(4):363–7. [PubMed: 3555011]
11. Finkelstein FO, Shirani S, Wuerth D, Finkelstein SH. Therapy Insight: sexual dysfunction in patients with chronic kidney disease. Nat Clin Pract Nephrol. 2007 Apr; 3(4):200–7. [PubMed: 17389889]
12. Kushnir VA, Lewis W. Human immunodeficiency virus/acquired immunodeficiency syndrome and infertility: emerging problems in the era of highly active antiretrovirals. Fertility and Sterility Elsevier Ltd. 2011 Sep 1; 96(3):546–53.
13. Clark RA, Mulligan K, Stamenovic E, Chang B, Watts H, Andersen J, et al. Frequency of anovulation and early menopause among women enrolled in selected adult AIDS clinical trials group studies. J Infect Dis. 2001 Nov 15; 184(10):1325–7. [PubMed: 11679923]
14. Laronda MM, Burdette JE, Kim JJ, Woodruff TK. Recreating the female reproductive tract in vitro using iPSC technology in a linked microfluidics environment. Stem Cell Research & 2013
15. Shulman LP. Transdermal hormone therapy and bone health. Clinical interventions in aging. Dove Press. 2008; 3(1):51.
16. Baudry M, Bi X, Aguirre C. Progesterone-estrogen interactions in synaptic plasticity and neuroprotection. Neuroscience IBRO. 2013 Jun 3; 239(C):280–94.
17. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest. 2005 Dec 1; 115(12):3318–25. [PubMed: 16322775]
18. Hall G, Phillips TJ. Estrogen and skin: The effects of estrogen, menopause, and hormone replacement therapy on the skin. Journal of the American Academy of Dermatology. 2005 Oct; 53(4):555–68. [PubMed: 16198774]
19. Larsen EC, Müller J, Schmiegelow K. Reduced Ovarian Function in Long-Term Survivors of Radiation-and Chemotherapy-Treated Childhood Cancer. Obstetrical & 2004
20. Donnez J, Dolmans M-M, Pellicer A, Diaz-Garcia C, Serrano MS, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. Fertility and Sterility Elsevier Inc. 2013 May 1; 99(6):1503–13.
21. Donnez J, Jadoul P, Pirard C, Hutchings G, Demylle D, Squifflet J, et al. Live birth after transplantation of frozen-thawed ovarian tissue oophorectomy for benign disease. Fertility and Sterility Elsevier Inc. 2012 Sep 1; 98(3):720–5.
22. Ernst E, Kjærsgaard M, Birkebæk NH, Clausen N, Andersen CY. Case report: Stimulation of puberty in a girl with chemo- and radiation therapy induced ovarian failure by transplantation of a small part of her frozen/thawed ovarian tissue. European Journal of Cancer Elsevier. 2013 Mar 1; 49(4):911–4.
23. Donnez J, Silber S, Andersen CY, Demeestere I, Piver P, Meirow D, et al. Children born after autotransplantation of cryopreserved ovarian tissue. A review of 13 live births. Ann Med. 2011 Sep; 43(6):437–50. [PubMed: 21226660]
24. Rosendahl M, Andersen MT, Ralfkiær E, Kjeldsen L, Andersen MK. Sc CYAMSDM. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. Fertility and Sterility Elsevier Ltd. 2010 Nov 1; 94(6):2186–90.

25. Meirow D, Hardan I, Dor J, Fridman E, Elizur S, Ra'anani H, et al. Searching for evidence of disease and malignant cell contamination in ovarian tissue stored from hematologic cancer patients. *Human Reproduction*. 2008 May 1; 23(5):1007–13. [PubMed: 18344563]
26. Sørensen SD, Greve T, Wielenga VT, Wallace WHB, Andersen CY. Safety considerations for transplanting cryopreserved ovarian tissue to restore fertility in female patients who have recovered from Ewing's sarcoma. *Future Oncology*. 2014 Feb; 10(2):277–83. [PubMed: 24490613]
27. Petersen TH, Calle EA, Zhao L, Lee EJ, Gui L, Raredon MB, et al. Tissue-engineered lungs for in vivo implantation. *Science*. 2010 Jul 30; 329(5991):538–41. [PubMed: 20576850]
28. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C, et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med Nature Publishing Group*. 2010 Jun 13; 16(7):814–20.
29. Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. *Hepatology*. 2011 Jan 10; 53(2):604–17. [PubMed: 21274881]
30. Lee S, Kang D-W, Hudgins-Spivey S, Krust A, Lee E-Y, Koo Y, et al. Theca-specific estrogen receptor-alpha knockout mice lose fertility prematurely. *Endocrinology*. 2009 Aug 1; 150(8):3855–62. [PubMed: 19423761]
31. West ER, Xu M, Woodruff TK, Shea LD. Physical properties of alginate hydrogels and their effects on in vitro follicle development. *Biomaterials*. 2007 Oct 1; 28(30):4439–48. [PubMed: 17643486]
32. Woodruff TK, Battaglia J, Bowdidge A, Molskness TA, Stouffer RL, Cataldo NA, et al. Comparison of functional response of rat, macaque, and human ovarian cells in hormonally defined medium. *Biology of Reproduction*. 1993 Jan; 48(1):68–76. [PubMed: 8418917]
33. Tagler D, Makanji Y, Tu T, Bernabé BP, Lee R, Zhu J, et al. Promoting extracellular matrix remodeling via ascorbic acid enhances the survival of primary ovarian follicles encapsulated in alginate hydrogels. *Biotechnol Bioeng*. 2014 Jan 22.;n/a–n/a.
34. Cui W, Kong NR, Ma Y, Amin HM, Lai R, Chai L. Differential expression of the novel oncogene, SALL4, in lymphoma, plasma cell myeloma, and acute lymphoblastic leukemia. *Mod Pathol*. 2006 Sep 22; 19(12):1585–92. [PubMed: 16998462]
35. Ma Y, Cui W, Yang J, Qu J, Di C, Amin HM, et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. *Blood*. 2006 Oct 15; 108(8):2726–35. [PubMed: 16763212]
36. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials*. 2011 Apr 1; 32(12):11–1.
37. Laronda MM, Duncan FE, Hornick JE, Xu M, Pahnke JE, Whelan KA, et al. Alginate encapsulation supports the growth and differentiation of human primordial follicles within ovarian cortical tissue. *J Assist Reprod Genet*. 2014 Aug 1; 31(8):1013–28. [PubMed: 24845158]
38. Sayoko, M.; van Blerkom, J.; Nottola, SA.; Naguro, T. *Atlas of Human Female Reproductive Function*. CRC Press; 2006. p. 1
39. Badylak SF, Liang A, Record R, Tullius R, Hodde J. Endothelial cell adherence to small intestinal submucosa: an acellular bioscaffold. *Biomaterials*. 1999 Dec 1; 20(23–24):2257–63. [PubMed: 10614932]
40. Allen RA, Seltz LM, Jiang H, Kasick RT, Sellaro TL, Badylak SF, et al. Adrenal Extracellular Matrix Scaffolds Support Adrenocortical Cell Proliferation and Function In Vitro. *Tissue Engineering Part A*. 2010 Nov; 16(11):3363–74. [PubMed: 20528677]
41. Song, JJ.; Guyette, JP.; Gilpin, SE.; Gonzalez, G.; Vacanti, JP.; Ott, HC. *Nat Med. Nature Publishing Group*; 2013 Apr 14. Regeneration and experimental orthotopic transplantation of a bioengineered kidney; p. 1-8.
42. Ott HC, Matthiesen TS, Goh S-K, Black LD, Kren SM, Netoff TI, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008 Jan 13; 14(2):213–21. [PubMed: 18193059]
43. Baiguera S, Del Gaudio C, Kuevda E, Gonfiotti A, Bianco A, Macchiarini P. *Biomaterials. Biomaterials Elsevier Ltd*. 2014 Aug 1; 35(24):6344–50.

44. Awe JP, Lee PC, Ramathal C, Vega-Crespo A, Durruthy-Durruthy J, Cooper A, et al. Generation and characterization of transgene-free human induced pluripotent stem cells and conversion to putative clinical-grade status. *Stem Cell Research & Therapy* Stem Cell Research & Therapy. 2013 Jul 26; 4(4):1–1.
45. Durruthy-Durruthy, J.; Briggs, SF.; Awe, J.; Ramathal, CY.; Karumbayaram, S.; Lee, PC., et al. Rapid and Efficient Conversion of Integration-Free Human Induced Pluripotent Stem Cells to GMP-Grade Culture Conditions. In: Zambidis, ET., editor. *PLoS ONE*. Vol. 9. 2014 Apr 9. p. e94231
46. Clark AT. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet*. 2004 Jan 28; 13(7):727–39. [PubMed: 14962983]
47. Lan C-W, Chen M-J, Jan P-S, Chen H-F, Ho H-N. Differentiation of Human Embryonic Stem Cells Into Functional Ovarian Granulosa-like Cells. *J Clin Endocrinol Metab*. 2013 Sep; 98(9): 3713–23. [PubMed: 23884780]

Fig. 1. Human ovarian tissue information and SALL4 staining. Table of age, diagnosis and treatment information for participants in this study (a). ALL: acute lymphoblastic leukemia. Representative images of native ovarian cortical tissue stained with an oncogene expressed in ALL patients. Participant D was diagnosed with pancreatic cancer and the patient's ovarian cortex contained no SALL4-positive cells (b, DNA, blue), while participant E was diagnosed with ALL and contained SALL4-positive cells (c, SALL4, green, arrows). Scale bar: 50 μ m.

Fig. 2.

Decellularization of bovine and human tissue. (a–h) Ovarian cortical tissues from four participants with ALL were selected. Primordial and primary follicles are visible within hematoxylin and eosin (H&E) stained native tissue sections (arrows in a,c,e,g) and nuclear material is stained blue. Tissue from these same participants that were decellularized with 0.1% SDS for 24 hours do not contain nuclear material, but only contain pink-staining extracellular matrix (ECM; b,d,f,h). This technique works for different ages and whether or not the patient has undergone chemotherapy. (a–b: A = 6yo, ALL, chemo, radiation; c–d: B = 14yo, ALL, chemo; e–f: C = 17yo, ALL; unknown treatment; g–h: E = 34yo, ALL, unknown treatment) Scale bar: 50 μ m. (i–m) Tissue slices from cortex and medulla regions of bovine ovary were obtained. Follicles are visible within H&E stained native tissue sections (arrows in i,k) and nuclear material are stained blue. Tissue from these same animals that were decellularized with 0.1% SDS for 24 hours do not contain nuclear material (j,l). Scale bar: 50 μ m. A whole bovine ovary was decellularized in 0.1% SDS for 28 days (m). Scale bar, 5 mm. Visible pores where a large recruitable follicle (3: 2.3mm), maturing and mature follicles (1: 4.1mm, 2: 3.6mm, 4: 4.3mm) are maintained within the ECM.

Fig. 3.

Microstructures of the ovarian cortex and medulla architecture. Decellularized bovine ovarian cortical tissue (a–c,g,h) and medullary tissue (d–g,i,j), were imaged with a scanning electron microscope. The ovarian surface epithelium is visible in the cortical region (a, green arrow). Organized collagen fibers are visible within pore walls of cortical ECM (h, black arrows). (d–g) Tissue strips from medulla regions contain larger follicle pores and more vascular pores (d, red arrows). Some organized collagen fibers are visible within medulla pore walls (J, black arrow). These walls also contain flexible fibronectin fibers (j, white arrow). Organized collagen bundles are visible within the decellularized human ovary (k,l, white arrow). Follicle pore diameters were measured to be 1: 33.6 μm , 2: 119.2 μm , 3: 169.4 μm , 4: 24.8 μm , 5: 162.3 μm . Scale bar in a,d: 100 μm . Scale bar in b–d,e–f: 20 μm . Scale bar in g–l: 1 μm .

Fig. 4.

ECM components in the decellularized human and bovine ovarian tissue. Decellularized bovine ovarian cortical tissue stained with collagen IV (a), laminin (c) and fibronectin (inset, d). Decellularized bovine ovarian medullary tissue stained with collagen IV (b), laminin (inset, c) and fibronectin (d). Decellularized human ovarian cortical tissue stained with collagen I (e), collagen IV (f–g) and fibronectin (h). Scale bar: 50 μ m.

Fig. 5.

Primary ovarian cells used to recellularize scaffold are FOXL2 or CYP17-positive, and create pores and blebs. Decellularized bovine ovary scaffold seeded with primary mouse ovarian cells and cultured for 2 days (a–e). Many primary ovarian stroma cells (blue, DNA, a–b) are positive for FOXL2 (red, a) or CYP17 (green, b). Several somatic cells have penetrated the decellularized bovine medulla scaffold (white dotted line, a–b). Scale bar in a–c: 50 μ m. SEM images of primary cells seeded onto bovine scaffold (d–f). Sheets of cells are visible on top of bovine ECM (c,d, ECM is dark purple). False-colored granulosa cells form a pore (yellow, green and blue, d) and secrete blebs (d, light purple) characteristic of mural granulosa cells. Scale bar in c–e: 2 μ m.

Fig. 6.

Primary ovarian cells cultured on decellularized ovary scaffold produce estradiol and support follicle growth in vivo. (a–b) Renal grafts of decellularized bovine scaffolds with primary murine ovarian cells in ovariectomized mice produce comparable levels of estradiol (mean $\pm$ SEM: 9.17 ± 1.30 pg/ml, N=7) and inhibin A (4.03 ± 1.18 pg/ml, N=6) to age-matched cycling females (estradiol: 8.39 ± 0.57 pg/ml, N=8; inhibin A: 3.55 ± 1.84 pg/ml, N=8), while ovariectomized mice with sham grafts do not (“*” estradiol: 5.90 ± 0.24 pg/ml, N=6, ANOVA significant at $p < 0.05$; inhibin A: 0.96 ± 0.84 pg/ml, N=5, 2-tailed t-test $p = 0.040$; ANOVA ns; a-b, black x’s represent ovariectomized animals without sham grafts). Gross histology of grafts with cells or sham grafts under kidney capsule, 2 weeks post-transplant (c). H&E of sagittal section through graft containing 2 follicles (d–e, stars), one of which is a large antral follicle with a distinct cumulus-oocyte complex (top). Granulosa cells within transplanted decellularized scaffold form pore-like structures (f, arrows) of 2–5 cells. Scale bar in c: 1mm; d: 100 μ m; e: 50 μ m; f: 10 μ m.