Human Reproduction, Vol.34, No.8, pp. 1523–1535, 2019
Advance Access Publication on July 9, 2019 doi:10.1093/humrep/dez077

human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

Initial steps in reconstruction of the human ovary: survival of pre-antral stage follicles in a decellularized human ovarian scaffold

S.E. Pors[1,*], M. Ramløse[1], D. Nikiforov[1,2], K. Lundsgaard[1], J. Cheng[1,3], C. Yding Andersen[1], and S.G. Kristensen[1]

[1]Laboratory of Reproductive Biology, The Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, Faculty of Health Science, University of Copenhagen, Blegdamsvej 9, 2100 Copenhagen, Denmark [2]Current address: University of Teramo, Teramo, Via Renato Balzarini, 1, 64100 Italy [3]Current address: People's Hospital of Guangxi Autonomous Region, 6 Taoyuan Rd, Qingxiu Qu, Nanning City, Guangxi province, 530005, China Via Renato Balzarini, 1, 64100 Teramo

*Corresponding address. Laboratory of Reproductive Biology, The Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark.
E-mail: susanne.elisabeth.pors@regionh.dk

Submitted on February 1, 2019; resubmitted on April 4, 2019; editorial decision on April 25, 2019

STUDY QUESTION: Can a reconstructed ovary using decellularized human ovarian tissue (DCT) support survival of pre-antral stage follicles?

SUMMARY ANSWER: We have demonstrated an effective protocol for decellularization of human ovarian tissues and successful recellularization with isolated human ovarian cells and pre-antral follicles.

WHAT IS KNOWN ALREADY: Survivors of leukemia or ovarian cancer run a risk of reintroducing malignancy when cryopreserved ovarian tissue is transplanted to restore fertility. A reconstructed ovary free of malignant cells could provide a safe alternative. Decellularization of ovarian tissue removes all cells from the extracellular matrix (ECM) including possible malignancies and leaves behind a physiological scaffold. The ECM offers the complex milieu that facilitates the necessary interaction between ovarian follicles and their surroundings to ensure their growth and development. Previous studies have shown that decellularized bovine ovarian scaffolds supported murine follicle growth and restoration of ovarian function in ovariectomized mice.

STUDY DESIGN, SIZE, DURATION: Optimizing a decellularization protocol for human ovarian tissues and testing biofunctionality of the decellularized scaffolds *in vitro* and *in vivo* by reseeding with both murine and human pre-antral follicles and ovarian cells.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Donated human ovarian tissue and isolated pre-antral follicles were obtained from women undergoing ovarian tissue cryopreservation for fertility preservation. Ovarian cortical and medullary tissues were decellularized using 0.1% sodium dodecyl sulfate (SDS) for 3, 6, 18 and 24 hours followed by 24 hours of 1 mg/mL DNase treatment and washing. Decellularization of ovarian tissues and preservation of ECM were characterized by morphological evaluation using Periodic Acid–Schiff (PAS) staining, DNA quantification, histochemical quantification of collagen content and immunofluorescence analysis for collagen IA, laminin, fibronectin and DNA. Human ovarian stromal cells and isolated human pre-antral follicles were reseeded on the DCT and cultured *in vitro*. Isolated murine ($N = 241$) and human ($N = 20$) pre-antral follicles were reseeded on decellularized scaffolds and grafted subcutaneously to immunodeficient mice for 3 weeks.

MAIN RESULTS AND THE ROLE OF CHANCE: Incubation in 0.1% SDS for 18–24 hours adequately decellularized both human ovarian medullary and cortical tissue by eliminating all cells and leaving the ECM intact. DNA content in DCT was decreased by >90% compared to native tissue samples. Histological examination using PAS staining confirmed that the cortical and medullary tissues were completely decellularized, and no visible nuclear material was found within the decellularized sections. DCT also stained positive for collagen I and collagen quantities in DCT constituted 88–98% of the individual baselines for native samples. Human ovarian stroma cells were able to recellularize the DCT and isolated human pre-antral follicles remained viable in co-culture. Xenotransplantation of DCT reseeded with human or murine

pre-antral follicles showed, that the DCT was able to support survival of human follicles and growth of murine follicles, of which 39% grew to antral stages. The follicular recovery rates after three weeks grafting were low but similar for both human (25%) and murine follicles (21%).

LARGE SCALE DATA: N/A

LIMITATIONS, REASONS FOR CAUTION: Further studies are needed to increase recovery and survival of the reseeded follicles. Longer grafting periods should be evaluated to determine the developmental potential of human follicles. Survival of the follicles might be impaired by the lack of stroma cells.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first time that isolated human follicles have survived in a decellularized human scaffold. Therefore, this proof-of-concept could be a potential new strategy to eliminate the risk of malignant cell re-occurrence in former cancer patients having cryopreserved ovarian tissue transplanted for fertility restoration.

STUDY FUNDING/COMPETING INTEREST(S): This study is part of the ReproUnion collaborative study, co-financed by the European Union, Interreg V ÖKS. Furthermore, Project ITN REP-BIOTECH 675526 funded by the European Union, European Joint Doctorate in Biology and Technology of the Reproductive Health, the Research Pools of Rigshospitalet, the Danish Cancer Foundation and Dagmar Marshalls Foundation are thanked for having funded this study. The funders had no role in the study design, data collection and interpretation, or in the decision to submit the work for publication.

Key words: artificial ovary / decellularization / extracellular matrix (ECM) / fertility preservation / *in vitro* culture / ovary transplantation / pre-antral follicles

Introduction

Ovarian tissue cryopreservation (OTC) followed by transplantation of thawed tissue is currently the only available fertility preserving option for pre-pubertal girls and young women who cannot delay gonadotoxic treatment (Schmidt *et al*., 2010; Donnez and Dolmans., 2017). To date, more than 100 children have been conceived following transplantation of frozen/thawed ovarian tissue (Donnez and Dolmans., 2017; Gellert *et al*., 2018). However, for patients with leukemia or cancers originating in the ovary this technique is potentially unsafe and inadvisable, due to a risk of reintroducing malignancy when the ovarian tissue is transplanted to restore fertility. Two studies have demonstrated that cryopreserved ovarian tissue may harbor leukemic cells in >50% of cases using PCR analysis (Dolmans *et al*., 2010; Rosendahl *et al*., 2010), and possible transmission of leukemia and borderline ovarian tumor is observed in the xenografting model (Dolmans *et al*., 2010; Gook *et al*., 2018; Masciangelo *et al*., 2018). However, with the presence of specific genetic markers, transplantation of ovarian tissue from women with a former leukemic diagnosis has been perform and a few children conceived so far (Shapira *et al*., 2018; Silber *et al*., 2018). Moreover, two live births after transplantation of frozen-thawed ovarian tissue in patients with early stage ovarian cancers have been reported, in which the grafted ovarian tissues were removed soon after delivery for safety reasons (Dittrich *et al*., 2015; Kristensen *et al*., 2017).

To date, safety issues surrounding transplantation of ovarian tissue from cancer patients have been the subject of concern and debate, and the need for a safe, alternative method of fertility restoration is evident. Two approaches have been suggested: (i) *in vitro* culture of follicles with subsequent *in vitro* oocyte maturation and fertilization (Smitz *et al*., 2010; Telfer *et al*., 2013; McLaughlin *et al*., 2018), and (ii) *in vivo* grafting of isolated pre-antral stage follicles in a transplantable artificial ovary (Dolmans *et al*., 2006; Amorim *et al*., 2009; Amorim *et al*., 2016). In recent years, the 'artificial ovary' has received much attention from the field of tissue engineering, as several research teams have been developing transplantable bioengineered ovaries using different biological materials, including three-dimensional matrices like alginate and fibrin, whose main goal is to mimic the natural organ (Xu *et al*., 2009a; Luyckx *et al*., 2014; Vancker *et al*., 2014). Isolated human pre-antral follicles have survived and grown in these hydrogels both *in vitro* and *in vivo* (Xu *et al*., 2009b; Xiao *et al*., 2015; Paulini *et al*., 2016). A bioengineered ovary can also be generated by the use of a decellularized scaffold in which cells and cellular components, including possible malignancies, are removed. In contrast to gel matrices, this approach retains the extracellular matrix (ECM) that offers the complex milieu that facilitates the necessary interaction between ovarian follicles and their surroundings to ensure their growth and development. ECM constructs of different origin have been shown to support human follicle growth in ovarian tissue fragments both *in vitro* and *in vivo* (Oktay *et al*., 2016; Jakus *et al*., 2017). Recently, Laronda *et al.* (2015) showed that a decellularized bovine ovarian scaffold was able to support the growth of isolated murine follicles and reestablish menstrual cyclicity in mice. The follicular basal lamina encapsulating the granulosa cells (GCs) excludes capillaries, white blood cells and nerve processes from the granulosa compartment (Rodgers *et al*., 2003) and grafting of fully isolated follicles into an ovarian scaffold would be considered as a safe alternative for cancer patients at high risk of ovarian involvement (Dolmans *et al*., 2006). A bioengineered ovary could thus facilitate the growth and development of reseeded early stage follicles free of malignancies (Amorim *et al*., 2016).

The aim of this study was to develop and evaluate a protocol for decellularization of human ovarian tissue, and to investigate if this scaffold supports viability and development of both murine and human ovarian cells and pre-antral stage follicles *in vitro* and *in vivo*, hereby moving a step closer to reconstructing a human ovary.

Materials and Methods

Human ovarian tissue

Donated human ovarian tissue was obtained from patients undergoing OTC for fertility preservation at the Laboratory of Reproductive Biology, University Hospital of Copenhagen, Denmark. The ovarian cortical tissue was slow-frozen according to standard practice

(Rosendahl *et al*., 2011). Ovarian cortical tissue was donated with written consent from patients with various malignant diseases. The use of donated surplus ovarian tissue for research had been approved by the Minister of Health as well as the ethical committee of Copenhagen (Journal no H-2-2011-044).

In total, donated cortical and medullary tissues were obtained from 21 patients (mean age 29.5 years, range 15–37). The diagnosis of the patients included breast cancer ($n = 10$), cerebral cancer ($n = 3$), colon cancer ($n = 1$), Wegener's granulomatosis ($n = 1$), cerebral vasculitis ($n = 1$), sclerosis ($n = 1$), esophageal cancer ($n = 1$), chondrosarcoma ($n = 1$), metachromatic leukodystrophy ($n = 1$) and sclerodermia ($n = 1$).

Experimental animals

Female mice (Naval Medical Research Institute (NMRI)-NU and NMRI, Taconic, Denmark) were housed in groups until ovariectomy. Mice were fed pellets and water *ad libitum* and kept under controlled 12-hour light/12-hour dark cycles at 20–22°C. Mice were anesthetized by use of either fentanyl/fluanison and midazolam (Vetapharma Ltd, France) or zoletil (Virbac, France), xylazin (Scanvet, Denmark) and butorphanol (Zoetis, New Jersey). Post-operative analgesia was provided by use of buprenorphine (ReckittBenckiser, England, UK) and carprofen (Norbrook, England, UK). Following grafting, mice were single housed up until euthanasia, which was done by cervical dislocation. The use of animals was approved by the Animal Experiments Inspectorate (case file 2015-15-0201-00505) under Danish legislation.

Decellularization of human ovarian tissue

Cryopreserved ovarian cortical tissue from 9 patients and fresh medullary tissue from 11 patients were used for optimizing the decellularization procedure. Initial and final wet weights were obtained for individual tissue pieces for normalization of DNA content. The size of the tissue pieces was ~5 × 5 × 1–2 mm for cortical tissue and 5 × 5 × 1–3 mm for medullary tissue. Cryopreserved human ovarian tissue pieces were thawed according to standard procedure in multiple steps to avoid osmotic damage to the tissue and to eliminate cryoprotective agents (Rosendahl *et al*., 2011). Immediately after thawing, the decellularization process was initiated. Ovarian tissue pieces were placed in excess 0.1% sodium dodecyl sulfate (SDS) in PBS. Medullary and cortical tissue pieces were then placed on a rotating table and agitated at room temperature for 3, 6 or 18/24 hours (medulla/cortex). Remaining SDS was removed by washing tissue pieces in PBS under agitation for 1 hour at room temperature. Tissue pieces were then placed in 1 mg/mL DNase1 in PBS and incubated on a rotating table at 37°C for 24 hours. To remove residual chemicals, tissue pieces went through six agitated PBS washing steps. Subsequently, the decellularized human ovarian tissue (DCT) was either fixed for histology or immunohistochemistry (IHC), analyzed for DNA and collagen content, or cryopreserved in 10% dimethyl sulfoxide in fetal bovine serum (FBS) at −80°C for xenografting studies.

Quantification of DNA content

Quantitative evaluation of DNA remnants in the DCT and untreated tissue from the same patients as baseline controls (native tissue) was performed. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) with an optimized protocol. Instead of heat digestion step, as described in the kit protocol, the tissue was manually sliced followed by mechanical disruption using a Tissuelyser II (Qiagen) with 2.8-mm ceramic beads at up to 30 Hz for up to 2 minutes in multiple cycles until homogenization was achieved. Triple elution of DNA was performed. DNA content was determined in each sample in triplicates using a spectrophotometer (DU-730, Beckman Coulter, USA). DNA content in the DCT was calculated on basis of average DNA content in the sample and the DNA content was then normalized to initial wet weight for proper estimation of tissue components (Bruyneel *et al*., 2017).

Quantification of collagen content

To evaluate the integrity of the ECM for each patient after the decellularization process, a comparison between decellularized and native tissue from the same patient was done by collagen quantification by histomorphometric analysis. This was performed on cortical ($N = 7$ patients) and medullary ($N = 8$ patients) tissue and compared to native tissue controls of the corresponding origin. Tissue sections were stained in one batch with picrosirius red (PSR; Pearse, 1985). Briefly, tissue was processed for histology and stained with 0.1% Sirius red in a saturated aqueous picric acid solution (pH 2.0) for 60 minutes. Slides were flushed for 2 × 1 minute in 0.01 M HCl. Slides were dehydrated in ascending concentrations of ethanol and mounted in xylene. The overall concept of computer-aided histomorphometric analysis has been described by others (Caetano *et al*. 2016; Morais *et al*., 2017). For this study, a modified version was used. Briefly, 15 tissue sections from both decellularized and native tissue from each patient were analyzed. In each section, 15 random fields within the tissue section were selected as region of interest (ROI) at ×10 magnification. A microscope (Leica, Germany) equipped with a polarizing filter, and a camera (SDPA72, Olympus, Japan) were used, and processing performed in Olympus cell-A software. Images obtained had a ROI that constituted a minimum of 50% of the total image. ImageJ software v. 1.51 k (US National Institutes of Health, USA) was used to quantify collagen using the threshold function. RGB images were stacked to separate channels, and a threshold was defined for each patient, using native tissue from the same patient as the baseline. The outcome of the analysis corresponding to collagen, i.e. red, yellow and green, was expressed as area fraction in percentage of total ROI.

Histological evaluation

Histological processing was performed using standard methods. Tissue pieces were cut into serial sections of 5 µm in thickness. For general evaluation of tissue morphology, microscopic slides were stained with Periodic acid–Schiff (PAS; Mayer's hematoxylin, Ampliqon, Denmark. AMPQ00254.1000; Periodic acid, Ampliqon, AQ15290.1000; Schiff's reagent, Ampliqon, AMPQ16582.1000). This was done by heat deparaffinization of slides, followed by periodic acid, Schiff's reagent and Mayer's hematoxylin treatment for 10, 10 and 3 minutes, respectively. Microscopic slides were evaluated on a Zeiss Axiophot microscope mounted with a Leica DFC420C digital microscope camera, and images processed in LAS software V4.9 (Leica).

Immunostainings for nuclear remnants and ECM components

Immunohistochemistry and immunofluorescence were performed for a qualitative evaluation of possible nuclear remnants ($N = 7$ patients) and various ECM components ($N = 12$ patients). Following histological processing, slides which were to be stained for DAPI and collagen, or laminin and fibronectin, were treated in citrate buffer (pH 6.0) or Tris-EGTA buffer with Tween20, respectively. Slides were blocked using 1% bovine serum albumin (BSA; Sigma Aldrich Chemie GmbH, Germany) for 15 minutes and endogenous peroxidase blocked with 30% hydrogen peroxidase solution (Sigma). Slides were incubated overnight at 4°C with primary antibody (COL1A, Santa Cruz, Texas, USA; cat#sc-59 772, 1:100; Anti-LAMB2, Sigma Atlas antibodies, cat#HPA001895, 1:300; Anti-FN1, Sigma Atlas antibodies, cat#HPA027066, 1:300). Controls included substitution of the primary antibody with mouse or rabbit immunoglobulin applied in the same concentration as the antibody (X0910 and X0903; Agilent;California, USA). Furthermore, sections with no primary antibody were included in all stainings. Subsequently, slides were incubated for 1 hour with a secondary antibody (FITC Donkey anti-mouse, Jackson Immuno Research, England, UK, cat#715–096-151, 1:500; polyclonal swine-anti rabbit HRP, DAKO, Agilent, 1:300). DAPI staining (Life Technologies, Thermo Fisher Scientific, Massachusetts, USA, cat#D1306, 1:5000) for nuclear remnants was performed. All washes were performed in PBS. Images were visualized with fluorescence using the same microscope, camera and software as for PAS stained sections.

Isolation of GCs from human preovulatory ovarian follicles

GCs were obtained from follicular fluid from preovulatory follicles from patients at the Fertility Clinic, Rigshospitalet, University Hospital of Copenhagen, Denmark. The follicular fluid was centrifuged generating a pellet consisting of GCs and blood cells. The pellet was then resuspended in a follicle flush buffer (Sidney IVF, K-SIFB-100) and added on a Lymphoprep gradient (Axis-Shield, Scotland, UK). Following centrifugation, the blood cells were located in the bottom of the gradient and GCs at the interphase of the gradient. The GCs were collected and resuspended in alpha-MEM medium (Gibco, Thermo Fisher Scientific) with 5% FBS (Gibco). GCs were co-cultured with cortical DCT for 12 days in four-well dishes and fixed for histological analysis.

Isolation of human ovarian stroma cells

Medullary tissue pieces were manually dissected with a scalpel into smaller fragments, followed by homogenization to pieces of 0.5 mm^3 by use of a tissue sectioner for 30 minutes (McIlwain Tissue Chopper, UK). Tissue was then enzymatically digested for 30 minutes as previously described (Kristensen *et al*., 2011; Yin *et al*., 2016). Briefly, tissue was incubated at 37°C under mild agitation in a digestion solution consisting of McCoy's 5α (Gibco), Liberase TM (Roche Diagnostics, Germany), Collagenase IV (Gibco) and DNase I (Worthington, USA). The enzymatic process was terminated by addition of equal volumes of McCoy's 5α containing 10% human serum albumin (HSA; CSL Behring, Germany). The tissue suspension was then filtered through a 100-µm cell strainer (Falcon, USA) and washed twice in a culture medium, consisting of alpha-MEM with 1% Glutamax (Gibco), 1% penicillin–streptomycin (PS; Gibco), 1% insulin–transferrin–selenium (ITS; Gibco), 1% HSA, 1% MEM non-essential amino acids (Gibco) and then left in culture flasks overnight. On the following day, non-attaching cells were washed away, and half the medium was changed every second day. Isolated ovarian stroma cells (OSCs) were cultured to full confluency in a 50-mL culture flask for one passage (6–7 days) before being included in re-seeding experiments.

Isolation of murine and human pre-antral stage follicles

Murine pre-antral follicles were isolated mechanically from 2–4-week-old female NMRI mice. Using a previously described procedure, ovaries were micro-dissected and follicles isolated using 27 gauge needles (Newton *et al*., 2001). Selected follicles were transferred individually by pipette to DMEM/F12 growth medium (Gibco, cat#11330057) containing 1% ITS, 1% PS, 0.1% BSA and 0.1% Albumax II (Gibco). Human follicles were isolated enzymatically from surplus ovarian tissue from two patients as described previously (Kristensen *et al*., 2011; Yin *et al*., 2016). Briefly, pieces of medullary tissue were rinsed in PBS and homogenized to pieces of 0.3–0.5 mm^3 by use of a tissue chopper (McIlwain Tissue Chopper, UK). Tissue pieces were transferred to a culture dish containing 37°C warm McCoy media with 25 mM HEPES (Invitrogen, UK, Gibco), 0.1% HSA (CSL Behring), 2 mM Glutamax, 0.05 mg/ml 1% PS, 1% ITS, 50 mg/ml ascorbic acid (Sigma Aldrich) and a mixture of Liberase TM (0.04 mg/ml, Roche) and Collagenase IV (0.2 mg/ml, Sigma Aldrich). Tissue was then incubated at 37°C for 70–80 minutes under mild agitation. The enzymatic process was terminated by addition of equal volumes of PBS containing 10% FBS. Repeated pipette aspiration was used for further mechanical disruption. Isolated follicles were then transferred to culture media containing DMEM, FBS, PS, ITS and BSA. All isolated pre-antral follicles were morphologically evaluated and only selected for reseeding experiments if they possessed an intact basal membrane and a well-preserved follicle integrity. Furthermore, the diameter of the follicles was measured to estimate the developmental stage.

Reseeding follicles and grafting of reconstructed 'ovaries' *in vivo*

Isolated murine and human pre-antral follicles were reseeded onto DCT either with or without the use of Matrigel hESC-qualified matrix (BD Bioscience, New Jersey, USA) as cell delivery vehicle. Follicles reseeded without Matrigel were placed into small pockets in the DCT created with a scalpel ($N = 1$). Matrigel was prepared according to manufacturer's instructions and isolated follicles were placed in a 30-µL Matrigel drop and pipetted on top of the DCT surface ($N = 4$), or isolated follicles were seeded in between two layers of DCT and 'sealed' with Matrigel ($N = 2$). The scaffolds used in the studies consisted of cortical DCT ($N = 1$) or medullary DCT ($N = 6$). The different seeding techniques and the type of DCT scaffold are outlined in Supplementary Fig. S1. Grafting of follicle-seeded DCT was performed immediately to NMRI-NU mice that had been ovariectomized. Grafting was performed subcutaneously ($N = 6$) or under the kidney capsule ($N = 1$) using DCT with or without isolated follicles. In the six murine studies, medullary DCT and cortical DCT were each reseeded with ~40 isolated pre-antral follicles. In the human study, medullary DCT

was reseeded with 20 isolated pre-antral stage follicles using Matrigel. Grafts were recovered after 2–3 weeks.

Co-culture of stromal cells and isolated pre-antral follicles with DCT

Ovarian stromal cells were detached from culture flasks by use of Accutase (Invitrogen), followed by washing twice in culture medium. They were then seeded onto DCT slices in a four-well dish (Nunc, Denmark) in 750-µL culture medium with a 75 000 cell/mL concentration. Reseeded DCT was cultured at 37°C, in a 5% CO2 humidified environment. Half of the medium was replaced every other day. Co-culturing was performed for 10, 20 and 30 days, after which parts of tissue were prepared for histology. Following co-culturing for 30 days, ∼20 freshly isolated pre-antral follicles were added to the culture system. To ensure proper placement, follicles were wrapped in an OSC monolayer by placing follicles in the center of an OSC monolayer, then detaching the edges of the OSC monolayer and covering the follicles with the sheets of cells. After overnight culture for aggregation of the cells these 'packages' were then placed in the center of the scaffold by use of 32G needles and scalpels. The scaffolds were then cultured for 1 month. Morphology and viability of the reseeded scaffold were evaluated by hematoxylin/eosin (HE) staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Merck, Germany), respectively. Tissue used was fixed in 4% paraformaldehyde overnight and prepared for histology and sectioned in 5 µm. Every other slide was stained with HE and the remaining analyzed with the TUNEL assay in accordance with manufacturer instructions.

Statistical analysis

Reduction of DNA and comparison of collagen content between native and decellularized tissue was analyzed using a linear mixed model including a random intercept to account for repeated measurements on the same samples. All analysis was done using the statistical program R version 3.4.4. (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.) Differences were considered statistically significant when the p-value was below 0.05.

Results

Human ovarian tissue can be decellularized leaving the extracellular components intact

Cryopreserved ovarian cortical tissue from nine patients and fresh medullary tissue from 11 patients were used for optimizing the decellularization procedure. Incubation with 0.1% SDS for over 6 hours followed by DNase treatment (1 mg/mL) for 24 hours adequately decellularized both human ovarian cortical and medullary tissues by eliminating all cells and leaving the ECM intact (Fig. 1). The importance of adequate DNase treatment to remove nuclear remnants in the DCT was evident when DCT, with or without following DNase treatment, was stained with DAPI (Supplementary Fig. S2). Histological examination using PAS staining confirmed that the cortical and medullary tissues were completely decellularized after 6 and 18 hours, respectively, as no visible nuclear material was found within the decellularized sections (Fig. 1A+B).

Immunofluorescence was used to detect collagen, fibronectin and nuclear remnants within the native and decellularized tissues. In native tissue, collagen and demarcated nuclear remnants were visible. In cortical tissue treated with 0.1% SDS for 3 hours and 1 mg/mL DNase for 24 hours, collagen and nuclear remnants were still visible, although nuclear remnants appeared smeared out. After 6 and 24 hours of decellularization only the collagen matrix was left, and no nuclear remnants were visible in the DCT (Fig. 1B). For the medullary tissue, immunostainings for fibronectin and nuclear material (DAPI) showed that nuclear remnants were present after 3 and 6 hours of decellularization. After 18 hours only the fibronectin was left in the matrix (Fig. 1D). Additionally, immunohistochemical staining of medullary native tissue and DCT for laminin showed consistent staining of the decellularized matrix after 3, 6 and 18 hours of SDS treatment (Supplementary Fig. S3).

Quantitative evaluation of DNA remnants in the DCT and native tissue (control) was measured and the relative DNA content in the native cortical (Fig. 1E; $N = 9$) and medullary (Fig. 1F; $N = 11$) tissues was compared to decellularized corresponding tissues treated for 3, 6 and 18/24 hours. DNA quantification demonstrated increasing tissue decellularization at increasing time points 3, 6 and 18/24 hours for both cortical and medullary tissues (Fig. 1E+F). DNA quantities in cortical DCT were significantly lower compared to matched native samples after 3, 6 and 24 hours of decellularization, and for the medullary DCT, DNA quantities were significantly lower compared to native samples after 6 and 18 hours of decellularization, which corresponds nicely to the immunofluorescent staining. DNA content was decreased by >90% compared to native tissue after 18 and 24 hours decellularization with 0.1% SDS for medullary and cortical tissues, respectively (Fig. 1E+F).

To evaluate the integrity of the ECM after the decellularization process comparative collagen quantification by histomorphometric analysis was performed on decellularized cortical ($N = 7$ patients) and medullary ($N = 8$ patients) tissue and compared to native tissue controls. Quantification and comparison of collagen content in native cortical and medullary tissues and DCT showed no significant changes in area fraction constituted by collagen (Fig. 2). Overall, collagen quantities in DCT constituted over 88% of the individual baselines for native samples.

Human ovarian cells and follicles recellularize the DCT *in vitro*

Decellularized ovarian scaffolds were reseeded with OSCs (Fig. 3A) and 20 pre-antral follicles isolated from one patient followed by culture *in vitro*. Initial experiments showed that co-culture of DCT with mature GCs from stimulated human preovulatory follicles was able to gradually migrate into decellularized scaffolds during culture for up to 12 days and re-populated whole areas within the DCT (Supplementary Fig. S4). Co-culture of DCT with OSC showed an attachment of the cells to the surface of the DCT scaffold making up a cellular monolayer after 1 week (Fig. 3B). After 1 month of co-culture, OSC were well redistributed in the internal fibrous matrix of the scaffold (Fig. 3C+D); however, some areas of the DCT remained cell-free (Fig. 3C). These results show biocompatibility between scaffolds and the OSC and prove the

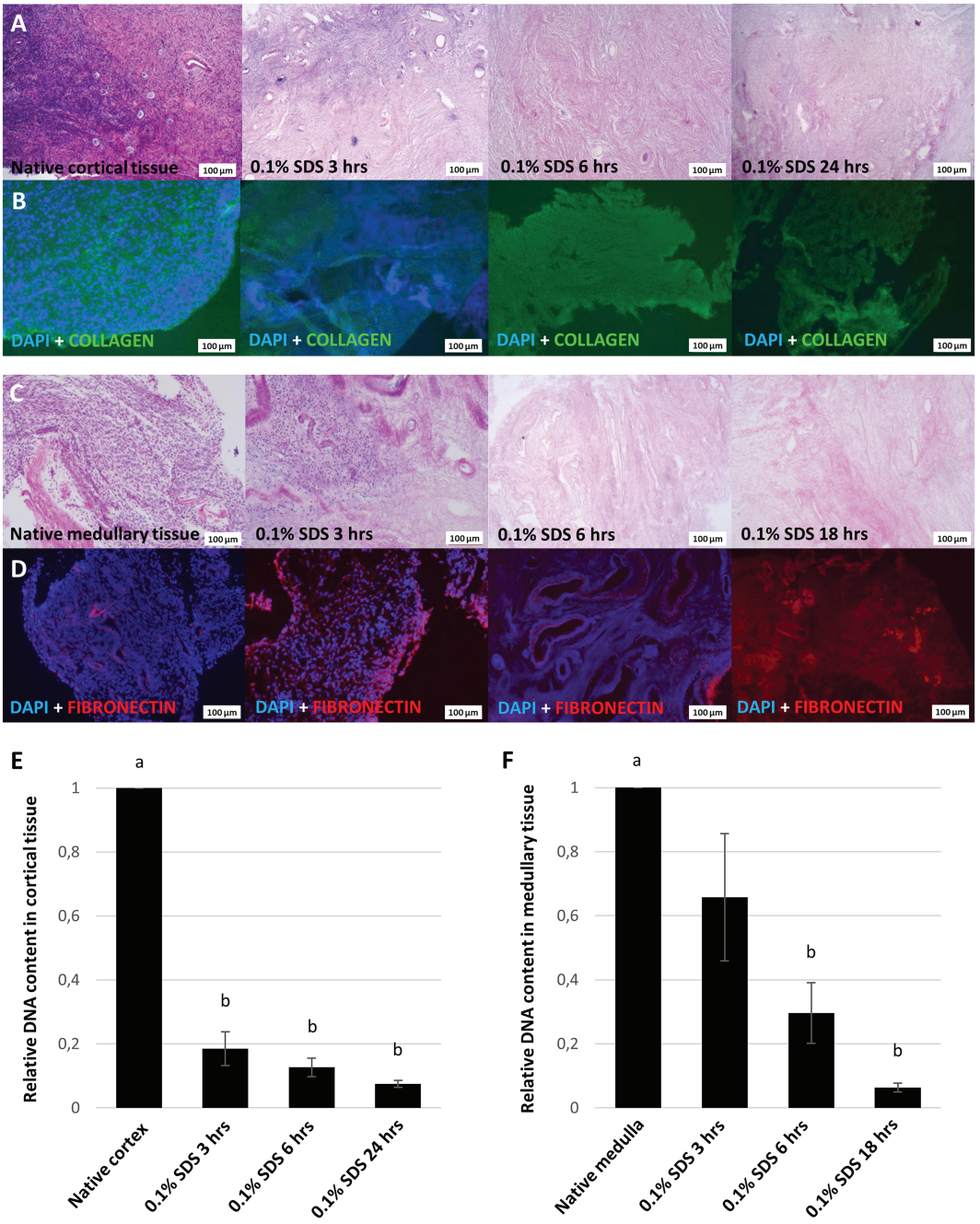

Figure 1 Optimizing decellularization protocol for human ovarian tissues. (**A**) PAS stainings and (**B**) Immunofluorescent stainings (collagen in green and DAPI in blue) of human cortical ovarian tissue. Both panels left to right: Native tissue followed by decellularized tissue after 3, 6 and 24 hours (0.1% SDS and subsequent DNase treatment for 24 h) ($N = 5$). Nuclear remnants are present after 3 hours of decellularization, but after 6 and 24 hours only the collagen matrix is left. (**C**) PAS stainings and (**D**) Immunofluorescent stainings (fibronectin in red and DAPI in blue) of ovarian medullary tissue. Both panels left to right: Native tissue followed by decellularized tissue after 3, 6 and 18 hours (0.1% SDS with subsequent DNase treatment for 24 hours) ($N = 5$). Nuclear remnants are present after 3 and 6 hours of decellularization, but after 18 hours only the fibronectin is left in the matrix. (**E and F**) Relative DNA content in the native cortical (E; $N = 9$) and medullary (F; $N = 11$) tissues compared to decellularized corresponding tissues treated for 3, 6 and 18/24 hours. Significant differences between the groups are marked with different letters. Scale bars: 100 µm.

concept that scaffolds provide suitable extracellular environment for OSC. When analyzed, scaffolds reseeded with OSC and co-cultured with isolated human follicles appeared completely repopulated after 1 month of culture with the pre-antral stage follicles present inside the reseeded scaffold. Twelve of the 20 seeded human follicles survived during the culture period and healthy-looking follicles with absence of pyknotic nuclei and homogeneous cytoplasm were found within the recellularized DCT (Fig. 3E+F+G+K). TUNEL staining confirmed the

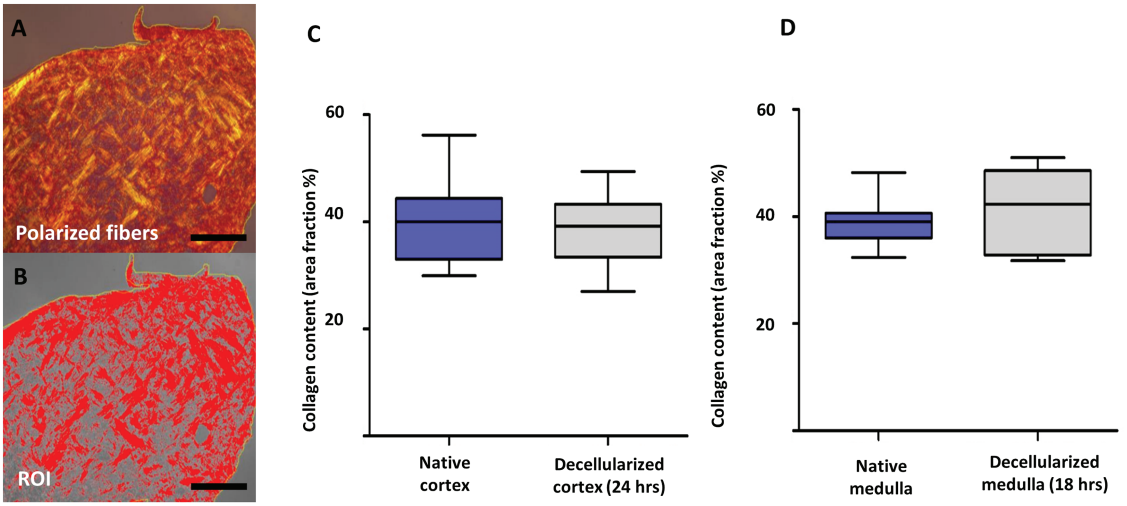

Figure 2 Quantification of collagen content in DCT and native tissues using PSR. (**A**) Native ovarian tissue viewed under polarized light after PSR staining. Scalebar: 100 µm. (**B**) Obtained image for quantification of collagen content within the ROI using ImageJ. (**C + D**) Boxplots showing collagen content in decellularized cortical (C; *N* = 7) and medullary (D; *N* = 8) tissues compared to native control tissues. Statistical analysis showed no significant changes in collagen content between DCTs and native tissues.

survival of the follicles following one month of co-culture on scaffolds reseeded with OSC. Follicles with a good morphology (*n*=4) did not show any apoptotic signal with TUNEL (Fig. 3H+I+L+M), whereas degenerated follicles (*n*= 4) (Fig. 3J) had a clear positive signal in all GCs after staining with TUNEL (data not shown).

Human pre-antral follicles survive in decellularized scaffolds *in vivo*

Twenty follicles isolated from one patient were reseeded on top of medullary DCT with the use of Matrigel as cell delivery vehicle and grafted immediately after to an immunodeficient mouse. Following 3 weeks of xenografting, 5 out of the 20 transplanted human follicles were recovered in the graft (recovery rate = 25%). The initial mean diameter of the 20 transplanted human follicles was 89.7 ± 25.9 µm (mean ± SD), ranging from 54.5–162.2 µm in diameter (representative images are shown in Fig. 4A). Follicle stages included primordial/primary follicles and different stages of secondary follicles. Following 3 weeks xenografting, the mean diameter of the five surviving follicles was 74.4 ± 8.1 (mean ± SD), ranging from 64.7–87.9 µm in diameter. Thus, the diameter of the grafted human pre-antral follicles did not increase during the grafting period, and only follicles with one layer of cuboidal GCs appeared to have survived in the decellularized scaffolds (Fig. 4D–F). The distribution of follicle stages before and after 3 weeks of grafting is shown in Fig. 4G. Recovered follicles were located near the edge of the scaffold and remnants of Matrigel were still present.

Murine pre-antral follicles survive and grow in human decellularized scaffolds *in vivo*

Serving as a negative control, DCT without follicles was also xenografted for 3 weeks (Fig. 5A), and grafts (*N* = 3) were very pale/white upon recovery and showed no signs of recellularization or vascularization macroscopically (Fig. 5A). Microscopically the control grafts had been recellularized with murine cells during the grafting period, but no follicles were found in the grafts (Fig. 5B). Murine follicles reseeded to the human decellularized scaffold (*N* = 6) survived and grew during the 3 weeks grafting period in the immunodeficient mice. Upon recovery, the grafts appeared recellularized and murine blood vessels were surrounding the grafts (Fig. 5C+E). Of the 241 isolated pre-antral stages murine follicles reseeded on 6 separate decellularized scaffolds, 51 surviving follicles with oocytes ranging from secondary to antral stage were recovered after 3 weeks grafting (recovery rate=21%, ranging from 2.5–47.5% per graft). In the majority of cases the surviving follicles were located within the decellularized matrix (76.5%) and in the remaining cases the follicles were found at the edges of the scaffold (23.5%). No notable differences were observed between the reseeding techniques for the isolated follicles, but the number of grafts were also too low in the groups to perform a proper comparison. The use of Matrigel appeared to provide the highest recovery rates. The initial diameter of isolated murine pre-antral follicles was 115±28 µm (mean ± SD, *N* = 241), and following 3 weeks of grafting the mean diameter of surviving secondary to antral stage follicles was 208 ±147 µm (mean ± SD). Of the 51 recovered follicles 39% had formed an antrum (N = 20) with a mean follicle diameter of 344 ±118 µm (mean±SD), ranging from 201–608 µm. The distribution of follicle stages before and after 3 weeks of grafting is shown in Fig. 5J. Examples of antrum forming follicles are shown in Fig. 5D+F+G. In some of the antrum forming follicles the oocytes were compromised to some degree as shown for a large antral follicle in Fig. 5H. In addition, the lack of a clear layer of theca cells surrounding the antral follicles was also evident in the vast majority of follicles as shown in Fig. 5I. In total, the number of antrum-forming follicles was 26, but 6 of the antral follicles did not contain an oocyte (Fig. 5K). Furthermore, in 6 of the antral follicles

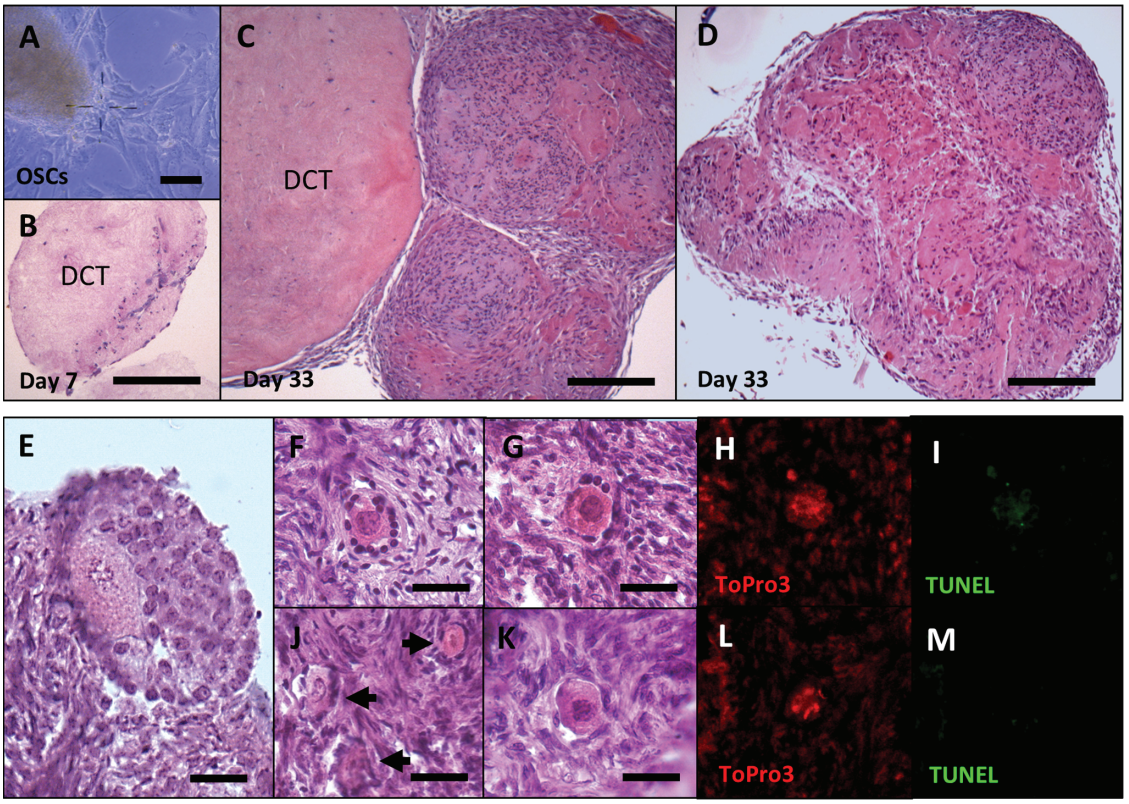

Figure 3 Re-cellularization of DCT with human OSCs and survival of re-seeded isolated human follicles in vitro. (**A**) Human OSCs in culture. Unstained. Scale bar: 50 µm. (**B**) A low degree of recellularization of DCT following 7 days of co-culture with OSCs. HE stain. Scale bar: 200 µm. (**C**) Partial and (**D**) complete recellularization of DCT following 33 days of co-culture with OSCs. HE. Scale bar: 200 µm. (**E–M**) Isolated human pre-antral follicles survived co-culture with OSCs in the recellularized DCT for 1 month. HE and terminal deoxynucleotidyl TUNEL. Scale bars: 50 µm. (E) Surviving secondary stage human follicle with homogeneous cytoplasm and absence of pyknotic nuclei. (F + G + K) Surviving primordial/primary stage human follicles, HE. (J) Degenerated primordial/primary stage human follicles within the recellularized DCT. (H + I) Images depict follicle (shown in G) stained with nuclear-marker (H) TO-PRO^{TM}-3 and (I) TUNEL showing no apoptosis. (L + M) Images depict follicle (shown in K) stained with nuclear-marker (L) ToPro3 and (M) TUNEL showing no apoptosis.

the nuclei of the oocytes were difficult to define, and in 6 of the follicles the ZP was very thin or permeated. Thus, only 8 of the recovered antral follicles contained morphologically healthy oocytes (Fig. 5K).

Discussion

This study has demonstrated an effective protocol for decellularization of human ovarian tissues while maintaining an intact ECM that was capable of supporting survival and growth of isolated pre-antral follicles *in vitro* and *in vivo*. This is the first time that isolated human follicles have survived in a decellularized human scaffold. Thus, this proof-of-concept could be a potential new strategy, to eliminate the risk of malignant cell reoccurrence in former cancer patients having cryopreserved ovarian tissue transplanted for fertility restoration.

In correspondence to the results reported by Laronda *et al.* (2015), we were able to decellularize human ovarian tissue using SDS as a cellular detergent. However, removing all malignant cells are of outmost importance and therefore, in addition to the detergent, we added DNase to the protocol to remove remnant nuclear material. Elimination of cells was verified with DNA quantification, histochemistry and immunofluorescence. DNA quantification showed that after 3 and 6 hours, the cortical and the medullary ovarian tissue, respectively, had a significant reduction of DNA. Examining the tissue histologically showed the remnants of cells until later stages and therefore decellularization should be performed 18 hours for medullary tissue and 24 hours for cortical tissue.

Human primordial follicles require a highly specialized environment to grow and mature and this environment regulates survival and function of the follicle profoundly (Woodruff and Shea, 2011). As the physical properties of the ovary direct follicle activation and growth, the ECM is a key component of the bioengineered ovary from decellularized tissue, and characterization of the DCT scaffold is imperative. Therefore, major components, collagen, laminin and fibronectin were chosen as evaluation parameters. Irrespective of whether medullary or cortical tissue was evaluated, no significant difference was found in collagen content either qualitatively or quantitatively in the two types of tissue after decellularization. But the variation in collagen content between patients were quite high. Overall, the ECM components were well preserved after decellularization as no apparent changes in the

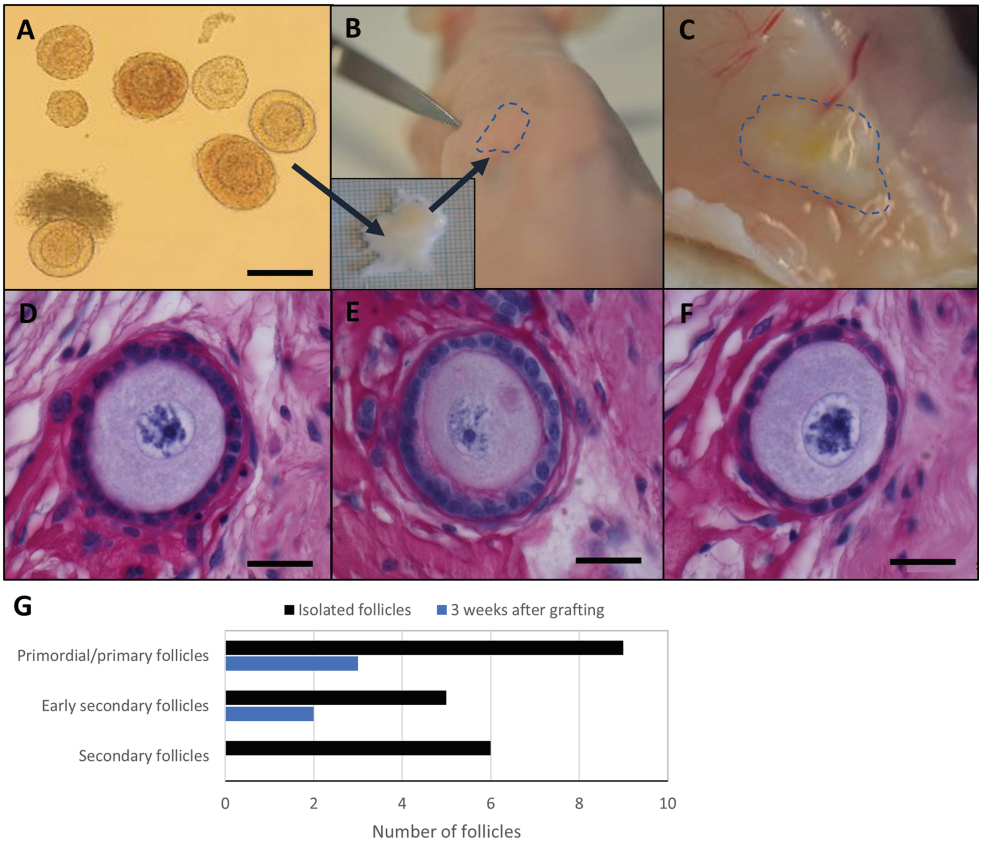

Figure 4 Survival of isolated human pre-antral follicles in human DCT following 3 weeks xenografting. (**A**) Enzymatically isolated pre-antral stage follicles. Scale bar: 100 µm. (**B**) Insert shows the decellularized ovarian scaffold reseeded with isolated pre-antral follicles, which were subsequently grafted to a subcutaneous pocket in the immunodeficient mouse. (**C**) The skin of the immunodeficient mouse with the xenograft/scaffold marked in the middle following 3 weeks of grafting. (**D–F**) Surviving pre-antral stage human follicles in the scaffold. PAS. Scale bar: 25 µm. (**G**) Distribution of follicle stages before (black) and after 3 weeks of grafting (blue). Primordial/primary follicles: 55–75 µm; Early secondary follicles: 75–100 µm; Secondary follicles: 100–160 µm.

distribution of laminin and fibronectin in decellularized medulla were observed. One unexpected observation was that the cortical tissue was cleared for DNA remnants faster than the medullary tissue. One possible explanation could be that medullary tissue contains more complex and heterogenous tissue structures than cortical tissue. Furthermore, the medullary pieces used in this study were more heterogenous in thickness compared to the cortical pieces, and this might contribute to the slower decellularization and higher variability observed during decellularization of medullary tissues.

A mixture of human ovarian cells and follicles was cultured in the DCT to assess the ability of the decellularized tissue to support survival and growth of ovarian cells *in vitro*. The results showed that cell adherence and proliferation was supported by the decellularized tissue. Thereby, proving the biofunctionality of the reseeded scaffolds. However, some areas of the tissue did not become recellularized highlighting that the recellularization is heterogenous and physical properties can lead to different potentials for supporting the cell growth.

Likewise, the studies of recellularization *in vivo* also supported these observations. Besides survival of both human and murine follicles, an evident growth of the murine follicles from pre-antral into antral follicles was observed. Thirty-nine percent of the murine follicles developed to antral follicles within the DCT, as their size and morphology differed considerably from the follicles reseeded. This is consequently the result of *in vivo* development, confirming previous studies where a decellularized bovine ovary (Laronda *et al.* 2015) and a gelatinous three-dimensional printed ovary supported murine follicle growth and restoration of ovarian function in ovariectomized mice (Laronda *et al.*, 2017).

The majority of recovered murine follicles contained oocytes with good morphology, but some of the oocytes were degenerating to some degree and a fairly large portion of follicles without oocytes was also observed in the retrieved grafts. This may be normo-physiological according to the concept of atresia. It is also plausible that lack of hormonal support led to degeneration of the more developed follicles. According to the whole concept of the bioengineered ovary, follicles are isolated without their theca cell layer or with very few surrounding theca cells. It is possible that the lack of supporting theca cells and hence lack of estrogen-precursors, renders the last, and gonadotropin-dependent, stages of folliculogenesis difficult. Although this would limit our current study set up, it may not be an issue in a clinical aspect. Just as is the case when autotransplanting cryopreserved ovarian tissue, a bioengineered ovary could be orthotopically grafted

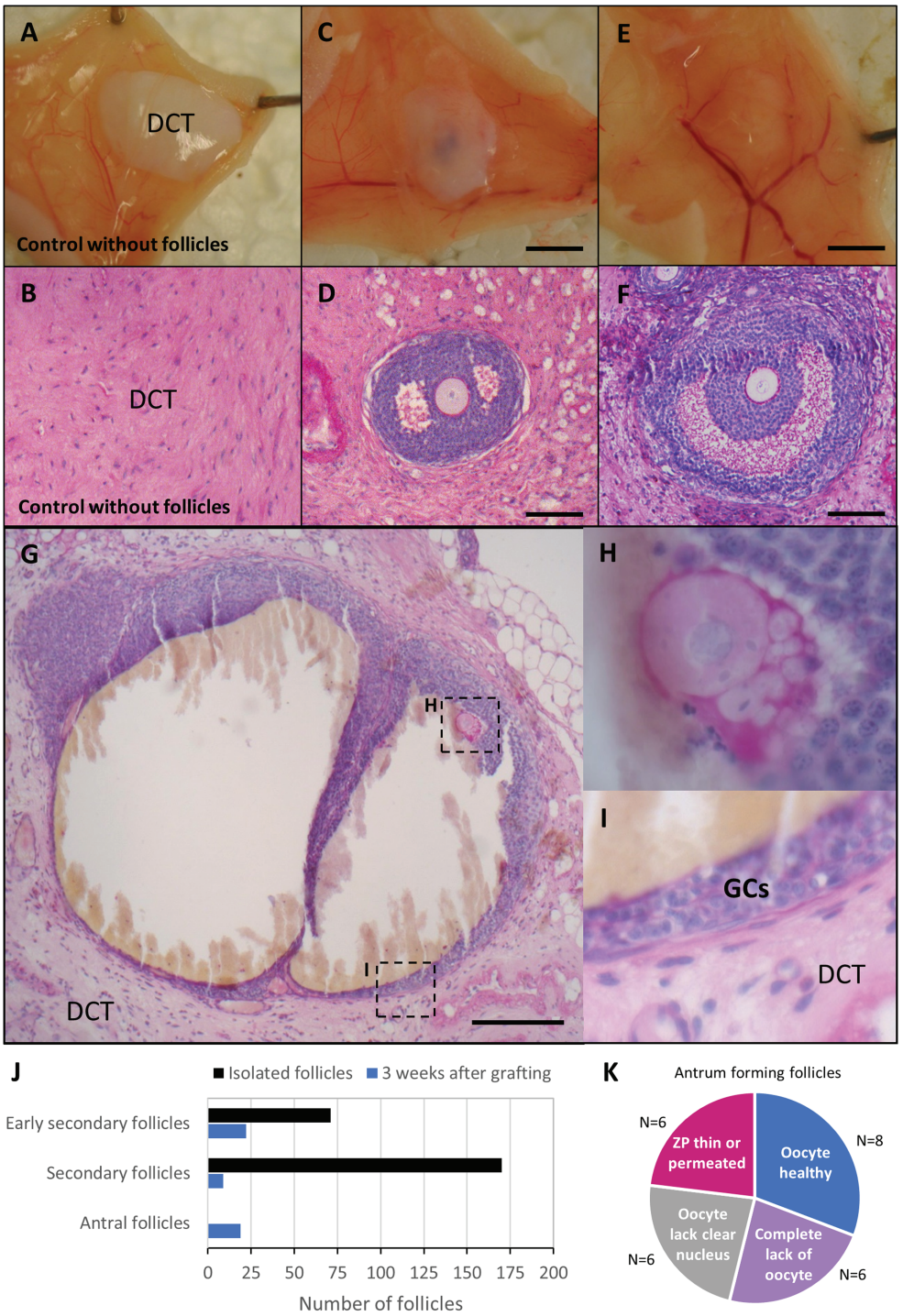

Figure 5 Graft recovery and antrum formation in isolated murine follicles reseeded on human DCT and xenografted for 3 weeks. (**A**) Sham-graft serving as a negative control comprising DCT without isolated follicles. (**B**) Histology of sham-graft found recellularized with murine cells following 3 weeks of grafting. PAS. (**C + E**) Examples of recovered grafts reseeded with murine pre-antral follicles. Scalebar: 3 mm. (**D + F**) Small antral stage murine follicles with good morphology oocytes found in the middle of the scaffolds. PAS staining. Scale bar: 100 µm. (**G**) Two large antral murine follicles. PAS. Scale bar: 200 µm. (**H**) Magnifcation of a compromised oocyte. (**I**) Magnification of the follicle wall showing the GC layer, the basal membrane and the lack of theca cells. (**J**) Distribution of murine follicle stages before (black) and after 3 weeks grafting (blue). Early secondary follicles: 68–100 µm; Secondary follicles: 100–180 µm; Antral follicles; >200 µm with antrum. (**K**) Number of antrum forming follicles with healthy oocytes, lacking oocytes, or compromised oocytes with lacking nuclei or permeated zona pellucida (ZP).

to a subcortical pocket in the remaining contralateral ovary. Void of cancerous cells and functional follicles, the remaining ovary still harbors stromal cells for theca cell recruitment by the bioengineered ovary. The potential fibrotic nature of the residual postmenopausal ovary might compromise stromal cell recruitment and could be considered a suboptimal environment for follicle growth. As an alternative, ovarian stromal cells could be harvested in a fresh ovarian biopsy from the contralateral ovary of the treated cancer patient in complete remission. Following adjacent reseeding with the isolated follicles, the assembled bioengineered ovary can then be autotransplanted to the patient. The feasibility of this has been demonstrated in an *in vitro* study, where isolated bovine ovarian stromal cells were shown to differentiate into theca cells after co-culturing with GCs (Orisaka *et al*., 2006).

Good general morphology and survival were observed in human follicles when grafted with Matrigel on human DCT. However, longer grafting periods are called for. Three-week grafting periods are sufficient for murine folliculogenesis but not for the protracted human folliculogenesis. The question of human follicles being able to survive and develop on DCT is of special interest. Murine studies are undoubtedly of use, and benefit from being faster with more easily accessible tissue material. However, murine follicles are more easily grown than isolated human follicles, which makes studies using isolated human follicles necessary. Isolated murine follicles have been shown to develop in non-ovarian follicle culture systems and to be capable of producing viable offspring (Eppig and O'Brien, 1996; Xu *et al*., 2009a; Mochida *et al*., 2013; Morohaku *et al*., 2016). One of the main issues of the bioengineered ovary is to determine which type of culture system and matrix is optimal to isolated human follicles for survival and continued follicular growth. The decellularized native matrix suggested in this study might not be optimal in its current state as supporting cells are needed to better mimic the follicular micro-environment and provide essential factors for survival and growth. In the current study survival and growth of only growing stages of pre-antral follicles were examined. It is reasonable to think that smaller stages of pre-antral follicles might survive better in cortical matrices and growing pre-antral follicles would survive better in medullary matrices. The current study provides a platform for such studies and it is of course especially interesting as a potential application for the smallest and most abundant stages of resting follicles.

Reseeding of isolated follicles proved to be a critical and difficult step in the reconstruction process and different seeding techniques were attempted; however, further developments of such techniques are needed to increase recovery and survival of the reseeded follicles. In the current study, recovery rates ranged from 2.5–47.5% per graft and on average 21% of the follicles were recovered, thus the need for a more effective reseeding technique is evident. Laronda *et al*. (2017) seeded isolated murine pre-antral follicles by micropipetting them directly onto gelatinous three-dimensional printed scaffolds followed by *in vitro* culturing for 4 days before xenografting. Whereas in their decellularization study, primary cultures of murine ovarian cells were seeded directly onto decellularized bovine ovarian scaffolds and cultured for 2 days before xenografting (Laronda *et al*., 2015). Thus, in both studies reseeded follicles/ovarian cells were cultured *in vitro* with the scaffolds to allow establishment of cell–cell and cell–matrix interactions before xenografting; however, no precise recovery rates were reported in the two studies (Laronda *et al*., 2015; 2017). The use of Matrigel in our study was helpful as we performed xenografting immediately after seeding; however, the reseeding is a technical challenge that needs to be overcome, especially when working with precious human material. Additionally, longer grafting studies are needed to evaluate growth of human follicles and the endocrine function of the recellularized scaffolds.

Looking further into the future of a safe bioengineered ovary for human use, a decellularized ovarian scaffold, cultured with patient-derived induced pluripotent stem cells, could be the key to personalized ovarian transplants for cancer patients and potentially for women of advanced reproductive age with a poor ovarian reserve.

In conclusion, these data provide the first steps toward creating an artificial human ovary in which the extracellular follicle niche is preserved, and viability and growth of early stages of isolated follicles are supported in a natural malignant-free three-dimensional scaffold.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

The authors wish to thank biomedical laboratory scientist Marianne Sguazzino for her excellent help in processing the ovarian tissue for histology. Dr Anne-Mette Bay Bjørn, Prof. Erik Ernst and Dr Kirsten Tryde Macklon are thanked for having consulted and recruited patients for the OTC program in Denmark, and all personnel involved in the clinical activities in fertility preservation are thanked for their passionate work. The women donating their ovarian tissue for research are also greatly appreciated.

Authors' roles

SEP, CYA and SGK planned the study. SEP, SGK, MR, DN, JC and KL executed the study and collected the data. SEP and SGK analyzed data, produced figures and wrote the first draft of the manuscript. All authors participated in critical discussion of the findings and revision of the manuscript.

Funding

This study is part of the ReproUnion collaborative study, co-financed by the European Union, Interreg V ÖKS. Furthermore, Project ITN REP-BIOTECH 675526 funded by the European Union, European Joint Doctorate in Biology and Technology of the Reproductive Health, the Research Pools of Rigshospitalet, the Danish Cancer Foundation and Dagmar Marshalls Foundation are thanked for having funded this study. The funders had no role in the study design, data collection and interpretation, or decision to submit the work for publication.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

Amorim CA, Shikanov A. The artificial ovary: current status and future perspectives. *Future Oncol* 2016;**12**:2323–2332.

Amorim CA, Van Langendonckt A, David A, Dolmans MM, Donnez J. Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix. *Hum Reprod* 2009;**24**:92–99.

Bruyneel AAN, Carr CA. Ambiguity in the presentation of decellularized tissue composition: the need for standardized approaches. *Artif Organs* 2017;**41**:778–784.

Caetano GF, Fronza M, Leite MN, Gomes A, Frade MAC. Comparison of collagen content in skin wounds evaluated by biochemical assay and by computer-aided histomorphometric analysis. *Pharm Biol* 2016;**54**:2555–2559.

Dittrich R, Hackl J, Lotz L, Hoffmann I. Beckmann MW pregnancies and live births after 20 transplantations of cryopreserved ovarian tissue in a single center. *Fertil Steril* 2015;**103**:462–468.

Dolmans MM, Michaux N, Camboni A, Martinez-Madrid B, Van Langendonckt A, Nottola SA, Donnez J. Evaluation of Liberase, a purified enzyme blend, for the isolation of human primordial and primary ovarian follicles. *Hum Reprod* 2006;**21**:413–420.

Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. *Blood* 2010;**116**:2908–2914.

Donnez J, Dolmans MM. Fertility preservation in women. *N Engl J Med* 2017;**377**:1657–1665.

Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 1996;**54**:197–207.

Gellert SE, Pors SE, Kristensen SG, Bay-Bjørn AM, Ernst E, Yding Andersen C. Transplantation of frozen-thawed ovarian tissue: an update on worldwide activity published in peer-reviewed papers and on the Danish cohort. *J Assist Reprod Genet* 2018;**35**:561–570.

Gook D, Westerman D, McBean M, Hughes V, Stern K. Potential leukemic contamination in cryopreserved ovarian tissue. *Proceedings of European Society of Human Reproduction and Embryology*. Barcelona, Spain, 2018; *O–81*.

Jakus AE, Laronda MM, Rashedi AS, Robinson CM, Lee C, Jordan SW, Orwig KE, Woodruff TK, Shah RN. 'Tissue papers' from organ-specific decellularized extracellular matrices. *Adv Funct Mater* 2017;**13**:27.

Kristensen SG, Giorgione V, Humaidan P, Alsbjerg B, Bjørn AB, Ernst E, Andersen CY. Fertility preservation and refreezing of transplanted ovarian tissue-a potential new way of managing patients with low risk of malignant cell recurrence. *Fertil Steril* 2017;**107**:1206–1213.

Kristensen SG, Rasmussen A, Byskov AG, Andersen CY. Isolation of pre-antral follicles from human ovarian medulla tissue. *Hum Reprod* 2011;**26**:157–166.

Laronda MM, Jakus AE, Whelan KA, Wertheim JA, Shah RN, Woodruff TK. Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials* 2015;**50**:20–29.

Laronda MM, Rutz AL, Xiao S, Whelan KA, Duncan FE, Roth EW, Woodruff TK, Shah RN. A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nat Commun* 2017;**8**: 15261.

Luyckx V, Dolmans MM, Vanacker J, Legat C, Fortuno Moya C, Donnez J, Amorim CA. A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold. *Fertil Steril* 2014;**101**:1149–1156.

Masciangelo R, Bosisio C, Donnez J, Amorim CA, Dolmans MM. Safety of ovarian tissue transplantation in patients with borderline ovarian tumors. *Hum Reprod* 2018;**33**:212–219.

McLaughlin M, Albertini DF, Wallace WHB, Anderson RA, Telfer EE. Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system. *Mol Hum Reprod* 2018;**24**:135–142.

Mochida N, Akatani-Hasegawa A, Saka K, Ogino M, Hosoda Y, Wada R, Sawai H, Shibahara H. Live births from isolated primary/early secondary follicles following a multistep culture without organ culture in mice. *Reproduction* 2013;**146**:37–47.

Morais GB, Viana DA, Silva FMO, Xavier Júnior FAF, Farias KM, Pessoa CD, Silveira JAM, Alves APNN, Mota MRL, Silva FDO *et al.* Polarization microscopy as a tool for quantitative evaluation of collagen using picrosirius red in different stages of CKD in cats. *Microsc Res Tech* 2017;**80**:543–550.

Morohaku K, Tanimoto R, Sasaki K, Kawahara-Miki R, Kono T, Katsuhiko Hayashi K, Hirao Y, Obata Y. Complete in vitro generation of fertile oocytes from mouse primordial germ cells. *Proc Natl Acad Sci USA* 2016;**113**:9021–9026.

Newton H, Illingworth P. In vitro growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. *Hum Reprod* 2001;**16**:423–429.

Oktay K, Bedoschi G, Pacheco F, Turan V, Emirdar V. First pregnancies, live birth, and in vitro fertilization outcomes after transplantation of frozen-banked ovarian tissue with a human extracellular matrix scaffold using robot-assisted minimally invasive surgery. *Am J Obstet Gynecol* 2016;**214**:94.e1–94.e9.

Orisaka M, Tajima K, Mizutani T, Miyamoto K, Tsang BK, Fukuda S, Yoshida Y, Kotsuji F. Granulosa cells promote differentiation of cortical stromal cells into theca cells in the bovine ovary. *Biol Reprod* 2006;**75**:734–740.

Paulini F, Vilela JM, Chiti MC, Donnez J, Jadoul P, Dolmans MM, Amorim CA. Survival and growth of human pre-antral follicles after cryopreservation of ovarian tissue, follicle isolation and short-term xenografting. *Reprod Biomed Online* 2016;**33**:425–432.

Pearse AGE. *Histochemistry. Theoretical and Applied*, Vol. 2, 4th edn. Edinburgh: Churchill Livingstone, 1985,157–158

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rodgers RJ, Irving-Rodgers HF, Russell DL. Extracellular matrix of the developing ovarian follicle. *Reproduction* 2003;**126**:415–424.

Rosendahl M, Schmidt KT, Ernst E, Rasmussen PE, Loft A, Byskov AG, Andersen AN, Andersen CY. Cryopreservation of ovarian tissue for a decade in Denmark: a view of the technique. *Reprod Biomed Online* 2011;**22**:162–171.

Rosendahl M, Andersen MT, Ralfkiaer E, Kjeldsen L, Andersen MK, Andersen CY. Evidence of residual disease in cryopreserved ovarian cortex from female patients with leukemia. *Fertil Steril* 2010;**94**:2186–2190.

Schmidt KT, Larsen EC, Andersen CY, Andersen AN. Risk of ovarian failure and fertility preserving methods in girls and adolescents with a malignant disease. *BJOG* 2010;**117**:163–174.

Shapira M, Raanani H, Barshack I, Amariglio N, Derech-Haim S, Marciano MN, Schiff E, Orvieto R, Meirow D. First delivery in a leukemia survivor after transplantation of cryopreserved ovarian tissue, evaluated for leukemia cells contamination. *Fertil Steril* 2018;**109**:48–53.

Silber SJ, DeRosa M, Goldsmith S, Fan Y, Castleman L, Melnick J. Cryopreservation and transplantation of ovarian tissue: results from one center in the USA. *J Assist Reprod Genet* 2018;**35**:2205–2213.

Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, Picton HM, Plancha C, Shea LD, Stouffer RL *et al*. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. *Hum Reprod Update* 2010;**16**:395–414.

Telfer EE, Zelinski MB. Ovarian follicle culture: advances and challenges for human and nonhuman primates. *Fertil Steril* 2013;**99**:1523–1533.

Vanacker J, Dolmans MM, Luyckx V, Donnez J, Amorim CA. First transplantation of isolated murine follicles in alginate. *Regen Med* 2014;**9**:609–619.

Woodruff TK, Shea LD. A new hypothesis regarding ovarian follicle development: ovarian rigidity as a regulator of selection and health. *J Assist Reprod Genet* 2011;**28**:3–6.

Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. In vitro follicle growth supports human oocyte meiotic maturation. *Sci Rep* 2015;**27**: 17323.

Xu M, Banc A, Woodruff TK, Shea LD. Secondary follicle growth and oocyte maturation by culture in alginate hydrogel following cryopreservation of the ovary or individual follicles. *Biotechnol Bioeng* 2009a;**103**:378–386.

Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK. In vitro grown human ovarian follicles from cancer patients support oocyte growth. *Hum Reprod* 2009b;**24**:2531–2540.

Yin H, Kristensen SG, Jiang H, Rasmussen A, Andersen CY. Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture. *Hum Reprod* 2016;**31**:1531–1539.