Reconstruction of the ovary microenvironment utilizing macroporous scaffold with affinity-bound growth factors

Shani Feldera, Hila Masasaa, Ayelet Orenbuchb, Noam Levaotb,c, Michal Shachar Goldenberg d, Smadar Cohena,c,e,∗

a Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel
b Department of Physiology and Cell Biology, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel
c Regenerative Medicine and Stem Cell (RMSC) Research Center, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel
d Department of Chemical Engineering, Shamoon College of Engineering, Ashdod, 77245, Israel
e The Ilse Katz Institute for Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel

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ABSTRACT

Implementing ovarian tissue engineering for the maturation of primordial follicles, the most abundant follicle population in the ovary, holds great potential for women fertility preservation. Here, we evaluated whether macroporous alginate scaffolds with affinity-bound bone morphogenetic protein-4 (BMP-4) could mimic the ovary microenvironment and support the culture and growth of primordial follicles seeded with supporting ovarian cells. Porcine primordial follicles developed in the alginate scaffolds up to the pre-antral stage within 21 days. Affinity-bound BMP-4 significantly contributed to follicular maturation, as evident by the 5-fold increase in the number of developing follicles and enhanced estradiol secretion in these cultures compared to when BMP-4 was added to cultures with no affinity binding. After 21 days in culture, an increase in GDF-9/AMH gene expression, which is correlated with follicular development, was statistically significant when BMP-4 was affinity bound, compared to all other scaffold groups. When developed in-vivo, after xeno-transplantation of the follicle devices supplemented with additional angiogenic factors, the follicles reached antral size and secreted hormones at levels leading to restoration of ovarian function in ovariectomized severe combined immunodeficiency (SCID) mice. Altogether, our results provide first affirmation for the applicability of macroporous alginate scaffolds as a suitable platform for promoting follicle maturation in-vitro and in-vivo, and lay the foundations for the advantageous use of affinity binding presentation of growth factors to cultured follicles.

1. Introduction

Preservation of female fertility, in light of pathological situations such as cancer, is among the main challenges engaged by researchers and clinicians over the last two decades. Unfortunately, the leading clinical fertility preservation treatments as of today - embryo or mature oocyte cryopreservation - are not always applicable, such as in cases when patients are pre-pubertal or require immediate treatment. Today, the prominent alternative in such cases is the cryopreservation and re-transplantation of ovarian tissue. Over the last decade, 130 births were obtained world-wide by this technique, and the birth rate per year is exponentially increasing [1]. However, this method includes an inherent risk of reseeding cancer cells to the patient, especially in cases of blood malignancies [2]. To avoid this risk, and to improve the yields of fertilizable oocytes, an emerging approach is the in-vitro maturation of early stage follicles (primary and primordial) which account for the vast majority of follicles in females at all ages [3].

Thus far, culture of primordial follicles has been performed either by cortical tissue culture (‘in-situ’), or by culture of the isolated follicles within a material matrix [4]. In spite of encouraging results, in-situ culture entails a difficulty in controlling the follicle environment, and the size to which follicles can grow within the pieces of tissue is limited to that of secondary follicles [5,6]. The birth of live pups, originating from a two-step in-vitro culture of primordial follicles was reported in 1996 in mice, yet success has not been translated to other species [7]. Recently, fertilizable metaphase II oocytes were obtained from human unilaminar follicles cultured in a multi-step culture system [8].

Over time, the importance of recapitulating the follicular natural
environment has been highlighted [4,9], shifting efforts from individual encapsulation of follicles, to cultures of follicles in large groups [10] with the addition of supporting ovarian cells [11]. While alginate hydrogel was widely used for individual or group encapsulation of several follicles, utilization of the robust macroporous alginate scaffold, obtained by the freeze-dry technique, has hardly been explored. The macroporous alginate scaffold is structurally different from the hydrogel; it is characterized with a pore size of 80–100 μm in diameter and a greater pore interconnectivity, thus potentially allowing the growth of follicle in size, the cultivation of multiple follicles in one device and importantly, it enables the device vascularization after implantation. In addition, the macroporous scaffolds display a greater stiffness (by at least one order of magnitude) and stability in culture and after transplantation compared to the alginate hydrogel [12,13]. All these beneficial effects were the main motivation of our group to utilize these scaffolds as 3D culture systems for various soft tissues [14–17], and for the culture of cortical tissue [18].

In the present paper, we investigated the appropriateness of the macroporous alginate scaffold for the cultivation and maturation of primordial follicles. As in this early stage, the follicles rely on the supplementation of growth factors for viability and growth, the alginate scaffold was constructed with alginate sulfate, to incorporate the growth factors by affinity binding and present them to the cultured follicles in a similar manner to their natural presentation by extracellular matrix (ECM). Previously, we used this strategy for the promotion of myocardial repair [19], osteochondral regeneration [20] and immunomodulation in-vivo [21].

The growth factors (GFs) secreted by the immediate cellular environment of primordial follicles dominate the follicular survival, latency and activation [22]. Many of these GFs belong to the transforming growth factor-β (TGF-β) superfamily, also known as heparin-binding proteins, due to their prefered presentation as affinity bound to heparan-sulfate (HS) in ECM [23,24]. Growth and differentiation factor-9 (GDF-9) is an oocyte-derived GF, shown to increase survival of early stage follicles and promote maturation to secondary stage from primordial follicles in goat [25]. Bone morphogenetic protein-4 (BMP-4) is expressed by stromal cells, and its receptors are present in follicles starting from fetal stages in both pigs [26] and humans [27]. BMP-4 has been shown to enhance primordial to primary follicular transition in rodents [28,29], and to stimulate growth of primary follicles in bovine ovarian tissue culture [30]. BMP-4 also promoted granulosa cell survival when exogenously added to culture [31]. Anti-mullerian hormone (AMH) is a growth factor with an inhibitory role. It is secreted by granulosa of developing follicles to inhibit activation of neighboring follicles and maintain the follicular reserve [32].

Herein, we aimed to reconstruct a physiologically relevant cultivation system for the in-vitro maturation of porcine primordial follicles, by employing a macroporous alginate scaffold with relevant pore size and interconnectivity, and impregnated with BMP-4 as affinity bound to the matrix via interaction with alginate sulfate (AlgS). The primordial follicles, co-seeded with stromal ovarian cells in the scaffold, denoted a follicle device, were cultivated and followed for 21 days. In a proof-of-concept implantation study, the follicle device included additional affinity bound angiogenic factors, vascular endothelial growth factor (VEGF) and platelet derived growth factor-β (PDGF-β), to accelerate device vascularization and enhance the viability of the transplanted cells. The in-vivo results indicated restoration of the ovarian function in ovariectomized mice, as evidenced by the high hormone levels found in serum and appearance of the vaginal area.

2. Materials and methods

2.1. Materials and animals

Sodium alginates (VLVG, LVG, > 65% guluronic acid monomer content) were from FMC Biopolymers (Drammen, Norway). Alginate sulfate (AlgS) was synthesized from sodium alginate (VLVG) as previously described [33]. Human recombinant Bone Morphogenetic Protein-4 (BMP-4), Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF-β) were purchased from Protech (Rocky Hill, NJ). Growth and Differentiation factor-9 (GDF-9) was purchased from Sigma Chemical Co. (St. Louis, MO). BMP-4 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems Inc. (Minneapolis, MN). Menopur, containing follicle stimulating hormone (FSH) was kindly donated by Ferring Pharmaceuticals LTD (Kiel, Germany). Culture media and supplements were purchased from Biological Industries, (Kibbutz Be’er Ha’Emek, Israel), unless otherwise specified. DNAase type 1 was purchased from Worthington (Lakewood, NJ). Gibco ACK lysis buffer was purchased from Thermo Fisher Scientific (Grand Island, NY) All other salts, reagents and enzymes were of analytical grade from Sigma-Aldrich (Rehovot, Israel), unless specified otherwise. Culture medium was Minimum Essential Medium Eagle alpha (α-MEM) supplemented with ribonucleotides (0.2% v/v), sodium pyruvate (2% v/v) and penicillin-streptomycin amphotericin (1% v/v).

Seven week old female C.B-17/IcrHsd severe combined immunodeficiency (SCID) mice were purchased from Envigo (Jerusalem, Israel). Mice were housed at the animal facility of the Ben-Gurion University Medical Center (Beer-Sheva, Israel) in autoclaved cages, with autoclaved bedding, food, and water. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ben-Gurion University of the Negev.

2.2. Affinity binding of BMP-4 to AlgS

Surface Plasmon Resonance (SPR) analysis for BMP-4 binding onto immobilized AlgS was carried out as previously described [20,33] using the BIACore 3000 instrument (Pharmacia, Uppsala, Sweden). Biotinylated samples of AlgS and pristine alginate were immobilized onto a streptavidin (SA)-modified sensor chip. Binding measurements were performed over a range of BMP-4 concentrations (50–250 nM). Dissociation equilibrium constants were calculated by nonlinear curve fitting of the primary sensorgrams using the Langmuir binding model with respect to mass transfer and drifting baseline available in the BIA evaluation 3.2 Software. Goodness of fit was indicated by chi 2 < 10.

2.3. Co-assembly of AlgS with BMP-4

BMP-4 in citric acid (10 mM, pH 3) was incubated with AlgS solution for 1.5 h at 37 °C to enable binding and spontaneous co-assembly, as previously described [34,35]. The BMP-4:AlgS molar ratio was 1:10, with concentrations of 1.5 μM:15 μM for examination in Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) and concentration of 4.2 μM:42 μM for Nano tracking analysis and surface charge measurements. TEM imaging was conducted at room temperature (RT) using a FEI Tecnai 12 G2 TWIN TEM (Gatan, Pleasanton, CA) at acceleration voltage of 120 kV. AFM images (Scanning Probe Microscopy SPM, Dimension – 3100, Veeco), were taken in tapping mode with a scan size of up to 2 μm, at a scan rate of 1 Hz. The apparent diameters of the particles were determined by automated grid analysis using SPM software of the instrument.

Nanoparticle tracking analysis (NTA) was utilized to evaluate the co-assembly to complexes between AlgS and GFs with an emphasis on volume concentration and wet size distribution. Complexes were analyzed with the NanoSight NS300 instrument (Malvern Instruments Ltd., Worcestershire, UK). All samples were analyzed under × 20 objective, and 5 separate 60s video clips were taken. Software version NTA 2.3 was used for capture and analysis. The surface charge (ζ potential, mV) of the complexes was measured on a Zetasizer Nano ZS (Malvern Instruments Ltd.), three times, each run at 11 25 °C, 10 to 100 measurements were taken, depending on standard deviation. The zeta cell was used was DTS 1060 (produced by Malvern Instruments Ltd.).
2.4. Fabrication of BMP-4/Alg-S affinity binding alginate scaffolds

Four types of macroporous scaffolds were fabricated from: (i) pristine alginate; (ii) alginate/AlgS (no factor); (iii) pristine alginate with adsorbed BMP-4; and (iv) alginate/AlgS with affinity bound BMP-4. Recombinant human BMP-4 has previously displayed bioactivity in porcine models [36]. The scaffolds, 5 mm in diameter and 1-mm thickness, were fabricated by the freeze-dry technique as previously described [16]. In brief, sodium alginate (L VG) was crosslinked by gradual addition of g-glutamic acid/hemicalcium salt solution (D-glucosamine hydrochloride (4%) in Dulbecco's Modified Eagle Medium (DMEM), v/v) for 7 min, washed twice in DMEM buffer, and permeabilized using Triton X-100 (0.2% v/v in DMEM buffer). For morphological visualization, the F-actin fibers were stained using Alexa-Fluor 546-conjugated phalloidin (1:200, Molecular Probes, Invitrogen, Karlsruhe, Germany). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:180; Molecular probes, Invitrogen, Karlsruhe, Germany). Imaging of the cell constructs was performed with Nikon C1si laser scanning confocal microscope.

2.8. Viability of cultured follicles

For viability assessment, follicles were isolated from scaffolds by dissolving the scaffold as described above. A fraction (0.25) of the scaffold was transferred to new tube and stained with live/dead kit (Calcine AM/ethidium homodimer-1, Molecular probes, Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Follicles were imaged under Nikon C1si laser scanning confocal microscope. Only fully alive follicles were counted as live. Follicles with either a dead oocyte, over 10% dead granulosa cells, or fully dead follicles were counted as dead. Counting was performed using the NIS-Elements software (Nikon).

2.9. mRNA gene expression analysis

Gene expression levels were evaluated using quantitative PCR (qPCR). RNA extraction was done using the EZ-RNA kit (Biological industries). Reverse transcription (cDNA synthesis) was performed using High Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems) according to the manufacturer's protocol, with an initial amount of 500 ng RNA (measured by NanoDrop™). GDF-9, and Anti-Müllerian hormone (AMH) Taqman gene expression assays were conducted, and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). mRNA levels were determined by real-time PCR using StepOnePlus™ Applied detection system according to the manufacturer's instructions (Applied Biosystems). Each 10 μL reaction contained 2 μL cDNA (50 ng).

2.10. Evaluation of estradiol-2 (E2) and progesterone-4 (P4) secretion

Steroid hormone secretion was evaluated by enhanced estradiol and progesterone chemi-immunofluorescence assay (ADVIA-Centaur chemistry analyzer, Siemens Healthcare Diagnostics, Los Angeles, CA). Thresholds of sensitivity for E2 and P4 levels were 11.7 pg/mL and 0.3 ng/mL, respectively. The assays were conducted in the endocrinology unit, Soroka Health Center (Beer Sheva, Israel), according to the clinical protocol.

2.11. Ovariectomy and grafting under the kidney capsule

The experiments were conducted at the animal facility of Ben-Gurion University of the Negev, under an ethical committee approved protocol, in accordance with local legislation and guidelines.

Table 1

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<th>Taqman probes for gene expression assay.</th>
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Bi-lateral ovariectomy (OVX) was performed on 8-week old female SCID mice, to reduce native ovarian function and simulate ovarian failure. To validate successful ovariectomy, mice were examined for vaginal closing every other day for the duration of 14 days [38].

Twenty-four hours prior to transplantation, porcine primordial follicles and ovarian cells (OCs) were isolated as described above and seeded in two scaffold types: (i) Alginate/AlgS, and (ii) Alginate/AlgS with affinity bound BMP-4 and GDF-9 (recombinant human, which showed bioactivity on porcine oocytes [39]), 200 ng of each GF. VEGF and PDGF-ββ (200 ng of each per scaffold) were added to both scaffold types to encourage vascularization, as was done in a similar implant study [21]. Third group consisted of ovarioctomized mice that were not grafted (untreated). The ovarioctomized mice were randomly assigned to one of two transplantation groups, or to the untreated control group. Mice were anesthetized and the follicle devices were implanted under the capsule of the left kidney (n = 4 per treatment). Kidney was previously shown to be an advantageous site for grafting of follicles [40]. All mice presented a quick recovery and remained healthy throughout the experiment.

Fourteen days post transplantation, the kidney, together with the implanted follicle devices were surgically removed and immediately fixed in 4% paraformaldehyde (4% w/v in DMEM-based buffer). Blood was collected by cardiac puncture, serum was separated and analyzed for steroid hormone levels as described in 2.10.

2.12. Histological analysis of the graft

The kidney with the grafted follicle devices in 4% Paraformaldehyde (PFA) were transferred to sucrose solution (30% in DMEM based buffer) for 48 h, embedded in Optimal Cutting Temperature compound (OCT) and stored at −80 °C. Horizontal cross-sections (12 μm thick) were cut with a cryostat (Leica CM3050 S) and kept at −20 °C until use. Prior to staining, the primary antibody diluting buffer was used to block nonspecific binding. Sections were then incubated with a primary monoclonal anti-mouse CD31 antibody (Abcam; cat ab28364) to visualize vascularization or FOXO-3A to visualize primordial follicles (Cells signaling, cst2497S) and stained with Alexa Flour 633-conjugated anti-rat IgG (Biolegend; cat 405416).

2.13. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA). All variables are expressed as mean ± SEM. Differences in surface ζ-potential were compared by a two-tailed unpaired t-test. Statistical analysis of the Gene expression and in-vitro estradiol secretion data was performed by a two-way analysis of variance (ANOVA); Sidak’s post hoc test was carried out to determine differences between the treatments. Statistical analysis of the amount of developing follicles and hormone secretion was performed by a one-way ANOVA, Dunnet’s post hoc test was carried out to determine differences between the treatments. p < 0.05 was considered statistically significant.

3. Results

3.1. Affinity binding of BMP-4 to AlgS and complex assembly

BMP-4 binding to alginate-sulfate (AlgS) was evaluated by Surface Plasmon Resonance analysis (SPR, Fig. 1a and b). Reversible high-affinity binding of BMP-4 to AlgS was demonstrated over a range of BMP-4 concentrations (Fig. 1a), while BMP-4 did not show affinity binding to pristine alginate (Fig. 1b). The equilibrium dissociation constant (K_D), after fitting the data to the Langmuir 1:1 binding model, was 47 nM, indicative of high affinity binding of BMP-4 to AlgS, at the same magnitude as BMP-4 binding to its native presenter in the ECM, heparin [20].

In solution, BMP-4 spontaneously co-assembled with AlgS to form nano-complexes, as observed by both TEM (Fig. 1c and d) and AFM (Fig. 1e) analyses. The TEM images reveal near spherical particles for complexes of BMP-4 and AlgS (Fig. 1d), while free AlgS showed weak, spread-out linear patterns, probably due to the low contrast of the spread polymer (Fig. 1c). AFM images also demonstrated the formation of spherical particles.

In their wet-state, the complexes had a relatively narrow size distribution with an average diameter of 107 nm, as observed by the nanotracking analysis (NTA) (Fig. 1f). Complexes were not observed when evaluating each of the components separately in solution. The co-assembly of AlgS with BMP-4 into particles also resulted in a significant change in their surface charge (Fig. 1g); the ζ-potential increased from −46 ± 4 mV for the free AlgS polymer, to −33 ± 4 mV for the AlgS/BMP-4 complexes, due to the complexion with the slightly positively charged protein (pI = 7.2, as calculated from http://isoelectric.ohv.org based on amino-acid sequence).

3.2. Scaffold characteristics: porosity, pore size, stiffness and BMP-4 content

The internal structure of the macroporous alginate scaffolds, visualized by SEM, resembled that of a decellularized porcine cortical piece (Fig. 2). The average pore diameter found in alginate scaffolds was 75 ± 26 μm, similar in magnitude to the average value of 93 ± 47 μm for pores located in cortical areas of the ovary (Fig. 2a and b). The incorporation of AlgS (10% w/w total polymer) into scaffolds did not change the internal porous morphology, yielding similar porosity, with a mean diameter of 75 ± 37 μm (Fig. 2c). AlgS was homogenously spread throughout the entire scaffold volume, as verified using elemental analysis for the presence of sulfur through different cross-sections of the scaffold by energy-dispersive x-ray spectroscopy (EDS, Fig. S1).

The Young’s modulus of the macroporous scaffold was ~2 kPa and it was similar for pristine alginate and alginate/AlgS scaffolds (Shaley, unpublished results), and 10 fold greater compared to alginate hydrogel (without lyophilization) [41]. In comparison, the Young modulus measured for native porcine cortical tissue, known for its rigidity, was two orders of magnitude higher, approaching 600 kPa. Young’s modulus of human ovaries was evaluated in-vivo in a recent study to circle 6–9 kPa, yet the measurements were done while excluding the cortex, leading to the assumption that the actual stiffness is higher [42].

The amount of BMP-4 loaded into the scaffold was significantly greater in the alginate/AlgS scaffolds, 65.73 ± 1.42 ng per scaffold, compared with that in pristine alginate scaffold, 51.84 ± 5.62 ng BMP-4, due to the affinity binding of BMP-4 to the matrix (Fig. 2d). The affinity binding to scaffold also resulted in a greater retention of the BMP-4 and its prolonged presentation over 21 days in culture, compared to the pristine alginate scaffold (p < 0.05, two-way ANOVA) (Fig. 2e).

3.3. Culture of primordial follicles in BMP-4 enriched macroporous scaffold

Four types of macroporous scaffolds were investigated as culture systems for the primordial follicles; they were fabricated from (i) pristine alginate (no GFs), (ii) alginate/AlgS (no GF), (iii) pristine alginate with supplementation of BMP-4, and (iv) alginate/AlgS with affinity bound BMP-4.

Isolated porcine primordial follicles (a representative image in Fig. 3a) were seeded together with supporting ovarian cells (OCs) into the macroporous scaffolds, and visualized for cytoskeleton (F-actin, red) GDF-9 (green) and nuclei (blue) using LSCM to observe their location in the matrix pores, at seeding (Fig. 3b) and after culture (Fig. 3c). The primordial follicles were distributed throughout the entire scaffold volume, mainly located in the pores, and the scaffold interconnected porous structure supported the culture of follicles in high
proximity to each other and with the support of surrounding ovarian cells. In the ovarian cortex, the primordial to primary follicles (labeled with foxo3A (green)) are nested in clusters, surrounded by supporting ovarian cells, mainly stromal cells (Fig. 3d). Fig. 3e and f shows that the two macroporous scaffolds, made of pristine alginate and alginate/AlgS, supported a similar arrangement of the primordial follicles. The cell packing density approached \(40 \times 10^6\) cells/mL, a value close to the cell density in the porcine ovary and comparable to ovaries of other domestic animals [43].

Initially, the seeded follicular population was comprised exclusively...
of follicles under 30 μm in size (Fig. 3b). Over 21 days in culture, the scaffolds successfully supported the gradual growth of follicles, resulting in a broad distribution of sizes, ranging from the initial average diameter of 25 μm and up to over 100 μm (Fig. 4a and b). Still, after 21 days in culture, the majority of follicles were under 35 μm in all culture systems (Fig. 4b). Analysis of follicle development was done based on morphological inspection, and a follicle was considered “developing” if the follicular diameter was larger than 70 μm, the characteristic size for porcine primary follicles [44]. Based on this criterion, the number of developing follicles was much greater (p < 0.01) in scaffolds containing BMP-4 as affinity bound to AlgS, compared to the other culture groups (Fig. 4c).

Maturation of the cultured follicles was also assessed by visualizing their three dimensional inner morphology, while in scaffold and after their release from the dissolved scaffolds. We observed follicular development starting from early stages, when follicles are characterized with only a few, sparse cells surrounding a centered oocyte (Fig. 4d) and up to developed stages, when cells surrounding the oocyte proliferate and acquire a cuboidal shape (Fig. 4e). More developed follicles reached the secondary stage, indicated by the formation of several layers surrounding the oocyte (Fig. 4f). Follicles released from the scaffolds were intact, and maintained spherical appearance (Fig. 4g and h). Yet, while the large follicles revealed layers of surrounding GCs, no budding of antral cavity was observed, and oocytes were still centered (Fig. 4i).

3.4. Follicular viability during culture

Roughly > 80% of follicles remained viable after 21 days in culture, for all examined scaffold compositions (Fig. 5). Only completely alive follicles were evaluated as alive (Fig. 5a), while those with over 10% dead granulosa cells (Fig. 5b, marked red) or oocytes (Fig. 5c, marked red), were referred to as dead. Scaffold composition did not seem to affect follicular viability; ~87% follicles cultured in pristine alginate scaffolds were viable after 14 days, similar to the average value obtained when scaffolds were supplemented with affinity bound BMP-4 (91%). A moderate decrease in viability was observed between days 14 and 21, for all culture groups (Fig. 5d). The reduction may be a result of limited transport of the assay reagents into large follicles, as displayed weak signal of calcein was evident in developed follicles (Fig. S2).

3.5. Gene expression and hormone induction during culture

The functionality of the developing follicles cultured in-vitro, was evaluated by following the gene expression pattern of selected follicle markers, as well as the ability to produce and secrete estradiol-17β (E2). GDF-9 and AMH mRNA gene expression levels are known to rise during early stages of follicular development [45], and were hence chosen for the evaluation of follicle development and functionality (Fig. 6).

On day 14 in culture, the transcript expression levels of GDF-9 (Fig. 6a) and AMH (Fig. 6b) were not significantly different in the follicles cultured within the different scaffold types, with or without the addition of BMP-4. By day 21 in culture, the effect of the affinity bound BMP-4 on GDF-9/AMH gene expression was statistically significant (p < 0.0005) compared to all other scaffold groups, showing 15.3-fold and 35.9-fold increase for GDF-9 and AMH, compared to follicle culture within pristine alginate. The increase was lesser but significantly different compared to culture in alginate/AlgS scaffold with no BMP-4 supplementation.

E2 levels secreted to culture media (Fig. 6c) measured on day 14 in culture, were 50 pg/mL in the media of follicle culture in scaffolds made of pristine alginate (with or without the addition of BMP-4), and two-fold higher levels were detected in the alginate/AlgS scaffold culture, with or without BMP-4. On day 21 in the pristine alginate scaffold culture, E2 secretion was negligible, while it significantly increased, reaching an average of 228 pg/mL in the alginate/AlgS scaffold culture with affinity bound BMP-4 and 201 pg/mL in the alginate/AlgS scaffold culture with no exogenously supplemented BMP-4.
3.6. Transplantation of GF enriched macroporous follicle device for restoration of hormonal activity in-vivo

Next, we evaluated the development and functionality of primordial follicles seeded in the scaffold, under the complexity of the physiological environment, in-vivo. Two scaffold types, which exhibited the best follicle maturation in-vitro were chosen, the alginate/AlgS (no added BMP-4 or GDF-9), and alginate/AlgS with affinity bound BMP-4, and with GDF-9 (each 200 ng per scaffold). A third group of ovariectomized mice was not grafted and used as control. The addition of GDF-9 was due to the fact that the follicle devices were implanted 24 h after follicle isolation and seeding. It is known that GDF-9 has anti-apoptotic activity during early follicular growth [46], and promotes primordial follicle survival in-vivo [47]. To enhance vascularization and integration of the follicle devices, VEGF and PDGF-ββ (each 200 ng per scaffold) were affinity-bound to the constructs. The primordial follicles were seeded onto the scaffolds after isolation and cultured for 24 h prior to transplantation.

SCID mice were ovariectomized two weeks prior to xenografting the follicular devices, in order to reduce steroid hormone production. Success of ovariectomy was evident through closing of the vaginal orifice, dryness and lack of swelling for at least 4 days prior to transplantation, as familiar for the diestrus phase of the estrus cycle [38]. Estradiol was not detected in serum of these mice, 2 weeks after ovariectomy.

Xenotransplantation of the two follicle-seeded devices resulted in restoration of systemic estradiol and progesterone levels in the mice serum (Table 2), measured 14 days after transplantation. This was a statistically significant effect when compared to the untreated ovariectomized mice group. The hormone levels were undetectable or minimal for all of the untreated mice, thus providing further evidence for the success of the ovariectomy process. In the treated mice, the estradiol levels were restored to the 10–25 pg/mL levels, as found in healthy reproductive SCID mice in the estrus cycle [48]. Ovarian function after grafting was also indicated by changes in the vaginal area. All mice resumed cyclic appearance, evident by re-opening of the vaginal orifices, swelling and wetness (Fig. S3). Notably, no significant difference in all parameters was observed between the two treatment groups.

In an attempt to locate the follicles within the graft, the kidney with the follicle devices attached to it were excised, fixed, cryosectioned and immunostained (Fig. 7). Most of the grafted devices were found intact in proximity to the kidney (Fig. 7a and b). Histological section of the graft (a representative image of BMP-4 and GDF-9 enriched graft is presented in 7b) demonstrates graft appearance and proximity to the kidney 14 days after transplantation. Cellularization is demonstrated by the red staining of cytoskeletal F-actin (Phalloidin) and blue staining of nuclei (DAPI), kidney is green due to host auto fluorescence.

Fox0-3A is known to be present within oocytes of early stage follicles [49] (Fig. 7c, positive control ovary) and we used this staining to
Fig. 5. Survival of primordial follicles cultured in alginate based scaffolds. Follicles were isolated from scaffolds and double stained by two markers: calcein which labels viable cells (green), and ethidium homodimer-1 which labels DNA of dead cells (red). (a) Example of fully live follicles. Bar = 50 μm (left), 25 μm (right). (b) Follicle with over 10% dead granulosa cells (arrowhead). Bar = 50 μm. (c) Degenerating follicle with dead oocyte. Bar = 10 μm. (d) Viability rate, expressed as the percent of viable follicles from all measured follicles (Mean ± SE). Over 100 follicles were analyzed per culture system. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Functionality of cultured cells and follicles. (a) GDF-9 and (b) AMH gene expression levels after 21 days in culture. For all experiments, GAPDH served as housekeeping reference. Results are relative to the expression on day 7 and to expression of pristine scaffolds to eliminate inter-experiment variability. (c) E2 levels in the culture medium of scaffolds after 14 and 21 days of culture. *, p < 0.05, **, p < 0.005, ***, p < 0.0005, ****, p < 0.0001 (multiple comparisons two-way ANOVA, Sidak post-test).

Table 2
Hormone serum levels. Blood serum levels of estradiol and progesterone were evaluated 14 days after transplantation. Total of 4 animals were evaluated per group.

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<th>O VX + scaffolds w/GF</th>
<th>O VX + scaffolds w/O GF</th>
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<tr>
<td>Estradiol (pg/mL)</td>
<td>ND</td>
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<td>Progesterone (ng/mL)</td>
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<sup>a</sup> p < 0.05, one-way ANOVA.
locate the seeded primordial follicles within the graft (Fig. 7d). The histological examination demonstrated that seeded pre-antral follicles could develop from the early, primary stage (Fig. 7d) up to the early antral stages within the grafts. The follicles reached sizes of over 200 μm in diameter (Fig. 6e), with a clear apparent antral cavity formation, and multiple layers of surrounding cells, depicted by the red staining of the cytoskeletal fibers of F-actin, used due to weakening of Foxo-3 staining in developed follicles. These developed follicles were found in both graft types.

Steroid hormone production over time and development of antral follicles demand a continuous supply of blood to the graft, as well as endocrine signals such as the GsRH gonadotropin [50]. Indeed, early vasculature was visible in both the implanted follicle devices, as depicted by CD-31 staining of the endothelial cells lining the vessels (Fig. 7e–g). On day 14 of transplantation, the vessels inside the graft were not yet matured, as seen by the negative results of staining with anti-smooth muscle actin (SMA).

4. Discussion

In this work, we aimed to reconstruct the ovarian microenvironment of primordial follicles by developing a synthetic ECM-mimicking platform, seeded with follicles and supporting ovarian cells. The macroporous alginate/AlgS scaffold with its unique internal porous structure and matrix stiffness as well as the affinity-bound BMP-4 proved to be an effective microenvironment, supporting the growth and development of primordial follicles to the pre-antral stage, their viability and steroid production, when cultured in-vitro.

The manner GF is presented to cells affects its bioactivity. Matrix systems have been developed with GFs, either covalently tethered or sequestered through physico-chemical affinity to the matrix, as an alternative to soluble GFs [51]. The affinity binding mechanism is bio-inspired, and contrary to the covalent binding strategy does not require a special chemistry or poses risks as altering the natural structure of the factor. To enable BMP-4 affinity binding to the matrix, we employed AlgS, previously synthesized in our lab with the intention to mimic the biological limitations associated with the use of HS, such as rapid degradation and anti-coagulation activity in-vivo [52]. The equilibrium dissociation constant, obtained by SPR, indicated high affinity binding of BMP-4 to AlgS, at the same order of magnitude as binding of GFs from TGF-β family to HS [33]. Another supporting evidence for the specific interactions is the spontaneous co-assembly of AlgS with TGF-β GFs, yielding spherical complexes, with ζ potential value less negative, due to AlgS complexation with the slightly positive protein [53].

The inclusion of the nanocomplexes of BMP-4/AlgS or just the AlgS into the scaffold during fabrication did not affect the open-pore structure of the alginate scaffold, its average pore diameter and pore interconnectivity as well as the matrix stiffness. The macroporous alginate scaffold with its pore interconnectivity structure provided the cultured follicles with a physical environment relevant to the ovarian cortex. The cortical area of the ovary is highly porous, and it is inhabited by clusters of primordial follicles populated in cavities of varying sizes [54]. The average pore size of 75 ± 26 μm enabled the accommodation and development of the follicle in the pore, while the pore interconnectivity contributed to the arrangement of follicles as proximate clusters. This arrangement contributed to efficient cross-talk between neighboring follicles, and the exchange of paracrine signals, such as steroid hormones and GFs. We believe that this cross-talk, maybe by influencing the Akt signaling pathway and a possible disruption of the hippo signaling mechanism [58], was a main contributor to the ovary-like dynamics of follicular development within the culture. Within 21 days,
the follicles experienced an up to 4-fold increase in their diameter, due to complex processes, such as proliferation and assembly of multi-layer granulosa cell support and joining of theca. Our results are in agreement with recent studies noting the importance of the porous architecture surrounding the follicles, as a critical parameter in the design of a functional culture system [56,57].

Notably, as in the natural ovary, only a small fraction of the seeded follicles were developing, while the rest remained in the primordial state [50,58]. In the ovary, this phenomenon is related to the action of AMH, a cytokine secreted by granulosa and theca cells of developing follicles (primary up to early antral) as a mean to prevent the robust maturation of surrounding follicles [59]. Indeed, in all our culture groups, AMH expression was increased after 21 days in culture, which may explain the high proportion of primordial follicles present in culture on this day [60]. Alongside the primordial follicles, our studies showed the presence of developing follicles in culture, supported by the increase in GDF-9 gene expression, which is associated with proliferation of granulosa cells and development of follicles [25,61].

The presentation of BMP-4 as affinity bound to the matrix had a significant effect on the follicular maturation in culture. Our studies showed that the affinity binding mechanism resulted in a significantly greater retention and prolonged presentation of BMP-4 compared to pristine alginate scaffolds. Thus, developing follicles reached a greater size (> 70 μm) when BMP-4 was presented as affinity bound to the matrix. The prolonged bioactivity of BMP-4 also resulted in a statistically significant elevation in the transcript levels of AMH and GDF-9. BMP-4 was previously shown to strongly upregulate AMH expression in a culture of cow and sheep GCs [62]. GDF-9 gene was also upregulated in a dose-dependent manner following addition of BMP-4 to cultured bovine tissue fragments [30]. In these studies, BMP-4 was added to the medium daily (50 ng and 100 ng, respectively). By contrast, in our study, the BMP-4 effective concentration in the scaffold (51.84 ± 5.62 ng BMP-4 for pristine alginate scaffolds and 65.73 ± 1.42 ng BMP-4 for alginate/AlgS scaffolds) was sufficient for 21 days in culture. An important validation for the functionality of our follicle device is the production and secretion of estradiol (E2). High E2 levels were present in the medium of follicle devices, where BMP-4 was affinity bound, and the levels measured are characteristic of several antral follicles [63] or a few million GCs [64]. Interestingly, similar levels were also found in the medium of follicle devices where only AlgS (no exogenous BMP-4) was integrated to culture matrix. Most likely, the integrated AlgS is accessible for binding GFs secreted by the seeded cells, and sustains their presentation. The GFs could be secreted by the ovarian cells co-cultured with the follicles, thus substantiating the advantage of using these accessory cells in the follicle microenvironment. E2 secretion also marks another important advantage for co-culturing the follicles with ovarian cells, since theca cells provide the substrate for the aromatase enzyme present in granulosa cells, hence enabling E2 production [65]. E2 secretion was shown to be diminished when theca cells were not present in culture of goat GCs [11]. An important consideration, when incorporating ovarian cells from cancer patients into culture, is the issue of safety. However, in the future, these cells can be safely obtained, for example by purging of malignant cells prior to incorporation to the culture system, or by isolating these cells from ovarian tissue after anti-cancer treatments [66].

The follicular development in scaffolds was restricted to the secondary stages. We assume this is the result of three main consequences. One may be the study duration. Culture systems vary in the rate of maturation, and it is possible that due to the enhanced AMH secretion, follicle growth is slower in our culture. The second is mechanical resistance applied by the scaffold walls; the third is the absence of pituitary-originated gonadotropins, such as GnRH and HCG, which are critical modulators of follicular maturation from secondary stages onward [67]. Future studies should address these limitations by extending culture time, modulating scaffold degradation and the addition of necessary factors to promote maturation. Also, the culture system, as described here, presents a robust reconstruction of the ovary, which will have to be adapted to the limited amount of human follicles available for research and treatment. Even so, the ability to culture cells in close proximity and present them with affinity bound GFs are both important features for small scale culture of follicles, as required for human follicle culture.

Importantly, the alginate scaffolds enabled the development and maturation of primordial follicles in-vitro, leading to restoration of ovarian function in ovarioctomized mice. Functionality was evident through both external visualization of the vaginal area and the statistically significant serum hormone levels, which correspond to hormone levels of reproductively healthy mice (10–25 pg/mL) [48]. Follicles, at the antral stage, were found already 14 days after transplantation, in agreement with the restoration of the ovarian function. The exogenous addition of BMP-4 and GDF-9 as affinity bound to the matrix has not contributed to further E2 secretion beyond the level measured in animals treated with follicle devices containing only AlgS. Two explanations could be postulated: 1) secretion of GFs by the transplanted cells in the construct is sufficient and as the hormone levels reach the normal value, there is feedback inhibition on the production; and 2) both constructs contained VEGF, known to be involved in follicular maturation [68,69]. Independent of which explanation is the correct one, the in-vitro results indicate that the inclusion of follicles with supporting OCs in the device contribute to the viability and maturation of the transplanted follicles up to the antral stage. Further, it is most likely that the inclusion of angiogenesis factors, VEGF/PDGF-Bβ, contributed to cell viability and follicle development after transplantation. These factors exhibit an extended half-life when affinity bound to alginate-sulfate [33,70], and their inclusion probably promoted vessel formation in the construct, thus allowing efficient mass transport of nutrients to the transplanted cells, and specifically the arrival of pituitary-secreted LH and FSH, important for steroidogenesis. In future studies, we will extend the transplantation time to longer periods in aim to substantiate whether the exogenous addition of GFs is required or sufficiently provided by the OCs.

Our results further indicate that the exogenous addition of GFs, such as BMP-4 and others as follicle develops, may be an important affecting factor, particularly in the in-vitro follicle cultures, which require the continuous supplementation of these factors. The affinity binding of the factors in close proximity to the cultured cells is an attractive way to achieve the ultimate goal of in-vitro follicle development.

5. Conclusions

We describe herein a promising strategy for the in-vitro recapitulation of the ovarian microenvironment, by employing macroporous alginate scaffolds impregnated with affinity-bound BMP-4 to AlgS. In-vitro culture of ovarian primordial follicles within this ovarian-mimicking microenvironment supported their healthy development and maturation up to the pre-antral stage within 21 days in culture. The development of limited numbers of follicles in this construct mimics, to a large extent, the natural course of follicle development in the ovary. This simple, bio-inspired approach could be implemented outside the field of reproductive biology, for example for spheroid cultivation. The proof-of-concept study in ovarioctomized mice demonstrated the potential of our system to serve as a transplantable platform for the restoration of lost ovarian function, and for regaining female fertility. Additional studies and longer transplantation times are needed to substantiate whether the exogenous addition of GFs is required or whether it is sufficiently provided by the OCs.

Conflicts of interest

The authors declare no conflict of interests.
Author contribution

S.F. and S.C. wrote this manuscript. S.F., M.S.G. and S.C. envisioned the ovary microenvironment and designed the in-vitro and in-vivo ovarian follicle experiments. S.F. performed all experiments in the paper, including follicle culture, immunostaining and confocal imaging of follicles seeded within scaffolds, transplantation of the follicle device, and histological analysis of follicle culture and surgical tissue sections. A.O and N.L. supervised and performed the Ovariectomy in mice. S.F., M.S.G. and S.C. contributed to experimental design and interpretation.

Data availability

The data that support this study are available within the article and its Supplementary data files or available from the authors upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2019.03.013.

References


