Interpenetrating fibrin–alginate matrices for in vitro ovarian follicle development

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ABSTRACT

In this report, we investigate the fibrin–alginate interpenetrating network (FA-IPN) to provide dynamic cell-responsive mechanical properties, which we apply to the in vitro growth of ovarian follicles. The mechanical properties and polymerization rate of the gels were investigated by rheology, and the fiber structure was imaged by electron microscopy. Using a mouse model, two-layered secondary follicles were encapsulated in FA-IPNs, and growth, morphology, hormone production, fibrin degradation rate and the numbers of competent eggs were assessed. The initial mechanics of the FA-IPN are determined by the composite material, and subsequent degradation of fibrin by the encapsulated cells would produce a material with mechanical properties due to the alginate alone. The rate of meiotically competent oocytes produced by culture in FA-IPN was 82%, which was significantly greater than in alginate alone. This increase in oocyte quality is an important step in identifying 3D culture systems that can provide a fundamental tool to investigate follicle maturation, and may be applied to promote the growth of human follicles, which can be used to provide reproductive options for women facing a cancer diagnosis.

1. Introduction

The objective of this study was to develop an interpenetrating network of natural biomaterials that have dynamic cell-responsive mechanical properties, which provides a culture environment that supports tissue growth. An interpenetrating network (IPN) is a combination of polymers in network form, where at least one polymer is synthesized and/or crosslinked in the presence of the other, either simultaneously or sequentially [1]. The chains of the individual polymers are completely entangled, and there may or may not be chemical bonds between the combined networks. This structure results in characteristics from each individual polymer being evident in overall IPN behavior [2]. For example, fibrin and alginate have been used individually as scaffolds in biomaterial applications, and we investigate their utility as an IPN. Fibrin forms a biomatrix with multiple ECM components and entrapped growth factors. Fibrinogen is a soluble 340 kDa protein that is polymerized into fibrin through the action of thrombin in the presence of

calcium. Factor XIIIa, activated by thrombin, then crosslinks fibrin by linking a glutamine residue on the fibrinogen to a lysine on another. By comparison, alginate is a relatively inert scaffold and does not interact with integrins of mammalian cells [3], yet forms a hydrogel under mild conditions, provides mechanical support to the tissue, and can be modified to present specific adhesion sequences [4–9]. Alginate, a naturally derived polysaccharide produced by brown algae, has been widely used to support tissue growth for numerous applications.

The physical properties of a hydrogel, such as mechanical stiffness, regulate tissue growth through directly impacting cellular processes such as cell proliferation and differentiation, and growth factor and ECM production [10,11]. A model in which the physical properties affect tissue development is ovarian follicle culture, which is being developed as a means to preserve reproductive function for women facing premature infertility due to cancer therapies or other disorders [12–14]. The ovarian follicle consists of an oocyte surrounded by layers of granulosa cells, a basement membrane composed of ECM, and an outer layer of theca cells. As follicles develop, the somatic cells surrounding the oocyte proliferate and differentiate, and the oocyte grows in preparation for ovulation and fertilization. Communication between the multiple cellular compartments is essential for follicle development and oocyte maturation; thus, we have employed hydrogels for culture

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of ovarian follicles to support and maintain the normal follicular architecture [6,7,12–16]. Follicles encapsulated and cultured in alginate hydrogels have been able to grow, produce fluid-filled antral cavities, and meiotically competent oocytes, which yielded multiple live births of healthy mouse pups. With this success, the opportunity remains to further increase the number and quality of oocytes. One hypothesis that emerged from the previous studies is that the alginate hydrogels, which are not degradable, become more rigid in the region adjacent to the follicle as the follicle increases in size. Previous studies indicated soft hydrogels provide a more permissive environment relative to more rigid hydrogels [8,14,15].

In this report, we investigate a fibrin–alginate IPN (FA-IPN) to provide dynamic cell-responsive mechanical properties, which we apply to the in vitro growth of ovarian follicles. A matrix for follicle culture must enable easy encapsulation, mild gelation and diges-tion options with retention of viability, allow transport of nutrients and hormones toward and away from the follicle, permit follicle expansion, and enable follicle collection at the end of culture [13]. These requirements are satisfied with an IPN based on fibrin and alginate, which can be gelled simultaneously using thrombin and calcium, the latter of which is required for thrombin activity. Fibrin–alginate IPNs (FA-IPNs) were formed at multiple concen-trations of thrombin. The mechanical properties and polymeriza-tion rate of the gels were investigated by rheology, and the fiber structure was imaged by electron microscopy. Using a mouse model, two-layered secondary follicles were encapsulated in FA-IPNs, and growth, morphology, hormone production, fibrin degra-dation rate and the numbers of competent eggs were assessed. Control cultures were performed in hydrogels formed with fibrin alone. This FA-IPN, which combines degradable and non-degrad-able components, may provide a dynamic mechanical environment that facilitates the growth of organized cell clusters in 3D culture.

2. Materials and methods

2.1. Animals and materials

Two-layered secondary follicles were mechanically isolated from 12-day-old female F1 hybrids (C57BL/6j × CBA/Ca). Animals were purchased (Harlan, Indian-apolis, IN), housed in a temperature and light controlled environment (12 L:12 D) and provided with food and water ad libitum. Animals were fed Teklad Global irradiated 2919 chow, which does not contain soybean or alfalfa meal and therefore contains minimal phytoestrogens. Animals were treated in accordance with NIH guide for the Care and Use of Laboratory Animals, and protocols were approved by the IACUC at Northwestern University. Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO), stains and antibodies from Molecular Probes (Eugene, OR), and media formulations from Invitrogen (Carlsbad, CA). Sodium alginate (55–65% guluronic acid) was provided by FMC BioPolymers (Phil-adelphia, PA) and Tisseel™, kindly provided by Baxter International Inc. (Baxter Healthcare, BioScience Division, Westlake Village, CA), was used for fibrin gels preparation.

2.2. FA-IPN preparation

The fibrinogen-containing component of Tisseel® was reconstituted in aprotinin (3000 KIU/mL) solution and the thrombin component was reconstituted in 40 mM CaCl$_2$, according to the Baxter kit instructions. Both solutions were diluted to the appropriate concentrations by diluting the fibrinogen-containing component in Tris-buffered saline solution (TBS) and thrombin in 40 mM CaCl$_2$ in TBS. Alginate aliquots were prepared as previously described [7,12] and diluted to 0.5% w/v. IPNs were prepared by mixing fibrinogen solution (50 mg/mL) with alginate solution 0.5% in 1:1 ratio, and then adding thrombin solutions of 5, 50 and 500 IU/mL to the mixture at 1:1 ratio. The fibrinogen–alginate mix and the thrombin solutions were filled with equal volumes in 1 mL syringes and injected using the Duploject System provided with the kit, while mixing in the needle. Thus, the final concentrations in the gels of fibrinogen and alginate were 12.5 mg/mL and 0.125%, respectively.

2.3. FA-IPN characterization

FA-IPNs shear elastic modulus was measured at 25 °C using a Paar Physica MCR Rheometer (Anton Paar, Graz, Austria) using a parallel plate geometry (diameter of

25 mm, gap of 0.5 mm) and Paar Physica US200 software. For each of the 3 different concentrations of thrombin the FA gels were extruded on the lower plate of the rheometer. The upper plate was immediately lowered, and the gels crosslinked between the plates for 10 min. The limits of linear viscoelasticity were determined in strain sweep experiment with strain range from 0.1% to 100% at a constant angular frequency of 10 rad/s. Storage and loss moduli were determined in amplitude sweep experiments at constant strain of 0.5% and angular frequency range from 100 to 0.1 rad/s. The gels were formed and let crosslink for 10 min before the experiment started. For the gelation rate measurement the gels were extruded on the lower plate and the experiment was started immediately. The gelation profiles for 3 different concentrations of thrombin were performed at 10 rad/s angular frequency and 0.5% strain for 30 min. Fibrin gel prepared with 25 mg/mL fibrinogen and 50 IU/mL thrombin concentrations, and alginate gels at concentrations of 0.125% and 0.5% were used as a control conditions.

2.4. Follicle isolation, encapsulation and culture

Two-layered secondary follicles (100–130 μm, type 4) were mechanically iso-lated as described before [7,8,12,14] and encapsulated in FA-IPNs or fibrin gels. Fibrinogen–alginate solution (7.5 μL, 25 mg/mL fibrinogen, 0.25% alginate) were pipetted on alcohol wiped glass slide with 3 mm spacers and covered with parafilm and individual follicles were pipetted into the droplets. Thrombin solutions (7.5 μL of
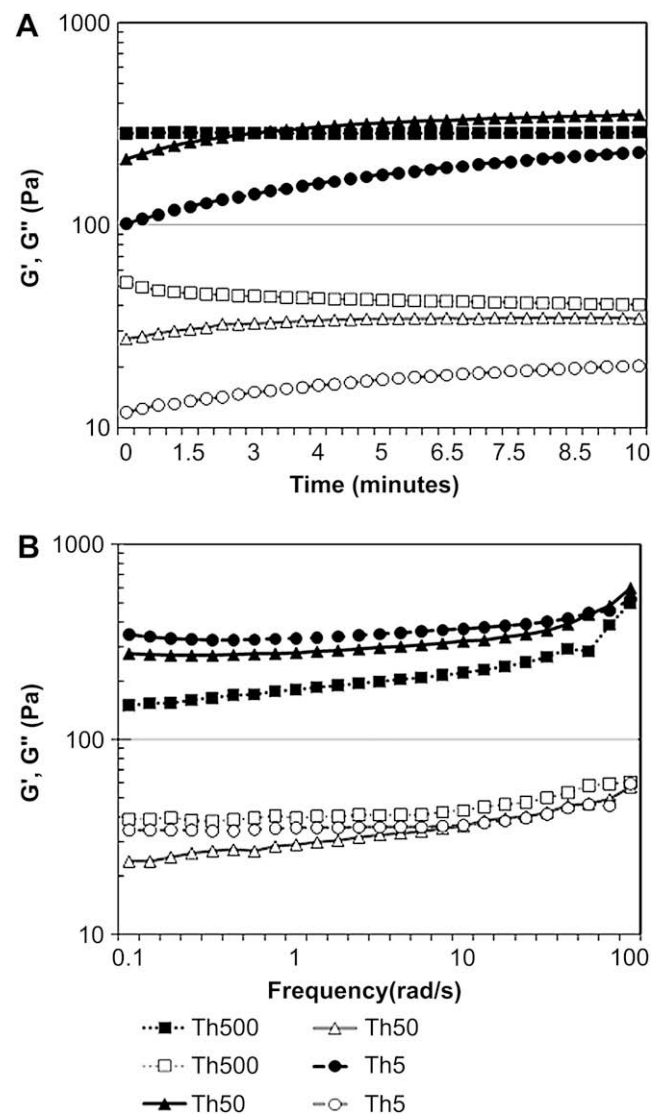

Fig. 1. Rheometric characterization of gelation rate and gel mechanics. The storage modulus *G*′ (black symbols) and loss modulus *G*″ (white symbols) during the cross-linking reaction (A) and amplitude sweep test (B) of IPNs with different concentrations of thrombin. For the amplitude sweep test the gels were crosslinked for 10 min and then tested.

Table 1
Hydrogel characterization: storage modulus data represented as average from three or more independent measurements.

Gel	Storage modulus (G') at 0.5% strain, 10 s^{-1} angular frequency
ALG/Fibrin Th5	370 Pa
ALG/Fibrin Th50	300 Pa
ALG/Fibrin Th500	235 Pa
Fibrin	300 Pa
ALG 0.5%	300 Pa
ALG 0.125%	42 Pa

G' = storage modulus, Pa = Pascal.

5, 50 or 500 IU/mL) were pipetted on top of each droplet with the follicle, covered with the second glass slide covered with alcohol wiped parafilm and transferred to the 37 °C incubator for 5 min. The beads with the follicles were washed in maintenance media (αMEM, 1 mg/mL bovine serum albumin and penicillin–streptomycin) and transferred to 96-well plates with 150 μl of culture media (αMEM, 3 mg/mL bovine serum albumin [MP Biomedicals, Inc, SOLON, OH], 10 mIU/mL rFSH [A.F. Parlow, NHPP, NIDDK], 1 mg/mL bovine fetuin, 5 μg/mL insulin, 5 μg/mL transferrin and 5 ng/mL selenium). As a control, follicles were encapsulated in fibrin only beads or fibrin gels formed in culture plate inserts (0.4 μm, 12 mm diameter, Millipore, Billerica, MA). Beads made of fibrin only were formed in the same manner as previously described for FA. For cultures in the insert, fibrinogen solution (25 mg/mL, 50 μL) was pipetted into the insert, and 5–7 follicles were added with thrombin solution (50 IU/mL, 50 μL) pipetted over the follicles. The gels were allowed to form for 10 min. The inserts were transferred to 24 well plates and covered with culture media. Throughout the isolation, encapsulation and plating, follicles were maintained at 37 °C and pH 7.

Encapsulated follicles were cultured at 37 °C in 5% CO$_2$ for 12 days. Every other day, half of the media (75 μL) was exchanged and stored at −80 °C for steroid assay. Follicle survival and diameter were assessed using an inverted Leica DM IRB microscope with transmitted light (Leica, Bannockburn). The diameter of follicles containing oocytes was measured in duplicate from the outer layer of theca cells using ImageJ 1.33U (NIH, http://rsb.info.nih.gov/ij/) and based on a calibrated ocular micrometer.

2.5. Oocyte meiotic competence

Oocyte meiotic competence was assessed by maturation after 12 days of culture. Follicles were removed from the beads by 10 min incubation of the beads in a 10 IU/mL solution of alginate lyase, which enzymatically degrades the alginate, in pre-warmed L-15 media. Antral follicles were transferred to αMEM containing 10% FBS,

5 ng/mL epidermal growth factor and 1.5 IU/mL human chorionic gonadotropin and were matured at 37 °C in 5% CO$_2$ for 14–16 h. Oocytes were denuded then from the surrounding cumulus cells by treating with 0.3% hyaluronidase. Oocyte state was assessed from the light microscopy images, and characterized as follows: germinal vesicle breakdown (GVBD) if the germinal vesicle was not present, GV if there was an intact germinal vesicle, metaphase II (MII) if a polar body was present in the peri-vitelline space and degenerated (DG) if the oocyte was fragmented or shrunken.

2.6. Follicle fixation and H&E staining

Follicles were fixed inside FA-IPN bead. The fixation was performed at 4 °C for overnight in a 4% paraformaldehyde (PFA), dehydrated in ascending concentrations of ethanol (50–100%), and embedded in paraffin by an automated tissue processor (Leica, Manheim, Germany). Serial 5 μm sections were cut and stained with hematoxylin and eosin (PO1 Ovarian Histology Core facility).

2.7. Oocyte preparation for confocal microscopy and imaging

Matured oocytes were fixed in 4% PFA for 2 h at room temperature and stored in wash solution containing 0.2% azide, 2% normal goat serum, 1% BSA, 0.1 M glycine, and 0.1% Triton X-100 at 4 °C until further processing. Oocytes were immunolabeled to ascertain maturation state, centrosome, spindle and polar body position and shape. A total of 15 oocytes per gel condition were incubated in primary antibody (α/β-tubulin cocktail 1:100; mouse; Sigma) in 4 °C overnight with gentle agitation, followed by three 10-min washes in wash buffer, followed by 1-h incubation of secondary antibody (Alexa 488 goat anti-mouse IgG 1:500; Molecular Probes) with rhodamine–phalloidin (1:500; Molecular Probes) at room temperature. Oocytes were mounted in 2 μL of a 50% glycerol/PBS solution containing 1 μg/mL Hoechst 33258 to label chromatin. Samples were analyzed on an inverted Nikon C1Si Multispectral Laser Scanning Confocal Microscope (Nikon Instruments, NY) equipped with a 100-W mercury arc lamp and were imaged using 40× and 63× objectives. A triple band pass dichroic and automated excitation filter selection specific for fluorescein (Alexa 488), rhodamine (Alexa 568) and bisbenzimides (Hoechst 33258) permitted the collection of in-frame images and z axis data sets at 0.5 μm intervals.

2.8. Steroid assays

Androstenedione, 17β-estradiol and progesterone were measured in collected media from 12-day individual follicle culture using commercially available radio-immunoassay kits (androstenedione and 17β-estradiol, Diagnostic Systems Laboratories, Inc., Webster, TX; progesterone, Diagnostic Products Corporation, Los Angeles, CA). The media from the same condition and time point were pulled together, triplicates for each condition. The sensitivities for the androstenedione, estradiol and progesterone assays are 0.1 ng/mL, 10 pg/mL and 0.1 ng/mL,
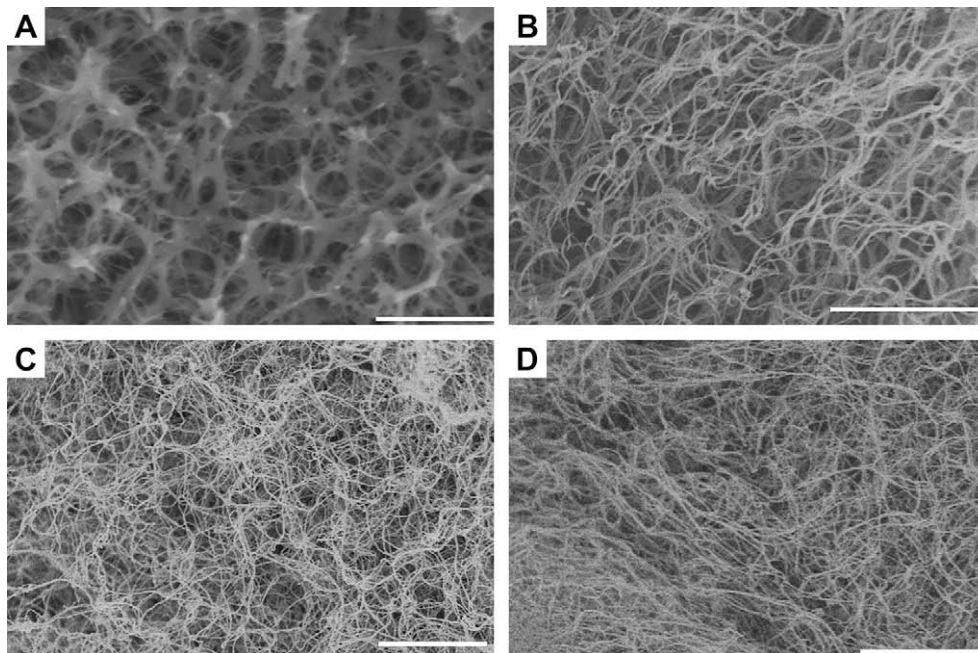

Fig. 2. SEM images of (A) fibrin gel 50 IU/mL thrombin, (B) FA-IPN with 5 IU/mL thrombin, (C) FA-IPN with 50 IU/mL thrombin, (D) FA-IPN with 500 IU/mL thrombin. The fibrin gel and FA-IPNs were prepared with 25 mg/mL fibrinogen. The scale bar is 3 μm.
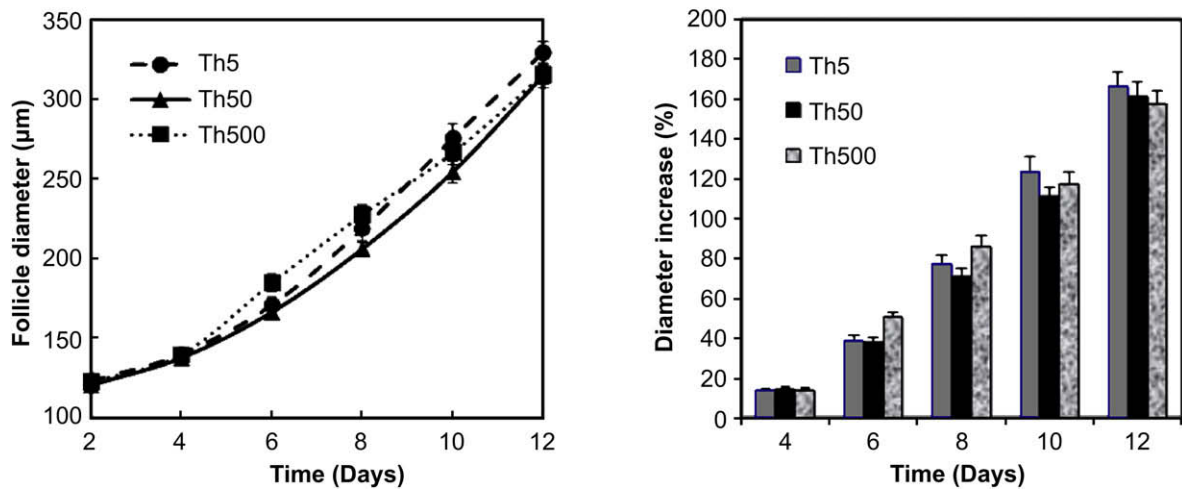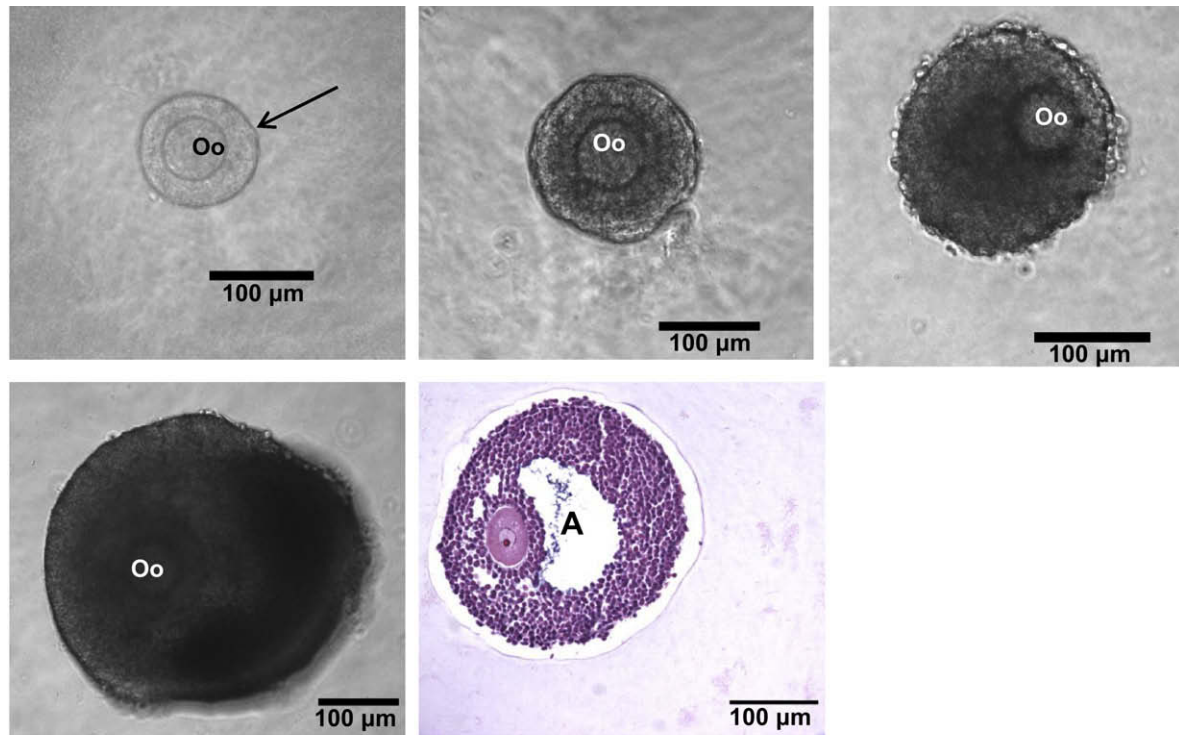
Fig. 3. Two-layered secondary follicle growth in FA-IPNs: (A–D) a representative encapsulated follicle at day 2 (A), 4 (B), 8 (C) and 12 (D); (E) fixed and H&E stained follicle after 12 day culture; growth curve over a 12-day culture period (F) and percent increase in follicle diameter relative to day 0 (G) in FA-IPNs with 5, 50 and 500 IU/mL thrombin.

respectively. The hormone assays were performed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core and was supported by the Eunice Kennedy Shriver NICHD/NIH (SCCPIR) Grant U54-HD28934.

2.9. Statistical analysis

Statistical calculations were performed using JMP 4.0.4 software (SAS Institute, Cary, NC). Statistical significance for follicle size measurements and steroid levels was analyzed using a two-way ANOVA with repeated measures, or one-way ANOVA followed by Tukey-HSD for single time points. Values of $p < 0.05$ were considered significant.

3. Results

3.1. FA-IPN characterization

Fibrin–alginate IPNs were formed with three concentrations of thrombin. Both components of the IPN, the fibrinogen and the alginate, start to crosslink immediately as they are mixed with the crosslinker, thrombin and Ca^{2+}, and a longer duration of cross-linking results in stronger gels. Gelation kinetic studies indicate that as crosslinking is initiated, the storage modulus (G') of the IPN increases with thrombin content (Fig. 1A). The storage modulus was 100 Pa, 220 Pa and 280 Pa for thrombin concentrations of 5 IU/mL, 50 IU/mL and 500 IU/mL. With increasing time, the

Table 2
Ratios of estradiol concentration to androstenedione and progesterone concentrations at end of culture.

	E	A	P
Th5	1	0.09	0.11
Th50	1	0.17	0.14
Th500	1	0.10	0.15

E = estradiol, A = androstenedione, P = progesterone.

dependence of the storage modulus on the thrombin content decreased. After 30 min, the moduli were equal to 350, 370 and 300 Pa for 5, 50 and 500 IU/mL thrombin, respectively. The low thrombin concentration resulted in a slower rate of crosslinking, yet produced a higher storage modulus after 30 min. A 100-fold higher thrombin concentration formed a gel immediately with no increase in storage modulus over time. In amplitude sweep experiments, IPNs were crosslinked for 10 min and then tested at constant strain of 0.5% with decreasing angular frequency from 100 to 0.1 s^{-1} (Fig. 1B). Higher thrombin concentration resulted in IPNs with lower storage modulus: 235 Pa for Th500, 300 Pa for Th50 and 370 Pa for Th5. The similar amplitude sweep experiment was used to determine the storage modulus for fibrin gel (25 mg/mL fibrinogen; 50 IU/mL thrombin), alginate 0.5% and 0.125% (Table 1).

We subsequently investigated the architecture of the FA-IPN that would be produced at a range of thrombin concentrations. For the three thrombin concentrations IPN gels have a network of fibers, with the fiber diameter ranging from 15 to 120 nm (Fig. 2). IPNs prepared with 500 IU/mL of thrombin resulted in more visually dense and compact matrix with thinner fibers compared to IPN formed at lower thrombin concentrations.

3.2. Two-layered secondary follicle encapsulation and growth

Follicle development was investigated in FA-IPNs prepared with increasing (5, 50 and 500 IU/mL) concentrations of thrombin. A minimum of 80 follicles were encapsulated and cultured for 12 days. Follicles maintained their spherical 3D structure while growing from small follicle with an oocyte surrounded by two layers of granulosa cells on day 2 of the culture (Fig. 3A), with growing number of granulosa cells layers on days 4 and 8 (Fig. 3B and C). A fluid-filled antrum cavity was observed on day 12 (Fig. 3D), which is consistent with in vivo morphology. Antral follicle fixed on day 12 from the culture, sectioned and stained with H&E had a spherical shape with a central antrum and oocyte surrounded by cumulus cells (Fig. 3E). The survival rate of the follicles was the same for all the conditions (77–81%, Table 2) and was similar to the previously reported rates in alginate cultured follicles [14]. In all groups, follicles increased in their diameter from 120 μm on day 2 to 330 μm on day 12, which is an increase of 160% (Fig. 3F and G).

3.3. Fibrin degradation

The fibrin component of the IPN gradually disappeared from the hydrogel, with the initial clearance occurring adjacent to the follicle and then progressing toward the edge of the hydrogel bead (Fig. 4). The follicles were initially difficult to image due to light diffraction in the FA-IPN, which makes the hydrogel appear cloudy (Fig. 4A). On the second day of a culture, the region immediately adjacent to the follicle became clear, consistent with the degradation of the fibrin component of the IPN. The non-degradable alginate component remained and supported the 3D architecture of the growing follicle (Fig. 4B–D). The distance from the edge of the encapsulated follicle to the edge of the degradation front was measured. Fibrin in Th5 degraded fastest relative to the other two conditions, reaching a distance of 690 μm in 6 days of culture. For Th50 and Th500 the degradation occurred at slower rates, reaching 470 μm and 540 μm, respectively (Fig. 4E). Follicles cultured for 2 and 4 days (Fig. 4F and G) had degradation around the follicle, with the fibrin–alginate matrix remaining intact beyond the degradation front.

Control studies were performed with fibrin hydrogels without alginate to investigate whether fibrin alone would be sufficient to promote follicle growth. Note that studies using alginate alone have been previously reported and were the basis for the development of
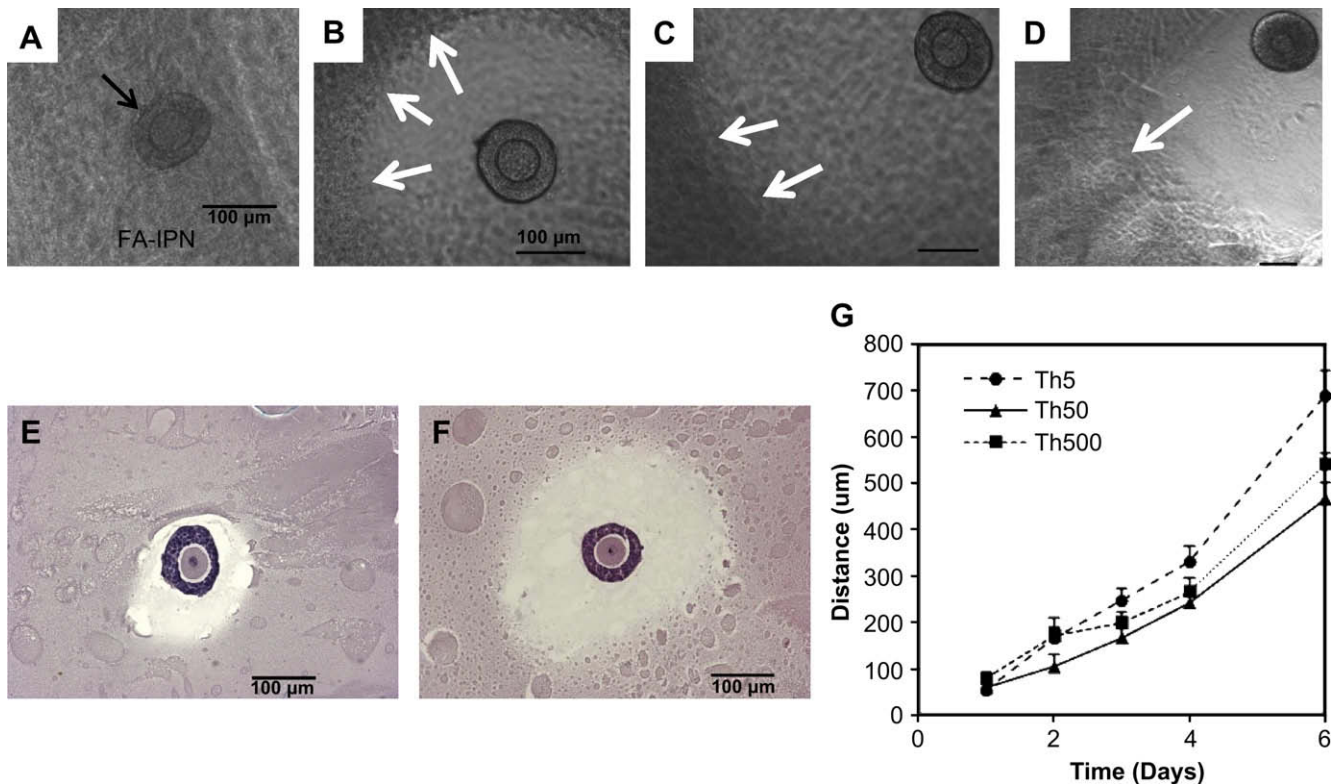

Fig. 4. The degradation process of the fibrin appears as a clearing ring in a matrix around the encapsulated growing follicle (black arrow). (A–D) Bright field images of the degradation ring on day 0 (A), day 2 (B), day 4 (C) and day 6 (D) during in vitro culture in FA-IPNs; (E) the distance from the follicle to the edge of the degradation ring during the culture in FA-IPNs with 5, 50 and 500 IU/mL thrombin; (F–G) H&E staining of the FA-IPN with degradation ring of the fibrin on day 2 (F) and day 3 (G). Scale bar 100 μm.
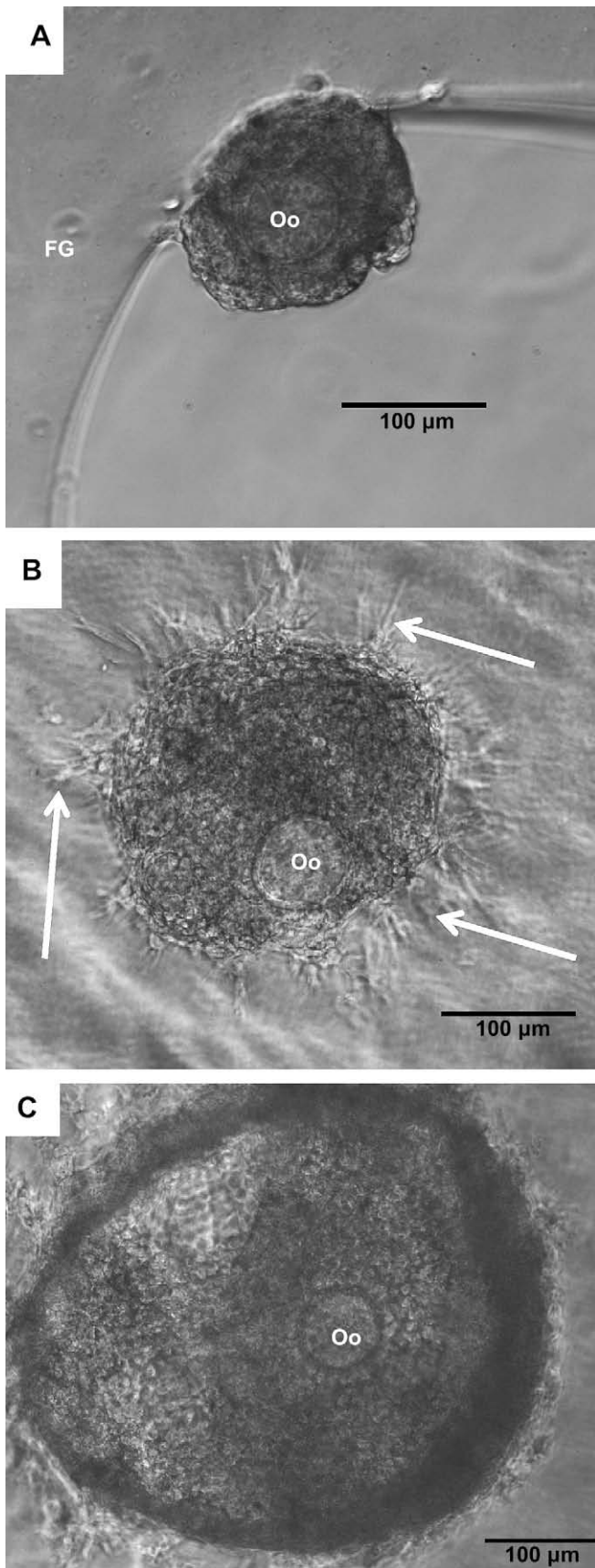
the IPN [8,14]. Follicles were cultured in fibrin gels using two different methods of encapsulation: beads and culture plate inserts, or transwells. Follicles cultured in fibrin beads degraded the surrounding fibrin, and by day 6, the follicles either lost their spherical shape or were no longer within the fibrin (Fig. 5A). Upon degradation of the fibrin, the mechanical support of the hydrogel was lost leading to changes in shape of the follicle. At day 6, the follicles stopped growing and were no longer viable. Due to this loss of support, follicles were subsequently cultured within the transwell inserts, which prevented loss of the follicle from the bead. In this condition the granulosa cells spread and migrated into the surrounding fibrin and follicles lost their typical spherical shape (Fig. 5B). At the end of a culture in inserts, follicles reached 600 μm in their diameter, but their shape was similar to 2D cultured follicles (i.e., flattened with poorly connected granulosa cells) (Fig. 5C).
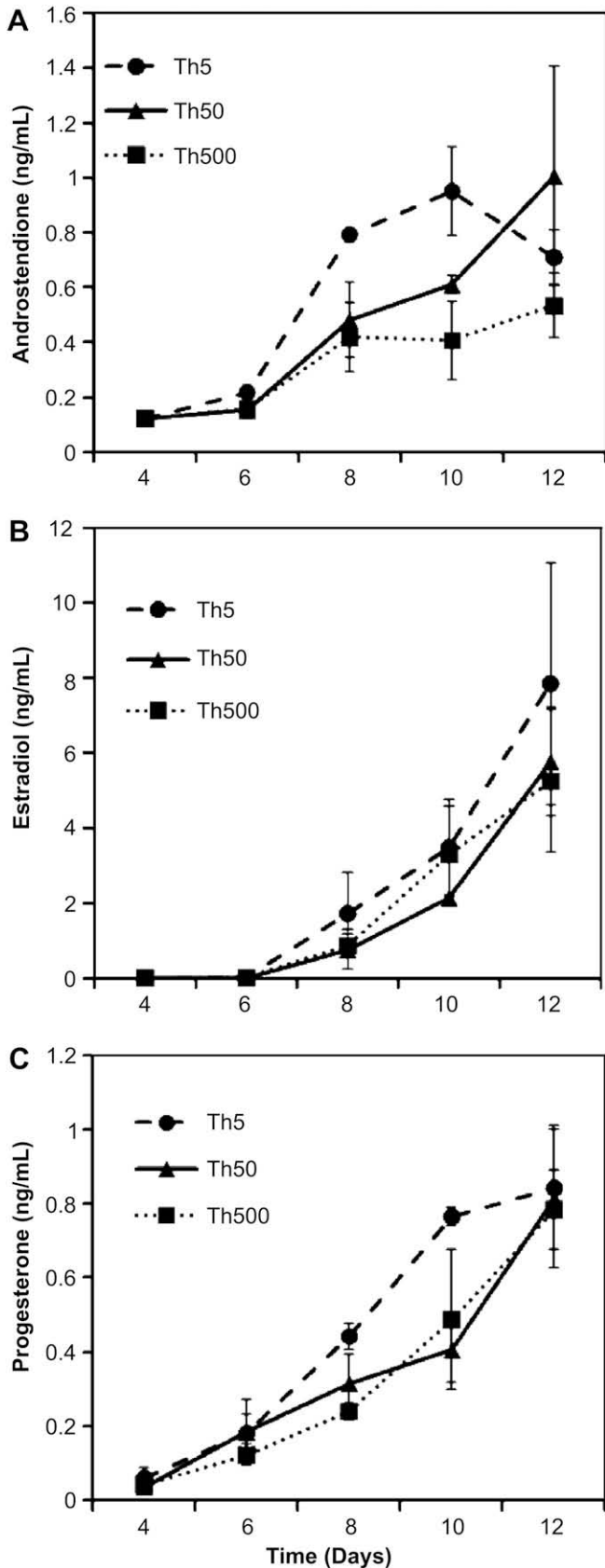
3.4. Functional analysis

We subsequently investigated the functional development of the follicles within the FA-IPN. Steroid production by the follicle, which confirms that follicles are performing their steroidogenic roles, was quantified. Androstenedione (A) levels in all three conditions increased from baseline on day 6 and was significantly greater in Th5 (0.8 and 1 ng/mL on days 8 and 10) than in Th50 and Th5 (Fig. 6A). However, on the last day of culture no significant difference was observed between conditions. Progesterone (P) levels, similar to androstenedione, increased on day 6 with greater levels in Th5 (0.8 ng/mL) on day 10 compared to other two conditions (0.8 ng/mL and 0.5 ng/mL, respectively). On day 12 of culture, all groups had similar progesterone concentration of 0.8 ng/mL (Fig. 6B). Estradiol (E) levels increased on day 8 and reached maximum concentration on day 12 (6–8 ng/mL) with no significant difference between the conditions (Fig. 6C). Appropriate quantities of steroid biosynthesis are reflected in the ratio of secreted estradiol, androstenedione and progesterone (Table 2). In all thrombin conditions described here the ratio A/E and P/E was 0.1–0.2, suggesting that follicle development is supported in FA-IPNs.

The quality of oocytes obtained from follicles cultured within FA-IPNs was subsequently measured by their ability to resume meiosis. Oocytes from follicles cultured in all thrombin conditions demonstrated high rate (75–82%, Table 3) of Metaphase II (MII) stage and polar body extrusion (Fig. 7A). This rate of MII stage oocytes obtained from follicles cultured in FA-IPNs was significantly higher than previously reported for 0.25% alginate system (67.2%). The percentage of GV oocytes was similar for all conditions, but in Th500, greater percentage of degenerated oocytes was observed (16% in Th500 versus 6% in Th50). For the analysis of spindle structure and chromosome positioning, MII stage oocytes obtained from cultured follicles were stained with a fluorescent antibody to β-tubulin and DAPI, and imaged with confocal microscope (Fig. 7B and C). Oocytes that extruded the first polar body exhibited a normal MII configuration, with microtubules organized into a bipolar spindle and the chromosomes tightly aligned on the spindle equator.

4. Discussion

We report on the development of FA-IPN to create a hydrogel with dynamic cell-responsive mechanical properties that were

Fig. 5. Follicles cultured in fibrin gel (25 mg/mL fibrinogen, 50 mg/mL thrombin). (A) Follicle cultured in fibrin gel on day 6 degraded the matrix around it and had support only on one side (FG: fibrin gel); (B) granulosa cells in follicle cultured in a transwell at day 6 started to migrate away from the oocyte (white arrows); (C) follicle cultured in fibrin gel in transwell reached 500 μm in diameter, but appeared flat similar to 2D culture (Oo: oocyte).

Fig. 6. Steroid secretion profiles of two-layered secondary follicles cultured in FA-IPNs with 5, 50 and 500 IU/mL thrombin. Androstenedione (A), estradiol (B) and progesterone (C) increased as follicles developed in the culture, no significant difference was observed between the different conditions on day 12.

Table 3
Survival rates, follicle size measurement and meiotic competence rates from two-layered secondary follicles cultured in FA-IPNs in vitro.

Condition	Survival[a] (%)	Day 12 diameter	MII (%)	GVBD (%)	GV (%)	DG (%)
ALG 0.25%[b]	78	326.5	67[c]	88[c]	4[c]	9[c]
Th5	77	329	76[d]	72[d]	8[c]	11[c]
Th50	81.3	314.6	82[e]	88[e]	6[c]	6[d]
Th500	78.1	315.4	75[d]	78[d]	6[c]	16[e]

[a] Values are the average of multiple follicles from five independent cultures.
[b] Data from Xu et al. [14].
[c–e] Different superscripts within each column indicate statistically significant differences ($p < 0.05$).

employed to support tissue growth. Others have used IPNs to enhance the material properties, such as fibrin–collagen IPNs having enhanced mechanics relative to fibrin or collagen alone [17] or agar/collagen IPNs to having greater toughness and slower degradation relative to collagen alone [18]. Our studies focused on the growth of encapsulated cells, and thus focused on a combination of alginate and fibrin. Alginate has been widely used for cell encapsulation due to its crosslinking by calcium. Fibrin is degradable, natural biopolymer that gels in the presence of thrombin, whose activity is dependent on calcium. Both gels demonstrate mild and rapid gelation. The speed of fibrin clot formation is determined by fibrinogen and thrombin concentrations. High thrombin concentrations result in fast clot formation, but thinner fibers, while lower thrombin concentrations result in slower crosslinking, but thicker fibers. In this study we used 5, 50 and 500 IU/mL thrombin to form FA-IPNs in 5–6 min, because longer crosslinking time affected the viability of the follicle. In all three conditions clots formed, but as expected the slowest gelation rate was observed with Th5 and the fastest with Th500. Gels formed with Th50 reached their final storage modulus of 300 Pa after 3 min of crosslinking and had the same result in the amplitude sweep test.

The initial mechanics of the FA-IPN are determined by the composite material, and subsequent degradation of fibrin by the encapsulated cells would produce a material with mechanical properties due to the alginate alone. The fibrin matrix is degraded primarily through the action of plasmin, but other proteases, such as matrix metalloproteases and collagenase, are also able to degrade fibrin. Plasmin is an activated form of plasminogen and the combination of plasminogen and plasminogen activators (PAs) serve as unlimited supply of proteolytic capacity. It is known from animal experiments that mouse granulosa cells that line the interior of the follicle, as well as theca cells produce different PAs that activate plasmin [19,20]. In addition to plasmin's ability to degrade fibrin, it also generates active collagenase [21,22]. The degradation of the matrix surrounding the follicle starts early and was noticeable 24 h after encapsulation. The slowest degradation was observed in Th50 formulation. The Th500 condition degraded slightly faster, but the fastest degradation was observed in Th5 condition where the crosslinking time was insufficient to form strong fibers at this low concentration of thrombin. Fibrin degradation may be delayed by addition of aprotinin or other protease inhibitors, which may be useful for smaller follicles that require longer culture time in more rigid environments, yet the inhibitors can be removed to initiate follicle growth.

The dynamic mechanical properties obtained with FA-IPN have been proposed to be consistent with the natural developmental process for ovarian follicles [8,13,15]. Three-dimensional culture of ovarian follicles maintains the morphology of the follicle and the cell–cell and cell–matrix interactions. Follicle development is regulated by many endocrine and paracrine factors, as well as the ECM of the follicle [6,12]. Antrum formation and steroidogenesis
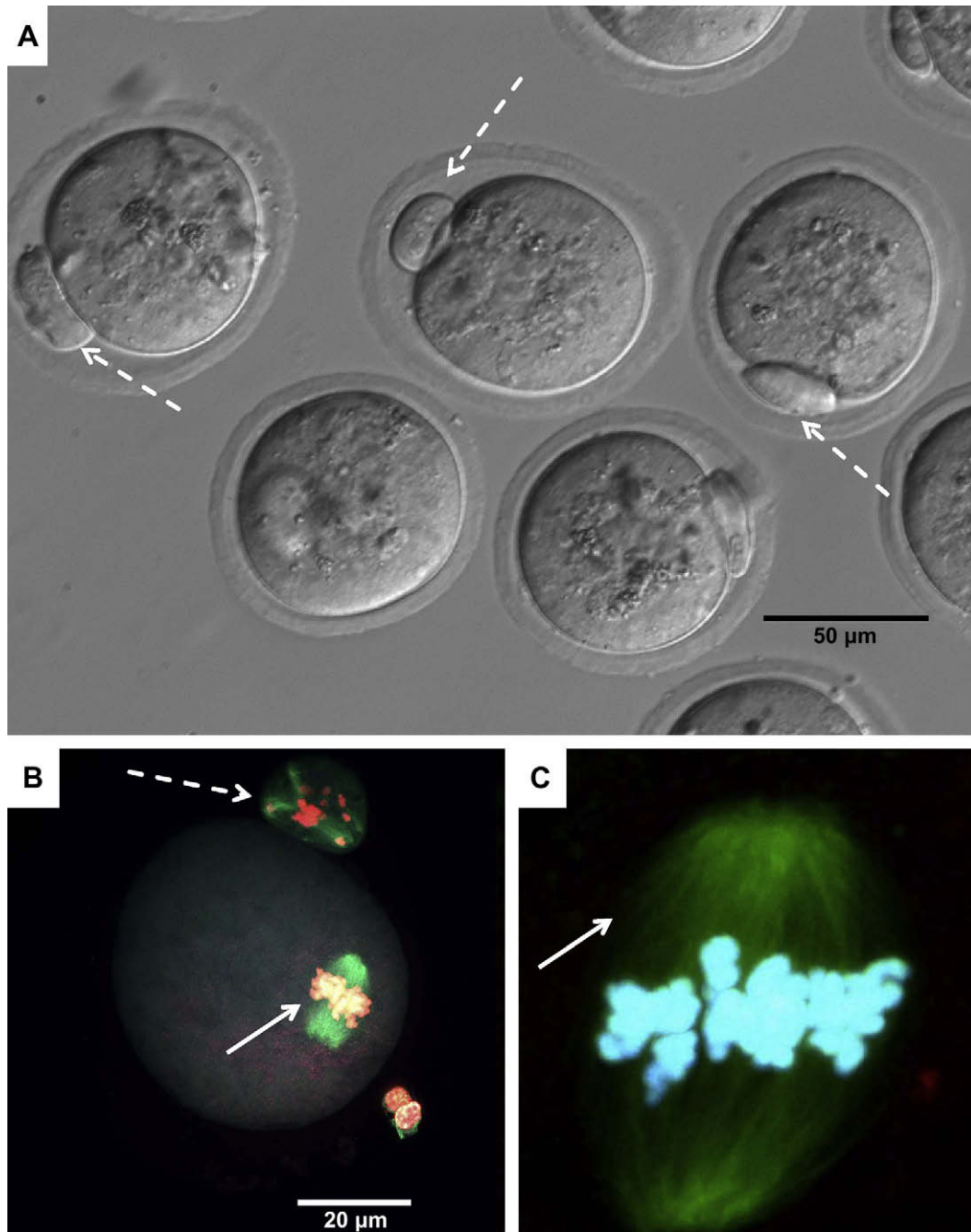
Fig. 7. Two-layered secondary follicles cultured for 12 days in FA-IPN were matured in vitro and oocytes resumed meiosis and extruded the first polar body (dashed arrow), bright field image, 40× (A) and confocal image of the MII stage follicle with the spindle (B, C, spindle is pointed with solid arrows).

are two aspects of this developmental process and are influenced by the matrix. Mechanical properties of the matrix have emerged as a significant regulator of follicle development. A model for mechanical regulation of ovarian function suggested that immature follicles reside in the "less permissive" region of the ovary – the cortex [8]. As immature follicles develop, they migrate to the medulla of the ovary, which is proposed to have a "more permissive" biomechanical environment. We hypothesized that small two-layered follicles cultured in a mechanically dynamic environment will mimic this in vivo environment and increase the rate of oocyte maturation. Previous studies using alginate could not recapitulate this dynamic environment. FA-IPNs have a modulus of 300 Pa immediately after formation, which is similar

to the maximal modulus that was considered permissive [8]. However, degradation of the fibrin component decreases the modulus of the hydrogel. At the end of culture, the modulus would be determined by the alginate gel, which has a modulus on the order of 40 Pa. Importantly, the final gel has a solids content of 0.125% alginate, and this solids content cannot form a gel by itself without the IPN.

The production of meiotically competent oocytes from the culture system was enhanced with the FA-IPN relative to the previous reports with alginate alone. Follicle growth and survival rate of the cultured two-layered follicles were similar for FA and alginate hydrogels. Follicles cultured in FA-IPNs and in the most permissive alginate condition [14] had a survival rate of 80% and

grew from 110 μm diameter on day 0 to 320 μm on day 12, which is significantly greater than in less permissive environment of 1% and 1.5% alginate. Relative hormone levels expressed as the ratio of progesterone and androstenedione to estrogen suggest appropriate steroidogenesis. Permissive environments ($G' < 300\,Pa$) have produced ratios of P/E and A/E of 0.38 and 0.23 respectively [8]. Although no significant difference between different thrombin conditions was observed, all ratios for P/E and A/E for follicles cultured in FA-IPNs ranged between 0.11–0.15 for P/E and 0.17–0.09 for A/E, suggesting healthy development of theca and granulosa cells. Although many of the growth characteristics are similar for the follicles cultured in FA-IPN and alginate alone, the rate of meotically competent oocytes produced by culture in FA-IPNs was between 75 and 82%, which improved upon our prior alginate only system of 67% [14]. Jin et al. [unpublished data] reported on 88% of MII stage oocytes matured from secondary follicles derived from primordial follicles after 4 days of ovarian organ culture of 8 days old mice. Additionally, 54% reached two-cell embryos after fertilization comparing to 33% after culture in alginate alone [14]. This increase in oocyte quality and fertilization rate of earlier stage follicles is an important step in engineering 3D culture systems, which can provide a fundamental tool to investigate follicle maturation, and may be applied to promote the growth of human follicles that can provide reproductive options for women facing a cancer diagnosis.

These hydrogels are proposed to be desirable for growing cell aggregates or clusters, in which cell–cell contacts can be retained yet the aggregate can degrade a matrix component to create space for expansion of the aggregate. We illustrate the potential of this approach using an ovarian follicle culture system, and demonstrate an enhancement of the quality of the follicle and oocyte at the completion of culture. However, the described two-component IPN may be applied to organized cell clusters other than ovarian follicle biological systems, such as matrix-directed cardioprogenitor cells [23], embryoid bodies (EBs) and primary cell co-cultures [24]. The biologically degradable natural fibrin component of the IPN degrades as cells grow, expand, and secrete ECM component. The inert slow-degradable alginate component of the gel maintains the integrity of the matrix in order to provide the needed physical support. The initial integrin concentration and the gel strength are likely determined by the fibrin component, yet alginate can be modified with ECM components to direct cell function after fibrin degradation. These dynamic properties of biomaterials may be able to directly influence the differentiation of cells at the exterior, and maintenance of the architecture of the cell aggregates can impact the subsequent growth of the group [25].

5. Conclusions

We developed FA-IPNs that have dynamic mechanical properties that can be employed to enhance tissue development relative to a single hydrogel. Fibrin and alginate can be gelled simultaneously under mild conditions to facilitate cell encapsulation, and the initial mechanics depending upon the properties of each component. Dynamic mechanical properties of the hydrogel can be imparted through the degradation of fibrin by the cell-secreted proteases, thereby leaving the non-degradable alginate to provide a physical support to the encapsulated cells. We demonstrated that FA-IPNs promote follicle growth and increase the number of meiotically competent oocytes relative to either fibrin or alginate alone. This system may generally be useful for the culture of cell aggregates or clusters, such as embryoid bodies, in which cell–cell contact should be maintained yet expansion of the cell aggregate can be facilitated.

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