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Adipose-derived stem cell-secreted factors promote early stage follicle development in a biomimetic matrix†

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Abstract

Development of primary follicles *in vitro* benefits from a three-dimensional matrix that is enriched with paracrine factors secreted from feeder cells and mimics the *in vivo* environment. In this study, we investigated the role of paracrine signaling from adipose-derived stem cells (ADSCs) in supporting primary follicle development in a biomimetic poly(ethylene glycol) (PEG) based matrix. Follicles co-cultured with ADSCs and follicles cultured in conditioned medium from ADSCs encapsulated in gels (3D CM) exhibited significantly ($p<0.01$ and $p=0.09$, respectively) improved survival compared to follicles cultured in conditioned medium collected from ADSCs cultured in flasks (2D CM) and follicles cultured without paracrine support. Gene expression of ADSCs suggested that the stem cells maintained their multipotency in the 3D PEG environment over the culture period, regardless of the presence of the follicle, while in the 2D condition the multipotency markers were downregulated. The differences in cytokine signatures of follicles exposed to 3D and 2D ADSC paracrine factors suggest that early cytokine interactions are key for follicle survival. Taken together, the biomimetic PEG scaffold provides a three-dimensional, *in vivo*-like environment to induce ADSCs to secrete factors which promote early stage ovarian follicle development and survival.

Introduction

Common cancer therapies, including chemotherapy and radiation, are gonadotoxic and frequently result in premature ovarian failure and infertility. For prepubertal girls preparing to undergo fertility compromising therapies, current fertility preservation treatment involves the experimental cryopreservation and later autotransplantation of ovarian tissue, which carries a risk of re-introducing occult malignant cells [1–5]. The ability to isolate and mature follicles *in vitro*, resulting in cryopreservation of meiotically competent oocytes, would allow the preservation of future fertility in prepubertal girls without the risk of re-exposure to malignant cells. While later stage follicles showed somewhat promising outcomes from *in vitro* culture, primary and primordial follicles, the most abundant follicles in cryopreserved ovarian tissue, suffer from low rates of activation and growth *in vitro*, possibly due to the absence of paracrine signaling from the ovarian environment [6–8].

Primary follicle development is gonadotropin-independent and therefore requires locally-produced factors to support granulosa cell proliferation [9], which in turn enables oocyte development and survival. A variety of follicle culture methods have been proposed to mimic the paracrine environment of the ovary through feeder cell co-culture and conditioned medium. Tagler et al. reported similar growth and survival outcomes when follicles were co-cultured with mouse embryonic fibroblasts (MEFs) or cultured with MEF conditioned medium [10], indicating paracrine support from MEFs is unidirectional and unaffected by feedback from the follicle. Alternatively, follicles co-cultured with ovarian stromal cells had improved folliculogenesis outcomes, possibly the result of bidirectional crosstalk between the follicles and stromal cells [11]. Follicles co-cultured

in groups of five or ten also show improved follicle growth outcomes over follicles cultured individually in a number dependent manner [12] stressing the importance of crosstalk between follicles and changes in the secretome [13]. These studies suggest that depending on the type of feeder cells and the paracrine mediators they secrete, unidirectional or bidirectional communication may be effective in promoting folliculogenesis.

Despite their success in promoting folliculogenesis, MEFs, ovarian cells, and embryonic tissue present significant limitations, such as availability and xeno-immunocompatibility, for clinical translation [10,11,14–16]. Adipose-derived stem cells (ADSCs), on the other hand, have been shown to be effective for a variety of human therapeutic applications and safe, especially in an *in vitro* setting [17–19]. Their abundance, ease of procurement, and ability to be easily isolated from either autologous or allogeneic sources makes them an optimal resource for co-culture purposes. Furthermore, the paracrine and cytokine profiles of multipotent ADSCs overlap with what is currently known of early follicle signaling [19–23]. We hypothesize that ADSCs will participate in bidirectional crosstalk when co-cultured with the developing follicle, closely mimicking the *in vivo* paracrine signaling environment.

In addition to the *in vivo* paracrine signaling environment, the structure of growing follicles allows in- and outward gradients of oocyte-derived and paracrine factors, justifying the use of three-dimensional (3D) biomimetic matrices to mimic functional interactions [24,25]. Biodegradable and tunable poly(ethylene-glycol) (PEG)-based hydrogels have been shown to accommodate the significant volumetric expansion of follicles using plasmin-sensitive "YKNS" peptide crosslinkers, which are cleaved by plasmin activated by follicle-secreted plasminogen activator

[26,27]. PEG hydrogels are stiffer than traditional alginate and fibrin matrices used for follicle culture, but the degradable nature mimics the process by which follicles remodel the ovarian extracellular matrix during expansion. In consideration of co-encapsulation of follicles and ADSCs, we must accommodate the conditions needed for successful growth of follicles while maintaining ADSC viability. Therefore, we have incorporated dual-degrading peptide crosslinkers (Supplemental Figure S1) via the addition of "VPMS", a matrix metalloprotease (MMP)-sensitive peptide sequence, which is cleaved by ADSC-secreted MMPs [28]. A system which supports both follicles and ADSCs allows us to study the bidirectional interaction between cells for promoting folliculogenesis.

While co-culture of follicles and ADSCs in a hydrogel will facilitate bidirectional crosstalk, the physical environment affects the multipotent state and the secretome of ADSCs [29,30], and will likely vary between 2D or 3D systems. Several studies investigating mesenchymal stem cells (MSCs) have observed maintenance of stemness markers and proliferation capacity in 3D environments[31–36]. Furthermore, a study of murine bone marrow-derived MSCs in hydrogels illustrated increased secretion of cytokines to promote angiogenesis when MSCs were cultured in hydrogel scaffolds[37]. For these reasons, the efficacy of ADSCs as a feeder cell source in follicle culture may differ in 2D and 3D environments.

Use of conditioned media collected from ADSC cultures, either 2D or 3D, presents a more practical approach, yet co-culture of follicles with ADSCs allows bidirectional crosstalk and may produce a unique cytokine signature. In this study, we investigated the effects of conditioned media obtained from ADSCs cultured in flasks (2D) or 3D hydrogels on supporting growth and survival

of the cultured follicles and determined whether the bidirectional communication prevailing between ADSCs and follicles grown in a 3D co-culture system significantly improves the outcomes of folliculogenesis over those grown in the presence of conditioned media.

Experimental Methods

Approach

Murine primary follicles were co-cultured with human ADSCs in a PEG hydrogel system to create culture conditions that closely mimic the *in vivo* structural and paracrine signaling environment prevailing during early folliculogenesis. Growth and survival of follicles co-encapsulated with ADSCs, where follicles and ADSCs can exchange signals continuously (bidirectional signaling) were compared with follicles grown in the presence of conditioned media (unidirectional signaling) obtained by culturing ADSCs in 2D and 3D systems (Supplemental Figure S2). The PEG hydrogel is not modified with integrin-binding sequences to prevent migration of granulosa cells away from the follicle and the loss of the spherical structure of the follicle. The absence of integrin-binding sequences also prevents migration of ADSCs and physical contact with the follicle, which allows us to decouple the effects of soluble paracrine signaling and direct cell-cell interactions over the culture period (Fig 1). Given their multipotent nature and to better understand the role of ADSC-derived factors in promoting follicle growth, we compared the expression of multipotency and paracrine factor genes of ADSCs cultured in 2D and 3D systems. To further characterize the paracrine signaling milieu that supports *in vitro* growth and survival of primary follicles, cytokine profiles of bidirectional and unidirectional signaling were also compared.

Hydrogel materials and preparation

8-arm PEG vinyl sulfone (PEG-VS) (40 kDa, >99% purity, JenKem Technology) and trifunctional (3 cysteine groups) MMP- and plasmin-sensitive crosslinker Ac-G**C**RDVPMS↓MRGGDR**C**GYK↓NS**C**G (YKNS/VPMS) (2391.8 g/mol, >90%, Celtek Peptides, cleavage site indicated by ↓) were used to form all hydrogels for follicle and ADSC cultures. All gels were formed using a 5% PEG-VS composition and crosslinked with YKNS/VPMS at a 1:1 stoichiometric ratio of –VS to thiol (-SH) groups. PEG and crosslinker were dissolved in isotonic 50 mM HEPES buffer (pH=7.4 at 37˚C) and 10μL gels were formed between parafilm-coated glass slides. Solution was kept at 37°C to allow gelation, which was terminated after 7 minutes by transferring gels to follicle maintenance media.

Animals

Female C57BL/6 and male CBA/J mice were purchased from Envigo (6-8 weeks old) and all procedures were performed in compliance with the Guidelines for the Care and Use of Animals at the University of Michigan and approved by the Animal Care and Use Committee at the University of Michigan (PRO00008465).

Follicle isolation, encapsulation, and culture

Primary follicles (single layer of cuboidal granulosa cells surrounding oocyte) were mechanically isolated from the ovaries of 10 to 12 day old B6CBAF1 mice (Envigo) in pre-warmed dissection

medium composed of Leibovitz's L-15 medium (Gibco, Thermo Fisher) supplemented with 1% (v/v) fetal bovine serum (Thermo Fisher) and 0.5% (v/v) penicillin-streptomycin (Thermo Fisher), then transferred to maintenance medium composed of minimal essential medium Eagle – alpha modification [αMEM] (Gibco, Thermo Fisher) supplemented with 1% (v/v) fetal bovine serum and 0.5% (v/v) penicillin-streptomycin. Individual primary follicles (90-110 μm in diameter) were transferred to hydrogel precursor mix, with or without ADSCs, and crosslinked at 37° for 7 minutes. The gels were then transferred to pre-equilibrated maintenance medium to recover for up to 1 hour. Encapsulated follicles were cultured for 10 days in individual wells of a 96-well plate containing 150 μL of growth medium (50% αMEM and 50% F-12 (Gibco) medium supplemented with 10 mIU/mL recombinant human FSH (Gonal-F, EMD Serono), 1 mg/mL bovine fetuin (Sigma-Aldrich), 3 mg/mL bovine serum albumin (Thermo Fisher), 5 μg/mL insulin (Sigma), 5 μg/mL transferrin (Sigma), and 5 μg/mL selenium (Sigma)). Half (75 μL) of the growth medium was exchanged every two days. Follicle survival was determined based on follicle morphology and growth. Follicle diameter was measured every two days in ImageJ by taking the average of two perpendicular measurements across the follicle. Follicles were considered dead if the oocyte was extruded and more than half of the oocyte was denuded of granulosa cells, or if follicle diameter did not change or decreased since the previous time point. Only the follicles which were alive and growing on day 10 of culture were used to generate the follicle growth curves across all time points.

Adipose-derived stem cell culture

Human ADSCs (Zen-Bio) were used in this study to gain a better understanding of their behavior in this system and to allow subsequent translation to support growth of human follicles, in view of the wide array of human gene markers and cytokines available for investigation. We used ADSCs collected from female patients of reproductive age (25 - 38) and within the normal range of body mass index (BMI) (18.8 - 21.6). All ADSCs were obtained under informed donor consent. ADSCs were expanded according to the manufacturer's protocol. Briefly, ADSCs were seeded in a flask at 8.9 x 10^3 cells/cm^2 and cultured in Preadipocyte Medium (PM-1) (Zen-Bio) until they were 85-90% confluent, with medium changes every two days. ADSCs were not used beyond passage 4 to ensure stemness. On the day of encapsulation, cells were trypsinized and centrifuged at 280*g* for 5 minutes. After aspirating the supernatant, the pellet was re-suspended in PEG gel precursor solutions at a concentration of 1 x 10^6 cells/mL. For the follicle and ADSC co-culture condition, gel precursors with ADSCs were formed into 10 μL drops and individual follicles were transferred to gels.

ADSC-conditioned medium

ADSC-conditioned medium was collected from cells cultured in PEG gels (3D CM) and from cells cultured in traditional flasks (2D CM) to determine changes in cytokine signatures resulting from different physical environments. For the 3D CM condition, ADSCs were encapsulated in 10 μL PEG-VS gels at a concentration of 1 x 10^6 cells/mL and cultured in individual wells of a 96-well plate in 150 μL of follicle growth medium. For the 2D CM condition, ADSCs were seeded at a density of 1.33 x 10^4 cells/cm^2 in a T25 flask with 5mL of follicle growth medium to keep consistent with the cell to medium ratio used for the co-culture condition to ensure that differences

in cytokine concentrations in the media would not arise from differences in cell concentration. Half (2.5 mL) of the medium was collected every two days for follicle culture and replaced with fresh follicle growth medium. Equilibrated conditioned medium was supplemented with an additional 10mIU/mL FSH before culture with follicles.

Hormone measurements

Follicle-secreted sex hormones androstenedione, estradiol, and progesterone were analyzed from the culture medium of follicles and ADSCs from days 4 – 10 of culture. For each replicate, media was pooled from two follicles or ADSC-loaded gels of the same condition. Three replicates of each condition were analyzed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Eunice Kennedy Shriver NICHD/NIH (NCTRI) Grant P50-HD28934).

RNA isolation and Real-Time qPCR

ADSC were cultured in PEG hydrogels with and without follicles for predetermined time points (4 and 10 days of culture). When co-cultured, hydrogels containing follicles were bisected under the microscope using insulin needles and the follicle was manually removed with a pipet. Up to 16 hydrogels containing ADSCs were placed in 2 mL of phenol-free alpha-MEM (Atlanta Biologicals) with 200 uL Liberase (Sigma, working concentration at 1.3 wunsch units/mL) and incubated at 37˚ C and 5% CO2 for 60 minutes. Pipetting was performed every 10 minutes through the incubation period. Two hundred microliters of (10%, v/v) fetal bovine serum (FBS, Thermo

Fisher) was added at the conclusion of incubation to discontinue the enzymatic digestion and the solution was centrifuged at 300*g* for 10 minutes. Supernatant was removed and mRNA was isolated using the RNeasy Mini Kit (Qiagen) following manufacturer's protocol. The mRNA was eluted with 30uL RNase-free water, flash frozen in liquid nitrogen and stored at -80˚ C. Total RNA (500 ng) was reverse transcribed into cDNA by the University of Michigan DNA Sequencing Core (Ann Arbor, MI). Real time PCR was performed using a 96 well TaqMan Array Plate (Thermo Fisher) and standard pre-designed human primers (Thermo Fisher) for 29 target genes consisting of a representative sample of candidate genes for ADSC stemness and lineage differentiation as well as paracrine factors known to be common between the ADSC and ovarian follicle secretomes (Supplementary Table 1). Amplification and fluorescence detection were carried out using the ABI 7900HT Fast Real Time PCR System (Applied Biosystems) with standard cycling conditions. Relative quantification of the amount of mRNA for each sample was normalized to 18S RNA, B-actin, GAPDH, and GUSB (ΔCT)[38]. Data was presented as the fold change ($\Delta\Delta$CT) in mRNA expression relative to ADSCs in standard 2D cell culture conditions on day 1 of culture.

Cytokine measurements and analyses

Spent media was collected every two days from follicles co-cultured with ADSCs, follicles cultured in 3D CM, and follicles cultured in 2D CM starting on day 4 of culture. Each replicate contains media pooled from two follicles of the same condition. Soluble cytokines (Supplementary Table 2) were measured from the culture media using the mouse angiogenesis/growth factor kit (MAGPMAG-24 K-27, Milliplex, EMD Millipore Corporation) on a Luminex FlexMAP 3D™ system (Millipore Sigma). Assays were run according to manufacturer's guidelines in 384-well

plates with minor changes including a 4x bead dilution as described previously [39]. All samples were measured in duplicate. For univariate analysis, we subtracted the lower detection limit (LDL) from the measured concentrations to normalize concentrations across conditions. For multivariate analysis, we excluded cytokines where the LDL varied greatly between background media used in different conditions.

The following mathematical conditions were applied to eliminate cytokines with variability in lower detection limits across plates and media conditions. For each cytokine, if $\frac{stdev(LDL1,LDL2,LDL3)}{min\ detected\ value} > 2$, it was removed. Additionally, to minimize the detection of false trends in the multivariate analysis, we eliminated cytokines if over half the measurements were below the LDL and required that each of the media conditions had at least one measurement above the detection limit. After this evaluation, seven cytokines (endoglin, angiopoietin-2, leptin, prolactin, IL-6, KC, VEGF-A) met criteria for inclusion in multivariate analysis.

Statistical analyses

Statistical analysis for follicle survival was performed in GraphPad Prism 7 using a Log-Rank test to determine significance between survival curves of each condition. We confirmed normality of follicle growth measurements on day 10 using a D'Agostino and Pearson test in Prism. Statistical analysis for follicle growth, univariate cytokine data, and differential gene expression was performed in Prism and involved a two-way ANOVA with repeated measures followed by Sidak's multiple comparisons tests, with significance determined as $p<0.05$. A principal component analysis (PCA) was performed to visualize differences in paracrine cytokine profiles across

conditions over time. A PCA model was created using cytokine data measured from 3D CM, 2D CM, and co-cultured follicles at day 4, 6, 8 and 10. All data was mean-centered and variance-scaled prior to analysis. Each sample was plotted on the first two principal components. Correlation networks were created for each condition using Pearson correlation coefficients computed for pairwise correlations between cytokine measurements. All multivariate statistical analysis was performed using Matlab (Mathworks).

Results and Discussion

Follicle survival and growth

Co-encapsulation of primary follicles ranging from 90 – 110 μm in diameter with ADSCs in PEG hydrogels and culture of follicles in 3D ADSC conditioned medium significantly improved follicle survival *in vitro* compared to follicles cultured alone (Fig 2a). As expected, follicles cultured individually in PEG hydrogels without any paracrine support from ADSCs (control) exhibited low survival rates, with only 44.4% of follicles surviving after 10 days of culture. However, when co-cultured with ADSCs (bidirectional communication), follicle survival increased significantly ($p<0.01$) to 70.0%, and follicles cultured in 3D CM (unidirectional communication) exhibited improved survival to 66.7% ($p=0.09$). In contrast, follicles cultured in 2D CM (unidirectional communication) exhibited survival rates (36.8% survival on day 10) comparable ($p=0.91$) to that of follicles cultured in the absence of CM.

Growth of follicles which survive to day 10 is significantly improved by paracrine support from ADSCs (Fig 2b). Follicles co-encapsulated with ADSCs grew from an average diameter of 103.9 ± 0.6 μm (average ± SEM) on day 0 to 212.7 ± 4.6 μm on day 10, a significant ($p<0.0001$) increase over control in which follicles grew from 104.8 ± 1.4 μm to 164.5 ± 10.0 μm after 10 days of culture. The surviving follicles cultured in both 3D CM and 2D CM also demonstrated significantly increased growth over control ($p<0.05$ and $p<0.0001$, respectively). Follicles cultured in 3D CM grew from 106.8 ± 1.1 μm to 190.2 ± 6.7 μm on day 10. Follicles cultured in 2D CM with an average starting diameter of 106.5 ± 1.5 μm exhibited a similar growth pattern to the 3D CM condition until day 8, at which point growth was accelerated and analogous to the co-culture condition, growing to a final diameter of 214.1 ± 17.4 μm. Follicles co-cultured with ADSCs and cultured in 2D CM both exhibited signs indicating the start of antrum formation, such as darkening of granulosa cells (Fig 2c), however follicles cultured in 3D CM did not show signs of antrum formation given that follicles in this condition did not grow as large. Culture medium was collected from follicles and ADSCs for functional analysis of sex hormones androstenedione, estradiol, and progesterone (Supplementary Figure S3). The results were consistent with the observed follicle growth patterns and confirmed that ADSCs do not secrete sex hormones. Primary follicles isolated from the ovary have fewer theca cells compared to larger follicles, resulting in attenuated androstenedione and delayed estradiol production.

Co-culture of follicles with ADSCs and culture of follicles in 3D CM significantly improved follicle survival compared to 2D CM and control. However, the follicles which survive in the co-culture and condition media groups showed similar growth patterns. The similarity in growth patterns of surviving follicles suggests that the cytokines secreted by ADSCs activate growth-

related mechanisms independent of the unidirectional or bidirectional culture environment (co-culture or CM). In contrast, survival-related cytokines are different depending on the 2D and 3D culture conditions of ADSCs. Here our data suggests that only ADSCs cultured in a biomimetic 3D and not 2D environment secrete factors which promote follicle survival.

ADSC gene expression

ADSCs were selected to serve as feeder cells because they represent an abundant, autologous and translational source of cells for applications involving human follicles and have the potential to differentiate in response to follicle-secreted factors. Compared to 2D controls, analysis of key stem cell multipotency markers expressed on day 10 in ADSCs co-encapsulated with follicles revealed a 4-fold increase in *NANOG* - a gene involved with self-renewal of undifferentiated embryonic stem cells ($p=0.02$), 3.8-fold increase in *SNAI2* - a Zinc finger protein coding gene ($p<0.0001$), 7.7-fold increase in octamer-binding transcription factor 4 (*Oct4*) - homeodomain transcription factor ($p<0.0001$), and greater than 10-fold increase in *SRY* (sex determining region Y)-box 2 (*SOX2)*- a transcription factor regulating cell fate ($p=0.016$) mRNA expression (Fig 3a-d). ADSCs cultured in 3D PEG hydrogels in the absence of the follicle displayed a 10-fold increase in *NANOG* ($p<0.0001$), 2.5-fold increase in *SNAI2* ($p<0.0001$), 5-fold increase in *Oct4* ($p=0.0001$), and 10-fold increase in *SOX2* ($p=0.025$) mRNA expression. ADSCs in PEG, with and without follicles, did not show up-regulation of early stage differentiation makers, such as *PPAR-g*, *PLIN4*, *ACAN,* or *ALPL,* compared to controls (Supplementary Figure S4), suggesting that ADSCs did not undergo adipogenic, chondrogenic, or osteogenic differentiation. Up-regulation of undifferentiated stem cell markers and insignificant changes in lineage differentiation markers in

ADSCs cultured with and without follicles indicate that the 3D culture condition of ADSCs maintains stem cell multipotency over the course of the culture period, consistent with previous mesenchymal stem cell studies[31–36], and that co-culture with follicles does not interfere with their multipotent potential.

In addition, ADSCs in a 3D environment exhibit up-regulation of genes for paracrine signaling molecules known to be important in early gonadotropin-independent ovarian follicular development (Fig 3e-g). After 10 days of culture, ADSCs co-encapsulated with follicles show a 3-fold increase in vascular endothelial growth factor A (*VEGFa*; $p<0.0001$), 18-fold increase in transforming growth factor beta 2 (*TGFB-2*; $p<0.0001$), and a greater than 3.5-fold increase in hepatocyte growth factor (*HGF*; $p=0.0005$) when compared to the baseline expression of ADSCs cultured in 2D. Furthermore, ADSCs cultured alone in PEG exhibit a 3-fold increase in *VEGFa* ($p<0.0001$), 15-fold increase in *TGFB-2* ($p<0.0001$), and 3-fold increase in *HGF* ($p=0.023$) mRNA expression. These results further support that the 3D environment induces changes in gene expression of paracrine markers in ADSCs. mRNA expression of *VEGF-A*, *TGFB-2*, and *HGF* were uniformly downregulated in ADSCs grown in 2D culture and significantly up-regulated in 3D culture of ADSCs with and without follicles at all time points. Differences in expression between ADSCs co-cultured with follicles versus 3D culture of ADSCs alone, if present, occurred on Day 4 of culture only. The significant upregulation of these paracrine factor genes may explain the nominally improved growth seen in follicles co-cultured with ADSCs compared to 3D CM beginning on day 4 of culture. These results further confirm the importance of physical environment in regulating paracrine gene expression of ADSCs and demonstrate that bidirectional crosstalk between ADSCs and follicles is not the impetus for changes in ADSC gene expression.

Cytokine analysis

To understand the signaling environment of the crosstalk between follicles and ADSCs, we investigated soluble cytokines and growth factors (Supplementary Table 2). Data-driven multivariate analysis was carried out to examine interactions between soluble paracrine factors. Principal component analysis (PCA) performed on cytokine measurements identified two principal components that captured 69.2% of the variance and revealed cytokine signatures that were associated with follicles co-encapsulated with ADSCs versus those cultured in ADSC conditioned medium (Fig 4a-b). Follicles co-cultured with ADSCs and follicles cultured in 3D CM form distinct clusters on the scores plot illustrating differences in soluble cytokine profiles; however, the co-culture condition exhibits spreading along the first principal component over time, indicating changes in the signature over the course of the culture period. Early 2D CM signatures are similar to 3D CM but differentiate over time, consistent with the trend of follicle growth in 2D CM in which follicles in 2DCM and 3DCM grow similarly at early time points but 2DCM growth diverges from 3DCM after day 6.

The loadings plot indicates that IL-6, KC, and VEGF-A may be influencing the separation of the co-culture and 2D CM points from 3D CM along the first principle component, which is consistent with individual trends (Supplementary Figure S5, Supplementary File E1). Concentrations of IL-6, KC and VEGF-A increase over time in the co-culture and 2D CM profiles and spreading of the later time points of these conditions in the PCA plot can be attributed to this dynamic activity. Concentrations of these three cytokines were higher in the 2D CM condition, which corresponds

to more exaggerated spreading in the PCA plot for 2D CM compared to co-culture. In contrast, the 3D CM profile shows consistent concentrations of these factors over time, which corresponds to clustering of all time points in the second quadrant of the scores plot. These factors have important roles in regulating folliculogenesis [13,40] and promoting angiogenesis [41,42], and therefore association of these factors with the co-culture and 2D CM points may account for slightly increased growth in these conditions.

Correlation maps of all factors included in the PCA revealed positive and negative interactions across time points (Fig 5a-c). The correlation map for follicles co-cultured with ADSCs shows a mix of positive and negative correlation for factors across all time points. Follicles cultured in 2D CM shows more dense correlation between later time points, and correlation is primarily positive between factors except between leptin on day 4 with IL-6 on day 8 and between VEGF-A on day 4 with endoglin on day 10. Follicles cultured in 3D CM exhibit less significant correlation between factors, but ANG2, IL-6, and leptin are strongly correlated from day 4 to day 8.

The correlation maps are in agreement with the trends observed in the PCA scores and loadings plots. The strength of correlations, depicted by line thickness, is similar between the co-culture and 2D CM conditions while 3D CM showed less significant correlations. This difference in correlation significance alludes to the dynamic cytokine signatures depicted in the PCA plot for co-culture and 2D CM, compared to the more static signature of 3D CM, possibly because of differentiation in 2D and bi-directional crosstalk in co-culture. Furthermore, the strongest correlations in the 3D CM map are between leptin and ANG-2, the factors which drive spreading of the 3D CM points along the second principle component in the scores plot.

These results further demonstrate how ADSC-secreted factors are responsive to physical environments, leading to different trends for follicle survival and growth between conditions. The presence of positive and negative correlation between factors in the co-culture condition, in conjunction with differences in follicle growth and survival, may indicate bidirectional crosstalk between follicles and ADSCs, whereas the absence of strong negative correlation in the 3D CM and 2D CM maps could be a result of unidirectional signaling and the inability of the follicle to affect ADSC secretome. Additionally, the co-culture and 3D CM conditions show uniform correlation density across time points, indicating the importance of paracrine signaling spanning the culture period and potentially explaining the overall improved survival in these conditions. Conversely, the 2D CM correlation map shows fewer correlations between factors on days 4 and 6 than the later time points, suggesting that poor interactions between factors early in the culture led to a drop in follicle survival on day 8.

The identified signatures capture small differences in the three conditions based on the seven factors which we found significant. PCA indicates that the cytokine signature of 2D CM is more dynamic over time than the co-culture and 3D CM conditions, which may correlate with downregulation of multipotency markers of ADSCs in 2D and indicate that ADSCs in 2D are differentiating, and as a result the cytokine signature is changing. Furthermore, correlation maps indicate that paracrine signaling in the early time points may lead to better survival in the co-culture and 3D CM conditions. These results again suggest that the 3D environment has a strong impact on ADSC secretome, leading to differences in follicle survival.

Conclusions

In this report, we have demonstrated improved primary folliculogenesis outcomes when follicles are co-cultured with ADSCs or cultured in ADSC-conditioned medium in a dual-degradable PEG matrix. Follicles co-cultured with ADSCs and follicles cultured in 3D ADSC conditioned medium exhibited significantly improved growth over 2D CM and control, indicating that ADSCs in a 3D environment secrete factors which promote follicle survival. However, follicles which survived until day 10 in the co-culture and conditioned medium groups exhibited comparable growth and reached sizes ranging between 190 and 215 μm, which is not sufficient for producing mature oocytes [43]. We determined that ADSC gene expression is dependent on physical environment, where ADSCs in PEG, in the presence and absence of the follicle, maintain their stemness over the course of the culture period. Furthermore, ADSC secretome is dependent on physical environment, leading to different folliculogenesis outcomes. The cytokine signatures of follicles co-cultured with ADSCs and follicles cultured in 3D CM remain consistent over the culture period and exhibit correlation between factors across all time points. The cytokine signature of follicles in 2D CM was more dynamic, possibly as a result of ADSC differentiation, and inadequate correlation between factors at early time points may have resulted in decreased follicle survival. In this study, we determined that culturing ADSCs in a 3D environment induces the expression of factors to promote follicle growth and survival, resulting in overall improved outcomes. We further demonstrated that follicles and ADSCs can be cultured in follicle growth medium to allow for paracrine signaling without the complications of multiple media formulations. This system represents a promising first step for a fertility preservation option for women and girls facing primary ovarian insufficiency as a result of gonadotoxic anti-cancer therapies. Future studies need

to address the importance of bidirectional versus unidirectional communication in promoting preovulatory follicle growth and oocyte quality.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Graphical abstract: ADSCs encapsulated in biomimetic PEG hydrogels maintain stemness and secrete survival- and growth-promoting factors that support *in vitro* folliculogenesis**.**

Fig 1. Fluorescent labeling of follicles co-cultured with and without adipose-derived stem cells. GFP-labeled follicles (green) encapsulated in PEG hydrogels with and without (red) fluorescently labeled ADSCs on day 0 of culture (scale bars are 100μm). Without integrin-binding sequences, ADSCs remain isolated from the follicle when co-cultured in PEG.

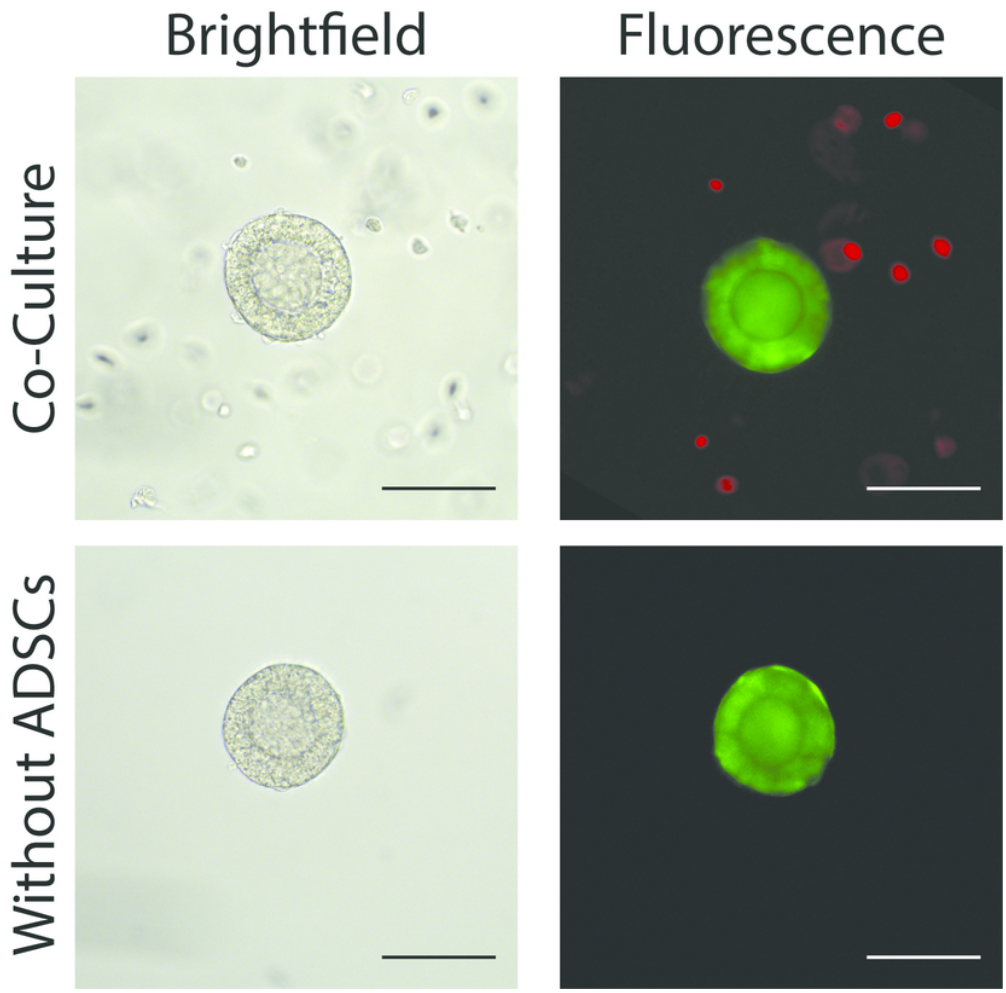
Fig 2. Growth and survival of follicles co-cultured with ADSCs and follicles cultured in ADSC-conditioned medium. (a) Survival and (b) growth of follicles co-cultured with ADSCs (green), cultured in conditioned medium from ADSCs cultured in a 3D environment (black), cultured in conditioned medium from ADSCs cultured in 2D flasks (blue), and cultured without paracrine support from ADSCs (red). (c) Representative images of follicles in each condition on days 0, 6, and 10 (scale bars are 100μm). White arrow heads indicate ADSCs in co-culture with follicles. Black arrows indicate granulosa cell darkening before antrum formation. Survival sample sizes: n = 100 (co-culture), 24 (3D CM), 19 (2D CM), 27 (control). Growth sample sizes: n = 65 (co-culture), 16 (3D CM), 7 (2D CM), 12 (control).

Fig 3. Gene expression of ADSCs cultured in 2D and 3D environments. Up-regulation and down-regulation of gene expression in human adipose derived stem cells (ADSCs) cultured in standard 2D conditions (blue), encapsulated in 3D PEG without follicles (red), and encapsulated in 3D PEG with primary murine follicles (green) on days 4 and 10 of culture relative to gene

expression of ADSCs in 2D culture on Day 1 (mean + SEM, n=48, $p<0.05$, vs ADSCs in 2D culture control group). (a-d) Markers of stem cell multipotency Oct4, NANOG, SOX2, and SNAI2 were up-regulated in both 3D encapsulation without follicle and 3D encapsulation with primary murine follicle conditions. (e-g) Vascular endothelial growth factor A (VEGFA), transforming growth factor beta 2 (TGFB2), and hepatocyte growth factor (HGF) were up-regulated in both 3D encapsulation without follicle and 3D encapsulation with primary murine follicle conditions.

Fig 4. Analysis of cytokine signatures in co-culture, 3D CM, and 2D CM. (a) PCA scores and (b) loadings plots of cytokines in co-culture (n=3, green), 3D CM (n=3, grey), and 2D CM (n=3, blue) samples at all 4 time points (days 4, 6, 8, & 10; light to dark). The first two PCs accounted for 69.2% of total variance; 48.3% and 20.9% respectively.

Fig 5. Bivariate cytokine correlations in co-culture, 3D CM, and 2D CM. Correlation networks for (a) co-culture, (b) 3D CM, and (c) 2D CM systems were generated using the cytokines on days where all measurements (n=3) were detectable in the given condition. Each node (dot) represents a cytokine on a specific day, and each edge (line connecting nodes) represents the correlation between the two nodes. Nodes are colored by day (day 4 as dark blue, day 6 as cyan, day 8 as green, and day 10 as orange). Edge thickness represents the significance of the bivariate correlation, thicker being more significant. Edge color represents the correlation coefficient, indicating the direction of correlation (red denoting positive and blue denoting negative). All correlations with a p-value below 0.1 are not shown.



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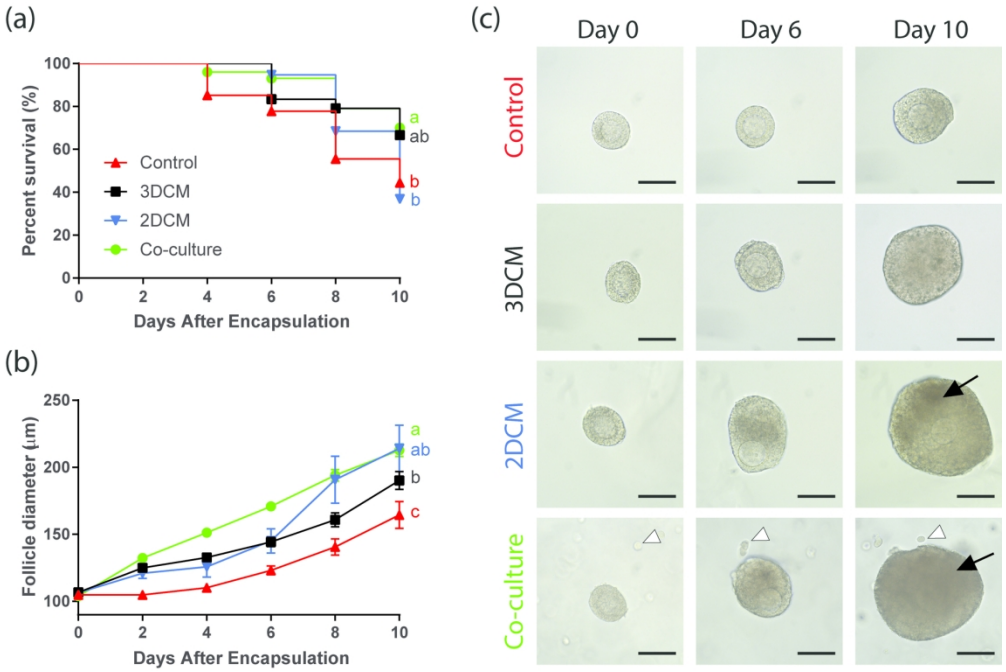

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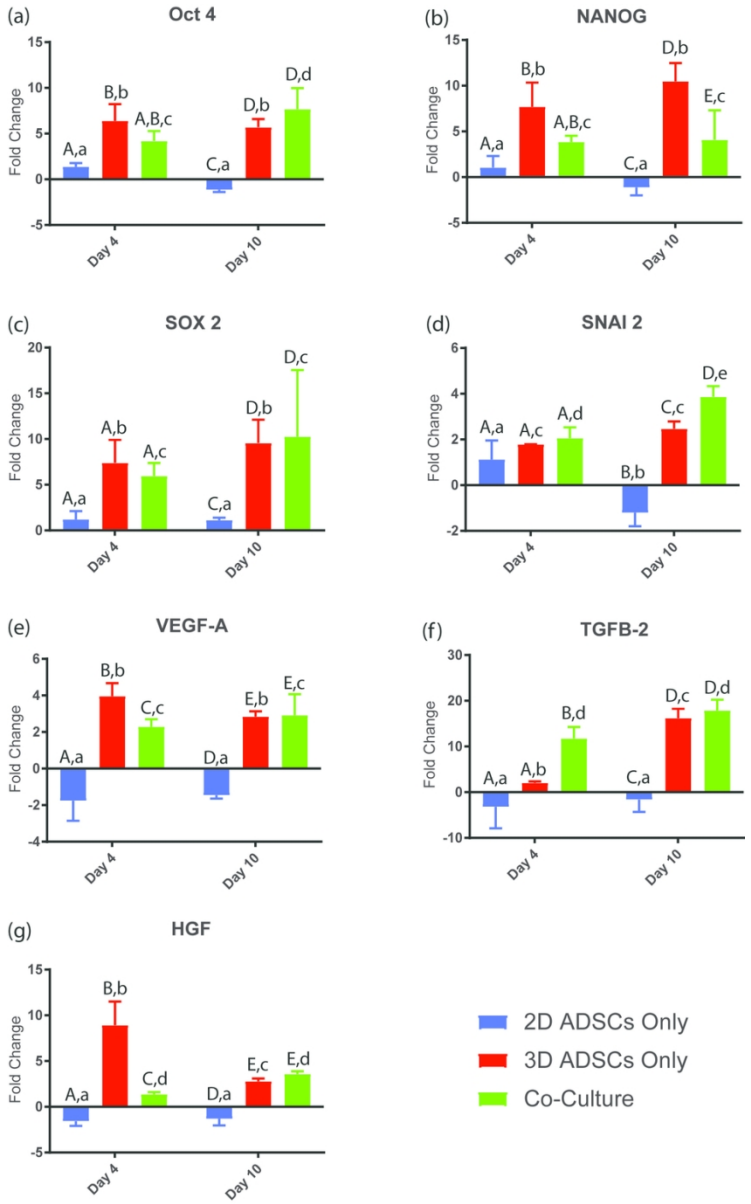

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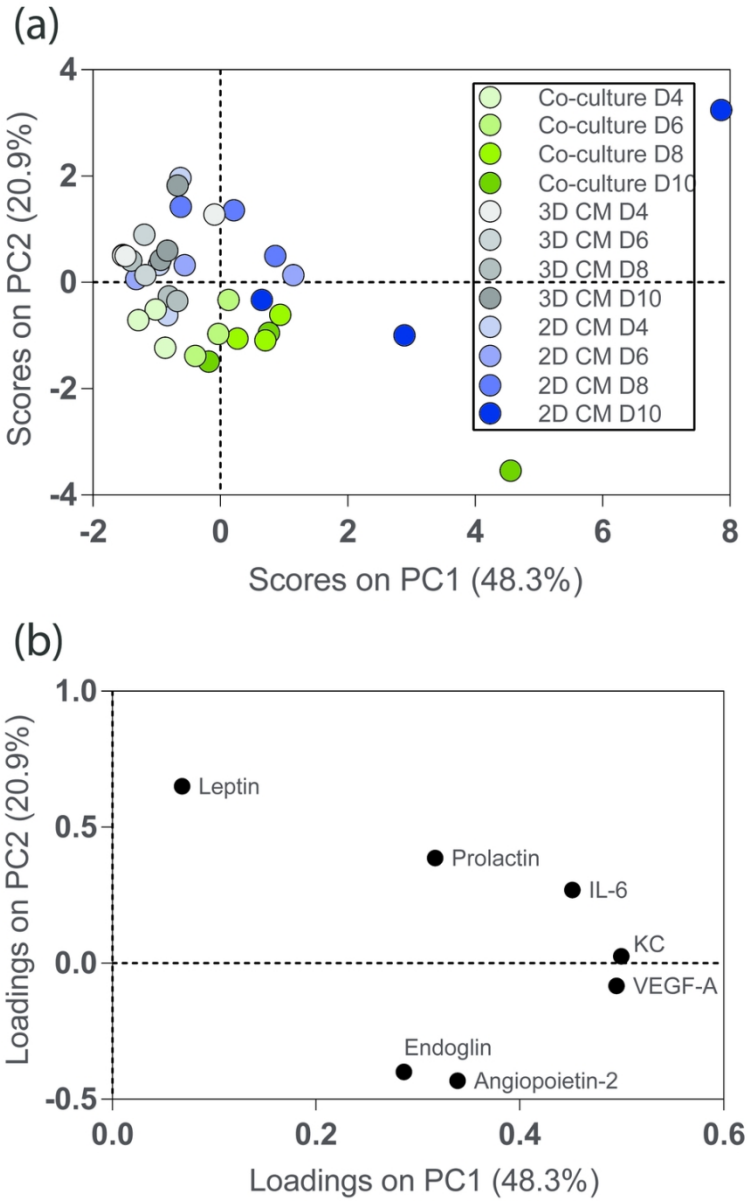

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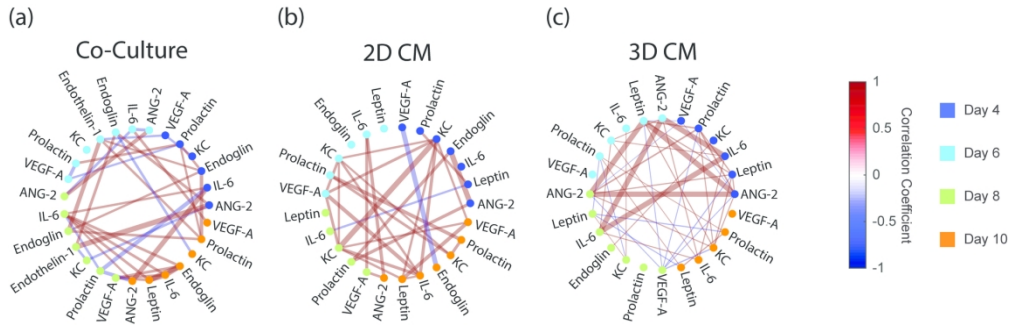

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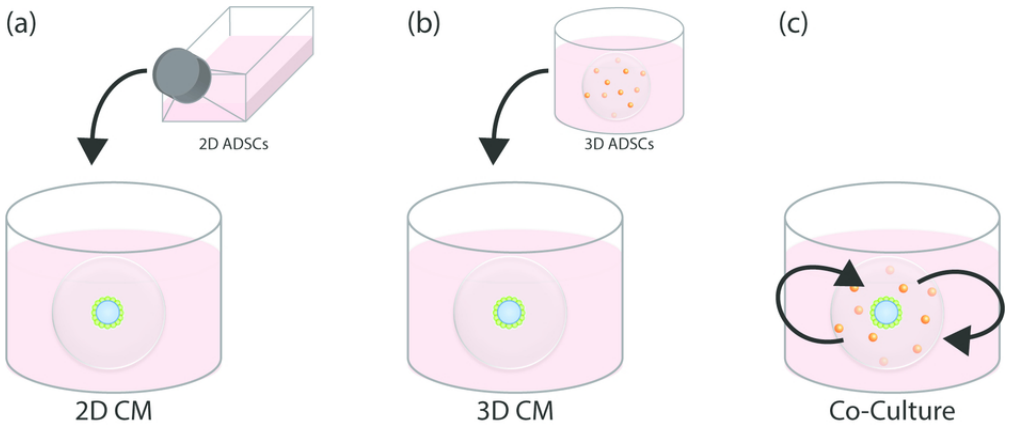

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