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Proangiogenic Activity of Endometrial Epithelial and Stromal Cells in Response to Estradiol in Gelatin Hydrogels

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Abstract

Biomaterial vascularization remains a major focus in the field of tissue engineering. Biomaterial culture of endometrial cells is described as a platform to inform the design of proangiogenic biomaterials. The endometrium undergoes rapid growth and shedding of dense vascular networks during each menstrual cycle mediated via estradiol and progesterone in vivo. Cocultures of endometrial epithelial and stromal cells encapsulated within a methacrylamide-functionalized gelatin hydrogel are employed. It is reported that proangiogenic gene expression profiles and vascular endothelial growth factor production are hormone dependent in endometrial epithelial cells, but that hormone signals have no effect on human telomerase reverse transcriptase (hTERT)-immortalized endometrial stromal cells. This study subsequently examines whether the magnitude of epithelial cell response is sufficient to induce changes in human umbilical vein endothelial cell network formation. Incorporation of endometrial stromal cells improves vessel formation, but coculture with endometrial epithelial cells leads to a decrease in vascular formation, suggesting the need for stratified cocultures of endometrial epithelial and stromal cells with endothelial cells. Given the transience of hormonal signals within 3D biomaterials, the inclusion of sex hormone binding globulin (SHBG) to alter the bioavailability of estradiol within the hydrogel is reported, demonstrating a strategy to reduce diffusive losses via SHBG-mediated estradiol sequestration.

Keywords

endometrium; estradiol; hydrogels; progesterone; vasculogenesis

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Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

1. Introduction

Nutrient transport has long been identified as a key limitation for the field of tissue engineering.^[1] Physiologically, capillaries with a maximal spacing of 200 μm facilitate nutrient and waste transport within tissues,^[2] suggesting the eventual need to generate similar vessel network densities in engineered biomaterials. While native proangiogenic processes can facilitate new vessel ingrowth within implanted biomaterials, the process can take weeks.^[1a,3] As a result, many biomaterials are either implanted in an acellular state or with a reduced density of encapsulated cells. Biotransport concerns can be particularly significant in the case of large hydrogel constructs or for highly metabolically active cells. While recent efforts using 3D printing have demonstrated avenues to generate on-demand vessel architectures,^[4] concerns with these approaches include the achievable network density as well as whether it is necessary to define network architecture and anastomosis sites as opposed to providing signals to support in situ vessel formation. There is a need to identify approaches to facilitate the rapid development of vascular structures that can readily incorporate into existing host vasculature, as well as approaches to promote remodeling of the vessel network to achieve increased vessel network density.

Tissue engineering efforts have increasingly explored a wide design parameter space regarding the use of biomaterial carriers (e.g., hydrogels, scaffolds), cells, and biomolecular cues in an attempt to improve in situ vascularization processes. For example, cocultures of endothelial cells with primarily stromal cell population have been shown to stabilize newly formed endothelial tubes.^[5] Mesenchymal stem cells (MSCs) have also received special interest due to their potential to secrete proangiogenic factors that may enhance endothelial cell proliferation and migration in a manner similar to that seen in vivo with pericytes.^[6] MSCs also offer the potential to differentiate into tissue-specific stromal cells for a wide variety of musculoskeletal tissues.^[7] However, a primary approach for promoting proangiogenic or provasculogenic responses remains delivery and presentation of biomolecule agonists and antagonists. While vascular endothelial growth factor (VEGF) on its own has been shown to increase proangiogenic phenotype in a wide range of biomaterials,^[8] response typically requires high dosages and can often promote rapid formation of small and relatively immature vessels. An increasingly common strategy, inspired by native angiogenic processes in response to regional hypoxia, relies on rapid release of an initiator of vessel formation such as VEGF followed by delayed release of factors, such as platelet-derived growth factor, that recruit supporting cells to stabilize the endothelial structures.^[9] These previous efforts now motivate additional bioinspired strategies to facilitate proangiogenic processes in biomaterials, some inspired by the dynamic set of cues required to promote vessel formation, growth, and maturation.

The endometrium, the lining of the uterus and site of embryo implantation, is an intriguing candidate model for inspiring proangiogenic biomaterials. As opposed to cancer-inspired models for vascularization, the endometrium is the site of cyclical growth, maturation, and shedding of dense vessel structures over the course of each menstrual cycle.^[10] Further, rapid changes in vessel architecture during and after embryo implantation take place in order to support the growing embryo until placentation is well developed at the end of the first trimester.^[11] The dynamics of endometrial vessel development, remodeling, and shedding

are tightly regulated by temporally varying levels of the sex hormones estradiol and progesterone.^[12] Hormonal regulation of the endometrium motivates exploring the use of sex hormones and endometrial-derived cells as novel means to initiate and control proangiogenic processes in vitro. Recent efforts in our lab showed that soluble estradiol was sufficient to induce VEGF secretion by endometrial epithelial cells at a level sufficient to induce changes in human umbilical vein endothelial cells (HUVECs) metabolic activity in a model collagen scaffold.^[13] The open pore architecture of the collagen scaffold made it difficult to monitor subsequent vessel formation events. Here, we describe the use of estradiol and progesterone to alter the individual and combined proangiogenic activities of endometrial epithelial and stromal cells (endoEpCs and endoSCs, respectively), as well as subsequent endothelial cell vessel formation, within a methacrylamide-functionalized gelatin (GelMA) hydrogel recently described by our laboratory.^[14] All experiments were performed in three-dimensions, with cells encapsulated directly within the hydrogel matrix from the start of the experiment. Inspired by the temporal presentation of sex steroids within the native endometrium, we also explore a method using sex hormone binding globulin (SHBG) to sequester estradiol within the hydrogel as a means to alter residence time.

2. Results

2.1. Hormone Supplementation Alters Angiogenic Gene Expression in EndoEpC Laden Hydrogels but Not EndoSC Laden Hydrogels

Expression profiles of three proangiogenic (*ANG1*, *ANG2*, *VEGF*) genes were examined in endoEpCs encapsulated within GelMA hydrogels as a function of media supplementation (control growth media; 1×10^{-6} M E2 added; 1×10^{-6} M P4 added). Expression of *ANG2* in endoEpCs, typically upregulated at sites of vascular remodeling,^[15] was hormone dependent ($p = 0.028$) (Figure 1B). Here, 1×10^{-6} M E2 had an overall effect of inducing significantly higher *ANG2* expression levels than 1×10^{-6} M P4 supplementation ($p = 0.04$), and trended toward higher expression compared to the nonsupplemented control ($p = 0.064$). EndoEpCs also showed a significant increase in *VEGF* expression with time ($p < 0.001$ for all time comparisons) (Figure 1C); however P4 supplemented media led to consistently significantly lower *VEGF* gene expression compared to E2-supplemented media ($p = 0.001$) or control media ($p = 0.012$). No observable trend in response to time or hormone supplementation was observed for *ANG1* (Figure 1A), typically associated with vessel maturation.^[15]

In contrast, while expression of *ANG1* (Figure 2A) and *VEGF* (Figure 2C) increased significantly with time at day 7 ($p < 0.01$) in endoSCs within the GelMA hydrogel, neither E2 nor P4 supplementation significantly altered angiogenic (*ANG1*, *ANG2*, *VEGF*) or stromal remodeling (*MMP11*) gene expression profiles (Figure 2).

2.2. EndoEpCs Produced More VEGF than EndoSCs

We subsequently quantified the effect of hormone supplementation on the number, metabolic activity, and VEGF protein secretion of epithelial and stromal cells within the GelMA hydrogel. By day 4 in culture, significant differences were observed between endometrial stromal and endoEpCs in GelMA hydrogels. Notably, while hormone

supplementation did not alter responses for each cell type, endoEpCs showed significantly ($p < 0.001$) increased proliferation, reduced ($p < 0.001$) metabolic activity per cell (Figures 3A,B), but most notably enhanced ($p < 0.001$) VEGF secretion into the media (Figure 3C).

2.3. Exogenous VEGF and EndoSCs, but Not EndoEpCs, Improves Vascularization in Cocultures with Endothelial Cells

To confirm the angiogenic potential of cocultures of endoEpCs and endoSCs with nonendometrial endothelial cells, GelMA hydrogels were seeded with HUVECs alone, HUVECs plus endoSCs, or HUVECs plus endoSCs and endoEpCs. Specimens were either maintained in conventional growth or VEGF supplemented (100 ng mL^{-1}) media for up to 10 d. Visible differences in vasculature were apparent between all culture conditions via fluorescent imaging following endothelial staining (CD31) (Figure S1, Supporting Information).

Mean metrics of vascular network complexity were acquired from measurements taken from each side of the hydrogel. Notably, addition of large dosages of soluble VEGF significantly ($p < 0.001$) increased the area, number of junctions, total vessel length and average vessel length. Further, the addition of endoSCs led to an increase in vessel area, total length, and average length over HUVEC cultures alone (Figure 4, $p < 0.05$). Additionally, direct addition of endoEpCs to form a triculture adversely affected metrics of vessel formation.

2.4. E2 Supplementation Did Not Alter Endothelial Cell Organization

We subsequently examined the potential influence of exogenous estradiol ($(0-1) \times 10^{-6} \text{ M}$) on network formation in GelMA hydrogels containing cocultures of endothelial cells and endoSCs (Figure S2, Supporting Information). Here, metrics of vessel formation showed no effect of estradiol concentration on vessel area, number of junctions, as well as total and average vessel length (Figure 5).

2.5. Incorporation of SHBG Alters the Retention and Release of E2 from the GelMA Hydrogel

We next examined an approach to alter the retention of E2 within the hydrogel network via the inclusion of SHBG. Here, the heat treatment (60°C , 1 h) sufficient to release E2 bound by SHBG did not affect the ability to measure total dose of E2 remaining within the hydrogel network (Figure S3, Supporting Information). However, covalent incorporation of the SHBG into the network via PEGylation^[14d,16] rendered SHBG unable to bind exogenous E2 (data not shown). However, SHBG added to the hydrogel precursor suspension prior to photoimmobilization significantly ($p = 0.004$) increased the capacity to retain E2 within the hydrogel over time (Figure 6). Examining E2 retention, while almost 85% of the initially loaded $1 \times 10^{-6} \text{ M}$ E2 dose was released from the GelMA network, retention was significantly ($p < 0.005$) higher at 6, 12, and 24 h for the GelMA hydrogel also incorporating $1 \times 10^{-6} \text{ M}$ SHBG (Figure 6A). Here, greater than 30% of the E2 dose was still retained in the SHBG containing hydrogel after 24 h. Further, increasing the dose of SHBG initially incorporated within the GelMA hydrogel ($(0-5) \times 10^{-6} \text{ M}$) led to a significant ($p < 0.001$) increase in initial sequestration of E2 ($1 \times 10^{-6} \text{ M}$ dose for all conditions) within the network (Figure 6B).

3. Discussion

In this study, we sought to leverage endometrial physiology to explore the vascularization of biomaterials in vitro. Few studies have previously sought to study the effects of sex hormones on endometrial angiogenesis in vitro.^[17] While these studies sought to understand specific endometrial angiogenic phenomena, they did not try to use endometrial-inspired signals to promote proangiogenic processes in biomaterial constructs. Additionally, none of these studies used a 3D biomaterial culture platform to explore the potential to translate the concept for regenerative medicine applications. This project builds on previous effort by our laboratories where we reported the effects of estradiol on proangiogenic signaling on cocultures of endometrial epithelial and nonendometrial endothelial cells in a model collagen scaffold.^[13] While vessel supporting cells direct angiogenesis through many means, we chose to focus primarily on how hormones affect angiogenic gene expression and VEGF production to parallel the angiogenic factor supplementation used in other angiogenic biomaterial research.^[18] A primary motivator of the transition from a collagen-based scaffold with pores of order 150 μm in size to a (gelatin-based) hydrogel was the ability to rapidly visualize de novo formation of vessel networks (as an early marker of angiogenesis) within the hydrogel (4–7 d), while similar studies in porous collagen scaffolds can take significantly longer (order weeks).^[19]

EndoSCs, like MSCs, have the potential for use in tissue engineering applications.^[20] EndoSCs offer a further advantage in regulating vessel formation as their vascular promoting factors have been shown to be regulated by sex hormone treatments. Proangiogenic factors within the endometrium, particularly VEGF and the angiopoietins (ANG1 and ANG2), have been found to vary temporally and spatially.^[15a,21] Previous research by Tsuzuki et al. found that estrogen treatment of isolated endoSCs led to decreased *ANG1* gene expression and increased VEGF production while medroxyprogesterone acetate (MPA; used as a progesterone mimetic) reduced *ANG2* gene expression and VEGF production.^[22] These finding suggest that E2 and P4 could be used in a coordinated manner in a tissue engineering setting to regulate vasculogenic processes within cocultures of endoSCs and endothelial cells. Interestingly, we observed angiogenic gene expression profiles in endoSCs were not significantly altered by either E2 or P4 hormonal supplementation in the GelMA hydrogel. It is possible that the nuanced effects of E2 and P4 may have been overwhelmed by other proangiogenic signals, specifically heightened levels of hypoxia within the hydrogel due to both its three-dimensional nature^[23] as well as the stromal cell-mediated hydrogel contraction which could heightened regional levels of hypoxia. Though E2 supplementation was sufficient to induce a response in open-cell collagen scaffolds,^[13] ongoing efforts are exploring an expanded dose range using hydrogels more resistant to contraction-based remodeling.

While we hypothesized that endoSCs could stabilize endothelial tube formation and provide angiogenic cues to promote angiogenesis, we were also interested whether endoEpCs could provide additional proangiogenic signals to enhance this response. Previous efforts in our laboratory using collagen scaffolds showed that VEGF within conditioned media from endoEpCs exposed to E2 in the scaffold were able to alter the metabolic response of endothelial cells. Increased VEGF expression has also been seen for a wider range of

epithelial cells in both 2D and 3D in vitro cultures as well as in vivo.^[17b,22] Our observed finding of temporal and hormone dependent changes in gene expression for endoEpCs in the GelMA hydrogel, specifically increased *VEGF* and *ANG1* expression with E2 suggests proangiogenic signals can be produced by endoEpCs in biomaterial culture.

While hormone signals were sufficient to alter gene expression profiles, the lack of measurable changes in soluble VEGF production in response to E2 by either epithelial or stromal cells within the hydrogel was curious. Previous research with 2D cultures of endoSCs demonstrated that VEGF production was increased by E2 supplementation, while MPA or P4 attenuated the response.^[22,24] Classen-Linke et al. also reported that E2 increased VEGF production in endometrial epithelial cells and fibroblasts.^[25] Additionally, we also previously reported that E2 supplementation of endometrial adenocarcinoma cells increased VEGF production in a collagen scaffolds.^[13] The potential that hydrogel-induced changes in hypoxia may mask the effects of hormone signals at the dosages tested suggests a need to further optimize the GelMA hydrogel for endometrial cell culture such as changes in macromere and photoinitiator concentration in order to test a wider library of hydrogel environments.^[14a,b] Given the known significance of hypoxia on vascular network formation as well as our demonstration of SHBG-based approaches to increase the bioavailability of estradiol within the hydrogel, ongoing efforts are exploring the coordinated effect of hypoxia (1% O₂) and the presence of enhanced E2 availability on proangiogenic signals, notably proteases and secreted biomolecules.^[6a,26]

We subsequently examined the formation of vessel networks within the GelMA hydrogel as a function of endometrial cell coculture. Previous studies using HUVECs have shown incorporation of VEGF can improve vessel network formation.^[8b,d,27] Here, we showed that while VEGF improves endothelial cell network formation, the incorporation of endoSCs also improved vessel formation. These results suggest endoSCs can be used as an alternative to MSCs for both structural support and to mediate hormone-signaling. Having found that endoSCs could improve endothelial cell network formation, we subsequently sought to assess whether addition of E2 could promote an increase in network formation. Our findings that E2 supplementation in the presence of endoSCs did not improve vascular network formation are consistent with results from indirect cocultures of endothelial cells and endoSCs that found no change in endothelial tube formation, though this work now extends these studies to fully 3D biomaterials.^[17b]

These findings led us to investigate whether addition of endoEpCs to the coculture of endoSCs and HUVECs could promote vessel network formation. We had previously shown that endoEpCs in 3D biomaterial culture produce endogenous VEGF in response to E2 that was sufficient to enhance the activity of endothelial cultures.^[13] While endoEpCs were found to alter angiogenic gene expression to hormone supplementation, the addition of endoEpCs to generate tricultures with endothelial cells and endoSCs led to diminished metrics of vessel network formation. These findings suggest that while endoEpCs are capable of generating proangiogenic factors in response to exogenous E2 stimulation, direct coculture of endometrial epithelial cells and endothelial cells is detrimental. Ongoing efforts are exploring the use of stratified hydrogel cultures where endoEpCs and cocultures of endothelial and endoSCs can be maintained, a degree of complexity that mirrors the native

stratification in the endometrium.^[10,28] Such studies, paired with quantification of secreted soluble factors via multiplex analyses, constitute important next steps in evaluating crosstalk between endoEpCs and endoSCs and may suggest additional exogenous factors that promote angiogenic response to hormone signals.^[29]

While the incorporation of growth factors such as VEGF into biomaterials via a variety of delivery and immobilization methods has been widely explored,^[9b,30] there has been only a few studies examining controlled presentation of sex hormones. E2 are most commonly incorporated into polymer coatings or have been released from nano- and microparticles to enhance MSC osteogenic differentiation^[31] and for therapies associated with menopause and osteoporosis.^[32] Here we show the application of SHBG to control the sequestration and release of E2 within our gelatin hydrogel. The small size of E2 led us to examine mechanisms by which bioavailability of sex hormones are regulated within the body. Although the majority of E2 in plasma is bound by albumin, SHBG is found readily in the endometrium^[33] and E2 binds more readily to SHBG than albumin, with an association constant four magnitudes higher.^[34] Excitingly, we demonstrate here the addition of SHBG into the hydrogel as a means to regulate E2 bioavailability. The concentration of SHBG within the hydrogel can be used to tune the total amount of E2 sequestered, providing a pathway to alter either the sequestration of exogenous E2 or E2 produced endogenously by cells within the hydrogel. Further, the presence of SHBG led to significantly increased E2 retention within the hydrogel (6.2-fold increased E2 retention after 24 h). The loss of E2 from SHBG laden hydrogels is hypothesized to result primarily from the release of E2 from SHBG and subsequent rapid loss of free E2 from the hydrogel matrix. Diffusive loss of SHBG-bound E2 likely also contributes to some loss of E2, but the significantly enhanced E2 retention in SHBG modified hydrogels suggests a new opportunity to tune the bioavailability of SHBG over significantly longer culture times. Ongoing studies are therefore exploring alternative approaches for binding SHBG within biomaterials such as the incorporation fibulin, which has been shown to bind SHBG natively,^[35] or antibodies for SHBG into the constructs. These new approaches will be used in cellular experiments under optimized angiogenic culture conditions.

4. Conclusion

Tissue engineering approaches present the exciting opportunity to develop models that explore a wider range of natively angiogenic tissues as a means toward uncovering new routes to promote angiogenesis within biomaterials. Here we explored the degree to which concepts associated with a physiological angiogenic process that occurs rapidly and cyclically in the endometrium can be translated to support early angiogenic events within a gelatin hydrogel. We find that endoSCs are capable of promoting vessel network formation by a nonendometrial derived endothelial cell population (HUVECs). Further, while E2 hormone supplementation enhances proangiogenic gene expression profiles in endometrial epithelial cell laden hydrogels, direct coculture of endoEpCs and endothelial cells interferes with network formation and necessitates development of stratified biomaterials for future studies. Finally, we describe a bioinspired strategy that incorporates SHBG within the hydrogel to alter the retention and release of E2, a critical step associated with the use of hormones as signaling molecules within 3D biomaterials. These findings suggest hydrogels

containing endometrial derived cells and sex steroid hormones may be a valuable model system for studying processes associated with endometrial physiology or pathology (e.g., endometriosis), but that they may also be useful for studying alternative pathways associated with proangiogenic processes valuable for a wider range of tissue engineering applications.

5. Experimental Section

Methacrylamide-Functionalized Gelatin Macromer Synthesis

GelMA was synthesized as previously described.^[14b] Briefly, Gelatin (Type A, 300 bloom from porcine skin) solution was dissolved in phosphate buffered saline (PBS) (Gibco, Grand Island, NY) to a concentration of 10 wt% (60 °C, stirring). MA (Sigma-Aldrich, St. Louis, MO) was then added dropwise and allowed to react (60 °C, 1 h). The mixture was diluted in PBS (60 °C) prior to dialysis (12 000–14 000 M.W., Fisherbrand, Pittsburgh, PA) against distilled water to remove salts and excess MA. The GelMA was then lyophilized prior to use. Sufficient MA was added to generate a final GelMA macromer with 47–57% degree of MA functionalization, as verified via ¹H NMR (Varian INOVA).

Lithium Acylphosphinate Photoinitiator Synthesis

Lithium acylphosphinate (LAP) photoinitiator was synthesized as previously described.^[36] 2,4,6-Trimethylbenzoyl chloride (Sigma-Aldrich) was added in a dropwise manner to equimolar dimethyl phenylphosphonite (Sigma Aldrich) at room temperature under continuous stirring and argon. A precipitate was formed by addition of fourfold excess of lithium bromide in 2-butanone (Sigma-Aldrich) and heating to 50 °C. The mixture was returned to room temperature prior to filtration and then washed 3 times in 2-butanone to remove unreacted lithium bromide. Excess solvent was subsequently removed by vacuum.

Cell Culture

EndoEpCs, Ishikawa 3-H-12 cells, and hTERT immortalized human endometrial stromal cells, endoSCs, were cultured in Dulbecco Modified Eagle Medium (DMEM)/F-12 medium supplemented with in-house carbon stripped fetal bovine serum (FBS) (5%; Invitrogen, Carlsbad, CA) as well as penicillin:streptomycin (100 U mL⁻¹:100 µg mL⁻¹; Invitrogen) and L-glutamine (2 × 10⁻³ M; Invitrogen) (37 °C, 5% CO₂). At confluence, cells were trypsinized and passaged (1.5 × 10⁶ epithelial cells or 2.5 × 10⁵ endoSCs per T 75 flask), cryopreserved, or seeded into hydrogels. HUVECs (2.5 × 10⁵ cells per T 75 flask; Invitrogen) were seeded in phenol red free endothelial basal medium (EBM) (Lonza) supplemented with endothelial cell growth medium-2 (EGM-2) SingleQuots (Lonza) (37 °C, 5% CO₂). HUVECs were used at passage 4 for all experiments.

GelMA Hydrogel Synthesis and Culture

A GelMA solution (4 wt%) with LAP photoinitiator (0.1 wt%) was prepared in EBM (Lonza) (60 °C). Cells were suspended in the GelMA solution prior to photopolymerization. The cell-hydrogel suspension was pipetted (40 µL) into the wells (5 mm diameter, 2 mm thick) in a Teflon mold attached to a glass slide with binder clips. The cell-impregnated hydrogel suspension was then exposed to UV light (10 mW cm⁻², 365 nm, 30 s)^[14d] to form a polymerized hydrogel which was then extracted from the molds. Hydrogel specimens were

incubated (37 °C, 5% CO₂) in 48 well culture plates in phenol-red free EBM media supplemented with EGM-2 SingleQuots supplemented with in-house carbon-stripped FBS (replaced every 2 d).^[37] Additionally, some experiments utilized epithelial cells pre-seeded into well plates before adding the hydrogel specimens (identified in Section 2). For experiments reporting the effect of sex steroid hormone and VEGF, media was supplemented with E2 ((10–1000) × 10^{−9} M; Sigma), P4 (1000 × 10^{−9} M; Sigma), or VEGF (100 ng mL^{−1}; ProSpec).

Profiling Proangiogenic Gene Expression

Cell-seeded hydrogels were generated using endometrial epithelial or endometrial stromal cell (5 MM cells mL^{−1}); after 1 d in growth media to allow for initial cell–matrix interactions, the culture media was replaced with media supplemented with no E2/P4, E2 (1 × 10^{−6} M), or P4 (1 × 10^{−6} M), and maintained for up to 7 d. RNA was isolated from epithelial or stromal cell-laden hydrogels (*n* = 4 hydrogels per cell/treatment/time) using an RNeasy Plant Mini kit (Qiagen, Valencia, CA), then quantified via a NanoDrop Lite (Thermo Scientific, Waltham, MA).^[38] RNA was then reverse transcribed with a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) using a Bio-Rad S1000 thermal cycler. Real-time PCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR System (Carlsbad, CA) to measure gene expression levels for *ANG1*, *ANG2*, *VEGF*, and *MMP11* with *ACTB* as a housekeeping gene (Table 1). Sequence Detection Systems software v2.4 (Applied Biosystems, Carlsbad, CA) was used to complete analysis. Results are expressed as fold changes relative to expression levels of cells with two days of control treatment.

Quantifying VEGF Secretion

For experiments quantifying VEGF secretion as a function of E2 or P4 supplementation, cell-seeded hydrogels were generated using endoEpCs (1 MM cells mL^{−1}) or endoSCs (2 MM cells mL^{−1}); after 1 d to allow for initial cell–matrix interactions, normal culture media was replaced with growth media where VEGF aliquots were omitted. EndoEpC and endoSC seeded hydrogels (*n* = 6) were subsequently grown for 4 d with daily media changes in either VEGF-free media, E2 (1 × 10^{−6} M) supplemented VEGF-free media, P4 (1 × 10^{−6} M) supplemented VEGF-free media, or E2 (1 × 10^{−6} M) plus P4 (1 × 10^{−6} M) supplemented VEGF-free media. Conditioned media at day 4 was collected and subsequently frozen (−80 °C) until analysis. The concentration of secreted VEGF was determined via the human VEGF ELISA duoset kit (R&D systems, Minneapolis, MN) using an F200 spectrophotometer (Tecan, Männedorf, Switzerland).

Quantifying Cell Number and Metabolic Activity

The number of endoEpCs and endoSCs encapsulated within the hydrogels is determined via DNA quantification (*n* = 6).^[41] Briefly, cell-seeded hydrogels were digested in papain solution (Sigma-Aldrich) (60 °C, 24 h), with DNA then tagged via Hoechst 33528 (Invitrogen). Fluorescent intensity of each sample was measured using a F200 spectrophotometer (Tecan) at 360(35)/465(35) nm (excitation/emission) and compared to a standard curve of known numbers of cells. The total metabolic activity within gels was assessed by a nondestructive alamarBlue assay as previously described.^[41,42] Hydrogels

were rinsed in PBS then incubated at 37 °C in alamarBlue (Invitrogen) under mild shaking. The metabolic reduction of resazurin to the fluorescent byproduct resorufin was measured on a F200 spectrophotometer (Tecan) at 540(52)/580(20) nm (excitation/emission). Relative metabolic activity was determined by comparing the measured fluorescent intensities against a prepared standard curve generated via known numbers of cells.

Analysis of Vessel Networks

Cell-seeded hydrogels of increasingly cellular complexity were used to quantify spontaneous vessel network formation within the hydrogel.^[43] GelMA hydrogels were seeded with either: HUVECs (1 MM cells mL⁻¹); HUVECs and endoSCs (3 MM total cells mL⁻¹, 2:1 HUVEC:stromal cell ratio) previously reported to enhance vessel network formation,^[43] or HUVECs, endoSCs, and endoEpCs (4 MM total cells mL⁻¹, 2:1:1 HUVEC:stromal:epithelial cell ratio). Hydrogels were maintained in growth or VEGF supplemented media for 7 or 10 d. Spontaneous vessel network formation within the hydrogel was analyzed as previously described.^[43] Hydrogels were fixed in 10% formalin (20 min, RT), blocked with 2% BSA (Sigma-Aldrich) and 0.1% Tween (Fischer Scientific), then incubated overnight in mouse antihuman CD31 antibody (1:200; Dako, Carpinteria, CA, USA) followed by Alexa Fluor 488-conjugated goat antimouse IgG (1:500; Invitrogen), all at 4°C. Vessel networks were imaged via fluorescent microscopy (Leica DMI4000B fluorescence microscope, Qimaging camera) at 4× and 10×, with six images per sample side analyzed via AngioTool in order to quantify average and total vessel length, vessel area, and total vessel junctions per field of view.^[44]

E2 Controlled Release Studies

Sex hormone binding globulin (Fitzgerald, Acton, MA, USA) with or without E2 (Sigma Aldrich) was incorporated into the hydrogel precursor suspension prior to UV crosslinking. The hydrogels were placed in PBS for varying lengths of time (1, 6, 12, 24 h) to allow for elution of E2 from the network. At the experiment endpoint, the hydrogels were subsequently heat treated in fresh PBS with intermittent vortexing (60 °C, 1 h) to release the remaining bound E2, which was then quantified via Estradiol Parameter Assay Kit (R&D systems).

Statistics

Statistical analyses were performed using SPSS software (IBM). Statistical significance was assumed at $p < 0.05$ unless otherwise stated. Error bars are reported as standard error of the mean unless otherwise noted. Main effects were assessed with ANOVAs with Tukey post hoc tests. *VEGF* gene expression within epithelial cells was evaluated further using one-way ANOVAs at each time point. Comparison of vessel formation for mono-, co-, and tricultures was performed via one-way ANOVAs; differences between E2 and SHBG with E2 groups were compared by independent sample tests at each time point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effect of 1×10^{-6} M estradiol or 1×10^{-6} M progesterone on proangiogenic gene expression of endoEpCs in GelMA hydrogels: A) *ANG1*, B) *ANG2*, C) *VEGF*. Expression levels are normalized to unsupplemented (control) media at day 1. *Significantly ($p < 0.05$) different between time points. #Significantly ($p < 0.05$) different versus control media at a given time. &Significantly ($p < 0.05$) different vs. estradiol supplemented media at a given time.

Figure 2. Effect of 1×10^{-6} M estradiol or 1×10^{-6} M progesterone on proangiogenic gene expression of endoSCs in GelMA hydrogels: A) *ANG1*, B) *ANG2*, C) *VEGF*, D) *MMP11*. Expression levels are normalized to unsupplemented (control) media at day 1. *Significantly ($p < 0.05$) different between time points.

Figure 3.

A) Increase in the overall number of viable of endoSCs versus endoEpCs, normalized to number of initially seeded cells, after 4 d within the GelMA hydrogel. B) Changes in the metabolic activity per cell, after 4 d within the GelMA hydrogel. C) Secreted VEGF per 10^5 endoSCs versus endoEpCs after 4 d within GelMA hydrogels. Results are reported as in response to unsupplemented (control), 1×10^{-6} M estradiol, 1×10^{-6} M progesterone, or the combination of 1×10^{-6} M estradiol and 1×10^{-6} M progesterone.

Figure 4.

Quantification of A) total vessel area, B) number of vessel junctions, C) total vessel length, D) and average vessel length per image of endothelial cell networks formed after 10 d within GelMA hydrogels in the presence or absence of soluble 100 ng mL⁻¹ VEGF for monolithic cultures of endothelial cells, cocultures of endothelial cells with endoSCs, or tricultures of endothelial cells with endoSCs and endoEpCs. *Significantly ($p < 0.05$) different as a function of VEGF. &Significantly ($p < 0.05$) different versus monolithic endothelial cell culture. #Significantly ($p < 0.05$) different versus coculture of endothelial cells and endoSCs.

Figure 5. Quantification of A) total vessel area, B) number of vessel junctions, C) total vessel length, and D) average vessel length per image of endothelial cell networks formed after 7 d within GelMA hydrogels in the presence of endoSCs and soluble (0×10^{-9} , 10×10^{-9} , 100×10^{-9} , 1000×10^{-9} M estradiol).

Figure 6.

A) Effect of including sex hormone binding globulin (SHBG) within the GelMA hydrogel on the retention of 1×10^{-6} M E2 within the hydrogel network. A) E2 retained within the hydrogel (100% corresponds to original E2 dosage) following incubation in a PBS wash solution for 6, 12, and 24 h. B) E2 retained in hydrogel (after 1 h) as a function of the concentration of SHBG incorporated within the GelMA hydrogel. #Significantly ($p < 0.05$) different versus hydrogels containing SHBG. &Significantly ($p < 0.05$) different versus other time points. *Significantly ($p < 0.05$) different versus other SHBG concentrations.

Table 1

Primers used for gene expression.

Transcript	Sequence	Reference
<i>ANG1</i>	Forward: 5'-CAGCGCCGAAGTCCAGAAAAC-3' Reverse: 5'-CACATGTTCCAGATGTTGAAG-3'	[22]
<i>ANG2</i>	Forward: 5'-GTCCACCTGAGGAAGTGTCT-3' Reverse: 5'-TTGTGACAGCAGCGTCTGTA-3'	[22]
<i>ACTB</i>	Forward: 5'-AGGCACCAGGGCGTGAT-3' Reverse: 5'-GCCCACATAGGAATCCTTCTGAC-3'	[39]
<i>MMP11</i>	Forward: 5'-ATTTGGTTCTTCCAAGGTGCTCAGT-3' Reverse: 5'-CCTCGGAAGAAGTAGATCTTGTCT-3'	[40]
<i>VEGF</i>	Forward: 5'-CAGGACCATGAACCTTCTGC-3' Reverse: 5'-CCTCAGTGGGCACACACTCC-3'	[22]