ORIGINAL ARTICLE

Mechanical and Biochemical Effects of Progesterone on Engineered Cervical Tissue

Michael House, MD,^{1,2,4} Jeannie Kelly, MD,¹ Nikolai Klebanov, BS,² Kyoko Yoshida, PhD,³ Kristin Myers, PhD,³ and David L. Kaplan, PhD²

Preterm birth is a leading cause of morbidity and mortality in newborns. Babies born prematurely are at increased risk of lifelong health problems, including neurodevelopmental abnormalities. Cervical shortening precedes preterm birth in many women. Cervical shortening is caused, in part, by excessive softening of the extracellular matrix (ECM) of the cervical stroma. In clinical obstetrics, cervical shortening prompts treatment with supplemental progesterone to prevent preterm birth. However, progesterone-mediated effects on the cervical ECM are not well understood. This research sought to study progesterone-mediated remodeling of ECM produced by human cervical fibroblasts *in vitro*. A previously developed three-dimensional (3D) engineered model of the cervical ECM was used for experiments. Cervical fibroblasts were seeded on porous scaffolds and cultured in spinner flasks to promote ECM synthesis. Scaffolds were exposed to two conditions: 10^{-8} M estradiol versus 10^{-8} M estradiol + 10^{-6} M progesterone for 4 weeks. To measure ECM strength, two scaffolds were mounted end-to-end on a wire and cultured such that ECM filled the gap between the scaffolds. The force required to pull the scaffolds apart was measured. Collagen content and collagen crosslinks were measured with ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry. Whole-transcriptome RNA sequencing (RNA-seq) was used to quantify gene expression between the two experimental conditions. Zymography was used to study the quantity and activity of matrix metalloproteinase-2 (MMP2) in the scaffolds. The study found that exposure to progesterone increased tissue softness of the engineered ECM over 28 days. Increased tissue softness correlated with decreased collagen content. With RNA-seq, progesterone exposure resulted in gene expression changes consistent with known progesterone effects. Pathway analysis of the RNA-seq data suggested MMPs were significantly dysregulated in progesterone-exposed engineered ECM. Increased expression of active MMP2 was confirmed in the progesterone-exposed engineered ECM. In summary, progesterone increased the softness of the ECM, which was correlated with decreased collagen production and altered histology. These results are important for deciphering the role of progesterone in preventing preterm birth.

Keywords: preterm birth, tissue engineering, cervix, progesterone

Introduction

PRETERM BIRTH IS a leading cause of morbidity and mortality in newborns, affecting >15 million babies worldwide.¹ In the United States, preterm birth is associated with more than half of all infant deaths, and healthcare costs attributed to preterm birth are an estimated \$26 billion per year according to the Institute of Medicine.² Preterm infants can develop lifelong health problems, including cerebral palsy, psychomotor dysfunction, cardiopulmonary compli-

cations, and developmental delay.^{3–5} Current therapies can decrease the rate of preterm birth by 30%.^{6–8} However, these therapies are as effective as they could be because biological mechanisms leading to preterm birth are heterogeneous and poorly understood.

Although preterm birth mechanisms are heterogeneous, cervical shortening is strongly associated with preterm birth in many patients.^{9–11} In clinical obstetrics, cervical shortening is an indication to initiate supplemental progesterone treatment, which aims to prevent preterm birth. Whether the cervix

¹Department of Obstetrics and Gynecology, Tufts Medical Center, Boston, Massachusetts.

²Department of Biomedical Engineering, Tufts University, Medford, Massachusetts.

³Department of Mechanical Engineering, Columbia University, New York, New York.

⁴Mother Infant Research Institute, Tufts Medical Center, Boston, MA.

shortens or maintains its length depends in part on softening of the extracellular matrix (ECM) of the cervical stroma.^{12,13} Excessive softening leads to undesired cervical shortening and preterm birth.^{14,15} It is known that cervical softening occurs throughout pregnancy,¹⁶ and softening is correlated with complex biochemical changes in the cervical ECM.^{17,18} Yet, the precise biological mechanisms of cervical softening remain elusive.

A central hypothesis of the current research is that the hormonal environment of pregnancy, specifically rising serum progesterone levels, leads to remodeling of the cervical ECM, which is detected clinically as softening. A long-term goal of this research is to elucidate the biologic mechanism by which progesterone mediates cervical softening in normal pregnancy and preterm birth.

Previously, a hormonally responsive, three-dimensional (3D) engineered cervix was developed using human cervical fibroblasts and porous silk protein scaffolds.^{19–21} This engineered cervix produced 3D ECM that was inhibited after exposure to progesterone.²⁰ Inhibition of ECM formation was mitigated by the addition of mifepristone, a progesterone receptor antagonist, suggesting the involvement of the progesterone receptor. However, no data correlating ECM inhibition to changes in softness were presented. Also, the mechanism by which progesterone exposure led to remodeling of engineered ECM was not explored.

Motivated by the clinical importance of the cervical ECM and its relationship to preterm birth, the objective was to study progesterone effects on tissue softness using the 3D engineered cervix. It was hypothesized that (1) exposure of engineered cervical ECM to progesterone would increase tissue softness, and (2) RNA-seq could be used to explore the mechanism of progesterone effects on engineered cervical ECM. To test these hypotheses, the effect of progesterone on engineered cervical ECM was evaluated by mechanical testing, collagen density, and crosslink analysis. Whole-transcriptome RNA sequencing (RNA-seq) together with functional pathway analysis of the RNA-seq data set was used to study potential mechanisms. When RNA-seq suggested progesterone effects were mediated by matrix metalloproteinases (MMPs), MMPs were studied directly in the 3D culture system. The present report shows the potential for using the 3D engineered cervix to study the biologic mechanisms by which progesterone mediates cervical remodeling during pregnancy.

Materials and Methods

Ethical approval

This study was approved by the Institutional Review Board at Tufts Medical Center (IRB #8315). Informed consent was obtained for cervical tissue biopsy from all subjects before surgery.

Sample collection

Nonpregnant women undergoing total hysterectomy for benign indications were approached before surgery to obtain consent for participation. Subjects denied a history of preterm birth or abnormal pap smears and were premenopausal. Cervical biopsies were performed, as previously described.¹⁹ Briefly, a 1 cm × 1 cm × 1 cm biopsy of the cervical stroma was obtained from the midcanal region of the surgical specimen

and washed with sterile saline. The specimen was then stored in cold media on ice for transport to cell culture. Biopsies from three different women were used for experiments (age range 33–44 years; indication for hysterectomy was dysfunctional uterine bleeding [$n = 1$] and fibroid uterus [$n = 2$]).

Cell media

Expansion media and phenol red-free media were used for experimentation, as previously described.²⁰

Expansion media. Expansion media comprised Dulbecco's modified Eagle's medium (DMEM; 11995065; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; 16000044; Thermo Fisher Scientific), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (15240062; Thermo Fisher Scientific).

Phenol red-free media. The phenol red-free media comprised DMEM without phenol red (31053036; Thermo Fisher Scientific) supplemented with 10% charcoal-stripped FBS (12676029; Thermo Fisher Scientific), 1 mM sodium pyruvate (11360070; Thermo Fisher Scientific), 2 mM L-glutamine (25030081; Thermo Fisher Scientific), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (15240062; Thermo Fisher Scientific), and 50 µg/mL ascorbic acid 2-phosphate (A8960; Sigma-Aldrich, St. Louis, MO). Supplementation with estradiol (E2758; Sigma-Aldrich) and progesterone (P8783; Sigma-Aldrich) was performed with 10 mM stock solutions in 200-proof ethanol over serial dilution to experimental concentrations, and ethanol did not exceed 0.01% in the final concentrations.

Cell culture

Cervical fibroblasts were cultured from the biopsy specimen, as previously described.²⁰ Briefly, the cervical biopsy was minced and cultured in expansion media incubated at 37°C, 5% CO₂, 95% air, and 95% humidity for 10 days until confluency. The cells were then trypsinized, culture expanded with expansion media, and passaged per standard protocol. Passages 5 and 6 were used for experiments.

3D culture

A hormonally responsive 3D culture was established per a previously described protocol²⁰ with several modifications. Briefly, silk protein was extracted from *Bombyx mori* silkworm cocoons (Tajima Shoji Co. Ltd., Yokohama, Japan) by boiling in a 0.02 M sodium carbonate solution, solubilized in 9.3 M lithium bromide solution, and dialyzed with distilled water to achieve a purified silk solution of 6% (w/w) concentration. The silk solution (2 mL) underwent gelation in 24-well plates by slowly pouring 4 g granular sodium chloride (NaCl; 500–600 µm) into each well containing silk solution. The plate was incubated at room temperature for 24 h and then at 60°C for 12 h. To remove the NaCl, the entire plate was then immersed in 4 L of distilled water, which was agitated with a magnetic stir bar for three days with water changes every 12–24 h, thereby creating the porous texture. Scaffolds were removed from the plate, punch-biopsied (33–37 Millex; Integra LifeSciences, Plainsboro, NJ) into cylinders of 8 mm in diameter, and cut to approximately 12 mm height. Cell

seeding followed previous publications^{20,21} with several variations. The scaffold cylinder was divided in half to create two identical scaffolds, 6 mm in height and 8 mm in diameter. The two cylinder scaffolds were mounted on straight, 0.020 inch stainless steel wire (Malin Company, Inc., Cleveland, OH), and C-flex tubing was used to keep the two cylinders stationary and positioned end to end (Fig. 1). The scaffolds on the wire were autoclaved. To promote cell attachment, the scaffolds were collagen coated by applying a sterile collagen solution (0.5 mg/mL rat tail collagen in 0.02 N glacial acetic acid; #354236, Corning, Inc., Corning, NY) until saturation of the scaffold and aspirating the remaining solution after 10 min. The process of saturation and aspiration was repeated once. The collagen coated scaffolds were washed in media containing phenol red until no color change was seen, indicating normal pH. The sterile scaffolds were seeded with 125 μ L of a concentrated fibroblast solution (20 million cells/mL) in expansion media (prepared as described above), placed in a T185 flask, and incubated for 1 h to promote cell attachment. Expansion media was then added to the flask, and the scaffolds were cultured for 1 week with one media change.

After 1 week, the scaffolds were transferred to sterile spinner flasks. The steel wire with seeded scaffolds was inserted into the rubber stopper (Thermo Fisher Scientific, Pittsburgh, PA) at the top of the spinner flask (#1967-10250; Bellco Glass,

Vineland, NJ) under sterile conditions. Scaffolds were cultured with 250 mL of media in each flask and the spinner bar rotating at 50 rpm. Phenol red-free media was used with two hormonal conditions: estradiol 10^{-8} M or estradiol 10^{-8} M + progesterone 10^{-6} M. Scaffolds were cultured for 4 weeks, and paired scaffolds were then removed and either tested directly (mechanical testing and histology) or flash frozen with liquid nitrogen (gene expression and protein assays). The experiment was performed in triplicate using the samples from the three different women.

Mechanical testing

To determine the effect of steroid hormones on mechanical properties of cervical tissue, tensile testing was performed on scaffolds from each condition. Two surgical sutures (4-0 Vicryl on an SH-1 needle) were passed through the distal ends of the paired scaffolds. The sutures were attached to tensile grips of a material testing machine with pneumatic clamps (Model 5948 MicroTester; Instron, Norwood, MA). A 10 N load cell with a 20 mN preload was used at an extension speed of 5 mm/min while recording force and displacement. The tension test was performed until failure, defined as the two scaffolds breaking apart (Fig. 1). After mechanical testing, samples were fixed in formalin for paraffin embedding and hematoxylin and eosin histology.

FIG. 1. Mechanical testing, collagen content, and histology of three-dimensional (3D) scaffolds. **(A)** Schematic showing the gap between the two scaffolds during culture. After 5 weeks of culture, the gap was filled with newly synthesized extracellular matrix (ECM). **(B)** For mechanical testing, the scaffolds were tested in tension until failure. Two representative load-displacement curves are shown. **(C)** The peak load at failure for scaffolds cultured in estradiol + progesterone (Est + Prog) was significantly lower compared to scaffolds cultured in Est (*, $p = .005$). Decreased mechanical strength was correlated to changes in histology and collagen content. **(D)** Scaffolds in Est + Prog showed significantly decreased collagen formation. **(E)** Immature collagen crosslinks were no different between the Est versus Est + Prog scaffolds. There were fewer mature crosslinks; mature crosslinks were increased in Est + Prog scaffolds (*, $p < .001$). **(F)** On histology, scaffolds cultured in Est showed more uniform ECM on the scaffold surface. Scaffolds cultured in Est + Prog showed areas of scant tissue formation. Color images available online at www.liebertpub.com/tea

Collagen density and crosslink analysis

Collagen density and crosslink analysis of the scaffolds was performed by ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) using a previously described protocol.²² Briefly, frozen scaffolds were lyophilized and weighed to determine dry weight. Samples were reduced with sodium borohydride to measure divalent crosslinks, and hydrolyzed under vacuum with 6 N hydrochloric acid at 110°C for 18–24 h. Reduced and hydrolyzed samples were then re-suspended in heptafluorobutyric acid and lyophilized for UPLC-ESI-MS/MS analysis. UPLC-ESI-MS/MS was used to quantify the divalent immature crosslinks dihydroxylysionorleucine (DHLNL), the trivalent mature crosslinks pyridinoline (PYD) and deoxypyridinoline (DPD), and hydroxyproline (OHP), as previously described.²² Total collagen content was determined by assuming 14% OHP content per collagen by weight and normalized by tissue dry weight (mg/mg). Total immature crosslink density was calculated by dividing the concentration of DHLNL by the concentration of collagen (nM/nM). Total mature crosslink density was calculated by dividing the concentration of PYD + DPD by the collagen concentration (nM/nM).

Next-generation sequencing

To determine differential gene expression between the two steroid conditions, RNA-seq was performed from RNA isolated from one tissue engineering experiment. Three scaffolds per steroid condition were analyzed. A 50 mg piece of the scaffold was homogenized and RNA was isolated using RNeasy Fibrous Tissue Kit (#74704, RNeasy Fibrous Tissue Kit; Qiagen, Valencia, CA), as previously described.²⁰ RNA quality was analyzed using the RNA 6000 Nano Kit for the Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). RNA concentration for all samples was 0.1–0.5 µg/µL. RNA quality was established by the RNA integrity number (RIN). All samples showed high quality (RIN >9.0).

The RNA-seq libraries were prepared using 1 µg of total RNA per sample using TruSeq RNA Sample Preparation Kit (cat. no. RS-122-2001; Illumina, San Diego, CA). The resulting libraries were quantified and subjected to quality check using a fragment analyzer (Advanced Analytical Technologies, Inc., Akeny, IA). The libraries were then mixed together at equal molar concentration for sequencing with single-read 50 bases format on an Illumina HiSeq 2500 instrument using High Output V3 chemistry. There were six files of sequenced data, which represented two steroid conditions (estradiol 10⁻⁸ M or estradiol 10⁻⁸ M + progesterone 10⁻⁶ M) and three replicates per condition.

The open-source software programs of the Tuxedo Suite were used to analyze differential expression between the two steroid conditions.²³ Briefly, sequenced reads for each steroid condition were mapped to human reference genome hg19 using TopHat2. Differential gene expression was calculated using Cuffdiff. CummeRbund for R was used for data visualization and analysis, as previously described.²³ A cutoff limit of 1.0 fragments per kilobase of exon per million fragments mapped (FPKM) was used as the minimum detection value. Genes were considered differentially expressed at a twofold or greater expression difference, with a false discovery rate (*q*-value) set at <0.05.

Functional pathway analysis

Functional analysis of differentially expressed genes (DEGs) was performed using Ingenuity Pathway Analysis (September 2016 release, Ingenuity Systems, www.ingenuity.com). The following settings were used: Ingenuity Knowledge Base (genes only); human species; molecules per network: 35; networks per analysis: 25. The Canonical Pathways feature was used to identify overrepresented physiologic functional pathways within the DEGs. A stringent corrected Fisher's exact test *p*-value of 0.01 was used for statistical significance.

The Upstream Analysis feature was used to predict differential activation or inhibition of upstream regulators between the two steroid conditions. This feature uses previously published literature of known target genes to predict upstream activity and gives a *z*-score of activation based on comparison with random regulation. The Diseases and Functions feature was used to determine which general biological processes were associated with progesterone exposure. An activation *z*-score >2 or <-2 was considered statistically significant.

Real-time quantitative reverse transcription polymerase chain reaction

To determine the validity of RNA-seq data, gene expression was compared to gene expression from separate scaffolds using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). An RNeasy Fibrous Tissue Kit (Qiagen) was used for RNA extraction, a High-Capacity cDNA Reverse Transcription Kit (#4368814; Applied Biosystems, Foster City, CA) was used for reverse transcription reactions (50 µL RNA and 50 µL reverse transcription master mix), and the Stratagene Mx 3000P QPCR System (Agilent) was used to determine quantitative gene expression from cDNA, as previously described.²⁰ A 50 µL reaction consisted of 25 µL TaqMan® Gene Expression Master Mix (4369016; Thermo Fisher Scientific) +5 µL cDNA template +17.5 µL RNase-free water +2.5 µL TaqMan Gene Expression Assay (Thermo Fisher Scientific). The A260/A280 ratio was >2.0, and the RNA concentration was 30–100 ng/µg for all the samples tested (Nanodrop 2000; Thermo Fisher Scientific). Genes that were differentially regulated from the MMP pathway or of clinical interest were included: *MMP3* (assay ID Hs00968305_m1), *MMP14* (Hs01037003_g1), anthrax toxin receptor 2 (*ANTXR2*, Hs00292467_m1), ADAM metalloproteinase with thrombospondin Type 1 Motif 1 (*ADAMTS1*, Hs00199608_m1), thrombospondin 1 (*THBS1*, Hs00962908_m1), the progesterone receptor (*PGR*, Hs01556702_m1), collagen type 1 alpha 1 (*COL1A1*, Hs00164004_m1), and collagen type 3 alpha 1 (*COL3A1*, Hs00943809_m1). Gene expression was normalized by the geometric mean of housekeeping genes ribosomal protein, large, P0 (*RPLP0*, Hs00420895_gH) and tumor protein, translationally controlled 1 (*TPT1*, Hs02621289_g1). These two genes were chosen due to known stability across physiologic states in the human female reproductive tract.²⁴ Cycle threshold was calculated from baseline-corrected, normalized fluorescence data using instrument software. The data were expressed as fold change relative to the gene expression of vehicle controls using the 2^{ΔΔCt} method.

Gelatin zymography

RNA-seq data suggested MMPs, specifically *MMP14*, was significantly upregulated. To determine the MMP14 activity in the cervical fibroblasts of the 3D engineered cervix, gelatin zymography of MMP2 was performed.^{25,26} Briefly, 50 mg of the scaffold from the 4-week time point was homogenized under liquid nitrogen and weighed. The scaffold was then centrifuged with 500 μ L of cold zymography lysis buffer (1.25 mL 1 M Tris-HCl solution, 2.5 mL 2 M NaCl solution, 5 mL 10% IGEPAL, and 41.25 mL deionized water) mixed with protease inhibitors (1 μ L 5 mg/mL aprotinin, 0.5 μ L 2 mg/mL leupeptin, and 1 μ L 2 M benzamide) at 16,000 *g* for 10 min. The supernatant was harvested and the protein content quantified using the Bradford Assay. Electrophoresis was performed with 5 μ g of protein per lane in 8.0% sodium dodecyl sulfate polyacrylamide gels and 0.1% gelatin at 125 V for 90 min. After washing and overnight incubation in developing buffer (0.01 M Tris base, 0.03 M Tris-HCl, 0.2 M NaCl, 6.6 mM CaCl_2 , 0.02% Tween 20), gels were stained with 0.5% Coomassie Brilliant Blue R250 for 30 min. After de-staining (Coomassie R-250 de-staining solution, methanol: acetic acid: water, 50:10:40), gels were imaged and quantified using Image J software. Ethylenediaminetetraacetic acid (15 mM) was used for positive control of MMP inhibition and recombinant MMP2 (MilliporeSigma, Burlington, MA) was used as a positive control. Four replicates from each hormone condition were used, and zymograms were performed in triplicate.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Means were calculated from four to five replicates as indicated. Statistical tests were Student's *t*-test or Pearson's correlation coefficient as indicated, with $p < 0.05$ considered statistically significant.

Results

3D culture

Cells proliferated in the gap between the two scaffolds. Newly formed tissue bridged the gap and essentially bonded the two scaffolds together. There was no difference in the gross appearance of the scaffolds cultured with estradiol (E) alone compared to the scaffolds cultured with estradiol + progesterone (E + P; Fig. 1A).

Mechanical testing

The strength of the newly formed tissue in the gap between the scaffolds was significantly weaker in the scaffold cultured with E+P compared to the scaffolds cultured with E (Fig. 1C). The mean peak load at failure $\pm$ the standard error of the mean (SEM) was 0.43 ± 0.02 N and 0.12 ± 0.003 N for the E and E+P scaffolds, respectively ($p = 0.005$). As a negative control, unseeded scaffolds were cultured under identical conditions. The unseeded scaffolds could not be mechanically tested; the scaffolds fell apart before testing. It was concluded that the measured mechanical response was solely due to the newly synthesized ECM; the silk scaffold itself did not influence the mechanical results.

Histology

Mechanical responses were correlated to differences in the histological appearance of the ECM. Scaffolds cultured with E showed a uniform ECM on the surface (Fig. 1F). In the scaffolds cultured with E + P, however, the newly formed ECM was not uniform; some areas of the scaffold showed scant ECM.

Collagen analysis

There was significantly more collagen in the scaffolds cultured with E compared to E+P scaffolds (Fig. 1D). The

TABLE 1. TOP 20 MOST EXPRESSED GENES

Symbol	Gene name	FPKM Est + Prog	FPKM Est	Log ₂ fold change
MIR4461	MicroRNA 4461	83,526	103,295	-0.31
TMSB4X	Thymosin beta 10	6390	5782	0.14
TPT1	Tumor protein, translationally-controlled 1	5289	5210	0.02
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	4048	4896	-0.27
COL1A1	Collagen type I alpha 1 chain	3859	4446	-0.20
RPL41	Ribosomal protein L41	3735	3961	-0.09
LGALS1	Galectin 1	3645	3696	-0.02
ACTB	Actin beta	3587	2056	0.80
FTH1	Ferritin heavy chain 1	3537	4039	-0.19
RPLP1	Ribosomal protein lateral stalk subunit P1	3500	4518	-0.37
TIMP1	TIMP metalloproteinase inhibitor 1	3227	2614	0.30
RPS18	Ribosomal protein S18	3219	3405	-0.08
COL3A1	Collagen type III alpha 1 chain	3098	3893	-0.33
RPS27	Ribosomal protein S27 like	3081	3279	-0.09
MT2A	Metallothionein 2A	2915	1114	1.39*
COL1A2	Collagen type I alpha 2 chain	2851	3865	-0.44
TAGLN	Transgelin	2821	1095	1.37*
DCN	Decorin	2813	5853	-1.06
RPS29	Ribosomal protein S29	2384	2394	-0.01
RNU86	SNORD139	2370	977	1.28

FPKM, fragments per kilobase of exon per million fragments mapped. Est + Prog, estradiol (10^{-8} M) + progesterone (10^{-6} M). Est, estradiol (10^{-8} M).

*Significantly upregulated.

mean collagen content $\pm$ SEM was 0.028 ± 0.004 and 0.011 ± 0.001 mg collagen/mg dry weight for the E and E+P scaffolds, respectively ($p=0.002$). The mean immature crosslink density $\pm$ SEM was 0.30 ± 0.03 and 0.36 ± 0.04 nM/nM collagen for the E and E+P scaffolds, respectively. There was no significant difference between E and E+P scaffolds ($p=0.3$). Compared to immature crosslinks, there were fewer mature crosslinks (Fig. 1E). The mean mature crosslink density $\pm$ SEM was 0.05 ± 0.004 and 0.10 ± 0.008 nM/nM collagen for the E and E+P scaffolds, respectively, with significantly more mature crosslinks in the E+P scaffolds ($p<0.001$).

RNA-seq and functional pathway analysis

With a minimal detection limit of FPKM >1.0 , there were 11,633 genes expressed in both steroid conditions. Among the most highly expressed genes (Table 1) were type 1 and type 3 collagen (*COL1A1*, *COL1A2*, *COL3A1*), decorin (*DCN*), and TIMP metalloproteinase inhibitor 1 (*TIMP1*), which highlights the importance of ECM remodeling in cervical fibroblasts. With differential expression defined as a fold change >2.0 and a false discovery rate of 0.05, there were 331 DEGs; 200 genes were upregulated, and 131 genes were downregulated. The 10 most highly up- and downregulated genes are shown in Table 2. Among the highly upregulated genes, two of the genes (*C10orf10* and *CRISPLD2*) are known to be upregulated by increased progesterone.^{27,28} Among the highly downregulated genes was the gene for the progesterone receptor (*PGR*). Progesterone downregulation of the progesterone receptor is consistent with the effect of progesterone in most target tissues.²⁹ These results suggested progesterone-mediated responses of the 3D engineered cervix were consistent with previously published reports.

Canonical pathways significantly associated with the data are shown in Table 3. Of note, inhibition of MMPs was most strongly associated with the data (16% of the DEGs overlapping in the data set; $p<0.001$). Canonical signaling pathways that were activated include signaling for high mobility group box 1, leukocyte extravasation, ERK/MAPK, and interleukin (IL)-6. The only inhibited signaling pathway was interferon signaling.

The most significant upstream regulators and diseases/functions associated with the data are shown in Table 4. Among the upstream regulators predicted to be activated is human chorionic gonadotrophin, which is consistent with its known role in pregnancy. Diseases/functions predicted to be increased include cell growth, cell movement, and cell proliferation. The only disease/function predicted to be decreased was cell death and apoptosis.

Validation of RNA-seq with RT-qPCR

For each gene, RT-qPCR results of fold change after progesterone exposure strongly correlated with the RNA-seq results (Pearson $r=0.98$, $p<0.0001$; Fig. 2). In addition, the housekeeping genes *RPLP0* and *TPT1* were not differentially regulated in the RNA-seq data set.

Gelatin zymography

RNA-seq data suggested the MMP pathway was significantly dysregulated (Table 3), so this pathway was explored in more detail. Specifically, *MMP14* was significantly upregulated (Fig. 3). *MMP14* is of particular interest because MMP2 processing is regulated by *MMP14* and *MMP2* is an essential gelatinase involved in ECM remodeling in the mouse cervix during pregnancy.³⁰ Zymography revealed

TABLE 2. TOP 10 MOST UP- AND DOWNREGULATED GENES

Symbol	Gene name	FPKM Est + Prog	FPKM Est	Log ₂ fold change
<i>Upregulated genes</i>				
RMRP	RNA component of mitochondrial RNA processing endoribonuclease	87.4	5.1	4.1
C13orf15	Regulator of cell cycle	55.6	3.8	3.9
GPX3	Glutathione peroxidase 3	64.4	5.0	3.7
RPPH1	Ribonuclease P RNA component H1	17.4	1.4	3.7
PRG2	Proteoglycan 2, bone marrow	11.4	1.1	3.4
C10orf10	Chromosome 10 open reading frame 10	240	22.8	3.4
MIR663A	MicroRNA 663a	2207	235.7	3.2
CRISPLD2	Cysteine rich secretory protein LCCL domain containing 2	24.0	3.0	3.0
IL8	C-X-C motif chemokine ligand 8	39.3	5.2	2.9
A2M	Alpha-2-macroglobulin	43.5	5.9	2.9
<i>Downregulated genes</i>				
FAM46C	Family with sequence similarity 46 member C	1.8	12.8	-2.8
SFRP4	Secreted frizzled related protein 4	81.8	513.1	-2.6
CLDN1	Claudin 1	1.0	5.9	-2.6
OLFML1	Olfactomedin like 1	4.0	23.4	-2.6
CXCR6	C-X-C motif chemokine receptor 6	1.4	8.3	-2.5
PGR	Progesterone receptor	5.6	30.4	-2.4
COL21A1	Collagen type XXI alpha 1	4.4	23.4	-2.4
SFRP1	Secreted frizzled related protein 1	96.9	489.5	-2.3
SLC47A1	Solute carrier family 47 member 1	1.1	5.6	-2.3
CPA4	Carboxypeptidase A4	4.6	20.1	-2.1

Upregulated genes are defined as upregulated genes in scaffolds cultured in Est + Prog compared to Est only.

TABLE 3. MOST SIGNIFICANT CANONICAL PATHWAYS

	$-\log$ (p-value)	Ratio	Activation z-score
<i>Canonical pathways</i>			
Inhibition of matrix metalloproteases	4.4	0.16	
Hematopoiesis from pluripotent stem cells	4.1	0.14	
Coagulation system	3.5	0.14	
Interferon signaling	3.5	0.14	−2.2
IL-17A signaling in fibroblasts	2.6	0.11	
<i>Canonical signaling pathways</i>			
HMGB1 signaling	4.8	0.08	2.3
Leukocyte extravasation signaling	2.1	0.04	2.1
ERK/MAPK signaling	2.7	0.05	1.9
IL-6 signaling	2.2	0.06	1.9
Colorectal cancer metastasis signaling	3.0	0.05	1.7
Interferon signaling	3.5	0.14	−2.2

Activated pathways refer to pathways activated in scaffolds cultured in Est + Prog compared with Est only. Ratio is defined as the number of differentially expressed genes in the pathway divided by the total number of genes in that pathway. The activation score measures the activation (or inhibition) of the signaling pathway; a positive activation score indicates activation and a negative score indicates inhibition.

IL, interleukin.

two bands corresponding to the pro- and active forms of MMP2 (Fig. 3). Densitometric quantification revealed total MMP2 was significantly higher in the scaffolds exposed to estrogen only (Fig. 3). The ratio of active MMP2 to pro-MMP2, however, demonstrated a significantly higher ratio in the progesterone-exposed samples (Fig. 3), suggesting progesterone leads to increased activation of MMP2. These results suggested MMP14 via its effect on MMP2 could have an important role in softening of the ECM in the 3D engineered cervix.

Discussion

In the 3D engineered cervix, exposure to progesterone increased tissue softness. Increased tissue softness corre-

lated with decreased collagen content and changes in histology of the engineered ECM. With RNA-seq, progesterone exposure resulted in gene expression changes consistent with known progesterone effects. In addition, pathway analysis of the RNA-seq data set suggested MMPs were significantly dysregulated. Indeed *MMP14*, a known regulator of collagen turnover in the mouse cervix,³⁰ was upregulated after exposure to progesterone. Increased expression of MMP14, which regulates the conversion of pro-MMP2 to active-MMP2, was confirmed in the 3D engineered cervix.

Whether the cervix shortens as in preterm birth, or remains long and closed in normal pregnancy, depends in part on the strength of the cervical stroma,^{12,14} which arises from its collagen-rich connective tissue.¹⁵ Softening of the stroma begins in early pregnancy and progresses continuously during the first two trimesters.¹⁶ Cervical insufficiency represents an extreme phenotype in which the cervix dilates during normal fetal growth.³¹ There is a need to decipher biochemical mechanisms of cervical softening, which could provide essential information regarding the cervical component of preterm birth.

In the current study, progesterone exposure correlated with decreased collagen content, less uniform collagen distribution on histology, and decreased tissue strength. It is noteworthy that increased mature collagen crosslinks were seen in the progesterone-exposed tissue, and mature crosslinks are generally associated with stronger tissue. The observed discrepancy requires further study to assess the relative contributions of histological appearance and collagen parameters to tissue mechanics.

The 3D engineered cervix is a useful model for studying cervical remodeling. Much like animal models and biopsy studies of human cervical tissue, engineered cervical ECM permits an alternate technique for studies of cervical remodeling. Different models offer unique opportunities to test specific hypotheses regarding cervical remodeling. With the 3D engineered cervix model, the specific effect of progesterone on the properties of the ECM can be studied in long-term culture. In addition, the properties of the ECM can be correlated to macroscopic mechanical properties of the tissue. With animal models, however, multiple mechanisms—for example inflammatory cells,³² stroma/epithelial interactions,³³ and changes in matricellular proteins³⁴—all occur simultaneously. Isolating cause and effect can be challenging. The observed decline in collagen content and

TABLE 4. MOST SIGNIFICANT UPSTREAM REGULATORS AND DISEASES AND FUNCTION

<i>Upstream regulator</i>			<i>Diseases and functions</i>		
<i>Upstream regulator</i>	<i>Activation/inhibition</i>	<i>Activation z-score</i>	<i>Diseases or functions annotation</i>	<i>Prediction</i>	<i>Activation z-score</i>
PDGF BB	Activated	3.8	Cellular development: epithelial-mesenchymal transition	Increased	3.0
NEDD9	Activated	3.6	Cellular growth and proliferation: outgrowth of cells	Increased	2.9
IL1A	Activated	3.4	Cellular development: differentiation of tumor cell lines	Increased	2.6
EGFR	Activated	3.3	Cellular movement: cell movement of tumor cell lines	Increased	2.5
HCG	Activated	3.2	Proliferation of vascular smooth muscle cells	Increased	2.4
IFNB1	Inhibited	−2.4	Metabolism of reactive oxygen species	Increased	2.3
IgG	Inhibited	−2.4	Transmigration of mononuclear leukocytes	Increased	2.2
IFNA2	Inhibited	−2.6	Cellular movement: migration of cells	Increased	2.1
PRL	Inhibited	−2.7	Inflammatory response	Increased	2.1
Hdac	Inhibited	−3.1	Cell death and survival: apoptosis of carcinoma cell lines	Decreased	−2.1

Activated regulators refer to regulators activated in scaffolds cultured in Est + Prog compared with Est only.

FIG. 2. Validation of RNA sequencing (RNA-seq) with real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The direction and magnitude of fold change differences for RNA-seq correlated with RT-qPCR results. Pearson $r=0.98$, $p<0.0001$.

increased transcripts encoding MMPs in the E+P group relative to E motivate further studies to evaluate these molecular processes in cervical ECM remodeling in pregnancy.

MMPs are suspected to be key mediators of cervical remodeling during human^{35,36} and animal^{37,38} pregnancy. Previous work in the pregnant mouse showed that (1) reduced MMP2 was associated with cervical and uterine fibrosis, and (2) reduced MMP2 correlated with reduced MMP14, which is an essential regulator of MMP2.³⁰ RNA-seq is a powerful tool that allows identification of all expressed transcripts within a sample to compare differential gene expression between experimental conditions. Functional analyses of the RNA-seq data via a bioinformatics tool can identify biologic pathways that are activated or inhibited in the experiment.

In the current experiment, pathway analysis of RNA-seq data pointed to the MMP pathway as being significantly dysregulated. More specifically, *MMP14* was upregulated in the scaffolds

exposed to progesterone. At the protein level, the ratio of active MMP2 to pro-MMP2 was increased, suggesting that increased MMP14 played a role in ECM remodeling in the scaffolds. The present study shows the potential for studying biological mechanisms of cervical remodeling in the 3D engineered cervix. Further work will be necessary to prove that MMP2 and MMP14 are essential biological mechanisms involved in progesterone-mediated ECM remodeling in this system.

Progesterone supplementation to prevent preterm birth is indicated for women with a history of preterm birth and for women with a short cervix.³⁹ It is likely that progesterone prevents preterm birth through pleiotropic effects, including its effects on the myometrium,⁴⁰ membranes,⁴¹ and decidua.⁴² The therapeutic effects of progesterone may not be related to ECM remodeling in the cervical stroma. Whether insights from the 3D engineered cervix can be used to decipher the therapeutic effect of progesterone in pregnancy is

FIG. 3. Matrix metalloproteinase (MMP) gene expression and protein activity. (A) RNA-seq showed MMP2 was not differentially regulated but MMP14 was significantly upregulated. (B) On zymography, bands corresponding to pro MMP2 and active MMP2 were seen. The Std lane corresponds to recombinant MMP2. Inhibition with ethylenediaminetetraacetic acid shows absence of activity. (C) Total MMP2 was increased in estradiol exposed scaffolds. However, the ratio of active MMP2 to pro MMP2 was increased for scaffolds exposed to Est + Prog, suggesting increased activity of MMP14. Data are presented as mean $\pm$ standard deviation. Color images available online at www.liebertpub.com/tea

not known. The present research with the 3D engineered cervix suggests increased progesterone concentration leads to decreased collagen concentration. Further experimentation is needed to determine if effects seen in the 3D cervix are relevant for progesterone supplementation during pregnancy.

An unbiased transcriptomics approach with RNA-seq can reveal novel pathways relevant for future study of cervical remodeling. Canonical signaling pathways activated after exposure to progesterone in the 3D engineered cervix included High-mobility group box protein 1 (HMGBP1) signaling, ERK/MAPK signaling, and IL-6 signaling (Table 3). These three signaling pathways have been implicated in studies of cervical remodeling. In studies using biopsies of the human cervix, HMGBP1 and its receptors are produced during pregnancy and labor is associated with distinct changes.⁴³ Biopsy studies of the human cervix also showed phosphorylated MAPK activation at term and postpartum.⁴⁴ Increased IL-6 concentration measured at 10–18 weeks in the serum was associated with subsequent cervical shortening.⁴⁵ Taken together, the unbiased transcriptomics approach highlighted signaling pathways relevant for cervical function and promising for future studies with the 3D engineered cervix.

The 3D engineered cervix model is novel in being able to study correlations between the mechanical properties of fibroblast-derived ECM and different culture environments. There are, however, several limitations of the present study. A single progesterone concentration was studied; no information about a dose–response effect was determined. Also, RNA-seq was studied at the 4-week time point, and different biological mechanisms could be important at earlier time points. Also, the 3D engineered cervix may not be representative of the complexity of cervical remodeling *in vivo* due to the absence of a blood supply or immune system inputs, among other factors. Nevertheless, the 3D engineered cervix model is an important alternate system for studying cervical remodeling in pregnancy.

In summary, progesterone had a softening effect on ECM produced *in vitro* with human cervical fibroblasts. These results are important for deciphering the role of progesterone in preventing preterm birth. Further development of the 3D engineered cervix model will allow more detailed study of the mechanism of progesterone-mediated effects on the cervix in pregnancy.

Acknowledgments

This article was previously presented in abstract form at the Annual Meeting of the Society for Maternal Fetal Medicine, 2016.

Disclosure Statement

No competing financial interests exist.

References

- Howson, C.P., Kinney, M.V., and Lawn, J.E. March of Dimes, PMNCH, Save the Children, WHO. Born Too Soon: The Global Action Report on Preterm Birth. Geneva, Switzerland: World Health Organization, 2012.
- Institute of Medicine. Preterm Birth: Causes, Consequences, and Prevention. Washington, DC: National Academies Press, 2006.
- Marlow, N., Wolke, D., Bracewell, M.A., Samara, M.; EPICure Study Group. Neurologic and developmental disability at six years of age after extremely preterm birth. *N Engl J Med* **352**, 9, 2005.
- Kwint, P., and Pietrzyk, J.J. Preterm birth and respiratory disease in later life. *Expert Rev Respir Med* **4**, 593, 2010.
- Serenius, F., Kallen, K., Blennow, M., *et al.* Neurodevelopmental outcome in extremely preterm infants at 2.5 years after active perinatal care in Sweden. *JAMA* **309**, 1810, 2013.
- Romero, R., Nicolaides, K.H., Conde-Agudelo, A., *et al.* Vaginal progesterone decreases preterm birth ≤ 34 weeks of gestation in women with a singleton pregnancy and a short cervix: an updated meta-analysis including data from the OPPTIMUM study. *Ultrasound Obstet Gynecol* **48**, 308, 2016.
- Society for Maternal Fetal Medicine. The choice of progesterone for the prevention of preterm birth in women with singleton pregnancy and prior preterm birth. *Am J Obstet Gynecol* **216**, B11, 2017.
- Owen, J., Hankins, G., Iams, J.D., *et al.* Multicenter randomized trial of cerclage for preterm birth prevention in high-risk women with shortened midtrimester cervical length. *Am J Obstet Gynecol* **201**, 375.e1, 2009.
- Owen, J., Yost, N., Berghella, V., *et al.* Mid-trimester endovaginal sonography in women at high risk for spontaneous preterm birth. *JAMA* **286**, 1340, 2001.
- ACOG Practice Bulletin No. 101: ultrasonography in pregnancy. *Obstet Gynecol* **113**, 451, 2009.
- Esplin, M.S., Elovitz, M.A., Iams, J.D., *et al.* Predictive accuracy of serial transvaginal cervical lengths and quantitative vaginal fetal fibronectin levels for spontaneous preterm birth among nulliparous women. *JAMA* **317**, 1047, 2017.
- Myers, K.M., Feltovich, H., Mazza, E., *et al.* The mechanical role of the cervix in pregnancy. *J Biomech* **48**, 1511, 2015.
- Westervelt, A.R., Fernandez, M., House, M., *et al.* A parameterized ultrasound-based finite element analysis of the mechanical environment of pregnancy. *J Biomech Eng* **139**, 2017.
- Myers, K.M., Paskaleva, A.P., House, M., and Socrate, S. Mechanical and biochemical properties of human cervical tissue. *Acta Biomater* **4**, 104, 2008.
- House, M., Kaplan, D.L., and Socrate, S. Relationships between mechanical properties and extracellular matrix constituents of the cervical stroma during pregnancy. *Semin Perinatol* **33**, 300, 2009.
- Badir, S., Mazza, E., Zimmermann, R., and Bajka, M. Cervical softening occurs early in pregnancy: characterization of cervical stiffness in 100 healthy women using the aspiration technique. *Prenat Diagn* **33**, 737, 2013.
- Mahendroo, M. Cervical remodeling in term and preterm birth: insights from an animal model. *Reproduction* **143**, 429, 2012.
- Yellon, S.M. Contributions to the dynamics of cervix remodeling prior to term and preterm birth. *Biol Reprod* **96**, 13, 2017.
- House, M., Sanchez, C.C., Rice, W.L., Socrate, S., and Kaplan, D.L. Cervical tissue engineering using silk scaffolds and human cervical cells. *Tissue Eng Part A* **16**, 2101, 2010.
- House, M., Tadesse-Telila, S., Norwitz, E.R., Socrate, S., and Kaplan, D.L. Inhibitory effect of progesterone on cervical tissue formation in a three-dimensional culture system with human cervical fibroblasts. *Biol Reprod* **90**, 18, 2014.

21. House, M., Daniel, J., Elstad, K., Socrate, S., and Kaplan, D.L. Oxygen tension and formation of cervical-like tissue in two-dimensional and three-dimensional culture. *Tissue Eng Part A* **18**, 499, 2012.
22. Yoshida, K., Jiang, H., Kim, M., *et al.* Quantitative evaluation of collagen crosslinks and corresponding tensile mechanical properties in mouse cervical tissue during normal pregnancy. *PLoS One* **9**, e112391, 2014.
23. Trapnell, C., Roberts, A., Goff, L., *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* **7**, 562, 2012.
24. Cheng, W.C., Chang, C.W., Chen, C.R., *et al.* Identification of reference genes across physiological states for qRT-PCR through microarray meta-analysis. *PLoS One* **6**, e17347, 2011.
25. Toth, M., and Fridman, R. Assessment of Gelatinases (MMP-2 and MMP-9 by Gelatin Zymography. *Methods Mol Med* **57**, 163, 2001.
26. Farhat, Y. Lysis of collagen gels for zymography. *Protocol Place*. 2012. <http://protocol-place.com/> (accessed May 1, 2016).
27. Yoo, J.Y., Shin, H., Kim, T.H., *et al.* CRISPLD2 is a target of progesterone receptor and its expression is decreased in women with endometriosis. *PLoS One* **9**, e100481, 2014.
28. Watanabe, H., Nonoguchi, K., Sakurai, T., Masuda, T., Itoh, K., and Fujita, J. A novel protein Depp, which is induced by progesterone in human endometrial stromal cells activates Elk-1 transcription factor. *Mol Hum Reprod* **11**, 471, 2005.
29. Graham, J.D., and Clarke, C.L. Physiological action of progesterone in target tissues. *Endocr Rev* **18**, 502, 1997.
30. Reeves, C.V., Wang, X., Charles-Horvath, P.C., *et al.* Anthrax toxin receptor 2 functions in ECM homeostasis of the murine reproductive tract and promotes MMP activity. *PLoS One* **7**, e34862, 2012.
31. ACOG Practice Bulletin No.142. Cerclage for the management of cervical insufficiency. *Obstet Gynecol* **123**, 372, 2014.
32. Mackler, A.M., Iezza, G., Akin, M.R., McMillan, P., and Yellon, S.M. Macrophage trafficking in the uterus and cervix precedes parturition in the mouse. *Biol Reprod* **61**, 879, 1999.
33. Timmons, B.C., Mitchell, S.M., Gilpin, C., and Mahendroo, M.S. Dynamic changes in the cervical epithelial tight junction complex and differentiation occur during cervical ripening and parturition. *Endocrinology* **148**, 1278, 2007.
34. Akins, M.L., Luby-Phelps, K., Bank, R.A., and Mahendroo, M. Cervical softening during pregnancy-regulated changes in collagen cross-linking and composition of extracellular proteins in the mouse. *Biol Reprod* **84**, 1053, 2011.
35. Ledingham, M.A., Denison, F.C., Riley, S.C., and Norman, J.E. Matrix metalloproteinases-2 and -9 and their inhibitors are produced by the human uterine cervix but their secretion is not regulated by nitric oxide donors. *Hum Reprod* **14**, 2089, 1999.
36. Sennstrom, M.B., Brauner, A., Bystrom, B., Malmstrom, A., and Ekman, G. Matrix metalloproteinase-8 correlates with the cervical ripening process in humans. *Acta Obstet Gynecol Scand* **82**, 904, 2003.
37. Gonzalez, J.M., Dong, Z., Romero, R., and Girardi, G. Cervical remodeling/ripening at term and preterm delivery: the same mechanism initiated by different mediators and different effector cells. *PLoS One* **6**, e26877, 2011.
38. Payne, K.J., Clyde, L.A., Weldon, A.J., Milford, T.A., and Yellon, S.M. Residency and activation of myeloid cells during remodeling of the prepartum murine cervix. *Biol Reprod* **87**, 106, 2012.
39. ACOG Practice Bulletin No. 130: Prediction and prevention of preterm birth. *Obstet Gynecol* **120**, 964, 2012.
40. Mesiano, S. Myometrial progesterone responsiveness and the control of human parturition. *J Soc Gynecol Investig* **11**, 193, 2004.
41. Luo, G., Abrahams, V.M., Tadesse, S., *et al.* Progesterone inhibits basal and TNF-alpha-induced apoptosis in fetal membranes: a novel mechanism to explain progesterone-mediated prevention of preterm birth. *Reprod Sci* **17**, 532, 2010.
42. Goldman, S., Weiss, A., Almalah, I., and Shalev, E. Progesterone receptor expression in human decidua and fetal membranes before and after contractions: possible mechanism for functional progesterone withdrawal. *Mol Hum Reprod* **11**, 269, 2005.
43. Dubicke, A., Andersson, P., Fransson, E., *et al.* High-mobility group box protein 1 and its signalling receptors in human preterm and term cervix. *J Reprod Immunol* **84**, 86, 2010.
44. Wang, H., and Stjernholm, Y.V. Plasma membrane receptor mediated MAPK signaling pathways are activated in human uterine cervix at parturition. *Reprod Biol Endocrinol* **5**, 3, 2007.
45. Venkatesh, K.K., Cantonwine, D.E., Ferguson, K., Arjona, M., Meeker, J.D., and McElrath, T.F. Inflammatory and oxidative stress markers associated with decreased cervical length in pregnancy. *Am J Reprod Immunol* **76**, 376, 2016.

Address correspondence to:

Michael House, MD
 Mother Infant Research Institute
 Tufts Medical Center, Box 394
 800 Washington Street
 Boston, MA 02111

E-mail: mhouse@tuftsmedicalcenter.org

Received: February 2, 2018

Accepted: May 31, 2018

Online Publication Date: July 18, 2018