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Oxygen Tension and Formation of Cervical-Like Tissue in Two-Dimensional and Three-Dimensional Culture

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Cervical dysfunction contributes to a significant number of preterm births and is a common cause of morbidity and mortality in newborn infants. Cervical dysfunction is related to weakened load bearing properties of the collagen-rich cervical stroma. However, the mechanisms responsible for cervical collagen changes during pregnancy are not well defined. It is known that blood flow and oxygen tension significantly increase in reproductive tissues during pregnancy. To examine the effect of oxygen tension, a key mediator of tissue homeostasis, on the formation of cervical-like tissue *in vitro*, we grew primary human cervical cells in both two-dimensional (2D) and three-dimensional (3D) culture systems at 5% and 20% oxygen. Immunofluorescence studies revealed a stable fibroblast phenotype across six passages in all subjects studied ($n = 5$). In 2D culture for 2 weeks, 20% oxygen was associated with significantly increased collagen gene expression ($p < 0.01$), increased tissue wet weight ($p < 0.01$), and increased collagen concentration ($p = 0.046$). 3D cultures could be followed for significantly longer time frames than 2D cultures (12 weeks vs. 2 weeks). In contrast to 2D cultures, 20% oxygen in 3D cultures was associated with decreased collagen concentration ($p < 0.01$) and unchanged collagen gene expression, which is similar to cervical collagen changes seen during pregnancy. We infer that 3D culture is more relevant for studying cervical collagen changes *in vitro*. The data suggest that increased oxygen tension may be related to significant cervical collagen changes seen in pregnancy.

Introduction

Preterm birth affects 12%–13% of pregnancies in the United States[1] and is a leading cause of morbidity and mortality in newborn infants. In 2006, more than one half (54%) of all infant deaths were associated with preterm birth before 32 weeks.[2] Among survivors of preterm birth born before 27 weeks, 25% have significant neurological disability.[3] The Institute of Medicine estimates health care costs related to preterm birth are $26 billion.[4] Few treatments for preterm birth are available[5,6] and those treatments have not made a large impact on the frequency of preterm birth.[1]

Although preterm birth is a complex disorder, a dysfunctional cervix is associated with a significant number of cases.[7–9] The cervix is a cylindrical structure with a central canal that forms the bottom part of the uterus. In normal pregnancy, the cervix remains long and closed until labor begins. In cases of preterm birth, undesired cervical deformation occurs—the cervix becomes short and dilated, often without clinically obvious uterine contractions. Cervical shortening is a well-recognized risk factor for preterm birth.[7–9] In addition, a short cervix is used to target treatment to prevent preterm birth.[5,6,10]

Preterm cervical deformation is related, in part, to weakened load bearing properties of the cervical stroma.[11,12] Cervical mechanical properties arise from its extracellular matrix (ECM) that is composed of fibrous connective tissue. Biopsies of cervical tissue show over 50% of cervical dry weight is fibrillar collagen.[13] Pregnancy is associated with important changes in cervical collagen—the concentration of cervical collagen concentration decreases and the extractability increases.[13] These collagen changes result in substantially softer cervical tissue in the third trimester.[11] The molecular mechanisms causing collagen changes are not well understood, although hormonal changes, inflammation, mechanical deformation, and stromal–epithelial interactions are likely important.[14,15]

During pregnancy, oxygen delivery to reproductive tissues significantly increases to meet the nutritional demands of the growing fetus. Uterine artery blood flow increases

Work performed in the Department of Biomedical Engineering, Tufts University, Medford, Massachusetts.
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from 9 mL/min in nonpregnant women to 142 mL/min by 21 weeks, a 15-fold increase.[16] Increases in blood flow have been correlated to increases in placental PO2. Measurement of intervillous PO2 in the first trimester revealed a significant increase from 17.9 to 60.7 mmHg when values at 8–10 weeks were compared with values at 12–13 weeks.[17]

Increased tissue PO2 could play a significant role in cervical remodeling during pregnancy. Cervical fibroblasts are the most prevalent cell type in the cervical stroma and oxygen tension is known to affect ECM production in placental,[18] renal,[19] and dermal fibroblasts.[20] In engineered tissue, oxygen diffusion limitations are known to limit size and viability of engineered constructs.[21] In our previous work, we demonstrated a system for producing three-dimensional (3D) cervical-like tissue using cervical cells and porous silk scaffolds.[22] Production of cervical-like tissue was significantly improved in dynamic versus static culture that likely relates to improved mass transport and delivery of dissolved oxygen.

Current model systems for studying cervical remodeling have important limitations. Studies of the human cervix are challenging because cervical biopsies are difficult to perform during pregnancy.[23] Animal models of cervical remodeling[24–26] may not reflect changes in the human cervix. The rationale for developing our system to produce 3D cervical-like tissue was to study cervical remodeling in a controlled, *in vitro* environment.[22] However, the advantages of our 3D system over a simple two-dimensional (2D) culture system are not known.

The objective of the present study was to determine the importance of oxygen tension on the production of cervical-like tissue in 2D and 3D culture with a goal toward gaining insight into cervical remodeling that occurs during pregnancy. We hypothesized that, compared with 2D culture, our 3D culture system for producing cervical-like tissue would be more relevant for studying cervical remodeling. We further hypothesized that increased oxygen tension would be associated with decreased collagen concentration in 3D cervical-like tissue that is similar to cervical collagen changes seen in human pregnancy.

Materials and Methods

Subject demographics

Cervical cells were isolated from human cervical tissue obtained from hysterectomy specimens. Informed consent was obtained from nonpregnant women scheduled for total hysterectomies for benign gynecological disorders. A total of 11 subjects were enrolled (Caucasian $n=6$, African American $n=2$, and Asian $n=3$). The age range was 34–50 years (median, 42 years). The indication for hysterectomy was fibroid uterus ($n=6$), dysfunctional uterine bleeding ($n=4$), and adenomyosis ($n=1$). Institutional Review Board approval from Tufts Medical Center was obtained before starting the study.

Cell culture

Cervical cells were isolated using an explant culture method as previously described.[22] Briefly, a biopsy of the cervical stroma (nearly 300 mg) was obtained in the operating room after removal of the hysterectomy specimen. The biopsy was obtained from the middle of the cervix taking care to avoid the uterine wall and external cervical os. The endocervical epithelium was removed and the biopsy was brought to the tissue culture hood in cold culture medium. The biopsy was divided into two parts. One part was flash frozen and used as a positive control for biochemical assays. The second part was minced and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B.[27] The minced pieces were maintained in a humidified tissue culture incubator at 37°C, 5% CO_2/95% air, and 95% relative humidity. After 10 days, cells were confluent around the explants at which point cells were trypsinized and culture-expanded. Cells from passage 4 to 5 were used for all experiments.

Cell phenotype

To determine the cell phenotype and phenotype stability of cells cultured from cervical explants, indirect immunofluorescence experiments were performed on cells from five subjects. Cells from passage 2 to 6 were used. At each passage, cells at 30% confluence were fixed and permeabilized. After blocking with 1% bovine serum albumin, cells were incubated with marker antibodies. Two marker antibodies were used for fibroblasts: vimentin (1:100 dilution; ab28028, Abcam Inc.) and prolyl-4-hydroxylase beta (1:50 dilution of 0.2 mg/mL stock; AF0910-1, Acris Antibodies). Pan cytokeratin was used for an epithelial cell marker (1:200 dilution of 1 mg/mL stock; ab7753, Abcam Inc.). Alpha smooth muscle actin was used for a smooth muscle cell marker (1:50 dilution of 0.2 mg/mL stock; ab7817, Abcam Inc.). Negative controls were incubated with mouse IgG (1:2000 dilution of 2 mg/mL stock; I2000, Vector Labs). Cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (ab6785, Abcam Inc.). Last, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) and examined with a confocal microscope (DM IRE2 microscope, Leica). To estimate the percentage marker-positive cells, the number of cells with strong signal was divided by the total number of cells in the field (DAPI positive) averaged over three fields.

Two-dimensional culture

Cervical cells were seeded in six well plates (Falcon, 35-3046) at a density of 1×10^4 cells/cm². Three culture conditions were used (Fig. 1):

1. 20% oxygen: Cells were cultured in an incubator (Thermo Scientific) with air circulation (95% air/5%) in static conditions.
2. 5% oxygen: Cells were cultured in a 5% oxygen environment (MCO-5M, Sanyo) in static conditions. We chose to use 5% oxygen tension because the oxygen tension in the cervix of nonpregnant women ranges from 1.7% (parous) to 6.3% (nulliparous).[28]
3. 5% rotator: Cells were cultured in a 5% oxygen environment and the six well plates were placed on a lab rotator (Thermo Scientific, #2309) set at 50 revolutions per minute. The rationale for using a lab rotator was to study the effect of convective mixing in 2D culture.

Culture media was supplemented with freshly prepared 50 μg/mL ascorbic acid 2-phosphate (A8960, Sigma-Aldrich)
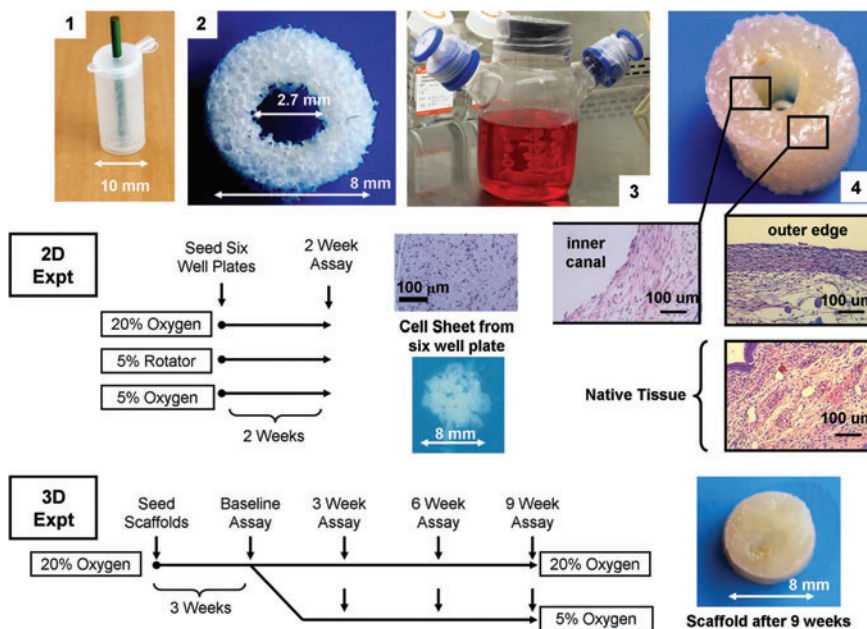
FIG. 1. Top panel: polytetra-fluoroethylene-coated, stainless steel wire was press fit into a polyethylene vial to create the scaffold mold (1). The scaffold was shaped like a cylinder with a central canal (2). The scaffolds were cultured in a spinner flask bio-reactor (3). Cervical-like tissue was present on the scaffold exterior and interior after 5 weeks (4). The morphology of cervical-like tissue resembled the stroma of native cervical tissue. Middle panel: Timeline for two-dimensional experiment. At the end of the 2 week period, the cell sheet was scraped from the well and assayed. Bottom panel: Timeline for the three-dimensional experiment. After 9 weeks, cervical-like tissue covered the scaffold and grew into the central canal. 2D, two-dimensional; 3D, three-dimensional. Color images available online at www.liebertonline.com/tea

because scant ECM synthesis occurred when ascorbic acid was absent (data not shown). Culture media was changed three times per week for 2 weeks at which point a cell sheet formed. Culture times longer than 2 weeks were not possible because the cell sheet detached from the well and contracted. The cell sheet was removed from the well by gentle scraping and assayed (Fig. 1).

Three-dimensional culture

Silk sponge scaffold. Silk fibroin protein was purified as previously described.[29] Briefly, cocoons of the *Bombyx mori* silkworm (Tajima Shoji Co., Ltd.) were cut into 2 mm pieces and boiled for 30 min in an aqueous solution of 0.02 M Na_2CO_3. Fibrous silk protein was solubilized in 9.3 M LiBr solution at 60°C for 4 h, dialyzed against distilled water for 2 days and diluted to obtain a 6% (w/v) silk fibroin solution.

Fabrication of the silk scaffold was improved from a previous report.[22] The human cervix is shaped like a cylinder with a central canal. Thus, silk scaffolds were formed into this shape for the present study (Fig. 1). A polyethylene vial (Fisherbrand, #033381B, Fisher Scientific) was modified by having a hole drilled through the top and bottom. A polytetrafluoroethylene-coated, stainless steel wire (diameter .106," # 1749T76, McMaster Carr) was press fit into the hole. Silk fibroin solution (2 mL) was poured into the vial followed by granular NaCl (4 gm, particle size 500–600 micron). The silk solution solidified after 24 h at room temperature and 4 h at 60°C. The lid was removed and the scaffold was immersed in water for 2 days to extract the NaCl. The scaffold was removed from the vial and washed for an additional 24 h in water. Next, an 8 mm cylindrical punch biopsy (33–37, Miltex) was used to cut the scaffold to the needed outside diameter and a blade was used to cut the scaffold to the needed height (4 mm). The scaffolds were autoclaved, collagen-coated, and seeded with cervical cells (2.5 million cells per scaffold, 125 μL of a concentrated cell solution of 20×10^6 cells/mL) as previously described.[22] Cells were allowed to attach for 2 h in six well plates, after which 5 mL of culture

media was added and the scaffolds were cultured overnight. After 24 h, scaffolds were moved to new six well plates. After 2 days, scaffolds were mounted on steel wire and suspended in a 250 mL spinner flask bioreactor (#1967-10250, Bellco Glass). All scaffolds were cultured in dynamic conditions (stir bar rotating at 50 rpm) because we have shown improved tissue synthesis in these conditions compared with static culture.[22] After 5 weeks, cervical-like tissue was seen on the outside and inside of the scaffold. Cervical-like tissue resembled native tissue at both the gross and microscopic scale (Fig. 1).

Two culture conditions were used: 20% and 5% oxygen (Fig. 1). At first, all scaffolds were cultured for 3 weeks at 20% oxygen and baseline assays were performed. After this baseline culture period, one half of the scaffolds were moved to the 5% oxygen incubator and one half stayed at 20% oxygen. Of note, the composition of the culture media for the 3D experiment was the same as the 2D experiment. Scaffolds were harvested at 3, 6, and 9 weeks and processed for histology or stored at −80°C until biochemical analysis. The experiment was stopped at 9 weeks because this time period was 4 weeks longer than previous experiments.[22] Histology was performed on 5 μm thick slices taken from formalin fixed, paraffin embedded blocks. Hematoxylin and eosin staining were performed using standard procedures.

Cell viability

The viability of the cervical cells at the end of the 2D and 3D experiments was confirmed with a two-color fluorescence assay (LIVE/DEAD viability/cytotoxicity kit, Invitrogen). The scaffold was incubated with two probes: 2 mM calcein AM and 1 mM ethidium homodimer-1 for 30 min at room temperature. Live cells convert calcein AM to calcein, a green fluorescent product. Ethidium homodimer-1 is a red-fluorescent stain that binds to both silk scaffold and DNA of membrane compromised dead cells (Fig. 2). The fluorescence signal was visualized using a confocal microscope (DM IRE2 microscope, Leica).

FIG. 2. Left panel: cervical cells demonstrated strong immunofluorescence signal for fibroblast markers for all subjects ($n = 5$) and all passages (up to passage 6). Right panel: cervical cells remained viable (green signal) on the silk scaffolds during the 12 week culture period. The cells in the 2D experiment appeared the same as in the 3D experiment (not shown). Color images available online at www .liebertonline.com/tea
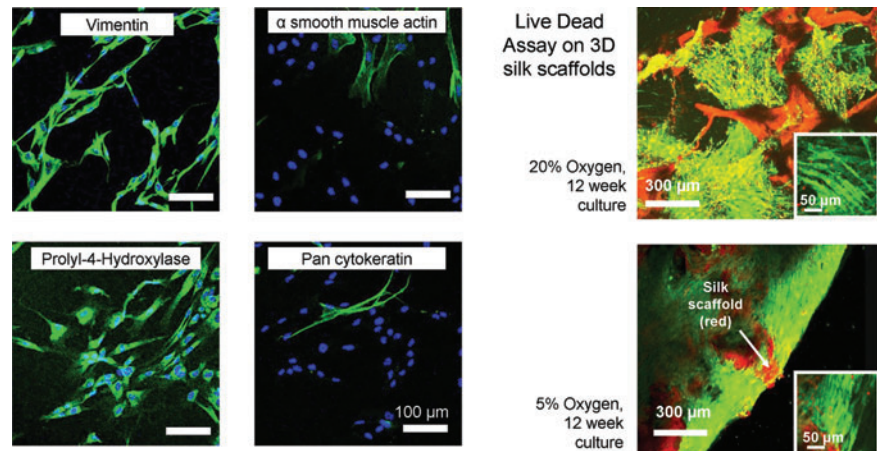
Biochemical characterization

The 2D cell sheets were assayed for cell metabolic activity. Both 2D cell sheets and 3D scaffolds were assayed for (1) hydration, (2) collagen concentration, (3) sulfated glycosaminoglycan (S-GAG) concentration, and (4) DNA concentration.

Cell metabolic activity. The alamarBlue® reagent (Invitrogen) was used to assess cell metabolic activity. Growth medium was used to dilute $10 \times$ alamarBlue dye and 2.5 mL was added to each well of a six well plate. The cells were incubated for 1 h. One hundred microliters of the culture medium was transferred to an opaque plate reader and fluorescence was read at 560 nm excitation/590 nm emission.

Hydration. Pieces of 3D scaffold or 2D cell sheet (15–30 mg) were homogenized in a Bessman tissue pulverizer precooled with liquid nitrogen. The frozen, crushed powder was placed in a preweighed 1.5 mL microcentrifuge tube and weighed. The powder was lyophilized overnight and reweighed. Hydration was determined by calculating hydration = (wet weight–dry weight)/wet weight.

Collagen concentration. Lyophilized sample were hydrolyzed in sealed Kimax tubes in 6 M HCl at 115°C overnight (1 mL acid per 2.5 mg dry weight). Acid was evaporated and the hydrolysate was resuspended in 1.5 mL water. Collagen concentration was determined according to hydroxyproline concentration.[30]

S-GAG and DNA concentration. Scaffolds or cell sheets (20–30 mg wet weight) were minced with scissors and digested in 400 μL of 1.0 mg/mL Proteinase K (#03115887001, Roche Diagnostics) in digestion buffer (50 mM Tris HCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0) at 50°C overnight. The digested solutions were clarified by centrifugation at 10,000 g for 10 min. The supernatants were assayed for either S-GAG or DNA. S-GAG concentrations was determined using a 1,9 dimethylmethylene blue dye label (Blyscan Assay kit, Biocolor). Chondroitin-6-sulfate was used for the standard curve. DNA concentration was determined using a fluorescent nucleic acid stain (Quant-iT PicoGreen dsDNA kit, Invitrogen). Lambda DNA was used for the standard curve.

Real-time quantitative reverse transcription–polymerase chain reaction

For 2D experiments, gene expression was determined at the end of the 2 week experiment. For 3D experiments, gene expression was evaluated at baseline and at the 3, 6, and 9 week time points (the baseline time point refers to cervical-like tissue cultured on a scaffold for 3 weeks as shown in Fig. 1, bottom panel). For both experiments, total RNA was extracted using the RNeasy Fibrous Tissue Kit (Qiagen). Tissue samples were stored at −80°C until time of assay. Small pieces of frozen scaffold or cell sheet (20–30 mg) were homogenized using a precooled Bessman tissue pulverizer. The frozen, crushed powder was placed in a guanidine-based lysis solution (Buffer RLT containing β-Mercaptoethanol) and further homogenized using an 18 gauge needle. Proteinase K incubation of the lysate was performed for 10 min at 55°C following the manufacturer's protocol. RNA was purified on the RNeasy spin column with an on-column DNase digestion. Purified RNA was eluted with 50 μL of water and stored at −80°C. The A_{260}/A_{280} ratio was above 2.0 for all samples tested (Nanodrop 2000, Thermo Scientific). The RNA concentration was 0.1–0.3 μg/μL. Reverse transcription reactions (50 μL RNA: 50 μL reverse transcription Master Mix) were performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the cDNA was stored at −20°C.

Quantitative gene expression was measured with the Stratagene Mx 3000P QPCR System (Stratagene) and primer and probe sets with TaqMan® chemistry (Applied Biosystems). A 50 μL reaction consisted of 25 μL TaqMan Gene Expression Master Mix + 5 μL cDNA template + 17.5 μL RNase-free water + 2.5 μL TaqMan Gene Expression Assay (product number 4331182). The following thermal profile was used: 50°C for 2 min, 95°C for 10 min, followed by 50 amplification cycles consisting of a denaturation step at 95°C for 15 s, and an extension step at 60°C for 1 min. The following four genes associated with cervical ECM remodeling were measured: collagen type 1 (collagen type I, alpha 1 (*COL1A1*), assay ID Hs00164004_m1), collagen type 3 (collagen type III, alpha 1 (*COL3A1*), assay ID Hs00164103_m1), decorin (*DCN*, assay ID Hs00370384_m1), and hyaluronan synthase 2 (*HAS2*, assay ID Hs00193435_m1). ECM-related gene expression was normalized by the geometric mean of two housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (assay ID Hs99999905_m1) and beta actin (*ACTB*, assay ID

Hs99999903_m1). The cycle threshold was calculated from baseline-corrected, normalized fluorescence data using instrument software. Each data point represented the mean ± SD of four biological replicates. For 3D cultures, quantification was expressed as fold change relative to gene expression at the baseline time point using the $2^{\wedge}$-(delta delta Ct) method.

Statistical analysis

Data are expressed as mean values ± standard deviation. Mean values were calculated from 3 to 5 replicates as indicated. Comparisons between means were performed using Student's *t*-test or two-way analysis of variance with Bonferroni post-tests (GraphPad Prism version 5.04 for Windows, GraphPad Software) as appropriate. Results were considered significant when $p < 0.05$.

Results

Gross morphology

Cervical-like tissue was produced in both 2D and 3D culture. In 2D culture, a cell sheet formed after 2 weeks at which point the sheet detached from the culture well and spontaneously contracted. In 3D culture, cervical-like tissue filled the pores (500–600 µm) of the scaffold after 3 weeks. After 5 weeks, cervical-like tissue began to grow into the central canal. After 8–10 weeks, the central canal was filled with cervical-like tissue (Fig. 1). No gross morphological differences were seen between 5% and 20% oxygen in 2D or 3D culture (Figs. 1 and 4).

Cell phenotype and viability

Immunofluorescence experiments showed vimentin and prolyl-4-hydroxylase beta signal for the majority of cells (Fig. 2). These fibroblast markers were seen at all passages stud-ied. Fewer than 10% of the cells showed signal for alpha smooth muscle actin. Less than 1% of cells showed signal for pancytokeratin. Thus, cervical cells maintained a stable fibroblast phenotype. It is likely epithelial cells were not seen because (1) the cervical epithelium was discarded and (2) the growth media was optimized for fibroblasts. The LIVE/DEAD assay showed viable cells at the end of the experiments.

Two-dimensional culture

2D cell sheets were assayed after 2 weeks in culture (Fig. 3). Compared with 5% oxygen, 20% percent oxygen was associated with significantly increased cell metabolic activity ($p < 0.01$), DNA content ($p = 0.01$), tissue wet weight ($p < 0.01$), and collagen production ($p = 0.046$). Comparing the two groups cultured at 5% oxygen, cells cultured on a lab rotator showed increased tissue wet weight ($p < 0.01$) and increased S-GAG production ($p = 0.05$) compared with cells cultured in static conditions. No differences in hydration were seen for the three culture conditions.

Three-dimensional culture

Compared with 5% oxygen, 20% oxygen was associated with significantly decreased collagen production ($p < 0.01$; Fig. 4). Histological appearance of cervical-like tissue was similar to native tissue in both 5% and 20% oxygen (Fig. 4). Tissue morphology was similar to native tissue in the first 100 µm of the scaffold surface, which is similar to our previous report.[22] Cervical-like tissue production was maximal between weeks 3 and 6 of the experiment after which a significant decline in tissue production was observed. Table 1 shows the biochemical constituents of cervical-like tissue at week 3 of the experiment.
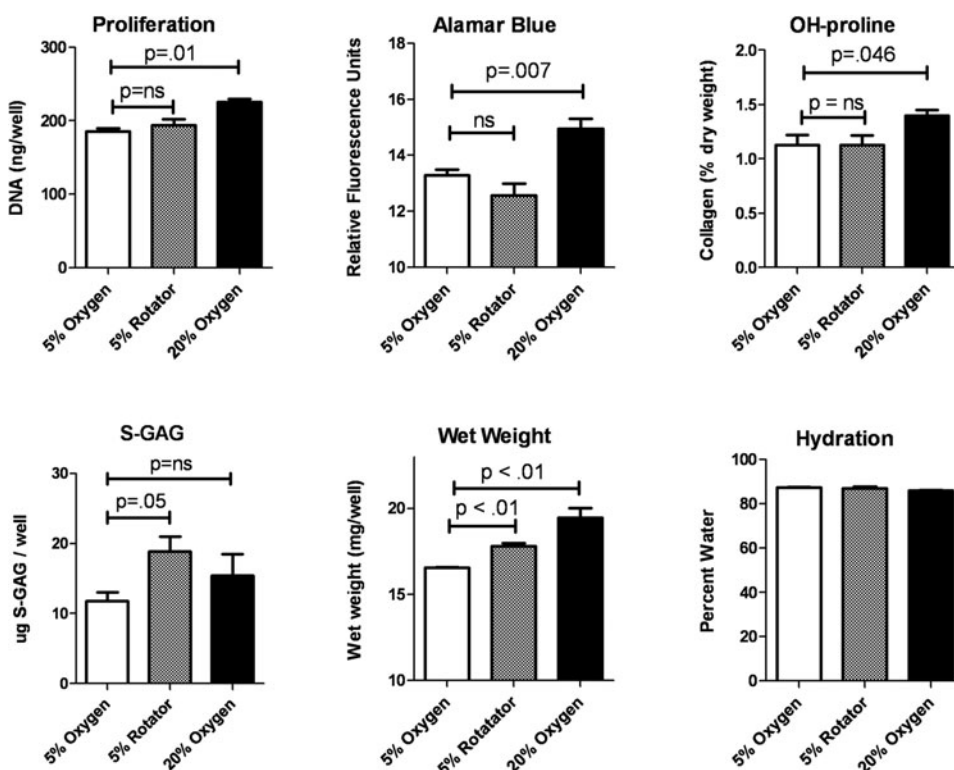

FIG. 3. Two-dimensional culture. Compared with 5% oxygen, 20% oxygen was associated with increased cell proliferation ($p = 0.01$), increased cell metabolism ($p = 0.007$), increased collagen concentration ($p = 0.046$), and increased tissue wet weight ($p < 0.01$). Compared with 5% oxygen in static conditions, 5% oxygen on a lab rotator was associated with increased tissue wet weight ($p < 0.01$) and increased S-GAG ($p = 0.05$). No differences in construct hydration were seen for the three experimental conditions. Data expressed as mean ± SD of four replicates. Experimental results are representative of experiments from three subjects. S-GAG; sulfated glycosaminoglycan; SD, standard deviation.

FIG. 4. Three-dimensional culture. Left panel shows construct histology as a function of time and oxygen tension. Tissue production peaked between the 3 and 6 week time points at both oxygen tensions. At the 6 week time point, histological appearance suggests increased tissue production at 5% oxygen tension. The scale bar is 100 μm. The top, right panel shows gross morphology at the 5 week time point. No gross differences were seen between 5% and 20% oxygen at this time point or any other time point. The bottom right panel shows collagen production as a function of time and oxygen tension. Twenty percent oxygen was associated with nearly 15% decrease in collagen production ($p < 0.01$, two-way analysis of variance). Data expressed as mean ± SD of five biological replicates. Color images available online at www.liebertonline.com/tea
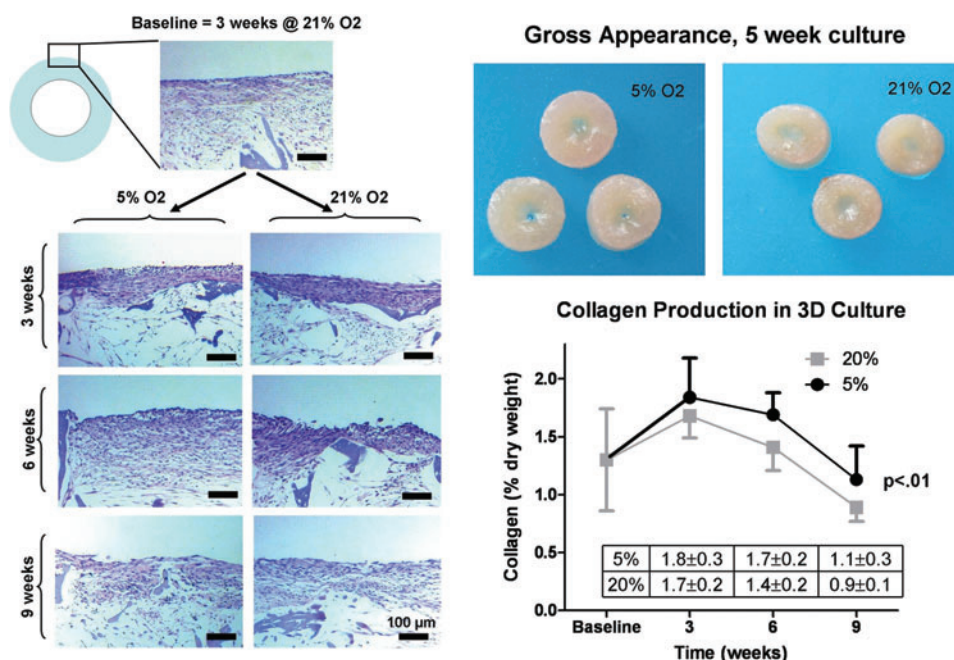
Gene expression

In 2D culture, *COL1A1* and *COL3A1* were significantly ($p < 0.01$) upregulated in 20% oxygen compared with 5% oxygen (Fig. 5). There were no differences in collagen gene expression between 5% and 5% oxygen (rotator) in 2D culture (Fig. 5). In 3D culture, *COL1A1* and *COL3A1* gene expression were significantly downregulated during the course of the experiment compared with the baseline time point ($p < 0.01$). There were no differences in collagen gene expression between 20% and 5% oxygen in 3D culture. Oxygen tension was not

FIG. 5. Real-time quantitative reverse transcription–polymerase chain reaction of matrix associated genes. In the 2D experiment (left panel), *COL1A1* and *COL3A1* were significantly upregulated in 20% compared with 5% oxygen ($p < 0.01$). There were no differences in collagen gene expression between 5% oxygen in static culture versus 5% oxygen on a lab rotator. In the 3D experiment (right panel). Collagen gene expression was significantly downregulated at all time points compared with the baseline time point. There were no differences in collagen gene expression between 5% and 20% oxygen in 3D culture. No differences were seen in gene expression of Decorin or *HAS2*. Gene symbols: *COL1A1*, collagen type I, alpha 1; *COL3A1*, collagen type III, alpha 1; *HAS2*, hyaluronan synthase 2; Gene expression was normalized by the geometric mean of two housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta actin. Data are expressed as mean ± SEM of four biological replicates. SEM, standard error of the mean.
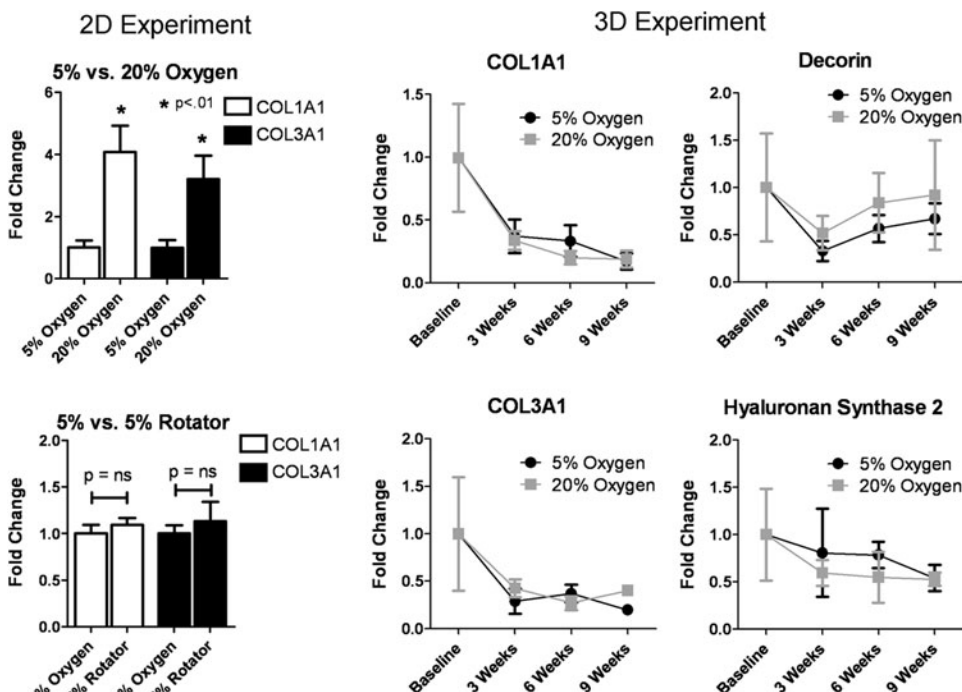
TABLE 1. BIOCHEMICAL CONSTITUENTS AT THE 3 WEEK TIME POINT OF THE THREE-DIMENSIONAL MODEL

Culture conditions	Hydration (% water) n=5	DNA concentration (µg/mg wet weight) n=4	Sulfated GAG (µg/mg wet weight) n=4	% Collagen (mg/mg dry weight) n=5
5% oxygen	90.1±0.25	0.17±0.027	0.31±0.07	1.84±0.3
20% Oxygen	89.3±0.61	0.15±0.061	0.35±0.1	1.68±0.2

Data presented as mean±standard deviation of four or five replicates as indicated.
GAG, glycosaminoglycan.

associated with differences in *DCN* or *HAS2* gene expression in 3D culture.

Discussion

In the present study, cervical fibroblasts produced cervical-like tissue in both 2D and 3D culture. However, 3D culture was associated with significant advantages. Cells in 2D could only be cultured for 2 weeks before the cell sheet detached from the culture well and spontaneously contracted. In contrast, cells in 3D culture maintained their shape on silk scaffolds for over 12 weeks. Prolonged culture time was important because culture time was a key factor in detecting biologically relevant collagen changes. In 3D culture, 20% oxygen was associated with significantly decreased collagen concentration compared with 5% oxygen over the 9 week culture period. In contrast, 2D culture was associated with increased collagen concentration in 20% versus 5% oxygen. Since pregnancy is associated with both (1) increased oxygen tension in reproductive tissues and (2) decreased collagen concentration in the cervix, we infer 3D culture is a more relevant system compared with 2D culture.

Human pregnancy is associated with significant cervical softening that has been correlated to changes in cervical collagen. Cervical collagen concentration decreases from 54%–77% (nonpregnant) to 23%–44% in the third trimester.[13] As collagen concentration decreases, collagen extractability after pepsin digestion increases.[13] It has been proposed that pregnancy is a state of increased collagen turnover leading to increased proportion of newly synthesized, uncross-linked collagen, and thus tissue softening.[24,31] Indeed, studies of the mouse cervix correlated decreased intermolecular collagen cross-links to progressive cervical softening as gestational age advanced.[24] The present study is novel for using a tissue engineering strategy to study collagen changes in a controlled *in vitro* environment with primary human cervical fibroblasts.

In our 3D culture system, oxygen tension increase from 5% to 20% was associated with nearly a 15% decrease in collagen concentration. No change in collagen gene expression was detected in 3D culture, which is also similar to studies of collagen gene expression in human pregnancy.[31] We did not see changes in sulfated GAGs, DNA concentration or hydration as a function of oxygen tension in 3D culture. In 2D culture, 20% oxygen was associated with increased tissue wet weight and increased collagen concentration. These changes were associated with increased cell metabolism, cell proliferation, and collagen gene expression.

Differences in 3D versus 2D culture may be related to differences in oxygen delivery. 3D cultures were performed in dynamic conditions whereas 2D cultures were performed in static conditions.[32] To study whether convective mixing was an important variable in 2D culture, cells at 5% oxygen were also cultured on a lab rotator. Compared with 5% oxygen in static conditions, cells at 5% oxygen on a lab rotator showed increased tissue wet weight and S-GAG, thereby suggesting that production of cervical-like tissue is dependent on not only oxygen tension but also oxygen delivery.

An environment of high oxygen is a biologically plausible environment for decreased collagen production as low oxygen conditions promote fibrogenesis in other mesenchymal cell systems. Placental fibroblasts cultured at 3% oxygen showed increased fibronectin, collagen I, and collagen IV at both the protein and mRNA level.[18] Dermal fibroblasts at 2% oxygen showed increased transcription of *COL1A1* gene and increased production of collagen protein.[20] In renal fibroblasts, hypoxia promotes ECM production and decreases ECM turnover.[19] We speculate that during pregnancy, oxygen delivery to cervical tissue is increased leading to decreased fibrogenesis and decreased collagen concentration. Further work will be needed to study the mechanisms responsible for oxygen related changes in cervical-like tissue.

Both hyaluronan (HA) and *DCN* were studied because of their suspected role in regulating cervical ECM composition and organization.[13] HA is known to increase at the end of pregnancy.[33] Increased HA contributes to increased cervical hydration that could influence collagen organization.[33] *HAS2* was studied because HA biosynthesis is transcriptionally regulated by *HAS2* and *HAS2* levels rise near parturition in women.[34] *DCN* is the dominant proteoglycan in cervical stroma.[35] *DCN* levels decrease by 40% near the end of pregnancy[36] and *DCN* is known to influence collagen fibrillogenesis. Others have suggested that decreased *DCN* levels could be responsible for increased disorganization seen in the cervical collagen during pregnancy.[37] The main objective in studying these markers was to determine whether our 3D culture system detected changes in HA or *DCN* at the transcript level as a function of oxygen tension. However, no differences were seen in expression of *HAS2* or *DCN* as a function of oxygen tension in our system.

In other 3D culture systems, oxygen can have positive and deleterious effects on ECM formation. Both hypoxia and hyperoxia can change the natural history of the wound healing response.[38] In cartilage tissue engineering, low oxygen tension influences proliferation, differentiation, and ECM synthesis of chondrocytes.[39–44] In the present study, only two oxygen tensions were studied and future work will expand the range of oxygen tensions to define better the role of oxygen on formation of cervical-like tissue.

The ability to perform 3D culture with primary cervical fibroblasts will allow a range of future studies important for cervical remodeling. Cervical fibroblasts were isolated from women with a range of gynecological conditions and no

differences in growth rate or phenotype were seen. The cells reliably attach to scaffolds and produce cervical-like tissue. In the future, it will be important to study not only collagen production but also collagen extractability because increased extractability is consistently associated with cervical softening in both human[13] and animal pregnancy.[45] Studies of collagen cross-links will be important for relating cervical remodeling to changes in tissue mechanical properties.[46] Mechanical properties arise not only from matrix production but also from matrix turnover and it will be important to study regulation of matrix metalloproteinases (MMP's) and tissue inhibitor of metalloproteinases (TIMP's) to clarify oxygen-related effects on cervical remodeling. Further, it is likely oxygen tension is not the only regulator of cervical remodeling and other causes (endocrine, mechanical) need to be studied.

In summary, we report an improved method of producing cervical-like tissue in 3D culture. The 3D culture system was superior to 2D culture for studying cervical remodeling in a controlled, *in vitro* environment. Increased oxygen tension was associated with decreased collagen concentration, a finding that may have relevance for cervical ECM changes that occur during pregnancy. In future studies, we expect to explore the mechanism of oxygen-related changes in the ECM of cervical-like tissue.

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Disclosure Statement

No competing financial interests exist.

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