Research Article

Maternal and fetal intrauterine tissue crosstalk promotes proinflammatory amplification and uterine transition[†]

Kelycia B. Leimert¹, Angela Messer², Theora Gray², Xin Fang², Sylvain Chemtob³ and David M. Olson^{1,2,*}

¹Department of Physiology, University of Alberta, Edmonton, Alberta, Canada; ²Departments of Obstetrics & Gynecology, and Pediatrics, University of Alberta, Edmonton, Alberta, Canada and ³Department of Pediatrics, Ophthalmology, and Pharmacology, CHU Sainte-Justine Research Center, Montreal, Quebec, Canada

***Correspondence:** Departments of Obstetrics & Gynecology, and Pediatrics, University of Alberta, 220 Heritage Medical Research Centre, Edmonton, AB T6G 2S2, Canada. Tel: +1-780-492-2765; Fax: +1-780-492-1308; E-mail: dmolson@ualberta.ca

[†]**Grant Support:** This research was funded by Canadian Institutes of Health Research (CIHR) #119513, Global Alliance for the Prevention of Prematurity and Stillbirth (GAPPS), an initiative of Seattle Children's #12005, and the March of Dimes (MOD) #21FY12-161. KB Leimert received a graduate studentship from the Women and Children's Health Research Institute, through the generosity of the Stollery Children's Hospital Foundation and supporters of the Lois Hole Hospital for Women.

Conference Presentation: Presented in part at the 64th Annual Scientific Meeting of the Society for Reproductive Investigation, March 15–18, 2017, Orlando, Florida, and the 65th Annual Scientific Meeting of the Society for Reproductive Investigation, March 6–10, 2018, San Diego, California.

Edited by Dr. Haibin Wang

Received 18 August 2018; Revised 6 October 2018; Accepted 29 October 2018

Abstract

Birth is a complex biological event requiring genetic, cellular, and physiological changes to the uterus, resulting in a uterus *activated* for completing the physiological processes of labor. We define the change from the state of pregnancy to the state of parturition as *uterine transitioning*, which requires the actions of inflammatory mediators and localized paracrine interactions between intrauterine tissues. Few studies have examined the in vitro interactions between fetal and maternal gestational tissues within this proinflammatory environment. Thus, we designed a co-culture model to address this gap, incorporating primary term human myometrium smooth muscle cells (HMSMCs) with human fetal membrane (hFM) explants to study interactions between the tissues. We hypothesized that crosstalk between tissues at term promotes proinflammatory expression and uterine transitioning for parturition. Outputs of 40 cytokines and chemokines encompassing a variety of proinflammatory roles were measured; all but one increased significantly with co-culture. Eighteen of the 39 cytokines increased to a higher abundance than the sum of the effect of each tissue cultured separately. In addition, *COX2* and *IL6* but not *FP* and *OXTR* mRNA abundance significantly increased in both HMSMCs and hFM in response to co-culture. These data suggest that synergistic proinflammatory upregulation within intrauterine tissues is involved with uterine transitioning.

Summary Sentence

Intrauterine tissue crosstalk, measured in vitro by co-culture of human fetal membrane tissue explants with primary human myometrium smooth muscle cells, promotes proinflammatory amplification and uterine transition for labor.

Key words: myometrium, fetal membrane, inflammation, crosstalk, parturition, amplification.

Introduction

During the transition from pregnancy to parturition, intrauterine maternal and fetal tissues lie adjacent to one another communicating via paracrine processes and diffusion. These tissues, the maternal myometrium and decidua and the fetal amnion and chorion, are responsible for both the signals and the physiological responses to these signals that evolve into the process of labor. The primary mediators, prostaglandins, growth factors, steroid hormones, proinflammatory cytokines, and chemokines [1–6] are produced by these tissues and participate in the sequence of events we call the birth cascade. It culminates in an *activated* uterine myometrium that produces the coordinated and forceful contractions necessary for birth of the fetus(es). This derives from the altered expression of many genes, including uterine activation proteins (UAPs), responsible for transitioning the uterus from pregnancy to parturition [7–11]. We track three of these mRNA species and their proteins as proxies for the changing UAPs that comprise activation [12, 13]: cyclooxygenase (COX)-2, an inducible enzyme catalyzing a key intermediate step in the synthesis of prostaglandins [14], the prostaglandin (PG) $F_{2\alpha}$ receptor (FP) [13], and the oxytocin receptor (OXTR) [15]; these are receptors whose responsiveness to uterotonic contractile stimulators increases late in gestation.

Despite their juxtaposition within the uterine ultrastructure, no studies have examined the interactions between fetal and maternal gestational tissues in regard to developing this proinflammatory cascade so essential for labor to occur. We therefore designed a co-culture model to address this gap, incorporating primary human myometrium smooth muscle cells (HMSMCs) with full thickness human fetal membrane (hFM, amnion and chorion with adherent maternal *decidua vera*) explants in transwells to study paracrine crosstalk between the tissues at term. The transwell allows free passage of paracrine mediators between upper and lower compartments with consequent interactions between maternal and fetal tissues. A separate analysis of responses in each tissue, or combined analysis of inflammatory outputs in shared supernatant, can be made at the end of an experiment.

The intent of this project was to establish our model for studying communication between term HMSMC and hFM explants and then use it to examine the interaction between these tissues in terms of mediator expression and output and responsiveness to IL-1 β or PGF $_{2\alpha}$ treatment. We selected these agonists as our first test substances due to their central importance in the birth cascade [16–18]. We hypothesized that paracrine crosstalk between tissues at term promotes proinflammatory expression in the birth cascade. Our data demonstrated that co-incubation of these tissues led to a very large increase of inflammatory mediators in the medium and tissues, suggesting a significant contribution to the uterine “inflammatory load” [19] necessary for uterine transition to labor. We surmise that in vivo the myometrium and adjacent fetal membrane tissues interact via paracrine signaling to amplify proinflammatory signals to a level necessary to activate the uterus and trigger labor onset.

Materials and methods

Cell culture of primary human myometrium smooth muscle cells

We used a validated and published protocol to isolate and culture HMSMCs obtained from lower uterine segment myometrial biopsies collected from nonlaboring pregnant women undergoing elective cesarean section at term (>37 weeks’ gestational age) at the Royal Alexandra Hospital in Edmonton, Alberta [16, 20–22]. Ethics approval for these studies was received from the University of Alberta Research Ethics Board. After the removal of any visible blood vessels, myometrial tissue was dissected into small pieces and dissociated using the Hank balanced salt solution (HBSS, Gibco, Thermo Fisher Scientific, Waltham, MA) containing 2.0 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO), 200 ng/mL DNase I (Roche Diagnostics, Basel, Switzerland), and 1x antibiotic/antimycotic (100 U/mL penicillin G sodium, 100 μ g/mL streptomycin sulfate, and 0.25 μ g/mL amphotericin B, HyClone, GE Healthcare Life Sciences, Mississauga, Ontario, Canada). Following 20 min of digestion at 37°C with agitation, supernatant was discarded and replaced with 10 mL fresh dissociation medium. Incubation was then continued for 2.5 h before the remaining solution was filtered through a 100- μ m filter and centrifuged at 1250 $\times$ g for 5 min. The resulting cell pellet was washed twice with Dulbecco modified Eagle medium (DMEM) (HyClone, GE Healthcare Life Sciences) before resuspension. The cell solution was then plated in a 25 cm² flask, and maintained at 37°C and 5% CO₂ in DMEM containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific) and 1x antibiotic/antimycotic as described above. After 15-min incubation at 37°C, the HMSMC-containing solution was moved to a new flask. Upon reaching confluence, cells were passaged using 0.05% trypsin-EDTA (Gibco, Thermo Fisher Scientific). At the seventh passage, cells were plated into six-well plates at a density of 2 $\times$ 10⁵ cells/mL; once reaching approximately 80–90% confluency, they were starved in serum-free DMEM for 24 h before undergoing cell treatments. At time of stimulation, HMSMCs were treated alone in monoculture or combined with fetal membrane explants in co-culture. The tissue pairings were heterologous, as it was not possible to obtain both myometrial biopsies and placenta from each patient.

Extraction of human fetal membrane explants

Intact placentas were obtained with consent from pregnant women (>37 weeks’ gestational age) undergoing elective cesarean sections at term (term nonlaboring, TNL) or spontaneous vaginal deliveries (term laboring, TL). Following a protocol outlined by Yin et al. [23, 24], intact fetal membranes were removed from the placenta. Human FM tissue explants were excised with a 6-mm tissue punch and washed in HBSS. As demonstrated with histology in Yin et al. [23], and confirmed by us with H&E staining (unpublished), the hFM explants contain intact amnion, chorion, and some *decidua vera*. This methodology has been validated to maintain tissue structural and metabolic integrity for at least 72 h [25]. Human FM explants were plated with choriodecidua facing the myometrium to best represent

Figure 1. Methodology: co-culture of human myometrial smooth muscle cells (HSMCs) and human fetal membrane (hFM) explants.

tissue orientation in vivo in 12-well Netwell transwells (Corning Life Sciences, Tewksbury, MA) in DMEM Nutrient Mixture F12 (HyClone, GE Healthcare Life Sciences) containing 15% FBS and 1x antibiotic/antimycotic. Fortunato et al. found that cytokine release in response to the tissue punch returned to baseline after 48 h in culture [26]. Therefore, hFM explants underwent a 48 h acclimation period at 37°C and 5% CO₂ (with fresh medium every 24 h) before 6–24 h experimental treatments, well within the timeframe of tissue viability.

Treatment of co-cultured HMSMC and hFM explants

Transwells containing hFM were placed into 12-well plates containing HMSMC with a mesh filter allowing for free passage between compartments via a shared culture medium (Figure 1). Monocultures and co-cultures were incubated (i) in the absence of exogenous stimulation (serum-free DMEM F12), (ii) with 10 μM PGF_{2α} (Cayman Chemical Company, Ann Arbor, MI), or (iii) 5 ng/mL IL-1β (Millipore Sigma, Etobicoke, Ontario, Canada) for 6 h or 24 h periods. These concentrations of agonists produce an optimal response in HMSMC and hFM [16, 27, 28]. Treatments were cultured in duplicate, and supernatants were collected and pooled with supernatant from the matching sample's duplicate. Human FM tissues were removed, weighed, and snap frozen, and HMSMCs were washed twice with phosphate-buffered saline (PBS, Gibco, Thermo Fisher Scientific) before RNA extraction in Trizol reagent (Ambion, Thermo Fisher Scientific, Waltham, MA). All sample sizes indicated in the figure legends represent the number of replicates of tissues from different individuals.

Quantitative RT-PCR

Trizol reagent was used for RNA extraction following HMSMC or hFM treatments using the manufacturer-supplied protocol. Total RNA (500 ng) was reverse-transcribed using qScript cDNA Super-

Mix (Quanta Biosciences, Beverly, MA) using the manufacturer-supplied protocol, resulting in a total reaction volume of 20 μL. We used the resulting cDNA in real-time quantitative PCR reactions (25 ng/μL). Human primer sequences, PCR product sizes, and accession numbers for *IL6*, *COX2*, *FP*, *OXTR*, and *GAPDH* are listed in Table 1. To ensure amplification of template cDNA and not genomic DNA, all 3' and 5' primers were designed to span exon–exon boundaries, therefore impeding the primer binding to genomic DNA due to the intron presence. Each 20 μL reaction was run in duplicate and included 1 μL of cDNA, 10 μL of 2x PerfeCTa SYBR Green FastMix for iQ (Quanta Biosciences), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, and 8 μL water. With the use of iCycler technology (Bio-Rad Laboratories, Hercules, CA), two-step quantitative RT-PCR was completed using the following conditions: 10 min at 95°C, 45 cycles of 15 s at 95°C, and 1 min at the annealing temperature. The annealing temperature of all primers was 60°C, except for IL-6, which was 58°C. Following amplification, melt curve analysis was performed for each plate to ensure that amplification of nonspecific products did not occur. PCR products from the primers used in this study have been confirmed previously in our lab by gel electrophoresis followed by sequencing to verify amplification of the correct products. Standard curves for target genes and *GAPDH* were generated by serial dilutions of cDNA samples and analyzed with iCycler IQ software (Bio-Rad Laboratories). The amplification efficiency for each primer set was determined by converting the slope of the standard curve using the algorithm $E = 10^{-1/\text{slope}}$. The mean threshold cycle for each gene was calculated from duplicate reactions, and then corrected for the efficiency of the reaction and expressed relative to a control sample for each experiment. Target gene levels were then expressed relative to *GAPDH* levels using the following formula [29]:

$$\text{Ratio} = \frac{E_{\text{FP}}^{\Delta Ct(\text{Control-Sample})}}{E_{\text{GAPDH}}^{\Delta Ct(\text{Control-Sample})}}$$

Table 1. Primer sequences used in quantitative polymerase chain reaction (qPCR).

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Size of PCR product (bp)	Accession number
<i>GAPDH</i>	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTTC	226	BC025925
<i>COX2 (PGHS2)</i>	GCTGGAACATGGAATTACCCA	CTTTCTGTACTGCGGGTGGAA	98	NM000963
<i>IL6</i>	CAAAGATGGCTGAAAAAGATGGA	CTGTTCTGGAGGTACTCTAGGT	118	NM000600
<i>FP</i>	TCCTGTATTGTTGGAGCCCATTTTC	TCCATGTTGCCATTCCGAGAGCA	115	BC112965
	TGGTAC	AAAAG		
<i>OXTR</i>	ATGGACAAGAACGAGTGTGCGTGAG	GAGTGGCATTCTGGGTCATATGG	155	X64878

Multiplex assay

Supernatants were collected at the end of treatment and pooled with their experimental duplicate, and then immediately stored at -80°C . The Bio-Rad custom human cytokine multiplex kits were used as per the manufacturer's instructions with a Bio-Plex 200 suspension array system and corresponding Bio-Plex 200 software, version 6.1 (Bio-Rad Laboratories). Briefly, magnetic beads coated with antibodies targeting the cytokines of interest were added to each well of the 96-well plate and incubated with supernatant samples and cytokine standards provided by the manufacturer. The beads were incubated with biotinylated detection antibodies followed by streptavidin tagged with a phycoerythrin fluorescent reporter, which strongly binds to the biotinylated detection antibody. Beads were resuspended in assay buffer for quantification of analytes using the Luminescence-based reader in the Bio-Plex system. Each analyte's concentration was calculated by measuring the median fluorescence intensity (MFI) signal of the phycoerythrin fluorescent reporter per bead (at least 50 beads per analyte). MFI signals were compared to a standard curve generated by the manufacturer-supplied cytokine standards. Concentration outputs were then normalized for each tissue; for hFM, outputs were normalized to a calculated ratio of total explant weight (with the median explant weight per patient group set as 1), and for HMSMC, outputs were normalized to a ratio of cell density (2×10^5 cells/mL).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Data were $\log_{10}$ -transformed to conform to normality and analyzed by two-way analysis of variance (ANOVA) using Prism statistical software (GraphPad Prism, La Jolla, CA) to determine significance of the main effects: culture condition and treatment condition. In certain cases, a significant interaction was identified between main effects. In these circumstances, Bonferroni post hoc testing was performed, and the degree of significance was depicted by asterisks over the applicable bar graphs ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). In most figures, no significant interaction was identified between the main effects, and therefore only the statistical outcomes of the main effects are presented. For multiplex outputs, when the culture condition (monoculture vs co-culture) reached significance ($P < 0.05$), Bonferroni post hoc testing was used to indicate whether both monoculture groups were significantly different from the co-culture group.

Results

Co-culture increased *COX2* and *IL6* mRNA expression in both HMSMC and hFM

After 48 h acclimation in culture for each tissue, HMSMC and hFM were combined in culture for 6 or 24 h to assess whether communi-

cation between compartments was sufficient to induce a change in *IL6* abundance or UAP mRNA in each tissue. As demonstrated in Figure 2A, HMSMC co-cultured with hFM explants increased relative abundance of *COX2* and *IL6* in HMSMC compared to HMSMC cultured alone: 6 h of co-culture upregulated *COX2* 3.9-fold, and 24 h in co-culture increased *COX2* 18-fold from the respective monoculture controls. *IL6* was upregulated 12.5-fold in HMSMC after both 6 and 24 h of co-culture: 6 h in co-culture increased *OXTR* expression 2.7-fold, but after 24 h there was no difference in *OXTR* between culture conditions (Figure 2A). *FP* expression in HMSMC did not change with the addition of hFM.

Human FM explants also increased relative mRNA abundance of *COX2* and *IL6* when co-cultured with HMSMC compared to hFM alone: 6 and 24 h of co-culture resulted in a 4.4-fold and 5-fold increase in *COX2* mRNA, respectively (Figure 2B). Six hours of co-culture upregulated *IL6* 22.4-fold, and 24 h increased *IL6* 6.4-fold from respective monoculture controls. *FP* and *OXTR* did not change whether hFM were cultured alone or in co-culture.

Eighteen inflammatory mediators are synergistically upregulated in HMSMC/hFM co-cultures without exogenous stimulation

We determined the concentration of 40 cytokines and chemokines in cell culture supernatants of HMSMC monocultures, hFM monocultures, and HMSMC/hFM co-cultures. After 6 h, we measured a 0.2 ± 0.1 ng/mL IL-6 output by HMSMC alone and a 1.9 ± 1.1 ng/mL output by hFM alone (Table 2). Co-cultures released a synergistic 11.6 ± 4.4 ng/mL IL-6, $5.6\times$ higher than the sum of each tissue alone (2.1 ng/mL). After 24 h, the synergy was even more pronounced: 41.4 ± 15.4 ng/mL IL-6 was released by co-cultures, $15.3\times$ higher than the additive effect of both monocultures (2.7 ng/mL). In addition to IL-6, co-culture induced synergistic outputs of cytokines TNF α , IL-2, and a series of 15 chemokines: CCL2, CCL3, CCL13, CXCL5, CXCL6, CXCL2, CXCL13, CCL22, CCL20, CCL11, CX3CL1, CCL7, CXCL11, CCL8, and CCL15. For example, after 24 h in co-culture the CXCL6 output was 590.0 ± 131.3 pg/mL, $46.9\times$ higher than the sum of the outputs of each monoculture (12.6 pg/mL). Out of the 40 cytokines and chemokines measured, 39 out of 40 (all except CCL24) were significantly upregulated by co-culture. We define synergism as an output that is greater in the co-cultures than the sum of the constituent cultures. However, unlike the first 18, for the other 22 cytokines Bonferroni post hoc testing depicted that inflammatory outputs from HMSMC and hFM monocultures were not both found to be statistically different from co-culture outputs (Supplemental Table S1). IL-1 β was included in this group of 22, with an output of 0.3 ± 0.05 pg/mL from HMSMC, 14.1 ± 8.7 pg/mL from hFM, and 42.4 ± 12.7 pg/mL from co-culture.

(A) Myometrium

(B) Fetal membranes

Figure 2. Co-culture of HMSMC and fetal membrane explants (hFM) upregulates mRNA abundance of *IL6* (i) and *COX2* (ii) but not *FP* (iii) and *OXTR* (iv) in both tissues. HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions received fresh serum-free media for 6 or 24 h before collection, $n = 7$ different subjects. HMSMC mRNA is displayed in (A) and hFM mRNA in (B). Data are presented as relative change (x-fold) from 6 h monoculture values, mean $\pm$ SEM. Two-way ANOVA was performed on $\log_{10}$ -transformed data; significance of main effects is presented in the axes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. When the interaction between culture condition and time was significant, Bonferroni post hoc testing outcomes are indicated on the figure over the applicable bars.

Table 2. Co-cultures of HMSMC and fetal membrane explants (hFM) produce synergistic outputs of 18 inflammatory mediators, as compared to HMSMC and hFM monocultures.

Cytokine	Time in culture: 6 h (pg/mL)				Time in culture: 24 h (pg/mL)			
	HMSMC	hFM	Co-culture	Fold change co-culture effect	HMSMC	hFM	Co-culture	Fold change co-culture effect
IL-6	221.7 ± 118.8	1861.8 ± 1059.8	11554.4 ± 4375.8	5.6	177.9 ± 56.5	2529.5 ± 1186.6	41359.9 ± 15422.0	15.3
CCL2	445.3 ± 266.7	2605.3 ± 810.4	18684.8 ± 7329.3	6.1	358.0 ± 127.0	4894.7 ± 1605.4	29256.6 ± 7185.6	5.6
TNF α	4.6 ± 3.0	16.7 ± 8.5	206.4 ± 87.4	9.7	3.1 ± 1.1	33.7 ± 4.7	139.0 ± 19.6	3.8
IL-2	0.8 ± 0.4	3.1 ± 1.0	6.5 ± 1.4	1.7	1.1 ± 0.1	3.1 ± 1.0	9.6 ± 1.8	2.3
CCL3	1.8 ± 0.6	41.4 ± 12.4	397.9 ± 68.7	9.2	0.9 ± 0.2	88.6 ± 45.2	1169.2 ± 282.0	13.1
CCL13	1.2 ± 0.5	20.6 ± 9.5	175.8 ± 76.0	8.1	1.0 ± 0.2	36.6 ± 18.1	296.4 ± 58.2	7.9
CXCL5	253.2 ± 39.8	869.8 ± 247.0	3090.1 ± 692.7	2.8	235.1 ± 14.5	1171.1 ± 481.3	6375.6 ± 1682.5	4.5
CXCL6	3.6 ± 3.4	6.3 ± 2.8	87.9 ± 26.5	8.9	3.0 ± 1.3	9.6 ± 4.3	590.0 ± 131.3	46.9
CXCL2	2.7 ± 2.7	38.9 ± 18.7	394.2 ± 188.8	9.5	0.4 ± 0.4	71.7 ± 37.0	1368.3 ± 801.9	19.0
CXCL13	< 0.1	0.3 ± 0.2	1.4 ± 0.2	4.5	< 0.1	0.5 ± 0.2	2.2 ± 0.6	5.0
CCL22	< 0.5	7.6 ± 4.0	20.0 ± 7.3	2.6	< 0.5	12.0 ± 6.4	79.1 ± 0.4	6.6
CCL20	< 0.1	6.1 ± 4.3	17.0 ± 10.2	2.8	< 0.1	2.8 ± 1.5	96.9 ± 84.3	34.7
CCL11	4.5 ± 2.0	16.7 ± 4.5	41.8 ± 8.7	2.0	1.7 ± 0.8	17.9 ± 6.1	337.4 ± 80.0	17.3
CX3CL1	5.0 ± 1.6	52.1 ± 31.5	105.0 ± 43.0	1.8	5.6 ± 0.2	33.8 ± 13.3	318.9 ± 192.4	8.1
CCL7	16.7 ± 2.6	20.5 ± 4.2	602.4 ± 465.2	16.2	13.0 ± 1.8	23.4 ± 6.7	2423.3 ± 1670.7	66.5
CXCL11	< 0.05	0.7 ± 0.2	1.7 ± 0.3	2.4	< 0.05	0.4 ± 0.2	3.4 ± 1.5	9.0
CCL8	1.3 ± 0.8	75.7 ± 47.4	170.0 ± 34.0	2.2	0.6 ± 0.2	71.0 ± 35.5	1829.5 ± 1451.8	25.5
CCL15	3.2 ± 0.5	15.1 ± 4.1	33.5 ± 4.4	1.8	3.3 ± 0.2	19.1 ± 8.0	56.5 ± 2.9	2.5

HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 or 24 h. Cytokine protein outputs were measured via multiplex; data are presented as concentration output (pg/mL), mean $\pm$ SEM. $N = 6$ for IL-6 and CCL2, $n = 3$ for remaining 16 analytes. Fold change co-culture effect is calculated as the co-culture output divided by the sum of HMSMC and hFM monoculture outputs. All data have the same statistical outcome as measured by post hoc Bonferroni testing and two-way ANOVA: HMSMC alone and hFM alone are both significantly different from the co-culture output group, * $P < 0.05$.

PGF $_{2\alpha}$ upregulates COX2 and IL6 mRNA further in HMSMC in co-culture but not hFM

Since we published that myometrial cells increase expression of cytokines, chemokines, and UAPs following PGF $_{2\alpha}$ stimulation [16, 20], we selected PGF $_{2\alpha}$ as a stimulus for the co-culture model. When both culture conditions were stimulated with PGF $_{2\alpha}$, myometrial IL6 and COX2 abundance were increased further, whereas FP and OXTR were not. The interaction between PGF $_{2\alpha}$ treatment and culture condition was not, however, found to be statistically significant. HMSMC monocultures upregulated COX2 mRNA 4.9-fold in response to PGF $_{2\alpha}$ (Figure 3A). PGF $_{2\alpha}$ -stimulated co-cultures increased myometrial COX2 10.1-fold, 2.1 $\times$ higher than PGF $_{2\alpha}$ -treated monocultures and 2.9 $\times$ higher than co-cultures without exogenous PGF $_{2\alpha}$. HMSMC incubated with PGF $_{2\alpha}$ increased IL6 9.8-fold, whereas co-cultures stimulated with PGF $_{2\alpha}$ upregulated IL6 in HMSMC by 23.1-fold, 2.4 $\times$ more. FP mRNA abundance was downregulated by more than half in response to PGF $_{2\alpha}$ regardless of culture condition. OXTR did not respond to PGF $_{2\alpha}$ treatment in monoculture or co-culture.

Monocultures of hFM stimulated with PGF $_{2\alpha}$ did not change expression of COX2, IL6, or OXTR. Human FM cultured with HMSMC increased COX2 by 5.6-fold, and co-cultures stimulated with PGF $_{2\alpha}$ increased COX2 8.1-fold, 1.4 $\times$ more (Figure 3B). Co-cultures with and without PGF $_{2\alpha}$ increased IL6 25.3 and 24.7-fold, respectively. Similar to HMSMC, FP was downregulated in response to PGF $_{2\alpha}$ in both hFM monocultures and co-cultures, and fetal membrane OXTR did not change.

IL-1 β influences COX2 and IL6 mRNA in HMSMC in co-culture but not hFM

We, and others, have previously shown that IL-1 β upregulates a range of proinflammatory mediators and UAPs in the myometrium

[16, 30–32], which promoted our interest in testing IL-1 β in co-culture. IL-1 β significantly increased COX2, IL6, and FP in HMSMC, and COX2, IL6, FP, and OXTR in hFM. COX2 mRNA abundance increased 15.6-fold in response to IL-1 β in HMSMC monocultures (Figure 4A). Co-cultures stimulated with IL-1 β upregulated COX2 43.6-fold in HMSMC, 12.5 $\times$ higher than the effect of co-culture alone and 2.8 $\times$ higher than the effect of IL-1 β alone. IL6 in HMSMC increased 49.6-fold with IL-1 β stimulation in co-culture, 4.1 $\times$ higher than the effect of co-culture alone and 2.3 $\times$ higher than IL-1 β alone. The interaction between culture condition and IL-1 β stimulation was not statistically significant. FP mRNA abundance in HMSMC was not affected by culture condition, but increased 2.7-fold and 1.6-fold with IL-1 β in monoculture and co-culture, respectively. OXTR did not respond to IL-1 β treatment.

IL-1 β had similar effects on hFM with or without the presence of HMSMC in culture (Figure 4B). COX2 mRNA increased 8.3-fold in response to IL-1 β in hFM alone and 10.7-fold in response to IL-1 β in co-culture (Figure 4B). IL6 was upregulated 16.8-fold and 26.9-fold with IL-1 β in monoculture and in co-culture, respectively, but no more than the effect of co-culture alone on IL6. IL-1 β treatment induced a small but significant increase in fetal membrane FP and OXTR, while culture condition had no effect.

IL-1 β but not PGF $_{2\alpha}$ increases co-culture inflammatory protein outputs

Cytokine/chemokine outputs were also measured in supernatants of HMSMC, hFM, and co-cultures when stimulated with PGF $_{2\alpha}$ or IL-1 β for 6 h (Tables 3 and 4, Supplemental Tables S2 and S3). Outcomes of PGF $_{2\alpha}$ stimulation were dependent on tissue. HMSMC monocultures stimulated with PGF $_{2\alpha}$ led to either no change or increased cytokine and chemokine outputs (Table 3). PGF $_{2\alpha}$

(A) Myometrium

(B) Fetal membranes

Figure 3. PGF $_2\alpha$ stimulation of co-cultures induced further upregulation of *IL6* (i) and *COX2* (ii) but not *FP* (iii) and *OXTR* (iv) in HMSMC but not hFM. HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 10 μ M PGF $_2\alpha$, $n = 5$. HMSMC mRNA is displayed in (A) and hFM mRNA in (B). Data are presented as relative change (x-fold) from 6 h monoculture values, mean $\pm$ SEM. Two-way ANOVA was performed on $\log_{10}$ -transformed data; significance of main effects is presented in the axes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There was no significant interaction between main effects, so no post hoc testing was completed.

(A) Myometrium

(B) Fetal membranes

Figure 4. IL-1 β stimulation of co-cultures induced further upregulation of *IL6* (i) and *COX2* (ii) but not *FP* (iii) and *OXTR* (iv) in HMSMC but not hFM. HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both culture conditions were treated for 6 h with serum-free DMEM F12 or 5 ng/mL IL-1 β , $n = 5$. HMSMC mRNA is displayed in (A) and hFM mRNA in (B). Data are presented as relative change (x-fold) from 6 h monoculture values, mean $\pm$ SEM. Two-way ANOVA was performed on log₁₀-transformed data; significance of main effects is presented in the axes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There was no significant interaction between main effects, so no post hoc testing was completed.

Table 3. PGF_{2α} stimulation of HMSMC monocultures, hFM monocultures, and HMSMC/hFM co-cultures.

Cytokine	HMSMC (pg/mL)		hFM (pg/mL)		Co-culture (pg/mL)	
	Control	PGF _{2α}	Control	PGF _{2α}	Control	PGF _{2α}
IL-6	221.7 ± 118.8	449.6 ± 246.9	1861.8 ± 1059.8	604.4 ± 148.7	11554.4 ± 4375.8	10638.1 ± 3141.2
CXCL1	83.1 ± 36.1	80.5 ± 26.4	839.5 ± 189.8	755.3 ± 299.3	4714.7 ± 724.3	5059.9 ± 1029.6
IL-10	1.0 ± 0.8	1.8 ± 1.2	12.7 ± 3.7	9.8 ± 2.3	41.7 ± 12.8	36.3 ± 10.6
GM-CSF	145.3 ± 6.7	147.5 ± 7.4	154.3 ± 14.3	167.0 ± 17.3	214.7 ± 33.7	211.4 ± 18.2
CXCL5	253.2 ± 39.8	257.6 ± 37.2	869.8 ± 247.0	617.7 ± 59.3	3090.3 ± 692.7	2714.0 ± 535.0
CCL11	4.5 ± 2.0	5.0 ± 1.5	16.7 ± 4.5	11.3 ± 0.3	41.8 ± 8.7	38.1 ± 5.2
CXCL2	2.7 ± 2.7	5.7 ± 2.9	38.9 ± 18.7	17.7 ± 6.8	394.2 ± 188.8	303.8 ± 74.4
IFN γ	1.9 ± 1.2	0.8 ± 0.7	8.3 ± 3.5	6.0 ± 1.4	23.0 ± 5.6	23.7 ± 4.0
IL-2	0.8 ± 0.4	1.0 ± 0.3	3.1 ± 1.0	1.9 ± 0.3	6.5 ± 1.4	5.4 ± 0.8
IL-16	1.5 ± 1.5	2.8 ± 2.8	32.8 ± 7.6	18.4 ± 8.3	66.8 ± 16.8	69.3 ± 8.3
CCL20	< 0.1	0.3 ± 0.3	6.1 ± 4.3	1.8 ± 0.4	17.0 ± 10.2	12.6 ± 4.1

HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both co-cultures and monocultures were treated in duplicate for 6 h with (i) serum-free DMEM F12 or (ii) 10 μ M PGF_{2α}. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean $\pm$ SEM. N = 6 for IL-6, CXCL1, IL-10, n = 3 for remaining eight analytes. Two-way ANOVA was performed on log₁₀-transformed data. Culture condition had a statistically significant effect on concentration output for every cytokine, ***P* < 0.01, but the effect of PGF_{2α} was not statistically significant *P* > 0.05. Bonferroni post hoc testing was not completed as there was no significant interaction between culture condition and PGF_{2α} treatment.

Table 4. IL-1 β stimulation of HMSMC monocultures, hFM monocultures, and HMSMC/hFM co-cultures.

Cytokine	HMSMC (pg/mL)		hFM (pg/mL)		Co-culture (pg/mL)	
	Control	IL-1 β	Control	IL-1 β	Control	IL-1 β
IL-6	221.7 ± 118.8	1337.5 ± 609.5	1861.8 ± 1059.8	2904.6 ± 328.4	11554.4 ± 4375.8	36862.2 ± 17216.0
CXCL1	83.1 ± 36.1	444.5 ± 152.4	839.5 ± 189.8	1618.3 ± 409.7	4714.7 ± 724.3	10048.7 ± 2167.8
IL-10	1.0 ± 0.8	6.0 ± 2.2	12.7 ± 3.7	17.1 ± 2.3	41.7 ± 12.8	44.5 ± 10.9
GM-CSF	145.3 ± 6.7	153.2 ± 14.8	154.3 ± 14.3	198.6 ± 10.9	214.7 ± 33.7	300.7 ± 46.0
CCL11	4.5 ± 2.0	11.0 ± 3.7	16.7 ± 4.5	21.9 ± 2.3	41.8 ± 8.7	52.5 ± 5.3
CXCL2	2.7 ± 2.7	34.4 ± 31.1	38.9 ± 18.7	158.8 ± 74.3	394.2 ± 188.8	1078.1 ± 318.0
IFN γ	1.9 ± 1.2	6.9 ± 0.8	8.3 ± 3.5	15.6 ± 1.9	23.0 ± 5.6	39.4 ± 4.6
IL-2	0.8 ± 0.4	1.7 ± 0.7	3.1 ± 1.0	4.1 ± 0.3	6.5 ± 1.4	8.6 ± 0.8
IL-16	1.5 ± 1.5	11.0 ± 4.8	32.8 ± 7.6	56.5 ± 11.7	66.8 ± 16.8	106.1 ± 8.0
CCL20	< 0.1	2.9 ± 2.0	6.1 ± 4.3	8.8 ± 2.3	17.0 ± 10.2	44.8 ± 18.1

HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture). Both co-cultures and monocultures were treated for 6 h in duplicate with serum-free DMEM F12 or 5 ng/mL IL-1 β . Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean $\pm$ SEM. N = 6 for IL-6, CXCL1, IL-10, n = 3 for remaining eight analytes. Two-way ANOVA was performed on log₁₀-transformed data. Culture condition had a statistically significant effect on concentration output for every cytokine, ***P* < 0.01, and the statistical output representing the main effect of IL-1 β is displayed here: **P* < 0.05; CXCL1, GM-CSF, CXCL2, IFN γ , IL-2, CCL20, ***P* < 0.01; IL-10, CCL11, IL-16, ****P* < 0.001; IL-6. No post hoc testing was completed as there was no significant interaction between culture condition and IL-1 β treatment.

stimulation of hFM alone instead suppressed many cytokine outputs. Co-cultures seemingly combine the responses of the two individual tissues, resulting in cytokine outputs that were mostly unchanged in response to PGF_{2α}. Unlike PGF_{2α}, the majority of IL-1 β 's effects were stimulatory in all culture conditions. IL-1 β treatment was found to significantly affect IL-6, CXCL1, IL-10, GM-CSF, CCL11, CXCL2, IFN γ , IL-2, IL-16, and CCL20 (Table 4). IL-1 β stimulated a 6-fold increase in IL-6 output by HMSMC from 0.2 ± 0.1 to 1.3 ± 0.6 ng/mL, and a 1.6-fold increase in IL-6 output by hFM from 1.9 ± 1.1 to 2.90 ± 0.3 ng/mL. In co-culture, IL-1 β induced a 3.2-fold increase in IL-6 output, from 11.6 ± 4.4 to 36.9 ± 17.2 ng/mL. Similarly, CXCL1 outputs increased 5.3-fold by HMSMC with IL-1 β stimulation (0.08 ± 0.04 ng/mL to 0.4 ± 0.2), 1.9-fold by hFM (0.8 ± 0.2 to 1.62 ± 0.4 ng/mL), and 2.1-fold in co-culture (4.71 ± 0.7 to 10.0 ± 2.2 ng/mL). Many of the remaining 29 cytokines and chemokines demonstrated increasing trends with IL-1 β treatment (NS) (Supplemental Table S3).

Co-culture of HMSMC with term non-laboring hFM versus term laboring hFM

As both HMSMC and hFM were isolated from elective cesarean sections (TNL), we repeated the experiment using the same TNL HMSMC but instead paired the cells with hFM extracted from spontaneous TL births to investigate whether this change influenced the upregulation of *IL6* and *COX2*. HMSMC co-cultured with TNL hFM explants for 24 h increased *COX2* 25.1-fold from HMSMC monocultures, whereas HMSMC co-cultured with TL hFM explants increased *COX2* 23.5-fold (Figure 5A). HMSMC with TNL hFM increased *IL6* 25.4-fold and HMSMC with TL hFM increased *IL6* 48.3-fold from their respective HMSMC monoculture controls. *FP* and *OXT* myometrial mRNA expression did not change significantly whether co-cultured with TNL or TL hFM.

There were no basal differences in mRNA abundance of *IL6*, *COX2*, *FP*, or *OXT* in hFM explants cultured alone from TNL and TL placentas (Figure 5B). Fetal membrane *IL6* and *COX2* upregulation with co-culture was slightly less in TL hFM upregulating

(A) Myometrium

(B) Fetal membranes

Figure 5. *IL6* (i), *COX2* (ii), *FP* (iii), and *OXTR* (iv) mRNA in HMSMC monocultures, hFM monocultures, and HMSMC/hFM co-cultures using hFM extracted from term nonlaboring (TNL) placentas collected from elective caesarean sections or term laboring (TL) placentas collected from spontaneous deliveries. HMSMC (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) for 24 h. HMSMC mRNA is displayed in (A) and hFM mRNA in (B). Messenger RNA was quantified using real-time RT-PCR in each tissue; target gene mRNA levels were first normalized to housekeeping gene GAPDH and then expressed relative to a pooled sample for (B) and the monoculture control for (A). Data are presented as relative change (x-fold) from a pooled sample, mean $\pm$ SEM. N = 5, two-way ANOVA was performed on log₁₀-transformed data, *** $P < 0.001$.

COX2 and *IL6* 12.9-fold and 4.4-fold instead of 15.5-fold and 7.3-fold, respectively (NS). *FP* and *OXTR* in hFM did not change when co-cultured with HMSMC, whether hFM were extracted from TNL or TL placentas.

Discussion

To our knowledge, this is the first demonstration of co-culturing of human intrauterine tissues comprising maternal myometrium and fetal chorion and amnion. Our primary finding is that mRNA abundance and protein output of many cytokines and chemokines are considerably amplified when the tissues are co-cultured in comparison to when the tissues are separately cultured. The data strongly suggest that a tissue “cooperativity” exists within the intrauterine tissues that significantly increase the levels of proinflammatory mediators. We surmise that, if similar events occur in vivo, this large increase in mediators could reflect a critical event in the transition of the uterus from the state of pregnancy to the state of parturition.

Every birth, whether term or preterm, is an inflammatory event. Infection is associated with only 11% of all preterm births and about 2% of term births [33]; hence, in the majority of cases the inflammatory events of parturition occur without an infectious process [33, 34]. In most cases at term, it appears that damage-associated molecular patterns (DAMPs) released by the ageing placenta and fetal membranes and the increasingly physiologically stressed uterus [35] promote expression of proinflammatory cytokines, chemokines, prostaglandins, and their receptors via stimulation of Toll-like receptors [13, 14, 36]. These events lead to the physiological change, or *transition*, of the uterus from the state of pregnancy to the state of parturition or delivery characterized by increased myometrial contractile activity. Human microarray studies support this concept that the transition to labor is an inflammatory event and not limited to a single tissue [7–11]. There is a substantial change in gene expression at term labor in human myometrium, with 86% of the altered pathways involving inflammation, leukocyte movement, intercellular communication, and cytokine signaling [8, 9]. The activation of immune pathways dominates changes to the choriodecidual transcriptome for term labor resulting in 796 altered genes [7]. Although TL fetal membranes upregulate genes involved in leukocyte recruitment, no inflammatory gene expression changes occur with labor in peripheral blood [11]. These studies affirm that uterine transition for labor is not limited to the uterine musculature alone, but rather encompasses multiple gestational tissues and paracrine interactions in a localized intrauterine inflammatory response. As hFM contains enzymes for steroid hormone metabolism and produces growth factors and cytokines in vivo, the tissues are likely a determinant of myometrial transitioning and contractility. We paired term hFM explants with HMSMC, a primary cell model that our group has validated to model the myometrium in vitro [16, 20, 22].

Placing hFM explant transwells into culture wells containing HMSMC had an immediate and expansive proinflammatory effect without any exogenous stimulation; after only 6 h, the co-cultures had released synergistic outputs of 18 different cytokines and chemokines, and those outputs increased further in 24 h. The highest response levels ($>15\times$ higher than the sum of the monoculture outputs) were exhibited by CCL7, CXCL6, CCL20, CCL8, IL-8 (CXCL8), CXCL2, and IL-6. Three of these mediators, IL-8, IL-6, and CCL7, also had the highest total concentration outputs alongside CCL2, CXCL1, and CXCL5. Interestingly, microarray analysis of the myometrium also characterized IL-8 and CXCL6, which

share homology, as the most upregulated genes for labor [8]. Gene ontology biological process categories identified six genes that were upregulated in concert (by 5-fold to 6.5-fold) in term labor in fetal membranes [11]; these six were all CXC chemokines involved in neutrophil recruitment, including IL-8, CXCL6, CXCL1, CXCL2, CXCL3, and CXCL5, a very similar list to what we see upregulated by our co-culture model [11]. The upregulation of chemokines also dominated gene changes in myometrium and cervix [9], and CC chemokines that increase with labor (involved in migration of monocytes, eosinophils, basophils, and T lymphocytes) were also upregulated by co-culture [7, 9, 11]. Therefore, the most profound genetic changes measured in the intrauterine environment at term labor are upregulated in vitro by the crosstalk between term hFM and HMSMC, corroborating that hFM/HMSMC co-culture is a valid model for gestational transition to labor. The upregulated cytokines and chemokines may possibly interact for further inflammatory amplification; IL-8 synergizes with CC and CXC chemokines, including CCL2, to induce increased chemotaxis [37], and CXCL6 synergizes with CCL2 in gastrointestinal tumors to increase neutrophil migration by more than 10-fold [38].

Coinciding with the increase in chemotactic chemokines in reproductive tissues nearing parturition, peripheral maternal leukocytes upregulate markers of functional activation and extravasate into intrauterine tissues [39–43]. In rats, increased chemotactic ability of peripheral leukocytes coincides with increased expression of *Ccl2* [41], and CCL2 increases expression of integrins involved in facilitation of leukocyte migration [44]. In addition to the chemotactic roles of CXC and CC chemokines, IL-6 has been identified as a key regulator of T-cell populations in the pregnant decidua in preparation for parturition [45]. Mice with a null mutation in *Il6* had decreased numbers of CD8 + Foxp3 + regulatory T cells and an increase in IL9 + CD4 + Th9 cells; both populations recovered with exogenous recombinant IL-6 treatment [45]. Additionally, mice with a null mutation in *Il6* deliver 24 h later than wild-type mice due to a 24 h delay in the upregulation of UAPs [46], confirming a rate-limiting role for IL6 in the transition to labor. In HMSMC/hFM co-cultures, *IL6* mRNA was highly upregulated in each tissue as well as IL-6 protein release into cell culture supernatant. Uterine myocytes cultured in the presence of primary monocytes isolated from peripheral blood samples of term pregnant women also synergistically release IL-6 and IL-8 [47], demonstrating another circumstance of tissue co-culture resulting in a greater inflammatory effect. Corticotropin-releasing hormone-induced upregulation of myometrial UAPs CX-43, *OXTR*, and *FP* was also further upregulated with the presence of monocytes (THP-1) in co-culture [48]. In addition, vascular smooth muscle cells produce synergistic outputs of IL-6 (and CCL2) when co-cultured with monocytes, an effect that was suppressed entirely with a combination of inhibitors for STAT3, p38, and NF κ B, leading the authors to conclude that amplified production of IL-6 and CCL2 was mediated by IL-1, IL-6, and TNF α signaling [49]. IL-6 outputs of vascular smooth muscle cell/monocyte co-cultures were also completely inhibited by combinations of indomethacin (a COX inhibitor) and statins [50]. In HMSMC/hFM co-cultures, IL-1 β treatment had some stimulatory effects on cytokine outputs, but not to the same degree as the monoculture responses, possibly due to co-culture upregulation of inflammatory outputs in part involving IL-1-dependent signaling mechanisms. It is likely that inflammatory amplification induced by HMSMC/hFM co-cultures is not due to a single mediator but a cocktail of soluble factors. Further work is required to unravel the underlying molecular mechanisms using selective inhibitors and the analysis of intracellular pathways.

COX-2, the rate-limiting step in prostaglandin synthesis, is induced by inflammatory mediators [31, 51–53], and is responsible for changes in prostaglandin levels in the human fetal membranes amid the transition from pregnancy to labor [54–56]. PGF_{2α} stimulated COX2 in HMSMC monocultures and induced further COX2 mRNA upregulation in HMSMC when co-cultured with hFM, corroborating previous results depicting a positive feedback loop between PGF_{2α} and COX-2 [16, 57]. PGF_{2α} stimulation did not however increase COX2 mRNA or cytokine outputs in hFM explants, when cultured alone or with HMSMC. This is likely due to the high metabolic activity of the chorion. 15-Hydroxy prostaglandin dehydrogenase (PGDH), the initial enzymatic step in the predominant metabolic pathway of prostaglandins, is expressed more highly by chorion than decidua, myometrium, or amnion [58–60]. The chorion can be viewed as a barrier that sequesters hFM-produced prostaglandins to prevent their access to the myometrium and decidua due to its high PGDH activity [61].

In the birth cascade, upstream inflammatory amplification increases the inflammatory load to reach the inflammatory threshold required to trigger functional progesterone withdrawal [62] and activation of the uterus for labor. The ratio of progesterone receptor isoforms (PR-A/PR-B) and the abundance and stability of PR-A are increased by inflammatory mediators including PGF_{2α} and IL-1β [63–65], contributing to functional progesterone withdrawal and pro-labor transitioning. Contractile mediators and uterotonic receptors, especially FP and OXTR, are upregulated in the final step of the birth cascade [13, 66]; in rats, OXTR does not increase until just a few hours before delivery [67]. For this reason, it is not surprising that mRNA expression of FP and OXTR were unaltered by co-culture conditions, while upstream COX2, IL6, and many other cytokines and chemokines were amplified. Inflammatory mediators have differing effects on FP and OXTR expression, including some of the only negative feedback mechanisms observed in uterine transition. Like many G-protein coupled receptors, PGF_{2α} negatively regulates its receptor FP [16], and has been shown to either have no effect or upregulate OXTR [16]. IL-1β upregulates the PGF_{2α} receptor FP [16, 32], and has been shown to downregulate OXTR [68, 69]. Rat uterine explants from late term pregnancies stimulated with IL-6 upregulated OXTR, but explants from nonpregnant rats did not [70]. We predicted that physiological differences exist between TNL and TL tissues as well, and compared the co-culture responses of TNL HMSMC paired with TL hFM to TNL hFM pairs to explore the differences in tissue crosstalk before and after spontaneous labor. Co-cultures still significantly upregulated IL6 and COX2 from monoculture levels, but there was no significant difference in IL6, COX2, FP, or OXTR mRNA abundance in either tissue whether HMSMCs were cultured with TNL vs TL hFM. As the birth cascade culminates in an endpoint of a pro-contractile and activated myometrium for labor, comparing co-culture outputs of TNL and TL HMSMC instead of just hFM would be especially valuable, notably in reference to uterotonic receptors like FP and OXTR. Unfortunately, we were not able to collect spontaneous laboring myometrium samples at our institution. Future studies should include co-culture pairs extracted from before and after spontaneous labor onset as well as co-culture of hFM explants with intact myometrial tissue explants.

The model we have described has considerable potential, but is just getting established and needs additional controls and more detailed mechanistic study to reach its full potential. Now that we have characterized the reciprocal stimulatory relationship between HMSMC and hFM, future work should focus on identifying the

mechanism and regulation of induced factors, the directionality of interactions, and the specific contributions of each tissue. In addition, this model may have utility for studying the processes of autophagy, cell and tissue apoptosis and necrosis, and the production of DAMPs or PAMPs that stimulate the birth cascade and lead to term and preterm birth [33, 35]. Co-culture supernatants should be tested for the presence of exosomes, as exosome-mediated signaling from fetal membranes may contribute to the timing of human parturition. In the future, replication of this model with tissue pairs isolated from multiple time points during pregnancy (as well as preterm laboring and term laboring deliveries) would provide an important and more complete picture of the physiology of pregnancy and parturition. Finally, this combined primary human tissue method may be a valuable in vitro model for the testing of therapeutic candidates for preclinical trial characterization. Upstream inflammatory amplification is inherent to uterine transitioning and the birth cascade, so identifying therapeutic targets involved in this process with the potential to suppress both contraction and inflammatory amplification would be valuable in the pursuit of commercialization.

Supplementary data

Supplementary data are available at [BIOLRE](#) online.

Supplemental Table S1. Concentration outputs of remaining 22 cytokines and chemokines from HMSMC and fetal membrane explant (hFM) monocultures and co-cultures. HMSMCs (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 or 24 h. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean $\pm$ SEM. N = 6 for IL-8, CXCL1, and IL-10, n = 3 for other 19 cytokines. Fold change co-culture effect is calculated as the co-culture output divided by the sum of HMSMC and hFM monoculture outputs. Two-way ANOVA was performed on log₁₀-transformed data. The data presented here all show the same statistical significance as measured by post hoc Bonferroni testing: HMSMC monoculture is significantly different from co-culture, * $P < 0.05$, but hFM monocultures are not, $P > 0.05$.

Supplemental Table S2. Concentration outputs of 29 cytokines and chemokines in HMSMC and hFM monocultures and HMSMC/hFM co-cultures when stimulated with PGF_{2α}. HMSMCs (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) in duplicate for 6 h, treated for 6 h with serum-free DMEM F12 or 10 μ M PGF_{2α}. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean $\pm$ SEM. N = 6 for IL-8, CCL2, n = 3 for remaining cytokines. Two-way ANOVA was performed on log₁₀-transformed data. The data presented here all show the same statistical significance: culture condition had a statistically significant effect on concentration output for every cytokine, ** $P < 0.01$, but the effect of PGF_{2α} was not statistically significant $P > 0.05$. There was no significant interaction between culture condition and PGF_{2α} treatment.

Supplemental Table S3. Concentration outputs of 29 cytokines and chemokines in HMSMC and hFM monocultures and HMSMC/hFM co-cultures when stimulated with IL-1β. HMSMCs (2×10^5 cells/mL) and hFM (6 mm) were cultured alone (monoculture) or together (co-culture) for 6h in duplicate, treated for 6h with serum-free DMEM F12 or 5 ng/mL IL-1β. Cytokine/chemokine protein concentrations were measured via multiplex, data are presented as concentration output (pg/mL), mean $\pm$ SEM. N = 6 for IL-8,

CCL2, $n = 3$ for remaining cytokines. Two-way ANOVA statistical testing on $\log_{10}$ -transformed data. The data presented here all show the same statistical significance: culture condition had a statistically significant effect on concentration output for every cytokine, $**P < 0.01$, but the effect of IL-1 β was not statistically significant $P > 0.05$. There was no significant interaction between culture condition and IL-1 β treatment.

Acknowledgments

The authors would like to thank the pregnant women who volunteered to participate in this study, as well as Donna Dawson, BScN, for her assistance in the recruitment and collection of myometrial biopsies and placentas at the Royal Alexandra Hospital in Edmonton, Alberta.

Conflict of Interest: The authors have declared that no conflict of interest exists.

References

- Chibbar R, Miller FD, Mitchell BF. Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. *J Clin Invest* 1993; **91**(1):185–192.
- Mitchell B, Cruickshank B, McLean D, Challis J. Local modulation of progesterone production in human fetal membranes. *J Clin Endocrinol Metab* 1982; **55**(6):1237–1239.
- Mitchell BF, Challis JR, Lukash L. Progesterone synthesis by human amnion chorion, and decidua at term. *Am J Obstet Gynecol* 1987; **157**(2):349–353.
- Romano WM, Lukash LA, Challis JR, Mitchell BF. Substrate utilization for estrogen synthesis by human fetal membranes and decidua. *Am J Obstet Gynecol* 1986; **155**(6):1170–1175.
- Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res* 2000; **20**(3):173–177.
- Pollard JK, Thai D, Mitchell MD. Evidence for a common mechanism of action of interleukin-1 beta, tumor necrosis factor-alpha, and epidermal growth factor on prostaglandin production in human chorion cells. *Am J Reprod Immunol* 1993; **30**(2-3):146–153.
- Stephen GL, Lui S, Hamilton SA, Tower CL, Harris LK, Stevens A, Jones RL. Transcriptomic profiling of human choriondecidua during term labor: inflammation as a key driver of labor. *Am J Reprod Immunol* 2015; **73**(1):36–55.
- Mittal P, Romero R, Tarca AL, Gonzalez J, Draghici S, Xu Y, Dong Z, Nhan-Chang CL, Chaiworapongsa T, Lye S, Kusanovic JP, Lipovich L et al. Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term. *J Perinat Med* 2010; **38**(6):617–643.
- Bollapragada S, Bollopragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. *Am J Obstet Gynecol* 2009; **200**:104.e101–111.
- Migale R, MacIntyre DA, Cacciatore S, Lee YS, Hagberg H, Herbert BR, Johnson MR, Peebles D, Waddington SN, Bennett PR. Modeling hormonal and inflammatory contributions to preterm and term labor using uterine temporal transcriptomics. *BMC Med* 2016; **14**(1):86.
- Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Mazor M, Romero R. Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. *Am J Obstet Gynecol* 2006; **195**(2):394–405.e12.
- Arthur P, Taggart MJ, Zielnik B, Wong S, Mitchell BF. Relationship between gene expression and function of uterotonic systems in the rat during gestation, uterine activation and both term and preterm labour. *J Physiol* 2008; **586**(24):6063–6076.
- Cook JL, Zaragoza DB, Sung DH, Olson DM. Expression of myometrial activation and stimulation genes in a mouse model of preterm labor: myometrial activation, stimulation, and preterm labor. *Endocrinology* 2000; **141**(5):1718–1728.
- Cook JL, Shallow MC, Zaragoza DB, Anderson KI, Olson DM. Mouse placental prostaglandins are associated with uterine activation and the timing of birth. *Biol Reprod* 2003; **68**(2):579–587.
- Fuchs AR, Fields MJ, Freidman S, Shemesh M, Ivell R. Oxytocin and the timing of parturition. Influence of oxytocin receptor gene expression, oxytocin secretion, and oxytocin-induced prostaglandin F2 alpha and E2 release. *Adv Exp Med Biol* 1995; **395**:405–420.
- Xu C, Long A, Fang X, Wood SL, Slater DM, Ni X, Olson DM. Effects of PGF2 α on the expression of uterine activation proteins in pregnant human myometrial cells from upper and lower segment. *J Clin Endocrinol Metab* 2013; **98**(7):2975–2983.
- Goupil E, Tassy D, Bourguet C, Quiniou C, Wisheart V, Pétrin D, Le Guillou C, Devost D, Zingg HH, Bouvier M, Saragovi HU, Chemtob S et al. A novel biased allosteric compound inhibitor of parturition selectively impedes the prostaglandin F2alpha-mediated Rho/ROCK signaling pathway. *J Biol Chem* 2010; **285**(33):25624–25636.
- Nadeau-Vallée M, Quiniou C, Palacios J, Hou X, Erfani A, Madaan A, Sanchez M, Leimert K, Boudreault A, Duhamel F, Rivera JC, Zhu T et al. Novel noncompetitive IL-1 receptor-biased ligand prevents infection- and inflammation-induced preterm birth. *J Immunol* 2015; **195**(7):3402–3415.
- Talati AN, Hackney DN, Mesiano S. Pathophysiology of preterm labor with intact membranes. *Semin Perinatol* 2017; **41**(7):420–426.
- Xu C, Liu W, You X, Leimert K, Popowycz K, Fang X, Wood SL, Slater DM, Sun Q, Gu H, Olson DM, Ni X. PGF2 α modulates the output of chemokines and pro-inflammatory cytokines in myometrial cells from term pregnant women through divergent signaling pathways. *Mol Hum Reprod* 2015; **21**(7):603–614.
- Aguilar HN, Tracey CN, Zielnik B, Mitchell BF. Rho-kinase mediates diphosphorylation of myosin regulatory light chain in cultured uterine, but not vascular smooth muscle cells. *J Cell Mol Med* 2012; **16**(12):2978–2989.
- Mosher AA, Rainey KJ, Bolstad SS, Lye SJ, Mitchell BF, Olson DM, Wood SL, Slater DM. Development and validation of primary human myometrial cell culture models to study pregnancy and labour. *BMC Pregnancy Childbirth* 2013; **13**(Suppl 1):S7.
- Yin N, Wang H, Zhang H, Ge H, Tan B, Yuan Y, Luo X, Olson DM, Baker PN, Qi H. IL-27 induces a pro-inflammatory response in human fetal membranes mediating preterm birth. *Int Immunopharmacol* 2017; **50**:361–369.
- Yin N, Takeda J, Fang X, Qi H, Olson D. An *in vitro* model for studying the regulation of uterine chemotactic factor. In: *Society for Reproductive Investigation Annual Meeting*, vol. 22. San Francisco, CA, USA: Reproductive Sciences; 2015:141–142A.
- Mitchell BF, Powell WA. Progesterone production by human fetal membranes: an *in vitro* incubation system for studying hormone production and metabolism. *Am J Obstet Gynecol* 1984; **148**(3):303–309.
- Fortunato SJ, Menon R, Swan KF, Lyden TW. I. Organ culture of amnio-chorionic membrane *in vitro*. *Am J Reprod Immunol* 1994; **32**(3):184–187.
- Parkington HC, Tonta MA, Davies NK, Brennecke SP, Coleman HA. Hyperpolarization and slowing of the rate of contraction in human uterus in pregnancy by prostaglandins E2 and f2alpha: involvement of the Na⁺ pump. *J Physiol* 1999; **514**(1):229–243.
- Li R, Ackerman WE, Summerfield TL, Yu L, Gulati P, Zhang J, Huang K, Romero R, Kniss DA. Inflammatory gene regulatory networks in amnion cells following cytokine stimulation: translational systems approach to modeling human parturition. *PLoS One* 2011; **6**(6):e20560.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**(9):45e–45.
- Belt AR, Baldassare JJ, Molnár M, Romero R, Hertelendy F. The nuclear transcription factor NF-kappaB mediates interleukin-1beta-induced expression of cyclooxygenase-2 in human myometrial cells. *Am J Obstet Gynecol* 1999; **181**(2):359–366.

31. Chevillard G, Derjuga A, Devost D, Zingg HH, Blank V. Identification of interleukin-1beta regulated genes in uterine smooth muscle cells. *Reproduction* 2007; 134(6):811–822.
32. Zaragoza DB, Wilson RR, Mitchell BF, Olson DM. The interleukin 1beta-induced expression of human prostaglandin F2alpha receptor messenger RNA in human myometrial-derived ULTR cells requires the transcription factor, NFkappaB. *Biol Reprod* 2006; 75(5):697–704.
33. Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Yeo L. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol* 2014; 72(5):458–474.
34. Elliott CL, Loudon JA, Brown N, Slater DM, Bennett PR, Sullivan MH. IL-1beta and IL-8 in human fetal membranes: changes with gestational age, labor, and culture conditions. *Am J Reprod Immunol* 2001; 46(4):260–267.
35. Nadeau-Vallée M, Obari D, Palacios J, Brien M, Duval C, Chemtob S, Girard S. Sterile inflammation and pregnancy complications: a review. *Reproduction* 2016; 152(6):R277–R292.
36. Ishiguro T, Takeda J, Fang X, Bronson H, Olson DM. Interleukin (IL)-1 in rat parturition: IL-1 receptors 1 and 2 and accessory proteins abundance in pregnant rat uterus at term - regulation by progesterone. *Physiol Rep* 2016; 4(14):pii: e12866.
37. Gouwy M, Struyf S, Catusse J, Proost P, Van Damme J. Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. *J Leukocyte Biol* 2004; 76(1):185–194.
38. Gijsbers K, Gouwy M, Struyf S, Wuyts A, Proost P, Opendakker G, Penninx F, Ectors N, Geboes K, Van Damme J. GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors. *Exp Cell Res* 2005; 303(2):331–342.
39. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, Greer IA, Norman JE. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Hum Reprod* 1999; 14(1):229–236.
40. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, Norman JE. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol Hum Reprod* 2003; 9(1):41–45.
41. Gomez-Lopez N, Tanaka S, Zaem Z, Metz GA, Olson DM. Maternal circulating leukocytes display early chemotactic responsiveness during late gestation. *BMC Pregnancy Childbirth* 2013; 13(Suppl 1):S8.
42. Yuan M, Jordan F, McInnes IB, Harnett MM, Norman JE. Leukocytes are primed in peripheral blood for activation during term and preterm labour. *Mol Hum Reprod* 2009; 15(11):713–724.
43. Takeda J, Fang X, Olson DM. Pregnant human peripheral leukocyte migration during several late pregnancy clinical conditions: a cross-sectional observational study. *BMC Pregnancy Childbirth* 2017; 17(1):16.
44. Vaddi K, Newton RC. Regulation of monocyte integrin expression by beta-family chemokines. *J Immunol* 1994; 153:4721–4732.
45. Gomez-Lopez N, Olson DM, Robertson SA. Interleukin-6 controls uterine Th9 cells and CD8 + T regulatory cells to accelerate parturition in mice. *Immunol Cell Biol* 2016; 94(1):79–89.
46. Robertson SA, Christiaens I, Dorian CL, Zaragoza DB, Care AS, Banks AM, Olson DM. Interleukin-6 is an essential determinant of on-time parturition in the mouse. *Endocrinology* 2010; 151(8):3996–4006.
47. Rajagopal SP, Hutchinson JL, Dorward DA, Rossi AG, Norman JE. Crosstalk between monocytes and myometrial smooth muscle in culture generates synergistic pro-inflammatory cytokine production and enhances myocyte contraction, with effects opposed by progesterone. *Mol Hum Reprod* 2015; 21(8):672–686.
48. You X, Liu J, Xu C, Liu W, Zhu X, Li Y, Sun Q, Gu H, Ni X. Corticotropin-releasing hormone (CRH) promotes inflammation in human pregnant myometrium: the evidence of CRH initiating parturition? *J Clin Endocrinol Metab* 2014; 99(2):E199–E208.
49. Chen L, Frister A, Wang S, Ludwig A, Behr H, Pippig S, Li B, Simm A, Hofmann B, Pilowski C, Koch S, Buerke M et al. Interaction of vascular smooth muscle cells and monocytes by soluble factors synergistically enhances IL-6 and MCP-1 production. *Am J Physiol-Heart Circ Physiol* 2009; 296(4):H987–H996.
50. Loppnow H, Zhang L, Buerke M, Lautenschläger M, Chen L, Frister A, Schlitt A, Luther T, Song N, Hofmann B, Rose-John S, Silber RE et al. Statins potentially reduce the cytokine-mediated IL-6 release in SMC/MNC cocultures. *J Cell Mol Med* 2011; 15(4):994–1004.
51. Bartlett SR, Sawdy R, Mann GE. Induction of cyclooxygenase-2 expression in human myometrial smooth muscle cells by interleukin-1beta: involvement of p38 mitogen-activated protein kinase. *J Physiol* 1999; 520(2):399–406.
52. Erkinheimo TL, Saukkonen K, Narko K, Jalkanen J, Ylikorkala O, Ristimäki A. Expression of cyclooxygenase-2 and prostanoic receptors by human myometrium. *J Clin Endocrinol Metab* 2000; 85:3468–3475.
53. Rauk PN, Chiao JP. Interleukin-1 stimulates human uterine prostaglandin production through induction of cyclooxygenase-2 expression. *Am J Reprod Immunol* 2000; 43:152–159.
54. Hirst JJ, Teixeira FJ, Zakar T, Olson DM. Prostaglandin endoperoxide-H synthase-1 and -2 messenger ribonucleic acid levels in human amnion with spontaneous labor onset. *J Clin Endocrinol Metab* 1995; 80:517–523.
55. Mijovic JE, Zakar T, Nairn TK, Olson DM. Prostaglandin-endoperoxide H synthase-2 expression and activity increases with term labor in human chorion. *Am J Physiol* 1997; 272:E832–840.
56. Grigsby PL, Poore KR, Hirst JJ, Jenkin G. Inhibition of premature labor in sheep by a combined treatment of nimesulide, a prostaglandin synthase type 2 inhibitor, and atosiban, an oxytocin receptor antagonist. *Am J Obstet Gynecol* 2000; 183:649–657.
57. Guo CM, Kasaraneni N, Sun K, Myatt L. Cross talk between PKC and CREB in the induction of COX-2 by PGF2α in human amnion fibroblasts. *Endocrinology* 2012; 153:4938–4945.
58. Mitchell BF, Rogers K, Wong S. The dynamics of prostaglandin metabolism in human fetal membranes and decidua around the time of parturition. *J Clin Endocrinol Metab* 1993; 77:759–764.
59. Cheung PY, Walton JC, Tai HH, Riley SC, Challis JR. Immunocytochemical distribution and localization of 15-hydroxyprostaglandin dehydrogenase in human fetal membranes, decidua, and placenta. *Am J Obstet Gynecol* 1990; 163:1445–1449.
60. Keirse MJ, Turnbull AC. Metabolism of prostaglandins within the pregnant uterus. *BJOG* 1975; 82:887–893.
61. van Meir CA, Matthews SG, Keirse MJ, Ramirez MM, Bocking A, Challis JR. 15-hydroxyprostaglandin dehydrogenase: implications in preterm labor with and without ascending infection. *J Clin Endocrinol Metab* 1997; 82:969–976.
62. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. *J Clin Endocrinol Metab* 2002; 87:2924–2930.
63. Madsen G, Zakar T, Ku CY, Sanborn BM, Smith R, Mesiano S. Prostaglandins differentially modulate progesterone receptor-A and -B expression in human myometrial cells: evidence for prostaglandin-induced functional progesterone withdrawal. *J Clin Endocrinol Metab* 2004; 89:1010–1013.
64. Amini P, Michniuk D, Kuo K, Yi L, Skomorovska-Prokvolit Y, Peters GA, Tan H, Wang J, Malesud CJ, Mesiano S. Human parturition involves phosphorylation of progesterone receptor-A at serine-345 in myometrial cells. *Endocrinology* 2016; 157:4434–4445.
65. Peters GA, Yi L, Skomorovska-Prokvolit Y, Patel B, Amini P, Tan H, Mesiano S. Inflammatory stimuli increase progesterone receptor-A stability and transrepressive activity in myometrial cells. *Endocrinology* 2017; 158:158–169.
66. Brodt-Eppley J, Myatt L. Prostaglandin receptors in lower segment myometrium during gestation and labor. *Obstet Gynecol* 1999; 93:89–93.

67. Fang X, Wong S, Mitchell BF. Relationships among sex steroids, oxytocin, and their receptors in the rat uterus during late gestation and at parturition. *Endocrinology* 1996; **137**:3213–3219.
68. Schmid B, Wong S, Mitchell BF. Transcriptional regulation of oxytocin receptor by interleukin-1beta and interleukin-6. *Endocrinology* 2001; **142**:1380–1385.
69. Rauk PN, Friebe-Hoffmann U. Interleukin-1 beta down-regulates the oxytocin receptor in cultured uterine smooth muscle cells. *Am J Reprod Immunol* 2000; **43**:85–91.
70. Fang X, Wong S, Mitchell BF. Effects of LPS and IL-6 on oxytocin receptor in non-pregnant and pregnant rat uterus. *Am J Reprod Immunol* 2000; **44**:65–72.