Supplementary Information

Materials and Methods

Primary endometrial epithelial and stromal cell isolation and purification

Primary endometrial epithelial and stromal cell biopsies were dissociated and cells purified as described by Osteen and coworkers[38] with some modifications. Biopsy specimens were collected using a pipelle and immediately placed in 10 mL of ice-cold 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 (Gibco) supplemented with 1% penicillin/streptomycin (Gibco) and 10% *v*/*v* dextran/charcoal treated fetal bovine serum (Atlanta Biologicals) (DMEM/F12/FBS) and transported on ice. The tissue was washed twice by centrifugation at 400xg in DMEM/F12 and dissected into small pieces (1-2 mm^3). Tissue pieces were incubated for 1h at 37°C in DMEM/F12 supplemented with 0.5% collagenase Type IV (Worthington Biochemical Corporation LS004188), 0.02% DNAase (Sigma-Aldrich DN25) and 2% chicken serum (Sigma-Aldrich C5405) and vortexed every 30 min. As a result of this first dissociation, stromal cells are present as single cells while epithelial cells remain aggregated. The cell suspension was then filtered twice through a 70 µm membrane filter (Falcon 352350) in order to separate the stromal cells from the epithelial cell clumps. The latter were collected on the surface of the filters and washed by centrifugation with sterile PBS. The stromal fraction flow through was further purified by two rounds of differential sedimentation at unit gravity as follows. Stromal cell were washed twice by centrifugation at 400xg for 5min and resuspended in 10 mL of DMEM/F12/FBS. After the second centrifugation, stromal cells were resuspended in 2 mL DMEM/F12/FBS and layered slowly drop by drop over 10 mL of DMEM/F12/FBS in a sterile 15 mL conical tube, and the tube was incubated in an upright position at 37°C for 30 min. The top 8 mL of sedimentation medium were collected, centrifuged at 400xg for 5min and resuspended in 2 mL DMEM/F12/FBS, and the sedimentation step was repeated for an additional 30 minutes. The top 8 mL of sedimentation medium was collected, filtered through a 40 µm membrane filter (Falcon 352340), and stromal cells were counted and seeded at 2-5 x 10^4 cells/cm^2 in DMEM/F12/FBS. The medium was replaced the following day and every 2-3 days thereafter until further use.

Further dissociation of the epithelial aggregates was achieved by incubation with an enzyme mixture supplemented with 0.5% collagenase, 0.1% hyaluronidase (Sigma-Aldrich H3506), 0.1% pronase (Sigma-Aldrich P5147), 0.02% DNAase and 2% chicken serum in PBS for 15-20 min at 37°C in a water bath. The cell preparation was filtered through a 70 µm membrane filter in order to get rid of the remaining stromal cells that were released during this digestion and epithelial cell clumps were collected again and further digested with fresh enzyme mixture for 30-45 min at 37°C. This final digestion resulted in small epithelial cell clumps of 50-100 cells that were purified by differential sedimentation at unit gravity as follows. Cells were centrifuged and resuspended in 2 mL DMEM/F12/FBS. Cells were layered slowly over 10 mL of DMEM/F12/FBS in a sterile 15 mL conical tube and the tube was incubated in an upright position at 37°C for 30 min. The top 10 mL of sedimentation medium were discarded and the sedimentation step was repeated with the bottom 2 mL. The top 10 mL of sedimentation medium were discarded again and 5 mL of DMEM/F12/FBS were added. Final purification was achieved by selective attachment of any remaining stromal cells to plastic substrate as follows. Cells were seeded in a 75 cm^2 tissue culture flask and incubated at 37°C, 95% air, 5% CO2 for 1h. Non-attached epithelial cells were collected and cultured in DMEM/F12/FBS at a density of 3 X 10^5 cells/mL for 2-3 days. After this plating period, medium was changed every other day for 2-3 days until further use.

ELISAs for IGFBP-1 and prolactin

Briefly, plates were coated with capture antibody (23 µL) overnight at 4 °C at concentration of 4.0 µg/mL and 0.8 µg/mL for IGFBP-1 and prolactin, respectively. Wells were rinsed three times with 0.1% Tween-20 in PBS wash buffer then blocked in 46 µL 5% Tween-20 in PBS for IGFBP-1 and 1% BSA in PBS for prolactin for 1 h at RT. Wells were again rinsed three times in wash buffer, then 23 µL of sample or standard was added and incubated overnight at 4 °C. Plates were rinsed three times with wash buffer then incubated with 23 µL detection antibody at 400 ng/mL in blocking buffer for prolactin and in blocking buffer plus 2% goat serum for IGFBP-1 for 2 h at RT. Wells were rinsed three times in wash buffer, then incubated with 23 µL streptavidin-

HRP (1:200) in blocking buffer for 20 minutes at RT. HRP substrate solution (23 μL) was added until color developed followed by addition of 11.5 μL of 2N H_2SO_4 to stop the reaction. Absorbance values were immediately read at 450 nm and 540 nm. Absorbance values 450 nm were corrected by subtracting absorbance values at 540 nm to account for discrepancies in the plate. Eight-point standard curves plus blanks (apical medium) were included for quantification. For each protein, 4-parameter logistic curves were fit to the standards, including blanks (Cardillo G. (2012) Four parameters logistic regression - There and back again, http://www.mathworks.com/matlabcentral/fileexchange/38122). Curves were used to calculate concentrations for each sample replicate.

Tables and Figures

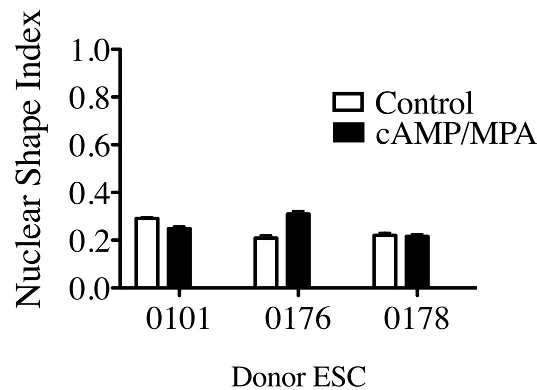
Supplementary Table S1: Primary endometrial biopsy donor data.

Donor	Age	Cycle Day	Diagnoses
0101	31	5	unilateral ovarian cyst
0176	27	7	left salpingitis
0178	20	4	bilateral ovarian cyst
0188	41	12	adenomyosis
0190	49	7	leiomyoma
0191	33	14	bilateral, paratubal cyst

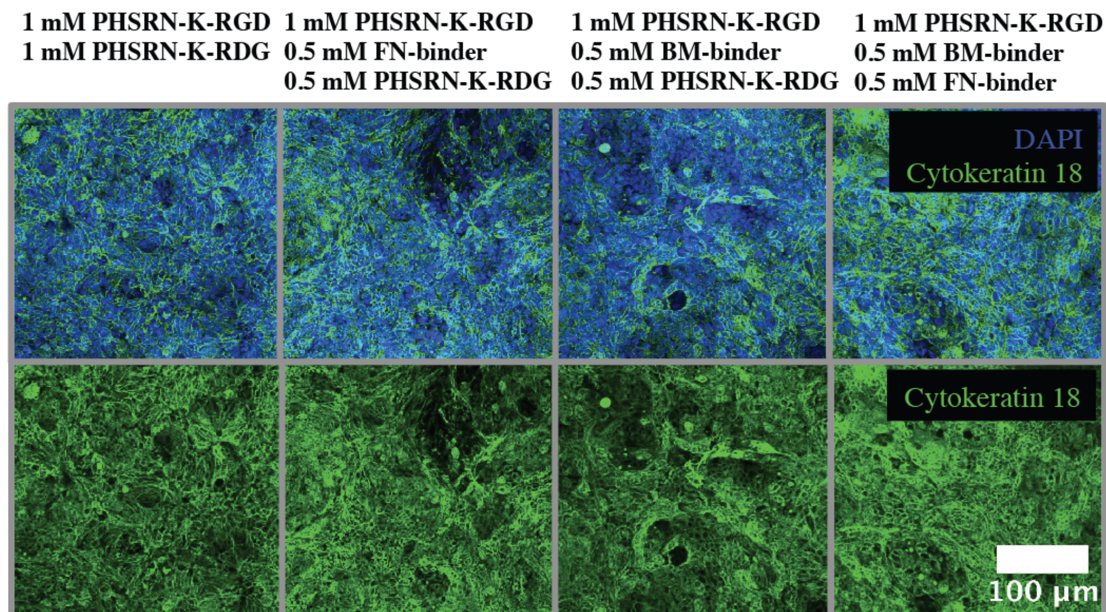
Supplementary Table S2: Cytokine concentrations (pg/mL) in apical medium collected on days 7 and 15 from monoculture (Ishikawa (75K cells) or tHESC (50K cells)) or co-culture gels (125K cells), with medium conditioned for 48 hours. In gels made with the cell lines, Ishikawa cells are the primary secretors of most cytokines, with the exception of eotaxin, IL-8, and MCP-1. $^{\&}$Below the assay detection limit (blank + 2 standard deviations).

	Day 7 (pg/mL)			Day 15 (pg/mL)		
	tHESC	Ishikawa	Co-culture	tHESC	Ishikawa	Co-culture
Eotaxin	136.6	77.5	80.6	76.7	42.2	79.9
FGF Basic (FGF-2)	6.7	0.0	0.0	0.0	0.0	0.0
G-CSF (CSF-3)	12.0	0.1	0.0	0.0	0.0	0.0
GM-CSF (CSF-2)	1.5$^{\&}$	2.5$^{\&}$	0.0	0.0	0.4$^{\&}$	0.0
IFN-γ	330.1	284.5	290.5	2.1$^{\&}$	268.8	259.6
IL-10	10.8	97.0	91.5	0.0	81.3	76.5
IL-12p70	29.3	577.0	566.6	0.0	579.9	514.2
IL-13	6.8	23.2	23.1	0.0	21.3	21.2
IL-15	0.9$^{\&}$	0.1$^{\&}$	0.1$^{\&}$	0.5$^{\&}$	0.0$^{\&}$	0.1$^{\&}$
IL-17A	78.4	22.9	5.8	0.0	3.5	12.3
IL-1β	1.5	0.0	0.0	0.0	0.0	0.0
IL-1ra	79.5	49.9	51.0	0.8$^{\&}$	43.0	45.1
IL-2	4.1	2.0$^{\&}$	1.6$^{\&}$	0.8$^{\&}$	1.3$^{\&}$	1.8$^{\&}$
IL-4	3.8	1.9	2.1	0.0	1.6	1.6
IL-5	14.9	0.0	0.0	0.0	0.0	0.0
IL-6	7.7	4.0	6.1	3.0$^{\&}$	4.1	10.7
IL-7	72.9	123.9	127.7	0.0	100.6	128.5
IL-8	19.1	8.8	28.4	16.6	11.3	26.9
IL-9	18.8	27.9	24.0	0.0	22.9	22.2
IP-10	259.5	253.1	290.2	17.3	211.6	270.8
MCP-1 (CCL2)	165.2	1.2	6.9	189.6	1.0	28.4
MIP-1α (CCL3)	1.6	0.2	0.1	0.0	0.0	0.3
MIP-1β (CCL4)	12.0	14.1	14.0	0.0	12.7	12.0
PDGF-BB	87.7	377.4	253.2	0.0	341.2	68.5
RANTES (CCL5)	9.2	0.0	0.0	0.0	0.0	0.0
TNF-α	81.5	0.0	0.0	0.0	0.0	0.0
VEGF	15.2	10231.4	13081.2	0.0	11545.8	7344.5

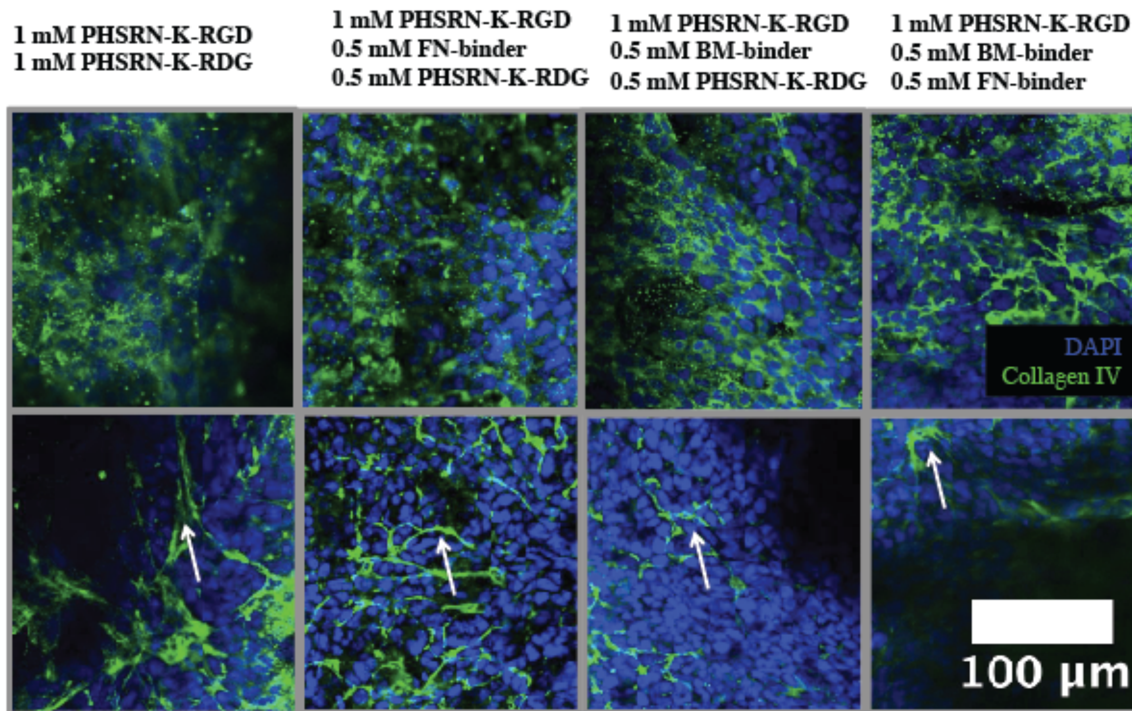
Supplementary Figure S1: Nuclear shape index of decidualized cells in hydrogels does not distinguish between control and cAMP/MPA stimulated primary endometrial stromal cells cultured in 3D. DAPI-stained nuclei were imaged and quantified using ImageJ software to quantify the nuclear shape index (NSI), where NSI = 4π x area / perimeter^2 for at least 50 nuclei counted per each ROI (n=3). A shape index of 1 indicates a perfect circle, whereas a shape index of 0 indicates a straight line.



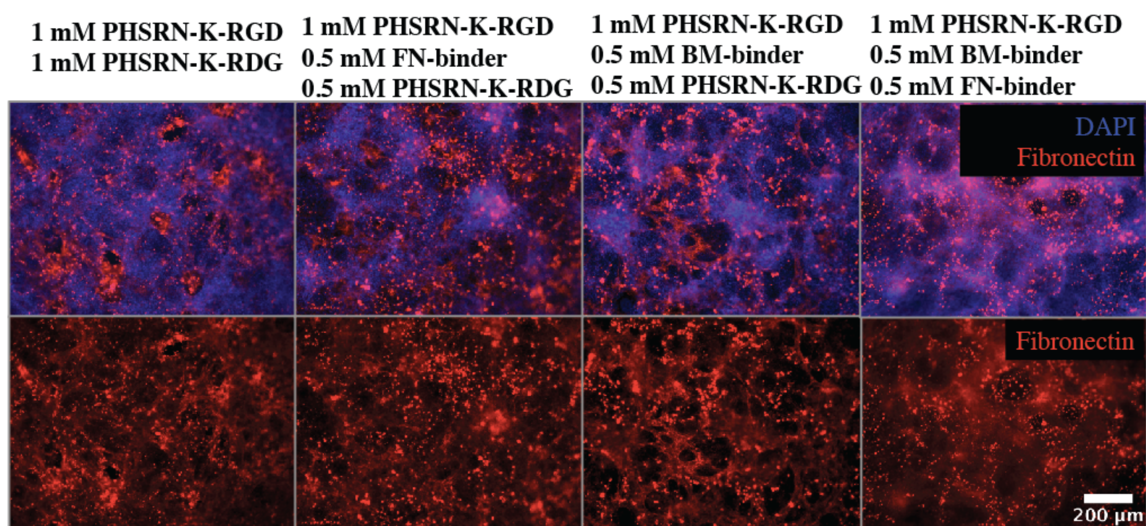
Supplementary Figure S2: Immunofluorescence images of epithelial sheet phenotype +/- matrix-binding peptides after 7 days in co-culture; confocal (maximum Z projection, 30 μm) (cytokeratin 18 (green) and DAPI-stained nuclei (blue) merged in top panel, cytokeratin 18 only in bottom panel) shows a layer of epithelial cells in all conditions.



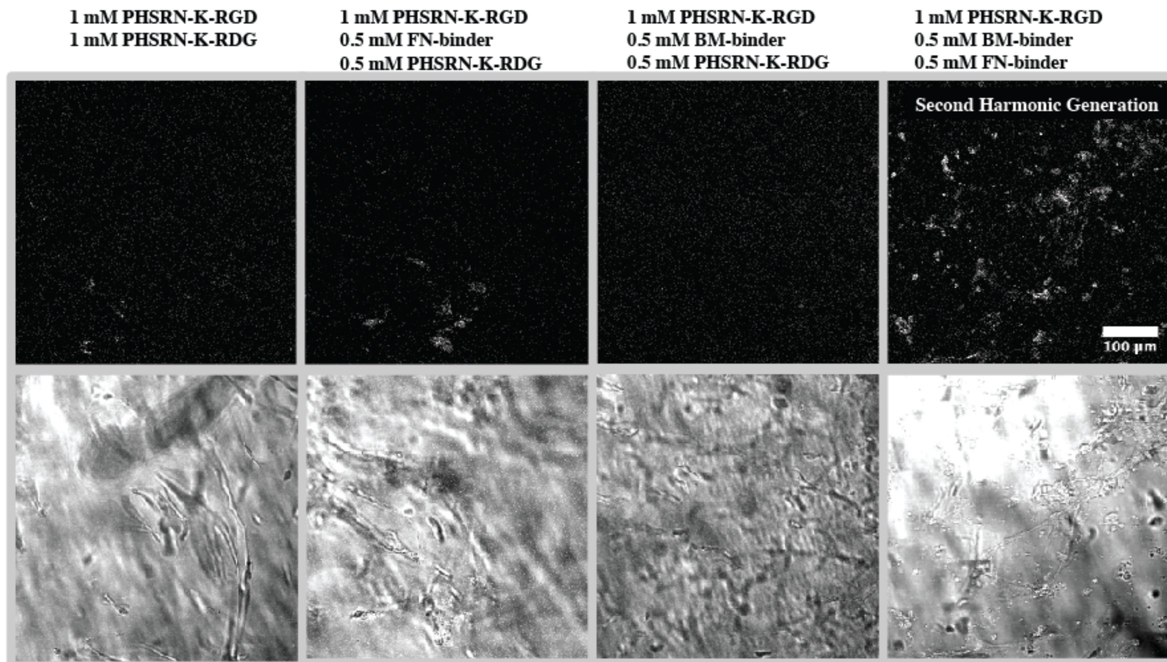
Supplementary Figure S3: Immunofluorescence images of collagen IV (green) staining at the top of the epithelial layer (top images) and 15 μm below (bottom images) for conditions +/- matrix-binding peptides after 7 days in co-culture and DAPI-stained nuclei (blue)). Single Z-planes show that the epithelial layer has organized, cell-associated collagen IV when BM-binder is present (top images) and fibroblasts that have migrated to the base of the Ishikawa layer show collagen IV staining in all conditions (arrows).



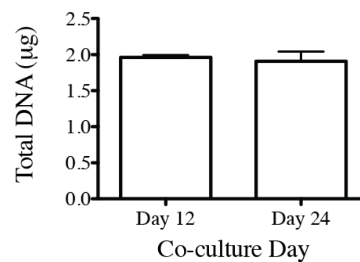
Supplementary Figure S4: Immunofluorescence images on day 16 of co-culture showing fibronectin (red) accumulation of encapsulated stromal cells (DAPI-stained nuclei (blue) and fibronectin merged in top panel, fibronectin only in bottom panel) occurs in all conditions with *PHSRN-K-RGD*.



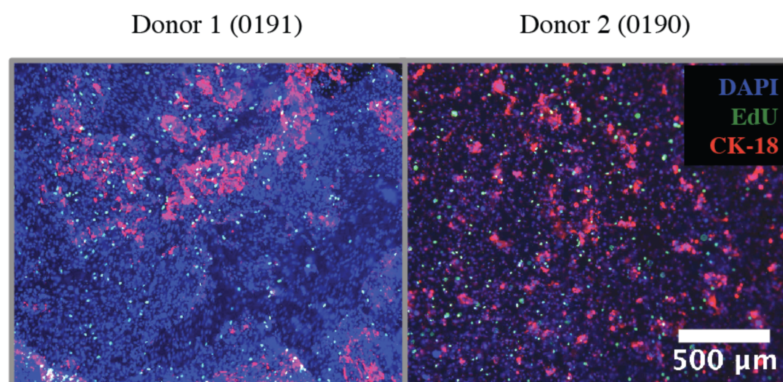
Supplementary Figure S5: Second harmonic imaging shows fibrillar collagen matrix deposition (white) in conditions with *FN-binder* after 14 days of culture, with significant enhancement in the presence of both *FN-binder* and *BM-binder* but no apparent deposition with *BM-binder* alone (top panel is a maximum Z projection of 250 µm for unfixed hydrogels imaged while hydrated on a coverslip) and the bottom panel shows a phase image (from a single z plane to illustrate the presence of stromal cells in all conditions) merged with the second harmonic signal.



Supplementary Figure S6: Total DNA in co-culture does not change between co-culture days 12 and 24 where n=2 and 6 hydrogels analyzed on days 12 and 24, respectively.



Supplementary Figure S7: Epithelial layer for two primary donor co-cultures (50K cells seeded) show incomplete monolayer formation after 15 days in culture where epithelial cells are visualized by cytokeratin-18 (red), DAPI stained nuclei (blue), and EdU positive nuclei (green).



Supplementary Table S3: Cytokine concentrations (pg/mL) in apical medium collected at 1 week for cell lines (Ishikawa and tHESC) and 1 and 2 weeks for primary endometrial cells. [&]Concentration below the assay detection limit (blank + 2 standard deviations). ^Concentrations outside the range of the standard curve are reported as > highest standard concentration; no statistics were calculated. NaN represents cytokines not quantified because of low bead counts. Total DNA from hydrogels lysed on day 15 for cell line co-cultures was 1944±470 ng and primary cell hydrogels were 559±292 ng, 1255±110 ng, and 1212±82 ng for donors 0188, 0190 and 0191, respectively.

	Week 1, 48 hr medium (pg/mL)				Week 2, 72 hr medium (pg/mL)		
	Cell Lines (n=8)	0188 (n=12)	0190 (n=4)	0191 (n=10)	0188 (n=6)	0190 (n=1)	0191 (n=5)
Eotaxin	274.1	1445.8	5291.7	10971.0	15636.3	10496.2	6449.7
FGF Basic (FGF-2)	0.0	12.7	0.0	21.7	19.5	NaN	3.0
G-CSF (CSF-3)	8.5	2130.5	1338.1	2268.2	1492.2	1523.5	1542.6
GM-CSF (CSF-2)	0.0	87.3	98.7	157.7	63.1	36.7	66.4
IFN-γ	101.1	303.8	215.0	360.7	244.0	301.8	235.4
IL-10	78.7	134.8	0.0	74.6	161.9	0.0	32.4
IL-12p70	239.4	38.6	0.0	100.2	49.7	0.0	8.3
IL-13	8.7	0.0	0.0	4.1[&]	0.0	0.0	0.0
IL-15	0.1[&]	21.5	19.5	34.2	24.3	56.0	34.7
IL-17A	95.8	172.4	112.2	233.0	126.6	152.7	119.3
IL-1β	0.0	11.9	4.5	20.2	5.0	7.3	5.8
IL-1ra	0.0	156.7	109.1	266.2	135.5	197.2	131.3
IL-2	0.0	1.6	0.0	5.2	0.1	0.0	1.7
IL-4	7.6	19.8	14.8	23.0	15.9	15.1	15.3
IL-5	0.0	0.5	0.0	1.5	0.0	0.0	0.0
IL-6	58.5	2131.1	1787.8	1977.8	2131.1	2131.1	1563.2
IL-7	79.1	71.4	58.1	85.0	97.0	88.1	75.1
IL-8	107.4	2690.7	2316.9	2523.1	2690.7	2690.7	2152.6
IL-9	9.0	9.7	5.1	30.9	9.3	0.0	7.4
IP-10^	245.2	21311.0	21311.0	21311.0	21311.0	21311.0	21311.0
MCP-1 (CCL2)	29.9	601.1	396.6	486.1	603.2	859.4	431.4
MIP-1α (CCL3)	0.7	17.0	6.4	60.8	9.2	6.3	16.2
MIP-1β (CCL4)	14.0	74.7	120.4	130.3	45.2	109.8	71.1
PDGF-BB	1666.6	380.5	151.5	1129.1	214.4	147.3	187.4
RANTES (CCL5)	0.7	96.3	253.0	90.7	453.3	1180.2	244.4
TNF-α	38.6[&]	945.4	928.9	2123.6	831.8	548.8	578.9
VEGF	7584.1	1391.1	27.9	4035.9	2164.0	46.3	617.7

Supplementary Table S4: Cytokine concentrations (pg/mL) in apical medium collected on day 15 from cAMP+MPA-stimulated and unstimulated primary endometrial cells and conditioned for 72 hours. Reported p-values were Benjamini-Hochberg adjusted for each subject. Four cytokines were significantly different for subject 0188 (eotaxin, IL-10, IL-15, and MIP-1β), of which two (IL-10 and IL-15) behaved consistently across all three subjects. [&]Below the assay detection limit (blank + 2 standard deviations). ^Concentrations outside the range of the standard curve are reported as > highest standard concentration; no statistics were calculated. NaN also represents p-values not calculable due to sample size (subject 0190) or comparisons for which both means and standard deviations were 0. Total DNA values were from 1-2 hydrogels per condition.

	Subject 0188			Subject 0190			Subject 0191		
Cytokine (pg/mL)	Unstim (n=6)	Stim (n=6)	BH-adj. P-value	Unstim (n=2)	Stim (n=1)	BH-adj. P-value	Unstim (n=5)	Stim (n=5)	BH-adj. P-value
Eotaxin	15636.3	1892.2	**0.003**	10496.2	13960.1	NaN	6449.7	4976.1	0.685
FGF-b (FGF-2)	19.5	11.8	0.722	NaN	NaN	NaN	3.0	0.8	0.685
G-CSF (CSF-3)	1492.2	2237.5	0.317	1523.5	1024.7	NaN	1542.6	1965.0	0.685
GM-CSF (CSF-2)	63.1	72.6	0.684	36.7	0.0	NaN	66.4	69.1	0.897
IFN-γ	244.0	291.1	0.317	301.8	239.8	NaN	235.4	270.3	0.685
IL-10	161.9	482.0	**0.006**	0.0	206.9	NaN	32.4	310.5	0.224
IL-12p70	49.7	134.8	0.129	0.0	0.0	NaN	8.3	5.5	0.710
IL-13	0.0	5.8[&]	0.143	0.0	0.0	NaN	0.0	0.0	NaN
IL-15	24.3	115.6	**0.009**	56.0	70.8	NaN	34.7	54.7	0.685
IL-17A	126.6	146.6	0.426	152.7	119.6	NaN	119.3	143.6	0.685
IL-1 β	5.0	7.2	0.302	7.3	3.9	NaN	5.8	7.2	0.685
IL-1ra	135.5	198.4	0.256	197.2	121.7	NaN	131.3	228.2	0.685
IL-2	0.1	3.7	0.317	0.0	0.0	NaN	1.7	0.0	0.685
IL-4	15.9	17.8	0.446	15.1	12.8	NaN	15.3	18.4	0.685
IL-5	0.0	1.5	0.446	0.0	0.0	NaN	0.0	1.4	0.685
IL-6	2131.1	1953.5	0.446	2131.1	2131.1	NaN	1563.2	1885.8	0.685
IL-7	97.0	90.4	0.722	88.1	112.1	NaN	75.1	88.0	0.685
IL-8	2690.7	2466.5	0.446	2690.7	2479.8	NaN	2152.6	2584.0	0.685
IL-9	9.3	16.3	0.325	0.0	0.0	NaN	7.4	1.7	0.685
IP-10	> 21311	> 21311	NaN	> 21311	> 21311	NaN	> 21311	> 21311	NaN
MCP-1 (CCL2)	603.2	612.7	0.921	859.4	582.9	NaN	431.4	356.3	0.685
MIP-1 α (CCL3)	9.2	15.7	0.256	6.3	3.0	NaN	16.2	31.4	0.685
MIP-1 β (CCL4)	45.2	118.5	**0.026**	109.8	52.3	NaN	71.1	90.1	0.685
PDGF-BB	214.4	297.7	0.302	147.3	106.6	NaN	187.4	246.4	0.685
RANTES (CCL5)	453.3	475.3	0.921	1180.2	575.0	NaN	244.4	410.4	0.685
TNF-α	831.8	968.8	0.446	548.8	NaN	NaN	578.9	664.5	0.685
VEGF	2164.0	6032.0	0.134	46.3	81.5	NaN	617.7	691.0	0.816

Supplementary Figure S8: Secondary immunostaining control for extracellular matrix where the merged image shows DAPI stained nuclei (blue), Dk anti-Ms IgG (red), and Dk anti-Rb IgG (green).