Thrombin Alters Human Endometrial Stromal Cell Differentiation During Decidualization

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Abstract

Vaginal bleeding and subchorionic hematomas are associated with increased risk of both early and late pregnancy loss. Thrombin generation may play a pivotal role in the development of these complications. To determine the effects of thrombin on human endometrial stromal cells (hESCs), cells were treated with thrombin at baseline or during decidualization with cyclic adenosine monophosphate (cAMP)+medroxyprogesterone acetate (MPA). Next-generation RNA sequencing revealed that markers of decidualization (IGF-1, IGFBP-1, and prolactin [PRL]) were induced after the initiation of decidualization, whereas thrombin suppressed insulin-like growth factor (*IGF-1*), Insulin-like growth factor binding protein (*IGFBP-1*), and *PRL* gene expression at baseline and during decidualization. These effects were mediated through protease activated receptor (PAR)-1- and PAR-1-independent pathways. Thrombin decreased the secretion of a key marker of decidualization (PRL), altered the morphological transformation of decidualizing hESCs, and activated genes involved in matrix degradation and proinflammatory chemokines (*Interleukin-8* and *Interleukin-6*). Genes encoding factors important for matrix stability (*Coll α1*, *LOX*) were suppressed. We suggest that intrauterine bleeding and generation of thrombin accentuates leukocyte extravasation and endometrial inflammation, impairs decidualization, and endometrial support of early pregnancy.

Keywords

endometrium, menstrual bleeding, prolactin, inflammation, RNAseq, protease

Introduction

Endometrial stromal cells undergo an interesting transformation of morphology and cell function during a process termed decidualization. This cellular change is induced by a mixture of ovarian hormones (mainly progesterone) prior to pregnancy in preparation for implantation.¹ During this process, fibroblast-like mesenchymal cells undergo epithelioid transformation and² acquisition of specialized cell functions including the secretion of a multiplicity of new gene products, including IGF-1, IGFBP-1, prolactin, and a myriad of cytokines,³ all of which are suggested to play a role in embryo implantation and maintenance of pregnancy.⁴

Failure of embryo implantation has been identified as a major cause of pregnancy loss particularly with embryo transfer after in vitro fertilization. Recent meta-analysis of 5 studies demonstrated lower clinical pregnancy rates after challenging embryo transfers suggesting that trauma to the endometrial lining during transfer may result in implantation failure. Even when pregnancy is established, vaginal bleeding and subchorionic hematomas are associated with increased risk of adverse pregnancy outcomes,^{5,6} and it has been suggested that thrombin generation plays a pivotal role in the development of these

complications.⁷⁻⁹ Prothrombin (coagulation factor II) is proteolytically cleaved to form thrombin in the coagulation cascade, which ultimately results in the reduction of blood loss.

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Thrombin in turn acts as a serine protease that converts soluble fibrinogen into insoluble strands of fibrin as well as catalyzing many other coagulation-related reactions.¹⁰ Decidual hemorrhage and thrombin formation have been implicated as a mediator of decidual extracellular matrix (ECM) breakdown,¹¹⁻¹³ a potential cause of failed implantation. Decidual hemorrhage and thrombin formation have been implicated as a mediator of decidual ECM breakdown.¹¹⁻¹³ Thrombin has been demonstrated to increase generation of matrix metalloproteinases (MMP)-1, MMP-3, and MMP-9 in decidual cells.¹²⁻¹⁴ Nonetheless, little is known regarding the effects of nondecidualized endometrial stromal cells before implantation and whether thrombin alters the decidualization process. Here, we tested the hypothesis that thrombin not only acts to alter the cellular microenvironment but also affects endometrial stromal cells during, and after, decidualization that may bring about adverse pregnancy outcomes. The objectives were to assess whether thrombin alters decidualization, activates MMPs, and disrupts the expression of genes involved in matrix homeostasis of the endometrium.

Materials and Methods

Reagents

DMEM/F12 (Ham, 11320) was from Invitrogen. The antibiotic-antimycotic solution (A5955), charcoal-stripped fetal bovine serum (F6765), and thrombin from human plasma (T6884) were purchased from Sigma-Aldrich. Recombinant thrombin was from R&D Systems. One NIH unit/mL of human plasma thrombin activity was equivalent to 0.324 $\mu\text{g/mL}$ (ie, 9 nM). PAR-1-activating peptide (TFLLRN, 61530) was purchased from Anaspec (Fremont, California). PAR-1 selective receptor antagonist, SCH79797 was obtained from Tocris (Ellisville, Missouri).

Isolation and Culture of human endometrial stromal cells

The study was approved by the institutional review board of University of Texas Southwestern Medical Center. After written informed consent, endometrial tissues were obtained from 16 patients in the proliferative phase, aged 30 to 43 years with regular menstrual cycles and no evidence of endometriosis or submucosal fibroids, who underwent hysterectomies for benign reasons (eg, pelvic relaxation or leiomyomas). A portion of each endometrial specimen was confirmed to be histologically normal. Human endometrial stromal cells were purified by the standard enzyme digestion method as described previously.¹⁵ Briefly, tissue scrapings were cut into 1- to 2-mm pieces and incubated with collagenase (200 IU/mL) and DNase (150 $\mu\text{g/mL}$) in Hank's-balanced solution with stirring for 1 hour at 37°C. The dispersed endometrial epithelial and stromal cells were separated by filtration through a 70 μm sterile cell strainer (BD Biosciences, San Jose, CA), which allowed the stromal cells to pass through while intact glands were retained. After washing 3 times, the cells were transferred to culture flasks at a

density of 1×10^6 cells/mL in phenol red-free D-MEM/F12 medium supplemented with 10% dextran-coated charcoal stripped (DCS)- fetal calf serum (FCS) and 1% antibiotic/antimycotic. The culture medium was replaced 30 minutes after plating to reduce epithelial cell contamination. Thereafter, culture medium was replaced every 3 days. Cultures were incubated at 37°C under a humidified atmosphere of 5% CO₂ in air. Cells isolated from each individual patient were used for 1 experiment at a time. Each experiment was performed in triplicate and repeated at least 3 times in different cell preparations.

Treatments

Nearly confluent cells were passaged 1 time before they were used for the experiments. All of the experiments were performed in subconfluent cells with the medium replaced by serum-free and phenol red-free DMEM/F-12 with added 1% antibiotic/antimycotic. Stromal cell decidualization was achieved using medroxyprogesterone acetate (MPA, 0.1 $\mu\text{mol/L}$) and 8-bromoadenosine 3', 5'-cyclic monophosphate (cAMP; 0.5 mmol/L; Sigma-Aldrich, St. Louis, MO). Ethanol was used as a vehicle control.

RNA Sequencing and Analysis

Total RNA samples were processed with the TruSeq Stranded Total RNA LT Sample Prep Kit from Illumina. Total RNA was isolated from 2 biological replicates of human endometrial stromal cells (hESCs) treated with vehicle, cAMP+MPA (dec), thrombin (2 U/mL), or cAMP+MPA+thrombin. Cyclic AMP+MPA treatment was for 72 hours with thrombin added in the final 24 hours. Samples were processed for whole-genome polyadenylated RNA sequencing (polyA+ RNA-Seq). Total RNA samples were subjected to the enrichment of polyA⁺ RNA using DynabeadsOligo(dT)25 (Invitrogen). Thereafter, strand-specific RNA-Seq libraries were prepared as described previously¹⁶ and sequenced using an IlluminaHi-Seq 2500 using SBS v3 reagents for 100 bp paired-end reads. Reads were trimmed to remove adaptor sequences and low-quality bases using fastq-mcf (v1.1.2-806, <http://code.google.com/p/ea-utils>), followed by mapping to human genome (hg19) using Tophat (v2.0.10, doi:10.1038/nprot.2012.016) with igene annotations (<https://ccb.jhu.edu/software/tophat/igenomes.shtml>). Duplicate reads were marked but not removed. FeatureCounts (doi:10.103/bioinformatics/btt656) was used for read counting and edgeR (doi: 10.1093/nar/gks042) for expression abundance estimation and differential expression test. Pathways enriched in differentially expressed genes were identified by ingenuity pathway analysis. RNA-sequencing data sets generated for this study are available at <https://www.ncbi.nlm.nih.gov/geo/>.

Quantitative Real-Time polymerase chain reaction

Quantitative RT-PCR was used to determine the relative levels of gene expression as described previously.^{17,18} Primer

Table 1. Genes in Which Thrombin Altered Signaling in hESCs.

Gene	Involved in Pathways
<i>IGFBP1</i>	Estrogen receptor signaling; IGF-I signaling; PXR/RXR activation; VDR/RXR activation
<i>IGFBP5</i>	Myometrial relaxation and contraction pathways; regulation of IGF transport
<i>CCL8</i>	Agranulocyte adhesion and diapedesis; granulocyte adhesion and diapedesis
<i>PRL</i>	Dopamine receptor signaling; glucocorticoid receptor signaling; prolactin signal in role of JAK2 in hormone-like cytokine signaling
<i>LEFTY2</i>	Human embryonic stem cell pluripotency
<i>MMP3</i>	Breakdown of extracellular matrix; tissue remodeling; embryonic development
<i>MMP10</i>	Agranulocyte adhesion and diapedesis; axonal guidance signaling; Bladder cancer signaling; colorectal cancer metastasis signaling; granulocyte adhesion and diapedesis; HIF1 α signaling; inhibition of matrix metalloproteases; leukocyte extravasation signaling
<i>ICAM1</i>	leukocyte adhesion; cell proliferation; differentiation; motility; trafficking; apoptosis; tissue architecture
<i>IL6</i>	inflammation; maturation of B cells; acute phase response
<i>IL8</i>	neutrophil chemotaxis; angiogenesis
<i>CCL2</i>	monocyte chemotaxis; inflammation
<i>MGP</i>	Inhibition of ectopic tissue calcification
<i>C3</i>	Inflammation; classical and alternative complement activation
<i>SLCO2A1</i>	Inflammation; uptake and clearance of prostaglandins
<i>TNC</i>	Extracellular matrix homeostasis
<i>WFDC1</i>	Protease inhibitor; inhibition of cell proliferation
<i>HPSE</i>	Extracellular matrix homeostasis; cell migration
<i>KIAA1199</i>	Encodes cell migration inducing hyaluronan binding protein
<i>ADRA2A</i>	Adrenergic neurotransmission
<i>COL28A1</i>	Extracellular matrix homeostasis; coagulation

Abbreviation: hESCs, human endometrial stromal cells.

sequences for amplifications are shown in Supplemental Table 1. Taqman probe with FAM dye label was used for COX2, and SYBR green was used for other genes. Gene expression was normalized to the mean of 2 housekeeping genes, *GAPDH* and *h36B4*, both of which were invariant in stromal cells under the treatment conditions used in this study.

Analysis of Secreted PRL

Media supernatant of treated cells were analyzed in duplicate to determine the concentration of PRL using a commercially available multiplex assay (HIGFBMAG-53 K and HPTP2MAG-66 K, EMD Millipore, Billerica, Massachusetts) and a LuminexMagPix magnetic bead system. Media supernatants were stored at -80°C until analysis. Manufacturer-supplied controls were included to measure assay variation. A minimum of 100 beads were collected for each analyte. Unknown sample values were calculated offline using Milliplex Analyst Software (EMD Millipore). The measured interassay (<15%) and intraassay (<10%)

variability were within the normal limits reported by the manufacturer.

Statistical Analysis

In addition to the analysis of RNA sequencing data sets described above, Student *t* test was used to compare 2 independent groups and analysis of variance (ANOVA) followed by Dunnett's (if all compared with control) or Student-Newman-Keuls (for all comparisons) post hoc testing for experiments with multiple groups. In experiments of time-dependent changes in 2 treatment groups (Figures 3 and 4), statistical analysis was by 2-way ANOVA. *P* values $\leq .05$ were considered statistically significant.

Results

Effect of Thrombin on Decidualization-Induced Gene Expression Profiles

The effect of thrombin on gene expression profiles of baseline hESCs and decidualized cells on a global scale was determined using RNA-sequencing analysis. Baseline cells were treated for 24 hours with vehicle or thrombin. Cells were decidualized for 72 hours with cAMP+MPA with or without thrombin for 24 hours (Supplemental Figures S1 and S2). Hierarchical clustering analysis revealed that control and decidualized cells clustered with dramatic changes in gene expression profiles (Figure 1, data deposited at NCBI GEO). The following cell signaling pathways were affected: cell cycle repair, embryonic stem cell pluripotency, ILK signaling, cAMP-mediated signaling, STAT3 pathways, β -adrenergic signaling, inhibition of MMPs, leukocyte extravasation, and oxidative stress response genes (Supplemental Figure 1). As expected, PRL was induced 9-fold, as well as PRLR, IRS1, and JAK2. Likewise, upregulation of IGF-1 and IGFBPs was confirmed including cell survival pathways and STAT3 signaling. Leukocyte activation and extravasation pathways were affected by induction of JAM2, ICAM-1, and CXCR4. Browser track views of IGFBP-1, IGF-1, PRL, ER α , and PR confirmed that cells underwent changes in gene expression consistent with decidualization (Figure 1).

Thrombin Affects Markers of Decidualization

Thrombin altered the transcriptome of baseline hESCs and decidualized cells (Table 1) including expression of genes that serve as markers of decidualization. To confirm the global gene profiling, hESCs were treated with vehicle or thrombin (1-6 units/mL) at baseline or during decidualization with cAMP+MPA. Specifically, RNA was isolated from nondecidualized cells treated for 24 hours or from cells treated with cAMP+MPA for 48 hours with media change to cAMP+MPA+vehicle or cAMP+MPA+thrombin for an additional 24 hours (total time of decidualization = 72 hours; Figure 2). Treatment with thrombin resulted in decreased expression of all 3 genes in nondecidualized hESCs (Figure 2 A, B, and C).

Figure 1. RNA-SeqData Analysis in hESCs. A, Principal component analysis demonstrating BCV of different treatment groups in which duplicates of two control preparations (Ctla and Ctla';Ctla and Ctla') were compared with decidualized cells (72 hours MPA+cAMP) (Dec-a and Dec-a'; Dec-b and Dec-b'). Volcano plots providing false discovery rate (FDR) values and fold change for gene transcripts comparing control versus thrombin-treated cells (B), Control versus decidualized cells (C), or control versus decidualized cells + thrombin (D). Differentially expressed genes (1.5-fold) with FDR < 0.1 and log counts per million (CPM) > 1 are indicated in red. E-I, Browser tracks of genes regulated by cAMP + MPA in endometrial stromal cells. Cells treated with vehicle (controls 1 and 2) were compared with those treated with cAMP + MPA for 72 hours (Dec 1 and 2). Genes shown are *IGF-1* (E), *ERα* (*ESR1*, F), *IGFBP1* (G), *PGR* (H), *PRL* (I). BCV indicate biological coefficient of variation; hESCs, human endometrial stromal cells.

Figure 2. Effect of pThr on basal and decidualization-induced expression of *IGF-I*, *PRL*, and *IGFBP-1* in hESCs. Cells were treated under baseline conditions with vehicle (CTL) or pThr for 24 hours. To initiate decidualization, cells were treated with 8-Bromo cyclic adenosine monophosphate + medroxy-progesterone acetate (cAMP+MPA) for 72 hours. During the last 24 hours, cells were treated with vehicle (dCTL) or various concentrations of pThr. Thereafter, relative gene expression of *IGF-I* (A), *PRL* (B), and *IGFBP-1* (C) were quantified using quantitative PCR (qPCR) as described in Materials and Methods section. Data represent mean $\pm$ standard error of the mean of 3 to 4 cell preparations from different patients. * $P < .05$; ** $P < .01$. (D-F) Extended time course of thrombin effects on molecular markers of decidualization in hESCs treated with 8-Bromo cAMP+MPA for 3 to 6 days $\pm$ thrombin (2 U/mL). Levels of *IGF-I* (D), *PRL* (E), or *IGFBP-1* (F) were quantified in control (open symbols) or thrombin-treated (solid squares) cells by qPCR. Data represent mean $\pm$ standard error of the mean of triplicates conducted in 1 cell preparation repeated in cells from another patient with identical results. * $P < .05$ compared with time point without thrombin. hESCs indicate human endometrial stromal cells; pThr, plasma thrombin.

Figure 3. Effects of thrombin on secreted prolactin, a marker of decidualization. Cells were treated with cAMP+MPA + vehicle (open bars) or thrombin (solid bars) for 1 to 6 days. Media was harvested and analyzed for prolactin content. A, Effect of decidualization for 24 hours (1 day). Data represent mean $\pm$ standard error of the mean of triplicates conducted in 3 cell preparations from different participants. * $P < .05$ compared with vehicle controls.

As expected, initiation of decidualization with cAMP+MPA resulted in 8-fold increases in *IGF-1*. Interestingly, however, treatment with thrombin (from 1 to 6 U/mL) dramatically forestalled decidualization-induced upregulation of *IGF-1* to levels less than baseline (Figure 2A). *Prolactin* was induced 40-fold 72 hours after initiation of decidualization (Figure 2B). Although the magnitude of suppression was not as impressive as with *IGF-1*, thrombin dose-dependently suppressed *PRL* mRNA in decidualized cells (Figure 2B). Finally, the magnitude of induction of *IGFBP-1* during decidualization was striking (120-fold, Figure 2C). Nonetheless, thrombin suppressed both baseline and decidualization-induced increases in *IGFBP-1* with maximal suppression at 2 U/mL (Figure 2C). Thrombin-mediated decreases in *PRL* and *IGFBP-1* were more profound in baseline ESCs compared with decidualized cells. Unless indicated otherwise, for the remaining studies, 2 U/mL of thrombin was utilized (~ 18 nM).

Thrombin Time Dependently Suppressed Markers of Decidualization

To determine the time course of thrombin-induced suppression of gene expression during decidualization, cells were treated with cAMP+MPA to induce decidualization and then treated with thrombin (2 U/mL) at the beginning of induction (ie, for 72 hours), the final 48 hours, or final 24 hours (Supplemental Figure 2A). Although not sufficient to suppress *IGF-1* at 24 hours, thrombin (2 U/mL) suppressed *IGF-1* mRNA to 50% at 48 to 72 hours (Supplemental Figure 2B). Similarly, 72 hours was required to suppress *PRL* (Supplemental Figure 2C). In contrast, *IGFBP-1* was sensitive to thrombin

as early as 24 hours (Supplemental Figure 2D), and the magnitude of suppression (80%) was greater than with *IGF-1* or *PRL*.

In general, 5 to 9 days of progestin/cAMP treatment is required to accomplish full morphological transformation of stromal cells into decidualized cells.^{15,19,20} Hence, we conducted an extended time course of gene expression in stromal cells undergoing decidualization with or without thrombin (Figure 2D,F, and G). In control cells, *IGF-1* mRNA declined after 72 hours of cAMP+MPA treatment (Figure 2D). Thrombin treatment suppressed *IGF-1* mRNA at most time points (from 10% to 25%), but the greater impact was early in the decidualization process with maximal suppression of 50% at 3 days (72 hours; Figure 2D). In contrast to *IGF-1*, both *PRL* and *IGFBP-1* increased as a function of time during decidualization with values at 6 days 2- to 2.8-fold that of the 72 hours time point (Figure 2E and F). It should be emphasized, however, that *IGFBP-1* increased 120-fold within 72 hours of induction of decidualization such that the further 2-fold increase with extended time is rather modest. Nonetheless, thrombin suppressed both *PRL* and *IGFBP-1* at all time points (Figure 2E and G).

Secreted PRL

Prolactin is a well-recognized marker of decidualization. We determined whether *PRL* mRNA expression correlated with the levels of *PRL* in the media. In control cells, treatment with cAMP+MPA resulted in 22-fold net increases in secreted *PRL* within 24 hours (Figure 3A). Thereafter, *PRL* level increased progressively to 80 ng/mL at 6 days (Figure 3B). Although thrombin did not affect the initial burst in *PRL* secretion with

Figure 4. Thrombin mediates effects of decidualization through PAR-1 and non-PAR-1 mechanisms. Human ESCs were treated with dbcAMP+MPA for 72 hours. Treatments were begun during the last 24 hours of decidualization (pThr, plasma thrombin, 2 U/mL; inactive Thr, heat inactivated pThr; PAR-1 agonist, or rThr, recombinant Thr, 2 U/mL). Thereafter, expression of *IGF-1* (A), *Prolactin* (B), or *IGFBP-1* (C) was expressed relative to a decidualized control present on each plate. Data represent mean $\pm$ standard error of the mean of triplicates with consistent results in 2 cell preparations. * $P < .05$ compared with Ctl, ANOVA, dunnett post hoc testing. ANOVA indicates analysis of variance.

cAMP+MPA (24 hours), it suppressed PRL content at every time point thereafter (Figure 3B). The results therefore indicate that thrombin inhibits PRL mRNA and protein in cells undergoing decidualization.

Role of PAR-1 in Suppression of Markers of Decidualization

To assess whether activation of PAR-1 is the primary mechanism by which thrombin suppresses markers of decidualization, several experimental approaches were employed. First, decidualized hESC were treated with a PAR-1 agonist (TRAP-6, SFLLRN-NH₂) to mimic the effects of thrombin. Second, since PAR-1 activation requires thrombin's protease activity, purified plasma thrombin was heat inactivated. Finally, recombinant thrombin (rThr) was used to make sure that inhibition of decidualization was not due to contamination of thrombin purified from plasma (Figure 4).

Thrombin (24 hours) consistently decreased IGF-1 mRNA >80% in cells decidualized for 72 hours, and heat inactivation obliterated this response (Figure 4A). The PAR-1 agonist also decreased IGF-1 mRNA in decidualized cells. Recombinant thrombin also decreased *IGF-1* gene expression to the same

degree as the PAR-1 agonist but not to the same extent as pThr. Since rThr is not glycosylated and has lower activity than the fully mature glycosylated protein,⁷ this result is not surprising. Overall, the results suggest that PAR-1 is the likely mechanism by which thrombin inhibits *IGF-1* in decidualized cells. In contrast, the PAR-1 agonist did not suppress the expression of *PRL*, and inhibition of heat inactivation of thrombin did not destroy thrombin-induced suppression of *PRL* mRNA (Figure 4B). Finally, as in *PRL*, thrombin-induced suppression of *IGFBP-1* mRNA was also PAR-1-independent (Figure 4C).

Thrombin Alters Interleukin 6, Interleukin 8, and Matrix Homeostasis in hESCs

Relative expression of *IL-6* and *IL-8* gene expression was quantified in thrombin-treated cells (Figure 5). Decidualization for 72 hours did not alter the expression of interleukin 6 (IL-6). Thrombin, however, upregulated IL-6 gene expression 2- to 3-fold in both baseline and decidualized cells. In contrast with IL-6, thrombin treatment resulted in marked induction of IL-8 gene expression (35-fold) in control cells and ~12-fold in decidualized cells (Figure 5). The results suggest that thrombin not only alters the process of decidualization, it also increases

Figure 5. Effect of thrombin on *IL-6*, *IL-8*, and genes that regulate matrix homeostasis. Nondecidualized control cells were treated with vehicle or thrombin for 24 hours. Cells were treated with CAMP+MPA for 72 hours to induced decidualization. Vehicle or thrombin (2 U/mL) was added in the last 24 hours. A, *IL-6*, B, *IL-8*. Data represent mean $\pm$ standard error of the mean in triplicates obtained in two cell preps. C, *MMP1*, D, *LOX*, E, *MMP9*, F, *Cola1a*. Data represent mean $\pm$ standard error of the mean of triplicate determinations in one cell preparation. * $P < .01$ compared with control, ANOVA indicates analysis of variance; IL indicates interleukin.

the expression of major chemokines involved in leukocyte trafficking in the endometrium.

The impact of thrombin on matrix homeostasis was determined (Figure 5). Interestingly, in nondecidualized stromal

cells, thrombin increased the major interstitial collagenase *MMP1* by 7-fold and *MMP9* 3-fold (Figure 5C and D). Induction of decidualization for 72 hours decreased the baseline expression of both *MMP1* and *MMP9* and protected the cells

Figure 6. Effect of thrombin on early decidualization-induced changes in cell shape. Endometrial stromal cells were treated with vehicle or cAMP+MPA $\pm$ thrombin (2 U/mL) for 72 hours. Phalloidin-immunofluorescent staining of filamentous actin was captured and merged with DAPI staining for cell nuclei. Arrows denote persistent fibroblast morphology in thrombin-treated cells undergoing decidualization (Panel D compared with Panel B). Ten Hpf images were taken from triplicate wells in each treatment group (one cell preparation).

from thrombin-mediated induction of *MMP9*. Nonetheless, thrombin increased *MMP1* significantly even in decidualized cells (Figure 5C). The major cross-linking enzyme lysyl oxidase (LOX) regulates maturation of collagen from immature soluble fibrils to mature cross-linked insoluble collagen rendering tissue integrity and matrix stability. *LOX* gene expression was increased significantly in endometrial stromal cells undergoing decidualization (Figure 5D). Thrombin suppressed not only baseline levels but also decidualization-induced increases in *LOX*. Thrombin also suppressed mRNA levels of collagen type 1a (*Col1a1*) in baseline and decidualized cells (Figure 5F). Together, these results indicate that thrombin alters gene expression involved in matrix stabilization in nondecidualized and decidualized ESCs with increases in degradation and compromise of collagen mRNA and cross-linking.

Thrombin Affects Morphological Changes in Decidualized hESC

To assess the impact of thrombin on cell morphology of decidualized hESC, phalloidin staining of F-actin was employed (Figure 6). Decidualization of hESCs for 3 days resulted in characteristic morphologic changes from elongated spindle-shaped cells to polygonal cells with random distribution of F-actin filaments (Figure 6 A and B). This change is consistent in all cell preparations. Although thrombin did not alter the fibroblast morphology of nondecidualized stromal cells (Figure 6C), it altered the morphology of decidualizing stromal

cells (Figure 6D) with the retention of longitudinally oriented actin filaments (Figure 6B and D).

Discussion

During pregnancy, increased progesterone from the corpus luteum and later from the placenta differentiates endometrial stromal cells into decidual cells that support implantation and growth of the fetus. The unique location between the maternal myometrium and fetal implantation site serves to position decidual cells as mediators of implantation and fetal development. The cells are known to be a rich source of proteases, cytokines, and eicosinoids. Furthermore, injury of the endometrium possibly through difficult embryo transfers during in vitro fertilization (IVF) would increase tissue factor/factor VIIa complexes that promote hemostasis through cleavage of prothrombin to thrombin. In human pregnancy, decidual cell-expressed tissue factor-derived thrombin prevents decidual hemorrhage (abruption).²¹⁻²⁵ In this investigation, we found that thrombin affects endometrial stromal cells at baseline and also alters decidualization of these stromal cells in vitro. Specifically, thrombin-suppressed *IGF-1*, *PRL*, and *IGFBP-1* expression and changes in cell shape during decidualization, suggesting that thrombin is not only involved in thrombosis of the endometrium and spiral arterioles but also affects cellular pathways leading to endometrial differentiation.

In general, thrombin activates protease-activated receptors (PARs) that act as exquisite sensors for extracellular proteases serving to bring about cell signaling pathways that adapt to the new microenvironment altered during proteolysis. The 4 different PARs (PAR 1-4) each respond to a select group of proteases.²⁶ In hESCs, thrombin acts via protease-activated receptors to induce MMPs and inflammatory factors such as IL-8, which lead to immune cell recruitment and degradation of the cellular matrix.^{4,27} Previously, we demonstrated that thrombin acts not only through PAR-1 but also through toll-like receptor 4 to cause matrix degradation in the fetal membranes.⁷ Here, using TRAP-6, a 6-amino acid peptide previously demonstrated to mimic thrombin actions in endothelial cells, and rThr to rule out the effect of possible contaminants of plasma thrombin preparations, we found that, like amnion, thrombin acts in both PAR-1- and PAR-1-independent mechanisms to alter gene expression of hESCs. Similar effects of plasma thrombin, rThr, and TRAP-6 on IGF-1 levels suggest that these responses are mediated primarily through the thrombin receptor, PAR-1. The more modest effects of thrombin on *PRL* and *IGFBP-1*, on the other hand, were clearly independent of PAR-1. In the case of suppressed *PRL*, thrombin protease activity was not required suggesting either indirect regulation or binding of thrombin to other cell receptors that do not require proteolysis. This has been reported previously in other cell types.⁷

Thrombin decreased secreted *PRL*, a classic marker of decidualization. Interestingly, the full decidual cell morphologic phenotype requires long-term treatment with progestin+cAMP. Here, cells underwent partial morphological change to polygonal cells with only 3 days treatment. Thrombin altered this

morphological change. Together with changes in secreted PRL, the results suggest a rather dramatic effect of thrombin not only on baseline cells that support the endometrium during the menstrual cycle but also during early pregnancy.

Decidual hemorrhage and thrombin formation have been implicated as a mediator of decidual ECM breakdown.¹¹⁻¹³ Thrombin has been demonstrated to increase generation of MMP-1, MMP-3, and MMP-9 in decidual cells.¹²⁻¹⁴ In the present study, we confirmed these findings and extended these observations to nondecidualized hESCs. Thrombin acts via PAR-1 in endometrial cells to induce mRNA and protein expression of matrix MMP-1 and -3.²⁸ Here, we show that thrombin induced *MMP1* and *MMP9* mRNA even at baseline. Further, mRNA of these proteases decreased during decidualization suggesting that decidualization is a process that fosters endometrial stability rather than menstruation. These findings are also relevant to the pathophysiology of matrix degradation in endometriosis in which thrombin-mediated interactions with PAR-1 and PAR-4 have been implicated.^{29,30} Interestingly, thrombin prevented decidualization-induced suppression of the major collagenase MMP1. Further, this upregulation of decidual MMP1 was accompanied by suppression of *LOX*, the gene encoding the major enzyme of collagen cross-linking. We believe that this is the first study to report the suppression of *LOX* by thrombin. Taken together, our data indicate that thrombin has overall suppressive effect on the extracellular matrix through the induction of matrix degradation and inhibition of collagen processing.

In addition to matrix homeostasis, inflammation is another tightly orchestrated process during decidualization. Leukocytes represent a large portion of endometrium especially during decidualization, with the highest number of leukocytes detected in the mid-secretory phase, around the implantation window.³¹ It has been hypothesized, therefore, that cytokine and chemokine production in the endometrium play an essential role in the mechanism of implantation in humans. Previous reports indicate that thrombin stimulates production of IL-8, *GRO α* , and MCP-1 via PAR-1 involving the MAP kinase system in ESCs.²⁷ These molecules play a key role in attraction and activation of immune cells, immunological tolerance, regulation of trophoblast invasion, and efficient disposal of blood and cellular debris during menstruation.³² Using our RNAseq data and data from primary cultures, we found that thrombin decreased well-recognized markers of decidualization but enhanced stromal-derived chemoattractants IL-6 and IL-8. It would be predicted therefore that enhanced stromal chemokines would amplify immune cell infiltration and intensify protease release by invading neutrophils resulting in a feed-forward loop of matrix degradation and endometrial shedding even during early pregnancy and decidualization.

In summary, thrombin adversely affects decidualization and matrix homeostasis in endometrial stromal cells. Furthermore, thrombin not only activates genes involved in matrix degradation (MMPs) and inflammation (ILs) but also suppresses factors important for collagen formation (*LOX*). An important caveat is that the study emphasizes the role of highly

differentiated stromal cells of the endometrium rather than the complex multicellular interactions that occur in endometrium in vivo. Nonetheless, findings from the present study support the hypothesis that intrauterine bleeding and generation of thrombin impairs matrix homeostasis, decidualization, and endometrial support of early pregnancy.

Authors' Notes

S.B. conducted the cell culture experiments, analyzed data, generated figures, and assisted in writing the manuscript. Statement describing each author's contributions to the manuscript. M.K. and C. X. analyzed the RNA sequence data, conducted bioinformatics, and assisted in figure preparation. B.C. assisted in developing the concept and design. R.A.W. directed the experimental design, analyzed the data, assisted in generation of figures and interpretation, and in writing of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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