GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Thrombin-Induced Decidual Colony-Stimulating Factor-2 Promotes Abruption-Related Preterm Birth by Weakening Fetal Membranes

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Preterm premature rupture of membranes (PPROM) and thrombin generation by decidual cell—expressed tissue factor often accompany abruptions. Underlying mechanisms remain unclear. We hypothesized that thrombin-induced colony-stimulating factor-2 (CSF-2) in decidual cells triggers paracrine signaling via its receptor (CSF2R) in trophoblasts, promoting fetal membrane weakening and abruption-associated PPRM. Decidua basalis sections from term ($n = 10$), idiopathic preterm birth (PTB; $n = 8$), and abruption-complicated pregnancies ($n = 8$) were immunostained for CSF-2. Real-time quantitative PCR measured *CSF2* and *CSF2R* mRNA levels. Term decidual cell (TDC) monolayers were treated with 10^{-8} mol/L estradiol $\pm$ 10^{-7} mol/L medroxyprogesterone acetate (MPA) $\pm$ 1 IU/mL thrombin pretreatment for 4 hours, washed, and then incubated in control medium with estradiol $\pm$ MPA. TDC-derived conditioned media supernatant effects on fetal membrane weakening were analyzed. Immunostaining localized CSF-2 primarily to decidual cell cytoplasm and cytotrophoblast cell membranes. CSF-2 immunoreactivity was higher in abruption-complicated or idiopathic PTB specimens versus normal term specimens ($P < 0.001$). *CSF2* mRNA was higher in TDCs versus cytotrophoblasts ($P < 0.05$), whereas *CSF2R* mRNA was 1.3×10^4 -fold higher in cytotrophoblasts versus TDCs ($P < 0.001$). Thrombin enhanced CSF-2 secretion in TDC cultures fourfold ($P < 0.05$); MPA reduced this effect. Thrombin-pretreated TDC-derived conditioned media supernatant weakened fetal membranes ($P < 0.05$), which MPA inhibited. TDC-derived CSF-2, acting via trophoblast-expressed CSF2R, contributes to thrombin-induced fetal membrane weakening, eliciting abruption-related PPRM and PTB. (*Am J Pathol* 2020, 190: 388–399; <https://doi.org/10.1016/j.ajpath.2019.10.020>)

During the luteal phase of the human menstrual cycle, increasing circulating progesterone (P4) levels induce endometrial stromal cells to undergo decidualization, converting fibroblast-like stromal cells to decidual cells. Sustained circulating P4 levels, accompanied by continuation of decidualization, are required to establish and maintain pregnancy.¹ Previously, we discovered that a key feature of decidualization is increased expression of tissue factor, a transmembrane 45-kDa glycoprotein.² In response to

vascular injury, plasma-derived factor VII binds to cell membrane-bound tissue factor, forming a tissue factor/factor VIIa complex that fosters hemostasis by activating

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factor X or IX to ultimately convert prothrombin to thrombin (THR). Newly formed THR triggers the clotting cascade by converting fibrinogen to fibrin and activating platelets.³ Therefore, decidual cell-expressed THR mitigates decidual hemorrhage (ie, abruptio) throughout human pregnancy. Among the cells in human term placenta, decidual cells express the highest tissue factor levels, which contrasts with the virtual absence of tissue factor immunostaining in adjacent interstitial or extravillous cytotrophoblasts (CTBs).⁴

As pregnancy progresses, bleeding into the decidua basalis may clinically manifest as an abruptio, vaginal bleeding, or a subchorionic hematoma.^{5,6} Vaginal bleeding during pregnancy is associated with a 74% increased risk of preterm birth (PTB), many of which are attributable to preterm premature rupture of membranes (PPROM).⁷ This correlation is greatest between 22 and 32 weeks of gestation, when abruptio occurs in greater than one-third of PPRM cases compared with only 0.8% of women experiencing term delivery.^{8–10}

We previously reported that P4, medroxyprogesterone acetate (MPA), or 17 α -hydroxyprogesterone treatment inhibits THR-induced fetal membrane weakening by inhibiting colony-stimulating factor (CSF)-2 production and action.¹¹ Because the cellular source, the relevant intracellular mechanism(s), and/or paracrine target(s) underlying this finding are not known, we hypothesized that THR-induced CSF-2 in term decidual cells (TDCs) triggers paracrine signaling via its receptor (CSF2R) on trophoblast cell membranes, promoting fetal membrane weakening and abruptio-associated PTB. Therefore, the current study evaluates a functional role of THR-enhanced CSF-2 secretion by human TDCs in mediating fetal membrane weakening and rupture as a prelude to PTB.

Materials and Methods

Tissues

Previously banked placental paraffin blocks containing decidua basalis, obtained from the Yale New Haven Hospital (New Haven, CT), were used in this study. Term decidua basalis biopsies were obtained from scheduled repeated cesarean deliveries (precontraction, $n = 5$) and after the onset of labor (post-contraction, $n = 5$). Preterm decidua basalis specimens were obtained from gestational age-matched idiopathic ($n = 8$) and abruptio-complicated PTB ($n = 8$). Abruptio-associated PTB specimens had a clinical diagnosis of abruptio, whereas idiopathic PTB specimens displayed no clinical evidence of abruptio, chorioamnionitis, or preeclampsia. This study was conducted after approval from the Yale University Human Investigation Committee and subsequently the University of South Florida Institutional Review Board (number 19472).

Immunohistochemistry and HSCORE Analysis

Paraffin sections (5 μ m thick) from formalin-fixed placentas of term and gestational age-matched idiopathic and abruptio-complicated PTB pregnancies were deparaffinized in xylene and rehydrated in a descending series of ethanol, then boiled in antigen retrieval solution using citrate buffer (pH 6.0), as described previously.¹² After several washes in Tris-buffered saline (pH 7.2) and endogenous peroxidase quenching in 3% hydrogen peroxide solution, the samples were blocked with 5% normal goat serum (Vector Labs, Burlingame, CA) for 30 minutes and incubated overnight with a rabbit polyclonal CSF-2 antibody (Abcam, Cambridge, MA). After several washes in Tris-buffered saline, the slides were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Labs) for 30 minutes, then incubated with streptavidin-conjugated peroxidase complex (Vector Labs) for 30 minutes. After several rinses in Tris-buffered saline, CSF-2 immunoreactivity was developed using diaminobenzidine (3, 3'-diaminobenzidine tetrahydrochloride dihydrate; Vector Labs) as the chromogen, and sections were counterstained with hematoxylin. To identify trophoblasts and decidual cells, a second set of serial sections were double immunostained sequentially for cytokeratin-7 (a trophoblast marker; Dako Cytomation, Carpinteria, CA) and vimentin (a decidual marker; Abcam), as described.¹³ Diaminobenzidine (brown) for cytokeratin and Vector RED for vimentin (Vector Labs) were used as chromogens. Serial sections of fetal membranes ($n = 4$) and decidua basalis ($n = 3$) of term pregnancies were also immunostained using mouse monoclonal CSF2R α (Santa Cruz Biotechnology, Inc., Dallas, TX) or rabbit monoclonal phosphorylated STAT5 (Cell Signaling, Danvers, MA) antibodies. For negative controls, appropriate normal rabbit or mouse IgG isotype was used at the same concentration as each primary antibody (Table 1). Immunoreactivity for CSF-2 was assessed by histologic score (HSCORE) analysis, a semiquantitative method that evaluates the intensity and the number of immunostained cells.¹² Three blinded observers (R.G.S., S.A., N.S.) assigned HSCOREs, and the mean score was used for each sample.

Term Decidual Cell Culture

Aliquots of frozen leukocyte-free TDCs, isolated from term placentas from uncomplicated pregnancies undergoing repeated cesarean deliveries, were grown to confluence, as described.^{4,14} Confluent TDCs were primed in serum-containing medium with 10^{-8} mol/L estradiol used as control (E_2 ; Sigma-Aldrich, St. Louis, MO) or 10^{-8} mol/L $E_2 + 10^{-7}$ mol/L MPA, a mixed progestin-glucocorticoid (Sigma-Aldrich) for 7 days, then exchanged to serum free-defined medium containing E_2 or $E_2 + MPA \pm 1$ U/mL THR (Sekisui Diagnostics, Stamford, CT) for 6 and 24 hours. In parallel experiments, primed TDCs were also

Table 1 Detailed Description of Antibodies Used for IHC and Immunoblotting (WB Analysis)

Antibody name	Catalog no./company	Species	Dilution/procedure
CSF-2	ab9741/Abcam	Rabbit	5 µg/mL/IHC
Cytokeratin -7	M7018/Dako Cytomation	Mouse	1:600/IHC
Vimentin	ab39376/Abcam	Chicken	1:300/IHC
CSF2Rα	sc21764/Santa Cruz Biotechnology, Inc.	Mouse	1:50/IHC
Phosphorylated STAT5	9359/Cell Signaling	Rabbit	1:250/IHC and 1:1000/WB analysis
Total STAT5	94205/Cell Signaling	Rabbit	1:1000/WB analysis
Total STAT3	9139/Cell Signaling	Mouse	1:1000/WB analysis
Phosphorylated STAT3	9145/Cell Signaling	Rabbit	1:1000/WB analysis
p65 NF-κB	8242/Cell Signaling	Rabbit	1:1000/WB analysis
Phosphorylated p65 NF-κB	3033/Cell Signaling	Rabbit	1:1000/WB analysis
ERK1/2 MAPK	4348/Cell Signaling	Rabbit	1:1000/WB analysis
Phosphorylated ERK1/2 MAPK	9106/Cell Signaling	Mouse	1:1000/WB analysis
p38 MAPK	9212/Cell Signaling	Rabbit	1:1000/WB analysis
Phosphorylated p38 MAPK	4511/Cell Signaling	Rabbit	1:1000/WB analysis
AKT	8596/Cell Signaling	Rabbit	1:1000/WB analysis
Phosphorylated AKT	4060/Cell Signaling	Rabbit	1:2000/WB analysis
β-Actin	5125/Cell Signaling	Rabbit	1:5000/WB analysis

CSF2Rα, CSF-2 receptor α; ERK, extracellular signal-regulated kinase; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; WB, Western blot.

treated with 10^{-8} mol/L E_2 + 10^{-7} mol/L dexamethasone, a pure glucocorticoid (Sigma-Aldrich), or 10^{-7} mol/L Organon 2058, a pure progestin (Merck, Whitehouse Station, NJ) ± 1 U/mL THR for 6 hours. RNA was isolated after 6 hours of incubation of TDCs, whereas after 24 hours of treatment of TDCs, the conditioned medium supernatant (CMS) was collected for enzyme-linked immunosorbent assay analysis. Modeled after our previous fetal membrane weakening studies,¹¹ TDCs were incubated with E_2 ± THR or E_2 + MPA ± THR for 4 hours, then washed with phosphate-buffered saline to remove residual THR, then incubated for an additional 24 hours in serum free—defined medium containing the aforementioned steroids. Collected CMS was used for fetal membrane strength testing to analyze the direct effect of TDCs on fetal membrane weakening.

In a set of parallel experiments, TDCs primed with E_2 + MPA for 7 days were preincubated with 2×10^{-5} mol/L PD98059 [an extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK)

inhibitor] or $\pm 2 \times 10^{-5}$ mol/L SB203580 (a p38 MAPK inhibitor) or $\pm 10^{-6}$ mol/L Bay-III (NF-κB activation inhibitor III; Millipore, San Diego, CA) for 45 minutes, then treated with 1 U/mL THR for 6 hours for real-time quantitative PCR (qPCR) analyses to identify relevant signaling pathways involved in THR-induced CSF-2 expression.

Trophoblast Cell Cultures

Total RNA from previously isolated and cultured CTBs and syncytiotrophoblasts (STBs^{15–17}; obtained from uncomplicated pregnancies and repeated cesarean deliveries at term; kindly provided by Dr. Seth Guller, Yale College of Medicine, New Haven, CT) were used to compare endogenous expression of CSF-2 and its receptor CSF2RA in CTBs, STBs, and TDCs.

To analyze CSF-2—mediated signaling pathway(s), BeWo cells (a trophoblastic cell line provided by Dr. Hana Totary-Jain, University of South Florida, Morsani College of Medicine, Tampa, FL) were cultured in Dulbecco's modified Eagle's medium and Ham F12 with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Confluent BeWo cells were incubated for 24 hours in serum-free media, then treated for 15 minutes with control or 10 ng/mL recombinant human (rh)-CSF-2 (R&D Inc., Minneapolis, MN), followed by protein extraction for immunoblotting analysis of signaling molecules.

RRT and Real-Time Quantitative PCR

Using an RNAqueous-Micro isolation kit (Ambion, Austin, TX), total RNA was isolated from primary cultures of human TDCs, CTBs, and STBs as well as choriodecidual explant cultures, per manufacturer's instructions. Reverse

Table 2 Detailed Description of Primers Used for Real-Time Quantitative PCR

Primer/probe name	TaqMan assay ID
CSF-2	Hs00929873_m1
CSF2RA	Hs00531296_g1
IL-1β	Hs0155410_m1
IL-6	Hs00985639_m1
IL-8	Hs00174103_m1
MMP-3	Hs00968305_m1
MMP-7	Hs01042796_m1
MMP-9	Hs00234579_m1
β-Actin	Hs99999903_m1

CSF2RA, CSF-2 receptor A; ID, identifier; MMP, matrix metalloproteinase.

Figure 1 *In situ* regulation of CSF-2 immunostaining at the maternal-fetal interface. **A–C:** Expression levels of CSF-2 immunoreactivity in decidua and trophoblasts at the maternal-fetal interface in term (**A**), idiopathic preterm birth (PTB; **B**), and abruption-associated PTB (**C**) specimens. **D:** Vimentin and cytokeratin double immunostaining is seen in red and brown to identify decidua and trophoblast cells, respectively. **E:** Negative control immunostaining for CSF-2 in term specimens using appropriate non-specific IgG isotype as the same concentration as CSF-2 primary antibody. **F:** Higher magnification of CSF-2 immunoreactivity in the cytoplasm of decidua cells (**arrowheads**) and trophoblasts cell membrane (**arrows**) in abruption-associated PTB specimens. **G** and **H:** Histologic score (HSCORE) levels of CSF2 immunoreactivity in decidua cells (**G**) and trophoblasts (**H**) at the maternal-fetal interface in term specimens or idiopathic PTB (iPTB) and abruption-associated PTB specimens. Data are expressed as means ± SEM (**G** and **H**). $n = 10$ (**A**); $n = 8$ (**B** and **C**). *** $P < 0.001$ versus term in decidua cells; † $P < 0.05$ versus term in trophoblasts. Scale bars = 30 μ m (**A–F**).

transcription was performed using the Retroscript (Invitrogen), as follows: template RNA and random primers were incubated at 65°C for 5 minutes (to eliminate any secondary structures). After adding the buffer and enzyme, the reaction was performed at 42°C for 1 hour. qPCR was performed using TaqMan gene expression assays for *CSF2*, *CSF2RA*, matrix metalloproteinase (*MMP*) 3, 7, or 9, as well as *IL-1B*, *IL-6*, or *IL-8* (Applied Biosystems, Foster City, CA) (Table 2). All samples were run in triplicate. Expression of the target mRNAs was normalized to β -actin mRNA levels and the $2^{-\Delta\Delta CT}$ (cycle threshold) method was used to calculate relative expression levels.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (R&D Inc.) was performed, as described,¹⁸ to measure CSF-2 secretion levels in the CMS collected from TDC ($n = 8$) cultures treated with 10^{-8} mol/L E_2 or 10^{-8} mol/L $E_2 + 10^{-7}$ mol/L MPA ± 1 U/mL THR for 24 hours.

Immunoblotting

Whole cell lysates from BeWo cells treated for 15 minutes with control or 10 ng/mL rh-CSF-2 (R&D Inc.) were

subjected to SDS-PAGE with 7.5% or 10% Tris-HCl gel (Bio-Rad, Hercules, CA) and then transferred to a nitrocellulose membrane. Blocking was performed with 10% nonfat dried milk, then membrane was incubated overnight with rabbit anti-phosphorylated or total (T) antibodies against STAT5, STAT3, p65 NF- κ B, ERK1/2, p38 MAPKs, or AKT (Cell Signaling) (Table 1). After washing with Tris-buffered saline with 0.1% Tween 20 (pH 7.2), secondary antibody labeling was performed by incubating the membrane with horseradish peroxidase-conjugated goat anti-rabbit IgG or horse anti-mouse IgG (Vector Labs). An enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ) was used to detect immunoblot bands. All membranes were stripped and reprobed with horseradish peroxidase-labeled rabbit anti- β -actin antibody (Cell Signaling).

Fetal Membrane Strength Testing

Portions of fetal membranes ($n = 4$), obtained from uncomplicated term repeated cesarean deliveries, were strength tested within transwell inserts using a rupture testing apparatus. To test fetal membrane tensile strength, fetal membranes were secured between horizontal plates. A 1-cm, motor-driven, spherical-head plunger was driven

Figure 2 *In vitro* comparative analysis of *CSF2* and its receptor, *CSF2RA*, expression. **A:** *CSF2* mRNA levels in primary cultured term decidual cells (TDCs), cytotrophoblasts (CTBs), and syncytiotrophoblasts (STBs) by real-time quantitative PCR (qPCR). **B:** Expression of *CSF-2* receptor, *CSF2RA*, mRNA in TDCs, CTBs, and STBs by qPCR. *CSF2* (**A**) and *CSF2RA* (**B**) mRNA levels were determined and normalized to β -actin. Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 3$ (**A** and **B**). * $P < 0.05$, *** $P < 0.001$ versus TDCs; † $P < 0.05$ versus CTBs.

perpendicular to the fetal membrane against the fetal amnion. Fetal membrane displacement and force in Newtons were recorded continuously until rupture. Fetal membrane strength testing was performed on term, prelabor specimens incubated in CMS collected from TDCs cultured in $E_2 \pm$ THR or $E_2 +$ MPA $\pm$ THR, as described in *Term Decidual Cell Culture*.

Choriodecidual Explant Culture

The fetal membrane (three experimental repeats) was dissected from the placenta collected from a nonlaboring woman with intact membrane at 33 weeks who underwent caesarean delivery for presumptive placenta accreta, which was not confirmed by histopathologic examination, then rinsed with phosphate-buffered saline to remove residual blood. After removing the amnion, the choriodecidual tissue was minced into small pieces and randomly distributed into

a 12-well culture plate containing Dulbecco's modified Eagle's medium and Ham F12 supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin solution for 24 hours. The following day, the medium was carefully removed and treated with control or 10 or 100 ng/mL rh-CSF-2 for 6 hours for RNA isolation and subsequent qPCR analysis.

Statistical Analysis

Normality of data was investigated using the Kolmogorov-Smirnov test. Immunohistochemistry HSCOREs and qPCR and fetal membrane strength results were normally distributed and analyzed by one-way analysis of variance, followed by post-hoc Tukey testing. Enzyme-linked immunosorbent assay results for CSF-2 secretion were not normally distributed and were analyzed using the Kruskal-Wallis test, followed by the post-hoc Student-Newman-Keuls test. Immunoblotting data from BeWo cell cultures and qPCR data from choriodecidual explants were analyzed using a *t*-test. Analyses were performed using SigmaStat version 3.0 (Systat Software, San Jose, CA). $P < 0.05$ was considered statistically significant.

Results

Increased CSF-2 Immunostaining at the Maternal-Fetal Interface, Obtained from Abruption-Associated PTB

Representative CSF-2 immunostaining from term specimens ($n = 10$) and gestational age-matched idiopathic (spontaneous; $n = 8$) and abruption-associated ($n = 8$) PTB samples are shown in *Figure 1*, A–C. Vimentin (red color) and cytokeratin (brown color) double immunostaining were used to differentiate decidual cells and trophoblasts, respectively (*Figure 1D*). Compared with term specimens (*Figure 1A*), abruption-associated PTB samples displayed stronger CSF-2 immunostaining, whereas CSF-2 immunostaining was similar in idiopathic and abruption-associated PTB samples (*Figure 1*, B and C). No immunoreactivity was observed in negative control slides (*Figure 1E*). HSCORE analysis confirmed significantly higher CSF-2 HSCOREs in idiopathic and abruption-associated PTB versus term specimens with respect to decidual cells ($P < 0.001$) (*Figure 1G*) and trophoblasts ($P < 0.05$) (*Figure 1H*). No differences in CSF-2 expression were observed at the maternal-fetal interface between term precontraction and term post-contraction specimens (data not shown). However, examination of immunostained slides under $\times 100$ magnification localized CSF-2 immunostaining primarily in the cytoplasm of decidual cells and the cell membrane of trophoblasts (*Figure 1F*), suggesting a potential *in situ* paracrine interaction mediated by binding of decidual-secreted CSF-2 to trophoblast-expressed CSF2R.

In Vitro Comparative Analysis of *CSF2* and *CSF2RA* Expression in TDCs, CTBs, and STBs

To extend the immunohistochemical observations of cytoplasmic and membranous CSF-2 immunostaining in decidual cells and trophoblasts, respectively (Figure 1F), mRNA expression levels of *CSF-2* and its receptor *CSF2RA* were evaluated in TDC, CTB, and STB cultures by qPCR. This analysis revealed 29-fold higher *CSF2* mRNA levels in TDCs compared with SBTs ($P < 0.05$), and *CSF2* mRNA levels were barely detectable in CTBs ($P < 0.05$) (Figure 2A). In contrast, *CSF2RA* mRNA levels are 13,000- and 20,000-fold higher in CTB and STB cultures, respectively, compared with TDC cultures ($P < 0.001$) (Figure 2B). Immunostaining of fetal membranes ($n = 4$) and decidua basalis ($n = 3$) from human placental specimens, obtained from term uncomplicated pregnancies, confirmed *in situ* expression of CSF2R α in chorionic extravillous trophoblasts in fetal membrane (Supplemental Figure S1) and interstitial cytotrophoblasts and multinucleated trophoblastic cells in decidua basalis (Supplemental Figure S2).

In Vitro Regulation of *CSF2* mRNA and Protein Levels by THR in TDCs

Increased abruption-related immunostaining for CSF-2 and higher *CSF2* expression in TDC cultures led to examination of the influence of THR, as a marker for abruption on *CSF2* mRNA levels in TDC cultures. Compared with E_2 as a control, THR enhanced *CSF2* mRNA levels in TDC cultures. Compared with E_2 as a control, THR enhanced *CSF2* mRNA levels by fivefold and fourfold in E_2 and $E_2 + MPA$ incubations, respectively ($n = 7$; $P < 0.05$) (Figure 3A). Although the addition of MPA did not affect basal *CSF2* mRNA levels, it significantly reduced THR-induced *CSF2* expression compared with $E_2 + THR$ [fold changes, 3.43 ± 0.47 versus 5.42 ± 0.60 (means $\pm$ SEM); $P < 0.05$].

In cultured TDCs, compared with E_2 , $E_2 + MPA$ did not affect CSF-2 protein levels. However, THR induced CSF-2 protein secretion by 25- and 10-fold in cultures treated with E_2 and $E_2 + MPA$, respectively ($P < 0.05$) (Figure 3B). To investigate the effect of steroids in THR-induced *CSF2* mRNA expression in TDCs, cells were also treated with dexamethasone, a pure glucocorticoid, or Organon 2058, a pure progestin.¹² Similar to MPA treatment, coinubation with dexamethasone or Organon 2058 significantly reduced THR-induced *CSF2* mRNA levels 2.2- or 1.9-fold, respectively ($n = 6$; $P < 0.05$) (Figure 3C) compared with $E_2 + THR$ -treated TDC cultures, suggesting that MPA-mediated inhibition of THR-induced *CSF2* gene expression is facilitated by either glucocorticoid or progesterone actions.

Fetal Membrane Strength Testing

Addition of the human recombinant CSF-2 on the choriondecidual side of the fetal membranes causes fetal membrane

Figure 3 Thrombin (THR)-mediated *in vitro* regulation of *CSF2* mRNA and protein levels in term decidual cell cultures. **A:** Confluent leukocyte-free term decidual cells were primed with estradiol (E_2 ; 10^{-8} mol/L) or $E_2 +$ medroxyprogesterone acetate (MPA; 10^{-7} mol/L), then treated with $E_2 + THR$ (1 U/mL) or $E_2 + MPA + THR$. *CSF2* mRNA levels were measured by real-time quantitative PCR and normalized to β -actin. **B:** Secreted CSF-2 levels were measured by enzyme-linked immunosorbent assay in conditioned media collected from primed term decidual cells treated with E_2 (10^{-8} mol/L), $E_2 + THR$ (1 U/mL), $E_2 + MPA$ (10^{-7} mol/L), or $E_2 + MPA + THR$ and normalized to total protein. **C:** In parallel conditions, thrombin-mediated *in vitro* regulation of *CSF2* mRNA in term decidual cells were treated with E_2 (10^{-8} mol/L), $E_2 + THR$ (1 U/mL), $E_2 +$ dexamethasone (Dex; 10^{-7} mol/L), $E_2 + Dex + THR$, $E_2 +$ Organon 2058 (Org; 10^{-7} mol/L), or $E_2 + Org + THR$. Data are expressed as means $\pm$ SEM (A–C). $n = 7$ (A); $n = 8$ (B); $n = 6$ (C). * $P < 0.05$ versus E_2 ; † $P < 0.05$ versus $E_2 + MPA$; ‡ $P < 0.05$ versus $E_2 + THR$; § $P < 0.05$ versus $E_2 + Dex$; ¶ $P < 0.05$ versus $E_2 + Org$.

Figure 4 Thrombin (THR)—induced fetal membrane weakening. Condition media obtained from term decidua cells, as mentioned in [Materials and Methods](#), treated with 10^{-8} mol/L estradiol (E₂) used as a control, E₂ + 1 U/mL THR, E₂ + 10^{-7} mol/L medroxyprogesterone acetate (MPA), or E₂ + MPA + THR, were applied to fetal membranes obtained from term placental specimens for 24 hours and fetal membrane strength was measured. Data are expressed as means $\pm$ SEM. $n = 4$. * $P < 0.05$ versus E₂ as a control; † $P < 0.05$ versus E₂ + THR.

weakening, showing that CSF-2 mediates both THR- and tumor necrosis factor- α —induced fetal membrane weakening.¹¹ To determine whether THR-induced TDC-secreted CSF-2 contributes to fetal membrane weakening, TDCs were treated with 10^{-8} mol/L E₂ as a control or E₂ + 10^{-7} mol/L MPA to simulate the hormonal milieu of pregnancy, with or without 1 U/mL of THR for only 4 hours. To eliminate the direct effect of THR in TDC cultures, after 4 hours, TDCs were washed with phosphate-buffered saline and then incubated in control media, expanding on our previous results.¹⁹ CMS obtained from TDC-treated conditions after 24 hours of incubation revealed that THR pretreatment causes fetal membrane weakening ($P < 0.05$) and that the addition of MPA reversed this THR-mediated action ([Figure 4](#)). These results showing THR-induced TDC-secreted protein(s) (ie, CSF-2 reduces fetal membrane tensile strength) complement our previous observation that preincubation with CSF-2 neutralizing antibody blocks THR-induced fetal membrane weakening.¹⁹

ERK1/2 and NF- κ B Signaling Pathways Mediate THR-Induced CSF-2 Expression in TDC Cultures

THR activates several intracellular signaling pathways, including NF- κ B, ERK1/2, and p38 MAPKs, in TDCs.¹³ To identify signaling pathways responsible for THR-induced CSF2 expression, TDCs were treated with specific inhibitors of these potential intracellular signaling pathways. qPCR analysis in TDCs treated with 10^{-8} mol/L E₂ + 10^{-7} mol/L MPA + 1 U/mL THR $\pm$ 2×10^{-5} mol/L PD98059, an ERK1/2 MAPK inhibitor, 2×10^{-5} mol/L SB203580, a p38 MAPK inhibitor, or 10^{-6} mol/L Bay-III, an NF- κ B activation inhibitor, revealed that both the ERK1/2 MAPK and NF- κ B inhibitors, but not the p38 MAPK inhibitor,

significantly reduced THR-induced CSF2 mRNA levels in TDCs ($n = 3$; $P < 0.05$) ([Figure 5](#)).

TDC-Secreted CSF-2 Activates STAT5 and Inhibits p38 MAPK Pathways in Trophoblasts

To identify mechanisms associated with TDC-secreted CSF-2—mediated paracrine activation of intracellular signaling pathways in trophoblasts, BeWo cells (a trophoblast cell line) were treated with control or rh-CSF-2 (10 ng/mL) for 15 minutes and then subjected to immunoblotting to determine activation status of several crucial intracellular signaling molecules by evaluating both the total and phosphorylated forms. Immunoblotting results revealed a significant 3.7-fold increase in STAT5 phosphorylation ($P < 0.05$) and a 1.5-fold reduction in p38 MAPK phosphorylation ($P < 0.05$) in BeWo cells treated with rh-CSF-2 versus control, whereas STAT3, ERK1/2, AKT, and NF- κ B p65 phosphorylation levels were not changed ([Figure 6](#)). To determine the presence of active STAT5 levels in chorionic extravillous trophoblasts, fetal membranes ($n = 4$) and decidua basalis ($n = 3$) were immunostained for active (phosphorylated) STAT5, which revealed phosphorylated STAT5 immunoreactivity in chorionic extravillous trophoblasts ([Supplemental Figure S3](#)), supporting induction of phosphorylated STAT5 by CSF-2 in choriodecidual explants.

In Vitro Regulation of IL1 β and MMP9 mRNA Levels by CSF-2 in Choriodecidual Explants

Previous studies reported CSF-2 levels in amniotic fluid ranging from undetectable to 20 ng/L in pregnancies with

Figure 5 Thrombin (THR)—induced CSF2 expression in term decidua cultures is mediated by extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) and NF- κ B signaling pathways. CSF2 mRNA levels in term decidua cells treated with 1 U/mL THR, THR + 2×10^{-5} mol/L ERK1/2 MAPK inhibitor PD98059 (PD), THR + 2×10^{-5} mol/L p38 MAPK inhibitor SB203580 (SB), or THR + 10^{-6} mol/L NF- κ B inhibitor Bay II-7082 (Bay) on thrombin-induced CSF2 mRNA expression. Data are expressed as means $\pm$ SEM. $n = 3$. * $P < 0.05$ versus E₂ + medroxyprogesterone acetate (MPA) + THR.

Figure 6 CSF-2-mediated activation of STAT5 and inhibition of p38 mitogen-activated protein kinase (MAPK) pathways in BeWo trophoblastic cell lines. Representative immunoblotting of BeWo cells, which were treated with control (Con) or 10 ng/mL recombinant CSF-2 for 15 minutes, displays phosphorylated (Ph-) or total (T-) levels of STAT5, p38 MAPK, p65 NF-κB subunit, AKT, extracellular signal-regulated kinase (ERK) 1/2 MAPK, and STAT3, as well as β-actin. Graphs display immunoblot densitometry readings obtained from experimental incubations of cells for phosphorylated/total forms of each protein after being normalized to β-actin. Data are expressed as means ± SEM. $n = 3$. * $P < 0.05$ versus control. Exp., experiment.

intact membranes before 33 weeks of gestation, whereas CSF-2 levels in PPRM cases are significantly elevated, reaching up to 400 ng/L during the same period.^{20,21} To elucidate the pivotal role of TDC-secreted CSF-2 in

abruption-associated PTBs, explant cultures of choriodecidual membranes obtained from a placenta at 33 weeks of pregnancy were treated with control or rh-CSF-2 (10 or 100 ng/mL) for 6 hours to evaluate mRNA levels of *MMP3*,

Figure 7 CSF-2 up-regulates IL-1β and matrix metalloproteinase (MMP)-9 expression in chorio-decidual explant culture. Explant cultures were treated with vehicle [control (Cont)] or 100 ng/mL recombinant CSF-2 for 6 hours. Real-time quantitative PCR for *IL1B*, *IL6*, *IL8*, *MMP9*, *MMP3*, and *MMP7* mRNA levels was measured and normalized to β-actin. **A:** Treatment of 100 ng/mL recombinant CSF-2 significantly increases *IL1B* ($P < 0.05$) mRNA levels versus control. **B** and **C:** Expression levels of *IL6* (**B**) and *IL8* (**C**) do not change by CSF-2. **D:** CSF-2 significantly increases *MMP9* ($P < 0.05$) mRNA levels in chorio-decidual explant culture versus control. **E** and **F:** CSF-2 treatment does not change expression levels of *MMP3* (**E**) and *MMP7* (**F**) mRNAs. Data are expressed as means ± SEM (**A–F**). * $P < 0.05$ versus Cont.

Figure 8 Bidirectional cross talk between decidual cells and trophoblasts induces abruption-associated preterm birth (PTB) and preterm premature rupture of the membranes (PPROM). In the presence of decidual hemorrhage, thrombin (Thr), generated from decidual cell–expressed tissue factor (TF), binding to protease-activated receptor (PAR)-1 receptors activates NF-κB and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase signaling pathways. Both pathways enhance production of several cytokines (IL-8 and CSF-2) to recruit neutrophils that elaborate various matrix metalloproteinases (MMPs) as well as other proteinases that promote fetal membrane rupture. Thr also suppresses decidual cell progesterone receptor (PR) levels by increasing ERK1/2 phosphorylation, resulting in functional progesterone withdrawal in the decidua that further induces MMP expression and decidual inflammation. Secreted CSF-2 (paracrine or exosome mediated) binding to CSF-2 receptor (CSF2R) expressing trophoblast cells activates the STAT5 signaling pathway to induce IL-1β and MMP-9 expression. In decidual cells, IL-1β activates NF-κB and other signaling pathways by enhancing expression of proinflammatory cytokines and MMPs and reduces PR levels to cause functional progesterone (P4) withdrawal. Taken together, functional P4 withdrawal, increased inflammatory cytokines (IL-8, IL-1β, and CSF-2), and MMPs directly or indirectly trigger fetal membrane rupture, leading to PPROM and/or abruption-associated PTB. ECM, extracellular matrix; Ph-, phosphorylated.

MMP7, and *MMP9* as well as proinflammatory cytokines *IL1B*, *IL6*, and *IL8*. This qPCR analysis revealed that 10 ng/mL rh-CSF-2 treatment did not significantly change mRNA levels of any of these genes (Supplemental Figure S4), whereas 100 ng/mL rh-CSF-2 treatment significantly increased *IL1B* (fourfold; $P < 0.05$) and *MMP9* (threefold; $P < 0.05$) levels in explant cultures versus control treatment (Figure 7, A and D); *IL6*, *IL8*, *MMP3*, and *MMP7* levels did not change (Figure 7, B, C, E, and F).

Discussion

These results elucidate pathways contributing to abruption-associated PTB. Our observations at the maternal-fetal interface demonstrate CSF-2 immunoreactivity in decidual cell cytoplasm and its receptor, CSF2RA, in trophoblast cell membranes, suggesting the existence of a potential paracrine mechanism whereby decidual cell–secreted CSF-2 binds to trophoblast-expressed CSF2R. *In vitro* observations confirming significantly higher expression of *CSF2* mRNA in TDCs and *CSF2RA* mRNA in CTB and STB cultures support this proposed mechanism. Furthermore, THR-treated TDC-secreted CSF-2 significantly weakened fetal membranes obtained from term placentas; and using

signaling pathway inhibitors, NF-κB and ERK1/2 MAPK, documented inducers of inflammatory gene expression,²² were identified as main signaling mediators of THR-induced *CSF2* gene expression in TDCs.

Strong evidence supports the association of decidual hemorrhage with approximately 10% of all bleeding-related PTBs. In most cases, vaginal bleeding arises from hemorrhage into the decidua basalis (placental abruption) or decidua parietalis (subchorionic hematoma).¹⁷ Decidual hemorrhage complicates almost 40% of PTBs due to preterm labor with intact membranes and PPROM between 22 and 38 weeks versus 0.8% post-term delivery.¹⁰ Despite its large public health impact, the precise mechanisms causing PTB are incompletely understood, limiting development of preventive and therapeutic interventions.

In most mammals, labor is initiated by a rapid decrease in circulating maternal P4 levels. In humans, however, elevated P4 levels in maternal plasma as well as fetal and amniotic fluid are sustained until delivery of the fetal-placental unit, suggesting that parturition is initiated in response to functional P4 withdrawal elicited by a decline in progesterone receptor levels and/or a shift in the progesterone receptor isoforms.^{12,13,18,23–25} THR contributes to PPROM, preterm labor, and abruption-associated PTB by multiple pathways²⁶ summarized in Figure 8. These include

THR: i) activation of ERK1/2 MAPK signaling and subsequent inhibition of progesterone receptor mRNA and protein expressions as well as reduction of the binding of progesterone receptor to its DNA response element in TDCs, leading to functional P4 withdrawal¹³; ii) up-regulation of extracellular matrix-degrading *MMP1* and *MMP9*^{27–29} mRNA transcription and protein secretion, contributing to fetal membrane weakening; and iii) up-regulation of IL-8 expression levels by promoting neutrophil trafficking into the hemorrhagic decidua by activating NF-κB signaling pathways³⁰ (Figure 8).

Mounting evidence indicates that exosomes are one of the key mediators of indirect cell-cell interactions in the pathophysiological regulation of the placenta.^{31–33} Thus, exosomes may mediate paracrine CSF2-CSF2R interactions between decidual cells and trophoblasts at the maternal fetal interface and/or choriondecidua of the fetal membrane. As the role played by increased neutrophil infiltration into the maternal-fetal interface in PTB became more evident, the current findings shed light on the involvement of THR-induced TDC-secreted CSF-2 in fetal membrane weakening, PPRM, and PTB.^{11,19,34} The growing body of evidence supporting the association between elevated THR levels with abruptio-associated PTB^{13,26} prompted this study to explore the involvement of CSF-2 in abruptio-associated PTB and the associated intracellular signaling pathways involved in the pathophysiological role played by CSF-2.

Colony-stimulating factors are well-established hematopoietic growth factors. Recent studies showed that CSF-2 also acts on nonhematopoietic cell types, including astrocytes,³⁵ cardiomyocytes,³⁶ and trophoblasts.³⁷ Chemotactic properties of CSF-2 include recruitment of monocytes³⁸ as well as neutrophils.^{39,40} The current results revealed the presence of significantly higher CSF-2 expression at the maternal-fetal interface of abruptio-associated or idiopathic PTBs versus term specimens. Similar CSF-2 levels in abruptio-associated and idiopathic PTB specimens suggest that CSF-2-mediated paracrine signaling is a common contributor to PTB. In cases of abruptio-associated PTB, the current *in vitro* observations suggest that THR is the primary factor responsible for the increase in CSF-2 expression. On the other hand, the factors responsible for increased CSF-2 expression in idiopathic PTB are unknown and require further investigation but could reflect occult hemorrhage.^{41,42}

The current *in vitro* results confirm our immunohistochemical results that TDC-derived THR induces *CSF2* mRNA and protein secretion. THR-induced CSF-2 levels were significantly blunted by treatment of either MPA or dexamethasone, pure glucocorticoid, or Organon 2058 as a pure progestin, suggesting the potential protective effect of P4 or glucocorticoid against abruptio-associated PTB; and THR-treated TDC-secreted CSF-2 significantly weakened fetal membranes obtained from term placentas. The current results support our previous study demonstrating that in the choriondecidua, THR induces fetal membrane weakening by

elevating CSF-2 levels, which is inhibited by blockade of CSF-2 action with a neutralizing antibody.¹¹

By using specific signaling pathway inhibitor(s), the current study identified NF-κB and ERK1/2 MAPK, documented inducers of inflammatory gene expression,²² as main signaling mediators of THR-induced *CSF2* gene expression in TDCs. Moreover, the current *in vitro* observations in BeWo cells, demonstrating activation of STAT5 and inhibition of p38 MAPK signaling by rh-CSF-2, suggest that THR-induced TDC-secreted CSF-2 acts as a paracrine inducer in trophoblasts that may contribute to fetal membrane weakening and thus induce abruptio-associated PTB. This suggestion was confirmed in choriondecidua explant cultures in which treatment with rh-CSF-2 enhances *IL1B* and *MMP9* mRNA expression. These results suggest that excess CSF-2 acts as a critical common mediator of PTB by contributing to abruptio-induced inflammation and fetal membrane weakening (Figure 8).

In conclusion, current immunohistochemical and *in vitro* results suggest that THR-induced excess CSF-2 levels trigger a paracrine signaling in adjacent trophoblast that induces inflammation and fetal membrane weakening, contributing to abruptio-associated PTB. Considered together, these observations suggest that CSF-2-mediated signaling pathways in TDCs or trophoblasts (ie, ERK1/2, NF-κB, or STAT5) are likely to be one of the mechanisms involved in abruptio-associated PTB.

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Supplemental Data

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