Novel anti-inflammatory actions of TIPE2 in human primary amnion and myometrial cells

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Abstract

Inflammation plays a pivotal role in the terminal process of human labor and delivery, including myometrial contractions and membrane rupture. TNF-alpha-induced protein 8-like-2 (TIPE2) is a novel inflammation regulator; however, there are no studies on the role of TIPE2 in human labor. We report that in myometrium, there is decreased *TIPE2* mRNA expression during late gestation which was further decreased in labor. In fetal membranes, *TIPE2* mRNA expression was decreased with both term and preterm labor compared to no labor samples. Knockdown of TIPE2 by siRNA in primary myometrium and amnion cells was associated with an augmentation of IL1B and TNF-induced expression of pro-inflammatory cytokines and chemokines; expression of contraction-associated proteins and secretion of the uterotonic prostaglandin PGF_{2α} and expression of extracellular matrix degrading enzymes. In TIPE2-deficient myometrial cells treated with inhibitors of NF-κB or ERK1/2, the secretion of pro-labor mediators was reduced back to control levels. In conclusion, these *in vitro* experiments indicate that loss of TIPE2 exacerbates the inflammatory response.

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Introduction

Preterm birth continues to be the main causation of neonatal morbidity and mortality (Blencowe *et al.* 2013). The dearth of effective strategies for idiopathic preterm labor currently in effect is due to only partial knowledge of the underlying mechanisms that drive human labor and delivery. It is widely recognized that inflammation contributes to the processes of human labor and delivery (Christiaens *et al.* 2008, Shynlova *et al.* 2013). Activation of the maternal immune system can occur physiologically (at term) or pathologically (at preterm) to induce an influx of inflammatory cells into fetal membranes, placenta and uterus (Thomson *et al.* 1999, Osman *et al.* 2003). Interleukin 1 beta (IL1B) and tumor necrosis factor alpha (TNF) are two pro-inflammatory cytokines that are secreted by activated leukocytes. They activate the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) (Lappas *et al.* 2002, 2003, Lindstrom & Bennett 2005, Lappas & Rice 2007) and mitogen-activated protein kinases (MAPKs) (Sooranna *et al.* 2005, Lappas *et al.* 2007, 2011b) to regulate the expression of many genes involved in the terminal effector pathways of human labor and delivery in myometrium and fetal membranes. These include, the pro-inflammatory cytokines TNF, IL1A, IL1B and IL6, the chemokines CCL2, CXCL1 and CXCL8, contraction-associated proteins such as prostaglandin-endoperoxide

synthase 2 (PTGS2), prostaglandin F receptor (PTGFR), connexin 43 (Cx43) and the oxytocin receptor (OXTR) and extracellular matrix-(ECM) degrading enzymes such as matrix metalloproteinase 9 (MMP9).

TNF-alpha-induced protein 8-like-2 (TNFAIP8L2 or TIPE2), a member of the TNFAIP8/TIPE family, negatively regulates inflammation (Sun *et al.* 2008, Lou *et al.* 2013, Goldsmith & Chen 2017, Lin *et al.* 2018). TIPE2 was originally identified in lymphoid tissues in mice (Sun *et al.* 2008); however, human TIPE2 is more widely expressed in a wide variety of hematopoietic and non-hematopoietic cell types (Zhang *et al.* 2011). TIPE2 has a distinct death effector domain-like domain that is essential for maintaining innate and adaptive immune homeostasis (Freundt *et al.* 2008, Sun *et al.* 2008). Deletion of TIPE2 in mice leads to multiorgan inflammation, splenomegaly, premature death and hypersensitivity to inflammatory insults (Sun *et al.* 2008, 2012). Likewise, knockdown of TIPE2 augments the expression of pro-inflammatory cytokines, chemokines and ECM-degrading enzymes in response to various inflammatory insults (Lou *et al.* 2013, Suo *et al.* 2016, Sun *et al.* 2017a, Zhang *et al.* 2017, Lin *et al.* 2018). Conversely, TIPE2 overexpression suppresses the expression of these inflammatory genes (Zhang *et al.* 2015, Sun *et al.* 2017a, Lin *et al.* 2018). Emerging evidence suggests that TIPE2 negatively regulates inflammation by inhibiting the activation of NF-κB (Sun

et al. 2008, Lou *et al.* 2013, Goldsmith & Chen 2017, Lin *et al.* 2018) and MAPK (Sun *et al.* 2008, Lou *et al.* 2013, Zhang *et al.* 2017, Lin *et al.* 2018).

The expression of TIPE2 is downregulated in a number of diverse immunological diseases including cancer (Gus-Brautbar *et al.* 2012), systemic lupus erythematosus (Li *et al.* 2009), diabetic nephropathy (Zhang *et al.* 2010), hepatitis B virus infection (Xi *et al.* 2011), respiratory infection (Zhang *et al.* 2017), asthma (Ma *et al.* 2013, Sun *et al.* 2017a), atherosclerosis (Lou *et al.* 2013) and colitis (Lou *et al.* 2014). With respect to pregnancy, TIPE2 expression is lower in decidual tissues of patients with missed abortion than healthy controls (Sun *et al.* 2017b). There are, however, no studies on the role of TIPE2 in human labor. To address this issue, we characterized TIPE2 expression in human myometrium and fetal membranes from laboring and non-laboring women and examined the effects of TIPE2 knockdown on pro-inflammatory and pro-labor mediators. For the functional studies we used IL1B and TNF as these are the two pro-inflammatory cytokines that play critical roles in stimulating pro-inflammatory and pro-labor mediators (Lappas 2017, Lim *et al.* 2017). Notably, they have also been shown to induce preterm labor in various animal species (Romero *et al.* 1991, Sadowsky *et al.* 2006).

Materials and methods

Tissue collection

Placenta (with attached fetal membranes) and myometrium were obtained, with Institutional Research and Ethics Committee approval, from women at the time of delivery. All women provided written informed consent. Exclusion criteria included women with any underlying medical conditions such as diabetes, asthma, polycystic ovary syndrome, preeclampsia and macrovascular complications, multiple pregnancies and obese women (BMI > 30).

Myometrium was obtained from the upper margin of the lower uterine segment incision during Cesarean section.

Placenta and myometrium was brought to the research laboratory and processed within 15 min of delivery. Fetal membranes were removed from placenta, washed extensively in PBS and then dissected into smaller pieces. Tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for expression studies or used immediately for cell culture experiments.

To characterize temporal-associated changes in TIPE2 expression, myometrium and fetal membranes were obtained from women at preterm (<37 week gestation) or term (37–41 weeks gestation) Cesarean section in the absence of labor ($n=9$ patients per group). In the term group, indications for Cesarean section in the absence of labor were breech presentation and/or previous Cesarean section. In the preterm group, indications for Cesarean section in the absence of labor were placenta praevia, placental abruption and antepartum hemorrhage. The relevant clinical characteristics of the patients used are detailed in Table 1.

To characterize labor-associated changes in TIPE2 expression in myometrium, samples were obtained from women at term Cesarean section (i) in the absence of labor or (ii) during active spontaneous labor ($n=8$ patients per group) as previously described (Lim *et al.* 2018). Labor was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation. None of the patients received any medications to augment or induce labor. Indications for Cesarean section in the absence of labor were breech presentation and/or previous Cesarean section. Indications for Cesarean section in the laboring samples were for fetal malpresentation, fetal distress and delayed or failure to progress. The relevant clinical characteristics of the patients used are detailed in Table 2.

To characterize labor-associated changes in TIPE2 expression in fetal membranes, samples were obtained from women at preterm or term (i) undergoing elective Cesarean section in the absence of labor or (ii) after spontaneous labor and vaginal delivery ($n=9$ patients per group). Fetal membranes from the non-laboring group were obtained from the area overlying the cervix (i.e. supracervical site, SCS) as previously described (Lappas *et al.* 2011a). In the after labor group, fetal membranes were obtained from the

Table 1 Clinical characteristics of the patients used for temporal-associated changes in TIPE2.

	Fetal membranes		Myometrium	
	Preterm ($n=9$)	Term ($n=9$)	Preterm ($n=9$)	Term ($n=9$)
Maternal age (years)	32.3 (2.33)	30.6 (2.1)	32.7 (1.6)	31.6 (1.5)
Pre-pregnancy maternal BMI	23.5 (1.8)	24.2 (1.6)	21.8 (0.9)	23.5 (1.2)
Gravida	2.6 (0.6)	3.6 (0.7)	2.0 (0.2)	2.1 (0.2)
Parity	2.0 (0.4)	2.7 (0.4)	1.6 (0.2)	2.0 (0.2)
Gestational age (weeks)	33.3 (0.8)	39.3 (0.3)*	34.4 (0.2)	38.8 (0.2)*
Fetal gender	5 female; 4 male	5 female; 4 male	7 female; 2 male	2 female; 7 male
Birthweight (g)	1647 (252)	3429 (120)*	2156 (124)	3416 (148)*
Labor				
No labor (%)	100	100	100	100
Spontaneous (%)	0	0	0	0
Membrane Rupture				
SROM (%)	0	0	0	0
ARM (%)	100	100	100	100

Values represent mean ($\pm$ S.E.M.) unless otherwise specified.

* $P < 0.05$ vs preterm (Student's *t*-test).

Table 2 Clinical characteristics of the patients used for term labor-associated changes in TIPE2.

	Fetal membranes		Myometrium	
	Term no labor (n=9)	Term after labor (n=9)	Term no labor (n=8)	Term in labor (n=8)
Maternal Age (years)	30.6 (2.1)	29 (2.3)	32.9 (1.3)	31.5 (1.6)
Pre-pregnancy maternal BMI	24.2 (1.6)	24.1 (1.2)	22.6 (1.1)	22.4 (1.0)
Gravida	3.6 (0.7)	2.8 (0.6)	2.6 (0.3)	2.0 (0.4)
Parity	2.3 (0.2)	1.6 (0.3)	1.3 (0.2)	0.5 (0.3)
Gestational age (weeks)	39.3 (0.3)	40.4 (0.2)	38.9 (0.2)	39.6 (0.5)
Fetal gender	5 female; 4 male	2 female; 7 male	4 female; 4 male	3 female; 5 male
Birthweight (g)	3429 (120)	3466 (133)	3318 (216)	3582 (77)
Labor				
No labor	100%	0%	100%	0%
Spontaneous	0%	100%	0%	100%
Duration of labor (min)	N/A	511 (127)	N/A	668 (90)
Membrane Rupture				
SROM	0%	100%	0%	12.5%
PROM	0%	0%	0%	25%
ARM	100%	0%	100%	62.5%

Values represent mean ($\pm$ S.E.M.) unless otherwise specified.

site of membrane rupture as previously described (Lappas et al. 2011a). None of the patients received any medications to augment or induce labor or had prelabor rupture of membranes. The relevant clinical characteristics of the patients used are detailed in Tables 2 and 3.

Primary myometrial and amnion epithelial and mesenchymal cell culture

Fresh myometrium and amnion were obtained from women who delivered healthy, singleton infants at term undergoing elective Cesarean section in the absence of labor (n=6–8 patients). Myometrial cells were isolated and cultured as previously described (Lim et al. 2013a). Isolation of amnion epithelial cells and mesenchymal cells was performed as described (Moore et al. 2009) with minor modifications. Briefly, approximately 3 g of amnion fragments were incubated in 10 mL of DMEM/F-12 with 1% penicillin-streptomycin and 0.25% trypsin for 35 min at 37°C with gentle agitation. After straining through a 100 μ m cell strainer, the eluate was neutralized with 1% FBS and remaining fragments were further digested in another 10 mL of DMEM/F-12 with 0.25% trypsin for 35 min at 37°C. Eluate was combined with previous digest and epithelial cells were pelleted by centrifugation at 500g for 10 min, resuspended in complete media (DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin) and plated in tissue culture flasks. Flasks were incubated at 37°C, 5% CO₂ and 8% O₂ with media changes 4 h after initial plating, and then 24–48 h thereafter until confluent. The de-epithelialized amnion fragments were washed extensively in PBS and then incubated in 10 mL DMEM/F-12 containing 1% penicillin-streptomycin and 0.125% collagenase A at 37°C for 1 h with gentle agitation, until fragments had dissolved. After straining, eluate was centrifuged at 500g for 10 min. Pelleted mesenchymal cells were plated in complete media (DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin) in tissue culture flasks. Upon confluency (3–5 days), epithelial and mesenchymal cells were trypsinized and plated in 48-well plates in complete media for siRNA transfection experiments.

Transfection of primary myometrial and amnion cells

Transfection of myometrial and amnion cells with TIPE2 siRNA (siTIPE2; Ambion (Thermo Fisher Scientific)) or negative control siRNA (siCONT; Ambion (Thermo Fisher Scientific)) was performed using RNAiMax (Life Technologies) according to manufacturer's guidelines. Myometrial cells (n=8 patients), amnion mesenchymal cells (n=6 patients) and amnion epithelial cells (n=6 patients) were transfected with 25 nM siTIPE2 or 25 nM siCONT for 48 h, followed by treatment with 1 ng/mL IL1B or 10 ng/mL TNF for an additional 20 h. Myometrial cells (n=5 patients) were also transfected with 25 nM (equivalent to 83 ng) siTIPE2 or 25 nM (83 ng) siCONT for 48 h, followed by treatment with 1 ng/mL IL1B

Table 3 Clinical characteristics of the patients used for preterm labor-associated changes in TIPE2.

	Fetal membranes	
	Preterm no labor (n=9)	Preterm after labor (n=9)
Maternal age (years)	32.3 (2.3)	33.1 (0.8)
Pre-pregnancy maternal BMI	23.5 (1.8)	25.0 (1.5)
Gravida	2.6 (0.6)	2.6 (0.6)
Parity	2.0 (0.4)	1.6 (0.2)
Gestational age (weeks)	33.3 (0.8)	33.6 (0.7)
Fetal gender	5 female; 4 male	2 female; 7 male
Birthweight (g)	1647 (252)	1859 (177)
Delivery mode		
Vaginal delivery	0%	100%
Cesarean section	100%	0%
Labor		
No labor	100%	0%
Spontaneous	0%	100%
Duration of labor (min)	N/A	357 (92)
Membrane Rupture		
SROM	0%	44.4%
PROM	0%	0%
ARM	100%	55.6%
Steroids	100%	77.8%

Values represent mean ($\pm$ S.E.M.) unless otherwise specified.

in the absence or presence of 10 μ M BAY 11-7082, 5 μ M U0126 or 400 μ M naringenin for an additional 20h. After final incubation, cells were collected and stored at -80°C until assayed for mRNA expression by qRT-PCR and protein expression by Western blotting as detailed below. Media was collected and stored at -80°C until assayed for cytokine and prostaglandin release as detailed below. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay as we have previously described (Lim et al. 2014).

RNA extraction and qRT-PCR

RNA extractions, cDNA synthesis and qRT-PCR were performed as previously described (Lim et al. 2013b) using 100nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen). Target gene Ct values were normalized to the average YWHAZ and succinate dehydrogenase (SDHA) Ct values of the same cDNA sample and fold differences determined using the comparative Ct method. Of note, there was no effect of treatment on YWHAZ or SDHA Ct values.

Cytokine and prostaglandin assays

The levels of IL6, CCL2, CXCL1 and CXCL8 in the incubation media was measured by sandwich ELISA from R&D Systems according to the manufacturer's instructions. The release of $\text{PGF}_{2\alpha}$ into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Cayman Chemical Company). The interassay and intraassay coefficients of variation for all assays were less than 10%.

Gelatin zymography

Incubation media were also collected and assessment of MMP9 was performed by gelatin zymography as previously described (Lim et al. 2013b). Gels were scanned using a ChemiDoc XRS system (Bio-Rad Laboratories), inverted and densitometry performed using Quantity One image analysis software (Bio-Rad Laboratories).

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software). Normality of the data was assessed using the Shapiro–Wilk test. For two sample comparisons, unpaired Student's *t*-test was used to assess the statistical significance between normally distributed data; otherwise, the nonparametric Mann–Whitney *U* (unpaired) test was used. For all other comparisons, non-normalized data were logarithmically transformed before analysis by a repeated-measures one-way ANOVA (with LSD *post hoc* testing to discriminate among the means). Statistical significance was ascribed to a *P* value ≤ 0.05 . Data are expressed as mean $\pm$ S.E.M.

Results

Temporal and labor-associated changes in *TIPE2* expression in myometrium and fetal membranes

As shown in Fig. 1A, in myometrium collected from women in the absence of labor, there was a significant decrease in *TIPE2* mRNA expression in myometrium from women at term compared to preterm gestation. Fig. 1B demonstrates that at term, there is a significant decrease in *TIPE2* mRNA expression in myometrium collected in labor compared to myometrium collected before the onset of labor. In fetal membranes, there was no change in *TIPE2* mRNA expression with regards

Figure 1 Temporal and labor-associated changes in *TIPE2* expression in myometrium and fetal membranes. (A) Myometrium was obtained from women at preterm Cesarean section in the absence of labor (preterm, *n*=9 patients) or from women at term Cesarean section in the absence of labor (term, *n*=9 patients). (B) Myometrium was obtained from women at term Cesarean section in the absence of labor (term no labor, *n*=8 patients) or from women at term Cesarean section during labor (term in labor, *n*=8 patients). (C) Fetal membranes were obtained from women at preterm Cesarean section in the absence of labor (preterm, *n*=9 patients) or from women at term Cesarean section in the absence of labor (term, *n*=9 patients). (D) Fetal membranes were obtained from women at term Cesarean section in the absence of labor (term no labor, *n*=9 patients) or from women after term spontaneous labor onset and delivery (term after labor, *n*=9 patients). (E) Fetal membranes were obtained from women at preterm Cesarean section in the absence of labor (preterm no labor, *n*=9 patients) or from women after preterm spontaneous labor onset and delivery (preterm after labor, *n*=9 patients). *TIPE2* mRNA expression was analyzed by qRT-PCR. Individual data points represent different patients and the horizontal line represents the mean $\pm$ S.E.M. of each group. **P* ≤ 0.05 , unpaired Student's *t*-test (panels A, B and C) or Mann–Whitney *U* test (panels D and E).

to term or preterm gestation (Fig. 1C). On the other hand, there were significant decreases in *TIPE2* mRNA expression after spontaneous labor and delivery at both term (Fig. 1D) and preterm (Fig. 1E) gestations when compared to non-laboring samples.

Effect of *TIPE2* on pro-inflammatory cytokines and chemokines in human primary myometrial and amnion cells

To determine if *TIPE2* regulates cytokine-induced expression of pro-labor mediators, primary cells isolated from human myometrium and amnion (epithelial and mesenchymal cells) were transfected with *TIPE2* siRNA (siTIPE2). The efficacy of siTIPE2 transfection was assessed by qRT-PCR. When compared to siCONT-transfected cells, *TIPE2* mRNA expression was significantly decreased in siTIPE2 transfected myometrial cells (82% decrease), amnion mesenchymal cells (76% decrease) and amnion epithelial cells (83% decrease). There was no effect of siTIPE2 on cell viability as determined by MTT cell viability assay (data not shown).

Figure 2 demonstrates the effect of siTIPE2 transfection on IL1B and TNF induced pro-inflammatory cytokines in myometrial cells and amnion mesenchymal cells. Table 4 demonstrates the effect of siTIPE2 transfection on TNF-induced pro-inflammatory cytokines in amnion epithelial cells. In myometrial cells and amnion mesenchymal cells,

there was an expected increase in *IL1A* and *IL6* mRNA expression and release of IL6 in siCONT-transfected cells treated with IL1B and an increase in *IL1A*, *IL1B* and *IL6* mRNA expression and release of IL6 in siCONT cells treated with TNF (Fig. 2A, B, C, D, E, F and G for myometrium, Fig. 2H, I, J, K, L, M and N for amnion mesenchymal cells). Likewise, in siCONT-transfected amnion epithelial cells, TNF induced significant increases in *IL1A* and *IL6* mRNA expression and release of IL6. In myometrial cells, there was a significant increase in *IL1A* and *IL6* mRNA expression and release of IL6 in siTIPE2-transfected myometrial cells treated with IL1B, compared to IL1B-treated siCONT-transfected cells (Fig. 2A, B and C). There was also an increase in *IL1A* and *IL1B* mRNA expression in TNF-stimulated siTIPE2-transfected cells (Fig. 2D and E); however, there was no effect of siTIPE2 on TNF-induced IL6 mRNA expression and secretion (Fig. 2F and G). Similar results were obtained in amnion mesenchymal and epithelial cells, where siTIPE2-transfected cells significantly increased IL1B-induced *IL1A* and *IL6* mRNA expression and IL6 secretion (Fig. 2H, I, J and Table 4) and TNF-induced *IL1A* and *IL1B* mRNA expression (Fig. 2K, L and Table 4); there was no effect of siTIPE2 on TNF-induced IL6 mRNA expression and secretion (Fig. 2M, N and Table 4).

Figure 3 depicts the effect of siTIPE2 on chemokine expression and secretion in myometrium cells. Treatment of siCONT-transfected cells with both IL1B and TNF

Figure 2 Effect of *TIPE2* siRNA transfection on pro-inflammatory cytokines in myometrial and amnion mesenchymal cells. (A, B, C, D, E, F and G) Human primary myometrial cells or (H, I, J, K, L, M and N) amnion mesenchymal cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with 1 ng/mL IL1B or 10 ng/mL TNF ($n=6$ patients). (A, B, D, E, F, H, I, K, L and M) *IL1A*, *IL1B* and *IL6* mRNA expression was analyzed by qRT-PCR. (C, G, J and N) The concentration of IL6 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to siCONT+IL1B- or siCONT+TNF-transfected cells and data displayed as mean $\pm$ s.e.m. Individual data points represent six independent experiments. * $P \leq 0.05$ (repeated measures one-way ANOVA).

Table 4 Effect of siTIPE2 on expression and secretion of pro-labor mediators in human primary amnion epithelial cells.

	mRNA expression					Secretion			
	IL1A	IL6	CCL2	CXCL1	CXCL8	IL6	CCL2	CXCL1	CXCL8
siCONT	0.0±0.0*	0.0±0.0*	0.0±0.0*	0.0±0.0*	0.1±0.0*	0.0±0.0*	0.0±0.0*	0.01±0.0*	0.0±0.0*
siCONT+TNF	1.0±0.2	1.0±0.7	1.0±0.2	1.0±0.3	1.0±0.3	1.0±0.4	1.0±0.3	1.0±0.5	1.0±0.2
siTIPE2+TNF	1.8±0.4*	1.0±0.8	1.1±0.3	2.0±0.4*	2.0±0.5*	1.1±0.4	1.1±0.5	1.7±0.4*	1.6±0.3*

For all data, the fold change was calculated relative to siCONT+TNF-transfected cells and data displayed as mean±S.E.M.

* $P \leq 0.05$ vs siTIPE2+TNF-transfected cells (repeated-measures one-way ANOVA).

significantly increased CCL2, CXCL1 and CXCL8 mRNA expression and secretion. There was no effect of siTIPE2 on CCL2 mRNA expression or secretion when treated with IL1B or TNF (Fig. 3A, B, G and H). In siTIPE2-transfected cells there was a significant augmentation in both IL1B- and TNF-induced CXCL1 mRNA expression and secretion (Fig. 3C, D, I and J). In siTIPE2-transfected cells, there was a significant augmentation of CXCL8 mRNA expression when treated with both IL1B and TNF (Fig. 3E and K); however, there was only a significant increase in CXCL8 secretion in siTIPE2 cells treated with TNF (Fig. 3L), not IL1B (Fig. 3F).

In amnion mesenchymal cells (Fig. 4), siTIPE2-transfected cells significantly increased both IL1B- and TNF-induced CXCL1 and CXCL8 mRNA expression and secretion (Fig. 4C, D, E, F, I, J, K and L) and CCL2 secretion (Fig. 4B and H). There was a significant increase IL1B-induced CCL2 mRNA expression in siTIPE2-transfected cells (Fig. 4A), but there was no change in TNF-induced

CCL2 mRNA expression (Fig. 4G). Similarly, in amnion epithelial cells (Table 4), the effect of siTIPE2 was a significant augmentation of TNF-induced CXCL1 and CXCL8 mRNA expression and CXCL1 and CXCL8 secretion. There was, however, no effect of siTIPE2 on TNF-induced CCL2 mRNA expression or CCL2 secretion.

The effect of siTIPE2 on the expression of pro-labor mediators, in the absence of inflammation, was also determined in myometrial cells, shown in Table 5. Compared to siCONT-transfected cells, there was no effect of siTIPE2 transfection on cytokine and chemokine mRNA expression (TNF, IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8) or secretion (IL6, CCL2, CXCL1 and CXCL8).

Effect of TIPE2 on expression of contraction-associated proteins in myometrium

The effect of siTIPE2 on contraction-associated proteins and prostaglandins in myometrial cells is demonstrated

Figure 3 Effect of TIPE2 siRNA transfection on chemokines in myometrial cells. Human primary myometrial cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with 1 ng/mL IL1B or 10 ng/mL TNF ($n=6$ patients). (A, C, E, G, I and K) CCL2, CXCL1 and CXCL8 mRNA expression was analyzed by qRT-PCR. (B, D, F, H, J and L) The concentration of CCL2, CXCL1 and CXCL8 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to siCONT+IL1B- or siCONT+TNF-transfected cells and data displayed as mean±S.E.M. Individual data points represent six independent experiments. * $P \leq 0.05$ (repeated measures one-way ANOVA).

Figure 4 Effect of TIPE2 siRNA transfection on chemokines in amnion mesenchymal cells. Human primary amnion mesenchymal cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with 1 ng/mL IL1B or 10 ng/mL TNF ($n=6$ patients). (A, C, E, G, I and K) *CCL2*, *CXCL1* and *CXCL8* mRNA expression were analyzed by qRT-PCR. (B, D, F, H, J and L) The concentration of CCL2, CXCL1 and CXCL8 in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to siCONT + IL1B- or siCONT + TNF-transfected cells and data displayed as mean $\pm$ S.E.M. Individual data points represent six independent experiments. * $P \leq 0.05$ (repeated measures one-way ANOVA).

in Fig. 5. siCONT-transfected cells treated with IL1B showed an expected increase in *PTGS2* and *PTGFR* mRNA expression (Fig. 5A and C), *PTGS2* protein expression (Fig. 5B) and *PGF_{2α}* secretion (Fig. 5E) and a decrease in *Cx43* mRNA expression (Fig. 5D). There was a significant augmentation of IL1B-induced *PTGS2* mRNA and protein expression (Fig. 5A and B) and secretion of *PGF_{2α}* (Fig. 5E) in siTIPE2 transfected cells. These cells also displayed a significant increase in IL1B-induced *Cx43* mRNA expression (Fig. 5D). The effect of siTIPE2 on TNF-induced expression of contraction-associated proteins and prostaglandins is shown in Fig. 5F, G, H and I. There was a significant augmentation of TNF-induced *PTGS2* and *Cx43* mRNA expression (Fig. 5F and H; *PTGS2* protein expression was too low for analysis) and *PGF_{2α}* secretion (Fig. 5I) in siTIPE2 cells. There was no effect of siTIPE2 on TNF-induced *PTGFR* mRNA expression (Fig. 5G). In addition, there was no effect of siTIPE2 on *OXTR* mRNA expression (data not shown).

Effect of TIPE2 on the expression of ECM components in human primary myometrial and amnion cells

Myometrial growth and remodeling during pregnancy depends on increased synthesis of interstitial matrix proteins. In myometrium, there is increased *MMP9* mRNA expression in humans (Roh et al. 2000) and fibronectin (*FN1*), laminin $\beta 2$ (*LAMB2*) and collagen IV mRNA expression (Shynlova et al. 2004) during labor in rat. Thus, it was also of interest to determine if TIPE2 is involved in their regulation. In siCONT-transfected myometrial cells, there was no effect of IL1B or TNF on *FN1* or *LAMB2* mRNA expression, while both IL1B and TNF significantly increased *MMP9* mRNA expression and the secretion of pro *MMP9* (Fig. 6A, B, C, D, E, F, G and H). *FN1*, *LAMB2*, *MMP9* mRNA expression and secretion of pro-MMP9 was further exaggerated in siTIPE2-transfected cells when treated with IL1B or TNF.

MMPs also plays an important role in the degradation of the fetal membranes during labor (Vadillo-Ortega

Table 5 Effect of siTIPE2 on basal mRNA expression and secretion of pro-labor mediators in human primary myometrial cells.

	TNF	IL1A	IL1B	mRNA expression				Secretion			
				IL6	CCL2	CXCL1	CXCL8	IL6	CCL2	CXCL1	CXCL8
siCONT	1.0 $\pm$ 0.1	1.0 $\pm$ 0.7	1.0 $\pm$ 0.6	1.0 $\pm$ 0.5	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3	1.0 $\pm$ 0.4	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2
siTIPE2	1.3 $\pm$ 0.5	0.8 $\pm$ 0.5	1.1 $\pm$ 0.6	0.9 $\pm$ 0.4	1.2 $\pm$ 0.3	0.8 $\pm$ 0.1	0.8 $\pm$ 0.2	1.1 $\pm$ 0.5	1.0 $\pm$ 0.3	1.1 $\pm$ 0.3	1.0 $\pm$ 0.2

For all data, the fold change was calculated relative to siCONT-transfected cells and data displayed as mean $\pm$ S.E.M.

Figure 5 Effect of TIPE2 siRNA transfection on myometrial contraction-associated proteins. Human primary myometrial cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with (A, B, C, D and E) 1 ng/mL IL1B or (F, G, H and I) 10 ng/mL TNF ($n=6$ patients). (A, C, D, F, G and H) *PTGS2*, *PTGFR* and *Cx43* mRNA expression was analyzed by qRT-PCR. (B) *PTGS2* protein expression was analyzed by Western blotting. A representative Western blot image from one patient is also shown. (E and I) The concentration of $PGF_{2\alpha}$ in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to siCONT + IL1B- or siCONT + TNF-transfected cells and data displayed as mean $\pm$ S.E.M. Individual data points represent six independent experiments. * $P \leq 0.05$ (repeated measures one-way ANOVA).

& Estrada-Gutierrez 2005). Thus, we examined the effect of siTIPE2 on the expression of MMPs in amnion mesenchymal cells and epithelial cells. In mesenchymal cells, IL1B and TNF treatment significantly increase in *MMP1* and *MMP8* mRNA expression (Fig. 7A, B, C and D). The effect of siTIPE2 was a significant augmentation of IL1B or TNF induced *MMP1* and *MMP8* mRNA expression. In epithelial cells, IL1B and TNF increased *MMP9* mRNA expression and the secretion of pro *MMP9* (Fig. 7E, F, G and H). There was no effect of siTIPE2 on IL1B-induced *MMP9* mRNA expression or release of pro-*MMP9* (Fig. 7E and F). On the other hand, there was a significant increase in *MMP9* mRNA expression and pro-*MMP9* secretion in TNF-induced siTIPE2-transfected cells (Fig. 7G and H).

Anti-inflammatory actions of TIPE2 are mediated through NF- κ B and MAPK signaling pathways

NF- κ B (Lappas et al. 2002, 2003, Lindstrom & Bennett 2005, Lappas & Rice 2007) and the MAPK protein

ERK1/2 (Sooranna et al. 2005, Lappas et al. 2007, 2011b) have been shown to regulate pro-labor mediators in intrauterine tissues. Thus, we wanted to investigate whether TIPE2 regulates pro-inflammatory and pro-labor mediators through NF- κ B and ERK1/2. To do this, we used selective pharmacological inhibitors of NF- κ B and ERK1/2, BAY 11-7082 and U0126, respectively. We also used the polyphenol naringenin, which we have shown to regulate pro-inflammatory and pro-labor mediators via NF- κ B (Lim et al. 2013b). As shown in Fig. 8, BAY 11-7082, naringenin and U0126 treatment of siTIPE2-transfected myometrial cells, in the presence of IL1B, significantly reduced the secretion of IL6, CXCL1, CXCL8 and $PGF_{2\alpha}$ back to levels similar to IL1B-transfected siCONT cells.

Discussion

The novel findings of this study are that in myometrium, advancing gestation is associated with decreased *TIPE2*

Figure 6 Effect of TIPE2 siRNA transfection on the expression of ECM proteins in myometrial cells. Human primary myometrial cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with (A, C, E and F) 1 ng/mL IL1B or (B, D, G and H) 10 ng/mL TNF ($n = 6$ patients). (A, B, C, D, E and G) *FN1*, *LAMB2* and *MMP9* mRNA expression was analyzed by qRT-PCR (F and H). For all data, the fold change was calculated relative to siCONT + IL1B- or siCONT + TNF-transfected cells and data displayed as mean $\pm$ S.E.M. Individual data points represent six independent experiments. * $P \leq 0.05$ (repeated measures one-way ANOVA).

expression. Furthermore, at term, labor is associated with decreased *TIPE2* expression in myometrium. In fetal membranes, while there was no difference in *TIPE2* expression with advancing gestation, *TIPE2* expression was decreased with labor at both term and preterm. In order to elucidate the role of TIPE2 in myometrium and fetal membranes, we performed loss-of-function studies. We found that, in the presence of pro-inflammatory stimuli, loss of TIPE2 was associated with a significant augmentation of pro-inflammatory cytokines, chemokines, ECM degrading/remodeling enzymes, contraction-associated proteins and the uterine prostaglandin $\text{PGF}_{2\alpha}$. NF- κ B and the MAPK protein ERK1/2 are required for TIPE2 to mediate its anti-inflammatory effects as the NF- κ B inhibitor BAY 11-7082 and the ERK1/2 inhibitor U0126 significantly inhibited siTIPE2-induced expression of pro-labor mediators.

The finding of decreased *TIPE2* mRNA expression at term compared to preterm suggests that the loss of TIPE2 may be involved in the shift of the myometrium from a quiescent into a contractile state in preparation for labor. A number of contraction-associated proteins have been shown to play a role in this process including Cx43 (Chow & Lye 1994, Sparey et al. 1999, Renthal et al. 2010), *PTGS2* (Slater et al. 1999) and *PTGFR* (Brodthppley & Myatt 1999). $\text{PGF}_{2\alpha}$ also plays an important role in myometrial contractility; $\text{PGF}_{2\alpha}$ levels are increased in the amniotic fluid in late pregnancy (Romero et al. 1996) and intrauterine injection of $\text{PGF}_{2\alpha}$ induces human labor (Lindberg 1977). To assess if TIPE2 is involved in the expression of contraction-associated proteins, human

primary myometrial cells were transfected with TIPE2 or control siRNA and then treated with either IL1B or TNF. We found that when compared to control siRNA-transfected cells, *Cx43*, *PTGS2* and *PTGFR* expression and $\text{PGF}_{2\alpha}$ release were significantly augmented. These findings suggest that TIPE2 may suppress myometrial contractility by negatively regulating the expression of Cx43 and the biosynthesis and activity of $\text{PGF}_{2\alpha}$. We also assessed the expression of ECM components involved in the growth and remodeling of the myometrium during pregnancy and labor, namely *MMP9*, *FN1* and *LAMB2* (Roh et al. 2000, Shynlova et al. 2004). We found that siTIPE2 increased their expression in myometrial cells suggesting a shift toward a more contractile state. Further functional studies are required to determine the effect of TIPE2 silencing on myometrial contractility and cytoskeleton reorganization.

Pro-inflammatory cytokines, chemokines and ECM proteins have all been shown to be increased during labor in the uterus and fetal membranes (Thomson et al. 1999, Roh et al. 2000, Osman et al. 2003, Shynlova et al. 2004, Bollapragada et al. 2009, Weiner et al. 2010, Singh et al. 2017). At or around the time of labor, leukocytes infiltrate uterine tissues (myometrium, cervix, fetal membranes, decidua), leading to this increased expression of pro-inflammatory cytokines and chemokines. In turn, these cytokines and chemokines regulate the expression of MMPs to facilitate rupture of fetal membranes. Decreased TIPE2 expression in both myometrium and fetal membranes during labor suggests that TIPE2 may be involved in propagating the labor

Figure 7 Effect of TIPE2 siRNA transfection on the expression of ECM proteins in amnion cells. Human primary amnion (A, B, C and D) mesenchymal cells or (E, F, G and H) epithelial cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with 1 ng/mL IL1B or 10 ng/mL TNF. (A, B, C, D, E and G) *MMP1*, *MMP8* and *MMP9* mRNA expression was analyzed by qRT-PCR. (F and H) The concentration of pro MMP9 in the incubation medium was assayed by zymography. Representative zymography image from one patient is also shown. For all data, the fold change was calculated relative to siCONT + IL1B- or siCONT + TNF-transfected cells and data displayed as mean $\pm$ S.E.M. Individual data points represent 5–6 independent experiments. * $P \leq 0.05$ (repeated-measures one-way ANOVA).

processes. In order to assess this, we examined the effect of siTIPE2 on the expression of pro-labor mediators. We found that in primary myometrial and amnion cells deficient in TIPE2, there was a significant augmentation of IL1B or TNF-induced pro-inflammatory cytokines (IL1A, IL1B, IL6), chemokines (CCL2, CXCL1, CXCL8) and MMPs. Interestingly, in myometrial cells and amnion epithelial cells, and in contrast to amnion mesenchymal cells, CCL2 mRNA expression and secretion was not regulated by TIPE2. Likewise, TIPE2 regulates *MMP9* mRNA expression and secretion of pro MMP9 induced by TNF not IL1B. This suggests that TIPE2 regulation of pro-inflammatory mediators is cell and stimulus specific.

TIPE2 has been found to inhibit NF- κ B and the MAPK protein ERK (Sun *et al.* 2008, Lou *et al.* 2013, Goldsmith & Chen 2017, Zhang *et al.* 2017, Lin *et al.* 2018). These signaling pathways have been shown to play important roles in regulating pro-inflammatory and pro-labor mediators in human myometrium and fetal membranes (Lappas *et al.* 2002, 2003, 2007, 2011b, Lindstrom & Bennett 2005, Sooranna *et al.* 2005,

Lappas & Rice 2007). Thus, we sought to determine if NF- κ B and ERK are involved in the upregulation of pro-labor mediators observed in TIPE2-deficient cells. This was assessed by determining the effect of inhibitors of NF- κ B (pharmacological inhibitor BAY 11-7082 and polyphenol naringenin) and ERK1/2 (pharmacological inhibitor U0126) activation on IL1B-induced pro-labor mediators in siTIPE2-transfected myometrial cells. We found that in siTIPE2-transfected cells, treatment with BAY 11-7082, naringenin or U0126 significantly reduced IL1B-induced secretion of pro-labor mediators. Collectively, these findings suggest that TIPE2 acts as a negative regulator of pro-labor mediators by interfering with NF- κ B and ERK1/2.

A limitation of this study is that the temporal and labor-associated changes observed in *TIPE2* mRNA expression could not be validated at the protein level. We tested numerous TIPE2 commercial antibodies but were unable to confirm specificity. Further studies examining the regional localization of TIPE2 in fetal membranes (i.e. site over the cervix prone to rupture

Figure 8 Effect of NF- κ B and MAPK inhibitors on TIPE2 siRNA transfection induced pro-labor mediators in myometrial cells. Human primary myometrial cells were transfected with 25 nM siCONT or 25 nM siTIPE2 and then treated with 1 ng/mL IL1B in the absence or presence of 10 μ M BAY 11-7082 (BAY), 400 μ M naringenin (nar) or 5 μ M U0126 for an additional 20 h ($n=5$ patients). (A, B, C and D) The concentration of IL-6, CXCL1, CXCL8 and PGF_{2 α} in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to siCONT+IL1B or siCONT+TNF transfected cells and data displayed as mean $\pm$ S.E.M. Individual data points represent five independent experiments. * $P \leq 0.05$ vs siTIPE2+IL1B-transfected cells (repeated-measures one-way ANOVA).

versus distal areas) would shed further light on the role of TIPE2 in fetal membrane rupture. In addition, due to laboratory restrictions, we were unable to confirm our siRNA results using overexpression of TIPE2. Finally, for the expression studies, we used fetal membranes overlying the cervix. This site is known to be inflamed due to infiltrating immune cells (Marcellin *et al.* 2017) and thus any changes in this area may not truly reflective of fetal membrane physiology away from cervix and on top of placental bed.

In summary, we have identified a novel role of TIPE2 in myometrium and fetal membranes. *TIPE2* is lower in myometrium at term gestation and further decreased during labor suggesting a possible role in the transition of the myometrium from a quiescent to contractile state. In support, siRNA-knockdown experiments demonstrated that TIPE2 regulates contraction-associated proteins (Cx43, PTGS2, PTGFR) and the uterotonic prostaglandin PGF_{2 α} . TIPE2 expression was also lower in myometrium and fetal membranes after active labor onset at term and/or preterm, which points to a potential mechanistic role for TIPE2 in human labor and delivery. This hypothesis is supported by our *in vitro* studies, whereby inhibition of TIPE2 in primary cells isolated from myometrium and amnion demonstrated that TIPE2 is a negative regulator of inflammation induced expression of mediators involved in propagating labor including pro-inflammatory cytokines, chemokines and ECM remodeling enzymes. Our conclusions are limited to mRNA expression studies and siRNA knockdown *in vitro* studies in primary myometrial amnion cells. Further studies are required to examine the potential role of TIPE2 in preterm birth.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L designed the study, performed some experiments, analyzed the data and wrote the manuscript. R L performed experiments and wrote the manuscript.

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