Slit2 is decreased after spontaneous labour in myometrium and regulates pro-labour mediators

Short title: Slit2 and human labour

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ABSTRACT

Preterm birth, a global healthcare problem, is commonly associated with inflammation. As Slit2 plays an emerging role in inflammation, the purpose of this study was to determine the effect of Slit2 on labour mediators in human gestational tissues. Slit2 mRNA and protein expression were assessed using qRT-PCR and immunohistochemistry in fetal membranes and myometrium obtained before and after labour. Slit2 silencing was achieved using siRNA in primary myometrial cells. Pro-inflammatory and pro-labour mediators were evaluated by qRT-PCR, ELISA and gelatin zymography. Slit2 mRNA and protein expression were found to be significantly lower in myometrium after labour onset. There was no effect of term or preterm labour on Slit2 expression in fetal membranes. Slit2 mRNA expression was decreased in myometrium treated with LPS and IL-1 β . Slit2 siRNA in myometrial cells increased IL-1 β -induced pro-inflammatory cytokine gene expression and release (IL-6 and IL-8), COX-2 expression and prostaglandin PGE₂ and PGF_{2 α} release, and MMP-9 gene expression and pro MMP-9 release. There was no effect of Slit2 siRNA on IL-1 β -induced NF- κ B transcriptional activity. Our results demonstrate that Slit2 is decreased in human myometrium after labour and our knock-down studies describe an anti-inflammatory effect of Slit2 in myometrial cells.

Highlights

- Slit2 expression is decreased in labouring term myometrium
- Proven inducers of preterm labour – bacterial endotoxin LPS and the pro-inflammatory cytokine IL-1 β – decrease Slit2 expression in myometrium
- Knockdown of Slit2 using siRNA is associated with an increase in pro-inflammatory and pro-labour mediators in human myometrial cells

Keywords: Slit2; myometrium; fetal membranes; human labour; inflammation

List of Abbreviations

ae – amnion epithelium; APH – antepartum haemorrhage; cl – chorionic trophoblast layer; COX-2 – cyclooxygenase-2; ct – connective tissue layer; Ct – threshold cycle; dec – decidua; ECM – extracellular matrix; HUVECs – human umbilical vein endothelial cells; IHC – immunohistochemistry; LPS – lipopolysaccharide; MMP – matrix metalloproteinase; NC – negative control; NF- κ B – nuclear transcription factor- κ B; PG – prostaglandin; qRT-PCR – quantitative reverse transcriptase polymerase chain reaction; Robo – roundabout; siRNA – small interfering ribonucleic acid

1. INTRODUCTION

Premature birth is the most significant problem facing contemporary clinical obstetrics in the developed world. Potential sequelae include fetal respiratory distress and neonatal sepsis. The problems are not limited to the perinatal period, as those babies who do survive may have long-term problems related to contracture deformities, brain damage and learning disabilities (Wood *et al.* 2000, Marlow *et al.* 2005, Saigal and Doyle 2008). Approximately 20 % of preterm deliveries are iatrogenic (e.g. intrauterine growth restriction, preeclampsia, placenta praevia) while the remaining 80 % of preterm deliveries are spontaneous, related to preterm labour or preterm premature rupture of the membranes. In the absence of useful prediction or prevention, current treatments for preterm labour involve suppression of uterine activity with tocolytics and preventive antibiotic therapy (Norman and Shennan 2013). However, these therapies are ineffective and of concern as they may be harmful; such circumstances include increased functional impairment and risk of cerebral palsy among the children at 7 years of age from women who were administered antibiotics for spontaneous preterm labour with intact membranes (Kenyon *et al.* 2008). Thus, to develop clinically useful interventions and improve neonatal outcome, elucidation of the mechanisms involved in the initiation and progression of labour, both at term and preterm, is essential.

Although the physiological factors that trigger the initiation of human labour and delivery at term or preterm are not fully known, many factors are thought to be involved in regulating parturition. Likely contributors include the phospholipid-derived mediators (such as eicosanoids and including prostaglandins), pro-inflammatory cytokines, and extracellular matrix (ECM) remodelling enzymes. Collectively, they participate in the regulation of myometrial contractility, cervical ripening and rupture of fetal membranes (Bowen *et al.* 2002, Olson 2003, Lappas and Rice 2004, Weiss *et al.* 2007, Christiaens *et al.* 2008). Infection and inflammation are the biggest factors contributing to both term and preterm births (Bastek *et al.* 2011), with the activation of the innate immune system

initiating a pro-inflammatory cascade that participates in activating these terminal effector pathways. Preterm labour can be due to untimely activation of these processes (Goldenberg *et al.* 2008) or the consequence of pathological activation, such as intrauterine infection, uterine ischaemia, allergy and hormonal disorders (Romero *et al.* 1994, 2006). Indeed, animal models have shown us that expression of cytokines by either fetal or maternal tissues can upregulate the activity of the mediators that lead to the terminal effector pathways of labour (Kemp *et al.* 2010), and that administration of bacterial endotoxin lipopolysaccharide (LPS) can induce preterm birth (Burd *et al.* 2010, Ernst *et al.* 2010).

The secretory Slit proteins (Slit1-3) and their receptors Robo (Roundabout; Robo1-4) have well-described functional roles in cell migration/invasion, apoptosis and proliferation (Dickinson and Duncan 2010, Shi *et al.* 2014). Given these functional roles of the Slit/Robo system, these proteins have been implicated as regulators of placental angiogenesis and trophoblast function. Human umbilical cord vascular endothelial cells (HUVECs) deficient in Robo4 displayed impaired migration (Kaur *et al.* 2008). Also, syncytiotrophoblasts and placental endothelium expression of Slit2, Slit3, Robo1 and Robo4 are significantly increased during hypoxia and in preeclamptic placentas compared with healthy controls (Liao *et al.* 2012). Recently, however, pro-inflammatory functions have been attributed to Slit/Robo proteins. We have recently shown that Slit3 is increased in the supracervical fetal membranes and in labouring myometrium (Lim *et al.* 2014). In addition, siRNA knockdown of Slit3 was associated with a decrease in IL-1 β -induced pro-inflammatory cytokine gene expression and secretion and in MMP-9 gene expression and secretion (Lim *et al.* 2014).

In contrast, the majority of studies have shown Slit2 to play an anti-inflammatory role. Slit2 is a negative regulator of chemokine-induced chemotaxis of leukocytes *in vitro* (Guan *et al.* 2003, Liu *et al.* 2006, Ye *et al.* 2010), and has been demonstrated to alleviate experimental crescentic

glomerulonephritis in rats *in vivo* (Kanellis *et al.* 2004). Of particular interest is a recent study in endothelial cells whereby Slit2 repressed LPS-induced secretion of pro-inflammatory cytokines via inhibiting the NF- κ B pathway (Zhao *et al.* 2014). Furthermore, bacterial endotoxin LPS and the pro-inflammatory cytokines TNF- α and IL-1 β induced significant downregulation of Slit2 in endothelial cells *in vitro* (Zhao *et al.* 2014). Similarly, *in vivo*, endotoxaemia decreased Slit2 expression in endothelial cells and in the liver (Zhao *et al.* 2014).

Slit2 has not been investigated in fetal membranes and myometrium. Given the central role of inflammation in the processes of human labour and delivery, and the above data implicating Slit2 as a regulator of inflammation, the aims of this study were (i) to establish labour-associated changes in Slit2 expression in human fetal membranes and myometrium; and (ii) to determine the effect of Slit2 knockdown by siRNA in both amnion and myometrial primary cells on pro-labour mediators.

2. MATERIALS AND METHODS

2.1 Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Informed written consent was obtained from all participating women. All tissues were obtained from women who delivered healthy, singleton infants. All tissues were obtained within 15 min of delivery. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, and fetuses with chromosomal abnormalities were excluded.

Fetal membranes were obtained from (i) term no labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section) (n=6 patients) and (ii) term after spontaneous labour, spontaneous membrane rupture, and normal vaginal delivery (n=6 patients). Clinical details of the patients are detailed elsewhere (Lappas *et al.* 2011). For these studies, fetal membranes were obtained approximately 2 cm from the periplacental edge. There was no difference in maternal age and body mass index, parity, or gestational age in the patients recruited. Tissue samples were fixed and paraffin-embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen and immediately stored at -80°C for gene expression analysis by qRT-PCR.

Fetal membranes were also obtained from women at preterm (i) no labour undergoing Caesarean section (n=8 patients); and (ii) after spontaneous labour and normal vaginal delivery (n=8 patients). All placentas collected at preterm gestations were swabbed for microbiological culture investigations and histopathological examination. Patients with chorioamnionitis were excluded from analyses. Women with preeclampsia, preexisting diabetes, asthma, multiple pregnancies, and

fetuses with chromosomal abnormalities were also excluded. Indications for preterm delivery (in the absence of labour) were placenta praevia, placental abruption, antepartum haemorrhage (APH) or Rhesus isoimmunisation. For the preterm labour study, fetal membranes from both the non-labouring and after labour preterm groups were obtained 2 cm from the peri-placental edge. Tissue samples were snap frozen in liquid nitrogen and immediately stored at -80°C for expression analysis of Slit2 mRNA expression by qRT-PCR.

Myometrium was obtained from consenting women at the time of term Caesarean section (≥ 37 weeks' gestation). Myometrial biopsies were collected from two groups of women: (i) pregnant women undergoing elective Caesarean section in the absence of labour ($n=6$ patients); and (ii) pregnant women who delivered during active labour (labour was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement and dilation; $n=6$ patients).

Women were excluded from the study if they had a multiple pregnancy or evidence of an active infection. A myometrial biopsy was obtained from the upper margin of the lower uterine segment incision during the Caesarean section. Tissue samples were fixed and paraffin embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen and immediately stored at -80°C for gene expression analysis by qRT-PCR.

2.2 Tissue explant culture

Tissue explants were performed to determine the effect of LPS and the pro-inflammatory cytokine IL-1 β on Slit2 expression ($n=6$ patients). Fresh fetal membranes and myometrium (collected as detailed above) were placed in DMEM at 37°C in a humidified atmosphere of 8% O_2 (fetal

membranes) or 21% O₂ (myometrium) and 5% CO₂ for 1 h. Tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight per well for fetal membranes, and 50 mg wet weight per well for myometrium). The explants were incubated in 1 ml of DMEM containing 100 U/ml penicillin G and 100 µg/ml streptomycin. Tissues were incubated in the absence or presence of 10 µg/ml LPS or 10 ng/ml IL-1β for 20 h.

2.3 Gene silencing of Slit2 with siRNA

Primary myometrial cells were used to investigate the effect of siRNA-mediated gene silencing of Slit2 on the pro-labour mediators. Myometrium were obtained from women who delivered healthy, singleton infants at term (37–39 weeks' gestation) undergoing elective Caesarean section in the absence of labour. Cells were isolated and cultured as previously described (Lappas 2013, Lim *et al.* 2013b). Cells at approximately 50% confluence were transfected using SilenceMag reagent according to the manufacturer's guidelines (Oz Biosciences, Marseille, France) and as we have previously described (Lim *et al.* 2013b). Slit2 and negative control (NC) siRNA were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). An initial dose response was performed using 50–200 nM siRNA. The decrease in Slit2 gene expression (as assessed by qRT-PCR) was highest using 200 nM (data not shown); thus, all subsequent experiments were performed using this dose. Cells were transfected with 200 nM Slit2 or NC siRNA in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA [w/v]) with or without 1 ng/ml IL-1β, and the cells were incubated at 37°C for an additional 24 h. Cells were collected and stored at –80°C until assayed for mRNA expression by qRT-PCR and Slit2 protein expression by IHC as detailed above. Media were collected and stored at –80°C until assayed for cytokine, prostaglandin, and MMP release, as detailed below. The response to IL-1β between patients varied greatly, as we have previously reported (Lim *et al.* 2013b). Thus, data are presented as fold change in expression relative to the expression level in the IL-1β-stimulated NC siRNA transfected cells, which was set at 1. Experiments were performed from myometrium obtained from six patients.

2.4 Immunohistochemistry (IHC)

To determine the expression of Slit2 in fetal membranes and myometrium, IHC was performed on paraffin sections as previously described (Lim *et al.* 2013b). Briefly, sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM citrate buffer, pH 6.0 for 10 min followed by 20-min incubation). Endogenous peroxidases were inactivated by adding 3% hydrogen peroxide for 10 min. Sections were incubated with goat polyclonal anti-Slit2 (sc-31597; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 µg/ml in 1% (wt/vol) bovine serum albumin in phosphate buffered saline (PBS) and incubated in a humidity chamber for 60 min. Binding sites were labelled with biotin conjugated rabbit anti-goat IgG antibody (Merck Millipore, Billerica, MA, USA) then streptavidin-HRP (Merck Millipore). Negative control slides, where primary antibody was replaced with diluent, were also performed.

To determine Slit2 protein expression in myometrial cells after siRNA knockdown, cells grown on glass coverslips were transfected as described above. After 48 h, cells were fixed in 4% formalin in PBS for 15 min. IHC staining was performed as described above for tissue sections.

2.5 RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells and tissues using TRIsure reagent according to the manufacturer's instructions (Bioline, Alexandria, NSW, Australia), as previously described (Lim *et al.* 2013a, 2013b). RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA (0.5 µg for tissue and 0.15 µg for cells) was converted to cDNA using the Tetro cDNA synthesis kit (Bioline, Alexandria, NSW, Australia) according to the manufacturer's instructions. The cDNA was diluted 50-fold, and 4 µl of this was used to perform RT-PCR using Sensimix Plus SYBR green (Bioline) and 200 nM of pre-designed and validated primers (QuantiTect Primer Assays, Qiagen, Germantown, MD,

USA). The RT-PCR was performed using a CFX384 real-time PCR detection system from Bio-Rad Laboratories (Hercules, CA, USA). Average gene CT values were normalised to the average GAPDH Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method (Livak and Schmittgen 2001). This method compares test samples with a calibrator sample and uses results obtained with a uniformly expressed control gene to correct for differences in the amount of RNA present in the two samples being compared to generate a Ct value. The calibrator samples were from the no labour group for the expression studies, basal for the explant studies and NS siRNA + IL-1 β for the transfection studies.

2.6 Cytokine and prostaglandin assays

The release of IL-6 and IL-8 was performed using CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). The limits of detection for the IL-6 and IL-8 assays were 16 and 12 pg/ml respectively. The release of PGE₂ and PGF_{2 α} into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, Waterloo, NSW, Australia). The limit of detection for the PGE₂ and PGF_{2 α} assays was 16 and 60 pg/ml respectively. The inter- and intra-assay coefficients of variation (CV) for all assays were less than 10%. Fold change was calculated relative to IL-1 β -stimulated NC siRNA-transfected cells, and was set at 1.

2.7 NF- κ B luciferase assay

A luciferase assay was utilised to determine possible interactions between Slit2 and NF- κ B, as previously described (Lim *et al.* 2014), with some modifications. Briefly, primary myometrial cells at approximately 70% confluence were transfected with FuGENE HD transfection reagent (Promega, Madison, WI, USA) according to manufacturer's guidelines. Cells were first transfected with 0.25 μ g of NF- κ B reporter construct (Qiagen) for 6 h. Cells were then transfected with 200 nM

NC siRNA or Slit2 siRNA (as described above) for 48 h. The medium was then replaced with DMEM/F-12 with or without 1 ng/ml IL-1 β , and the cells were incubated at 37°C for an additional 24 h. The cells were harvested in lysis buffer, and luminescence activity was measured using a Luciferase Reporter Assay Kit (Life Research, Scoresby, VIC, Australia) and Renilla Luciferase Flash Assay kit (Thermo Fisher Scientific) as instructed. The ratio of the firefly luciferase level to the Renilla luciferase level was determined and the results expressed as a ratio of the normalised luciferase activity of IL-1 β -stimulated NF- κ B reporter plus NC siRNA transfected cells, which was set as 1. The experiments were performed on myometrium obtained from six patients.

2.8 Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Two sample comparisons, either a paired or an unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann–Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett's test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to *P* value <0.05. Data were expressed as mean $\pm$ standard error of the mean (SEM).

3. RESULTS

3.1 Effect of term labour on Slit2 expression in fetal membranes

Fetal membranes were obtained at term Caesarean section in the absence of labour (no labour) or after spontaneous labour and membrane rupture (after labour; n=6 patients per group). Slit2 mRNA expression remained unchanged in fetal membranes with labour onset (Figure 1A). Representative immunohistochemistry image demonstrating the localisation of Slit2 in fetal membranes is shown in Figure 1B; Slit2 staining was present in amnion epithelium, chorionic trophoblasts, and decidua. No staining was observed in the negative control. There was no change in Slit2 expression in fetal membranes after labour.

3.2 Effect of human term labour on Slit2 expression in myometrium

Myometrium was obtained at term Caesarean section in the absence of labour (no labour) and after spontaneous labour onset (after labour; n=6 patients per group). Slit2 mRNA expression was significantly lower after labour in myometrium (Figure 2A). Immunohistochemistry showed positive Slit2 staining in longitudinal and transverse muscle fibres. Slit2 staining was higher in non-labouring myometrium; only faint staining was observed in myometrium after labour (Figure 2B). No staining was observed in the negative control.

3.3 Effect of preterm labour and pro-inflammatory mediators on Slit2 expression

Fetal membranes were obtained from women at preterm Caesarean section in the absence of labour or after spontaneous preterm labour and normal vaginal delivery (n=8 patients per group). Women with preterm deliveries were matched for maternal age, BMI, gravidity, parity and gestational age. Slit2 mRNA expression did not change in fetal membranes with preterm labour onset (Figure 3A).

Given that infection and inflammation are the biggest factors for term and/or preterm birth, the next aim was to determine the effect of the endotoxin LPS and the pro-inflammatory cytokine IL-1 β on Slit2 expression in fetal membranes and myometrium. There was no effect of LPS or IL-1 β on Slit2 expression in fetal membranes (Figure 3B). In contrast, LPS and IL-1 β significantly decreased Slit2 expression in myometrium (Figure 3C).

3.4 Slit2 gene silencing in primary myometrium cells by siRNA

Having shown that Slit2 is decreased in term labouring myometrium, the final aims of this study were to determine the effect of Slit2 knockdown using siRNA on the expression of mediators that are associated with human labour. The efficacy of transfection was analysed by qRT-PCR and IHC. Compared with NC siRNA transfected primary cells, transfection with Slit2 siRNA resulted in a significant decrease in Slit2 mRNA (Figure 4A) and protein expression (Figure 4B).

The effect of Slit2 siRNA on IL-1 β -induced pro-inflammatory cytokine gene expression and release in primary myometrial cells was also assessed (Figure 5). As expected, treatment with IL-1 β induced a significant increase in both IL-6 and IL-8 mRNA expression and secretion in NC siRNA-transfected cells. Additionally, in Slit2-deficient cells stimulated with IL-1 β , both IL-6 and IL-8 mRNA and release were also significantly increased.

The effect of Slit2 siRNA on the COX-prostaglandin pathway in myometrium is demonstrated in Figure 6. As expected, myometrial cells treated with IL-1 β significantly increased COX-2 mRNA expression (Figure 6A), and release of PGE₂ (Figure 6B) and PGF_{2 α} (Figure 6C). The loss of Slit2 was associated with significantly higher COX-2 mRNA expression, and PGE₂ and PGF_{2 α} release. There was no effect of IL-1 β or Slit2 siRNA on COX-1 mRNA expression (data not shown).

A luciferase assay was used to determine whether Slit2 regulates pro-labour mediators in primary myometrial cells through the NF- κ B pathway. For these studies, cells were transfected with the NF- κ B reporter plasmid. Cells were then transfected with either NC or Slit2 siRNA, and treated with IL-1 β . The cellular luciferase activity was subsequently measured using luminescence. As shown in Figure 7, IL-1 β significantly increased the luciferase activity in myometrial cells that were transfected with the NF- κ B-luc reporter plasmid. In contrast, no effect was observed in the luciferase activity of Slit2 siRNA knockdown in myometrial cells.

4. DISCUSSION

The present study describes, for the first time, tissue-specific and temporal labour-associated changes in the expression of Slit2 in human myometrium and fetal membranes. The data presented in this study demonstrate that Slit2 expression is decreased in myometrium after spontaneous term labour. In addition, the expression of Slit2 was also decreased in the presence of bacterial endotoxin LPS and the pro-inflammatory cytokine IL-1 β . Loss-of-function studies were also performed in primary myometrial cells to determine the effect of Slit2 siRNA on the expression and release of pro-labour mediators. The effect of Slit2 knockdown on primary myometrial cells was a significant increase in the expression and release of the pro-inflammatory cytokines IL-6 and IL-8 in the presence of IL-1 β . Furthermore, COX-2 mRNA expression and subsequent PGE₂ and PGF_{2 α} release were also significantly increased with Slit2 siRNA. Slit2 knockdown did not affect NF- κ B luciferase activity in myometrial cells. In fetal membranes, there was no change in Slit2 expression after both term and preterm labour.

In this study, Slit2 expression was decreased in myometrium after spontaneous term labour and delivery; a process characterised by increased inflammation. For example, coincident with labour, there is a significant increase in IL-1 β , IL-6 and IL-8 mRNA expression in myometrium (Osman *et*

al. 2003, Tattersall *et al.* 2008). Within labouring myometrium, the up-regulation of pro-inflammatory cytokines stimulates and potentiates uterine contractions (Tothova *et al.* 2007). In this study, IL-1 β decreased the expression of Slit2, suggesting a mechanism by which Slit2 may be decreased during normal labour. While Slit2 expression was decreased in myometrium after term

labour, there was no change in fetal membranes after spontaneous term and preterm labour and delivery. One explanation may be that the fetal membranes used in this study were obtained from deliveries after spontaneous labour, rather than from membranes that ruptured before labour onset, i.e. PROM. Slit2 may exert its effects in gestational tissues at an appropriate time, as in the normal course of labour, contractions occur before membrane rupture.

It is now well-established that term and preterm labour share a common terminal pathway of uterine contractility, cervical ripening and membrane activation. However, preterm labour is the consequence of pathological activation, such as intrauterine infection, uterine ischaemia, allergy and hormonal disorders (Romero *et al.* 1994, 2006). In this study, there was no effect of preterm labour or LPS on Slit2 expression in fetal membranes. No myometrium samples from preterm deliveries were available for this study. However, bacterial endotoxin LPS significantly decreased Slit2 expression in myometrium, suggesting a mechanism by which Slit2 might be decreased during preterm labour. Of note, these findings are in support of studies in HUVECs whereby LPS, TNF- α and IL-1 β decreased Slit2 expression (Zhao *et al.* 2014), and in mice where endotoxaemia decreased Slit2 expression in endothelial cells and in the liver (Zhao *et al.* 2014).

In keeping with the above studies, the loss-of-function studies in primary myometrial cells demonstrate that Slit2 has anti-inflammatory actions in these tissues. Specifically, Slit2-deficient cells had increased IL-1 β -induced pro-inflammatory cytokines IL-6 and IL-8, and COX-2 and prostaglandins. To our knowledge, this is the first study that has shown that knockdown of Slit2 is associated with an increase in pro-inflammatory and pro-labour mediators in human gestational tissues. However, and in support of the findings in this study, Slit2 signalling is important in regulating LPS-induced endothelial inflammation (Zhao *et al.* 2014).

In HUVECs, Slit2 represses inflammatory responses by inhibiting the NF- κ B pathway downstream of LPS-TLR4 interaction (Zhao *et al.* 2014). The archetypal inflammatory transcription factor, NF- κ B can be activated in the presence of infection and inflammatory cytokines, such as those present during labour. Activation of NF- κ B in myometrium (Lindstrom and Bennett 2005, Khanjani *et al.* 2011) is associated with both term and preterm labour. NF- κ B regulates pro-labour mediators, such as pro-inflammatory cytokines, MMP-9, and COX-2 and prostaglandins (Lappas *et al.* 2002, 2004, Lappas and Rice 2009, Lim *et al.* 2013a). While Slit2 inhibition increased pro-labour mediators, there was no effect on NF- κ B transcriptional activity. It is possible that other transcription factors are involved. Indeed, studies have shown that NeuroD1, a transcription factor involved in neurogenesis and tumorigenesis, which is reduced in an inflammatory model of autoimmune disease (Brose *et al.* 1999), down-regulates Slit2 expression in neuroblastoma tissue (Liang *et al.* 1999).

During inflammation, Slit2 can inhibit chemotaxis of leukocytes induced by chemotactic factors (Wu *et al.* 2001, Guan *et al.* 2003, Chen *et al.* 2004), thus exerting an anti-inflammatory effect. The onset of labour is coincident with a large influx of leukocytes, such as neutrophils and macrophages, in the myometrium, an event that has become a hallmark of the developing uterus from a quiescent to a contractile organ (Thomson *et al.* 1999). This occurs from the leukocytes stimulating the production of uterotonic prostaglandins (Casey *et al.* 1990). Increased expression of chemotactic factors may, in part, be accountable for the accumulation of leukocytes in the uterus (Kemp *et al.* 2010, Bastek *et al.* 2011). IL-8 mRNA is overexpressed in human myometrium with spontaneous labour (Mittal *et al.* 2010). IL-8 is a potent chemotactic, mediating neutrophil chemotaxis and activation. Inducible by IL-1 β and TNF- α (Mittal *et al.* 2010), it is thought that the main role of IL-8 is to maintain the incursion of neutrophils and perpetuate the inflammatory response (Gomez-Lopez *et al.* 2010). The findings of our current study demonstrate that IL-1 β decreases Slit2 mRNA in myometrium and that Slit2 silencing is also associated with an increase in

IL-8 mRNA expression and release, suggesting that Slit2 might play a regulatory role during inflammatory chemotaxis.

In primary HUVECs, Slit2 represses LPS-induced secretion of pro-inflammatory cytokines and chemokines via its dominant endothelial-specific receptor Robo4 (Zhao *et al.* 2014). It is unknown whether the actions of Slit2 are dependent on the Robo pathway; preliminary studies in our laboratory have detected the expression of Robo proteins in fetal membranes and myometrium; however, further work is required to determine if these proteins are necessary to potentiate the anti-inflammatory actions of Slit2. Slit2 has been shown to bind laminin, glypican-1 and netrin 1 (Brose *et al.* 1999, Liang *et al.* 1999, Ronca *et al.* 2001); however, the significance of these associations remains unclear. While specific ligand–receptor interactions and downstream signalling was not examined in these cells, our unpublished data demonstrate that Robo1 and Robo4 are expressed in human gestational tissues, and could perhaps be instrumental in the anti-inflammatory role of Slit2.

We have previously described a pro-inflammatory effect of Slit3 in human gestational tissues, where Slit3 is increased in labouring myometrium and in non-labouring fetal membranes overlying the cervix, which is prone to rupture (Lim *et al.* 2014). In contrast, we found that Slit2 has an anti-inflammatory effect in myometrium. Without knowing the nature of (any) Slit/Robo interactions, as mentioned above, we can currently only speculate as to how these Slit proteins exert such different responses in human gestational tissues. Hohenester has suggested that Slit signalling might be a result of changing the oligomeric state of Robo, where Slit binding may convert an inactive Robo dimer into an active monomer, or Slit binding may convert an inactive Robo monomer into an active dimer (Hohenester 2008). It would be a vast undertaking to fully comprehend how these Slit proteins elicit their effects; however, our combined results indicate an interesting signalling network that would contribute to our understanding of the mechanisms of human labour.

5 CONCLUSIONS

In summary, the results presented here demonstrate the potentially important role of endogenous Slit2 in modulating the inflammatory response in human gestation tissues. Numerous pathways culminate in preterm birth, including infection and/or inflammation (MacIntyre *et al.* 2012). The development of therapeutics to manage and prevent preterm labour can only arise from a complete understanding of the role of inflammation in initiating uterine contractions and membrane rupture. The above data presents a novel finding, indicating that Slit2 plays a role in myometrium in, either suppressing the inflammatory response associated with term labour, or in the mechanisms that contribute to labour onset. Indeed, systemic Slit2 administration ameliorates inflammation *in vivo* (Kanellis *et al.* 2004). Collectively, these observations make Slit2 an exciting therapeutic candidate for the prevention and treatment of preterm labour.

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DISCLOSURE SUMMARY

The authors have nothing to declare.

REFERENCES

- Bastek, J.A., et al., 2011. The role of inflammation and infection in preterm birth. *Clin. Perinatol* 38, 385-406.
- Bowen, J.M., et al., 2002. Cytokines of the placenta and extra-placental membranes: Biosynthesis, secretion and roles in establishment of pregnancy in women. *Placenta* 23, 239-256.
- Brose, K., et al., 1999. Slit proteins bind robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96, 795-806.
- Burd, I., et al., 2010. Inflammation-induced preterm birth alters neuronal morphology in the mouse fetal brain. *J. Neurosci. Res.* 88, 1872-1881.
- Casey, M.L., et al., 1990. Cytokines and infection-induced preterm labour. *Reprod. Fertil. Dev.* 2, 499-509.
- Chen, B., et al., 2004. Cutting edge: Bone morphogenetic protein antagonists *drn/gremlin* and *dan* interact with slits and act as negative regulators of monocyte chemotaxis. *J. Immunol.* 173, 5914-5917.
- Christiaens, I., et al., 2008. Inflammatory processes in preterm and term parturition. *J. Reprod. Immunol.* 79, 50-57.
- Dickinson, R.E., Duncan, W.C., 2010. The slit-robo pathway: A regulator of cell function with implications for the reproductive system. *Reproduction* 139, 697-704.
- Ernst, L.M., et al., 2010. Inflammation-induced preterm birth in a murine model is associated with increases in fetal macrophages and circulating erythroid precursors. *Pediatr. Dev. Pathol.* 13, 273-281.
- Goldenberg, R.L., et al., 2008. Epidemiology and causes of preterm birth. *Lancet* 371, 75-84.
- Gomez-Lopez, N., et al., 2010. The role of chemokines in term and premature rupture of the fetal membranes: A review. *Biol. Reprod.* 82, 809-814.
- Guan, H., et al., 2003. Neuronal repellent slit2 inhibits dendritic cell migration and the development of immune responses. *J. Immunol.* 171, 6519-6526.
- Hohenester, E., 2008. Structural insight into slit-robo signalling. *Biochem. Soc. Trans.* 36, 251-256.
- Kanellis, J., et al., 2004. Modulation of inflammation by slit protein in vivo in experimental crescentic glomerulonephritis. *Am. J. Pathol.* 165, 341-352.
- Kaur, S., et al., 2008. Silencing of directional migration in roundabout4 knockdown endothelial cells. *BMC Cell Biol.* 9, 61. (doi: 10.1186/1471-2121-9-61.)
- Kemp, M.W., et al., 2010. Preterm birth, infection, and inflammation advances from the study of animal models. *Reprod. Sci.* 17, 619-628.
- Kenyon, S., et al., 2008. Childhood outcomes after prescription of antibiotics to pregnant women with spontaneous preterm labour: 7-year follow-up of the oracle ii trial. *Lancet* 372, 1319-1327.
- Khanjani, S., et al., 2011. Nf-kappab regulates a cassette of immune/inflammatory genes in human pregnant myometrium at term. *J. Cell. Mol. Med.* 15, 809-824.
- Lappas, M., 2013. Forkhead box o1 (*foxo1*) in pregnant human myometrial cells: A role as a pro-inflammatory mediator in human parturition. *J. Reprod. Immunol.* 24-32.
- Lappas, M., Rice, G.E., 2004. Phospholipase a2 isozymes in pregnancy and parturition. *Prostaglandins Leukot. Essent. Fatty Acids* 70, 87-100.
- Lappas, M., Rice, G.E., 2009. Transcriptional regulation of the processes of human labour and delivery. *Placenta* 30 Suppl A, S90-95.
- Lappas, M., et al., 2002. Nuclear factor kappa b regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biol. Reprod.* 67, 668-673.
- Lappas, M., et al., 2004. Effect of nuclear factor-kappa b inhibitors and peroxisome proliferator-activated receptor-gamma ligands on pthrp release from human fetal membranes. *Placenta* 25, 699-704.

- Lappas, M., et al., 2011. Sirt1 is a novel regulator of key pathways of human labor. *Biol. Reprod.* 84, 167-178.
- Liang, Y., et al., 1999. Mammalian homologues of the drosophila slit protein are ligands of the heparan sulfate proteoglycan glypican-1 in brain. *J. Biol. Chem.* 274, 17885-17892.
- Liao, W.X., et al., 2012. Human placental expression of slit/robo signaling cues: Effects of preeclampsia and hypoxia. *Biol. Reprod.* 86, 111. (doi: 10.1095/biolreprod.110.088138.)
- Lim, R., et al., 2013a. Sirt6 is decreased with preterm labor and regulates key terminal effector pathways of human labor in fetal membranes. *Biol. Reprod.* 88, 17. (doi: 10.1095/biolreprod.112.105163)
- Lim, R., et al., 2013b. Apelin is decreased with human preterm and term labor and regulates prolabor mediators in human primary amnion cells. *Reprod. Sci.* 20, 957-967.
- Lim, R., et al., 2014. Slit3 is increased in supracervical human foetal membranes and in labouring myometrium and regulates pro-inflammatory mediators. *Am. J. Reprod. Immunol.* 71, 297-311.
- Lindstrom, T.M., Bennett, P.R., 2005. The role of nuclear factor kappa b in human labour. *Reproduction* 130, 569-581.
- Liu, D., et al., 2006. Neuronal chemorepellent slit2 inhibits vascular smooth muscle cell migration by suppressing small gtpase rac1 activation. *Circ. Res.* 98, 480-489.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 25, 402-408.
- MacIntyre, D.A., et al., 2012. Prevention of preterm labour via the modulation of inflammatory pathways. *J. Matern. Fetal Neonatal Med.* 25 Suppl 1, 17-20.
- Marlow, N., et al., 2005. Neurologic and developmental disability at six years of age after extremely preterm birth. *N. Engl. J. Med.* 352, 9-19.
- Mittal, P., et al., 2010. Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term. *J. Perinat. Med.* 38, 617-643.
- Norman, J.E., Shennan, A.H., 2013. Prevention of preterm birth--why can't we do any better? *Lancet* 381, 184-185.
- Olson, D.M., 2003. The role of prostaglandins in the initiation of parturition. *Best Pract. Res. Clin. Obstet. Gynaecol.* 17, 717-730.
- Osman, I., et al., 2003. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol. Hum. Reprod.* 9, 41-45.
- Romero, R., et al., 1994. The preterm labor syndrome. *Ann. N. Y. Acad. Sci.* 734, 414-429.
- Romero, R., et al., 2006. The preterm parturition syndrome. *BJOG.* 113 Suppl 3, 17-42.
- Ronca, F., et al., 2001. Characterization of slit protein interactions with glypican-1. *J. Biol. Chem.* 276, 29141-29147.
- Saigal, S., Doyle, L.W., 2008. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet* 371, 261-269.
- Shi, R., et al., 2014. Knockdown of slit2 promotes growth and motility in gastric cancer cells via activation of akt/beta-catenin. *Oncol. Rep.* 31, 812-818.
- Tattersall, M., et al., 2008. Pro-labour myometrial gene expression: Are preterm labour and term labour the same? *Reproduction* 135, 569-579.
- Thomson, A.J., et al., 1999. Leukocytes infiltrate the myometrium during human parturition: Further evidence that labour is an inflammatory process. *Hum. Reprod.* 14, 229-236.
- Tothova, Z., et al., 2007. Foxos are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* 128, 325-339.
- Weiss, A., et al., 2007. The matrix metalloproteinases (MMPs) in the decidua and fetal membranes. *Front. Biosci.* 12, 649-659.
- Wood, N.S., et al., 2000. Neurologic and developmental disability after extremely preterm birth. Epicure study group. *N. Engl. J. Med.* 343, 378-384.

- Wu, J.Y., et al., 2001. The neuronal repellent slit inhibits leukocyte chemotaxis induced by chemotactic factors. *Nature* 410, 948-952.
- Ye, B.Q., et al., 2010. Slit2 regulates attractive eosinophil and repulsive neutrophil chemotaxis through differential srgap1 expression during lung inflammation. *J. Immunol.* 185, 6294-6305.
- Zhao, H., et al., 2014. Slit2-robo4 pathway modulates lipopolysaccharide-induced endothelial inflammation and its expression is dysregulated during endotoxemia. *J. Immunol.* 192, 385-393.

FIGURE LEGENDS

Figure 1. Effect of term labour on Slit2 expression in fetal membranes.

(A) Human fetal membranes were obtained from women not in labour at term Caesarean section and women after term spontaneous labour onset and delivery (n=6 patients per group). Slit2 gene expression was analysed by qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to the no labour group. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. no labour (Student's t-test). (B) Representative immunohistochemical expression of Slit2 in human fetal membranes without labour and with labour. ae, amnion epithelium; cl, connective tissue layer; ct, chorionic trophoblast layer; dec, decidua. Magnification x100.

Figure 2. Effect of term labour on Slit2 expression in myometrium.

(A) Human myometrium was obtained from non-labouring and labouring women at term Caesarean section (n=6 patients per group). Slit2 gene expression was analysed by qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to the no labour group. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. no labour (Student's t-test). (B) Representative immunohistochemical expression of Slit2 in human myometrium without labour and with labour. Arrows indicate positive Slit2 staining. Magnification x100.

Figure 3. Effect of preterm labour and pro-inflammatory mediators on Slit2 expression

(A) Human fetal membranes were obtained from women not in labour at preterm Caesarean section and women after preterm spontaneous labour onset and delivery (n=6 patients per group). Slit2 gene expression was analysed using qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to the no labour group. (B) Term fetal membranes and (C) myometrium were incubated in the absence or presence of 10 μ g/ml of LPS and

10 ng/ml of IL-1 β for 20 h (n=6 patients). Slit2 gene expression was analysed by qRT-PCR. Slit2 gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to basal. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. basal (paired-sample comparison).

Figure 4. Transfection efficiency of Slit2 gene knockdown by siRNA in primary myometrial cells.

Human primary myometrial cells were transfected with or without 200 nM NC or Slit2 siRNA for 48 h (n=6 patients). Transfection efficacy was determined by qRT-PCR and IHC. **(A)** Slit2 mRNA expression was normalised to GAPDH mRNA expression, and the fold change was calculated relative to NC siRNA transfected cells. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. NC siRNA transfected cells (paired sample comparison). **(B)** Cells grown on coverslips were transfected with or without 200 nM of NC or Slit2 siRNA for 48 h and IHC performed. Positive Slit2 staining is denoted by black arrows. Representative images from one patient.

Figure 5. Effect of Slit2 gene knockdown by siRNA in primary myometrial cells on pro-inflammatory cytokine expression and secretion.

Human primary myometrial cells were transfected with or without 200 nM of Slit2 or NC siRNA for 48 h and then treated with 1 ng/ml of IL-1 β for an additional 24 h (n=6 patients). **(A,B)** Gene expression for IL-6 and IL-8 was analysed using qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to IL-1 β -stimulated NC siRNA transfected cells. Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β -stimulated NC siRNA transfected cells (one-way ANOVA). **(C,D)** The incubation medium was assayed for the concentration of IL-6 and IL-8 release by ELISA. The fold change was calculated relative to IL-1 β -stimulated NC siRNA transfected cells. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β -stimulated NC siRNA transfected cells (one-way ANOVA).

Figure 6. Effect of Slit2 gene knockdown by siRNA in primary myometrial cells on the COX-2-prostaglandin pathway.

Human primary myometrial cells were transfected with or without 200 nM NC or Slit2 siRNA for 48 h and then treated with 1 ng/ml of IL-1 β for an additional 24 h (n=6 patients). **(A)** COX-2 mRNA expression was analysed by qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to IL-1 β -stimulated NC siRNA transfected cells. Data displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β -stimulated NC siRNA transfected cells (one-way ANOVA). **(B,C)** The incubation medium was assayed for concentration of PGE₂ and PGF_{2 α} release by ELISA. The fold change was calculated relative to IL-1 β -stimulated NC siRNA transfected cells. Data are displayed as mean $\pm$ SEM. * P <0.05 vs. IL-1 β -stimulated NC siRNA transfected cells (one-way ANOVA).

Figure 7. Effect of Slit2 inhibition on NF- κ B transcriptional activity.

Human myometrial cells were co-transfected with 0.25 μ g of NF- κ B reporter construct, along with 200 nM of NC or Slit2 siRNA for 48 h, and then treated with 1 ng/ml of IL-1 β for an additional 24 h (n=6 patients). Promoter activity (normalised with Renilla expression) is expressed as a ratio of luciferase activity of NF- κ B reporter plus NC siRNA plus the IL-1 β group. Each bar represents the mean $\pm$ SEM. * P < 0.05 vs. cells transfected with NF- κ B reporter plus NC siRNA plus IL-1 β (one-way ANOVA).

Figure 1

B)

Figure 2

B)

Figure 3

Figure 4

Figure 5

[PF1]

Figure 6

[PF2]

Figure 7

Conflict of Interest

The authors have nothing to declare.

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