KLF5 regulates infection- and inflammation-induced pro-labour mediators in human myometrium

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Abstract

The transcription factor Kruppel-like factor 5 (KLF5) has been shown to associate with nuclear factor kappa B (NFκB) to regulate genes involved in inflammation. However, there are no studies on the expression and regulation of KLF5 in the processes of human labour and delivery. Thus, the aims of this study were to determine the effect of i) human labour on KLF5 expression in both foetal membranes and myometrium; ii) the pro-inflammatory cytokine interleukin 1 beta (*IL1β*), bacterial product flagellin and the viral dsRNA analogue poly(I:C) on KLF5 expression and iii) KLF5 knockdown by siRNA in human myometrial primary cells on pro-inflammatory and pro-labour mediators. In foetal membranes, there was no effect of term or preterm labour on KLF5 expression. In myometrium, the term labour was associated with an increase in nuclear KLF5 protein expression. Moreover, KLF5 expression was also increased in myometrial cells treated with *IL1β*, flagellin or poly(I:C), likely factors contributing to preterm birth. KLF5 silencing in myometrial cells significantly decreased *IL1β*-induced cytokine expression (*IL6* and *IL8* mRNA expression and release), *COX2* mRNA expression, and subsequent release of prostaglandins PGE₂ and PGF_{2α}. KLF5 silencing also significantly reduced flagellin- and poly(I:C)-induced *IL6* and *IL8* mRNA expression. Lastly, *IL1β*-, flagellin- and poly(I:C)-stimulated NFκB transcriptional activity was significantly suppressed in KLF5-knockout myometrial cells. In conclusion, this study describes novel data in which KLF5 is increased in labouring myometrium, and KLF5 silencing decreased inflammation- and infection-induced pro-labour mediators.

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Introduction

Kruppel-like factor (KLF) transcription factors are involved in the development of various human diseases, such as obesity, cardiovascular disease, cancer and inflammatory conditions (Turner & Crossley 1999, Dang *et al.* 2000, Black *et al.* 2001, Kaczynski *et al.* 2003, McConnell & Yang 2010). In humans, the KLF family consists of ~20 members which are structurally characterised by three tandem zinc-finger domains at the C-terminus. KLF5, also named BTEB2 (Sogawa *et al.* 1993), is ubiquitously expressed (Sogawa *et al.* 1993, Shi *et al.* 1999) and is primarily expressed in the nucleus (Shi *et al.* 1999). KLF5 is a transcription factor and thought to be active only in the nucleus where it binds directly to a specific recognition motif in the promoters of target genes. KLF5 has been demonstrated to regulate many genes involved in cell cycle regulation, apoptosis, migration, differentiation and inflammation (Ghaleb *et al.* 2005, Chancevalap *et al.* 2006, Kumekawa *et al.* 2008, Shinoda *et al.* 2008, Wan *et al.* 2008). The KLF5 protein has been shown to associate with numerous transcription factors, including nuclear factor

kappa B (NFκB), to regulate gene transcription (Sur *et al.* 2002, Aizawa *et al.* 2004).

There is limited data on the role of KLF5 in the regulation of inflammation; however, the available evidence suggests that KLF5 exerts pro-inflammatory actions. *In vitro*, knockdown of KLF5 by siRNA inhibits tumor necrosis factor alpha (TNFα)-induced expression of the chemokine MCP1 (Kumekawa *et al.* 2008) and lipopolysaccharide (LPS)-induced NFκB expression and its downstream target genes, including *TNFα* and interleukin 6 (*IL6*) (Chancevalap *et al.* 2006, Chen *et al.* 2014). *In vivo*, KLF5 initiates the accumulation of M1 macrophages and regulates the inflammation of tubulointerstitium in the unilateral ureteral obstruction (UUO) mouse (Fujiu *et al.* 2011), and the overexpression of KLF5 in distal tubule cells is associated with increased production of pro-inflammatory cytokines in kidney tissues of young rats fed with melamine and cyanuric acid (Huang *et al.* 2013).

Human labour is associated with inflammation. During labour there is increased expression of pro-inflammatory mediators. These include pro-inflammatory cytokines, such as *IL1β*, *IL6* and *IL8* in the cervix,

myometrium, foetal membranes and placenta (Denison *et al.* 1998, Young *et al.* 2002, Osman *et al.* 2003); prostaglandins PGE₂ and PGF_{2α} in the foetal membranes and myometrium (Olson 2003); and matrix metalloproteinase 9 (MMP9) in the placenta and foetal membranes (Xu *et al.* 2002). Regulation of these mediators facilitates tissue remodelling of the cervix, myometrial contractions and rupture of membranes, leading to successful delivery at term. It is untimely activation of these processes that can lead to spontaneous preterm birth (Goldenberg *et al.* 2008). In gestational tissues, expression of cytokines and prostaglandins is regulated by the transcription factor NFκB (Belt *et al.* 1999, Lappas *et al.* 2002). The activation of NFκB is tightly controlled, with both intracellular and extracellular feedback control; inflammatory stimuli such as cytokines and bacterial products (e.g. LPS) activate NFκB, which enhances cytokine production that further amplifies the original inflammatory signal (Hayden & Ghosh 2004). It is in fully understanding the mechanisms that drive human labour that we can begin to develop therapeutics for the prevention or management of preterm labour.

KLF5 is expressed in human placenta (Bai *et al.* 2012), and in the mouse uterus where it plays an essential role in implantation (Sun *et al.* 2012). However, there are no studies on KLF5 in foetal membranes and myometrium and its role in the regulation of inflammation. In non-gestational tissues, KLF5 is induced by a number of inflammatory stimuli (Bafford *et al.* 2006, Kumekawa *et al.* 2008, Noto *et al.* 2013, Chen *et al.* 2014) where it exerts pro-inflammatory actions (Chanchevalap *et al.* 2006, Kumekawa *et al.* 2008, Chen *et al.* 2014). Thus, the hypothesis to be tested is that KLF5 is increased in foetal membranes and myometrium with human labour, and inhibition of KLF5 is associated with decreased expression of pro-inflammatory and pro-labour mediators. The aims of this study were to determine the effect of i) human term and preterm spontaneous labour on KLF5 expression in human foetal membranes and myometrium and ii) KLF5 siRNA knockdown on pro-inflammatory and pro-labour mediators in primary cells isolated from human myometrium.

Materials and methods

Tissue collection

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

For expression studies by western blotting, foetal membranes were obtained from women at i) term no labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section; $n=6$ patients; mean gestational age 38.8 ± 0.3 weeks) and ii) term after spontaneous labour, spontaneous membrane rupture and normal vaginal delivery ($n=6$ patients; mean gestational age 38.7 ± 0.4 weeks). Foetal membranes from the non-labouring group were obtained from the supracervical site (SCS). Identification of the SCS was performed as previously detailed (Lappas *et al.* 2011). Briefly, Bonneys blue dye was introduced through the cervix before Caesarean section. Upon delivery of the placenta, a blue mark was obvious on the chorion-facing membrane where the dye had been applied. In the after-labour group, foetal membranes from the site of membrane rupture (SOR) as previously described (Lappas *et al.* 2011). Amnion and underlying choriodecidua were collected from along the line of foetal membrane rupture. There was no difference in maternal age and BMI, parity or gestational age of the patients recruited. In the term after-labour group, none of the patients received any medications to augment or induce labour, and the average length of labour was $6 \text{ h } 40 \text{ min} \pm 1 \text{ h } 40 \text{ min}$. Tissue samples were snap frozen in liquid nitrogen and immediately stored at -80°C for western blotting analysis as detailed below. Full thickness extra-placental membranes (obtained $\sim 2 \text{ cm}$ from the peri-placental edge) were also collected, fixed and paraffin embedded for immunohistochemical analysis.

Foetal membranes were also obtained from women at preterm (without histological chorioamnionitis) from two groups: i) no labour undergoing Caesarean section ($n=6$ patients; mean gestational age 32.8 ± 0.7 weeks) and ii) after spontaneous labour and normal vaginal delivery ($n=6$ patients; mean gestational age 32.5 ± 0.8 weeks). All placentas collected from the two preterm groups were swabbed for microbiological culture investigations and histopathological examination. Histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes. Indications for preterm delivery (in the absence of labour) were placenta praevia, placental abruption or antepartum haemorrhage. For these studies, foetal membranes from both the non-labouring and after-labour preterm groups were obtained 2 cm from the peri-placental edge. The tissue samples were snap frozen in liquid nitrogen and immediately stored at -80°C for western blotting analysis as detailed below.

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; mean gestational age 39.4 ± 0.3 weeks) and ii) pregnant women who were 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; mean gestational age 39.8 ± 0.2 weeks). Women were excluded from the study if they had a multiple pregnancy, evidence of active infection. A myometrial biopsy was obtained from the upper margin of the lower uterine segment

incision during the Caesarean section. There was no difference in maternal age and BMI, parity or gestational age of the patients recruited. In the term laboring group, none of the patients received any medications to augment or induce labour, and the average length of labour was $10 \text{ h} \pm 6 \text{ h}$ 40 min. Tissue samples were fixed and paraffin embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen and immediately stored at -80°C for western blotting analysis as detailed below.

Gene silencing of KLF5 with siRNA

Primary myometrial cells were used to investigate the effect of KLF5 siRNA knockdown on pro-labour mediators. Myometrium was obtained from women who delivered healthy, singleton infants at term (37–41 weeks gestation) undergoing elective Caesarean section in the absence of labour. The cells were isolated and cultured as previously described (Lim *et al.* 2013a). Briefly, myometrium was minced and digested for 1 h in DMEM/nutrient mixture F-12 Ham (DMEM/F-12) with 3 mg/ml type 1 collagenase (Worthington Biochemical, Freehold, NJ, USA) and 80 $\mu\text{g}/\text{ml}$ DNase 1 (Roche Diagnostics) at 37°C . The cells were centrifuged at 400 *g* for 10 min and grown in DMEM/F-12 enriched with 10% heat-inactivated FCS (containing 100 U/ml penicillin G and 100 mg/ml streptomycin). Myometrial cells from passages 1 to 4 were used. The cells at $\sim 50\%$ confluence were transfected using Lipofectamine 3000 reagent according to the manufacturer's guidelines (Life Technologies). KLF5 siRNA (HSC.RNAI.N001730.12.1) was obtained from Integrated DNA Technologies (Coralville, IA, USA) and the negative control (NC) siRNA was obtained from Sigma. The cells were transfected with 200 nM KLF5 or 200 nM NC siRNA in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA) with or without 1 ng/ml IL1 β , 5 $\mu\text{g}/\text{ml}$ poly(I:C) or 1 $\mu\text{g}/\text{ml}$ flagellin, and the cells were incubated at 37°C for an additional 24 h. The cells were collected and stored at -80°C until assayed for mRNA expression by quantitative RT-PCR (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. The response to IL1 β , poly(I:C) or flagellin between patients varied greatly, as previously reported (Lim *et al.* 2013a). Thus, data are presented as fold change relative to IL1 β -, poly(I:C)- or flagellin-stimulated NC siRNA-transfected cells, which was set at 1. Experiments were performed from myometrium obtained from five individual patients.

NF κ B luciferase assay

A luciferase assay was used to determine possible interactions between KLF5 and NF κ B, as previously described (Lim *et al.* 2013a). Primary myometrial cells, prepared as described previously, at $\sim 70\%$ confluence, were transfected with 0.75 ng NF κ B reporter construct (Qiagen) using FuGENE HD Transfection Reagent (Promega). After 6 h, cells were transfected with 200 nM KLF5 or NC siRNA (as detailed above) for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA), with or without 1 ng/ml IL1 β , 5 $\mu\text{g}/\text{ml}$

poly(I:C) or 1 $\mu\text{g}/\text{ml}$ flagellin, 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 the Luciferase Reporter Assay Kit (Life Research, Scoresby, VIC, Australia) and Renilla Luciferase Flash Assay Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) as instructed. The ratio of the firefly luciferase level to the Renilla luciferase level was determined and the results are expressed as a ratio of normalised luciferase activity of IL1 β -, poly(I:C)- or flagellin-stimulated NF κ B reporter plus NC siRNA transfected cells, which was set as 1. The experiments were performed from myometrium obtained from five patients.

Immunohistochemistry

To determine the localisation of KLF5 in foetal membranes and myometrium, immunohistochemistry was performed on paraffin sections as described previously (Lim *et al.* 2013a) using the IHC Select HRP Detection Set (Merck Millipore, Billerica, MA, USA). Briefly, sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM Tris, 1 mM EDTA, pH 9.0 for 10 min followed by 20 min incubation) and then endogenous peroxidases were inactivated by adding 3% hydrogen peroxide for 10 min. After blocking (Blocking Reagent: normal goat serum in PBS) for 5 min, sections were incubated with 2 $\mu\text{g}/\text{ml}$ rabbit polyclonal anti-KLF5, #GTX103289 (GeneTex, Inc., Irvine, CA, USA) in 1% (wt/vol) BSA in PBS and incubated in a humidity chamber for 60 min. Binding sites were labelled with biotin-conjugated rabbit anti-goat IgG antibody followed by the streptavidin–HRP. NC slides, where primary antibody was replaced with rabbit IgG, were also performed.

RNA extraction and qRT-PCR

RNA extraction and qRT-PCR were performed as previously described (Lim *et al.* 2013a). Briefly, total RNA was extracted from cells using the TRIsure reagent according to manufacturer's instructions (Bioline, Alexandria, NSW, Australia). RNA concentration and purity were measured using a NanoDrop ND1000 Spectrophotometer (Thermo Fisher Scientific). RNA quality was determined via the $A_{260}:A_{280}$ ratio. RNA was converted to cDNA using the Tetro cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. The cDNA was diluted 50-fold, and 4 μl of this was used to perform RT-PCR using the SensiFAST SYBR NO-ROX Kit (Bioline) and 100 nM of pre-designed and validated QuantiTect primers (Qiagen). The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories). Average gene Ct values were normalised to the average β -actin Ct values of the same cDNA sample. Of note, there was no effect of experimental treatment on β -actin gene expression. Fold differences were determined using the comparative Ct method.

Western blotting

Extraction of cytoplasmic and nuclear protein by western blotting was performed as previously described (Lim *et al.* 2013b). Forty micrograms of protein was separated onto 10%

polyacrylamide gels (Bio-Rad Laboratories) and transferred to PVDF. The blots were incubated with 0.8 µg/ml rabbit polyclonal anti-KLF5, #GTX103289 (GeneTex, Inc.) diluted in blocking buffer (5% skimmed milk in TBS with 0.05% Tween-20) for 16 h at 4 °C. The membranes were viewed and analysed using the ChemiDoc XRS system (Bio-Rad Laboratories). Semi-quantitative analysis of the relative density of the bands in western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories). The data were normalised to β -actin or Ponceau S staining as previously described (Lim *et al.* 2013b). For data normalisation, a section of the Ponceau S stained membrane was chosen. Notably, the section chosen was one that did not show variation with labour status.

Cytokine and prostaglandin assays

The release of IL6 and IL8 was performed using CytoSet sandwich ELISA according to the manufacturer's instructions (Life Technologies). The limit of detection of the IL6 and IL8 assays was 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 of the PGE₂ and PGF_{2 α} assays was 16 and 60 pg/ml respectively. For all assays, the interassay and intraassay coefficients of variation were <10%.

Statistical analysis

Statistics was performed on the normalised data unless otherwise specified. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For data on the effect of human labour on KLF5 expression in foetal membranes and myometrium, unpaired Student's *t*-test was used to assess statistical significance between normally distributed data; otherwise, the non-parametric Mann-Whitney *U* was used. For assessing effect of KLF5 siRNA on basal expression of pro-inflammatory cytokines, a one-sample *t*-test, against the constant of 1, was used. For assessing effect of KLF5 siRNA on IL β -, poly(I:C)-, flagellin- induced pro-inflammatory cytokines; on COX2-prostaglandin pathway and on NF κ B p65 transcriptional activity the homogeneity of data was assessed by Bartlett's test, and when significant, the data were logarithmically transformed before further analysis. The data were analysed by a one-way ANOVA using Fisher's least significant difference *post hoc* test to allow multiple comparisons between groups. Statistical significance was ascribed to *P* value <0.05. Data were expressed as mean $\pm$ S.E.M., unless otherwise specified.

Results

Localisation of KLF5 in foetal membranes and myometrium

Immunohistochemistry was performed on term non-labouring foetal membranes and myometrium to identify

Figure 1 Localisation of KLF5 in term foetal membranes and myometrium. KLF5 expression in human term (A) foetal membranes and (B) myometrium. Negative control for (C) foetal membranes and (D) myometrium is also displayed. ae, amniotic epithelium; cl, connective tissue layer; ct, cytotrophoblast layer; dec, decidua. Original magnification $\times 100$.

the cellular localisation of KLF5 (Fig. 1). In foetal membranes, positive cytoplasmic and nuclear KLF5 expression was detected in amnion epithelial cells, cytotrophoblast cells and decidua (Fig. 1A). In addition, some KLF5 expression was also observed in the fibroblast cells of the connective tissue layer. In myometrium, positive KLF5 cytoplasmic and nuclear staining was detected in the smooth muscle fibres (Fig. 1C). There was no staining present in the NC for foetal membranes (Fig. 1B) or myometrium (Fig. 1D).

Effect term and preterm labour on KLF5 expression in human foetal membranes and myometrium

The next aim of this study was to determine the effect of human labour on KLF5 nuclear protein expression in foetal membranes and myometrium. Foetal membranes were obtained at term Caesarean section in the absence of labour (term no labour; *n*=6 patients) and after spontaneous labour and membrane rupture (term after labour; *n*=6 patients). To determine the effect of spontaneous preterm birth (without histological chorioamnionitis) on KLF5 expression, foetal membranes were obtained from women at preterm Caesarean section with no labour (preterm no labour; *n*=6 patients), and after spontaneous preterm labour and normal vaginal delivery (preterm after labour; *n*=6 patients). Myometrium was obtained at term Caesarean section in the absence of labour (term no labour; *n*=6 patients) and after spontaneous labour onset (term in labour; *n*=6 patients).

Figure 2 Effect of human labour on KLF5 expression in foetal membranes and myometrium. (A) Human foetal membranes were obtained from women not in labour at term Caesarean section and women after term spontaneous labour onset and delivery ($n=6$ patients/group). (B) Human foetal membranes were obtained from women not in labour at preterm Caesarean section and women after preterm spontaneous labour onset and delivery ($n=6$ patients/group). (C) Human myometrium was obtained from non-labouring and labouring women at term Caesarean section ($n=6$ patients/group). (A, B and C) Nuclear and cytoplasmic protein expression of KLF5 was analysed by western blotting analysis. KLF5 protein expression was normalised to either loading control (Ponceau S) or β -actin and the fold change was calculated relative to the no labour group. Data are displayed as mean $\pm$ s.e.m. * $P<0.05$ vs no labour (Student's t -test). The western blot from the six patients per group is also shown.

In foetal membranes, KLF5 cytoplasmic and nuclear protein expression was similar between non-labouring and labouring tissues at both term (Fig. 2A) and preterm (Fig. 2B). On the other hand, in myometrium, KLF5 nuclear protein expression was

significantly higher in labouring myometrium when compared with non-labouring samples (Fig. 2C). Although cytoplasmic KLF5 was lower in labouring myometrium, this did not reach statistical significance ($P=0.06$).

Figure 3 Effect of KLF5 siRNA on basal expression of pro-inflammatory cytokines. Human primary myometrial cells were transfected with or without 200 nM KLF5 or NC siRNA for 48 h ($n=3$ patients). (A, C and D) *KLF5*, *IL6* and *IL8* gene expression was analysed by qRT-PCR. Gene expression was normalised to β -actin mRNA expression and the fold change was calculated relative to the NC siRNA-transfected cells. Data are displayed as mean $\pm$ S.E.M. * $P<0.05$ vs NC siRNA (one-sample t -test). (B) Representative western blot images demonstrating KLF5 protein expression. Ponceau S staining was used as loading control. (E and F) The incubation medium was assayed for concentration of IL6 and IL8 by ELISA. The fold change was calculated relative to NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M.

Effect of KLF5 siRNA on pro-inflammatory cytokines in primary myometrium cells

Having shown that human term labour increases KLF5 expression in myometrium; the next aim of this study was to determine the effect of *KLF5* knockdown using siRNA on pro-labour mediators. For these studies, primary myometrial cells isolated from fresh myometrial tissue were used. The efficacy of transfection was analysed by qRT-PCR and western blotting and the data presented in Fig. 3A and B; both the gene and protein expression of KLF5 were decreased in KLF5 siRNA transfected cells when compared with NC siRNA transfected cells. Also presented in Fig. 3 is the effect of *KLF5* siRNA

knockdown on *IL6* and *IL8* mRNA expression and release under basal conditions. There was no effect of KLF5 siRNA knockdown on basal mRNA expression (Fig. 3C and E) or release (Fig. 3D and F) of *IL6* and *IL8*.

For subsequent experiments, after siRNA transfection, cells were treated with the pro-inflammatory cytokine *IL1 β* , the viral dsRNA analogue poly(I:C) and the bacterial product flagellin to mimic preterm labour in order to define the relative importance of KLF5 in the expression of pro-inflammatory cytokines and prostaglandin synthesis. Figure 4 demonstrates the effect of siRNA knockdown of *KLF5* on *IL1 β* -induced gene expression and secretion of pro-inflammatory cytokines. When compared with basal NC siRNA, there was a significant increase in both the mRNA expression (Fig. 4A and C) and release (Fig. 4B and D) of *IL6* and *IL8*. However, in cells deficient in KLF5, this increase in cytokine expression and secretion was significantly reduced.

The effect of *KLF5* siRNA knockdown on the expression and release of pro-inflammatory cytokines in the presence of poly(I:C) is shown in Fig. 5. In NC siRNA transfected cells, *IL6* and *IL8* mRNA expression

Figure 4 Effect of KLF5 siRNA on *IL1 β* -induced pro-inflammatory cytokines. Human primary myometrial cells were transfected with or without 200 nM KLF5 or NC siRNA 48 h and then treated with 1 ng/ml *IL1 β* for an additional 24 h ($n=5$ patients). (A and C) Gene expression for *IL6* and *IL8* was analysed by qRT-PCR. Gene expression was normalised to β -actin mRNA expression and the fold change was calculated relative to *IL1 β* -stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs *IL1 β* -stimulated NC siRNA-transfected cells (one-way ANOVA). (B and D) The incubation medium was assayed for concentration of IL6 and IL8 by ELISA. The fold change was calculated relative to *IL1 β* -stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs *IL1 β* -stimulated NC siRNA-transfected cells (one-way ANOVA).

Figure 5 Effect of KLF5 siRNA on poly(I:C)-induced pro-inflammatory cytokines. Human primary myometrial cells were transfected with or without 200 nM KLF5 or NC siRNA 48 h and then treated with 5 µg/ml poly(I:C) for an additional 24 h ($n=5$ patients). (A and C) Gene expression for *IL6* and *IL8* was analysed by qRT-PCR. Gene expression was normalised to β -actin mRNA expression and the fold change was calculated relative to poly(I:C)-stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs poly(I:C)-stimulated NC siRNA-transfected cells (one-way ANOVA). (B and D) The incubation medium was assayed for concentration of IL6 and IL8 by ELISA. The fold change was calculated relative to poly(I:C)-stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs poly(I:C)-stimulated NC siRNA-transfected cells (one-way ANOVA).

(Fig. 5A and C) and release (Fig. 5B and D) was significantly augmented by poly(I:C). This increase in poly(I:C)-induced cytokine production was significantly reduced in cells transfected with KLF5 siRNA.

The effect of KLF5 siRNA silencing on the expression and secretion of pro-inflammatory cytokines in the presence of flagellin is depicted in Fig. 6. As expected, in NC siRNA transfected myometrial cells, flagellin induced a significant increase in *IL6* and *IL8* mRNA expression (Fig. 6A and C) and release (Fig. 6B and D). The effect of KLF5 silencing was a significant decrease in flagellin-induced cytokine gene expression and secretion.

Effect of KLF5 siRNA on the COX2–prostaglandin pathway in primary myometrium cells

The effect of KLF5 silencing on the COX–prostaglandin pathway in the presence of IL1 β , TNF α or poly(I:C) was also assessed and the data presented in Fig. 7. As expected, in NC siRNA transfected myometrial cells, treatment with IL1 β (Fig. 7A), poly(I:C) (Fig. 7D) or flagellin (Fig. 7E) induced a significant increase in *COX2*

mRNA expression. Likewise, the release of PGE₂ and PGF_{2 α} was significantly augmented by IL1 β in NC siRNA transfected cells (Fig. 7B and C). The concentration of prostaglandins was below the sensitivity of the curve for poly(I:C)- or flagellin-stimulated primary human myometrial cells. The effect of *KLF5* silencing was a significant decrease in IL β -induced *COX2* gene expression, and PGE₂ and PGF_{2 α} release (Fig. 7A, B and C). Similarly, *KLF5* knockout was associated with a significant decrease in poly(I:C)- and flagellin-induced *COX2* gene expression (Fig. 7D and E). Of note, there was no effect of IL1 β , poly(I:C), flagellin, or KLF5 siRNA on *COX1* mRNA expression (data not shown).

Silencing of KLF5 represses NF κ B p65 transcriptional activity

To determine whether KLF5 regulates pro-labour mediators in primary myometrial cells through NF κ B, the effect of *KLF5* gene silencing on NF κ B transcriptional activity was determined using a luciferase activity assays. As shown in Fig. 8, treatment with IL1 β (Fig. 8A), poly(I:C)

Figure 6 Effect of KLF5 siRNA on flagellin-induced pro-inflammatory cytokines. Human primary myometrial cells were transfected with or without 200 nM KLF5 or NC siRNA 48 h and then treated with 1 µg/ml flagellin (flag) for an additional 24 h ($n=5$ patients). (A and C) Gene expression for *IL6* and *IL8* was analysed by qRT-PCR. Gene expression was normalised to β -actin mRNA expression and the fold change was calculated relative to flag-stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs flag-stimulated NC siRNA-transfected cells (one-way ANOVA). (B and D) The incubation medium was assayed for concentration of IL6 and IL8 by ELISA. The fold change was calculated relative to flag-stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs flag-stimulated NC siRNA-transfected cells (one-way ANOVA).

Figure 7 Effect of KLF5 siRNA on COX2–prostaglandin pathway. Human primary myometrial cells were transfected with or without 200 nM KLF5 or NC siRNA 48 h and then treated with (A, B and C) 1 ng/ml IL1β, (D) 5 μg/ml poly(I:C) or (E) 1 μg/ml flagellin (flag) for an additional 24 h ($n=5$ patients). (A, D and E) Gene expression for COX2 was analysed by qRT-PCR. Gene expression was normalised to β -actin mRNA expression and the fold change was calculated relative to IL1β-, poly(I:C)- or flag-stimulated NC siRNA-transfected cells. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs IL1β-stimulated NC siRNA-transfected cells (one-way ANOVA); § $P<0.05$ vs poly(I:C)-stimulated NC siRNA-transfected cells (one-way ANOVA) and # $P<0.05$ vs flag-stimulated NC siRNA-transfected cells (one-way ANOVA). (B and C) The incubation medium was assayed for the concentration of PGE₂ and PGF_{2α} release by EIA. Data displayed as mean $\pm$ S.E.M. * $P<0.05$ vs IL1β-stimulated NC siRNA-transfected cells (one-way ANOVA).

(Fig. 8B) or flagellin (Fig. 8C) significantly increased NFκB p65 luciferase activity in NC siRNA-transfected myometrial cells. However, luciferase activity was significantly decreased in KLF5 siRNA-transfected cells.

Discussion

The novel findings of this study are KLF5 expression is higher in term labouring myometrium when compared with term non-labouring myometrium. Moreover,

loss-of-function studies using siRNA in primary myometrial cells revealed that KLF5 is required for the expression and secretion of pro-inflammatory and pro-labour mediators in the presence of mediators of preterm birth. Specifically, in cells transfected with KLF5 siRNA, there was a significant decrease in IL1β-, flagellin- and poly(I:C)-stimulated expression and secretion of pro-inflammatory cytokines, and COX2 expression and prostaglandin release. Furthermore, a luciferase assay also demonstrated a significant decrease in NFκB p65 transcriptional activity in KLF5 siRNA-transfected cells, suggesting that KLF5 may mediate its pro-inflammatory effects via the NFκB pathway.

Human labour is considered an inflammatory response as an influx of leukocytes into the myometrium and cervix increases the production of pro-inflammatory cytokines IL1β and TNFα in these tissues as well as foetal membranes (Osman *et al.* 2003, Romero *et al.* 2006, Bollapragada *et al.* 2009). Contributing to the onset of labour, these pro-inflammatory cytokines can increase the production of pro-inflammatory cytokines and chemokines; promote the production of prostaglandins via COX2, mediators of uterine contractions; and MMPs,

Figure 8 Effect of KLF5 siRNA on NFκB p65 transcriptional activity. Human myometrial cells were transfected with 0.1 μg NFκB reporter construct. After 6 h, cells were transfected with 200 nM KLF5 or NC siRNA for 48 h, then treated with (A) 1 ng/ml IL1β, (B) 5 μg/ml poly(I:C) or (C) 1 μg/ml flagellin (flag) for an additional 24 h ($n=5$ patients). Promoter activity (normalised with Renilla expression) is expressed as a ratio of luciferase activity of IL1β-, poly(I:C)- or flag-stimulated NC siRNA-transfected cells. Each bar represents the mean $\pm$ S.E.M. * $P<0.05$ vs IL1β stimulated NC siRNA-transfected cells (one-way ANOVA); § $P<0.05$ vs poly(I:C)-stimulated NC siRNA-transfected cells (one-way ANOVA) and # $P<0.05$ vs flag-stimulated NC siRNA-transfected cells (one-way ANOVA).

which instigate cervical ripening and foetal membrane rupture, events culminating in successful labour and delivery (Fata *et al.* 2000, Bowen *et al.* 2002, Keelan *et al.* 2003, Lappas & Rice 2004, Christiaens *et al.* 2008). While nuclear KLF5 expression was increased in myometrium with spontaneous term labour, there was no change in KLF5 expression in foetal membranes after spontaneous labour and delivery, at term or preterm. It may be that KLF5 acts in the myometrium during labour to induce uterine contractions, upstream from events that lead to rupture of membranes.

It is possible that increased KLF5 expression in term labouring myometrium may be a consequence of the processes of labour. Indeed, it has been shown that TNF α induces KLF5 expression in human umbilical vein endothelial cells, venous smooth muscle cells and intestinal epithelial cells (Bafford *et al.* 2006, Kumekawa *et al.* 2008). Whether KLF5 expression is also increased in labouring myometrium from preterm deliveries with or without infection is not known and could not be determined due to lack of suitable samples. However, bacterial endotoxin LPS has been shown to induce KLF5 expression *in vitro* (Chanchevalap *et al.* 2006, Chen *et al.* 2014) and *in vivo* (Chen *et al.* 2014). Likewise, the Gram-negative, microaerophilic bacterium *Helicobacter pylori* promotes the expression of KLF5 in human gastric epithelial cells (Noto *et al.* 2013).

Term and preterm labour share the common terminal pathway of labour that includes increased myometrial contractility, cervical ripening and decidual/membrane activation (Romero *et al.* 2006). Term labour is the result of physiological activation of this pathway, while preterm labour is due to pathological insults (Romero *et al.* 2006). There appears to be a causal role for IL1 β in the processes of parturition; there is elevated expression of IL1 β in the third trimester during normal pregnancies without signs of infection (Elliott *et al.* 2001) as well as in pregnancies that display infection and in preterm deliveries (Goldenberg *et al.* 2000, Menon & Fortunato 2004, Farina & Winkelman 2005). Intrauterine infection caused by bacteria is a common and important mechanism that leads to preterm birth (Goldenberg *et al.* 2000). Bacterial products, such as flagellin which is isolated from both Gram-positive and Gram-negative bacteria, stimulate the production of pro-inflammatory cytokines and prostaglandins, leading to myometrial contractions and thus preterm labour (Schaefer *et al.* 2004, Herbst-Kralovetz *et al.* 2008, Lim *et al.* 2014). Associations between viral infections and chorioamnionitis and/or spontaneous preterm birth have been also reported (Gomez *et al.* 2008, Tsekoura *et al.* 2010). Furthermore, the viral dsRNA analogue poly(I:C) induces pro-inflammatory cytokines in foetal membranes (Bakaysa *et al.* 2014). It was thus of interest to determine if KLF5 regulates pro-inflammatory and pro-labour mediators in the presence of IL1 β , flagellin and the poly(I:C) in human myometrium.

Loss-of-function studies revealed that KLF5 has a pro-inflammatory and pro-labour role in human myometrium. Specifically, KLF5 silencing attenuated IL1 β -stimulated expression of pro-inflammatory cytokines IL6 and IL8, COX2 mRNA expression and release of prostaglandins PGE₂ and PGF_{2 α} . In addition, flagellin- and poly(I:C)-induced IL6 and IL8 mRNA expression was also significantly reduced in KLF5 knockout cells. Concurrent with these findings, knockdown of KLF5 by siRNA inhibits TNF α -induced MCP1 expression in endothelial cells (Kumekawa *et al.* 2008), LPS-induced TNF α and IL6 in intestinal epithelial cells (Chanchevalap *et al.* 2006); and microsomal PGE₂ synthase 1 (mPGES1) mRNA and protein expression and subsequent PGE₂ synthesis in breast cancer cells (Xia *et al.* 2013). *In vivo*, epithelial COX2 expression is absent with loss of KLF5 (Sun *et al.* 2012); KLF5 heterozygous knockout mice show an attenuated induction of hyperproliferative responses after bacterial infection (McConnell *et al.* 2008) and KLF5 haploinsufficient (Klf5^{+/-}) mice are protected from renal injury and inflammation induced by UUO (Fujiu *et al.* 2011). The zinc finger E-box binding homeobox protein ZEB2 is increased in KLF5 deficient embryonic stem cells (Parisi *et al.* 2010). Interestingly, in uterine tissues, the ZEB1 and ZEB2 proteins, which are downregulated during human labour and in mouse models of preterm labour (Renthal *et al.* 2010), have been shown to regulate uterine quiescence and contractility during pregnancy and labour (Renthal *et al.* 2010).

It is now well established that the pro-inflammatory nuclear transcription factor, NF κ B, plays a crucial role in the processes of human labour and delivery (Belt *et al.* 1999, Lappas *et al.* 2002, 2003, 2004, 2006, Lappas & Rice 2007). NF κ B has been shown to regulate the expression of numerous pro-labour genes including IL6, IL8 and COX2 in the presence of infection or inflammation (Belt *et al.* 1999, Lappas *et al.* 2002, 2003, 2004, 2006, Lappas & Rice 2007, Bakaysa *et al.* 2014). Studies in non-gestational tissues demonstrate that NF κ B is required for KLF5 signalling. For example, in intestinal epithelial cells, knockdown of KLF5 by siRNA reduces the expression of p50 and p65 subunits of NF κ B in response to LPS (Chanchevalap *et al.* 2006). Thus, in order to determine if NF κ B plays a role in the pro-inflammatory actions of KLF5 in response to IL1 β , flagellin or poly(I:C), a luciferase assay was performed. As expected, the pro-inflammatory cytokine IL1 β , the bacterial product flagellin and the viral dsRNA analogue poly(I:C) induced NF κ B transcriptional activity in NC siRNA transfected primary myometrial cells. Notably, in cells transfected with KLF5 siRNA, there was a significant decrease in NF κ B transcriptional activity in the presence of IL1 β , flagellin or poly(I:C), suggesting that KLF5 may be exerting its pro-inflammatory and pro-labour actions via NF κ B. These findings suggest that KLF5 is an important transcription factor required for NF κ B promoter activity. That is, KLF5 may interact with

the NF κ B promoter in myometrial cells upon IL1 β , flagellin or poly(I:C) stimulation. Indeed, KLF5 has also been shown to directly interact with NF κ B in epidermal epithelial cells (Sur *et al.* 2002).

Members of the KLF family of transcription factors share a high degree of sequence homology in their zinc-finger regions; however their non-DNA binding portions are diverse in composition and thus function (Bieker 2001). Several of the 17 mammalian KLF proteins have been implicated in inflammation, with both pro- and anti-inflammatory actions reported. For example, overexpression of KLF4 mediates pro-inflammatory signalling in macrophages (Feinberg *et al.* 2005, Liu *et al.* 2012); overexpression of KLF2 downregulates pneumococci-induced NF κ B-dependent gene expression and IL8 release; siRNA silencing of KLF2 provoked an enhanced inflammatory response (Zahlten *et al.* 2010); and mice with systemic and smooth muscle-specific deficiency of KLF15 exhibit aggressive inflammatory vasculopathy (Lu *et al.* 2013). On the other hand, overexpression of KLF6 in HEK293 cells enhances TNF α and IL1 β -induced activation of NF κ B and transcription of downstream genes; knockdown of KLF6 had the opposite effects (Zhang *et al.* 2014). In cultured 3T3-L1 adipocytes, treatment with TNF α significantly reduced the mRNA levels of *KLF9* and *KLF15* (Enomoto *et al.* 2013). Thus, it would also be of interest to elucidate the role that other KLF family members play in human myometrium.

In conclusion, KLF5 has a pro-inflammatory role in human myometrium. KLF5 expression was increased in labouring term myometrium. Furthermore, silencing of KLF5 in myometrial cells decreased inflammation-induced expression of pro-inflammatory cytokines, COX2 mRNA expression and subsequent prostaglandin release. In addition, bacterial and viral infection-induced pro-inflammatory cytokine release was also significantly reduced in *KLF5* siRNA knockout cells. These findings are of significance given the central role of these mediators in the terminal processes of human preterm labour and delivery (Bowen *et al.* 2002, Keelan *et al.* 2003, Lappas & Rice 2004, Christiaens *et al.* 2008). Further studies are required to further elucidate the role of KLF5 in the processes of human labour and delivery and its potential as a therapeutic target for the treatment of inflammation- and/or infection-induced 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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