Binding loci of RelA-containing nuclear factor-kappaB dimers in promoter regions of PHM1-31 myometrial smooth muscle cells

Victoria J. Cookson1,5,†, Sarah L. Waite1,†, Paul R. Heath2, Paul J. Hurd3, Saurabh V. Gandhi4, and Neil R. Chapman1,*

1Academic Unit of Reproductive and Developmental Medicine, Department of Human Metabolism, University of Sheffield, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF, UK 2Sheffield Institute for Translational Neuroscience, University of Sheffield, 385a Glossop Road, Sheffield S10 2HQ, UK 3School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK 4Department of Obstetrics and Gynaecology, Sheffield Teaching Hospitals NHS Foundation Trust, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF, UK 5Present address: Academic Unit of Clinical Oncology, University of Sheffield, Medical School, Beech Hill Road, Sheffield S10 2RX, UK

*Correspondence address. Tel: +44 114 2268530; E-mail: n.r.chapman@sheffield.ac.uk

Submitted on April 22, 2015; resubmitted on July 24, 2015; accepted on September 3, 2015

ABSTRACT: Human parturition is associated with many pro-inflammatory mediators which are regulated by the nuclear factor-kappaB (NF-κB) family of transcription factors. In the present study, we employed a ChIP-on-chip approach to define genomic loci within chromatin of PHM1-31 myometrial cells that were occupied by RelA-containing NF-κB dimers in response to a TNF stimulation of 1 h. In TNF-stimulated PHM1-31 cells, anti-RelA serum enriched 13 300 chromatin regions; importantly, 11 110 regions were also enriched by anti-RelA antibodies in the absence of TNF. DNA sequences in these regions, from both unstimulated or TNF-stimulated PHM1-31 cultures, were associated with genic regions including IkBα, COX-2, IL6RN, Jun and KCNMB3. TNF-induced binding events at a consensus κB site numbered 1667; these were represented by 112 different instances of the consensus κB motif. Of the 1667 consensus κB motif occurrences, 770 (46.2%) were identified within intronic regions. In unstimulated PHM1-31 cells, anti-RelA-serum-enriched regions were associated with sequences corresponding to open reading frames of ion channel subunit genes including CACNB3 and KCNB1. Moreover, in unstimulated cells, the consensus κB site was identified 2116 times, being defined by 103 different sequence instances of this motif. Of these 2116 consensus κB motifs, 1089 (51.5%) were identified within intronic regions. Parallel expression array analyses in PHM1-31 cultures demonstrated that TNF stimulated a 2.2-fold induction in 51 genes and a fold repression of 1.5 in 18 others. We identified 14 anti-RelA-serum-enriched genomic regions that correlated with 17 TNF-inducible genes, such as COX2, Egr-1, Jun, IkBα and IL6, as well as five regions associated with TNF-mediated gene repression, including Col1A2.

Key words: NF-kappaB / chromatin / labour / microarray / preterm birth

Introduction

In the developed world, premature birth (that before 37 weeks completed gestation) complicates 6–12% of pregnancies (Khashan et al., 2010). Annually it is estimated that 1.1 million babies worldwide die from being born prematurely (Blencowe et al., 2012; Chang et al., 2013); surviving infants having an elevated risk of major long-term mental and physical handicap (Marlow et al., 2005; Costeloe et al., 2012). Moreover, such infants also have a disproportionate effect on health-care budgets worldwide: a recent UK estimate of the total cost of preterm birth to the public sector was £2.95 billion (Mangham et al., 2009). Tocolytic therapies (drugs which stop premature contractions of the womb) are few in number and are associated with complications for both infant and mother (Oei, 2006). This problem is compounded by the fact that, despite many years of research, we remain ignorant of the fundamental biological principles governing uterine function during pregnancy and labour.

NF-κB biology and the myometrium

Regulatory networks between transcription factors and DNA ensure cells function normally. The nuclear factor-kappaB (NF-κB) family are
one set of transcription factors which govern a wide variety of cellular activities (reviewed in Perkins, 2007, 2012; Cookson and Chapman, 2010; Hayden and Ghosh, 2012). NF-κB, which is rapidly induced by over 400 different stimuli including TNF (Perkins, 2007; Cookson and Chapman, 2010; Hayden and Ghosh, 2012), is present in virtually every cell type within the body. NF-κB is composed of dimeric complexes formed from five distinct subunits: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Perkins, 2007, 2012; Cookson and Chapman, 2010; Hayden and Ghosh, 2012). DNA binding by NF-κB dimers is mediated by a conserved N-terminal domain termed the Rel Homology Region (Chen and Ghosh, 1999). Combinations of subunits determine the specificity of transcriptional activation (Perkins et al., 1992; Chen and Ghosh, 1999); indeed NF-κB can modulate prolonged gene expression through the exchange of NF-κB dimers at a given promoter (Saccani et al., 2003).

There are predicted to be in excess of 3000 κB sites within the human genome with the consensus NF-κB binding site generally viewed as S'-G...G-A-G...G-A-N-2, Y-1, Y-1, Y-3C-G-3C-G-A/G-T (where R = A or G, N = A, C, T or G and Y = C or T; Natoli et al., 2005). Importantly, there are a great many functional variants of this consensus κB motif and there is now a wealth of studies describing how κB motifs associate with various NF-κB dimers (Ghosh et al., 1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997, 2005; Chen et al., 1998a; Chen and Ghosh, 1999; Phelps et al., 2000; Hoffmann et al., 2003; Leung et al., 2004; Moorthy et al., 2007; Trinh et al., 2008; Wan and Lenardo, 2009; Wang et al., 2012).

At term, the smooth muscle of the uterus, the myometrium, is exposed to a complex milieu of inflammatory signalling factors (Aguilar and Mitchell, 2010; Cookson and Chapman, 2010; Golightly et al., 2011; Webster et al., 2013). Moreover, there is now a body of evidence that NF-κB dimers containing the RelA NF-κB subunit play a pivotal role in regulating human parturition (Belt et al., 1999; Allport et al., 2001; Eliot et al., 2001; Yan et al., 2002a, b; Lappas et al., 2003, 2004; Lee et al., 2003; Chapman et al., 2004, 2005; Lappas and Rice, 2004; Soloff et al., 2004, 2006; Lindström and Bennett, 2005; Terzidou et al., 2006; Mohan et al., 2007; Lindström et al., 2008). Consequently, it would seem highly likely that, based on the evidence above, the myometrial smooth muscle cell could have evolved suitable mechanisms to ensure those NF-κB-regulated promoters are expressed only at the correct spatio-temporal juncture. Consistent with this notion, we have previously demonstrated that temporal changes in NF-κB subunit composition and associated DNA-binding activity occurs between non-pregnant (NP), pregnant (P) and spontaneously labouring (SL) myometrium (Chapman et al., 2004). At present, the importance of this change in NF-κB dimer composition within the uterine smooth muscle is unclear. Temporal changes in NF-κB subunit composition on NF-κB-regulated promoters, however, can permit fine-tuning of the transcriptional response ensuring the gene is expressed at the correct level for the appropriate length of time (Saccani et al., 2003).

The obvious corollary to these observations, therefore, is that it is highly likely that a similar temporal manner of regulation is being employed in the uterus ensuring parturition occurs at the correct juncture. The study described herein examines where NF-κB complexes bind to chromatin in myometrial cells and whether this binding influences gene expression in such cells. Essentially this allows us to determine if NF-κB promoter occupancy is associated with transcriptional activation, transcriptional repression or homeostasis.

Materials and Methods

PHM1-31 cell passaging

PHM1-31 immortalized human myometrial myocytes were the kind gift of Prof. Barbara Sanborn, Colorado State University, USA (Monga et al., 1996). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) FCS and 2 mM L-glutamine and 0.1 mg/ml Geneticin using published cell culture procedures (Chapman et al., 2005; Webster et al., 2013; Waite et al., 2014).

Transient transfections, plasmids and luciferase assays

Transient transfection of PHM1-31 myometrial cells was performed using the LT-1 reagent from Miras (Genflow, Staffordshire UK) as described by Chapman et al. (2005) for primary myometrial cells. The 3x-kB-ConA-luciferase (3x-kB-ConA-Luc) and enh-ConA-luciferase (ΔkB-ConA-Luc) vectors were the generous gift of Prof. Ron Hay (University of Dundee, UK) and the construction of these has been reported in detail (Rodriguez et al., 1996). All transfection experiments were performed a minimum of three times and results are expressed as the mean ± SEM. All data analyses were conducted on GraphPad Prism Version 5.02 (GraphPad Software, San Diego, CA, USA). Data from two matched samples were compared using a paired, two-tailed t-test; P < 0.05 was considered statistically significant.

Rela immunocytochemistry in PHM1-31 cells following TNF stimulation

PHM1-31 cells were cultured in a 24-well plate, washed in PBS and fixed in 1% (v/v) formaldehyde overnight at 4°C. Endogenous cellular peroxidase was quenched with 1% (v/v) hydrogen peroxide for 10 minutes. The Vectastain® Elite ABC kit (Vector Labs) was used for the following reactions. Endogenous biotin was blocked with PBS containing horse serum and avidin for one hour at room temperature, followed by incubation with primary antibody (anti-RelA, #sc-372, Santa Cruz Biotechnology Inc.) in antibody diluent and avidin. Following four washing steps in PBS, sections were incubated with the ABC reagent (Vector Labs) for 30 min at room temperature before the addition of the ABC reagent for 30 min at room temperature and finally DAB (3, 3-dianisobenzindole). Cells were stored in PBS and photographed. Negative control experiments included the substitution of the primary antibody with an isotype control (Abcam, #ab64450).

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared essentially as described in Dignam et al. (1983). In this study, the electrophoretic mobility shift assay (EMSA) utilized an oligonucleotide consisting of the HIV-1 3’long terminal repeat (LTR) κB site (in bold; 5’-GATCCGCTGGGACTTTCGAGCCG-3’). The EMSA was carried out as detailed in Chapman et al. (2002, 2005).

Western immunodetection

Expression of the RelA NF-κB subunit was examined using western analysis with immunoblots probed with antibodies that recognize either the amino terminal or carboxy terminal of RelA (p65) (Santa Cruz Biotechnology Inc. Santa Cruz, CA; #sc-109 and #sc-372, respectively) and developed using EZ-ECL detection reagents (Genflow, Staffs., UK) as detailed in Chapman et al. (2004).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed on eight T-75 flasks of PHM1-31 cells (~2.6–2.8 × 10^6 cells/flask) grown to 100% confluence using the Magna-ChIP ChIP assay kit (#17–611, Millipore UK).
Ltd, Dundee) following the manufacturer’s guidelines and detailed in Webster et al. (2013). ChIP antibodies used were RelA and RNA Polymerase II (#sc-372 and #sc-899 respectively; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Briefly, four flasks were stimulated with 10 ng/ml TNF for 1 h while the remaining four were unstimulated controls. TNF has been demonstrated to be present in myometrium at term (Opsjøn et al., 1993; Fitzgibbon et al., 2009; reviewed in Golightly et al., 2011) and has been used regularly by our group studying cytokine-induced myometrial NF-κB function (Chapman et al., 2005, Webster et al., 2013; Waite et al., 2014). The rationale for this time point was that it would represent an early response to TNF. We believe such early binding events play pivotal roles in the cell’s choice of subsequent signalling pathway usage (reviewed in Perkins, 2007). It was accepted that TNF-induced gene regulation events occurring after one hour would not be investigated (Campbell et al., 2001; Rocha et al., 2003). Three biological replicates of these ChIP assays were completed. The workflow utilized to generate the appropriate chromatin samples is illustrated in Fig. 1.

**Quality control PCR of immunoprecipitated DNA**

Prior to microarray analyses, ChIP efficacy was determined by enrichment of RelA on the IkBα promoter. PCR was carried out on the immunoprecipitated DNA using primers flanking the κB sites within the IkBα promoter as a positive control (Chapman et al., 2005). The Gasp promoter, which is not regulated by RelA was chosen as a negative control (Webster et al., 2013). Once it was determined that the chromatin was of sufficiently high quality, it was then prepared ready to probe Affymetrix 1.0R Human promoter Arrays (Affymetrix, Santa Clara, CA, USA).

---

**Figure 1** Schematic representation of the workflow used to generate chromatin for this study.
Affymetrix microarrays

Full details of both arrays employed in this study can be found at the manufacturer’s web site:

GeneChip Human Promoter 1.0R Array: http://www.affymetrix.com/estore/catalog/131461/ AFFY/Human+Promoter+1.0R+Array#I_1


ChiP DNA amplification, fragmentation and labelling

ChiP DNA was amplified using the Whole Genome Amplification Kit (WGA; Sigma) as detailed in the manufacturer’s instructions with slight modifications. Briefly, 1 μl of ChiP DNA was diluted with 9 μl of ultrapure water. Then 2 μl of library preparation buffer together with 1 μl of library stabilization solution was added to this and heated at 95 °C for 2 min before cooling on ice. After that, 1 μl of library preparation enzyme was added and the reaction was incubated in the thermal cycler for the following times: 20 min at 16 °C (pre-cooled to this temperature), 20 min at 20 °C, 20 min at 37 °C, 5 min at 75 °C, and on hold at 4 °C. The amplified DNA was re-amplified using the WGA re-amplification kit to generate the 7.5 μg required. This method is described in the manufacturer’s guidelines (Sigma). The re-amplified DNA was then fragmented and labelled according to the Affymetrix ChiP Protocol using the GeneChip WT Double-Stranded DNA Terminal Labelling Kit.

Human Promoter 1.0R Array procedures

Hybridization of amplified DNA to Affymetrix Human Promoter 1.0R arrays was carried out using the GeneChip Hybridization, Wash and Stain Kit (Affimetrix) according to the Affymetrix ChiP Protocol. The hybridization cocktail (7.5 μg fragmented labelled DNA, 50 pM control oligonucleotide B2, hybridization mix, 7% (v/v) DMSO) was hybridized in the Affimetrix GeneChip Hybridization Oven 640. Washing and staining was carried out using the GeneChip Fluidics Station 450 as described in the GeneChip Expression Wash, Stain and Scan User manual (Affimetrix). The GeneChip Scanner 3000 7G, operated by the GeneChip Operating Software (GCOS, Affimetrix), was used to scan the Human Promoter 1.0R Arrays.

Data analysis in Partek Genomics Suite 6.6

The raw data (.CEL) files, generated by the GCOS software, were imported into Partek Genomics Suite (PGS; Version 6.6; www.partek.com/pgs) and subjected to Robust Multi-array Average (RMA) background correction, quantile normalization and Log (base 2) transformation utilizing the software’s tiling workflow. Prior to invoking an ANOVA, the raw data were normalized to the baseline by subtraction of all values ascribed to IgG samples from both unstimulated controls and TNF-treated samples since these values would represent non-specific binding events (Fig. 1: illustration of workflow). A two-way ANOVA was then completed within PGS to undertake multiple comparisons and determine the difference between unstimulated controls and TNF-treated samples at probe-level. Differences between control and TNF-stimulated samples were considered significant if P ≤ 0.05. Upon completion of the ANOVA, the Model-based Analysis of Tiling (MAT) algorithm (T statistic) was employed to detect enriched regions of chromatin in un-stimulated and TNF-stimulated data sets (Johnson et al., 2006). The MAT algorithm allowed a rapid method of detecting regions enriched by a given transcription factor (in this study RelA-containing NF-kB dimers). The MAT algorithm was then applied across a sliding window of 600 bp, using a minimum of 10 probes per region with ChiP-enriched regions deemed to be statistically significant when P ≤ 0.01: this output was the MAT score. Those regions with a positive MAT score and P-value ≤ 0.05 from the ANOVA indicated significant enrichment by anti-RelA antisera in those samples treated with TNF compared with untreated controls. Conversely, a negative MAT score and P-value ≤ 0.05 from the ANOVA represented those samples where enrichment of genomic loci was greater in unstimulated controls compared with those exposed to TNF. Promoters of known genes within the significantly enriched regions were then identified using the RefSeq database, based on the hg18 build of the human genome. All of the array data has been archived with the NCBI Gene Expression Omnibus (NCBI GEO) with the accession number GSE65721 (www.ncbi.nlm.nih.gov/geo); this number covers all associated experimental sub-series. All tables of original data sets can be accessed and downloaded from the folder entitled Cookson et al 2015 Public Access MHR Original Data Sets at the following hyperlink: https://drive.google.com/folderview?id=0B4bwcdSzbnm8OXdLWEtKemxtb0k &usp=sharing.

RNA extraction

On reaching 90% confluence, PHM1-31 cells were stimulated with 10 ng/ml TNF for one hour with non-stimulated flasks serving as controls. RNA was extracted using the EZ-RNA extraction system (Geneflow, Staffs., UK) and quantified using the nanophotometer (Implen; supplied by Geneflow, Staffs., UK). Prior to first strand synthesis, the quality of isolated RNA was verified using Agilent’s Eukaryote Total RNA Nano Chip (5067-1511) in conjunction with the Agilent 2100 bioanalyzer following the manufacturer’s guidelines. Three biological replicates were performed.

Affymetrix U133 plus2 human expression array procedures

RNA was reverse transcribed using Bio-Rad iScript cDNA synthesis Kit according to the manufacturer’s guidelines (Bio-Rad Laboratories Ltd, Hertfordshire, UK). The preparation and hybridization of cDNA to U133 plus2 expression arrays was performed according to published protocols (Kirby et al., 2011; Simpson et al., 2011; Brockington et al., 2013; Raman et al., 2015).

Data analysis in Partek Genomics Suite 6.6

The raw data files (.CEL) were imported into PGS V6.6 and analysed following the software’s Gene Expression workflow. Differentially expressed genes were identified using ANOVA to generate p values. Linear contrast was used to calculate fold-change and mean ratio from the contrast between unstimulated and TNF stimulated samples. Genes with fold change ≥ 2 or fold change ≤ 1.5 and with P-values ≤ 0.05 were identified using RefSeq.

Data analysis of combined human promoter 1.0R and U133 plus2 human expression arrays

Using PGS, the enriched region list from both Criteria-A and Criteria-C were merged with the respective gene list from the expression array analyses. This provided data defining NF-kB-enriched regions that were either expressed or repressed.

Validation of microarray results by qRT–PCR

Quantitative RT–PCR using SYBR Green Jumpstart Taq ReadyMix (Sigma, UK) was undertaken following MIQE guidelines (Bustin et al., 2009). GAPDH and β-Actin were selected as housekeeping genes. The primer sequences were as follows: TNFAIP3 Forward: 5′-TGAGCCCTTGGGCGTGGAACC-3′; TNFAIP3 Reverse: 5′-AAAGGGGCTGGTGCTTGGAC-3′; NFKBIA Forward: 5′-CGCCCGAAGCAACGCGGACGAC-3′; NFKBIA Reverse: 5′-GGGCGCTGTCCCTCTGGTA-3′; GAPDH Forward: 5′-TTGTTGAGATGTCGCGCATC-3′; GAPDH Reverse: 5′-CGGGGCGCGAATACGACCAATGC-3′; β-Actin Forward: 5′-CGAGCACAGAGCCCTGCGCT-3′; β-Actin Reverse: 5′-CGAGCACAGAGCCCTGCGCT-3′.
qPCR was performed in triplicate using a 7900HT fast qPCR Machine (Applied Biosystems) and gene expression data were analysed using ΔΔCT using SDS 2.0 Software (Applied Biosystems).

Results

TNF induces RelA nuclear localization and occupancy of the IkBα promoter in PHM1-31 cells

A number of pro-inflammatory cytokines, including TNF, are associated with the onset of both normal and preterm birth (Aguilar and Mitchell, 2010; Golightly et al., 2011). Prior to the ChIP-on-chip experiments, it was important to confirm that in PHM1-31 cells, NF-κB RelA was activated by stimulation with TNF. RelA immunocytochemistry of PHM1-31 cells showed diffuse staining in the control, unstimulated cells and those stained with control IgG, (Fig. 2A; Panels I and II) whilst translocation of the RelA subunit into the nucleus (black arrows) was apparent following 1 h TNF stimulation (Fig. 2A; Panel III). Moreover, increased RelA binding to the consensus κB site in EMSA was also seen and that binding activity could be specifically super-shifted with anti-RelA antiserum (Fig. 2B). Furthermore, when PHM1-31 cells were transiently transfected with the RelA-responsive 3x-κB-luc reporter and subsequently exposed to TNF for one hour, increased reporter activity was seen in those cells harbouring the 3x-κB-luc vector, but not those with the ΔκB-luc control (Fig. 2C).

Immunoprecipitation using the RelA antiserum (sc-372) showed specific binding to the RelA protein, while the control IgG failed to precipitate any RelA complexes (Fig. 2D). To demonstrate the specificity of the ChIP, the RelA antiserum detected low level binding of RelA complexes to the IkBα promoter region without TNF stimulation; as predicted, this increased upon TNF exposure. No binding was seen at the RelA-insensitive Gαs promoter region (Fig. 3A; Original Data Set - Table 3). While we cannot rule out the possibility that some RelA-enriched regions may be non-specific, the removal of the IgG-associated regions will minimize such interference.

RelA-enriched regions encoding or juxtaposed to genes associated with parturition

Enrichment of chromatin from TNF-treated cells by anti-RelA serum was seen to contain or be juxtaposed to regions encoding many genes believed to play a significant role in myometrial quiescence or labour itself including, but not limited to, PTGS2, Jun, I6RN, I6, CACNB3, KCNMB3, TRPC2 and VCAM-1 (Original Data Set - Table 4; Criteria-A and Original Data Set - Table 5; Criteria-C). Figure 4 illustrates examples of genes identified in RelA-enriched chromatin after cells were stimulated with TNF. Of these, regions harbouring PTGS2 and Jun were enriched in the presence of TNF (Figs 4A and B; Criteria-A regions are red bars) and those regions also encoded κB motif(s) that were in agreement with the published κB consensus site. For PTGS2 a region was also enriched in the absence of TNF but no κB motif was identified therein. Interestingly, genes encoding I6RN and KCNMB3 were also enriched in the presence of TNF but no discernible consensus κB motif could be identified in those respective Criteria-A enriched regions, suggesting a non-consensus κB motif was being utilized (Figs 4C and D; red bars). Moreover, we also observed that intronic regions of both I6RN and KCNMB3 were also enriched by anti-RelA NF-κB in the absence of TNF stimulation suggesting that RelA-containing dimers do have a function in governing expression of these genes (Figs 4C and D; blue bars). Finally, regions that were only enriched by RelA-containing dimers in the absence of TNF (i.e. unstimulated) were also examined. Examples of such regions were those encoding regulatory subunits of calcium and potassium channels such as CACNB3 and KCNB1 (Fig. 4E and F; blue bars). Significantly, regions encoding these ion channel subunits also harboured κB motifs corresponding to the consensus κB sequence (Figs 4E–F).

To ensure our experimental system was functioning correctly, we also examined the NF-κB-regulated the IkBα promoter region (Le Bail et al., 1993). As expected, in TNF-treated cells, aspects of the IkBα promoter were enriched by anti-RelA serum. Interestingly, however, no enrichment of the IkBα promoter was observed in the absence of TNF (Fig. 5A) despite this region being weakly amplified in the control ChIP assay (Fig. 2E). Increasing the resolution of the schematic representation

TNF induces RelA NF-κB promoter occupancy at a variety of promoters

In this study of PHM1-31 myometrial myocytes, statistically significant differences in promoter occupancy between unstimulated and TNF-treated cultures were observed in 24 410 genomic regions enriched by the anti-RelA antiserum (P ≤ 0.05); these data were termed Criteria-B and represent all enriched regions from both unstimulated and TNF-treated cultures (Fig. 3A; Original Data Set - Table 1). Within the set Criteria-B, defining the MAT algorithm parameters to enriched values of >0 and P ≤ 0.01 (i.e. a positive MAT score) generated 13 300 genomic loci that were significantly more enriched by the anti-RelA antiserum in TNF-treated cells compared with unstimulated controls; this subset was termed Criteria-A (Fig. 3A; Original Data Set - Table 2) and represents TNF-induced enrichment.

Similarly within the Criteria-B dataset, defining the MAT algorithm parameters to enriched values of <0 and P ≤ 0.01 (i.e. a negative MAT score), we identified 11 110 genomic regions that were significantly more enriched by the anti-RelA antiserum in the unstimulated control set compared with the TNF-treated samples. This subset was termed Criteria-C and represents unstimulated enrichment. Therefore, in this study, the Criteria-C dataset identifies genomic loci bound by NF-κB dimers containing the RelA subunit (homo- or heterodimers of RelA) when the cell population is not exposed to an exogenous stimulant such as TNF (Fig. 3A; Original Data Set - Table 3). While we cannot rule out the possibility that some RelA-enriched regions may be non-specific, the removal of the IgG-associated regions will minimize such interference.

RelA-enriched loci from both Criteria-A and Criteria-C datasets represent given regions of chromatin and thus may map to different aspects of a gene including exons and introns. Figure 3B illustrates a schematic representation of chromosome 14 (the IkBα locus) illustrating such differentially enriched regions listed in either Criteria-A (TNF-induced; red vertical lines) or Criteria-C (unstimulated; blue vertical lines) datasets.
TNF induces RelA NF-κB activity in PHM1-31 myometrial cells. Immunostaining was used to demonstrate TNF-mediated induction of RelA nuclear localization in PHM1-31 myometrial cell lines (A; Panel-I, unstimulated; Panel-II, negative control; Panel-III, TNF-stimulated; scale bar = 100 μm). Nuclear extracts were prepared from PHM1-31 cells and incubated with α-32P-labelled oligonucleotide harbouring the 3′-HIV-LTR κB site. Three main complexes were seen to form and, using supershift analyses, these were demonstrated to be p50:RelA heterodimers, RelA homodimers and a lower non-specific complex. An increased shift in RelA in TNF-treated cells illustrates TNF was inducing RelA NF-κB translocation to the nucleus. Specificity of the experiment was confirmed by including an excess (100 ng) of cold, HIV κB DNA (B). PHM1-31 cells were transfected with 200 ng of either 3x-κB-ConA-luc (NF-κB responsive; C; Panel-I) or DκB-ConA-luc (NF-κB unresponsive; C; Panel-II). After 24 h cells were stimulated with TNF (10 ng/ml) for 1 h. Promoter activity was quantified using a Berthold Sirius tube luminometer. All experiments were performed three times in triplicate. Data were analysed using an unpaired, two-tailed t-test and results are expressed as the mean ± SEM (error bars); *P < 0.05 was considered statistically significant. As expected, TNF induced NF-κB activity (C; Panel-I; *P = 0.0001). No NF-κB activity was observed in a control reporter lacking the κB site (C; Panel-II). Nuclear extracts were prepared from PHM1-31 cells and subjected to immunoprecipitation with anti-RelA antiserum. RelA was recovered from both control and TNF-stimulated samples, illustrating the effectiveness of the antiserum. Minimal non-specific binding was observed with IgG (D). RelA occupancy of the IκBα promoter was seen under basal conditions. In the presence of TNF, both RelA and RNA Pol II were seen to be associated with the IκBα promoter (E; Upper Panels). No RelA occupancy of the control Gαs promoter was observed, illustrating the specificity of the ChIP assay (E; Lower Panels).
in Fig. 5A illustrates two regions within the IκBα gene enriched in TNF-treated cells (Fig. 5B). The region upstream of the IκBα transcription start site was also seen to encode the three κB motifs believed to be responsible for governing IκBα expression (Le Bail et al., 1993). A second intra-genic region within IκBα open reading frame was seen to be enriched in TNF-treated cells although no consensus κB motifs were identified therein (Fig. 5B). In contrast to genes in close proximity to the anti-RelA serum enriched loci, other enriched regions were decidedly more remote from the nearest genes (Original Data Set - Table 4, Criteria-A and Original Data Set - Table 5, Criteria-C, full annotated gene lists associated with these enriched regions can also be viewed therein).

**Figure 3** Comparison of RelA-enriched chromatin regions. The Criteria-B dataset represents all regions with a P-value < 0.05. Within this, the Criteria-A dataset (red circle) represents those regions where P < 0.05 and the MAT score is >0; essentially, these regions are enriched over the control in response to TNF. The Criteria-C dataset (blue circle) represents those regions where P < 0.05 and the MAT score is <0; essentially, these regions are enriched in the absence of TNF stimulation (A). An illustrative heat map of chromosome 14 illustrating the loci of Criteria-A regions (red bars) and Criteria-C regions (blue bars). Known transcripts from each strand are represented by green blocks (B).

**Frequency of κB site motif occurrence in Criteria-A and Criteria-C datasets**

In the Criteria-A dataset (i.e. RelA-enriched regions in response to TNF), 1667 occurrences of the κB consensus sequence were identified, defined by 112 different sequence representations (Original Data Set - Table 6). Of the 1667 occurrences, 1604 resided in non-repetitive genomic regions. To aid clarity, we focused the study on those motifs from the non-repetitive regions. These motifs were defined by 65 different sequence instances (Table I). The remaining 63 occurrences, whilst not studied further, were defined by 47 different sequence instances many of which were also observed in the non-repetitive dataset (data not...
Genes within Criteria-A and Criteria-C enriched regions and associated \(\kappa B\) motif loci. Schematic representation of the loci of both Criteria-A (TNF-induced NF-\(\kappa B\) enrichment) and Criteria-C (unstimulated NF-\(\kappa B\) enrichment) regions around selected genes including COX-2 (A); Jun (B); IL6RN (C); KCNMB3 (D); CACNB3 (E) and KCNB1 (F). For COX-2 and Jun, \(\kappa B\) motifs corresponding to the consensus were found in the TNF-induced RelA-enriched regions (Fig. 3A and B). With IL6RN and KCNMB3, both TNF-induced RelA enriched regions and unstimulated RelA-enriched regions were observed. The former were not associated with a consensus \(\kappa B\) motif while the latter, unstimulated RelA-enriched, both harboured consensus \(\kappa B\) motifs (Fig. 3C and D). Regions around CACNB3 and KCNB1 were not enriched by RelA in the presence of TNF but were enriched by RelA in unstimulated cells, possibly by the consensus \(\kappa B\) motifs identified (Fig. 3E and F). Arrows indicate the direction of transcription, not actual transcription start sites.
shown). Of the 1667 consensus kB motif occurrences in the Criteria-A dataset, 770 (46.2%) were identified within intronic regions (Original Data Set - Table 6).

In the Criteria-C data set (i.e. RelA-enriched regions in absence of TNF), the consensus kB motif occurred 2116 times defined by 103 different sequence representations (Original Data Set - Table 7). Of the 2116 occurrences, 2064 were seen to reside in non-repetitive elements of the genome. These were chosen for further study. In turn, these motifs were also represented by 65 different sequence instances (Table I). The remaining 52 occurrences, whilst not studied further as they were from highly repetitive sequences, were defined by 38 different sequence instances many of which were also observed in the non-repetitive dataset (data not shown). Of the 2116 consensus kB motif occurrences in the Criteria-C dataset, 1089

**Figure 5** Differential enrichment of regions around the NFKBIA (IkBa) gene locus on chromosome 14. Low resolution schematic illustration of the IkBa locus and surrounding regions. In the absence of TNF, no enrichment around the IkBa promoter is observed (A; blue bars). In the presence of TNF, a number of regions around the promoter are enriched by the anti-RelA antiserum (A; red bars). High resolution schematic illustration of the IkBa promoter illustrating TNF-induced regions enriched by the anti-RelA antiserum. One region encompasses the IkBa promoter and harbours the three reported kB motifs in that region. A second intra-genic region, encompassing the 3' portion of exon 2 and all of exons 3 and 4, is also enriched but no consensus kB motifs were identified therein (B).
(51.5%) were identified within intronic regions (Original Data Set - Table 7).

Overall, the consensus κB motif was observed a total of 3783 times in anti-RelA serum-enriched genomic regions with 3720 κB motifs identified in non-repetitive elements of the genome of PHM1-31 myometrial myocytes. Alignment of the 65 representations of the κB sequence was performed using WebLogo 3 open access software (Crooks et al., 2004; http://weblogo.threeplusone.com/). Consensus κB sequence variability was seen to be 5′GGG(A/G)(A/C/T/G)(C/T)(C/T)(C/T)3′ but no difference was observed with this between Criteria-A (TNF-induced enrichment) and Criteria-C (un-stimulated enrichment) (Fig. 6).

### Expression array analysis of PHM1-31 gene expression induced by TNF

As illustrated thus far, promoter occupancy by RelA-containing NF-κB dimers per se, does not provide information on associated transcriptional levels (i.e. expressed, repressed or quiescent; akin to chromatin-bound RNA polymerase II and promoter-proximal stalling (Core and Lis, 2009)). Consequently, we undertook expression array analyses in PHM1-31 cells utilizing total RNA extracted from cells exposed to TNF for one hour (an identical time course to those used to isolated chromatin for ChIP-on-chip studies).

A statistically significant change in expression of 2963 genes was induced by TNF (Original Data Set - Table 8, P-value region <0.05).

<table>
<thead>
<tr>
<th>κB motif (5′-3′)</th>
<th>Frequency (+ TNF)</th>
<th>Frequency (unstimulated)</th>
<th>κB motif (5′-3′)</th>
<th>Frequency (+ TNF)</th>
<th>Frequency (unstimulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGGCCTCCCC</td>
<td>29</td>
<td>83</td>
<td>GGGATTTTCC</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>GGGGCTCCCC</td>
<td>37</td>
<td>75</td>
<td>GGGATCCCCC</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>GGGGCTTCCCC</td>
<td>47</td>
<td>60</td>
<td>GGGGTTTCC</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>GGGGGCTTCCC</td>
<td>17</td>
<td>60</td>
<td>GGGGATTCCC</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>GGGGGCTTCCC</td>
<td>28</td>
<td>60</td>
<td>GGGGACTTCCC</td>
<td>49</td>
<td>23</td>
</tr>
<tr>
<td>GGGAGCTTCCC</td>
<td>32</td>
<td>58</td>
<td>GGGAACCTCCC</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>GGGGACCTCCC</td>
<td>21</td>
<td>53</td>
<td>GGGGGTTCCC</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>22</td>
<td>52</td>
<td>GGGGATTCCC</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>37</td>
<td>51</td>
<td>GGGGTTTCCC</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>GGGACCTCCC</td>
<td>12</td>
<td>50</td>
<td>GGGGCTTCCC</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>32</td>
<td>50</td>
<td>GGGGACTTCCC</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>32</td>
<td>47</td>
<td>GGGGATTTCCC</td>
<td>41</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>17</td>
<td>47</td>
<td>GGGATCCTCC</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>13</td>
<td>47</td>
<td>GGGAACCTCCC</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>29</td>
<td>43</td>
<td>GGGGACTTCCC</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>10</td>
<td>43</td>
<td>GGGGCTTCCC</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>33</td>
<td>40</td>
<td>GGGATCCCCC</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>GGGGCTCTCCC</td>
<td>30</td>
<td>39</td>
<td>GGGGGTTCCC</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>28</td>
<td>39</td>
<td>GGGAGTTCCC</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>20</td>
<td>37</td>
<td>GGGGACTCCC</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>19</td>
<td>37</td>
<td>GGGGACCTCCC</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>21</td>
<td>37</td>
<td>GGGGACTTCCC</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>24</td>
<td>35</td>
<td>GGGGACTTCCC</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>26</td>
<td>34</td>
<td>GGGGACTTCCC</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>27</td>
<td>31</td>
<td>GGGGACTTCCC</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>21</td>
<td>30</td>
<td>GGGGACTTCCC</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>22</td>
<td>30</td>
<td>GGGGACTTCCC</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>18</td>
<td>28</td>
<td>GGGGACTTCCC</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>20</td>
<td>28</td>
<td>GGGGACTTCCC</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>31</td>
<td>26</td>
<td>GGGGACTTCCC</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>GGGGCTTCCC</td>
<td>28</td>
<td>26</td>
<td>GGGGACTTCCC</td>
<td>32</td>
<td>11</td>
</tr>
</tbody>
</table>

kB motifs in bold typeface represent examples of those appearing less frequently in unstimulated RelA-enriched dataset. The italicized, underlined consensus κB motif represents that employed by Chen et al. (1998a) defining the crystallographic structure of the RelA:p50 heterodimer bound to κB DNA.

Table I Comparison of consensus κB motif instances and frequencies between the TNF-stimulated (Criteria-A) and unstimulated (Criteria-C) enriched region datasets.
Expression of six genes remained unchanged between unstimulated and TNF-treated cells (SLC35F5, DEFB106A, RFTN1, DTWD2, SLC34A3 and SEZ6L). Removal of duplicates from the original data gave a total of 2223 genes that were differentially expressed when PHM1-31 cells were stimulated with TNF for one hour (Fig. 7; Criteria-B1, $P < 0.05$; and Original Data Set - Table 8). Of this total, 51 genes were seen to have a fold induction of $>2$. Of these, two were discounted as they were not annotated, leaving a total of 49 genes; these were termed Criteria-A1. Physiologically relevant genes identified in this dataset were EGR 1–4, FOS, FOSB, JUN, JUNB, ATF3, NFKBIA, NFKBIZ, TNFAIP3, COX2, CXCL2, CXCL3, CCL20, LIF, IL-6, MAP3K8, THBS1 and TNF. Eighteen genes were seen to have a fold repression of $>1.5$. Of these, one was not annotated and was thus excluded leaving a total of 17 genes; these were termed Criteria-C1 (Fig. 7). Included in this dataset were HOXA11, COL1A2 and STAT2. To summarize, Table II lists those genes whose expression was induced by at least 2-fold in response to TNF while Table III lists those genes subject to repression by at least 1.5-fold in the presence of TNF. Further details from the expression array analyses can be viewed in Original Data Set - Table 8–10.

**Combined analysis of PHM1-31 promoter occupancy and gene expression**

Merging the Criteria-A TNF-enriched promoter dataset with Criteria-A1 (TNF-induced gene expression), 14 TNF-induced, RelA-enriched genomic regions from the promoter array screen also encoded TNF-inducible genes (Table II; genes annotated with * and Original Data Set - Table 11). These included known NF-κB-regulated genes IL-6, Jun, NFKBIA, PTGS2, TNC and TNFAIP3. Genes not yet conclusively demonstrated to be under NF-κB control included DUSP-2, DUSP-5, ERRFI1, THBS1 and CTGF (Table II; genes annotated with * and Original Data Set - Table 11). Moreover, by combining the Criteria-A promoter array dataset with the expression array Criteria-C1 dataset (TNF-repressed genes), we also identified five RelA-enriched
genomic regions that harboured TNF-repressed genes, including COL1A2 (Table III; genes annotated with * and Original Data Set - Table 12).

Gene ontology and validation of selected RelA-enriched regions modulated by TNF

PGS Gene Ontology analysis identified two functional groups of interest in the Biological Processes category: *Intra-cellular Signal Transduction* and *Cell Surface Receptor Protein Signalling*. These included TNFAIP3 (*Cytokine-Induced Signalling*) and IκBα (*NIK/NF-κB Signalling*; Fig. 8A). These targets were subsequently used to validate the expression array data because both have important roles in governing TNF signalling and NF-κB function respectively (Chen and Ghosh, 1999; Perkins, 2007; Hayden and Ghosh, 2012). Real-time qRT–PCR on total RNA from PHM1-31 cells treated with TNF for one hour demonstrated that both IκBα and TNFAIP3 were expressed in response to TNF. Good agreement was observed for induction of gene expression for IκBα (Fig. 8B; 2.94 on array versus 4.43 for qRT–PCR) while a slightly greater margin of difference was noted for TNFAIP3 expression (Fig. 6B; 7.96 on array versus 3.85 for qRT–PCR).

**Discussion**

**NF-κB binding and distribution in the genome of TNF-stimulated and unstimulated cells**

This report is the first to describe a promoter array-based approach to define chromatin regions of myometrial myocytes occupied by RelA-containing NF-κB dimers. Our data demonstrated that in PHM1-31 myometrial cells, NF-κB-mediated enrichment of 13 300 chromatin regions in the presence of TNF and 11 110 in unstimulated cells. Some of these regions were juxtaposed to genes known to function in human labour, for example PTGST2 (Chan et al., 2014) and KCNMB3.

NF-κB-controlled regulation of PTGS2 in the myometrium and amnion is well documented (Allport et al., 2001; Soloff et al., 2004; Lindström and Bennett, 2005) while a putative role in governing gene activity of the potassium channel subunit KCNMB3 is less so. In contrast, other RelA-enriched regions were decidedly more remote from the nearest transcription start with distances being measured in numbers of kilobases. Many of these loci were also noted to be within intronic regions as discussed below. We did not examine the influence of such remote binding events in this study but we cannot rule out that they represent enhancer sequences or non-coding RNA transcriptional units. Indeed,

---

**Table II** List of TNF-induced genes with expression greater than 2-fold in PHM1-31 cells.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Transcript ID</th>
<th>Fold change</th>
<th>Gene symbol</th>
<th>Transcript ID</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR3</td>
<td>NM_001199880</td>
<td>32.2827</td>
<td>IL6*</td>
<td>NM_000600</td>
<td>3.06508</td>
</tr>
<tr>
<td>FOSB</td>
<td>NM_00114171</td>
<td>15.7702</td>
<td>FAM43A*</td>
<td>NM_153690</td>
<td>3.00641</td>
</tr>
<tr>
<td>EGR1*</td>
<td>NM_001964</td>
<td>14.405</td>
<td>DUSP5*</td>
<td>NM_004419</td>
<td>2.95136</td>
</tr>
<tr>
<td>FOS</td>
<td>NM_005252</td>
<td>13.7368</td>
<td>NFKBIA*</td>
<td>NM_020529</td>
<td>2.94068</td>
</tr>
<tr>
<td>LOC100653132</td>
<td>XR_133404</td>
<td>11.1993</td>
<td>TNF</td>
<td>NM_000594</td>
<td>2.70207</td>
</tr>
<tr>
<td>EGR4</td>
<td>NM_001965</td>
<td>9.42917</td>
<td>LIF</td>
<td>NM_001257135</td>
<td>2.64715</td>
</tr>
<tr>
<td>NR4A2</td>
<td>NM_006186</td>
<td>9.22167</td>
<td>KLF6</td>
<td>NM_001008490</td>
<td>2.62646</td>
</tr>
<tr>
<td>ATFB3</td>
<td>NM_00103287</td>
<td>8.60664</td>
<td>RASD1</td>
<td>NM_001199899</td>
<td>2.45863</td>
</tr>
<tr>
<td>TNFAIP3*</td>
<td>NM_006290</td>
<td>7.96588</td>
<td>CXCL3</td>
<td>NM_002090</td>
<td>2.45687</td>
</tr>
<tr>
<td>NR4A3</td>
<td>NM_006981</td>
<td>7.69277</td>
<td>THBS1*</td>
<td>NM_003246</td>
<td>2.43686</td>
</tr>
<tr>
<td>EGR2</td>
<td>NM_000399;</td>
<td>6.60315</td>
<td>BIRC3</td>
<td>NM_001165</td>
<td>2.36586</td>
</tr>
<tr>
<td>DUSP2*</td>
<td>NM_004418</td>
<td>5.57734</td>
<td>CSRNP1</td>
<td>NM_003027</td>
<td>2.23011</td>
</tr>
<tr>
<td>NFKBIZ</td>
<td>NM_001005474</td>
<td>5.20843</td>
<td>IER3</td>
<td>NM_003897</td>
<td>2.22675</td>
</tr>
<tr>
<td>C8orH*</td>
<td>NM_020130</td>
<td>4.15299</td>
<td>CCL20</td>
<td>NM_001130046</td>
<td>2.17664</td>
</tr>
<tr>
<td>MAP3K8</td>
<td>NM_001244134</td>
<td>3.98537</td>
<td>TRIB1</td>
<td>NM_025195</td>
<td>2.1427</td>
</tr>
<tr>
<td>AKIP1; NUAK2</td>
<td>NM_001206645</td>
<td>3.95817</td>
<td>TIPARP</td>
<td>NM_001184717</td>
<td>2.12245</td>
</tr>
<tr>
<td>KBTBD8</td>
<td>NM_032505</td>
<td>3.73984</td>
<td>JUN*</td>
<td>NM_002228</td>
<td>2.12162</td>
</tr>
<tr>
<td>PTGS2*</td>
<td>NM_000963</td>
<td>3.45309</td>
<td>CTGF*</td>
<td>NM_001901</td>
<td>2.1202</td>
</tr>
<tr>
<td>ERRFI1*</td>
<td>NM_018948</td>
<td>3.43108</td>
<td>PLK2</td>
<td>NM_001252226</td>
<td>2.09449</td>
</tr>
<tr>
<td>NR4A1</td>
<td>NM_001202233</td>
<td>3.33043</td>
<td>KLF2</td>
<td>NM_0016270</td>
<td>2.05193</td>
</tr>
<tr>
<td>IER2</td>
<td>NM_004907</td>
<td>3.2552</td>
<td>RCAN1</td>
<td>NM_004414</td>
<td>2.04372</td>
</tr>
<tr>
<td>ZFP36</td>
<td>NM_003407</td>
<td>3.19889</td>
<td>RRAD</td>
<td>NM_001128850</td>
<td>2.04022</td>
</tr>
<tr>
<td>JUNB</td>
<td>NM_002229</td>
<td>3.19488</td>
<td>TNC*</td>
<td>NM_002160</td>
<td>2.03576</td>
</tr>
<tr>
<td>CXCL2</td>
<td>NM_002089</td>
<td>3.14873</td>
<td>MCLI</td>
<td>NM_001197320</td>
<td>2.03424</td>
</tr>
<tr>
<td>NEDD9</td>
<td>NM_001142393</td>
<td>3.0767</td>
<td>EDNI1</td>
<td>NM_001168319</td>
<td>2.02957</td>
</tr>
</tbody>
</table>

Genes highlighted in bold type have been documented to be up-regulated in labouring human myometrium (Chan et al., 2014). Fourteen genes (labelled *) were seen to be associated with or juxtaposed to NF-κB-enriched regions (Original Data Set - Table 11).
Table III  List of TNF-repressed genes with repression greater than 1.5-fold in PHM1-31 cells.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Transcript ID</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID1</td>
<td>NM_002165</td>
<td>-1.89317</td>
</tr>
<tr>
<td>HOXA11</td>
<td>NM_005523</td>
<td>-1.72856</td>
</tr>
<tr>
<td>ZNF57*</td>
<td>NM_173480</td>
<td>-1.72129</td>
</tr>
<tr>
<td>TXNIP*</td>
<td>NM_006472</td>
<td>-1.65709</td>
</tr>
<tr>
<td>ARRDCC4*</td>
<td>NM_020801</td>
<td>-1.63565</td>
</tr>
<tr>
<td>TMEPE64</td>
<td>NM_001008495</td>
<td>-1.61349</td>
</tr>
<tr>
<td>ID2; ID2B</td>
<td>NM_001039082</td>
<td>-1.59068</td>
</tr>
<tr>
<td>ZNF785</td>
<td>NM_152458</td>
<td>-1.57708</td>
</tr>
<tr>
<td>MBNL2</td>
<td>NM_144778</td>
<td>-1.57392</td>
</tr>
<tr>
<td>COL1A2*</td>
<td>NM_000089</td>
<td>-1.56247</td>
</tr>
<tr>
<td>ZNF420</td>
<td>NM_144689</td>
<td>-1.55821</td>
</tr>
<tr>
<td>ZNF14*</td>
<td>NM_021030</td>
<td>-1.55443</td>
</tr>
<tr>
<td>TET1</td>
<td>NM_030625</td>
<td>-1.53925</td>
</tr>
<tr>
<td>TXNIP</td>
<td>NM_006472</td>
<td>-1.53412</td>
</tr>
<tr>
<td>ZNF555</td>
<td>NM_001172775</td>
<td>-1.51617</td>
</tr>
<tr>
<td>ZNF823</td>
<td>NM_001080493</td>
<td>-1.51568</td>
</tr>
<tr>
<td>STAT2</td>
<td>NM_005419</td>
<td>-1.50378</td>
</tr>
<tr>
<td>ZNF709</td>
<td>NM_001145647</td>
<td>-1.50255</td>
</tr>
</tbody>
</table>

The gene highlighted in bold type has been documented to be down-regulated in labouring human myometrium (Chan et al., 2014). Five genes (labelled *) were seen to be associated with or juxtaposed to NF-κB-enriched regions (Original Data Set - Table 12).

Supporting this notion are the observations that many transcription factor binding sites are arranged many kilobases from the transcription start site of the genes they regulate (Deaton and Bird, 2011).

**κB binding site loci**

Intriguingly, RelA appeared to exhibit stimulus dependent binding to different loci of the same gene; this was evidenced with PTGS2, IL6RN and KCNMB3. In contrast, however, a section of the promoter region for Jun was only enriched in the presence of TNF while for CACNB3 and KCNMB3, sections of promoter regions for these respective genes were only enriched by RelA-containing dimers in the absence of TNF; the molecular mechanisms accounting for these observations remain to be established. We did, however, determine that in both datasets, the consensus κB motif was represented a total of 3783 times, including 1859 (49.14%) sites identified within intronic regions. A number of studies in various cell lines have reported such intronic binding by NF-κB (Martone et al., 2003; Schreiber et al., 2006; Lim et al., 2007; Wong et al., 2011; Satoh, 2013; Xing et al., 2013) and, given that the early work describing its function demonstrated it was bound to the first intron of the κ-light chain enhancer (Schjerven et al., 2001), our observations support such previous data. Interestingly there are reports of transcription of certain regulatory proteins initiating from within the 3′-intronic regions of the parental gene. The calcium channel associated transcriptional regulator (CCAT), for example, is generated through initiation of independent transcription of exons 46 and 47 at a 3′-intronic site of the parental Cα1.2 calcium channel gene (Gomez-Ospina et al., 2013). Clearly, our data does not illustrate if such NF-κB binding directly modifies gene activity in this manner but it offers a likely rationale for such intronic binding, perhaps as a means of governing post-transcriptional RNA splicing, and a further avenue for investigating the complexities of myometrial gene activity as labour commences. Due to the apparent promiscuity of RelA binding to multiple loci and limited sensitivity of the ChIP-on-chip methodology, ChIP-exo (see below) would be a superior means by which to narrow down the precise binding location of RelA-containing dimers to near single base pair resolution (Rhee and Pugh, 2012).

**Selection of κB motifs**

The consensus κB binding motif is viewed as 5′-G(G/A)-G(G/A)-G(G/A)-R-B-N-B-B with many functional variants on this being reported (Chen and Ghosh, 1999; Perkins, 2007; Hayden and Ghosh, 2012). This motif does offer a level of subunit selectivity and crystallographic studies of various NF-κB dimers bound to different κB DNA sequences support this (Ghosh et al., 1995; Müller et al., 1995; Cramer et al., 1997; Huang et al., 1997, 2005; Chen et al., 1998a, b; Phelps et al., 2000; Moorthy et al., 2007; Trinh et al., 2008). The actual κB DNA sequence clearly does impose binding constraints upon certain dimers; for example, within the κB motif, the 5′-G(G/A)-G(G/A)-G(G/A)-R-B-N-B3′ half site is bound by p50; in contrast, the 5′-Y(Y/C)-Y(Y/C)-Y(Y/C)-Y(Y/C)-Y(Y/C)-C′-C′-3′ half site is necessary for RelA binding (Huang et al., 2005 and references therein). Whether region enrichment is dimer-specific and dependent on the nature of the stimulus (in this case TNF or not) could not be determined herein. Since we focused on immunoprecipitated RelA, this would recover four possible groups of RelA-containing dimers; RelA:RelA, RelA:c-Rel, RelA:p50 and RelA:p52. (RelA:RelB heterodimers are not thought to bind DNA; Marienfeld et al., 2003). Given the published physical constraints imposed upon certain NF-κB dimer:κB motif interactions (Phelps et al., 2000; Huang et al., 2005) one could propose that, at this time point of one hour, it is likely the RelA:p50 or RelA:p52 heterodimers are being physiologically favoured. Given that we focused on RelA, the obvious confounder of our work is that those enriched regions would likely reflect only contributions from such RelA-containing dimers; other non-RelA dimers would be missed. As above, further studies employing ChIP-seq would be required to provide the higher resolution data defining whether dimer composition on individual promoters/loci changed over multiple time points.

**Limitations of ChIP-on-chip**

While ChIP-on-chip is a robust, well-documented method to analyse transcription factor-mediated chromatin enrichment on a whole genome scale, it is associated with limitations when compared with more recent sequencing technologies including ChIP-seq (reviewed in Hurd and Nelson, 2009; Park, 2009). The obvious corollary, therefore, is that we cannot rule out the possibility that bias occurred in our system and influenced the data presented herein. A comparison between both ChIP-on-chip and ChIP-seq methodologies has been highlighted in Ho et al. (2011) who address the limitation and benefits associated with both technologies. With whole genome sequencing now available in most institutions, and methods including ChIP-exo (essentially ChIP-seq but using lambda exonuclease to trim the immunoprecipitated DNA to within a few base pairs of the binding residues on a given transcription factor) being developed, the next step would be to conduct ChIP-seq with all NF-κB subunit antisera, at different time points to
obtain an unbiased genome-wide signature of binding events taking place within myometrial cells.

In our study, we employed the GeneChip 1.0R Human Promoter array. This contains 25,500 human promoter regions but it lacks full genomic coverage of the corresponding tiling arrays. Consequently, we cannot rule out the possibility that the low correlation between region enrichment and subsequent transcription expression/repression could arise because the Human 1.0R Promoter array was not fully representative of the complete human genome.

PHM1-31 cultures were not cell-cycle synchronized prior to the ChIP assay because the induction of synchronization itself (serum starvation) has been documented to effect expression of key genes involved in cell function and ion transport including the MaxiK potassium channel (Woodfork et al., 1995; Panner et al., 2005; Patel et al., 2005). Essentially, potassium channels are responsible for hyperpolarising the plasma membrane, an event necessary for the cells to move from G1 to S (Wonderlin and Strobl, 1996; Ouadid-Ahidouch and Ahidouch, 2013). Calcium channels are thought

![Figure 8](https://example.com/figure8.png)

**Figure 8** GO enrichment—biological process and validation of expression array analyses by qRT–PCR. Within the Biological Processes group, two functional groups of interest were *Intra-cellular Signal Transduction* and *Cell Surface Receptor Protein Signalling*. This included TNFAIP3 (*Cytokine-mediated Signalling*) and IκBα (*NIK/NF-κB Cascade*) (A). qRT–PCR on total RNA from PHM1-31 cells treated with TNF for 1 h demonstrated that both IκBα and TNFAIP3 were expressed in response to TNF (B). A close agreement was seen for induction of gene expression for IκBα (4.43 for qRT–PCR; stippled bars versus 2.94 on array; diagonal stripes) while a slightly greater margin of difference was noted for TNFAIP3 expression (3.85 for qRT–PCR; stippled bars versus 7.96 on array; diagonal stripes).
to provide transient signals at checkpoints within the cell cycle which are necessary for the cell to continue cycling (Whitaker, 2006). We believe it highly likely that these events would have influenced those TNF-induced effects reported herein (Perkins, 2012).

Promoter occupancy and transcriptional activity

Occupancy of a given promoter by NF-κB does not necessarily mean transcriptional activity directed by that promoter will change and there is robust data to support this notion (Hoffmann et al., 2003; Leung et al., 2004; Wan and Lenardo, 2009; Wang et al., 2012). Of the TNF-induced NF-κB-enriched regions identified in our work, 14 were correlated with an increase in gene expression while five targets were repressed. Therefore, one must ask why the disparity between the number of enriched regions and the number of genes with altered activity? Clearly under normal physiological conditions, the myometrium would be bathed in a milieu rich in cytokines including IL-1β, IL-6, IL-8 and TNF to name but a few (Aguilar and Mitchell, 2010; Golightly et al., 2011; Webster et al., 2013). The manner by which these other pro-inflammatory stimuli influence NF-κB activity was not examined herein but it is reasonable, based on published evidence, to assume these factors would also moderate myometrial gene activity. Secondly, it is without doubt that we will have missed many key binding events at promoters at immediate early time points, ranging from seconds to minutes, as well as more prolonged stimuli after a number of hours. Indeed, this approach may underline why we did not observe enrichment of regions that encoded genes for IL-1β, oxytocin or oxytocin receptor, all of which have previously been shown to require NF-κB for induction of expression (Belt et al., 1999; Lee et al., 2003; Soloff et al., 2006; Terzidou et al., 2006).

Differential gene expression

In the context of premature birth research, many authors have published expression array studies in attempts to define genes responsible for promoting myometrial quiescence and myometrial contraction. Such studies have focused on native tissues (amnion, decidua and myometrium) as well as both immortalized and primary cell lines (Bethin et al., 2005; Charpigny et al., 2003; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Esplin et al., 2005; Havelock et al., 2005; Han et al., 2008; Khanjani et al., 2011; Lim et al., 2012).

A recent study by Chan et al. (2014), used a robust RNA-seq-based approach to define 764 differentially expressed genes in human myometrium from pregnant, non-labouring women and those women in active labour. Salient examples of up-regulated genes from that list included IL6, IL8, IL13, MCP, enzymes governing prostaglandin biosynthesis (PTGS2), THBS2, DUSP family members, members of the NF-κB family of proteins, and intermediates in the TGF-β and TNF-signalling pathway (Chan et al., 2014). Significantly, our study of TNF-stimulated differential gene expression identified 49 induced genes; of these expressed genes, 17 were also represented in the list of genes up-regulated in labouring human myometrium documented by Chan et al. (2014). Furthermore, 14 of the TNF-induced genes were also seen to be in regions of chromatin enriched by RelA. This is a key observation because it indirectly validates our own expression array work. Moreover, it supports the notion that NF-κB plays a pivotal role in controlling expression of genes involved in human parturition since many of those targets identified by Chan et al. are documented to be regulated by NF-κB (www.bu.edu/nf-κb/gene-resources/target-genes/).

Interestingly, a recent meta-analysis of gestational tissue-based transcriptomic studies highlighted significant variation in expression of individual genetic regions; essentially only 23 common sites were identified out of 10 993 unique transcriptionally active units (Eidem et al., 2015). Focussing on studies of myometrial gene expression, Eidem et al. identified 15 genes present in four or more studies. In our study, four TNF-induced genes were also present in the myometrial group identified by Eidem et al., including FOSB, NRAI1, LIF and PTGS2. Moreover, those genes reported to act as biomarkers of preterm birth, including IL6 and TNF, were also represented in our work giving further validation of the TNF-induced gene expression data presented herein (Table II and Eidem et al., 2015). The meta-analysis described by Eidem et al., does not, however, consider changes in promoter occupancy of those gene targets, essentially because, other than our work, there are no data in the reproductive field describing such investigations.

TNF-induced gene expression

In terms of NF-κB function, the NFKBIA gene, which encodes the IkBα protein, was induced 2.9-fold by TNF. As our positive control, this observation also validates the data presented here. A second IkB family member, NFKBIZ, which encodes the IkBζ protein, was induced 5.2-fold in response to TNF. Significantly, other groups (Eto et al., 2003) have observed that IkBζ induction is not TNF-mediated. The differences in these observations may be accounted for through cell-type-specific effects (macrophage or kidney versus myometrium) but it is noteworthy that one effector, TNF, can exert diametrically opposed effects on the same gene in different cell types, suggesting other nuclear-based factors are influencing the NF-κB-mediated gene regulation process. Significantly, IkBζ is known to bind specifically to p50 homodimers forming a robust ternary complex on the IL6 promoter activating expression of this gene (Trinh et al., 2008). Indeed, in our study TNF stimulation also caused a three-fold induction of IL-6 expression and we speculate this is mediated by p50 homodimer:IkBζ complex although further experimental analyses would be required to confirm this. TNFAIP3 (also termed A20) was also up-regulated. This protein also plays a significant role in termination of the NF-κB signal by inhibiting NF-κB DNA binding (Hayden and Ghosh, 2012; Perkins, 2012).

Our study also identified various transcription factors that were significantly up-regulated in response to TNF, including members of the early growth response transcription factor family, Egr-1, -2, -3 and -4, as well as those of the API family, namely ATF3, Fos, FosB, Jun, JunB. The Egr family are well-described zinc-finger containing proteins recognizing the consensus sequence of 5-GCGG/TGGGCG-3’ (Christy and Nathans, 1989). The function of the Egr family in human myometrium is not clear but they have been shown to co-operate with the RelA subunit of NF-κB in embryonic kidney cells through an interaction between the RelA Rel Homology Domain and the zinc-finger region of Egr-1 (Chapman and Perkins, 2000), as well as competing for Sp1 sites in pro-inflammatory promoters such as PDGF-B (Khachigian et al., 1996). Many of the promoters identified in our study were GC-rich and up-regulation of factors which readily bind to such regions offers a...
potential insight into the control of complex myometrial gene expression networks.

Members of the AP1 transcription factor family, including Fos, FosB and Jun, bind to the consensus AP1 motif 5′-TGAG/CTCA-3′ or 5′-TGACGTCA-3′ (Shaullian and Karin, 2002) and have been shown to be differentially expressed in premature versus labouring rat myometrium. Importantly, studies in rat myometrium have described the differential expression of members of this family, between pregnant and labouring states. The salient observation is that peak levels of Fos, FosB, Jun and JunB occur during active labour (Mitchell and Lye, 2002; Mohan et al., 2007); importantly, those observations are in keeping with our data from human cells. Moreover, since Fos/Jun are immediate early genes (Shaullian and Karin, 2002), they are likely regulators of more extensive transcriptional networks within the cell and it is therefore interesting to speculate that NF-κB dimers may orchestrate a hierarchy of transcriptional activity within the myometrial cell. Interestingly, NF-κB has also been shown to play a key regulatory role in JunB expression as part of the JunB-mediated induction of VEGF in response to hypoxia (Schmidt et al., 2007). It is well documented that uterine contractions during labour do induce local hypoxic regions (Bugg et al., 2006); whether such myometrial hypoxia initiates NF-κB-induced AP1 family expression remains unclear at present but induction of such factors may be how the uterus responds to such a hypoxic stress and utilizes NF-κB to instigate this protective mechanism.

Conclusions

In conclusion, our data demonstrate that RelA-containing dimers of NF-κB bind to numerous loci throughout the genome of PHM1-31 myometrial myocytes. For some promoters, this binding occurred in the presence of TNF as well as in unstimulated cells; this was mediated by different regions of the same promoter. Occupancy of other promoters was seen either only in unstimulated conditions or only after cells were stimulated by TNF. The consensus κB motif was identified 3783 times in this study with over 100 different sequence instances potentially mediating NF-κB DNA binding. Of these κB motifs, 41% were found within intronic regions of the PHM1-31 cell chromatin. Of the 49 TNF-induced genes, 17 were shown to have promoters enriched by NF-κB in response to TNF. Interestingly, five genes with promoters occupied by NF-κB were repressed by TNF. Together, our data illustrate that NF-κB influences a wide range of regulatory gene networks within myometrial cells; we must decipher how these interactions govern myometrial function during pregnancy and labour if we are to begin to understand the syndrome of premature birth.

Acknowledgements

We are grateful to Prof. Barbra Sanborn for generously providing the PHM1-31 cells utilized in this study. We would like to thank Prof. Nick Europe-Finner and Dr Gaynor Miller for their support and critical reviews of the manuscript prior to submission.

Authors’ roles

V.J.C. and S.L.W. performed the experimental work, undertook initial data analyses and read and helped edit the manuscript. P.R.H. performed the array work (promoter and expression) and read the manuscript. P.J.H. and S.V.G. assisted with the data analysis and manuscript preparation. N.R.C. conceived the study, obtained study funding, designed the experiments, undertook the data analyses and prepared the manuscript.

Funding

This work was funded by: the Sheffield Hospitals Charitable Trust (grant no. 7858); the Jessop Wing Small Grants Scheme (Ellen Webster/Legacy; grant no. OGN/06/03); the Department of Human Metabolism, University of Sheffield; and the Faculty of Medicine Research and Innovation Fund, University of Sheffield.

Conflict of interest

The authors declare they have no competing interests, financial or otherwise, that would affect the publication of this data.

References


Chapman NR, Europe-Finner GN, Robson SC. Expression and DNA-binding activity of the nuclear factor kappab (NF-κB) family in the human myometrium during pregnancy and labour. J Clin Endocrinol Metab 2004;89:5683–5693.

Chapman NR, Smyrnias I, Anumba DOC, Europe-Finner GN, Robson SC. Expression of the GTP-binding protein (Gαs) is repressed by the nuclear factor kappab (NF-κB) RelA subunit in human myometrium. Endocrinology 2005;146:4994–5002.


Yan X, Sun M, Gibb W. Localisation of Nuclear Factor-kB (NF-kB) and Inhibitory Factor-kB (IkB) in human fetal membranes and decidua at term and preterm delivery. Placenta 2002b; 23:288–293.