Regulation and Function of the MAFF Transcription Factor in Myometrial Cells

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List of Abbreviations

AP-1	activator protein-1
ARE	antioxiodant response element
ATF	cyclic AMP-dependent transcription factor (activating transcription factor)
ATP	Adenosine triphosphate
bZIP	basic leucine zipper
C/EBP	CCAAT-enhancer-binding proteins
Ca^{2+}	calcium
CaM	calmodulin or calcium-modulated protein
CAM	cell adhesion molecule
CAPs	contraction-associated-proteins
CBP	CREB binding protein
CCL2,3,5	chemokine C-C Motif ligand 2/3/5
CNC	Cap'n'Collar
COX-2	cycloxygenase-2
cPLA2	cytoplasmic phospholipase A2
cpms	counts per million
CRE	cyclic AMP response element
CREB	cyclic AMP responsive element binding protein
CX-43	connexin-43
CXCL10	chemokine C-X-C motif ligand 10
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham
E1	estrone
E2	estradiol
E3	estriol

ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EHR	extended homology region
EP	prostaglandin E_2 receptors
EpRE	electrophile response element
ER-alpha	estrogen receptor alpha
ERE	estrogen response element
ERK1/2	extracellular-signal regulated kinases 1/2
ESR1	estrogen receptor 1
FM	fetal membrane
FOS v-fos	FBJ murine osteosarcoma viral oncogene homolog
FP	prostaglandin $F_{2\alpha}$ receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$GABA_A$	gamma-aminobutyric acid type A
GAGs	glycosaminoglycans
G_i G_i	alpha subunit
GPCR	G-protein coupled receptor
GPR30	G protein-coupled receptor 30
$G_{q/11}$ G_q	alpha subunit
HA	hyaluronan
HAS2	hyaluronan synthase 2
IKB-alpha	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK-beta	inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1 beta	interleukin-1 beta
IL-1R1	IL-1 Receptor I

IL-1R3	IL-1 Receptor 3
IL-33	interleukin-33
IL-6	interleukin-6
IL-8	interleukin-8
IL-R2	IL-1 Receptor II
IP_3	inositol triphosphate
IRAK-1,2,4	interleukin-1 receptor-activated protein kinase 1,2,4
JNK	c-jun N-terminal kinase
JUN	v-jun avian sarcoma virus 17 oncogene homolog
K^+	potassium ion
LPS	lipopysaccharide
MAF	avian muscoloaponeurotic fibrosarcoma oncogene homologue
MAFF	avian muscoloaponeurotic fibrosarcoma oncogene homologue F
MAFG	avian muscoloaponeurotic fibrosarcoma oncogene homologue G
MAFK	avian muscoloaponeurotic fibrosarcoma oncogene homologue K
MAPK	mitogen-activated protein kinase
MARE	Maf-recognition element
MIP	MAFF interacting protein
MLCK	myosin light chain kinase
MMP-9	matrix metalloproteinase-9
mPR-alpha	membrane progestin receptor alpha
mPR-beta	membrane progestin receptor beta
mPR-gamma	membrane progestin receptor gamma
MyD88	myeloid differentiation primary response gene 88
Na^+	sodium ion

Na+/K+	pump sodium-potassium adenosine triphosphatase
NALP3	NACHT, LRR and PYD domains-containing protein 3
NF-E2	transcription factor NF-E2 45 kDa subunit
NFE2L1	nuclear factor, erythroid 2-like 1
NFE2L2	nuclear factor, erythroid 2-like 2
NFE2L3	nuclear factor, erythroid 2-like 3
NFKB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NK	natural killer
nPR	nuclear progesterone receptor
OT	oxytocin
OTXR	oxytocin receptor
P2X7R	purinergic receptor P2X, ligand-gated ion channel 7
PAR	proline and acid rich
PGDH	15-hydroxy-PG-dehydrogenase
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGRMC1	progesterone receptor membrane component 1
PGRMC2	progesterone receptor membrane component 2
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
pRL	plasmid Renilla Luciferase
PRR	pattern recognition receptor
PTGFR	prostaglandin $F_{2\alpha}$ receptor
PTGS1	prostaglandin-endoperoxide synthase 1

PTGS2	prostaglandin-endoperoxide synthase 2
RIN	RNA integrity number
RNA-seq	RNA sequencing
SP-A	pulmonary surfactant-associated protein A
SP-D	pulmonary surfactant-associated protein D
StRE	stress response element
TAB-1	TAK-binding protein 1
TAB-2	TAK-binding protein 2
TAK-1	TGF-beta-actiaved kinase 1
TBP	TATA box binding protein
THBS1	thrombospondin-1
TIMP1	tissue inhibitor of metalloproteinase 1
TIMP2	tissue inhibitor of metalloproteinase 2
TIMP3	tissue inhibitor of metalloproteinase 3
TIR	toll IL-1 receptor
TK	thymidine kinase
TLR	toll-like-receptor
TNF-alpha	tumor necrosis factor alpha
TPA	12-O-tetradecanoate 13-acetate
TRAF-6	tumor necrosis factor-associated factor 6
TRE	12-O-tetradecanoate 13-acetate responsive element
US-2	ggaatgattactcagctaga; corresponding to nucleotides -1433 to -1414 of the human OXTR gene

Abstract

Preterm birth is one of the most important causes of neonatal mortality and morbidity. Recent evidence suggests that proinflammatory cytokines play a role in a variety of reproductive processes including preterm birth as well as normal parturition. Using immortalized human myometrial cells, we showed that interleukin-1 beta (IL-1 beta) rapidly induces avian muscoloaponeurotic fibrosarcoma homologue F (MAFF) transcription factor expression in a concentration and time dependent manner. We performed *MAFF* siRNA mediated knockdown using RNA interference in the presence and absence of IL-1 beta. Using RNA sequencing (RNA-seq) analysis, we identified a series of genes, whose expression is controlled by the MAFF transcription factor and/or IL-1 beta. Inhibition of MAFF expression in the presence/absence of IL-1 beta resulted in a series of expression changes in groups of genes involved in activation of several pathways as well as modulation of genes linked to parturition. We found that prostaglandin-endoperoxide synthase 2 (PTGS2), a key regulator of labor and parturition, was upregulated in the absence of MAFF, suggesting negative control of the expression of this enzyme by MAFF. Using a luciferase reporter under the control of the PTGS2 promoter, we showed that this regulation is, at least in part, mediated at the transcriptional level. Moreover, other genes that have been associated with normal birth, thrombospondin-1 (THBS1) and tissue metallopeptidase inhibitor 3 (TIMP3), are also modulated by MAFF. Our studies provide valuable insights into the MAFF dependent transcriptional network governing myometrial cell function in the presence and absence of proinflammatory stimuli.

Résumé

La prématurité est une des causes les plus importantes de mortalité et de morbidité néonatale. Des données récentes suggèrent que les cytokines pro-inflammatoires jouent un rôle dans divers processus de reproduction, y compris dans l'accouchement prématuré ainsi que dans l'accouchement normal. En utilisant des cellules humaines immortalisées du myomètre, nous avons montré que l'IL-1 beta induit rapidement l'expression du facteur de transcription aviaire musculo-aponeurotic fibrosarcome homologue F (MAFF) d'une manière dépendante de la concentration et du temps. Nous avons utilisé des petits ARN interférents (siRNA) afin de diminuer l'expression de MAFF en présence et en absence d'IL- 1 beta. Grâce à une analyse de séquençage de l'ARN, nous avons identifié une série de gènes dont l'expression est contrôlée par le facteur de transcription MAFF et/ou l'IL- 1 beta. L'inhibition de l'expression de MAFF en présence/absence d'IL- 1 beta a conduit à une série de changements d'expression de gènes impliqués dans l'activation de plusieurs voies de signalisation ainsi que dans la modulation de gènes liés à la parturition. Nous avons constaté que l'expression de la prostaglandine endoperoxyde synthase 2 (PTGS2), un régulateur clé de la parturition et de l'accouchement, a été augmentée en l'absence de MAFF, suggérant que MAFF exerce un contrôle négatif sur l'expression de cet enzyme. A l'aide d'un gène rapporteur de la luciférase sous le contrôle du promoteur *PTGS2,* nous avons montré que la modulation est, au moins en partie, médiée au niveau transcriptionnel. De plus, d'autres gènes qui ont été associés à un accouchement normal comme la thrombospondine-1 (THBS1) et l'inhibiteur des métallopeptidases 3 (TIMP3) sont aussi modulés par MAFF. Nos études fournissent ainsi des informations précieuses sur le réseau transcriptionnel de MAFF, régissant la fonction des cellules du myomètre en présence et en absence de stimuli pro-inflammatoires.

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I acknowledge the work of Dr. Gregory Chevillard and Fangshi Lu in the optimization of *MAFF* knockdown conditions in myometrial cells using Amaxa Technologies System.

Preface

In accordance with McGill University guidelines for thesis preparation, the candidate chose to present her work in the standard format. An introduction with a detailed literature review is presented in the first section, followed by materials and methods, then manuscript-based results, and finally a detailed discussion with the conclusion.

Contribution of Authors:

All research presented in Chapter IV was performed by the candidate with the exception of:

- The optimization of the *MAFF* knockdown conditions using Amaxa transfection reagent as well as parts of the time point analysis of siRNA mediated knockdown were performed by Fangshi Lu.

INTRODUCTION

Parturition is the process of childbirth after a period of gestation. Although much progress has been made with years of research on the topic, the inability for direct testing leaves the mechanisms by which parturition initiates and progresses unclear. The most supported hypotheses are hormone level changes, paracrine molecule action, as well presence of inflammatory processes. These aspects are all important for parturition, and links between them have been established. Below is a literature review summarizing the knowledge in the field of initiation of parturition. After the initiation of parturition section follows a summary of the literature on the basic leucine zipper (bZIP) family of proteins, focusing on the MAFF transcription factor that may play a role in the initiation of childbirth.

A. Human Parturition Events and Hormonal Regulation

A.1 Events leading to Childbirth

There are 5 distinct physiological events that lead to childbirth: "fetal membrane rupture, cervical dilation, myometrial contractions, placental separation and uterine involution" [1]. Fetal membrane rupture is a term used to describe the breaking of amniotic sac and the release of water prior to labor. Cervical dilation is necessary for the passage of the fetus while myometrial contractility is required for the expulsion. Placental separation from the uterus follows and uterine involution is the return of the uterus to its normal state.

A.1.1 Myometrial Activation

Myometrial contractility is the central event that leads to the expulsion of the baby and this process is regulated by a variety of factors. For the contractility to occur the myometrium must transition from the quiescent state in which it spends most of the pregnancy to a contractile state. Events leading to activation of the myometrium are mediated by "contraction-associated-proteins" (CAPs). Different CAPs participate in priming the myometrium for contractions. They can be involved in 1) promoting interaction between actin and myosin to activate the cellular contractile apparatus, in 2) mediating myometrial excitability for action potential to occur, or in 3) increasing intracellular myometrial connectivity for better transmission of action potential [2].

Myometrium is a smooth muscle and contractions happen by shortening of the muscle, an event for which actin and myosin proteins need to interact. Contractions require actin in filamentous form to attach to focal points located at the cell membrane in order to link the intracellular cytoskeleton to the membrane [3, 4]. "Activated" myosin will slide over actin filaments and create shortening of the muscle. The "activation" of myosin happens by phosphorylation of a myosin head which will induce a conformational change and activate myosin's ATPase activity, the driving force of sliding over [5]. Myosin is phosphorylated by myosin light chain kinase (MLCK) that needs to be activated. An increase of calcium levels in the cell leads to an augmentation of calcium binding to the regulatory protein, calmodulin that changes conformation and becomes active. The calcium-calmodulin complex then binds to the CaM-binding domain of MLCK and sequesters the inhibitory region of MLCK, making it an active kinase [5, 6] that will phosphorylate myosin.

The increase of calcium ions inside the myometrium can occur in two ways: by direct entry from outside of the cell and by its release from intracellular stores. It was reported however that the largest Ca^{2+} contribution is due to the entry of calcium by the voltage-dependant channels [7]. The resting membrane potential of myometrial cells is such that the interior is more negatively charged than the exterior because of the action of the sodium-potassium adenosine triphosphatase (Na^+/K^+ pump) [8]. Potassium channels play a role in the resting potential – the calcium-gated and voltage-gated potassium channels allow exit of the ions, making the cell interior more negative and preventing depolarization. In neuronal cells, sodium is usually the ion causing depolarization of the cell and ultimately leading to action potential, but in the myometrium, an increase in calcium ions is needed for the contractile activity of the smooth muscle [2, 9]. At labor, a change in the uterine smooth muscle sensitivity to CAPs occurs (through modulation of CAP receptors, for example) for a more efficient rise in the intraceullular calcium levels in order to allow depolarization and activation of contractile machinery. The first mode of calcium increase is its release from intracellular stores, regulated by the action of a subset of CAPs including lipid compounds prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) or by oxytocin hormone action [2, 10]. The interaction of the CAPs with the receptor on myometrial cells leads to an intracellular signaling cascade resulting in the release of calcium. For instance, the oxytocin protein, upon binding to its G-protein coupled receptor (GPCR), activates phospholipase C (PLC) which then activates protein kinase C (PKC) leading to release of inositol triphosphate (IP_3) and finally to Ca^{2+} release from the sarcoplasmic stores [11]. The second mode of calcium increase in myometrium at labor is mediated by voltage-gated channels that open due to depolarization. Once again, $PGF_{2\alpha}$ and oxytocin hormone play the role of CAPs by regulating the opening of channels [12].

During parturition, myometrial cell connectivity is important for electrical activity passage to create synchronous contractions [13]. The myocytes are connected to each other through gap junction proteins (such as gap-junction alpha-1 protein, previously known as connexin-43 (CX-43) and channels that facilitate propagation of the action potential through myofibrils. The electrical activity helps depolarization of the cells for resulting in calcium influx, creating a "calcium wave" through the myometrium. In this wave, the action potential travels, contracting individual cells on the way. The myocytes remain contracted as long as the intracellular concentration of calcium is elevated [12]. The higher concentration of calcium inside the cell then acts on calcium-gated potassium channels that allow the efflux of K^+ ions, making the cell repolarize. Chlorine ions also contribute to the membrane potential of myometrial cells by aiding the potassium to repolarize the myocyte [9]. With the repolarization, calcium concentrations drop, making the cell ready for another depolarization and repeated contractions.

A.1.2 Fetal Membrane Rupture

What exactly triggers the rupture of chorioamniotic membranes is not completely known to this date. Evidence suggests that the action of matrix remodeling enzymes might play a role in this process as the quantity of collagen and other extracellular matrix (ECM) components decrease prior to gestation [14]. The amnion contacts directly the amniotic fluid which contains many factors involved in initiation of parturition including inflammatory modulators. It was reported that tumor necrosis factor alpha (TNF-alpha) and interleukin-1 beta (IL-1 beta) treatment of fetal membrane [15] fragments are able to induce weakening of the tissue by decreasing strength and work needed for rupture. The treatment with cytokines coincided with an increase in protein levels of matrix metalloproteinase-9 (MMP-9) and a decrease in protein levels

of TIMP-3 [16]. MMP-9 is involved in collagenolysis whereas TIMP-3 is an inhibitor of the matrix metalloproteinases hinting at a possibility of increased extracellular matrix degradation in the fetal membranes. Moreover, apoptosis happens in amniotic epithelium at term, which might be contributing to fetal membrane rupture [17]. There is an increase of monocytes and T cells at fetal membranes during labor hence increasing production of cytokines such as IL-1 beta. These T cells were also shown to express higher MMP-9 levels [18]. Therefore the proinflammatory cytokine network at the fetal membranes contribute to extracellular matrix remodeling and collagenolysis by either secreting matrix remodeling enzymes or by inducing the FM to do so [14].

A.1.3 Cervical Remodeling

For successful parturition, cervical remodeling must occur to allow passage of the fetus. There are several stages in the remodeling process – 1) softening of the cervix which occurs during pregnancy, 2) cervical ripening when the tissue loses its integrity and becomes disorganized, 3) cervical dilation for passage of the fetus and finally 4) postpartum repair of the cervix [19].

Since the 1980s, when it was first proposed that the leukocytes that infiltrate the cervix are responsible for secretion of matrix remodelling enzymes it was believed that cervical ripening is the process of collagen destruction and disorganization. However recent evidence suggests that infiltration of leukocytes to the cervical region is important for the postpartum repair of the cervix rather than for cervical ripening and initiation of parturition. A microarray study of ripe and unripe cervices revealed that there is only a small increase in proinflammatory gene expression at the moment of cervical ripening [20] suggesting that inflammatory

modulators might be upregulated after the onset of labor for the purpose of preventing infection associated with birth and for repairing the cervical tissue postpartum.

The hypothesis that cervical ripening is not the result of action of inflammatory modulators is supported by a series of studies. It was reported that there is conflicting data on the subject whether cervical ripening happens due to degradation or changes in structure of collagen, which is the most abundant protein in the ECM of the cervix [19]. As noted more than 30 years ago, collagen passes from organized fibrils in human non-pregnant cervix to highly disorganized fragments with irregular outlines [21]. Although a degradation hypothesis is not totally rejected, many findings suggest that changes in extracellular matrix protein expression might be responsible for remodelling of the ECM [19]. For example, glycosaminoglycans (GAGs) content in the cervix changes in composition with the progression of pregnancy. Hyaluronan (HA) increases prior to parturition in the uterine cervix, which corresponds to the increase of the hyaluronan synthase 2 (HAS2) in the mouse [22]. Moreover, it was reported that with the progression of pregnancy, HA is found in lower molecular weight towards the end of pregnancy, a change that might be involved in the decrease of tensile strength and integrity of the tissue, which is required for the childbirth process [19].

A.2 Hormonal and Paracrine Regulation of Labor Onset

A.2.1 Progesterone Withdrawal

It has been known for some time that throughout pregnancy, the steroid hormone progesterone promotes uterine relaxation and maintains the myometrium in a quiescent state. In the 1950s, the “progesterone block” hypothesis was first proposed, and it states that during

pregnancy the uterus is under a “block” and that the excitability of the smooth muscle is suppressed by progesterone [23], suggesting that at the end of pregnancy the progesterone block is released allowing parturition to occur. Since the first series of studies in late 1990s, it is now accepted that progesterone withdrawal contributes to the initiation of parturition.

A.2.1.1 Factors regulated by Progesterone

During human pregnancy, progesterone is mainly produced by the placenta and it is thought to exert its action through genomic and nongenomic mechanisms. Genomic pathways are long-acting changes in contraction-associated gene expression whereas nongenomic pathways are rapid and affect directly the machinery responsible for the contractile phenotype. On the genomic level, progesterone negatively modulates the expression of genes encoding CAPs, which keeps the myometrium in a relaxed state. CAPs involved in parturition, whose gene expression is negatively affected by progesterone, include prostaglandin $F_{2\alpha}$ receptor (PTGFR or FP), oxytocin receptor (OTXR), the prostaglandin metabolizing enzyme 15-hydroxy-PG-dehydrogenase (PGDH), and the gap-junction protein CX-43. Nongenomic pathways affected by progesterone are poorly characterized, but evidence suggests that progesterone participates in the regulation of signalling pathways involved in the regulation of intracellular free calcium, which can in turn affect the actin-myosin contractile apparatus [24, 25]. It is also worth mentioning that progesterone has been shown to partially prevent cholesterol transport from and to the plasma membrane as well as negatively interfering with cholesterol esterification. Cholesterol normally increases binding affinity of oxytocin to oxytocin receptor, an important event for myometrial contractions [26].

A.2.1.2 Functional Progesterone Withdrawal at Parturition

If progesterone is required to maintain pregnancy, then the withdrawal from progesterone must be the mechanism responsible for initiation of parturition [24]. Half a century of research on the topic supports this hypothesis and strong evidence to this comes from the studies where administration of mifepristone (RU486), a progesterone receptor antagonist, terminates pregnancy at early gestation periods or serves as an inducer of labor towards the end of gestation [27-31]. In most species of laboratory animals, a decrease in progesterone levels in maternal circulation at the onset of parturition is observed, and progesterone withdrawal is said to be "systemic" [32]. In humans however, progesterone levels are high and constant during pregnancy and they remain unchanged even at labor, suggesting that the progesterone withdrawal might be functional, by a change in the receptor expression or cellular sensitivity to it [24]. Therefore the differences between the species is significant and as noted previously, murine models might not represent accurately the complexity of events happening in humans, as a network of several players have a role in human parturition [33].

The proposed mechanisms behind functional progesterone withdrawal may include sequestration of progesterone in circulation, changes in progesterone receptor expression or production of an endogenous progesterone antagonist [25]. Evidence suggests that although all the described mechanisms are plausible, many researchers decided to focus their studies on progesterone receptor expression.

A.2.1.3 Progesterone Receptors

There are several progesterone receptors that mediate the actions of progesterone in the cell. Ligand-activated nuclear progesterone receptors (nPRs) exist in 3 isoforms – PR-A, PR-B

and PR-C. The PR-B isoform is believed to be responsible for the relaxatory phenotype of myometrium during pregnancy. PR-A is transcribed from the same gene, but is shorter at the N-terminus and is transcriptionally less active than PR-B. The PR-C isoform was discovered not so long ago and is even shorter in length at the N-terminus than PR-A.

Evidence suggests that there is an increase in PR-A (PR-C)/PR-B ratio at the onset of parturition in human myometrium where PR-A/PR-B ratios are around 0.5 at 30 weeks of gestation, with a ratio of 1 at the onset of labor and a ratio of 3 in labouring myometrium [34]. It was also shown that there is a shift in rhesus Macaques from nPR-B to nPR-A expression between mid-pregnancy to labouring myometrium [35]. It was demonstrated that there are increased ratios of nPR-A/nPR-B mRNA in term human myometrium [36]. Of interest, it is now known that nPR-A and PR-B bind to different gene promoters leading to the hypothesis that nPR-B supports the transcription of relaxatory proteins whereas nPR-A is less active, and promotes the transcription of CAPs [24]. As evidence, it was shown that progesterone was able to decrease proinflammatory gene expression when receptor ratios favoured nPR-B. They argued that nPR-B acts through an increase in expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IKB-alpha) and decrease expression of liposaccharide-induced proinflammatory genes [37]. IKB-alpha is involved in keeping the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB) transcription factor in the cytoplasm, thus preventing it to exert its role in induction of pro-inflammatory genes. It was proposed in 2001 that nPR-A inhibits the nPR-B mediated transcription in term human amnion suggesting that nPR-A might be a repressor of nPR-B [38].

nPR functions similarly to other ligand-dependant transcription factors – upon ligand binding, dimerization occurs, followed by translocation to the nucleus where they bind

progesterone-response elements and regulate gene expression. Ligand-bound nPRs have also been shown to function indirectly to modulate gene expression by binding and activating Src-Kinase which activates downstream signaling cascades ultimately leading to activation of extracellular-signal regulated kinases 1/2 (ERK1/2) and modulation of many transcription factors. Moreover, progesterone is also believed to exert its action through non-genomic pathways by interacting with receptors that are not part of the nPR family. There are progesterone receptors that are part of the G-protein-coupled receptor family, namely membrane progestin receptor alpha, beta or gamma (mPR-alpha, mPR-beta and mPR-gamma, also known as progestin and adipoQ receptor family members VII, VIII and V, respectively) that act through inhibitory G proteins. Progesterone receptor membrane component 1 and 2 (PGRMC1 and PGRMC2) are receptors of the tyrosine-kinase family and have been identified as membrane-bound progesterone receptors as well. Recently, Wu *et al.* [39], suggested that PGRMC1 may be one of the key players of the non-genomic action of progesterone. They reported that PGRMC1 expression is lower in term human (in labor or not) compared to pre-term non-labor myometrium. Finally, progesterone might be mediating the non-genomic action by binding to other membrane receptors. Progesterone metabolites such as 5β-dihydro-progesterone or 3α-hydroxy-5α-pregnan-20-one have been reported to interact with gamma-aminobutyric acid type A ($GABA_A$) receptor, a member of ligand-gated ion channels. It was suggested that the action of the metabolite is mediated at least in part through the $GABA_A$ receptor. $GABA_A$ receptors are present in many tissues including human uterus and treatment of myometrium with $GABA_A$ antagonists have been shown to block the inhibition of contractility of the tissue [24, 40]. Progesterone has also been reported to interact with rodent OXTR, but not with the human protein. However, in humans a progesterone metabolite, 5β-dihydro-progesterone, interacts with

OXTR and might be involved in decreasing oxytocin-induced myometrial contractility [24, 41-43].

A.2.2 Estrogen Activation

During pregnancy, the uterus is exposed to high levels of estrogens, which are of several types – estrone (E1), estradiol (E2) and estriol (E3). Estrogen responsiveness plays an important role in parturition as E2 increases the expression of several CAPs – the gap junction protein CX-43, OXTR and PTGFR as well as the prostaglandin-endoperoxidase synthase 2 (PTGS2, previously known as cycloxygenase-2 or COX-2) [44]. The action of estrogen on oxytocin receptor as well as on the production of prostaglandins was shown by administration of the estrogen antagonist tamoxifen, which blocked the increase in these two factors and delayed parturition for 24h in rats [45]. Levels of estrogens do not change towards the end of pregnancy, but the expression of estrogen-controlled genes increases [44], suggesting an increase in myometrial responsiveness to estrogen, hence functional estrogen activation. It appears that estrogen responsiveness correlates with progesterone withdrawal – when ratios of PR-A to PR-B increase [36]. It was therefore proposed that during pregnancy, progesterone inhibits the expression of estrogen receptor alpha (ER-alpha, or also known as estrogen receptor 1, (ESR1)), a receptor believed being involved in activation of CAPs, and once functional progesterone withdrawal occurs, the inhibition is relieved leading to higher expression of ESR1 [17]. A study was done to assess main estrogen receptors in human pregnant uterus by RT-PCR. It was found that the transcripts *ESR1*, a nuclear receptor for estrogen, as well as G protein-coupled receptor 30 (*GPR30*) were detected in pregnant myometrium. However, no protein was detected, but upon inhibition of 26S proteasome, increased ESR1 protein levels were found, suggesting that ESR1 has a high turnover rate [46]. This finding supports the idea that progesterone might be inhibiting

ERS1 during pregnancy by targeting the receptor for degradation. It was also suggested that E2 effect on myometrium is exerted by ERK1/2 as its phosphorylation (and thus activation) is inhibited by antagonists to estrogen.

A.2.3 Oxytocin in Parturition

Another important hormone is oxytocin, although its role during parturition has been controversial. Oxytocin (OT) is mostly produced by the pituitary gland, but it has been found that human fetal membranes and uterus are able to produce oxytocin as well [44]. It has been also linked to prostaglandins, as OT stimulates $PGF_{2\alpha}$, PGE_2 production in decidua, PGE_2 production in the amnion as well as arachidonic acid (prostaglandin building block) release from the decidual cells [47, 48]. Oxytocin induces the prostanoid synthesis, as shown in rabbit amnion by increasing cytoplasmic phospholipase A2 (cPLA2) and prostaglandin-endoperoxide synthase 1 (PTGS1) and PTGS2 [49]. Oxytocin exerts a direct effect on myometrial cells by binding to its receptor, a G-protein coupled receptor that functions through G_q alpha subunit ($G_{q/11}$) and G_i alpha subunit (G_i) proteins. In human myometrium, they stimulate PLC isoform beta – isoforms beta 1 and 3 are stimulated by $G_{q/11}$ and isoforms beta 2 and 3 by G_i [7]. Subsequenty, PLC mediates inositol triphosphate production that mobilizes calcium and contributes to the contraction of the myocyte. The controversy in oxytocin action comes from the fact that it was believed that OT is indispensible for human parturition until knockout mice showed otherwise by delivering viable pups [50].

Oxytocin is believed to be important for the precise timing of the labor onset. In late pregnancy, there is an increase in OXTR in humans [51] as well as in other species such as rats, mice and sheep [11]. *OXTR* mRNA expression was assessed in primary human myometrial cells

from 3 sets of patients – non-pregnant, pregnant before labor onset and pregnant after labor onset and found that the receptor's transcript levels are lowest in non-pregnant, and highest in pregnant, after labor onset samples. Moreover, they analyzed *OXTR* expression in response to stretch and determined that stretch affected pregnant before labor onset samples by increasing their transcript levels [52]. *OXTR* upregulation corresponds to progesterone withdrawal and increase in estrogen responsiveness, however there is no direct evidence that steroid hormones directly regulate OXTR expression [7]. Human *OXTR* promoter does not contain an obvious estrogen response element (ERE) sequence but has binding sites for activator protein-1 (AP-1), CCAAT-enhancer-binding proteins (C/EBP and NFKB) transcription factors. It was reported that expression of both C/EBP and NFKB synergistically increase *OXTR* mRNA expression by 55-fold with interleukin-1 beta induction [53].

A.2.4 Prostaglandins in Parturition

Prostaglandins have numerous functions in various reproductive tissues during all stages of parturition – they are involved in fetal membrane rupture, cervical dilation, contractions of the myometrium, separation of the placenta as well as uterine involution [54]. Of interest to the initiation of labor, it has been shown that PGE_2 and $PGF_{2\alpha}$ are increased before the onset of spontaneous labor [55].

Arachidonic acid is the precursor for prostaglandins – it is released from the plasma membrane by the action of the phospholipase A2 (PLA2). In women in labor, a significant increase in free arachidonic acid is observed when compared to women that are not in labor. Moreover, intra-amniotic injection of the prostaglandin precursor can cause abortion in the second trimester of pregnancy, suggesting that it plays a role in parturition initiation [44]. The

rate limiting step in prostaglandin synthesis is the conversion of arachidonic acid into an unstable endoperoxidase intermediate. The action is carried out by prostaglandin-endoperoxide synthase, an enzyme previously known as cycloxygenase. Two different isoforms of the enzymes exist, PTGS1 and PTGS2; PTGS1 being the constitutively expressed and ubiquitous while PTGS2 is inducible. Upregulation of PTGS2 in uterus is associated with human parturition [56]. PTGS1 expression does not change, but it was reported that levels of PTGS2 were increased before the onset of labor in human decidua, myometrium and fetal membranes [44]. More precisely, IL-1 beta and TNF-alpha has been shown to increase PTGS2 expression and therefore production of PG in myometrial cells by NFKB activity [56, 57]. Moreover, it has been shown that the activity of the enzyme mediating prostaglandin catabolism, PGDH in the chorion is decreased at the initiation of labor [58].

Prostaglandins exert their effect on reproductive tissues by binding to their G-protein coupled receptors, generating second messenger molecules and inducing cAMP formation, Ca^{2+} mobilization or other cellular responses. $PGF_{2\alpha}$ binds to prostaglandin F2 alpha receptor, which exists in two isoforms. The engagement of PTGFR receptor by $PGF_{2\alpha}$ leads to activation of G_i protein and increase in Ca^{2+} concentration by mobilization [54], therefore activating the myometrium for contraction. The action of PGE_2 is mediated by prostaglandin E_2 receptors [59] that have been classified by subtypes. EP1 acts through G_i protein and stimulates contraction by increasing intracellular calcium levels, just like the FP receptor. EP3 is also known to stimulate contraction, but it works by inhibiting the action of adenylate cyclase therefore decreasing cAMP formation. The action of EP2 and EP4 receptors is relaxatory as they lead to increasing cAMP levels by the action of adenylate cyclase [54, 60]. During pregnancy, some contractile receptors, such as FP and EP3 are lower in expression while relaxatory receptors like EP2 are higher in

order to maintain uterine quiescence, but at labor the expression of the contractile receptors is upregulated in a number of species. For example in the longitudinal section of the rat myometrium, *FP* transcripts are increased from late gestation to delivery whereas relaxatory receptors EP2 are decreased [59]. Expression of EP3 receptor in the upper part of the uterus increases at parturition, while in the lower part of the uterus it is the relaxatory EP2 receptor that has shown to be increased. To explain differential receptor expression throughout the myometrium, it has been proposed that the contractility of the upper segment contributes to the expulsion of the baby at labor, and the relaxatory phenotype of the lower segment allows the passage of the fetal head [61].

Prostaglandins have been shown to regulate some of the CAPs such as oxytocin receptor, gap junction protein CX-43 as well as the enzyme that controls their own synthesis, PTGS2 [54]. It was shown that $PGF_{2\alpha}$ treatment of cultured myometrial cells increased expression of CX-43 as well as of PTGS2 protein, while decreasing PTGFR protein levels. The decrease of PTGFR could serve as a negative feedback loop to attenuate the effect of $PGF_{2\alpha}$. At the same time, it was also shown that the upregulation of OXTR protein levels in the lower segment of the myometrium while downregulating OXTR levels in the upper segment in response to $PGF_{2\alpha}$. The differential expression observed were enhanced by IL-1 beta and reversed by inhibition of PTGFR. Moreover, PTGFR knockout mice abolished the effect of $PGF_{2\alpha}$ treatment on PTGS2 and OXTR but not on CX-43. [62]. There has also been reports of $PGF_{2\alpha}$ effect on progesterone receptors – the prostaglandin increases expression of PR-A but not PR-B, possibly helping in progesterone withdrawal [44]. Finally, prostaglandins have an effect also on matrix remodelling enzymes. A group analyzed decidua for its MMP-2 and MMP-9 secretion as well as for tissue inhibitor of matrix metalloproteinases 1 (TIMP1) protein expression in response to $PGF_{2\alpha}$

treatment. It was determined that $PGF_{2\alpha}$ caused an increase of 2.4 fold of the inactive form of MMP-2 and 11.3 fold on the active form while increasing MMP-9 by 1.9 fold. The protein production of the inhibitor of the matrix proteinase, TIMP1 was decreased by 70% in the decidua [63], suggesting that $PGF_{2\alpha}$ creates conditions for matrix remodeling at the decidua.

B. Inflammation at the Onset of Normal Parturition

Although the precise mechanism of childbirth is still a mystery, inflammation is considered as one of the main mediators of parturition. Inflammation is not necessarily a pathology in reproduction as several instances illustrate the presence of inflammation at different stages such as in ovulation or implantation [64]. Studies confirm that inflammatory mediators participate in parturition in different tissues of the reproductive system, including the chorioamniotic membranes, the uterine cervix as well as in the myometrium [65].

B.1 Infiltration of Leukocytes

The inflammation process is characterized by the presence of cytokines, which are often associated with leukocytes. In fact, at parturition there is an infiltration of different subpopulations of leukocytes to the uterine tissues [14]. The myometrium recruits neutrophils, macrophages as well as T cells at the onset of term labor [66] whereas the uterine cervix attracts neutrophils and macrophages prior to labor onset [67]. The recruitment of leukocytes depends on their attraction by chemokines, low molecular weight proteins that are secreted by many cells. There are several important chemokines that act as early mediators of the inflammatory response,

namely chemokine (C-C Motif) ligand 2/3/5 (CCL2,3,5) and interleukin-8 (IL-8). Later towards the end of pregnancy, an increase of *CCL13,19,21* transcripts has been observed as well. There are also other chemokines that increase only at labor, precisely CCL3 and chemokine (C-X-C motif) ligand 10 (CXCL10) [14]. Leukocytes attracted to the uterine tissue extravasate from the local circulation. Once they are on the site, they are held in place, which is accomplished through the expression of cell adhesion molecules (CAMs) by local cells. [14].

Although the function of the presence of leukocytes in uterine tissues remains to be confirmed, evidence suggests that the attracted leukocytes exert their effect by two main mechanisms. First, they secrete pro-inflammatory cytokines such as IL-1 beta, interleukin-6 (IL-6) as well as TNF-alpha. Second, they are thought to directly contribute to the secretion of parturition mediator molecules such as matrix remodelling enzymes like MMPs [14].

B.2 Leukocytes and Proinflammatory Cytokines

B.2.1 IL-1 beta Cytokine

The IL-1 family of cytokines is associated with inflammation and immune response. There are 11 members in the family of IL-1, divided by subfamilies. The subfamily comprising IL-1 beta also includes IL-1 alpha and interleukin-33 (IL-33) cytokines [68]. Cytokine secretion has been attributed to many cells in events unrelated to childbirth. Macrophages are the main secreting cells of IL-1 beta, as well as of TNF-alpha and IL-6 cytokines. However, many other cells produce IL-1 beta cytokine such as monocytes, B lymphocytes and natural killer (NK) cells [69, 70]. IL-1 beta is an inducible cytokine, which is transcribed under the control of regulatory regions which are scattered over a few thousand base pairs upstream of the start site. There are

cAMP response element (CRE), AP-1 [71] recognition sites. The induction and transcription of IL-1 beta can be mediated by Toll-Like-Receptor (TLR) ligands or by non-TLR ligands like blood clotting, or hypoxia, but without the increase in protein levels, therefore without protein translation. Indeed, IL-1 beta is transcribed into an inactive 31kDa precursor, which needs to be cleaved by caspase-1 to release the C-terminal active and mature cytokine of 18kDa [68]. Under normal conditions, caspase-1 is also found as an inactive precursor, procaspase-1, inside the cell. The processing of the procaspase-1 into its active form requires several steps: 1) under specific conditions, extracellular ATP activates the transmembrane receptor Purinergic Receptor P2X, Ligand-Gated Ion Channel 7 (P2X7 receptor), which allows efflux of potassium through K channels, and decreases the K^+ levels inside the cell; 2) lower levels of potassium activate the assembly of the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome, which in turn will 3) process the procaspase-1 into caspase-1. The active caspase will then proceed to cleave IL-1 beta in the secretory lysosome or the cytosol. The secretion of the mature form of IL-1 beta is dependent on the calcium concentrations inside the cell. Upon Ca^{2+} increase, IL-1 beta is secreted by exocytosis with the lysosomal contents into the ECM [70].

The effect of IL-1 beta on other cells depends on the expression of its receptor on the surface of the cell. IL-1 beta binds to the IL-1 Receptor I (IL-1R1) or IL-1 Receptor II (IL-R2) on cell surface. IL-1R1 is a ligand binding (alpha chain) receptor which requires dimerization with the co-receptor IL-1 Receptor 3 (IL-1R3) [68]. As shown (Figure 1), upon IL-1 beta binding (step 1), IL-1R1 heterodimerizes with IL-1R3 (step 2), and their two intracellular Toll IL-1 receptor (TIR) domains come into proximity. The TIR domain is present in most Toll-like receptor proteins and it received its name from its homology to the *Drosophila* Toll proteins, which are, as explained below a class of proteins playing an important role in immunity [70].

Figure 1

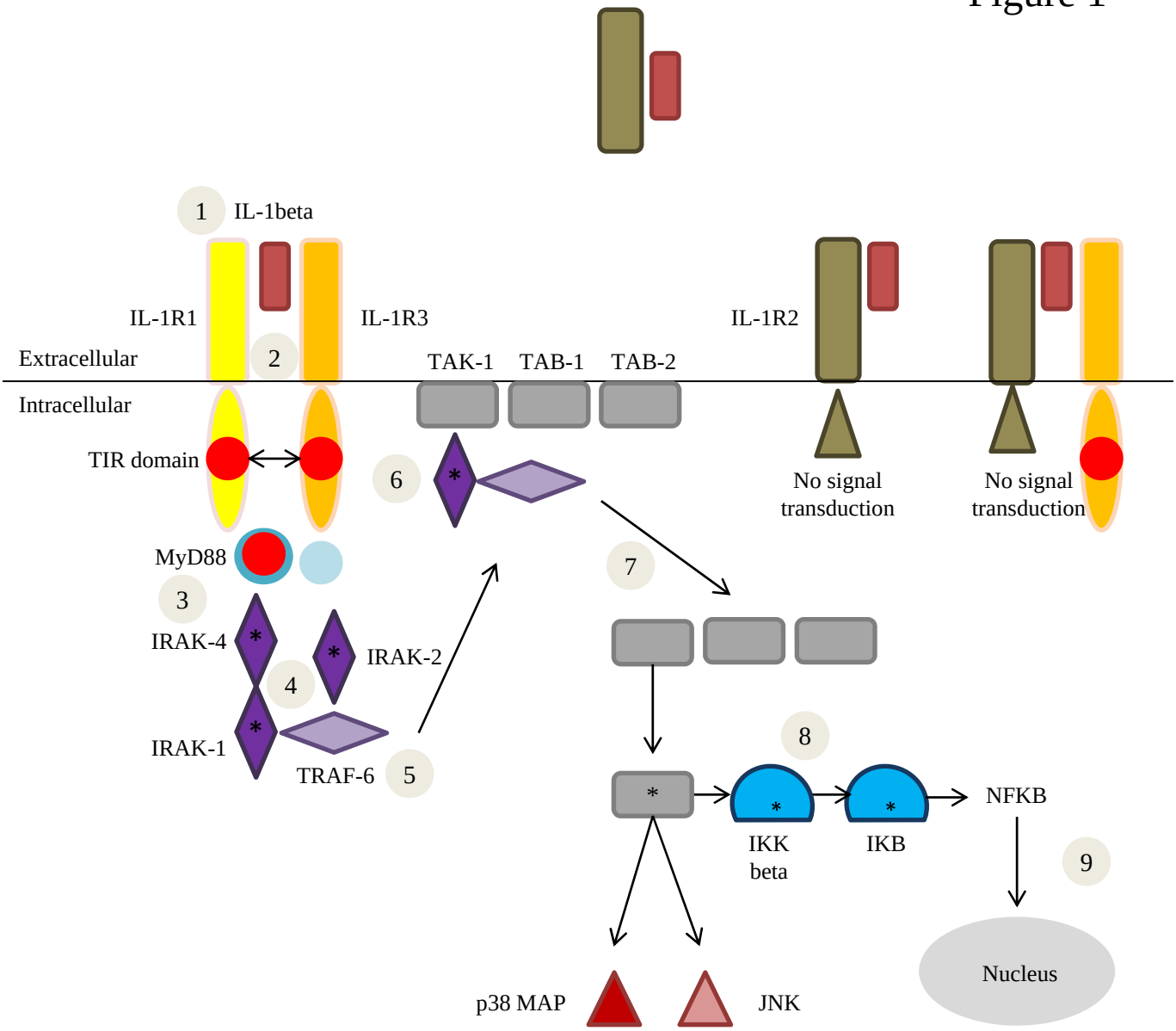


Figure 1: IL-1 beta signal transduction pathway: * represents phosphorylation. Upon IL-1 beta binding (1), IL-1R1 heterodimerizes with IL-1R3 (2) which allow the two intracellular TIR domains to come into proximity. This event leads to the recruitment of adaptor proteins MyD88 and IRAK-4 (3). IRAK-4 is autophosphorylated and recruits and phosphorylates IRAK-1 and IRAK2 , forming a stable complex (4). The recruitment of TRAF-6 (5) is followed by its migration, together with IRAK-1 to the intracellular membrane, where the complex interacts with TAK-1 in association with TAB-1 and TAB-2 (6). The ubiquitin E3 ligase activity of TRAF-6 leads to the ubiquitination and dissociation of TAK-1 and TAB-1/2 from the membrane (7). In the cytosol, TAK-1 is phosphorylated and then proceeds to activate p38 MAP, JNK and more importantly, IKK beta by phosphorylation. The phosphorylation of IKB by IKK (8) leads to the release of NFKB from its inhibitor, allowing it to migrate to the nucleus (9). Moreover, IL-1 beta can also bind to IL-1R2 which lacks the TIR domain in the extracellular part resulting in no signal transduction. Figure modified from (70).

The link between the domain and immunity has been established when, in 1996 it was shown that the TIR domain is homologous to the *Drosophila* TLR domain and is necessary for its survival in response to fungal infection [15]. The TIR domains of the receptor IL-1R1 and co-receptor IL-1R3 recruit two adaptor protein myeloid differentiation primary response gene 88 (MyD88) (step 3) and interleukin-1 receptor-activated protein kinase (IRAK-4) (step 4), forming a stable complex. IRAK-4 is autophosphorylated and it further recruits and phosphorylates IRAK-1 and IRAK-2 (step 4). The recruitment of the tumor necrosis factor-associated factor 6 (TRAF-6) follows (step 5). IRAK-1 together with TRAF-6 then migrate to the cellular membrane where they interact with TGF-beta-actiaved kinase 1 (TAK-1) in association with TAK-binding protein 1 and 2 (TAB-1 and TAB-2) (step6) where TRAF-6 acts as ubiquitin E3 ligase and ubiquitinates TAK-1, promoting its release from the membrane (step 7). In the cytosol TAK-1 is phosphorylated and it allows activation of inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-beta) by phosphorylation (step 8). Activated IKK-beta in turn phosphorylates the inhibitor of NFKB, IKB, which releases the NFKB transcription factor from it and allows its migration to the nucleus (step 9). The phosphorylated TAK-1 has also been shown to activate mitogen-activated protein kinase (MAPK) p38 and c-jun N-terminal kinase (JNK). The interaction of IL-1 beta with the other receptor IL-1R2 does not lead to signal transduction, as the cytoplasmic domain of IL-1R2 has no TIR domain. Moreover, IL-1R2 receptor can be proteolytically processed and the extracellular part of the receptor is able to be secreted into the ECL. In this case, the soluble (sIL-1R2) receptor sequesters IL-1 beta and prevents it from exerting its action on the cell [70, 72].

B.2.2 Cytokines and Labor

The TNF-alpha levels were compared in the amnion during labor to the amnion before the onset of labor and it was found that TNF-alpha was significantly increased in amniotic fluid during labor [73]. A series of studies support the notion that proinflammatory cytokines control the expression of proteins involved in the promotion of parturition. It was reported that OXTR, PTGS2, CX-43, PTGFR, as well as some matrix remodeling enzymes are regulated by the cytokines [69]. Many cytokines, including TNF-alpha and IL-1 beta regulate expression of PTGS2 and hence prostaglandin production. It was reported that mice lacking the receptors for IL-1 beta and TNF-alpha have a lower concentration of *PTGS2* transcripts in the myometrium when stimulated by *E. coli*, a bacteria that, in wild type animals, induces *PTGS2* production and stimulates preterm labor [74]. The expression of certain proinflammatory transcripts in myometrial term compared to preterm labor samples was assessed. Using qPCR, it was found that in the lower segment of the myometrium there is higher expression of *PTGS2*, *IL1B*, *IL-6* as well as *IL-8* mRNA. In the upper segment of the myometrium they showed higher *PTGS2* and *IL1B* expression in term labor samples, as compared to no labor samples. It was concluded that the term and preterm labor transcriptome was significantly different in the myometrium, and suggested that these changes might be mediated by the inflammatory cytokines produced by the infiltrated leukocytes [75]. Moreover, in cultured chorion, placental trophoblasts cells and in fetal membrane disks, IL-1 beta cytokine, and TNF-alpha to a lesser extent, were shown to decrease the transcript levels and activity of prostaglandin 15-hydroxy dehydrogenase (*PGDH*), the enzyme that catalyzes prostaglandins into inactive metabolites [76]. Another important effect of cytokines in human myometrium is the increase in transcript expression of PTGFR. It was

shown that incubation of a myometrium-derived cell line with IL-6 or IL-1 beta increase PTGFR transcript levels [77], possibly priming the myometrium for contraction.

B.3 Leukocytes and Parturition Effector Molecules

Although leukocytic function is hypothesized to be primarily the secretion of proinflammatory cytokines, it was reported that the white blood cells might also be involved in synthesis of parturition effector molecules like matrix remodeling enzymes and prostaglandins. It has been shown that IL-1 beta and TNF-alpha lead to an increased production of several matrix metalloproteinases. For example it was found that in response to IL-1 beta and TNF-alpha cytokines, fetal membranes (amnion and chorion) induce pro-MMP-9 secretion and its activation in the amnion which contributes to collagenolysis [78]. A quantification of cervical stromal neutrophil count and MMP-8,9 revealed that there is a parallel increase in neutrophils and matrix metalloproteinases, which also correlated with an increase in IL-8, the chemokine that attracts and activates neutrophils. What concerns the uterine cervix, it was previously hypothesized that neutrophils and macrophages that infiltrate the tissue secrete matrix remodeling enzymes which degrade collagen and create an “opening”. However, recent studies revealed that cervical ripening happens by changes in the ECM, rather than degradation of collagen and that leukocytes in the cervix are necessary for the postpartum repair of the tissue [19].

B.4 NFKB in Parturition

Members of the 11 dimeric complexes of NFKB family are associated with inflammation and induced by proinflammatory cytokines. Evidence suggests that the Nuclear Receptor Kappa B (NFKB), family of transcription factors participates in processes leading to labor. IL-1 beta and TNF-alpha, as well as IL-6 stimulate NFKB translocation into the nucleus [69]. A study in human choriocarcinoma cells JEG-3 demonstrated that inflammatory mediators, TNF-alpha and lipopysaccharide (LPS), a component present on bacterial cell wall, increase expression of p50 and p65 subunits of NFKB as well as of cytoplasmic IKK-beta which is responsible for the phosphorylation and inactivation of IKB-alpha, an inhibitor of NFKB. These changes were associated with increased PTGS2 protein expression and increased $PGF_{2\alpha}$ release in the placental cells [79]. Another study showed that in Rhesus Monkeys, contractions that are induced by IL-1 beta can be suppressed by interleukin-10, an anti-inflammatory mediator which inhibits NFKB activity [80]. Many factors involved in parturition such as PTGS2, OXTR, IL-8, phospholipase-A2, as well as IL-1 beta and TNF-alpha themselves are positively regulated by NFKB in fetal membranes, myometrium and cervix [81].

The inflammatory microenvironment present in uterine tissue at parturition is complex (Figure 2). Briefly, unknown triggering mechanisms induce choriodecidua and amnion to secrete chemokines, mostly IL-8 and CCL2, 3 and 10 at the onset of labor. The chemokines induce extravasation of different leukocyte population into uterine tissues. Subsequently, leukocytes then secrete mediator molecules – cytokines which will in turn induce local cells to produce labor effector molecules such as prostaglandins and matrix metalloproteinases. The consequences of the effector molecules leads to fetal membrane rupture, cervical dilation and myometrial contractility for the expulsion of the fetus.

Figure 2

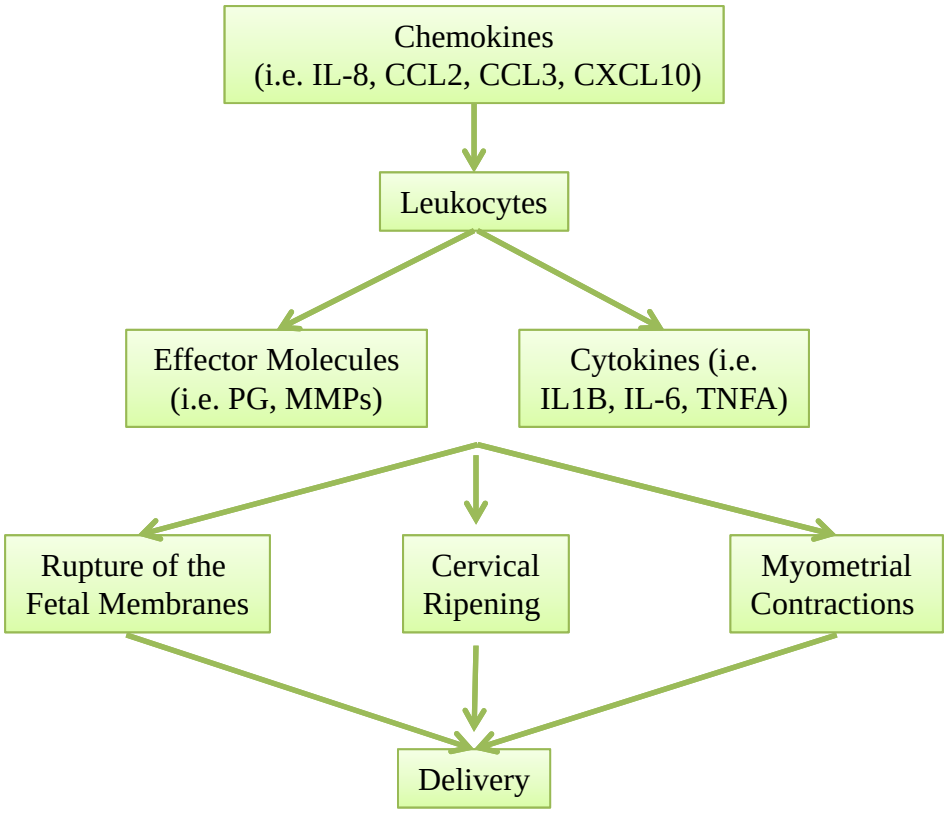


Figure 2: Simplified schematic representation of the inflammatory microenvironment at parturition in the uterine tissues: An unknown triggering mechanism induces chemokine secretion by the uterine tissues at the end of pregnancy to attract leukocytes. At the site, the white blood cells secrete cytokines, which induce the local cells to produce parturition effector molecules (such as prostaglandins (PG), oxytocin receptor, matrix metalloproteinases (MMPs)). Finally, effector molecules mediate direct events of delivery – rupture of the fetal membranes, cervical ripening and myometrial contractions. Figure modified from (14).

B.5 Preterm Labor

Preterm labor is one of the most common causes of neonatal morbidity and mortality. Preterm is considered as any birth before 37 weeks of gestation. In the United States, 12% of pregnancies terminate in preterm childbirth, and 25% - 40% of those pregnancies are related to intrauterine infection [82]. There are many factors that are involved in preterm birth such as socioeconomic factors, nutritional factors or the use of tobacco, but of interest to our research is the presence of systemic or local intrauterine infections. Infection can cause premature activation of the inflammatory pathways and accelerate the labor initiation process.

The most common pathway through which microorganisms cause intrauterine infection is through the vaginal and cervix ascending route. The infection could also be caused by transmission of maternal infection through the placenta, or peritoneal cavity microorganisms could migrate by the fallopian tube to the uterine tissue. Finally, infection can be accidently introduced by invasive procedures like amniocentesis [83].

B.5.1 Defence Mechanisms against Pathogens in Pregnancy and Parturition

There are many defense mechanisms that are present to fight the infection. For example, the epithelium is the first line of defence and it produces anti-microbial peptides [84]. Fetal lungs also have a defence mechanism by secreting surfactant proteins (SP-A and SP-D) that facilitate phagocytosis of microorganisms [85]. The immune system however is the major player in recognition of pathogenic organisms. It recognizes microorganisms by binding to their components through pattern recognition receptors (PRRs) that are either soluble, transmembranal or intracellular [83]. Important components of the innate response are toll-like receptors (TLRs) that are present on the cellular surface of many leukocytes. They recognize diverse pathogens

and usually dimerize and bind to other proteins. There are 10 different TLRs that have been recognized in humans and many of them were identified in gestational tissues. For example TLR-1,2,3,5,6 have been identified in epithelium of the vagina, cervix and endometrium [86]. Trophoblast cells as well as amniotic epithelium have also been shown to recognize pathogens through toll-like receptors-2 and -4 [87, 88]. TLR-2 recognizes Gram-positive bacteria, mycoplasmas and fungi, TLR-4 recognizes lipopolysaccharides that are present on Gram-negative bacteria, and TLR-3 recognizes double-stranded RNA from viruses [82]. The role that TLRs play in premature birth related to infection comes from from animal studies: TLR-4-deficient mice show less probability of delivering preterm following intrauterine infection with heat-killed *E. coli* or LPS administration [65, 89, 90]. The engagement of TRLs by dimerization or binding other proteins results in activation of NFKB which leads to a cascade of production of proinflammatory cytokines and chemokines [83], which in turn leads to the activation of the inflammatory pathways and possibly initiation of labor.

B.5.2 Cytokines in Infection-induced Parturition

Changes in expression levels of proinflammatory cytokines and chemokines such as IL-1, IL-6, TNF and IL-8 are observed in the onset of human preterm labor with elevation of cytokine expression within amniotic cavity [82, 91]. The current view of the infection-induced expression of inflammatory molecules is that when ascending infection coming from the cervical-vaginal areas reaches the decidua, it stimulates production of inflammatory mediators including cytokines and chemokines. If the reaction does not stimulate preterm labor, infection can spread to the amniotic cavity, where additional inflammatory mediators are produced. Even in the case that there is no initiation of labor, the last point of infection spreading would be the fetus that will induce systemic inflammatory response syndrome, and further increase inflammatory

cytokine production [83]. IL-1 beta was the first cytokine to be implicated in preterm labor related to infection [83]. Numerous publications support the notion that IL-1 beta is involved in preterm parturition. Under normal conditions, IL-1 beta is involved in prostaglandin production and myometrial contraction stimulation which both lead to parturition. In addition, in 1989 it was determined that in response to bacterial products, human decidual produces IL-1 beta [92] and that in the amniotic fluid of women with preterm labor and infection, the concentration and bioavailability of IL-1 beta is increased [93], suggesting faster stimulation of labor with the presence of infection. Moreover, administration of IL-1 beta to pregnant mice induce preterm labor and birth [94], an effect that could be blocked by administration of the IL-1 beta receptor antagonist, IL-1ra [95]. TNF-alpha also plays a role in preterm labor related to infection. Like in the case of IL-1 beta, TNF-alpha is produced by human decidua, among other tissues, in response to bacterial products [96], and its concentrations in amniotic fluid are also elevated in women in preterm labor with intra-amniotic infection [97]. In addition, in guinea pigs, the local application to the cervix of TNF-alpha alone, or with IL-1 beta induces cervical ripening [30]. Finally, a study examining IL-1 and TNF receptor double knockout mice confirmed that IL-1 and TNF play important roles in preterm infection induced parturition, as the rates of preterm birth were significantly lower in double knockout mice stimulated with *E. coli* before gestation [74].

C. Transcription Factors

C.1 Basic-Leucine-Zipper (bZip) Transcription Factors

The first protein with a leucine zipper structure was described more than 20 years ago as a factor binding to enhancer core and CCAAT box motif in various gene promoters and was named C/EBP [98]. Later, many other proteins were identified possessing a common structure to the leucine zipper [99]. In 1992 the crystal structure of the first bZip protein was reported for GCN4 yeast transcriptional activator [100]. The basic leucine zipper region comprises two distinct regions – heptad repeats that mediate the dimer formation between two α-helix motifs and the N-terminal basic region that interacts with the DNA major groove [101]. Different families belong to the bZIP superfamily of transcription factors including AP-1, ATF/CREB, C/EBP, CNC, MAF, and PAR. All members of the families are summarized (Figure 3) [102] . Among the early classifications of the family members were the Jun/Fos and the ATF/CREB families. They were first considered as homogs binding to their respective DNA sequences and therefore classified by their preferential binding sites: the Jun/Fos (AP-1 factor) was claimed to possess higher affinity to the 12-O-tetradecanoate 13-acetate (TPA) responsive element (TRE); while the ATF/CREB family preferentially recognize (CRE) binding sites. It was later determined that factors that are part of these families could form intrafamily (homodimers) as well as interfamily (heterodimers) interactions and hence depending on the dimerization couple, interaction with either of these sequences is possible [103]. A wide range of possible interactions exit between different bZIP factors.

Figure 3

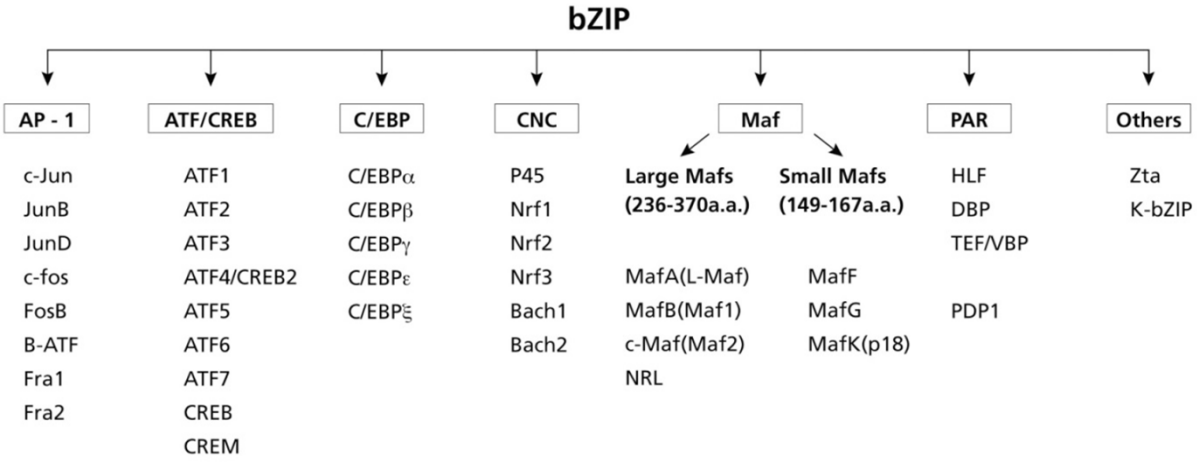


Figure 3: Overview of the bZIP superfamily of transcription factors: Subfamily members of the bZIP superfamily of transcription. Reproduced with permission from (102).

C.2 The MAF Family

The founding member of the MAF family, the *v-maf* oncogene, was discovered as the transforming retrovirus AS42 isolated from chicken spontaneous musculoaponeurotic fibrosarcoma [104]. The cellular counterpart of the viral oncogene, *c-maf*, was classified as a basic leucine zipper (bZip) transcription factor family [105]. Other homologues proteins were later found and the MAF family was expanded into two subgroups – large and small MAFs. Large MAFs include c-MAF [105], MAFA [106], Kreisler/MAFB [107] and NRL [108] while small MAFs are composed of 3 members, including MAFF, MAFK [109] and MAFG [110] (Figure 3). The difference between the two subgroups lies in the structure – large MAFs possess an N-terminus activation domain which is absent in small MAFs [111]. In addition to the leucine zipper domain that allows formation of homo/heterodimers, MAFs contain a basic DNA binding domain and a region called extended homology region [112] [113], the latter also interacting with DNA [114] (Figure 4). The individuality of the MAF family members' DNA binding domain lies in the ability of MAFs to recognize longer palindromic sequences than other bZIP factors [115, 116]. MAFs bind to MARE (Maf-recognition element) sites that are composed of the TRE/CRE core and flanked on both sides by GC (guanine/cytosine) elements [117]. The crystal structure of MAFG/DNA monomer led to the identification of crucial residues in the basic region domain – the Arg57 (arginine) and Asn61 (aspargine) that interact with the GC elements of MARE. Depending if a TRE or a CRE core is present, it is called either T-MARE or C-MARE. The CRE/TRE core of the MARE site can be more variable as long as the flanking GC sequences are conserved. Moreover, MAF proteins can bind to half-MARE sites if the sequence preceding this MARE is rich in A/T bases [118]. A visual representation of MAF binding sites is depicted (Figure 5).

Figure 4

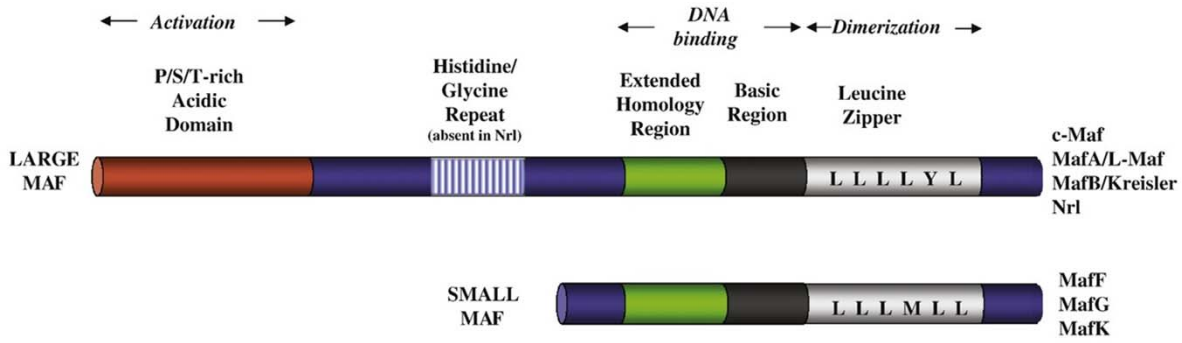


Figure 4: Structural representation of functional domains of a linearized large and small MAF protein sequences: Small and large MAFs share three homologous domains – a Leucine Zipper domain for dimerization, a basic region for DNA recognition and an Extended Homology Region for more specific interaction with DNA. Large MAFs also contain an acid transactivation domain on their N-terminus (red), and an additional Histidine/Glycine rich domain. Figure from (111).

Figure 5

TRE	TGACTCA
CRE	TGACGTCA
T-MARE	GCTGACTCAGC
C-MARE	GCTGACGTCAGC
Degenerate MARE	GCNNNNNNNNGC
Half MARE + A/T-rich	$(A/T)_N$GCNNNN

Figure 5: MAF binding sites: MAF-recognition elements (MAREs) sequences are composed of a TRE or CRE core, flanked on both sides by GC elements. MAFs can also bind to degenerate MAREs or half MAREs that are preceded by A/T rich sequence. Figure modified from (121).

C.3 Large MAFs

As mentioned before, large MAFs possess an N-terminal activation domain. They can activate transcription by recruiting p300, CBP (CREB binding protein) [119] or TBP (TATA box binding protein) [120] to gene promoters. They can form homodimers, or heterodimers with other bZIP factors, but they cannot heterodimerize with small MAFs [121]. The expression pattern of large MAFs is complex, they are expressed in spatial and time regulated manner in development [122]. They are also involved in the regulation of genes that play a role in terminal differentiation and they have been shown to be involved in human angioimmunoblastic T-cell lymphonas and in multiple myelomas [121].

C.4 Small MAFs

Small MAFs, MAFF, MAFG and MAFK are nuclear [123, 124] 18 kDa [113] bZIP factors without a trans-activational domain [109, 125]. Although lacking the ability to trans-activate genes, small MAFs are important proteins for cellular function – they been associated with hematopoiesis, CNS function, inflammation and most importantly with stress signaling [111]. Since their discovery, small MAFs have been linked to oncogenesis, and in recent years new pathologies such as diabetes, thrombocytopenia and neuronal diseases were found to be associated with aberrant small MAF expression [126].

C.4.1 Small MAF Interactome

Small MAFs interact with different proteins such as CNC (cap'n'collar) bZIP members p45 NFE2 [127], NFE2L1 [128], NFE2L2 [129], NFE2L3 [130, 131] and BACH1/2 [132]. They

also have the ability to interact with other bZIP proteins as well as non-bZIP factors [126]. A complete list of the small MAF interactome is shown (Table I). Small MAFs form homodimers with each other and by doing so, they are believed to repress transcription, possibly by competing with large MAFs for binding [133, 134]. By heterodimerizing with NFE2L2, MAFs have been found to regulate many ARE-dependant genes that are important for cellular response to chemical or oxidative stress [135-138] . Depending on the small MAF dimerization partner, DNA binding specificity is different for several dimer combinations, as determined by plasmon resonance-microarray [139]. Small MAFs, in combination with bZIP factors, bind MARE, NF-E2 (Transcription factor NF-E2 45 kDa subunit) binding elements, ARE (antioxiodant response element), StRE (stress response element) and EpRE (electrophile response element) [111, 134, 139].

C.4.2 Small MAF Expression

Small MAFs expression profiles have been examined. *MAFG, MAFK* and *MAFF* are expressed in many different tissues, but at different levels [109, 129]. For example *MAFG* mRNAs were most abundant in the brain, while *MAFK* was detected in higher levels in the placenta [129]. In fact, *MAFK* expression profiles were examined in developmental stages in more details and it was determined that it is tightly regulated in a space dependent and time dependent manner [140]. Studies of knockout mice provided additional insights into the function of small MAFs. Single knockouts of the MAFs did not show any phenotype except for a mild megakaryocytic phenotype in $MAFG^{-/-}$ [141-143] suggesting that other small MAFs can compensate for the loss of one. Double knockouts revealed that $MAFG^{-/-}/K^{-/-}$ exhibit many blood-related problems such as severe thrombocytopenia, anemia and red cell abnormalities [144]. Animals heterozygous for either *MAFG* or *MAFK* while homozygous for a null allele of the

Table I: Small MAF interactome: Small MAF proteins are known to interact with many bZIP factors as well as other proteins as summarized. Modified from Kannan, M. B., Solovieva, V., and Blank, V. (2012) The small MAF transcription factors MAFF, MAFG and MAFK: Current knowledge and perspectives. Bba-Mol Cell Res 1823, 1841-1846

Protein	Interaction with small MAF
Leucin zipper proteins	+
MAFF	+
MAFG	+
MAFK	+
NFE2 p45	+
NFE2L1	+
NFE2L2	+
NFE2L3	+
BACH1	+
BACH2	+
MIP	+
FOS	+
FOSB	+
JUN	-
MAFB	-
v-MAF	-
Non-leucine zipper proteins	
HIF1A	+
HOX12	+
MATIIA	+
PAX6	+
MHOX	+

other gene showed considerable improvement in their condition [144, 145]. Finally, triple knockouts revealed that MAFs are indispensible for murine embryonic development after E9.5. Mutant embryos developed normally until E9.5, but did not survive past E13.5 and had severe liver hypoplasia with reduced expression of cytoprotective genes that are under the control of AREs [146].

C.4.3 MAFF

The *MAFF* gene has been first isolated in 1993 by screening cDNA from chicken embryo fibroblasts [109]. The human gene consists of three exons and murine *MAFF* was found to have three different promoters about 8kbp upstream of the first exon. The expression levels of murine *MAFF* was quantified by qPCR and it was determined that lung tissue contain the highest abundance of *MAFF*, as well as *MAFG* and *MAFK* transcripts [141].

The human *MAFF* gene is expressed on chromosome 22 and has 4 different transcription variants – 1, 3, 4 and 5. All except variant 5 code for a 164 amino acid protein and is referred to as isoform a. The variant 5 codes a shorter protein at the N-terminus composed of 135 amino acids.

It was shown that the MAFF binds to the US-2 motif of the promoter of oxytocin [147]. The same US-2 (ggaatgattactcagctaga; corresponding to nucleotides -1433 to -1414 of the human OXTR gene) element from the *OXTR* promoter in a yeast two-hybrid screening was used to identify a novel MAFF interaction partner – MIP (MAFF interacting protein). The function of MIP has not been determined to this date, but it was shown that co-expression of MIP and MAFF could activate US-2-driven transcription in a reporter assay, suggesting that MIP is a co-activator

of MAFF. Moreover, interaction of MIP with MAFF through its coiled-coil domain caused translocation of MIP from the cytoplasm into the nucleolus of HELA cells [148, 149].

D. Hypothesis/Rationale

The MAFF transcription factor has previously been linked to human pregnancy, as it was shown that its transcripts are highly expressed in term myometrium obtained from delivery patient, but not in samples of non-pregnant or early gestation period myometrium. Furthermore, a yeast one-hybrid screen assay has been performed using a US-2 element from *OXTR* promoter as bait. They identified a 2.3 kb clone encoding MAFF protein [147]. An additional link of MAFF to the process of parturition was revealed by research on inflammatory cytokines. Our laboratory showed that MAFF transcript and protein levels are upregulated by the cytokines IL-1 beta and TNF in a time and dose dependant manner in PHM1-31 myometrial cells. Moreover, MAFF was the only member of the small MAF family to be upregulated by these cytokines.

We therefore decided to investigate MAFF's role in myometrial cell function as we hypothesized that it might play a role in parturition, specifically when upregulated by inflammatory cytokines.

D.1 Specific Research Aims

The objectives of the following research project were to assess MAFF-dependent gene regulation in myometrium using a cell model. To attain the objective, we used the following approaches in consecutive steps:

1) Assessment of optimal conditions for *MAFF* knockdown as well as concentration and time points of MAFF induction by the cytokine IL-1 beta in the hTERT-C3 myometrial cell line.
2) RNA-sequencing of hTERT-C3 myometrial cells comparing cells transfected with negative control (scrambled) siRNA or *MAFF*-specific siRNA, both in the presence or absence of IL-1 beta.
3) Bioinformatics analysis of gene expression and comparisons between conditions to identify differential gene expression to determine a) genes dependent on the presence of MAFF b) genes responsive to IL-1 beta and c) genes modulated by MAFF in response to IL-1 beta treatment.
4) Follow up studies to determine important genes regulated by MAFF in myometrial cells.

D.2 Cell model – immortalized Myometrial Cells

Immortalized human myometrial cells, hTERT-C3 (provided by Dr. Zingg, McGill University) were used for the project. Generation of hTERT-C3 was described previously [150, 151]: a piece of the anterior wall of the uterine fundus from women undergoing hysterectomy was obtained and retroviral vector containing the catalytic subunit of telomerase was used to infect the cells. Selection of colonies was made by puromycin antibiotic [150]. A cell population from a mix of the colonies was propagated and a subclone 3 of the selected population was made by serial dilutions [152]. Original colonies were verified for smooth muscle expression, and smooth muscle specific proteins calponon, *h*-caldesmon and alpha-actin were found to be expressed [150].

MATERIALS AND METHODS

A. Cell Culture and Treatment Conditions

Generation of hTERT-C3 myometrial cells (provided by Dr Zingg, McGill University) was described previously [150, 151]. Human embryonic kidney 293T cells were obtained from ATCC. All cells were cultured in 5% CO_2 at 37°C using DMEM/F12 media (Invitrogen, 11330-057) for myometrial cells and DMEM media for 293T (Invitrogen, 11965-118) containing 10% fetal bovine serum (Invitrogen, 12483-020) and 2% antibiotic-antimycotic solution containing 5000 U/ml penicillin and 5000 U/ml streptomycin (Invitrogen, 15070-063). Myometrial cells were passaged using 0.05% trypsin-EDTA (Invitrogen, 25300-054) and 293T cells using 0.25% trypsin-EDTA (Invitrogen, 25200-056).

Treatment with recombinant human IL-1 beta cytokine (Invitrogen, PHC0816) was performed in DMEM/F12 (10% FBS, 2% antibiotic-antimycotic) with cells reaching 80-90% confluency. For time course studies, we used 10ng/ml IL-1 beta and collected the cells by scraping in PBS after 1, 2, 3, 4, 8 or 24 hours of treatment.

293T human embryonic kidney cells were transfected using calcium phosphate co-precipitation method. Cells were plated at 30-50% confluency 24 hours prior to transfection. On transfection day, the basic pGL3 reporter vector (Promega, E1751) containing *PTGS2* (−2390 to + 34) promoter, pRL reporter vector containing the cDNA for *Renilla* luciferase under the control of a TK promoter (Promega, E2241), and increasing concentrations of pcDNA3.1 containing MAFF coding regions were mixed in water to a total volume of 210µL. 30µl of 2M $CaCl_2$ was added to the mixture and the mixture was vortexed. 240µL of 2x HeBS were then added drop-wise prior to a 20 minute incubation at room temperature. Following the incubation, the solution was added to the respective 60mm dish.

The basic pGL3 plasmid containing a 2kb fragment of the *PTGS2* promoter was kindly provided by Dr. John A. Di Battista from McGill University [153].

B. siRNA mediated Knockdown

For inhibition of MAFF expression, hTERT-C3 cells were transfected by electroporation with 10nM *MAFF* siRNA #7 (Qiagen, SI03026611), 10nM *MAFF* siRNA #9 (Qiagen, SI03116456), or the negative control scrambled siRNA (Qiagen, 1022076) using Amaxa Basic Nucleofector Kit for primary mammalian smooth muscle cells (Lonza, VPI-1004). Cells were detached from plates, pooled and counted using Trypan blue (Invitrogen, 15250-061) and a hematocytometer. Transfections of $2x10^6$ cells in 100 μL of nucleofector solution mix with 10nM scrambled siRNA or 10nM of *MAFF* siRNA were performed by electroporation using an Amaxa instrument (program D-033). Transfected cells were transferred to dishes containing freshly prepared and pre-heated DMEM/F12 (10%FBS, 2% antibiotic-antimycotic). For the RNA-seq experiments, two sets of 6 cm dishes were transfected with either scrambled or MAFF siRNA #7 were prepared. After 24h, media was removed and new media (10% FBS; 2% antibiotic-antimycotic) was added. One set (scrambled and *MAFF* siRNA #7) was treated with 10ng/ml IL-1 beta, the other set was not treated. Cells were collected 3h post IL-1 beta treatment in TRIzol Reagent (Invitrogen, 15596-018). Three independent experiments were performed for RNA-seq.

C. RNA sequencing

RNA-sequencing was performed at the McGill University and Génome Québec Innovation Centre, Montréal, Québec. Cells in presence/absence of *MAFF* siRNA and presence/absence of 10ng/ml IL-1 beta were collected for preparation of RNA using TRIzol Reagent following the instructions by the manufacturer. The concentration and integrity of the RNA was determined using a Bioanalyzer. RIN (RNA Integrity Number) values were obtained for each sample. Samples with RIN values > 8.5 were selected and RNA from three independent experiments was analyzed by high-throughput sequencing at the McGill University and Génome Québec Innovation Centre. TruSeq RNA Sample Preparation Kit was used to prepare the mRNA-Seq libraries: mRNA was purified from total RNA, fragmented into smaller pieces, converted to doublestranded cDNA, purified, subjected to end-repair and phosphorylation and finally 3'adenylated with adenosine for adapter-ligation. To enrich the adaptor-ligated cDNA, PCR was used to amplify the library, followed by quality control on Bioanalyzer. Quantitative PCR was then performed to quantify the library and equal ratios of each library were used on Illumina HiSeq 2000 platform with 100 Paired End cycles and 4 samples per lane.

D. Bioinformatics and Statistical Analysis of RNA-sequencing Data

Depending on the sample, sequencing of the libraries generated between 47 to 65 million raw paired-end reads of 100 bp length. The raw read-outs were filtered by trimming reads for quality and length using fastx software. Subsequently, a Tuxedo protocol [154] was used to obtain expression levels of different genes. Sequence aligment of the reads was performed against the Ensembl Homo Sapiens reference transcriptome using Tophat software (Appendix

Table I). Mapped reads were assembled into transcripts with Cufflinks and then merged into longer transcripts with Cuffmerge. In order for the samples to be comparable, two important corrections were made – the normalization for the transcript length, and the normalization for the total yield of the sample. To obtain differential gene expression, samples were compared using the Cuffdiff program which also provides statistical significance between the compared samples [154]. The boxplots and heat maps were realized using R software, ggplot2 and pheatmap packages.

To determine differentially expressed genes, comparisons were made between the following: 1) scrambled (sc) siRNA,-IL-1 beta versus *MAFF* siRNA,-IL-1 beta (transcripts modulated by MAFF); 2) sc siRNA,+IL-1 beta versus *MAFF* siRNA,+IL-1 beta (transcripts modulated by IL-1 beta in the presence of MAFF); 3) sc siRNA,-IL-1 beta versus sc siRNA,+IL-1 beta (transcripts modulated by IL-1 beta); 4) *MAFF* siRNA,-IL-1 beta versus *MAFF* siRNA,+IL-1 beta (transcripts modulated by IL-1 beta in absence of MAFF).

E. Quantitive PCR

Following RNA extraction using TRIzol, DNase I (Roche, 04 716 728 001) digestion was performed using 2 units per 10µg RNA followed by reverse transcription of 3µg total RNA using oligo[59] and random hexamer primers following the instructions of the manufacturer using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Transcript levels were measured by real-time quantitive polymerase chain reaction (qPCR) using LightCycler FastStart DNA Master SYBR Green I for PTGS2 TIMP3, THBS1 and 18S using 5µl of cDNA in a total of 20µl reaction mixture. Primers were selected to prevent genomic amplification and were used at a

final concentration of 0.2pmol/μL. The following primer sets (left primer, right primer) were used: PTGS2 (5'- CGAGGTGTATGTATGAGTGTG -3', 5'-TCTAGCCAGAGTTTCACCGTA -3'), TIMP3 (5'-GTGCAACTTCGTGGAGAGGT-3', 5'-AGCAGGACTTGATCTTGCAGT-3'), THBS1 (5'- CCACATTCAGGAGTGTGACAA 3', 5'-AGAGCCGGATCCTTGTGAT-3'), 18S (5'- CTCAACACGGGAAACCTCAC -3', 5'-CGCTCCACCAACTAAGAACG -3').

The internal control 18S amplification was performed with a first step of 10 minutes at 95ºC followed by 40 cycles of 10 seconds at 95ºC, 10 seconds at 60ºC and 5 seconds at 72ºC. PCR cycles for target genes were as follows: PTGS2 (30 seconds at 95ºC, 30 seconds at 58ºC and 60 seconds at 72ºC); THBS1 (10 seconds at 95ºC, 5 seconds at 56ºC and 5 seconds at 72ºC) and TIMP3 (10 seconds at 95ºC, 10 seconds at 59ºC and 5 seconds at 72ºC). Cycle threshold values of genes were normalized against the internal control 18S rRNA. Each experiment was performed independantly at least three times, samples were run in duplicates and data were analyzed using the LightCycler Relative Quantification Software.

F. Luciferase Reporter Assay

F.1 Human Myometrial hTERT-C3 Cells

Dual Luciferase Reporter Assay (Promega, E1980) was performed by co-transfecting ~2,000,000 hTERT-C3 cells by electroporation (Amaxa technologies described above) with 500ng of basic pGL3 plasmid containing the *PTGS2* promoter with negative control (scrambled) or *MAFF* siRNA. Transfection efficiencies were controlled by co-transfecting 20ng of pRL-TK

Renilla. For MAFF knockdown 10nM *MAFF* siRNA #7 (Qiagen, SI03026611) or 10nM *MAFF* siRNA #9 (Qiagen, SI0311646) were used.

Luciferase values were measured as relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold Technologies), normalized to pRL-TK *Renilla* luminescence as an internal control.

F.2 Human Kidney Embryonic 293T Cells

Dual luciferase reporter assay on 293T cells was performed by co-transfecting ~500,000 cells by calcium phosphate method with 350ng of basic pGL3 plasmid containing the *PTGS2* promoter and increasing amounts of pcDNA3.1 containing *MAFF* coding region. The amount of pcDNA3.1 with *MAFF* used were 0, 1, 10, 100 ng. Empty pcDNA3.1 of 100, 99, 90 and 0 ng was used to balance total quantities of the vector. Transfection efficiencies were controlled by co-transfecting 14ng of pRL reporter vector containing the cDNA for *Renilla* luciferase under the control of a TK promoter.

G. Cell lysis and Immunoblot Analysis

Whole-cell extracts were prepared by scraping cells using 1xPBS and cells were lysed for 10 minutes in whole-cell lysis buffer (10 mM Tris-HCl pH 8.0, 420 mM NaCl, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton-X100) supplemented with Complete protease inhibitor cocktail (Roche, 04 693 116 001), and then centrifuged at 13,000 rpm for 10 minutes at 4ºC. Supernatants were collected and protein concentrations determined using a protein assay kit (Bio-Rad, 500-0006). 20 to 30µg of the total protein lysate were separated by electrophoresis on

a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (PVDF) (Millipore). Blots were blocked using 5% milk in TBST (500mM Tris pH 7.6, 2M NaCl, 0.5% Tween) at room temperature for at least 1h and then incubated overnight at 4°C with primary antibodies specific for MAFF (1:1000) [155], GAPDH (1:450 000; Research Diagnostics Inc, RDI-TRK5G4-6C5) or PTGS2 (1:200; Cayman Chemicals, 160107) proteins. Horseradish-peroxidase-conjugated antibodies were used for 1h at room temperature. A goat anti-mouse secondary (1:40 000; Thermo Scientific, 31430) was used to detect GAPDH; a goat anti-rabbit antibody (1:40 000; Thermo Scientific, 31460) was used to detect MAFF, PTGS-2 and a rabbit anti-goat antibody (1:40 000, Thermo Scientific, 31402) to detect LDH-A. The antigen-antibody complexes were visualized using the chemiluminescent HRP substrate (Millipore, WBKLS0500) following the manufacturer's instructions and exposed to Hyperfilm (GE Healthcare, 28-9068-35). Quantification of immunoblot experiments was performed using Image Lab.

H. Statistical Analysis

Data are presented as mean ± S.E.M. Differences between experiments were calculated using a two-tailed Student's *t*-test, for at least 3 independent experiments and statistically significant data are indicated (one asterisk indicates $p < 0.05$, two asterisks, $p<0.01$).

RESULTS

A. Optimization of MAFF Induction and Knockout Conditions

A.1 siRNA-mediated Knockdown of MAFF in Myomtrial Cells

We first optimized transient transfection conditions of hTERT-C3 myometrial cells with *MAFF* siRNA. Three commercially available siRNAs against *MAFF* were selected and tested, #6, #7 and #9. The siRNA sequences were analyzed using the BLAST (NCBI) program to ensure that there is no unspecific binding. We found that 10nM of *MAFF* siRNA under the same conditions generated ~ 90% knockdown with all siRNAs (Figure 6). MAFF siRNA #7 yielded the best knockdown effect and was selected for further experiments (RNA-seq and validation of RNA-seq); the other experiments were done with 2 different ones, 7 and 9).

A.2 Induction of MAFF Protein Levels by IL-1 beta in hTERT-C3 Myometrial Cells

It was previously shown that proinflammatory cytokines have an effect on MAFF levels in human myometrial cells [155, 156]. We examined whether MAFF expression is modulated by IL-1 beta in hTERT-C3 myometrial cells. We found a dose-dependent regulation of MAFF protein levels in hTERT-C3 cells, with expression levels peaking and reaching a plateau at 1ng/ml of IL-1 beta (Figure 7). Our time course studies showed that induction of MAFF transcription factor is visible starting from 2 hours, reaching maximal levels at 3-4 hours following exposure to IL-1 beta (Figure 8).

To assess MAFF dependent gene regulation in myometrial cells, we performed transfections with either scrambled or *MAFF*#7 siRNA, both in the presence or in the absence of

Figure 6

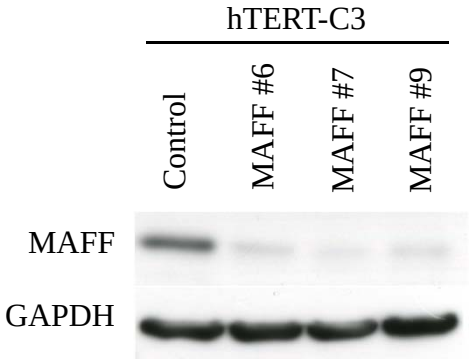


Figure 6: Standardization of knock down of MAFF protein levels in hTERT-C3 myometrial cells by transfection of *MAFF* siRNAs complementary to three different regions Western blot analysis of total protein extract from hTERT-C3 cells transfected with either scrambled siRNA (negative control) or *MAFF* siRNA #6, 7, or 9 at a concentration of 10nM for 24h. 25 micrograms of total protein extract per lane were loaded. MAFF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) proteins are indicated. The data shown are representative of two independent experiments.

Figure 7

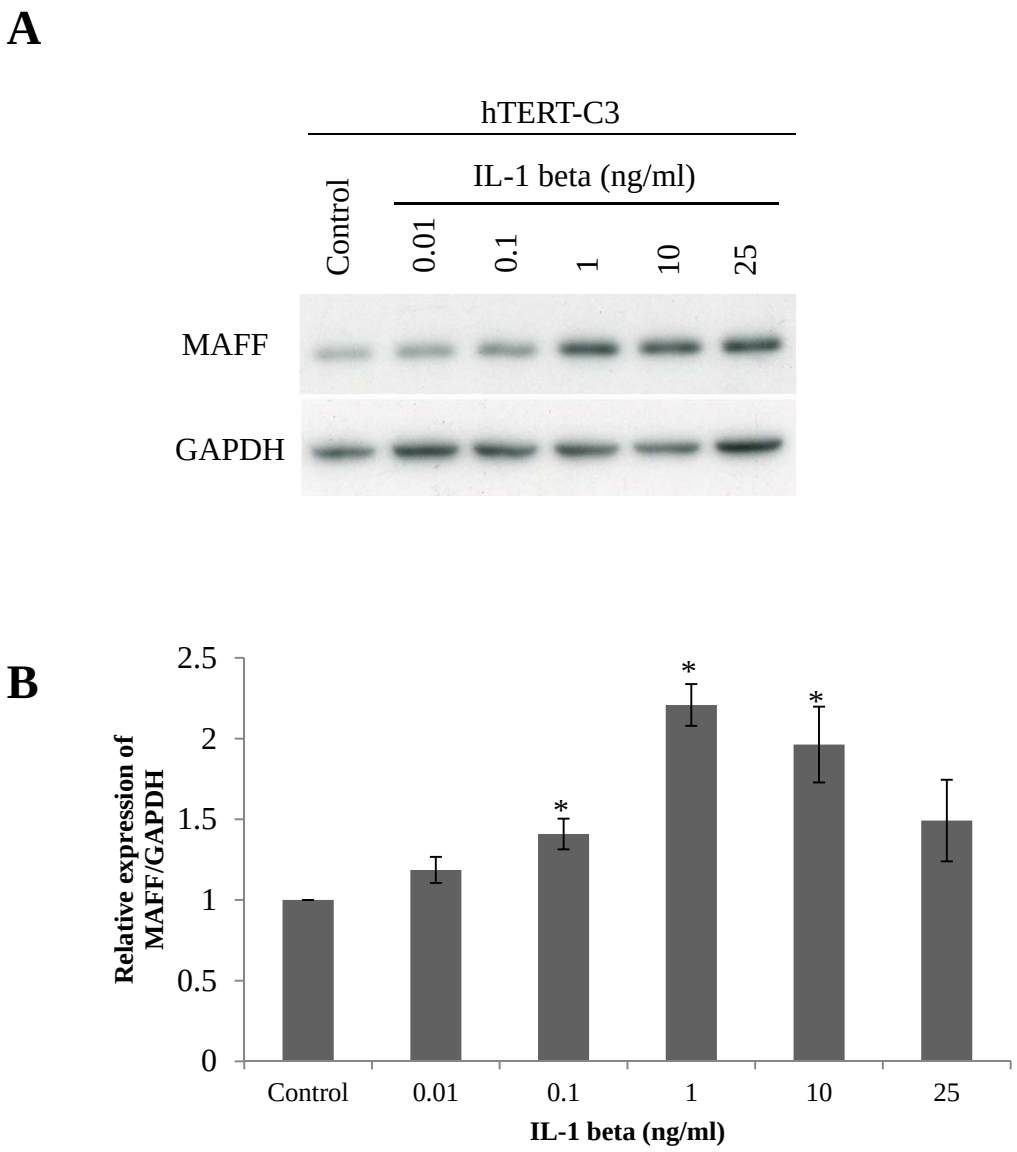
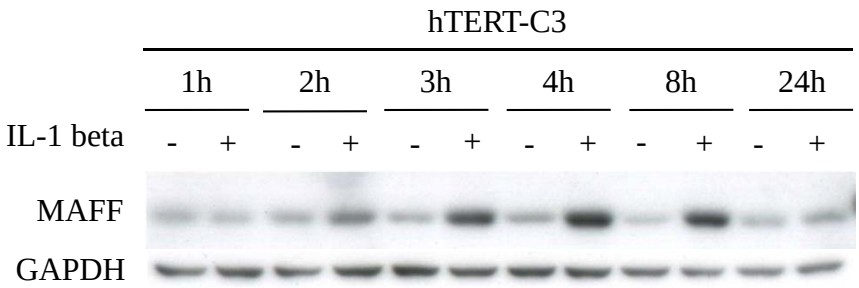


Figure 7: Dose dependent upregulation of MAFF protein levels in hTERT-C3 myometrial cells by IL-1 beta. A) MAFF protein levels in response to IL-1 beta (0.01-25ng/ml) treatment. 25 micrograms of total protein per lane was loaded. MAFF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) proteins are indicated. The data shown are representative of three independent experiments. B) Quantification of three different experiments is shown. Error bars represent standard error of the mean (S.E.M) and statistically significant differences are indicated by an asterisks ($P<0.05$).

Figure 8

A


hTERT-C3
1h
2h
3h
4h
8h
24h
IL-1 beta
- + - + - + - + - + - +
MAFF
GAPDH

B

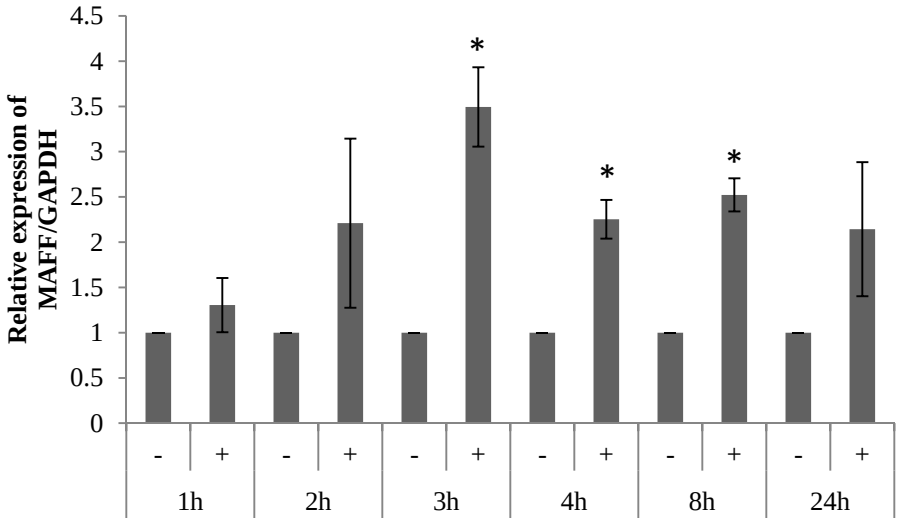

Relative expression of MAFF/GAPDH
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
- + - + - + - + - + - +
1h
2h
3h
4h
8h
24h

Figure 8: Time-dependent upregulation of MAFF protein levels in hTERT-C3 myometrial cells by IL-1 beta. A) Western blot analysis of total protein extract from hTERT-C3 cells induced with 10ng/ml IL-1 beta for 1h, 2h, 3h, 4h, 8h and 24h. 25 micrograms of total protein extract per lane were loaded. MAFF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) proteins are indicated. B) Quantification of three different experiments is shown. Error bars represent standard error of the mean (S.E.M) and statistically significant differences are indicated by an asterisks ($P<0.05$).

IL-1 beta and subsequent RNA-sequencing (RNA-seq) analysis. Prior to RNA analysis, the knockdown of *MAFF* by siRNA and its induction by IL-1 beta were verified at the protein level (Figure 9).

B. MAFF and IL-1 beta dependent Transcripts in Myometrial Cells

The comparisons between different conditions allowed us to determine 1) transcripts regulated by MAFF transcription factor in the presence or absence of IL-1 beta, by comparing scrambled to *MAFF* transfected siRNAs; 2) transcripts regulated by treatment of myometrial cells with 10ng/ml IL-1 beta for 3h, by comparing scrambled-transfected with no IL-1 beta induction to scrambled-transfected induced with IL-1 beta and 3) transcripts regulated by MAFF in response to IL-1 beta induction, by comparing the differentially expressed genes between the above mentioned two groups.

B.1 MAFF dependent Transcripts

To verify our hypothesis that the MAFF transcription factor is involved in uterine gene regulation at the onset of parturition we transfected myometrial cells with scrambled or *MAFF* siRNA by electroporation. Using RNA-sequencing and differential transcript expression analysis, we identified transcripts that were differentially expressed in the absence of MAFF transcription factor mediated by *MAFF*-siRNA knockdown. Three independent experiments were carried out and analyzed. The genes whose transcripts are modulated by two fold or more (total of 27 genes) upon *MAFF* knockdown are listed (Table II, appendix Figure 1). We used Ingenuity Pathway Analysis software to assess the biological functions and pathways of the

Figure 9

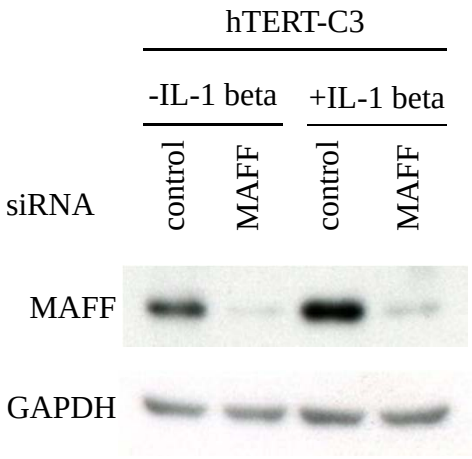


Figure 9: siRNA mediated knockdown of MAFF protein levels in the presence or absence of IL1B. MAFF protein expression in hTERT-C3 myometrial cells transfected for 24h with scrambled or MAFF siRNA in the presence of 10ng/ml IL-1 beta or absence of IL-1 beta (for 3h). 30 micrograms of total protein extract were loaded in each lane. MAFF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) proteins are indicated. The data shown are representative of three independent experiments.

Table II: Differentially expressed transcripts of genes that are modulated by two-fold or more in the absence of MAFF transcription factor (mediated by MAFF siRNA knockdown). Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included. P-value and false-discover for knockdown versus control conditions are 0.05 or less.

Symbol	Fold Change	Gene Description
KIAA1199	3.73	colon cancer secreted protein 1
PTGS2	3.45	prostaglandin-endoperoxide synthase 2
SLC7A8	2.66	solute carrier family 7 (amino acid transporter, L-type), member 8
GDF15	2.53	growth differentiation factor 15
IL36RN	2.39	interleukin 36 receptor antagonist
LTB	2.11	lymphotoxin beta
DDIT4	2.08	DNA-damage-inducible transcript 4
MGC12916	2.06	uncharacterized protein
KCNN3	-2.03	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3
ADCY3	-2.07	adenylate cyclase 3
COPB1	-2.09	coatomer protein complex, subunit beta 1
HMGCS1	-2.14	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
TGFB2	-2.14	transforming growth factor, beta 2
FAM83A	-2.17	family with sequence similarity 83, member A
MYPN	-2.25	myopalladin
NEURL1B	-2.26	neuralized homolog 1B
RCN2	-2.28	reticulocalbin 2, EF-hand calcium binding domain
BORA	-2.29	bora, aurora kinase A activator
SYT1	-2.33	synaptotagmin I
KLF17	-2.43	Kruppel-like factor 17
TXNDC12	-2.49	thioredoxin domain containing 12 (endoplasmic reticulum)
CRKL	-2.58	v-crk sarcoma virus CT10 oncogene homolog (avian)-like
LMAN2	-2.83	lectin, mannose-binding 2
TOP2A	-2.84	topoisomerase (DNA) II alpha 170kDa
GALNT1	-2.87	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)
SCN4B	-3.23	sodium channel, voltage-gated, type IV, beta subunit
MAFF	-5.53	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)

identified genes. We performed Ingenuity knowledge base canonical pathway analysis for association of the biological functions of involved with the knockdown of *MAFF*. The dataset used to carry out IPA analysis comprised 880 genes that were upregulated or downregulated by 1.3 fold or more in the absence of MAFF. To determine the significance of the association Fisher's Exact test was performed by the IPA software and only statistically significant groups are mentioned. With respect to MAFF dependent transcripts, we found that in the absence of MAFF, the gene products that are upregulated, participate in the pathways that are involved in inhibition of matrix metalloproteinases, PPARalpha/RXRalpha activation, cell cycle damage checkpoint regulation and the modulation of cell cycle control of chromosomal replication among many other pathwas (Figure 10). The top pathways modulated by MAFF as well as gene products known to be involved in these pathways are shown (Table III).

B.2 IL-1 beta dependent Transcripts

It was previously shown that myometrial cells are responsive to IL-1 beta [155, 156]. As part of the experimental setup, we had a set of myometrial cells transfected with negative control (scrambled) siRNA that were treated or not with IL-1 beta, allowing us to analyze genes modulated by cytokine treatment. As previously mentioned, three independent experiments were analyzed and only statistically significant transcripts were retained. We found that 74 gene-corresponding transcripts are regulated by IL-1 beta by at least 4 fold and expressed by at least 2 cpms (counts per million) are shown (Table IV, appendix Figure 2). The transcript levels of an additional 1043 genes were modulated by the cytokine from a range of 1.3 to 4 fold (data not shown). Ingenuity analysis of the entire dataset of 1114 genes (71 + 1043) revealed gene

Figure 10

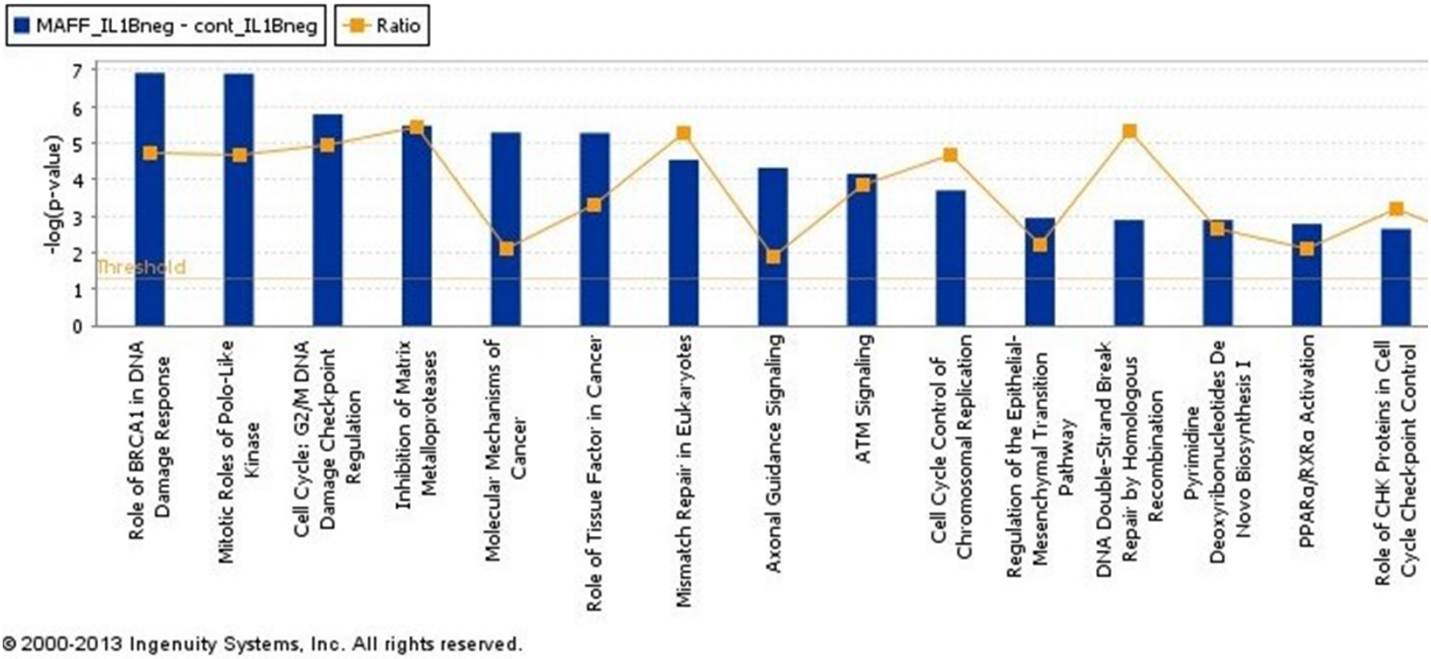


Figure 10: Ingenuity canonical pathway analysis of biological functions associated with genes differentially expressed in hTERT-C3 cells in the absence of the MAFF transcription factor. Bar graph representing the top pathways associated with the genes modulated by absence MAFF transcription factor. The orange line shows the ratio of number of gene products that are modulated by MAFF transcription factor to the total number of molecules known to be present in the pathway. Threshold line is set at a p-value of 0.05.

Table III: Ingenuity canonical pathway analysis of biological functions associated with genes differentially expressed in hTERT-C3 cells in the absence of the MAFF transcription factor. List of molecules involved in the top pathways.

Ingenuity Canonical Pathways	Molecules Involved
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	CDC25C, CKS2, CCNB2, PLK1, CDK1, CCNB1, CDC25B, CKS1B, TOP2A, PKMYT1, BTRC, BRCA1, CHEK2
Inhibition of Matrix Metalloproteases	TIMP3, ADAM17, SDC2, MMP16, MMP14, MMP15, THBS2, TFPI2, MMP17, LRP1, MMP1, MMP19
Mismatch Repair in Eukaryotes	PCNA, RFC4, RFC2, FEN1, MLH1, EXO1, RFC3
ATM Signaling	CDC25C, CCNB2, CBX5, MAPK11, CDK1, CCNB1, RAD51, SMC2, FANCD2, H2AFX, BLM, BRCA1, CHEK2
Cell Cycle Control of Chromosomal Replication	MCM6, CDC45, ORC2, CDC6, CDC7, ORC6, CHEK2, DBF4
Regulation of the Epithelial-Mesenchymal Transition Pathway	ID2, ADAM17, MAP2K7, FGF2, EGR1, NFKB2, FGF1, PIK3R3, FZD8, CDH2, FZD4, RRAS2, WNT7B, TGFB2, FZD6, AKT3, JAG1, NOTCH1, FGF7, WNT5B, TCF7L2, WNT5A, FGF5
DNA Double-Strand Break Repair by Homologous Recombination	RAD51, GEN1, POLA1, BRCA2, BRCA1
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	TYMS, DUT, RRM2, DTYMK, RRM1
PPARα/RXRα Activation	MAP2K7, PRKAB2, ADCY3, GNAQ, CKAP5, PRKAR2A, ADCY6, PLCG1, NFKB2, GK, ABCA1, ACVR1B, RRAS2, PLCE1, HELZ2, IRS1, PRKACA, TGFB2, PRKAG2, PLCB1, IL1B, SLC27A1
Role of CHK Proteins in Cell Cycle Checkpoint Control	PCNA, CDC25C, RFC4, RFC2, CLSPN, PLK1, BRCA1, CDK1, CHEK2, RFC3

Table IV: Differentially expressed transcripts of genes that are modulated by 4-fold or more in response to IL-1 beta treatment. Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included. P-value and false-discover for control versus IL-1 beta induction conditions are 0.05 or less.

Symbol	Fold Change	Gene Description
SLC12A7	151.06	solute Carrier Family 12 (Potassium/Chloride Transporters), Member 7
OLR1	129.71	oxidized low density lipoprotein (lectin-like) receptor 1
CSF2	62.72	colony stimulating factor 2 (granulocyte-macrophage)
CXCL2	50.47	chemokine (C-X-C motif) ligand 2
CCL2	44.78	chemokine (C-C motif) ligand 2
TNF	38.71	tumor necrosis factor
EFNA1	35.33	ephrin-A1
CSF3	34.39	colony stimulating factor 3 (granulocyte)
IL36G	33.84	interleukin 36, gamma
SLC7A2	32.33	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
CXCL3	31.95	chemokine (C-X-C motif) ligand 3
PTX3	30.84	pentraxin 3, long
TRAF1	30.21	TNF receptor-associated factor 1
IL6	27.18	interleukin 6 (interferon, beta 2)
C8orf4	24.42	chromosome 8 open reading frame 4
NR4A3	21.37	nuclear receptor subfamily 4, group A, member 3
TNFAIP6	21.03	tumor necrosis factor, alpha-induced protein 6
CXCL1	20.42	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
TNFAIP3	19.95	tumor necrosis factor, alpha-induced protein 3
BIRC3	19.12	baculoviral IAP repeat containing 3
RHCG	18.72	Rh family, C glycoprotein
LIF	18.56	leukemia inhibitory factor
CCL20	18.45	chemokine (C-C motif) ligand 20
IL1A	15.32	interleukin 1, alpha
RCSD1	14.69	RCSD domain containing 1
ADORA2A	14.28	adenosine A2a receptor
TSLP	12.26	thymic stromal lymphopoietin
ICAM4	12.10	intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)
IL8	12.02	interleukin 8
MMP12	11.98	matrix metallopeptidase 12 (macrophage elastase)
PTGS2	10.78	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)

IL23A	9.98	interleukin 23, alpha subunit p19
TNFAIP2	9.67	tumor necrosis factor, alpha-induced protein 2
IL1B	9.58	interleukin 1, beta
ELOVL7	9.30	ELOVL fatty acid elongase 7
NFKBIZ	9.15	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NFKBIA	8.88	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
MRGPRX3	8.55	MAS-related GPR, member X3
RNF144B	8.40	ring finger protein 144B
ZC3H12A	8.31	zinc finger CCCH-type containing 12A
TNIP3	8.31	TNFAIP3 interacting protein 3
SERPINB4	8.21	serpin peptidase inhibitor, clade B (ovalbumin), member 4
PPAP2B	7.51	phosphatidic acid phosphatase type 2B
ITGB8	6.86	integrin, beta 8
ICAM1	6.70	intercellular adhesion molecule 1
LTB	6.60	lymphotoxin beta (TNF superfamily, member 3)
BCL2A1	6.56	BCL2-related protein A1
SOD2	6.51	superoxide dismutase 2, mitochondrial
IER3	6.51	immediate early response 3
IRAK2	6.21	interleukin-1 receptor-associated kinase 2
MAP3K8	6.11	mitogen-activated protein kinase kinase kinase 8
CEBPD	6.07	CCAAT/enhancer binding protein (C/EBP), delta
C11orf96	5.73	chromosome 11 open reading frame 96
NFKB1	5.56	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
PPP4R4	5.52	protein phosphatase 4, regulatory subunit 4
TNFAIP8	5.40	tumor necrosis factor, alpha-induced protein 8
RBM20	5.26	RNA binding motif protein 20
MCF2L2	4.89	MCF.2 cell line derived transforming sequence-like 2
TNFRSF9	4.84	tumor necrosis factor receptor superfamily, member 9
EDNRB	4.70	endothelin receptor type B
EBF1	4.59	early B-cell factor 1
GCH1	4.40	GTP cyclohydrolase 1
C15orf48	4.33	chromosome 15 open reading frame 48
NKX3-1	4.31	NK3 homeobox 1
PRDM1	4.26	PR domain containing 1, with ZNF domain
STX11	4.25	syntaxin 11
INHBA	4.12	inhibin, beta A
TCTEX1D4	4.09	Tctex1 domain containing 4
MIR143HG	-4.21	MIR143 host gene (non-protein coding)
RASD2	-4.57	RASD family, member 2

products participating in pathways such as NFKB, TNFR2 and TWEAK death receptor signaling (Figure 11). A more complete list of the pathways is illustrated (Table V).

B.3 Transcripts modulated by MAFF in Response to IL-1 beta Induction

Finally, to uncover genes regulated by the MAFF transcription factor in response to IL-1 beta, we compared the differentially expressed genes between the group of genes that were modulated by *MAFF* absence and the group of genes that were induced by IL-1 beta treatment of myometrial cells. We report here 20 genes that are upregulated or downregulated by at least 1.5 fold (Table VI, appendix Figure 3).

C. Genes modulated by MAFF Transcription Factor

We selected genes identified in our RNA-seq experiment for further analysis as they have been shown to be either directly or indirectly involved in the processes prior to parturition. Boxplots $\log_2$ of counts per million reads of *PTGS2, TIMP3* and *THBS1* and *PGRMC1* in control versus *MAFF* siRNA conditions are shown (Figure 12) and the corresponding fold change represented in the boxplots is shown (Figure 12E). To confirm results obtained in the RNA-seq experiment, we assessed the expression levels of *PTGS2,* and *THBS1* by quantitative PCR using the same biological samples. Indeed, we confirmed the data of the regulation of the *PTGS2,* and *THBS1* genes obtained in the RNA-seq studies by qPCR (Figure 13). We found that *PTGS2* was upregulated in the presence of *MAFF* siRNA in presence and abence of IL-1 beta and that *THBS1* was downregulated in the presence of *MAFF* siRNA (Figure 13). The obtained results follow the similar patter as the RNA-seq analsys (Figure 12E).

Figure 11

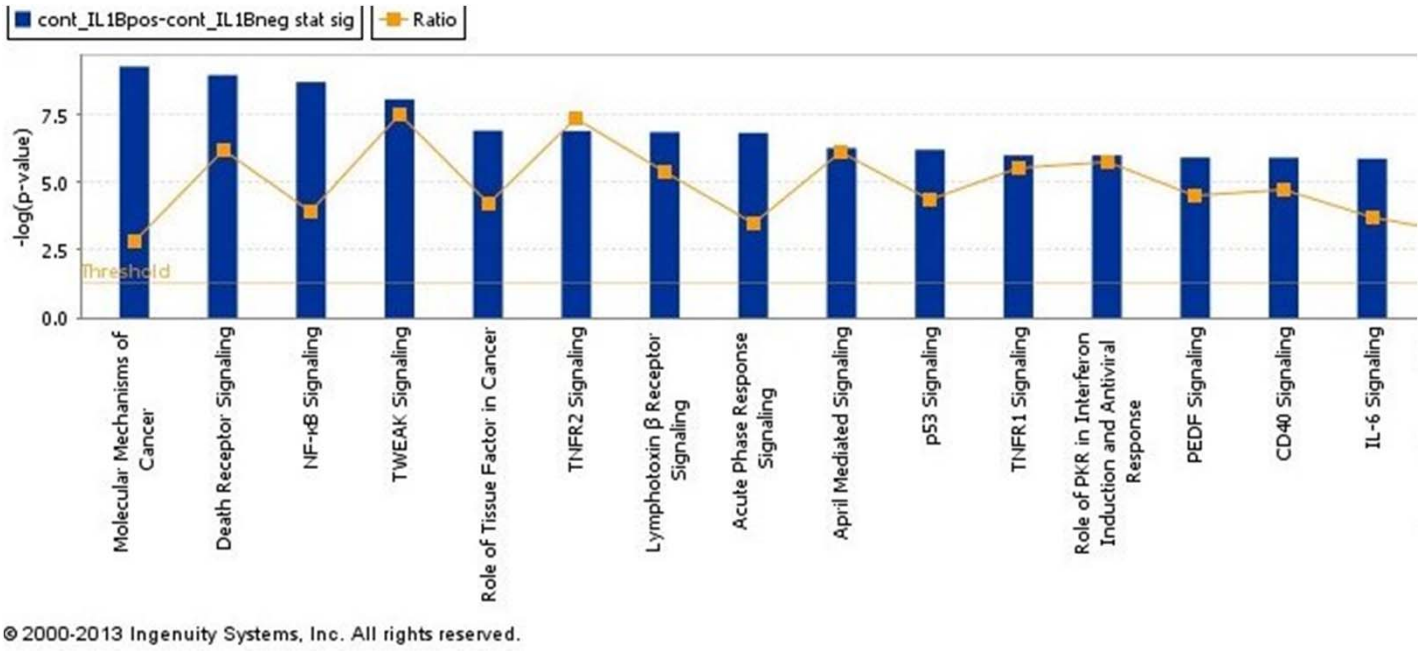


Figure 11: Ingenuity canonical pathway analysis of biological functions associated with genes regulated in response to IL-1 beta treatment. Bar graph representing the top pathways associated with the genes modulated by IL-1 beta treatment. The orange line shows the ratio of number of molecules gene products that are modulated by the treatment to the total number of molecules known to be present in the pathway. Threshold line is set at a p-value of 0.05.

Table V: Ingenuity canonical pathway analysis of biological functions associated with genes regulated in response to IL-1 beta treatment. List of molecules involved in the top pathways.

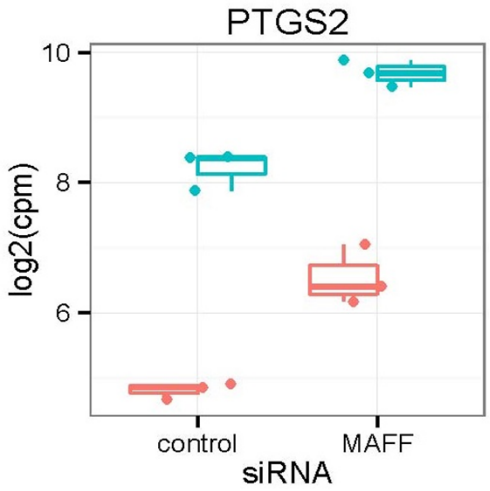
Ingenuity Canonical Pathways	Molecules Involved
Death Receptor Signaling	TNFRSF21, MAP3K14, TNFRSF10B, IKBKE, MAP3K5, NFKB2, NFKB1, DAXX, CASP6, TRAF2, NFKBIA, BID, CFLAR, NFKBIB, TNFRSF1B, BIRC3, CASP7, BIRC2
NF-κB Signaling	TRAF3, BMP2, TAB2, EGF, TNFAIP3, NFKB1, TNIP1, NFKBIA, CARD10, IGF1R, AKT3, GSK3B, TRAF5, TNFRSF1B, NFKBIB, EGFR, MAP3K14, RELB, MALT1, IL1R1, NFKB2, IRAK3, IGF2R, TAB3, PIK3R3, TRAF2, BCL10, IL36RN, TGFA, IL1B, PIK3CD
TWEAK Signaling	CASP6, MAP3K14, TRAF2, TRAF3, NFKBIA, BID, IKBKE, NFKB2, NFKBIB, NFKB1, BIRC3, CASP7, BIRC2
TNFR2 Signaling	MAP3K14, TRAF2, NFKBIA, TNFAIP3, IKBKE, NFKB2, TNFRSF1B, NFKBIB, NFKB1, BIRC3, BIRC2
Lymphotoxin β Receptor Signaling	MAP3K14, TRAF3, RELB, CXCL1, IKBKE, NFKB2, NFKB1, PIK3R3, BCL2L1, TRAF2, NFKBIA, AKT3, PIK3CD, TRAF5, BIRC2
Acute Phase Response Signaling	IL6ST, SOCS3, TCF4, IL6, MAP3K5, NFKB1, NR3C1, HMOX1, SOD2, NFKBIA, CFB, AKT3, OSMR, TNFRSF1B, NFKBIB, MAP3K14, C3, IKBKE, CEBPB, IL1R1, NFKB2, MAPK12, PIK3R3, TRAF2, IL36RN, IL1B, MAP2K3, PIK3CD
April Mediated Signaling	MAP3K14, TRAF2, TRAF3, NFKBIA, NFATC3, IKBKE, NFATC4, TRAF5, NFKB2, MAPK12, NFKBIB, NFKB1
p53 Signaling	WT1, PMAIP1, GADD45B, THBS1, TNFRSF10B, C12orf5, TP53BP2, SERPINE2, PIK3R3, CASP6, BCL2L1, CCND2, GADD45A, SNAI2, AKT3, PIK3CD, GSK3B, PML, DRAM1
TNFR1 Signaling	CASP6, MAP3K14, TRAF2, NFKBIA, BID, TNFAIP3, IKBKE, NFKB2, NFKBIB, NFKB1, BIRC3, CASP7, BIRC2
Role of PKR in Interferon Induction and Antiviral Response	TRAF2, TRAF3, NFKBIA, TAB2, BID, IKBKE, MAP2K3, TRAF5 ,NFKB2, NFKBIB, NFKB1, IRF1
PEDF Signaling	TCF4, BDNF, IKBKE, NFKB2, MAPK12, NFKB1, TCF7, PIK3R3, BCL2L1, NFKBIA, SOD2, AKT3, PIK3CD, CFLAR, NFKBIB, CASP7
IL-6 Signaling	IL6ST, IL8, SOCS3, MAP3K14, IKBKE, IL6, NFKB2, IL1R1, CEBPB, NFKB1, MAPK12, PIK3R3, TRAF2, NFKBIA, IL36RN, AKT3, IL1B, MAP2K3, PIK3CD, NFKBIB, TNFRSF1B

Table VI: Differentially expressed transcripts of genes that are modulated by 1.5-fold or more by the MAFF transcription factor in response to IL-1 beta treatment. Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included. P-value and false-discover for control versus IL1B induction conditions are 0.05 or less.

Symbol	Fold Change	Gene Description
GPR183	1.91	G protein-Coupled Receptor 183
NR4A2	1.89	Nuclear Receptor subfamily 4, group A, member 2
IDO1	1.69	Indoleamine 2,3-dioxygenase 1
RASD2	1.62	RASD family, member 2
ASPRV1	1.54	Aspartic peptidase, retroviral-like 1
ELOVL7	1.52	ELOVL fatty acid elongase 7
CARD16	1.51	Caspase recruitment domain family, member 16
LY6G5B	-1.52	Lymphocyte antigen 6 complex, locus G5B
PTX3	-1.53	Pentraxin 3, long
CXCL1	-1.53	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
HLA-G	-1.54	Major histocompatibility complex, class I, G
OLR1	-1.56	Oxidized low density lipoprotein (lectin-like) receptor 1
SNORD48	-1.58	Small nucleolar RNA, C/D box 48
TNFAIP2	-1.59	Tumor necrosis factor, alpha-induced protein 2
CSF3	-1.62	Colony stimulating factor 3 (granulocyte)
EDN1	-1.97	Endothelin 1
SLC12A7	-2.18	Solute carrier family 12 (potassium/chloride transporters), member 7
LTB	-2.73	Lymphotoxin beta (TNF superfamily, member 3)
RMRP	-6.02	RNA component of mitochondrial RNA processing endoribonuclease
RN5-8S1	-17.94	RNA, 5.8S ribosomal 5

Figure 12

A



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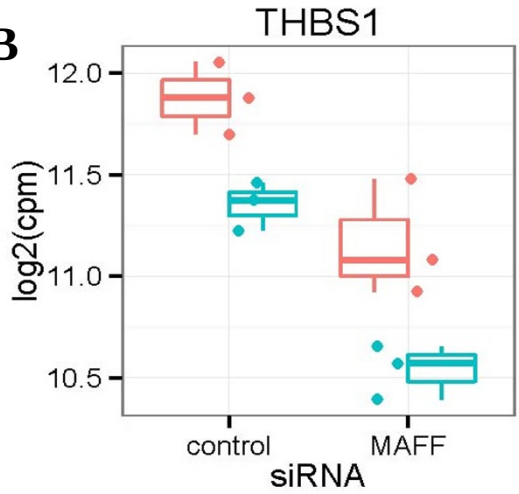

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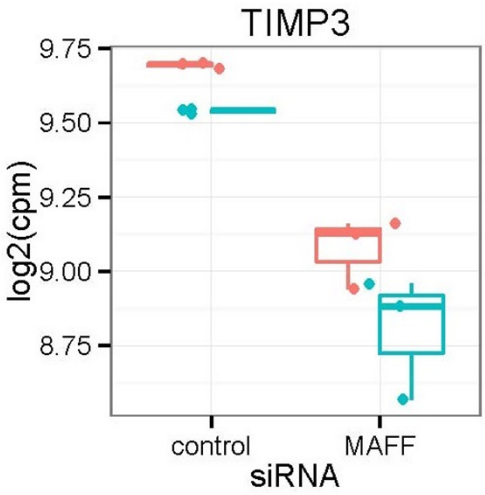

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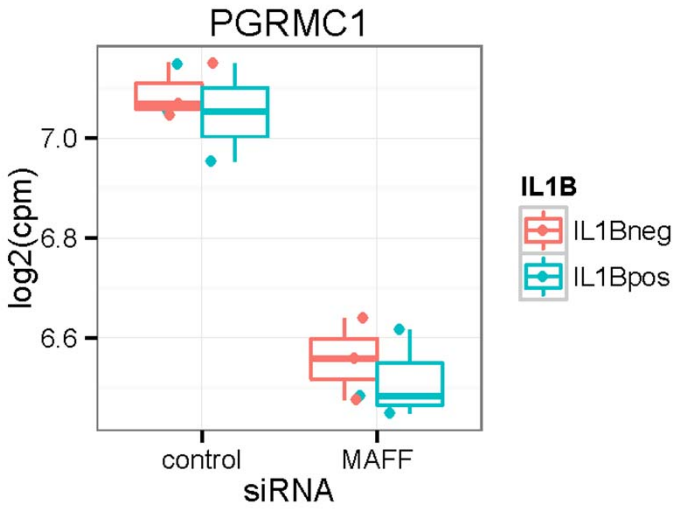

E

IL-1 beta	Comparison	*PTGS2*	*THBS1*	*TIMP3*	*PGRMC1*
Negative IL-1 beta	*MAFF* siRNA vs Control	3.45	-1.63	-1.53	-1.45
Positive IL-1 beta	*MAFF* siRNA vs Control	2.74	-1.75	-1.66	-1.45

Figure 12: Modulation of selected genes as observed in the RNA-sequencing analysis.
Boxplots representing A) *PTGS2*, B) *THBS1*, C) *TIMP3* and D) *PGRMC1* changes in control (scrambled) versus *MAFF* siRNA knockdown. The table (E) shows the values of modulation in the absence or presence of IL-1 beta.

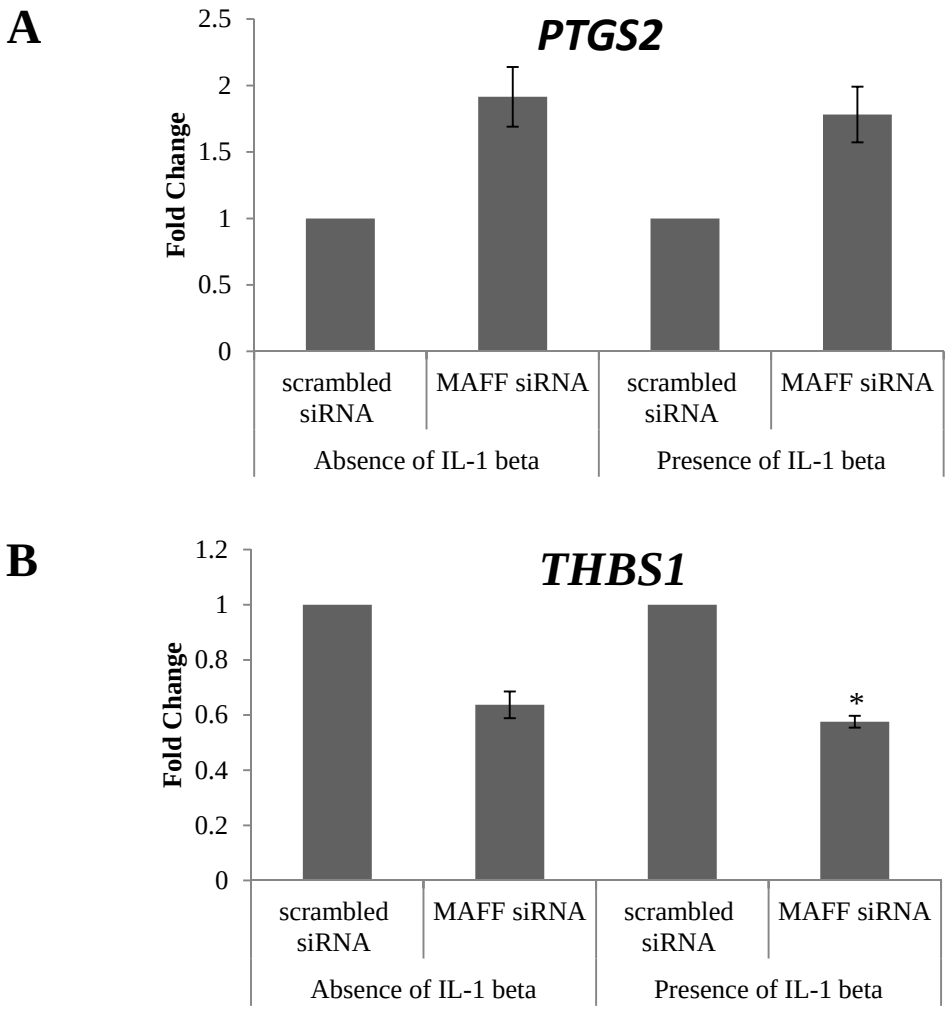

Figure 13: Quantitative PCR to confirm RNA-seq expression changes of genes that have a possible link to parturition in hTERT-C3 myometrial cells. cDNA levels were measured in samples transfected with scrambled or *MAFF* siRNA in presence of IL-1 beta for (A) *PTGS2* and (B) *THBS1*. Three independent experiments were analyzed.

D. MAFF – Control of PTGS2

We analyzed whether the presence of the MAFF transcription factor has also an effect on PTGS2 protein levels. We performed MAFF knockdown using two distinct siRNAs (*MAFF* siRNA # 7 and # 9) and subsequently assessed protein levels by immunoblot analysis (Figure 14). Our data showed that MAFF negatively controls PTGS2 in a significant fashion, as absence of the MAFF leads to a considerable increase in PTGS2 protein either with or without exposure to IL-1 beta. To analyze whether regulation of *PTGS2* gene is mediated at the transcriptional level, we carried out reporter assays. Myometrial cells were transfected with a pGL3 basic vector comprising a 2kb fragment of the *PTGS2* promoter [153]. We observed an increased in luciferase activity upon *MAFF* knockdown, suggesting that transcription of the *PTGS2* gene is negatively regulated by MAFF (Figure 15).

Moreover, we analyzed whether overexpression of MAFF would decrease luciferase activity under the promoter of *PTGS2*, using a reporter assay. We thus overexpressed MAFF by co-transfection of increasing amounts of pcDNA3.1 expression vector containing *MAFF* cDNA with pGL3 construct containing 2kb *PTGS2* promoter and measured luciferase activity. However, we did not observe the expected decrease in luciferase activity with higher MAFF concentrations when compared to cells transfected with empty pcDNA3.1 vector (Figure 16).

Figure 14

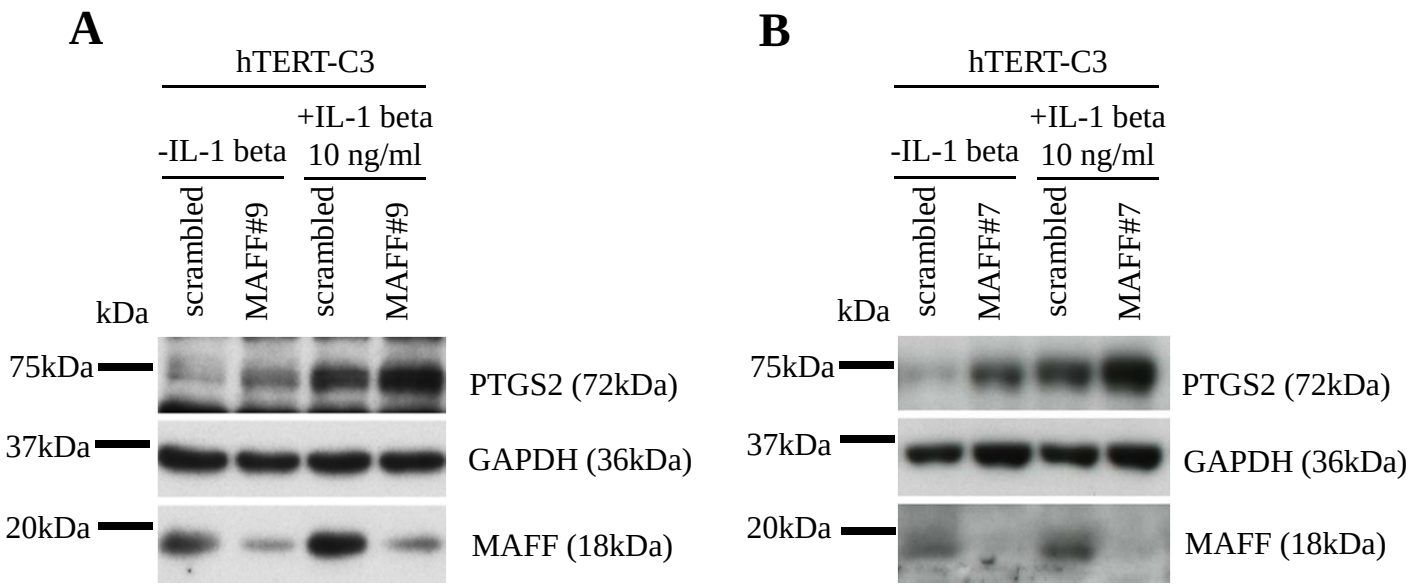

Figure 14: PTGS2 protein upregulation in the absence of MAFF. (A) and (B) PTGS2 protein expression in hTERT-C3 myometrial cells transfected for 24h with scrambled or MAFF siRNA #9 or MAFF siRNA in the presence of 10ng/ml IL-1 beta or absence of IL-1 beta (for 3h). 30 micrograms of total protein extract were loaded in each lane. MAFF (transfection control) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) are indicated. The data shown are representative of three independent experiments.

Figure 15

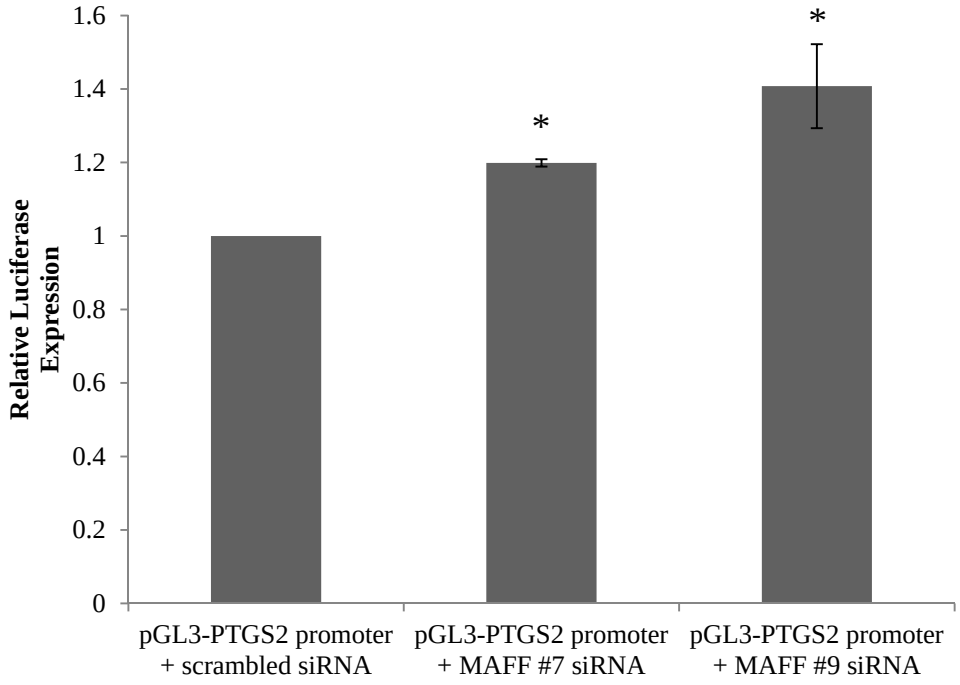

Figure 15: Relative luciferase activity expressed from a luciferase-human PTGS2 promoter construct. hTERT-C3 myometrial cells co-transfected with basic pGL3-PTGS2 promoter-luciferase construct, pRL-TK Renilla (internal control) and either control (scarmbled) siRNA or MAFF-specific siRNA (#7 or #9). Bar graph representing normalized relative luciferase activity. At least 3 independent experiments were analyzed.

Figure 16

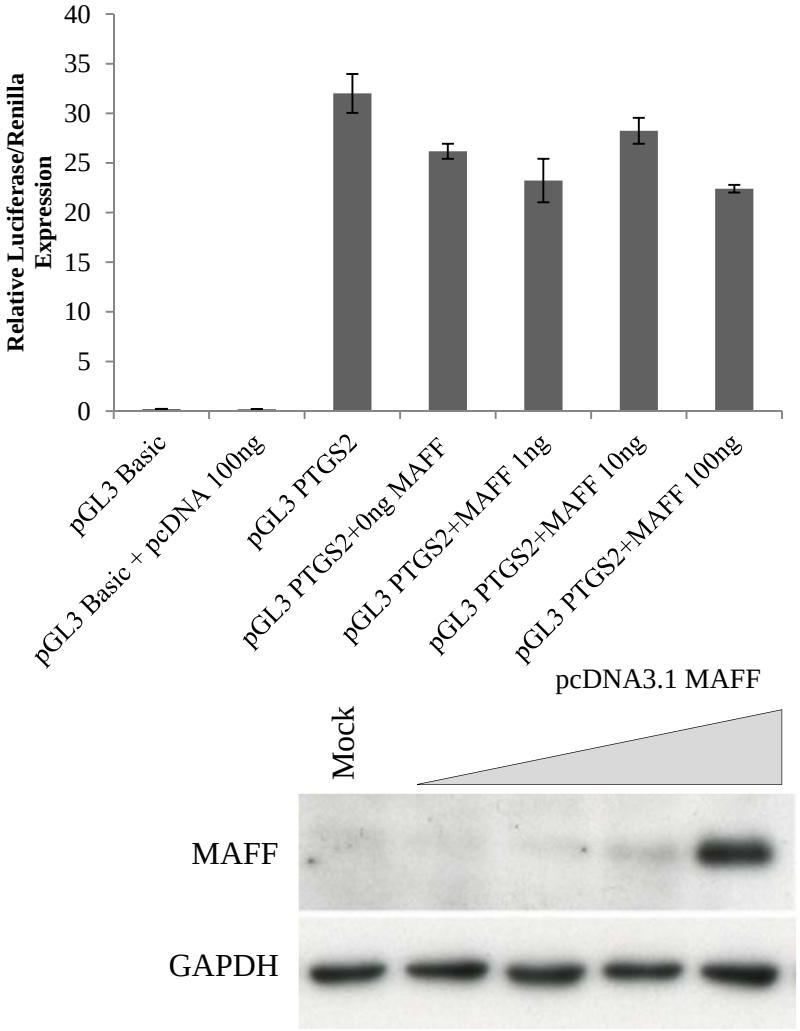

Figure 16: Relative luciferase activity expressed from a luciferase-human *PTGS2* promoter construct. (A) 293T HEK cells co-transfected with basic pGL3-PTGS2 promoter-luciferase construct, pRL-TK Renilla (internal control) and increasing concentrations of pcDNA3.1-MAFF construct (balanced with empty pcDNA3.1 vector). Bar graph representing relative luciferase activity. (B) Corresponding confirmation of MAFF protein overexpression in 293T cells with 0, 1, 10 and 100 nanograms of pcDNA3.1 MAFF vector. 30 micrograms of total protein extract were loaded in each lane. MAFF and GAPDH, loading control are indicated.

DISCUSSION

Our previous data linked the MAFF transcription factor to proinflammatory cytokine signaling in uterine smooth muscle cells [155, 156]. In the present study, we elucidated the MAFF dependent transcription network in hTERT-C3 myometrial cells, suggesting that this transcription factor controls important regulators of parturition, including PTGS2, the key enzyme in the synthesis of prostaglandins. In addition, we obtained data with respect to IL-1 beta regulated genes in this cellular model.

It has been established that at parturition, leukocytes invade the uterus that together with the cells of the reproductive tissues secrete proinflammatory cytokines creating an inflammatory microenvironment in the reproductive tissues [14]. This inflammatory process participates in membrane rupture, cervical dilation, myometrial contraction and expulsion of the fetus [17]. In earlier studies, it has been reported that MAFF is present in high levels in pregnant myometrium at term [147]. Using PHM1-31 uterine smooth muscle cells as well as primary myometrial cells as models, our laboratory previously showed that MAFF transcript and protein levels are upregulated by proinflammatory cytokines TNF-alpha and IL-1 beta [155, 156], which have been linked to the onset of labor [81]. We also found that MAFF is the only member of the small MAF family, to be regulated by pro-inflammatory cytokines in this cell type [155]. Together, these studies suggested a specific role for MAFF in myometrial cell during labor and/or parturition. Here, we confirmed cytokine induction of MAFF in hTERT-C3 cells and we performed siRNA-mediated knockdown studies (Figure 9) in combination with RNA-sequencing.

The first step of the project consisted of optimization of the siRNA mediated knockdown of MAFF with respect to timing, dose and siRNA species. The knockdown of *MAFF* was carried out by electroporation as this was the method that yielded best results in hTERT-C3 myometrial

cells (data not shown). Based on immunoblot results (Figure 6) we performed RNA-sequencing experiment with *MAFF* siRNA #7 and subsequent experiments using *MAFF* siRNA #7 and #9. Since we wanted to identify genes that are regulated directly by IL-1 beta, we chose a concentration of 10 ng/ml (Figure 7) and a treatment period of 3 hours (Figure 8). A concentration of 10ng/ml was selected as it is the one that showed consistant and statistically significant high upregulation of MAFF and a time point of 3 hours was chosen as it is the shortest time in which MAFF reaches high exression levels.

Complementary to our previous studies on myometrial cells [156] we further dissected IL-1 beta dependent gene pathways in the hTERT-C3 myometrial cell model. As expected, a large number of genes (1114) was found to be modulated by this cytokine by 1.3 fold or more (Table V and Appendix Figure 2). Among the modulated genes, there were more upregulation rather than downregulation, which confirm previous findings in myometrial, endometrial and synovial sarcoma cell lines respectively [156-158]. Data presented comprise only genes that were expressed by at least 2 counts per million, excluding genes with expression close to background levels that may not represent induction or repression events. IL-1 beta treatment of myometrial cells regulates pathways involved in many processes as for instance in apoptosis and cell proliferation, differentiation (Figure 11). Canonical pathways identified by IPA suggest that myometrial cells respond to IL-1 beta by activating genes signaling the cell to initiate apoptosis through death receptor signaling pathway, TNF Receptor (TNFR1/2) or proliferation inducing ligand (April) mediated signaling. The modulation of TNF signaling is not surprising as IL-1 beta and TNF signaling share many biological activities [159]. This confirms earlier data since IL-1 beta has been shown to act not only as a negative, but also as a positive regulator of apoptosis [160]. On the other hand, IL-1 beta is also activating signaling pathways that lead to an

inflammatory response that generally constitutes a defense mechanism against injury or microorganism invasion. Such pathways are illustrated by the upregulation of genes that are part of the TWEAK Acute phase Response Signaling of the activation of protein kinase R (PKR) or NFKB signaling. The modulation of these pathways is consistent with the role of IL-1 beta as an inflammatory cytokine involved in cell protection [161].

To gain further insights into the role of MAFF in uterine smooth muscle cells, we used siRNA mediated knockdown combined with genome wide analysis of the transcriptome to identify MAFF regulated genes in the absence and presence of IL-1 beta. Differentially expressed genes in negative control (scrambled) siRNA and *MAFF* siRNA in the absence of IL-1 beta were identified (Table II and appendix Figure 1A) and in the presence of IL-1 beta (appendix Figure 1B). Our RNA-seq data revealed that knockdown of *MAFF* has an impact on cell cycle or chromosomal replication checkpoint regulations, mismatch repair and ATM signaling (Figure 10). These results were unexpected as MAFF has been characterized as a stress-induced transcription factor [162]. The expression of genes involved in peroxisome proliferator-activated receptor alpha/retinoid x receptor alpha (PPARalpha/RXRalpha) pathway is affected when MAFF is present and this is observed as a change in the transcript levels of these genes when MAFF is reduced. PPARs are ligand dependent nuclear transcriptional factors [163] found to be expressed in human gestational tissues and have been linked to preterm birth [164]. Activators of PPARs have been shown to have anti-inflammatory properties [165] and it was proposed that downregulation of their expression or activity can lead to preterm birth [166].

Of note, we found that the MAFF transcription factor controls a series of genes that play important roles in the labor and birth processes. Intriguingly, our RNA-sequencing data, showed that absence of MAFF leads to a significant increase of *PTGS2* levels (Figure 12A). This result

has been confirmed by qPCR at the transcript level (Figure 13A) and further analysis showed that PTGS2 protein levels are equally increased upon MAFF knockdown (Figure 14). PTGS2, an enzyme, previously known as cyclooxygenase 2 (COX-2) mediates the rate-determining step in the synthesis of prostaglandins and it is induced by IL-1 beta in the myometrium [167]. The upregulation of PTGS2 in the absence of MAFF suggests a negative regulation of PTGS2 by MAFF. This could be explained by the need of signals attenuating the inflammatory processes leading to parturition. The induction of MAFF by IL-1 beta would be consistent with the hypothesis that MAFF functions a delayed early response factor acting as a negative feedback regulator. This notion is supported by a previous finding, showing that MAFF might be involved in a negative control of the EGF-mediated response in different epithelial cell lines [168]. We showed that knockdown of MAFF also leads to a moderate but statistically significant increase of luciferase activity using a reporter comprising a 2 kb of the *PTGS2* promoter (Figure 14), suggesting at least a partial transcriptional control of PTGS2 by MAFF. However, it is possible that this fragment does not contain all the regulatory elements needed for appropriate control of the promoter by MAFF. In the future, we will carry out studies to dissect the exact molecular mechanisms of how MAFF controls *PTGS2* transcription. Overexpressing MAFF in increasing concentrations with luciferase reporter vector under *PTGS2* promoter using 293T cell line as a model did not result in the expected decrease of luciferase activity with higher concentrations of MAFF. The expression of MAFF levels was confirmed by immunoblotting (Figure 16B). It is possible that we did not observe the expected decrease of luciferase activity because the model system may have not been optimal, for example MAFF may have been forming heterodimers with other bZIP transcription factors not allowing it to bind to the promoter of *PTGS2*.

Furthermore, MAFF also modulates the expression of *TIMP3*. Our RNA-seq and qPCR data revealed that the knockdown of the *MAFF* leads to a decrease of *TIMP3* levels both in the absence and presence of IL-1 beta, suggesting a positive regulation of *TIMP3* by MAFF (Figure 12C). TIMP3 belongs to the family of tissue inhibitor of metalloproteinases, which are, as discussed in the literature review, involved in remodeling of the extracellular matrix for cervical dilation and fetal membrane rupture during parturition. Comparing the expression of MMPs and TIMPs involved in extracellular degradation and remodelling demonstrated that matrix metalloproteases (specifically MMP-9) as well as plasminogen activation cascade proteins are present in different concentrations before, during and after labor [169]. An imbalance in these enzymes or an improper expression of their tissue inhibitors may cause preterm birth, as it has been reported that TIMPs, especially TIMP1 and TIMP2 concentrations, were lower in preterm gestation tissues [170]. Moreover, it has been found that increased IL-1 beta and TNF cytokine levels in the amniotic fluid at the time of parturition cause FM weakening. Treatment of FM with TNF showed an increase of MMP9 protein in parallel to a decrease of TIMP3 [16].

From our data on *PTGS2* and *TIMP3* we propose a hypothetical model (Figure 17) in which MAFF acts as a delayed early gene and negative feedback regulator. Upregulation of MAFF by IL-1 beta cytokine leads to inhibition of *PTGS2* expression and an increase in *TIMP3* levels resulting in a negative feedback loop to attenuate the response of the myometrium to proinflammatory cytokines.

In addition, we found that thrombospondin 1 transcript levels are decreased in the absence of MAFF transcription factor, both in untreated and IL-1 beta treated cells (Figure 12B and Figure 13B). This is of interest, as it has been shown that THBS1 is a glycoprotein modulating interaction between ECM and cells. Increased THBS1 in the myometrium at labor

Figure 17

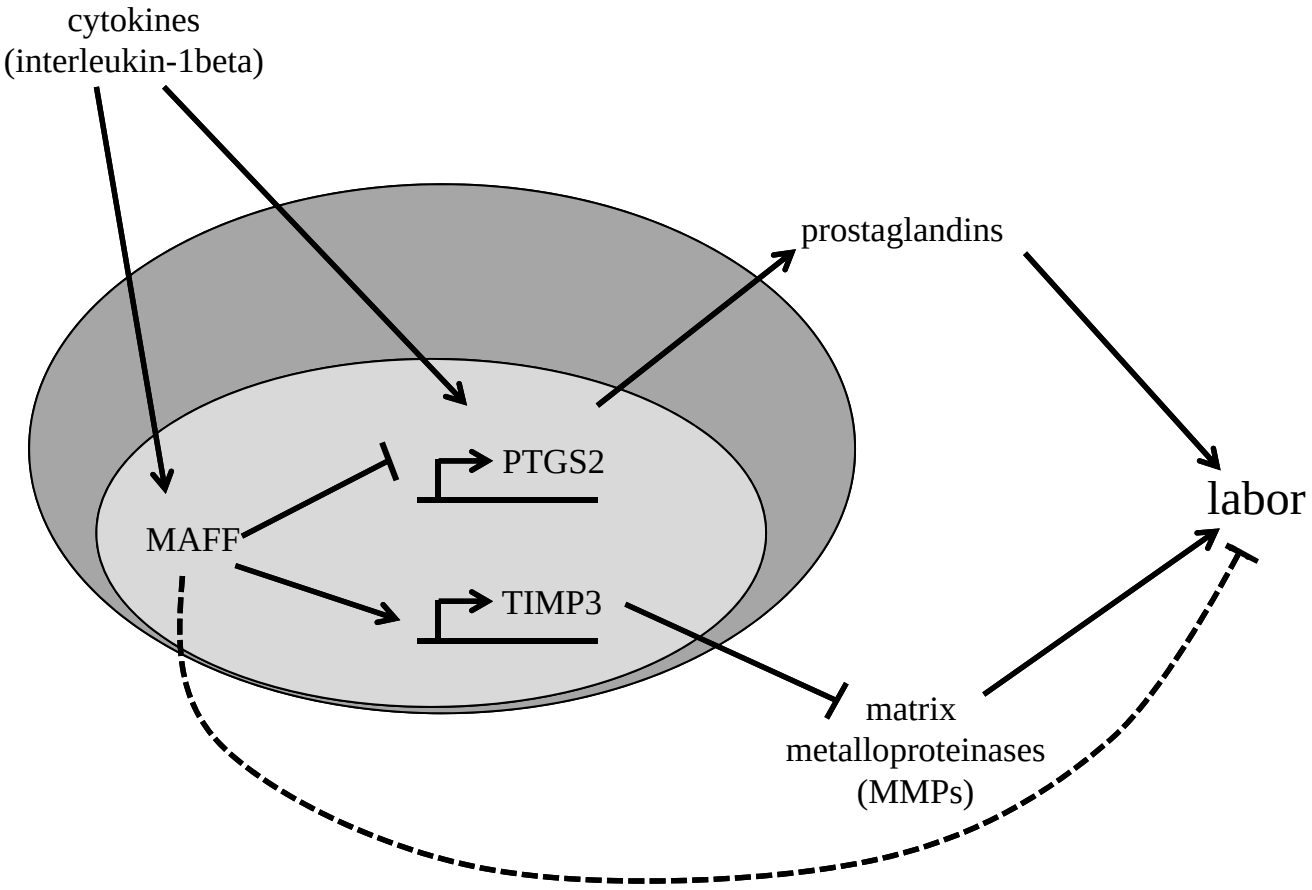

Figure 17: Hypothetical model showing the attenuation of cytokine induced labor by inhibition of *PTGS2* and induction of *TIMP3* through the MAFF transcription factor. MAFF acts like a delayed early gene, downregulating *PTGS2* (a positive regulator of labor) expression and increasing *TIMP3* (a negative regulator of labor) expression, resulting in a negative feedback loop (dashed line) to attenuate the response of the myometrium to proinflammatory cytokines.

has been shown in human and sheep [171, 172], but its role in parturition has not yet been established. Analysis of genes expressed in the activated amnion (assessed by measuring NFKB activity) revealed that *THBS1* transcripts were upregulated 3 fold in activated amnion when compared to non-activated amnion [173]. Thrombospondin 2, another member of the family, is believed to be involved in the tissue repair process after cervical remodeling of parturition, as it is induced by several fold in the postpartum human cervix [19]. This is further evidence that MAFF might act as negative feedback regulator by repressing expression of *THBS1*, which is normally upregulated during labor.

More studies are required to determine whether MAFF directly regulates PTGS2, TIMP3 or THBS1. This can be verified by doing a chromatin immunoprecipitation (ChIP) experiment. Moreover, on a more global scale, MAFF binding sites can be determined by ChIP-sequencing. The role of MAFF can also be studied on functional basis in myometrial cells using the contractility assay developed previously for the PHM1-31 and hTERT-C3 cellular models [152, 156].

Future directions

A series of follow-up experiments can be proposed. It is important to determine whether MAFF transcription factor binds directly to regulatory regions of the *PTGS2* gene by a using chromatin immunoprecipitation (ChIP) approach.. In parallel, ChIP sequencing experiments will be performed to identify the DNA binding site occupancy of MAFF on a genome-wide scale. This approach, in combination with the data obtained from RNA-sequencing will help to determine whether genes are regulated directly, and whether they are rcontrolled indirectly via downstream effector molecules of MAFF transcription factor. Classical promoter analyses will

be used to further define the binding sites of MAFF. In addition, the role of possible DNA binding partners, the CNC transcription factors Nrf1, Nrf2 and Nrf3, in myometrial cells will be examined by using expression, protein-protein interactions and DNA binding studies. As we are identified many genes that are controlled by Il-1 beta and/or MAFF, additional pathways that play a role in myometrial cell function will be explored.

Conclusion

In conclusion, we uncovered the transcriptional network governed by the MAFF transcription factor and/or IL-1 beta in myometrial cells. The identified targets likely play a role in uterine smooth muscle cell function, contractions and may be involved in preterm labor, labor and/or parturition. Our initial hypothesis that MAFF plays a role in parturition is supported by our data showing that genes specifically involved in childbirth are modulated by MAFF. Moreover, we formulated a novel hypothesis that MAFF plays a role in attenuation of labor-associated genes, which is supported by the fact that MAFF controls the expression of *PTGS2*, *TIMP3* and *THBS1*. Our data shed new light on the molecular mechanisms regulating normal parturition as well infection induced preterm labor. The further elucidation of the MAFF dependent gene network will help to gain important insights into the molecular mechanisms that regulate inflammation induced labor. In the future, these studies may contribute to the prevention of early labor, through the development of novel diagnostic and treatment tools.

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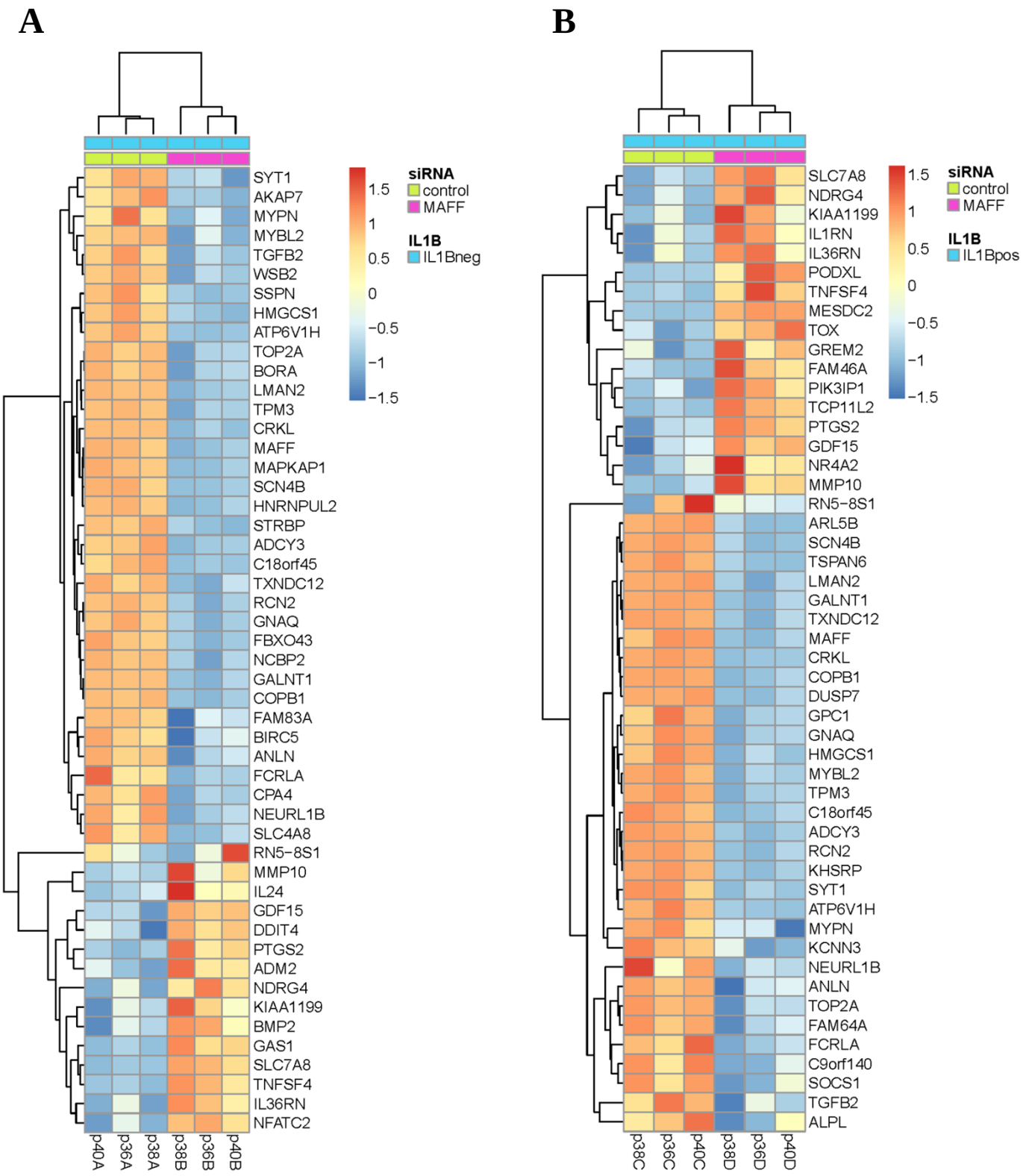
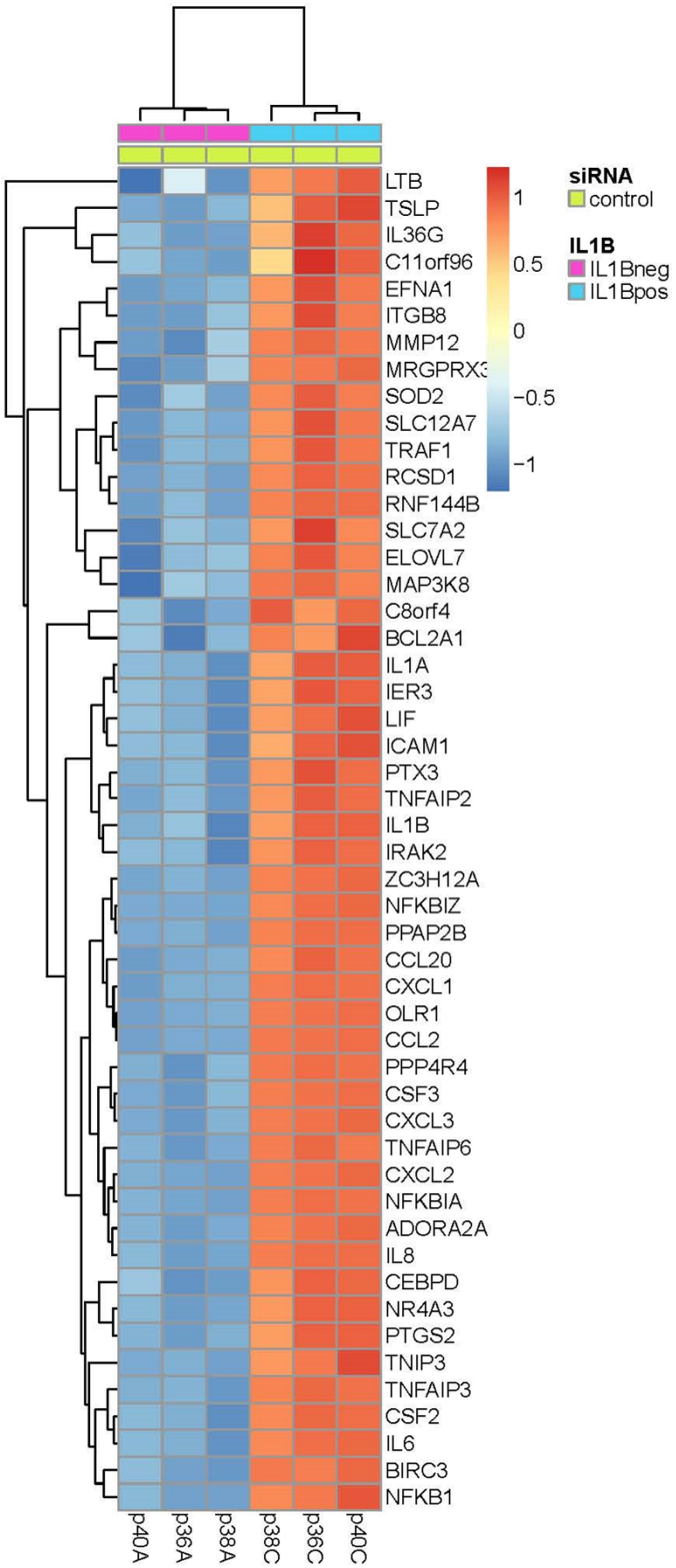
Appendix Figure 1



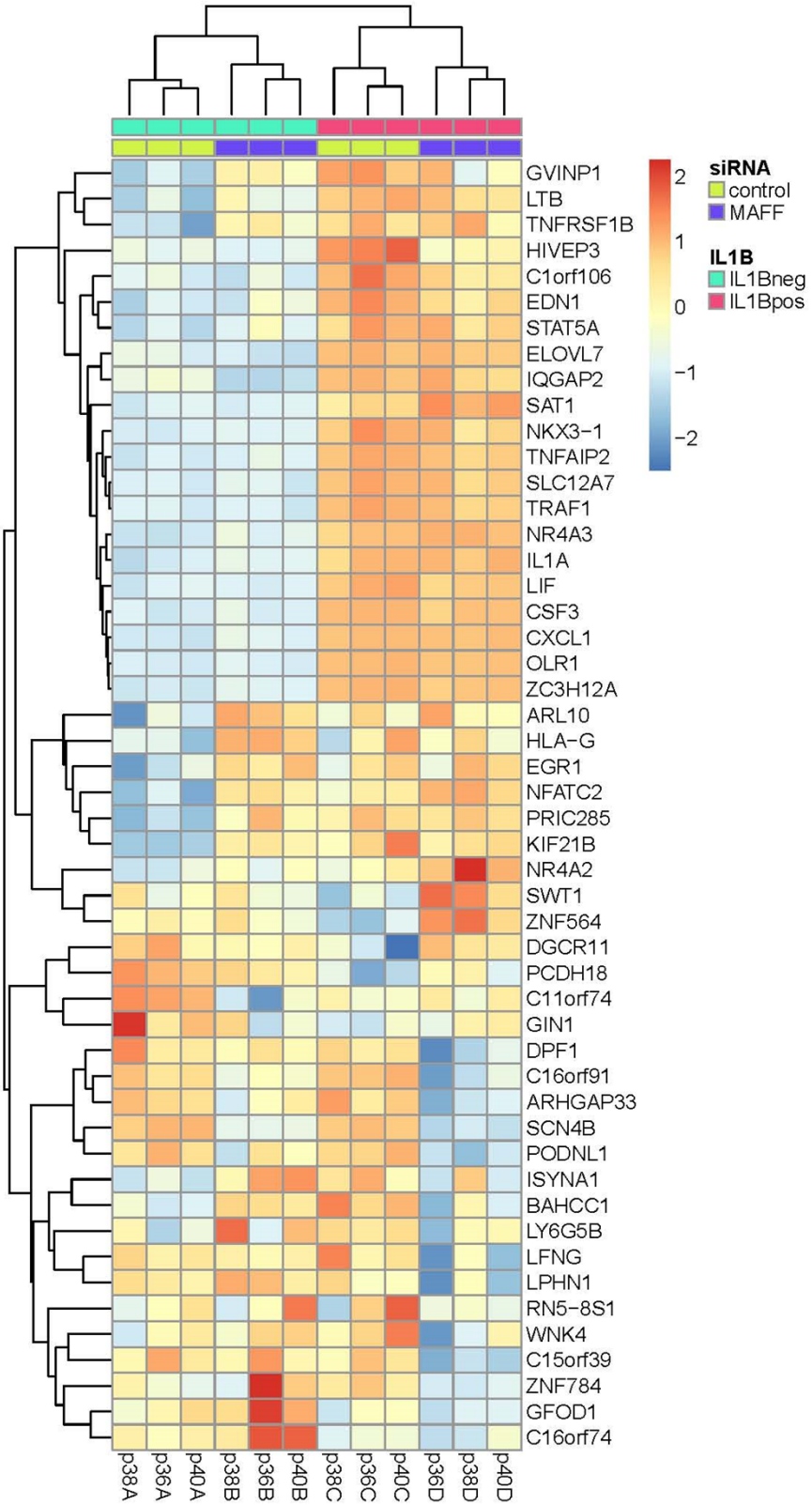
Appendix Figure 1: Heatmap of transcripts regulated by MAFF knockdown in hTERT-C3 myometrial cells in the presence and absence of IL-1 beta.
Statistically significant genes (p-value > 0.05) that have the highest fold change in transcript levels modulated by the absence of MAFF. Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included **A)** In the absence of IL-1 beta; **B)** In the presence of IL-1 beta.

Appendix Figure 2



Appendix Figure 2: Heatmap of transcripts regulated upon induction with IL-1 beta in hTERT-C3 myometrial cells. Statistically significant genes (p-value > 0.05) that have the highest fold change in transcript levels modulated by the IL-1 beta treatment. Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included.

Appendix Figure 3



Appendix Figure 3: Heatmap of transcripts regulated by MAFF in response to IL-1 beta treatment. Statistically significant genes (p-value > 0.05) that have the highest fold change in transcript levels modulated by IL-1 beta through MAFF. Only transcripts expressed by at least 2 counts per million (cpm) in one of the comparison condition are included.

Appendix Table I: Statistics of reads corresponding to the RNA-sequencing data. Raw column shows the number of reads sequenced. Filtered reads number is obtained after quality and length trimming and aligned reads show the reads that were successfully mapped to the Homo Sapiens reference genome. p36/p38/p40 represent 3 independent experiments. Condition A: scrambled siRNA – IL-1 beta; B: MAFF siRNA – IL-1 beta; C: scrambled siRNA + IL-1 beta; D: MAFF siRNA + IL-1 beta.

Sample	Raw	% sequenced	Filtered	% filtered	Aligned	% aligned
p36A	48,953,915	100	46,887,455	95.8	42,006,713	89.6
p36B	47,920,395	100	45,808,721	95.6	41,027,957	89.6
p36C	52,461,302	100	49,729,199	94.8	44,438,990	89.4
p36D	60,896,338	100	58,424,340	95.9	52,408,960	89.7
p38A	50,011,008	100	48,020,388	96.0	42,678,812	88.9
p38B	48,686,023	100	46,707,008	95.9	41,755,904	89.4
p38C	65,027,817	100	62,383,002	95.9	55,977,796	89.7
p38D	64,310,754	100	61,513,130	95.6	55,018,571	89.4
p40A	55,200,671	100	52,996,789	96.0	47,250,729	89.2
p40B	42,134,369	100	40,449,129	96.0	35,970,029	88.9
p40C	65,321,994	100	61,520,121	94.2	54,429,403	88.5
p40D	65,453,985	100	63,010,614	96.3	56,511,670	89.7