REGULATION OF EXPRESSION OF KEY GENES IN UTERINE MYOCYTES

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Declaration

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10.10.2017

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Signed: Marina Ilicic Date

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List of Publications for Inclusion

Journal Articles (Original Research) published

M. Ilicic, T. Butler, T. Zakar, & J.W. Paul. Contraction-associated gene expression changes during ex situ culture of pregnant human uterine smooth muscle. *Journal of Smooth Muscle Research (2017) 53: 73-89.*

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M. Ilicic, T. Zakar, & J.W. Paul. Modulation of Progesterone Receptor (*PR*) isoform expression in pregnant human myometrium. *BioMed Research International (2017).*

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J.W. Paul*, **M. Ilicic***, T. Zakar, R. Smith. Expression of *KCNH2* (hERG1) and *KCNE2* correlates with expression of key myometrial genes in human pregnant myometrium. *J Hum Endocrinol (2017) 2: 008.*

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J.W. Paul, S. Hua, **M. Ilicic**, T. Butler, J. Tolosa, S. Robertson, R. Smith. Drug Delivery to the Human and Mouse Uterus using Immunoliposomes Targeted to the Oxytocin Receptor. *Am J Obstet Gynecol* (2017) 216: 283.e1-14.

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Journal Articles (Original Research) in preparation:

M. Ilicic, T. Zakar, R. Smith, & J.W. Paul. Regulation of *KCNH2* (hERG1) and *KCNE2* expression in human pregnant myometrium. *Original Research.*

Additional Publications

Journal Articles (Original Research) published

S.Y. Chai, R. Smith, J.T. Fitter, C. Mitchell, X. Pan, **M. Ilicic**, K. Maiti, T. Zakar, G. Madsen. Increased progesterone receptor A expression in labouring human myometrium is associated with decreased promoter occupancy by the histone demethylase JARID1A. *Molecular Human Reproduction 20(5):442-453, 2014*

I made a significant contribution to the above manuscript during my PhD. I performed the immunohistochemistry and captured all the confocal microscopy images presented in Figure 3, which demonstrate the localisation of JARID1A and SMYD3 in term laboring human myometrial tissue. I also wrote the corresponding section of the manuscript.

Report published

J.W. Paul, S. Hua, **M. Ilicic**, T. Butler, J. Tolosa, S. Robertson, R. Smith. Applying nanopharmacology to obstetrics: A novel targeted drug delivery system for the uterus. *Atlas of Science (2017).*

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

∆Ct	delta ct
20αHSD	20α hydroxysteroid dehydrogenase
4-AP	4-aminopyridine
8-Br-cAMP	8-bromoadenosine 3', 5'-cyclic monophosphate
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AP	activator protein
AUC	area under the curve
BMI	body mass index
BSA	bovine serum albumin
C	cytosine
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CAPs	contractile associated proteins
cDNA	complementary deoxyribonucleic acid
CHD1	chromodomain-helicase-DNA-binding I protein
ChIP	chromatin immunoprecipitation
CO_2	carbon dioxide
CpG	sequence CG
CRE	cAMP regulatory element

CRH	corticotropin releasing hormone
CRH-BP	corticotropin releasing factor binding protein
CS	caesarean section
Ct	threshold cycle
Cx43	connexin 43
DAG	diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMEM	dulbecco’s modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DOF	dofetilide
DSPC	1, 2-distearoyl-sn-glycero-2-phosphocholine
EE	encapsulation efficiency
EP	E prostanoid receptor
ER	estrogen receptor
ERα	estrogen receptor α
ERE	estrogen responsive element
ERG1	ether-à-go-go-related gene 1
ERK	extracellularly regulated kinase
ESR1	estrogen receptor 1

FP	prostaglandin F receptor
$G_{\alpha s}$	G alpha subunit
GA	gestation age
GAPPS	global alliance to prevent prematurity and stillbirth
GPCR	G protein-coupled receptor
GPR30	G protein-coupled estrogen receptor 1
H_2O	water
H3K4	histone 3 lysine 4
H3K4me3	histone 3 lysine 4 trimethylation
H3K9me3	histone 3 lysine 9 trimethylation
H3K27me3	histone 3 lysine 27 trimethylation
H3R2me2a	histone H3 asymmetric di methyl R2
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HDM	histone demethylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hERG1	human ether-à-go-go-related gene 1
HMRI	hunter medical research institute
HPLC	high performance liquid chromatography
ICAM-1	intercellular adhesion molecule-1
IL	in-labor
IgG	immunoglobulin-G
IP	intraperitoneal

IP_3	inositol triphosphate
IPTG	isopropyl b-D-thiogalactopyranoside
IND	indomethacin
IV	intravenous
IVIS-100	*in vivo* imaging system
JARID1A	jumonji AT-rich interactive domain 1A
K^+	potassium ion
K_V	voltage-dependent K^+
KAT	lysine (K)-acetyltransferases
KCl	potassium chloride
KCNH2	potassium voltage-gated channel subfamily H member 2
KCNE2	potassium voltage-gated channel subfamily E regulatory subunit 2
KH_2PO_4	monopotassium phosphate
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
$MgSO_4$	magnesium sulfate
MLC	myosin light chains
MLCP	myosin light chains phosphatase
mRNA	messenger ribonucleic acid
mERG	mouse ether-à-go-go-related gene 1
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate

NHMRC	national health and medical research council
NIF	nifedipine
NIL	not-in-labor
no-RT	no-reverse transcription
nPR	nuclear progesterone receptor
nPR-A	nuclear progesterone receptor A
nPR-B	nuclear progesterone receptor B
NTC	no template control
O_2	oxygen
OTR	oxytocin receptor
OXTR	oxytocin receptor
P450c17	17-α-hydroxylase/ 17, 20 lyase
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDE4	phosphodiesterase 4
PG	prostaglandin
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGR	progesterone receptor
PHM1-31	pregnant human myometrial cell line
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PKA	protein kinase A
PLC	phospholipase C
Pol-II	RNA polymerase-II

PPH	postpartum hemorrhage
PR	progesterone receptor
PR-A	progesterone receptor isoform A
PR-B	progesterone receptor isoform B
PR-T	progesterone receptor total
PSS	physiological saline solution
PTB	preterm birth
PTGS2	prostaglandin-endoperoxidase synthase 2
qRT-PCR	quantitative real time polymerase chain reaction
R	relative abundance
RNA	ribonucleic acid
ROL	rolipram
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RU486	mifepristone
SAL	salbutamol
SEM	standard error of mean
SMYD3	SET and MYND domain-containing protein 3
SPDP	N-succinimidyl-3-(2-pyridyldithio) propionate
TBS	tris-buffered saline
TBS-T	tris-buffered saline with tween 20
TEA	tetraethylammonium
TRPV4	transient receptor potential vanilloid 4

TSA	trichostatin A
TSS	transcription start site
ZEB	zinc finger E-box binding homeobox protein

Units

Da	dalton
s	second/s
min	minute(s)
hr	hour(s)
U	units
cm^2	square centimetre
g	gram
µg	microgram
pg	pictogram
mg	miligram
µL	microliter
mL	mililiter
V	volt
nM	nanomolar
mM	milimolar
µM	micromolar

Abstract

Background: As term pregnancy approaches, the human uterus is transformed from a relaxed quiescent phenotype to a contractile phenotype capable of the powerful coordinated contractions of labor. Regulation of this transformation is yet to be fully understood, however, key contraction-associated genes have been identified. Understanding the regulation of these genes is a high priority. Ethical consideration of human pregnancy primarily limits researchers to *in vitro* investigations using human cell lines and biopsied tissues. Whilst informative, concerns have been raised about the ability of cells and tissues in culture to remain representative of the tissue of origin. This concern was realised in relation to pregnant human myometrium upon observing that expression of progesterone receptor (PR) isoforms in non-laboring tissue transitioned to a laboring state *in vitro*. This confounded the ability to study PR regulation *in vitro*, and raised questions as to whether other key contraction-associated genes underwent culture-induced expression changes.

Hypotheses and Aims: It was hypothesised that the key contraction-associated genes; estrogen receptor 1 (*ESR1),* prostaglandin-endoperoxide synthase 2 (*PTGS2),* oxytocin receptor *(OXTR), PR,* human ether-à-go-go-related gene (*KCNH2*) and potassium voltage-gated channel subfamily E regulatory subunit 2 (*KCNE2)* underwent significant expression changes upon culturing term non-laboring human myometrium *in vitro*. Furthermore, it was hypothesised that these culture-induced changes were consistent with transition toward a laboring myometrial phenotype.

The aim of this thesis was to determine whether expression of *ESR1, PTGS2, OXTR, PR*, *KCNH2* and *KCNE2* changed upon placing non-laboring human

myometrium into culture. In addition, this research aimed to identify culture conditions that blocked or minimized phenotypic transition of these genes *in vitro*. As newly identified contraction-associated genes, this thesis also aimed to determine whether expression of *KCNH2* and *KCNE2* in term non-laboring human myometrium correlate with the expression of *ESR1, PTGS2, OXTR* and *PR*, which was yet to be examined in pregnant human myometrium.

Methods: For gene expression studies, quantitative RT-PCR was used to determine *ESR1*, *PTGS2, OXTR, PR, KCNH2* and *KCNE2* mRNA abundance in fresh term non-laboring human myometrial tissue and tissue cultured for 48 h under conditions routinely used within the field. Effects of specific treatments were examined, including; serum, progesterone, estrogen, cAMP, PMA, stretch, NF-κB inhibitors, $PGF_{2\alpha}$ and trichostatin-A (TSA). For contraction studies, strips of term non-laboring human myometrium were suspended in organ baths. Effects of hERG channel activators and inhibitors on spontaneous contractions were examined.

Results: Placing myometrial tissue in culture for 48 h resulted in significant culture-induced up-regulation of *ESR1*, *PTGS2, PR-T*, *PR-A* and *KCNE2* mRNA expression, as well as significant down-regulation of *OXTR* mRNA expression, relative to fresh tissue. Expression of *PR-B* and *KCNH2* mRNA remained unchanged. Progesterone and estrogen prevented culture-induced increase in *ESR1* mRNA expression; PMA maintained *KCNE2* and *OXTR* mRNA expression; while TSA prevented culture-induced increase in *PR-A* mRNA expression and maintained the original *PR-A/PR-B* expression ratio. In term non-laboring human myometrium, *KCNH2* and *KCNE2* expression were found

to correlate with expression of *ESR1, PTGS2, OXTR* and *PR.* Additionally, the hERG channel activators had no effect on spontaneous contractions *in vitro*, whereas inhibitors significantly increased contraction duration.

Conclusion: Standard conditions routinely employed to perform *in vitro* culture of human myometrium results in significant changes in key contraction-associated genes, including the newly identified contraction-associated genes, *KCNH2* and *KCNE2*. Overall, the altered expression of these genes was consistent with transition of non-laboring tissue toward a laboring phenotype *in vitro*. No single supplement was able to prevent all culture-induced changes of the genes examined. Culture-induced changes are anticipated to extend well beyond the limited cohort of genes examined. These findings have serious implications for *in vitro* studies attempting to advance our understanding of human parturition, and highlight the need to develop and validate robust *in vitro* models for the future.

Chapter One: Introduction

The regulation of uterine function during pregnancy and parturition: an update and recent advances

Marina ILICIC, Tamas ZAKAR and Jonathan W. PAUL

This Chapter contains a manuscript submitted to Reproductive Sciences (Appendix D).

There is now a substantial body of evidence highlighting key myometrial genes involved in the transformation of the uterus from a relaxed, quiescent state to a contractile and excitable phenotype to facilitate parturition. Despite continued advances in our understanding myometrial biology, the exact mechanisms that regulate parturition are yet to be fully understood.

This review examines the published evidence that consolidates the current paradigm for the regulation of human parturition, as well as more recent findings that are yet to be integrated into the existing body of research.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Marina Ilicic **(1st Author)**	**Manuscript writing** **Figure creations**
Tamas Zakar	Manuscript editing
Jonathan W. Paul	Manuscript editing Figure creations

1.1 Abstract

Successful pregnancy necessitates that the uterus is maintained in a relaxed, quiescent state for the majority pregnancy, before being transformed to a contractile and excitable phenotype to facilitate parturition. There is now a substantial body of evidence highlighting key genes involved in this transformation. Despite our rapidly advancing knowledge of myometrial biology, the exact mechanisms that regulate parturition are not yet understood and further work is necessary to define the complex interactions that form the key regulatory pathways controlling uterine quiescence, contractility and the transition between the two states. Furthermore, new evidence continues to emerge implicating novel mechanisms that regulate uterine activity at normal and preterm birth. This review examines current evidence regarding the key myometrial genes that have been implicated in human parturition over the past few decades, as well as more recent findings that are yet to be integrated into the current paradigm of uterine regulation.

1.2 Introduction

Preterm birth (PTB), defined as birth occurring before 37 weeks of gestation, affects 11.1% of pregnancies worldwide [1]. In recent decades, the rate of PTB has continue to increase in most countries despite advances in medical technology as well as the introduction of medical interventions designed to reduce PTB [1-4].This is cause for alarm given that PTB is a leading cause of neonatal death and is responsible for respiratory illness, neurodevelopmental disorders, such as cerebral palsy, intellectual disabilities, vision and hearing

impairments, as well as possible increased risk of cancer [3, 5]. Little progress has been made addressing PTB due to a poor understanding of the underlying mechanisms that initiate myometrial contractions. Nevertheless, Challis and colleagues proposed that parturition is divided into four distinct physiological phases (Figure 1.1) [6]. Phase 0 comprises 95% of pregnancy, and is a state of quiescence maintained through the separate or combined autocrine-paracrine actions of inhibitors of uterine contraction, such as corticotrophin-releasing hormone (CRH) and progesterone [5-9]. As term approaches the uterus undergoes myometrial activation, Phase 1 [5-9]. During this phase there is a shift from progesterone to estrogen dominance, increased expression of contraction-associated proteins (CAPs), including receptors for oxytocin and prostaglandins (PGs), increased myometrial gap junction formation and increased influx of calcium ions (Ca^{2+}) into myocytes [5-9]. This leads to increased responsiveness to uterotonins, such as oxytocin and PGs, which stimulate the uterus, Phase 2, and generate frequent, high intensity, co-ordinated phasic contractions [5, 6, 10-12]. Post-partum involution, Phase 3, is a period of placenta expulsion as well as remodelling of the uterus and is primarily influenced by maternal oxytocin [5, 6, 11]. Reviewed herein are the key myometrial genes that have been implicated in human parturition over the past few decades, as well as more recent findings in the field that are yet to be integrated into the current paradigm of uterine regulation.

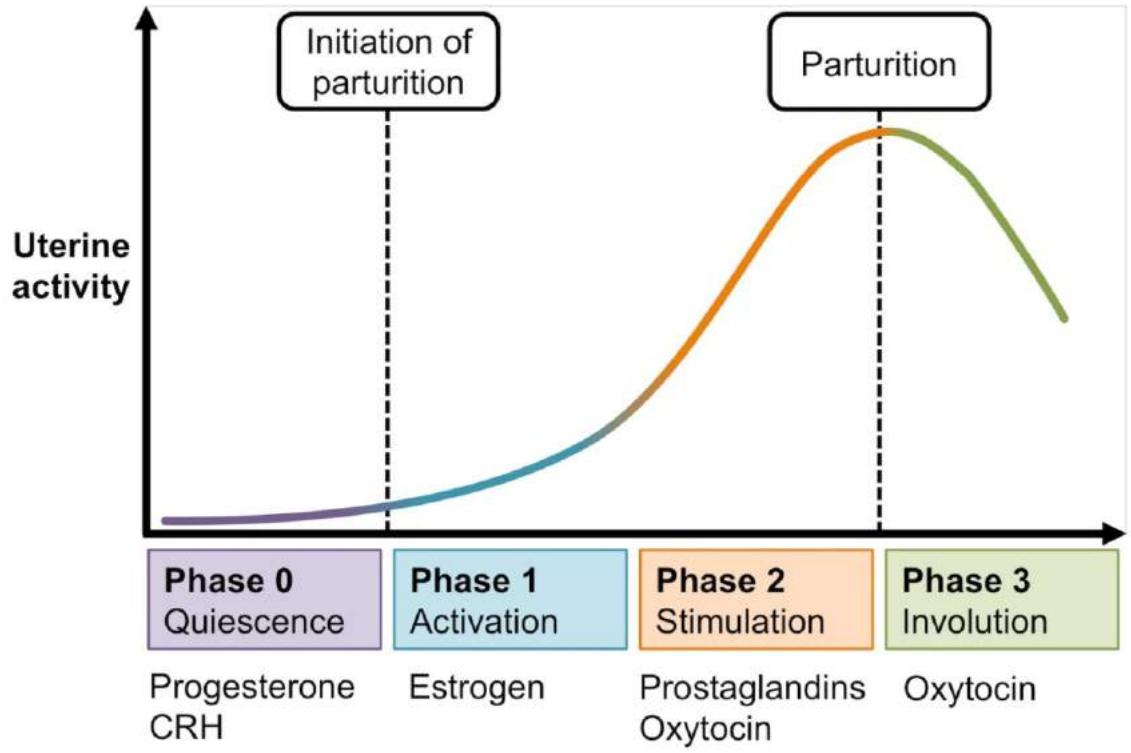

Figure 1.1. Phases of parturition.
Throughout pregnancy, the uterus is maintained in a state of quiescence (phase 0) via the actions of inhibitors, such as progesterone and CRH. Toward the end of pregnancy, the uterus undergoes activation (phase 1) and adopts a pro-contractile phenotype. Uterotonins, such as prostaglandins and oxytocin, then stimulate powerful coordinated contractions of labor (phase 2). After delivery, oxytocin mediates expulsion of the placenta and is followed by involution of the uterus (phase 3). (Modified from Norwitz *et al.* [13])

1.3 Key Myometrial Genes

Corticotrophin-releasing hormone

CRH is a peptide hormone purported to play a role in the control of myometrial quiescence and stimulation as well as fetal maturation. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the maternal pituitary, and may also acts at peripheral, non-pituitary CRH receptors [14]. Throughout human pregnancy, the placenta produces large amounts of CRH [15-17]. Placental CRH production increases progressively with advancing gestation and maternal plasma CRH concentration peaks at term [15-17]. High

CRH concentrations have been detected in umbilical cord plasma as well as amniotic fluid at term [15, 16, 18]. Following the discovery of a high-affinity binding protein for CRH (CRH-BP) [19], Linton *et al.* [14] measured plasma CRH-BP during human pregnancy. For the majority of pregnancy, CRH-BP was found to be present at high concentrations in the maternal plasma, effectively rendering circulating CRH biologically inactive [14]. In the final weeks of pregnancy, however, CRH-BP levels decreased significantly, thus increasing levels of biologically active circulating CRH in maternal plasma [14]. Linton *et al.* [14] also reported that CRH-BP returned to normal non-pregnant concentrations within 48 h of delivery. These findings indicate that the last few weeks of gestation are associated with an increase in the availability of biologically active CRH. These results are consistent with findings by Perkins *et al*. [20], who reported high levels of CRH-BP in non-pregnant women as well as in pregnant women during the majority of pregnancy (Phase 0). Perkins *et al*. also found that CRH-BP levels dropped significantly in the final few weeks of pregnancy and then returned to normal non-pregnant levels within 48 h of delivery, and suggested a role for the fetoplacental unit in CRH-BP production [20]. In addition, Perkins *et al*. examined CRH-BP levels in pregnancies complicated by diabetes, preterm labor or preeclampsia. Diabetes had no effects on CRH-BP levels at each gestational age, however, CRH-BP levels were significantly reduced in pregnancies complicated by preterm labor or pre-eclampsia [20].

Significantly decreased CRH-BP levels during the final 3 weeks of gestation, reported by Linton *et al.* [14] and Perkins *et al*. [20], suggests that the increase in free CRH availability in late pregnancy might be linked to the mechanism responsible for the initiation of parturition in humans. These results

led to a prospective, longitudinal cohort study performed by McLean *et al*. [21] where CRH and CRH-BP levels were measured in 485 pregnant women during pregnancy. This study showed that women who labored preterm had an early rise in CRH, while women who labored post-term had a later rise in CRH compared to women that labored at term [21]. This discovery led to proposal of the 'placental clock'. Placental production of CRH was hypothesized as a marker of the 'placental clock', which appeared to be active from the early stages of human pregnancy and determines the timing of parturition and delivery [21]. Furthermore, McLean *et al.* reported an association between the exponential increase in maternal plasma CRH concentrations with advancing pregnancy and the significant decrease in maternal plasma CRH-BP concentrations in late pregnancy [21]. Following this hypotheses that CRH is a placental clock that determines the length of gestation as well as the timing of parturition, Inder *et al.* [22] performed a prospective longitudinal study to determine if a single measurement of plasma CRH could predict PTB. The study found that a single CRH measurement of more than 90 pM at 26 weeks of gestation had a sensitivity of 45% and a specificity of 94% to predict a group of women at risk of PTB, nevertheless over 50% of PTBs remained unpredictable. The measurement of plasma CRH is therefore not sensitive enough to be part of the routine clinical practise to predict PTB.

Following the finding that PTB was associated with an early rise in CRH, Hobel *et al.* [23] looked for a link between maternal stress during pregnancy and elevated maternal levels of CRH, as well as the activation of the placental-adrenal axis (described as a complex set of direct influences and feedback interactions that exist between the hypothalamus, the pituitary

gland and the adrenals [24]) before PTB. They also found that women who delivered preterm had significantly higher maternal plasma levels of CRH at 18 – 20 weeks and 28 – 30 weeks of gestation compared to women that delivered at term. They did not find any difference between stress levels (reported by an abbreviated version of the Perceived Stress Scale) at 18 – 20 weeks and 28 – 30 weeks of gestation, however, significant differences were found in CRH levels at 28 – 30 weeks of gestation following maternal stress at 18 – 20 weeks of gestation. Thus, a substantial body of indirect associative evidence indicates that placental-derived CRH might play a role in the initiation of labor. However, there is still a lack of data regarding the direct cellular, as well as molecular, action of CRH in the myometrium, fetal membranes and placenta. It is known that placental CRH release is stimulated by regulatory factors, such as norepinephrine, acetylcholine, epinephrine, angiotensin II, interleukin-1, oxytocin, and arginine-vasopressin, [25] and that these ligands often act on various cells via cAMP-signalling pathways [26]. As such Cheng *et al.* [27] examined the role of cAMP in the regulation of CRH expression in primary cultures of human placental trophoblasts. They found that forskolin and 8-bromo-cAMP, both activators of protein kinase A (PKA), increased the activity of the CRH promoter in a dose-dependent manner [27]. Furthermore, they found that cAMP stimulated the activity of the CRH promoter in human placental cells via the cAMP regulatory element (CRE) in the CRH promoter region. Recent studies in primary placental cells have shown that progesterone is also involved in the regulation of CRH gene transcription via the CRE in the CRH promoter [28].

In an earlier study Grammatopoulos *et al*. [29] reported that CRH increases cAMP production by myometrial membrane preparations. Following up on these findings, Tyson *et al.* [30] investigated the role of CRH by determining the effect of CRH, together with progesterone, on contracting strips of preterm and term human myometrium. They found that CRH caused dose-dependent relaxation of spontaneously contracting myometrial strips in both preterm and term samples [30]. Furthermore, CRH with progesterone pre-treatment reduced the contractility of myometrial strips and thus promoted myometrial quiescence [30]. In addition, CRH with Rolipram (ROL) pre-treatment, a phosphodiesterase 4 (PDE4) inhibitor that increases intercellular cAMP concentrations, also reduced the contractility of myometrial strips and led to myometrial relaxation [30]. These results suggest that CRH might act to relax the uterus in late pregnancy. Grammatopoulos *et al*. [29] reported, however, that the ability of CRH to stimulate cAMP production by myometrial membranes was reduced at term. Furthermore, findings by Europe-Finner *et al.* [31] indicate that the ability of cAMP, and thus CRH, to promote relaxation is dependent on the coupling of adenylyl cyclase to the G alpha subunit ($G_{\alpha s}$), and that down-regulation of $G_{\alpha s}$ occurs at the time of labor decreasing the relaxatory effect of cAMP. The relaxing action of CRH, therefore, appears to diminish at labor, and it has been proposed that a switch of receptor coupling under the influence of oxytocin redirects CRH action towards pathways that stimulate contractions [32].

Previous studies have demonstrated that the increased expression of Connexin 43 (Cx43) in uterine muscle is crucial for uterine contraction [33, 34]; therefore Wu *et al.* [35] investigated the effects of CRH on Cx43 expression in

human myometrial smooth muscle cells. They have found that CRH facilitated Cx43 mRNA and protein expression by up-regulating the expression of c-Fos, the activator protein (AP)-1 subunit that plays a crucial regulatory function in Cx43 expression [35, 36]. In addition, Wu *et al.* found that CRH-mediated up-regulation of Cx43 can be neutralised by blocking AP-1 sites of the Cx43 promoter in human myometrial smooth muscle cells [35]. More recently, Zhang *et al.* [37] investigated the effect of CRH on contractility of human term myometrium and compared those effects in laboring and non-laboring myometrial strips. They found that CRH had no effect on spontaneous contractions in laboring myometrium, however, in non-laboring term myometrial strips, CRH displayed dose-dependent decrease in the amplitude of contractions rather than a change in the frequency of contractions [37]. This CRH-mediated decrease in contraction amplitude was blocked by pre-treating myometrial strips with a CRH-R1 antagonist, antalarmin, but not by pre-treating myometrial strips with a CRH-R2 antagonist, astressin 2B [37].

Progesterone

In the 1950s, Arpad Csapo performed studies in a variety of species and provided the first evidence that the steroid hormone progesterone (‘pro’: in favor; ‘-gest’: gestation; “-ster”: a steroid ‘-one’: ketone chemical structure) maintains pregnancy by promoting myometrial relaxation and quiescence [38]. This discovery led to the “progesterone block” hypothesis, which hypothesized that progesterone maintains pregnancy by blocking myometrial contractions, while its withdrawal leads to the end of pregnancy [38]. Consistent with this hypothesis, in non-primate mammals a fall in maternal progesterone levels leads to the initiation of labor [7, 39-42]. For instance, prior to the onset of labor

in mice, rats, rabbits, pigs and goats there is a decline in the activity of the corpus luteum, which is the source of progesterone during pregnancy, while in sheep there is a decrease in placental progesterone secretion, which leads to reduced circulating levels of maternal progesterone in these species [7, 39-42]. In humans and higher primates, however, maternal, fetal and amniotic concentrations of progesterone do not decline prior to labor; instead the levels remain elevated during parturition and delivery (Figure 1.2) [8, 9, 43]. Interestingly, although circulating progesterone levels do not decline in humans and higher primates at the end of pregnancy, the administration of RU486 (a synthetic progesterone antagonist) to Rhesus monkeys or humans at any stage of pregnancy promotes cervical ripening and parturition [44-49]. This suggests that progesterone is essential in maintaining primate pregnancy and that parturition involves a functional, rather than systemic, progesterone withdrawal where myometrial cells become refractory to nuclear progesterone receptor (nPR)-mediated relaxatory action.

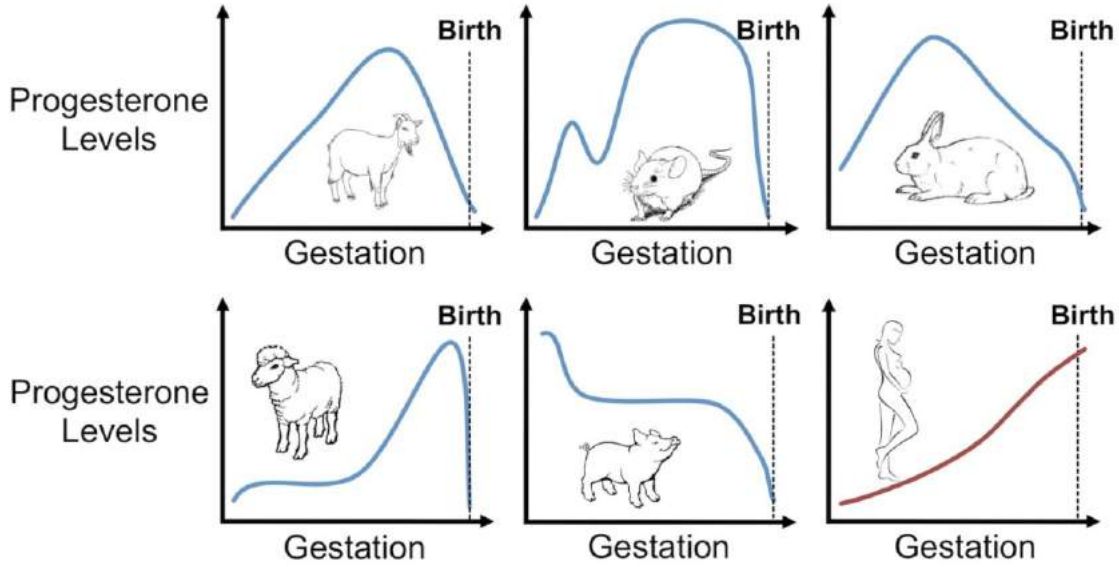

Figure 1.2. Progesterone levels across fetal gestation.
In most mammals, parturition is preceded by a drop in maternal progesterone levels. In humans, however, circulating progesterone levels remain elevated up to and during delivery.

The nPRs have distinct functions; full length nPR-B is the principal mediator of progesterone action and maintains uterine quiescence, whereas truncated nPR-A represses the transcriptional activity of nPR-B, and therefore, decreases progesterone responsiveness [4, 44, 45, 49]. The degree to which nPR-B activity is decreased by nPR-A depends on the amount of nPR-A relative to nPR-B. Thus, genomic progesterone responsiveness is believed to be regulated by the opposing actions of nPR-A and nPR-B and is inversely associated with the nPR-A/nPR-B ratio [4, 44, 45, 49]. Several research groups have tested the hypothesis that functional progesterone withdrawal in human parturition could be mediated by an increase in the nPR-A/nPR-B ratio to effectively reduce responsiveness to progesterone [50-57]. Studies performed by Pieber *et al.* [54] on human myometrial samples showed that nPR-A protein abundance increased during labor, while levels of nPR-B were not altered by the labor status. In addition, Merlino *et al.* [55] found that the nPR-A/nPR-B protein ratio in pregnant human myometrium was 0.5 (a PR-B dominant state) at 30 weeks gestation, which increased to 1.0 at term prior to the onset of labor, and at the time of the labor the ratio increased further to 3.0 (a PR-A dominant state). All these findings support the nPR-A/nPR-B hypothesis for functional progesterone withdrawal.

More recently, several studies have been looking at epigenetics and the possibility that epigenetic regulation of nPRs is responsible for functional progesterone withdrawal. Li *et al*. [58] explored the possibility that DNA methylation is involved in regulation of myometrial nPR isoform expression. They found that nPR-A gene expression significantly increased during normal labor, while nPR-B gene expression remained unchanged [58]. Furthermore,

they found that increased nPR-A gene expression was associated with decreased DNA methylation in its promoter as well as increased gene expression of both DNA methyltransferase (DNMT) 1 and DNMT3a [58]. These results imply that DNA methylation is a crucial epigenetic mechanism of labor-related differential expression of nPRs, thus regulating the biological process of functional progesterone withdrawal. Chai *et al.* [59] have also examined the methylation profile of the CpG island of the nPR gene in pregnant myometrial tissues. In both preterm and term samples, they found a significant decrease in methylated CpGs with labor downstream of the PR-B transcription start site. Previous studies performed in endometrial cells in endometriosis have shown that CpG methylation in this region is associated with the down-regulation of PR-B expression [60]. Additionally, Chai *et al.* [61] found significantly higher levels of acetylated histones at the nPR-A promoter compared with the nPR-B promoter in term human myometrial samples in labor. Therefore, it is possible that the combination of increased histone acetylation and the decreased DNA methylation leads to the increased expression of nPR at the time of labor, with a greater increase in nPR-A. Furthermore, Condon *et al.* [62] found a noticeable decrease in histone H3 acetylation in myometrium obtained from women in labor as well as in term mouse uterine tissue. To analyse the significance of this decrease in histone acetylation in the pregnant uterus at term, they administered Trichostatin A (TSA), a specific and potent histone deacetylase inhibitor (HDACi), to pregnant mice late in gestation and found that that histone acetylation was increased and that labor was delayed by 24 – 48 hours [62]. These results suggest that the decrease in histone acetylation in the uterus near term could impair nPR function and the subsequent decrease in the

expression of nPR-responsive genes would be anticipated to enhance sensitivity of the uterus to contractile stimuli [62].

Histone acetylation can be modified by histone acetylases (Lysine (K)-acetyltransferases [KATs]) and histone deacetylases (HDACs) [63, 64]. Studies of breast cancer cells have previously shown that HDACs, particularly HDAC1, regulates estrogen receptor (ER) α expression by binding to the ERα promoter [65, 66]. Ke *et al*. [67] found that low expression of HDAC1 was associated with increased expression of nPR-A in myometrium during labor [67]. Furthermore, they found that HDAC1 down-regulated nPR-A expression by binding to the promoter region of nPR-A. These results suggest that HDAC1 may play a role in regulating ER and PR isoform expression promoting functional progesterone withdrawal and estrogen activation.

Trimethylation of lysine 4 of histone H3 (H3K4me3) is believed to be a gene-activating histone modification that marks the transcription start sites (TSS) of actively transcribed genes and is associated with binding of ribonucleic acid (RNA) polymerase II (Pol II) [68-70]. Therefore, multiple studies have explored the H3K4me3 modification. Pavri *et al.* [71] observed that the promoter and coding regions became trimethylated on H3K4 upon transcription *in vitro* and *in vivo*. Furthermore, they found that H3K4me3 is responsible for recruitment of factors that coordinate effects downstream of transcription [71]. This is consistent with a study by Sims *et al.* [69] which found that H3K4me3 recruits the human chromodomain-helicase-DNA-binding I protein (CHD1) through specific tandem CHD1-chromodomains, and thus facilitates pre-mRNA maturation. More recently, Chai *et al.* [61] looked at the involvement of several

activating modifications at the PR promoter in myometrium obtained from term women in-labor (IL) and not-in-labor (NIL). They found significantly higher levels of the activating modifications (aH3, aH4, and H3K4me3) at the PR-A promoter compare to the PR-B promoter regardless of labor status [61]. Furthermore, they found that H3K4me3 increased at both promoters in laboring term samples; however, the increase was significantly greater at the PR-A promoter compared to the PR-B promoter [61]. In addition, they also examined repressive modifications (H3K9me3, H3K27me3 and H3R2me2a) and have found modest but significant levels of repressive modifications at both PR promoters regardless of labor status [61]. This suggests that both PR-A and PR-B promoters are in a permissive chromatin state, and are primed for transcription by activating histone modifications.

Given that the activating modification H3K4me3 appears to play a role in regulating PR gene expression, Chai *et al.* [59] examined the H3K4me3 methyltransferases SET and MYND domain-containing protein 3 (SMYD3) and demethylases Jumonji AT-rich interactive domain 1A (JARID1A) at the PR promoters in the myometrial tissues obtained from term and preterm women. They found significant binding of SMYD3 near the TSS of both nPR-A and nPR-B, however, there was a significantly higher enrichment at the nPR-A promoter compared to nPR-B, regardless of labor status [59]. They also found that prior to term labor; JARID1A bound to the nPR-A promoter but not the nPR-B promoter, and that there was a significant decrease of this occupancy at the onset of labor [59]. This suggests that H3K4 demethylase activity is reduced at the nPR-A promoter in the myometrium at labor [59]. These results are consistent with their previous findings where they found increased H3K4

trimethylation and transcriptional activity [61]. Nevertheless, the factors controlling JARID1A targeting to the nPR-A promoter remain unknown; however, one possible explanation is that it may be estrogen driven.

More recently, Nadeem *et al*. [72] found that during pregnancy progesterone-liganded nPR-B forms a complex with JUN/JUN homodimers and P54nrb/Sin3A/HDAC to inhibit transcription of *GJA1*, which encodes Cx43. In contrast, during labor nPR-A dissociates from progesterone, thus becoming unliganded, and in this unliganded state nPR-A interacts with FRA2/JUND heterodimers to activate *GJA1* transcription [72]. This data offers a mechanistic explanation of the 'functional progesterone withdrawal' hypothesis where human labor occurs despite the presence of high circulating levels of progesterone (Figure 1.3). In addition, this data might be relevant to other labor associated genes, such as Prostaglandin-Endoperoxide Synthase 2 *(PTGS2), OXTR*, *NFκB2* and several pro-inflammatory cytokines, chemokines and extracellular matrix proteins, which are also known to be regulated by AP-1 transcription factors. Furthermore, they found that the dissociation of nPR-A from progesterone in the myometrium is due to a decrease in intracellular progesterone levels as a result of increased expression of the metabolizing enzyme, 20α hydroxysteroid dehydrogenase (20αHSD) [72]. These results signify a paradigm shift in our understating of the mechanism by which progesterone regulates myometrial contractility via its receptors throughout pregnancy and labor. In addition, these results propose that a more effective therapeutic approach to PTB prevention is the use of non-metabolizable progestogen.

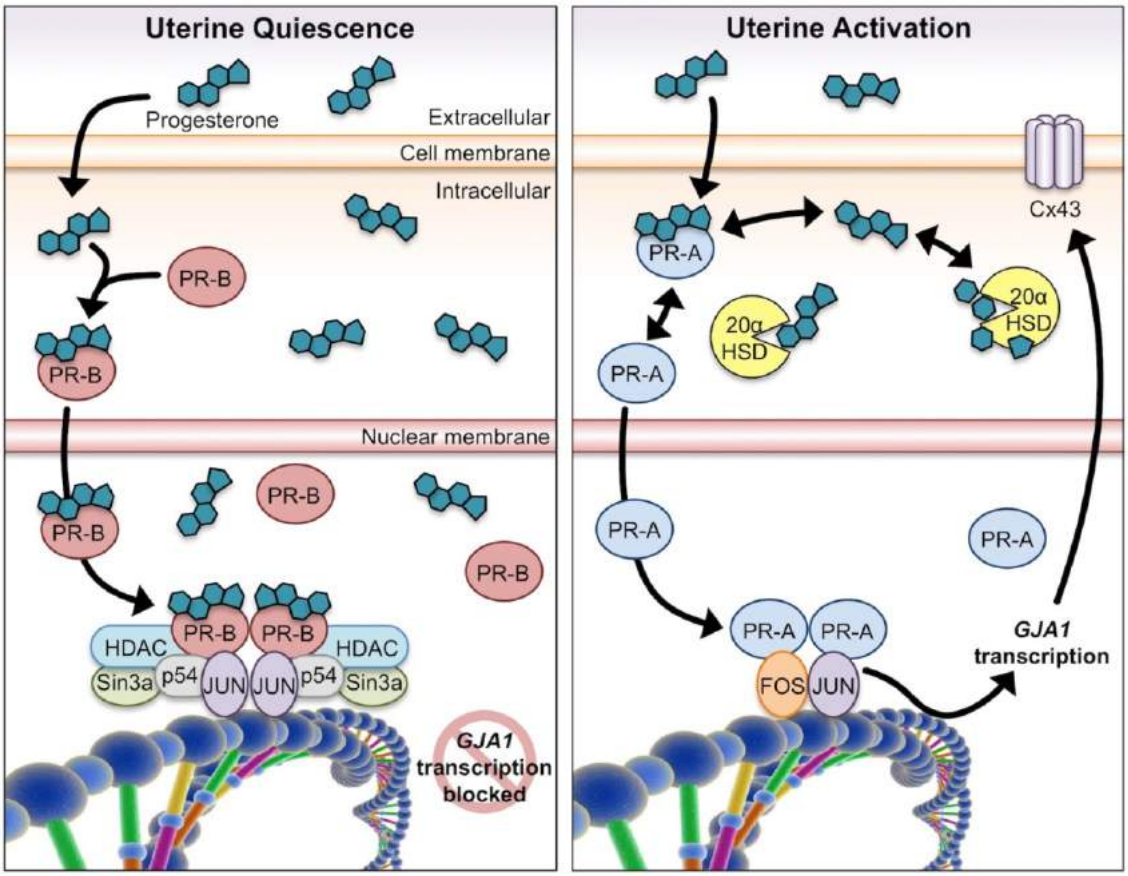

Figure 1.3. New paradigm for the 'functional progesterone withdrawal' hypothesis.
During pregnancy, progesterone-liganded PR-B forms a complex with JUN/JUN homodimers and P54nrb/Sin3A/HDAC to inhibit *GJA1* transcription, promoting uterine quiescence. At labor, PR-A is dissociated from progesterone and in this unliganded state translocates to the nucleus to interact with FRA2/JUN heterodimers to activate *GJA1* transcription, promoting uterine activation.

Estrogens

Transformation of the myometrium from the quiescent to a contractile state necessitates the presence of estrogen prior to the onset of labor [5-9, 44, 45]. Previous studies on various species have shown that estrogens are responsible for the increase in production of PGE_2 and $PGF_{2\alpha}$ [73], increased expression of genes encoding CAPs, including *OXTR* [74], α-adrenergic agonists, which regulate membrane calcium channels [75], increased synthesis of Cx43 and gap junction formation in myometrium [76], up-regulation of enzymes crucial for muscle contraction, such as myosin light chain kinase

(MLCK) and calmodulin [77-80]. All of these myometrial changes promote coordinated uterine contractions and it is generally accepted that estrogen actions are mediated by the ERs. The specific ERs and associated signalling pathways are not well characterized in the pregnant human myometrium; nevertheless we do know that there are three major forms of ERs in humans: two classical nuclear ER subtypes, ERα and ERβ, and a seven-transmembrane domain G protein-coupled receptor known as GPR30 [81-84].

In the majority of species, estrogen activation is mediated by an increase in circulating estrogen levels. In humans, however, maternal estrogen levels are high for the majority of pregnancy and remain elevated during parturition and delivery (Figure 1.4) [85, 86]. This has led to the concept of functional estrogen activation in human parturition, which is mediated by changes in expression as well as transcriptional activity in target cells. It has been shown that the ERα and ERβ subtypes, depending on the ligand and response elements, have opposite effects on gene transcription [87]. In the presence of low sub-saturating concentrations of estrogen, ERβ acts as a transdominant repressor on transcriptional activity of ERα, however, as hormone levels increase, so too does ERα expression, which leads to transcription of ERα target genes [87]. Mesiano *et al.* [56] found that the expression of ERα was low in non-laboring term myometrium and that the levels rise with the onset of labor, whilst the expression of ERβ was barely detectable and did not change with the onset of labor. This suggests that increased ERα expression mediates functional estrogen activation [56]. Furthermore, they reported a correlation between ERα mRNA levels and the PR-A/PR-B mRNA ratio, which is indicative of a functional link between the PR and ERα systems [56]. In addition, this link is in agreement

with studies performed in a range of species demonstrating that progesterone decreases expression of ERα, thus decreasing uterine responsiveness to estrogen [88, 89]. This suggests that the interaction between progesterone and PR-B suppresses ERα expression, therefore rendering the myometrium refractory to circulating estrogen [49]. However, with advancing gestation there is an increase in the expression of PR-A, which in turn represses the transcriptional activity of PR-B, and as a result the PR-B-mediated inhibition of ERα expression is withdrawn. This theory explains why inhibition of PR-mediated progesterone action is responsible for initiation of the full parturition cascade, especially since estrogen levels are high for the majority of the pregnancy. Additionally, the theory implies that human parturition is activated by events that increase myometrial expression of PR-A, and thus induce functional progesterone withdrawal and estrogen activation.

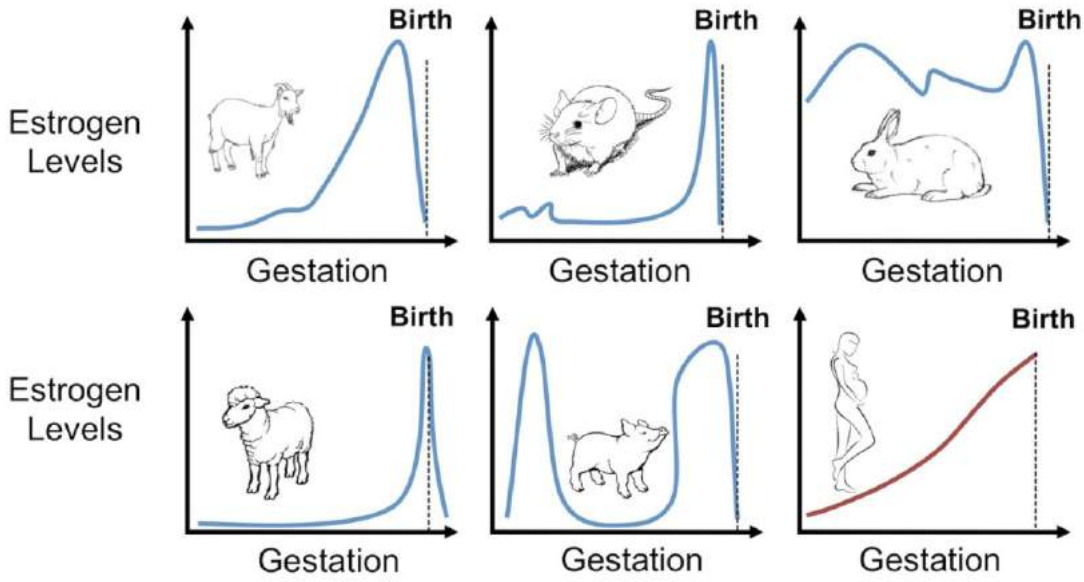

Figure 1.4. Estrogen levels across fetal gestation.
In most mammals, estrogen activation is mediated by an increase in maternal estrogen levels approaching term. In humans, however, circulating estrogen levels rise steadily across gestation.

At variance with others, Wu *et al.* [90] found that expression of ERβ increased in human term myometrial tissues compared to non-pregnant tissue and that the expression of ERα was high in non-pregnant tissues compared to term myometrial tissues. They suggest that there is a dramatic switch from ERα to ERβ expression in the myometrium throughout pregnancy [90]. In addition, they found that ERβ might inhibit AP-1 activity during pregnancy and therefore prevent the expression of Cx43 and other labor-associated genes. This suggests that once the myometrial expression of ERβ decreases, the expression of Cx43 increases, thereby promoting labor [90]. Based on these results there is a lack of consensus regarding the expression of ERα and ERβ in the human myometrium during pregnancy as well as the role ERα and ERβ play in mediating the actions of estrogen. Furthermore, the discovery of GPR30 introduced new complexity to myometrial estrogen signalling.

Welsh *et al.* [91] examined which ERs are expressed in the pregnant human myometrium and explored the signalling pathways through which estrogen regulates the expression of *OXTR*. The group has shown that ERα is the most abundantly expressed ER in the pregnant myometrium and that mRNA abundance of ERα is much higher in laboring myometrial tissues when compared to non-laboring myometrial tissues [91]. These results are consistent with previous results reported by Mesiano *et al*. [56]. Furthermore, the results showed that mRNA abundance of ERβ is extremely low, which is also consistent with previous results reported by Mesiano *et al*. [56], in contradiction to the data reported by Wu *et al.* [90]. In addition to examining ERα and ERβ, Welsh *et al.* [91] analysed GPR30 and found that GPR30 mRNA and protein are indeed present in the term pregnant human myometrium. This is consistent

with a previous study by Maiti *et al*. [92] where GPR30 was reported in term human myometrium samples. The exact role of GPR30 in uterine function during parturition is still unknown, but notably, GPR30 knockout mice have unaffected fertility, retain normal uterine estrogenic responses and appear to have normal pregnancies and labor [93-96].

The interaction between estrogens and their receptors can affect target cell function through genomic and non-genomic pathways (Figure 1.5) [97-99]. In the genomic pathway, binding of estrogens to ERs induces a conformational change in the receptors that instigates dissociation from chaperones, dimerization as well as activation of the receptor transcriptional domain [97-99]. The ER-mediated regulation of gene expression involves the direct binding of dimeric ER to DNA sequences called an estrogen response elements (ERE) [100]. Nevertheless, some pro-contraction genes, such as *OXTR* and *GJA1* (Cx43), are regulated by estrogens but do not contain EREs in their promoter regions [101, 102]. This suggests that estrogen regulates the expression of these genes by non-genomic/extranuclear pathways and/or through intervening factors that lead to downstream changes in gene expression [103-105]. In non-genomic/extranuclear pathways, cell function is affected by regulating cytoplasmic signalling cascades, which in turn affect the activity of multiple cellular processes as well as transcription factors [103-105]. The non-genomic/extranuclear pathways activated by estrogens are the extracellularly regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PKB/AKT) pathways, as well as Ca^{2+} influx and G protein signalling [103-105]. All three ERs have been found to stimulate extranuclear signalling; however, the involvement of different

receptors seems to be tissue and cell-type specific [103-105]. In the pregnant human myometrium there is very little known about extranuclear pathways, nevertheless studies performed in animals highlight the significance of this pathway in the regulation of myometrial smooth muscle contractility. In pregnant rats there is an increase in the abundance of activated ERK with gestation [106, 107] and onset of labor [108]. The administration of RU486 (a synthetic progesterone antagonist) to pregnant rats, which triggers preterm birth, caused an increase in ERK phosphorylation levels [109]. Furthermore, treatment of rats with an agent that prevents ERK activation, U-0126, caused a delay in the onset of RU-486-induced preterm labor as well as decreased the RU-486-induced increase in myometrial contractility and ERK phosphorylation [109]. This suggests that ERK activation is a part of a complex pathway that leads to development of contractions and labor in this rat model.

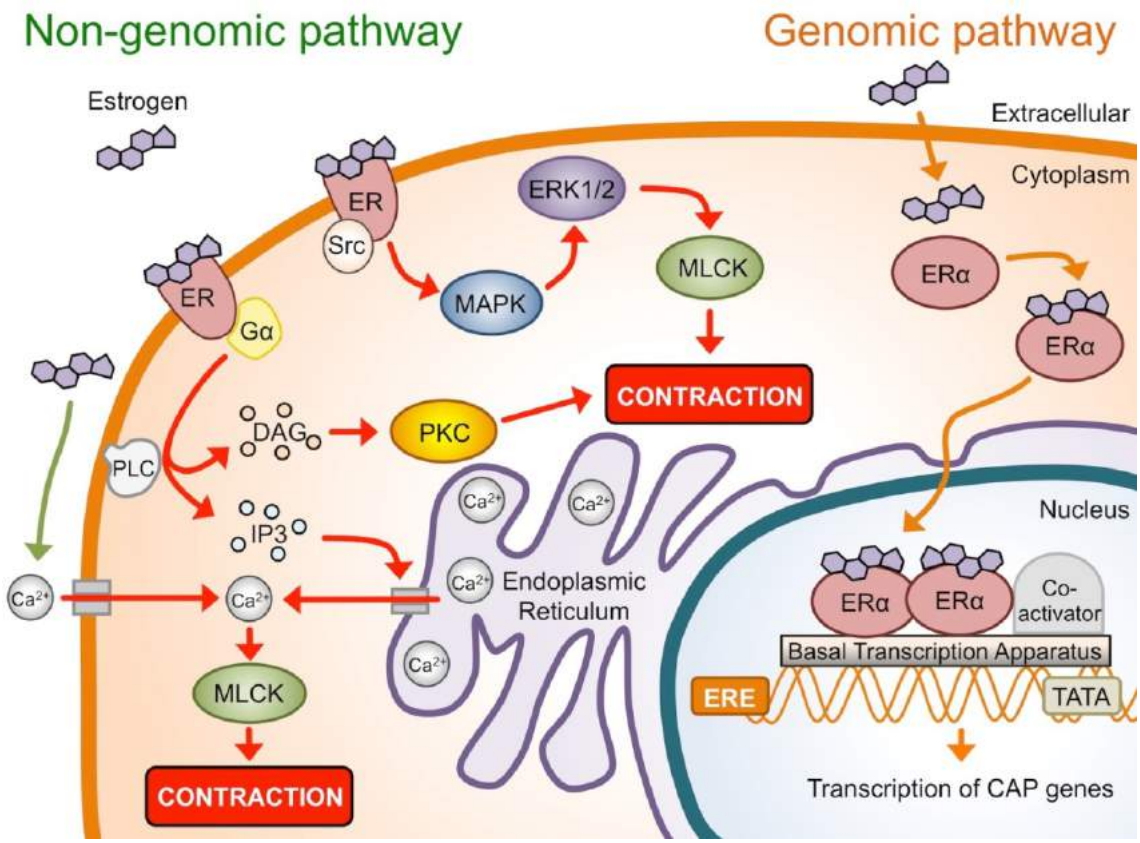

Figure 1.5. Genomic and non-genomic pathways of estrogen signalling.

In the genomic pathway, estrogen interacts with ERs to induce a conformational change in the receptors that instigates dissociation from chaperones, dimerization as well as activation of the receptor transcriptional domain. The non-genomic pathways activated by estrogens are: i) ERK/MAPK pathway, ii) PLC/PKC pathway and iii) Ca^{2+} influx. These interactions directly affect the contractile capacity of uterine myocytes.

Welsh *et al.* [91] have also examined the importance of ERs in the activation of extranuclear ERK signalling cascade in response to estrogens in term human myometrium. They found that classical ERs, but not GPR30, mediated E2-stimulated ERK1/2 phosphorylation in human myometrial samples [91]. Consistent with previous results, where they found that the expression of ERβ was extremely low in human myometrium samples, they concluded that this effect was mediated exclusively by ERα [91]. Nevertheless, Maiti *et al*. [92] revealed that the specific activator of GPR30, G1, increased ERK1/2 phosphorylation in human myometrial explants, however they did not investigate the contribution of GPR30 to estrogen action. Furthermore, Welsh *et al.* [91] examined importance of the ERK pathway in the induction of pro-contraction gene expression in the myometrium. They found that U-0126, highly selective inhibitor of MEK, totally blocked the ability of estrogens to stimulate increased expression of *OXTR* in human myometrial explants [91]. This suggests that the action of estrogens in human myometrium is mediated at least in part by extranuclear signalling via ERs through activation of the MEK/ERK cascade [91]. In addition, Welsh *et al.* [91] found that there was no change in ERK1/2 phosphorylation with labor in human myometrium. This is consistent with previous results by Oldenhof *et al*. [106] in rat myometrium; however, it varies from results done by Li *et al*. [108] who found an increase in ERK2 phosphorylation in the rat myometrium with labor. Li *et al*. [108] also reported that there was no change in total ERK protein levels in the rat myometrium with

labor; however, Welsh *et al.* [91] have found that ERK protein levels decreased in human myometrium with advancing gestation. In addition, Welsh *et al.* [91] measured phospho-ERK2 levels and found that the activity of that pathway increased with advancing gestation. This is consistent with the study performed by Paul *et al.* [110] where they found that total ERK2 protein levels increased slightly with labor in term human myometrium and that there was no change in the ratio of pERK2/total ERK2. This was the first report of ERK1/2 phosphorylation levels in human myometrium further confirming that the ERK pathway is an integral component of the human parturition cascade.

Oxytocin

Oxytocin is a potent uterotonic agent which historically was thought to be crucial for the initiation of parturition as clinical administration of oxytocin can initiate labor in pregnant women late in gestation [111]. In addition, the labor pattern induced by oxytocin was identical to the normal spontaneous labor. Therefore, many initial experiments were performed to determine the plasma levels of oxytocin during labor. In all placental mammals studied so far, such as rabbit [112], sheep [113], cow [114], rat [115], goat [116], pony [117], rhesus monkey [118], pig [119] and human [120, 121], it was observed that maternal plasma oxytocin concentrations increased at some stage of labor. This coincides with a dramatic increase in OXTR expression during parturition in guinea pigs [122], rabbits [123, 124], rats [125, 126], sheep [127] and humans [128-130]. Furthermore, studies in rat [131-133], rhesus monkeys [134, 135], baboons [136] and humans [137, 138] indicate that oxytocin antagonists can inhibit uterine contractions in late gestation and delay delivery. Based on this evidence, several investigators subscribed to the view that oxytocin functions as

the crucial factor in the initiation of labor. However, oxytocin does not stimulate cervical softening, and endogenous plasma levels of oxytocin are much lower than levels obtained by oxytocin infusion to induce labor [139, 140]. The majority of studies are therefore consistent with the possibility that the significance of oxytocin is limited to the last stage of parturition where the placenta is expelled and the uterus undergoes remodelling.

Some of the controversy concerning the role of oxytocin in labor might exist because of the technical difficulties measuring oxytocin concentrations at the time of parturition. First, oxytocin is released in a pulsatile manner and the release is maximal with fetal expulsion. There are also species differences as maximal concentration of oxytocin in the rabbit occurs at the time of delivery of the first foetus [112], while in sheep [113], cow [114] and rhesus monkey [118], maximal concentration of oxytocin occurs at the birth of the singleton. In the pig, a pulse of oxytocin occurs at the expulsion of each fetus [119], while in the rat baseline plasma oxytocin is high during labor with large pulses overlapping with pup expulsion [115]. Therefore, any future studies done to determine oxytocin levels need to have serial plasma samples as well as have in place a rapid sampling strategy. Second, the presence of oxytocinase, which degrades oxytocin, increases during gestation in human plasma [141, 142]. Therefore, to accurately measure oxytocin concentration in plasma samples researchers need to inhibit oxytocinase activity [143]. The third challenge measuring oxytocin concentration is the use of antibodies that cross react with oxytocin precursors and thus yield inaccurate results [144].

Studies performed in humans support the animal data in that plasma oxytocin concentration increases during the expulsion phase of labor. Fuchs *et al.* [120] observed the release of oxytocin in discrete pulses of short duration and these discrete pulses significantly increased during labor. In addition, Burd *et al*. [143] observed pulsatile plasma oxytocin release during the first stage of labor. Thornton *et al*. [121] found that serum oxytocin concentrations were very low in pregnant women and that levels did not increase during the second stage of labor. Nevertheless, they did find that a minority of women produced a large surge of oxytocin immediately prior to delivery. Based on these results they concluded that oxytocin is not crucial for labor unless uterine activity is regulated by low plasma oxytocin concentration or the uterus develops sensitivity to constant oxytocin concentration [121]. This is consistent with additional studies where increases in maternal serum oxytocin concentrations could not be measured until after the onset of labor [118, 145]. In addition, some studies have reported that oxytocin peak concentrations occur at the time of fetal expulsion [146, 147]. Therefore, the role of maternal plasma oxytocin in human parturition is still unclear.

The discovery of oxytocin production in intrauterine tissues led to a hypothesis that oxytocin might play a paracrine role rather than, or in addition to, an endocrine role in parturition [148]. Wathes *et al*. [149] were first to suggest that oxytocin synthesis occurs in peripheral tissues after discovering large amounts of oxytocin in the ovine corpus luteum. Following this discovery, Ivell *et al*. [150] demonstrated synthesis of mRNA encoding oxytocin in luteal tissues, and Flint *et al.*[151] found that ovine luteolysis is regulated by ovarian oxytocin and uterine $PGF_{2\alpha}$. Chibbar *et al*. [148] found synthesis of oxytocin

mRNA in amnion, chorion and decidua as well as significantly higher concentrations of oxytocin mRNA in tissues following the onset of labor at term when compared to tissues prior to the onset of labor. Intrauterine oxytocin has also been found in uterine epithelium of the rat prior to the labor [152, 153]. In this species, the total oxytocin mRNA in the intrauterine tissues was approximately 70-fold greater than in the hypothalamus three days prior to labor [152]. In addition, Fang *et al*. [154] measured oxytocin mRNA in rats and detected a 3- to 4-fold increase in uterine oxytocin mRNA between days 16 and 19 of pregnancy with no additional change throughout gestation. These results indicate that translation, in addition to transcription of the oxytocin gene, is a crucial regulatory phase in labor.

It is well documented that oxytocin acts through its G-protein-coupled receptor (GPCR) to stimulate myometrial contractions. The binding of oxytocin to its receptor activates the heterotrimeric G-proteins $G_{\alpha q/11}$ and $G_{\alpha i/o}$ which in turn activate phospholipase C (PLC), thus hydrolysing phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). Signalling through $G_{\alpha i/o}$ leads to reduced activity of adenylate cyclase and thus decreases intracellular cAMP levels, whilst signalling through $G_{\alpha q/11}$ leads to increase in intracellular concentrations of Ca^{2+} [155-157]. Increased intracellular Ca^{2+} can activate MLCK to phosphorylate myosin light chains (MLC) and instigate contraction of the myocytes (Figure 1.6) [158]. A process known as "Ca^{2+} sensitization" may also occur in response to oxytocin, where a greater contractile force generated from a given increase in cytosolic Ca^{2+} [158]. This phenomenon arises when there is an inhibition of MLC phosphatase (MLCP), an enzyme that reverses the phosphorylation of MLC [158].

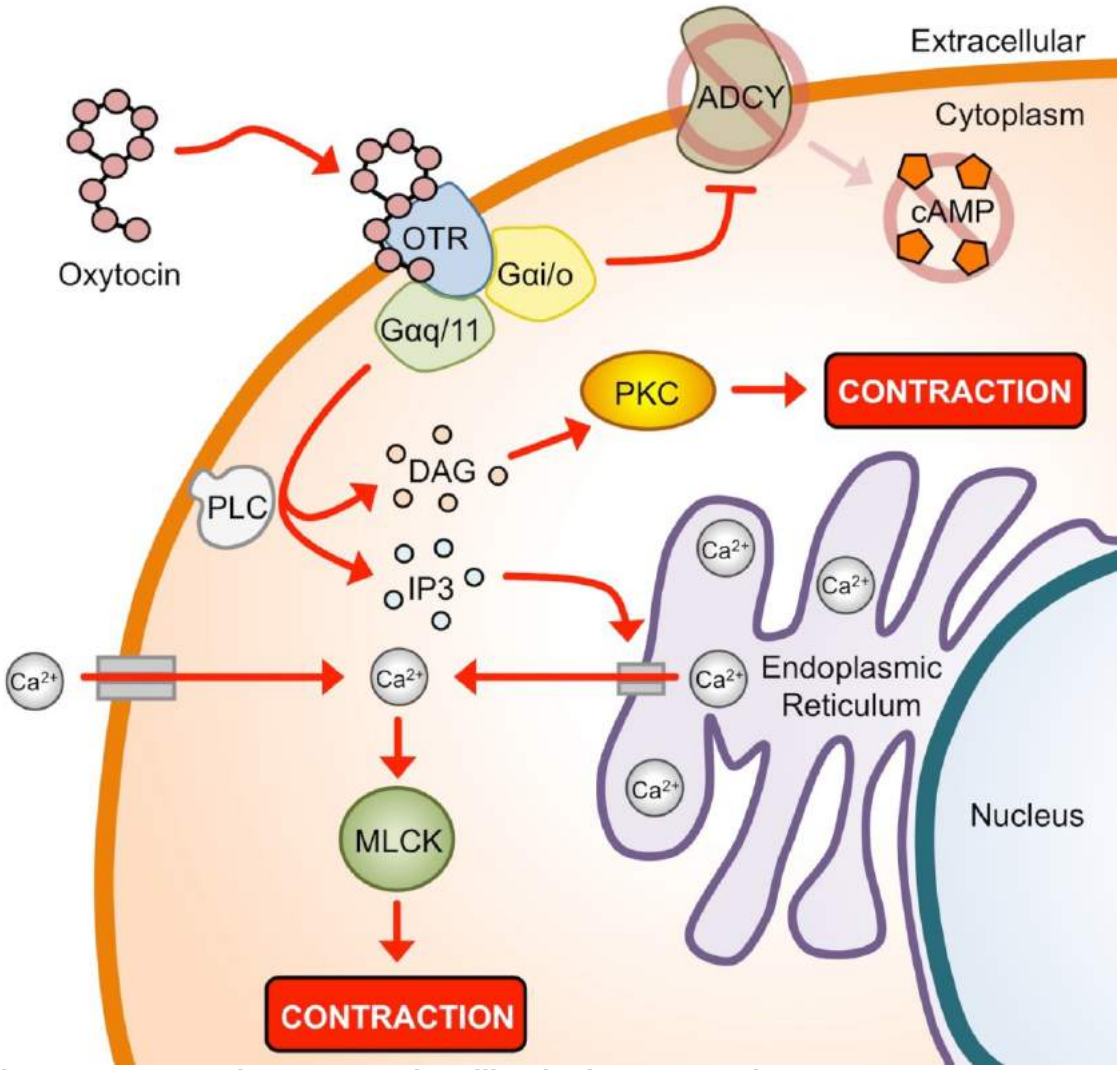

Figure 1.6. Oxytocin receptor signalling in the myometrium.
Oxytocin binding its receptor activates $G_{\alpha q/11}$ and $G_{\alpha i/o}$, which in turn activate PLC/PKC pathways. Signalling through $G_{\alpha q/11}$ raises intracellular Ca^{2+} levels, which in turn activates MLCK to phosphorylate MLC and instigate contraction. Signalling through $G_{\alpha i/o}$ reduces adenylyl cyclase activity to reduce intracellular cAMP levels.

Studies on human chorio-decidua explants showed that oxytocin increased the production of $PGF_{2\alpha}$ and PGE_2 as well as leukotrienes while in amnion oxytocin mainly increased the production of PGE_2 [159-161]. Furthermore, in the cow and the sheep, oxytocin induced the release of $PGF_{2\alpha}$ from the endometrium during pregnancy and increased the level of OTRs [162, 163]. This increase in $PGF_{2\alpha}$ and OTR was maximal at the time of parturition [162, 163]. In recent years, studies in human tissues and myometrial cell lines

showed that the binding of oxytocin to its receptor led to nuclear factor-κB (NF-κB) activation, which subsequently increased the production of PGs, inflammatory chemokines and cytokines, which are known to be involved in fetal membrane remodelling, cervical ripening and myometrial activation [164, 165]. More recently, Kim *et al.* [166] found that Atosiban, an oxytocin antagonist, failed to inhibit the oxytocin-stimulated activation of NF-κB and subsequent PG synthesis. Instead, Atosiban increased the expression of PGs and inflammatory cytokines via $G_{\alpha i-2}$ and $G_{\alpha i-3}$ [166]. This is not a desirable effect of tocolytics, thus these results suggest that therapeutic modulation of oxytocin and its receptor should take into account the effects of differential G-protein coupling of the OTR in the activation of pro-inflammatory pathways.

Prostaglandins

There is strong evidence that PGs are involved in the initiation and maintenance of human labor [167, 168]. In all mammalian species tested so far, PGs induce progesterone withdrawal and initiate parturition [169-171]. Studies in rats, mice, goats, pigs and rabbits reveal that the uterine tissues increase the production of $PGF_{2\alpha}$ late in gestation, which in turn causes luteolysis and thus induces systemic progesterone withdrawal [169-171]. In the sheep, placental PGE_2 increases prepartum cortisol release, which leads to high placental expression of the enzyme 17-α-hydroxylase/ 17,20 lyase (P450c17), and thus leads to increased pregnenolone metabolism [172, 173]. This subsequently leads to decreased secretion of progesterone and increased production of estrogen, thus promoting the onset of labor [172, 173]. In humans, $PGF_{2\alpha}$ is produced mainly by the maternal decidua and is involved in the up-regulation of oxytocin receptor levels and gap junctions in the myometrium, thus promoting

uterine contractions [174]. PGE_2 is produced by the fetus and placenta and is involved in collagen degradation and dilation of small blood vessels in the cervix, thus promoting cervical ripening as well as spontaneous rupture of the fetal membranes [175]. Furthermore, PG levels in humans increase prior to labor and throughout labor in the uterus and the fetal membranes [174, 176]. Administration of PGs or synthetic analogues at any stage of human pregnancy have also been shown to promote the pro-contractile transformation of the myometrium and the ripening of the cervix, thus inducing birth [171, 177-180]. PGs administered to women that are already in active labor leads to potent and rapid uterotonic action, whilst PG administration to women who are in the quiescent stage of pregnancy leads to labor following a latency of 15 – 20 hours. These results indicate that PGs first act as uterotrophins to transform the myometrium to a contractile phenotype, and then as uterotonics to induce contractions [171, 177-180].

PGs exert their effect through specific GPCRs using various intracellular signalling pathways [181, 182]. $PGF_{2\alpha}$ mediates its effects via a seven-transmembrane GPCR, FP. The FP receptor is linked to the PLC/protein kinase C (PKC) pathway, which upon activation increases intracellular levels of Ca^{2+} and promotes smooth muscle contraction [183, 184]. PGE_2 effects are mediated via four related receptors termed E prostanoid receptor $(EP)_1$, EP_2, EP_3 and EP_4. All four receptors are present in the human uterus. EP_1 activation increases intracellular levels of Ca^{2+} and thus promotes contraction, whilst EP_2 and EP_4 activation raises intracellular cAMP levels, thus promoting relaxation [183, 184]. The EP_3 receptor is unique in that there are multiple isoforms and depending on the particular isoform can either stimulate contractions, by

increasing intracellular levels of Ca^{2+}, or promote relaxation via cAMP/PKA signalling [185, 186].

Several studies demonstrated that EP and FP receptors are regulated by progesterone and estrogen [187-189]. Lim *et al.* [187] found that EP_2 expression in the mouse uterus was up-regulated by progesterone and further increased by estrogen. Furthermore, they found that EP_2 was expressed selectively in the luminal epithelium mainly on day 4, which is the day of implantation, and day 5, which is early implantation, of pregnancy [187]. In addition, Dong *et al*. [188] reported that in rat uterus EP_2 expression increased throughout pregnancy and decreased during term labor, whilst FP expression increased throughout pregnancy and reached maximal levels during labor. Furthermore, they found that when ovariectomized rats were treated with progesterone the expression of EP_2 increased while FP expression stayed the same [188]. In contrast, when ovariectomized rats were treated with estrogen, the expression of FP increased whilst EP_2 expression stayed the same [188]. These results suggest that progesterone and estrogen regulate the PG responsiveness of the uterus by controlling the expression of relaxant EP_2 and contractile FP receptors [188]. More recently, Blesson *et al.* [189] reported that in rat uterus ERα down-regulated all EPs and FP expression, while ERβ down-regulated only EP_2 and EP_4 expression. Furthermore, they found that estradiol up-regulated the expression of EP_2 and EP_4 proteins, while the combination of progesterone and estradiol treatment up-regulated the expression of EP_1 and EP_3 proteins in the myometrium [189]. This suggests that steroid hormones regulate the uterine expression of EPs and FP to promote relaxation during pregnancy and contractility at labor [189].

Several studies explored the localization of EP and FP receptors in the gestational tissues assessed their role in normal labor [190-193]. Grigsby *et al*. [191] examined paired upper and lower segment myometrium to determine the localization of EP and FP receptors during human pregnancy. They found that all receptor subtypes were present in all myometrial layers, however, a change was observed in intracellular localization in term labor samples where EP_1 and EP_4 receptors were mainly located in the nucleus [191]. They found no change in the expression of PG receptor subtypes in relation to gestational age, labor status, or between the upper and lower myometrial segments [191]. They did find, however, that the expression of PG receptor subtypes differ in maternal and fetal tissues; EP_1 and EP_4 were mostly expressed in the fetal membranes, EP_2 expression was the highest in the myometrium, while EP_3 and FP were equally expressed in the myometrium and the fetal membranes [191]. This suggests that the myometrial activation is mediated by the balance between one or more PG receptor subtypes in addition to other known CAPs [191]. Studies on baboons showed that expression of EP_1 and EP_3 was significantly higher in myometrium from the fundus compared to the lower segment, whilst the expression of EP_2 was significantly lower in the fundus [190]. The study also found that labor was associated with a decrease in the regional variation of EP_2, but not with EP_1 and EP_3 expression [190]. This suggests that regional and labor-related localization of PG receptors in the uterus might be crucial for primate parturition [190].

The presence of PG receptors in the cervix have been reported in humans, baboons, rodents and goats [194-198], which is consistent with the role of PGs in cervical ripening and the initiation of labor in these species [199-

203]. Blesson *et al.* [198] examined the expression and localization of all PG receptors in the cervix of non-pregnant, term pregnant and postpartum women. They found that expression of the relaxation promoting EPs, EP_2 and EP_4, was lowest in the term pregnant women just prior to final cervical ripening [198]. In addition, contraction-inducing PG receptors were absent in the majority of cell types, thus suggesting they might not play a role in cervical ripening [198]. This is in agreement with the study by Hinton *et al*. [194] in rats.

Recent work explored the possibility that PGs are involved in the mechanism of functional progesterone withdrawal [204]. Immortalised human pregnant myometrial cells, PHM1-31 cell line, were treated with PGE_2 and $PGF_{2\alpha}$ to test the hypothesis that PGs induce functional progesterone withdrawal by altering myometrial PR expression. They found that $PGF_{2\alpha}$ produced a dose-dependent increase in PR-A expression, but not PR-B expression, thereby increasing the PR-A/PR-B ratio [204]. PGE_2, however, dose-dependently increased the both PR-A and PR-B expression causing a biphasic effect on the PR-A/PR-B ratio [204]. Low PGE_2 concentrations increased the abundance of PR-A relative to PR-B while high PGE_2 concentrations restored the ratio to basal levels [204]. This biphasic response is characteristic of PGE_2 action since PGE_2 has four EP receptors. Therefore, when more than one EP receptor is present, one can mediate relaxation while another can mediate contractions [204]. PHM1-31 cells were also treated with cyclic-8-bromoadenosine monophosphate (8-Br-cAMP), an agonist for the PKA pathway, and phorbol 12-myristate 13-acetate (PMA), a potent PKC activator [204]. They found that 8-Br-cAMP increased both PR-A and PR-B expression, with no net effect on the PR-A/PR-B ratio [204]. These results are consistent

with the interaction between PGE_2 and its EP_2 and/or EP_4 receptors [204]. PMA mimicked the effects of $PGF_{2\alpha}$ and selectively increased PR-A expression but not PR-B expression, thus increasing the PR-A/PR-B ratio [204]. These results suggest that PGs, acting via specific receptors using different intracellular signalling pathways, can differentially regulate PR-A and PR-B expression (Figure 1.7) [204].

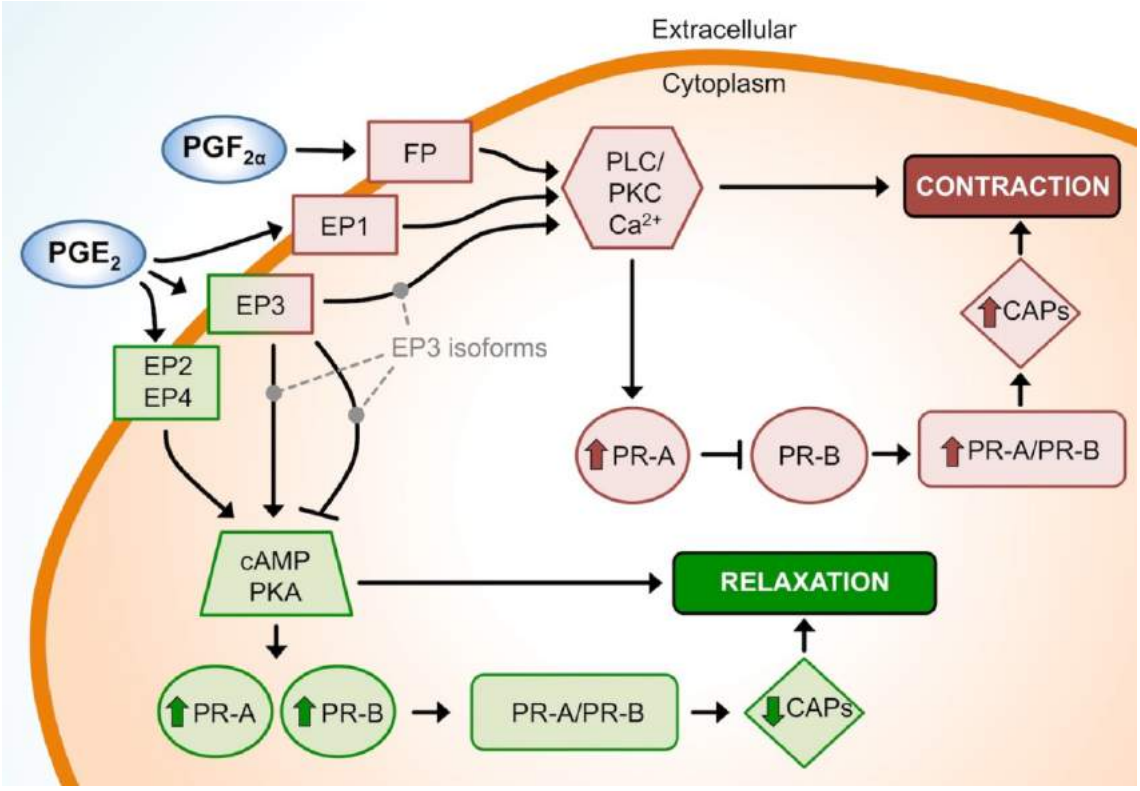

Figure 1.7. Model for prostaglandin-induced functional progesterone withdrawal in human myometrium.
$PGF_{2\alpha}$ action is mediated by the PLC/PKC-linked FP receptors, which selectively increase the expression of PR-A, thus increasing PR-A/PR-B expression ratio leading to CAP expression. PGE_2 acts through four receptor subtypes, EP_1, EP_2, EP_3 and EP_4. EP_1 mediates smooth muscle contraction. EP_2 and EP_4 increase intracellular cAMP levels, which in turn increases both PR-A and PR-B expression, with no change in the PR-A/PR-B expression ratio. This represses the expression of CAPs and maintains relaxation in the myometrium. EP_3 receptor has at least nine isoforms. Depending on the isoform expressed, EP_3 signalling can inhibit and/or stimulate production of cAMP, or increase levels of intracellular Ca^{2+}, causing contraction or relaxation, respectively.

Milne *et al*. [205] used human first-trimester decidua to determine the separate and combined effects of Mifepristone and the PGE analogue

(Gameprost) on leukocyte populations and steroid receptor expression. They found that administration of both Mifepristone and Gameprost significantly increased macrophage and neutrophil numbers, while natural killer cell numbers remained unchanged [205]. Furthermore, Mifepristone and Gameprost significantly decreased immunoreactive PR and ERα levels but had no effect on androgen receptor or ERβ receptor expression [205]. They also found that Gameprost alone decreased PR expression in first-trimester decidua [205]. Subsequently, Goldman *et al.* [206] examined the effect of $PGF_{2\alpha}$ on the expression of the PRs in the term human decidua and found that $PGF_{2\alpha}$ decreased the expression of all forms of PR isoforms. Based on these results they theorised that functional progesterone withdrawal along with decreased expression of PR responsive genes in the decidua ultimately lead to increased sensitivity to contractile stimuli [206].

Recently, Welsh *et al.* [207] used the guinea pig, another mammalian species that lacks systemic progesterone withdrawal, to study PR-mediated functional progesterone withdrawal during late pregnancy and labor *in vivo*. There was a significant decrease of both PR-A and PR-B protein abundance as well as *PR* mRNA expression during the last third of gestation and in labor [207]. This decrease in both PR-A and PR-B was also detected in guinea pig pregnancies that were experimentally manipulated to induce growth restriction as well as render the dames prone to preterm birth [208]. Earlier work done in term guinea pig myometrium found a decrease of high affinity progesterone binding in the nuclei of myocytes, thus further supporting declining expression of PR as a possible mechanism of functional progesterone withdrawal in term guinea pigs [209, 210]. More importantly, this study investigated whether PGs

promote functional progesterone withdrawal by altering myometrial PR expression *in vivo* [207]. Following the treatment with Sulprostone, a PGE-analogue with high potency to induce parturition in guinea pigs [211], there was a significant decrease in PR-A protein abundance and *PR* mRNA expression in the uterus at day 45 of pregnancy (which corresponds to the last third of gestation), thus mimicking the changes that happen prior to normal birth [207]. This down-regulation of PR-A expression by labor-stimulating PGs may decrease uterine progesterone responsiveness, thus contributing to functional progesterone withdrawal in the guinea pig [207]. In conclusion, the mechanism of receptor-mediated functional progesterone withdrawal in the guinea pig uterus is similar to that in the human decidua, but different from what is observed in the human myometrium. Prostaglandins facilitate the receptor-mediated changes in both species and tissues suggesting that guinea pigs may be a useful model to study the hormonal regulation of pregnancy and parturition in experimental settings that are not feasible in women.

1.4 Recent Advances

A recent study by Chan *et al*. [212] used high throughput RNA sequencing to compare human myometrial samples isolated from women who were NIL with women who were IL. They documented a significant number of novel transcripts of both protein-coding mRNA and microRNA. This list of differentially expressed genes is largely, but not completely, consistent with previous microarray data [213-219]. Furthermore, their data is consistent with previous gene expression studies performed on human myometrium. For instance, they reported a down-regulation of *OXTR* mRNA transcripts in

samples that were collected during labor [212], which is consistent with the study performed by Phaneuf *et al.* [220] where *OXTR* mRNA and protein expression reportedly increased prior to labor and then decreased during the labor. In addition, Chan *et al*. [212] reported a labor-associated decrease in mRNA encoding the zinc finger E-box binding homeobox protein (ZEB1). Renthal *et al.* [221] previously reported that ZEB1 is directly up-regulated by the action of progesterone/PR at the ZEB1 promoter. Furthermore, they have found that ZEB1 and ZEB2 decreased the expression of contraction-associated genes, *OXTR* and *GJA1*, as well as inhibited oxytocin-induced contractility in human myometrial cells [221]. These results suggest that ZEB1 and ZEB2 are important progesterone/PR-mediated regulators of uterine quiescence and contractility throughout pregnancy and parturition (Figure 1.8) [221]. The study by Chan *et al*. [212] provides an excellent resource for further work to define the regulatory network controlling uterine activity at term parturition.

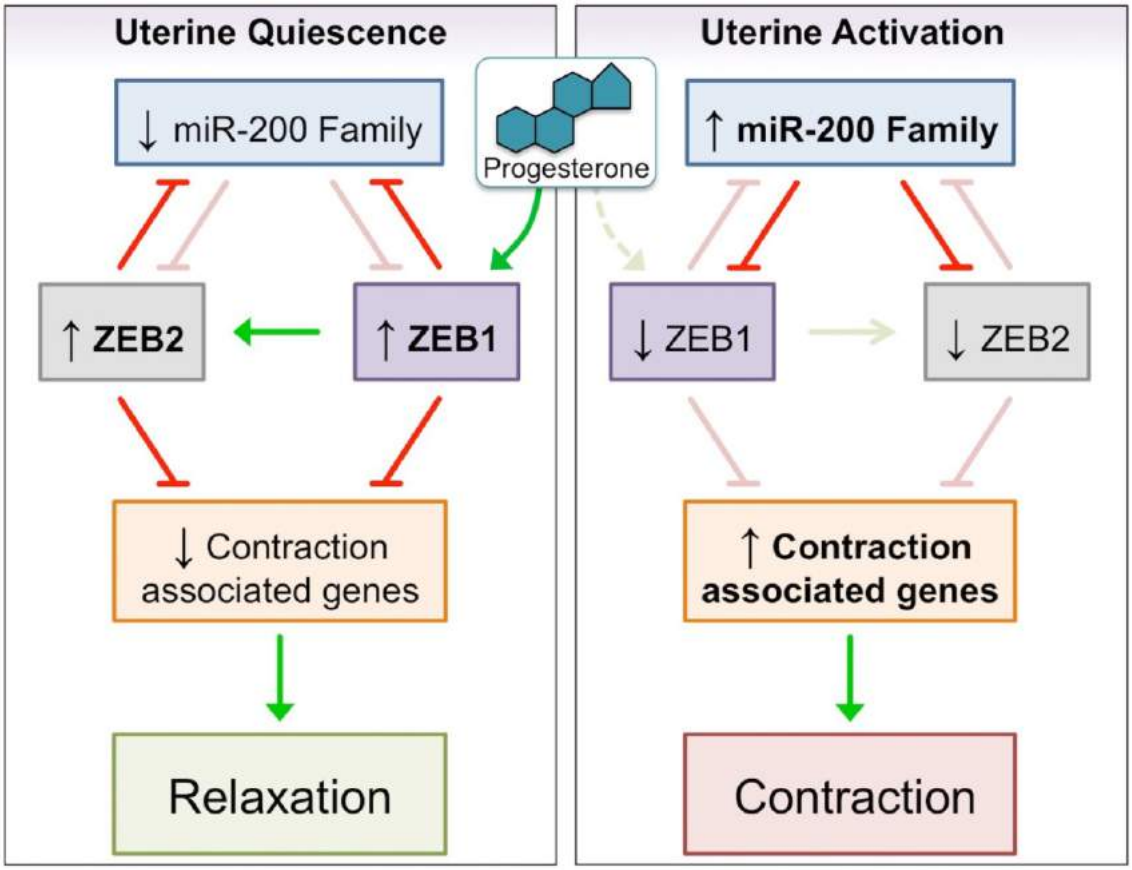

Figure 1.8. The role of ZEB1 and ZEB2 during pregnancy and labor.
Throughout pregnancy, progesterone drives expression of ZEB1, which acts to suppress the miR-200 family as well as contraction-associated genes. Decreased expression of the miR-200 family reduces suppression of ZEB2 (as well as ZEB1), resulting in further down-regulation of contraction-associate genes further promoting relaxation. Progesterone withdrawal results in down-regulation of ZEB1 expression and up-regulation of the miR-200 family. Increased miR-200 family expression supresses ZEB1 and ZEB2 leading to increased expression of contraction-associated genes.

The human ether-a-go-go-related gene

Gene products of the ether-à-go-go-related gene (ERG) constitute the major component of the delayed rectifier potassium channel, $K_v11.1$, which conducts potassium ions (K^+) out of the cell. In humans, ERG (hERG) is best known for its role in cardiac myocytes where it mediates the delayed rectifier current (I_{Kr}) [222]. Following an action potential (AP), this K^+ outflux functions to repolarize the cardiac myocyte membrane, which terminates the AP and the associated contraction. hERG thus plays an important role in regulating the

electrical activity of the heart (reviewed in Vandenberg *et al.* [222]). ERG has now been investigated in numerous smooth muscle tissues and has been shown in rat stomach and murine portal vein [223-225] as well as in opossum oesophagus [226]. In addition, studies have used selective ERG channel blockers to increase contractility in rat stomach [223], mouse portal vein [225], opossum oesophagus [226], mouse and guinea pig gall bladder [227], bovine epididymis [228] and human and equine jejunum [229, 230]. All of these smooth muscles exhibit spontaneous contractile activity.

Aaronson *et al*. [231] provided the first evidence that tetraethylammonium (TEA)- and 4-aminopyridine (4-AP)-sensitive voltage-dependent K^+ (K_v) channels are crucial in limiting the activity of an AP once it has been initiated in rat myometrium. Greenwood *et al*. [232] examined whether ERG1-3 genes were present in the murine myometrium and sought to determine the functional impact of the ion channels encoded by these genes. They found that both ERG1a and ERG1b were detected in whole myometrial tissue, with ERG1a expression being more abundant than ERG1b [232]. Furthermore, they found that the expression of ERG1 did not change throughout the gestation or with the onset of labor, however, expression of genes encoding auxiliary regulatory subunits (KCNE) of the respective K^+ channels were up-regulated considerably [232]. Human myometrium also exhibits spontaneous contractile activity [233], and recently our group provided compelling evidence that hERG not only regulates the electrical activity of uterine myocytes but plays a role in maintaining quiescence of the uterus prior to labor [234]. This was the first study to show the presence of hERG protein and the auxiliary subunit KCNE2 in pregnant human myometrium in late

gestation as well as in labor [234]. The study found that hERG channel activity is responsible for supressing contraction amplitude and duration, thus limiting the capacity for contraction propagation [235] and promoting uterus quiescence [234]. In addition, we found labor was associated with increased expression of the inhibitory auxiliary subunit, KCNE2, causing a decrease in hERG activity, which in turn leads to increased duration of uterine APs and the associated contractions [234]. These results suggest that changes in hERG channel activity contribute to electrophysiological mechanisms that generate contractions during labor [234]. Furthermore, a connection between obesity and hERG function in human myometrium was also determined [234]. Previous studies have linked maternal obesity with increased rates of labor induction, dysfunctional labor, which requires caesarean delivery, longer pregnancies, as well as postpartum haemorrhage (PPH) [236-238]. Our data demonstrates increased hERG channel activity and decreased inhibitory subunit expression in women with high body mass index (BMI). This translates into rapidly terminated, weak contractions with diminished capacity to propagate [235], and could thus contribute to poor labor outcomes observed in many obese women [234]. The discovery linking BMI with K^+ channel activity is a significant advancement in understanding the molecular mechanisms responsible for dysfunctional labor.

The transient receptor potential vanilloid 4 (TRPV4) channel

A recent study by Ying *et al.* [239] revealed that the transient receptor potential vanilloid 4 (TRPV4) Ca^{2+} channel might play an important role in regulating myometrial contractility. *TRPV4* gene and protein expression was found to increase in myometrial smooth muscle cells with gestation, as well as that TRPV4-mediated Ca^{2+} entry and contractility increased in pregnant rats

when compared to non-pregnant rats [239]. Furthermore, they found that the expression of TRPV4 increased in cell membranes, whilst the expression of β-arrestin-1 and β-arrestin-2, molecules that can isolate TRPV4 in the cytoplasm, decreased [239]. Activation of TRPV4 increased myometrial contractility whilst inhibition of TRPV4 and global deletion of TRPV4 in mice blocked myometrial contractions [239]. Furthermore, inhibiting TRPV4 led to prolonged pregnancy in two mouse models of preterm labor, suggesting that the TRPV4 channel might be a potential tocolytic target in preterm labor in humans [239].

Targeted drug delivery

A recent advancement for the field has been the development of a targeted drug delivery system for the uterus. Paul *et al*. [240] developed nanoliposomes targeted by an antibody to an extracellular domain of the OTR. When loaded with the tocolytic agents nifedipine (NIF), salbutamol (SAL) and ROL, the targeted nanoliposomes were effective in blocking human and mouse uterine contractions *in vitro,* and when loaded with the hERG channel inhibitor dofetilide (DOF) contraction duration was significantly increased. Non-targeted liposomes loaded with these agents had no effect. When injected into live pregnant mice at term, OTR-targeted nanoliposomes showed strong localization to the uterus and low-level localization to mammary tissue, but were not detected in the brain, heart, kidney or lungs, nor were they detected in neonates. Finally, OTR-targeted nanoliposomes loaded with indomethacin (IND) were effective in reducing rates of PTB in mice, whereas non-targeted liposomes containing IND had no effect [240]. These findings were supported by Refuerzo *et al.* [241] who generated IND-loaded nanoliposomes coupled to the OTR antagonist, Atosiban. OTR-targeted nanoliposomes therefore appear

to be a versatile drug delivery platform for the uterus that may one day be available for tocolytic applications.

1.5 Summary

There is now a substantial body of evidence implicating a complex network of endocrine and paracrine factors in the transformation of the myometrium from a quiescent to a contractile state at the end of gestation. Progesterone is responsible for maintaining pregnancy by promoting myometrial quiescence and estrogen is considered a principal endocrine factor instigating myometrial activation. Essential components of the regulatory changes are increased responsiveness to uterotonins, such as oxytocin and prostaglandins, which stimulate the uterus to generate frequent, high intensity, co-ordinated phasic contractions and promote cervical remodelling. Key to the underlying mechanism is the changing pattern of gene expression in the uterine myocytes, which transforms the phenotype from quiescent to excitatory and contractile. Alterations in steroid receptor levels, increasing expression of CAPs, including receptors for oxytocin and PGs, increased myometrial gap junction formation and increased influx of Ca^{2+} into myocytes are firmly established. Genome wide data are accumulating and demonstrate numerous further gene expression changes and intricate gene regulatory networks. It is still unknown; however, what exact molecular mechanisms control the complex interactions between the myometrial genes that determine the uterine phenotype. Meanwhile, new evidence is emerging to expand the range of the regulatory mechanisms involved, such as hERG1 and TRPV4, which play previously unanticipated roles in controlling uterine activity at term. Further research will integrate these new

findings into the current paradigm of uterine regulation. As the understanding of myometrial biology improves it will become possible to develop effective therapies to prevent or block premature labor.

1.6 Hypotheses and Aims

Hypotheses

1. Human myometrial tissue pieces and strips undergo culture-induced changes in the expression of key contraction-associated gene. The change in expression of these genes is consistent with transition toward a laboring phenotype.
2. The level of expression of different contraction-associated genes in freshly biopsied term, non-laboring human myometrium can be differentially preserved *in vitro* through exposing the tissue to specific culture supplements and conditions, such as physiological concentrations of steroids, stretch and signalling pathway inhibitors.
3. Expression of the newly identified contraction-associated genes, *KCNH2* and *KCNE2,* correlate with the expression of other key contraction-associated genes in pregnant human myometrium.
4.

Aims

To investigate the above hypotheses, the research outlined herein was conducted to address 3 primary aims:

1. To determine whether non-laboring myometrial tissue pieces and strips undergo culture-induced changes in the mRNA abundance for key

myometrial genes, including Estrogen Receptor 1 *(ESR1), PTGS2, OXTR, PR, KCNH2* and *KCNE2,* which are consistent with transition toward a pro-contractile, laboring phenotype. Furthermore, this work aimed to identify culture conditions that could be implemented to block or minimize phenotypic transition *in vitro*, thus providing researchers with a stable platform on which to conduct experimental studies.

2. To determine whether the mRNA abundance for *KCNH2* and *KCNE2* in term non-laboring human myometrium correlate with the mRNA abundance for *ESR1, PTGS2, OXTR* and *PR.*
3. To develop a means of targeting therapeutic agents to uterine myometrial tissue, in order to allow therapeutic modification of myometrial contractions in obstetric settings, such as preterm labor, labor induction and PPH.

Chapter Two: Extended Methods

This Chapter explains the methods for the studies in extended detail to that provided in individual data Chapters due the word restrictions in submitted/published manuscripts.

2.1 Samples

Human Studies

These studies were performed in Newcastle, New South Wales, Australia and were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent. Human myometrial samples (5 × 5 × 10 mm) were obtained from lower uterine segment during elective caesarean section (CS) of singleton pregnancies. Preterm samples ranged from 31 – 34 weeks gestation while and term samples were 38.2 – 39.6 weeks gestation. Patient BMI range was 18.3 – 38.0, and all patients were NIL. The indications for elective NIL CS were previous CS, previous $3^{rd}/4^{th}$ degree tear, placenta praevia, fetal distress or breach presentation. Women were excluded if they were given steroids or had signs of infection. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of PPH. Samples were therefore exposed to oxytocin for a brief period of time (3 min). Myometrial samples used for contractility studies were placed on ice in ice-cold physiological saline, whilst myometrial samples used for tissue culture studies were placed on ice in

serum-free medium containing Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin and 10 mM HEPES for the transfer to the laboratory.

Mouse In Vitro Studies

Mouse uterine horns were dissected from pregnant CD1 Swiss mice (8 – 10 weeks of age) at term gestation prior to the onset of labor (fetal gestation day 18). Mouse studies were approved by the University of Newcastle Animal Ethics Committee (A-2014-400 / A-2014-429). All mice were housed under SPF/PC2 conditions under a 12 h light day cycle and had food and water available *ad libitum.*

2.2 Liposome Manufacture

Liposomes containing NIF, SAL, ROL, DOF (each at approximately 4 mg/mL) or IND (approximately 5.5 mg/mL), as determined by high performance liquid chromatography (HPLC), were manufactured as previously outlined [242]. Liposomes were composed of 1,2-distearoyl-sn-glycero-2-phosphocholine (DSPC) and cholesterol in a molar ratio of 2:1, and contained 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG(2000) maleimide) at 1.5 mol percent of DSPC as a coupling lipid (Avanti Polar Lipids). The resulting multilamellar dispersions were reduced in size and lamellarity to approximately 200 nm in diameter by high-pressure extrusion. The activated liposome suspension was then mixed with thiolated polyclonal anti-OTR antibody (Abcam, Cat# ab115664), which was prepared by first conjugating 25 µg of OTR antibody with a heterobifunctional reagent N-

succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Figure 2.1). The OTR antibody recognizes an extracellular domain of the human OTR. Non-targeted liposomes were coated with rabbit immunoglobulin G (IgG). All liposomes incorporated the membrane stain 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) for fluorescent detection. Unconjugated antibody and non-encapsulated drug was removed by centrifugal filtration of the liposomes through a 100 kDa molecular weight filter (Amicon Ultra-15). Amicon Ultra-15 filters were washed with Milli-Q H_2O before 500 µL of liposome suspension was loaded into the filter reservoir. Liposomes were diluted with 5 mL of sterile 0.9% saline and centrifuged at 4000 x g until retentate volume was <250 µL. Liposomes were then resuspended in a futher 5 mL of 0.9% saline and centrifuged until retentate volume was <250 µL. Filtered liposomes were then collected, transferred to a fresh Eppendorf tube and redispersed to an original volume of 500 µL.

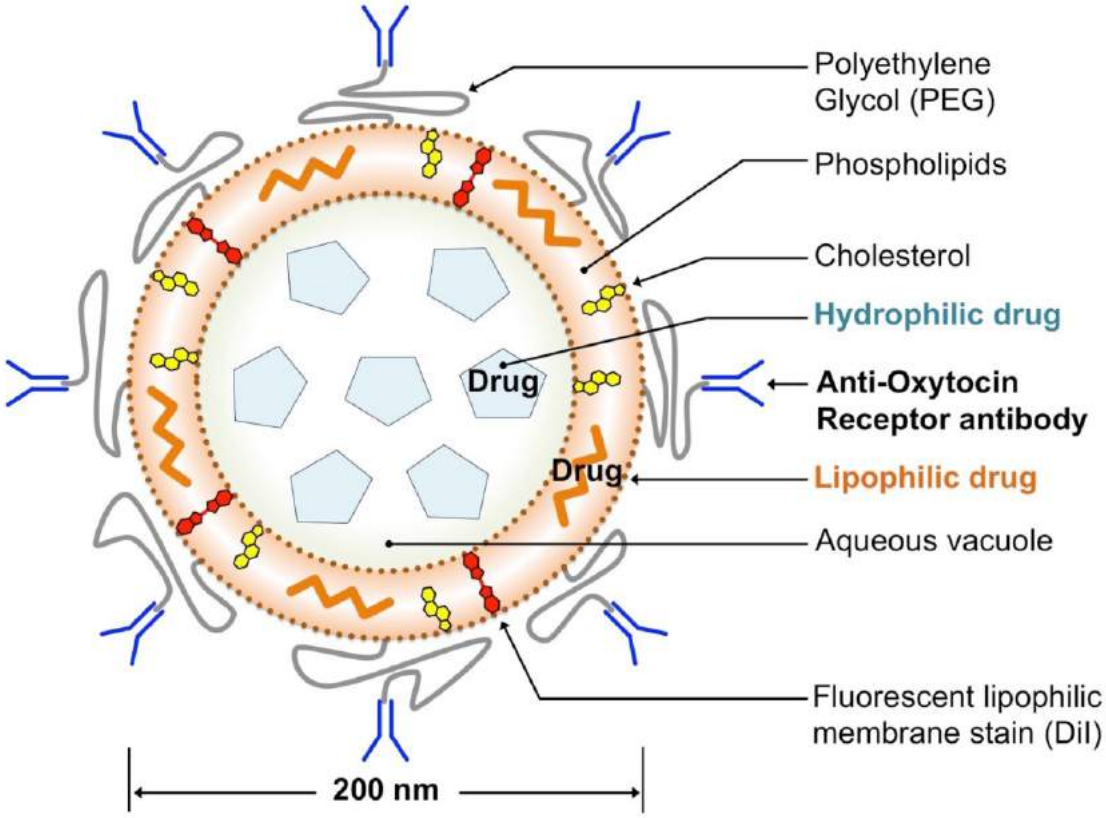

Figure 2.1. Schematic of OTR-targeted liposome structure

The size distribution of the liposomal dispersion was determined by dynamic laser light scattering (Zetasizer Nano S^{TM}, ATA Scientific). Encapsulation efficiency (EE%) was determined by disrupting the vesicles with ethanol and evaluating drug concentration using HPLC. Quantification of the amount of antibody associated with liposomes was determined using the CBQCA protein assay (ThermoFisher Scientific Inc. Watham, MA, USA), using bovine serum albumin for the preparation of the standard curve.

2.3 Myometrial Contractility Studies

Non-laboring human myometrial samples, or uterine horns obtained from pregnant CD1 Swiss mice, were dissected into strips (10 × 2 × 2 mm) and suspended in organ baths containing 30 mL physiological saline solution (PSS)

containing (in mM) NaCl 120, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11, and continuously gassed with carbogen (95% O_2, 5% CO_2), at pH 7.4. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1 g passive tension applied (1 g was calibrated to equal 1 V). PSS was replaced five times during the first hour, with strips re-tensioned to 1 g passive tension following each wash. Thereafter strips were maintained at 37°C until spontaneous rhythmic contractions developed. Data were digitized using a MacLab/8E data-acquisition system and contraction status visualized in real time using Chart software (ADInstruments, NZ). For each strip a contraction baseline was acquired to serve as reference [243].

To administer hERG channel activators and inhibitors, 600 µL of PSS buffer was carefully extracted from an organ bath and tranferred to an Eppendorf tube. The appropriate volume of the treatment was pipetted into the PSS to pre-dilute the treatment. The total volume of pre-diluted treatment (600 µL PSS + treatment) was then carefully reinjected back into the appropriate organ bath. Final concentrations of each drug were; PD-118057 10.0 µM, NS-1643 10.0 µM, DOF 1.0 µM and E-4031 1.0 µM.

To administer liposome treatments, 600 µL of PSS buffer was carefully extracted from an organ bath and tranferred to an Eppendorf tube. The appropriate volume of liposome preparation (mixed by inversion) was pipetted into the PSS to pre-dilute the liposomes. The total volume of pre-diluted liposomes (600 µL PSS + liposomes) was then carefully reinjected back into the appropriate organ bath. Final concentrations of each drug were; NIF 7.7 µM, SAL 9.25 µM, ROL 19.4 µM and DOF 3.0 µM. Doses were based on prior

investigations of the non-encapsulated drug (*in vitro* contraction assays using human myometrium). Where treated tissue was not washed, tissue strips remained in the presence of the liposomes for the duration of the assay. Where washout studies were performed, organ baths were twice drained of buffer and refilled with 37°C PSS. Human tissue strips were washed after 1 h 25 min whereas mouse tissue strips were washed after 15 min.

Tension generated by tissue strips is indicated in the results and representative contraction traces. The effect of treatments is interpreted relative to the pre-treatment contraction baseline, which consisted of 3 or more contractions of consistent frequency and amplitude. Figure 2.2. illustrates the experimental set-up for the contraction bioassay experiments.

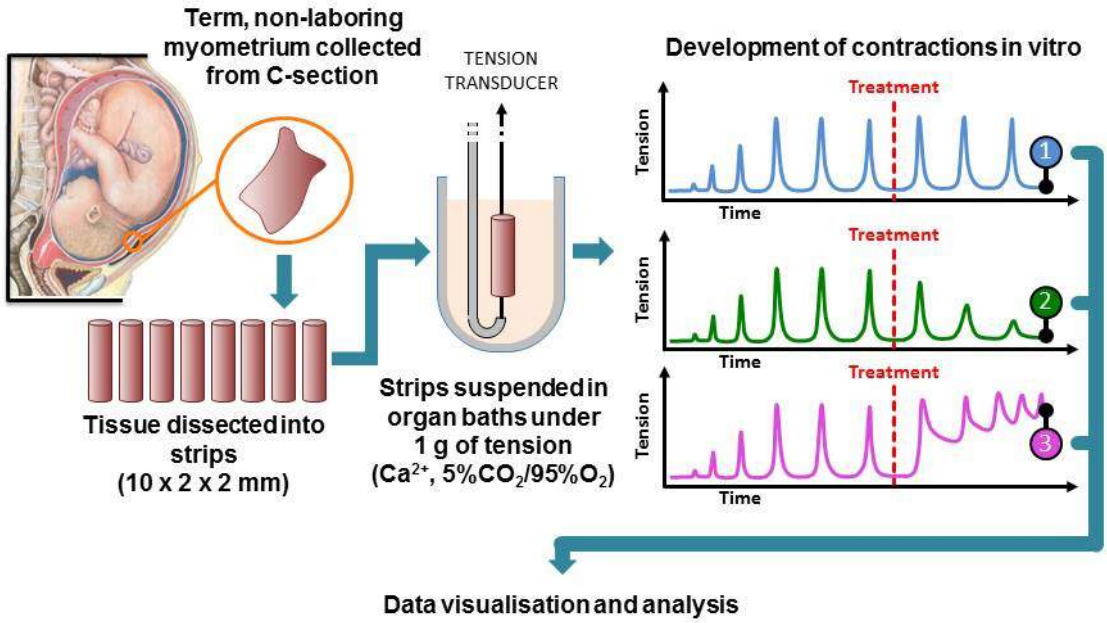

Figure 2.2. Contraction bioassay.
Myometrial samples are collected from the lower uterine segment of term singleton pregnancies undergoing caesarean section. The myometrial sample is then dissected into strips and connected to a Grass force transducer inside an organ bath. Following the development of spontaneous regular contractions desired treatments are applied to individual strips.

2.4 Myometrial Tissue (Explant) Culture

Myometrial tissue that was placed on ice in serum-free media was transferred to the laboratory and approximately 100 mg of tissue was snap frozen in liquid nitrogen for subsequent analysis. The remaining tissue was cleared of serosa, fibrous or damaged tissue and visible blood vessels, dissected into smaller pieces (10 – 15 mg explants) and washed in serum-free media to remove excess blood. Figure 2.3. illustrates the experimental set-up for the myometrial tissue (explant) culture experiments.

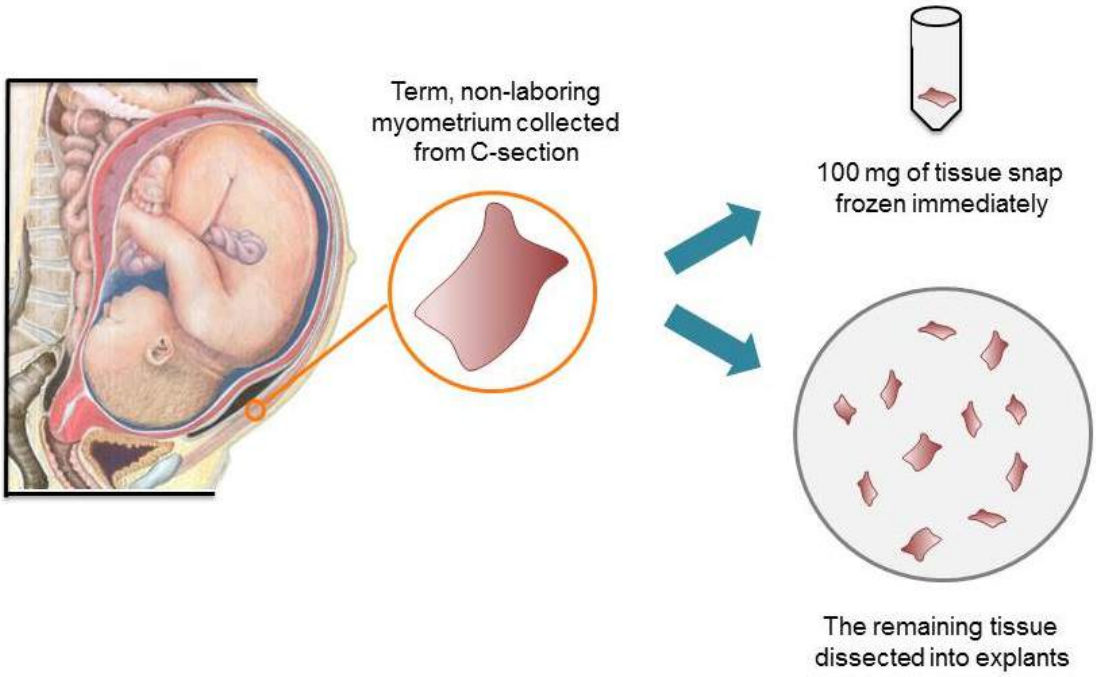

Figure 2.3 Myometrial tissue (explant) culture.
Human myometrial samples were obtained from lower uterine segment during elective CS of singleton pregnancies. Tissues were transferred to the laboratory and approximately 100 mg of tissue was snap frozen immediately. The remaining tissue was cleared of serosa, fibrous or damaged tissue and visible blood vessels, dissected into smaller pieces (explants).

To determine the differences between the fresh samples and samples kept in explant culture, myometrial tissue samples were incubated in serum-free media in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed

after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To obtain a better indication of when culture-induced changes in *PR* isoform expression occur, myometrial tissue samples were incubated in serum-free media for 0, 1, 2, 6, 24 or 48 h in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of different media, myometrial tissue samples were incubated in serum-free media as well as 5% charcoal stripped serum (CSS) media in a 37°C, 95% air/5% CO_2 humidified incubator. 5% CSS media contained DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentemicin and 10 mM HEPES. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of steroids, myometrial tissue samples were incubated in 5% CSS media in physiological concentrations of progesterone (P4) (500 nM) and estrogen (E2) (100 nM and 10 µM) alone or in a combination (500 nM P4 + 400 nM E2) in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effect of stretch on human myometrium, myometrial tissue strips were cultured in 5% CSS media in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of tension. To determine the effect of stretch and steroids on human myometrium, myometrial strips were cultured in 5% CSS media with P4 and E2 in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of tension. Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 mL of culture media in 50 mL tubes (strips subjected to 0 g only were tied at one end). Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, tissue strips snap frozen in liquid nitrogen and stored at -80 for subsequent analyses. Figure 2.4. illustrates the experimental set-up for the stretch experiments.

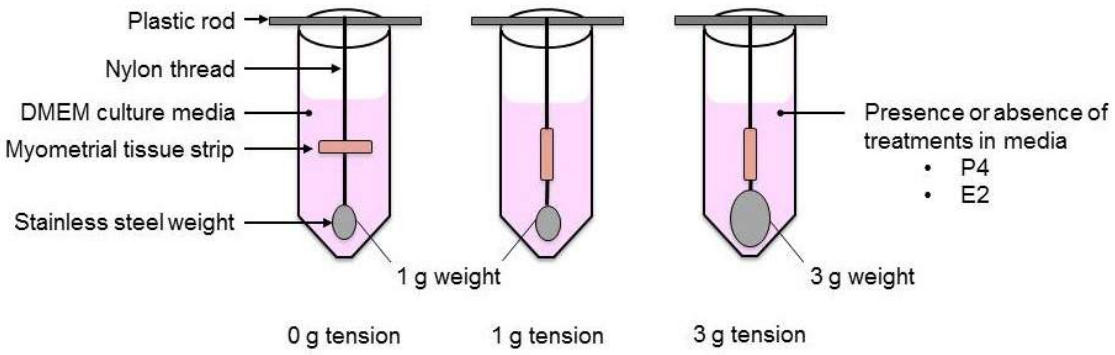

Figure 2.4. Stretch experiments.
Human myometrium tissues were obtained from lower uterine segment during elective CS of singleton pregnancies and transferred to the laboratory. Tissues were dissected into strips (10 x 2 x 2 mm) and weight was attached to the tissue (1 g and 3 g weight). Myometrial tissue strips were then cultured in 5% CSS media in presence or absence of steroids, P4 and E2, in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, tissue strips snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial tissues were incubated in 5% CSS media with cAMP analogue 8-Br-cAMP (250 µM) or vehicle and PMA (0.1, 1.0 and 5.0 µM) or vehicle in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of PGs, myometrial tissues were incubated in 5% CSS media with $PGF_{2\alpha}$ (1, 10, 100 and 1000 nM) or vehicle in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of NF-κB inhibitor, myometrial tissue samples were incubated in 5% CSS media with MG-132 (2.0, 5.0 and 10.0 µM) or vehicle and BAY-11-7085 (2.0, 5.0 and 10.0 µM) or vehicle in a 37°C, 95% air/5% CO_2 humidified incubator. Additionally, myometrial tissues were incubated in presence of both NF-κB inhibitors, 10.0 µM MG-132 + 10.0 µM BAY-11-7085, in a 37°C, 95% air/5% CO_2 humidified incubator. Following 48 h incubation, the media was decanted, explants snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

To determine the effects of HDACi, myometrial tissue samples were incubated in 5% CSS media with TSA (0.5, 1.0, 2.5 and 5.0 µM) or vehicle in a 37°C, 95% air/5% CO_2 humidified incubator. Media was refreshed after 24 h

incubation. Following 48 h incubation, the media was decanted, myometrial tissues snap frozen in liquid nitrogen and stored at -80 for subsequent analyses.

2.5 Molecular Analysis

2.5.1 RNA Extraction Using TRizol Reagent

RNA was extracted using TRizol Reagent (Thermo Fisher) according to the manufacturer's instructions (Figure 2.5). Briefly, 1 mL of TRIzol Reagent was added to 100 mg of tissue in 2 mL 2.8 mm ceramic bead tubes (CK28-R). Tissues were then homogenised using a Precellys24 homogenizer (Bertin Instruments, France). Following homogenization, samples were centrifuged at 12,000 rpm for 10 min on 4°C and clear supernatant from each ceramic bead tube was transferred to a new Eppendorf tube. Samples were then homogenized for 5 min at room temperature to ensure complete dissociation of the nucleoprotein complex. Chloroform (0.2 mL per 1 mL of TRIzol Reagent) was added for homogenization and tubes were shaken vigorously by hand for 15 sec. Samples were then incubated for 15 min at room temperature and following incubation centrifuged at 12,000 rpm for 15 min on 4°C. This allowed separation of the mixture into lower red-phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phases were removed and placed into clean tubes, since RNA remains exclusively in this phase. As the RNA was precipitated from small sample quantities, 5 µL of RNase-free glycogen was added as a carrier to the aqueous phases. Glycogen co-precipitates with the RNA; however it does not inhibit first-strand synthesis and thus does not inhibit PCR. The RNA was then precipitated by adding 100%

isopropanol (0.5 mL per 1 mL of TRIzol Reagent) to the aqueous phases. Samples were then incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were discarded and pellets washed with 75% ethanol (1 mL per 1 mL of TRIzol Reagent). Samples were vortexed and centrifuged at 7,500 rpm for 5 min at 4°C. Supernatants were discarded and RNA pellets air dried. The extracted RNA pellets were resuspended in RNase-free water.

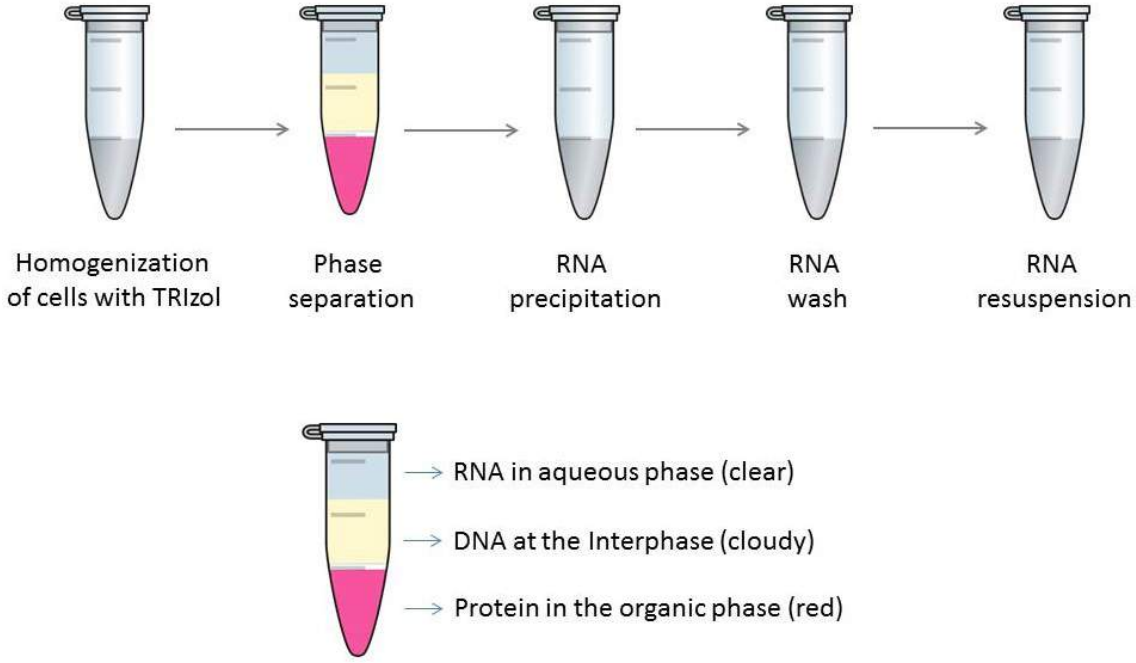

Figure 2.5. RNA extraction using TRIzol Reagent.
Cells were disrupted by adding TRIzol Reagent and lysates homogenized. Chloroform was added to the lysate, thus creating conditions to allow separation of the mixture into three phases. The RNA was precipitated with RNase-free glycogen and isopropanol. Samples were centrifuged and RNA pellets resuspended in RNase-free water.

2.5.2 DNase Treatment

DNase treatment was performed using the TURBO DNA-free DNase (Ambion) according to the manufacturer's instructions (Figure 2.6). The kit is designed to digest contaminating DNA; remove the DNase and divalent cations from RNA samples to a level below the detection limit of PCR. Briefly, DNase I

buffer 10X (0.1 volume) and 1 µL TURBO DNAase were added to RNA samples (20 µL). Tubes were flicked gently to ensure mixing, pulse centrifuged and incubated for 30 min at 37°C. Following incubation, resuspended DNase inactivation reagent (0.1 volume) was added. DNase inactivation reagent removes the DNase enzymes as well as divalent cations such as magnesium and calcium that can catalyse RNA degradation once RNA is heated with the sample. Samples were then flicked gently to ensure mixing and incubated for 5 min at room temperature. During the incubation period, tubes were flicked 2-3 times to redisperse the DNase inactivation reagent. After incubation, samples were centrifuged at 13,000 rpm for 2 min and supernatants (~20 µL) were transferred into clean tubes.

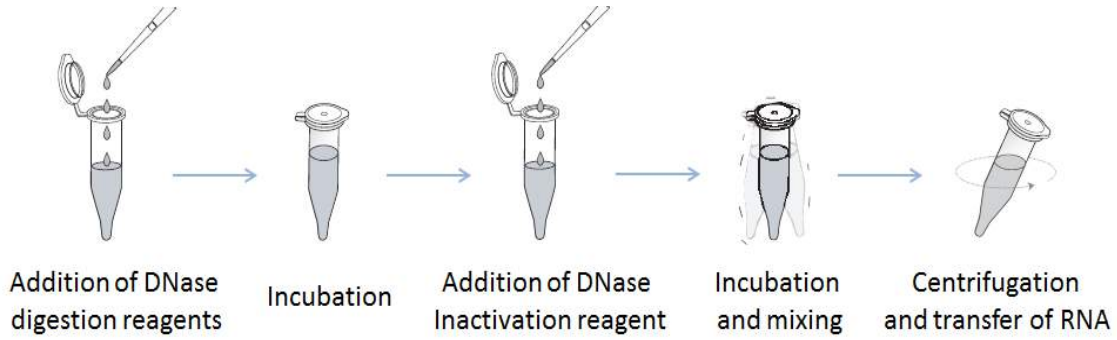

Figure 2.6. DNase treatment.
DNase digestion reagents were added to samples; tubes were mixed and incubated at 37°C for 30 min. Subsequently, resuspended DNase inactivation reagent was added; samples were incubated at room temperature for 5 min and mixed 2-3 times during incubation to redisperse the DNase inactivation reagent. Samples were then centrifuged for 2 min at 13,000 rpm and supernatants containing the RNA were transferred into new tubes.

An ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) was used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. Our results showed no difference between RNA concentrations in fresh tissues and tissues incubated for 48 h. RNA integrity

was checked by agarose gel electrophoresis. Remaining RNA samples were kept at -80°C until needed.

2.5.3 Alien Reference RNA

The Alien Reference RNA QRT-PCR Detection Kit was used as a high quality external reference RNA control system for quantitative real time PCR (qRT-PCR). All qRT-PCR experiments have been done with the Alien RNA transcript since it is an *in vitro* transcribed RNA molecule that is non homologous to any known nucleic acids. The stock tube of the Alien RNA transcript (containing 3×10^{10} copies/µL) was thawed and aliquoted into 1 µL aliquots. For each experiment, a fresh 1 µL aliquot was used and diluted with 59 µL of MQ water to obtain a concentration of 5×10^{8} copies/µL. Each aliquot was then further diluted 1:200 (2 µL of 1:60 dilution and 398 µL of MQ water) to obtain a final concentration of 0.25×10^{7} copies/µL. This final concentration of 0.25×10^{7} copies/µL was added to RNA samples prior to reverse transcription.

2.5.4 Reverse Transcription (First-strand cDNA Synthesis)

SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) was used to synthesise first-strand cDNA from total RNA with random hexamers. This process involved 5 stages: I) denature, II) anneal, III) cDNA synthesis, IV) termination of reaction, and V) RNA removal.

The reverse transcription (RT) samples had a volume of 8 µL containing 0.5 µg of RNA, 2 µL of the Alien RNA transcript (0.25×10^{7} copies/µL) and MilliQ water to the final volume. To confirm the absence of genomic DNA

contamination, the no-reverse transcription (no-RT) samples acted as a negative control for each sample. The no-RT samples also had a volume of 8 µL (0.5 µg of RNA and MilliQ water to the final volume), however they did not contain Alien transcript. Denaturation mix (2 µL) was added to both RT and no-RT samples (Table 2.1). Samples were incubated at 65°C for 5 min in the GeneAmp PCR system 9700 (Applied Biosystem) to allow denaturation and then placed on ice for 1 min. While the samples were incubating, cDNA synthesis mix and no-RT mix were prepared, adding each component in the specified order (Table 2.1). cDNA synthesis mix (10 µL) was added to RT samples and no-RT mix (10 µL) was added to no-RT samples. Samples were then mixed gently by flicking and collected by pulse centrifugation. Following centrifugation, samples were incubated for 10 min at 25°C (anneal), 50 min at 50°C (cDNA synthesis) and 5 min at 85°C (termination) in the GeneAmp PCR system 9700 (Applied Biosystem). After first-strand synthesis, reactions were collected by pulse centrifugation and *E.Coli* RNase H (1 µL) was added to samples. RNase H removed the RNA template from the cDNA-RNA hybrid molecule and therefore increased the sensitivity of the PCR. Samples were incubated for 20 min at 37°C to ensure that all RNA was removed and synthesised cDNA was stored at -20°C until needed.

Table 2.1. Reverse transcription mixes.

Denaturation Mix: Components	Per tube (µL)
Primers (50 ng/µL random hexamers)	1
10 mM dNTP mix	1
Total	2 µL

cDNA Synthesis Mix: Components	Per tube (µL)
10X Reverse Transcription (RT) Buffer	2
25 mM Magnesium Chloride ($MgCl_2$)	4
0.1 M Dithiothreitol (DTT)	2
RNase OUT (40 U/µL)	1
SuperScript III Reverse Trnascriptase (200 U/µL)	1
Total	10 µL

No-RT Mix: Components	Per tube (µL)
10X Reverse Transcription (RT) Buffer	2
25 mM Magnesium Chloride ($MgCl_2$)	4
0.1 M Dithiothreitol (DTT)	2
RNase OUT (40 U/µL)	1
MQ water	1
Total	10 µL

2.5.5 PCR Primers

PCR primers were designed using Primer Express and Sigma Design Tool. *ESR1*, *PGR* and *OXTR* PCR primers have been previously optimised by the different members of the laboratory and multiple manuscripts have been published using these PCR primers [59, 61, 91]; nevertheless the new batch of every PCR primer was purchased from Sigma and re-optimised for the use in these experiments. The first step in the PCR primer optimisation was to determine the optimal PCR primer concentrations (100 nM, 200 nM, 300 nM,

400 nM, 500 nM and 600 nM) using the calibrator sample. Following the PCR, gel electrophoresis was used to visual the PCR products. Both PCR results (melt curves and clean no template control (NTC)) and gel electrophoresis results (one band of the desired size) were used to check for unspecific products. Based on these results, the minimal concentration of each primer that gives a clean band of desired size was used for further optimisation. The second step in the PCR primer optimisation was to take the optimal concentration of each primer and perform PCR using different concentrations of the calibrator sample (1, 5, 10, 15, 20 and 25 ng/µL). Following the PCR, gel electrophoresis was used to visual the PCR products. Once again, both PCR results and gel electrophoresis results were used to check for unspecific products. If one band of the desired size was observed for each concentrations of the calibrator sample and PCR showed no primer dimers, the primers were considered optimised and are used for these experiments (Table 2.2).

Table 2.2. cDNA primer sequences for *ESR1, KCNH2, KCNE2, OXTR, PR-T, PR-B* and *PTGS2.*

Primer	Primer Sequence	Amplicon Size	GenBank #
ESR1	F: TGAAAGGTGGGATACGAAAAGAC R: CATCTCTCTGGCGCTTGTGTT	66	NM_000125.3
KCNH2	F: ACCTCATCGTGGACATCA R: CTCCTCGTTGGCATTGAC	77	NM_000238.3
KCNE2	F: CACGAGGCAAATCCAAAT R: CTCCAACAAGCAAGCATAA	141	NM_172201.1
OXTR	F: CTGGACGCCTTTCTTCTTCGT R: GAAGGCCGAGGCTTCCTT	73	NM_000916.3
PR-T	F: GTGGGAGCTGTAAGGTCTTCTTTAA R: AACGATGCAGTCATTTCTTCCA	83	NM000926.4
PR-B	F: TCGGACACCTTGCCTGAAGT R: CAGGGCCGAGGGAAGAGTAG	68	NM000926.4
PTGS2	F: ATGTTCCACCCGCAGTACAGA R: CAGCATAAAGCGTTTGCGGTA	101	NM_000963

ESR1: Estrogen Receptor 1; *KCNH2:* Potassium Voltage-Gated Channel Subfamily H Member 2; *KCNE2:* Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2; *OXTR:* Oxytocin Receptor; *PR-T:* Progesterone Receptor total; *PR-B*: Progesterone Receptor Isoform B; *PTGS2:* Prostaglandin-Endoperoxide Synthase 2.

2.5.6 Quantitative Real Time PCR

ABI 7500 Sequence Detector (Applied Biosystems) was used to perform qRT-PCR and measure messenger RNA (mRNA) abundance of key myometrial genes such as *ESR1*, *PGR*, *OXTR* and *PTGS2*, as well as *KCNH2* and *KCNE2* [244]. The PCR was carried out in a reaction volume of 20 µL containing 10 µL of 2x SYBR Green PCR master mix (Applied Biosystems), cDNA template

(corresponding to 10 ng of reverse transcribed RNA), forward and reverse primers and MilliQ water to the final volume. SYBR Green dye is a highly specific, double-stranded DNA binding dye that detects PCR products by binding to the minor groove double-stranded DNA. During PCR, complementary DNA strands or amplicons are synthesized by polymerases. The SYBR Green dye binds to each new replica of double-stranded DNA. As the PCR continues, more replicates are produced. As a result there is an increase in fluorescence intensity, which is directly proportional to the amount of DNA produced.

All samples were assayed in duplicate and no-RT control samples were included. NTC samples were also included to detect primer-dimer formation as well as calibrator cDNA, reference samples, to ensure that there are no plate-to-plate variations. Amplification mixtures were loaded into a 96-well plate and sealed with adhesive film to decrease well-to-well contamination and sample evaporation. The 96-well plate was mixed and centrifuged in a microplate carrier (Beckman) at 500 g for 1 min, then loaded into the sequence detector. The standard amplification cycling protocol was as follows: (1) 2 min at 50°C, (2) 10 min at 95°C denaturation (3) 50 cycles of 15 sec at 95°C to denature DNA template by disrupting the hydrogen bonds amongst complementary bases, therefore generating single-stranded DNA molecules. Following denaturation, (4) the reaction temperature was lowered to 60°C for 1 min to allow annealing of the primers and probe to the single-stranded DNA template. Formation of stable DNA-DNA hydrogen bonds occurs when the primer sequence closely matches the template sequence. Polymerisation of a new DNA strand increases fluorescence which is detected and measured by the PCR machine. The dissociation curve stage was included to run immediately

following the PCR run, with cycling conditions as follows: (1) 15 sec at 95°C, (2) 1 min at 60°C, (3) 15 sec at 95°C and (4) 15 sec at 60°C (Figure 2.7). The dissociation curve analysis measures the melting temperature (Tm) at which the double-stranded DNA denatures into two single strands. The measurement is taken when 50% of double-stranded DNA molecules are dissociated. When the run was completed, data were exported into excel and analysed.

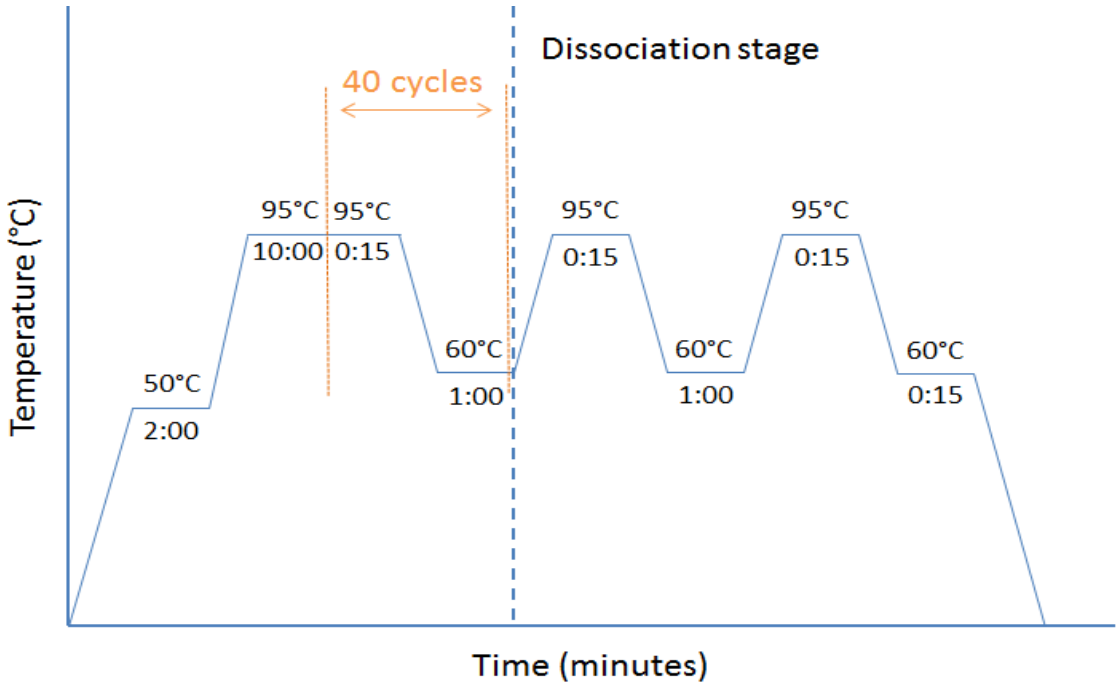

Figure 2.7. qRT-PCR cycling conditions.
The cycling conditions included 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The final dissociation stage included 15 sec at 95°C, 1 min at 60°C, 15 sec at 95°C and final 15 sec at 60°C.

2.6 *In Vivo* Biodistribution Study

Timed-mated CD1 Swiss pregnant mice were injected with drug-free, DiI-labelled preparations of either non-targeted or OTR-targeted liposomes on fetal gestation days 17 and 18 at 4:00pm. Mice that labored overnight were euthanized on the morning of day 19 (9:00 – 11:00am) by CO_2 asphyxiation. Maternal internal organs of interest (heart, brain, liver, lung, kidney, uterus and

mammary tissue) were harvested and transferred to a Petri dish along with a sacrificed neonate. The Petri dish was loaded into an *In Vivo* Imaging System (IVIS-100) (Xenogen, CA, USA) and a light image captured. Tissues were then imaged under conditions appropriate for the detection of DiI (Excitation: 554 nm; Emission: 583 nm; Filter: DsRed; Exposure: 4 sec; Field of view: 10; Binning: 4). Organs were imaged 17 – 19 h after the second injection, following labor. Nanoliposomes were previously determined to be detectable at tissue depths (within whole mice) greater than the diameter of neonate bodies. Background signal was subtracted from the detected signal to produce the final fluorescence image. Fluorescence signal is reported as radiance (p/sec/cm^2/sr). The radiance range was kept constant across all images (min = 2.0×10^8 : max = 1.8×10^9).

2.7 Preterm Birth Study

Establishing LPS Mouse Model

To establish a preterm labor mouse model, time-mated pregnant CD1 Swiss mice were administered 0, 0.5, 0.7, 1.0, 1.5 and 3.0 µg/g of lipopolysaccharide (LPS) from *E. coli* (0111:B4) via intraperitoneal (IP) injection at 12:00pm on gestation day 15 (GA15) (one-time injection). Total IP injection volume was 150 µL in saline. Animals were then monitored every 6 h for the onset of labor. Animals that labored within 48 h of LPS injection were deemed to have labored preterm (term labor = GA19 – 21). Optimal dose for achieving preterm birth rates between 50 – 70% was determined to be 0.7 µg/g.

Prevention of LPS-induced Preterm Birth

Time-mated pregnant CD1 Swiss mice were administered 0.7 µg/g LPS from *E. coli* (0111:B4) (Sigma-Aldrich) via IP injection at 12:00pm on GA15 (one-time injection). Total IP injection volume was 150 µL in saline. At 4:00pm on GA15, mice began receiving daily intravenous (IV) injections of IND free-drug or liposomal preparations according to assigned treatment groups. Treatment groups are indicated in Table 2.3. Total IV injection volume was 150 µL. Mice were monitored for onset of labor every 6 h. Treatments were repeated daily at 4:00pm until all mice labored. Term gestation was 19 – 22 days. Mice that labored within 48 h of receiving LPS (GA17) were deemed to have labored preterm.

Table 2.3. Treatment groups for in preterm labor study

Group (n)	**One-time IP Injection** (12:00pm on GA15)	**Daily IV injections** (4:00pm, GA15 onwards)
1	saline	saline
2	0.7 µg/g LPS	50% DMSO
3	0.7 µg/g LPS	1.0 mg/kg/day IND in 50% DMSO
4	0.7 µg/g LPS	2.0 mg/kg/day IND in 50% DMSO
5	0.7 µg/g LPS	OTR-targeted, drug-free liposomes in saline
6	0.7 µg/g LPS	2.0 mg/kg/day IND via non-targeted liposomes in saline
7	0.7 µg/g LPS	2.0 mg/kg/day IND via OTR-targeted liposomes in saline

LPS, lipopolysaccharide; *OTR*, oxytocin receptor; *IP*, intraperitoneal; *IV*, intravenous; *GA15*, pregnancy day 15

2.8 Data and Statistical Analysis

For contraction traces, LabChart software (ADInstruments, NZ) was used to determine the area under the curve (AUC) (g tension × sec) for the 30 min

prior to treatment (pre-treatment) and 30 min after treatment (post-treatment). AUC before and after treatment was compared by two-tailed paired t-test (GraphPad Prism).

For hERG channel activators and inhibitors studies, contraction plateau duration (sec) was determined for four contractions pre- and post-treatment using LabChart software (ADInstruments, NZ). Plateau duration was determined as the time between the point of highest amplitude and point where contraction force declined sharply. Contraction duration data were obtained for 3 individual tissues (n=3 women). Pre- and post-treatment measurements (n=12 each) were compared by two-tailed unpaired t-test.

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the delta C_t (ΔC_t) method [245]. The relative mRNA abundance of *PR-A* was calculated by subtracting the relative mRNA abundance of *PR-B* from that of *PR-T*. All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, paired Student's *t* test was used. For multiple, matched comparisons a one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett multiple comparisons was used. For correlation studies Pearson's product moment correlation coefficient was used (Pearson's *r*). P-values ≤ 0.05 were considered statistically significant.

Average radiance (p/sec/cm^2/sr) was determined for each organ of interest using Living Image software (v2.5). Where fluorescence was detected, regions of interest (ROIs) were applied automatically (contour). Where detection was low or absent, ROIs were specified manually (circles or squares) to tightly encompass the tissue being analysed. Data were tested for normality by the Shapiro-Wilk normality test (GraphPad Prism). Average radiance for each organ/tissue was compared between treatment groups (n=4 animals per group) by 1-way ANOVA with multiple comparisons (Holm-Sidak) (GraphPad Prism).

For the preterm labor studies, rate of PTB was compared between treatment groups by Chi-squared analysis. Time (h) between LPS injection and labor was calculated. Data were transformed ($Y=Y^2$) to obtain normal distribution (D'Agostino & Pearson normality test) and analysed by 1-way ANOVA with multiple comparisons (Tukey). Data recorded for number of pups for term deliveries was normally distributed (Shapiro-Wilk normality test) and analysed by 1-way ANOVA with multiple comparisons (Tukey). Preterm deliveries did not yield any viable pups.

Chapter Three: Contraction-associated gene expression changes during *ex situ* culture of pregnant human uterine smooth muscle

Marina ILICIC, Trent BUTLER, Tamas ZAKAR and Jonathan W. PAUL

This Chapter contains a manuscript published in the Journal of Smooth Muscle Research. The original manuscript is included in appendix E.

Concerns have previously been raised about the ability of primary cells in culture to remain representative of their tissues of origin. In this manuscript we report that non-laboring human myometrial tissues in culture undergo culture-induced changes in the expression of key contraction-associated genes. These changes are consistent with a spontaneous *in vitro* transition toward a laboring phenotype. We further report that implementing culture conditions approaching the *in vivo* environment was successful in preventing some of these culture-induced changes. Consistent with other studies in the field, we examined changes in gene expression as quantitative assessment of mRNA was preferential to semi-quantitative estimates of protein levels by western blot. Recent studies have also shown that changes in mRNA plays a dominant role in determining protein levels during periods of dynamic change, such as during myometrial phenotype transition.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Marina Ilicic (1st Author)	**Conceived and designed experiments** **Sample collections** **Performed explant experiments** **Performed molecular analyses** **Data analyses** **Manuscript writing**
Trent Butler	Provided advice on data analysis
Tamas Zakar	Conceived and designed experiments Provided advice on data analyses Provided reagents and materials Manuscript editing
Jonathan W. Paul	Conceived and designed experiments Performed explant experiments Data analyses Provided reagents and materials Manuscript editing

3.1 Abstract

Background: *Ex situ* analyses of human myometrial tissue has been used to investigate the regulation of uterine quiescence and transition to a contractile phenotype. Following concerns about the validity of cultured primary cells, we examined whether myometrial tissue undergoes culture-induced changes *ex situ* that may affect the validity of *in vitro* models.

Objectives: To determine whether human myometrial tissue undergoes culture-induced changes *ex situ* in estrogen receptor 1 (*ESR1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) and oxytocin receptor (*OXTR*) expression. Additionally, to determine whether culture conditions approaching the *in vivo* environment influence the expression of these key genes.

Methods: Term non-laboring human myometrial tissues were cultured in the presence of specific treatments, including; serum supplementation, progesterone and estrogen, cAMP, PMA, stretch or NF-κB inhibitors. *ESR1*, *PTGS2* and *OXTR* mRNA abundance after 48 h culture was determined using quantitative RT-PCR.

Results: Myometrial tissue in culture exhibited culture-induced up-regulation of *ESR1* and *PTGS2* and down-regulation of *OXTR* mRNA expression. Progesterone prevented culture-induced increase in *ESR1* expression. Estrogen further up-regulated *PTGS2* expression. Stretch had no direct effect, but blocked the effects of progesterone and estrogen on *ESR1* and *PTGS2* expression. cAMP had no effect whereas PMA further up-regulated *PTGS2* expression and prevented decline of *OXTR* expression.

Conclusion: Human myometrial tissue in culture undergoes culture-induced gene expression changes consistent with transition toward a laboring phenotype. Changes in *ESR1*, *PTGS2* and *OXTR* expression could not be controlled simultaneously. Until optimal culture conditions are determined, results of *in vitro* experiments with myometrial tissues should be interpreted with caution.

3.2 Introduction

Parturition requires that myometrial smooth muscle undergo a phenotypic transition, remaining quiescent for the majority of gestation and then transforming to a tissue capable of generating forceful, co-ordinated contractions to expel the fetus and the placenta [5, 6]. Transformation of the myometrium from the quiescent to a contractile state necessitates the presence of estrogen prior to the onset of labor [5-9]. In humans and higher primates, however, maternal estrogen levels are already high for most of pregnancy and remain elevated during parturition [85, 86]. This has led to the concept of functional estrogen activation whereby myometrium becomes sensitive to estrogen through changes in ER expression, encoded by the genes *ESR1* and *ESR2*. Our group has reported that expression of *ESR1* is low in non-laboring term myometrium and that *ESR1* levels rise with the onset of labor, whilst expression of *ESR2* was barely detectable and did not change with labor [56, 91]. This suggests that increased *ESR1* expression mediates functional estrogen activation. Furthermore, we found that U-0126, a highly selective inhibitor of mitogen-activated protein kinase kinase (MEK), blocked the ability of

estrogens to stimulate the expression of the oxytocin receptor (OTR) in human myometrial samples *in vitro* [91]. Expression of the *OXTR* in the myometrium increases after 37 weeks of gestation [127], which is followed by a fall in advanced labor [128, 246, 247]. Furthermore, a genome-wide study found that the high *OXTR* mRNA abundance in term non-laboring myometrium samples decreased with labor [212]. Studies using human tissues and myometrium-derived cell lines have demonstrated that the binding of oxytocin to its receptor led to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which subsequently increased the production of PGs, inflammatory chemokines and cytokines that are involved in fetal membrane remodelling, cervical ripening and myometrial activation [164, 165]. *PTGS2* encodes a key enzyme responsible for the biosynthesis of PGs. *PTGS2* mRNA abundance is low in term non-laboring human myometrium and increases with the onset of labor [212, 248]. Increased expression of *ESR1* and *PTGS2* and decreased expression of *OXTR* is therefore associated with transition toward a laboring phenotype. Characterizing the regulation of these key myometrial genes is essential to understanding normal human birth, as well as obstetric complications, including preterm labor.

Outside of clinical trials, researchers are primarily limited to observational studies on human pregnancy and as such rely heavily on animal models of pregnancy and *in vitro* studies using human cell lines and tissues. Primary myocyte cultures have been used extensively to study myometrial biology, but concerns have been raised about the lifespan of cultured primary cells [249] and their ability to remain to be representative of the tissue of origin [250-253]. Incubation of tissue *ex vivo* as small pieces or strips may represent the *in vivo*

phenotype more closely. Tissue strips are routinely used to analyze the effect of treatments on contractility [30, 254, 255], and have even been used to capture dynamic phosphorylation events that occur in phase with contractions [110, 256].

Incubating pieces and strips of myometrium has emerged a popular experimental model for interrogating myometrial biology. Both approaches involve artificial *in vitro* conditions and rely on the assumption that the tissue phenotype remains stable across the course of the study. Non-laboring myometrial tissue strips, however, spontaneously develop rhythmic contractions *in vitro* over the course of just 1 – 2 h, suggesting that the tissue strips may rapidly transition away from a non-contractile phenotype [30, 110, 254]. Moreover, with tissue incubation studies routinely performed for 48 h or more, the transition from the *in vivo* phenotype may be even more pronounced. The implications are potentially significant as findings made using 'non-laboring' tissue may in fact have been generated using tissue that may not be representative of the non-laboring phenotype and could respond differently to treatments.

Aims

Experimental models of myometrium that are in a state of flux have the potential to confound the results when researchers seek to elucidate the trigger(s) for labor. Through characterizing the expression of key myometrial genes, the present study aimed to explore whether non-laboring myometrial tissue pieces and strips undergo culture-induced changes *in vitro* that are consistent with transition to a pro-contractile, laboring phenotype. We further

aimed to identify culture conditions that could prevent or attenuate phenotypic changes thereby providing researchers with a stable platform to conduct experimental studies.

Here we report evidence suggesting that non-laboring human myometrium undergoes a culture-induced transition to a labor-like phenotype *in vitro*. We further report that different aspects of the non-laboring phenotype can be differentially preserved by supplementing the medium with physiological concentrations of progesterone and estrogen, applying NFκB inhibitors and by exposing the uterine muscle samples to constant stretch.

3.3 Materials and Methods

Consumables and reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA–free 50 reactions were from Ambion (Austin, USA). Alien QRT-PCR Inhibitor Alert was purchased from Integrated Sciences Pty (Sydney, Australia). (R)-MG132, BAY-11-7085 and Phorbol Myristate Acetate (PMA) were obtained from Cayman Chemical Company (Michigan, USA). 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), PCR primers, Progesterone and Estradiol were purchased from Sigma (St Louis, USA). The 2 mL 2.8 mm CK28-R Ceramic Bead Kit was acquired from Bertin Technologies (Montigny-le-Bretonneux, France). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES, DMEM and Charcoal

Stripped Fetal Bovine Serum were obtained from Gibco (Carlsbad, USA). SYBR Green 2× Master mix was from Applied Biosystems (Carlsbad, USA).

Myometrial Tissue acquisition

These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). Human myometrial samples (5 × 5 × 10 mm) were obtained from the lower uterine segment during elective CS of singleton term pregnancies (38.2 – 39.6 weeks gestation). Patient BMI range was 18.3 – 38.0, and none of the patients were in-labor. The indications for elective CS were previous CS, placenta praevia, fetal distress or breach presentation. Women were excluded if they were given steroids. Following delivery of the placenta, all women immediately received 5 units of oxytocin (Syntocinon) into an intravenous line. Administration of oxytocin was part of the standard care for the prevention of postpartum hemorrhage. Myometrial biopsies were excised within 3 minutes after oxytocin administration. Samples were placed on ice in serum-free medium containing DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin and 10 mM HEPES for the transfer to the laboratory.

Myometrial tissue culture

Approximately 100 mg tissue from each sample was immediately snap frozen in liquid nitrogen for subsequent analysis. The remaining myometrium was dissected into approximately 2 × 2 × 2 mm pieces and washed in serum-free media. Samples were then incubated in serum-free or 5% (v/v) CSS-supplemented media in a 37°C, 95% air/5% CO_2 humidified incubator for 48 h.

The 5% CSS-supplemented culture media contained DMEM with high glucose, 5% CSS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin and 10 mM HEPES. To determine the effects of steroids, myometrial samples were incubated in 5% CSS-containing media with physiological concentrations of progesterone (P4; 500 nM) and/or estradiol (E2; 400 nM) [8] in a 37°C, 95% air/5% CO_2 humidified incubator for 48 hours. To determine the effect of stretch, myometrial tissue strips (2 × 2 × 10 mm) were cultured in 5% CSS-containing media for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of constant stretch (0, 2.45 or 7.35 kN/m^2, respectively). Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 mL of culture media in 50 mL tubes (strips subjected to 0 g only were tied at one end). To determine the effect of stretch and steroids, myometrial strips were cultured in 5% CSS-containing media with 500 nM P4 and 400 nM E2 for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of stretch. To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial pieces were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with the cAMP analogue 8-Br-cAMP (250 µM), or PMA (0.1, 1.0 µM), or the NF-κB inhibitors MG-132 (2.0, 5.0, 10.0 µM) and BAY-11-7085 (2.0, 5.0, 10.0 µM) individually or in combination (10.0 µM MG-132 + 10.0 µM BAY-11-7085). Vehicle was DMSO (0.1%). Following each incubation, the tissue pieces or strips were snap frozen using liquid nitrogen and stored at -80°C for subsequent analyses.

RNA extraction, reverse transcription and real-time quantitative PCR

RNA was extracted using TRizol Reagent (Ambion, USA) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Ambion, USA). An ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) was used to measure RNA concentration (absorbance at 260 and 280 nm) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5 x 10^7 copies of Alien RNA (Integrated Sciences Pty, Australia) and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers (Invitrogen, USA). Quantitative RT-PCR was performed using an ABI 7500 Sequence Detector (Applied Biosystem, USA). No-reverse transcription (no-RT) negative controls were prepared for each sample. The final volume of each PCR reaction was 20 µL, containing 10 µL of 2× SYBR Green PCR Master Mix (Applied Biosystems, USA), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 µL with 1.0 µL of 2.5 µM of Alien Primer Mix, 10 µL of 2× SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. NTCs were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express and are shown in the Table 3.1.

Table 3.1. ***cDNA primer sequences for ESR1, PTGS2 and OXTR.***

Primer	Primer Sequence	Amplicon Size	GenBank #
ESR1	F: TGAAAGGTGGGATACGAAAAGAC R: CATCTCTCTGGCGCTTGTGTT	66	NM_000125.3
PTGS2	F: ATGTTCCACCCGCAGTACAGA R: CAGCATAAAGCGTTTGCGGTA	101	NM_000963
OXTR	F: CTGGACGCCTTTCTTCTTCGT R: GAAGGCCGAGGCTTCCTT	73	NM_000916.3

ESR1: Estrogen Receptor 1; *PTGS2:* Prostaglandin-Endoperoxide Synthase 2; *OXTR:* Oxytocin Receptor; *PGR:* Progesterone Receptor.

Data and statistical analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the ΔCt method [245]. All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t* test was used. For multiple comparisons a one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett multiple comparisons was used. P-values ≤ 0.05 were considered statistically significant.

3.4 Results

Gene Expression Changes in Myometrium during 48 h of Culture

Myometrial tissue samples were incubated for 48 h in serum-free media to determine gene expression changes that the tissue undergoes upon being removed from the *in vivo* environment and cultured *in vitro*. There was a statistically significant increase in mRNA abundance for *ESR1* (p<0.0001) and *PTGS2* (p<0.0001) (Figure 3.1A and B), whilst *OXTR* mRNA abundance significantly decreased (p<0.0001) (Figure 3.1C). *ESR2* mRNA abundance was detected at extremely low levels in fresh tissue, and remained low and relatively unchanged following 48 h incubation (not shown).

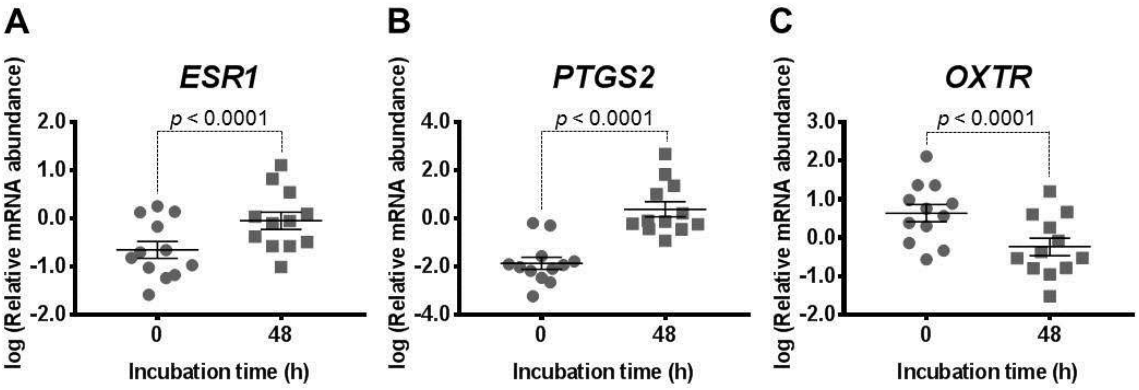

Figure 3.1. Culture-induced change in myometrial expression of *ESR1*, *PTGS2* and *OXTR* mRNAs following 48 h culture.
Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples immediately after biopsy (0 h) as well as following 48 h incubation (n=12), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then analysed by paired t-test*. *Data are mean ± SEM.*

Serum-free Media versus 5% CSS-containing Media

Having observed culture-induced changes in the expression of *ESR1*, *PTGS2* and *OXTR* mRNAs, we examined whether the presence of 5% CSS, often included in myometrial culture media [257], affected the phenotypic change of myometrium *in vitro*. Following 48 h incubation, there was no significant difference in *ESR1* mRNA abundance between serum-free media

and 5% CSS media (Figure 3.2A). Similarly, the presence or absence of 5% CSS in media had no effect on culture-induced change of *PTGS2* mRNA abundance (Figure 3.2B) or *OXTR* mRNA abundance (Figure 3.2C) following 48 h incubation.

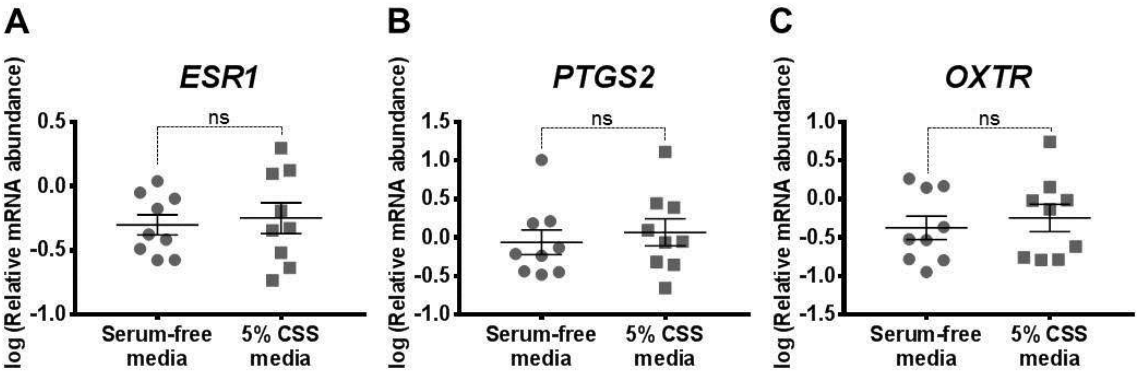

Figure 3.2. Effect of serum on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA levels *in vitro.*
Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples following 48 h incubation in serum-free media or media supplemented with 5% CSS (n=9), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then analysed by paired t-test*. *Data are mean ± SEM.*

The Effect of Steroids

Relative abundance of *ESR1*, *PTGS2* and *OXTR* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 500 nM P4 or 500 nM P4 + 400 nM E2, which are hormone concentrations in term maternal plasma [8].

A statistically significant decrease in *ESR1* mRNA abundance was observed between DMSO- and P4-treated tissue after 48 h (p=0.0015), indicating that supplementing media with 500 nM P4 blocked the culture-induced increase in *ESR1* mRNA abundance compared to fresh tissue (Figure 3.3A). The combination of 500 nM P4 + 400 nM E2 had no further effect as the

500 nM P4 + 400 nM E2 treatment significantly reduced mRNA levels at 48 h relative to vehicle treatment (*p*=0.0008), but not relative to P4 treatment alone (Figure 3.3A).

Supplementing media with 500 nM P4 had no statistically significant effect on *PTGS2* mRNA abundance relative to the vehicle (Figure 3.3B). Supplementing media with 500 nM P4 + 400 nM E2, however, significantly increased *PTGS2* mRNA abundance at 48 h relative to the vehicle (*p*=0.0482), indicating that P4 + E2 in combination exacerbated the up-regulation of *PTGS2* expression compared to the fresh tissue (Figure 3.3B).

Supplementing media with 500 nM P4 alone, or 500 nM P4 + 400 nM E2, had no effect on the culture-induced change in *OXTR* mRNA abundance relative to the control (Figure 3.3C).

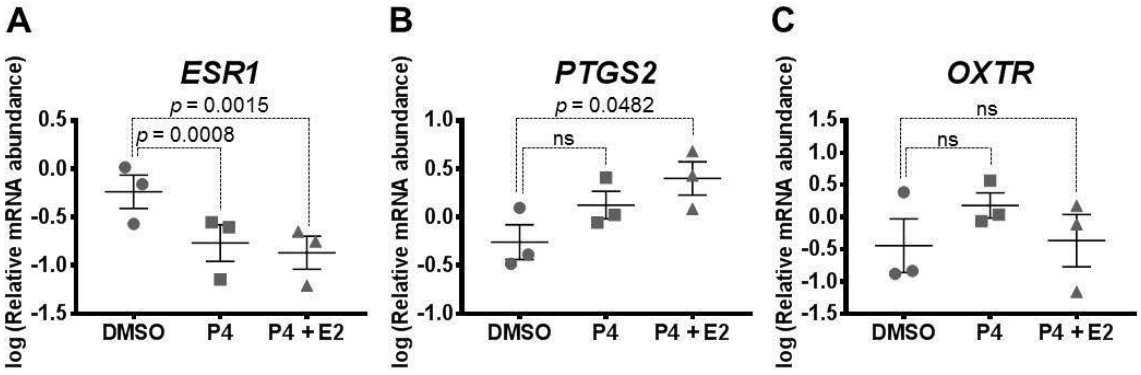

Figure 3.3. Effect of steroids on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNAs *in vitro.*
Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples following 48 h incubation in the presence of 500 nM progesterone (P4) or 500 nM P4 + 400 nM estradiol (E2) (n=3), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data were logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then compared by 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effect of Stretch on Human Myometrial Gene Expression in vitro

Myometrial tissue strips were subjected to 0, 1 or 3 g of stretch (0, 2.45 or 7.35 kN/m^2, respectively) for 48 h in absence or presence of steroids (500 nM P4 + 400 nM E2) to determine whether applying stretch to the muscle influenced the culture-induced changes in *ESR1*, *PTGS2* or *OXTR* mRNA levels *in vitro*.

As seen in Figure 3.4A, 0 – 3 g stretch had no effect on *ESR1* expression in the tissue strips. Interestingly, stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *ESR1* mRNA levels relative to the control (Figure 3.4B), indicating that P4 + E2 was no longer effective in preventing culture-induced increase in *ESR1* mRNA abundance compared to fresh tissue.

Similarly, stretch applied to myometrial strips for 48 h had no effect on *PTGS2* mRNA expression relative to the control (Figure 3.4C). Stretch applied in the presence of P4 + E2 likewise had no effect (Figure 3.4D). Thus, the P4 + E2 treatment no longer exacerbated the increase in *PTGS2* mRNA abundance that occurred after 48 h *in vitro*.

Applying stretch to myometrial strips for 48 h had no effect on *OXTR* expression (Figure 3.4E). Stretch applied in the presence of 500 nM P4 + 400 nM E2 also had no effect on *OXTR* mRNA abundance after 48 h culture relative to the control (Figure 3.4F).

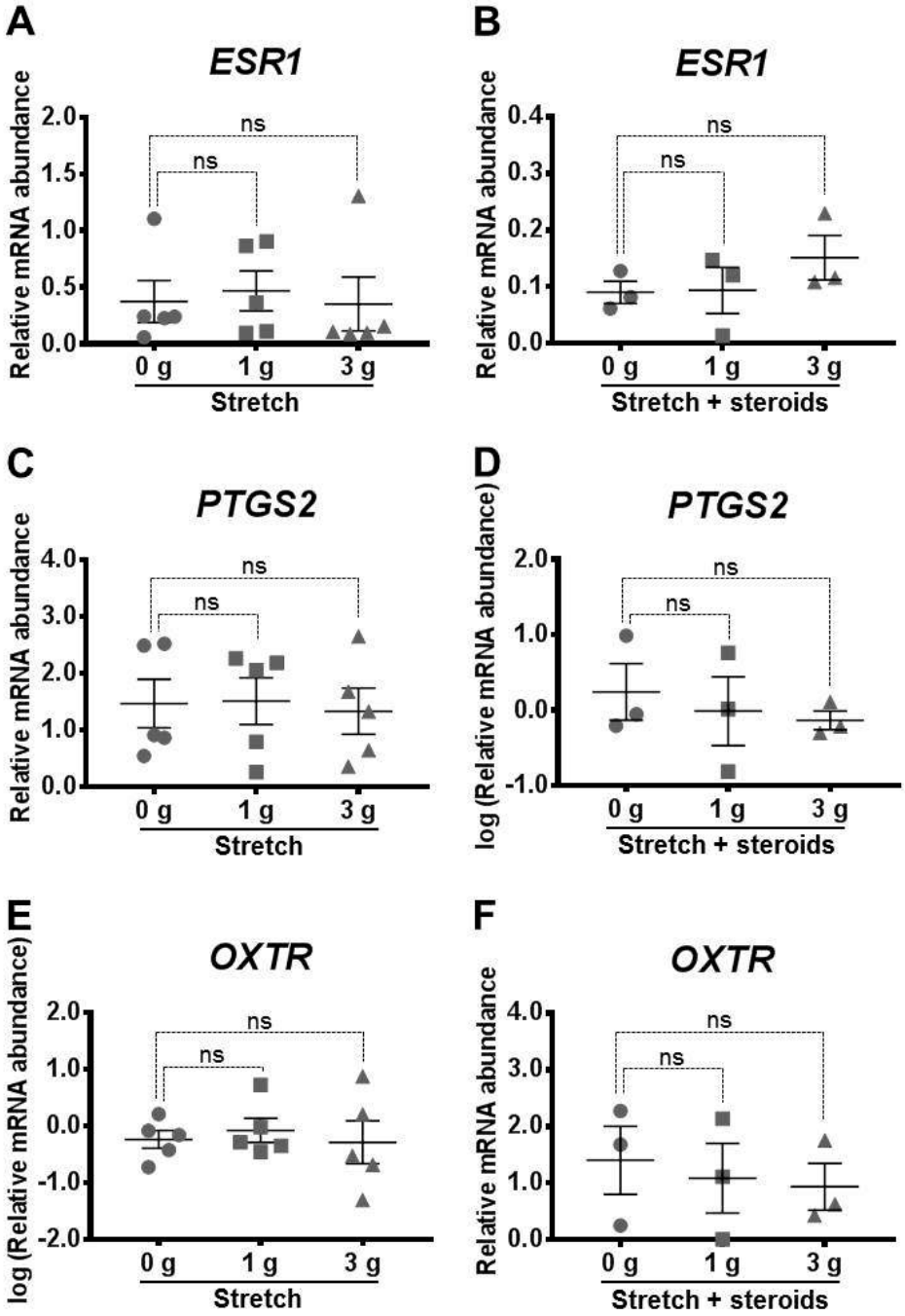

Figure 3.4. Effect of stretch in the absence or presence of steroids on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA levels *in vitro.*
Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial strips whilst applying 0, 1 and 3 g of stretch for 48 h (n=5), as well as in presence of steroids (500 nM P4 + 400 nM E2) whilst applying 0, 1 and 3 g of stretch for 48 h (n=3), and expressed relative to Alien reference. (A) Effect of stretch on *ESR1* mRNA abundance. (B) Effect of stretch and steroids on *ESR1* mRNA abundance. (C) Effect of stretch on *PTGS2* mRNA abundance. (D) Effect of stretch and steroids on *PTGS2* mRNA abundance. (E) Effect of stretch on *OXTR* mRNA abundance. (F) Effect of stretch and steroids on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effects of Activating Intracellular Signalling Pathways

Relative abundance of *ESR1*, *PTGS2* and *OXTR* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 8-Br-cAMP (250 µM), PMA (0.1, 1.0 µM) or vehicle (DMSO).

Adding 250 µM 8-Br-cAMP to the culture media had no effect on *ESR1* mRNA expression relative to the control (Figure 3.5A). Similarly, supplementing media with 0.1 or 1.0 µM PMA, a PKC activator, had no effect on *ESR1* mRNA levels relative to the control (Figure 3.5B).

Adding 250 µM 8-Br-cAMP had no effect on *PTGS2* mRNA abundance relative to the control (Figure 3.5C), but supplementing the culture media with 1.0 µM PMA increased *PTGS2* expression significantly (p=0.047) relative to the control (Figure 3.5D).

Adding 250 µM 8-Br-cAMP to the culture media had no significant effect on *OXTR* mRNA abundance after 48 h of culture relative to the control (Figure 3.5E). *OXTR* mRNA abundance was significantly increased in response to 1.0 µM PMA (p=0.0099) relative to the control (Figure 3.5F).

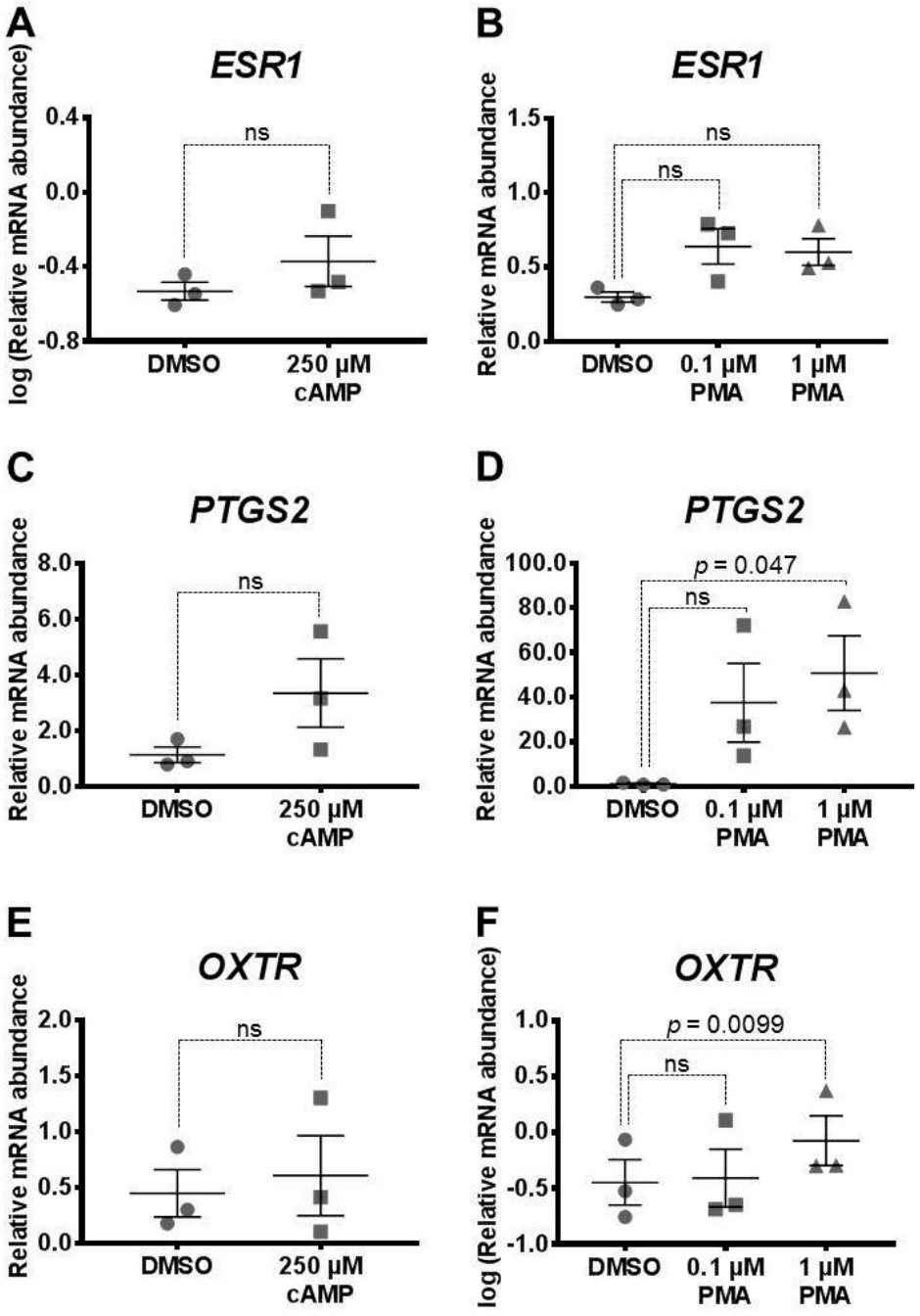

Figure 3.5. Effect of cAMP and PMA on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA *in vitro.*

Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of 8-Br-cAMP (250 µM) or PMA (0.1 and 1.0 µM), and expressed relative to Alien reference. (A) Effect of 8-Br-cAMP on *ESR1* mRNA abundance. (B) Effect of PMA on *ESR1* mRNA abundance. (C) Effect of 8-Br-cAMP on *PTGS2* mRNA abundance. (D) Effect of PMA on *PTGS2* mRNA abundance. (E) Effect of 8-Br-cAMP on *OXTR* mRNA abundance. (F) Effect of PMA on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). 8-Br-cAMP data was analysed using paired t-test, whilst 1-way ANOVA with multiple comparisons (Dunnett) was used to analyse PMA data. Data are mean ± SEM.*

The Effect of NF-κB Inhibitors

The NF-κB inhibitors, MG-132 and BAY-11-7085, were employed to test whether NF-κB pathway activation was involved in the gene expression changes induced by the *in vitro* conditions.

Incubating myometrial samples with 0, 2, 5 or 10 μM MG-132 or BAY-11-7085 individually or in combination (10 μM each) had no effect on *ESR1* mRNA abundance relative to the control (Figures 3.6A – 3.6C).

Neither inhibitor affected culture-induced changes in *PTGS2* mRNA abundance (Figures 3.6D and 3.6E), nor was *PTGS2* mRNA abundance affected by a combination of the two compounds relative to the control (Figure 3.6F).

Similarly, supplementing culture media with MG-132 or BAY-11-7085 had no effect on *OXTR* mRNA abundance following 48 h of culture relative to the control (Figures 3.6G and 3.6H), and *OXTR* mRNA abundance was not affected by a combination of the two compounds (Figure 3.6I).

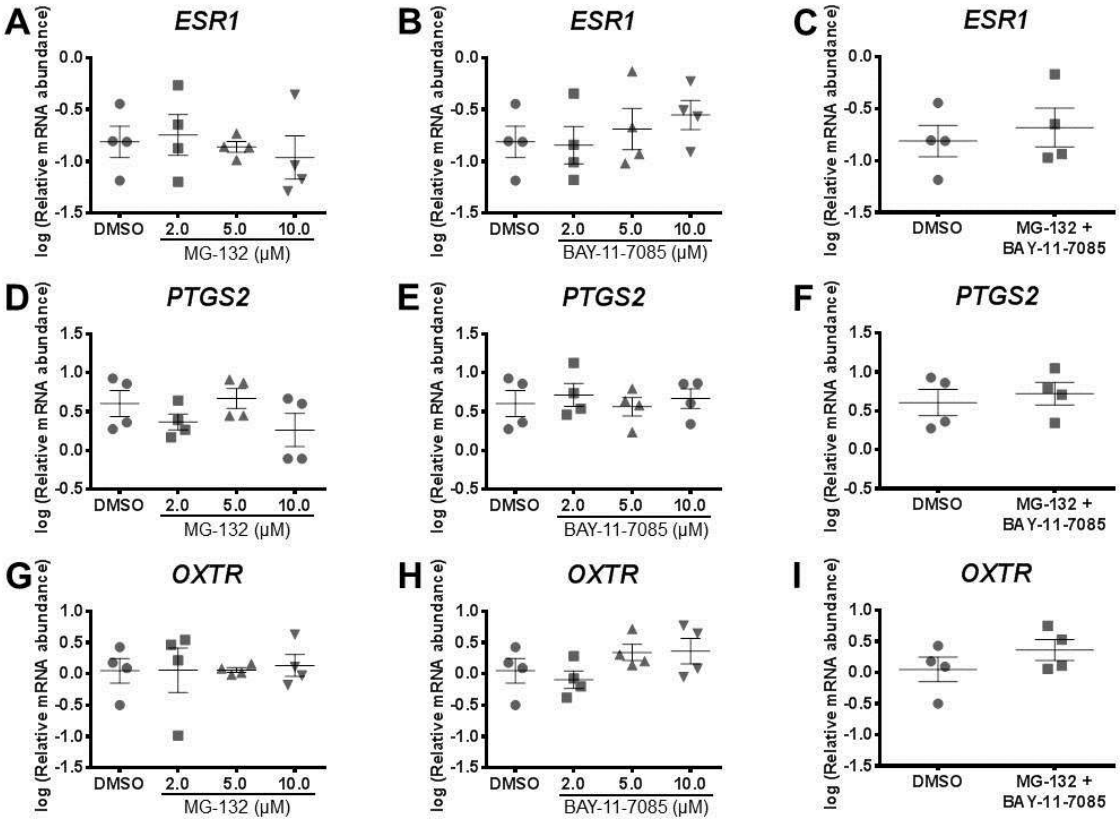

Figure 3.6. Effect of NF-κB inhibitors, MG-132 and BAY-11-7085, on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA *in vitro.*

Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial samples (n=4) following 48 h incubation in the presence of different NF-κB inhibitors, and expressed relative to Alien reference RNA. (A) Effect of MG-132 on *ESR1* mRNA abundance. (B) Effect of BAY-11-7085 on *ESR1* mRNA abundance. (C) Effect of MG-132 and BAY-11-7085 on *ESR1* mRNA abundance. (D) Effect of MG-132 on *PTGS2* mRNA abundance. (E) Effect of BAY-11-7085 on *PTGS2* mRNA abundance. (F) Effect of MG-132 and BAY-11-7085 on *PTGS2* mRNA abundance. (G) Effect of MG-132 on *OXTR* mRNA abundance. (H) Effect of BAY-11-7085 on *OXTR* mRNA abundance. (I) Effect of MG-132 and BAY-11-7085 on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). MG-132 and BAY-11-7085 data was analysed by 1-way ANOVA with multiple comparisons (Dunnett). MG-132 + BAY-11-7085 data was analysed using paired t-test. Data are mean ± SEM.*

3.5 Discussion

In the context of human parturition, *in vitro* models such as myometrial cell lines and *ex situ* tissues have been an important tool for investigating the maintenance of uterine quiescence and the mechanisms by which the myometrium transforms to an actively contracting organ at labor. This study

examined whether non-laboring myometrial tissues and strips undergo changes in culture that are consistent with transition to a pro-contractile, laboring phenotype. We determined gene expression changes as quantitative assessment of mRNA in preference to semi-quantitative estimates of protein levels by Western blot [258]. Notably, recent studies examining protein profiles in mammalian cells have found that transcription, not translation, chiefly determines protein abundance [259], and that during periods of dynamic change, such as that occurring during phenotype transition, changes in mRNA abundance play a particularly dominant role in controlling changes in protein levels [260].

Here we showed that the abundance of both *ESR1* and *PTGS2* mRNAs was significantly increased after 48 h of culture of myometrial tissues (Figures 3.1A and 1B). Our group previously reported that *ESR1* expression increased in the myometrium with the onset of labor (6- to 7-fold) [56, 91, 261]. *PTGS2* expression was also reported to increase in the human myometrium with labor (9-fold) [212, 248]. Our observation that *ESR1* (4.5-fold) and *PTGS2* (88-fold) mRNA abundance increase during culture is thus consistent with the possibility that the myometrium has been transitioning to a laboring state *in vitro*. Moreover, *OXTR* expression was high in term non-laboring myometrium and significantly decreased after 48 h incubation (6.9-fold) (Figure 3.1C). As shown previously, *OXTR* mRNA abundance increases in uterine tissue after 37 weeks of gestation [127] and there is an apparent fall in *OXTR* expression in advanced labor [128, 246, 247]. Chan Y.W. *et al.* [212] characterized the human myometrial transcriptome during the transition from term, not-in-labor to in-labor state and confirmed that *OXTR* mRNA expression decreased with labor (5.7-

fold) [212]. Therefore, our observation that *OXTR* expression spontaneously declines during culture further supports a transition toward a laboring phenotype *in vitro*.

Performing *in vitro* experiments using myometrium that is in a state of flux is not ideal and raises the possibility that the relevant literature may contain findings reported for non-laboring tissue were in fact obtained with myometrium transitioning to a laboring phenotype. To address this problem we sought to identify culture conditions that could be implemented to prevent culture-induced changes in *ESR1*, *PTGS2* and *OXTR* expression, which are markers of the pro-contractile transformation. We examined whether the presence of CSS (5%) in the media affected the culture-induced changes of *ESR1*, *PTGS2* and *OXTR* in myometrium (Figure 3.2). Supplementing media with serum is common practice during *in vitro* culture, however, our results indicate that supplementing with serum alone is not sufficient to prevent culture-induced changes in these key myometrial genes.

The balance between progesterone and estrogen plays a key role in transformation of the myometrium from the quiescent to a contractile state. Progesterone maintains pregnancy by promoting myometrial quiescence [7, 38], while estrogen is considered a principal endocrine factor responsible for instigating myometrial activation [5-7, 56]. Both progesterone and estrogen maternal plasma levels are high during human pregnancy and remain elevated during parturition [8, 9, 43, 85, 86]. Previous studies have shown that the interaction between progesterone and PR-B suppresses *ESR1* expression, thereby rendering the myometrium refractory to circulating estrogen [49, 56].

With advancing gestation, however, PR-A expression increases, which in turn represses the transcriptional activity of PR-B, and as a result the PR-B-mediated inhibition of *ESR1* expression is withdrawn [49, 56]. Once myometrial tissue is removed from *in vivo* environment and cultured *in vitro*, the high plasma levels of progesterone and estrogen are no longer present, thereby removing the functional link between progesterone and estrogen [56], which could possibly account for the observed culture-induced changes in *ESR1*, *PTGS2* and *OXTR* expression (Figure 3.1). Consistent with the existing literature [262, 263], we found that supplementing media with 500 nM P4 prevented the culture-induced up-regulation of *ESR1* mRNA abundance (Figure 3.3A) over 48 h. Further, *ESR1* expression remained repressed when 500 nM P4 was administered in combination with 400 nM E2. While culture-induced up-regulation of *ESR1* expression was prevented by 500 nM P4, P4 alone was unable to prevent the culture-induced increase in *PTGS2* expression *in vitro*. It was anticipated that P4 alone would prevent the culture-induced increase in *PTGS2* expression based on previous reports that P4 inhibits *PTGS2* expression in myometrial cells via direct interaction of the PR with NF-κB p65, as well as by progesterone-induced expression of the NF-κB inhibitor, IκB-α [264, 265]. The reason for this discrepancy is unclear. Interestingly, supplementing media with P4 + E2 significantly increased *PTGS2* mRNA abundance beyond the level of culture-induced increase in the absence of steroids (Figures 3.1B and 3.3B).

A study using immortalized human myocytes reported that progesterone binding to its receptor up-regulated zinc finger E-box binding homeobox protein 1 and suppressed miR-200b/429, which led to down-regulated *OXTR*

expression [221]. We found that supplementing media with P4 alone was unable to prevent the culture-induced decline in *OXTR* expression during culture (Figure 3.3C). Furthermore, it was anticipated that supplementing media with E2 would increase *OXTR* expression, as our group has previously shown that E2 treatment for 6 h significantly increased *OXTR* expression in myometrial tissue [91]. Possibly, E2 was unable to up-regulate *OXTR* expression in the present study due to being administered in combination with P4.

In normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [266, 267]. Previous studies in the sheep, rat and wallaby have shown that stretch increased *PTGS2* and *OXTR* expression, whereas *ESR1* expression was not significantly affected [268-270]. In humans, uterine wall tension was found to increase across gestation, as calculated by measuring uterine wall thickness and intrauterine pressure [271]. Tension rose markedly beyond 30-weeks, and at 37-weeks ranged from approximately 10 – 75 kN/m^2. This study examined stretch as a culture condition potentially affecting the *in vitro* changes in *ESR1*, *PTGS2* or *OXTR* expression. We found that constant stretch, applied by means of attaching a hanging weight to tissue strips, did not influence the changes in *ESR1, PTGS2* or *OXTR* mRNA levels (Figures 3.1 and 3.4). This is at variance with previous reports that stretch has up-regulated *PTGS2* and *OXTR* expression in the uterus; however, it should be noted that those studies were performed in animals [268-270]. It should also be noted that maximum tension applied in our assay (3 g; 7.35 kN/m^2) was slightly below the range calculated by Sokolowski *et al*. at 37-weeks [271] (10 – 75 kN/m^2). Applying tension >7.35 kN/m^2 in preliminary experiments was found to cause muscle

tearing in our system, whereas 7.35 kN/m^2 stretched the tissue strips sustainably for the 48 h culture period. It has been reported that in a P4-dominated endocrine environment, moderate stretch maintains relaxation and quiescence; however, in the absence of P4 or excessive stretch, the uterus contracts [266, 267]. Interestingly, although stretch did not directly affect the culture-induced changes in *ESR1*, *PTGS2* or *OXTR* expression, it prevented steroids (P4 + E2) from blocking the culture-induced increase in *ESR1* mRNA abundance, and prevented steroids from increasing *PTGS2* mRNA abundance (beyond the increase *in vitro)*.

It is well documented that the cAMP signalling pathway is up-regulated in the human myometrium throughout pregnancy to maintain uterine quiescence [10, 31, 272]. Supplementing media with a cAMP analogue 8-Br-cAMP failed to prevent culture-induced changes in *ESR1*, *PTGS2* or *OXTR* mRNA abundance. We also examined effects of PMA, a pro-contractile agent, on *ESR1*, *PTGS2* and *OXTR* expression. PMA activates PKC, which stimulates signalling pathways mediated by ERK and NF-κB, both involved in labor [273, 274]. Furthermore, PKC activity is crucial for oxytocin-stimulated myometrial contractions [275]. PMA was effective in modulating expression of both *PTGS2* and *OXTR*, but had no effect on *ESR1*. As expected for a pro-contractile agent, PMA up-regulated *PTGS2* expression *in vitro*, which was consistent with augmenting the transition toward a laboring phenotype. Interestingly, PMA prevented the culture-induced decline in *OXTR* expression. Actions of PMA on *OXTR* were therefore consistent with preserving the term not-in-labor phenotype, which is characterised by high *OXTR* expression.

Previous studies have shown that labor is an inflammatory process associated with increased production of pro-inflammatory mediators, increased expression of *PTGS2* mRNAs and increased NF-κB activity [248, 276-278]. Supplementing media with NF-κB inhibitors such as MG-132 or BAY-11-7085, therefore, represented a feasible means of preventing culture-induced changes in *ESR1*, *PTGS2* or *OXTR* expression. These inhibitors, added either separately or in combination, had no effect on *ESR1, PTGS2* and *OXTR* mRNA abundance. This argues against the possibility that our culture conditions activated NFκB-dependent inflammatory pathways causing transition to a laboring phenotype. Nevertheless, preliminary data using two NF-κB inhibitors do not prevent the need for further detailed studies to fully explore the effects of various culture conditions on inflammatory pathway activation and the associated functional consequences in myometrial explant systems.

Conclusion

Term non-laboring human myometrial tissue undergoes culture-induced changes in expression of *ESR1*, *PTGS2* and *OXTR* that are consistent with transitioning toward a laboring phenotype. Culture-induced transition toward a laboring phenotype calls for caution as to whether non-laboring myometrial biopsies remain representative of non-laboring myometrial tissue throughout the course of experiments, and as such may cast doubt on findings gleaned from not-in-labor tissue. Through examining various culture conditions, we found progesterone blocked the culture-induced increase in *ESR1*, but failed to prevent culture-induced increase in *PTGS2*. Culture-induced decline in *OXTR* expression was prevented by supplementing media with PMA, however, this was concurrent with further stimulation of *PGTS2* expression toward a laboring

phenotype. Culture-induced increase of *PTGS2* expression *in vitro* was not counteracted by any of the treatments examined, including supplementation with NF-κB inhibitors. Additional studies are warranted to determine appropriate culture conditions that prevent the changes of key myometrial genes *in vitro,* thereby providing a stable platform on which to investigate the regulation of myometrial biology. Before that, *in vitro* studies using myometrial cells or tissues should be interpreted cautiously regarding the relevance to myometrial regulation during pregnancy and labor *in vivo*.

3.6 Acknowledgements

The authors wish to thank the obstetricians from the John Hunter Hospital, NSW, our research midwife, Anne Wright, and the research participants who donated samples toward this study.

Chapter Four: Modulation of Progesterone Receptor isoform expression in pregnant human myometrium

Marina ILICIC, Tamas ZAKAR and Jonathan W. PAUL

This Chapter contains a manuscript published in BioMed Research International. The original manuscript is included in appendix F.

Concerns have been raised about the ability of cells and tissues in culture to remain representative of the tissues of origin. In this manuscript we report that non-laboring human myometrial tissue undergoes 'culture-induced' changes in the expression of progesterone receptor (*PR*) isoforms. These changes were consistent with non-laboring myometrium transitioning toward a laboring phenotype *in vitro*. These findings have important ramifications for the field as culture-induced changes occurred under conditions routinely used to culture myometrial explants, therefore raising questions as to the meaningfulness of the *in vitro* model.

Furthermore, we report that implementing culture conditions approaching the *in vivo* environment was successful in preventing these culture-induced changes.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Marina Ilicic (1st Author)	**Conceived and designed experiments** **Sample collections** **Performed explant experiments** **Performed molecular analyses** **Data analyses** **Manuscript writing**
Tamas Zakar	Conceived and designed experiments Provided reagents and materials Manuscript editing
Jonathan W. Paul	Conceived and designed experiments Performed explant experiments Data analyses Provided reagents and materials Manuscript editing

4.1 Abstract

Background: Regulation of myometrial PR expression is an unresolved issue central to understanding the mechanism of functional progesterone withdrawal and initiation of labor in women.

Objectives: To determine whether pregnant human myometrium undergoes culture-induced changes in *PR* isoform expression *ex situ*. Furthermore, to determine if conditions approaching the *in vivo* environment stabilise *PR* isoform expression in culture.

Methods: Term non-laboring human myometrial tissues were cultured under specific conditions: serum supplementation, steroids, stretch, cAMP, PMA, $PGF_{2\alpha}$, NF-κB inhibitors or TSA. Following 48 h culture, *PR-T*, *PR-A* and *PR-B* mRNA levels were determined using qRT-PCR. *PR-A/PR-B* ratios were calculated.

Results: *PR-T, PR-A* expression and the *PR-A/PR-B* ratio significantly increased in culture. Steroids prevented the culture-induced increase in *PR-T and PR-A* expression. Stretch blocked the effects of steroids on *PR-T* and *PR-A* expression. PMA further increased the *PR-A/PR-B* ratio, while TSA blocked culture-induced increases of *PR-A* expression and the *PR-A/PR-B* ratio.

Conclusion: Human myometrial tissue in culture undergoes changes in *PR* gene expression consistent with transition toward a laboring phenotype. TSA maintained the non-laboring PR isoform expression pattern. This suggests that preserving histone and/or non-histone protein acetylation is critical for

maintaining the progesterone dependent quiescent phenotype of human myometrium in culture.

4.2 Introduction

Preterm birth is a major societal and economic problem that affects approximately 9.6% of pregnancies worldwide, and accounts for 80 - 90% of neonatal morbidity and death [2, 279-281]. The prevention of preterm birth continues to be an important health priority. There is a substantial body of evidence highlighting the importance of progesterone in maintaining the pregnant state by promoting myometrial quiescence and relaxation [282-284]. The withdrawal of progesterone action signals the end of pregnancy, and in most mammalian species happens by a rapid fall in circulating levels of progesterone [276, 285-288]. In humans and higher primates, however, maternal, fetal and amniotic concentrations of progesterone remain elevated during parturition and delivery, suggesting that systemic progesterone withdrawal does not occur at the initiation of labor [289-291]. Nonetheless, the administration of a synthetic progesterone antagonist, RU486, to humans at any stage of pregnancy promotes cervical ripening and parturition [46-49, 282, 283]. As such a “functional” withdrawal of progesterone action has been proposed to explain the loss of pro-pregnancy progesterone actions despite circulating levels of progesterone remaining elevated. The exact mechanism of functional progesterone withdrawal is unclear and in recent years it has been the focus of intense research. One proposed mechanism is that functional progesterone withdrawal occurs through a decrease in myometrial responsiveness caused by

a change in PR isoform expression. Two major isoforms, PR-A and PR-B, exist in humans. PR-B is the principal transcriptional mediator of progesterone action and maintains uterine quiescence, while PR-A represses the transcriptional activity of PR-B and therefore decreases progesterone responsiveness [49, 280, 282, 283]. Recent work has also shown that PR-A ligand-independently stimulates the expression of the key labor promoting gene *Cx43* [72]. Thus, genomic progesterone responsiveness is believed to be regulated by the opposing actions of PR-A and PR-B and is inversely associated with the PR-A/PR-B ratio [49, 280, 282, 283]. Indeed, several studies, including our own, have shown that myometrial expression of *PR-A* has significantly increased late in human pregnancy and with the onset of labor [261, 292-294].

Elucidating the mechanism of functional progesterone withdrawal is therefore important for understanding the mechanisms regulating the balance between uterine quiescence and contractions. Outside of clinical trials, researchers are primarily limited to observational studies on human pregnancy. Interventional studies rely on animal models of pregnancy as well as on *in vitro* experiments using human myometrial smooth muscle cell lines and tissues. Human cell cultures are a valuable *in vitro* tool used to gain insight into numerous physiological and pathological processes; however, concerns have been raised about the lifespan of cultured primary cells [249] as well as their ability to remain to be representative of the tissue of origin [250-253]. The use of *ex vivo* myometrial tissue may represent the *in vivo* phenotype more closely and can involve utilizing smooth muscle biopsy samples as small pieces or dissecting the tissue into strips. Tissue strips are primarily utilized to examine

myometrial contractility [30, 254, 255, 295] such as the dynamic phosphorylation events that occur in phase with contractions [110, 256].

Although the use of *ex vivo* tissues pieces and strips has greatly facilitated studies into gene expression and regulation, both approaches rely on the assumption that the tissue phenotype remains stable across the course of the study. For instance, it is assumed that non-laboring myometrium retains a non-laboring phenotype *ex vivo* providing an experimental system to induce labor-associated changes. Myometrial strips from non-laboring pregnant women, however, spontaneously develop contractions *ex vivo* over the course of just 1 – 2 h, suggesting a rapid transition away from the non-laboring *in vivo* phenotype [30, 110, 254]. Furthermore, tissue incubation studies are routinely performed for 48 h or more, therefore the transition away from the original phenotype may be even more pronounced after 48 h culture *in vitro*.

Aims

The aim of this study was to determine if non-laboring myometrial tissue pieces and strips undergo culture-induced changes in PR expression that are consistent with transition to a PR isoform expression pattern similar to labor. We further aimed to identify culture conditions that could be implemented to block or minimize such transition *in vitro*, presenting researchers with a stable platform on which to conduct experimental studies.

Here we report that non-laboring human myometrium undergoes culture-induced changes in *PR* isoform expression *in vitro* comparable with the changes attributed to functional progesterone withdrawal at labor. We further report that supplementing media with the histone deacetylase inhibitor (HDACi),

TSA, prevents the culture-induced functional progesterone withdrawal phenomenon by maintaining a low *PR-A/PR-B* ratio, consistent with maintenance of a non-laboring phenotype.

4.3 Materials and Methods

Consumables and Reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA–free 50 reactions were from Ambion (Thermo Fisher). Alien QRT-PCR Inhibitor Alert 400 Reactions were purchased from Integrated Sciences Pty (Sydney, Australia). (R)-MG132, BAY-11-7085, PMA and $PGF_{2\alpha}$ were obtained from Cayman Chemical Company (Michigan, USA). 8-Br-cAMP, PCR primers, Progesterone and Estradiol were purchased from Sigma (St Louis, USA). 2 mL 2.8 mm ceramic bead kits (CK28-R) for the Precellys homogenizer (Bertin Instruments, France) were purchased from Thermo Fischer Scientific (Melbourne, Australia). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES, DMEM and Charcoal Stripped Fetal Bovine Serum were obtained from Gibco (Carlsbad, USA). SYBR Green 2× Master mix was from Applied Biosystems (Carlsbad, USA). TSA was supplied by Bio-Scientific Pty. Ltd. (Sydney, Australia).

Myometrial Tissue Acquisition

These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). Human myometrial samples were obtained

from the lower uterine segment during elective CS of singleton term pregnancies (38.2 – 39.6 weeks gestation). Patient BMI range was 18.3 – 38.0, and none of the patients were in-labor. The indications for elective CS were previous CS, placenta praevia, fetal distress or breach presentation. Women were excluded if they were given steroids. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of postpartum hemorrhage. Samples were therefore exposed to oxytocin for a brief period of time (3 min). All samples were placed on ice in serum-free medium containing DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/mL Gentamicin and 10 mM HEPES for the transfer to the laboratory.

Myometrial tissue (explant) culture

Approximately 100 mg tissue from each sample was immediately snap frozen in liquid nitrogen for subsequent analysis. The remaining myometrium was dissected into approximately 2 × 2 × 2 mm pieces and washed in serum-free media. Samples were then incubated in serum-free or 5% (v/v) CSS-supplemented media in a 37°C, 95% air/5% CO_2 humidified incubator for 48 h. The 5% CSS-supplemented media contained DMEM with high glucose, 5% CSS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/mL Gentemicin and 10 mM HEPES. To determine the effects of steroids, myometrial samples were incubated with physiological concentrations of progesterone (P4; 500 nM) and/or estradiol (E2; 400 nM) [290] in a 37°C, 95% air/5% CO_2 humidified incubator for 48 hours. To determine the effect of stretch on human myometrium, myometrial tissue strips (2 × 2 × 10 mm) were cultured in 5% CSS-containing media for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator

whilst being subjected to 0, 1 or 3 g of constant stretch. Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 mL of culture media in 50 mL tubes (strips subjected to 0 g only were tied at one end). To determine the effect of stretch and steroids on human myometrium, myometrial strips were cultured in 5% CSS-containing media with 500 nM P4 and 400 nM E2 for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of stretch. To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial tissues were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with the cAMP analogue 8-Br-cAMP (250 µM), PMA (0.1, 1.0 µM), $PGF_{2\alpha}$ (1, 10, 100 and 100 nM), or the nuclear factor-κB (NF-κB) inhibitors MG-132 (2.0, 5.0, 10.0 µM) and BAY-11-7085 (2.0, 5.0, 10.0 µM) individually or in combination (10.0 µM MG-132 + 10.0 µM BAY-11-7085). Furthermore, myometrial tissues were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with TSA (0.5, 1.0, 2.5 or 5.0 µM). Vehicle was DMSO (0.1%). Following each incubation, the media was decanted, tissue pieces or strips were snap frozen using liquid nitrogen and stored at -80°C for subsequent analyses.

RNA extraction, Reverse transcription and Real-time quantitative PCR

RNA was extracted from 100 mg of tissue using TRizol Reagent (Thermo Fisher) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys 24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Thermo Fisher). An ND-1000 spectrophotometer was

used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5 $x10^7$ copies of Alien RNA and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers. Quantitative RT-PCR was performed using an ABI 7500 Sequence Detector. no-RT negative controls were prepared for each sample to ensure there was no DNA contamination. The final volume of each PCR reaction was 20 µL containing 10 µL of 2x SYBR Green PCR Master Mix (Thermo Fisher), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 µL with 1.0 µL of 2.5 µM of Alien Primer Mix, 10 µL of 2x SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. NTCs were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express and are shown in the Table 4.1.

Table 4.1. cDNA primer sequences for PR-T and PR-B.

Primer	Primer Sequence	Amplicon Size	GenBank #
PR-T	F: GTGGGAGCTGTAAGGTCTTCTTTAA R: AACGATGCAGTCATTTCTTCCA	83	NM000926.4
PR-B	F: TCGGACACCTTGCCTGAAGT R: CAGGGCCGAGGGAAGAGTAG	68	NM000926.4

PR-T: Progesterone Receptor total; *PR-B:* Progesterone Receptor Isoform B

Data and Statistical Analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the ΔC_t method [245]. The relative mRNA abundance of *PR-A* was calculated by subtracting the relative mRNA abundance of *PR-B* from that of *PR-T*. All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t* test was used. For multiple comparisons a one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett multiple comparisons was used. P-values ≤ 0.05 were considered statistically significant.

4.4 Results

Culture-induced changes in myometrial PR isoform expression over time

Myometrial tissues were incubated for 0, 1, 2, 6, 24 or 48 h in serum-free media to determine changes in *PR* isoform expression that occurred upon being removed from the *in vivo* environment and cultured *in vitro*. *PR-T*, *PR-B* and *PR-A* mRNA abundance were determined across the timeline and the *PR-A/PR-B* ratio calculated.

PR-T mRNA abundance was significantly increased after 48 h culture (p=0.0301) (Figure 4.1A) and was attributable to increased *PR-A* mRNA abundance, which was significantly increased beyond 24 h culture (p=0.0121)

(Figure 4.1B). *PR-B* mRNA abundance remained relatively constant (Figure 4.1C). The *PR-A*/*PR-B* ratio was significantly increased after 6 h *in vitro* culture (p=0.0487), and highly significant beyond 24h culture (p<0.0001) (Figure 4.1D).

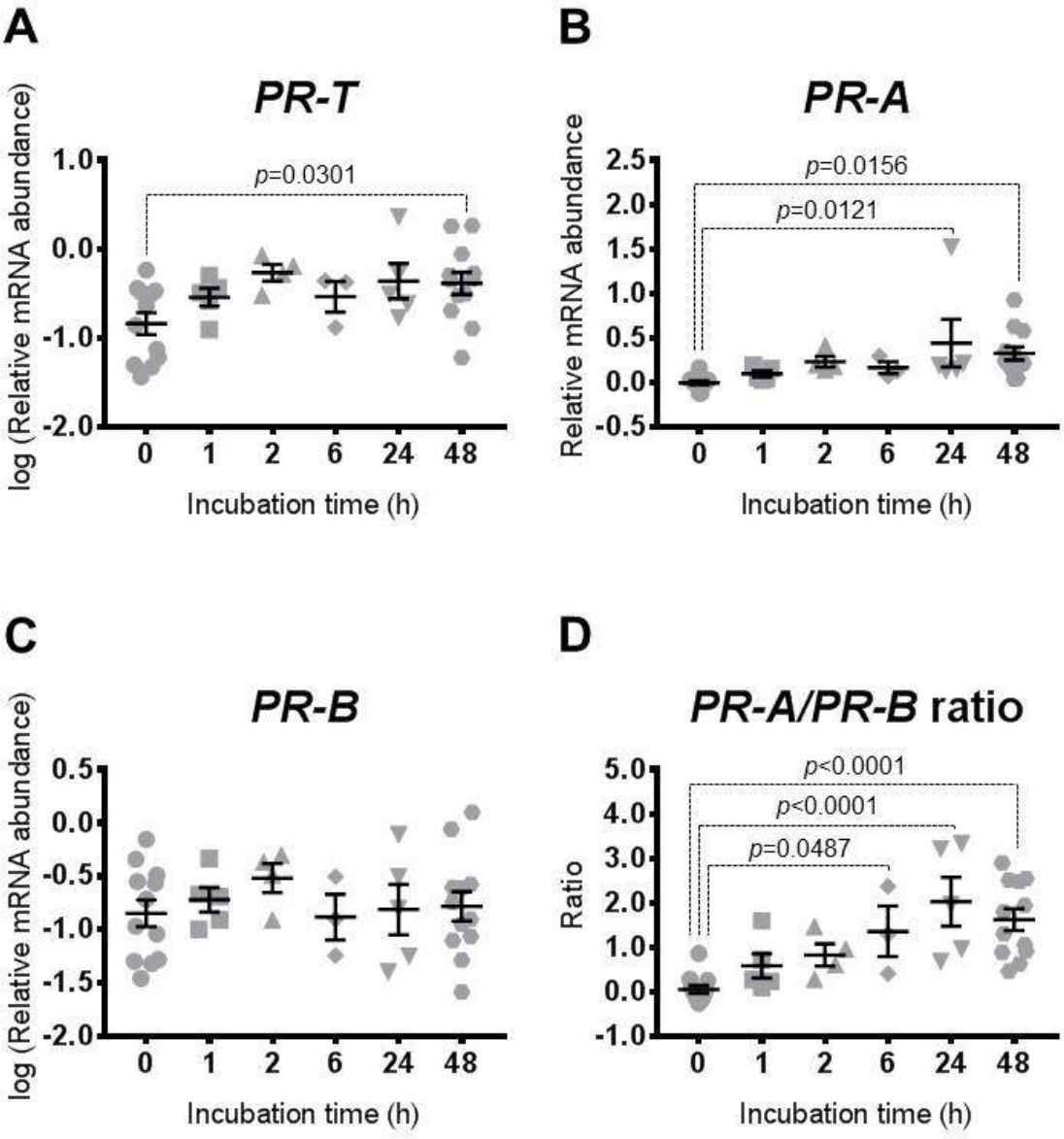

Figure 4.1. Culture-induced change in myometrial *PR* isoform expression over time.
Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples at different time points (0, 1, 2, 6, 24 and 48 h), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes in PR Isoform Expression Using Serum

CSS is often used in myometrial culture media [257, 296, 297]. Having observed a culture-induced increase in *PR-A* expression (and thus *PR-T* expression*),* we examined whether supplementing media with 5% CSS affected culture-induced changes in *PR* expression. CSS supplementation had no effect on culture-induced changes in *PR* expression. After 48 h culture, there was no significant difference in *PR-T, PR-A* or *PR-B* mRNA abundance. Furthermore, there was no significant difference in *PR-A/PR-B* expression ratio between myometrial tissues cultured in serum-free media versus 5% CSS-supplemented media (Figure 4.2).

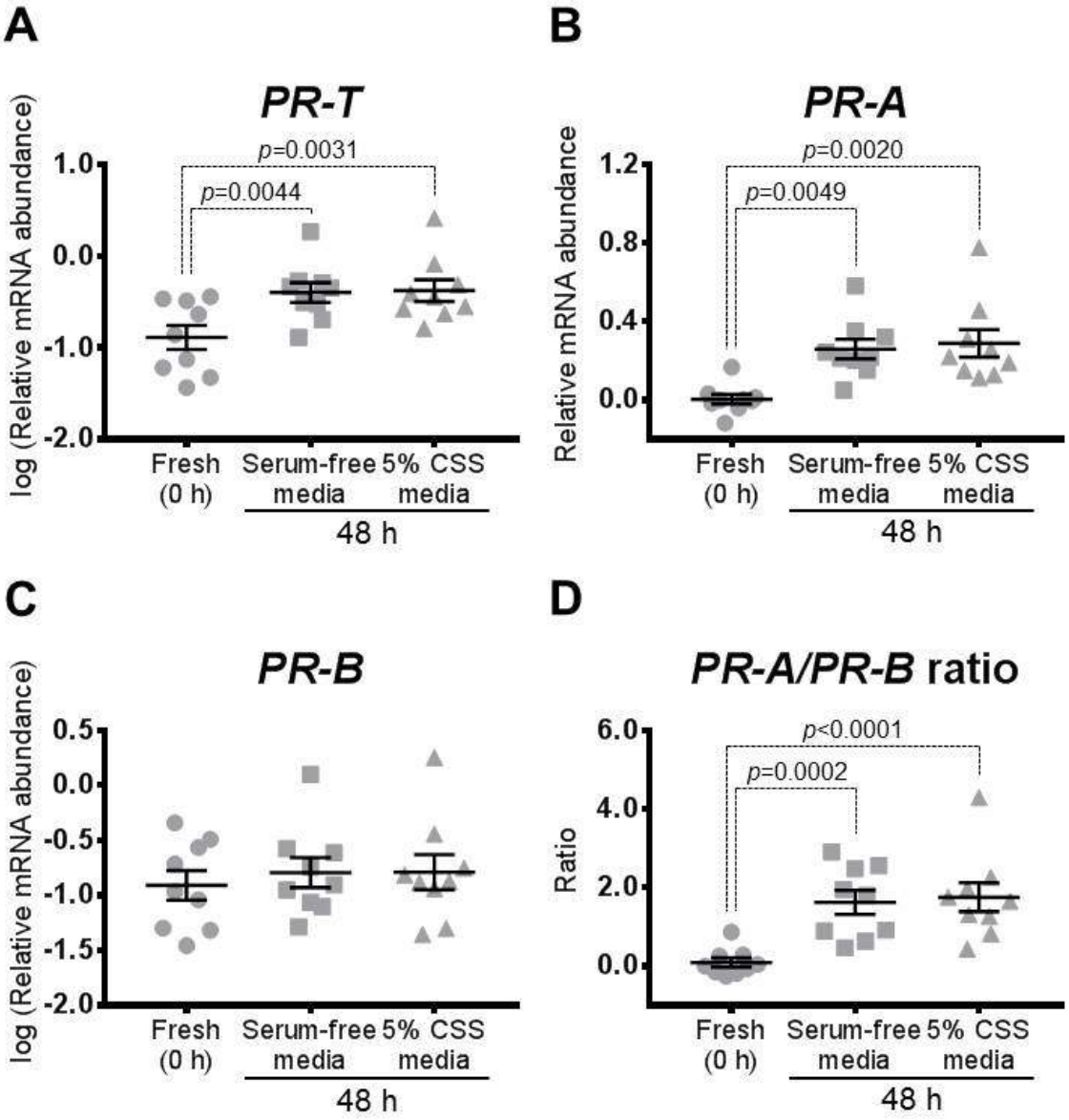

Figure 4.2. Effect of serum on culture-induced changes in *PR* isoform expression *in vitro.*

Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples following 48 h incubation in serum-free media or media supplemented with 5% CSS (n=9), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes in PR Isoform Expression Using Steroids

Relative abundance of *PR-T, PR-A* and *PR-B* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 500 nM P4 or 500 nM

P4 + 400 nM E2, which are hormone concentrations in term maternal plasma [290].

PR-T mRNA abundance significantly increased in DMSO-treated (control) tissues following 48 h incubation (p=0.0317) (Figure 4.3A). Upon supplementing media with 500 nM P4, *PR-T* mRNA abundance was reduced after 48 h culture relative to the control, however, the effect did not reach statistical significance (p=0.2457) (Figure 4.3A). Supplementing media with the combination of 500 nM P4 + 400 nM E2 for 48 h prevented the increase in *PR-T* mRNA abundance to the extent that there was a significant difference relative to 48 h DMSO-treated control tissues (p=0.0232) (Figure 4.3A).

Similarly, *PR-A* mRNA abundance significantly increased in DMSO-treated (control) tissues following 48 h incubation (p=0.0036) and supplementing media with 500 nM P4 reduced *PR-A* mRNA abundance after 48 h relative to the control; however, the difference was not statistically significant (p=0.3234) relative to 48 h DMSO-treated control tissues (Figure 4.3B). Supplementing media with the combination of 500 nM P4 + 400 nM E2 prevented the increase in *PR-A* mRNA abundance to the extent that there was a significant difference relative to 48 h DMSO-treated control tissues (p=0.0175) (Figure 4.3B).

PR-B mRNA abundance remained unchanged over 48 h of culture and supplementing media with 500 nM P4 alone, or 500 nM P4 + 400 nM E2, had no significant effect on *PR-B* mRNA abundance (Figure 4.3C).

The *PR-A/PR-B* expression ratio significantly increased in DMSO-treated (control) tissues following 48 h incubation (p=0.0054) (Figure 4.3D). P4-

supplementation had no effect on the *PR-A/PR-B* expression ratio relative to DMSO-treated control tissues and remained significantly elevated compared to fresh tissues (p=0.0006) (Figure 4.3D). Similarly, the combination of 500 nM P4 + 400 nM E2 had no significant effect on the *PR-A/PR-B* expression ratio after 48 h relative to DMSO-treated control tissues (p>0.9999), and the *PR-A/PR-B* expression ratio remained significantly elevated relative to the fresh tissues (p=0.0053) (Figure 4.3D).

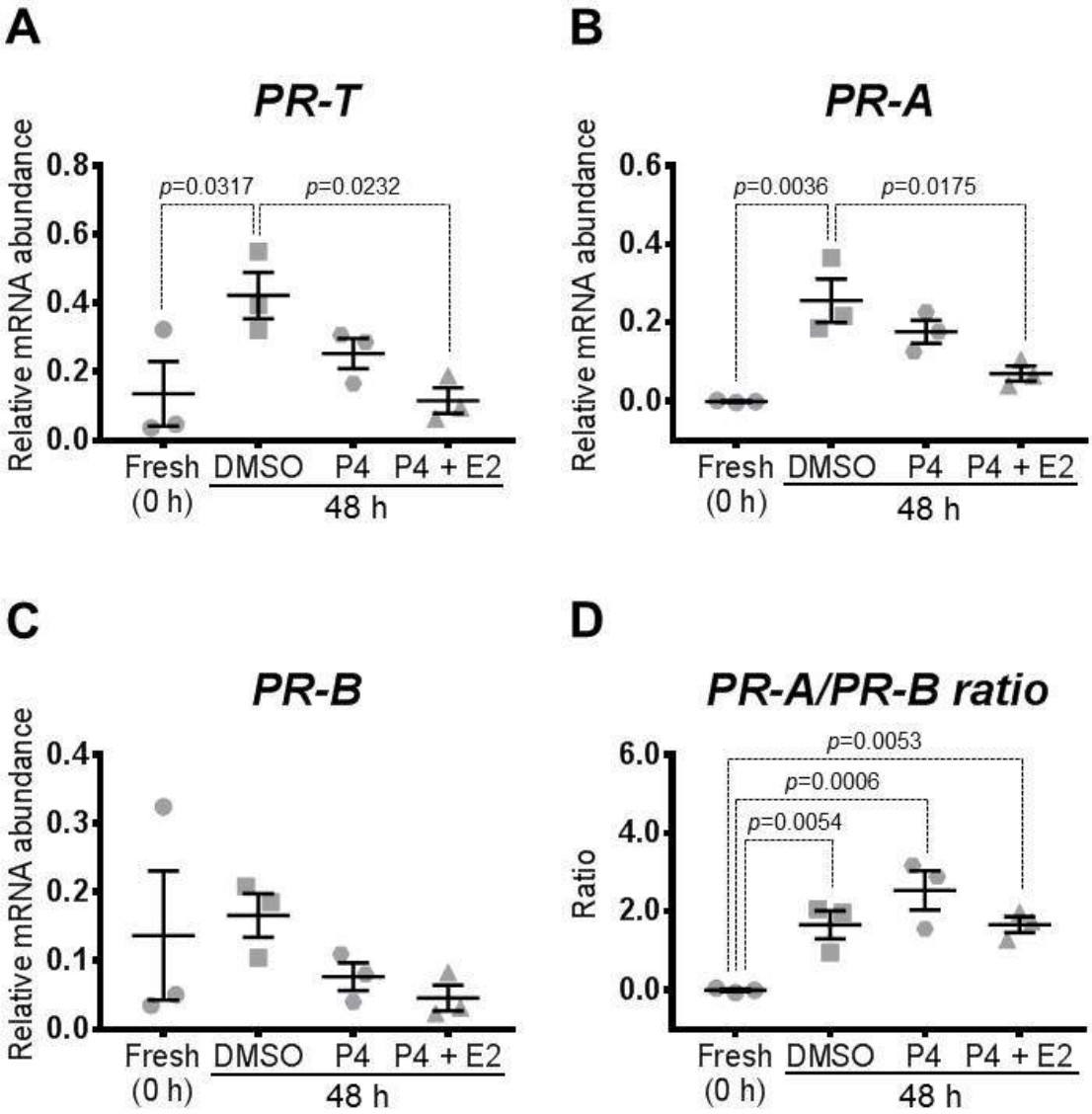

Figure 4.3. Effect of steroids on culture-induced changes in *PR* isoform expression *in vitro.*

Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples following 48 h incubation in the presence of 500

nM P4 or 500 nM P4 + 400 nM E2 (n=3), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes in PR Isoform Expression Using Stretch

Myometrial tissue strips were subjected to 0, 1 or 3 g of stretch for 48 h to determine whether applying stretch to the muscle influenced culture-induced changes in *PR* isoform expression. The effect of stretch was investigated in the absence and presence of steroids (500 nM P4 + 400 nM E2).

Stretch (1 or 3 g) applied to myometrial strips for 48 h had no effect on *PR-T* expression (Figure 4.4A) relative to non-stretched (0 g) control strips. Interestingly, stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *PR-T* mRNA levels (Figure 4.4B), indicating that P4 + E2 was no longer effective in decreasing *PR-T* mRNA abundance compared to fresh tissues.

Similarly, stretch applied to myometrial strips for 48 h had no effect on *PR-A* mRNA expression (Figure 4.4C). Stretch applied in the presence of 500 nM P4 + 400 nM E2 likewise had no effect (Figure 4.4D), indicating that P4 + E2 was no longer effective in preventing culture-induced increase in *PR-A* mRNA abundance compared to fresh tissues.

As seen in Figure 4.4E, 0 – 3 g stretch had no effect on *PR-B* expression in the tissue strips. Stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *PR-B* mRNA levels (Figure 4.4F).

The *PR-A/PR-B* expression ratio was calculated and there was significant increase following 48 h incubation in non-stretched (0 g) control strips when compared to fresh tissues (p=0.0164) (Figure 4.4G). Applying stretch (1 or 3 g) to myometrial strips for 48 h had no effect on expression ratio when compared to non-stretched strips (Figure 4.4G). Further, there was significant increase in *PR-A/PR-B* expression ratio following 48 h incubation in non-stretched (0 g) control strips in the presence of 500 nM P4 + 400 nM E2 when compared to fresh tissues (p=0.0067) (Figure 4.4H). Stretch (1 or 3 g) applied in the presence of 500 nM P4 + 400 nM E2 had no effect on expression ratio after 48 h culture when compared to control strips (Figure 4.4H).

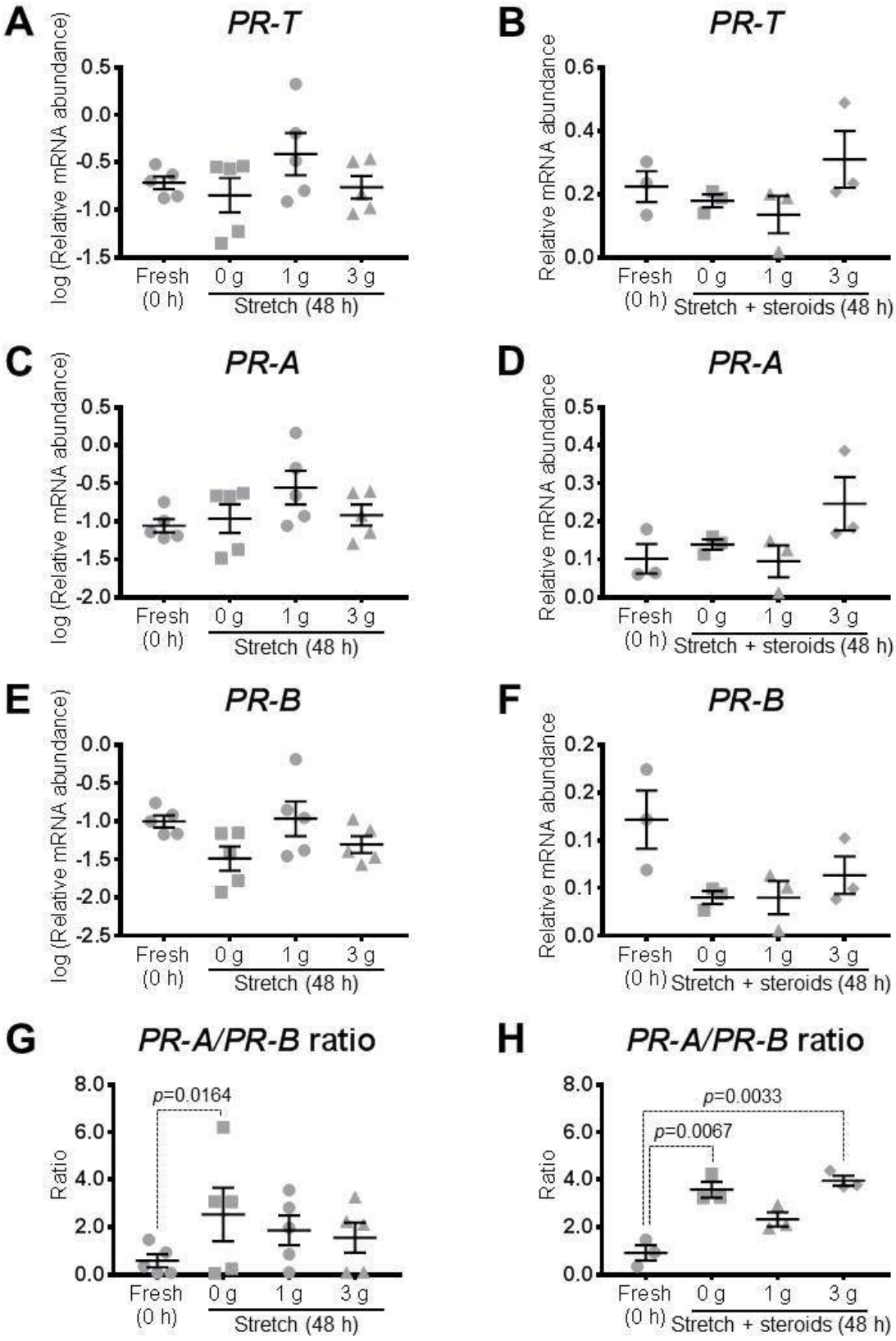

Figure 4.4. Effect of stretch in the absence or presence of steroids on culture-induced changes in *PR* isoform expression *in vitro.*

Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial strips whilst applying 0, 1 and 3 g of stretch for 48 h (n=5), as well as in presence of steroids (500 nM P4 + 400 nM E2) whilst applying 0, 1 and 3 g of stretch for 48 h (n=3), and expressed relative to Alien reference. In addition, *PR-A/PR-*

B expression ratio was calculated. (A) Effect of stretch on *PR-T* mRNA abundance. (B) Effect of stretch and steroids on *PR-T* mRNA abundance. (C) Effect of stretch on *PR-A* mRNA abundance. (D) Effect of stretch and steroids on *PR-A* mRNA abundance. (E) Effect of stretch on *PR-B* mRNA abundance. (F) Effect of stretch and steroids on *PR-B* mRNA abundance. (G) Effect of stretch on *PR-A/PR-B* expression ratio. (H) Effect of stretch and steroids on *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes in PR Isoform Expression Using cyclic-AMP and PMA

Relative abundance of *PR-T, PR-A* and *PR-B* mRNA was measured in myometrial tissue incubated for 48 h in the presence of 8-Br-cAMP (250 µM), PMA (0.1 and 1.0 µM) or vehicle (DMSO).

Supplementing culture media with 250 µM 8-Br-cAMP had no effect on mRNA abundance for *PR-T*, *PR-A* or *PR-B* (Figures 4.5A – C). The *PR-A/PR-B* expression ratio in DMSO-treated tissues was significantly elevated following 48 h incubation when compared to fresh tissues (p=0.0236) (Figure 4.5D). Supplementing culture media with 250 µM 8-Br-cAMP did not prevent the increase in the *PR-A/PR-B* expression ratio (p=0.0141) (Figure 4.5D).

Supplementing media with 0.1 or 1.0 µM PMA, a protein kinase C (PKC) activator, had no significant effect on mRNA abundance for *PR-T* or *PR-A,* relative to 48 h DMSO-treated control tissues (Figures 4.5E and F). *PR-B* mRNA abundance was reduced by both PMA treatments relative to DMSO-treated control tissues, however, the decreases did not reach statistical significance (p=0.3659 and p=0.5259, respectively) (Figure 4.5G). The *PR-A/PR-B* expression ratio was significantly elevated in DMSO-treated control tissues at 48 h, relative to fresh tissues (p=0.0428) (Figure 4.5H). Supplementing media with 0.1 µM PMA significantly increased the *PR-A/PR-B*

expression ratio beyond levels detected in the 48 h DMSO-treated control tissues (*p*=0.0451) (Figure 4.5H) and was attributable to the, albeit non-significant, decline in *PR-B* mRNA abundance. The *PR-A/PR-B* expression ratio was significantly elevated after 48 h in 1.0 µM PMA-treated tissues compared to fresh tissues (*p*=0.0023) (Figure 4.5H).

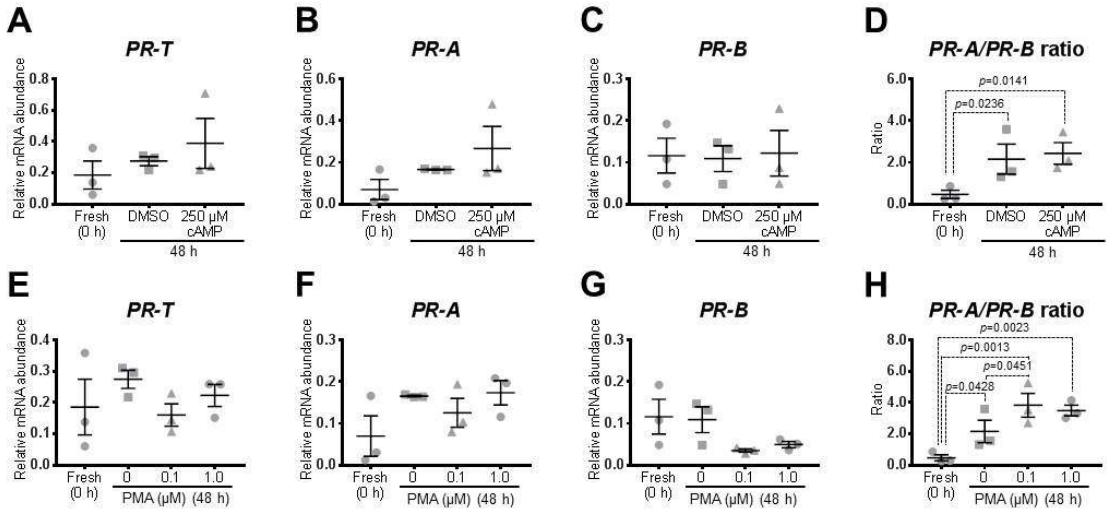

Figure 4.5. Effect of cAMP and PMA on culture-induced changes in *PR* isoform expression *in vitro.*
Relative mRNA abundance of *PR-T*, *PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of 8-Br-cAMP (250 µM) or PMA (0.1 and 1.0 µM), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) Effect of 8-Br-cAMP on *PR-T* mRNA abundance. (B) Effect of 8-Br-cAMP on *PR-A* mRNA abundance. (C) Effect of 8-Br-cAMP on *PR-B* mRNA abundance. (D) Effect of 8-Br-cAMP on *PR-A/PR-B* expression ratio. (E) Effect of PMA on *PR-T* mRNA abundance. (F) Effect of PMA on *PR-A* mRNA abundance. (G) Effect of PMA on *PR-B* mRNA abundance. (H) Effect of PMA on *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes PR Isoform Expression Using $PGF_{2\alpha}$

Relative abundance of *PR-T, PR-A* and *PR-B* mRNA was measured in myometrial tissue incubated for 48 h in the presence of $PGF_{2\alpha}$ (1, 10, 100 or 1000 nM) or vehicle (DMSO).

Following 48 h incubation *PR-T* mRNA abundance in DMSO-treated control tissues was elevated relative to fresh tissues but did not reach statistical significance (Figure 4.6A). $PGF_{2\alpha}$ treatments had no effect on *PR-T* mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 4.6A).

PR-A mRNA abundance was significantly increased in DMSO-treated control tissues relative to fresh tissues (p=0.0451) (Figure 4.6B). $PGF_{2\alpha}$ treatments had no effect on PR-A mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 4.6B).

PR-B mRNA abundance remained unchanged following 48 h incubation and was not affected by $PGF_{2\alpha}$ treatments (Figure 4.6C).

In 48 h DMSO-treated tissue the *PR-A/PR-B* expression ratio was significantly elevated relative to fresh tissues (p=0.0294) (Figure 4.6D). $PGF_{2\alpha}$ supplementation (1, 10, 100 or 1000 nM) had no effect on the *PR-A/PR-B* expression ratio relative to DMSO-treated control tissues (Figure 4.6D).

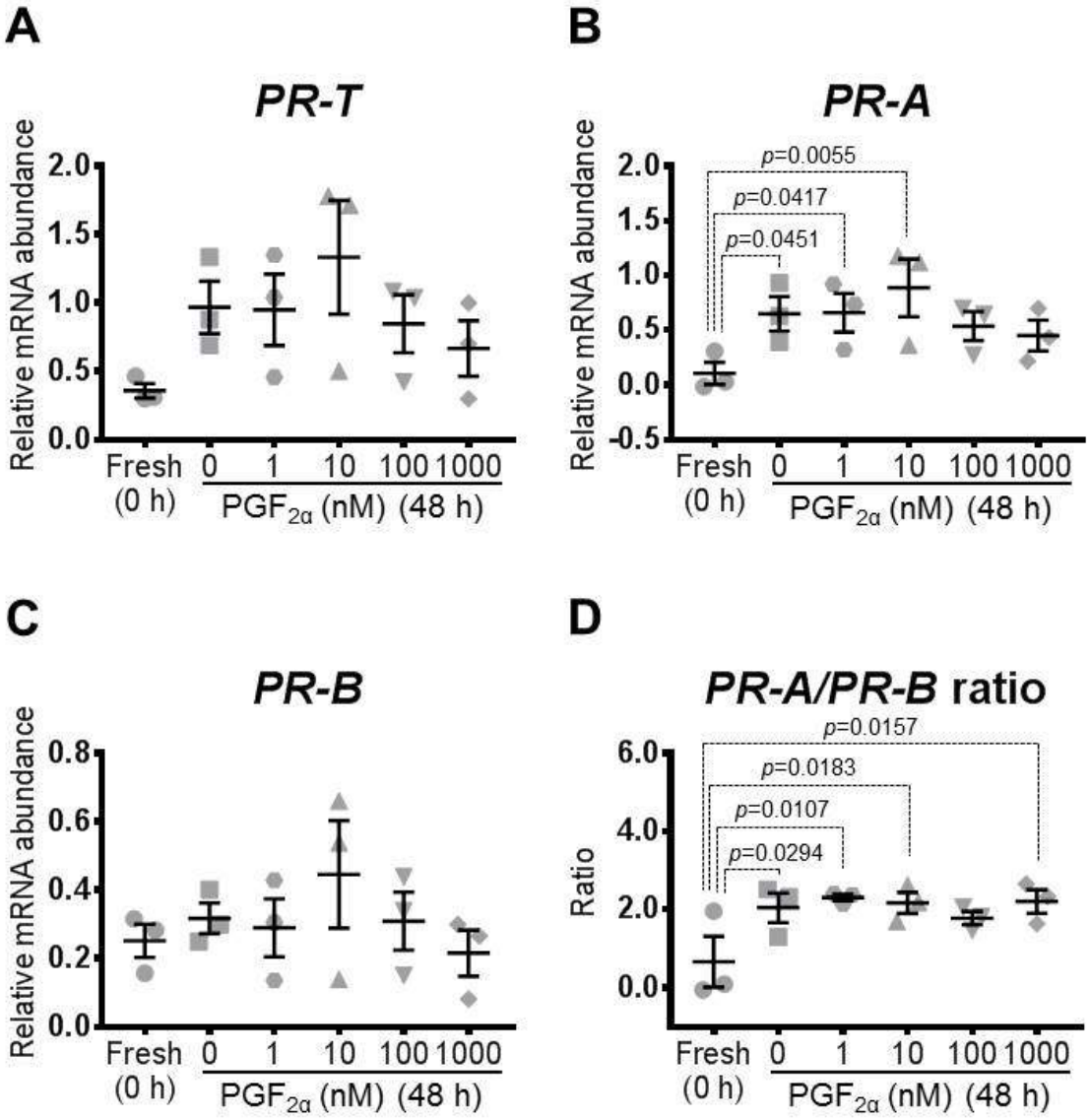

Figure 4.6. Effect of $PGF_{2\alpha}$ on culture-induced changes in *PR* isoform expression *in vitro.*

Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of $PGF_{2\alpha}$ (1, 10, 100 and 1000 nM), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

Controlling Changes in PR Isoform Expression Using NF-κB Inhibitors

The NF-κB inhibitors, MG-132 and BAY-11-7085, were employed to test whether NF-κB pathway activation was involved in the *PR* isoform expression changes induced by *in vitro* culture.

Following 48 h incubation, there was no significant difference in *PR-T* mRNA abundance between vehicle-treated tissues and fresh tissues (Figure 4.7A). Incubating myometrial samples with 2.0, 5.0 or 10.0 µM MG-132 or BAY-11-7085 individually or in combination (10 µM each) had no effect on *PR-T* mRNA abundance relative to vehicle-treated tissues (Figure 4.7A).

PR-A mRNA abundance in vehicle-treated tissues significantly increased compared to fresh tissues following 48 h incubation (p=0.0170) (Figure 4.7B). Supplementing culture media with 2.0, 5.0 or 10.0 µM MG-132 or BAY-11-7085 individually or in combination (10 µM each) had no significant effect on *PR-A* mRNA abundance relative to 48 h vehicle-treated tissues (Figure 4.7B).

Following 48 h incubation, there was no significant difference in *PR-B* mRNA abundance between vehicle-treated tissues and fresh tissues (Figure 4.7C). Incubating myometrial samples with 2.0, 5.0 or 10.0 µM MG-132 or BAY-11-7085 individually or in combination (10 µM each) had no effect on *PR-B* mRNA abundance relative to vehicle-treated tissues (Figure 4.7C).

Following 48 h incubation, the *PR-A/PR-B* expression ratio was significantly elevated in vehicle-treated tissues compared to fresh tissues (p=0.0011) (Figure 4.7D). Supplementing culture media with 2.0, 5.0 or 10.0 µM MG-132 or BAY-11-7085 individually or in combination (10 µM each) had no effect on *PR-A/PR-B* expression ratio compared to the vehicle-treated tissues (Figure 4.7D).

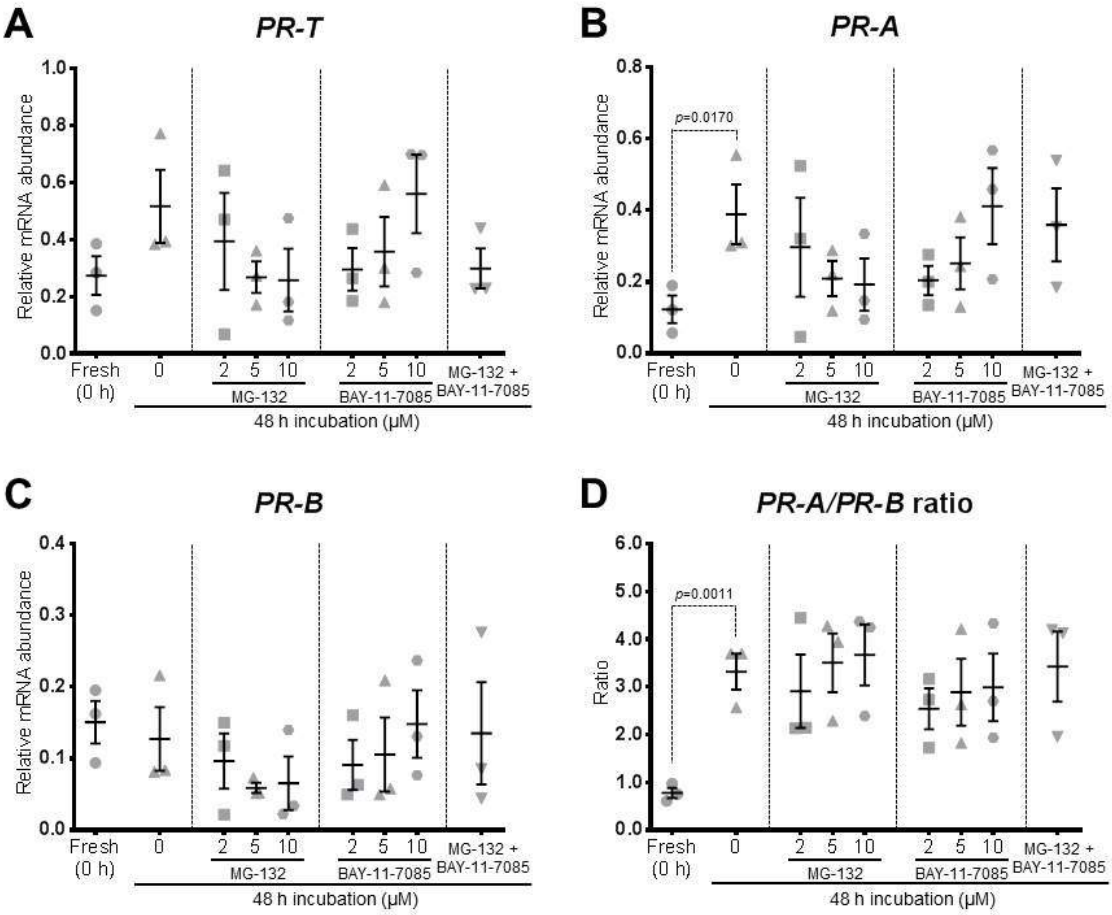

Figure 4.7. Effect of NF-κB inhibitors, MG-132 and BAY-11-7085, on culture-induced changes in *PR* isoform expression *in vitro.*
Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial samples (n=3) following 48 h incubation in the presence of different NF-κB inhibitors, and expressed relative to Alien reference RNA. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effect of TSA

Relative abundance of *PR-T, PR-A* and *PR-B* mRNA was measured in myometrial tissue incubated for 48 h in the presence of TSA (0.5, 1.0, 2.5 and 5.0 μM) or vehicle (DMSO).

There was no significant difference in *PR-T* mRNA abundance between 48 h vehicle-treated tissues and fresh tissues (Figure 4.8A). Incubating

myometrial samples with 0.5, 1.0, 2.5 and 5.0 µM TSA had no effect *PR-T* mRNA abundance relative to 48 h vehicle-treated tissues (Figure 4.8A).

PR-A mRNA abundance was significantly increased in 48 h vehicle-treated tissues relative to fresh tissues (p=0.0431) (Figure 4.8B). Culture-induced increases in *PR-A* mRNA abundance were inhibited by supplementing media with TSA. The extent of inhibition reached statistical significance, relative to 48 h vehicle-treated tissue, at 5.0 µM TSA (p=0.0305) (Figure 4.8B).

PR-B mRNA abundance did not change following 48 h incubation and was unaffected by TSA treatments relative to vehicle-treated tissues (Figure 4.8C).

Following 48 h incubation, the *PR-A/PR-B* expression ratio was significantly elevated in vehicle-treated tissues compared to fresh tissues (p=0.0002) (Figure 4.8D). The *PR-A/PR-B* expression ratio was significantly lower in tissue treated with 2.5 and 5.0 µM TSA (p=0.0003 and p<0.0001, respectively) relative to 48 h vehicle-treated tissues (Figure 4.8D). TSA dose-dependently prevented culture-induced increases in the *PR-A/PR-B* expression ratio.

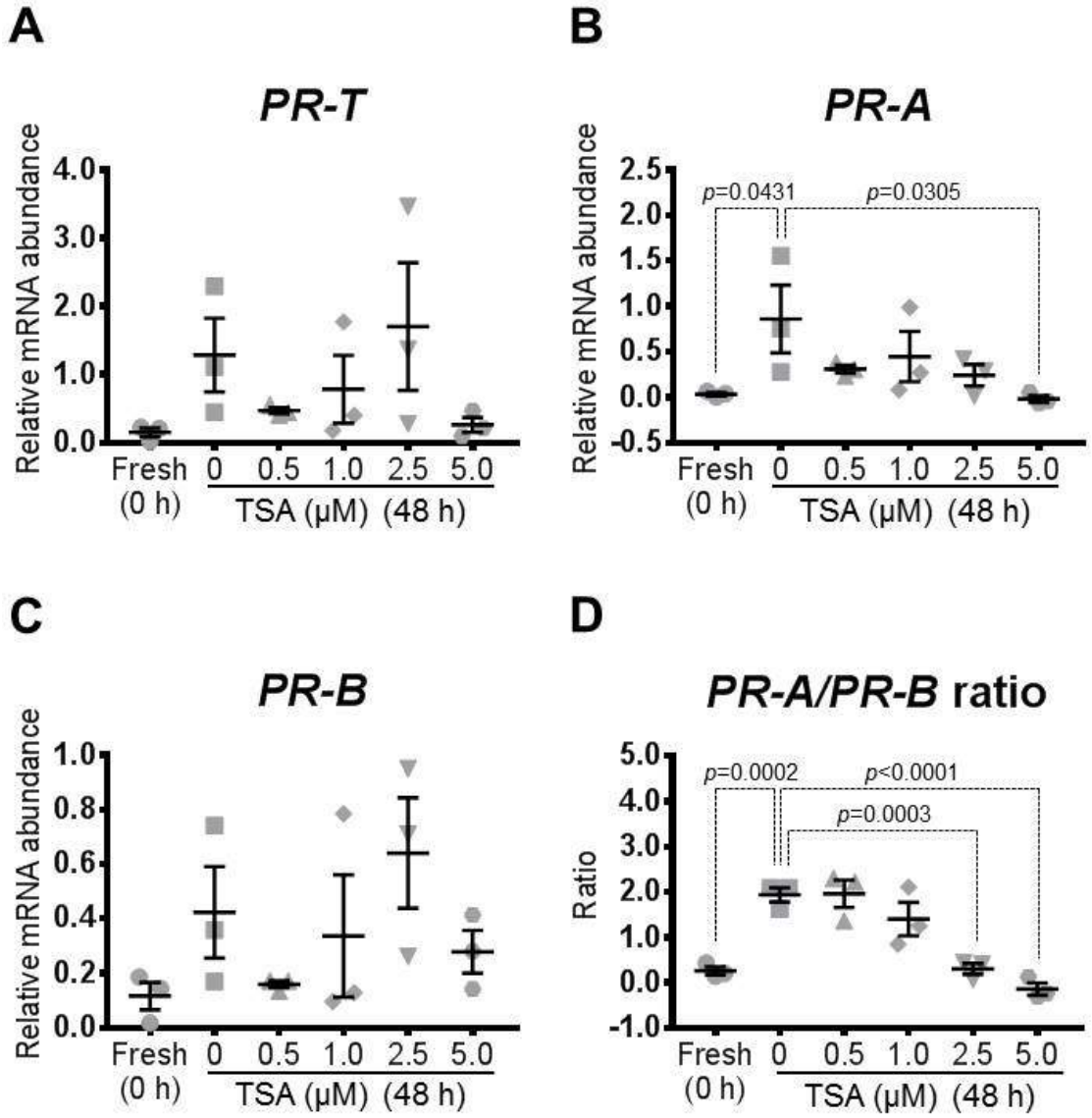

Figure 4.8. Effect of TSA on culture-induced changes in *PR* isoform expression *in vitro.*

Relative mRNA abundance of *PR-T, PR-A* and *PR-B* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of TSA (0.5, 1.0, 2.5 and 5.0 μM), and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (A) *PR-T* mRNA abundance. (B) *PR-A* mRNA abundance. (C) *PR-B* mRNA abundance. (D) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

4.5 Discussion

Human tissue and cell cultures are a valuable *in vitro* tool used to investigate the maintenance of uterine quiescence and the mechanisms by which the myometrium transforms to an actively contracting organ at labor. Our

previous results show that upon culturing non-laboring myometrial tissues *in vitro*, the tissue undergoes culture-induced changes in expression of the key myometrial genes *ESR1*, *PTGS2* and *OXTR,* which are consistent with transition toward a pro-contractile, laboring phenotype [298]. In light of this evidence, we further examined whether *PR* isoform expression undergoes culture-induced changes that are consistent with transition to a pro-contractile, laboring phenotype.

In this study we examined changes in *PR* isoform expression via determining mRNA levels, an approach which is consistent with other studies in the field [258]. Nevertheless, we are aware that PR isoform protein levels may reflect PR function more closely than mRNA abundance especially in pregnancies complicated by intrauterine inflammation [299]. Previous studies have demonstrated, however, that there is close correspondence between PR isoform mRNA and protein expression changes in the human myometrium at normal term labor [261, 294], which is the context of this study. Furthermore, recent studies examining protein profiles in mammalian cells have found that transcription, not translation, mostly determines protein abundance [259], and that during periods of dynamic change, such as phenotype transition, changes in mRNA abundance play a dominant role in determining changes in protein levels [260]. Overall, assessing myometrial PR function by determining *PR-A* and *PR-B* mRNA levels appears a reliable approach in the patient population we examined.

Tissue incubation studies are routinely performed for 48 h or more [91, 296, 297]. Considering that non-laboring human myometrium develops

contractility *in vitro* in just 1 – 2 h [30, 110, 240, 254], the transition away from the original phenotype may be even more pronounced after such 48 h incubations. Our results illustrate that in human myometrial pieces, *PR-A* mRNA abundance begins increasing after just 1 h culture. With *PR-B* mRNA abundance remaining constant, a statistically significant increase in the *PR-A/PR-B* expression ratio was evident after just 6 h culture (Figure 4.1). Previous studies using enzyme-immunoassays found that samples collected from the upper segment myometrium during labor had higher total PR concentrations than samples collected prior to labor [300]. Haluska *et al.* [293] used rhesus monkey, another genus that lacks a systemic progesterone withdrawal, to look at the changes in PR isoform concentrations. They found that there was no change in total PR expression during the transition from late pregnancy to labor, however, they did find a significant shift in the ratio of PR isoforms [293]. More specifically, the myometrial *PR-A/PR-B* ratio increased significantly from late pregnancy to spontaneous labor at term [293]. Furthermore, Pieber *et al.* [301] performed immunoblot analyses on lower segment myometrium from pregnant women and reported an increase in the PR-A protein abundance during labor, while levels of PR-B were not altered by labor status. Recently, our group showed that the onset of labor is associated with increased abundance of *PR-A* mRNA (18- to 19-fold) and an increase in the *PR-A/PR-B* expression ratio in term human myometrium [261]. Our group has also found that the PR-A/PR-B protein ratio in pregnant human myometrium was 0.5 (a PR-B dominant state) at 30 weeks gestation, which then increased to 1.0 at term prior to the onset of labor, and at the time of the labor the ratio increased further to 3.0 (a PR-A dominant state) [294]. These results indicate that *PR* mRNA levels reflect PR

protein levels in human myometrium. Our observation that *PR-T* and *PR-A* mRNA abundance (23-fold) as well as the *PR-A/PR-B* expression ratio increased during culture is therefore consistent with the tissue transitioning to a labor-like state as a consequence of *in vitro* conditions.

This finding has implications for the interpretation *in vitro* of studies performed on non-laboring myometrium, which may have in fact already transitioned to a labor-like phenotype during the early stages of the study, and may therefore have affected the outcome of the study. To address this, we sought to identify culture conditions that could be implemented to maintain a non-laboring state whereby human myometrium retained a low *PR-A/PR-B* expression ratio (a *PR-B* dominant state), thereby providing a more appropriate *in vitro* model for conducting studies into myometrial biology.

Previous studies utilising myometrial culture (explants) often included CSS in their media [257, 296, 297]. Therefore, we examined whether supplementing culture media with 5% CSS affected culture-induced changes in *PR* isoform expression. Surprisingly, 5% CSS had no significant effect on *PR-T*, *PR-A* or *PR-B* mRNA abundance after 48 h culture, and consequently had no effect on the *PR-A/PR-B* expression ratio (Figure 4.2). While supplementing media with serum is common practice during *in vitro* culture, our results indicate that this practice is not sufficient to prevent culture-induced changes in *PR* isoform expression.

The steroid hormone progesterone plays a crucial role in maintaining pregnancy by promoting myometrial quiescence and relaxation [282-284]. In contrast to most mammalian species [276, 285-288], no decrease in maternal

serum levels of progesterone can be observed in humans and higher primates prior to the onset of labor [289-291]. Thus, the term “functional progesterone withdrawal” has been used to describe the withdrawal of progesterone action. Once myometrial tissue is removed from *in vivo* environment and cultured *in vitro*, the high plasma levels of progesterone are no longer present, which could possibly account for culture-induced changes in *PR* expression *in vitro*. To explore this, we incubated myometrial tissues in media that contained physiological concentrations of progesterone. Supplementing media with progesterone alone was not sufficient to prevent the culture-induced increases in *PR-T* and *PR-A* mRNA abundance. Moreover, progesterone decreased *PR-B* mRNA abundance; however it was not statistically significant, nevertheless this further exacerbated the increase in the *PR-A/PR-B* expression ratio (Figure 4.3). A previous study using myometrial strips showed that progesterone exerts rapid inhibition of the amplitude of myometrial contractions *in vitro* [254]. More recently, Baumbach *et al.* [255] investigated the suppression of uterine contractility using progesterone alone and in a combination with various tocolytics and found that progesterone alone had little effect inhibiting contractility [255]. This is consistent with our results where progesterone alone did not prevent culture-induced increases in *PR-T* and *PR-A* mRNA abundance.

In numerous mammalian species, the process of parturition, especially transformation of the myometrium from the quiescent to a contractile state, necessitates an increase in circulating estrogen concentrations prior to the onset of labor [13, 287, 290, 302]. In humans and higher primates, however, maternal estrogen levels are high for most of pregnancy and remain elevated during parturition and delivery [290, 303, 304]. Furthermore, our group reported

a correlation between *ESR1* mRNA levels and the *PR-A/PR-B* mRNA ratio, which is indicative of a functional link between the PR and ESR1 systems [261]. In addition, this link between the two systems is in agreement with studies performed in a range of species demonstrating that progesterone decreases expression of ESR1, thus decreasing uterine responsiveness to estrogen [305, 306]. These results imply that the interaction between progesterone and PR-B suppresses ESR1 expression, therefore rendering the myometrium refractory to circulating estrogen [49]. However, with advancing gestation there is an increase in the expression of PR-A, which in turn represses the transcriptional activity of PR-B, and as a result the PR-B-mediated inhibition of ESR1 expression is withdrawn [49]. Once myometrial tissue is removed from *in vivo* environment and cultured *in vitro*, the high plasma levels of progesterone and estrogen are no longer present, thereby removing the functional link between progesterone and estrogen [261] which could possibly account for the observed culture-induced changes in *PR* expression *in vitro.* To explore this, we incubated myometrial tissue in media that contained physiological concentrations of P4 and E2. The combination of P4 and E2 prevented culture-induced increase in *PR-T* and *PR-A* mRNA abundance observed *in vitro*. However, P4 in combination with E2 also decreased *PR-B* mRNA abundance; nevertheless this decrease was not statistically significant. As such, after 48 h culture the *PR-A/PR-B* expression ratio had still increased relative to fresh tissue and adopted a *PR-A* dominant state (Figure 4.3).

Throughout normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [266, 267]. A previous study using term non-laboring human

myometrium tissue showed that stretch applied to myometrial cells in culture resulted in decreased *PR-T* and *PR-B* mRNA expression [307]. We found that constant stretch, applied by means of hanging 1 or 3 g weights from tissue strips, had no effect on culture-induced changes in *PR* isoform expression (Figure 4.4). This is inconsistent with a previous report where stretch down-regulated *PR-T* and *PR-B* expression; however, it should be noted that those studies used myometrial cells while our study uses myometrial tissue strips [307]. Previous animal studies suggest that progesterone is responsible for maintaining uterine quiescence and promoting myometrial hyperplasia and hypertrophy to inhibit any increase in uterine wall tension [269, 270, 308, 309]. In addition, human studies show that in a progesterone-dominated endocrine environment, moderate stretch possibly maintains relaxation and quiescence, however, in the absence of progesterone or excessive stretch, the uterus starts to contract [266, 267]. Interestingly, although stretch did not directly affect the culture-induced changes in *PR-T*, *PR-A* or *PR-B* expression, the application of stretch prevented steroids (P4 + E2) from blocking culture-induced increases in *PR-T* and *PR-A* mRNA expression and prevented steroids (P4 + E2) from decreasing *PR-B* mRNA expression (Figures 4.3 and 4.4).

There is now extensive evidence to suggest that components of the cAMP signalling pathway are up-regulated in the human myometrium throughout pregnancy to maintain uterine quiescence until term [31, 272, 310-312]. Moreover, our group showed that in PHM1-31 cells, a pregnant human myometrial cell line, 8-Br-cAMP, an agonist for the PKA pathway, increased the expression of both *PR-A* and *PR-B* but had a net effect of decreasing the *PR-A/PR-B* expression ratio [313]. Supplementing media with a cAMP analogue

was therefore examined as a potential means to prevent culture-induced changes in *PR* isoform expression. Although cAMP has a well-defined role in promoting myometrial relaxation, supplementing media with 8-Br-cAMP failed to prevent culture-induced changes in *PR-T* or *PR-A* mRNA abundance and increased *PR-A/PR-B* expression ratio (Figure 4.5).

In contrast to previously discussed treatments that attempted to prevent culture-induced changes in *PR* isoform expression, we also examined the effect of the pro-contractile agent, PMA, to determine whether *PR* expression would be driven further toward a labor-like state. Previous studies by our group show that PKC activation by PMA increased the *PR-A/PR-B* expression ratio by selectively increasing expression of *PR-A* [313]. This study found that supplementing culture with PMA further increased the *PR-A/PR-B* expression ratio *in vitro*, which was consistent with this pro-contractile agent driving further transition toward a laboring phenotype. Interestingly, PMA did not increase expression of *PR-A,* but rather decreased expression of *PR-B* over the course of the myometrial culture (Figure 4.5).

There is increasing evidence that locally produced immune/inflammatory cytokines, particularly PGs, are involved in normal term labor as well as infection-associated preterm labor [170, 171, 211]. In human pregnancy, administration of PGs or PG analogues at any stage of pregnancy transform the myometrium and cervix and induces labor [171, 314-317]. Previously, our group has tested the hypothesis that PGs, specifically $PGF_{2\alpha}$, induce functional progesterone withdrawal by altering myometrial *PR* expression in PHM1-31 cells [313]. $PGF_{2\alpha}$ produced a dose-dependent increase in expression of *PR-A*,

but not *PR-B*, thereby resulting in an increase in the *PR-A/PR-B* expression ratio [313]. In this study, supplementing media with $PGF_{2\alpha}$ had no effect on *PR* mRNA abundance and therefore did not prevent culture-induced changes in the *PR-A/PR-B* expression ratio (Figure 4.6). This is not consistent with previous results where $PGF_{2\alpha}$ increased the *PR-A/PR-B* expression ratio by increasing PR-A expression [313].

Romero *et al.* [318] have shown that tissue-level inflammation in the myometrium, decidua, and fetal membranes plays a crucial role in the human parturition. In recent years, studies have demonstrated that myometrium in pregnant women at term exhibits biochemical and histological characteristics of inflammation, including increased expression of PGs, increased NF-κB activity, increased infiltration of neutrophils, and macrophages, which may precede the onset of active labor and is independent of infection [219, 276-278, 283, 319-321]. Furthermore, studies using human myometrial cells have shown that progesterone inhibits the pro-inflammatory NF-κB transcription factor complex as a result of PR-induced expression of inhibitor-κB-α (NFKB1A), a major NF-κB repressor [265]. Supplementing media with NF-κB inhibitors therefore represented a potential means of preventing spontaneous changes in *PR* isoform expression. Supplementing media with MG-132 or BAY-11-7085 had no effect on *PR-T, PR-A* and *PR-B* mRNA abundance and therefore did not prevent culture-induced changes in the *PR-A/PR-B* expression ratio (Figure 4.7).

Condon *et al.* [322] administered TSA, a specific and potent HDACi, to pregnant mice late in gestation and found increased histone H3 acetylation as

well as a delay in the initiation of parturition by 24 – 48 h. Decreased histone acetylation in the pregnant uterus near term, caused by a marked decrease in expression of uterine co-activators with intrinsic histone acetyltransferase activity, might serve an important role in the loss of PR function, thus instigating a functional progesterone withdrawal and the initiation of labor [322]. Furthermore, Wilson *et al*. [323] used the mouse mammary tumor virus promoter to examine the impact of TSA on PR activated transcription and found that TSA removed the transcription factor nuclear factor 1 from the promoter and decreased PR-induced transcription [323]. Based on these results we hypothesised that TSA may modulate *PR* isoform expression, and supplemented culture media with TSA in anticipation of maintaining a low *PR-A/PR-B* expression ratio *in vitro*. Excitingly, TSA produced a dose-dependent inhibition of culture-induced up-regulation of *PR-A* mRNA abundance. With no effect of *PR-B* mRNA abundance, TSA was successful in maintaining a low *PR-A/PR-B* expression ratio over 48 h culture, consistent with freshly isolated term non-laboring myometrium and consistent with preventing *in vitro* transformation to a laboring phenotype (Figure 4.8). Using TSA to maintain a low *PR-A/PR-B* ratio could have important clinical ramifications in that progesterone therapy is currently a leading strategy for the prevention of preterm birth (reviewed by van Zijl *et al*. [324]). Efficacy of progesterone administration may be enhanced if an agent such as TSA could be administered to preserve or even restore progesterone sensitivity in women with threatened preterm labor.

Conclusion

Concerns have previously been raised about the ability of primary cells in culture to remain representative of their tissues of origin. Adding to this concern,

our previous study shows that term non-laboring human myometrial tissue undergoes culture-induced changes in expression of *ESR1*, *PTGS2* and *OXTR* that are consistent with transitioning toward a laboring phenotype [325]. In this study we examined *PR* isoform expression and found that *PR-T* and *PR-A* mRNA expression increased in untreated tissue over 48 h culture. Additionally, the *PR-A/PR-B* expression ratio significantly increased, consistent with transition to a laboring phenotype. Through examining various culture conditions, we were able to maintain a non-laboring state of PR isoform expression by supplementing culture media with TSA, which prevented the culture-induced increase in *PR-A* mRNA abundance and maintained a low *PR-A/PR-B* expression ratio. In summary, this study demonstrates that:

i) Human myometrial tissues undergo culture-induced up-regulation of *PR-T* and *PR-A* mRNA expression, which significantly increases the *PR-A/PR-B* expression ratio *in vitro*, even in non-treated tissue;

ii) The combination of progesterone and estrogen down-regulated *PR-T* and *PR-A* mRNA expression;

iii) Stretch had no direct effect on *PR-T*, *PR-A* or *PR-B* expression, however, stretch blocked the effects of progesterone and estrogen on *PR-T and PR-A* expression;

iv) cAMP was unable to control culture-induced changes in *PR* expression;

v) PMA further up-regulated *PR-A/PR-B* expression ratio;

vi) $PGF_{2\alpha}$ had no effect of *PR* expression *in vitro*;

vii) NF-κB inhibitors were unable to control culture-induced changes in *PR* expression;

viii) TSA down-regulated *PR-A* mRNA expression as well as down-regulated *PR-A/PR-B* expression ratio.

4.6 Acknowledgements

The authors wish to thank the obstetricians from the John Hunter Hospital, NSW, our research midwife, Anne Wright, and the research participants who donated samples toward this study.

Chapter Five: Expression of *KCNH2* (hERG1) and *KCNE2* correlates with expression of key myometrial genes in human pregnant myometrium

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Previously our group has reported that *KCNH2* (hERG1) and *KCNE2*, which collectively encode the hERG potassium channel, play a role in regulating uterine contractility, and is dysregulated in obese women (Nature Comms. 2014). This manuscript reports novel correlations in the expression of *KCNH2* and *KCNE2* with key contraction-associated genes in the myometrium, including estrogen, oxytocin and progesterone receptors. Furthermore, we also performed an initial investigation into the expression of *KCNH2* and *KCNE2* within myometrial biopsies obtained from preterm caesarean deliveries.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Jonathan W. Paul	Conceived and designed experiments Data analyses Provided reagents and materials Manuscript writing
Marina Ilicic **(Co-first Author)**	**Conceived and designed experiments** **Sample collections** **Performed molecular analyses** **Data analyses** **Manuscript writing**
Tamas Zakar	Provided advice on data analyses Provided reagents and materials Manuscript editing
Roger Smith	Manuscript editing

5.1 Abstract

Background: Loss of activity of K_v11.1 potassium channels (encoded by *KCNH2)* facilitates labor, and is associated with expression of an inhibitory subunit encoded by *KCNE2.*

Objective: To determine whether *KCNH2* and *KCNE2* expression was linked to expression of key genes involved in myometrial contractility, including the *OXTR*, *ESR1*, progesterone receptor (*PGR*) and *PTGS2*. We further aimed to examine *KCNH2* and *KCNE2* expression in preterm samples.

Study Design: Biopsies of term or preterm, non-laboring human myometrium were analysed by qPCR to determine *KCNH2* and *KCNE2* mRNA abundance, as well as abundance of *OXTR*, *ESR1*, *PGR* and *PTGS2* mRNAs.

Results: *KCNH2* and *KCNE2* expression were significantly correlated at term, but not correlated with BMI. *KCNH2* expression significantly correlated with *OXTR*, *ESR1, PGR* and *PTGS2* expression, while *KCNE2* expression correlated with the expression of *ESR1, PGR* and *PTGS2.* Preterm samples revealed no difference in *KCNH2* and *KCNE2* expression compared to term.

Conclusion: *KCNH2* and *KCNE2* expression correlate with the expression of key myometrial genes implicated in parturition, further strengthening a role for K_v11.1 channels in human pregnancy. Neither gene was correlated with BMI, suggesting that previously reported effects of obesity likely affect protein levels rather than gene expression.

5.2 Introduction

A number of studies have demonstrated that ion channels encoded by the *ERG1* play a role in regulating the contractile activity of cardiac myocytes (reviewed by Vandenberg *et al.,* [222]). Moreover, there is increasing evidence demonstrating a role for *ERG1* in regulating smooth muscle cell contractility. *ERG1* encodes the pore-forming α-subunit of the delayed rectifier voltage-gated potassium channel, K_v11.1. Herein we refer to the *ERG* gene and the cognate mRNA as '*KCNH2'*, and '*KCNH2* mRNA', respectively, to the channel protein as ERG and to the functional channel as K_v11.1. In addition to ERG, K_v11.1 channels contain regulatory β-subunits [326, 327], such as the single transmembrane domain protein potassium voltage-gated channel subfamily E members 2, encoded by the gene *KCNE2* (mRNA referred to as '*KCNE2* mRNA', and channel protein referred to as KCNE2).

K_v11.1 channels are activated following action potentials and function to repolarize the cell membrane by conducting potassium ions (K^+) out of the cell, which constitutes the rapid component of the delayed rectifier current I_{Kr} [328, 329]. Repolarization terminates the action potential and the associated contraction.

K_v11.1 channels have been shown in rat stomach and murine portal vein [223-225], as well as in opossum oesophagus [226]. In addition, selective K_v11.1 channel blockers have been shown to increase contractility in rat stomach [223], mouse portal vein [225], opossum oesophagus [226], mouse and guinea pig gall bladder [227], bovine epididymis [228] and human and

equine jejunum [229, 230]. These smooth muscles all exhibit spontaneous contractile activity, which is also a property of uterine smooth muscle [233].

K_v11.1 channels were initially linked to myometrial contractility by Aaronson *et al*., who demonstrated that tetraethylammonium (TEA)- and 4-aminopyridine (4-AP)-sensitive voltage-dependent K^+ (K_v) channels played a role in regulating action potential duration in rat myometrium [231]. Greenwood *et al*. later examined mouse ERG (mERG) expression and function, and confirmed the presence of both the ERG1a and ERG1b splice variants, with ERG1a expression being more abundant than that of ERG1b [232]. They found that *KCNH2* mRNA abundance did not change throughout gestation or with the onset of labor, however, mRNAs encoding auxiliary subunits, namely *KCNE2*, were significantly up-regulated approaching term [232].

More recently, our group reported that human ERG1 (hERG1) and KCNE2 were present in pregnant human myometrium during late gestation and labor [234]. We found that K_v11.1 activity supressed the amplitude and duration of contractions prior to labor, thereby supporting a role for K_v11.1 activity in helping to maintain uterine quiescence [234]. Previous reports have indicated that KCNE2 co-expression with hERG1 was not necessarily inhibitory of K_v11.1 activity [330], however, we found that the onset of labor was associated with the increased expression of KCNE2 and the decreased responsiveness of myocytes to the K_v11.1 inhibitor, dofetilide [234]. Together these findings support an inhibitory role for KCNE2 in the context of regulating hERG1 in the myometrium. The evidence also suggests that K_v11.1 activity contributes to the electrophysiological mechanisms that regulate uterine contractions and that

inhibition of the α-subunit, ERG1, by the β-subunit, KCNE2, may facilitate labor [234]. Interestingly, the mechanism for reducing K_v11.1 activity to facilitate labor appears to be dysregulated in obese women. Maternal obesity is associated with increased rates of labor induction, dysfunctional labor requiring CS delivery, longer pregnancies, as well as PPH [236-238]. We reported that high BMI was associated with increased levels of hERG1 and reduced levels of KCNE2 in the myometrium [234]. Furthermore, high BMI was associated with heightened K_v11.1 activity *in vitro,* suggesting that the delayed and protracted labor often observed in obese women is linked to elevated K_v11.1 activity in the myometrium [234].

Genes encoding the *PGR* [4, 6, 49], *ESR1* [49, 91], *OXTR* [6, 331, 332] and *PTGS2* [6, 333] have been identified as key genes involved human parturition. There is now extensive published literature linking the myometrial regulation of these genes, among others, to the maintenance of uterine quiescence and the transition to a contractile phenotype at labor [212, 219, 334]. Uncovering an association between expression of these key genes and genes encoding the K_v11.1 channel would ascertain the involvement of this channel in the increased contractility of the myometrium at term and as part of the myometrial transformation leading to labor.

Aims

The aim of this study therefore was to determine whether expression of *KCNH2* and *KCNE2* in term non-laboring human myometrium correlate with the expression of *PGR, ESR1, OXTR* and *PTGS2*. We report that in term pregnancy, *KCNH2* and *KCNE2* are expressed co-ordinately with these key

parturition-associated genes, thus strengthening the link between uterine contractility and K^+ channel abundance in myometrial cells. Furthermore, this study examined the relationship of myometrial *KCNH2* and *KCNE2* mRNA abundance with BMI, and explored the expression of both genes in myometrial biopsies from preterm deliveries.

5.3 Materials and Methods

Consumables and reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA–free 50 reactions were from Ambion (Austin, USA). Alien QRT-PCR Inhibitor Alert 400 Reactions were purchased from Integrated Sciences Pty (Sydney, Australia). PCR primers were purchased from Sigma (St Louis, USA). The 2 mL 2.8 mm CK28-R Ceramic Bead Kit for the Precellys homogenizer (Bertin Instruments, France) were purchased from Thermo Fischer Scientific (Melbourne, Australia). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES and DMEM were obtained from Gibco (Carlsbad, USA). SYBR Green 2× Master mix was from Applied Biosystems (Carlsbad, USA).

Myometrial Tissue acquisition

These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). All participants gave informed written consent. Human myometrial samples (5 × 5 × 10 mm) were obtained from the

lower uterine segment during elective CS of singleton pregnancies. Preterm samples ranged from 31 – 34 weeks gestation while term samples were 38.2 – 39.6 weeks gestation. Patient BMI range was 18.3 – 38.0, and all patients were not-in-labor. The indications for elective not-in-labor term CS were previous CS, placenta praevia, fetal distress or breach presentation. The indications for elective not-in-labor preterm CS were placenta increta, pre-eclampsia and low levels of amniotic fluid. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of post-partum hemorrhage. Samples were therefore exposed to oxytocin for a brief period of time (3 min). All myometrial samples were placed on ice in a serum-free media containing DMEM with high glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 40 µg/mL gentamicin and 10 mM HEPES for the transfer to the laboratory. Myometrial tissues were then cleared of serosa, fibrous or damaged tissue and visible blood vessels before being dissected into smaller pieces and washed in serum-free media to remove excess blood. Approximately 100 mg of tissue was snap frozen in liquid nitrogen for subsequent analysis.

RNA extraction, Reverse transcription and Real-time quantitative PCR

RNA was extracted from 100 mg of tissue using TRizol Reagent (Ambion, USA) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Ambion, USA). An ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) was used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. RNA integrity was

checked by agarose gel electrophoresis. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5 x10^7 copies of Alien RNA (Integrated Sciences Pty, Australia) and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers (Invitrogen, USA). The Alien RNA transcript is an *in vitro* transcribed RNA molecule that is non-homologous to any known nucleic acids and as such was used as a housekeeping gene for these studies [335-337]. Quantitative RT-PCR was performed using an ABI 7500 Sequence Detector (Applied Biosystem, USA). no-RT negative controls were prepared for each sample to ensure there was no DNA contamination. The final volume of each PCR reaction was 20 µL containing 10 µL of 2x SYBR Green PCR Master Mix (Applied Biosystems, USA), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 µL with 1.0 µL of 2.5 µM of Alien Primer Mix, 10 µL of 2x SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. NTCs were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express, optimized and validated by confirming that single amplicons of appropriate size and sequence were generated (Table 5.1).

Table 5.1. ***cDNA primer sequences for KCNH2, KCNE2, OXTR, ESR1, PGR and PTGS2.***

Primer	Primer Sequence	Amplicon Size	GenBank #
KCNH2	F: ACCTCATCGTGGACATCA R: CTCCTCGTTGGCATTGAC	77	NM_000238.3
KCNE2	F: CACGAGGCAAATCCAAAT R: CTCCAACAAGCAAGCATAA	141	NM_172201.1
OXTR	F: CTGGACGCCTTTCTTCTTCGT R: GAAGGCCGAGGCTTCCTT	73	NM_000916.3
ESR1	F: TGAAAGGTGGGATACGAAAAGAC R: CATCTCTCTGGCGCTTGTGTT	66	NM_000125.3
PGR	F: GTGGGAGCTGTAAGGTCTTCTTTAA R: AACGATGCAGTCATTTCTTCCA	83	NM000926.4
PTGS2	F: ATGTTCCACCCGCAGTACAGA R: CAGCATAAAGCGTTTGCGGTA	101	NM_000963

KCNH2: Potassium Voltage-Gated Channel Subfamily H Member 2; *KCNE2:* Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2; *OXTR:* Oxytocin Receptor; *ESR1:* Estrogen Receptor 1; *PGR:* Progesterone Receptor; *PTGS2:* Prostaglandin-Endoperoxide Synthase 2.

Data and Statistical Analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the ΔC_t method [245]. All mRNA relative abundance values were then logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t* test was used. For correlation studies Pearson's product moment correlation coefficient was used (Pearson's *r*). Preterm and term samples were

compared by 1-way ANOVA with Bonferroni's multiple comparisons test. P-values ≤ 0.05 were considered statistically significant.

5.4 Results

KCNH2 and KCNE2 Expression in Human Myometrium at Term Pregnancy

Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in non-laboring term myometrial tissues (n=18). Expression of *KCNH2* was much higher than of *KCNE2* (over two orders of magnitude, Figure 1A). A statistically significant positive correlation was found between *KCNH2* and *KCNE2* mRNA abundance (r^2=0.34, *p*=0.01)(Figure 5.1B).

There was no statistically significant relationship between *KCNH2* mRNA abundance and gestational age (r^2=0.06, *p*=0.31)(Figure 5.1C), or *KCNE2* mRNA abundance and gestational age (r^2=0.04, *p*=0.41)(Figure 5.1D) within the term gestation range of 38.2 - 41.0 weeks.

Since we have found previously that hERG1 levels increase whilst KCNE2 levels decrease with increasing BMI of term pregnant women, we have correlated *KCNH2* mRNA as well as *KCNE2* mRNA abundance to BMI. The BMI in our patients group ranged from 18.3 to 38.0. There was no statistically significant correlation between *KCNH2* mRNA abundance (r^2=0.0002, *p*=0.96)(Figure 5.1E) or *KCNE2* mRNA abundance and BMI (r^2=0.07, *p*=0.29)(Figure 5.1F).

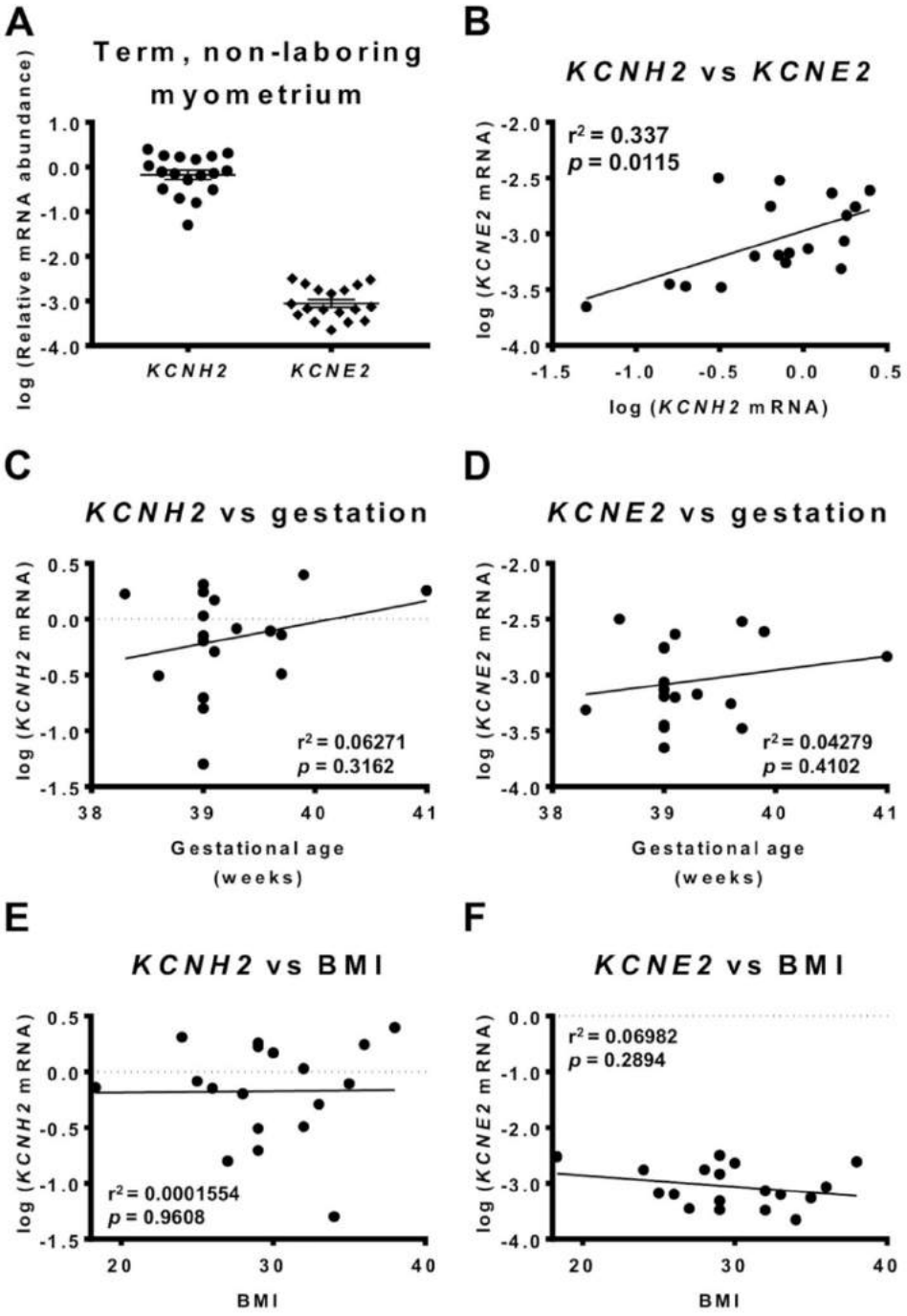

Figure 5.1. Expression of *KCNH2* and *KCNE2* mRNA in pregnant human myometrium.

Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in term non-laboring myometrial tissues (n=18) and expressed relative to Alien reference RNA. (A) *KCNH2* and *KCNE2* mRNA abundance at term. (B) Correlation between *KCNH2* and *KCNE2* mRNA abundance. (C) *KCNH2* mRNA abundance across term gestation time points. (D) *KCNE2* mRNA abundance across term gestation time points. (E) *KCNH2* mRNA abundance against BMI. (F) *KCNE2* mRNA abundance against BMI. *(Pearson's r)*

Correlations Between the Expression of KCNH2, KCNE2 and Contraction-associated Myometrial Genes

We measured the relative abundance of *OXTR, ESR1, PGR* and *PTGS2* mRNAs (n=18 each) and correlated their abundance with *KCNH2* and *KCNE2* mRNA abundance. A statistically significant positive correlation was found between *KCNH2* and *OXTR* (r^2=0.42, *p*=0.0039)(Figure 5.2A) but not between *KCNE2* and *OXTR* mRNA abundance (r^2=0.11, *p*=0.19)(Figure 5.2B). *ESR1* mRNA abundance was positively correlated with *KCNH2* (r^2=0.3117, *p*=0.0160)(Figure 5.2C) and *KCNE2* mRNA abundance (r^2=0.62, *p*=0.0001)(Figure 5.2D). Moreover, *PGR* mRNA also correlated positively with *KCNH2* (r^2=0.67, *p*=0.0001)(Figure 5.2E) and *KCNE2* mRNA abundance (r^2=0.51, *p*=0.0009)(Figure 5.2F). In our term non-laboring samples *PR-A* expression was barely detectable, meaning correlations with PGR were predominantly in relation to *PR-B*. Finally, the key contraction-associated gene product, *PTGS2* mRNA, also exhibited significant positive correlation with *KCNH2* (r^2=0.37, p=0.0076)(Figure 5.2G) and *KCNE2* mRNA abundance (r^2=0.50, *p*=0.0011)(Figure 5.2H).

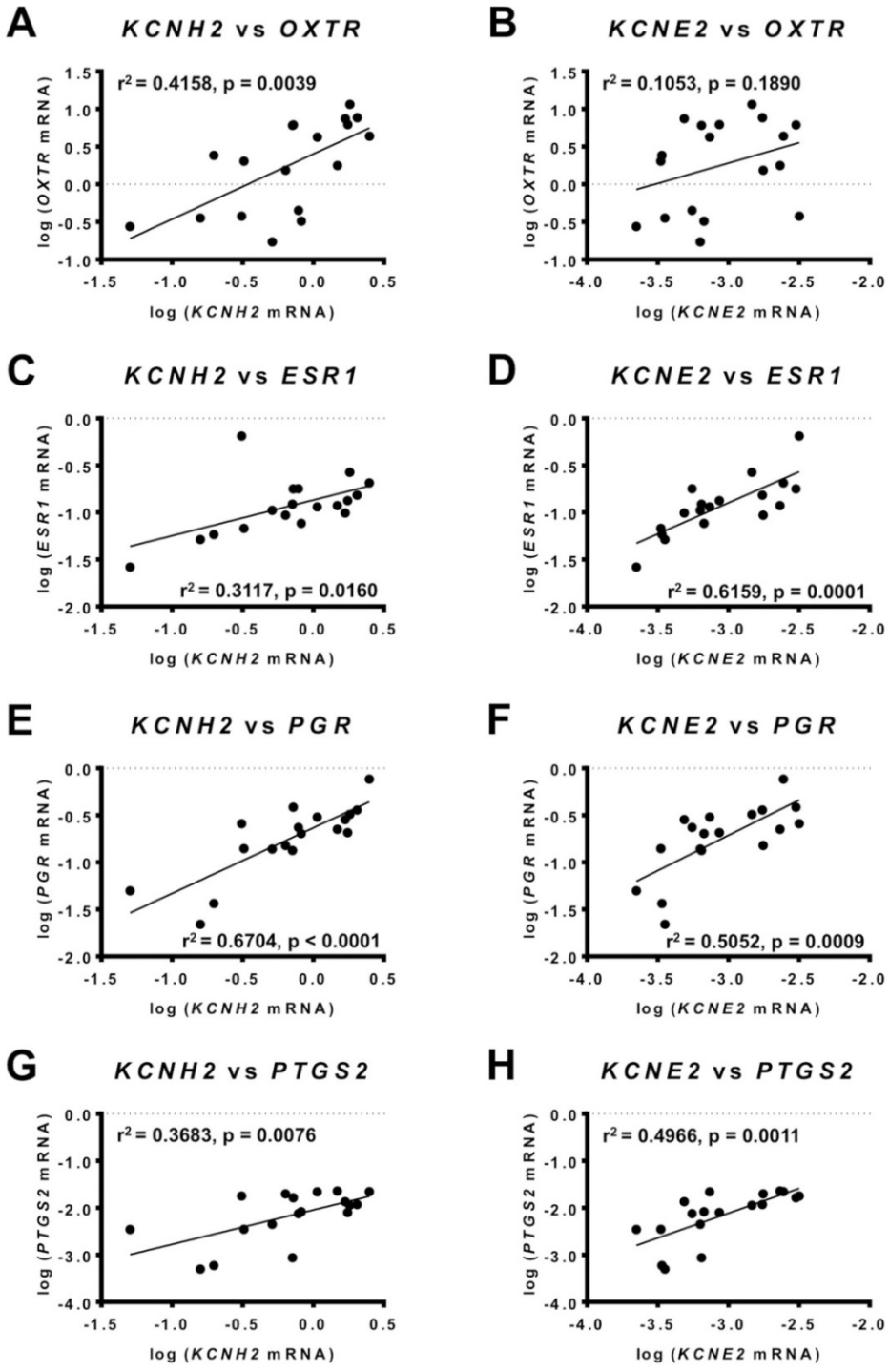

Figure 5.2. *KCNH2* and *KCNE2* mRNA expression correlates with expression of key myometrial genes at term.
Relative mRNA abundance of *KCNH2*, *KCNE2, OXTR, ESR1, PGR* and *PTGS2* were measured in term non-laboring myometrial tissues (n=18) and expressed relative to Alien reference RNA. Correlations of mRNA abundance between (A) *KCNH2* and *OXTR*; (B) *KCNE2* and *OXTR*; (C) *KCNH2* and *ESR1*; (D) *KCNE2* and *ESR1*; (E) *KCNH2* and *PGR*; (F) *KCNE2* and *PGR*; (G) *KCNH2* and *PTGS2*; (F) *KCNE2* and *PTGS2*. *(Pearson's r)*

KCNH2 and KCNE2 Expression in Preterm Human Myometrium

The relative abundance of *KCNH2* and *KCNE2* mRNA was determined in a limited number of non-laboring preterm myometrial tissues (n=3). No difference was found for *KCNH2* (*p*=0.47) or *KCNE2* (*p*=0.92) mRNA abundance compared to term non-laboring samples (n=18)(Figure 5.3).

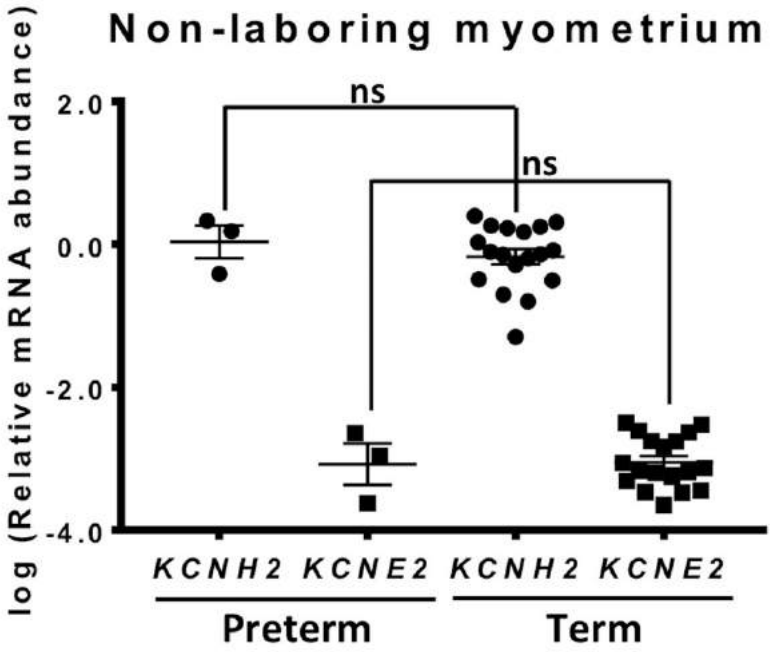

Figure 5.3. *KCNH2* and *KCNE2* expression in preterm and term non-laboring human myometrium.
Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in preterm (n=3) and term (n=18) non-laboring myometrial tissues, and expressed relative to Alien reference RNA. *(ANOVA)*

5.5 Discussion

We previously reported the presence of hERG1 and KCNE2 in pregnant human myometrium in late gestation, and demonstrated that labor onset is associated with diminished $K_v11.1$ activity in association with enhanced expression of the inhibitory subunit, KCNE2 [234]. Furthermore, we reported a significant positive correlation between $K_v11.1$ activity and BMI, which was

attributable to increased hERG1 levels and decreased KCNE2 levels [234]. In follow up to that functional study, we examined *KCNH2* and *KCNE2* gene expression in term non-laboring myometrium and found that *KCNE2* expression was relatively low, and correlated with *KCNH2* expression. Moreover, the levels of both mRNAs correlated positively with the expression of myometrial genes that are key regulators of parturition, but showed no relationship with the BMI of the participants. Patients comprising our term non-laboring cohort ranged from underweight (BMI 18.3) to Class II obese (BMI 38.0)(World Health Organisation guidelines). The lack of correlation suggests that within the examined BMI range, the effect of obesity is not on *KCNH2* or *KCNE2* gene expression, but rather on protein levels. This finding refines the link between K_v11.1 activity and BMI, and suggests that future studies should focus on the effect of obesity on *KCNH2* and *KCNE2* mRNA translation as well as protein turnover.

Another potassium channel that plays a crucial role in pregnancy is the ATP-sensitive K+ (K_{ATP}) channel. K_{ATP}–mediated K^+ efflux plays a role in maintaining the resting membrane potential of myocytes [338]. Several studies have found that in late pregnancy, the number of myometrial K_{ATP} channels is reduced, which increases uterine excitability, thereby promoting the establishment of labor [339-341]. Du *et al.* [342] found that all K_{ATP} channel subunits, apart from the SUR2A subunit, were down-regulated in the late pregnant uterus compared to the non-pregnant uterus. More importantly, expression of SUR2B/Kir6.1 in term pregnant human myometrium was found to be increased in women older than 35 years [342]. Advanced maternal age is associated with increased obstetric risks in general, as well as increased risk of elective and emergency CS [343, 344]. The study therefore suggests that

increased risk of birth complications in women aged over 35 years may be linked to reduced myocyte excitability attributable to increased numbers of K_{ATP} channels in the myometrium [342]. This is consistent with our previous study on hERG1, which similarly links poor labor outcomes for obese women with the elevated activity of a potassium channel [234]. Both studies illustrate the importance of understanding how different potassium channels regulate uterine contractility, as dysregulation can lead to obstetric complications.

In pregnant human myometrium, we observed a significant positive correlation between *KCNH2* and *KCNE2* mRNA abundance at term (Figure 5.1B). This correlation, if it translates to protein abundance, could suggest an underlying subunit stoichiometry in myometrial K_v11.1 channels. Given that all term samples analysed were non-laboring, preservation of the high *KCNH2*:*KCNE2* ratio may play a role in maintaining quiescence in non-laboring tissue.

The uterine quiescence, which persists for the majority of pregnancy, is maintained by inhibitors of uterine contraction, such as progesterone [5-9]. As term approaches there is a shift from progesterone to estrogen dominance and the uterus undergoes a phenotypic transition. This transition is characterised by up-regulated expression of a series of contraction-associated genes (reviewed by Smith [345]), including receptors for oxytocin and prostaglandins, increased expression of genes encoding myometrial gap junctions, such as connexin 43, which facilitates synchronous contractions, and alterations in resting membrane potential of myocytes, which renders myocytes more prone to excitation [5-9, 128]. Collectively these changes increase the likelihood that sporadic

contractions of the uterus will propagate in synchrony and lead to established labor [235]. In fulfilling a role as a regulator of myometrial contractility, it would therefore be reasonable to expect K_v11.1 activity, and thus *KCNH2* and *KCNE2* expression, to correlate with the expression of key genes that are known to play a role in this phenotypic transition.

OXTR expression in the myometrium is reportedly constant between 24 – 36 weeks gestation, but rises significantly in term samples (>37 weeks) prior to the onset of labor [128, 346]. Within this latter time frame (term), we found that *KCNH2* expression correlated positively with *OXTR* expression, whereas *KCNE2* did not. Given that *KCNH2* mRNA abundance is significantly correlated with both *KCNE2* and *OXTR* mRNA abundance, a correlation should be expected between *KCNE2* and *OXTR* mRNA levels. It is possible that with greater sample numbers a positive correlation would be demonstrated between *KCNE2* and *OXTR* expression. Given that the role of K_v11.1 within the myometrium is to rapidly terminate contractions in order to prevent propagation of uterine contractility and the establishment of labor, it is reasonable to conjecture that *KCNH2* expression is co-regulated with *OXTR* expression as a counter-measure to offset increased sensitivity to oxytocin. Furthermore, Welsh *et al*. [91] found that U-0126, highly selective inhibitor of MEK, completely blocked the ability of estrogens to stimulate increased expression of *OXTR* in human myometrial explants. This suggests that the action of estrogens in human myometrium is mediated, at least in part, by extranuclear signalling via ERs operating through activation of the MEK/ERK cascade [91]. Afrasiabi *et al.* [347] found that there was a decrease in the phosphorylation of MAP kinase after a short treatment of cells with the hERG inhibitor, but the long-term

phosphorylation was not significantly changed. Since both *OXTR* and *KCNE2* can be regulated through MEK/ERK cascade, this may account for significant correlation between these two genes.

Similar considerations may apply to the observed positive correlation of *KCNH2* and *KCNE2* with *ESR1* and *PTGS2*. *ESR1* encodes ERα, which drives the estrogen-dependent expression of contraction-associated proteins, such as connexin 43 [76, 348-350]. *PTGS2* is responsible for the biosynthesis of PGs. $PGF_{2\alpha}$ is produced mainly by the maternal decidua and is involved in the up-regulation of *OXTR* levels and gap junctions in the myometrium, thus promoting uterine contractions [174]. PGE_2 is produced by the fetus and placenta and is involved in collagen degradation and dilation of small blood vessels in the cervix, thus promoting cervical ripening as well as spontaneous rupture of the fetal membranes [175]. It is important to remain mindful, however, that non-laboring tissues were analysed in our study suggesting the possibility that women with high expression of *ESR1* and *PTGS2* may require higher levels of *KCNH2* in order to maintain uterine quiescence than women with lower *ESR1* and *PTGS2* expression. Moreover, in the heart KCNE2 expression is up-regulated by estrogen [351]. Taking into consideration that in humans circulating levels of estrogen are high for most of pregnancy, and remain elevated during parturition [9, 352], it is likely that estrogen regulation of KCNE2 expression, and thus $K_v11.1$ activity, plays an important role in modulating the transition from quiescence to contractility. As previously discussed in the literature review (Chapter 1), in the genomic pathway, binding of estrogens to ERs induces a conformational change in the receptors that instigates dissociation from chaperones, dimerisation, as well as activation of the receptor

transcriptional domain [97-99]. The ER-mediated regulation of gene expression involves the direct binding of dimeric ER to DNA sequences, called ERE [100]. Kundu *et al.* [351] found that KCNE2 was highly up-regulated by E2 due to the presence of an ERE in the *KCNE2* promoter. The presence of an ERE within both the *KCNE2* and *ESR1* promoters may explain the significant correlation of expression between the two genes.

KCNH2 and *KCNE2* mRNA levels were both positively correlated with *PGR* expression. We report *PGR* total expression [212] as within our term non-laboring cohort *PR-A* expression was barely detectable. The strong positive correlations between *KCNH2* and *KCNE2* expression with *PGR* expression are therefore in relation to *PR-B*, which is consistent with the observed *KCNH2*:*KCNE2* ratio potentially contributing to pregnancy maintenance. Activators of PKA, stimulate cAMP production, which in turn increase PGR protein levels. Recently, Chen *et al*. [353] found that activation of the PKA pathway increased hERG protein phosphorylation, which facilitated hERG incorporation into the plasma membrane. Furthermore, Cui *et al.* [354] found that PKA influenced the activity of *KCNE2*. Since *PGR*, *KCNE2* and *KCNH2* all interact with PKA pathway, this could potentially explain the significant correlation detected between *PGR* and *KCNE2* and *PGR* and *KCNH2*. A follow up analyses determining whether *KCNH2* and *KCNE2* expression continue to correlate with *PGR* expression in laboring tissue would provide valuable insight into whether progesterone regulates hERG1 and KCNE2 levels as means of maintaining uterine quiescence.

In mice, *KCNH2* expression remains constant across gestation and there is no change in ERG1 protein levels [232]. *KCNE2,* however, is gestationally regulated in mice in that mRNA abundance is significantly up-regulated by day 14 of a 20 day pregnancy, and KCNE2 protein levels are significantly up-regulated by day 17 [232]. Our analyses of preterm non-laboring samples showed no change in *KCNH2* or *KCNE2* gene expression earlier in gestation suggesting that, unlike mice, *KCNE2* gene expression may not be gestationally regulated in humans. If confirmed by analysis of additional preterm samples, this could indicate that alternative auxiliary subunits need to be explored in the context of human myometrium. To date, studies exploring the role of K_v11.1 channels in human myometrium have focused on KCNE2 co-expression alongside hERG1. However, a variety of auxiliary subunits are known to modulate K_v11.1 activity [222], and studies by Greenwood *et al.* [232] indicate that *KCNE4* expression is gestationally regulated in mouse uterine tissue in addition to *KCNE2*. As such it is possible that KCNE4, or another regulatory subunit, is gestationally regulated in humans.

Conclusions

We have previously provided functional data examining the role of K_v11.1 in regulating myometrial contractility at term labor. Here we have followed up the functional study with an analysis of *KCNH2* and *KCNE2* gene expression in term non-laboring women. We have shown that *KCNH2* and *KCNE2* expression correlate with the expression of key myometrial genes implicated in parturition. Our data suggest that *KCNH2* and *KCNE2* are participants in the gene network controlling myometrial contractility at term. Uncovering this association advances our understanding of the mechanisms that underpin myometrial

transformation, and reiterates the complexity of the parturition process. A complementary analysis using laboring myometrium would assist with the further interpretation of these results as alterations of the correlative network with the onset of labor would provide insight into which relationships are critical in maintaining uterine quiescence. We have also performed an initial investigation into *KCNH2* and *KCNE2* expression in preterm samples, which did not show any robust association between the preterm status and *KCNH2* or *KCNE2* expression. This investigation should be followed up with additional preterm samples to strengthen the study, as well as the analysis of preterm in-labor samples. Furthermore, future studies include examining whether these correlations translate into correlations at the protein level.

5.6 Acknowledgments

The authors wish to thank the obstetricians from the John Hunter Hospital, NSW, our research midwife, Anne Wright, and the research participants who donated samples toward this study.

Chapter Six: Regulation of *KCNH2* (hERG1) and *KCNE2* Expression in Human Pregnant Myometrium

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This Chapter contains a manuscript in preparation for submission to the Journal of Smooth Muscle Research.

Recently, we found novel correlations in the expression of *KCNH2* and *KCNE2* with the key contraction-associated genes, including *ESR1, OXTR, PR* and *PTGS2* (Chapter 5). In addition, our previous studies showed that human non-laboring myometrial tissues undergo culture-induced changes in the expression of key contraction-associated genes which is consistent with transition toward a laboring phenotype (Chapter 3 and 4).

In this manuscript, we report that *KCNE2* mRNA expression undergoes culture-induced increase which is also consistent with transitioning toward a laboring phenotype. We further report that implementing culture conditions approaching the *in vivo* environment was successful in preventing this culture-induced increase. Finally, we report that hERG channel inhibitors increased myometrial contraction duration *in vitro*, while hERG channel activators had no effect on myometrial contraction duration.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Marina Ilicic (1st Author)	**Conceived and designed experiments** **Sample collections** **Contraction bioassay** **Performed explant experiments** **Performed molecular analyses** **Data analyses** **Manuscript writing**
Tamas Zakar	Conceived and designed experiments Provided reagents and materials Manuscript editing
Roger Smith	Manuscript editing
Jonathan W. Paul	Conceived and designed experiments Performed explant experiments Contraction bioassay Data analyses Provided reagents and materials Manuscript editing

6.1 Abstract

Background: Labor onset is associated with diminished activity of $K_v11.1$ potassium channels, encoded by *KCNH2,* in association with increased expression of the inhibitory subunit, *KCNE2*. Furthermore, during term pregnancy, *KCNH2* and *KCNE2* are expressed co-ordinately with key contraction-associated myometrial genes, thus strengthening the link between uterine contractility and K^+ channel abundance in myometrial cells.

Objectives: This study aimed to examine the effect of hERG channel activators and inhibitors on spontaneous human uterine contractions *in vitro* within an organ bath setting. Furthermore, the study aimed to determine whether the expression of *KCNH2* and *KCNE2* changed upon placing non-laboring human myometrium in culture, and if so, to determine whether these culture-induced changes could be prevented by implementing culture conditions that approached the *in vivo* environment.

Methods: For contractility studies, term non-laboring human myometrial tissue strips were incubated in presence of hERG activators (PD-118057 and NS-1643) or inhibitors (DOF and E-4031). Myometrial contraction plateau duration was measured for four contractions pre- and post-treatment. For gene expression studies, term non-laboring human myometrial tissues were cultured in the presence of specific treatments, including; serum supplementation, progesterone and/or estrogen, cAMP, PMA, stretch or NF-κB inhibitors. qRT-PCR was used to determine *KCNH2* and *KCNE2* mRNA abundance in fresh and 48 h cultured tissue. *KCNH2* and *KCNE2* mRNA abundance was then compared.

Results: Both hERG channel inhibitors significantly increased myometrial contraction duration *in vitro,* whereas both activators had no effect. Expression of *KCNE2* mRNA significantly increased when myometrial tissue was placed in culture whereas *KCNH2* mRNA expression remained unchanged. Supplementing media with progesterone and estrogen prevented culture-induced up-regulation of *KCNE2* expression and down-regulated *KCNH2* expression relative 48 h control tissue. Stretch had no direct effect, but blocked the effects of progesterone and estrogen on *KCNH2* and *KCNE2* expression. PMA prevented culture-induced increase in *KCNE2* expression without affecting *KCNH2* expression.

Conclusion: hERG channel activity plays a role in regulating the duration of contractions in term non-laboring human myometrium. Although channel activity could be inhibited to significantly increase contraction duration, channel activity could not be activated to reduce contraction duration. Significantly up-regulated *KCNE2* expression following 48 h culture is consistent with transition toward a laboring phenotype and indicates that standard conditions routinely used to culture human myometrium are not optimal for maintaining *in vivo* expression levels of *KCNE2.* However, culture-induced changes in *KCNE2* can be prevented by supplementing media with PMA, thereby preserving this aspect of the non-laboring phenotype *in vitro*.

6.2 Introduction

The hERG encodes the pore-forming subunit of a delayed rectifier voltage gated potassium channel, $K_v11.1$, which conducts K^+ out of the cell

[222]. A number of studies have demonstrated that ERG plays a role in regulating the contractile activity of cardiac myocytes (reviewed by *Vandenberg et al.,* [222]). Furthermore, ERG has been investigated in numerous smooth muscle tissues and has been shown in rat stomach and murine portal vein [223-225] as well as in opossum oesophagus [226]. In addition, studies have used selective ERG channel blockers to increase contractility in rat stomach [223], mouse portal vein [225], opossum oesophagus [226], mouse and guinea pig gall bladder [227], bovine epididymis [228] and human and equine jejunum [229, 230]. These smooth muscles all exhibit spontaneous contractile activity concurrent with the generation of AP. Human myometrium also exhibits spontaneous contractile activity underpinned by AP discharged [233].

Aaronson *et al*. [231] were the first to demonstrate that TEA- and 4-AP-sensitive voltage-dependent K^+ (K_v) channels played a role in regulating AP duration in rat myometrium. Greenwood *et al*. [232] later examined mERG expression and function, and confirmed the presence of both the ERG1a and ERG1b splice variants, with ERG1a expression being more abundant than that of ERG1b. Furthermore, they found that ERG1 protein levels as well as *KCNH2* mRNA expression did not change throughout the gestation or with the onset of labor; however, expression of the gene encoding the β-auxiliary inhibitory subunit, *KCNE2*, were significantly up-regulated approaching term [232].

Recently, our group reported that hERG protein and the auxiliary subunit KCNE2 are present in pregnant human myometrium in late gestation and labor [234]. In addition, we found that hERG channel activity is responsible for supressing contraction amplitude and duration, thus limiting the capacity for

contraction propagation [235] and thereby promoting uterus quiescence [234]. Furthermore, we found that labor was associated with increased expression of the inhibitory auxiliary subunit, KCNE2, which caused a decrease in hERG activity that led to increased duration of uterine APs and the associated contractions [234]. These results suggest that changes in hERG channel activity contribute to electrophysiological mechanisms that generate contractions during labor [234].

Given that hERG and KCNE2 are newly identified contraction-associated proteins in human myometrium, a component of this thesis was to examine whether expression of *KCNH2* and *KCNE2* correlate with the expression of other key contraction-associated genes. It was determined that at term pregnancy, *KCNH2* and *KCNE2* were expressed co-ordinately with the parturition-associated genes: *OXTR*, *ESR1*, *PGR* and *PTGS2*. This finding strengthened the link between uterine contractility and K^+ channel abundance in myometrial cells (Chapter 5, under review). In addition, a previous study outlined that term non-laboring human myometrial tissue undergoes culture-induced changes in expression of *ESR1*, *PTGS2* and *OXTR* (Chapter 3) as well as *PR* and the *PR-A/PR-B* expression ratio (Chapter 4), and that changes are consistent with transition toward a laboring phenotype (both manuscripts are under review).

Aims

In light of the above, the first aim of this study was to examine the effect of hERG channel activators and inhibitors on spontaneously contracting pregnant human myometrium in an organ bath setting, as prior contractility

studies published by our group were on patch clamp analysis. Second, the study aimed to determine whether term, non-laboring human myometrial tissue undergoes culture-induced changes in the expression of *KCNH2* and *KCNE2* that are consistent with transition toward a pro-contractile, laboring phenotype. Third, the study aimed to identify culture conditions that could be implemented to block or minimize changes in expression of *KCNH2* and *KCNE2 in vitro*, thereby providing researchers with a stable platform on which to investigate these newly discovered contraction-associated genes.

This component of research reports that hERG channel inhibitors significantly increased myometrial contraction duration *in vitro*, while hERG channel activators had no effect. In addition, upon being placed into culture, non-laboring human myometrium underwent significant changes in *KCNE2* expression whereas *KCNH2* expression remained unchanged. Finally, it is reported that *KCNE2* expression was able to be maintained at levels consistent with term, non-laboring human myometrium; however, the culture conditions also affected *KCNH2* expression.

6.3 Materials and Methods

Consumables and Reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA–free 50 reactions were from Ambion (Thermo Fisher). Alien QRT-PCR Inhibitor Alert 400 Reactions were purchased from Integrated Sciences Pty (Sydney, Australia).

(R)-MG132, BAY-11-7085 and PMA were obtained from Cayman Chemical Company (Michigan, USA). 8-Br-cAMP, PCR primers, Progesterone and Estradiol were purchased from Sigma (St Louis, USA). 2 mL 2.8 mm ceramic bead kits (CK28-R) for the Precellys homogenizer (Bertin Instruments, France) were purchased from Thermo Fischer Scientific (Melbourne, Australia). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES, DMEM and Charcoal Stripped Fetal Bovine Serum were obtained from Gibco (Carlsbad, USA). SYBR Green 2× Master mix was from Applied Biosystems (Carlsbad, USA). DOF, E-4031, NS-1643 and PD-118057 were purchased from Tocris (Bristol, UK).

Myometrial Tissue Acquisition

These studies were performed in Newcastle, New South Wales, Australia and were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent. Human myometrial samples (5 × 5 × 10 mm) were obtained from the lower uterine segment during elective CS of singleton term pregnancies (38.2 – 39.6 weeks gestation). All women were examined clinically and none of the patients were in-labor. The clinical indications for elective cesarean delivery were previous CS, placenta praevia, fetal distress, breach presentation or previous $3^{rd}/4^{th}$ degree tear. Women were excluded if they were given steroids or had signs of infection. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of postpartum hemorrhage. Samples were therefore exposed to oxytocin for a brief period of time (3 min). Myometrial samples used for contractility studies were placed on ice in ice-cold

physiological saline, whilst myometrial samples used for tissue culture studies were placed on ice in serum-free medium containing DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin and 10 mM HEPES for the transfer to the laboratory.

Myometrial Contractility Studies

Myometrial strips were set up as previously described [110]. Briefly, non-laboring human myometrial samples were dissected into strips (10 × 2 × 2 mm) and suspended in organ baths containing 30 mL PSS containing (in mM) NaCl 120, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11, and continuously gassed with carbogen (95% O_2, 5% CO_2), at pH 7.4. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1 g passive tension applied (1 g was calibrated to equal 1 V). PSS was replaced five times during the first hour, with strips re-tensioned to 1 g passive tension following each wash. Thereafter strips were maintained at 37°C until spontaneous rhythmic contractions developed. Data were digitized using a MacLab/8E data-acquisition system and contraction status visualized in real time using Chart software (ADInstruments, NZ). For each strip a contraction baseline was acquired to serve as reference [243].

To administer various treatments, 600 µL of PSS buffer was carefully extracted from an organ bath and tranferred to an Eppendorf tube. The appropriate volume of treatment was pipetted into the PSS to pre-dilute the treatment. The total volume of pre-diluted treatment (600 µL PSS + treatment) was then carefully reinjected back into the appropriate organ bath. Final

concentrations of each drug were; PD-118057 10.0 μM, NS-1643 10.0 μM, DOF 1.0 μM and E-4031 1.0 μM.

Tension generated by tissue strips is indicated in the results and representative contraction traces. The effect of treatments is interpreted relative to the pre-treatment contraction baseline, which consisted of 4 contractions of consistent frequency and amplitude.

Myometrial Tissue (Explant) Culture

Approximately 100 mg tissue from each sample was immediately snap frozen in liquid nitrogen for subsequent analysis. The remaining myometrium was dissected into approximately 2 × 2 × 2 mm pieces and washed in serum-free media. Samples were then incubated in serum-free or 5% (v/v) CSS-supplemented media in a 37°C, 95% air/5% CO_2 humidified incubator for 48 h. The culture media contained DMEM with high glucose, 5% CSS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/mL Gentemicin and 10 mM HEPES. To determine the effects of steroids, myometrial samples were incubated with physiological concentrations of P4 (500 nM) and/or E2 (100 nM, 400 nM and 10 μM) [8] in a 37°C, 95% air/5% CO_2 humidified incubator for 48 hours. To determine the effect of stretch on human myometrium, myometrial tissue strips (2 × 2 × 10 mm) were cultured in 5% CSS-containing media for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of constant stretch. Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 mL of culture media in 50 mL tubes (strips subjected to 0 g only were tied at one end). To determine the effect of stretch and steroids on human

myometrium, myometrial strips were cultured in 5% CSS-containing media with 500 nM P4 and 400 nM E2 for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of stretch. To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial tissues were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with the cAMP analogue 8-Br-cAMP (250 µM), PMA (0.1, 1.0, 5.0 µM), NF-κB inhibitors MG-132 (2.0, 5.0, 10.0 µM) or BAY-11-7085 (2.0, 5.0, 10.0 µM). Vehicle was DMSO (0.1%). Following each incubation, the media was decanted, tissue pieces or strips were snap frozen using liquid nitrogen and stored at -80°C for subsequent analyses.

RNA Extraction, Reverse Transcription and Real-time Quantitative PCR

RNA was extracted from 100 mg of tissue using TRizol Reagent (Thermo Fisher) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Thermo Fisher). An ND-1000 spectrophotometer was used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5 $x10^7$ copies of Alien RNA and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers. qRT-PCR was performed using an ABI 7500 Sequence Detector. no-RT negative controls were prepared for each sample to ensure there was no DNA contamination. The final volume of each PCR reaction was 20 µL containing 10 µL of 2x SYBR Green PCR

Master Mix (Thermo Fisher), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 µL with 1.0 µL of 2.5 µM of Alien Primer Mix, 10 µL of 2x SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. NTC samples were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express and are shown in the Table 6.1.

Table 6.1. ***cDNA primer sequences for KCNH2 and KCNE2.***

Primer	Primer Sequence	Amplicon Size	GeneBank #
KCNH2	F: ACCTCATCGTGGACATCA R: CTCCTCGTTGGCATTGAC	77	NM_000238.3
KCNE2	F: CACGAGGCAAATCCAAAT R: CTCCAACAAGCAAGCATAA	141	NM_172201.1

KCNH2, Potassium Voltage-Gated Channel Subfamily H Member 2; *KCNE2*, Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2

Data and Statistical Analysis

For contraction traces, contraction plateau duration (sec) was determined for four contractions pre- and post-treatment using LabChart software (ADInstruments, NZ). Plateau duration was determined as the time between the point of highest amplitude and point where contraction force declined sharply. Contraction duration data were obtained for 3 individual tissues (n=3 women). Pre- and post-treatment measurements (n=12 each) were compared by two-tailed unpaired t-test.

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the ΔC_t method [245]. All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t* test was used. For multiple comparisons a one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett multiple comparisons was used. P-values ≤ 0.05 were considered statistically significant.

6.4 Results

Human Myometrial Contractility

Contraction bioassays were performed to determine not only the effect of hERG channel inhibitors on spontaneous contractions in term, non-laboring human myometrium *in vitro,* but also the effect of hERG channel activators. Consistent with the existing literature, contraction duration was examined [232, 234, 240]. DMSO controls are reported for each inhibitor/activator as the different compounds were investigated across different days.

Administering DMSO (control) to spontaneously contracting tissue strips (n=3) (Figure 6.1Ai) had no significant effect on contraction plateau duration (p=0.2171; pre-treatment = 44.58 ± 3.10; post-treatment = 38.25 ± 3.91 sec) (Figure 6.1Bi). Administration of 10.0 µM PD-118057 (a hERG activator) to spontaneously contracting tissue strips (n=3) (Figure 6.1Aii) had no significant

effect on contraction plateau duration (p=0.2885; pre-treatment = 47.17 ± 8.21; post-treatment = 36.67 ± 5.09 sec) (Figure 6.1Bii). Higher PD-118057 doses were also examined (up to 30.0 µM) but had no effect (Data not shown. Data is shown for 10.0 µM PD-118057 as this is consistent with existing literature) [232].

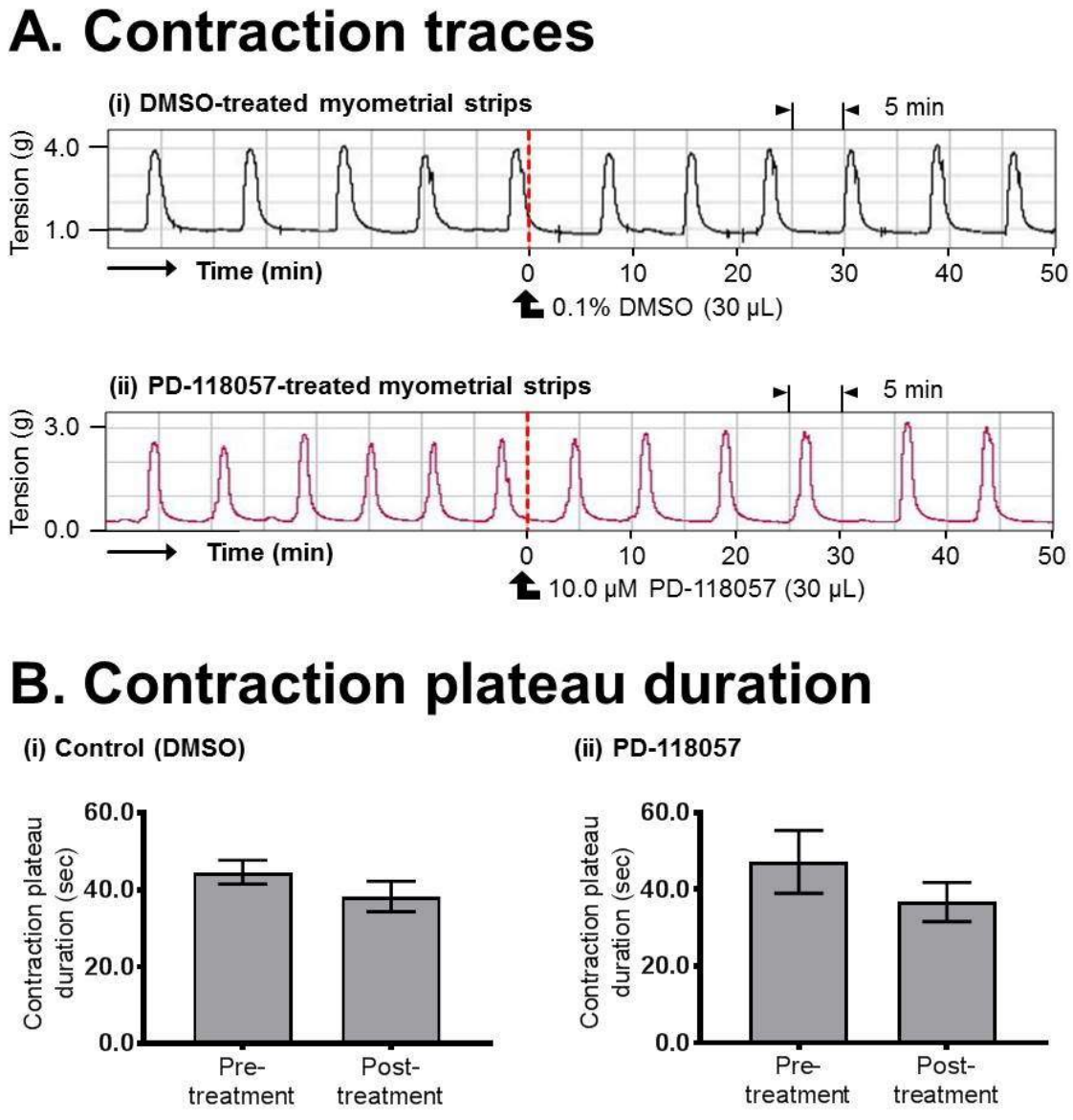

Figure 6.1. Effect of PD-118057 on myometrial contractility *in vitro*.
Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of DMSO (vehicle) or 10.0 µM PD-118057 on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with DMSO or PD-118057 (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

Administering DMSO (control) to spontaneously contracting tissue strips (n=3) (Figure 6.2Ai) had no significant effect on contraction plateau duration (p=0.6275; pre-treatment = 34.33 ± 4.50; post-treatment = 31.00 ± 5.07 sec) (Figure 6.2Bi). Administration of 10.0 µM NS-1643 (a hERG activator) to spontaneously contracting tissue strips (n=3) (Figure 6.2Aii) had no significant effect on contraction plateau duration (p=0.2720; pre-treatment = 35.08 ± 3.61; post-treatment = 30.25 ± 2.31 sec) (Figure 6.2Bii). Higher NS-1643 doses were also examined (up to 30.0 µM) but had no effect (Data not shown. Data is shown for 10.0 µM E-4031 as this is consistent with existing literature) [232].

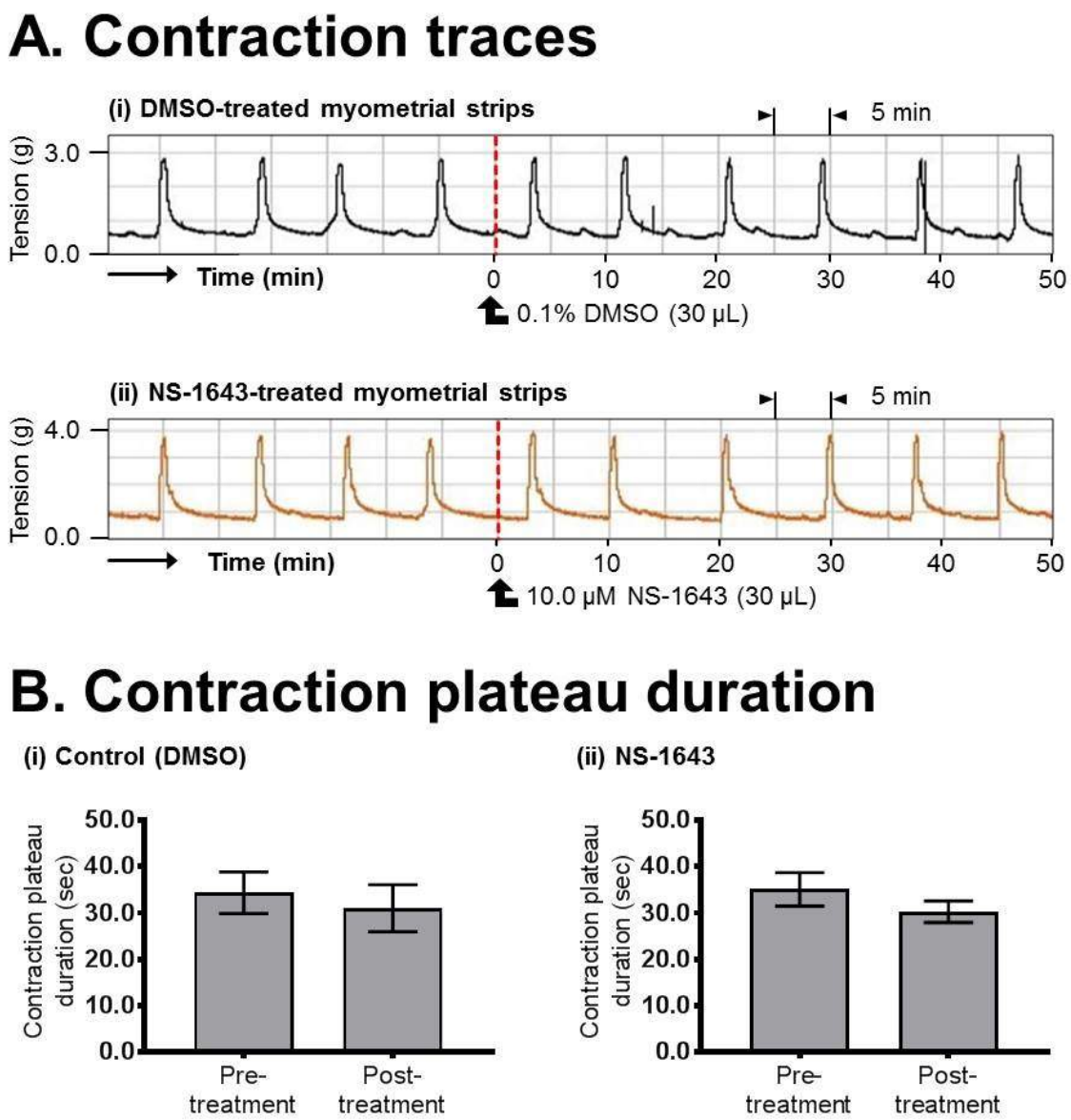

Figure 6.2. Effect of NS-1643 on myometrial contractility *in vitro*.
Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of DMSO (vehicle) or 10.0 µM NS-1643 on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with DMSO or NS-1643 (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

Administering DMSO (control) to spontaneously contracting tissue strips (n=3) (Figure 6.3Ai) had no significant effect on contraction plateau duration (p=0.7662; pre-treatment = 36.92 ± 5.24; post-treatment = 34.83 ± 4.52 sec) (Figure 6.3Bi). Administration of 1.0 µM DOF (a hERG inhibitor) to spontaneously contracting tissue strips (n=3) (Figure 6.3Aii) significantly

increased contraction plateau duration (p<0.0001; pre-treatment = 20.50 ± 3.45; post-treatment = 88.75 ± 13.95 sec) (Figure 6.3Bii). Increased contraction plateau duration is consistent with previous reports from our group on DOF action on human myometrium [234].

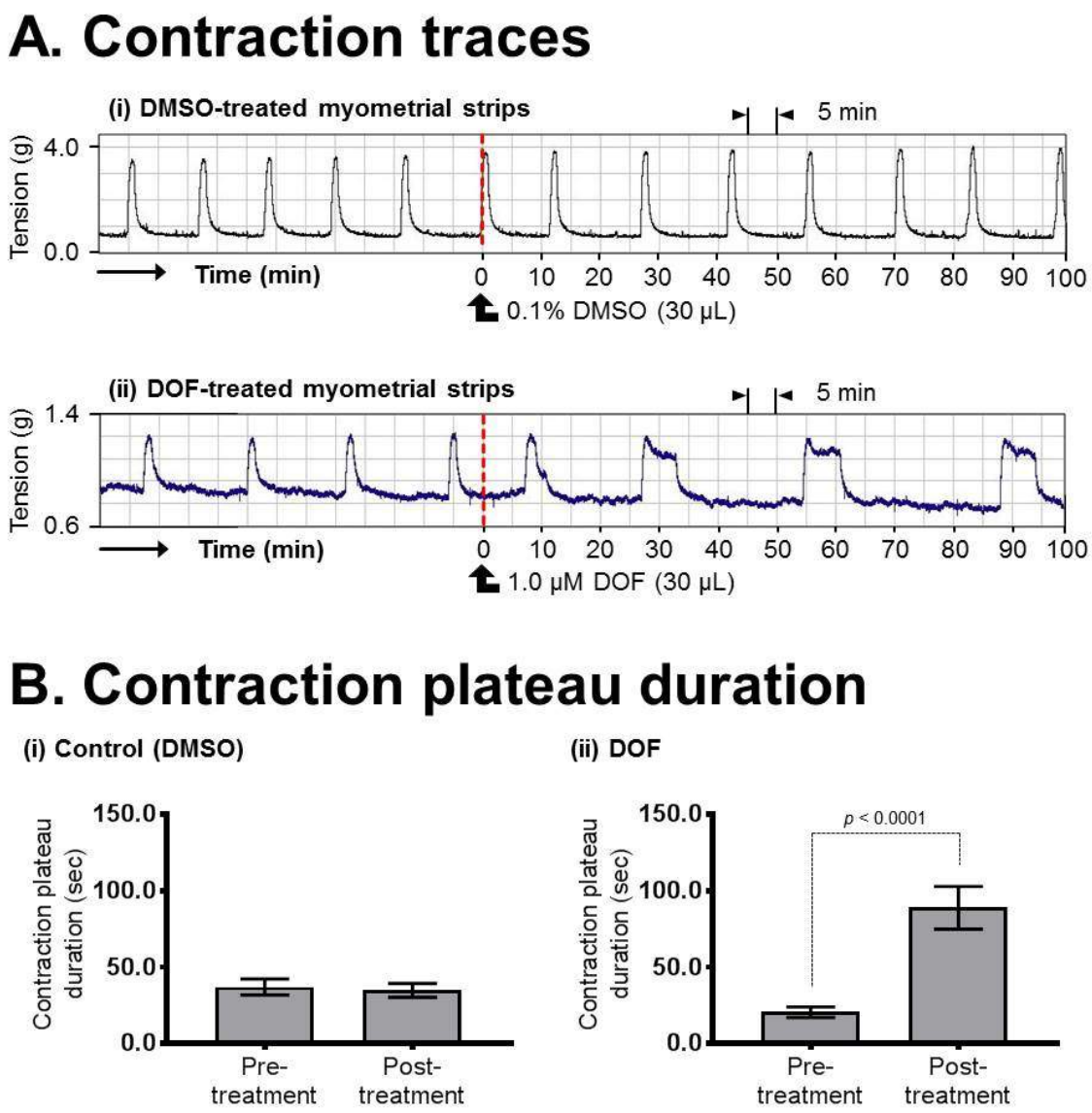

Figure 6.3. Effect of DOF on myometrial contractility *in vitro*.
Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of DMSO (vehicle) or 1.0 µM DOF on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with DMSO or DOF (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

Administering DMSO (control) to spontaneously contracting tissue strips (n=3) (Figure 6.4Ai) had no significant effect on contraction plateau duration (p=0.1292; pre-treatment = 48.25 ± 2.45; post-treatment = 42.75 ± 2.49 sec) (Figure 6.4Bi). Administration of 1.0 µM E-4031 (a hERG inhibitor) to spontaneously contracting tissue strips (n=3) (Figure 6.4Aii) significantly increased contraction plateau duration (p<0.0001; pre-treatment = 50.75 ± 4.15; post-treatment = 112.80 ± 10.31 sec) (Figure 6.4Bii).

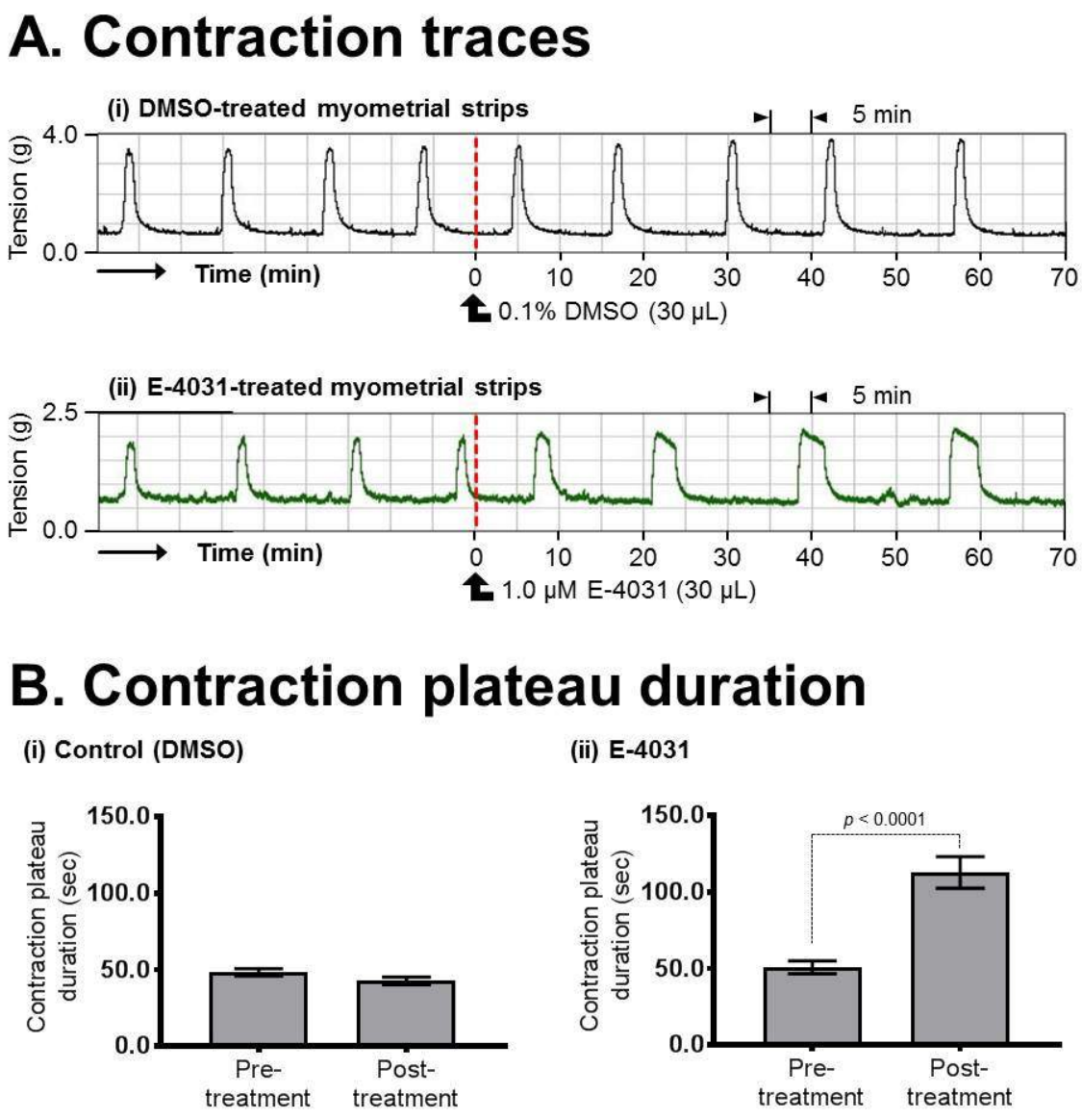

Figure 6.4. Effect of E-4031 on myometrial contractility *in vitro*.
Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of DMSO (vehicle) or 1.0 µM E-4031 on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with DMSO

or E-4031 (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

Gene Expression Changes in Myometrium during 48 h of Culture

Myometrial tissue samples were incubated for 48 h in serum-free media to determine gene expression changes that the tissue undergoes upon being removed from the *in vivo* environment and cultured *in vitro*. Following 48 h incubation there was no statistically significant change in *KCNH2* mRNA abundance (Figure 6.5A); however, there was a statistically significant increase in *KCNE2* mRNA abundance (*p*=0.0002) (Figure 6.5B).

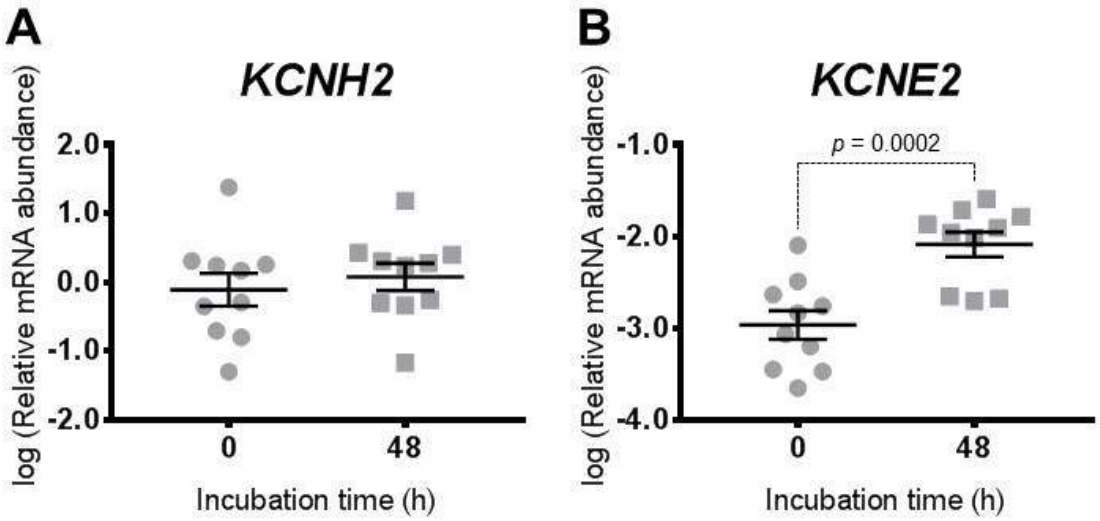

Figure 6.5. *KCNH2* and *KCNE2* mRNA abundance in fresh and 48 h cultured non-laboring human myometrium.
Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in term non-laboring myometrial tissue samples immediately after biopsy (0 h) as well as following 48 h incubation (n=10), and expressed relative to Alien reference. (A) *KCNH2* mRNA abundance. (B) *KCNE2* mRNA abundance. *Data was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then analysed by paired t-test*. *Data are mean ± SEM.*

Serum-free Media versus 5% CSS-containing Media

During myometrial culture (explants), CSS is often included in the culture media [257, 296, 297]. Having determined that *KCNE2* expression significantly

changed upon myometrial tissue being placed into culture, we examined whether supplementing media with 5% CSS affected this aspect of phenotypic transformation *in vitro*. Following 48 h incubation, there was no significant difference in *KCNH2* or *KCNE2* mRNA abundance between myometrium cultured in serum-free or 5% CSS-supplemented media (Figure 6.6), indicating that media supplementation with CSS did not prevent increased *KCNE2* expression during *in vitro* culture.

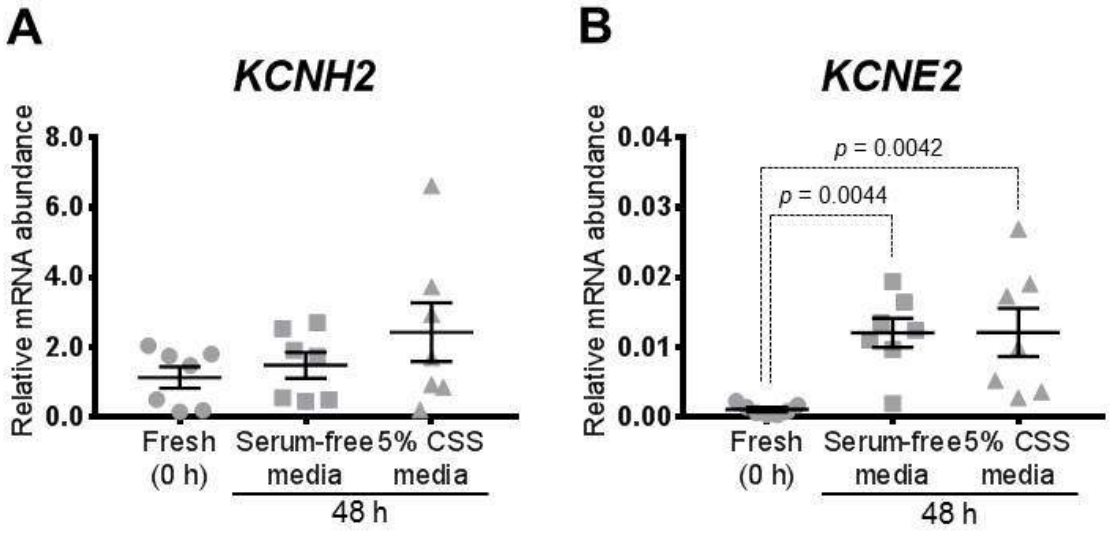

Figure 6.6. Effect of serum supplementation on culture-induced changes in *KCNH2* and *KCNE2* mRNA abundance.
Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in term non-laboring myometrial tissue samples following 48 h incubation in serum-free media or media supplemented with 5% CSS (n=7), and expressed relative to Alien reference. (A) *KCNH2* mRNA abundance. (B) *KCNE2* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effect of Steroids

Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in myometrial tissues incubated for 48 h in the presence of P4 (500 nM) and E2 (100 nM and 10 µM) alone or in a combination (500 nM P4 + 400 nM E2).

No significant change was detected in *KCNH2* mRNA abundance following 48 h culture in vehicle (DMSO). Supplementing media with P4 (500 nM) or E2 (100 nM and 10 µM) individually had no statistically significant effect on *KCNH2* mRNA abundance (Figure 6.7A and B). However, supplementing media with 500 nM P4 + 400 nM E2 in combination significantly decreased *KCNH2* mRNA abundance relative to 48 h DMSO-treated control tissues (p=0.0322) (Figure 6.7C).

KCNE2 mRNA abundance significantly increased in DMSO-treated (control) tissues following 48 h incubation (p=0.0013). Supplementing media with P4 (500 nM) or E2 (100 nM and 10 µM) individually had no statistically significant effect on *KCNE2* mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 6.7D and E). Supplementing media with the combination of 500 nM P4 + 400 nM E2, however, prevented the increase in *KCNE2* mRNA abundance to the extent that there was a significant difference relative to 48 h DMSO-treated control tissues (p=0.0083) (Figure 6.7F).

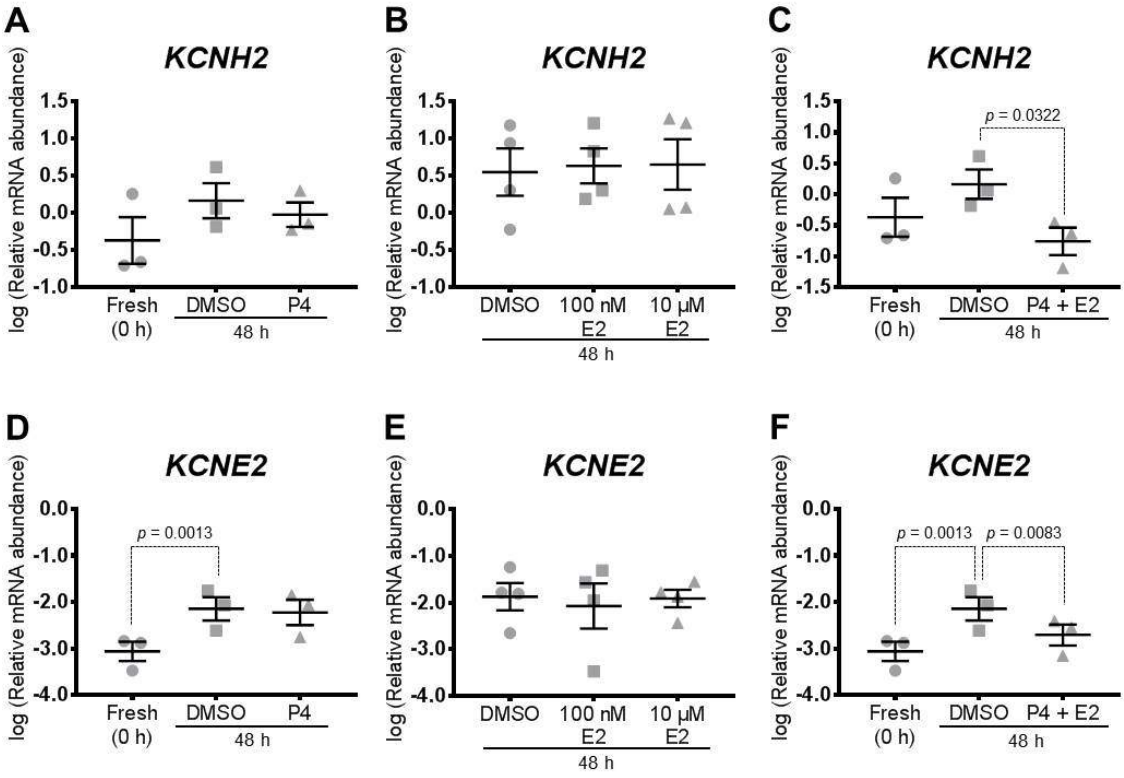

Figure 6.7. Effect of progesterone and estrogen on culture-induced changes in *KCNH2* and *KCNE2* mRNA abundance.
Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in term non-laboring myometrial tissue samples following 48 h incubation in the presence of 500 nM P4 (n=3) or 100 nM and 10 µM E2 (n=4) or 500 nM P4 + 400 nM E2 (n=3), and expressed relative to Alien reference. (A) Effect of P4 on *KCNH2* mRNA abundance. (B) Effect of E2 on *KCNH2* mRNA abundance. (C) Effect of P4 + E2 on *KCNH2* mRNA abundance. (D) Effect of P4 on *KCNE2* mRNA abundance. (E) Effect of E2 on *KCNE2* mRNA abundance. (F) Effect of P4 + E2 on *KCNE2* mRNA abundance. *Data were logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effect of Stretch on Human Myometrial Gene Expression in vitro

Myometrial tissue strips were subjected to 0, 1 or 3 g of stretch for 48 h to determine whether applying stretch to the muscle influenced the culture-induced changes in *KCNE2* mRNA levels. Additionally, the effect of stretch was also investigated in the presence of steroids (500 nM P4 + 400 nM E2).

Stretch (1 or 3 g) applied to myometrial strips for 48 h had no effect on *KCNH2* mRNA abundance (Figure 6.8A) relative to non-stretched (0 g) control

strips and fresh tissue. Interestingly, stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *KCNH2* mRNA levels (Figure 6.8B), indicating that P4 + E2 was no longer effective in decreasing *KCNH2* mRNA abundance compared to fresh tissues.

KCNE2 mRNA abundance significantly increased following 48 h incubation in non-stretched (0 g) control strips when compared to fresh tissues (p=0.0236) (Figure 6.8C). Applying stretch (1 or 3 g) to myometrial strips for 48 h had no effect on *KCNE2* mRNA expression when compared to 48 h non-stretched strips (Figure 6.8C). Stretch applied in the presence of P4 + E2 likewise had no effect (Figure 6.8D), indicating that P4 + E2 was no longer effective in preventing culture-induced increase in *KCNE2* mRNA abundance compared to fresh tissue.

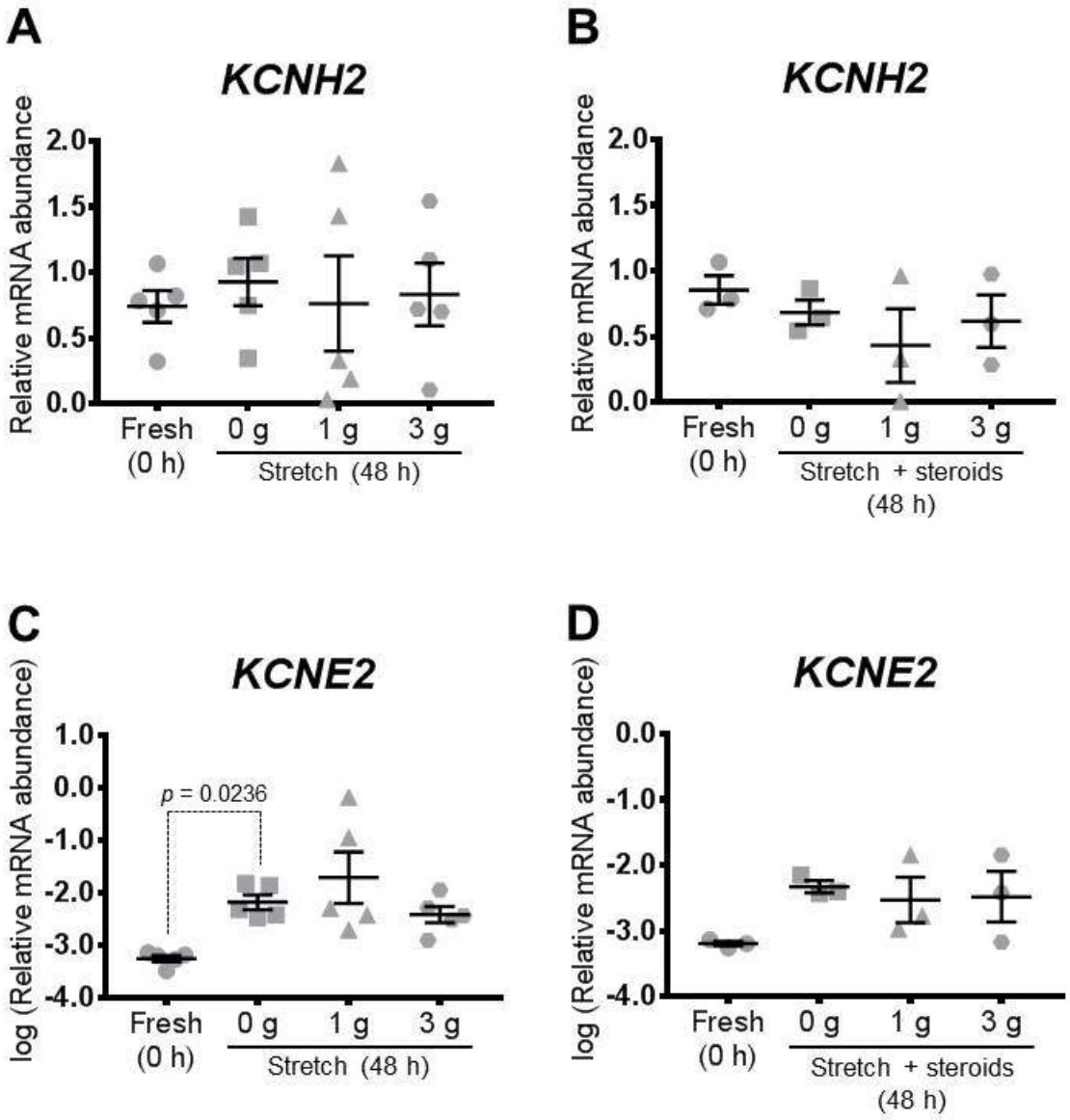

Figure 6.8. Effect of stretch in the absence or presence of steroids on culture-induced changes in *KCNH2* and *KCNE2* mRNA abundance.
Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in term non-laboring myometrial strips whilst applying 0, 1 and 3 g of stretch for 48 h (n=5), as well as in presence of steroids (500 nM P4 + 400 nM E2) whilst applying 0, 1 and 3 g of stretch for 48 h (n=3), and expressed relative to Alien reference. (A) Effect of stretch on *KCNH2* mRNA abundance. (B) Effect of stretch and steroids on *KCNH2* mRNA abundance. (C) Effect of stretch on *KCNE2* mRNA abundance. (D) Effect of stretch and steroids on *KCNE2* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effects of Activating Intracellular Signalling Pathways

Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in myometrial tissue incubated for 48 h in the presence of 8-Br-cAMP (250 µM), PMA (0.1, 1.0 and 5.0 µM) or vehicle (DMSO).

KCNH2 mRNA abundance remained unchanged over 48 h of culture (Figure 6.9A). Adding 250 µM 8-Br-cAMP to the culture media had no effect on *KCNH2* mRNA expression relative to 48 h DMSO-treated control tissues and fresh tissues (Figure 6.9A).

KCNE2 mRNA abundance significantly increased in DMSO-treated control tissues following 48 h incubation (p=0.0167) (Figure 6.9B). Adding 250 µM 8-Br-cAMP had no effect on *KCNE2* mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 6.9B).

KCNH2 mRNA abundance remained unchanged over 48 h of culture and supplementing media with 0.1, 1.0 and 5.0 µM PMA, a PKC activator, had no effect on *KCNH2* mRNA abundance (Figure 6.9C).

KCNE2 mRNA abundance significantly increased in DMSO-treated control tissues following 48 h incubation (p=0.0043) (Figure 6.9D). PMA dose-dependently inhibited this increase, and at a final PMA concentration of 5.0 µM, *KCNE2* mRNA abundance was significantly decreased relative to the 48 h DMSO-treated control tissue (p=0.0450) (Figure 6.9D). High-dose PMA therefore blocked the statistically significant increase in *KCNE2* mRNA abundance that occurs when term non-laboring myometrium is placed into culture under standard conditions.

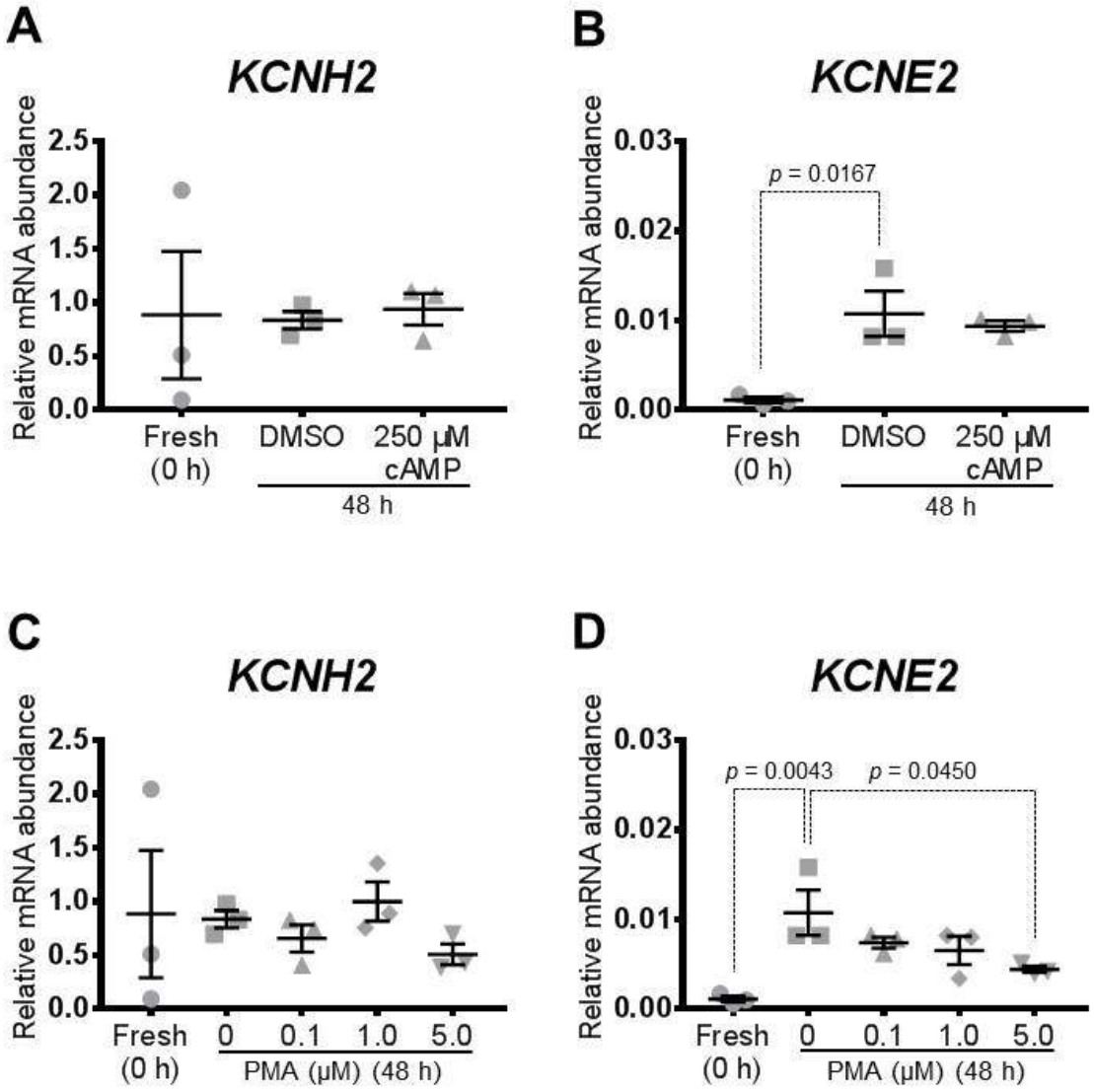

Figure 6.9. Effect of cAMP and PMA on culture-induced changes in *KCNH2* and *KCNE2* mRNA abundance.
Relative mRNA abundance of *KCNE2* and *KCNH2* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of 8-Br-cAMP (250 µM) or PMA (0.1, 1.0 and 5.0 µM), and expressed relative to Alien reference. (A) Effect of 8-Br-cAMP on *KCNH2* mRNA abundance. (B) Effect of 8-Br-cAMP on *KCNE2* mRNA abundance. (C) Effect of PMA on *KCNH2* mRNA abundance. (D) Effect of PMA on *KCNE2* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

The Effect of NF-κB Inhibitors

The NF-κB inhibitors, MG-132 and BAY-11-7085, were employed to test whether NF-κB pathway activation was involved in the *KNCE2* mRNA

expression changes induced by *in vitro* culture. The effect of these inhibitors on *KCNH2* mRNA expression were also examined.

Following 48 h incubation, there was no significant difference in *KCNH2* mRNA abundance between vehicle-treated tissues and fresh tissues (Figure 6.10A). Incubating myometrial samples with 2.0, 5.0 or 10.0 µM MG-132 or BAY-11-7085 had no effect on *KCNH2* mRNA abundance relative to vehicle-treated tissue or fresh tissue (Figure 6.10A).

Following 48 h incubation, *KCNE2* mRNA abundance in 48 h vehicle-treated tissue increased relative to fresh tissue; however, the increase did not reach statistical significance for this cohort of samples (Figure 6.10B). Similarly, neither MG-132 or BAY-11-7085 produced a statistically significant effect on *KCNE2* mRNA abundance. However, the trend in the data suggests that both inhibitors increased *KCNE2* mRNA levels above that of the 48 h vehicle-treated tissue, and that with additional replicates a statistically significant difference would be detected (Figure 6.10B).

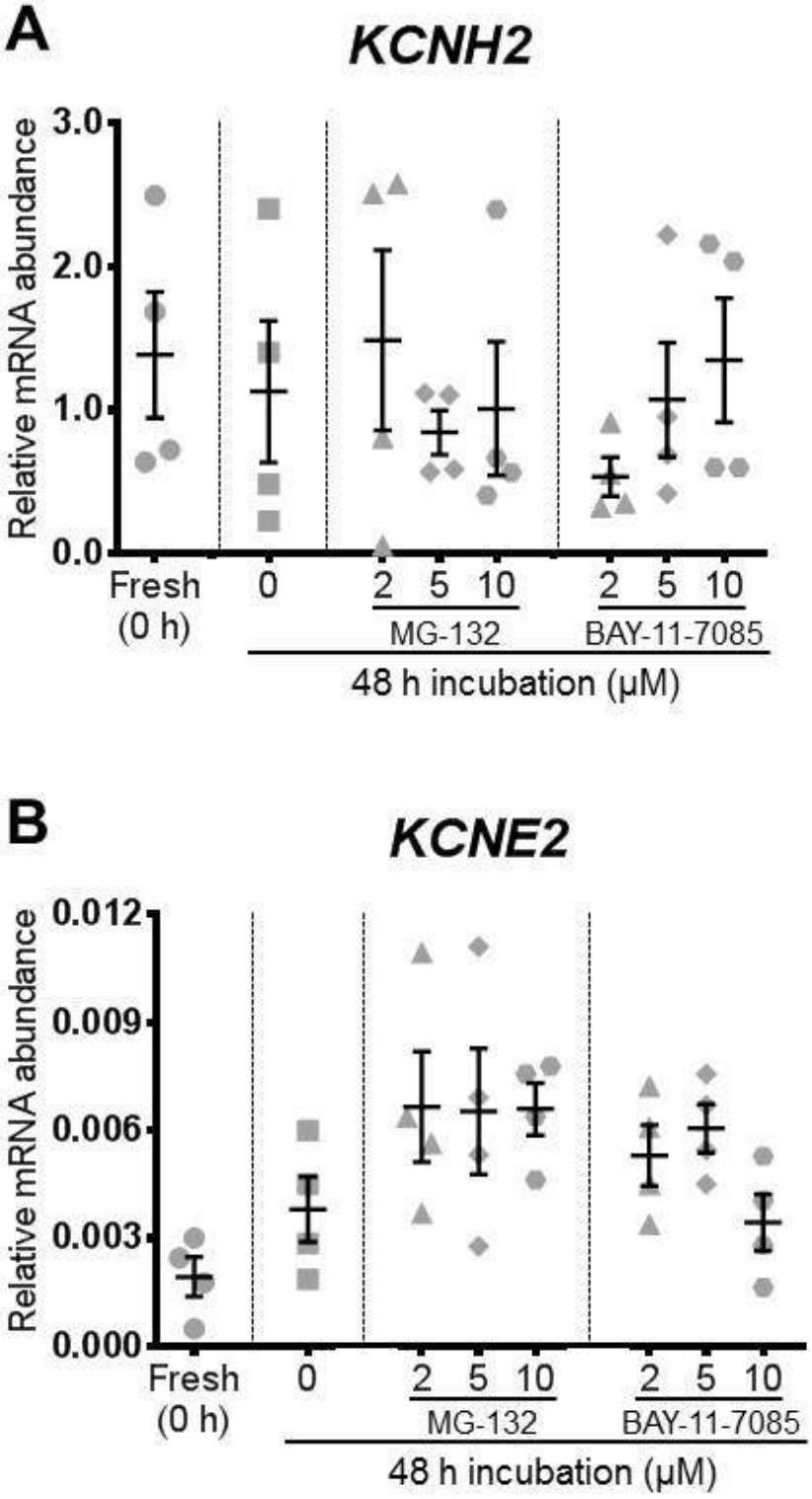

Figure 6.10. Effect of MG-132 and BAY-11-7085 on culture-induced changes in *KCNH2* and *KCNE2* mRNA abundance.

Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in term non-laboring myometrial samples (n=4) following 48 h incubation in the presence of different NF-κB inhibitors, and expressed relative to Alien reference RNA. (A) *KCNH2* mRNA abundance. (B) *KCNE2* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed by 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

6.5 Discussion

Recently our group provided evidence that hERG and KCNE2 are present in pregnant human myometrium in late gestation, and that hERG channel activity is responsible for supressing contraction amplitude and duration [235]. The implication is that by rapidly terminating contractions, hERG channel activity limits the capacity for contractions to propagate, thereby promoting uterus quiescence [234]. To complement the patch clamp analysis where myocytes were transiently exposed to hERG activators and inhibitors, this study used organ bath analyses to expose term myometrial non-laboring strips to hERG activators and inhibitors over extended period. This long term exposure may be more representative of the clinical situations where hERG modulators are administrated to pregnant women *in vivo*. The results showed that PD-118057 and NS-1643 had no effect on myometrial contraction duration *in vitro* (Figures 6.1 and 6.2). This is inconsistent with our earlier study where a hERG channel activator decreased AP and contraction duration [234]; however, it should be noted that the previous study utilised patch clamp analyses and the activator, ICA-195574, whereas this study reports the results of organ bath analyses and the activators, NS-1643 and PD-118057. Nevertheless, our results are consistent with findings published by Greenwood *et al.* [232] where ERG channel activators had no effect on the contractile activity of uterine tissue from pregnant mice. Interestingly, Greenwood *et al.* [232] found that ERG channel activators did decrease spontaneous contractility of uterine tissue from non-pregnant mice. In light of the parallels with our own study, this raises the question as to whether hERG activators would similarly decrease contractile activity of non-pregnant human myometrium in our organ bath setting.

The hERG channel inhibitors DOF and E-4031 both significantly increased contraction plateau duration in term non-laboring human myometrial tissues (Figures 6.3 and 6.4). These findings are consistent with previous studies where ERG channel blockers increased contractility in rat stomach [223], mouse portal vein [225], opossum oesophagus [226], mouse and guinea pig gall bladder [227], bovine epididymis [228] and human and equine jejunum [229, 230], as well as non-pregnant mouse myometrium [232] and term non-laboring human myometrial tissues [234]. Interestingly, while both inhibitors significantly increased the contraction duration, they produced slightly different contraction plateau profiles (Figure 6.11). DOF produced an elongated contraction where there was a sharp decline in amplitude following the peak of contraction then a relatively flat plateau. For E-4031, however, there was no sharp decline following the peak of contraction, and instead contraction amplitude decreased steadily across the entire plateau. The underlying reason the different plateau profiles is unclear, however, it suggests the compounds have slight differences in the way they affect membrane repolarization, which in turn manifests as slightly different contraction profiles.

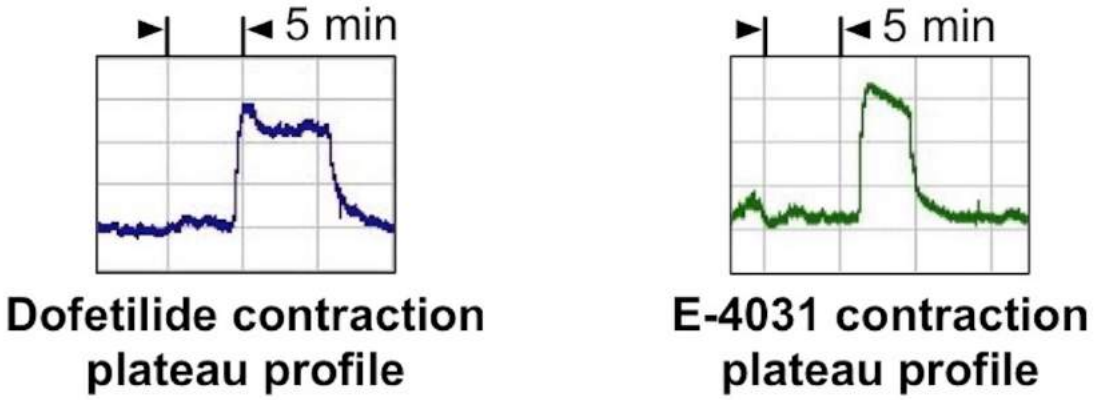

Figure 6.11. Contraction plateau profiles produced by DOF and E-4031 when applied to term non-laboring human myometrium *in vitro*.

In light of the demonstrated capacity for hERG activity to affect human myometrial contractility, investigations were warranted to determine factors that affect expression of the hERG gene, *KCNH2*, and that of the auxiliary subunit, *KCNE2*. In this regard, *in vitro* cell and tissue (explant) culture has been a valuable tool for investigating the regulation of genes involved in human parturition. However, as previously outlined, the contraction-associated genes *ESR1*, *PTGS2, OXTR and PR* all undergo changes in expression during *in vitro* culture that are consistent with transition toward a pro-contractile, laboring phenotype [325, 355]. Given that *KCNH2* and *KCNE2* expression correlate with expression of these contraction-associated genes [356], we examined whether *KCNH2* and *KCNE2* undergo similar changes in expression during *in vitro* culture. Such changes would render it difficult to examine the regulation of these genes *in vitro*.

Consistent with the other contraction-associated genes examined, *KCNE2* expression increased (8-fold) when term non-laboring myometrium was placed into culture. Up-regulation of *KCNE2* expression is consistent with transition toward a pro-labor phenotype in that KCNE2 protein is a regulatory subunit that inhibits hERG channel activity.

Similar to results previously observed for *PR-B*, *KCNH2* expression did not change when term non-laboring tissue was placed in culture (1.5-fold increase). This is particularly interesting as *KCNH2* and *PR-B* are both pro-gestation/pro-quiescence genes; progesterone acting through PR-B to suppress CAP expression, while hERG channel activity terminates APs/contractions, thereby limiting the capacity for contractions to propagate and give rise to labor.

This thesis has therefore raised the question as to why the expression of pro-labor genes changed *in vitro* whereas pro-gestation genes were unaffected. This suggests there is a fundamental deficiency in the standard culture conditions used to culture myometrium (DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/mL Gentamicin and 10 mM HEPES). Whatever this deficiency is, it promotes activation of multiple pro-labor genes, and highlights the need to develop more appropriate culture systems.

Supplementing myometrial culture media with 5% CSS, as often used during myometrial culture [257, 296, 297], was unable to prevent up-regulation of *KCNE2* expression *in vitro* (Figure 6.6). This was consistent with previous findings where 5% CSS failed to prevent changes in expression of *ESR1, PTGS2, OXTR* and *PR* [325, 355].

Interestingly, P4 alone and E2 alone both had no effect on *KCNH2* or *KCNE2* expression. In combination, however, P4 + E2 resulted in significantly reduced *KCNH2* expression after 48 h, relative to the 48 h DMSO control. A study by Chen *et al.* [357] similarly examined the effect of P4 and E2 on ERG1 expression in rat uterus. They found that P4 failed to stimulate *ERG1* mRNA expression and that E2 significantly increased *ERG1* mRNA expression, while the combination of P4 and E2 significantly decreased E2-mediated *ERG1* expression by more than 60%. These results suggested that P4 antagonizes the action of E2, and that *ERG1* expression in the uterus is regulated by a complex interplay of E2 and P4. The results obtained in this study are inconsistent with the findings of Chen *et al.* [357], however it should be noted that this study was performed on human myometrium rather than rat uterus.

Kundu *et al*. [351] found that in mice, E2 treatment stimulated *KCNE2* promoter activity in a dose-dependent manner [351], indicating that *KCNE2* was regulated by direct genomic action of E2. In these studies, culture-induced up-regulation of *KCNE2* expression was neither exacerbated nor suppressed by treatment with E2 alone. The combination of P4 + E2 did however significantly affect *KCNE2* expression, in that after 48 h culture the combination of the steroids successfully maintained *KCNE2* mRNA abundance at levels comparable to fresh tissue. These differences may be due to species variation; however, it is also important to consider that the treatments were being applied to non-laboring human myometrium. Within non-laboring pregnant human myometrium, steroids such as P4 and E2 may function to repress expression of pro-labor *KCNE2*, thereby promoting uterine quiescence. It would be interesting to determine whether P4 and E2 applied to laboring tissue has this same effect, or whether *KCNE2* expression would be up-regulated rather than repressed.

Throughout normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [266, 267]. Stretch is an important stimulus to uterine growth and previous animal studies showed that progesterone is responsible for maintaining uterine quiescence and promoting myometrial hyperplasia and hypertrophy to inhibit any increase in uterine wall tension [269, 270, 308, 309]. In the absence of progesterone, stretch was found to increase *PTGS2* and *OXTR* expression [268-270, 309]. In addition, human studies show that in a progesterone-dominated endocrine environment, moderate stretch possibly maintains relaxation and quiescence, however, in the absence of progesterone or when subjected to excessive stretch, the uterus starts to contract [266, 267].

We found that constant stretch did not influence the culture-induced changes in *KCNE2* expression, either in the presence or absence of steroids (P4 + E2). Interestingly, although stretch did not directly affect the culture-induced changes in *KCNE2* mRNA expression, the application of stretch prevented the combination of P4 + E2 from blocking the culture-induced increase in *KCNE2* mRNA expression, as well as prevented P4 + E2 from decreasing *KCNH2* mRNA expression.

There is now extensive evidence to suggest that cAMP activation of PKA pathways is involved in the human myometrium throughout pregnancy to maintain uterine quiescence until term, and that components of PKA signalling are down-regulated during labor [10, 31, 272, 310, 312, 358]. Recently, Chen *et al*. [353] found that activation of the PKA pathway increased hERG protein phosphorylation, which facilitated hERG incorporation into the plasma membrane. Furthermore, Cui *et al.* [354] found that PKA influenced the activity of *KCNE2*. Supplementing media with a cAMP analogue was therefore examined as a potential means to prevent culture-induced changes in *KCNE2* expression. Although cAMP has a well-defined role in promoting myometrial relaxation, we found that supplementing media with 8-Br-cAMP had no effect on *KCNH2* mRNA abundance. Additionally, 8-Br-cAMP failed to prevent culture-induced changes in *KCNE2* mRNA abundance (Figure 6.9).

In parallel to cAMP treatment, we also examined effects of supplementing media with a pro-contractile agent, the purpose of which was to determine whether *KCNH2* and *KCNE2* expression would be further driven toward the laboring phenotype. The phorbol ester, PMA, activates PKC, which

in turn stimulates signalling pathways mediated by ERK and NF-κB, both which are involved in labor [273, 274]. Furthermore, PKC activity is crucial during oxytocin-stimulated myometrial contractions [275]. Our results showed that PMA had no effect on *KCNH2* mRNA expression; however, PMA dose-dependently suppressed the increase in *KCNE2* mRNA expression (Figure 6.9). This was unexpected as PMA previously further up-regulated *PTGS2* expression as well as increased the *PR-A/PR-B* expression ratio *in vitro*. These effects are consistent with PMA driving the tissue toward a pro-contractile, laboring phenotype, whereas PMA-mediated suppression of *KCNE2* expression is pro-quiescence. Given that the tissue examined was non-laboring, down-regulation of *KCNE2* in response to pro-labor signals may be a mechanism that operates to attenuate the effects of any pro-labor signals present during gestation. Studies examining the effect of PMA on *KCNE2* expression in laboring human myometrium would therefore be very interesting.

There is increasing evidence that locally produced immune/inflammatory cytokines, particularly PGs, are involved in normal term labor as well as infection-associated preterm labor [169-171]. This is based on the evidence that in other species PGs initiate parturition by inducing progesterone withdrawal [169-171]. In rodents, the uterine tissues increase the production of $PGF_{2\alpha}$ during late gestation, which causes luteolysis and induces systemic progesterone withdrawal. In human pregnancy, administration of PGs or PG analogues at any stage of pregnancy transforms the myometrium and cervix and induces labor [171, 177-180]. In recent years, studies using human tissues and myometrium-derived cell lines have demonstrated that the binding of oxytocin to its receptor led to activation of NF-κB, which subsequently increased

the production of PGs [164, 165]. Supplementing media with NF-κB inhibitors therefore represented a feasible means of preventing non-laboring tissue transitioning toward a laboring phenotype. Despite the above, supplementing media with MG-132 or BAY-11-7085 had no effect on *KCNH2* or *KCNE2* mRNA abundance (Figure 6.10).

Conclusion

The hERG channel and its regulatory subunit, KCNE2, are recently identified additions to the list of contraction-associated proteins expressed within pregnant human myometrium. Studies investigating factors that affect the expression of their encoding genes were therefore warranted. Similar to results from our previous study, expression of pro-contractile *KCNE2* increased when non-laboring tissue was placed in culture, whereas expression of pro-quiescence *KCNH2* did not change. Upon examining culture conditions that may affect *KCNH2* and *KCNE2* expression, culture-induced increase of *KCNE2* expression was prevented by supplementing media with steroids (P4 + E2); however the combination of steroids also affected *KCNH2* expression. PMA, however, was effective in preventing culture-induced increase in *KCNE2* expression without affecting *KCNH2* expression. Future studies should consider whether supplementing culture media with PMA may be appropriate when investigating KCNH2 and KCNE2 expression in non-laboring human myometrium. In summary, this study demonstrated that:

i) hERG channel activators had no effect on term non-laboring myometrial tissues;

ii) hERG channel inhibitors significantly increased the contraction plateau duration in term non-laboring myometrial tissues;

iii) Myometrial tissues undergo culture-induced up-regulation of *KCNE2* mRNA expression *in vitro,* whereas *KCNH2* expression is unchanged.

iv) The combination of progesterone and estrogen successfully prevented culture-induced up-regulation of *KCNE2* expression, and significantly repressed *KCNH2* expression relative to the 48 h vehicle culture tissue;

v) Stretch had no direct effect on *KCNH2* or *KCNE2* expression, however, stretch blocked the effects of progesterone and estrogen on *KCNH2* and *KCNE2* expression;

vi) cAMP was unable to control culture-induced changes in *KCNE2* expression;

vii) PMA prevented culture-induced up-regulation of *KCNE2* mRNA expression;

viii) NF-κB inhibitors were unable to control culture-induced changes in *KCNE2* expression.

6.6 Acknowledgements

The authors wish to thank the obstetricians from the John Hunter Hospital, NSW, our research midwife, Anne Wright, and the research participants who donated samples toward this study.

Chapter Seven: Drug Delivery to the Human and Mouse Uterus using Immunoliposomes Targeted to the Oxytocin Receptor

Jonathan W. PAUL*, Susan HUA*, **Marina ILICIC**, Jorge M. Tolosa, Trent BUTLER, Sarah ROBERTSON and Roger SMITH

* *These authors contributed to the work equally*

This Chapter contains a manuscript published in American Journal of Obstetrics & Gynecology. The original manuscript is included in appendix H.

This manuscript demonstrates that nanoliposomes targeted to the oxytocin receptor can used to deliver therapeutic agents to human and mouse uterine tissue in order to modulate uterine contractions *in vitro*. The manuscript further demonstrates that nanoliposomes targeted to the oxytocin receptor localise to the uterine tissue upon injection into live pregnant mice.

In addition, this manuscript demonstrate the potential to use this novel drug delivery system to achieve targeted delivery of tocolytic and uterotonic agents to the myometrium, and discuss the clinical significance of oxytocin receptor-targeted nanolipsomes in relation to obstetric complications such as preterm labor and PPH.

The format of the manuscript has been altered for the purposes of this thesis.

Author	Contribution
Jonathan W. Paul	Conceived and designed experiments Animal studies Sample collections Contraction bioassay Data analyses Provided reagents and materials Manuscript writing
Susan Hua	Conceived and designed experiments Liposome manufacture Provided reagents and materials Manuscript editing
Marina Ilicic **(2nd Author)**	**Animal studies** **Sample collections** **Contraction bioassay** **Data analyses** **Manuscript editing**
Jorge M. Tolosa	Animal studies Manuscript editing
Trent Butler	Sample collections Contraction bioassay Manuscript editing
Sarah Robertson	Provided advice on animal studies
Roger Smith	Conceived and designed experiments Provided reagents and materials Manuscript editing

Certification

I, Dr. Jonathan W. Paul, do hereby certify that the PhD candidate, Marina Ilicic, made a significant contribution to the published manuscript titled, ‘Drug Delivery to the Human and Mouse Uterus using Immunoliposomes Targeted to the Oxytocin Receptor’ during the course of her PhD studies.

Marina’s contributions included performing sample collections, contraction bioassays, designing and assisting with animal studies, as well as performing data analyses and contributing to the writing of the final manuscript.

Signed

Jonathan W. Paul

7.1 Abstract

Background: The ability to provide safe and effective pharmacotherapy during obstetric complications, such as preterm labor or PPH, is hampered by the systemic toxicity of therapeutic agents leading to adverse side effects in mother and fetus. Development of novel strategies to target tocolytic and uterotonic agents specifically to uterine myocytes would improve therapeutic efficacy while minimizing the risk of side effects. Ligand-targeted liposomes have emerged as a reliable and versatile platform for targeted drug delivery to specific cell types, tissues or organs.

Objective: Our objective was to develop a targeted drug delivery system for the uterus utilizing an immunoliposome platform targeting the OTR.

Study Design: We conjugated liposomes to an antibody that recognizes an extracellular domain of the OTR. We then examined the ability of OTR-targeted liposomes to deliver contraction blocking (NIF, SAL and ROL) or contraction enhancing (DOF) agents to strips of spontaneously contracting myometrial tissue *in vitro* (human and mouse). We evaluated the ability of OTR-targeted liposomes to localize to uterine tissue *in vivo*, and assessed if targeted liposomes loaded with IND were capable of preventing lipopolysaccharide-induced preterm birth in mice.

Results: OTR-targeted liposomes loaded with NIF, SAL or ROL consistently abolished human myometrial contractions *in vitro*, while oxytocin receptor-targeted liposomes loaded with DOF increased contraction duration. Non-targeted control liposomes loaded with these agents had no effect. Similar results were observed in mouse uterine strips. Following *in vivo* administration

to pregnant mice, OTR-targeted liposomes localized specifically to the uterine horns and mammary tissue. Targeting increased localization to the uterus 7-fold. Localization was not detected in the maternal brain or fetus. Targeted and non-targeted liposomes also localized to the liver. OTR-targeted liposomes loaded with IND were effective in reducing rates of preterm birth in mice, whereas non-targeted liposomes loaded with IND had no effect.

Conclusion: Our results demonstrate that OTR-targeted liposomes can be used to either inhibit or enhance human uterine contractions *in vitro*. *In vivo*, the liposomes localize to the uterine tissue of pregnant mice and were effective in delivering agents for the prevention of inflammation induced preterm labor. The potential clinical advantage of targeted liposomal drug delivery to the myometrium is reduced dose and reduced toxicity to both mother and fetus.

7.2 Introduction

Complications arising from PTB are the leading cause of death among children under 5 years of age, accounting for nearly 1 million deaths in 2013 [359], while PPH is the leading cause of maternal mortality worldwide, accounting for up to 27.1% of maternal deaths [360]. Given that both can arise from dysregulation of uterine contractility, the need exists for safe and effective clinical interventions capable of modifying myometrial contractions to improve treatment of women in preterm labor, to induce or facilitate labor and to prevent or treat PPH, without adverse off-target effects on either the mother or fetus.

When a woman presents with preterm labor attempts are often made to halt contractions by administering tocolytics that inhibit or block components of

the contraction cascade. A recent study proposed that “The ideal tocolytic agent should be myometrium-specific, easy to administer, inexpensive, effective in preventing PTB and improve neonatal outcomes, with few maternal, fetal, and neonatal side effects, and without long-term adverse effects” [361]. Standard therapy varies from country to country, but tocolysis may involve the administration of calcium channel blockers, such as NIF, or β_2-adrenergic receptor agonists, such as SAL, an oxytocin receptor antagonist, such as atosiban, or a PG synthetase inhibitor, such as IND [362-367]. Unfortunately, the systemic administration of these therapies and lack of specificity means that large doses need to be administered in order to achieve a therapeutic effect at the target tissue, the myometrium. Maternal side effects of β_2-adrenergic receptor agonists include tremors, heart palpitations and tachycardia, as well as myocardial ischemia and pulmonary oedema [366, 368-370]. NIF has been associated with fewer side effects, however approximately 1% of women experience a severe side effect and a further 1% experience mild adverse side effects [371]. Atosiban is associated with the lowest side effect risk but the efficacy of this agent is disputed [372, 373]. Usefulness of IND is limited by fetal side effects, such as premature closure of the ductus arteriosus [374-376]. Achieving targeted drug delivery to the myometrium would reduce the quantity of drug required to achieve therapeutic efficacy, reduce the likelihood of maternal and fetal side effects, and would therefore represent a significant advancement for maternal-fetal medicine [370, 377-379].

Targeted liposomes have emerged as a platform for achieving the delivery of drugs to specific tissues. Liposomes are artificial vesicles that range in size from 50 – 1000 nm, and are comprised of one or more phospholipid

bilayers [380, 381]. Liposomes are able to encapsulate both lipophilic and/or hydrophilic drugs, and are non-toxic and biodegradable with minimal immunogenicity [379, 382, 383]. Liposomal encapsulation improves the pharmacokinetics of drugs, particularly if the liposome surface is PEGylated, which reduces uptake by the reticuloendothelial system and prolongs half-life [384]. This has led to the development of liposomal-based preparations of various agents, including doxorubicin, amphotericin B, daunorubicin, and verteporfin [385]. Ligand-targeted liposomes offer the potential for site-specific delivery of drugs to designated cell types or organs *in vivo* that selectively express specific cell surface cognate receptors [384]. Although many types of targeting molecules are available, such as peptides/proteins and carbohydrates, the coupling of antibodies to the liposome surface to create immunoliposomes has many advantages. One advantage of using antibodies is their stability and higher binding avidity because of the presence of dual binding sites [384]. For example, liposomes coated with antibodies to intercellular adhesion molecule-1 (ICAM-1) have been developed for the treatment inflammatory diseases [242]. Administration of ICAM-1-targeted immunoliposomes loaded with an analgesic agent demonstrated specific localization and therapeutic efficacy exclusively in peripheral inflammatory tissue. All control groups (free drug solution, empty non-targeted liposomes, drug-loaded non-targeted liposomes, and empty ICAM-1-targeted immunoliposomes) showed no significant therapeutic response [386].

Aims

The aim of this study was to develop a means of targeting therapeutic agents to uterine myometrial tissue, in order to allow therapeutic modification of

myometrial contractions in obstetric settings, such as preterm labor, labor induction and PPH. The expression of the OTR is significantly up-regulated in myometrial cells approaching term [128, 346]. Here we report the development of OTR-targeted PEGylated immunoliposomes loaded with traditional tocolytics, such as NIF and SAL, as well as ROL, a PDE4 inhibitor and potent inhibitor of myometrial contractions [387-389]. Moreover, we report enhancement of human myometrial contraction duration *in vitro* through liposomal delivery of DOF, a hERG channel blocker that increases myometrial contraction duration [390, 391], demonstrating that this delivery platform can be used to either inhibit or enhance contractions in human myometrial tissue. We demonstrate that intravenously administered OTR-targeted liposomes localize specifically to the uterine tissue of pregnant mice *in vivo*. Finally, using an inflammatory mouse model of preterm birth (LPS administration), we show that OTR-targeted liposomes loaded with IND are effective in preventing preterm birth, while IND loaded non-targeted liposomes have no effect.

7.3 Materials and Methods

Myometrial Tissue Acquisition

Human Studies: These studies were performed in Newcastle, New South Wales, Australia and were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent. Collection of myometrial samples (5 × 5 × 10 mm) occurred from the lower uterine segment of term singleton pregnancies. All women were examined clinically, and those with signs of

infection were excluded. Women were undergoing term elective cesarean delivery and were NIL; the clinical indications for elective NIL cesarean delivery were previous CS or previous $3^{rd}/4^{th}$ degree tear. All participants ranged from 37 – 40 completed weeks of gestation. Following delivery of the placenta, all women immediately received 5 units of oxytocin (syntocinon) into an intravenous line, which was administered as standard care. Myometrial biopsies were excised 3 – 5 min after oxytocin administration, thus all samples were briefly exposed to oxytocin. After biopsy, myometrial samples were dissected from connective tissue and washed in ice-cold physiological saline.

Mouse *In Vitro* Studies: Mouse uterine horns were dissected from pregnant CD1 Swiss mice (8 – 10 weeks of age) at term gestation prior to the onset of labor (fetal gestation day 18). Mouse studies were approved by the University of Newcastle Animal Ethics Committee (A-2014-400 / A-2014-429). All mice were housed under SPF/PC2 conditions under a 12 h light day cycle and had food and water available *ad libitum.*

Liposome Manufacture

Liposomes containing NIF, SAL, ROL, DOF (each at approximately 4 mg/mL) or IND (approximately 5.5 mg/mL), as determined by HPLC, were manufactured as previously outlined [242]. Liposomes were composed of DSPC and cholesterol in a molar ratio of 2:1, and contained 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG(2000) maleimide) at 1.5 mol percent of DSPC as a coupling lipid (Avanti Polar Lipids). The resulting multilamellar dispersions were reduced in size and lamellarity to approximately 200 nm in diameter by high-pressure extrusion. The

activated liposome suspension was then mixed with thiolated polyclonal anti-OTR antibody (Abcam, Cat# ab115664), which was prepared by first conjugating 25 µg of OTR antibody with a heterobifunctional reagent SPDP (Figure 7.1). The OTR antibody recognizes an extracellular domain of the human OTR. Non-targeted liposomes were coated with rabbit IgG. All liposomes incorporated the membrane stain DiI for fluorescent detection. Unconjugated antibody and non-encapsulated drug was removed by centrifugal filtration of the liposomes through a 100 kDa molecular weight filter (Amicon Ultra-15). Amicon Ultra-15 filters were washed with Milli-Q H_2O before 500 µL of liposome suspension was loaded into the filter reservoir. Liposomes were diluted with 5 mL of sterile 0.9% saline and centrifuged at 4000 x g until retentate volume was <250 µL. Liposomes were then resuspended in a futher 5 mL of 0.9% saline and centrifuged until retentate volume was <250 µL. Filtered liposomes were then collected, transferred to a fresh Eppendorf tube and redispersed to an original volume of 500 µL.

The size distribution of the liposomal dispersion was determined by dynamic laser light scattering (Zetasizer Nano S^{TM}, ATA Scientific). EE% was determined by disrupting the vesicles with ethanol and evaluating drug concentration using HPLC. Quantification of the amount of antibody associated with liposomes was determined using the CBQCA protein assay (ThermoFisher Scientific Inc. Watham, MA, USA), using bovine serum albumin for the preparation of the standard curve.

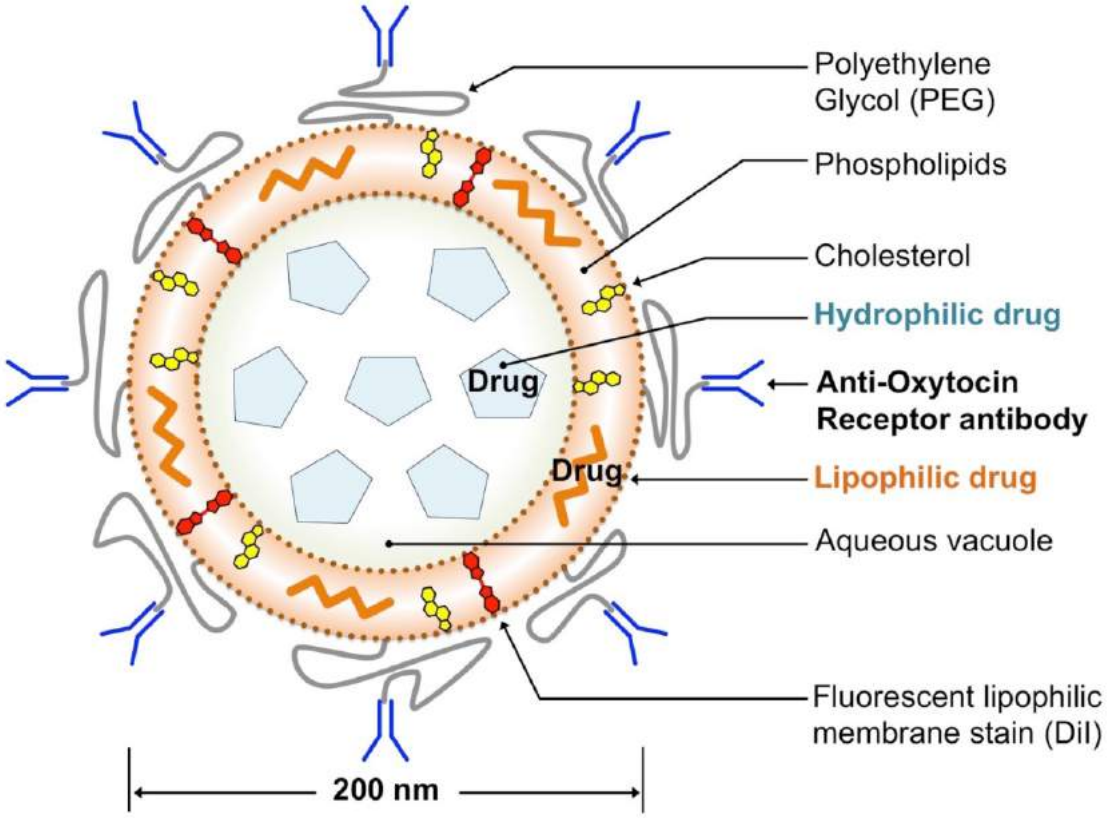

Figure 7.1. Schematic of OTR-targeted liposome structure

Myometrial Contractility Studies

Myometrial strips were set up as previously described [110]. Briefly, non-laboring human myometrial samples, or uterine horns obtained from pregnant CD1 Swiss mice, were dissected into strips (10 × 2 × 2 mm) and suspended in organ baths containing 30 mL PSS containing (in mM) NaCl 120, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11, and continuously gassed with carbogen (95% O_2, 5% CO_2), at pH 7.4. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1 g passive tension applied (1 g was calibrated to equal 1 V). PSS was replaced five times during the first hour, with strips re-tensioned to 1 g passive tension following each wash. Thereafter strips were maintained at 37°C until spontaneous rhythmic contractions developed. Data were digitized using a

MacLab/8E data-acquisition system and contraction status visualized in real time using Chart software (ADInstruments, NZ). For each strip a contraction baseline was acquired to serve as reference [243].

To administer liposome treatments, 600 µL of PSS buffer was carefully extracted from an organ bath and tranferred to an Eppendorf tube. The appropriate volume of liposome preparation (mixed by inversion) was pipetted into the PSS to pre-dilute the liposomes. The total volume of pre-diluted liposomes (600 µL PSS + liposomes) was then carefully reinjected back into the appropriate organ bath. Final concentrations of each drug were; NIF 7.7 µM, SAL 9.25 µM, ROL 19.4 µM and DOF 3.0 µM. Doses were based on prior investigations of the non-encapsulated drug (*in vitro* contraction assays using human myometrium). Where treated tissue was not washed, tissue strips remained in the presence of the liposomes for the duration of the assay. Where washout studies were performed, organ baths were twice drained of buffer and refilled with 37°C PSS. Human tissue strips were washed after 1 h 25 min whereas mouse tissue strips were washed after 15 min.

Tension generated by tissue strips is indicated in the results and representative contraction traces. The effect of treatments is interpreted relative to the pre-treatment contraction baseline, which consisted of 3 or more contractions of consistent frequency and amplitude.

In Vivo Biodistribution Study

Timed-mated CD1 Swiss pregnant mice were injected with drug-free, DiI-labelled preparations of either non-targeted or OTR-targeted liposomes on fetal gestation days 17 and 18 at 4:00pm. Mice that labored overnight were

euthanized on the morning of day 19 (9:00 – 11:00am) by CO_2 asphyxiation. Maternal internal organs of interest (heart, brain, liver, lung, kidney, uterus and mammary tissue) were harvested and transferred to a Petri dish along with a sacrificed neonate. The Petri dish was loaded into an *In Vivo* Imaging System (IVIS-100) (Xenogen, CA, USA) and a light image captured. Tissues were then imaged under conditions appropriate for the detection of DiI (Excitation: 554 nm; Emission: 583 nm; Filter: DsRed; Exposure: 4 sec; Field of view: 10; Binning: 4). Organs were imaged 17 – 19 h after the second injection, following labor. Background signal was subtracted from the detected signal to produce the final fluorescence image. Fluorescence signal is reported as radiance (p/sec/cm^2/sr). The radiance range was kept constant across all images (min = 2.0×10^8: max = 1.8×10^9).

Preterm Birth Study

Time-mated pregnant CD1 Swiss mice were administered 0.7 μg/g LPS from *E. coli* (0111:B4) (Sigma-Aldrich) via IP injection at 12:00pm on gestation day 15 (GA15) (one-time injection). LPS dose had been previously optimized to result in PTB rates of 50 – 70%. Total IP injection volume was 150 μL in saline. At 4:00pm on GA15, mice began receiving daily IV injections of IND free-drug or liposomal preparations according to assigned treatment groups. Treatment groups are indicated in Table 7.1. Total IV injection volume was 150 μL. Mice were monitored for onset of labor every 6 h. Treatments were repeated daily at 4:00pm until all mice labored. Term gestation was 19 – 22 days. Mice that labored within 48 h of receiving LPS (GA17) were deemed to have labored preterm.

Table 7.1. Treatment groups for in preterm labor study

Group (n)	One-time IP Injection (12:00pm on GA15, 150 µL)	Daily IV injections (4:00pm, GA15 onwards, 150 µL)
1	saline	saline
2	0.7 µg/g LPS	50% DMSO
3	0.7 µg/g LPS	1.0 mg/kg/day IND in 50% DMSO
4	0.7 µg/g LPS	2.0 mg/kg/day IND in 50% DMSO
5	0.7 µg/g LPS	OTR-targeted, drug-free liposomes in saline
6	0.7 µg/g LPS	2.0 mg/kg/day IND via non-targeted liposomes in saline
7	0.7 µg/g LPS	2.0 mg/kg/day IND via OTR-targeted liposomes in saline

LPS, lipopolysaccharide; *OTR*, oxytocin receptor; *IP*, intraperitoneal; *IV*, intravenous; *GA15*, pregnancy day 15

Statistical Analyses

For contraction traces, LabChart software was used to determine the AUC (g tension × sec) for the 30 min prior to treatment (pre-treatment) and 30 min after treatment (post-treatment) (ADInstruments, NZ). AUC before and after treatment was compared by two-tailed paired t-test (GraphPad Prism).

For DOF studies, contraction plateau duration (sec) was determined for four contractions pre- and post-treatment using LabChart software (ADInstruments, NZ). Plateau duration was determined as the time between the point of highest amplitude and point where contraction force declined sharply. Contraction duration data were obtained for 3 individual tissues (n=3 women). Pre- and post-treatment measurements (n=12 each) were compared by two-tailed unpaired t-test.

Average radiance (p/sec/cm^2/sr) was determined for each organ of interest using Living Image software (v2.5). Where fluorescence was detected, ROIs were applied automatically (contour). Where detection was low or absent, ROIs were specified manually (circles or squares) to tightly encompass the tissue being analysed. Data were tested for normality by the Shapiro-Wilk normality test (GraphPad Prism). Average radiance for each organ/tissue was compared between treatment groups (n=4 animals per group) by 1-way ANOVA with multiple comparisons (Holm-Sidak) (GraphPad Prism).

For the preterm labor studies, rate of PTB was compared between treatment groups by Chi-squared analysis. Time (h) between LPS injection and labor was calculated. Data were transformed ($Y=Y^2$) to obtain normal distribution (D'Agostino & Pearson normality test) and analysed by 1-way ANOVA with multiple comparisons (Tukey). Data recorded for number of pups for term deliveries was normally distributed (Shapiro-Wilk normality test) and analysed by 1-way ANOVA with multiple comparisons (Tukey). Preterm deliveries did not yield any viable pups.

Consumables and Reagents

Mice were supplied by the University of Newcastle Animal Support Unit (ASU). NIF (cat#1075), salbutamol hemisulfate (cat#0634), ROL (cat#0905) and DOF (cat#3757) were purchased from Tocris (Bristol, UK). IND (cat#L2630) was purchased from Sigma-Aldrich Pty. Ltd (Sydney, Australia). Anti-OTR antibody (ab115664) was purchased from Abcam (Cambridge, MA, USA). Other miscellaneous reagents were purchased from Sigma-Aldrich Pty. Ltd and ThermoFisher Scientific Inc. (Watham, MA, USA).

7.4 Results

Characteristics of the Liposomal Delivery System

OTR-targeted PEGylated immunoliposomes had a mean particle size of 197 ± 6.8 nm with a polydispersity index of 0.243 ± 0.043 (Mean ± S.D.; n=3). The size and polydispersity of the control liposome formulations were similar. Encapsulation of therapeutic agents into the liposomes did not significantly affect the size or polydispersity index. Mean antibody coupling ratio for the OTR-targeted liposomes was 1.86 ± 0.17 µg of antibody per µmol of phospholipid. With a starting antibody concentration of 25 µg and a phospholipid concentration of 2.03×10^{-5} mol this equates to a conjugation efficiency of >99%. The liposomes have a neutral net charge and a drug loading efficiency of >95%, which equates to ~4 mg/mL of drug encapsulated per mL of liposome suspension composed of 16 mg DSPC and 4 mg cholesterol (molar ratio 2:1). *In vitro* dialysis studies have demonstrated highly stable vesicles upon dilution in an aqueous phase (PBS pH 7.4) and in serum (50% FCS) at 37^{o}C (data not included).

Human Myometrial Contractility

Contraction bioassays were performed to assess whether targeted liposomes were capable of delivering encapsulated therapeutic agents to modulate spontaneous human uterine contractions *in vitro*. Treating the uterine strips with OTR-targeted liposomes that contained no therapeutic agent (n=3) (Figure 7.2Ai) had no effect on myometrial contractility in that AUC was not affected (p=0.08; pre-treatment = 1790.0 ± 19.5; post-treatment = 1704.0 ± 38.8 g.sec) (Figure 7.2Aii). For each therapeutic agent examined *in vitro*, we

prepared non-targeted (IgG-coated) and OTR-targeted (anti-OTR-coated) liposomes (at 4 mg/mL). Administering 7.7 μM NIF to human uterine strips via non-targeted NIF-loaded liposomes (Figure 7.2Bi) (n=5) had no effect on contractility in that AUC the curve was not affected (p=0.4136; pre-treatment = 1701.4 ± 55.9; post-treatment = 1643.8 ± 19.6 g.sec) (Figure 7.2Bii). Administering 7.7 μM NIF via OTR-targeted NIF-loaded liposomes (Figure 7.2Biii) (n=4) resulted in abolition of myometrial contractions and a significant reduction in AUC (p=0.0277; pre-treatment = 1767.8 ± 15.5; post-treatment = 1137.5 ± 24.8 g.sec) (Figure 7.2Biv).

Administering 9.25 μM SAL to spontaneously contracting human uterine strips via non-targeted SAL-loaded liposomes (Figure 7.2Ci) (n=3) had no effect on contractility with AUC being unaffected by the treatment (p=0.2022; pre-treatment = 1775.3 ± 24.8; post-treatment = 1749.3 ± 16.4 g.sec) (Figure 7.2Cii). Administering 9.25 μM SAL via OTR-targeted SAL-loaded liposomes (Figure 7.2Ciii) (n=3) resulted in complete abolition of contractions and significant reduction in AUC (p=0.0293; pre-treatment = 1749.7 ± 27.3; post-treatment = 1292.0 ± 77.1 g.sec) (Figure 7.2Civ). Similar results were observed when ROL was encapsulated in non-targeted and OTR-targeted liposomes (Figure 7.4).

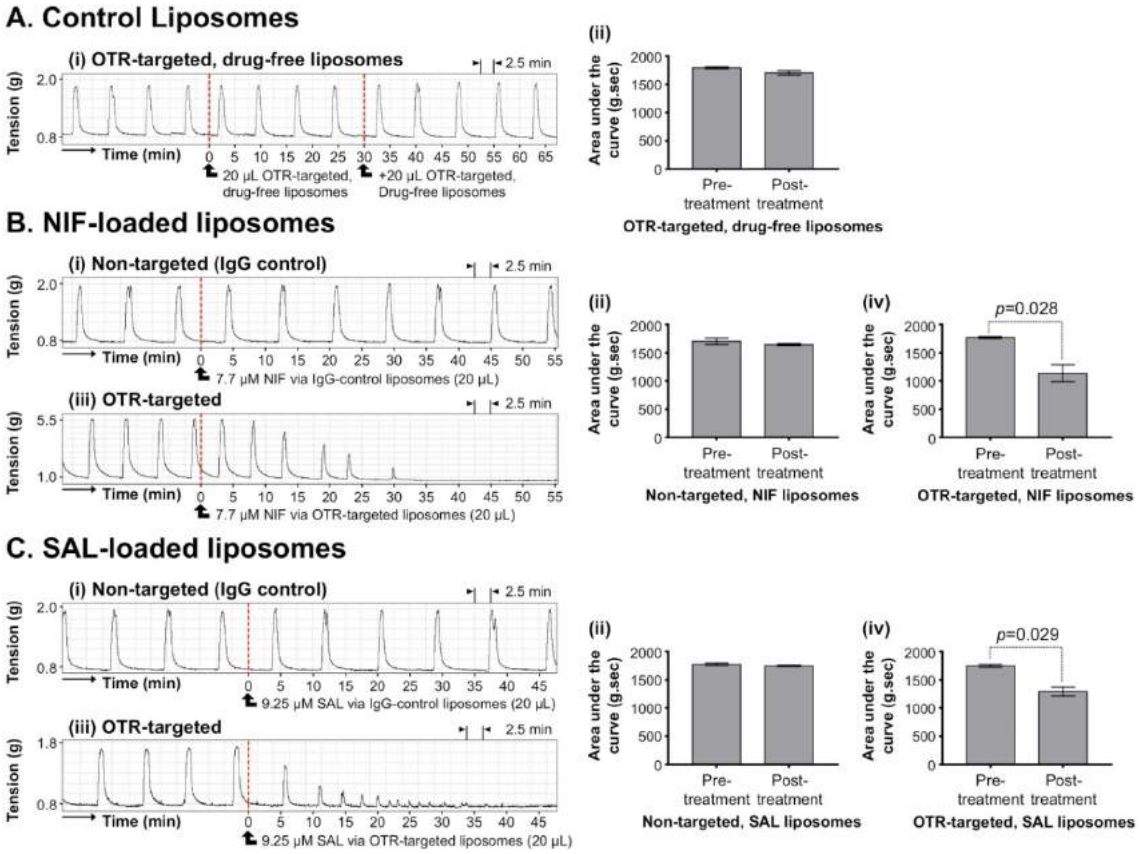

Figure 7.2. Use of targeted liposomes to inhibit human uterine contractility *in vitro*.
Data are contraction traces for strips of human myometrial tissue and corresponding AUC analyses. (A) Effect of OTR-targeted, drug-free control liposomes on myometrial contractions *in vitro* (n=3). (B) Effect of non-targeted (n=5) and OTR-targeted (n=4) NIF-loaded liposomes on myometrial contractions *in vitro*. (C) Effect of non-targeted (n=3) and OTR-targeted (n=3) SAL-loaded liposomes on myometrial contractions *in vitro*. Average AUC analyses covers 30 min immediately prior to and 30 min after treatment with NIF- or SAL-loaded liposomes (pre- and post-treatment, respectively). *AUC analyses are paired t-tests*.

To demonstrate that our OTR-targeted liposomes are capable of functioning as a drug delivery system for different obstetric applications, such as treating PPH, liposomes were prepared that contained the hERG channel blocker, DOF. When administered to human myometrial tissue, DOF increases the contraction duration and reduces contraction frequency by delaying repolarization of the myocyte membrane [391]. Administering 3.0 µM DOF to spontaneously contracting tissue strips (n=3) via non-targeted DOF-loaded liposomes (Figure 7.3Ai) had no significant effect on contraction plateau

duration (p=0.083; pre-treatment = 27.0 ± 0.8; post-treatment = 39.0 ± 3.7 sec) (Figure 7.3Bi). When administered via OTR-targeted DOF-loaded liposomes (Figure 7.3Aii), 3.0 μM DOF significantly increased contraction plateau duration (p=0.0001; pre-treatment = 66.4.0 ± 9.8; post-treatment = 162.4 ± 35.4 sec) (Figure 7.3Bii). Increased contraction plateau duration is consistent with our previous report of DOF action on human myometrium [391]. These results demonstrate that a single delivery system, OTR-targeted liposomes, can be utilized to deliver either contraction blocking or contraction promoting therapeutics to uterine myocytes.

The effect of non-targeted and OTR-targeted DOF-loaded liposomes on the AUC was analysed. Neither treatment significantly affected AUC (data not shown).

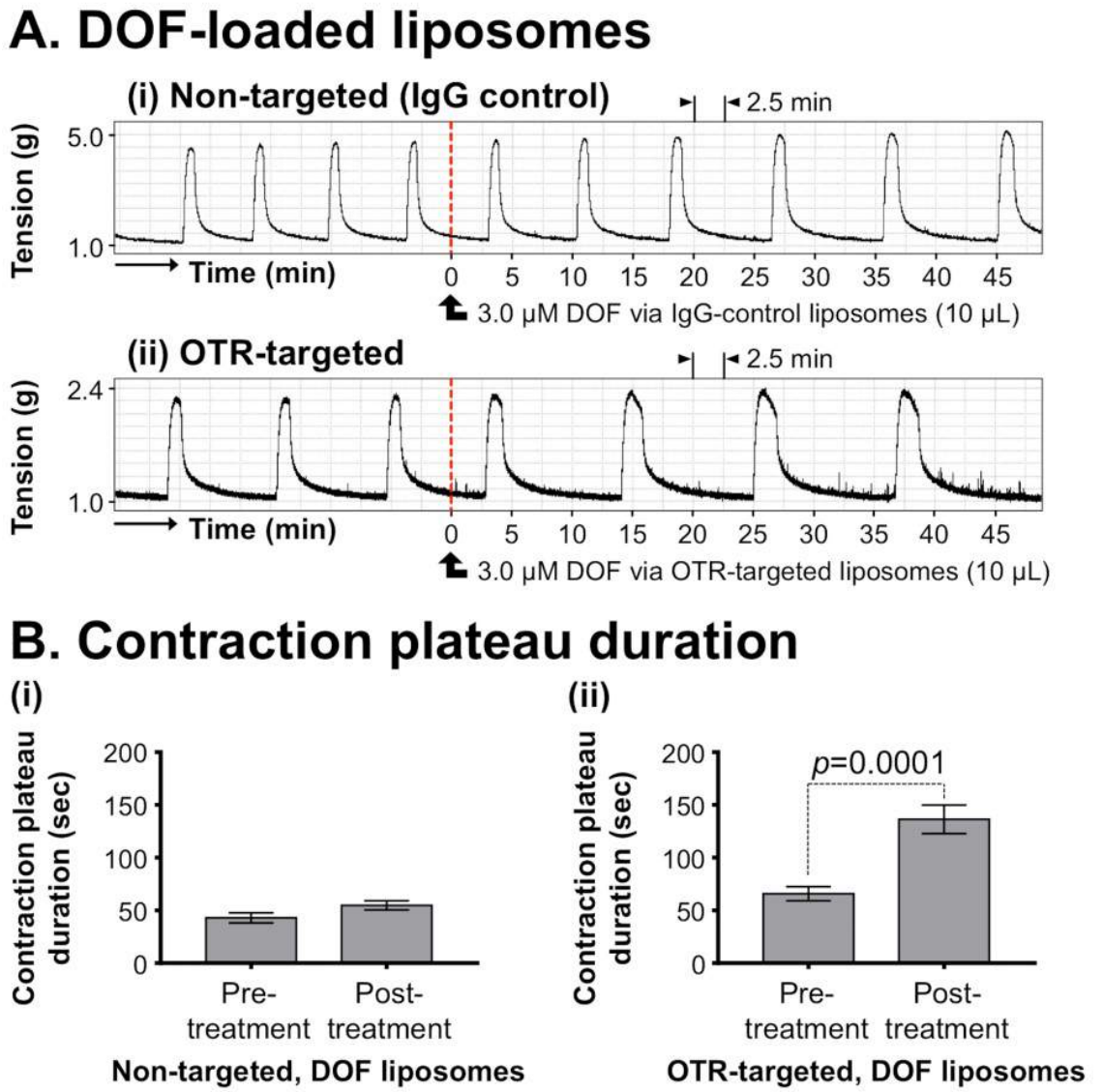

Figure 7.3. Use of targeted liposomes to enhance human myometrial contractions *in vitro*.
Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of 3.0 µM DOF administered via non-targeted (n=3) or OTR-targeted (n=3) DOF-loaded liposomes on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with non-targeted or OTR-targeted DOF-loaded liposomes (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

Effect of Targeted Liposomes is Reversible

To demonstrate that the effects on contractility were due to pharmacological actions of the drugs and not the result of toxic effects of OTR-targeted liposomes, washout experiments were performed. ROL is a reversible

inhibitor of PDE4 that induces myometrial relaxation [392, 393]. Administering 19.4 μM ROL to contracting strips via non-targeted ROL-loaded liposomes (Figure 7.4Ai) (n=3) had no effect. When 19.4 μM ROL was administered via OTR-targeted ROL-loaded liposomes (Figure 7.4Aii) (n=3) contractions were abolished. Analyses of contraction data indicated no reduction in AUC following treatment with non-targeted ROL-loaded liposomes (p=0.061; pre-treatment = 1657.0 ± 21.0; post-treatment = 1611.7 ± 14.9 g.sec) (Figure 7.4Bi), whereas AUC was significantly reduced following treatment with 19.4 μM ROL administered via OTR-targeted ROL-loaded liposomes (p=0.0023; pre-treatment = 1648.1 ± 14.3; post-treatment = 1155.7 ± 36.3 g.sec) (Figure 7.4Bii). Once contractions had been inhibited for 1 h 25 min, tissue strips were washed twice in PSS and monitored. Spontaneous, rhythmic contractions resumed in myometrial strips previously treated with OTR-targeted ROL-loaded liposomes (Figure 6.4Aii), indicating that the tissue remained viable.

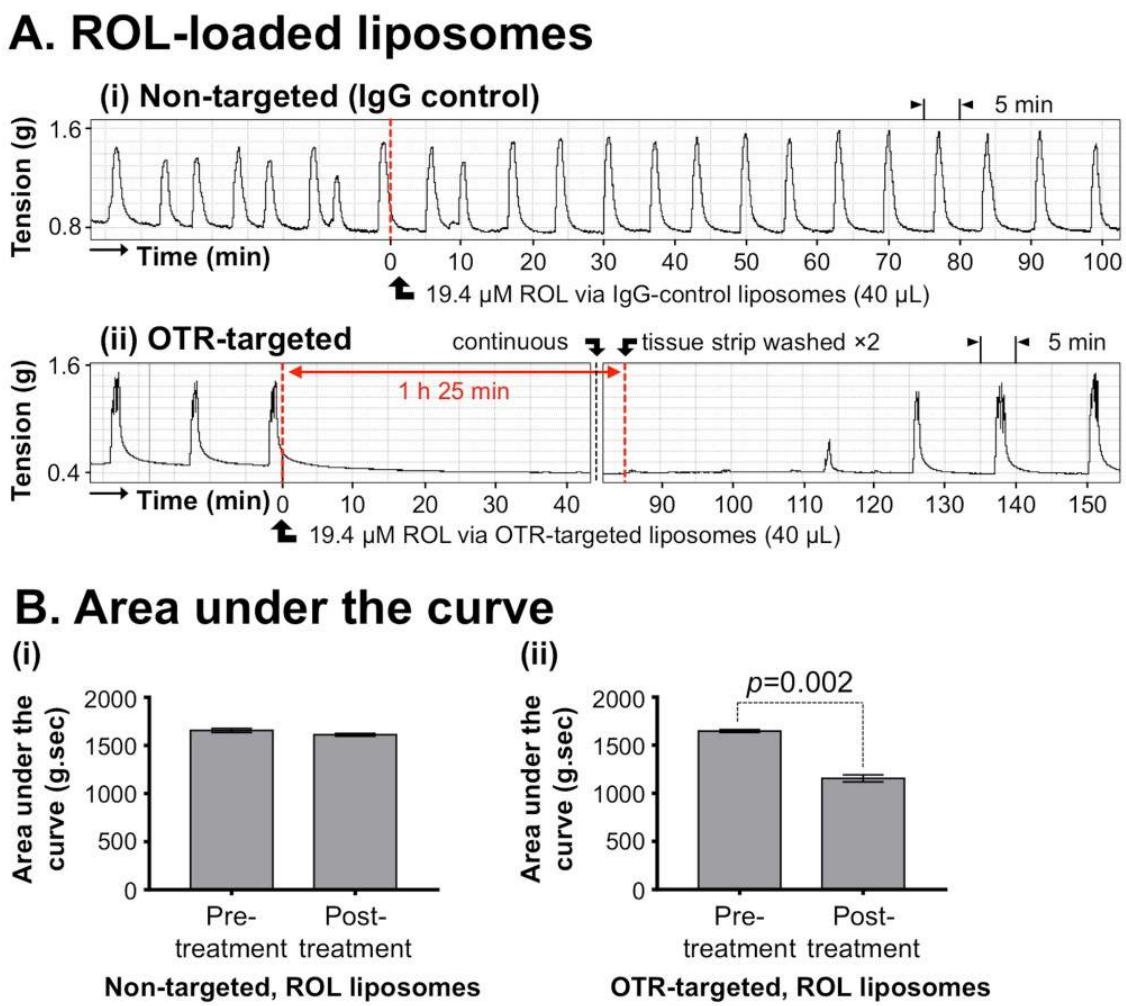

Figure 7.4. Modulation of uterine contractility by targeted liposomes is reversible.
Data are contraction traces for individual strips of human myometrial tissue (A) Effect of non-targeted (n=3) and OTR-targeted ROL-loaded liposomes (n=3) on myometrial contractions *in vitro*. (Aii) demonstrates restoration of contractions after washout. (B) Average AUC for 30 min immediately prior to and 30 min after treatment with ROL-loaded liposomes (pre- and post-treatment, respectively). *AUC analyses were paired t-tests*.

Mouse Myometrial Contractility

Prior to commencing mouse *in vivo* studies, we confirmed that liposomes were effective in delivering therapeutic agents to mouse uterine tissue *in vitro*. Results observed in the mouse were consistent with human myometrial contractility studies. OTR-targeted, drug-free liposomes (a control preparation) had no effect on mouse uterine contractions (Figure 7.5A) (n=3). Administering 9.25 µM SAL via non-targeted (IgG-coated control) SAL-loaded liposomes had

no effect on contractility (Figure 7.5Bi) (n=3), whereas the same SAL dose administered via OTR-targeted SAL-loaded liposomes abolished mouse myometrial contractions *in vitro* (Figure 7.5Bii) (n=3). Spontaneous contractions resumed following washing of tissue strips, demonstrating that the mouse uterine tissue remained viable following administration of the liposomes. Similar results were obtained for liposomes loaded with NIF (data not shown). These results demonstrated that OTR-targeted liposomes were effective in modulating mouse myometrial contractility.

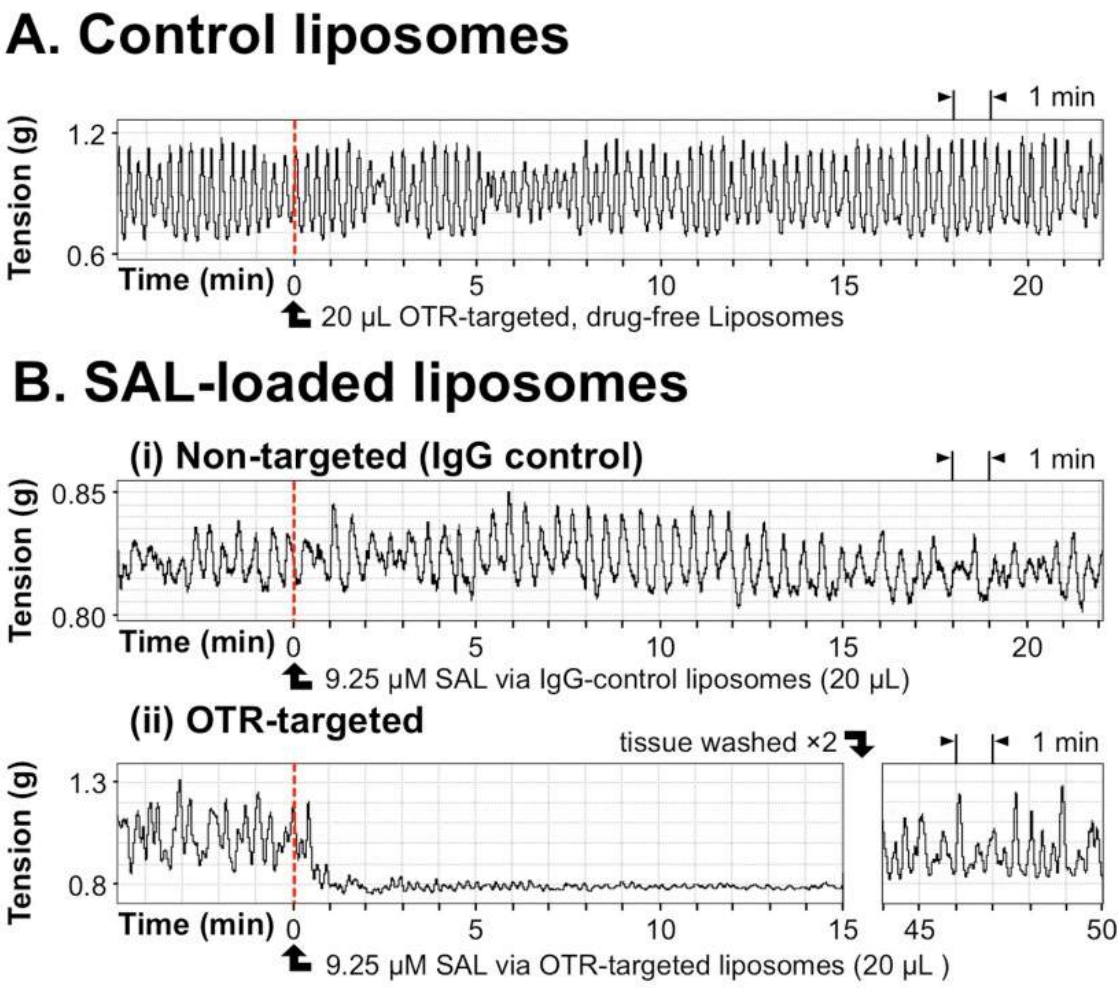

Figure 7.5. Use of targeted liposomes to modulate mouse uterine contractility *in vitro*.
Data are contraction traces for individual strips of mouse uterine tissue and illustrate effect of treatments on contraction amplitude and frequency. (A) Effect of OTR-targeted, drug-free liposomes (n=3). (Bi) Effect of 9.25 µM SAL administered via non-targeted SAL-loaded liposomes (n=3). (Bii) Effect of 9.25 µM SAL administered via OTR-targeted SAL-loaded liposomes (n=3).

Liposome Biodistribution

We examined the biodistribution of DiI-labelled non-targeted (naked) and OTR-targeted liposomes that occurred *in vivo*. Pregnant mice were injected with liposomes approaching term (GA17 and 18) then scarified shortly after labor (GA19) (17 – 19 h after injection). Whole organs (liver, brain, heart, kidney, lung, mammary tissue and uterus) were placed on Petri dishes, along with a euthanized neonate, and imaged. The arrangement of tissues (Figure 7.6A) was kept consistent when imaging tissues from different mice. DiI does not readily exchange out of liposomes into cell membranes or other lipid-containing structures, and therefore is an appropriate marker to assess the biodistribution of liposomes.

Fluorescence detection (p/sec/cm^2/sr) of non-targeted liposomes injected into pregnant mice consistently revealed liposome accumulation in the liver, which is the site of liposome clearance from the blood stream and metabolism [394]. Accumulation of non-targeted liposomes was not detected in the brain, heart, kidney, lung, mammary tissue or uterus nor in the neonates (n=4 each) (Figure 7.6Bi). Organs isolated from mice injected with OTR-targeted liposomes showed accumulation of the OTR-targeted liposomes in the uterus and the mammary glands. As expected, there were also high levels of liposome localization in the liver. OTR-targeted liposome accumulation was not detected in the brain, heart, kidney or lung, nor in the neonates (n=4 each) (Figure 7.6Bii).

DiI fluorescence was quantified for each organ (Table 7.2). OTR targeting of liposomes resulted in significantly increased localization to the

uterus compared to non-targeted liposomes (p<0.0001; non-targeted = 5.04 × 10^7 ± 7.5 × 10^6; OTR-targeted = 3.57 × 10^8 ± 3.05 × 10^7 p/sec/cm^2/sr) (Figure 7.6C). On average this equalled a 7-fold increase in uterine localization. Furthermore, the level of OTR-targeted liposome localization in the uterus was significantly greater than that of brain (p=0.0002), lung (p=0.0003), kidney (p=0.0005), heart (p<0.0001) and neonate (p<0.0001) (Figure 7.6C). For both non-targeted and OTR-targeted liposomes, accumulation in the liver was significantly greater than all other organs examined (p<0.0001), however there was no difference in liposome accumulation in the liver between non-targeted and OTR-targeted liposomes (p>0.9999; non-targeted = 7.90 × 10^8 ± 9.15 × 10^7; OTR-targeted = 7.37 × 10^8 ± 9.05 × 10^7) (Figure 7.6C).

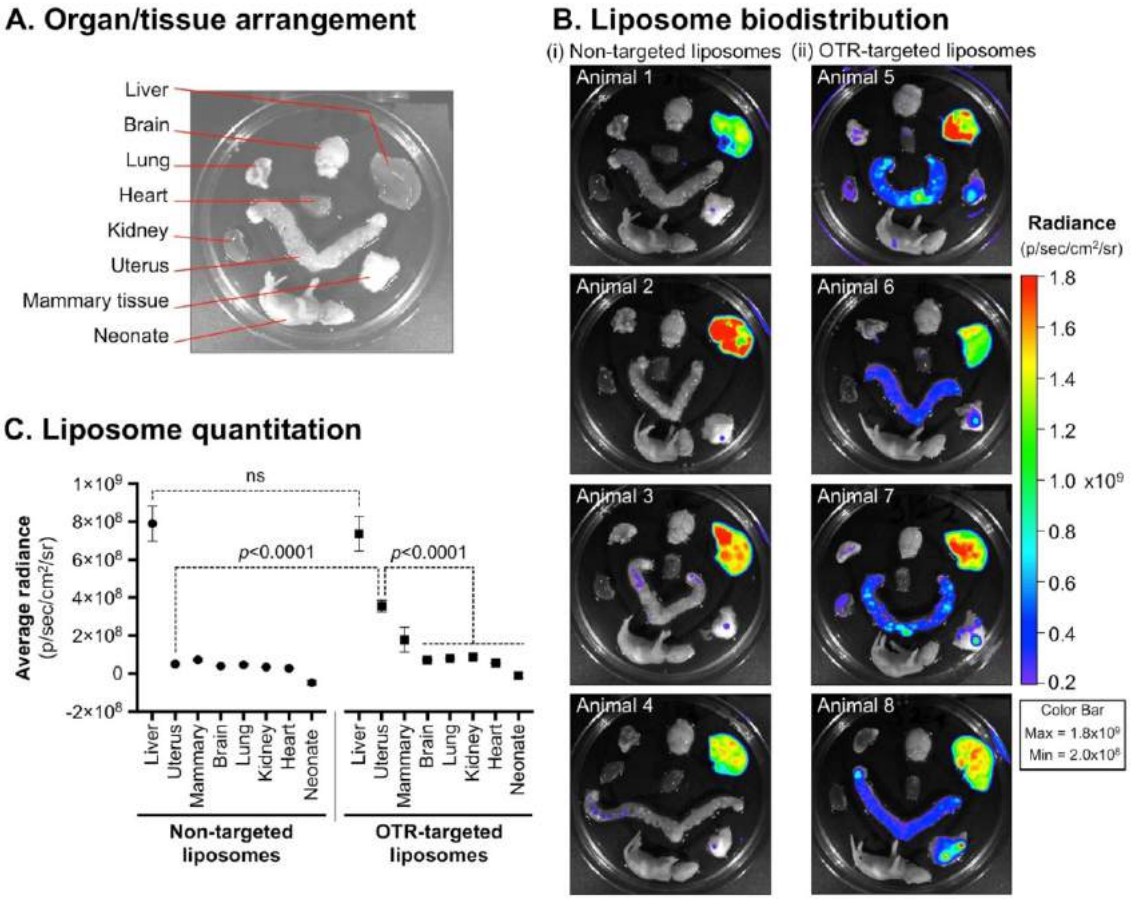

Figure 7.6. OTR-targeted liposomes accumulate in the uterus *in vivo*.
Data are light or fluorescence images captured shortly after labor by an IVIS-100 illustrating liposome biodistribution that occurred *in vivo*. (A) Representative image

demonstrating the arrangement of organs and tissues of interest (liver, brain, lung, heart, kidney, uterus, mammary tissue and a neonate). (B) Fluorescent detection of liposome biodistribution that occurred *in vivo*. (Bi) Biodistribution of non-targeted liposomes (n=4). (Bii) Biodistribution of OTR-targeted liposomes (n=4). (C) Quantitation of liposomal detection in different organs. Average radiance (p/sec/cm^2/sr) was determined for each organ and compared across treatment groups (n=4 for each organ per group). *Data were confirmed to be normally distributed (Shapiro-Wilk normality test) then compared by 1-way ANOVA with multiple comparisons (Holm-Sidak). Not all statically significant comparisons are indicated.*

Table 7.2 Average radiance of DiI-labelled liposomes detected in organs and neonates

Organ / tissue	Average radiance (p/sec/cm2/sr) (mean ± SEM)	
	Non-targeted liposomes (n=4 animals)	**OTR-targeted liposomes** (n=4 animals)
Liver	$9.73 \times 10^8 \pm 9.1 \times 10^7$	$7.37 \times 10^8 \pm 9.05 \times 10^7$
Uterus	$5.04 \times 10^7 \pm 7.5 \times 10^6$	$3.57 \times 10^8 \pm 3.05 \times 10^7$
Mammary tissue	$7.29 \times 10^7 \pm 6.05 \times 10^6$	$1.78 \times 10^8 \pm 6.47 \times 10^7$
Brain	$3.97 \times 10^7 \pm 2.51 \times 10^6$	$7.03 \times 10^7 \pm 4.51 \times 10^6$
Lung	$4.65 \times 10^7 \pm 4.82 \times 10^6$	$7.97 \times 10^7 \pm 2.94 \times 10^6$
Kidney	$3.46 \times 10^7 \pm 1.78 \times 10^6$	$8.79 \times 10^7 \pm 9.51 \times 10^6$
Heart	$2.79 \times 10^7 \pm 1.65 \times 10^6$	$5.52 \times 10^7 \pm 1.65 \times 10^6$
Neonate	$-4.83 \times 10^7 \pm 1.30 \times 10^7$	$-1.03 \times 10^7 \pm 1.46 \times 10^7$

OTR, oxytocin receptor

Preventing Preterm Birth

We used an LPS model of PTB to access whether targeted liposomes could be used to administer IND for the prevention of LPS-induced PTB in mice. Non-targeted and OTR-targeted liposomes loaded with 5.5 mg/mL IND were compared against IND administered as free-drug (1.0 or 2.0 mg/kg/day). Observed PTB rates are indicated in Table 7.3. Chi-squared analyses were performed.

Table 7.3. Rates of LPS-induced preterm birth and time between LPS injection and labor

Group No.	Treatment Group	n	PTB rate (%)	Time between LPS injection and observed labor (h) (mean ± SEM)
1	Control (no LPS, no liposomes)	12	0/12 (0)	109.7 ± 4.1
2	LPS control (+ 50% DMSO)	18	12/18 (67)	50.8 ± 8.9
3	LPS + 1.0 mg/kg IND (50% DMSO)	10	4/10 (40)	77.5 ± 14.1
4	LPS + 2.0 mg/kg IND (50% DMSO)	13	4/13 (31)	86.6 ± 12.7
5	LPS + OTR-targeted, drug-free liposomes	16	9/16 (56)	65.0 ± 9.9
6	LPS + Non-targeted 2.0 mg/kg IND liposomes	12	7/12 (58)	55.6 ± 12.4
7	LPS + OTR-targeted 2.0 mg/kg IND liposomes	11	2/11 (18)	101.3 ± 12.4

OTR, oxytocin receptor; *PTB*, preterm birth; *LPS*, lipopolysaccharide; *IND*, indomethacin; DMSO, dimethyl sulfoxide

PTB rates in control mice (Group 1) and the LPS control group (Group 2) were 0 (n=12) and 67% (n=18), respectively. At 2.0 mg/kg/day, IND administered as free-drug significantly reduced rates of PTB from 67% down to 31% (Group 2 (n=18) vs Group 4 (n=13); p=0.0484) (Figure 7.7A). PTB rate for OTR-targeted, drug-free control liposomes (Group 5) was 56% (n=16), and was not different to PTB rate observed for LPS control animals (Group 2) (p=0.532). IND administered at 2.0 mg/kg/day via non-targeted liposomes (Group 6) (n=12) had no effect as the observed PTB rate of 58% was not significantly different to PTB rates for LPS control animals (Group 2) (p=0.643) or animals treated with OTR-targeted, drug-free liposomes (Group 5) (p=0.91) (Figure 7.7A).

IND administered at 2.0 mg/kg/day via OTR-targeted liposomes (Group 7) (n=11) resulted in a PTB rate of 18%, which was a significant reduction

compared to the PTB rate of 67% for the LPS control animals (Group 2) (p=0.0112) (Figure 7.7A). Furthermore, PTB rate for 2.0 mg/kg/day IND administered via OTR-targeted liposomes was significantly reduced compared to the same dose administered by non-targeted liposomes (Group 6) (p=0.048). No significant difference was observed between 2.0 mg/kg/day IND admnistrered as free-drug compared to when administered via OTR-targeted liposomes (Group 4 vs Group7; p=0.4780) (Figure 7.7A).

The time between LPS injection and labor was calculated for each animal (average ± SEM shown in Table 7.3). Analysis of the normalised data showed that IP administration of LPS (0.7 µg/g) significantly advanced the time of labor, compared to control animals (Group 1 vs Group 2; p=0.0017) (Figure 7.7B). IND administered as free-drug at 1.0 and 2.0 mg/kg/day dose-dependently increased the average time between LPS injection and labor (77.5 ± 14.1 and 86.6 ± 12.7 h, respectively) compared to the LPS control (50.8 ± 8.9 h), however neither dose reached statistical significance (Group 2 vs Group 3; p=0.53) (Group 2 vs Group 4; p=0.08) (Figure 7.7B). OTR-targeted, drug-free liposomes had no effect on time between LPS injection and labor, compared to LPS control animals (Group 2 vs Group 5; p=0.92). 2.0 mg/kg/day IND administered via non-targeted liposomes had no significant effect on the time between LPS injection and labor (Group 2 vs Group 6; p=0.99), however, when administered via OTR-targeted liposomes, time between LPS injection and labor was significantly increased (Group 2 vs Group 7; p=0.0048). The time between LPS injection and labor was significantly different between 2.0 mg/kg/day IND delivered via non-targeted liposomes compared to OTR-targeted liposomes (Group 6 vs Group 7; p=0.0438).

Number of live pups was recorded for term deliveries (no viable pups arose from preterm deliveries). Data were normally distributed (Shapiro-Wilk normality test) and analysed by 1-way ANOVA with multiple comparisons (Tukey). There was no significant difference in the number of live pups from term deliveries in the different groups (Figure 7.7C).

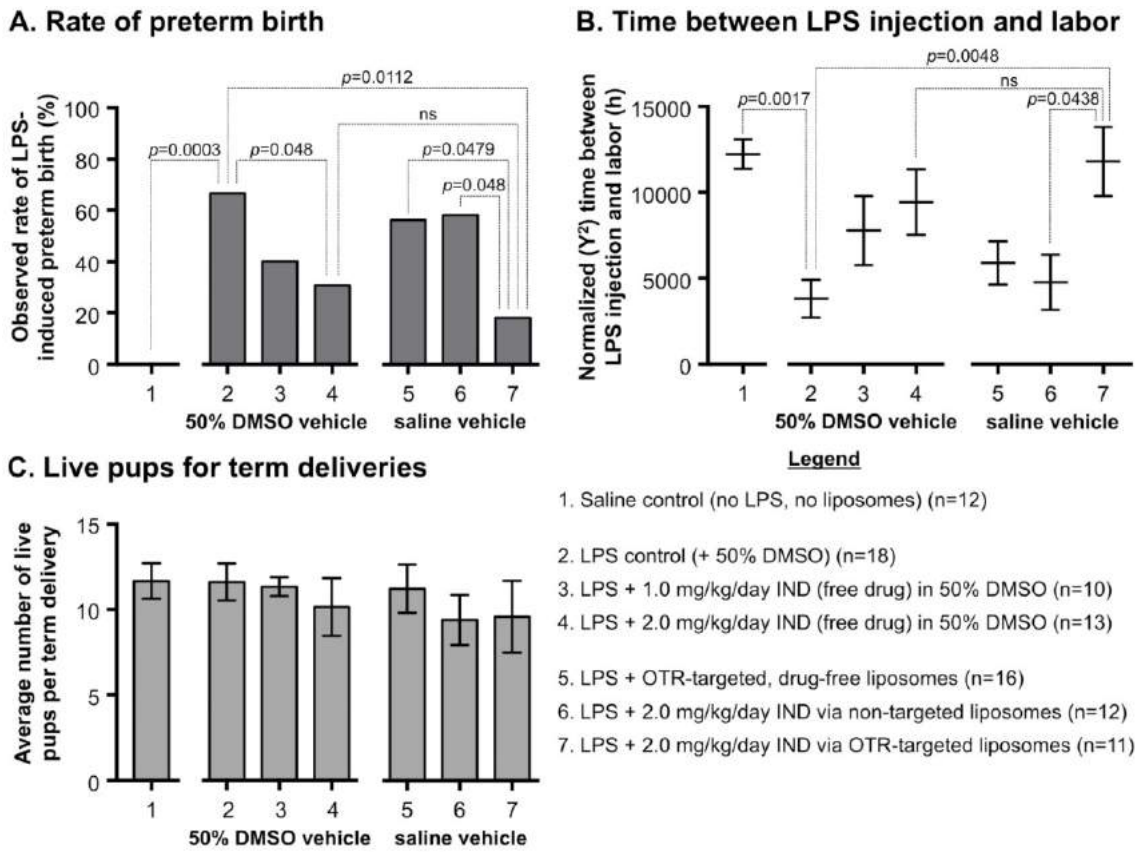

Figure 7.7. Preventing preterm birth using OTR-targeted indomethacin-loaded liposomes.
Efficacy of targeted liposomes was assessed using a LPS mouse model of preterm birth. (A) The effect of IND administered as free drug (1.0 or 2.0 mg/kg/day) or via liposomal preparations (2.0 mg/kg/day) on rates of LPS-induced preterm birth. (B) Time (h) between LPS injection and observed labor. (C) The number of live pups born for term deliveries. No significant differences were recorded in the number of live pups. *Preterm birth rates were analysed by Chi-square analysis. Data for time (h) between LPS injection and labor were normalized ($Y=Y^2$) (D'Agostino & Pearson normality test) then analysed by 1-way ANOVA with multiple comparisons (Tukey). Number of live pups was normally distributed and analysed by 1-way ANOVA with multiple comparisons (Tukey).*

7.5 Discussion

Principal Findings

OTRs are expressed at low levels on various tissues toward the end of pregnancy, including the brain and mammary tissue [395]. Expression in the pregnant uterus however is high approaching term [128, 346], indicating that the OTR is an excellent candidate for the development of a targeted drug delivery system for the uterus. This study represents an initial analysis of OTR-targeted liposomes as a drug delivery system, and demonstrates that:

(i) conjugation of the OTR antibody to the surface of liposomes confers the ability for NIF-, SAL- and ROL-loaded liposomes to significantly reduce human myometrial contractions *in vitro*, as confirmed by AUC analyses,

(ii) enhancement of myometrial contractility can be achieved through encapsulation of uterotonic agents, as confirmed by use of OTR-targeted DOF-loaded liposomes to significantly increase contraction plateau duration,

(iii) non-targeted liposomes loaded with these same therapeutic agents do not affect myometrial contractions *in vitro*, as confirmed by AUC and contraction plateau duration analyses,

(iv) the effects are reversible (depending on the therapeutic), as confirmed by the spontaneous resumption of contractions in both human and mouse myometrial tissue *in vitro*,

(v) the OTR-targeted liposomes themselves have no apparent effect on myometrial contractions, as confirmed by AUC analyses for

myometrial contractions in vitro, and lack of effect on PTB rates in mice or time between LPS injection and labor,

(vi) *in vivo,* OTR-targeted liposomes localize to the uterus and breast of pregnant mice whereas non-targeted liposomes do not. Uterine localization was increased 7-fold by OTR targeting, as confirmed by quantitation of average radiance for key organs of interest,

(vii) no evidence of transplacental passage of the liposomes to the fetus was observed, as determined quantitative evaluation of DiI fluorescence in neonates,

(viii) OTR-targeted liposomes loaded with IND are effective in reducing rates of LPS-induced PTB in mice whereas non-targeted IND-loaded liposomes have no effect.

Clinical Significance

Many current tocolytics have been associated with adverse effects on the mother (β-sympathomimetics) [364, 396, 397] and on the fetus (NIF, IND) [374-376, 398, 399] or have no evident effect on prolongation of pregnancy (atosiban) [372, 373]. NIF is capable of providing some clinical benefit, with a systematic review and meta-analysis indicating a significant reduction in the risk of delivery within 7 days of initiation of NIF treatment [361]. However, the high doses required to achieve relaxation of the myometrium increases the risk of adverse systemic effects [398-400]. IND has been explored as a tocolytic agent for preterm labor [401-403]; however, systematic review indicates that IND is associated with an increased risk for severe intraventricular hemorrhage, necrotizing enterocolitis, and periventricular leukomalacia [404]. A recent study by Refuerzo *et al*. (2015) demonstrated that encapsulation of IND inside non-

targeted liposomes can reduce IND levels in the foetus 7.6-fold, suggesting the potential for reduced fetal side effects [405].

Here we have demonstrated in mice that IND encapsulated inside OTR-targeted liposomes was effective in reducing rates of LPS-induced preterm birth, whereas non-targeted IND-loaded liposomes were not. These results, in conjunction with our biodistribution studies, suggest that OTR-targeting confers upon liposomes the ability to target therapeutics to the uterus. The clinical implications are that OTR-targeted liposomes may enable existing tocolytics, such as NIF and IND, to be administered with improved efficacy and improved safety.

The restricted biodistribution of OTR-targeted liposomes raises the possibility of introducing into clinical practice therapeutic agents that are known to be highly effective tocolytics, yet are known to have adverse off-target effects. One such group of candidates are the PDE4 inhibitors, such as ROL, which have been demonstrated to be highly effective in controlling inflammation-driven preterm delivery in mice [406]. Mounting evidence indicates that PTB in humans is also an inflammation driven event, and evidence that ROL is highly effective in abolishing spontaneous contractions in human myometrium [388] suggests that PDE4 inhibitors may be an excellent cargo for OTR-targeted liposomes in the setting of preterm labor.

Clinical implications also include the prospect of encapsulating uterotonic agents, and here we demonstrate that possibility through the use of DOF. DOF is not a traditional uterotonic agent, but when administered via OTR-targeted liposomes DOF significantly increased the duration of human myometrial

contractions *in vitro*, consistent with previous findings [391]. Targeted delivery of uterotonics may be useful to promote contractions, including during failure of labor to progress, expulsion of the placenta after labor, expulsion of retained products after miscarriage, or to control PPH. PPH is a leading cause of maternal mortality world-wide and is linked with major morbidities such as peripartum hysterectomy and massive transfusion [407-409]. First line therapy for PPH is uterine massage and oxytocin administration; however rates of atonic PPH after oxytocin use are increasing in many developed countries [410, 411]. When refractory uterine atony occurs, second line therapy may include administration of uterotonic agents such as methylergonovine and carboprost. Methylergonovine was recently identified as the more effective of the two [412], however both agents could effectively be encapsulated in OTR-targeted liposomes. Evidence indicates that post-receptor contractile signalling pathways are maintained in oxytocin desensitized primary myocytes *in vitro* [413], however oxytocin desensitization occurs, at least in part, by down-regulation of OTR protein levels [414]. Uterotonic-loaded liposomes targeted to the oxytocin receptor may therefore be of reduced effectiveness in patients with prolonged exposure to oxytocin.

Future Research

These data provide the first evidence that OTR-targeted liposomes are a drug delivery system that affords flexibility in delivery of different classes of therapeutic agents to human uterine tissue in order to modulate myometrial contractility. Furthermore, these data provide the first evidence that OTR-targeted liposomes can be used to administer therapeutic agents for the prevention of preterm birth in mice.

Further studies are necessary to determine the mechanism of OTR-targeted liposome uptake in myocytes, the quantitative biodistribution of therapeutic agents achieved in the uterus compared to other organs, and the rate of liposome clearance. Additional studies have been planned to determine whether the use of OTR-targeted liposomes to administer therapeutics for prevention of PTB is effective in reducing fetal side effects, such as premature closure of the ductus arteriosus in response to IND exposure in utero.

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Chapter Eight: Conclusion

Successful pregnancy necessitates that the uterus is maintained in a relaxed, quiescent state for the majority pregnancy, before being transformed to a contractile and excitable phenotype to facilitate parturition [5-9, 44, 45]. There is now a substantial body of evidence highlighting *PR, ESR1, OXTR* and *PTGS2* as key contraction-associated genes involved in this transformation. Nevertheless, new evidence continues to emerge implicating novel mechanisms that regulate uterine activity during parturition. Recently, our group reported the presence of hERG1 and KCNE2 in pregnant human myometrium in late gestation and demonstrated that labor onset is associated with diminished $K_v11.1$ activity in association with enhanced expression of the regulatory subunit, KCNE2 [234]. This thesis has built upon this work and further reports that *KCNH2* and *KCNE2* are expressed co-ordinately with the key parturition-associated genes, *OXTR*, *ESR1*, *PR* and *PTGS2.* Uncovering this association advances our understanding of the mechanisms that underpin myometrial transformation, and reiterates the complexity of the parturition process.

To define the complex interactions that underpin parturition, it is necessary to perform in depth investigations into the associated signalling pathways, gene expression regulation, and more. For human pregnancy, ethical considerations largely restrain these investigations to *in vitro* studies, and this often entails experimentation with biopsied gestational tissues in culture. During the initial stages of this project, efforts to investigate the regulation of *PR* expression in pregnant human myometrium were confounded by observations that the low *PR-A/PR-B* ratio of non-laboring tissue significantly increased in

untreated tissue across the course of 48 h cultures. This phenomenon was interesting, as it was consistent with a transition toward a laboring phenotype *in vitro*, and yet also troubling, as these initial studies utilised culture conditions that were routine within the field and therefore called into question the meaningfulness of the *in vitro* model. Previous studies have already demonstrated that animal smooth muscle cells are susceptible to undergoing phenotypic changes *in vitro* [415, 416]. In the case of pregnant human myometrium, this is particularly well exemplified by the fact that non-contractile tissue biopsied from a woman that is NIL spontaneously develops rhythmic contractions *in vitro* over the course of a 1 – 2 h equilibration period, thus indicating a rapid transition away from the *in vivo* phenotype [30, 110, 254]. Researchers must remain mindful of potential pitfalls of *in vitro* tissue culture, and as such the focus of this work shifted to examine whether other key myometrial genes undergo culture-induced changes, and determine whether these changes are also consistent with transition toward a pro-contractile, laboring phenotype. In addition, the project sought to identify supplements or conditions that prevented culture-induced changes in key parturition-associated genes, which would not only provide a more stable *in vitro* platform on which to perform future studies, but also provide insight into the regulation of the genes.

Following 48 h incubation, significant culture-induced increases were observed for *ESR1*, *PTGS2, PR-T*, *PR-A* and *KCNE2* mRNA expression, as well as a significant increase in the *PR-A/PR-B* expression ratio. *OXTR* expression significantly decreased following 48 h incubation, while expression of *PR-B* and *KCNH2* remained unchanged. It is interesting to note that the genes susceptible to culture-induced changes were the pro-labor genes (*ESR1,*

OXTR, PTGS2, PR-A and *KCNE2*), whereas the pro-gestation genes (*PR-B* and *KCNH2)* were unaffected. This suggests there is a fundamental deficiency in the standard culture conditions used to culture human myometrium, which leads primarily to pro-labor gene changes, further highlighting the need to develop more appropriate culture systems.

Experimental models of myometrium that are in a state of flux have the potential to confound results when researchers seek to elucidate the trigger(s) for labor. Through examining culture conditions that could be implemented to prevent or minimize phenotypic transition *in vitro,* it was determined that *ESR1* expression was maintained by supplementing media with steroids (P4 alone or P4 + E2 in combination); *KCNE2* and *OXTR* expression were maintained by PMA; *PR-A* expression and the *PR-A/PR-B* expression ratio were maintained by TSA. *PTGS2* expression was unable to be maintained, and was exacerbated by both steroids (P4 + E2) and PMA. Additionally, the pro-gestation genes, *PR-B* and *KCNH2*, which were previously unaffected by the culture, were down-regulated by steroids, P4 and E2. The effects of various treatments are summarised in the table below.

Table 8.1. Summary of culture-induced gene expression changes and effects of culture supplements conditions

Gene	Culture-induced change (48 h)	Effect of various treatments (48 h)
ESR1	Increased	Prevented by P4 Prevented by P4 + E2 Effects of P4 + E2 were blocked by stretch
KCNH2	No change	Down-regulated by P4 + E2 The effects of P4 + E2 blocked by stretch
KCNE2	Increased	Prevented by P4 + E2 Effects of P4 + E2 were blocked by stretch Prevented by PMA
OXTR	Decreased	Prevented by PMA
PR-T	Increased	Prevented by P4 + E2 The effects of P4 + E2 blocked by stretch
PR-A	Increased	Prevented by P4 + E2 The effects of P4 + E2 blocked by stretch Prevented by TSA
PR-B	No change	Down-regulated by P4 + E2
PR-A/PR-B ratio	Increased	Up-regulated by PMA Prevented by TSA
PTGS2	Increased	Up-regulated by P4 + E2 Up-regulated by PMA

No single supplement was successful in controlling culture-induced changes across all the genes examined. Furthermore, culture-induced increase in *PTGS2* expression was unable to be controlled by any of the supplements examined. Additional studies are therefore necessary. One possibility is to

examine the effect of multiple supplements applied in combination to control the respective gene changes. Whilst this makes sense in theory, applying a complex cocktail of supplemental agents may actually serve to drive further overall gene expression changes, due to the complex regulatory network that exists. Another option is to supplement culture media with pregnant human serum. Exploring this possibility was a high priority for this project, but was unable to be completed due to time constraints. Nonetheless, it is feasible that pregnancy-related factors present in pregnant human serum may offer some benefit for maintaining the non-laboring phenotype *in vitro*. This raises additional complications, however, as serum contents would vary between donors. Furthermore, commercially available serums are from 3rd trimester pregnancies, which could range from 28 – 40 weeks. Previous studies have shown that *OXTR* expression rises significantly in term samples but prior to labor (>37 weeks gestation); therefore, serum from 28 weeks gestation might lack the necessary factors for maintaining *OXTR* expression, which declined in culture.

Additional work is therefore necessary to devise more appropriate culture media that is capable of maintaining the non-laboring myometrial phenotype *in vitro*. Future studies may one day reveal drugs or agents that are highly effective in preventing myometrial transformation toward a laboring phenotype. Such drugs would be ideal candidates for targeted drug delivery to the uterus. This recent development of OTR-targeted nanoliposomes permits a broad range of drugs to be encapsulated and delivered to uterine tissue, thus opening up the possibility of controlling the various separately-regulated, yet inter-connected, components of the parturition web.

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Appendix A: Materials

Table A.1. List of general material used and their suppliers

Material(s)	Supplier
(R)-MG132	Cayman Chemical Company Michigan, USA
2ML 2.8mm-CK28-R Ceramic Bead Kit	Bertin Technologies Montigny-le-Bretonneux, France
8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP)	Sigma-Aldrich Pty. Ltd Sydney, Australia
Absolute Ethanol	UNIVAR Ingleburn, Australia
Alien QRT-PCR Inhibitor Alert 400 Reactions	Integrated Sciences Pty Sydney, Australia
Anti-OTR antibody	Abcam Cambridge, USA
Antigen Recovery Solution	ImmunoSolution Everton Park, Australia
BAY-11-7085	Cayman Chemical Company Michigan, USA
Boric acid	Sigma-Aldrich Pty. Ltd Sydney, Australia
Bovine serum albumin	Research Organics Inc Cleveland, USA
Bromophenol blue	Sigma-Aldrich Pty. Ltd Sydney, Australia
Charcoal Stripped Fetal Bovine Serum	Gibco Carlsbad, USA

Chloroform	BDH Ltd Dorset, UK
Cell culture 6-well plates	Nunc Denmark
Dofetilide	Tocris Bristol, UK
Dulbecco's Modified Eagle Medium (DMEM)	Gibco Carlsbad, USA
Ethidium bromide	Sigma-Aldrich Pty. Ltd Sydney, Australia
Ethylendiamine-tetra-acetic acid (EDTA)	Sigma-Aldrich Pty. Ltd Sydney, Australia
Estrogen	Sigma-Aldrich Pty. Ltd Sydney, Australia
Gentamicin	Gibco Carlsbad, USA
Glycerol	Fisher Scientific Massachusetts, United States
HEPES	Gibco Carlsbad, USA
Indomethacin	Sigma-Aldrich Pty. Ltd Sydney, Australia
Isopropanol	BDH Ltd Dorset, UK
JARID1A	Novus Biologicals Colorado, USA
L-Glutamine	Gibco Carlsbad, USA

Nifedipine	Tocris Bristol, UK
PCR primers	Sigma-Aldrich Pty. Ltd Sydney, Australia
Phorbol Myristate Acetate (PMA)	Cayman Chemical Company Michigan, USA
Progesterone	Sigma-Aldrich Pty. Ltd Sydney, Australia
Rabbit anti-JARID1A/RBP2	Bethyl Laboratories Inc. Montgomery, USA
Rabbit anti-SMYD	Abcam Cambridge, USA
Rabbit IgG	Santa Cruz Biotechnology Texas, USA
Rolipram	Tocris Bristol, UK
Salbutamol hemisulfate	Tocris Bristol, UK
Sodium chloride (NaCl)	Chem-Supply Pty Ltd Port Adelaide, AUS
Sodium citrate	Fisher Scientific Massachusetts, United States
Sodium hydroxide	UNIVAR Ingleburn, Australia
Sodium pyruvate	Gibco Carlsbad, USA
Superscript III First Strand Synthesis System	Invitrogen Carlsbad, USA

SYBR Green 2x Master mix	Applied Biosystems Carlsbad, USA
Trackit 100 BP DNA ladder	Invitrogen Carlsbad, USA
Triton X-100	Sigma-Aldrich Pty. Ltd Sydney, Australia
TRizol Reagent	Ambion Austin, USA
Turbo DNA –free 50 reactions	Ambion Austin, USA
Tween 20	Sigma-Aldrich Pty. Ltd Sydney, Australia
UltraPure Agarose	Invitrogen Carlsbad, USA
UltraPure Glycogen	Invitrogen Carlsbad, USA
UltraPure Tris	Invitrogen Carlsbad, USA

Appendix B: Solutions

Table B.1. List of solutions and their components

Solution	Components
TE buffer	1M Tris-Cl (10 mL) (pH 7.5) and 500 mM EDTA (2 mL) (pH 8.0), MiliQ H_2O to 1 L
10X loading dye	Glycerol (7.5 mL), TE buffer pH 8.0 (2.5 mL), Bromophenol blue (0.005 g)
2M Sodium Hydroxide	Sodium hydroxide pallets (80 g), MiliQ H_2O to 1 L
Ethidium bromide	Ethidium bromide (1 tablet), MiliQ H_2O (10 mL)
5X TBE buffer	0.09 M Tris base (54 g), 0.09 M Boric acid (27.5 g), 0.5 M EDTA pH 8.2 - 8.4 (20 mL), MiliQ H2O to 1 L
1X TBE buffer	5X TBE buffer (200 mL), MiliQ H2O (800 mL)
1% Agarose gel	Agarose powder (1 g), 1X TBE buffer (100 mL)
20X Tris-buffered saline (TBS) (1L)	3 M Sodium chloride (175.32 g), 0.4 M Tris Base (48.46 g), MiliQ H_2O to 1 L. Adjust the pH to 7.5 with Hydrochloric acid (~50 mL).
1X TBS-Tween 20 (TBS-T)	20X TBS (100 mL), Tween-20 (2 mL), MiliQ H2O to 2 L
1% Bovine serum albumin (BSA)	Bovine serum albumin (0.1 g), TBS-T (10 mL)
0.01 M Sodium citrate buffer pH 6.0	Sodium citrate (2.94 g), Tween 20 (0.5 mL), adjust the pH to 6.0 with Hydrochloric acid (5 M), MiliQ H2O to 1 L
01 M Tris-EDTA buffer pH 9.0	Tris base (1.21 g), 1 mM EDTA (2 mL), Tween 20 (0.5 mL), adjust the pH to 9.0 with Hydrochloric acid (5 M), MiliQ H2O to 1 L

10X Krebs' buffer	Potassium chloride (2.24 g), Magnesium sulfate (2.46 g), Monopotassium phosphate (1.63 g), Sodium chloride (56.7 g), Sodium Bicarbonate (20.5 g), MiliQ H_2O to 1 L
1X Krebs' buffer	10x Kreb's buffer (200 mL), Glucose (1.982 g), 1.89 M Calcium chloride (2 mL), MiliQ H_2O to 2 L

Appendix C: Equipment

Table C.1. List of general equipment and their suppliers

Equipment	Supplier
Analytical balance AS 310/X	ScaleLogic Limited *Palmerston North*, New Zealand
Centrifuge Thermo Heraeus *Multifuge*	DJB Labcare Ltd. Buckinghamshire, England
Confocal microscope	Nikon Instruments Inc. Melville, USA
Dry block heater	Thermoline Scientific Wetherill Park, Western Sydney
Fume Hood	Contamination Control Laboratories Pty Ltd Ingleburn, Australia
Gel electrophoresis equipment	Bio-Rad Hercules, USA
Hybridization oven	Thermoline Scientific Wetherill Park, Western Sydney
Heraeus humidified incubator	Thermoline Scientific Wetherill Park, Western Sydney
In vitro contraction bioassay	Grass Instruments and ADInstruments Bella Vista, Australia
Microcentrifuge Heraeus Biofuge Pico	DJB Labcare Ltd. Buckinghamshire, England)
Microscope	Nikon Instruments Inc. Melville, USA
Milestone RHS-1 Microwave Tissue Processor	Milestone SRL Sorisole, Northern Italy

PCR machine 7500 Sequence Detector	Applied Biosystem Carlsbad, USA
Pipettes	Gilson, Inc. Middleton, USA
Precellys homogenizer	Bertin Instruments France
Spectrophotometer	NanoDrop Technologies, Inc. Delaware, USA
Water bath	Julabo Seelbach, Germany

Appendix D: Submitted manuscript

"The regulation of uterine function during pregnancy and parturition: an update and recent advances"

Reproductive Sciences - Manuscript ID RSCI-17-091

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Appendix E: Published manuscript

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Original

The expression of genes involved in myometrial contractility changes during *ex situ* culture of pregnant human uterine smooth muscle tissue

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Abstract

Background: *Ex situ* analyses of human myometrial tissue has been used to investigate the regulation of uterine quiescence and transition to a contractile phenotype. Following concerns about the validity of cultured primary cells, we examined whether myometrial tissue undergoes culture-induced changes *ex situ* that may affect the validity of *in vitro* models. Objectives: To determine whether human myometrial tissue undergoes culture-induced changes *ex situ* in Estrogen receptor 1 (*ESR1*), Prostaglandin-endoperoxide synthase 2 (*PTGS2*) and Oxytocin receptor (*OXTR*) expression. Additionally, to determine whether culture conditions approaching the *in vivo* environment influence the expression of these key genes. Methods: Term non-laboring human myometrial tissues were cultured in the presence of specific treatments, including; serum supplementation, progesterone and estrogen, cAMP, PMA, stretch or NF-κB inhibitors. *ESR1*, *PTGS2* and *OXTR* mRNA abundance after 48 h culture was determined using quantitative RT-PCR. Results: Myometrial tissue in culture exhibited culture-induced up-regulation of *ESR1* and *PTGS2* and down-regulation of *OXTR* mRNA expression. Progesterone prevented culture-induced increase in *ESR1* expression. Estrogen further up-regulated *PTGS2* expression. Stretch had no direct effect, but blocked the effects of progesterone and estrogen on *ESR1* and *PTGS2* expression. cAMP had no effect whereas PMA further up-regulated *PTGS2* expression and prevented decline of *OXTR* expression. Conclusion: Human myometrial tissue in culture undergoes culture-induced gene expression changes consistent with transition toward a laboring phenotype. Changes in *ESR1*, *PTGS2* and *OXTR* expression could not be controlled simultaneously. Until optimal culture conditions are determined, results of *in vitro* experiments with myometrial tissues should be interpreted with caution.

Key words: myometrium, phenotype, transition, *ESR1*, *OXTR*, *PTGS2*

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Introduction

Parturition requires that myometrial smooth muscle undergo a phenotypic transition, remaining quiescent for the majority of gestation and then transforming to a tissue capable of generating forceful, co-ordinated contractions to expel the fetus and the placenta (1, 2). Transformation of the myometrium from the quiescent to a contractile state necessitates the presence of estrogen prior to the onset of labor (1–5). In humans and higher primates, however, maternal estrogen levels are already high for most of pregnancy and remain elevated during parturition (6, 7). This has led to the concept of functional estrogen activation whereby myometrium becomes sensitive to estrogen through changes in estrogen receptor (ER) expression, encoded by the genes *estrogen receptor 1 (ESR1)* and *estrogen receptor 2 (ESR2)*. Our group has reported that expression of *ESR1* is low in non-laboring term myometrium and that *ESR1* levels rise with the onset of labor, whilst expression of *ESR2* was barely detectable and did not change with labor (8, 9). This suggests that increased *ESR1* expression mediates functional estrogen activation. Furthermore, we found that U-0126, a highly selective inhibitor of mitogen-activated protein kinase kinase (MEK), blocked the ability of estrogens to stimulate the expression of the oxytocin receptor (OTR) in human myometrial samples *in vitro* (9). Expression of the *oxytocin receptor* gene *(OXTR)* in the myometrium increases after 37 weeks of gestation (10), which is followed by a fall in advanced labor (11–13). Furthermore, a genome-wide study found that the high *OXTR* mRNA abundance in term non-laboring myometrium samples decreased with labor (14). Studies using human tissues and myometrium-derived cell lines have demonstrated that the binding of oxytocin to its receptor led to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which subsequently increased the production of prostaglandins (PGs), inflammatory chemokines and cytokines that are involved in fetal membrane remodelling, cervical ripening and myometrial activation (15, 16). *Prostaglandin-endoperoxide synthase 2 (PTGS2)* encodes a key enzyme responsible for the biosynthesis of PGs. *PTGS2* mRNA abundance is low in term non-laboring human myometrium and increases with the onset of labor (14, 17). Increased expression of *ESR1* and *PTGS2* and decreased expression of *OXTR* is therefore associated with transition toward a laboring phenotype. Characterizing the regulation of these key myometrial genes is essential to understanding normal human birth, as well as obstetric complications, including preterm labor.

Outside of clinical trials, researchers are primarily limited to observational studies on human pregnancy and as such rely heavily on animal models of pregnancy and *in vitro* studies using human cell lines and tissues. Primary myocyte cultures have been used extensively to study myometrial biology, but concerns have been raised about the lifespan of cultured primary cells (18) and their ability to remain to be representative of the tissue of origin (19–22). Incubation of tissue *ex vivo* as small pieces or strips may represent the *in vivo* phenotype more closely. Tissue strips are routinely used to analyze the effect of treatments on contractility (23–25), and have even been used to capture dynamic phosphorylation events that occur in phase with contractions (26, 27).

Incubating pieces and strips of myometrium has emerged a popular experimental model for interrogating myometrial biology. Both approaches involve artificial *in vitro* conditions and rely on the assumption that the tissue phenotype remains stable across the course of the study. Non-laboring myometrial tissue strips, however, spontaneously develop rhythmic contractions *in vitro* over the course of just 1–2 h, suggesting that the tissue strips may rapidly transition away from a non-contractile phenotype (23, 25, 26). Moreover, with tissue incubation studies routinely performed for 48 h or more, the transition from the *in vivo* phenotype may be even more pronounced. The implications are potentially significant as findings made using 'non-laboring' tissue may in fact have been generated using tissue that may not be representative of the non-laboring phenotype and could respond differently to treatments.

Experimental models of myometrium that are in a state of flux have the potential to confound the results when researchers seek to elucidate the trigger(s) for labor. Through characterizing the expression of key myometrial genes, the present study aimed to explore whether non-laboring myometrial tissue pieces and strips undergo culture-induced changes *in vitro* that are consistent with transition to a pro-contractile, laboring phenotype. We further aimed to identify culture conditions that could prevent or attenuate phenotypic changes thereby providing researchers with a stable platform to conduct experimental studies.

Here we report evidence suggesting that non-laboring human myometrium undergoes a culture-induced transition to a labor-like phenotype *in vitro*. We further report that different aspects of the non-laboring phenotype can be differentially preserved by supplementing the medium with physiological concentrations of progesterone and estrogen, applying NFκB inhibitors and by exposing the uterine muscle samples to constant stretch.

Materials and Methods

Consumables and reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA–free 50 reactions were from Ambion (Austin, USA). Alien QRT-PCR Inhibitor Alert was purchased from Integrated Sciences Pty (Sydney, Australia). (R)-MG132, BAY-11–7085 and Phorbol Myristate Acetate (PMA) were obtained from Cayman Chemical Company (Michigan, USA). 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), PCR primers, Progesterone and Estradiol were purchased from Sigma (St Louis, USA). The 2 ml 2.8 mm CK28-R Ceramic Bead Kit was acquired from Bertin Technologies (Montigny-le-Bretonneux, France). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES, Dulbecco's Modified Eagle Medium (DMEM) and Charcoal Stripped Fetal Bovine Serum were obtained from Gibco (Carlsbad, USA). SYBR Green 2× Master mix was from Applied Biosystems (Carlsbad, USA).

Myometrial Tissue acquisition

These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). Human myometrial samples (5 × 5 × 10 mm) were obtained from the lower uterine segment during elective Caesarean section (CS) of singleton term pregnancies (38.2–39.6 weeks gestation). Patient BMI range was 18.3–38.0, and none of the patients were in-labor. The indications for elective CS were previous CS, placenta praevia, fetal distress or breach presentation. Women were excluded if they were given steroids. Following delivery of the placenta, all women immediately received 5 units of oxytocin (Syntocinon) into an intravenous line. Administration of oxytocin was part of the standard care for the prevention of postpartum hemorrhage. Myometrial biopsies were excised within 3 min after oxytocin administration. Samples were placed on ice in serum-free medium containing DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/ml Gentamicin and 10 mM HEPES for the transfer to the laboratory.

Myometrial tissue culture

Approximately 100 mg tissue from each sample was immediately snap frozen in liquid nitrogen for subsequent analysis. The remaining myometrium was dissected into approximately 2 × 2 × 2 mm pieces and washed in serum-free media. Samples were then incubated in serum-free or 5% (v/v) charcoal stripped fetal

bovine serum (CSS)-supplemented media in a 37 °C, 95% air/5% CO_2 humidified incubator for 48 h. The 5% CSS-supplemented culture media contained DMEM with high glucose, 5% CSS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 µg/ml Gentamicin and 10 mM HEPES. To determine the effects of steroids, myometrial samples were incubated in 5% CSS-containing media with physiological concentrations of progesterone (P4; 500 nM) and/or estradiol (E2; 400 nM) (4) in a 37 °C, 95% air/5% CO_2 humidified incubator for 48 h. To determine the effect of stretch, myometrial tissue strips (2 × 2 × 10 mm) were cultured in 5% CSS-containing media for 48 h in a 37 °C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of constant stretch (0, 2.45 or 7.35 kN/m^2, respectively). Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 ml of culture media in 50 ml tubes (strips subjected to 0 g only were tied at one end). To determine the effect of stretch and steroids, myometrial strips were cultured in 5% CSS-containing media with 500 nM P4 and 400 nM E2 for 48 h in a 37 °C, 95% air/5% CO_2 humidified incubator whilst being subjected to 0, 1 or 3 g of stretch. To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial pieces were incubated for 48 h a 37 °C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with the cAMP analogue 8-Br-cAMP (250 µM), or PMA (0.1, 1.0 µM), or the NF-κB inhibitors MG-132 (2.0, 5.0, 10.0 µM) and BAY-11–7085 (2.0, 5.0, 10.0 µM) individually or in combination (10.0 µM MG-132 + 10.0 µM BAY-11–7085). Vehicle was DMSO (0.1%). Following each incubation, the tissue pieces or strips were snap frozen using liquid nitrogen and stored at -80 °C for subsequent analyses.

RNA extraction, reverse transcription and real-time quantitative PCR

RNA was extracted using TRizol Reagent (Ambion, USA) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Ambion, USA). An ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) was used to measure RNA concentration (absorbance at 260 and 280 nm) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 µg of total RNA) was spiked with 0.5×10^7 copies of Alien RNA (Integrated Sciences Pty, Australia) and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers (Invitrogen, USA). Quantitative real-time PCR was performed using an ABI 7500 Sequence Detector (Applied Biosystem, USA). No-reverse transcription (no-RT) negative controls were prepared for each sample. The final volume of each PCR reaction was 20 µl, containing 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems, USA), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 µl with 1.0 µl of 2.5 µM of Alien Primer Mix, 10 µl of 2× SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. No-template control samples (NTCs) were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express and are shown in the Table 1.

Data and statistical analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the delta Ct (ΔCt) method (28). All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, USA). Graphical data are presented as mean ± S.E.M. For compari-

Table 1. cDNA primer sequences - *ESR1*, *PTGS2* and *OXTR*

Primer	Primer sequence (5'-3')	Amplicon size	GenBank #
ESR1	F: TGAAAGGTGGGATACGAAAAGAC R: CATCTCTCTGGCGCTTGTGTT	66	NM_000125.3
PTGS2	F: ATGTTCCACCCGCAGTACAGA R: CAGCATAAAGCGTTTGCGGTA	73	NM_000916.3
OXTR	F: CTGGACGCCTTTCTTCTTCGT R: GAAGGCCGAGGCTTCCTT	101	NM_000963

ESR1, Estrogen Receptor; *OXTR*, Oxytocin Receptor; *PTGS2*, Prostaglandin-endoperoxide synthase 2.

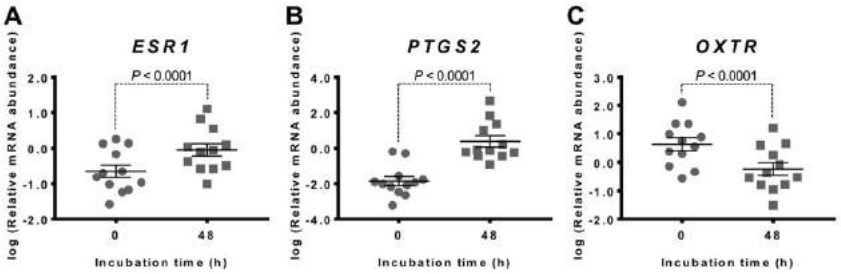

Fig. 1. Culture-induced changes in myometrial expression of *ESR1*, *PTGS2* and *OXTR* mRNAs following 48 h culture. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples immediately after biopsy (0 h) as well as following 48 h incubation (n=12), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then analysed by paired t-test. Data are mean ± S.E.M.*

son between two groups, Student's t test was used. For multiple comparisons a one-way analysis of variance (ANOVA) followed by post-hoc test of Dunnett multiple comparisons was used. P-values ≤ 0.05 were considered statistically significant.

Results

Gene expression changes in myometrium during 48 h of culture

Myometrial tissue samples were incubated for 48 h in serum-free media to determine gene expression changes that the tissue undergoes upon being removed from the *in vivo* environment and cultured *in vitro*. There was a statistically significant increase in mRNA abundance for *ESR1* (P<0.0001) and *PTGS2* (P<0.0001) (Fig. 1A and B), whilst *OXTR* mRNA abundance significantly decreased (P<0.0001) (Fig. 1C). *ESR2* mRNA abundance was detected at extremely low levels in fresh tissue, and remained low and relatively unchanged following 48 h incubation (not shown).

Serum-free media versus 5% CSS-containing media

Having observed culture-induced changes in the expression of *ESR1*, *PTGS2* and *OXTR* mRNAs, we examined whether the presence of 5% CSS, often included in myometrial culture media (29), affected the phe-

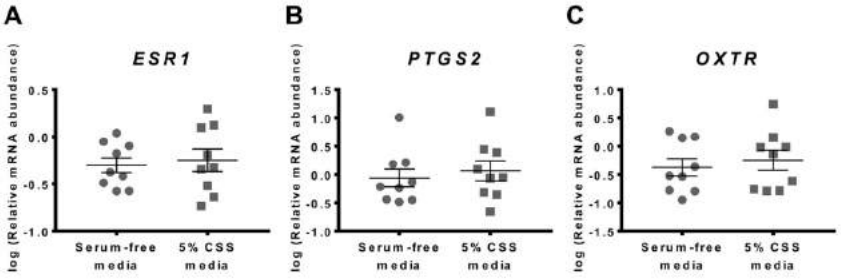

Fig. 2. Effect of serum on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA levels *in vitro*. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples following 48 h incubation in serum-free media or media supplemented with 5% CSS (n=9), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then analysed by paired t-test. Data are mean ± S.E.M.*

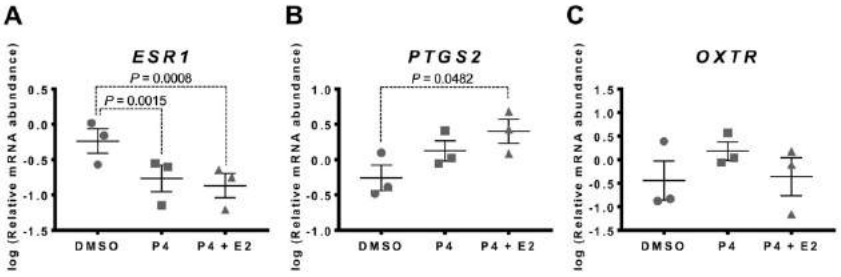

Fig. 3. Effect of steroids on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNAs *in vitro*. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples following 48 h incubation in the presence of 500 nM progesterone (P4) or 500 nM P4 + 400 nM estradiol (E2) (n=3), and expressed relative to Alien reference. (A) *ESR1* mRNA abundance. (B) *PTGS2* mRNA abundance. (C) *OXTR* mRNA abundance. *Data were logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test) then compared by 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± S.E.M.*

notypic change of myometrium *in vitro*. Following 48 h incubation, there was no significant difference in *ESR1* mRNA abundance between serum-free media and 5% CSS media (Fig. 2A). Similarly, the presence or absence of 5% CSS in media had no effect on culture-induced change of *PTGS2* mRNA abundance (Fig. 2B) or *OXTR* mRNA abundance (Fig. 2C) following 48 h incubation.

The effect of steroids

Relative abundance of *ESR1*, *PTGS2* and *OXTR* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 500 nM P4 or 500 nM P4 + 400 nM E2, which are hormone concentrations in term maternal plasma (4).

A statistically significant decrease in *ESR1* mRNA abundance was observed between DMSO- and P4-treated tissue after 48 h (P=0.0015), indicating that supplementing media with 500 nM P4 blocked the culture-induced increase in *ESR1* mRNA abundance compared to fresh tissue (Fig. 3A). The combination of 500 nM

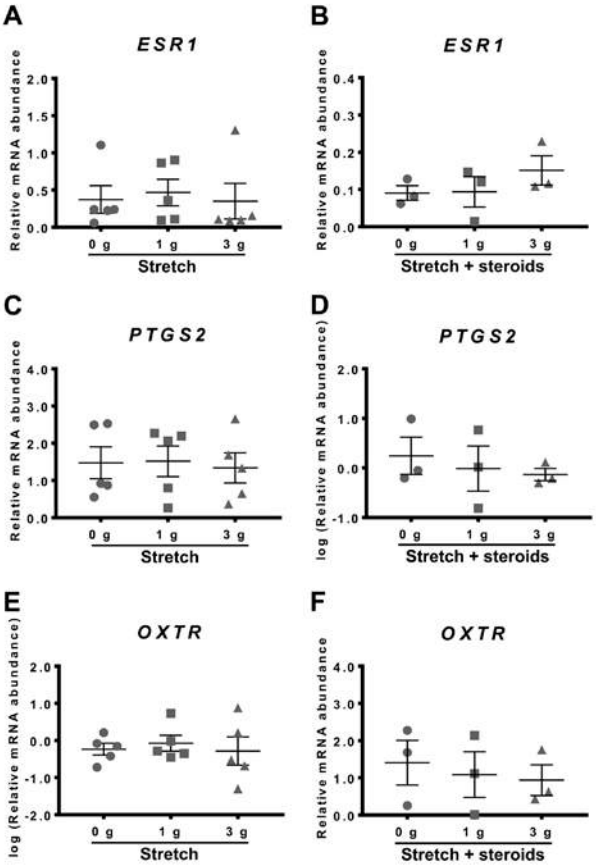

Fig. 4. Effect of stretch in the absence or presence of steroids on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA levels *in vitro*. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial strips whilst applying 0, 1 and 3 g of stretch for 48 h (n=5), as well as in presence of steroids (500 nM P4 + 400 nM E2) whilst applying 0, 1 and 3 g of stretch for 48 h (n=3), and expressed relative to Alien reference. (A) Effect of stretch on *ESR1* mRNA abundance. (B) Effect of stretch and steroids on *ESR1* mRNA abundance. (C) Effect of stretch on *PTGS2* mRNA abundance. (D) Effect of stretch and steroids on *PTGS2* mRNA abundance. (E) Effect of stretch on *OXTR* mRNA abundance. (F) Effect of stretch and steroids on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± S.E.M.*

P4 + 400 nM E2 had no further effect as the 500 nM P4 + 400 nM E2 treatment significantly reduced mRNA levels at 48 h relative to vehicle treatment (P=0.0008), but not relative to P4 treatment alone (Fig. 3A).

Supplementing media with 500 nM P4 had no statistically significant effect on *PTGS2* mRNA abundance relative to the vehicle (Fig. 3B). Supplementing media with 500 nM P4 + 400 nM E2, however, significantly

increased *PTGS2* mRNA abundance at 48 h relative to the vehicle (P=0.0482), indicating that P4 + E2 in combination exacerbated the up-regulation of *PTGS2* expression compared to the fresh tissue (Fig. 3B).

Supplementing media with 500 nM P4 alone, or 500 nM P4 + 400 nM E2, had no effect on the culture-induced change in *OXTR* mRNA abundance (Fig. 3C).

The effect of stretch on human myometrial gene expression in vitro

Myometrial tissue strips were subjected to 0, 1 or 3 g of stretch (0, 2.45 or 7.35 kN/m^2, respectively) for 48 h in absence or presence of steroids (500 nM P4 + 400 nM E2) to determine whether applying stretch to the muscle influenced the culture-induced changes in *ESR1*, *PTGS2* or *OXTR* mRNA levels *in vitro*.

As seen in Fig. 4A, 0–3 g stretch had no effect on *ESR1* expression in the tissue strips. Interestingly, stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *ESR1* mRNA levels (Fig. 4B), indicating that P4 + E2 was no longer effective in preventing culture-induced increase in *ESR1* mRNA abundance compared to fresh tissue.

Similarly, stretch applied to myometrial strips for 48 h had no effect *PTGS2* mRNA expression (Fig. 4C). Stretch applied in the presence of P4 + E2 likewise had no effect (Fig. 4D). Thus, the P4 + E2 treatment no longer exacerbated the increase in *PTGS2* mRNA abundance that occurred after 48 h *in vitro*.

Applying stretch to myometrial strips for 48 h had no effect on *OXTR* expression (Fig. 4E). Stretch applied in the presence of 500 nM P4 + 400 nM E2 also had no effect on *OXTR* mRNA abundance after 48 h culture (Fig. 4F).

The effects of activating intracellular signalling pathways

Relative abundance of *ESR1*, *PTGS2* and *OXTR* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 8-Br-cAMP (250 μM), PMA (0.1, 1.0 μM) or vehicle (DMSO).

Adding 250 μM 8-Br-cAMP to the culture media had no effect on *ESR1* mRNA expression (Fig. 5A). Similarly, supplementing media with 0.1 or 1.0 μM PMA, a protein kinase C (PKC) activator, had no effect on *ESR1* mRNA levels (Fig. 5B).

Adding 250 μM 8-Br-cAMP had no effect on *PTGS2* mRNA abundance (Fig. 5C), but supplementing the culture media with 1.0 μM PMA increased *PTGS2* expression significantly (P=0.047) (Fig. 5D).

Adding 250 μM 8-Br-cAMP to the culture media had no significant effect on *OXTR* mRNA abundance after 48 h of culture (Fig. 5E). *OXTR* mRNA abundance was significantly increased in response to 1.0 μM PMA (P=0.0099) (Fig. 5F).

The effect of NF-κB Inhibitors

The NF-κB inhibitors, MG-132 and BAY-11–7085, were employed to test whether NF-κB pathway activation was involved in the gene expression changes induced by the *in vitro* conditions.

Incubating myometrial samples with 0, 2, 5 or 10 μM MG-132 or BAY-11–7085 individually or in combination (10 μM each) had no effect on *ESR1* mRNA abundance (Fig. 6A–6C).

Neither inhibitor affected culture-induced changes in *PTGS2* mRNA abundance (Fig. 6D and 6E), nor was *PTGS2* mRNA abundance affected by a combination of the two compounds (Fig. 6F).

Similarly, supplementing culture media with MG-132 or BAY-11–7085 had no effect on *OXTR* mRNA abundance following 48 h of culture (Fig. 6G and 6H), and *OXTR* mRNA abundance was not affected by a combination of the two compounds (Fig. 6I).

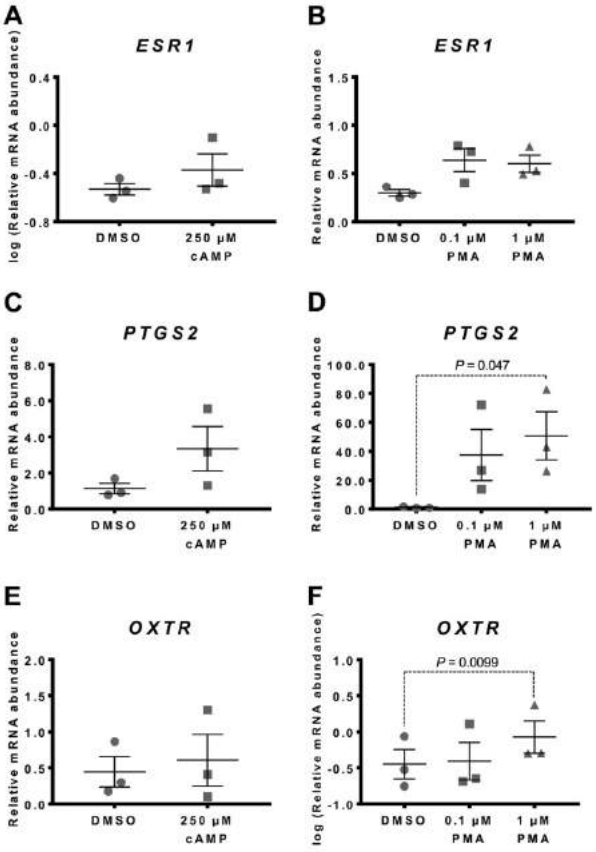

Fig. 5. Effect of cAMP and PMA on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA *in vitro*. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial tissue samples (n=3) following 48 h incubation in the presence of 8-Br-cAMP (250 µM) or PMA (0.1 and 1.0 µM), and expressed relative to Alien reference. (A) Effect of 8-Br-cAMP on *ESR1* mRNA abundance. (B) Effect of PMA on *ESR1* mRNA abundance. (C) Effect of 8-Br-cAMP on *PTGS2* mRNA abundance. (D) Effect of PMA on *PTGS2* mRNA abundance. (E) Effect of 8-Br-cAMP on *OXTR* mRNA abundance. (F) Effect of PMA on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). 8-Br-cAMP data was analysed using paired t-test, whilst 1-way ANOVA with multiple comparisons (Dunnett) was used to analyse PMA data. Data are mean ± S.E.M.*

Discussion

In the context of human parturition, *in vitro* models such as myometrial cell lines and *ex situ* tissues have been an important tool for investigating the maintenance of uterine quiescence and the mechanisms by which the myometrium transforms to an actively contracting organ at labor. This study examined whether

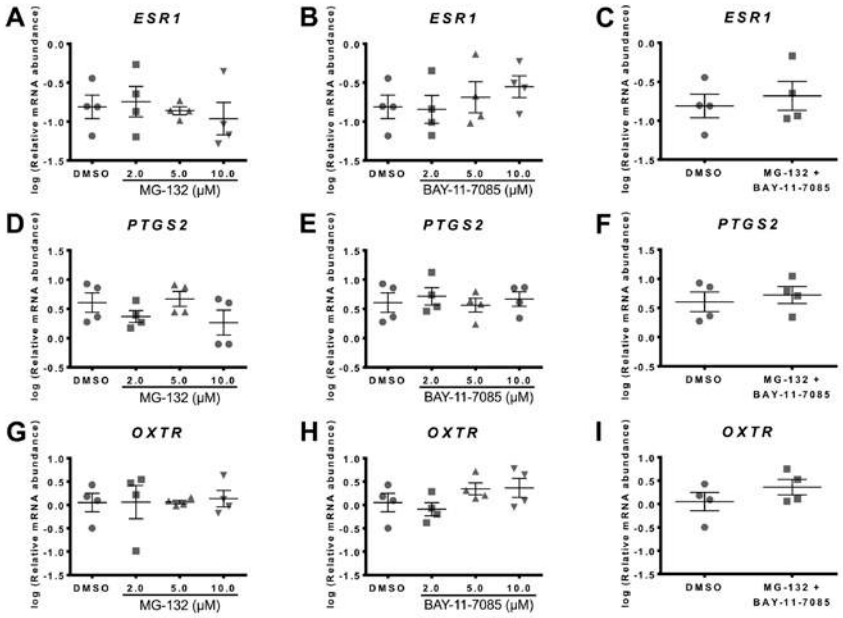

Fig. 6. Effect of NF-κB inhibitors, MG-132 and BAY-11-7085, on culture-induced changes in *ESR1*, *PTGS2* and *OXTR* mRNA *in vitro*. Relative mRNA abundance of *ESR1*, *PTGS2* and *OXTR* was measured in term non-laboring myometrial samples (n=4) following 48 h incubation in the presence of different NF-κB inhibitors, and expressed relative to Alien reference RNA. (A) Effect of MG-132 on *ESR1* mRNA abundance. (B) Effect of BAY-11-7085 on *ESR1* mRNA abundance. (C) Effect of MG-132 and BAY-11-7085 on *ESR1* mRNA abundance. (D) Effect of MG-132 on *PTGS2* mRNA abundance. (E) Effect of BAY-11-7085 on *PTGS2* mRNA abundance. (F) Effect of MG-132 and BAY-11-7085 on *PTGS2* mRNA abundance. (G) Effect of MG-132 on *OXTR* mRNA abundance. (H) Effect of BAY-11-7085 on *OXTR* mRNA abundance. (I) Effect of MG-132 and BAY-11-7085 on *OXTR* mRNA abundance. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). MG-132 and BAY-11-7085 data was analysed by 1-way ANOVA with multiple comparisons (Dunnett). MG-132 + BAY-11-7085 data was analysed using paired t-test. Data are mean + S.E.M.*

non-laboring myometrial tissues and strips undergo changes in culture that are consistent with transition to a pro-contractile, laboring phenotype. We determined gene expression changes as quantitative assessment of mRNA in preference to semi-quantitative estimates of protein levels by Western blot (30). Notably, recent studies examining protein profiles in mammalian cells have found that transcription, not translation, chiefly determines protein abundance (31), and that during periods of dynamic change, such as that occurring during phenotype transition, changes in mRNA abundance play a particularly dominant role in controlling changes in protein levels (32).

Here we showed that the abundance of both *ESR1* and *PTGS2* mRNAs was significantly increased after 48 h of culture of myometrial tissues (Fig. 1A and 1B). Our group previously reported that *ESR1* expression increased in the myometrium with the onset of labor (8, 9). *PTGS2* expression was also reported to increase in the human myometrium with labor (14, 17). Our observation that *ESR1* and *PTGS2* mRNA abundance increase

during culture is thus consistent with the possibility that the myometrium has been transitioning to a laboring state *in vitro*. Moreover, *OXTR* expression was high in term non-laboring myometrium and significantly decreased after 48 h incubation (Fig. 1C). As shown previously, *OXTR* mRNA abundance increases in uterine tissue after 37 weeks of gestation (10) and there is an apparent fall in *OXTR* expression in advanced labor (11–13). Chan Y.W. et al. (14) characterized the human myometrial transcriptome during the transition from term, not-in-labor to in-labor state and confirmed that *OXTR* mRNA expression decreased with labor (14). Therefore, our observation that *OXTR* expression spontaneously declines during culture further supports a transition toward a laboring phenotype *in vitro*.

Performing *in vitro* experiments using myometrium that is in a state of flux is not ideal and raises the possibility that the relevant literature may contain findings reported for non-laboring tissue were in fact obtained with myometrium transitioning to a laboring phenotype. To address this problem we sought to identify culture conditions that could be implemented to prevent culture-induced changes in *ESR1*, *PTGS2* and *OXTR* expression, which are markers of the pro-contractile transformation. We examined whether the presence of CSS (5%) in the media affected the culture-induced changes of *ESR1*, *PTGS2* and *OXTR* in myometrium (Fig. 2). Supplementing media with serum is common practice during *in vitro* culture, however, our results indicate that supplementing with serum alone is not sufficient to prevent culture-induced changes in these key myometrial genes.

The balance between progesterone and estrogen plays a key role in transformation of the myometrium from the quiescent to a contractile state. Progesterone maintains pregnancy by promoting myometrial quiescence (3, 33), while estrogen is considered a principal endocrine factor responsible for instigating myometrial activation (1–3, 8). Both progesterone and estrogen maternal plasma levels are high during human pregnancy and remain elevated during parturition (4–7, 34). Previous studies have shown that the interaction between progesterone and progesterone receptor B (PR-B) suppresses *ESR1* expression, thereby rendering the myometrium refractory to circulating estrogen (8, 35). With advancing gestation, however, progesterone receptor A (PR-A) expression increases, which in turn represses the transcriptional activity of PR-B, and as a result the PR-B-mediated inhibition of *ESR1* expression is withdrawn (8, 35). Once myometrial tissue is removed from *in vivo* environment and cultured *in vitro*, the high plasma levels of progesterone and estrogen are no longer present, thereby removing the functional link between progesterone and estrogen (8), which could possibly account for the observed culture-induced changes in *ESR1*, *PTGS2* and *OXTR* expression (Fig. 1). Consistent with the existing literature (36, 37), we found that supplementing media with 500 nM P4 prevented the culture-induced up-regulation of *ESR1* mRNA abundance (Fig. 3A) over 48 h. Further, *ESR1* expression remained repressed when 500 nM P4 was administered in combination with 400 nM E2. While culture-induced up-regulation of *ESR1* expression was prevented by 500 nM P4, P4 alone was unable to prevent the culture-induced increase in *PTGS2* expression *in vitro*. It was anticipated that P4 alone would prevent the culture-induced increase in *PTGS2* expression based on previous reports that P4 inhibits *PTGS2* expression in myometrial cells via direct interaction of the progesterone receptor (PR) with NF-κB p65, as well as by progesterone-induced expression of the NF-κB inhibitor, IκB-α (38, 39). The reason for this discrepancy is unclear. Interestingly, supplementing media with P4 + E2 significantly increased *PTGS2* mRNA abundance beyond the level of culture-induced increase in the absence of steroids (Fig. 1B and 3B).

A study using immortalized human myocytes reported that progesterone binding to its receptor up-regulated zinc finger E-box binding homeobox protein 1 and suppressed miR-200b/429, which led to down-regulated *OXTR* expression (40). We found that supplementing media with P4 alone was unable to prevent the culture-induced decline in *OXTR* expression during culture (Fig. 3C). Furthermore, it was anticipated that

supplementing media with E2 would increase *OXTR* expression, as our group has previously shown that E2 treatment for 6 h significantly increased *OXTR* expression in myometrial tissue (9). Possibly, E2 was unable to up-regulate *OXTR* expression in the present study due to being administered in combination with P4.

In normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta (41, 42). Previous studies in the sheep, rat and wallaby have shown that stretch increased *PTGS2* and *OXTR* expression, whereas *ESR1* expression was not significantly affected (43–45). In humans, uterine wall tension was found to increase across gestation, as calculated by measuring uterine wall thickness and intrauterine pressure (46). Tension rose markedly beyond 30-weeks, and at 37-weeks ranged from approximately 10–75 kN/m². This study examined stretch as a culture condition potentially affecting the *in vitro* changes in *ESR1*, *PTGS2* or *OXTR* expression. We found that constant stretch, applied by means of attaching a hanging weight to tissue strips, did not influence the changes in *ESR1*, *PTGS2* or *OXTR* mRNA levels (Figs. 1 and 4). This is at variance with previous reports that stretch has up-regulated *PTGS2* and *OXTR* expression in the uterus; however, it should be noted that those studies were performed in animals (43–45). It should also be noted that maximum tension applied in our assay (3 g; 7.35 kN/m²) was slightly below the range calculated by Sokolowski et al. at 37-weeks (46) (10–75 kN/m²). Applying tension >7.35 kN/m² in preliminary experiments was found to cause muscle tearing in our system, whereas 7.35 kN/m² stretched the tissue strips sustainably for the 48 h culture period. It has been reported that in a P4-dominated endocrine environment, moderate stretch maintains relaxation and quiescence; however, in the absence of P4 or excessive stretch, the uterus contracts (41, 42). Interestingly, although stretch did not directly affect the culture-induced changes in *ESR1*, *PTGS2* or *OXTR* expression, it prevented steroids (P4 + E2) from blocking the culture-induced increase in *ESR1* mRNA abundance, and prevented steroids from increasing *PTGS2* mRNA abundance (beyond the increase *in vitro*).

It is well documented that the cAMP signalling pathway is up-regulated in the human myometrium throughout pregnancy to maintain uterine quiescence (47–49). Supplementing media with a cAMP analogue 8-Br-cAMP failed to prevent culture-induced changes in *ESR1*, *PTGS2* or *OXTR* mRNA abundance. We also examined effects of PMA, a pro-contractile agent, on *ESR1*, *PTGS2* and *OXTR* expression. PMA activates PKC, which stimulates signalling pathways mediated by extracellular signal-regulated kinase (ERK) and NF-κB, both involved in labor (50, 51). Furthermore, PKC activity is crucial for oxytocin-stimulated myometrial contractions (52). PMA was effective in modulating expression of both *PTGS2* and *OXTR*, but had no effect on *ESR1*. As expected for a pro-contractile agent, PMA up-regulated *PTGS2* expression *in vitro*, which was consistent with augmenting the transition toward a laboring phenotype. Interestingly, PMA prevented the culture-induced decline in *OXTR* expression. Actions of PMA on *OXTR* were therefore consistent with preserving the term not-in-labor phenotype, which is characterised by high *OXTR* expression.

Previous studies have shown that labor is an inflammatory process associated with increased production of pro-inflammatory mediators, increased expression of *PTGS2* mRNAs and increased NF-κB activity (17, 53–55). Supplementing media with NF-κB inhibitors such as MG-132 or BAY-11–7085, therefore, represented a feasible means of preventing culture-induced changes in *ESR1*, *PTGS2* or *OXTR* expression. These inhibitors, added either separately or in combination, had no effect on *ESR1*, *PTGS2* and *OXTR* mRNA abundance. This argues against the possibility that our culture conditions activated NFκB-dependent inflammatory pathways causing transition to a laboring phenotype. Nevertheless, preliminary data using two NF-κB inhibitors do not prevent the need for further detailed studies to fully explore the effects of various culture conditions on inflammatory pathway activation and the associated functional consequences in myometrial explant systems.

Conclusion

Term non-laboring human myometrial tissue undergoes culture-induced changes in expression of *ESR1*, *PTGS2* and *OXTR* that are consistent with transitioning toward a laboring phenotype. Culture-induced transition toward a laboring phenotype calls for caution as to whether non-laboring myometrial biopsies remain representative of non-laboring myometrial tissue throughout the course of experiments, and as such may cast doubt on findings gleaned from not-in-labor tissue. Through examining various culture conditions, we found progesterone blocked the culture-induced increase in *ESR1*, but failed to prevent culture-induced increase in *PTGS2*. Culture-induced decline in *OXTR* expression was prevented by supplementing media with PMA, however, this was concurrent with further stimulation of *PGTS2* expression toward a laboring phenotype. Culture-induced increase of *PTGS2* expression *in vitro* was not counteracted by any of the treatments examined, including supplementation with NF-κB inhibitors. Additional studies are warranted to determine appropriate culture conditions that prevent the changes of key myometrial genes *in vitro*, thereby providing a stable platform on which to investigate the regulation of myometrial biology. Before that, *in vitro* studies using myometrial cells or tissues should be interpreted cautiously regarding the relevance to myometrial regulation during pregnancy and labor *in vivo*.

Author Contributions

Conceived and designed experiments: MI, TZ, JWP. Sample collection: MI. Performed experiments: MI, JWP. Data analysis: MI, JWP. Assisted with data analysis: TZ, TB. Provided reagents and materials: TZ, JWP. Manuscript Writing: MI, JWP. Manuscript Editing: TB, TZ.

Ethical approval

All studies involving human participants were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent.

Conflict of Interest

The Author(s) declare(s) that there is no conflict of interest.

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Appendix F: Published manuscript

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Research Article

Modulation of Progesterone Receptor Isoform Expression in Pregnant Human Myometrium

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Background. Regulation of myometrial progesterone receptor (PR) expression is an unresolved issue central to understanding the mechanism of functional progesterone withdrawal and initiation of labor in women. *Objectives.* To determine whether pregnant human myometrium undergoes culture-induced changes in *PR* isoform expression ex situ and, further, to determine if conditions approaching the in vivo environment stabilise *PR* isoform expression in culture. *Methods.* Term nonlaboring human myometrial tissues were cultured under specific conditions: serum supplementation, steroids, stretch, cAMP, PMA, $PGF_{2\alpha}$, NF-κB inhibitors, or TSA. Following 48 h culture, *PR-T*, *PR-A*, and *PR-B* mRNA levels were determined using qRT-PCR. *PR-A*/*PR-B* ratios were calculated. *Results.* *PR-T* and *PR-A* expression and the *PR-A*/*PR-B* ratio significantly increased in culture. Steroids prevented the culture-induced increase in *PR-T* and *PR-A* expression. Stretch blocked the effects of steroids on *PR-T* and *PR-A* expression. PMA further increased the *PR-A*/*PR-B* ratio, while TSA blocked culture-induced increases of *PR-A* expression and the *PR-A*/*PR-B* ratio. *Conclusion.* Human myometrial tissue in culture undergoes changes in *PR* gene expression consistent with transition toward a laboring phenotype. TSA maintained the nonlaboring PR isoform expression pattern. This suggests that preserving histone and/or nonhistone protein acetylation is critical for maintaining the progesterone dependent quiescent phenotype of human myometrium in culture.

1. Introduction

Preterm birth is a major societal and economic problem that affects approximately 9.6% of pregnancies worldwide and accounts for 80–90% of neonatal morbidity and death [1–4]. The prevention of preterm birth continues to be an important health priority. There is a substantial body of evidence highlighting the importance of progesterone in maintaining the pregnant state by promoting myometrial quiescence and relaxation [5–7]. The withdrawal of progesterone action signals the end of pregnancy and in most mammalian species happens by a rapid fall in circulating levels of progesterone [8–12]. In humans and higher primates, however, maternal, fetal, and amniotic concentrations of progesterone remain elevated during parturition and delivery, suggesting that systemic progesterone withdrawal does not occur at the initiation of labor [13–15]. Nonetheless, the administration of a synthetic progesterone antagonist, RU486, to humans at any stage of pregnancy promotes cervical ripening and parturition [5, 6, 16–19]. As such a "functional" withdrawal of progesterone action has been proposed to explain the loss of propregnancy progesterone actions despite circulating levels of progesterone remaining elevated. The exact mechanism of functional progesterone withdrawal is unclear and in recent years it has been the focus of intense research. One proposed mechanism is that functional progesterone withdrawal occurs through a decrease in myometrial responsiveness caused by a change in progesterone receptor (PR) isoform expression. Two major isoforms, PR-A and PR-B, exist in humans. PR-B is the principal transcriptional mediator of progesterone action and maintains uterine quiescence, while PR-A represses the transcriptional activity of PR-B and therefore

decreases progesterone responsiveness [3, 5, 6, 19]. Recent work has also shown that PR-A ligand-independently stimulates the expression of the key labor promoting gene *Cx43* [20]. Thus, genomic progesterone responsiveness is believed to be regulated by the opposing actions of PR-A and PR-B and is inversely associated with the PR-A/PR-B ratio [3, 5, 6, 19]. Indeed, several studies, including our own, have shown that myometrial expression of *PR-A* has significantly increased late in human pregnancy and with the onset of labor [21–24].

Elucidating the mechanism of functional progesterone withdrawal is therefore important for understanding the mechanisms regulating the balance between uterine quiescence and contractions. Outside of clinical trials, researchers are primarily limited to observational studies on human pregnancy. Interventional studies rely on animal models of pregnancy as well as on in vitro experiments using human myometrial smooth muscle cell lines and tissues. Human cell cultures are a valuable in vitro tool used to gain insight into numerous physiological and pathological processes; however, concerns have been raised about the lifespan of cultured primary cells [25] as well as their ability to remain to be representative of the tissue of origin [26–29]. The use of ex vivo myometrial tissue may represent the in vivo phenotype more closely and can involve utilizing smooth muscle biopsy samples as small pieces or dissecting the tissue into strips. Tissue strips are primarily utilized to examine myometrial contractility [30–33] such as the dynamic phosphorylation events that occur in phase with contractions [34, 35].

Although the use of ex vivo tissues pieces and strips has greatly facilitated studies into gene expression and regulation, both approaches rely on the assumption that the tissue phenotype remains stable across the course of the study. For instance, it is assumed that nonlaboring myometrium retains a nonlaboring phenotype ex vivo providing an experimental system to induce labor-associated changes. Myometrial strips from nonlaboring pregnant women, however, spontaneously develop contractions ex vivo over the course of just 1-2 h, suggesting a rapid transition away from the nonlaboring in vivo phenotype [30, 33, 35]. Furthermore, tissue incubation studies are routinely performed for 48 h or more; therefore the transition away from the original phenotype may be even more pronounced after 48 h culture in vitro.

The aim of this study was to determine if nonlaboring myometrial tissue pieces and strips undergo culture-induced changes in PR expression that are consistent with transition to a PR isoform expression pattern similar to labor. We further aimed to identify culture conditions that could be implemented to block or minimize such transition in vitro, presenting researchers with a stable platform on which to conduct experimental studies.

Here we report that nonlaboring human myometrium undergoes culture-induced changes in *PR* isoform expression in vitro comparable with the changes attributed to functional progesterone withdrawal at labor. We further report that supplementing media with the histone deacetylase inhibitor (HDACi), trichostatin A (TSA), prevents the culture-induced functional progesterone withdrawal phenomenon by maintaining a low *PR-A/PR-B* ratio, consistent with maintenance of a nonlaboring phenotype.

2. Materials and Methods

2.1. Consumables and Reagents. Superscript III First-Strand Synthesis System, Ultrapure Glycogen, UltraPure Agarose, and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA-free 50 reactions were from Ambion (Thermo Fisher). Alien QRT-PCR Inhibitor Alert 400 Reactions were purchased from Integrated Sciences Pty (Sydney, Australia). (R)-MG132, BAY-11-7085, Phorbol Myristate Acetate (PMA), and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were obtained from Cayman Chemical Company (Michigan, USA). 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), PCR primers, progesterone, and estradiol were purchased from Sigma (St Louis, USA). 2 mL 2.8 mm ceramic bead kits (CK28-R) for the Precellys homogenizer (Bertin Instruments, France) were purchased from Thermo Fischer Scientific (Melbourne, Australia). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES, Dulbecco's Modified Eagle Medium (DMEM), and Charcoal Stripped Fetal Bovine Serum were obtained from Gibco (Carlsbad, USA). SYBR Green 2x Master mix was from Applied Biosystems (Carlsbad, USA). TSA was supplied by Bio-Scientific Pty. Ltd. (Sydney, Australia).

2.2. Myometrial Tissue Acquisition. These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). Human myometrial samples were obtained from the lower uterine segment during elective Caesarean section (CS) of singleton term pregnancies (38.2–39.6 weeks' gestation). Patient body mass index (BMI) range was 18.3–38.0, and none of the patients were in-labor. The indications for elective CS were previous CS, placenta praevia, fetal distress, or breach presentation. Women were excluded if they were given steroids. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of postpartum hemorrhage. Samples were therefore exposed to oxytocin for a brief period of time (3 min). All samples were placed on ice in serum-free medium containing DMEM with high glucose, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/mL Gentamicin, and 10 mM HEPES for the transfer to the laboratory.

2.3. Myometrial Tissue (Explant) Culture. Approximately 100 mg tissue from each sample was immediately snap-frozen in liquid nitrogen for subsequent analysis. The remaining myometrium was dissected into approximately $2 \times 2 \times 2$ mm pieces and washed in serum-free media. Samples were then incubated in serum-free or 5% (v/v) charcoal stripped serum- (CSS-) supplemented media in a 37°C, 95% air/5% CO_2 humidified incubator for 48 h. The 5% CSS-supplemented media contained DMEM with high glucose, 5% CSS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 40 μg/mL Gentamicin, and 10 mM HEPES. To determine the effects of steroids, myometrial samples were incubated with physiological concentrations of progesterone (P4; 500 nM) and/or estradiol (E2; 400 nM) [14] in a 37°C, 95% air/5% CO_2 humidified incubator for 48 hours. To determine the effect of stretch

Table 1: cDNA primer sequences for *PR-T* and *PR-B*.

Primer	Primer sequence (5′-3′)	Amplicon size	GeneBank #
PR-T	F: GTGGGAGCTGTAAGGTCTTCTTTAA R: AACGATGCAGTCATTTCTTCCA	83	NM000926.4
PR-B	F: TCGGACACCTTGCCTGAAGT R: CAGGGCCGAGGGAAGAGTAG	68	NM000926.4

PR-T, progesterone receptor total; *PR-B*, progesterone receptor isoform B.

on human myometrium, myometrial tissue strips (2 × 2 × 10 mm) were cultured in 5% CSS-containing media for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator while being subjected to 0, 1, or 3 g of constant stretch. Constant stretch was applied by using nylon thread to attach stainless steel weights to the ends of strips and then suspending the strips in 30 mL of culture media in 50 mL tubes (strips subjected to 0 g only were tied at one end). To determine the effect of stretch and steroids on human myometrium, myometrial strips were cultured in 5% CSS-containing media with 500 nM P4 and 400 nM E2 for 48 h in a 37°C, 95% air/5% CO_2 humidified incubator while being subjected to 0, 1, or 3 g of stretch. To determine the effects of the signalling pathways involved in myometrial relaxation and contraction, myometrial tissues were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with the cAMP analogue 8-Br-cAMP (250 μM), PMA (0.1, 1.0 μM), $PGF_{2\alpha}$ (1, 10, 100, and 100 nM), or the nuclear factor-κB (NF-κB) inhibitors MG-132 (2.0, 5.0, and 10.0 μM) and BAY-11-7085 (2.0, 5.0, and 10.0 μM) individually or in combination (10.0 μM MG-132 + 10.0 μM BAY-11-7085). Furthermore, myometrial tissues were incubated for 48 h a 37°C, 95% air/5% CO_2 in 5% CSS-containing media supplemented with TSA (0.5, 1.0, 2.5, or 5.0 μM). Vehicle was DMSO (0.1%). Following each incubation, the media were decanted and tissue pieces or strips were snap-frozen using liquid nitrogen and stored at −80°C for subsequent analyses.

2.4. RNA Extraction, Reverse Transcription, and Real-Time Quantitative PCR. RNA was extracted from 100 mg of tissue using TRizol Reagent (Thermo Fisher) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys 24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-*free* kit (Thermo Fisher). An ND-1000 spectrophotometer was used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 μg of total RNA) was spiked with 0.5×10^7 copies of Alien RNA and reverse-transcribed using the SuperScript III First-Strand Synthesis System with random hexamer primers. Quantitative RT-PCR was performed using an ABI 7500 Sequence Detector. No-reverse transcription (no-RT) negative controls were prepared for each sample to ensure there was no DNA contamination. The final volume of each PCR reaction was 20 μL containing 10 μL of 2x SYBR Green PCR Master Mix (Thermo Fisher), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 μL with 1.0 μL of 2.5 μM of Alien Primer Mix, 10 μL of 2x SYBR Green PCR, and the same amount of cDNA as the target genes and MilliQ water. No-template control samples (NTCs) were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express and are shown in the Table 1.

2.5. Data and Statistical Analysis. All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the delta C_t (ΔC_t) method [36]. The relative mRNA abundance of *PR-A* was calculated by subtracting the relative mRNA abundance of *PR-B* from that of *PR-T*. All mRNA relative abundance values were checked for normal distribution using Shapiro-Wilk normality test and if data was not normally distributed, then it was logarithmically transformed to approach normal distribution. Statistical analyses were conducted with GraphPad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t*-test was used. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by post hoc test of Dunnett multiple comparisons was used. p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Culture-Induced Changes in Myometrial PR Isoform Expression over Time. Myometrial tissues were incubated for 0, 1, 2, 6, 24, or 48 h in serum-free media to determine changes in *PR* isoform expression that occurred upon being removed from the in vivo environment and cultured in vitro. *PR-T*, *PR-B*, and *PR-A* mRNA abundance were determined across the timeline and the *PR-A*/*PR-B* ratio calculated.

PR-T mRNA abundance was significantly increased after 48 h culture ($p = 0.0301$) (Figure 1(a)) and was attributable to increased *PR-A* mRNA abundance, which was significantly increased beyond 24 h culture ($p = 0.0121$) (Figure 1(b)). *PR-B* mRNA abundance remained relatively constant (Figure 1(c)). The *PR-A*/*PR-B* ratio was significantly increased after 6 h in vitro culture ($p = 0.0487$) and highly significant beyond 24 h culture ($p < 0.0001$) (Figure 1(d)).

3.2. Controlling Changes in PR Isoform Expression Using Serum. CSS is often used in myometrial culture media [37–39]. Having observed a culture-induced increase in *PR-A* expression (and thus *PR-T* expression), we examined whether supplementing media with 5% CSS affected culture-induced

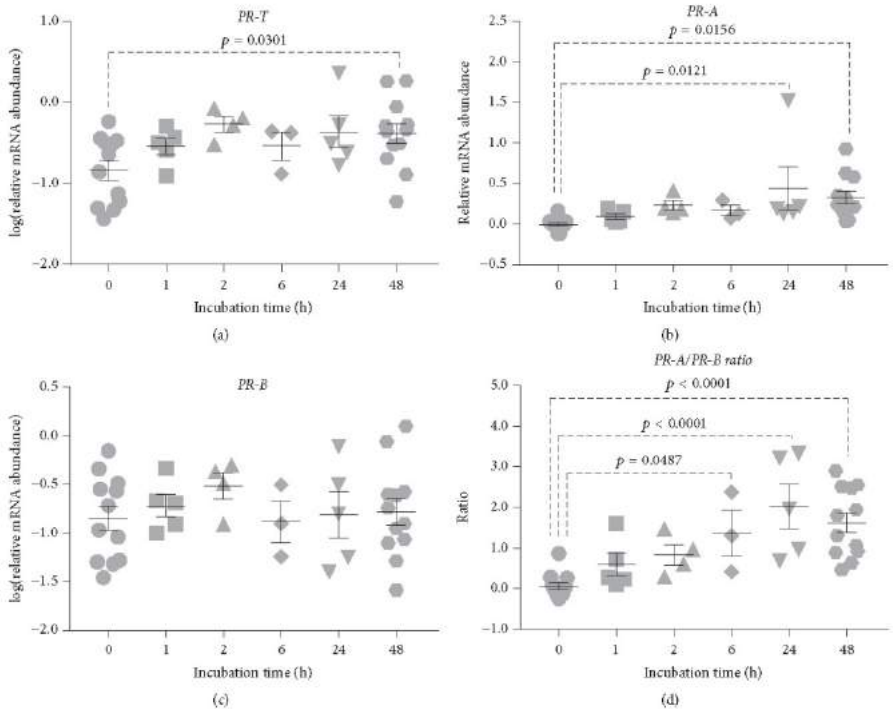

FIGURE 1: Culture-induced change in myometrial PR isoform expression over time: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples at different time points (0, 1, 2, 6, 24, and 48 h), and expressed relative to Alien reference. In addition, *PR-A*/*PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A*/*PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

changes in *PR* expression. CSS supplementation had no effect on culture-induced changes in *PR* expression. After 48 h culture, there was no significant difference in *PR-T*, *PR-A*, or *PR-B* mRNA abundance. Furthermore, there was no significant difference in *PR-A*/*PR-B* expression ratio between myometrial tissues cultured in serum-free media versus 5% CSS-supplemented media (Figure 2).

3.3. Controlling Changes in PR Isoform Expression Using Steroids. Relative abundance of *PR-T*, *PR-A*, and *PR-B* mRNA was measured in myometrial tissues incubated for 48 h in the presence of 500 nM P4 or 500 nM P4 + 400 nM E2, which are hormone concentrations in term maternal plasma [14].

PR-T mRNA abundance significantly increased in DMSO-treated (control) tissues following 48 h incubation ($p = 0.0317$) (Figure 3(a)). Upon supplementing media with 500 nM P4, *PR-T* mRNA abundance was reduced after 48 h culture relative to the control; however, the effect did not reach statistical significance ($p = 0.2457$) (Figure 3(a)). Supplementing media with the combination of 500 nM P4 + 400 nM E2 for 48 h prevented the increase in *PR-T* mRNA abundance to the extent that there was a significant difference relative to 48 h DMSO-treated control tissues ($p = 0.0232$) (Figure 3(a)).

Similarly, *PR-A* mRNA abundance significantly increased in DMSO-treated (control) tissues following 48 h incubation ($p = 0.0036$) and supplementing media with 500 nM P4 reduced *PR-A* mRNA abundance after 48 h relative to the control; however, the difference was not statistically significant ($p = 0.3234$) relative to 48 h DMSO-treated control tissues (Figure 3(b)). Supplementing media with the combination of 500 nM P4 + 400 nM E2 prevented the increase in *PR-A* mRNA abundance to the extent that there was a significant

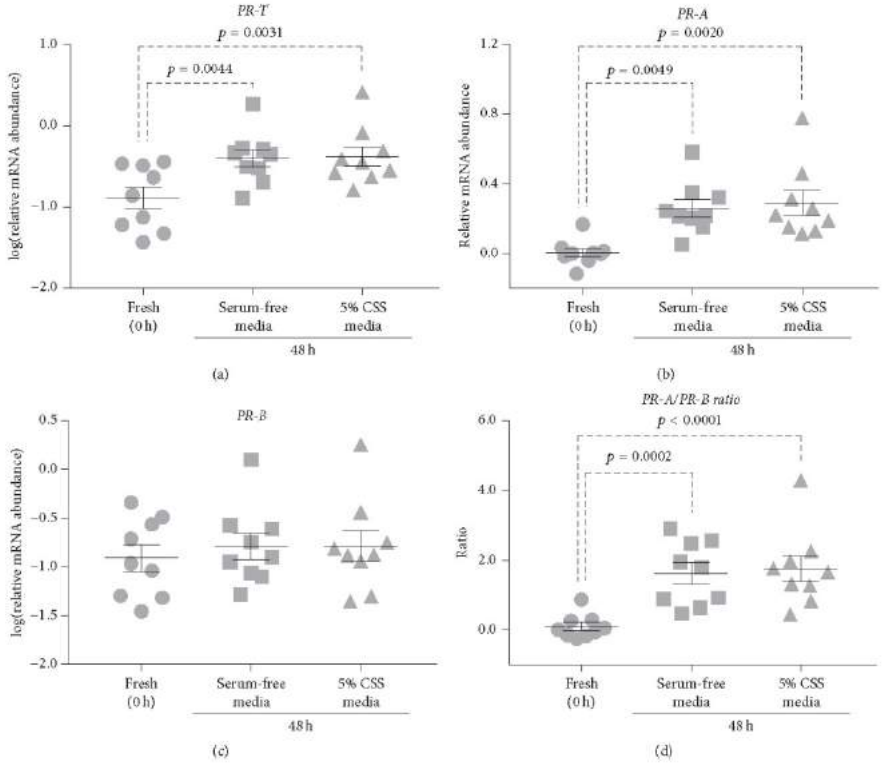

Figure 2: Effect of serum on culture-induced changes in PR isoform expression in vitro: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples following 48 h incubation in serum-free media or media supplemented with 5% CSS ($n = 9$) and expressed relative to Alien reference. In addition, *PR-A*/*PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A*/*PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

difference relative to 48 h DMSO-treated control tissues ($p = 0.0175$) (Figure 3(b)).

PR-B mRNA abundance remained unchanged over 48 h of culture and supplementing media with 500 nM P4 alone, or 500 nM P4 + 400 nM E2, had no significant effect on *PR-B* mRNA abundance (Figure 3(c)).

The *PR-A*/*PR-B* expression ratio significantly increased in DMSO-treated (control) tissues following 48 h incubation ($p = 0.0054$) (Figure 3(d)). P4-supplementation had no effect on the *PR-A*/*PR-B* expression ratio relative to DMSO-treated control tissues and remained significantly elevated compared to fresh tissues ($p = 0.0006$) (Figure 3(d)). Similarly, the combination of 500 nM P4 + 400 nM E2 had no significant effect on the *PR-A*/*PR-B* expression ratio after 48 h relative to DMSO-treated control tissues ($p > 0.9999$), and the *PR-A*/*PR-B* expression ratio remained significantly elevated relative to the fresh tissues ($p = 0.0053$) (Figure 3(d)).

3.4. Controlling Changes in PR Isoform Expression Using Stretch. Myometrial tissue strips were subjected to 0, 1, or 3 g of stretch for 48 h to determine whether applying stretch to the muscle influenced culture-induced changes in *PR* isoform expression. The effect of stretch was investigated in the absence and presence of steroids (500 nM P4 + 400 nM E2).

Stretch (1 or 3 g) applied to myometrial strips for 48 h had no effect on *PR-T* expression (Figure 4(a)) relative to nonstretched (0 g) control strips. Interestingly, stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *PR-T* mRNA levels (Figure 4(b)),

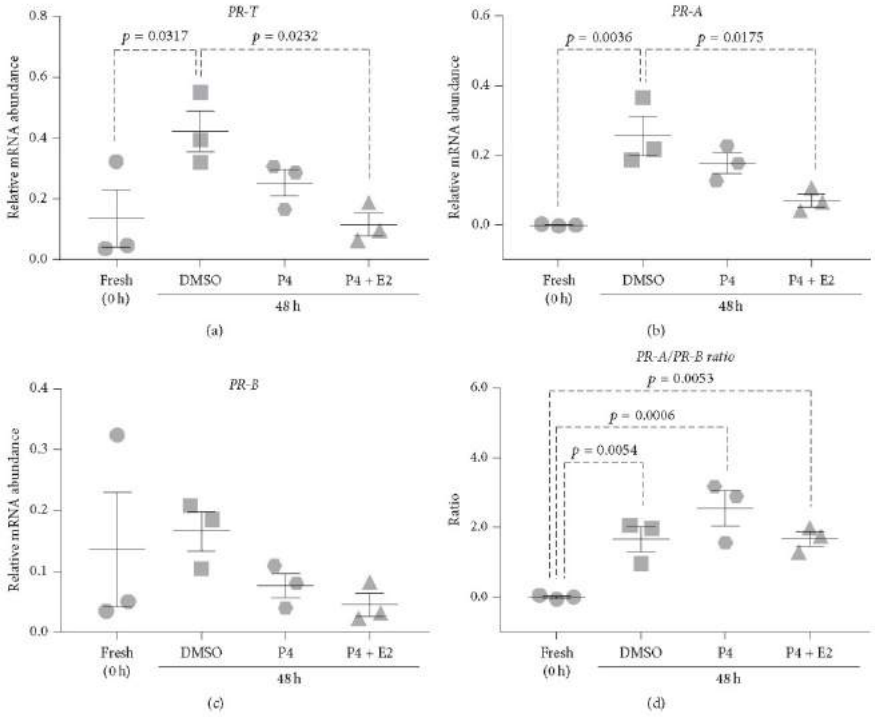

FIGURE 3: Effect of steroids on culture-induced changes in PR isoform expression in vitro: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples following 48 h incubation in the presence of 500 nM progesterone (P4) or 500 nM P4 + 400 nM estradiol (E2) ($n = 3$) and expressed relative to Alien reference. In addition, *PR-A/PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

indicating that P4 + E2 was no longer effective in decreasing *PR-T* mRNA abundance compared to fresh tissues.

Similarly, stretch applied to myometrial strips for 48 h had no effect on *PR-A* mRNA expression (Figure 4(c)). Stretch applied in the presence of 500 nM P4 + 400 nM E2 likewise had no effect (Figure 4(d)), indicating that P4 + E2 was no longer effective in preventing culture-induced increase in *PR-A* mRNA abundance compared to fresh tissues.

As seen in Figure 4(e), 0–3 g stretch had no effect on *PR-B* expression in the tissue strips. Stretch in the presence of 500 nM P4 + 400 nM E2 for 48 h also had no significant effect on *PR-B* mRNA levels (Figure 4(f)).

The *PR-A/PR-B* expression ratio was calculated and there was significant increase following 48 h incubation in nonstretched (0 g) control strips when compared to fresh tissues ($p = 0.0164$) (Figure 4(g)). Applying stretch (1 or 3 g) to myometrial strips for 48 h had no effect on expression ratio when compared to nonstretched strips (Figure 4(g)). Further, there was significant increase in *PR-A/PR-B* expression ratio following 48 h incubation in nonstretched (0 g) control strips in the presence of 500 nM P4 + 400 nM E2 when compared to fresh tissues ($p = 0.0067$) (Figure 4(h)). Stretch (1 or 3 g) applied in the presence of 500 nM P4 + 400 nM E2 had no effect on expression ratio after 48 h culture when compared to control strips (Figure 4(h)).

3.5. Controlling Changes in PR Isoform Expression Using Cyclic-AMP and PMA. Relative abundance of *PR-T*, *PR-A*, and *PR-B* mRNA was measured in myometrial tissue incubated for 48 h in the presence of 8-Br-cAMP (250 μM), PMA (0.1 and 1.0 μM), or vehicle (DMSO).

Supplementing culture media with 250 μM 8-Br-cAMP had no effect on mRNA abundance for *PR-T*, *PR-A*, or *PR-B* (Figures 5(a)–5(c)). The *PR-A/PR-B* expression ratio in DMSO-treated tissues was significantly elevated following 48 h incubation when compared to fresh tissues ($p = 0.0236$)

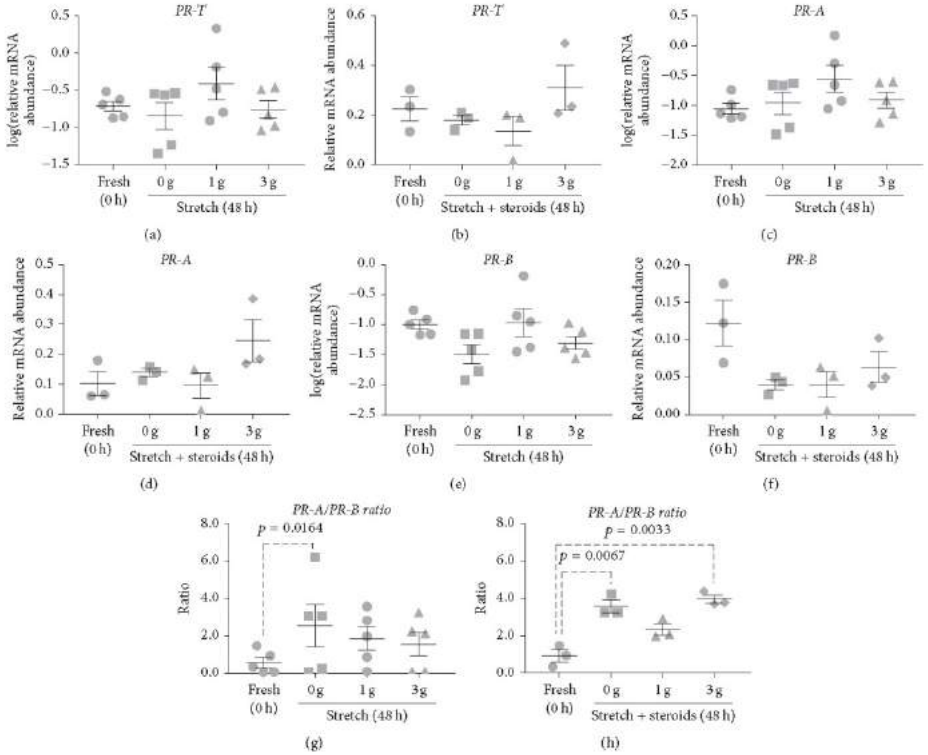

FIGURE 4: Effect of stretch in the absence or presence of steroids on culture-induced changes in PR isoform expression in vitro: Relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial strips while applying 0, 1, and 3 g of stretch for 48 h ($n = 5$), as well as in presence of steroids (500 nM P4 + 400 nM E2) while applying 0, 1, and 3 g of stretch for 48 h ($n = 3$), and expressed relative to Allen reference. In addition, *PR-A/PR-B* expression ratio was calculated. (a) Effect of stretch on *PR-T* mRNA abundance. (b) Effect of stretch and steroids on *PR-T* mRNA abundance. (c) Effect of stretch on *PR-A* mRNA abundance. (d) Effect of stretch and steroids on *PR-A* mRNA abundance. (e) Effect of stretch on *PR-B* mRNA abundance. (f) Effect of stretch and steroids on *PR-B* mRNA abundance. (g) Effect of stretch on *PR-A/PR-B* expression ratio. (h) Effect of stretch and steroids on *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and if necessary was logarithmically transformed to approach normal distribution (Shapiro-Wilk normality test). Data was analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

(Figure 5(d)). Supplementing culture media with 250 μM 8-Br-cAMP did not prevent the increase in the *PR-A/PR-B* expression ratio ($p = 0.0141$) (Figure 5(d)).

Supplementing media with 0.1 or 1.0 μM PMA, a protein kinase C (PKC) activator, had no significant effect on mRNA abundance for *PR-T* or *PR-A*, relative to 48 h DMSO-treated control tissues (Figures 5(e) and 5(f)). *PR-B* mRNA abundance was reduced by both PMA treatments relative to DMSO-treated control tissues; however, the decreases did not reach statistical significance ($p = 0.3659$ and $p = 0.5259$, resp.) (Figure 5(g)). The *PR-A/PR-B* expression ratio was significantly elevated in DMSO-treated control tissues at 48 h, relative to fresh tissues ($p = 0.0428$) (Figure 5(h)). Supplementing media with 0.1 μM PMA significantly increased the *PR-A/PR-B* expression ratio beyond levels detected in the 48 h DMSO-treated control tissues ($p = 0.0451$) (Figure 5(h)) and was attributable to the, albeit nonsignificant, decline in *PR-B* mRNA abundance. The *PR-A/PR-B* expression ratio was significantly elevated after 48 h in 1.0 μM PMA-treated tissues compared to fresh tissues ($p = 0.0023$) (Figure 5(h)).

3.6. Controlling Changes in PR Isoform Expression Using $PGF_{2\alpha}$. Relative abundance of *PR-T*, *PR-A*, and *PR-B* mRNA

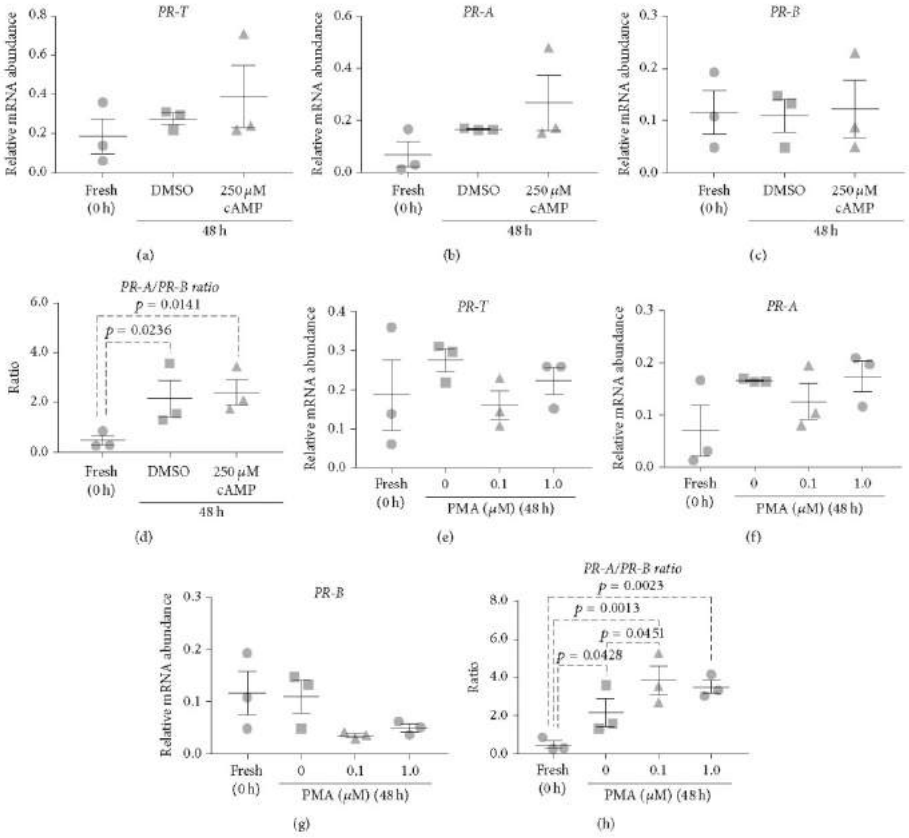

FIGURE 5: Effect of cAMP and PMA on culture-induced changes in PR isoform expression in vitro: Relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples ($n = 3$) following 48 h incubation in the presence of 8-Br-cAMP (250 μM) or PMA (0.1 and 1.0 μM) and expressed relative to Alien reference. In addition, *PR-A*/*PR-B* expression ratio was calculated. (a) Effect of 8-Br-cAMP on *PR-T* mRNA abundance. (b) Effect of 8-Br-cAMP on *PR-A* mRNA abundance. (c) Effect of 8-Br-cAMP on *PR-B* mRNA abundance. (d) Effect of 8-Br-cAMP on *PR-A*/*PR-B* expression ratio. (e) Effect of PMA on *PR-T* mRNA abundance. (f) Effect of PMA on *PR-A* mRNA abundance. (g) Effect of PMA on *PR-B* mRNA abundance. (h) Effect of PMA on *PR-A*/*PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

was measured in myometrial tissue incubated for 48 h in the presence of $PGF_{2\alpha}$ (1, 10, 100, or 1000 nM) or vehicle (DMSO).

Following 48 h incubation *PR-T* mRNA abundance in DMSO-treated control tissues was elevated relative to fresh tissues but did not reach statistical significance (Figure 6(a)). $PGF_{2\alpha}$ treatments had no effect on *PR-T* mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 6(a)).

PR-A mRNA abundance was significantly increased in DMSO-treated control tissues relative to fresh tissues ($p = 0.0451$) (Figure 6(b)). $PGF_{2\alpha}$ treatments had no effect on PR-A mRNA abundance relative to 48 h DMSO-treated control tissues (Figure 6(b)).

PR-B mRNA abundance remained unchanged following 48 h incubation and was not affected by $PGF_{2\alpha}$ treatments (Figure 6(c)).

In 48 h DMSO-treated tissue the *PR-A*/*PR-B* expression ratio was significantly elevated relative to fresh tissues ($p = 0.0294$) (Figure 6(d)). $PGF_{2\alpha}$ supplementation (1, 10, 100, or

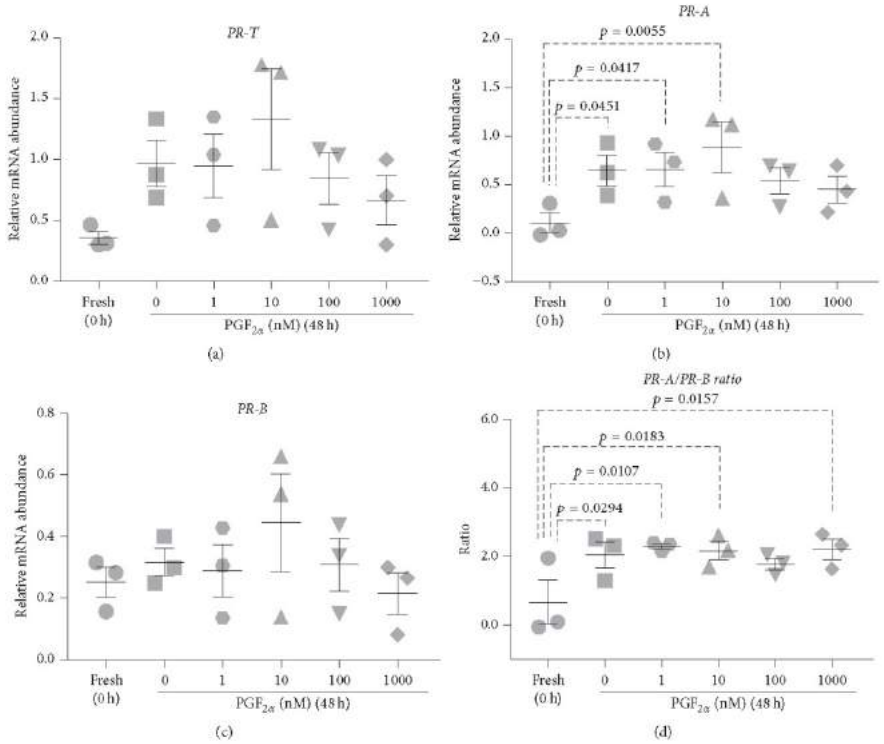

FIGURE 6: Effect of $PGF_{2\alpha}$ on culture-induced changes in PR isoform expression in vitro: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples ($n = 3$) following 48 h incubation in the presence of $PGF_{2\alpha}$ (1, 10, 100, and 1000 nM) and expressed relative to Alien reference. In addition, *PR-A*/*PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A*/*PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

1000 nM) had no effect on the *PR-A*/*PR-B* expression ratio relative to DMSO-treated control tissues (Figure 6(d)).

3.7. Controlling Changes in PR Isoform Expression Using NF-κB Inhibitors. The NF-κB inhibitors, MG-132 and BAY-11-7085, were employed to test whether NF-κB pathway activation was involved in the *PR* isoform expression changes induced by in vitro culture.

Following 48 h incubation, there was no significant difference in *PR-T* mRNA abundance between vehicle-treated tissues and fresh tissues (Figure 7(a)). Incubating myometrial samples with 2.0, 5.0, or 10.0 μM MG-132 or BAY-11-7085 individually or in combination (10 μM each) had no effect on *PR-T* mRNA abundance relative to vehicle-treated tissues (Figure 7(a)).

PR-A mRNA abundance in vehicle-treated tissues significantly increased compared to fresh tissues following 48 h incubation ($p = 0.0170$) (Figure 7(b)). Supplementing culture media with 2.0, 5.0, or 10.0 μM MG-132 or BAY-11-7085 individually or in combination (10 μM each) had no significant effect on *PR-A* mRNA abundance relative to 48 h vehicle-treated tissues (Figure 7(b)).

Following 48 h incubation, there was no significant difference in *PR-B* mRNA abundance between vehicle-treated tissues and fresh tissues (Figure 7(c)). Incubating myometrial samples with 2.0, 5.0, or 10.0 μM MG-132 or BAY-11-7085 individually or in combination (10 μM each) had no effect on *PR-B* mRNA abundance relative to vehicle-treated tissues (Figure 7(c)).

Following 48 h incubation, the *PR-A*/*PR-B* expression ratio was significantly elevated in vehicle-treated tissues

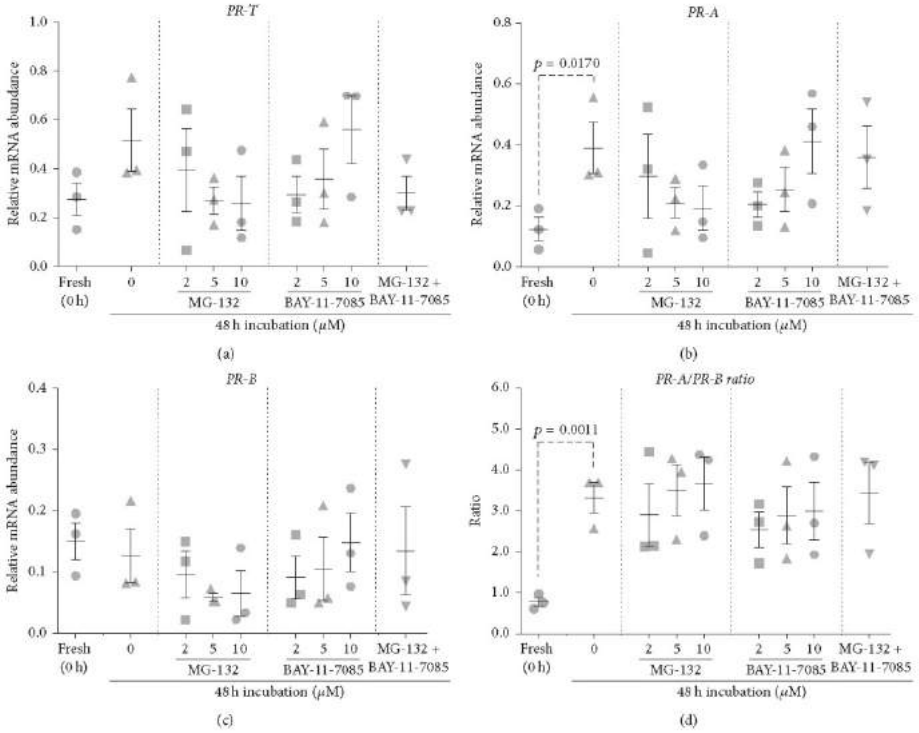

FIGURE 7: Effect of NF-κB inhibitors, MG-132 and BAY-11-7085, on culture-induced changes in PR isoform expression in vitro: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial samples ($n = 3$) following 48 h incubation in the presence of different NF-κB inhibitors and expressed relative to Allen reference RNA. In addition, *PR-A/PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A/PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

compared to fresh tissues ($p = 0.0011$) (Figure 7(d)). Supplementing culture media with 2.0, 5.0, or 10.0 μM MG-132 or BAY-11-7085 individually or in combination (10 μM each) had no effect on *PR-A/PR-B* expression ratio compared to the vehicle-treated tissues (Figure 7(d)).

3.8. Controlling Changes in PR Isoform Expression Using TSA. Relative abundance of *PR-T*, *PR-A*, and *PR-B* mRNA was measured in myometrial tissue incubated for 48 h in the presence of TSA (0.5, 1.0, 2.5, and 5.0 μM) or vehicle (DMSO).

There was no significant difference in *PR-T* mRNA abundance between 48 h vehicle-treated tissues and fresh tissues (Figure 8(a)). Incubating myometrial samples with 0.5, 1.0, 2.5, and 5.0 μM TSA had no effect *PR-T* mRNA abundance relative to 48 h vehicle-treated tissues (Figure 8(a)).

PR-A mRNA abundance was significantly increased in 48 h vehicle-treated tissues relative to fresh tissues ($p = 0.0431$) (Figure 8(b)). Culture-induced increases in *PR-A* mRNA abundance were inhibited by supplementing media with TSA. The extent of inhibition reached statistical significance, relative to 48 h vehicle-treated tissue, at 5.0 μM TSA ($p = 0.0305$) (Figure 8(b)).

PR-B mRNA abundance did not change following 48 h incubation and was unaffected by TSA treatments relative to vehicle-treated tissues (Figure 8(c)).

Following 48 h incubation, the *PR-A/PR-B* expression ratio was significantly elevated in vehicle-treated tissues compared to fresh tissues ($p = 0.0002$) (Figure 8(d)). The *PR-A/PR-B* expression ratio was significantly lower in tissue treated with 2.5 and 5.0 μM TSA ($p = 0.0003$ and $p < 0.0001$, resp.) relative to 48 h vehicle-treated tissues (Figure 8(d)). TSA dose-dependently prevented culture-induced increases in the *PR-A/PR-B* expression ratio.

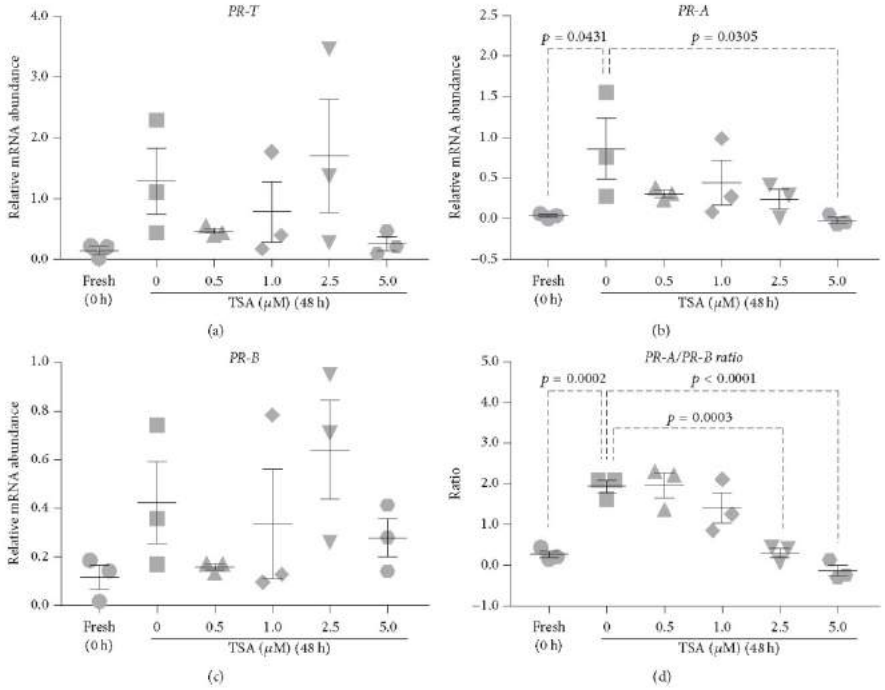

FIGURE 8: Effect of TSA on culture-induced changes in PR isoform expression in vitro: relative mRNA abundance of *PR-T*, *PR-A*, and *PR-B* was measured in term nonlaboring myometrial tissue samples ($n = 3$) following 48 h incubation in the presence of TSA (0.5, 1.0, 2.5, and 5.0 μM) and expressed relative to Allen reference. In addition, *PR-A*/*PR-B* expression ratio was calculated. (a) *PR-T* mRNA abundance. (b) *PR-A* mRNA abundance. (c) *PR-B* mRNA abundance. (d) *PR-A*/*PR-B* expression ratio. *Data was checked for normality (Shapiro-Wilk normality test) and then analysed using 1-way ANOVA with multiple comparisons (Dunnett). Data are mean ± SEM.*

4. Discussion

Human tissue and cell cultures are a valuable in vitro tool used to investigate the maintenance of uterine quiescence and the mechanisms by which the myometrium transforms to an actively contracting organ at labor. Our previous results show that, upon culturing nonlaboring myometrial tissues in vitro, the tissue undergoes culture-induced changes in expression of the key myometrial genes *ESR1*, *PTGS2*, and *OXTR*, which are consistent with transition toward a procontractile, laboring phenotype (under review). In light of this evidence, we further examined whether *PR* isoform expression undergoes culture-induced changes that are consistent with transition to a procontractile, laboring phenotype.

In this study we examined changes in *PR* isoform expression via determining mRNA levels, an approach which is consistent with other studies in the field [40]. Nevertheless, we are aware that PR isoform protein levels may reflect PR function more closely than mRNA abundance especially in pregnancies complicated by intrauterine inflammation [41]. Previous studies have demonstrated, however, that there is close correspondence between PR isoform mRNA and protein expression changes in the human myometrium at normal term labor [23, 24], which is the context of this study. Furthermore, recent studies examining protein profiles in mammalian cells have found that transcription, not translation, mostly determines protein abundance [42] and that during periods of dynamic change, such as phenotype transition, changes in mRNA abundance play a dominant role in determining changes in protein levels [43]. Overall, assessing myometrial PR function by determining *PR-A* and *PR-B* mRNA levels appears a reliable approach in the patient population we examined.

Tissue incubation studies are routinely performed for 48 h or more [38, 39, 44]. Considering that nonlaboring human myometrium develops contractility in vitro in just 1-2 h [30, 33, 35, 45], the transition away from the original phenotype may be even more pronounced after such 48 h

incubations. Our results illustrate that, in human myometrial pieces, *PR-A* mRNA abundance begins increasing after just 1 h culture. With *PR-B* mRNA abundance remaining constant, a statistically significant increase in the *PR-A/PR-B* expression ratio was evident after just 6 h culture (Figure 1). Previous studies using enzyme-immunoassays found that samples collected from the upper segment myometrium during labor had higher total PR concentrations than samples collected prior to labor [46]. Haluska et al. [22] used rhesus monkey, another genus that lacks a systemic progesterone withdrawal, to look at the changes in PR isoform concentrations. They found that there was no change in total PR expression during the transition from late pregnancy to labor; however, they did find a significant shift in the ratio of PR isoforms [22]. More specifically, the myometrial *PR-A/PR-B* ratio increased significantly from late pregnancy to spontaneous labor at term [22]. Furthermore, Pieber et al. [47] performed immunoblot analyses on lower segment myometrium from pregnant women and reported an increase in the PR-A protein abundance during labor, while levels of PR-B were not altered by labor status. Recently, our group showed that the onset of labor is associated with increased abundance of *PR-A* mRNA and an increase in the *PR-A/PR-B* expression ratio in term human myometrium [24]. Our group has also found that the PR-A/PR-B protein ratio in pregnant human myometrium was 0.5 (a PR-B dominant state) at 30 weeks' gestation, which then increased to 1.0 at term prior to the onset of labor, and at the time of the labor the ratio increased further to 3.0 (a PR-A dominant state) [23]. These results indicate that *PR* mRNA levels reflect PR protein levels in human myometrium. Our observation that *PR-T* and *PR-A* mRNA abundance as well as the *PR-A/PR-B* expression ratio increased during culture is therefore consistent with the tissue transitioning to a labor-like state as a consequence of in vitro conditions.

This finding has implications for the interpretation in vitro of studies performed on nonlaboring myometrium, which may have in fact already transitioned to a labor-like phenotype during the early stages of the study and may therefore have affected the outcome of the study. To address this, we sought to identify culture conditions that could be implemented to maintain a nonlaboring state whereby human myometrium retained a low *PR-A/PR-B* expression ratio (a *PR-B* dominant state), thereby providing a more appropriate in vitro model for conducting studies into myometrial biology.

Previous studies utilizing myometrial culture (explants) often included CSS in their media [37–39]. Therefore, we examined whether supplementing culture media with 5% CSS affected culture-induced changes in *PR* isoform expression. Surprisingly, 5% CSS had no significant effect on *PR-T*, *PR-A*, or *PR-B* mRNA abundance after 48 h culture and consequently had no effect on the *PR-A/PR-B* expression ratio (Figure 2). While supplementing media with serum is common practice during in vitro culture, our results indicate that this practice is not sufficient to prevent culture-induced changes in *PR* isoform expression.

The steroid hormone progesterone plays a crucial role in maintaining pregnancy by promoting myometrial quiescence and relaxation [5–7]. In contrast to most mammalian species [8–12], no decrease in maternal serum levels of progesterone can be observed in humans and higher primates prior to the onset of labor [13–15]. Thus, the term "functional progesterone withdrawal" has been used to describe the withdrawal of progesterone action. Once myometrial tissue is removed from in vivo environment and cultured in vitro, the high plasma levels of progesterone are no longer present, which could possibly account for culture-induced changes in *PR* expression in vitro. To explore this, we incubated myometrial tissues in media that contained physiological concentrations of progesterone. Supplementing media with progesterone alone was not sufficient to prevent the culture-induced increases in *PR-T* and *PR-A* mRNA abundance. Moreover, progesterone decreased *PR-B* mRNA abundance; however it was not statistically significant; nevertheless this further exacerbated the increase in the *PR-A/PR-B* expression ratio (Figure 3). A previous study using myometrial strips showed that progesterone exerts rapid inhibition of the amplitude of myometrial contractions in vitro [30]. More recently, Baumbach et al. [31] investigated the suppression of uterine contractility using progesterone alone and in a combination with various tocolytics and found that progesterone alone had little effect inhibiting contractility [31]. This is consistent with our results where progesterone alone did not prevent culture-induced increases in *PR-T* and *PR-A* mRNA abundance.

In numerous mammalian species, the process of parturition, especially transformation of the myometrium from the quiescent to a contractile state, necessitates an increase in circulating estrogen concentrations prior to the onset of labor [11, 14, 48, 49]. In humans and higher primates, however, maternal estrogen levels are high for most of pregnancy and remain elevated during parturition and delivery [14, 50, 51]. Furthermore, our group reported a correlation between estrogen receptor 1 *(ESR1)* mRNA levels and the *PR-A/PR-B* mRNA ratio, which is indicative of a functional link between the PR and ESR1 systems [24]. In addition, this link between the two systems is in agreement with studies performed in a range of species demonstrating that progesterone decreases expression of ESR1, thus decreasing uterine responsiveness to estrogen [52, 53]. These results imply that the interaction between progesterone and PR-B suppresses ESR1 expression, therefore rendering the myometrium refractory to circulating estrogen [19]. However, with advancing gestation there is an increase in the expression of PR-A, which in turn represses the transcriptional activity of PR-B, and as a result the PR-B-mediated inhibition of ESR1 expression is withdrawn [19]. Once myometrial tissue is removed from in vivo environment and cultured in vitro, the high plasma levels of progesterone and estrogen are no longer present, thereby removing the functional link between progesterone and estrogen [24] which could possibly account for the observed culture-induced changes in *PR* expression in vitro. To explore this, we incubated myometrial tissue in media that contained physiological concentrations of P4 and E2. The combination of P4 and E2 prevented culture-induced increase in *PR-T* and *PR-A* mRNA abundance observed in vitro. However, P4 in combination with E2 also decreased *PR-B* mRNA abundance; nevertheless this decrease was not statistically significant.

As such, after 48 h culture the *PR-A/PR-B* expression ratio had still increased relative to fresh tissue and adopted a *PR-A* dominant state (Figure 3).

Throughout normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [54, 55]. A previous study using term nonlaboring human myometrium tissue showed that stretch applied to myometrial cells in culture resulted in decreased *PR-T* and *PR-B* mRNA expression [56]. We found that constant stretch, applied by means of hanging 1 or 3 g weights from tissue strips, had no effect on culture-induced changes in *PR* isoform expression (Figure 4). This is inconsistent with a previous report where stretch downregulated *PR-T* and *PR-B* expression; however, it should be noted that those studies used myometrial cells while our study uses myometrial tissue strips [56]. Previous animal studies suggest that progesterone is responsible for maintaining uterine quiescence and promoting myometrial hyperplasia and hypertrophy to inhibit any increase in uterine wall tension [57–60]. In addition, human studies show that, in a progesterone-dominated endocrine environment, moderate stretch possibly maintains relaxation and quiescence; however, in the absence of progesterone or excessive stretch, the uterus starts to contract [54, 55]. Interestingly, although stretch did not directly affect the culture-induced changes in *PR-T*, *PR-A*, or *PR-B* expression, the application of stretch prevented steroids (P4 + E2) from blocking culture-induced increases in *PR-T* and *PR-A* mRNA expression and prevented steroids (P4 + E2) from decreasing *PR-B* mRNA expression (Figures 3 and 4).

There is now extensive evidence to suggest that components of the cAMP signalling pathway are upregulated in the human myometrium throughout pregnancy to maintain uterine quiescence until term [61–65]. Moreover, our group showed that, in PHM1-31 cells, a pregnant human myometrial cell line, 8-Br-cAMP, an agonist for the protein kinase A (PKA) pathway, increased the expression of both *PR-A* and *PR-B* but had a net effect of decreasing the *PR-A/PR-B* expression ratio [66]. Supplementing media with a cAMP analogue was therefore examined as a potential means to prevent culture-induced changes in *PR* isoform expression. Although cAMP has a well-defined role in promoting myometrial relaxation, supplementing media with 8-Br-cAMP failed to prevent culture-induced changes in *PR-T* or *PR-A* mRNA abundance and increased *PR-A/PR-B* expression ratio (Figure 5).

In contrast to previously discussed treatments that attempted to prevent culture-induced changes in *PR* isoform expression, we also examined the effect of the procontractile agent, PMA, to determine whether *PR* expression would be driven further toward a labor-like state. Previous studies by our group show that PKC activation by PMA increased the *PR-A/PR-B* expression ratio by selectively increasing expression of *PR-A* [66]. This study found that supplementing culture with PMA further increased the *PR-A/PR-B* expression ratio in vitro, which was consistent with this procontractile agent driving further transition toward a laboring phenotype. Interestingly, PMA did not increase expression of *PR-A* but rather decreased expression of *PR-B* over the course of the myometrial culture (Figure 5).

There is increasing evidence that locally produced immune/inflammatory cytokines, particularly prostaglandins (PGs), are involved in normal term labor as well as infection-associated preterm labor [67–69]. In human pregnancy, administration of PGs or PG analogues at any stage of pregnancy transforms the myometrium and cervix and induces labor [69–73]. Previously, our group has tested the hypothesis that PGs, specifically $PGF_{2\alpha}$, induce functional progesterone withdrawal by altering myometrial *PR* expression in PHM1-31 cells [66]. $PGF_{2\alpha}$ produced a dose-dependent increase in expression of *PR-A*, but not *PR-B*, thereby resulting in an increase in the *PR-A/PR-B* expression ratio [66]. In this study, supplementing media with $PGF_{2\alpha}$ had no effect on *PR* mRNA abundance and therefore did not prevent culture-induced changes in the *PR-A/PR-B* expression ratio (Figure 6). This is not consistent with previous results where $PGF_{2\alpha}$ increased the *PR-A/PR-B* expression ratio by increasing PR-A expression [66].

Romero et al. [74] have shown that tissue-level inflammation in the myometrium, decidua, and fetal membranes plays a crucial role in the human parturition. In recent years, studies have demonstrated that myometrium in pregnant women at term exhibits biochemical and histological characteristics of inflammation, including increased expression of PGs, increased NF-κB activity, increased infiltration of neutrophils, and macrophages, which may precede the onset of active labor and is independent of infection [6, 8, 75–80]. Furthermore, studies using human myometrial cells have shown that progesterone inhibits the proinflammatory NF-κB transcription factor complex as a result of PR-induced expression of inhibitor-κB-α (NFKBIA), a major NF-κB repressor [81]. Supplementing media with NF-κB inhibitors therefore represented a potential means of preventing spontaneous changes in *PR* isoform expression. Supplementing media with MG-132 or BAY-11-7085 had no effect on *PR-T*, *PR-A*, and *PR-B* mRNA abundance and therefore did not prevent culture-induced changes in the *PR-A/PR-B* expression ratio (Figure 7).

Condon et al. [82] administered TSA, a specific and potent HDACi, to pregnant mice late in gestation and found increased histone H3 acetylation as well as a delay in the initiation of parturition by 24–48 h. Decreased histone acetylation in the pregnant uterus near term, caused by a marked decrease in expression of uterine coactivators with intrinsic histone acetyltransferase activity, might serve an important role in the loss of PR function, thus instigating a functional progesterone withdrawal and the initiation of labor [82]. Furthermore, Wilson et al. [83] used the mouse mammary tumor virus promoter to examine the impact of TSA on PR activated transcription and found that TSA removed the transcription factor nuclear factor 1 from the promoter and decreased PR-induced transcription [83]. Based on these results we hypothesised that TSA may modulate *PR* isoform expression and supplemented culture media with TSA in anticipation of maintaining a low *PR-A/PR-B* expression ratio in vitro. Excitingly, TSA produced a dose-dependent inhibition of culture-induced upregulation of *PR-A* mRNA

abundance. With no effect of *PR-B* mRNA abundance, TSA was successful in maintaining a low *PR-A/PR-B* expression ratio over 48 h culture, consistent with freshly isolated term nonlaboring myometrium and consistent with preventing in vitro transformation to a laboring phenotype (Figure 8). Using TSA to maintain a low *PR-A/PR-B* ratio could have important clinical ramifications in that progesterone therapy is currently a leading strategy for the prevention of preterm birth (reviewed by van Zijl et al. [84]). Efficacy of progesterone administration may be enhanced if an agent such as TSA could be administered to preserve or even restore progesterone sensitivity in women with threatened preterm labor.

5. Conclusion

Concerns have previously been raised about the ability of primary cells in culture to remain representative of their tissues of origin. Adding to this concern, our previous study shows that term nonlaboring human myometrial tissue undergoes culture-induced changes in expression of *ESR1*, *PTGS2*, and *OXTR* that are consistent with transitioning toward a laboring phenotype. In this study we examined *PR* isoform expression and found that *PR-T* and *PR-A* mRNA expression increased in untreated tissue over 48 h culture. Additionally, the *PR-A/PR-B* expression ratio significantly increased, consistent with transition to a laboring phenotype. Through examining various culture conditions, we were able to maintain a nonlaboring state of PR isoform expression by supplementing culture media with TSA, which prevented the culture-induced increase in *PR-A* mRNA abundance and maintained a low *PR-A/PR-B* expression ratio. In summary, this study demonstrates that

(i) human myometrial tissues undergo culture-induced upregulation of *PR-T* and *PR-A* mRNA expression, which significantly increases the *PR-A/PR-B* expression ratio in vitro, even in nontreated tissue;

(ii) the combination of progesterone and estrogen downregulated *PR-T* and *PR-A* mRNA expression;

(iii) stretch had no direct effect on *PR-T*, *PR-A*, or *PR-B* expression, but it blocked the effects of progesterone and estrogen on *PR-T and PR-A* expression;

(iv) cAMP was unable to control culture-induced changes in *PR* expression;

(v) PMA further upregulated *PR-A/PR-B* expression ratio;

(vi) $PGF_{2\alpha}$ had no effect of *PR* expression in vitro;

(vii) NF-κB inhibitors were unable to control culture-induced changes in *PR* expression;

(viii) TSA downregulated *PR-A* mRNA expression and downregulated *PR-A/PR-B* expression ratio.

Disclosure

The funding providers had no involvement in the study or production of this article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Marina Ilicic, Tamas Zakar, and Jonathan W. Paul conceived and designed the experiments. Marina Ilicic contributed to sample collection. Marina Ilicic and Jonathan W. Paul performed the experiments and contributed to data analysis and manuscript writing. Tamas Zakar and Jonathan W. Paul provided reagents and materials. Tamas Zakar contributed to manuscript editing.

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Appendix G: Published manuscripts

Paul JW, et al., J Hum Endocrinol 2017, 2: 008
DOI: 10.24966/HHE-9640/10008

HSOA Journal of Human Endocrinology

Research Article

Expression of KCNH2 (hERG1) and KCNE2 Correlates With Expression of Key Myometrial Genes in Term Pregnant Human Myometrium

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Abstract

Background

Loss of activity of K_v11.1 potassium channels (encoded by *KCNH2*) facilitates labor, and is associated with expression of an inhibitory subunit encoded by *KCNE2*.

Objective

To determine whether *KCNH2* and *KCNE2* expression was linked to expression of key genes involved in myometrial contractility, including the oxytocin receptor (*OXTR*), estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*) and prostaglandin-endoperoxidase synthase 2 (*PTGS2*). We further aimed to examine *KCNH2* and *KCNE2* expression in preterm samples.

Study Design

Biopsies of term or preterm, non-laboring human myometrium were analysed by qPCR to determine *KCNH2* and *KCNE2* mRNA abundance, as well as abundance of *OXTR*, *ESR1*, *PGR* and *PTGS2* mRNAs.

Results

KCNH2 and *KCNE2* expression were significantly correlated at term, but not correlated with BMI. *KCNH2* expression significantly correlated with *OXTR*, *ESR1*, *PGR* and *PTGS2* expression, while *KCNE2* expression correlated with the expression of *ESR1*, *PGR* and *PTGS2*. Preterm samples revealed no difference in *KCNH2* and *KCNE2* expression compared to term.

Conclusion

KCNH2 and *KCNE2* expression correlate with the expression of key myometrial genes implicated in parturition, further strengthening a role for K_v11.1 channels in human pregnancy. Neither gene was correlated with BMI, suggesting that previously reported effects of obesity likely affect protein levels rather than gene expression.

Keywords: *ESR1*; hERG; *KCNE2*; *KCNH2*; Key myometrial genes; Myometrium; *OXTR*; *PGR*; *PTGS2*

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Introduction

A number of studies have demonstrated that ion channels encoded by the ether-à-go-go-related gene 1 (ERG1) play a role in regulating the contractile activity of cardiac myocytes (reviewed by Vandenberg *et al.* [1]). Moreover, there is increasing evidence demonstrating a role for *ERG1* in regulating smooth muscle cell contractility. *ERG1* encodes the pore-forming α-subunit of the delayed rectifier voltage-gated potassium channel, K_v11.1. Herein we refer to the *ERG* gene and the cognate mRNA as '*KCNH2*', and '*KCNH2* mRNA', respectively, to the channel protein as ERG and to the functional channel as K_v11.1. In addition to ERG, K_v11.1 channels contain regulatory β-subunits [2,3], such as the single transmembrane domain protein potassium voltage-gated channel subfamily E members 2, encoded by the gene *KCNE2* (mRNA referred to as '*KCNE2* mRNA', and channel protein referred to as KCNE2).

K_v11.1 channels are activated following action potentials and function to repolarize the cell membrane by conducting potassium ions (K^+) out of the cell, which constitutes the rapid component of the delayed rectifier current I_{Kr} [4,5]. Repolarization terminates the action potential and the associated contraction.

K_v11.1 channels have been shown in rat stomach and murine portal vein [6-8], as well as in opossum oesophagus [9]. In addition, selective K_v11.1 channel blockers have been shown to increase contractility in rat stomach [6], mouse portal vein [8], opossum oesophagus [9], mouse and guinea pig gall bladder [10], bovine epididymis [11] and human and equine jejunum [12,13]. These smooth muscles all exhibit spontaneous contractile activity, which is also a property of uterine smooth muscle [14].

K_v11.1 channels were initially linked to myometrial contractility by Aaronson *et al.*, who demonstrated that tetraethylammonium (TEA)- and 4-aminopyridine (4-AP)-sensitive voltage-dependent K^+ (K_v) channels played a role in regulating action potential duration in rat myometrium [15]. Greenwood *et al.* later examined mouse ERG (mERG) expression and function, and confirmed the presence of both the ERG1a and ERG1b splice variants, with ERG1a expression being more abundant than that of ERG1b [16]. They found that *KCNH2* mRNA abundance did not change throughout gestation or with the onset of labor, however, mRNAs encoding auxiliary subunits, namely *KCNE2*, were significantly up-regulated approaching term [16].

More recently, our group reported that human ERG1 (hERG1) and KCNE2 were present in pregnant human myometrium during late gestation and labor [17]. We found that K_v11.1 activity supressed the amplitude and duration of contractions prior to labor, thereby

supporting a role for $K_v11.1$ activity in helping to maintain uterine quiescence [17]. Previous reports have indicated that KCNE2 co-expression with hERG1 was not necessarily inhibitory of $K_v11.1$ activity [18], however, we found that the onset of labor was associated with the increased expression of KCNE2 and the decreased responsiveness of myocytes to the $K_v11.1$ inhibitor, dofetilide [17]. Together these findings support an inhibitory role for KCNE2 in the context of regulating hERG1 in the myometrium. The evidence also suggests that $K_v11.1$ activity contributes to the electrophysiological mechanisms that regulate uterine contractions and that inhibition of the α-subunit, ERG1, by the β-subunit, KCNE2, may facilitate labor [17]. Interestingly, the mechanism for reducing $K_v11.1$ activity to facilitate labor appears to be dysregulated in obese women. Maternal obesity is associated with increased rates of labor induction, dysfunctional labor requiring Caesarean section (CS) delivery, longer pregnancies, as well as postpartum haemorrhage [19-21]. We reported that high body mass index (BMI) was associated with increased levels of hERG1 and reduced levels of KCNE2 in the myometrium [17]. Furthermore, high BMI was associated with heightened $K_v11.1$ activity *in vitro*, suggesting that the delayed and protracted labor often observed in obese women is linked to elevated $K_v11.1$ activity in the myometrium [17].

Genes encoding the progesterone receptor (*PGR*) [22-24], estrogen receptor 1 (*ESR1*) [23,25], oxytocin receptor (*OXTR*) [24,26,27] and prostaglandin-endoperoxidase synthase 2 (*PTGS2*) [24,28] have been identified as key genes involved human parturition. There is now extensive published literature linking the myometrial regulation of these genes, among others, to the maintenance of uterine quiescence and the transition to a contractile phenotype at labor [29-31]. Uncovering an association between expression of these key genes and genes encoding the $K_v11.1$ channel would ascertain the involvement of this channel in the increased contractility of the myometrium at term and as part of the myometrial transformation leading to labor.

The aim of this study therefore was to determine whether expression of *KCNH2* and *KCNE2* in term non-laboring human myometrium correlate with the expression of *PGR*, *ESR1*, *OXTR* and *PTGS2*. We report that in term pregnancy, *KCNH2* and *KCNE2* are expressed coordinatedly with these key parturition-associated genes, thus strengthening the link between uterine contractility and K^+ channel abundance in myometrial cells. Furthermore, this study examined the relationship of myometrial *KCNH2* and *KCNE2* mRNA abundance with BMI, and explored the expression of both genes in myometrial biopsies from preterm deliveries.

Materials and Methods

Consumables and reagents

Superscript III First Strand Synthesis System, Ultrapure Glycogen, Ultra Pure Agarose and Trackit 100 BP DNA ladder were purchased from Invitrogen (Carlsbad, USA). TRizol Reagent and Turbo DNA-free 50 reactions were from Ambion (Austin, USA). Alien QRT-PCR Inhibitor Alert 400 Reactions were purchased from Integrated Sciences Pty (Sydney, Australia). PCR primers were purchased from Sigma (St Louis, USA). The 2 mL 2.8 mm CK28-R Ceramic Bead Kit for the Precellys homogenizer (Bertin Instruments, France) were purchased from Thermo Fischer Scientific (Melbourne, Australia). L-Glutamine, Sodium Pyruvate, Gentamicin, HEPES and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco (Carlsbad, USA). SYBR Green 2x Master mix was from Applied Biosystems (Carlsbad, USA).

Myometrial tissue acquisition

These studies were approved by the Hunter and New England Area Human Research Ethics Committee and the University of Newcastle Human Ethics Committee (02/06/12/3.13). All participants gave informed written consent. Human myometrial samples (5 × 5 × 10 mm) were obtained from the lower uterine segment during elective CS of singleton pregnancies. Preterm samples ranged from 31 - 34 weeks gestation while term samples were 38.2 - 39.6 weeks gestation. Patient BMI range was 18.3 - 38.0, and all patients were not-in-labor. The indications for elective not-in-labor term CS were previous CS, placenta praevia, fetal distress or breach presentation. The indications for elective not-in-labor preterm CS were placenta increta, pre-eclampsia and low levels of amniotic fluid. Following delivery of the placenta, 5 units of syntocinon were administrated directly into an intravenous line as part of standard care for the prevention of postpartum hemorrhage. Samples were therefore exposed to oxytocin for a brief period of time (3 min). All myometrial samples were placed on ice in a serum-free media containing DMEM with high glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 40 μg/mL gentamicin and 10 mM HEPES for the transfer to the laboratory. Myometrial tissues were then cleared of serosa, fibrous or damaged tissue and visible blood vessels before being dissected into smaller pieces and washed in serum-free media to remove excess blood. Approximately 100 mg of tissue was snap frozen in liquid nitrogen for subsequent analysis.

RNA extraction, Reverse transcription and Real-time quantitative PCR

RNA was extracted from 100 mg of tissue using TRizol Reagent (Ambion, USA) according to the manufacture's protocol. Homogenization of tissue in TRizol Reagent was performed using a Precellys24 homogenizer (Bertin Instruments, France). Following extraction, RNA samples were purified using the TURBO DNA-free kit (Ambion, USA). An ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) was used to measure RNA concentration (absorbance at 260 nm (A_{260}) and 280 nm (A_{280})) and purity. RNA integrity was checked by agarose gel electrophoresis. Each RNA sample (0.5 μg of total RNA) was spiked with 0.5 x 10^7 copies of Alien RNA (Integrated Sciences Pty, Australia) and reverse-transcribed using the Super Script III First-Strand Synthesis System with random hexamer primers (Invitrogen, USA). The Alien RNA transcript is an *in vitro* transcribed RNA molecule that is non-homologous to any known nucleic acids and as such was used as a housekeeping gene for these studies [32-34]. Quantitative real-time PCR was performed using an ABI 7500 Sequence Detector (Applied Biosystem, USA). No-reverse transcription (no-RT) negative controls were prepared for each sample to ensure there was no DNA contamination. The final volume of each PCR reaction was 20 μL containing 10 μL of 2x SYBR Green PCR Master Mix (Applied Biosystems, USA), master mix cDNA template (corresponding to 10 ng of reverse transcribed RNA), target cDNA-specific forward and reverse primers, and MilliQ water. For the reference gene (Alien primer), the final volume was also 20 μL with 1.0 μL of 2.5 μM of Alien Primer Mix, 10 μL of 2x SYBR Green PCR and the same amount of cDNA as the target genes and MilliQ water. No-template control samples (NTCs) were included in each PCR plate to detect any contamination and primer-dimers. PCR primers were designed using Primer Express, optimized and validated by confirming that single amplicons of appropriate size and sequence were generated (Table 1).

Primer	Primer Sequence (5'-3')	Amplicon Size	GenBank #
KCNH2	F: ACCTCATCGTGGACATCA R: CTCCTCGTTGGCATTGAC	77	NM_000238.3
KCNE2	F: CACGAGGCAAATCCAAAT R: CTCCAACAAGCAAGCATAA	141	NM_172201.1
OXTR	F: CTGGACGCCTTTCTTCTTCGT R: GAAGGCCGAGGCTTCCTT	101	NM_000983
ESR1	F: TGAAAGGTGGGATACGAAAAGAC R: CATCTCTCTGGCGCTTGTGTT	66	NM_000125.3
PGR	F: GTGGGAGCTGTAAGGTCTTCTTTAA R: AACGATGCAGTCATTTCTTCCA	83	NM000926.4
PTGS2	F: ATGTTCCACCCGCAGTACAGA R: CAGCATAAAGCGTTTGCGGTA	73	NM_000916.3

Table 1: cDNA primer sequences for *KCNH2*, *KCNE2*, *OXTR*, *ESR1*, *PGR* and *PTGS2*.

KCNH2: Potassium Voltage-Gated Channel Subfamily H Member 2

KCNE2: Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2

OXTR: Oxytocin Receptor

ESR1: Estrogen Receptor 1

PGR: Progesterone Receptor

PTGS2: Prostaglandin-Endoperoxide Synthase 2

Data and statistical analysis

All mRNA abundance data were expressed relative to the Alien reference RNA. The relative mRNA abundance was calculated using the delta C_t (ΔC_t) method [35]. All mRNA relative abundance values were then logarithmically transformed to approach normal distribution. Statistical analyses were conducted with Graph Pad Prism software (San Diego, CA, USA). Graphical data are presented as mean ± SEM. For comparison between two groups, Student's *t*-test was used. For correlation studies Pearson's product moment correlation coefficient was used (Pearson's *r*). Preterm and term samples were compared by 1-way ANOVA with Bonferroni's multiple comparisons test. *P*-values ≤ 0.05 were considered statistically significant.

Results

KCNH2 and *KCNE2* expression in human myometrium at term pregnancy

Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in non-laboring term myometrial tissues (n=18). Expression of *KCNH2* was much higher than of *KCNE2* (over two orders of magnitude, Figure 1A). A statistically significant positive correlation was found between *KCNH2* and *KCNE2* mRNA abundance (r^2=0.34, p=0.01) (Figure 1B).

There was no statistically significant relationship between *KCNH2* mRNA abundance and gestational age (r^2=0.06, p=0.31) (Figure 1C), or *KCNE2* mRNA abundance and gestational age (r^2=0.04, p=0.41) (Figure 1D) within the term gestation range of 38.2-41.0 weeks.

Since we have found previously that hERG1 levels increase whilst KCNE2 levels decrease with increasing BMI of term pregnant women, we have correlated *KCNH2* mRNA as well as *KCNE2* mRNA abundance to BMI. The BMI in our patients group ranged from 18.3 to 38.0. There was no statistically significant correlation between *KCNH2* mRNA abundance (r^2=0.0002, p=0.96) (Figure 1E) or *KCNE2* mRNA abundance and BMI (r^2=0.07, p=0.29) (Figure 1F).

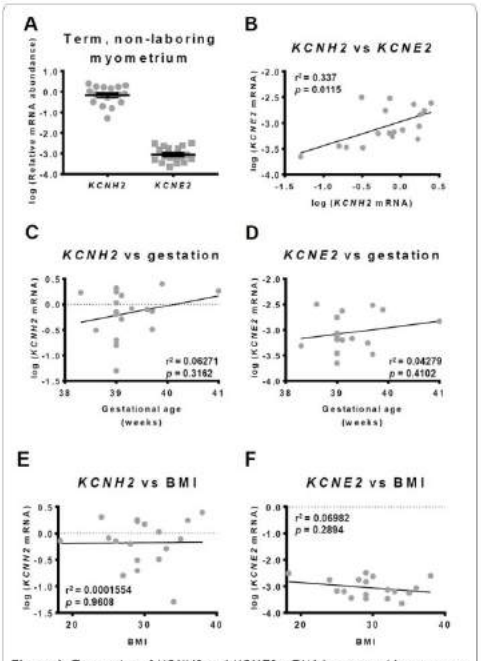

Figure 1: Expression of *KCNH2* and *KCNE2* mRNA in pregnant human myometrium. Relative abundance of *KCNH2* and *KCNE2* mRNA was measured in term non-laboring myometrial tissues (n=18) and expressed relative to Alien reference RNA. (A) *KCNH2* and *KCNE2* mRNA abundance at term. (B) Correlation between *KCNH2* and *KCNE2* mRNA abundance. (C) *KCNH2* mRNA abundance across term gestation time points. (D) *KCNE2* mRNA abundance across term gestation time points. (E) *KCNH2* mRNA abundance against BMI. (F) *KCNE2* mRNA abundance against BMI (Pearson's *r*).

Correlations between the expression of *KCNH2*, *KCNE2* and contraction-associated myometrial genes

We measured the relative abundance of *OXTR*, *ESR1*, *PGR* and *PTGS2* mRNAs (n=18 each) and correlated their abundance with *KCNH2* and *KCNE2* mRNA abundance. A statistically significant positive correlation was found between *KCNH2* and *OXTR* (r^2=0.42, p=0.0039) (Figure 2A) but not between *KCNE2* and *OXTR* mRNA abundance (r^2=0.11, p=0.19) (Figure 2B). *ESR1* mRNA abundance was positively correlated with *KCNH2* (r^2=0.3117, p=0.0160) (Figure 2C) and *KCNE2* mRNA abundance (r^2=0.62, p=0.0001) (Figure 2D). Moreover, *PGR* mRNA also correlated positively with *KCNH2* (r^2=0.67, p<0.0001) (Figure 2E) and *KCNE2* mRNA abundance (r^2=0.51, p=0.0009) (Figure 2F). In our term non-laboring samples progesterone receptor A (*PR-A*) expression was barely detectable, meaning correlations with *PGR* were predominantly in relation to progesterone receptor B (*PR-B*). Finally, the key contraction-associated gene product, *PTGS2* mRNA, also exhibited significant positive correlation with *KCNH2* (r^2=0.37, p=0.0076) (Figure 2G) and *KCNE2* mRNA abundance (r^2=0.50, p=0.0011) (Figure 2H).

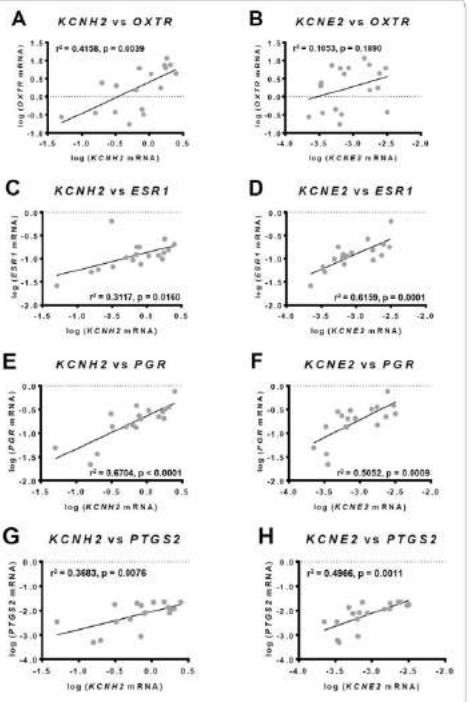

Figure 2: *KCNH2* and *KCNE2* mRNA expression correlates with expression of key myometrial genes at term. Relative mRNA abundance of *KCNH2*, *KCNE2*, *OXTR*, *ESR1*, *PGR* and *PTGS2* were measured in term non-laboring myometrial tissues (n=18) and expressed relative to Alien reference RNA. Correlations of mRNA abundance between (A) *KCNH2* and *OXTR*; (B) *KCNE2* and *OXTR*; (C) *KCNH2* and *ESR1*; (D) *KCNE2* and *ESR1*; (E) *KCNH2* and *PGR*; (F) *KCNE2* and *PGR*; (G) *KCNH2* and *PTGS2*; (F) *KCNE2* and *PTGS2* (Pearson's *r*).

KCNH2 and *KCNE2* expression in preterm human myometrium

The relative abundance of *KCNH2* and *KCNE2* mRNA was determined in a limited number of non-laboring preterm myometrial tissues (n=3). No difference was found for *KCNH2* (p=0.47) or *KCNE2* (p=0.92) mRNA abundance compared to term non-laboring samples (n=18) (Figure 3).

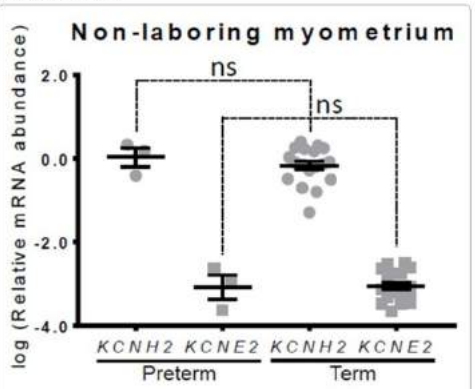

Figure 3: *KCNH2* and *KCNE2* expression in preterm and term non-laboring human myometrium. Relative mRNA abundance of *KCNH2* and *KCNE2* was measured in preterm (n=3) and term (n=18) non-laboring myometrial tissues, and expressed relative to Alien reference RNA (ANOVA).

Discussion

We previously reported the presence of hERG1 and KCNE2 in pregnant human myometrium in late gestation, and demonstrated that labor onset is associated with diminished K_v11.1 activity in association with enhanced expression of the inhibitory subunit, KCNE2 [17]. Furthermore, we reported a significant positive correlation between K_v11.1 activity and BMI, which was attributable to increased hERG1 levels and decreased KCNE2 levels [17]. In follow up to that functional study, we examined *KCNH2* and *KCNE2* gene expression in term non-laboring myometrium and found that *KCNE2* expression was relatively low, and correlated with *KCNH2* expression. Moreover, the levels of both mRNAs correlated positively with the expression of myometrial genes that are key regulators of parturition, but showed no relationship with the BMI of the participants. Patients comprising our term non-laboring cohort ranged from underweight (BMI 18.3) to Class II obese (BMI 38.0) (World Health Organisation guidelines). The lack of correlation suggests that within the examined BMI range, the effect of obesity is not on *KCNH2* or *KCNE2* gene expression, but rather on protein levels. This finding refines the link between K_v11.1 activity and BMI, and suggests that future studies should focus on the effect of obesity on *KCNH2* and *KCNE2* mRNA translation as well as protein turnover.

Another potassium channel that plays a crucial role in pregnancy is the ATP-sensitive K+ (K_{ATP}) channel. K_{ATP}-mediated K^+ efflux plays a role in maintaining the resting membrane potential of myocytes [36]. Several studies have found that in late pregnancy, the number of myometrial K_{ATP} channels is reduced, which increases uterine excitability, thereby promoting the establishment of labor [37-39]. Du *et al.* [40]

found that all K_{ATP} channel subunits, apart from the SUR2A subunit, were down-regulated in the late pregnant uterus compared to the non-pregnant uterus. More importantly, expression of SUR2B/Kir6.1 in term pregnant human myometrium was found to be increased in women older than 35 years [40]. Advanced maternal age is associated with increased obstetric risks in general, as well as increased risk of elective and emergency CS [41,42]. The study therefore suggests that increased risk of birth complications in women aged over 35 years may be linked to reduced myocyte excitability attributable to increased numbers of K_{ATP} channels in the myometrium [40]. This is consistent with our previous study on hERG1, which similarly links poor labor outcomes for obese women with the elevated activity of a potassium channel [17]. Both studies illustrate the importance of understanding how different potassium channels regulate uterine contractility, as dysregulation can lead to obstetric complications.

In pregnant human myometrium, we observed a significant positive correlation between *KCNH2* and *KCNE2* mRNA abundance at term (Figure 1B). This correlation, if it translates to protein abundance, could suggest an underlying subunit stoichiometry in myometrial K_v11.1 channels. Given that all term samples analysed were non-laboring, preservation of the high *KCNH2*:*KCNE2* ratio may play a role in maintaining quiescence in non-laboring tissue.

The uterine quiescence, which persists for the majority of pregnancy, is maintained by inhibitors of uterine contraction, such as progesterone [24,43-46]. As term approaches there is a shift from progesterone to estrogen dominance and the uterus undergoes a phenotypic transition. This transition is characterised by up-regulated expression of a series of contraction-associated genes (reviewed by Smith [47]), including receptors for oxytocin and prostaglandins, increased expression of genes encoding myometrial gap junctions, such as connexin 43, which facilitates synchronous contractions, and alterations in resting membrane potential of myocytes, which renders myocytes more prone to excitation [24,43-46,48]. Collectively these changes increase the likelihood that sporadic contractions of the uterus will propagate in synchrony and lead to established labor [49]. In fulfilling a role as a regulator of myometrial contractility, it would therefore be reasonable to expect K_v11.1 activity, and thus *KCNH2* and *KCNE2* expression, to correlate with the expression of key genes that are known to play a role in this phenotypic transition.

OXTR expression in the myometrium is reportedly constant between 24 - 36 weeks gestation, but rises significantly in term samples (>37 weeks) prior to the onset of labor [48,50]. Within this latter time frame (term), we found that *KCNH2* expression correlated positively with *OXTR* expression, whereas *KCNE2* did not. Given that *KCNH2* mRNA abundance is significantly correlated with both *KCNE2* and *OXTR* mRNA abundance, a correlation should be expected between *KCNE2* and *OXTR* mRNA levels. It is possible that with greater sample numbers a positive correlation would be demonstrated between *KCNE2* and *OXTR* expression. Given that the role of K_v11.1 within the myometrium is to rapidly terminate contractions in order to prevent propagation of uterine contractility and the establishment of labor, it is reasonable to conjecture that *KCNH2* expression is co-regulated with *OXTR* expression as a counter-measure to offset increased sensitivity to oxytocin.

Similar considerations may apply to the observed positive correlation of *KCNH2* and *KCNE2* with *ESR1* and *PTGS2*. *ESR1* encodes estrogen receptor α (ERα), which drives the estrogen-dependent expression of contraction-associated proteins, such as connexin 43 [51-54]. *PTGS2* is responsible for the biosynthesis of prostaglandins (PGs). Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) is produced mainly by the maternal decidua and is involved in the up-regulation of *OXTR* levels and gap junctions in the myometrium, thus promoting uterine contractions [55]. Prostaglandin E_2(PGE_2) is produced by the fetus and placenta and is involved in collagen degradation and dilation of small blood vessels in the cervix, thus promoting cervical ripening as well as spontaneous rupture of the fetal membranes [56]. It is important to remain mindful, however, that non-laboring tissues were analysed in our study suggesting the possibility that women with high expression of *ESR1* and *PTGS2* may require higher levels of *KCNH2* in order to maintain uterine quiescence than women with lower *ESR1* and *PTGS2* expression. Moreover, in the heart KCNE2 expression is up-regulated by estrogen [57]. Taking into consideration that in humans circulating levels of estrogen are high for most of pregnancy, and remain elevated during parturition [46,58], it is likely that estrogen regulation of KCNE2 expression, and thus K_v11.1 activity, plays an important role in modulating the transition from quiescence to contractility.

KCNH2 and *KCNE2* mRNA levels were both positively correlated with *PGR* expression. Consistent with previous studies we report *PGR* total expression [30] as within our term non-laboring cohort *PR-A* expression was barely detectable. The strong positive correlations between *KCNH2* and *KCNE2* expression with *PGR* expression are therefore in relation to *PR-B*, which is consistent with the observed *KCNH2*:*KCNE2* ratio potentially contributing to pregnancy maintenance. A follow up analyses determining whether *KCNH2* and *KCNE2* expression continue to correlate with *PGR* expression in laboring tissue would provide valuable insight into whether progesterone regulates hERG1 and KCNE2 levels as means of maintaining uterine quiescence.

In mice, *KCNH2* expression remains constant across gestation and there is no change in ERG1 protein levels [16]. *KCNE2*, however, is gestationally regulated in mice in that mRNA abundance is significantly up-regulated by day 14 of a 20 day pregnancy, and KCNE2 protein levels are significantly up-regulated by day 17 [16]. Our analyses of preterm non-laboring samples showed no change in *KCNH2* or *KCNE2* gene expression earlier in gestation suggesting that, unlike mice, *KCNE2* gene expression may not be gestationally regulated in humans. If confirmed by analysis of additional preterm samples, this could indicate that alternative auxiliary subunits need to be explored in the context of human myometrium. To date, studies exploring the role of K_v11.1 channels in human myometrium have focused on KCNE2 co-expression alongside hERG1. However, a variety of auxiliary subunits are known to modulate K_v11.1 activity [1], and studies by Greenwood *et al.* [16] indicate that *KCNE4* expression is gestationally regulated in mouse uterine tissue in addition to *KCNE2*. As such it is possible that KCNE4, or another regulatory subunit, is gestationally regulated in humans.

Conclusion

We have previously provided functional data examining the role of K_v11.1 in regulating myometrial contractility at term labor. Here we have followed up the functional study with an analysis of *KCNH2* and *KCNE2* gene expression in term non-laboring women. We have shown that *KCNH2* and *KCNE2* expression correlate with the expression of key myometrial genes implicated in parturition. Our data suggest that *KCNH2* and *KCNE2* are participants in the gene network controlling myometrial contractility at term. Uncovering this association advances our understanding of the mechanisms that underpin

myometrial transformation, and reiterates the complexity of the parturition process. A complementary analysis using laboring myometrium would assist with the further interpretation of these results as alterations of the correlative network with the onset of labor would provide insight into which relationships are critical in maintaining uterine quiescence. We have also performed an initial investigation into *KCNH2* and *KCNE2* expression in preterm samples, which did not show any robust association between the preterm status and *KCNH2* or *KCNE2* expression. This investigation should be followed up with additional preterm samples to strengthen the study, as well as the analysis of preterm in-labor samples. Furthermore, future studies include examining whether these correlations translate into correlations at the protein level.

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Conflict of Interests Disclosure

Roger Smith holds a patent through the University of Newcastle in relation to the hERG potassium channel, titled "Compositions and methods for modulating uterine contractions". The remaining authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix H: Published manuscript

Original Research ajog.org

OBSTETRICS

Drug delivery to the human and mouse uterus using immunoliposomes targeted to the oxytocin receptor

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BACKGROUND: The ability to provide safe and effective pharmacotherapy during obstetric complications, such as preterm labor or postpartum hemorrhage, is hampered by the systemic toxicity of therapeutic agents leading to adverse side effects in the mother and fetus. Development of novel strategies to target tocolytic and uterotonic agents specifically to uterine myocytes would improve therapeutic efficacy while minimizing the risk of side effects. Ligand-targeted liposomes have emerged as a reliable and versatile platform for targeted drug delivery to specific cell types, tissues or organs.

OBJECTIVE: Our objective was to develop a targeted drug delivery system for the uterus utilizing an immunoliposome platform targeting the oxytocin receptor.

STUDY DESIGN: We conjugated liposomes to an antibody that recognizes an extracellular domain of the oxytocin receptor. We then examined the ability of oxytocin receptor–targeted liposomes to deliver contraction-blocking (nifedipine, salbutamol and rolipram) or contraction-enhancing (dofetilide) agents to strips of spontaneously contracting myometrial tissue in vitro (human and mouse). We evaluated the ability of oxytocin receptor–targeted liposomes to localize to uterine tissue in vivo, and assessed if targeted liposomes loaded with indomethacin were capable of preventing lipopolysaccharide-induced preterm birth in mice.

RESULTS: Oxytocin receptor–targeted liposomes loaded with nifedipine, salbutamol or rolipram consistently abolished human myometrial contractions in vitro, while oxytocin receptor–targeted liposomes loaded with dofetilide increased contraction duration. Nontargeted control liposomes loaded with these agents had no effect. Similar results were observed in mouse uterine strips. Following in vivo administration to pregnant mice, oxytocin receptor–targeted liposomes localized specifically to the uterine horns and mammary tissue. Targeting increased localization to the uterus 7-fold. Localization was not detected in the maternal brain or fetus. Targeted and nontargeted liposomes also localized to the liver. Oxytocin receptor–targeted liposomes loaded with indomethacin were effective in reducing rates of preterm birth in mice, whereas nontargeted liposomes loaded with indomethacin had no effect.

CONCLUSION: Our results demonstrate that oxytocin receptor–targeted liposomes can be used to either inhibit or enhance human uterine contractions in vitro. In vivo, the liposomes localized to the uterine tissue of pregnant mice and were effective in delivering agents for the prevention of inflammation-induced preterm labor. The potential clinical advantage of targeted liposomal drug delivery to the myometrium is reduced dose and reduced toxicity to both mother and fetus.

Key words: contraction, drug delivery, human, immunoliposomes, labor, liposomes, mouse, myometrium, oxytocin receptor, preterm, birth, targeted, tocolysis, uterotonic

Introduction

Complications arising from preterm birth (PTB) are the leading cause of death among children age <5 years, accounting for nearly 1 million deaths in 2013,[1] while postpartum hemorrhage (PPH) is the leading cause of maternal mortality worldwide, accounting for up to 27.1% of maternal deaths.[2] Given that both can arise from dysregulation of uterine contractility, the need exists for safe and effective clinical interventions capable of modifying myometrial contractions to improve treatment of women in preterm labor, to induce or facilitate labor and to prevent or treat PPH, without adverse off-target effects on either the mother or fetus.

When a woman presents with preterm labor, attempts are often made to halt contractions by administering tocolytics that inhibit or block components of the contraction cascade. A recent study proposed that "the ideal tocolytic agent should be myometrium-specific, easy to administer, inexpensive, effective in preventing PTB and improve neonatal outcomes, with few maternal, fetal, and neonatal side effects, and without long-term adverse effects."[3] Standard therapy varies from country to country, but tocolysis may involve the administration of calcium channel blockers, such as nifedipine (NIF); β_2-adrenergic receptor agonists, such as salbutamol (SAL); an oxytocin receptor (OTR) antagonist, such as atosiban; or a prostaglandin synthetase inhibitor, such as indomethacin (IND).[4–9] Unfortunately, the systemic administration of these therapies and lack of specificity means that large doses need to be administered to achieve a therapeutic effect at the target tissue, the myometrium. Maternal side effects of β_2-adrenergic receptor agonists include tremors, heart palpitations, and tachycardia, as well as myocardial ischemia and pulmonary edema.[8,10–12] NIF has been associated with fewer side effects, however approximately 1% of women experience a severe side effect and a further 1% experience mild adverse side effects.[13] Atosiban is associated with the lowest side effect risk but the efficacy of this agent is disputed.[14,15] Usefulness of IND is limited by fetal side effects, such as premature closure of the ductus arteriosus.[16–18] Achieving

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targeted drug delivery to the myometrium would reduce the quantity of drug required to achieve therapeutic efficacy, reduce the likelihood of maternal and fetal side effects, and would therefore represent a significant advancement for maternal-fetal medicine.[12,19-21]

Targeted liposomes have emerged as a platform for achieving the delivery of drugs to specific tissues. Liposomes are artificial vesicles that range in size from 50-1000 nm, and are composed of ≥1 phospholipid bilayers.[22,23] Liposomes are able to encapsulate both lipophilic and/or hydrophilic drugs, and are nontoxic and biodegradable with minimal immunogenicity.[21,24,25] Liposomal encapsulation improves the pharmacokinetics of drugs, particularly if the liposome surface is PEGylated, which reduces uptake by the reticuloendothelial system and prolongs half-life.[26] This has led to the development of liposomal-based preparations of various agents, including doxorubicin, amphotericin B, daunorubicin, and verteporfin.[27] Ligand-targeted liposomes offer the potential for site-specific delivery of drugs to designated cell types or organs in vivo that selectively express specific cell surface cognate receptors.[26] Although many types of targeting molecules are available, such as peptides/proteins and carbohydrates, the coupling of antibodies to the liposome surface to create immunoliposomes has many advantages. One advantage of using antibodies is their stability and higher binding avidity because of the presence of dual binding sites.[26] For example, liposomes coated with antibodies to intercellular adhesion molecule (ICAM)-1 have been developed for the treatment of inflammatory diseases.[28] Administration of ICAM-1-targeted immunoliposomes loaded with an analgesic agent demonstrated specific localization and therapeutic efficacy exclusively in peripheral inflammatory tissue. All control groups (free drug solution, empty nontargeted liposomes, drug-loaded nontargeted liposomes, and empty ICAM-1-targeted immunoliposomes) showed no significant therapeutic response.[29]

The aim of this study was to develop a means of targeting therapeutic agents to uterine myometrial tissue, to allow therapeutic modification of myometrial contractions in obstetric settings, such as preterm labor, labor induction, and PPH. The expression of the OTR is significantly up-regulated in myometrial cells approaching term.[30,31] Here we report the development of OTR-targeted PEGylated immunoliposomes loaded with traditional tocolytics, such as NIF and SAL, as well as rolipram (ROL), a phosphodiesterase (PDE)4 inhibitor and potent inhibitor of myometrial contractions.[32-34] Moreover, we report enhancement of human myometrial contraction duration in vitro through liposomal delivery of dofetilide (DOF), a hERG channel blocker that increases myometrial contraction duration,[35,36] demonstrating that this delivery platform can be used to either inhibit or enhance contractions in human myometrial tissue. We demonstrate that intravenously (IV) administered OTR-targeted liposomes localize specifically to the uterine tissue of pregnant mice in vivo. Finally, using an inflammatory mouse model of PTB (lipopolysaccharide [LPS] administration), we show that OTR-targeted liposomes loaded with IND are effective in preventing PTB, while IND-loaded nontargeted liposomes have no effect.

Materials and Methods

Myometrial tissue acquisition

Human studies

These studies were performed in Newcastle, Australia, and were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent. Collection of myometrial samples (5 × 5 × 10 mm) occurred from the lower uterine segment of term singleton pregnancies. All women were examined clinically, and those with signs of infection were excluded. Women were undergoing term elective cesarean delivery and were not in labor (NIL); the clinical indications for elective NIL cesarean delivery were previous cesarean delivery or previous third-/fourth-degree tear. All participants ranged from 37-40 completed weeks of gestation. Following delivery of the placenta, all women immediately received 5 U of oxytocin (Syntocinon) into an IV line, which was administered as standard care. Myometrial biopsies were excised 3-5 minutes after oxytocin administration, thus all samples were briefly exposed to oxytocin. After biopsy, myometrial samples were dissected from connective tissue and washed in ice-cold physiological saline.

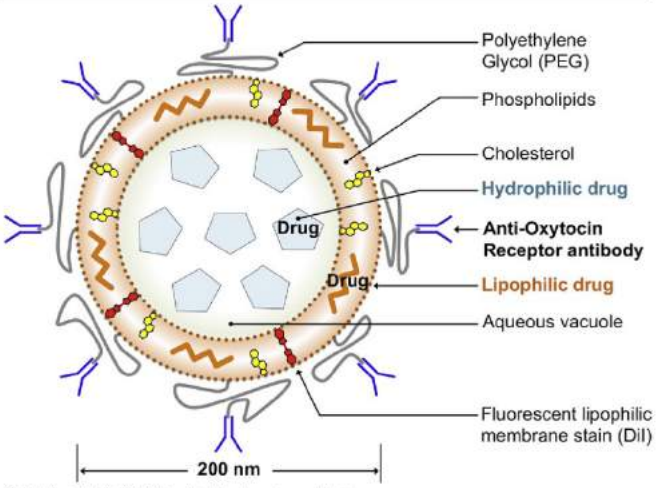
Mouse in vitro studies

Mouse uterine horns were dissected from pregnant CD1 Swiss mice (8-10 weeks of age) at term gestation prior to the onset of labor (fetal gestation day [GA] 18). Mouse studies were approved by the University of Newcastle Animal Ethics Committee (A-2014-400/A-2014-429). All mice were housed under SPF/PC2 conditions under a 12-hour light-day cycle and had food and water available ad libitum.

Liposome manufacture

Liposomes containing NIF, SAL, ROL, DOF (each at approximately 4 mg/mL), or IND (approximately 5.5 mg/mL), as determined by high-performance liquid chromatography, were manufactured as previously outlined.[28] Liposomes were composed of 1,2-distearoyl-sn-glycero-2-phosphocholine (DSPC) and cholesterol in a molar ratio of 2:1, and contained 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG(2000) maleimide) at 1.5 mol percent of DSPC as a coupling lipid (Avanti Polar Lipids). The resulting multilamellar dispersions were reduced in size and lamellarity to approximately 200 nm in diameter by high-pressure extrusion. The activated liposome suspension was then mixed with thiolated polyclonal anti-OTR antibody (catalog no. ab115664; Abcam, Cambridge, MA), which was prepared by first conjugating 25 μg of OTR antibody with a heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio) propionate (Figure 1). The OTR antibody recognizes an extracellular domain of the human OTR.

FIGURE 1
Schematic of oxytocin receptoretargeted liposome structure



DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.
Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

Nontargeted liposomes were coated with rabbit IgG. All liposomes incorporated the membrane stain 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) for fluorescent detection. Unconjugated antibody and nonencapsulated drug was removed by centrifugal filtration of the liposomes through a 100-kd molecular weight filter (Amicon Ultra-15). Amicon Ultra-15 filters were washed with Milli-Q H_2O before 500 μL of liposome suspension was loaded into the filter reservoir. Liposomes were diluted with 5 mL of sterile 0.9% saline and centrifuged at 4000 x g until retentate volume was <250 μL. Liposomes were then resuspended in a further 5 mL of 0.9% saline and centrifuged until retentate volume was <250 μL. Filtered liposomes were then collected, transferred to a fresh Eppendorf tube, and redispersed to an original volume of 500 μL.

The size distribution of the liposomal dispersion was determined by dynamic laser light scattering (Zetasizer Nano S, ATA Scientific). Encapsulation efficiency was determined by disrupting the vesicles with ethanol and evaluating drug concentration using high-performance liquid chromatography. Quantification of the amount of antibody associated with liposomes was determined using the CBQCA protein assay (ThermoFisher Scientific Inc, Waltham, MA), using bovine serum albumin for the preparation of the standard curve.

Myometrial contractility studies

Myometrial strips were set up as previously described.[37] Briefly, NIL human myometrial samples, or uterine horns obtained from pregnant CD1 Swiss mice, were dissected into strips (10 × 2 × 2 mm) and suspended in organ baths containing 30 mL of physiological saline solution (PSS) containing 120 mmol/L of NaCl, 5 mmol/L of KCl, 25 mmol/L of $NaHCO_3$, 1 mmol/L of KH_2PO_4, 1.2 mmol/L of $MgSO_4$, 2.5 mmol/L of $CaCl_2$, and 11 mmol/L of glucose, and continuously gassed with carbogen (95% O_2, 5% CO_2) at pH 7.4. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1g passive tension applied (1g was calibrated to equal 1 V). PSS was replaced 5 times during the first hour, with strips retensioned to 1g passive tension following each wash. Thereafter strips were maintained at 37°C until spontaneous rhythmic contractions developed. Data were digitized using a MacLab/8E data-acquisition system and contraction status visualized in real time using Chart software (ADInstruments, Dunedin, New Zealand). For each strip a contraction baseline was acquired to serve as reference.[38]

To administer liposome treatments, 600 μL of PSS buffer was carefully extracted from an organ bath and transferred to an Eppendorf tube. The appropriate volume of liposome preparation (mixed by inversion) was pipetted into the PSS to predilute the liposomes. The total volume of prediluted liposomes (600 μL PSS + liposomes) was then carefully reinjected back into the appropriate organ bath. Final concentrations of each drug were: NIF 7.7 μmol/L, SAL 9.25 μmol/L, ROL 19.4 μmol/L, and DOF 3.0 μmol/L. Doses were based on prior investigations of the nonencapsulated drug (in vitro contraction assays using human myometrium). Where treated tissue was not washed, tissue strips remained in the presence of the liposomes for the duration of the assay. Where washout studies were performed, organ baths were twice drained of buffer and refilled with 37°C PSS. Human tissue strips were washed after 1 hour and 25 minutes whereas mouse tissue strips were washed after 15 minutes.

Tension generated by tissue strips is indicated in the results and representative contraction traces. The effect of treatments is interpreted relative to the pretreatment contraction baseline, which consisted of ≥3 contractions of consistent frequency and amplitude.

In vivo biodistribution study

Timed-mated CD1 Swiss pregnant mice were injected with drug-free, DiI-labeled preparations of either nontargeted or OTR-targeted liposomes on fetal GA 17

TABLE 1
Treatment groups for in preterm labor study

Group	One-time IP injection (12:00 PM on GA 15, 150 μL)	Daily IV injections (4:00 PM, GA ≥15, 150 μL)
1	Saline	Saline
2	0.7 μg/g LPS	50% DMSO
3	0.7 μg/g LPS	1.0 mg/kg/d IND in 50% DMSO
4	0.7 μg/g LPS	2.0 mg/kg/d IND in 50% DMSO
5	0.7 μg/g LPS	OTR-targeted, drug-free liposomes in saline
6	0.7 μg/g LPS	2.0 mg/kg/d IND via nontargeted liposomes in saline
7	0.7 μg/g LPS	2.0 mg/kg/d IND via OTR-targeted liposomes in saline

DMSO, dimethyl sulfoxide; *GA*, gestation day; *IND*, indomethacin; *IP*, intraperitoneal; *IV*, intravenous; *LPS*, lipopolysaccharide; *OTR*, oxytocin receptor.
Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

and 18 at 4:00 PM. Mice that labored overnight were euthanized on the morning of day 19 (9:00-11:00 AM) by CO_2 asphyxiation. Maternal internal organs of interest (heart, brain, liver, lung, kidney, uterus, and mammary tissue) were harvested and transferred to a Petri dish along with a sacrificed neonate. The Petri dish was loaded into an in vivo imaging system (IVIS-100) (Xenogen, Alameda, CA) and a light image captured. Tissues were then imaged under conditions appropriate for the detection of DiI (excitation: 554 nm; emission: 583 nm; filter: DsRed; exposure: 4 seconds; field of view: 10; binning: 4). Organs were imaged 17-19 hours after the second injection, following labor. Background signal was subtracted from the detected signal to produce the final fluorescence image. Fluorescence signal is reported as radiance (p/s/cm^2/sr). The radiance range was kept constant across all images (min = 2.0×10^8; max = 1.8×10^9).

PTB study

Time-mated pregnant CD1 Swiss mice were administered 0.7 μg/g LPS from *Escherichia coli* (0111:B4) (Sigma-Aldrich) via intraperitoneal (IP) injection at 12:00 PM on GA 15 (1-time injection). LPS dose had been previously optimized to result in PTB rates of 50-70%. Total IP injection volume was 150 μL in saline.

At 4:00 PM on GA 15, mice began receiving daily IV injections of IND free-drug or liposomal preparations according to assigned treatment groups. Treatment groups are indicated in Table 1. Total IV injection volume was 150 μL. Mice were monitored for onset of labor every 6 hours. Treatments were repeated daily at 4:00 PM until all mice labored. Term gestation was 19-22 days. Mice that labored within 48 hours of receiving LPS (GA 17) were deemed to have labored preterm.

Statistical analyses

For contraction traces, LabChart software (ADInstruments) was used to determine the area under the curve (AUC) (g tension × seconds) for the 30 minutes prior to treatment (pretreatment) and 30 minutes after treatment (posttreatment). AUC before and after treatment was compared by 2-tailed paired *t* test (GraphPad Prism).

For DOF studies, contraction plateau duration (seconds) was determined for 4 contractions pretreatment and posttreatment using LabChart software. Plateau duration was determined as the time between the point of highest amplitude and point where contraction force declined sharply. Contraction duration data were obtained for 3 individual tissues (n = 3 women). Pretreatment and posttreatment measurements (n = 12 each) were compared by 2-tailed unpaired *t* test.

Average radiance (p/s/cm^2/sr) was determined for each organ of interest using Living Image software (v2.5). Where fluorescence was detected, regions of interest were applied automatically (contour). Where detection was low or absent, regions of interest were specified manually (circles or squares) to tightly encompass the tissue being analyzed. Data were tested for normality by the Shapiro-Wilk normality test (GraphPad Prism). Average radiance for each organ/tissue was compared between treatment groups (n = 4 animals per group) by 1-way analysis of variance (ANOVA) with multiple comparisons (Holm-Sidak) (GraphPad Prism).

For the preterm labor studies, rate of PTB was compared between treatment groups by χ^2 analysis. Time (hours) between LPS injection and labor was calculated. Data were transformed ($Y = Y^2$) to obtain normal distribution (D'Agostino and Pearson normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). Data recorded for number of pups for term deliveries were normally distributed (Shapiro-Wilk normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). Preterm deliveries did not yield any viable pups.

Consumables and reagents

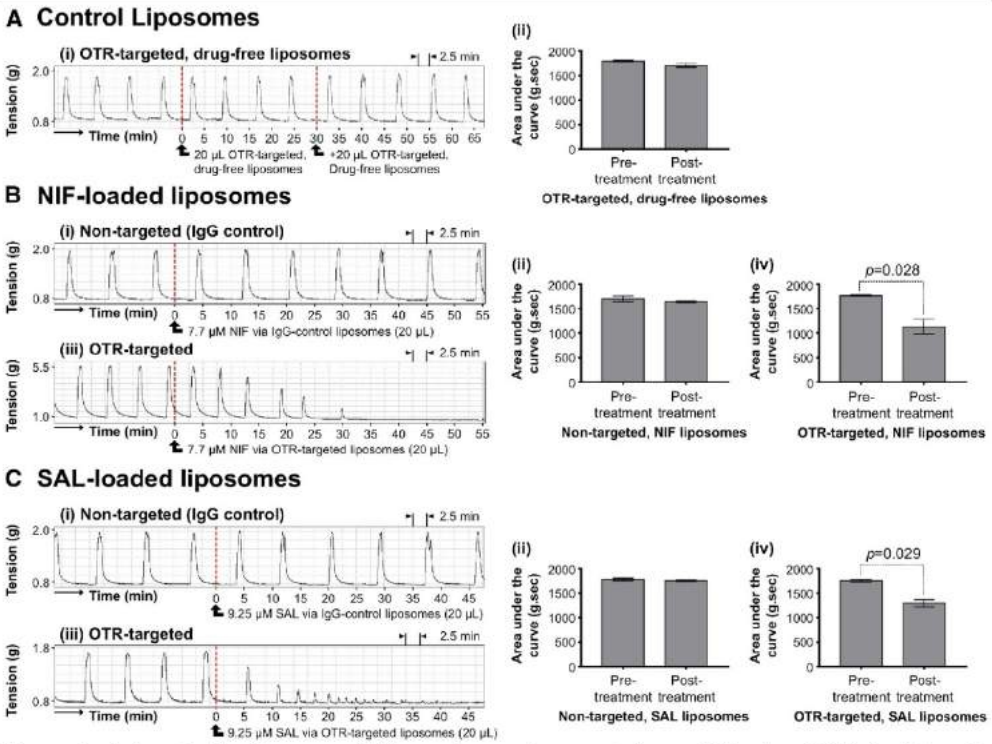
Mice were supplied by the University of Newcastle Animal Support Unit. NIF (catalog no. 1075), SAL hemisulfate (catalog no. 0634), ROL (catalog no. 0905), and DOF (catalog no. 3757) were purchased from Tocris (Bristol, United Kingdom). IND (catalog no. L2630) was purchased from Sigma-Aldrich Pty Ltd (Sydney, Australia). Anti-OTR antibody (ab115664) was purchased from Abcam. Other miscellaneous reagents were purchased from Sigma-Aldrich Pty Ltd and ThermoFisher Scientific Inc.

Results

Characteristics of the liposomal delivery system

OTR-targeted PEGylated immunoliposomes had a mean particle size of 197 ± 6.8 nm with a polydispersity index of

FIGURE 2
Use of targeted liposomes to inhibit human uterine contractility in vitro



Data are contraction traces for strips of human myometrial tissue and corresponding area under the curve (AUC) analyses. **A**, Effect of oxytocin receptor (OTR)-targeted, drug-free control liposomes on myometrial contractions in vitro (n = 3). **B**, Effect of nontargeted (n = 5) and OTR-targeted (n = 4) nifedipine (NIF)-loaded liposomes on myometrial contractions in vitro. **C**, Effect of nontargeted (n = 3) and OTR-targeted (n = 3) salbutamol (SAL)-loaded liposomes on myometrial contractions in vitro. Average AUC analyses covers 30 minutes immediately prior to and 30 minutes after treatment with NIF- or SAL-loaded liposomes (pretreatment and posttreatment, respectively). AUC analyses are paired *t* tests.

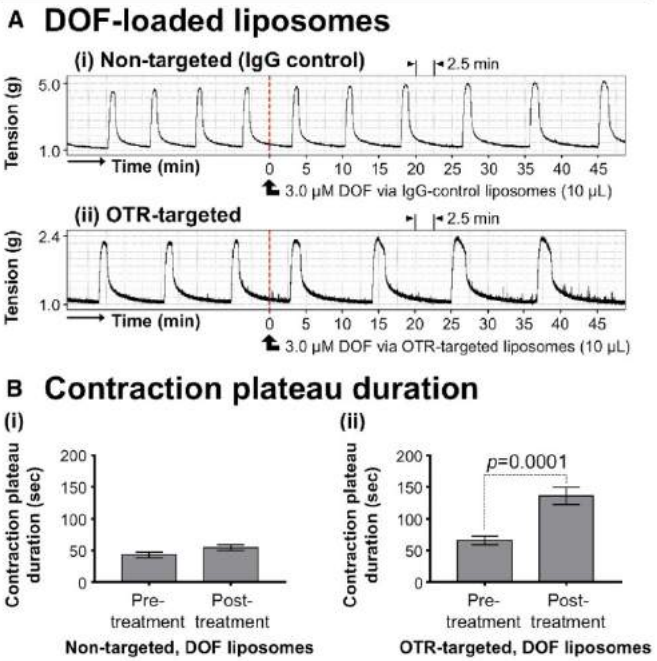
Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

0.243 ± 0.043 (mean ± SD; n = 3). The size and polydispersity of the control liposome formulations were similar. Encapsulation of therapeutic agents into the liposomes did not significantly affect the size or polydispersity index. Mean antibody coupling ratio for the OTR-targeted liposomes was 1.86 ± 0.17 µg of antibody/µmol of phospholipid. With a starting antibody concentration of 25 µg and a phospholipid concentration of 2.03×10^{-5} mol this equates to a conjugation efficiency of >99%. The liposomes have a neutral net charge and a drug loading efficiency of >95%, which equates to ~4 mg/mL of drug encapsulated/mL of liposome suspension composed of 16 mg DSPC and 4 mg cholesterol (molar ratio 2:1). In vitro dialysis studies have demonstrated highly stable vesicles upon dilution in an aqueous phase (PBS pH 7.4) and in serum (50% FCS) at 37°C (data not included).

Human myometrial contractility

Contraction bioassays were performed to assess whether targeted liposomes were capable of delivering encapsulated therapeutic agents to modulate spontaneous human uterine contractions in vitro. Treating the uterine strips with OTR-targeted liposomes that contained no therapeutic agent (n = 3) (Figure 2, Ai) had no effect on myometrial contractility in that AUC was not affected ($P = .08$; pretreatment = 1790.0 ± 19.5; posttreatment = 1704.0 ± 38.8 *g/s*) (Figure 2, Aii). For each therapeutic agent examined in vitro, we prepared nontargeted (IgG-coated) and OTR-targeted (anti-OTR-coated) liposomes

FIGURE 3
Use of targeted liposomes to enhance human myometrial contractions in vitro



Data are contraction trace analyses for strips of human myometrial tissue. **A**, Effect of 3.0 μmol/L dofetilide (*DOF*) administered via nontargeted (n = 3) or oxytocin receptor (*OTR*)-targeted (n = 3) DOF-loaded liposomes on contractility in vitro. **B**, Average contraction plateau duration for 4 contractions immediately prior to and after treatment with nontargeted or OTR-targeted DOF-loaded liposomes (pretreatment and posttreatment, respectively) (n = 3 tissues strips each). Unpaired *t* test (12 pretreatment plateau durations vs 12 posttreatment plateau durations).

Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

(at 4 mg/mL). Administering 7.7 μmol/L NIF to human uterine strips via nontargeted NIF-loaded liposomes (Figure 2, Bi) (n = 5) had no effect on contractility in that AUC was not affected (P = .4136; pretreatment = 1701.4 ± 55.9; posttreatment = 1643.8 ± 19.6 *g/s*) (Figure 2, Bii). Administering 7.7 μmol/L NIF via OTR-targeted NIF-loaded liposomes (Figure 2, Biii) (n = 4) resulted in abolition of myometrial contractions and a significant reduction in AUC (P = .0277; pretreatment = 1767.8 ± 15.5; posttreatment = 1137.5 ± 24.8 *g/s*) (Figure 2, Biv).

Administering 9.25 μmol/L SAL to spontaneously contracting human uterine strips via nontargeted SAL-loaded liposomes (Figure 2, Ci) (n = 3) had no effect on contractility with AUC being unaffected by the treatment (P = .2022; pretreatment = 1775.3 ± 24.8; posttreatment = 1749.3 ± 16.4 *g/s*) (Figure 2, Cii). Administering 9.25 μmol/L SAL via OTR-targeted SAL-loaded liposomes (Figure 2, Ciii) (n = 3) resulted in complete abolition of contractions and significant reduction in AUC (P = .0293; pretreatment = 1749.7 ± 27.3; posttreatment = 1292.0 ± 77.1 *g/s*) (Figure 2, Civ). Similar results were observed when ROL was encapsulated in nontargeted and OTR-targeted liposomes (Figure 4).

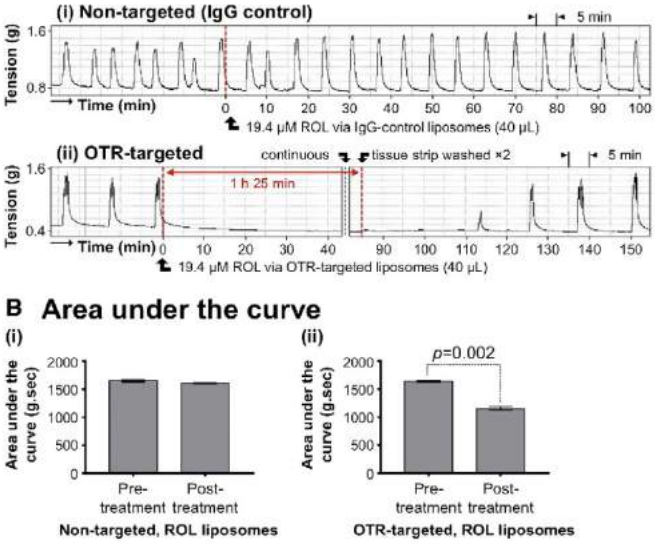
To demonstrate that our OTR-targeted liposomes are capable of functioning as a drug delivery system for different obstetric applications, such as treating PPH, liposomes were prepared that contained the hERG channel blocker, DOF. When administered to human myometrial tissue, DOF increases the contraction duration and reduces contraction frequency by delaying repolarization of the myocyte membrane.[36] Administering 3.0 μmol/L DOF to spontaneously contracting tissue strips (n = 3) via nontargeted DOF-loaded liposomes (Figure 3, Ai) had no significant effect on contraction plateau duration (P = .083; pretreatment = 27.0 ± 0.8; posttreatment = 39.0 ± 3.7 seconds) (Figure 3, Bi). When administered via OTR-targeted DOF-loaded liposomes (Figure 3, Aii), 3.0 μmol/L DOF significantly increased contraction plateau duration (P = .0001; pretreatment = 66.4.0 ± 9.8; posttreatment = 162.4 ± 35.4 seconds) (Figure 3, Bii). Increased contraction plateau duration is consistent with our previous report of DOF action on human myometrium.[36] These results demonstrate that a single delivery system, OTR-targeted liposomes, can be utilized to deliver either contraction-blocking or contraction-promoting therapeutics to uterine myocytes.

The effect of nontargeted and OTR-targeted DOF-loaded liposomes on AUC was analyzed. Neither treatment significantly affected AUC (data not shown).

Effect of targeted liposomes is reversible

To demonstrate that the effects on contractility were due to pharmacological actions of the drugs and not the result of toxic effects of OTR-targeted liposomes, washout experiments were performed. ROL is a reversible inhibitor of PDE4 that induces myometrial relaxation.[39,40] Administering 19.4 μmol/L ROL to contracting strips via nontargeted ROL-loaded liposomes

FIGURE 4
Modulation of uterine contractility by targeted liposomes is reversible



Data are contraction traces for individual strips of human myometrial tissue. **A**, Effect of nontargeted (n = 3) and oxytocin receptor (OTR)-targeted (n = 3) reliparin (ROL)-loaded liposomes on myometrial contractions in vitro. **Aii**, Restoration of contractions after washout. **B**, Average area under the curve (AUC) for 30 minutes immediately prior to and 30 minutes after treatment with ROL-loaded liposomes (pretreatment and posttreatment, respectively). AUC analyses were paired *t* tests.

Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

(Figure 4, Ai) (n = 3) had no effect. When 19.4 μmol/L ROL was administered via OTR-targeted ROL-loaded liposomes (Figure 4, Aii) (n = 3), contractions were abolished. Analyses of contraction data indicated no reduction in AUC following treatment with nontargeted ROL-loaded liposomes (P = .061; pretreatment = 1657.0 ± 21.0; posttreatment = 1611.7 ± 14.9 *g/s*) (Figure 4, Bi), whereas AUC was significantly reduced following treatment with 19.4 μmol/L ROL administered via OTR-targeted ROL-loaded liposomes (P = .0023; pretreatment = 1648.1 ± 14.3; posttreatment = 1155.7 ± 36.3 *g/s*) (Figure 4, Bii). Once contractions were inhibited for 1 hour and 25 minutes, tissue strips were washed twice in PSS and monitored. Spontaneous, rhythmic contractions resumed in myometrial strips previously treated with OTR-targeted ROL-loaded liposomes (Figure 4, Aii), indicating that the tissue remained viable.

Mouse myometrial contractility

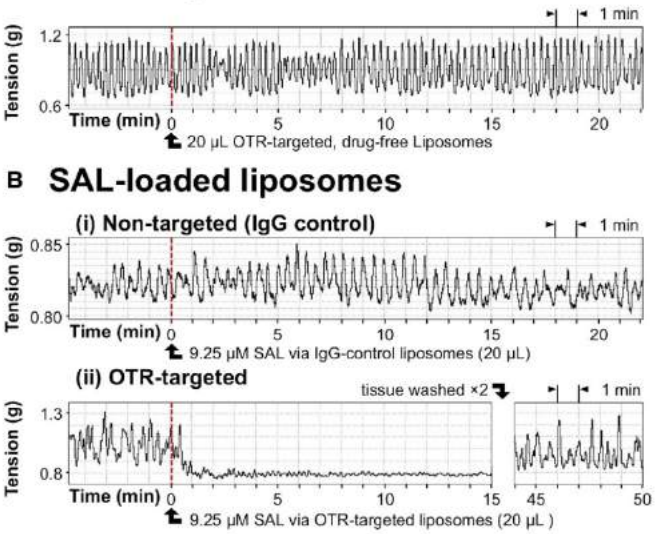
Prior to commencing mouse in vivo studies, we confirmed that liposomes were effective in delivering therapeutic agents to mouse uterine tissue in vitro. Results observed in the mouse were consistent with human myometrial contractility studies. OTR-targeted, drug-free liposomes (a control preparation) had no effect on mouse uterine contractions (Figure 5, A) (n = 3). Administering 9.25 μmol/L SAL via nontargeted (IgG-coated control) SAL-loaded liposomes had no effect on contractility (Figure 5, Bi) (n = 3), whereas the same SAL dose administered via OTR-targeted SAL-loaded liposomes abolished mouse myometrial contractions in vitro (Figure 5, Bii) (n = 3). Spontaneous contractions resumed following washing of tissue strips, demonstrating that the mouse uterine tissue remained viable following administration of the liposomes. Similar results were obtained for liposomes loaded with NIF (data not shown). These results demonstrated that OTR-targeted liposomes were effective in modulating mouse myometrial contractility.

Liposome biodistribution

We examined the biodistribution of DiI-labeled nontargeted (naked) and OTR-targeted liposomes that occurred in vivo. Pregnant mice were injected with liposomes approaching term (GA 17 and 18) then sacrificed shortly after labor (GA 19) (17-19 hours after injection). Whole organs (liver, brain, heart, kidney, lung, mammary tissue, and uterus) were placed on Petri dishes, along with a euthanized neonate, and imaged. The arrangement of tissues (Figure 6, A) was kept consistent when imaging tissues from different mice. DiI does not readily exchange out of liposomes into cell membranes or other lipid-containing structures, and therefore is an appropriate marker to assess the biodistribution of liposomes.

Fluorescence detection (p/s/cm^2/sr) of nontargeted liposomes injected into pregnant mice consistently revealed liposome accumulation in the liver, which is the site of liposome clearance from the blood stream and metabolism.[41] Accumulation of nontargeted liposomes was not detected in the brain, heart, kidney, lung, mammary tissue, or uterus nor in the neonates (n = 4 each) (Figure 6B; animals 1 - 4). Organs isolated from mice injected with OTR-targeted liposomes showed accumulation of the OTR-targeted liposomes in the uterus and the mammary glands. As expected, there were also high levels of liposome localization in the liver. OTR-targeted liposome accumulation was not detected in the brain, heart, kidney,

FIGURE 5
Use of targeted liposomes to modulate mouse uterine contractility in vitro



Data are contraction traces for individual strips of mouse uterine tissue and illustrate effect of treatments on contraction amplitude and frequency. **A**, Effect of oxytocin receptor (OTR)-targeted, drug-free liposomes (n = 3). **Bi**, Effect of 9.25 µmol/L salbutamol (SAL) administered via non-targeted SAL-loaded liposomes (n = 3). **Bii**, Effect of 9.25 µmol/L SAL administered via OTR-targeted SAL-loaded liposomes (n = 3).

Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

or lung, nor in the neonates (n = 4 each) (Figure 6B; animals 5 - 8).

DiI fluorescence was quantified for each organ (Table 2). OTR targeting of liposomes resulted in significantly increased localization to the uterus compared to nontargeted liposomes ($P < .0001$; nontargeted = $5.04 \times 10^7 \pm 7.5 \times 10^6$; OTR-targeted = $3.57 \times 10^8 \pm 3.05 \times 10^7$ p/s/cm^2/sr) (Figure 6, C). On average this equaled a 7-fold increase in uterine localization. Furthermore, the level of OTR-targeted liposome localization in the uterus was significantly greater than that of brain ($P = .0002$), lung ($P = .0003$), kidney ($P = .0005$), heart ($P < .0001$), and neonate ($P < .0001$) (Figure 6, C). For both nontargeted and OTR-targeted liposomes, accumulation in the liver was significantly greater than all other organs examined ($P < .0001$), however there was no difference in liposome accumulation in the liver between nontargeted and OTR-targeted liposomes ($P > .9999$; nontargeted = $7.90 \times 10^8 \pm 9.15 \times 10^7$; OTR-targeted = $7.37 \times 10^8 \pm 9.05 \times 10^7$) (Figure 6, C).

Preventing PTB

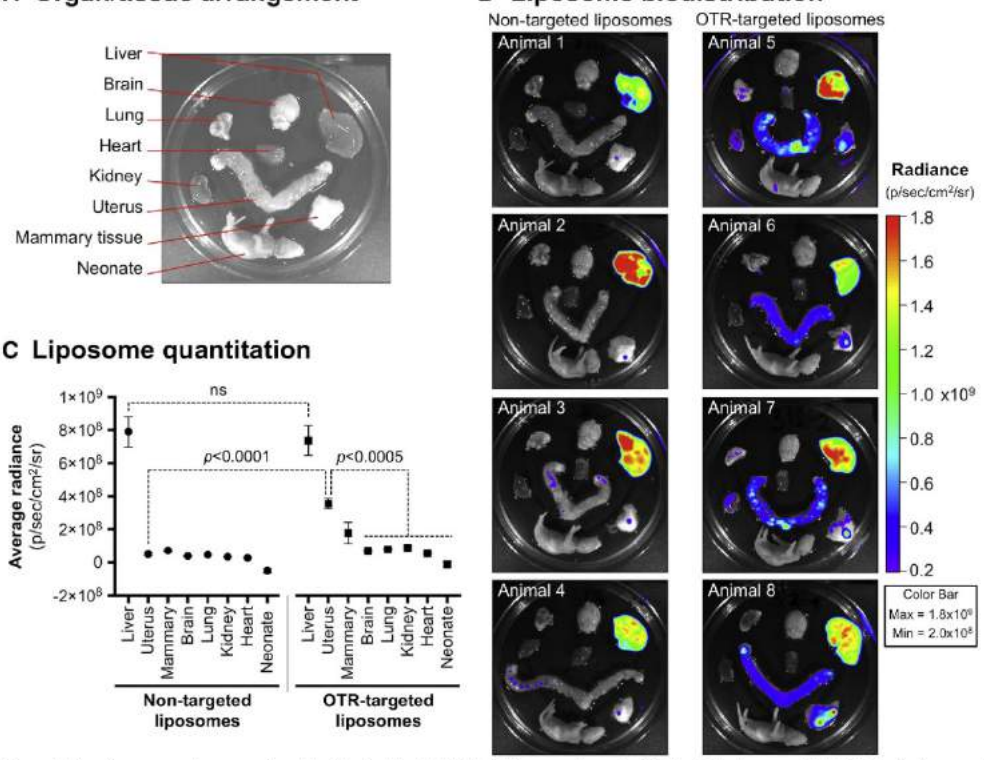
We used an LPS model of PTB to assess whether targeted liposomes could be used to administer IND for the prevention of LPS-induced PTB in mice. Nontargeted and OTR-targeted liposomes loaded with 5.5 mg/mL IND were compared against IND administered as free drug (1.0 or 2.0 mg/kg/d). Observed PTB rates are indicated in Table 3. The χ^2 analyses were performed.

PTB rates in control mice (group 1) and the LPS control group (group 2) were 0 (n = 12) and 67% (n = 18), respectively. At 2.0 mg/kg/d, IND administered as free drug significantly reduced rates of PTB from 67% down to 31% (group 2 [n = 18] vs group 4 [n = 13]; $P = .0484$) (Figure 7, A). PTB rate for OTR-targeted, drug-free control liposomes (group 5) was 56% (n = 16), and was not different to PTB rate observed for LPS control animals (group 2) ($P = .532$). IND administered at 2.0 mg/kg/d via nontargeted liposomes (group 6) (n = 12) had no effect as the observed PTB rate of 58% was not significantly different from PTB rates for LPS control animals (group 2) ($P = .643$) or animals treated with OTR-targeted, drug-free liposomes (group 5) ($P = .91$) (Figure 7, A).

IND administered at 2.0 mg/kg/d via OTR-targeted liposomes (group 7) (n = 11) resulted in a PTB rate of 18%, which was a significant reduction compared to the PTB rate of 67% for the LPS control animals (group 2) ($P = .0112$) (Figure 7, A). Furthermore, PTB rate for 2.0 mg/kg/d IND administered via OTR-targeted liposomes was significantly reduced compared to the same dose administered by nontargeted liposomes (group 6) ($P = .048$). No significant difference was observed between 2.0 mg/kg/d IND administered as free drug compared to when administered via OTR-targeted liposomes (group 4 vs group 7; $P = .4780$) (Figure 7, A).

The time between LPS injection and labor was calculated for each animal (average ± SEM shown in Table 3). Analysis of the normalized data showed that IP administration of LPS (0.7 µg/g) significantly advanced the time of labor, compared to control animals (group 1 vs group 2; $P = .0017$) (Figure 7, B). IND administered as free drug at 1.0 and 2.0 mg/kg/d dose-dependently increased the average time between LPS injection and labor (77.5 ± 14.1 and 86.6 ± 12.7 hours, respectively) compared to the LPS control (50.8 ± 8.9 hours), however neither dose reached statistical significance (group 2 vs group 3; $P = .53$, and group 2 vs group 4; $P = .08$) (Figure 7, B). OTR-targeted, drug-free liposomes had no effect on time between LPS injection and labor, compared to LPS

FIGURE 6
Oxytocin receptor (OTR)-targeted liposomes accumulate in uterus in vivo



Data are light or fluorescence images captured shortly after labor by IVIS-100 (Xenogen, Alameda, CA) illustrating liposome biodistribution that occurred in vivo. **A**, Representative image demonstrating arrangement of organs and tissues of interest (liver, brain, lung, heart, kidney, uterus, mammary tissue, and neonate). **B**, Fluorescent detection of liposome biodistribution that occurred in vivo. Biodistribution of nontargeted liposomes (n = 4; animals 1–4) and OTR-targeted liposomes (n = 4; animals 5–8). **C**, Quantitation of liposomal detection in different organs. Average radiance (p/s/cm^2/sr) was determined for each organ and compared across treatment groups (n = 4 for each organ per group). Data were confirmed to be normally distributed (Shapiro-Wilk normality test) then compared by 1-way analysis of variance with multiple comparisons (Holm-Sidak). Not all statically significant comparisons are indicated.

Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

control animals (group 2 vs group 5; $P = .92$). A total of 2.0 mg/kg/d IND administered via nontargeted liposomes had no significant effect on the time between LPS injection and labor (group 2 vs group 6; $P = .99$), however, when administered via OTR-targeted liposomes, time between LPS injection and labor was significantly increased (group 2 vs group 7; $P = .0048$). The time between LPS injection and labor was significantly different between 2.0 mg/kg/d IND delivered via nontargeted liposomes compared to OTR-targeted liposomes (group 6 vs group 7; $P = .0438$).

Number of live pups was recorded for term deliveries (no viable pups arose from preterm deliveries). Data were normally distributed (Shapiro-Wilk normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). There was no significant difference in the number of live pups from term deliveries in the different groups (Figure 7, C).

Comment

Principal findings

OTRs are expressed at low levels on various tissues toward the end of pregnancy, including the brain and

TABLE 2
Average radiance of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–labeled liposomes detected in organs and neonates

Organ/tissue	Average radiance, p/s/cm²/sr Mean ± SEM: Nontargeted liposomes n = 4 animals	OTR-targeted liposomes n = 4 animals
Liver	$9.73 \times 10^8 \pm 9.1 \times 10^7$	$7.37 \times 10^8 \pm 9.05 \times 10^7$
Uterus	$5.04 \times 10^7 \pm 7.5 \times 10^6$	$3.57 \times 10^8 \pm 3.05 \times 10^7$
Mammary tissue	$7.29 \times 10^7 \pm 6.05 \times 10^6$	$1.78 \times 10^8 \pm 6.47 \times 10^7$
Brain	$3.97 \times 10^7 \pm 2.51 \times 10^6$	$7.03 \times 10^7 \pm 4.51 \times 10^6$
Lung	$4.65 \times 10^7 \pm 4.82 \times 10^6$	$7.97 \times 10^7 \pm 2.94 \times 10^6$
Kidney	$3.46 \times 10^7 \pm 1.78 \times 10^6$	$8.79 \times 10^7 \pm 9.51 \times 10^6$
Heart	$2.79 \times 10^7 \pm 1.65 \times 10^6$	$5.52 \times 10^7 \pm 1.65 \times 10^6$
Neonate	$-4.83 \times 10^7 \pm 1.30 \times 10^7$	$-1.03 \times 10^7 \pm 1.46 \times 10^7$

OTR, oxytocin receptor.
Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

mammary tissue.[42] Expression in the pregnant uterus however is high approaching term,[30,31] indicating that the OTR is an excellent candidate for the development of a targeted drug delivery system for the uterus. This study represents an initial analysis of OTR-targeted liposomes as a drug delivery system, and demonstrates that:

(i) conjugation of the OTR antibody to the surface of liposomes confers the ability for NIF-, SAL-, and ROL-loaded liposomes to significantly reduce human myometrial contractions in vitro, as confirmed by AUC analyses;
(ii) enhancement of myometrial contractility can be achieved through encapsulation of uterotonic agents, as confirmed by use of OTR-targeted DOF-loaded liposomes to significantly increase contraction plateau duration;
(iii) nontargeted liposomes loaded with these same therapeutic agents do not affect myometrial contractions in vitro, as confirmed by AUC and contraction plateau duration analyses;
(iv) the effects are reversible (depending on the therapeutic), as confirmed by the spontaneous resumption of contractions in both human and mouse myometrial tissue in vitro;
(v) the OTR-targeted liposomes themselves have no apparent effect on myometrial contractions, as confirmed by AUC analyses for myometrial contractions in vitro, and lack of effect on PTB rates in mice or time between LPS injection and labor;
(vi) in vivo, OTR-targeted liposomes localize to the uterus and breast of pregnant mice whereas nontargeted liposomes do not. Uterine localization was increased 7-fold by OTR targeting, as confirmed by quantitation of average radiance for key organs of interest;
(vii) no evidence of transplacental passage of the liposomes to the fetus was observed, as determined quantitative evaluation of DiI fluorescence in neonates;
(viii) OTR-targeted liposomes loaded with IND are effective in reducing rates of LPS-induced PTB in mice whereas nontargeted IND-loaded liposomes have no effect.

Clinical significance

Many current tocolytics have been associated with adverse effects on the

TABLE 3
Rates of lipopolysaccharide-induced preterm birth and time between lipopolysaccharide injection and labor

Group no.	Treatment group	n	PTB rate (%)	Time between LPS injection and observed labor, h, mean ± SEM
1	Control (no LPS, no liposomes)	12	0/12 (0)	109.7 ± 4.1
2	LPS control (+50% DMSO)	18	12/18 (67)	50.8 ± 8.9
3	LPS + 1.0 mg/kg IND (50% DMSO)	10	4/10 (40)	77.5 ± 14.1
4	LPS + 2.0 mg/kg IND (50% DMSO)	13	4/13 (31)	86.6 ± 12.7
5	LPS + OTR-targeted, drug-free liposomes	16	9/16 (56)	65.0 ± 9.9
6	LPS + nontargeted 2.0 mg/kg IND liposomes	12	7/12 (58)	55.6 ± 12.4
7	LPS + OTR-targeted 2.0 mg/kg IND liposomes	11	2/11 (18)	101.3 ± 12.4

DMSO, dimethyl sulfoxide; *IND*, indomethacin; *LPS*, lipopolysaccharide; *OTR*, oxytocin receptor; *PTB*, preterm birth.
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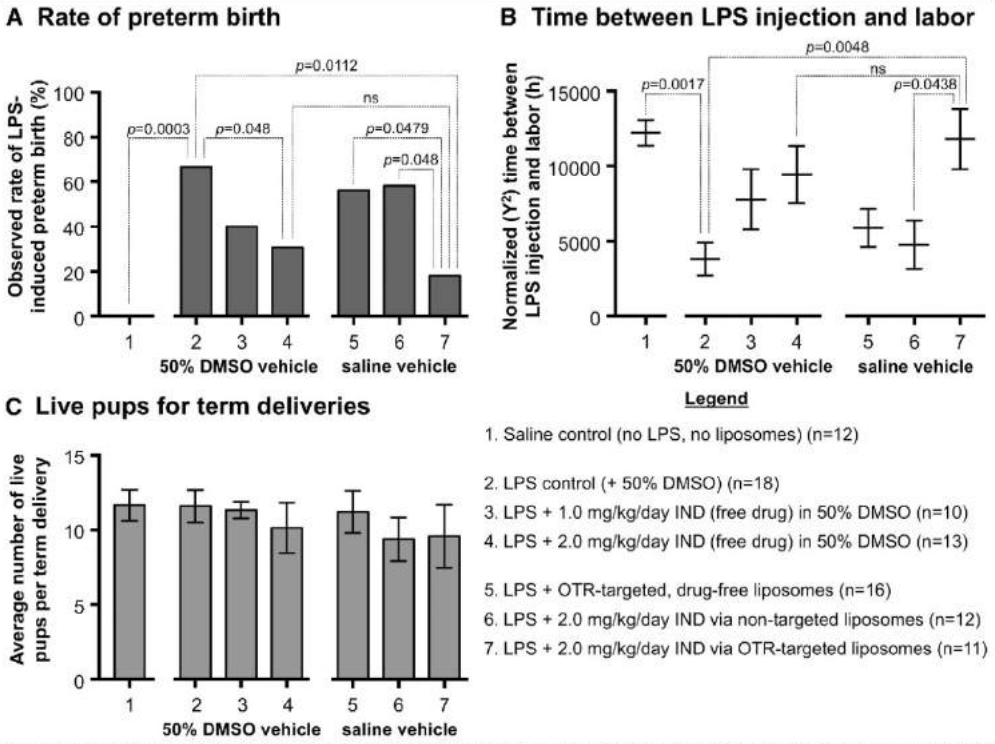
FIGURE 7
Preventing preterm birth (PTB) using oxytocin receptor (OTR)-targeted indomethacin (IND)-loaded liposomes



Efficacy of targeted liposomes was assessed using lipopolysaccharide (LPS) mouse model of PTB. **A**, Effect of IND administered as free drug (1.0 or 2.0 mg/kg/d) or via liposomal preparations (2.0 mg/kg/d) on rates of LPS-induced PTB. **B**, Time (hours) between LPS injection and observed labor. **C**, Number of live pups born for term deliveries. No significant differences were recorded in number of live pups. PTB rates were analyzed by χ^2 analysis. Data for time (hours) between LPS injection and labor were normalized ($Y = Y^2$) (D'Agostino and Pearson normality test) then analyzed by 1-way analysis of variance (ANOVA) with multiple comparisons (Tukey). Number of live pups was normally distributed and analyzed by 1-way ANOVA with multiple comparisons (Tukey).

DMSO, dimethyl sulfoxide.

Paul et al. Targeted uterine drug delivery system. Am J Obstet Gynecol 2016.

mother (β-sympathomimetics)[6,43,44] and on the fetus (NIF, IND)[16-18,45,46] or have no evident effect on prolongation of pregnancy (atosiban).[14,15] NIF is capable of providing some clinical benefit, with a systematic review and meta-analysis indicating a significant reduction in the risk of delivery within 7 days of initiation of NIF treatment.[3] However, the high doses required to achieve relaxation of the myometrium increases the risk of adverse systemic effects.[45-47] IND has been explored as a tocolytic agent for preterm labor,[48-50] however, systematic review indicates that IND is associated with an increased risk for severe intraventricular hemorrhage, necrotizing enterocolitis, and periventricular leukomalacia.[51] A study in 2015 by Refuerzo et al[52] demonstrated that encapsulation of IND inside nontargeted liposomes can reduce IND levels in the fetus 7.6-fold, suggesting the potential for reduced fetal side effects.

Here we have demonstrated in mice that IND encapsulated inside OTR-targeted liposomes was effective in reducing rates of LPS-induced PTB, whereas nontargeted IND-loaded liposomes were not. These results, in conjunction with our biodistribution studies, suggest that OTR targeting confers upon liposomes the ability to target therapeutics to the uterus. The clinical implications are that OTR-targeted liposomes may enable existing tocolytics, such

as NIF and IND, to be administered with improved efficacy and improved safety.

The restricted biodistribution of OTR-targeted liposomes raises the possibility of introducing into clinical practice therapeutic agents that are known to be highly effective tocolytics, yet are known to have adverse off-target effects. One such group of candidates are the PDE4 inhibitors, such as ROL, which have been demonstrated to be highly effective in controlling inflammation-driven preterm delivery in mice.[53] Mounting evidence indicates that PTB in human beings is also an inflammation-driven event, and evidence that ROL is highly effective in abolishing spontaneous contractions in human myometrium[33] suggests that PDE4 inhibitors may be excellent cargo for OTR-targeted liposomes in the setting of preterm labor.

Clinical implications also include the prospect of encapsulating uterotonic agents, and here we demonstrate that possibility through the use of DOF. DOF is not a traditional uterotonic agent, but when administered via OTR-targeted liposomes DOF significantly increased the duration of human myometrial contractions in vitro, consistent with previous findings.[36] Targeted delivery of uterotonics may be useful to promote contractions, including during failure of labor to progress, expulsion of the placenta after labor, expulsion of retained products after miscarriage, or to control PPH. PPH is a leading cause of maternal mortality worldwide and is linked with major morbidities such as peripartum hysterectomy and massive transfusion.[54–56] First-line therapy for PPH is uterine massage and oxytocin administration, however rates of atonic PPH after oxytocin use are increasing in many developed countries.[57,58] When refractory uterine atony occurs, second-line therapy may include administration of uterotonic agents such as methylergonovine and carboprost. Methylergonovine was recently identified as the more effective of the two,[59] however both agents could effectively be encapsulated in OTR-targeted liposomes. Evidence indicates that post-receptor contractile signaling pathways are maintained in oxytocin desensitized primary myocytes in vitro,[60] however oxytocin desensitization occurs, at least in part, by down-regulation of OTR protein levels.[61] Uterotonic-loaded liposomes targeted to the OTR may therefore be of reduced effectiveness in patients with prolonged exposure to oxytocin.

Future research

These data provide the first evidence that OTR-targeted liposomes are a drug delivery system that affords flexibility in delivery of different classes of therapeutic agents to human uterine tissue to modulate myometrial contractility. Furthermore, these data provide the first evidence that OTR-targeted liposomes can be used to administer therapeutic agents for the prevention of PTB in mice.

Further studies are necessary to determine the mechanism of OTR-targeted liposome uptake in myocytes, the quantitative biodistribution of therapeutic agents achieved in the uterus compared to other organs, and the rate of liposome clearance. Additional studies have been planned to determine whether the use of OTR-targeted liposomes to administer therapeutics for prevention of PTB is effective in reducing fetal side effects, such as premature closure of the ductus arteriosus in response to IND exposure in utero. ■

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