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Comparative and functional analysis of β-adrenoceptors in human myometrium

by

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Abstract

There are three subtypes of the β-adrenoceptors (β_1, β_2 and β_3). All the β-adrenoceptor subtypes couple to $G_{\alpha s}$ subunit of G protein. All the β-adrenoceptor subtypes couple mainly to $G_{\alpha s}$ subunit of G protein. β_2-adrenoceptor agonists have been used clinically to suppress myometrial contractions during the management of preterm labour. β_2-adrenoceptor agonists lose their effectiveness in a short time and their use causes several side effects. Evidence in the literature supports the expression of functional β_2- and β_3-adrenoceptors in the lower pregnant human myometrial tissues. However, no definitive understanding in terms of regional and temporal expression and function of the β-adrenoceptors in the uterus was obtained by reviewing the literature. Experiments of this work have confirmed the expression of the three β-adrenoceptor subtypes in term pregnant (upper and lower) human myometrial tissues by real-time RT-PCR analysis. Poor antibody specificity was an obstacle to determine and localize the β-adrenoceptor proteins in the myometrium by immunohistochemistry. β_2- and β_3-adrenoceptor agonists did not affect the expression of *CRISPLD2*, *RGS2* and *NA4A3* in primary MSM cells after 2 and 6 h of incubation. Further research must be done to determine and localize the expression of the β-adrenoceptors in the myometrium.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC	Adenylate cyclase
ANOVA	Analysis of variance
B2M	β-2-microglobulin gene
BEAS-2B	Human bronchial epithelial cell line
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CREB	cAMP response element-binding protein
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2 gene
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GRK2	G protein-coupled receptor kinase 2
HBSS	Hanks' Balanced Salt Solution
HRP	Horseradish-peroxidase
IL-1β	Interleukin-1β
IL-6	Interleukin 6 gene
IL-8	Interleukin 8
kDa	Kilodalton
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
MSM	Myometrial smooth muscle

$NaHCO_3$	Sodium bicarbonate
NR4A3	Nuclear receptor subfamily 4 group A member 3 gene
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PPROM	Preterm pre-labour rupture of membrane
RGS2	Regulator of G protein signaling 2 gene
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFM	Serum free media
TBS-T	Tris-buffered saline-tween
TL	Term labour
TNL	Term non-labour
β-AR	β-adrenoceptor

Chapter One: Introduction

1.1 Significance

Preterm birth is a major cause of neonatal morbidity and mortality (Hamilton & Tower, 2013). Clinical intervention for the management of preterm labour includes agents called tocolytic agents. Tocolysis is the suppression of uterine contraction (Simhan & Cariti, 2007). Tocolytic agents are medications that either inhibit myometrial contraction or activate myometrial relaxatory mechanisms (Simhan & Cariti 2007; Hamilton & Tower, 2013). A recent study indicated that tocolytic agents are not effective to prolong pregnancy for more than 7 days (Miyazaki *et al.*, 2016). β_2-Adrenoceptor agonists are clinically used tocolytic agents that promote myometrial relaxation. During the management of preterm labour, β_2-adrenoceptor agonists rapidly lose their effectiveness and, in addition, have several side-effects (Jp *et al*., 2014). The β_3-adrenoceptors lack phosphorylation sites for PKA and GRK2 making them less susceptible to receptor desensitization. Therefore, the β_3-adrenoceptors may offer more advantages than β_2-adrenoceptors as potential therapeutic targets for mediating utero-relaxation and gene expression (Nantel *et al*., 1993; Takeda *et al.*, 2002). Thus, a clearer understanding of the distribution of β-adrenoceptors and their potential roles in the human myometrium might reveal new strategies for the management of preterm labour, which could include the dual use of β_2- and β_3-adrenoceptor agonists.

1.2 Preterm labour

In humans, labour normally occurs between 37-41 weeks of pregnancy, while preterm labour is defined as labour that occurs prior to 37 weeks and after 24 weeks of gestation (Behrman *et al.*, 2007). Preterm delivery is associated with approximately 80% of neonatal

morbidity and mortality (Jeyabalan & Caritis, 2002). The severity of outcomes in preterm neonates is inversely proportional to the degree of developmental maturity. A major problem of preterm birth is the immaturity of the neonate's lung which may lead to pulmonary hyperplasia and respiratory distress syndrome. Neonates born prematurely also have an increased risk of neurodevelopmental impairments, which extend from cerebral palsy and cognitive impairment to visual and hearing disorders (Behrman *et al.*, 2007).

The pathophysiology of preterm labour is still poorly understood; however, research has shown that several factors increase the risk of preterm labour including: stress, poor nutrition, smoking, ethnicity, age, and low body mass index (Hamilton & Tower, 2013). Moreover, 70% of preterm labours occur spontaneously in response to pathological conditions including intra-amniotic infection, such as chorioamnionitis, and preterm pre-labour rupture of membrane (PPROM) (Goldenberg *et al.*, 2008). Intrauterine infection is usually chronic but sub-clinical due to lack of symptoms other than preterm labour (Goldenberg *et al*., 2000). Cytokines and chemokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are increased in response to intrauterine infection and they may lead to the progression of labour (Friebe-Hoffmann *et al.*, 2001; Rauk *et al.*, 2001; Peltier, 2003).

1.3 The uterus and parturition

The uterus is a hollow muscular organ whose function is to hold the fetus during gestation. The myometrium is the layer of the uterine muscle responsible for uterine contraction and birth of neonates (Seeley *et al.*, 2008). Anatomically, the human uterus is divided into two parts, the upper and the lower uterine segments. During pregnancy the myometrium

maintains a relatively quiescent state, but inn late pregnancy it transforms to a contractile state and generates contractions of labour. The mechanism by which myometrium transforms from a quiescent to a contractile phenotype is not clearly understood. It has been suggested that the control of this transformation could be mechanical, maternal, local (endocrine or paracrine) or fetal (Callis *et al.,* 2000).

G protein-coupled receptors expressed in the myometrium have an important role in the regulation of myometrial activity during labour. For example, oxytocin receptors (OTRs), prostaglandin $F_{2\alpha}$ receptors (FPs), prostaglandin E receptors ($EP_{1\text{-}4}$), corticotropin releasing hormone receptors (CRH-$R_{1\text{-}2}$) and β-adrenoceptors are all G protein-coupled receptors that couple to different G protein subunits ($G_{\alpha s}$, $G_{\alpha i}$ or $G_{\alpha q}$) to elicit different effects in the myometrium **(Figure 1.1.)**. Evidence from the literature shows that some of the GPCRs and other proteins are not distributed equally through the upper and lower uterine segments during pregnancy and labour (Sparey *et al.,* 1999; Astle *et al.,* 2005; Grigsby *et al.,* 2006; Cong *et al.,* 2009). Therefore, it has been hypothesized that the upper and lower uterine segments of the uterus have different roles during labour; after the onset of labour, the upper segment of the uterus contracts to push the fetus downward while the lower uterine segment becomes more relaxed to allow passage of the fetus. In support of this hypothesis, studies have demonstrated differential expression of several genes in the upper and lower segment of the pregnant human uterus. For example, prostaglandin-endoperoxide synthase-1 and 2 (PTGS-1 and 2), enzymes that stimulate prostaglandin synthesis, protein expression was higher in the lower compared to the upper myometrial segment and this difference is not affected by labour (Sparey *et al.,* 1999), whereas connexin-43, a gap junction protein, protein expression was higher in the upper compared to the lower

myometrial segment and the increase in the expression of connexin-43 protein in the upper segment was greater after the onset of labour (Sparey *et al.*, 1999). In addition, after the onset of labour, the lower myometrial segment expressed a higher level of corticotrophin-releasing hormone receptor 1 protein (CRH-R1) in comparison to the upper segment (Cong *et al.*, 2009), while prostaglandin E_2 receptor, EP_3, mRNA expression was higher in the upper compared to the lower myometrial segment (Astle *et al.*, 2005; Grigsby *et al.*, 2006). However, whether there are differences in the expression of β-adrenoceptors with labour is not yet known.

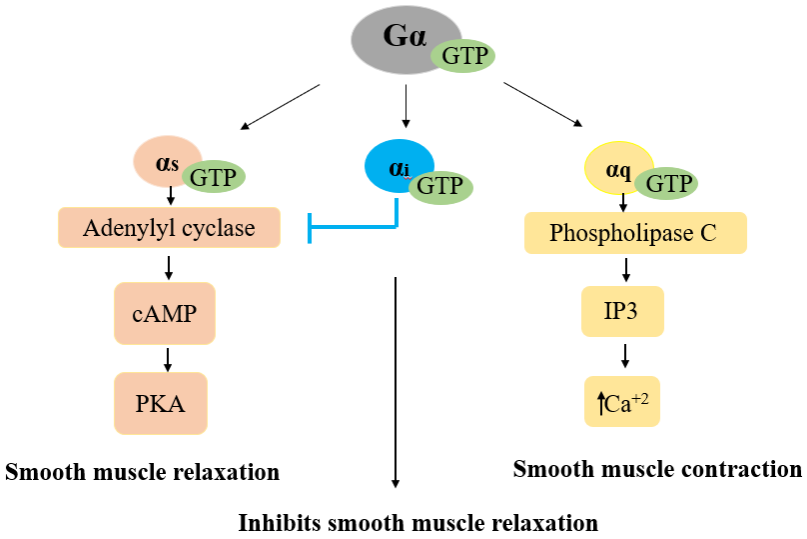


Figure 1.1. Different G_α subunits and their effect on smooth muscle. Different G_α subunits ($G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$) regulate different effectors, initiate downstream signaling and evoke different effects. $G_{\alpha s}$ activates adenylyl cyclase system which lead to smooth muscle relaxation, $G_{\alpha i}$ inhibits adenylyl cyclase system, thus inhibit smooth muscle relaxation while $G_{\alpha q}$ activates phospholipase C, which activate synthesis of biomolecules that increase calcium level in the cells leading to smooth muscle contractions.

1.4 Myometrial contraction-relaxation control

The myometrium is a layer of smooth muscle cells in the uterine wall. The contractile apparatus of a single uterine smooth muscle cell, a myocyte, works by attachment and detachment of two structural microfilamentous proteins called actin and myosin (Blanks *et al.*, 2007). There are two isoforms of actin that contribute to contraction mechanism, α and γ-actin (Aguilar & Mitchell, 2010). Myosin consists of two heavy chains (MHC) and two pairs of light chains (MLC) (Aguilar & Mitchell, 2010). The process of actin and myosin interaction is called crossbridge cycling. This process is activated by phosphorylating myosin light chain by the enzyme myosin light change kinase (MLCK) and inhibited by the enzyme myosin light chain phosphatase. Myosin light chain kinase is activated through the protein calmodulin, which in turn requires binding to four calcium ions for activation (Blanks *et al.*, 2007). (**Figure 1.2.**).

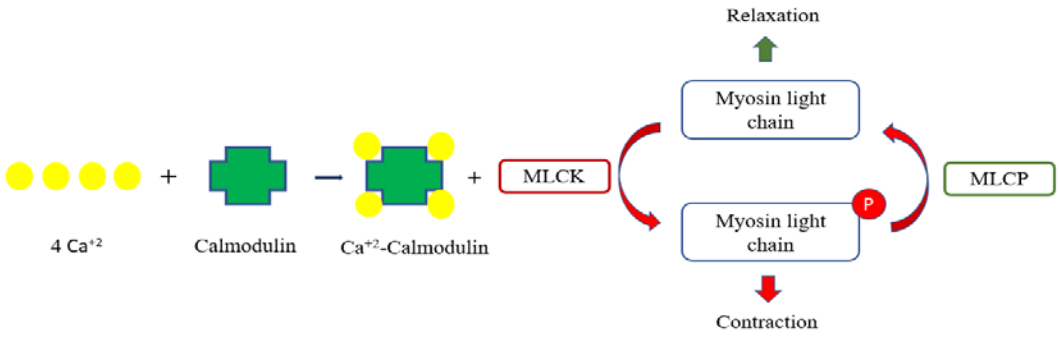


Figure 1.2. Myometrial contraction-relaxation control. Calmodulin binds to four calcium ions and activates to form a complex that activates the enzyme myosin light chain kinase (MLCK), which phosphorylates myosin light chain causing contraction. Myosin light chain phosphatase (MLCP) is the enzyme that reverses the action of myosin light chain kinase (MLCK).

1.5 The β-adrenoceptors

The β-adrenoceptors are G-protein coupled receptors (GPCRs) that mediate the physiological effects of adrenaline and noradrenaline (Hall, 2004). There are 3 distinct β-adrenoceptor subtypes, β_1, β_2, and β_3 encoded by the genes *ADRB1*, *ADRB2* and *ADRB3* respectively (**Table 1.1**). Catecholamines have different potency order on these β-adrenoceptor subtypes. (**Table 2.1**).

1.6 β-adrenoceptors canonical signaling pathway:

Agonist binding to β-adrenoceptors results in activation of downstream signaling pathways. The β-adrenoceptors signal primarily through the heterotrimeric G protein, $G_{\alpha s}$ (Hall, 2004). Coupling of $G_{\alpha s}$ to adenylyl cyclase (AC) increases the synthesis of the second messenger cAMP, which activates protein kinase A (PKA). Protein kinase A activation promotes smooth muscle relaxation by phosphorylating proteins that affect smooth muscle contractility. Myosin light chain kinase is phosphorylated by PKA resulting in reduced affinity of MLCK to calcium-calmodulin complex required for activation (Challis *et al.*, 2000). Protein kinase A also phosphorylates calcium-activated potassium channels (K_{Ca}-channels) increasing their activity and leading to cell membrane hyperpolarization. Protein kinase A also phosphorylates phospholipase C enzyme reducing its activity thus reducing formation of inositol triphosphate (IP_3). Inositol triphosphate activates inositol triphosphate receptors (IP_3R) in the endoplasmic reticulum leading to the release of calcium. Therefore, phosphorylating PLC-β enzyme would lead to a decrease in the release of calcium, which is required for smooth muscle contraction. Protein kinase A also affects the intracellular calcium level by phosphorylating inositol triphosphate receptors (IP_3R) that reduces their

activity (Yuan & Bernal, 2007). Additionally, PKA can phosphorylate the transcription factor, cAMP-responsive element binding protein (CREB) **(Figure 1.3.)**, which binds to cAMP response elements (CRE) in the promoter region of target genes to affect gene transcription (Summers *et al.*, 1997). Microarray data show an increase in the expression of genes in BEAS-2B cells following cAMP-elevating treatments including β_2-adrenoceptor agonists. The β-adrenoceptors may thus play a role in modulating gene transcription during parturition. The following sections illustrate and describe examples of genes that are regulated in the BEAS-2B cells, bronchial epithelial cell line, by β_2-adrenoceptor agonists and may have clinical significance in the management of preterm labour:

1.6.1 Cysteine-rich secretory protein LCCL domain containing 2 (*CRISPLD2*)

Lipopolysaccharide (LPS) is a toxic component of gram-negative bacteria such as *Escherichia coli*. Lipopolysaccharides from different gram-negative bacteria have a similar general structure, in which lipid A is the major toxic component (Erridge *et al.*, 2002). Lipopolysaccharides are recognized by toll-like receptors (TLR2 and TLR4) which initiate signaling pathways to stimulate the production of pro-inflammatory cytokines (Janeway *et al.*, 2005). Cytokines such as interleukin-1β stimulate synthesis of prostaglandins, which activate myometrial contraction. Production of cytokines also leads to infiltration of neutrophils and release of metalloproteinases, which activate rupture of chorioamniotic membrane and cervical ripening (Goldenberg *et al.*, 2000). Collectively, the immunological response leads to labour by activating myometrial contractions, rupture of chorioamniotic membranes and cervical ripening.

CRISPLD2 encodes a secretory protein, which has a high affinity for lipid A thereby impairing its function and reducing the immunological response, which leads to progression of labour. Impairing the immunological response evoked by gram-negative bacteria toxin by CRISPLD2 protein may prevent preterm labour. Microarray-based gene expression profiling in uterine smooth muscle cells identified *CRISPLD2* as progesterone responsive gene (Lei *et al.,* 2011). In BEAS-2B, *CRISPLD2* mRNA expression increased in response to cAMP-elevating agents including β_2-adrenoceptor agonists (BinMahfouz *et al.,* 2015; Joshi *et al.,* 2015). The expression of *CRISPLD2* in myometrial tissues was confirmed by conventional RT-PCR.

1.6.2 Nuclear receptor subfamily 4 group A member 3 (*NR4A3*)

NR4A3 is an orphan nuclear receptor. The expression of *NR4A3* increased in response to cAMP-elevating agents in BEAS-2B cells. Nomiyama *et al.,* (2006) illustrated that *NR4A3* works as a regulator for smooth muscle proliferation in rat aortic vascular smooth muscles. In human vascular smooth muscle cells, *NR4A3* prevented activation of the NF-κB pathway (Calvayrac *et al.,* 2015). NF-κB is a transcription factor that regulates the transcription of some pro-inflammatory cytokines such as IL-1β, which are thought to have a role in the initiation of labour (Lindstrom & Bennett, 2005). Evidence in the literature shows that the transcription factor NF-κB has a constitutive activity in amnion cells and this activity causes an increase in the expression of the enzyme prostaglandin-endoperoxide synthase (PTGS-2) and this activity also contributes to functional progesterone withdrawal (Allport *et al.,* 2001). Increased expression of PTGS-2 and functional progesterone withdrawal are both factors that lead to labour. Thus, preventing the activation of NF-κB through *NR4A3* could be a clinical approach in the management of preterm labour.

1.6.3 Regulator of G-protein signaling (*RGS2*)

RGS2 encodes a protein that switches off $G_{\alpha q}$-coupled GPCRs by activating $G_{\alpha q}$-GTPase (Heximer, 2004). The expression of *RGS2* is increased by long acting β_2-adrenoceptor agonists treatments in primary human airway smooth muscle cells and bronchial epithelial cells (BEAS-2B) (Holden *et al.*, 2011; Holden *et al.*, 2014). The myometrium expresses $G_{\alpha q}$-coupled GPCRs such as oxytocin receptors (OXTRs), prostaglandin $F_{2\alpha}$ receptors (FPs) and prostaglandin E_2 receptors 1 and 3 ($EP_{1,3}$). Activation of $G_{\alpha q}$-coupled receptors leads to activation of the contractile machinery of the myometrium and subsequently to labour. OXTR inhibitors such as atosiban and prostaglandin synthesis inhibitors such as indomethacin are used clinically for tocolysis (Hamilton & Tower, 2013) whereas oxytocin and prostaglandin analogues are used to induce labour (Alfirevic *et al.*, 2016). OXTR, FP and $EP_{1,3}$ signaling might be targeted by RGS2 to reduce the contractile activity of the myometrium thus delaying labour.

1.6.4 Interleukin-6 (*IL-6*)

IL-6 is a cytokine which gives rise to the production of cytokines. The expression of *IL-6* increased in BEAS-2B following β_2-adrenoceptor agonists treatments. In adipocytes, β_3-adrenoceptor agonist treatment resulted in increased expression of *IL-6* and this increase was independent on PKA, rather PKC was involved (Tchivileva *et al.*, 2009). Evidence suggests that *IL-6* causes an increase in the expression of oxytocin receptors and enhances the secretion of the oxytocin in human myometrial cells (Friebe-Hoffmann *et al.*, 2001; Rauk *et al.*, 2001). Increased expression of oxytocin and oxytocin receptors would lead to labour.

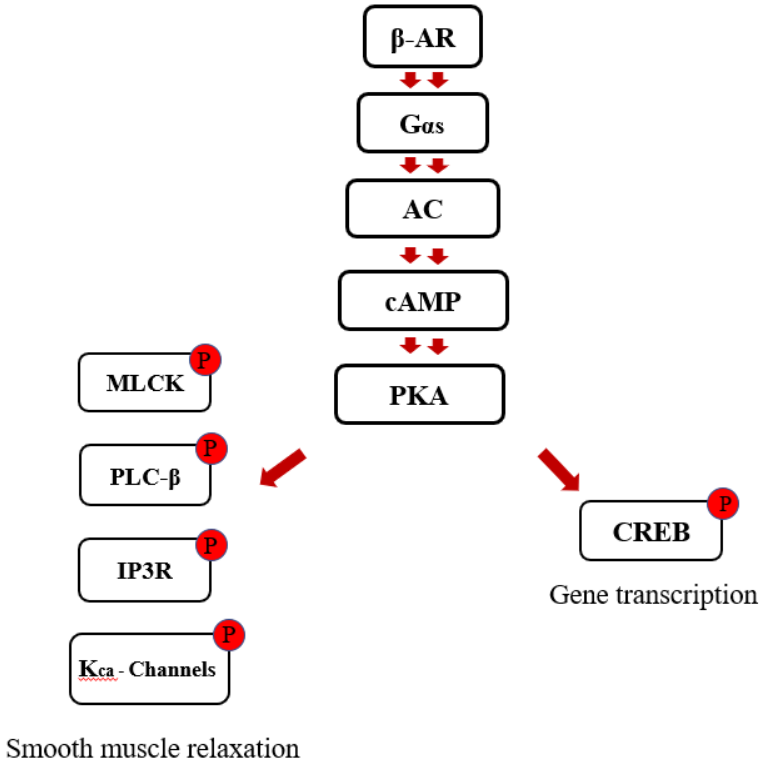


Figure 1.3. A simplified schematic showing β-adrenoceptor-mediated signaling and response. Ligand binding to the β-adrenoceptors leads to a conformational change in the receptor, which activates $G_{\alpha s}$ subunit of G protein. $G_{\alpha s}$ subunit activates the enzyme adenylyl cyclase (AC), which in turn activates the biosynthesis of the second messenger cAMP. The second messenger cAMP activates protein kinase A (PKA) which phosphorylates proteins to elicit smooth muscle relaxation and modulate gene transcription. **MLCK:** myosin light chain kinase, **PLC-β:** phospholipase C-β, **IP_3R:** inositol triphosphate receptor, **K_{Ca}-Channel**: Calcium activated potassium channels, **CREB:** cAMP-response element binding protein.

1.7 Other β-adrenoceptor signaling pathways:

In addition to the canonical signaling pathway of the β-adrenoceptors, there is evidence for the ability of these receptors to signal through other more complex pathways, which could

be cAMP-independent pathways. For example, in airways smooth muscle, β_2-adrenoceptors activate tyrosin kinase (Src) through coupling to $G_{\alpha s}$ or $G_{\alpha i}$ subunits of G-protein. Activation of Src leads to the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2). In addition, the β_2-adrenoceptors activate exchange proteins directly activated by cAMP (Epac), a pathway that is independent on the activation of PKA. The β_2-adrenoceptor activation can also lead to activation of protein kinase G (PKG) through cross-over activation by cAMP (Giembycz & Newton, 2006). Moreover, in HEK 293 cells stably transfected by β_2-adrenoceptors, β_2-adrenoceptors could elicit β-arrestin-biased activation which result in ERK1/2 dependent signaling pathway as well as activation of epidermal growth factor receptor (EGFR) transactivation (Carr *et al.,* 2016).

The β_3-adrenoceptors have also shown an ability to signal through multiple pathways in different tissues. In adipocytes, β_3-adrenoceptor agonists activated ERK1/2-dependent signaling pathway through coupling to $G_{\alpha i}$ subunit of G-proteins (Cao *et al.,* 2000), while activated mitogen-activated protein kinase P38-dependent signaling pathway through coupling to $G_{\alpha s}$ subunit in the same tissue (Cao *et al.,* 2001). In primary myometrial cells, a novel β_3-adrenoceptor signaling pathway was introduced by Hadi *et al.,* (2013), in which a selective β_3-adrenoceptor agonist elicited cell proliferation through $G_{\alpha s}$ and PKA-dependent ERK1/2 activation after 3 minutes of activation. However, after 8 hours, β_3-adrenoceptor agonist activated ERK1/2 signaling pathway which was $G_{\alpha i}$ and Src-dependent.

1.8 The β_2-adrenoceptor in the management of preterm labour (clinical observations)

β_2-Adrenoceptor agonists, such as ritodrine, were given intravenously to suppress myometrial contractions during the management of preterm labour, but their action is accompanied by maternal and fetal systemic and metabolic side-effects (Jp *et al.*, 2014). Maternal side effects include tremor, palpitation, tachycardia, hypotension, hyperglycaemia, and hypokalaemia. The β_2-adrenoceptor agonists can cross the placenta and cause fetal tachycardia and hyperinsulinemia (Jp *et al.*, 2014). Moreover, β_2-adrenoceptor agonists lose their effectiveness after a short period of time (typically 48 hours after administration). Delaying labour for 2 days is usually sufficient to complete a course of antenatal corticosteroids required to enhance fetal lung maturity, and to ensure safe transport of the mother to a tertiary intensive care unit where care for premature neonates is available (Simhan & Caritis, 2007). However, the use of β_2-adrenoceptor agonists in tocolysis is controversial due to their side-effects. One possible reason for β_2-adrenoceptor agonist loss of effectiveness is β_2-adrenoceptor desensitization.

1.9 β-Adrenoceptor desensitization

Desensitization is a reduction in the effect produced by a drug after repeated administration (Summers *et al.*, 1997). The two main mechanisms that can produce receptor desensitization are (i) receptor phosphorylation leading to its inability to couple to $G_{\alpha s}$, and (ii) downregulation or internalization of the receptor (Wallukat, 2002). There is also evidence that β_2-adrenoceptor-mediated signaling can be desensitized through the up-regulation of the phosphodiesterase enzymes with a resultant increase in cAMP breakdown (Giembycz, 1996).

Generally, GPCRs have 7 transmembrane spanning domains, which form a ligand binding pocket; these domains are connected by 3 extracellular and 3 intracellular loops and ending by an intracellular carboxyl group and an extracellular amino group (Summers *et al.,* 1997) (**Figure 1.4.**).

In β_2-adrenoceptors, the third intracellular loop and the carboxyl terminus contain phosphorylation sites for PKA and G protein-coupled receptor kinase 2 (GRK2), which are involved in receptor desensitization. Significantly, β_3-adrenoceptors lack these phosphorylation sites and are, therefore, less susceptible to agonist-induced desensitization (Nantel *et al.,* 1993).

The mechanism of desensitization differs among cell types, and may depend on the level of GRK2 expression (Johnson & Kingdom, 2006). For example, in the myometrium, expression of GRK2 is observed in term pregnant but not non-pregnant myometrial tissue (Brenninkmeijer *et al.,* 1999); higher expression of GRK2 may explain, in part, the loss of effectiveness of β_2-adrenoceptor agonists after a short period of time of repeated administration during the treatment of preterm labour.

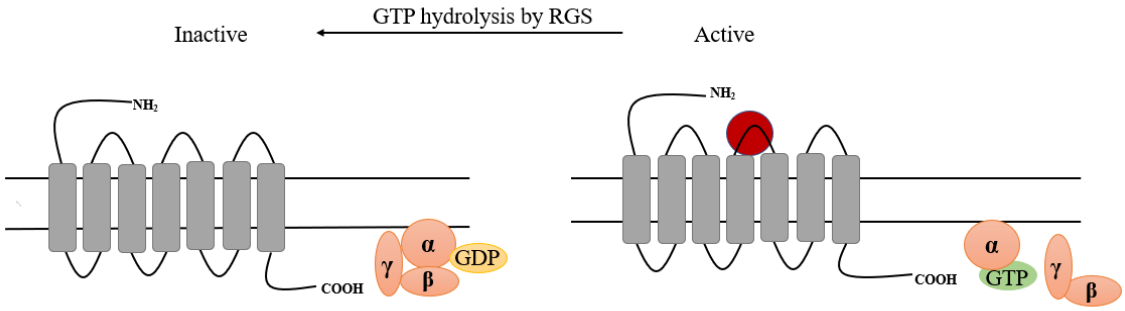


Figure 1.4. Schematic showing the structure of β-adrenoceptors along with a heterotrimeric G protein. The β-adrenoceptors consist of 7 transmembrane-spanning domains connected to each other by 3 extracellular and 3 intracellular loops and ending in an intracellular carboxyl group and an extracellular amino group. G proteins consist of 3 subunits: α, which in its inactive state binds to GDP while binds to GTP molecule in its active state, β and γ subunits.

1.10 Systematic review: The β-adrenoceptors as targets for the management of preterm labour; what is the evidence?

1.10.1 Main question

What is the evidence for targeting the β-adrenoceptors during the management of preterm labour?

1.10.2 Rationale

The β_2-adrenoceptors were targeted for the management of preterm labour, but β_2-adrenoceptor agonists lose their effectiveness in inhibiting myometrial contractions shortly after initiation of treatment. In addition, usage of β_2-adrenoceptor agonists is accompanied with several side-effects. The β_3-adrenoceptor subtype has been suggested as an alternative target to inhibit myometrial contractions during preterm labour. I reviewed the literature systematically to find evidence for targeting the β-adrenoceptors during preterm labour including their special and temporal expression and function in the uterus during pregnancy.

1.10.3 Aims

- To review the literature systematically and assess the data suggesting expression and function of β-adrenoceptors in the uterus.
- To collect available information about β-adrenoceptors functional response in the uterus.

1.10.4 Search terms

An electronic search was performed using MEDLINE on July 30th, 2017. Search terms included: ADRB1 or ADRB2 or ADRB3 or beta-adrenoceptor* or beta1-adrenoceptor* or beta2-adrenoceptor*or beta3-adrenoceptor* or beta-AR* or beta1-AR or beta2-AR or beta3-AR or beta 1-AR or beta 2-AR or beta 3-AR or beta-adrenergic receptor* or beta1-adrenergic receptors or beta2-adrenergic receptors or beta3-adrenergic receptors or beta 1-adrenergic receptors or beta 2-adrenergic receptors or beta 3-adrenergic receptors AND uterus or uterine or myometrium or myometrial or pregnancy or pregnant or labor or labour or parturition.

1.10.5. Search results and data extraction

The search resulted in 1202 articles from which 793 articles were irrelevant and excluded based on material provided in the abstract. The remaining articles were skimmed, their full text assessed, and compiled according to the context and the model studied. All articles which studied expression, signaling pathways and function of the β-adrenoceptors in the uterus were included for data extraction. Data was extracted from the articles as follow: article title, authors, methods utilized, species, sample type, sample number and results. Results of relevant papers were analyzed and evaluated. Please find the data analysis section in the Appendix (p. 104).

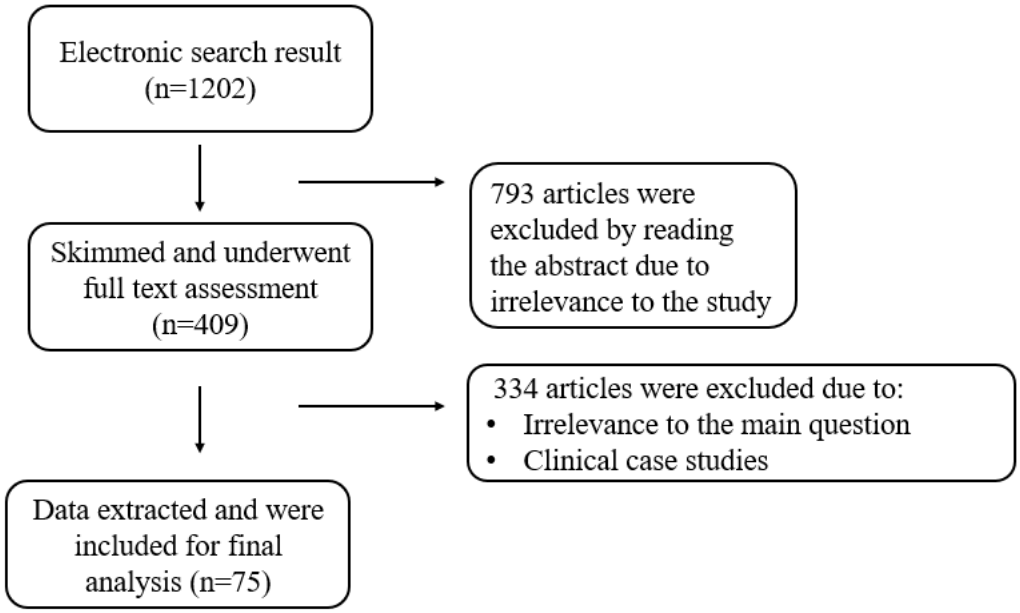


Figure 1.5. Data extraction flow diagram

1.10.6 Key findings of systematic literature review

Three subtypes of the β-adrenoceptors (β_1-, β_2-, and β_3-adrenoceptors) have been identified by molecular and pharmacological approaches. The expression and function of β-adrenoceptors in the uterus have been most extensively studied using radioligand binding assays and contractility studies. Based on radioligand binding assays, the β_2-adrenoceptor subtype appears to be the predominant β-adrenoceptor subtype over β_1-adrenoceptors in pregnant myometrial tissues of human and rodents (McPherson *et al.,* 1984; Bottari *et al.,* 1985; Pennefather & Molenaar 1986; Breuiller *et al.,* 1987; Legrand *et al.,* 1987; Elalj *et al.,* 1988; Handberg *et al.,* 1988; Maltier *et al.,* 1988; Maltier *et al.,* 1989; Whitaker *et al.,* 1989; Chen *et al.,* 1994; Kaneko *et al.,* 1996 and Brauer & Burnstock 1998). The β_3-adrenoceptor subtype is a more novel subtype of the β-adrenoceptors which has been first described by Emorine *et al.,* (1989). Non-selective β-adrenoceptor antagonists generally

have lower affinities to the β_3-adrenoceptors when compared to the other β-adrenoceptor subtypes (Emorine *et al.,* 1989). Therefore, they have not been detected previously in the uterus. For example, the affinity of [^{125}Iodo] cyanopindolol (ICYP) to β_3-adrenoceptors is very low (PK_d=9.2) while the same ligand has higher affinity to β_1- and β_2-adrenoceptors (PK_d=10.4-11.3 and 11.1 respectively). ICYP, a radioligand which is commonly used to quantify the β-adrenoceptor binding sites in the uterus, was used in concentration ranges that were too low to detect β_3-adrenoceptors. The expression of β_3-adrenoceptors was determined in human myometrium using radioligand binding assay by Bardou *et al.*, (2000). Later, and contrary to all binding assay studies conducted to determine the β-adrenoceptor subtypes in human myometrium, Rouget *et al.,* (2005) illustrated the predominance of β_3-adrenoceptor subtype in human near term pregnant myometrium.

The β_2-adrenoceptors were considered the β-adrenoceptor subtype that elicit myometrial smooth muscle relaxation and that was based on binding and functional studies before detection of the β_3-adrenoceptors in the myometrium. However, the experimental design of functional studies did not include selective β-adrenoceptor antagonists as controls to help identify the β-adrenoceptor subtype involved in response modulation such as myometrial strip relaxation and cAMP production. Contractility studies were designed based on radioligand binding assay results, which indicated the predominance of β_2-adrenoceptors. Isoprenaline (a non-selective β-adrenoceptor agonist) has been extensively used to examine β-adrenoceptor-induced smooth muscle relaxation. However, the effects of isoprenaline on myometrial activity and on cAMP production were not verified using selective β_1-, β_2- and β_3-adrenoceptor antagonists and were exclusively considered to be β_2-adrenoceptor-mediated effects (Johansson *et al.*, 1978; Johansson *et al.*, 1981; Meisheri

et al., 1979; Levin *et al.* 1980; Izumi *et al.*, 1982; Tougui *et al.*, 1981; Bryman *et al.*, 1984, Berg *et al.,* 1984; Ikeda *et al.,* 1984; McPherson *et al.*, 1984; Bryman *et al.,* 1986; Khac *et al.,* 1986; Litime *et al.*, 1989; Story *et al.,* 1988; Bramuglia *et al.,* 1992; Ohashi *et al.*, 1996; Engstrom *et al.,* 1997; Le´crivain *et al.*, 1998; Liu *et al.,* 1998; Shinkai *et al.,* 2000; Engstrøm *et al.*, 2001; Dennedy *et al.,* 2001; Sakakibara *et al.,* 2002; Rouget *et al.,* 2004; Chanrachakul *et al.,* 2005; Ga´spa´r *et al.*, 2005; Rouget *et al*., 2005; Bardou *et al.*, 2007; Croci *et al.,* 2007; Spiegl *et al.,* 2009; Klukovits *et al.,* 2010; Wrzal *et al.,* 2012; Parida *et al.*, 2013; Verli *et al.,* 2013). However, since selective β-adrenoceptor antagonists were not used in those studies, the action of the non-selective β-adrenoceptor agonists cannot be considered to be β_2-adrenoceptors.

Functional roles for the small fraction of β_1-adrenoceptors in the pregnant myometrium have not been investigated. Only one study demonstrated that a selective β_1-adrenoceptor antagonist did not affect the relaxatory action of isoproterenol on term pregnant human myometrium (Liu *et al.*, 1998). This suggests no effect for the β_1-adrenoceptor subtype on uterine activity. However, the effect of selective β_1-adrenoceptor agonists was not examined on myometrial activity at different ages of pregnancy nor at different stages of labour. Also, the functions of β_1-adrenoceptors other than smooth muscle relaxation such as modulating gene transcription were not investigated. Therefore, elimination of functional roles of β_1-adrenoceptors in human myometrium is not based on concrete scientific data.

After the detection of the β_3-adrenoceptors in the myometrium, functional studies demonstrated that both β_2- and β_3-adrenoceptor agonists can elicit pregnant human myometrial relaxation and that is based on the following observations (i) β_2- and β_3-

adrenoceptor selective agonists relaxed human lower segment myometrial strips (Bardou *et al.,* 2000; Dennedy *et al.,* 2002), and (ii) β_2- and β_3-adrenoceptor agonist treatments were associated with an increase in cAMP production in term pregnant human myometrial strips (Bardou *et al.,* 2000). β_2- and β_3-adrenoceptor agonists relaxed rat myometrial strips suggesting expression of these receptors in term pregnant rat myometrium (Yurtcu *et al.,* 2006) and this relaxation seems to be, at least in part, through a cAMP-dependent pathway (Yurtcu *et al.,* 2006; Kaya *et al.,* 2012). The specific biochemical mediators that are responsible for β_2- and β_3-adrenoceptor-mediated myometrial relaxation are not well defined in the literature. Selective β_2- and β_3-adrenoceptor agonists increase the production of cAMP in human and rat term pregnant myometrial tissues (Yurtcu *et al.,* 2006; Kaya *et al.,* 2012; Bardou *et al.,* 2000) suggesting PKA-dependent relaxatory mechanisms are present at term. Several mediators may be involved in the relaxatory effect of the β_2- and β_3-adrenoceptors. As described in the introduction of this thesis, the β_2-adrenoceptors can activate potassium channels which lead to cell membrane repolarization and relaxation in airway smooth muscle cells (Giembycz & Newton, 2006). Potassium channel activation by β-adrenoceptors could be through cAMP-dependent or cAMP-independent pathways. Evidence in the literature supports the effect of β_2- and β_3-adrenoceptor agonists on calcium-activated potassium channels (K_{Ca}-channels) and ATP-sensitive potassium channels (K_{ATP}-channels) in human myometrial cell membranes (Hamada *et al.,* 1994; Doheny *et al.,* 2006). However, in human myometrium, research showed differences in the expression of K_{Ca}-channels and K_{ATP}-channels with pregnancy and labour in human myometrium (Matharoo-Ball *et al.,* 2003; Xu *et al.,* 2011), but the effect of the differential expression of potassium channels on the relaxatory effect of β-adrenoceptors in the

myometrium has not been investigated. Evidence in the literature supports the ability of non-selective β-adrenoceptor agonist to activate CREB, transcription factor, in an immortalized myometrial cell line (ULTR cells) (Pearce *et al.,* 2017). However, the role of β-adrenoceptors in modulating gene transcription in primary myometrial cells and myometrial tissues was not investigated.

Differences in the expression of β-adrenoceptors with pregnancy and the onset of term labour were studied using molecular techniques such as northern blotting, conventional RT-PCR, real-time RT-PCR and western blotting. To obtain any concrete conclusion, in these molecular studies, however, the experimental design must include controls that are essential for appropriate data interpretation. For example, positive and negative control samples, loading controls and reverse transcriptase negative samples must be included and the experiments must be done under optimized conditions. For example, RT-PCR primer efficiency and specificity must be validated before conducting the actual experiment. This is achieved by generating standard curves, melting curves and by PCR product sequencing. The β-adrenoceptor subtypes have very similar protein structures. Therefore, it is very important to confirm specificity of antibodies used to determine the expression of the β-adrenoceptor proteins. However, none of the data obtained from articles included for analysis in this review used all proper controls. Therefore, data interpretation was not possible and results from molecular studies were not considered as evidence for the expression of the β-adrenoceptors in the uterus (Vivat *et al.,* 1992; Engstrom *et al.,* 1997; Principe *et al.,* 1997; Bardou *et al.,* 2000; Engstrøm *et al.,* 2001; Chanrachakul *et al.,* 2003; Rouget *et al.,* 2004; Ga´spa´r *et al.,* 2005; Rouget *et al.,* 2005; Minorics *et al.,* 2009; Spiegl *et al.,* 2009; Parida *et al.,* 2013; Yang *et al.,* 2015).

Expression of functional β_2- and β_3-adrenoceptors in human non-pregnant fundal myometrial strips has been also determined (Pędzińska-Betiuk *et al.,* 2011). β_2- and β_3-adrenoceptor agonists inhibited spontaneous contractions of fundal myometrial strips (Pędzińska-Betiuk *et al.,* 2011). However, data published in the literature regarding the β-adrenoceptors in human uterus were limited to lower myometrial tissues obtained during cesarean sections (Andersson *et al.,* 1979; Rydtn *et al.,* 1982; Bottari *et al.,* 1985; Story *et al.,* 1988; Litime *et al.,* 1989; Engelhard *et al.,* 1997; Bardou *et al.,* 1999; Bardou *et al.,* 2000; Dennedy *et al.,* 2002; Sakakibara *et al.,* 2002; Chanrachakul *et al.,* 2003; Chanrachakul *et al.,* 2004; Rouget *et al.,* 2004; Chanrachakul *et al.,* 2005; Frambach *et al.,* 2005; Rouget *et al.,* 2005; Doheny *et al.,* 2006; Bardou *et al.,* 2007; Verli *et al.,* 2013). There are no studies investigating the differences in receptor expression, signaling pathways, or functional responses in human pregnant fundal myometrial tissues.

Rat uterine tissues were used as a model to study the β-adrenoceptors. Contrary to human uterine tissues which are obtained only during cesarean sections, rat uterine tissues can be obtained at different ages of pregnancy and different stages of labour. However, results obtained from utilizing animal uterine tissues must be compared to the results obtained from human uterine tissues to gain concrete conclusions. Regarding the β-adrenoceptor expression and function in the uterus, different studies utilized different kinds of rat uterine tissues such as whole myometrial tissue homogenates, whole uterine horn homogenates, longitudinal muscle layer homogenates, uterine wall strips and myometrial strips without differentiation between these tissues. This leads to difficulties in achieving a definitive understanding of the expression and function of the β-adrenoceptors in rats. For example, Maltier *et al.,* (1989) illustrated that density of β_2-adrenoceptor binding sites was not

different in day 21 compared to day 22-10h of pregnant rat myometrium while it was sharply decreased after the onset of labour while Ga´spa´r *et al.,* (2005) found significantly higher density of β_2-adrenoceptors binding sites in uterine membrane preparations of day 22 pregnant rats when compared to day 20 pregnant rat uterine membrane samples. The two studies used different kinds of tissues with different results. However, definitive understanding of these results cannot be achieved unless the distribution of the β_2-adrenoceptors within the uterine wall tissues at different ages of pregnancy is identified.

Furthermore, none of the papers included in this review attempted to investigate if there are regional differences in the expression and function of β-adrenoceptors in rat uterine tissues. Differences or similarities between human and animal models regarding the temporal β-adrenoceptor expression and function in the uterus were not investigated (Meisheri *et al.,* 1979; Levin *et al.,* 1980; Izumi *et al.,* 1982; Cheng *et al.,* 1984; Khac *et al.,* 1986; Legrand *et al.,* 1987; Piercy 1987; Elalj *et al.,* 1988; Maltier *et al.,* 1988; Tolszczuk & Pelletier1988; Maltier *et al.,* 1989; Legrand *et al.,* 1991; Vivat *et al.,* 1992; Bramuglia *et al.,* 1993; Kaneko *et al.,* 1996; Ohashi *et al.,* 1996; Engstrom *et al.,* 1997; Principe *et al.,* 1997; Ruzycky & DeLoia 1997; Brauerb *et al.,* 1998; Le´crivain *et al.,* 1998; Bramuglia *et al.,* 2000; Shinkai *et al.,* 2000; Engstrøm *et al.,* 2001; Mihályi *et al.,* 2003; Klukovits *et al.,* 2004; Mhaouty-Kodja *et al.,* 2004; Ga´spa´r *et al.,* 2005; Yurtcu *et al.,* 2006; Minorics *et al.,* 2009; Spiegl *et al.,* 2009; Klukovits *et al.,* 2010; Kaya *et al.,* 2012).

In conclusion, all β-adrenoceptor subtypes (β_1-, β_2- and β_3-) are expressed in human and rat myometrial tissues. Differences in the expression and function of β_1-adrenoceptors have not been investigated in human and animal uterine tissues. Evidence in the literature

suggests differences in the expression of β_2-adrenoceptors in rat myometrium with term labour, but the there is no information regarding the differences in the expression and function of β_2-adrenoceptors with pregnancy and labour in human myometrium. The expression of β_3-adrenoceptors is identified only in the non-pregnant and term pregnant rat and human myometrium, and whether the expression and function of β_3-adrenoceptors differs with age of pregnancy and state of labour is not known. Moreover, information regarding the β-adrenoceptors in human myometrium is limited to data obtained using lower myometrial segments. Before studying the functions of β-adrenoceptors, the spesial and temporal expression of β-adrenoceptors at different ages of pregnancy and different stages of labour must be identified in human and then compared with that of animal models to help identify valid model to study the β-adrenoceptors. After identifying the molecular profiles of β-adrenoceptors, the functions of the β-adrenoceptors in the myometrium including evoking myometrial relaxation and modulating gene transcription should be investigated during pregnancy and labour. More understanding of the β-adrenoceptor expression and function in the myometrium will help identify their roles in the myometrium during pregnancy and parturition and whether they form potential targets for the management of preterm labour.

1.11 Hypothesis

We hypothesize that

- In humans, the β-adrenoceptors have distinct expression profile comparing the spatial location (upper *vs.* lower uterine segments) and the temporal state (labour *vs.* non-labour).
- The β-adrenoceptors have a role in regulating *CRISPLD2* in myometrial smooth muscle in pregnant women.

1.12 Aims

Aim 1 To determine the spatial and temporal profile of β-adrenoceptor subtype expression in pregnant human myometrium

Specific Objectives:

1. To determine the relative abundance of β-adrenoceptor mRNA in the upper and lower pregnant human myometrium.

2. To determine if there are any changes in β-adrenoceptor mRNA expression within term labour in the upper and lower pregnant human myometrium.

3. To localize the β-adrenoceptors in the upper and lower pregnant human myometrium.

Rationale

The expression of β-adrenoceptors in the upper and lower myometrium before and after labour has not been defined. Mapping the expression profile of the β-adrenoceptors in the upper and lower uterine segments of the myometrium, in term samples with and without the onset of labour may provide a more understanding of the process of parturition.

Aim 2 To examine the effect of β-adrenoceptor agonists on *CRISPLD2* expression in MSM cells.

Specific Objective:

To determine the role of β-adrenoceptor agonists on *CRISPLD2* expression in primary pregnant human myometrial cells.

Rationale

In other models, such as BEAS-2B cell line, mRNA expression of several genes including *CRISPLD2* was increased by cAMP-elevating agents including β_2-adrenoceptor agonists. Understanding potential roles of β-adrenoceptors such as regulating *CRISPLD2*, in the myometrium could lead to new strategies to prevent preterm labour.

Tables

Table 1.1. The β-adrenoceptor genes (Eisenach, 2010).

β-AR subtype	**β_1/*ADRB1***	**β_2/*ADRB2***	**β_3/*ADRB3***
Gene location	Chromosome 10q	Chromosome 5q	Chromosome 8p
Gene structure	Intronless/1 exon	Intronless/1 exon	1 intron / 2 exons
Encoded amino acids (aa)	477 aa	413 aa	408 aa

Table 1.2. Pharmacological properties of β-adrenoceptors (Badino & Odore, 2005) (IUPHAR/BPS, 2017).

	β_1-adrenoceptors	**β_2-adrenoceptors**	**β_3-adrenoceptors**
Potency order for endogenous ligands	noradrenaline = adrenaline	adrenaline > noradrenaline	noradrenaline > adrenaline
Selective agonists	xamoterol	indacaterol formoterol salmeterol	BRL 37344 L-755507 CL316243
Selective antagonists	carvedilol labetalol	ICI-118551	L-748328

Chapter Two: Materials and Methods

2.1 Tissue collection

Institutional ethics was obtained for the collection of human gestational tissues at caesarean delivery (Office of medical bioethics, and The Conjoint Health Research Ethics Board (CHREB), University of Calgary). Gestational tissues were collected from women who were undergoing caesarean section at term (37-40 weeks of gestation) prior the onset of labor (TNL), or after the onset of labour (TL); (**Table 2.1**). Women had healthy singleton pregnancies and caesarean sections were indicated for the following reasons: previous caesarean section, breech presentation, fetal distress and placenta praevia. Collected samples were either stored at -80°C for total RNA extraction, fixed in 10% formalin for paraffin embedding for immunohistochemistry, or processed immediately for cell isolation.

2.2 Primary myometrial cell culture and passage

Primary myometrial cells were isolated from lower myometrial segments from pregnant women in term (gestational age range (week+day) 37+0 – 38+6) who were undergoing caesarean section before the onset of labour as previously described (Mosher *et al.*, 2013). In brief, each myometrial biopsy was dissected into small pieces (1 mm^3), and washed with Hanks' Balanced Salt Solution (HBSS, Life Technologies Inc., Burlington, ON, Canada). After removal of excess blood by HBSS, cells were placed in smooth muscle medium containing 1 mg/ml collagenase XI, 1 mg/ml collagenase IA and 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Oakville, ON, Canada). Cells were digested for 60 minutes

at 37°C. The digestion solution was passed into a sterile pipette to disperse the cells and then treated with smooth muscle media containing 5% fetal bovine serum (FBS, Life Technologies). Cells then were passed through a 70 µm sieve and centrifuged for 5 minutes at 400 x g. Cells were resuspended in smooth muscle medium containing 1x antibiotic-antimycotic and 5% FBS. Cells were plated in 25 cm^2 flasks and incubated in a humidified incubator at 37°C in 95% air/5% CO_2. After cells reached 80-90% confluency, the cells were sub-cultured in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer Scientific, Waltham, MA, USA) containing 5% fetal bovine serum (FBS, Sigma-Aldrich Company, Oakville, ON, Canada), 0.5 µg/ml epidermal growth factor (EGF), 5 µg/ml insulin, 10000U penicillin / 10 mg/ml streptomycin, 2 mM L-glutamine and $NaHCO_3$. Cells were incubated in a humidified incubator at 37°C in 95% air/5% CO_2. When cells achieved approximately 90% confluency, the medium was aspirated, and cells rinsed with 10 ml of Hanks' Balanced Salt Solution (HBSS). Subsequently, 5 ml of 0.05% trypsin-EDTA was added to the flask and incubated in 37°C for ≤5 min to detach the cells from the surface of the flask. To stop the action of trypsin-EDTA, 10 ml DMEM containing 5% FBS was added. Cells were counted using a haemocytometer then centrifuged at 800 x g for 5 min and the pellet was resuspended in DMEM to be sub-cultured and seeded in 75 cm^2 flask or 12-well plates at density of 4.0 x 10^4 cells/ml. The medium was changed every 2-3 days. When cells reached approximately 80-90% confluency, the medium was replaced with serum free media (SFM) containing $NaHCO_3$ and 2 mM L-glutamine to be treated after 18~20 hours.

2.3 Cell treatments

Drugs used to treat MSM cells were diluted in fresh serum-free DMEM. Cells treated with the β_2-adrenoceptor antagonist, ICI-118,551 (100 nM) were incubated with the antagonist in a humidified incubator for 30 min. Subsequently, cells were treated with corresponding treatment and incubated in humidified incubator till they were ready to be harvested for RNA isolation. Controls (NS) or non-stimulated plates were treated with fresh serum-free DMEM only.

2.4 RNA isolation

RNA was extracted from primary myometrial cells using the Nucleospin® RNA mini kit (Macherey-Nagel, Geilenkirchen, Germany) according to the manufacturer's protocol. In brief, after medium aspiration, cells were lysed by 350 µl RA1 buffer containing 1% β-mercaptoethanol. Cell lysates in the plates were thawed, scraped, applied onto a Nucleospin filter placed in a collection tube and centrifuged for 1 min at 11,000 x g. Homogenized cell lysate was mixed with an equal volume of 70% ethanol (350 µl), transferred to a Nucleospin RNA column, and centrifuged for 30 s at 11,000 x g. Silica membrane was desalted using 350 µl membrane desalting buffer (MDB) and dried by centrifuging at 11,000 x g for 1 min. To remove any DNA, the column was incubated for 15 min with DNase reaction mixture containing 10 µl reconstituted DNase. After the incubation, the column was successively washed with 200 µl buffer RAW2, 600 µl buffer RA3 and 250 µl buffer RA3. Finally, total RNA was eluted by adding 30 µl RNase-free water to the column, incubating for 3 min followed by centrifugation at 11,000 x g for 1 min. Using a

NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.), total RNA concentration was measured, and 260/280 ratio was used to assess the RNA purity.

2.5 cDNA synthesis

250-1000 ng of total RNA isolated from pregnant myometrium tissue or myometrial cells were used to synthesize cDNA using the qScript^{TM} cDNA Synthesis Kit (Quanta Bioscience, Beverly, MA, USA) according to the manufacturer's guideline. The thermal cycler was programmed as: 1 cycle at 22°C for 5 min, 1 cycle at 42°C for 30 min and 1 cycle 85°C for 5 min. The resultant cDNA was then diluted using RNase-free water (1:5) and stored at 4°C or -20°C for long term storage.

2.6 Primer design and verification

Primers were designed using the NCBI primer designing tool. Primers were designed to be no more than 30 base pairs in length, have no more than 60% of guanine and cytosine content, minimum match repeats and their product size ranges from 60-200 base pairs; (**Table 2.2**). Products that gave single bands by RT-PCR were sequence verified. In brief, products were purified using MinElute PCR Purification Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol and sequenced by The University of Calgary sequencing facility. Sequence results were verified using the NCBI BLAST tool. *ADRB1* and *ADRB2* are intronless receptors while *ADRB3* has one intron. **Figure 2.1.** represents positions of each pair of primers in the genes.

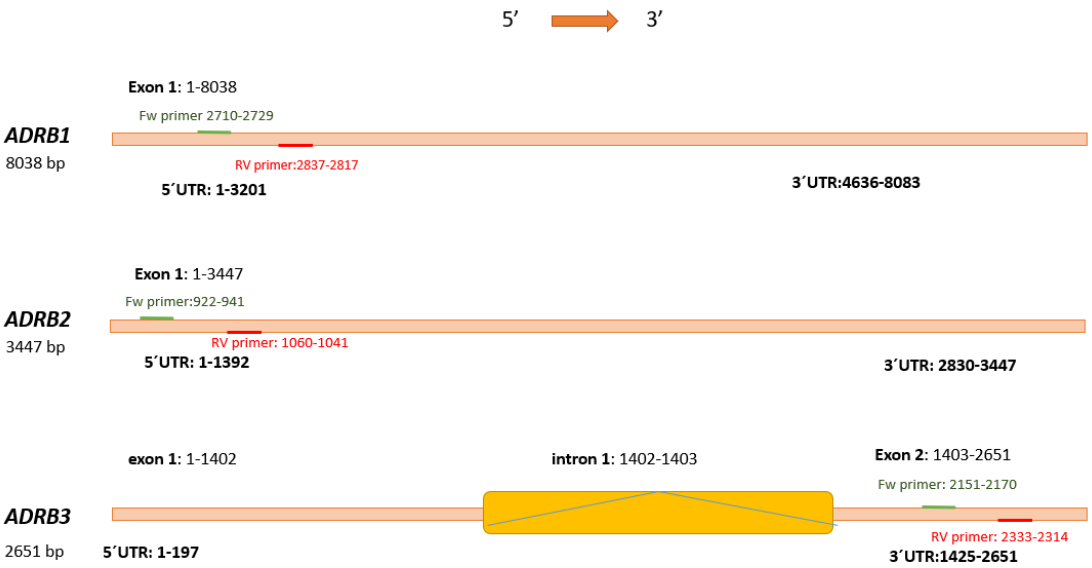


Figure 2.1 Positions of the real-time RT-PCR primers in *ADRB1*, *ADRB2* and *ADRB3*. *ADRB1* and *ADRB2* are intronless receptors while *ADRB3* has one intron.

2.7 Conventional reverse transcriptase - Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed in volume of 25 µl. Each reaction contained 2 µl template cDNA and 23 µl previously prepared master mix. This contains 0.25 µl MyTaq™ DNA Polymerase (50mU/µl (Bioline, London, UK), forward and reverse primers (400 nM of each primer), 1x PCR reaction buffer. An Eppendorf™ Mastercycler™ Pro PCR System thermocycler was adjusted as follows: initial denaturation for 2 min at 94°C; 35-38 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, and a final elongation step at 72°C for 5 min. RT-PCR products were stored at 4°C until gel analysis. 10% Orange G dye was added to the products before loading to agarose gel. Gel electrophoresis for data analysis was performed using 2% agarose gel (Amresco, Solon, OH, USA) containing Ethidium bromide for staining. 10 µl of each sample was

loaded into the gel and 5 µl of Hyperladder II (Bioline, London, UK) was used to estimate product size. β2-microglobulin (*B2M*) was used as a reference gene. RNA from different tissue samples (heart, lung, bladder, adipose, ovary, cervix and placenta) were used to synthesize cDNA using Ambion FirstChoice® Human Total RNA Survey Panel (Applied Bioscience, Foster, CA, USA) was used as positive controls. Negative reverse transcriptase samples were used as controls to check genomic DNA contamination. Gels were run in 1x tris base acetic acid EDTA buffer (TAE buffer) (40mM Tris, 20mM acetic acid, and 1mM EDTA) containing ethidium bromide at 80V for about 40 mins. Finally, gels were visualized and imaged using ChemiDoc™ instrument (Bio-Rad Laboratories, Inc).

2.8 Real-time RT-PCR

MicroAmp optical 96-well reaction plates and a StepOne instrument were used to perform real-time PCR. Fast SYBR® Green Master Mix. (Thermo Fischer Scientific, Waltham, MA, USA), water and forward and reverse primers (400 nM of each primer) were added to 2 µl of template cDNA to make a 10 µl reaction volume. Plates were spun before starting the following real-time RT-PCR conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 30 s, followed by a melt curve generation (95°C for 15 s, 60°C for 1 min). All samples were analyzed in duplicates. Water and negative reverse transcriptase samples were used as controls in each plate. Standard curves were constructed using serial dilutions of a standard sample (pooled placenta sample) cDNA which expresses all the relevant target genes. The reference gene, β2-microglobulin (*B2M*) was used to normalize all the results.

2.9 Protein isolation for western blotting

Protein from frozen day 19 pregnant mouse myometrial tissue was prepared by homogenizing the tissue for about 5 min in radio-immunoprecipitation assay buffer (RIPA buffer- 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris) (300 µl RIPA / 5 mg tissue). Protease inhibitors (40 µl / 1 ml RIPA) were added to the RIPA buffer before lysing the tissue. After the tissue was completely homogenized, samples were centrifuged for 20 mins at 12,000 x g at 4°C. The supernatant stored at -80°C.

Cells were seeded in 6-well plates and after they reached 90-95% confluency, the medium was aspirated, and the cells were lysed using 300 µl RIPA buffer for each well. Protease inhibitors were added to the RIPA buffer (40 µl / 1 ml RIPA). Plates containing cell lysates were incubated on ice for 30 mins before cell lysates were scraped and transferred to Eppendorf tubes to be centrifuged for 20 mins in 13,200 x g at 4°C. Supernatants were collected and stored at -80°C.

2.10 Western blotting

Western blots were performed using proteins isolated from frozen mouse myometrial tissue, BEAS-2B cells and HeLa cells were mixed with loading buffer 2xLaemmli (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.125 M Tris HCL and 0.004% bromophenol blue) in a 1:1 ratio then heated at 95°C for 2 min before loading onto the polyacrylamide gel.

Proteins were separated in 8% polyacrylamide gel (0.375M Tris HCl, PH 8.8, 1% SDS, 1% APS, 0.05% TEMED) electrophoresis (150 mV for 1.5 h in 1% Tris, glycine running

buffer). 6 µl BlueEye Prestained Protein Marker (Jena Bioscience, Jena, Germany) was loaded to monitor protein separation and to give an approximate for protein size. Proteins were transferred to nitrocellulose membranes in transfer buffer (Tris, Glycine, 0.1% SDS and 20% methanol) at 150 mV for 1 h. Membranes were first rinsed with Ponceau S to visualize the proteins followed by 3 washes with 0.05% Tris-buffered saline-Tween (TBS-T). Membranes were incubated with blocking solution for 2 h. Blocking solutions used were either (i) 5% bovine serum albumin (BSA), (ii) 5% skimmed milk (5 g of milk or BSA mixed in 100 ml TBS-T), (iii) iblock or (iv) 2% ECL™ Advance Blocking Reagent. Membranes were washed 3 times with TBS-T followed by incubation with the primary antibody overnight at 4°C with gentle shaking. Subsequently, an appropriate Horseradish peroxidase (HRP)-conjugated secondary antibody was applied to the membranes and incubated for 2 h at room temperature with gentle shaking. Membranes were washed 3 times with 0.05% TBS-T before they were developed with Enhanced chemiluminescence solution (ECL) for 3 min. Membranes were washed 3 times with TBS-T before incubation with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody, used as reference protein.

2.11 Immunohistochemistry

Paraffin-embedded tissue sections were washed twice by CitriSolv for deparaffinization, 5 mins each. Sections were rehydrated by washing them in decreasing concentrations (100%, 95%, 80%, and 70%) of ethanol. Sections were washed with ddH_2O 3 times. For antigen retrieval, tissue sections were immersed in 10 mM citrate buffer, pH 6.0 in a water bath adjusted at 95°C for 30 min. Tissue sections were washed in phosphate-buffered saline

(PBS), outlined by Pap pen, and then incubated with blocking serum for 30 min at room temperature. Subsequently, primary antibodies were applied, and tissue sections were incubated in a humidified chamber overnight at 4°C. Mouse, rabbit or goat ABC kit (Santa Cruz Biotechnology, Dallas, TX, USA) was used for staining as described by the manufacturer. In brief, tissue sections were incubated with biotinylated secondary antibody for 30 min at room temperature, and then washed three times with PBS; 5 min each. Tissue sections were incubated for 25 min in the dark with 3% hydrogen peroxide to block endogenous peroxidases activity. Sections were washed 3 times with PBS followed by incubation with avidin and biotinylated-HRP reagent in a humidified chamber at room temperature for 30 min. Sections were treated with peroxidase substrate (3,3'-diaminobenzidine (DAB)) for 10 min then washed in ddH_2O, counterstained by dipping them in Harris hematoxylin, washed with 1% acid alcohol to remove excess dye, dehydrated by washing in increasing concentrations of ethanol followed by 2 washes of citrisolv. Finally, sections were mounted and visualized using a Zeiss Axio Scope A1 microscope connected to an Axiocam ICc 3 camera. The AxioVision 4.7.2 software (Carl Zeiss Canada Ltd, Toronto, Ontario, Canada) was used to obtain Images. Controls included no primary antibody. α-actin was used as a reference protein.

2.12 Data presentation

For MSM cell culture experiments, data are presented as the mean with standard deviation (SD) of n independent experiments. For the tissue study, data presented as mean (SD) in scattered dot plots. One-way analyses of variance (ANOVA) followed by Bonferroni's multiple comparison test were used for statistical analysis. GraphPad Prism, version 6.04

software was used for statistical analysis. Results were considered significant when $P < 0.05$.

Tables

Table 2.1. Summary of patient information.

Labour Status	Number of patients	Gestational Age (Weeks + Days)	Cervical Dilation (cm)	Labour (Hours)ǂ	Maternal Age
Term Non-labour	Upper segment n=11 Lower segment n= 9	37 + 0 – 39 + 4 (259 – 277 days)	0 – 2	0	29 – 41
Term Labour	Upper segment n=9 Lower segment n=8	37 + 2 – 40 + 2 (261 – 282 days)	1 – 4	2 - 9	31 – 39

ǂ Number of hours, patients were in labour prior to caesarean section.

Table 2.2. Primers used in conventional and real-time RT-PCR

Genes (Accession number)	**Primers 5'-3'**	**Product size (bp)**
ADRB1 (NM_000684.2)	**F:** AAGTGCCCACGTGAATTTGC **R:** TGCACATGGACCAATTCCTCA	128
ADRB2 (NM_000024.5)	**F:** GGCAGCTCCAGAAGATTGAC **R:** GTCTTGAGGGCTTTGTGCTC	139
ADRB2 (NM_000024.5)	**F:** TTTTGGCAACTTCTGGTGCG **R:** AGGCCTGACACAATCCACAC	189
ADRB3 (NM_000025.2)	**F:** TGGCTTTTGACAGAGGCAGT **R:** AGCGTGGCTTTGCTACTCAA	183
CRISPLD2 (NM_031476.3)	**F:** CAAACCTTCCAGCTCATTCATG **R:** GGTCGTGTAGCAGTCCAAATCC	64
β2M (NM_004048.2)	**F:** CTTATGCACGCTTAACTATCTTAACAA **R:** TAGGAGGGCTGGCAACTTAG	127
NR4A3 (NM_001307989.1)	**F:** GTAGACAAGAGACGTCGAAACC **R:** CCTCTCCTCCCTTTCAGACTAT	110
RGS2 (NM_002923.3)	**F:** CCTCAAAAGCAAGGAAAATATATACTGA **R:** AGTTGTAAAGCAGCCACTTGTAGCT	131
IL-6 (NM_000600.4)	**F:** GAGTAGTGAGGAACAAGCCAG **R:** GTCAGGGGTGGTTATTGCATC	103

Table 2.3. Agonists and antagonist affinities and concentrations used in cell culture experiment (Baker, 2010; IUPHAR, 2017).

Drug	Drug action	Affinity for target receptor	Drug concentration used
Formoterol	β_2-Adrenoceptor agonist	pK_d 8.6	10 nM
Forskolin	Direct adenylyl cyclase activator	-	1µM
ICI-118,551	β_2-Adrenoceptor antagonist	pK_i 9.2 - 9.5	100 nM
Isoprenaline	Non-selective β-adrenoceptor agonist	β_1-Adrenoceptor pK_a 6.6 - 7.0 β_2-Adrenoceptor pK_a 6.4 β_3-Adrenoceptor pK_a 5.1 - 6.2	1µM
L-755,507	β_3-Adrenoceptor agonist	pK_d 8.6	10 nM

Table 2.4. Primary antibodies used for immunohistochemistry and western blotting

Antigen	Reactive species	Host species	Supplier	Catalog#	Dilution IHC	Dilution WB
β_2-adrenoceptor	Human, mouse and rat	Rabbit	Santa Cruz	Sc-9042	1:50	1:500
β_3-adrenoceptor	Human	Goat	Santa Cruz	Sc-1472	1:50	1:250
α-Actin	Human	Mouse	Santa Cruz	Sc- 58669	1:100	n/a
GAPDH	Human	Mouse	AbD Serotech	4699-9555	n/b	1: 10000

Table 2.5. Secondary antibodies used in western blotting

Antibody	Supplier	Catalog#	Dilution used in Western blotting
Goat anti-mouse HRP-conjugated	Jackson Immuno Research	115-035-003	1:10000
Goat anti-rabbit HRP-conjugated	Jackson Immuno Research	111-035-003	1:10000
Rabbit anti-goat HRP-conjugated	Dako	P0449	1:10000

Table 2.6. Staining kits used in immunohistochemistry

Kit name	**Supplier**	**Catalog#**
Immunocruz® Rabbit ABC Staining System	Santa Cruz	sc-2018
Immunocruz® Goat ABC Staining System	Santa Cruz	sc-2023
Immunocruz® Mouse ABC Staining System	Santa Cruz	sc-2017

Chapter Three: Spatial and temporal expression of β-adrenoceptor subtypes in pregnant human myometrium

3.1 β-adrenoceptor expression in the pregnant human myometrium.

RT-PCR was used to determine the expression of β-adrenoceptors in samples of pregnant human myometrium. All β-adrenoceptor subtypes were expressed in the upper and lower myometrial tissue. cDNA from placenta, lung, heart, urinary bladder, cervix and ovary were included as positive controls and a sample in which water was substituted for cDNA was included as a no template control. No reverse transcriptase (RT-ve) samples were used to check for genomic DNA contamination. *B2M* reference gene expression was assessed in each sample. All PCR products were sequence verified (**Figure 3.1.**).

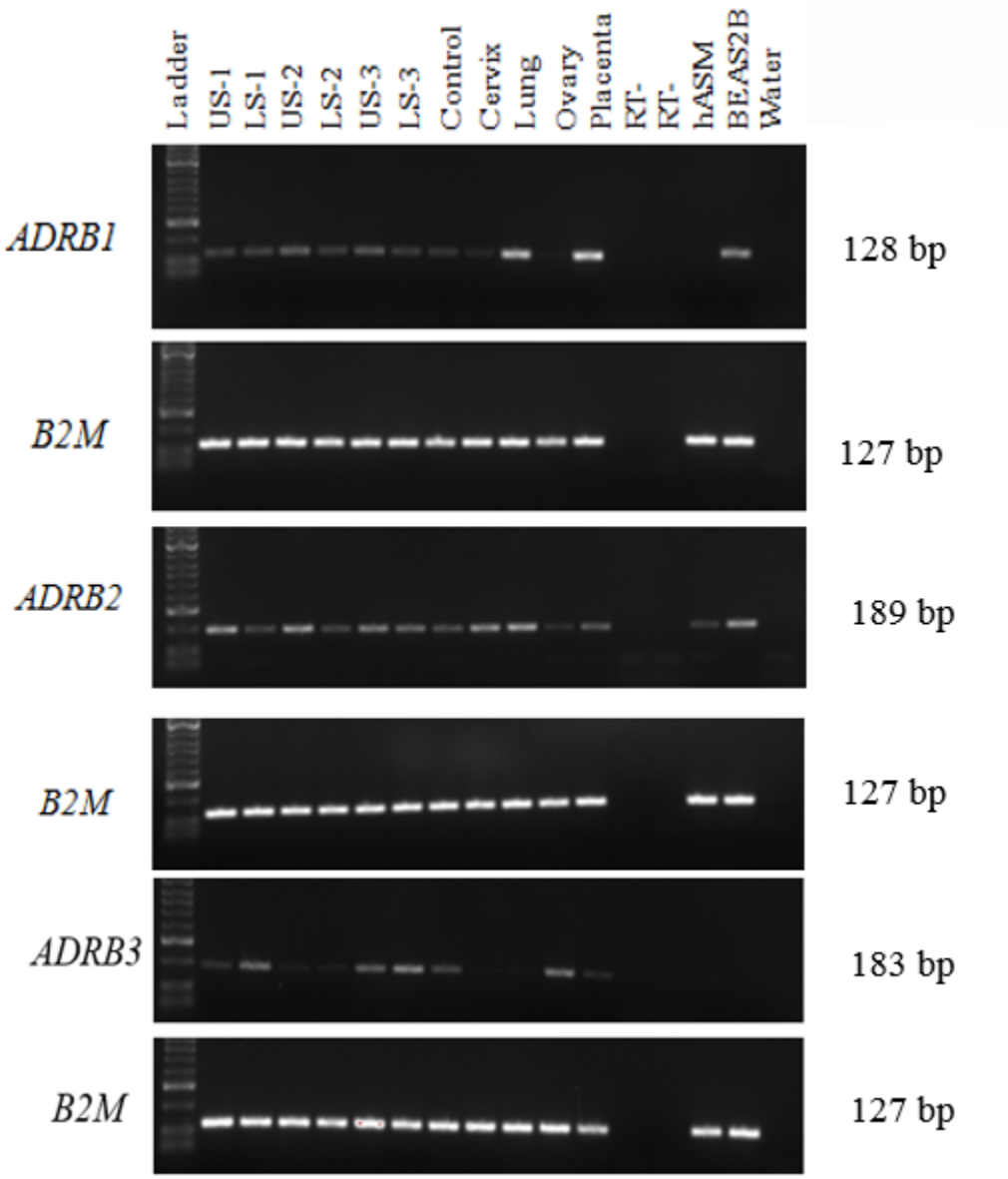


Figure 3.1. *ADRB1*, *ADRB2* and *ADRB3* gene expression in upper and lower human myometrial tissue. *ADRB1*, *ADRB2* and *ADRB3* expression in upper (US) and lower (LS) human myometrial tissues was assessed by conventional RT-PCR. The expression of *ADRB* was also determined in cervix, ovary, placenta, human airways smooth muscle cells and BEAS-2B cells. β2-Microglobulin (*β2M*) was used as a reference gene.

3.2 Real-time RT-PCR efficiency and specificity

Before conducting the real-time RT-PCR, *ADRB1*, *ADRB2* and *ADRB3* primer efficiencies were optimized. The three standard curves have similar slopes. Based on the equation Efficiency = $10^{(-1/\text{slope})} - 1$, *ADRB1*, *ADRB2*, and *ADRB3* primers have similar PCR efficiencies (**Figure 3.2.**).

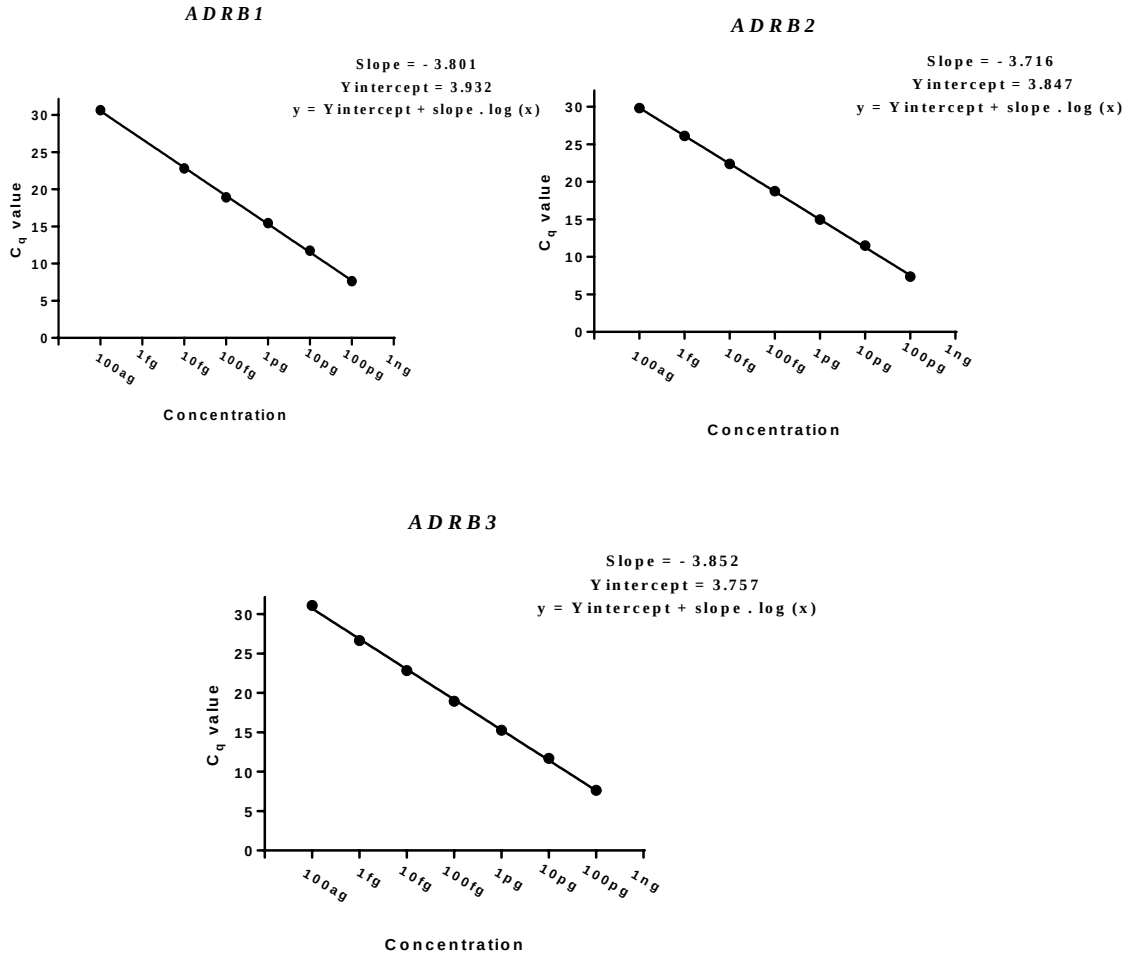


Figure 3.2. Measurement of PCR efficiency. Real-time RT-PCR was performed for each *ADBR1*, *ADBR2* and *ADBR3.* The products were purified and used as a template for real-time RT-PCR. The C_q values at the different product concentrations (1 ng – 100 ag) were plotted and the slope was recorded. Statistical analysis was performed by non-linear regression (Semi-log line)

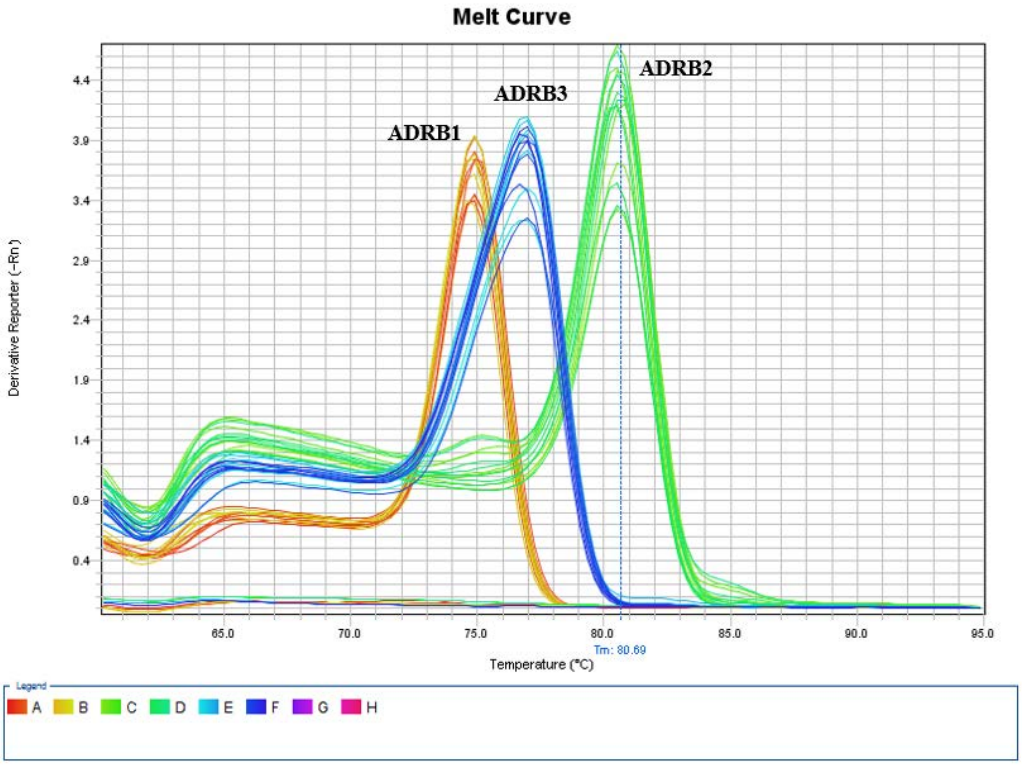


Figure 3.3. Melting curves of *ADRB1, ADRB2 and ADRB3*. Representative melting curves for *ADRB1*, *ADRB2* and *ADRB3*. One sharp peak was obtained from using each pair of primers. A sample in which water was substituted for cDNA was included as a no template control.

3.3 Spatial and temporal expression of β-adrenoceptor subtypes in pregnant human myometrium

Real time RT-PCR was used to determine the relative abundance of β-adrenoceptors in upper and lower myometrial tissues obtained from women at term before the onset of labour (term non-labour - TNL) and from women at term following the onset of labour (term labour - TL).

ADBR1: There were no significant differences observed in the expression of *ADRB1* comparing upper and lower or labour and non-labour samples.

ADRB2: There were no significant differences observed in the expression of *ADRB2* comparing upper and lower or labour and non-labour samples.

ADRB3: There were no significant differences observed in the expression of *ADRB3* comparing upper and lower or labour and non-labour samples.

(**Figure 3.4.**).

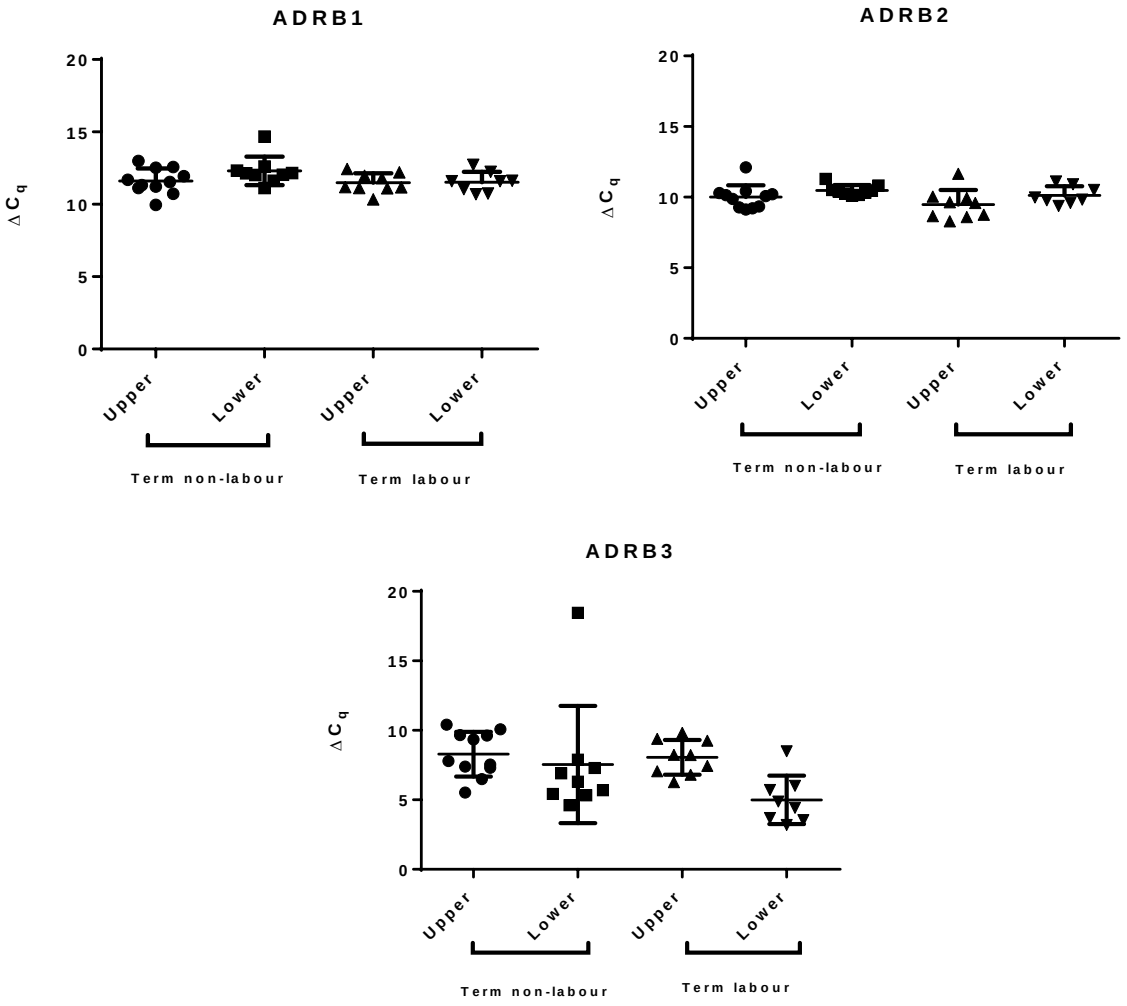


Figure 3.4. Expression of *ADRB1, ADRB2* and *ADRB3* in human myometrial tissue. Total RNA was isolated from upper (n=11) and lower (n=9) term non-labour (TNL) and from upper (n=9) and lower (n=8) term labour (TL) myometrial tissue and 250 ng was used to synthesize cDNA. Real-time RT-PCR was used to quantify (A) *ADBR1,* (B) *ADBR2* and (C) *ADBR3* mRNA expression, which was normalized to the reference gene β-2-microglobulin (*B2M*). Real-time RT-PCR was performed in technical duplicates. Duplicates were averaged by sample per gene. ΔC_q was obtained by substracting average C_q of reference gene (*B2M*) from average C_q of *ADRB2* for corresponding sample. Data are presented as means (SD) of the groups. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni's post-hoc test (* $P < 0.05$). No statistical differences were found between the groups.

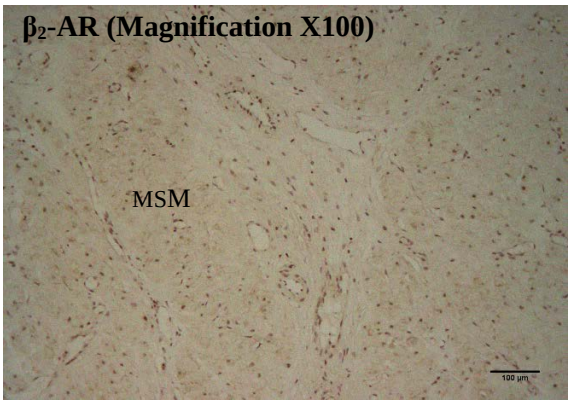
3.3 Localization of β_2-adrenoceptors in term non-labour and term labour upper and lower myometrial tissue by immunohistochemistry.

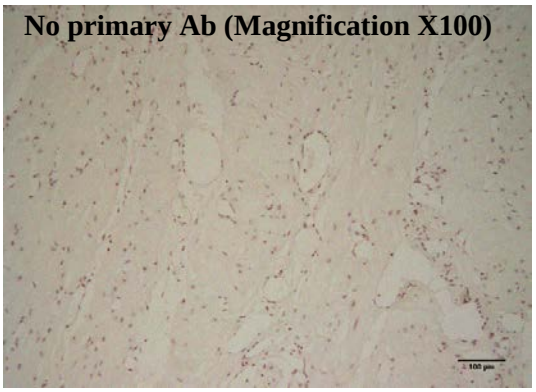
The expression of β_2-adrenoceptors in paired upper and lower myometrial sections obtained from pregnant women (term non-labour TNL) and (term labour TL) was determined using immunohistochemistry. All myometrial sections and placenta section were processed at the same experimental run. In the following figures, the brown color indicates positive staining.

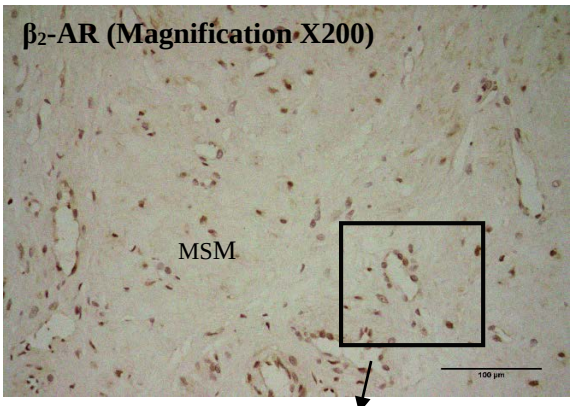
Positive staining was observed in the nuclei and cytoplasm of the myometrial smooth muscle cells. Positive staining was also observed in the nuclei of the endothelial cells of blood vessels.

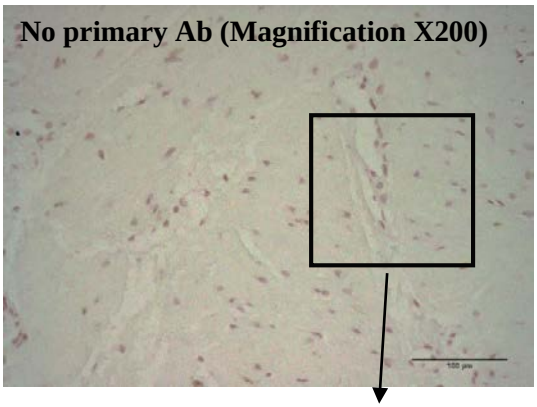
No staining was observed in the no primary antibody controls. All sections contained myometrial smooth muscle bundles and were positively stained for the reference protein, α-actin. Positive brown staining was observed in the placenta sample section which was used as a positive control.

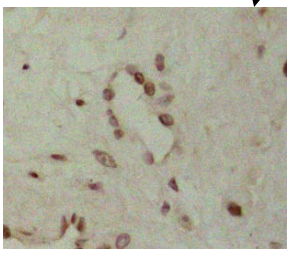
(A). Upper myometrial segment - (Term - Non-labour):

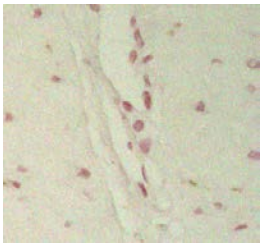


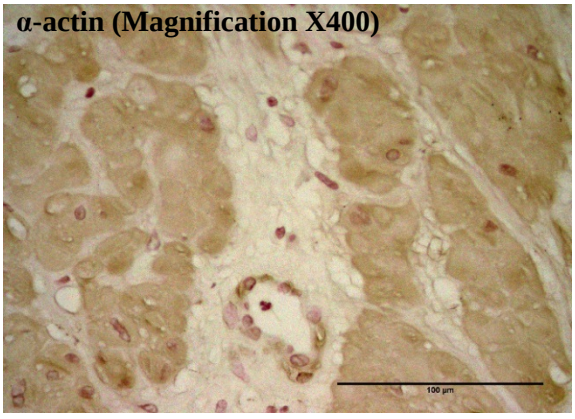


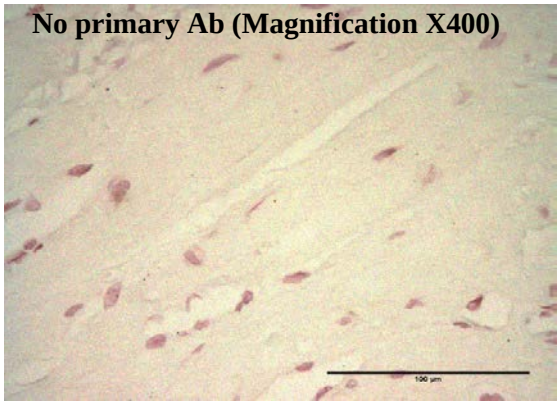




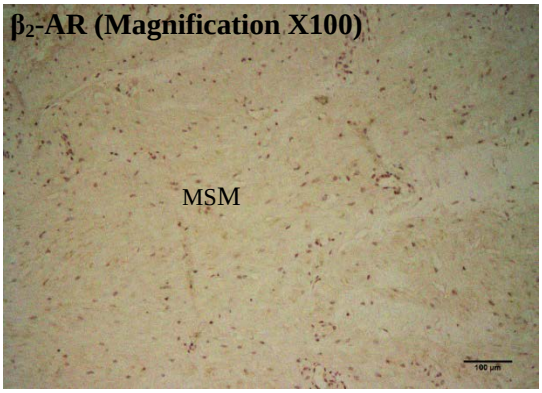


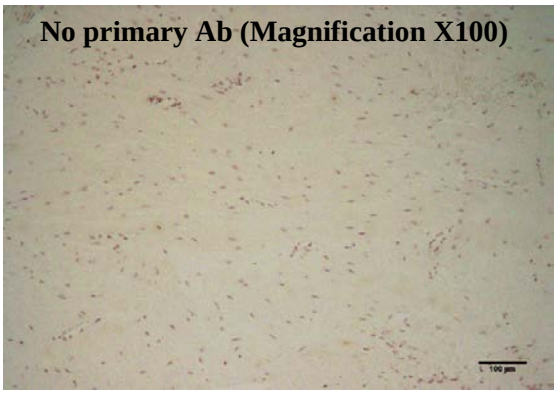


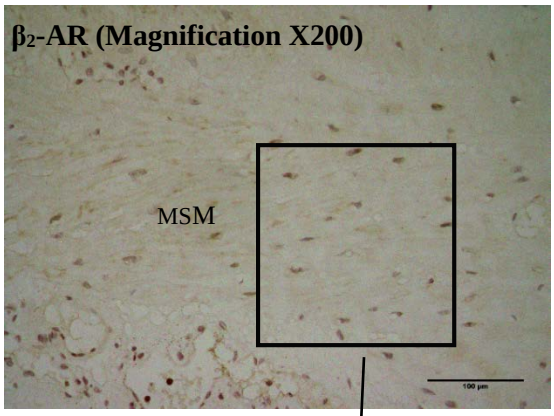


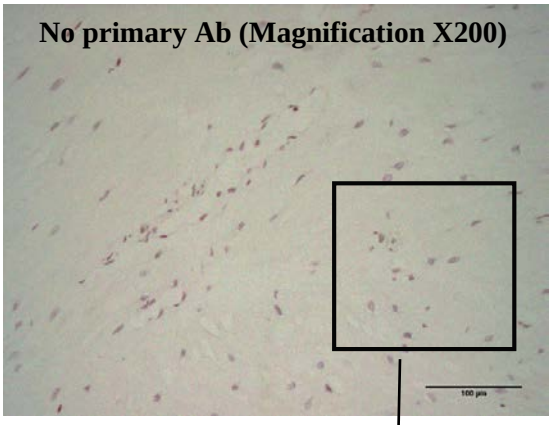


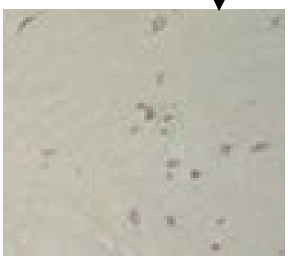
(B). Lower myometrial segment - (Term - Non-labour):

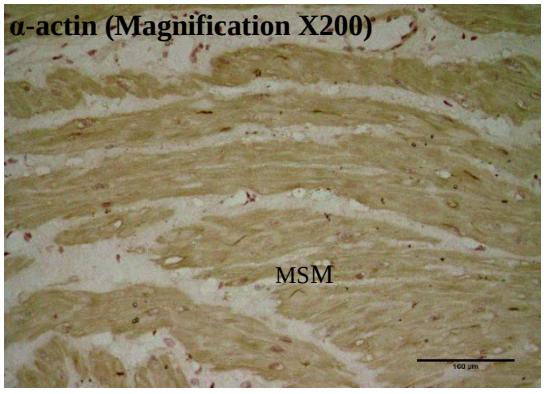


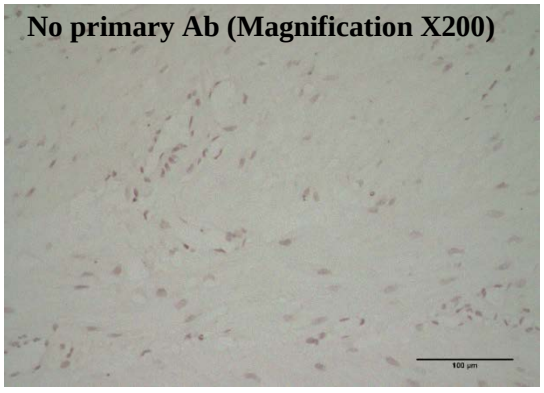




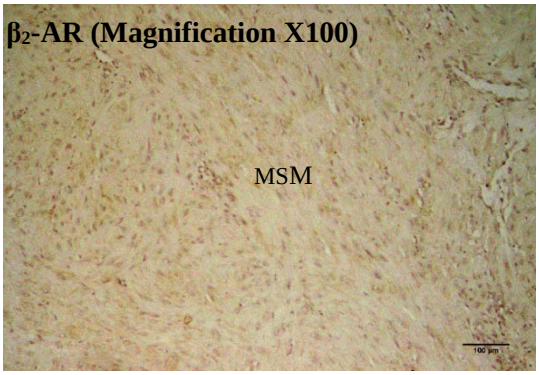


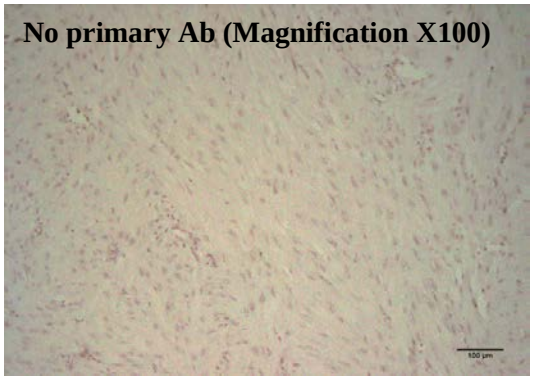


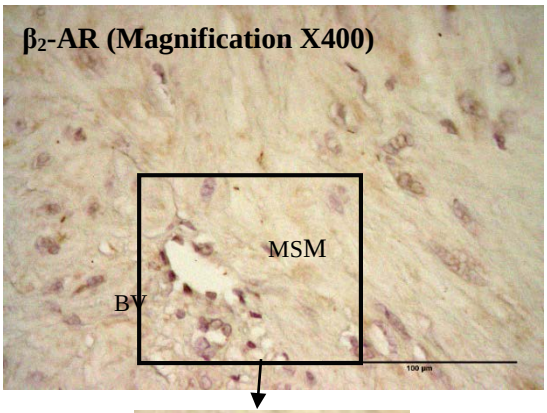


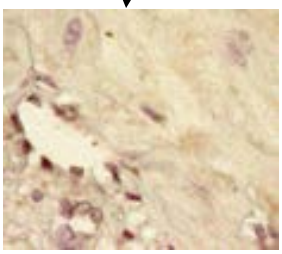


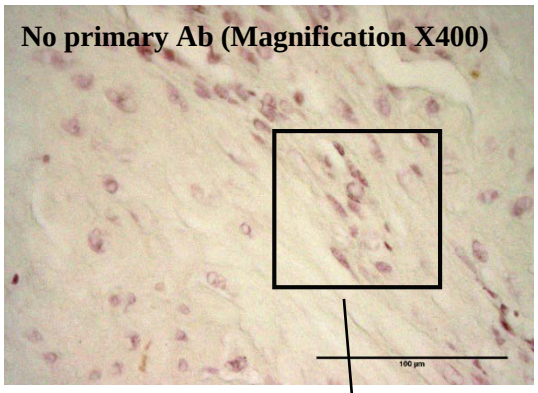
(C). Upper myometrial segment - (Term-Labour):

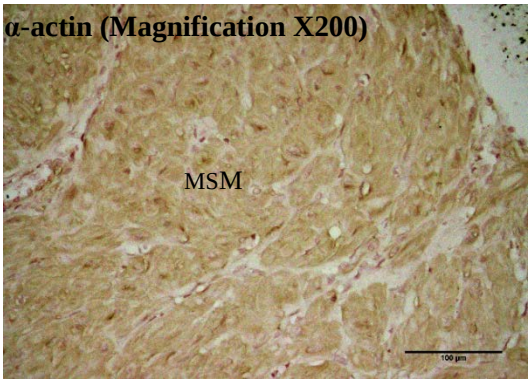


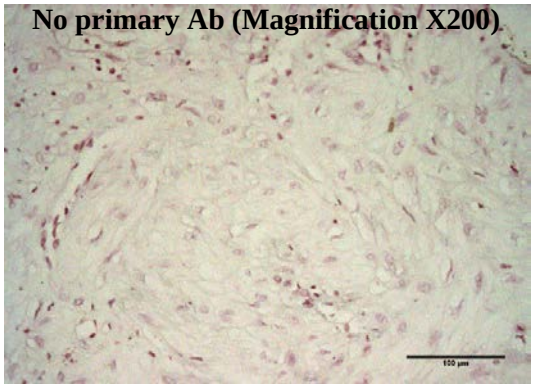




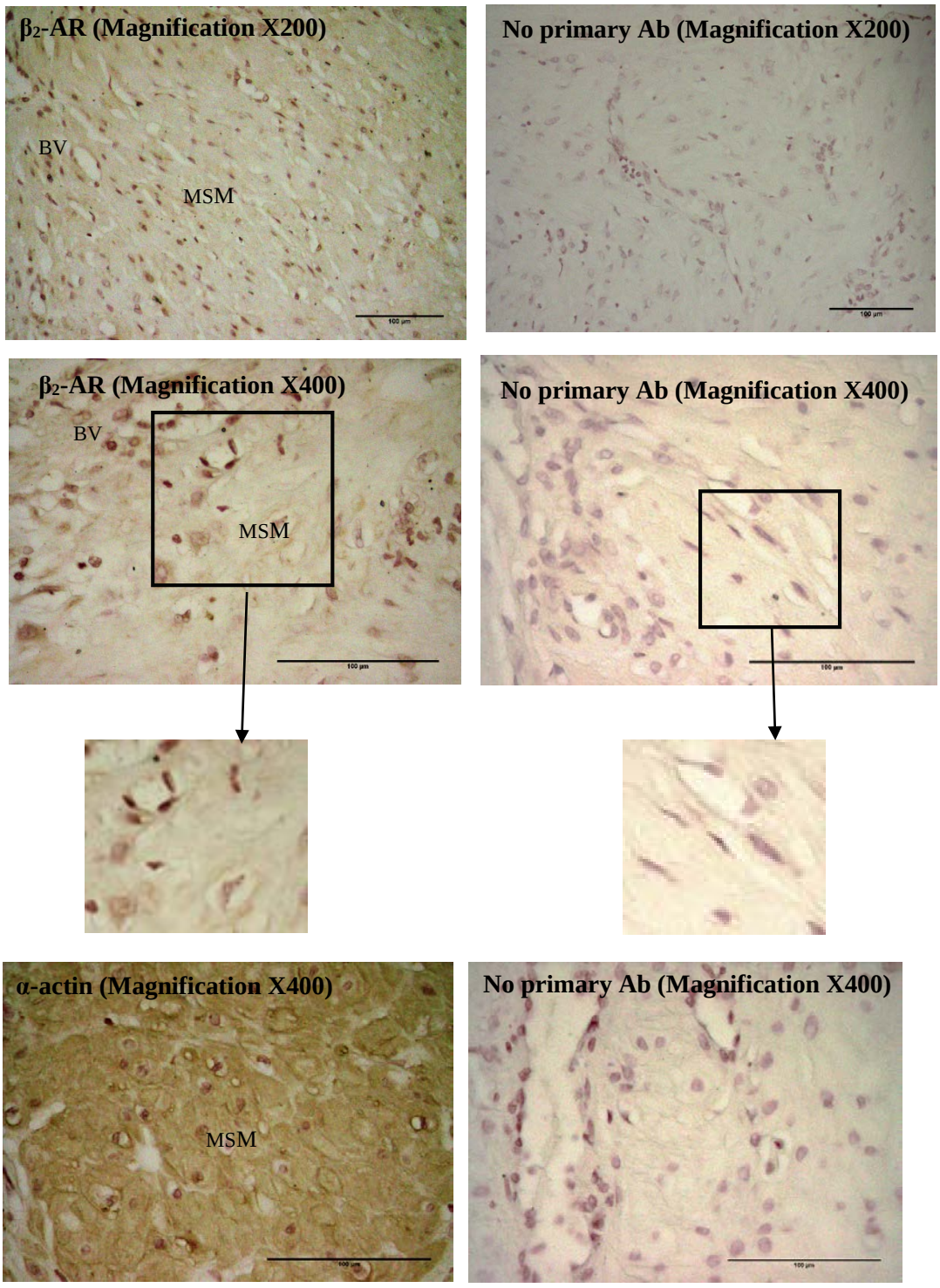








(D). Lower myometrial segment - (Term-Labour):



(E). Placenta – Positive control tissue:

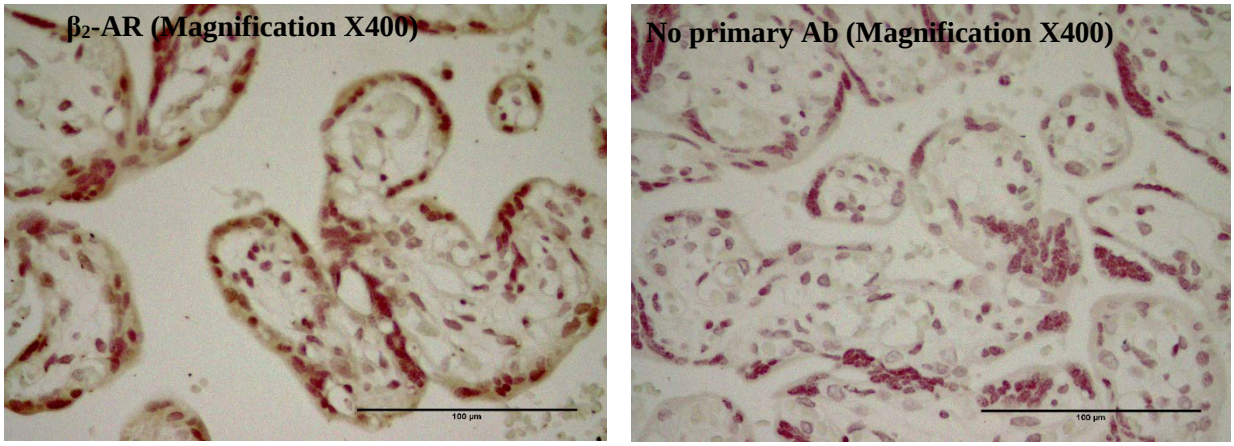


Figure 3.5 Immunohistochemistry localization of β2-adrenoceptors in pregnant human myometrium. Panels A, B, C, and D are myometrial tissue sections obtained from different position and different gestational ages as indicated. Each panel contains positive and negative control sections with different magnifications as indicated in the images. Panel E is a placenta section used as positive control tissue. Scale bars are 100 μm. MSM: myometrial smooth muscles, BV: blood vessels.

3.5 Optimization of β_2-adrenoceptor antibody working conditions

(A). Testing different blocking solutions

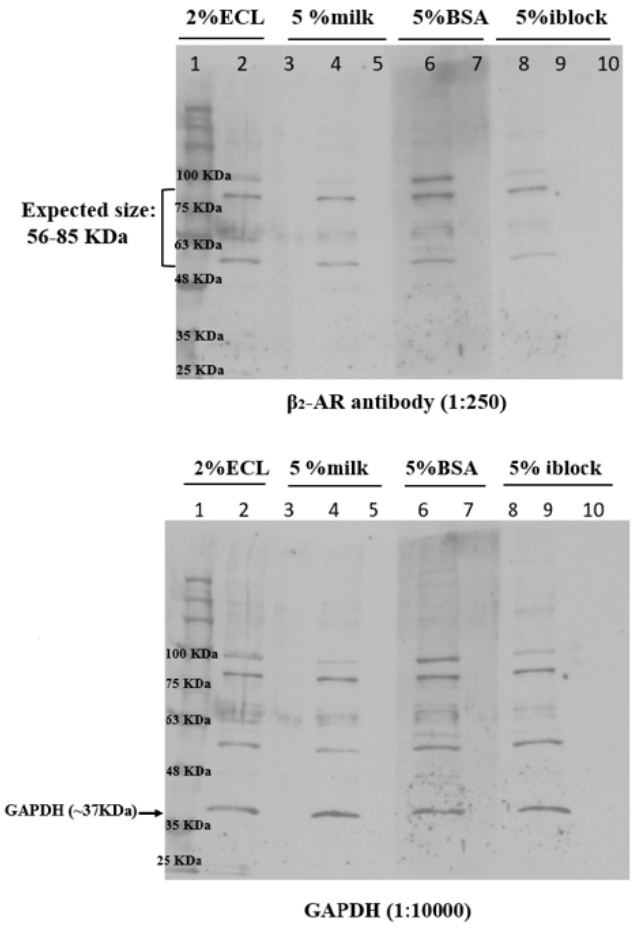


Figure 3.6. Testing different blocking solutions to optimize β_2-adrenoceptor antibody working condition. 1. Ladder, 2., 4., 6. and 8. are BEAS-2B cell lysate protein. 3., 5., 7., 9., and 10 are empty wells. β_2-adrenoceptor primary antibody used in 1:250 dilution. 8% running polyacrylamide gel was used. Expected molecular weight range for β_2-adrenoceptor protein is 56-85 KDa. The same membrane was washed 3 times with TBS-T before incubation with GAPDH primary antibody. Expected molecular weight for GAPDH is 37 KDa. (n=1)

(B). Optimizing β_2-adrenoceptor antibody dilution

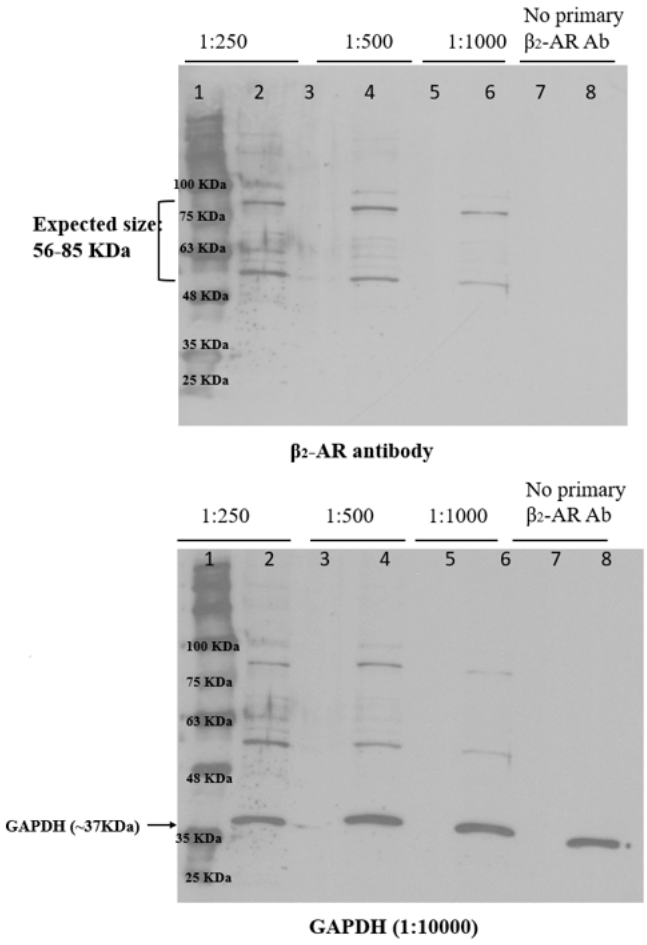


Figure 3.6. Testing different β_2-adrenoceptor antibody dilutions. β_2-adrenoceptor antibody was used in different dilutions: 1:250, 1:500 and 1:1000. **1.** Ladder, **2., 4., 6.** and **8.** are BEAS-2B cell lysate. 3., 5., and 7., are empty wells. 8% running polyacrylamide gel was used. Expected molecular weight range for β_2-adrenoceptor protein is 56-85 KDa. The same membrane was washed 3 times with TBS-T before incubation with GAPDH primary antibody Expected molecular weight for GAPDH is 37 KDa (n=1).

3.6 Assessment of β_2-adrenoceptor antibody specificity by western blotting

Positive control samples for the β_2-adrenoceptor were used to assess the specificity of the β_2-adrenoceptor antibody used in the immunohistochemistry experiments.

Multiple bands were observed at the expected molecular weight range (56-85 KDa) of the β_2-adrenoceptors in the positive control sample (BEAS-2B). Multiple non-specific bands were also observed in the mouse myometrium sample. No bands were detected in the no primary antibody control. A band of the appropriate size was observed in all samples with GAPDH antibody (~37 KDa) (**Figure 3.7.**).

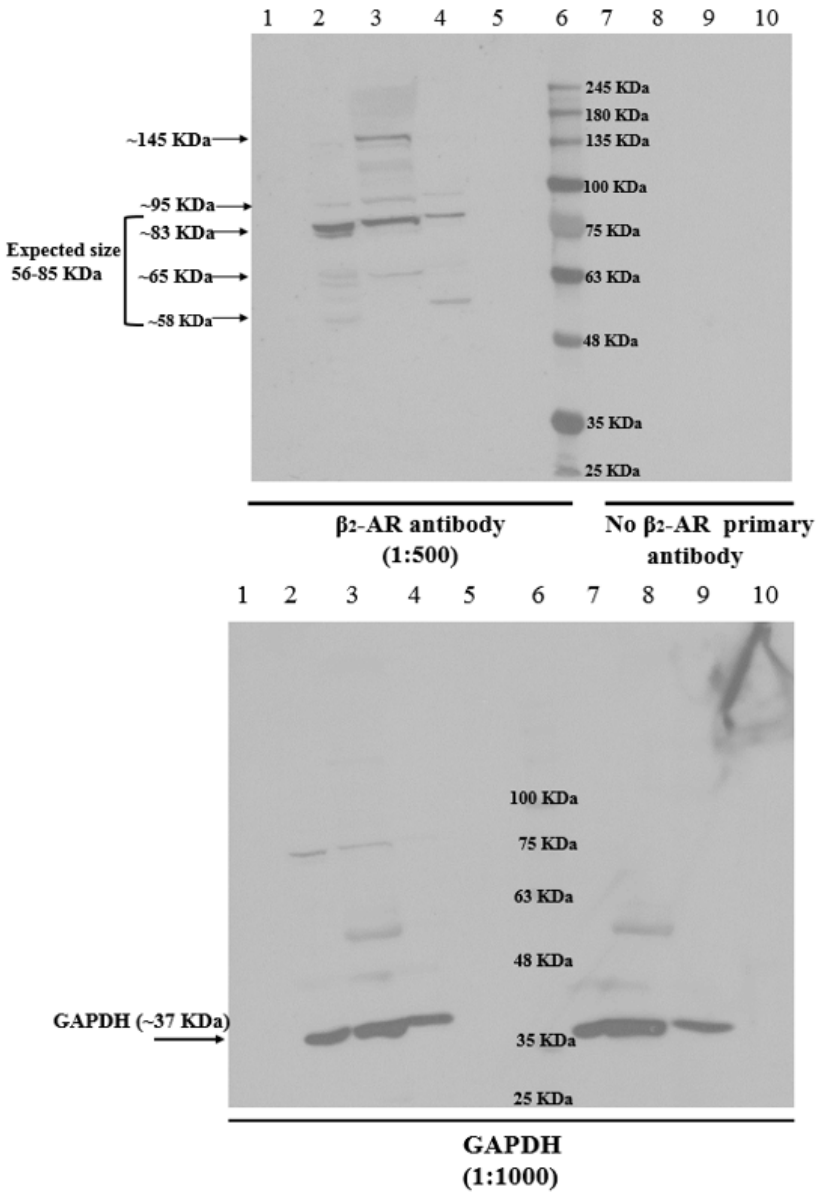


Figure 3.8. Staining pattern of β2-adrenoceptor antibody by western blot. 1. Loading buffer, **2.** HEK293 cell lysate, **3.** Mouse myometrium, **4.** BEAS-2B cell lysate (positive control), **5.** Loading buffer, **6.** Ladder, **7.** HEK293 cell lysate, **8.** Mouse myometrium, **9.** BEAS-2B cell lysate, **10.** Loading buffer. Arrows indicate the molecular weight of the observed bands. Expected molecular weight range for β2-adrenoceptor protein is 56-85 KDa. The same membrane was washed 3 times with TBS-T before incubation with GAPDH primary antibody. Expected molecular weight for GAPDH is 37 KDa. (n=1).

3.6 Assessment of β_3-adrenoceptor antibody specificity

β_3-adrenoceptor antibody specificity was assessed by western blotting. Similar staining was observed with the different blocking solutions (5% skimmed milk, 2% ECL, or 5% BSA). No bands were detected at the expected molecular weight sizes in both samples. A small molecular weight band (~35 KDa) was observed in HeLa whole cell lysate. No bands were detected in a mouse myometrium sample. A band of expected GAPDH molecular weight was observed in all samples (**Figure 3.9.**).

Placenta sample was used as a positive control for the β_3-adrenoceptor in immunohistochemistry. There was no difference between the placenta section incubated without antibody, with the primary antibody alone, and placenta incubated with the primary antibody and blocking peptide mixture (**Figure 3.10.**).

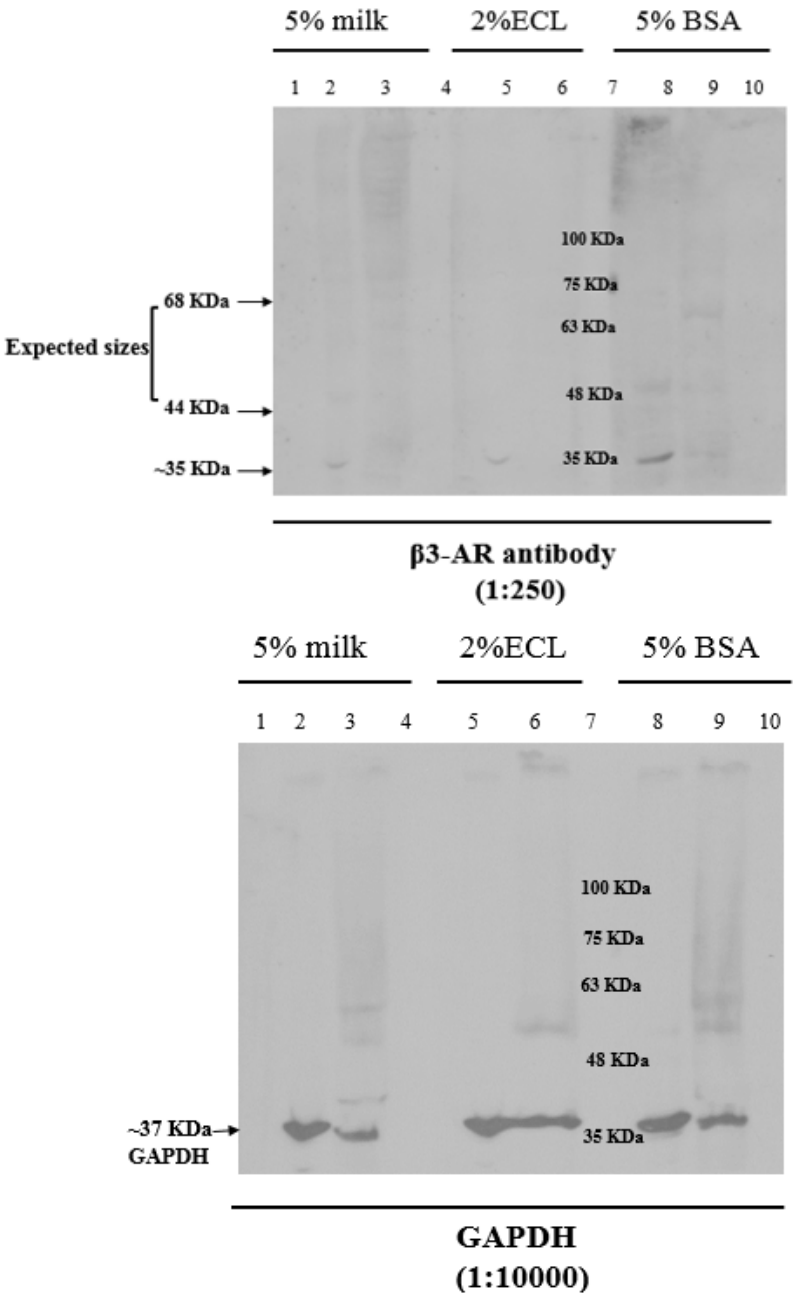


Figure 3.9. Staining pattern of β_3-adrenoceptor antibody on western blot. 1. Loading buffer, **2.** HeLa whole cell lysate (positive control), **3.** Mouse myometrium, **4.** Loading buffer, **5.** HeLa whole cell lysate, **6.** Mouse myometrium, **7.** Ladder, **8.** HeLa whole cell lysate, **9.** Mouse myometrium, **10.** Loading buffer. Different blocking solutions were used. Anticipated Molecular Weight of β_3-adrenoceptor is 44 KDa. Molecular Weight of glycosylated β_3-adrenoceptor is 68 KDa. Anticipated molecular weight for GAPDH is 37 KDa. (n=1)

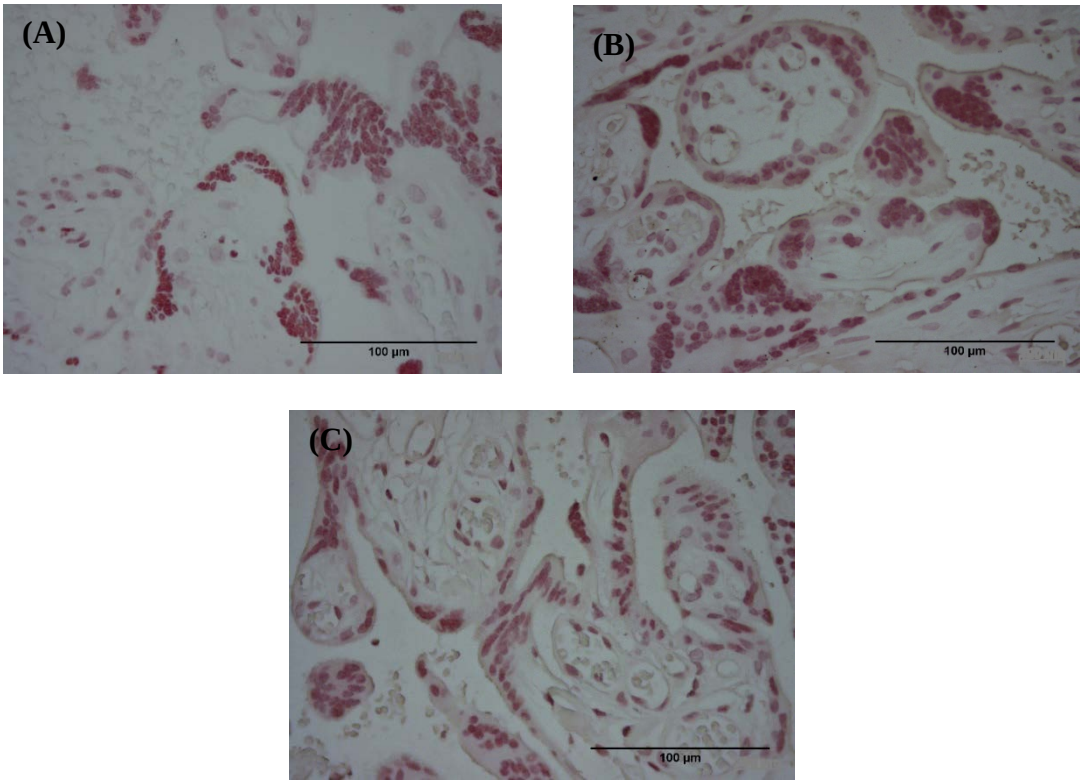


Figure 3.10. β_3-adrenoceptor antibody specificity assessment using immunohistochemistry. Placenta tissue sections used as a positive control for β_3-adrenoceptor. Section **(A)** is no primary antibody control. **(B).** placenta tissue section incubated with β_3-adrenoceptor antibody (1:50) overnight at 4^{o}C. **(C).** β_3-adrenoceptor antibody incubated with the relevant blocking peptide before it was applied to the placenta section. Scale bars are 100 µm.

Chapter Four: Effect of β-adrenoceptor agonists on *CRISPLD2* expression in MSM cells

4.1 Expression of *CRISPLD2* in the uterus

Expression of *CRISPLD2* was identified in upper and lower myometrial segments, decidua, placenta and BEAS-2B cells. A sample in which water was substituted for cDNA was included as a no template PCR control. *B2M* reference gene expression was present in each sample.

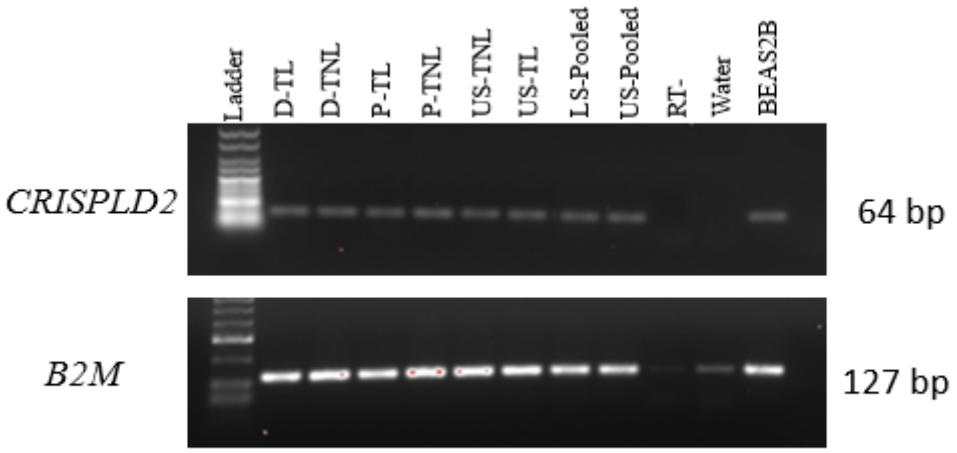


Figure 4.1. *CRISPLD2* mRNA expression in the human uterus. *CRISPLD2* expression in upper and lower pooled myometrium cDNA was assessed by RT-PCR. β2-Microglobulin (*β2M*) was used as a reference gene. **D-TL:** Decidua-labour, **D-TNL:** Decidua non-labour, **P-TL:** Placenta labour, **P-TNL:** Placenta non-labour, **US-TNL:** Upper myometrial segment non- labour, **US-TL:** Upper myometrial segment labour, **LS-Pooled:** Lower myometrial segment- Pooled cDNA, and **US-Pooled:** Upper myometrial segment- Pooled cDNA. (Water used to prepare B2M master mix is slightly contaminated) (n=1).

4.2 Effect of β-adrenoceptor agonists on *CRISPLD2* expression in MSM cells

Expression of *CRISPLD2* was about 1.8-fold and 2-fold higher in formoterol- and isoprenaline-treated cells respectively in comparison to untreated cells at 2 and 6 hours incubation. In the cells treated with the β_2-antagonist (ICI-118,551) plus formoterol or isoprenaline, no difference in the expression of *CRISPLD* was observed in comparison to non-treated cells. The expression of *CRISPLD2* was not different in β_3-agonist, (L-755507)-treated cells in comparison to non-treated cells at 2 and 6 hours incubation. Cells treated with forskolin, a cAMP elevating agent, had 3.5-fold and 4-fold higher *CRISPLD2* mRNA expression at 2 and 6 hours of incubation. These experiments were performed using n=5 different sets of MSM cells represented in 5 different colors.

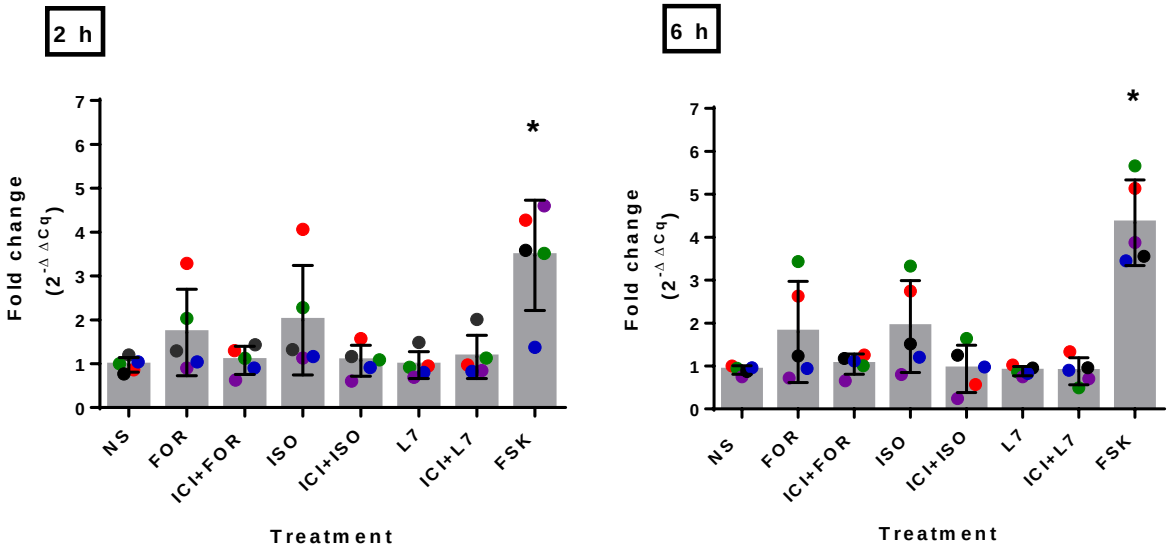


Figure 4.2. Expression of *CRISPLD2* in MSM cells - Effect of β-adrenoceptor agonists on the expression of *CRISPLD2* in primary MSM cells. Cells were treated with formoterol (FOR; 10 nM), isoprenaline (ISO; 1 µM), and L-755507 (L7; 10 nM) in the absence and presence of ICI-118,551 (ICI; 100 nM) and forskolin (FSK; 1 µM). Cells harvested after 2 or 6 hours for RNA extraction. Bars represent the mean (SD) of n = 5 different sets of MSM cells, and experiments were performed in duplicate. Different colors represent different sets of MSM cells; red: donor 1, green: donor 2, blue: donor 3, black: donor 4, purple: donor 5. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni's post-hoc test (* $P < 0.05$).

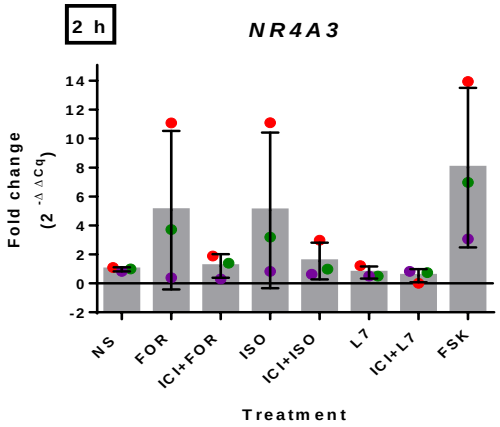
4.3 Effect of β-adrenoceptor agonists on *NR4A3, RGS2* and *IL-6* expression in MSM cells

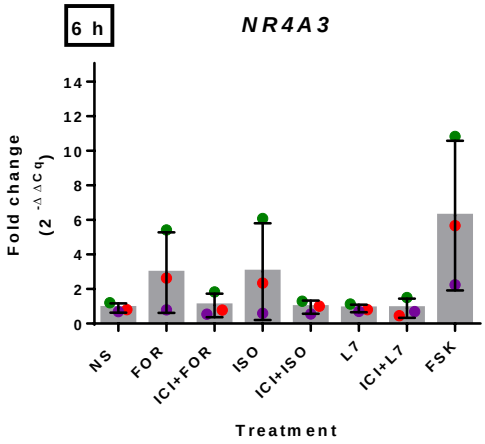
Effect of β-adrenoceptor agonists on *NR4A3*, *RGS2* and *IL-6* expression in MSM cells was determined by real-time RT-PCR. Samples from figure **4.2** were used.

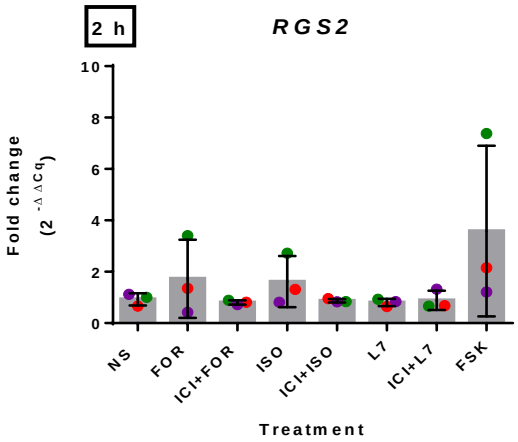
NR4A3: No significant differences were found in the expression of *RGS2* in formoterol-, isoprenaline-, L-755507- and forskolin-treated samples in comparison to the non-treated samples in both incubation periods.

RGS2: No significant differences were found in the expression of *RGS2* in formoterol-, isoprenaline-, L-755507- and forskolin-treated samples in comparison to the non-treated samples in both incubation periods.

IL-6: No differences were observed in the expression of *IL-6* in formoterol-, isoprenaline-, L-755507- and forskolin-treated samples in comparison to the non-treated samples in both incubation periods.


2 h
NR4A3
Fold change
(2 -ΔΔCq)
14
12
10
8
6
4
2
0
-2
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment


6 h
NR4A3
Fold change
(2 -ΔΔCq)
14
12
10
8
6
4
2
0
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment


2 h
RGS2
Fold change
(2 -ΔΔCq)
10
8
6
4
2
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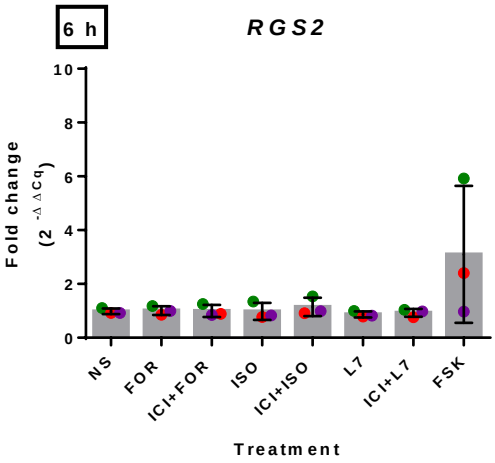
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment


6 h
RGS2
Fold change
(2 -ΔΔCq)
10
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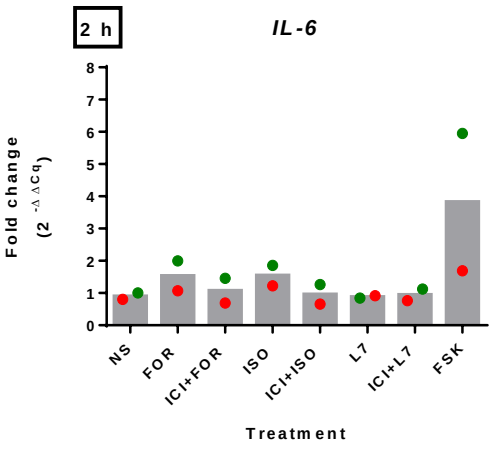
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment


2 h
IL-6
Fold change
(2 -ΔΔCq)
8
7
6
5
4
3
2
1
0
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment

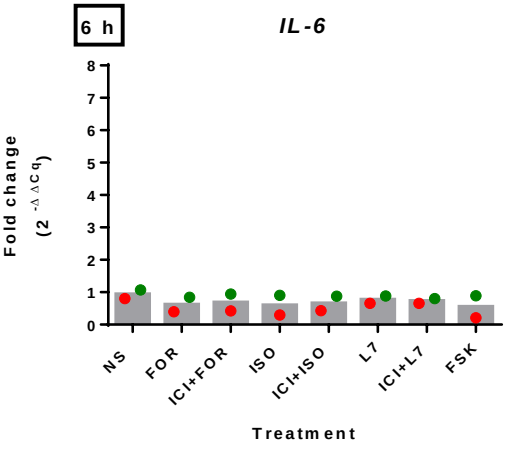

6 h
IL-6
Fold change
(2 -ΔΔCq)
8
7
6
5
4
3
2
1
0
NS
FOR
ICI+FOR
ISO
ICI+ISO
L7
ICI+L7
FSK
Treatment

Figure 4.3. Effect of β-adrenoceptor agonists on the expression of *NR4A3*, *RGS2* and *IL-6* in primary MSM cells.

Cells were treated with formoterol (FOR; 10 nM), isoprenaline (ISO; 1 µM), and L-755507 (L7; 10 nM) in the absence and presence of ICI-118,551 (ICI; 100 nM), and Forskolin (FSK; 1 µM). Cells harvested after 2 or 6 hours for RNA extraction. For *NR4A3* and *RGS2*, bars represent the mean (SD) of n = 3 different sets of MSM cells. For *IL-6*, data are presented as means of n = 2 different sets of MSM cells. Different colors represent different sets of MSM cells; red: donor 1, green: donor 2, purple: donor 5. Statistical analysis was performed by one-way ANOVA test.

4.4 Effect of the passage on the expression of *ADRB1, ADRB2,* and *ADRB3* in MSM cells (P1-P10)

MSM cells from 3 different MSM sets were sub-cultured from passage 1 (P1) through passage 10 (P10). Total RNA was isolated from each passage non-treated cells. There were no significant differences in the expression of *ADRB1*, *ADRB2*, or *ADRB3* comparing passage 1 through passage 10.

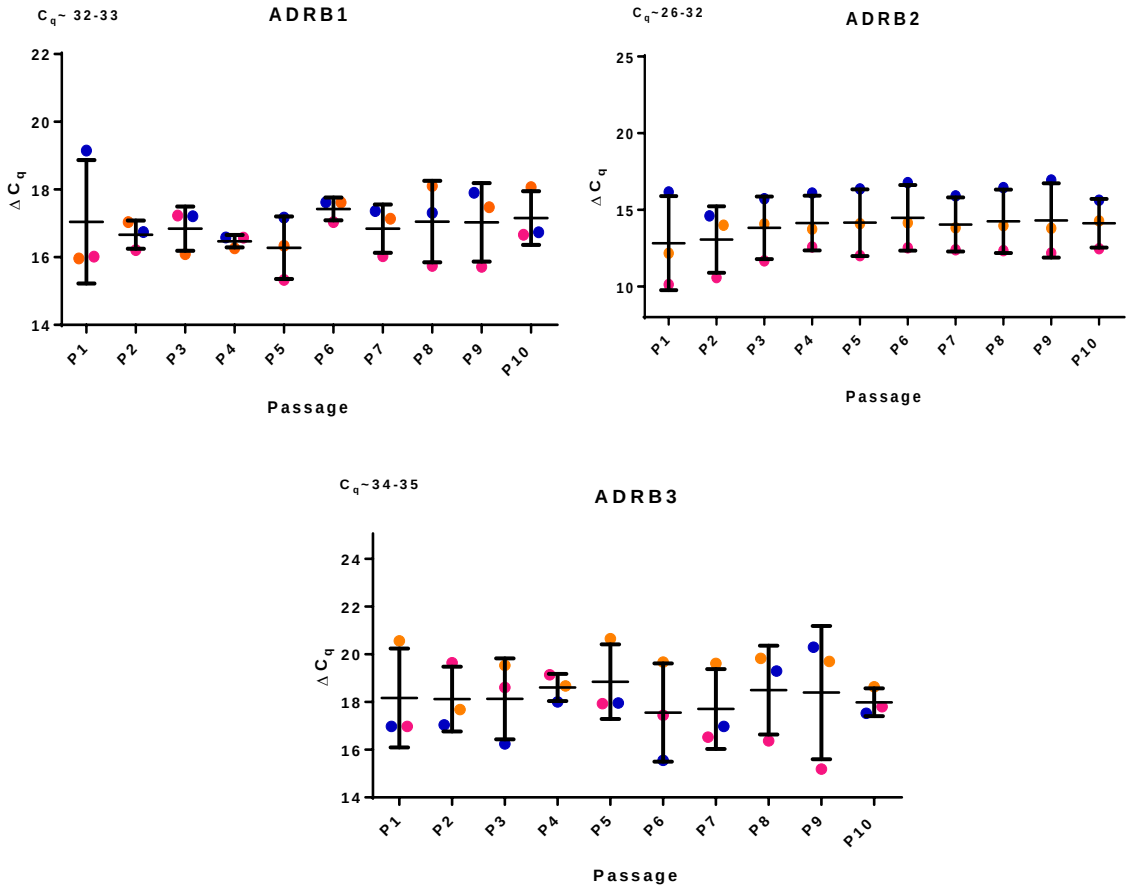


Figure 4.4. Relative expression of *ADRB1*, *ADRB2* and *ADRB3* in primary human MSM cells (P1-P10). Total RNA was isolated from primary untreated term non-labour myometrial cells from P1 to P10 and 1 µg was used to synthesize cDNA. Real-time RT-PCR was used to assess *ADBR1*, *ADBR2* and *ADBR3* mRNA expression, which was normalized to the reference gene β2-microglobulin (*B2M*). Bars represent the mean (SD) of n = 3 different sets of MSM cells. Real-time RT-PCR was performed in technical duplicates. Duplicates were averaged by sample per gene. ΔC_q was obtained by substracting average C_q of reference gene (*B2M*) from average C_q of *ADRB* for corresponding sample. Different colors represent different sets of MSM cells; blue: donor 3, orange: donor 6, pink: donor 7. X-axis represents passage number (P1-P10). Statistical analysis was performed by one-way ANOVA test.

4.5 Effect of formoterol, isoprenaline and forskolin treatments on the expression of *ADRB2* after 2 and 6 hours.

Real-time RT-PCR was used to determine the relative expression of *ADRB2* in non-treated, formoterol-, isoprenaline-, and forskolin-treated cells after 2 and 6 hours. The same samples shown in figure **4.2** were used. Expression of *ADBR2* was not significantly different comparing un-treated, formoterol-, isoprenaline-, and forskolin-treated cells after 2 and 6 hours.

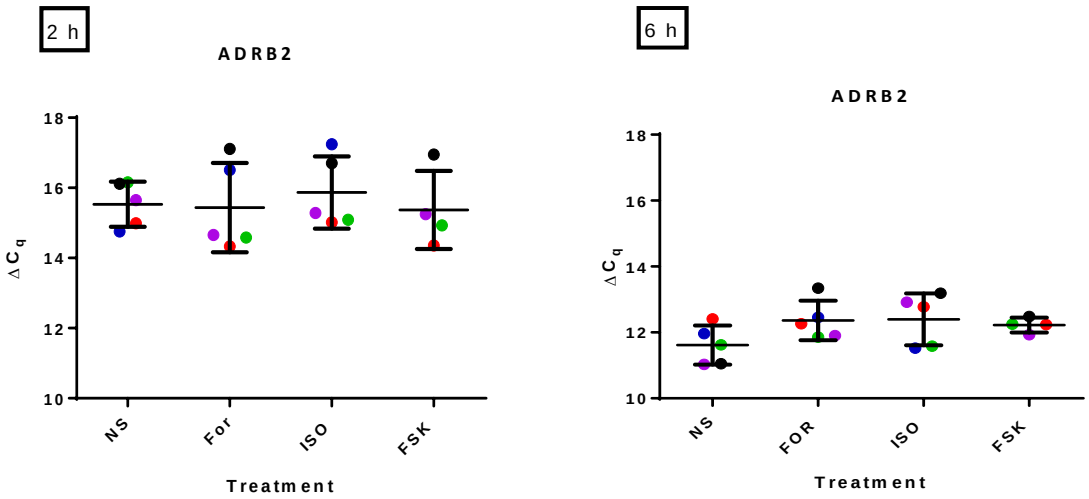


Figure 4.5. Effect of formoterol, isoprenaline and forskolin treatments on the expression of *ADRB2* after 2 and 6 hours. Expression of *ADRB2* in non-treated sample (NS), formoterol (For; 10 nM), isoprenaline (ISO; 1 µM), and forskolin (FSK; 1 µM) treated samples was determined by real-time RT-PCR. Real-time RT-PCR was performed in technical duplicates. Duplicates were averaged by sample per gene. Duplicates were averaged by sample per gene. ΔC_q was obtained by subtracting average C_q of reference gene (*B2M*) from average C_q of *ADRB2* for corresponding sample. Different colors represent different sets of MSM cells; red: donor 1, green: donor 2, blue: donor 3, black: donor 4, purple: donor 5. Statistical analysis was performed by one-way ANOVA test. Bars represent the mean (SD) of n = 5 different sets of MSM cells.

Chapter Five: Discussion

5.1 Summary of results

Experiments of this work have confirmed the expression of the three β-adrenoceptor subtypes (β_1, β_2 and β_3) in term pregnant human myometrial tissues by real-time RT-PCR analysis. No differences in the mRNA expression of β_1, β_2 and β_3-adrenoceptors in upper compared to the lower segment of term pregnant myometrium before and after the onset of labour were detected. I was unable to localize the β_2- and β_3-adrenoceptor proteins by immunohistochemistry due to poor specificity of antibodies used.

The effect of β-adrenoceptor agonists on the expression of *CRISPLD2, NR4A3, RGS2* and *IL-6* in primary MSM cells was tested. Overall, the expression of *CRISPLD2, NR4A3, RGS2* and *IL*-6 was not significantly different after treatment with β-adrenoceptor agonists whereas forskolin treatment was associated with approximately 4-fold higher *CRISPLD2* expression at 2 and 6 hours of incubation.

5.2 The β-adrenoceptors as targets for the management of preterm labour; what is the evidence?

I reviewed the literature systematically to find evidence to target the β-adrenoceptors during the management of preterm labour. I summarized and analyzed data from published literature about the expression of functional β-adrenoceptors in the uterus. Much of the available information regarding the expression and function of the β-adrenoceptors were obtained by utilizing rat tissues. Rat tissues can be obtained from different regions of the uterus at different stages of pregnancy and labour. Moreover, *in vitro* functional studies could be done using rats at different stages of pregnancy and labour. However, results obtained from animals must be compared with those obtained from human tissues to

understand similarities and differences between human and animal models. Rat uterine anatomy is different than human uterine anatomy. There were no attempts to identify whether there are regional differences in the expression and function of the β-adrenoceptors in rat and mice myometrial tissues. Therefore, there is no definitive understanding of the model used in studying the β-adrenoceptors in the uterus. Several weaknesses were observed in the published data regarding the β-adrenoceptors in the uterus; specifically previous molecular studies, lack proper controls and/or were performed under unoptimized conditions (Vivat *et al.*, 1992; Engstrom *et al.,* 1997; Principe *et al.,* 1997; Bardou *et al.,* 2000; Engstrøm *et al.,* 2001; Chanrachakul *et al.,* 2003; Rouget *et al.,* 2004; Ga´spa´r *et al.,* 2005; Rouget *et al.,* 2005; Minorics *et al.,* 2009; Spiegl *et al.,* 2009; Parida *et al.,* 2013; Yang *et al.,* 2015). Also, results obtained from animal studies were variable due to diverse kinds of tissues and/or different experimental conditions used for analysis. For example, using rats as a model to study the β-adrenoceptors, myometrial tissue preparations were used in several studies while uterine wall preparations, which include the myometrium and other uterine wall tissues such as the decidua were used in others. Different results were obtained, and it is not known whether different experimental conditions or different tissues is the reason for such variability (Legrand *et al.,* 1987; Kaneko *et al.,* 1996; Ohashi *et al.,* 1996; Engstrom *et al.,* 1997; Principe *et al.,* 1997; Ga´spa´r *et al.,* 2005; Minorics *et al.,* 2009). (iii). Several studies, which used human tissues did not consider age of pregnancy during sample collection (Rydtn G, *et al.,* 1982; Engelhard *et al.,* 1997; Liu *et al.,* 1998; Gsell *et al.,* 2000; Dennedy *et al.,* 2001; Chanrachakul *et al.,* 2003; Frambach T, *et al.,* 2005)., and the samples obtained from pregnant women were limited to the lower uterine segment (as it is easier to access during cesarean sections), whereas the expression of

functional β-adrenoceptors in the upper myometrial tissue was studied only in non-pregnant uterus (Andersson *et al.,* 1979; Rydtn *et al.,* 1982; Bottari *et al.,* 1985; Story *et al.,* 1988; Litime *et al.,* 1989; Engelhard *et al.,* 1997; Bardou *et al.,* 1999; Bardou *et al.,* 2000; Dennedy *et al.,* 2002; Sakakibara *et al.,* 2002; Chanrachakul *et al.,* 2003; Chanrachakul *et al.,* 2004; Rouget *et al.,* 2004; Chanrachakul *et al.,* 2005; Frambach *et al.,* 2005; Rouget *et al.,* 2005; Doheny *et al.,* 2006; Bardou *et al.,* 2007; Pędzińska-Betiuk *et al.,* 2011; Verli *et al.,* 2013). Finally, the majority of the articles did not use selective β-adrenoceptor agonists and antagonists to specify the β-adrenoceptor subtype involved in functional response such as mediating myometrial relaxation (Johansson *et al.,* 1978; Johansson *et al.,* 1981; Meisheri *et al.,* 1979; Levin *et al.* 1980; Izumi *et al.,* 1982; Tougui *et al.,* 1981; Bryman *et al.,* 1984, Berg *et al.,* 1984; Ikeda *et al.,* 1984; McPherson *et al.,* 1984; Bryman *et al.,* 1986; Khac *et al.,* 1986; Litime *et al.,* 1989; Story *et al.,* 1988; Bramuglia *et al.,* 1992; Ohashi *et al.,* 1996; Engstrom *et al.,* 1997; Le´crivain *et al.,* 1998; Liu *et al.,* 1998; Shinkai *et al.,* 2000; Engstrøm *et al.,* 2001; Dennedy *et al.,* 2001; Sakakibara *et al.,* 2002; Rouget *et al.,* 2004; Chanrachakul *et al.,* 2005; Ga´spa´r *et al.,* 2005; Rouget *et al*., 2005; Croci *et al.,* 2007; Spiegl *et al.,* 2009; Klukovits *et al.,* 2010; Wrzal *et al.,* 2012; Parida *et al.,* 2013; Verli *et al.,* 2013).

5.3 Spatial and temporal expression of the β-adrenoceptors in term pregnant human myometrial tissue

Based on real-time RT-PCR experiments within this study, there were no differences in the expression of *ADRB1, ADRB2* and *ADRB3* comparing upper to lower myometrial segment. Also, no differences were observed in the expression of *ADRB1, ADRB2 and ADRB3* comparing labour to non-labour samples. Unfortunately, specificity of the antibodies toward their targets was not confirmed in this research project. Therefore, whether there

are differences in the β-adrenoceptor protein levels between the protein samples studied is not known with certainty

It is important to stress that the myometrial tissue contains other structural components in addition to the myometrial smooth muscle cells, which include blood vessels (containing vascular smooth muscles and endothelial cells) and secretory glands. The β-adrenoceptors may have different functional roles in different components of the myometrium. Real-time RT-PCR data identified the expression of the β-adrenoceptors in the myometrial tissues. Specific localization for the β-adrenoceptor subtypes is required to help identify their roles in the myometrium.

The β-adrenoceptor subtype distribution was studied using immunohistochemistry and autoradiographic studies. Chanrachakul *et al.,* (2003) used immunohistochemistry to localize the β-adrenoceptors in human myometrium. However, data from Chanrachakul *et al.,* (2003) were hard to interpret for reasons mentioned previously in the systematic review within this thesis (p. 102). The distribution of the β-adrenoceptors in non-pregnant human cervix was studied by Whitaker *et al.,* (1989) using autoradiographic studies. In autoradiography tissue distribution of receptors is determined using radiolabeled ligands. Based on Whitaker *et al.,* (1989), the β_2-adrenoceptors were predominantly expressed in the surface columnar epithelium and glandular epithelium of non-pregnant human cervix; therefore, the β_2-adrenoceptors have been suggested to have a secretory function in this tissue. Whitaker *et al.,* (1989) did not investigate the distribution of the β_3-adrenoceptor and used a low concentration of ^{125}ICYP (25 pM – no affinity for the β_3-adrenoceptors) in their study.

It is not known where exactly the β-adrenoceptors are expressed within pregnant myometrial tissues during the different stages of pregnancy. A specific antibody toward each β-adrenoceptor subtype is required to localize these receptors within the myometrium.

Western blotting was used as a first step to assess the antibody specificity of a commercially available β_2-adrenoceptor antibody. Regarding the β_2-adrenoceptors, the results presented for western blotting clearly show 2 bands at the expected molecular weight range in the BEAS2B cells lysate (positive control). Numerous bands were also detected in the pregnant mouse myometrium tissue protein. HEK293 cell lysate was used as a negative control. HEK293 cells is a cell line that used in the literature to study the β_2-adrenoceptors after transfecting the cells with these receptors. There are different clones of HEK293 cell line, which might be different regarding the expression of the β_2-adrenoceptors. HEK 293 cells protein lysate used in this study was available in Giembycz laboratory as a control sample. Functional studies were not performed on the HEK293 cells to validate the existence of the β-adrenoceptors in this clone.

The expected molecular weight of the β_2-adrenoceptor protein is 46.4 KDa. However, a wide range for the molecular mass of the β_2-adrenoceptor was given by the manufacturer for the antibody (56-85 KDa) used here; presumably as the β_2-adrenoceptors have multiple sites for posttranslational modification. Posttranslational modifications for the β_2-adrenoceptors include: 3 sites for N-glycosylation at amino acids 6, 15 and 187, a glycosylation site at amino acid 187 and a palmitoylation site at amino acid 341 (McGraw & Liggett, 2005). A range of antibody dilutions (1:250, 1:500, 1:1000) and multiple blocking solutions were tested with no difference in the interpretation of the results. Negative control samples were not available since the β_2-adrenoceptors are ubiquitously

expressed throughout the tissues of the body. Genetically modified animals lacking these receptors are not available currently at the University of Calgary. Receptor knock-down or overexpression in BEAS-2B cell line might give a clue as to the specificity of this antibody, but this was not performed. Until the antibody specificity is well-validated, the immunohistochemistry data cannot be appropriately interpreted. Alternative methods to localize the β_2-adrenoceptors within the myometrium are *in situ* hybridization and autoradiographic studies. *In situ* hybridization is used to localize RNA using probed nucleotides and it does not depend on the use of antibodies, but localizing the β-adrenoceptor proteins would be still required. Autoradiographic studies require specific ligands for each β-adrenoceptor subtype to distinguish between them. Highly selective β-adrenoceptor ligands are available and could be used to localize these receptors under highly optimized experimental conditions.

The expression of β_3-adrenoceptor protein in pregnant human myometrial tissues was not investigated, and it is unknown whether it follows the same pattern of the mRNA expression or not. Western blotting experiments were performed to assess the specificity of the available antibody before conducting the immunohistochemistry experiments. The antibody did not show any band at the expected molecular weight with the positive control sample suggested by the manufacturer (HeLa whole cell lysate). Rather, a band at a low unexpected molecular weight was observed. This small band was also observed in BEAS-2B cell lysate protein which does not express the β_3-adrenoceptors. These data suggest non-specificity of the antibody for β_3-adrenoceptors. The β_3-adrenoceptor antibody was tested on placenta tissue section using immunohistochemistry. Under my experimental conditions, there was no staining in the experimental control and the β_3-adrenoceptor

stained placental tissue. Due to the lack of specificity of the antibody, immunohistochemistry experiments to localize the β_3-adrenoceptors in the myometrium were not performed.

5.4 The effect of β-adrenoceptor agonists on the expression of *CRISPLD2, NR4A3, RGS2,* and *IL-6* in human MSM cells.

The effect of β-adrenoceptor agonists on the expression of *CRISPLD2* in primary myometrial cells was examined at 2 and 6 hours. The β-adrenoceptor agonists had no significant effect on *CRISPLD2* expression at 2 and 6 hours. There was a significant increase in the expression of *CRISPLD2* following forskolin treatment indicating cAMP-dependent induction for this gene at the same time points (**Figure 5.1**). β_2-Adrenoceptor mRNA was expressed in the same level in all treated cells. Concentrations of reagents used were dependent on ligands affinities to their targets. However, whether the increase in cAMP by the β-adrenoceptor agonists was sufficient to modulate gene transcription in these cells or not is not known. cAMP analysis would allow determination of cAMP induction by the β-adrenoceptors in MSM cells.

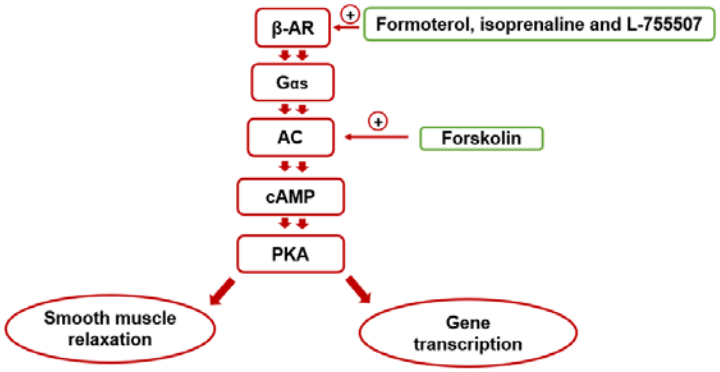


Figure 5.1. Schematic shows targets of treatments used in cell culture experiment. Formoterol, isoprenaline and L-755507 bind to β_2- and β_3-adrenoceptors while forskolin directly activates the enzyme adenylyl cyclase to activate cAMP synthesis.

The expression of *CRISPLD2* was not different comparing non-treated and L-755507-treated samples in this work. Different explanations are provided in the following points:

i. Functional β_3-adrenoceptors might not be expressed in MSM cells. To examine this possibility, the mRNA expression of the β_3-adrenoceptors and other β-adrenoceptor subtypes was determined in MSM cells from passage 1 through passage 10. The β_3-adrenoceptor mRNA was expressed and maintained from passage 1 to passage 10 in in the used MSM model. Moreover, based on functional studies by Bardou *et al.* (2000) and Dennedy *et al.*, (2002), β_3-adrenoceptor agonists relaxed lower myometrial strips obtained from pregnant women before the onset of labour, tissues that are from same region and gestational stage of the MSM cells used in this study. However, functional studies should be done to examine if the β_3-adrenoceptor mRNA translates to functional protein in the MSM cells.

ii. L-755507 does not regulate the expression of *CRISPLD2*. The effect of L-755507 and other β-adrenoceptor agonists (Formoterol and isoprenaline) on the expression of other

genes including *NR4A3*, *RGS2* and *IL-6* was examined. None of the tested ligands including forskolin affected the expression of *NR4A3, RGS2* and *IL-6.* However, more number of ends is required to test the expression of *NR4A3, RGS2* and *IL-6* following β-adrenoceptor agonists and forskolin treatment. Also, the expression of each gene should be tested after multiple time points of treatment. The β_3-adrenoceptor agonist, L-755507 did not affect the expression of any of the tested genes at any time points (2 and 6 hours). It has been suggested that the β_3-adrenoceptors couple to $G_{\alpha s}$ subunits after short times of activation (minutes) then and couple to $G_{\alpha i}$ subunit switch in the steady state in primary human myometrial cells (Hadi *et al.,* 2013). That might provide an explanation for the absence of the effect of L-755507 on cAMP inducible genes at 2 and 6 hours. This can be tested in our model by examining the effect of specific $G_{\alpha i}$ or $G_{\alpha s}$ inhibitors on downstream signaling mediators of $G_{\alpha s}$ and $G_{\alpha i}$ pathways at different time points.

5.5 Strengths and limitations

One of the strengths of this work is that the effect of β-adrenoceptor agonists on the expression of *CRISPLD2* has been studied using primary human myometrial cells isolated from the lower segment of the myometrium of women in term prior to the onset of labour. This cultured cell model has been validated and has been found to maintain the expression of smooth muscle markers, pregnancy associated markers and responds to inflammatory activators up to passage 10 (Mosher *et al.,* 2013). Moreover, these myometrial cells maintain the mRNA expression of the β-adrenoceptors from passage 1 through passage 10. Furthermore, cell culture experiment was performed using n=5 different sets of myometrial cells and each experiment was performed in duplicate.

One of the major limitations of this work is the poor specificity of the β_2 and β_3-adrenoceptor antibodies employed. I reviewed published data and company descriptions for β-adrenoceptor antibodies, but I could not find a clear data in the literature that show highly specific β-adrenoceptor antibodies. Commercially available antibodies against the β-adrenoceptors are polyclonal antibodies. Determination of the β-adrenoceptor protein expression may require development of specific antibodies for each β-adrenoceptor subtype. The full sequence of the β-adrenoceptor proteins is available in the literature (molecular weight 46.4 KDa). However, the amino acid sequence of β-adrenoceptor subtypes shares high degree of identity. For example, the percentage of identity in the amino acid sequence between the β_2- and β_3-adrenoceptors is about 46% (Coman *et al.,* 2009)

The first step in designing an antibody is to identify a unique peptide within the protein structure to be used as a targeted antigen for each subtype. As discussed in the β-adrenoceptor desensitization in the introduction of this thesis, the C-terminus of the β_2- and β_3-adrenoceptors have different amino acid sequences. β_2- and β_3-adrenoceptor antibodies used in this study were directed against peptides in the C-terminus of each subtype (peptides 338-413 for the β_2-adrenoceptor, unspecified for the β_3-adrenoceptor). However, these antibodies are polyclonal antibodies that were generated from multiple B cells, thus recognizing more than one epitope in the antigen is anticipated. Generating specific monoclonal antibodies from single B cell as described in Carvalho *et al.,* (2017) would allow identification of protein expression and localization of each β-adrenoceptor subtype.

5.6 Future directions

This chapter has discussed multiple experiments that could be done to improve our understanding about the expression and function of the β-adrenoceptors in the human uterus.

More understanding to the β-adrenoceptors in human myometrium and other gestational tissues such as the decidua would lead to better understanding for the process of human parturition. It would also help identify potential targets for the management of preterm labour.

Cell culture is a very beneficial technique for studying signaling pathways of the receptors. However, it is essential first to localize the β-adrenoceptors within the myometrium to identify which type of cells to be cultured. A validation for the cultured cells is crucial to identify any factors that might affect the expression of these receptors in the cultured cells such as passaging and growth medium contents.

Since human myometrial samples from different regions of the pregnant myometrium in different stages of gestation are mainly obtained from women undergoing caesarean section, collection of human myometrial samples would need long time. The molecular profile of these receptors in other species such as rats can be determined and compared to that of human to produce an experimental model that enables further studies on the β-adrenoceptors.

In conclusion, further work is required to fully determine the expression of the β-adrenoceptors in the pregnant myometrium. Full determination to the molecular profile of these receptors might help identify potential targets for the management of preterm labour. It would also improve the general understanding for the process of parturition.

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Appendix

Data analysis for the systematic review within this thesis

1. Molecular (mRNA/protein) expression of β-adrenoceptors in the uterus

In this section, all articles resulting from the electronic search investigating the existence of β-adrenoceptors in different tissues of human uterus were included. Reference lists of selected articles were also reviewed to try to identify additional relevant articles.

The expression of β-adrenoceptors in human uterus has been investigated using different molecular techniques such as conventional RT-PCR, real-time RT-PCR, western blotting and immunohistochemistry. The existence of the β-adrenoceptor subtypes has been also investigated using pharmacological methods including binding assays and autoradiography.

Chanrachakul *et al.*, (2003) used western blotting, immunohistochemistry, and immunofluorescence to localize and identify β_2-adrenoceptor expression in human non-pregnant, term non-labour, and term labour myometrial tissue. Chanrachakul *et al.*, (2003) concluded that the β_2-adrenoceptor protein expression in the term-labour was lower when compared to β_2-adrenoceptor protein expression in term non-labour myometrium and that the non-pregnant tissue expressed higher levels of β_2-adrenoceptor protein than the pregnant myometrium. However, the presented western blotting data did not contain negative control samples, nor a loading control was included to ensure equal loading.

Moreover, myometrial tissue samples from non-pregnant women were obtained from the upper while from the pregnant myometrium were obtained from the lower uterine segment. As the samples were obtained from different regions of the uterus, the comparison between the expression of β-adrenoceptors in non-pregnant and the pregnant myometrium may not

be appropriate. Fluorescence micrographs to localize the β_2-adrenoceptors in Chanrachakul *et al.,* (2003) show positive staining in the membrane and the cytoplasm of the myometrial cells, but the data are hard to interpret since validation for the specificity of the used antibodies has not been performed.

Bottari *et al.,* (1985) showed predominance (>80%) of β_2- over β_1-adrenoceptor subtype in human non-pregnant myometrial tissues. The predominance of β_2-adrenoceptor subtype in pregnant human myometrium has also been suggested by Breuiller *et al.,* (1987). Breuiller *et al.,* (1987) concluded that the inner myometrial layer contained 50% higher β_2-adrenoceptor binding sites than the outer myometrial layer at preterm while both myometrial layers had the same density of the β_2-adrenoceptors at term. In both studies the existence of the β_3-adrenoceptors was not investigated as they were published before the identification of the β_3-adrenoceptor subtype.

Whitaker *et al*., (1989) suggested predominance of β_2- (>95%) over β_1-adrenoceptor subtype in human cervix. Whitaker *et al*., (1989) used autoradiographical studies to identify the distribution of β-adrenoceptors in non-pregnant human cervix. The radioligand [^{125}I] iodocyanopindolol (^{125}ICYP) was detected around blood vessels, glands and surface columnar epithelium. Selective β_1-, β_2- and β_3-adrenoceptor ligands were not used separately with radioligand to identify the receptors involved in ^{125}ICYP binding.

Vivat *et al.,* (1992) identified the expression of the β_2-adrenoceptor mRNA in rat myometrium at day 21 of pregnancy. The expression of β_2-adrenoceptor mRNA was increasing by longer incubation with progesterone. However, northern blotting data presented in Vivat *et al.,* (1992) did not contain loading control nor positive and negative controls. Principe *et al.,* (1997) using conventional RT-PCR argued that the expression of

β_2-adrenoceptors was not different in rat myometrium through days 12, 14, 16, 20, 21, and 22 and on day 1 postpartum. However, conventional RT-PCR data presented in Principe *et al.,* (1997) represent results from only one sample for each examined day of pregnancy. There was no densitometric analysis to show all results obtained from all samples they have used (2-5 samples) nor statistical analysis to examine the significant differences between the groups. Moreover, the identity of the RT-PCR product was examined based on restriction endonuclease enzymes, but the experiment did not include important controls to identify sample contamination such as negative reverse transcriptase samples.

Radioligand binding assay and autoradiography conducted by Brauer & Burnstock, (1998) showed predominance of β_2- over β_1-subtype and that the β_2-adrenoceptors were localized in the endometrial epithelium, longitudinal and circular myometrial muscles of non-pregnant rats. Chen *et al.,* (1994) also suggested predominance of β_2- over β_1-subtype in non-pregnant mice myometrium. However, selective β_3-adrenoceptor ligands were not included in these studies. Therefore, the data still missing information which may include the existence of the β_3-adrenoceptor subtype.

Yang *et al.,* (2015) used western blotting to examine protein expression of β_2-adrenoceptors in control and oxytocin-induced mice. However, based on data presented, the β_2-adrenoceptor protein expression cannot be confirmed due to lack of proper controls. Legrand *et al.,* (1987) demonstrated that the number of β_2-adrenoceptor binding sites was not different in rat myometrium during the last 5 days of pregnancy while it was significantly decreased by the onset of labour. In their work, Legrand *et al.,* (1987) used pooled samples of myometrial tissue membrane preparations for each examined age of

pregnancy. Variation in the number of binding sites between rat myometrial tissues of each age of pregnancy cannot be assessed.

Maltier *et al.,* (1988) used binding assay to determine the β_2-adrenoceptors density in rat myometrial membrane preparations. Number of β_2-adrenoceptors binding sites was not different in day 21-9h and day 22-9h, but it was significantly lower in day 22-16h compared to day 22-9h. Maltier *et al.,* (1989) used radioligand binding assay to assess the density of β_2-adrenoceptors in term pregnant rat myometrium. Based on Maltier *et al.,* (1989) results, the density of β_2-adrenoceptors was not different in day 21 compared to day 22-10h of pregnancy while it was sharply decreased by the onset of labour (when vaginal bleeding was started).

Kaneko *et al.,* (1996) demonstrated that the number of β-adrenoceptor binding sites in rat myometrium was higher in day 21 when compared to day 15 and day 18 of pregnancy. However, Kaneko *et al.,* (1996) assumed predominance of β_2-adrenoceptors at all days they examined. No statistical analysis was performed to identify the significant differences in between the groups in Kaneko *et al.,* (1996).

In guinea pigs, Handberg *et al.,* (1988) and Pennefather & Molenaar P, (1986) determined the density of β_2-adrenoceptors in uterine membrane samples of guinea pigs. All three papers suggested predominance of β_2- over β_1-adrenoceptor subtype. However, presence of β_3-adrenoceptor subtype has not been investigated in these articles.

Pennefather & Molenaar (1986) found that the β_2-adrenoceptors were the predominant subtype in oestrogen-treated guinea pig circular and longitudinal myometrial tissues. Handberg *et al.,* (1988) demonstrated the density of β_2-adrenoceptors in virgin and post-partum guinea-pig uterine membranes. In post-partum animals, circular and longitudinal

myometrial layers contained β_2-adrenoceptors. β_2-adrenoceptor density was similar in post-partum and virgin animals.

In rabbits, Vallieres *et al.,* (1978) concluded that number of β-adrenoceptor binding sites in pregnant rabbit myometrium decreased by half at the end of pregnancy (day 3 compared to day 30). Falkay (1990) demonstrated that the density of β-adrenoceptors in pregnant rabbit myometrial and cervical tissues was not different in antiprogesterone (RU-48) treated samples when compared to non-treated samples. In both studies there was no specification for which subtypes of the β-adrenoceptor were expressed; selective β-adrenoceptor ligands were not used in binding experiments.

All the above-mentioned articles studied the existence of β-adrenoceptor in human, rats, rabbits and guinea pig uteri without correlating the expression of β-adrenoceptors to any functional responses.

2. Molecular expression of β-adrenoceptors with functional responses:

The molecular expression of β-adrenoceptors is not necessarily translated to a functional response. Therefore, the identification of the β-adrenoceptors should be done using functional methods using pharmacological ligands. All articles studing functional responses evoked by β-adrenoceptor agonists were included in this section.

Sakakibara *et al.,* (2002) studied the expression and function of β_2-adrenoceptors in term-pregnant myometrium before the onset of labour using radioligand binding assays and contractility studies. According to Sakakibara *et al.,* (2002), β_2-adrenoceptor selective agonists including ritodrine and KUR-1246 and the non-selective β-adrenoceptor, isoproterenol, inhibited spontaneous myometrial contraction. The inhibitory responses to isoproterenol varied between complete to moderate inhibition between different samples.

Forskolin inhibited myometrial contractions in all examined myometrial strips. The difference in the magnitude of myometrial contraction inhibition by isoproterenol was correlated to the number of the β_2-adrenoceptor binding sites in different strips. However, the β_3-adrenoceptor subtype has not been taken in consideration during the binding study experiments and result interpretations. For example, β_2- and β_3-adrenoceptor selective antagonists have not been used to confirm the action of isoproterenol on β_2-adrenoceptors. This makes the data hard to interpret especially that the used agent, isoproterenol can stimulate all β-adrenoceptor subtypes. Also, statistical analysis was not performed to correlate the ritodrine functional response to the number of the β_2-adrenoceptor binding sites.

The mRNA expression of the β_2- and β_3-adrenoceptors in human non-pregnant and term non-labour myometrial tissue has been studied by Rouget *et al.*, (2005). In their study, Rouget *et al.*, (2005) concluded that the mRNA expression of β_2-adrenoceptors is higher in non-pregnant compared to the pregnant myometrium whereas the protein expression was not different between the two groups. They also argued that β_3-adrenoceptor mRNA and protein expression were higher in term pregnant compared to the non-pregnant human myometrium. However, as presented, the conventional RT-PCR data lack appropriate controls such as negative reverse transcriptase, positive and negative control samples. Moreover, no loading controls were used to ensure equal protein loading between the samples in the western blotting study. Conventional RT-PCR and western blotting were performed using four and five tissues from different patients respectively, but no statistical analysis was mentioned on the result figures. In Rouget *et al.*, (2005), number of β_2-adrenoceptor binding sites was not different between pregnant and non-pregnant

myometrium whereas number of β_3-adrenoceptor binding sites were significantly higher in term pregnant myometrium compared to non-pregnant myometrium (6-8 samples). Based on their binding study, the β_3-adrenoceptor was the predominant subtype in term pregnant and non-pregnant myometrium. Salbutamol, a selective β_2-adrenoceptor, had higher efficacy in inhibiting the spontaneous myometrial contractions in non-pregnant compared to term pregnant tissues. In contrast, SAR 59119A, β_3-adrenoceptor agonist, had higher efficacy in term pregnant compared to the non-pregnant tissues. Selective β_2- and β_3-adrenoceptor antagonists have not been used to examine whether salbutamol and SAR 59119A exerted their actions through β_2- and β_3-adrenoceptors.

Rouget *et al.*, (2004) compared β_2- and β_3-adrenoceptor resistance to desensitization in term pregnant human myometrial tissues following sustained treatment with selective β_2- and β_3-adrenoceptor agonist. Exposure of myometrial strips to salbutamol for 15h significantly reduced the number of β_2- but not β_3-adrenoceptor binding sites. Salbutamol pre-treatment inhibited the potency but not efficacy of salbutamol inhibitory action on spontaneous myometrial contraction while SAR 59119A pre-treatment did not affect the subsequent SAR 59119A inhibitory effect on myometrial strip contraction. Exposure of myometrial strip to SAR59119A for 15h had no effect on the number of β_2- and β_3-adrenoceptor binding sites. Salbutamol and SAR 59119A had no effect on the mRNA expression of β_2- and β_3-adrenoceptors. 15h salbutamol pre-treatment significantly reduced cAMP production by further treatment with salbutamol while 15 h SAR59119A pre-treatment had no effect on cAMP production by SAR59119A. Salbutamol and SAR59119A pre-treatments did not affect phosphodiesterase activity. Effects of salbutamol and SAR 59119A was not verified using β_2- and β_3-adrenoceptor antagonists.

Liu *et al.,* (1998) demonstrated the inhibition of KCl-induced myometrial contractions in term pregnant women by isoproterenol. The selective β_2-adrenoceptor antagonist, ICI - 118551, caused a 189-fold right shift in the concentration-response curve of isoproterenol. A selective β_1-adrenoceptor antagonist did not affect isoproterenol concentration response curve. However, the effect of a selective β_3-adrenoceptor antagonist was not tested for this isoproterenol effect. Therefore, it cannot be concluded that the effect of isoproterenol was only through the β_2-adrenoceptors.

Andersson *et al.,* (1979) demonstrated the inhibitory action of isoprenaline and terbutaline on oxytocin-induced myometrial contraction. In Andersson *et al.,* (1979) isoprenaline and terbutaline inhibited contractions in pregnant human myometrium *in vivo* and *in vitro*. The effect of isoprenaline and terbutaline was blocked by the non-selective β_1- and β_2-adrenoceptor antagonist propranolol while was not affected by a selective β_1-adrenoceptor, atenolol, suggesting a β_2-adrenoceptor-mediated action for isoprenaline.

Story *et al.,* (1988) studied the inhibitory action of isoprenaline on term pregnant human myometrium. In this study, isoprenaline inhibited contraction in field-stimulated myometrial strips. 3 out of 11 myometrial strips were unresponsive to the inhibitory action of isoprenaline while all the strips responded to the treatment with forskolin. Responsiveness of all the samples to forskolin but not isoprenaline suggests same activity of adenylyl cyclase; the difference between the strips could be in the density of the β-adrenoceptor binding sites or ability of the receptors to couple to the G-protein. Samples used in Story *et al.,* (1988) were obtained from women in different ages of pregnancy (31-40 weeks of pregnancy), which means some samples were obtained from preterm while others from term pregnant myometrium. It was not specified which samples did not

respond to isoprenaline. However, in their study, differences between the responsive and unresponsive strips such as differences in the density of β-adrenoceptors and G-protein activation were not investigated.

Dennedy *et al.,* (2001) and Dennedy *et al.,* (2002) studied the function of β_2- and β_3-adrenoceptor agonists in the lower uterine segment of term pregnant human myometrium. In Dennedy *et al.,* (2001), the β_3-adrenoceptor agonist BRL 37344 had the same potency and efficacy of the β_2-adrenoceptor agonist, ritodrine in relaxing oxytocin-induced myometrial contraction. Action of both ligands was not validated by selective β_2- and β_3-adrenoceptor antagonists. In Dennedy *et al.,* (2002), BRL 37344 and ritodrine inhibited oxytocin-induced myometrial contraction. The action of BRL 37344 was not affected by propranolol but was antagonised by bupranolol, a non-selective β-adrenoceptor blocker. The action of ritodrine was antagonised by both propranolol and bupranolol. Collectively, Dennedy *et al.,* (2002) results suggest functional β_2- and β_3-adrenoceptors in term pregnant human myometrium.

Pędzińska-Betiuk *et al.,* (2011) investigated the effect of BRL 37344 and ritodrine on fundal (upper uterine segment) non-pregnant myometrial contraction. BRL 37344 and ritodrine inhibited spontaneous myometrial contractions. The action of ritodrine was antagonised by propranolol, bupranolol and butoxamine while the action of BRL 37344 was partially inhibited by propranolol and bupranolol and not altered by butoxamine. These results suggest other mediators for the action of BRL 37344 in addition to β_3-adrenoceptors.

Chanrachakul *et al.,* (2005) studied the effect of progesterone on the ritodrine relaxatory action. Incubating term pregnant human myometrial tissues with progesterone led to more

potent inhibitory response for ritodrine. Progesterone effect on the density of β_2-adrenoceptor binding sites was not investigated and action of ritodrine was not validated using a selective β-adrenoceptor antagonist.

Bryman *et al.,* (1984) and Bryman *et al.,* (1986) studied effects of isoprenaline and terbutaline on cervical tone of non-pregnant and early pregnant women. Isoprenaline and terbutaline inhibited cervical tone and the inhibition was more pronounced in non-pregnant cervixes. In both studies, there was not verification for the effects of theses ligands using selective β-adrenoceptor antagonists.

Spiegl *et al.,* (2009) determined the expression of β_2-adrenoceptors in pregnant rat myometrium using conventional RT-PCR. In their study, Spiegl *et al.,* (2009) compared the expression of β_2-adrenoceptors in control and diabetic day 15 pregnant rats. There were no differences in the expression of β_2-adrenoceptors between the control and diabetic rats of non-pregnant rats. The actual gels for the conventional RT-PCR were not presented and proper controls were not mentioned. Terbutaline was less potent in inhibiting myometrial contractions in diabetic rat compared to its efficacy in control group in non-pregnant rats. Reasons why terbutaline had different efficacies in control and diabetic non-pregnant rats have not been speculated. In the same study, the potency of terbutaline was not different between control and diabetic rats during pregnancy. No comparison between the expression of β_2-adrenoceptors in control and diabetic rats during pregnancy was performed. Also, action of terbutaline was not examined in the presence of selective β_2-adrenoceptor antagonist.

The effect of pregnancy and gestational age on the expression of β_2-adrenoceptor mRNA has been tested in rat myometrium by Engstrom *et al.,* (1997) using conventional RT-PCR.

Based on their study, the mRNA expression of the β_2-adrenoceptors was lower in the pregnant myometrium toward term (days 7-14-21 of pregnancy) and the expression of the β_2-adrenoceptors was approximately 8-fold higher at 5 days postpartum myometrium when compared to the level before delivery. Only densitometric analysis obtained from four different rats in each age of pregnancy was presented. As presented and as described in the methods section, the data of the RT-PCR lack proper controls and the identity of the amplified product has not been confirmed. In the same study, isoproterenol inhibited KCl-induced myometrial contractions. Isoproterenol had reduced potency in the term pregnant myometrium (day 21) compared to days 7, day 14 of pregnancy and day 5 postpartum myometrium. It has been suggested in Engstrom *et al.,* (1997) that reduced potency of isoproterenol in day 21 of pregnancy was due to the decreased β_2-adrenoceptor mRNA synthesis at that day. However, number of β_2-adrenoceptor binding sites was not determined in the samples. Selective β-adrenoceptor antagonists were not used to investigate the effect of isoproterenol, which is a non-selective β-adrenoceptor. Therefore, it is not known if isoproterenol caused the inhibition of the myometrial tissues through one, a combination of two or all the β-adrenoceptor subtypes.

Engstrøm *et al.,* (2001) studied the expression of β_2-adrenoceptor mRNA in non-pregnant rat myometrium in control and *in vivo* steroid hormones-treated animals using conventional RT-PCR. The β_2-adrenoceptor mRNA with progesterone-treated rats was not different from the control group (treated with saline) while the β_2-adrenoceptor mRNA was significantly higher in estrogen- and estrogen plus progesterone-treated animals compared to the control group. Progesterone did not affect β_2-adrenoceptor mRNA. Proper controls were not included in the RT-PCR of Engstrøm *et al.,* (2001). *In vitro* isoprenaline treatment

inhibited myometrial contraction in all used animal groups, but with different pharmacological parameters. Isoprenaline had significantly lower potency and efficacy in estrogen-treated myometrial strips when compared to the control strips. Progesterone alone had no effect on the E_{max} of the isoprenaline relaxtory action on uterine strips. Engstrøm *et al.,* (2001) suggested estrogen dominance during the end of pregnancy as an inducer for β_2-adrenoceptor desensitization. This cannot be concluded because it is not known if the action of isoprenaline was through the β_2-adrenoceptors since selective β_2-adrenoceptor antagonists were not used for isoprenaline action verification.

Piercy (1987) investigated the relaxatory effect of β-adrenoceptor agonists on non-pregnant rat uterine muscles throughout the oestrous cycle. The actions of selective β_2-adrenoceptor agonists including fenoterol and salbutamol, a selective β_3-adrenoceptor agonist, BRL 28410 and non-selective β-adrenoceptor agonists including isoprenaline and noradrenaline were tested. All the used ligands had the same potencies throughout the oestrous cycle suggesting no change in the receptor subtypes population. Isoprenaline and fenoterol had the same and the highest potency in relaxing the uterine muscles. Salbutamol was more potent than BRL 28410 in relaxing the uterine muscles. Pharmacological analysis using atenolol and ICI 118551 to test responses of fenoterol and noradrenaline resulted in the same PA_2 values. It has been suggested that the samples express homogenous population of receptor subtype, which is β_2-adrenoceptor subtype.

Radioligand binding assay conducted by Elalj *et al.,* (1988) showed predominance of β_2-over the β_1-adrenoceptor subtype in non-pregnant rat myometrium. In the same study, contractility study demonstrated that antiprogestin (RU 486) treatment did not alter the pharmacological parameters (potency and efficacy) of salbutamol, a selective β_2-

adrenoceptor agonist, in relaxing circular and longitudinal non-pregnant rat myometrial strips.

In rats, the expression of β_3-adrenoceptors was studied by Minorics *et al.*, (2009) using conventional RT-PCR and western blotting in pregnant rat uterine tissues. There were no changes in the mRNA and protein expression through days 18, 20, 21, 22 of pregnancy. However, neither RT-PCR nor western blotting presented data contained proper positive, negative and experimental controls. Moreover, no optimization processes for the primers and antibodies specificity were performed. BRL 37344, a selective β_3-adrenoceptor agonist, inhibited KCl-induced myometrial contractions in pregnant rat myometrium and its action was not altered by a selective β_2-adrenoceptor antagonist. The effect of a selective β_3-adrenoceptor antagonist was not tested. The potency of BRL 37344 was significantly higher in day 18 compared to its potency in the day 20, 21 and 22 pregnant myometrium, which were not different from each other. However, BRL 37344 had significantly less efficacy in inhibiting myometrial contraction in day 21 when compared to its efficacy in days 20 and 22. Progesterone treatment reduced the potency of BRL 37344 in inhibiting myometrial contraction in day 22. Conventional RT-PCR data suggested no difference in the expression of β_3-adrenoceptor in control and progesterone-treated animals.

Parida *et al.,* (2013) suggested the predominance of β_2- over β_3-adrenoceptor in late pregnant mice by real-time RT-PCR. There were no details about real-time RT-PCR experiments in Parida *et al.,* (2013). The expression of β_3-adrenoceptors was examined by conventional RT-PCR. The representative presented conventional RT-PCR gel shows a large band of primer-dimer along with a band in the desired product size. The specificity and efficiency of the primers used were not validated. The product sizes for the β_2-

adrenoceptor and β_3-adrenoceptor primers were 468 and 473 bp respectively, which are very large to be used in real-time RT-PCR technique. Parida *et al.,* (2013) showed that salbutamol, a selective β_2-adrenoceptor was more potent than SAR150640, a selective β_3-adrenoceptor in relaxing late pregnant mouse myometrium. However, effects of selective β_2- and β_3-adrenoceptor antagonists on salbutamol, and SAR150640 were not tested. Therefore, presence of functional β_2- and β_3-adrenoceptor cannot be confirmed.

Ohashi *et al.,* (1996) showed higher number of β_2-adrenoceptor binding sites in term pregnant when compared to non-pregnant rat myometrium. HSR 81 and ritodrine, selective β_2-adrenoceptor agonists were more potent in relaxing myometrial strips obtained from pregnant than non-pregnant myometrium. Forskolin had the same potency in relaxing the pregnant and non-pregnant myometrium. Ohashi *et al.,* (1996) suggested that the increase in the potency of the β_2-adrenoceptor agonist was due to the higher reserve of β_2-adrenoceptors in the pregnant myometrium and there is no alteration in the other components of adenylyl cyclase signaling pathway. However, selective β_2-adrenoceptor antagonist was not used to confirm the action of terbutaline on β_2-adrenoceptors. Isoprenaline was more potent than the selective β_2-adrenoceptor agonists in inhibiting the spontaneous myometrial contraction, but selective β-adrenoceptor antagonists were not used to check the source of isoprenaline action.

Shinkai *et al.,* (2000) argued the existence of β_2-adrenoceptors in late pregnant rat uterine strips (day 18 and 19 of pregnancy). β_2-adrenoceptor agonists including formoterol and ritodrine relaxed oxytocin-induced myometrial contraction in pregnant rat uterine muscles. The action of formoterol and ritodrine was not verified by selective β_2-adrenoceptor antagonists.

Meisheri *et al.,* (1979), Ikeda *et al.,* (1984) concluded that isoproterenol treatment inhibited the spontaneous myometrial contraction in pregnant rats. In this study, the action of isoproterenol was not validated using selective and non-selective β-adrenoceptor antagonist. In Ikeda *et al.,* (1984) isoproterenol was about 100 times more potent than the selective β_2-adrenoceptor agonist, ritodrine. However, the action of the β-adrenoceptor agonists has not been validated using selective β-adrenoceptor antagonists.

Mihályi *et al.,* (2003) indicated potentiation of terbutaline inhibitory action (potency and efficacy) on electrical field-induced myometrial contraction in term pregnant rats when terbutaline was combined with α_1-adrenoceptor antagonist, WB 4101.

The action of β_2-adrenoceptor agonist, terbutaline, on cervical resistance in non-pregnant and late pregnant rats was investigated by Ga´spa´r *et al.,* (2005). Based on Ga´spa´r *et al.,* (2005) mechanical stretching test, the effect of terbutaline on day 22 pregnant rat cervical resistance was antagonized by propranolol, but the effect of a selective β_2-adrenoceptor antagonist on terbutaline action was not tested. β_2-adrenoceptor protein and mRNA expression in non-pregnant and pregnant rat cervical tissues were investigated using western blotting and conventional RT-PCR. β_2-adrenoceptor protein and mRNA expression were significantly higher in day 18 compared to non-pregnant cervical tissue. β_2-adrenoceptor protein and mRNA were not different through days 18, 20, 21 and 22 of pregnancy. However, western blotting and conventional RT-PCR experiments did not include any proper controls other than loading controls. Ga´spa´r *et al.,* (2005) also investigated G-protein activation following *in vitro* terbutaline treatment using [^{35}S] GTPγS binding assay. Terbutaline decreased [^{35}S] GTPγS binding of pregnant cervixes while did not alter [^{35}S] GTPγS binding in non-pregnant cervixes. This would suggest

uncoupling of β_2-adrenceptors from G-protein during pregnancy to increase the resistance of the cervix only if β_2-adrenceptor antagonist antagonised the effect of terbutaline.

In guinea pigs, McPherson *et al.,* (1984) demonstrated the dominance of β_2- over β_1-adrenoceptors in uterine membrane samples of guinea pigs. Isoprenaline was approximately 400 times more potent than RO363, a selective β_1-adrenoceptor agonist. However, action of both ligands was not verified using selective and non-selective β-adrenoceptor antagonists. Therefore, target for isoprenaline is not known, Also RO363 action on β_1-adrenoceptors cannot be confirmed without testing the effect of selective β_1-adrenoceptor antagonist on RO363 action.

3. The β-adrenoceptors function, signaling pathways and regulation in the uterus:

In this section, articles that studied β-adrenoceptor functional responses in the uterus and investigated the signaling pathways that might influence the response were included.

Ability of β-adrenoceptor agonists to enhance cAMP production and activate cAMP-dependent signaling pathway to elicit myometrial relaxation is extensively studied in the literature. Specifications for the outcomes of papers studied this pathway is described in the following paragraphs:

Litime *et al.,* (1989) studied the effect of isoprenaline on the activity of adenylyl cyclase (AC) enzyme in preterm and term pregnant inner and outer myometrial tissue preparations. In Litime *et al.,* (1989), as presented, isoprenaline increased AC activity in both preterm and term samples. The increase in AC activity was more pronounced in the preterm samples. It was interpreted in the results of Litime *et al.,* (1989) that isoprenaline increases the activity of AC in preterm but not term sample. AC activity was similar in forskolin,

NaF and GPP(NH)P- treated samples in both preterm and term samples, suggesting intact AC and G-protein systems in both stages. Nevertheless, action of isoprenaline was not verified using selective β-adrenoceptor antagonists. Also, statistical analysis was not performed to investigate the significance in the differences between the AC activity in differently treated samples in different stages.

Bardou *et al.,* (2000) demonstrated mRNA expression of β_3-adrenoceptors in human term pregnant myometrium by conventional RT-PCR. Even though, as presented, the conventional RT-PCR conditions of Bardou *et al.,* (2000) were not optimized, functional β_3-adrenoceptors in term pregnant myometrium were identified. In Bardou *et al.,* (2000), selective β_3-adrenoceptor agonists, SR 59119A and SR 59104A, and selective β_2-adrenoceptor agonists, salbutamol and terbutaline, inhibited spontaneous myometrial contractions. All the tested ligands had comparable potencies while the β_3-adrenoceptor agonists had higher efficacies than the tested β_2-adrenoceptor agonists. Propranolol and ICI 118551 did not affect the relaxatory action of SR 59119A and SR 59104A, but significantly inhibited the action of salbutamol and terbutaline. A selective β_3-adrenoceptor antagonist, SR59230A, inhibited the action of SR 59119A and SR 59104A. Both SR 59119A and salbutamol enhanced cAMP level in the myometrial tissues. The action of SR 59119A was not affected by propranolol, but antagonised by SR59230A while the action of salbutamol was antagonised by propranolol and was not affected by SR59230A. Collectively, these data demonstrate the existence of functional β_2- and β_3-adrenoceptors in term pregnant human myometrium and suggest cAMP-dependent signaling pathway in exerting myometrial relaxation by β_2- and β_3-adrenoceptors.

Croci *et al.,* (2007) studied the effect of β_3-adrenoceptor agonists on cAMP production in human uterine smooth muscle cells. Effect of SAR150640, a selective β_3-adrenoceptor agonist, on cAMP production was examined in membrane preparations of the cells. SAR150640 significantly increased production of cAMP compared to the basal level. SAR150640 also inhibited term pregnant myometrial spontaneous activity and inhibited ERK1/2 phosphorylation in human uterine smooth muscle cells. However, all mentioned actions of SAR150640 were not verified using β_3-adrenoceptor antagonist, therefore it cannot be argued that action of SAR150640 was through β_3-adrenoceptors.

Rydtn *et al.,* (1982); Berg *et al.,* (1984); Martin *et al*., (1989); Engelhardt *et al.,* (1997) and Frambach *et al.,* (2005) studied the ability of β_2-adrenocepor agonists to enhance cAMP production in the myometrium. Rydtn *et al.,* (1982) found significantly higher basal cAMP level in myometrial tissues obtained from women who did not receive tocolysis (control group) compared to myometrial tissues obtained from terbutaline for tocolysis. *In vitro* terbutaline treatment was associated with a significant increase in cAMP production in control group but not in the group treated with terbutaline *in vivo* for tocolysis. Estimation of phosphodiesterase activity in both groups revealed higher phosphodiesterase activity (faster cAMP degradation) in the group received terbutaline *in vivo.* The author has suggested increase in phosphodiesterase activity as a mechanism for β_2-adrenoeceptor desensitization.

Berg *et al.,* (1984) concluded that myometrial strips obtained from women received β_2-adrenoceptor agonists for tocolysis had lower AC activity compared to myometrial strips obtained from women did not receive tocolysis (control). Frambach *et al.,* (2005) did not find correlation between gestational age and AC activity in myometrial tissues obtained

from women did not received fenoterol for tocolysis, received fenoterol for less than 48h and received fenoterol for more than 48h. Isoproterenol enhanced AC activity in the control group and the increase in AC activity was significantly less in the samples obtained from women received fenoterol. Forskolin and GTP treatments enhanced AC activity in all the groups in the same level, suggesting intact AC and $G_{\alpha s}$ systems in all groups. Action of isoproterenol was not verified using selective β-adrenoceptor antagonists.

Engelhardt *et al.,* (1997) has identified the mRNA expression of the β_2-adrenoceptors in human myometrium using conventional RT-PCR. Engelhard *et al.,* (1997) concluded that, β_2-adrenoceptor mRNA expression in myometrial tissue obtained from women received fenoterol (β_2-agonist) for tocolysis was not significantly different from β_2-adrenoceptor mRNA expression in myometrial tissues obtained from women who did not receive fenoterol. However, fenoterol-treated samples had significantly lower density of β_2-adrenoceptor binding sites compared to non-treated samples as examined by radioligand binding assay. Moreover, both groups had same levels of β-adrenoceptor kinase (βARK or GRK) mRNA and the activity of βARK was not different between the two groups. The protein expression of $G_{\alpha s}$ and $G_{\alpha i}$ subunits was not different between the groups. Treating the samples of both groups with NaF, GPP(NH)P or forskolin was associated with increase in cAMP levels in all samples and this increase was not different between the groups suggesting intact G-protein and AC systems in all groups. The only difference was observed between *in vivo* fenoterol-treated and untreated myometrial samples was the density of β_2-adrenoceptor binding sites. The ability of β_2-adrenoceptors to couple to G-protein by testing G-protein activity after addition of β_2-adrenoceptor selective agonists and antagonist was not tested in this study.

Bardou *et al.,* (1999) and Verli *et al.,* (2013) studied the effect of β_2-adrenoceptor agonists and phosphodiesterase inhibitors (PDE) on human myometrial contractions. In Bardou *et al.,* (1999), PDE4 inhibitors including rolipram and Ro 20-1724 had higher efficacy than salbutamol and formoterol in relaxing near term myometrial spontaneous activity. Verli *et al.,* (2013) used myometrial strips obtained from preterm and term pregnant women. Terbutaline relaxed oxytocin-induced myometrial contraction and its inhibitory action was potentiated by addition of rolipram (PDE4 inhibitor). Immunoblotting showed higher expression of PDE4B in preterm myometrial samples while PDE4D had higher expression in term pregnant human myometrium. No selective β-adrenoceptor antagonists were used in this study.

Wrzal *et al.,* (2012) studied β-adrenoceptor signaling in hTERT-C3 cells. Wrzal *et al.,* (2012) suggested interaction between oxytocin receptors (OTR) and β_2-adrenoceptors to promote ERK1/2-dependent effect. Isoprenaline and oxytocin both activated ERK1/2 phosphorylation with maximum activation at 5 minutes. $G_{\alpha i}$ and PKC inhibitors both inhibited oxytocin and isoprenaline action. These results suggest that isoprenaline can mediate ERK1/2-dependent effect in hTERT-C3 and this effect involve coupling to $G_{\alpha i}$ and activating PKC. It is not known which subtype of β-adrenoceptors was involved in this pathway since selective agonists and antagonists of β-adrenoceptor subtypes were not used in this study.

Hadi *et al.,* (2013) studied the role of β_3-adrenoceptor in cell proliferation in primary human myometrial cells. Treating myometrial cells with selective β_3-adrenoceptor agonist, SAR150640 for 48h was associated with significant increase in the number of the cells. Phosphorylated ERK1/2 in SAR 150640-treated cells was significantly higher compared

to non-treated cells. ERK1/2 phosphorylation was significantly high at 3,4, 5 minutes and 8h incubations. Propranolol antagonized the action of SAR 150640 on the cell number in higher concentration while did not alter the action of SAR 150640 in low concentration, this was argued as β_3-adrenoceptor effect. However, the effect of a selective β_3-adrenoceptor antagonist was not tested on the action of SAR 150640. ERK1/2 activation by SAR 150640 at 3 minutes was pertussis toxin insensitive while ERK1/2 activation by SAR 150640 at 8h was pertussis toxin sensitive. Also, activation of ERK1/2 at 3 min was inhibited by PKA inhibitor, H89, and the tyrosine kinase (Src) inhibitor, PP2. Activation of ERK1/2 after 8h incubation period with SAR150640 was inhibited by pertussis toxin, suggesting $G_{\alpha i}$ coupling. After 8h of incubation with SAR150640, ERK1/2 activation was Src but not PKA-dependent. Inhibiting either $G_{\alpha s}$ or $G_{\alpha i}$ proteins led to loss in the β_3-adrenoceptor proliferative effect, so both signaling pathways were required.

Hamada *et al.,* (1994) studied the effect of β_2-adrenoceptor activation on potassium channels, the event that cause cell membrane hyperpolarization and smooth muscle relaxation. Hamada *et al.,* (1994) used patch-clamp technique to assess the opening probability of calcium-activated potassium (K_{Ca}) channels and ATP-sensitive potassium (K_{ATP}) channels following ritodrine treatment in pregnant myometrial cells. Ritodrine activated both K_{Ca} and K_{ATP} channels. β_2-adrenoceptor antagonist was not used to verify the action of ritodrine. However, forskolin and GTP treatments activated K_{Ca} channels, suggesting G-protein direct activation for K_{Ca} channels and cAMP-dependent mechanism in the activation of these channels in the myometrial cells. However, PKA but not G-protein activation activated K_{ATP} channels, suggesting PKA-dependent mechanism involved in the activation of K_{ATP} channels. The effect of ritodrine was not directly related

to the suggested mechanisms for opening both kinds of channels. For example, testing the effect of G-protein inhibitor on the effect of ritodrine activation for potassium channels would allow identifying if ritodrine activate these channels through direct G-protein activation. Also, selective antagonist for each biochemical parameter must be included in every test to help identify specific mediators contributing in responses.

Doheny *et al.*, (2006) suggested functional coupling between β_3-adrenoceptors and calcium-activated potassium (K_{Ca}) channels in lower segment of term pregnant myometrial cells. BRL 37344 activated K_{Ca} channels; the effect of PRL 37344 was antagonised by the selective β_3-adrenoceptor antagonist, SR59230a and non-selective β-adrenoceptor antagonist, bupranolol. BRL 37344 increased the whole cell current and this effect was antagonised by selective K_{Ca} channel blocker, IbTX. BRL 37344 relaxed oxytocin-induced and spontaneous myometrial contraction and this effect was attenuated with IbTX. Collectively, the data suggest that BRL 37344 activates β_3-adrenoceptors and mediate myometrial relaxatory effect through, at least in part, activating K_{Ca} channels.

Pearce *et al.*, (2017) studied β_2-adrenoceptor signaling pathway involved in cAMP response element (CRE) activation in human ULTR myometrial cell line. Isoprenaline activated CREB and P38 phosphorylation in time and concentration-dependent manner and its action was significantly decreased by the selective β_2-adrenoceptor antagonist, ICI188,551, suggesting β_2-adrenoceptor dependent phosphorylation. CREB phosphorylation was significantly high at 5, 10, 20 and 30 minutes of incubation with isoprenaline. CREB phosphorylation at 5 minutes was inhibited by PKA inhibitor but not Src and P38 inhibitors. Thus, CREB phosphorylation was PKA-dependent at 5 minutes of isoprenaline incubation period. CREB phosphorylation at 20 minutes of isoprenaline

incubation was not affected by PKA inhibitor while it was antagonised by Src and P38 inhibitors.

In the same study, Pearce *et al.,* (2017) examined the role of β-arrestin2 and β-arrestin3 on isoprenaline mediated CREB and P38 phosphorylation. β-arrestin3 knocking down led to increase in CREB phosphorylation at all time points. While depletion of β-arrestin2 and β-arrestin3 led to decreased CREB phosphorylation at 5 minutes. P38 phosphorylation by isoprenaline (5 minutes) was significantly increased in β-arrestin2-knocked-down cells at 5-minutes while significantly reduced at 10 minutes. Collectively, isoprenaline treatment leads to biphasic CREB phosphorylation; PKA-dependent at 5 minutes incubation and Src, β-arrestin2 and P38-dependent at 20 minutes incubation.

In rats, Johansson *et al.,* (1978); Levin *et al.,* (1980); Tougui *et al.,* (1981); Izumi *et al.,* (1982); Khac *et al.,* (1986); Bramuglia *et al.,* (1992); Bramuglia *et al.,* (2000); Yurtcu *et al.,* (2006) and Kaya *et al.,* (2012) studied the effect of β-adrenoceptor agonists on cAMP production in rat uterine muscles. In Johansson *et al.,* (1978), isoprenaline increased cAMP production and relaxed non-pregnant rat myometrium. cAMP level was maximum after 3 minutes of isoprenaline treatment then started declining; however, the muscle remained relaxed for 60 minutes after isoprenaline treatment.

Levin *et al.,* (1980) demonstrated that uterine muscle treated *in vivo* or *in vitro* with isoprenaline had approximately 50% less β_2-adrenoceptor binding sites compared to vehicle treated rats. AC activity was lower in the uterine muscle preparations obtained from isoprenaline-treated compared to vehicle-treated animals. The effect of isoprenaline was not verified by selective β-adrenoceptor antagonists. Tougui *et al.,* (1981) results were consistent with Levin *et al.* (1980) where isoproterenol treatment led to a reduction in β_2-

adrenoceptor in non-pregnant rat myometrial tissue preparation. However, there was no verification for the action of isoprenaline using selective β-adrenoceptor antagonists.

Izumi *et al.,* (1982) studied effect of ritodrine on cAMP production and contractility of longitudinal and circular rat myometrium through different ages of pregnancy including days 17, 19 and 22 of pregnancy. Izumi *et al.,* (1982) interpreted that at 22 days of pregnancy, ritodrine increased cAMP production and relaxed myometrial strips. Results of longitudinal and circular muscles were similar at day 22. At days 16 and 17 of pregnancy, the longitudinal muscle strips response to ritodrine was not different than that of day 22, but there was no increase in cAMP production in the circular muscles. However, statistical analysis was not performed in this study. Therefore, data interpretation was not valid. Also, action of ritodrine was not verified using a selective β_2-adrenoceptor antagonist.

Khac *et al.,* (1986) suggested cAMP-independent mechanism in addition to the cAMP-dependent pathway responsible for isoproterenol-mediated rat myometrial muscle relaxation. Based on Khac *et al.,* (1986), forskolin caused myometrial relaxation only with large increase in cAMP while isoproterenol caused myometrial relaxation with modest increase in cAMP suggesting another pathway mediated by isoproterenol beside the cAMP pathway. Specific targets for isoproterenol were not known in this study since selective β-adrenoceptor antagonists were not used to verify its action.

Bramuglia *et al.,* (1992) used uterine muscles of virgin rats to examine the effect of clenbuterol pre-treatment on isoproterenol effect on rat uterine muscles. In this study, rats were treated with clenbuterol, β_2-adrenoceptor agonist, *in vivo* then uterine muscle tissues were collected after 24 h. Isoproterenol had less potency and efficacy in clenbuterol-treated compared to untreated rat uterine muscles, while forskolin relaxatory effect was not

different between the 2 groups. Moreover, isoproterenol treatment led to significantly higher cAMP production in untreated compared to clenbuterol-treated rat uterine muscles.

Bramuglia *et al.,* (2000) used membrane preparations of myometrial strips obtained from non-pregnant rats to investigate effect of clenbuterol pre-treatment on the isoproterenol relaxatory action. Bramuglia *et al.,* (2000) results were consistent with Bramuglia *et al.,* (1992). However, in Bramuglia *et al.,* (2000) investigated the differences in β_2-adrenoceptor density between the two groups. Myometrial preparations obtained from clenbuterol-treated rats had significantly lower density of β_2-adrenoceptors compared to myometrial preparation of non-treated rats.

Yurtcu *et al.,* (2006) compared effects of formoterol and BRL37344 on isolated days 19 and 21 pregnant myometrial tissues in terms of contractility, cAMP and GMP production. Formoterol and BRL37344 had the same potencies in relaxing rat myometrial strips but formoterol had higher maximal effect (E_{max}). The action of formoterol on the myometrial tissues was antagonised by ICI118,551, but was not affected by metoprolol, β_1-adrenoceptor antagonist, and the selective β_3-adrenoceptor antagonist, SR 59230A. In contrast, the action of BRL37344 was antagonised by SR 59230A but not by ICI118,551 and metoprolol. Formoterol had no effect on cGMP level while BRL 37344 treatment was associated with a significant increase in cGMP level and this increase was significantly antagonized by a selective β_3-adrenoceptor antagonist. Both formoterol and BRL 37344 significantly increased cAMP and their actions were antagonized by β_2- and β_3-adrenoceptor antagonists respectively. This suggest existence of functional β_2- and β_3-adrenoceptor pregnant rat myometrium.

Kaya *et al.,* (2012) studied the effect of the selective β_3-adrenoceptor agonist, CL 316243, on term pregnant rat myometrial contraction, cAMP and cGMP production. CL 316243 relaxed oxytocin-induced contraction in term pregnant rat myometrial strips. The relaxatory action of CL 316243 was antagonised by the selective β_3-adrenoceptor antagonist, SR 59230A while was not affected by ICI 118,551 and metoprolol. Also, CL 316243 significantly increased cAMP and cGMP levels in comparison to the basal level. CL 316243 effect on cAMP and cGMP levels was antagonised by SR 59230A but not affected by ICI 118,551 and metoprolol. These data suggest that, CL 316243 relaxatory action is mediated, at least in part, through β_3-adrenoceptor activation to cAMP and/or cGMP production.

Johansson *et al.,* (1981) studied mechanisms that affect β-adrenoceptor responsiveness and contribute to β-adrenoceptor desensitization in non-pregnant rat myometrial tissues. In Johansson *et al.,* (1981), rats were received isoprenaline, saline, or nothing for four days then myometrial tissues were collected. Isoprenaline relaxatory action was markedly lower in isoprenaline pre-treated animals when compared to isoprenaline relaxant effect in the control groups. The number of β-adrenoceptor binding sites was about 50% lower in isoprenaline pre-treated animals compared to control groups. The subsequent isoprenaline treatment led to significantly lower cAMP production and lower PKA activity in isoprenaline pre-treated group compared to control groups. AC activity was significantly higher following isoprenaline or NaF treatments in control groups compared to isoprenaline pre-treated group. Finally, phosphodiesterase (PDE) activity was significantly higher in isoprenaline pretreated group compared to control groups when cAMP was used as a substrate, while PDE activity was not significantly different between the groups when

cGMP was used as a substrate for PDE. These data suggested multiple parameters that affect myometrial strips responsiveness to isoprenaline. However, there was no verification for the effect of isoprenaline using selective β_2- and β_3-adrenceptor ligands in this study. The suggested changed parameters need to be investigated during pregnancy in presence and absence of β-adrenoceptor selective agonists and antagonists to help identify specific changed parameters affecting each β-adrenoceptor subtype.

Klukovits *et al.,* (2010) studied the effect of rolipram, a phosphodiesterase 4 (PDE4) inhibitor, on the relaxatory effect of terbutaline on day 20 and day 22 pregnant rat myometrial strips. They also investigated the effect of LPS treatment on rolipram potentiating effect on terbutaline action. Rolipram enhanced the effect of terbutaline in reducing KCl-induced contractions in day 22 pregnant rats. Rolipram potentiated the relaxatory action of terbutaline on myometrial contractions in day 20 LPS-treated rats. Rolipram significantly enhanced cAMP production by terbutaline in day 20 LPS-treated rats and day 22 untreated rats. The effect of rolipram on terbutaline effect on myometrial relaxation and cAMP production have not been investigated in day 20 untreated rats. Therefore, it can only be concluded that rolipram potentiated the effect of terbutaline on myometrial relaxation and cAMP production on day 22 of pregnancy in rat. Effect of LPS treatment on rolipram need to be further investigated. The effect of selective β_2-adrenoceptor antagonist on terbutaline action must be tested to suggest β_2-adrenoceptor mediated response.

Le´crivain *et al.,* (1998) investigated mechanisms that might involve β-adrenoceptor-induced adenylyl cyclase desensitization in late pregnant rat myometrial tissues. In Le´crivain *et al.,* (1998), rats were treated with isoproterenol *in vivo* and myometrial tissues

were collected after 1 or 76h after isoproterenol treatment initiation (multiple treatments). AC activity was significantly lower after isoproterenol, forskolin and GTP in 76h isoproterenol treated animals compared to control animals (Day 21 of pregnancy). Pertussis toxin abolished the effect of isoproterenol on the AC activity. The number of β_2-adrenoceptor binding sites was significantly lower in 76h isoproterenol-treated samples compared to control samples (non-treated). Samples from animals were treated by isoproterenol for 76h expressed significantly higher $G_{\alpha i}$ and $G_{\alpha s}$ proteins. It was suggested that AC desensitization in the studied model occur because of the β_2-adrenoceptor uncoupling and increased $G_{\alpha i}$ activity. However, this interpretation cannot be made because: AC activity was also reduced by forskolin treatment, increase in $G_{\alpha i}$ expression does not necessarily mean increase in its activity, effect of $G_{\alpha s}$ inhibitor was not tested and the action of isoprenaline was not verified using selective β_2-adrenoceptor antagonist.

Ga´spa´r *et al.,* (2005) demonstrated the effect of progesterone on β_2-adrenoceptor modulated G-protein activating effect in uterine strips of pregnant rats. In Ga´spa´r *et al.,* (2005)-2, terbutaline potency and efficacy in relaxing uterine strip were decreasing toward term (days 15,18, 20 and 22). Progesterone level was significantly lower in day 18 compared to day 15 and significantly lower in day 22 compared to day 18 of pregnancy. The number of β_2-adrenoceptor binding sites was significantly higher in day 22 compared to day 20 pregnant myometrium while no differences in mRNA expression of β_2-adrenoceptors were observed. Progesterone treatment led to increase in the density of β_2-adrenoceptor binding sites but not the mRNA level. Progesterone also shifted concentration-response curve of terbutaline in relaxing myometrial strips in day 22 to the right (increased both potency and efficacy). Activation of G-protein by terbutaline was

decreasing toward term and it was below the basal level ([^{35}S] GTPγS without stimulation) in day 22 of pregnancy. Progesterone treatment increased G-protein activating action of terbutaline. This study suggested pregnancy-induced decrease in the activity of G-protein and progesterone ability to restore the G-protein activity in rats.

Engstrøm *et al.,* (1997) and Klukovits *et al.,* (2004) suggested β-adrenoceptor modulated regulatory effect on oxytocin receptors as a mechanism of β-adrenoceptor desensitization process. Engstrøm *et al.,* (1997) found a significant increase in the mRNA expression and number of binding sites of oxytocin receptors in myometrial tissues isolated from isoproterenol-treated compared to vehicle-treated non-pregnant rats. Oxytocin efficacy, but not potency in generation myometrial contractions was higher in isoproterenol-treated rats compared to vehicle-treated rats. Klukovits *et al.,* (2004) results were consistent with Engstrøm *et al.,* (1997). Klukovits *et al.,* (2004) examined the effect of fenoterol and hexoprenaline on the expression of oxytocin receptors in term pregnant rats. Myometrial tissues obtained from fenoterol or hexoprenaline-treated animals had significantly higher oxytocin receptor mRNA expression. Oxytocin efficacy, but not potency in generating myometrial contractions was significantly higher in fenoterol-treated animals compared to control group. The specific mechanism for oxytocin receptor expression and action differences after β-adrenoceptor agonists stimulation were not speculated.