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**Regulation Of Myometrial Gene Expression By  
Oestrogen And Progesterone: The Use Of A Cell  
Culture Model To Mimic The *In Vivo* Conditions  
Of Pregnant Human Uterus**

*A thesis submitted to NUI – Galway in fulfilment of the requirement for the  
degree of*

**Doctor of Philosophy**

**By**

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**National Centre for Biomedical Engineering Science, NUI – Galway**

**Thesis Supervisor: Prof. Terry J Smith**

**Date of Submission: September 2012**

आचार्यात् पादमादत्ते पादं शिष्यः स्वमेधया ।  
सब्रह्मचारिभ्यः पादं पादं कालक्रमेण च ॥

*Āchāryath pādamādathé pādam sishya swamedhaya /  
Sa bhrahmachāribhya pādam pādam kālakramena cha //*

The first quarter of the knowledge is passed by the teacher

Students must themselves gain the second quarter

Next quarter is gained from peers and colleagues

Experience teaches the last quarter

## Table of Contents

<b>Abstract</b>	<b>i</b>
<b>Acknowledgement</b>	<b>ii</b>
<b>Abbreviations</b>	<b>iii</b>
<b>List of tables</b>	<b>vi</b>
<b>List of figures</b>	<b>viii</b>
<b>Chapter 1 - Introduction</b>	
<b>1.1 Pregnancy</b>	<b>2</b>
<b>1.2 Female reproductive system</b>	<b>2</b>
<b>1.3 Fertilisation</b>	<b>4</b>
<i>1.3.1 Blastocyst development</i>	5
<i>1.3.2 Embryo development</i>	6
<i>1.3.3 Foetal and placental development</i>	6
<b>1.4 Gestation length</b>	<b>7</b>
<i>1.4.1 Preterm (premature) delivery</i>	8
<i>1.4.2 Post-term (postmature) delivery</i>	9
<b>1.5 Utero-placental tissues in pregnancy</b>	<b>9</b>
<i>1.5.1 Amnion</i>	9
<i>1.5.2 Chorion</i>	10
<i>1.5.3 Placenta</i>	10
<i>1.5.4 Cervix</i>	11
<i>1.5.5 Decidua</i>	11
<i>1.5.6 Myometrium</i>	12

1.5.6.1 Smooth muscle and its structure	12
1.5.6.2 Uterine smooth muscle contraction and relaxation	13
<b>1.6 Hormones and peptides in pregnancy and labour</b>	<b>16</b>
<b><i>1.6.1 Oxytocin (OT)</i></b>	<b>17</b>
<b><i>1.6.2 Prostaglandins (PG)</i></b>	<b>18</b>
<b><i>1.6.3 Corticotropin-releasing hormone (CRH)</i></b>	<b>19</b>
<b><i>1.6.4 Relaxin (RLX)</i></b>	<b>21</b>
<b><i>1.6.5 Oestrogen</i></b>	<b>22</b>
1.6.5.1 Oestrogen synthesis during pregnancy	22
1.6.5.2 Oestrogen levels during parturition	25
1.6.5.3 Role of oestrogen in human parturition	26
<b><i>1.6.6 Progesterone</i></b>	<b>29</b>
1.6.6.1 Progesterone synthesis during human pregnancy	30
1.6.6.2 Changes in the progesterone levels at parturition	30
1.6.6.3 Role of progesterone in human parturition	31
<b>1.7 Steroid hormone receptor (SHR) dynamics during pregnancy and labour</b>	<b>32</b>
<b><i>1.7.1 Oestrogen receptors (ER)</i></b>	<b>35</b>
<b><i>1.7.2 Progesterone receptors (PR)</i></b>	<b>35</b>
<b>1.8 Parturition</b>	<b>37</b>
<b><i>1.8.1 Phases of parturition</i></b>	<b>38</b>
<b><i>1.8.2 Parturition in humans and other animals</i></b>	<b>38</b>
<b>1.9 Functional genomic studies of pregnant and labouring human myometrium</b>	<b>41</b>
<b>1.10 Uterine smooth muscle cell culture system</b>	<b>47</b>
<b>1.11 Aims of Study</b>	<b>49</b>

## Chapter 2 – Materials and Methods

<b>2.1 Materials</b>	51
<b>2.2 Methods</b>	51
<b>2.2.1 Culture of human uterine smooth muscle cells</b>	51
<b>2.2.2 Cryopreservation</b>	52
<b>2.2.3 Resuscitation</b>	52
<b>2.2.4 Preconditioning the cells for serum starvation</b>	52
<b>2.2.5 Serum starvation</b>	52
<b>2.2.6 17<math>\beta</math>-Estradiol (E2) treatment</b>	53
<b>2.2.7 Progesterone (P4) and Mifepristone (RU486) treatment</b>	55
<b>2.2.8 Cell lysate preparation</b>	57
<b>2.2.9 Protein assay</b>	57
<b>2.2.10 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)</b>	57
<b>2.2.11 Western blotting</b>	58
<b>2.2.12 Isolation of RNA</b>	60
<b>2.2.13 Quantitation of RNA</b>	60
<b>2.2.14 Quality control of RNA</b>	61
<b>2.2.15 cDNA synthesis</b>	61
<b>2.2.16 Polymerase chain reaction (PCR)</b>	62
<b>2.2.17 Agarose gel electrophoresis</b>	63
<b>2.2.18 Quantitative real-time PCR</b>	63
<b>2.2.19 TaqMan small RNA assay</b>	65
<b>2.2.20 Microarray analysis</b>	66
2.2.20.1 First strand synthesis	67
2.2.20.2 Second strand cDNA synthesis and purification	68
2.2.20.3 <i>In vitro</i> transcription to synthesise amino allyl-modified	

aRNA	68
2.2.20.4 Amino allyl-modified aRNA purification	69
2.2.20.5 Amino allyl-modified aRNA quality control	69
2.2.20.6 Amino allyl-modified aRNA labelling	69
2.2.20.7 Labelled amino allyl-modified aRNA purification	69
2.2.20.8 Labelled amino allyl-modified aRNA fragmentation	70
2.2.20.9 Pre-hybridisation, hybridisation and washing	70
2.2.20.10 Scanning	72
2.2.20.11 Image analysis	72
2.2.20.12 GeneSpring GX analysis	74
<b>2.2.21 Gene Ontology analysis</b>	75
<b>2.2.22 Pathway analysis</b>	76
<b>2.2.23 Statistical analysis</b>	77

## **Chapter 3: Characterisation and Optimisation of Human Uterine Smooth Muscle Cell Culture System**

<b>3.1 Overview</b>	79
<b>3.2 Results</b>	80
<b>3.2.1 Characterisation of hUtSMCs</b>	80
<b>3.2.2 Expression of ER-<math>\alpha</math> in hUtSMCs</b>	82
<b>3.2.3 Assaying the responsiveness of hUtSMC to E2 treatment</b>	83
3.2.3.1 Effect of E2 treatment on CD38 expression	83
3.2.3.2 Effect of E2 treatment on CX43 expression	86
3.2.3.3 Estimation of optimal E2 dose for hUtSMC treatment	88

3.2.3.4 Optimisation of hUtSMC cell culture conditions	89
3.2.3.5 Effect of progesterone and mifepristone treatment on BCL2 gene expression	93
<b><i>3.2.4 Analysis of the quality of RNA samples</i></b>	94
<b><i>3.2.5 Quality control of labelled RNA</i></b>	99
<b>3.3 Discussion and Conclusion</b>	102
<b>Chapter 4: Effect of 17<math>\beta</math>-Estradiol on Gene Expression in Cultured Human Uterine Smooth Muscle Cells: Global Transcriptomic Profiling</b>	
<b>4.1 Overview</b>	105
<b>4.2 Results</b>	106
<b><i>4.2.1 Analysis of the global gene expression profile of hUtSMC upon E2 treatment</i></b>	106
<b><i>4.2.2 Time course analysis of differentially expressed genes identified after E2 treatment</i></b>	108
<b><i>4.2.3 Gene Ontology analysis</i></b>	112
<b><i>4.2.4 Real-time RT-PCR validation of microarray results</i></b>	117
<b><i>4.2.5 Comparison of the differentially expressed genes identified in the present study with oestrogen responsive genes reported in the ERGDB</i></b>	118
<b>4.3 Discussion</b>	121
<b>4.4 Conclusions</b>	137

## **Chapter 5: Developing a Cell Culture Model System to Understand the Transcriptomic Effect of Oestrogen and Progesterone Treatment on Cultured Uterine Smooth Muscle Cells to Study Myometrial Functions During Human Pregnancy**

<b>5.1 Overview</b>	140
<b>5.2 Results</b>	141
<b><i>5.2.1 Developing a cell culture model system mimicking labouring and non-labouring human myometrium</i></b>	141
<b><i>5.2.2 Effect of progesterone treatment and functional progesterone withdrawal on gene expression in cultured hUtSMCs</i></b>	143
5.2.2.1 Effect of progesterone treatment on gene expression in cultured hUtSMCs	143
5.2.2.2 Effect of functional progesterone withdrawal using RU486 on gene expression in cultured hUtSMCs	145
5.2.2.3. Comparison between progesterone-responsive genes and functional progesterone withdrawal responsive genes	149
<b><i>5.2.3 Mimicking steroid hormone condition in pregnant and non-pregnant myometrium – (P4+E2 vs. Control)</i></b>	149
<b><i>5.2.4 Mimicking steroid hormone conditions in the labouring myometrium – (P4+E2+RU486 vs. P4+E2)</i></b>	156
<b><i>5.2.5 Comparison of differentially expressed genes identified in cultured hUtSMCs between mimicking pregnant myometrium and labouring myometrium</i></b>	165
<b>5.3 Discussion</b>	166
<b>5.4 Conclusions</b>	183

<b>Chapter 5 – Conclusion and Future Direction</b>	186
<b>Chapter 6 – Bibliography</b>	195
<b>Appendix – 1</b>	239
<b>Appendix – 2</b>	244
<b>Appendix – 3</b>	266

## Abstract

Pregnancy and labour are physiological events controlled by a complex interplay between maternal and foetal factors. These processes are diverse and specific to each species, hence simple extrapolation from model organisms to humans is challenging. Nonetheless, understanding the underlying regulatory mechanisms involved is of utmost importance in the diagnosis and treatment of preterm and post-term labour. Throughout pregnancy the uterus maintains a quiescent state and develops a strong and rhythmic contraction towards parturition. Progesterone and oestrogen play vital roles in this transformation. In most mammals, high concentrations of progesterone dominate pregnancy (pro-relaxatory), while oestrogen (pro-contraction) levels take over towards labour. However, in humans the circulating levels of both progesterone and oestrogens remain high throughout pregnancy. Hence, functional withdrawal of progesterone and responsiveness to oestrogen is believed to occur more at the molecular level than at the circulating level. This study, therefore, focused on developing a myometrial smooth muscle cell culture model to evaluate the transcriptomic effect of steroid hormone treatment on cultured human uterine smooth muscle cells (hUtSMCs).

Microarray-based gene expression analysis was performed on RNA isolated from hUtSMCs, treated with  $17\beta$ -estradiol (E2) and progesterone (P4) (separately or in combination) in addition to functional withdrawal of P4 with mifepristone (RU486) in order to identify differentially expressed genes associated with each treatment. Functional P4 withdrawal by RU486 altered the expression of these genes favouring myometrial contraction, whereas (P4+E2)-treatment favoured myometrial quiescence. The findings of these studies are in line with published reports on labouring and non-labouring myometrium across various species, confirming that the cultured cell model system developed in the present study responds to hormonal treatments in a similar manner to that of the myometrium *in vivo*. The myometrial cell culture system is therefore an additional research tool that can be used for functional studies of human pregnancy and labour.

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## Abbreviations

ACTH	Adrenocorticotropic hormone
AF	Activation function element
ALLN	N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine
APS	Ammonium persulfate
b2-AR	b2-adrenergic receptor
BKCa	Ca <sup>2+</sup> -activated K <sup>+</sup> channel
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium ions
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CAP	Contraction associated proteins
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	Complimentary DNA
cGMP	Cyclic guanosine monophosphate
ChIP	Chromatin immunoprecipitation
CO <sub>2</sub>	Carbon dioxide
COX1	Cyclo-oxygenase type 1
COX2	Cyclo-oxygenase type 2
CRH	Corticotropin-releasing hormone
CRHBP	CRH binding protein
CS-FBS	Charcoal stripped foetal bovine serum
Cy3	Cyanine3
Cy5	Cyanine5
DCS-FBS	Double charcoal stripped foetal bovine serum
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
E2	17β-Estradiol
EDD	Expected delivery date

EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER- $\alpha$	Oestrogen receptor-alpha
ER- $\beta$	Oestrogen receptor-beta
FBS	Foetal bovine serum
FSH	Follicle-stimulating hormone
GPCR	G-protein coupled receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hanks' balanced salt solution
HSC70	Heat Shock Cognate protein 70 kDa
hUTSMC	Human uterine smooth muscle cells
IVH	Intraventricular haemorrhage
K <sup>+</sup>	Potassium ion
LH	Luteinizing hormone
LNMP	Last normal menstrual period
M	Molar
MgCl <sub>2</sub>	Magnesium chloride
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRNA	messenger Ribonucleic acid
Na <sup>2+</sup>	Sodium ion
NaCl	Sodium chloride
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NP-40	Nonyl phenoxy polyethoxy ethanol
OD	Optical density
OTr	Oxytocin receptor
P4	Progesterone
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCOS	Polycystic ovary syndrome
PGE2	Prostaglandin E2
PGF2a	Prostaglandin F2a
PR-A	Progesterone receptor A

PR-B	Progesterone receptor B
PR-C	Progesterone receptor C
PRF-DMEM	Phenol red free Dulbecco's modified Eagle medium
qPCR	Quantitative PCR (Real-time PCR)
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RU486	Roussel Uclaf – 486 (Mifepristone)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethyl- ethane-1,2-diamine
T <sub>m</sub>	Melting temperature
UCP	Uncoupling protein

All genes are referred by their official gene symbol approved by HUGO Gene Nomenclature Committee (HGNC)

<b>List of Tables</b>		
<b>Table no.</b>	<b>Title</b>	<b>Page no.</b>
1.1	Published microarray studies in human myometrium during pregnancy and labour	46
2.1	Treatments and corresponding <i>in vivo</i> hormone conditions mimicked	55
2.2	Preparation of SDS-PAGE running gel	58
2.3	Preparation of SDS-PAGE stacking gel	58
2.4	Primary antibodies and working dilutions	60
2.5	PCR reaction setup	62
2.6	Thermocycler program settings	62
2.7	Genes selected for PCR reaction	63
2.8	Quantitative real-time PCR setup	64
2.9	ABI StepOne Plus real-time PCR system program settings	64
2.10	Genes verified by quantitative qPCR	64
2.11	TaqMan® Small RNA Assays RT reaction setup master mix	65
2.12	Thermal cycler parameters for miRNA RT reaction.	65
2.13	TaqMan® Small RNA Real-time RT-PCR Assays reaction setup master mix.	66
2.14	miRNAs whose expression was verified by quantitative real-time PCR	66
2.15	ABI StepOne Plus real-time PCR system program settings for miRNA	66
2.16	Reverse transcription master mix	68
2.17	Second strand master mix	68
2.18	IVT master mix	69
2.19	Post-hybridisation washing solutions	72
3.1	RIN values of all RNA samples used for microarray experiments	96
4.1	Summary of genes with altered expression (>5.0-fold) following E2 treatment	107
4.2	Differentially expressed genes common to all timepoints (6, 24 and 72 h) post E2 treatment	110
4.3	Summary of enriched GO biological process terms identified under muscle contraction	114
4.4	Summary of enriched GO terms identified in data enrichment analysis	115
4.5	Summary of qPCR validation of microarray results	117
4.6	Comparison of E2-responsive genes from hUtSMCs with the ERGDB	119
5.1	Treatment condition and abbreviations explained	142
5.2	Table summarising the microarray results	142

5.3	Summary of genes with altered expression (>2.5-fold) following P4 treatment	144
5.4	Summary of genes with altered expression (>2.5-fold) following functional P4 withdrawal	146
5.5	Comparison of expression profiles of common genes between P4 treatment and functional progesterone withdrawal	149
5.6	Summary of genes with altered expression (>5-fold) following P4+E2 treatment	150
5.7	Summary of genes with altered expression (>5-fold) following P4+E2+RU486 treatment	156
5.8	Summary of PCR validations	161
5.9	Summary of expression profile of common genes differentially expressed genes identified in cultured hUtSMCs mimicking pregnant myometrium versus labouring myometrium	165

<b>List of Figures</b>		
<b>Figure no.</b>	<b>Title</b>	<b>Page no.</b>
1.1	Anatomy of the human female reproductive organ	4
1.2	The processes of ovulation and fertilisation	5
1.3	Schema depicting different stages of pregnancy	7
1.4	Schema of the mechanism of smooth muscle contraction and relaxation	16
1.5	Structural formula of various oestrogens	23
1.6	Steroidogenesis pathway of oestrogens and progesterone	23
1.7	Circulating levels of unconjugated steroids in the maternal system during different stages of pregnancy	26
1.8	Structural formula of progesterone and RU486	30
1.9	Steroid hormone receptor family	34
1.10	Model for the role of the myometrial ER and PR in regulating human pregnancy and parturition	37
1.11	Proposed mechanism of labour initiation at term	41
2.1	Experiment workflow for 17 $\beta$ -estradiol (E2) treatment	54
2.2	Experiment workflow for progesterone, 17 $\beta$ -estradiol and RU486 treatment	56
2.3	Workflow of microarray experiment	73
3.1	Characterisation of smooth muscle cells	81
3.2	Analysis of the morphology of human uterine smooth muscle cells	82
3.3	Assay of ER- $\alpha$ expression in hUtSMCs	83
3.4	Induction of CD38 mRNA and protein expression by E2 treatment	85
3.5	Protein and transcript expression of Connexin43 upon E2 treatment	87
3.6	Dose response curve indicating an increase in CD38 mRNA expression upon E2 treatment	89
3.7	Demonstration of the oestrogenic effect of serum and Phenol Red in the media	91
3.8	Effect of hormonal starvation of hUtSMC cells prior to E2 treatment on CD38 expression	92
3.9	Effect of progesterone and mifepristone treatment on BCL2 gene expression	94
3.10	Examples of low and high quality RNA samples from hUtSMC	97
3.11	Electropherogram of RNA for quality assessment	98
3.12	Quality analysis of RNAs used in microarray experiments	99
3.13	Bioanalyzer analysis of amplified and labelled RNA shows even distribution of Cy5 dye	100
3.14	The Bioanalyzer electropherogram of the labelled RNA (P4 treatment)	101
4.1	Venn diagram comparing the differentially expressed genes between 6, 24 and 72 h post E2 treatment	109

4.2	Expression profile of the 15 genes identified common to all time points (6, 24 and 72 h) post E2 treatment	111
4.3	Enriched GO biological processes identified following E2 treatment of hUtSMCs	113
4.4	Real-time RT-PCR validation of differentially expressed genes following 72 h of E2 treatment.	118
5.1	Enriched GO biological processes identified following progesterone treatment of hUtSMCs	145
5.2	Enriched GO biological processes identified following functional progesterone withdrawal	147
5.3	Validation of microarray results	148
5.4	Enriched GO biological processes identified following P4+E2 combined treatment of hUtSMCs	151
5.5	qPCR validation of differentially expressed genes following P4+E2 treatment	152
5.6	Apoptosis and survival pathway by antiapoptotic action of membrane bound ESR1 identified by MetaCore pathways analysis	154
5.7	Figure legend for the pathway provided by MetaCore	155
5.8	Enriched GO biological processes identified following P4+E2+RU486 treatment of hUtSMCs	158
5.9	qPCR validation of differentially expressed genes following P4+E2+RU486 treatment	160
5.10	Muscle contraction by oxytocin signalling in uterus and mammary gland identified by pathways analysis	163
5.11	Immune and inflammatory response pathway identified by pathways analysis –MIF – the neuroendocrine – macrophage connector	164

# **Chapter 1: Introduction**

### **1.1 Pregnancy**

Pregnancy is defined as the process of carrying one or more developing embryo or spermatozoa forming a zygote and ends once the foetus and the placenta are expelled out of the uterus (Mahendroo, 2012). The gestation length, physiological and molecular events leading to foetal development, maturation and subsequently labour vary among species. Throughout this thesis emphasis is given to human pregnancy and parturition unless otherwise specified.

### **1.2 Female reproductive system**

The internal female reproductive organs are the vagina, the uterus and the ovaries (Figure 1.1). The vagina is the female copulatory organ - a fibromuscular tube that connects the external genitals to the cervix of the uterus (O'Connell et al., 2008). In the reproductive process, the vagina accepts the penis and semen during sexual intercourse and also acts as a passageway for the expulsion of the menstrual blood and the foetus.

The uterus is the major female reproductive organ, located deep within the pelvis. It is in this pear-shaped thick muscular organ that foetal development occurs during gestation. The uterus in the non-pregnant state is approximately 7.5 cm long and at the top (the widest part) is approximately 5 cm wide. The thickness of the wall varies from 2 – 2.5 cm and the weight ranges between 30 and 40 g. The uterus consists of three main segments, the cervix – a conical neck that connects the uterus to the vagina; the isthmus – the lower uterine segment and the corpus uteri – the main body of the uterus (Chapter 6: Mader, 1992). The reproductive role of the uterus is to accept the fertilised ovum, to protect and nourish the developing foetus during gestation and eventually expel the foetus during parturition (Blanks et al., 2007; Impey and Boylan, 1999).

The three tissue layers that constitute the uterus are the endometrium, the myometrium and the perimetrium. The endometrium is the inner lining of the uterus and if fertilisation fails to occur this lining periodically sheds in a process called menstruation. If the egg is fertilised the endometrium forms the decidua, a modified

## ***Chapter 1: Introduction***

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mucous membrane that lines the uterine wall. This membrane is rich in hormones, cytokines and peptides that can regulate uterine contractility. The decidua is shed at parturition along with the foetus. The myometrium is the middle layer of the uterus, whose major constituent is smooth muscle. This is the most active layer during pregnancy, especially during parturition. The loose surrounding tissue around the uterus is called the perimetrium. Externally, the uterus is covered by peritoneum, to which the uterine arteries supply the blood. The endometrial and myometrial layers receive the blood from this lining. During pregnancy, the uterus undergoes vast changes in shape and consistency to accommodate the growing foetus. Hypertrophy, where the organ or tissue size increases due to the enlargement of the constituent cells, and hyperplasia, where organ or tissue size increases due to an increase in the cell proliferation rate of the myocytes, are the two main mechanisms by which the uterus accommodates the growing foetus. This transforms the uterus into a thin-walled muscular organ capable of accommodating the growing foetus, placenta and the amniotic fluid. After parturition, the uterus shrinks and returns to its original size and shape (Devedeux et al., 1993).

In normal cases, there are two ovaries in women that are situated on either side of the uterus. Their function is to secrete female sex hormones (oestrogens, luteinising hormone (LH), follicle-stimulating hormone (FSH) and progesterone) and the ova (egg) (Adashi, 1994). The rest of the female reproductive organs only play a role in transporting and nurturing the eggs or the developing foetus. The ovarian follicles, where the eggs develop, rupture after maturation and eject the eggs to the fallopian tube in a process known as ovulation. Ovulation can occur randomly from either of the ovaries and generally happens during the middle of the menstrual cycle (14 days).

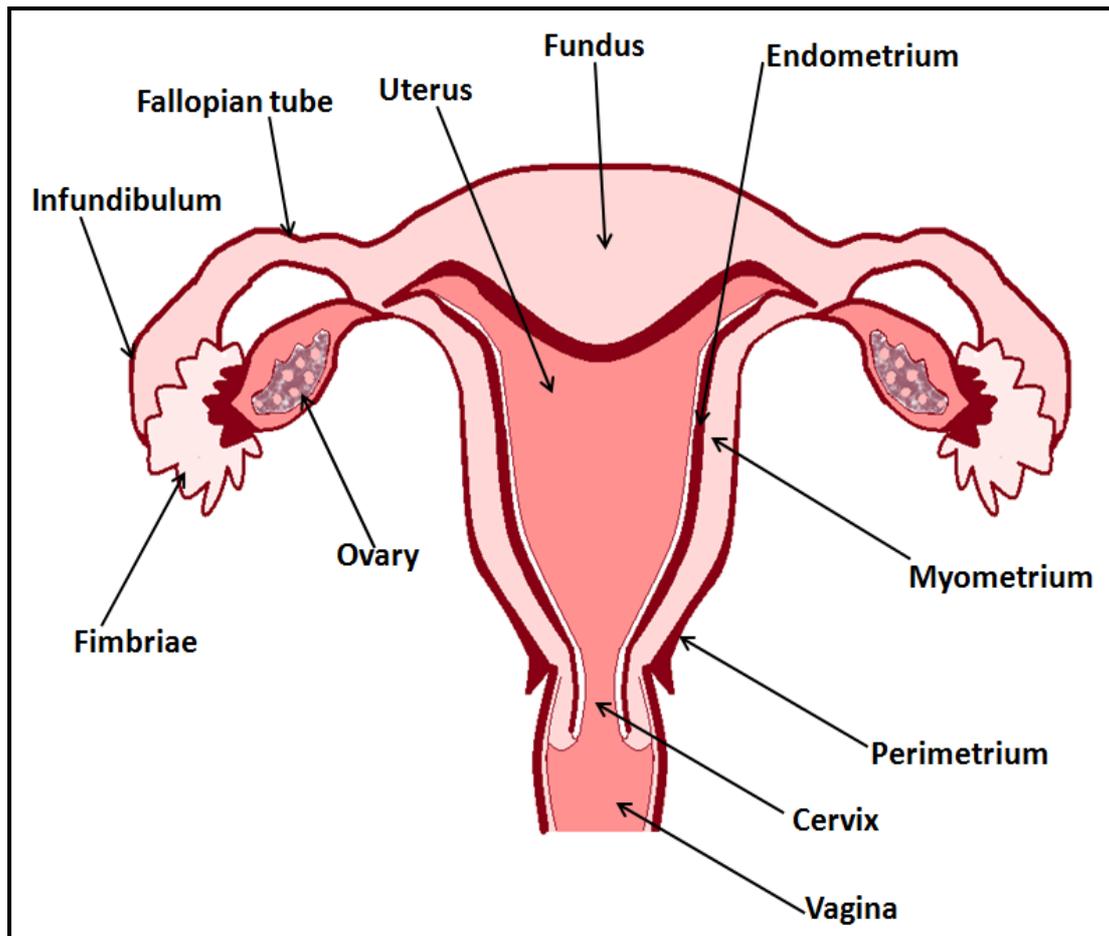
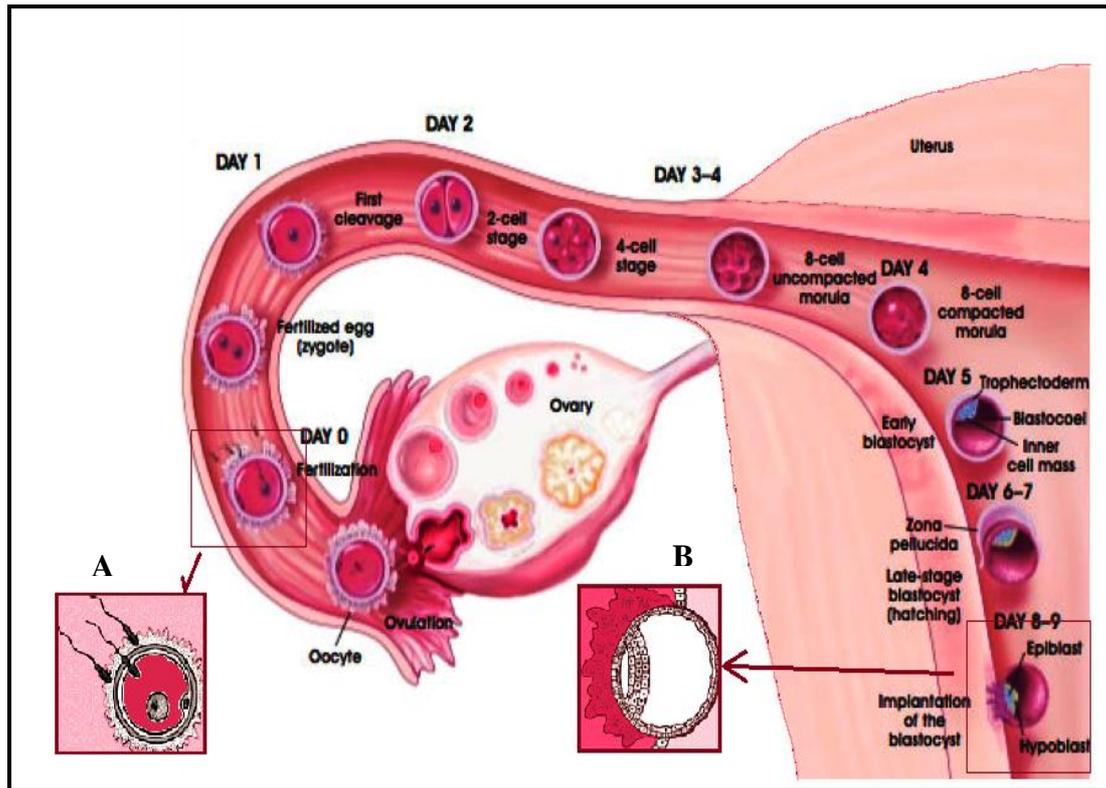


Figure 1.1: Anatomy of the human female reproductive organ. Image adapted from (Chapter 6: Mader, 1992).

### 1.3 Fertilisation

During ovulation the ovary releases the ovum or egg, which travels down the oviducts (fallopian tubes) to the uterus. Fertilisation occurs when a spermatozoan fuses with the ovum, usually in the fallopian tube, producing a zygote. After fertilisation, the zygote is propelled down to the uterus where it implants itself and develops into a foetus (Figure 1.2). If fertilisation fails to occur the ovum is expelled in the next menstrual cycle. In a successful pregnancy a zygote passes through various stages of development namely, blastocyst, embryo and foetus before the fully developed foetus is ejected out of the uterus during labour (De Jonge, 1998; Pepperell, 1997).



**Figure 1.2: The processes of ovulation and fertilisation.** The ovary produces the egg that falls into the fallopian tube and travels through the tube to the uterus. Usually the egg fuses with the sperm in the fallopian tube, a process known as fertilisation. After fertilisation, the zygote starts dividing and travels through the fallopian tube to the uterus, where it attaches itself to develop into a foetus. In the inset shown are (A) the sperms fertilising the egg and (B) the blastocyst attaching itself to the uterine wall. Image adapted from Encyclopaedia Britannica (<http://media-2.web.britannica.com/eb-media/98/26998-004-554F159B.jpg>) and stem cell information (<http://stemcells.nih.gov/info/scireport/appendixa>).

### 1.3.1 Blastocyst development

After fertilisation the single celled zygote divides through mitosis to produce two genetically identical daughter cells. This process takes about 10 to 15 hours. Cell division continues and by the third day after fertilisation a ball of cells is formed called a blastocyst. By definition the blastocyst is a mammalian conceptus in post-morula stage consisting of an embryoblast (inner cell mass), a central fluid-filled blastocyst cavity and a trophoblast (a layer of cells surrounding the embryoblast and the blastocyst cavity). The blastocyst wall is one cell thick except for one region where it can be three or four cells thick. This inner cell mass separates and forms three distinct layers – the ectoderm, the mesoderm and the endoderm. The ectoderm later forms including hair, skin and sweat glands, the mesoderm forms including blood, bones and muscles, and the ectoderm forms the internal organs such as heart,

## ***Chapter 1: Introduction***

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lung, liver and intestine. The outer cells in the blastocyst attach to the uterine wall, forming the placenta. Some cells from the placenta develop to form the chorion (outer membrane) and the amnion (inner membrane) a membranous covering around the developing embryo. Implantation of the blastocyst occurs within 10 days of fertilisation (Foulk, 2001).

### ***1.3.2 Embryo development***

A conceptus within eight weeks of fertilisation (10 weeks of pregnancy) is referred to as an embryo. The cells in the embryo multiply taking on specialised functions in a process called differentiation. The organ development in the conceptus begins about three weeks after fertilisation. The neural tube, the precursor of the central nervous system is the first to begin development, later it will form brain and spinal cord. The heart and major blood vessels also start to develop around 20 days of embryo development. Within 8 weeks after fertilisation most of the organs are completely formed in the embryo. Most birth defects occur during this stage, making it the most vulnerable period during pregnancy (Brinster, 1974; Motta et al., 1995).

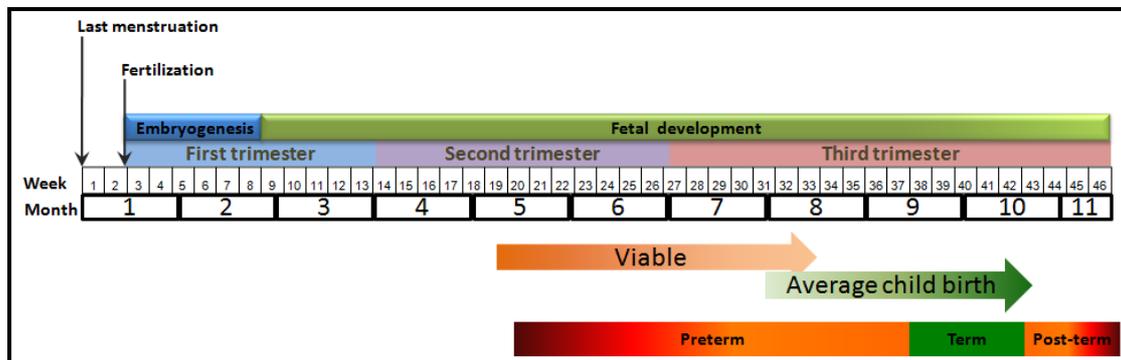
### ***1.3.3 Foetal and placental development***

By the end of 8 weeks after fertilisation the embryo is considered a foetus. By this stage the embryo has a recognisable shape. By 12 weeks the foetus fills the uterus and at about 21 weeks the mother starts to feel the foetus move. The development and maturation of the organs continue throughout pregnancy. After about 25 weeks of development, the foetus prepares for *ex utero* viability by improving the brain, muscle and lung function, thereby increasing the chance of foetal survival when labour occurs.

During pregnancy the placenta develops more villi (finger-like appendages) that attach to the uterine wall, increasing the area of surface contact with the uterus. This helps in the exchange of nutrients and waste materials between the foetus and the mother, a vital process during foetal development. The placenta is fully developed by 20 weeks after its formation, and continues to grow through pregnancy (Brinster, 1974; Motta et al., 1995).

## 1.4 Gestation length

In humans, the expected delivery date (EDD) is about 38 weeks from conception, which is approximately 40 weeks from the last normal menstrual period (LNMP), assuming a regular 28-day menstrual cycle. The World Health Organization defines term delivery as childbirth between 37 and 42 weeks (between 259 and 294 days). Delivery before 37 weeks or 259 days is considered preterm (earlier known as premature delivery) and after 42 weeks or 294 days as post-term (postmature delivery) (Kieler et al., 1995) (Figure 1.3).



**Figure 1.3: Schema depicting different stages of pregnancy.** Weeks/months of gestation are indicated by numbers (1 to 46 weeks and 1 to 11 months). The last menstruation represents menstruation cycle prior to fertilisation. Upon successful fertilisation the mother pauses menstruation cycles (amenorrhoea) (Master-Hunter and Heiman, 2006) until a few weeks after labour. The trimesters are based on maternal changes, which are shown below. Underneath the time-line the viability of the foetus when pregnancy occurs is represented. Preterm, term and post-term labour is marked in red/orange and green bars. Here intensity of the red colour denotes the high risk of neonatal morbidity and mortality with the green colour representing the average time for mature childbirth. Image adapted from (Tucker and McGuire, 2004) and (Cardonick and Iacobucci, 2004).

In relation to the maternal development and changes, pregnancy is divided into three trimesters. The embryo attaches itself to the uterus and the initial development of the embryo occurs in the first trimester. In the second trimester (months 4 to 6) the uterus expands considerably and the foetus takes a recognisable shape. The placenta becomes fully functional during this trimester. The final trimester (months 7 to 9) is when the foetus undergoes active growth. Towards the end of this trimester the foetus prepares for delivery by rotating and positioning the head facing the birth canal. The foetal organs become more functional improving the postpartum rate of foetal survival (Campbell and Reece, 2002). Although human pregnancy is the most studied of all mammalian pregnancies, our understanding of the molecular events that

maintain pregnancy and initiate parturition is still vague and needs to be further investigated.

### ***1.4.1 Preterm (premature) delivery***

Preterm delivery refers to the birth of an infant before the developing organs are mature enough to allow normal *ex utero* survival. Infants born prior to 37 weeks or 259 days of gestation are considered preterm (Steer, 2005). As mentioned earlier, it is in the final trimester that the foetal organs mature, preparing the foetus for *ex utero* survival. In preterm delivery the maturation of vital organs such as the lungs fail to occur, increasing the risk of infant mortality and birth defects. Some 5 – 15% of all deliveries occur as preterm (Slattery and Morrison, 2002) and a strong reciprocal link is found between perinatal death rate and the gestational age (Challis, 2001; Challis et al., 2001; Challis and Smith, 2001). Neonatal death rates increases from 2 in one thousand deliveries at 38 to 40 weeks to 18 at 32–36 weeks and 216 at 24–31 weeks (Lopez Bernal and TambyRaja, 2000). About 70% of neonatal deaths and 75% of neonatal morbidity is associated with births of gestational age less than 37 weeks (Challis, 2001; Challis et al., 2001; Challis and Smith, 2001; Lopez Bernal and TambyRaja, 2000). Studies demonstrate that severe impairments such as cognitive, perceptual and behavioural problems (Hall, 1995; McCarton et al., 1997; Schothorst and van Engeland, 1996), intraventricular haemorrhage (IVH) (Paneth, 1990; Paneth et al., 1990) and associated cerebral palsy (Paneth, 1994) result from preterm labour. Several factors and pathways are identified that could lead to preterm labour. However, its treatment and management remains limited (Simhan and Caritis, 2007). Despite extensive research, there has been no significant decrease in the number of preterm births in the past few decades (Goldenberg et al., 2008). The healthcare cost implications of preterm births are significant, considering the huge costs involved in short-term neonatal care and long-term medical and special aid required by many surviving children (Goldenberg et al., 2008). Thus, preterm birth and its associated complications is one of the greatest health concerns in obstetrics. An effective method to diagnose and treat preterm labour is of utmost importance for reducing the cost involved in preterm labour and to improve foetal and maternal health. A key step to this is a deeper understanding of the molecular and biochemical events that regulate

## ***Chapter 1: Introduction***

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parturition. The known molecular mechanisms and key regulators of parturition will be discussed in detail later in this chapter.

### ***1.4.2 Post-term (postmature) delivery***

At the other end of the spectrum is post-term labour, where gestational age exceeds 42 weeks or 294 days (Rohwer et al., 2012). About 5% to 10% of all pregnant women continue pregnancy beyond 42 weeks (Norwitz et al., 2007; Olesen et al., 2003). This condition also poses a major concern in obstetrics because the intra-uterine condition becomes hostile for foetal survival once the foetus attains maturity inside the uterus. A high rate of foetal death and impairment is associated with post-term pregnancy (Froen et al., 2001; Heimstad et al., 2008; Smith, 2001). The underlying cause of post-term labour also lies in the dysregulation of myometrial contraction. Here the myometrium fails to initiate contraction despite attaining foetal development and reaching gestational age. Induction of labour or surgical removal of the foetus, are the main methods to treat post-term labour (Neilson, 2007).

## **1.5 Utero-placental tissues in pregnancy**

During pregnancy, the uterus contains both foetal (placenta, amnion and chorion) and maternal (myometrium, cervix and decidua) tissues (Garfield et al., 1998). These tissues undergo various adaptations to accommodate the growing foetus, supply the foetus with vital nutrients and also aid in propelling the mature foetus out from the uterus (Garfield et al., 1998). Several studies have been performed in these tissues to understand the complex molecular events that precede parturition. These studies are discussed later in this chapter.

### ***1.5.1 Amnion***

The tough yet elastic vascular membrane lining the innermost layer of the foetal sac is called the amnion (Chapter 7 : Carlson, 2009; Egarter and Husslein, 1992). This membrane builds the amniotic sac, whose main function is to protect the developing embryo (Chapter 7 : Carlson, 2009; Egarter and Husslein, 1992). At term, the amnion has five distinguishable layers. The layer adjacent to the chorion is the spongy layer

## ***Chapter 1: Introduction***

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of coelomic reticulum, which is covered in mucin. The inner layer is strongly attached to the chorion. The next layer is a fibroblast layer, embedded in a mass of reticular fibre, which forms the bulk of the amnion. This layer later thickens to become an acellular dense layer. This along with the amniotic basal membrane forms the strongest structural component of the amnion. The amniotic epithelium covers the amniotic basal membrane (McLaren et al., 1999).

### ***1.5.2 Chorion***

The chorion is composed of three layers, namely: the trophoblast layer, reticular layer and a fibroblast layer (Chapter 7 : Carlson, 2009). The part of the chorion that is in contact with the decidua undergoes atrophy leaving no trace of this membrane within a few months of gestation, leaving the chorion smooth, named the chorion laeve; as it is not involved in the formation of the placenta (Chapter 7 : Carlson, 2009). As the chorion grows, the chorion laeve comes in contact with the decidua and fuse. The villi on the embryonic pole, which is in contact with the decidua basalis, increase greatly in size and complexity. This part is named the chorion frondosum (McLaren et al., 1999). Due to the difficulty of isolating chorion tissue from the decidual tissue less research has been carried out on this tissue.

### ***1.5.3 Placenta***

The placenta separates the foetus from the maternal endometrium. The placenta originates partly from the foetus (chorion frondosum) and partly from maternal desidualised endometrium (decidua basalis) (Chapter 7 : Carlson, 2009). The foetal and maternal bloodstreams connect here, allowing vital exchange of substances including nutrients, oxygen, and carbon dioxide (Chapter 7 : Carlson, 2009). Typically, at term placenta weighs nearly a pound (500g), 20cm to 22cm long and is 2cm to 2.5cm thick. It is dark maroon in colour in the maternal side and shiny translucent gray in colour in foetal side (Chapter 6: Mader, 1992). It is attached to the foetus by the umbilical cord. The placenta contains a heterogeneous mixture of lymphoid and non-lymphoid cells (Chapter 7 : Carlson, 2009; Egarter and Husslein, 1992). Apart from its function of transportation, the placenta also acts as an endocrine organ. The main hormones produced by placenta include progesterone, oestrogen and relaxin (Chapter 7 : Carlson, 2009; Egarter and Husslein, 1992).

## ***Chapter 1: Introduction***

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### ***1.5.4 Cervix***

The lower cylindrical portion of the uterus is called the cervix, which is composed of connective tissue (Danforth, 1954; Rorie and Newton, 1967). The connective tissue consists of cells (fibroblasts), fibres (type I and II collagen (Leppert, 1995), the proteoglycans – elastin (Leppert et al., 1983) reticulin, and ground substance (Norman et al., 1993a).

Cervical ripening is an important step in pregnancy and labour, which is increasingly viewed as an inflammatory process (Kelly, 2002; Leppert, 1995; Lopez Bernal et al., 1993; Norman et al., 2007). The biochemical changes of the cervix start early during pregnancy but a noticeable change occurs only a few days before labour. At term, the cervix produces cytokines and enzymes, including elastase and collagenase to prepare for labour (Kelly, 2002; Lopez Bernal et al., 1993; Winkler and Rath, 1999). These enzymes help in the transformation of the rigid cervix to a soft one, a process known as cervical ripening. During labour the cervix dilates aiding in the ejection of the foetus from the uterus.

### ***1.5.5 Decidua***

The decidua is a highly modified endometrium, where the blastocyst attaches and the development of the placenta occurs (Chapter 6: Mader, 1992; Chapter 7 : Carlson, 2009). The growing blastocyst and surrounding trophoblasts are covered by decidua. A large population of leucocytes and lymphocytes are evident in this tissue, which is capable of producing cytokines and growth factors that support placental development (Terzidou, 2007). At term, the decidua can be divided into three regions based on its relationship with the implanted embryo, namely: the decidua basalis, decidua capsularis and decidua parietalis. The decidua basalis is the decidualised endometrium adjacent to the site of placentation that protects the underlying smooth muscle from syncytiotrophoblast invasion. The outer layer of the growing conceptus is the decidua capsularis, which fuses with the decidua parietalis, once the foetus fully occupies the space within the uterus. The lining of the uterine lumen is called the decidua parietalis (Terzidou, 2007). The cells that form the decidua are derived from endometrial stromal cells, which are affected by sex steroid hormones and other stimuli.

### ***1.5.6 Myometrium***

The myometrium is the muscular middle layer of the uterus (Chapter 6: Mader, 1992). This layer forms the functional contractile tissue called the fasciulata. Smooth muscle fibres are the main constituents of the myometrium giving it a firm nature (Chapter 6: Mader, 1992). Apart from smooth muscle fibres, blood vessels, lymph vessels, and nerves are also present in this layer. The muscle is organised into three layers of fibres, arranged in an irregular order. The outermost fibres are arranged longitudinally whereas the middle layer, which is the thickest of the three layers, does not show any specific arrangement pattern. The innermost fibres are longitudinal and circular in arrangement. During labour the smooth muscle rich myometrium is the main site of contraction (Chapter 6: Mader, 1992). The uterine enlargement towards the later stages of gestation is mainly by stretch-induced myometrial hypertrophy, but hyperplasia also occurs in the early weeks of gestation (Shynlova et al., 2009). The myocytes can independently generate force in any direction making the uterus capable of producing multidirectional forces during pregnancy. This helps in accommodating, positioning and propelling the foetus during different stages of gestation. Thus, throughout pregnancy and during labour the myometrium is the most active part of the uterus and regulates the parturition event. Hence in the present study it is the myometrial smooth muscle cells that are used to study the gene expression effects of steroid hormones.

Topographically, the myometrium is separated into the fundal, corpus (upper) and the isthmal (lower) segments (Chapter 6: Mader, 1992). The fundal region actively contracts during labour, whereas the lower segment relaxes and thins out distinctly (Challis, 2000). At term the myometrial protein expression pattern varies markedly, with increased pro-contractile protein expression in the fundus compared to the lower segments (Fuchs et al., 1984; Sparey et al., 1999).

#### **1.5.6.1 Smooth muscle and its structure**

Smooth muscle, a non-striated involuntary muscle found within the walls of hollow organs including stomach, intestines, bronchi, uterus, urethra, bladder, and blood vessels, is responsible for the contraction of these organs. Smooth muscle is so called because it lacks the banding pattern seen in other types of muscles. Smooth muscle

cells are small spindle shaped cells that have the ability to tense and relax. They are often classified as single-unit or multi-unit smooth muscle (Small, 1995). In single-unit smooth muscle, the fibres are roughly arranged parallel to each other in sheets or bands. The plasma membranes of adjacent fibres in single-unit smooth muscle are rich in gap junctions that act as low resistant channels for the transmission of electrical impulses. Multi-unit smooth muscle has no interconnecting cross-bridges and the fibres are arranged in a loose fashion. In this arrangement connective tissues are interspersed between the smooth muscle fibres (Barany, 1996; Small, 1995).

At the cytoskeletal level actin filaments are attached to the sarcolemma by focal adhesion in a spiral corkscrew fashion (Small, 1995). The filament proteins vimentin and desmin are alternatively positioned next to actin filaments and the contractile proteins organise themselves into zones of actin and myosin along the axis of the cell (Barany, 1996; Small, 1995). The sarcolemma has caveolae – specialised microdomains for cell-signalling and ion channels. These invaginations host an array of receptors (adrenergic receptors, muscarinic receptors, prostacyclin, endothelin, serotonin), second messengers (phospholipase C, cyclase, adenylate), G-proteins (RhoA), kinases (protein kinase A & C, rho kinase-ROCK) and ion channels (ATP sensitive Potassium channels, L type Calcium channels, Calcium sensitive Potassium channels) in very close proximity. The caveolae are located close to the sarcoplasmic reticulum or mitochondria and are believed to organise signalling molecules in the membrane (Barany, 1996; Small, 1995). The protein troponin, a complex of three regulatory proteins, regulate contraction in skeletal muscle and cardiac muscle is absent in smooth muscle. However, proteins calmodulin (CaM), caldesmon and calponin are highly expressed. Calmodulin plays a major role in regulating uterine smooth muscle function (Barany, 1996; Means et al., 1991; Walsh, 1994).

#### 1.5.6.2 Uterine smooth muscle contraction and relaxation

Milenov and Goldenhofen (1969) identified two types of contractions in stomach smooth muscle cells – phasic and tonic (Golenhofen et al., 1969; Milenov and Golenhofen, 1969). The onset of labour is facilitated by the phasic myometrial contraction and is necessary for successful progression of labour (Young, 2007). During labour, the uterus develops a stronger and more frequent contraction that begins in the fundus and spreads rapidly across the body of the uterus (Wray and

## ***Chapter 1: Introduction***

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Noble, 2008; Young, 2007). A variety of physiological mechanisms control myometrial contractility including intracellular signalling, cell membrane receptors, ion channels, hormones, gap junctions and metabolic and neuronal factors (Burszty et al., 2007; Grammatopoulos, 2007; Hertelendy and Zakar, 2004; Mendelson, 2009; Mesiano and Welsh, 2007).

Smooth muscle contraction is achieved by the sliding of actin and myosin filaments in the cells (Huxley, 1971a, b). For this interaction to occur, conversion of actin from a globular form to a more active filamentous form is necessary (Huxley, 1971a; Huxley and Brown, 1967). Attachment of actin to the cytoskeleton at focal points of the cell is a key for developing tension in the myocytes (Barany, 1996). Like other muscle types, smooth muscle contraction is also initiated through  $\text{Ca}^{2+}$  mediated changes in the thick filament.

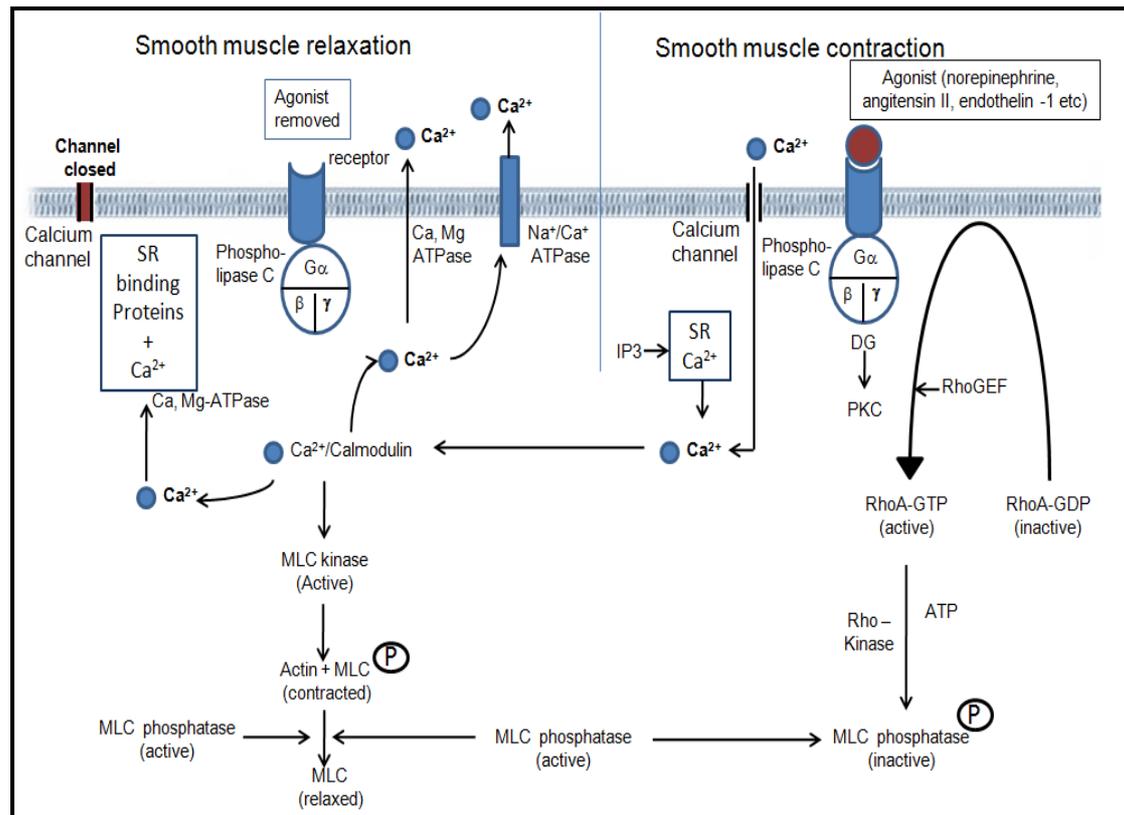
Stimuli in the smooth muscle increase the intracellular  $\text{Ca}^{2+}$  levels, which in turn promotes the association of  $\text{Ca}^{2+}$  ions with calmodulin (CaM). The  $\text{Ca}^{2+}$ -CaM complex then activates myosin light chain kinase (MLCK). MLCK then phosphorylates a 20-kDa protein - myosin light chain (MLC), which in turn enhances the interaction between actin and myosin to facilitate smooth muscle contraction (Barany, 1996; Hartshorne, 1998; Stull, 1996). Agonists such as angiotensin II, endothelin and norepinephrine activate phospholipase C by binding to serpentine receptors. Activation of phospholipase C results in the formation of two secondary messengers called: inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DG).  $\text{IP}_3$  binds to its receptors in the sarcoplasmic reticulum to stimulate more  $\text{Ca}^{2+}$  release. This cytosolic  $\text{Ca}^{2+}$  binds to DG to activate protein kinase C (PKC), which in turn stimulates several contraction proteins (Barany, 1996). Phosphorylation of myosin by myosin-light chain kinase (MLCK) activates myosin, which is the key regulatory step in smooth muscle contraction. In addition to MLCK, MLC phosphatase (MLCP) also regulates MLC phosphorylation (Barany, 1996; Fukata et al., 2001). MLCP removes the phosphate group from the active myosin promoting smooth muscle relaxation. Phosphorylation of the myosin-binding domain of MLCP by RhoA/Rho kinase inhibits the action of MLCP and increases the phosphorylated form of MLC (Sah et al., 2000; Solaro, 2000; Somlyo, 1999; Somlyo and Somlyo, 1998, 2000; Somlyo et al., 1999) and thus induces muscle contraction. Additional regulators of MLCK and

## ***Chapter 1: Introduction***

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MLCP have been reported including calmodulin-dependent protein kinase II that promotes smooth muscle relaxation (Sah et al., 2000; Solaro, 2000; Somlyo, 1999; Somlyo and Somlyo, 1998, 2000; Somlyo et al., 1999).

Myometrial smooth muscle relaxation is achieved either by the removal of the contractile stimulus or by the action of substances that can inhibit the contractile machinery (e.g. atrial natriuretic factor). Decreases in the  $\text{Ca}^{2+}$  concentration and increased activity of MLCP are key steps in achieving smooth muscle relaxation (Adelstein et al., 1980; Fukata et al., 2001; Morgan and Suematsu, 1990; Sah et al., 2000).  $\text{Ca}^{2+}$ -ATPases in the sarcoplasmic reticulum (SERCA), the plasma membrane (PMCA) and  $\text{Mg}^{2+}$ -ATPase help in maintaining the intercellular  $\text{Ca}^{2+}$  levels by releasing the cytosolic  $\text{Ca}^{2+}$ . Various sarcoplasmic reticular  $\text{Ca}^{2+}$ -binding proteins such as calsequestrin and calreticulin bind to cytosolic  $\text{Ca}^{2+}$  thereby reducing intercellular  $\text{Ca}^{2+}$  (Uehata et al., 1997).  $\text{Na}^+/\text{Ca}^{2+}$  exchangers (NCX) (Yu and Choi, 1997) and receptor-regulated and voltage-gated  $\text{Ca}^{2+}$  channels (Yamakage and Namiki, 2002) present in the plasma membrane also help to reduce the cytosolic  $\text{Ca}^{2+}$  concentration (Woodrum and Brophy, 2001). Cytosolic  $\text{Ca}^{2+}$  binds to these exchangers and channels and releases  $\text{Ca}^{2+}$  in exchange for  $\text{Na}^+$  (Yu and Choi, 1997). The affinity of these channels for  $\text{Ca}^{2+}$  is more than that of  $\text{Na}^+$  hence more  $\text{Ca}^{2+}$  is released from the cell than  $\text{Na}^+$  enters (Carafoli et al., 2001). The schema of smooth muscle contraction and relaxation is provided in Figure 1.4.



**Figure 1.4: Schema of the mechanism of smooth muscle contraction and relaxation.** Different agonists bind to their specific receptors to activate the contraction of smooth muscle. Following the binding of agonist to receptor, the phospholipase C activity is increased through coupling of G proteins. Phospholipase C produces phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>). IP<sub>3</sub> binds to its receptors on the SR, releasing Ca<sup>2+</sup>. DG and Ca<sup>2+</sup> activates PKC, which phosphorylates PKC target proteins. Activator Ca<sup>2+</sup> binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase) thus phosphorylating the light chain of myosin initiating shortening of the smooth muscle cell. The contractile response is maintained by the inhibition of myosin phosphatase activity by Rho kinase. This along with the activation of phospholipase C leads to the activation of the small GTP-binding protein RhoA. Once activated, RhoA increases Rho kinase activity, and hence inhibition of myosin phosphatase promoting contraction. Image adapted from (Webb, 2003)

## 1.6 Hormones in pregnancy and labour

A complex cascade of autocrine and paracrine events involving mother, foetus, and the placenta controls pregnancy and labour. Despite the discovery of important hormones and proteins that regulate this mechanism, little is known about their exact role during labour, making it difficult to predict the exact time of birth (McLean, 2001).

Two main changes prerequisite for labour are (i) conversion of the quiescent myometrium to an actively and rhythmically contracting organ and (ii) transformation of the cervix from a rigid structure to a soft one, known as cervical ripening (Casey

and MacDonald, 1997; Castracane, 2000; Snegovskikh et al., 2006; Weiss, 2000). Fine balances of these two events are key in maintaining optimal gestational length. Complementary pathways that involve changes in prostaglandin and calcium metabolism control these changes (McCarron et al., 2002; Norwitz et al., 1999; Sanborn, 2000). However, different interactive mechanisms that can tilt this balance towards contractility include progesterone withdrawal, dominance of oestrogen over progesterone in the uterus, increased oxytocin sensitivity, gap junction formation, increase in the prostaglandins and relaxin action and degradation of collagens (Bernal, 2001; Holt et al., 2011; Navitsky et al., 2000; Vidaeff and Ramin, 2008). However, it should be noted that a defect or change in one of these mechanisms could be overcome by alterations in another pathway. A brief description of various hormones that regulate pregnancy and labour is provided below with more emphasis on oestrogen and progesterone (topics of the thesis).

### ***1.6.1 Oxytocin: (OT)***

Oxytocin (OT) is a nanopeptide hormone that plays an important role in parturition and lactation. Oxytocin is known for its important role in the initiation of labour. No marked increase in the levels of oxytocin is observed until late pregnancy and during labour its level does not increase until after full cervical dilation (Leake et al., 1981). Oxytocin is locally synthesised in the uterus, which causes uterine contraction (Chibbar et al., 1993; Miller et al., 1993). Research has shown how oxytocin induces labour in two ways – by stimulating the release of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and F<sub>2α</sub> (prostaglandin F<sub>2α</sub>) and by activating calcium channels (Depoortere et al., 2000; Sniekers et al., 2010). Increase in foetal membrane PGE<sub>2</sub> and PGF<sub>2α</sub> levels activate Phospholipase C (PLC). PLC increases the release of calcium from myocytes leading to contraction (Arthur et al., 2007; Carsten and Miller, 1987; Rivera et al., 1990). Oxytocin is clinically used to induce uterine contraction and labour, where it is found to be more effective at term (Thornton and Smith, 1995; Zeeman et al., 1997).

Oxytocin receptor (OTR), is a typical G-protein coupled receptor with seven transmembrane domains (Gimpl and Fahrenholz, 2001). OTR is expressed in both pregnant and non-pregnant uteri. As mentioned earlier, the uterine sensitivity to OT is directly proportional to the density of OTR in the myometrium at labour and the

density of OTR in myometrium increases towards parturition (Kimura et al., 1996). The level of OTR increases in the myometrium towards late pregnancy resulting in increased efficiency of oxytocin in labour progression (Fuchs et al., 1984). OTR is also expressed in fetoplacental tissues such as amnion and chorodecidua where expression also significantly increases at labour (Benedetto et al., 1990). However, it has been demonstrated that mice with defects in OTR genes undergo a normal parturition cascade (Takayanagi et al., 2005).

### ***1.6.2 Prostaglandins (PG)***

Prostaglandins are non-peptide messenger molecules that exert their action locally (Brodt-Eppley and Myatt, 1999; Challis et al., 2002). The major stimulatory prostaglandins are prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and F<sub>2α</sub> (PGF<sub>2α</sub>), synthesised from their precursor, arachidonic acid (Chan et al., 1982). During the biosynthesis of prostaglandins, arachidonic acid is first converted to prostaglandin H<sub>2</sub> by the enzyme prostaglandin H<sub>2</sub> synthetase (PGHS) (also known as cyclooxygenase 2 - COX<sub>2</sub>) and then to the various prostaglandins by specific synthase enzymes (Allport et al., 2001). Prior to labour, increased expression of COX<sub>1</sub> and COX<sub>2</sub> has been reported in the lower segment of the uterus aiding cervical ripening (Sparey et al., 1999). Unlike in other species, the role of prostaglandins as regulators of labour in humans is debatable (Kamel, 2010). However, an increase in prostaglandin bioactivity prior to labour is reported in humans (Romero et al., 1996). Evidence suggests that the production of intrauterine and membrane prostaglandins increases during term and preterm labour (Challis et al., 2000; Fischer et al., 2008; Kniss, 1999; Olson et al., 1983; Skinner and Challis, 1985). Interleukins and cytokines induce prostaglandin synthesis (Ishihara et al., 1995; Olson et al., 1983; Pomini et al., 1999). Nitric oxide (NO) is demonstrated to increase the COX<sub>2</sub> concentration thereby increasing prostaglandin levels during inflammation. Contradictions remain as to whether the increase in prostaglandins induces inflammation or if the increase is an after-effect of inflammation (Lee et al., 2008; MacDonald and Casey, 1993; Romero et al., 1994; Romero et al., 1996). However, Lee and co-workers reported that the increase in amniotic fluid prostaglandins occurs prior to the onset of labour (Lee et al., 2008). Clinically, PGE<sub>1</sub> and PGE<sub>2</sub> are used to induce cervical ripening, where the action of PGE<sub>2</sub> is thought to be associated with collagen degradation (Leppert, 1995).

## ***Chapter 1: Introduction***

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Prostaglandin receptors are classified based on their response to specific classes of PGs. For example the receptor for thromboxane is the thromboxane receptor (TP), prostaglandin E is prostaglandin E receptors (EP), prostaglandin F is prostaglandin F receptor (PF), prostaglandin D<sub>2</sub> is prostaglandin D<sub>2</sub> receptor (DP<sub>1</sub>) and prostacyclin is prostaglandin I receptor (IP) (Tsuboi et al., 2002). Subtypes for EP are EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> and for IP are IP<sub>1</sub> and IP<sub>2</sub> (Coleman et al., 1994; Negishi et al., 1995; Sugimoto et al., 1995). EP<sub>1</sub> and EP<sub>3</sub> are pro-contractile agents and their activation induces contraction by increasing the intercellular calcium concentration and by inhibiting adenylate cyclase activity (Funk et al., 1993; Kotani et al., 1995). The receptors EP<sub>2</sub> and EP<sub>4</sub> induce uterine relaxation by activating adenylate cyclase, thereby increasing the cAMP concentration (Bastien et al., 1994; Regan et al., 1994a; Regan et al., 1994b). All four types of prostaglandin receptors are expressed in the pregnant myometrium (Brodt-Eppley and Myatt, 1999). Compared to non-pregnant uterus, the mRNA expression levels of FP, EP<sub>2</sub> and EP<sub>2</sub> are downregulated in pregnant uterus (Matsumoto et al., 1997). The expression of EP<sub>2</sub> is reported to further reduce with gestational age suggesting its role in maintaining uterine quiescence (Brodt-Eppley and Myatt, 1999). Although the role of the fine balance between the expression of prostaglandin receptors in regulating myometrial quiescence and contractility in mice is proven (Sugimoto et al., 1997), its role in controlling human parturition needs further investigation.

### ***1.6.3 Corticotropin-releasing hormone (CRH)***

Corticotropin-releasing hormone (CRH) is a 41 amino acid polypeptide that exerts a wide spectrum of hypothalamic and extrahypothalamic functions. CRH regulates the production of adrenocorticotrophic hormone (ACTH). A complex feedback mechanism modulates CRH and ACTH by adrenal cortisol. Maternal ACTH stimulates the adrenal glands to secrete cortisol, which reduces the production of CRH, resulting in the reduction of ACTH production. In humans, unlike most other species, the timing of birth is connected with the development of the placenta – especially with placental expression of CRH (McLean et al., 1995). Maternal plasma CRH levels increase considerably as pregnancy advances and peaks during foetal delivery (McLean et al., 1995; Smith, 2007). The increase in maternal plasma CRH levels is rapid in preterm delivery and vice versa for post-term delivery, indicating the key role of the placental

## ***Chapter 1: Introduction***

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clock in determining timing of delivery (McLean et al., 1995). CRH binding protein (CRHBP) binds to the circulating CRH thus making it unavailable to bind to its receptor (Linton et al., 1990a). Evidence suggests that in humans and in great apes there is a decrease in circulating levels of CRHBP towards term, thus increasing the bioactivity of CRH (Chrousos et al., 1998; Wadhwa et al., 1998).

Previous reports show that CRH regulates the expression of several uterotonic genes such as oxytocin and prostaglandins (Benedetto et al., 1994; Jones et al., 1989; Jones and Challis, 1990; Quartero and Fry, 1989). CRH also increases the circulating level of corticotropin in mother and foetus as the gestational age increases. This increase in corticotropin and CRH stimulates the production of dehydroepiandrosterone sulfate (DHEA-S) by the adrenal glands. DHEA-S acts as a precursor for oestrogen – a key player in regulating pregnancy (Grammatopoulos and Hillhouse, 1999b; Smith, 1998) and is responsible for creating an oestrogen-dominant environment prior to labour (Majzoub et al., 1999). Yang and colleagues have proven that CRH inhibits progesterone synthesis in human placental trophoblasts. Circulating CRH is also present in the foetus and is demonstrated to increase with gestational age (Yang et al., 2006). However, the concentration of circulating CRH is not as high as seen in the mother. CRH stimulates the production of corticotropin in the foetus, which helps in foetal lung maturation, which in turn increases the cortisol levels that enhances the production of surfactant protein and phospholipids. Surfactant protein and phospholipids have pro-inflammatory actions and may induce myometrial contraction (Speroff and Fritz, 2005). In short, a positive feed-forward system both in mother and foetus leads to an increase in the CRH concentration. The increase in CRH levels reflects in the increase in cortisol levels, foetal lung maturation and production of proteins, phospholipids and receptors that stimulate various pathways that helps myometrial contraction.

Three distinct types of CRH receptors are found to be upregulated in human myometrium during pregnancy (Grammatopoulos et al., 1998). CRH acts mainly through its membrane bound CRH type 1 receptor, a G protein-coupled receptor family member, which is present in the pituitary and the myometrium in the mother, and in the pituitary and the adrenal glands in the foetus (Linton et al., 1990b; Stevens et al., 1998). At term, these receptors stimulate the G $\alpha$ q pathway, which activates

protein kinase C, which in turn activates the contractile pathway (Grammatopoulos and Hillhouse, 1999a). During pregnancy myometrial quiescence is achieved through the dissociation of the  $\alpha$  subunit of CRH1 $\alpha$ – a primary form of CRH receptor in the myometrium - that sends signal to the intracellular effectors to prolong relaxation. Towards term, there is a reduced coupling of the  $G_{\alpha s}$  regulatory protein of the CRH-receptor complex with the catalytic component of adenylate cyclase (Daroqui et al., 2012). Oxytocin influences this process in two ways by inhibiting the activity of adenylate cyclase and by decreasing the affinity of CRH to adenylate cyclase (Grammatopoulos and Hillhouse, 1999a). Towards labour the lower segment of the uterus expresses more CRH type 1 receptor favouring cervical ripening (Stevens et al., 1998).

#### **1.6.4 Relaxin (RLX)**

Relaxin is a peptide hormone and a member of the relaxin peptide family comprising of several members (Bathgate et al., 2003). Structurally, relaxin has two polypeptide chains A and B that are linked together by a disulfide bond. The corpus luteum is the primary site of production for relaxin in women (Weiss et al., 1976). Circulating relaxin levels increase until mid-gestation and remain constant, or slightly reduced throughout the entire duration of pregnancy (Weiss and Goldsmith, 2005; Weiss et al., 1976). Pregnancy by egg donation (i.e. IVF), where circulating relaxins are absent, reach term and eventually undergo labour proving that circulating relaxin is not essential for maintaining pregnancy and initiating labour (Quagliarello et al., 1979). The placenta and decidua also produce relaxin that may act locally during pregnancy (Adashi et al., 1996; Quagliarello et al., 1979).

Preterm labour is associated with high circulating levels of relaxin in the maternal system (Petersen et al., 1992). It is found that women with higher circulating relaxin levels at week 30 of pregnancy are predicted to have high chances of preterm labour (Petersen et al., 1992). This effect could be related to the role of relaxin on cervical softening. The human cervix expresses receptors for relaxin (Palejwala et al., 1998) and their activation upon relaxin binding results in induction of pro-collagenase and pro-stromelysin activity and a decrease in metallopeptidase activity (Palejwala et al., 1998). These changes help in cervical ripening, an important maternal adaptation for successful parturition.

### ***1.6.5 Oestrogen***

Oestrogen is the key female sex hormone that controls female reproductive development and function. The uterus is highly responsive to oestrogen. Oestrogen plays an important role from the growth and development of the uterus in prepubescent girls to the contractility of the pregnant uterus during labour.

#### ***1.6.5.1 Oestrogen synthesis during pregnancy***

Four different isoforms of oestrogen have been identified in the blood of pregnant women – estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4) (Figure 1.5) (Coelingh Bennink, 2004; Siiteri and MacDonald, 1966). In maternal circulation, estrone is present mainly in its sulphated form, estradiol in unconjugated form (70 – 80 %) and sex hormone-binding globulin forms (0.5 – 1.0 %), estriol mostly in conjugated form, and estetrol only in unconjugated form (Ahmed and Kellie, 1973; Coelingh Bennink, 2004; Siiteri and MacDonald, 1966). The principal source of oestrogen in human pregnancy is the placenta (Baulieu and Dray, 1963; Bolte et al., 1964; Brodie et al., 1969). Although there is a large supply of C<sub>21</sub>-progestins (precursors of oestrogen synthesis) in human placenta during pregnancy, oestrogen cannot be synthesised from these precursors due to the reduced expression of steroid 17 $\alpha$ -hydroxylase/17,20 lyase (Cytochrome P450c17) that catalyses steroid hormone synthesis (Hum and Miller, 1993; Ryan, 1959). Therefore, oestrogen synthesis depends on the supply of C<sub>19</sub> androgens in the form of dehydroepiandrosterone sulphate (DHEA-S) from foetal and maternal adrenals (Figure 1.6) (Siiteri and MacDonald, 1966; Valle et al., 2006). The placenta extracts circulating DHEA-S and converts it to DHEA with the help of a DHEA-sulphatase. DHEA is then converted to androstenedione and testosterone with the help of the enzyme 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) (Ryan, 1959). Androstenedione and testosterone are finally converted to estradiol and estrone respectively by the aromatase enzyme. Due to the efficiency of the placenta in converting DHEA-S and DHEA to oestrogens, the ratio of DHEA-S to DHEA does not vary much throughout pregnancy, although their production significantly increases as pregnancy advances (Gant et al., 1971; Hoedemaker et al., 1990; Siiteri and MacDonald, 1966).

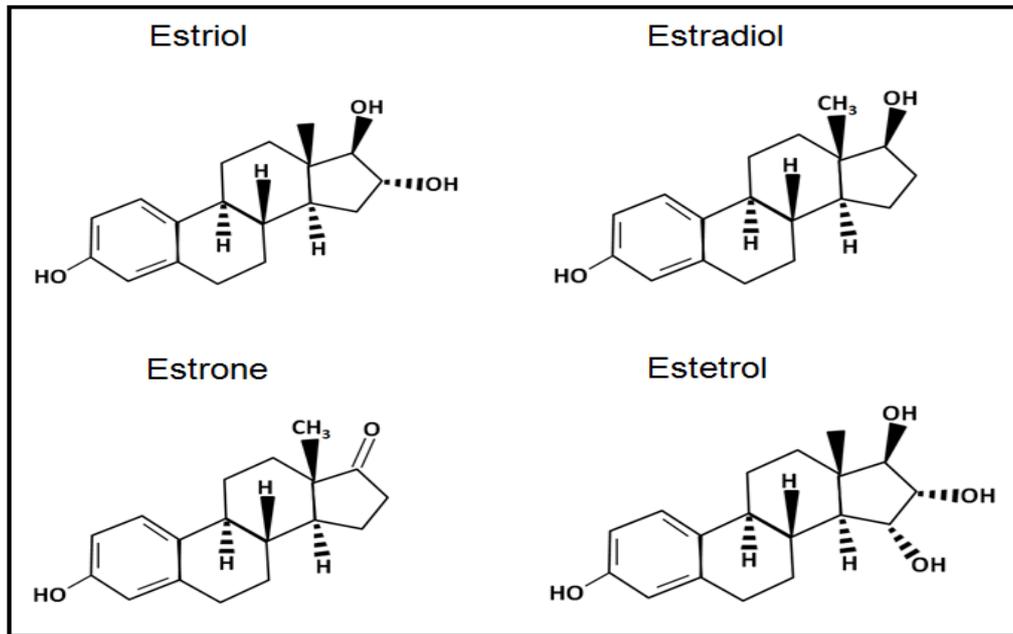


Figure 1.5: Structural formula of various oestrogens adapted from (Ali et al., 2011)

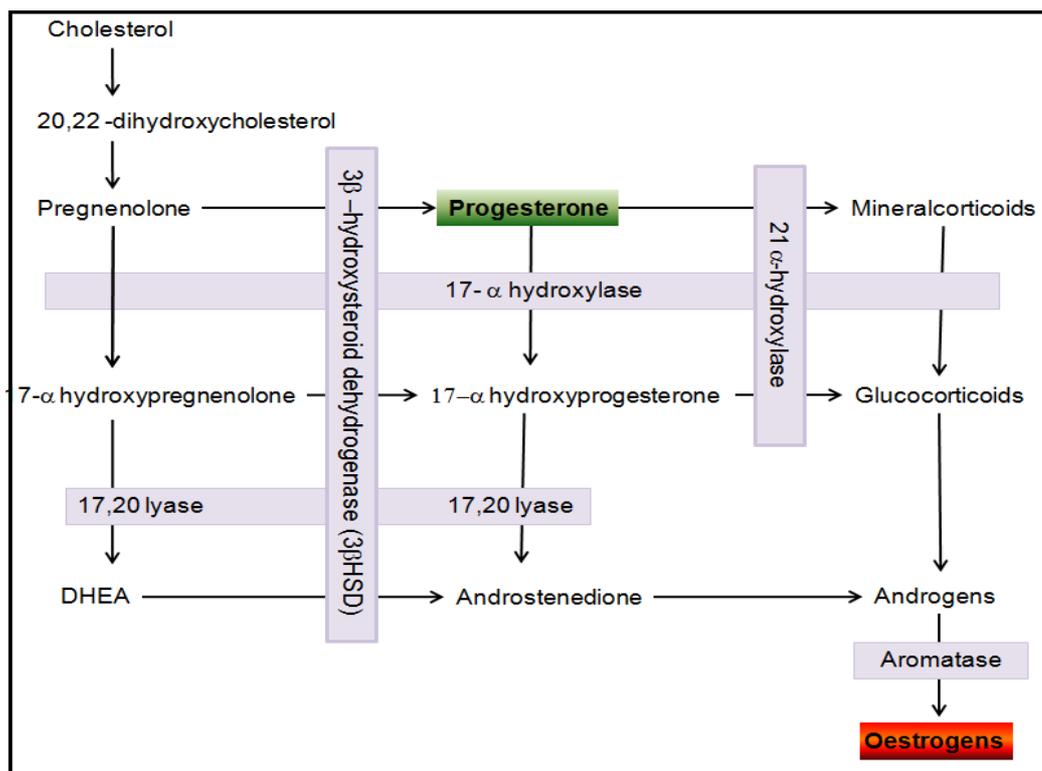


Figure 1.6: Steroidogenesis pathway of oestrogens and progesterone. Cholesterol is converted to pregnenolone, a precursor of progesterone. Progesterone is then converted to either androstenedione or glucocorticoids that are later converted into androgens. The aromatase enzyme converts androgens to oestrogens. Pregnenolone is converted to 5-dehydroepiandrosterone (DHEA) by the enzyme 17,20 lyase. DHEA produced from pregnenolone is converted to androstenedione then androgen and finally oestrogen. Figure adapted from (Scott et al., 2009) and (Albrecht and Pepe, 1990).

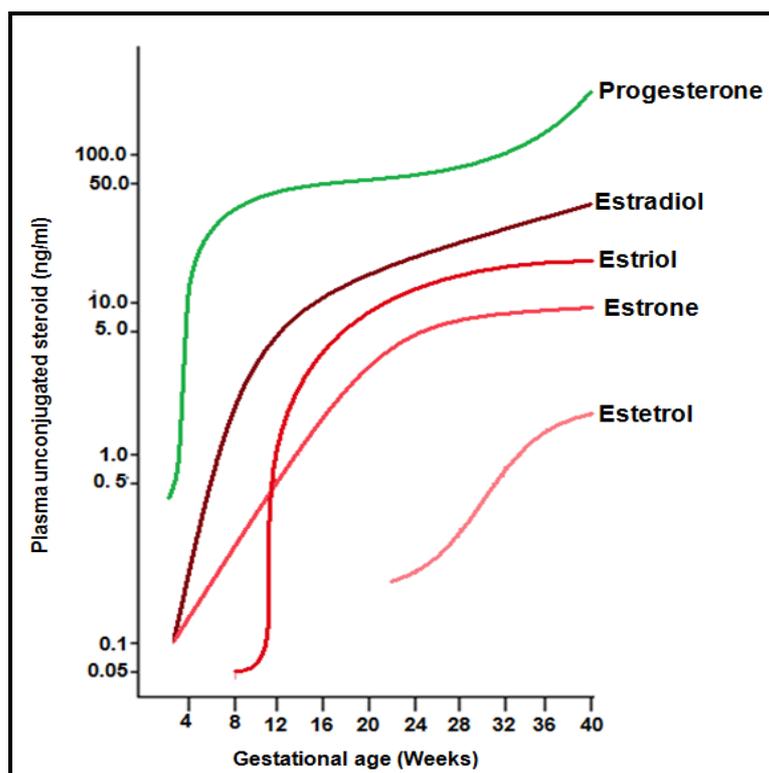
The production of estriol by the placenta progressively increases with gestation and exceeds the levels of estradiol and estrone by late gestation (Hobson, 1971; Siiteri and MacDonald, 1966). During pregnancy the urinary excretion of estradiol and estrone is increased by over 100-fold, whereas that of estriol is increased by over 1000-fold, suggesting that estriol is the predominant form of oestrogen during pregnancy (Bolte et al., 1964). However, despite estriol being the major oestrogen produced by the placenta during pregnancy, about 90-95% of estriol is found in the conjugated form, rendering it inactive. The amount of unconjugated estriol (the active form) present in maternal circulation is therefore far less than the level of estradiol, in fact similar to that of estrone (De Hertogh et al., 1975; Yen, 1973). Thus, most oestrogenic effects on the target tissues are mediated by estradiol.

Estriol synthesis is dependent on foetal C<sub>19</sub>-androgen as its sole precursor. The human placenta needs a supply of 16-hydroxylated C<sub>19</sub> precursors for the production of estriol (a 16-hydroxylated oestrogen) as it lacks the 16-hydroxylase. DHEA-S produced from the foetal adrenal glands is converted into 16-OH-DHEA-S by the 16-hydroxylase produced by the foetal liver (Bolte et al., 1966; Slaunwhite et al., 1965). 16-OH-DHEA-S is then converted to 16-OH-DHEA and aromatised to estriol by the placenta. There is little contribution of 16-OH-DHEA-S from the maternal side for the production of estriol (Belisle et al., 1977; Siiteri and MacDonald, 1966). Thus any block in DHEA-S results in reduced estriol synthesis and lower estradiol and estrone levels than normal (Diczfalusy, 1964; Frandsen and Stakemann, 1961). An increase in the levels of estriol around the 10<sup>th</sup> – 15<sup>th</sup> week of gestation is due to the increased steroidogenic activity of the inner cortisol compartment of foetal adrenals, which is the primary site for DHEA-S production (Mesiano and Jaffe, 1997). Thus, maternal estriol level is used as a marker for foetal hypothalamic-pituitary-adrenal activity and the well-being of the foetus (Mesiano and Jaffe, 1997).

The fourth form of oestrogen found in human pregnancy is estetrol, which is also produced exclusively from foetal DHEA-S. Estetrol is synthesised from 15-OH-DHEA-S by 15-hydroxylase, an enzyme expressed only in the foetal liver (Gurpide et al., 1966). As in estriol, the levels of estetrol also increases from mid-gestation (Giebenhain et al., 1972), but the exact physiological role of estetrol during pregnancy is yet to be identified.

1.6.5.2 Oestrogen levels during parturition

In all mammals except humans, parturition is associated with a sharp increase in the levels of circulating oestrogens (Yen, 1973). In humans, this rise is small and gradual over an extended period of pregnancy, indicating that the role played by oestrogen in parturition is in some way different in humans (Figure 1.7) (Casey and MacDonald, 1997). The ovine system is considered a model system to understand mammalian pregnancy; therefore key events of parturition in humans are often compared to those in sheep. An important event in initiating labour in sheep is the synthesis of 21-hydroxylase (Cytochrome P450c21) by the placenta, which can convert progesterone to testosterone and androstenedione that are later converted to oestrogen (Silver, 1994). Increased production of Cytochrome P450-C21 in sheep therefore, helps to tip the uterine balance of progesterone to oestrogen, in favour of oestrogen (Silver, 1994; Thorburn, 1978). In humans at term, foetal adrenal cortisol production increases, due to an increase in foetal hypothalamic-pituitary-adrenal axis activity; this leads to organ maturation which is a major pre-requisite for labour (Anderson et al., 1978). Levels of maternal oestrogen towards the end of gestation in humans are a direct result of increased DHEA-S synthesis by the mature foetal adrenals (Mesiano and Jaffe, 1997). Thus, in sheep the uterus is exposed to more oestrogen/less progesterone, thus helping uterine contraction, whereas in humans a rise in oestrogen levels is controlled by the increased foetal adrenal supply of DHEA-S (Mesiano and Jaffe, 1997) and there is no reciprocal decrease in progesterone.



**Figure 1.7: Circulating levels of unconjugated steroids in the maternal system during different stages of pregnancy.** Both oestrogen and progesterone levels in the plasma gradually increase from early weeks of gestation and peak towards mid-gestation. Only a very slight increase in the levels of circulating plasma steroids are observed from mid-gestation. Thus, in humans labour occurs in the presence of high levels of steroids, an event that does not occur in other primates or non-primates. (Figure adapted from (Yen and Jaffe, 1991).

#### 1.6.5.3 Role of oestrogen in human parturition

During parturition oestrogen is believed to oppose the action of progesterone, stimulating physical and biochemical changes in the uterus and foetal membranes, in preparation for labour and delivery (reviewed in (Pepe and Albrecht, 1995). Evidence from different species suggests that oestrogen induces uterine contraction by altering the resting membrane potential (Boyle et al., 1987), increasing myometrial cell gap junction formation (Boyle et al., 1987; Kilarski et al., 1996; Kilarski et al., 2000; Lye et al., 1993; Petrocelli and Lye, 1993), increasing uterotonic agent responsiveness of myometrium (Nissenson et al., 1978; Pinto et al., 1966; Pinto et al., 1967) and increasing foetal membrane production and release of prostaglandins (Saitoh et al., 1984). Furthermore, oestrogen stimulates the expression of proteolytic enzymes such as collagenases in the cervix, thus helping cervical degradation and dilation (Rajabi et al., 1991a; Rajabi et al., 1991b).

As discussed earlier, the increase in circulating oestrogens in the human system is gradual, compared to other model organisms, and this increase is foetal adrenal dependent (Casey and MacDonald, 1997; Yen, 1973). In contrast to the ovine system, the increase in oestrogen during human term is due to increased foetal adrenal production of DHEA-S (Mesiano et al., 1997). It was proposed that this event is analogous to foetal cortisol action (increasing the oestrogen levels) in sheep and therefore is a key event in regulating primate parturition (Nathanielsz et al., 1998). This hypothesis was tested in rhesus monkeys by treatment with androstenedione at mid-gestation to increase placental oestrogen synthesis (Wu et al., 1995). Maternal oestrogen and oxytocin were found to increase in treated monkeys, inducing premature labour, thus proving that the timing of birth was influenced by foeto-placental unit action (the foetus and the placenta as a single physiological unit). In another study, pregnant rhesus monkeys were treated with dexamethasone, a strong anti-inflammatory drug, and found that pregnancy was significantly prolonged due to the decrease in the basal level of estradiol and cortisol, but not progesterone (Novy and Walsh, 1983). The treatment prevented the prepartum increase in oestrogen levels, supporting the role of oestrogen in primate parturition. However, foetectomy during mid-gestation in rhesus monkeys was shown to cause post-term placental delivery (Nathanielsz et al., 1992) and also altered uterine activity (Umezaki et al., 1993), suggesting that normal parturition in rhesus monkey requires an intact foeto-placental unit.

Several congenital abnormalities in humans that can affect the function of the foeto-placental unit have allowed the evaluation of the role played by placental oestrogen in regulating parturition (Mesiano and Jaffe, 1997). The effect of increased placental oestrogen production during parturition can be seen in pregnancies where the foetus has congenital adrenal hyperplasia due to mutation in the gene coding for 21-hydroxylase (White, 1994). In these pregnancies, foetal adrenal capacity to synthesise cortisol is reduced due to the lack of the 21-hydroxylase (New, 1995). The foetal pituitary produces an abnormally high level of corticotropin, resulting in hyperstimulation of the foetal adrenal cortex, which increases DHEA-S synthesis, leading to higher oestrogen production. Interestingly, these individuals do not deliver prematurely despite the higher activity of the foeto-placental unit, suggesting that higher levels of foeto-placental unit activity are not responsible for initiating

## ***Chapter 1: Introduction***

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parturition in human (Price et al., 1971). Studies of abnormalities in placental oestrogen synthesis, such as anencephaly (Diczfalusy, 1964; Frandsen and Stakemann, 1961), placental aromatase deficiency (Mullis et al., 1997; Shozu et al., 1992; Shozu et al., 1991), and deficiency in placental sulphate (France, 1979; France and Liggins, 1969; France et al., 1973) suggest that placental oestrogen synthesis has a minuscule role to play in birth timing. Study of placental deficiency also suggests that placental oestrogen is not needed for normal pregnancy and parturition (Grumbach, 2004).

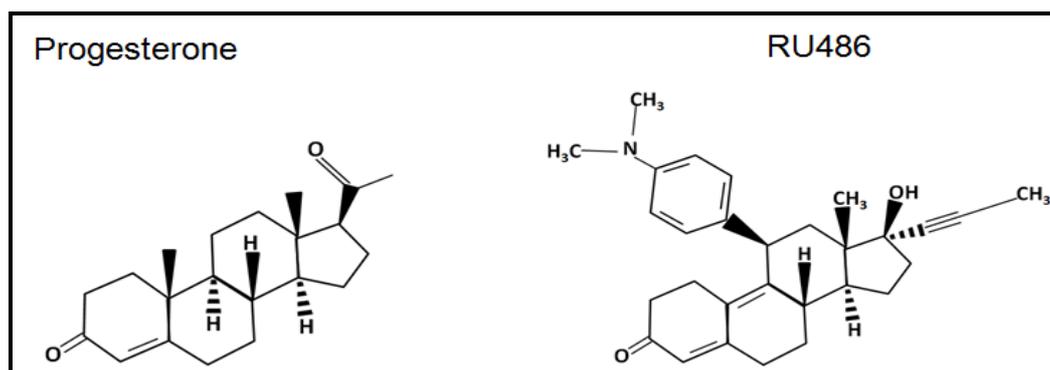
The above observations suggest that the initiation of human parturition is not associated with an increase in placental oestrogen production. However, this does not completely eliminate oestrogen from a role in the human parturition process. Maternal oestrogen (produced from ovaries), although less in concentration compared to amount of oestrogen produced by the placenta during normal pregnancy, is present in the target tissue (Mesiano, 2001). Thus, the oestrogen target tissue (stroma, luminal epithelial, glandular epithelial, and smooth muscle) is exposed to a reasonable level of estradiol even when the placenta fails to produce oestrogen (Mesiano, 2001). This suggests that the action of oestrogen in human parturition could be mediated by the responsiveness of its receptors rather than by the change in the concentration of circulating oestrogens in the maternal system. Interestingly, more cases of preterm birth were observed in humans, rhesus monkeys and guinea pigs when raised levels of oestrogen were detected in the maternal system (Mesiano and Jaffe, 1997). A reason for this could be the availability of high levels of oestrogens in the body to initiate the parturition cascade. Thus the oestrogen responsiveness of target tissues could be mediated by changes in oestrogen receptor expression and switching of oestrogen between active and inactive forms (Mesiano et al., 2002).

It is reported that human foetal membranes have 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD) activity and it can convert estrone, a weak form of oestrogen, to estradiol, an active form (Mitchell and Wong, 1993). In their study, Mitchell and Wong (1993) noted that this conversion was much greater in foetal membranes obtained from women in spontaneous term labour, compared to membranes obtained from women undergoing caesarean section. These data suggest that throughout pregnancy the foetal membranes selectively convert estradiol to estrone, and that as parturition

approaches estrone is converted back to the more potent estradiol via a change in 17 $\beta$ HSD activity. Various studies have proven that this activity of 17 $\beta$ HSD can be connected to at least seven isoenzymes, which are encoded by different genes, having precise enzyme activity, substrate preference and tissue localisation (Andersson and Moghrabi, 1997; Isomaa et al., 1993; Peltoketo et al., 1999). No studies have been carried out to examine specific expression patterns of different 17 $\beta$ HSD isoenzymes but a few studies show that 17 $\beta$ HSD-II, an enzyme that specifically converts estrone to estradiol, is expressed in the foetal membranes (Milewich et al., 1979; Milewich et al., 1977). The above findings suggest that the changes in 17 $\beta$ HSD activity could mediate foetal maturation and parturition. Thus, during most of gestation, oestrogen action on foetal membranes could be inhibited by inactivation of estradiol by converting it to estrone. Hence, the foetal maturation and parturition cascade could be as a result of decreased expression of 17 $\beta$ HSD-II thereby making more active estradiol available to interact with its receptors. It was proved that in human endometrium, progesterone augments net oxidative 17 $\beta$ HSD activity proposing that this would increase 17 $\beta$ HSD-II expression (Gurpide and Tseng, 1974; Tseng and Gurpide, 1975). Thus, high progesterone levels during most of pregnancy help in the conversion of estradiol to the less active estrone, thereby maintaining uterine quiescence (Grow, 2002; Resnik, 1983). An increase in cortisol levels can block this effect of progesterone on 17 $\beta$ HSD-II thereby allowing more estradiol to act and initiate the parturition cascade. This hypothesis highlights the connection between foetal maturation, progesterone withdrawal, oestrogen activation and the timing of birth.

### ***1.6.6 Progesterone***

Progesterone (P4) is a C-21 steroid hormone that plays a crucial role in pregnancy. Figure 1.8 illustrates the molecular structure of progesterone and mifepristone (RU486 – progesterone inhibitor).



**Figure 1.8: Structural formula of progesterone and RU486** (mifepristone – progesterone inhibitor) (Leonhardt and Edwards, 2002).

#### 1.6.6.1 Progesterone synthesis during human pregnancy

In women the corpus luteum is the main source of progesterone at the beginning of pregnancy (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994). However after a few weeks of gestation the placenta takes over the production of progesterone to become the main source of progesterone in pregnancy and labour (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994). Like other steroid hormones, cholesterol is the precursor for progesterone synthesis. Cholesterol is converted to pregnenolone, which is then converted to progesterone (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994) (Figure 1.6).

#### 1.6.6.2 Changes in the progesterone levels at parturition

Circulating progesterone in the system maintains uterine quiescence during pregnancy (Mesiano, 2007). A fall in the circulating levels of progesterone leads to labour in species where pregnancy is luteal dependent (Mesiano, 2007). However, this does not appear to be true in humans (Csapo et al., 1973). Serum progesterone levels do not vary to a great extent between women in labour and those not-in-labour (Buster and Abraham, 1975; Buster et al., 1976; Lindberg et al., 1974; Tulchinsky et al., 1972; Yen and Jaffe, 1991) (Figure 1.7). In humans the removal of the corpus luteum during early pregnancy results in abortion (Csapo et al., 1973; Mesiano, 2001). It was previously reported that the use of the progesterone receptor inhibitor, mifepristone (RU486) initiates labour (Spitz and Bardin, 1993) and the mechanism by which this is accomplished is by increasing the effect of CRH (Karalis et al., 1996).

### ***1.6.6.3 Role of progesterone in human parturition***

The timing of birth is a result of a fine balance between the dynamic effects of progesterone and oestrogen on uterine contractility. Progesterone maintains uterine quiescence by regulating several molecules that control myometrial contractility (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994). Progesterone downregulates prostaglandin production, oxytocin receptor expression and inhibits calcium channel formation, all favouring uterine quiescence (Fuchs et al., 1984; Garfield et al., 1998; Ishihara et al., 1995; Smith and Kelly, 1987). Progesterone prevents cervical softening by increasing the expression of matrix metalloproteinase 1 (TIMP-1) inhibitors, which, in turn blocks the degradation of collagen fibres (Leppert and Woessner, 1991). *In vitro* progesterone is demonstrated to inhibit myometrial gap junction formation by downregulating gap junctions, thereby reducing myometrial contractility (Garfield et al., 1998).

At parturition when the uterus transforms to a rhythmically contracting organ, the activity of 17,20 hydroxysteroid dehydrogenase increases resulting in increased 17 $\beta$ -estradiol and 20-dihydroprogesterone synthases (Mitchell and Wong, 1993). This helps to tip the balance of the uterus from a progesterone-dominant environment to an oestrogen-dominant one, a key to uterine contractility (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994). The progesterone levels in maternal, foetal and amniotic fluids do not vary in humans (Boroditsky et al., 1978; Tulchinsky et al., 1972; Walsh et al., 1984), suggesting progesterone levels do not play a major role in labour. However it was found that the progesterone receptor inhibitor mifepristone (RU486) treatment induced labour at any stage of pregnancy. Mifepristone exposure and induced labour were associated with significant increase in plasma cortisol without alterations of systemic CRH or ACTH levels (Byrne et al., 2004). This leads to the conclusion that parturition in humans involves some form of progesterone withdrawal other than the reduction in circulating progesterone levels. There are many suggested methods of progesterone withdrawal including, (1) binding of circulating progesterone-binding-protein to free active progesterone, (2) inactivation of local progesterone activity within the cell, (3) production of an endogenous progesterone antagonist, and (4) decreased myometrial responsiveness to progesterone (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994).

However, none of these theories have been proven definitively. The most studied mechanism is functional withdrawal of progesterone, mediated by specific changes in the expression of nuclear progesterone receptors (nPRs) in the myometrium (Brown et al., 2004; Mesiano, 2004; Zakar and Hertelendy, 2007).

### **1.7 Steroid hormone receptor (SHR) dynamics during pregnancy and labour**

Steroid hormone receptors (SHR) are intracellular proteins that have binding sites for a specific steroid hormone. Steroid hormone receptors belong to the nuclear receptor (NR) superfamily and specifically come under the title of nuclear receptor subfamily 3 (NR3). The SHR subfamily is subdivided into 3 further subgroups namely subfamily 3A, 3B and 3C. Subfamily 3A contains oestrogen receptors (ER) and subfamily 3C includes glucocorticoid receptors (GR) that bind cortisol, the aldosterone-binding mineralocorticoid receptors (MR), the progesterone receptors (PR) and dihydrotestosterone (DHT) binding androgen receptors (AR). Subfamily 3B contains three orphan receptors, which are closely similar to oestrogen receptors hence they are named oestrogen-related receptors (ERR) (Escriva et al., 2000). The SHRs are ligand-inducible transcription factors and function as modulators of target gene expression by binding to specific palindromic DNA sequences in the target gene promoter (Beato et al., 1989). These sequences are called the hormone response elements (HRE). The binding of the hormone to its receptors activates the receptor, triggering dimerisation, an important step for HRE binding of the hormone receptor (Cairns et al., 1991; Eriksson and Wrangé, 1990; Fawell et al., 1990; Kumar and Chambon, 1988; Lees et al., 1990).

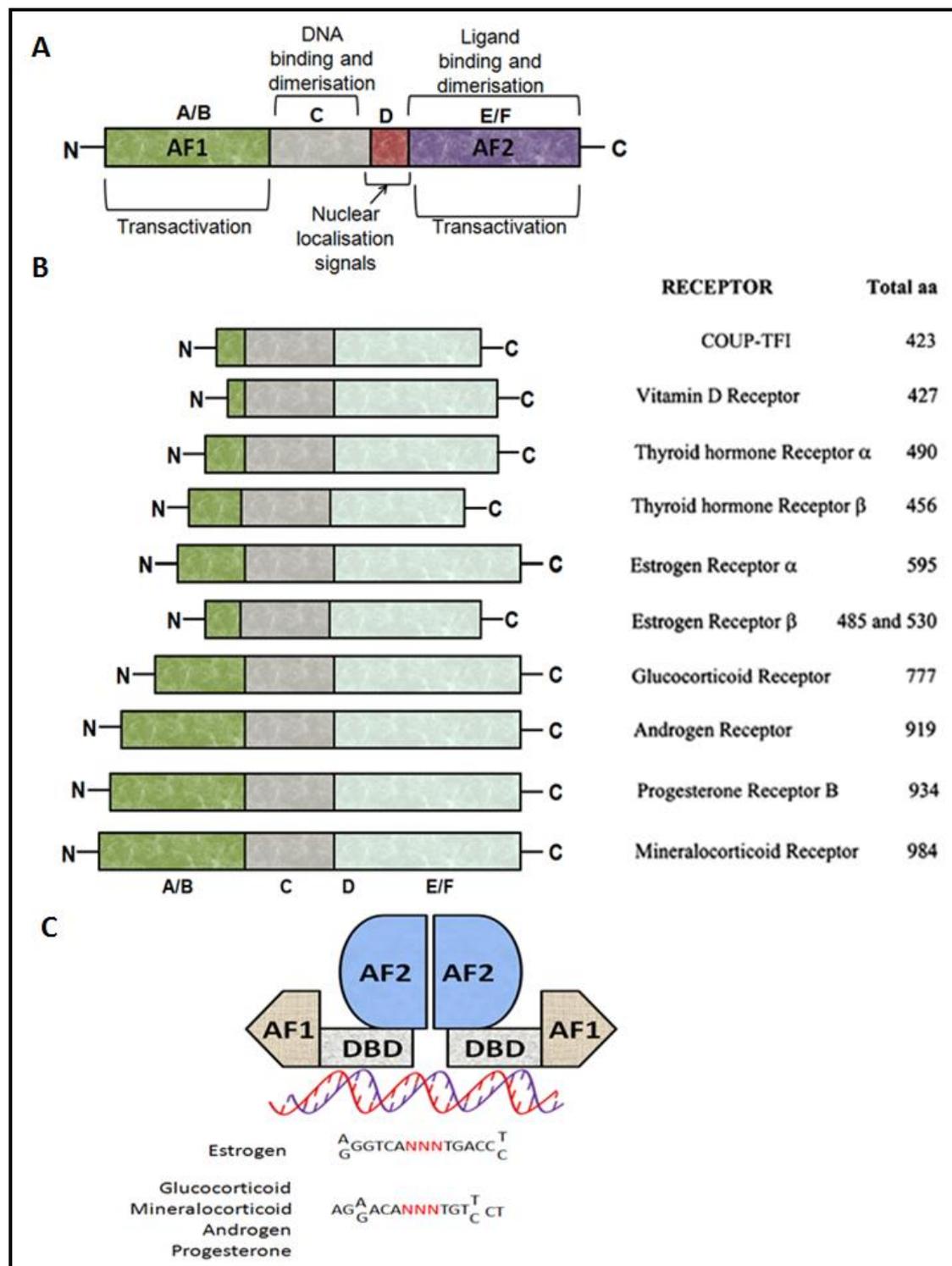
Structurally, SHRs share a similar modular architecture with all other NRs. SHRs have a variable N-terminal domain (NTD A/B), a well-conserved DNA binding domain (C), a flexible hinge that connects the DNA binding domain and the C-terminal domain (D) and a C-terminal ligand-binding domain (E) (Bain et al., 2007; Evans, 1988; Parker, 1990). A unique additional C-terminal domain F of unknown function is found in ER $\alpha$  (Schwabe et al., 1993). The N-terminal domain (NTD A/B) is a highly diverse sequence of variable length across all SHRs and houses an autonomous transcription activation function known as AF-1. AF-1 is a ligand-independent transcriptional activator, but can also function with AF-2 (ligand-dependent activator). The C region in the DNA binding domain (DBD-C) is highly

## ***Chapter 1: Introduction***

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conserved and gives the sequence-specific DNA binding ability to the SHR. The D region, the flexible hinge, contains the nuclear localisation signal. The ligand-binding domain (LBD) in the C-terminus binds to hormones and ligands that contain ligand-dependent transcriptional activation function (AF-2). AF-2 controls the transcription machinery by recruiting transcriptional co-activators that interact with chromatin remodelling proteins (Bain et al., 2007; Evans, 1988; Parker, 1990). Ligands such as heat shock chaperones in the cytoplasm (heat shock protein 90 - Hsp90) interact with the SHRs through the C-terminal LBD (Pratt and Toft, 1997). Upon ligand binding, the nuclear localisation signal (NLS) in the ligand-binding domain (LBD) aids in the translocation of the SHRs to the nucleus. The ligand binding domain together with the DNA binding domain contains sequence for receptor dimerisation with its partners (Kumar et al., 2006). Once in the nucleus the chaperone dissociates from the receptor and the ligand-bound receptor can now bind to specific sequence in the promoter of the target gene hormone response element (HRE) (Edwards, 2005). This complex initiates chromatin remodelling and sends signals for activation or repression of target gene transcription (Edwards, 2005).

Figure 1.9A is a schematic representation of the structure of SHRs showing their different domains. Figure 1.9B highlights the structural homology between various steroid hormone receptors. Figure 1.9C represents how the homodimeric steroid hormone receptor binds to the palindromic or hemi-palindromic sequence of the hormone response element in the target gene.



**Figure 1.9: Steroid hormone receptor family** **A.** Schematic representation of the structure of the steroid hormone receptor showing the various domains of the receptor. **B.** Structural homology within the steroid hormone receptor superfamily. This figure illustrates the difference in the length of various domains within various SHRs. **C.** Pictorial representation of the binding of homodimeric steroid hormone receptor to the palindromic sequence (hormone response element) in the promoter region of the target gene. The palindromic or hemi-palindromic steroid hormone response element sequence of oestrogen receptors and other steroid hormone receptors are also shown. Adapted from (Wahli and Martinez, 1991).

### ***1.7.1 Oestrogen receptors (ER)***

Oestrogen receptors are a group of receptors to which oestrogens bind to exert their action on target cells. There are two types of ERs namely, oestrogen receptor alpha ( $ER\alpha$ / NR3A1) (Green et al., 1986) and beta ( $ER\beta$ / NR3A2) (Mosselman et al., 1996). Upon ligand activation these receptors bind to specific DNA sequences on the target gene promoter called oestrogen response elements (ERE) to activate the transcription of the target gene.  $ER\alpha$  and  $ER\beta$  can also bind to the activating protein-1 (AP-1) response element where  $ER\alpha$  acts as a transcriptional activator and  $ER\beta$  as a repressor (Gustafsson, 2000).

Both ERs are expressed in the human myometrium and foetal membranes (Khan-Dawood and Dawood, 1984). Evidence shows that  $ER\alpha$  levels are quite low in non-labouring myometrium compared to labouring myometrium and the increase occurs towards parturition (Hegele-Hartung et al., 1992; Tan et al., 1999). Thus,  $ER\alpha$  is believed to be the modulator for oestrogen refractoriness of the myometrium. Mesiano and Welsh in their study found a correlation between the mRNA levels of  $ER\alpha$  and the ratio of progesterone receptor A (PR-A)/B (PR-B), suggesting the role of that nuclear progesterone receptor (nPRs) have a role in regulating  $ER\alpha$  expression (Mesiano and Welsh, 2007).

### ***1.7.2 Progesterone receptors (PR)***

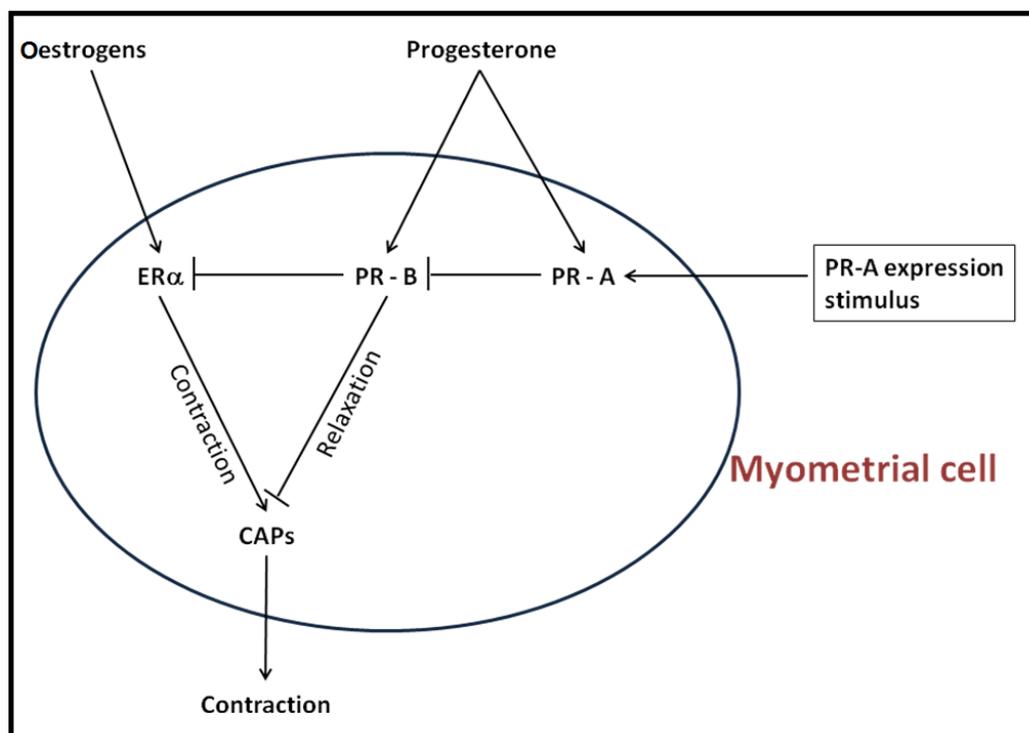
Like the other steroid hormones, progesterone acts primarily via ligand-activated nuclear transcription factors. Receptors to which progesterone binds to exert their actions are called progesterone receptors (PRs). Humans have three types of PR namely, PR-A, PR-B and PR-C that are products of the same gene with two distinct promoters (Kastner et al., 1990; Ogle et al., 1998). PR-A and B are the most functionally active receptors while PR-C represses PR-B activity (Ogle et al., 1998). Of the two receptors PR-A and PR-B, PR-B is the main effector of progesterone activity, whereas PR-A is a shorter transcript variant of PR-B that acts as a dominant negative repressor. PR-B contains three activation function (AF) elements in it whereas PR-A contains only two AFs, explaining the difference in its function (Hovland et al., 1998). Pieber and colleagues in their study demonstrated that PR-A can dose-dependently repress PR-B activity in cultured human myometrial cells

(Pieber et al., 2001). Smith and co-workers analysed the expression of PR-A and PR-B in myometrial biopsies at term and found that both PR-A and PR-B mRNA levels increased in tissue samples at term compared to samples not at term, while the rate of increase of PR-A was greater than for PR-B (Smith et al., 2002). They also found an increase in ER $\alpha$  mRNA but not ER $\beta$ . To prove that PR expression change is associated with changes in myometrial progesterone responsiveness, the expression of two progesterone responsive genes, ER $\alpha$  (Haluska et al., 1990; Leavitt and Clark, 1979; Wilson and Williams, 1998) and HOXA10 (Cermik et al., 2001) were examined and a positive association of their expression with PR-A/PR-B mRNA ratio was established. Thus, the increase in PR-A/PR-B ratio in the myometrium at term plays a role in functional progesterone withdrawal during parturition in woman. Another study suggests that PR-C also contributes to repressing PR-B activity in labouring myometrium (Condon et al., 2006). Thus, research so far points to differences in the ratio of PR-A or (PR-C) to PR-B in the regulation of the functional withdrawal of progesterone during labour.

To investigate the relationship between PR-A/PR-B ratio and ER $\alpha$  expression, the levels of the oestrogen-responsive genes COX2 and oxytocin receptor (OTR) were investigated in labouring myometrium (Slonina et al., 2009). ER $\alpha$  mRNA levels correlated positively with COX2 and OTR mRNA levels (Slonina et al., 2009). This finding suggests that the PR-A/PR-B ratio modulates the level of ER $\alpha$  expression, which in turn switches on the functional activation of oestrogen responsiveness of the human myometrium at term. Overall, PRs play a vital role in the transformation of the human myometrium from a relaxed tissue to an actively contracting one by changing its responsiveness to progesterone and oestrogen at a functional level (Slonina et al., 2009).

Evidence also indicates that the interaction of nPRs (nuclear PRs) with their substrates decreases towards parturition (Merlino et al., 2007). The main mechanism by which this occurs is by reduction of nPR co-activators (Mesiano, 2007) and/or by the inhibition of transactivation of nPR by the protein polypyrimidine tract-binding protein-associated splicing factor (PSF) (Dong et al., 2005; Mesiano, 2007). PSF also controls myosin phosphatase activity, an important regulator of myometrial contraction, by regulating the splicing of myosin phosphatase targeting protein

mRNAs (Dong et al., 2005). Thus, the genomic effect of progesterone's role in the regulation of myometrial contractility, functions through a complex mechanism involving multiple levels of progesterone withdrawal. A schematic representation of the regulation of myometrial contraction by steroid hormone receptors is provided in Figure 1.10.



**Figure 1.10: Model for the role of the myometrial ER and PR in regulating human pregnancy and parturition.** For most of pregnancy progesterone, acting through PR-B, inhibits expression of ER $\alpha$ . At term, expression of PR-A increases, leading to functional progesterone withdrawal. As a consequence, expression of ER $\alpha$  is co-ordinately increased leading to increased myometrial responsiveness to circulating oestrogens, which in turn increase expression of genes encoding contraction-associated proteins (CAPs) that increase myometrial contractility and excitability (Smith et al., 2002).

## 1.8 Parturition

Parturition is a result of the complex interaction between both maternal and foetal factors, which is marked by the initiation of synchronised and rhythmic uterine contraction that propels the foetus and the placenta out of the uterus. This is a crucial step in pregnancy and the timely activation of parturition is very important for both foetal and maternal health and survival (Hilder et al., 1998).

### ***1.8.1 Phases of parturition***

Pregnancy and parturition can be divided into four independent but overlapping phases (Challis et al., 2000; Marshall and Lamming, 1994). Various studies point that key regulators such as progesterone, relaxin, CRH, nitric oxide, prostacyclin (PGI<sub>2</sub>) and parathyroid hormone-related peptide (PTH-rP) regulate uterine quiescence and the initiation of contraction during parturition, in an endocrine and/or paracrine fashion (Challis et al., 2002; Challis et al., 2000; Mesiano and Welsh, 2007; Norwitz et al., 1999; Zakar and Mesiano, 2011). Phase 0, the pregnant state – is the longest phase where the uterus remains relatively dormant. In Phase 1, mechanical stretch of the uterus occurs due to foetal growth. This leads to the activation of a group of genes called contraction-associated proteins (CAPs), which include Connexin 43, agonist receptors and ion channel proteins. CAPs are activated through two independent pathways – one by the activation of foetal hypothalamic-pituitary-adrenal (HPA) axis and the other by expansion of the uterus, triggering stress-related up-regulation of CAP genes (Whittle et al., 2001). During Phase 2 of parturition, uterotonins such as prostaglandins, corticotrophin-releasing hormone (CRH) and oxytocin stimulate the uterus to initiate uterine contraction leading to labour. The last phase of parturition, Phase 3 marks the discharge of the placenta and involution of the uterus mainly due to the effect of oxytocin.

### ***1.8.2 Parturition in humans and other animals***

The endocrine events during parturition are unique to each species (Chaim and Mazor, 1998; Jenkin and Young, 2004; Kamel, 2010; Mitchell and Taggart, 2009; Navitsky et al., 2000; Vidaeff and Ramin, 2008). Various theories have been proposed for the control and onset of human parturition including the theory that the foetus is the determinant factor for the initiation of labour. The latest and the most accepted theory suggest that the joint action of steroid hormones, paracrine molecules and inflammatory responses controls parturition (Chaim and Mazor, 1998; Jenkin and Young, 2004; Kamel, 2010; Mitchell and Taggart, 2009). Enormous efforts have been invested to understand the cascade of events leading to parturition in humans, but with limited results (Chaim and Mazor, 1998; Jenkin and Young, 2004; Kamel, 2010; Mitchell and Taggart, 2009). The reason lies in two key issues: firstly, the hormonal

control of parturition in humans is considerably different from that of other animals, and secondly, ethical issues limit experimental studies on humans.

However, a substantial wealth of information and understanding is available about the molecular mechanisms that regulate parturition in animals, especially in sheep (Ellwood and Anderson, 1981; Liggins et al., 1973). In most animals studied progesterone withdrawal is a quintessential prerequisite for parturition (Csapo, 1956; Csapo and Wiest, 1969). In animals, where the corpus luteum is the primary source of progesterone, luteolysis initiates progesterone withdrawal. However, in humans, placenta is the main source of progesterone during late pregnancy (Chaim and Mazor, 1998; Jenkin and Young, 2004; Kamel, 2010; Mitchell and Taggart, 2009). Also, there is very little difference seen in circulating maternal serum progesterone levels towards term (Boroditsky et al., 1978; Tulchinsky et al., 1972). Thus progesterone withdrawal in the human system is believed to happen at a functional level prior term.

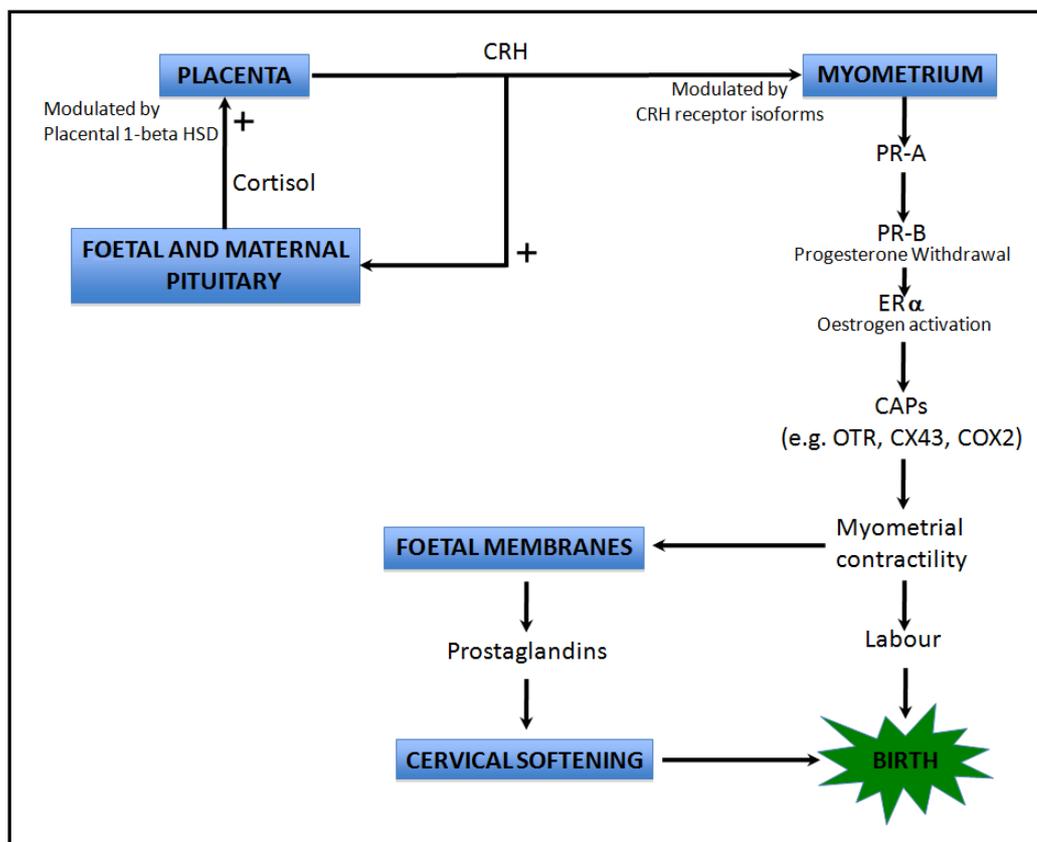
During parturition, in sheep, CRH secreted by the hypothalamus controls pituitary adrenocorticotrophic hormone synthesis, which in turn controls the production of foetal cortisol. Foetal cortisol synthesised by the adrenal gland of sheep controls the placental production of steroid hormones oestrogen and progesterone (Cox, 1975; Liggins, 1974; Liggins et al., 1973; Thorburn, 1978). An increase in the foetal cortisol level switches the steroid synthesis pathway from progesterone synthesis to that of oestrogen (Liggins et al., 1973). Thus, an increase in the placental oestrogen level induces contraction-associated proteins (CAPs) and progesterone withdrawal from the system (Brown et al., 2004; Chwalisz and Garfield, 1994). The cascade of events in human parturition is quite different from this. Unlike in other mammals, it is the placenta and not the adrenals that produce corticotropin-releasing hormone (CRH) and chorionic gonadotropin (Abou-Seif et al., 2012; Grammatopoulos, 2007; Lalmansingh and Uht, 2008; Norman et al., 1993b; Sengupta et al., 2008). Therefore foetal maturation does not play a vital role in human parturition. Also the molecular structures of ovine CRH and chorionic gonadotropin vary from that of human. In sheep, damage to the foetal hypothalamus, pituitary or adrenal would terminate pregnancy, whereas in humans this is not the case.

Some degree of similarity can be found in the process of pregnancy and parturition among non-human primates. However extrapolation of this information to the human

system becomes difficult due to the variation of events and controls in humans compared to lower primates. For example, despite the similarity in the molecular structure of placental CRH and oestrogen in baboons and humans, there is a marked difference in the pattern of their production during pregnancy (Goland et al., 1992; Smith et al., 1993). Only chimps and gorillas have been shown to have similar hormonal changes during pregnancy (Smith et al., 1999). Limited animal studies are performed on these species since the ethical issues are similar to those for human studies, and also because of the threat of spread of potentially serious viral illness to humans as a result of the studies on primates.

Evidence suggests that in humans there is an increase in the expression of PGHS-2 and increased synthesis of prostaglandins within the uterus prior to labour (Gyomory et al., 2000). However, the role of prostaglandins and PGHS-2 in parturition is unclear. In animals, the production of  $\text{PGF}_{2\alpha}$  has a crucial role in causing luteolysis and subsequently progesterone withdrawal, thus initiating labour (Gross et al., 1998). Evidence also suggests that stimulation of PGHS is an important part of the immune response that accompanies labour (Garfield et al., 1980). In animals where gestation is corpus luteum dependent, immune response stimulates the production of  $\text{PGF}_{2\alpha}$  followed by luteolysis and parturition (Garfield et al., 1980). However, this cascade of events does not occur in animals where progesterone is predominantly synthesised in the placenta.

A proposed mechanism for the induction of labour in humans is depicted in Figure 1.11. CRH produced by the placenta increases the expression of PR-A in the myometrium. This induces the inactivation of PR-B by PR-A, hence causing functional progesterone withdrawal despite the presence of high circulating progesterone in the system. PR-A also increases the expression of  $\text{ER}\alpha$  thereby increasing the responsiveness of the myometrium to circulating oestrogen. These events induce the expression of various contraction-associated proteins initiating myometrial contraction and subsequent labour.



**Figure 1.11: Proposed mechanism of labour initiation at term.** CRH induces the expression of progesterone receptor A in the myometrium. PR-A mediates inactivation of PR-B (active receptor for progesterone) and thereby progesterone withdrawal. This activates the alpha type oestrogen receptor increasing the myometrial sensitivity to circulating oestrogen. Oestrogen activation induces the expression of contraction-associated proteins (CAPs), subsequently myometrial contractility, labour and finally childbirth. Myometrial contraction induces immune response that increases the production of prostaglandins, which help in cervical remodelling. Figure adapted from (Smith et al., 2002).

### 1.9 Functional genomic studies of pregnant and labouring human myometrium

With the availability of near entire genome sequences for humans and many model organisms, high throughput global transcriptional analysis has gained popularity (White, 2001). Several methods are in place for assessing the expression of a gene both at mRNA and protein levels. These include northern blotting, western blotting, reverse transcription polymerase chain reaction (RT-PCR), fluorescent *in situ* hybridisation and suppression subtractive hybridisation (SSH). These traditional one-gene one-experiment methods are used to understand the role of a specific gene expression under particular conditions at a specific time. However, the limitation of these experiments include, (1) do not provide a global view of the transcriptomic or proteomic profile of a cell, (2) long time taken for the experimental procedures, and

(3) the cost of the experiment. These reasons have limited scientists from understanding complex physiological conditions where multiple gene products are involved in regulating cellular transformations. DNA microarray technology is a relatively new and powerful technique used in profiling the transcriptome of a cell or tissue sample under various physiological or treatment conditions (Dunckley et al., 2005). This technique also exploits the relation between the function of a gene product and its expression in a cell at a particular time. Except in the case of housekeeping genes, a gene is expressed in a tissue only when its product make a functional contribution to the survival of a cell under that condition (Brown and Botstein, 1999). The microarray approach allows simultaneous comparison of multiple genes in one experiment improving the experimental efficiency and reducing the time and cost involved in the study (Schena et al., 1995).

As evident from earlier sections, the transformation of a relatively quiescent uterus during pregnancy to a highly contractile organ in labour is the end result of several complex processes. Studying the transcriptomic changes of the uterus during pregnancy and labour would provide a comprehensive and unbiased insight into physiologic events during parturition (Hu et al., 2006; Khoury and Romero, 2006; Kolialexi et al., 2008; Romero et al., 2006; Romero et al., 2010; Tarca et al., 2006).

Since the dawn of high-throughput transcriptomic studies many attempts have been made to understand the molecular mechanisms involved in parturition in other animals (Girotti and Zingg, 2003; Haddad et al., 2008; Hashizume et al., 2007; Helguera et al., 2009; Mason et al., 2006; Salomonis et al., 2005; Weiner et al., 2006; Zhao et al., 2007). Many studies were also performed to identify differentially expressed genes and proteins in the human labouring gestational membranes (Haddad et al., 2006; Lee et al., 2010a; Lee et al., 2010b; Li et al., 2011; Marvin et al., 2002a, b; Sitras et al., 2008; Tromp et al., 2004), amniotic fluid (Buhimschi et al., 2008; Bujold et al., 2008; Cho et al., 2007; Cobo et al., 2009; Gravett et al., 2004; Michaels et al., 2007; Park et al., 2008; Park et al., 2006; Romero et al., 2008; Ruetschi et al., 2005; Vuadens et al., 2003), umbilical cord blood (Madsen-Bouterse et al., 2010), uterine cervix (Bollapragada et al., 2009; Bukowski et al., 2006; Hassan et al., 2006; Hassan et al., 2010; Hassan et al., 2007; Hassan et al., 2009; Huber et al., 2005; Mowa et al., 2008; Read et al., 2007; Wang et al., 2001), and human myometrium

(Aguan et al., 2000; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Bethin et al., 2003; Bukowski et al., 2006; Chan et al., 2002; Charpigny et al., 2003; Cordeaux et al., 2010; Esplin et al., 2005a; Esplin et al., 2005b; Havelock et al., 2005; Khanjani et al., 2011; Mittal et al., 2010; O'Brien et al., 2008; Rehman et al., 2003) shedding more insight into the processes of parturition. This section compiles all microarray studies that were performed in pregnant human myometrium to understand the transcriptomic changes between labouring and non-labouring myometrial samples (Table 1.1).

Aguan and colleagues were the first to use functional genomics to study the regulation of uterine quiescence and contractility during pregnancy (Aguan et al., 2000). In their study they used cDNA array blots to compare the expression profile of labouring and non-labouring myometrial tissues at term. They reported 21 genes to be differentially regulated (12 upregulated genes and nine downregulated genes) between the samples (Aguan et al., 2000). Soon after Chan and his colleagues used suppression subtractive hybridisation (SSH) to identify genes whose expression changed as a result of labour (Chan et al., 2002). Dot blot screening of 400 positive clones were analysed of which 30 clones were differentially regulated with 14 clones upregulated in term labouring myometrium. Several of the identified genes were previously associated with labour (Chan et al., 2002).

Three microarray studies were published in 2003 comparing labouring and non-labouring myometrial samples from preterm and term pregnancy. Charpigny and co-workers compared preterm, term not in labour and term in labour myometrial samples using DNA microarrays to identify their gene expression profile (Charpigny et al., 2003). They reported parturition was associated with a down-regulation of a large panel of developmental, cell adhesion molecules (CAMs) and proliferation-related genes, along with the up-regulation of inflammatory, contraction and apoptosis-associated genes (Charpigny et al., 2003). Bethin and colleagues used oligonucleotide arrays to analyse gene expression in murine and human uterus during term pregnancy (Bethin et al., 2003). Several genes were identified to be differentially regulated both in human and mouse samples. They reported that the pattern of gene regulation within functional groups were similar in human preterm and term labouring samples. However, the magnitude of changes among these groups varied. They also reported that mouse and human shared few genes that changed their expression significantly

during pregnancy (Bethin et al., 2003). In a separate study Rehman and colleagues analysed the gene expression profile of pregnant and non-pregnant human term myometrium using cDNA microarray (Rehman et al., 2003). Several genes were reported to be upregulated in the study, but only eight transcripts were repressed in pregnant myometrium compared to non-pregnant samples (Rehman et al., 2003).

In 2005, Havelock and colleagues published results where they compared the gene expression profile of human fundal myometrium isolated from patients before and after the onset of labour (Havelock et al., 2005). Several genes including the previously reported oxytocin receptor (OXTR) were found to be differentially regulated in myometrial samples obtained from labouring patients (Havelock et al., 2005). Esplin and co-workers also compared the transcriptome of myometrial samples from patients in spontaneous labour and that not in labour (Esplin et al., 2005a). Expression of only 56 genes changed with an increase in expression of 22 transcripts and the rest were decreased. The four upregulated genes whose expression confirmed by RT-PCR were thrombospondin-1 (THBS1), pre-B-cell colony enhancing factor 1 (PBEF1), superoxide dismutase 2, mitochondrial (SOD2) and nicotinamide N-methyl transferase (NNMT) (Esplin et al., 2005a).

O'Brien and colleagues used DNA microarray experimental analysis to compare the expression profile of human myometrium at term pregnancy to that in labour (O'Brien et al., 2008). Differential expression of many genes was identified which included a few genes previously reported to be upregulated in labouring myometrium. Several novel genes were also reported to be upregulated in labouring myometrium including pleckstrin homology, Sec 7 and coiled coil domains, binding protein (PSCDBP), endothelin- $\beta$  receptor (EDNRB), toll-like receptor 2 (TLR2), TWIST1 and regulator of G-protein signaling-12 (RGS12) (O'Brien et al., 2008).

Mittal and colleagues also analysed the human myometrial transcriptome during spontaneous labour at term (Mittal et al., 2010). The comparison was made between women in term labour and term not in labour using a microarray platform. The study reported 471 genes differentially expressed in labouring myometrium. Gene ontology analysis of these genes identified 103 biological processes and 18 molecular functions. Some of the main functions were cytokine activity, inflammatory response and chemokine activity. Six pathways including cytokine-cytokine receptor

## ***Chapter 1: Introduction***

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interaction, Jak-STAT signalling, complement and coagulation cascades, NOD-like receptor signalling pathway, systemic lupus erythematosus, and chemokine signalling pathways were identified by Pathway analysis (Mittal et al., 2010).

There are only very few common genes observed in these above studies. The reason for this could be due to: (1) the limited number of patient samples considered for each study; (2) differences in the region of the uterus from which the samples were collected; and (3) dissimilarities in the approach and stringency in the informatics analysis. In spite of this, each of these studies has improved our understanding about the complex mechanism that regulates human pregnancy and parturition. However, tight ethical regulations and difficulty in obtaining human samples have made *in vivo* studies more difficult. Availability of a myometrial cell culture system would facilitate performance of more high-throughput experiments generating more data that could be analysed to gain a better understanding on myometrial contraction and labour. Less stringent ethical regulation, wider availability of cells and simple experimental procedures makes cell culture systems a good choice in addressing such complex physiological mechanisms. This thesis focuses on developing a human myometrial smooth muscle cell culture system to understand the effect of progesterone and 17 $\beta$ -estradiol treatment on these cells. Transcriptomic profiling of this system should shed more light on the molecular events that regulate pregnancy and parturition.

Table 1.1: Published microarray studies in human myometrium during pregnancy and labour.

No	Name of the research article	Author, year and experiment type	Brief description of the study
1	Application of a functional genomics approach to identify differentially expressed genes in human myometrium during pregnancy and labour	Aguan et al., 2000, cDNA array blot	Comparison between labouring myometrium versus non-labouring myometrium. Expression of 9 genes decreased whereas 12 genes increased in labouring myometrium (more than 50% difference compared to non-labouring myometrium).
2	Human myometrial genes are differentially expressed in labour: a suppression subtractive hybridization study	Chan et al., 2002, SSH	Dot blot screening of 400 clones from labouring and non-labouring myometrial samples was performed. 30 of these were found to be differentially regulated of which 14 were upregulated in labour.
3	A functional genomic study to identify differential gene expression in the preterm and term human myometrium	Charpigny et al., 2003, cDNA microarray	Comparison between pre-term, full term-not-in-labour and full term-in-labour. 82 genes were downregulated between pre-term and term-in-labour and 35 genes were upregulated between pre-term and term-in-labour ( $P < 0.05$ selected).
5	Human myometrial adaptation to pregnancy: cDNA microarray gene expression profiling of myometrium from non pregnant and pregnant women	Rehman et al., 2003, cDNA microarray	Human pregnant versus non-pregnant myometrium were compared. Of the 7075 cDNA elements in the array 602 transcripts were found to be upregulated (>2-fold) and nine were found downregulated (<2-fold).
6	Human myometrial gene expression before and during parturition	Havelock et al., 2005, DNA microarray	Gene expression profiling of human fundal myometrium before and after the onset of labour. Two sets of arrays were performed, one a pooled sample array and the other single sample arrays. In at least one array 42 transcripts were found to be upregulated (>3-fold) in labouring myometrium and only three transcripts were differentially expressed (>3-fold) in both arrays. Only two transcripts were found to be downregulated (>3-fold) at least in one of the arrays and none was downregulated in both.

7	The use of cDNA microarray to identify differentially expressed labour-associated genes within the human myometrium during labour	Esplin et al., 2005, cDNA microarray	mRNA from myometrium, foetal membrane and placenta were collected from labouring and non-labouring patients to perform a cDNA array. A total of 56 transcripts were observed to be differentially expressed of which 22 transcripts were increased and the rest were decreased in expression ( $P < 0.01$ selected).
8	Up-regulation of PSCDBP, TLR2, TWIST1, FLJ35382, EDNRB and RGS12 gene expression in human myometrium at labour.	O'Brien et al., 2008, Oligonucleotide microarray	mRNA array of non-labouring and labouring myometrium were carried out. 698 genes were differentially expressed with a confidence level of $P < 0.05$ and 105 genes with $P < 0.01$ .
9	Characterization of the myometrial transcriptome and biological pathways of spontaneous human labour at term	Mittal et al., 2010, cDNA microarray	Transcriptomics of human myometrium during spontaneous labour at term was analysed using cDNA microarray. 471 genes were differentially expressed. Gene ontology analysis was performed to enrich the gene list with 103 biological processes and 18 molecular functions ( $> 1.5$ fold difference).
10	Effects of progesterone treatment on expression of genes involved in uterine quiescence.	Soloff et al, 2011, cDNA microarray	The effect of progesterone treatment on immortalised human myometrial cells cultured from a patient near the end of pregnancy. They demonstrate that progesterone treatment alters a variety of cellular mechanisms that favour myometrial relaxation including increased expression of calcium and voltage-operated $K^+$ channels, increased cellular $Ca^{2+}$ concentration and down-regulation of proteins involved in actin and myosin cross-link formation ( $>1.8$ fold difference).

### **1.10 Uterine smooth muscle cell culture system**

Uterine smooth muscle cells can be successfully cultured *in vitro* either by an enzymatic isolation method (Chamley-Campbell et al., 1979) or by an explant method (Chen et al., 1973; Kawaguchi et al., 1985; Moss and Benditt, 1975). Telomerase can be used to immortalise myometrial cells increasing the life span of these cells in

culture (Condon et al., 2002; Soloff et al., 2004). Several studies using primary human myometrial smooth muscle cells in culture were performed to identify regulation of response to retinoids (Boettger-Tong et al., 1997), effect of protein kinase C (PKC) on connexin 43 expression and cell proliferation (Taniguchi et al., 2001), endothelin induced myometrial contraction (Dallot et al., 2003), activation of potassium channels (Adelwoehrer and Mahnert, 1993) and expression of various receptors (Brown et al., 1997). Despite these studies, no efforts have been made to develop a cell culture model system that mimics *in vivo* uterine hormonal conditions to understand the complex changes these cells undergo during pregnancy and parturition. The most closely related study is a recent study where the effect of progesterone treatment was examined in immortalised human myometrial cells cultured from a patient nearing the end of pregnancy (Soloff et al., 2011). Here immortalised myometrial cells (engineered to express oestrogen receptor  $\alpha$  (ER-  $\alpha$ ) primed with oestrogen were subsequently treated with increasing doses of progesterone to elucidate the genomic effect of progesterone. The authors reported that progesterone treatment altered a variety of cellular mechanisms that favour myometrial relaxation including increased expression of calcium and voltage-operated  $K^+$  channels, increased cellular  $Ca^{2+}$  concentration and downregulation of proteins involved in actin and myosin cross-link formation (Soloff et al., 2011).

There is a marked difference in the events that precedes onset of labour in humans and well studied model organisms (Jenkin and Young, 2004; Mitchell and Taggart, 2009) making extrapolation of known facts from model organisms difficult. Hence, developing a model cell culture system to understand the role of progesterone and oestrogen regulating myometrial function is important to understand the transition of relatively quiet myometrium during pregnancy to an actively contracting tissue during labour.

### **1.11 Aims of Study**

Pregnancy and labour are events controlled by a complex interplay of maternal and foetal factors. These events are diverse and specific to each species. Human studies are limited due to ethical issues associated with collecting patient samples. However, understanding these underlying mechanisms is of utmost importance to diagnose and treat preterm and post-term labour. This would help significantly in improving the health of both mother and infant and also would reduce associated healthcare costs.

Progesterone and oestrogen are regarded as the two vital steroid hormones that play an important role in maintaining pregnancy and initiating labour (Brown et al., 2004). The aim of the study was to establish a myometrial cell culture model to understand the effect of steroid hormone treatment on myometrial function in cultured human uterine smooth muscle cells. Human uterine smooth muscle cells from non-pregnant myometrium were used to develop the cell culture model system since these cells were not exposed to high amount of progesterone and oestrogen.  $17\beta$ -estradiol is the most active form of oestrogen present in the maternal circulatory system and the main effector of oestrogenic action during pregnancy and labour. The aim of the first study was to analyse the gene expression profile of cultured hUtSMCs after treatment with  $17\beta$ -estradiol. This would help in identifying oestrogen-responsive genes in the human myometrium and also in understanding the role of these genes in parturition.

In the second study, hUtSMCs were treated (either separately or in combination) with oestrogen, progesterone and RU486 (progesterone inhibitor) to simulate *in utero* hormonal conditions during various stages of pregnancy. Transcript profiling of hUtSMCs following treatment was performed to understand the effect of these hormones in regulating myometrial function. Development of a suitable model cell culture system would provide better insight into the complex mechanisms that initiate and control myometrial contraction.

## **Chapter 2: Materials and Methods**

### **2.1 Materials**

All chemicals and reagents were of the highest grade obtainable and supplied by Ambion (CA, USA), Applied Biosystems (California, USA), Bio-Rad Laboratories (CA, USA), Fisher Scientific (Loughborough, UK), Invitrogen (Paisley, UK), Promega (Madison, USA), Qiagen (Hilden, Germany), Sigma-Aldrich (Poole, UK), Thermo Scientific (Erembodegem, Belgium) and Tocris Bioscience (Bristol, UK) as stated. Cell culture reagents were procured from Sigma-Aldrich (Poole, UK) and Gibco (Paisley, UK), unless otherwise stated. Foetal bovine serum (FBS) and double charcoal-stripped FBS (DCS-FBS) was supplied by Biosera (East Sussex, UK). Human uterine smooth muscle cells were purchased from Lonza (Slough, UK). Reagents and slides for microarray were procured from Operon (Cologne, Germany).

### **2.2 Methods**

#### ***2.2.1 Culture of human uterine smooth muscle cells***

Human uterine smooth muscle cells (hUtSMC), from the myometrium of normal non-pregnant women, were purchased from Lonza (UK). hUtSMCs were in passage 2 while procured from the company. Cells from non-pregnant myometrium were used because unlike myometrial cells from pregnant myometrium these cells will not be pre-exposed to high levels of circulating oestrogen or progesterone. Cells were maintained in T75 cm<sup>2</sup> tissue culture flasks (Sarstedt AG, Nümbrecht, Germany) with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS) (Biosera) and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich) at 37 °C, 5% CO<sub>2</sub> in a humidified incubator. Henceforth, this medium composition will be referred to as 'normal medium'. On reaching 80-85% confluence, cells were detached from cell culture flasks by incubation with 3 ml trypsin-EDTA (Ethylene diamine tetra acetic acid) solution in Hanks balanced salt solution (HBSS) (Sigma-Aldrich) for 3 min. Cells were collected by centrifuging at 1200 rpm, re-suspended in 'normal medium' and counted prior to reseeding in a new T75 cm<sup>2</sup> flask at 40,000 cells/ml 'normal medium'.

### **2.2.2 Cryopreservation**

hUtSMCs were frozen in 1 ml aliquots, at  $1 \times 10^6$  cells per ml of DMEM, supplemented with 20% FBS and 10% DMSO (Dimethyl sulfoxide), in 1.5 ml cryovials (Nunc, Wiesbaden, Germany). The vials were placed in a Nalgene Cryo 1 °C freezing container (Rochester, NY, USA) containing room temperature 100% isopropanol, and stored at -80 °C overnight. The freezing container is specified to freeze at a rate of 1 °C per min. Vials were transferred to a liquid nitrogen container (Jencons-PLS, Bedfordshire, UK) the following day for long-term storage.

### **2.2.3 Resuscitation**

Frozen cells were thawed by placing the vial at 37 °C water bath following removal from liquid nitrogen. The thawed cell suspension was added to 2 ml of pre-warmed medium in a 15 ml sterile tube. The cell suspension was incubated at room temperature for 2 min before an additional 2 ml media was added. The total cell suspension was then placed in a 25 cm<sup>2</sup> flask (Sarstedt). The cells were incubated in the conditions previously outlined, and the culture medium was changed the next day of resuscitation.

### **2.2.4 Preconditioning the cells for serum starvation**

For experiments cells within passage 5 and 8 were used to maintain the characteristics of the human uterine smooth muscle cell. The cells were serum starved prior to steroid hormone treatment to remove any endogenous steroids present in the cell culture medium. All serum-starved cells cultured in 'normal medium' were preconditioned overnight with 'preconditioning medium' preparing the cells for serum starvation and subsequent steroid hormone treatment. 'Preconditioning media' contained: Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% double charcoal-stripped foetal bovine serum (DCS-FBS) (Biosera) and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich). Cells were maintained at 37 °C, 5% CO<sub>2</sub> in a humidified incubator.

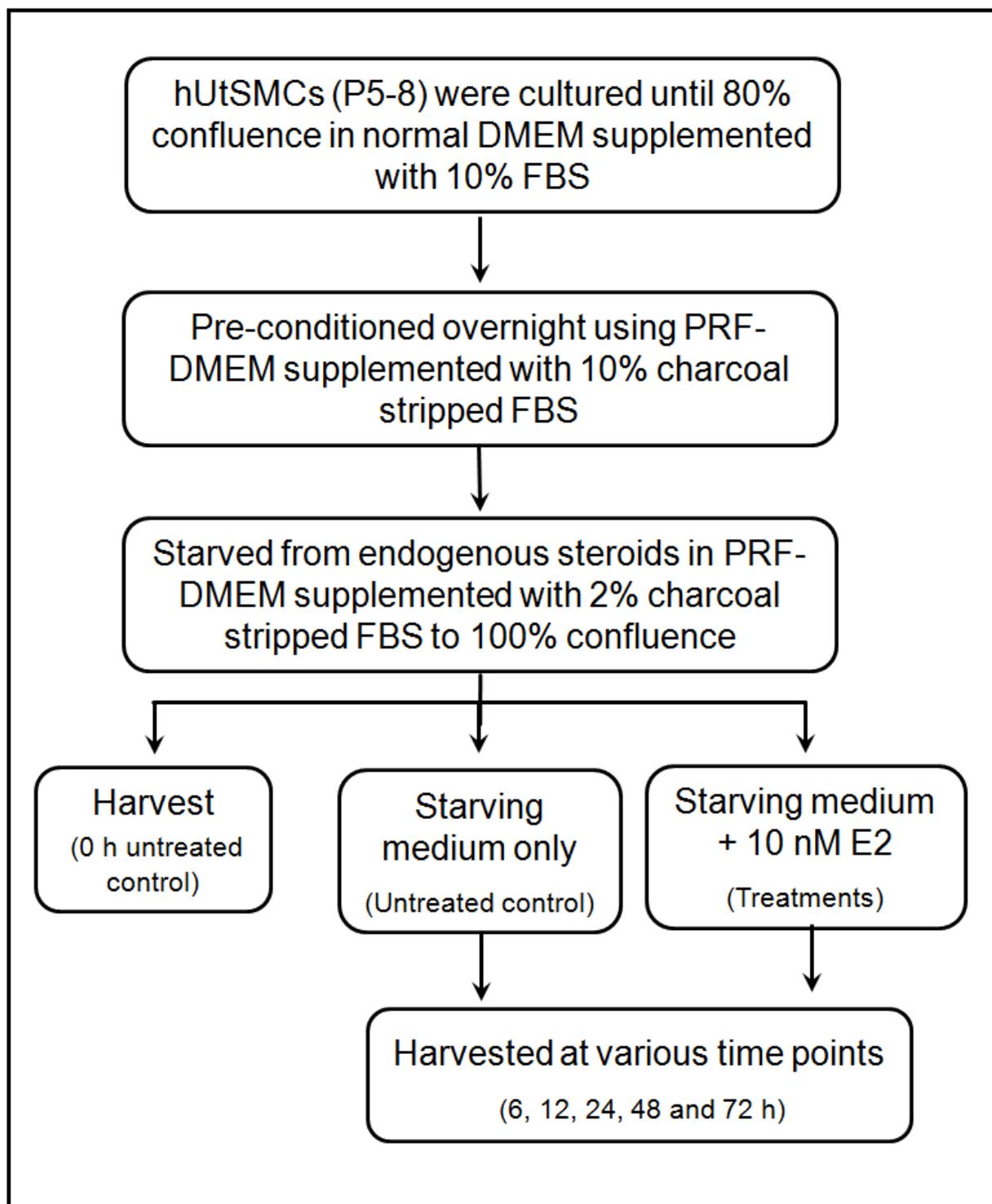
### **2.2.5 Serum starvation**

Since the aim of the experiments is to analyse the effect of steroid hormone treatment in hUtSMCs, cells were cultured in Phenol red-free (PRF) DMEM and serum starved to remove endogenous steroids from the system. Phenol red, a pH indicator used in cell culture media, is

proven to have weak oestrogenic effects (Berthois et al., 1986; Hubert et al., 1986; Wesierska-Gadek et al., 2007). Also serum supplements used in the media have traces of hormones (Cao et al., 2009). Preconditioned cells were starved from endogenous steroids by changing the medium to PRF-DMEM supplemented with 2% DCS-FBS and 100 units/ml penicillin and 0.1 mg/ml streptomycin ('starvation medium'). Cells were cultured in the starvation medium until treatment with relevant hormones.

### ***2.2.6 17 $\beta$ -Estradiol (E2) treatment***

For each 17 $\beta$ -estradiol (E2) (Sigma-Aldrich) treatment experiment, hUTSMCs (within passage 5 and 8) were cultured in 11 T25 cm<sup>2</sup> flasks with complete medium. Upon reaching 80-85% confluence these cells were preconditioned overnight as mentioned above. The cells were then cultured in 'starvation medium' until the cells attained 100% confluence. Cells in one flask were harvested at the time of E2 treatment (0 h control). Cell culture medium was changed (5 ml per T25 flask) in all the flasks with 'starvation medium', where only the treatment flasks were further supplemented with 10 nM E2. E2 was diluted in DMSO for the stock concentration and was further diluted in 'starvation medium' for making the working concentration (<0.001 % DMSO in the treatment media; Appendix 1). The cells were cultured until the desired time point and harvested along with their corresponding controls. Different time points were chosen to study the change in the time-dependent expression profile of E2-responsive genes. Each set of experiments was carried out on three different dates to generate statistically significant results. This was the experimental setup for Chapter 3. The schema of this experiment is explained in a flow diagram (Figure 2.1).



**Figure 2.1 Experiment workflow for 17β-estradiol (E2) treatment.** hUtSMCs (within passage 5 and 8) were cultured in ‘normal medium’. Upon 80% confluence, the cells were pre-conditioned overnight and prepared for serum starvation by changing the culture medium to ‘preconditioning medium’. Following day the medium was changed to ‘starvation medium’ and the cells were cultured until they attained 100% confluence. Cells cultured in one flask were harvested at E2 treatment (0 h control). The remaining flasks were divided into two sets and medium in the one set of flasks was changed to fresh ‘starvation medium’ (control cells) and in the other set of cells ‘starvation medium’ was supplemented with 10 nM E2 (E2-treated samples). Cells were cultured until desired time points and harvested along with their controls.

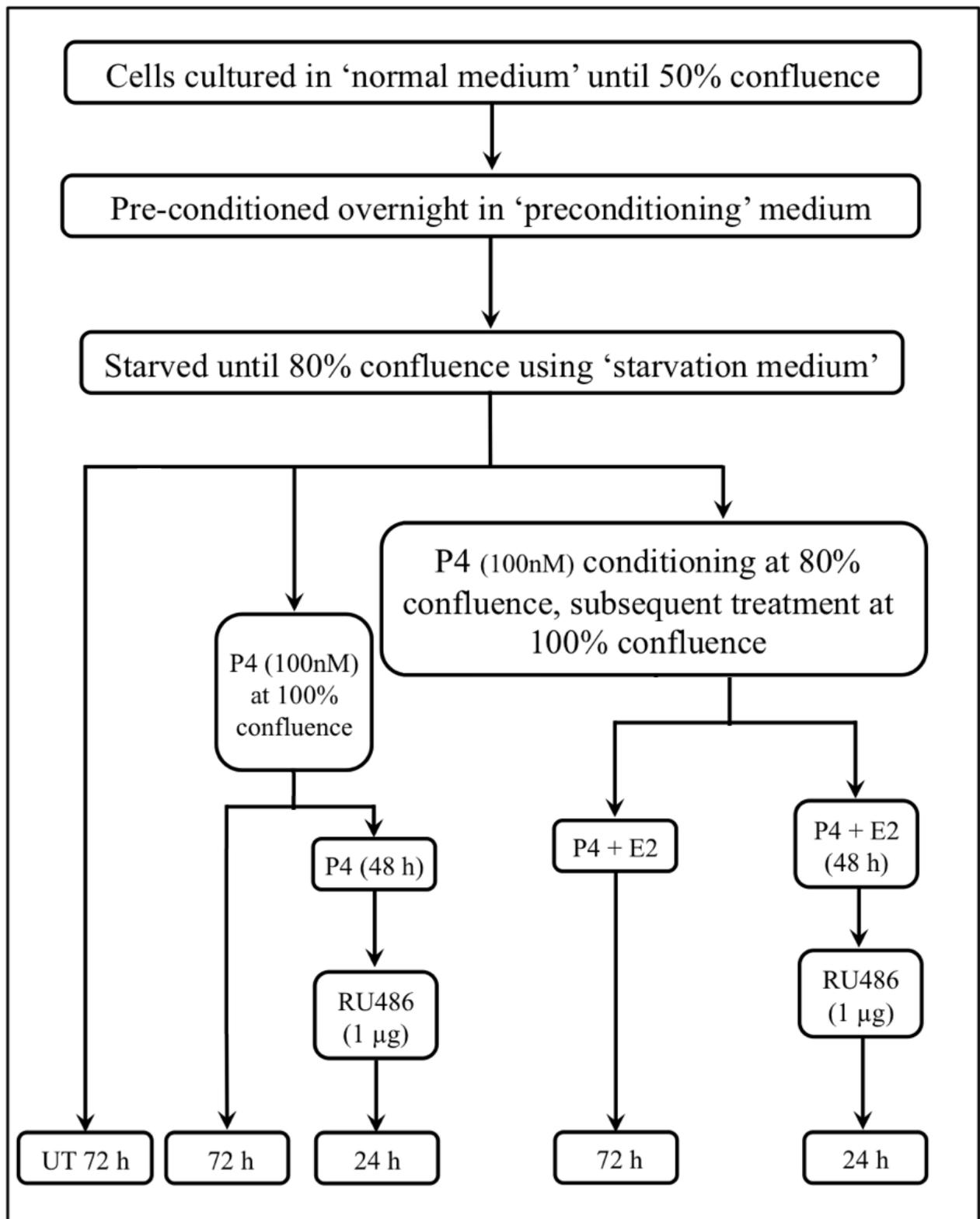
**2.2.7 Progesterone (P4) and Mifepristone (RU486) treatment**

For P4 and RU486 treatment, cells were cultured in 5 T25 cm<sup>2</sup> flasks with ‘complete medium’ until they reached 50% confluence. The cells were preconditioned overnight and serum-starved as mentioned earlier (Sections: 2.2.4 and 2.2.5) until the cells reached 80% confluence. Cells in one flask were left untreated (control) and were harvested along with the other treated cells. Cells in the second and third flask were treated with 100 nM P4 (Sigma-Aldrich) alone at 100% confluence. Cells from one of these flasks were harvested after 72 h (P4 only treated). Cells in the other flask was co-treated with 1µM of RU486 48 h after P4 treatment and harvested after 24 h of RU486 co-treatment (to study the effect of functional progesterone withdrawal). At 80% confluence cells in 2 flasks (out of 5) were P4 pre-treated (with 100 nM P4) and cultured until 100% confluence. At 100% confluence, cells from the one of the flasks were co-treated with 10 nM E2 and harvested after 72 h of E2 co-treatment (to study the effect of high progesterone and oestrogen on hUtSMCs). At 100 % confluence, cells from the other P4 pre-treated samples were co-treated with 10 nM E2 followed by addition of 1µM of mifepristone (RU486; Sigma-Aldrich) 48 h after E2 treatment. The cells from this flask were harvested 24 h after RU486 treatment (study the effect of high E2 in the presence of functional progesterone withdrawal). Each set of experiment was carried out on three different dates to generate statistically significant results. This was the experimental setup used in Chapter 4. Workflow of the experiment is illustrated in Figure 2.2.

The treatment and the corresponding *in vivo* hormone conditions mimicked are tabulated in Table 2.1.

**Table 2.1. Treatments and corresponding *in vivo* hormone conditions mimicked.**

<b>Flask number</b>	<b>P4</b>	<b>E2</b>	<b>RU486</b>	<b><i>In vivo</i> condition mimicked</b>
1	-	-	-	Untreated sample
2	+	-	-	P4 treated sample
3	+	+	-	Steroid hormone condition observed in pregnant myometrium
4	+	-	+	Functional P4 withdrawal
5	+	+	+	High E2 and functional P4 withdrawal; steroid hormone condition observed in labouring myometrium



**Figure 2.2 Experiment workflow for progesterone, 17 $\beta$ -estradiol and RU486 treatment.** hUtsMCs (between passage 5 and 8) were cultured in 'normal medium'. At 50% confluence cells were preconditioned overnight and further serum-starved until cells reached 80% confluence. Two flasks were P4 pre-treated until cells reached 100% confluence. One of these flasks was further co-treated with E2 and the other with both E2 and RU486. Two flasks were treated only with P4 once the cells reached 100% confluence. One set was harvested 72 h after P4 treatment and the other was co-treated with RU486, harvested 24 h post-RU486 treatment. Another flask was left untreated and harvested 72 h after the cells reached 100% confluence.

### **2.2.8 Cell lysate preparation**

At the time of harvest following cell treatment, cells were washed twice in cold phosphate buffer saline (PBS). Cell lysis buffer (50  $\mu$ l) (20 mM HEPES pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 1 mM  $MgCl_2$ , 0.1 mM EGTA and 1% NP-40), containing protease and phosphatase inhibitors (2.5  $\mu$ g/ml Aprotinin, 100  $\mu$ M PMSF, 0.5 mM DTT, 25  $\mu$ M ALLN, 10  $\mu$ M Leupeptin, 2  $\mu$ g/ml Pepstatin) was then added to tissue culture flasks. Cells were scraped in lysis buffer, and transferred to 1.5 ml tubes, vortexed and incubated on ice for 15 min. Protein lysates were stored at  $-80^\circ C$ , and allowed to defrost on ice prior to use.

### **2.2.9 Protein assay**

Protein concentration was determined using the Bradford assay (Bradford, 1976). BSA standards were prepared in protein lysis buffer 1 mg/ml. Each cell lysate was vortexed for 5 s before a 2  $\mu$ l aliquot was pipetted into triplicate wells of a 96-well plate (Sarstedt) containing 100  $\mu$ l of Bradford Reagent. Five microlitres of each standard was pipetted into triplicate wells. The colour was allowed to develop for 5 min, after which the absorbance was read at 595 nm on a Victor<sup>2</sup> 1420 plate reader (Wallac, MA, USA). A standard curve of absorbance versus protein concentration was constructed from the absorbance of the BSA standards. The protein concentration in the cell lysates was calculated using the equation of the standard curve.

### **2.2.10 Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-polyacrylamide gels were prepared according to Tables 2.2 and 2.3 below. The running gel was prepared (Tables 2.2) depending on the polyacrylamide percentage required and smoothly pipetted into the space between the plates. Isopropanol was gently pipetted on top (to level the gel mixture) and the gel was allowed to polymerise. The stacking gel was prepared according to Table 2.3. Once the running gel was set the excess isopropanol was drained and the stacking gel was gently pipetted into the top of the running gel. The combs were positioned into the stacking gel and the gel was allowed to polymerise.

Cell lysates were separated on SDS-PAGE gels using the Bio-Rad Mini-Protean® 3 Cell System or the Bio-Rad Criterion® system. Protein lysate (30  $\mu$ g), prepared as described above (Section 2.2.8), was mixed with 5X Laemmli buffer. The samples were heated to  $95^\circ C$  for 5 min to denature the sample and were put on ice. Each sample was loaded on the gel, which was submerged in 1 X running buffer (0.31% Tris base, 1.88% glycine and 0.1% SDS). Pre-stained

broad-range protein marker (Fermantas) was loaded (5 µl) in one well of each gel. Samples were separated on the gel by electrophoresis in 1X running buffer. Until the dye front crossed the stacking gel the applied voltage was set at 80 V, thereafter the voltage was increased to 110 V until the dye front had just reached the end of the gel. The proteins separate according to the difference in molecular weight, with the smaller protein migrating fastest.

**Table 2.2 Preparation of SDS-PAGE running gel**

<b>10% Running Gel</b>	
<b>Reagents</b>	<b>Volume*</b>
H <sub>2</sub> O	2
30% acrylamide mix	1.7
1.5M Tris pH8.8	1.3
10%SDS	0.05
10% ammonium persulphate (APS)	0.05
TEMED	0.002

\* For 5 ml of running gel. All volumes are in ml.

**Table 2.3 Preparation of SDS-PAGE stacking gel.**

<b>Stacking Gel</b>	
<b>Reagents</b>	<b>Volume*</b>
H <sub>2</sub> O	1.36
30% acrylamide mix	0.34
1M Tris pH6.8	0.26
10%SDS	0.02
10% ammonium persulphate (APS)	0.02
TEMED	0.002

\* For 2 ml of stacking gel. All volumes are in ml.

### **2.2.11 Western blotting**

While electrophoresis progressed, transfer buffer was prepared by adding 100 ml of 0.1 M CAPS (Sigma-Aldrich) pH 11 to 200 ml of methanol (Sigma-Aldrich) and 700 ml of distilled water. The transfer buffer was stored at 4 °C. When the samples had fully separated and the dye front had migrated to the end of the gel, the gel was taken out of the casting tray, the stacking gel carefully removed and immersed in transfer buffer. Filter paper and sponges were soaked in

transfer buffer while the nitrocellulose membrane was activated by immersing in distilled water.

The gel was placed on top of sponge and filter paper. Nitrocellulose membrane (Whatman, UK) was placed above the gel and on top another filter paper and sponge was placed. Air bubbles were carefully removed. The transfer sandwich was placed in the transfer tank with the gel facing the anode side. The tank was filled with transfer buffer and transfer was carried out at 110 V for 90 min at 4 °C. The principle of the transfer is that electrical current is passed horizontally forcing the proteins to migrate from the gel onto the nitrocellulose membrane, where they become irreversibly bound. After completion of transfer, the nitrocellulose membrane was removed from the sandwich and stained with Ponceau S to visually ensure that all proteins were transferred to the membrane.

After washing off the Ponceau S, the membrane was blocked for 1 h to at room temperature prevent unspecified protein binding. Blocking solution was 5% milk (made up by dissolving 2.5 g of powdered milk, Marvel, Premier Foods) in 50 ml of PBS/(0.05 %)Tween. The primary antibody was prepared to its standard working ratio either in milk or BSA/PBS/(0.05 %) Tween (Table 2.4). The membrane was incubated in the primary antibody for 2 h at room temperature or overnight at 4 °C. The membrane was washed thrice for 5 min in PBS/(0.05 %)Tween to remove any excess primary antibodies. Binding of the primary antibody to target protein was detected using horseradish peroxidase conjugated secondary antibody. The secondary antibody (Jackson ImmunoResearch) specific to the Primary antibody was diluted either in milk or BSA/PBS/Tween to obtain 1:10,000 dilution. The membrane was incubated in secondary antibody at room temperature for 2 h. The membrane was again washed thrice for 5 min.

Pierce Supersignal Solution (ThermoScientific) was pipetted onto the membrane and was incubated for 5 min. This allows chemi-luminescent product to form on reaction with horseradish peroxidase, which can be detected on X-ray film (Agfa). The membrane was placed in a cassette. In the dark room, X-ray film was placed over the membrane. The film was exposed for the desired time and developed, washed and fixed.

**Table 2.4 Primary antibodies and working dilutions.**

<b>Antibody</b>	<b>Supplier</b>	<b>Species of origin</b>	<b>Dilution</b>
Connexin 43	Abcam (ab11369)	Mouse	1:1000
CD 38	Abcam (ab108403)	Rabbit	1:1000
HSC70	Enzo life science (ALX-804-067)	Mouse	1:2000

### ***2.2.12 Isolation of RNA***

All surfaces and equipment was treated with RNase Zap (Ambion) before use and sterile RNase-free filter tips, tubes and reagents were used for all RNA work. Treated and untreated cells (from all biological replicates) were washed using Hanks balanced salt solution and the cells were harvested. Total RNA was isolated from the cells using a combination of Trizol (Ambion) and Qiagen RNeasy Mini Kit (Qiagen). Cells were lysed in Trizol reagent (1 ml Trizol per  $1 \times 10^7$  cells) in a 1.5 ml tube. Two hundred microlitres chloroform (Sigma-Aldrich) per millilitre of Trizol reagent was added, vortexed for 2 min and incubated at room temperature for 10 min. The mixture was centrifuged at 13,000 rpm for 15 min at 4 °C and upper phase was taken for RNA isolation. The upper layer was mixed with equal volume of 70% ethanol, loaded onto the RNeasy column (Qiagen) and centrifuged at 10,000 rpm for 15 s in a microfuge. The column was washed with 700  $\mu$ l of RW1 buffer and the buffer removed by centrifuging at 10,000 rpm for 15 s. Seventy microlitres of DNase (Qiagen) was added to the column and incubated at room temperature for 15 min. The column was then centrifuged at 10,000 rpm for 15 s to remove the DNase. The column was again washed twice with 500  $\mu$ l RPE buffer, each time centrifuging at 10,000 rpm for 15 s to remove the buffer from the column. RNA was eluted in 70  $\mu$ l RNase-free water.

### ***2.2.13 Quantitation of RNA***

The quantity of the total RNA in each sample was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The instrument was set to measure the optical density (OD) at 260 nm (optimal absorption for RNA and DNA) and blanked using water. One microlitre of each sample was placed on the sampling arm and the OD values for 260/280. 260/280 ratio indicates protein contamination since proteins and other contaminants absorb at 280 nm (optimal reading  $\sim$  1.8). 260/230-absorbance ratio was also noted to detect phenolic contamination, since absorption for phenols are at 230 nm (optimum value between 1.8 and

2.2). Readings for 260/280 and 260/230 along with the quantity of the RNA detected were recorded.

#### **2.2.14 Quality control of RNA**

RNA quality was assessed using the Agilent 2100 Bioanalyzer and RNA 6000 Series II Nano Kit (Agilent). RNA 6000 Nano gel matrix (550  $\mu$ l) was centrifuged at 1500 rpm for 10 min and 65  $\mu$ l of gel were aliquoted. The gels were used within 4 weeks of preparation. RNA 6000 Nano dye concentrate blue was allowed to equilibrate to room temperature for 30 min. The dye was vortexed for 10 s and centrifuged. Dye (1  $\mu$ l) was added to 65  $\mu$ l of filtered gel. The solution was vortexed well and the tube was centrifuged at 13,000 rpm for 10 min at room temperature. Gel - dye mix (9  $\mu$ l) was pipette to well marked 'G' of the RNA chip mounted on the chip priming station. The chip priming station was closed and plunger was pressed until the clip held it. The clip was released after exactly 30 s. Gel - dye mix (9  $\mu$ l) was again pipetted to the second well marked-'G'. RNA sample (5  $\mu$ l) to be quantified were prepared at a final concentration of 100 ng/ $\mu$ l. All the samples and the RNA ladder were denatured at 72 °C for 2 min. RNA 6000 Nano Marker (5  $\mu$ l) was pipetted to well marked-'L' and in all 12 sample wells. RNA 6000 ladder (1  $\mu$ l) was loaded to the well marked-'L' and samples to the 12 sample wells. The chip was put into the adapter and vortexed for 1 min at 2400 rpm. The chip was processed in the Agilent 2100 Bioanalyzer within 5 min from preparation.

#### **2.2.15 cDNA synthesis**

cDNA synthesis was performed using Superscript II® reverse transcriptase (Invitrogen). RNA (2  $\mu$ g) was mixed with 1  $\mu$ l Oligo dT (50  $\mu$ M) (Invitrogen), 1  $\mu$ l dNTP Mix (10 mM each) and made up to a final volume of 10  $\mu$ l using nuclease-free water. The mixture was incubated at 65 °C for 2 min to reduce secondary structures. This was followed by incubation at 42 °C for 2 min for annealing of the Oligo dT to the RNA and to reduce the temperature of reaction mixture to the optimal temperature for Superscript II® reverse transcriptase. The reaction mixture was further incubated with 4  $\mu$ l 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl<sub>2</sub>) and 2  $\mu$ l of 0.1 M DDT (0.1 M). The final volume was made to 20  $\mu$ l using nuclease-free water and the mixture was incubated at 42 °C for 50 min followed by incubation at 75 °C for 10 min to inactivate Superscript II® reverse transcriptase. The cDNA produced was used for semi-quantitative and qPCR reactions.

**2.2.16 Polymerase chain reaction (PCR)**

Polymerase chain reaction (PCR) was performed using the GoTaq® DNA polymerase Kit (Promega). Each reaction was prepared in a sterile, nuclease-free microcentrifuge tube according to Table 2.5. The reaction mixtures were mixed gently and placed in a thermal cycler (MJ Research PTC-200 DNA engine Thermal cycler PCR) that had been preheated to 95 °C. The PCR cycle was set according to Table 2.6. The list of genes and corresponding primers sequences obtained from PrimerDepot (Cui et al., 2007) (<http://primerdepot.nci.nih.gov/>) are tabulated in Table 2.7. Reactions were carried out for all the three sets of experiments mentioned above (Section 2.2.6 and 2.2.7).

**Table 2.5 PCR reaction setup.**

<b>Component</b>	<b>Final Volume (µl)</b>	<b>Final concentration</b>
5X Green GoTaq® Reaction Buffer	10	1X (1.5mM MgCl <sub>2</sub> )
dNTP Mix, 10mM each	1	0.2 mM each dNTP
Forward primer	2	1 µM
Downstream primer	2	1 µM
GoTaq® DNA Polymerase (5u/µl)	0.25	1.25 units
Template DNA	2	0.5 µg
Nuclease free water	32.75	
<b>Final Volume</b>	<b>50</b>	

**Table 2.6 Thermocycler program settings**

<b>Step</b>	<b>Temperature</b>	<b>Time (min)</b>	<b>Number of cycles</b>
Initial denaturation	95 °C	2	1
Denaturation	95 °C	0.5	25 – 40 cycles
Annealing	65 °C	0.5	
Extension	72 °C	1	
Final Extension	72 °C	10	1 cycle
Halt	4 °C	Infinite	1 cycle

Table 2.7 Genes selected for PCR reaction.

Gene Symbol	RefSeq ID	Forward primer	Reverse primer
PLCB1	NM_015192	CCCCACATCCAAAAGTTCAC	AGGACTGACCCTCAGGGATT
LMNB1	NM_005573	TGCAGTAGCAAGAGCTGCAT	GATCGAGCTGGGCAAGTG
ID1	NM_002165	CGTTCATGTCGTAGAGCAGC	GTCTGTCTGAGCAGAGCGTG
DHRS7C	NM_001105571	GCTGATGCTCCCCCTGCTGC	CACCAGCCTTGCCCCACCTG
LEPROT	NM_017526	GAGATGGCGTGAAAATCAG	CCCCAGTTCGGGAGACAT
C10orf99	NM_207373	GTTCTCCGCTGTCTGGAGTC	TAGCCCCAACTCAACAAACC
SCN4A	NM_000334	AGGTTGTTTCATCTCGCCATC	CTCACCGTCTTCCTCATGGT
NDUFA10	AF453834	GTACCCAGGGCTGTACTIONG	CTGCGATTACTGGTTCAGGA
MYL6	NM_079425	ATTCACACAGGGAAAGGCAC	TATGAAGCGTTTGTGAGGCA
SRD5A2	NM_000348	ATTTCCAGTGCAGAAGGCAG	GACGGTACTTCTGGGCCTCT
PLCD4	NM_032726	TCAGTGGTCAGCTGGTCTTG	CACAGCTCACAGACACAGGAA
GAPDH	NM_002046	TTGAGGTCAATGAAGGGGTC	GAAGGTGAAGGTCGGAGTCA

### 2.2.17 Agarose gel electrophoresis

The size of the PCR products were analysed by gel electrophoresis. Agarose (Sigma-Aldrich) gels (1 %) were prepared in Tris-acetate-EDTA (TAE) pH 7.4 buffer. DNA ladder and PCR products were loaded in individual wells and electrophoresis was performed at 100 V for the appropriate time until the dye front reached the end of the gel. The image was captured using a gel documentation system (Syngene) and densitometric analysis was performed using Genesnap software (Syngene).

### 2.2.18 Quantitative real-time PCR

Quantitative (real-time) PCRs (qPCRs) were performed using the QuantiTect SYBR Green PCR Kit. The 2X QuantiTect SYBR Green PCR master mix (Qiagen), QuantiTect primers (Qiagen), template cDNA and RNase-free water were thawed on ice. The master mix was prepared as described in Table 2.8. All reagents except cDNA were added as a mix directly to the PCR plate. Later the cDNA was added to appropriate wells in the plate. The PCR plate was sealed and centrifuged at 1000 rpm for 2 min at 4 °C. The plate was then placed in ABI StepOne Plus real-time PCR system (Applied Biosystems) that was programmed as given in Table 2.9. Genes selected for quantitative real-time PCR with the QuantiTect primer accession numbers are tabulated in Table 2.10 (primer sequences of QuantiTect primers are not disclosed

**Chapter 2: Materials and Methods**

by the company). Reactions were carried out in triplicates for all the three sets of experiments mentioned earlier (Section 2.2.6 and 2.2.7).

**Table 2.8 Quantitative real-time PCR setup.**

Component	Volume per reaction (μl)	Final concentration
2x QuantiTect SYBR Green PCR Master Mix (Tris·Cl, KCl, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 5 mM MgCl <sub>2</sub> , pH 8.7)	12.5	1X
Forward primer	2.5	0.5 μM
Reverse primer	2.5	0.5 μM
RNase free water	2.5	
Template DNA	5	0.5 μM
<b>Final volume</b>	<b>25</b>	

**Table 2.9 ABI StepOne Plus real-time PCR system program settings.**

Step	Temperature	Time	Number of cycles
PCR initiation (initial denaturing)	95 °C	15 min	1 cycle
Denaturation	94 °C	15 s	40 cycles
Annealing	55 °C	30 s	
Extension	72 °C	30 s	

**Table 2.10 Genes verified by quantitative qPCR.**

Gene Symbol	REFSEQ ID	QuantiTect Primer accession number
BCL2	NM_000633	QT00025011
CD38	NM_001775	QT00073192
CREB3L2	NM_194071	QT00074361
EMILIN2	NM_032048	QT00092246
ESR2	NM_001437, NM_001040275, NM_001040276	QT00060641
F2	NM_000506	QT00013314
FEM1C	NM_020177	QT01156225
GJA1	NM_000165	QT00012684
KCNMA1	NM_002247, NM_001014797, NM_001161352, NM_001161353	QT00024157
KCNN3	NM_002249	QT00070966
KIF17	NM_020816, NM_001122819	QT00022645
MMP19	NM_002429, XM_001129299	QT00027286
PRMT5	NM_006109, NM_001039619	QT00049938
RET	NM_020630, NM_020975, NM_020629, NM_000323, NM_000323, NM_020629	QT00047985
SEMA3A	NM_006080	QT00040936
TTPA	NM_000370, XM_001128514	QT00012040

2.2.19 TaqMan small RNA assay

TaqMan® Small RNA Assays are designed to detect and quantify microRNAs. This assay uses a stem-looped primer for reverse transcription and a sequence-specific TaqMan assay to accurately detect mature miRNAs.

**Table 2.11 TaqMan® Small RNA Assays RT reaction setup master mix**

Component	Master mix volume per 15 µl reaction (µl)
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µl	1.00
10X Reverse Transcription Buffer	1.50
RNase Inhibitor, 20 U/µl	0.19
Nuclease-free water	4.16
Total volume	7.00

For 15 µl RT reaction setup, 7 µl of master mix (Table 2.11) was mixed with 5 µl total RNA (100ng/ µl) in a 96-well reaction plate. The appropriate RT primer (3 µl, 5X) for each assay was added to each well. The wells were sealed thoroughly and centrifuged briefly. The plate was kept on ice for 5 min before loading to the thermal cycler. The thermal cycler (MJ Research PTC-200 DNA engine Thermal cycler PCR) parameters were set according to Table 2.12.

**Table 2.12 Thermal cycler parameters for miRNA RT reaction.**

Step	Temperature (°C)	Time (min)
Hold	16	30
Hold	42	30
Hold	85	5
Hold	4	Infinity

To run the TaqMan® Small RNA Assay, the reaction mix was set up according to Table 2.13 and the samples placed, in triplicate, in MicroAmp® fast optical 96-well reaction plates. miRNAs selected for the TaqMan® Small RNA Assay with the primer accession numbers are tabulated in Table 2.14. The PCR reaction was set up according to the Table 2.15 and was run on an Applied Biosystem StepOne Plus Real-Time PCR system.

Table 2.13 TaqMan® Small RNA Real-time RT-PCR Assays reaction setup master mix.

Component	Volume per 20 µl Reaction (µl)
TaqMan® Small RNA Assay (20X)	1.00
Product from RT reaction	5.00
TaqMan® Universal PCR Master Mix II (2X), no UNG	10.00
Nuclease-free water	2.67
Total volume	20.00

Table 2.14 miRNAs whose expression was verified by quantitative real-time PCR.

miR name	Mature miR sequence	Accession No: (Applied Biosystems)
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG	MIMAT0000080
hsa-miR-128	UCACAGUGAACCGGUCUCUUU	MIMAT0000424
hsa-miR-135a	UAUGGCUUUUUUAUCCUAUGUGA	MIMAT0000428
RNU43 (Mature miRNA Control)	GAACTTATTGACGGGCGGACAGAACTGTGTGCTGAT TGTCACGTTCTGATT	568909

Table 2.15 ABI StepOne Plus real-time RT-PCR system program settings for miRNA.

Step	AmpErase UNG activity	Enzyme Activation	PCR	
	Hold	Hold	Cycle (40 Cycles)	
			Denature	Anneal/extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 s	60 s

### 2.2.20 Microarray analysis

DNA microarray analysis is an important technique used to capture a global snapshot of the transcriptional events in cells or tissues under various physiological or pathophysiological conditions (Dunckley et al., 2005). The rationale behind this technique is that there is a relation between the function of a gene product and its expression pattern (Brown et al., 1999). Uses of DNA microarray vary from understanding differential gene expression, identifying novel functions for known genes, comparative genomic hybridisation, detecting single nucleotide

polymorphism (SNP), identifying alternative spliced forms of genes and in chromatin immunoprecipitation (ChIP) on chip studies. Thus DNA microarray analysis has a wide field of application in discovering novel disease subtypes, developing new diagnostic kits, and understanding the molecular mechanism in various diseases and drug response.

The main physiochemical reaction in the DNA microarray is the hybridisation of complementary strands of DNA on the slide to the sample (Knudsen, 2004). This has been the principal for its ancestral techniques such as Southern and Northern blots. In DNA microarrays the probes (short oligonucleotide sequences specific to each gene) are printed on an inert surface such as glass, silicon or plastic biochips or on a nylon membrane (Knudsen, 2004; Maskos and Southern, 1992). mRNA from different samples are isolated, reverse transcribed, amplified (optional) and labelled with a fluorescent dye. This labelled cDNA is later hybridized to the microarray slide and washed under stringent condition to minimize cross-hybridisation by similar genes. Using laser-induced fluorescent imaging, an image of the hybridized slides is captured. The relative concentration of each mRNA is calculated depending on the intensity of the fluorescence at the corresponding spot on the microarray. However, these levels do not indicate the true expression of a specific gene but is a helpful tool to compare the expression of genes between different conditions, for example, treated versus untreated or diseased versus normal samples.

Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion) was used for modifying and labelling the RNA samples prior to hybridisation. The detailed procedure is given below.

#### 2.2.20.1 First strand synthesis

Total RNA (1 µg) and 1 µl T7 Oligo (dT) primer were mixed in a nuclease-free microcentrifuge tube, and the final volume adjusted to 12 µl. The mixture was denatured by incubating at 70 °C for 10 min and then kept on ice to stop the secondary structure formation. To this mixture 8 µl of reverse transcription master mix (Table 2.11) was added, mixed gently and incubated at 42 °C for 2 h. The tubes were placed on ice and immediately taken forward to second strand cDNA synthesis.

Table 2.16 Reverse transcription master mix.

Component	Volume ( $\mu$ l)
Nuclease free water	1
T7 oligo(dT) primer	1
10X first strand buffer	2
dNTP mix	4
RNase inhibitor	1
ArrayScript	1
<b>Total volume</b>	<b>10</b>

### 2.2.20.2 Second strand cDNA synthesis and purification

Second strand master mix (78  $\mu$ l) was mixed with 2  $\mu$ l of each sample (Table 2.12) and incubated at 16 °C for 2 h. The mixture was placed on ice (same day experiment continuation) or frozen (storage) before proceeding to the next step.

Table 2.17 Second strand master mix.

Component	Volume ( $\mu$ l)
Nuclease free water	63
10X second strand buffer	10
dNTP mix	4
DNA polymerase	2
RNase H	1
<b>Total volume</b>	<b>80</b>

The cDNA was purified by adding 250  $\mu$ l cDNA binding buffer to the samples and passing them through a cDNA filter cartridge. The cartridge was washed with 500  $\mu$ l wash buffer and cDNA was eluted in 18  $\mu$ l of nuclease free-water at 55 °C.

### 2.2.20.3 In vitro transcription to synthesise amino allyl-modified aRNA

Amino allyl-modified amplified RNA (aRNA) was synthesized by *in vitro* transcription (IVT) of the cDNA. IVT master mix (26  $\mu$ l, Table 2.13) was added to each eluted sample and mixed by vortexing. The mixture was incubated at 37 °C in a hybridisation chamber for 8 h. The reaction was stopped by adding 60  $\mu$ l of nuclease-free water.

Table 2.18 IVT master mix.

Component	Amount ( $\mu$ l)
aaUTP (50 mM)	3
ATP, CTP, GTP mix	12
UTP solution (50 mM)	3
T7 10X Reaction Buffer	4
T7 Enzyme Mix	4
<b>Total volume</b>	<b>26</b>

#### 2.2.20.4 Amino allyl-modified aRNA purification

The synthesised aRNA was mixed with 350  $\mu$ l of aRNA binding buffer. Ethanol (250  $\mu$ l, 100 %) was added immediately to the mixture and gently mixed by pipetting. Each sample was transferred to the aRNA filter cartridge and centrifuged for 1 min at 10,000 rpm. The filter was washed with 650  $\mu$ l wash buffer. The aRNA was eluted with 100  $\mu$ l preheated (55 °C) nuclease-free water.

#### 2.2.20.5 Amino allyl-modified aRNA quality control

The aRNA quality and yield was assessed using NanoDrop spectrophotometer by measuring its absorbance at 260 nm. The distribution profile of aRNA was evaluated using the Agilent 2100 Bioanalyzer.

#### 2.2.20.6 Amino allyl-modified aRNA labelling

Quantified aRNA (5  $\mu$ g) was vacuum-dried on a low heat. The dried aRNA was re-suspended in 9  $\mu$ l coupling buffer. Cyanine 3 (Cy3) dye (11  $\mu$ l) previously re-suspended in DMSO was added to the aRNA and mixed by vortexing. The mixture was incubated in the dark at room temperature for 30 min. Hydroxylamine (4.5  $\mu$ l, 4 M) was added and the mixture was further incubated in the dark for 15 min in room temperature. To each sample 5.5  $\mu$ l nuclease-free water was added to bring the final volume to 30  $\mu$ l.

#### 2.2.20.7 Labelled amino allyl-modified aRNA purification

The dye-labelled aRNA was purified to remove excess dye. aRNA binding buffer (105  $\mu$ l) was added to each sample. Ethanol (75  $\mu$ l, 100 %) was added immediately and mixed well by pipetting. The samples were passed through a labelled aRNA filter cartridge by centrifuging for

1 min at 10,000 rpm. The columns were washed once with 500 µl wash buffer. The labelled aRNAs were eluted by adding preheated nuclease-free water at 50-60 °C. The column was held at room temperature for 2 min and centrifuged for 1.5 min at 10,000 rpm. The elution step was repeated with an additional 10 µl of preheated nuclease-free water. The labelled aRNA was quantified with a NanoDrop at OD. 260 nm. The incorporation of dye within the nucleotide was calculated using the formula:

$$\left( \frac{\text{\#dye molecules}}{1000 \text{ nt}} \right) = \left( \frac{A_{\text{dye}}}{A_{260}} \right) \times \left( \frac{9010 \text{ cm}^{-1} \text{ M}^{-1}}{\text{dye extinction coefficient}} \right) \times 1000$$

Where, A dye is absorption of sample at 260 nm, A 260 is the maximum absorption of Cy3 at 260 nm (= 650) and dye extinction coefficient for Cy3 is 250000. The purified labelled aRNA was stored at -20 °C.

#### 2.2.20.8 Labelled amino allyl-modified aRNA fragmentation

RNA fragmentation was carried out using RNA fragmentation reagent (Ambion). The volume of aRNA sample was brought down to 4.5 µl by vacuum drying and 0.5 µl of 10X fragmentation buffer was added. The mixture was mixed, microfuged briefly and kept at 70 °C for 15 min in a heating block. Stop solution (0.5 µl) was added and the reaction mix was stored at -80 °C.

#### 2.2.20.9 Pre-hybridisation, hybridisation and washing

The Operon Human Genome Array-Ready Oligo Set Microarrays™ ((AROS) Version 4.0) were used for hybridisation. These arrays contain 35,035 oligonucleotide probes, representing approximately 25,100 unique genes and 39,600 transcripts excluding control oligos. The array was constructed based on Ensembl human database build (NCBI-35c), covering the complete NCBI human RefSeq dataset. In addition, these chips contain a large portion of unique full-length cDNA clone sequences from the H-Invitational database (release 8). Multiple data sources were used to cover genes from the human mitochondrial genome, RNA genes, microRNA genes, the endogenous human viral genes the exogenous reporter genes. The arrays are designed in an exon-centric fashion to provide the microarray detection precision at the transcript gene levels. 31,387 exon oligos are present in this array set, of which 26,283 are computationally classified as CGH oligos for comparative genome hybridisation.

Pre-hybridisation was carried out using the OpArray Pre-Hyb Kit (Operon). An appropriate volume of OpArray Pre-Hyb solution was pre-warmed to 42 °C for 30 min. The slides were incubated in OpArray Pre-Hyb solution for 60 min at 42 °C, ensuring that the slides were completely immersed in the solution. During the pre-hybridisation step, wash solution was prepared by diluting OpArray wash B with water in the ratio 1:40. The slides were washed in wash buffer at room temperature for 5 min. The slides were immediately transferred to sterile distilled water and rinsed for 30 s. This step was repeated twice. The slides were dried by centrifugation at 200 rpm for 5 min.

The hybridisation cassettes were rinsed with sterile distilled water and dried thoroughly. Sterile dH<sub>2</sub>O (15 µl) was added to the lower groove inside the cassette chamber and the OpArray was inserted to the chamber, DNA side up. The LifterSlip (Thermo Scientific) was carefully placed on the microarray slide. The labelled sample solution was denatured by incubating in the tube at 65 °C for 5 min on a heating block. The denatured target solution was applied directly to one end of the LifterSlip and was let to disperse across the surface. The clear plastic cassette lid was quickly placed on top of the cassette chamber and locked in place. The cassettes were placed into the hybridisation oven set at 42 °C and were hybridized for 16 h.

The post-hybridisation solutions were prepared as given in Table 2.14. Wash solution 2 was pre-warmed to 42 °C. The cassette was removed from the oven after hybridisation; the slides were removed from the cassettes and were placed in individual 50 ml tubes containing wash solution 2 at 42 °C for 10 min. The slide trays were transferred to fresh 50 ml tubes containing wash solution 2 and were incubated at room temperature for 10 min. The slides were again transferred to fresh 50 ml tubes with wash solution 4 and were incubated at room temperature for 5 min. This step was repeated twice to wash away any traces of wash solution from the slides. All the above solutions were kept in the dark by wrapping the tubes in tinfoil to avoid light desensitising the dye. The slides were dried by centrifuging at 200 rpm for 5 min.

Table 2.19 Post-hybridisation washing solutions.

Wash solution 2	
Contents	Volume (ml)
OpArrays wash A solution	50
OpArrays wash B solution	25
Sterile distilled water	425
Wash solution 3	
Contents	Volume (ml)
OpArrays wash A solution	50
Sterile distilled water	450
Wash solution 3	
Contents	Volume (ml)
OpArrays wash A solution	5
Sterile distilled water	495

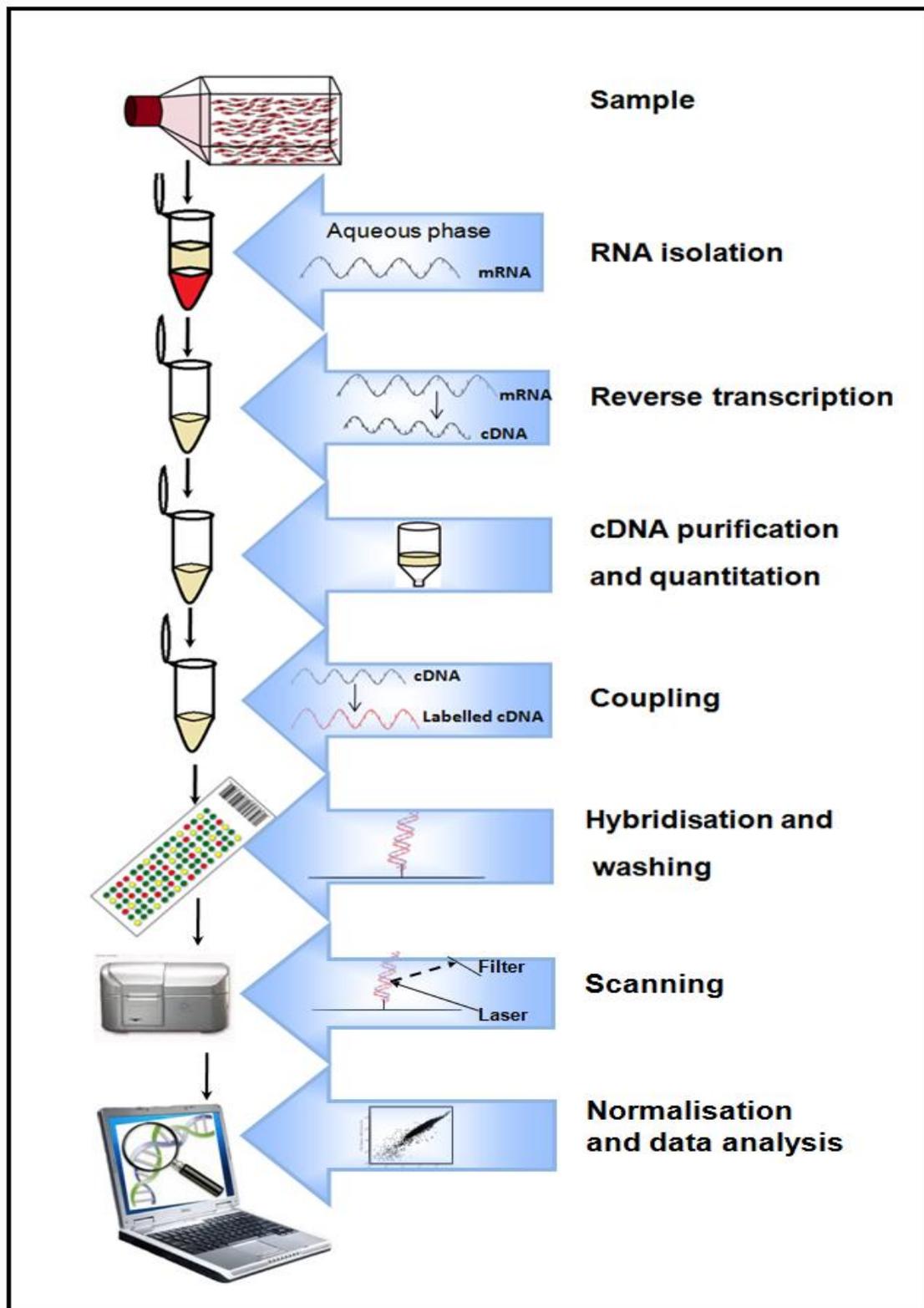
#### 2.2.20.10 Scanning

A Perkin Elmer ScanArray Express HT scanner was used to scan the slides. The slides were scanned at 543 nm. Various photomultiplier (PMT) gains (the power of the laser) were tested (60, 70 and 80) and the optimal PMT was decided as 70 since the best signals were obtained with this setting. Each slide was then scanned thrice at 70 PMT and 10 micron resolution.

#### 2.2.20.11 Image analysis

The image obtained after scanning was analyzed using Axon GenePix 6.0 software. The image along with the corresponding GAL (GenePix Array) file were loaded into the software and all spots were aligned properly. Background intensity for each slide and the intensity of the signal for each spot were then calculated. Median intensity of each spot was calculated from the three images obtained at 70 PMT of the same slide and this value was subtracted from the background to obtain the raw value. This raw value was used for further analysis.

Figure 2.3 summarises the workflow for a typical microarray experiment.



**Figure 2.3 Workflow of microarray experiment.** RNA was isolated from cells the cDNA was synthesised and purified. cDNA was labelled using Cy5 dye and the microarray slides were hybridised and washed. The slides were scanned and image was generated using the Perkin Elmer ScanArray Express HT scanner. The image was analysed using Axon GenePix 6.0 software to generate the raw data. Raw data was normalised and analysed to generate the differentially expressed genes using GeneSpring GX 12.0 programme.

**2.2.20.12 GeneSpring GX analysis**

GeneSpring GX 12.0 (Agilent Technologies) was used for data normalisation and downstream analysis.

A ‘new technology’ (an experimental setup in GeneSpring GX) was created for each of the experiment (17 $\beta$ -estradiol [E2] treatment and progesterone [P4] treatment) using the .gpr files (GenePix Results file) obtained from GenePix analysis. Columns listing the name, Operon probe IDs, median of main signal intensity, median of background signal intensity and values corresponding to median background signal subtracted from median signal intensity were selected from the .gpr files. Correct identifiers were tagged to corresponding columns to finish the creation of the new technology used for the analysis. A ‘new experiment’ was created under each technology created with the threshold raw signal value as 1.0 and the raw values normalised using percentile shift method (75% with baseline set to median).

**E2 TREATMENT**

The samples were named – untreated as control (C), 6 h, 24 h and 72 h untreated samples as 6C, 24C and 72C and 6, 24 and 72 h E2-treated samples as 6E, 24E and 72E respectively. Three interpretations were created (1) ‘Type’ – based on the treatment condition – control (C) and E2-treated (E), (ii) ‘Time’ – based on time of treatment – 0 for untreated control, 6 for 6 h treated and untreated samples, 24 for 24 h treated and untreated samples and 72 for 72 h treated and untreated samples, and (3) ‘Experiment’ – based on the biological replicate (replicate 1, 2 and 3).

Intensity values for all entities (35,357) were initially filtered to exclude values less than 50 in both conditions – control and oestrogen-treated (technically, this was set such that only 8 of the 9 samples in both conditions had to be below 50 for removal). Following this, two separate two-way ANOVAs were carried out: the first was the standard analysis with treatment (control and oestrogen-treatment) against time (6, 24 and 72 h) as variables. A second ANOVA was performed with treatment and experiment (biological replicates 1, 2 and 3) as variables. All significant differentially expressed entities relating to oestrogen-treatment from both analyses were pooled to make a common list. The list of genes was further refined by accepting only genes whose expression was altered >1.5-fold (to include more number of genes): this list was subsequently used for Gene Ontology and pathway analyses. In the running thesis only genes

with high expression values are mentioned, the complete list of differentially expressed genes is provided in the appendix.

### **P4 TREATMENT**

The samples were named – control (C) progesterone-treated (P4), progesterone and 17 $\beta$ -estradiol [E2]-treated (P4E2), progesterone and RU486-treated (P4RU) and progesterone, 17 $\beta$ -estradiol and RU486-treated (ALL). ‘Type’ interpretation was created based on the treatment condition – as mentioned above. Intensity values for all entities (35,357) were initially filtered to exclude values less than 50 in all conditions. Unpaired *t*-tests using ‘Type’ interpretations (C vs. P4, P4 vs. P4RU, C vs. P4E2 and P4E2 vs. ALL) were performed to identify differentially expressed genes. The lists of genes were further restricted by allowing only genes whose expression was altered >1.5-fold (to include more number of genes): these lists were subsequently used for Gene Ontology and pathway analyses. In the running thesis only genes with high expression values are mentioned, the complete list of differentially expressed genes is provided in the appendix.

#### **2.2.21 Gene Ontology analysis**

Gene Ontology (GO) annotations represent a link between the gene product and the role that that product plays functionally, in terms of the biological processes it contributes in a cell. To gain more knowledge on the biological significance of the differentially expressed genes, GO analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Dennis et al., 2003) and Onto-Express (OE) (Khatri et al., 2004; Khatri et al., 2002).

### **DAVID ANALYSIS**

Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Dennis et al., 2003) was used for this analysis. Official gene symbol lists for the differentially expressed genes identified in each study were separately created and saved as unformatted text files. Genbank accession IDs for all 35,357 entities represented on the slide was used as the background for GO analysis. Both the files were uploaded to the DAVID site (<http://david.abcc.ncifcrf.gov/tools.jsp>). The annotation clustering tool was used for GO analysis. All the default settings were left unchanged except for search parameters where only Gene Ontology (GO biological process – ‘full’, GO molecular function – ‘full’ and GO cellular

component – ‘full’) was selected. DAVID provides GO results in clusters where GO terms with similar functions are grouped under one cluster. Each cluster is provided with a one-tail Fisher Exact *P*-Value used for gene-enrichment analysis (called EASE score in DAVID). The higher this number the more enriched each cluster is. Clusters and enriched terms within the clusters were selected based on the EASE score and the *P*-Value for specific enriched GO term. Once significant GO terms were identified, they were grouped based on common biological processes. Redundant genes were removed and the final list was used for the thesis.

### **ONTO-EXPRESS ANALYSIS**

Onto-Express (Draghici et al., 2003; Khatri et al., 2002) is available as a part of Onto-Tools provided by the Intelligent System and Bioinformatics Laboratory, Wayne State University. This software is available at <http://vortex.cs.wayne.edu/ontoexpress/>. The same input files (gene list and background lists) were used in this analysis as above. Default settings were left unchanged. GO terms associated with biological process, molecular function and cellular component were search in the analysis. In Onto-Express the GO results are given as a Direct Acyclic Graph (DAG). DAG is a generalised representation of tree in which certain subtrees are shared by the main part of the tree. This means for any vertex *v*, there is no non-empty directed path that starts and ends on *v*. Significant GO terms were selected and the genes involved in those processes were identified. This list was then merged with the list obtained from DAVID analysis to obtain a single list of enriched GO terms and associated genes.

#### **2.2.22 Pathway analysis**

Differentially expressed genes identified in the present study were analysed to identify the role of these genes in any important pathway using MetaCore (Ekins et al., 2006) and DAVID (Dennis et al., 2003).

In MetaCore pathway analysis (Gene Go, Inc., St. Joseph, MI, USA), gene list with RefSeq IDs were uploaded online. With default settings left unchanged, pathway maps were analysed with *P*-Value set at 0.05. The most highly represented pathways from each experimental set were selected.

For pathway analysis using DAVID, the official gene symbol for the differentially expressed genes identified in each study were separately created and saved as unformatted text files. Genbank accession IDs for all 35,357 entities represented in the slide was used as the

background for pathway analysis. The files were uploaded to the DAVID site (<http://david.abcc.ncifcrf.gov/tools.jsp>). In DAVID under Onto-Tools (pathway analysis) both KEGG pathway and Biocarta pathway were selected.

#### **2.2.24 Statistical analysis**

All the experiments were done in triplicate. Results are expressed as mean +/- standard deviation. *P*-Value was used as a measure of the magnitude of the change and inter-subject variability among the biological replicates. *P*-Value was calculated by Student's *t*-test using either Graphpad program (<http://www.graphpad.com/quickcalcs/ttest2.cfm>) or an online program available at [http://www.physics.csbsju.edu/stats/t-test\\_bulk\\_form.html](http://www.physics.csbsju.edu/stats/t-test_bulk_form.html).

**Chapter 3: Characterisation and  
Optimisation of Human Uterine Smooth  
Muscle Cell Culture System**

### **3.1 Overview**

The aim of the research described in this thesis is to understand the transcriptomic effect of steroid hormone treatment in cultured human uterine smooth muscle cells (hUtSMCs) with emphasis on regulation of myometrial functions during pregnancy and labour. hUtSMCs from non-pregnant human myometrium (purchased from Lonza) were characterised for human uterine smooth muscle characteristics before they using in the experiments. The responsiveness of these cells to steroid hormone treatment was also tested and the cell culture system was optimised prior to the main experiment. The results proved that the cells used in this study maintained uterine smooth muscle cell characteristics and were responsive to the hormone treatments making this an ideal cell culture system to investigate the aims of the research.

## **3.2 Results**

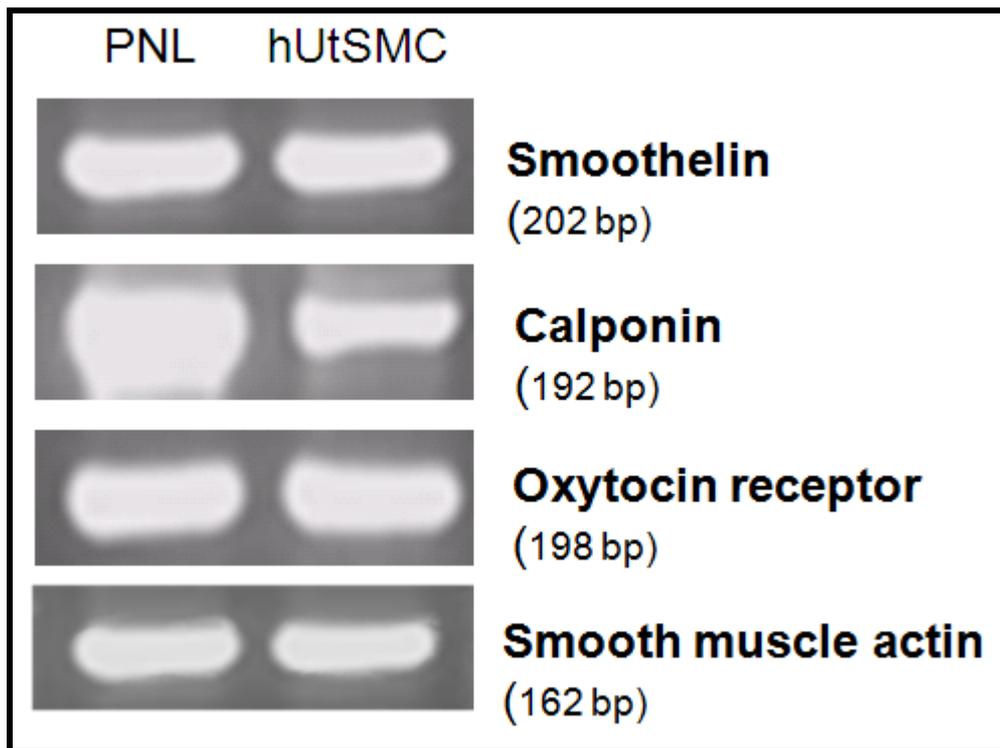
### ***3.2.1 Characterisation of hUtSMCs***

hUtSMCs from normal non-pregnant human uteri (Lonza, UK) were used in all experiments. These cells were selected as they are not pre-exposed to high progesterone or oestrogen like their counterparts during pregnancy or labour. hUtSMCs were assayed for the characteristic features of smooth muscle cells to ensure the quality of cells. All the characterisation studies had been performed previously in our lab (Dr. Margaret O'Brien, personal communication).

The oxytocin receptor (OTR) is a member of the G-protein coupled receptor family and acts as a receptor for oxytocin (Gimpl and Fahrenholz, 2001). OTRs are expressed in myometrial smooth muscle cells and are used as a marker to characterise these cells (Jeng et al., 2003; Lee et al., 1998; Loddenkemper et al., 2003; Moore et al., 2010).

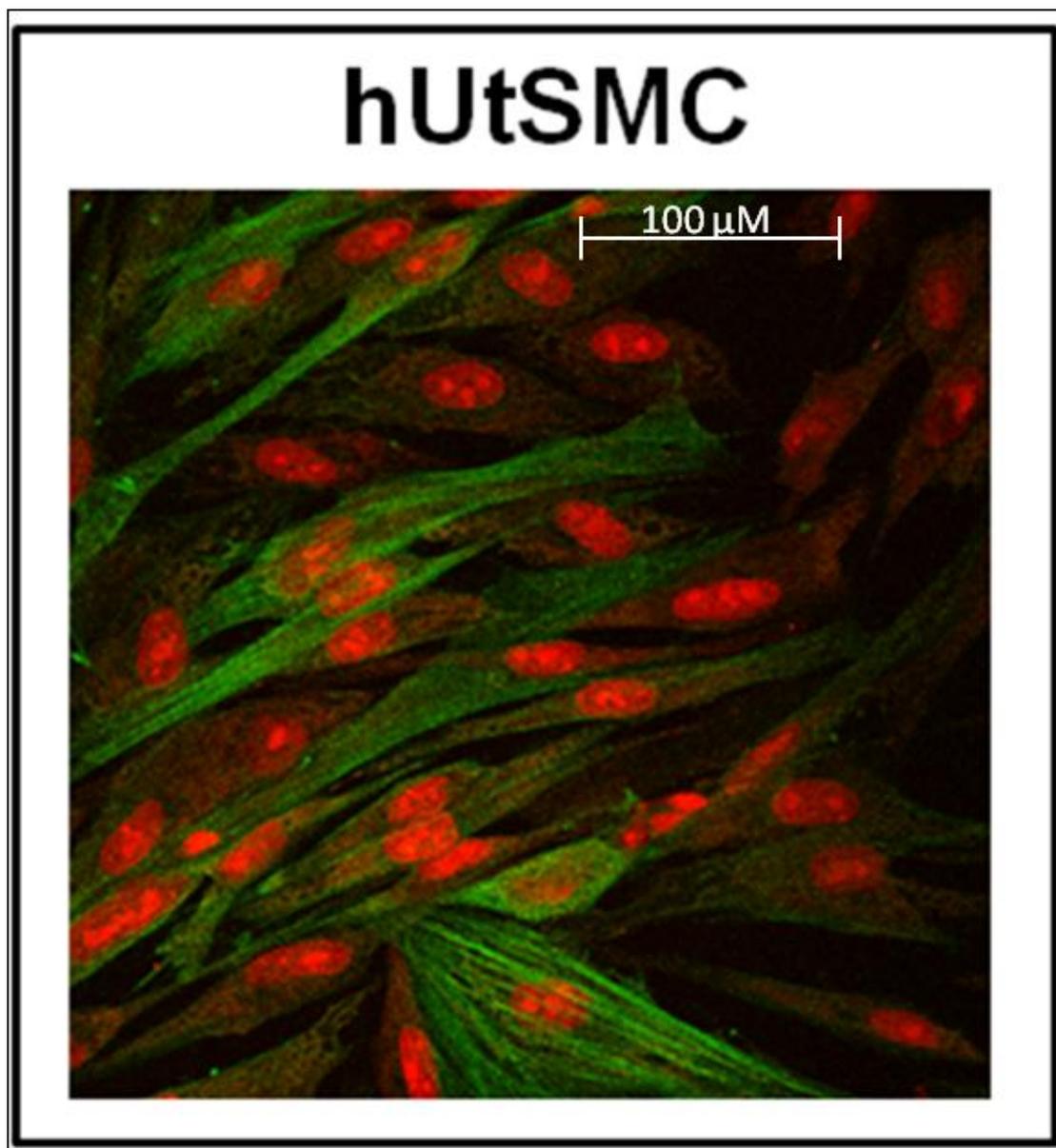
Calponin is an actin- and calmodulin-binding protein of smooth muscle cells. It regulates the interaction between actin and myosin in the smooth muscle (el-Mezgueldi, 1996). As calponin is present only in smooth muscle and not in skeletal or cardiac muscle (Chalovich, 1992; Walsh et al., 1995), expression of this gene can be used as a positive marker for the characterisation of smooth muscle cells. Similarly, smoothelin is a protein found exclusively in smooth muscle cells, where primary cells express smoothelin and immortalised smooth muscle cells lack smoothelin expression (van Eys et al., 2007), making this a very useful marker of active, contractile smooth muscle tissue.

To characterise the uterine smooth muscle cell phenotype, expression of calponin, smoothelin and oxytocin receptor (OTR) mRNA in human uterine smooth muscle cells procured from Lonza (passage 6; cells used in this study) was assayed using semi-quantitative RT-PCR. Myometrial smooth muscle cells obtained from pregnant non-labouring (pnl) women (passage 3) were also used. All the mRNAs investigated were expressed in myometrial smooth muscle cells (procured from Lonza and used for further experiments) confirming that the cells used for the subsequent experiments are uterine smooth muscle and have the characteristics of primary UtSMCs (Figure 3.1).



**Figure 3.1: Characterisation of smooth muscle cells.** Semi-quantitative RT-PCR was performed to investigate the expression of smoothelin, calponin and oxytocin receptor mRNA in hUtSMCs. Myometrial smooth muscle cells obtained from pregnant non-labouring (PNL) women were used as positive control (+ve). Smooth muscle actin was used as housekeeping gene.

The morphology of the myometrial smooth muscle cells (procured from Lonza) were also characterised in our lab using immunofluorescence staining (Dr. Margaret O'Brien, personal communication). The hUtSMCs were stained using a primary antibody to smooth muscle  $\alpha$ -actin and a FITC-conjugated secondary antibody.  $\alpha$ -actin is present in high amounts in smooth muscle cells and is localised to microfilament bundles exerting contractile functions and it is commonly used as a marker for characterising smooth muscle cells (Skalli et al., 1989). Nuclei were counterstained with propidium iodide (O'Brien et al., 2008a). Myometrial smooth muscle cells in culture were characterised by a long, fusiform shape and a central nucleus with cells growing in parallel arrays (Figure 3.2).

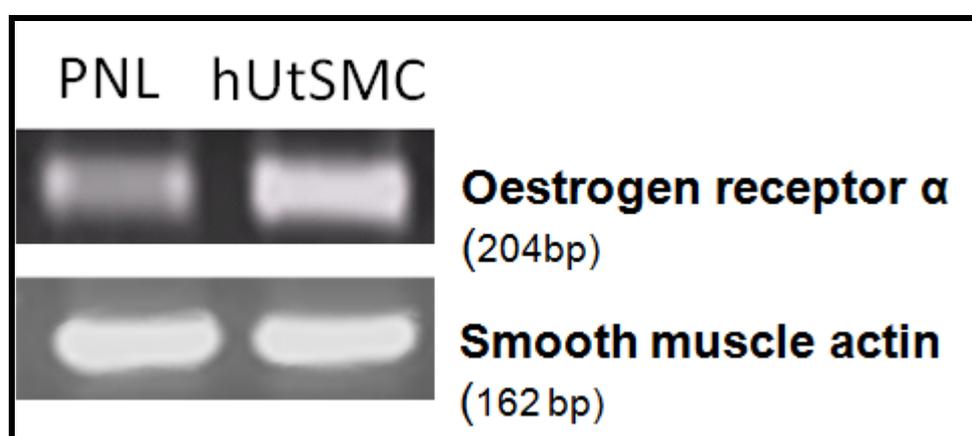


**Figure 3.2: Analysis of the morphology of human uterine smooth muscle cells.** hUtSMCs were stained using anti-smooth muscle  $\alpha$ -actin ( $\alpha$ SMA) antibody (green) and the nucleus was stained with propidium iodide (red). Images were captured using an Olympus fluorescence microscope, at 40X magnification. hUtSMCs in culture were characterised by a long, fusiform shape and a central nucleus.

### 3.2.2 Expression of ER- $\alpha$ in hUtSMCs

The aim of the study was to investigate the effect of E2 treatment on transcription in cultured human myometrial smooth muscle cells. Oestrogen exerts its actions via its two receptors – oestrogen receptors (ER) alpha ( $\alpha$ ) and beta ( $\beta$ ) (Cheng et al., 2004). ER- $\alpha$  is the active form of the oestrogen receptor, while ER- $\beta$  regulates the expression of ER- $\alpha$  (Imamov et al., 2005; Weihua et al., 2000). Since this study aims

at understanding the transcriptomic effect of E2 treatment in cultured hUtSMCs, it was important to identify the presence of transcripts of ER- $\alpha$  in the myometrial smooth muscle cells used in experiment. The expression of ER- $\alpha$  mRNA in human uterine smooth muscle cells procured from Lonza (passage 6; cells used in this study) was assayed using semi-quantitative RT-PCR. Myometrial smooth muscle cells obtained from pregnant non-labouring (pnl) women (passage 3) were also used (O'Brien et al., 2008b). ER- $\alpha$  was expressed in both the hUtSMCs procured from Lonza and the primary smooth muscle cells obtained from pregnant non-labouring woman (Figure 3.3).



**Figure 3.3: Assay of ER- $\alpha$  expression in hUtSMCs.** The expression of ER- $\alpha$  mRNA in hUtSMCs (from Lonza, passage 6) and myometrial smooth muscle cells obtained from pregnant non-labouring women (pnl) (+ve control) was assayed by semi-quantitative RT-PCR. ER- $\alpha$  mRNA is expressed both in hUtSMCs and in PNL myometrial samples. Smooth muscle actin was used as housekeeping gene.

### 3.2.3 Assaying the responsiveness of hUtSMC to E2 treatment

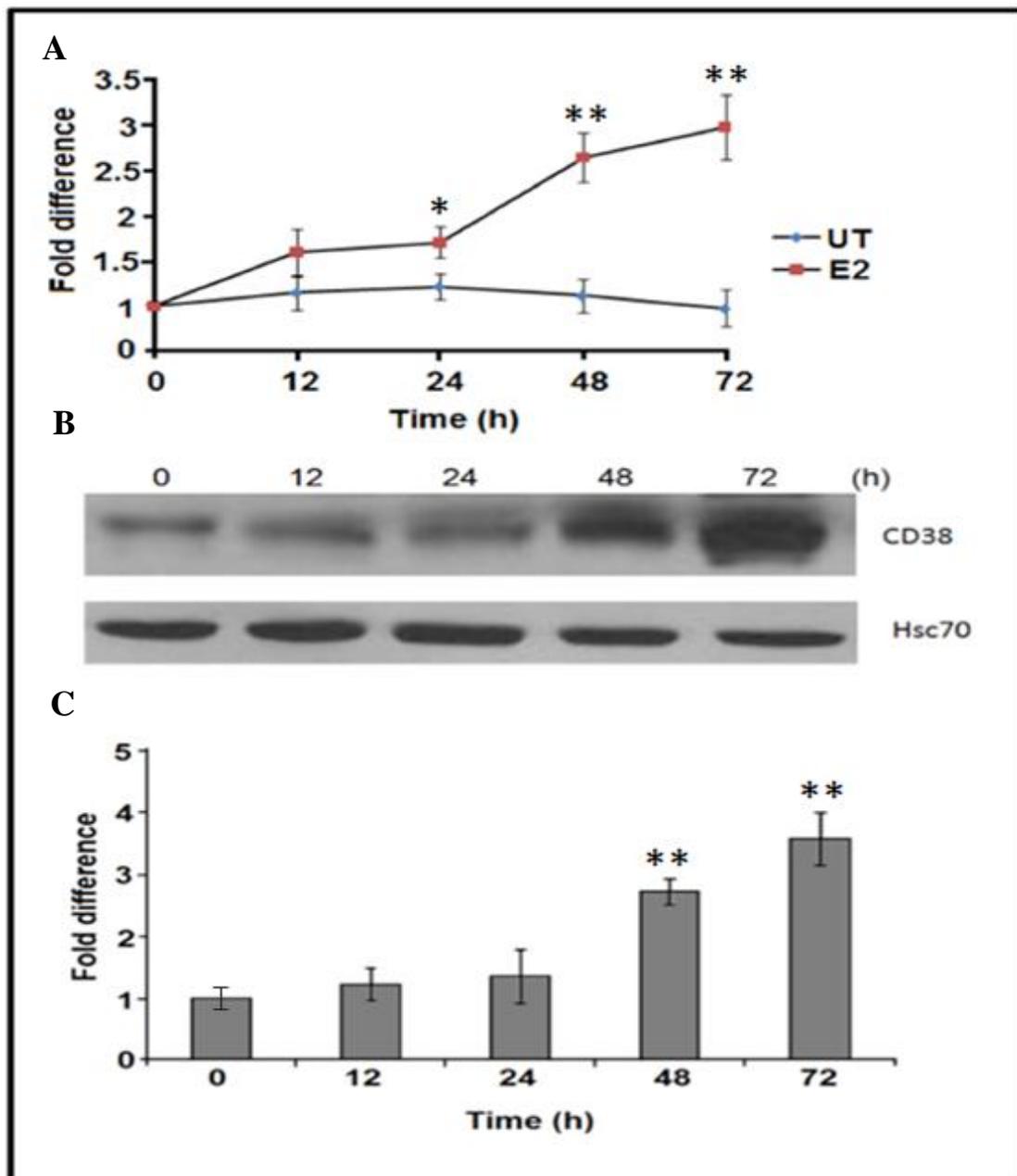
In order to confirm that the E2 supplement approach to be adapted in the system was active and effective in our model, the mRNA and protein expression of two previously reported E2 responsive genes, CD38 (Dogan et al., 2006) and CX43 (Andersen, 2000; Di et al., 2001), was first evaluated.

#### 3.2.3.1 Effect of E2 treatment on CD38 expression

CD38 is a multifunctional nucleotide metabolising enzyme that catalyses the synthesis and hydrolysis of cyclic ADP-ribose (cADPR) from NAD<sup>+</sup> to ADP-ribose. These reaction products are essential for the regulation of intracellular Ca<sup>2+</sup> (Malavasi

et al., 2008). Studies conducted in rat myometrium demonstrated an increase in CD38 expression after E2 treatment (Dogan et al., 2006). To validate the effectiveness of E2 treatment in our hUtSMC system, the mRNA and protein levels of CD38 were measured after 12, 24, 48 and 72 h in hUtSMCs treated with 10 nM E2 (concentration identified from previous reports) (Fiorelli et al., 2002; Kawaguchi et al., 1985; Wang et al., 2003). An increase in the level of CD38 mRNA and/or protein expression would be a positive indicator of the effect of E2 treatment. The results showed an induction of CD38 both at the mRNA (real-time PCR, Figure 3.4A) and protein levels (Figure 3.4B and C), demonstrating the effectiveness of E2 treatment in the cell culture system. The highest induction (3.0-fold) of CD38 mRNA expression level was observed following 72 h of E2 treatment ( $P < 0.005$ ). No significant increase in the mRNA expression level of CD38 was observed between 12 h and 24 h of E2 treatment, whereas the mRNA was induced by over 2.5-fold after 48 h of E2 treatment ( $P < 0.005$ ). The protein expression of CD38 was also highest (3.5-fold) after 72 h of E2 treatment ( $P < 0.005$ ). Similar to the mRNA expression, protein levels were also very similar at 12 h and 24 h after E2 treatment. CD38 protein expression increased by 2.6-fold following 48 h of E2 treatment ( $P < 0.005$ ). In the present study, both protein and mRNA expression of CD38 increased in E2-treated samples in a time-dependent manner, consistent with increased CD38 mRNA and protein expression in oestrogen-treated ovariectomised mice (Dogan et al., 2006). The fold changes were calculated by normalising the values to the housekeeping gene (Hsc70, see below) and comparing the values to corresponding untreated samples. All results presented are the average from three biological replicates. Statistical significance of the difference in the expression levels of CD38 between E2-treated and untreated control samples was determined using the Student *t*-test.

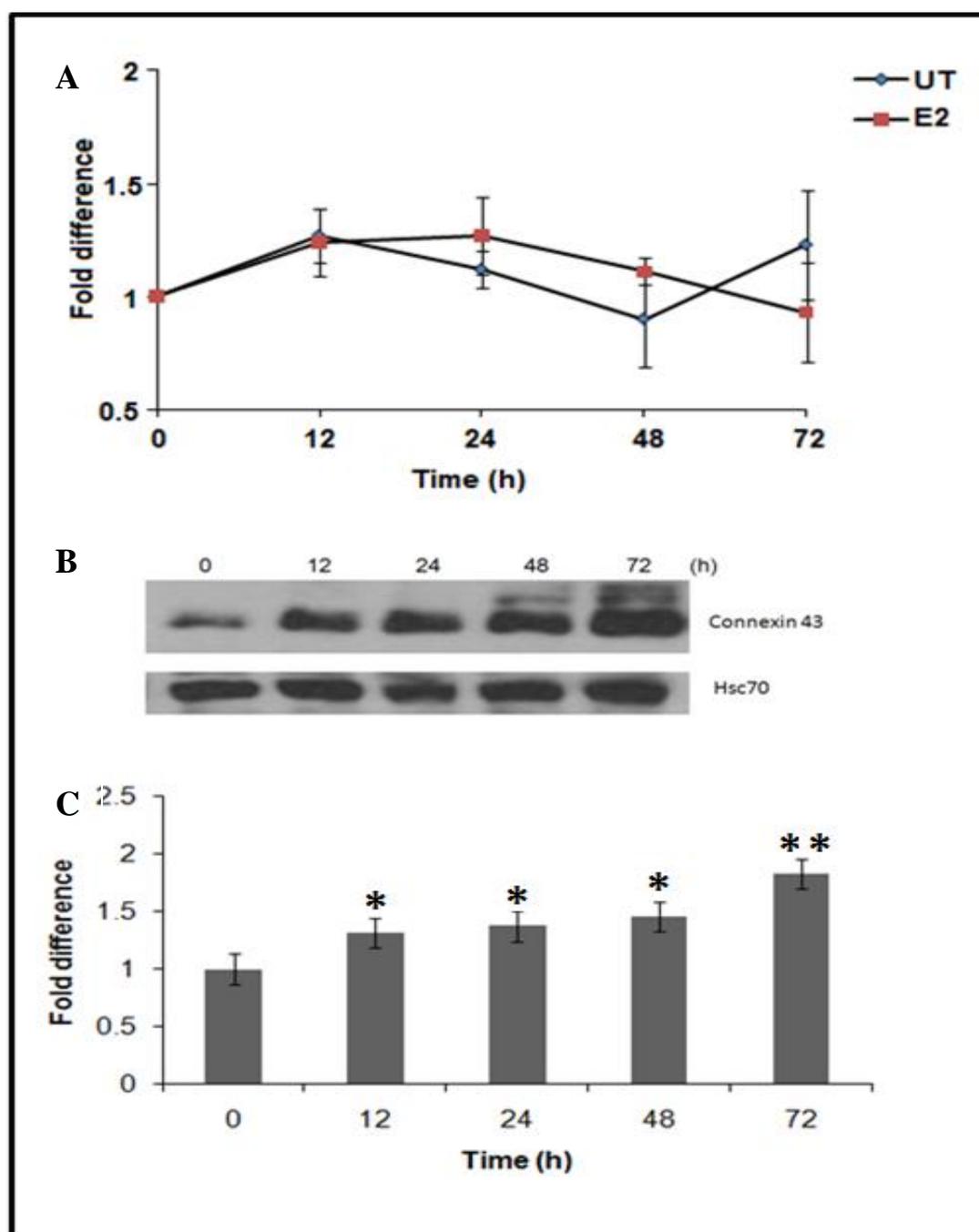
Heat shock cognate protein 70 (Hsc70) is a member of Hsp70 (heat shock protein 70) family. Hsc70 protein is constitutively expressed in all cells where its expression does not alter during stress or drug treatment (Paroo et al., 1999). Henceforth, Hsc70 was used as the housekeeping gene for all western blot experiments. The optimal concentration of E2 was obtained from a dose response study conducted (results provided in Section 3.4.3.3) and the optimal dosage identified was in agreement with previously published data (Fiorelli et al., 2002; Kawaguchi et al., 1985; Wang et al., 2003).



**Figure 3.4: Induction of CD38 mRNA and protein expression by E2 treatment.** (A) CD38 mRNA expression analysed by real-time PCR in hUtSMCs after treatment with 10 nM E2 for 0, 12, 24, 48 and 72 h. Untreated cells (UT) harvested at corresponding timepoints were also plotted. The average of three independent experiments (n=3) was plotted ( $\pm$ SD). Constitutively expressed HSC70 was used as housekeeping gene. (B) Western blot analysis of 10 nM E2-treated hUtSMCs for 0, 12, 24, 48 and 72 h. The membranes were probed with antibodies against CD38 and Hsc70. Hsc70 was used as loading control. Representative figures from three independent experiments are shown. (C) Graph depicting the densitometry analysis of the western blot for CD38 (B). Each value plotted corresponds to the average from three independent experiments (n=3), plotted ( $\pm$ SD). The fold changes in the expression were calculated by normalising the values to the housekeeping gene and comparing the values to corresponding untreated samples. Statistical significance of the difference in the expression levels of CD38 between E2-treated and untreated control samples were determined using the Student *t*-test \* - *P*-value <0.05 and \*\* - *P*-value <0.005.

### 3.2.3.2 Effect of E2 treatment on CX43 expression

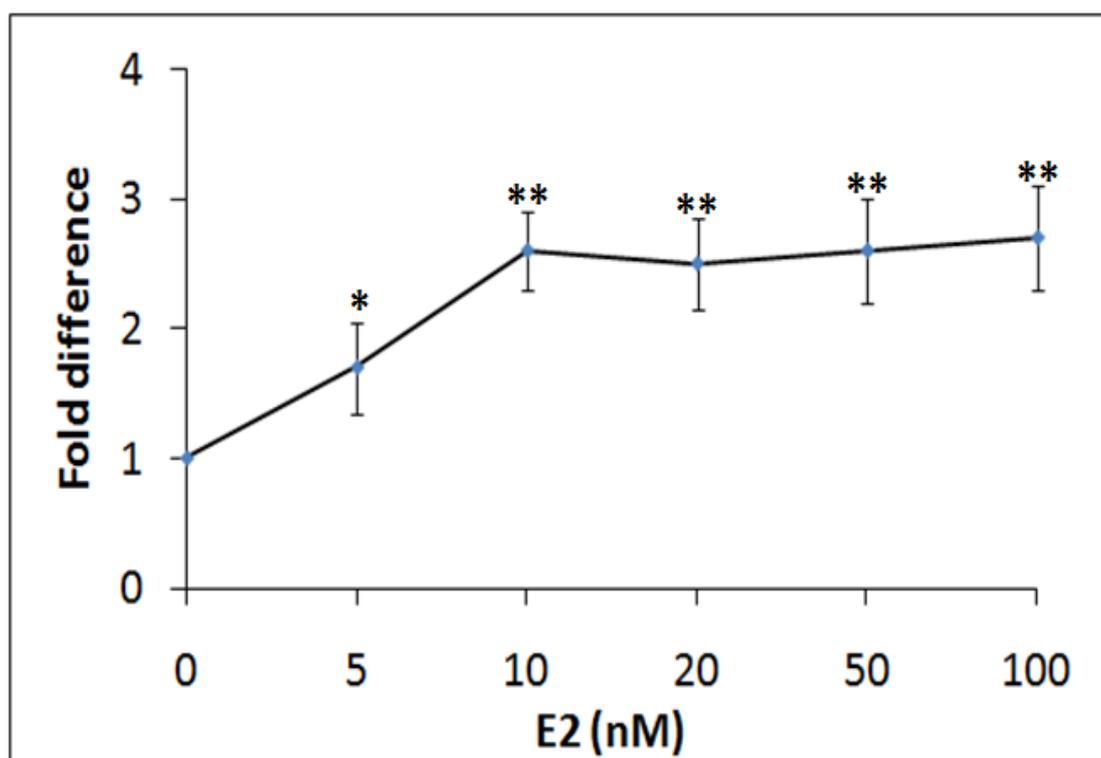
An increase in myometrial gap junction communication is a key event associated with the onset of labour (Helguera et al., 2009). It has been observed in non-primates that the expression of Connexin43 (Cx43), a gap junction protein, increases in response to E2 treatment (Di et al., 2001). However, in humans (*in vivo*) the mRNA levels of Cx43 does not alter, but an increase in the protein concentration was reported (Di et al., 2001; Hertelendy and Zakar, 2004). To verify this result in the current system, hUtSMCs were treated with 10 nM E2 for 12, 24, 48 and 72 h and Cx43 mRNA and protein levels were analysed by comparing the values to corresponding untreated samples. As published previously (Andersen, 2000), an increase in the protein level was observed (Figure 3.5B). Expression of Cx43 protein was induced ~1.8-fold following 72 h of E2 treatment ( $P < 0.05$ ), whereas the induction was less than 1.5-fold for all the other timepoints ( $P < 0.05$ ) (Figure 3.5B and C). Heat shock cognate protein 70 (Hsc70) was used as the housekeeping gene for western blots. At the mRNA level no significant changes were observed in any of the timepoints analysed (Figure 3.5A). All results presented are the average from three biological replicates. Statistical significance of the difference in the expression levels of Cx43 between E2-treated and untreated control samples was determined using the Student *t*-test.



**Figure 3.5: Protein and transcript expression of Connexin43 upon E2 treatment.** (A) Cx43 mRNA expression was measured by qPCR in hUtSMCs treated with 10 nM E2 for 0, 12, 24, 48 and 72 h (E2). Untreated cells (UT) harvested at corresponding timepoints were also analysed. The average of three independent experiments ( $n=3$ ) was plotted ( $\pm$  SD). HSC70 was used as housekeeping gene. (B) Western blot analysis of hUtSMCs treated with 10 nM E2 for 0, 12, 24, 48 and 72 h. Membranes were probed with antibodies against Cx43 and Hsc70. Hsc70 was used as loading control. Representative figures from three independent experiments are shown. (C) Graph depicting the densitometry analysis of the above western blot for Cx43 (B). Each value corresponds to the average from three independent experiments ( $\pm$  SD). The fold changes in the expression were calculated by normalising the values to the housekeeping gene and comparing the values to corresponding untreated samples. Statistical significance of the difference in the expression levels of Cx43 between E2-treated and untreated control samples was determined using the Student *t*-test. \* - *P*-value <0.05; \*\* - *P*-value <0.005.

### 3.2.3.3 Estimation of optimal E2 dose for hUtSMC treatment

To identify the optimal concentration of E2 for further treatments, a dose response study was performed on cultured hUtSMC using CD38 mRNA as a marker. hUtSMCs were treated with 5, 10, 20, 50 and 100 nM E2 for 72 h and qPCR analysis was performed to determine CD38 mRNA induction. The levels of CD38 mRNA increased with increased concentration of E2 (Figure 3.6). CD38 mRNA was induced by over 1.5-fold when treated with 5 nM E2 and by over 2.6-fold with 10 nM of E2 ( $P < 0.005$ ). However, no significant change in the induction of CD38 mRNA was observed between the E2 treated samples beyond 10 nM E2 concentration. All values were normalised to the housekeeping gene (GAPDH). The results presented are an average from three biological replicates. Statistical significance of the difference in the expression levels of CD38 between E2-treated and untreated control samples was determined using the Student *t*-test. Since the concentration of E2 used in similar reported experiments was also 10 nM (Fiorelli et al., 2002; Kawaguchi et al., 1985; Wang et al., 2003) the 10 nM E2 concentration was chosen for future experiments.

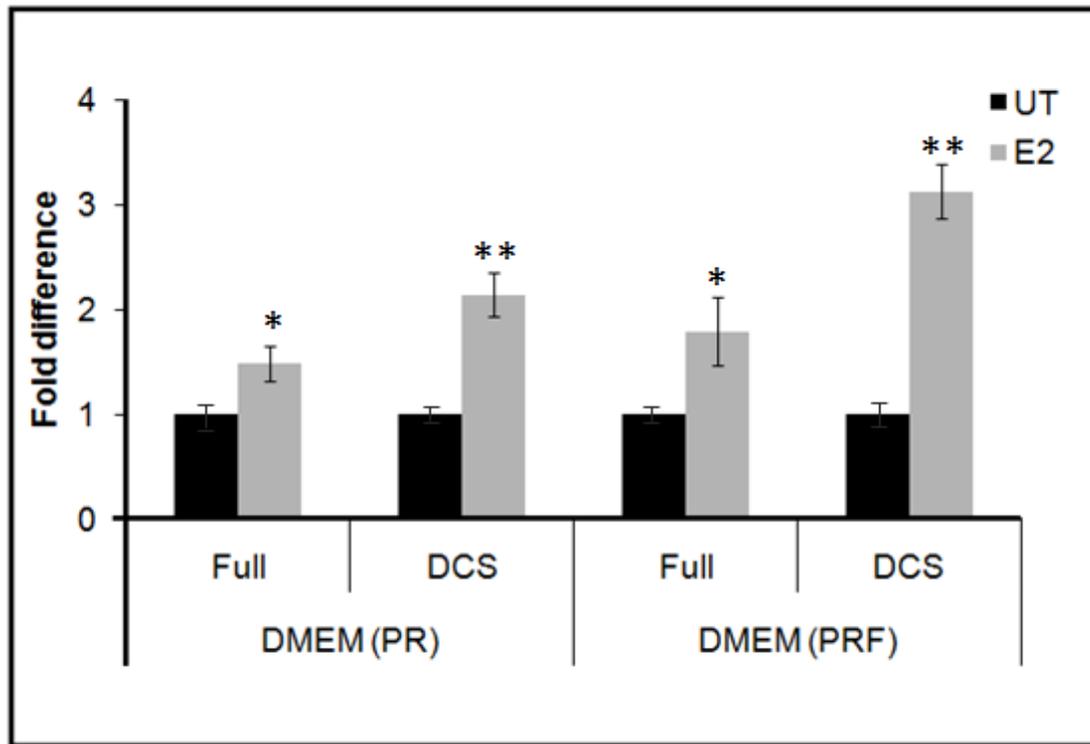


**Figure 3.6: Dose response curve indicating an increase in CD38 mRNA expression upon E2 treatment.** CD38 mRNA expression in hUtSMCs was measured by qPCR upon hUtSMCs treatment with various concentrations of E2 for 72 h. No significant change in the expression of CD38 mRNA was detected between the E2 treated samples beyond 10 nM E2 treatment. The average of three independent experiments was plotted ( $\pm$  SD). The fold changes in the expression were calculated by normalising the values to the housekeeping gene (GAPDH) and comparing the values to corresponding untreated samples. Significant differences in the expression levels of CD38 between E2-treated and untreated control samples were determined using the Student *t*-test. \* - *P*-value <0.05 and \*\* - *P*-value <0.005.

#### 3.2.3.4 Optimisation of hUtSMC cell culture conditions

Endogenous compounds in cell culture media and supplements can exert hormonal effects and alter gene and protein expression in cultured cells. For example, Phenol Red, a pH indicator used in cell culture media has been proven to have weak oestrogenic effects (Berthois et al., 1986; Hubert et al., 1986; Wesierska-Gadek et al., 2007). Also serum supplements used in cell culture media have traces of hormones (Cao et al., 2009). Since the aim of the current study was to investigate the effect of E2 hormone treatment in a human uterine smooth muscle cell culture system, care had to be taken to remove endogenous hormones present in the cell culture medium and supplements to avoid false positive results. In order to avoid the oestrogenic effect of Phenol Red in the cell culture medium, Phenol Red-free Dulbecco's modified Eagle

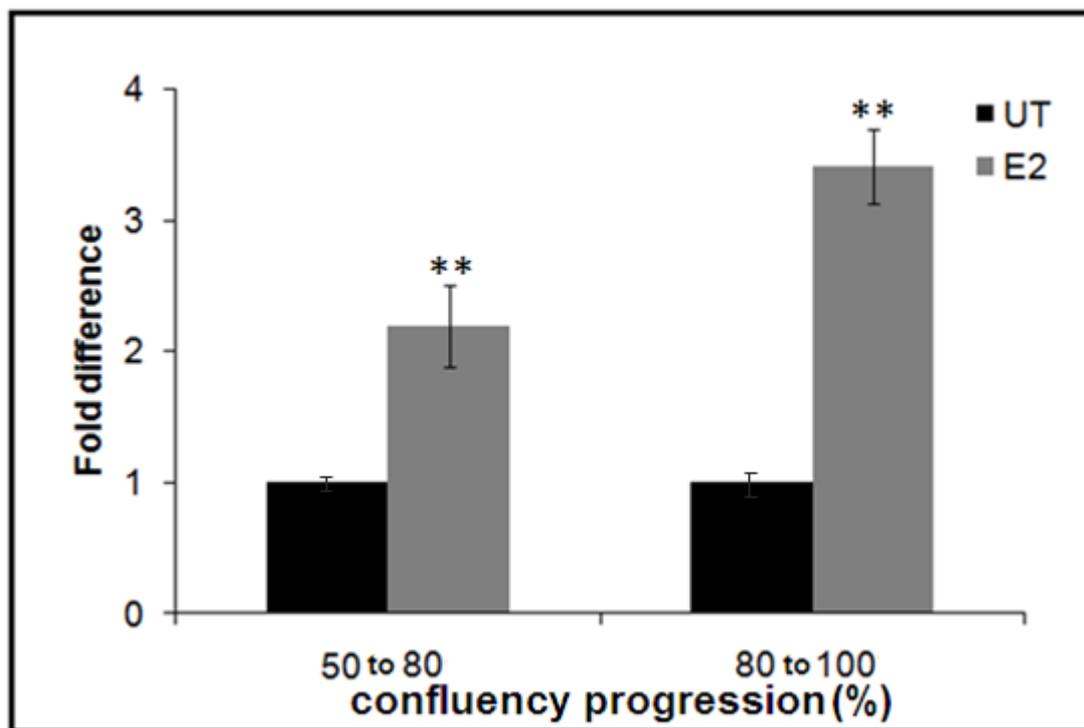
media (PRF-DMEM) was used to culture the cells. Double charcoal-stripped foetal bovine serum (DCS-FBS) was used as the medium supplement to avoid the effect of hormonal factors from the serum (Cao et al., 2009; Dang and Lowik, 2005; Gstraunthaler, 2003). To optimise culturing conditions, hUtSMCs were cultured in various cell culture conditions. Cells were cultured in Phenol Red DMEM and 10 % unstripped FBS until 80 % confluence, starved overnight and media changed to various combinations of Phenol Red DMEM or Phenol Red-free DMEM and 2 % unstripped FBS or double charcoal-stripped FBS. All the flasks were treated with 10 nM of E2 at 100 % confluence and harvested after 72 h. Since the maximum change in CD38 mRNA induction was observed after 72 h of E2 treatment, this timepoint was used for treatment experiments. mRNA levels of CD38 were checked in each sample to identify the optimal culturing conditions (Figure 3.7). The maximum difference in CD38 mRNA expression was observed in cells cultured in Phenol Red free DMEM supplemented with 2 % double charcoal-stripped FBS and treated with 10 nM E2 (3.25-fold,  $P < 0.005$ ). For all other treatments, the differences in the expression of CD38 mRNA compared to their controls were: 1.5-fold for cells grown in complete DMEM and FBS ( $P < 0.05$ ); 2.2-fold for cells grown in complete DMEM and DCS FBS ( $P < 0.005$ ); and 1.78-fold for cells cultured in PFR DMEM ( $P < 0.05$ ). All results obtained were an average from three biological replicates. Statistical significance of the difference in the expression levels of CD38 between E2-treated and untreated control samples was determined using the Student *t*-test.



**Figure 3.7: Demonstration of the oestrogenic effect of serum and Phenol Red in the media.** mRNA expression of CD38 was evaluated by qPCR with reference to the CD38 mRNA levels of untreated samples. Cells were either cultured in Phenol Red (PR) DMEM or Phenol Red-free (PRF) DMEM from 80 % confluence. Double charcoal-stripped (DCS)-FBS or unstripped FBS (Full) was used as serum supplement. At 100 % confluence all flasks were treated with 10 nM of E2 and harvested after 72 h. The CD38 mRNA expression levels for untreated samples are represented by black bars (expression values considered as 1) and the E2-treated samples are represented by grey bars. The average of three independent experiments ( $n=3$ ) is plotted ( $\pm$  SD). The fold changes in the expression were calculated by normalising the values to the housekeeping gene (GAPDH) and comparing the values to corresponding untreated samples. Statistical significance of the difference in the expression levels of CD38 between E2-treated and untreated control samples was determined using the Student *t*-test. \* - *P*-value <0.05 and \*\* - *P*-value <0.005.

The hUtSMCs were cultured in media with DMEM and FBS to provide the optimal growth conditions. The cells were starved in PRF-DMEM supplemented with 2 % DCS-FBS prior to E2 treatment to remove any endogenous hormones that are present in the cell culture media. To find the optimum cell confluence for starvation and E2 treatment same number of cells were seeded per flask, cells were starved from ~50 % and ~80 % confluence and were treated with 10 nM of E2 at ~80 and 100 % confluence. The mRNA levels of CD38 were analysed after 72 h to check the scale of induction compared to corresponding untreated samples. A maximum difference in the mRNA expression (~3.3-fold) was observed in cells starved from ~80 % confluence followed by E2 treatment at ~100 % confluence ( $P<0.005$ ). Cells starved

from ~50 % confluence and treated with E2 at ~80 % confluence showed an mRNA induction of 2.2-fold ( $P < 0.005$ ). hUtSMCs starved at ~50 % confluence showed senescence and failed to reach 100 % confluence within seven days of starvation (Figure 3.8). The fold changes were calculated by normalising the values to the housekeeping gene and comparing the values to corresponding untreated samples. Statistical significance of the difference between E2-treated and untreated control samples was determined using the Student *t*-test. Hence, for further experiments hUtSMCs were serum starved from ~80 % confluence and treated with 10 nM E2 at ~100 % confluence.



**Figure 3.8: Effect of hormonal starvation of hUtSMC cells prior to E2 treatment on CD38 expression.** hUtSMCs were cultured in Phenol Red DMEM supplemented with 10 % unstripped FBS till 50 or 80 % confluence. Culture medium was changed to Phenol Red free DMEM supplemented with 2 % double charcoal-stripped serum and harvested at 100 % confluence. The graph shows the profile of CD38 mRNA expression between cells starved at 50 % (black bar) and E2-treated at 80 % (grey) and cells starved at 80 % confluence (black) and E2-treated at 100 % confluence (grey). Differences in the expression of CD38 mRNA were analysed by qPCR. The fold changes in the expression were calculated by normalising the values to the housekeeping gene (GAPDH) and comparing the values to corresponding untreated samples (set as 1). Statistical significance of the difference between E2-treated and untreated control samples was determined using the Student *t*-test. \*\* -  $P$ -value  $< 0.005$ .

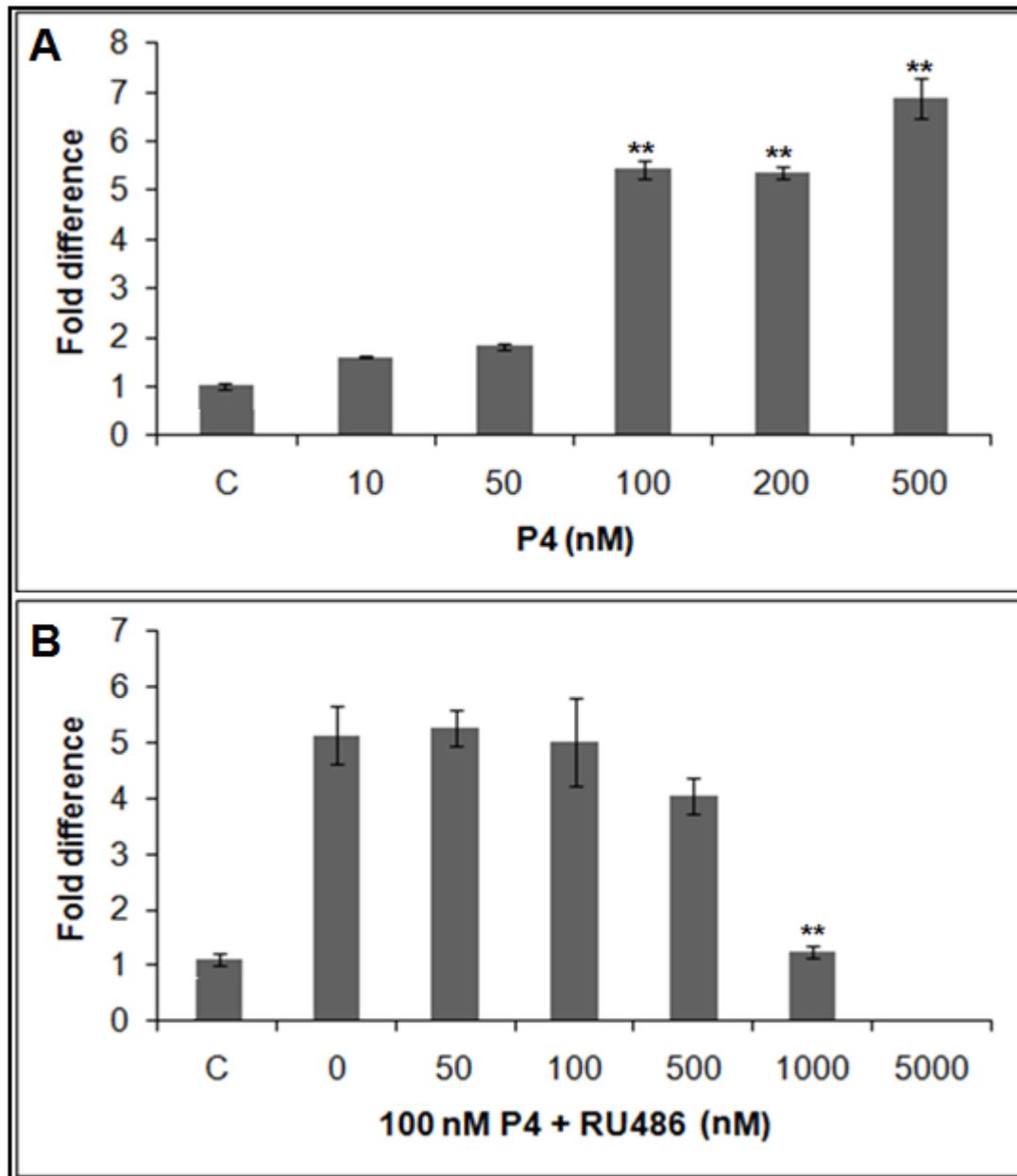
### 3.2.3.5 Effect of progesterone and mifepristone treatment on BCL2 gene expression

The B-cell lymphoma 2 (BCL2) gene encodes for the anti-apoptotic protein Bcl2. The expression of BCL2 gene has been proven to be responsive to progesterone treatment (Liszewska et al., 2005; Matsuo et al., 1997; Yin et al., 2007). Upregulation of BCL2 mRNA could act as a positive control for efficacy of P4 treatment of hUtSMCs (Ogle, 2002).

To identify the optimal dose of progesterone for cell culture treatment, cultured hUtSMCs were treated with varying concentrations of P4 (10, 50, 100, 200 and 500 nM). Control cells were left untreated. Cells were harvested 72 h after P4 treatment and BCL2 mRNA expression was analysed using real-time PCR. Statistically significant induction in BCL2 mRNA expression was seen in samples treated with 100 nM (5.75 fold,  $P < 0.005$ ), 200 nM (5.70 fold,  $P < 0.005$ ) and 500 nM (6.8 fold,  $P < 0.005$ ) progesterone (Figure 3.9A). Since the induction of BCL2 gene expression did not continue to increase at higher doses of progesterone beyond 100 nM, the optimum dose for P4 treatment in the study was fixed at 100 nM. This concentration was consistent with previously published P4 data (Yin et al., 2007).

To investigate the effect of the P4 inhibitor RU486, and to identify the optimal dose for RU486 treatment, hUtSMCs in culture supplemented with 100 nM P4 were treated with varying doses of RU486 (50, 100, 500, 1000 and 5000 nM) 24 h prior to harvesting. Two sets of controls were used: one control was untreated cells and one was of cells treated with 100 nM P4. Compared to the untreated samples expression of BCL2 mRNA was induced by over 5-fold in P4 treated samples (+ve control). The maximum inhibition of BCL2 mRNA expression induction was observed in samples treated with 1  $\mu$ M RU486 (4 fold,  $P < 0.005$ ) (Figure 3.9B). Other concentrations above 1  $\mu$ M showed marginal reduction in BCL2 mRNA expression upon RU486 treatment and 5  $\mu$ M concentration was lethal for the cell.

The result demonstrated that RU486 treatment inhibited the P4-induced increase of BCL2 gene expression and that the maximum inhibition by RU486 was observed with 1  $\mu$ M RU486 treatment. Hence for further analysis 1  $\mu$ M RU486 and 100 nM P4 were used (Lewis-Tuffin et al., 2007; Ma et al., 2001; Makrigiannakis et al., 1999).



**Figure 3.9: Effect of progesterone and mifepristone treatment on BCL2 gene expression. A.** BCL2 mRNA expression in hUtMSCs in response to P4 treatment. **B.** Effect of RU486 treatment on the expression of BCL2 in P4-treated hUtMSCs. The expression level of BCL2 was determined using qPCR. All values were normalised to the housekeeping gene (GAPDH). Results were an average from three biological replicates. Statistically significant differences in the treated samples (compared to the Control (C) in A or P4 alone in B) were determined using the Student *t*-test \*\*-  $P < 0.005$ .

### 3.2.4 Analysis of the quality of RNA samples

The quantity and the quality of RNA are very crucial in studies where gene expression changes are being evaluated. Although RNA is a thermodynamically

stable molecule, the ubiquitously present RNase enzyme digests RNA molecules thereby potentially compromising the results of downstream analyses (Auer et al., 2003; Imbeaud et al., 2005). All the RNA samples that were used in this study were tested both for quantity (using Thermo Scientific NanoDrop) and for quality (using an Agilent 2100 Bioanalyzer). Only RNA samples of a high standard as indicated by their RNA integrity numbers (RIN) were used in downstream analyses.

RNA integrity number (RIN) is an indicator of the quality of RNA (Schroeder et al., 2006). The Agilent 2100 Bioanalyzer software calculates the integrity of eukaryotic RNA from the entire electrophoresis trace of the RNA sample unlike conventional methods (electrophoresis), where only the ratio of 28S:18S is considered (Schroeder et al., 2006). Since the whole spectrum of degraded RNA is considered in calculating the RIN value, this method gives a greater accuracy in determining the quality of RNA. A value between 1 and 10, where 1 is the most degraded form of RNA and 10 the most intact form, is attributed to each sample to denote the integrity of the sample. All the RNA samples used in the experiments were assessed for their quality. A high quality control cut-off of RIN value more than 9 was used to select RNA for downstream analyses. Table 3.1 summarises the RIN values for all the RNA samples from three biological replicates.

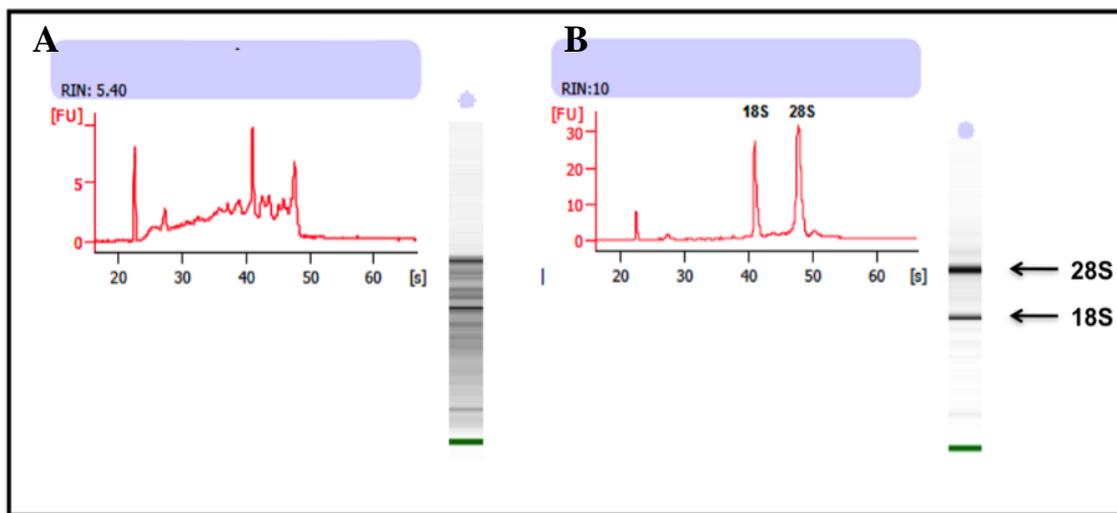
Table 3.1: RIN values of all RNA samples used for microarray experiments.

<b>E2 treatment</b>			
<b>Sample Name</b>	<b>Biological Replicate 1</b>	<b>Biological Replicate 2</b>	<b>Biological Replicate 3</b>
0 h Untreated sample	9.7	9.8	9.8
6 h Untreated sample	10.0	9.6	9.9
6 h 10 nM E2-treated sample	9.7	9.7	9.5
24 h Untreated sample	10.0	9.8	9.1
24 h 10 nM E2-treated sample	10.0	9.8	9.9
72 h Untreated sample	9.6	9.8	9.8
72 h 10 nM E2-treated sample	10.0	9.9	10.0
<b>P4 treatment</b>			
<b>Sample Name</b>	<b>Biological Replicate 1</b>	<b>Biological Replicate 2</b>	<b>Biological Replicate 3</b>
Control (C)	10.0	9.5	10.0
P4	9.9	10.0	10.0
E2	9.9	9.6	9.6
P4+RU486	9.7	9.6	9.5
P4+E2	9.4	10.0	9.0
P4+E2+RU486	9.9	10.0	9.5

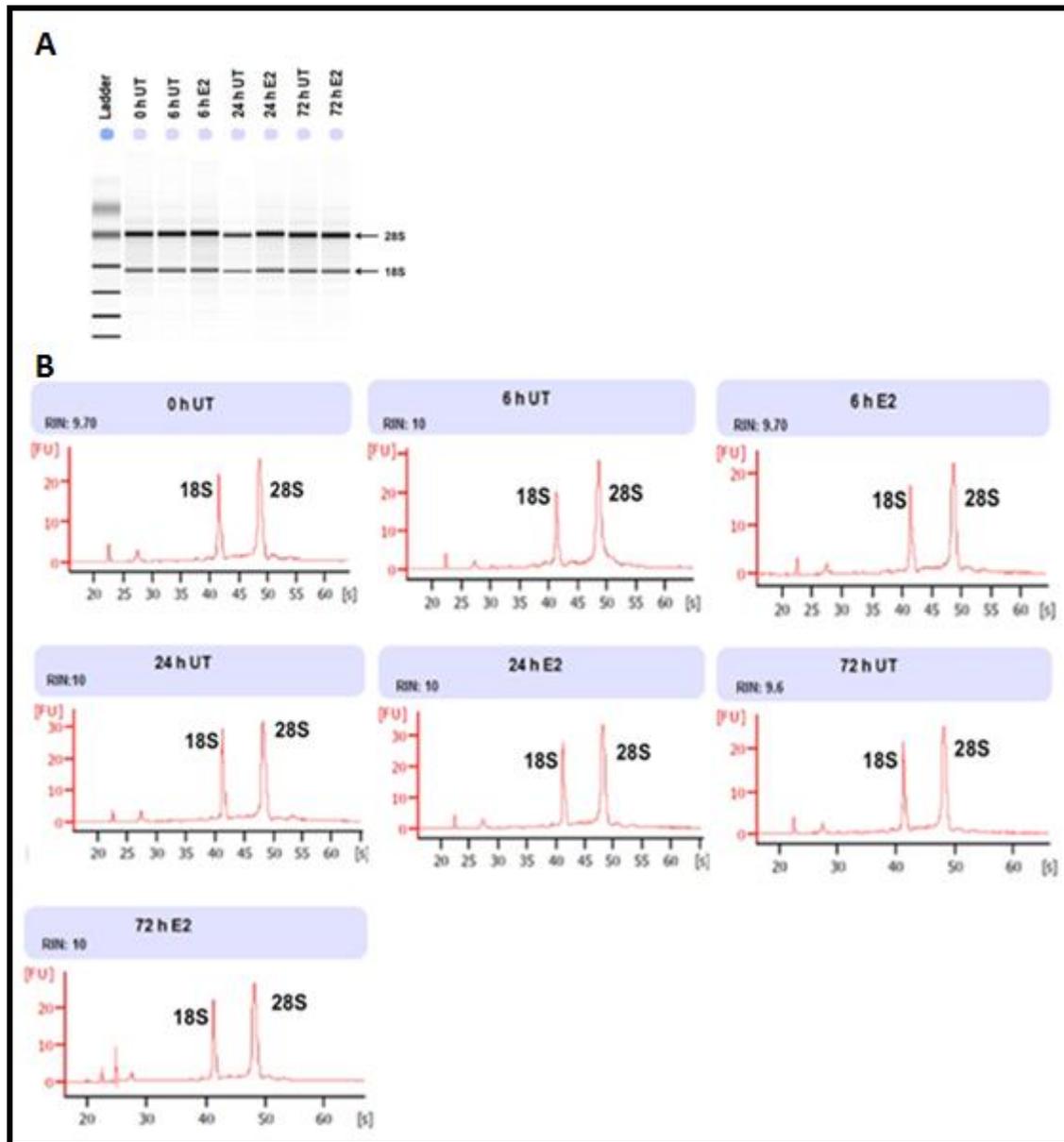
A sample electropherogram and corresponding gel image of intact and degraded RNA sample is presented in Figure 3.9. Figure 3.10A depicts a degraded RNA with a RIN value of 5.4. The multiple peaks in the graph correspond to smaller fragments of rRNA arising due to RNA degradation. The corresponding gel image shows a smear. Figure 3.10B depicts intact RNA with a perfect 10 RIN value. In the graph the peaks corresponding to the marker, the 5S, 18S and 28S ribosomal bands are clearly seen. The corresponding gel image shows the bands corresponding to 18S and 28S fragments of the rRNA. Figures 3.11 and 3.11 shows representative gel image and

electropherograms respectively of RNA samples, isolated from one of the experimental samples used in the downstream analyses. In each figure both the graphs and the gel images show the distinct peaks and bands corresponding to 28S and 18S rRNA.

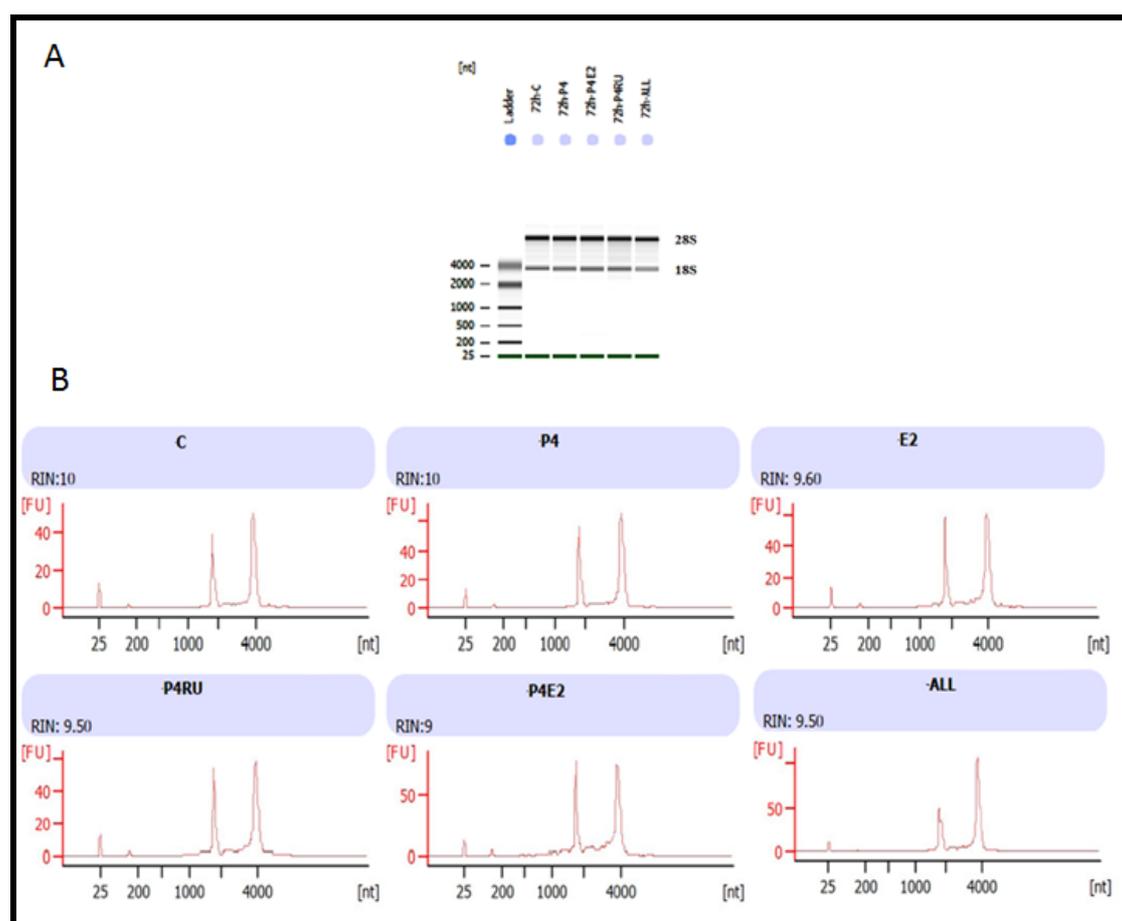
Once the RNA was found to be of high quality, each sample was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion). This step amplifies all RNA equally in the sample thereby increasing the chances of detecting low copy number RNAs. The amplified RNA samples were purified and labelled using Cyanine 5 (Cy5) dye. The labelled RNA was then assessed for quantity and quality before it was used in DNA microarray hybridisation analysis.



**Figure 3.10: Examples of low and high quality RNA samples from hUtSMC.** Bioanalyzer chip electropherogram of total RNA (extracted) and the corresponding gel images are shown. (A) Image of a low quality RNA with a RIN of 5.4. Multiple peaks in the graph indicate the degraded RNA. Gel image shows smears instead of distinctive bands. (B) Electropherogram of high quality RNA sample with distinct peaks corresponding to 18S and 28S. The gel image shows distinct band patterns as opposed to a smear.



**Figure 3.11: Electropherogram of RNA for quality assessment.** **A.** Gel image from Bioanalyzer chip for total RNA from a representative biological replicate. **B.** Electropherogram for each corresponding RNA sample is shown. Distinct peaks for 18S and 28S rRNA are marked. The RIN value for each corresponding RNA sample is shown above each graph. (The samples are named with the harvested hour followed by the treatment. UT represents untreated sample and E2 represents 10 nM E2-treated sample).

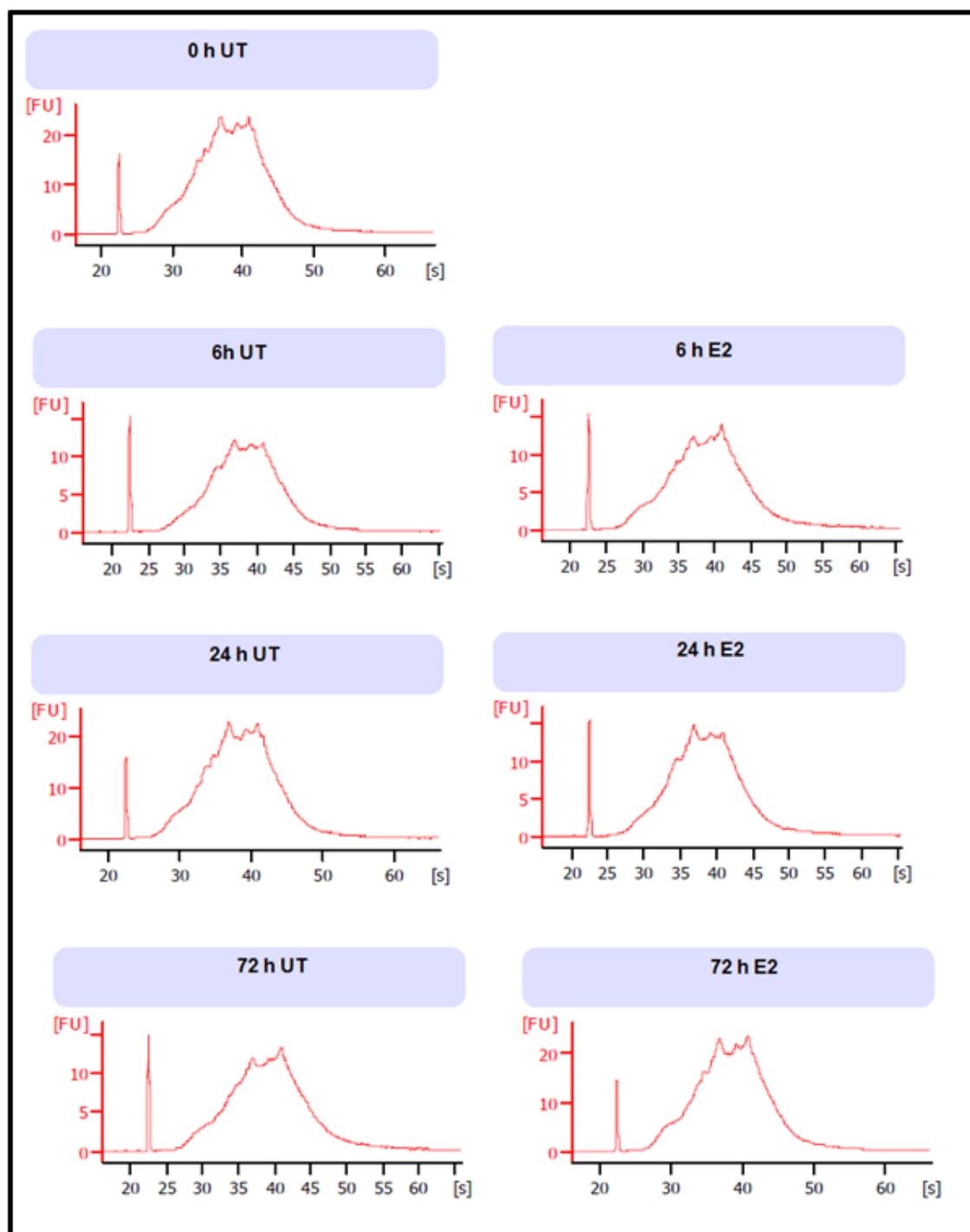


**Figure 3.12: Quality analysis of RNAs used in microarray experiments.** **A.** Gel image from Bioanalyzer chip for total RNA from a representative biological replicate. **B.** Electropherograms for representative RNA samples used for further experiments. The RIN value for each corresponding RNA sample is shown above each graph. The distinct peaks depict 18S and 28S rRNA. Control, C; progesterone only, P4; 17 $\beta$ -estradiol, E2; progesterone plus RU486, P4RU; progesterone plus 17 $\beta$ -estradiol, P4E2; and progesterone plus 17 $\beta$ -estradiol plus RU486, ALL.

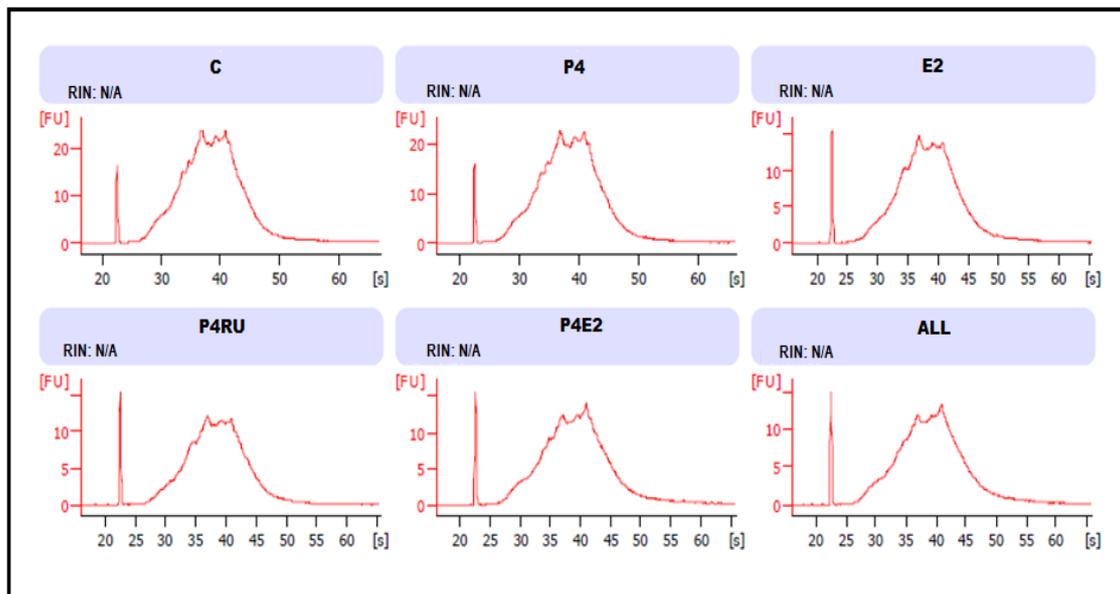
### 3.2.5 Quality control of labelled RNA

Even distribution of the dye (Cy5) in the sample is important to generate uniform signals after DNA microarray hybridisation. The labelled RNA samples from both experiments were checked for uniform dye distribution using the Agilent Bioanalyzer. An optimal labelling condition would be when 30 – 60 Cy5 dye molecules are incorporated per 1000 nt of labelled aRNA. The number of Cy5 dye molecules incorporated per 1000 nucleotide of labelled aRNA was calculated as explained in Section 2.2.20.7. Quality control of all labelled aRNA were performed and all samples used for hybridisation had an average of 45 Cy5 dye molecules incorporated per 1000 nt of labelled aRNA. The electropherograms of the labelled aRNA samples

from representative E2 experiments are presented in Figure 3.12 and representative figure from P4 experiment is given in Figure 3.13.



**Figure 3.13: Bioanalyzer analysis of amplified and labelled RNA shows even distribution of Cy5 dye.** Isolated and purified RNA was amplified and Cy5-labelled using Agilent Amino Allyl Messenger Amp II aRNA Amplification Kit™. The electropherograms from the Bioanalyzer analyses of labelled RNAs are shown above. The absorption peak between 35 and 50 s in each graph indicates the incorporation of 30 – 50 Cy5 dye molecules/1000 nt of labelled aRNA. Samples are named as treatment hour followed by UT for untreated sample and E2 for 10 nM E2-treated sample.



**Figure 3.14: The Bioanalyzer electropherogram of the labelled RNA (P4 treatment).** Isolated and purified RNA was amplified and Cy3-labelled RNA using the Amino Allyl Messenger Amp II aRNA Amplification Kit™ (Ambion). The absorption peak between 35 and 50 in each graph indicates the incorporation of 30 – 50 Cy3 dye molecules/1000 nt of labelled aRNA. Control, C; progesterone only, P4; 17 $\beta$ -estradiol, E2; progesterone plus RU486, P4RU; progesterone plus 17 $\beta$ -estradiol, P4E2; and progesterone plus 17 $\beta$ -estradiol plus RU486, ALL.

### **3.3 Discussion and Conclusion**

The research in this thesis focuses in understanding the transcriptomic changes in cultured hUtSMCs in response to steroid hormone changes. Hence, the characterisation of the cells for smooth muscle characteristics is of utmost importance. Even though Lonza performs routine characterisation studies including immunofluorescent staining on hUtSCMs procured (cells used in the present study), we also conducted separate experiments to confirm that the characteristics of myometrial smooth muscle cells used in this study. RT-PCR experiments for the expression of smooth muscle marker genes smoothelin, calponin, and oxytocin receptor confirmed that these cells exhibited smooth muscle characteristics.

Steroid hormones exert their action on target cells through their receptors (Mesiano et al., 2011). Cultured myometrial cells rapidly lose their ability to express oestrogen receptors (Zaitseva et al., 2006). Semi-quantitative PCR analysis confirmed that the cells used in this experiment expressed oestrogen receptor alpha ( $ER\alpha$ ).  $ER\alpha$  is shown to induce the expression of progesterone receptors (Mesiano et al., 2011).

Prior to the main experiments, the responsiveness of hUtSMCs to E2, P4 and RU486 treatment were tested. The mRNA expression of CD38 was analysed to verify the responsiveness to E2 treatment, whereas the mRNA expression of BCL2 was tested to confirm the responsiveness to P4 and RU486 treatment. The change in the expression of CD38 and BCL2 mRNA in response to hormone treatment was in line with previous reports. Although, no significant change in the mRNA expression of CX43 was observed in response to E2 treatment, there was an induction in CX43 protein expression (Di et al., 2001; Hertelendy and Zakar, 2004). This could be due to the fast translation to protein before rapid mRNA degradation possibly through microRNA mediated decay mechanism. Overall, myometrial smooth muscle cells used in this study were responsive to the hormone treatments.

Cell culture media and supplements are proven to have hormone effect (Cao et al., 2009; Dang and Lowik, 2005; Gstraunthaler, 2003). Since this study was aimed to understand the effect of steroid hormones on myometrial functions during pregnancy and labour, it is important to confirm that the influence of endogenous hormones in the cell culture system was removed. In the present study, this was achieved by using

phenol-red free (PRF) DMEM supplemented with double charcoal stripped (CS) serum for endogenous hormone starvation and subsequent hormone treatment. The induction in the expression of CD38 gene in response to E2 treatment was maximal in cells cultured in PRF-DMEM supplemented with CS-FBS. Hence, for further experiments hUtSMCs were starved from endogenous hormones in PRF-DMEM supplemented with CS-FBS prior to hormone treatment.

Since the quality of the RNA is very crucial in microarray analysis, the quality and quantity of the total RNA isolated and also of the labelled aRNA was assayed. To maximise the reliability of the experiments only RNA with highest quality was used for further downstream analysis.

In conclusion, the uterine smooth muscle cells used in the study expressed the characteristics of smooth muscle cells. These cells were tested and proven responsive to the steroid hormone treatment. The cell culturing conditions were also optimised to maximise the treatment effect, thus making this cell culture model system suitable for further studies.

**Chapter 4: Effect of 17 $\beta$ -Estradiol on  
Gene Expression in Cultured Human  
Uterine Smooth Muscle Cells: Global  
Transcriptomic Profiling.**

#### **4.1 Overview**

Oestrogen is a pro-contractile steroid hormone that regulates myometrial function during parturition and labour. Oestrogen also has other functions in the female system, but this study emphasises the role of oestrogen in relation to pregnancy and labour. 17- $\beta$  Estradiol (E2) is the most active form of oestrogen present in the circulatory system of labouring women (Mendelson and Condon, 2005). Towards the onset of labour the uterus, especially, the myometrium becomes more responsive to circulating oestrogen (Mesiano, 2001). Hence, the active contraction of the myometrium during labour is partly attributed to oestrogen. The study initially verified if cultured hUtSMCs would react to hormonal treatment in a way similar to that reported in *in vivo* physiological conditions. The focus of this study was thus to identify genes that were differentially expressed following E2 treatment in cultured hUtSMCs using DNA microarray-based global gene expression analysis. Identifying estradiol responsive genes would provide a valuable insight into the various molecular mechanisms regulated by these genes in the myometrium. This information combined with published *in vivo* data would help to understand the role played by oestrogen in regulating myometrial functions.

## **4.2 Results**

hUtSMCs were cultured in steroid-free medium and were treated with 10 nM E2 and harvested at specific timepoints (6, 24 and 72 h) with their corresponding untreated controls. Different timepoints were selected to understand the time-dependent transcriptomic effect of E2 treatment on cultured hUtSMCs. RNA from these samples was isolated and differentially expressed genes following E2 treatment were identified using DNA microarray technology. Each set of experiments was performed in triplicate to obtain statistically significant results. Data enrichment analyses such as gene ontology and pathway analysis were performed on all classified lists of differentially expressed genes to understand the biological function and significance of each gene. The expression profiles of selected genes identified from the microarray experiment were validated using real-time RT-PCR (qPCR).

### ***4.2.1 Analysis of the global gene expression profile of hUtSMC upon E2 treatment***

The expression of 25,100 unique genes and 39,600 transcripts, excluding control oligos, represented by 35,357 oligonucleotide probes on the Operon array were analysed in hUtSMCs for differential expression following E2 treatment for 6, 24 and 72 h. Three biological replicates were used for each experimental set. For each biological replicate separate slides were used (6 conditions X 3 replicated = 18 slides). Functional genomics analysis was performed using GeneSpring GX12.0 software (elaborated in Chapter 2, Section 2.2.20).

Intensity values for all entities (35,357) were initially filtered to exclude values less than 50 in both conditions – control and oestrogen treated. Following this, two separate two-way ANOVAs were carried out: the first was the standard analysis with treatment (control or oestrogen-treatment) and time (6, 24 or 72 h) as variables. As explained in Section 2.2.20 a second ANOVA was performed with treatment and experiment (biological replicates 1, 2 and 3) as variables. All statistically significant differentially expressed entities relating to oestrogen treatment from both analyses were pooled to make a common list of 1,189 entities. Restricting this further to include only entities that are differentially expressed >1.5-fold returned 868 entities. Annotation of the oligonucleotide probes on the Operon microarray chip used in the present study (build date: April 4, 2005) was updated; the list of 868 entities was

#### Chapter 4: Effect of 17- $\beta$ estradiol on hUiSMCs

manually checked and corresponding RefSeq entities were either modified or deleted. This process eliminated a further 319 genes leaving a total of 509 fully annotated genes. Only a shortened list of genes whose expression was altered by >5-fold is presented in table 4.1. The complete list of all differentially expressed genes (>1.5-fold) is given in an appended table (A3.1).

**Table 4.1 Summary of genes with altered expression (>5.0-fold) across all timepoints following E2 treatment.**

Gene Symbol	Time (h)	Fold Change <sup>1</sup>	P-Value	RefSeq ID	Gene Name
MAML3	24	5.2	3.36E-02	NM_018717	Mastermind-like 3 (Drosophila)
MAGEA4	24	5.3	2.05E-02	NM_002362	Melanoma antigen family A, 4
MYL1	24	5.3	1.32E-02	NM_079422	Myosin, light polypeptide 1, alkali; skeletal, fast
MYBPC1	24	5.4	9.22E-05	NM_206820	Myosin binding protein C, slow type
KIAA0895	24	6.6	2.46E-02	NM_015314	KIAA0895 protein
GOLGA6B	24	6.7	9.97E-04	NM_018652.4	Similar to Golgi autoantigen, golgin subfamily A member 6 (Golgin linked to PML) (Golgin-like protein)
AP3M1	24	6.8	1.21E-02	NM_207012	Adaptor-related protein complex 3, mu 1 subunit
BRIP1	24	7.4	1.94E-03	NM_032043	BRCA1 interacting protein C-terminal helicase 1
FBXO31	24	8.4	1.22E-02	NM_024735.3	F-box protein 31
SCGB1D4	24	9.6	1.46E-02	NM_206998	Secretoglobin family 1D member 4
SCN5A	24	9.6	1.82E-02	NM_198056	Sodium channel, voltage-gated, type V, alpha (long QT syndrome 3)
CT47A11	24	10.7	8.51E-03	NM_173571	Hypothetical protein LOC255313
KL	24	10.8	2.16E-03	NM_004795	Kelch-like ECT2 interacting protein
RAB37	72	12.1	1.12E-02	NM_175738	RAB37, member RAS oncogene family
GIT1	24	-14.3	1.71E-05	NM_014030	G protein-coupled receptor kinase interactor 1
PTPRF	24	-8.9	7.95E-03	NM_130440.2	Protein tyrosine phosphatase, receptor type, F
LBX2	72	-8.4	2.05E-02	NM_001009812	Similar to LP3727
CHRM3	24	-8.2	1.71E-02	NM_000740.2	Cholinergic receptor, muscarinic 3

#### Chapter 4: Effect of 17- $\beta$ estradiol on hUtSMCs

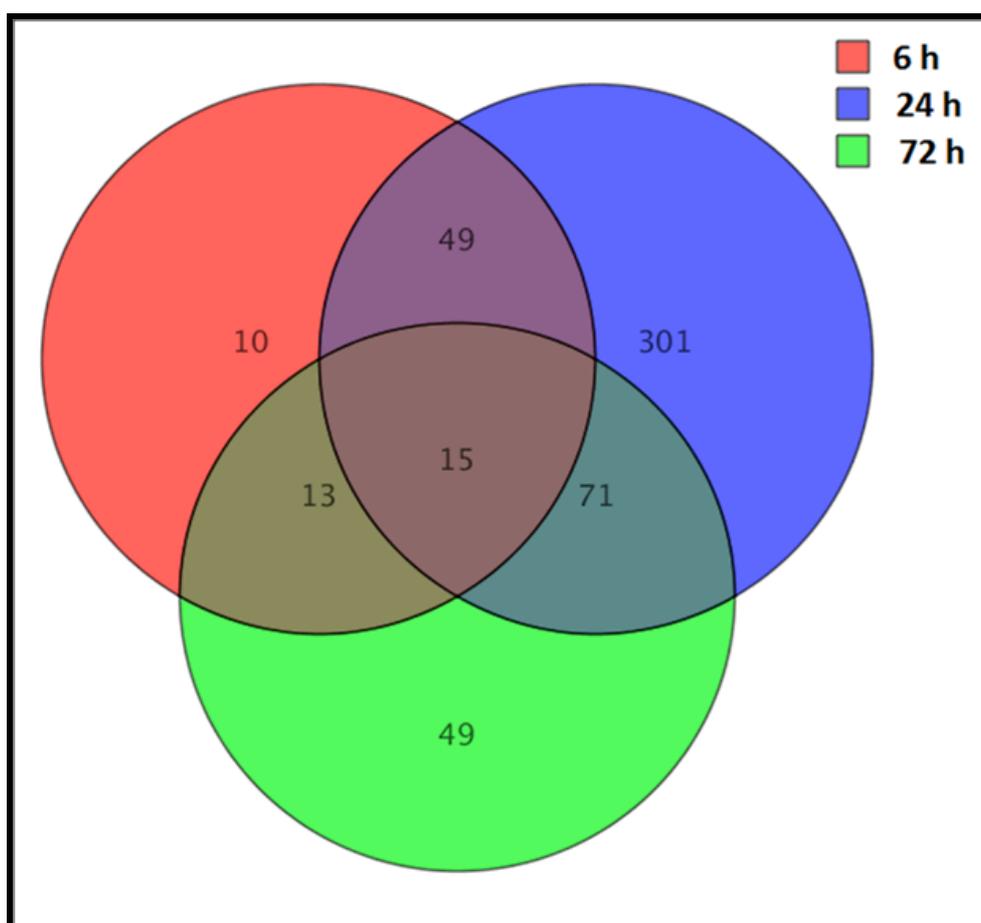
RALGPS1	24	-7.4	5.56E-03	NM_014636	Ral GEF with PH domain and SH3 binding motif 1
ACSM1	24	-7.1	3.72E-02	NM_052956	Butyryl Coenzyme A synthetase 1
USH1C	24	-6.9	1.04E-02	NM_005709	Usher syndrome 1C (autosomal recessive, severe)
SLC26A5	24	-6.8	3.88E-02	NM_198999	Prestin (motor protein)
KRTAP11-1	24	-6.6	1.08E-02	NM_175858	Keratin associated protein 11-1
PBX1	24	-6.4	8.36E-03	NM_002585	Pre-B-cell leukemia transcription factor 1
GPR52	24	-6.3	4.36E-02	NM_005684	G protein-coupled receptor 52
TRPM2	24	-6.1	2.78E-02	NM_001001188	Transient receptor potential cation channel, subfamily M, member 2
ARHGAP32	24	-5.7	6.92E-08	NM_014715	Rho GTPase-activating protein
CD8A	24	-5.5	2.58E-02	NM_001768	CD8 antigen, alpha polypeptide (p32)
DIDO1	24	-5.5	3.96E-02	NM_080796	Death associated transcription factor 1
KCNJ5	24	-5.3	1.59E-04	NM_000890	Potassium inwardly-rectifying channel, subfamily J, member 5
KNG1	24	-5.3	1.01E-02	NM_000893.3	Kininogen 1
AKT3	24	-5.2	3.65E-03	NM_181690	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)

<sup>1</sup> Positive values (in red) indicate upregulation and negative values (in green) represent downregulation.

#### 4.2.2 Time course analysis of differentially expressed genes identified after E2 treatment

In the present study, the hUtSMCs were treated with E2 for three different periods of time (6, 24 and 72 h). A comparison of all the differentially expressed genes identified in this study across all timepoints (509 genes) was performed to identify a time-dependent expression profile of genes. Identification of any such pattern would give further insight into how the expression of genes change according to the duration of E2 exposure.

From the analysis, 64 genes were identified to be common between 6 and 24 h, 28 genes between 6 and 72 h, 86 genes between 24 and 72 h and 15 genes were common between all the timepoints analysed (Figure 4.1). Figure 4.2 represents the level of genes differentially expressed across all timepoints analysed. Table 4.2 lists the expression profiles of these 15 genes that are common to all three timepoints analysed.



**Figure 4.1: Venn diagram comparing the differentially expressed genes between 6, 24 and 72 h post E2 treatment.** Shaded areas denote differentially expressed genes from 6 h (red), 24 h (green) and 72 h (blue). Shared area between 6 h and 24 h are shaded in purple, 6 h and 72 h in brown and 24 h and 72 h in aquamarine. The area shaded in light plum colour represents common genes shared between all three timepoints. Numbers within each area represents total number of genes in that group.

Table 4.2: Differentially expressed genes common to all timepoints (6, 24 and 72 h) post E2 treatment.

Gene Symbol	Gene Name	Fold change <sup>1</sup>		
		6 h	24 h	72 h
CD86	CD86 molecule	1.5	3.4	1.8
RBBP9	Retinoblastoma binding protein 9	1.6	2.5	2.1
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	1.7	2.1	1.9
F2	Coagulation factor II (thrombin)	2.1	2.1	4.7
SLC13A2	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2	-1.6	-1.5	-3.4
GCKR	Glucokinase (hexokinase 4) regulator	-1.6	-2.3	-1.7
RAI1	Retinoic acid induced 1	-1.5	-2.2	-2.3
VAV3	Vav 3 guanine nucleotide exchange factor	-1.8	-1.7	-1.6
RALGPS1	Ral GEF with PH domain and SH3 binding motif 1	-1.8	-7.4	-2.1
CCDC75	Coiled-coil domain containing 75	-2.0	-4.3	-1.8
GJD2	Gap junction protein, alpha 9	-2.7	-2.7	-3.4
LBX2	Ladybird homeobox 2	-1.6	-3.4	-8.4
MEPE	Matrix extracellular phosphoglycoprotein	-1.7	-2.2	-2.4
CD8A	CD8a molecule	1.6	-5.5	-2.0
AKT3	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	1.6	-5.2	-2.3

<sup>1</sup> Positive values indicate upregulation and negative values represent downregulation

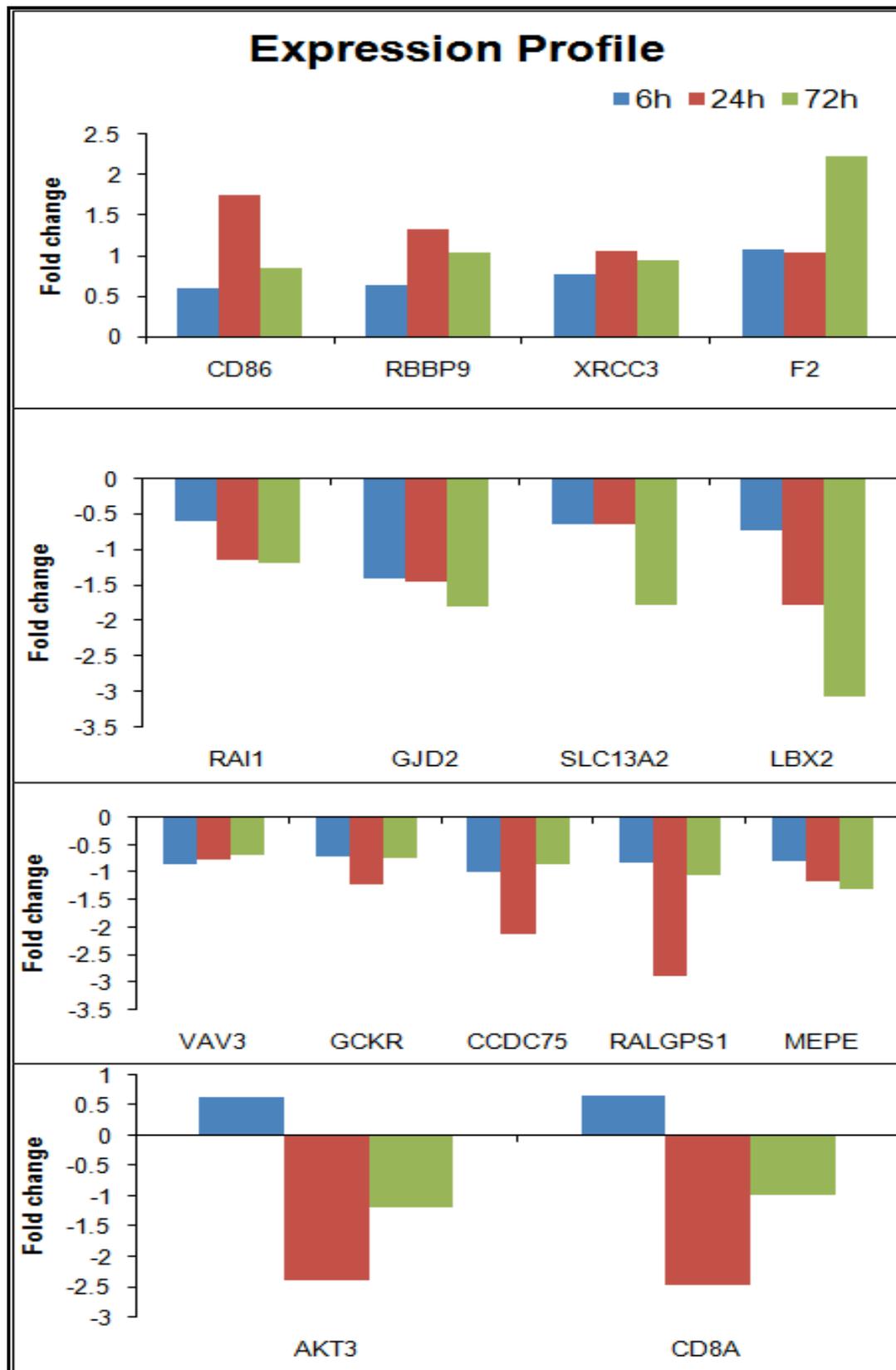


Figure 4.2: Expression profile of the 15 genes identified common to all timepoints (6, 24 and 72 h) post E2 treatment. Graph plotted using  $\log_2$  values.

### **4.2.3 Gene Ontology analysis**

Gene Ontology (GO) annotation represents a link between the gene product and the role that product plays functionally, in terms of the biological processes it contributes to a cell or organism. To gain more knowledge on the biological significance of the differentially expressed genes (509 genes), GO analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Dennis et al., 2003) and Onto-Express (OE) (Khatri et al., 2004; Khatri et al., 2002). Each of the differentially expressed genes identified in the present study (509 genes) was also manually checked in GeneCard ([www.genecards.org](http://www.genecards.org)) (Safran et al., 2010) and in published literature (PubMed) to identify biological processes that DAVID or OntoExpress analysis might have missed. GO analysis identifies over-represented gene ontology categories (biological processes, molecular functions and cellular component) in a given set of genes. This information, along with the experimental conditions from which the genes are generated, will provide insight into the biological relevance of the genes that are differentially expressed in a cell under a specific condition. Default settings in the software were used for all analyses (explained in 2.2.21).

Of the 509 genes identified to be differentially expressed, GO analysis identified biological processes for 251 genes. The most over-represented biological processes identified in the analysis were muscle contraction, developmental process, immune and inflammatory response, ion transport, metabolism, cell adhesion, cell signalling, cell differentiation, cell proliferation, apoptosis, transcription and transport. Identified biological process terms were grouped based on common function and genes annotated under each biological process term were grouped to remove all repeated genes. The graph (Figure 4.3) represents the main biological processes identified with the total number of genes (grouped) identified under each category.

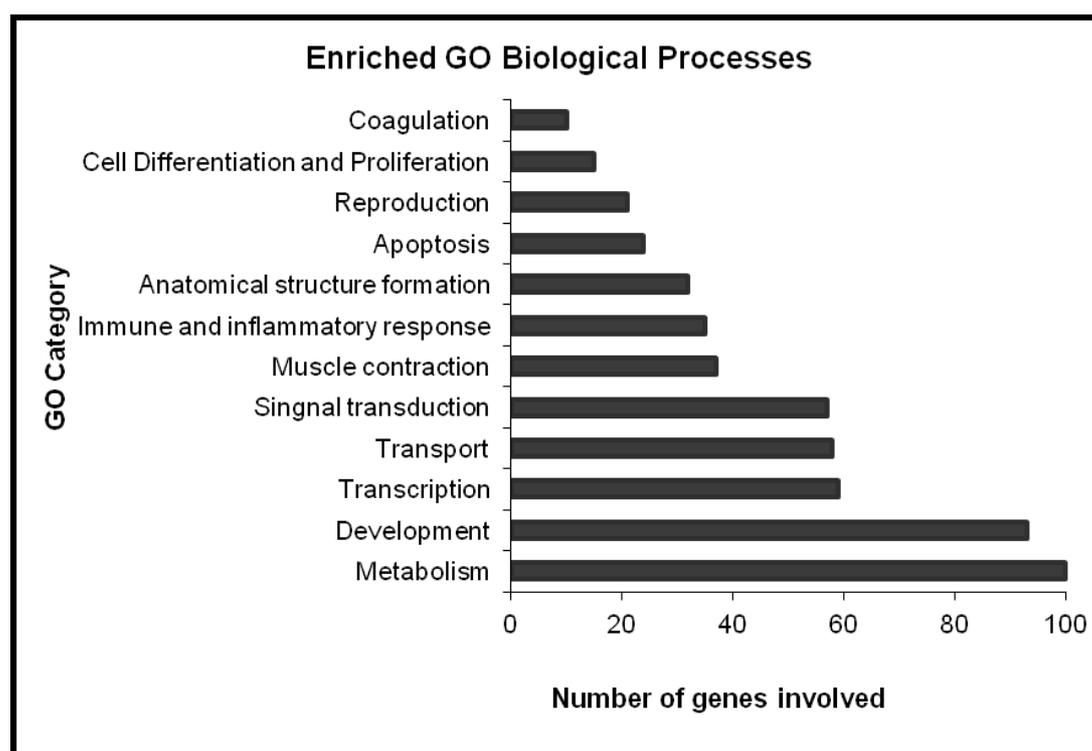


Figure 4.3: Enriched GO biological processes identified following E2 treatment of hUtSMCs.

GO analysis organises enriched GO terms in a Directed Acyclic Graph (DAG), a directed graph with no directed cycles (explained in Chapter 2, Section 2.2.21). Thus, a single parent biological process term can have multiple sub-biological (child) processes associated with it and a sub-biological (child) process may share multiple ancestors. It should therefore be noted that there are common genes within different enriched GO biological process terms and the categories listed - e.g. under the category ‘muscle contraction’ there are four enriched GO biological process terms, namely, (a) muscle contraction, (b) G-protein coupled receptor activity (c) elevation of cytosolic calcium concentration and (d) regulation of muscle contraction. Each of these GO biological process terms has 11, 24, 7 and 6 genes annotated under them respectively (Table 4.3). Calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G) gene is common among all the three enriched biological process, Cholinergic receptor, muscarinic 3 (CHRM3) and Myosin light chain kinase 2 (MYLK2) are common between muscle contraction and regulation of muscle contraction, Calcitonin-related polypeptide alpha (CALCA) is common between elevation of cytosolic calcium concentration and regulation of muscle contraction and Kininogen 1 (KNG1) between muscle contraction and elevation of cytosolic calcium

## Chapter 4: Effect of 17- $\beta$ estradiol on hUtSMCs

concentration. Similarly, different biological process categories listed below also share common genes. Thus, a single gene identified to be differentially expressed in hUtSMCs post E2-treatment may be involved in multiple biological processes.

**Table 4.3: Summary of enriched GO biological process terms identified under muscle contraction.**

Category	Biological Processes	No of Genes	Gene Symbol <sup>1</sup>
Muscle contraction	Muscle contraction	11	MYL6, <b>KNG1</b> , <b>CHRM3</b> , MYBPC1, MYL1, <b>CACNA1G</b> , <b>MYLK2</b> , PGAM2, CHRND, TTN, SCN5A
	G-protein coupled receptor activity	24	OR2M7, OR7A17, TAS2R5, F2RL1, RXFP2, OR10H1, OR1E2, OR5M9, TAS1R1, OR10G4, OR10H3, SORCS3, FZD10, GRM2, <b>CHRM3</b> , OR8J3, GPR113, GPR52, OR8B4, OR7D4, OPN4, OR2C1, OPRD1, GPR116
	Elevation of cytosolic calcium concentration	7	<b>CALCA</b> , <b>KNG1</b> , SAA1, IL6ST, F2, F2RL1, <b>CACNA1G</b>
	Regulation of muscle contraction	6	<b>CALCA</b> , <b>CHRM3</b> , SPHK1, <b>CACNA1G</b> , <b>MYLK2</b> , NKX2-5

<sup>1</sup> Genes that are common within different biological processes are highlighted.

Statistically significant enriched biological process categories identified in this analysis along with selected sub-biological processes; number of genes identified in the sub-biological process and *P*-value is tabulated (Table 4.4). The full list of 251 genes (across all timepoints) identified in GO analysis (compiled GO terms from DAVID, OntoExpress and GeneCard) with associated GO terms is tabulated in appendix table (A3.2).

Table 4.4: Summary of enriched GO terms identified in data enrichment analysis.

Category	Enriched GO Term	No of Genes Involved	p-Value <sup>1</sup>
<b>REPRODUCTION</b>	Embryo implantation	3	4.65E-03
	Parturition	1	1.41E-01
	Pregnancy	1	2.95E-01
<b>DEVELOPMENT</b>	Gland development	11	2.68E-03
	Endocrine system development	7	9.00E-03
	Embryonic organ development	7	2.71E-01
<b>IMMUNE AND INFLAMMATORY RESPONSES</b>	Immune response	16	2.02E-02
	Coagulation	9	4.31E-03
	Haemostasis	9	5.85E-03
	Platelet activation	5	7.20E-03
	Response to wound healing	17	2.26E-01
	Defence response	13	8.38E-01
	Negative regulation of cytokine secretion	1	9.67E-02
	Positive regulation of immune response	1	2.37E-01
	Positive regulation of adaptive immune response	1	2.39E-01
	Antigen processing and presenting	2	1.55E-01
	Positive regulation of angiogenesis	1	3.83E-01
<b>METABOLISM</b>	Steroid biosynthesis	4	4.50E-02
	Branched chain amino acid catabolism	1	1.41E-01
	Positive regulation of collagen biosynthesis	1	1.20E-01
	Positive regulation of biosynthesis	1	7.34E-02
	Positive regulation of protein catabolism	1	7.34E-02
	Negative regulation of low-density lipoprotein receptors biosynthesis	1	7.34E-02
<b>ANATOMICAL STRUCTURE FORMATION</b>	Cell substrate adhesion	7	3.70E-02
	Cell matrix adhesion	6	7.28E-02
	Cell adhesion	20	2.01E-02
	Actin filament bundle formation	1	2.63E-01
	Apical cell junction assembly	1	4.95E-02
	Focal adhesion formation	5	2.44E-01
	Calcium independent cell-cell adhesion	1	3.99E-01
	Calcium dependent cell-cell adhesion	1	4.43E-01
	Positive regulation of cell adhesion	3	4.08E-02

Chapter 4: Effect of 17- $\beta$  estradiol on hU $i$ SMCs

<b>MUSCLE CONTRACTION</b>	Muscle contraction	11	7.31E-03
	G-protein coupled receptor activity	24	2.60E-02
	Elevation of cytosolic calcium concentration	7	1.50E-02
	Regulation of muscle contraction	6	4.21E-02
<b>TRANSPORT</b>	Regulation of ion transport	6	1.09E-01
	Metal ion transport	4	3.91E-01
	Calcium ion transport	3	6.30E-01
	Transmembrane transporter	23	2.98E-02
	Intracellular mRNA localization	1	7.34E-02
	Cell migration	3	5.69E-02
	Regulation of cell migration	5	5.29E-02
<b>TRANSCRIPTION</b>	Positive regulation of gene expression	17	5.56E-01
	Positive regulation of transcription - RNA pol II dependent	9	4.96E-02
	Positive regulation of transcription DNA dependent	11	1.35E-03
<b>ENZYMES</b>	GTPase activity	6	1.49E-01
	Hydrolase activity	11	2.32E-01
	Regulation of phosphorylation	19	5.51E-02
	Activation of protein kinase activity	7	7.10E-02
<b>CELL DEATH</b>	Regulation of apoptosis	29	6.32E-02
	Regulation of programmed cell death	29	6.77E-02
	Regulation of cell death	29	7.19E-02
	Positive regulation of apoptosis	17	8.83E-02
	Positive regulation of programmed cell death	17	9.09E-02
	Positive regulation of cell death	17	9.39E-02
	Negative regulation of apoptosis	8	8.14E-01
<b>CELL PROLIFERATION AND DIFFERENTIATION</b>	Regulation of mononuclear cell proliferation	3	6.52E-01
	Regulation of cell differentiation	15	3.75E-01
	Cell differentiation	41	6.08E-01
	Muscle cell differentiation	3	6.32E-01
	Negative regulation of cell differentiation	7	4.67E-01
<b>COAGULATION</b>	Coagulation	9	4.31E-03
	Blood coagulation	9	4.31E-03
	Regulation of blood coagulation	3	2.13E-01

<sup>1</sup> p-values less than 0.05 is highlighted. GO category, enriched GO biological processes term, number of genes involved and corresponding *p*-value is given.

**4.2.4 Real-time RT-PCR validation of microarray results**

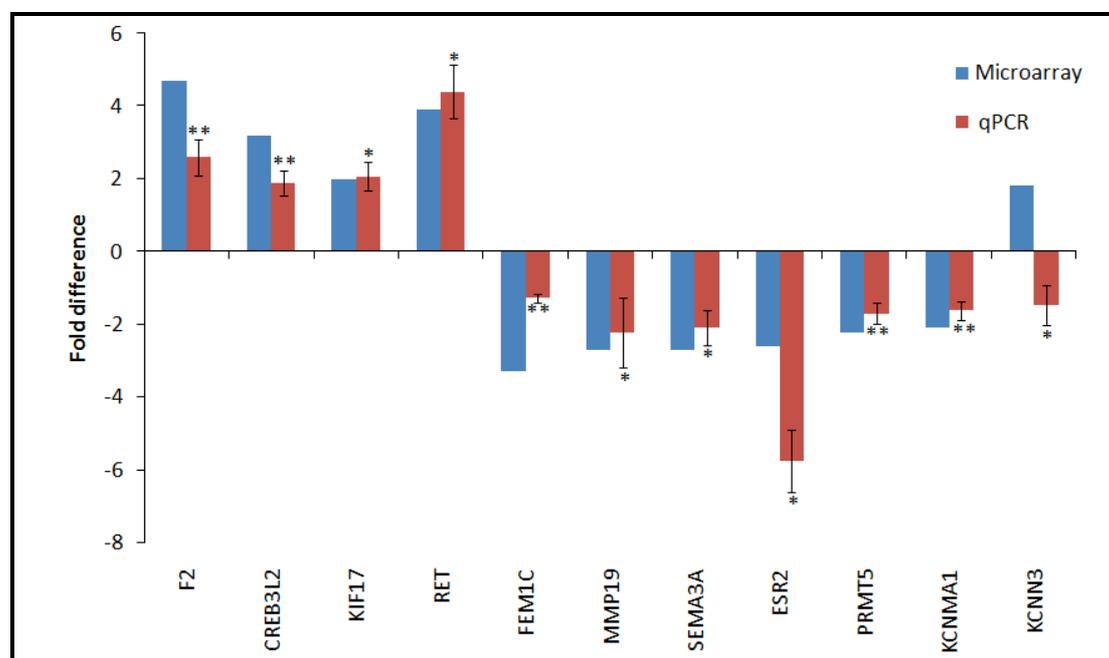
To confirm the microarray results, random selected genes from 72 h of E2 treatment (longest exposure to E2) were validated using a complementary gene expression profiling system - real-time RT-PCR (or qPCR). qPCR is a robust and accurate technique used to simultaneously amplify and quantify a target DNA sequence. SYBR-Green chemistry was used as the detection method for monitoring PCR amplification.

11 genes were selected for validation (Figure 4.4), four genes upregulated (F2, CREB3L2, KIF17 and RET), and seven downregulated genes (KCNM3, MMP19, SEMA3A, ESR2, PRMT5, FEM1C and KCNMA1). Table 4.5 below summarises the results from qPCR validation of these genes. All qPCR results agreed with the microarray data with the exception of KCNN3 whose expression was downregulated (approximately 1.5-fold) in qPCR contrary to what was identified in microarray (~2-fold upregulated).

**Table 4.5: Summary of qPCR validation of microarray results.**

<b>Gene Symbol</b>	<b>Gene Name</b>	<b>FC* Micro array</b>	<b>FC* qPCR</b>	<b>Description</b>
F2	Coagulation factor II	4.7	2.6	Prothrombin precursor
CREB3L2	cAMP responsive element binding protein 3-like 2	3.2	1.9	B-ZIP transcription factor
KIF17	Kinesin family member 17	2	2.1	Mitotic kinesin motor protein
FEM1C	Fem-1 homolog c	-3.3	-1.3	Signalling pathway that controls sex determination
RET	Ret proto-oncogene	3.9	4.4	Tyrosine kinase receptor
MMP19	Matrix metalloproteinase 19	-2.7	-2.2	Breakdown of extracellular matrix
SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	-2.7	-2.1	Innervations during gestation
ESR2	Oestrogen receptor 2 (ER beta)	-2.6	-5.8	Cellular signalling of Oestrogen
PRMT5	Protein arginine methyltransferase 5	-2.25	-1.7	Regulation of signal transduction & transcription
KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	-2.1	-1.6	MaxiK channel protein
KCNN3	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	1.8	-1.5	Ca <sup>2+</sup> - activated K channel protein 3

<sup>1</sup> Positive value indicates upregulation and negative value represents downregulation. \*FC = Fold change.



**Figure 4.4: Real-time RT-PCR validation of differentially expressed genes following 72 h of E2 treatment.** hUtSMCs were treated with 10 nM E2 and were harvested 72 h post-treatment. Extracted Total RNA was subjected to Real-Time RT-PCR together with untreated samples as control. Fold differences were calculated based on untreated samples. Each value corresponds to the average from three independent experiments (n=3) plotted ( $\pm$  SD). All expression levels were normalised to the corresponding housekeeping gene (GAPDH) levels. Statistical significance of the difference between the E2-treated and untreated controls was determined based on untreated samples using the Student *t*-test. \*- $P < 0.05$  and \*\*- $P < 0.005$ .

#### 4.2.5 Comparison of the differentially expressed genes identified in the present study with oestrogen responsive genes reported in the ERGDB

The “Estrogen Responsive Gene Database” (ERGDB) is a database of oestrogen-responsive genes integrated from published literatures (Tang et al., 2004). All differentially expressed genes identified in the present study (509 genes) were checked in ERGDB to verify if the genes were previously reported as differentially expressed upon oestrogen treatment. Of the 509 differentially expressed genes identified in the current study 36 (7.3 %) were previously reported as oestrogen-responsive genes in ERGDB (Table 4.6).

Table 4.6: Comparison of E2-responsive genes from hUtSMCs with the ERGDB.

Gene Symbol	Species (ERGDB) <sup>1</sup>	Effect of E2 on hUtSMC		Gene Name
		Regulation <sup>2</sup>	Time (h)	
MCM7	Hs/Mm	↓	24	MCM7 minichromosome maintenance deficient 7 (S. cerevisiae)
CD86	Hs	↑	6, 24, 72	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)
IDS	Hs	↑	24, 72	iduronate 2-sulfatase
ANKRD44	Hs	↓	24	ankyrin repeat domain 44
GTF2I	Hs	↓	72	general transcription factor II, i
CCNT2	Hs	↑	24, 72	cyclin T2
ABCB1	Hs	↓	24	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ISGF3G	Hs	↑	24	ring finger protein 31
TFPI2	Hs	↓	72	tissue factor pathway inhibitor 2
KRT23	Hs	↓	24, 72	keratin 23 (histone deacetylase inducible)
SYNGR2	Hs	↓	24	synaptogyrin 2
CALCA	Mm	↓	24	calcitonin/calcitonin-related polypeptide, alpha
PRLR	Hs	↓	24, 72	prolactin receptor
CENPA	Hs	↑	24	chromosome 2 open reading frame 18
KYNU	Hs	↑	6, 72	kynureninase (L-kynurenine hydrolase)
ALDH1A2	Ms	↓	72	aldehyde dehydrogenase 1 family, member A2
KL	Hs	↑	6, 24	klotho
SAA1	Hs	↑	24, 72	serum amyloid A1
TIMM17A	Hs	↑	24	translocase of inner mitochondrial membrane 17 homolog A (yeast)
BRIP1	Hs	↑↓	24 (↑), 72 (↓)	BRCA1 interacting protein C-terminal helicase 1
CYR61	Hs/Mm	↑	24, 72	cysteine-rich, angiogenic inducer, 61
CRH	Hs	↓	24, 72	corticotropin releasing hormone
PRSS23	Hs	↓	72	protease, serine, 23
THRB	Rn	↓	24	hypothetical gene supported by AK096885; AK098084
PTPRO	Hs	↓	6, 24	protein tyrosine phosphatase, receptor type, O
BICD1	Hs	↑	24, 72	bicaudal D homolog 1 (Drosophila)
CD8A	Hs	↑↓↓	6 (↑), 24 (↓), 72 (↓)	CD8 antigen, alpha polypeptide (p32)
TSC22D3	Hs	↓	24	delta sleep inducing peptide, immunoreactor
ZNF230	Hs	↓	24, 72	zinc finger protein 230
COL3A1	Mm	↑	24	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
NBEA	Hs	↓	24	neurobeachin

**Chapter 4: Effect of 17- $\beta$  estradiol on hU<sub>i</sub>SMCs**

F2	Rn	↑	6, 24, 72	coagulation factor II (thrombin)
CREB3L2	Hs	↑	72	cAMP responsive element binding protein 3-like 2
CSF1R	Mm	↓	24	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
PBX1	Mm	↓	24, 72	pre-B-cell leukemia transcription factor 1
DIO2	Mm	↓	6, 72	deiodinase, iodothyronine, type II

<sup>1</sup> Hs = *Homo sapiens*, Mm = *Mus musculus*, Rn = *Rattus norvegicus*. <sup>2</sup> ↑ = Upregulated, ↓ = Downregulated

### **4.3 Discussion**

As discussed in the introduction to this chapter (Section 3.2), oestrogen is the primary sex steroid hormone in females. Oestrogen plays an important role in regulating pregnancy and parturition (Mesiano, 2001), in determining the growth of hormone-dependent tumours (Fuhrman et al., 2012; Green et al., 2012; Zhang et al., 2012) and in regulating the oestrous cycle (Nakamura et al., 2010), and it contributes to the development of secondary sex characteristics in women (Lavranos et al., 2006). Encompassing these multiple roles, this study's purpose was to identify the transcriptomic effects of 17 $\beta$ -estradiol treatment on human uterine smooth muscle cells, while primarily focusing on the expression profiles of genes associated with myometrial contractility. Several studies have extended our understanding of the molecular mechanisms implicated in the action of pregnant and labouring myometrium (Aguan et al., 2000; Bailey and Europe-Finner, 2005; Bailey et al., 2005; Bethin et al., 2003; Bukowski et al., 2006; Chan et al., 2002; Charpigny et al., 2003; Cordeaux et al., 2010; Esplin et al., 2005a; Esplin et al., 2005b; Havelock et al., 2005; Khanjani et al., 2011; Mittal et al., 2010; O'Brien et al., 2008a, b; Rehman et al., 2003). However, to the best of our knowledge, no efforts have been made to understand the transcriptomic effect of E2 treatment on human uterine smooth muscle cells in culture. The present study identified differentially expressed genes in cultured human myometrial smooth muscle cells following E2 treatment. Several genes that have been previously identified as oestrogen-responsive in cell culture and animal studies have been confirmed as such in the present study. Of the differentially expressed genes approximately 60 % were downregulated and 40 % upregulated. Time course analysis identified differential regulation of several genes across two timepoints analysed, and 15 genes across all three timepoints. Biological processes relating to the oestrous cycle, pregnancy, parturition and the growth of hormone-dependent tumours are discussed below together with some of the most relevant and interesting genes.

#### *MUSCLE CONTRACTION:*

Central to the function of myometrial smooth muscle cells is their role in muscle contraction. Uterine tissue undergoes contraction both during the non-pregnant and pregnant state. During the menstrual cycle, in the follicular phase, uterine contraction

increases reaching its peak at ovulation. In the luteal phase, the frequency and amplitude of uterine contraction decreases and in the absence of implantation, during menstruation, the amplitude of contraction increases leading to labour-like contractions (Aguilar and Mitchell, 2010; Ijland et al., 1996; van Gestel et al., 2003). The levels of oestrogen are at their highest in labouring myometrium (Mesiano, 2001) and in the uterus during the late follicular stage of the oestrous cycle (Nakamura et al., 2010). During pregnancy and labour, cytoskeletal remodelling of myometrial cells is essential for contraction and relaxation of the uterus (Breuiller-Fouche and Germain, 2006; Shynlova et al., 2005). With the onset of labour, signalling pathways which regulate muscle relaxation are repressed and those regulating contraction are induced (Price and Bernal, 2001).

Several factors regulate the contraction of uterine smooth muscles. Intracellular  $\text{Ca}^{2+}$  concentration is the most important factor controlling contraction of myometrial smooth muscle (Saleh et al., 2005; Wray, 1993). An increase in myometrial  $\text{Ca}^{2+}$  levels is responsible for the phosphorylation of myosin light chain, activity of myosin ATPase and subsequent contraction (Sanborn et al., 2005). Release of  $\text{Ca}^{2+}$  from intracellular vesicular stores to the cytoplasm contributes to the phosphorylation of myosin light chain and induces uterine contraction during labour (Chini et al., 1997). For  $\text{Ca}^{2+}$  ions to have a coordinated effect across the tissue, cells must act in a synchronous manner requiring tight control of cell-to-cell communication. Gap junctions have an essential role in coordinating myometrial contraction and are highly regulated during labour onset in order to maintain myometrial quiescence or contraction (Helguera et al., 2009). It is reported in rats that rapid formation of gap junctions terminates pregnancy and initiates labour (Garfield et al., 1978). Although  $\text{Ca}^{2+}$  ions are the main ion regulators of muscle contraction, they cannot function without the maintenance of a cell membrane potential.  $\text{K}^+$  and  $\text{Na}^+$  ions are instrumental in this role.  $\text{K}^+$  channels in myometrium change the impulsiveness of the myometrium during labour (Wang et al., 1998) and the  $\text{Na}^+$  current is reported to increase during pregnancy (Kimura et al., 1967). In the present study, 11 genes differentially expressed post-E2 treatment were identified by GO analysis as having a potential role in regulating muscle contraction ( $P=0.007$ ). Among these, GO analysis identified seven genes (CALCA, KNG1, SAA1, IL6ST, F2, F2RL1, CACNA1G)

having a role in increasing cytosolic calcium levels. Expression of all genes except CALCA and KNG1 was increased in cultured hUtSMCs post-E2 treatment.

When hUtSMCs were treated with 17β-estradiol, the expression of Regulator of G-protein Signalling 4 (RGS4) was downregulated by over 2-fold. The activation of G-protein coupled receptors (GPCRs) is important for the contraction of myometrium, where they target the activation of phospholipase C thereby leading to release of internal Ca<sup>2+</sup> stores and stimulation of calcium influx (Brenninkmeijer et al., 1999). RGS4 plays an important role in regulating gastrointestinal smooth muscle contraction by binding to and increasing the intrinsic GTPase activity of the Gαq subunit thereby inhibiting agonist-induced contraction (Hu et al., 2008; Hu et al., 2007; Huang et al., 1997). In addition to RGS4 other GPCR-related genes (GPR52, GPR113, GRM2 [downregulated], and GPR116 [upregulated]) were also differentially expressed in the present study. Although downregulation of RGS4 would appear to be linked to increased contractility, agreeing with the role of E2 in contraction, levels of RGS4 have been shown to increase in term labouring samples relative to the non-pregnant, preterm and term non-labouring groups (Ladds et al., 2009).

Sphingosine kinase 1 (SPHK1) catalyses the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P), was downregulated at 24 h in the present study when hUtSMCs were treated with E2. The resulting product (S1P) is a bioactive sphingolipid metabolite that is known to play a key role in vascular, gastric and airway smooth muscle contraction (Watterson et al., 2005) through the action of a family of G-protein coupled receptors. A pharmacological inhibitor of SPHK1 dose-dependently decreased oestrogen-stimulated transcriptional activity through oestrogen response elements and thereby diminished mRNA levels of oestrogen receptor (ER)-regulated genes (Antoon et al., 2011). Although the current study shows downregulation of SPHK1, E2 has been shown to stimulate SPHK1 activity (Sassoli et al., 2011; Sukocheva et al., 2006). Furthermore, SPHK1 protein and its activity increase with gestational age in human decidua parietalis (Yamamoto et al., 2010), while in rat myometrium the levels and activity of SPHK1 increase from mid-gestation through day 19 (that is, in physiological state where oestrogen is at their highest concentration) before declining postpartum (Serrano-Sanchez et al., 2008).

The observed downregulation of SPHK1 by estradiol seems contradictory. In itself this would suggest reduced S1P signalling yet estradiol activates SPHK1 activity, and inhibitors of SPHK1 activity effectively decrease oestrogen-related responses (Antoon et al., 2011). In addition it is known that RGS4 (mentioned above) is known to inhibit S1P signalling therefore the E2-induced downregulation seen in the current study should be associated with increased S1P signalling (Cho et al., 2003). What is clear is that oestrogen is involved in S1P signalling, that S1P signalling is involved in muscle contraction, and that S1P levels are regulated during pregnancy. An added level of complexity is introduced as it has been shown that progesterone upregulates SPHK1 (Jeng et al., 2007; Yamamoto et al., 2010) and that E2 effects may be orchestrated through the EGFR as well as the ER.

Expression of the neuropeptide somatostatin (SST) gene was downregulated in the present study after both 24 and 72 h of E2 treatment. Smooth muscle cells are infiltrated with neurons which take the form of varicosities, swollen regions filled with neurotransmitter vesicles. Somatostatin was able to induce contraction of saphenous vein smooth muscle and colonic smooth muscle cells (Corleto et al., 1998; Dimech et al., 1995). Vascular smooth muscle cells incubated with somatostatin also showed reduction in planar cell surface area, decrease in cAMP levels and increase in intracellular  $Ca^{2+}$  concentration indicating somatostatin's role in positively regulating smooth muscle contraction (Torrecillas et al., 1999). At least one of a number of putative receptors for somatostatin is responsive to oestrogen therefore possibly the reduced level of SST and the increased level of the receptor combine to activate a specific function through that receptor.

#### *CELL STRUCTURE AND MORPHOLOGY:*

Several myosins and related genes were differentially expressed in hUtSMCs following E2 treatment (MYO15A, MYL6, MYL1 and MYBPC1 [upregulated], and MYOG and MTMR1 [downregulated]). Also downregulated at 24 h (2.1-fold) was the expression of myosin light chain kinase 2 (MYLK2). Myosins are motor proteins, which are responsible for actin-based muscle movement (Mello and Thomas, 2012). In pregnant myometrium, where the muscles are relaxed, myosin genes are highly expressed which leads to cell hypertrophy (Word et al., 1993). Activation of uterine smooth muscle contraction initiates with the phosphorylation of myosin light chain,

which is catalysed by myosin light chain kinase (Bresnick, 1999; Moore and Bernal, 2001; Wray, 1993). Microtubule-related protein 2 (MAP2), whose expression decreased by 2-fold in hUtSMCs following 24 h of E2 treatment, interacts with the myosin-binding subunit of myosin phosphatase (Amano et al., 2003). Myosin phosphatase is implicated in the phosphorylation state of myosin light chain to induce smooth muscle contraction (Amano et al., 2003). Myogenin (MYOG) mRNA expression was downregulated by over 3-fold in the present study following 24 h of E2 treatment. Myogenin is a transcription factor important in muscle development and is involved in differentiation of fibroblasts to myoblasts (Tseng et al., 1999). Myogenin knockout mice die at birth due to severe deficiencies in skeletal muscle (Hasty et al., 1993) and mice in which MYOG was conditionally removed before embryonic muscle development show myofiber deficiency (Knapp et al., 2006). Myogenin in skeletal muscle increases in expression in post-menopausal women on hormone replacement therapy (HRT) suggesting regulation of the gene by oestrogen (Hasty et al., 1993). Estradiol is clearly affecting several components of the muscle and non-muscle machinery within smooth muscle cells. It may allow the muscle cells to grow while attenuating differentiation until a certain critical point of development.

COL3A1, the gene encoding the pro-alpha1 chain of type III collagen, is upregulated by over 2-fold (24 h) in the present study. It is a fibrillar collagen found in extensible connective tissues such as skin, lung, uterus, intestine and the vascular system, and is frequently associated with type I collagen (Dalglish et al., 1985). Collagen fibres impart tensile strength and transfer tension across smooth muscle cells (Stevenson et al., 2006). 17 $\beta$ -Estradiol significantly stimulated COL3A1 in human skin cells (Gopaul et al., 2012) and in preimplantation mouse uterus (Diao et al., 2011). Another collagen related gene, COL8A1, was downregulated by 1.9-fold following 72 h of E2 treatment in hUtSMCs. This gene is necessary for the migration and proliferation of vascular smooth muscle cells (Desronvil et al., 2010).

Inter-alpha-trypsin inhibitor heavy chain family, member 5 (ITIH5) gene expression was downregulated at 24 h by over 4-fold following E2 treatment. ITIH5 mRNA expression was downregulated in multiple human tumour types including breast tumours (Hamm et al., 2008; Himmelfarb et al., 2004), and this reduced ITIH5 level was associated with the positive expression of oestrogen receptors (Veeck et al.,

2008), indicating the role of oestrogen in regulating the expression of ITIH5. ITIH5 is involved in stabilisation of the extracellular matrix (Himmelfarb et al., 2004).

Expression of Vinculin (VCL), an actin filament-binding protein involved in cell-matrix adhesion and cell-cell adhesion (Demali, 2004), was downregulated following 24 h of E2 treatment. In myocytes VCL is part of a large protein complex that attaches the contractile apparatus to the cell membrane (sarcolemma). Vinculin is important in muscular function as it has been shown that VCL knockout mice die *in utero* due to impaired cardiac muscle contraction (Zemljic-Harpf et al., 2007). Interestingly, in these knockout mice some junctional proteins are downregulated and the gap junction protein connexin 43 is mislocated. Downregulation by oestrogen has been shown in another model system of smooth muscle: in a rat model of prostate cancer, E2 treatment led to reduced levels of vinculin expression in smooth muscle cells (Yuen et al., 2005).

Integrin beta 3 (ITGB3) is one polypeptide chain of two which make up a number of integral cell surface proteins acting as cell surface receptors. The receptors act as adhesion proteins for other cellular and extracellular matrix proteins. The expression of this gene was upregulated at 24 h and 72 h after E2 treatment of hUtSMCs. Polymorphism in this gene is associated with recurrent pregnancy loss in humans (Jeddi-Tehrani et al., 2011).

#### *DIFFERENTIATION, PROLIFERATION AND APOPTOSIS:*

Oestrogen has been shown to induce cell proliferation aiding tumour progression in a wide variety of tumours (Chang, 2011; Tyson et al., 2011). Elevated oestrogen in the follicular phase of the oestrous cycle is responsible for the proliferation of stratum functionalis (the layer which is shed during menstruation), endometrial glands and the stromal connective tissues (Felix and Farahmand, 1997; Ferenczy et al., 1979). During early stages of pregnancy, oestrogen mediates rapid myometrial cell proliferation in order to accommodate the growing foetus (Shynlova et al., 2006), which reaches a hypertrophic phase by mid-gestation (Harkness and Harkness, 1954; Shynlova et al., 2010; Shynlova et al., 2004). At late pregnancy, an increase in apoptosis is observed in rat myometrium (Leppert, 1995, 1998; Leppert and Yu, 1994).

GO analysis for biological processes revealed 18 genes with functions related to cell differentiation ( $P=0.06$ ) and proliferation ( $P=0.03$ ) and 17 genes that are involved in apoptosis ( $P=0.06$ ).

In the present study, KIF17 gene expression was upregulated by over 2-fold following 24 and 72 h of E2 treatment. Kinesin family member 17 (KIF17) has been shown to stabilise microtubules, thereby aiding cell division (Jaulin and Kreitzer, 2010). Tyrosine phosphorylation was another significant ( $P=0.03$ ) GO term identified. Protein tyrosine phosphatase, receptor type F (PTPRF), whose mRNA expression was reduced by over 8-fold following 24 h of E2 treatment, is a key player in the insulin signalling pathway. Genes involved in insulin signalling are important regulators of growth and cellular proliferation (Jones and Clemmons, 1995) and an upregulation of insulin growth factor signalling is noted in pregnant myometrium (Leppert, 1998; Rehman et al., 2003).

The expression levels of histones HIST2H2AB (1.8-fold at 72 h) and HIST3H2BB (1.5-fold at 24 h) were increased following E2 treatment. Histones are important components of chromatin acting as the core for DNA winding (Lehninger, 1970; Lehninger et al., 2005). Elevated levels of histone proteins are observed in the S-phase of cell cycle and monitoring levels of histone proteins by histological localisation is a method to identify cell proliferation (Hewitson et al., 2006). Thus, an increase in histone mRNA is indicative of increased cell division.

In the present study Transforming Growth Factor-beta (TGFB2) was downregulated by over 2-fold following 24 h of E2 treatment. TGFB2 is a main regulator of morphogenesis and differentiation, and it is known to regulate myoblasts (Lopez-Casillas et al., 2003) and induce differentiation of cardiomyocytes (Singla and Sun, 2005). TGFB2 induced dose-dependent apoptosis in cultured endometrial cells (Caron et al., 2009) and its expression in endometrial culture is itself reduced following E2 treatment (Gaide Chevronnay et al., 2008). The reduced expression of TGFB2 in the present study therefore agrees with similar experiments in endometrial culture and suggests reduced apoptosis in our model. TGFB2 also acts as a cytokine and has been shown to have a suppressive effect on interleukin-dependent growth of T-cells (de Martin et al., 1987). In the bovine endometrium TGFB2 has been shown to respond to oestrogen but only after being primed by progesterone (Shimizu et al., 2010).

Cysteine-rich, angiogenic inducer, 61 (CYR61) mRNA expression, upregulated at 24 h post E2 treatment in the present study, is involved in cell proliferation, cell migration and cell adhesion (Chen et al., 2001). It is believed that this secreted protein interacts with ECM components and integrins of cells possibly inducing the expression of metalloproteinases which allow proliferation and migration of cells through the matrix. E2 treatment induced expression of this gene in an endometrial cancer cell line and this induction was blocked by anti-oestrogens (Collins et al., 2009). An increase in a number of angiogenesis-related factors was noted in uterine tissue undergoing spontaneous term labour and preterm labour: these included CYR61, RGS4 (mentioned earlier) and WISP1 (Haddad et al., 2008).

Expression of TSC22 domain family, member 3 (TSC22D3), a putative apoptosis-regulating transcription factor, was downregulated in hUtSMCs following 24 h of E2 treatment. This protein is known to protect T-cells from apoptosis induced by IL2 deprivation by downregulation of the pro-apoptotic factor Bim (Asselin-Labat et al., 2004). TSC22D3 is rapidly downregulated by oestrogen in breast cancer cells and antagonists of oestrogen block this effect (Tynan et al., 2004). Interestingly, E2 acts through a segment of the promoter that does not contain an ERE, and although E2 downregulates TSC22D3 in breast cancer cell lines the same promoter segment is responsible for E2-induced upregulation in other cell lines. This shows how regulation per se by oestrogen is relevant and not the direction of the regulation – whether it is up or downregulated might be tissue-specific, or condition-specific.

On the whole, E2 treatment of hUtSMCs regulated the expression of genes favouring cell proliferation and opposing apoptosis. In addition downregulation of ITIH5 (mentioned in the previous section) leads to weakening of the extracellular matrix allowing proliferation. Several of these genes have been shown in the literature, either directly by E2, or indirectly by ER status, to be regulated by the hormone in other smooth muscle cells, in the myometrium/uterus or in cancer cell lines.

#### *COAGULATION:*

Coagulation is a property of blood and has obvious importance in tissues such as the myometrium, which is supplied with blood and infiltrated with blood vessels. In our *in vitro* cell culture system, the hUtSMCs are the only cells present and the carrier

medium becomes the culture medium. In effect the hUtSMCs can send out signals in response to estradiol treatment but the appropriate responses cannot be expected in the absence of other cell types.

In humans, systematic analysis of coagulation factors during the oestrous cycle identified the menstrual and early follicular phases as having the lowest concentration of coagulation factors in women: this is in line with the concentration of circulating oestrogen in women (Knol et al., 2012). During parturition dramatic changes in the circulating levels of coagulation factors are observed, where clotting factors are increased and anti-coagulants are decreased, thereby preventing maternal blood loss (Bremme, 2003; Chi and Kadir, 2012; Jurus et al., 2010; O'Brien et al., 2008a). Two coagulation-related genes (KNG1 and F2) showed large fold changes (in opposing directions) upon estradiol treatment. Kininogen (KNG1), whose expression was downregulated by over 5-fold following 24 h of E2 treatment, plays an important role in blood coagulation by helping to co-localise prekallikrein and factor XI with factor XII. It also inhibits the thrombin- and plasmin-induced aggregation of thrombocytes (Schmaier and McCrae, 2007; Webb, 2011). Since KNG1 inhibits thrombin-induced aggregation of thrombocytes, it is probably not surprising that the regulation of KNG1 and F2 are in opposing directions. Naturally occurring mutations of this gene in humans have been shown to result in coagulation defects (Houlihan et al., 2010) and knockout KNG1 mice demonstrated prolonged activated partial thromboplastin time (aPTT) and delayed arterial thrombosis (Merkulov et al., 2008).

The F2 gene that codes for coagulation factor prothrombin, was upregulated at all timepoints post-E2 treatment (by over 2-fold at 6 and 24 h, and by over 4.5-fold at 72 h). Thrombin is reported to play an important role in coagulation by inducing the aggregation of thrombocytes, converting fibrinogen to fibrin, activating coagulation factors (V, VIII and XIII) and stimulating platelet aggregation (Lancellotti and De Cristofaro, 2009). Other studies have also reported that during pregnancy, F2 mRNA levels increase and remain unchanged until term (Clark et al., 1998; Nilsson and Kullander, 1967; Stirling et al., 1984). Although well-recognised as a coagulation factor, thrombin has also been shown to stimulate contractility in both human and rat myometrium (Elovitz et al., 2000; O'Sullivan et al., 2004; Shintani et al., 2000). A thrombin-like receptor (F2RL1) coupled to G proteins, also known as PAR2

(protease-activated receptor 2), was 1.6-fold downregulated in the present study following 24 h of E2 treatment. F2RL1 senses protease levels many of which are involved in the coagulation cascade. O'Brien and co-workers observed no significant changes in F2RL1 myometrial expression (localized to the smooth muscle cells) at term pregnancy or labour where oestrogen might be expected to be at its most influential (O'Brien et al., 2008a).

*IMMUNE AND INFLAMMATORY RESPONSES:*

During pregnancy, suppression of immune and inflammatory responses is necessary for toleration of the developing foetus in the uterus (Siiteri and Stites, 1982) but towards the end of pregnancy and during the onset of labour this effect reverses, activating immune and inflammatory responses (Helguera et al., 2009). Infiltration of leucocytes into the myometrium is considered a hallmark event in preparation for labour initiation (Osman et al., 2003; Thomson et al., 1999). GO biological processes analysis identified 35 genes ( $P=0.1$ ) in the current list of differentially expressed genes with functions related to immune and inflammatory response, of which the expression of 27 genes (77 %) was downregulated. The four genes detailed below show the general trend of downregulation upon estradiol treatment.

The expression of interferon regulatory factor 5 (IRF5) was downregulated at 24 h by over 2-fold following E2 treatment in the present study. It is a transcription factor involved in the induction of inflammatory cytokines - interferon  $\alpha$  and  $\beta$  (Barnes et al., 2001; Schoenemeyer et al., 2005). This gene is involved in TLR signalling (as is F2RL1 mentioned above in relation to coagulation) and is an activator of signal transducer of transcription 4 (STAT4) that interacts with the type I IFN receptor (Ronnlom, 2011). Hence, downregulation of IRF5 may function by suppressing immune and inflammatory response.

ELF3, whose expression is downregulated by 2.5-fold in the present study following 24 h of E2 treatment, is a transcriptional activator involved in many functions, which include mediating vascular inflammation (Oliver et al., 2012). This gene has been shown to be upregulated by day 19 in the gravid mouse uterus suggesting a role for steroids hormones in its regulation and a role in myometrial function (Bethin et al., 2003). TNFSF11 mRNA was downregulated by 1.6-fold (24 h) in the present study.

TNFSF11 is a cytokine in the same superfamily as TNF $\alpha$  and is an important regulator of interaction between dendritic cells and T cells (Anderson et al., 1997). This gene was upregulated in pigs treated with the progesterone inhibitor RU486 during early pregnancy: this treatment increases the plasma levels of oestrogen (Mathew et al., 2011). The expression of interleukin 11 (IL11) was downregulated by 1.5-fold at 24 h on E2 treatment. IL11 has a protective effect against inflammation of muscle associated with the gut. Recombinant human IL11 has been shown to restore smooth muscle function in both rabbits and rats with intestinal inflammation (Depoortere et al., 2000; Greenwood-Van Meerveld et al., 2001). E2 downregulated the IL11 transcript in an osteoblast-like cell line (Wang et al., 2006). Therefore IL11 has known effects on smooth muscle cells and evidence suggests it might be regulated by oestrogen.

One of very few upregulated genes was the Interleukin 6 signal transducer (IL6ST), which was upregulated by 1.5-fold at 6 h upon E2 treatment. It functions as part of a receptor complex responsive to a range of cytokines and also a key player in ER $\alpha$  signalling pathway (Kishimoto, 1989; Wilson and Giguere, 2008). Production of IL6ST increased on E2-treatment and combined treatment with E2 and P4 further increased the production in human granulosa tumour cell lines (Deura et al., 2005).

#### *REPRODUCTION:*

The tocopherol (alpha) transfer protein (TTPA), downregulated (1.7-fold at 24 h) in hUtSMCs following E2 treatment, transfers  $\alpha$ -tocopherol to adipose tissue and liver.  $\alpha$ -Tocopherol is a form of vitamin E, which is an essential nutrient for reproduction (Debier, 2007).  $\alpha$ -Tocopherol negatively regulates protein kinase C (PKC) activity in smooth muscle cells (Singh et al., 2005). Also, vitamin E inhibits cell adhesion, inflammation, coagulation and proliferation of smooth muscle cells (Azzi et al., 1998; Boscoboinik et al., 1991a; Boscoboinik et al., 1991b; Freedman et al., 1996; Rimbach et al., 2002). Thus, a reduction in expression of this gene would activate PKC and also induce functions such as cell adhesion, inflammation, coagulation and proliferation of smooth muscle cells.

Also downregulated (at 24 h and 72 h) in hUtSMCs treated with E2 was PRLR, the receptor for prolactin. Classically, prolactin is released as a hormone from the

pituitary, however, prolactin is expressed and acts as a cytokine in many other tissues (Dogusan et al., 2001). Apart from milk production, prolactin is involved in many key functions in humans including reproduction, metabolism and immune response (Ben-Jonathan et al., 2008; Freeman et al., 2000; Grattan and Kokay, 2008). E2 reduces the level of PRLR mRNA in the uterus of ovary intact pigs compared to saline treated animals (Trott et al., 2009).

*TRANSPORT:*

Some gene ontology categories are large and rather overarching. Although they may be highly significant and point to changes in a broad range of effects (e.g. metabolism, cellular process, development) it is the “children” of these terms that point more directly to specific effects. Transport, defined as “the directed movement of substances (such as macromolecules, small molecules, ions) into, out of, or within a cell, or between cells, or within a multicellular organism by means of some agent such as a transporter or pore” is one such broad range term. The child term “ion transport” points to key processes involved, but the broader term is much more significant and indicates the involvement of other transport proteins.

Gene Ontology analysis of differentially expressed genes in hUtSMCs upon E2 treatment identified 69 genes potentially involved in transport. Of the 69 genes annotated, transmembrane transport accounted for 31 genes ( $P=0.02$ ), ion transport for 23 ( $P=0.2$ ) and 10 genes were involved in protein transport  $P=0.2$ ). Apolipoprotein A-I (APOA1) (involved in transmembrane transport and ion transport) was increased in hUtSMCs treated with E2 for 6 and 24 h. APOA1 is a major component of plasma high density lipoprotein (HDL) and is responsible for the promotion of cholesterol efflux from tissues to the liver (Remaley et al., 2001; Ritter and Scanus, 1977). Potentially E2, by increasing APOA1 levels, could be providing more cholesterol for steroid biosynthesis. Post-menopausal women, who had undergone oestrogen therapy, exhibit increased levels of APOA1 as well as HDL suggesting a link of APOA1 with E2 (Christodoulakos et al., 2004; Creatsas et al., 2003).

The cell surface protein, Synaptophysin (SYP) (involved in ion transport), was also upregulated following 24 h of E2 treatment. This protein of pre-synaptic vesicles is

normally associated with neural tissue and has a role in the intracellular transport of neuroendocrine hormones but its presence has also been confirmed in the gastrointestinal tract (Portela-Gomes et al., 1999) and the pancreas (Brudzynski and Martinez, 1993). The SYP transcript was induced in a neuroblastoma cell line upon E2 treatment (Chamniansawat and Chongthammakun, 2010) and the reduced level of SYP protein in ovariectomised mice (compared to controls) was upregulated by the administration of E2 (Sharma et al., 2007).

Sodium channel, voltage-gated, type V, alpha subunit (SCN5A) mRNA expression was upregulated in hUtSMCs treated with E2 for 24 h. In excitable membranes this protein mediates voltage-dependent sodium ion permeability. In response to the voltage difference across the membrane, SCN5A forms a sodium-selective channel assuming opened or closed conformations through which sodium ions pass (Chagot et al., 2009). Another gene upregulated following 24 h, which acts as an ion transporter, is Calcium channel, voltage-dependent, T type, alpha 1G subunit (CACNA1G). This protein is involved in many calcium-dependent processes such as muscle contraction, cell motility, cell division and cell death (Mittman et al., 1999; Monteil et al., 2000). T type channels frequently carry out pacemaker functions such as those required in smooth muscle cells. Oestrogen has been shown to upregulate a variety of T-type calcium channels in the hypothalamus and pituitary (Qiu et al., 2006), and both oestrogen and tamoxifen have effects on T-type channels in vascular smooth muscle cells (Song et al., 1996; Zhang et al., 1994).

#### *DEVELOPMENT:*

Gene Ontology defines development as the process of progress of the system for the formation of a mature functional structure (Hill et al., 2008). GO analysis identified 93 of the differentially expressed genes to be involved in development ( $P=0.05$ ). Among these, three genes were annotated for reproductive structural development (RXFP2, LHX9, NR5A1), 8 in reproductive developmental process (AMHR2, SPANXB2, RNF17, RXFP2, PBX1, LHX9, TGFB2, NR5A1), 7 in endocrine system development (ALDH1A2, APOA1, ALDH1A3, CRH, PBX1, NKX2-5, NR5A1), 12 in neuron development (GPRIN1, FSCN2, RTN4RL1, NEDD4, MAP2, CNTN2, LGI4, EN2, SLIT1, GRK1, CDH23, TGFB2), and 7 in vasculature development (CALCA, LAMA4, COL3A1, ROBO4, NKX2-5, CYR61, TGFB2). Although

‘Development’ is an overarching term, the child terms like ‘reproductive’ and ‘endocrine’ point to the aspect of development that is relevant.

*TRANSCRIPTION:*

Upon stimulation with oestrogen, ERs dissociate from nuclear chaperones, dimerise, and bind to DNA sequences known as oestrogen response elements (EREs) (Heldring et al., 2007). Transcriptional regulation of ERs through the ERE, occurs in combination with cofactors. The cofactors function with ERs modifying histones, altering chromatin structure, and recruiting the RNA polymerase II transcriptional machinery (Pan et al., 2008). Oestrogen’s regulation of transcription is tissue specific. Studies on transcriptional regulation in different mouse tissues showed that the highest number of differentially regulated genes were identified in the uterus (Leitman et al., 2012).

GO analysis identified 59 genes to be involved in transcription, of which 29 were transcription factors ( $P=0.1$ ). Oestrogen-related receptor  $\gamma$  (ERR $\gamma$ /ESRRG) is a transcriptional activator (Hentschke et al., 2009) and modulating factor for oestrogen signalling pathways (Ijichi et al., 2011). It binds to the same response element as the estrogen receptor. The expression of this gene was downregulated following E2 treatment in hUtSMCs. ESRRG modulates cell proliferation and oestrogen signalling in breast cancer (Ijichi et al., 2011). Elevated levels of ESRRG mRNA are reported in ER $\alpha$ -positive endometrial adenocarcinomas suggesting that oestrogen regulates ESRRG transcript levels (Gao et al., 2006). Furthermore, ESRRG binds calmodulin, suggesting a direct effect on muscle contraction (Hentschke et al., 2009).

PBX1 mRNA was downregulated following 24 and 72 h of E2 treatment. PBX1 is essential for the ER $\alpha$ -mediated transcriptional response in aggressive breast cancer tumours (Magnani et al., 2011). It appears that PBX1 interacts with histones thereby opening up chromatin structure at specific sites (a so-called pioneer factor) to allow subsequent binding of ER $\alpha$ . PBX1 expression correlated with ER $\alpha$  in primary breast cancers where PBX1-depleted breast cancer cells failed to proliferate following oestrogen stimulation (Magnani et al., 2011). The pioneer factor role may also be present in muscle cells where PBX1 allows MyoD to promote expression of

myogenin (Berkes et al., 2004). Therefore PBX1 is intimately related to ER-responses and may have a role in muscle differentiation.

Expression of GTF3C1 (24 h) and GTF2I (72 h) was downregulated in hUtSMC following E2 treatment. GTF2I coordinates the formation of a multiprotein complex at the *c-Fos* promoter thereby interacting with the basal transcription machinery (Novina et al., 1999). GTF2I is also required for the activation of immunoglobulin heavy-chain transcription during activation of B-lymphocyte and hence the activation of immune and inflammatory responses (Egloff and Desiderio, 2001; Novina et al., 1999; Yang and Desiderio, 1997). Hence, reduction of GTF2I transcription may also reduce immune and inflammatory responses in hUtSMCs in response to E2. Two more transcription factors, POU6F2 (24 h) and POU3F1 (72 h) were downregulated in cultured hUtSMCs following E2 treatment. The POU3F1 promoter has been shown to contain an oestrogen response-like enhancer sequence. Although the levels of POU3F1 increased in glial cells with oestrogen treatment (Renner et al., 1996), in a different tissue with a different set of co-activators/repressors possibly the response to oestrogen might be different.

The first of two upregulated genes in this section is NKX2-5, whose transcript was upregulated in hUtSMCs treated with E2 for 24 and 72 h. This gene encodes a homeobox-containing transcription factor, which plays a role in formation and development of the heart (Pashmforoush et al., 2004; Zhao et al., 2012). It has also been shown that NKX2-5 is upregulated in pregnancy (Genead et al., 2012).

Twist homolog 1 (TWIST1), a transcriptional regulator, was upregulated in hUtSMCs treated with E2 for 24 h. TWIST1 inhibits myogenesis and reverses myotube formation by sequestering E proteins and inhibiting DNA-binding by the muscle transcription factor MYOD1 (Hjiantoniou et al., 2008). TWIST1 was observed to be upregulated in human myometrium at labour (O'Brien et al., 2008b). In breast cancer cells, TWIST1 represses ER $\alpha$  expression and clearly shows a reciprocal expression pattern with ER $\alpha$  in either forced overexpression or knockdown systems of TWIST1 expression (Fu et al., 2012; Vesuna et al., 2012). Therefore upregulation of TWIST1 as seen in hUtSMCs should downregulate oestrogen responses through ER $\alpha$  or vice versa. TWIST1 also represses expression of proinflammatory cytokines such as

TNF $\alpha$  and IL1 $\beta$  (Li et al., 2012) and could therefore have a role in the oestrogen-induced downregulation of immune responses seen in the study.

Oestrogen is known to alter the expression of its target genes by altering the action of transcription factors. In the present study, following E2 treatment, the expression of genes that aid in induced cell proliferation, metabolism and microtubule formation were achieved by modulating the expression of transcription factors that regulate these transcripts. All these functions are of importance in pregnancy and parturition.

#### **4.4 Conclusions**

The principal output of this study was the identification of a panel of 509 differential expressed genes in response to E2 treatment. Approximately 60 % of these genes were downregulated. Thorough analysis of the transcriptome and the use of high-dimensional systems biology techniques implicated different biological processes which included muscle contraction, cell proliferation, differentiation, coagulation and immune and inflammatory responses. E2 treatment also altered the expression of several structural molecules. Change in the expression of genes involved in muscle contraction generally favoured smooth muscle relaxation. In the present study, genes with cell proliferative functions were mostly upregulated following E2 treatment. This is in line with reports of enhanced proliferation of cancer or normal cells after treatment with oestrogen. Elevated oestrogen in the follicular phase of the oestrous cycle is responsible for the proliferation of functionalis (the layer of cells that sloughs off of the uterus during a menstrual cycle), endometrial glands and the stromal connective tissues. However, in the gravid uterus, smooth muscle cells proliferate rapidly during early periods of gestation but growth moderates towards parturition. It was also found in the current study that the expression of genes involved in immune and inflammatory responses and apoptosis were mostly downregulated following E2 treatment. This is in line with reports of reduced function upon oestrogen treatment in other cell culture systems, but is contrary to *in vivo* studies of labouring myometrium.

It is clear that this microarray based global gene expression analysis approach has identified oestrogen effects on transcription in hUtSMCs that generally agree with results from *in vivo* studies. Here, the results from the present study is compared with published oestrogen responses from myometrium in the human oestrous cycle, with the uterus of different species during pregnancy and parturition, and with human cell lines (often breast cancer cell lines because oestrogen effects have been exhaustively studied in these). In many cases regulation of a gene is in the opposing direction to the cited example but it could be that oestrogen-responsiveness is key and that up or downregulation may be a function of many other factors including the array of co-activating factors present in the tissue and the duration of oestrogen exposure. The hUtSMC cell system is not a uterus: there is no foetus present to stretch these cells or to initiate an immune response, there are no blood vessels to carry signals from one

tissue to another. Nor is oestrogen the only player in pregnancy. Having set up the system and confirmed its usefulness in this chapter, this cell culture model was used in a more complex situation, which would reflect some of the transcriptomic events in myometrial smooth muscle cells associated with pregnancy and labour.

**Chapter 5: Developing a Cell Culture  
Model System to Understand the  
Transcriptomic Effect of Oestrogen and  
Progesterone Treatment on Cultured  
Uterine Smooth Muscle Cells to Study  
Myometrial Functions During Human  
Pregnancy**

## **5.1 Overview**

Progesterone maintains the quiescent state of the uterus throughout pregnancy and the withdrawal of circulating progesterone in late pregnancy is one of the important steps in initiating labour. In the previous chapter the transcriptomic effect of  $17\beta$ -estradiol on hUtSMCs were investigated. In this chapter, the effects of both progesterone and progesterone withdrawal (in the presence and absence of  $17\beta$ -estradiol) on gene expression in cultured human myometrial smooth muscle cells were examined. Functional progesterone withdrawal was mimicked in the cell culture system by co-treating the cells with the progesterone inhibitor RU486 (Mifepristone). By culturing hUtMCSs in the presence of progesterone and  $17\beta$ -estradiol and by functionally withdrawing progesterone from the system, may reflect the physiological hormonal conditions of the myometrium during pregnancy and labour. Identification of differentially expressed genes in this system would provide insights into various molecular mechanisms regulated by these genes, and shed light on their role in initiating and regulating myometrial contractility.

## **5.2 Results**

hUtSMCs from non-pregnant myometrium were used in all the experiments since these cells were not pre-exposed to high oestrogen or progesterone and are suitable for understanding the transcriptomic effect of these steroid hormone treatment. All experiments were performed with cells within passage 5 and 8 to maintain the phenotypic characteristics of the myometrial smooth muscle cells. hUtSMCs were cultured in steroid-free medium and were treated with 100 nM progesterone, 100 nM progesterone (P4) plus 10 nM 17 $\beta$ -estradiol (E2), 100 nM progesterone (P4) plus 1  $\mu$ M RU486 (mifepristone) or 100 nM P4 plus 10 nM E2 plus 1  $\mu$ M RU486. One flask of cells was left as an untreated control. Cells were harvested 72 h following treatment to understand the effect of prolonged exposure of these hormones. RNA from cells was isolated and was used in a microarray-based gene expression study to identify differentially expressed genes. Each experiment was performed in triplicate to obtain statistically significant results. Data enrichment analyses were performed on all differentially expressed genes identified to understand the biological function and significance of each gene. The expression profiles of selected genes identified from the microarray experiments were subsequently validated by qPCR or semi-quantitative RT-PCR.

### ***5.2.1 Developing a cell culture model system mimicking labouring and non-labouring human myometrium***

The aim of this study was to develop a cell culture model system that mimics *in vivo* steroid hormone conditions in pregnant and labouring myometrium and to understand the effect of these hormones on the regulation of gene expression. To achieve this, cultured human uterine smooth muscle cells (hUtSMCs) were treated with variable combinations of P4, E2 and RU486. The treatment conditions and the abbreviations designated for each treatment are summarised in Table 5.1. Cells were harvested (in triplicates; Section 2.2.7) after 72 h of treatment and total RNA was extracted from all samples. Whole genome (human) DNA microarray analysis was performed to identify differentially expressed genes. For details in methodology refer to Chapter 2, Section 2.2.7.

Table 5.1: Treatment conditions and abbreviations explained.

Treatment Condition	Condition Mimicking	Abbreviation
Untreated sample	Control	C
100 nM P4-treated sample	P4 treatment	P4
100 nM P4 + 10 nM E2-treated sample	Pregnant myometrium	P4+E2
100 nM P4 + 1 $\mu$ M RU486-treated	Functional P4 withdrawal	P4+RU486
100 nM P4 + 10 nm E2 + 1 $\mu$ M RU486-treated sample	Labouring myometrium	P4+E2+RU486

The expression of 25,100 unique genes and 39,600 transcripts, excluding control oligos, represented by 35,357 oligonucleotide probes were analysed in hUtSMCs for differential expression following 72 h of steroid hormone treatment (either separately or in combination). Differential expression of genes was analysed between the following treatment conditions: (1) control versus P4-treated (to identify progesterone responsive genes); (2) P4-treated versus P4+RU486-treated (to identify progesterone withdrawal responsive genes); (3) control versus P4+E2-treated (non-pregnant versus pregnant myometrium); and (4) P4+E2 versus P4+E2+RU486-treated (non-labouring versus labouring myometrium).

Differentially expressed genes (>1.5-fold) with a *P*-Value cut-off of 0.05 (few genes whose *P*-Value was >0.05 were included because of their high fold change in expression) were identified (Table 5.2). Annotation of the oligonucleotide probes on the Operon microarray chip used in the present study (built date: April 4, 2005) was updated. Therefore, each gene from the list of differentially expressed genes identified was manually checked and corresponding RefSeq entities were either modified or deleted.

Table 5.2: Table summarising the microarray results.

Sample	Differentially expressed genes	Upregulated	Downregulated
P4-treated against Control	32	16	16
P4+RU486-treated against P4 treated	49	32	17
P4+E2-treated against Control	62	36	26
P4+E2+RU486-treated against P4+E2 treated	123	47	76

For ease of understanding the results section is subdivided into: (1) progesterone (P4) and functional progesterone withdrawal (P4+RU486 vs. P4); (2) cultured hUtSMC model for pregnant myometrium (P4+E2 vs. C); and (3) labouring myometrium model (P4+E2+RU486 vs. P4+E2).

### ***5.2.2 The effect of progesterone treatment and functional progesterone withdrawal on gene expression in cultured hUtSMCs***

In order to understand the effect of progesterone treatment and its functional withdrawal, cultured hUtSMCs (starved of endogenous steroids) were treated with 100 nM progesterone and harvested 72 h post-treatment. To mimic functional progesterone withdrawal, samples were co-treated with 1 $\mu$ M RU486 (mifepristone) 24 h prior to harvest. Total RNA was isolated for whole genome human DNA microarray analysis to identify differentially expressed genes.

#### **5.2.2.1 The effect of progesterone treatment on gene expression in cultured hUtSMCs**

A total of 55 genes were differentially expressed in progesterone-treated sample compared to untreated controls of which 22 were removed after updating the Operon array genome (2005 build) with current annotation from NCBI as a part of their standard update. Of the remaining 32 genes 16 genes were upregulated and 16 genes were downregulated. In the thesis only a short list of genes whose expression was altered by over 2.5-fold is shown (Table 5.3). The full list of differentially expressed genes identified following P4 treatment is appended (Appendix Table A3.1).

In the preliminary analysis of the cell culture system, B-cell lymphoma 2 (BCL2), an anti-apoptotic protein, was used as a marker to identify the effect of P4 treatment. Following P4 treatment the expression of this gene was induced by over 5-fold in cultured hUtSMCs (Figure 3.9A). The microarray study also identified the expression of BCL2 induced over 2-fold following P4 treatment (Appendix Table A3.1).

In the previous study, where hUtSMCs were treated with E2, the F2 (Prothrombin) mRNA expression was upregulated upon 72 h of E2 treatment (Table 4.5). In the present study, the expression of F2 mRNA expression was downregulated by 2-fold post progesterone treatment (Appendix Table A3.1). Matrilin 3 (MATN3) was the most upregulated gene (>31-fold)

identified post-P4 treatment whereas Zinc finger protein 600 (ZNF600) was the most downregulated gene (>4-fold) identified post-P4 treatment.

**Table 5.3 Summary of genes with altered expression (>2.5-fold) following P4 treatment.**

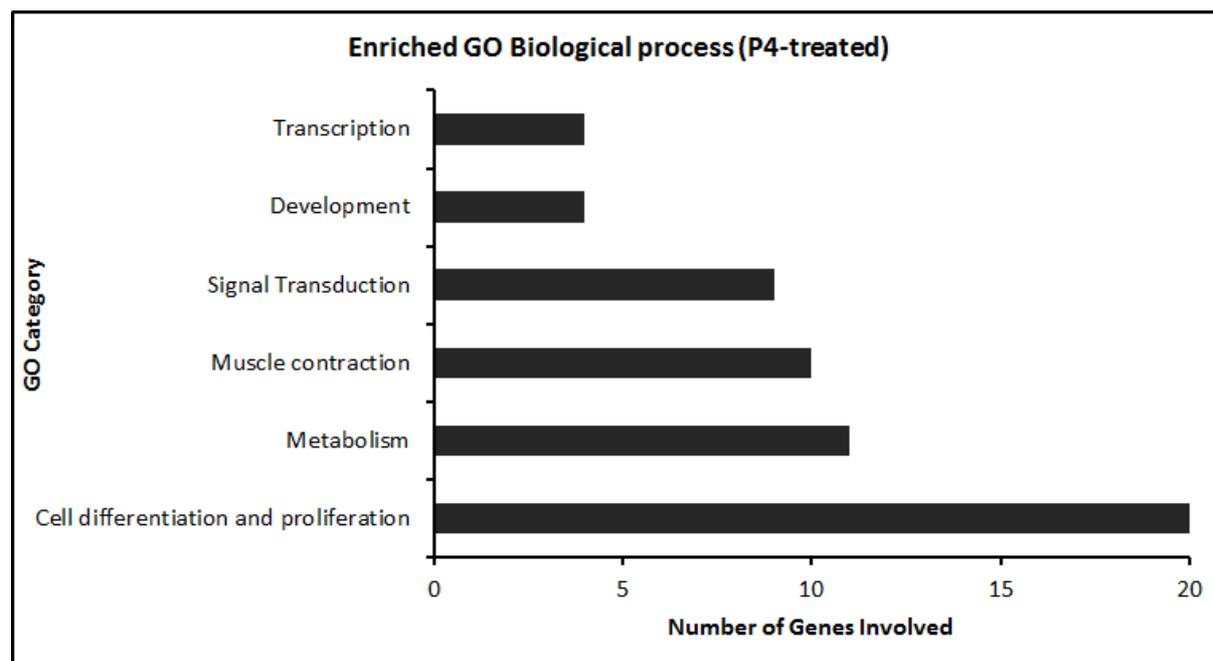
Gene Symbol	Fold Change	P-value	RefSeq ID	Operon ID	Gene Name
MATN3	31.5	1.78E-01	NM_002381	H200017287	Matrilin-3 precursor
LRP2BP	4.4	6.02E-03	NM_018409	H200004595	LRP2 binding protein
IFT122	4.0	6.02E-01	NM_052989	opHsV0400007587	Intraflagellar transport 122 homolog
JPH3	3.0	1.62E-02	NM_020655	opHsV0400000706	Junctophilin type 3
SSTR2	2.6	6.79E-03	NM_001050	opHsV0400001022	Somatostatin receptor type 2
ZNF600	4.2	4.16E-02	NM_198457	opHsV0400008855	Zinc finger protein 600
CCDC88	3.3	3.61E-02	NM_032251	H300012917	Coiled-coil domain containing 88
OR2T10	3.3	1.37E-02	NM_001004693	opHsV0400002014	Olfactory receptor, family 2, subfamily T, member 10
PAF1	2.9	9.48E-01	NM_019088	H300010878	Paf1, RNA polymerase II associated factor, homolog
CMTM1	2.8	3.03E-02	NM_181269	H200019968	CKLF-like MARVEL transmembrane domain-containing protein 1
SST	2.8	3.91E-02	NM_001048	H200002126	Somatostatin precursor
MYLK3	2.7	2.66E-02	NM_182493	H200019888	Myosin light chain kinase 3
ALDH3A2	2.6	2.90E-02	NM_000382	H300000003	Aldehyde dehydrogenase 3 family, member A2

<sup>1</sup>Positive values (in red) indicate upregulation and negative values (in green) represent downregulation.

#### GENE ONTOLOGY ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED FOLLOWING P4 TREATMENT

To gain more knowledge on the biological significance of the differential expression of the identified genes, GO analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Dennis et al., 2003). Each of the differentially expressed genes identified in the present study was also manually checked in GeneCards ([www.genecards.org](http://www.genecards.org)) (Safran et al., 2010) and in published literature (PubMed) to identify biological processes that DAVID analysis might have missed. GO analysis identifies overrepresented gene ontology categories (biological processes, molecular functions and cellular component) in a given set of genes. This information, along with the experimental conditions from which the genes are generated, provides insights into the biological relevance of the genes that are differentially expressed in a cell under a specific condition. Default settings in the software were used for all analyses (explained in Section 2.2.21). Throughout the chapter the same procedure was followed for all GO analyses.

Of the 32 differentially expressed genes identified (no longer fold-change limited) after P4 treatment GO analysis identified biological processes for 25 genes (Appendix Table A.3.2). Identified biological process terms were grouped based on common function and genes annotated under each biological process term were combined to avoid repeats. The most overrepresented biological process categories were cell differentiation and proliferation, metabolism, muscle contraction and signal transduction (Figure 5.1).



**Figure 5.1: Enriched GO biological processes identified following progesterone treatment of hUtSMCs.**

#### 5.2.2.2 Effect of functional progesterone withdrawal using RU486 on gene expression in cultured hUtSMCs

The expression of 75 genes was altered in cultured myometrial smooth muscle cells following functional progesterone withdrawal using the progesterone inhibitor RU486 (mifepristone) (P4+Ru486 vs P4). Of the differentially expressed genes identified, 26 were removed from NCBI as a part of their standard updating procedure. Of the remaining 49 genes, the expression of 32 genes was upregulated and 17 genes were downregulated. Selected genes with fold changes over 3-fold are tabulated (Table 5.4).

Elastin microfibril interfacier 3 (EMILIN3) was the most upregulated (4.3-fold) gene identified after functional progesterone withdrawal. Angiopoietin-like 4 (ANGPTL4) was the

most downregulated gene (4.2-fold). The full list of differentially expressed genes identified following functional P4 withdrawal is appended (Appendix Table A.3.3).

**Table 5.4 Summary of genes with altered expression (>2.5-fold) following functional P4 withdrawal.**

Gene Symbol	Fold Change	P-value	RefSeq ID	Operon ID	Gene Name
EMILIN3	4.3	3.79E-02	NM_052846	H200003572	Elastin microfibril interfacier 3
FLJ32165 fis	4.1	1.82E-02	AK056727	opHsV0400000973	cDNA FLJ32165 fis, clone PLACE6000424
LONRF2	3.9	4.64E-02	NM_198461	opHsV0400011464	LON peptidase N-terminal domain and ring finger 2
ASB7	3.7	3.20E-02	NM_198243	opHsV0400002484	Ankyrin repeat and SOCS box protein 7
ZNF404	3.3	2.28E-02	NM_001033719	opHsV0400001303	Zinc finger protein 404
ZNF71	3.1	2.55E-02	NM_021216	opHsV0400001552	Zinc finger protein 71
ANGPTL4	4.2	4.42E-02	NM_139314	H300021145	Angiopoietin-related protein 4
IFT122	3.1	9.54E-01	NM_052989	opHsV0400007587	Intraflagellar transport 122 homolog

<sup>1</sup>Positive values (in red) indicate upregulation and negative values (in green) represent downregulation.

#### GENE ONTOLOGY ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED FOLLOWING FUNCTIONAL PROGESTERONE WITHDRAWAL

All analyses were performed as explained earlier in Gene Ontology analysis of differentially expressed genes identified following p4 treatment.

Of the 49 differentially expressed genes following treatment GO analysis identified biological processes for 22 genes (Appendix Table A.3.4). The most overrepresented biological process categories were cell differentiation and proliferation, and transcription (Figure 5.2). Both after P4 treatment and functional P4 withdrawal the most overrepresented GO term was cell differentiation and proliferation.

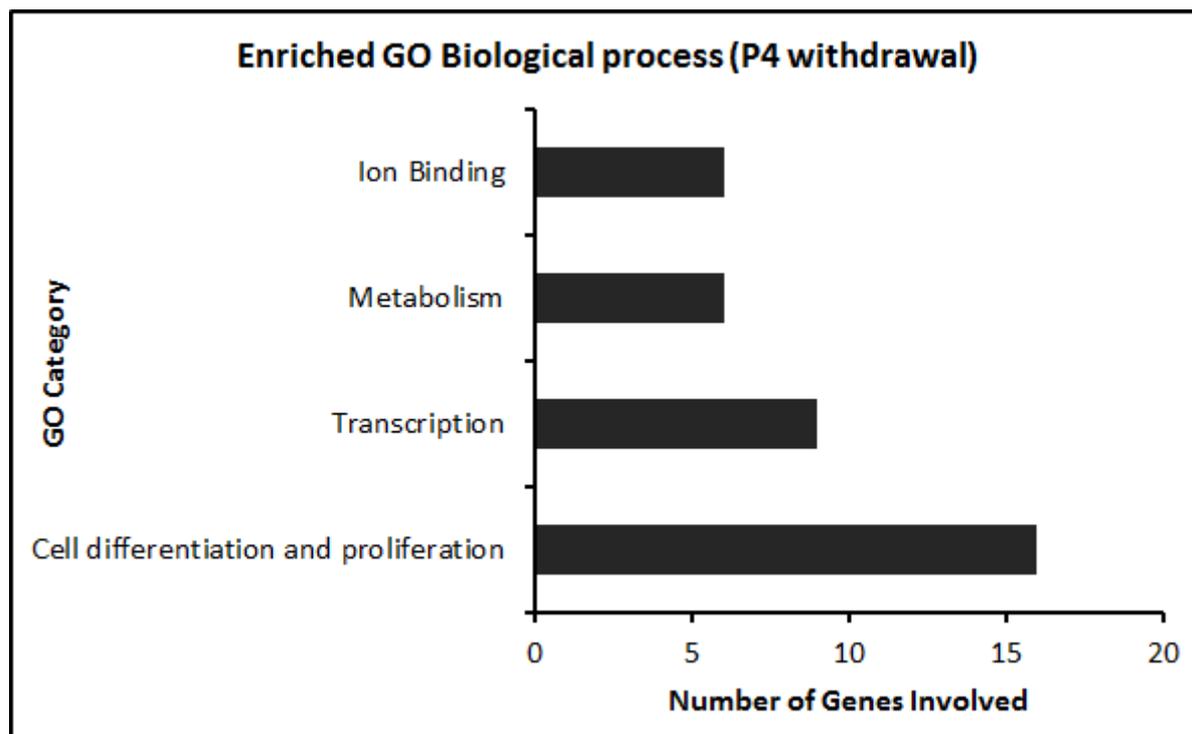
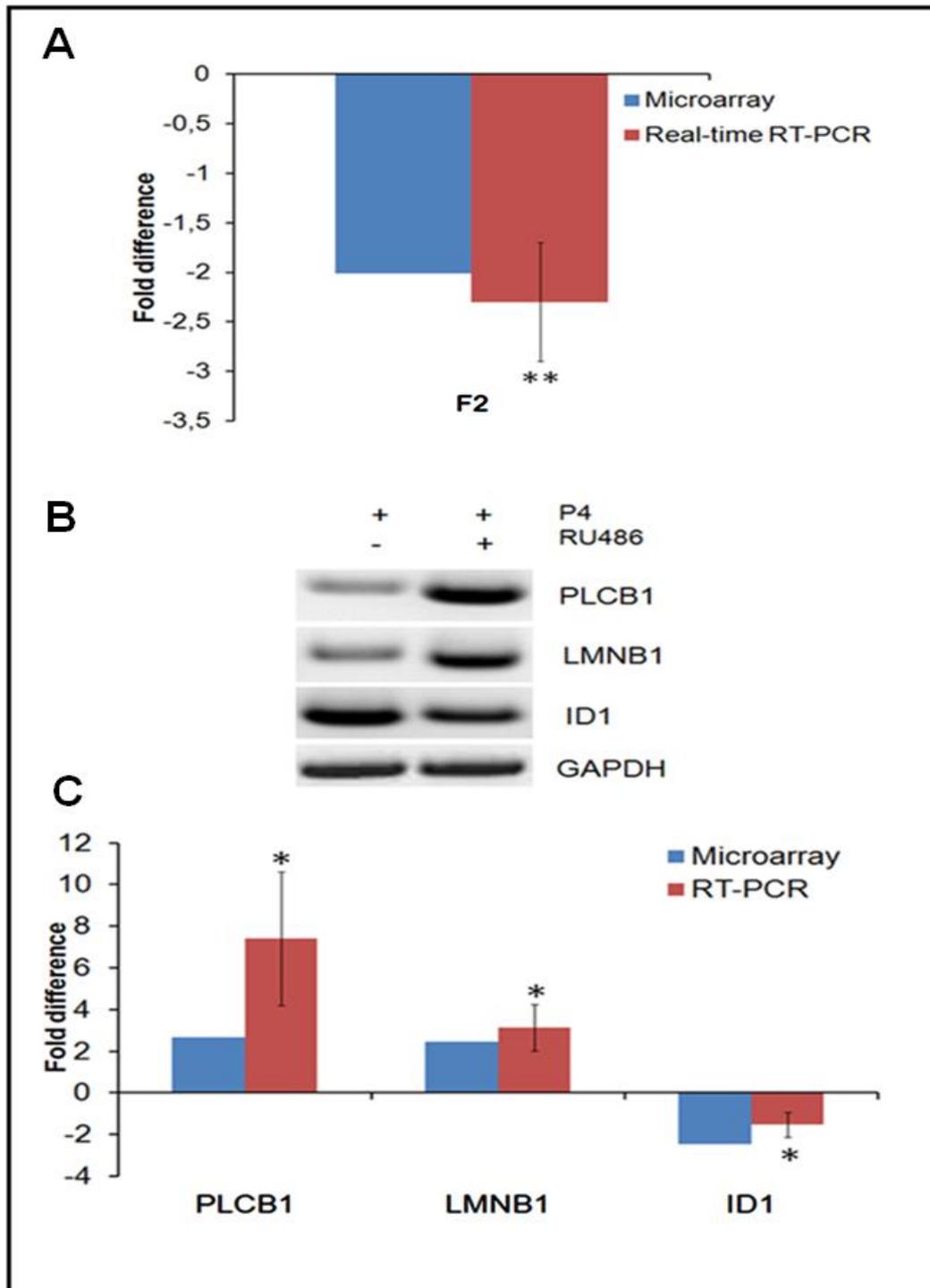


Figure 5.2: Enriched GO biological processes identified following functional progesterone withdrawal.

#### PCR VALIDATION OF MICROARRAY RESULTS

Microarray analysis of cells treated with P4 showed a decrease in expression of the F2 gene (2-fold) compared to untreated samples. qPCR results demonstrated that P4 treatment of hUtSMCs for 72 h downregulated the expression of F2 mRNA by over 2-fold ( $P < 0.05$ ) (Figure 5.3A). This result was similar to the result obtained in the microarray analysis.

Functional P4 withdrawal from hUtSMCs upregulated the expression of Phospholipase C, beta 1 (PLCB1, 2.6-fold) and Lamin B1 (LMNB1, 2.4-fold), and downregulated the expression of DNA-binding protein inhibitor ID-1 (ID1) (2-fold). Semi-quantitative RT-PCR analysis confirmed that the expression of PLCB1 and LMNB1 was upregulated and ID1 was downregulated in cells when P4 was functionally withdrawn using RU486 (Figure 5.3B&C).



**Figure 5.3: Validation of microarray results.** **A.** hUtSMCs were treated with 100 nM P4 and were harvested 72 h post-treatment. Extracted total RNA was subjected to qPCR along with untreated control samples. The fold difference was calculated by normalising the values to the housekeeping gene (GAPDH) and comparing it with corresponding untreated sample (set as 1). Each value corresponds to the average from three independent experiments (n=3) plotted (+/-SD). Statistically significant differences between the P4-treated and untreated controls were determined using the Student *t*-test (\*\*- $P < 0.005$ , \*- $P < 0.05$ ). **B.** hUtSMCs were treated with 100 nM P4 for 48 h and co-treated with 1  $\mu$ M of RU486. Cells were harvested after 24 h of RU486 treatment. Total RNA extracted from P4-withdrawn sample and control (P4-only treatment) was subjected to semi-quantitative PCR. Constitutively expressed GAPDH was used as a control gene for normalisation of mRNA across the samples. PCR amplification cycles were controlled to avoid the plateau effect. Samples were separated by agarose gel (1%) electrophoresis. **C.** Images analysed by densitometry.

5.2.2.3 Comparison between progesterone-responsive genes and functional progesterone withdrawal responsive genes

Comparison of the differentially expressed genes in response to progesterone treatment and functional progesterone withdrawal was performed to identify any common genes and also to note the change in expression profile among those genes as a result of the different treatments. Two genes were identified in this analysis - LRP2 binding protein (LRP2BP) and Intraflagellar transport 122 homolog (IFT122). Both these genes were upregulated following P4 treatment but the functional withdrawal of progesterone from the cell system downregulated their expression (Table 5.5).

**Table 5.5: Comparison of expression profiles of common genes between P4 treatment and functional progesterone withdrawal.**

Gene Symbol	Gene Name	Fold Change <sup>1</sup>	
		P4 Treatment	P4 Withdrawal
LRP2BP	LRP2 binding protein	4.4	-2.5
IFT122	Intraflagellar transport 122 homolog	4	-3.1

<sup>1</sup> Positive values indicate upregulation and negative values represent downregulation.

**5.2.3 Mimicking the steroid hormone conditions in pregnant and non-pregnant myometrium – (P4+E2 vs. Control)**

Unlike other primate and non-primate model organisms, the human myometrium is exposed to high concentration of oestrogen and progesterone through most of pregnancy (Albrecht et al., 2000). Hence, to mimic this steroid hormone environment, cultured hUtSMCs were exposed to 10 nM E2 and 100 nM P4 (concentrations correlating to circulating levels) for 72 h. Combined treatment of P4 and E2 altered the expression of 103 genes compared to control samples. 41 genes were removed after updating the Operon array genome (2005 build) with current annotation from NCBI as a part of their standard update. Of these 62 genes 36 were upregulated and 26 downregulated (Appendix Table A3.5). Only a shortened list of genes

whose expression was altered by >5-fold is presented here (Table 5.6). Coiled-coil domain containing 42B (CCDC42B) was the most upregulated gene (~43-fold) whereas, Growth arrest-specific 8 (GAS8) was the most downregulated gene (~101-fold).

**Table 5.6 Summary of genes with altered expression (>5-fold) following P4+E2 treatment.**

Gene Symbol	Fold Change	P-value	RefSeq ID	Operon ID	Gene Name
CCDC42B	42.9	6.66E-03	NM_001144872	opHsV0400002986	Coiled-coil domain containing 42B
MAGEE2	8.1	6.67E-03	NM_138703	H300004354	Melanoma-associated antigen E2
CCDC87	7.0	2.11E-02	NM_018219	H300009298	Coiled-coil domain containing 87
VPS39	6.9	7.86E-03	NM_015289	opHsV0400004231	Vacuolar protein sorting 39 homolog
FCGBP	5.2	2.65E-02	NM_003890	H300018727	IFc fragment of IgG binding protein
C2orf57	5.1	3.24E-02	NM_152614	H300008206	Chromosome 2 open reading frame 57
GAS8	101.1	8.64E-03	NM_001481	H300015231	Growth-arrest-specific protein 8
C20orf132	16.8	8.39E-03	NM_152503	H300018088	Chromosome 20 open reading frame 132
TTPA	16.3	3.61E-02	NM_000370	H200005658	Alpha-tocopherol transfer protein
MACC1	14.4	6.19E-03	NM_182762	opHsV0400002508	Metastasis associated in colon cancer 1
LEPROT	11.5	4.41E-03	NM_017526	opHsV0400003795	Leptin receptor precursor
FAM71A	10.3	1.06E-02	NM_153606	H200012813	Family with sequence similarity 71, member A
MAN1C1	10	3.51E-03	NM_020379	H200001706	Mannosidase, alpha, class 1C, member 1
SERPINA9	5.6	1.05E-02	NM_175739	opHsV0400003059	Serpin A9 precursor
C22orf33	5.5	1.75E-02	NM_178552	opHsV0400001926	Chromosome 22 open reading frame 33
SRL	5.3	3.24E-03	NM_001098814	opHsV0400001924	Sarcalumenin

<sup>1</sup>Positive values (in red) indicate upregulation and negative values (in green) represent downregulation.

#### GENE ONTOLOGY ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED FOLLOWING P4+E2 CO-TREATMENT

All analysis was performed as explained earlier in Gene Ontology analysis of differentially expressed genes identified following p4 treatment.

Of the 62 genes analysed for biological processes, GO analysis retrieved overrepresented GO terms for 28 genes (Appendix Table A.3.6). The most overrepresented biological process categories were cell differentiation and proliferation, muscle contraction, transcription and development. Identified biological process terms were grouped based on common function and genes annotated under each biological process term were combined to avoid repeats. The graph (Figure 5.4) represents the main biological processes identified with the total number of genes identified under each category.

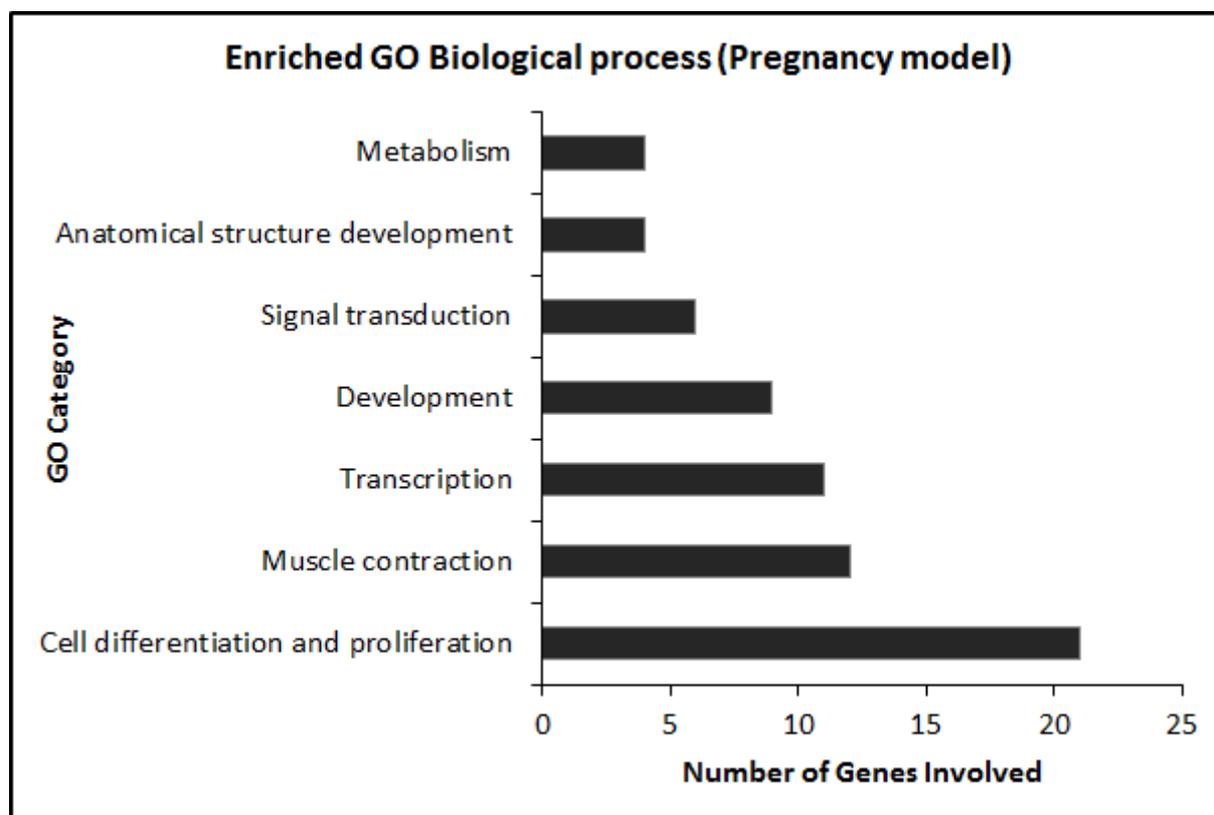
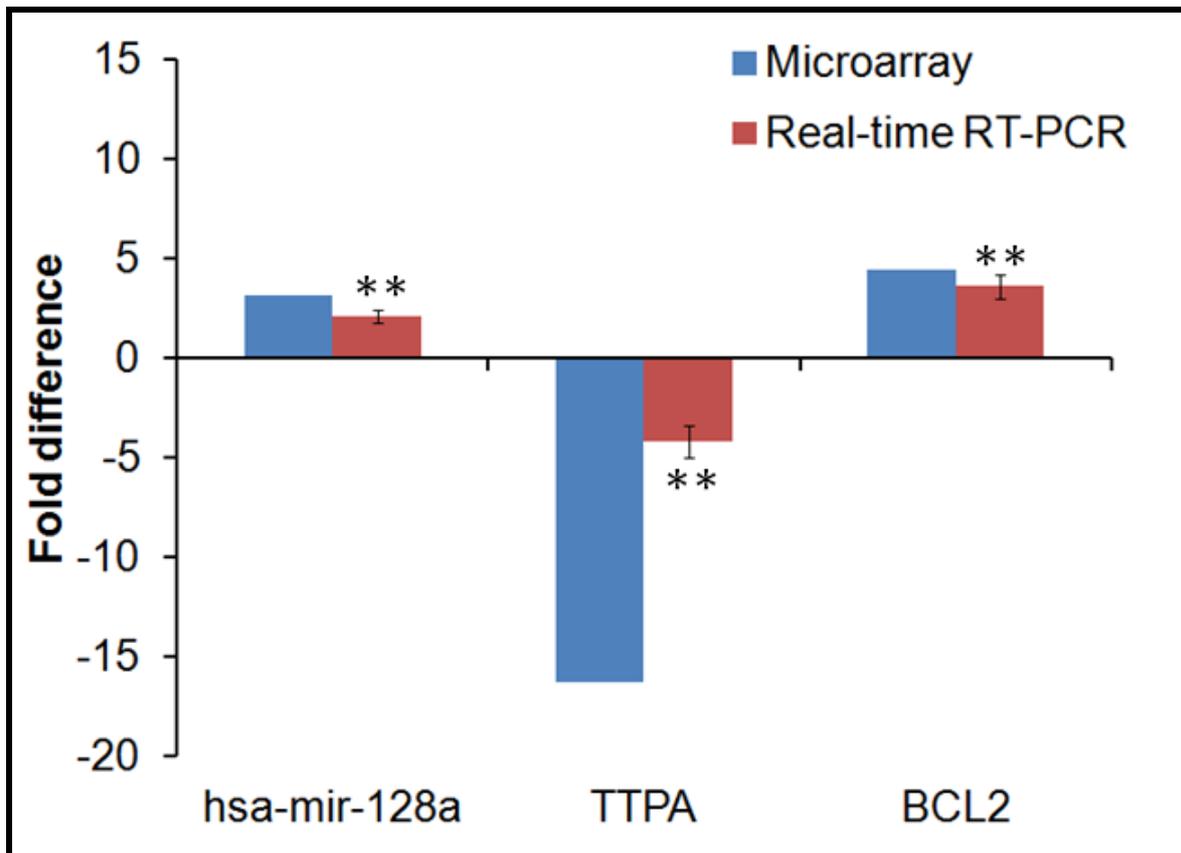


Figure 5.4: Enriched GO biological processes identified following P4+E2 combined treatment of hUtSMCs.

#### REAL-TIME RT-PCR VALIDATION OF MICROARRAY RESULTS

To validate the results obtained from the microarray study, qPCR analysis of TTPA and miR128A was performed. The downregulation of TTPA (>3-fold in microarray and >5-fold in qPCR,  $P<0.005$ ) and upregulation of miR128A (>3-fold in microarray and >3-fold in qPCR,  $P<0.005$ ) was confirmed by qPCR (Figure 5.5).

In section 3.1.3.5, BCL2 was used as a marker to confirm the P4 effect on hUtSMCs. The qPCR analysis showed ~5-fold upregulation of BCL2 confirming the microarray findings (Figure 5.5). Expression of BCL2 was also induced following P4 treatment (>2-fold, appendix list for P4). However, co-treatment of hUtSMC with P4 and E2 increased the expression of this gene by ~5-fold compared to untreated controls.



**Figure 5.5: qPCR validation of differentially expressed genes following P4+E2 treatment.** hUtSMCs were treated with 100 nM P4+10 nM E2 and harvested after 72 h. Total RNA extracted from the sample and control (untreated) was subjected to qPCR. The fold differences were calculated by normalising the values to the housekeeping gene (GAPDH) and comparing with the corresponding untreated sample (set as 1). Each value corresponds to the average from three independent experiments (n=3) plotted (+/-SD). Statistically significant differences between the treated and controls samples were determined using the Student *t*-test (\*\*- $P < 0.005$ ).

## PATHWAY ANALYSIS

Differentially expressed genes identified following P4+E2 treatment (P4+E2 vs. Control) were analysed to identify the role of these genes in important pathways using MetaCore (Ekins et al., 2006). MetaCore is an integrated knowledge database and software suite for pathway analysis of experimental data and gene lists. All human protein-protein, protein-

DNA and protein-compound interactions, metabolic and signalling pathways were analysed using MetaCore. The most statistically significant pathways are identified by the ratio of genes differentially regulated that are involved in the pathway to the total number of genes involved in the pathway. The most statistically significant pathway identified for P4+E2 treatment was the “Anti-apoptotic action of membrane bound ESR1 pathway” (Figure 5.6). Genes involved in “Anti-apoptotic action of membrane bound ESR1 pathway” were CACNA1C (Voltage-dependent L-type calcium channel subunit alpha-1C) and BCL2 (B-cell CLL/lymphoma 2). Expression of these genes was induced in hUtSMCs following P4+E2 treatment. Other pathways identified relating to immune responses were the “IL5 pathway” and the “JAK-STAT cascade pathway” (Appendix Figure A3.1). A legend for the figure (descriptions of each symbol used) is shown (Figure 5.7).

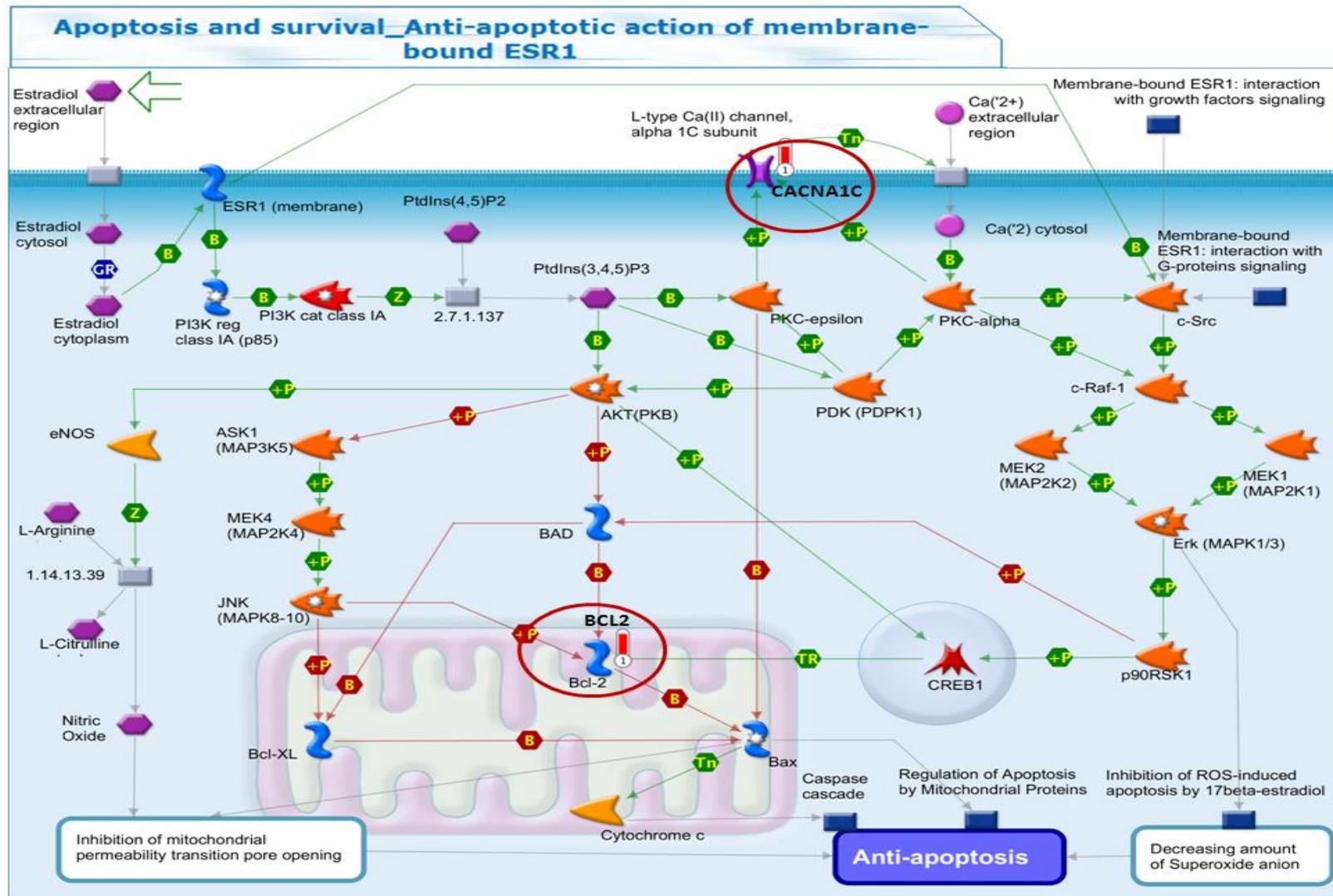


Figure 5.6: Apoptosis and survival pathway/Anti-apoptotic action of membrane bound ESR1 identified by MetaCore pathways analysis. Genes identified in the present study are circled and red line used for circling indicate upregulation of the gene in the present study.



Figure 5.7: Figure legend for the pathway provided by MetaCore.

*Chapter 5: Cell culture model for labouring and non-labouring myometrium*

**5.2.4 Mimicking steroid hormone conditions in the labouring myometrium – (P4+E2+RU486 vs. P4+E2)**

The expression of 207 genes was altered in cultured hUtSMC treated with P4+E2+RU486 compared to cells treated with P4+E2 only. Of these, 84 genes were removed as part of the NCBI standard annotation update. The expression of 123 genes (47 upregulated and 76 downregulated) was altered (>1.5-fold) in hUtSMCs treated with P4+E2+RU486 (Appendix Table A3.7). A short list of genes whose expression was altered by over 5-fold is given in Table 5.7. Elastin microfibril interfacier 2 (EMILIN2) was the most upregulated (9.6-fold) gene identified and Tocopherol (alpha) transfer protein (TTPA) the most downregulated (>28-fold) gene.

**Table 5.7 Summary of genes with altered expression (>5-fold) following P4+E2+RU486 treatment.**

Gene Symbol	Fold Change	P-value	RefSeq ID	Operon ID	Gene Name
EMILIN2	9.6	2.54E-02	NM_032048	H300017224	Elastin microfibril interface-located protein 2
NDUFA10	8.5	3.65E-02	NM_004544	H300016572	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10, mitochondrial precursor
FLJ21991 fis	7.5	6.39E-02	AK025644	opHsV04000 13396	FLJ21991 fis, clone HEP06475, highly similar to HSPEX3P Homo sapiens mRNA for Pex3 protein
MYL6	6.6	3.59E-02	NM_079423	H300022343	Myosin, light chain 6, alkali, smooth muscle and non-muscle
C15orf27	5.7	1.56E-01	NM_152335	H200021222	Chromosome 15 open reading frame 27
KIAA0652	5.6	5.71E-02	NM_014741	H300015133	KIAA0652 (KIAA0652), mRNA
HNRNPA3	5.6	2.53E-03	NM_194247	opHsV04000 01916	Heterogeneous nuclear ribonucleoprotein A3
PROKR2	5.5	9.13E-03	NM_144773	H200017845	Prokineticin receptor 2
RPL23	5.4	2.55E-02	NM_000978	H300005578	Ribosomal protein L23
TAAR6	5.3	2.68E-02	NM_175067	H300005298	Trace amine-associated receptor 6

*Chapter 5: Cell culture model for labouring and non-labouring myometrium*

SRD5A2	5.28	3.15E-02	NM_000348	H200000509	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)
CRH	5.3	1.79E-01	NM_000756	H200006062	Corticotropin releasing hormone
TTPA	28.7	1.88E-02	NM_000370	H200005658	Tocopherol (alpha) transfer protein
DHRS7C	19.6	7.07E-02	NM_001105571	opHsV0400001964	Dehydrogenase/reductase (SDR family) member 7C
LEPROT	15.1	9.99E-03	NM_017526	opHsV0400003795	Leptin receptor
C10orf99	9.7	1.20E-01	NM_207373	opHsV0400003625	Chromosome 10 open reading frame 99
RNF217	9.3	2.74E-02	NM_152553	H300011309	Ring finger protein 217
FAM78A	8.8	1.06E-01	NM_033387	opHsV0400000177	Family with sequence similarity 78, member A
GRIA3	8.4	1.98E-03	NM_007325	opHsV0400007800	Glutamate receptor ionotropic, AMPA 3
C20orf132	7.1	1.86E-02	NM_152503	H300018088	Chromosome 20 open reading frame 132
YIPF7	6	8.06E-03	NM_182592	opHsV0400002536	Yip1 domain family, member 7
FAM71A	5.9	2.65E-02	NM_153606	H200012813	Family with sequence similarity 71, member A
SERPINA9	5.8	2.18E-02	NM_175739	opHsV0400003059	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9
SPAG17	5.7	1.93E-02	NM_206996	H300017001	Sperm associated antigen 17
SCN4A	5.5	3.16E-02	NM_000334	H200004755	Sodium channel protein type 4 subunit alpha
CLDN2	5.2	4.97E-02	NM_020384	H200002539	Claudin 2

<sup>1</sup> Positive values (in red) indicate upregulation and negative values (in green) represent downregulation.

GENE ONTOLOGY ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED FOLLOWING P4+E2+RU486 TREATMENT

All analyses were performed as explained earlier in Gene Ontology analysis of differentially expressed genes identified following P4 treatment.

GO analysis identified 55 genes with associated biological processes. Ion binding was the most overrepresented GO term identified in the study. The other major processes identified were transport, transcription and metabolism (Figure 5.8).

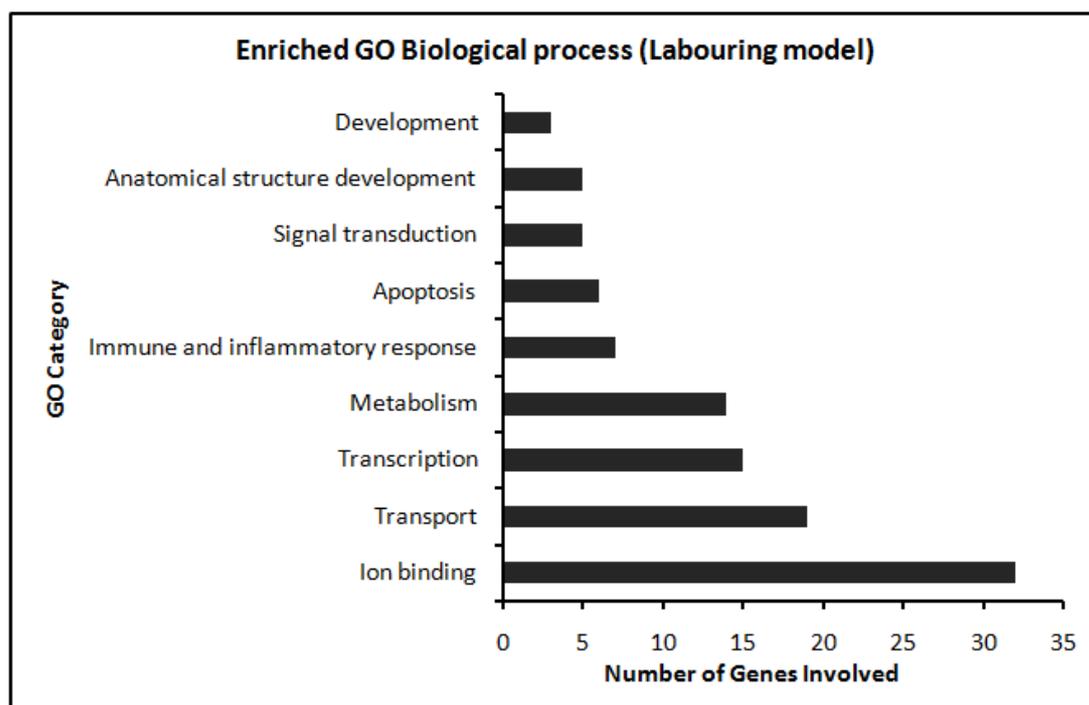


Figure 5.8: Enriched GO biological processes identified following P4+E2+RU486 treatment of hUtSMCs.

#### VALIDATION OF MICROARRAY RESULTS

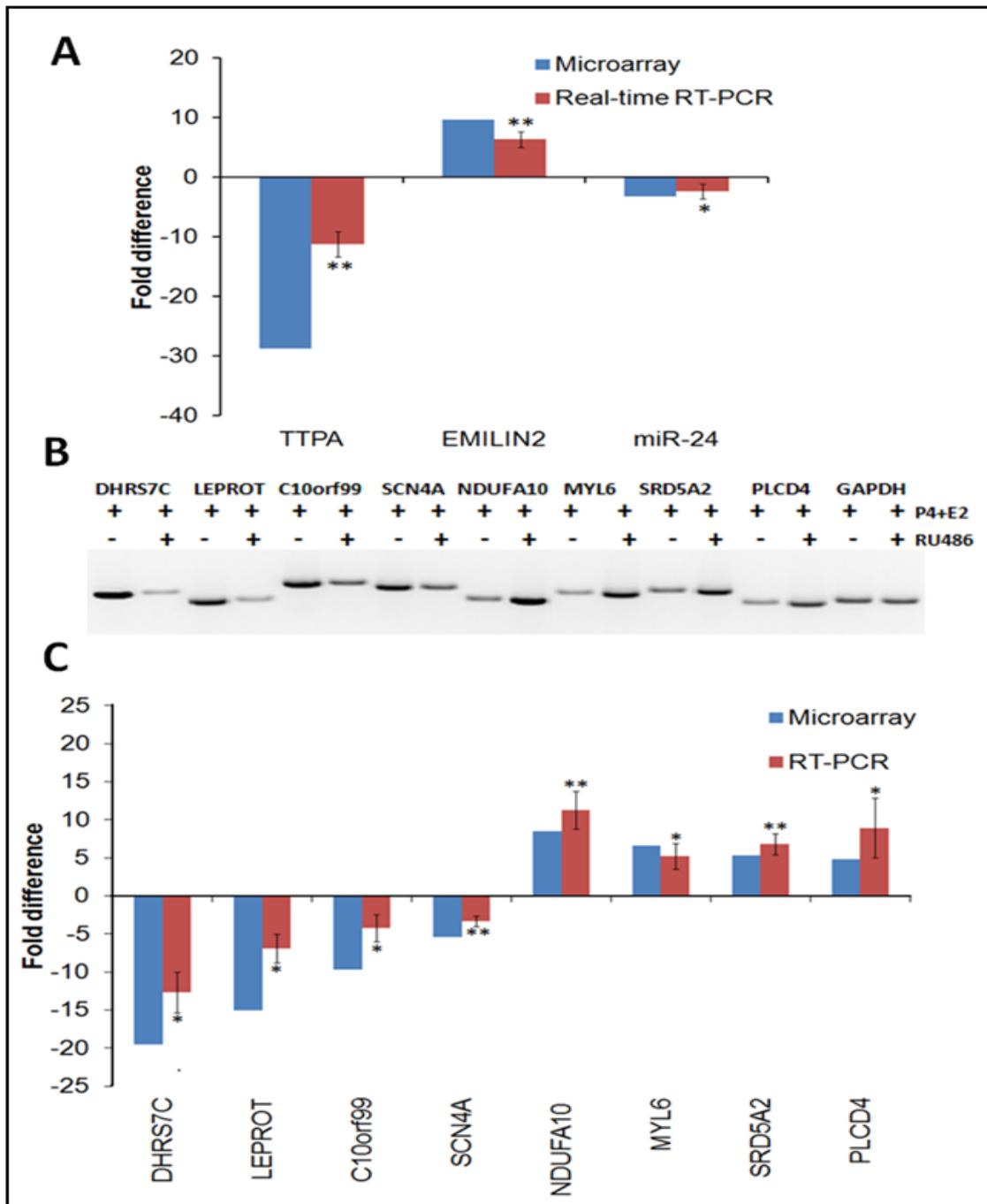
To validate the microarray results obtained from the study qPCR and semi-quantitative RT-PCR were carried out on selected genes. The genes analysed were selected based on their functions and relevance to the present study. Real-time RT-PCR was performed to confirm the differential gene expression of EMILIN2 (highest upregulated gene identified), TTPA (highest downregulated gene identified) and miR-24 (Figure 5.9A).

In the present study Elastin microfibril interface located protein 2 (EMILIN2) expression was increased 9.6-fold in P4+E2+RU486 treated hUtSMCs. In qPCR analysis the expression of EMILIN2 was increased 6.3-fold ( $P < 0.005$ ) (Figure 5.9A).  $\alpha$ -Tocopherol expression was downregulated 28.7-fold in hUtSMCs following P4+E2+RU486 treatment. qPCR confirmed this finding, where the expression of TTPA was reduced by 11.2 fold ( $P < 0.005$ , Figure 5.9A). Expression of miR-24 was downregulated (3.3-fold) in hUtSMCs treated with P4+E2+RU486. qPCR confirmed that expression of miR-24 mRNA was reduced by 2.4-fold ( $P < 0.05$ ) in cells treated with P4+E2+RU486 compared to cells treated with P4+E2 alone (Figure 5.9A).

## *Chapter 5: Cell culture model for labouring and non-labouring myometrium*

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Semi-quantitative RT-PCR was carried out on 8 genes (4 upregulated - NDUFA10, MYL6, SRD5A2 and PLCD4, and 4 downregulated - DHRS7C, LEPROT, C10orf99 and SCN4A) identified as differentially expressed in P4+E2+RU486 treated samples compared to cells treated with P4+E2 alone (Figure 5.9B). Densitometry analysis was also performed to obtain an accurate value for the differential expression identified by semi-quantitative RT-PCR experiment (Figure 5.9C). Table 5.8 summarises the results from PCR analysis.



**Figure 5.9: qPCR validation of differentially expressed genes following P4+E2+RU486 treatment.** hUtSMCs were treated with 100 nM P4, 10 nM E2 and co-treated with 1 $\mu$ M RU486 24 h prior to harvest. Cells were harvested after 72 h. **A.** qPCR validation of EMILIN2 and TTPA **B.** Gel image from semi-quantitative PCR validation of the remaining genes. **C.** Densitometry analysis of intensity values. The fold differences were calculated by normalising the values to the housekeeping gene (GAPDH) and comparing with corresponding untreated sample (set as 1). Each value corresponds to the average from three independent experiments (n=3) plotted (+/-SD). Statistically significant differences between the treated and control samples were determined using the Student *t*-test (\*- $P$ <0.05, \*\*- $P$ <0.005). For semi-quantitative PCR amplification cycles were controlled to avoid the plateau effect. Samples were separated by agarose (1%) gel electrophoresis. Gel images were analysed by densitometry.

Table 5.8 Summary of PCR validations.

Gene name	Description	Fold change <sup>1</sup>	
		Microarray	PCR
EMILIN2	Elastin microfibril interface-located protein 2	9.6	6.3*
NDUFA10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	8.5	11.2
MYL6	Myosin light polypeptide 6	6.6	5.2
SRD5A2	Steroid 5- alpha-reductase 2	5.3	6.8
PLCD4	Phospholipase C, delta 4	4.8	8.9
TTPA	Tocopherol (alpha) transfer protein	-28.7	-11.2*
DHRS7C	dehydrogenase/reductase (SDR family) member 7C	-19.6	-12.7
LEPROT	Leptin receptor precursor	-15.1	-6.9
C10orf99	Novel protein	-9.7	-4.2
SCN4A	Sodium channel protein type 4 subunit alpha	-5.5	-3.3
miR-24	micro RNA	-3.3	-2.4*

\* All validations were by semi-quantitative PCR except these three, which were by real-time qPCR.

<sup>1</sup> Positive values indicate upregulation and negative values indicate downregulation.

## PATHWAY ANALYSIS

Differentially expressed genes identified in the microarray analysis of the treatment conditions aimed at mimicking the hormonal conditions of labouring myometrium were analysed to identify their roles in important physiological/biological pathways using both MetaCore (Ekins et al., 2006) and Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 tools (Dennis et al., 2003). Using MetaCore all human protein-protein, protein-DNA and protein-compound interactions, metabolic and signalling pathways were analysed. All molecular interaction and reaction networks for *Homo sapiens* provided in Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000; Kanehisa et al., 2010), BioCarta proteomic pathway analysis (Hsu et al., 2005) and Biological Biochemical Image Database (BBID) (Becker et al., 2000) were analysed using DAVID. The most important pathways identified by MetaCore analysis were “Muscle contraction by oxytocin signalling in uterus and mammary gland” (Figure 5.10) and “Immune response by MIF – the neuroendocrine – macrophage connector” (Figure 5.11). Genes

involved in the “Muscle contraction pathway” were – IFNG (Interferon, gamma) and PRKCB1 (Protein kinase C, beta) whose mRNA expression was upregulated upon functional withdrawal of progesterone. Genes involved in the “Immune response by MIF” were CRH (Corticotropin releasing hormone) and PRKCB1. Expression of all these genes was also upregulated following P4+E2+RU486 treatment indicating an activation of these pathways thereby activating immune and inflammatory responses in the cell culture model system.

Several signalling pathways were identified by DAVID analysis including calcium signalling, oestrogen receptor signalling, GnRH signalling, Jak-STAT signalling and MAPK signalling. Other pathways identified were cell adhesion, axon guidance, gap junctions, focal adhesion, cell communication, apoptosis and various metabolic pathways. The pathways identified and the genes involved in each pathway are tabulated and appended (Appendix Table A3.9).

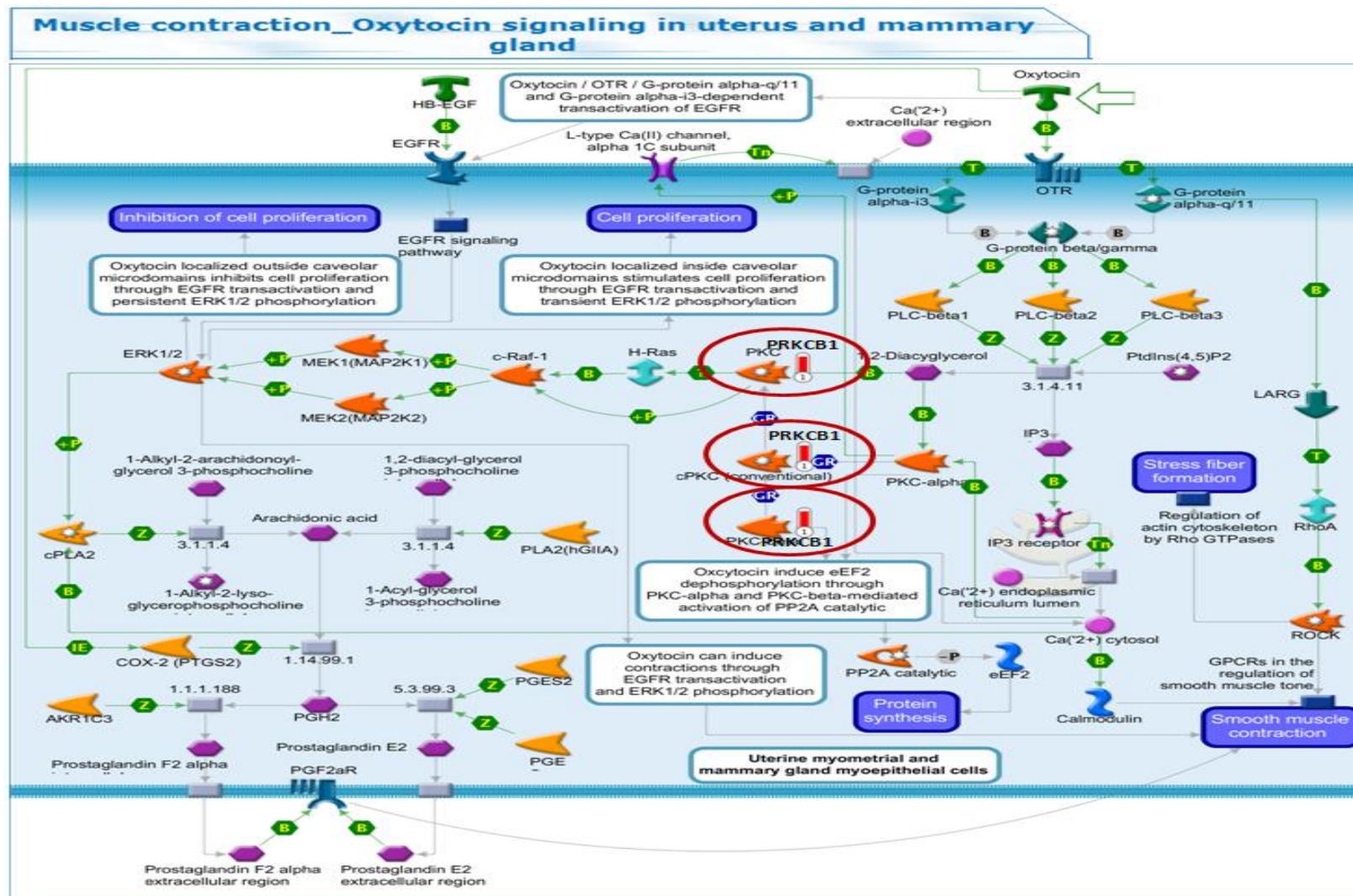
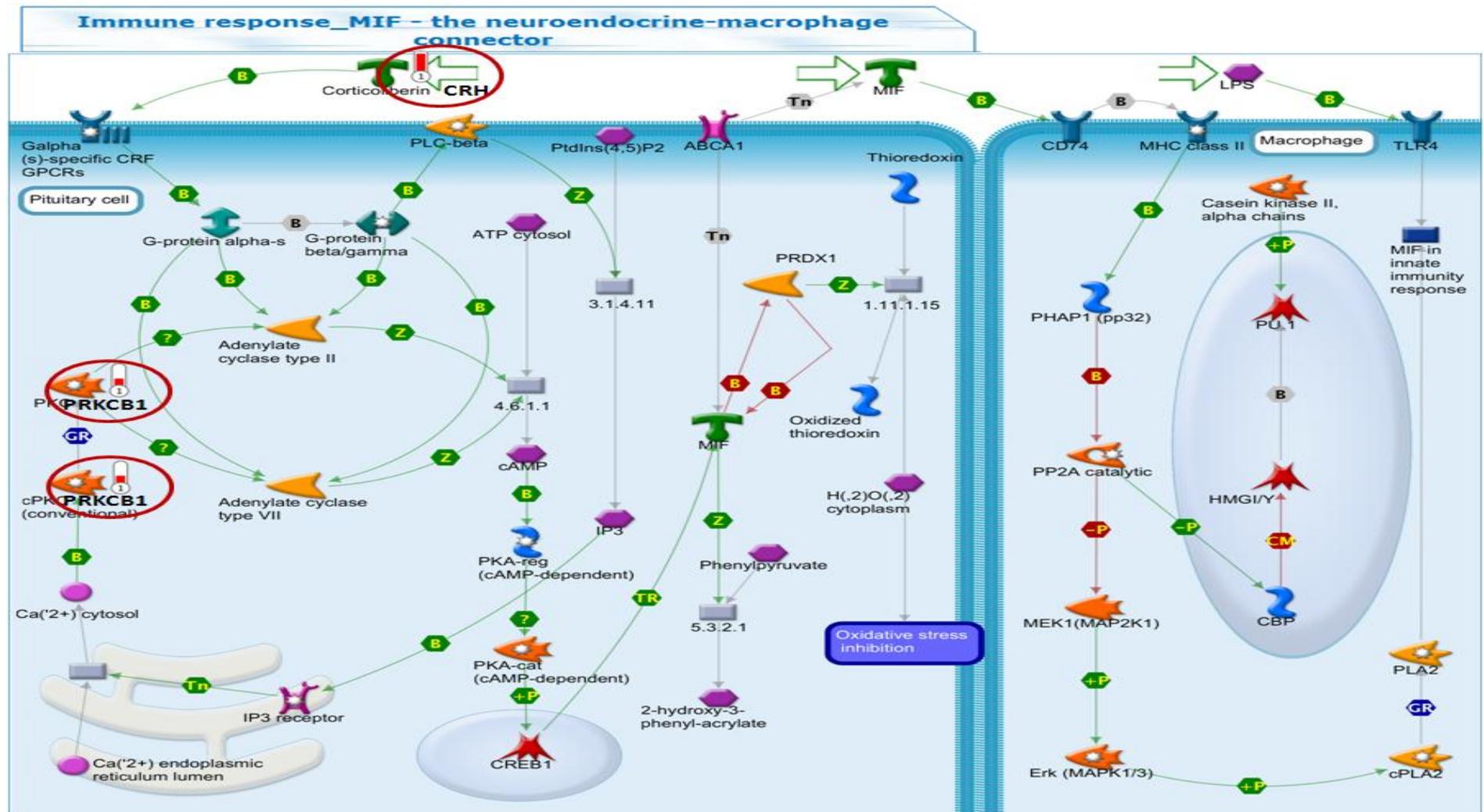


Figure 5.10: Muscle contraction/Oxytocin signalling in uterus and mammary gland identified by pathways analysis. Genes identified in the present study are circled and red line used for circling indicate upregulation of the gene in the present study. The figure legend is provided in Figure 5.7.



**Figure 5.11: Immune and inflammatory response pathway identified by pathways analysis** – Genes identified in the present study are circled and red line used for circling indicate upregulation of the gene in the present study. The figure legend is given in Figure 5.7.

**5.2.5 Comparison of differentially expressed genes identified in cultured hUtSMCs between mimicking pregnant myometrium and labouring myometrium**

Comparison of the differentially expressed genes identified in response to P4+E2 treatment (pregnant myometrium) and P4+E2+RU486 treatment (labouring myometrium) was performed to identify any common genes and also to note the change in expression pattern across those genes. Eight genes were identified that were common to both groups. The findings are summarised in Table 5.9. Fold changes for (P4+E2+RU486)-treated samples were calculated compared to (P4+E2)-treated samples and for (P4+E2)-treated samples were calculated compared to untreated controls. So, the downregulation seen in (P4+E2+RU486)-treated samples are further reduced under this treatment condition compared to (P4+E2)-treated samples, i.e. the expression of TTPA is reduced ~16-fold in hUtSMCs co-treated with P4 and E2 and the expression of this gene is further reduced by ~29-fold in (P4+E2+RU486)-treated samples.

**Table 5.9 Summary of expression profile of common genes differentially expressed genes identified in cultured hUtSMCs mimicking pregnant myometrium versus labouring myometrium.**

Gene symbol	Description	Fold change*	
		P4E2RU486	P4E2
TTPA	Tocopherol (alpha) transfer protein	-28.7	-16.3
LEPROT	Leptin receptor	-15.1	-11.5
C10orf99	Chromosome 10 open reading frame 99	-9.7	-4.9
SERPINA9	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	-5.8	-5.6
C20orf132	Chromosome 20 open reading frame 132	-7.1	-16.8
FAM71A	Family with sequence similarity 71, member A	-5.9	-10.3
YIPF7	Yip1 domain family, member 7	-6.0	-4.3
MTMR11	Myotubularin related protein 11	-2.5	-3.6

\*Negative symbol indicates downregulation

### **5.3 Discussion**

The present study is aimed at developing a cell culture model system that mimics the physiological steroid hormone conditions of pregnant and labouring human myometrium. To achieve this, hUtSMCs were cultured in the presence of progesterone alone and with  $17\beta$ -estradiol and/or RU486. Cultured hUtSMCs were treated with four different combinations of steroid hormones: (1) P4 only - to study the effect of progesterone on the cells; (2) P4+RU486 - to study changes in the gene expression due to functional progesterone withdrawal; (3) P4+E2 to investigate the expression profile of genes in hUtSMCs exposed to steroid hormonal conditions similar to those in the pregnant uterus; and (4) P4+E2+RU486 to study the effects on gene expression of hormonal conditions similar to those in labouring myometrium.

Pregnancy in most mammals studied to date does not proceed in the absence of progesterone, proving the important role of progesterone in maintaining pregnancy (Brown et al., 2004). In humans, the circulating progesterone levels do not decrease towards labour, hence progesterone withdrawal is believed to occur at a functional level (Mesiano, 2001). RU486 (Mifepristone) is a progesterone receptor antagonist used as an abortifacient during early pregnancy (Van Look and von Hertzen, 1995), a contraceptive (Piaggio et al., 2003) and as a labour inducer in late pregnancy (Bartley et al., 2000; Fairley et al., 2005). In the present study RU486 was used to mimic functional progesterone withdrawal. Identifying differentially expressed genes in this system should therefore provide insights into the complex molecular processes that are regulated by these hormones, thereby improving our understanding the processes involved in parturition. The main biological processes altered in the uterus during pregnancy and parturition are muscle contraction, metabolism, cell proliferation, cell adhesion, immune and inflammatory responses and coagulation (Helguera et al., 2009). Hence, differentially expressed genes identified in this study were examined in relation to these functions in order to understand the molecular changes happening in the myometrial smooth muscle cells that could lead to contraction or relaxation of these cells.

*REGULATION OF CONTRACTION AND RELAXATION:*

Progesterone is generally referred to as the hormone that promotes myometrial relaxation (Albrecht, 1980; Brown et al., 2004; Chwalisz and Garfield, 1994). Furthermore progesterone withdrawal at any stage of pregnancy initiates muscle contraction leading to labour (Mesiano et al., 2011). In most model organisms studied (primates and non-primates) the transformation of the uterus from a quiescent state to an active and rhythmically contracting state involves an increase in the levels of circulating oestrogen along with systemic progesterone withdrawal (Lopez Bernal et al., 1995; Wolstenholme and Knight, 1969a; Wolstenholme and Knight, 1969b). However, in humans this change in concentration is not observed and functional withdrawal of progesterone is believed to be the basis of uterine contraction (Brown et al., 2004; Mesiano, 2004; Zakar and Hertelendy, 2007).

MYL6 (Myosin light chain 6), which encodes the smooth muscle isoform of myosin light chain (MLC) (Hailstones and Gunning, 1990) was upregulated (6.6-fold) in hUtSMCs exposed to the hormonal conditions simulating those in the labouring myometrium (P4+E2+RU486). Reversible  $\text{Ca}^{2+}$ -dependent MLC phosphorylation is a major determinant of smooth muscle contraction (Ierardi et al., 1996; Zhang et al., 1994). MLC phosphorylation leads to increased activity of actin-activated  $\text{Mg}^{2+}$ -ATPase, which leads to interaction of actin with myosin, and it is this interaction that results in smooth muscle contraction (Ikebe and Hartshorne, 1985; Khalil, 2010). In addition, the expression NUA1 (NUAK family of SNF1-like kinase 1) mRNA was upregulated (3.2-fold) in cultured hUtSMCs following functional P4 withdrawal in P4 and E2 co-treated cells. NUA1 induces phosphorylation of myosin phosphatase targeting-1 (MYPT1) protein, thereby enhancing the phosphorylation of myosin light chain 2 (Zagorska et al., 2010). NUA1 also interacts with several myosin phosphatases inhibiting cell adhesion (Zagorska et al., 2010). This increases cell detachment, which is indicative of smooth muscle contraction (Helguera et al., 2009). NUA1 is also responsible for the phosphorylation of p53 which has been shown to inhibit cell proliferation by arresting cells in G1/S phase (Hou et al., 2011).

The Phospholipase C (PLC) family of proteins plays an important role in the induction of labour (Dupuis et al., 2008). During labour, oxytocin activates PLCs,

which are responsible for induction of myometrial contractility (Otsuki et al., 1993; Shojo and Kaneko, 2000). Activation of any of these phospholipase C forms releases two secondary messengers, namely inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG). IP3 binds to its receptors in the sarcoplasmic reticulum stimulating  $\text{Ca}^{2+}$  release. The cytosolic  $\text{Ca}^{2+}$  binds to DG leading to activation of contraction-associated proteins (Barany, 1996; Lodge and Leach, 1973). Phospholipase C, delta 4 (PLCD4; 4.8-fold) and Phospholipase C, epsilon 1 (PLCE1, 3.2-fold) were upregulated in the present study when cultured hUtSMCs were exposed to P4+E2+RU486 (labouring hormonal condition). Furthermore an additional PLC form, Phospholipase C beta-1 (PLCB1) was upregulated 2.6-fold in hUtSMCs treated with P4+RU486 (i.e. in the absence of E2). This increase in PLCB1 (Phospholipase C beta 1) in hUtSMCs following functional P4 withdrawal may be indicative of smooth muscle contraction in the present experimental system.

As mentioned earlier, myometrial contraction also involves the activation of G protein-coupled receptors (Brenninkmeijer et al., 1999). Following (P4+E2+RU486) treatment in hUtSMCs the expression of several GPCRs including OR1G1 (2-fold), OR2B11 (2.5-fold), OR5AT1 (2.6-fold), TAAR6 (5.3-fold) and PROKR2 (5.5-fold) were upregulated. An increase in the expression of these receptors could induce G-protein receptor mediated signalling in cultured hUtSMCs.

The expression of  $\beta$ -adrenergic receptor kinase 2 (ADRBK2) was upregulated (3.6-fold) in hUtSMCs treated with P4+E2+RU486.  $\beta$ -Adrenergic receptor ( $\beta$ -AR) induction by progesterone plays an important role in uterine relaxation during pregnancy (Simon et al., 2001).  $\beta$ -adrenergic receptor kinase acts as a negative feedback enzyme regulating the stimulation of  $\beta$ -AR (Pippig et al., 1993; Pitcher et al., 1992).

PKC- $\beta$  (PRKCB1) mRNA expression was upregulated (2-fold) in cultured hUtSMCs following treatment with P4+E2+RU486. Protein kinase C (PKC) is an important protein in the regulation of smooth muscle contraction (Rasmussen et al., 1990; Rasmussen et al., 1987; Takuwa, 1996).  $\text{Ca}^{2+}$  ions activate PKC, which in turn phosphorylates specific target proteins like myosin light chain kinases and mitogen

activated protein kinases to promote contraction (Rasmussen et al., 1987; Webb, 2003).

The main pathway identified after analysing the differentially expressed genes in hUtSMCs treated with P4+E2+RU486 was 'Muscle contraction via Oxytocin signalling'. Oxytocin, a strong uterotonic hormone, is a cyclic peptide, which plays a key role in sustaining myometrial smooth muscle contraction (Blanks and Thornton, 2003; Reversi et al., 2005; Russell and Leng, 1998). Oxytocin binds to oxytocin receptors (OTR) that are coupled to G-proteins  $\alpha$ -q/11 and  $\alpha$ -i3 (Strakova and Soloff, 1997). Activation of G-protein  $\alpha$ -q/11 leads to G-protein  $\beta/\gamma$  complex release, which in turn binds to Phospholipase C $\beta$  (PLC- $\beta$ 1) (Gimpl and Fahrenholz, 2001; Hoare et al., 1999; Phaneuf et al., 1996; Zhong et al., 2003; Zingg, 1996). PLC- $\beta$  activity releases IP3 (Gimpl and Fahrenholz, 2001; Phaneuf et al., 1993; Rhee, 2001), which then binds to its receptor resulting in the release of cytosolic Ca<sup>2+</sup> (from the sarcoplasmic reticulum) (Gimpl and Fahrenholz, 2001; Strunecka et al., 2009). Oxytocin also can increase the influx of extracellular Ca<sup>2+</sup> into the myometrial cells by activation of PKC $\alpha$  (Arnaudeau et al., 1994; Gimpl and Fahrenholz, 2001; Monga et al., 1999). These Ca<sup>2+</sup> ions bind to calmodulin to form a Ca<sup>2+</sup>-calmodulin complex that induces smooth muscle contraction (Pfitzer, 2001; Sanborn et al., 2005). One of the main regulatory steps in uterine smooth muscle contraction is the influx of Ca<sup>2+</sup> to the smooth muscle cells. This step is co-regulated by PRKCB1, whose expression was induced (1.8-fold) in cultured hUtSMCs following P4+E2+RU486 treatment. Increased PRKCB1 expression would lead to increased IP3 secretion and increased cytosolic Ca<sup>2+</sup> release, which subsequently result in increased smooth muscle contraction.

A number of genes in the contraction and relaxation category were also identified under the pregnancy condition (P4+E2 treatment). The expression of GJA5 (Connexin40) was upregulated (3.5-fold) in hUtSMCs treated with P4+E2. Connexin40 is involved in relaxation of endothelial smooth muscles as well as skeletal muscles; reduction of GJA5 contributed to the loss of relaxatory capacity of mouse aorta and skeletal muscles (Alonso et al., 2010; Milkau et al., 2010). Activation of G protein-coupled receptors is important during myometrial contraction, where GPCRs target the activation of phospholipase C, thereby leading to release of

Ca<sup>2+</sup> and stimulation of Ca<sup>2+</sup> influx (Brenninkmeijer et al., 1999). OR7A17 is an olfactory receptor, which has GPCR function (Spehr and Munger, 2009). This gene was found to be downregulated (3.6-fold) in cultured hUtSMCs treated with P4+E2 compared to untreated control cells.

GO analysis of differentially expressed genes in response to P4-treatment of hUtSMCs identified 10 genes to be involved in muscle contraction ( $P=0.04$ ; AGFG2, F2, MCTP1, MYLK3, OR2T10, SST, SSTR2, TRIM67, ZNF20, ZNF600). OR2T10, which belongs to the olfactory receptor family of proteins, acts as a G protein-coupled receptor (DeMaria and Ngai, 2010). In this study, expression of this gene was shown to be downregulated (3.3-fold) in hUtSMCs following P4-treatment.

Somatostatin precursor (SST) was shown to be downregulated (2.8-fold) in hUtSMCs following P4-treatment. Somatostatin induces contraction of colonic smooth muscle and saphenous vein smooth muscle cells (Corleto et al., 1998; Dimech et al., 1995). The association of somatostatin with its receptor (SSTR) regulates the effects of somatostatin on target cells. Vascular smooth muscle cells incubated with somatostatin showed reduction in planar cell surface area, increase in intracellular calcium concentration and decrease in cAMP levels indicating somatostatin's role in positively regulating smooth muscle contraction (Torrecillas et al., 1999). Expression of SSTR2, a receptor for somatostatin, was upregulated (2.6-fold) in hUtSMCs treated with P4. SSTR2 is another G protein-coupled receptor and is shown to have either contractile or relaxatory effects depending on the tissue (Corleto et al., 1998; Corleto et al., 1997; Feniuk et al., 1993). Since an increase in expression of SSTR2 has been observed in pregnant myometrium where the myometrial smooth muscles are more relaxed (De Leo et al., 2003), the upregulation of this GPCR is still consistent with a relaxed phenotype.

In summary, P4-treatment of cultured hUtSMCs, either alone or in combination with E2, altered the expression of several genes involved in contraction and relaxation favouring relaxation of the cells. On the other hand, functional progesterone withdrawal through progesterone inhibitor RU486 altered expression of smooth muscle contraction-associated genes favouring contraction, as has been reported for the human myometrium during labour (Brown et al., 2004).

*CELL ADHESION:*

During parturition, digestion of extracellular matrix proteins by specific proteases is observed (Fata et al., 2000). In addition, a reduction of cell adhesion and communication is associated with contraction of smooth muscle (Dahm and Bowers, 1998).

GO analysis of differentially expressed genes in response to P4+E2+RU486 treatment identified five genes (ITGA9, FREM2, PCDH20, CLDN2, EMILIN2) involved in cell adhesion ( $P=0.04$ ). FRAS1-related extracellular matrix protein 2 (FREM2) expression was downregulated (3.6-fold) following P4+E2+RU486 treatment. FREM2 is an extracellular matrix protein localised to the epithelial basement membranes (Chiotaki et al., 2007; Dalezios et al., 2007). Loss of this gene leads to collapse of structural protein assembly, which results in reduced cell attachment (Petrou et al., 2008).

In the present study, integrin alpha 9 (ITGA9) was downregulated 4-fold in hUtSMCs treated with P4+E2+RU486 compared to (P4+E2)-treated cells. ITGA9 belongs to the integrin family of transmembrane cell adhesion molecules that mediate cell-cell and cell-matrix adhesion (Alberts, 2002; Palmer et al., 1993). The reduction of ITGA9 identified in the present study, indicates reduced cell adhesion following P4+E2+RU486 treatment. CLDN2 mRNA expression was also downregulated (5.2-fold) in hUtSMCs treated with P4+E2+RU486. CLDN2 is a member of the claudin protein family involved in the formation of tight junctions (Sakaguchi et al., 2002). This family of proteins is important for  $Ca^{2+}$ -independent cell adhesion (Kubota et al., 1999).

In the pregnancy-mimicking condition (P4+E2), Spleen tyrosine kinase (SYK), was upregulated (4.7-fold) in cultured hUtSMCs. SYK regulates cell proliferation and cell adhesion (Coopman et al., 2000). Fostamatinib, a SYK inhibitor, attenuated the adhesion of inflammatory cells in mice (Hilgendorf et al., 2011). Also, transfection of SYK into SYK-negative breast cancer cell lines inhibited metastasis (Coopman et al., 2000). Hence, an induction of the SYK gene in the present study may be indicative of increased cell adhesion in hUtSMC cell culture system.

On the whole, GO analysis identified cell adhesion as a significant biological process for differentially expressed genes identified following P4+E2 and P4+E2+RU486 treatments. The increased expression of SYK identified in (P4+E2)-treated hUtSMCs favoured cell adhesion in these cells, whereas the expression of genes identified following P4+E2+RU486 treatment prohibited cell adhesion. Eventhough cell adhesion and tissue remodelling are not directly involved in the initiation of parturition cascade, the above-mentioned genes might play an important role in parturition (Bethin et al., 2003). Both these findings are in line with previous reports where cell adhesion is increased during pregnancy and a sharp decline in cell adhesion is reported in labouring myometrium (Macphee and Lye, 2000).

*REGULATION OF IMMUNE AND INFLAMMATORY RESPONSES:*

During pregnancy, suppression of immune and inflammatory responses is necessary to tolerate the developing foetus in the uterus (Siiteri and Stites, 1982), but towards the end of pregnancy and during the onset of labour this is reversed, with the activation of immune and inflammatory responses (Helguera et al., 2009).

GO analysis of differentially expressed genes following (P4+E2+RU486) treatment of hUtSMCs identified seven genes to be involved in immune and inflammatory responses ( $P=0.02$ ) (C7, IL23R, GRIK2, TAP2, IFNG, CRH, KIR3DL1). IFNG, which was upregulated (2.3-fold) following (P4+E2+RU486) treatment in the current study, encodes IFN $\gamma$  (Interferon- $\gamma$ ), which is produced predominantly by natural killer cells and T-cells as part of an immune response (Schoenborn and Wilson, 2007). IFN $\gamma$  also activates the production of inducible nitric oxide synthase and hence of nitric oxide (NO), a key player in inflammatory responses and apoptosis (Key et al., 1992; Schoenborn and Wilson, 2007; Schroder et al., 2004).

Regulator of calcineurin 2 (RCAN2) was downregulated (1.8-fold) in hUtSMCs treated with P4+E2+RU486. Induction of RCAN2 was previously found to reduce many pro-inflammatory responses in HUVECs (Canaider et al., 2010). Thus the reduction in the expression of this gene in the present condition may induce inflammatory responses by upregulating pro-inflammatory genes.

Killer cell immunoglobulin-like receptor (KIR) genes play an important role in regulating natural killer (NK) cell function. KIR3DL1, a member of the KIR family, was downregulated (2.6-fold) in hUtSMCs mimicking hormonal conditions of labour (P4+E2+RU486). Downregulation of KIR3DL1 in NK cells by shRNA was reported to enhance the *in vitro* function of the NK cells (Qin et al., 2011). Downregulation of KIR3DL1 may therefore be indicative of enhanced immune response in the present study.

Pathway analysis (MetaCore) of differentially expressed genes identified ‘Immune response by HMGB1 release from the cell’ as a pathway potentially involved in regulating cellular functions in response to hormonal conditions mimicking labouring myometrium. HMGB1 is a ubiquitous nuclear protein, which is secreted into the cytoplasm mediating an inflammatory reaction, in response to exogenous and endogenous stimuli (Chen et al., 2004; Tang et al., 2007; Wahamaa et al., 2007; Wang et al., 1999; Wu et al., 2012). In addition, HMGB1 can be released passively during cell apoptosis (Bell et al., 2006) and necrotic cell death (Ditsworth et al., 2007; Rovere-Querini et al., 2004). Release by any of these means results in the activation of NF- $\kappa$ B (Hayden and Ghosh, 2008). In the present study the expression of IFNG was increased (2.2-fold) following P4+E2+RU486 treatment. Increase in the expression of IFNG could lead to activation of the IFNG signalling cascade and subsequent increased release of HMGB1.

The IFNG pathway can also induce  $Ca^{2+}$  release from the endoplasmic reticulum through activation of the IP3 receptor. Cytosolic  $Ca^{2+}$  activates PKC $\gamma$  and calmodulin-dependent CaMK4 thereby phosphorylating HMGB1 (Zhang et al., 2008a). HMGB1 can also be phosphorylated and released to the cytosol by calcium-dependent PKC- $\alpha$  and PKC- $\beta$  (Zhang et al., 2008a). In (P4+E2+RU486)-treated hUtSMCs the expression of PKC- $\beta$  (PRKCB1) was increased 2-fold. Both increase of  $Ca^{2+}$  through IFNG signalling and induced PRKCB1 could together mediate increased phosphorylation of HMGB1 and hence increased cytosolic HMGB1 concentration. Thus, the increased expression of IFNG and PRKCB1 following P4+E2+RU486 treatment may, through increased levels of cytosolic HMGB1, induce immune and inflammatory responses.

Differentially expressed genes identified following functional progesterone withdrawal from P4-treated hUtSMCs (in the absence of E2) were also implicated in immune and inflammatory responses. Progestagen-associated endometrial protein (PAEP), which was downregulated 2.3-fold, is a glycoprotein with potent immunosuppressant action blocking the activation of B-cell associated receptor (Dell et al., 1995). Downregulation of PAEP in the present system may, therefore, favour activation of the immune response. Interferon regulatory factor 4 (IRF4), which is downregulated 2-fold in hUtSMCs treated with P4+RU486, is necessary for the development of T-helper cells (Huber et al., 2008; Staudt et al., 2010). Suppression of this gene was necessary for tumour elimination in association with NOXA (a proapoptotic Bcl-2 family protein) (Piya et al., 2011). Also, mice lacking IRF4 showed a strong and lethal inflammatory response (Negishi et al., 2005). Hence, a reduction in the expression of IRF4 could indicate an increase in immune and inflammatory response. Overall, functional withdrawal of P4 by RU486 results in an increase in the immune and inflammatory response.

Pathway analysis (MetaCore) of genes differentially expressed in conditions mimicking pregnancy, i.e. (P4+E2) treatment, identified that Spleen tyrosine kinase (SYK) and B-cell Cell/lymphoma 2 (BCL2) are involved in 'IL15 signalling pathway' ( $P=0.04$ ; Appendix Figure A3.1). IL15 binds to its receptors and phosphorylates SYK (Ratthe and Girard, 2004). Activated SYK subsequently activates T-cells thereby inducing an immune response. SYK activation also leads to stimulation of neutrophil phagocytosis (Ratthe and Girard, 2004) and activation of PLC- $\gamma$ 1 in B cells (Bulanova et al., 2001). In the present study the expression of SYK was downregulated (4.6-fold) following (P4+E2) treatment, which could indicate reduced availability of SYK to activate T-cells thereby diminishing the immune response. Membrane bound IL15 activates Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) cascades, which in turn induces the transcription of anti-apoptotic gene BCL2 (upregulated (4.4-fold) in hUtSMCs exposed to P4+E2) (Qin et al., 2001) thereby preventing apoptosis. Both reduced immune response and cell death are characteristics of pregnant myometrium.

In the present study, myosin light chain kinase 3 (MYLK3) was downregulated 2.7-fold upon P4 treatment. Non-muscle MYLKs are known to have a role in

inflammatory responses and are regarded as major players in acute lung injury susceptibility, inflammatory bowel disease and sepsis (Mirzapoiiazova et al., 2011; Roy et al., 2007; Xu et al., 2008). Inhibition of MYLK attenuated the inflammatory response in mice (Mirzapoiiazova et al., 2011).

Overall, it may be concluded that the more pronounced activation of immune and inflammatory response (based on the number of genes involved in this processes and the role of these genes in the process) was identified in hUtSMCs treated with hormonal conditions mimicking that of the labouring myometrium (P4+E2+RU486) as opposed to those conditions in the absence of E2. This is supported by previous report that during parturition immune and inflammatory responses are stimulated (Helguera et al., 2009).

*REGULATION OF CELL PROLIFERATION, GROWTH AND DIFFERENTIATION:*

During the early stages of pregnancy, myometrial cells proliferate rapidly to accommodate the growing foetus (Shynlova et al., 2006) and reach a hypertrophic phase by mid-gestation (Harkness and Harkness, 1954; Shynlova et al., 2010; Shynlova et al., 2004). Late pregnancy in rat myometrium is marked by an increase in apoptosis (Leppert, 1995, 1998; Leppert and Yu, 1994). Caspases are also activated in the rat uterus during mid-gestation leading to the onset of apoptosis and uterine contraction (Shynlova et al., 2006).

GO analysis of those genes differentially expressed in hUtSMCs exposed to hormonal conditions similar to the labouring myometrium identified six genes involved in apoptosis ( $P=0.05$ ) (C7, GRIK2, IFNG, CRH, PAX2, LOC440577). A literature search identified several additional genes that were differentially expressed in response to P4+E2+RU486 treatment involved in cell proliferation though GO annotation did not report this function.

Elastin microfibril interface-located protein 2 (EMILIN2) was upregulated in this study where P4 was functionally withdrawn from hUtSMCs treated with P4 and E2 (P4+E2+RU486). EMILIN2 binds to TRAIL receptor DR4 to induce apoptosis (Mongiati et al., 2007; Mongiati et al., 2010). Knockdown of EMILIN2 increased cell survival while overexpression led to extensive apoptosis and impaired clonogenicity

(Mongiati et al., 2007). The induction of EMILIN2 in hUtSMCs under these hormonal conditions may therefore lead to reduced cell survival.

Ribosomal protein L23 (RPL23) was upregulated (5.4-fold) in hUtSMCs treated with P4+E2+RU486. RPL23 blocks the interaction between p53 (a tumour suppressor gene) and MDM2, activating p53 and thereby inhibiting cell proliferation (Dai et al., 2006; Zhang et al., 2010a). Expression of the ribosomal protein encoded by gene RPL18A was also induced in (P4+E2+RU486)-treated hUtSMCs (2.8-fold); this protein too has been shown to have anti-proliferative effects. Knockdown of the RPL18A gene suppressed mesothelioma cell proliferation (Sudo et al., 2010). Another tumour suppressor gene MFSD2A (Major facilitator superfamily domain-containing protein 2A) blocks cell division in G1 (Spinola et al., 2010). The expression of MFSD2A was induced 2-fold in (P4+E2+RU486)-treated hUtSMCs.

Aquaporin 5 (AQP5) was downregulated (2.7-fold) in (P4+E2+RU486)-treated hUtSMCs. Upregulation or overexpression of AQP5 enhances cell proliferation whereas siRNA targeting AQP5 reduced cell proliferation in a number of cancer cell lines (Chae et al., 2008; Zhang et al., 2010b). Fucosyltransferase 1 (FUT1) was downregulated 2.6-fold following RU486-treatment of hUtSMCs exposed to P4 and E2 (P4+E2+RU486). Rat colon adenocarcinoma cells overexpressing FUT1 showed resistance to apoptosis (Goupille et al., 2000). Gene silencing of FUT1 in bovine and human endothelial cells inhibited cell proliferation (Palumberi et al., 2010). Downregulation of this gene suggests reduced cell proliferation and increased apoptosis. Under the same conditions CYP26A1 (Cytochrome P450, family 26, subfamily A, polypeptide 1) was also downregulated (2.8-fold). Upregulation of this gene is known to attenuate apoptosis in uterine stromal cells (Xia et al., 2010).

Myoblast determination protein family inhibitor (MDFI) was also upregulated (2.1-fold) in (P4+E2+RU486)-treated hUtSMCs. MDFI is an inhibitor of MYOD family members, where it interacts with MYOD inhibiting myogenesis (Chen et al., 1996).

Several genes that were involved in cell growth and proliferation were also identified in progesterone-withdrawn samples in the absence of E2. ANGPTL4 (angiopoietin-related protein 4), whose expression was downregulated 4.2-fold in hUtSMCs treated with P4+RU486, acts as a pro-survival protein promoting tumour growth: suppression

of ANGPTL4 enhances apoptosis (Zhu et al., 2011). ID1 (Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein), which was downregulated 2.4-fold in hUtSMCs treated with P4+RU486, is associated with cell proliferation in cancer. Anaplastic thyroid cancer expresses the highest levels of ID1 and normal cells expressed the lowest level of the same gene compared to other cancers (Hara et al., 1994; Kebebew et al., 2004). Reduction in ID1 gene expression observed in hUtSMCs following P4+RU486 treatment could be indicative of reduced cell proliferation.

In summary, in cultured hUtSMCs, when progesterone was functionally withdrawn following treatment with either progesterone alone or progesterone and 17 $\beta$ -estradiol in combination, resulted in an overall reduction of cell proliferation and an increase in apoptosis being observed.

Several genes that were differentially expressed in response to P4-treatment (i.e. P4 alone) were also involved in cell proliferation. GO identified 20 genes involved in cell differentiation and proliferation. Three genes were involved in regulation of cell proliferation ( $P=0.03$ ) (SSTR2, NPPC, SST) while 19 genes were involved in cell cycle progression ( $P=0.009$ ) (CMTM1, JPH3, LRP2BP, TTLL6, ZNF20, FAM9C, ALDH3A2, MCTP1, SSTR2, TRIM67, RAB30, ZNF600, OR2T10, F2, PAF1, CHST15, OSTALPHA, PES1, SST).

SH3-binding domain kinase 1 (SBK1) was upregulated in hUtSMCs treated with P4 in the present study. Even though GO analysis failed to identify that this gene was involved in cell proliferation and apoptosis, a literature search showed that SBK1 was involved in tumour development (Wang et al., 2011). Overexpression of SBK1 in ovarian cancer cells SK-OV-3 protected cells against apoptosis (Wang et al., 2011).

RNA polymerase II associated factor (PAF1) was downregulated 2.9-fold in hUtSMCs treated with progesterone. Mutations in the Paf1 complex or its deletion led to alterations in gene expression promoting uncontrolled cell proliferation (Jaehning, 2010).

In (P4+E2)-treated hUtSMCs, GO analysis identified 21 genes to be involved in cell proliferation and differentiation. Four genes were involved in regulation of cell proliferation ( $P=0.03$ ) (BCL2, GAS8, IHH, SYK), three genes in negative regulation

of apoptosis ( $P=0.02$ ) (PAX7, BCL2, IHH), 18 genes in cell cycle regulation ( $P=0.01$ ) (BCL2, TTN, TXNL4A, OR7A17, CUBN, KLB, CDHR3, GJA5, SLC26A3, NTRK2, PALM2, OR8I2, EFNA5, LEPROT, CACNA1C, SYK, IHH, GAS8) and five genes were involved in cell differentiation ( $P=0.008$ ) (BCL2, PAX7, POU4F3, NTRK2, EFNA5).

BCL2 mRNA expression was induced (>4-fold) in hUtSMCs exposed to hormonal conditions of the pregnant myometrium (P4+E2). The expression of this gene was also induced (2.2-fold) in hUtSMCs following progesterone treatment alone. BCL2 mRNA expression was induced by 2.5-fold in 'pregnant' human myometrium compared to 'non-pregnant' myometrium (Rehman et al., 2003). Expression of BCL2, as discussed in several previous sections, has been associated with tumour survival pathways and Bcl2 is an anti-apoptotic protein, which acts as an important determinant of cell proliferation, differentiation and tumorigenesis (Craig, 2002).

Neurotrophic tyrosine kinase, receptor, type 2 (NTRK2, also known as TRKB) was upregulated (4.7-fold) in hUtSMCs treated with P4+E2. NTRK2 controls the Ras-PI3 kinase-AKT1 signalling cascade that mainly regulates growth and survival of cells (Meakin et al., 1999; Yeo et al., 2004). NTRK2 is a potent suppressor of caspase-associated anoikis - a type of programmed cell death - demonstrating its pro-survival properties (Douma et al., 2004).

Paired box protein 7 (PAX7) was upregulated (3.3-fold) in hUtSMCs treated with P4+E2. PAX genes play an important role in organogenesis and formation of tissues during embryogenesis. PAX7 is responsible for the marking of myogenic progenitor cells and in regulating their differentiation to muscles (Buckingham and Relaix, 2007). Absence of PAX7 arrested postnatal muscle formation and cells died or assumed a non-myogenic fate (Relaix et al., 2006; Relaix et al., 2005).

ST6-(alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GALNAC1) expression was downregulated (3-fold) in hUtSMCs treated with P4+E2. Even though this gene has no related annotation in the GO database, a literature search indicates that ST6GALNAC1 negatively regulates cell growth, proliferation and migration in breast cancer cell lines (Julien et al., 2001).

Indian hedgehog protein precursor (IHH) was upregulated by 4.4-fold in hUtSMCs treated with P4+E2. This gene is highly expressed in uterine epithelium in the presence of progesterone (Lee et al., 2006a; Lee et al., 2006b; Matsumoto et al., 2002) and during pregnancy in mice, IHH level increased from day 1 of implantation reaching a peak by day 4 (Lee et al., 2006b; Matsumoto et al., 2002). IHH has also been shown to regulate proliferation in a variety of tumours and tissues (Berman et al., 2003; Minina et al., 2001; Niemann et al., 2003; St-Jacques et al., 1999). Mice deficient in IHH exhibited reduced proliferation of chondrocytes during development (Karp et al., 2000; Pearse et al., 2001; St-Jacques et al., 1999) and an antibody targeting IHH quenched the proliferation of secondary chondrocytes (Ericson et al., 1996; Long et al., 2001).

Small proline-rich protein 3 (SPRR3) was upregulated (3.9-fold) in hUtSMCs exposed to hormonal conditions mimicking pregnancy (P4+E2). SPRR3 is highly expressed during epithelial cell and keratinocyte differentiation (Fischer et al., 1999; Zhang et al., 2008b). An increase in SPRR3 promotes tumourigenicity in colorectal cancer (Cho et al., 2010) and overexpression of SPRR3 in breast cancer cells promoted cell proliferation by the enhancement of p53 degradation (Kim et al., 2012). Another gene involved in cell proliferation and apoptosis, Tumour protein D52 (TPD52) (Cao et al., 2006), was upregulated 3-fold in hUtSMCs treated with P4+E2. In prostate and breast cancers this gene is highly induced compared to borderline tumours (Byrne et al., 2010; Ummanni et al., 2008).

The main pathway identified after analysing the differentially expressed genes in hUtSMCs treated with P4+E2 was 'Anti-apoptotic action pathway by membrane-bound ESR1'. 17 $\beta$ -Estradiol (E2) is well known for its anti-apoptotic effect on a wide variety of tissues and cells (Chang, 2011; Felix and Farahmand, 1997; Ferenczy et al., 1979; Tyson et al., 2011). E2 exerts this action through the activation of its membrane-bound receptor – oestrogen receptor 1 (ESR1 or ER- $\alpha$ ) where it triggers the activation of several anti-apoptotic pathways (Honda et al., 2001; Kousteni et al., 2001; Wang et al., 2006). In this pathway 17 $\beta$ -estradiol activates protein kinase C (PKC) (Sovershaev et al., 2006; Yang et al., 2005), which then phosphorylates CACNA1C (Voltage-dependent calcium channel L-type alpha 1C subunit. CACNA1C was upregulated (3.7-fold) in hUtSMCs exposed to P4+E2 and promotes

Ca<sup>2+</sup> transport into the cytosol (Wu et al., 2005; Yang et al., 2005). Increased cytosolic Ca<sup>2+</sup> levels activate PKC, which activates the ‘Mitogen-activated protein kinases 1 and 3 (Erk (MAPK1/3)) pathway’ (Cordey et al., 2003; Kolch et al., 1993; Schonwasser et al., 1998). Activated Erk phosphorylates and activates p90RSK1 (Ribosomal protein S6 kinase 90kDa polypeptide 1) (Bonni et al., 1999; Fernando and Wimalasena, 2004), which in turn activates cAMP responsive element binding protein 1 (CREB1). Activation of CREB1 promotes transcription of BCL2 (upregulated (4.4-fold) in hUtSMCs exposed to P4+E2) (Nilsen and Brinton, 2004; Subramanian and Shaha, 2007; Wu et al., 2005). Also, under the action of E2, BCL2 is activated by its dissociation from BAD (BCL2 antagonist of cell death) thereby suppressing Bax. Suppression of Bax prevents formation of mitochondrial permeability transition pore and the release of cytochrome *c* from the mitochondria into the cytoplasm, thus preventing activation of the caspase cascade (Nilsen and Brinton, 2004; Nilsen et al., 2006).

On the whole, progesterone treatment (either alone or in combination with 17 $\beta$ -estradiol) induced cell proliferation and decreased apoptosis. However, functional withdrawal of progesterone from either of the above-mentioned systems reversed this effect. These findings support previous reports indicating that progesterone favours uterine quiescence and functional progesterone withdrawal facilitates myometrial contraction (Lee et al., 2012; Tan et al., 2012; Xie et al., 2012).

#### *GENES REGULATING PREGNANCY, LABOUR AND PARTURITION:*

Corticotropin releasing hormone (CRH), which was upregulated in the present study (5.3-fold; P4+E2+RU486), determines the duration of pregnancy. A rapid increase of circulating levels of CRH occurs at the onset of labour suggesting its role in regulating parturition (Ellis et al., 2002; McLean and Smith, 1999; Tyson et al., 2009; Wadhwa et al., 1998). CRH increases the levels of dehydroepiandrosterone and prostaglandins, and it is these that initiate myometrial contraction (Bocking and Harding, 2001; Tyson et al., 2009). In trophoblast culture, the expression of CRH was inhibited following progesterone treatment suggesting the role of functional withdrawal of progesterone in inducing this gene (Jones et al., 1989).

Steroid 5-alpha-reductase (SRD5A2) gene expression was upregulated (>5-fold) in human myometrial smooth muscle cells treated with P4+E2+RU486. The same gene was reported to be upregulated in 'term-labouring' human myometrium compared to 'term non-labouring' myometrium (Charpigny et al., 2003). SRD5A2 knockouts in mice lead to defective parturition with 70% of mice failing to undergo delivery at term due to failure in cervical ripening and elevated progesterone concentration in the cervix (Mahendroo et al., 1996; Mahendroo et al., 1999).

TTPA, or alpha-tocopherol transfer protein, was downregulated (>3-fold) in uterine smooth muscle cells treated with P4+E2 compared to untreated control cells. TTPA is important for the maintenance of pregnancy as it has been shown that TTPA knockout mice failed to reach term after implantation due to severe defects in placental development which could be rescued by supplementing with  $\alpha$ -tocopherol (Jishage et al., 2001). TTPA also has a role to play in implantation in mice (Jishage et al., 2001; Kaempf-Rotzoll et al., 2002). TTPA-null mice showed activation of cell proliferation pathways, where genes such as apoptosis inhibitor 6 and mitogen-activated protein kinase 3 were induced (Gohil et al., 2003). Hence, downregulation of TTPA in this cultured hUtSMC study could be linked to increased cell proliferation. In Chapter 3 (cultured hUtSMCs treated with E2), expression of TTPA was downregulated 1.7-fold following 24 h of E2 treatment. Here, when hUtSMCs were co-treated with P4+E2 the expression of TTPA was further reduced. This indicates an additive effect on the downregulation of TTPA by P4/E2 co-treatment in the hUtSMC system.

Gonadotropin-releasing hormone receptor (GNRHR) was downregulated (4.4-fold) in hUtSMCs exposed to conditions mimicking labouring myometrium (P4+E2+RU486). The receptor's ligand, peptide hormone-gonadotropin-releasing hormone (GnRH), also known as luteinising-hormone-releasing hormone (LHRH) regulates the release of both follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Pawson and McNeilly, 2005). GnRH caused a significant decrease in the contraction intensity of non-pregnant and pregnant uterine muscle strips and GnRH treatment of pregnant rats was able to delay parturition (Gohar et al., 1996). In addition, GnRH significantly inhibited the release of placental prostaglandins E and F, and thromboxane B2 (proteins that regulate myometrial contraction) in a dose-dependent fashion (Gohar et al., 1996). GnRH agonists have been shown to reduce progesterone synthesis

(Sridaran et al., 1999). Like other hormones, GnRH exerts its action through its receptor – GNRHR (Sengupta et al., 2008). A reduction of GNRHR expression in hUtSMCs following P4+E2+RU486 treatment might therefore reduce the effects of GnRH by reducing the receptor-bound response in the present cell culture model system and may play a role in inducing smooth muscle contraction.

#### **5.4 Conclusions**

This study was aimed to develop a model cell culture system of pregnant and labouring myometrium. This was achieved by co-treating hUtSMCs with variable combinations of P4, E2 and RU486 and analysing the effect of these treatments on the transcriptomic profile in cultured hUtSMCs. The principal findings of the study demonstrated differential expression of genes in cultured hUtSMCs in response to co-treatment with P4 and E2 (P4+E2), and also in response to functional P4 withdrawal (by addition of the inhibitor RU486) in hUtSMCs either treated with progesterone alone (P4+RU486) or both progesterone and estradiol (P4+E2+RU486). Thorough analysis of the resulting transcriptome profiles identified different biological processes and pathways regulated in cultured hUtSMCs following treatment with various combinations of P4, E2 and RU486. This analysis confirmed a strong association of progesterone with uterine quiescence by altering the expression of genes favouring myometrial relaxation. Mimicking the steroid hormonal conditions of the term pregnant non-labouring uterus (hUtSMCs co-treated with P4+E2), the expression of genes was altered that would aid the suppression of smooth muscle contraction and inflammation, and increase cell proliferation and cellular adhesion. These changes in the molecular events identified in (P4+E2)-treated hUtSMCs were similar to studies on pregnant non-labouring myometrium (Charpigny et al., 2003; Helguera et al., 2009; Mittal et al., 2010).

Functional withdrawal of progesterone in this cell culture model altered the expression of genes involved in molecular events regulating parturition such as: increased contraction, decreased cell proliferation and cell adhesion. Treatment of hUtSMCs with P4+E2+RU486 (mimicking steroidal hormone conditions present in labouring myometrium) altered the expression of genes favouring events such as apoptosis, coagulation and immune and inflammatory responses. Processes such as cell proliferation and cellular adhesion were repressed, which also aid in initiating myometrial contractions. These changes in molecular events in (P4+E2+RU486)-treated hUtSMCs were in line with changes that are identified in the labouring myometrium *in utero*. Potentially this model could add to the debate whether labour is an inflammatory response or whether the inflammatory response is a reaction to the foetus (Bollapragada et al., 2009; Kelly, 2002; Marvin et al., 2002). In the present cell

culture model system although the foetal effects are absent, the hormone combinations still induced an inflammatory responses by altering the expression of genes involved in immune and inflammatory responses. This would, therefore, argue that the inflammatory response elicited during parturition is an effect of functional progesterone withdrawal and E2 treatment (effect of E2 treatment as described in the previous chapter).

In conclusion the human uterine smooth muscle cell culture system developed here to mimic the steroid hormonal conditions of labouring and pregnant myometrium behaved in a similar manner with reported *in vivo* responses. Thus this model cell culture system may be used for further studies of various aspects of pregnancy and parturition.

# **Chapter 6: Conclusions and Future Directions**

## ***Chapter 6: Conclusions and future directions***

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The aims of this study were, (1) to establish a myometrial cell culture model that would simulate the *in utero* steroid hormone conditions during pregnancy and labour, and (2) to investigate the effects of steroid hormone treatment on the global gene expression profile of cultured human uterine smooth muscle cells. To achieve these goals hUtSMCs were treated with 17 $\beta$ -estradiol and/or progesterone, the two most important steroid hormones that regulate pregnancy and labour. Adding mifepristone, a progesterone antagonist, to the system was used to mimic functional progesterone withdrawal during labour.

Myometrial smooth muscle from non-pregnant uterus (procured from Lonza) was used in this study. Non-pregnant myometrial cells were used since these cells were not pre-exposed to high oestrogen and progesterone environment unlike the cells from pregnant myometrium. Thus, any change observed in the expression profile of hUtSMCs following steroid hormone treatment would be purely an effect of the treatment of the cells with oestrogen and/or progesterone. These cells were characterised for smooth muscle characteristics by verifying the expression of marker genes smoothelin, calponin, oxytocin receptor and oestrogen receptor alpha. The morphology of the cells were also confirmed by immunofluorescence staining. Prior to hormone treatment the responsiveness of the cells to progesterone and 17 $\beta$ -estradiol were also confirmed. Both the characterisation and optimisation studies proved that the myometrial smooth muscle cells used in the present study retained smooth muscle characteristics and was responsive to steroid hormonal treatment.

In the first study, where cultured hUtSMCs were treated with 17 $\beta$ -estradiol, the differentially regulated genes identified demonstrated a strong association with pregnancy and the oestrous cycle. A total of 509 differentially expressed genes identified following treatment were analysed to identify their key functions related to pregnancy and labour. In line with previously published reports analysing pregnant uterine samples, E2-treatment downregulated the expression of genes involved in muscle contraction, cell adhesion, coagulation and immune and inflammatory responses (Bethin et al., 2003; Helguera et al., 2009). An upregulation of cell growth and proliferation-associated genes was also observed. Elevated oestrogen levels are also commonly associated with malignancies and benign tumours in several tissues including the uterus, where it aids in tumour progression by altering cell proliferation

## ***Chapter 6: Conclusions and future directions***

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and adhesion (Andersen and Barbieri, 1995; Robboy et al., 2000; Swartz et al., 2005; Tiltman, 1997). In the present study, 17 $\beta$ -estradiol-treated hUtSMCs, also showed an increase in cell proliferation and reduction of cell adhesion.

The second study evaluated the transcriptomic changes induced by progesterone alone or in combination with 17 $\beta$ -estradiol and mifepristone in cultured hUtSMCs. Different combinations of these compounds were used in order to simulate the *in utero* hormonal conditions of pregnancy and labour. The most important analyses in this study were the identification of differentially expressed genes in hUtSMCs co-treated with progesterone/17 $\beta$ -estradiol and mifepristone (hormonal conditions aimed at mimicking the labouring myometrium) compared to progesterone/17 $\beta$ -estradiol (myometrial hormonal conditions mimicking the pregnant uterus) in comparison to untreated control cells (non-pregnant myometrium). Functional progesterone withdrawal in the presence of high 17 $\beta$ -estradiol altered the expression of genes involved in several biological processes that are associated with labour, including smooth muscle contraction, immune and inflammatory responses (both increased) and cell proliferation and cell adhesion (both decreased). On the other hand, hormonal treatment mimicking pregnant myometrium (progesterone and 17 $\beta$ -estradiol treatment) decreased the expression of genes involved in biological processes such as smooth muscle contraction, inflammation and apoptosis, and cell adhesion, communication and proliferation, indicating myometrial quiescence. Transcriptomic analysis of the model system mimicking only functional progesterone withdrawal (i.e. co-treatment of progesterone and mifepristone in the *absence* of 17 $\beta$ -estradiol) also altered the expression of genes involved in all biological processes that were identified in treatments mimicking labouring myometrium (i.e. co-treatment of progesterone and mifepristone in the *presence* of 17 $\beta$ -estradiol). However, the numbers of genes identified that were involved in each biological processes were considerably fewer in cells co-treated with progesterone and mifepristone in the absence of 17 $\beta$ -estradiol. It would therefore appear that the changes in these biological processes were less pronounced in the functional progesterone withdrawal study compared to the study mimicking labouring myometrium (functional progesterone withdrawal in the presence of 17 $\beta$ -estradiol). The lesser response in the

## ***Chapter 6: Conclusions and future directions***

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functional progesterone withdrawal study (in the absence of 17 $\beta$ -estradiol) would indicate the important role 17 $\beta$ -estradiol plays in the cell culture system.

Overall, these studies identified several novel genes that were regulated by steroid hormones in the human myometrium. In this cell culture model system, oestrogen, when treated alone or in combination with progesterone, favoured myometrial quiescence. But when progesterone was functionally withdrawn from this co-treatment system (P4+E2+RU486), oestrogen acted as a hormone favouring contractility. This indicates a complex mechanism of action by which oestrogen regulates myometrial contraction that is yet to be understood. Progesterone treatment of cultured hUtSMCs in the absence of 17 $\beta$ -estradiol also altered the transcriptomic profile of the cells favouring quiescence. In line with several studies conducted on non-primates, where removal of progesterone from the system induced labour (Mesiano, 2004, 2007; Mesiano et al., 2002; Mesiano et al., 2011; Mesiano and Welsh, 2007; Pasqualini et al., 1985), functional withdrawal of progesterone by mifepristone in the current model altered biological processes, by altering the expression of genes involved, favouring myometrial contraction. Thus it may be concluded that the human uterine smooth muscle cell culture system developed in this study responds to hormonal treatment in a similar manner to the myometrium *in vivo* making this a suitable model system in which to conduct future studies on various aspects of pregnancy and labour.

Downstream analysis of potential regulators of myometrial function has to be carried out to better understand the exact role of these genes in regulating myometrial contraction. For example, Phospholipase C, beta 1 (PRKCB), phospholipase C, delta 4 (PLCD4), and protein phosphatase 3, regulatory subunit B, beta (PPP3R2) were differentially regulated in human myometrial smooth muscle cells treated with hormones simulating the conditions of labouring myometrium. All three genes are associated with calcium signalling, a main regulator of muscle contraction (Adelstein et al., 1980; Fukata et al., 2001; Morgan and Suematsu, 1990; Sah et al., 2000). PPP3R2 is also involved in gap junction and WNT signalling. These genes have not previously been reported to be associated with pregnancy or labour. Gene silencing or overexpression of these genes in the cultured cell model could provide useful

## *Chapter 6: Conclusions and future directions*

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information on how essential these genes are in the regulation of calcium signalling in myometrial smooth muscle cells.

It must be borne in mind that increased mRNA expression need not necessarily result in increased protein production. Proteins are the chief mediators within the cell that carry out the biological functions, specified by the information encoded in genes (Lodish, 2004). Hence, translation of mRNA to protein is necessary to mediate cellular responses regulated by these genes. It will be very important to verify if the differences in gene expression identified between each treatment is mirrored by changes at the protein level. One approach to investigate this question is to use a multiparallel protein expression profiling system such as an antibody array (Chaga, 2008). In antibody arrays, collections of antibodies are spotted and fixed onto a protein chip for the purpose of detecting proteins of interest. This method detects expression of hundreds of proteins simultaneously from cell lysates and hence comparison of the levels of these proteins with the differentially expressed genes identified in the present study would provide a better understanding of the gene-protein functional connection. Individual Western blot analysis of the proteins expressed by the highest and most relevant differentially expressed genes identified in the present study would be a simpler approach, though would lack the power of the multiparallel approach.

For certain proteins, especially enzymes, post-translational modification regulates their biological functions. One of the most important mechanisms by which a protein is activated or deactivated is by phosphorylation and dephosphorylation, carried out by kinases and phosphatases (Anderson et al., 2006; Becker, 1974; Burnell and Hatch, 1986). At the genomic level, the identification of differential expression of genes encoding for phosphatases and kinases provides insight into the potential activation or deactivation of their target proteins. Identification of substrates for these kinases and phosphatases is important in order to understand the exact role played by these proteins in pregnancy and labour. At the proteomic level, specific antibodies for these substrate proteins could be used to identify their phosphorylation status. In pathway analysis 'Muscle contraction by oxytocin signalling in uterus and mammary gland' was identified as a significant pathway relating to labouring myometrium condition. PRKCB1, which was upregulated (P4+E2+RU486 treatment), is necessary for the

## ***Chapter 6: Conclusions and future directions***

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phosphorylation and activation of its downstream targets. Phosphorylation of c-Raf-1 by PRKCB1 and subsequent phosphorylation of MAP2K2, MAP2K1, ERK1 and ERK2 through c-Raf-1 are key steps for the proper execution of contraction of uterine smooth muscle cells. In addition, phosphorylation of HMGB1 by PRKCB1 is needed for HMGB1 release to the cytosol and subsequent induction of immune responses. The present study is limited to the information it can provide on the differential regulation of genes related to the pathways and cannot indicate the activation/deactivation status of these pathways. Western blot analysis targeted at phosphorylated substrates of the above mentioned kinases should therefore be carried out in order to confirm whether these pathways are activated or not.

KEGG and BioCarta pathway analyses of the differentially expressed genes identified in the present study revealed several signalling, metabolic pathways and apoptotic pathways that may have a potential role in regulating myometrial function. Other pathways identified include cell adhesion, tight junctions, gap junctions, axon guidance and actin cytoskeleton regulation pathways and the coagulation cascade. A detailed understanding of the mechanisms by which the differentially expressed genes regulate each pathway, in the context of myometrial contraction and relaxation, is crucial to identify the exact regulatory role played by these genes in maintaining uterine function. The present study identified only a limited number of genes involved in each pathway, of which some are key regulators. However, it is unclear if the differential expression of the identified genes results in activation or deactivation of the corresponding pathway. Hence thorough analysis of each gene specific to each pathway (key pathways only) is essential. One approach would be to conduct a PCR array analysis of all regulatory genes for a specific pathway. PCR Arrays are reliable tools for analysing the expression of a focused panel of genes (<http://www.superarray.com/manuals/pcrarraydataanalysis.xls>). This would help to identify the expression profile of all the genes in a given pathway. Translation of these findings to the protein level and assaying the posttranslational modification state of key gene products would considerably clarify whether the pathway is activated or deactivated.

Alterations in processes such as cellular adhesion and communication in the myometrium are observed during pregnancy, and reduced cell adhesion is a hallmark

## ***Chapter 6: Conclusions and future directions***

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of smooth muscle contraction and parturition (Dahm and Bowers, 1998; Macphee and Lye, 2000). Several cell adhesion and signalling molecules were differentially expressed in the present study, when hUtSMCs were treated with P4+E2+RU486 (hormonal conditions of labouring myometrium). Even though these genes were differentially expressed, their roles in maintaining cell and adherence junctions are unknown. To better understand cell-cell adhesion and the expression of adherence junction proteins at the cell junctions, immuno-fluorescence staining and confocal microscopy can be carried out. hUtSMCs cultured on coverslips, treated as per the present experimental conditions, can be immune-stained using antibodies against specific cell junction proteins and probed using fluorescent-tagged secondary antibody. A 3D image of immune-stained cells will provide the expression and localisation profile of the specific proteins targeted. This information would provide an insight into the maintenance of cell junctions in response to various hormonal treatments.

Smooth muscle contraction is the most important event in successful labour. Several genes were differentially expressed in hUtSMCs exposed to hormonal conditions of labour, which favour smooth muscle contraction. In addition, hUtSMCs exposed to hormonal conditions of pregnant myometrium ((P4+E2)-treatment) indicated a relaxatory phenotype based on the gene expression profile. Even though the gene expression profile provides an indication about the contractile phenotype of the smooth muscle cells, this needs to be verified. A contraction assay of the smooth muscle cells post-hormonal treatment would help to identify the exact state of contraction in these cells. hUtSMCs can be embedded in collagen or silicon-based 2D or 3D matrigels and treated accordingly with hormones and/or inhibitors (Fitzgibbon et al., 2009). A change in the surface area of the gel can be recorded over time; a reduction in diameter (2D matrigel) or volume (3D matrigel) of the gel indicates contractile phenotype while an increase in the diameter or volume indicates a relaxatory phenotype of the smooth muscle cells (Bell et al., 1979; Kim et al., 2009; Kobayashi et al., 2005). However cultured cells and the corresponding tissue do not necessarily respond in the same way to drug treatment (Jamieson, 1975; Lazarow et al., 1973; Rajan, 1974). It is important to understand the effects of these treatments on myometrial tissue strips to identify whether these molecular functions are regulated in tissue strips in a manner similar to that of cells in culture. Studies using tissue strip

## *Chapter 6: Conclusions and future directions*

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culture in the presence of steroid hormones to understand complex physiological processes like pregnancy, where multifaceted molecular mechanisms regulate the events, would add more value to the limited knowledge currently available in the field.

MicroRNAs (miRNAs) are post-transcriptional regulators that base-pair to complementary sequences in the 3'-untranslated region of target mRNA, resulting in translational repression or targeted degradation and gene silencing (Bartel, 2004, 2009). miRNAs show tissue-specific expression and function, and are evidently expressed in utero-placental tissues (Bentwich et al., 2005). Interest in understanding the role of miRNAs in pregnancy is increasing (Avisar-Whiting et al., 2010; Chim et al., 2008; Enquobahrie et al., 2011; Hu et al., 2011; Li et al., 2011; Maccani et al., 2010; Maccani et al., 2011; Mouillet et al., 2010a; Mouillet et al., 2010b; Pineles et al., 2007). Although this study did not specifically target miRNAs, several miRNAs were identified as differentially expressed in response to steroid hormone treatment. Since the RNA isolation method used in the present study employed a column-based approach, most of the microRNAs would have been lost in the isolation process. Despite this several miRNAs were identified. A more stringent isolation procedure and subsequent analysis to identify differentially expressed miRNAs would provide more detailed results and would shed more light on the target proteins and key pathways. Next-generation sequencing technology can be used to identify novel miRNAs expressed in a particular experiment set (Zhou et al., 2011). This would open new avenues to understand the role of miRNA in regulating myometrial contraction during labour.

Pregnancy is a complex event where the interactions of different cell types (both maternal and foetal) are crucial. Although the myometrium is the most actively contracting tissue in the uterus during pregnancy and labour (Blanks et al., 2007; Grimm, 1977; Huszar and Roberts, 1982), the present model is limited by being a single cell-type culture system. In response to the changing hormonal conditions these uterine smooth muscle cells in culture would be sending and receiving signals to and from neighbouring cells and tissues to activate/deactivate specific physiological processes (e.g. coagulation and immune response). Isolation and analysis of proteins from the culture medium would give insight into the signals that these cells send out.

## ***Chapter 6: Conclusions and future directions***

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2D gel electrophoresis of the concentrated media following various treatments could be compared. Mass spectrometry of isolated proteins from the 2D gel could be used to identify these proteins many of which will be small soluble cytokines, chemokines and interleukins.

Also to understand the role of other cell types in the myometrium during pregnancy and labour similar studies on other components of the myometrium such as stromal cells and vascular cells are necessary. Co-culture experiments can be carried out using different cell types involved in pregnancy and parturition. Using layer-by-layer micropatterned deposition of extracellular matrix components, different cells can be embedded to the culturing surface. This is achieved through an affinity based adhesion mechanism, where cells are attached to polymeric layers that are prepared using electrostatic forces (Fukuda et al., 2006). Membrane inserts, which are coated with extracellular matrix proteins using the above technique, can be used to build layer over layer of cell types to mimic tissues. Another approach is by magnetically labelling cells and embedding them in specific region to be studied using a magnetic field (Ito et al., 2004). hUtSMCs could also be co-cultured with leukocytes and lymphocytes to study the immune and inflammatory response (Duell et al., 2011). In this case, the cell culture media acts as the circulating system mimicking the blood.

In the present study only 17 $\beta$ -estradiol was used as an oestrogen. The role of other oestrogens, especially E3 (the most abundant oestrogen in the maternal system during pregnancy) can also be investigated. In addition to oestrogen and progesterone several other hormones including oxytocin, prostaglandins, CRH and relaxin also play an important role in regulating this complex event. It is therefore difficult to ascribe specific effects of steroid hormones to myometrial gene expression. Hence, a more comprehensive cell culture model system including other hormones would be an advantage in understanding the complex events that regulate labour.

Progesterone and oestrogen exert their actions on the target cells through receptor mediated signalling (Mesiano, 2001, 2004; Mesiano et al., 2002; Mesiano and Welsh, 2007). It has been shown that the expression of these receptors is modulated in human myometrium throughout pregnancy (Mesiano, 2001, 2004; Mesiano et al., 2002; Mesiano and Welsh, 2007). The quiescent state of the myometrium, throughout pregnancy, is regulated by progesterone action (Mesiano, 2001). Unlike in other

## ***Chapter 6: Conclusions and future directions***

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mammals, in humans the circulating levels of progesterone in the maternal system do not drop towards term pregnancy (Mesiano, 2001). Therefore, the myometrial refractiveness to circulating progesterone and responsiveness to oestrogens towards term is believed to be mediated through progesterone receptors (Mesiano, 2001, 2004; Mesiano et al., 2002; Mesiano and Welsh, 2007). In the present study, functional progesterone withdrawal was mimicked by the use of the progesterone antagonist – mifepristone. A more sensitive method to study the effect of progesterone receptors in mediating steroid effects in myometrial cells would be by regulating progesterone receptor expression in the cells. A detailed study by silencing of specific progesterone and/or oestrogen receptor types in hUtSMCs exposed to hormonal conditions would provide a better understanding of how receptor levels control the onset and progress of labour. Since gene-silencing studies are impossible in humans, developing such an experimental model system would provide a key advantage in the study of hormones in relation to pregnancy and parturition.

Steroid hormone receptors, such as those for progesterone and oestrogen, bind to specific sequences in the promoter regions of their target genes called hormone response elements (HREs) (Gruber et al., 2004; Klinge, 2001; Lieberman et al., 1993) (Burns et al., 2011; Klinge, 2001). Promoter analysis of progesterone- and oestrogen-responsive co-regulated genes can identify other common transcription activation sites. Having developed a ‘signature motif’ this allows searching across the entire human genome to identify novel/unreported progesterone- or oestrogen-responsive genes.

The purpose of this study was to develop a cell culture model system to identify the transcriptomic effects of steroid hormone treatment on human myometrial smooth muscle cells. Even though the present cell culture model system is a single cell-type system, the responses of this system to steroid hormone treatment were similar to the results published from *in vivo* studies. The current study thus confirms that the human uterine smooth muscle cell culture system developed here is suitable to study the complex biochemical changes that precede the transformation of the quiescent myometrium to an active and rhythmically contracting tissue during labour.

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## Chapter 7: Bibliography

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# **Appendix 1**

## **Buffers and Master Mixes**

## Polymerase Chain Reaction

<b>1st Strand Master Mix</b>	
<b>Reagents</b>	<b>Volume (µl)</b>
Molecular grade water	2.6
1st Strand buffer	4
DTT	2
dNTPs (10 mM)	1
Superscript II	0.4
<b>PCR Master Mix</b>	
<b>Reagents</b>	<b>Volume (µl)</b>
5X Go Taq PCR buffer (green)	4
10 mM dNTPs	0.75
Forward primer (10 mM)	3
Reverse primer (10 mM)	3
Molecular grade water	7
Taq DNA polymerase (Promega GoTaq)	0.25

<b>Agarose gel electrophoresis</b>	
<b>TAE buffer</b>	
<b>Reagents</b>	<b>Quantity</b>
Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	18.6 g
Distilled water	1 l ml

### Western Blotting

Phosphate buffer Saline (PBS)		
Reagents	Final Concentration (mM)	Weight (g; for 10X)
NaCl	137	80.9
KCl	27	2
Na <sub>2</sub> HPO <sub>4</sub>	10	14.2
KH <sub>2</sub> PO <sub>4</sub>	1.76	2.4

Lysis Buffer		
Stock solution	Volume per 5ml (µl)	Final concentration
Hepes (0.5M; pH 7.5)	200	20
NaCl (1M)	1750	350
MgCl <sub>2</sub> (150mM)	35	1
EDTA (100mM)	25	0.5
EGTA (20mM)	25	0.1
NP40 (10%)	500	1% Igepal-630
H <sub>2</sub> O	2470	

Protease inhibitors		
Stock solution	Volume per ml (µl)	Final concentration
0.5M DTT	1	0.5 mM
PMSF (100mM)	1	1.0 mM
Pepstatin, (1 mg/ml)	1	1.0 mg/ml
Leupeptin (10 mM)	1	10 mM
Aprotinin (2.3 mg/ml)	1	2.5 mg/ml
ALLN (250 mM)	1	250 mM

## Appendix 1

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<b>5X Laemmli buffer</b>	
<b>Reagents</b>	<b>Volume</b>
Tris-HCl (1M, pH6.8)	1.5 ml
SDS	500 mg
Glycerol	1.0 ml
Mercaptoethanol	1.25 ml
PMSF (100mM)	0.25 ml
Bromphenolblue (0.5%)	1.0 ml
Water	Make upto 5 ml

<b>Running buffer</b>		
<b>Reagents</b>	<b>Weight (for 10X; g)</b>	<b>Weight (for 1X; g)</b>
Tris base	31	3.1
Glycine	188	18.8
SDS	10	1
Water	Make upto 1 l	Make upto 1 l

<b>CAPS buffer</b>	
<b>Reagents</b>	<b>For 10X</b>
CAPS	22.13 g
Water	Make upto 1 l (final volume)
pH	Set pH to 11, by adding approx. 10 ml 5 N NaOH

<b>Transfer buffer</b>	
<b>Reagents</b>	<b>Volume (ml)</b>
Water	700
10x CAPS (pH 11)	100
High purity methanol	200

## Appendix 1

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<b>Hormone treatment and concentrations</b>				
	<b>Stock concentration (in DMSO)</b>	<b>Working concentration (in starvation media)</b>	<b>Final concentration</b>	<b>% DMSO</b>
E2	1 M	20 $\mu$ M	10 nM	0.001
P4	1 M	100 $\mu$ M	100 nM	0.001
RU486	20 mg/ml	100 $\mu$ g/ml	1 $\mu$ g/ml	0.005

## **Appendix 2**

Appendix 2

**Table A2.1 Differentially expressed genes identified post-E2 treatment.** FC = Fold change. <sup>1</sup> Highlighted negative value represent downregulation.

Gene Symbol	FC <sup>1</sup> 6 h	FC <sup>1</sup> 24 h	FC <sup>1</sup> 72 h	RefSeq ID	Operon ID	Gene name
TNFSF11	-1.0	-1.7	-1.1	NM_033012	H200011975	tumor necrosis factor (ligand) superfamily, member 11
ZNF3	1.1	-1.6	-1.1	NM_017715	H200018845	zinc finger protein 3 (A8-51)
OR9A4	1.0	-1.9	-1.0	NM_001001656	H300003262	olfactory receptor, family 9, subfamily A, member 4
VSTM5	-1.2	-1.9	-1.0	NM_001144871.1	H300004649	hypothetical LOC387804
SLC35F1	-1.3	-1.7	-1.2	NM_001029858.3	H300005080	solute carrier family 35, member F1
PPP1R1A	1.1	1.8	1.1	NM_006741	H300005331	protein phosphatase 1, regulatory (inhibitor) subunit 1A
GSTTP2	-1.6	4.7	1.2	NR_003082.1	H300009788	similar to RIKEN cDNA 4930583C14
AKT1S1	-1.1	-1.7	-1.7	NM_032375	H300010462	AKT1 substrate 1 (proline-rich)
C8orf44	-1.4	-1.9	-1.3	NM_019607	H300011359	hypothetical protein FLJ11267
EPB41L4A	1.4	4.4	3.4	NM_022140	H300012199	erythrocyte membrane protein band 4.1 like 4A
MCM7	-1.4	-1.9	1.2	NM_182776	H300013915	MCM7 minichromosome maintenance deficient 7 (S. cerevisiae)
ARHGAP32	-1.1	-5.7	1.0	NM_014715	H300014878	Rho GTPase-activating protein
SPHK1	1.1	-2.0	-1.0	NM_021972.3	H300015885	sphingosine kinase 1
ITIH5	1.1	-4.4	1.1	NM_001001851	H300016742	inter-alpha (globulin) inhibitor H5
CD86	1.5	3.4	1.8	NM_006889	opHsV0400000337	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)
KRT222	-1.4	-2.4	-1.2	NM_152349	opHsV0400000340	hypothetical protein MGC45562
CLDN5	-2.2	3.4	1.4	NM_003277	opHsV0400000396	claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)
CDH23	-2.2	-1.7	1.0	NM_052836	opHsV0400000587	cadherin-like 23
IFITM3	-1.0	1.5	1.6	NM_021034	opHsV0400000692	interferon induced transmembrane protein 3 (1-8U)
ADAM28	1.2	-2.1	1.4	NM_021777	opHsV0400000607	a disintegrin and metalloproteinase domain 28
KRTAP10-11	1.1	-2.1	-1.2	NM_198692	opHsV0400000626	keratin associated protein 10-11
C11orf88	-1.0	-2.2	-1.1	NM_207430	opHsV0400000695	FLJ46266 protein
ENO4	1.1	-3.4	-1.0	NM_001242699.1	opHsV0400000773	similar to RIKEN cDNA 6430537H07 gene
LOC391322	1.1	1.9	2.0	NM_001144931.1	opHsV0400000737	similar to D-dopachrome tautomerase (Phenylpyruvate tautomerase II)
LIG3	-1.2	-1.9	-1.1	NM_002311	H300014378	ligase III, DNA, ATP-dependent

Appendix 2

CACNA1G	-1.2	3.9	1.3	NM_198386	opHsV04000068 11	calcium channel, voltage- dependent, alpha 1G subunit
USH1C	1.3	-6.9	-1.2	NM_005709	opHsV04000061 85	Usher syndrome 1C (autosomal recessive, severe)
SLC13A2	-1.6	-1.5	-3.4	NM_003984	H200011166	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
IDS	-1.0	2.1	1.6	NM_006123.4	H300011385	iduronate 2-sulfatase
NR1H4	-2.0	-3.5	1.0	NM_005123	H200007983	nuclear receptor subfamily 1, group H, member 4
C14orf130	-1.4	-1.7	1.0	NM_018108	H300015695	chromosome 14 open reading frame 130
ZMYND11	-1.3	-1.9	-1.7	NM_006624	H300019781	zinc finger, MYND domain containing 11
SRCIN1	1.2	1.5	1.3	NM_025248	H300019925	SNAP25-interacting protein
CXorf56	1.6	1.5	-1.1	NM_022101.3	H300014893	chromosome X open reading frame 56
VCL	-1.0	-1.6	1.2	NM_014000	H300015147	vinculin
SLC18A1	-1.2	-1.4	-1.6	NM_003053	H300020046	solute carrier family 18 (vesicular monoamine), member 1
CCDC99	1.0	1.0	-1.9	NM_017785	H200004166	hypothetical protein FLJ20364
PHF23	-1.2	2.3	1.0	NM_024297	H300018450	hypothetical protein MGC2941
ATP6V1H	1.1	1.5	1.0	NM_213619	H200002840	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
SCML1	1.1	2.7	1.1	NM_006746	H300010551	sex comb on midleg-like 1 (Drosophila)
FAM184B	1.0	-1.8	-1.1	NM_015688.1	H200008606	KIAA1276 protein
ITGB3	-1.1	1.7	1.6	NM_000212	H200010214	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
U2AF2	-1.3	2.1	1.1	NM_007279	H200001469	U2 (RNU2) small nuclear RNA auxiliary factor 2
MTMR1	1.0	-2.4	-1.1	NM_176789	H300021040	myotubularin related protein 1
SLC9A3R2	-1.2	-1.7	-1.5	NM_004785	H200011144	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 2
ANKRD44	1.0	-3.2	-1.1	NM_153697.2	H300011003	ankyrin repeat domain 44
NFYC	-1.2	1.7	1.6	NM_014223.4	H300011779	nuclear transcription factor Y, gamma
GPR116	-1.3	4.3	1.2	NM_015234	H300020010	G protein-coupled receptor 116
NEDD4	1.0	-1.6	-1.2	NM_198400	H200000406	neural precursor cell expressed, developmentally down- regulated 4
NEDD4	-1.1	-2.2	1.1	NM_006154	H300014825	neural precursor cell expressed, developmentally down- regulated 4
SIDT1	-1.1	2.2	1.5	NM_017699	H200011924	SID1 transmembrane family, member 1
FRY	1.2	-2.2	1.1	NM_023037	H300011717	hypothetical protein CG003
NSF	1.0	1.8	1.1	NM_006178	H200011596	N-ethylmaleimide-sensitive factor
NOX3	-1.3	-2.8	-1.2	NM_015718	H200015913	NADPH oxidase 3

Appendix 2

KCNQ2	1.1	1.0	1.5	NM_172109	opHsV04000040 68	potassium voltage-gated channel, KQT-like subfamily, member 2
KLHL20	1.1	1.0	1.6	NM_014458	H200011436	kelch-like ECT2 interacting protein
GTF3C1	-1.1	-1.5	1.1	NM_001520	H300018509	general transcription factor IIIC, polypeptide 1, alpha 220kDa
GTF2I	-1.1	-1.1	-1.5	NM_001518	H200017316	general transcription factor II, i
ITGA8	-1.2	-3.6	-1.0	NM_003638	H200010481	integrin, alpha 8
MAP2	-1.1	-2.1	-1.0	NM_002374.3	H300013992	microtubule-associated protein 2
HOXA9	-1.3	-4.2	-1.5	NM_152739.3	H300014188	homeobox A9
CDC14A	1.6	1.1	1.2	NM_033312	H300008591	CDC14 cell division cycle 14 homolog A (S. cerevisiae)
CADPS2	1.3	3.4	1.4	NM_017954	H300015562	Ca <sup>2+</sup> -dependent activator protein for secretion 2
CCNT2	1.2	1.6	1.4	NM_001241	H300019888	cyclin T2
BCKDHB	-1.2	-1.5	1.1	NM_183050	H200000325	branched chain keto acid dehydrogenase E1, beta polypeptide (maple syrup urine disease)
GCKR	-1.6	-2.3	-1.7	NM_001486	H200010391	glucokinase (hexokinase 4) regulator
ABCB1	-1.2	-3.1	-1.2	NM_000927	H200003004	ATP-binding cassette, sub-family B (MDR/TAP), member 1
TTC39A	-1.4	4.1	-1.3	NM_00108049 4.2	H300019708	chromosome 1 open reading frame 34
CNOT3	1.1	-2.7	-1.2	NM_014516.3	H300017080	CCR4-NOT transcription complex, subunit 3
TMEM40	1.5	2.2	1.3	NM_018306	H300011409	transmembrane protein 40
RBBP9	1.6	2.5	2.1	NM_006606	opHsV04000033 20	retinoblastoma binding protein 9
ANKRD24	-1.2	-2.1	1.3	NM_133475.1	H300010554	ankyrin repeat domain 24
SPG21	-1.1	-1.4	-1.5	NM_016630	H200005745	spastic paraplegia 21 (autosomal recessive, Mast syndrome)
MYO15A	1.3	1.5	1.0	NM_016239	opHsV04000055 03	myosin XVA
ISGF3G	1.1	2.0	1.4	NM_017999	H300018037	ring finger protein 31
MYL6	1.5	1.3	4.0	NM_021019	H300022343	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle
CLSPN	-1.0	2.5	-1.4	NM_022111.3	H200008233	claspin
TGFB2	1.1	-2.2	-1.1	NM_003238	H200007807	transforming growth factor, beta 2
ULK4	-1.1	-1.9	-1.1	NM_017886.2	H300011608	unc-51-like kinase 4 (C. elegans)
NANS	2.2	2.2	-1.2	NM_018946.3	H300010784	N-acetylneuraminic acid synthase
IL11	-1.0	-1.6	-1.0	NM_000641	H200000450	interleukin 11
IL12RB1	-1.2	-1.6	-1.0	NM_005535	H200012331	interleukin 12 receptor, beta 1
ABL1	-1.4	1.0	-1.7	NM_005157.4	H300012008	c-abl oncogene 1, non-receptor tyrosine kinase
SEZ6L	1.1	-1.5	-1.0	NM_021115	H300018283	seizure related 6 homolog (mouse)-like

Appendix 2

SEPT3	-1.1	-1.8	1.3	NM_145733	opHsV0400001608	septin 3
SLC5A1	-1.0	2.3	1.5	NM_000343	H300009809	solute carrier family 5 (sodium/glucose cotransporter), member 1
KDEL3	-1.0	1.2	1.7	NM_016657	opHsV0400006317	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
SMCR7L	-1.2	-3.9	1.1	NM_019008	H300018639	hypothetical protein FLJ20232
APOL1	1.3	-3.7	1.0	NM_145343	H300015933	apolipoprotein L, 1
SAMD15	1.1	1.5	1.2	NM_001010860	opHsV0400008784	similar to CG15625-PA
MMP9	1.5	2.2	2.7	NM_004994	H200013790	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
SGK2	-1.0	-2.6	1.6	NM_170693	H300002272	serum/glucocorticoid regulated kinase 2
DIDO1	1.1	-5.5	1.2	NM_080796;NM_022105;NM_080797	H300022113	death associated transcription factor 1
C20orf59	1.5	4.9	1.5	NM_022082	H300015434	chromosome 20 open reading frame 59
C20orf58	1.4	-3.0	-1.8	NM_152864	H300001409	chromosome 20 open reading frame 58
ARFRP1	1.1	-2.0	-1.8	NM_003224.3	H300015899	ADP-ribosylation factor related protein 1
SEL1L2	-1.0	3.9	2.1	NM_025229.1	H200017169	chromosome 20 open reading frame 50
MYLK2	-1.4	-2.2	-1.7	NM_033118	H200007178	myosin light chain kinase 2, skeletal muscle
VSIG1	1.0	-2.7	1.0	NM_182607	H200015918	V-set and immunoglobulin domain containing 1
SYP	-1.2	3.2	-1.1	NM_003179	H300009929	synaptophysin
TEX28	1.1	-2.2	1.2	NM_001586	opHsV0400001904	chromosome X open reading frame 2
KLHL4	-1.1	1.8	-1.5	NM_019117	opHsV0400005771	kelch-like 4 (Drosophila)
ITIH5L	-1.1	-2.1	-1.0	NM_198510	H200015881	inter-alpha (globulin) inhibitor H5-like
DGKH	-1.0	2.4	1.1	NM_178009	H300019576	diacylglycerol kinase, eta
MAPK3	-1.0	1.6	1.2	NM_002746	H300019761	mitogen-activated protein kinase 3
CCL22	2.6	2.2	1.1	NM_002990	H300007445	chemokine (C-C motif) ligand 22
ABCC1	1.1	-2.0	-1.2	NM_019899	opHsV0400004193	ATP-binding cassette, subfamily C (CFTR/MRP), member 1
FBXO31	-1.0	8.4	-1.1	NM_024735.3	H300014448	F-box protein 31
FAM38A	-1.1	2.8	2.2	NM_014745	H200010190	family with sequence similarity 38, member A
PIEZO1	-1.0	2.3	1.3	NM_001142864.2	opHsV0400001419	piezo-type mechanosensitive ion channel component 1
LILRA1	-2.0	1.0	-2.2	NM_006863.1	H300017289	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 1
URI1	-1.6	-2.0	-1.4	NM_003796.3	H300016965	prefoldin-like chaperone

Appendix 2

SULT2A1	1.1	4.2	-1.1	NM_003167	H200006859	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1
PTPRS	1.0	-1.9	1.0	NM_130854	H300019753	protein tyrosine phosphatase, receptor type, S
TFPI2	1.1	-1.3	-1.7	NM_006528	H200009647	tissue factor pathway inhibitor 2
PLEKHA8	1.1	1.6	1.3	NM_032639	opHsV0400000257	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8
PTCD1	-1.1	-2.1	-1.1	NM_015545.3	H200019287	pentatricopeptide repeat domain 1
POU6F2	-1.1	-2.1	-1.2	NM_007252	H200015474	POU domain, class 6, transcription factor 2
TBL2	1.4	3.7	1.0	NM_032988	H300020343	transducin (beta)-like 2
ELAVL2	-1.0	2.6	2.1	NM_004432	H200007658	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)
KRT23	-1.1	-1.8	-2.3	NM_015515	H200001726	keratin 23 (histone deacetylase inducible)
GIT1	-1.4	-14.3	1.3	NM_014030	opHsV04000002936	G protein-coupled receptor kinase interactor 1
FAM18B2	1.5	3.1	1.6	NM_145301	H300001507	similar to CGI-148 protein
RAI1	-1.5	-2.2	-2.3	NM_030665	H300017048	retinoic acid induced 1
SYNGR2	-1.2	-1.5	1.0	NM_004710	H300017048	synaptogyrin 2
ABI3	-1.5	-2.1	-1.2	NM_016428	H200012916	ABI gene family, member 3
SMURF2	-1.2	3.4	1.1	NM_022739	opHsV04000000301	SMAD specific E3 ubiquitin protein ligase 2
NRXN2	1.0	-2.1	-1.1	NM_015080	H200012485	neurexin 2
CD5	-1.2	-3.2	1.5	NM_014207	H200005344	CD5 antigen (p56-62)
CALCA	-1.5	-1.7	1.2	NM_001741	H300018828	calcitonin/calcitonin-related polypeptide, alpha
VWF	-1.0	-2.1	1.3	NM_000552.3	H300013679	von Willebrand factor
FZD10	1.0	3.5	1.1	NM_007197	H300002878	frizzled homolog 10 (Drosophila)
CPSF6	-1.0	-1.5	-2.1	NM_007007.2	H300012867	cleavage and polyadenylation specific factor 6, 68kDa
UHRF1BP1L	-1.0	-1.7	-1.3	NM_001006947	H300020450	KIAA0701 protein
C6orf60	2.5	2.7	1.5	NM_024581	H300022686	chromosome 6 open reading frame 60
CRSP3	-1.0	-1.8	-1.2	NM_004830	H200010141	cofactor required for Sp1 transcriptional activation, subunit 3, 130kDa
LAMA4	1.2	1.9	1.0	NM_002290.4	H300011178	laminin, alpha 4
RNF130	1.1	-1.1	-1.9	NM_018434	H200011196	ring finger protein 130
CNOT6	1.4	-1.7	-1.4	NM_015455	H200001104	carbon catabolite repression 4 protein
PRLR	-1.1	-2.9	-1.6	NM_000949	H300022611	prolactin receptor
KNG1	-1.1	-5.3	1.3	NM_000893.3	H300012555	kininogen 1
PFKFB4	-1.4	-1.9	-1.1	NM_004567	H200014799	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
PLCH1	-1.1	-1.9	-1.6	NM_014996.2	H300017390	phospholipase C, eta 1
CENPA	1.2	2.0	1.1	NM_017877	opHsV04000005120	chromosome 2 open reading frame 18

Appendix 2

ACADL	-1.6	-1.9	-1.2	NM_001608	H200000312	acyl-Coenzyme A dehydrogenase, long chain
TPO	-1.2	2.0	-1.3	NM_175721	H300022041	thyroid peroxidase
KYNU	1.5	1.2	1.6	NM_003937	H300017766	kynureninase (L-kynurenine hydrolase)
PAPPA2	-3.4	-3.8	-1.1	NM_021936.2	opHsV0400012424	pappalysin 2
KIAA1324	-1.1	2.1	2.2	NM_020775	opHsV0400000123	maba1
OPRD1	-1.5	-1.6	-1.7	NM_000911	H200000100	opioid receptor, delta 1
AKT3	1.6	-5.2	-2.3	NM_181690	opHsV04000003020	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)
RGS4	-1.2	1.0	-2.1	NM_005613	H200015387	regulator of G-protein signalling 4
C1orf181	1.0	1.5	-1.5	NM_017953	H300008315	hypothetical protein FLJ20729
KIF17	1.1	2.5	2.1	NM_020816	H300013228	kinesin family member 17
NSL1	-1.5	-2.1	1.5	NM_001042549.1	opHsV0400011935	MIND kinetochore complex component, homolog (S. cerevisiae)
STAG1	1.1	3.2	1.0	NM_005862	H300018107	stromal antigen 1
APOA1	1.6	1.5	1.3	NM_000039	H200010566	apolipoprotein A-I
RPS25	1.0	2.3	1.5	NM_001028	H300006732	ribosomal protein S25
ABCG2	-1.1	-2.8	1.4	NM_004827	H200014669	ATP-binding cassette, subfamily G (WHITE), member 2
RAB3GAP2	1.1	2.2	-1.2	NM_012414	H300018974	rab3 GTPase-activating protein, non-catalytic subunit (150kD)
TNFSF18	-1.0	-2.2	-1.1	NM_005092	H200016118	tumor necrosis factor (ligand) superfamily, member 18
KCNJ5	1.1	-5.3	1.1	NM_000890	H200014536	potassium inwardly-rectifying channel, subfamily J, member 5
KIAA1217	-1.2	-1.5	-1.5	NM_019590	H300022108	KIAA1217
PLS1	-1.6	2.7	-1.1	NM_002670.2	H300019956	plastin 1
TNFRSF8	-1.5	-1.7	-1.0	NM_152942	H200000344	tumor necrosis factor receptor superfamily, member 8
FYTTD1	1.1	1.7	1.0	NM_032288	H200008268	forty-two-three domain containing 1
MYOG	-1.0	-3.1	-1.1	NM_002479	H300009924	myogenin (myogenic factor 4)
OPN4	-1.0	-1.6	-1.1	NM_033282.3	H300014263	opsin 4
ZNF644	1.3	1.2	1.9	NM_201269	H300019034	zinc finger protein 644
TWIST1	1.1	1.6	-1.0	NM_000474	H200010175	twist homolog 1 (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) (Drosophila)
ECD	-1.4	-1.2	-1.6	NM_007265	H200002844	suppressor of S. cerevisiae gcr2
CCDC70	-1.2	-1.7	1.0	NM_031290	H300010105	hypothetical protein DKFZp434K1172
ITIH5	1.1	-3.4	1.2	NM_030569	H300015449	inter-alpha (globulin) inhibitor H5
SEMG1	-1.3	-1.6	-1.1	NM_198139	opHsV0400004082	semenogelin I
TREM1	1.1	2.0	1.0	NM_018643	H200017640	triggering receptor expressed on myeloid cells 1

Appendix 2

ATP5S	-1.1	2.9	1.1	NM_00100380 3.2	H300017867	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit s (factor B)
KIR2DL1	1.2	-1.6	-2.6	NM_012313	H300021830	killer cell immunoglobulin- like receptor, two domains, short cytoplasmic tail, 3
TXNDC13	-1.4	-2.0	-1.1	NM_021156	opHsV04000064 00	hypothetical protein DJ971N18.2
CEP250	-1.7	-3.3	-1.4	NM_007186	opHsV04000052 46	centrosomal protein 2
XRCC3	1.7	2.1	1.9	NM_005432	H200010988	X-ray repair complementing defective repair in Chinese hamster cells 3
PRRG2	-1.1	-1.7	-1.0	NM_000951	H200004292	proline rich Gla (G- carboxyglutamic acid) 2
HIVEP3	-1.7	-1.7	-1.0	NM_024503	H300002950	human immunodeficiency virus type I enhancer binding protein 3
TAS2R5	-1.2	-4.5	1.2	NM_018980	H200016897	taste receptor, type 2, member 5
OR1E2	1.2	-2.5	1.2	NM_003554	H300005519	olfactory receptor, family 1, subfamily E, member 2
LRRC4	-1.4	-1.7	-1.6	NM_022143	H200011588	leucine rich repeat containing 4
IRF5	1.0	-2.1	-1.2	NM_002200	H200019515	interferon regulatory factor 5
ALDH1A2	-1.3	-1.2	-1.6	NM_003888	H200010688	aldehyde dehydrogenase 1 family, member A2
MPDU1	1.6	-2.7	-1.2	NM_004870.3	H300017103	mannose-P-dolichol utilization defect 1
SIGLEC9	1.3	2.5	1.8	NM_014441	H200015832	sialic acid binding Ig-like lectin 9
GALNT8	-1.2	-2.5	-1.2	NM_017417	H200018218	UDP-N-acetyl-alpha-D- galactosamine:polypeptide N- acetylgalactosaminyltransf erase 8 (GalNAc-T8)
C9orf121	-1.1	1.5	1.5	NM_145283	H300008573	chromosome 9 open reading frame 121
NXNL2	1.2	1.8	1.7	NM_145283.2	opHsV04000001 99	nucleoredoxin-like 2
CYorf15B	1.2	-2.3	-1.3	NM_032576	H200014065	chromosome Y open reading frame 15B
ZNF141	-1.2	-1.7	-2.2	NM_003441	H200014583	zinc finger protein 141 (clone pHZ-44)
AOC3	1.6	1.8	1.0	NM_003734	H200014785	amine oxidase, copper containing 3 (vascular adhesion protein 1)
TNS4	-1.4	-1.6	1.1	NM_032865	H200009623	C-terminal tensin-like
LHX1	-1.4	-2.1	-1.1	NM_005568	H200007266	LIM homeobox 1
MUTYH	1.1	-1.5	1.1	NM_012222	H200016735	mutY homolog (E. coli)
RNF17	-1.0	4.3	1.3	NM_031277	H200019387	ring finger protein 17
CHRM3	-4.8	-8.2	-1.3	NM_000740.2	opHsV04000123 28	cholinergic receptor, muscarinic 3
TMCC2	1.0	-1.7	-1.0	NM_014858.3	opHsV04000020 47	transmembrane and coiled- coil domain family 2
RXFP2	-1.6	-1.9	-1.3	NM_130806	H300002851	leucine-rich repeat- containing G protein- coupled receptor 8
KL	2.0	10.8	1.2	NM_004795	H200010653	klotho
TBC1D8B	1.6	1.1	1.5	NM_017752	H300016076	FLJ20298 protein
EBF2	-1.4	3.1	1.1	NM_022659	H200013635	early B-cell factor 2

Appendix 2

VAV3	-1.8	-1.7	-1.6	NM_006113	H200016605	vav 3 oncogene
CMPK2	-1.3	-1.6	-1.4	NM_207315.3	H200001373	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial
SAA1	1.0	2.5	1.9	NM_199161.3	H300009198	serum amyloid A1
IL6ST	1.6	1.1	1.1	NM_175767.2	opHsV0400012641	interleukin 6 signal transducer (gp130, oncostatin M receptor)
TIMM17A	-1.1	1.8	1.1	NM_006335	H200002924	translocase of inner mitochondrial membrane 17 homolog A (yeast)
GRP	1.1	-1.6	-1.5	NM_002091	H200000383	gastrin-releasing peptide
PHC2	-1.0	1.8	1.1	NM_004427	H200007617	polyhomeotic-like 2 (Drosophila)
DSG3	1.2	-2.9	-1.4	NM_001944	H200000493	desmoglein 3 (pemphigus vulgaris antigen)
PION	-1.2	2.0	1.2	NM_017439.3	H200002213	hypothetical protein LOC54103
CCDC146	1.3	1.9	2.2	NM_020879	H300010610	KIAA1505 protein
CSN2	1.3	2.6	1.1	NM_001891	H200000566	casein beta
AMHR2	-1.3	-4.3	-1.3	NM_020547	H200012416	anti-Mullerian hormone receptor, type II
SLC26A10	-1.6	-1.1	-1.7	NM_133489.2	H300016043	solute carrier family 26, member 10
PCNXL2	-1.0	-2.0	-1.1	NM_014801.3	H300021793	pecanex-like 2 (Drosophila)
CHRND	-1.1	-2.2	1.0	NM_000751	H200011037	cholinergic receptor, nicotinic, delta polypeptide
TMBIM1	1.2	-1.1	-2.2	NM_022152	H200014249	PP1201 protein
ARMC9	-1.1	1.7	1.3	NM_025139.3	opHsV0400009907	hypothetical gene supported by BC017229
TNS3	1.3	3.5	1.1	NM_022748	H300020339	tensin-like SH2 domain containing 1
CIDEB	-1.2	-1.8	1.1	NM_014430	H200009376	cell death-inducing DFFA-like effector b
BRIP1	1.0	7.4	-1.5	NM_032043	H200010810	BRCA1 interacting protein C-terminal helicase 1
RALGPS1	-1.8	-7.4	-2.1	NM_014636	H300019463	Ral GEF with PH domain and SH3 binding motif 1
DPM2	1.1	2.1	1.0	NM_152690	H300011976	dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit
NR5A1	-1.1	-2.4	-1.0	NM_004959	H200007234	nuclear receptor subfamily 5, group A, member 1
TTPA	-1.4	-1.7	-1.4	NM_000370	H200005658	tocopherol (alpha) transfer protein (ataxia (Friedreich-like) with vitamin E deficiency)
TMPRSS4	1.0	1.0	1.6	NM_019894	H200005518	transmembrane protease, serine 4
NOX5	1.0	-1.8	-1.4	NM_024505	opHsV0400006445	NADPH oxidase, EF hand calcium-binding domain 5
C15orf15	-1.8	2.0	1.1	NM_016304	H200008739	chromosome 15 open reading frame 15
CYP2C8	-1.3	2.0	1.2	NM_000770.3	H300012156	cytochrome P450, family 2, subfamily C, polypeptide 8
ZNF365	-1.1	-3.0	-1.3	NM_199452	opHsV0400004100	zinc finger protein 365
TMTC3	-1.1	-2.0	-1.0	NM_181783	H300018325	SMILE protein
CBLN3	1.1	-2.2	1.0	NM_001039771.2	H300008761	cerebellin 3 precursor

Appendix 2

ZADH1	1.0	-1.7	-1.1	NM_152444	opHsV04000066 15	zinc binding alcohol dehydrogenase, domain containing 1
DUOX2	-1.2	-1.6	1.1	NM_014080	H200005741	dual oxidase 2
FANCI	-1.3	2.9	1.1	NM_018193.2	H300015476	Fanconi anemia, complementation group I
SH3GL3	-1.0	-2.1	1.1	NM_003027	H300020141	SH3-domain GRB2-like 3
TAF4B	-1.2	-2.9	-1.5	NM_005640.1	H200004902	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa
C18orf43	1.1	2.4	1.1	NM_006553	H300013095	chromosome 18 open reading frame 43
MEP1B	-1.7	-1.6	1.2	NM_005925	H200014690	mep1in A, beta
CARD14	-1.5	1.7	-1.3	NM_052819	H300016444	caspase recruitment domain family, member 14
FAM125A	1.0	1.3	1.5	NM_138401	H200011457	hypothetical protein BC011840
TRPM2	-1.2	-6.1	-1.2	NM_00100118 8	H300013011	transient receptor potential cation channel, subfamily M, member 2
DOPEY2	-1.1	-1.4	-1.6	NM_005128	H200012853	chromosome 21 open reading frame 5
CYR61	-1.0	2.1	1.5	NM_001554	H300020208	cysteine-rich, angiogenic inducer, 61
PTPRF	-1.0	-8.9	1.4	NM_130440.2	opHsV04000027 17	protein tyrosine phosphatase, receptor type, F
LHX9	-1.5	-1.3	-3.1	NM_020204	H300004733	LIM homeobox 9
CTSK	-1.1	2.0	-1.5	NM_000396	H200007076	cathepsin K (pyncnodysostosis)
POGZ	1.3	1.9	2.8	NM_145796	H300011834	pogo transposable element with ZNF domain
GABPB2	-1.1	-1.0	-1.6	NM_144618	H300005454	hypothetical protein MGC29891
VASH2	1.1	-1.5	-1.3	NM_024749	H200012574	hypothetical protein FLJ12505
ANKMY1	1.1	-2.0	1.4	NM_016552	H300020278	ankyrin repeat and MYND domain containing 1
EAF1	-1.6	-2.2	-1.1	NM_033083	H200020512	ELL associated factor 1
ZNF660	-1.2	-2.0	-1.3	NM_173658	H300001854	hypothetical protein FLJ36870
COL8A1	1.1	-1.2	-2.0	NM_001850	H200011925	collagen, type VIII, alpha 1
PLAC8	1.0	1.3	3.0	NM_016619	H300020304	placenta-specific 8
FAM105A	1.1	1.5	1.4	NM_019018	H200010476	hypothetical protein FLJ11127
FEM1C	1.3	-1.3	-3.3	NM_020177	H200004840	fem-1 homolog c (C.elegans)
FARS2	-1.0	-1.8	-1.0	NM_006567	H300013935	phenylalanine-tRNA synthetase 2 (mitochondrial)
C6orf170	1.0	2.1	1.3	NM_152730.4	H200007216	chromosome 6 open reading frame 170
IBRDC1	-1.1	-1.7	1.1	NM_152553	H200018794	IBR domain containing 1
ZNF673	1.2	1.1	1.5	NM_017776	opHsV04000040 47	hypothetical protein FLJ20344
MAGEA4	1.1	5.3	-1.1	NM_002362	opHsV04000060 83	melanoma antigen family A, 4
CSGALNAC T1	-1.9	-1.8	-1.0	NM_018371	opHsV04000063 47	chondroitin beta1,4 N- acetylgalactosaminyltransf erase
CRH	-1.1	-2.4	-2.5	NM_000756	H200006062	corticotropin releasing hormone

Appendix 2

ADAMTSL1	1.0	1.6	1.1	NM_052866	H200017196	ADAMTS-like 1
WDR31	1.1	1.8	2.0	NM_001006615	opHsV0400006012	WD repeat domain 31
NACC2	-1.2	2.2	-1.1	NM_144653.4	opHsV0400011283	NACC family member 2, BEN and BTB (POZ) domain containing
TAF5	1.1	2.2	-1.9	NM_006951	opHsV0400004259	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa
KIAA1755	-2.2	-2.3	-1.1	NM_001029864.1	H200011905	KIAA1755 protein
TAOK2	-1.5	-1.4	-1.1	NM_016151	H200005599	TAO kinase 2
OR5M9	1.1	1.7	1.1	NM_001004743	H300004494	olfactory receptor, family 5, subfamily M, member 9
PRSS23	-1.4	-1.2	-2.1	NM_007173	H200003514	protease, serine, 23
C11orf53	-1.1	-1.9	-1.4	NM_198498	H300005050	Similar to RIKEN cDNA 1810046K07 gene
THRB	1.1	-1.8	1.2	NM_000461.4	opHsV0400008577	hypothetical gene supported by AK096885; AK098084
PLBD2	-1.1	-1.6	1.2	NM_173542	H300000054	hypothetical protein LOC196463
VIPAS39	1.0	-2.4	1.0	NM_022067.3	H300012095	VPS33B interacting protein, apical-basolateral polarity regulator, spe-39 homolog
PTPRO	-1.6	-1.8	1.3	NM_002848	H200016468	protein tyrosine phosphatase, receptor type, O
AMN1	1.8	1.6	1.2	NM_207337	H300016757	hypothetical protein LOC196394
BICD1	-1.0	3.6	1.5	NM_001003398	opHsV0400005965	bicaudal D homolog 1 (Drosophila)
GABRA2	-1.3	-2.3	-1.1	NM_000807	H200010483	gamma-aminobutyric acid (GABA) A receptor, alpha 2
CCDC75	-2.0	-4.3	-1.8	NM_174931	H300001543	hypothetical protein FLJ38348
FAM151B	-1.1	2.8	-1.1	NM_205548	H300007982	AASA9217
MEPE	-1.7	-2.2	-2.4	NM_020203	H300011412	matrix, extracellular phosphoglycoprotein with ASARM motif (bone)
IFLTD1	-1.3	-1.6	1.4	NM_152590	H300005591	hypothetical protein FLJ36004
C13orf16	3.4	3.0	1.3	NM_152324	H300006701	hypothetical protein MGC35169
CD8A	1.6	-5.5	-2.0	NM_001768	H200007145	CD8 antigen, alpha polypeptide (p32)
TUBGCP5	1.8	1.3	1.2	NM_052903	H300017697	tubulin, gamma complex associated protein 5
LGI4	-1.1	-1.7	-1.3	NM_139284	H300020538	leucine-rich repeat LGI family, member 4
AK5	1.0	3.0	-1.0	NM_174858.2	H300013247	adenylate kinase 5
ROBO4	-1.2	1.3	-1.7	NM_019055.5	H300016925	roundabout, axon guidance receptor, homolog 4 (Drosophila)
FAM134B	1.1	1.8	2.2	NM_019000	H200006918	hypothetical protein FLJ20152
LASS3	-4.8	-2.6	-1.0	NM_178842	opHsV0400006727	LAG1 longevity assurance homolog 3 (S. cerevisiae)
ABCA10	1.2	3.2	-1.2	NM_080282	H200017657	ATP-binding cassette, subfamily A (ABC1), member 10

Appendix 2

CNTNAP3	-1.1	2.4	1.3	NM_033655.3	H300007870	similar to cell recognition molecule CASPR3
OXNAD1	1.0	-2.6	1.0	NM_138381	opHsV0400000503	hypothetical protein BC008322
USP25	3.7	1.5	2.1	NM_013396.3	H300012111	ubiquitin specific peptidase 25
TTN	1.4	3.0	2.8	NM_133378	opHsV0400003863	titin
SPAG17	-1.5	-3.2	-1.4	NM_206996	H300017001	projection protein PF6
ALX3	-1.0	-1.0	-1.6	NM_006492	opHsV0400000514	aristaless-like homeobox 3
RPGR	-2.4	-1.1	-1.3	NM_001034853.1	H300010420	retinitis pigmentosa GTPase regulator
SORCS3	1.2	2.1	1.1	NM_014978	H300005735	sortilin-related VPS10 domain containing receptor 3
SFXN2	-1.0	-2.4	-1.5	NM_178858	H300013605	sideroflexin 2
ITGAD	1.2	-3.6	-1.2	NM_005353.2	H300020144	integrin, alpha D
SST	-1.3	-1.8	-2.3	NM_001048	H200002126	somatostatin
TSC22D3	1.1	-2.9	1.1	NM_004089	H300015152	delta sleep inducing peptide, immunoreactor
C21orf25	-1.0	-4.0	-1.1	NM_199050	H300002832	chromosome 21 open reading frame 25
SNX22	-1.4	-2.7	-1.1	NM_024798	H200007271	sorting nexin 22
FMNL2	1.2	-2.5	-1.2	NM_001004417	H300011094	formin-like 2
LDLRAP1	-1.1	-1.7	1.2	NM_015627	H300002046	LDL receptor adaptor protein
EDA	-1.1	2.7	-1.2	NM_001005609	H300004287	ectodysplasin A
C21orf66	1.1	1.8	1.2	NM_058191	opHsV0400003750	chromosome 21 open reading frame 66
GJD2	-2.7	-2.7	-3.4	NM_020660	H200017836	connexin-36
CES7	1.5	4.8	1.1	NM_145024	H200020805	hypothetical protein FLJ31547
ABR	1.0	-2.6	-1.2	NM_001092	H300013539	active BCR-related gene
ZNF230	1.0	-3.7	-1.9	NM_006300	opHsV0400006207	zinc finger protein 230
ABCF3	1.2	-1.0	-1.9	NM_018358	H200010480	ATP-binding cassette, sub-family F (GCN20), member 3
DISC1	1.2	2.3	1.7	NM_001012958	H300010967	Homo sapiens disrupted in schizophrenia 1 (DISC1), transcript variant Es, mRNA
DUSP19	-1.4	1.7	-1.2	NM_080876	H300020839	dual specificity phosphatase 19
PCDP1	-1.0	-1.8	-1.1	NM_001029996.3	H300000411	similar to hypothetical protein
MRPS18C	1.0	1.7	1.0	NM_016067	opHsV0400006299	mitochondrial ribosomal protein S18C
HIPK1	-1.1	1.8	1.3	NM_152696	H300017147	homeodomain interacting protein kinase 1
LRRC58	1.1	-3.3	-1.8	NM_001099678.1	H200020562	hypothetical protein LOC116064
ELF3	1.1	-2.6	-1.1	NM_004433.4	H200007656	E74-like factor 3 (ets domain transcription factor, epithelial-specific )
CCDC141	-1.0	1.7	1.0	NM_173648.3	H300009725	coiled-coil domain containing 141
CTLA4	-1.0	2.0	-1.1	NM_005214	H300020878	cytotoxic T-lymphocyte-associated protein 4

Appendix 2

PPP4R2	-1.3	-1.7	-1.2	NM_174907.2	opHsV04000015 91	protein phosphatase 4, regulatory subunit 2
CD200R1	-1.2	-2.2	1.1	NM_170780	H300020879	CD200 receptor 1
CDCP1	-1.1	-1.6	-1.4	NM_022842	opHsV04000067 07	CUB domain-containing protein 1
GRM2	-1.3	-2.2	1.1	NM_000839	H200012321	glutamate receptor, metabotropic 2
F2RL1	1.2	-1.7	-1.1	NM_005242	H200013958	coagulation factor II (thrombin) receptor-like 1
STXBP5	-1.1	-1.1	-1.6	NM_139244	H200014270	syntaxin binding protein 5 (tomosyn)
KIAA0895	-1.1	6.6	-1.1	NM_015314	H300020950	KIAA0895 protein
SLC13A4	-2.6	-1.4	-1.9	NM_012450	H200001741	solute carrier family 13 (sodium/sulfate symporters), member 4
PGAM2	1.3	-2.5	-2.5	NM_000290	H200004756	phosphoglycerate mutase 2 (muscle)
EN2	-1.3	-2.3	-1.3	NM_001427	opHsV04000060 57	engrailed homolog 2
ANKS6	-1.0	-3.1	-1.0	NM_173551	opHsV04000066 60	sterile alpha motif domain containing 6
AQP3	1.1	1.6	1.2	NM_004925	opHsV04000039 95	aquaporin 3
FOLR2	1.1	3.2	1.1	NM_000803	H300020999	folate receptor 2 (fetal)
PCF11	1.0	-2.0	-1.8	NM_015885	H300021003	pre-mRNA cleavage complex II protein Pcf11
C10orf25	-1.1	-1.5	-1.5	NM_00103938 0.2	opHsV04000102 21	chromosome 10 open reading frame 25
ZNF503	-1.1	2.2	1.3	NM_032772	H300021010	zinc finger protein 503
RAG1	-1.1	-1.7	-1.1	NM_000448	opHsV04000006 79	recombination activating gene 1
PKD1L2	1.1	1.8	-1.2	NM_052892	H300002702	polycystic kidney disease 1-like 2
ACSM1	2.0	-7.1	1.2	NM_052956	H300011500	butyryl Coenzyme A synthetase 1
GLYATL1	-1.9	2.2	1.3	NM_080661	H300021086	similar to RIKEN cDNA 0610008P16 gene
RCCD1	1.2	-1.3	-1.8	NM_00101791 9.1	H200002420	similar to cyclin-E binding protein 1 (H. sapiens)
CCDC103	-1.3	1.0	-1.6	NM_213607	opHsV04000069 30	similar to RIKEN 4933439F11
GOLGA6B	1.5	6.7	1.2	NM_018652.4	opHsV04000005 54	similar to Golgi autoantigen, golgin subfamily A member 6 (Golgin linked to PML) (Golgin-like protein)
OR8J3	-1.1	1.8	-1.1	NM_00100406 4	H300007016	olfactory receptor, family 8, subfamily J, member 3
CD300A	-1.1	-2.8	1.1	NM_007261	H300021149	leukocyte membrane antigen
TMEM88	1.1	-2.1	-1.0	NM_203411	H300005680	similar to RIKEN cDNA 2600017H02
OR2C1	1.2	-2.5	1.3	NM_012368	opHsV04000062 52	olfactory receptor, family 2, subfamily C, member 1
MYL1	1.2	5.3	-1.3	NM_079422	H300021245	myosin, light polypeptide 1, alkali; skeletal, fast
COL3A1	1.1	2.1	1.1	NM_000090	opHsV04000058 87	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
AXIN2	1.2	-1.5	-1.2	NM_004655	H200012672	axin 2 (conductin, axil)
GPRIN1	-1.1	-2.3	1.1	NM_052899	H300004321	G protein-regulated inducer of neurite outgrowth 1

Appendix 2

PDILT	-1.1	-3.3	-1.2	NM_174924	H300001483	protein disulfide isomerase-like protein of the testis
SLC33A1	1.2	1.7	1.3	NM_004733	H200008817	solute carrier family 33 (acetyl-CoA transporter), member 1
HTRA4	-1.4	-1.3	-1.8	NM_153692	H300008859	HtrA serine peptidase 4
GUSB	1.6	-1.1	1.5	NM_000181.2	opHsV0400002519	zinc finger protein 479
PFKFB3	-2.6	1.3	-2.0	NM_004566	opHsV0400005632	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
FOXA3	1.5	1.5	1.1	NM_004497	H200004320	forkhead box A3
SLC26A5	-1.0	-6.8	1.8	NM_198999	H300009706	prestin (motor protein)
FPRL1	-1.5	4.8	-1.2	NM_001462	H200011000	formyl peptide receptor-like 1
JAGN1	1.1	-1.1	-2.0	NM_032492	H200003360	jagunal homolog 1 (Drosophila)
KRT38	-1.3	2.2	1.0	NM_006771	H200016113	keratin, hair, acidic, 8
CLCN5	-1.2	-1.7	-1.1	NM_000084.2	opHsV0400010489	chloride channel, voltage-sensitive 5
ZNF318	-1.7	-2.1	-1.3	NM_014345	H300021241	zinc finger protein 318
TIGD1	1.1	-2.9	-1.1	NM_145702	H200021092	tigger transposable element derived 1
PTCRA	-3.2	-1.0	-1.0	NM_138296	H300015807	pre T-cell antigen receptor alpha
LRRC34	-1.0	2.7	-1.0	NM_153353	H300021228	hypothetical protein MGC27085
TXNL6	1.0	-2.8	1.3	NM_138454	H200020207	thioredoxin-like 6
OR10H3	1.0	1.7	1.2	NM_013938	H300003383	olfactory receptor, family 10, subfamily H, member 3
IL17D	1.0	-1.2	-1.6	NM_138284	H300008939	interleukin 17D
MUS81	-1.1	-1.1	-1.6	NM_025128	H200009361	MUS81 endonuclease homolog (yeast)
RAB37	2.0	-1.1	12.1	NM_175738	H300021438	RAB37, member RAS oncogene family
NBEA	1.0	-3.3	1.2	NM_015678	H300019586	neurobeachin
BNC2	-1.3	-2.1	-2.2	NM_017637.5	H300012571	basonuclin 2
LTA	1.1	-1.8	-1.5	NM_000595	H200000010	lymphotoxin alpha (TNF superfamily, member 1)
C2orf70	-1.2	3.5	1.3	NM_001105519.1	opHsV0400001850	hypothetical protein LOC339778
GPR113	-1.2	-2.5	-1.1	NM_153835	H300009856	G protein-coupled receptor 113
TAS1R1	-1.1	2.6	-1.3	NM_138697	H300022659	taste receptor, type 1, member 1
XXYLT1	-1.2	-2.4	-1.0	NM_152531.4	H300012101	xyloside xylosyltransferase 1
RGMB	-1.0	-1.1	-1.9	NM_001012761	opHsV0400011190	Homo sapiens RGM domain family, member B (RGMB), transcript variant 1, mRNA
OR7D4	-1.3	-1.6	-1.3	NM_001005191	H300009010	olfactory receptor, family 7, subfamily D, member 4
FAM116A	1.1	1.7	1.4	NM_152678	H300011067	hypothetical protein FLJ34969
CDRT4	1.2	1.6	1.0	NM_173622	H300005963	hypothetical protein FLJ36674
UCP3	1.1	2.5	1.3	NM_022803	H300012387	uncoupling protein 3 (mitochondrial, proton carrier)
FOSL1	-1.5	-1.4	-2.1	NM_005438	H200017744	FOS-like antigen 1

Appendix 2

ZNF135	-1.1	-2.5	1.1	NM_003436.3	opHsV04000007 40	zinc finger protein 135
GJE1	1.1	-2.3	-1.3	NM_181538	H300004421	gap junction protein, epsilon 1, 29kDa
WDR25	1.1	2.1	-1.0	NM_024515	opHsV04000029 83	pre-mRNA splicing factor- like
C15orf56	-1.2	-2.3	-1.1	NM_00103990 5.1	H300004970	chromosome 15 open reading frame 56
MAMSTR	1.0	1.1	1.5	NM_182574	opHsV04000067 66	hypothetical protein FLJ36070
OR2M7	-1.2	-2.7	-1.2	NM_00100469 1	H300007742	olfactory receptor, family 2, subfamily M, member 7
SPACA4	1.2	3.2	1.3	NM_133498	H300006385	sperm acrosome associated 4
FBXO39	1.1	2.1	1.1	NM_153230	opHsV04000066 36	F-box protein 39
TOP3A	1.6	1.0	1.8	NM_004618	H300005258	topoisomerase (DNA) III alpha
ST8SIA3	-1.6	-4.0	-1.1	NM_015879	H300002206	sialyltransferase 8C (alpha2,3Galbeta1,4GlcNA calpha 2,8-
TPRX1	-1.0	-1.7	-1.6	NM_198479	H300003914	FLJ40321 protein
C17orf68	-1.2	-1.6	-1.0	NM_025099	H200009296	hypothetical protein FLJ22170
MAGEL2	-1.3	-3.9	1.3	NM_019066	H300000470	MAGE-like 2
LBX2	-1.6	-3.4	-8.4	NM_00100981 2	H300002781	similar to LP3727
AKAP5	-1.3	-2.5	-1.1	NM_004857	H200004897	A kinase (PRKA) anchor protein 5
CRSP2	1.5	2.9	2.6	NM_004229	H200003043	cofactor required for Sp1 transcriptional activation, subunit 2, 150kDa
F2	2.1	2.1	4.7	NM_000506	opHsV04000058 96	coagulation factor II (thrombin)
DEFB124	1.2	2.6	1.4	NM_00103750 0.1	H300001942	defensin, beta 124
OR10G4	1.1	-2.2	1.1	NM_00100446 2	H300007470	olfactory receptor, family 10, subfamily G, member 4
CREB3L2	1.3	1.0	3.2	NM_194071	opHsV04000127 51	cAMP responsive element binding protein 3-like 2
MRGPRG	-1.5	3.9	1.2	NM_00116437 7.1	opHsV04000023 87	MAS-related GPR, member G
IZUMO1	-2.7	-2.0	-1.1	NM_182575	opHsV04000025 30	hypothetical protein MGC34799
CSF1R	-1.2	-2.6	1.1	NM_005211	H300011116	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
KRTAP11-1	1.0	-6.6	-1.2	NM_175858	opHsV04000023 98	keratin associated protein 11-1
SLC25A18	1.6	1.8	-1.3	NM_031481	opHsV04000014 31	solute carrier family 25 (mitochondrial carrier), member 18
CNOT10	-1.7	-1.2	-1.4	NM_015442	opHsV04000016 96	CCR4-NOT transcription complex, subunit 10
SLC25A10	1.1	-1.2	-1.6	NM_012140	opHsV04000042 54	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10
CAMK1D	-1.5	1.6	1.2	NM_153498	opHsV04000042 46	calcium/calmodulin- dependent protein kinase ID

Appendix 2

NKX2-5	1.4	2.4	1.7	NM_004387	opHsV0400004089	NK2 transcription factor related, locus 5 (Drosophila)
C21orf67	1.5	1.4	2.2	NM_058188	opHsV0400002022	chromosome 21 open reading frame 67
RGAG4	-1.1	-3.3	-1.0	NM_001024455.3	opHsV0400001537	retrotransposon gag domain containing 4
PCBP3	1.0	2.4	1.1	NM_020528	opHsV0400006376	poly(rC) binding protein 3
KCTD16	-1.1	-3.6	-1.4	NM_020768	opHsV0400001520	potassium channel tetramerisation domain containing 16
SCN5A	-1.2	9.6	1.1	NM_198056	opHsV0400009392	sodium channel, voltage-gated, type V, alpha (long QT syndrome 3)
CNTN2	1.0	-2.2	1.3	NM_005076	H300007429	contactin 2 (axonal)
ALDH1A3	-1.1	-1.1	-1.7	NM_000693	opHsV0400005908	aldehyde dehydrogenase 1 family, member A3
HIST2H2AB	-1.1	-1.2	-1.9	NM_175065	H300008668	histone 2, H2ab
MUC6	-1.1	3.1	1.4	NM_005961.2	opHsV0400002267	mucin 6, gastric
GMCL1	1.2	1.6	-1.2	NM_178439	H300006042	germ cell-less homolog 1 (Drosophila)
AP3M1	1.2	6.8	1.2	NM_207012	opHsV0400002029	adaptor-related protein complex 3, mu 1 subunit
KLHL33	1.1	-1.7	-1.3	NM_001109997.2	opHsV0400004088	similar to RIKEN cDNA C530050O22
OR7A17	-1.0	-2.3	-2.4	NM_030901	opHsV0400001432	olfactory receptor, family 7, subfamily A, member 17
PBX1	1.3	-6.4	-2.6	NM_002585	opHsV0400006090	pre-B-cell leukemia transcription factor 1
SYN3	-1.7	-1.3	-1.1	NM_133633	opHsV0400003381	synapsin III
POU3F1	-1.4	-1.3	-2.0	NM_002699	opHsV0400001644	POU domain, class 3, transcription factor 1
EVI2B	-1.0	-1.7	-1.2	NM_006495	H200001067	ecotropic viral integration site 2B
KLHL34	1.1	1.9	2.0	NM_153270	H300003232	hypothetical protein FLJ34960
RTN4RL1	1.0	3.5	1.6	NM_178568	opHsV0400002169	reticulon 4 receptor-like 1
GRK1	1.0	-3.6	-1.4	NM_002929	opHsV0400006100	G protein-coupled receptor kinase 1
LCE5A	-1.6	-3.8	1.0	NM_178438	opHsV0400006715	late cornified envelope 5A
FSD2	1.0	-2.2	-2.1	NM_001007122	H300008567	hypothetical protein LOC123722
OR10H1	1.4	-2.9	-1.7	NM_013940	H300003102	olfactory receptor, family 10, subfamily H, member 1
FSCN2	1.0	1.6	1.0	NM_012418	opHsV0400002872	fascin homolog 2, actin-bundling protein, retinal (Strongylocentrotus purpuratus)
SERPINA11	-2.4	-4.9	-1.2	NM_001080451.1	opHsV0400002860	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 11
TCEB2	-1.1	2.5	1.2	NM_007108	H200008077	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)
SLIT1	-1.2	2.2	1.1	NM_032900	H300022728	Rho GTPase activating protein 19
SLC23A3	1.2	-1.2	-3.4	NM_024782	H200013544	hypothetical protein FLJ12610

Appendix 2

AGAP1	1.1	1.7	1.2	NM_00103713 1.2	opHsV04000071 17	similar to Centaurin gamma 2
TYW1	1.1	-1.6	-1.2	NM_018264	opHsV04000090 16	radical S-adenosyl methionine and flavodoxin domains 1
FAM83G	1.1	-1.7	-1.3	NM_00103999 9.2	opHsV04000047 53	family with sequence similarity 83, member G
TMEM198	-1.0	-1.5	1.1	NM_00100520 9	opHsV04000102 65	similar to RIKEN cDNA A230078105 gene
ASTL	1.1	-1.6	-1.0	NM_00100203 6	H300003637	astacin-like metalloendopeptidase (M12 family)
AADACL3	1.0	2.2	1.0	NM_00110317 0.1	opHsV04000053 73	similar to arylacetamide deacetylase
KCTD21	-1.1	-2.5	1.0	NM_00102985 9.1	H300002117	hypothetical protein LOC283219
ALKBH2	1.0	1.7	1.2	NM_00100165 5	opHsV04000038 72	similar to hypothetical protein 9530023G02
TRIM64	1.2	-4.4	-1.6	NM_00113648 6.1	opHsV04000004 52	similar to tripartite motif- containing 43
MYBPC1	1.0	5.4	1.2	NM_206820	H300018086	myosin binding protein C, slow type
CPNE4	-1.4	-2.7	1.1	NM_130808	H300013389	copine IV
ZNF658B	-1.2	-1.9	-1.3	NM_033160	opHsV04000100 65	LOC441401
ZNF124	1.6	1.8	1.0	NM_003431	opHsV04000056 02	zinc finger protein 124 (HZF-16)
ESRRG	-1.1	-2.3	1.1	NM_206595	H300019878	estrogen-related receptor gamma
C1orf186	1.1	-1.3	-1.8	NM_00100754 4.1	opHsV04000049 32	chromosome 1 open reading frame 186
MAML3	1.9	5.2	1.3	NM_018717	opHsV04000013 95	mastermind-like 3 (Drosophila)
ZFP28	1.6	1.9	-1.2	NM_020828	H200002411	zinc finger protein 28 homolog (mouse)
FAM163B	1.0	2.0	-1.1	NM_00108051 5.2	opHsV04000036 34	family with sequence similarity 163, member B
ZNF498	1.1	-2.3	1.1	NM_145115	H200012740	zinc finger protein 498
SLC6A17	1.2	-1.7	-1.1	NM_00101089 8	H300016719	solute carrier family 6 (neurotransmitter transporter), member 17
C20orf112	1.0	-1.6	-1.0	NM_080616	opHsV04000000 26	chromosome 20 open reading frame 112
GRRP1	-1.0	-1.9	-1.1	NM_024869	H200014922	hypothetical protein FLJ14050
SYNGAP1	1.1	1.8	-1.2	NM_006772	H300016305	synaptic Ras GTPase activating protein 1 homolog (rat)
C17orf102	1.0	-1.5	-1.4	NM_207454	opHsV04000050 35	FLJ44815 protein
DACT3	-1.3	-1.7	-1.3	NM_145056	opHsV04000007 02	thymus expressed gene 3- like
HIST3H2BB	1.0	-1.6	-1.0	NM_175055	opHsV04000103 09	histone 3, H2bb
CSAG3B	-1.1	-2.1	1.2	NM_203311	opHsV04000057 77	similar to Taxol resistant associated protein 3 (TRAG-3)
SERPINB13	1.0	-2.5	-1.1	NM_012397	H300009118	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 13
ZNF460	-1.3	-1.7	-1.5	NM_006635	H200011036	zinc finger protein 272
SCGB1D4	1.1	9.6	1.2	NM_206998	opHsV04000052 30	secretoglobulin family 1D member 4

Appendix 2

ZNF71	-1.3	-1.8	-1.9	NM_021216	opHsV0400001552	zinc finger protein 71 (Cos26)
NOL8	1.3	1.5	1.1	NM_017948	H300022411	nucleolar protein 8
ZNF485	1.6	-3.3	-1.2	NM_145312	opHsV0400006597	zinc finger protein 485
ZNF568	-1.2	1.7	1.2	NM_198539	H300021408	zinc finger protein 568
OR8B4	-1.9	-2.4	1.0	NM_001005196	H300010111	olfactory receptor, family 8, subfamily B, member 4
SPANXB2	1.3	1.9	1.3	NM_145664	H200017908	SPANX family, member B2
hsa-mir-422a	1.3	3.7	1.0	-	opHsV0400013379	-
SNORD36B	1.3	-2.7	1.1	NR_000017.1	opHsV0400006945	small nucleolar RNA, C/D box 36B
SNORD42B	1.1	-3.3	1.3	NR_000013.1	opHsV0400006942	small nucleolar RNA, C/D box 42B
GPR52	1.1	-6.3	-1.6	NM_005684	opHsV0400006184	G protein-coupled receptor 52
C6orf163	-1.0	-1.7	-1.1	NM_001010868	H200012602	chromosome 6 open reading frame 163
CT47A11	1.1	10.7	-1.9	NM_173571	H300006689	hypothetical protein LOC255313
LRRC22	1.0	-2.9	-1.0	NM_001017924.2	opHsV0400007371	leucine rich repeat containing 22
TRNP1	-1.2	-3.1	-1.2	NM_001013642.2	opHsV0400007546	hypothetical LOC388610
PRAMEF1	1.0	-3.1	-2.9	NM_023013	H300003409	hypothetical protein similar to preferentially expressed antigen of melanoma
PRAMEF9	-2.4	-2.8	1.3	NM_001009611.2	opHsV0400008863	hypothetical protein LOC343070
DUXA	1.0	-4.3	-1.5	NM_001012729.1	opHsV0400001666	similar to double homeobox, 4; double homeobox protein 4
ZNF468	1.1	3.9	1.1	NM_199132	opHsV0400006040	zinc finger protein ZNF468
MOG	1.2	-1.9	-1.4	NM_001008229	H300011659	myelin oligodendrocyte glycoprotein
KRTAP10-1	1.1	2.0	1.5	NM_198696	opHsV0400006829	keratin associated protein 10-3
KRTAP5-6	-1.0	-4.3	-1.0	NM_001012416.1	opHsV0400009613	hypothetical gene supported by AB126075
C3HC4	-1.4	-2.6	1.3	NM_001102562.1	opHsV0400002411	similar to hypothetical protein BC009489
HLA-DPA1	-1.1	-1.1	-1.5	NM_033554	H300001485	major histocompatibility complex, class II, DP alpha 1
MCCD1	-1.0	1.6	1.0	NM_001011700.2	opHsV0400008280	similar to coiled-coil domain 1 protein precursor
HLA-F	1.4	1.3	1.8	NM_018950	H300021953	major histocompatibility complex, class I, F
hsa-mir-203	-1.5	1.2	-2.6	-	opHsV0400013330	-
hsa-mir-130b	-1.3	-1.8	-1.9	-	opHsV0400013299	-
hsa-mir-219-2	-2.2	-2.4	-1.2	-	opHsV0400013341	-
DIO2	-1.8	-1.0	-2.7	NM_001007023;NM_013989;NM_000793	opHsV0400005825	deiodinase, iodothyronine, type II

**Table A2.2 GO analysis of biological process.** Biological processes of differentially expressed genes identified post-E2 treatment.

GO category	No. of genes	Gene Symbol
Metabolism	100	CCNT2, ELF3, THRB, IL6ST, FOXA3, POU6F2, CNOT3, ZNF673, TTN, CNOT6, IL11, TGFB2, APOA1, MCM7, CREB3L2, ALX3, FOSL1, GABPB2, TWIST1, TIGD1, POGZ, ZNF644, RXFP2, ZNF141, ZNF503, ZFP28, ZNF3, FRY, CARD14, CD86, GRM2, HIPK1, GTF2I, ZNF135, F2, EDA, ZNF485, ZMYND11, ZNF468, SCML1, TNFRSF8, NFYC, ITGB3, ZNF230, ZNF660, CALCA, TSC22D3, DUSP19, RGMB, AKT1S1, LHX1, ECD, ZNF71, ZNF124, MYOG, MAML3, POU3F1, LHX9, NKX2-5, TPRX1, NACC2, VAV3, ZNF568, TAOK2, TAF5, SPHK1, ESRRG, BRIP1, ELAVL2, DGKH, EN2, ZNF498, GMCL1, DIO2, EAF1, IRF5, LASS3, NEDD4, RGS4, BNC2, EBF2, HIVEP3, TCEB2, ZNF460, ZNF318, SMURF2, ABL1, OPRD1, NR5A1, GRK1, DUOX2, TPO, ALDH1A2, ALDH1A3, CRH, PRLR, SULT2A1, PBX1, LDLRAP1, NR1H4
Development	93	GPRIN1, ELF3, CD8A, SLC5A1, POU6F2, MMP9, DUOX2, TTPA, TTN, MOG, IL11, TGFB2, SYP, APOA1, ROBO4, LGI4, KCNQ2, ALX3, LTA, DISC1, CYR61, TWIST1, CDH23, PHC2, SH3GL3, POGZ, PTPRF, RXFP2, LIG3, MYLK2, ZNF141, MEPE, SLC9A3R2, SLIT1, BICD1, ZNF3, TNS3, CTSK, CHRM3, HIPK1, F2, CNTN2, EDA, MYL6, CCDC99, RNF17, RTN4RL1, LCE5A, SCML1, ARFRP1, COL3A1, MYL1, RAG1, NFYC, CALCA, ALDH1A2, LHX1, CENPA, MYO15A, ALDH1A3, PDILT, MYOG, DOPEY2, POU3F1, COL8A1, AXIN2, LHX9, NKX2-5, NR1H4, CSF1R, AMHR2, NOX3, FSCN2, KL, EN2, CSGALNACT1, SPANXB2, VWF, LAMA4, FZD10, GMCL1, TNFSF11, PRLR, NEDD4, EBF2, ITGA8, MAP2, CRH, CHRND, PAPP2, PBX1, NR5A1, GRK1
Transcription	59	CCNT2, THRB, ELF3, FOXA3, POU6F2, CNOT3, CNOT6, MCM7, CREB3L2, GABPB2, TWIST1, ZNF644, ZNF141, ZNF503, ZFP28, ZNF3, FRY, HIPK1, ZNF135, GTF2I, ZNF485, ZMYND11, ZNF468, NFYC, ZNF230, DIDO1, ZNF660, LHX1, ECD, ZNF124, MAML3, POU3F1, GTF3C1, ZNF568, TAF5, ZNF498, IRF5, EAF1, BNC2, EBF2, TCEB2, ZNF460, HIVEP3, PBX1, ZNF318, NR5A1, SCML1, TSC22D3, MYOG, ZNF71, NKX2-5, ALX3, LHX9, FOSL1, NR1H4, TPRX1, ESRRG, EN2, LASS3
Transport	58	IL6ST, MMP9, F2RL1, ABI3, ITGB3, VCL, TGFB2, IL11, CALCA, SAA1, ROBO4, NKX2-5, LTA, NSF, SPHK1, MYLK2, LDLRAP1, LAMA4, NEDD4, F2, CRH, EDA, SST, TIMM17A, SLC5A1, ATP6V1H, ABCB1, SFXN2, SLC26A10, TRPM2, AQP3, APOA1, SLC26A5, UCP3, FOLR2, SLC23A3, SLC25A10, CACNA1G, SLC13A2, ABCC1, SLC18A1, SLC13A4, KCNQ2, SCN5A, SLC25A18, PKD1L2, SYP, ATP5S, GJD2, GABRA2, NOX5, ABCG2, KCNJ5, PLEKHA8, CHRND, KCTD16, CSN2, CLCN5
Signal transduction	57	OR7A17, CD8A, IL6ST, TAS2R5, F2RL1, OR1E2, TAS1R1, TGFB2, APOA1, LILRA1, SPG21, KNG1, PTPRF, RXFP2, OR5M9, GRP, GRM2, CHRM3, HIPK1, F2, OR8J3, GPR52, OR8B4, OR7D4, COL3A1, ITGB3, SIGLEC9, OR10G4, PKD1L2, SORCS3, CALCA, RGMB, IL12RB1, MAML3, AXIN2, CSF1R, OR2C1, AMHR2, OR2M7, GABRA2, VAV3, KL, SPHK1, OR10H1, DGKH, OR10H3, FZD10, PRLR, ITGA8, CRH, GPR113, SMURF2, SST, GPR116, OPRD1, GRK1, OPN4
Muscle contraction	37	OR2M7, OR7A17, TAS2R5, F2RL1, RXFP2, OR10H1, OR1E2, OR5M9, TAS1R1, OR10G4, OR10H3, SORCS3, FZD10, GRM2, CHRM3, OR8J3, GPR113, GPR52, OR8B4, OR7D4, OPN4, OR2C1, OPRD1, GPR116, MYL6, KNG1, MYBPC1, MYL1, CACNA1G, MYLK2, GAM2, CHRND, TTN, SCN5A, CALCA, SPHK1, NKX2-5

Appendix 2

Immune and inflammatory response	35	F2RL1, CD86, APOA1, IL6ST, CTLA4, ATP6V1H, SPG21, TGFB2, AQP3, CALCA, CD8A, TNFSF11, CD5, KNG1, IL17D, CCL22, ELF3, KL, SAA1, F2, CRH, AOC3, KIR2DL1, ITGAD, CD300A, TNFSF18, RAG1, IFITM3, BPRD1, EDA, LILRA1, CD38, HLA-F, HLA-DPA1, COL3A1,
Anatomical structure formation	32	VWF, TAOK2, COL3A1, CNTN2, EDA, VAV3, SAA1, TGFB2, CNTN2, CD300A, IZUMO1, VCL, PTPRS, CYR61, PTPRF, CDH23, RGMB, SIGLEC9, DSG3, AOC3, NRXN2, CNTNAP3, SPACA4, FPRL1, ABL1, MYBPC1, LAMA4, CLDN5, ITGAD, ITGA8, ITGB3, CALCA
Apoptosis	24	IL6ST, CNOT6, TGFB2, FOSL1, CARD14, HIPK1, TNFRSF8, TSC22D3, VAV3, TAOK2, SPHK1, PRLR, ABL1, CRH, ALDH1A3, MMP9, LTA, PTPRF, NOX3, SST, CD5, KNG1, ABR, CIDEB
Reproduction	21	ADAM28, AMHR2, RNF17, SEMG1, RXFP2, LIG3, TTPA, PGAM2, TGFB2, CALCA, SPANXB2, GMCL1, PRLR, NEDD4, CRH, PDILT, PBX1, IZUMO1, LHX9, FOSL1, NR5A1
Cell Differentiation and Proliferation	15	ELF3, CNOT6, IL11, TGFB2, APOA1, MCM7, POGZ, ZNF3, CD86, ZMYND11, ITGB3, ECD, LHX9, NACC2, GMCL1,
Coagulation	10	IL11, F2, ITGB3, DGKH, COL3A1, VWF, F2RL1, SAA1, KNG1, TFPI2

## **Appendix 3**

*Appendix 3*

**Table A3.1 Differentially expressed genes identified post-P4 treatment.**

<sup>1</sup>Negative value represents downregulation.

<b>Gene Symbol</b>	<b>Fold Changes <sup>1</sup></b>	<b>p-Value</b>	<b>RefSeq ID</b>	<b>Operon ID</b>	<b>Gene Name</b>
MATN3	31.5	1.78E-01	NM_002381	H200017287	Matrilin-3 precursor
LRP2BP	4.4	6.02E-03	NM_018409	H200004595	LRP2 binding protein
IFT122	4	6.02E-01	NM_052989	opHsV0400007587	Intraflagellar transport 122 homolog
JPH3	3	1.62E-02	NM_020655	opHsV0400000706	Junctophilin type 3
MYLK3	2.7	2.66E-02	NM_182493	H200019888	Myosin light chain kinase 3
SSTR2	2.6	6.79E-03	NM_001050	opHsV0400001022	Somatostatin receptor type 2
PRAM1	2.4	1.73E-02	NM_032152	opHsV0400006486	PML-RARA-regulated adapter molecule 1
TTLL6	2.2	2.53E-02	NM_173623	opHsV0400006664	Tubulin tyrosine ligase-like family, member 6
BCL2	2.2	2.66E-02	NM_000657	H300021315	Apoptosis regulator Bcl-2
SBK1	2.2	1.66E-02	NM_001024401	opHsV0400003661	SH3-binding domain kinase 1
FLJ39780 fis	2.2	4.31E-02	AK097099	H300007951	CDNA FLJ39780 fis, clone SPLEN2002007
RAB30	2	3.14E-02	NM_014488	H200007455	Ras-related protein Rab-30
ZNF20	2	6.92E-03	NM_021143	H200011728	Zinc finger protein 20
NPPC	2	4.98E-02	NM_024409	H200015979	C-type natriuretic peptide precursor
FAM9C	2	5.85E-01	NM_174901	opHsV0400002731	Family with sequence similarity 9, member C
TRIM67	2	8.76E-01	NM_001004342	opHsV0400011322	Tripartite motif-containing 67
ZNF600	-4.2	4.16E-02	NM_198457	opHsV0400008855	Zinc finger protein 600

Appendix 3

CCDC88	-3.3	3.61E-02	NM_032251	H300012917	Coiled-coil domain containing 88
OR2T10	-3.3	1.37E-02	NM_001004693	opHsV0400002014	Olfactory receptor, family 2, subfamily T, member 10
PAF1	-2.9	9.48E-01	NM_019088	H300010878	Paf1, RNA polymerase II associated factor, homolog
CMTM1	-2.8	3.03E-02	NM_181269	H200019968	CKLF-like MARVEL transmembrane domain-containing protein 1
SST	-2.8	3.91E-02	NM_001048	H200002126	Somatostatin precursor
ALDH3A2	-2.6	2.90E-02	NM_000382	H300000003	Aldehyde dehydrogenase 3 family, member A2
FAM135A	-2.2	7.10E-03	NM_020819	H300016360	Family with sequence similarity 135, member A
AGFG2	-2.2	4.95E-02	NM_006076	H300022014	ArfGAP with FG repeats 2
PES1	-2	4.26E-02	NM_014303	H300014995	Pescadillo homolog 1, containing BRCT domain
BEND2	-2	4.06E-02	NM_153346	opHsV0400001066	BEN domain containing 2
LCN6	-2	3.81E-02	NM_198946	H300021242	Lipocalin-6
MCTP1	-2	4.32E-02	NM_024717	opHsV0400008092	Multiple C2-domains with two transmembrane regions 1 isoform L
F2	-2	2.39E-02	NM_000506	opHsV0400005896	Prothrombin precursor
CHST15	-2	4.42E-02	NM_015892	H300017161	Carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15
OSTalpha	-2	4.12E-02	NM_152672	H300008456	Organic solute transporter alpha

### Appendix 3

**Table A3.2 GO analysis of biological process.** Biological processes of differentially expressed genes identified post-P4 treatment.

Process	No:of Genes	Genes Involved
Cell differentiation and proliferation	20	ALDH3A2, CHST15, CMTM1, F2, FAM9C, JPH3, LRP2BP, MCTP1, NPPC, OR2T10, OSTALPHA, PAF1, PES1, RAB30, SST, SSTR2, TRIM67, TTL6, ZNF20, ZNF600
Metabolism	11	ALDH3A2, CHST15, F2, MYLK3, NPPC, PAF1, PES1, SSTR2, TTL6, ZNF20, ZNF600
Muscle contraction	10	AGFG2, F2, MCTP1, MYLK3, OR2T10, SST, SSTR2, TRIM67, ZNF20, ZNF600
Signal Transduction	9	CMTM1, F2, MCTP1, NPPC, OR2T10, PRAM1, RAB30, SST, SSTR2
Development	4	MATN3, F2, NPPC, ALDH3A2
Transcription	4	ZNF600, PAF1, PES1, ZNF20

**Table A3.3 Differentially expressed genes identified upon functional P4 withdrawal.**

<sup>1</sup>Negative value represents downregulation.

Gene Symbol	Fold Changes <sup>1</sup>	p-Value	RefSeq ID	Operon ID	Gene Name
EMILIN3	4.3	3.79E-02	NM_052846	H200003572	Elastin microfibril interfacier 3
FLJ32165 fis	4.1	1.82E-02	AK056727	opHsV0400000973	cDNA FLJ32165 fis, clone PLACE6000424
LONRF2	3.9	4.64E-02	NM_198461	opHsV0400011464	LON peptidase N-terminal domain and ring finger 2
ASB7	3.7	3.20E-02	NM_198243	opHsV0400002484	Ankyrin repeat and SOCS box protein 7
ZNF404	3.3	2.28E-02	NM_0010337 19	opHsV0400001303	Zinc finger protein 404
ZNF71	3.1	2.55E-02	NM_021216	opHsV0400001552	Zinc finger protein 71
IFT57	2.9	1.21E-03	NM_018010	H200007935	Intraflagellar transport 57 homolog (Estrogen-related receptor beta like 1)
ELMO2	2.9	7.15E-01	NM_133171	H300019232	Engulfment and cell motility protein 2
EDA	2.9	3.35E-02	NM_0010056 10	opHsV0400003180	Ectodysplasin-A

Appendix 3

HFE	2.8	4.01E-02	NM_139003	opHsV0400003033	Hemochromatosis (Hereditary hemochromatosis protein precursor (HLA-H))
BBS9	2.8	5.94E-03	NM_001033605	H300018307	Bardet-Biedl syndrome 9 protein (Parathyroid hormone-responsive B1 gene protein (PTHB1))
EPM2A	2.7	2.60E-02	NM_005670	H300019743	Epilepsy, progressive myoclonus type 2A, Lafora disease (laforin)
SHANK3	2.7	3.35E-02	NM_001080420	opHsV0400003072	SH3 and multiple ankyrin repeat domains protein 3
PLCB1	2.7	2.24E-02	NM_015192	opHsV0400001576	Phospholipase C, beta 1 (phosphoinositide-specific)
FAM129C	2.7	2.78E-02	NM_173544	opHsV0400004267	Family with sequence similarity 129, member C
UBQLNL	2.6	1.50E-02	NM_145053	H300004433	Ubiquilin-like
LMNB1	2.4	3.92E-02	NM_005573	H200010335	Lamin-B1
XKR4	2.4	7.98E-03	NM_052898	opHsV0400001413	XK, Kell blood group complex subunit-related family, member 4
SNX18	2.4	1.40E-03	NM_052870	H300001966	Sorting nexin-18
ABHD1	2.4	4.44E-02	NM_032604	H300010535	Abhydrolase domain containing 1 (alpha/beta hydrolase domain containing protein 1)
SYDE2	2.4	4.71E-03	NM_032184	H300004220	Synapse defective 1, Rho GTPase, homolog 2
INO80E	2.4	2.82E-02	NM_173618	H300021254	INO80 complex subunit E (Coiled-coil domain-containing protein 95)
ITIH5	2.3	2.58E-02	NM_030569	H300015449	Inter-alpha (globulin) inhibitor H5
BFSP1	2.3	2.05E-03	NM_001195	H200012828	Beaded filament structural protein 1, filensin
TYMP	2.1	3.30E-02	NM_001953	H200005893	Thymidine phosphorylase precursor
ADRBK2	2.1	3.63E-02	NM_005160	H300018631	Adrenergic, beta, receptor kinase 2

Appendix 3

GMCL1L	2.1	4.16E-02	NM_178439	opHsV0400001512	Germ cell-less protein-like 1-like
SFTPC	2.1	2.58E-02	NM_003018	H300021316	Pulmonary surfactant-associated protein C precursor (SP-C or SP5)
SIPA1L2	2.1	4.02E-02	NM_020808	H200002761	Signal-induced proliferation-associated 1-like protein 2
TNN	2.1	7.50E-01	NM_022093	H300010970	Tenascin-N precursor
RNGTT	2.1	3.45E-02	NM_003800	H300020025	RNA guanylyltransferase and 5'-phosphatase
FAM154B	1.7	5.00E-02	NM_001008226	H300001480	Family with sequence similarity 154, member B
ANGPTL4	-4.2	4.42E-02	NM_139314	H300021145	Angiopoietin-related protein 4
IFT122	-3.1	9.54E-01	NM_052989	opHsV0400007587	Intraflagellar transport 122 homolog
MYO7B	-3	9.81E-01	NM_001080527	opHsV0400001517	Myosin VIIB
TMEM161B	-2.9	2.96E-02	NM_153354	opHsV0400008725	Transmembrane protein 161B
MNX1	-2.6	4.88E-02	NM_005515	H200004364	Motor neuron and pancreas homeobox 1 (Homeobox protein HB9 (HLXB9))
CLVS1	-2.5	7.22E-03	NM_173519	opHsV0400004432	Clavesin 1
LRP2BP	-2.5	2.82E-02	NM_018409	H200004595	LRP2 binding protein
ID1	-2.4	1.61E-03	NM_181353	H200006097	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
TMCO5A	-2.4	2.47E-02	NM_152453	H300011039	Transmembrane and coiled-coil domains 5A
PAEP	-2.3	6.05E-01	NM_001018049	H200006917	Progesterone-associated endometrial protein
SLC6A4	-2.3	2.23E-02	NM_001045	H200000140	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Sodium-dependent serotonin transporter)
C16orf89	-2.2	9.07E-01	NM_152459	H300020528	Chromosome 16 open reading frame 89

**Appendix 3**

NR2E3	-2.1	2.34E-02	NM_016346	H200014369	Nuclear receptor subfamily 2, group E, member 3
FRMD3	-2.1	2.13E-02	NM_174938	H300001773	FERM domain containing 3
IRF4	-2.1	5.17E-01	NM_002460	opHsV0400004447	Interferon regulatory factor 4
CHST14	-2.1	3.76E-02	NM_130468	H200003457	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 14 (Carbohydrate sulfotransferase (D4ST1))
INHBA	-2	2.25E-02	NM_002192	opHsV0400004610	Inhibin beta A chain

**Table A3.4 GO analysis of biological process.** Biological processes of differentially expressed genes identified following functional P4 withdrawal.

Process	No: of genes	Genes involved
Cell differentiation and proliferation	16	BBS9, BFSP1, EDA, EPM2A, FRMD3, GMCL1L, HFE, IFT57, IRF4, LMNB1, NR2E3, PLCB1, RNGTT, SLC6A4, SNX18, ZNF71
Transcription	9	EDA, GMCL1L, ID1, IFT57, INHBA, IRF4, MNX1, NR2E3, ZNF71
Metabolism	6	ID1, INHBA, IRF4, MNX1, NR2E3, ZNF71
Ion Binding	6	CHST14, HFE, ZNF71, NR2E3, PLCB1, LONRF2

**Table A3.5 Differentially expressed genes identified post-P4+E2 cotreatment.**

<sup>1</sup>Negative value represents downregulation.

Gene Symbol	Fold Changes <sup>1</sup>	p-Value	RefSeq ID	Operon ID	Gene Name
CCDC42B	42.9	6.66E-03	NM_001144872	opHsV0400002986	Coiled-coil domain containing 42B
MAGEE2	8.1	6.67E-03	NM_138703	H300004354	Melanoma-associated antigen E2
CCDC87	7.0	2.11E-02	NM_018219	H300009298	Coiled-coil domain containing 87
VPS39	6.9	7.86E-03	NM_015289	opHsV0400004231	Vacuolar protein sorting 39 homolog
FCGBP	5.2	2.65E-02	NM_003890	H300018727	IFc fragment of IgG binding protein
C2orf57	5.1	3.24E-02	NM_152614	H300008206	Chromosome 2 open reading frame 57
AKAP14	5.0	8.75E-03	NM_001008534	H300003624	A kinase (PRKA) anchor protein 14

Appendix 3

NTRK2	4.7	8.28E-03	NM_006180	H200004858	Neurotrophic tyrosine kinase, receptor, type 2
SOHLH2	4.6	6.95E-03	NM_017826	H300012282	Spermatogenesis and oogenesis specific basic helix-loop-helix 2
BCL2	4.4	3.04E-02	NM_000657	H300021315	Apoptosis regulator Bcl-2
TXNL4A	4.4	3.17E-03	NM_006701	opHsV0400007533	Thioredoxin-like protein 4A
IHH	4.4	8.08E-03	NM_002181	opHsV0400006077	Indian hedgehog protein precursor
EPB41L4A	4.2	1.07E-02	NM_022140	H300012199	Erythrocyte membrane protein band 4.1 like 4A
EML6	4.2	2.23E-03	NM_001039753	H300006719	Echinoderm microtubule associated protein like 6
ZNF727	4.1	5.45E-04	NM_001159522	opHsV0400004719	Zinc finger protein 727
SPRR3	3.9	3.60E-03	NM_005416	H300001971	Small proline-rich protein 3
CACNA1C	3.8	2.50E-03	NM_000719	opHsV0400003004	Voltage-dependent L-type calcium channel subunit alpha-1C
GJA5	3.5	5.40E-03	NM_005266	opHsV0400001817	Gap junction protein, alpha 5, 40kDa (CX40)
KLB	3.4	3.29E-03	NM_175737	opHsV0400000248	Klotho beta
PAX7	3.3	2.01E-02	NM_013945	H200011868	Paired box protein Pax-7
CNGB1	3.3	2.31E-03	NM_001297	H200010619	Cyclic nucleotide gated channel beta 1
TPD52L3	3.2	5.16E-05	NM_001001874	H300012731	Tumor protein D52-like 3
hsa-mir-128a	3.1	3.80E-03		opHsV0400013296	hsa-mir-128a
ZSWIM2	3.1	4.59E-03	NM_182521	H300002846	Zinc finger SWIM domain-containing protein 2
POU4F3	3.0	5.76E-03	NM_002700	H300002195	POU class 4 homeobox 3
CUBN	2.9	4.79E-04	NM_001081	H300020055	Cubilin precursor (Intrinsic factor-cobalamin receptor)
PALM2	2.9	5.19E-04	NM_053016	H300019459	Paralemmin 2
TTN	2.8	2.21E-02	NM_133379	opHsV0400000806	Titin
GANC	2.8	1.31E-02	NM_198141	opHsV0400006799	Glucosidase, alpha; neutral C
LGALS14	2.7	6.64E-04	NM_203471	opHsV0400000002	Lectin, galactoside-binding, soluble, 14

Appendix 3

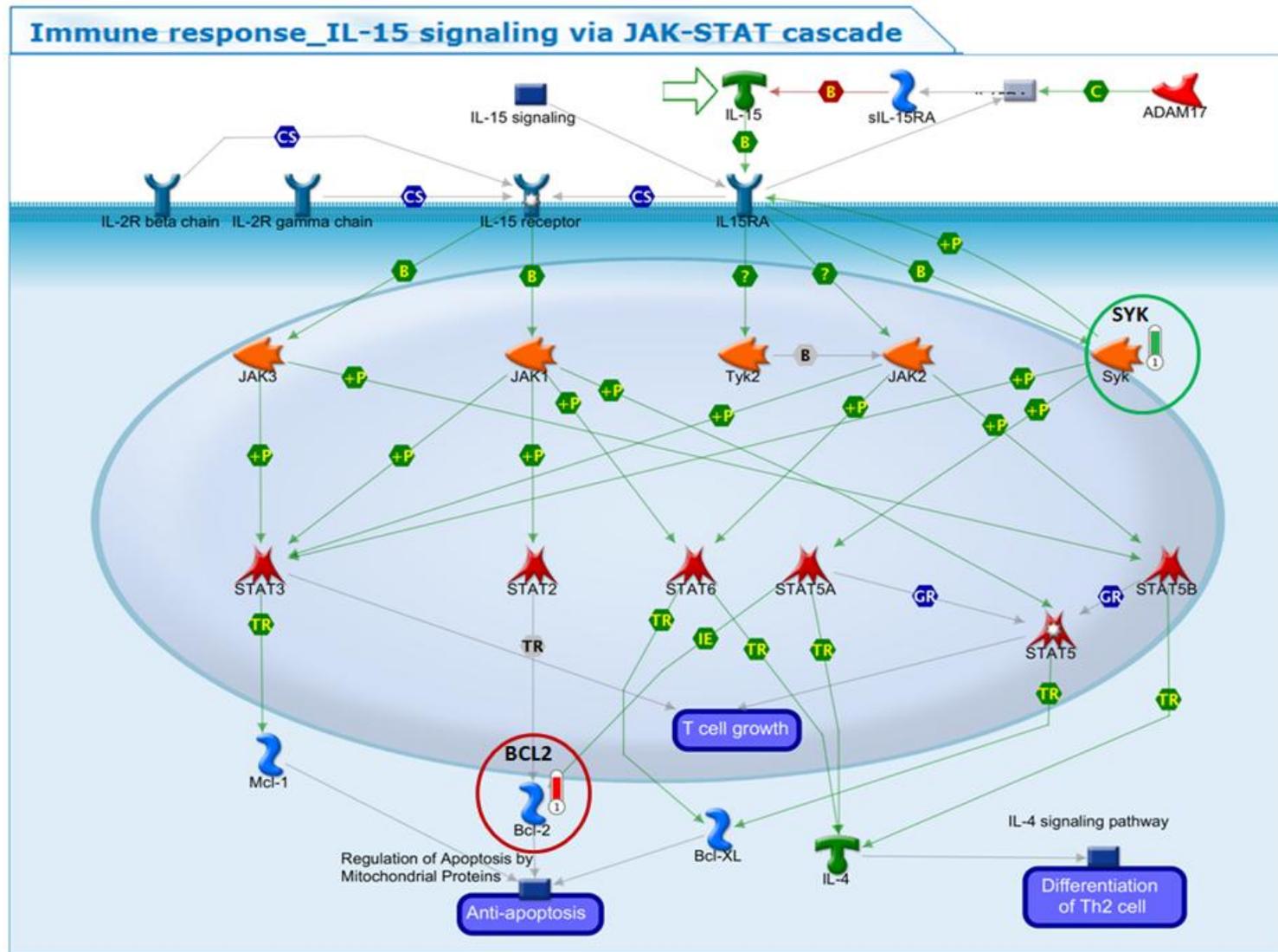
BSPRY	2.7	7.20E-03	NM_017688	H300014369	B box and SPRY domain-containing protein
DNAJB5	2.7	6.81E-03	NM_012266	H300015084	DnaJ (Hsp40) homolog, subfamily B, member 5
OR8I2	2.7	3.26E-04	NM_001003750	H300004923	Olfactory receptor, family 8, subfamily I, member 2
BDP1	2.6	4.88E-03	NM_018429	H300022202	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB
PURG	2.6	2.36E-02	NM_001015508	opHsV0400000836	Purine-rich element binding protein G
GLYATL2	2.5	4.26E-05	NM_145016	opHsV0400006589	Glycine-N-acyltransferase-like 2
EPS8L1	2.3	5.08E-03	NM_017729	H300018568	Epidermal growth factor receptor kinase substrate 8-like protein 1
MUC17	2.3	3.92E-03	NM_001040105	H300009273	Mucin 17
GAS8	-101.1	8.64E-03	NM_001481	H300015231	Growth-arrest-specific protein 8
C20orf132	-16.8	8.39E-03	NM_152503	H300018088	Chromosome 20 open reading frame 132
TTPA	-16.3	3.61E-02	NM_000370	H200005658	Alpha-tocopherol transfer protein
MACC1	-14.4	6.19E-03	NM_182762	opHsV0400002508	Metastasis associated in colon cancer 1
LEPROT	-11.5	4.41E-03	NM_017526	opHsV0400003795	Leptin receptor precursor
FAM71A	-10.3	1.06E-02	NM_153606	H200012813	Family with sequence similarity 71, member A
MAN1C1	-10.0	3.51E-03	NM_020379	H200001706	Mannosidase, alpha, class 1C, member 1
SERPINA9	-5.6	1.05E-02	NM_175739	opHsV0400003059	Serpin A9 precursor
C22orf33	-5.5	1.75E-02	NM_178552	opHsV0400001926	Chromosome 22 open reading frame 33
SRL	-5.3	3.24E-03	NM_001098814	opHsV0400001924	Sarcalumenin
C10orf99	-4.9	5.22E-03	NM_207373	opHsV0400003625	Chromosome 10 open reading frame 99
ZBTB10	-4.7	1.04E-02	NM_023929	H300020960	Zinc finger and BTB domain-containing protein 10
SYK	-4.7	4.41E-02	NM_003177	H200005918	Tyrosine-protein kinase
YIPF7	-4.3	4.37E-02	NM_182592	opHsV0400002536	Yip1 domain family, member 7

**Appendix 3**

CRYBA1	-4.1	2.02E-03	NM_005208	H200004757	Crystallin, beta A1
PLA2G2F	-3.9	1.01E-03	NM_022819	H200018022	Group IIF secretory phospholipase A2
CDHR3	-3.6	2.62E-03	NM_152750	H300016992	Cadherin-related family member 3
MTMR11	-3.6	7.40E-04	NM_181873	H300018925	Myotubularin related protein 11
OR7A17	-3.6	2.99E-03	NM_030901	opHsV0400001432	Olfactory receptor, family 7, subfamily A, member 17
SLC26A3	-3.3	8.78E-04	NM_000111	H300008103	Solute carrier family 26, member 3
NCKAP5	-3.3	1.97E-04	NM_207481	opHsV0400004006	NCK-associated protein 5
ZBTB48	-3.1	7.82E-03	NM_005341	H300014251	Zinc finger and BTB domain-containing protein 48
ST6GALNA C1	-3.0	3.29E-03	NM_018414	H200011361	ST6 (alpha-N-acetylneuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminidase alpha-2,6-sialyltransferase 1
EFNA5	-2.6	3.04E-02	NM_001962	opHsV0400002585	Ephrin A5

**Table A3.6 GO analysis of biological process.** Biological processes of differentially expressed genes identified following P4+E2 cotreatment.

<b>Process</b>	<b>No: of Genes</b>	<b>Genes Involved</b>
Cell differentiation and proliferation	21	BCL2, CACNA1C, CDHR3, CUBN, EFNA5, GAS8, GJA5, IHH, KLB, LEPROT, NTRK2, OR7A17, OR8I2, PALM2, PAX7, POU4F3, SLC26A3, SYK, TTN, TXNL4A, ZBTB10
Muscle contraction	12	BSPRY, CACNA1C, CDHR3, CUBN, KLB, MAN1C1, PLA2G2F, SLC26A3, TTN, ZBTB10, ZBTB48, ZSWIM2
Transcription	11	BCL2, BDP1, IHH, MACC1, PAX7, POU4F3, PURG, SLC26A3, SOHLH2, ZBTB10, ZBTB48
Development	9	BCL2, CRYBA1, EFNA5, GJA5, IHH, NTRK2, PAX7, POU4F3, TTN
Signal transduction	6	NTRK2, EFNA5, CACNA1C, GJA5, IHH, SYK
Anatomical structure Development	4	BCL2, CDHR3, FCGBP, SYK
Metabolism	4	BCL2, NTRK2, TTN, SYK



**Figure A3.1: Immune response by IL-15 signalling.** Genes identified in the present study is circled and red circles represent upregulation and green circle represents downregulation of the gene in the present study.

### Appendix 3

**Table A3.7 Differentially expressed genes identified post-P4+E2+RU486 cotreatment.**

<sup>1</sup>Negative value represent downregulation.

Gene Symbol	Fold Changes <sup>1</sup>	p-Value	RefSeq ID	Operon ID	Gene name
EMILIN2	9.6	2.54E-02	NM_032048	H300017224	Elastin microfibril interface-located protein 2
NDUFA10	8.5	3.65E-02	NM_004544	H300016572	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10, mitochondrial precursor
FLJ21991 fis	7.5	6.39E-02	AK025644	opHsV0400013 396	FLJ21991 fis, clone HEP06475, highly similar to HSPEX3P Homo sapiens mRNA for Pex3 protein
MYL6	6.6	3.59E-02	NM_079423	H300022343	Myosin, light chain 6, alkali, smooth muscle and non-muscle
C15orf27	5.7	1.56E-01	NM_152335	H200021222	Chromosome 15 open reading frame 27
KIAA0652	5.6	5.71E-02	NM_014741	H300015133	KIAA0652 (KIAA0652), mRNA
HNRNPA3	5.6	2.53E-03	NM_194247	opHsV0400001 916	Heterogeneous nuclear ribonucleoprotein A3
PROKR2	5.5	9.13E-03	NM_144773	H200017845	Prokineticin receptor 2
RPL23	5.4	2.55E-02	NM_000978	H300005578	Ribosomal protein L23
TAAR6	5.3	2.68E-02	NM_175067	H300005298	Trace amine-associated receptor 6
SRD5A2	5.3	3.15E-02	NM_000348	H200000509	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)
CRH	5.3	1.79E-01	NM_000756	H200006062	Corticotropin releasing hormone
RPS7	5	1.19E-02	NM_001011	opHsV0400001 653	Ribosomal protein S7
PLCD4	4.8	4.78E-03	NM_032726	H200019500	Phospholipase C, delta 4
GPATCH2	4.6	1.39E-03	NM_018040	opHsV0400005 032	G patch domain-containing protein 2
EFTUD1	4.1	6.21E-02	NM_024580	opHsV0400008 802	Elongation factor Tu GTP binding domain containing 1
DDX18	3.9	1.31E-02	NM_006773	opHsV0400001 587	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
STXBP4	3.9	1.06E-01	NM_178509	opHsV0400000 677	Syntaxin-binding protein 4
ADRBK2	3.6	3.78E-02	NM_005160	H300018631	Adrenergic, beta, receptor kinase 2
C1orf161	3.5	3.20E-02	NM_152367	opHsV0400005 003	Chromosome 1 open reading frame 161
SLC26A1	3.5	6.61E-03	NM_134425	H300016012	Solute carrier family 26 (sulfate transporter), member 1

Appendix 3

PCDH20	3.5	3.77E-02	NM_022843	opHsV0400004 484	Protocadherin 20
ZNF229	3.3	5.00E-02	NM_014518	H300003325	Zinc finger protein 229
NUAK1	3.2	3.82E-02	NM_014840	H300019565	NUAK family SNF1-like kinase 1
PLCE1	3.2	1.50E-02	NM_016341	H300008936	Phospholipase C, epsilon 1
PURB	3.1	6.89E-02	NM_033224	H200013406	Purine-rich element binding protein B
RTDR1	3.1	2.66E-02	NM_014433	H200004315	Rhabdoid tumor deletion region protein 1
DHX57	3	3.90E-02	NM_198963	opHsV0400004 525	DEAH (Asp-Glu-Ala- Asp/His) box polypeptide 57
RPL18A	2.8	4.88E-02	NM_000980	H300006101	Ribosomal protein L18a
GP2	2.8	1.27E-02	NM_001502	H300010914	Glycoprotein 2 (zymogen granule membrane)
C9orf6	2.6	4.30E-02	NM_017832	H300002940	Chromosome 9 open reading frame 6
OR5AT1	2.6	2.94E-02	NM_0010019 66	H300007306	Olfactory receptor 5AT1 (Olfactory receptor, family 14, subfamily A, member 16 (OR14A16))
C19orf77	2.5	1.88E-02	NM_0011365 03	H300018607	Chromosome 19 open reading frame 77
ZNF407	2.5	1.41E-02	NM_017757	opHsV0400006 331	Zinc finger protein 407
OR2B11	2.4	1.39E-02	NM_0010044 92	opHsV0400001 126	Olfactory receptor, family 2, subfamily B, member 11
KLK10	2.3	4.44E-02	NM_002776	opHsV0400003 035	Kallikrein-related peptidase 10
KANK2	2.3	2.73E-02	NM_015493	opHsV0400004 813	KN motif and ankyrin repeat domains 2 (Ankyrin repeat domain-containing protein 25 (ANKRD25))
ENTPD3	2.3	2.84E-02	NM_001248	H300021162	Ectonucleoside triphosphate diphosphohydrolase 3
IFNG	2.3	4.12E-02	NM_000619	H200000229	Interferon gamma
LOC44057 7	2.1	3.63E-02	XM_496355	opHsV0400008 867	Similar to nucleophosmin 1
MDFI	2.1	3.93E-02	NM_005586	H300001204	MyoD family inhibitor
MFSD2A	2.1	7.76E-03	NM_032793	H300013801	Major facilitator superfamily domain-containing protein 2A
MANEA	2	4.59E-02	NM_024641	H300015291	Mannosidase, endo-alpha
ATP6V0E2	2	3.21E-02	NM_145230	H300013798	ATPase, H <sup>+</sup> transporting V0 subunit e2
LRRC2	2	4.98E-02	NM_024512	H200012580	Leucine-rich repeat- containing protein 2
PRKCB1	2	6.00E-03	NM_002738	H300021219	Protein kinase C beta
OR1G1	2	3.55E-02	NM_003555	H200016108	Olfactory receptor, family 1, subfamily G, member 1
TTPA	-28.7	1.88E-02	NM_000370	H200005658	Tocopherol (alpha) transfer protein
DHRS7C	-19.6	7.07E-02	NM_0011055 71	opHsV0400001 964	Dehydrogenase/reductase (SDR family) member 7C

Appendix 3

LEPROT	-15.1	9.99E-03	NM_017526	opHsV0400003 795	Leptin receptor
C10orf99	-9.7	1.20E-01	NM_207373	opHsV0400003 625	Chromosome 10 open reading frame 99
RNF217	-9.3	2.74E-02	NM_152553	H300011309	Ring finger protein 217
FAM78A	-8.8	1.06E-01	NM_033387	opHsV0400000 177	Family with sequence similarity 78, member A
GRIA3	-8.4	1.98E-03	NM_007325	opHsV0400007 800	Glutamate receptor ionotropic, AMPA 3
C20orf132	-7.1	1.86E-02	NM_152503	H300018088	Chromosome 20 open reading frame 132
YIPF7	-6	8.06E-03	NM_182592	opHsV0400002 536	Yip1 domain family, member 7
FAM71A	-5.9	2.65E-02	NM_153606	H200012813	Family with sequence similarity 71, member A
SERPINA9	-5.8	2.18E-02	NM_175739	opHsV0400003 059	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9
SPAG17	-5.7	1.93E-02	NM_206996	H300017001	Sperm associated antigen 17
SCN4A	-5.5	3.16E-02	NM_000334	H200004755	Sodium channel protein type 4 subunit alpha
CLDN2	-5.2	4.97E-02	NM_020384	H200002539	Claudin 2
PAX2	-4.8	3.42E-02	NM_003989	H300018793	Paired box protein
EPN3	-4.8	3.04E-02	NM_017957	H200014857	Epsin 3
LOC28503 3	-4.7	2.10E-01	NM_0010372 28	opHsV0400008 561	Hypothetical protein LOC285033
SPATA22	-4.7	2.17E-02	NM_032598	H200011360	Spermatogenesis- associated protein 22
RNF175	-4.6	3.98E-02	NM_173662	H300020309	RING finger protein 175
C11orf9	-4.5	4.16E-02	NM_013279	opHsV0400001 501	Chromosome 11 open reading frame 9
GNRHR	-4.4	7.10E-02	NM_000406	H200005827	Gonadotropin-releasing hormone receptor
DGKK	-4.3	2.26E-03	NM_0010137 42	H300007767	Diacylglycerol kinase kappa
IL23R	-4.2	2.84E-02	NM_144701	H300020818	Interleukin-23 receptor
GALNT13	-4.1	3.83E-02	NM_052917	H200002289	UDP-N-acetyl-alpha-D- galactosamine:polypeptide N- acetylgalactosaminyltransf erase 13
FAM150B	-4	3.61E-02	NM_0010029 19	opHsV0400004 065	Family with sequence similarity 150, member B
ITGA9	-4	2.53E-02	NM_002207	H300013985	Integrin alpha 9
XIRP2	-3.9	4.30E-02	NM_152381	opHsV0400008 931	Xin actin-binding repeat containing 2
CCDC60	-3.9	1.68E-02	NM_178499	opHsV0400001 451	Coiled-coil domain- containing protein 60
NUP210	-3.9	2.55E-02	NM_024923	H300010964	Nucleoporin 210kDa
CAMSAP1	-3.8	1.33E-02	NM_015447	H300022657	Calmodulin regulated spectrin-associated protein 1
C7	-3.7	1.82E-02	NM_000587	opHsV0400005 900	Complement component 7

Appendix 3

GJA8	-3.7	2.23E-02	NM_005267	H200007263	Gap junction alpha-8 protein (Connexin-50)
CNR1	-3.7	3.84E-02	NM_033181	H300018963	Cannabinoid receptor 1 (CB1)
TTLL9	-3.6	3.54E-02	NM_001008409	H300019228	Tubulin tyrosine ligase-like family, member 9
CA2	-3.6	6.97E-03	NM_000067	H200014027	Carbonic anhydrase 2
FREM2	-3.6	3.72E-02	NM_207361	opHsV0400003816	FRAS1-related extracellular matrix protein 2
ZNF337	-3.6	4.01E-02	NM_015655	opHsV0400004189	Zinc finger protein 337
TMOD4	-3.5	3.18E-02	NM_013353	H200016253	Tropomodulin 4
TAP2	-3.4	1.47E-02	NM_018833	H300014785	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP
CREB5	-3.3	3.14E-02	NM_182898	H200000042	cAMP response element-binding protein 5
hsa-mir-24-2	-3.3	3.12E-02		opHsV0400013349	hsa-mir-24-2
SLC4A5	-3.2	4.64E-02	NM_021196	H300021879	Solute carrier family 4, sodium bicarbonate cotransporter, member 5
SP140L	-3.1	4.32E-02	BC004921	H200016714	Nuclear body protein SP140-like protein
ALLC	-2.9	4.61E-02	NM_018436	H200010839	Allantoicase
CLRN3	-2.9	2.73E-02	NM_152311	H300010160	Clarin 3
C1orf227	-2.9	4.66E-02	NM_001024601	opHsV0400002518	Chromosome 1 open reading frame 227
PRDM12	-2.9	3.07E-02	NM_021619	H200008918	PR domain zinc finger protein 12
ZSCAN4	-2.8	4.50E-02	NM_152677	H300005461	Zinc finger and SCAN domain-containing protein 4
CYP26A1	-2.8	1.37E-02	NM_057157	H200013714	Cytochrome P450, family 26, subfamily A, polypeptide 1
LOC729159	-2.8	2.88E-02	XM_001129515	opHsV0400004181	Similar to nuclear pore membrane protein 121 (LOC392364) (UPF0607 protein ENSP00000381418-like)
GRB14	-2.8	4.72E-02	NM_004490	H200007000	Growth factor receptor-bound protein 14
NR2C1	-2.8	1.91E-03	NM_001032287	opHsV0400002051	Nuclear receptor subfamily 2, group C, member 1
RRP1B	-2.7	2.46E-02	NM_015056	H300020735	Ribosomal RNA processing 1 homolog B
AQP5	-2.7	1.96E-02	NM_001651	H200009849	Aquaporin 5
SCN2B	-2.7	3.12E-02	NM_004588	H200012855	Sodium channel, voltage-gated, type II, beta
LYPD6	-2.7	1.47E-02	NM_194317	H300002872	LY6/PLAUR domain-containing protein 6
KIR3DL1	-2.6	3.31E-02	NM_013289	H300022329	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1

Appendix 3

FUT1	-2.6	1.23E-02	NM_000148	H200005691	Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)
SMG6	-2.5	4.95E-03	NM_017575	H300022022	Smg-6 homolog, nonsense mediated mRNA decay factor (Telomerase-binding protein EST1A)
MTMR11	-2.5	2.10E-02	NM_181873	H300018925	Myotubularin related protein 11
ZNF667	-2.5	3.33E-02	NM_022103	H300014772	Zinc finger protein 667
TRIM42	-2.3	4.13E-02	NM_152616	H300012331	Tripartite motif-containing protein 42
GRIK2	-2.3	3.99E-02	NM_175768	opHsV0400005190	Glutamate receptor, ionotropic kainate 2
OCM2	-2.2	1.97E-02	NM_006188	H300008785	Oncomodulin 2
PPP3R2	-2.2	1.95E-02	NM_147180	opHsV0400004040	Protein phosphatase 3, regulatory subunit B, beta
SYT16	-2.2	1.38E-02	NM_031914	H300006906	Synaptotagmin 16
REPS2	-2	2.23E-02	NM_004726	opHsV0400012490	RALBP1 associated Eps domain containing 2
HPS1	-2	2.08E-02	NM_000195	H300008011	Hermansky-Pudlak syndrome 1
SLC16A11	-2	2.63E-02	NM_153357	opHsV0400005676	Solute carrier family 16, member 11 (monocarboxylic acid transporter 11)
C6orf103	-2	3.59E-02	NM_024694	H300022013	Chromosome 6 open reading frame 103
TMEM105	-2	4.09E-02	NM_178520	H300008738	Transmembrane protein 105
NAP1L2	-1.9	3.68E-02	NM_021963	opHsV0400002947	Nucleosome assembly protein 1-like 2
OVCH2	-1.9	1.77E-02	NM_198185	opHsV0400002243	Ovochymase 2
RCAN2	-1.9	9.75E-03	NM_005822	H300002477	Calcineurin 2
ANKRD2	-1.8	4.92E-02	NM_020349	H300013293	Ankyrin repeat domain-containing protein 2

### Appendix 3

**Table A3.8 GO analysis of biological process.** Biological processes of differentially expressed genes identified following P4+E2+RU486 cotreatment.

Process	No:of Genes	Genes Involved
Ion binding	32	MYL6, REPS2, NUA1, OVCH2, PCDH20, RNF217, PPP3R2, OCM2, NR2C1, PRDM12, TRIM42, TAP2, ZNF407, PLCD4, ENTPD3, GALNT13, DHX57, SLC4A5, SP140L, SCN2B, SMG6, ZNF337, ZNF667, CYP26A1, CREB5, RNF175, ZSCAN4, ITGA9, PLCE1, FREM2, CA2, SCN4A
Transport	19	AQP5, ATP6V0E2, CLDN2, GJA8, GRIA3, GRIK2, IFNG, LOC440577, MDFI, MYL6, NUP210, RPL23, SCN2B, SCN4A, SLC26A1, SLC4A5, SMG6, STXBP4, TAP2
Transcription	15	PRDM12, MDFI, C11ORF9, ZNF667, PURB, IFNG, ZNF337, ZSCAN4, NR2C1, CREB5, PAX2, ZNF407, LOC440577, SP140L, SMG6
Metabolism	14	C11ORF9, CREB5, CRH, CYP26A1, IFNG, LOC440577, MDFI, NR2C1, PAX2, PURB, SRD5A2, ZNF337, ZNF667, ZSCAN4
Immune and inflammatory response	7	C7, IL23R, GRIK2, TAP2, IFNG, CRH, KIR3DL1
Apoptosis	6	C7, GRIK2, IFNG, CRH, PAX2, LOC440577
Signal transduction	5	C11ORF9, SCN2B, GRIK2, CRH, SRD5A2
Anatomical structure development	5	ITGA9, FREM2, PCDH20, CLDN2, EMILIN2
Development	3	FREM2, CA2, PAX2

**Table A3.9 List of genes identified to be involved in various pathways.**

Pathway Name	C Vs P4+E2		P4+E2 Vs P4+E2+RU486	
	Gene	Differential regulation	Gene	Differential regulation
Fc Epsilon R1 Signalling	SYK	-4.69	PRKCB1	2
	PLA2G2F	-3.86		
Glycan Biosynthesis	MAN1C1	10	FUT1	-2.62
	ST6GALNAC1	3.04		
GnRH Signalling	CACNA1C	3.79	GNRHR	-4.38
	PLA2G2F	-3.86	PRKCB1	2
Focal Adhesion	BCL2	4.42	ITGA9	-3.98
			PRKCB1	2
Natural Killer Cell Mediated Cytotoxicity	SYK	-4.69	KIR3DL1	-2.64
			IFNG	2.27

Appendix 3

			PPP3R2	-2.2
			PRKCB1	2
			PLCD4	4.81
			PPP3R2	-2.2
Calcium Signalling	CACNA1C	3.79	PRKCB1	2
Apoptosis	BCL2	4.42	PPP3R2	-2.2
Axon Guidance	EFNA5	2.56	PPP3R2	-2.2
	CACNA1C	3.79	PRKCB1	2
	PLA2G2F	-3.86	PPP3R2	-2.2
MAPK signalling	NTRK2	4.65		
			PRKCB1	2
VEGF Signalling	PLA2G2F	-3.86	PPP3R2	-2.2
B-cell Signalling	SYK	-4.69		
Olfactory Transduction			ADRBK2	3.59
Nitrogen Metabolism			CA2	-3.62
			CLDN2	-5.17
Cell Adhesion Molecules (CAMs)			ITGA9	-3.98
			CLDN2	-5.17
Leukocyte Transendothelial Migration			PRKCB1	2
Tight Junction			CLDN2	-5.17
			PRKCB1	2
Pyrimidine Metabolism			ENTPD3	2.31
			ENTPD3	2.31
Purine Metabolism			ALLC	-2.94
Gap Junction			GJA8	-3.66
Glycosphingolipid Biosynthesis Globoseries			FUT1	-2.62
Glycosphingolipid Biosynthesis Lactoseries			FUT1	-2.62
Glycosphingolipid Biosynthesis Neo-lactoseries			FUT1	-2.62
Regulation of Autophagy			IFNG	2.27
TGF-beta Signalling			IFNG	2.27
Type-I Diabetes Mellitus			IFNG	2.27
			IFNG	2.27
Cytokine-Cytokine Receptor Interaction			IL23R	-4.22
			IFNG	2.27
Jak-STAT Signalling			IL23R	-4.22

*Appendix 3*

ECM Receptor Interaction			ITGA9	-3.98
Regulation of Actin Cytoskeleton			ITGA9	-3.98
Antigen Processing and Presentation			KIR3DL1	-2.64
Oxydative Phosphorylation			NDUFA10	8.5
Phosphatidylinositol Signalling System			PCLD4	4.81
			PRKCB1	2
Inositol Phosphate Metabolism			PLCD4	4.81
T-cell Receptor Signalling			PPP3R2	-2.2
			IFNG	2.27
Long-term Potentiation			PRKCB1	2
			PPP3R2	-2.2
Wnt Signalling			PRKCB1	2
			PPP3R2	-2.2
ErbB Signalling			PRKCB1	2
Gap Junction			PRKCB1	2
Glioma			PRKCB1	2
Melanogenesis			PRKCB1	2
B Cell Receptor Signalling			PRKCB1	2
			PPP3R2	-2.2
Ribosome			RPL18A	2.84
Androgen and Oestrogen Metabolism			SRD5A2	5.28

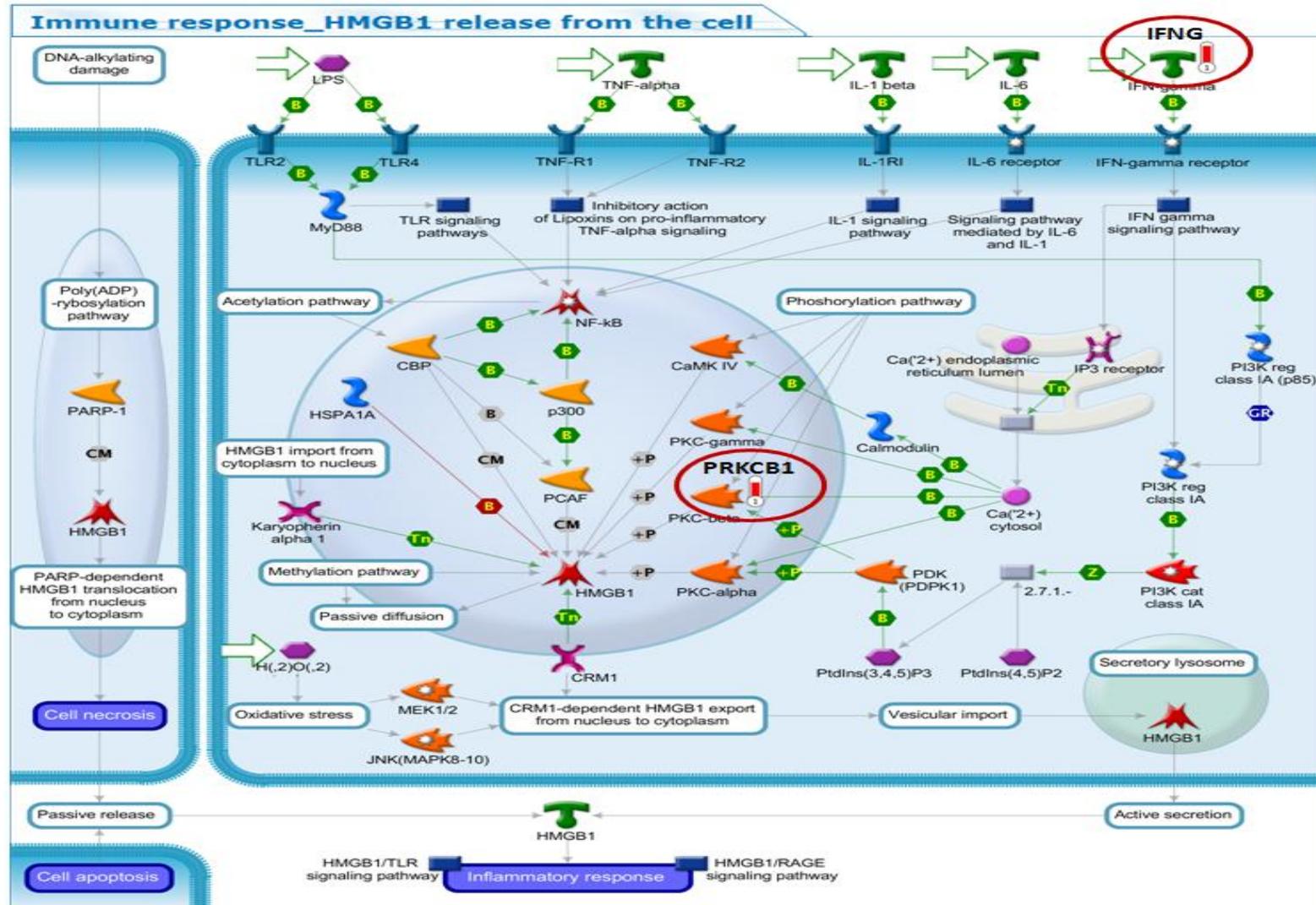


Figure A3.2: Immune response pathway by HMGB1 release from the cell. Genes identified in the present study in response to P4+E2+RU486-treatment is circled. Red circles represent upregulation of the gene in the present study.