Innate Immune Molecules in Preterm Birth and Disease

A thesis submitted for the degree of Doctor of Philosophy

By

Georgios Sotiriadis

Department of Life Sciences

College of Health and Life Sciences

Brunel University

January 2016

Declaration

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified, and has not been submitted for any other degree.

Georgios Sotiriadis

Dedication

To my parents, Alexis and Anastacia, to whom I owe everything; who I am, what I have achieved so far, and who I will be in the future. Σας αγαπώ and I always will, even if I don't say it enough. I promise I'll do it more!

Abstract

Preterm labour is one of the leading causes of perinatal mortality and morbidity. Most premature babies die before the age of five, and if they survive they face a high risk of disabilities and impairments. SP-A and SP-D are pattern recognition, innate immune molecules that are responsible for the maintenance of pulmonary immunity and surfactant homeostasis. They have been localised in the human reproductive tract and implicated in pregnancy and the initiation of labour. We hypothesised that SP-A and SP-D could play an active role in the activation of the myometrium and the timing of parturition.

In this study we investigated the effects of the recombinant forms of SP-A and SP-D (rhSP-A and rhSP-D) on contractile events in the myometrium. We validated the expression of surfactant proteins A and D in the *in vitro* model of myometrium cell line, ULTR, using qPCR, immunofluorescence and ImageStream technology. We demonstrate that rhSP-A and rhSP-D treatments led to an increase in cell motility and had an effect on the contractile response of ULTR cells when grown on collagen matrices showing reduced surface area. We studied this effect further by measuring the expression of contraction-associated protein genes. rhSP-A and rhSP-D led to an increase in the expression of oxytocin receptor and connexin 43. We reported that both rhSP-A and rhSP-D induce the secretion of pro-inflammatory cytokines, such as IL-6 and IL-8. We also propose a new model for functional progesterone withdrawal showing a cross-talk between progesterone (including non-genomic effects) and SP-A.

Emerging evidence has linked shredding of the uterine wall with ovarian cancer cases, suggestive of an interaction between myometrium and ovaries. We investigated the effects of rhSP-D in ovarian cancer *in vitro* using SKOV3 cells as a model. Conditioned media from ULTRs treated with rhSP-D resulted in growth arrest of SKOV3. We also demonstrated that rhSP-D led to a decrease in cell motility and cell proliferation in these cells. This was followed by an inhibition of the mTOR pathway activity. We expanded on our observations by measuring expression of SP-A and SP-D in human myometrial samples as well as the expression of SP-D in human ovaries. Collectively, this study presents novel interactions of SP-A and SP-D at both myometrial and ovarian level, rendering them key molecules for conditions such as preterm labour as well as implicating them as therapeutic targets for ovarian cancer.

Acknowledgments

To my parents and brother, who I can't thank enough for their drive and support all these years. I couldn't have done any of this without you.

A huge thank you to my supervisor, Dr. Karteris, who took me under his wing since I moved to the UK to do my MSc degree. We have collaborated perfectly all these years and I couldn't have asked for a better supervisor. I hope we get to work together again in the future!

A big thank you to my second supervisor, Dr. Kishore, who has given me guidance throughout this project and made the whole thing happen.

To Dr. Julie Davies and Dr. Karly Rogers-Broadway, I love you both and I thank you for the help, the laughter and the tears. You have made this PhD a little more bearable, if that can be true!

To Amanda Sara Rozeik, Dimple Chudasama, Eswari Dodagatta-Marri, Amir Jassim and Rooban Jeyaneethi, who I enjoyed working with. Thank you for your help and for making the lab, a bit more fun.

A massive thanks to my friends: Chris, Kostas, Marc, Panos, Robin, Stefan for listening to me, for their support, and for the good times. You are all very dear to me.

A special thank you to Marc Pritchard, who has spent the last four and a half years being there for me, supporting me and making me smile when I needed it the most.

And last but not least, to me. It has been a rollercoaster but I wouldn't change a thing! Thank you for all the choices, mostly right, and for allowing me to experience some of the best years of my life. Here's, to many more, Dr. Sotiriadis to be!

List of figures

List of tables

Abbreviations

2-ME	β-mercaptoethanol
ACTH	Adrenocorticotropic hormone
ALX	Lipoxin receptor
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CAP	Contraction-associated proteins
CDK1	Cyclin dependent kinase 1
cDNA	Complementary DNA
CL-K1	Collectin kidney 1
CL-L1	Collectin liver 1
CL-P1	Collectin placenta 1
CO_2	Carbon dioxide
COX ½	Cyclooxygenase ½
CRD	Collagen region domain
CRH	Corticotropin-releasing hormone
CT	Cycle threshold
CX43	Connexin 43
DAB	3,3'-Diaminobenzidine
DAG	Diaglycerol
DCs	Decidual cells
DEPTOR	Domain-containing mTOR-interacting protein
DHEA-S	Dehydroepiandrosterone sulphate
DMEM	Dulbecco's modified eagle's medium
DMs	Decidual macrophages
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNKs	Decidual natural killer cells
dNTP	Deoxynucleoside triphosphate
DPX	Distyrene plasticizer xylene

DTT	Dithiothreitol
E2	Estradiol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERα	Oestrogen receptor α
ERK	Extracellular-signal-regulated kinase
EVT	Extravillous trophoblast cells
FBS	Foetal bovine serum
fFN	Foetal fibronectin
FP	Prostaglandin F
GCs	Glucocorticoids
HIC	High income countries
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary axis
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IAV	Influenza A virus
ICD	International classification of diseases
IFN	Interferon
IL	Interleukin
IL-6 Ra	Interleukin 6 receptor alpha
IP_3	Inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Jak-STAT	Janus kinase-signal transducer and activator of transcription
LB	Luria broth
LIC	Low income countries
LPS	Lipopolysaccharide
Mφ	Macrophages
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKAP-1	Mitogen activated protein kinase associated protein 1

MBL	Mannan binding lectin
Mg^{2+}	Magnesium ion
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
Mn^{2+}	Manganese ion
mPRα-ε	Membrane progestin receptor α-ε
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa beta
NK	Natural killer
NSCLC	Non-small cell lung cancer
NTC	Non-template control
OD	Optical density
OT	Oxytocin
OTR	Oxytocin receptor
P_3R	Triphosphate receptor
P4	Progesterone
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PGs	Prostaglandins
PGHS-2	Prostaglandin H synthase 2
phIGFBP1	Phosphorylated insulin-like growth binding protein-1
PI3K-Akt	Phosphoinositide 3 kinase-Protein kinase B
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKCα	Protein kinase C alpha
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PRA/B	Progesterone receptor A/B
PREs	Progesterone-responsive elements
PROM	Premature rupture of membranes
PVDF	Polyvilidene fluoride
PWBCs	Peripheral white blood cells

qPCR	Quantitative polymerase chain reaction
Raptor	Regulatory associated protein of TOR
Rictor	Rapamycin insensitive companion of mTOR
RNA	Ribonucleic acid
RQ	Relative quantity
RSV	Respiratory syncytial virus
S6K	S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sHLA-G	Soluble HLA gamma
SMC	Smooth muscle cells
SP-A/D	Surfactant proteins A/D
TBE	Tris borate EDTA
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Treg	T regulatory cells
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organisation

Chapter 1 Introduction

1.1 Preterm birth

According to the World Health Organisation (WHO), preterm birth is defined as birth that occurs before the 37th week of gestation; whereas a full term pregnancy lasts 40 weeks (WHO, 2015). Approximately 15 million births are premature each year, a number which is rising (Blencowe *et al*., 2012). Preterm birth represents a financial burden to governments for the support of the preterm babies. In England and Wales, it costs approximately £3 billion per year (Mangham *et al*., 2009).

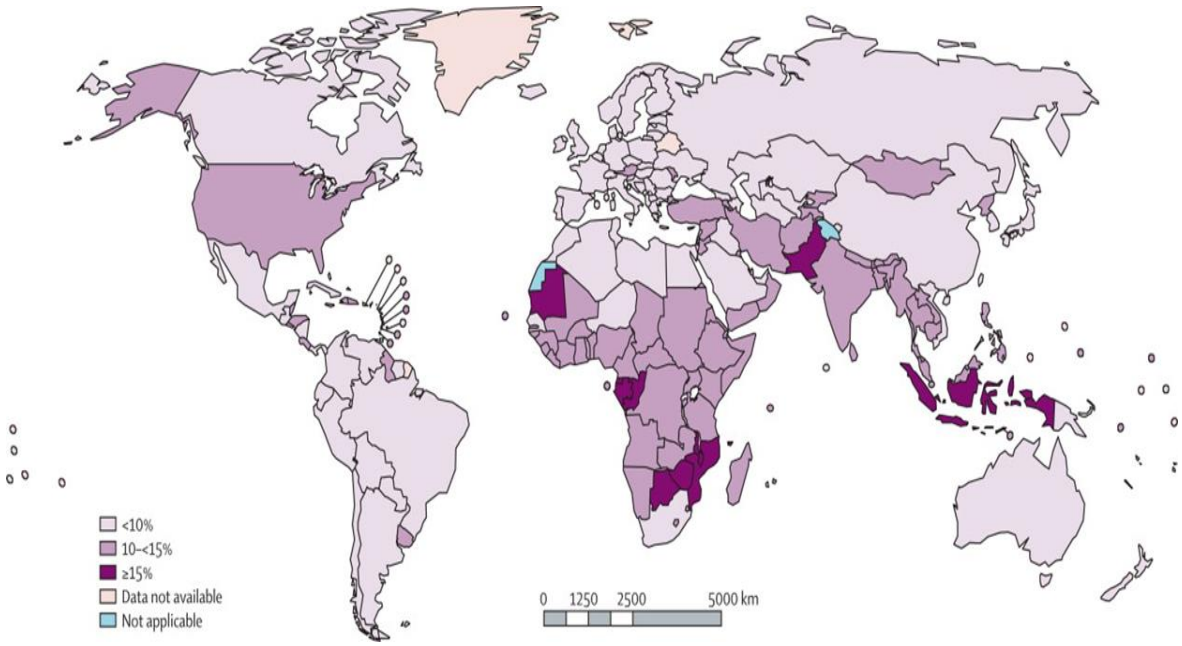


Figure 1.1 *Global estimated preterm birth rates in 2010. Higher rates of preterm birth are reported in Africa and some Asian countries. In the rest of the world, the United States have the highest preterm birth rates (Blencowe et al., 2012).*

There are three sub-categories of preterm birth depending on the timing of birth (Figure 1.2). Extreme preterm birth is when a baby is born prior to the 28th week of gestation, very preterm is when birth occurs between the 28th and 32nd week of gestation, and moderate to late preterm is birth that occurs between the 32nd and 37th week of gestation (WHO, 2015). Preterm labour is a major cause of neonatal deaths; approximately 35% of deaths are due to preterm birth, and it is the second cause of death in children aged up to 5 years old (Liu *et al*., 2012). Children that are born before the 37th week of gestation face a high risk of disabilities, some of which include respiratory illnesses, hearing and visual disabilities, and

cerebral palsy (Beck *et al*., 2009). Respiratory illnesses occur due to improper lung maturation which renders babies susceptible to infections (Mwaniki *et al*., 2012). Records show that the closer to term babies are born, the higher are the chances of survival; 50% chance of survival for babies born at the 24th week of gestation and 81% for those born after the 28th week (Costeloe *et al*., 2000).

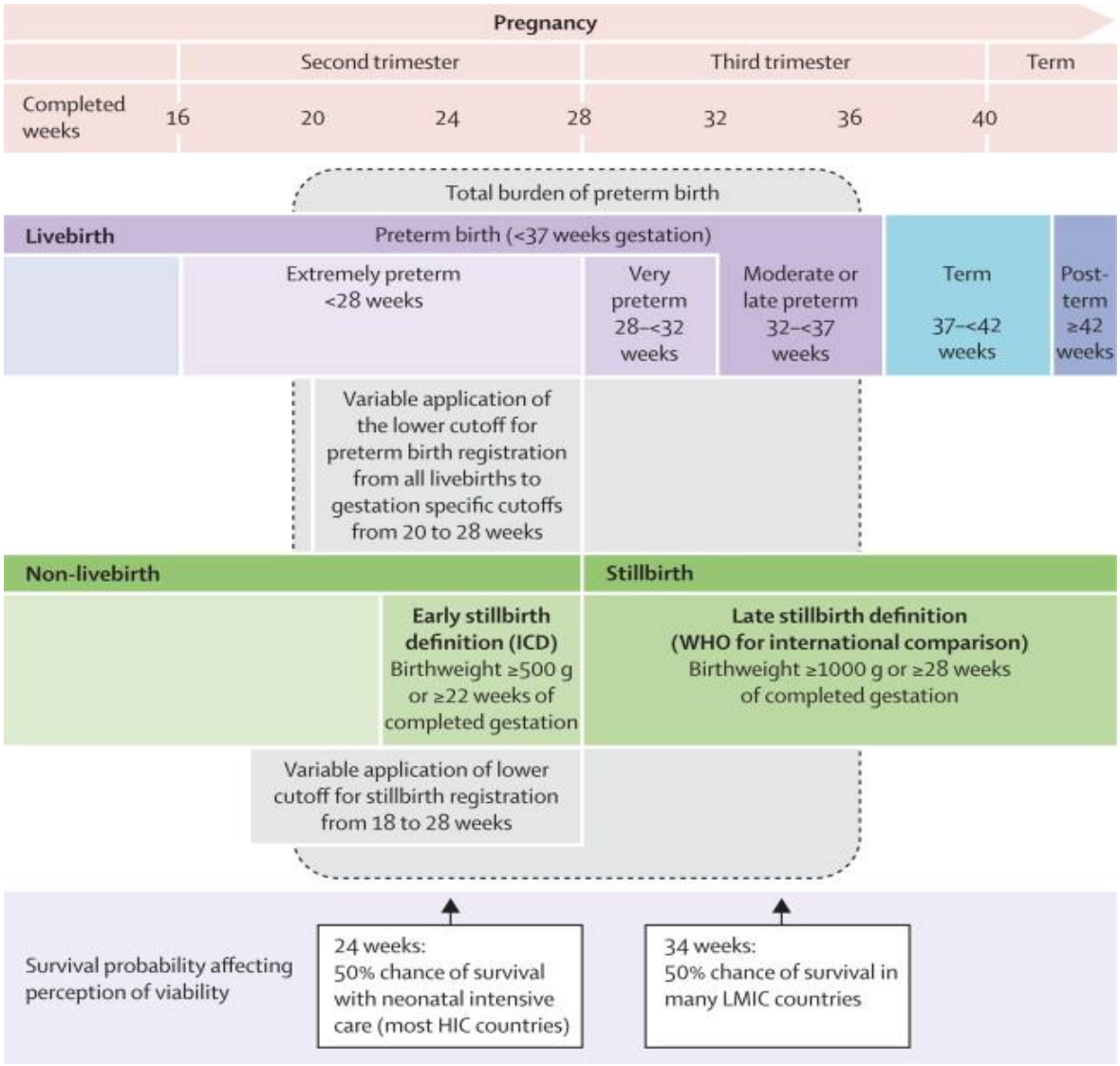


Figure 1.2 *Pregnancy, preterm birth definitions along with sub-categories (extreme, very preterm and moderate preterm) and survival probabilities in high and low-middle income countries (HIC and LMIC respectively). ICD: International Classification of Diseases, (Blencowe et al., 2012).*

To date, the actual causes of preterm birth have not been elucidated. Approximately 40% of the preterm births are idiopathic, premature rupture of membranes (PROM) accounts for

approximately 20% and the remaining 40% includes preterm births due to placental abruption, pre-eclampsia and infections (Henderson & Macdonald, 2004).

Approaches currently used to predict preterm birth include a small number of tests that could determine whether a woman is at risk of giving birth prematurely. First, there is an assessment of some risk factors. These include the demographic status of the pregnant woman, such as socioeconomic status, age and origin (Dekker *et al*., 2012). Other factors include the history of the woman, the outcome of previous pregnancies, genetic predisposition and the current status of the pregnancy, whether there are infections or bleeding and diabetes (Greco *et al*., 2012). Another measure is the cervical measurement. Some pregnant women due to genetics or other biological/developmental reasons can have a short cervix. The length of the cervix can be measured using a transvaginal ultrasound, although this test alone cannot be a competent indicator of preterm birth or used as a screening tool (Berghella *et al*., 2013). Another assessment used in conjunction to cervical measurements and risk assessment is the analysis of the biochemical milieu in samples from saliva, urine, blood, cervicovaginal and amniotic fluid. The aim is to pinpoint to specific biomarkers that can indicate risk of preterm birth. Biomarkers currently used are cytokines such as interleukins (IL-6, IL-6 Ra, IL-8, IL-1β, IL-10), foetal fibronectin (fFN) and phosphorylated insulin-like growth factor binding protein-1 (phIGFBP1), which are currently the most assessed biomarkers due to their effectiveness and specificity, and other biomarkers that arise via analysis of those bodily fluids using microarray analysis. The limitation of this approach is that each study identifies new biomarkers that do not necessarily apply to all pregnancies due to heterogeneity of the pregnant women and the factors that could lead to preterm birth. It is important therefore, to identify potent biomarkers for women that are both symptomatic and asymptomatic, and have high specificity and sensitivity (Parker and Brennecke, 1995; Paternoster *et al*., 2007; Khambay *et al*., 2012; Georgiou *et al*., 2015).

1.2 The placenta and the myometrium

The placenta is a ‘temporary’ organ of high complexity. For the placenta to be able to expand in order to house the foetus and allow its growth, its co-operation with the foetal membranes and the amniotic fluid is necessary (Gude *et al*., 2004). The placenta regulates several functions such as nutrient supply, protection from pathogens and clearing of waste, and the balance between pro and anti-inflammatory processes to prevent rejection of the

foetus which is considered as semi-allogenic (Jurisicova *et al*., 2005, Yadav *et al*., 2014). The mature placenta has the shape of a discus, a diameter of 22 cm and weighs approximately 500g (Burton and Fowden, 2015).

When the foetus implantation is successful, the placenta starts developing. It starts forming from the blastocyst from which the embryo is derived (Figure 1.3). During the initial few weeks, the development of the placenta occurs under low oxygen conditions. After the initial period, the placenta transforms drastically (Burton *et al*., 2010). The placental trophoblast cells form a layer between the maternal side and the foetus to allow blood transferring oxygen and nutrients to the foetus (Loke and King, 1995). Trophoblast cells differentiate to villous cells that regulate the nutrient transfer, and the extravillous cells which pass the myometrium and invade the uterine wall (Benirschke *et al*., 2005). The umbilical cord connects the placenta, through the villi that form the villous tree, to the foetus to allow the transfer of important components for the survival of the foetus. It consists of an umbilical vein that is wrapped by two arteries (Sood *et al*., 2006).

On the foetal side, the foetus is surrounded by the amniotic fluid that is enclosed by the amnion also called the 'amniotic sac'. The amniotic sac grows proportionally to the growth of the foetus to sustain pregnancy. The amniotic fluid passes through the foetal lungs and back to the amniotic sac, forming a communication between the mother and the foetus (Marieb and Hoehn, 2007).

The maternal part of the placenta is formed by the chorion and the decidua. The chorion is between the amnion and the cytotrophoblasts (Robbins *et al*., 2010). The villous trees are covered by the chorion and more specifically the synciotrophoblasts, which are thought to inhibit the circulation of pathogens from the maternal blood to the placenta (Robbins *et al*., 2010).

The decidua is adjacent to the placenta on the uterine wall and it derives from the endometrium. It consists of the decidua basalis, the decidua capsularis and the decidua parietalis (Burton and Jauniaux, 2015). Surrounding the decidua is a thick smooth muscle layer; the myometrium (Aguilar and Mitchell, 2010).

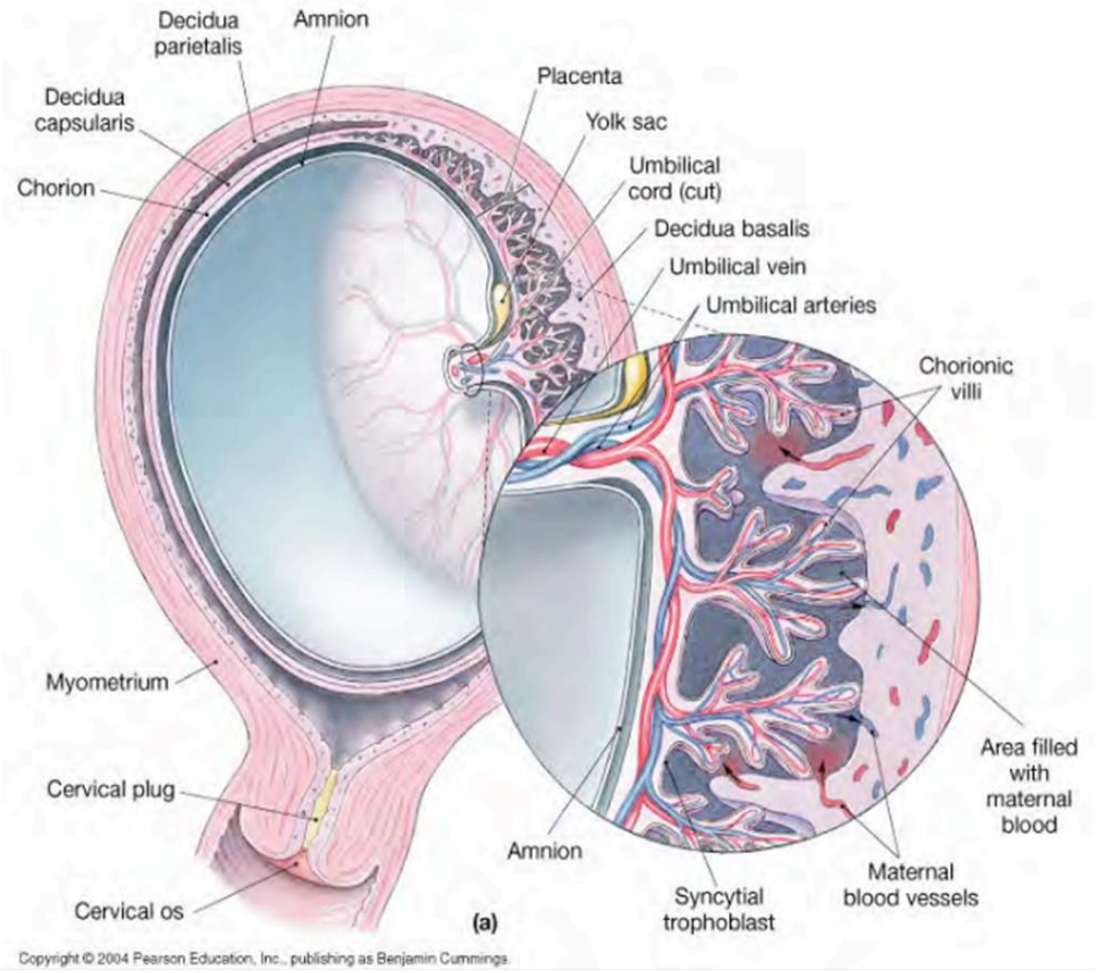


Figure 1.3 *Placenta and the foetus. After foetus implantation, the placenta starts developing. It starts forming from the blastocyst from which the embryo is derived. The placental trophoblast cells form a layer between the maternal side and the foetus to allow blood transferring oxygen and nutrients to the foetus. Trophoblast cells differentiate to villous cells that regulate the nutrient transfer, and the extravillous cells which pass the myometrium and invade the uterine wall. The foetus is housed inside the amniotic sac that contains the amniotic fluid and connects to the placenta with the umbilical cord. The maternal part of the placenta is formed by the chorion and the decidua. The myometrium surrounds the placenta and is adjacent to the decidua (Marieb and Hoehn, 2007).*

The myometrium is comprised of smooth muscle cells (SMCs) that express myosin and actin. SMCs are small and contain myofilaments that occupy most of the cell volume and are responsible for the contractility of the myometrium (Gunst and Zhang, 2008). During the early stages of pregnancy, the myometrium is in a quiescent, non-contracting state. Towards the end of pregnancy, it transitions from a quiescent to a contractile state prior to

delivery (Pehlivanoğlu *et al*., 2013). Early contraction of the myometrium could result in preterm birth.

The placenta, therefore, acts as a link between the maternal and the foetal side, allowing the transportation of oxygen and nutrients that are necessary for the development of the foetus, by diffusion. It can also act as a barrier by blocking pathogens from the maternal side and also allowing the infiltration of maternal antibodies to protect the foetus (Phillips *et al*., 2003). Expression of important hormones such as growth factors, insulin, progesterone (P4) and oestrogen takes place in the placenta. These hormones are necessary for the growth of the placenta to meet the demands of the foetus but also the maintenance of pregnancy (Fowden *et al*., 2009).

1.3 Phases of parturition

For most of the gestation period, the uterus is in an inactive state, also characterised as quiescence (Rabotti and Mischi, 2015) (Figure 1.4). During this stage, the myometrium remains inactive to allow the development of the foetus, and this is regulated by a variety of components such as progesterone, nitric oxide and relaxin (Norwitz and Robinson, 2001). Although they regulate this via distinct pathways, the end result is to reduce the amount of Ca^{2+} in the cells. Less Ca^{2+} means that myosin light chain-kinase (MLCK) remains in its non-phosphorylated inactive state; MLCK's main activity is to phosphorylate myosin, which then binds to actin leading to contractions due to Ca efflux (Arrowsmith and Wray, 2014). Occasionally, the myometrium contracts during this phase; these contractions have been called Braxton-Hicks and they tend to be mild and not lead to parturition (Harding *et al*., 1982).

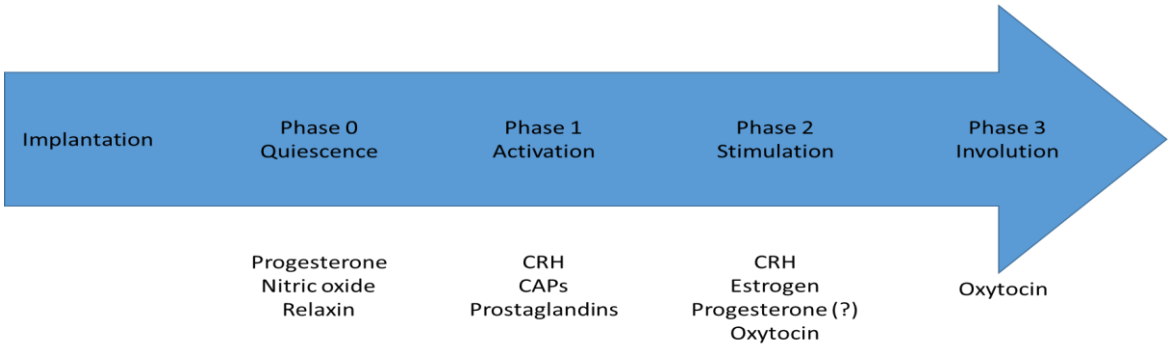


Figure 1.4 *Phases of pregnancy from implantation to involution. Several components mediate each phase and enable the transition to the next. After implantation the myometrium is in the quiescent state to allow foetal development and growth. When the foetus is ready, the myometrium is activated and stimulated to allow birth. The last stage is involution that allows cervix repairment. Progesterone, contraction-associated proteins (CAPs), steroids and prostaglandins are key mediators of labour.*

Towards the end of gestation there is a biochemical change in the uterine environment that leads to myometrial activation and birth. The main modulators of the activation of the myometrium are the contraction associated proteins (CAPs), which include connexin 43 (CX43), oxytocin receptor (OTR) and prostaglandins (Shynlova *et al*., 2013). The upstream signals that lead to the upregulation of these molecules may originate in the foetal hypothalamic-pituitary-adrenal axis (HPA) or can be a result of mechanical stretch. When the foetus is mature, corticotropin-releasing hormone (CRH) is released by the HPA axis and the human placenta, which results in the increased expression of cortisol and androgen that eventually lead to myometrial contractility (Smith *et al*., 2002).

Myometrial activation is followed by myometrial stimulation. During this phase the decidual and foetal membranes are activated, due to increased expression of prostaglandins and CAPs. The uterus begins to contract and the cervix ripens to prepare for labour (Arrowsmith and Wray, 2014). In rodents, this phase is also characterised by a progesterone withdrawal. The drop in circulating progesterone levels results in an increase in oestrogen (Mitchell and Wong, 1993). In humans, withdrawal of progesterone is not observed and both steroid levels remain high throughout pregnancy. Increasing levels of CRH and oestrogen (from androgens) lead to increased expression of prostaglandins that enhance myometrial contractility (Challis *et al*., 2000). Uterine contractility is regulated by the amount of Ca^{2+} in the cells. Ca^{2+} forms a complex with calmodulin which leads to the

phosphorylation of myosin that binds to actin to initiate contractions (Figure 1.5, Arrowsmith *et al*., 2014).

The last stage of the stimulation is the cervical ripening which can last several weeks. This stage is characterised of a drastic reconditioning and remodelling of the cervix which is the result of pro-inflammatory cytokines, prostaglandins and steroids (Romero *et al*., 2004). The last phase of labour is involution that consists of uterine contractions, the separation of the placenta from the uterine wall and cervical repair after birth (Jorge *et al*., 2014).

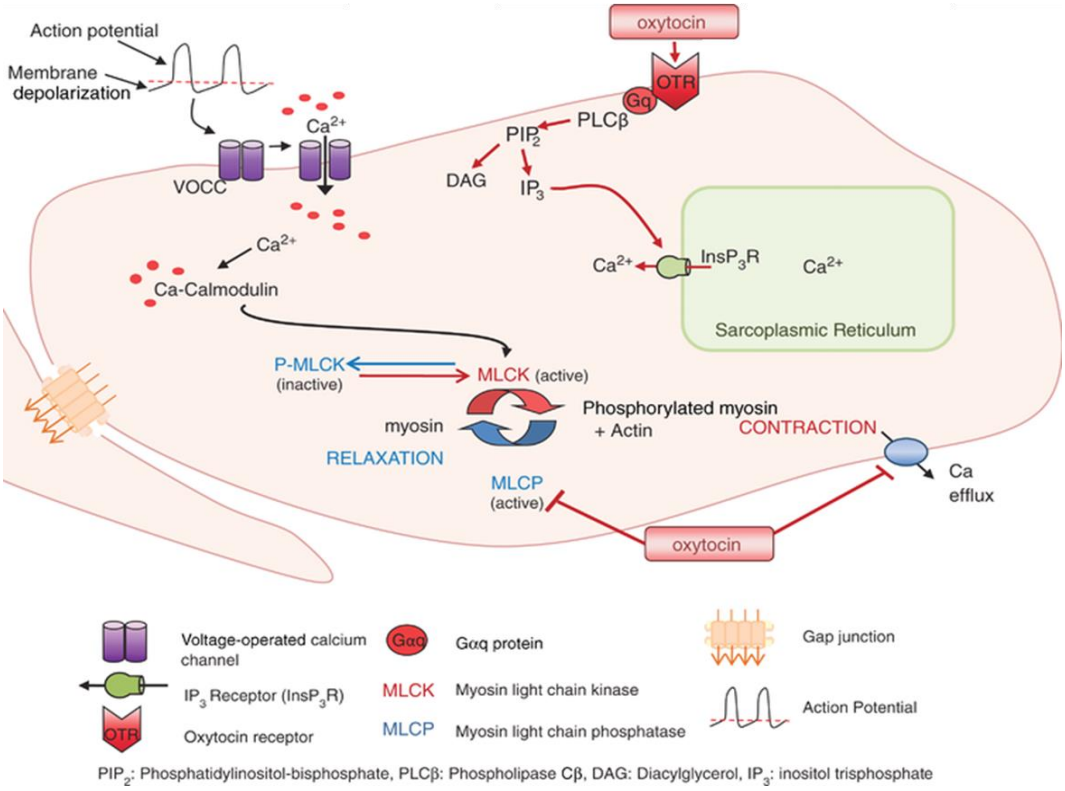


Figure 1.5 *Myometrial cell contractility during labour. Ca^{2+} channels allow the entrance of Ca^{2+} inside the cells which forms a complex with calmodulin that phosphorylates myosin light chain kinase and leads to contractions. Oxytocin binds to its receptor and activates the inositol 1,4,5-trisphosphate (IP_3) pathway that leads to the increase of Ca^{2+} inside the cell. Gap junction proteins regulate contractility cell synchronisation (Arrowsmith et al., 2014).*

1.4 Pathways leading to preterm birth

Over the last few years, research has focused on the signalling mechanisms that lead to preterm birth. There have been four main pathways identified that affect components similar to those that are activated during normal labour and lead to the initiation of parturition; 1) the premature activation of the HPA axis, 2) infections, 3) ischemia and decidual haemorrhage and 4) uterine distension (Lockwood, 2003). These pathways have an effect on the decidual and foetal membranes leading to preterm birth (Figure 1.6).

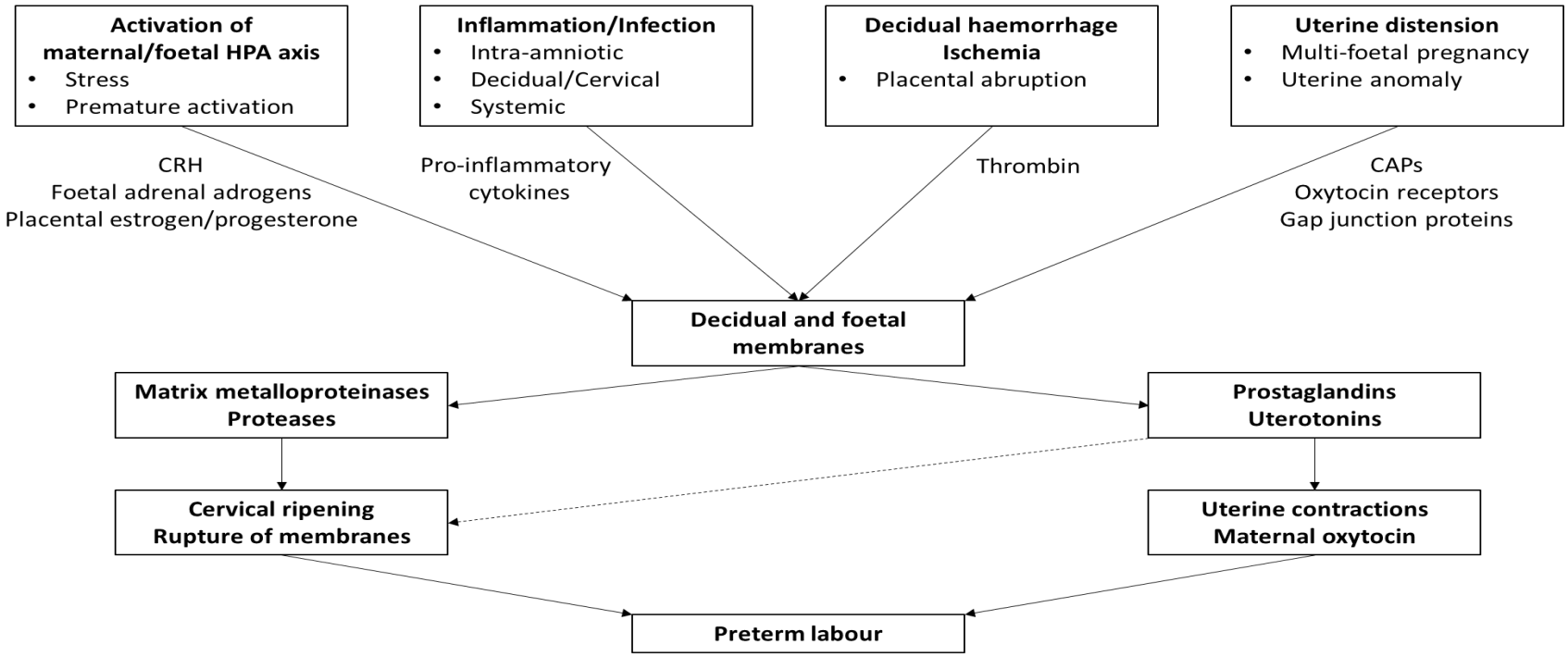


Figure 1.6 *Pathways that lead to preterm birth. Upstream effects (HPA activation, infection and inflammation, decidual haemorrhage and uterine distension), mediated by the action of steroids, pro-inflammatory cytokines and CAPs, lead to preterm activation of the decidual and foetal membranes which results in uterine contractility and cervical ripening via matrix metalloproteinases (MMPs) and uterotonins (adapted from Institute of Medicine US, 2007).*

1.4.1 HPA axis and Corticotropin-releasing hormone (CRH)

Towards term, CRH is progressively expressed in the foetal placenta and controls the HPA axis. CRH is a hormone that plays a role in the activation and stimulation of the myometrium (Smith *et al*., 2002). It has been shown that CRH has a circadian rhythm and potentially acts as a "clock" that regulates the extent of gestation and the timing of birth (McLean *et al*., 1995). CRH binds to CRH binding protein towards the end of term and as more CRH is produced its plasma levels increase, leading to parturition (McLean and Smith, 2001). Previous research has shown that in both term and preterm births placental CRH levels are similar (Torricelli *et al*., 2007). Maternal stress, whether that is psychological or social, has been reported to affect the timing of parturition (Wadhwa *et al*., 2001). CRH levels in the placenta affect the foetal HPA axis which leads to the expression of adrenocorticotropic hormone (ACTH) and regulates the expression of cortisol (Figure 1.7). Cortisol in a positive feedback loop upregulates the expression of CRH leading to myometrial activation and labour (Vrachnis *et al*., 2012). Pro-inflammatory cytokines such as IL-1β and tumour necrosis factor alpha (TNF-α), also induce expression of CRH thus affecting the timing of parturition (Dudley, 1999). Progesterone mainly acts as an inhibitor of CRH but due to the functional withdrawal observed in labour, it fails to control CRH expression thus leading to preterm birth (Smith, 2007).

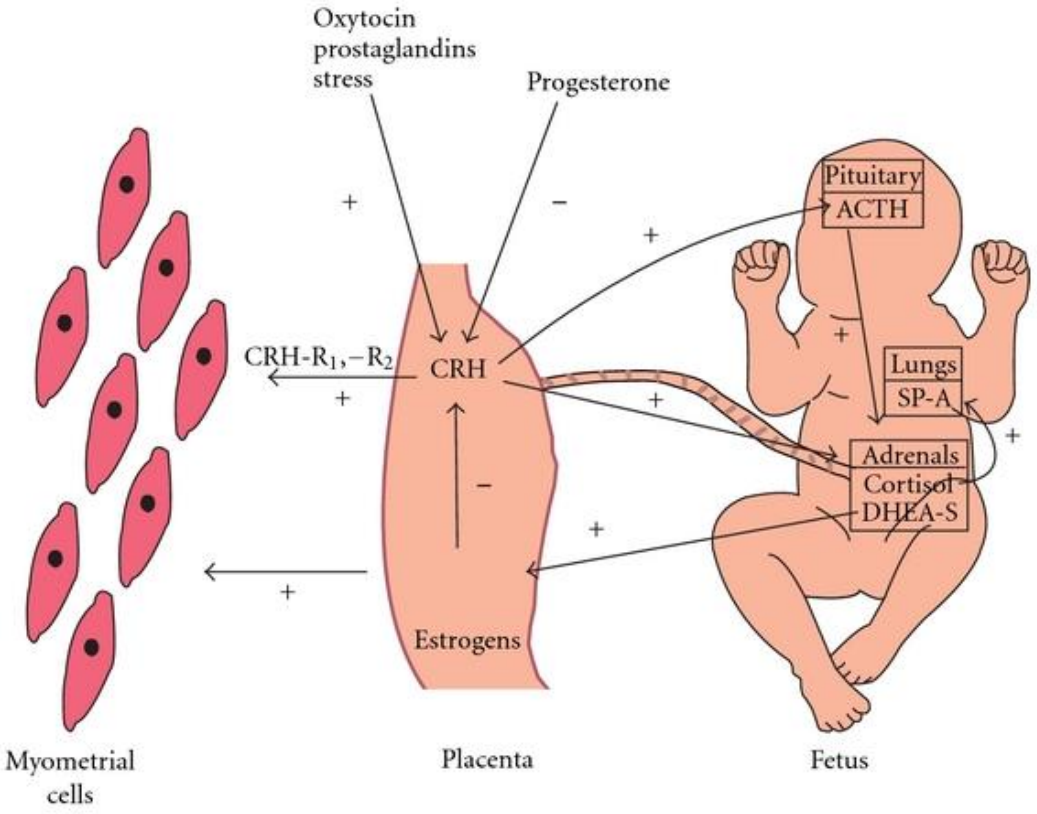


Figure 1.7 *CRH expression in the placenta and foetus. Stress causes CRH increase in the placenta which affects the foetal HPA axis. This results in an increase in the expression of CRH that leads to the expression of cortisol and surfactant protein A (SP-A). Increase in foetal CRH leads to further expression of CRH in the placenta in a positive feedback loop. CRH can also cause myometrial cells to initiate labour (Vrachnis et al., 2012).*

1.4.2 Uterine distension

Uterine stretching has been associated with preterm birth. Stretching of the myometrium leads to the expression of CAPs such as CX43, OTR and cyclo-oxygenase 2 (COX2) (Terzidou *et al*., 2005). Nitric oxide plays a key role in the relaxation of the myometrium. Nitric oxide increase in uterine smooth muscle cells leads to the inhibition of myosin phosphatase and therefore reduces the contraction of the cells (Buxton, 2004). Loudon *et al*., showed that uterine stretching can lead to the increase of pro-inflammatory cytokines that regulate the reconditioning of the myometrium and result in cervical ripening (Loudon *et al*., 2004). The

Mitogen-activated protein kinases (MAPK) pathway can induce the expression of IL-8 along with prostaglandin H synthase 2 (PGHS-2), leading to preterm labour (Souranna *et al*., 2005).

1.4.3 Decidual haemorrhage

Decidual haemorrhage accounts for approximately 10% of preterm birth incidents (Ananth *et al*., 1999). Ischemia is inadequate blood supply that is a result of haemorrhage due to placental abruption or retrochorionic haematoma (Han *et al*., 2011). A key mediator of decidual haemorrhage that leads to PROM is thrombin. Thrombin is responsible for uterine contractility; it binds to G protein-coupled receptors (GPCRs) which results in phospholipase C (PLC) activation (Macfarlane *et al*., 2001). PLC activity results in the increase of Ca^{2+} inside the cells, which can activate myosin and relaxin coupling and induce contractions (Arrowsmith *et al*., 2014).

Thrombin can also lead to the expression of MMPs, which in turn, promote degradation of the extracellular matrix and collagen, and result in PROM (Rose *et al*., 2002). Lockwood *et al*., have shown that thrombin can result in an increase of IL-8 levels and promote neutrophil infiltration in the decidua. This leads to an increase in MMP expression which has been associated with preterm birth (Lockwood *et al*., 2005).

1.4.4 Inflammatory environment in preterm birth

Infections, such as bacterial vaginosis (BV) and urinary tract infections (UTIs), can affect the inflammatory milieu in the placenta and eventually lead to preterm birth (Cappelletti *et al*., 2016). Infections have been associated with PROM that results in preterm birth (Gomez *et al*., 1998). Pro-inflammatory cytokines can induce the expression of glucocorticoids (GCs) via the HPA axis, which in turn, induce a negative-feedback loop reducing the expression of those cytokines (McEwen *et al*., 1997). Chronic stress dysregulates this negative feedback and leads to further expression of pro-inflammatory cytokines resulting in preterm birth.

Another pathway that has been suggested to lead to preterm birth are infections in the reproductive tract (Andrews *et al*., 2000). Intrauterine infections can lead to chorioamnionitis which is characterised by the infiltration of neutrophils in the amnion and chorion, and the

expression of pro-inflammatory cytokines such as IL-1 and IL-6 (Figure 1.8) (Kim *et al*., 2015). Pro-inflammatory cytokines produced by macrophages (Mφ) and decidual cells (DCs) due to infection from bacteria can induce the expression of prostaglandins and eventually lead to the activation of the myometrium and preterm birth (Romero *et al*., 2005). Lower genital tract infections, such as bacterial vaginosis, can also lead to preterm birth (Kimberlin and Andrews, 1998).

During infections, the innate immune system acting as the first line of defence, is able to fight pathogens that could lead to infections and the expression of pro-inflammatory cytokines. However, prolonged exposure to pathogens, results in continuous activity of the immune system leading to preterm birth (Kemp, 2014).

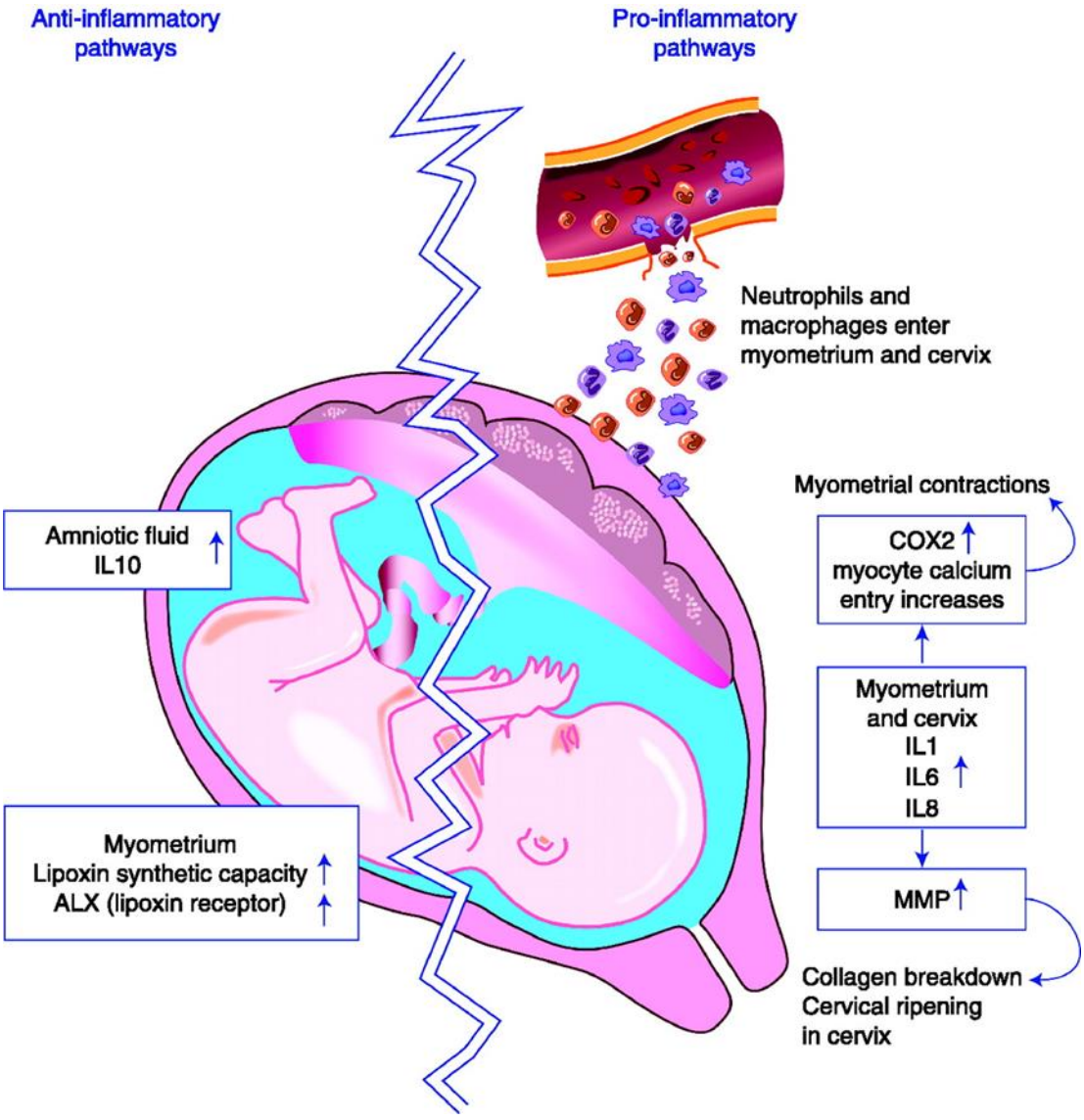


Figure 1.8 *Balance between pro and anti-inflammatory pathways in pregnancy. Invasion of neutrophils and macrophages leads to increased expression of pro-inflammatory cytokines such as IL-1, IL-6 and IL-8 which lead to the expression of MMPs and CAPs. The anti-inflammatory pathways are characterised by the expression of IL-10 in the amniotic fluid and lipoxin in the myometrium (Jabbour et al., 2009).*

1.5 Immunity during pregnancy and labour

As the foetus has been characterised to be semi-allogenic, it makes maternal tolerance very important for the maintenance of pregnancy and parturition (Yadav *et al*., 2014). The innate

immune system is activated early in pregnancy whereas the adaptive is restricted. The innate immune system consists of natural killer (NK) cells, monocytes, neutrophils, macrophages and pattern recognition molecules such as Toll-like receptors (TLRs) and collectins, whereas the adaptive immune system consists of B and T lymphocytes, trophoblasts and antigen-presenting cells (APCs) (Figure 1.9, Sykes *et al*., 2012). There is a cross-talk between pro and anti-inflammatory components and signals from both the maternal and foetal side in order to prevent rejection of the foetus, and also protect it from pathogens (Warning *et al*., 2011).

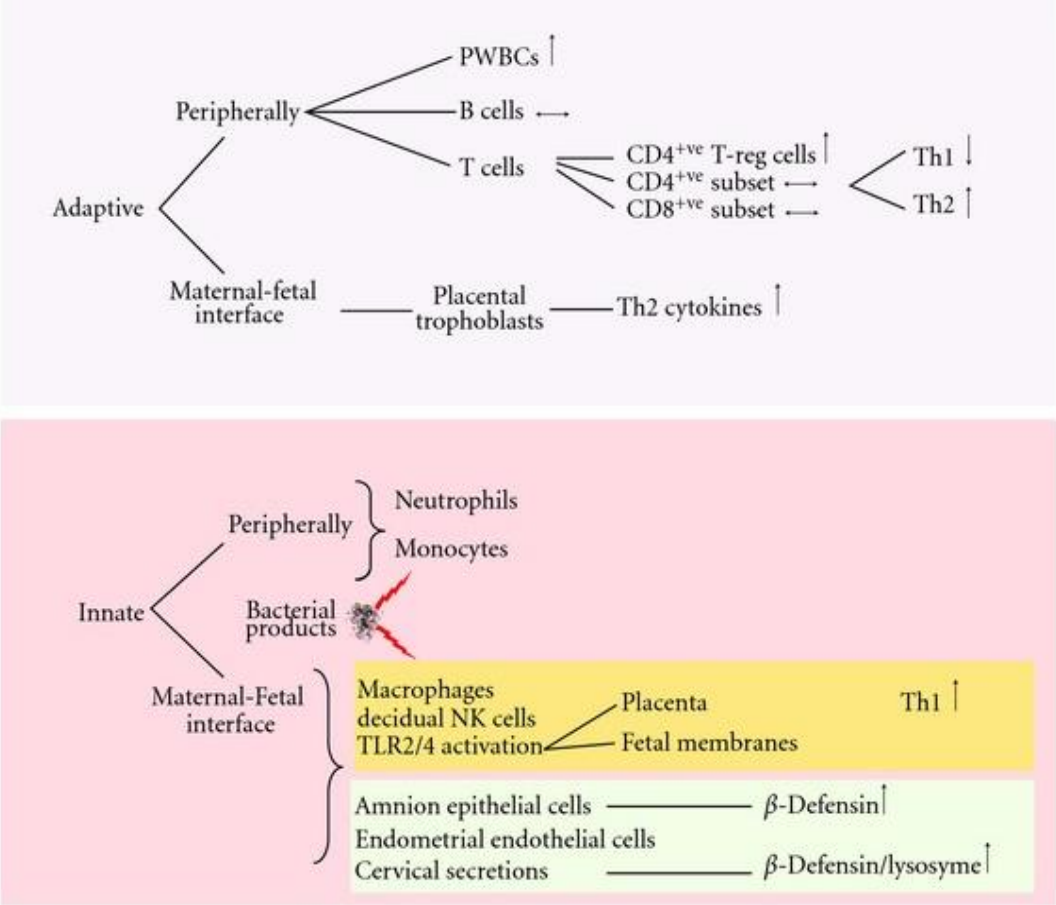


Figure 1.9 *Components and functions of the adaptive and immune system in pregnancy. There is a balance between pro and anti-inflammatory responses to promote maternal tolerance. The adaptive immune system is comprised of peripheral white blood cells (PWBCs), Treg cells and trophoblasts that are upregulated to lead to an anti-inflammatory state. The innate immune system however, comprised of neutrophils, monocytes, macrophages, NK cells and TLRs secrete pro-inflammatory cytokines (Sykes et al., 2012).*

Trophoblast cells are responsible for the sustainability of the maternal side towards the foetus (Hofmeister and Weiss, 2003). They regulate the expression of human leukocyte antigens (HLA) to suppress a pro-inflammatory response against the parental markers presented on trophoblasts that are produced from the foetal side (Hviid, 2006).

B and T lymphocytes are specific and could lead to the rejection of the foetus. For that reason, the adaptive immune system is restricted during pregnancy in order to sustain birth; an effect which is mainly regulated by Treg cells (Sasaki *et al*., 2004). Based on the antigen presenting cells and the type of cytokines in the environment, $CD4^+$ T cells can differentiate into T helper cells: Th1, Th2, Treg or Th17 (Figure 1.10, Warning *et al*., 2011). There is a balance between Th1 (pro-inflammatory, e.g. IL-6, 8, TNF-α) and Th2 (anti-inflammatory, e.g. IL-4, 5 and 10) responses that lead to the secretion of specific cytokines that enable clearing of pathogens but not triggering a prolonged immune response that could endanger the foetus (Soeters and Grimble, 2013).

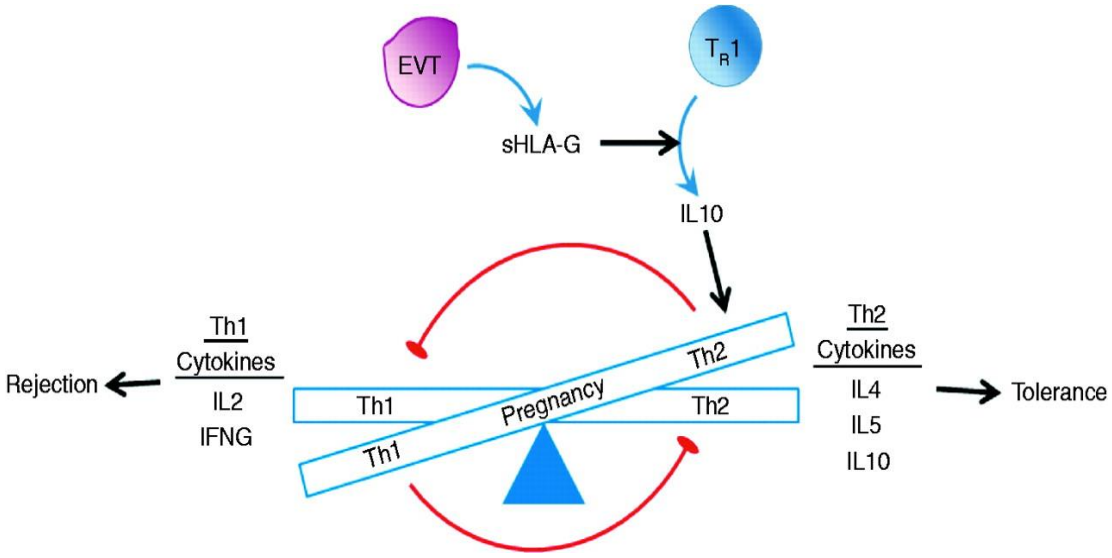


Figure 1.10 *Balance between Th1 and Th2 functions. Extravillous trophoblast cells (EVT) express HLA-G that induces the secretion of the anti-inflammatory cytokine IL-10 from Treg cells in order to sustain the foetus. A shift towards a Th1 response leads to foetal rejection. IL: interleukin, IFNG: interferon gamma, EVT: extravillous trophoblast, sHLA-G: soluble human leukocyte antigen gamma, T_R: Treg cells (Warning et al., 2011).*

In early pregnancy the production of Th17 is inhibited as it has a pro-inflammatory role, however it has been reported that Treg cells can transform into Th17 if there is a pro-inflammatory environment (Ito *et al*., 2010, Chaudry *et al*., 2009). Previous studies in mice have shown that depletion of Treg cells leads to pregnancy loss whereas injection of those cells can revert this effect and save the foetus (Aluvihare *et al*., 2004, Zenclussen *et al*., 2005).

During the first few months of gestation, the decidua consists of decidual NK cells, decidual macrophages (DMs), dendritic cells (DCs) and Treg cells (Figure 1.12). dNK cells' main role is to clear infected and tumour cells (Loke *et al*., 1995). They secrete various cytokines and growth factors to promote tissue remodelling and enable trophoblasts to invade the uterine wall (Hanna *et al*., 2006). Many diseases have been associated with low levels of dNK cells, such as preeclampsia and intrauterine growth restriction (Williams *et al*., 2009). DCs and DMs have anti-inflammatory effects. DMs express anti-inflammatory markers and cytokines such as IL-10 and transforming growth factor beta (TGF-β) in order to suppress immune responses (McIntire *et al*., 2004). They can, however, lead to a pro-inflammatory response when challenged with pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) (Singh *et al*., 2005). The same function is mediated by Treg cells that are also programmed to an anti-inflammatory state (Read and Powrie, 2001).

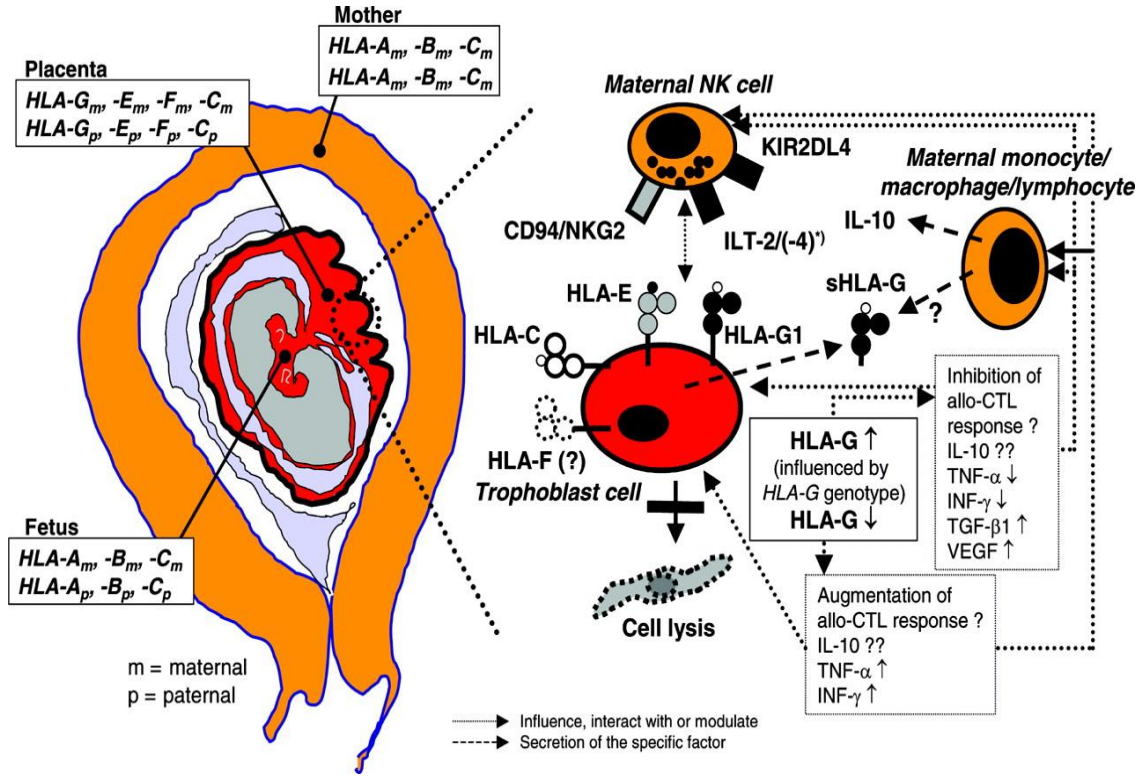


Figure 1.11 *Interaction between trophoblast cells, NK cells, macrophages and the secreted HLA class molecules from the maternal and paternal side, and cytokines to regulate inflammatory responses in the placenta. The expression of paternal antigens on trophoblast cells from the foetus poses a risk for its rejection, as they can be recognised by the maternal natural killer cells. Regulation of the maternal NK cells and expression of pro/anti-inflammatory cytokines by HLA-G and macrophages can prevent the lysis of trophoblast cells (Hviid, 2006).*

Monocytes and neutrophils recognise pathogens and clear them via phagocytosis. Continuous activation of those components has been associated with preeclampsia (Saito *et al*., 2007).

TLRs, a major part of the innate immune system, are transmembrane receptors that are able to identify PAMPs that are presented on pathogens such as bacteria, viruses and fungi (Koga *et al*., 2014). This family contains eleven members though only ten have been identified in the human placenta (Patni *et al*., 2009). Each receptor can recognise specific patterns, for example, TLR4 binds to LPS and TLR2 which can bind to a wider range of pathogens, but their actions can be expanded as they are able to form dimers; TLR1/2 and TLR2/6 (Poltorak

et al., 1998, Triantafillou *et al*., 2006). Inhibition of those receptors has been associated with preterm birth (Adams Waldorf *et al*., 2008).

Another family that recognises specific patterns on pathogens are the collectins, which are C-type lectins and they include the following: mannan binding lectin (MBL), collectin liver 1 (CL-L1), collectin kidney 1 (CL-K1), collectin placenta 1 (CL-P1) and surfactant proteins A and D (SP-A, SP-D respectively) (Ohtani *et al*., 2008).

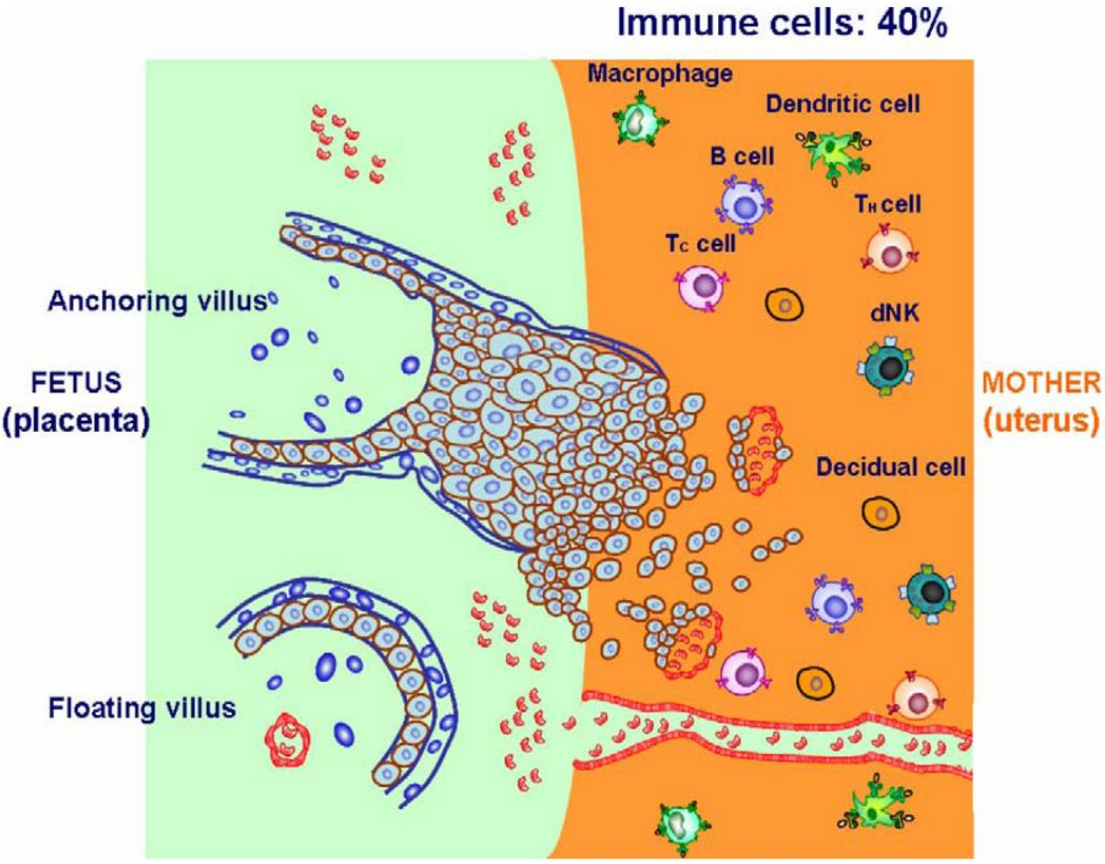


Figure 1.12 *Innate and adaptive immune cells in the decidua. Immune cells consist approximately 40% of the total cells in the decidua at the trophoblast invasion site (Weiss et al., 2009).*

1.6 Hormones and labour

There are a number of hormones that affect pregnancy and labour. The role of CRH and the HPA axis was described in section 1.4.1. Other hormones regulating pregnancy are: progesterone, oestrogen, prostaglandins and oxytocin.

1.6.1 Progesterone

Progesterone is a steroid hormone that was first identified in the 1930s in the corpus luteum and since then many studies have focused on this hormone, which is considered necessary for pregnancy and parturition. Apart from its role in the placenta, progesterone plays a role in the mammary glands, brain, and the central nervous and cardiovascular systems (Li *et al*., 2004).

In the uterus, progesterone acts mainly on the reproductive tract (Moutsatsou and Sekeris, 1997). When progesterone binds to progesterone receptors, those receptors bind to progesterone-responsive elements (PREs), thus regulating gene expression (Niswender *et al*., 2000). It also regulates the differentiation of stromal cells, creating a favourable environment for the development of the foetus (Maslar *et al*., 1986).

Progesterone plays a role during the quiescent state. It inhibits the uptake of extracellular Ca^{2+} by inhibiting the transcription of genes regulating the Ca^{2+} pathway, such as tachykinin and calmodulin, and therefore stops the contractility of the cells. It is, therefore, important throughout the duration of the quiescent state of the myometrium (Niswender *et al*., 2000). Another mechanism via which progesterone maintains quiescence is by inhibiting the binding of estradiol to α-adrenergic receptors and the expression of CAPs, which could lead to myometrial activation (Bottati *et al*., 1983, Mesiano *et al*., 2011). Progesterone can bind directly to OTR and inhibit the effects of oxytocin (Grazzini *et al*., 1998). Progesterone can also affect the immune system during pregnancy (Rinaldi *et al*., 2011). It has been shown to inhibit the expression of IL-8 and COX2 in the uterus, thus preventing labour (Loudon *et al*., 2003). Progesterone can also regulate antibody production and inhibit pro-inflammatory cytokine expression by decidual macrophages during pregnancy (Beagly and Gockel, 2003). It decreases the function of DCs and regulates NK cells and the transformation of T cells to Th2 cells that promote an anti-inflammatory environment (Szekeres-Batho *et al*., 2009).

Progesterone has been used in the clinical environment to inhibit preterm labour however, more studies need to be done to evaluate its prolific effects to inhibit preterm labour. Inhibitors of progesterone are used to induce birth whereas progesterone is used to arrest labour (Tita and Rouse, 2009). Progesterone is a product of cholesterol, and its production occurs in the mitochondria in the placenta. Cholesterol is cleaved to pregnenolone which 3β-hydroxysteroid dehydrogenase then converts to progesterone (Morel *et al*., 1997).

Progesterone mainly acts via two receptors; progesterone-receptor A and B (PRA and PRB, respectively) and they are members of the nuclear receptor superfamily. Nuclear receptors such as PR-B and PR-A are DNA-binding proteins that upon ligand binding recognize specific cis-acting hormone response elements typically located in the promoter region of target genes. The expression levels of these receptors change throughout the duration of pregnancy to enable support and development of the foetus but also lead to labour towards the end of term (Shynlova *et al*., 2013). PRB is the main receptor through which progesterone exerts its effects on gene transcription, whereas PRA acts primarily as a negative regulator of PRB functions (Karalis *et al*., 1996). Addition of IL-1β to myometrial cells leads to the expression of NF-kB which leads to the expression of PRs, suggesting that there is a cross-talk between progesterone functions and inflammatory components (Condon *et al*., 2006).

Across species, progesterone levels remain high throughout pregnancy but there is a significant decrease before labour. This is not however, the case with the human pregnancy, where progesterone levels remain high even during labour (Yen, 1991). The drop in functional progesterone levels is important for the contractility of the myometrium and successful parturition (Walsh *et al*., 1984). There have been many studies suggesting theories and models for a functional progesterone withdrawal towards term.

Karteris *et al*. reported of a new class of progesterone receptors, the membrane PRs (mPRs), which can transactivate PRB during pregnancy and enhance progesterone effects, but during parturition, they lead to a decrease of steroid receptor coactivator, and therefore, to a decrease in PRB effects (Figure 1.13). This results in a functional progesterone withdrawal that permits the contractility of the myometrium and birth (Karteris *et al*., 2006). It has been reported that progesterone exerts its non-genomic effects via mPRs (Zachariades *et al*., 2012). A non-genomic action defines any action that does not directly and initially influence gene

expression, as do the classical steroid receptors, but rather drives more rapid effects such as the activation of signalling cascades. These so-called 'non-genomic' effects do not depend on gene transcription or protein synthesis and involve steroid-induced modulation of cytoplasmic or cell membrane-bound regulatory proteins. There have been five isoforms identified: α, β, γ, δ, and ε (Gellerse *et al*., 2009).

mPRα has been localised in the placenta, the testes, the ovaries and the kidneys. It is the membrane receptor through which most rapid non-genomic effects of progesterone are exerted (Zhu *et al*., 2003a). mPRβ, however, has been localised in the brain, the spinal cord and the fallopian tubes (Zhu *et al*., 2003b). These receptors are crucial during the menstrual cycle, implantation of the foetus, its development throughout gestation and parturition. They can regulate the activation of the extracellular signal–regulated kinases (ERK) and MAPK signalling pathways, inhibit cyclic adenosine monophosphate (cAMP) production via coupling to an inhibitory Gα protein and activate Ca^{2+} channels (Gellersen *et al*., 2009). It has been reported that at term the levels of these two mPRs are decreased in the myometrium and that mPRα is the predominant isoform (Fernandes *et al*., 2005). It has also been proposed that mPRs enable the shift from myometrial quiescence to an active state (Karteris *et al*., 2006).

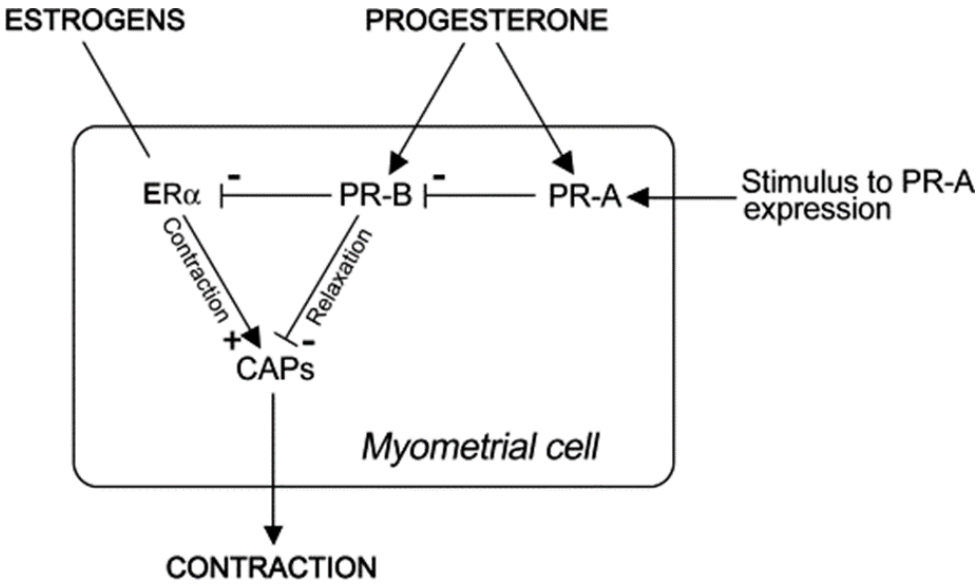


Figure 1.13 *Model for the functional progesterone withdrawal in the myometrium. Increase of PRA towards the end of term minimises the binding of progesterone to PRB, which in turn leads to expression of oestrogens and CAPs, resulting in the contractility of the myometrium (Smith et al., 2002).*

1.6.2 Prostaglandins

Prostaglandins (PGs) have been implicated in myometrial activation and labour. They are derived from arachidonic acid by COX. This enzyme, which has two isoforms; COX-1 and COX-2, converts arachidonic acid to prostaglandin H_2 and then, via the action of other synthases, to PGs (Helliwell *et al*., 2004). PGs mediate their effects via binding to GPCRs, with each PG having specific effects (Narumiya *et al*., 1999).

PGF_{2a} and PGE_2 have been characterised as the main PGs that regulate parturition and their main production site during labour is the amnion. These two PGs have been used for the last three decades as drugs to regulate the timing of labour (Thomas *et al*., 2014). PGF_{2a} binds to prostaglandin F (FP) receptor and enhances the uptake of Ca^{2+} in the cells leading to contractility (Parkington *et al*., 1999). PGE_2 leads to myometrial activation by decreasing the collagen content of the cervix, thus enabling its ripening, upregulating the expression of proteoglycans and MMPs (Figure 1.14) (Ekman *et al*., 1986, Normal *et al*., 1993, McLaren *et al*., 2000).

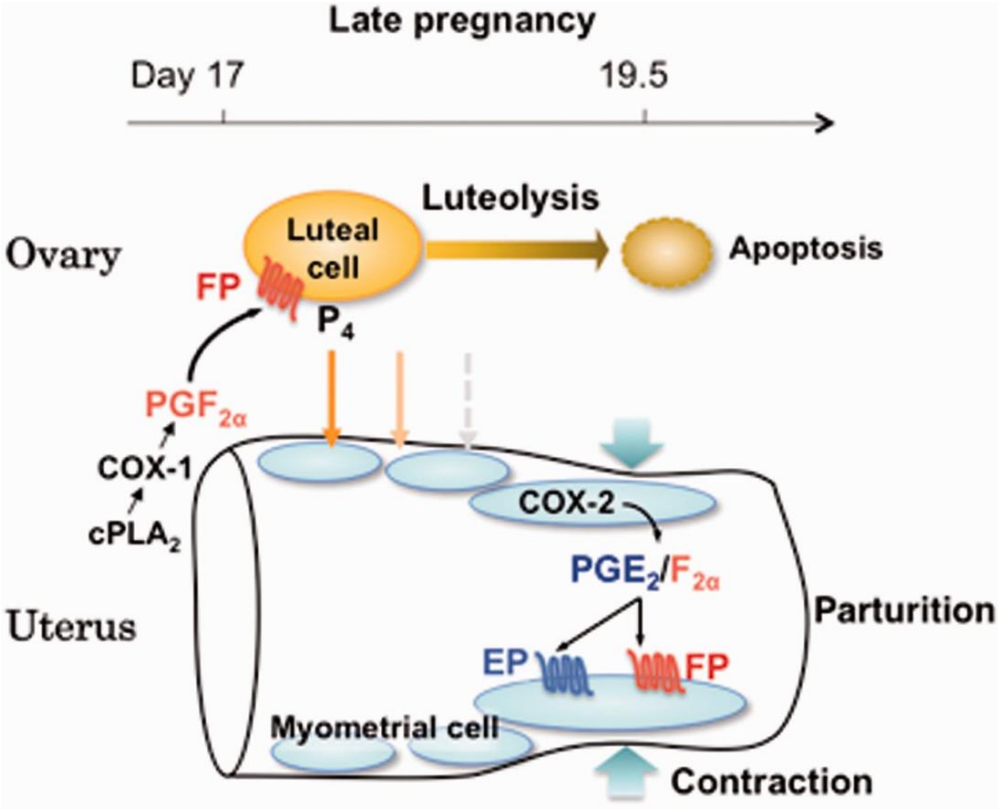


Figure 1.14 *Actions of PGs during parturition in mice. PGF_{2a} expression via COX-1 leads to FP binding and apoptosis of luteal cells that are a source of progesterone. Progesterone levels fall which results in further expression of PGs which leads to contraction and parturition (Sugimoto et al., 2015).*

PGs can regulate inflammation by interacting with various pro-inflammatory cytokines. These cytokines result in the increased expression of PG synthase which in a positive feedback loop can lead to expression of PGs (Aoki and Narumiya, 2012). PGF_{2a} has been shown to be upregulated by IL-1β via NF-κB in ULTR cells (Zaragosa *et al*., 2006). The same PG has been reported to activate IL-1β in the decidua, resulting in the expression of MMP9 (Christiaens *et al*., 2008).

Although PGs primarily have a pro-inflammatory effect, few studies have shown that they can also exert anti-inflammatory effects during pregnancy. PGD_2 in the placenta has been reported to shift the Th1/Th2 balance towards Th2 and also inhibit pro-inflammatory cytokines such as

IL-6 (Saito *et al*., 2002, Helliwell *et al*., 2006). PGJ_2 binds to specific receptors and inhibits the expression of pro-inflammatory cytokines (Keelan *et al*., 2003).

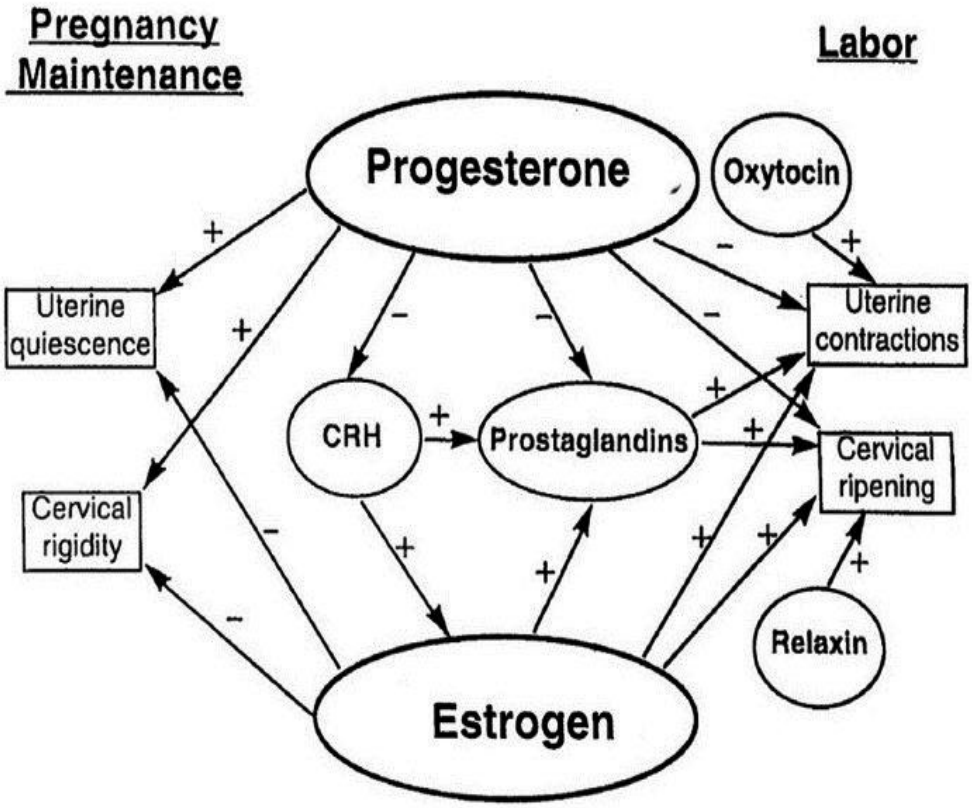


Figure 1.15 *Cross-talk of hormones regulating pregnancy and birth. Progesterone promotes myometrial quiescence and cervical rigidity by inhibiting the role/expression of oxytocin, CRH and prostaglandins. Oestrogen promotes activation by increasing the role/expression of those hormones leading to cervical ripening and parturition (Kota et al., 2013).*

1.6.3 Oxytocin

Oxytocin (OT) is a nonapeptide hypophysial neuropeptide hormone. The OT gene is located on chromosome 20 which is also the location of vasopressin (Rao *et al*., 1992). It was first discovered in 1906 in the human posterior pituitary gland when extracts of the gland were used to treat a pregnant cat. The extracts led to contractility induction in the feline uterus (Dale, 1909; Viero *et al*., 2010). Its main production site is the hypothalamus and it is released

from the posterior pituitary gland. It is also produced in peripheral tissues such as the amnion, the placenta and the testes (Viero *et al*., 2010).

OT plays a key role in several processes such as lactation, parturition, sexual behaviour and the regulation of the HPA axis (Neumann *et al*., 1996). Nishimori *et al*. reported that mice deficient in OT could not feed their offspring due to lack of contraction in the milk ducts, showing a role for OT in lactation (Nishimori *et al*., 1996). It acts through a single receptor, the OTR, which is a GPCR. This receptor needs Mn^{2+} and Mg^{2+} ions as well as cholesterol to be activated. The functions of OT are dependent on steroids although OTR genes do not have a steroid responsive element (Gimpl and Fahrenholz, 2001). Oestrogens have been reported to cause OTR increase in the uterus, whereas progesterone inhibits OTR expression (Arrowsmith and Wray, 2014). In the HPA axis, during stress, oxytocin leads to the expression of ACTH, which is inhibited by progesterone (Gibbs, 1986; Link *et al*., 1993).

One of the most important functions of OT is during the initiation of parturition. Before birth, levels of OT and OTR dramatically increase in the uterus (Blanks *et al*., 2003, Russell *et al*., 1998). Although this hormone has such important functions, it is not necessary for labour. Studies have shown that mice deficient in OT gave birth normally, and also women with deregulation of their pituitary gland had no issues during delivery (Otsuki *et al*., 1983, Young *et al*., 1996).

When OT binds to OTR, it leads to the dissociation of its subunits. Subunit α binds to ATP and activates PLC and diaglycerol (DAG). PLC then activates the IP3 pathway resulting in the release of Ca^{2+} (Gimpl and Fahrenholz, 2001; Lodish *et al.* 2007). In the uterus, activation of OTR leads to the cell membrane depolarization which allows the influx of more of Ca^{2+}. As described in section 1.3.1, this leads to smooth muscle cell contractions. OTR results in the activation of the MAPK pathway which leads to the transcription of genes that promote proliferation, and also to the expression of COX-2. Increased COX-2 levels further drive the expression of prostaglandins. OTR binding can activate the adenylyl cyclase pathway which promotes cAMP (Figure 1.16, Viero *et al*., 2010).

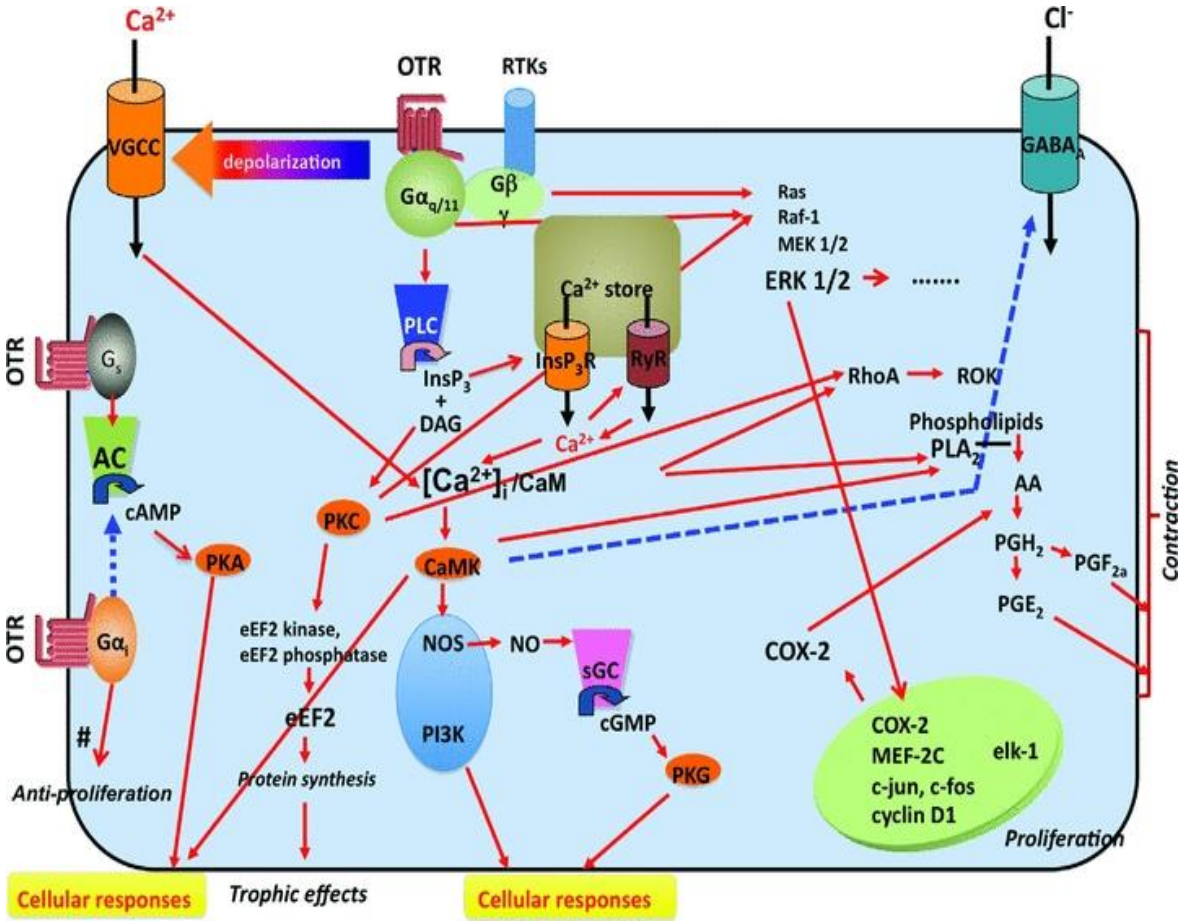


Figure 1.16 *OT/OTR regulated pathways. OT binding causes the dissociation of the G subunits leading to activation of DAG and IP3 resulting in the increase of* Ca^{2+} *in the cells which regulates cellular responses. Subunits β, and γ lead to the activation of the MAPK via ERK. PKC activity leads to Rho activation. COX-2 expression through ERK leads to the production of PGs, that results in contractility. Binding to subunit* α_i *leads to anti-proliferative effects. (Viero et al. 2010).*

Phaneuf *et al*., reported that prolonged exposure to OT leads to downregulation of OTR and its desensitisation, and also showed that if labour lasts for more than 12 hours, OTR levels decrease (Phaneuf *et al*., 1998).

Over the last few decades, oxytocin has been used to induce labour in conjunction with amniotomy (breaking of the amniotic membranes) or after cervical ripening. However, OTR antagonists have been used as tocolytic agents as a treatment to prevent labour for approximately 24-48 hours. Within that time period corticosteroids are administered to

accelerate foetal lung maturation and increase the chances of survival. Atosiban and Barusiban (Ferring Pharmaceuticals) have structures similar to that of oxytocin and bind to OTR, blocking the binding to OT. These drugs along with other OTR antagonists are used to delay preterm birth (Arrowsmith and Wrey, 2014).

1.7 Surfactant proteins A and D

1.7.1 Definition and structure of surfactant proteins

The pulmonary surfactant is a key component in the lungs that regulates breathing. Approximately 90% of the pulmonary surfactant consists of lipids and the remaining 10% of proteins. It lines the alveoli and forms a liquid phase that regulates the surface tension during breathing (Haagsman and van Golde, 1991). It is produced by alveolar type II cells and a monolayer lining covering the alveoli. Surfactant proteins, SP-A and SP-D, which are also called collectins, are expressed by alveolar type II cells and are Ca^{2+} dependent c-type lectins that contain collagen regions (Holmsklov *et al*., 2003).

There are four surfactant proteins identified: SP-A, SP-B, SP-C and SP-D. SP-B and SP-C have a mechanistic role in regulating the surface tension of the pulmonary surfactant. They are small and hydrophobic whereas SP-A and SP-D are larger molecules that are hydrophilic (Weaver and Whistsett, 1991). SP-A and SP-D are involved in maintaining pulmonary immunity and, along with neutrophils and macrophages, form the first line of defence of the innate immune system in the lungs. As mentioned previously, SP-A and SP-D recognise specific carbohydrate or charge patterns expressed on pathogens which results in their clearance via opsonisation or agglutination. They have been known to modulate both pro and anti-inflammatory properties (Ohtani *et al*., 2008).

Both SP-A and SP-D genes are located on chromosome 10q21-24. They can be formed by the assembly of one or more polypeptide chains forming subunits; each subunit consists of three chains. SP-A gene has two isoforms SP-A1 and SP-A2 that are transcribed by *SFTPA1* and *SFTPA2* and share 97% of their amino acid sequence (Floros *et al*., 2009). The SP-A protein consists of six subunits (total of 18 chains) that form a protein of approximately 630kDa. SP-D however, is transcribed by a single gene, *SFTPD,* which consists of four subunits (total of 12

chains). The human SP-D protein is approximately 520kDa and it has been reported that more molecules of SP-D can combine and make larger multimers of up to 96 chains (Holmsklov *et al*., 2003).

Both molecules have similar overall structure that is divided into four distinct regions: a cysteine-containing N-terminus which is responsible for the oligomerization of the protein, a triple-helical collagen region with multiple Gly-X-Y repeats (X can be any amino acid and Y is often hydroxyproline or hydroxylysine) that provide the shape and stability of the molecule, an α-helical coiled coil neck region that regulates trimerization, and a globular structure at the C-terminus consisting of carbohydrate that is responsible for the binding to pathogens (Figure 1.17, Kishore *et al*., 2006).

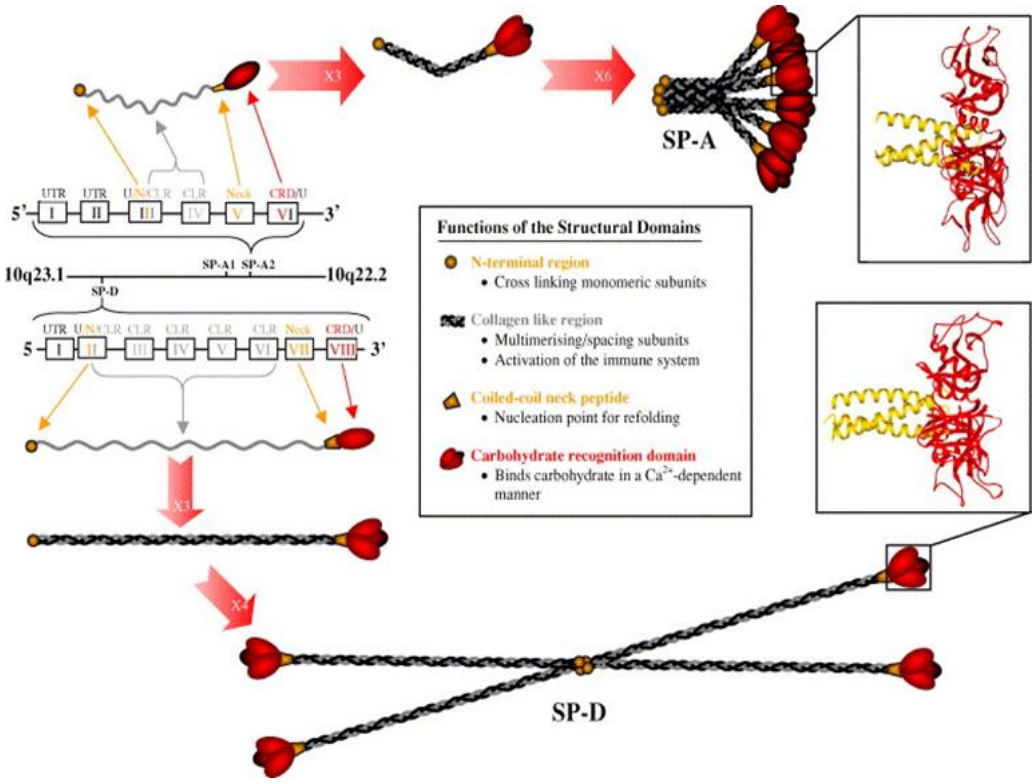


Figure 1.17 *SP-A and SP-D structure. Surfactant protein genes are located on chromosome 10. SP-A is formed of six subunits comprising of three chains whereas SP-D is formed of four subunits comprising of three chains. They have an N-terminal region (orange circle), a collagen like region (grey), a coiled-coil neck region (orange rectangle) and a CRD region (red). SP-A molecules have a bouquet shape whereas SP-D shows a cross formation (Kishore et al., 2006).*

1.7.2 Functions of SP-A and SP-D

The main role of SPs is to maintain immunity and regulate homeostasis. They bind to pathogens via their CRD domains which leads to their clearing (Kishore *et al*., 2006). After binding to a ligand, SP-A and SP-D can activate antigen-presenting cells such as DCs and DMs. They can also interact with neutrophils and lymphocytes and lead to the expression of cytokines that shift the balance to either a Th1 or Th2 profile (Ohtani *et al*., 2008). Targets can include bacteria, viruses, fungi, necrotic cells and apoptotic cells. SP-D, due to its cross-like structure, can bind to more pathogens simultaneously compared to SP-A (Kishore *et al*., 2006).

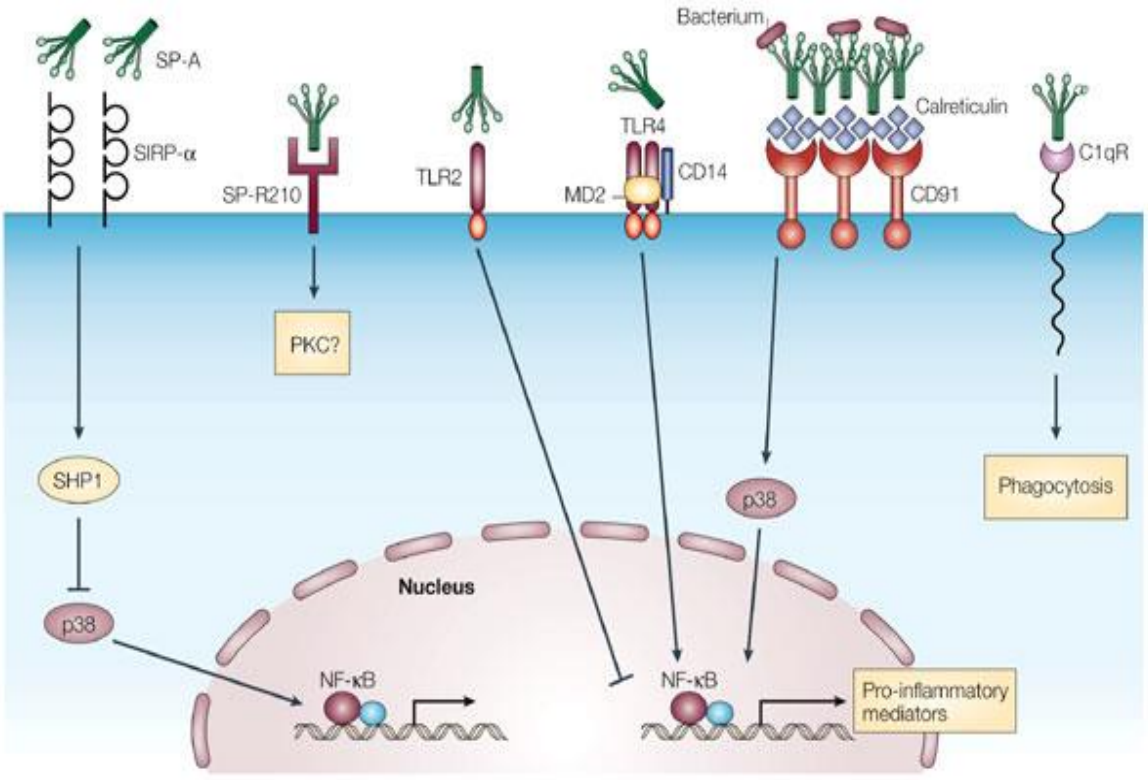


Figure 1.18 *Receptors of SP-A and SP-D. SP-A and SP-D can bind to SP-R210, Toll-like receptors 2 and 4, signal-inhibitory regulatory protein-α and CD91-calreticulin. When SP-A binds to SIRP- α and TLR2 it downregulates expression of pro-inflammatory cytokines, whereas when it binds to TLR4 and calreticulin, it promotes the expression of those cytokines. Binding to C1q leads to phagocytosis of pathogens (Wright, 2005).*

Due to some differences in their amino acid sequence, SP-A and SP-D bind to different pathogens (sharing only a small amount of targets) and exert distinct effects, examples of which are shown in Figure 1.19.

SP-A and SP-D can bind to various strains of the influenza A virus (IAV) and the respiratory syncytial virus (RSV) and neutralise them, thus preventing infection (Hartshorn *et al*., 1994; Benne *et al*., 1995; Hickling *et al*., 1999; Sano *et al*., 1999). SP-A has also been reported to bind to the herpes simplex virus (HSV) and activate macrophages for its phagocytosis. Both SPs have been found to bind to the human immunodeficiency virus (HIV) and inhibit viral infection and replication (Gaiha *et al*., 2008; Meschi *et al*., 2005). SPs can bind to Gram negative (via the LPS on their wall) and Gram positive bacteria. *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are some of the bacteria SP-A and SP-D bind to and mediate their clearance (Nayak *et al*., 2012). Regarding fungal pathogens, SP-A and SP-D have been shown to bind directly to fungi and lead to their phagocytosis. They bind to various species of aspergillus and candida, as well as *pneumocystis carinii* (Madan *et al*., 1997; van Rozendaal *et al*., 2000). SP-A and SP-D have also been reported to clear apoptotic and necrotic cells via direct binding, and modulate their phagocytosis and ingestion by macrophages (Vandivier *et al*., 2002).

SP-A and SP-D have been associated with many pulmonary diseases. Mice deficient in SP-A and SP-D are susceptible to infections from pathogens and have low survival rates (Nayak *et al*., 2012). Smoking leads to a low expression of SP-D which may provide an explanation for smokers having increased rates of lung infections (More *et al*., 2010). In cystic fibrosis, expression levels of SP-A and SP-D are low, which suggests an explanation for the increased infection incidents in patients with this condition (Noah *et al*., 2003).

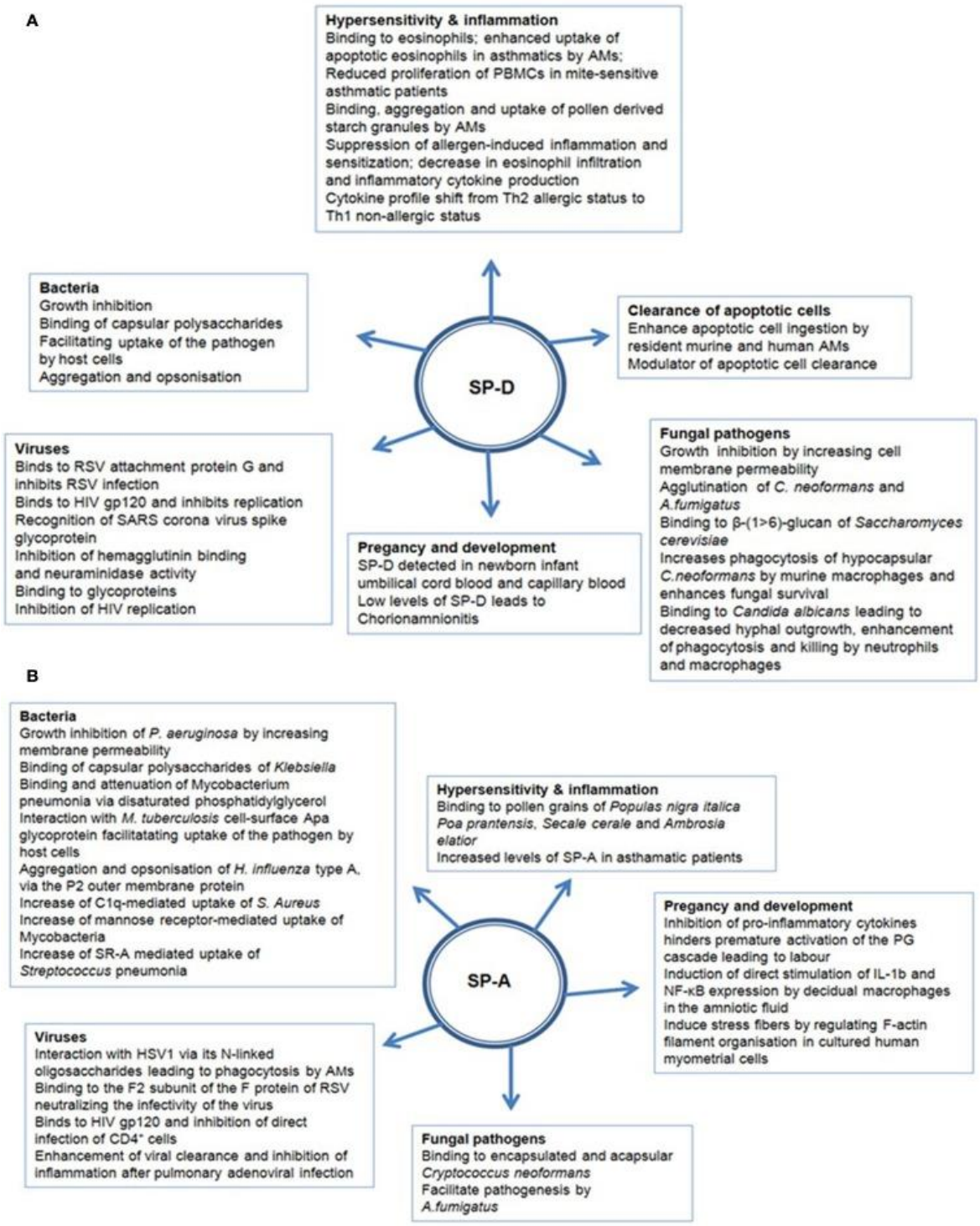


Figure 1.19 *Functions of SP-D (A) and SP-A (B). SP-A and SP-D bind to pathogens and lead to their and lead to their phagocytosis. More importantly they have roles in pregnancy and parturition, as well as lung diseases (Nayak et al., 2012).*

1.7.3 Regulation of surfactant protein transcription

Gene expression of surfactant proteins can be regulated by hormones, cytokines and GCs via cAMP levels. Analogues of AMP and other molecules such as beta-adrenergic agonists and PGE_2 have been reported to induce the expression of SP-A mRNA *in vitro* (Odom *et al*., 1987; Acarreugui *et al*., 1990; Kumar and Snyder, 1998).

GCs can either induce or inhibit the expression of SP-A dependent on the dose and the differentiation of the tissues (Boggaram and Mendelson, 1988; Khoor *et al*., 1993). When human foetal lung tissues from mid-trimester were incubated with GCs, the expression of SP-A mRNA was increased at low concentrations, whereas it was decreased at higher concentrations. The downregulation of SP-A was reversed when a glucocorticoid receptor antagonist, RU486, was added to the tissues (Odom *et al*., 1988; Boggaram *et al*., 1991).

GCs have also been reported to regulate the expression of SP-D mRNA. Studies in foetal rats and human lung tissues showed that injections of GCs in the mother led to an increase in the expression of SP-D in the foetal lungs (Mariencheck and Crouch 1994; Dulkerian *et al*., 1996).

Cytokines can also modulate the expression of surfactant proteins. Interferon-γ led to an increase in SP-A transcript levels in human foetal lung tissues *in vitro* (Ballard *et al*., 1990). TNF-α can decrease the SP-A mRNA levels in foetal lung tissues *in vitro* (Wispe *et al*., 1990). TGF-β led to a decrease in SP-A levels in human foetal lung tissues (Beers *et al*., 1998). IL-1β, however, can lead to an increase in SP-A mRNA expression in rabbits and sheep (Bry *et al*., 1997; Bachurski *et al*., 2001). A study in diabetic pregnant rats showed poor lung maturation in their offspring (Pignol *et al*., 1987). Diabetic pregnant women have been shown to have reduced levels of SP-A in their amniotic fluid (Snyder *et al*., 1988). Moreover, when lung explants were treated with insulin; this resulted in low expression of SP-A (Snyder *et al*., 1987).

Steroid hormone, cortisol, can induce expression of SP-A by increasing the expression of PGE_2 and COX-2 (Sun *et al*., 2006).

1.7.4 Extra-pulmonary localisation of surfactant proteins

In addition to the main production site of surfactant proteins, the lungs, SP-A and SP-D have been localised in several other tissues in which they exert different roles. These sites have higher chances of being exposed to pathogens, hence justifying the localisation of SP-A and SP-D.

SP-A has been localised in the intestines (Lin *et al*., 2001), the human lacrimal system (Brauer *et al*., 2007), the human prostate, the thymus, the colon and salivary glands and the human skin (Madsen *et al*., 2003; Mo *et al*., 2007). SP-D has been localised in the human trachea, the oesophagus, the kidney, the testes, the brain, the urinary tract and the spleen (Nayak *et al*., 2012).

Both SP-A and SP-D from the foetus have been localised in various sites of the reproductive system such as in foetal membranes, the placenta, the umbilical cord, the amniotic fluid (Madsen *et al*., 2003)

SP-A and SP-D are expressed in the foetal lungs, and can be located in the amniotic fluid during the second trimester of pregnancy. At that stage the expression ratio between SP-A and SP-D is 1:1. Towards the end of term, the levels of SP-A are highly increased (approximately 8-fold higher) whereas levels of SP-D increase more steadily. At term the ratio of SP-A and SP-D changes to 6:1 (Miyamura *et al*., 1994).

1.7.5 Role of SP-A and SP-D in pregnancy and parturition

In addition to their role in clearing pathogens and preventing infections that could be harmful for the foetus and lead to preterm birth, SP-A and SP-D have been implicated in foetal development and parturition (Lahra and Jeffery, 2004).

As SP-A and SP-D are produced in the foetal lungs, they are indicators of the maturation of the lungs (Mendelson and Condon, 2005). Condon *et al*., showed that in mice, SP-A from the foetal lungs is secreted in the amniotic fluid which then migrates to the maternal side and leads to prostaglandin and NF-κB expression which leads to myometrial activation and parturition towards term (Condon *et al*., 2004). NF-κB can lead to myometrial activation either via the increased expression of macrophages in the amniotic fluid, the direct regulation of genes such

as COX-2 that leads to PG production, or inhibiting the expression of genes that are promoted by progesterone, resulting in the transition from quiescence to contractility (Condon *et al*., 2004). SP-A and SP-D have also been reported to bind to smooth muscle cells and initiate contractions via the formation of stress fibres and the induction of prostaglandin expression via a pathway involving Rho-kinase (Breuiller-Fouche *et al*., 2010). SP-A has been found to bind to myometrial cells and activate the ERK pathway and COX-2, inducing myometrial cell contractility (Figure 1.20) (Garcia-Verdugo *et al*., 2007).

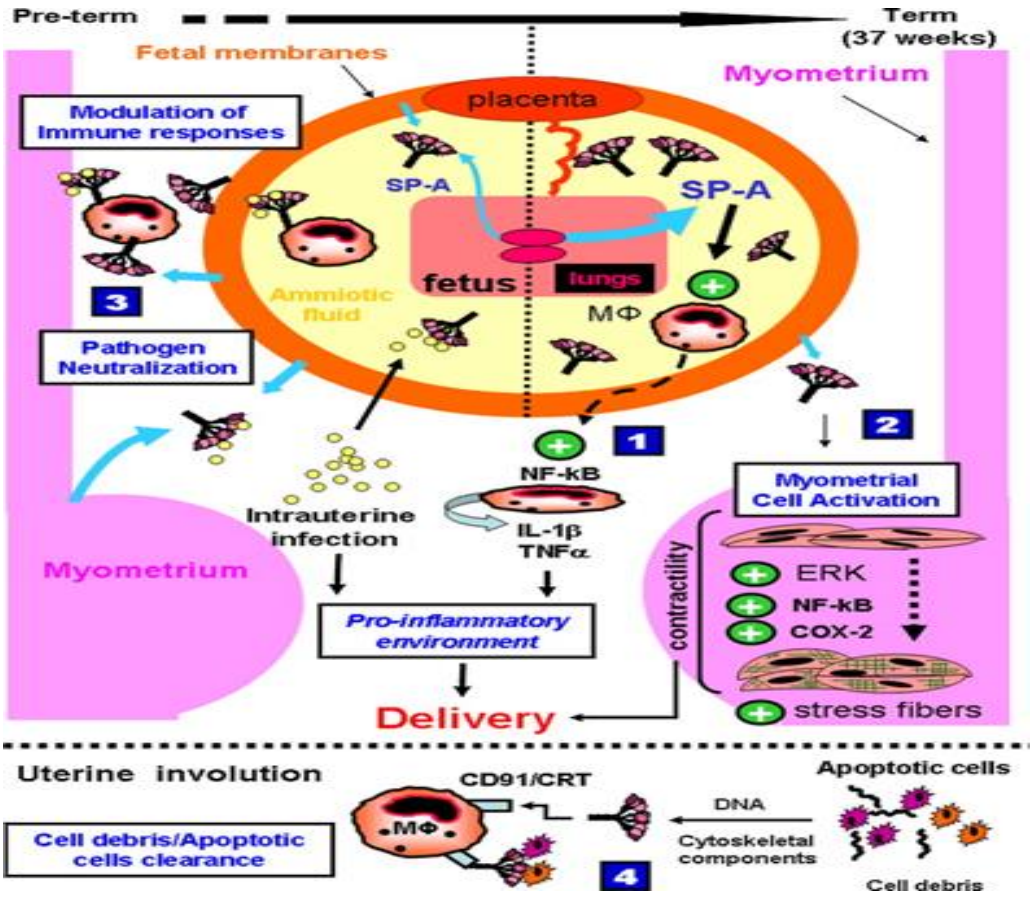


Figure 1.20 *SP-A functions during pregnancy and parturition in mice. SP-A from the foetal lungs activates macrophages and induces the expression of pro-inflammatory cytokines NF-κB, IL-1β and TNF-α leading to parturition (1). SP-A is also produced in other reproductive tissues and leads to cell contraction via the prostaglandins and the ERK pathway and the formation of stress fibres (2). SP-A binds to pathogens and inhibits infections (3). SP-A clears apoptotic cells and debris during uterine involution (4) (Garcia-Verdugo et al., 2010).*

Condon *et al*., showed that when pregnant mice were injected with exogenous SP-A in the amniotic fluid, this resulted in preterm birth. Interestingly, in pregnant mice that were injected with an antibody for SP-A, labour was delayed by up to 24 hours (Condon *et al*., 2004). Injection of pregnant mice with inhibitors for the pro-inflammatory cytokines TNF-α and IL-1β also led to the delay of labour (Condon *et al*., 2004).

When Sato *et al*., administered SP-D to preterm baby lambs, SP-D inhibited lung infection and the expression of pro-inflammatory cytokines (Sato *et al*., 2010). Nadesalingam *et al*., reported that SP-D interacts with decorin which is a proteoglycan that binds to collagen and collagen regions of C1q (first sub-component of the complement classical pathway), and a key component of the extracellular matrix (ECM) (Nadesalingam *et al*., 2003). It regulates the expression of TGF-β which affects cell proliferation (Schonherr *et al*., 1998). Decorin can also promote the expression of MMPs 1, 2 and 14 (Schonherr *et al*., 2001). This implicates SP-D in tissue remodelling *in vivo* (Nadesalingam *et al*., 2003).

SP-A and SP-D could be therefore used as potential indicators of lung maturation but also as biomarkers for diseases such as preterm birth.

1.8 Hypothesis and aims of this study

Previous research on SP-A and SP-D has shown that these surfactant proteins might be involved in the maintenance of pregnancy and the initiation of labour (Condon *et al*., 2004, Garcia-Verdugo *et al*., 2007; Sato *et al*., 2010). We hypothesised that SP-A and SP-D have further roles in the myometrium leading to its activation, apart from their roles regulating homeostasis and immunity. The main objectives of this study were to:

- Investigate the effects of SP-A and SP-D *in vitro* using a myometrium model; by examining their effects on cell motility, CAP gene and cytokine expression;
- Study the interactions of sex steroids with SP-A and SP-D by measuring the effects of sex steroids (oestrogen and progesterone) on the expression of SP-A and SP-D, and investigating further potential cross-talk mechanisms between mPRs and SP-A and SP-D;
- Investigate the role of SP-D in ovarian cancer by studying its effects on cell viability, proliferation, motility, and the caspase and mTOR pathway;
- Measure the expression of SP-A and SP-D in human myometrial samples (labour, non-labour, term, and pre-term) and the levels of SP-D in ovarian cancer clinical samples; investigate the protein expression and cellular distribution of SP-D in ovarian cancer using tissue microarray and analyse *in silico* data to evaluate SP-D effects on survival.

Chapter 2 Materials and methods

2.1 Cell lines

ULTR: This cell line is a uterine leiomyometous smooth muscle cell line that has been retroviral immortalised and took its name from the uterine long terminal repeats (ULTR). Cell line was a kind gift from Dr Xiaolan Cui (Department of Obstetrics and Gynaecology, University of Cincinnati, USA). ULTR cell line retains smooth muscle cell morphology as well as characteristics observed in primary human myometrium cells and it has been widely used in various studies to investigate myometrium cell functionality (Perez-Reyes *et al*., 1992, Belt *et al*., 1999, Zaragoza *et al*., 2006).

SKOV3: This is an aggressive ovarian cancer cell line that is p53 null and mTOR active. This cell line was purchased from ATCC (HTB-77) and was derived from ascites of a Caucasian patient aged 64, and it is of human clear cell adenocarcinoma type. This cell line has been previously used to assess signalling pathways involved in epithelial ovarian cancer (Rochefort *et al*., 1998; Foster *et al*., 2010, Bail *et al*., 2015).

2.1.1 Cell culture

Cells were grown in 75cm^2 non treated culture flasks (Nunc) under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37^{o}C). For the experiments, a Holten LaminAir Class II (Haeraus) Instrument hood was used under aseptic conditions. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/l) and L-glutamine (0.58 g/l) (Gibco, Life Technologies), supplemented with 1% v/v of penicillin and streptomycin along with 10% v/v heat inactivated foetal bovine serum (Gibco, Life Technologies). After cells reached confluency, they were subcultured in a 1:3 ratio after being washed with sterile 1x Phosphate buffer saline (PBS-Gibco) and detached by incubating with 2.5 ml of TryPLE Express (Invitrogen) as a trypsin alternative for approximately 3 minutes. Fresh DMEM was added to the flasks to allow cell survival.

2.1.2 Thawing cells

Prior to removing frozen cells from liquid nitrogen, a 75cm^2 flask containing 19ml of fresh media was incubated under standard culture conditions for a few hours, to allow the media to equilibrate. Next, a cryovial including the frozen cells, was removed from the liquid nitrogen and thawed in the water bath at 37^{o}C. When the cell suspension was defrosted, the solution was transferred into the flask and then moved into the incubator overnight to allow the cells to adhere. The next morning, media was replaced with fresh media in order to remove the excess of DMSO that was used to freeze the cells down.

2.1.3 Cryopreserving cells

In order to make stocks of cells, several flasks of cells were grown under standard culture conditions. First, the media was aspirated using Pasteur pipettes and the cells were incubated using TrypLE for a few minutes in order for the cells to detach from the flask surface. Cells were diluted in 5ml of media and then centrifuged at 12,000rpm for 5 minutes. Supernatant was discarded and the cell pellet was resuspended in 0.5ml of FBS and 0.4ml of fresh media, and moved to a cryovial. 100µl of DMSO were added at the end at the top of the solution. The cryovial was frozen gradually in a container (Nalgene), prior to being moved to liquid nitrogen after 24 hours.

2.2 Clinical samples

Myometrial clinical samples from women that had given birth at term (labour; n=10, non-labour; n=10) and preterm (labour; n=10, non-labour; n=10) were provided by the University Hospital of Crete in Greece. All pregnancies were healthy without any conditions and samples were collected at time of delivery and stored in RNAlater (Invitrogen). Ovarian tissue samples (x6) were acquired from healthy patients and were provided by the University of Thessaloniki, Greece.

Ethical approval was obtained from Brunel University, the department of Obstetrics and Gynaecology of University Hospital of Crete and the University Hospital of Thessaloniki.

2.3 Expression of the recombinant fragments of homotrimeric CRD regions of human SP-A (rhSP-A) and SP-D (rhSP-D)

Transformated BL21 (λDE3) pLysS colonies that contained the plasmid containing neck and CRD regions of human SP-A and SP-D were grown on ampicillin 100 µg/ml and chloramphenicol 50 µg/ml agar plates. Ten randomly selected colonies were inoculated in 5ml of Luria Broth (LB) (Sigma) supplemented with ampicillin and chloramphenicol (amp/chl) and grown in a 37^{o}C shaking incubator overnight. The next morning 500 µl of the primary cultures were transferred into 10 ml LB with amp/chl, and were grown in the 37^{o}C shaking incubator until the point that cells reached the late log phase, which was shown by an optical density (OD) at 600nm between 0.6-0.8. 1 ml sample, from each tube, was then transferred into eppendorf tubes to represent the un-induced control samples. The rest of the cultures were treated with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) and both groups of samples were incubated for 3 hours in the 37^{o}C shaking incubator. After the induction was complete, 1ml of the induced samples was transferred into new eppendorf tubes and along with the un-induced samples they were centrifuged at 13,000 rpm for 10 minutes. The supernatant was then discarded and the pellets were re-suspended in 100µl of 2x treatment buffer. Samples were heated at 100^{o}C for 10 minutes on a heating block to allow protein denaturisation. 10 µl of each sample were then loaded on a 12% SDS-PAGE. Expected size is approximately 18 kDa for rhSP-A and 20 kDa for rhSP-D. The colonies that showed the strongest bands, hence had better expression of the proteins of interest were streaked on LB amp/chl agar plates.

Single colonies from the above agar plates were then individually inoculated in 15 ml of LB with amp/chl and left to grow overnight in the 37^{o}C shaking incubator. The next morning, 12.5ml of the primary cultures were transferred into 500 ml LB with amp/chl and were incubated until the cells reached the late log phase and had an OD between 0.6-0.8. Un-induced samples were collected as described above. The rest of the samples were treated with 0.5 mM IPTG and left to grow for 3 hours. Induced samples were collected as previously described and then centrifuged at 13,000 rpm for 10 minutes. Pellets were then re-suspended in 2x treatment buffer and were denatured for 10 minutes. Samples were then again run on a 12% SDS-PAGE. The 500 ml cultures were centrifuged at 5,000 rpm for 30 minutes and the pellets were stored at -20^{o}C.

After observing the right bands on the agarose gels, the pellets were solubilised in 25 ml of lysis buffer (50 mM Tris-HCl, 200 nM NaCl, 5 mM EDTA, pH 7.4) with PMSF and lysozyme (Sigma-Aldrich). After lysis for 1 hour at 4 ^{o}C, the lysed cells were sonicated for 15 cycles at 40 kHz for 30 seconds with 2 minute intervals, on ice. The sample was then centrifuged at 13,000 rpm for 15 minutes and the pellet was collected.

The pellet was solubilised in 25 ml of 8 M Urea buffer (50 mM Tris-HCl, 100 nM NaCl, 5 mM EDTA, 8 M Urea, pH 7.5) containing 1.4 ml of β2-mercaptoethanol (Sigma-Aldrich) for 1 hour at 4^{o}C. The solubilised protein was centrifuged at 13,000 rpm for 15 minutes and transferred into a dialysis bag. The protein was dialysed against dialysis buffer containing 4 M Urea for 2 hours, then 2 M Urea for another 2 hours and finally 1 M Urea for another 2 hours, stirring constantly. The protein was then dialysed against 0 M Urea buffer overnight.

The next day the protein was dialysed against affinity buffer (50 mM Tris-HCl, 100 nM NaCl, 5 mM CaCl2, pH 7.5) to remove EDTA and to calcify the protein for the affinity chromatography. Proteins were purified by passing through a maltose – agarose matrix. The protein was passed through the column 3 times. The proteins of interest were bound in the matrix whereas unwanted ones were collected as waste since they were passing through. The proteins of interest were then collected using elution buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 7.5) in 20 x 1 ml fractions over ice. The absorbance of the fractions was then measured at 280 nm and then a 15µl of them was loaded on a 12% SDS-PAGE to examine the purity of the samples.

Since these proteins would be used for further studies and cells would be treated with them, the removal of any endotoxins present in the fractions is of high importance. Lipopolysaccharides are present in the outer cell membrane of gram – negative bacteria. The removal was performed using endotoxin removal resin spin columns. The spin column was initially incubated with 5 ml 0.2 N NaOH in 95% ethanol for 1 hour at room temperature. The column was washed with 5 ml 2 M NaOH and 5 ml of endotoxin free ultrapure water. The column was then equilibrated with phosphate buffer at room temperature. The previously collected protein fractions were added to the column and were incubated overnight at room temperature and eluted the next day using endotoxin free equilibration buffer. Flow through samples and fractions were loaded on a 12% SDS-PAGE to determine their purity.

2.4 Cell treatments

Prior to any experiment, cells were allowed to reach 80-100% confluency (usually after 48 hours). Cells were detached from the flask surface using TryPLE Express and then counted using a Countess Automated Cell Counter (Invitrogen, Life Technologies). An appropriate amount of cells was then transferred into either 6-well plates or petri dishes depending on the experiment, as described below:

Total number of cells = viable cells per mL x Volume mL
Number of cells per µL = Total number of cells/Volume mL
Volume of cells to add to well = Cells required/Number of cells per µL

Table 2.1 *Concentrations and time points for treatments of ULTR and SKOV3 cells in order to extract RNA and protein samples.*

Cell treatments for RNA and protein extraction	Final concentration	Time points
ULTR x rhSP-D	5, 10 and 20 µg/ml	4, 6, 12 and 24 hours
ULTR x rhSP-A	5, 10 and 20 µg/ml	4, 6,12 and 24 hours
ULTR x OD, P4, E2	10^{-7}M	6, 12, 24 and 48 hours
SKOV3 x rhSP-D	5, 10 and 20 µg/ml	6, 12, 24 and 48 hours

2.5 Total RNA extraction from cells

Cells were grown in 6-well plates and treated as mentioned above. RNA was extracted using the GenElute Mammalian Total RNA miniprep kit (Sigma-Aldrich), following the manufacturer's instructions. Cells were lysed directly in the wells using Lysis solution complemented with an appropriate amount of β-mercaptoethanol (2-ME) per RNA preparation (10µl of 2-ME for each 1ml). The side of the wells was tapped so that the solution would cover the surface of the well. The culture vessel was let to sit for approximately 2 minutes and then tilted on the side to allow collection of the cell lysate. To allow the removal of cellular debris and DNA, the lysed cells were pipetted into a filtration column and then centrifuged at

13,000 x g for 2 minutes. The filtration column was then discarded and an equal amount of 70% ethanol solution was added to the filtered lysate. After a brief vortex, approximately 700µl of solution was pipetted into the binding column and centrifuged at maximum speed for 15 seconds. The flow-through was discarded and this step was repeated. The column was then washed and centrifuged at maximum speed for 15 seconds. The flow-through was again discarded and the column was washed again with a wash solution diluted with ethanol. The column was centrifuged again at maximum speed for 15 seconds. The above step was repeated though this time the column was centrifuged at maximum speed for 2 minutes to dry the binding column. 50µl of elution solution was added to the column, which was centrifuged for 1 minute. RNA was stored at -70^{o}C.

2.6 Total RNA extraction from tissue (clinical samples)

To allow the RNA extraction from the clinical samples, the tissues were homogenised using TissueLyser II (Qiagen). The samples were previously stored and frozen in RNAlater (Invitrogen). Once thawed, the tissues were transferred to sterile tubes. Lysis solution and a steel bead were added to the tube to allow homogenisation. Each sample was incubated with Proteinase K (Sigma-Aldrich) at 55^{o}C for 10 minutes. RNA was extracted following the procedure described above (section 2.5)

2.7 cDNA synthesis

RNA was first quantified using NanoDrop 200C (Thermo Fisher Scientific). RNA concentration and purity was determined using the absorbance at 260 and 280nm. Ratio of the two absorbance readings was within 1.7-2.0. cDNA synthesis was performed using the Precision nanoScript 2 Reverse Transcription kit (Primerdesign). Following the manufacturer's protocol, RNA samples were diluted in water to normalise the concentration across all samples and reach a final volume of 9µl and incubated with 1µl of random nonamer primer at 65^{o}C for 5 minutes. Samples were transferred directly from 65^{o}C to the ice. 10µl of mastermix (nanoScript2 4x buffer, dNTP mix 10mM, RNAse free water and nanoScript2 enzyme) was added to the existing 10µl of the samples on ice. Samples were briefly vortexed, spun and incubated at 25^{o}C for 5 minutes and then at 42^{o}C for 20 minutes. The reaction was

heat inactivated by incubating at 75ºC for 10 minutes. cDNA samples were stored at -20ºC until use.

2.8 Real Time Polymerase chain reaction (qPCR)

PrecisionPlus mastermix premixed with SYBR green (Primerdesign) was used for the qPCR. An ABI PRISM 9000 was used for the experiment and the Sequence Detection System (SDS 2.4) software was used for the analysis. Samples were loaded on a 96-well plate containing cDNA, mastermix, water and primers specific for each gene, following the manufacturer's instructions.

Table 2.2 *Composition of a 20µl reaction per well for qPCR.*

Reagent	**Quantity per reaction (20µl)**
Mastermix with SYBR green (PrimerDesign)	10 µl
Nuclease-free water (Ambion)	8 µl
Forward and reverse primer mix (PrimerDesign)	1µl
cDNA	1µl

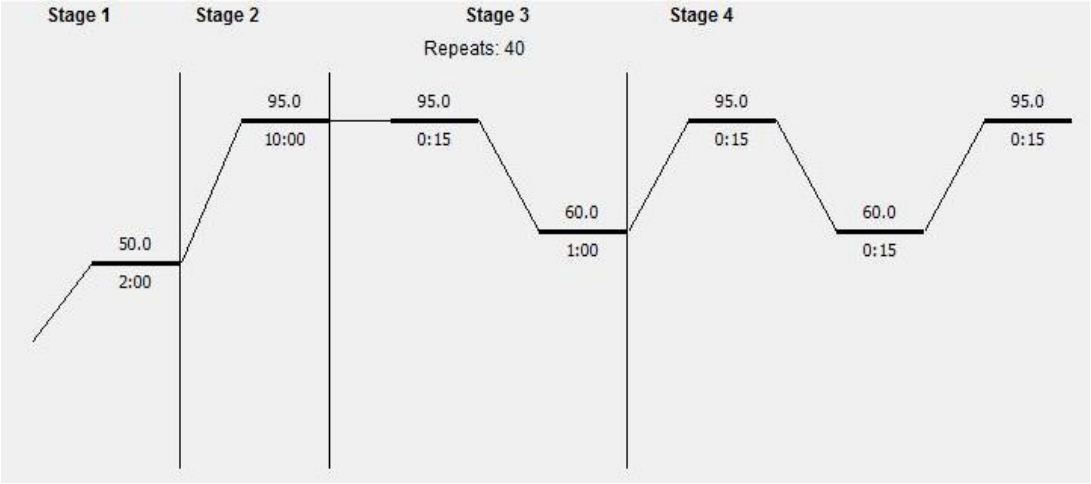


Figure 2.1 *Thermocycling conditions for qPCR.*

A relative quantification was performed and all values were normalised to the housekeeping gene. The relative quantification produces an amplification curve which shows the number of cycles versus the intensity of fluorescence. As the cycles increase, more double-stranded DNA is produced and binds to SYBR green which is the detected by the machine. The cycle that this happens is called the 'cycle threshold'. In order to distinguish between specific binding and unspecific binding to other DNA products or primer dimer complexes, a dissociation curve is acquired. This shows the melting curve of the primer products which are specific and previously known and other, if any, by-products. So to evaluate the quality of the results both curves need to be obtained and checked. In the graphs throughout this thesis, the control (no supplement) cell RQ value is set to 1.

Table 2.3 *Primer sequences of human gene targets used for qPCR experiments. Expected product sizes are shown in base pairs.*

Gene name	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'	Length in bp
SP-A1	TGGGTCGCTGATTTCTTGGA	CGCTGCTCTCACTGACTCA	82
SP-A2	TGAAAAGAAGGAGCAGCGACT	ACCAGGGCTTCCAACACAA	126
SP-D	AATGGCAAGTGGAATGACAGG	CACCCCAGTTGGCTCAGAA	73
OTR	TTACAATCACTAGGATGGCTACAA	CATTTACATTCCCACCAACAATTTAA	105
CX43	TGGATTCAGCTTGAGTGCTG	GGTCGCTCTTTCCCTTAACC	130
COX2	CAAATCATCAACACTGCCTCAAT	TCTGGATCTGGAACACTGAATG	89
mTOR	TGCCAACTATCTTCGGAACC	GCTCGCTTCACCTCAAATTC	114
DEPTOR	CACCATGTGTGTGATGAGCA	TGAAGGTGCGCTCATACTTG	101
Rictor	GGAAGCCTGTTGATGGTGAT	GGCAGCCTGTTTTATGGTGT	117
Raptor	ACTGATGGAGTCCGAAATGC	TCATCCGATCCTTCATCCTC	170
mPRα	GCTGTTCACTCACATCCC	TGGTGCAACCCCCAGA	289
mPRβ	GCGGCCCTGGTACTGCTGC	CACGGCCACCCCCACA	200
PRB	AGCAGTCCGCTGTCCTTTTCT	CCTGAAGTTTCGGCCATACCT	196

For cell culture samples the following equations were used:

$\Delta Ct = Ct_{(gene\ of\ interest)} - Ct_{(housekeeping\ gene)}$

$\Delta\Delta Ct = \Delta Ct_{(sample)} - \Delta Ct_{(calibrator)}$

Relative Quantity (RQ) = $2^{-\Delta\Delta Ct}$

For clinical samples: $\Delta Ct = Ct_{(\text{gene of interest})} - Ct_{(\text{housekeeping gene})}$

Relative Quantity (RQ) = $2^{-\Delta Ct}$

2.9 geNorm analysis

The geNorm array kit from Primerdesign, includes 12 human housekeeping genes, to assess their expression under different experimental conditions. MIQE guidelines have shown that the expression of reference genes can change under different experimental conditions. geNorm is a PCR using different samples across treatments and time points of the experimental design. Data is then analysed using the qBASE software to identify which genes are the most stable and the optimum number of housekeeping genes to be used for the experimental model. This analysis translates into an M value for each gene. The M value signifies the variance between samples. When M is lower, the reference gene is considered to be stable. Conditions were the same used for the q-PCR assay described above. The reference genes included in the geNorm kit are: ATP5B, UBC, TOP1, ACTB, CYC1, GAPDH, SDHA, RPL13A, 18S, YWHAZ, B2M, and EIF4A2. The housekeeping gene selected after the GeNorm analysis was GAPDH. GAPDH sequence is protected by Primerdesign.

Table 2.4 *Genes included in the geNorm kit that were used for the reference gene selection.*

ACTB*	Actin, beta (mRNA)
GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase (mRNA)
UBC*	Ubiquitin C (mRNA)
B2M*	Beta-2-microglobulin (mRNA)
YWHAZ*	Phospholipase A2 (mRNA)
RPL13A*	Ribosomal protein L13A (mRNA)
18S	18S RNA (rRNA)
CYC1	Cytochrome C-1 (mRNA)
EIF4A2	Eukaryotic translation initiation factor 4A isoform 2 (mRNA)
SDHA	Succinate dehydrogenase complex (mRNA)
TOP1	Topoisomerase (DNA) 1 (mRNA)
ATP5B	ATP synthase subunit (mRNA)

2.10 Agarose Gel electrophoresis

To check the quality of the DNA products a 2% agarose gel electrophoresis was performed. Agarose was dissolved in 1x TBE buffer (90Mm Tris HCL, 90Mm Boric acid, 2mM EDTA) and 0.05% (w/v) ethidium bromide. Samples were mixed with 10x loading buffer and then loaded in the wells. The gel was ran at 80V for approximately 2 hours. The bands were visualised on a short wave ultra-violet transluminator.

2.11 Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis and Western Blot

Protein was extracted from cells seeded on a 6-well plate using 2x Laemmli buffer [4% (w/v) SDS, 20% glycerol, 10% 2-mercaptoethanol (v/v), 0.004% bromophenol blue (w/v), 0.125 M Tris HCL pH 6.8 (w/v)], and boiled at 100^{o}C for 10 minutes. For the electrophoresis a 10%

SDS-PAGE gel was used. First, the resolving gel was made and put between the glass plates and left for about 20 minutes to allow polymerisation. Resolving gel (10%) consisted of: ddH2O, 30% acrylamide/bis-acrylamide solution (National Diagnostics), 1.5M Tris base (pH8.8), 10% SDS, 10% ammonium persulfate (APS), tetramethylethylenediamine (TEMED). To stop the gel from drying, isopropanol was added at the top. Once the resolving gel was set, the isopropanol was discarded. The stacking gel was then added on top of the resolving gel and a 1mm comb was put on top to form the wells. Stacking gel (5%) consisted of: ddH_2O, 30% acrylamide/bis-acrylamide solution, 1M Tris base (pH6.8), 10% SDS, 10% APS, TEMED.

The gel was again allowed to polymerise and once ready the glass plates were moved into a tank containing 1x SDS-PAGE Running buffer (14.4g glycine, 3.02g tris base, 1g SDS, 1L distilled H_2O). Samples were loaded in the wells along with a PageRuler^{TM} protein marker (Life Technologies). Gel was run at 40mA until the dye reached the bottom of the gel.

The gel was then taken out of the glass plates and was placed on filter papers. A Polyvilidene fluoride (PVDF) membrane (Thermo Scientific), previously activated by being placed in methanol and water, was put on top of the gel for the transfer of the proteins. Sponges were put on both sides of the gel-membrane to form a sandwich, which was placed in a tank containing 1x Wet transfer buffer (2.41g tris base, 11.25g glycine, 800ml ddH_2O, 200ml methanol). An ice pack was placed directly inside the tank and the tank was also surrounded by ice. The transfer was accomplished by running at 100V for an hour. Once the transfer had finished, the membrane was blocked in 5% (w/v) blocking solution (Marvel dried milk powder dissolved in 1x TBS Tween 20) for 2 hours on a shaker.

After blocking the membrane was briefly rinsed and incubated with the primary antibody which was dissolved in 5% Bovine Serum Albumin and 1x TBS Tween 20, overnight at 4^{o}C. The next day the membrane was rinsed in 1x TBS Tween 20, 3 times for 0 minutes each, and then incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) which was dissolved in the same manner to the primary antibody. Membrane was then rinsed as described above. To be able to visualise the protein bands specific to the antibody used, enhanced chemiluminescence (ECL) solutions, (containing Solution A: 100Mm Tris pH 8,

30% H_2O_2, and Solution B: 100Mm Tris pH 8, 250mM luminol and 90mM coumaric acid), were mixed and placed on the membrane. The membrane was placed in a cassette and an X-ray film was placed on top. The film was processed in the Curix60 (AGFA) automatic developer passing through developing solution, fixer solution and finally rinsed in water. Visualised bands were quantified on ImageJ and normalised to the housekeeping gene GAPDH. Experiments were done in collaboration with Rooban Jeyaneethi.

Table 2.5 *List of primary and secondary antibodies used for Western Blotting with their respective dilutions. Human SP-A and SP-D antibodies were provided by Prof KM Reid at the University of Oxford, MRC Immunohistochemistry Unit.*

Primary Antibodies			**Secondary Antibodies**	
Antibody	**Dilution**	**Species/Details**	**Antibody**	**Dilution**
SP-A	1:1000	Rabbit	Goat anti-rabbit	1:2000
SP-D	1:1000	Rabbit	Goat anti-rabbit	1:2000
OT	1:1000	Goat (Abcam)	Rabbit anti-goat	1:2000
CX43	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000
COX2	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000
Caspase 3	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000
Caspase 9	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000
S6 kinase	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000
GAPDH	1:1000	Rabbit (Cell Signalling)	Goat anti-rabbit	1:2000

2.12 Proliferation studies

Cells at 100% confluency were counted using a haemocytometer and a specific amount was seeded in 6-well plates. After 24 hours cells were treated with either rhSP-A or rhSP-D (10 µg/ml), or OD-02 (10^{-7}M) and cell counts were performed at 24 and 48 hours. The media was first aspirated and 200µl TryPLE Express was added to the cells. The plate was returned into the incubator for approximately 2 minutes. 800µl of media was added to all the wells and then a small amount of cell suspension was added to an equal amount of 0.4% Trypan Blue. Cells were loaded on counting slides which were placed in a Countess Automated Cell Counter

(Invitrogen, Life Technologies) to allow quantification. Cells without any treatment were used as a control.

2.13 Nimblegen microarray analysis

ULTR cells were grown in 75cm^2 flasks and treated with OD-02 (10^{-7}M). RNA was extracted as mentioned above and purified using an RNAeasy (Qiagen) following manufacturer's instructions. Transplex complete whole amplification kit (Sigma) was used to synthesise cDNA, which was then treated with RNase (Sigma) and precipitated following instructions provided with the Nimblegen Expression Array (Roche). cDNA was labelled with Cy3 and hybridised to a 12plex x 135k gene human transcriptome microarray. After a series of washes, the slide was scanned at 3µm using an InnoScan 700 Microarray Scanner. Images were generated using Magpix v. 5.1 and probe intensity values were acquired using the Nimblegen DEVA software. Experiment was done in collaboration with Dr. Julie Davies and the slide was scanned by Dr. Ryan Pink and Dr. David Carter at the Oxford Brookes University. Data was analysed using the KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway database and Gene Ontology (GO) terms that are parameters for biological processes. This allows the representation of genes in the pathways they are part of. GO terms used were specific for OD-02 and for its composition and function.

2.14 Magnetic Luminex Screening Assay

For this assay a custom kit of a human cytokine/chemokine magnetic bead panel was used (EMD Millipore). Prior to the experiment, all reagents were allowed to warm to room temperature. Wash buffer was added to each well of the plate and was mixed for 10 minutes on a plate shaker at room temperature. The wash buffer was then discarded, and assay buffer and controls were added to the appropriate wells. Matrix solution (control culture media) was added to background, standard and control wells. Conditioned media from treated cells was added in the appropriate wells. Premixed beads were then added to the wells and the plate was sealed and incubated overnight at 4^{o}C. The next day, the well contents were gently removed and the plate was washed twice. Detection antibodies were added to each well, and the plate was incubated for an hour at room temperature on a plate shaker. After the incubation,

streptavidin-phycoeythrin was added to each well and the plate was brought back to the plate shaker for an incubation of 30 minutes at room temperature. The wells were washed twice and sheath fluid was added to all of them. The beads were resuspended on a plate shaker for 5 minutes. The plate was then read using a Luminex 100/200 analyser (Luminex, U.S.A).

Table 2.6 *Human cytokines, chemokines and growth factors used in the Multiplex cytokine assay.*

CD40	Cluster of differentiation 40	**EGF**	Epidermal growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor	**GROα (CXCL1)**	Melanoma Growth Stimulating Activity, Alpha
ENA-78 (CXCL5)	Epithelial-derived neutrophil-activating peptide 78	**FGF basic**	Basic fibroblast growth factor
IFN-γ	Interferon gamma	**G-CSF**	Granulocyte-colony stimulating factor
IL-1α	Interleukin 1 alpha	**IL-1β**	Interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist	**IL-1 RII**	Interleukin 1 receptor type II
IL-2	Interleukin 2	**IL-3**	Interleukin 3
IL-4	Interleukin 4	**IL-5**	Interleukin 5
IL-6ra	Interleukin 6 receptor alpha	**IL-6**	Interleukin 6
IL-8	Interleukin 8	**IL-10**	Interleukin 10
IL-12 p70	Interleukin 12 heterodimer 35+40	**IL-15**	Interleukin 15
IL-17	Interleukin 17	**IL-19**	Interleukin 19
IL-27	Interleukin 27	**IL-31**	Interleukin 31
IP-10 (CXCL10)	Interferon gamma-induced protein 10	**TNF-α**	Tumour necrosis factor alpha
MIP-1α (CCL3)	Macrophage Inflammatory Protein 1 alpha	**MIP-1β (CCL4)**	Macrophage Inflammatory Protein 1 beta
VEGF	Vascular endothelial growth factor		

2.15 Cell live imaging

Cells were grown in petri dishes to a confluency between 30-40% and then treated with either rhSP-A, rhSP-D (10 μg/ml), or OD-02 (10^{-7}M) and then left to adhere for approximately 6 hours. The petri dish was placed under a Carl Zeiss Axiovert 200M microscope (Zeiss). A small incubator was placed on top of the microscope to allow cell survival under standard culture conditions (37ºC, 5% v/v CO_2). After an area with about 25-30 cells was found, images were captured every 5 minutes for 12 hours at 10x magnification. The images from the first 8 hours were used to analyse cell motility. ImageJ software was used to identify X and Y coordinates for 25 cells. Cell distance (starting point to final point) travelled was acquired using Pythagoras' theorem, and velocity and displacement (path travelled) were also identified for each cell. Values from treated cells were then compared to untreated cells.

2.16 Wound Healing assay

Cells were grown to 100% confluency in a petri dish. Using a 10μl pipette a gap was formed by scratching the cell surface. Cells were treated with either rhSP-A or rhSP-D (10 μg/ml) or OD-02 (10^{-7}M). The petri dish was placed under a Zeiss Axiovert 200M microscope and images were captured every 10 minutes for up to 48 hours, to visualise the distance between the cells and how quickly the gap closed. Treated cells were then compared to untreated cells.

2.17 Immunofluorescence

Cells were grown and seeded at a specific density in a 24-well plate that contained coverslips. Cells were allowed to adhere for 24 hours and then fixed with 4% PFA (Sigma-Aldrich) for 10 minutes at room temperature. Cells were then permeabilised with 0.5% Triton X-100 (Sigma-Aldrich) on ice for 5 minutes. Cells were blocked in 5% FBS-PBS (v/v) for 45 minutes and then incubated for 45 minutes with anti-human SP-A and SP-D polyclonal antibodies which were diluted in 5% FBS-PBS (v/v). Cells were washed and then incubated with a staining buffer that contained Alexafluor 568 secondary anti-rabbit antibody, phalloidin 488 (to stain f-actin, cytoskeleton) and Hoechst (to stain DNA, nucleus) (Invitrogen, Life Technologies). This incubation was done in the dark for 45 minutes. After another wash with 5% FBS-PBS

(v/v), the coverslips were mounted on slides and visualised on a HF14 Leica DM4000 microscope.

2.18 ImageStream

A confluent 75cm^2 flask of cells was used for ImageStream analysis. The cells were detached from the flask using TryPLE Express and an amount of fresh DMEM was added to the cells for an end volume of 5ml. This was split evenly into 5 eppendorf tubes and those were centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with sterile PBS, followed by another spin for 3 minutes at 2000 rpm. Cells were fixed in 4% PFA for 7 minutes on ice and then centrifuged for 5 minutes at 1500 rpm. The supernatant was again discarded and the pellet was washed with PBS and spun at 2000 rpm for 3 minutes. Cells were blocked for 30 minutes in FBS-PBS and then centrifuged at 2000 rpm for 3 minutes. The supernatant was removed and the cells were incubated overnight with primary antibody (SP-A or SP-D) diluted in FBS-PBS (1:2000) at 4^{o}C. The next day, cells were centrifuged at 2000 rpm for 3 minutes and then washed with PBS. Secondary HRP conjugated anti-rabbit antibody, diluted in FBS-PBS (1:1000) was added to the cells for a 30 minute-incubation. The cells were centrifuged and washed once more as described above. Accumax (Innovative Cell Technologies) and DRAQ5 were added before visualising in the ImageStream. 5 samples in total were made.

Table 2.7 *ImageStream samples composition used for the localisation of SP-A and SP-D. A and B samples contained cells incubated with the respective antibodies, whereas the rest (C, D and E) were used as compensation samples to normalise data.*

Sample name	Description	Consistency
A	Cells+SP-D	Primary+secondary+DRAQ5
B	Cells+SP-A	Primary+secondary+DRAQ5
C	Cells SP-D compensation	Primary+secondary (no DRAQ5)
D	Cells SP-A compensation	Primary+secondary (no DRAQ5)
E	Cells compensation	DRAQ5 only

After the machine was calibrated, 10,000 cells were counted for acquisition. Compensation samples as described in the table above were also used for data normalisation. Data and images were analysed using an ImageStream^{x} Mark II Imaging Flow Cytometer (Amnis, Merck Millipore).

2.19 Immunohistochemistry

To identify the expression and localisation of SP-D in normal ovarian and ovarian cancer tissue samples, Ovarian carcinoma test tissue microarrays, containing 10 cases of ovarian tumour with 2 non-neoplastic tissue, duplicated cores per case, were used (Biomax, U.S.).

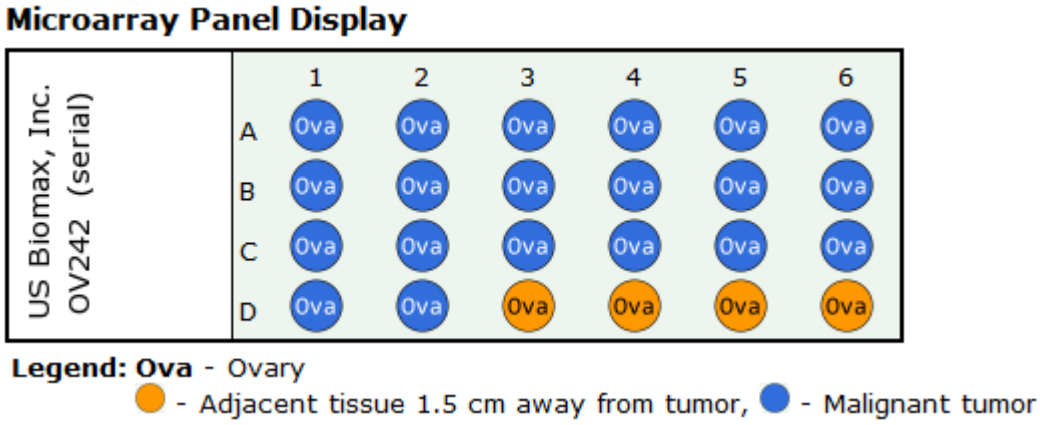


Figure 2.2 *Microarray panel display of the ovarian carcinoma tissue test slide. Figure showing the position of the malignant tissue as well as the normal ovarian tissue samples.*

Table 2.8 *Specifications showing the sample number, position on the slide, the age of the patient, the pathology diagnosis and the grade of ovarian cancer, as well as normal samples.*

Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade*	Type
A1	1	F	40	Ovary	Endometrioid adenocarcinoma	II	Malignant
A2	2	F	40	Ovary	Necrosis tissue	–	Malignant
A3	3	F	57	Ovary	Serous papillary cystadenocarcinoma	II	Malignant
A4	4	F	57	Ovary	Serous papillary cystadenocarcinoma	II	Malignant

Pos	No.	Sex	Age	Organ	Pathology diagnosis	Grade*	Type
A5	5	F	48	Ovary	Clear cell carcinoma	–	Malignant
A6	6	F	48	Ovary	Clear cell carcinoma	–	Malignant
B1	7	F	57	Ovary	Serous papillary cystadenocarcinoma	II	Malignant
B2	8	F	57	Ovary	Serous papillary cystadenocarcinoma	II	Malignant
B3	9	F	46	Ovary	Fibrofatty tissue	–	Malignant
B4	10	F	46	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
B5	11	F	42	Ovary	Endometrioid adenocarcinoma	I	Malignant
B6	12	F	42	Ovary	Endometrioid adenocarcinoma	I	Malignant
C1	13	F	66	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
C2	14	F	66	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
C3	15	F	53	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
C4	16	F	53	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
C5	17	F	35	Ovary	Serous papillary cystadenocarcinoma	II	Malignant
C6	18	F	35	Ovary	Serous papillary cystadenocarcinoma	II	Malignant
D1	19	F	52	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
D2	20	F	52	Ovary	Serous papillary cystadenocarcinoma	III	Malignant
D3	21	F	41	Ovary	Cancer adjacent ovary tissue	–	Adjacent
D4	22	F	41	Ovary	Cancer adjacent ovary tissue	–	Adjacent
D5	23	F	49	Ovary	Cancer adjacent ovary tissue	–	Adjacent
D6	24	F	49	Ovary	Cancer adjacent ovary tissue	–	Adjacent

*The grade 1-3 (or I-III) in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.
Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.
Grade 2 or moderately-differentiated: Cells appear slightly different than normal.

Grade 3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.
Grade 4 or undifferentiated: (for certain tumours), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.
Slides were deparaffinised by washing in coplin jars as follows:

Table 2.9 *Table showing the composition and duration for the washes in order to deparaffinise the tissue array slide.*

Histoclear	3 minutes (twice)
Histoclear:Ethanol (1:1)	3 minutes
100% Ethanol	3 minutes
95% Ethanol	3 minutes
70% Ethanol	3 minutes
50% Ethanol	3 minutes
Running tap water	1 minute

Sodium citrate (2.94g in 1L dH_2O, 500µl Tween, pH6) was warmed in the microwave. The slides were boiled for 20 minutes but were not allowed to boil dry. They were then washed with running tap water to cool and washed twice with TBS 0.025% Triton X on a shaker. Slides were incubated with 3% hydrogen peroxide in PBS for 30 minutes and then washed in TBS 0.025% Triton X 3 times for 5 minutes each. Slides were blocked for an hour by incubating with 5% goat serum in PBS Triton-X in a humidity chamber. Anti-human primary antibodies for SP-A and SP-D were diluted in PBS Triton-X with goat serum and loaded on the slides and spread using parafilm. The primary antibody incubation was performed overnight at 4^{o}C in a humidity chamber. The next day, the slides were washed in TBS 0.025% Triton X 3 times for 5 minutes each, and then incubated with anti-rabbit secondary antibody diluted in PBS Triton-X with goat serum, for 1 hour at room temperature in a humidity chamber. After a series of washes as mentioned above, a DAB solution (Vector Laboratories) containing hydrogen peroxide was loaded on the slides for 5 minutes at room temperature. Brown staining in the form of spots was evident on the slides. Slides were washed in dH_2O for 5 minutes and then incubated with haematoxylin for 30 seconds, to allow the staining of the

nucleus. The excess of solution was rinsed off under dH_2O. Slides were stained with 0.1% sodium bicarbonate for 30 seconds and then dehydrated as follows:

Table 2.10 *Table showing the composition and duration for the washes in order to dehydrate the tissue array slide.*

50% Ethanol	3 minutes
70% Ethanol	3 minutes
95% Ethanol	3 minutes
100% Ethanol	3 minutes
Histoclear:Ethanol (1:1)	3 minutes
Histoclear	3 minutes

A coverslip was placed on top of the slide with a few drops of DPX, and dried overnight at 4^{o}C. Images of the tissues were captured using an EOS 1200D Brunel Microscope Ltd. For the analysis, the tissue was split in three sections and positive stained cells were counted; an average was acquired for each tissue.

2.20 Collagen Assay

For this assay, ULTR cells were grown in phenol red free DMEM because phenol contains oestrogens that would affect cell contraction. DMEM was made using charcoal stripped FBS and penicillin/streptomycin. Charcoal stripped FBS was chosen as it is treated with activated carbon that removes non-polar, lipophilic material such as hormones, cytokines and growth factors, regardless of molecular weight. Therefore it has all the hormonal and amino acid components removed while still allowing cell growth and maximal contraction after incubation. For the collagen discs the following materials were added: collagen from bovine skin (Sigma-Aldrich), 10x PBS and water. The pH of the solution is required to be 7 in order to allow cell growth and survival. pH was measured using litmus paper and 0.1 M NaOH was added to adjust the pH. The volume added was subtracted from the initial volume of water needed. After the solution was ready, it was kept on ice. Cells were counted as described above and the required amount was added to the collagen solution which was then vortexed. 400 µl of cell-collagen solution was added in a 24 well plate that would eventually contain

about 150,000 cells. Wells with no treatments and no cells were used as a control. The plates were incubated for about 2 hours at 37^{o}C to allow polymerisation. Using a fine pipette tip the collagen discs were released from the sides of the wells, scoring around the edge carefully, as vigorous movement would cause the discs to fold and make them un-useable. Cells were treated in a dose dependant manner and all treatments were performed in triplicates. Cells were treated with rhSP-A and rhSP-D with a final concentration of 10 µg/ml. Using the BioRad machine, photos were taken after the addition of treatments at time intervals: 0 minutes (time before treatment), 30 minutes, 1 hour, 2 hours, 4 and 24 hours. The software Image J was used to measure the surface area changes.

2.21 Statistical analysis

Data from all the experiments were analysed using the f-test to determine whether the data is of equal or unequal variance. If the data was of equal variance, a student's *t*-test was performed in order to determine significance between groups of data. If the data had unequal variance the Mann-Whitney U test was performed in order to determine significance. Values were considered significant when *$p<0.05$, *$p<0.01$ and ***$p<0.001$.

Chapter 3

Study of the effects of rhSP-A and rhSP-D on the myometrium-derived cell line ULTR

3.1 Introduction

Preterm delivery is one of the leading causes of perinatal mortality and morbidity that affects thousands of babies and it is on the rise (Goldenberg *et al.*, 2008). Babies born prematurely have a high risk of disabilities, which include respiratory issues, cerebral palsy, and visual or hearing impairment (Beck *et al.*, 2010). Maintenance of pregnancy is of great importance and the uterus needs to host the foetus thus allowing protection against pathogens. The preservation of the balance between pro- and anti-inflammatory events will determine the outcome of the pregnancy and the time of parturition (Yadav *et al.*, 2014). Labour however, is thought to require a pro-inflammatory environment (Sykes *et al.*, 2014).

SP-A and SP-D are innate immune molecules and they have been localised in various areas of the reproductive tract and the uterus. These molecules are thought to play a protective role against pathogens (MacNeill *et al.*, 2004, Oberley *et al.*, 2004). They are also expressed in foetal lungs and play a role in lung maturation for post-natal survival (Nayak *et al.*, 2012). There is emerging data supporting a role for SP-A and SP-D in the regulation of pregnancy and parturition. They can have a dual role based on the molecules they bind to; they can have a pro- or anti- inflammatory effect (Nayak *et al.*, 2012). SP-A and SP-D have been localised in the amniotic fluid at 26 weeks of gestation and their levels are increased towards term (Miyamura *et al.*, 1994). They are produced in the lungs of the foetus in the third trimester (Haagsman and van Golde, 1994). Previous studies showed that an increase of SP-A in the amniotic fluid led to the expression of IL-1β and the activation of NF-κB, which involves SP-A in the initiation of parturition in mice. When pregnant mice were injected with SP-A, they gave birth prematurely whereas when pregnant mice were injected with polyclonal anti SP-A antibody, initiation of labour was delayed for up to 24 hours (Condon *et al.*, 2004). SP-D however, has been shown to interact with decorin, one of the most important proteoglycans of the foetal membranes and the cervix, via regulation of the collagen content in the ECM, which

potentially involves SP-D in tissue remodelling during labour and bringing the uterus to its pre-pregnancy state (Nadesalingam *et al*., 2003).

There are several events leading to myometrial activation (activation of HPA axis, inflammation, uterine distension, ischemia), but they all lead to a few well-understood downstream pathways that play a role in initiating labour which involve CAP genes (Behrman *et al*., 2007). Based on this data that involves SP-A and SP-D in the reconditioning of the uterus or the transitioning from the quiescent state to the active state before parturition we sought to further investigate their involvement in pregnancy and labour.

The human myometrial cell line ULTR was used as an *in vitro* model to study the myometrium. ULTR is a smooth muscle cell line that has many characteristics of a primary human myometrial cell line and has been validated as a mean to study several functions of the myometrium, including inflammatory events and cytokine expression (Perez-Reyes *et al*., 1992).

3.2 Objectives

-Identify effects of SP-A and SP-D on cell proliferation and motility;

-Assess the effects of SP-A and SP-D on key contractile genes (OXTR, CX43, and COX2) that play a major role in pregnancy and parturition;

-Assess the effects of SP-A and SP-D on components of the mTOR pathway (mTOR and DEPTOR) which are involved in the reconditioning of the myometrium;

-Investigate whether SP-A and SP-D produce a pro or anti-inflammatory milieu and how that could lead to induction of contractions and parturition by studying their effects on cytokine expression;

-To measure the expression of SP-A and SP-D in a range of myometrial tissue clinical samples from women that delivered at term and preterm, labour (contracting myometrium) and non-labour (non-contracting myometrium).

3.3 Results

Prior to any experiment the recombinant fragments of homotrimeric CRD regions of human SP-A (rhSP-A) and SP-D (rhSP-D) were expressed in transformated BL21 (λDE3) pLysS colonies. Expressed proteins were purified and were passed through columns for endotoxin removal.

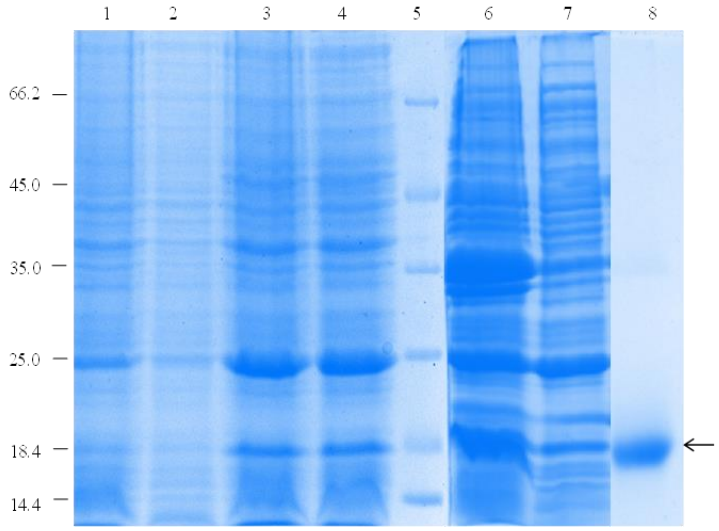


Figure 3.1 *SDS-PAGE for SP-A expression and purification samples. Lanes show: 1; before induction, 2; uninduced, 3; induced with IPTG, 4; induced with IPTG, 5; protein marker, 6; pellet after lysis, 7; supernatant after lysis, 8; purified peak fraction of rhSP-A (arrow) after maltose agarose column.*

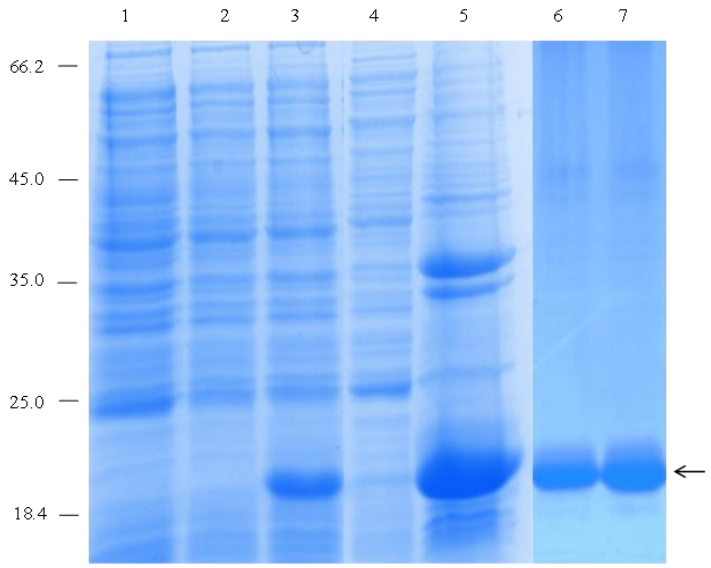


Figure 3.2 *SDS-PAGE for SP-D expression and purification samples. Lanes show: 1; before induction, 2; uninduced, 3; induced with IPTG, 4; supernatant after lysis, 5; pellet after lysis, 6 and 7; purified peak fraction of rhSP-D (arrow) after maltose agarose column.*

3.3.1 Proliferation studies to monitor effects of rhSP-A and rhSP-D on myometrium cells

ULTR myometrium cells were grown as described in the materials and methods section in 75cm^2 flasks and were seeded at a specific density (150,000) in 6 well plates. After cells were left to adhere, they were treated with rhSP-A or rhSP-D at 10 µg/ml, which was used as the optimal concentration for treatment. Cells were counted after 24 and 48 hours. Cell numbers were counted in 3 independent experiments.

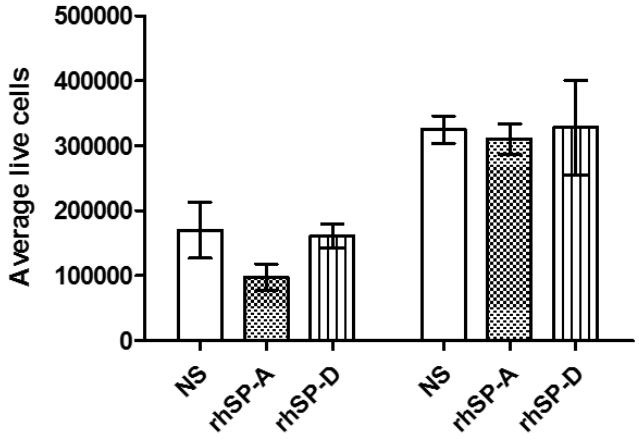


Figure 3.3 *Schematic representation showing average ULTR cell numbers after cells were treated with rhSP-A or rhSP-D at 24 and 48 hours ±SD, of three independent experiments. No significant difference in cells numbers appears at 24 hours and 48 hours between treated cells with SP-A/SP-D and untreated cells. Significance was calculated using the unpaired student's t-test (n=3). NS: no supplement.*

After 24 hours, there was not a significant difference in cell numbers under either treatment compared to the untreated cells. After 48 hours, live untreated cells had almost doubled and there was no significant difference between untreated cells and cells treated with rhSP-A or rhSP-D (Figure 3.3).

3.3.2 Immunofluorescence of ULTR cells using anti SP-A and anti SP-D antibodies

ULTR cells were seeded at a specific density (70,000) in 24-well plates and left to adhere for 24 hours. Cells were then incubated with antibodies for SP-A or SP-D for 24 hours and then with anti-rabbit secondary antibodies to enable visualisation. A HF14 Leica DM 4000 microscope was used to take images of the cells and to localise surfactant proteins.

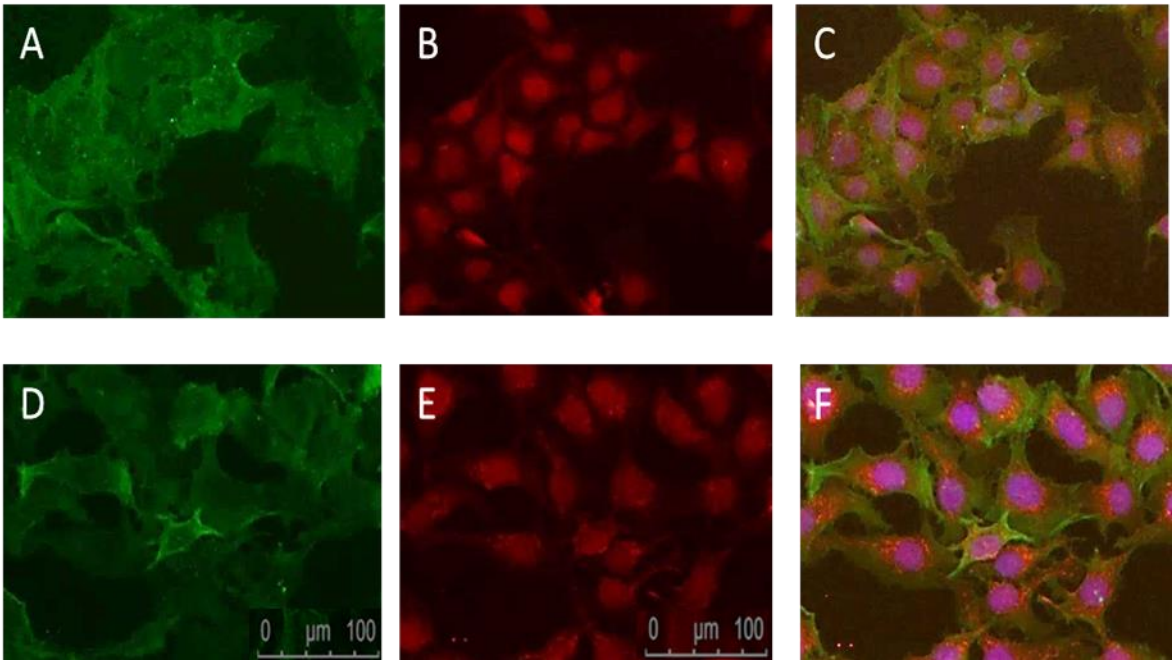


Figure 3.4 *Immunostaining of ULTR cells using antibodies for SP-A (B) and SP-D (E). Cytoskeleton staining using phalloidin 488 for SP-A (A) and SP-D (D). Nuclear staining using Hoechst. Merged images for SP-A (C) and SP-D (F), x 40 magnification.*

Based on the immunofluorescence, it is evident that ULTR cells express both surfactant proteins. It was also shown that surfactant proteins appear to be mainly localised in the cytoplasm (Figure 3.4).

3.3.3 ImageStream flow cytometry

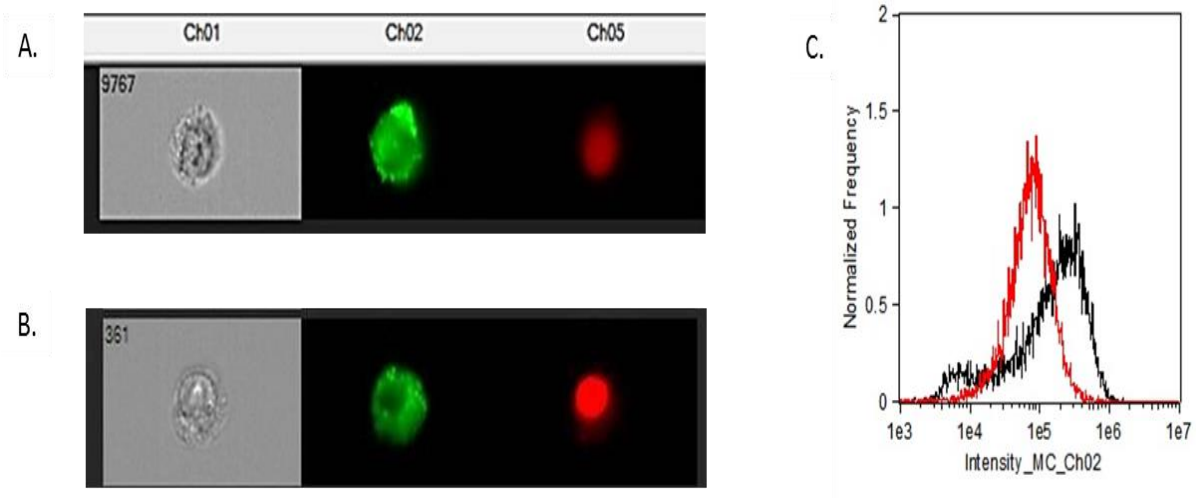


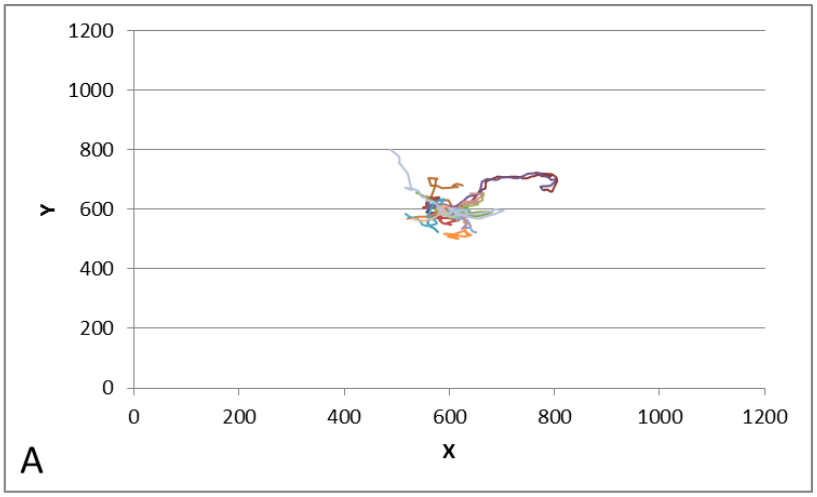
Figure 3.5 *ImageStream analysis showing localisation of surfactant proteins SP-A (A) and SP-D (B), in individual cells. Channel 1 shows the brightfield photos of cells, channel 2 shows surfactant protein localisation (green) and channel 5 shows the nucleus stained with DRAQ5 (red). SP-A and SP-D appear to be localised in the cytoplasm. There was a stronger staining for SP-D (black) compared to SP-A (red) when fluorescence intensity was measured and normalised (C).*

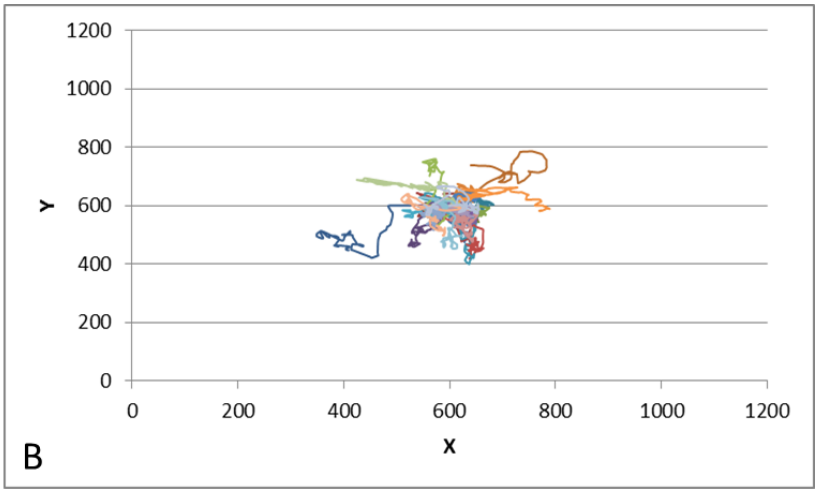
Using an ImageStreamx Mark II flow cytometer, 10,000 cells were counted and visualised. Fluorescence was normalised against cells incubated with antibodies and without DRAQ5 (nuclear stain), as well as cells incubated with just DRAQ5. ULTR cells express surfactant proteins and their localisation appeared to be cytoplasmic, confirming the data that was acquired using Immunofluorescence. When the intensity of staining was measured and normalised, it was shown that there was a higher staining for SP-D compared to SP-A (Figure 3.5).

3.3.4 Cell motility assay for ULTR cells treated with surfactant proteins rhSP-A and rhSP-D

ULTR cells were first cultured in a 75cm^2 flask and allowed to reach 100% confluency. They were then sub-cultured at approximately 30% confluency in a petri dish. Cells were allowed to adhere for 5-6 hours before being treated with rhSP-A or rhSP-D. The petri dishes were placed

under a Zeiss Axiovert 200M microscope which had an incubator attached to allow cell visualisation and survival under standard culture conditions. After an area with approximately 25-30 cells was located, images were captured every 5 minutes for up to 12 hours. Using ImageJ, the X and Y coordinates for 25 cells were acquired.





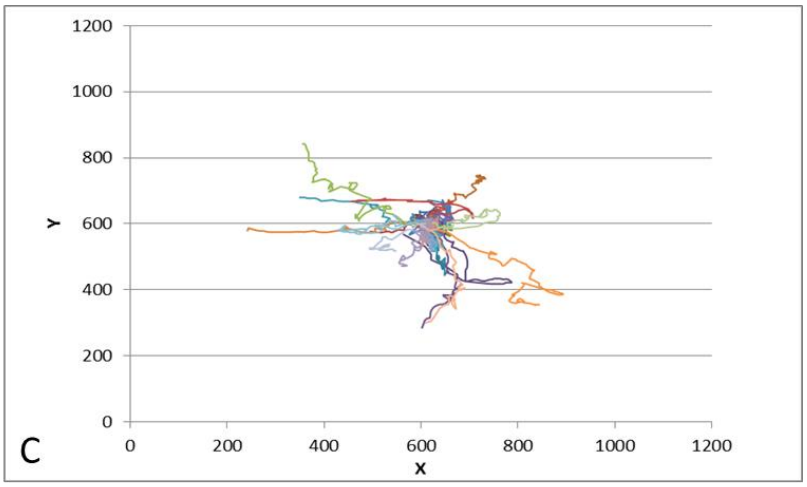


Figure 3.6 *Schematic representation showing the coordinates X and Y (pixels) across all time points of untreated (A), treated with rhSP-A (B) and treated with rhSP-D (C) ULTR cells. 25 cells were counted for each experiment. The point (600,600) was set as a starting point for each cell. Untreated cells did not appear to move considerably from their initial position, whereas cells treated with surfactant proteins appeared to move further away from their starting position. Each line represents a single cell (n=25).*

Based on the coordinates acquired using ImageJ, the start point for each cell, was set at (X=600, Y=600) in order to be able to compare all cells and in between experiments. Then at each time point, the position of each of the cells was determined in order to acquire all the coordinates. Cells treated with rhSP-A and rhSP-D moved further away from their initial position across time points. Untreated cells appeared to remain close to their initial position or move slightly across time points (Figure 3.6).

Using Pythagora's theorem for each point for each of the cells, the distance, velocity and displacement for all 25 cells were calculated.

Table 3.1 *Table showing the distance, velocity and displacement values for the cell motility of untreated, cells treated with rhSP-A and cells treated with rhSP-D. NS: no supplement (n=1).*

	Distance (pixels)	Velocity (pixels/minute)	Displacement (pixels)
ULTR NS	215.587	0.449	74.905
ULTR x rhSP-A	734.755	1.502	90.192
ULTR x rhSP-D	517.096	1.077	127.909

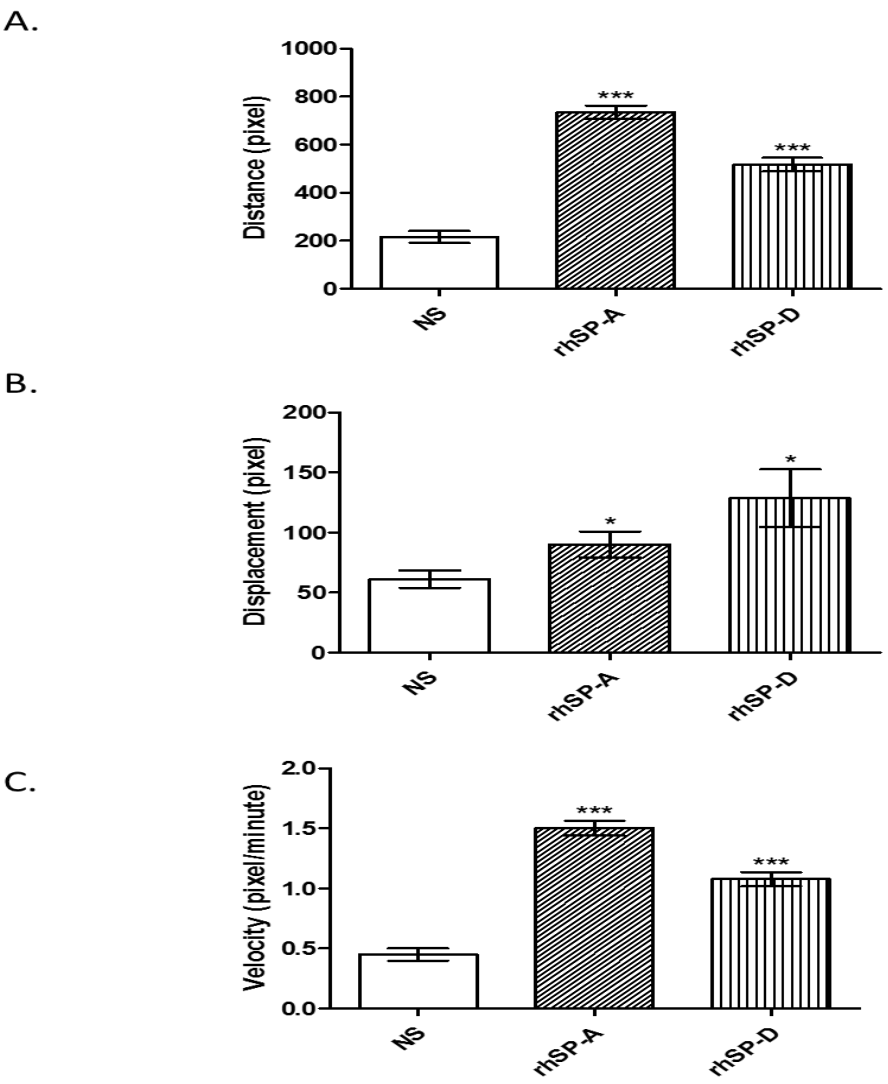


Figure 3.7 *Schematic representation of the distance (A), velocity (B) and displacement (C) comparisons ±SEM between untreated and treated ULTR cells with rhSP-A or rhSP-D at 10 µg/ml (Mann Whitney U test: *p<0.05, ***p<0.001). 25 cells were counted for each experiment. Treatments with surfactant proteins resulted in a significant increase in distance, velocity and displacement compared to untreated cells. rhSP-A resulted in a significant increase in distance and velocity compared to rhSP-D treated cells. There was no significant difference in displacement between treatments. NS: no supplement.*

Cells treated with rhSP-A or rhSP-D moved significantly further and faster compared to untreated cells. There was a significant increase in distance, velocity and displacement compared to untreated cells. Treatment with rhSP-A resulted in a significant increase in

distance and velocity compared to cells treated with rhSP-D (1.4 fold). In regards to displacement, there was no significant difference in between treatments (Figure 3.7).

3.3.5 Wound healing assay

The wound healing assay has been extensively used as an experimental model to study various cell lines and how specific molecules/conditions lead to the closing of an artificial gap created on the cell surface. We therefore used the same technique to study the reconditioning of the myometrium and the healing of scars created due to contractions. ULTR cells were grown in petri dishes and reached 100% confluency. Using a fine pipette tip, an artificial wound was created on the cell surface. The petri dishes were placed under a Zeiss Axiovert 200M microscope attached to an incubator to allow visualisation as well as cell survival. Pictures of the cells were acquired every 10 minutes for up to 48 hours.

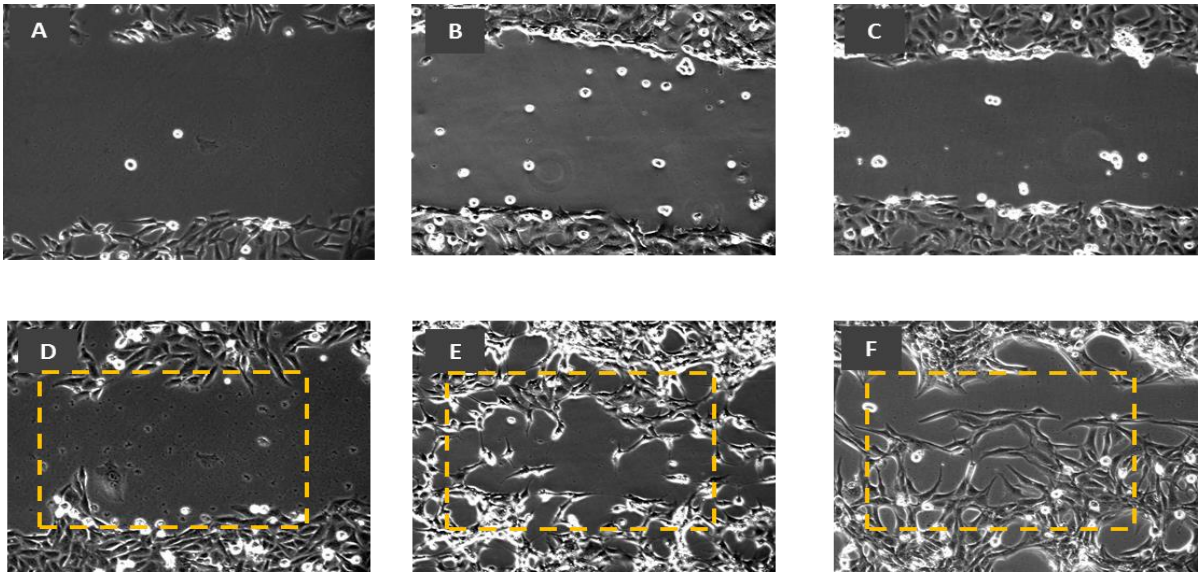


Figure 3.8 *Schematic representation showing the artificial wound created on the cell surface in untreated cells (A), treated cells with rhSP-A (10 µg/ml) (B) and treated cells with rhSP-D (10 µg/ml) (C) at 0 hours. Untreated cells did not close the gap after 48 hours (D), whereas rhSP-A treated cells closed the gap after 17 hours (E) and rhSP-D treated cells did so after 25 hours (F).*

Images acquired from the wound healing assay over 48 hours showed that untreated cells did not close the gap after 48 hours. ULTR cells treated with rhSP-A closed the gap after 17 hours

and rhSP-D treated cells closed the gap after 25 hours (Figure 3.8). This suggests that surfactant proteins affect cell migration.

3.3.6 Cell contractility assay

In order to identify whether SP-A and SP-D affect contractility in the myometrium *in vitro*, ULTR cells were seeded at a specific density and grown in 24-well plates. Cells were seeded in a collagen matrix and left to adhere for 2-3 hours and then the collagen disc was detached from the sides of the wells using a fine pipette tip. Cells were treated with rhSP-A or rhSP-D (10 µg/ml) and images of the discs were acquired at specific time points. The surface area of the discs was measured using ImageJ.

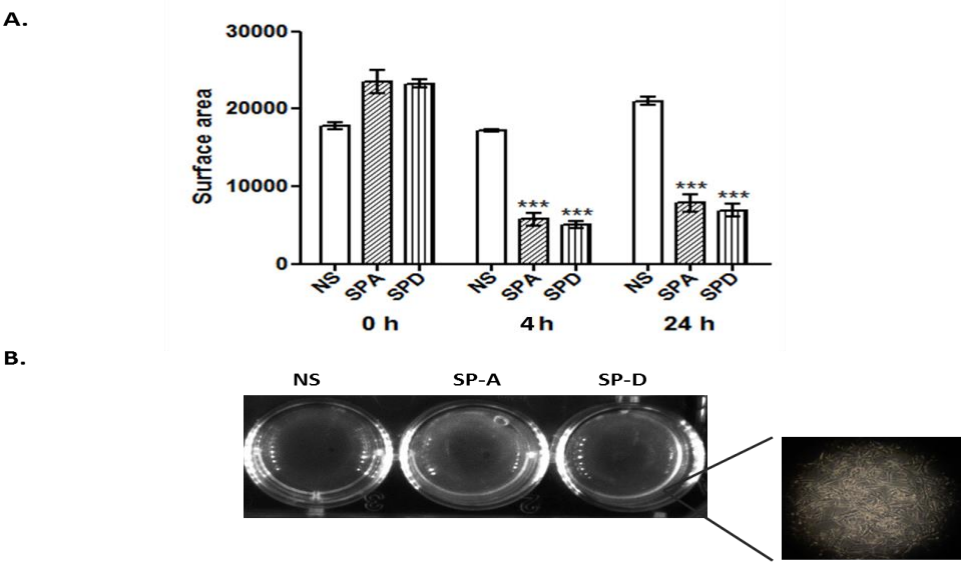


Figure 3.9 *Schematic representation showing the average surface area (pixels) ±SEM between untreated cells and treated cells with rhSP-A or rhSP-D (10 µg/ml) at 4 and 24 hours (A) (n=4). rhSP-A and rhSP-D significantly reduced the disc surface area compared to untreated cells at 4 and 24 h (A) (***p<0.001, unpaired student's t-test). Photos of collagen discs at 4 h (B; NS: no supplement). Insert: picture of ULTR cells growing within the collagen matrix.*

Images acquired were processed with ImageJ to measure the surface area of the discs for each experiment and time point. Treatments with both surfactant proteins led to a significant decrease in the surface area of the collagen discs compared to the untreated cells after 4 hours.

Treatment with rhSP-A resulted in a 66% reduction in disc surface area and with rhSP-D in a reduction of 70%. The surface area of the discs remained at a similar size even at 24 hours. There was no significance observed between treatments with rhSP-A and rhSP-D (Figure 3.9).

3.3.7 Expression of key contractile genes in ULTR cells

ULTR cells were treated with rhSP-A or rhSP-D for different times (4, 6, 12 and 24 hours) and at different concentrations (5, 10 and 20 μg/ml). RNA and protein samples were collected at the above mentioned time points and cDNA was produced following the procedure described in materials and methods.

cDNA samples were loaded on a 96-well plate along with PCR buffer and specific primers for each gene of interest. Non template controls were used to evaluate potential contamination of the samples. All values were normalised against the house-keeping gene GAPDH. Three biological experiments were performed for each study.

3.3.7.1 Study of the expression of CAP genes in rhSP-A treated ULTR cells

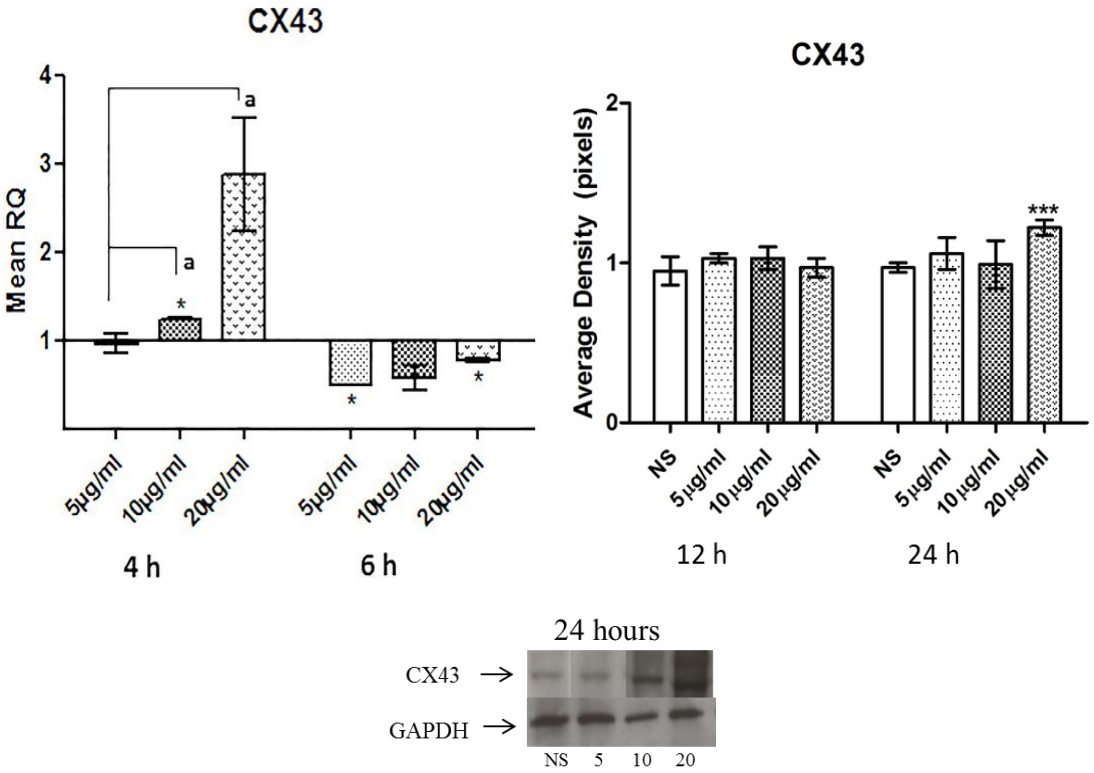


Figure 3.10 *Left: Schematic representation of mean relative quantities showing fold change of connexin 43 in ULTR cells treated with rhSP-A at 4 and 6 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-A resulted in an increase of the CX43 transcript after 4h (10 and 20 µg/ml), compared to untreated cells; levels were decreased after 6 hours. Right: Average density comparisons of the protein expression bands for CX43 in ULTR cells treated with SP-A ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for CX43 at 24 hours. CX43 protein expression was increased after 24 hours at 20 µg/ml, when compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, ***p<0.001), a; shows significance between values as shown by the horizontal and vertical lines. 10 and 20 µg/ml resulted in an increased expression of CX43 compared to 5 µg/ml at 4 hours. Values were normalised against GAPDH. NS: no supplement.*

After Ct values were obtained from an ABI 9000 Prism, they were analysed using the Sequence Detection System (SDS 2.4). Ct values were normalised against GAPDH and no

supplement controls. They were transformed to Relative Quantity values with the untreated controls set to 1, in order to acquire fold changes values for each of the treatments.

rhSP-A treatments at 10 and 20 µg/ml led to an increase in CX43 mRNA transcript after 4 hours when compared to control cells and when compared to 5 µg/ml. Lower concentrations of rhSP-A did not have a potent effect on CX43 gene expression. At 6 hours CX43 expression was lower than the 4 hour time point and it remained at the same levels after 12 hours (data not shown). At protein level, CX43 expression was significantly increased after 24 hours at 20 µg/ml (Figure 3.10).

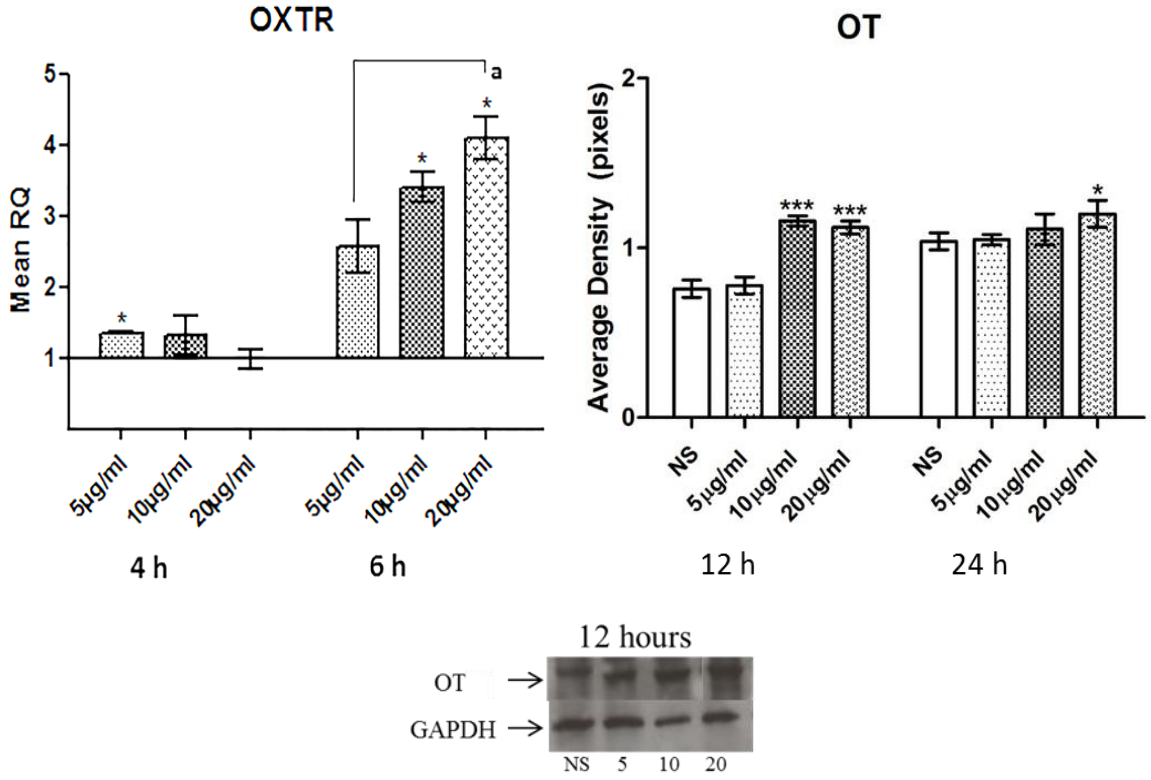


Figure 3.11 *Left: Schematic representation of mean relative quantities showing fold change of oxytocin receptor in ULTR cells treated with rhSP-A at 4 and 6 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-A resulted in an increase of the OXTR transcript after 6 hours at 10 and 20 µg/ml, compared to untreated cells. Right: Average density comparisons of the protein expression bands for OT in ULTR cells treated with SP-A ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for OT at 12 hours. OT protein expression was increased after 12 hours at 10 and 20 µg/ml, and after 24 hours at 20 µg/ml, when compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, ***p<0.001), a; shows significance between values as shown by the horizontal and vertical lines. 20 µg/ml treatment resulted in a higher expression of OXTR after 6 hours. Values were normalised against GAPDH. NS: no supplement.*

OXTR gene expression was not upregulated at all concentrations of rhSP-A at 4 hours. There was a significant increase of OXTR gene expression at 6 hours at all concentrations compared to untreated cells. The two higher doses appeared to result in a higher increase in OXTR gene

expression when compared to 5 µg/ml. At the protein level OT expression was significantly increased after 12 hours at 10 and 20 µg/ml and after 24 hours at 20 µg/ml (Figure 3.11). This implicates rhSP-A in the activation of the myometrium and initiation of labour, potentially acting through OXTR.

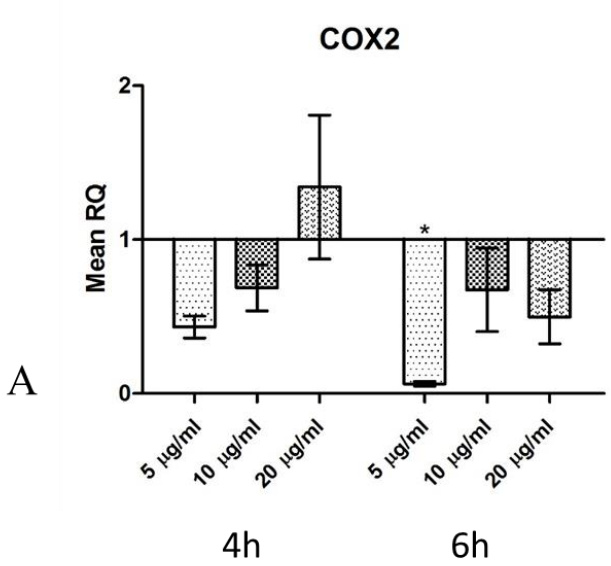


Figure 3.12 *Schematic representation of mean relative quantities showing fold change of cyclo-oxygenase 2 in ULTR cells treated with rhSP-A at 4 and 6 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-D resulted in a decrease of the COX2 transcript after 6 hours at 5 µg/ml compared to untreated cells, whereas higher treatments did not appear to have an effect. Treatments did not have an effect at 4 hours. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

Treatments with rhSP-A did not have a significant effect on COX2 mRNA expression at 4 hours whereas at 6 hours only the lower concentration appeared to decrease the expression of COX2 mRNA (Figure 3.12).

3.3.7.2 Study of the expression of CAP genes in rhSP-D treated ULTR cells

The same procedure as previously described was followed for rhSP-D treated cells.

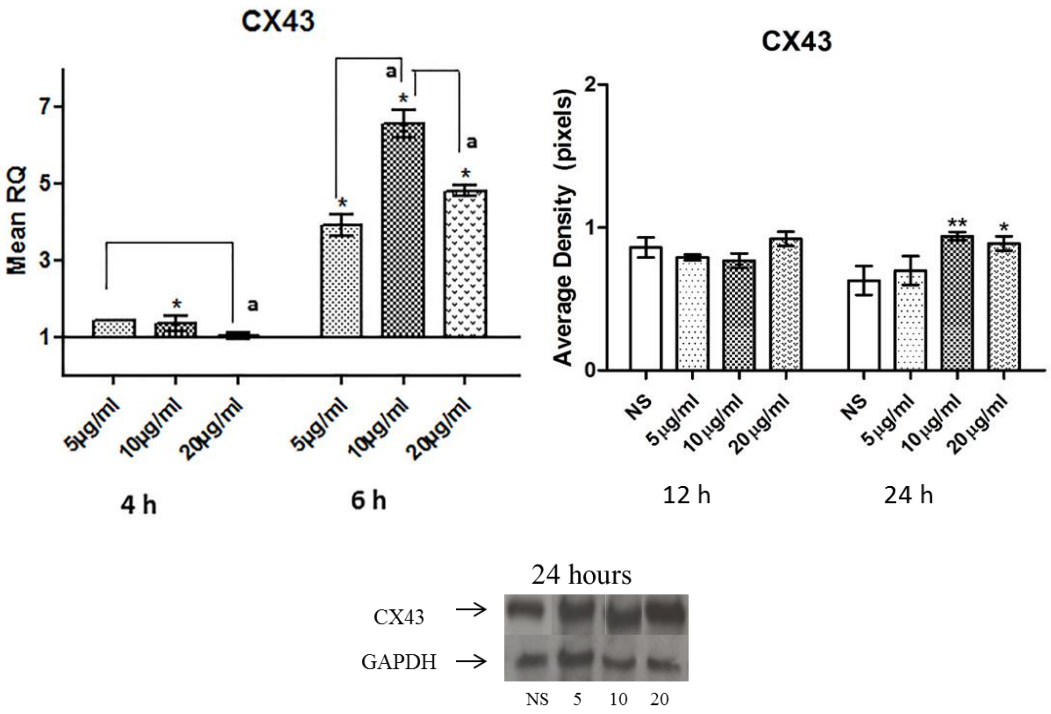


Figure 3.13 *Left: Schematic representation of mean relative quantities showing fold change of connexin 43 in ULTR cells treated with rhSP-D at 4 and 6 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-D resulted in an increase of the CX43 transcript after 6h (5, 10 and 20 µg/ml), when compared to untreated cells. Right: Average density comparisons of the protein expression bands for CX43 in ULTR cells treated with SP-D ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for CX43 at 24 hours. CX43 protein expression was increased after 24 hours at 10 and 20 µg/ml, compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, **p<0.01), a; shows significance between values as shown by the horizontal and vertical lines. 5 µg/ml treatment CX43 expression levels were higher compared to 20 µg/ml at 4 hours. 10 µg/ml treatment resulted in an increased expression of CX43 compared to 5 and 20 µg/ml at 6 hours. Values were normalised against GAPDH. NS: no supplement.*

rhSP-D treatments at all concentrations led to a moderate increase in CX43 transcript expression at 4 hours. The effect was more profound at 6 hours where rhSP-D treatments at 5, 10 and 20 µg/ml led to a significant increase even up to 6-fold of CX43 mRNA expression. Expression levels appeared to be decreased after 12 hours (data not shown). There was a

significant increase between 5 and 10 µg/ml, and 10 and 20 µg/ml, with 10 µg/ml being more potent compared to the other two concentrations. At the protein level, CX43 expression was significantly increased after 24 hours at 10 and 20 µg/ml (Figure 3.13).

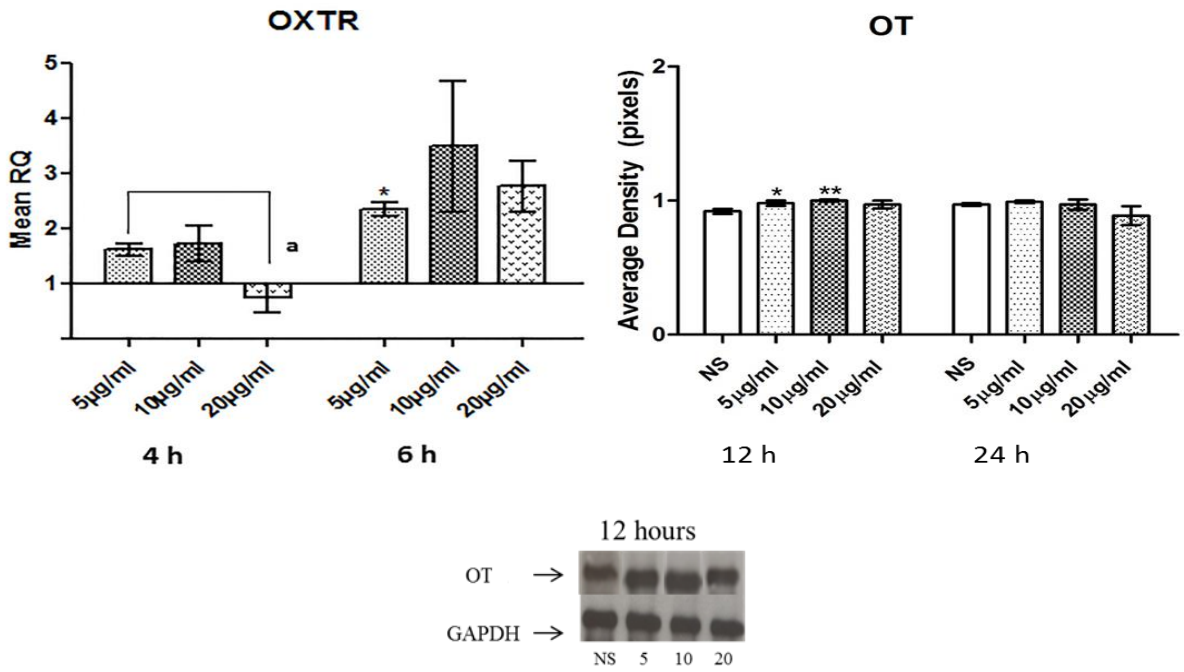


Figure 3.14 *Left: Schematic representation of mean relative quantities showing fold change of oxytocin receptor in ULTR cells treated with rhSP-D at 4 and 6 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-D resulted in an increase of the OXTR transcript after 6 hours at 5 µg/ml, when compared to untreated cells. Right: Average density comparisons of the protein expression bands for OT in ULTR cells treated with SP-D ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for OT at 12 hours. OT protein expression was increased after 12 hours at 5 and 10 µg/ml, when compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, **p<0.01), a; shows significance between values as shown by the horizontal and vertical lines. 5 µg/ml treatment resulted in an increased expression of OXTR mRNA compared to the 20 µg/ml treated samples. Values were normalised against GAPDH. NS: no supplement.*

rhSP-D treatments at 5 and 10 µg/ml had a moderate effect on the OXTR mRNA at 4 hours compared to untreated cells. The effect was more profound after 6 hours where the expression

was increased by 2.5-fold at 5 µg/ml. After 12 hours the expression levels appeared to decrease (data not shown). At the protein level, OT expression was increased after 12 hours at 10 and 20 µg/ml whereas no effect was evident after 24 hours (Figure 3.14).

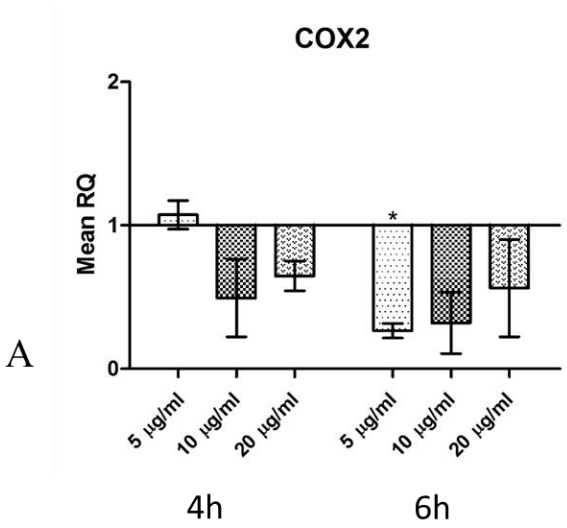


Figure 3.15 *Schematic representation of mean relative quantities showing fold change of cyclo-oxygenase 2 in ULTR cells treated with rhSP-D at 4 and 6 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-D resulted in a decrease of the COX2 transcript after 6 hours at 5 µg/ml, when compared to untreated cells, whereas higher treatments did not appear to have an effect. Treatments did not have an effect at 4 hours. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

rhSP-D treatments at all concentrations did not have a significant effect on the expression of the COX2 mRNA after 4 hours. At 6 hours, only the lower concentration appeared to decrease the expression of the COX2 transcript. The higher concentrations did not have a significant effect (Figure 3.15).

3.3.8 Auto-regulation of surfactant proteins

Surfactant proteins have several roles depending on the receptors they bind to. They can have pro or anti-inflammatory properties. Due to the nature of their functions, there needs to be a measure to regulate surfactant protein expression after a function is completed or there would

be implications, for example promoting a prolonged pro- or anti-inflammatory environment. Therefore, it was important to investigate the effects of surfactant proteins on surfactant mRNA expression.

3.3.8.1 Effects of rhSP-A on surfactant protein expression

Following the same method for growing ULTR cells and treating them with rhSP-A at different doses and time points, RNA was extracted from the cells and transcribed into cDNA. A qPCR was performed to measure the expression of SP-A1, SP-A2 and SP-D mRNA expression.

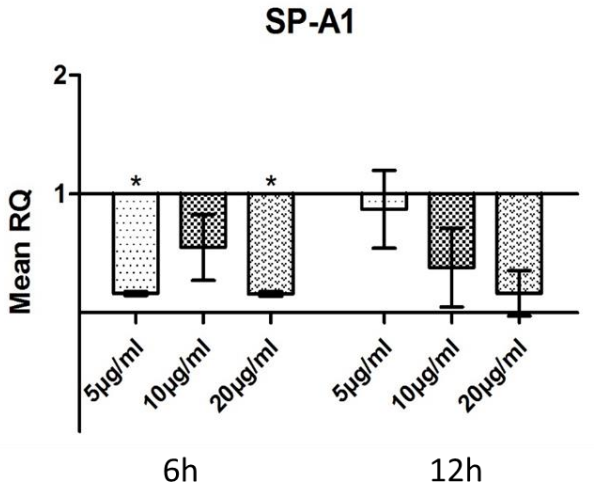


Figure 3.16 *Relative quantification comparisons showing fold change of SP-A1 in ULTR cells treated with rhSP-A at 6 and 12 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-A resulted in a decrease of the SP-A1 transcript at 5 and 20 µg/ml after 4 hours whereas it did not have any effect at 6 hours, compared to untreated cells. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

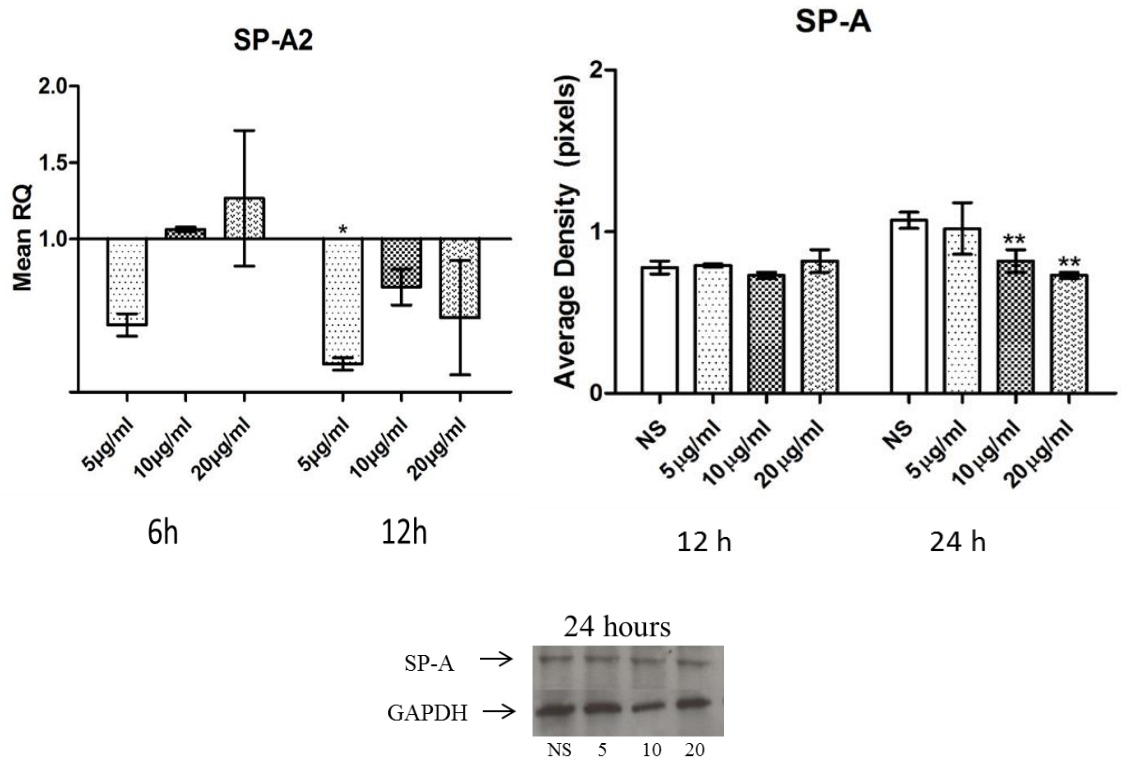


Figure 3.17 *Left: Relative quantification comparisons showing fold change of SP-A2 in ULTR cells treated with rhSP-A at 6 and 12 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-A did not have an effect after 6 hours. At 12 hours there was a significant decrease at 5 µg/ml though the higher concentrations did not have a significant effect compared to untreated cells. Right: Average density comparisons of the protein expression bands for total SP-A in ULTR cells treated with SP-A ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for SP-A at 24 hours. SP-A protein expression was significantly decreased after 24 hours at 10 and 20 µg/ml, when compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, **p<0.01). Values were normalised against GAPDH. NS: no supplement.*

Treatments with rhSP-A showed that there was a decrease in SP-A1 transcript expression after a 6-hour treatment with 5 and 20 µg/ml; there was not an effect at 12 hours (Figure 3.16). SP-A2 mRNA expression was only decreased at 12 hours after treatment with 5 µg/ml of rhSP-A. Other doses did not have an effect. At the protein level, SP-A expression was decreased after

24 hours at 10 and 20 µg/ml (Figure 3.17). There could be a negative feedback loop to control the expression of SP-A when exogenous SP-A is added to the cells.

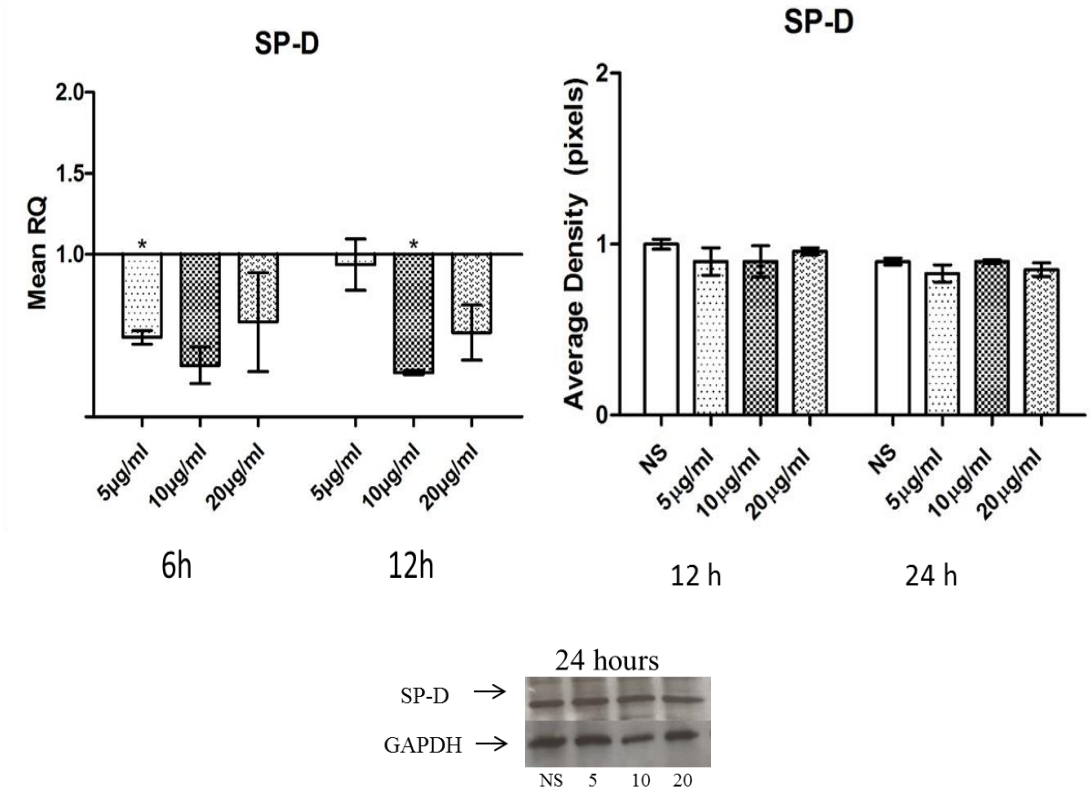


Figure 3.18 *Left: Relative quantification comparisons showing fold change of SP-D in ULTR cells treated with rhSP-A at 6 and 12 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-A resulted in a decrease of the SP-D transcript at 5 µg/ml after 6 hours and at 10 µg/ml after 12 hours, when compared to untreated cells. Right: Average density comparisons of the protein expression bands for SP-D in ULTR cells treated with SP-A ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for SP-D at 24 hours. SP-D protein expression was not affected throughout the time points or doses compared to untreated cells. Significance was obtained using the student's paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05). Values were normalised against GAPDH. NS: no supplement.*

A similar effect was observed when the mRNA expression of SP-D was measured in ULTR cells treated with rhSP-A (Figure 3.18). rhSP-A appeared to result in a decrease of SP-D transcript expression at 5 µg/ml after 6 hours and at 10 µg/ml after 12 hours. At protein level, our treatments did not have a significant effect. This is of great interest as although SP-A and SP-D have similar properties and functions, they also have their own distinct actions in the myometrium. The negative feedback loop in this case shows that excess of surfactant protein A leads to a decrease of both surfactant protein A and D in an attempt to regulate specific actions and prevent a longer time effect.

3.3.8.2 Effects of rhSP-D on surfactant protein expression

Following the same method for growing ULTR cells and treating them with rhSP-D at different doses and time points, RNA was extracted from the cells and transcribed into cDNA. A qPCR was performed to measure the expression of SP-A1, SP-A2 and SP-D mRNA.

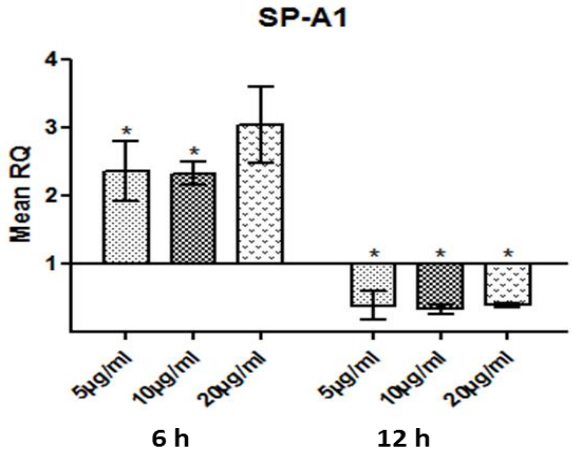


Figure 3.19 *Relative quantification comparisons showing fold change of SP-A1 in ULTR cells treated with rhSP-D at 6 and 12 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-D resulted in an increase of the SPA-1 transcript after 6 hours at 5 and 10 µg/ml, when compared to untreated cells. Expression levels were decreased at 12 hours at all concentrations. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

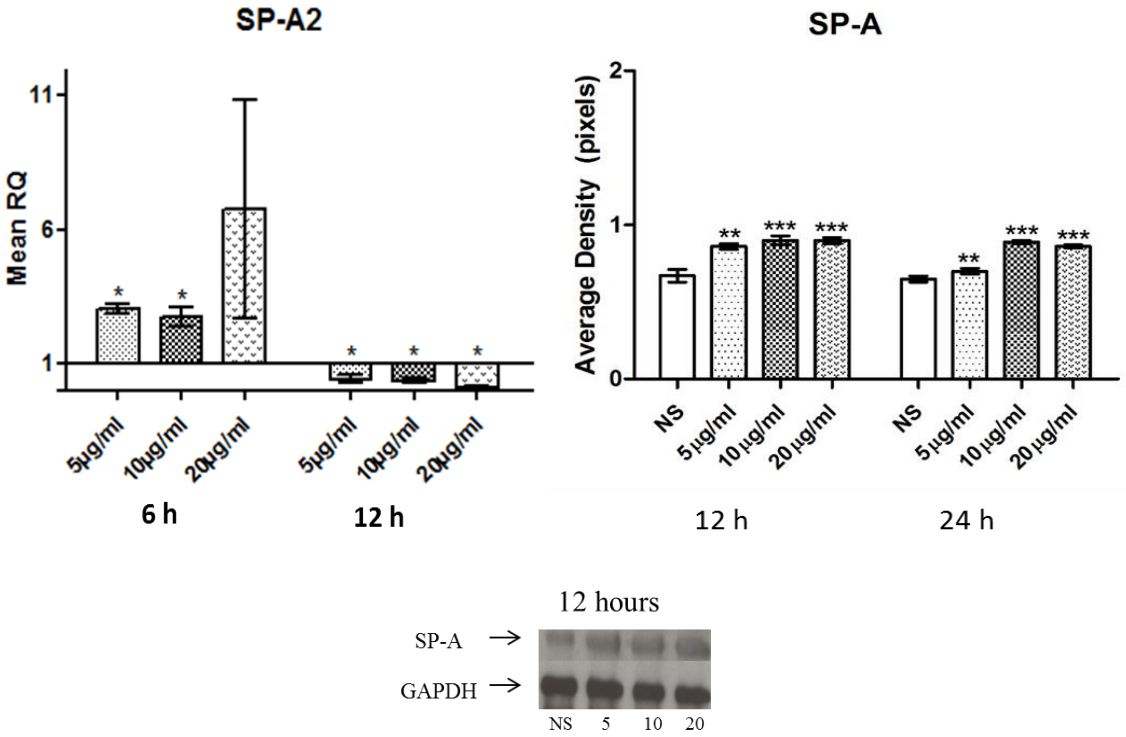


Figure 3.20 *Left: Relative quantification comparisons showing fold change of SP-A2 in ULTR cells treated with rhSP-D at 6 and 12 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-D resulted in an increase of the SPA-2 transcript after 6 hours at 5 and 10 µg/ml, when compared to untreated cells. Expression levels were decreased at 12 hours at all concentrations. Right: Average density comparisons of the protein expression bands for SP-A in ULTR cells treated with SP-D ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for SP-A at 12 hours. SP-A protein expression was increased at 5, 10 and 20 µg/ml at all time points. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, ***p<0.001). Values were normalised against GAPDH. NS: no supplement.*

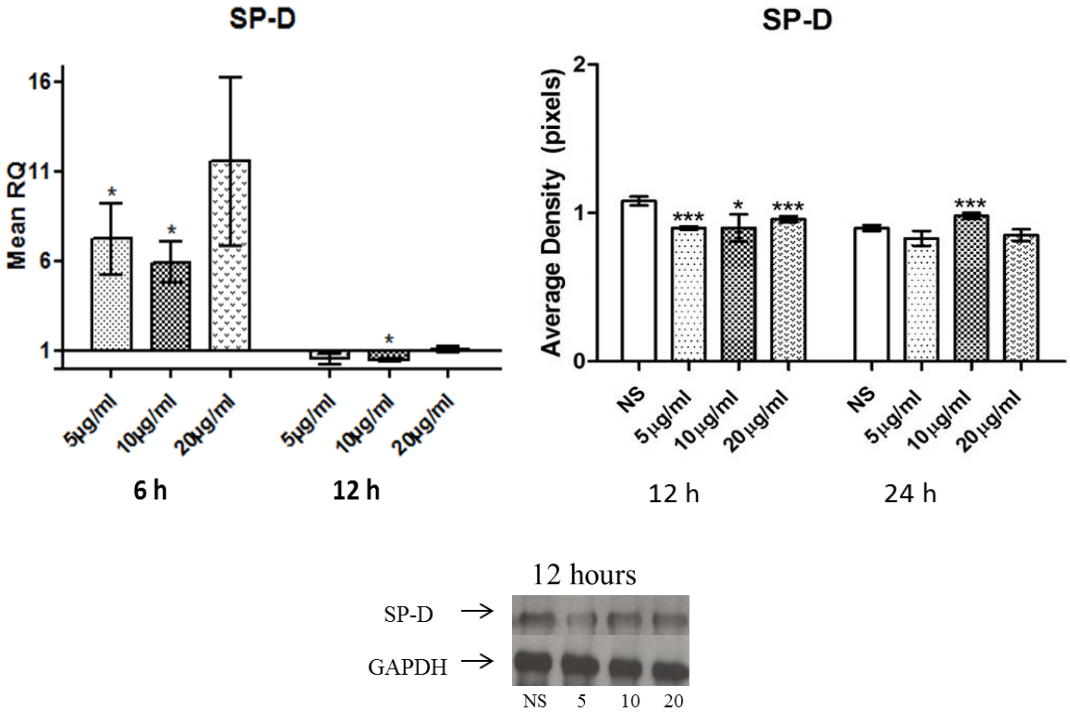


Figure 3.21 *Left: Relative quantification comparisons showing fold change of SP-D in ULTR cells treated with rhSP-D at 6 and 12 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-D resulted in an increase of the SP-D transcript after 6 hours at 5 and 10 µg/ml, when compared to untreated cells. Expression levels were significantly decreased at 12 hours at 10 µg/ml, compared to untreated cells. Right: Average density comparisons of the protein expression bands for SP-D in ULTR cells treated with SP-D ±SD at 12 and 24 hours which were acquired from the western blotting analysis. Bottom: Representative bands for SP-D at 12 hours. SP-D protein expression was decreased at 5, 10 and 20 µg/ml after 12 hours and was increased after 24 hours at 10 µg/ml, when compared to untreated cells. Significance was obtained using the paired student's t-test for the qPCR and unpaired for the WB data (*p<0.05, ***p<0.001). Values were normalised against GAPDH. NS: no supplement.*

From the observations of the surfactant protein A1 (Figure 3.19), A2 (Figure 3.20) and D (Figure 3.21) mRNA expression, it appears that rhSP-D treatments resulted in an increase of SP-A and SP-D expression at 6 hours. At the later time points, the levels significantly decreased. At protein level, SP-A expression remained increased compared to untreated ULTR

cells even at 24 hours, whereas SP-D levels were decreased at 12 hours. This is interesting as treatments with rhSP-A did not appear to lead to an increase in SP-A or SP-D at early time points, yet both molecules led to a decrease of surfactant protein mRNA expression at 12 hours at various concentrations.

3.3.9 Effects of surfactant proteins on the mechanistic target of rapamycin (mTOR) complex

mTOR is expressed in the human myometrium and one of its main roles is to regulate its reconditioning at the early stages of pregnancy to allow the accommodation of the foetus. mTOR forms complexes with DEPTOR, Rictor and Raptor (Foster *et al*., 2014). To date there has not been a study to investigate whether there is a connection between mTOR pathway and surfactant proteins. Therefore, levels of mTOR and DEPTOR were measured in ULTR samples treated with rhSP-A or rhSP-D.

3.3.9.1 mTOR and DEPTOR expression levels in rhSP-A and rhSP-D treated ULTR cells

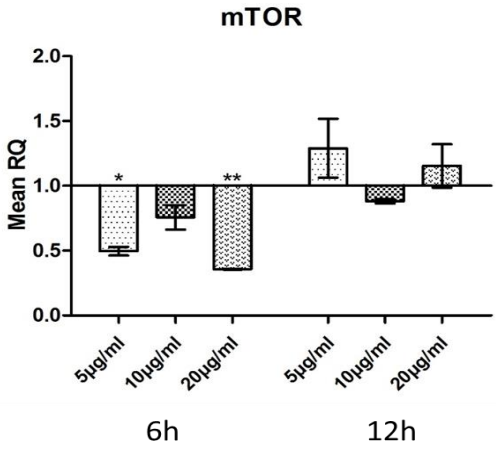


Figure 3.22 *Relative quantification comparisons showing fold change of mTOR in ULTR cells treated with rhSP-A at 6 and 12 hours ±SD (*p<0.05, **p<0.01, ***p<0.001). Results were obtained from three independent experiments. Treatments with rhSP-A resulted in a decrease of the mTOR transcript after 6 hours at 5 and 20 µg/ml, when compared to untreated cells. There was no effect after 12 hours Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

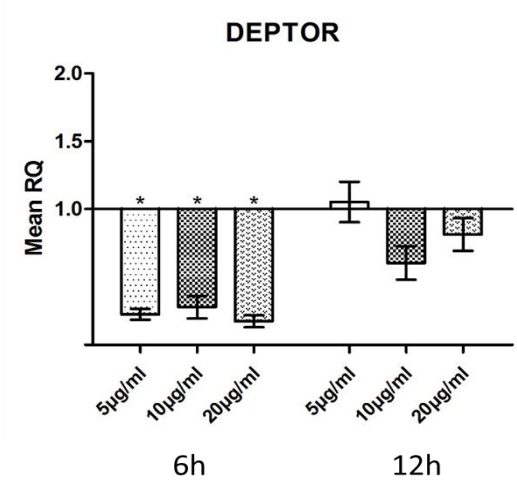


Figure 3.23 *Relative quantification comparisons showing fold change of DEPTOR in ULTR cells treated with rhSP-A at 6 and 12 hours ±SD (*p<0.05). Results were obtained from three independent experiments. Treatments with rhSP-A resulted in a decrease of the DEPTOR transcript after 6 hours at 5, 10 and 20 µg/ml whereas there was not an apparent effect at 12 hours, when compared to untreated cells. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

rhSP-A treatments resulted in a decrease of mTOR mRNA at 5 and 20 µg/ml and DEPTOR at all doses after 6 hours but they did not have an effect on the mRNA expression of mTOR (Figure 3.22) and DEPTOR (Figure 3.23) at 12 hours.

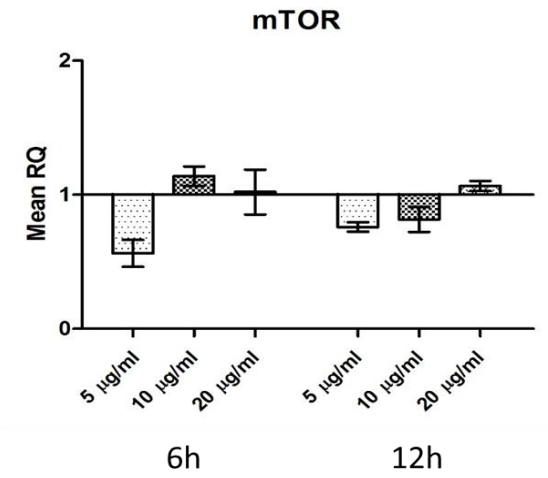


Figure 3.24 *Relative quantification comparisons showing fold change of mTOR in ULTR cells treated with rhSP-D at 6 and 12 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-D did not have an effect on mTOR expression at 6 or 12 hours at any concentration. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

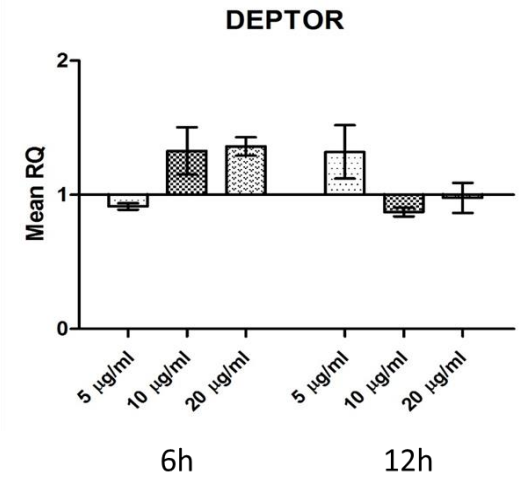


Figure 3.25 *Relative quantification comparisons showing fold change of DEPTOR in ULTR cells treated with rhSP-D at 6 and 12 hours ±SD. Results were obtained from three independent experiments. Treatments with rhSP-D did not have an effect on mTOR expression at 6 or 12 hours at any concentration. Significance was obtained using the paired student's t-test. Values were normalised against GAPDH.*

rhSP-D treated ULTR cells did not appear to express mTOR (Figure 3.24) or DEPTOR (Figure 3.25) differently to untreated cells. Treatments did not have a potent effect to alter mTOR and DEPTOR expression suggesting there is not a connection between the mTOR pathway and surfactant proteins in the myometrium.

3.3.10 Cytokine microarray analysis in ULTR cells treated with rhSP-A or rhSP-D

Conditioned media from ULTR cells treated with rhSP-A or rhSP-D at 10 and 20 µg/ml was collected, centrifuged and then used for a cytokine microarray. Media was loaded on a custom kit of a human cytokine/chemokine magnetic bead panel that consisted of 31 analytes to study their expression in ULTR samples.

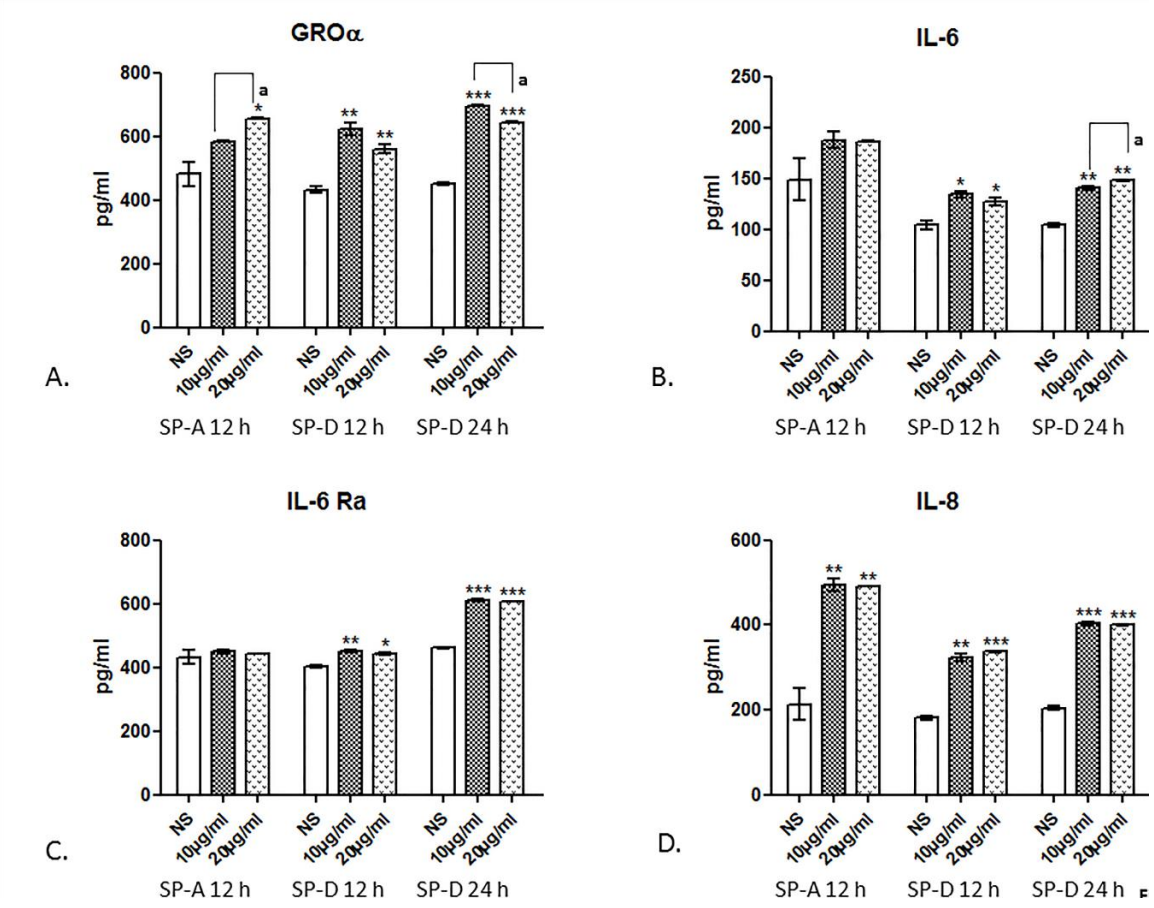


Figure 3.26 *Multiplex cytokine array analysis in conditioned media from ULTR cells treated with rhSP-A or rhSP-D at different concentration and time points. Secretion levels for GROα (A), IL-6 (B), IL-6 Ra (C) and IL-8 (D). rhSP-A treatments (10 and 20 µg/ml) resulted in an increase in the expression of GROα, IL-6 and IL-8, but did not have an effect on IL-6Ra, when compared to untreated cells. rhSP-D treated cells (10 and 20 µg/ml) showed an increase in GROα, IL-6 and IL-8 levels at 12 and 24 hours, when compared to untreated cells. ULTR cells treated with rhSP-D (10 and 20 µg/ml) also increased the levels of IL-6 Ra after 24 hours, compared to untreated cells. Significance was measured using the unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001), a; shows significance between values as shown by the horizontal and vertical lines. NS: no supplement.*

rhSP-A treated samples resulted in an increased secretion of GROα, IL-6 and IL-8 at both concentrations after 12 hours in a similar manner compared to untreated cells. A higher dose of rhSP-A seemed to increase the expression of GROα compared to the lower dose. rhSP-D

treatments led to an increase in the expression of the above mentioned cytokines at both concentrations in a similar manner. rhSP-D treatments also resulted in an increase of IL-6 Ra levels, which is a receptor of IL-6 and augments its activity in solution.

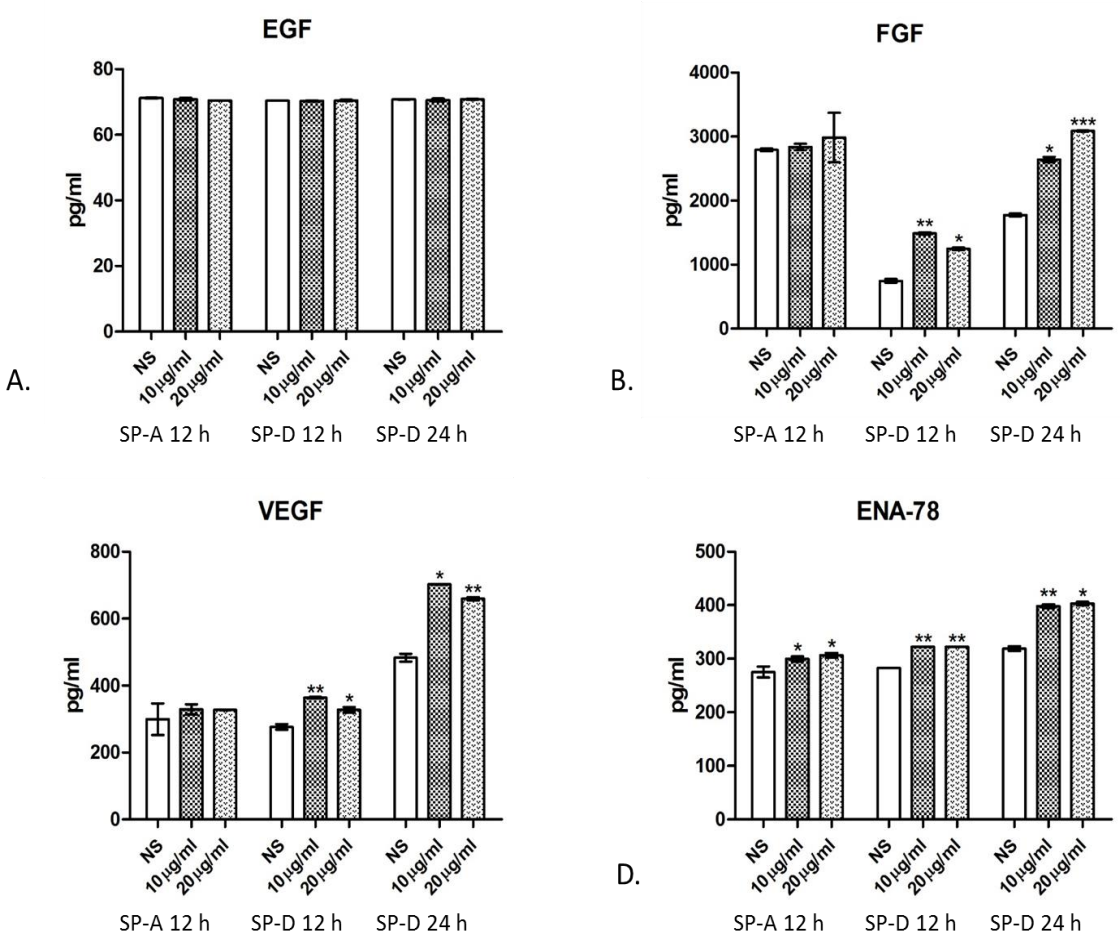


Figure 3.27 *Multiplex cytokine array analysis in conditioned media from ULTR cells treated with rhSPA or rhSPD at different concentration and time points. Secretion levels for EGF (A), FGF (B), VEGF (C) and ENA-78 (D). rhSP-A treatments (10 and 20 µg/ml) resulted in an increase in the expression of ENA-78, when compared to untreated cells, but did not have an effect on EGF, FGF and VEGF. rhSP-D treated cells (10 and 20 µg/ml) showed an increase in the levels of FGF, VEGF and ENA-78 at 12 and 24 hours compared to untreated cells. ULTR cells treated with rhSP-D (10 and 20 µg/ml) did not have an effect on EGF. Significance was measured using the unpaired student's t-test (*p<0.05, **p<0.01, ***p<0.001). NS: no supplement.*

None of the molecules had an effect on the expression of EGF. rhSP-D led to an increase in the secretion of VEGF and FGF at 10 and 20 µg/ml after 12 and 24 hours, which are growth factors that regulate several physiological changes in cells. rhSP-A did not affect the expression of these growth factors. Both treatments led to an increase of ENA-78 at all doses and time points.

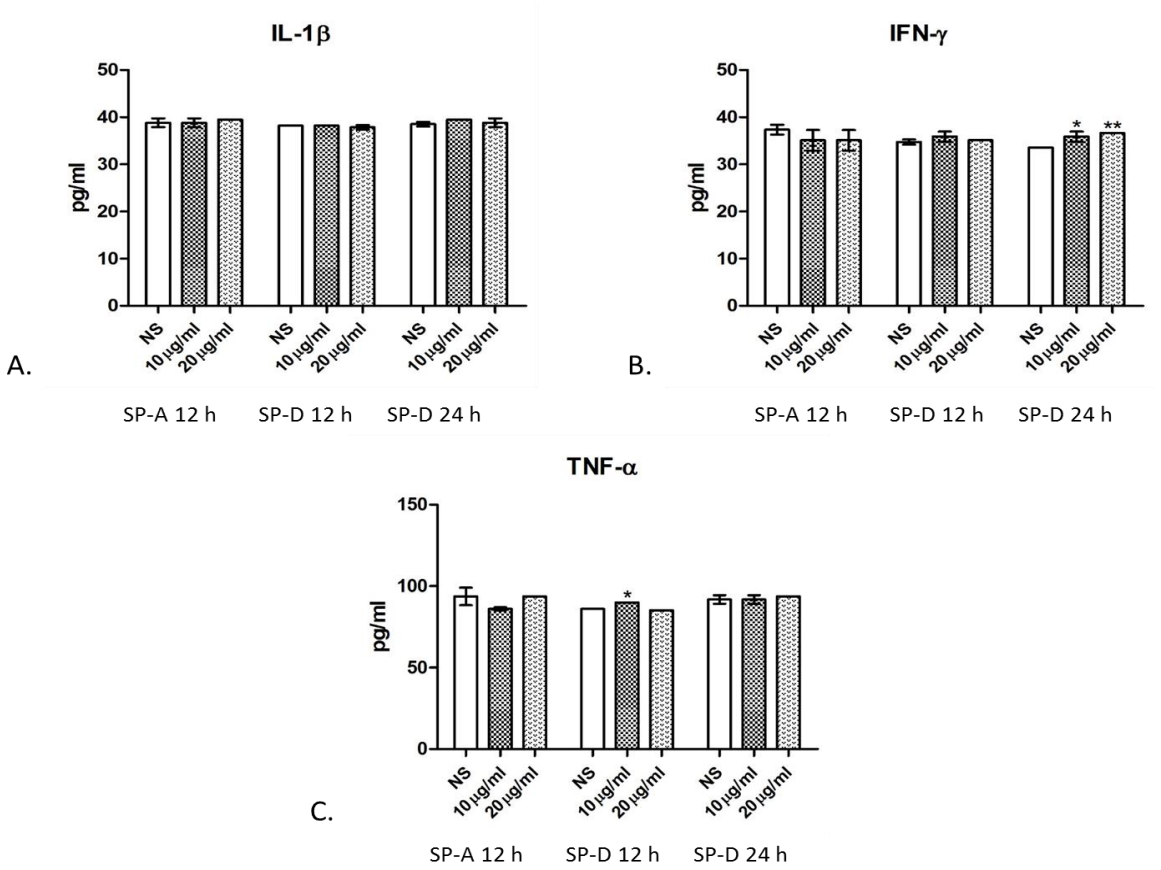


Figure 3.28 *Multiplex cytokine array analysis in conditioned media from ULTR cells treated with rhSPA or rhSPD at different concentration and time points. Secretion levels for IL-1β (A), IFN-γ (B) and TNF-α (C). rhSP-A and rhSP-D treatments (10 and 20 µg/ml) did not have an effect on IL-1β. rhSP-D treatments at (10 and 20 µg/ml) led to an increase of IFN-γ at 24 hours, compared to untreated cells. TNF-α expression was only significantly increased at 10 µg/ml after 12 hours, but neither protein had an effect at 24 hours. Significance was measured using the unpaired student's t-test (*p<0.05, **p<0.01). NS: no supplement.*

rhSP-A and rhSP-D did not have an effect on the expression of IL-1β. rhSP-D led to an increase of IFN-γ at both doses at 24 hours compared to untreated cells. rhSP-D treatment at 5 μg/ml resulted in an increase of TNF-α after 12 hours when compared to untreated cells.

Based on the data from the multiplex cytokine microarray it seems that SP-A and SP-D expression results in a pro-inflammatory environment in the myometrium as the cytokines that are mostly secreted promote inflammation.

3.3.11 Preterm birth clinical data – Study of the expression of SP-A and SP-D in the myometrium

Myometrium tissue was collected from women that gave birth at term (n=20) and preterm (n=20) either with caesarean or physical labour. Tissue samples were homogenised, RNA was extracted and cDNA was synthesised using the protocol previously described. SP-A1, SP-A2 and SP-D expression levels were measured using qPCR.

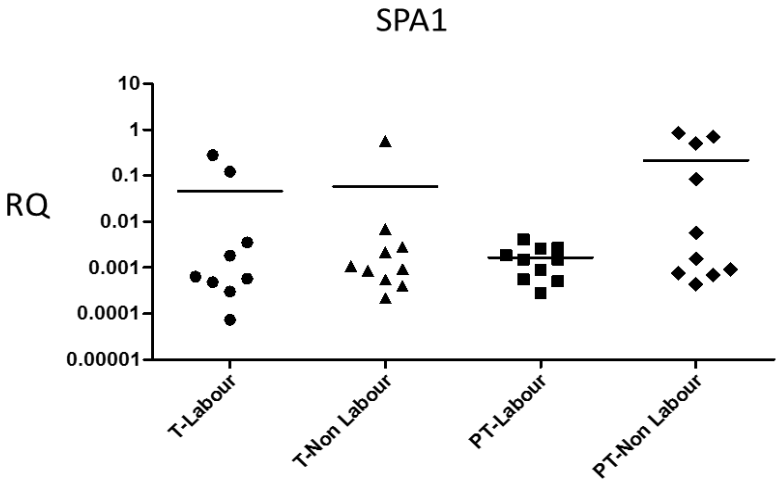


Figure 3.29 *Log relative quantity values of SP-A1 mRNA levels in myometrial tissue samples from women that gave birth at term or preterm, via normal or caesarean delivery. There was no difference in SP-A1 expression levels in term labour/non labour and preterm labour/non-labour. Line represents mean value, significance was acquired using the unpaired student's t-test (n=40). Values were normalised against GAPDH.*

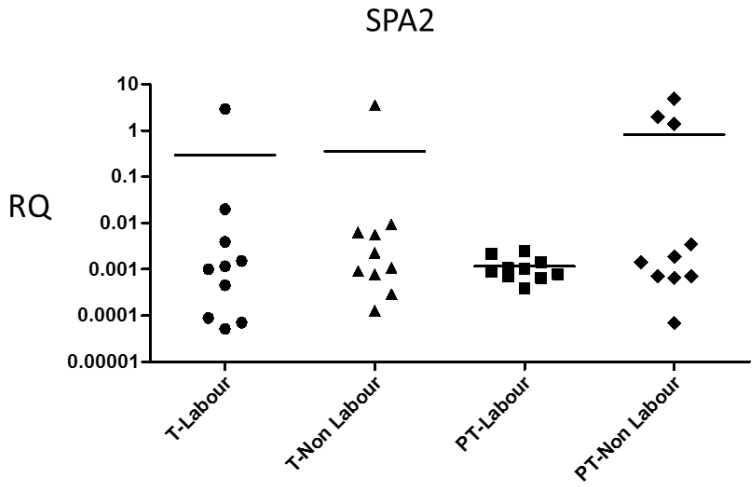


Figure 3.30 *Log relative quantity values of SP-A2 mRNA levels in myometrial tissue samples from women that gave birth at term or preterm, via physical or caesarean delivery. There was no difference observed in SP-A2 expression levels in term labour/non labour and preterm labour/non labour. Line represents mean value, significance was acquired using the unpaired student's t-test (n=40). Values were normalised against GAPDH.*

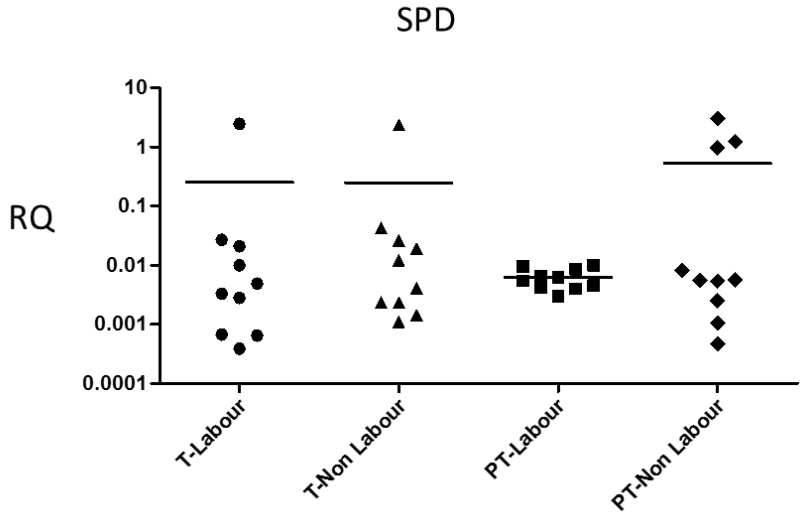


Figure 3.31 *Log relative quantity values of SP-D mRNA levels in myometrial tissue samples from women that gave birth at term or preterm, via physical or caesarean delivery. There was no difference in SP-D expression levels in term labour/non labour and preterm non labour. Line represents mean value significance, was acquired using the unpaired student's t-test (n=40). Values were normalised against GAPDH.*

Data from the qPCR showed that the levels of SP-A and SP-D in the myometrial samples tend to have high interpatient variability, but in a similar way across term samples suggesting that the way of delivery does not affect the surfactant protein levels at term. The preterm labour group showed the least interpatient variation.

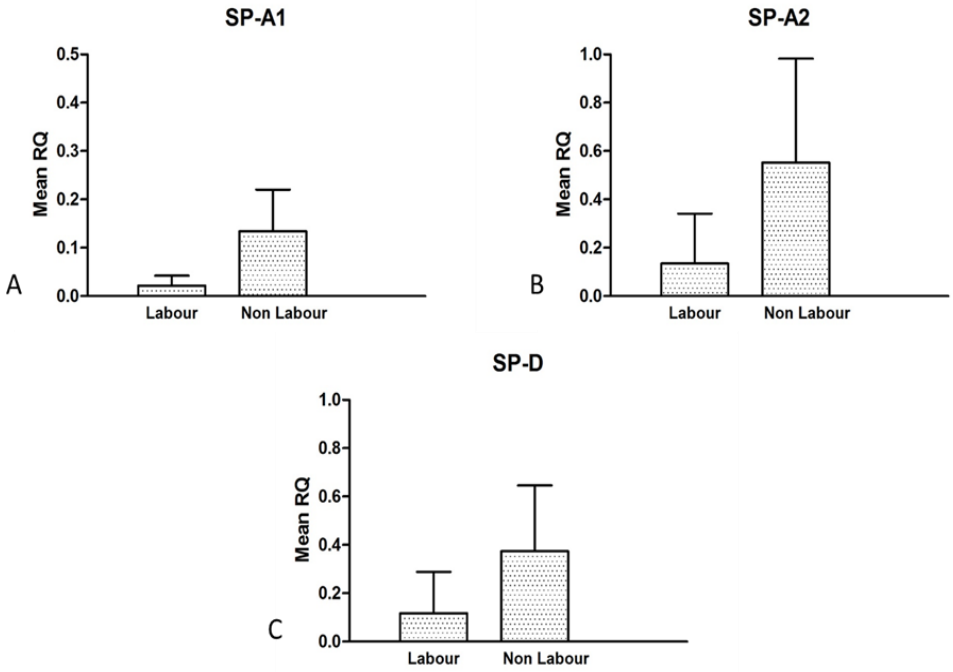


Figure 3.32 *Relative quantity values of SP-A1 (A), SP-A2 (B) and SP-D (C) mRNA levels ±SEM in myometrial tissue samples from women that gave birth via normal or caesarean delivery (term and preterm values combined, n=40). Values were normalised against GAPDH.*

We also split the groups based on the delivery method, in order to compare labour and non-labour values, and investigate whether the contractility state of the myometrium affects surfactant protein levels, it was evident that the contractility state of the myometrium does not have a significant effect on surfactant protein mRNA expression. However, there was a trend for all three mRNA transcripts where levels were lower in labour samples (term or preterm) compared to non-labour samples (term/preterm).

3.4 Discussion

In this chapter we validated the expression of surfactant proteins A and D in the myometrium cell line ULTR which was used as our *in vitro* model. The localisation of SP-A and SP-D in the ULTR cells was mainly cytoplasmic which corroborates with recently published data that studied the localisation of SP-A and SP-D in human trophoblast and decidua cells (Madhukaran *et al*., 2015). ULTR cells showed higher intensity of fluorescence when they were incubated with anti SP-D than when incubated with SP-A, which could potentially indicate a higher expression of SP-D in ULTR cells. Surfactant proteins did not have a significant effect on cell proliferation when ULTR cells were incubated with rhSP-A or rhSP-D. When the effects of surfactant proteins were studied on cell motility, the distance travelled by ULTR cells was significantly higher (triple in the case of rhSP-A and double in the case of rhSP-D) when compared to control cells. They also travelled faster and further, which shows a novel effect of surfactant proteins on cell motility. rhSP-A had a more potent effect compared to rhSP-D with the distance and velocity increasing by almost 50%. This was in agreement with the wound healing assay that showed that ULTR cells treated with rhSP-A or rhSP-D closed the gap much quicker compared to control cells. rhSP-A treated cells closed the gap after 17 hours and rhSP-D treated cells closed the gap after 25 hours (Sotiriadis *et al*., 2015). During pregnancy, the myometrium passes through different phases. In early pregnancy, there is a proliferative phase which is followed by hypertrophy and hyperplasia. This leads to the reconditioning of the myometrium, its activation and preparation for labour (Shynlova *et al*., 2009). SP-A and SP-D could, therefore, have a role in the reconditioning and tissue remodelling by increasing cell motility, thus enabling myometrial activation.

After validating the expression of SP-A and SP-D in ULTR cells and observing the functional effects they exerted, we decided to investigate whether surfactant proteins have a potential role in myometrium contractility. There have been recent studies suggesting that surfactant proteins have a more active role during pregnancy and parturition apart from protecting both the mother and the foetus from pathogens and assisting in the maintenance of pregnancy; surfactant proteins have been implicated in the initiation of labour and tissue remodelling (Condon *et al*. 2004, Nadesalingam *et al*., 2003). When ULTR cells were grown in collagen discs to mimic a 3D environment and treated with rhSP-A and rhSP-D, the collagen discs

contracted more and decreased in surface area compared to control cells (by almost 70%). The effect was still evident after 24 hours (Sotiriadis *et al*., 2015). There was no significant difference between rhSP-A and rhSP-D cells.

After observing the contractility of the collagen discs, we decided to elucidate how SP-A and SP-D exert that effect. We, therefore, decided to investigate their effects on the myometrial contractile mechanism. We studied the expression of contractility associated genes connexin 43, oxytocin receptor and cyclo-oxygenase 2. These genes affect the contractility of the human myometrium and are pro-labour mediators (Sotiriadis *et al*., 2015). Expression of CX43 and OXTR was significantly increased in ULTR cells treated with either rhSP-A or rhSP-D. rhSP-A had a more profound effect on the expression of OXTR, whereas rhSP-D had a more significant effect on the expression of CX43; its expression being higher by almost 2-fold compared to the expression of OXTR (Sotiriadis *et al*., 2015). Interestingly, when mice were injected with SP-A in the amnion labour was preterm, whereas when mice were injected with anti SP-A, labour was delayed by more than 24 hours (Condon *et al*., 2004). In another study, mice that were deficient in SP-A and SP-D were shown to express low levels of both CX43 and OXTR (Montalbano *et al*., 2013). COX2 levels were not affected by treatments with surfactant proteins suggesting there is not an effect mediated via COX2; Western blotting revealed low concentration of COX2 in our samples (data not shown). Further studies should be carried out to determine whether SP-A or SP-D have an effect on the expression of COX1 and the prostaglandin pathway. Collectively, the above data suggests that both SP-A and SP-D could play a role in myometrium contractility and cytoskeletal organisation, through regulation of CAP genes.

As SP-A and SP-D had an effect on contractility and organisation of ULTR cells, we decided to examine their effects on the mTOR pathway. We have previously shown that mTOR pathway components are expressed in the myometrium and can be regulated by steroids, more specifically, P4 can downregulate mTOR activity (Foster *et al*., 2014). Jaffer *et al*., (2009) have also showed that estradiol regulates the functionality of the PI3K/mTOR pathway in the myometrium in rats. When ULTR cells were treated with surfactant proteins, mTOR and DEPTOR levels were initially decreased by rhSP-A, whereas there was not an effect on their expression levels by rhSP-D. This means that SP-A can inhibit mTOR activation to enable

hypertrophy, in a similar manner to P4. Further studies should focus on other downstream components of the mTOR pathway, such as Rictor and Raptor, and investigate whether SP-A can affect S6K, since mTOR can regulate cell proliferation and gene transcription by phosphorylating S6K (Foster *et al*., 2014).

Due to the important roles and the effects SP-A and SP-D have in maintaining homeostasis by clearing pathogens or by regulating the contractility of the myometrium in this case, it was imperative to study how surfactant proteins can self-regulate. Previously, there have been reports showing feedback mechanisms through which surfactant proteins regulate each other (LeVine *et al*., 2002, Yadav *et al*., 2014). We therefore, studied the effects of rhSP-A and rhSP-D on the expression of SP-A and SP-D mRNA. Surfactant proteins had a biphasic response to treatment. rhSP-D treatment led to an increase of SP-A1, SP-A2 and SP-D transcripts after 6 hours and a decrease after 12 hours. rhSP-A treatment also led to a decrease of those transcripts (Sotiriadis *et al*., 2015).

To investigate whether SP-A and SP-D have a pro- or anti-inflammatory effect in the myometrium, we studied the secretion of 31 cytokines in ULTR cells treated with rhSP-A or rhSP-D. Conditioned media from the treated and control cells was used for a cytokine microarray. Both SP-A and SP-D led to increased secretion of GROα, IL-6, IL-8 and ENA-78 (Sotiriadis *et al*., 2015). GROα is a pro-inflammatory cytokine that has a chemotactic activity and it has been found to be decreased in babies that were born at term (Królak-Olejnik *et al*., 2006). IL-6 and 8 are both pro-inflammatory cytokines that promote immune infiltration and angiogenesis (Dimitriadis *et al*., 2005). rhSP-D treatments also led to the upregulation of IL-6 Ra which binds to IL-6 and promotes IL-6 activity. ENA-78 which is a C-X-C motif and is co expressed with IL-8, has the same role promoting reconditioning of tissue and angiogenesis (Imaizumi *et al*., 1997). Interestingly, rhSP-A and rhSP-D did not have an effect on the secretion levels of TNF-α, EGF and IL-1β, which are molecules that have been associated before, mainly with SP-A, and are crucial for the initiation of labour (Condon *et al*., 2004) and during pregnancy (Vasiliadis *et al*., 2006). rhSP-D resulted in increased secretion of FGF and VEGF which are growth factors that have a role in various cell functions and physiological changes (Reynolds and Redmer, 1998). rhSP-D also led to a moderate increase in the secretion of IFN-γ. In conclusion, the data from the cytokine microarray shows that SP-A and SP-D

have a pro-inflammatory effect in the myometrium, which suggests they may be important for the initiation of labour which is a pro-inflammatory event. Collectively, the data presented in this chapter evidence novel roles and interactions of SP-A and SP-D in the myometrium and could potentially lead to new therapeutic strategies for preterm birth, through modulation of their expression.

In this chapter, we also measured the expression of SP-A and SP-D mRNA in term and preterm clinical samples. There was no change between term labour, non-labour and preterm labour, non-labour. Interestingly, the preterm labour group showed the least interpatient variation. When we made a comparison based on the contractility state of the myometrium, whether that was at term or preterm, there was a trend showing that SP-A and SP-D mRNA expression was higher in the non-labour group, though it was not significant, due to interpatient variation. To our knowledge, this is the first study with such a large cohort of clinical samples that demonstrates the expression of SP-A and SP-D in the human myometrium at birth. Previous data has shown that levels of SP-A and SP-D are increased at term and preterm birth in the amniotic fluid; this could also be the case in the myometrial samples we have examined here.

Chapter 4

Study of the effects of sex steroids, progesterone and estradiol, on SP-A and SP-D and validation of non-genomic responses *in vitro*

4.1 Introduction

The exact signals that control the onset of parturition in humans remain to be defined. A number of studies have underpinned the role of oxytocin receptor in labour allowing an increased uterine responsiveness to oxytocin, thereby initiating contractions, in response to oestrogen (Niswender *et al*., 2000). The placenta-derived sex hormone, progesterone, appears to exert an opposite effect in the first and second trimesters of pregnancy, allowing the uterus to relax and enable foetal development (Walsh *et al*., 1984).

During late pregnancy, the withdrawal of progesterone is essential for initiating parturition. In humans, however, progesterone levels remain high until the end of pregnancy (Condon *et al*., 2003). Functional withdrawal of progesterone towards term involving changes of myometrial responsiveness to the hormone and causing a shift from the quiescent state of the uterus to a contractile state, can induce labour (Graham and Clarke, 2013). This suggests the existence of an alternative pathway that facilitates progesterone withdrawal in the myometrium. Deregulation of key cellular signals is thought to be involved in pathological conditions including preterm birth. To date, very little is known about the cross-talk between sex steroids and SP-A and SP-D.

In chapter 3, we evaluated the ULTR cell line to study the effects of SP-A and SP-D on key contractile events such as myometrial activation via CAPs and pro-inflammatory cytokine expression (Sotiriadis *et al*., 2015). Given that SP-A and SP-D were evidenced to affect myometrial tone we hypothesised that there might be a cross-talk between sex steroids (mainly progesterone) in the modulation of uterine tone. During pregnancy, the secretion of progesterone and oestrogens is increased, peaking at the onset of labour (Smith *et al*., 2002).

It has been previously shown that the human myometrium is a target for progesterone acting in a genomic and non-genomic manner (Falkenstein *et al*., 2000). A non-genomic action defines

any action that does not directly and initially influence gene expression, as do the classical steroid receptors, but rather drives more rapid effects such as the activation of signalling cascades. These so-called 'non-genomic' effects do not depend on gene transcription or protein synthesis and involve steroid-induced modulation of cytoplasmic or cell membrane-bound regulatory proteins. In addition to progesterone receptors PRA and PRB, there is another class of receptors (Mesiano *et al.*, 2002, Conneely *et al.*, 2002). mPRs (membrane progesterone receptors) have been localised in the myometrium and are regulated by steroids *in vitro*. They can transactivate nuclear receptor PRB and also bind to progesterone. It has been proposed that they play a role in a functional progesterone withdrawal (Karteris *et al*., 2006).

4.2 Aims

- To validate the non-genomic effects of progesterone on gene expression in ULTR cells treated with OD-02 (which is a selective agonist of membrane progesterone receptor) using microarray. Org OD-02 (10-ethenyl-19-norprogesterone, 19-CH(2)P4) is a mechanistic analog of progesterone and can bind to mRPs, but not nuclear PRs (Kelder *et al*., 2010, Zachariades *et al.*, 2012);

- To assess the effects of P4, OD-02 and E2 on SP-A and SP-D expression in ULTR cells using qPCR and western blotting;

- To validate non genomic functional responses of progesterone in myometrial cells;

- To study the effects of SP-A and SP-D on mPRs and PRB expression, in ULTR cells treated with rhSP-A and rhSP-D using qPCR.

4.3 Results

4.3.1 Effects of steroid hormones on SP-A and SP-D

ULTR cells were grown and seeded at a specific density. Cells were left to adhere and were treated with P4, OD-02 and E2 at a final concentration of 10^{-7}M. RNA and protein were collected at specific time points and used for cDNA synthesis and western blotting respectively. Three biological replicates were performed for this study.

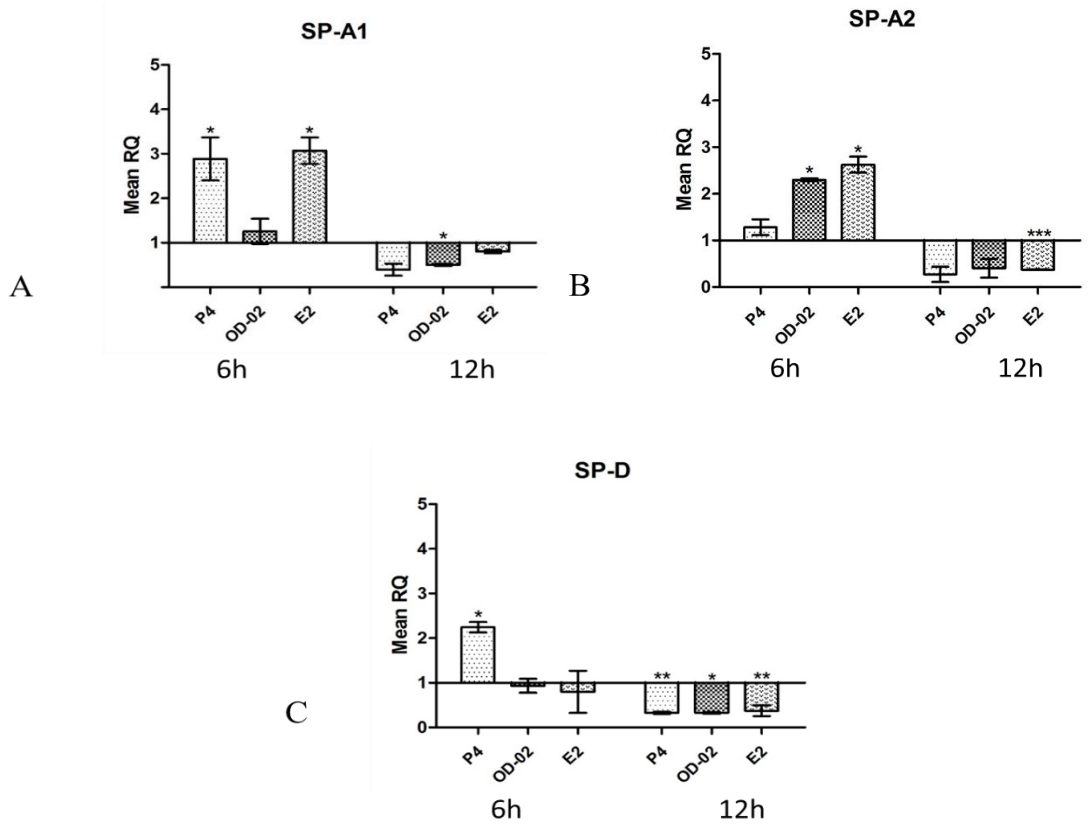


Figure 4.1 *Schematic representation showing the mean relative quantification of SP-A1 (A), SP-A2 (B) and SP-D (C) in cells treated with P4, OD-02 and E2, at 6 and 12 hours ±SD (significance was measured using the paired student's t-test *p<0.05, **p<0.01, ***p<0.001). Results were obtained from three independent experiments. Treatments with P4 and E2 led to an increased expression of SP-A1 at 6 hours whereas OD-02 and E2 led to an increase in the SP-A2 mRNA transcript and P4 led to an increase in the expression of SP-D at 6 hours, when compared to untreated cells. At 12 hours the expression levels of all mRNA transcripts were decreased. Values were normalised against GAPDH.*

P4 led to an increase in the SP-A1 (2.8-fold change) and SP-D (2.2-fold change) mRNA expression at 6 hours and a decrease of SP-D after 12 hours, whereas E2 led to an increase in the expression of SP-A1 (3-fold change) and SP-A2 (2.6-fold change) gene levels at 6 hours and decreased the levels of SP-A2 and SP-D at 12 hours, when compared to untreated cells. OD-02 treatment resulted in an increase of SP-A2 (2.3-fold change) after 6 hours whereas levels of SP-A1 and SP-D were decreased after 12 hours, when compared to untreated cells.

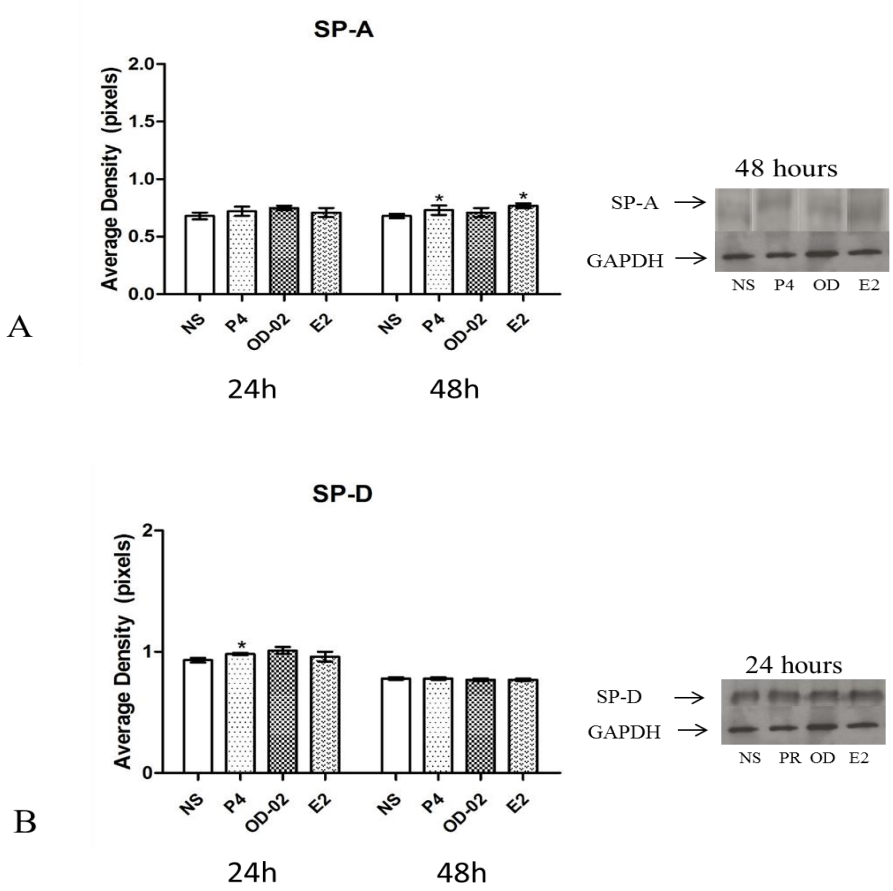


Figure 4.2 *Left: Average density of the protein expression bands for SP-A (A) and SP-D (B) ±SD (*p<0.05) of cells treated with P4, OD-02 and E2 at 24 and 48 hours from the western blotting analysis. Right: Representative bands for SP-A (A) and SP-D (B). There was no increase in the protein expression levels of SP-A at 24 hours, but there was an increase at 48 hours following treatments with P4 and E2. SP-D levels were only moderately increased at 24 hours after treatment with P4, whereas there was no effect at 48 hours. Significance was acquired using the unpaired student's t-test. Values were normalised against GAPDH. NS: no supplement.*

Protein detection using western blotting showed that, treatments with P4, OD-02 and E2 did not have an effect on the protein expression of SP-A at 24 hours, though at 48 hours there was an increase in SP-A protein expression in P4 and E2 treated samples compared to the untreated samples. SP-D protein levels were only slightly increased at 24 hours after treatment with P4. The treatments did not have an effect at 48 hours (Figure 4.2).

4.3.2.1 mPR expression in ULTR cells and binding to P4 and OD-02

Prior to elucidating the potential cross-talk between mPRs and SPs, the ULTR cell line was validated as an *in vitro* model that expresses –in addition to nuclear PRs- mPRs.

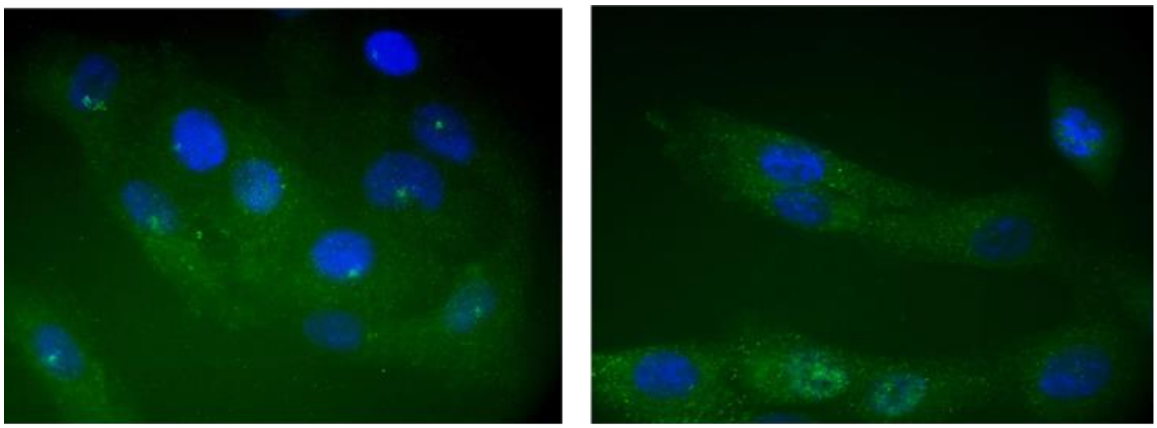


Figure 4.3 *Immunofluorescent analysis showing expression of mPRα and mPRβ in ULTR cells. The receptors were localised in the cytoplasm and the cell membrane (courtesy of Seda Turkcigdem).*

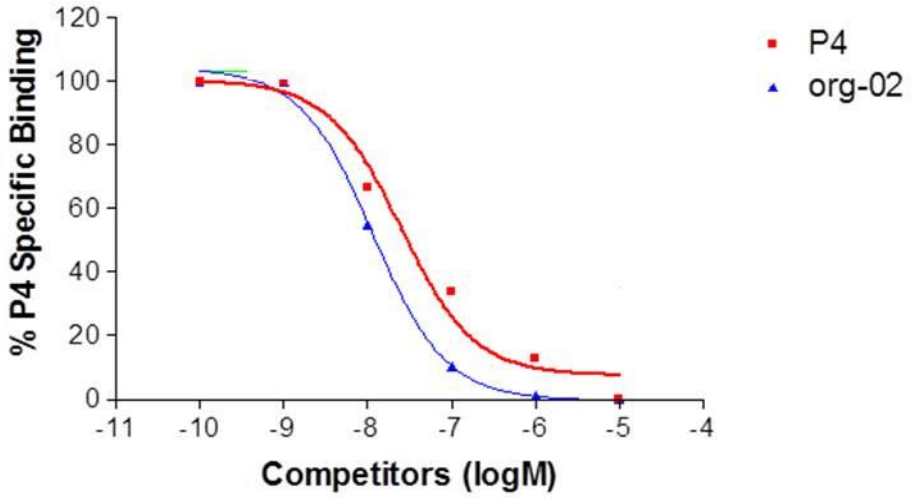


Figure 4.4 *Schematic representation showing the binding of P4 and OD-02. Binding was specific towards mRPs (courtesy of Professor Peter Thomas, University of Texas).*

Immunofluorescent analysis showed that the ULTR cells express mPRα and mPRβ, and their localisation was mainly cytoplasmic and in the cell membrane (Figure 4.3). Binding studies also showed that P4 and its mechanistic analog OD-02 specifically bind to mPRs to exert downstream functions (Figure 4.4).

4.3.2.2 Microarray analysis in ULTR cells treated with OD-02

ULTR cells were then treated with OD-02 at a final concentration of 10^{-7}M for 24 hours. RNA was extracted and used for microarray. Genes were selected depending on the fold change they showed (at least 2.5-fold) and the significance (p-value <0.05). The 40 genes that were most up-regulated or down-regulated are presented in the tables below.

Table 4.1 *20 highest up-regulated genes by OD-02 in ULTR cells as acquired from the microarray analysis. Table includes gene sequence ID, name, fold-change and p-value for each gene.*

SEQ_ID	Gene Name	Full name	Fold Change	p-Value
BC000183	TMSL8	Thymosin-like protein 8	3.7	0.0262
NM_001003679	LEPR	Leptin receptor	3.6	0.00251
DQ139833	ARHGEF10L	Rho Guanine Nucleotide Exchange Factor (GEF) 10-Like	3.3	0.029
BC032835	TMEM67	Transmembrane protein 67	3.3	0.00854
NM_002869	RAB6A	Ras-related protein Rab-6A	3.2	0.0241
AL832683	PTAR1	Protein prenyltransferase alpha subunit repeat containing 1	3.2	0.0268
NM_002748	MAPK6	Mitogen-Activated Protein Kinase 6	3.2	0.0359
NM_013387	UCRC	Ubiquinol-cytochrome c reductase complex	3.2	0.00387
NM_001009894	C12orf29	Chromosome 12 Open Reading Frame 29	3.1	0.00246

BX247969	AP4S1	Adaptor related protein complex 4, sigma 1 subunit	3.1	0.0145
NM_032456	PCDH7	Protocadherin 7	3.0	0.0326
AY437879	PXK	PX Domain Containing Serine/Threonine Kinase	3.0	0.0276
NM_001012968	RP11-93B10.1	Encoding hypothetical protein LOC139886	3.0	0.0472
AB209513	EVI5	Ecotropic Viral Integration Site 5	2.9	0.00967
NM_004229	CRSP2	Cofactor required for SP1 activation	2.9	0.00646
NM_012098	ANGPTL2	Angiopoietin-Like 2	2.9	0.00892
NM_018325	C9orf72	Chromosome 9 open reading frame 72	2.9	0.0485
NM_016150	ASB2	Ankyrin Repeat And SOCS Box Containing 2	2.9	0.0165
NM_001005753	VPS24	Vacuolar Protein Sorting-Associated Protein 24	2.9	0.0397
BC028369	FEM1C	Fem-1 homolog c	2.8	0.0124

Table 4.2 *Some of the most down-regulated genes by OD-02 in ULTR cells as acquired from the microarray analysis. Table includes gene sequence ID, name, fold-change and p-value for each gene.*

SEQ_ID	Gene Name	Full name	Fold Change	p-Value
XM_927187	LOC643926	Gene in cytogenetic band chr6q14	0.3	0.0237
XM_932719	LOC645194	Hypothetical gene on chromosome 5	0.3	0.00148
AK056275	FLJ31713	Uncharacterized Protein	0.3	0.0097
BC074960	RNASE7	Ribonuclease, RNase A Family, 7	0.3	0.0181
NM_013959	NRG1	Neuregulin 1	0.3	0.0373

NM_181614	KRTAP19-7	Keratin Associated Protein 19-7	0.3	0.0273
AK074124	TINAGL1	Tubulointerstitial Nephritis Antigen-Like 1	0.3	0.00816
BC104872	KRTAP20-1	Keratin associated protein 20-1	0.3	0.0189
AK130578	LOC400558	Uncharacterized Protein	0.3	0.00238
NM_018667	SMPD3	Sphingomyelin Phosphodiesterase 3	0.3	0.0195
BC105083	SPRR1A	Small Proline-Rich Protein 1A	0.3	0.00659
XM_932913	LOC645462	Hypothetical gene supported by AK024248	0.3	0.00895
NM_144974	RP11-5G9.1	Product of RP11-5G9.5 (Clone-based (Vega)	0.3	0.0408
NM_001836	CMA1	Chymase 1	0.4	0.0104
NM_144585	SLC22A12	Solute Carrier Family 22, member 12	0.4	0.0223
NM_003357	SCGB1A1	Secretoglobin, Family 1A, Member 1 (Uteroglobin)	0.4	0.0289
XM_930334	LOC647274	Similar to 60S acidic ribosomal protein P1	0.4	0.0332
XM_926054	LOC642568	Hypothetical gene on chromosome 11	0.4	0.0135
XM_928233	LOC645196	Hypothetical protein	0.4	0.00901
NM_032243	TXNDC2	Thioredoxin Domain Containing 2	0.4	0.0366

Based on the functions of several genes differentially regulated by OD-02 in ULTR cells, they were divided according to the pathway they are a part of or interact with. KEGG pathway software was used to generate the graphs as described in materials and methods.

MAPK Signalling Pathway

The MAPK signalling pathway consists of cytoplasmic proteins that form a communication channel within the cell, transferring signals from cell surface receptors to the nucleus, regulating gene transcription. Activation of this pathway is crucial for several cell functions such as growth, proliferation and differentiation which are important processes in the uterus during pregnancy. For this reason the effects of OD-02 on the MAPK pathway were further studied.

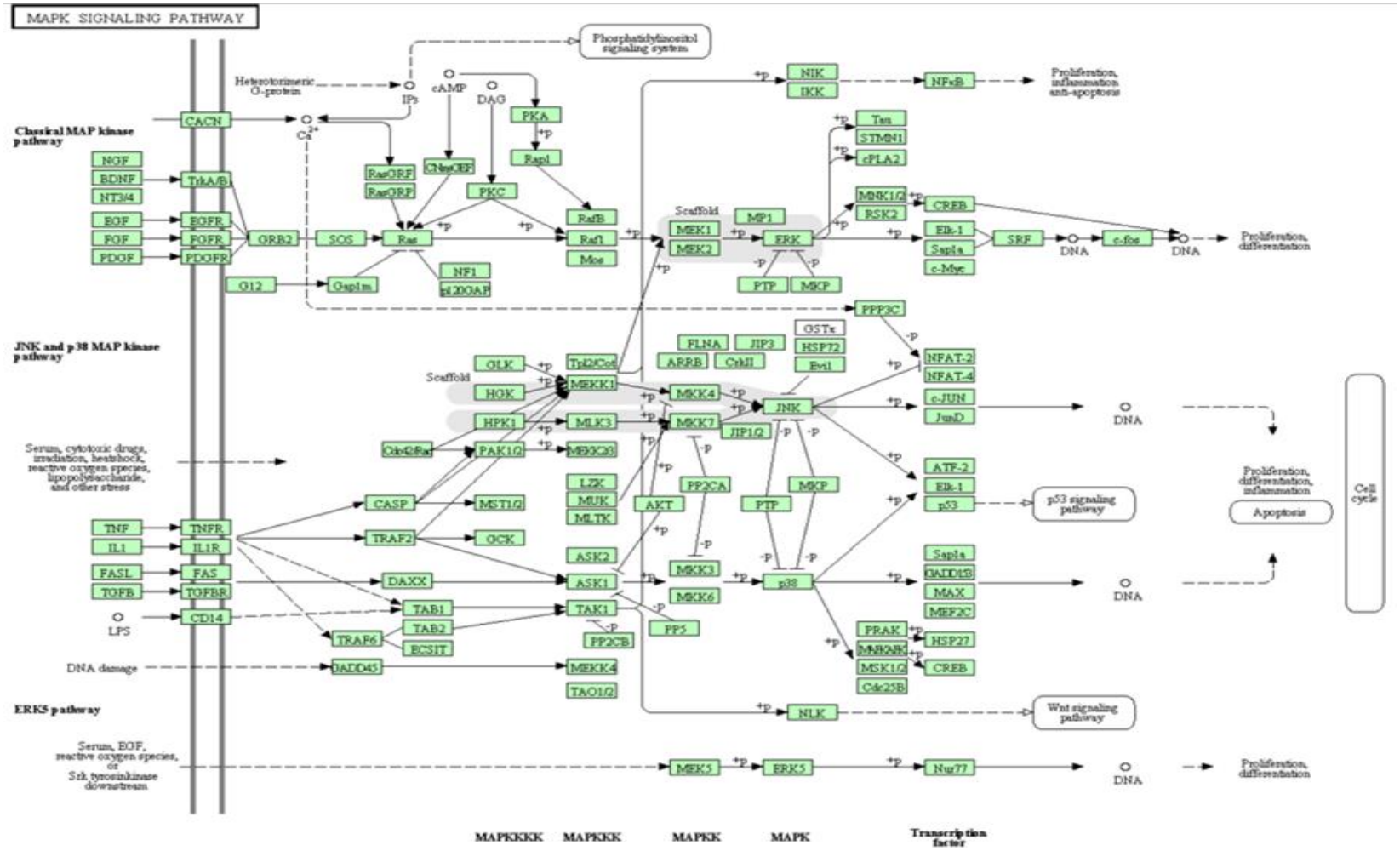


Figure 4.5 *Schematic representation showing components of the MAPK pathway.*

OD-02

OD-02 was able to induce the expression of several genes that are components of the MAPK signalling pathway. Genes found to be upregulated were: Mitogen-activated protein kinase 8 (MAP3K8) with a fold change increase of 2.01, Calcium channel, voltage-dependent, beta 2 subunit (CACNB2) with a fold change increase of 1.49, Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3) with a fold change increase of 1.53 and RAS guanyl releasing protein 1 (calcium and DAG-regulated) (RASGRP1) with a fold change increase of 1.27.

OD-02, however, resulted in the downregulation of two genes linked to MAPK pathway; the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFKB1) with a fold change of 0.83 and Platelet-derived growth factor beta polypeptide (PDGFB) with a fold change of 0.46.

PI3K-Akt Signalling Pathway

The PI3K-Akt signalling pathway is responsible for essential cellular functions that include gene transcription and translation, cell proliferation, growth and survival. This pathway could be related to the state of quiescence in the myometrium so we therefore, investigated the effects of OD-02 on components of this pathway.

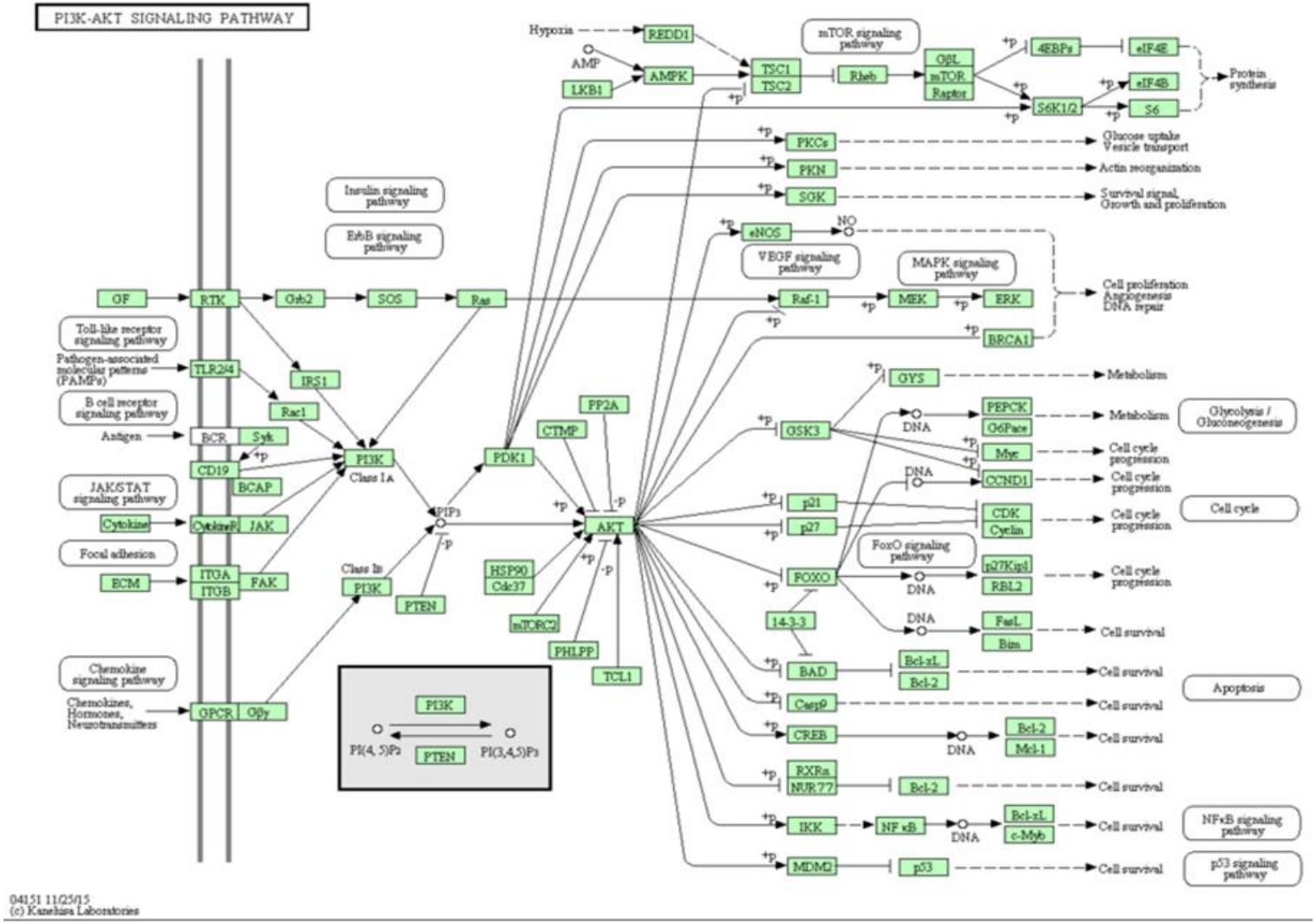


Figure 4.6 *Schematic representation showing components of the PI3K-Akt pathway.*

OD-02

The genes whose expression was increased due to OD-02 treatment were: Toll-like receptor 2 (TLR2) with a fold change of 1.29, Guanine nucleotide binding protein (G protein) gamma 11 (GNG11) with a fold change of 2.76, serum/glucocorticoid regulated kinase 2 (SGK2) with a fold change of 1.32, IL-2 with a fold change of 1.54, and Interferon (alpha, beta and omega) receptor 1 (IFNAR1) with a fold change of 1.34.

The genes whose expression was decreased due to OD-02 treatment were: PDGFB with a fold change of 0.46, Guanine nucleotide binding protein gamma 3 (GNG3) with a fold change of 0.52, Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFKB1) with a fold change of 0.83, and Insulin-like growth factor 1 (IGF1) with a fold change of 0.59.

Calcium Signalling Pathway

Calcium channels have a crucial role in signal transduction and can be activated directly by calcium ions or indirectly by G protein-coupled receptors via the phospholipase C pathway. These channels regulate the amount of intracellular Ca^{2+} and affect the contractility of smooth muscle cells. This is of great importance during pregnancy as premature contractions can lead to preterm birth, which is the reason why we investigated the effects of OD-02 on this pathway.

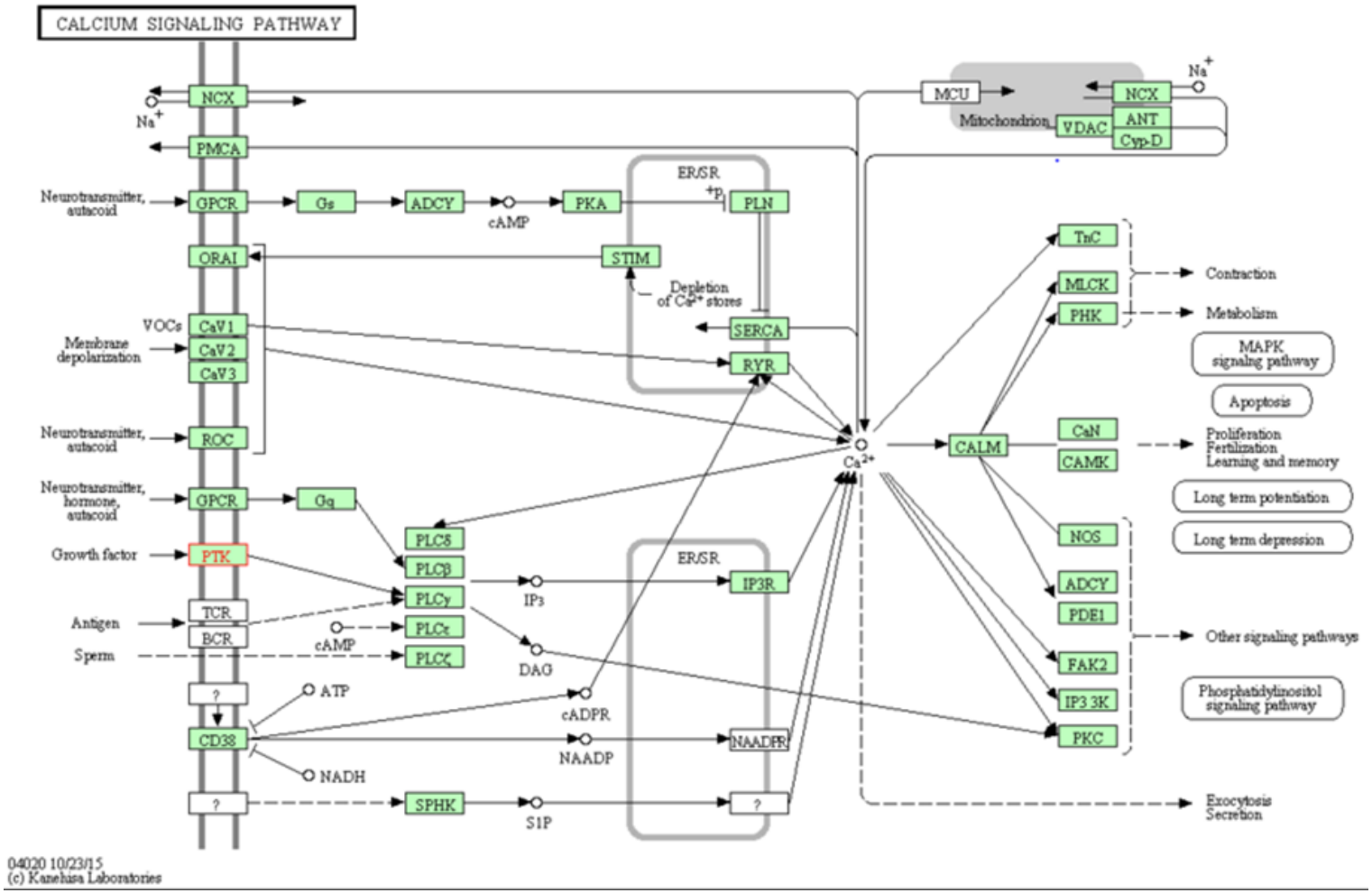


Figure 4.7 *Schematic representation showing components of the calcium signalling pathway.*

OD-02

OD-02 induced the expression of the purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7), with a fold change of 1.55, and decreased the expression of the gene encoding the Tachykinin receptor 1 (TACR1); with a fold change of 0.56.

JAK-STAT signalling Pathway

This pathway is responsible for the transportation of signals through the cell's plasma from receptors on the cell membrane, to promoters encoded in the nuclear DNA. It can, therefore, regulate gene transcription which is why we investigated the effects of OD-02 on its components.

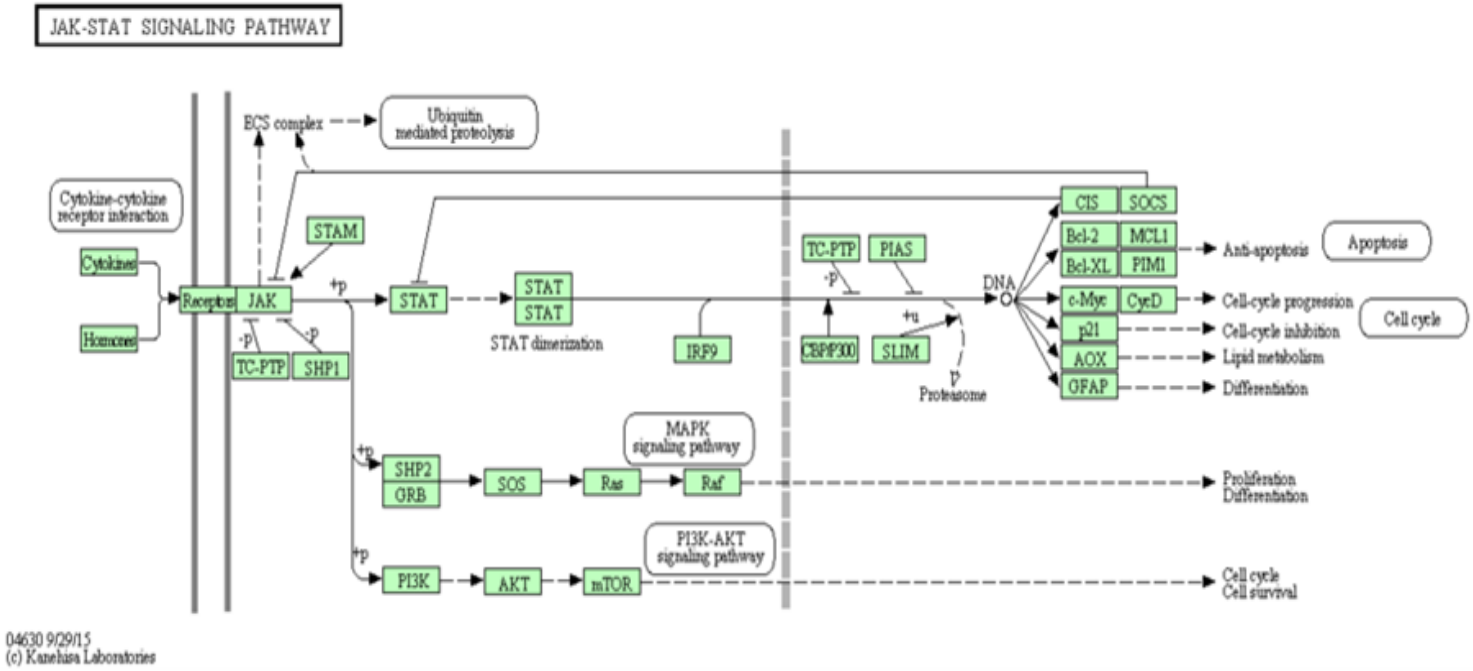


Figure 4.8 *Schematic representation showing components of the JAK-STAT pathway.*

OD-02

OD-02 treatment resulted in the upregulation of: the Leptin receptor (LEPR) gene with a fold change of 3.55, IL2 with a fold change of 1.54, Interleukin 10 (IL10) with a fold change of 1.49, and IFNAR1 with a fold change of 1.34. OD-02 reduced the expression of IL11 with a fold change of 0.56.

Regulation of Actin Cytoskeleton

The cytoskeleton consists of several compartments and molecules that form a complex network to regulate the structure of the cells as well as cell functions. It can affect several signalling pathways and cell contractions.

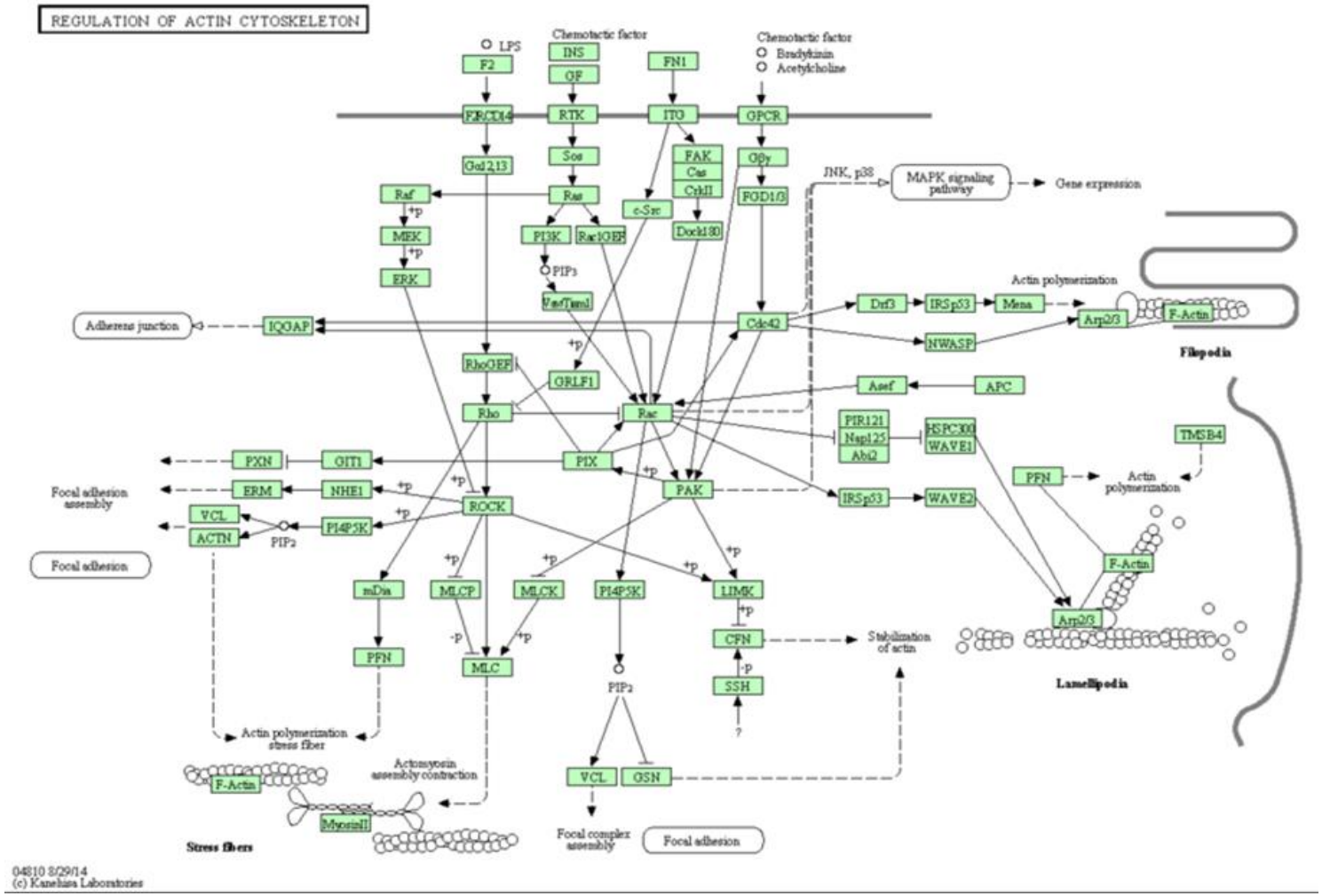


Figure 4.9 *Schematic representation showing components that regulate the actin cytoskeleton.*

OD-02

OD-02 resulted in an increase in the gene expression of vinculin (VCL) with a fold change of 1.27, and actin related protein 2/3 complex, subunit 5, 16kDa (ARPC5) and with a fold change of 1.23. OD-02 resulted in a decrease in the expression of the Platelet-derived growth factor subunit B (PDGFB) gene with a fold change of 0.46.

Adherens Junctions

Adherens junctions are formed between cells. They consist of proteins such as, cadherins and catenins, whose main role is to connect cells and regulate their interactions and contractions. This pathway was therefore studied in the treated with OD-02, ULTR cells.

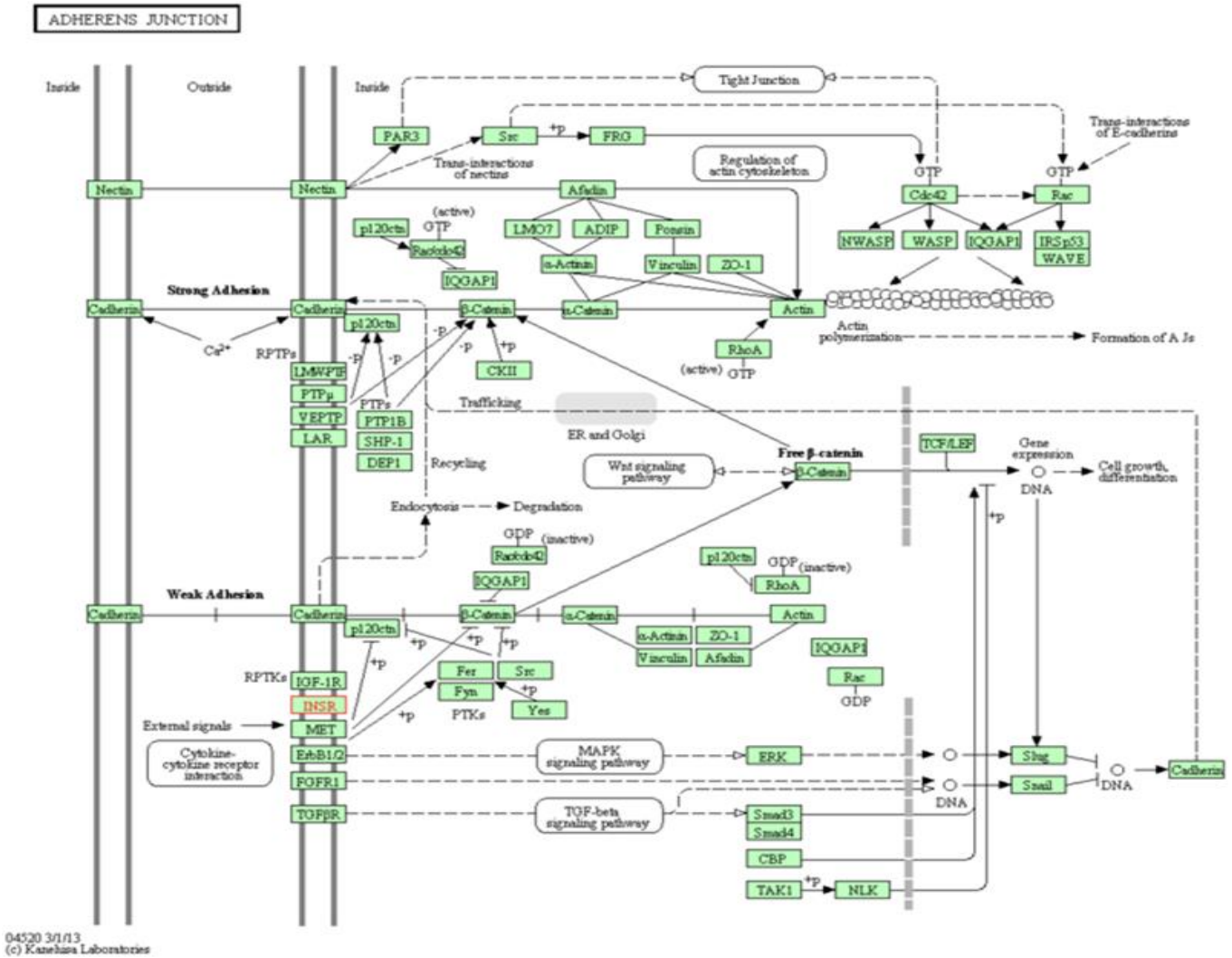


Figure 4.10 *Schematic representation showing components of the Adherens junction pathway.*

OD-02

OD-02 resulted in the upregulation of vinculin (VCL) with a fold change of 1.27, which is also a component of the actin cytoskeleton complex. OD-02 led to a decrease in the expression of the FERM, RhoGEF and pleckstrin domain-containing protein (FARP) with a fold change of 0.57.

Summary of OD-02 actions in term of functions

To summarise the actions of OD-02, we have grouped the actions of activated mPRs in terms of tissue remodelling, signalling, and inflammatory and immune responses.

Table 4.3 *Summary of functions of genes that were up or down-regulated by OD-02 in ULTR cells.*

Gene & Genbank	Fold Change	Functions
Tissue Remodelling:		
Actin related protein 2/3 complex, subunit 5, 16kDa (ARPC5) *BC057237*	↑ 1.23	Control of actin polymerisation regulating reconditioning and invasion (Nurnberg *et al*., 2011)
Vinculin (VCL) *BC039174*	↑ 1.27	Cytoskeletal protein associated with actin filaments and focal adhesion plaques (Bershadsky *et al*., 2003)
FERM, RhoGEF and pleckstrin domain protein 2 (FARP2) *NM_014808*	↓ 0.57	A guanine nucleotide exchange factor with roles in actin cytoskeleton remodelling, integrin signalling and neuronal development (He *et al*., 2013).
Caldesmon 1 (CALD1) *AF247820*	↑ 1.44	Protein that binds to actin and myosin and regulates smooth muscle contraction (Kim *et al*., 2012)
Adducin 3, gamma (ADD3) *BC062559*	↑ 1.35	Membrane skeletal protein that binds to calmodulin and regulates the Ca2+ pathway (Matsuoka *et al*., 2000).
Actinin, alpha 3 (ACTN3) *NM_001104*	↓ 0.63	Alpha-actin binding protein involved in binding actin to intracellular structures (Berman and North, 2010)

Gap junction protein, beta 7, 25kDa (GJB7) *NM_198568*	↓ 0.47	A connexin involved in the formation of gap junctions, also known as connexin 25 (Söhl *et al*., 2003)
Myosin regulatory light chain interacting protein (MYLIP) *NM_013262*	↑ 1.28	Mediates ubiquitination and proteasomal degradation of myosin regulatory light chain (Lindholm *et al*., 2009)
Signal Transduction:		
Guanine nucleotide binding protein (G protein), gamma 3 (GNG3) *BC015563*	↓ 0.52	Regulator of transmembrane signalling, required for GTPase activity and adenylyl cyclase signalling (Demokan *et al*., 2003)
Guanine nucleotide binding protein (G protein), gamma 11 (GNG11) *NM_004126*	↑ 2.76	Regulator of transmembrane signalling, required for GTPase activity and adenylyl cyclase signalling (Demokan *et al*., 2003)
RAS guanyl releasing protein 1 (calcium and DAG-regulated) (RASGRP1) *NM_005739*	↑ 1.27	Modulates Ras activity and activates the Erk/MAP kinase cascade. Can also bind to DAG and regulate Ca^{2+} activity (Stone, 2006)
Tachykinin receptor 1 (TACR1); also known as neurokinin receptor 1 (NK1R) *BC074912*	↓ 0.56	Tachykinin receptors interact with G proteins to regulate rat myometrial contractility (Pennefather *et al*., 1993; Magraner *et al*., 1998)
A kinase (PRKA) anchor protein 12 (AKAP12) *BC046095*	↑ 1.48	Interacts with protein kinases A and C and regulates cell migration and actin formation during foetal development (Weiser *et al*., 2007; Choi and Kim, 2008)
Calcium channel, voltage-dependent, beta 2 subunit (CACNB2) *AF465485*	↑ 1.49	Regulate oxygen and calcium channels in the foetal heart mediating vascular remodelling and smooth muscle contraction (Akaike *et al*., 2009; Waleh *et al*., 2010)
Progesterone receptor membrane component 2 (PGRMC2) *AK091741*	↑ 2.01	PGRMC2 is a progesterone receptor in the uterus (Pru and Clark, 2013)

Regulator of G-protein signalling 4 (RGS4) *NM_005613*	↑ 1.94	Regulate G protein signalling via GTPase activity (Ladds *et al*., 2009)
Serum/glucocorticoid regulated kinase 2 (SGK2) *AY987010*	↑ 1.32	Serine/threonine protein kinase involved in regulating ion channels. Down-regulation in decidua associated with spontaneous abortion (Alliston *et al.*, 2000)
Mitogen-activated protein kinase 8 (MAP3K8) *BC104833*	↑ 2.01	MAP3K8 can activate both MAP kinase and JNK kinase pathways and regulate progesterone synthesis via ERK (Liu *et al*., 2015)
Mitogen-activated protein kinase 8 interacting protein 3(MAPK8IP3) *NM_015133*	↑ 1.53	Scaffold protein that regulates proteins of the JNK signalling pathway (Tiwari *et al*., 2013)
Purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7 or P2X7) *AY847301*	↑ 1.55	P2RX7 activation results in uterine contraction in rats (Miyoshi *et al*., 2010)
CREB regulated transcription coactivator 1(CRTC1) *NM_025021*	↓ 0.69	Coactivator of cAMP response element binding protein (CREB1) that activates transcription through cAMP response elements (CRE). Myometrial quiescence during pregnancy is characterised by elevated cAMP (Sakamoto *et al.,* 2013)
Protein kinase, AMP-activated, beta 2 non-catalytic subunit (PRKAB2) *NM_005399*	↑ 1.83	Encodes a subunit of the AMP-activated protein kinase (AMPK), which acts as an energy sensor protein kinase. Regulation of cellular polarity via actin and potentially myosin. (Thorton *et al*., 1998)
Leptin receptor (LEPR) NM_001003679	↑ 3.55	Leptin receptors inhibit LPS induced apoptosis in chorioamnionitis (Wendremaire *et al.* 2011) and is thought to play a role in fertility, pregnancy (Herrid *et al*., 2014).

Inflammatory/Immune Response/ Cytokines:		
Toll-like receptor 2 (TLR2) *NM_003264*	↑ 1.29	TLRs mediate the expression of pro-inflammatory cytokines IL-1b, 6, 8 (Youssef *et al.*, 2009)
Toll-like receptor 8 (TLR8) NM_138636	↑ 1.40	Activates NK cells and promotes inflammation (Gorski *et al*., 2006)
Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) NM_052887	↓ 0.70	Induces expression of pro-inflammatory cytokines (Narayanan and Park, 2015)
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFKB1) *BC051765*	↓ 0.83	NFKB1is involved in the regulation of pro-inflammatory cytokines (Cookson and Chapman 2010).
Insulin-like growth factor 1 (somatomedin C) (IGF1) *M29644*	↓ 0.59	IGF1 is upregulated during the proliferative stage and again during postpartum involution (Shynlova *et al.*, 2009)
Interferon (alpha, beta and omega) receptor 1 (IFNAR1) *BC021825*	↑ 1.34	Receptor for interferons type I and protects the uterus from infections (Rosenfeld *et al*., 2002)
Interleukin 2 (IL-2) *BC066254*	↑ 1.54	Cytokine produced by Treg cells important in the regulation of immunity (Fainboim and Arruvito, 2011)
Interleukin 10 (IL-10) *NM_000572*	↑ 1.49	Anti-inflammatory cytokine and thought to regulate maternal tolerance in order to sustain pregnancy (Thaxton and Sharma, 2010)
Interleukin 11 (IL-11) *BC012506*	↓ 0.56	IL11 belongs to the gp130 family, is important for fertility, implantation and trophoblast invasion (Dimitriadis *et al*., 2005).
Platelet-derived growth factor alpha polypeptide (PDGFA)	- on KEGG	PDGFA is a mitogenic growth factor implicated in hypertrophy during pregnancy (Mendoza *et al.*, 1990)

Platelet-derived growth factor beta polypeptide (PDGFB) *X83705*	↓ 0.46	PDGFB regulates cell proliferation, differentiation and migration; loss of PDGFB is related to foetal diseases (Chhabra *et al*., 2010).
Latent transforming growth factor beta binding protein 1 (LTBP1) *M34057*	↑ 2.14	LTBP1 regulates the secretion of TGFβ which is important for foetal implantation and development (Maurya *et al.*, 2013).
Pregnancy-associated plasma protein A, pappalysin 1 (PAPPA) *NM_002581*	↑ 1.71	Metalloproteinase that regulates the activity of insulin-like growth factor binding proteins (IGFBPs) (Gyrup and Oxvig, 2007)

4.3.3 Effect of OD-02 on ULTR proliferation

ULTR cells were cultured and seeded in 6-well plates as mentioned in the materials and methods section. Cells were left to adhere for up to 6 hours and then treated with OD-02 at a final concentration of 10^{-7}M, which was selected as an optimal concentration based on the total binding from the binding study (Figure 4.4). Cell counts were performed using a Countess Automated Cell Counter (Invitrogen, Life Technologies) at 24 and 48 hours.

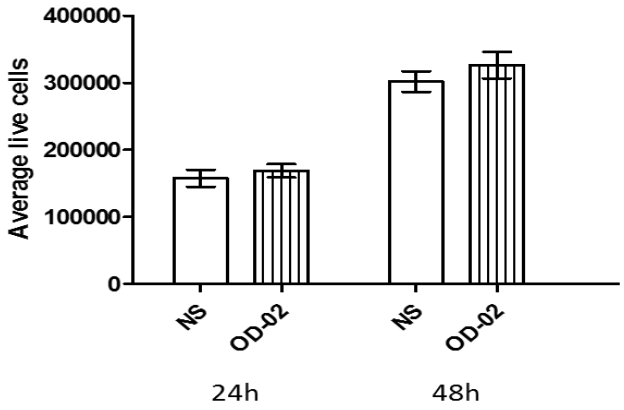


Figure 4.11 *Schematic representation showing the average number of live cells following treatment with OD-02 (10^{-7}M) at 24 and 48 hours ±SEM (n=4). No difference was identified between treated and untreated cells regardless of the time point. Significance was calculated using the unpaired student's t-test. NS: no supplement.*

Treatment with OD-02 did not have a significant effect on live cell numbers compared to untreated cells after 24 and 48 hours. Untreated as well as treated cells at 48 hours were doubled.

4.3.4 Wound healing assay

ULTR cells were grown in petri dishes and allowed to reach 100% confluency. Using a fine pipette tip, an artificial wound was created on the cell surface. The petri dishes were placed under a Zeiss Axiovert 200M microscope attached to an incubator to allow cell visualisation and survival. Images of the cells were acquired every 10 minutes for up to 48 hours.

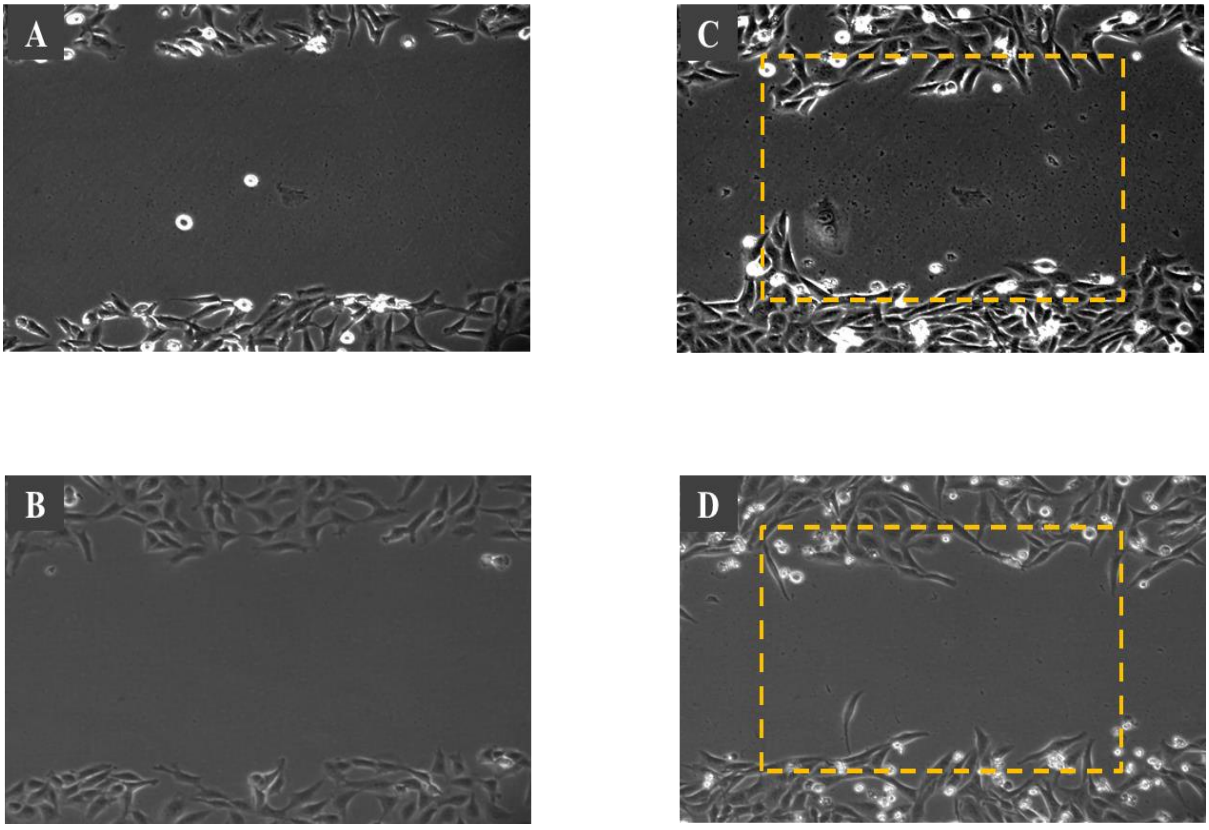


Figure 4.12 *Schematic representation showing the artificial wound created on the cell surface in untreated (A) and treated cells with OD-02 (B) at 0 hours. No difference was observed between the two groups after 48 hours (C, D).*

Images acquired from the wound healing assay over 48 hours showed that untreated and treated cells with OD-02 did not close the gap.

4.3.5 Cell motility assay for ULTR cells treated with OD-02

ULTR cells were sub-cultured at a 30% confluency in a petri dish and then allowed to adhere for approximately 6 hours before being placed under a Zeiss Axiovert 200M microscope to allow visualisation and tracking of cells. Cells were either untreated or treated with OD-02 (10^{-7}M). After an area of 25-30 cells was identified, images were captured every 5 minutes for up to 12 hours. Using ImageJ, the coordinates of the cells were acquired.

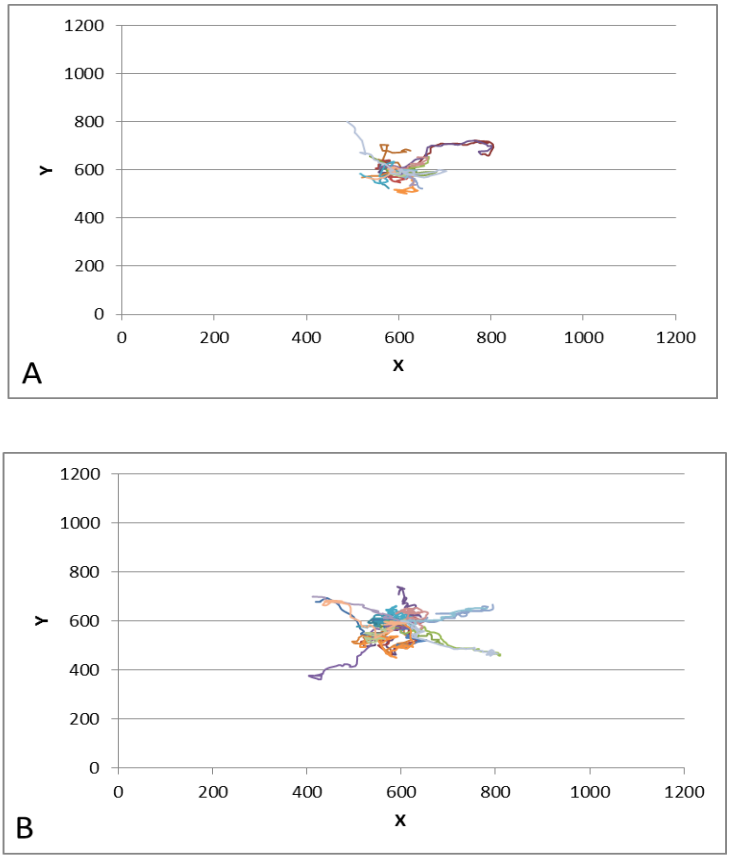


Figure 4.13 *Schematic representation showing the coordinates of all time points of untreated (A) and treated with OD-02 (B) ULTR cells. 25 cells were counted for each experiment. The point (600,600) was set as a starting point for each well. Treated cells with OD-02 appeared to move quicker and further from their initial position compared to untreated cells. Each line represents a single cell.*

From the images acquired, the starting point for the cells was set at (600,600). The position of the cells was then determined for each time point. Cells treated with OD-02 moved further

away from their initial position compared to control cells that did not move significantly across time points (Figure 4.13)

Using Pythagoras' theorem for each point for each cell, the distance, velocity and displacement for all 25 cells were calculated.

Table 4.4 *Table showing the distance, velocity and displacement value for the cell movements of untreated and cells treated with OD-02. NS: no supplement (n=1).*

	Distance (pixels)	**Velocity (pixels/minute)**	**Displacement (pixels)**
ULTR NS	215.587	0.449	74.905
ULTR xOD-02	433.044	0.902	122.965

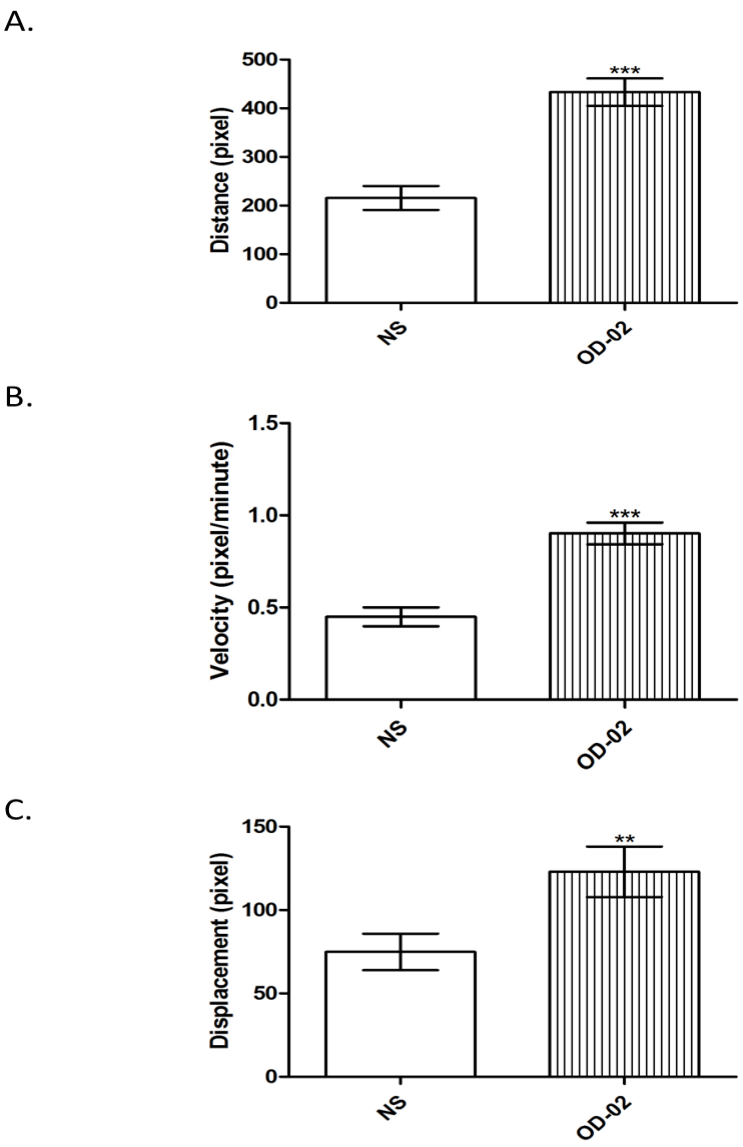


Figure 4.14 *Schematic representation of the distance (A), velocity (B) and displacement (C) comparisons ±SEM between untreated and treated ULTR cells with OD-02 (10^{-7} M) (Mann Whitney U test: **p<0.01, ***p<0.001). Treatment with OD-02 resulted in an increase in distance, velocity and displacement compared to untreated cells. NS: no supplement.*

ULTR cells treated with OD-02 moved significantly more and faster, when compared to untreated cells. There was a significant increase in distance, velocity and displacement compared to untreated cells. Distance was increased from approximately 220 to 430, velocity from 0.4 to 0.9 and displacement from 70 to 120 (Figure 4.14).

4.3.6 Effects of rhSP-A and rhSP-D on non-genomic functions

ULTR cells were treated with rhSP-A and rhSP-D at specific doses, as described previously, and RNA was collected at specific time points. cDNA was synthesised and qPCR was performed to investigate the effects of SP-A and SP-D on mPRα, mPRβ and PRB.

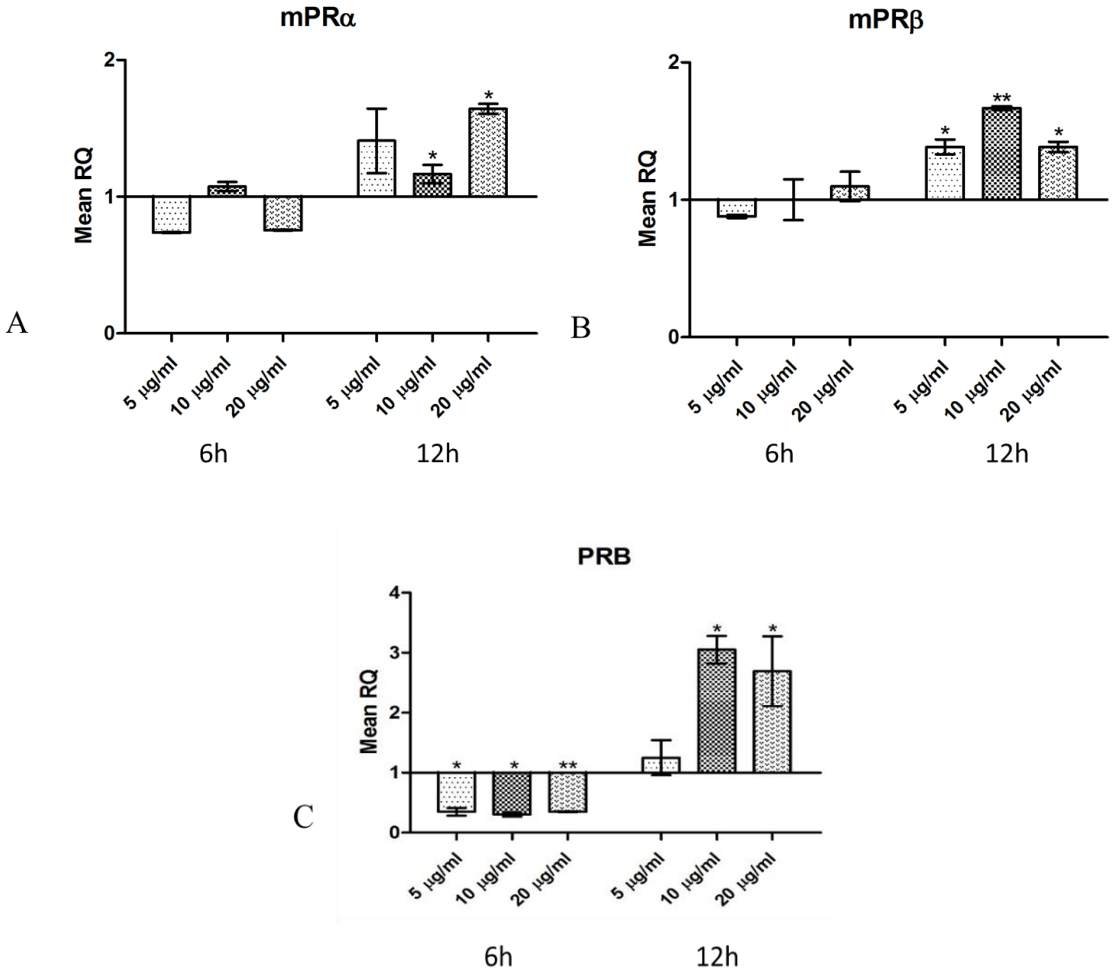


Figure 4.15 *Schematic representation showing the mean relative quantities of mPRα (A) mPRβ (B) and PRB (C) mRNA expression ±SD at 6 and 12 hours (paired student's t-test: *p<0.05, **p<0.01) from cells treated with rhSP-A (n=3). There was an upregulation of all three receptors at 12 hours, when compared to untreated cells. There was no evident effect on rhSP-A on mPRα and mPRβ at 6 hours, whereas there was a downregulation of PRB, when compared to treated cells. Values were normalised against GAPDH. NS: no supplement.*

Results from the qPCR analysis showed that rhSP-A at 10 and 20 µg/ml led to an upregulation of the mPRα gene expression after 12 hours (1.1-fold and 1.6-fold change respectively), when compared to treated cells. rhSP-A at 5, 10 and 20 µg/ml resulted in an upregulation of mPRβ at 12 hours (1.3-fold, 1.6-fold and 1.3-fold respectively), whereas no effect was evident at 6 hours. PRB expression was upregulated at 12 hours when cells were treated with rhSP-A at 10 and 20 µg/ml (3.1-fold and 2.8-fold respectively). At 6 hours the expression levels of treated samples were decreased.

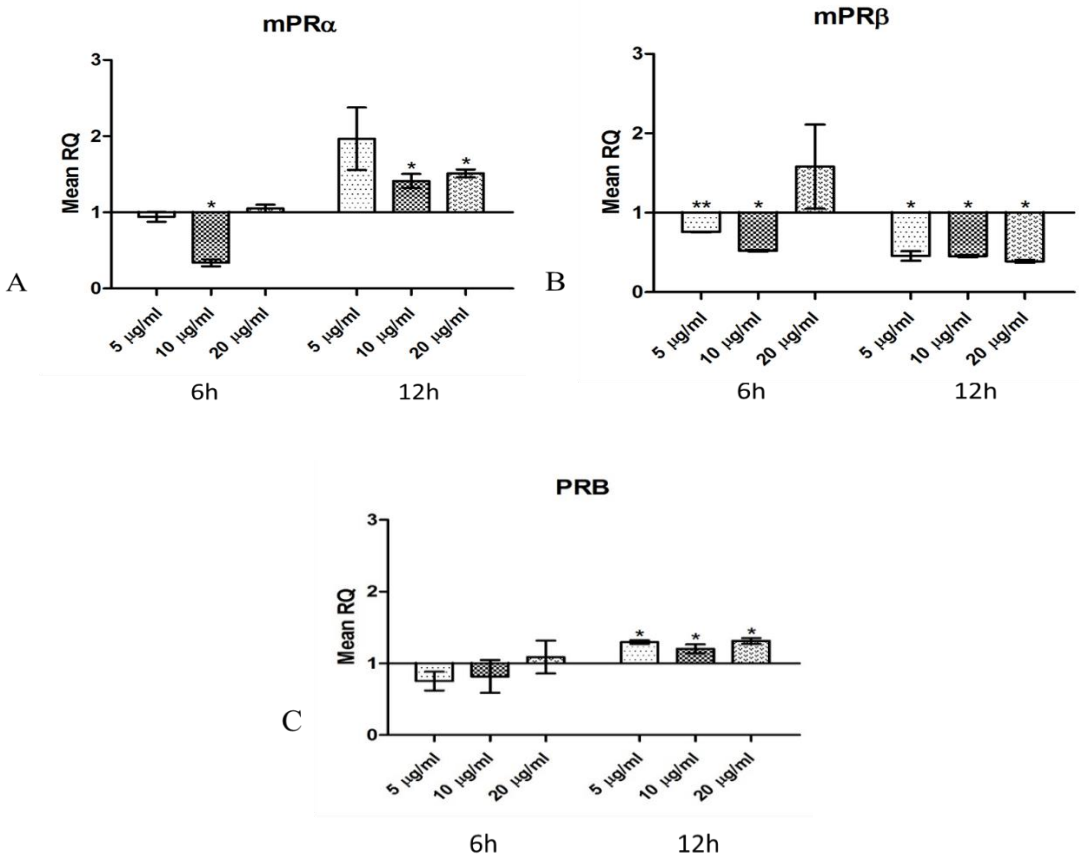


Figure 4.16 *Schematic representation showing the mean relative quantities of mPRα (A) mPRβ (B) and PRB (C) mRNA expression ±SD at 6 and 12 hours (paired student's t-test: *p<0.05, **p<0.01) from cells treated with rhSP-D (n=3). There was an upregulation of mPRα and PRB receptors at 12 hours, when compared to untreated cells. There was no evident effect on mPRα and PRB at 6 hours, whereas there was a downregulation of mPRβ at 6 and 12 hours. Values were normalised against GAPDH.*

Results from the qPCR analysis showed that rhSP-D at 10 and 20 µg/ml led to an upregulation of the mPRα gene expression after 12 hours (1.2 and 1.4-fold change respectively, compared to untreated cells), though there was no effect at 6 hours. rhSP-D at 5, 10 and 20 µg/ml resulted in an upregulation of PRB at 12 hours (1.2, 1.1 and 1.3-fold change respectively), whereas no effect was evident at 6 hours. mPRβ expression was downregulated at 6 and 12 hours at all three concentrations, when compared to untreated cells.

Combining the data from the ULTR cells treated with rhSP-A and rhSP-D, it is evident that surfactant proteins upregulate the expression of PRs and potentially exert further functions.

4.4 Discussion

Pregnancy is a complex event that requires an orchestrated cooperation of several components of the reproductive system. The signals that regulate the initiation of labour have not been fully elucidated (Zachariades *et al*., 2012). There has been increasing evidence implicating steroids in the maintenance of pregnancy and parturition. A successful pregnancy is dependent on the ability of the placenta to regulate steroid expression. Progesterone (P4) is expressed in the reproductive tract and is important for the maintenance of pregnancy and the timing of labour. In rodents there is a fall in circulating progesterone levels just before the initiation of labour (Condon *et al*., 2003). In humans, however, this withdrawal is not observed suggesting other means of reducing free progesterone. Estradiol is a hormone that can affect the expression of CAP genes and regulate the prostaglandin pathway which is crucial for pregnancy and the transition of the myometrium from the quiescent to an active state (Shynlona *et al*., 2009). To this date, there is no data available on the potential link of these steroid hormones to SP-A and SP-D. ULTR cells were treated with P4, E2 and OD-02 (mPR specific agonist) and we measured surfactant protein levels. P4 and E2 managed to increase the expression of SP-A, though they had little or no effect on the expression of SP-D. OD-02 did not have an effect on the expression of these proteins, suggesting that the expression of SP-A and SP-D is not controlled in a non-genomic manner. Interestingly, it is attractive to speculate that this cross-talk could be mediated in an autocrine or a paracrine manner.

Progesterone can act via different groups of receptors in a genomic and non-genomic way. It can bind to PRB and PRA in a genomic manner and mRPs (mPRα and mPRβ) in a non-genomic manner. Recently, it has been hypothesised that mPRs by binding to inhibitory proteins can participate in functional progesterone withdrawal (Karteris *et al*., 2006). In this study we validated the expression and localisation of mPR receptors in the human myometrial cell line ULTR. Since we identified a possible link between surfactant proteins and P4, we then decided to study the effects of surfactant proteins on mPRα, mPRβ and PRB. ULTR cells were treated with the two surfactant proteins and qPCR showed that rhSP-A treatments resulted in an increase of all three receptors, whereas rhSP-D resulted in an increase of mPRα and PRB. We, therefore propose a model including P4, SP-A, and these receptors, leading to myometrial transition from a quiescent state to a contractile/active state.

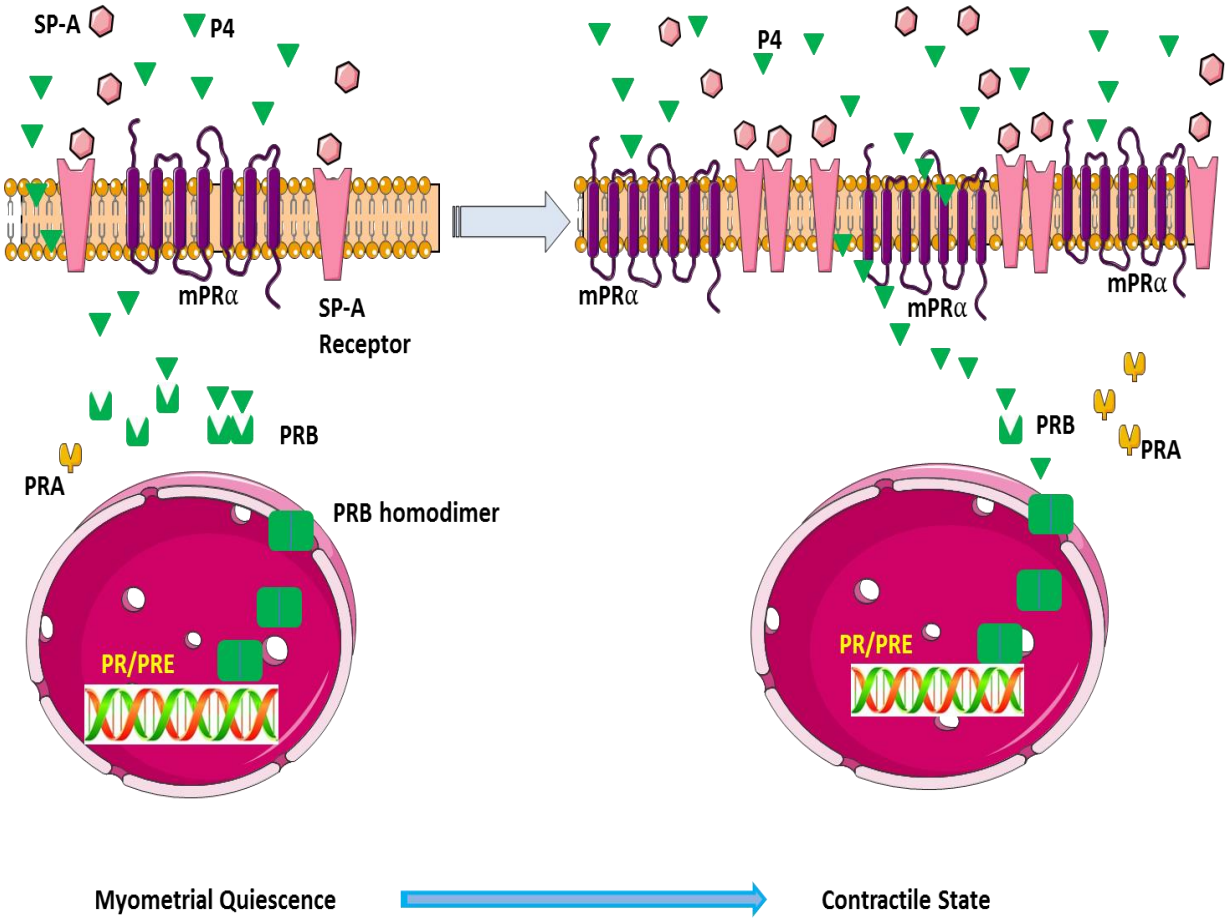


Figure 4.17 *Model proposed for the transition of the myometrium from quiescence to a contractile state.*

During the initial stages of pregnancy, P4 and SP-A are expressed at low levels. Towards the end of term P4 levels increase dramatically. A similar increase is observed in SP-A levels. We propose that P4 leads to increased expression of SP-A which in turn induces the expression of mPRα. PRA is an antagonist of PRB and its increase towards term leads to reduced binding of P4 to PRB. We have previously shown that, surfactant proteins also lead to the expression of OXTR and CX43 and pro-inflammatory cytokines. All these factors create an environment that facilitates the shift from a quiescent to a contractile state in the myometrium (Figure 4.17).

We, also, investigated further non-genomic effects of P4 by using OD-02, which is a mechanistic analog of P4 and binds to mPRs but not PRB. Microarray analysis in ULTR cells

treated with OD-02 showed a large number of genes whose expression was altered due to our treatments. Some of those genes are involved in important signalling pathways that play a role in pregnancy and parturition.

The microarray analysis showed several genes that were up or down-regulated due to non-genomic function of progesterone acting via the mPRs. Among which, were caldesmon 1 and adducin 3 which play a key role in tissue remodelling via interacting with actin, myosin and calmodulin leading to the increase in Ca^{2+} inside the smooth muscle cells leading to myometrial activation and contractility (Matsuoka *et al*., 2000; Kim *et al*., 2012). Genes that regulate signal transduction and were upregulated included GNG11 which regulates transmembrane signalling, PGRMC2 which acts as a progesterone receptor, MAP3K8 which regulates the MAPK signalling, P2RX7 which is involved in uterine contractility, and LEPR which is a receptor for leptin and is important during pregnancy (Demokan *et al*., 2003; Miyoshi *et al*., 2010; Pru and Clark, 2013; Herrid *et al*., 2014; Liu *et al*., 2015). Inflammatory/immune response genes that were upregulated were TLRs 2 and 4 which promote the expression of pro-inflammatory cytokines, IL-2 and 10 which regulate immune responses, and LTBP1 which mediates activity of TGF-β (Gorski *et al*., 2009; Youssef *et al*., 2009; Thaxton and Sharma, 2010; Fainboim and Arruvit, 2011; Maurya *et al*., 2013). NF-kB1 which promotes the expression of pro-inflammatory cytokines was downregulated (Cookson and Chapman, 2010). All this data shows that non-genomic functions of progesterone lead to expression of genes that modulate signal transduction pathway to allow myometrial contractility, and promote the expression of pro-inflammatory cytokines leading to myometrial activation.

When we studied the effects of OD-02 on cell functionality, OD-02 treatment had a moderate effect on cell proliferation, but it led to an increase in cell motility. This is crucial for the phases the myometrium goes through during pregnancy leading to parturition. The initial proliferative stage is followed by hypertrophy, hyperplasia and reconditioning (Foster *et al*., 2014). We, therefore, witness that P4 acting via mPRs plays a role during these later phases, thus enabling the activation of the myometrium in preparation of labour.

Chapter 5

Effect of a recombinant form of human SP-D (rhSP-D) on the ovarian cancer cell line SKOV3

5.1 Introduction

Hydrophilic surfactant proteins A (SP-A) and D (SP-D) are important for clearing pathogens, maintaining pulmonary homeostasis and inflammatory state. They have been localised in various parts of the reproductive tract, including the ovaries (Oberley *et al*., 2004). SP-D has recently been implicated in cancer; tumour cells can be affected by the inflammatory environment and that can have an effect on tumour proliferation and metastasis (Brigati *et al*., 2002), thus making surfactant proteins' role even more important. Many studies have focused on lung cancer, as it is the primary production site of SP-D where it plays a crucial role in homeostasis; a recent study showed that SP-D can downregulate the EGF pathway via direct binding to EGFR and inhibit cell proliferation, metastasis and invasion of the lung cancer cell line A549 (Hasegawa *et al.* 2015). Mutations in the SP-D gene have been correlated with lung cancer and other diseases such as pneumonia or emphysema (for example the single nucleotide polymorphism (SNP) rs721917, which results in methionine being exchanged for threonine at amino acid 11 (the Met11Thr variation)), where SP-D has been reported to have a specific role and effect in each of these diseases (Lin *et al.* 2007, Ishii *et al.* 2012). Moreover, circulating SP-D has been identified as a potential biomarker in chronic obstructive pulmonary disease (Sin *et al.* 2007). A clinical study in 71 patients showed that low levels of SP-D were associated with increased lung cancer incidence and that SP-D levels in bronchoalveolar lavage fluid (BALF) could be used as a biomarker (Sin *et al.* 2008). Similar data have been observed in the PANC pancreatic cancer cell line.

Ovarian cancer is one of the most common types of cancer in women; the second most common occurring in the reproductive tract and the fifth overall. It is usually called a 'silent killer' as it is diagnosed in later stages when the disease has spread to other organs and there is not a successful early diagnostic test to date (Rogers-Broadway *et al*., 2016).

The ovaries are in close proximity to the uterine wall which consists of the perimetrium, the myometrium and the endometrium. There is, therefore, an interaction between the different layers of the uterine wall and the surrounding organs. Shedding of the uterine wall has been connected with ovarian cancer cases (Sinaii *et al*., 2002). It is possible, therefore, that the uterine wall may affect ovarian physiology through secreted proteins and hormonal changes from the different layers. A previous study showed that secretions from normal tissues (ovarian, myometrial and peritoneal) affect the expression of matrix metalloproteinases (MMPs) in ovarian cancer cells. The same study also measured the expression of MMPs and their inhibitors and concluded that secretions of those normal tissues can regulate cancer cell invasion (Kikkawa *et al*., 1998).

5.2 Objectives

Based on the recent data in lung and pancreatic cancer and the lack of data in ovarian cancer, an involvement of SP-D in ovarian cancer was studied. We hypothesised that SP-D has a potential inhibitory role in ovarian cancer progression since it is expressed in various parts in the reproductive tract. The main aims were to:

- Identify the effects of myometrial tissue secreted proteins on cancer cell proliferation;
- Measure the effects of SP-D on gene expression of surfactant proteins and mTOR pathway components;
- Study the effects of SP-D on cell proliferation and motility;
- To measure the expression of SP-D in tissue clinical samples from normal ovaries;
- To investigate the expression of SP-D *in silico*, in normal and in ovarian cancer patients, and its potential role in survival.

5.3 Results

5.3.1 Effects of conditioned media from ULTR cells treated with rhSP-D on ovarian cancer cells

Conditioned media from ULTR cells treated with rhSP-D for 24 hours at 5, 10 and 20 µg/ml was used to treat SKOV3 cells seeded at a specific density and cell numbers were measured after 24 and 48 hours.

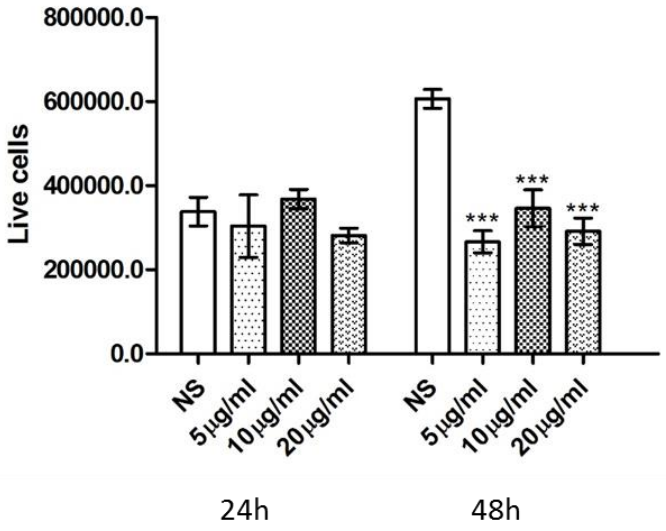


Figure 5.1 *SKOV3 live cells numbers at 24 and 48 hours. Cells were treated with conditioned media from ULTR cells treated with rhSP-D (5, 10 and 20 µg/ml) at 24 and 48 hours ±SEM, of three independent experiments. Cell numbers were not altered between treated and untreated cells at 24 hours whereas treated cells were approximately 40% less compared to untreated cells at 48 hours. Significance was calculated using the unpaired student's t-test (***p<0.001) (NS: no supplement; n=6).*

At 24 hours, there was no difference in cell numbers between treated and untreated cells. After 48 hours, untreated cell numbers were doubled whereas the number of cells treated with conditioned media from ULTR cells (at 5, 10 and 20 µg/ml of rhSP-D), was significantly lower compared to untreated cells (Figure 5.1).

5.3.2.1 Immunofluorescence of SKOV3 cells using anti-human SP-D antibody

The effects of rhSP-D on ovarian cancer cells were investigated in order to determine its role in reducing proliferation/increasing cell death. SKOV3 cells were seeded at a specific density (70,000) in 24-well plates and left to adhere for 24 hours. Cells were then treated with anti-human polyclonal SP-D for 24 hours and then with secondary antibody to enable visualisation. A HF14 Leica DM 4000 microscope was used to capture images of the cells and to localise SP-D.

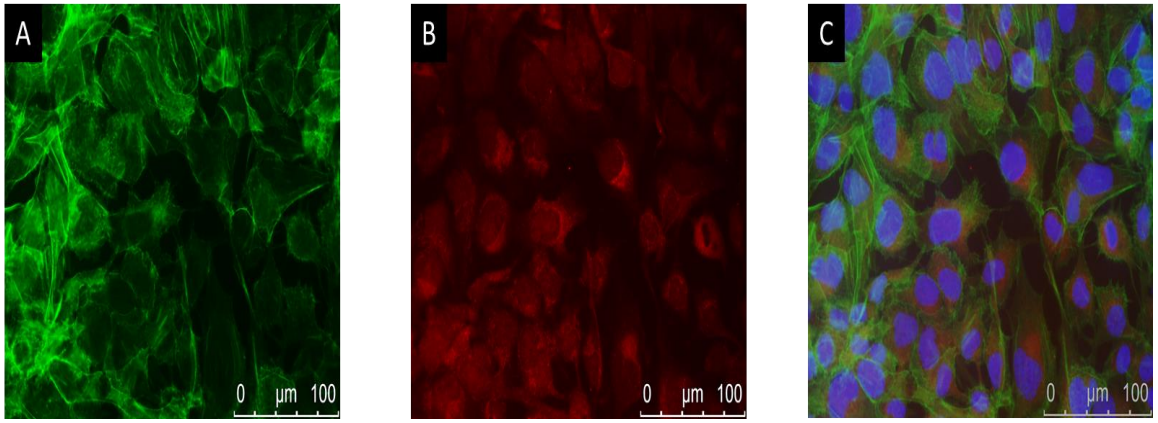


Figure 5.2 *Immunostaining of SKOV3xSP-D cells using anti-human SP-D. Cytoskeleton staining using phalloidin 488 (A) and anti SP-D (B). Hoechst was used for nuclear staining. Merged images for SP-D (C), x 40 magnification.*

Based on the immunofluorescence, it is evident that SKOV3 cells express SP-D. SP-D appears to be mainly localised within the cytoplasm (Figure 5.2).

5.3.2.2 ImageStream flow cytometry

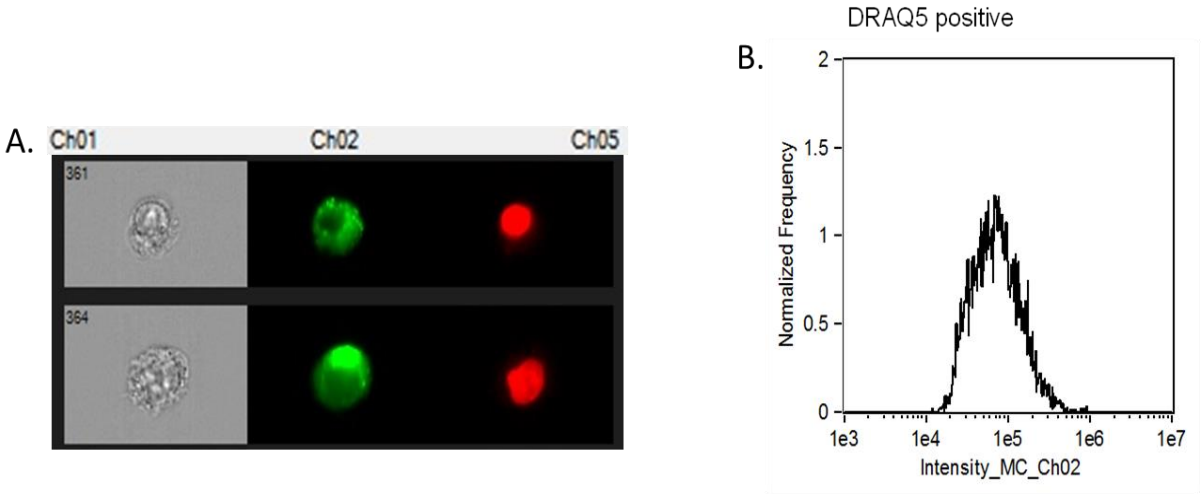


Figure 5.3 *ImageStream analysis showing localisation of surfactant protein SP-D (A), in SKOV3xSP-D individual cells. Channel 1 shows the brightfield pictures of cells, channel 2 shows surfactant protein localisation and channel 5 shows the nucleus stained with DRAQ5. SP-D is localised in the cytoplasm, with strong fluorescence intensity (B).*

Using an ImageStreamx Mark II flow cytometer, 10,000 cells were counted and visualised in order to validate the immunofluorescence results. Fluorescence was normalised against cells treated with antibodies but without DRAQ5 (nuclear stain), and those treated with DRAQ5 only. Localisation of SP-D was limited to the cytoplasm (Figure 5.3).

5.3.3 Proliferation study to evaluate the effects of rhSP-D on ovarian cancer cells

SKOV3 cells were cultured and seeded in 6-well plates as mentioned previously. After cells were left to adhere, they were treated with rhSP-D at 10 μg/ml. Cells were counted after 24 and 48 hours.

A proliferation assay using SKOV3 cells treated with rhSP-D showed that there was a small decline in treated cell numbers compared to untreated cells after 24 hours, though it was not statistically significant. However, it does show a trend of the effect that SP-D has on ovarian cancer cells. After 48 hours the cell numbers between the two groups were similar between the no supplement and treated cells (Figure 5.4), suggesting that SKOV3 cells eventually overcome the anti-proliferative effects of rhSP-D.

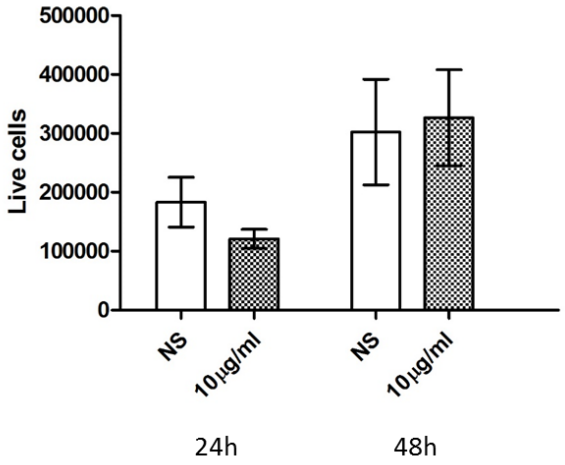


Figure 5.4 *SKOV3 live cell numbers after cells were treated with rhSP-D at 10 µg/ml at 24 and 48 hours ±SEM, of three independent experiments. Cell numbers slightly decreased after treatment with rhSP-D at 24 hours, compared to untreated cells (NS). Cell numbers were similar at 48 hours. Significance was calculated using the unpaired student's t-test (n=6).*

5.3.4 Wound healing assay

SKOV3 cells were grown in petri dishes and reached 100% confluency. Using a fine pipette tip, an artificial wound was created on the cell surface. The petri dishes were placed under a Zeiss Axiovert 200M microscope attached to an incubator to allow visualisation. Images of the cells were acquired every 10 minutes for up to 48 hours.

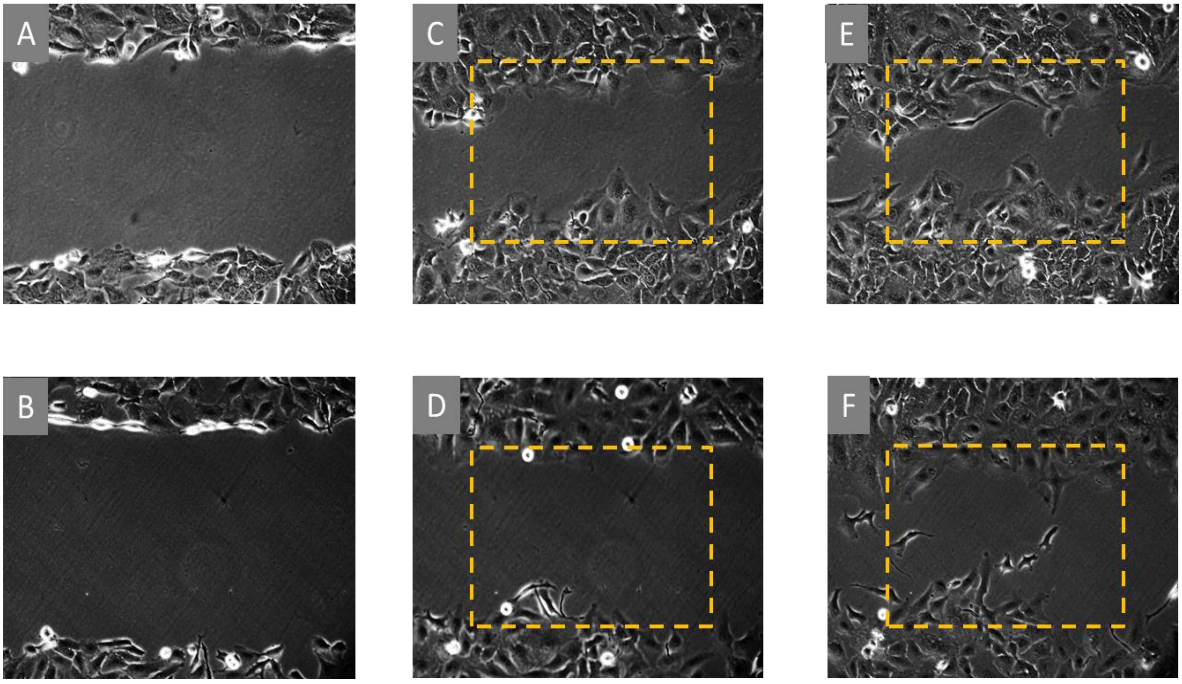


Figure 5.5 *Schematic representation showing the artificial wound created on the cell surface in untreated cells (A) and treated cells with rhSP-D (B) at 0 hours. Untreated cells (C) or treated cells with rhSP-D (D) did not appear to close the gap after 12 hours. At 24h untreated cells managed to close the gap (E) whereas rhSP-D treated cells did so after 30 hours (F).*

Images acquired from the wound healing assay over 48 hours showed that untreated cells closed the gap at 24 hours, more rapidly compared to treated cells with rhSP-D, which closed the gap at 30 hours (Figure 5.5).

5.3.5 Cell motility assay for SKOV3 cells treated with rhSP-D

SKOV3 cells were first cultured in a 75cm^2 flask and allowed to reach 100% confluency. They were then sub-cultured at a 30% confluency in a petri dish. Cells were allowed to adhere for 5-6 hours before being treated with rhSP-D. The petri dish was placed under a Zeiss Axiovert 200M microscope which had an incubator attached to allow cell visualisation and survival under standard culture conditions. After an area with approximately 25-30 cells was located, images were captured every 5 minutes for up to 12 hours. Using ImageJ, the coordinates for 25 cells were acquired.

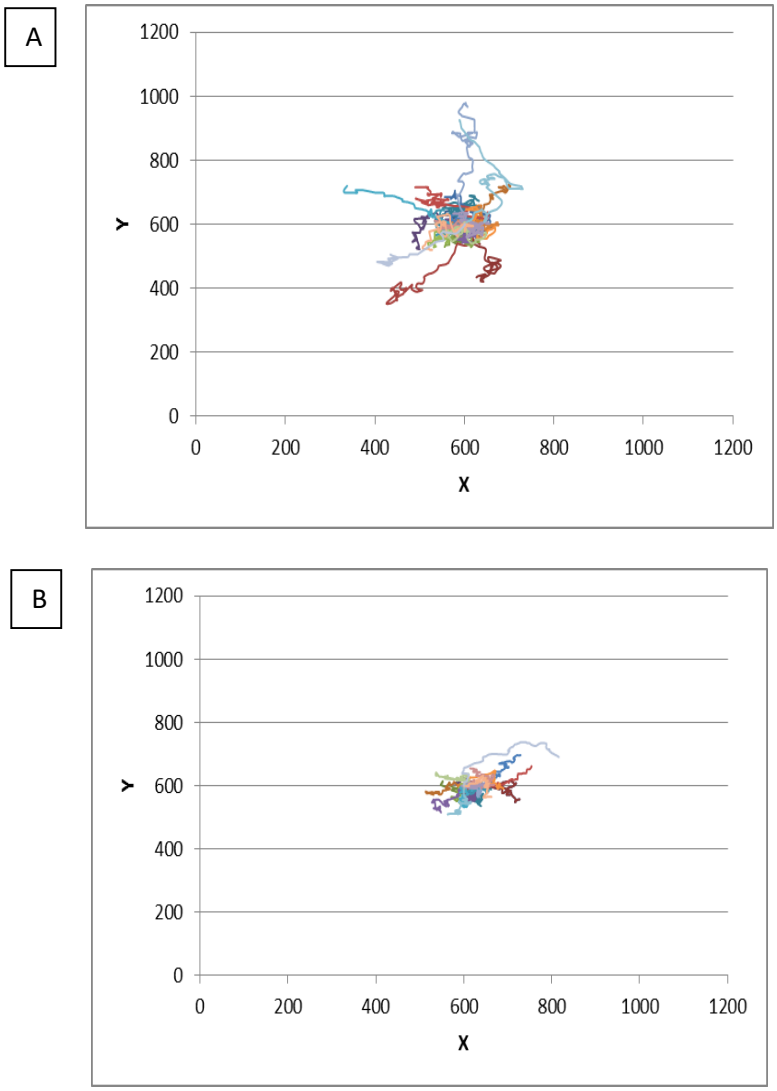


Figure 5.6 *Schematic representation showing the X and Y coordinates of all time points of untreated (A) and treated with rhSP-D (B) SKOV3 cells. 25 cells were counted for each experiment. The point (600,600) was set as a starting point for each cell. Cells treated with surfactant protein D were less motile compared to untreated cells. Each line represents a single cell.*

Based on the coordinates acquired using ImageJ, the start point for each cell of the 25 in total, was set at (600,600) in order to be able to compare more cells and in between experiments. At each time point, the position of each of the cells was determined in order to acquire all the coordinates. Cells treated rhSP-D remained close to their initial position at 0 hours across time

points. Untreated cells appeared to move further away from their initial position across time points (Figure 5.6), suggesting that rhSP-D treatment considerably affect SKOV3 motility.

Using Pythagora's theorem for each point for each of the cells, the distance, velocity and displacement for all 25 cells were calculated.

Table 5.1 *Table showing the distance, velocity and displacement values for the cell motility of untreated (NS) and cells treated with rhSP-D.*

	Distance (pixels)	**Velocity (pixels/minute)**	**Displacement (pixels)**
SKOV3 NS	586.69	1.22	116.56
SKOV3 x rhSP-D	336.62	0.70	72.23

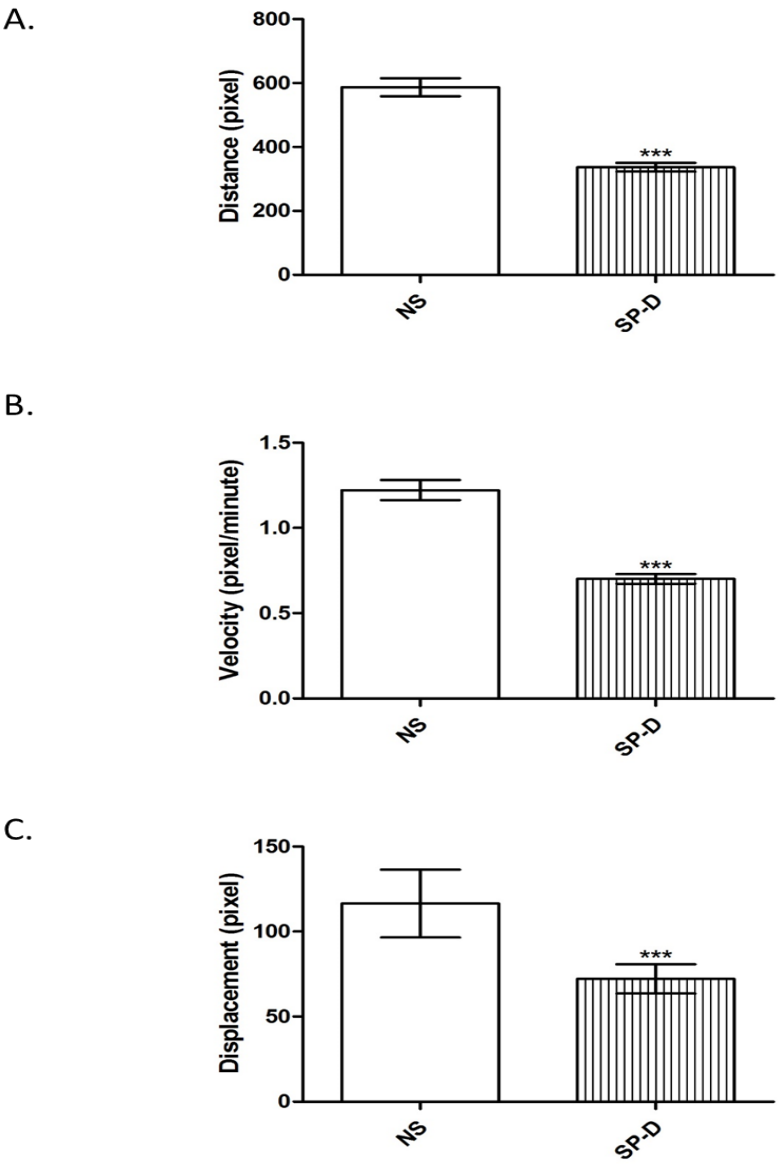


Figure 5.7 *Schematic representation of the distance (A), velocity (B) and displacement (C) comparisons ±SEM between untreated and treated SKOV3 cells with rhSP-D at 10µg/ml (Mann Whitney U test:***p<0.001). 25 cells were counted for each experiment. Treatments with surfactant SP-D resulted in a significant decrease in distance, velocity and displacement compared to untreated (NS) cells (n=25).*

Cells treated with rhSP-D moved significantly less and slower compared to untreated cells; there was a significant decrease in distance, velocity and displacement, compared to untreated cells (Figure 5.7).

5.3.6 Effects of rhSP-D on surfactant protein expression

SKOV3 cells were grown and treated with rhSP-D as described previously. RNA was extracted from the cells and transcribed into cDNA. qPCR was performed to quantify the expression of SP-A1, SP-A2 and SP-D mRNA.

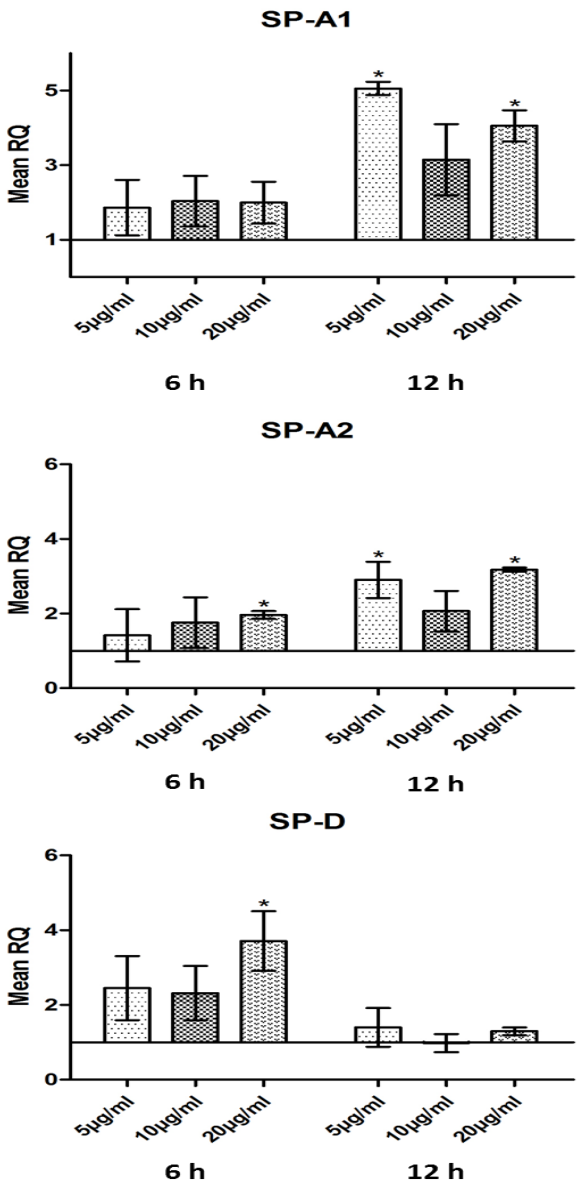


Figure 5.8 *Relative quantification comparisons of SP-A1 (A), SP-A2 (B), and SP-D (C) in SKOV3 cells treated with 5, 10 and 20 µg/ml of rhSP-D after 6 and 12 hours ±SD (paired student's t-test: *p<0.05). rhSP-D treatments resulted in an increase of SP-A2 and SP-D mRNA expression at 6 hours, compared to untreated cells. At 12 hours there was a further increase in SP-A1 and SP-A2 mRNA expression at 5 and 20 µg/ml, whereas the SP-D transcripts were decreased, when compared to untreated cells (n = 3). Values were normalised against GAPDH.*

The upregulation of SP-D takes places in the early phase of treatment when compared to untreated cells. However, its levels were decreased by 12 hours. On the contrast, SP-A1 and SP-A2 remain basal at 6 hours while a higher level of upregulation occurs at 12 hours, compared to untreated cells. It is possible that the two transcripts are differentially transcribed and stabilised (Figure 5.8).

5.3.7 Effects of rhSP-D on the components of the mTOR pathway

As treatments with SP-D led to a decrease in cell motility and cell growth, we assessed the effects of this molecule on several components of the mTOR pathway which acts as a sensor and can regulate cell proliferation, growth, autophagy and apoptosis (Laplante and Sabatini, 2012). Upregulation of the pathway has been associated with ovarian cancer progression (Zhou and Wong, 2006).

Cells were treated as mentioned previously and RNA was extracted. A qPCR was performed to measure the levels of the expression of mTOR, DEPTOR, Rictor and Raptor.

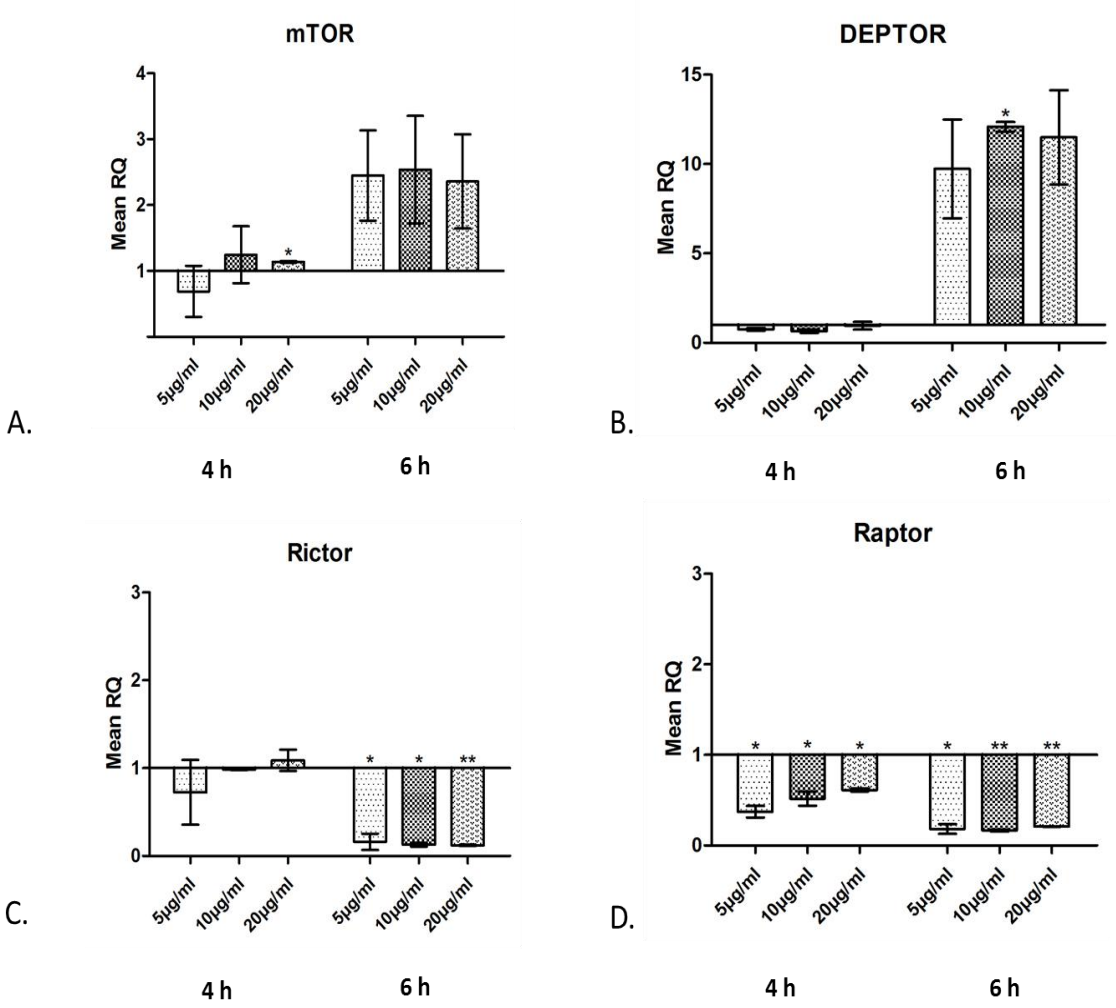


Figure 5.9 *Relative quantification comparisons of mTOR (A), DEPTOR (B), Rictor (C) and Raptor (D) mRNA expression in SKOV3 cells treated with 5, 10 and 20 μg/ml of rhSPD after 4 and 6 hours ±SD (paired student's t-test: *p<0.05, **p<0.01). Levels of mRNA transcripts of mTOR, DEPTOR and Rictor remained unchanged compared to untreated levels at 4 hours. Levels of Raptor decreased at all concentrations after a 4-hour treatment with rhSP-D. At 6 hours levels of mTOR and DEPTOR were increased, when compared to untreated cells. Levels of Rictor and Raptor were decreased at all concentrations. Values were normalised against GAPDH.*

qPCR results showed that treatments with rhSP-D did not have a profound effect on mTOR, DEPTOR and Rictor after 4 hours. Expression levels of the above mentioned genes remained

close to basal levels at all concentrations of rhSP-D. Raptor expression levels were significantly decreased after 4 hours at all concentrations.

At 6 hours, the expression of mTOR and DEPTOR was upregulated when compared to untreated cells, with rhSP-D treatments having a more profound effect on DEPTOR mRNA expression. With regards to Rictor and Raptor transcript expression, rhSP-D treatments resulted in a decrease of both genes simultaneously at all concentrations. This is of great significance as it means that the protein interactions in both mTOR complexes (mTORC1 and mTORC2) can be compromised with reduced expression of Rictor and Raptor, resulting in the downregulation of the mTOR pathway. Having a compromised mTOR signalling pathway might lead to an inhibition of protein synthesis, cell growth and proliferation (Figure 5.9).

To assess further the effects of SP-D on mTOR, we measured changes on the phosphorylation status of S6K as a downstream target of mTORC1 activity. When the mTOR pathway is active, it phosphorylates S6 kinase which in turn can regulate several cell functions such as cycle progression and protein synthesis (Fingar *et al*., 2004; Holz *et al*., 2005). An increase of S6Kphosphorylation is an indication of an active mTOR pathway and has been shown in late stage ovarian cancer (Rogers-Broadway *et al*., 2016).

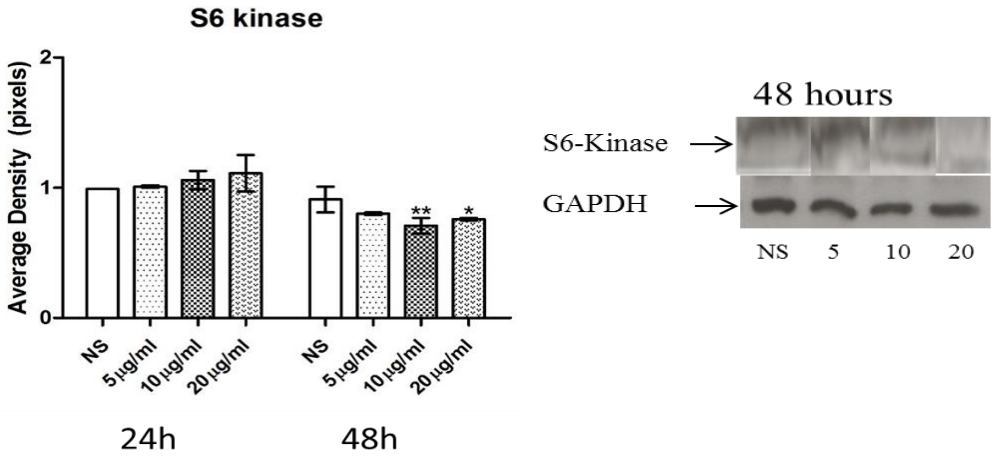


Figure 5.10 *Left: Average density comparisons of the protein expression for phospho-S6 kinase in SKOV3 cells treated with rhSP-D ±SD at 24 and 48 hours which were acquired from the western blotting analysis. Right: Representative bands for S6 kinase at 48 hours. Phospho-S6 kinase was not altered at 24 hours at any concentration whereas the expression was decreased after 48 hours at 10 and 20 µg/ml, compared to untreated cells. Significance was obtained using the unpaired student's t-test (*p<0.05, **p<0.01). Values were normalised against GAPDH. NS: no supplement.*

rhSP-D treatments did not have an effect on phosphorylated S6K (Ser) expression after 24 hours when cells were treated at 5, 10 or 20 µg/ml. After 48 hours there was a significant decrease in the phosphorylation levels of S6K protein at 10 and 20 µg/ml, compared to untreated cells (Figure 5.10).

5.3.8 Effects of rhSP-D on the caspase pathway

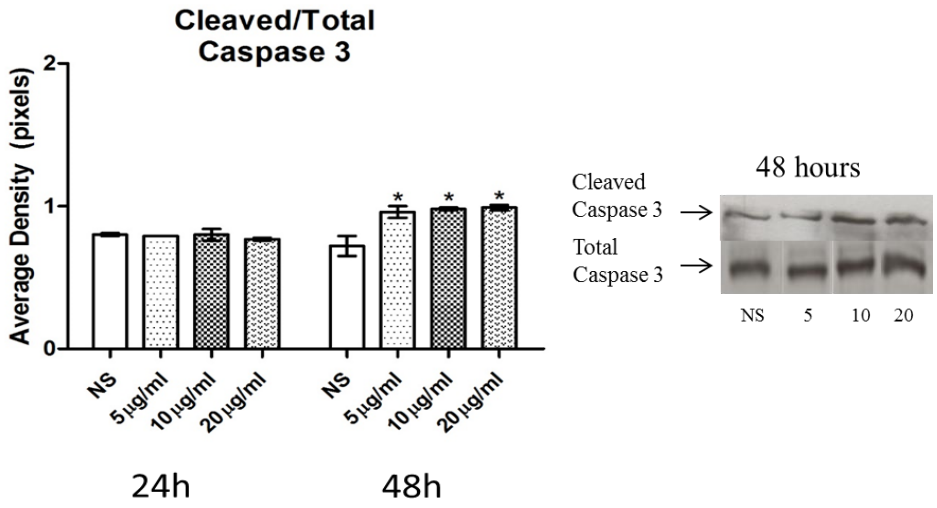


Figure 5.11 *Left: Average density comparisons of the protein expression bands for the ratio of cleaved over total caspase 3 in SKOV3 cells treated with rhSP-D ±SD at 24 and 48 hours which were acquired from the western blotting analysis. Right: Representative bands for cleaved and total caspase 3 at 48 hours. Cleaved over total ratio was not altered at 24 hours at any concentration whereas the ratio was increased after 48 hours at 5, 10 and 20 µg/ml, when compared to untreated cells. Significance was obtained using the unpaired student's t-test (*p<0.05). Values were normalised against GAPDH. NS: no supplement.*

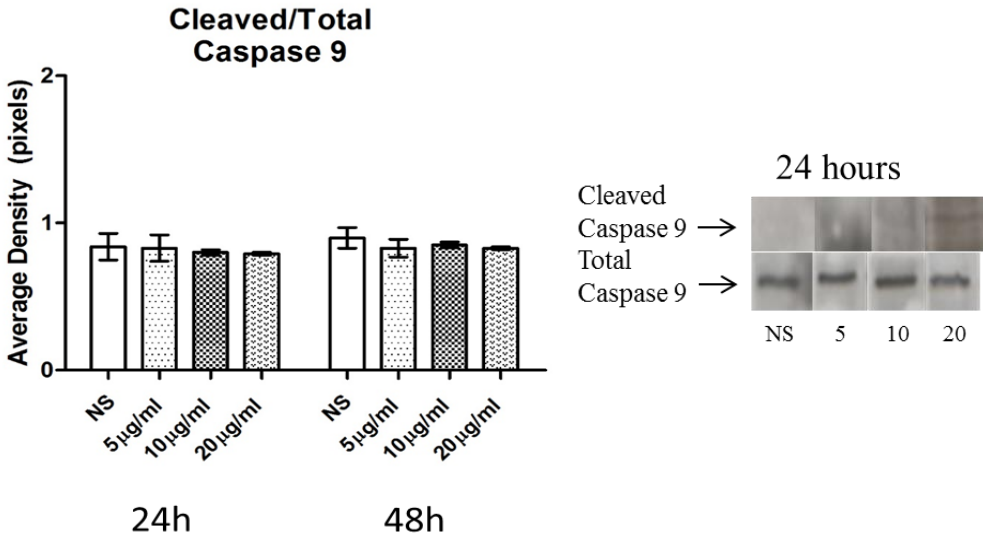


Figure 5.12 *Left: Average density comparisons of the protein expression bands for the ratio of cleaved over total caspase 9 in SKOV3 cells treated with rhSP-D ±SD at 24 and 48 hours which were acquired from the western blotting analysis. Right: Representative bands for cleaved and total caspase 9 at 24 hours. Cleaved over total ratio was not altered at 24 hours or 48 hours at any concentration (5, 10 and 20 µg/ml), when compared to untreated cells. Significance was obtained using the unpaired student's t-test (*p<0.05). Values were normalised against GAPDH. NS: no supplement.*

rhSP-D treatments resulted in differential expressions of the proteins caspase 3 and caspase 9. Cleaved caspase 3 expression remained unchanged after 24 hours but it was increased after 48 hours at 10 and 20 µg/ml, when compared to untreated cells. Cleaved caspase 9 on the other hand was not altered after 24 or 48 hours at any concentration, after SKOV3 cells were treated with rhSP-D (Figure 5.11, 5.12).

5.3.9 SP-D mRNA expression in normal ovarian tissue

In order to determine how the expression of SP-D affects ovarian cancer progression and metastasis, we aimed to determine at what levels normal ovaries express SP-D in 6 clinical samples.

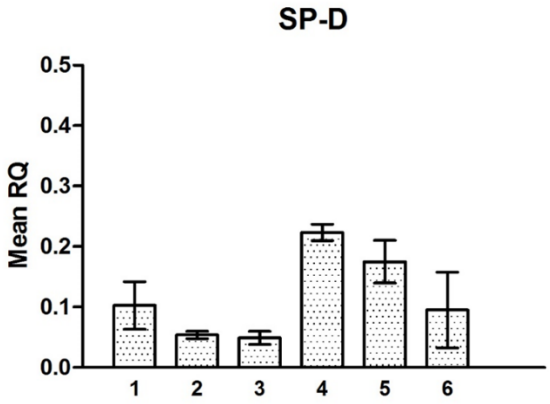


Figure 5.13 *Mean relative quantity values of SP-D mRNA in normal ovarian tissue samples (1-6) ±SD. SP-D is expressed in the ovaries differentially between the ovarian tissue clinical samples (n=6). Values were normalised against GAPDH.*

qPCR showed that SP-D is expressed in normal ovarian tissue in all our clinical samples. It is evident that there is interpatient variation in the expression of SP-D in normal ovaries. Due to ethical considerations, there was no further access to ovarian cancer tissues. We therefore decided to use online resources.

5.3.10 *In silico* analysis of SP-D expression in normal and ovarian cancer patients

After measuring the SP-D gene expression levels in a small number of samples, we sought to assess SP-D levels in a much larger cohort of samples. We, therefore, used Oncomine™ (oncomine.org), an online source that collects data and combines different microarray studies to enable study and overview in a large number of samples (Rhodes *et al*., 2004). For this study we selected 'Cancer vs Normal' analysis in all datasets.

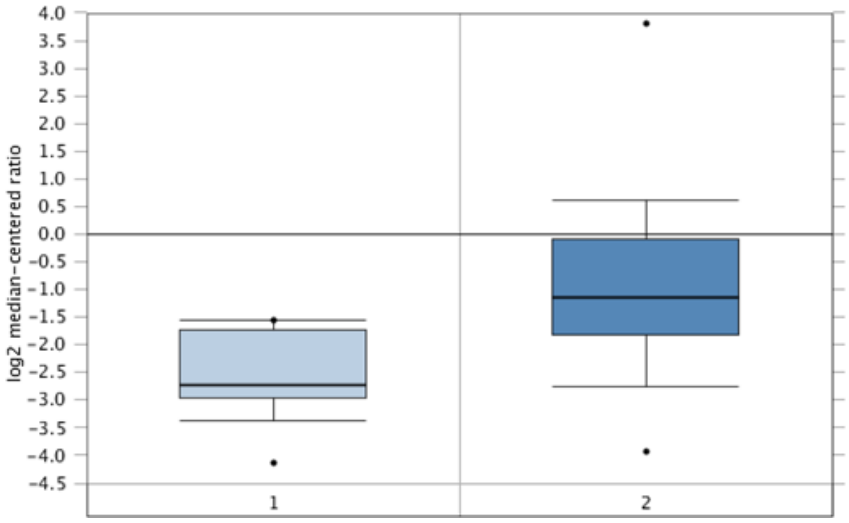


Figure 5.14 *SP-D gene expression from the Yoshihara dataset plotted by Oncomine™. Mean SP-D gene expression is shown for normal peritoneum (1, n=10) compared to ovarian serous adenocarcinoma (2, n=42). Boxes represent the 25th-75th percentile (with median line), bars show the 10th-90th percentile with circles (●) showing the absolute spread of data. Fold increase in SP-D expression was 3.059 (p=2.07E-5).*

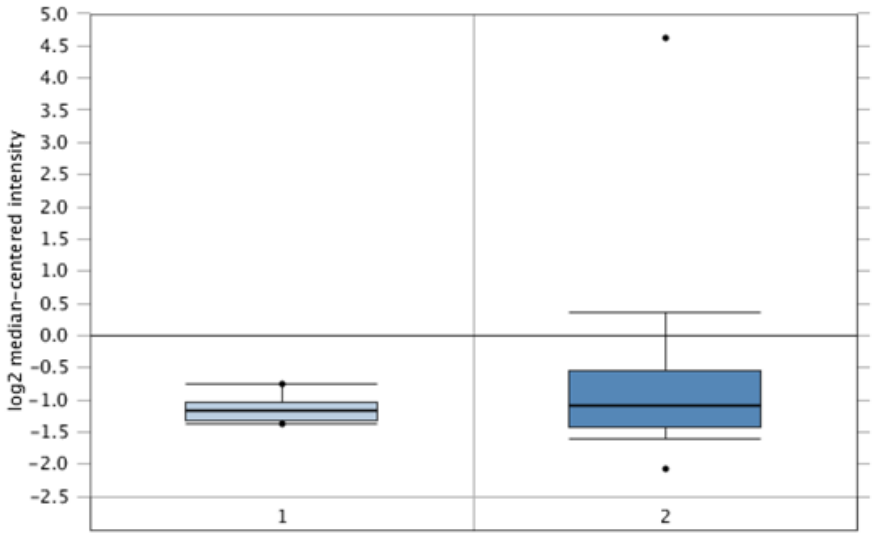


Figure 5.15 *SP-D gene expression from the TCGA dataset plotted by Oncomine™. Mean SP-D gene expression is shown for normal ovaries (1, n=8) compared to ovarian serous cystadenocarcinoma (2, n=586). Boxes represent the 25th-75th percentile (with median line), bars show the 10th-90th percentile with circles (●) showing the absolute spread of data. Fold increase in SP-D expression was 1.263 (p=9.00E-4).*

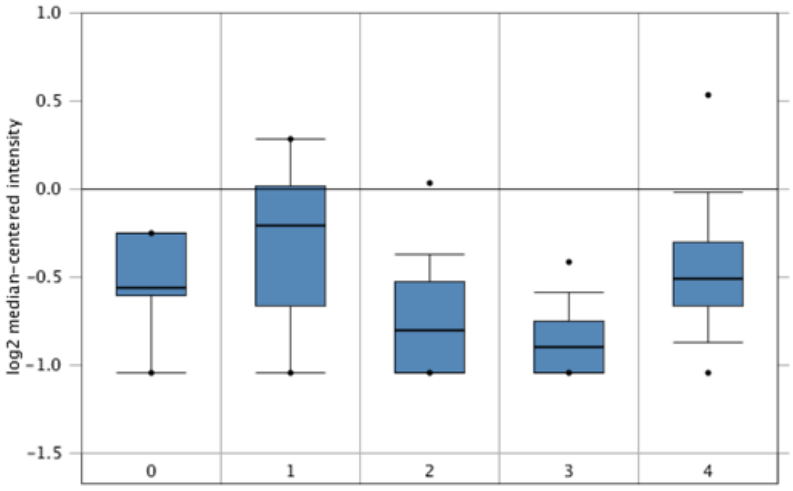


Figure 5.16 *SP-D gene expression from the Hendrix dataset plotted by Oncomine™. Mean SP-D gene expression is shown for normal (0, n=4), ovarian clear cell adenocarcinoma (1, n=8), ovarian endometrioid adenocarcinoma (2, n=37), ovarian mucinous adenocarcinoma (3, n=13) and ovarian serous adenocarcinoma (4, n=41). Boxes represent the 25th-75th percentile (with median line), bars show the 10th-90th percentile with circles (●) showing the absolute spread of data. Fold increase in SP-D expression was 1: 1.217 (p=0.123), 2: -1.109 (p=0.782), 3: -1.201 (p=0.897) and 4: 1.086 (p=0.266) when compared to normal respectively.*

Analysis using the online software tool Oncomine™ showed there is a differential expression in SP-D levels in different types of ovarian cancer compared to control/normal samples (Figure 6.6, 6.7, 6.8), suggesting a role for SP-D in ovarian cancer which could be type-specific. SP-D expression was increased in ovarian serous adenocarcinoma, ovarian serous cystadenocarcinoma and ovarian clear cell adenocarcinoma. SP-D levels were decreased in ovarian endometrioid adenocarcinoma and ovarian mucinous adenocarcinoma. For this reason, we sought to investigate further the expression and cellular distribution of SP-D in ovarian cancer using immunohistochemistry.

5.3.11 Protein expression analysis of SP-D in paraffin embedded ovarian tissue clinical samples

Commercially available ovarian cancer tissue array slides (Biomax, U.S.) were probed with anti SP-D using immunohistochemistry. Three areas of the tissue samples were randomly selected.

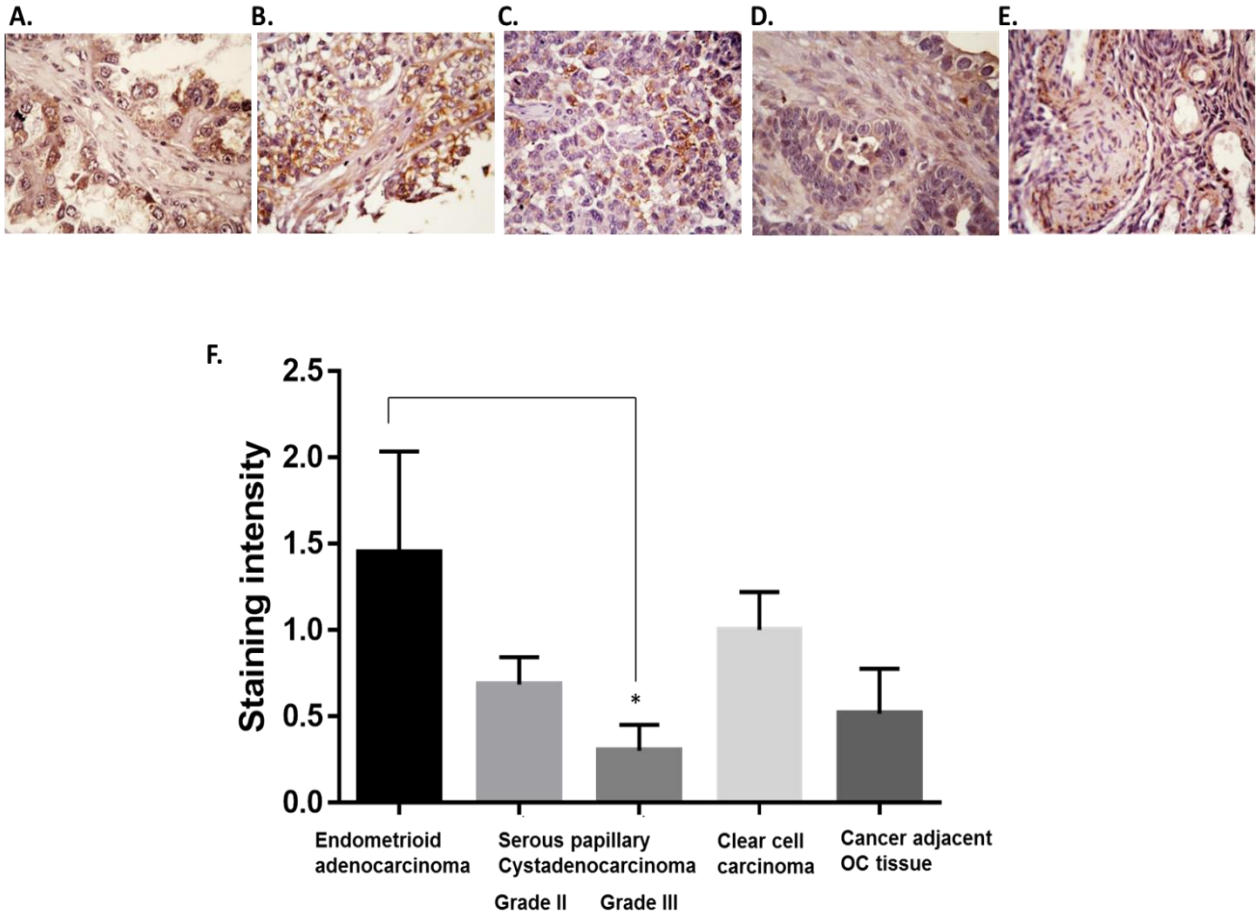


Figure 5.17 *Immunohistochemistry for SP-D expression in ovarian tissue array clinical samples (x40x magnification). Representative images of endometrioid adenocarcinoma (A), serous papillary cystadenocarcinoma grade II and grade III (C-D), clear cell carcinoma (E) and cancer adjacent ovarian cancer (OC) tissue. There was a clear down-regulation of SP-D serous papillary cystadenocarcinoma grade III compared to endometrioid adenocarcinoma (F; *p<0.05), and a notable decrease in staining intensity with advancing grade.*

After three areas were randomly selected, positive cells were counted in each image and an average for the tissue was acquired. SP-D expression was increased in endometrioid adenocarcinoma, clear cell carcinoma and serous papillary cystadenocarcinoma compared to the other types of ovarian cancer. Measuring staining intensity showed a significant difference between serous papillary cystadenocarcinoma Grade III and endometrioid adenocarcinoma, whereas it just failed to reach significance when compared to clear cell carcinoma ($p=0.0574$; Figure 5.17 A-E). It is also evident that there was a notable difference in staining amongst stage II and stage III of serous papillary cystadenocarcinomas, with the lower grade having a stronger staining. Grouping all ovarian cancers in stage II and III, showed there was a significant down-regulation of SP-D staining with higher grade.

5.3.12 *In silico* analysis of survival related to SP-D expression

In order to assess the relationship between SP-D expression and survival in ovarian cancer patients, we used the OvMark Kaplan-Meier Plotter, developed by the National Institute for Cellular Technology at Dublin City University. This online resource uses an algorithm and different sources of microarray and clinical data to provide survival rates in relation to prognostic biomarkers for ovarian cancer (Madden *et al.*, 2014).

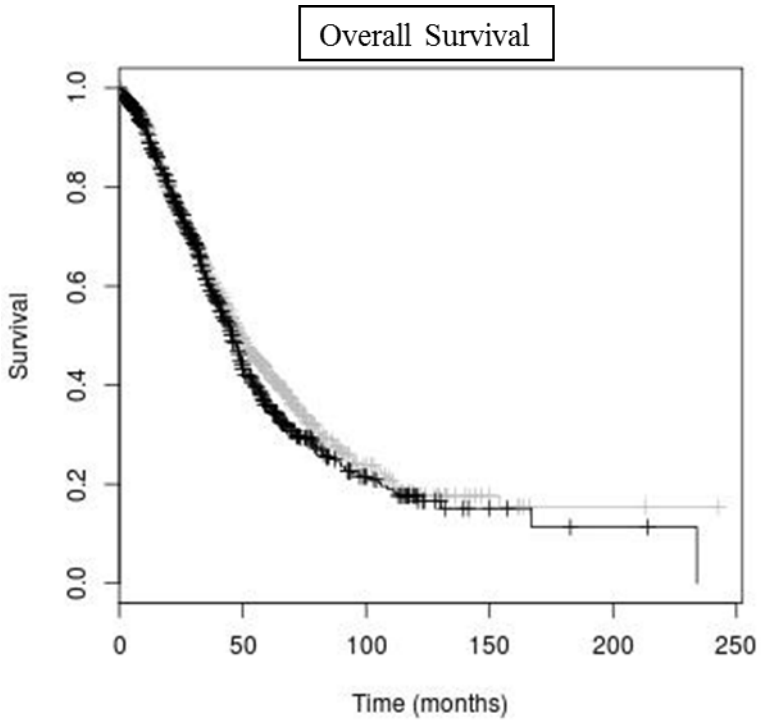


Figure 5.18 *Kaplan Meier plot showing Overall Survival probability of ovarian cancer patients over 250 months (n=1990). The two expression groups (higher expression-black n=919, lower expression-grey n=1071), have similar survival rates (p=0.1443).*

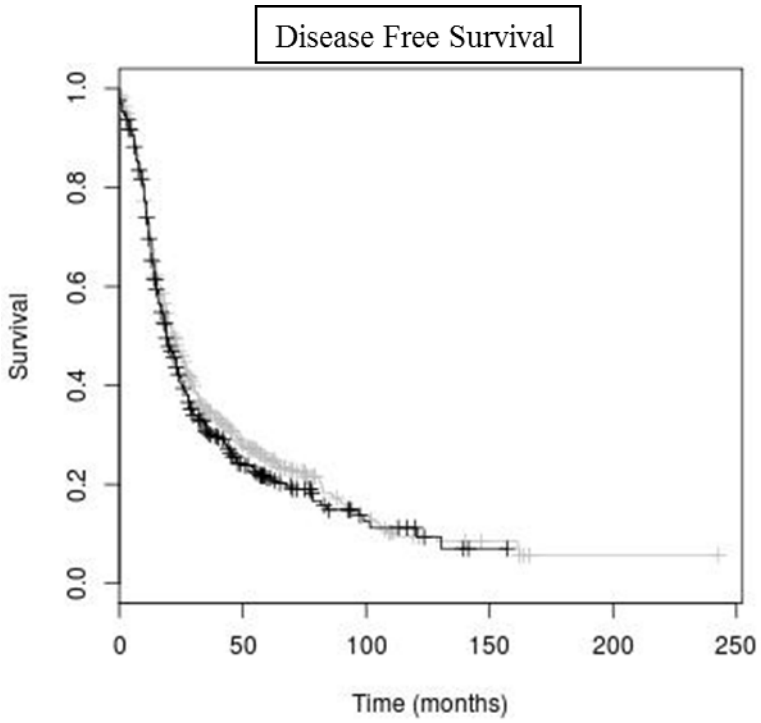


Figure 5.19 *Kaplan Meier plot showing Disease Free Survival probability of ovarian cancer patients over 250 months (n=996). The two expression groups (higher expression-black n=451, lower expression-grey n=545), have similar survival rates (p=0.1866).*

Based on the data acquired from OvMark, there was no significant difference between the higher and lower expression of SP-D groups, regarding to the overall survival and disease free survival rates (Figure 6.11, 6.12). At later stages (40-100 months of disease) where expression of SP-D is lower there is a slight increase in survival rates.

5.4 Discussion

SP-D has been reported to have differential properties that are cell and tissue specific. In chapter 3 we reported that SP-D has a pro-inflammatory effect in the myometrium (Sotiriadis *et al*., 2015). Kikkawa *et al*., (1998) used normal tissue secretions (from ovaries, myometrium and peritoneum) to study the effects on cancer cells. The study concluded that cancer cell invasion is regulated by the normal tissues, and showed an increased expression of MMPs and a decreased expression of their inhibitors in the ovarian cancer cell line NOM1 when it was treated with the normal tissue secretions. In this study we used conditioned media from the myometrium cell line ULTR which was treated with rhSP-D, to treat the ovarian cancer cell line SKOV3. The proliferation assay showed that cancer cell numbers were not affected at 24 hours but were decreased at 48 hours compared to control cells with a significant difference of approximately 40% which suggests that the conditioned media contained surfactant proteins and other hormones/cytokines following rhSP-D treatment, which can exert an inhibitory effect on cancer growth. When SKOV3 cells were treated with rhSP-D, there was a moderate decrease in cell proliferation, suggesting a potential anti-proliferative effect of SP-D. A recent study using the lung cancer cell line A549 showed that SP-D has an anti-inflammatory effect, inhibiting cancer cell progression and metastasis via inhibition of the EGF pathway through binding to EGFR and compromising the EGFR-EGF complex (Hasegawa *et al*., 2015). In contrast, a report on non-small cell lung cancers (NSCLC) showed that an increased expression of SP-D in NSCLC patients was correlated with metastasis and poor prognosis or clinical outcome (Chong *et al*., 2006). The effects of SP-D were more profound in the leukemic cell lines THP-1, AML14.3D10, Jurkat and Raji in the study published by Mahajan *et al*. The study also proposed a model for this particular effect of SP-D. They suggested that SP-D increases oxidative stress, expression of p21 and phosphorylation of p53 which leads to activation of caspase 9 (Mahajan *et al*., 2013).

Following the anti-proliferative effect of rhSP-D noted in the proliferation assays, we sought to investigate the effects of rhSP-D on cell migration and motility. The wound healing assay showed that control SKOV3 cells closed the artificial wound after 24 hours, whereas in SKOV3 cells treated with rhSP-D the gap closed after 30 hours. In conjunction with the wound healing assay, the cell motility assay showed that SKOV3 cells treated with rhSP-D

travelled less and much slower compared to control cells. There was a significant decrease in distance, velocity and displacement of SKOV3 treated cells. This shows that SP-D could have a cytostatic or cytotoxic effect that can ultimately inhibit cancer progression and subsequent metastasis. qPCR analysis showed that SKOV3 cells treated with rhSP-D, drove the expression of SP-A and SP-D transcripts. This positive feedback mechanism could have a prolonged inhibitory effect on tumour microenvironment. Further studies should investigate cyclin D, which is decreased when cells undergo apoptosis, and MMP expression, which promotes cytoskeletal degradation, in SKOV3 cells in order to gain better understanding of the mechanism(s) through which SP-D exerts these inhibitory effects.

To this date, little is known regarding the interactions of SP-D and mTOR. This particular pathway has been characterised as an important nexus through which cell growth, metabolism, autophagy, gene expression and protein synthesis are regulated (Gentilella *et al*., 2015). mTOR acts through two complexes mTORC1 and mTORC2. mTOR has been found to be activated in cancer cells that carry mutations in the p53 gene, which is the case with SKOV3 cells (Perl, 2015). SP-D can lead to p53 phosphorylation and an upregulation of p21 that results in cell cycle arrest, DNA repair or apoptosis (Mahajan *et al*., 2013). We, therefore, decided to study the effects of rhSP-D on the mTOR pathway. When we treated SKOV3 cells with rhSP-D and measured the expression of key components of the mTOR pathway, mTOR expression was not affected by SP-D. However, DEPTOR expression was increased and Rictor and Raptor mRNA were downregulated.

DEPTOR is part of the mTOR complexes and acts as an endogenous inhibitor of mTOR. When its expression is increased, it inhibits mTORC1 activity and down-regulates S6K phosphorylation (Peterson *et al*., 2009). Since rhSP-D treatment led to increased expression of DEPTOR, it is logical to speculate that DEPTOR can block mTORC1 activity and subsequently inhibit ovarian cancer cell growth.

Raptor is a component of the mTORC1 complex. It is necessary for its formation, and therefore, the mTOR pathway (Wang *et al*., 2012). In our experiment, rhSP-D downregulated the expression of Raptor, which suggests that the formation of the mTORC1 is compromised and the mTOR pathway is blocked. Rictor, however, is a key component of the mTORC2. mTORC2 can activate the Akt pathway as a negative feedback loop for mTOR (Beauchamp

and Platanias, 2013). SP-D treatments also led to down-regulation of Rictor, which might indicate that mTORC2 complex is also compromised. Upregulation of DEPTOR and downregulation of Rictor and Raptor suggest that the entire mTOR pathway could be inhibited downstream of both complexes. This could partially explain the effect of SP-D on SKOV3 cells.

Apart from the components of the mTOR complexes, we decided to study the effects of rhSP-D on the phosphorylation status of S6K, a downstream kinase that is a measure of mTORC1 activity. When the mTOR is phosphorylated, it leads to activation/phosphorylation of S6K (Schalm and Blenis, 2002). Since rhSP-D led to a down-regulation of mTOR activity, it was expected that there would be a downstream effect. Phosphorylated S6K (Ser) was not affected by rhSP-D at 24 hours but was significantly down-regulated at 48 hours, which is consistent with the notion that rhSP-D can inhibit the mTOR pathway in ovarian cancer.

We also aimed to investigate further, how SP-D exerted its anti-proliferative effects and whether SP-D had an effect on caspase activation. rhSP-D treatments did not have an effect on the caspase 9 cleaved/total ratio after 24 or 48 hours. Regarding caspase 3, there was no effect on the cleavage after 24 hours but there was an increase in the cleaved/total ratio after 48 hours. Caspase 3 can regulate cell death (Huang *et al*., 2011), which suggests another way through which SP-D exerts its cytotoxic effect as it is a pathway through which apoptosis can be modulated in SKOV3 cells, as previously described (Zhou *et al*., 2015).

Previously published data have shown that SP-D is expressed in human ovaries (Oberley *et al*., 2004, Oberley *et al*., 2007). In this chapter, we measured the expression of SP-D in clinical samples from ovarian tissues. There was interpatient variation but some ovarian tissues from patients appeared to express more SP-D than others.

Due to ethical considerations, we did not have access to ovarian cancer tissue samples. We, therefore, decided to investigate the expression and localisation of SP-D in a tissue microarray using immunohistochemistry. There was stronger staining in serous papillary cystadenocarcinoma. Interestingly, stage II of serous papillary cystadenocarcinoma showed higher expression of SP-D compared to stage III. SP-D lower expression could lift the inhibitory actions of SP-D and therefore, enable quicker progression of cancer.

SP-D expression was also evident in endometrioid adenocarcinoma, clear cell carcinoma, necrosis tissue and fibrofatty tissue. In the literature, there are no published data on the expression of SP-D in different types of ovarian cancer. For that reason we made use of Oncomine^{TM} to investigate SP-D gene expression levels in several types of ovarian cancer. The Yoshihara dataset showed a moderate, but not significant increase of SP-D levels in ovarian serous adenocarcinoma tissues compared to normal peritoneum tissues. The TCGA dataset showed similar levels in normal and ovarian serous cystadenocarcinoma tissue samples. The Hendrix dataset compared the levels of SP-D in different types of ovarian cancer. Levels were not significantly different but, there was a trend suggesting higher gene expression levels in ovarian clear cell adenocarcinoma. Low levels were reported in endometrioid adenocarcinoma which corroborates with our immunohistochemistry data.

Finally, we investigated whether SP-D expression has an effect on ovarian cancer patient survival. Using the OvMark Kaplan-Meier Plotter, which is a resource that correlates data from transcriptomic analyses to patient survival. Overall Survival figures over 20 years did not show a significant change between the low and the high expression groups. Between 4 and 8 years there was a trend showing increased survival probability in the low expression group. Disease Free Survival figures again showed no significant change in ovarian cancer patients survival rates.

To date, there has not been a study measuring the levels of SP-A and SP-D in clinical myometrial and ovarian tissues. Further research should be done in a larger amount of samples as well as ovarian cancer samples to investigate the roles of SP-A and SP-D in preterm birth and disease.

Collectively, these data show novel interactions of SP-D with components of the mTOR pathway. SP-D downregulates the activity of the mTOR pathway and leads to an increased cleavage of caspase 3, showing that it inhibits ovarian cancer cell growth and can potentially inhibit further cancer cell progression and metastasis. Further studies should make use of genomic/proteomic approach – as a non-biased screen – that could identify novel candidates through which SP-D mediates its effects at ovarian level. More studies should focus on the phosphorylation status of key components of the mTOR pathway in the SKOV3 cell line and how SP-D affects that status. Moreover, the effects of SP-D should be further validated in

other ovarian cancer cell lines, as well as, in normal ovarian cells and primary ovarian cancer cells.

Chapter 6 General Discussion

6.1 Preterm birth

Why is this research in preterm labour important?

Preterm birth is labour prior to the 37th week of gestation (WHO, 2015), one of the leading causes of perinatal mortality and morbidity. Its rates have been increasing over the last 20 years and it poses a sentimental burden to the families involved but also a financial one to the governments (Goldenberg *et al.*, 2008). Babies which are born prematurely do not survive after the age of five, and if they do they face a life full of risks of disabilities; some of which are respiratory problems, visual and hearing impairments, and cerebral palsy (Beck *et al.*, 2010). Respiratory illnesses are one of the major problems in preterm babies which are susceptible to infections and other lung-related diseases, due to the immaturity of the lungs. Records show that the closer to term a baby is born, the higher is the probability of its survival (Costeloe *et al*., 2000). The main causes of preterm birth are not yet fully understood. It can be caused by infections, premature rapture of the membranes, preeclampsia, but those account for approximately 50% of the cases (Henderson and Macdonald, 2004). Currently, there are no definite biomarkers to predict preterm birth and no screening methods for it, mainly due to the variation and the individuality of pregnancies.

What are SP-A and SP-D, and how are they implicated in preterm labour?

SP-A and SP-D are molecules of the innate immune system. They are c-type lectins that contain collagen, called collectins. Four types have been currently identified; SP-A, SP-B, SP-C and SP-D. Their main role is to maintain surfactant homeostasis and pulmonary immunity (Kishore *et al*., 2006). SP-A and SP-D have been localised in various parts of the human reproductive tract and have been identified as potential biomarkers in proteomic studies in preterm birth (Nayak *et al.* 2012). SP-A has been involved in the initiation of labour in mice acting via IL-1β and NF-κB (Condon *et al*., 2004). SP-D has been implicated in the remodelling of the foetal membrane by interacting with decorin (Nadesalingam *et al*., 2003). In this study we showed that SP-A and SP-D can lead to an increase in myometrial cell

motility and migration. Treatments with surfactant proteins also induced the expression of CAP genes, OXTR and CX43, which can regulate myometrial contractility and tissue remodelling. The effects of surfactant proteins on the contractility machinery of myometrial cells was also supported by the fact that when ULTR cells were grown in 3D cultures and treated with surfactant proteins, they acquired a contractile phenotype.

The foetus has been characterised to be semi-allogenic and labour to be a pro-inflammatory event. It is important therefore, for a balance between pro- and anti-inflammatory molecules to be maintained for the pregnancy to be successful (Warning *et al*., 2011). Surfactant proteins can exert both pro- and anti-inflammatory effects that are tissue specific (Yadav *et al*., 2014). In this study we showed that surfactant protein treatments lead to the secretion of pro-inflammatory cytokines, such as GROα, IL-6 and IL-8, ENA-78 thus leading to a favourable environment for labour, enabling the shift from myometrial quiescence to a contractile state (Dimitriadis *et al*., 2005; Królak-Olejnik *et al*., 2006; Montalbano *et al*., 2013; Rajagopal *et al*., 2015). Cytokines secreted in validated *in vitro* myometrial model (IL-8, ENA-78) also play a role in tissue reconditioning and angiogenesis which supports their role in contractile mechanisms. This study investigated the gene expression levels of surfactant proteins in a large cohort of myometrial tissue clinical samples from women that gave birth at term or preterm, labour or non-labour. Although gene expression was similar, we do not know how much is actually secreted in circulation from the same patients due to interpatient variation.

Why is the interaction of surfactant proteins and steroid hormones important?

Due to the complexity of pregnancy and parturition, there needs to be an orchestrated cooperation of several components of the reproductive system (Walsh *et al*., 1984). Steroid hormones play a key role in pregnancy maintenance and parturition. Oestradiol can regulate CAP gene expression and the prostaglandin pathway (Niswender *et al*., 2000). In this study we showed that oestradiol can induce SP-A expression. Progesterone is expressed in the reproductive tissues throughout pregnancy and has been implicated in the timing of labour. In this study we showed that progesterone treatments led to an increase in the expression of both SP-A and SP-D.

Towards term there is a clear progesterone withdrawal in rodents but this is not observed in humans (Condon *et al*., 2003). It has been suggested that in humans, a functional progesterone withdrawal must occur for labour to proceed. Previous data has implicated an alternative family of PRs, termed membrane PRs (mPRs) in this process (Karteris *et al*., 2006). In this study we showed that surfactant protein treatments led to an increase in the expression of mPRα and mPRβ *in vitro*. Due to this novel data, we proposed a model for the transition of the myometrium from a quiescent state to a contractile one, involving progesterone, surfactant proteins, mPRs and PRB. During the initial stages of pregnancy, P4 and surfactant proteins are expressed at low levels. Towards the end of term P4 and surfactant protein levels increase dramatically. We propose that P4 leads to increased expression of surfactant proteins which in turn induces the expression of mPRα. We have also shown that, surfactant proteins lead to the expression of OXTR and CX43 and pro-inflammatory cytokines. All these factors create an environment that facilitates the shift from a quiescent to a contractile state in the myometrium.

Why is the study on OD-02 important?

Progesterone can bind to PRA and PRB and exert its genomic functions, and also to mRPs to exert its non-genomic effects (Conneely *et al*., 2002). A lot of research has been done on the interaction of progesterone and PRs, but only in the recent years researchers focused on mPRs. In this study we showed that the myometrium expresses mPRα and mPRβ *in vitro*. OD-02 is a mechanistic analog of progesterone that binds to mPRs, but does not bind to PRA and PRB (Kelder *et al*., 2010; Zachariades *et al*., 2012). Since there is still a controversy as to the exact role on mPRs in the myometrium, we decided to investigate its functions using transcriptomics as a non-biased screen approach. Therefore, we investigated further non-genomic effects of progesterone mediated exclusively via mPRs. The microarray analysis showed a plethora of genes whose expression was altered due to OD-02 treatments. Some of those genes are members of pathways that are important for pregnancy and parturition, such as the MAPK, PI3K-Akt, Calcium, Jak-STAT, adherens' junction and actin cytoskeleton. The MAPK pathway regulates cell proliferation and differentiation which is important during pregnancy. PI3K-Akt pathway is responsible for transcription and translation and plays an important role during the quiescent stage of pregnancy. The calcium pathway is especially important during pregnancy and parturition as it regulates an array of cellular functions that affect cell

proliferation, muscle contraction and reconditioning. The latter three pathways all regulate important cell functions and also interact with the previously mentioned pathways, making them equally important for pregnancy. Some of the genes that were altered that are members of these pathways were: interleukins 2 and 10 that have an immune role clearing pathogens, adducing 3 and caldesmon 1 that interact with actin and regulate muscle contraction, and guanine nucleotide binding protein gamma 11 and leptin receptor that are expressed in myometrial cells and play a role in pregnancy (Matsuola *et al*., 2000; Demokan *et al*., 2003; Thaxton and Sharma, 2010; Fainboim and Arruvito, 2011; Kim *et al*., 2012; Herrid *et al*., 2014).

After identifying these novel signalling pathways, we investigated further the functional effects of OD-02. Cell motility was increased in cells treated with OD-02 and also, there was a moderate increase in cell proliferation. This is an important effect of progesterone acting via mPRs to potentially regulate hypertrophy, hyperplasia and reconditioning of the myometrium.

The above data combined shows the important roles surfactant proteins play during pregnancy and parturition apart from protecting both the foetus and the mother from infections. Their interaction with steroid hormones is also important due to the role progesterone and oestradiol play during pregnancy and the receptors they bind to.

What are the limitations of this research?

To our knowledge, this study is the first that used a large cohort of myometrial clinical samples to study the expression of surfactant proteins in preterm birth. It is evident that there is interpatient variation between the groups. Samples were taken from individual deliveries and surfactant protein levels are unlikely to be identical to other samples in the same group. Moreover, the samples were from a very homogeneous population. A larger number of samples from different ethnicities and background would therefore, give more accurate results.

Due to ethical considerations we were not able to acquire primary myometrial cell lines for this study so the effects that we witness *in vitro* might differ from cells obtained directly from patients in many ways, since there is cross-talk with neighbouring tissues and organs.

The KEGG database, although a very useful tool, was our only bioinformatics software for the transcriptomic analysis. Gene alterations acquired from the microarray data were not validated by qPCR.

What could be done as future work?

This study used the myometrial cell line ULTR as an *in vitro* model to study preterm labour. Although this cell line is well characterised and used to study several effects of pregnancy and preterm birth, a useful addition would be to use further myometrial cell lines including primary cells, to validate the effects seen in ULTR cells.

In this study we used microarray analysis to study the non-genomic effects of progesterone using OD-02. We identified a large number of genes whose expression was altered due to OD-02 and mPR signalling. Microarray data need to be validated using qPCR. We used a cytokine microarray to identify secreted molecules due to surfactant protein treatments and whether those are pro- or anti-inflammatory. It would be interesting to use microarray analysis for SP-A and SP-D and study genes that they interact with that are important for pregnancy and parturition. Future experiments should include proteomics and metabolomics studies which could lead to identifying novel interactions of surfactant proteins that would enable more targeted studies in the future.

For the purpose of this research, we used recombinant fragments of SP-A and SP-D, rhSP-A and rhSP-D respectively. It would therefore be important to use full length molecules to repeat these experiments.

This study had access to 40 myometrial tissue samples from women that gave birth at term (n=20) and preterm (n=20). It is necessary for more clinical samples from several backgrounds and ethnicities to be analysed, in order to identify true surfactant protein expression levels in myometrial tissues. Similarities or trends seen in our cohort, should be expanded as this could lead to using surfactant proteins as potential preterm birth biomarkers.

Proteomics studies using blood, saliva and cervicovaginal fluid over the last 40 years have failed to identify specific molecules to be used as biomarkers that could be used for all pregnancies. It is highly unlikely that a single molecule can be used as a biomarker to predict

preterm birth. A combination of multiple ones could lead to accurate prediction of preterm labour. Surfactant proteins have already been identified as potential biomarkers in some studies. As many factors can predict preterm birth, patients from a wide range of backgrounds and ethnicities should be included. Future studies should make use of bodily fluids to identify novel biomarkers and assess the validity of surfactant proteins, in combination with other biomarkers such as the human chorionic gonadotrophin (hGCP).

6.2 Ovarian cancer

Why is this research in ovarian cancer important?

Ovarian cancer is one of the most common types of cancer in women. It has been characterised as a 'silent killer' because it is one of the hardest types of cancer to diagnose and has high mortality rates. Each year approximately seven thousand women are diagnosed with ovarian cancer. In early stages it is asymptomatic or the symptoms are misdiagnosed as they resemble those of other diseases (Rogers-Broadway *et al*., 2016). To date, there is not a successful screening method for this type of cancer, or at least reliable biomarkers that can be used for its prognosis. Transvaginal ultrasound is the method used once there is suspicion of ovarian cancer, but that usually happens at later stages of the cancer progression, stages III or IV. Conventional methods of treatment, such as surgery and chemotherapy, can improve survival rates but a cure has not been developed yet. There is a need therefore, for an effective screening method and biomarkers with high specificity to enable detection of all cancer cell types at all stages. Stage I patients have increased survival rate, which shows how crucial and beneficial earlier diagnosis is.

How is surfactant protein D implicated in ovarian cancer?

It has been reported that cancer cells are affected by the inflammatory environment which can have an impact on tumour progression and metastasis (Brigati *et al*., 2002). Since surfactant proteins' primary production site is the lungs, many studies have focused in several types of lung cancer. SP-D has been suggested to identify with EGFR and downregulate the EGF pathway and have cytostatic and cytotoxic effects in the A549 lung cancer cell line (Hasegawa

et al., 2015). Mutations in the SP-D gene have been associated with increased rates of lung cancer, and higher expression levels with decreased cancer progression (Lin *et al.,* 2007, Ishii *et al.,* 2012). SP-D has also been characterised as a potential biomarker for chronic obstructive pulmonary disease and for lung cancer incidence (Sin *et al.,* 2007).

To date, there is little data on the effects of SP-D in ovarian cancer. In this study we demonstrated that SP-D is expressed in the ovarian cancer cell line SKOV3 which was used as our *in vitro* model. A previous study showed that secretions from normal tissues (ovarian, myometrial and peritoneal) affects the expression of MMPs and their inhibitors which regulates cancer cell invasion (Kikkawa *et al*., 1998). This study used conditioned media from ULTR cells treated with surfactant protein D and incubated SKOV3 cells. There was a 40-50% decrease in live cell numbers after 48 hours. When SP-D was used to treat SKOV3 cells, there was a moderate decrease in treated cells at 24 hours. Functional studies, including wound healing and live cell imaging, showed that there was a cytostatic effect of SP-D, which corroborates with effects seen in lung cancer.

In this study, for the first time, we showed the expression and localisation of SP-D in several ovarian cancer tissues using a tissue array including serous papillary cystadenocarcinoma, endometrioid adenocarcinoma and clear cell carcinoma. OvMark Kaplan-Meier plots showed that there was not a significant increase in overall or disease free survival rates of ovarian cancer patients, but there was a trend showing moderate increased survival rates in the low expression group between 4-10 years.

What is the role of the mTOR pathway in ovarian cancer?

The mTOR pathway regulates several cellular functions such as protein synthesis, growth, and differentiation of both normal and cancer cells. mTOR acts via mTOR complexes 1 and 2. mTOR is downstream of the PI3K/Akt pathway which is found to be active in higher stage tumours (Gentilella *et al*., 2015). It is activated in cancer cell lines that carry a p53 mutation and deregulated in *in vitro* and *in vivo* studies in ovarian cancer. Inhibitors of the Akt pathway or mTOR increase sensitivity to various drugs such as paclitaxel and have an anti-proliferative effect (Perl, 2015). To date, there is no data regarding any interaction between the mTOR pathway and SP-D. When SKOV3 cells were treated with SP-D, mTOR expression levels

were not affected but DEPTOR levels were increased. DEPTOR is a component of both mTOR complexes and can regulate mTOR activity acting as an inhibitor (Peterson *et al*., 2009). Raptor which is a component of mTOR complex 1 (Wang *et al*., 2012), was downregulated after treatment with SP-D. A similar effect was seen on the expression levels of Rictor, a component of mTORC 2 (Beauchamp and Platanias, 2013). Based on this study, since SP-D downregulated the expression of Rictor and Raptor and upregulated the expression of DEPTOR, it is attractive to speculate that the cytotoxic and cytostatic effects of SP-D seen in our previous experiments are a result of the downregulation of the mTOR pathway. We also report that SP-D treatments led to a decrease in the phosphorylation of S6K at 48 hours, which is a downstream kinase used as a measure of mTOR activity (Schalm and Blenis, 2002). SP-D did not affect cleavage of caspase 9 but there was an increase in caspase 3 cleavage after 48 hours, which suggests that prolonged exposure to SP-D can exert cytotoxic as well as cytostatic effects.

What are the limitations of this study?

In this study we used the ovarian cancer cell line SKOV3 as our *in vitro* model. Although this cell line has been previously used to study ovarian cancer, it would be important to use other cell lines that have originated from different types of ovarian cancer and study the cytotoxic and cytostatic effects of SP-D witnessed in this study. Due to ethical considerations, we did not have access to primary ovarian cancer tissues or blood samples. The *in silico* analysis showed high interpatient variation which could be attributed to the heterogeneity of cancer tumours. Research has shown that cancer cells of the same origin can be genetically and phenotypically distinct (Shalek *et al*., 2013). This can also be the case with cell lines, including SKOV3 (Bai *et al*., 2015). The ovarian cancer tissue array used in this study consisted of a small amount of samples and also the staining of the cells does not necessarily correlate to true expression in those tissues as DAB staining does not adhere to the Beer-Lambert law, which means that staining is not directly proportional to protein expression/concentration (van der Loos, 2008).

What could be done as future work?

Future experiments should focus on increasing the number of clinical samples, both tissue and blood. There are differences in the expression of mTOR, DEPTOR, Rictor and Raptor reported between stages, grades and types of ovarian cancer (Foster *et al*., 2010; Rogers-Broadway *et al*., 2016). It would therefore be important to study expression of SP-D in all these subtypes. For the purpose of this research we used the recombinant fragment of SP-D, rhSP-D. It would be of great interest to study the effects of the full length molecule to identify what effects are attributed to which part of surfactant protein D. It would be of interest to repeat these experiments to investigate the effects of SP-A in ovarian cancer.

For this study we used the adenocarcinoma derived cell line as our *in vitro* model. It would be useful to study other cell lines and see how SP-D would act under similar conditions. A normal ovarian cell line, such as the IOSE (immortalised ovarian surface epithelial) cell line, would be a great model to compare effects under normal and disease conditions.

SP-D exerted cytotoxic and cytostatic effects on SKOV3 cells and was documented to interact with the mTOR pathway. Further research should be done on molecules that regulate cell cycle progression, such as cyclin D and its kinase which regulate G2/M transition (Porter and Donoghue, 2003), and proliferating cell nuclear antigen (PCNA) which regulates G1/S transition (Stewart and Dell'orco, 1992). It would be useful to study the effect of SP-D on the phosphorylation status of mTOR and DEPTOR and how that impacts mTORC2 activity. There are a few mTOR pathway inhibitors currently tested in clinical trials such as the rapalogues; rapamycin, everolimus, deforolimus and temsirolimus, for ovarian cancer treatment. Future work should test the combination of rapalogues with SP-D and their ability to treat ovarian cancer.

Chapter 7 Bibliography

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Chapter 8 Appendix

8.1 ULTRxOD-02 Microarray data

Table 8.1 List of genes with altered expression due to treatment of ULTR cells with OD-02. Table includes the gene sequence ID number, the fold change in gene expression due to treatment and the *p* value to show statistical significance.

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_015305	0.798012674	0.0000114	NM_014712	0.751807332	0.0252
BC113845	0.770567954	0.0000151	XM_933480	0.7328251	0.0252
BC014067	0.757963717	0.0000566	NM_015931	0.616538584	0.0252
BC038301	0.612772703	0.0000861	AK093161	0.450853258	0.0252
NM_207333	0.476610541	0.000105	NM_032043	2.047175407	0.0253
XM_932271	0.569936872	0.000185	BC112367	1.339421749	0.0253
NM_024743	1.278391004	0.000226	Y10529	0.603276908	0.0253
NM_001014373	1.535208941	0.000245	BC114375	0.563227117	0.0253
NM_005908	2.265727282	0.00025	NM_006313	1.9104352	0.0254
BC074764	0.529768527	0.000294	BC060815	1.488007784	0.0254
NM_024575	0.567497194	0.00033	NM_024997	1.25363481	0.0254
NM_001039580	1.39289093	0.00035	XM_927498	0.688683391	0.0254
BX648919	1.781362176	0.000403	NM_001017967	0.603141427	0.0254
XM_372423	0.680273533	0.000423	NM_000591	0.6595276	0.0255
NM_031966	1.083644509	0.000462	NM_001025096	1.323314667	0.0256
NM_052889	2.367292643	0.000472	BC000819	1.323194027	0.0256
AK123565	0.745150387	0.000497	NM_017599	1.099425793	0.0256
AK000366	0.706303537	0.000504	NM_152251	0.718572855	0.0256
BC020514	2.167798042	0.000515	NM_152448	0.592545867	0.0256
NM_173657	1.360280871	0.000518	XM_294370	0.49594599	0.0256
NM_001004325	0.469982147	0.000535	NM_001039067	0.462405413	0.0256
NM_213598	1.536166787	0.000557	NM_181782	2.214885235	0.0257
AK131464	0.471604794	0.000612	NM_138729	1.881066322	0.0257
XM_929354	1.437391043	0.000638	NM_014028	1.720966339	0.0257
XM_932024	0.597854018	0.000652	BC101487	1.630846262	0.0257
NM_207443	0.548594236	0.000689	NM_004872	1.374628663	0.0257
NM_001005412	0.74040395	0.000769	NM_001037231	0.787438393	0.0257
BC073769	0.876748502	0.000782	AF289615	0.641411006	0.0257
NM_004828	0.534888923	0.000797	NM_152336	0.501683116	0.0257
XM_496546	1.572036028	0.000799	AY327595	1.931970596	0.0258
NM_001029999	0.804379821	0.000824	NM_006855	1.299055219	0.0258

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_004248	0.554140329	0.000844	XM_928994	0.65277946	0.0258
XM_933097	1.676128983	0.000854	AK022213	0.509918272	0.0258
XM_929356	0.639320493	0.000875	XM_929422	0.444658607	0.0258
XM_926759	0.584207833	0.000875	U47007	1.304358602	0.0259
BC022068	1.313298106	0.000907	BC000317	0.863479435	0.0259
NM_014571	0.661931753	0.000934	XM_929423	0.721332788	0.0259
NM_001013743	0.716957211	0.000944	XM_928965	0.666887999	0.0259
BC093706	0.574938357	0.000944	XM_496037	0.388119638	0.0259
AK056025	1.832048535	0.000975	NM_006868	1.454490781	0.026
NM_152386	1.483264446	0.000997	BC107750	1.232765198	0.026
BC056235	0.709983408	0.000999	NM_000835	0.602415204	0.0261
CR615710	1.565796018	0.00103	NM_015063	0.528708756	0.0261
CR594752	1.450679541	0.00108	BC000183	3.701253176	0.0262
BC021104	1.70155561	0.00111	BX647726	0.79152894	0.0262
AF161549	0.637169302	0.00117	AK131328	0.68845439	0.0262
XM_295062	1.308122516	0.00119	NM_002515	2.766606092	0.0263
BC111964	0.432169914	0.00122	BC009291	2.441901922	0.0263
AF090933	1.847724676	0.00124	U17278	1.596938491	0.0263
NM_016945	0.809313118	0.00125	NM_017846	1.302075863	0.0263
XM_170840	0.62272501	0.00126	NM_148968	0.663156927	0.0263
NM_001238	1.512014151	0.0013	NM_015424	0.64982146	0.0263
BC065928	1.417430043	0.00133	NM_001037666	0.590464234	0.0263
BC073765	0.459297925	0.0014	XM_932825	0.578024626	0.0263
NM_178349	0.601431489	0.00141	NM_024787	1.908153772	0.0264
BC058939	0.667161345	0.00144	NM_052966	1.806342602	0.0264
NM_002171	0.507725418	0.00146	NM_004856	1.252623081	0.0264
NM_025136	0.678872585	0.00148	NM_017763	0.898379326	0.0264
NM_000395	0.607796669	0.00148	BC027895	0.710117102	0.0264
XM_932719	0.273455679	0.00148	NM_001039907	0.692469478	0.0264
NM_012327	1.966504335	0.00153	AK131288	0.574307203	0.0264
NM_152694	1.855041265	0.00153	XM_497837	0.537898183	0.0264
NM_017820	0.522369683	0.00154	XM_371933	2.550323009	0.0265
AK125976	0.497943372	0.00159	AK091420	1.504264712	0.0265
NM_203281	0.387156665	0.0016	NM_001039772	1.335159063	0.0265
AB024691	1.477844	0.00163	NM_001004760	0.904617429	0.0265
BC104649	0.446073711	0.00168	AK124224	0.467911392	0.0265
XM_497639	0.646089494	0.0017	NM_006880	2.235740662	0.0266
NM_016376	1.376217246	0.00172	NM_002759	1.345374942	0.0266
AY704142	1.951168895	0.00177	NM_003264	1.292830348	0.0266
NM_014400	0.681359053	0.0018	BC032007	0.762923717	0.0266

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
AJ437318	0.651422918	0.00185	XM_063287	1.413915753	0.0267
NM_003596	1.179833651	0.00187	NM_001005168	1.16707325	0.0267
NM_018439	2.330361605	0.00188	CR456537	0.762832463	0.0267
NM_001009880	2.326977253	0.0019	XM_933551	0.701786995	0.0267
BC011034	1.761694312	0.0019	NM_138336	0.560578525	0.0267
BC047592	0.503230035	0.00199	AB209917	0.551288784	0.0267
NM_207337	1.738291264	0.00209	AL832683	3.226864338	0.0268
AL833283	0.735055327	0.0021	NM_004570	1.525372028	0.0268
NM_001004473	0.779419601	0.00214	XM_929566	1.147940874	0.0268
NM_173598	0.608675361	0.00214	XM_929001	0.462346613	0.0268
AK090886	1.305651069	0.00217	BC012304	1.267166257	0.0269
XM_933350	0.423974633	0.00219	AK125560	0.557872534	0.0269
NM_207325	1.891496062	0.00224	BC093795	0.887239516	0.027
XM_932824	0.473843008	0.00224	NM_178456	1.878512621	0.0271
AK054645	0.659020066	0.00225	AF286592	1.489559889	0.0271
NM_014309	1.161786795	0.00227	L01420	0.769100189	0.0271
XM_929188	1.303202748	0.0023	BC024010	0.723137379	0.0271
NM_152772	1.369786739	0.00234	NM_001040	0.696489573	0.0271
NM_001007464	1.162104249	0.00235	BC034992	2.167893171	0.0272
BC104752	0.545146763	0.00238	BC036080	1.896635652	0.0272
AK130578	0.337951571	0.00238	NM_015087	1.409670711	0.0272
AK024672	0.661055982	0.00239	AK124059	0.851617992	0.0272
XM_927485	0.640685201	0.00239	NM_001004064	0.696112931	0.0272
NM_001009894	3.107098341	0.00246	NM_032287	1.82343936	0.0273
BC064518	0.835629642	0.00246	NM_052846	1.639583826	0.0273
NM_207286	0.739385426	0.00248	NM_033640	1.245448709	0.0273
AK056753	1.5787673	0.00249	NM_001001691	0.758297205	0.0273
NM_001003679	3.554828882	0.00251	XM_932167	0.738467813	0.0273
NM_003460	1.427113056	0.00255	AK021957	0.703544378	0.0273
AK097325	0.695070386	0.00259	XM_935435	0.692706704	0.0273
NM_004770	0.504775047	0.00265	NM_181614	0.336791903	0.0273
AK056520	0.41604802	0.00266	AK057540	0.750023663	0.0274
NM_001004352	1.394597054	0.00269	XM_042936	0.709335029	0.0274
BC109197	0.580662072	0.00271	AF221846	0.529581189	0.0274
NM_001007098	1.170911431	0.00277	XM_927384	0.489425242	0.0274
NM_015481	1.335340619	0.00281	AL137703	0.470858306	0.0274
NM_181671	1.128564715	0.00285	NM_000313	2.63543272	0.0275
NM_004543	0.697574496	0.00285	NM_000153	2.298094511	0.0275
NM_006496	1.388846636	0.0029	NM_020845	1.900291324	0.0275
XM_934799	0.729516327	0.00291	NM_173060	1.535816193	0.0275

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_018037	1.602784395	0.00293	AK127692	0.514768422	0.0275
NM_002550	0.493044734	0.00293	AY437879	3.00560689	0.0276
NM_207408	1.429032445	0.00295	BC042496	1.258529067	0.0276
BC113118	1.350667953	0.00295	XM_933935	1.558189869	0.0277
NM_020336	1.78829968	0.00297	AK095136	0.717597187	0.0277
NM_002100	0.821597338	0.00298	AY700780	0.709436357	0.0277
BC112126	0.509266675	0.00298	BC111008	1.818354487	0.0278
DQ063579	2.13609767	0.00299	NM_022842	1.621147156	0.0278
XM_931704	0.47361356	0.00299	NM_022766	1.424822211	0.0278
BC037288	0.66667974	0.003	NM_001291	0.79233855	0.0278
NM_004626	0.457003832	0.00307	NM_178424	0.782353103	0.0278
NM_001017534	2.771005869	0.00309	NM_080830	0.740631044	0.0278
BX647638	2.14347434	0.00309	AK026675	0.601424456	0.0278
NM_006393	0.689476669	0.0031	BC062320	0.513921976	0.0278
NM_006725	0.736985624	0.00311	BC110084	1.694863081	0.0279
BC074912	0.562360525	0.00311	XM_931767	1.335074067	0.0279
BC020962	0.510468304	0.00311	NM_003890	1.766495347	0.028
NM_181717	0.562161088	0.00313	J00146	1.488349319	0.028
NM_199077	0.750696063	0.00314	NM_152382	0.64134419	0.028
NM_004217	1.100146413	0.00319	NM_002700	0.593776524	0.028
BC027920	0.833186269	0.00321	BC030539	1.835161924	0.0281
NM_001033604	1.19967556	0.00322	NM_198998	0.81425643	0.0281
BC071890	0.751012921	0.00324	BC014400	0.595251799	0.0281
AY168775	1.430893183	0.00325	NM_198568	0.472149968	0.0281
BC066254	1.54479754	0.00326	NM_138333	1.726750851	0.0282
XM_372097	1.817458391	0.00329	BC042884	1.259053111	0.0282
BC109269	1.387450695	0.0033	NM_079425	1.144979358	0.0282
BC075018	0.691063523	0.0033	NM_207426	0.671102762	0.0282
NM_004788	2.25492835	0.00334	BC033789	0.662239969	0.0282
XM_928694	0.563040972	0.00336	BC063705	0.562038898	0.0282
XM_067605	1.194000483	0.00339	XM_371722	0.461016685	0.0282
NM_006539	0.776086509	0.0034	NM_031293	1.427023888	0.0283
NM_144637	0.74780643	0.00344	NM_001926	0.759211004	0.0283
NM_012168	1.198446274	0.00346	NM_004178	0.687385201	0.0283
AY484516	0.589064896	0.00346	NM_139164	1.417890429	0.0284
XM_926750	0.589512467	0.00348	NM_005164	1.37710464	0.0284
NM_002501	2.053118229	0.00349	NM_197955	1.230679393	0.0284
AF193057	0.823249996	0.0035	BC047064	0.524133742	0.0284
XM_927009	2.620391846	0.00352	NM_030905	0.492738247	0.0284
NM_172313	1.303604007	0.00354	NM_080791	0.419358611	0.0284

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
AK097459	0.407746822	0.00355	NM_033633	1.968141913	0.0285
XM_925874	0.695744097	0.00356	NM_183374	1.766413212	0.0285
NM_198511	0.551976681	0.00359	BC001401	1.100018978	0.0285
NM_006235	0.70887816	0.0036	NM_001009992	0.856766105	0.0285
BC035978	0.671212614	0.00366	BC004205	0.822513998	0.0285
NM_012450	0.565640569	0.00368	BC026192	0.555245996	0.0285
BC067345	0.669453681	0.00369	BC017199	0.55233568	0.0285
NM_002581	1.711485863	0.00379	M34057	2.137239933	0.0286
NM_001008661	1.568284631	0.00379	NM_000051	1.642967224	0.0286
XM_499183	0.661465704	0.0038	BC067296	1.488996983	0.0286
NM_014772	2.753846884	0.00383	AJ275973	1.375628829	0.0286
AK172833	0.716037989	0.00386	NM_004950	0.731730819	0.0286
NM_013387	3.158229828	0.00387	BC039117	1.705546975	0.0287
BC012299	2.243537188	0.00388	NM_001010931	0.729228318	0.0287
NM_183050	1.660816908	0.0039	BC109091	0.684086442	0.0287
NM_031907	0.465933532	0.00392	AF130061	1.438932538	0.0288
BC059370	1.767730951	0.00393	NM_152484	1.224845052	0.0288
NM_000371	0.534853637	0.00393	NM_000777	0.767536938	0.0288
XM_926711	1.138987303	0.00396	AK075493	0.730530739	0.0288
NM_001010898	2.48037982	0.00398	NM_001006604	1.598528981	0.0289
NM_033213	1.280563474	0.00398	NM_001005472	1.147984147	0.0289
BC036554	1.29524076	0.00402	BC107099	0.909027994	0.0289
XM_930291	0.901714206	0.00402	NM_003357	0.355067194	0.0289
BC105956	1.536227942	0.00406	DQ139833	3.285107613	0.029
NM_001010979	1.384893298	0.00406	NM_004284	1.369692206	0.029
XM_497545	0.609861135	0.00406	BC001241	1.258958817	0.029
BX648976	1.846256733	0.00412	NM_014765	1.196649194	0.029
NM_013286	1.369172215	0.00412	NM_017714	0.772844911	0.029
BC006510	1.155144334	0.00412	BC001703	0.741770148	0.029
XM_931560	0.524522483	0.00412	NM_001005329	0.547581673	0.029
NM_178013	0.833550811	0.00413	BC011804	0.522437274	0.029
Z22551	1.560041904	0.00414	XM_927654	1.3785851	0.0291
NM_001004733	1.524046063	0.00418	XM_933381	0.667862833	0.0291
NM_144661	0.616801739	0.00422	NM_005867	0.564811289	0.0291
AF289589	0.714631498	0.00424	XM_927282	0.498937815	0.0291
XM_927557	1.26104176	0.00429	BC028101	0.446495622	0.0291
NM_178460	0.653900683	0.00434	BC016633	1.475805879	0.0292
NM_001004128	0.852949142	0.00435	BC034422	1.394114733	0.0292
NM_080739	1.357063532	0.00436	NM_014662	0.795229733	0.0292
AK129938	0.760057569	0.00444	XM_929962	0.675873399	0.0292

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
BC104436	1.633701682	0.00446	NM_003049	0.643279195	0.0292
NM_032958	0.843894124	0.00448	XM_934331	0.782223046	0.0293
BC093748	0.576624632	0.00451	XM_932198	0.562893569	0.0293
NM_013276	1.800519705	0.00454	XM_934667	0.559230804	0.0293
BC073794	0.645658553	0.00454	NM_001194	0.424321264	0.0293
NM_006378	1.699118495	0.00455	NM_004838	0.628298819	0.0294
AB163438	1.522236347	0.00457	NM_001037535	1.864798307	0.0295
NM_015336	1.864455819	0.00462	XM_931879	0.828762829	0.0295
NM_194449	1.622721791	0.00462	XM_932351	0.698231876	0.0295
XM_927945	0.658753157	0.00462	NM_152346	0.437408984	0.0295
NM_014520	0.844789684	0.00464	NM_024003	1.346278667	0.0296
XM_374379	0.482017845	0.00467	XM_935034	1.250548482	0.0296
NM_007276	1.332081795	0.00468	BC023234	1.166607261	0.0296
NM_175057	0.711279809	0.00469	AF118062	0.630742252	0.0296
NM_001009614	0.6640746	0.0047	BX648927	0.63015908	0.0296
NM_206883	1.36771822	0.00471	NM_017893	0.483342171	0.0296
AL080197	0.460381657	0.00474	AB046787	1.678721905	0.0297
M29644	0.593365371	0.00475	NM_014373	1.385337591	0.0297
NM_203282	1.501268625	0.00479	CR622376	0.827583075	0.0297
BC093749	0.706472337	0.00481	BC027865	0.542287469	0.0297
AK026773	0.685484588	0.00482	XM_931288	0.511937976	0.0297
AK001019	0.603436232	0.00484	NM_017694	1.698338628	0.0298
NM_021176	0.572615981	0.00484	NM_018418	1.663986802	0.0298
NM_144724	0.576491237	0.00493	AK124161	1.592813849	0.0298
BC023546	0.712089479	0.00494	XM_929357	0.763793707	0.0298
NM_024327	1.525635481	0.00496	AK124375	0.643274248	0.0298
NM_021603	0.429059595	0.00496	NM_001039717	1.619980931	0.0299
BC032520	1.312827826	0.00497	NM_001004127	1.446948767	0.0299
XM_928013	0.678769588	0.00501	AK125000	0.722101271	0.0299
BC012392	1.295639634	0.00502	NM_001004753	0.565685391	0.0299
BC020712	0.648378372	0.00503	NM_017639	0.55331403	0.0299
XM_928642	0.458162487	0.00503	BC017279	0.537012875	0.0299
XM_929610	0.40982601	0.00504	NM_018843	1.689910531	0.03
XM_931998	0.568643093	0.00509	AY847301	1.554467559	0.03
AK124170	0.436834097	0.0051	NM_000823	0.749222994	0.03
NM_001005471	0.486723185	0.00514	AK095107	0.530332386	0.03
NM_213605	0.473210216	0.00516	BC041666	0.522657573	0.03
NM_001024457	1.345759273	0.00519	NM_018140	1.888177514	0.0301
NM_001000	1.26431334	0.00521	NM_138288	1.610766649	0.0301
NM_002341	0.730887055	0.00523	BC000094	0.809297204	0.0301

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
BC047523	1.482849717	0.00524	AB042648	0.773779035	0.0301
XM_932032	0.644129753	0.00524	NM_001033551	0.747345984	0.0301
BC041961	0.610654712	0.00527	NM_032471	0.717065096	0.0301
NM_024920	2.376277447	0.0053	BC094004	0.471870542	0.0301
NM_012162	0.826683462	0.0053	NM_144633	1.288406253	0.0302
BC036083	1.676802397	0.00532	BC008718	1.191488266	0.0302
NM_023036	1.224511266	0.00533	NM_031911	0.610727429	0.0302
NM_173801	0.700148225	0.00533	XM_372239	0.520111144	0.0302
NM_006965	1.684354901	0.00534	NM_001025079	2.433493853	0.0303
AK056756	1.267614365	0.0054	U89329	1.74472487	0.0303
NM_173687	0.562968373	0.00543	BC053665	1.311112404	0.0303
NM_014300	1.322379827	0.00544	NM_013373	0.747970045	0.0303
AL833593	1.561557174	0.00548	AK097542	0.71402967	0.0303
DQ059646	0.635642111	0.00549	NM_004037	0.663160622	0.0303
BC021825	1.335222125	0.00554	NM_033427	0.755916178	0.0304
NM_021831	0.804782629	0.00559	BC063654	0.626881421	0.0304
NM_025009	1.708960652	0.0056	NM_012074	0.604572654	0.0304
NM_015383	0.923929214	0.00561	NM_030765	0.451972127	0.0304
XM_293352	0.615135074	0.00562	BC047469	1.368883491	0.0305
NM_152681	1.669116378	0.00566	BC070338	1.365065455	0.0305
BC112271	0.638272226	0.00566	NM_198720	0.724821925	0.0305
NM_152243	0.734763682	0.00569	BC074787	0.699965835	0.0305
NM_001001677	0.641786993	0.00573	NM_020682	2.105424881	0.0306
NM_001005243	0.658167064	0.0058	NM_015358	1.612194896	0.0306
NM_182398	1.455515981	0.00582	XM_497865	0.494477034	0.0306
XM_926850	1.631931424	0.00583	NM_001938	2.006769419	0.0307
AB016902	0.381225646	0.00584	XM_496217	1.778174758	0.0307
NM_018240	0.672856629	0.00586	BC007750	1.498910189	0.0307
NM_004341	0.776933134	0.00588	NM_020844	1.46144104	0.0307
AB007923	2.166883945	0.00592	NM_002076	1.356028318	0.0307
BC020718	1.765597224	0.00592	NM_024539	0.822248518	0.0307
XM_372255	1.5757581	0.00592	BC036354	0.752850115	0.0307
BC017329	0.749129951	0.00594	AK090617	0.74161154	0.0307
NM_001025252	2.50575757	0.00595	NM_000475	0.544154525	0.0307
BX649037	1.899043918	0.00595	BC023586	0.527943671	0.0307
NM_014246	1.411416173	0.00598	AK127277	2.233515978	0.0308
BC031589	1.914697409	0.00601	BC034432	1.818484306	0.0308
AY422168	0.590133667	0.00602	NM_000330	1.446885347	0.0308
NM_001010913	0.436056197	0.00603	XM_499190	0.718594074	0.0308
NM_005669	1.882704735	0.00604	AY696295	0.609353006	0.0308

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
D88587	1.606590509	0.00612	XM_933766	0.54850471	0.0308
BC003194	0.583135247	0.00613	XM_926554	0.424512357	0.0308
AK130404	0.637104154	0.00616	XM_928211	1.621496916	0.0309
NM_022918	1.917273164	0.0062	NM_133496	1.404192924	0.0309
BC009073	0.638508618	0.00623	NM_212559	0.648961067	0.0309
BC035598	1.316834092	0.00628	NM_015503	0.551307499	0.0309
BC021178	0.4704445	0.00629	BC041374	1.194727302	0.031
AL834515	0.702975631	0.0063	XM_048362	0.810351968	0.031
BC074920	2.750873327	0.00632	XM_373075	0.617009044	0.031
NM_014252	1.595537066	0.00635	NM_033222	1.855443954	0.0311
NM_152913	1.529871225	0.00636	NM_005428	1.426259875	0.0311
M64445	1.401875734	0.00643	XM_059341	0.701829672	0.0311
NM_004229	2.894977808	0.00646	NM_018088	0.621122599	0.0311
NM_001972	0.749328673	0.00653	NM_003140	0.604894042	0.0311
NM_057091	0.497949839	0.00655	BC035288	0.575219512	0.0311
AK123892	1.360542059	0.00656	BC033777	1.770299196	0.0312
BC105083	0.339198261	0.00659	BC040849	1.239411473	0.0312
NM_032135	1.314498425	0.00663	BC067736	0.743812501	0.0312
XM_927974	0.656345546	0.00665	NM_018287	1.590160012	0.0313
NM_182700	1.347593188	0.00671	AK130753	0.686527312	0.0313
XM_929379	0.47107181	0.00672	NM_018344	0.676248789	0.0313
BC047741	0.808268487	0.00678	BC012102	0.656924784	0.0313
NM_001004735	0.523993731	0.00679	AB231740	0.565373242	0.0313
XM_926131	0.859600425	0.0068	Y15718	1.744755983	0.0314
BC000844	0.693239868	0.0068	BX647096	1.693044782	0.0314
AK128158	0.68292737	0.00682	D21255	1.423177361	0.0314
XM_929912	0.901952386	0.00685	NM_014787	1.317754984	0.0314
XM_293225	1.246551037	0.00686	NM_022468	0.796524823	0.0314
BC107704	2.503301382	0.00691	NM_001039764	0.537539184	0.0314
NM_173583	1.917435169	0.00691	AK097342	1.237294078	0.0315
NM_001013673	1.999942899	0.00692	NM_006196	1.120820165	0.0315
NM_018388	1.617584705	0.00692	NM_001260	0.667389452	0.0315
AK094418	0.430644512	0.00705	XM_935373	1.08058548	0.0316
BC063647	1.720778227	0.00706	NM_014518	0.79868263	0.0316
XM_928118	0.784635723	0.00709	NM_015490	0.673797071	0.0316
XM_933650	0.548191369	0.00709	NM_004189	0.498427182	0.0316
NM_174941	0.814948559	0.00713	BC053886	0.664590716	0.0317
BC011635	1.725294709	0.00716	NM_000937	0.587487936	0.0317
BC040950	1.575423121	0.00731	BC029486	1.727405906	0.0318
BC007374	0.629629791	0.00738	AK091779	1.649168134	0.0318

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_198795	1.576058388	0.00739	NM_024632	1.647644997	0.0318
AK127393	0.380216181	0.00739	NM_001464	1.142804265	0.0318
NM_016563	0.628866255	0.00741	NM_001242	0.573200881	0.0318
NM_021010	1.372585773	0.00742	BC015563	0.521790266	0.0318
XM_496360	1.179985523	0.00745	BC063632	1.294203877	0.0319
NM_018128	1.210982442	0.00747	NM_001017434	1.290299416	0.0319
NM_006664	0.675709128	0.00749	XM_058931	0.750948966	0.0319
NM_002701	0.478098273	0.00749	BC020885	0.750293493	0.0319
NM_181522	1.637781024	0.00756	NM_133334	0.699384511	0.0319
NM_000253	0.767762303	0.00756	NM_032539	0.647989333	0.0319
AF329488	0.688502789	0.00756	AB051853	0.583132446	0.0319
XM_926158	1.131461859	0.00758	BC069134	1.96518743	0.032
NM_020407	0.642001152	0.00759	AF057170	1.35212636	0.032
BC056240	0.392181218	0.00763	NM_001008723	0.688240409	0.032
NM_016568	0.796273351	0.00765	BC066596	1.733367324	0.0321
NM_203454	2.790353775	0.0077	NM_001033523	1.399875522	0.0321
NM_005261	1.18075037	0.00773	BC043916	1.778612852	0.0322
NM_001004472	0.613607168	0.00773	NM_005468	0.63421607	0.0322
BC010128	0.646621943	0.00775	XM_933051	0.442492336	0.0322
BC030790	1.38121438	0.00778	NM_001010895	1.782262921	0.0323
NM_018931	0.694459021	0.00778	AK123617	1.692348242	0.0323
XM_927567	0.637434781	0.00778	NM_000458	0.756532371	0.0323
NM_017824	2.143326521	0.0078	NM_172139	0.576816201	0.0323
XM_925964	0.629627466	0.00782	BC023567	1.266925216	0.0324
NM_006602	0.799562752	0.00784	BC059387	0.851121485	0.0324
NM_001117	1.48321116	0.00786	BC056253	0.72643739	0.0324
BC015006	0.545538604	0.00787	NM_001013704	0.604391754	0.0324
XM_929132	0.683257282	0.00788	XM_927256	0.543737531	0.0324
AK127311	0.523743808	0.0079	NM_003122	0.448362708	0.0324
BC067125	0.778907657	0.00804	AF545864	0.435730994	0.0324
NM_005613	1.937201858	0.00806	NM_182762	1.798280001	0.0325
NM_019099	1.332599163	0.00808	AK054633	1.301591158	0.0325
NM_006175	0.619685769	0.00813	NM_006029	1.275280237	0.0325
AF023259	0.556377113	0.00813	BC012395	0.717402458	0.0325
AY462284	0.873539209	0.00814	BC012096	0.697270274	0.0325
AK074124	0.336949378	0.00816	NM_025087	0.685154557	0.0325
NM_012473	0.876545608	0.00818	NM_032456	3.032559156	0.0326
XM_930757	0.587850034	0.00818	NM_013444	1.74228406	0.0326
AK125034	0.465141892	0.00818	AK096474	1.571512818	0.0326
NM_015061	1.475977778	0.00825	NM_001950	0.806482375	0.0326

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_001241	1.458180904	0.00825	NM_001005169	0.541909218	0.0326
AJ272365	1.551478624	0.00826	NM_001039498	0.53412497	0.0326
NM_001584	0.534181118	0.00827	BC005327	1.6316607	0.0327
NM_005097	1.991970658	0.00828	NM_006261	1.096536279	0.0327
NM_006879	1.833544493	0.0083	NM_201625	0.769057989	0.0327
BC015944	1.59162271	0.00833	XM_933145	0.695912957	0.0327
AK094515	1.558116555	0.00837	BC004181	0.695545733	0.0328
L25616	1.493994594	0.00837	BC074922	0.672263443	0.0328
BC011886	1.454050899	0.00839	NM_000679	0.639412224	0.0328
NM_178343	0.449051648	0.0084	AF090937	0.596644044	0.0328
BC012506	0.560507536	0.00842	AK091101	0.518696427	0.0328
AL833417	0.583077431	0.00843	NM_052924	0.42906788	0.0328
NM_006840	0.548418701	0.00852	NM_004295	1.242451787	0.0329
BC032835	3.254400253	0.00854	NM_001061	1.731897354	0.033
BC064545	0.611162007	0.00857	BC041806	1.492627382	0.033
XM_370697	1.145484567	0.00859	NM_001002901	0.830303967	0.033
BC112021	0.524022341	0.00861	XM_930776	0.819678664	0.033
AF161541	1.904473305	0.00872	AK000249	0.67654258	0.033
AK057244	1.504809618	0.00874	AK127390	0.536456287	0.033
NM_022449	0.589125216	0.00874	NM_182525	0.490527928	0.033
NM_020061	0.729987681	0.00875	XM_928061	1.839765072	0.0331
NM_005255	0.690786898	0.00878	NM_014720	1.501173019	0.0331
NM_001005614	0.658748567	0.00878	XM_926528	1.151125431	0.0331
BC035808	0.466193885	0.00878	XM_929876	0.794290721	0.0331
NM_144646	2.302619934	0.00879	NM_000429	0.667383552	0.0331
NM_001007240	0.463240653	0.00881	NM_001001722	0.373341233	0.0331
XM_496581	1.785390973	0.00883	NM_002901	1.284565926	0.0332
AL117496	1.615072131	0.00883	NM_000257	0.652406752	0.0332
NM_002653	0.680172145	0.00883	XM_930334	0.355592906	0.0332
NM_032333	2.386789083	0.00887	AK058119	0.482864678	0.0333
XM_496836	1.59645164	0.00887	NM_173487	0.383966982	0.0333
XM_926695	0.629196048	0.00891	XM_930101	2.001690149	0.0334
NM_012098	2.882982731	0.00892	NM_016488	1.849941969	0.0334
BC096331	1.76803267	0.00892	NM_006795	0.857574046	0.0334
NM_018267	1.379165292	0.00894	NM_017433	0.738362968	0.0334
BC017714	0.44046858	0.00894	NM_001039905	0.55101943	0.0334
XM_932913	0.343497843	0.00895	BC012602	1.528311849	0.0335
XM_293680	0.687891483	0.00896	NM_006172	0.724032938	0.0335
BC015052	1.523267746	0.00899	NM_005586	0.647824824	0.0335
XM_373106	0.537896037	0.009	AK125281	0.632802308	0.0335

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_007058	0.837290227	0.00901	XM_929910	1.662472725	0.0336
NM_020131	0.75284636	0.00901	NM_001449	1.608464241	0.0336
XM_928233	0.358734667	0.00901	NM_001472	0.517087758	0.0336
NM_152232	0.742698312	0.00903	XM_928847	1.324066281	0.0337
XM_059104	0.467071593	0.00903	NM_006993	0.757916152	0.0337
NM_139179	0.75639993	0.00908	BC110639	0.685240448	0.0337
XM_932902	0.671964586	0.00908	NM_001013710	0.558000028	0.0337
NM_000705	0.768302798	0.00916	XM_928062	0.556267798	0.0337
BC005922	1.462682843	0.0092	BC101556	1.424486756	0.0338
NM_003478	1.431378126	0.00922	BC093670	1.324029207	0.0338
NM_001004739	0.685284019	0.00928	BC030612	1.213424087	0.0338
BC105057	1.578225255	0.00937	NM_006563	0.830593765	0.0338
NM_016356	1.466413617	0.00937	NM_003018	0.700101018	0.0338
AY234408	2.226819515	0.00944	NM_001005190	0.616530895	0.0338
BC045630	1.682946801	0.00946	NM_144677	0.568839788	0.0338
NM_152670	0.87230891	0.00948	AY792621	2.43438983	0.034
NM_002962	0.644803464	0.00948	NM_006903	1.639529467	0.034
AB209512	1.33752811	0.00952	BC068467	1.148536801	0.034
NM_052863	0.613995612	0.00952	NM_001708	0.766145349	0.034
NM_014929	1.206469417	0.00953	BC035419	0.764842749	0.034
XM_931864	1.41286099	0.00957	AK131282	0.590504587	0.034
BX648113	1.964891076	0.00958	CR619928	2.00798583	0.0341
BC058837	0.383205354	0.00958	XM_932327	1.465931654	0.0341
NM_001024656	0.521617949	0.00959	NM_024966	1.269659877	0.0341
NM_004650	1.577080488	0.00962	NM_001053	0.749991238	0.0341
BC061700	0.619823039	0.00962	AL832009	0.674659729	0.0341
NM_004126	2.760669947	0.00966	BC026186	1.168751597	0.0342
AB209513	2.911610842	0.00967	BC014423	0.670887828	0.0342
BC034141	0.648426712	0.00967	XM_933289	0.620522141	0.0342
BC111713	0.599296749	0.00967	NM_001005194	0.610749066	0.0342
AK126816	0.935210824	0.00968	XM_376281	0.539207995	0.0342
NM_001006939	0.494239092	0.00968	NM_021067	1.81539607	0.0343
NM_033130	1.816166162	0.0097	BC063881	1.347175956	0.0343
AK056275	0.288810998	0.0097	NM_024603	0.700354397	0.0343
NM_153486	0.658639193	0.00973	NM_139278	0.616977751	0.0343
BX648678	1.983107209	0.00975	NM_139211	1.432209015	0.0344
NM_003475	1.61066699	0.00975	XM_930608	0.643076897	0.0344
AK056484	1.315196514	0.00975	NM_024643	0.636627138	0.0344
AF520569	1.815468192	0.00977	AF447585	0.771762967	0.0345
AY987010	1.31553793	0.00978	BC040471	0.769735157	0.0345

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_153022	0.531146169	0.00978	NM_052925	0.694743395	0.0345
AK054978	0.52672857	0.0098	NM_016730	1.352924824	0.0346
NM_005739	1.265429497	0.00981	NM_003176	0.82584852	0.0346
NM_021708	1.279091477	0.00982	BC096699	0.746534944	0.0346
BC040480	0.704722583	0.00982	XM_933872	0.707076609	0.0346
NM_001003652	1.495693088	0.00983	AY513657	0.588852465	0.0346
NM_145798	0.540561438	0.00987	AF116706	0.529569745	0.0346
NM_006380	1.889760256	0.00989	NM_024306	0.849601805	0.0347
XM_932216	0.477272004	0.00994	XM_374919	0.7787292	0.0347
NM_184087	0.731275022	0.00995	XM_499314	0.638979435	0.0348
NM_006534	1.602101088	0.0101	NM_173039	0.523156285	0.0348
BC093861	1.510538697	0.0101	XM_932197	0.459981471	0.0348
NM_001039801	0.607584476	0.0101	NM_012433	1.195929646	0.0349
BC071181	1.301173687	0.0102	XM_929177	1.162211061	0.0349
BC032800	1.214844584	0.0102	NM_206919	0.803370714	0.0349
XM_934803	0.679596245	0.0102	BC034582	1.827281475	0.035
NM_001012264	0.557844341	0.0102	NM_032800	1.733751893	0.035
BC111550	0.526314735	0.0102	BC001283	1.621145844	0.035
BC020740	1.507961869	0.0103	NM_001003891	0.799223185	0.035
NM_016657	1.102007389	0.0103	BC074902	0.615135074	0.035
BC013375	0.868794501	0.0103	X04876	1.705678225	0.0351
NM_001335	0.622763395	0.0103	NM_152453	1.624516249	0.0351
AK126635	0.570018351	0.0103	AF212995	1.405380726	0.0351
XM_371312	0.401284069	0.0103	AF462446	0.766245663	0.0351
BC012907	1.483747482	0.0104	BC001451	0.66362524	0.0351
NM_002259	1.452555299	0.0104	NM_001004728	0.522237599	0.0351
NM_022752	0.725468814	0.0104	NM_018451	0.858199298	0.0352
NM_183059	0.552977741	0.0104	NM_031933	0.65048933	0.0352
NM_001836	0.350974619	0.0104	BC040558	0.414871663	0.0352
X95486	1.354806185	0.0105	NM_000864	1.230629563	0.0353
BC014315	1.191013932	0.0105	AK128370	0.729963064	0.0353
NM_001004476	0.381799549	0.0105	NM_176820	0.722870171	0.0353
NM_021634	1.626070499	0.0106	NM_173799	0.639685988	0.0353
NM_001018003	0.845188379	0.0106	NM_032953	0.517008781	0.0353
BC028388	0.760313272	0.0106	NM_001004300	1.657557964	0.0354
NM_001001872	0.651322544	0.0106	NM_032023	1.482706666	0.0354
XM_930049	0.423844367	0.0106	NM_015941	1.281687379	0.0354
NM_032947	1.75125742	0.0107	XM_927653	0.791725218	0.0354
NM_014363	0.712338746	0.0107	NM_013401	0.665588439	0.0354
BC030835	0.681238651	0.0107	NM_018111	0.552644491	0.0354

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_178548	0.603935897	0.0107	NM_012444	1.506602168	0.0355
NM_002711	1.493487835	0.0108	XM_374767	1.274984598	0.0355
XM_212241	1.302971601	0.0108	NM_000493	1.185122132	0.0355
BC018728	0.962256432	0.0108	XM_376822	0.432458431	0.0355
XM_926237	0.543196976	0.0108	BC012078	1.627472401	0.0356
BC012390	1.423067331	0.0109	NM_153812	1.395272493	0.0356
XM_929209	1.304817915	0.0109	NM_000089	1.245419741	0.0356
XM_933939	0.78679198	0.0109	BC008958	0.876096308	0.0356
BC040552	0.692885756	0.0109	BC003175	0.84092772	0.0356
AF332961	0.513398051	0.0109	XM_929851	0.827726901	0.0356
BC017591	1.819964409	0.011	NM_001004326	0.815220535	0.0356
BC062583	1.259080291	0.0111	NM_001003892	0.674659848	0.0356
NM_001386	1.6236763	0.0112	XM_379325	0.647077799	0.0356
BC013289	1.393275976	0.0112	XM_933035	0.419700414	0.0356
AY494983	1.204637289	0.0112	AK057061	1.594902992	0.0357
AK097760	0.68535012	0.0112	NM_032561	0.742065787	0.0357
NM_001013742	0.597661376	0.0112	XM_933787	0.72536701	0.0357
NM_138396	0.531533062	0.0112	NM_002944	1.578329921	0.0358
NM_201259	2.182397366	0.0113	BC108901	1.494711518	0.0358
NM_000167	1.486180305	0.0113	BC095441	1.409736991	0.0358
NM_014848	1.279790044	0.0113	BX640637	1.113651156	0.0358
BC075021	1.217128634	0.0113	AK125401	0.566507101	0.0358
BC069293	0.672897816	0.0113	AK022237	0.539588094	0.0358
NM_013349	2.503317356	0.0114	BC108724	0.48665145	0.0358
NM_000248	1.537596464	0.0115	NM_002748	3.167170286	0.0359
AK222768	1.228640676	0.0115	NM_000258	1.679374099	0.0359
NM_001009571	1.719250798	0.0116	BC036029	1.292961597	0.0359
NM_001012964	0.57538712	0.0116	NM_015106	0.852704406	0.0359
XM_928361	0.52904284	0.0116	NM_203422	1.644889832	0.036
NM_032427	0.80652684	0.0117	NM_032900	1.516316414	0.036
NM_003567	0.772406816	0.0117	BC063296	1.509080291	0.036
XM_934727	0.553857327	0.0117	NM_006763	1.341850638	0.036
XM_926927	0.533599615	0.0117	BC066644	0.871710718	0.036
BC000313	0.755677521	0.0118	XM_932065	0.473285079	0.036
NM_000641	0.706749499	0.0118	NM_198566	1.514296174	0.0361
NM_002984	0.699288726	0.0118	XM_379298	1.453709006	0.0361
NM_004751	0.640920401	0.0118	BC071933	0.789944947	0.0361
XM_932975	0.528615475	0.0118	XM_930702	0.558566451	0.0361
XM_927658	0.493846834	0.0118	AF161473	1.543067694	0.0362
BC100867	0.418068051	0.0118	NM_004624	1.458532333	0.0362

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_080675	1.755665898	0.0119	BC074795	0.720618725	0.0362
XM_926812	1.308182478	0.0119	XM_931341	0.596672118	0.0362
NM_016434	0.695737004	0.0119	XM_933409	0.497903913	0.0362
NM_152607	0.557747424	0.0119	NM_012463	1.833450913	0.0363
NM_145304	1.958147168	0.012	NM_020353	1.316710711	0.0363
BC062354	0.755070269	0.012	BC014666	0.773205459	0.0363
XM_926898	0.699319899	0.012	NM_020847	0.697035432	0.0363
AB096941	0.686897576	0.012	BC036569	1.805904865	0.0364
NM_016591	1.844150901	0.0121	BX648138	1.602112889	0.0364
BC000993	1.295549512	0.0121	NM_015540	0.79189837	0.0364
BC000180	1.130919099	0.0121	NM_153364	0.627637565	0.0364
AK124700	0.802055717	0.0121	AF027515	1.064344764	0.0365
NM_145652	0.645964503	0.0121	XM_925927	0.397158772	0.0365
NM_006863	2.300276041	0.0122	NM_017635	2.564743042	0.0366
NM_001004704	1.633920074	0.0122	NM_005382	0.541255713	0.0366
NM_007153	1.559466004	0.0122	AB040885	0.532682955	0.0366
NM_000301	0.71236372	0.0122	NM_001004726	0.4673931	0.0366
Z11898	0.405730754	0.0122	NM_032243	0.360889733	0.0366
NM_020134	1.66402626	0.0123	BC036399	1.988249898	0.0367
AB075848	0.753623128	0.0123	XM_933454	1.494265795	0.0367
NM_001005388	0.71595788	0.0123	NM_021177	0.89090097	0.0367
BC028369	2.839823484	0.0124	NM_018487	0.807631373	0.0367
NM_006734	1.498244047	0.0124	XM_927442	0.689448774	0.0367
NM_007061	0.811456621	0.0125	XM_059074	1.887245893	0.0368
NM_153268	0.717265129	0.0125	XM_926140	1.871939898	0.0368
NM_198275	0.703762233	0.0125	NM_017586	0.777170241	0.0368
NM_004466	0.515594304	0.0125	BC047720	0.716738939	0.0368
NM_172078	0.386929572	0.0125	XM_927445	0.550263941	0.0368
NM_138796	1.244185448	0.0126	D50924	0.535215616	0.0368
NM_183243	0.822991014	0.0126	NM_172131	0.514924169	0.0368
NM_014256	0.758398831	0.0126	BC062585	0.859519541	0.0369
NM_194251	0.696569681	0.0126	XM_934685	0.608414114	0.0369
NM_013979	0.74662143	0.0127	XM_932705	0.606690347	0.0369
NM_173351	0.693676353	0.0127	D50915	0.599247992	0.0369
AF282732	1.67357409	0.0128	NM_000149	0.438679814	0.0369
BC041628	0.763399601	0.0128	NM_152655	1.930248141	0.037
NM_005985	0.643740177	0.0128	BC057237	1.229921818	0.037
NM_007101	0.568271041	0.0128	BC066311	0.81726253	0.037
NM_173500	1.527656078	0.0129	XM_933799	0.752539814	0.037
NM_138636	1.401243091	0.0129	NM_001491	0.541127563	0.037

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_032680	0.77202189	0.0129	DQ124708	2.286149263	0.0371
XM_926889	0.527629673	0.0129	NM_001560	1.615385413	0.0371
AF276423	1.367488027	0.013	NM_001033925	1.576887965	0.0371
BC103831	1.242447615	0.013	BC005143	1.594395876	0.0372
NM_181575	0.936501443	0.013	AK097343	0.936156332	0.0372
NM_014877	0.883259058	0.013	NM_015251	1.706694722	0.0373
AL157435	0.458305031	0.013	X67697	0.652949929	0.0373
NM_152777	1.595436692	0.0131	AK126505	0.434315622	0.0373
BC003071	0.82234329	0.0131	NM_013959	0.328481227	0.0373
BC101414	0.74967736	0.0131	NM_198460	1.689200282	0.0375
BC022476	0.621798694	0.0131	BC023525	1.59317553	0.0375
BC050454	0.53047967	0.0131	NM_018355	1.495568991	0.0375
XM_928579	0.515673041	0.0131	NM_004091	0.84419018	0.0375
NM_031427	1.440365672	0.0132	X77633	0.823290467	0.0375
NM_001010912	1.47834897	0.0133	AF258585	0.657981634	0.0375
NM_000381	1.27709043	0.0133	NM_001004712	1.857141972	0.0376
BC013693	0.624143839	0.0133	AK128375	1.341901302	0.0376
NM_138316	1.996872425	0.0134	NM_018268	1.208946824	0.0376
NM_173627	1.96882987	0.0134	AK023650	1.130475163	0.0376
NM_004133	1.190457702	0.0134	AK000962	0.756959498	0.0376
AK131366	0.771035969	0.0134	AK092352	0.68741107	0.0376
NM_001004474	0.632476628	0.0134	AK128206	0.544871271	0.0376
NM_004350	0.614610791	0.0134	NM_145283	0.532166839	0.0376
NM_152610	0.494199336	0.0134	NM_145003	0.448666871	0.0376
NM_024528	1.699660301	0.0135	XM_929804	1.637655973	0.0377
NM_145248	0.651320994	0.0135	NM_006089	1.465285182	0.0377
XM_926054	0.358291715	0.0135	XM_927610	0.70983839	0.0377
NM_198562	1.452713132	0.0136	BC110513	0.614838958	0.0377
NM_018198	1.259405851	0.0136	AF340020	0.490454227	0.0377
NM_001009607	0.740565836	0.0136	NM_033387	0.364672899	0.0377
BC027970	0.714301705	0.0136	NM_003190	1.26626277	0.0378
NM_003323	0.680975854	0.0136	XM_372248	0.764567554	0.0378
AB018258	0.561953783	0.0136	BC112049	0.581294715	0.0378
AK022140	1.804319263	0.0137	NM_001034832	0.524895191	0.0378
NM_031890	1.65507853	0.0137	XM_935352	1.273159266	0.0379
BC039879	1.203471422	0.0137	BC006456	1.193305016	0.0379
NM_181690	1.379078984	0.0138	X78136	1.033993244	0.0379
AK223558	1.089161396	0.0138	BC030828	0.700992584	0.0379
NM_020175	0.56537807	0.0138	XM_928474	0.647991002	0.0379
NM_003771	0.373246998	0.0138	BC012617	0.525117993	0.0379

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_001039547	1.785367608	0.0139	NM_016248	1.556931376	0.038
NM_004520	1.298543334	0.0139	NM_019900	0.728838742	0.038
BC032581	0.540820301	0.0139	NM_002016	0.68492353	0.038
NM_152444	2.38949132	0.014	XM_929541	0.662676752	0.038
XM_496847	1.400782108	0.014	CR608708	0.544926524	0.038
BC025276	1.130650282	0.014	XM_290597	1.702420831	0.0381
AK021445	0.820627332	0.014	AJ698950	1.383249879	0.0381
AK127126	0.488420397	0.014	XM_209505	0.559515834	0.0381
NM_015076	1.411104321	0.0141	X83705	0.456555277	0.0381
XM_378545	0.753622711	0.0141	XM_926326	0.373186201	0.0381
AK025430	0.712548852	0.0141	NM_001024655	2.027560949	0.0382
XM_929881	0.696613014	0.0141	NM_003113	1.348258734	0.0382
NM_170776	0.643708229	0.0141	NM_001927	0.661371171	0.0382
NM_004246	1.255548596	0.0142	NM_147129	0.627124488	0.0382
NM_001003794	0.76714319	0.0142	BC101204	0.603899419	0.0382
NM_024303	0.746115386	0.0142	NM_006881	1.766762137	0.0383
BC057759	0.64630419	0.0142	AY054974	1.392749429	0.0383
NM_018297	1.449367642	0.0143	NM_052957	1.23676312	0.0383
BC034988	1.39124155	0.0143	BC100931	0.85376668	0.0383
AF259965	0.813714683	0.0143	NM_203311	0.693942904	0.0383
CR933674	0.761339962	0.0143	BC007333	0.560972333	0.0383
BC114468	0.752310574	0.0143	XM_370973	0.423665941	0.0383
XM_929838	0.445965469	0.0143	NM_002059	0.727636039	0.0384
NM_019598	0.687871397	0.0144	XM_498207	0.658920765	0.0384
NM_001287	0.546410143	0.0144	BC080599	0.59857887	0.0384
BX247969	3.095725298	0.0145	BC036049	0.423860252	0.0384
AF329495	0.640755355	0.0145	NM_016623	1.619452596	0.0385
XM_929585	2.036934376	0.0146	NM_170675	1.578917265	0.0385
BC048810	1.627361536	0.0146	AY349358	1.240796685	0.0385
BC009734	0.851875246	0.0146	XM_928629	1.200398564	0.0385
L14813	0.643002391	0.0146	BC104213	1.103588581	0.0385
NM_138344	0.623827279	0.0146	AK002004	0.823009312	0.0385
XM_933886	0.51813525	0.0146	NM_080733	0.476469338	0.0385
AL137368	1.889320374	0.0147	NM_032921	1.395549297	0.0386
NM_016075	1.518137336	0.0147	NM_016578	1.272292376	0.0386
XM_497889	1.46377492	0.0147	BC033437	0.398381352	0.0386
AF247820	1.436598063	0.0147	BC109075	1.390547633	0.0387
U12767	1.376343608	0.0147	NM_004383	0.837281764	0.0387
NM_014808	0.570183933	0.0147	NM_207404	0.644465804	0.0387
BC096105	1.57518363	0.0148	NM_198843	0.585334301	0.0387

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
XM_086761	1.482782006	0.0148	NM_004737	0.517312348	0.0387
BC063126	1.262555003	0.0148	NM_001033025	1.734155774	0.0388
NM_001007027	1.158005953	0.0148	NM_152271	1.715531349	0.0388
AK125094	1.655260205	0.0149	BC041014	1.636969447	0.0388
BC042676	1.646499276	0.0149	NM_001899	0.818442822	0.0388
BC007508	1.174295783	0.0149	NM_001276	0.724198282	0.0388
NM_006168	0.815052748	0.0149	NM_006001	0.402774245	0.0388
AY172962	0.776195526	0.0149	XM_371617	1.695969582	0.0389
BC104997	0.559918702	0.0149	NM_001007122	1.398454666	0.0389
BC033082	0.494043052	0.0149	NM_006355	1.303854227	0.0389
NM_181710	0.429003805	0.0149	NM_030673	0.887456059	0.0389
AB040895	1.68237114	0.015	CR610648	0.868208885	0.0389
BX648397	1.424157143	0.015	NM_130794	0.773140848	0.0389
NM_003986	1.29999733	0.015	NM_178841	0.700568736	0.0389
XM_930573	0.595438838	0.015	X73568	0.668732822	0.0389
NM_005251	0.594643056	0.015	AY279090	0.644651771	0.0389
BC053989	0.601312816	0.0151	NM_001005410	0.624987245	0.0389
AK074626	0.588559926	0.0151	BC071989	0.574118674	0.0389
BC069440	0.581015706	0.0151	AB102644	0.505928457	0.0389
NM_004610	0.562051713	0.0151	AK127153	0.425412774	0.0389
BC038857	2.082105398	0.0152	BC094779	1.635321975	0.039
NM_001024372	1.471976757	0.0152	AK130030	1.451826811	0.039
NM_205864	0.6765306	0.0152	NM_000895	1.226406097	0.039
BC009709	2.695126057	0.0153	NM_013378	0.746409059	0.039
NM_145013	1.800337911	0.0153	BC075840	0.446364969	0.039
X52008	1.587260365	0.0153	NM_006047	2.04707408	0.0391
AF465485	1.485573173	0.0153	NM_001039706	1.347940683	0.0391
NM_194324	1.475169063	0.0153	AK092861	0.75002861	0.0391
AX770999	0.810786605	0.0153	NM_003553	0.742482007	0.0391
NM_013374	2.238041162	0.0154	CR456436	1.084729195	0.0392
NM_030958	1.539710879	0.0154	BC014868	0.606315613	0.0392
NM_138778	0.733706176	0.0154	NM_001014291	0.498352468	0.0392
DQ321787	1.761159539	0.0155	NM_145284	1.480967045	0.0393
NM_006359	1.498975754	0.0155	NM_203497	1.302925944	0.0393
NM_173513	0.776654899	0.0155	NM_005192	1.184935451	0.0393
BC033735	0.687906742	0.0155	BC104976	0.6986081	0.0393
XM_932588	0.658453882	0.0155	AF290004	0.633397162	0.0393
BC033939	1.466159582	0.0156	NM_007034	1.650258183	0.0394
AK054581	1.206163764	0.0156	NM_014584	1.842891812	0.0395
AK074914	0.819331229	0.0156	BC046095	1.48049438	0.0395

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_212556	0.68575263	0.0156	AK125219	1.362281084	0.0395
AK090563	0.629115522	0.0156	BC033902	1.089284062	0.0395
BC098584	1.216630578	0.0157	CR749793	0.812401056	0.0395
XM_291716	0.488452643	0.0157	XM_933021	0.615715325	0.0395
NM_174978	1.257686615	0.0158	XM_930152	0.573062479	0.0395
NM_002635	1.077124715	0.0158	BX648845	2.273005009	0.0396
NM_016612	0.786514044	0.0158	NM_015091	1.661643624	0.0396
BC074918	0.564255893	0.0158	NM_032506	1.569250345	0.0396
AK094629	1.946585894	0.0159	BC101712	0.687438905	0.0396
DQ099384	1.322213888	0.016	BC064534	0.572072029	0.0396
BC032571	1.240963459	0.016	NM_001005753	2.844763756	0.0397
BX648310	0.692405224	0.016	AF370415	0.826479614	0.0397
NM_016257	0.649922311	0.016	NM_004428	0.774528027	0.0397
AK124321	0.632998586	0.016	NM_130782	0.654784262	0.0397
NM_145000	1.80807817	0.0161	XM_062872	0.572523773	0.0397
AK130204	1.445517898	0.0161	NM_004259	0.512457311	0.0397
XM_927388	0.729371428	0.0161	NM_173501	1.838192463	0.0398
NM_173574	0.54931736	0.0161	NM_013412	1.78233552	0.0398
NM_001039796	2.420336723	0.0162	AK093319	1.299415469	0.0398
NM_004429	1.303899765	0.0162	NM_002532	1.247249603	0.0398
BC012264	1.20367825	0.0162	NM_020482	1.204505324	0.0398
NM_002565	0.646523893	0.0162	NM_133280	0.880416095	0.0398
NM_001018059	0.636728108	0.0162	BC104897	0.705129147	0.0398
BC013682	1.951921344	0.0163	AF251047	1.310429931	0.0399
NM_173484	1.356555462	0.0163	NM_144676	0.820993841	0.0399
BC021822	1.351740837	0.0163	NM_144675	0.471633703	0.0399
NM_001631	0.693128407	0.0163	XM_051017	0.445322067	0.0399
BC033685	0.746986568	0.0164	NM_032342	1.588341594	0.04
NM_153337	0.735913098	0.0164	AB096952	1.286492109	0.04
NM_178177	0.610058486	0.0164	NM_145271	0.817186832	0.04
BC053655	0.483507752	0.0164	NM_000541	0.766980886	0.04
NM_016150	2.861176968	0.0165	XM_928863	0.713712335	0.04
NM_173808	1.379474163	0.0165	NM_020040	0.671030641	0.04
NM_012108	0.763346732	0.0165	AK026889	1.635792494	0.0401
BC018529	0.715643227	0.0165	BC071661	1.250801921	0.0401
AK130638	0.654791832	0.0165	BC093947	0.61620748	0.0401
NM_020724	2.103607893	0.0166	XM_379479	0.613058865	0.0401
AB008790	1.943235397	0.0166	XM_931118	1.834130049	0.0402
NM_006878	1.738335848	0.0166	BC104816	1.799641132	0.0402
BC107755	1.734993219	0.0166	AF542548	1.490685344	0.0402

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
BC015948	1.293088794	0.0166	NM_006947	1.361930728	0.0402
BC069608	0.708294988	0.0166	NM_015912	0.714176893	0.0402
NM_001004750	0.660423636	0.0166	NM_173089	0.559409857	0.0402
XM_932990	0.461335421	0.0166	NM_004694	0.548624158	0.0402
XM_934307	1.792472124	0.0167	BC042956	1.580518007	0.0403
BC098394	1.648866177	0.0167	BC001133	1.184889793	0.0403
BC067784	1.433303475	0.0167	Y14442	0.582823098	0.0403
NM_005704	0.791695237	0.0167	BC010011	1.510676861	0.0404
BC109292	1.829706907	0.0168	BC018143	0.800393522	0.0404
NM_031483	1.437182069	0.0168	NM_198526	0.761930287	0.0404
NM_198075	1.412934542	0.0168	NM_138412	0.754407942	0.0404
BC069570	1.218067646	0.0168	AK027158	0.647332966	0.0404
BX248778	0.491879821	0.0168	AF375481	0.635390759	0.0404
NM_153344	1.455244422	0.0169	AF403061	1.779229879	0.0405
NM_000160	0.639775276	0.0169	BC036101	1.413298726	0.0405
AK128390	0.441308886	0.0169	NM_175075	1.36760807	0.0405
NM_021944	1.513807893	0.017	BC060785	0.628775418	0.0405
BC029559	1.494277716	0.017	NM_001009568	0.427206039	0.0405
NM_194284	1.325798631	0.017	AK074256	1.673693299	0.0406
NM_148912	0.706719279	0.017	AY358442	1.470350981	0.0406
AK128263	0.474923164	0.017	NM_002264	1.389754772	0.0406
NM_172127	1.786422253	0.0171	XM_931829	1.329425335	0.0406
BC038108	1.659710526	0.0171	BC086308	0.808443487	0.0406
NM_003600	1.255804181	0.0171	AL834183	0.739674211	0.0406
XM_932374	0.894684732	0.0171	BC000544	0.56400919	0.0406
NM_153002	0.565209746	0.0171	BX648702	0.551630795	0.0406
NM_021936	0.545563221	0.0171	NM_001017972	1.28330338	0.0407
BC109390	2.159892321	0.0172	BC112102	0.804155409	0.0407
XM_926824	1.378228188	0.0172	NM_003482	0.678215861	0.0407
BC093853	0.611917973	0.0172	NM_173625	0.368322819	0.0407
NM_021015	0.495869607	0.0172	XM_933328	1.63171792	0.0408
AF354656	2.534319639	0.0173	NM_000531	1.528578043	0.0408
NM_005528	0.875330925	0.0173	NM_170699	0.642169774	0.0408
XM_931036	0.836489022	0.0173	XM_928154	0.58691591	0.0408
BC015558	0.802353919	0.0173	NM_022903	0.531853557	0.0408
XM_929701	0.714273334	0.0173	NM_021175	0.462879062	0.0408
AY358869	0.682094812	0.0173	NM_144974	0.344343573	0.0408
XM_932171	0.495119065	0.0173	NM_001008215	1.667531133	0.0409
NM_133505	1.625598669	0.0174	NM_007193	1.36456573	0.0409
NM_030907	1.555808783	0.0174	NM_020117	1.348014474	0.0409

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_080387	1.322832704	0.0174	NM_001004328	0.734740555	0.0409
NM_001032364	0.719979346	0.0174	NM_000608	0.725818276	0.0409
NM_173643	0.617968678	0.0174	BC008846	0.699454486	0.0409
BC111742	1.517306328	0.0175	NM_001004322	0.698357821	0.0409
BC036834	1.30158627	0.0175	NM_032902	0.617967784	0.0409
NM_019618	0.68644768	0.0175	NM_001001961	0.581310391	0.0409
BC025726	0.655262947	0.0175	NM_001046	2.677779913	0.041
NM_002517	0.640891552	0.0175	NM_032812	1.600482702	0.041
NM_177965	1.724941254	0.0177	NM_004654	0.826470554	0.041
CR611169	1.329797149	0.0177	AF411214	0.480876833	0.041
NM_001699	1.12760067	0.0177	NM_004396	1.322250485	0.0411
BC035124	0.792148054	0.0177	NM_014976	0.700633883	0.0411
NM_001037558	1.709426284	0.0178	XM_496659	0.634174824	0.0411
NM_020957	1.672255754	0.0178	AY372494	0.448800027	0.0411
AK055808	1.565953016	0.0178	L10403	1.509563923	0.0412
NM_144609	1.245350003	0.0178	XM_932150	0.630904675	0.0412
AK094286	0.760285556	0.0178	XM_934048	0.612675309	0.0412
AY453794	0.403467655	0.0178	AK131289	0.541619003	0.0412
NM_173587	0.663878441	0.0179	NM_015233	1.44837606	0.0413
BC054893	0.611632466	0.0179	NM_207379	0.71641773	0.0413
NM_015112	0.535530329	0.0179	M67468	2.226235151	0.0414
XM_927467	0.48832193	0.0179	BC105949	0.613907754	0.0414
NM_017890	2.467087746	0.018	XM_931389	1.445541024	0.0415
NM_015133	1.531474829	0.018	NM_001039649	1.342639685	0.0415
NM_001024465	1.387009382	0.018	BC109208	0.743033469	0.0415
XM_293416	0.777096987	0.018	NM_199046	0.678379834	0.0415
AY161136	1.917196512	0.0181	AK127597	0.595420539	0.0415
AB014605	1.829271793	0.0181	NM_005449	0.555659354	0.0415
L29216	0.823776782	0.0181	NM_032857	1.591822982	0.0416
BC027590	0.571441054	0.0181	BC039174	1.271185279	0.0416
BC074960	0.299225062	0.0181	NM_016357	1.265930176	0.0416
BC064694	1.954782367	0.0182	NM_013300	0.682621837	0.0416
AY766456	0.811593235	0.0182	BC031934	0.485970169	0.0416
XM_926241	0.580287457	0.0182	NM_015558	1.818007827	0.0417
AK124936	1.885539174	0.0183	NM_004645	1.112444997	0.0417
NM_018257	1.467370987	0.0183	AB032179	0.684913576	0.0417
BC096250	0.865004838	0.0183	NM_058199	0.459203929	0.0417
BC000091	0.753547728	0.0183	NM_033341	0.684739172	0.0418
AK056020	2.582198381	0.0184	NM_182606	1.627656579	0.0419
AL832935	2.21791172	0.0184	NM_033550	1.500255346	0.0419

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
BC016477	1.655292273	0.0184	BX648021	1.374908209	0.0419
NM_006439	0.65488708	0.0184	BC036381	1.274416566	0.0419
NM_015533	0.577998519	0.0184	NM_174983	0.710215092	0.0419
NM_014728	0.52935797	0.0184	AF260237	0.707566857	0.042
NM_004664	1.53747344	0.0185	NM_007056	0.697182953	0.042
NM_052887	0.703166425	0.0185	AF418289	0.607084572	0.042
XM_931989	0.568261027	0.0185	NM_145804	1.555369735	0.0421
NM_033316	1.362781763	0.0186	NM_198081	1.203171968	0.0421
NM_003278	1.283202171	0.0186	NM_006662	0.808267772	0.0421
BC105073	1.094187379	0.0186	AK130276	0.727395833	0.0421
XM_927587	0.748540223	0.0186	NM_001018112	0.689031303	0.0421
AF445026	0.577286601	0.0186	XM_928213	0.572226107	0.0421
NM_015224	1.589008212	0.0187	NM_004755	1.588779807	0.0422
AF458591	1.48901093	0.0187	BC069523	1.385176539	0.0422
NM_003588	1.187574506	0.0187	BC038398	0.675549388	0.0422
AK021536	0.757247448	0.0187	NM_012076	0.645116806	0.0422
NM_173557	0.615735352	0.0187	XM_372616	0.46897018	0.0422
AF289566	0.475277096	0.0187	NM_001010857	0.444748342	0.0422
BC112123	1.972657323	0.0188	NM_005545	1.302656531	0.0424
BC017187	1.433437705	0.0188	XM_931659	0.794556916	0.0424
XM_928551	0.621522248	0.0188	AB127078	0.769393146	0.0424
BC070352	0.576515555	0.0188	AY196783	0.717240393	0.0424
NM_003440	1.53917563	0.0189	NM_020704	1.380305529	0.0425
D87073	0.804060161	0.0189	BC062559	1.350767016	0.0425
NM_145662	0.785912216	0.0189	NM_024641	1.276789188	0.0425
NM_000790	0.777096927	0.0189	NM_030928	0.827039957	0.0425
AK126764	0.599173665	0.0189	NM_001097	0.643271863	0.0425
NM_004469	0.580706835	0.0189	BC032452	0.656252146	0.0426
BC104872	0.337065339	0.0189	NM_033106	0.578348815	0.0426
NM_133636	2.37574935	0.019	AF165281	2.355235577	0.0427
NM_000272	1.835358381	0.019	NM_007280	1.598316312	0.0427
NM_002399	1.27861321	0.019	AK124314	0.649386227	0.0427
NM_017739	0.736173749	0.019	XM_498195	0.601235449	0.0427
AF461897	0.36795637	0.019	AK094289	0.496471673	0.0427
NM_006122	1.615243077	0.0191	NM_003823	0.46367529	0.0427
AK092432	1.511577964	0.0191	NM_014805	2.626133919	0.0428
NM_001017431	1.407165885	0.0191	NM_001039656	1.465504766	0.0428
XM_378787	1.331621408	0.0191	AK056420	0.791164398	0.043
NM_182500	1.288936734	0.0191	BC002807	0.765908837	0.043
NM_032784	1.10633111	0.0191	NM_014861	0.592728019	0.043

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_001828	0.586526394	0.0191	NM_001024677	0.532520175	0.043
BC093960	0.528842628	0.0191	AK123255	0.523364604	0.043
BC009111	1.386475801	0.0192	XM_934146	0.455335408	0.043
AK122831	1.689221025	0.0193	BC014340	0.443239331	0.043
AK096925	1.412719131	0.0194	NM_004732	2.619584084	0.0431
NM_032213	0.717720628	0.0194	XM_930952	2.266005278	0.0431
BC072383	0.67318207	0.0194	NM_001009824	0.741168022	0.0431
XM_933408	0.663995922	0.0194	BC037829	0.698571384	0.0431
NM_144565	0.498085052	0.0194	BC110872	0.676751256	0.0431
NM_020314	1.791079998	0.0195	BC020499	0.62942493	0.0431
NM_024295	1.225453138	0.0195	NM_080860	0.514327466	0.0431
AF116601	0.626743436	0.0195	BC012006	1.420758009	0.0432
NM_018667	0.338966727	0.0195	NM_001006113	0.820321083	0.0432
AK074586	0.709110796	0.0196	NM_001024024	1.502000213	0.0433
NM_006043	0.707493663	0.0196	NM_015980	1.330503106	0.0433
XM_371535	0.496774793	0.0196	BC101499	1.327148914	0.0433
NM_002519	2.063100815	0.0197	AF332240	0.667904794	0.0433
NM_080819	0.775987685	0.0197	XM_926598	0.462643743	0.0433
BC012097	0.704212666	0.0197	NM_207481	0.593282938	0.0434
NM_021061	0.682200432	0.0197	NM_015896	0.484872699	0.0434
AK130020	0.584558606	0.0197	BC039107	1.408712029	0.0435
Y09858	0.555085301	0.0197	NM_001039762	1.233200431	0.0435
BC013171	0.363436371	0.0197	BC032889	0.865285754	0.0435
AJ278348	0.755032539	0.0198	AK075168	0.667447746	0.0435
BC050704	0.700793564	0.0198	NM_012174	1.912792921	0.0436
NM_003496	0.596834004	0.0198	BC013912	1.736222386	0.0436
NM_000572	1.48897016	0.0199	AF132203	1.660160542	0.0436
XM_927536	1.459644079	0.0199	BC017701	1.617046475	0.0436
NM_017993	1.458582759	0.0199	BC026089	1.164803505	0.0436
NM_015252	1.328114629	0.0199	BC005218	1.144037485	0.0436
NM_015915	1.297299027	0.0199	NM_020360	0.828142643	0.0436
AK093799	2.500697851	0.02	NM_015225	1.694442511	0.0437
NM_022132	1.355712175	0.02	BC039573	1.590263486	0.0437
NM_015541	1.188642144	0.02	NM_013445	1.201284409	0.0437
AY149296	0.672500968	0.02	XM_927713	0.585150361	0.0437
XM_929171	0.415314525	0.02	NM_002271	2.179948807	0.0438
BC091515	1.553214431	0.0201	NM_002999	1.380514383	0.0438
NM_173472	0.680852413	0.0201	BC110612	1.089481235	0.0438
AF285439	0.644039869	0.0201	XM_927491	0.773677528	0.0438
NM_182926	1.435984731	0.0202	NM_003675	1.592151165	0.0439

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
BC112209	0.635321975	0.0202	BC069700	0.665467799	0.0439
NM_023929	1.700764775	0.0203	NM_152389	0.633677781	0.0439
AF421361	1.66423583	0.0203	BC017020	1.508865714	0.044
AF440756	1.409321666	0.0203	NM_138352	0.806660116	0.044
NM_030783	0.761182547	0.0203	NM_001018096	0.723475337	0.044
AB088047	0.561582208	0.0203	NM_001001917	0.581581652	0.044
NM_001870	1.839805841	0.0204	BC056896	1.749695778	0.0441
XM_930821	0.790161669	0.0204	NM_005176	0.774973512	0.0441
NM_024320	0.514025509	0.0204	NM_178542	0.769269824	0.0441
NM_001012506	1.8877666	0.0205	NM_017621	0.76925087	0.0441
XM_927166	0.561238706	0.0205	XM_934348	0.537719429	0.0441
NM_022135	0.514377892	0.0205	XM_373772	0.445980936	0.0441
AK126941	1.657679558	0.0206	AF152527	0.426273406	0.0441
BC093870	0.704913974	0.0206	NM_013262	1.281461358	0.0442
XM_929992	0.630467772	0.0206	BC051765	0.830685735	0.0442
XM_926517	0.548822165	0.0206	BC093998	0.626615465	0.0442
AY358410	1.230803847	0.0207	NM_178565	0.621362805	0.0442
BC012183	0.82851404	0.0207	NM_001005387	0.432378262	0.0442
NM_003717	1.301437497	0.0208	NM_006517	0.876832247	0.0443
NM_030781	0.863737106	0.0208	XM_927940	0.49871552	0.0443
NM_001858	0.717803657	0.0208	BC031265	1.679195642	0.0444
XM_933429	1.517983079	0.0209	NM_030920	1.621363401	0.0444
NM_001012398	1.400462747	0.0209	U32331	1.518189788	0.0444
DQ099386	1.091877699	0.0209	BC108915	0.640934408	0.0444
NM_014807	0.750686884	0.0209	BC024184	2.563863993	0.0445
NM_003169	0.725448668	0.0209	AK092825	1.476545453	0.0445
XM_928006	0.610613704	0.0209	NM_001037553	1.412635684	0.0445
AK057318	0.55731827	0.0209	NM_007238	1.302083015	0.0445
NM_024898	0.534457505	0.0209	NM_004411	0.786169469	0.0445
NM_004051	1.871541619	0.021	XM_496643	0.690170705	0.0445
NM_025180	1.732644439	0.021	BC009317	0.674070537	0.0446
NM_175727	0.665356755	0.021	NM_007374	0.433907896	0.0446
NM_024744	2.10467124	0.0211	NM_033180	1.574399352	0.0447
BC112921	1.454287648	0.0211	NM_014709	0.934137225	0.0447
XM_927014	1.394419193	0.0211	BC062368	0.738416374	0.0447
AK126643	1.190741658	0.0211	NM_173050	0.55167675	0.0447
NM_001724	0.879620075	0.0211	BC036231	1.38163054	0.0448
AB037789	0.78180778	0.0211	NM_001006622	1.297679305	0.0448
AB110791	0.725712955	0.0211	NM_001004355	0.714837492	0.0448
NM_014212	0.707851768	0.0211	NM_032854	0.662001014	0.0448

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_020708	0.535693407	0.0211	NM_002193	0.620665431	0.0448
BC019087	2.161870718	0.0212	NM_198682	0.619619787	0.0448
AK130716	1.829300165	0.0212	AF289552	0.571212709	0.0448
NM_005463	1.574195147	0.0212	NM_015621	1.236933112	0.0449
NM_000391	1.489100575	0.0212	NM_033415	0.724824071	0.0449
BC101619	0.825579643	0.0212	NM_002686	0.613225818	0.0449
XM_934887	0.664735019	0.0212	AK058045	0.552031815	0.0449
BX647448	0.592983365	0.0212	NM_181607	0.387774616	0.0449
NM_002641	0.589251995	0.0212	NM_032521	1.296627998	0.045
NM_018364	2.452111721	0.0213	NM_014285	0.842872441	0.0451
NM_000717	0.915682912	0.0213	NM_152348	0.659521461	0.0451
BC014277	0.655615866	0.0213	NM_003985	0.556447089	0.0451
NM_134263	1.790590048	0.0214	NM_006785	2.115905523	0.0452
NM_021156	1.59698391	0.0214	NM_004313	0.754179299	0.0452
NM_024889	1.226523161	0.0214	AY065978	0.603639185	0.0452
NM_012182	0.593738854	0.0214	NM_001001343	1.626870751	0.0453
XM_932304	0.553407609	0.0214	XM_933689	2.575433493	0.0454
NM_001008394	1.901866555	0.0215	BC011913	1.593123198	0.0454
XM_371943	0.520441055	0.0215	NM_001004751	1.363714695	0.0454
BC060845	1.391096711	0.0216	NM_152265	1.222093344	0.0454
NM_021953	0.815781653	0.0216	NM_001013674	1.202972531	0.0454
NM_001001913	0.718877316	0.0216	BC015822	0.632831454	0.0454
NM_013306	0.621721447	0.0216	NM_001104	0.629992545	0.0454
BC012352	0.414731383	0.0216	XM_931440	0.731373727	0.0455
NM_005778	0.860457659	0.0217	NM_002069	2.743140697	0.0456
NM_025268	0.745823383	0.0217	NM_000459	1.450032711	0.0456
NM_018335	0.725520551	0.0217	NM_005618	1.361450791	0.0456
XM_933134	1.733498454	0.0218	NM_006419	1.246015429	0.0457
AK126677	0.688864589	0.0218	NM_032596	0.690528035	0.0457
XM_168055	0.600428998	0.0218	BC020666	0.63563925	0.0457
BC109047	0.571411073	0.0218	AJ487679	0.443000138	0.0457
AB158503	1.242491961	0.0219	XM_929074	0.408674777	0.0457
BC068050	0.845452607	0.0219	XM_059578	0.382707953	0.0458
BC110617	0.568945229	0.0219	AF119569	1.731978536	0.0459
XM_931290	1.25644052	0.022	NM_015018	1.81301558	0.046
XM_926737	1.180504084	0.022	NM_002959	1.509333253	0.046
XM_931000	0.844562292	0.022	BC000629	1.211013317	0.046
XM_497406	0.590709805	0.022	XM_931826	0.757623196	0.046
XM_933836	0.579013765	0.022	NM_025021	0.690636337	0.046
NM_133267	0.529447675	0.022	BC112120	0.638701737	0.046

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_002221	2.036212444	0.0221	NM_004598	2.238116741	0.0461
BC069557	1.831515789	0.0221	NM_001008389	0.699778438	0.0461
NM_012176	1.820711374	0.0221	NM_152542	1.446822643	0.0462
NM_003110	0.828770638	0.0221	AK124421	0.783321142	0.0462
NM_000300	0.588406324	0.0221	BC062711	0.727683723	0.0462
XM_377725	0.578139901	0.0221	BC109279	2.394278288	0.0463
AL833128	2.354409933	0.0222	AL136605	1.490994096	0.0463
NM_015170	2.274003029	0.0222	NM_153711	1.199903965	0.0463
NM_003932	1.782563448	0.0222	BC071753	0.919133186	0.0463
AK127730	0.657907307	0.0222	NM_004704	0.803504527	0.0463
XM_928465	0.579901457	0.0222	NM_012406	0.749729156	0.0463
BC044929	0.540437937	0.0222	NM_014839	2.13309145	0.0464
AK126822	1.792732716	0.0223	NM_214711	1.713399887	0.0464
NM_014679	1.481210828	0.0223	BC107742	1.476037621	0.0464
Z22970	1.298350215	0.0223	BC104834	1.223284841	0.0464
NM_198496	0.701022089	0.0223	NM_024658	0.888504028	0.0464
NM_001003938	0.61937505	0.0223	BC011877	0.642839909	0.0464
NM_182703	0.611378849	0.0223	BC069331	0.642567396	0.0464
NM_013389	0.592437863	0.0223	NM_080474	0.577267706	0.0464
AF269288	0.585479081	0.0223	BC027875	0.495781153	0.0464
BC022399	0.48765263	0.0223	AK057851	1.23267889	0.0465
NM_144585	0.354354143	0.0223	NM_001678	0.737704873	0.0465
AK091406	1.291690588	0.0224	XM_930536	0.717921257	0.0465
XM_926746	1.244156837	0.0224	NM_004445	0.66261524	0.0465
NM_176875	0.789070547	0.0224	NM_080552	0.546012402	0.0465
XM_927976	0.732391	0.0224	NM_002102	0.51627773	0.0465
XM_071013	0.661674142	0.0224	BC032362	0.462768614	0.0465
NM_005055	0.531741977	0.0224	NM_134425	1.265312433	0.0466
NM_001003811	1.49435091	0.0225	NM_003270	1.257155657	0.0466
BC050373	1.328035951	0.0225	AB117974	1.131655335	0.0466
NM_022342	0.606480837	0.0225	NM_005671	0.842886508	0.0466
NM_030583	1.241706014	0.0226	BC098109	0.561782062	0.0466
AK131375	1.204753518	0.0226	NM_001039756	0.544434845	0.0466
NM_019102	1.524199843	0.0227	NM_033031	0.527218759	0.0466
XM_926351	0.614852309	0.0227	NM_022166	0.728461921	0.0467
NM_021999	1.76128304	0.0228	DQ275472	0.651174366	0.0467
NM_014656	0.676525056	0.0228	NM_003387	1.395029783	0.0468
XM_934972	0.442800403	0.0228	AF167994	1.252498865	0.0468
BC060838	2.322581768	0.0229	BC049836	1.196916223	0.0468
BC009778	1.180637717	0.0229	NM_017810	0.801710427	0.0468

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
XM_931093	0.753253758	0.0229	BC028374	0.586562395	0.0468
XM_935369	0.470474303	0.0229	BC036124	1.694419026	0.0469
NM_012345	2.448552608	0.023	BC044938	1.440203071	0.0469
BC024253	1.76606214	0.023	XM_929523	0.44993332	0.0469
AK124214	1.545239449	0.023	AF107493	0.832757056	0.047
BC026362	1.110773921	0.023	NM_152406	0.777201891	0.047
AK131475	0.819221497	0.023	NM_015848	0.598816574	0.047
BC030813	0.621813953	0.023	BC014020	0.8091501	0.0471
AB209387	0.441644341	0.023	BC007637	0.790180564	0.0471
AK123797	0.44076246	0.023	XM_927588	0.73058784	0.0471
NM_032868	0.433465093	0.023	NM_001012968	3.004499197	0.0472
BC007732	1.393341064	0.0231	BC013422	1.569071174	0.0472
NM_001025357	0.792005837	0.0231	AX775941	0.702103019	0.0472
BC100932	0.786691904	0.0231	BC069075	0.505080879	0.0472
AK093659	0.447398692	0.0231	BC091490	1.562944531	0.0473
NM_004559	0.821762979	0.0232	XM_929734	0.709737182	0.0473
NM_002621	0.806745589	0.0232	NM_152343	0.53086853	0.0473
XM_929363	0.669034839	0.0232	AK123052	0.45686096	0.0473
NM_001010969	0.656433523	0.0232	AK123920	0.440255433	0.0473
AY841899	1.359424233	0.0233	NM_024930	1.473285437	0.0474
NM_000863	0.616199553	0.0233	NM_022463	1.284835577	0.0474
XM_927228	0.590600193	0.0233	BC069704	1.088990927	0.0474
BC053871	2.573528528	0.0234	XM_929883	0.732845724	0.0474
NM_032230	2.250483036	0.0234	NM_144505	0.708924711	0.0474
XM_932180	1.809938908	0.0234	NM_020361	0.70318234	0.0474
BC019883	0.680241108	0.0234	XM_934928	1.664152145	0.0475
BC060803	0.632241189	0.0234	NM_004043	1.601913095	0.0475
BC040264	0.444452047	0.0234	AK023413	1.396837234	0.0475
BC035060	0.630974472	0.0235	BC069344	0.836784005	0.0475
NM_033081	1.601413369	0.0236	NM_004088	0.735767782	0.0475
AY007161	0.713979602	0.0237	BC112395	0.622984171	0.0475
NM_138418	0.61617142	0.0237	AK127072	0.541363657	0.0475
NM_153007	0.592491031	0.0237	NM_001795	0.533816874	0.0475
XM_927187	0.26457727	0.0237	NM_019644	1.476652741	0.0476
BC111539	1.458922505	0.0238	NM_019036	1.298591137	0.0476
NM_001004315	0.597768128	0.0238	BC069474	0.431576163	0.0476
NM_178553	0.808848679	0.0239	BC042193	1.500165582	0.0477
AK130265	0.730974436	0.0239	XM_927891	1.434455395	0.0477
NM_018374	1.870489717	0.024	NM_005836	1.205158591	0.0477
BC025966	1.499361277	0.024	NM_148970	0.749419689	0.0477

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
NM_003251	0.710957408	0.024	BC093672	0.704312205	0.0477
NM_022573	0.691761136	0.024	BC105632	2.194403648	0.0478
BC107762	0.585693657	0.024	XM_933327	2.030615091	0.0478
AF370371	0.443298608	0.024	NM_001189	1.7324332	0.0478
NM_002869	3.231385231	0.0241	BC063439	1.363407731	0.0478
AK000177	2.258551598	0.0241	AK125720	1.299006462	0.0478
AF095771	2.233471155	0.0241	NM_006819	0.833541453	0.0478
BC075801	1.531310439	0.0241	NM_015727	0.562376201	0.0478
NM_021963	1.434504509	0.0241	NM_003339	2.342713356	0.0479
BC047231	1.403368354	0.0241	NM_001707	0.821628273	0.0479
NM_000444	0.609586239	0.0241	BC011875	0.779462039	0.0479
BC103888	1.98065567	0.0242	NM_207462	0.715328395	0.0479
BC044911	0.705379009	0.0242	NM_004734	2.785658836	0.048
NM_001004308	0.675481379	0.0242	NM_014819	1.806982875	0.048
XM_926409	0.578850389	0.0242	NM_133264	1.547192454	0.048
NM_152284	0.565005004	0.0242	BC021204	0.747672796	0.048
BC030833	2.683851004	0.0243	NM_174895	0.657960951	0.048
AK123288	1.201743364	0.0243	BC104866	0.513301909	0.048
AK000760	0.822860241	0.0243	NM_033670	1.848138571	0.0481
BC028235	0.679317236	0.0243	NM_012139	1.672466159	0.0481
BC041031	0.633750558	0.0243	NM_001012329	2.26303339	0.0482
AK091741	2.010754347	0.0244	NM_198141	1.667643547	0.0482
NM_014897	1.633131027	0.0244	NM_001037954	1.639343381	0.0482
NM_005975	0.747396767	0.0244	CR749363	1.56352067	0.0482
XM_926705	0.654211104	0.0244	NM_001447	0.821358562	0.0482
NM_145183	0.648325324	0.0244	NM_145039	2.548856497	0.0483
NM_032167	0.564814627	0.0245	BC045546	1.314815402	0.0483
BC005988	1.105754852	0.0246	NM_001032367	0.630227804	0.0483
NM_058242	0.73065418	0.0246	XM_928905	0.550402343	0.0483
AK093052	1.406701565	0.0247	NM_015253	0.546409249	0.0483
NM_004123	0.764296889	0.0247	XM_929450	0.838588595	0.0484
NM_004419	0.648509324	0.0247	NM_019852	0.799738944	0.0484
XM_071712	0.559067309	0.0247	NM_014272	0.620522976	0.0484
AK124987	0.632592738	0.0248	NM_018325	2.876763582	0.0485
NM_005332	0.617275536	0.0248	BC016326	1.513373852	0.0485
AY995211	0.593970478	0.0248	BC068587	2.122957945	0.0486
AK131378	0.592442155	0.0248	NM_001008224	1.591766119	0.0486
BC069522	0.449717581	0.0248	NM_213631	0.661707342	0.0486
NM_001008572	1.743604779	0.0249	NM_182520	0.60410893	0.0486
BC012919	1.540329814	0.0249	NM_005272	0.55237335	0.0486

SEQ_ID	Fold Change	p-Value	SEQ_ID	Fold Change	p-Value
AF145026	1.351503134	0.0249	NM_001015051	1.50541687	0.0487
XM_379543	0.482379526	0.0249	NM_005185	0.73912847	0.0487
AK127719	0.420353264	0.0249	NM_017711	0.434795022	0.0487
NM_173538	2.063655138	0.025	NM_001408	2.50877738	0.0488
NM_145263	1.310944438	0.025	AF116725	1.517647266	0.0488
AY358667	1.157944918	0.025	BC008590	1.471902013	0.0488
AK128836	0.759058058	0.025	NM_174920	0.848717928	0.0488
XM_931495	0.61503619	0.025	AK057364	0.759232521	0.0488
XM_934131	0.472247332	0.025	NM_005248	0.688583016	0.0488
XM_928757	0.428210646	0.025	BC053362	0.541387379	0.0488
NM_005006	1.980866075	0.0251	NM_032042	2.116215467	0.0489
BC074813	1.780861497	0.0251	NM_005920	1.574958444	0.0489
NM_173061	1.420737386	0.0251	XM_929976	0.782848001	0.0489
XM_499072	1.347909808	0.0251	NM_133455	0.680808783	0.0489
AY309006	1.216379046	0.0251	XM_929440	0.601975203	0.0489
XM_057107	0.795944214	0.0251	BC071656	0.444304556	0.0489
NM_017844	0.675067961	0.0251	BC038353	1.716191173	0.049
NM_005666	1.471807122	0.0252	NM_021072	1.3802526	0.049
NM_004709	1.425210834	0.0252	CR594281	0.886859059	0.049
AK058093	0.624492347	0.049	NM_020764	0.786537528	0.049