PROGESTERONE RECEPTORS IN THE HUMAN PLACENTA: EXPRESSION, SIGNALLING CHARACTERISTICS AND FUNCTIONAL RELEVANCE

A thesis submitted for the degree of Doctor of Philosophy by

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Abstract

The human placenta is a transient life sustaining organ which is responsible for mediating all the physiological exchanges between the mother and the fetus. The steroid hormone progesterone, often referred to as the hormone of pregnancy, is critical for the establishment and for maintaining the pregnancy. During the gestation period the human placenta produces progesterone which via interacting with the progesterone receptors exerts its many effects. Specific intracellular progesterone receptors (PRs) have been reported to mediate the genomic signalling of progesterone whereas recently two novel receptor families which are phylogenetically distinct to the nuclear receptor superfamily have been characterised, and shown to mediate progesterone's non genomic actions. These are the multiple membrane progestin receptors (mPRα, mPRβ, and mPRγ) and progesterone membrane receptor component 1 (PGMRC-1). The rapid progesterone actions mediated via these non-classical progesterone receptors have received attention with main focus on their reproductive functions. Our aim is to elucidate the expression of the receptors in the human placenta, further understand the signalling pathways via which progesterone mediates its effects and lastly examine the functional relevance of these receptors in this organ. Choriocarcinoma cell lines are used frequently as placental models for investigations of steroid hormone actions, but until now little is known about the expression of progesterone receptors (PRs) in these cell lines. Quantitative RT-PCR revealed that in fully syncytialized BeWo cells (treated with 50 μM forskolin for 72 h) there was a significant down-regulation of mPRα and up-regulation of mPRβ and of the PGRMC1 when compared with non-syncytialized BeWo cells. Expression of all the mPR and PGRMC1 mRNAs was significantly lower in JEG-3 cells compared to non-syncytialized BeWo cells. Expression of PR-B was unaltered between the two BeWo states but was significantly higher in JEG-3 cells. Immunofluorescence analysis revealed that mPR proteins are differentially expressed in these choriocarcinoma cell lines as well as in the human placenta. The functionality of mPRs was investigated *in vitro*, using BeWo and JEG-3 cells that were treated with Org OD-02 (a specific mPR agonist), progesterone (P4) and R5020 (a specific nuclear PR agonist) in the presence or absence of the pro-inflammatory cytokine inteleukin-1β (IL-1β) at a concentration of 10ng/μl. The effect was more exacerbated in JEG-3 cells, where IL-1β induced 40% cell death when compared to BeWo cells that reached a modest but significant 15% cell death.

When JEG-3 cells were treated with IL-1β and progesterone, there was a significant decrease in cell death at concentrations of 100nM and 1000nM. When cells were treated with 1000nM progesterone, IL-1β's effect was completely abolished. Progesterone was also able to induce phosphorylation of $ERK_{1/2}$ in these cells. Pretreatment of JEG-3 cells with a specific MAPK inhibitor (UO126) inhibited substantially the progesterone's proliferative effect. Moreover, using the specific mPR agonist Org OD 02-0, we have shown that that the progestin antagonism of apoptotic effects of IL-1β on BeWo cells is mediated through mPRs. Quantitative PCR in clinical samples revealed a 2.8 fold decrease of mPRβ in labouring comparing to non-labouring tissues and 4.6 fold higher levels of mPRγ in preterm mPRγ compared to term placentas. The ratio of mPRα to PR-B was increased in term compared to preterm samples, whereas it was decreased in labour compared to non-labour placentas. There was also a high correlation between mPRα and PGRMC1 expression irrespective of pathologies. This study addressed many fundamental questions regarding how progestins exert their effect at placental level. It is evident that there is a higher order of complexity and changes in the ratios of placental progesterone receptors rather than individual fluctuations might affect subsequent signalling events.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PUBLICATIONS

Below are the papers and presentations taken from the research presented in this thesis.

Zachariades, E., Mparmpakas, D., Pang, Y., Rand-Weaver, M., Thomas, P., Karteris, E. (2012) *"Changes in placental progesterone receptors in term and preterm labour."*. [Epub ahead of print].

Zachariades, E., Mparmpakas, D., Rand-Weaver, M., Karteris, E. (2012) *"Crucial cross-talk of interleukin-1β and progesterone in human choriocarcinoma"*. International Journal of Oncology. **40(5):**1358-64.

Zachariades, E., Foster, H., Goumenou, A, Thomas, P., Rand-Weaver, M., Karteris, E. (2011) *"Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BeWo): effects of syncytialization"*. International Journal of Molecular Medicine. **27(6):**767-74.

Mparmpakas, D., **Zachariades**, **E**., Foster, H., Kara, A., Harvey, A., Goumenou, A., Karteris, E. (2010) "*Expression of mTOR and downstream signalling components in the JEG-3 and BeWo human placental choriocarcinoma cell lines"*. International Journal of Molecular Medicine. **25(1):**65-9.

Other publications on placental cell model:

Mparmpakas, D., **Zachariades, E**., Goumenou, A., Gidron, Y., Karteris, E. (2011) *"Placental DEPTOR as a stress sensor during pregnancy"*. Clinical Science. In press.

Abstract presentations:

Zachariades, E., Mparmpakas, D., Rand-Weaver, M., Karteris, E. (2011) "*R5020 rescues Interleukin 1-beta (IL-1β) induced cell death via a MAPK induced pathway in BeWo and JEG-3 cells"*. Congress on Steroid Research, 27-29/3/2011, Chicago, USA.

Zachariades, E., Mparmpakas, D. & Karteris, E. (2010) "*Cell growth is differentially modulated by IL-1beta and progesterone in human choriocarcinoma cells"*.14th Congress of Gynaecological Endocrinology, 4-7/3/2010, Florence, Italy.

Zachariades, E., Foster, H., Goumenou, A., Thomas, P., Rand-Weaver, M. & Karteris, E. (2009) *"Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BeWo): effects of syncytialization"*. 6th International Meeting on Rapid Responses to Steroid Hormones, 2-5/9/2009, Elche, Spain.

Zachariades, E & Karteris, E. *"Expression and signalling characteristics of placental membrane progesterone receptors (mPRs)"*, 4th Annual Postgraduate Research Day, School of Health Sciences & Social Care, 2/7/2008.

Zachariades, E & Karteris, E. *"Expression and Signalling Characteristics of membrane Progesterone Receptors (mPRs) in a Human Placental (BeWo) Cell Line"*, Research Poster Conference, Brunel University, 30/4/2008- 1/5-2008.

Abbreviations

bp - Base Pairs

BSA - Bovine Serum Albumin

BeWo CT - BeWo Cytotrophoblasts

BeWo ST - BeWo Syncytiotrophoblasts

cAMP - Cyclic Adenosine Monophosphate

cDNA - Complementary Deoxyribonucleic Acid

Ct - Cycle Threshold

dH_2O - Deionized Water

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DNase - Deoxyribonuclease

dNTP - Deoxyribonucleotide Triphosphates

ERK - Extra Cellular Signal Regulated Kinase/MAPK

EtOH - Ethanol

FBS - Fetal Bovine Serum

GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase

GCM1 - Glial Cell Missing-1

GPCR - G-Protein Coupled Receptor

HSP 70 - Heat Shock Protein 70

HSP 90 - Heat Shock Protein 90

MAPK - Mitogen Activated Protein Kinases

MEK - MAPK Kinase/ ERK Kinase

MEM - Minimun Essential Medium

mPR - Membrane Progesterone Receptor

mRNA - Messenger Ribonucleic Acid

MW - Molecular Weight

P4	- Progesterone
PAGE	- Polyacrylamide Gel Electrophoresis
PBS	- Phosphate Buffered Saline
PCR	- Polymerase Chain Reaction
PGRMC-1	- Progesterone Receptor Membrane Component -1
PR	- Progesterone Receptor
PKA	- Protein Kinase A
SD	- Standard Deviation
SEM	- Standard Error of Mean
TBST	- TBT plus Tween 20
Tween 20	- Polyoxyethylene Sorbitan Monolaurat
UV	- Ultraviolet

Units

g	- Grams
M	- Molar
mg	- Milligrams
ml	- Millilitres
mm	- Milimeters
mM	- Milimolar
nm	- Nanometer
^{o}C	- Degree centigrade
µg	- Microgram
µl	- Microlitre
µM	- Micromolar
v/v	- Volume / Volume
w/v	- Weight / Volume

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1

CHAPTER 1

1. INTRODUCTION

1.1. Placental Physiology

1.1.1. Formation of the human placenta - Placentation

The placenta is the organ which a successful pregnancy outcome fully depends on. Therefore the successful development of this highly specialised organ is a very crucial step of pregnancy (Gude *et al.,* 2004). The placenta is a transient life - sustaining organ which mediates all the physiological exchanges such as oxygen and nutrient supply as well as waste removal, between the mother and the fetus (Jurisicova *et al.*, 2005; Gude *et al.,* 2004). Beyond these abilities, the placenta also poses endocrine activity and acts as an immunological barrier to prevent maternal rejection of the developing fetal allograft (Benirschke *et al*., 2005). During the gestation period which lasts 40 weeks approximately (Patel *et al.*, 2003), the development and normal growth of the fetus is supported by the placenta synergistically with the fetal membranes and the amniotic fluid (Gude *et al.,* 2004). There are different types of placentation and these are classified depending upon the number and types of layers present between the maternal and fetal circulations (Loke & King, 1995). The human placenta is classified as a haemochorial villous organ, where the maternal blood comes into direct contact with the placental trophoblast cells creating an intimate relationship between the developing fetus and the maternal nutrient source (Loke & King, 1995).

The development of the human placenta begins with a successful implantation where the outer trophoblast cells of the blastocyst invade the uterine lining to be embedded in the uterine stroma. The outer cell layer situated at the implantation pole of the blastocyst, proliferates to form the trophoblastic cell mass from which the cells infiltrate between the cells of the endometrial epithelium. These latter cells then degenerate and the trophoblast comes into direct contact with the endometrial stroma (Benirschke *et al*, 2005).

The cells of the trophoblastic cell mass may differentiate along two main pathways: the villous and extravillous pathway. Villous trophoblast cells will ultimately cover the chorionic villi of the definitive placenta and have as a major concern the transport of oxygen and nutrients from the mother to the fetus (Benirschke *et al*, 2005; Loke & King, 1995). The villi are bathed in blood and contact is only achieved with maternal cells present in the circulation. In contrast, the extravillous trophoblast cells will migrate deep into the uterine mucosa as far as the myometrium and infiltrate through uterine tissues (Benirschke *et al*, 2005; Loke & King, 1995).

1.1.1.1. Villous trophoblast cells

The appearance of buds of cytotrophoblasts protruding into the primitive syncytium in the second week of gestation is the earliest sign of villous development. The primary villi are then converted into secondary villi by penetration of the solid cytotrophoblast buds by the underlying mesenchymal cells. Their conversion into tertiary villi occurs when the mesenchymal core is supplied by embryonic blood vessels which are proximally connected to the umbilical arteries. The embryonic blood vessels which are connected with blood vessels in the developing fetus establish by the fourth week of gestation the feto-placental circulation (Benirschke *et al*, 2005; Loke & King, 1995).

During the second and third months of pregnancy, the villous tree, a characteristic of the mature placenta is achieved by rapid extension and branching of the villi. At first, the villi are covered by primitive syncytium which subsequently becomes the villous syncytiotrophoblast. During the first trimester, the villi are composed of two trophoblast layers: the inner cytotrophoblast cells situated on the basement membrane and are covered by the outer syncytiotrophoblast layer. This syncytiotrophoblast layer outlines the intervillous space and comes into direct contact with maternal blood (Fig. 1.1). The core of the villus contains loose mesenchymal cells, embryonic blood vessels as well as fetal macrophages (Benirschke *et al*, 2005; Loke & King, 1995).

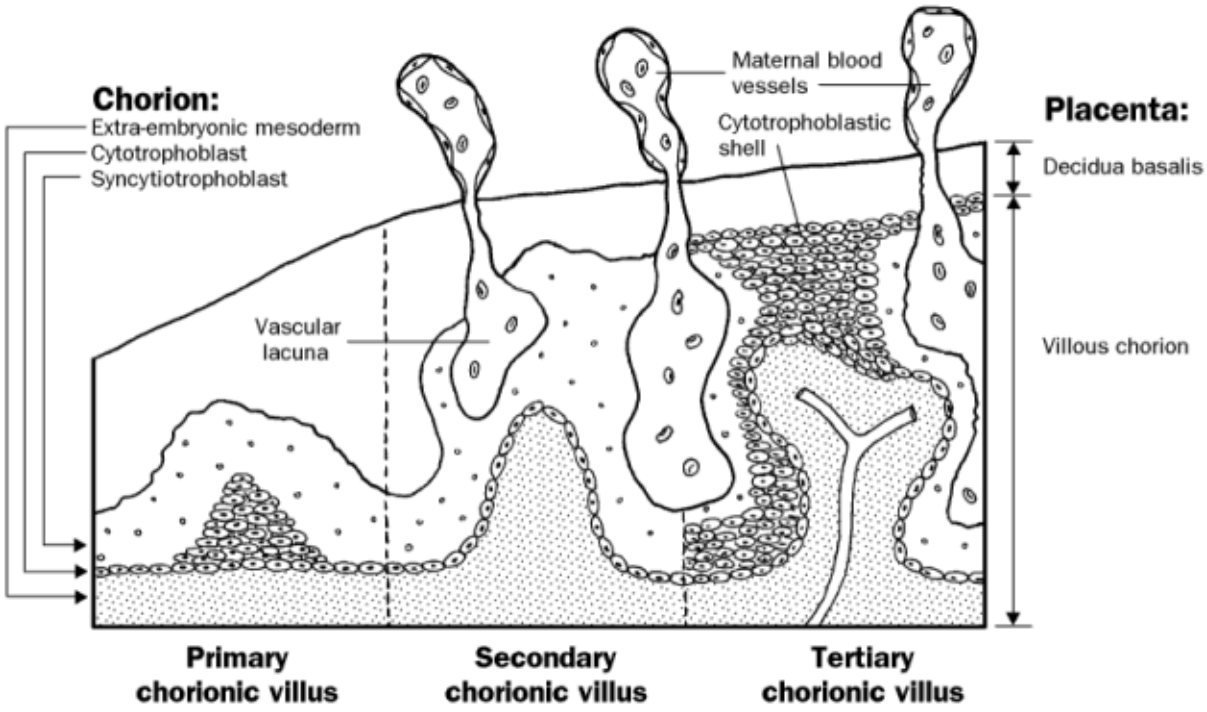


Figure 1.1: Development and structure of the placental chorionic villi. The primary chorionic villi are at the stage of outgrowths (columns) of cytotropoblast protruding into the syncytiotrophoblast. The secondary chorionic villi are formed as the extra embryonic mesenchyme invades the columns. The tertiary chorionic villi contain the embryonic blood vessels that develop from mesenchymal cells (Gest, 1999).

Through the progression of gestation the villi become smaller, whereas the cytotrophoblast layer becomes less prominent as the cells become extensively separated. During this time, the cells of the syncytiotrophoblast layer turn into more irregular and narrow cells with clustering of the nuclei. The fetal capillaries are outlined by a narrow rim of thinned syncytiotrophoblast layer. This is due to their transformation into vasculosyncytial membranes, the specialised layer responsible for gas transfer across the placenta (Loke & King, 1995).

1.1.1.2. Extravillous trophoblast cells

Following the second week of gestation, the budding cytotrophoblast cells which ultimately will form the villous tree, penetrate the primitive syncytium. At the tips of these newly formed villi the cytotrophoblast cells remain as a solid core known as the cytotrophoblast cell columns responsible for attaching the villi to the decidua. These cytotrophoblast columns are known as the anchoring villi (Loke & King, 1995). Once the penetration of the syncytiotrophoblast shell is completed, the proliferating cytotrophoblast from the proximal section of the cytotrophoblast cell columns of the anchoring villi differentiate into two distinctive subsets of extravillous cytotrophoblast. The phenotype that these cells pose enables them to invade, penetrate and remodel the maternal spiral arteries to redirect the maternal blood flow to the implantation site. This step is an important part of a successful pregnancy.

Two routes for extravillous cytotrophoblast invasion exist (Pijnenborg *et al*., 2009) with different end point results: interstitial where the extravillous cytotrophoblast invades the decidua stroma reaching up to the inner third of the myometrium where they encircle the spiral arteries and replace the vascular media with fibrinoid material converting the spiral arteries into large, dilated vessels of low resistance (Lyall, 2006) and endovascular where the

maternal blood vessels in the uterine decidua are penetrated by the extravillous trophoblasts in order to remodel the maternal blood arteries (Kaufmann *et al*., 2003).

The cytotrophoblast cell columns extend across and fuse with neighbouring columns forming a cytotrophoblast shell which encircles the entire embryonic sac. The invading trophoblast cells arise from the cytotrophoblast shell and once it has come into direct contact with the decidua, the isolated interstitial trophoblast cells penetrate the uterine mucosa. As the interstitial trophoblast cells lose contact with the cytotrophoblast shell their morphology changes where they appear as rounded uniform cohesive cells. Once the decidua has been invaded the interstitial trophoblast cells become isolated fusiform pleomorphic cells. The morphology of the nucleus changes from being a nucleus with prominent nucleoli in the columns to being a hyperchromatic pleomorphic nucleus with an irregular nuclear membrane (Loke & King, 1995).

On the maternal side of the cytotrophoblast shell and specifically in the region of the original primitive syncytium, two irregular narrow strips of fibrinoid necrosis appear and are known as the Rohr's layer and the Nitabuch's layer. Apart from these small foci of necrosis at the placental decidual junction, the invasion of maternal tissues by trophoblast cells lacks any further tissue destruction or inflammatory response. The movement of the invading interstitial trophoblast cells towards the decidual spiral arteries is a focused process as trophoblasts are located in the region of the arteries and a much less number of invading cells is found in the intervening areas. The arteries in the decidua basalis which are surrounded by trophoblasts exhibit endothelial swelling and a characteristic destruction of the muscular media which is replaced with "fibrinoid" material. This disruption of the walls of the vessels is only seen in decidual vessels in the proximity of the implantation site (Loke & King, 1995).

During the beginning of the second trimester of pregnancy, the interstitial trophoblast cells have colonised the full thickness of the uterine mucosa in an effort to reach the decidua-myometrial border (Fig.1.2). As the cells penetrate deeper layers, the trophoblast cells become multinucleated and more rounded and are known as placental bed giant cells. These cells can be regarded as the terminally differentiated end point of the extravillous pathway. During the second trimester there is further invasion into the inner myometrium. Destruction of the musculo-elastic tissues of the intra-myometrial segments of the spiral arteries occurs with a similar fibrinoid change of the existing vessel media (Loke & King, 1995).

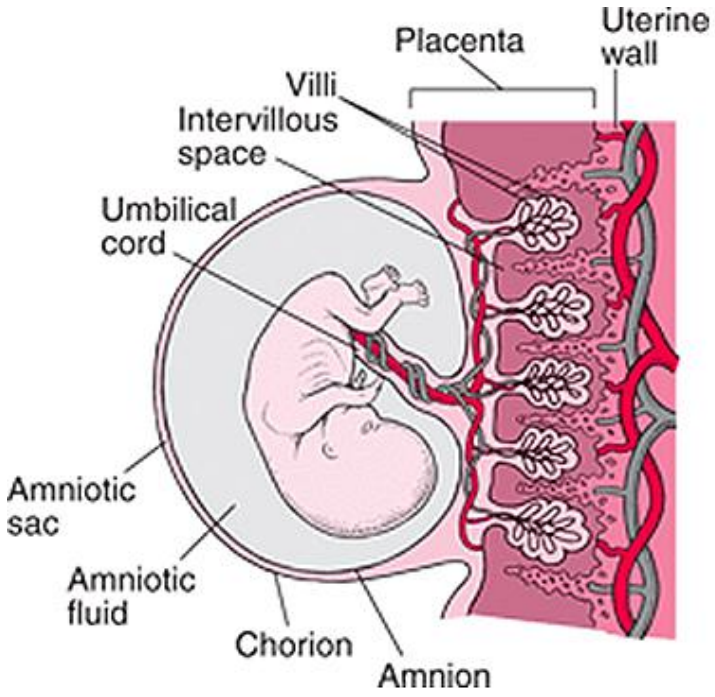


Figure 1.2: The placenta and the developing embryo, taken from: Brown, H.L. (2007); the merck manuals, stages of development of the fetus.

Endovascular extravillous cytotrophoblasts, being a separate pathway to the interstitial extravillous cytotrophoblasts, invade the lumen of the maternal spiral arteries were they form an "endovascular plug" to prevent the maternal blood from entering the intervillous space. Endovascular plugging helps to maintain a hypoxic environment in relation to that of the maternal tissues (Burton & Jauniaux, 2001) which is crucial for fetoplacental angiogenesis of the early formed tertiary villi, also for the regulation of important cellular events taking place during trophoblast differentiation and for the in-utero fetal organogenesis (Burton & Jauniaux, 2001; Caniggia *et al*., 2000). The endovascular plug eventually regresses to establish the maternal-fetal circulation, this take place around the 10th week of gestation (Chaddha *et al*., 2004).

To conclude the remodelling of the maternal spiral arteries, further differentiation of the extravillous cytotrophoblasts occurs where intramural extravillous cytotrophoblast infiltrate the walls of the spiral arteries thus transforming them into uteroplacental arteries. The endothelium is being replaced by intra-arterial extravillous cytotrophoblasts where they have adopted a vascular endothelial phenotype (Lyall, 2002). The vessels transform from being elastic, muscular, narrow and highly coiled to being widened exhibiting high flow, low-resistance and lacking vasomotor control thus allowing maximal maternal flow to reach the fetoplacental unit (Harris & Aplin, 2007).

1.1.2. Proliferation, differentiation and fusion of villous trophoblast.

There are two layers from which the villous trophoblast is composed of: the cytotrophoblast and the syncytiotrophoblast. The distinctive differences between the two define their function within the human placenta. The main characteristic of the cytotrophoblast layer is that the cells comprising it display high proliferative capacities. In contrast, the syncytiotrophoblasts have lost their generative ability and are unable to proliferate. The syncytiotrophoblast is a multinucleated layer also known as syncytium where it frames the outer surface of the

chorionic villi and it comes into direct contact with maternal blood (Huppertz & Borges, 2008). The syncytium serves many of the important functions of the placenta including the transport of oxygen, nutrients and waste products, production and secretion of steroid hormones such as progesterone and oestrogen, peptide hormones such as human chorionic gonadotrophin, human chorionic prolactin and also provide immune tolerance (Huppertz & Borges, 2008 ; Lunghi *et al*., 2007; Benirschke *et al*., 2005).

By having the ability to expand its surface area the syncytium meets successfully the increasing demands of the growing fetus. An important contributor of this is the process of trophoblast turnover which involves the proliferation, differentiation and fusion of the mononucleated cytotrophoblasts with the overlying syncytiotrophoblast (Gauster *et al*., 2009).

Cytotrophoblast proliferation is a continuous process that begins from the first trimester of gestation and continues to a lesser degree up to the end of the gestation period (Huppertz *et al.,* 1998). Throughout gestation, the growth and integrity of the placental villous trees is fully dependent on the continuous proliferation and fusion of villous trophoblast cells (Mayhew *et al*., 1994) as these progenitor cells are needed for the continual growth and regeneration of the overlying syncytium. The process of fusion is also very important as it maintains the post-mitotic syncytium alive as the cytotrophoblastic material provides organelles, enzymes and nucleic acid. If the input of cytotrophoblastic material is diminished the syncytium will become necrotic within a short period of time (Arnholdt *et al*., 1991).

As seen in Fig. 1.3, the process of trophoblast turnover takes place on two levels: on the mononucleated cell level and on the multinucleated cell level. On the first level, the cytotrophoblasts undergo proliferation from which daughter cells are produced. These cells then exit the cell cycle and start differentiating. The cells undergoing differentiation start to

accumulate high amounts of RNA, proteins and organelles. Once this is achieved the last differentiation step occurs which is the syncytial fusion with the syncytiotrophoblast layer (Benirschke *et al*., 2005; Tanaka *et al*., 1998). Evidence of the fusion includes localised confluence of cytotrophoblast and syncytiotrophoblast cytoplasms and intrasyncytial plasma membrane (Mayhew, 2001). On the second level, the incorporated materials integrate fully with the syncytiotrophoblast layer (Benirschke *et al*., 2005). Further differentiation leads to the production of hormones, transporters and other proteins which are important for maintaining the pregnancy (Huppertz, 2006).

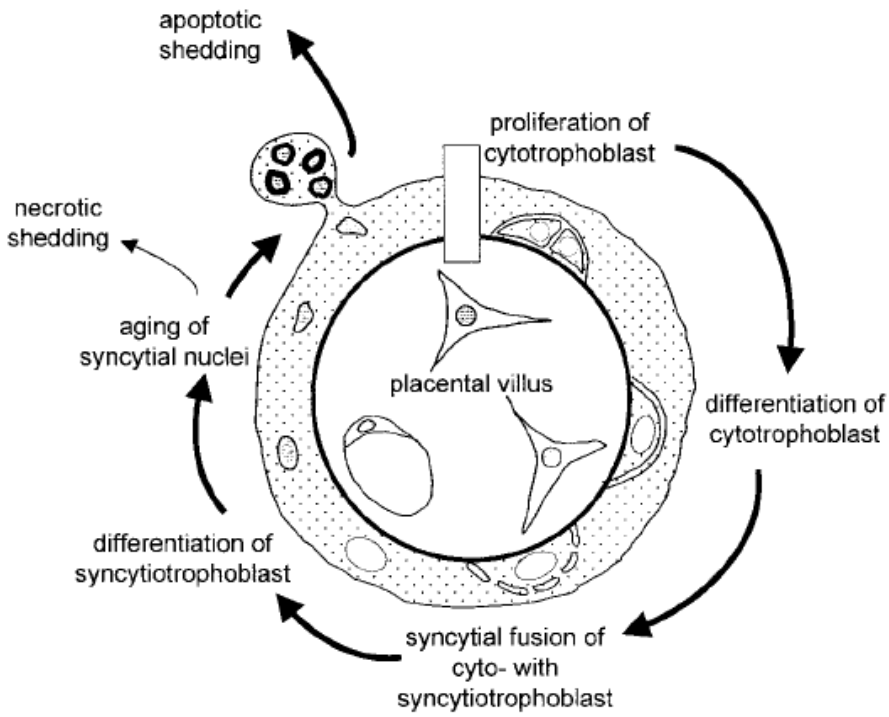


Figure 1.3 : Turnover of villous trophoblast. The cytotrophoblast cells in the stem cell layer proliferate, then undergo differentiation and finally fuse forming the syncytiotrophoblast layer. The cellular contents of the fused cells are incorporated into the syncytial layer and remain for 3-4 weeks. Following the differentiation and maturation of the cytotrophoblast cells within the syncytiotrophoblast layer the aged nuclei are extruded from the syncytial layer by apoptitic and to a lesser extent, by necrotic shedding of syncytial knots which are released into the maternal circulation. This process is performed in a continuous cycle which is tightly regulated through out gestation (Huppertz *et al*., 2002).

Finally, nuclei that demonstrate signs of ageing and late apoptosis (Huppertz *et al.*, 1998) are packed into protrusions of the apical plasma membrane of the syncytiotrophoblast layer, known as syncytial knots. The syncytial knots are released from the layer and enter the maternal circulation (Johansen *et al*., 1999) where they are engulfed and cleared in the lungs of the mother protecting her from an imminent inflammatory response (Delmis *et al*., 2000; Lee *et al*., 1986). The nuclei that have been discarded are then replaced by further fusion events (Fig. 1.3). Disruption in the homeostasis of trophoblast turnover can lead to potential increases or decreases in the material being incorporated or being removed from the syncytium layer. This will have a direct effect on the proper function of the villous membrane, where dysfunctions of the syncytium layer will result in compromised fetal growth and development seen in pathophysiological conditions including pre-eclampsia (Leung *et al*., 2001) and intra-uterine growth restriction (IUGR) (Smith *et al*., 1997).

1.1.3. Molecular basis of cytotrophoblast differentiation

1.1.3.1. Fusigenic proteins involved in cytotrophoblast differentiation

The exhaustion of the syncytial layer following limited fusion or depletion of fresh cellular components being provided within the syncytiotrophoblast must be avoided and the process of trophoblast turnover must be strictly regulated to avoid excess or restricted cytotrophoblast –syncytiotrophoblast fusion (Gauster *et al.*, 2009). To ensure the orderly progression of the differentiation process a number of different factors have been identified for regulating this process.

The regulation of the syncytialisation process remained a mystery for several years until recently where studies identified that envelope (Env) proteins derived from human endogenous retroviruses (HERVs) as key regulators of this important process (Rote *et al*.,

2004; Mi *et al*., 2000). A protein called syncytin-1 plays an essential role in the cellular fusion events in the placenta. It was shown that decreased expression of syncytin-1 was associated with the presence of pregnancy anomalies such as pre-eclampsia and hemolysis elevated liver enzymes (Wilson *et al*., 2003). However, unanswered questions remained as to the exact role played by syncytin-1 in trophoblast cell fusion and it was suggested that other HERV Env proteins might be involved in this event (Potgens *et al*., 2004; Potgens *et al*., 2002). The most attractive candidate was syncytin-2 (Malassine *et al*., 2006; Renard *et al*., 2005; Blaise *et al*., 2004; Blaise *et al*., 2003; de Parseval *et al*., 2003). It has been shown that the expression of syncytin-2 is exclusive in placental cells. Also the receptor binding to this protein has been shown to be placenta-specific opposing the findings for the receptor binding to syncytin-1 (Lavillette *et al*., 2002; Blond *et al*., 2000) thus making it a most suitable fusion candidate.

The hypothesis that syncytin-2 demonstrates a better correlation with cell fusion events and therefore might be more relevant was corroborated by recent experimental studies where it was shown that in primary trophoblast cells both were showed to be expressed at the onset of cell culturing, with a significant drop in the expression levels of syncytin-1 was observed compared to a significant increase in expression levels of syncytin-2 with time (Vargas *et al*., 2009). BeWo cells were used to perform similar experiments where cells were incubated with cell-fusion-inducing agents and a parallel increase in the level of mRNA of both syncytin-1 and syncytin-2 was detected. Notably, syncytin-2 was very poorly expressed in nonstimulated cells and presented a more drastic increase in its expression upon stimulation (Vargas *et al*., 2009).

Further studies have been conducted to expose any differences in the localisation of these two proteins in primary trophoblast cultures and in BeWo cells. In villous trophoblasts it was shown that syncytin-2 is expressed in the membrane and in the cytoplasm. In the membrane

the signals obtained where in regions of cell to cell contact showing specific localisation as being expected from its potential function in trophoblast cell fusion (Vargas *et al*., 2009). In second trimester placenta expression of syncytin-2 was more frequently seen in cytotrophoblastic cells at the level of the cell membrane and this staining occurred at the sites of contact with the overlying syncytiotrophoblast (Malassiné *et al*., 2006).

However, syncytin-1 appeared close to the region of cell to cell contact (Vargas *et al*., 2009). Also the expression was not affected by the differentiation stage of which the cells were in and an equal expression was observed in all trophoblastic cells, villous and extravillous trophoblast, independently of their differentiation stage (Muir *et al*., 2006; Malassine *et al*., 2005). Based on the localisation, an involvement in cell fusion might be speculated but the expression was equally presented in stimulated and unstimulated BeWo cells (Vargas *et al*., 2009).

The fusigenic activities of syncytin-2 are mediated trough the major facilitator superfamily domain containing 2A (MFSD2A) which is the receptor for syncytin-2 mediated cell-cell fusion. In the human placenta, both syncytin-2 and MFSD2A have been shown to be expressed (Esnault *et al*., 2008).

Esnault et al., have proposed an "in-fusion" cytotrophoblast model (Fig. 1.4) where the two partners involved in the cell fusion process – Env protein and concomitant receptor are finely regulated and not being expressed in parallel on the same cell type therefore allowing for the specific fusion between cytotrophoblast and syncytiotrophoblast to take place. This ability restricts fusion of mononucleated cytotrophoblast cells to "in-fusion" into the syncytiotrophoblast thus allowing the growth and maintenance of the syncytium layer and promoting an orderly organization of the syncytiotrophoblas (Esnault *et al*., 2008).

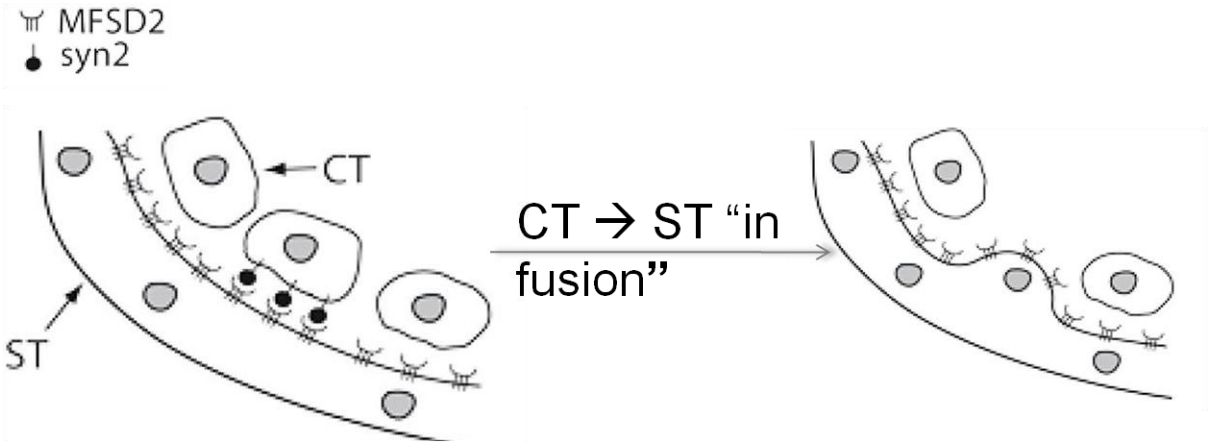


Figure 1.4: ''In-fusion'' model of the cytotrophoblasts (CT) into the syncytiotrophoblast (ST) layer. This modes proposes that syncytin-2 protein is expressed on a fraction of the cytotrophoblasts cells and major facilitator superfamily domain containing 2 (MFSD2) is expressed on the syncytiotrophoblast cells thus allowing for a controlled fusion and subsequently maintenance of the syncytiotrophoblast layer (Adapted from Esnault *et al*., 2008).

Recent studies devoted to understanding how the expression of syncytion-2 and MFSD2A are regulated in the placenta have shown that the expression of these genes is regulated by the glial cell missing-1 (GCM1) placental transcription factor (Liang *et al*., 2010). The functional importance of this transcription factor is stressed in studies performed in mice, where both syncytiotrophoblast differentiation and formation of the chorionic villi are regulated by GCM1. Mouse embryos that lack GCM1 die *in utero* mid-gestation as the syncytiotrophoblast fails to differentiate (Baczyk *et al*., 2004). Up- regulation of GCM1 results in less cytotrophoblast proliferation, induction of syncytial fusion and the subsequent formation of syncytiotrophoblast (Baczyk *et al*., 2009). The expression of GCM1 was confirmed on clustered trophoblast where each contained 3-4 cells that were uniformly distributed on the basal surface of the chorion. The majority of the chorionic trophoblast cells do not express GCM1 suggesting that the chorion does not consist of a uniform cell population (Anson-Cartwright *et al*., 2000).

Other fusogenic proteins that have been shown to be involved in the process of syncytial fusion in the human placenta include the ADAM proteins (disintegrin and metalloprotease) which belong to the family of integral membrane or secreted glycoproteins (Huppertz *et al*., 2006). A study performed by Ito et al., reported the presence of ADAM12 on the apical part of syncytiotrophoblasts in term placentae (Ito *et al*., 2004). Even though the localisation of this protein does not provide evidence of an involvement in syncytial fusion it may well provide a proteolytic activity needed for the cleavage of certain proteins thus preparing the process of fusion (Huppertz *et al*., 2006).

1.1.3.2. Signalling pathways involved in trophoblast fusion -MAPK

Apart from the fusogenic proteins discussed above that regulate the fusion of the trophoblast cells, the trophoblastic fusogenic machinery is also finely tuned by a number of other signalling molecules (Delidaki *et al*., 2010). The mitogen activated protein kinases (MAPKs) are a family of protein kinases responsible for converting extracellular stimuli into specific cellular responses; among them are the control of cell proliferation, differentiation, apoptosis, embryogenesis, regulation of inflammatory and stress responses (Kyriakis & Avruch, 2001 ; Pearson *et al*., 2001).

The extracellular-signal-regulated kinases **(**ERK) pathway (Fig.1.5) was the first MAPK pathway to be described in mammals. ERK_1 and ERK_2 ($ERK_{1/2}$) can be strongly activated in mammalian cells by growth factors, serum and phorbol esters. Ligands of heterotrimeric G protein-coupled receptors (GPCRs) such as hormones and neurotransmitters can activate to a lesser extent the $ERK_{1/2}$ along with cytokines and osmotic stress (Daoud *et al*., 2005). The p38 pathway, another member of the MAPK, in mammalian cells is strongly activated by environmental stress and by pro-inflammatory cytokines such as intereukin 1 (IL-1) and tumour necrosis factor α (TNFα) (Daoud *et al*., 2005).

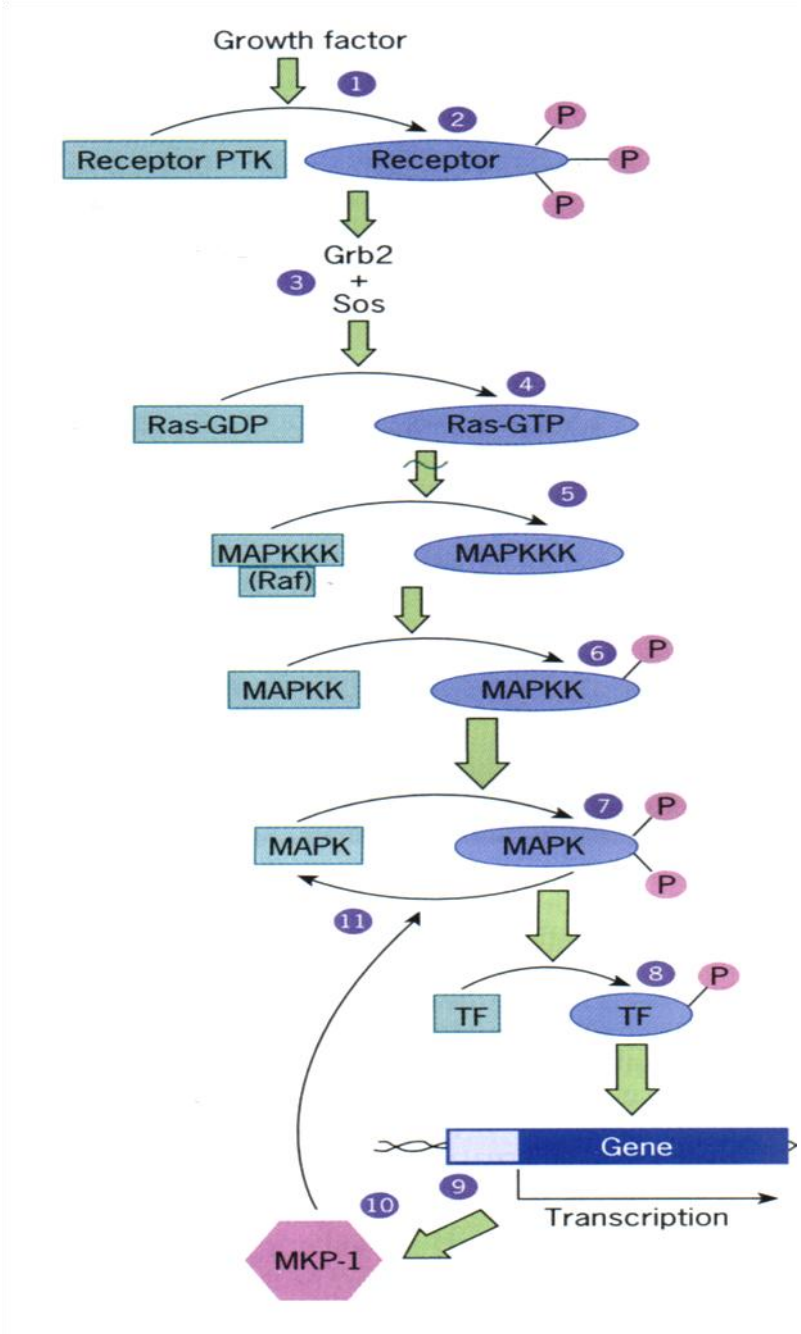


Figure 1.5: Schematic representation of the ERK pathway**.** The ERK pathway is activated through the binding of the adaptor proteins Growth factor receptor-bound protein 2 (Grb2) or Shc to the phosphorylated, which results in the recruitment of the son of sevenless (SOS) to the activated receptor SOS activates RAS thus leading to the activation of RAF. RAF subsequently phosphorylates MEK1 and MEK2 which activate ERK_1 and ERK_2 respectively.

Studies using choriocarcinoma cell models and primary villous trophoblasts have proven the important role of the MAPK cascade in the process of trophoblast differentiation (Delidaki *et al*., 2010). $ERK_{1/2}$ activation was shown to be essential in the initial stage of epithelial tubule development, the stage where the cells migrate and depolarize (O'Brien *et al*., 2004). Also the importance of $ERK_{1/2}$ was demonstrated in the sea urchin embryo where $ERK_{1/2}$ was essential in the stage where the cells repolarise and differentiate (Fernandez-Serra *et al.* 2004). However, the molecular actions adopted by $ERK_{1/2}$ and by p38 by which their activity in trophoblast fusion is coordinated, are not fully understood yet (Delidaki *et al*., 2010).

Apart from the involvement of the MAPK family in trophoblast fusion, the formation of the syncytiotrophoblast cell layer in humans had been shown to be regulated by the cAMP/PKA signalling cascade (Keryer *et al*., 1998), demonstrating an initial understanding of the signalling pathways involved in this process. Extending these first observations, studies performed in BeWo cells showed that the activation of the syncytin-1 gene is controlled by the cAMP pathway through regulation of glial cell missing (GCMa) (Chang *et al*., 2005; Knerr *et al*., 2005). Forskolin and PKA are able to stimulate GCMa mediated transcriptional activation while CREB-binding protein (CBP) directly interacts with GCMa. PKA is responsible for facilitating the interaction between CBP and GCMa promoting the acetylation of GCMa thus increasing the stability of the protein and enhancing the transcriptional activation mediated by GCMa (Chang *et al*., 2005). Thus the GCMa/ syncytin signalling cascade which directly regulated the fusion of trohoblast cells is contolled by the cAMP/ PKA signalling cascade (Knerr *et al*., 2005).

A potential signalling cross-talk between the cAMP/PKA and MAPK pathways was recently introduced by Delidaki and co workers where they demonstrate that $ERK_{1/2}$ and p38 pathways are activated downstream of adenylyl cyclase and play an essential role in the upregulation of specific fusogenic genes regulating the trophoblast differentiation process.

The cAMP – dependent PKA in trophoblasts is responsible for mediating the biological effects of cAMP influencing cell differentiation. $ERK_{1/2}$ and p38 MAPK activation induced by forskolin has also been shown to be mediated by PKA (Delidaki *et al*., 2010).

1.1.4. Models of human placental function

Understanding the development and the physiological processes of the human placenta has been pursued by scientists for many years. However, numerous difficulties arise in obtaining human placental samples and impede the research performed on human level. Overcoming the ethical issues and obtaining placental samples from vaginal deliveries or elective caesarean sections performed at term or pre-term is still not optimun as maintaining explants in culture is a restrictive step due to the limited viability of only 7 to 24 hours (Di Santo *et al*., 2003). Performing studies using placental tissue can be limited also by the fact that this tissue has been exposed to stress at birth and to ischaemia prior to reaching the researcher, therefore its normal physiological state has been compromised and barely reflect an *in vivo* situation (Di Santo *et al*., 2003).

In response to these difficulties, cell lines established from normal cells or cells from trophoblastic tumors of the human placenta have been widely used to study and understand key functions of the human placenta such as development, endocrine and immune function as well as signal transduction mechanisms of trophoblast cells (King *et al*., 2000).

1.1.4.1. BeWo cell line

The most popular and extensively used *in vitro* mode is the BeWo cell line which is derived from trophoblastic cells originated from human choriocarcinoma. Choriocarcinomas are defined as highly malignant tumours that arise from the trophoblast cells (Berkowitz & Goldstein, 1996). It has been documented that around 50% of choriocarcinomas follow a molar pregnancy where a mass is grown inside the uterus, 30% occur after a miscarriage and

20% after an apparently normal pregnancy (Fox, 1997). The hormonal and cellular metabolic functions exhibited by this tumor resemble the functions of the normal placenta.

In 1959, Hertz isolated the original choriocarcinoma specimen which was then successfully established by serial transplantations in the hamster cheek pouch (Hertz, 1959). Following this and using the same original specimen, Pattillo and Gey established the first continuous human hormone-synthesizing tissue in cell culture (Pattillo & Gey, 1968). This tissue has been reported to have maintained stable growth characteristics, with production of the multiple placental hormones secreted during pregnancy and are hallmarks of a normal pregnancy specifically, it exhibites a high degree of synthesis of the functional hormone hCG (human chorionic gonadotrophin). In addition, many aspects of placental-cell metabolism, growth, and differentiation are seen in this unique multipotential cell line (Pattillo *et al*., 1971; Patillo *et al*., 1968).

Wice *et al*., were the first to define the *in vitro* differentiation system where single cytotrophoblast cells of the human choriocarcinoma (BeWo) cell line underwent fusion and extensive morphological differentiation following stimulation with forskolin (Wice *et al*., 1990). Forskolin is produced by the plant coleus forskohlii which is the only known plant containing the diterpenoid forskolin. Once in the cell, forskolin activates adenylate cyclase, which subsequenlty activates cyclic adenosine monophoshate (cAMP), an intracellular second messenger which is responsible for inducing the cellular responses in response to hormonal activation (Bhat, 1983).

Incubation of cells with forskolin demonstrated a dose-dependent increase in intracellular and secreted cyclic AMP and a coordinated fusion of cells which yielded syncytia containing hundreds of nuclei per cytoplasm. Also these fused cells synthesized and secreted large amounts of human chorionic gonadotropin (Wice *et al*., 1990). A different inducer of cell

differentiation that can be used to promote differentiation is the 8- Bromoadenosine- 3', 5'-cyclic monophosphate (8-Br-cAMP) an exogenous cAMP analogue where the hydrogen in position 8 of the heterocyclic nucleobase is replaced by bromine making the compound more lipophilic able to readily diffuse through the plasma membrane (Chen *et al*., 2011).

As mentioned, the BeWo cell line has been extensively used for a variety of *in vitro* studies (Burleigh *et al*., 2007), including preventive and therapeutic agents for preeclampsia (Orendi *et al*., 2011; Gould *et al*., 2010), and placental transport of compounds and molecules (Sønnegaard Poulsen *et al.*, 2009 ; Jones *et al*., 2007; Schmid *et al*., 2003).These studies have established BeWo cells as a good *in vitro* model for studying placental functions, and the fusigenic ability of these cells enabling them to undergo fusion *in vitro* has further facilitated an increased scientific understanding of many aspects of the placental physiology and function.

1.1.4.2. JEG-3 cell line

The JEG-3 cell line was established by Kohler in 1971 and was a part of six clonal lines of human choriocarcinoma origin that were previously adapted to passage in the hamster cheek pouch by Hertz in 1959 (Kohler & Bridson, 1971). The morphology of JEG clones has been reported to vary among clones, but all clones including JEG-3 grew as individual cells but syncytia were not visible. All JEG clones were found to be active in an endocrine sense (Kohler & Bridson, 1971; Kohler *et al*., 1971).

It is well accepted that JEG-3 cells are unable to fuse upon stimulation with a cAMP analog (8-bromocyclic AMP) unlike BeWo cells (Coutifaris *et al*., 1991). More specifically, in a study where the Ca^{2+}-dependent cell adhesion molecule, E-cadherin, was examined during the morphological differentiation in trophoblastic cells including cells from JEG-3 and BeWo choriocarcinoma cell lines and cytotrophoblast cells isolated from human chorionic villi, it

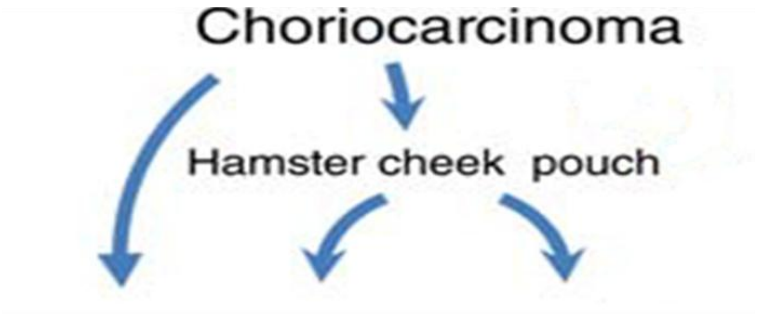
was shown that JEG-3 cells are unable to fuse and form multinucleated syncytia (Coutifaris *et al*., 1991).

The JEG-3 cell line has been widely used as a model for different placental actions such as placental metabolism of retinol (Blanchon *et al*., 2002), the effects of cytokine on steroid production (Pongcharoen & Supalap, 2009), and the transport of substances across the human placenta (Hardman *et al*., 2006; Müller *et al*., 2004).

1.1.4.3. Other trophoblast cell lines

Another trophoblastic cell line that is been used as a placental model is the JAR cell line. The JAR cell line was established by Pattillo and colleagues in 1971 and was established directly from a biopsy specimen of a trophoblastic tumor of the placenta. It has been shown to have secretory capabilities of various hormones including the steroid hormones estrogen and progesterone, the lactogen hormone hPL and the gonadotrophic hormone hCG. JAR cells were used in placental immunology studies (Apps *et al*., 2009 ; Jianga *et al*., 2006), apoptosis studies (Hallmann *et al*., 2004), and signalling studies (Boileau *et al*., 2001).

In our study we chose to use the BeWo and JEG-3 cell lines as their inherent difference in their fusigenic capacity were ultimate for the aims of this study.These being to evaluate the expression and signalling characteristics of progesterone receptors in first trimester and third trimester placental cells. This opportunity is provided by using BeWo cells in their unstimulated (first trimsester) and stimulated (third trimester). JEG-3 cells being unable to syncytialise provide an excellent comparison between the first trimester placental cells (unstimulated BeWo cells) and also provide an independent



JAR	BeWo	JEG-3	
+	+	+	hCG
+	+	+	hPL
+	+	+	Invasion
	+		Syncytialisation
Patillo *et al*., 1971	Patillo & Gey, 1968	Kohler & Bridson, 1971	Reference

Figure 1.6: Summary figure of the choriocarcinoma cell lines used as models along with the hormone secretion and characteristics of each cell line.

1.2. Steroid hormone Progesterone and its actions

1.2.1. Physiological actions of progesterone during pregnancy

Progesterone (P4) is a steroid hormone critical for the establishment and maintainance of pregnancy (Niswender *et al*., 2000). In the uterus, progesterone exerts its effects on the endometrium as a differentiation factor where it induces differentiation of the stromal cells of the endometrium; it stimulates glandular secretions along with the accumulation of basal vacuoles in the glandular epithelium (Maslar *et al*., 1986) and alters the secretion pattern of proteins by endometrial cells (Maslar *et al*., 1986; Strinden & Shapiro, 1983). These changes enforced on the uterus by progesterone are essential to provide a supportive environment for early embryonic development (Niswender *et al*., 2000). Progesterone is also responsible for preventing preterm labour by maintaining the myometrium in a quiescent state (Larsen, 2001;

Niswender *et al*., 2000). Electrical coupling is prevented and increased resting potential is provoked by progesterone in the myometrial cells to assure the myometreium remains in a non-contractile state (Parkington, 1983). Additionally, progesterone decreases the uptake of extracellular calcium that is necessary for myometrial cell contractions (Batra, 1986) through downregulation of genes encoding subunits of voltage-dependent calcium channels which are the entering points of extracellular calcium into the cell (Tezuka *et al*., 1995). Apart from its effects on the uterus, progesterone is responsible for mediating interactions between the endocrine and immune system to establish a friendly immunological environment for the developing fetus (Beagley & Gockel, 2003). This is achieved by progesterone induced blocking of mitogen stimulated lymphocyte proliferation, prolonging the survival of the allograft. Furthermore, progesterone exerts pluripotent effects during human gestation such as modulating the production of antibodies, reducing the production of macrophage induced secretion of proinflammatory cytokines and adapting the cytokine pattern during pregnancy (Beagley & Gockel, 2003).

1.2.2. Synthesis and secretion of progesterone by the human placenta

An essential role of the human placenta is to synthesise and secrete progesterone to maintain the pregnancy, where its secretion increases throughout pregnancy (Fig 1.7 Panel B) (Tuckey, 2005). After the 8^{th} week of gestation the placenta becomes the primary source of progesterone production by expressing cholesterol side chain cleavage activity (Brook & Marshall, 2001).The cholesterol side-chain cleavage reaction involves the conversion of cholesterol to pregnenolone by cytochrome P450scc (CYP11A1). This reaction takes place in the inner membrane of the placental mitochondria (Tuckey, 2005). The newly synthesised

pregnenolone is converted to progesterone by the action of type 1 3β-hydroxysteroid dehydrogenase (3β-HSD) (Fig.1.7 Panel A) (Beaudoin *et al*., 1997; Morel *et al*., 1997).

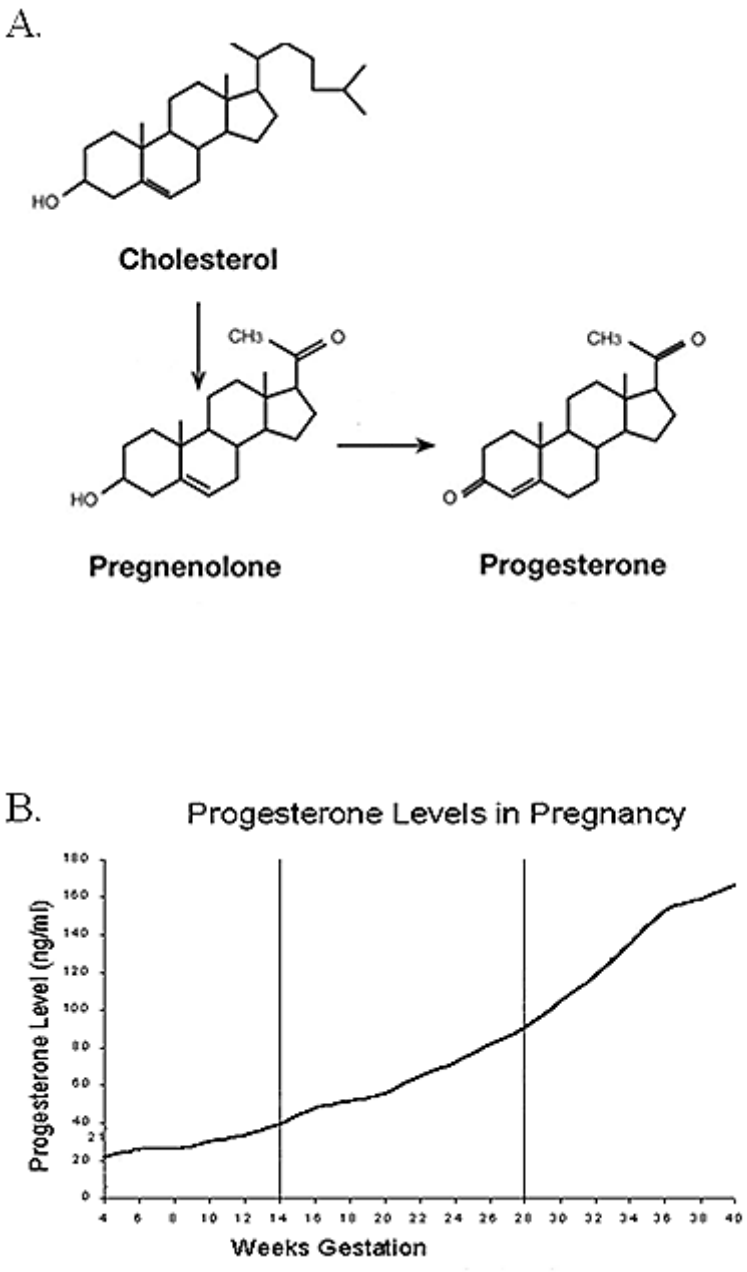


Figure 1.7: Panel A: Steroidogenesis – Progesterone is synthesised from pregnenolone, which is derived from cholesterol (The Hormones : Progestins, 1996), Panel B: Levels of Progesterone during pregnancy (Pope Paul VI Institute, National Reproductive Hormone Laboratory).

The synthesis of progesterone by the placenta shows some differences in comparison to other steroidogenic tissues such as the adrenal glands and the corpus luteum (Tuckey, 2005). A main difference is that the placenta does not express the steroidogenic acute regulatory (StAR) protein (Tuckey, 2005). This StAR protein regulates the binding and transport of cholesterol to the inner mitochondrial membrane where it can be taken up by P450scc, and subsequently converted to pregnenolone (Miller, 2006 ; Tuckey, 2005). Although the placenta does not express StAR, a successful pregnancy is not prevented (Miller, 1998), and metastatic lymph node 64 protein (MLN64), a protein closely related to the StAR protein, is expressed constitutively in the placenta and is involved in the transport of cholesterol in the mitochondria (Strauss *et al*., 2000). Even though research is in its infancy regarding this protein and its mode of action, MLN64 in the placenta is a key player in maintaining an unrestrictive supply of cholesterol to the inner mitochondrial membrane for placental progesterone synthesis (Tuckey, 2005).

Whilst in steroidogenic tissues the rate-limiting step to the production of steroid hormones is the delivery of cholesterol to the P450scc site in the mitochondria, in the placenta the rate-limiting step for P450scc catalysis is the reduction of adrenodoxin (Adx) by adrenodoxin reductase (AR). Therefore the reduced concentration of AR in the matrix of the mitochondria is insufficient to maintain the Adx pool in a fully reduced state and deliver the electron supply that is required to P450scc (Tuckey, 2005). Adrenodoxin reductase (AR) and its redox partner, adrenodoxin (Adx), are responsible for providing a short electron transport chain for the delivery of six electrons from three molecules of NADPH to P450scc which are required for the side chain cleavage of cholesterol. AR and Adx are located in the mitochondrial matrix whereas P450scc is attached to the matrix of the inner mitochondrial membrane (Tuckey *et al*., 1997).

The regulation of placental progesterone synthesis is not acutely regulated as it is in other steroid-producing organs, and this is evident from the absence of short term fluctuations in maternal progesterone during pregnancy (Tuckey, 2005). The progesterone production and secretion from the syncytiotrophoblasts during the first weeks of pregnancy is insufficient to maintain the healthy progression of gestation as luteal failure results in abortion (Miller, 1998). The transition from low blood supply and a hypoxic environment to a high blood and sufficient oxygen supply in the placenta stimulates the production of progesterone as oxygen is an essential substrate for the cholesterol side-chain cleavage reaction (Cannigia *et al*., 2000; Schneider, 2000).

1.2.3. Progesterone Receptors

Progesterone is a steroid hormone which plays a central role in the establishment and the maintenance of human pregnancy by regulating a number of reproductive effects. Besides from the placenta, progesterone has been proven to affect the uterus, ovaries, mammary glands and brain, but it also plays an important role in non-reproductive tissues such as the cardiovascular system, bone and the central nervous system, thus highlighting the extensive role of this hormone in humans (Li *et al*., 2004; Graham & Clarke, 2002; Conneely & Lydon, 2000; Graham & Clarke, 1997). Numerous physiological effects that progesterone exerts are mediated via interactions of the hormone with specific intracellular progesterone receptors (PRs) (Conneely *et al*., 2002).

1.2.3.1. Nuclear Progesterone Receptors

Nuclear progesterone receptor (PR) belongs to the super-family of nuclear receptors (Bramley, 2003; Aranda &Pascual, 2001) which are believed to be evolutionary maintained and derived from a common ancestor (Aranda & Pascual, 2001). The nuclear receptor superfamily is subdivided in six different subfamilies (Laudet, 1997). In the first subfamily

thyroid hormone receptors, retinoic acid receptors, vitamin D receptors and peroxisome proliferator-activated receptors are members. The second subfamily includes the retinoid X receptors, hepatocyte nuclear factor 4, testis receptors and the receptors involved in the eye development (TLX and PNR). The members of third subfamily (Fig.1.8) include the steroid receptors and the highly related orphan estrogen-related receptors (Aranda & Pascual, 2001). The fourth, fifth, and sixth subfamilies contain the orphan receptors NGFI-B, FTZ-1/SF-1, and GCNF, respectively (Aranda & Pascual, 2001; Giguere, 1999).

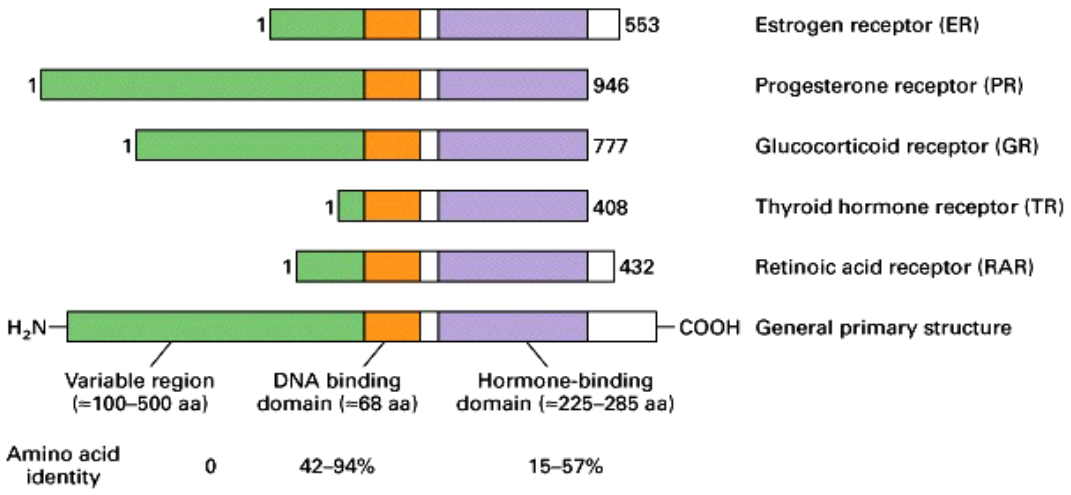


Figure 1.8: Primary structure of the human nuclear receptors and the major representatives of each subfamily (Adapted from Wahli & Martinez, 1991).

The nuclear receptors and hence the PR as shown in Fig 1.9, consist of a variable N-terminal domain (A/B domain), a conserved domain that binds DNA (DBD or domain C), a hinge domain (D domain), a ligand –binding domain (LBD) also known as domain E which is conserved among the nuclear receptors (Fig. 1.9) (Hirata *et al*, 2002; Aranda & Pascual,

2001). The N-terminus contains an activation-function-1 (AF-1) which contributes to the integral ligand- independent activation by the receptor. A second activation domain, known as AF-2 is situated on the C-terminus of the LBD but in contrast to the AF-1 domain, AF-2 is dependent upon the ligand and it is found to be conserved among all the members of the nuclear receptor superfamily (Fig.1.9) (Aranda & Pascual, 2001). AF-3, a third activation function is found within the N-terminus domain and it is specific to PR-B isoform (Fig. 1.10) (Li & O'Malley, 2003).

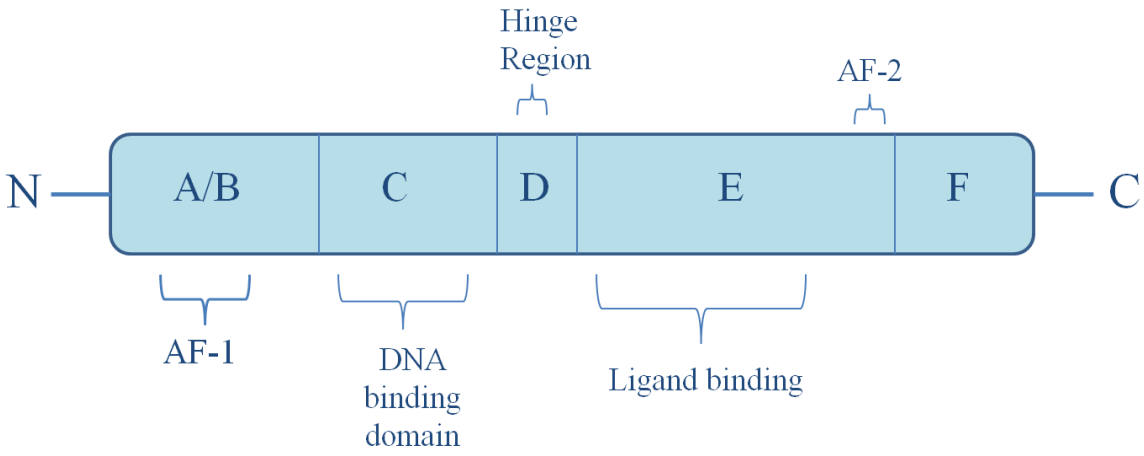


Figure 1.9: Schematic representation of a nuclear receptor. A typical nuclear receptor is composed of several functional domains. The variable NH2-terminal region (A/B) contains the ligand-independent AF-1transactivation domain. The conserved DNA-binding domain (DBD), or region C, is responsible for the recognition of specific DNA sequences. A variable linker region D connects the DBD to the conserved E/F region that contains the ligand-binding domain (LBD) as well as the dimerization surface. The ligand-independent transcriptional activation domains contained within the A/B region, and the ligand-dependent AF-2 core transactivation domain within the COOH-terminal portion of the LBD (Adapted from Aranda & Pascual, 2001).

Progesterone mediates its biological effects through two PR isoforms, PR-A and PR-B. In humans, a single gene consisting of eight exons (Misrahi *et al.*, 1993) generates both mRNA transcripts through two alternative promoters and translational initiation at two different AUG codons (Kastner *et al*., 1990; Conneely & Lydon, 2000). The structural difference between PR-B from PR-A is the additional extension of 164 amino acids residing in the N-terminus of the protein (Li & O'Malley, 2003).

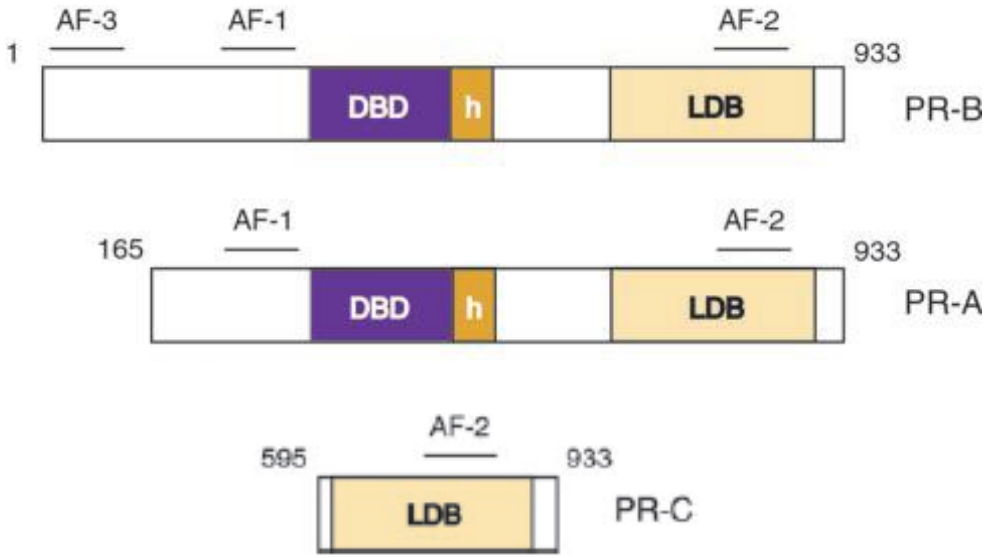


Figure 1.10: A schematic representation of the domain organization of the PR variants PR-A, PR-B & PR-C; h: hinge region; LDB: ligand binding domain; DBD: DNA binding domain. The numbers denote the positions of amino acids for each isoform proteins. AF-1, -2, and -3 are transcription activation domains (Li & O'Malley, 2003).

The two PR isoforms are able to elicit distinct physiological responses to progesterone as this has been demonstrated by selective ablation of these proteins in mice (Conneely *et al*., 2002) where pleiotropic reproductive abnormalities were observed (Lydon *et al*., 1995). The phenotypic mutations seen on the function of the female reproductive system proved the idea

that each PR isoform had an individual contribution, as distinct transcriptional responses to PR-A and PR-B were seen in cell-based transactivation assays (Conneely *et al*., 2002).

It was shown in mice lacking both nuclear PR isoforms that ovulation had failed to occur despite the presence of normally developed follicles that possess mature oocytes (Lydon *et al.,* 1995). In the endometrium, selective knockout of nuclear PR, revealed that progesterone drives the differentiation of stromal cells upon activation of the PR-A isoform. Female mice that lack PR-A isoform were shown to be sterile due to defective ovulation, implantation and decidualisation (Mulac- Jericevic & Conneely, 2004).

Progesterone also exerts its actions on the myometrium, and using anti-progestins (RU486) to induce parturition, it has been shown that nuclear PRs contribute to the relaxation of the myometrium during pregnancy (Avrech *et al*., 1991). The onset of labour is been regarded as the result of decreased myometrial progesterone responsiveness, also known as 'functional progesterone withdrawal' (Mesiano, 2007; Brown *et al*., 2004; Astle *et al*., 2003). In humans, the PR-B isoform is important for maintaining the myometrium in a relaxed state, whereas PR-A isoform upregulation at term can contribute towards adopting the contractile phenotype (Merlino *et al.,* 2007). In the breast the role of the nuclear PR mediating the progesterone actions are strongly supported by studies performed in PR knockout (PRKO) mice. Impaired mammary gland development which was characterized by decreased pregnancy-associated side branching of the ductal epithelium was the results of a null mutation for both nuclear PR isoforms (Lydon *et al.,* 1995). In PR-A knockout mice, progesterone is able to elicit side-branching and lobular–alveolar development, but this is not evident in mice with a PR-B ablation. In contrast, to the lower female reproductive tract, the expression of PR-B is sufficient for mediating the proliferation and differentiation actions of progesterone on the mammary gland (Mulac-Jericevic & Conneely, 2004; Mulac-Jericevic *et al.,* 2003; Mulac-Jericevic *et al.,* 2000).

Further to the two well characterised PR isoforms, a third isoform PR-C has also been described (Wei & Miner, 1994). The expression of this third isoform is regulated from alternate initiation sites of translation on PR mRNA in target tissues in humans. The functional role of PR-C is less well explored (Ogle, 2002). The first description of the presence of PR-C was received by studies using the human breast cancer cell line T47D (Wei & Miner, 1994). Since, it has been translated from a partial PR cDNA and it has been shown to be an N-terminally truncated form of PR-A and PR-B with a molecular mass of 60 kDa (Fig 1.10) (Samalecos & Gellersen, 2008; Wei *et al*., 1996). PR-C contains the PR domains found upstream of the second zinc finger motif of the DND-binding domain, including sequences necessary for dimerazitaion and for ligand binding. Although it is transcriptionally silent it can modulate the transcriptional activity of the other two PR isoforms (PR-A and PR-B) proposing an ability to form heterodimers (Wei *et al*., 1996).

The progesterone receptor is activated upon progesterone binding to the ligand binding domain. A conformational change in the structure of the receptor facilitates the dissociation of the receptor from the multiprotein sequestering complex that consists of protein chaperones including heat shock proteins (hsp 70 & hsp 90) and immunophilins (Li & O'Malley, 2003). Receptors then homodimerize and bind to progesterone – responsive elements (PRE) (Li & O'Malley, 2003; Allan *et al*., 1992) located in the promoter regions of target genes (Conneely & Lydon, 2000). Target gene transcription rates can be increased through additional interactions of the DNA-bound receptors with steroid coactivators and the transcription machinery, where the assembly of the preinitiation complex at the promoter is facilitated (Li & O'Malley, 2003).

The coactivators for the progesterone receptor steroid receptor coactivator-1 (SRC-1), steroid receptor coactivator-2 (SRC-2), steroid receptor coactivator-3 (SRC-3), CREB-binding protein (CBP) and the related protein p300 are the most important coregulators involved in

enhancing the effect on target gene transcription. This is achieved through multiple mechanisms, including stabilization of nuclear receptors and the basal transcription machinery at the promoter; covalent modification of histones, activators, other coregulators; and possibly turnover of activators and other proteins (Rowan & O'Malley, 2000).

SRC-1 interacts with the PR through the AF-2 containing LBD and also it interacts with the AF-1 domain through multiple regions of SRC-1 (Onate *et al*., 1998) thus bringing together both activation functions of the receptor in order to achieve maximal transcription at the target genes. The function of SRC-1 is mediated by the two activation domains which are important for the interactions with other activators or the basal transcription machinery (Onate *et al*., 1998), by the binding domains for CREB binding protein (CBP) and the histone acetyltransderase (HAT) p/CAF and an intrinsic HAT activity which gives SRC-1 a role in the remodelling of chromatin (Spencer *et al*., 1997). The biological importance of SRC-1 for the actions of progesterone has been documented in SRC-1 null mice which exhibited partial hormone resistance in the main target tissues of progesterone the mammary gland and uterus (Xu *et al*., 1998). Similarly to SRC-1, SRC-2 and SRC-3 are able to interact with the PR in a hormone-dependent manner and to coactivate it (Rowan & O'Malley, 2000; Li *et al*., 1997).

CREB-binding protein and the related protein p300 function as coactivators for a diverse group of transcription factors, including the PR (Rowan & O'Malley, 2000). CBP possesses an intrinsic HAT activity and recruits the HAT, p/CAF (Yang *et al*., 1996). CBP functionally interacts with all three SRC family members (Voegel *et al*., 1998; Torchia *et al*., 1997; Kamei *et al*., 1996) and acts synergistically with the SRC-1 to coactivate the PR (Smith *et al*., 1996). However, interaction between the CBP and either the SRC-1 or the PR was shown to be weak when compared to the interaction between the SRC-1 and the PR (McKenna *et al*., 1998). Collectively, these studies point to a model where an initial complex is formed

between the liganded PR and the SRC-1 prior to recruiting the CBP on site (Fig. 1.11) (McKenna *et al*., 1998).

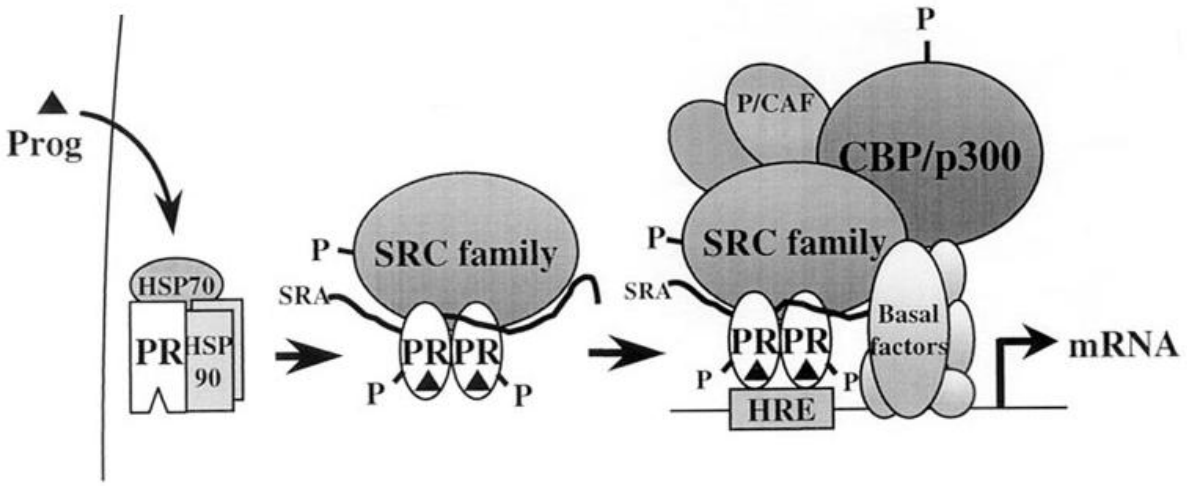


Figure 1.11 : Activation of progesterone receptor (PR). PR is an inactive complex with heat shock proteins when in the unbound state. Upon binding of progesterone, heat shock proteins dissociate, and the PR adopts a conformation that will first recruit the SRC family of coactivators, and subsequenlty other cofactors to the promoter which will open up local chromatin structure through histone acetyltransferase (HAT) activity and contribute to the stabilization of the preinitiation complex (Rowan & O'Malley, 2000).

1.2.3.2. Membrane Progesterone Receptors

Many of the physiological effects exerted by steroid hormones are too rapid to be mediated via the classical genomic mechanism, involving activation of the nuclear steroid receptors (Gu *et al*., 1999; Yang *et a*l., 1994). Thomas' lab in a series of elegant experiments discovered a putative membrane receptor as a steroid membrane receptor able to bind and respond to progestins. Seven important criteria were fulfilled to establish this designation: plausibility of its structure, tissue specificity, plasma membrane localization, steroidal

binding characteristics, ability to activate signal transduction pathways, hormonal regulation, and biological relevance (Zhu, *et al*., 2003[A]).

A cDNA clone was isolated from the ovaries of a spotted seatrout (*Cynoscion nebulosus*). The 1.7kb cDNA sequence comprised an open reading frame (ORF) of 1,059 base pairs and coded for a 352 amino acid protein (Fig.1.12) with an estimated molecular mass of 40,585Da. With the use of computational analysis based on the deduced amino acid sequence the prediction was that the protein locates in the plasma membrane and had seven transmembrane domains, a known characteristic of G-protein-coupled membrane receptors (Zhu, *et al*., 2003[A]).

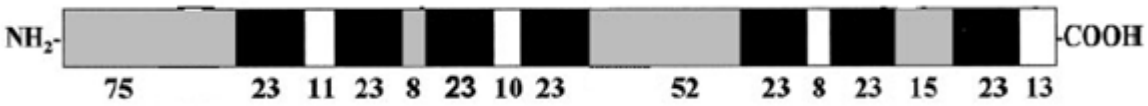


Figure 1.12: A schematic diagram of the encoded protein showing extracellular (gray), seven-transmembrane (solid black), and cytoplasmic (clear) domains predicted by several programs. Numbers indicate the amino acid number in each domain (Zhu, *et al*., 2003[A]).

Saturable and specific progesterone binding was reported with the use of a recombinant protein whereas no specific progesterone binding was observed with control proteins containing an empty vector. Also high affinity binding sites were confirmed (*K*d 30 nM). Steroid competition studies showed that the binding was highly specific for progesterone and 17-hydroxyprogesterone and no displacement of [^{3}H] progesterone was observed with estradiol-17β, cortisol, or 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S, a maturation inducing steroid) (Zhu, *et al*., 2003[A]).

The receptor was able to cause the rapid activation of intracellular signalling pathways, with $ERK_{1/2}$ activated within 5 min following stimulation with progesterone and 20β-S. No activation of $ERK_{1/2}$ was observed in control MDA-MB-231 cells transfected with an empty carrier vector. Following incubation with 20β-S both the mRNA of the membrane receptor and protein levels were shown to be up-regulated in reproductive tissue, specifically in the ovaries (Zhu *et al*., 2003[B]).

After the first set of experiments that introduced the membrane progesterone receptor (mPR), thirteen cDNAs similar to the mPR were identified in other vertebrate species, including human. By using computer - based structural analyses it was consistently predicted that all of these cDNAs encoded for plasma membrane proteins with seven, or six, transmembrane domains. The phylogenetic analysis showed that three major clades of mPRs existed, mPRα, mPRβ, mPRγ in mammals. These membrane progesterone receptors (mPRs) are structurally unrelated to the nuclear steroid receptor and pose characteristics similar to G-protein-coupled receptors (GPCRs), however they are phylogenetically distinct from the GPCR superfamily (Fig. 1.13) (Zhu, *et al*., 2003[A]).

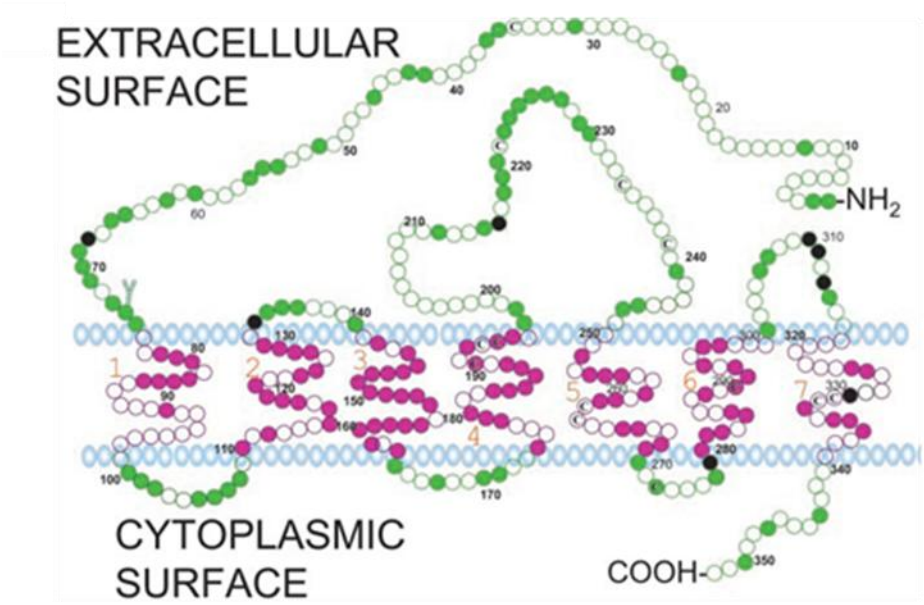


Figure 1.13: A proposed model for insertion of the seatrout mPR in the plasma membrane. Each circle represents one amino acid residue (Zhu, *et al*., 2003[B]).

There are 8, 16, and 38 amino acid substitutions that characterize and distinguish the three mPR clades, respectively. The amino acids unique to mPRα are found mainly in the extracellular loops, where those amino acids that are unique to mPRβ are found among the N-terminal, transmembrane domain, external loops and internal loops, those unique to mPRγ are in the transmembrane domain (Zhu, *et al*., 2003[B]).

Preliminary evidence obtained from array analysis revealed distinct tissue localization of the three mPR. mPRα was localized in reproductive tissues, particularly in the placenta, testis, and ovary and also in the kidney, suggesting it is the predominant subtype mediating the rapid non- genomic actions of progesterone in major reproductive tissues (Zhu *et al*, 2003[A]).

Early studies indicated that mPRβ was found to be exclusively localized in neural tissues and was detected throughout the brain and in the spinal cord whereas the –γ subtype was found to be present in the kidney, fetal kidney, colon, a lung carcinoma, and in HeLa 53 cells. The authors proposed that, non - overlapping tissue distribution of the human mPRα and mPRβ subtype mRNA is an indication that the two subtypes are responsible for regulating different physiological functions of progesterone (Zhu, *et al*., 2003[B]). Recent studies though have identified mPRβ in human fallopian tubes and mPRγ was identified also in the human ovary (Nutu *et al*., 2007).

Experiments to establish the steroid binding characteristics of these receptors where performed by using recombinant proteins produced in *E. Coli* for each of the three subtypes. The results indicated high affinity and saturable progesterone binding to [^{3}H] progesterone. Additionally, the kinetics of association and dissociation where shown to be rapid, occurring within a few minutes, a typical characteristic of membrane receptors. Also, several synthetic progestins and antiprogestins, which have relatively high binding affinities for nuclear progesterone receptors, displayed no binding affinity for the recombinant human mPRγ protein (Zhu, *et al*., 2003[B]).

Five years after the first reports another study from the Lyons lab presented data confirming that the human mPRα, mPRβ, mPRγ are capable of detecting and responding to progesterone when expressed in a yeast experimental model. This was carried out by expressing mPRs in the eukaryotic model organism *Saccharomyces cerevisiae*. Reasons for choosing this model as stated by the authors take account of the fact that progesterone has a very low biological activity in this model organism (Banerjee *et al*, 2004) and also proteins encoding the nuclear receptor family are not present in its genome (Phelps *et al*, 2006). Based solidly on their ability to sense and respond to progesterone, these groups presented two more types of mPRs which were renamed mPRδ and mPRε respectively (Smith *et al*, 2008).

Whilst the early studies on mPRs concentrated on oocyte maturation in fishes (Josefsberg *et al*., 2007; Zhu *et al*., 2003[A]; Thomas *et al*., 2002) establishing the progesterone induced oocyte maturation as the classic example of the rapid, non genomic effects that are mediated via steroid receptors present on the cell membrane (Josefsberg *et al*., 2007), more recent studies have attempted to establish the significance of the role of mPRs in human reproductive tissues.

Up to now it has been shown that mPRs, are involved in the progesterone regulated gamete transport in women. In particular, mPRβ and mPRγ are present in the ciliated cells of the fallopian tube whose main function is to facilitate gamete transport and fertilization in women. These receptors have been shown to be regulated by progesterone thus further strengthening the role of the ciliated cells of the fallopian tube as physiological targets of the action of progesterone (Nutu *et al*., 2009). mPRs have been shown to be expressed and able to activate G-protein signalling in the endometrium, the myometrium and in fetal tissues (Fernandes *et al*., 2005). Two individual studies have suggested that mPRs are regulated in uterine tissues throughout the estrous cycle and also may be involved in term and preterm labor. In the human endometrium, mPRα, mPRβ and mPRγ are regulated throughout estrous with the levels of mPRα and mPRβ increasing midway through the cycle while mPRγ levels decrease (Fernandes *et al*., 2005).

During labour, the expression levels of mPRs present in the myometrium change. In term and preterm labour it has been shown that mPRα levels decrease while the levels of mPRβ decrease only during term labour whereas mPRγ levels appear unregulated (Fernandes et *al*., 2005). Yet another study showed that only mPRα and mPRβ were expressed in human pregnant myometrium, with mPRα being the predominant isoform. In the same study performed by Karteris, an alternative model was proposed that implicated mPRs in the onset of parturition: mPRs binding to petrusis toxin sensitive G protein alpha subunits and inhibite

adenyl cyclise and increase the myosin light chain phosphorylation. These mPR mediated events promote a shift from the quiescent phase in which the myometrium is in and sensitize it towards a contractile state at term (Karteris *et al*., 2006).

The importance of the physiological actions of the multiple mPR isoforms throughout the female reproductive cycle have been acknowledged, including oocyte growth (Qiu *et al*., 2008) transport of the oocyte from the ovary to the uterus (Nutu *et al*., 2009), preparation of the uterus for implantation (Ashley *et al*., 2009; Ashley *et al*., 2006), gestation and fetal development (Fernandes *et al*., 2005), and parturition (Karteris *et al*., 2006).

From the research progress achieved in the field up to now, it is seen that in the reproductive tissues the expression of both PRs and mPRs is responsible for functional redundancy ensuring reproductive success via progesterone mediated actions.

1.2.3.3. Progesterone Receptor Membrane Component-1

Another protein able of mediating rapid non-genomic actions of progesterone is the progesterone receptor membrane component-1 (PGRMC-1). A cDNA encoding a progesterone membrane binding protein was isolated from a porcine vascular smooth muscle cell cDNA library (Falkenstein *et al*., 1996). This small progesterone binding protein consists of 194 amino acids (Falkenstein *et al*, 1996) and has a molecular weight of 28 kDa (Peluso *et al*, 2006). It comprises of a short N-terminal extracellular domain, single transmembrane domain and a cytoplasmic domain (Min *et al*., 2004) where the cytoplasmic domain contains three Src homology domains that may be involved in the ligand – dependent signal transduction (Peluso, 2006). It has been reported as being detected as a 56 kDa dimer or as a 28 kDa monomer by two different groups (Selmin *et al*., 1996; Meyer *et al*., 1996). PGRMC-1 molecule has been found to interact with plasminogen activator inhibitor mRNA binding

protein 1 (PAIRBP1) (Peluso *et al*., 2005), which localizes on the cell membrane and is involved in transducing progesterone membrane-initiated actions (Peluso *et al*., 2004),

PGRMC-1 has been reported to be present in the mouse granulosa cells (McRae *et al*., 2005), in luteal cells (Cai & Stocco, 2005), and spontaneously immortalized granulosa cells (SIGCs) (Peluso *et al*., 2005). Additionally, it is found on the plasma membrane (Peluso *et al*., 2009; Peluso *et al*., 2006; Krebs *et al*., 2000) as well as in several intracellular locations such as the endoplasmic reticulum, Golgi apparatus (Wu *et al*., 2011), inner acrosomal membrane (Losel *et al*., 2004).

A wide range of physiological roles have been attributed to this protein including steroid synthesis and metabolism (Raza *et al*., 2001), regulation of cholesterol uptake (Runko *et al*., 2002), endocytosis, alteration of reproductive behaviors (Krebs *et al*., 2000), by functioning as an adapter protein and also an ability to form complexes with other proteins that are involved in the cholesterol system (SCAP and Insig1) (Suchanek *et al*., 2005).

A recent study performed by Wu identified PGRMC-1 as the mediator of progesterone action on myometrial contractility (Wu *et al*., 2011). The antiapoptotic effects of progesterone in granulose and luteal cells demonstrated by Peluso in 2005 were shown to be rapid (within minutes) and initiated at the plasma membrane. These findings were extended by the same group where it was demonstrated that PGRMC-1 is an essential component of progesterone's membrane-initiated antiapoptotic action (Peluso *et al*., 2006).

1.2.4. Genomic vs. Non-genomic actions of progesterone.

As the actions of progesterone are essential for the orderly reproductive functions to occur, the mechanisms by which this steroid hormone mediates its actions has been an area of intense study for the last 20-30 years. It appears that there is an expanding PR repertoire involving multiple classes of receptors cpable of binding to progesterone (Fig.1.14).

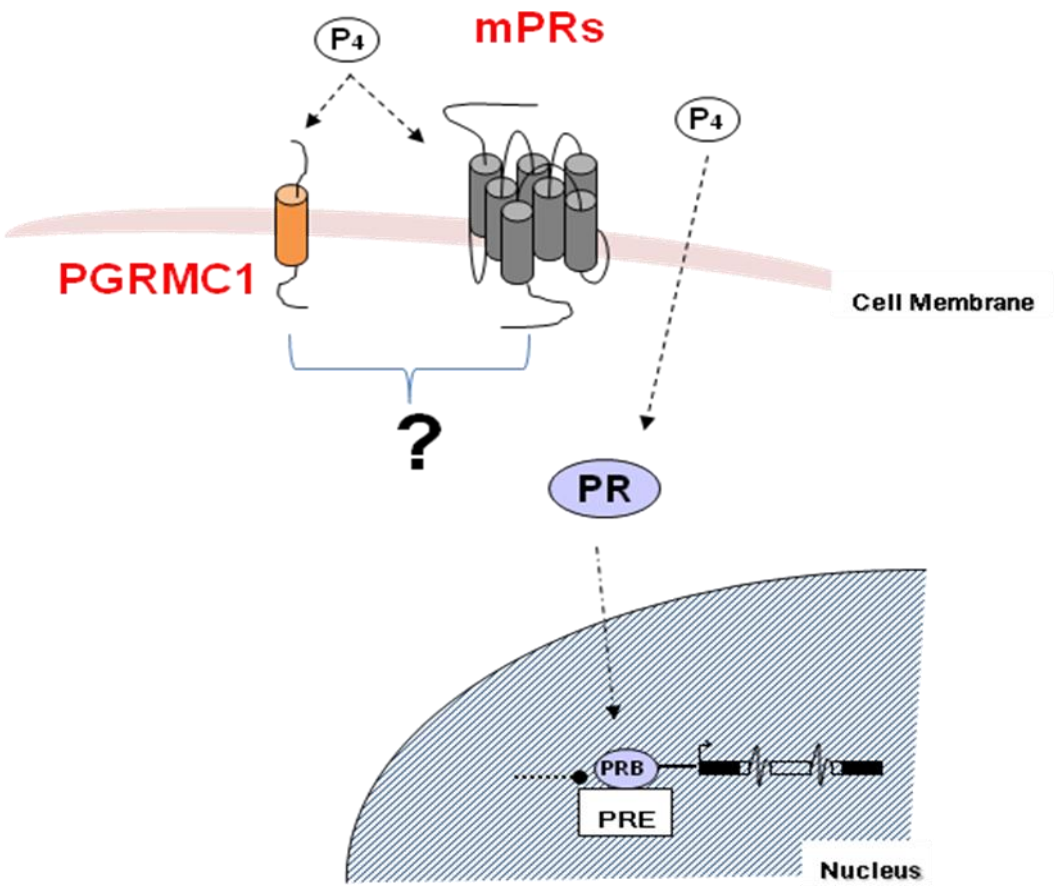


Figure 1.14: Diagram of the receptors involved in progesterone (P4) signalling. Receptors present on the membrane, mPRs and PGRMC-1 able to bind progesterone and mediate their rapid signalling through signalling cascades which are still under investigation. The intracellular nuclear PR mediates its actions through transcription and translation.

In accordance with the cnonical theory of steroid hormone action, steroids and thus progesterone affect the transcription of mRNA and subsequently protein synthesis (Revelii *et al.*, 1998; Wehling, 1997). This is accomplished by the ability of nuclear receptors to interact with DNA responding to hormone binding and recruiting multiprotein complexes controlling gene expression (Gellersen *et al.*, 2009). Gene transcription and translation occurs in the order of hours to days.

In the recent years, non-classical effects of steroid hormones have been examined that do not depend on gene transcription for protein synthesis and are too rapid to be mediated by gene transcription (Table 1.1). Terms like non-genomic or non-nuclear are used to describe these effects so as to underline that DNA binding of the receptors and subsequently RNA synthesis are not a necessity (Simoncini & Genazzani, 2003). Non - genomic steroidal effects are characterized by two properties; their insensitivity to transcriptional and protein synthesis inhibitors, and by their rapid onset of action which is within seconds to minutes (Norman *et al*., 2004 ; Losel & Wehling, 2003; Bramley, 2003 ; Falkenstein *et al*., 2000). These rapid effects can be replicated in cells that do not express genomic receptors such as spermatozoa and can be imitated by binding to receptors unable of activating transcriptional actions due to mutations (Simoncini & Genazzani, 2003).

Mechanisms of Genomic Vs. Nongenomic signalling	
Genomic	***Nongenomic***
Gene expression & new protein synthesis	Activation/ repression of pre-existing proteins
Relatively long latency of onset	Rapid onset of action
Medium and long term cell programme	Rapid adaptation to changes in the milieu
Organisation of cell networks for complex functions	Dynamic modifications of long term cell programme

Table 1.1: Genomic and non-genomic actions of steroid hormones: a tentative interpretation. (Adapted from Simocini & Genazzani, 2003).

There have been several studies performed in mammals that show the ability of nuclear PR to signal non - genomically by initiating rapid signalling both from the cytoplasm and the cell membrane. Studies with breast cancer cells have shown that the nuclear PR in both states – liganded and unliganded – can initiate and respond to growth factor signalling pathways. MAP kinase activation by nuclear PR is initiated by the interaction between a proline-rich motif on the nuclear PR and SH3 domains on the upstream kinase c-Src (Boonyaratanakornkit *et al*., 2007; Carnevale *et al*., 2007; Faivre *et al*., 2005; Skildum *et al*., 2005). Studies suggest that progesterone acts via interactions with signalling pathways mediated by growth factors to regulate the proliferation of breast cancer cells (Boonyaratanakornkit *et al*., 2007; Carnevale *et al*., 2007). Other studies have indicated the interactions of nPR with the nuclear estrogen receptor (ER) to indirectly activate MAP kinase (Migliaccio *et al*., 1998). These interactions were facilitated by two domains of the nPR that through interaction with the ER the c-Src and MAP kinase are activated (Ballare *et al*., 2003). These interactions between nPR and ER have been shown to mediate the progesterone induced proliferation of endometrial cells (Vallejo *et al*., 2005).

Except from the rapid non-genomic nPR mediated signalling initiated in the cytoplasm, nPR also contains highly conserved motif in the ligand binding domain which induces its membrane localization (Pedram *et al*., 2007).

The actions of progesterone mediated via the "classical" genomic pathway in the female reproductive tract received unequivocal attention through the years. Researchers have embarked on understanding the actions which are mediated non genomically via the nPR and the mPRs in order to further understand the multiple actions of progesterone (Hammes, 2003).

1.3. Research aims and objectives

To this day the knowledge of the receptors by which progesterone exerts its effects in the human placenta, are very limited. This study focuses on understanding the expression of progesterone receptors in the human placenta along with achieving an understanding on the mechanism of PR signalling and to assess the functionality of mPRs in this organ.

Objectives:

1. Validation of the synsytialization model of BeWo cells.
2. Elucidating the expression and cellular distribution of progesterone receptors (mPRs, PGRMC-1 and PR-A/B) in two human placental cell lines (BeWo and JEG-3) as well as in human placental tissues. Also examining the effect of syncytialization on the expression of these receptors.
3. Examine the progesterone-receptor mediated signalling in the human placental cell lines.
4. Investigating the involvement of progesterone-receptor signalling *in vitro* during an inflammation response.

2

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Solutions

Chemicals and Reagents

- Elution buffer: Molecular grade H_2O.
- Wash buffer 1: 1 M Tris (pH 7).
- DNase I containing buffer: Amplification Grade DNase I - 10mM Tris-HCl (pH 7.5), 10mM CaCl2, 10mM MgCl2 and 10X Reaction Buffer- 200mM Tris-HCl (pH 8.3), 20mM MgCl2 per 1ml.
- Stop buffer: 50mM EDTA per 1ml.
- 1x TBE buffer: 90mM Tris, 90mM Boric acid, 2 mM EDTA.
- 10x loading buffer : 20% Ficoll 400 (Fisher Scientific, Cat.No 26873-85-8), 0.1 M Na_2EDTA (pH 8), 1% SDS, 0.25% Bromophenol blue, 0.25% xylene cyanol.
- 5X first-strand buffer : 250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_{2.}$
- 2x Laemmli buffer: 4% SDS (w/v), 20% glycerol, 10% 2-mercaptoethanol (v/v), 0.004% bromphenol blue (w/v), 0.125 M Tris HCl (pH 6.8) (w/v).

Western Blot analysis:

- 12.5% SDS-PAGE Gels

- Resolving gel: 30% acrylamide/ bisacrylamide (v/v), 1.5M Tris base (pH 8.8), 10% SDS (w/v), 10% ammonium persulphate (w/v), TEMED.
- Stacking gel: 30% acrylamide/ bisacrylamide (v/v), 1M Tris base (pH 6.8), 10% SDS (w/v), 10% ammonium persulphate (w/v), TEMED.

- Blocking solution (5% w/v) dried milk powder in 1X TBS- Tween 20 (20mM Tris base, 140mM NaCl, 0.1% Tween 20 (v/v) (pH 7.6).
- 1x PBS 0.1% Tween 20

2.2. Cell lines

BeWo : Human trophoblastic cell line with endocrine properties and able to cellularly differentiate in vitro. This cell line was initiated from a malignant gestational choriocarcinoma of the fetal placenta (ECACC European Collection of Cell Culture; Liu *et al*., 1997; Rama Sastry 1999).

JEG-3: Human placental choriocarcinoma cell line with endocrine activity (Health Protection Agency, Salisbury, UK; Pattillo & Gey, 1968)

2.3. Cell culture

A Holten LaminAir Class II Haeraus Instruments hood was used for the routine culture of the cell lines. BeWo cells were cultured in Ham F12 Medium (Gibco) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco) and 100 µg/ml of Penicillin and 100 µg/ml of Streptomycin (Gibco). The cells were maintained in 75cm^2 non treated culture flasks (Nunc) under standard culture conditions in humidified atmosphere containing 5% CO_2 at 37˚C. The cells were confluent after 48 to 72 hours where subculturing of BeWo cells consisted in splitting the cells 1:3 into 19ml of complete Ham F12 medium per culture flask. The cells were firstly washed with 2.5 ml of 1x Phosphate- Buffered Saline (PBS) (Gibco) to

then detach the cells by incubating in 2.5 ml of 0.25 % Trypsin (Gibco) for a few minutes and by tapping the culture flask gently. The cell suspension was placed in a 50 ml tube and after adding 2.5 ml of Ham F12 medium to neutralise the activity of trypsin the cells were centrifuged for 5 minutes at 1500 RPM using a megafuge (Heraeus 1.0, Heraeus Instruments). The supernatant was then removed and the cells were resuspended in a known volume of fresh medium. BeWo cells were supplied with fresh media every 48 hours maximum according to supplier's instructions to prevent glucose exhaustion in the culture.

JEG-3 cells were maintained in Minimum Essential Medium (MEM) (Sigma Aldrich) supplemented with 2mM Glutamine (Gibco), 1% (v/v) Non Essential Amino Acids (Gibco), 1mM Sodium Pyruvate (Gibco), 100 μg/ml of Penicillin, 100 μg/ml of Streptomycin (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco). The procedure followed for subculturing JEG-3 cells was the same as for BeWo cells.

2.4. Placental Tissue Acquisition

Placental tissues were obtained from women at both at term (> 37 weeks) and preterm (<37 weeks) undergoing either normal vaginal delivery or elective caesarean section. All samples were collected from pregnancies without any known pathological problems. Immediately after delivery, the maternal and fetal surfaces were dissected off and the fetal membranes were peeled gently away from the placenta. Placental tissues were obtained from the maternal side of the placentas. The tissue samples taken were approximately 0.2-0.5cm^3 in size and were taken from the centre of the cotyledons evenly across the placenta. The tissues were dissected to remove any visible connective tissue and calcium deposits. Depending on the experimental analysis, some of the tissues were transferred to 10 volumes of RNAlater solution (Invitrogen). Others were homogenisated directly and RNA was obtained from them. Placenta samples were then washed five times with PBS and were snap frozen in liquid

nitrogen. Placental tissue sections were embedded in paraffin by the histology services of the University Hospital of Crete were the samples were obtained from. Ethical approval was obtained from the local ethical committees and also informed consent was obtained from all the patients.

2.5. Syncytialisation of BeWo cells

BeWo cytotrophoblast (CT) cells were induced to fuse forming multinuclear syncytia also known as syncytiotrophoblast (ST) cells. The syncytialisation process was performed using the chemical Forskolin (Sigma, Cat. No. F3917). Forskolin was dissolved in DMSO at a concentration of 0.1M. To assess the syncytialisation process, BeWo cells were treated with 50μM and 100μM over a total period of 72 hours. Forskolin treatment was renewed every 24 hours. The cells were maintained in 75cm^2 non treated culture flasks (Nunc) under standard culture conditions in humidified atmosphere containing 5% CO_2 at 37˚C.

2.6. RNA Extraction

RNA extraction was performed using GenElute^{TM} Mammalian Total RNA miniprep kit (Sigma-Aldrich, Cat. No. RTN70) which isolates total RNA from mammalian cells and tissues. The protocols followed for RNA extraction from mammalian cells and tissue samples differ as different cell lysis and disruption conditions apply.

2.7. RNA Extraction from cells

The cells of interest were cultured in 75cm^2 non treated culture flasks or in 6-well non treated multidishes (Nunc). The cells were maintained in culture to achieve 70% confluence, after which the cells were washed with sterile 1xPBS (Gibco) prior to starting the RNA extraction.

Since the cells were attached on the culture vessel the lysis/ RNase inactivation step was performed directly in the culture vessel. The mixture containing lysis buffer and 2-mercaptoethanol (10µl 2-mercaptoethanol added for each 1 ml) was left in the culture vessel for 2 minutes with repetitive shaking. The lysate was filtered by centrifugation using GenElute filtration columns for 2 minutes at 16,000 RPM. RNA was isolated by binding in the presence of 70% ethanol solution in a GenElute binding column. The isolated RNA was collected in the collection tubes and was washed three times with the provided wash buffers in order to obtain ethanol-free RNA and remove contaminants. The columns were further centrifuged to ensure that were completely dry before eluting the RNA using 40µl of elution buffer in RNase free tubes. RNA was maintained on ice at all times.

For removal of any residual DNA the extracted RNA preparations were further treated with DNase I (Sigma-Aldrich, Cat. No. AMPD1), whereby RNA was incubated with DNase I containing buffer for 15 minutes at room temperature. The action of DNase I was stopped by adding the stop buffer and denaturing the enzyme by incubating at 70ºC for 10 minutes.

2.8. RNA Extraction from placental tissue

Prior to extracting RNA form the samples, the placental tissues were homogenised using TissueLyser II (Qiagen). The snap-frozen placental samples stored in RNAlater were left to thaw on ice. Once thawed, the placental tissues were transferred to sterile eppendorf tubes containing the lysis mixture. A stainless steel bead was added to each tube to facilitate cell disruption. Proteinase K (Sigma-Aldrich, Cat. No. P4850) was added directly to each sample after homogenization to ensure efficient cell disruption and incubated at 55°C for 10 minutes. Next, the lysate was filtered and the same steps as above (section 2.6) were followed.

The quantity and quality of the extracted RNA was measured by analyzing 1µl of RNA sample on a Nanodrop-1000 Spectrophotometer (Fisher Scientific). As a blank reading, 1µl

of elution solution was used. Further, to ensure no RNA degradation, 2µl of 10x loading buffer were added to 5µl of RNA sample and run on a 1% agarose gel at 80V for 45 minutes and subsequently visually examines under UV conditions. Purified RNA was stored at -70 ºC until further use.

2.9. cDNA synthesis

SuperScriptTM II reverse transcriptase kit (Invitrogen, Cat.No. 18064-022) was used to generate complimentary DNA. In a nuclease-free 0.6ml microcentrifuge tube, 500ng of RNA sample, 100ng of random hexamers and 10mM deoxyribonucleotide triphosphate (dNTP) mix were added together. The mixture was centrifuged briefly before incubating at 65 ºC for 5 minutes and then placed on ice for 1 minute. Following, 5X first-strand buffer and 0.1M DTT were added to the above mixture and incubated for 2 minutes at room temperature. In each sample 200 units of SuperScript™ II reverse transcriptase enzyme were added and the mixture was incubated at 42ºC for 50 minutes. The activity of the enzyme was stopped by incubating the samples at 70ºC for 15 minutes. The newly synthesised cDNA was stored at -20ºC until further use.

2.10. Primer design

The design of the primers of interest began at: www.ncbi.nlm.nih.gov where the gene sequences were searched for the genes of our interest by reference number available from publishing. This target sequence was then inserted into Invitrogen's own primer design tool, OligoPerfect™ Designer at: www.invitrogen.com/content.cfm?pageid=9716, synthesizing a primer set of sense and antisense primer. The maximum primer size required was 25bp, the minimum product should be 200-500bp, and as extremes were preferably avoided, the percentage of GC (around 50%) content and temperature of the primer (around 60 ºC) was required to be the same for each one in the pair to prevented problems with annealing during PCR.

2.11. Reverse Transcriptase Polymerase chain reaction (RT-PCR)

PCR procedure along with the enzyme *Taq* DNA polymerase (Sigma-Aldrich, Cat. No. D4545) was used to amplify the genes of interest. The reaction mixture consisted of 1 µl of cDNA synthesised as described above, 5 µl of 10x PCR Buffer, 1 µl dNTP mix (10 mM), 1.5 µl $MgCl_2$ (25 mM), 1 µl of each specific forward and reverse primers (0.1 µg/µl; Table 2.1) ,0.5 µl of *Taq* DNA polymerase (5 U/ µl) and 39µl of ddH_2O. The constituents were mixed thoroughly before being subjected to PCR amplification procedure. Initially they were denatured at 94ºC for 4 minutes followed by 30 cycles at 94ºC for 30 seconds, primer specific annealing temperature for 30 seconds and 72ºC for 1 minute, the final cycle was followed by 72ºC extension for 1 minute.

Gene of Interest	Forward Primer	Reverse Primer	Product size (bp)
β-actin	AAGAGAGGCATCCTCACCCT	TACATGGCTGGGGTGTTGAA	216
mPRα	GCTGTTCACTCACATCCC	TGGTGCAACCCCCAGA	289
mPRβ	GCGGCCCTGGTACTGCTGC	CACGGCCACCCCCACA	200
mPRγ	ACTATGGTGCCGTCAACCTC	GTGGTACGAGGTGGCTTCAT	305
mPRδ	CCCCAACTTCTTCAAGTCCA	CTGGAAGGAGCTGAGGACAC	114
mPRε	CGCCTTGGAGGAGACTACTG	TTGGGATCCCTTTGTAGCAG	155
PCNA	GCCGAGATCTCAGCCATATT	ATGTACTTAGAGGTACAAAT	452

Table 2.1: List of genes studied by reverse transcriptase PCR. Sequences of primers are shown with the expected product size in base pairs (bp).

2.12. Real Time Polymerase chain reaction (Q-PCR)

SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, Cat. No. S4438) was used to perform the Q-PCR along with ABI PRISM® 7900HT Sequence Detection System (SDS 2.1)

software. A final reaction volume of 25 µl included 2x JumpStart*Taq* ReadyMix (1.25 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTP, stabilizers), 1 µl of each specific forward and reverse primers (0.1 µg/µl; Table 2.1), 1x reference dye and cDNA. The reaction mixture was then thoroughly mixed and 24 µl were aliquoted in each of the 96 wells of the plate (MicroAmp™ Fast Optical 96-Wells Reaction Plate, Applied Biosystems, Cat.No. 4314320). 1 µl of cDNA was added soon after and the plate was sealed using an optical adhesive film (MicroAmp® Optical Adhesive Film, Applied Biosystems, Cat.No. 4314320). The plate was then centrifuged for 1 minute at 1,000 RPM and loaded onto the Sequence Detection System. All reactions were performed in triplicates and control reactions where no cDNA was added in the wells, only dH_2O were included in the study design. The conditions for the Q-PCR reaction the following:

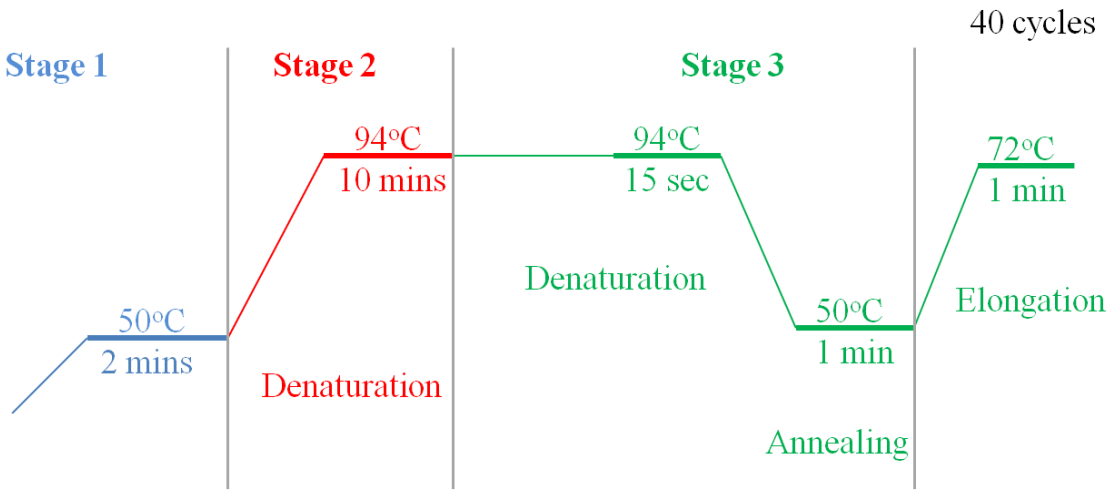


Figure 2.1: Real time PCR cycle conditions.

Q- PCR quantification method can be either relative or absolute. In absolute quantification the exact copy number per cell is determined whereas in the relative quantification, a comparison is performed between the expressions of a gene of interest to that of a housekeeping gene.

The relative quantification step produces a graph known as the ‘amplification curve’. This is plotted by the machine and indicates the number of cycles versus the fluorescence intensity. As the number of cycles progress the amount of double-stranded DNA also increases and fluoresce as the SYBR Green molecule binds to the newly formed double-strand DNA. A threshold is set automatically depending on the range of detection of the amplification. At the cycle where the fluorescence signal crosses the set threshold, that value is named ‘cycle threshold’ (Ct).

A limitation that occurs with relative Q-PCR is that SYBR Green also binds non-specifically to double stranded DNA and primer dimer complexes. A way to distinguish between specific and non-specific amplification was by performing a ‘dissociation-curve’ analysis. It is known that the melting temperature (Tm) of DNA depends upon the length of the strand as well as its GC’s content. These properties can be used to distinguish between the specific and non-specific products formed during the relative Q-PCR. The ‘dissociation-curve’ is a graph that shows the temperature versus the fluorescence and as the temperature gradually increases the laser of the machine is able to detect the loss of fluorescence signal as SYBR Green detaches as DNA melts at high temperature. Non-specific products would be expected to have different sizes and GC content compared to the specific product thus being able to melt at different temperatures. Therefore, a comparison was made to ascertain whether the obtained peak is of desired product. A representative amplification plot (panel A) and a representative dissociation curve (panel B) are shown in Fig.2.2.

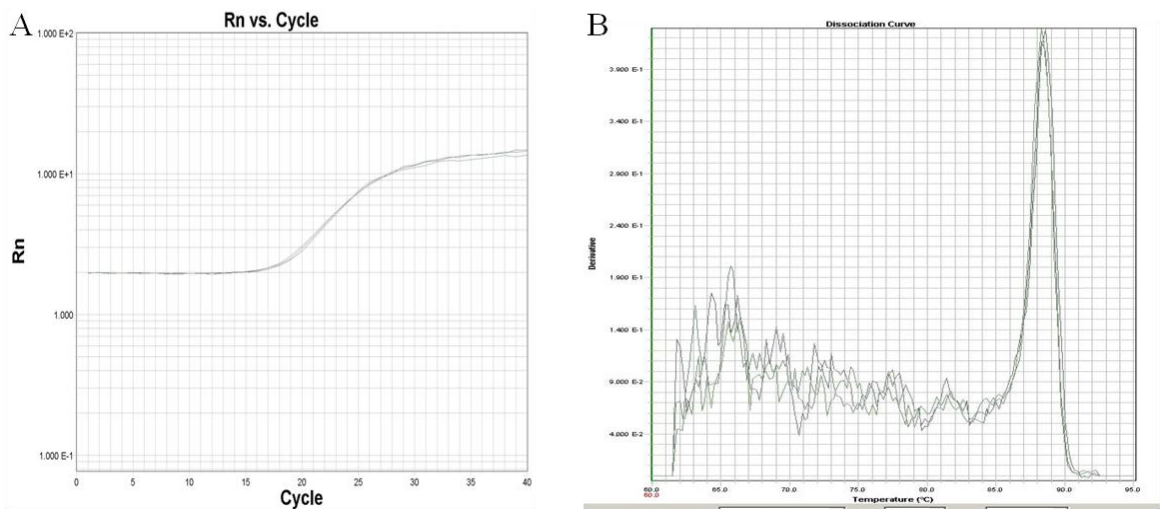


Figure 2.2: Representative amplification plot and dissociation curve.

In this study, the relative quantification approach was used with a number of genes of interest and a calibrator gene (Table 2.2).

Gene of Interest	Forward Primer	Reverse Primer	Product size (bp)
mPRα	CGCTCTTCTGGAAGCCGTACATC TATG	CAGCAGGTGGGTCCAGACATTCAC	100
mPRβ	AGCCTCCTACATAGATGCTGCCC	GGTGCCTGGTTCACATGTTCTTCA	194
mPRγ	CAGCTGTTTCACGTGTGTGTGAT CCTG	GCACAGAAGTATGGCTCCAGCTATC TGAG	120
PGRMC-1	TCTGGACTGCACTGTTGTCCTTG	GCAAACACCTGTTCCTATTCTG	290
PR-B	AGCAGTCCGCTGTCCTTTTCT	CCTGAAGTTTCGGCCATACCT	176
β-actin	AAGAGAGGCATCCTCACCCT	TACATGGCTGGGGTGTTGAA	216
Syncytin-2	ACACCTTGCCTGAAGTTTCG	CTGTCCTTTTCTGGGGGACT	129

Table 2.2: List of genes studied by real time. Sequences of primers are shown with the expected product size in base pairs (bp).

The following equations (*User Bulletin #2*, ABI PRISM 7700 Sequence Detection System) were used to analyse the results obtained from the Q-PCR:

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

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

Relative Quantity (RQ) = $2^{-\Delta\Delta Ct} \pm (2^{-\Delta\Delta SD}{}_{(+)} - 2^{-\Delta\Delta SD}{}_{(-)})$

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

Arbitary Value : $2^{-\Delta Ct}$

2.13. Agarose Gel electrophoresis

For DNA electrophoresis gels were prepared with agarose (Fisher, Cat.No. BP1356), the concentration of which was chosen based on the size of fragments to be resolved and it was dissolved in 1x TBE buffer containing 0.05% (w/v) ethidium bromide (Sigma, Cat. No. E1510). The PCR products were mixed with 10x loading buffer and an indicative sample of 20 µl was loaded into the well of the gel. 1Kb DNA ladder (Invitrogen, Cat.No. 10787-018) was loaded once all of the samples had been loaded into the gel. Then, the gel was electrophoresed for a suitable length of time at 80 volts. The DNA was visualised by placing the gels on a short wave ultra-violet transluminator and an image was captured using an Alpha Imager 2200 (AlphaInnotech Corporation).

2.14. Indirect Immunofluorescence analysis

2.14.1. Indirect Immunofluorescence on cells

BeWo CT, BeWo ST and JEG-3 cells were grown on sterile 0.13mm-0.17mm glass coverslips in 6-well multidishes and grown to a sufficient confluence. Cells were rinsed with 1x PBS, three times before fixing using 4% Paraformaldehyde (pfa) and incubating for 10 minutes at room temperature. Three washes with 1x PBS followed. The cells were then permeabilised using 0.2% PBS Tween 20 solution, at room temperature for 20 minutes, followed by three washes in 1x PBS solution containing 100mM Glycine. Cells were then incubated with 10% specific species serum (Jackson ImmunoResearch) in 1x PBS for 1 hour at room temperature in order to minimize any non-specific binding of IgG. The blocking serum used for each primary antibody was chosen based on the species in which the secondary antibody was raised.

Antibodies were diluted in 1.5% of specific serum in 1x PBS. Primary antibodies used in this study are listed in table 2.3 along with their dilution. The coverslips were incubated at 4°C overnight. Next, the coverslips were washed three times with 1x PBS and incubated with a respective secondary antibody (Table 2.3) for 1 hour at room temperature. Cells were then washed with 1x PBS and rinsed in distilled water before mounting in Vectashield® Mounting Medium containing DAPI. Negative controls were prepared by omitting the primary antibody. All the other steps were exactly the same.

Primary Antibodies			Secondary Antibodies	
Antibody	**Dilution**	**Species/ Details**	**Antibody**	**Dilution**
mPRα	1:50	Goat (Santa Cruz /sc-50111)	Donkey anti-goat (Invitrogen/A11055)	1:400
mPRβ	1:50	Goat (Santa Cruz / sc-50109)	Donkey anti-goat (Invitrogen/A11055)	1:400
mPRγ	1:50	Goat (Santa Cruz / sc-28019)	Donkey anti-goat (Invitrogen/A11055)	1:400
PGRMC-1	1:75	Rabbit (Gift from Prof. P. Thomas	Donkey anti-rabbit (Invitrogen/A10042)	1:400
PR-B	1:50	Mouse (Santa Cruz / sc-811)	Goat anti-mouse (Santa Cruz / sc-3796)	1:75
Pan-Cadherin	1:200	Mouse (Abcam / ab22744)	Goat anti-mouse (Santa Cruz / sc-3796)	1:200
Anti-PCNA	1:1000	Mouse	Rabbit anti-mouse (Zymed / 616520)	1:200

Table 2.3: List of primary and secondary antibodies used with their respective dilutions.

2.14.2. Indirect Immunofluorescence on paraffin-embedded placental tissues.

The specimens used were heated in an oven at 65^{o}C for 1 hour. Following, the slides were dewaxed in Histo-Clear (Fisher Scientific, Cat.No. HIS-010-010S) a non-hazardous substitute of xylene. The sections were then dehydrated in 100% ethanol, twice for 5 minutes each time. The sections were rehydrated through a graded series of ethanol in normal distilled H_2O over 20 minutes (95%, 70%, 50%, and 30%). The slides were rinsed in distilled H_2O once, and then in 1x TBS tween 20. The endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide for 10 minutes at room temperature. Following from this step the previously stated methodology (section 2.12.) was followed starting from the blocking step and onwards. Primary and secondary antibodies are detailed in table 2.4.

Primary Antibodies			Secondary Antibodies	
Antibody	**Dilution**	**Species/ Details**	**Antibody**	**Dilution**
mPRα	1:50	Goat (Santa Cruz /sc-50111)	Donkey anti-goat (Invitrogen/A11055)	1:400
mPRβ	1:50	Goat (Santa Cruz / sc-50109)	Donkey anti-goat (Invitrogen/A11055)	1:400
mPRγ	1:50	Goat (Santa Cruz / sc-28019)	Donkey anti-goat (Invitrogen/A11055)	1:400
PGRMC-1	1:75	Rabbit (Gift from Prof. P. Thomas	Donkey anti-rabbit (Invitrogen/A10042)	1:400
PR-B	1:50	Mouse (Santa Cruz / sc-811)	Goat anti-mouse (Santa Cruz / sc-3796)	1:75

Table 2.4: List of primary and secondary antibodies used with their respective dilutions.

2.15. Microscopy

The slides were examined and imaged on an using a Plan Apo Neofluor 63X NA 1.25 oil objective (Zeiss) on a Zeiss Axiovert 200M microscope and the images were viewed using the AxioVision software. Images were taken at a fixed exposure time. The data was derived after examining three independently treated coverslips for each sample along with the negative control.

2.16. Treatments of BeWo and JEG-3 cells

BeWo and JEG-3 cells were maintained in cell culture as described above. Cells were seeded in 6-well multi dishes for treatment purposes. Some BeWo cells were synsytialised for the following treatments to take place. Once confluence (~70%) was achieved, the media was aseptically removed and the cells were rinsed with 1xPBS. A known volume of phenol red free media containing only 100 μg/ml of Penicillin and 100 μg/ml of Streptomycin (Gibco)

was added to the cells 24 hours prior starting the treatments. BeWo CT, BeWo ST and JEG-3 cells were treated with the compounds shown below (Table 2.5). The time points were identical for all treatments and were the following: 1minute, 3 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, and 60 minutes.

Compound	Stock Concentration	Final concentration
Progesterone (P4) (Sigma, P0130)	10^{-1} M	100nM ± U0126 (1μM)
10-ethenyl-19-norprogesterone (OD 02-0) (Gift from Prof. P. Thomas)	10^{-3} M	100nM
Promegestone (R5020) (PerkinElmer, NLP00400)	10^{-2} M	30nM ± U0126
U0126 (Sigma, U120)	10^{-3} M	1μM

Table 2.5: Compounds used to treat cells, the final concentration used in the experiment is shown in the last column of the table.

2.17. Electrophoresis and Western blotting

Once the treatments were completed, BeWo CT, BeWo ST and JEG-3 cells where lysed in 2x Laemmli buffer and boiled at 100^{o}C for 3 minutes. The samples were then stored at -70^{o}C until further use. 12.5% SDS-PAGE gels were prepared. Firslty, the resolving polyacrylamide solution was poured between glass plates and was overlaid with isopropanol and allowed to polymerise for ~30minutes at room temperature. Once the gels had polymerised, the overlay was rinsed off with double distilled H_2O and the top of the gel was dried briefly using filter paper. The stacking gel was poured on top and the 10 well combs were placed in it. The stacking gel was left to polymerise for ~30minutes at room

temperature. Once ready to be used, the protein samples were firstly defrosted, then heated for 3 minutes using a heat block at 100^{o}C and then placed on ice. The samples were loaded onto the gel and were electrophoresed in 1X SDS-PAGE running buffer at 80V until the dye front reached the bottom of the gel. A ColorBurst^{TM} electrophoresis marker (Sigma-Aldrich, Cat.No. C1992) was used to monitor the progress of the electrophoretic run as well as to confirm that the protein transfer was completed. MagicMark™ XP Western Protein Standard was also used to detect protein size on film (Invitrogen, Cat. No. LC5602).

Before transferring the proteins onto a Polyvinylidene fluoride (PVDF) membrane (Immobilon-P Membrane, Millipore), the membrane was activated by incubating for 15 seconds in methanol, following by a 2 minute incubation in dH_2O and finally incubating for 5 minutes in wet-transfer buffer. Proteins were then electrophoretically transferred onto the activated PVDF membrane in wet-transfer buffer for 1 hour at 100V. The transblot sandwich was made up by placing a sponge pad on the upper and lower site of the apparatus, 2 pieces of Whatman paper on each site. The gel was placed on top of the paper on the lower site and a piece of membrane was placed on top of the paper. A note should be made that all components were soaked in wet-transfer buffer before making up the transblot sandwich. The transblot sandwich was positioned according to the manufacturer's instructions in a Biorad Transblot apparatus. To help minimise non-specific binding of the antibody to the membrane matrix, the membranes were incubated in blocking solution (5% w/v) dried milk powder in 1X TBS- Tween 20 for 3 hours at room temperature with gentle agitation.

The primary antibodies used are described in table 2.6. The membranes were incubated with the antibody of interest which was diluted in 5% Bovine Serum Albumin (Sigma-Aldrich, Cat. No. A7906) 1X TBS-Tween 20 overnight at 4^{o}C. Following three washes in 1X TBS-Tween 20 the membranes were incubated in horseradish peroxidase (HRP) conjugated

secondary antibody for 1 hour at room temperature. The secondary antibodies were diluted in 5% Bovine Serum Albumin in 1X TBS-Tween 20.

Primary Antibodies			Secondary Antibodies	
Antibody	**Dilution**	**Species/ Details**	**Antibody**	**Dilution**
mPRα	1:200	Goat (Santa Cruz /sc-50111)	Rabbit anti-goat (Sigma/A5420)	1:2000
mPRβ	1:200	Goat (Santa Cruz / sc-50109)	Rabbit anti-goat (Sigma/ A5420)	1:2000
Phospho-p44/42 MAPK (Erk1/2)	1:1000	Rabbit (Cell Signalling / 197G2)	Goat anti-rabbit (Sigma /A0545)	1:2000
p44/42 MAPK (Erk1/2)	1:1000	Rabbit (Cell Signalling / 9102)	Goat anti-rabbit (Sigma /A0545)	1:2000
Pan-Cadherin	1:1000	Mouse (Abcam / ab22744)	Rabbit anti-mouse (Zymed / 616520)	1:2000
Anti-PCNA	1:1000	Mouse	Rabbit anti-mouse (Zymed / 616520)	1:2000
GAPDH	1:2000	Rabbit (Sigma / G9545)	Goat anti-rabbit (Sigma /A0545)	1:2000

Table 2.6: List of primary and secondary antibodies used with their respective dilutions.

In order to detect the antibody complexes, the Enhanced Chemiluminescence (ECL) method was used. Solution A containing 100 mM Tris (pH 8.0) and 30% H_2O_2 was mixed with solution B containing 100 mM Tris (pH 8.0), 250 mM luminol and 90 mM coumaric acid and applied to the blots for 5 minutes under vigorous agitation. All the excess solution was drained and the blots were placed in a plastic case along with the x-ray film (Kodak, Biomax x-ray film). The x-ray film was developed using developer solution (Kodak, GBX developer & replinisher) and fixer solution (Kodak, GBX Fixer & Replenisher).

2.18. Densitometric Analysis

The Western blots were scanned and the digital images were uploaded using the AlphaEaseFC software.The band intensities were then normalised against the band intensity of GAPDH or in the case of phospho-ERK against, total ERK.

2.19. ImageStream^{x} Analysis

The ImageStream^{X} is an imaging flow cytometer providing scientists with the ability to visualise cells directly in flow, at rates exceeding 1,000 cells per second, and with the fluorescence sensitivity of the best conventional flow cytometers. The cellular morphology along with the intensity and location of fluorescent probes directed against antigens of interest can be quantitatively measured by using this instrument. Through microscopy detailed cellular images and morphologic information are offered to scientists, a useful scientific tool for the study of cell function. The interpretation of microscopic imagery though can be subjective and limited to qualitative information. Flow cytometry offers quantitative phenotyping and has the ability to yield statistically robust results by interrogating large numbers of cells. Flow cytometry lacks the ability to image, therefore the ability to measure sub-cellular localization and cell function are compromised and are measured indirectly. By combining the phenotyping abilities of flow cytometry with the detailed imagery and functional capabilities of microscopy, we chose to use ImageStream^{X} technology which overcomes the limitations of both techniques. Specifically, this approach was considered to be the most appropriate to use, apart from the reasons stated previously, but also due to the limitations arising by the chosen progesterone receptor isoforms to be studied as this are produced by a single gene.

Cells were grown to confluence (~70%) prior to being treated with 0.25% trypsin. The effect of trypsin was neutralized by addition of an equal volume of specific medium as mentioned

in section 2.2. The cell suspension was centrifuged at 1500 RPM for 5minutes prior to removing the supernatant and resuspending the cells in a known volume of medium. The cells were incubated with Progesterone-BSA complex labelled with fluorescein isothiocyanate (P4-BSA-FITC) (Sigma, Cat. No. P8779) for 5 minutes at 37^{o}C. Cells were then centrifuged for 5 minutes at 1500 RPM, the supernatant was removed and washed by gentle resuspension with 1x PBS 0.1% Tween 20. The cells were again centrifuged and removed the supernatant before fixing in 4% paraformaldehyde by incubation at room temperature for 10 minutes. Cells were centrifuged at 5000 RPM, the fixative was removed and washed by gentle resuspension with 1x PBS 0.1% Tween 20. Cells were centrifuged once more and fresh 1x PBS 0.1% Tween 20 was added. Counterstain DAPI (Vectashield® Mounting Medium) was added and the cells were placed at 4^{o}C for ~ 30 minutes prior to loading onto ImageStream^{x}.

The following protocol was performed using a non-BSA complexed antibody to the PR-B this time. Once cells were confluent (~70%) 0.25% of trypsin was used to detach the cells. The effect of trypsin was neutralized by addition of an equal volume of specific medium as mentioned in section 2.2. The cell suspension was centrifuged at 1500 RPM for 5minutes and the supernatant was removed. The cells were then resuspended in 1ml 1xPBS solution and transferred to a 1.5ml Eppendorf tube. The cells were then fixed by adding 500µl of 4% paraformaldehyde per tube and incubated at room temperature for 10mins. The cells were then centrifuged at 5000 RPM for 5 minutes, the fixative was removed and fresh 1x PBS solution was added. Another centrifugation step was performed and the supernatant was removed from the cells. 500µl of 0.5% Bovine Serum Albumin (Sigma Cat. No.A7906) in 1x PBS 0.1 % Tween 20 solution was added to the cells and incubated at room temperature for 1 hour on a roller to ensure a homogenous blocking. The cells were then centrifuged at 5000 RPM for 5 minutes and the supernatant was removed. Primary antibody, PR-B (Santa

Cruz, Cat.No sc-811) was added at a dilution of 1:100 made in 0.5% Bovine Serum Albumin in 1x PBS 0.1 % Tween 20 solution and incubated at room temperature for 1 hour. A centrifugation step followed where the supernatant was removed. Two washes with 1x PBS 0.1 % Tween 20 solution were performed. Then the cells were incubated with anti-mouse TRITC (Santa Cruz, Cat. No. sc-3796) secondary antibody at a dilution of 1:50 made up in 0.5% Bovine Serum Albumin in 1x PBS 0.1 % Tween 20 solution. The cells were incubated for 1 hour at room temperature. The cells were then centrifuged, the supernatant was removed and two washes with 1x PBS 0.1 % Tween 20 solution followed. Counterstain DAPI (Vectashield® Mounting Medium) was added and the cells were placed at 4^{o}C for ~ 30 minutes prior to loading onto ImageStream^{x}.

2.20. Cell Proliferation assay (MTT assay)

An MTT (Sigma, Cat.No.CGD-1) test was carried out in order to assess the viability of BeWo cells after exposing them to Forskolin treatments for set time points (48 hours and 72 hours). The number of cells per well was set to $5x10^4$ and the cells were plated in 96 multi well dishes. It is important to note that media without phenol red was used for this assay. MTT solution (Provided: 5mg/ml MTT in RPMI-1640 without phenol red) equal to 10% of the culture volume was added to each well and incubated for 3.5 hours. Following, the resulting MTT formazan crystals were dissolved in MTT solvent (0.1 N HCl in anhydrous Isopropanol). Absorbance was read at 560nm and 620nm using a spectrophotometric microplate reader and the absolute absorbance was calculated. The experiments were performed in triplicate wells and repeated 3 times.

2.21. Trypan Blue exlusion assay

BeWo and JEG-3 cells were maintained in cell culture as described above (2.2). Cells were seeded in 6-well multi dishes for treatment purposes. Once confluence was achieved, the

media was aseptically removed and the cells were rinsed with 1xPBS. A known volume of phenol red free media containing only 100 μg/ml of Penicillin and 100 μg/ml of Streptomycin (Gibco) was added to the cells 24 hours prior starting the treatments. The cells were incubated for 24 hours with the specific compounds (Table 2.7) prior to starting the trypan blue exclusion assay. It should be noted that U0126 compound was used to pre-treat the cells for 30 minutes before continuing with any other treatments.

Compound	Final Concentration	Combination treatments
Progesterone (P4) (Sigma, P0130)	1nM	• ± IL-1β ± U0126
	10nM	• ± IL-1β ± U0126
	100nM	• ± IL-1β ± U0126
	1000nM	• ± IL-1β ± U0126
10-ethenyl-19-norprogesterone (OD 02-0) (Gift from Prof. Thomas)	1nM	• ± IL-1β ± U0126
	10nM	• ± IL-1β ± U0126
	100nM	• ± IL-1β ± U0126
	1000nM	• ± IL-1β ± U0126
IL-1β (Sigma, I9401)	10ng/ μl	
Promegestone (R5020) (PerkinElmer, NLP00400)	30nM	• ± IL-1β ± U0126
U0126 (Sigma, U120)	1μM	

Table 2.7: Compounds used to treat cells. Final concentration used to treat cells is shown along with the combined treatments that were performed.

Once the incubation period was over, medium was removed and the cells were rinsed in 1xPBS. Following a 0.25% trypsin solution was added to the cells for ~ 3 minutes. The effect of trypsin was then neutralised by addition of an equal volume of medium. The cell suspension was centrifuged at 1000 RPM for 5 minutes. The supernatant was aseptically removed and the cells were resuspended in a known volume of medium. In order to determine the cell numbers an aliquot was removed from the cell suspension and mixed with trypan blue stain (Gibco) in a 5:1 ratio, total volume 100 µl. 20µl were removed and placed on a hemocytometer (Neubauer) with a chamber depth of 0.1 mm. The number of cells was recorded in 4 large squares under a 10x lens on a phase contrast light microscope. The number of dead cells that had been stained blue was recorded along with the total number of cells which was estimated by following the equation: *Average No. of cells* ***x*** *dilution factor* ***x*** 10^4.

2.22. Statistical Analysis

Data were expressed as mean± SEM. For the quantitative PCR, the following equations were used: ΔCt=Ct (gene of interest)-Ct (house keeping gene), ΔΔCt=ΔCt (sample) - ΔCt (calibrator), Relative Quantity (RQ) $=2^{-\Delta\Delta Ct}$. RQ value was set up as 1 for the untreated (no supplement) BeWo and JEG-3 cells. We have also calculated the gene expression levels as RQ values, using the untreated control for each cell line as a calibrator. We did so, since RQ values provide a more accurate comparison between the initial amounts of template in each sample, without requiring an exact copy number for analysis. Statistical analysis of the ΔCt and RQ value was performed using student's t-test. For the correlation studies, a two-tailed test using SPSS (Version 18) was used. For the western blotting studies, each band was quantified using AlphaEaseFC software followed by student's t-test. A p value of <0.05 was regarded as significant for all the above mentioned studies.

3

CHAPTER 3

3. RESULTS

3.1. Validation of BeWo syncytialisation model

BeWo cells are fusigenic thus they have the ability to syncytialise *in vitro*. Once they are fully syncytialised they resemble a third trimester placental model, whereas in the non-differentiated state their morphology is similar to that of primary trophoblast cultures (Pattillo & Gey, 1968; Hertz, 1959). This property of BeWo cells will be instrumental in providing a better insight of the 3rd trimester human placenta by allowing examination of two distinct functional cell types which are vital for the functionality of the human placenta. Prior to embarking on any further studies the syncytialisation model had to be validated, and in order to assess the incidence of syncytialisation in BeWo cells a number of different markers were used.

3.1.1. Expression of Proliferating Cell Nuclear Antigen (PCNA)

Proliferating Cell Nuclear Antigen (PCNA) was used as an indicator of cell proliferation as it is found in the nucleus of cells during the DNA synthesis phase. BeWo cells were treated with the chemical agent forskolin, an adenyl cyclase activator (Lyden *et al*., 1993), which was used to induce the syncytialisation process for 48 hours at a concentration of 50 μM and

100 μM. Expression of PCNA at mRNA level was significantly down-regulated in BeWo cells that were treated with forskolin (50 and 100 μM) for 48 hours when compared to the untreated BeWo cells (Fig.3.1). The results obtained from semi- quantitative RT-PCR were used to quantify the amount of DNA amplified for PCNA and were normalised against β-actin which was the housekeeping gene. The normalised signal intensities obtained are represented in Fig. 3.2.

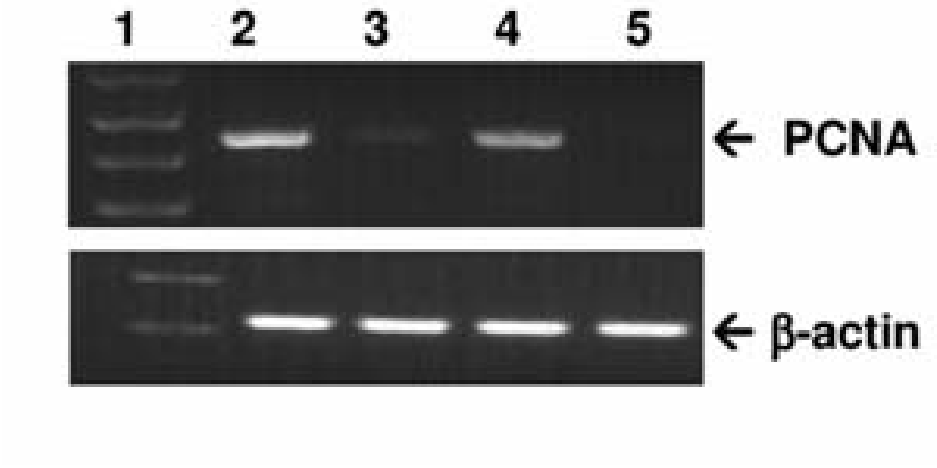


Figure 3.1: RT-PCR gel demonstrating the expression of PCNA and β-actin housekeeping gene in treated and untreated BeWo cells. Lane 1: DNA ladder; lane 2: cDNA from untreated BeWo cells (48 hours); lane 3: cDNA from stimulated BeWo cells (50 μM forskolin, 48 hours); lane 4: cDNA from untreated BeWo cells (48 hours); lane 5: cDNA from stimulated BeWo cells (100 μM forskolin, 48 hours).

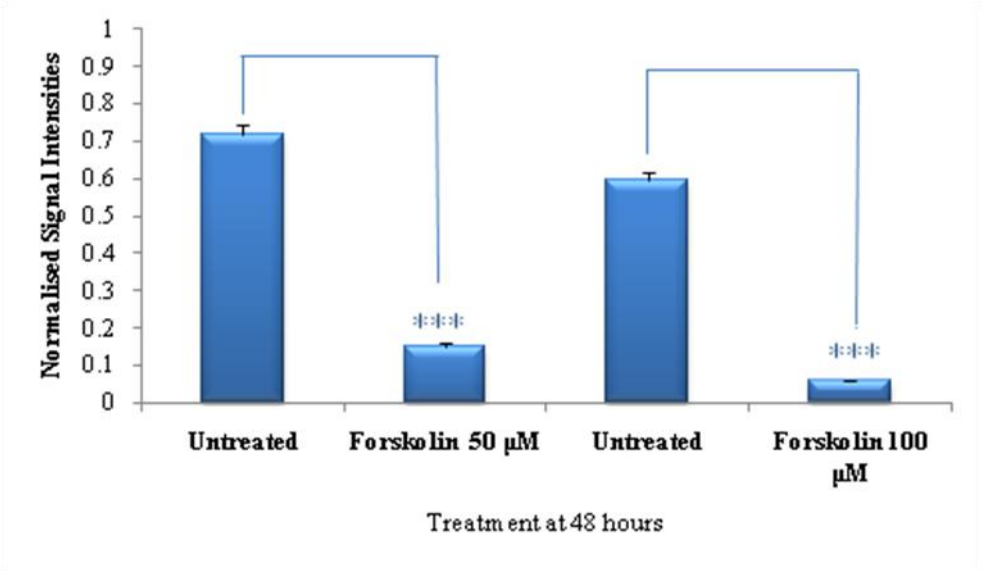


Figure 3.2: Densitometric analysis of PCNA in forskolin treated and untreated BeWo cells. Expression of PCNA was normalised against the expression of β-actin housekeeping gene. Each histogram represents the mean ± SEM of three individual experiments. Blue lines indicate significance, $p<0.0001$***.

The expression of PCNA was also examined at the protein level, (Fig. 3.3) demonstrating down-regulation following treatment with 50 µM and 100 µM Forskolin for 72 hours (lane 1 & 3) when compared with the untreated samples (lane 2 & 4). The membrane was reprobed with antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as shown in the lower panel of Fig.3.3. Densitometric analysis (Fig. 3.4) confirmed a significant reduction ($p<0.001$) in PCNA protein in the BeWo cells treated with 50 and 100 µM forskolin for 72 hours compared to untreated cells.

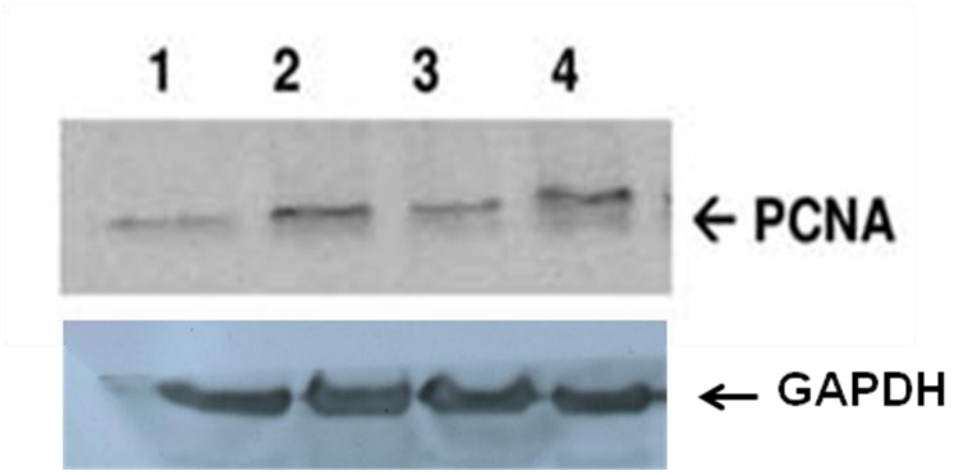


Figure 3.3: Protein expression of PCNA and GAPDH . Lane 1: BeWo cells treated with 50 μM forskolin for 72 hours; lane 2: control, unstimulated BeWo cells (72 hours), lane 3: BeWo cells treated with 100 μM forskolin for 72 hours; lane 4: control, unstimulated BeWo cells (72 hours).

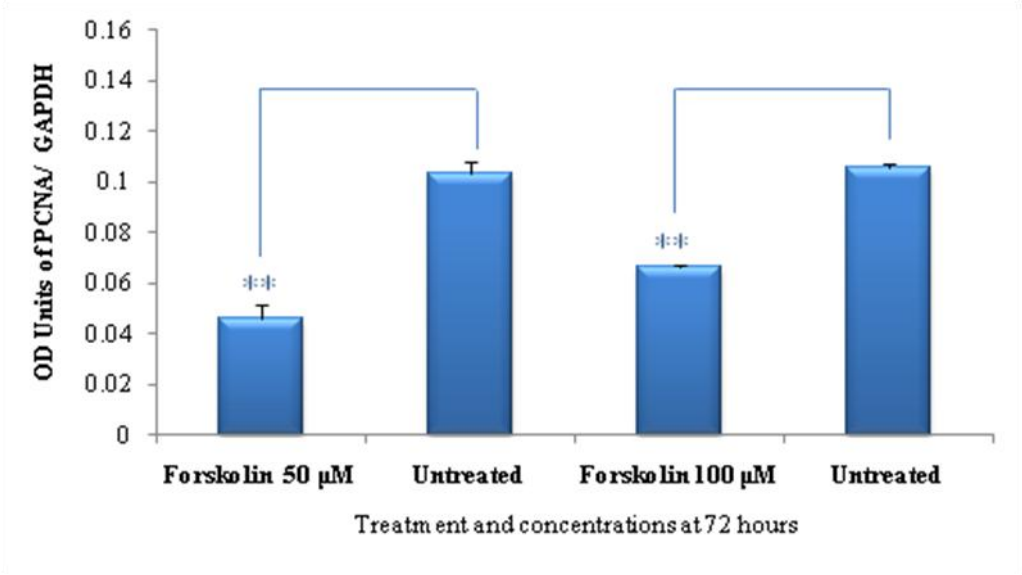


Figure 3.4: Densitometric analysis of PCNA in forskolin treated (50 and 100 μM) for 72 hours and untreated BeWo cells. Each histogram represents the optical density units. PCNA expression was normalised against the expression of GAPDH. Blue lines indicate significance, $p<0.001$**.

In order for the mononuclear cytotrophoblast cells to fuse and form the multinuclear syncytia layer, the cells must stop proliferating (Benirschke *et al*., 2005; Tanaka *et al*., 1998). As PCNA is only found in the nucleus of proliferating cells it is evident from the above results that BeWo cells undergoing chemical syncytialisation *in vitro* showed decreased levels of PCNA at both gene and protein level as a result of ceased proliferation.

3.1.2. Expression of Pan-Cadherin in syncytialised BeWo cells

During the process of syncytialisation the plasma membrane of the individual cytotrophoclast cells fuses in order to form the multinuclear syncytia layer. Therefore the decrease in plasma membrane content is an indication that the process of differentiation is in order. We have used a cell membrane marker, pan-Cadherin to examine the levels of the cadherin protein in treated and untreated cells to determine whether a decrease in total plasma membrane was achieved following treatment with forksolin. Western blot analysis revealed down-regulation of cadherin protein levels in BeWo cells that were treated with forskolin over a total of 72 hours at both concentrations (50 μM & 100 μM) when compared with the non-treated control BeWo cells (Fig.3.5). As shown in Fig. 3.6 the down regulation in the expression of cadherin in BeWo cell treated with 50μM forskolin for 72 hours was significant ($p < 0.001$) when compared to the untreated BeWo cells. Cells that were treated with 100 μM of forskolin showed a significant ($p < 0.05$) downregulation of cadherin when compared to the untreated BeWo cells (Fig.3.6).

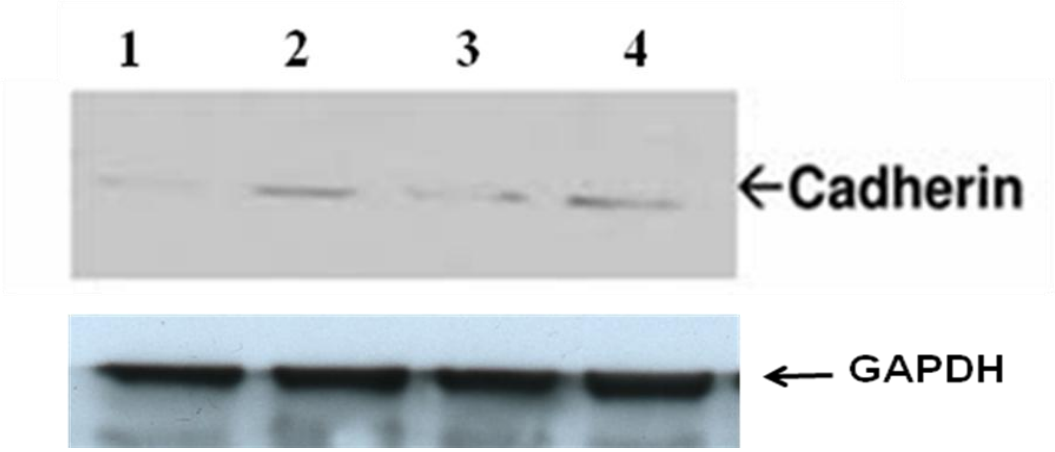


Figure 3.5: Protein expression of Cadherin and GAPDH. Lane 1: BeWo cells treated with 50 µM forskolin for 72 hours; lane 2: control, unstimulated BeWo cells (72 hours), lane 3: BeWo cells treated with 100 µM forskolin for 72 hours; lane 4: control, unstimulated BeWo cells.

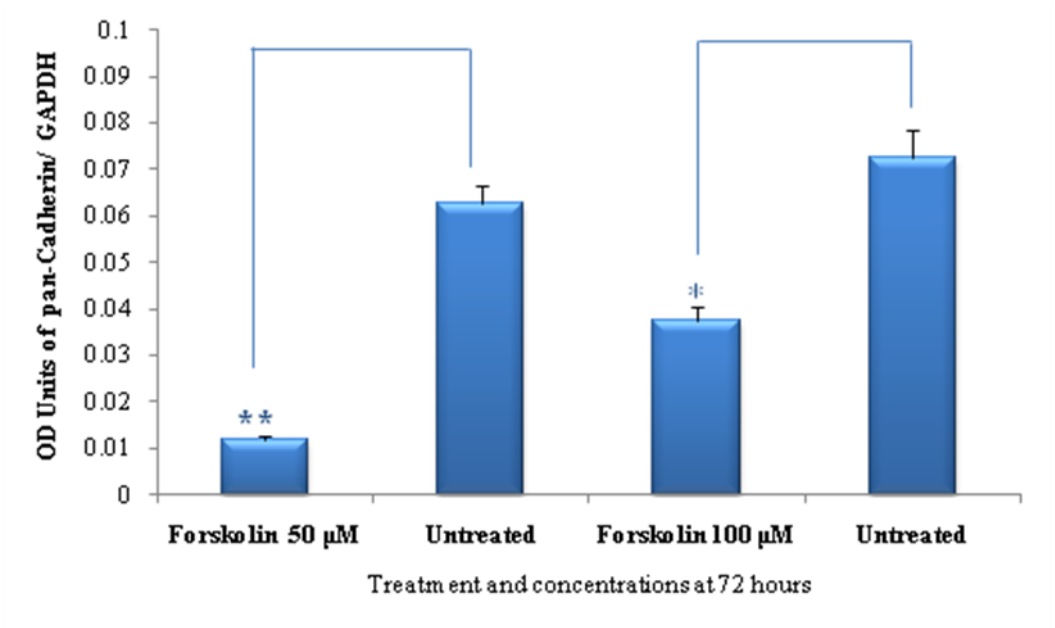


Figure 3.6: Densitometric analysis of Cahderin in forskolin treated (50 and 100 µM) and untreated BeWo cells. Each histogram represents the optical density units. Cadherin expression was normalised against the expression of GAPDH. Blue line indicate significance, $p < 0.001$**, $p < 0.05$*.

Immunostaining was performed on BeWo syncytialised cells using the same pan-cadherin antibody to visualize if the plasma membrane had started to fuse as it does during the process of syncytialisation. BeWo cells treated with 50 μM & 100 μM forskolin for 48 hours (Fig.3.7) and 72 hours (Fig.3.8) were subjected to immunofluoresence analysis.

It is clearly demonstrated that the cell membranes of the mononuclear BeWo cytotrophoblast cells started to fuse with each other 48 hours after treatment. Some cells still maintain their individual cell membrane at this stage. Following 72 hours of forskolin treatment it had become more difficult to visualize the cell borders of individual BeWo cytotrophoblast cell as the multinuclear syncytia had started to form.

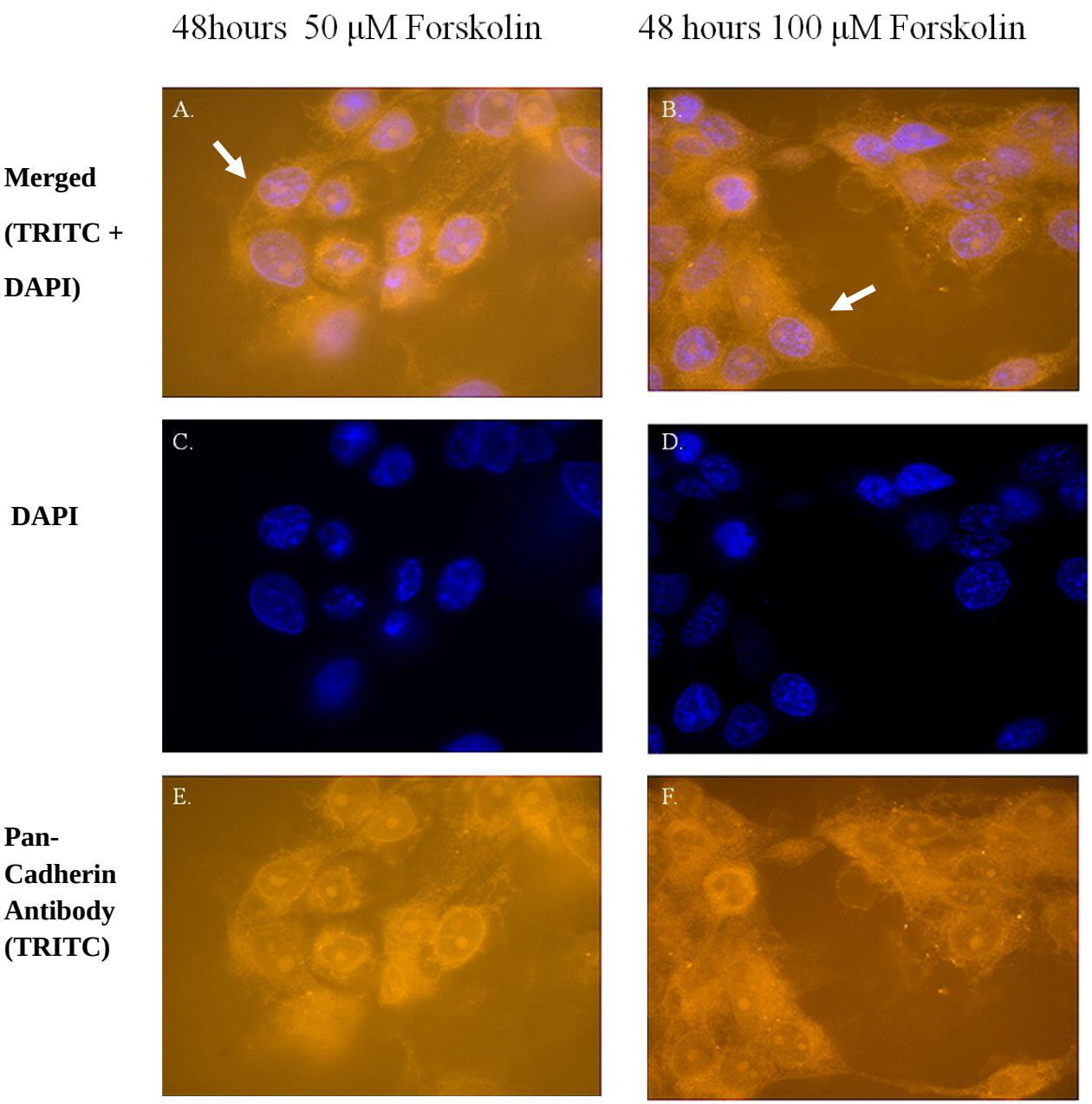


Figure 3.7: Immunostaining with cadherin in BeWo cells treated with 50 µM & 100 µM forskolin for 48 hours. White arrows in the panels A and B of the merged images of Pan-Cadherin and DAPI staining; indicate at both doses the points where the plasma membrane has fused. Panels C and D indicate the DAPI stained nuclei whereas E and F Pan-Cadherin panels indicate the specific staining of the plasma membrane.

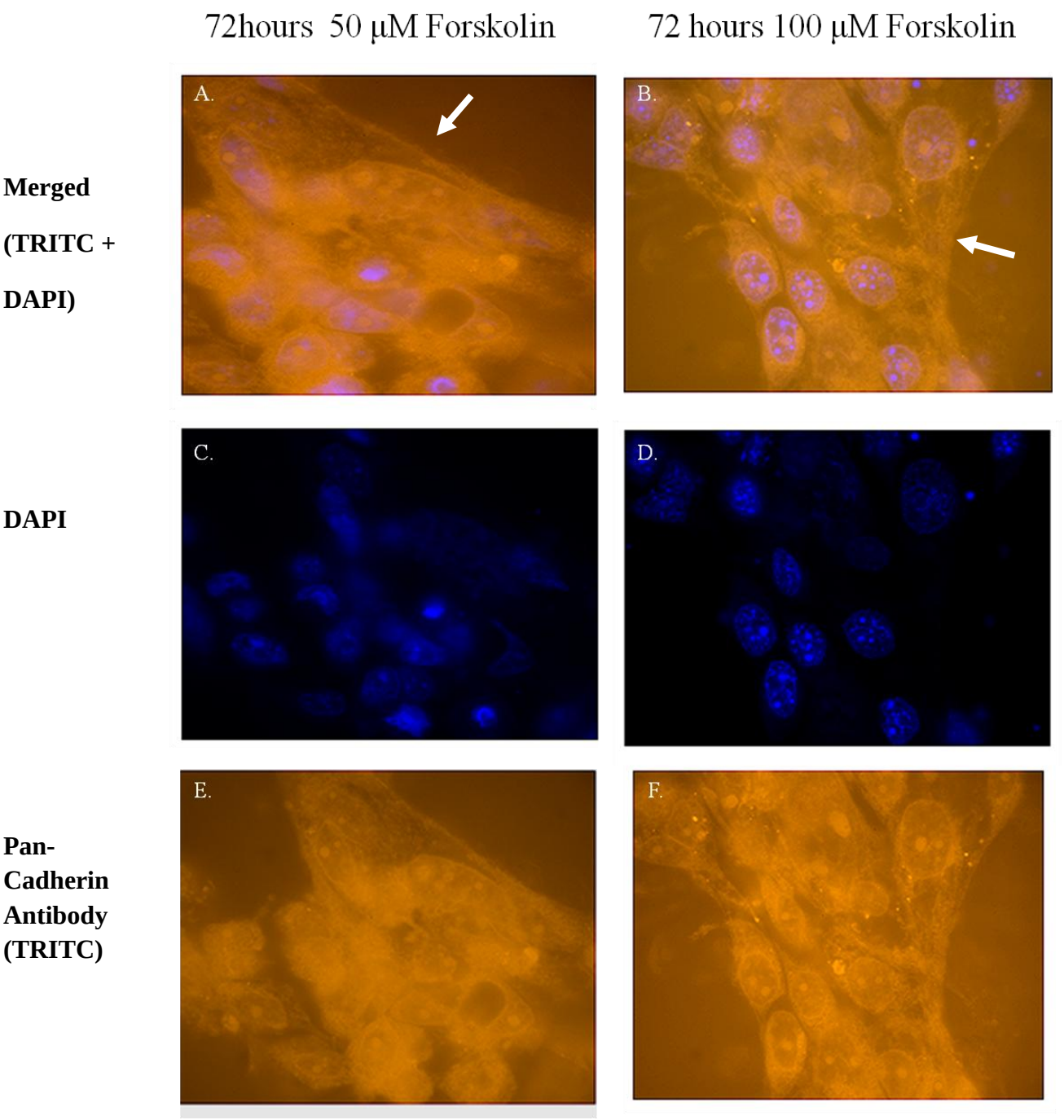


Figure 3.8: Immunostaining with cadherin in BeWo cells treated with 50 µM & 100 µM forskolin for 72 hours. White arrows in the panels A and B of the merged images of Pan-Cadherin and DAPI staining, indicate at both doses the points where the plasma membrane has fused. Panels C and D indicate the DAPI stained nuclei whereas E and F Pan-Cadherin panels indicate the specific staining of the plasma membrane.

Immunostaining with Pan- Cadherin antibody on non-treated control BeWo cytotrophoblast cells revealed no signs of fusion (Fig. 3.9). In contrast distinct cell borders of individual cells were visible.

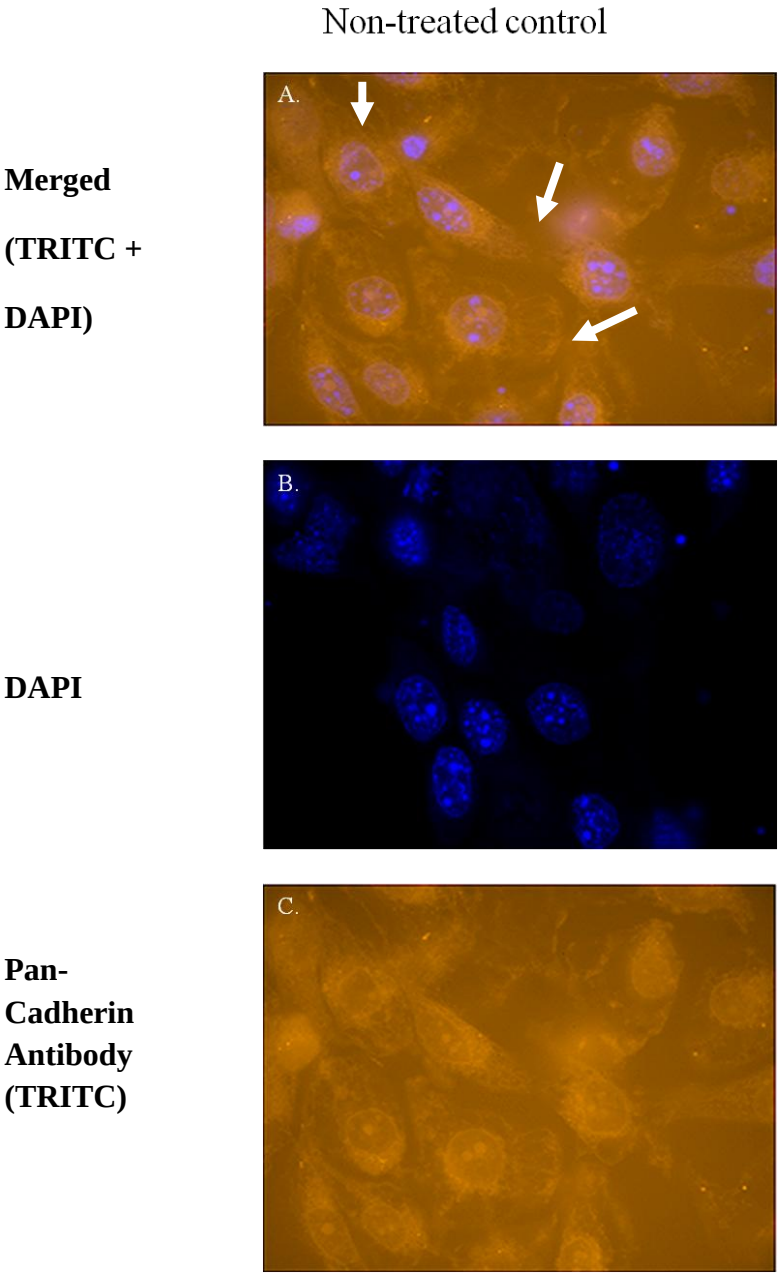


Figure 3.9: Immunostaining using Pan-Cadherin antibody in untreated BeWo cells. No signs of plasma membrane fusion are visible (white arrows) in panel A. Panel B indicates the staining with DAPI and panel C the cadherin staining.

3.1.3. Examining the proliferation and viability of BeWo cells undergoing syncytialisation.

During the process of syncytialisation it is required for the cells to stop proliferating in order to syncytialise. Trophoblastic cells though should remain viable during this process. Ceased proliferation of BeWo cytotrophoblast cells may also be attributed as cell death due to the addition of forkolin. To eliminate the possibility of cell death and to assess the proliferation and viability of BeWo cell undergoing chemical syncytialisation, two approaches were used – a cell proliferation assay based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and a trypan blue exclusion assay based on the dye trypan blue.

Once BeWo cells were plated and were chemically syncytialised with 50μM and 100 μM of forskolin the MTT assay was performed at both time points (48 hours and 72 hours) to assess proliferation of these cells. It would be anticipated for treated cells to show less proliferation that the control cells. It should be noted that the control cells were also grown in media containing DMSO as it was the vehicle for foskolin. Given that we also tested the effect of DMSO alone on cell proliferation. The vehicle (i.e. DMSO) did not exert any proliferative effects.

The principle of this test lays in the ability of the mitochondrial dehydrogenases of the viable cells to cleave the tetrazolium ring yielding purple MTT formazan crystals that are further dissolved in acidified isopropanol. This results in a purple solution being formed which is spectrophotometrically measured. If the cell number has increased the amount of MTT formazan formed would increase and subsequently there would be an increase in absorbance.

Absolute absorbance was calculated by subtracting the absorbance readings of 560nm from the absorbance readings of 620nm. A graph displaying the absolute absorbance readings of BeWo cells treated with forskolin for 48 and 72 hours with 50 and 100 μM is shown in Fig. 3.10. It is evident that the forskolin treated BeWo cells showed less proliferation. This is in

accordance with the syncytialisation process they were induced to undergo. When comparing the BeWo cells treated with 50 µM and 100 µM forskolin for 48 hours with the control BeWo cells, a significant decrease in proliferation was observed. Whereas when the BeWo cells were treated for 72 hours, a significant decrease was detected only with 100 µM forskolin upon comparison with the control BeWo cells.

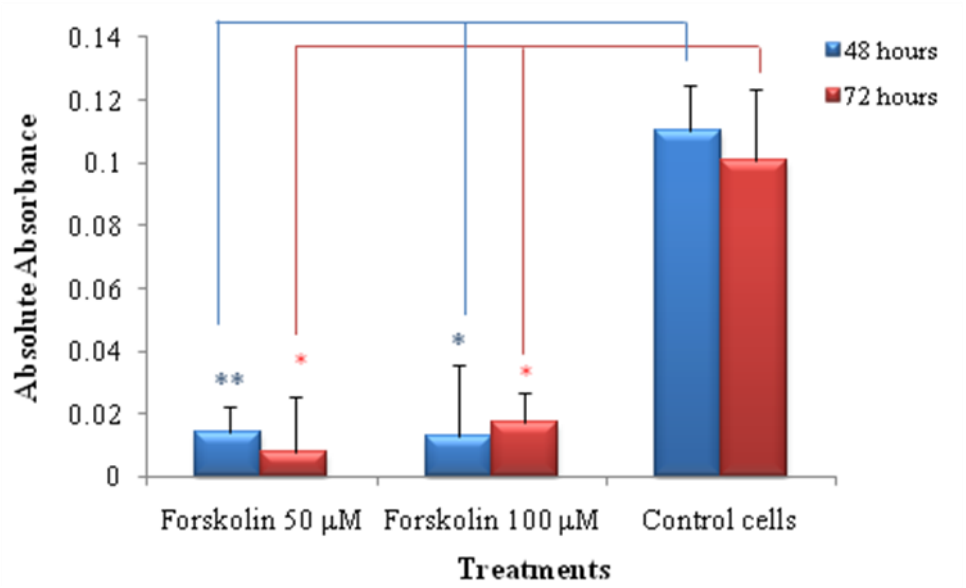


Figure 3.10: Graphical representation of the absolute absorbance of BeWo cells treated with forskolin for 48 hours and 72 hours with 50 µM and 100 µM. Each histogram represents the mean ± SEM of three individual experiments. Red lines indicate significance of treatment with 100 µM of forskolin for 72 hours when compared to the control BeWo cells, whereas blue line indicates significance of treatment with 50 µM & 100 µM of forskolin for 48 hours when compared to the control BeWo cells ($p < 0.001$ **, $p < 0.05$ *).

To determine the percentage of BeWo cells that had died during the syncytialisation process the trypan blue exclusion assay was also performed. The principle of this assay lays in the ability of the blue dye to be absorbed by dead cells as their cell membrane is not intact.

Therefore the dead cells are stained blue, can be visualised under a microscope and subsequently counted.

Graph displayed in Fig.3.11 shows the percentage of dead BeWo cells. No dead cells were seen when the cells were grown in media containing DMSO and BeWo cells maintained in culture in standard media indicating that no effect of DMSO is exerted on the cells. BeWo cells treated with 50 and 100 µM of forskolin for 48 and 72 hours had a percentage of dead cells between 20% - 30%.

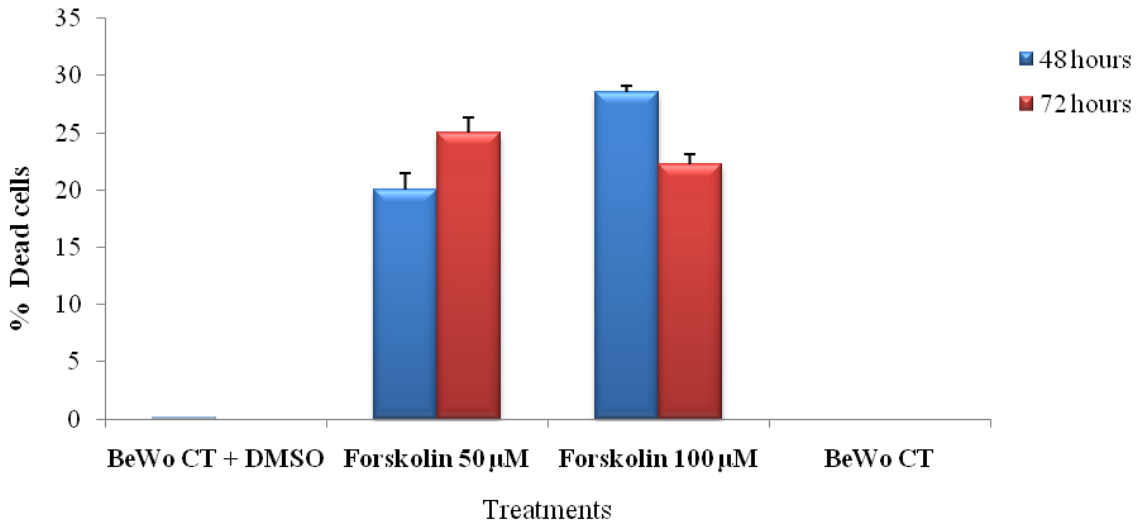


Figure 3.11: Percentage of dead BeWo CT cells after performing Trypan Blue assay. BeWo cells were treated with forskolin 50 & 100 µM, also dead cells were counted for untreated BeWo CT cells and BeWo cells cultured in the presence of DMSO. Each histogram represents the mean ± SEM of three individual experiments.

3.1.4. Examining the expression of syncytin-2

The expression of syncytin -2 was chosen to be studied as a syncytialisation marker as it has been identified recently to be a placental membrane protein with fusogenic properties

(Malassine *et al*., 2006). The expression was examined in BeWo cells that were treated with 50μM and 100μM of forskolin for 48 and 72 hours along with control samples of non-treated BeWo CT cells. The Q-PCR results presented in Fig.3.12 indicate that the expression detected in BeWo cells treated with 50μM and 100μM forkolin for 48 hours were significantly uperegulated when compared to the non-treated cells, a sign that the cells had started to fuse into syncytiotrophoblasts (Mparmpakas *et al*., 2010). After treating the cells for 72 hours with 50μM of forkolin a significant up-regulation in the expression of syncytin-2 was detected compared to the control cells. The relative quantity (RQ) value of the non-treated BeWo cells was set to 1 as these cells were the control samples (Mparmpakas *et al*., 2010).

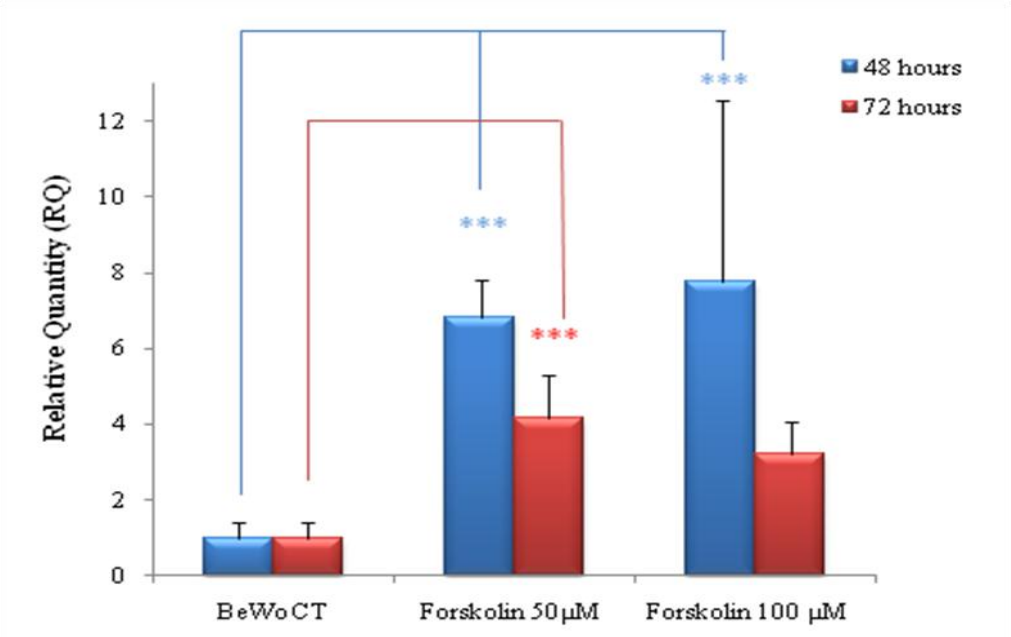


Figure 3.12: Expression of syncytin-2 in BeWo cells treated with 50 & 100μM forkolin for 48 & 72 hours. Each histogram represents the mean ± [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments and the expression is relative to β-actin housekeping gene. Red lines indicate significance of expression after treating cells with 50 μM for 72 hours, whereas blue line indicates significance of expression after treating cells with 50 & 100 μM for 48 hours ($p < 0.0001$ ***).

3.2. Discussion

3.2.1. Validation of BeWo syncytialisation model

BeWo and JEG-3, the two cell lines studied are widely used to investigate placental physiology and function (Novakovic *et al*., 2011; Burleigh *et al*., 2007). These cell lines have distinct fusigenic capacities. The JEG-3 cell line, despite being of choriocarcinoma origin, is unable to morphologically differentiate and therefore it resembles the undifferentiated cytotrophoblast cells unable to produce hormones thus making it an appropriate *in vitro* model to investigate first trimester placental events (Ntrivalas *et al*., 2006). BeWo cells are fusigenic and when they are fully differentiated (i.e. syncytialised) they resemble a third trimester placental model. At this stage the predominant feature of the human placenta is the hormonally active syncytiotrophoblast layer. The multinucleated syncytiotrophoblasts cover the external surface of the placental villous and are in direct interface with maternal blood (Das *et al*., 2004). Undifferentiated BeWo cells are similar in morphology to primary trophoblast cultures, with a monolayer and microvillar projections on the apical side. The capability of BeWo cells to differentiate has established these cells as an *in vitro* model to study immune, endocrine and developmental aspects (Heaton *et al*., 2008; Neelima & Rao, 2008; Ellinger *et al*., 1999), as well as placental transport mechanisms (Manley *et al*., 2005).

Validation of the syncytialisation model was performed using a wide range of morphological markers and examining different aspects of the syncytialisation process. In our hands, validating the BeWo syncytialisation model had been also achieved (Zachariades *et al*., 2011; Mparmpakas *et al*., 2010). Proliferating Cell Nuclear Antigen (PCNA) is present in the nucleus of replicating cells only. Cytotrophoblast cells and thus BeWo cells exit the cell cycle to being able to start differentiating (Benirschke *et al*., 2005). PCNA, a marker of proliferation which has been used in the past to assess proliferation of cytotrophoblast cells in

gestational trophoblastic diseases (Xue *et al*., 2003) when used in our BeWo cell preparations treated with forskolin (50μM & 100μM) for 48 hours demonstrated significant down-regulation of PCNA transcript, an indication of ceased proliferation in comparison to non treated BeWo cells. Antibodies against cadherin protein have been used as plasma membrane markers (Benaitreau *et al*., 2010). Staining of BeWo cells that were treated with 50μM and 100μM of forskolin using a pan-cadherin antibody showed signs of plasma membrane fusion and formation of multinucleated syncytia that became more intense after 72 hours of treatment, this is in agreement with previous studies performed confirming syncytia formation in *in vitro* differentiated BeWo cells (Al-Nasiry *et al*., 2006; Coutifaris *et al*., 1991). Cells that were maintained in standard cell culture conditions and were not exposed to forskolin when stained with pan-cadherin, had intact plasma membranes with distinct cell boundaries. Significant reductions in the levels of cadherin protein were also observed in immunoblots performed using cell preparation treated with forskolin (48 & 72 hours with 50μM & 100μM).

A well accepted characteristic of trophoblast differentiation is the arrest of mitosis, where the cells cease proliferating (Anson-Cartwright *et al*., 2000) without cell viability being endangered. Similarly, in the *in vitro* differentiation model BeWo cytotrophoblast cells will stop proliferating in order to form multinucleated syncytia. BeWo cytotrophoblast cells though should remain viable during this process. Ceased proliferation of BeWo cytotrphoblast cells may also be attributed as cell death due to the addition of forskolin. The proliferation assay confirmed that BeWo cells had ceased proliferating after treatment with forskolin for 48 and 72 hours with 50μM & 100μM. Significant reduction in MTT absorbance was observed in the treated cells when compared to the untreated thus leading to the conclusion that cells were still viable hence their mitochondria were able to produce formazan, corroborating earlier findings (Al-Nasiry *et al*., 2006).

The fusigenic protein syncytin-2 which recently was shown to hold a key role in trophoblast differentiation by mediating cell fusion (Vargas *et al*., 2009) was shown to be significantly up-regulated in forskolin treated BeWo cells when compared to the non treated cells (Zachariades *et al*., 2011). This is in accordance with previous studies indicating that syncytin-2 was found up regulated in placental cells that had been exposed to forskolin (Chen *et al*., 2008; Blaise *et al*., 2003).

3.3. Expression of Progesterone Receptors in placental cell lines: effects of syncytialisation on receptor expression.

3.3.1. Evaluation of the expression and the cellular localization of membrane progesterone receptors (mPRs) in BeWo and JEG-3 cell lines.

Up to now, there is no data about the expression of PRs in human choriocarcinoma cell lines, which are used extensively as placental experimental models. Therefore the expression of progesterone receptors which include the membrane progesterone receptors, the progesterone membrane component – 1 and the nuclear progesterone receptor (PR-B) were investigated in syncytialised and non syncytliased BeWo cells as well as in JEG-3 cells.

The expression of membrane progesterone receptors (mPRs) at the gene level was evaluated in detail in two cell lines, BeWo and JEG-3. The expression was also assessed in BeWo cytotrophoblast (CT) cells and BeWo syncytiotrophoblast (ST) cells.

Following the extensive evaluation of the syncytialisation model, and based on the following reasons, it was agreed that for consistency purposes throughout the study the fully syncytialised BeWo cells to be treated with 50μM forskolin for 72 hours.We have shown that at 72 hours and treated with 50μM forskolin BeWo cells significantly decreased the expression of PCNA and Cadherin proteins (Mparmpakas *et al*., 2010), both are important markers as PCNA is only found in the nucleus of proliferating cells, therefore it was confirmed that BeWo cells had ceased proliferating in order to syncytialise (O'Brien *et al*., 2003). Cadherin is another important marker and more specifically it is a plasma membrane marker. As the process of differentiation progresses the levels of plasma membrane decrease as a sign of fusion and this was confirmed in our model as a significantly decreased expression was detected. This was again confirmed at protein level, where antibodies to cadherin were used and the results once visualised confirmed the fusion of BeWo cells as no distinct cell boundaries were visible. The results obtained from the MTT assay confirmed that the cells showed significantly less proliferation than the untreated BeWo cells. Further, the

cells that where treated with the 50μM forskolin for the period of 72 hours, showed a 25% dead cells following the treatment a percentage that was found to be intermediary in comparison to the results seen from the percentage of dead BeWo cells of the 48 hour treatments. Lastly, the profound result of the expression of syncytin-2 mRNA was shown to be significantly ($p < 0.0001$) upregulated in comparison to the cells treated with 100 μM forskolin for the same time period (72 hours) (Mparmpakas *et al*., 2010). Q-PCR analysis revealed that there was a significant down regulation in the expression of mPRα, mPRδ and for mPRγ a more modest decrease was seen in BeWo ST when compared to the expression of the same genes in BeWo CT. The expression of mPRβ was found to be significantly upregulated in BeWo ST when compared to the expression seen in BeWo CT. The graph in Fig. 3.13 shows the results expressed in relative quantity (RQ) values where the expression of BeWo CT was set at 1. Table 3.1 below indicates the down regulation and upregulation of these genes in percentile.

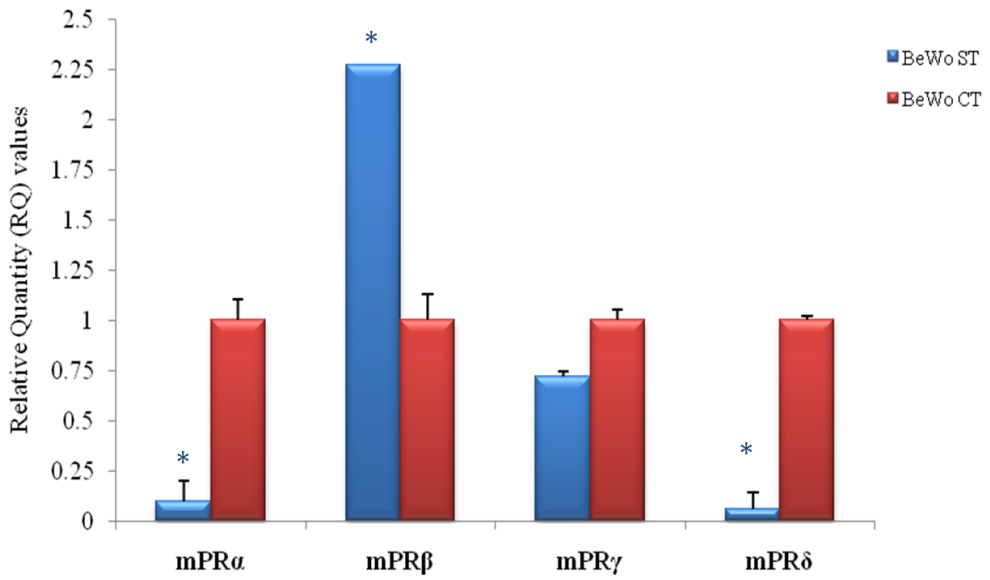


Figure 3.13: Expression of mPRα, mPRβ, mPRγ , mPRδ in control untreated BeWo cells (BeWo CT) vs. BeWo ST cells (forskolin 50 μM for 72 hours) (Zachariades *et al*., 2011).

Following, when the non-differentiated but fusigenic BeWo (CT) cells where compared to the non-fusigenic JEG-3 cells, it was found that there was a significantly lower expression of mPRα, mPRβ, mPRγ (Fig. 3.14), whereas there was no expression of mPRδ in JEG-3 cells. In table 3.1, the percentages in the down regulation of the mPRs can be seen.

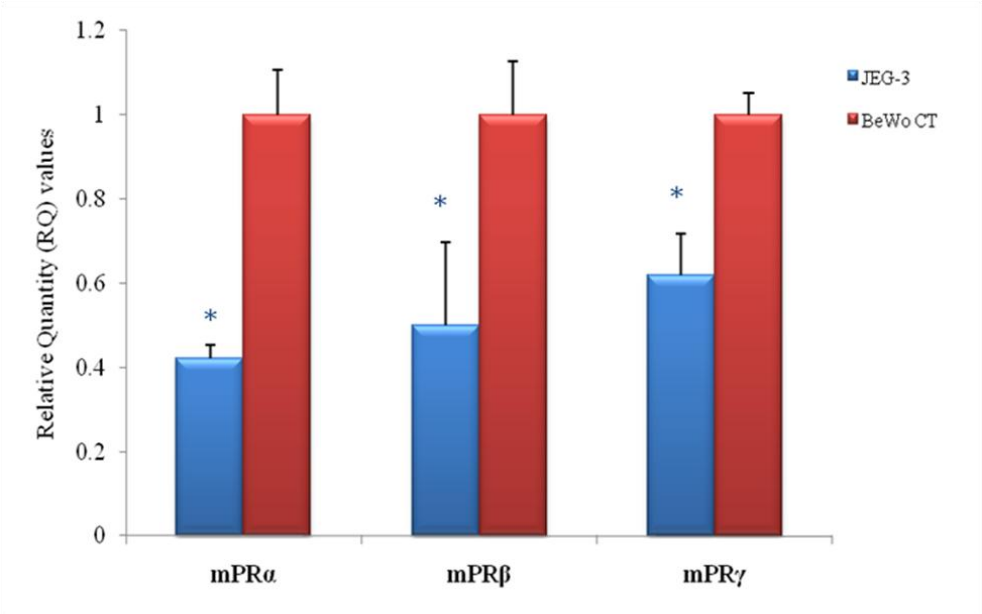


Figure 3.14: Expression of mPRα, mPRβ, mPRγ in control untreated BeWo cells (BeWo CT) vs. JEG-3 cells (Zachariades *et al*., 2011).

Cell line/ cell type comparison			
Gene	**BeWo ST versus BeWo CT**	**JEG-3 versus BeWo CT**	**BeWo ST versus JEG-3**
mPRα	90% ↓	58% ↓	28%
mPRβ	127% ↑	50% ↓	120%
mPRγ	28% ↓	38% ↓	80%
mPRδ	99% ↓	No expression	No expression
mPRε	No expression	No epxression	No expression

Table 3.1: Gene expression expressed in percentile in untreated BeWo cells (BeWo CT) vs. BeWo ST cells (forskolin 50 μM for 72 hours), in untreated BeWo cells vs. JEG-3 cells and in BeWo ST cells (forskolin 50 μM for 72 hours) vs. JEG-3 cells.

The protein expression and cellular localization of mPRs was assessed in JEG-3, non-syncytialised (CT) and syncytialised (ST) BeWo cells, using immunofluorescent analysis. The protein expression of mPRα in JEG-3 was detected on the plasma membrane and in the cytoplasm and it appears as a homogenous staining (Fig.3.15). mPRβ and mPRγ showed a cytoplasmic localisation as seen in Fig.3.15 with a dotted appearance. As observed in the panel of images below (Fig.3.15) in BeWo CT cells, mPRα, mPRβ and mPRγ are localised in the plasma membrane as well as in the cytoplasm and showed a dotted appearance. When observing the images collected for the expression of mPR proteins in BeWo ST cells it was seen that mPRα was predominantly localised in the cytoplasm homogeneously, whereas mPRβ was present also on the plasma membrane. mPRβ shows a peri – nuclear dotted staining. mPRγ was expressed intensely on the plasma membrane with some dotted staining being visible. (Fig.3.15) (Zachariades *et al*., 2011).

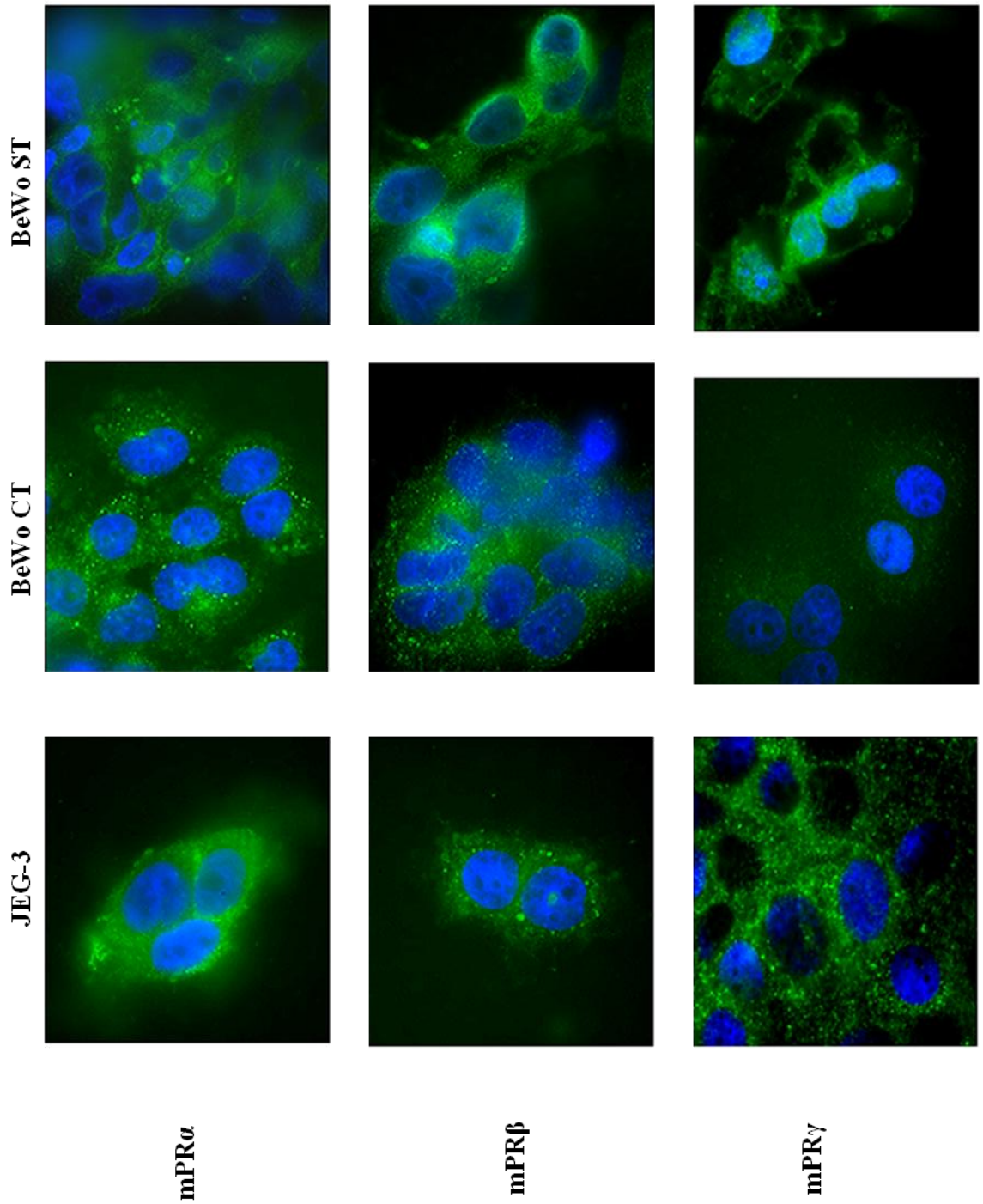


Figure 3.15: Immunofluorescence analysis for mPRα, mPRβ, mPRγ in JEG-3, untreated BeWo cells (BeWo CT) and syncytialised BeWo cells (BeWo ST).

Below, a summarising table indicating the cellular localisation of mPRs can be found (Table 3.2).

	Cellular localisation		
Receptor	**JEG-3**	**BeWo CT**	**BeWo ST**
mPRα	Cytoplasm + plasma membrane	Cytoplasm + plasma membrane	Cytoplasm
mPRβ	Cytoplasm	Cytoplasm + plasma membrane	Cytoplasm + plasma membrane
mPRγ	Cytoplasm	Cytoplasm + plasma membrane	plasma membrane

Table 3.2: Summary of the cellular localisation of mPRα, mPRβ, mPRγ proteins in JEG-3, untreated BeWo cells (BeWo CT) and syncytialised BeWo cells (BeWo ST).

3.3.2. Evaluation of the expression and the cellular localisation of progesterone receptor membrane component -1 (PGRMC-1) in BeWo and JEG-3 cell lines.

The expression of another membrane receptor able to bind progesterone was examined in our cell preparations. The expression of PGRMC-1 mRNA was significantly upregulated in BeWo ST cells when compared to BeWo CT cells. This upregulation was found to be of 77%. When the expression was assessed in JEG-3 cells compared to BeWo CT it was found that these non-fusigenic cells express PGRMC-1 at a lower level (68% less). Graph presented in Fig.3.16 shows the relative quantitiy (RQ) values of the expression levels. Expression of PGRMC-1 in BeWo CT was set to 1.

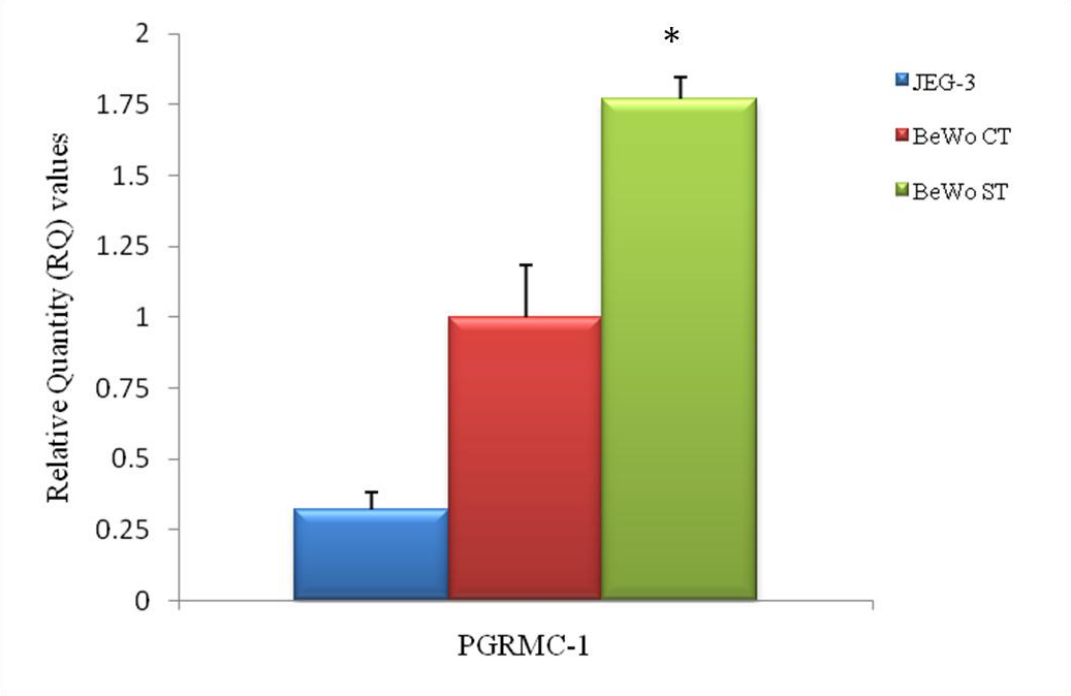


Figure 3.16: Expression of PGRMC-1 in control untreated BeWo cells (BeWo CT), treated BeWo cells (forskolin 50μM 72 hours) and JEG-3 cells.

Immunofluorescence analysis of the PGRMC-1 protein in JEG-3 cells revealed a strong homogeneous peri-nuclear staining as well as cytoplasmic staining (Fig.3.17). In BeWo CT cells this protein was localised mainly in the cytoplasm where the staining was dotted and some staining being seen on the plasma membrane with a more homogeneous appearance. Finally, in BeWo ST dotted staining was evident in the cytoplasm and in the plasma membrane of these cells (Fg.3.17).

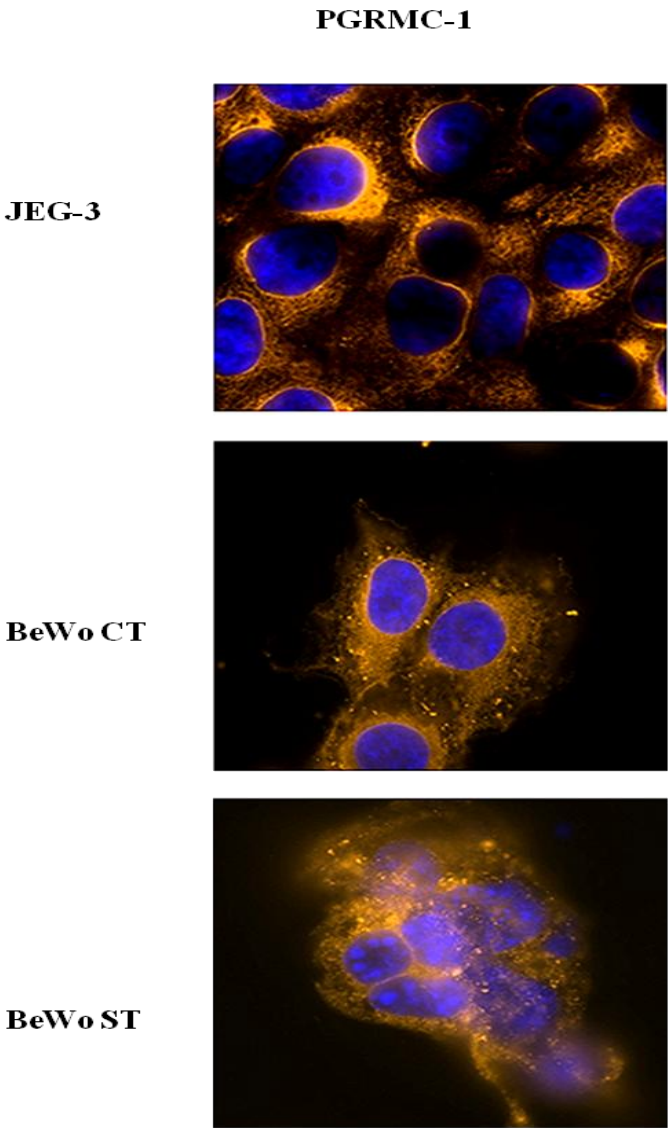


Figure 3.17: Immunofluorescence analysis for PGRMC-1 in JEG-3, untreated BeWo cells (BeWo CT) and syncytialised BeWo cells (BeWo ST).

3.3.3. Expression of nuclear progesterone receptor (PR-B) in BeWo and JEG-3 cell lines.

To help assess the expression of functional wild-type nuclear PR-B, semi-quantitative RT-PCR analysis was used. This approach was selected as the primers designed had the maximum length of the target gene therefore this restricted the use of Q-PCR. RT-PCR was chosen also because of the presence of multiple splice variants of the PR gene. RT-PCR analysis of this gene in BeWo CT and BeWo ST cells revealed that no change in the expression was present (Fig.3.18). In JEG-3 cells there was a 4-fold increase of PR mRNA when compared to both BeWo CT and ST cells.

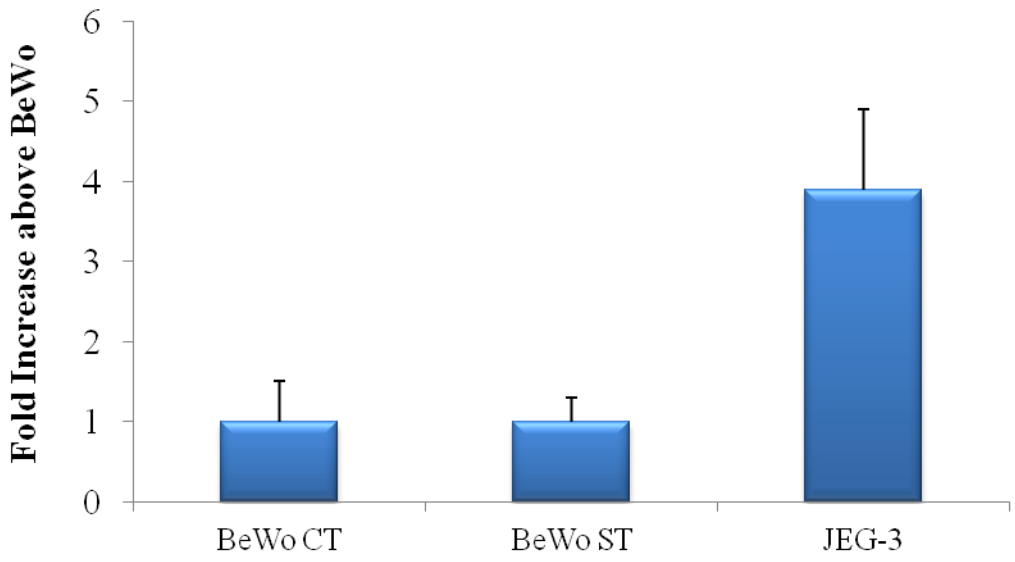


Figure 3.18: Illustration of the fold increase in expression of PR-B using semi-quantitative RT-PCR. Correcting over β-actin.

Western blotting was then utilised to investigate the expression of the protein PR isoforms in BeWo (Fig. 3.19A) and JEG-3 (Fig. 3.19B) cells. Specific bands at ~116 and 94 kDa were detected in all preparations which were indicative of the expression of the full-length PR-B as well as the truncated PR-A, respectively.

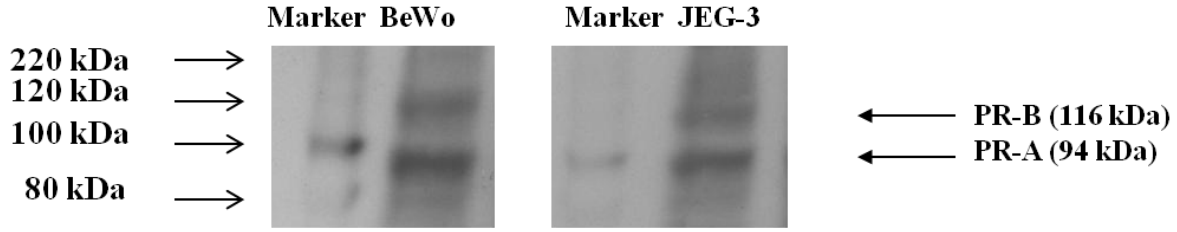


Figure 3.19 : Western blotting of the expression of PR-A (94) kDA and PR-B (116 kDA) in cell lysates from BeWo and JEG-3 cells.

Using antibodies against PR-B protein we observed in JEG-3 cells this protein to be found around the nucleus indicating a speckled peri- nuclear pattern of staining whereas in BeWo CT the localisation of this protein is mainly cytoplasmic but also some speckled nuclear staining is observed. The staining observed in BeWo ST cells was mainly granular nuclear as Fig.3.20 depicts.

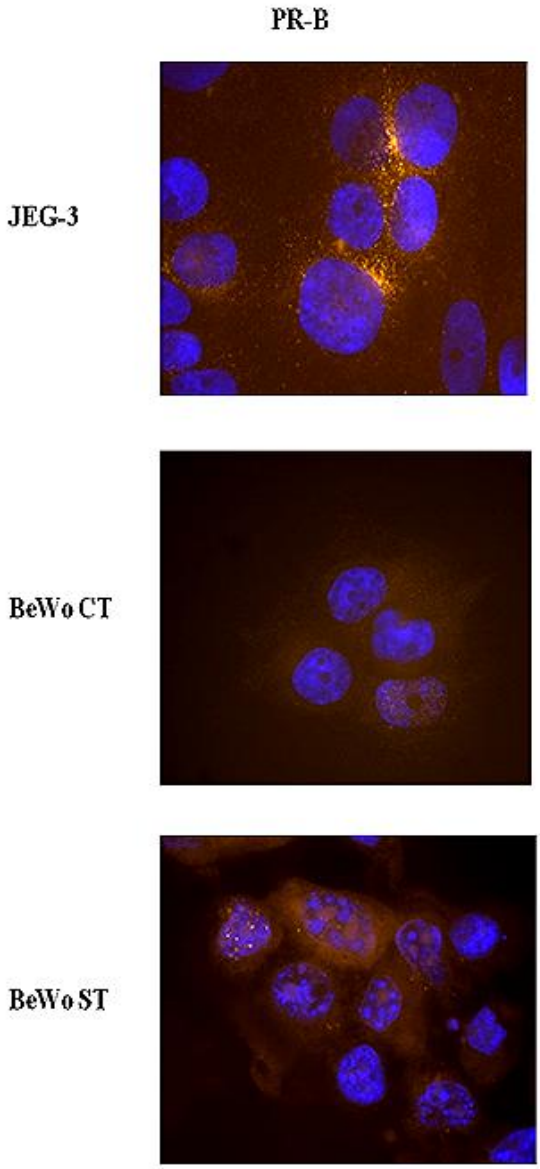


Figure 3.20: Immunofluorescence analysis for PR-B in in JEG-3, untreated BeWo cells (BeWo CT) and syncytialised BeWo cells (BeWo ST).

Further, analysis of PR-B localisation in BeWo and JEG-3 was conducted using ImageStream^{x}. Incubation of cells with fluorescently conjugated progesterone bound to the impermeable moiety BSA resulted in binding on the cell membrane of BeWo cells (white arrows), indicative of membrane progesterone binding sites (Fig.3.21).

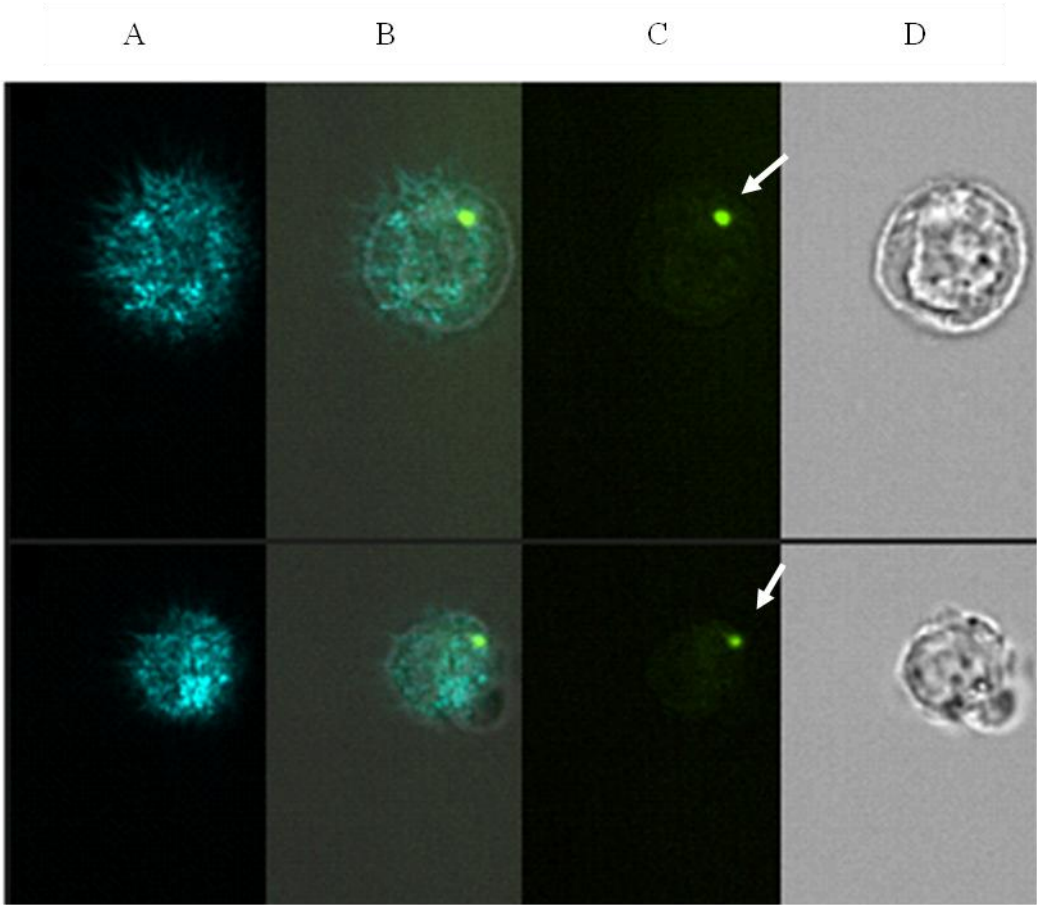


Figure 3.21: Binding of FITC-P4-BSA on the cell membrane of BeWo cells. A: scatter cell imaging, B/C: shows FITC-P4-BSA staining (white arrow; cell membrane), and D is the brightfield image of the cell.

Below an indicative result obtained is given where the intra-nuclear PR-A/B protein is seen in BeWo (white dotted arrows). Strong nuclear expression of PR was evident in most of the 4,448 single cells studied (Fig. 3.22).

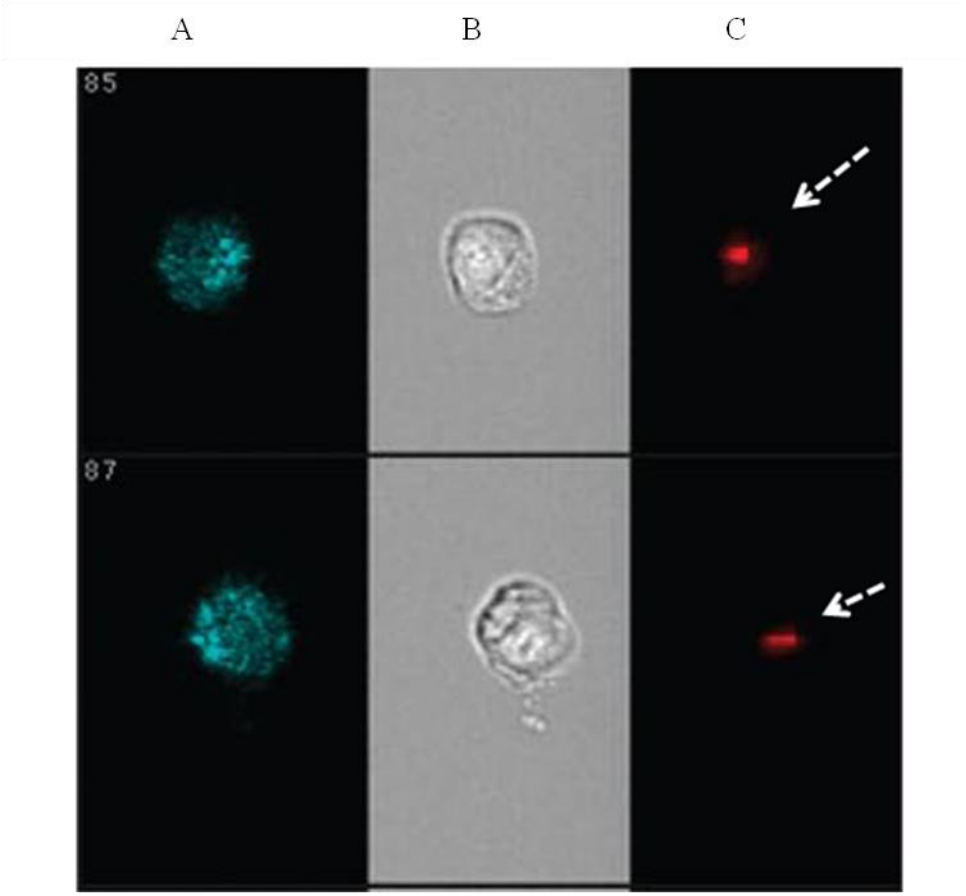


Figure 3.22: ImageStream analysis of PR-A/B in two representative BeWo cells. Panel A is scatter cell imaging, B is the brightfield image of the cells, and C is the specific staining (red) for PR-A/B (white-dotted arrows).

3.4. Discussion

3.4.1. Expression of Progesterone Receptors in placental cell lines: effects of syncytialisation on receptor expression.

Over the past 20 years many research groups have shown that steroids also act at the cell surface of many target tissues and cell types to initiate rapid responses, via binding to membrane receptors (Falkenstein *et al*., 2000). However, the lack of comprehensive information on the identity and molecular structure of any membrane steroid receptor has prevented understanding of critical molecular aspects of steroid actions via this alternative mechanism and a more widespread appreciation of its significance (Watson, 2003). Recently, new genes exhibiting characteristics of a membrane progestin receptor (mPR) have been discovered and separated into 5 subtypes, α, β, γ, (Zhu *et al*., 2003[B]) δ, and ε (Smith *et al*., 2008). Until now existing knowledge is limited regarding expression of membrane progesterone receptors in the human placenta. Results presented from this research comprise the first detailed mapping of progesterone receptors in the human placenta integrating signalling and functional studies of these receptors.

In this part of the study a detailed map of the progesterone receptors expression at gene and protein levels in two human choriocarcinoma cell lines has been provided. Moreover, the effect of the syncytialisation agent forskolin was assessed on the expression of these genes.

Examining the expression of mPRs and PGRMC-1 by Q-PCR in the pre- and differentiated state of BeWo cells it was revealed that in syncytialised BeWo cells, mPRα, mPRγ and mPRδ were down-regulated, whereas mPRβ and PGRMC-1 were up-regulated when compared to non-syncytialised BeWo cells (Zachariades *et al*., 2011). Upregulation of mPRβ was very distinctive in BeWo ST cells where a 2.25 fold increase over the expression of mPRβ in the non-treated BeWo cells was detected. The expression of PGRMC-1 was shown to be 1.75

fold above the expression recorded in the BeWo CT cells. These results corroborate a recent study where it was shown that forskolin altered the expression of several genes during the process of syncytialization in BeWo cells (Neelima & Rao, 2008). Using differential display RT-PCR analysis and Northern blot analysis the up-regulation of genes such as secretory leukocyte peptidase inhibitor (SLPI), elongation factor-1 alpha-1 (ELF-1α-1) and prolyl 4-hydroxylase β following treatment with forskolin was detected (Neelima & Rao, 2008). Also the elevation of cAMP can activate certain steroid receptors and augment the ligand-dependent activation (Rowan *et al*., 2000). For example, 8-bromo-cyclic AMP induces the phosphorylation of two sites in SRC-1 that facilitate the ligand-independent activation of the chicken PR and are critical for the functional cooperation between SRC-1 and the CREB binding protein (Rowan *et al*., 2000).

The JEG-3 cell line does not have a fusigenic capacity and responds poorly to inducers of syncytialization (Coutifaris *et al*., 1999). Phenotypically JEG-3 cells resemble the undifferentiated cytotrophoblasts, making it a suitable *in vitro* model to investigate first trimester placental function (Ntrivalas *et al*., 2006). When the expression of mPR and PGRMC-1 genes was compared between the non-syncytialised BeWo cells and the JEG-3 cells, mPRα, mPRβ, mPRγ, and PGRMC-1 mRNA levels were shown to be significantly down-regulated in JEG-3 cells. No expression of mPRε was detected using quantitative or standard RT-PCR analyses even after 40 cycles of amplification (Zachariades *et al*., 2011). Although both the JEG-3 and BeWo cell lines may represent a cytotrophoblastic state, there is evidence that they have inherent gene differences. Microarray analysis revealed that many transcripts were differentially expressed between BeWo and JEG-3 cells (Burleigh *et al*., 2007), suggesting that each cell line may vary in its capacity to respond to hormones.

The full length PR-B is shown from this study to be expressed in JEG-3 cells as well as in non - syncytialised BeWo cells and in syncytialised BeWo cells as detected by RT-PCR. Expression of PR-A and PR- B was also confirmed at protein level in BeWo CT cells, using western blot analysis and an antibody that recognises both isoforms (Zachariades *et al*., 2011). This data is in agreement with a recent study where both the PR-A and PR-B proteins where shown to be expressed in BeWo cells (Yasuda *et al*., 2009). The presence of multiple PR isoforms may be indicative of the diverse responses to progesterone as for example, in BeWo cells tranfected with PR these isoforms were found to differentially regulate the expression of the breast cancer resistance protein (Wang *et al*., 2008).

In BeWo CT, BeWo ST and JEG-3 cells, mPRα protein was localised in the cytoplasm of these cells as shown by immunofluorescence analysis. mPRβ showed a similar immunoreactivity pattern with an even stronger cytoplasmic signal in our cell lines. Whereas mPRγ revealed substantial cytoplasmic staining in JEG-3 cells where the intensity of the same protein in BeWo CT cells had the same localisation but a less intense signal intensity was detected. In BeWo ST, mPRγ was primarily expressed on the plasma membrane of the syncytiotrophoblasts (Zachariades *et al*., 2011). This is not the first time where differences in the expression level of mPRβ and mPRγ are reported between tissues with mPRβ demonstrating less flactuations (Nutu *et al*., 2009). Previous studies have identified mPRα on the cell surface of human myometrial cells (Foster *et al*., 2010) whereas mPR-β was reported to be localised on the plasma membrane of ocytes collected from pigs (Qiu *et al*., 2008). Furthermore, mPRβ was shown to be localised in the cilia of the epithelial cells (Nutu *et al*., 2009) where mPRγ was found in the apical membrane of ciliated cells facing the lumen in human fallopian tubes (Nutu *et al*., 2007).

PGRMC-1 showed strong perinuclear and moderate dispersed cytoplasmic staining in JEG-3 cells, where in BeWo CT cells the signal detected was mainly cytoplasmic with sporadic membrane staining. Upon treatment with forskolin in BeWo cells, the cellular distribution of PGRMC-1 showed no remarkable differences (Zachariades *et al*., 2011). This protein has been previously shown to be expressed in placental structures and more specifically in smooth muscle cells of the placental vasculature, villous capillaries and the syncytiotrophoblast layer (Zhang *et al*., 2008).

Following, the expression of the nuclear progesterone receptor (PR-B) in JEG-3 cells and in non-syncytialised BeWo cells was localised in the cytoplasm and the nucleus, whereas in the forskolin-treated BeWo cells the localisation of PR-B was primarily nuclear (Zachariades *et al*., 2011). The differential localisation of PR-B observed in BeWo cells upon treatment with forskolin may be a direct result of the alteration of specific genes as previously shown (Neelima & Rao, 2008). Also emerging data from a recent study have indicated that without hormone induction, the PR-B distributes between the cytoplasm and the nucleus (Ellmann *et al*., 2009).

The existing knowledge on progesterone mediated action in human choriocarcinoma cell lines is limited to a few studies only. For example, a study where it was shown that progesterone can have inhibitory effects on folic acid transport (Keating *et al*., 2007) and to down-regulate human organic anion transporter 4 (hOAT4) transport activity in human placental BeWo cells (Zhou *et al*., 2008). However, none of these studies clearly identifies the receptor mechanism via which these progesterone effects are mediated. Our data provide evidences for the expression of multiple PRs in human placental cell lines, suggestive of a higher order of complexity of progesterone-mediated responses.

3.5. Genomic or non-genomic signalling at placental level?

3.5.1. Expression of p-ERK1/2 and MAPK in BeWo CT, BeWo ST and JEG-3 cells.

Mitogen-activated protein kinase (MAPK) super family consists of three major groups of kinases: the extracellular-receptor kinases (ERK) which includes $ERK_1$and ERK_2 which are studied in this research and two further types of MAPK-related kinases: the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 MAPKinases (Kyriakis & Avruch, 2001). It has been proven that in the villous trophoblast pathway which promotes cytotrophoblast cell differentiation and proliferation, the group of MAPK that plays a role is the $ERK_{1/2}$ (Daoud *et al.*, 2005). Therefore in this part of the thesis the results of the expression of phospho-$ERK_{1/2}$ (p-$ERK_{1/2}$) and total MAPK in BeWo CT, BeWo ST and JEG-3 cells are shown after performing treatments with specific compounds (table below) able to activate the MAPK pathway and phosphorylate the $ERK_{1/2}$ protein.

Compound	Stock Concentration	Final concentration
Progesterone - a known activator of mPRs, PGRMC-1 and PRs	10^{-1} M	100nM ± U0126 (1μM)
10-ethenyl-19-norprogesterone (OD 02-0) – a selective agonist of mPRα	10^{-3} M	100nM
Promegestone (R5020) - a specific agonist of nPR	10^{-2} M	30nM ± U0126
U0126 – a selective $MEK_{1/2}$ inhibitor	10^{-3} M	1μM

Before embarking on the analysis of the activation of $ERK_{1/2}$ in our cells, representative western blots from the study are shown below.

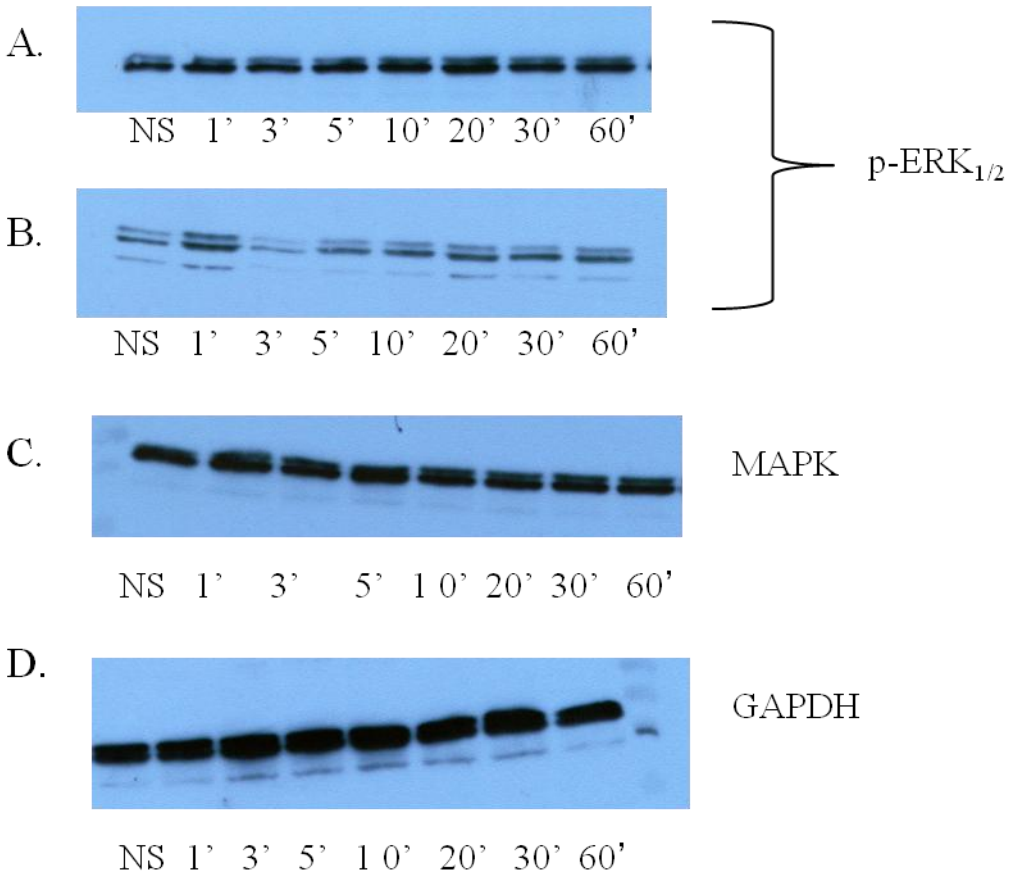


Figure 3.23: Cells were treated with either 100nM of OD 02-0, 100nM of P4 or 30nM of R5020 for a period of 1 hour. Following, protein was extracted by the cells and the samples were electrophoresed on 12.5% SDS-PAGE gels. Phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2) and GAPDH antibodies were used. The results are indicated above using representative blots from the study. Panel A: BeWo ST cells treated with R5020; Panel B: JEG-3 cells treated with P4; Panel C: BeWo ST cells treated with OD 02-0; Panel D & E: JEG-3 cells treated with R5020.

The cells were treated with 100 nM Progesterone (P4) to determine whether P4 caused $ERK_{1/2}$ activation in BeWo CT, BeWo ST and JEG-3 cells. $ERK_{1/2}$ activation was measured in a time dependent manner by assessing the expression of phospho-$ERK_{1/2}$ and total MAPK

proteins (Fig.3.24). This time - course was chosen as the aim was to establish the principal signalling type in the human placental cell lines i.e genomic or non-genomic. Using AlphaEase software the optical density values of the immunocomplexes were calculated. The expression of p-$ERK_{1/2}$ was normalised to total MAPK expression and the relative expression of p-$ERK_{1/2}$ expressed in optical density values are presented in the graph in Fig.3.24. The basal $ERK_{1/2}$ phosphorylation in each cell line was established and is indicated in the graph as non supplement (NS) where no treatment was performed on these cells. Western blot analysis showed that exposure of JEG-3 cells to P4 resulted in a significant increase of $ERK_{1/2}$ phosphorylation at 1 minute (Fig. 3.24). Mean $ERK_{1/2}$ activation was significantly increased 1.7 fold over non-supplement (NS) after 1 minute of treatment with 100 nM P4 whereas the mean activation of $ERK_{1/2}$ was significantly decreased 2 fold over NS after 5 minutes of treatment with 100 nM of P4. No significant increase or decrease in $ERK_{1/2}$ phosphorylation was detected in BeWo CT cells treated with 100 nM of P4 (Fig.3.24). Progesterone treatment of BeWo ST cells resulted in a significant 2.2 fold increase of $ERK_{1/2}$ phosphorylation over NS after 20 minutes of treatment. After 30 minutes of P4 treatment in the BeWo ST cells a significant decrease of the $ERK_{1/2}$ phosphorylation levels was detected compared to the phosphorylation levels detected in the non-treated BeWo ST cells (Fig.3.24).

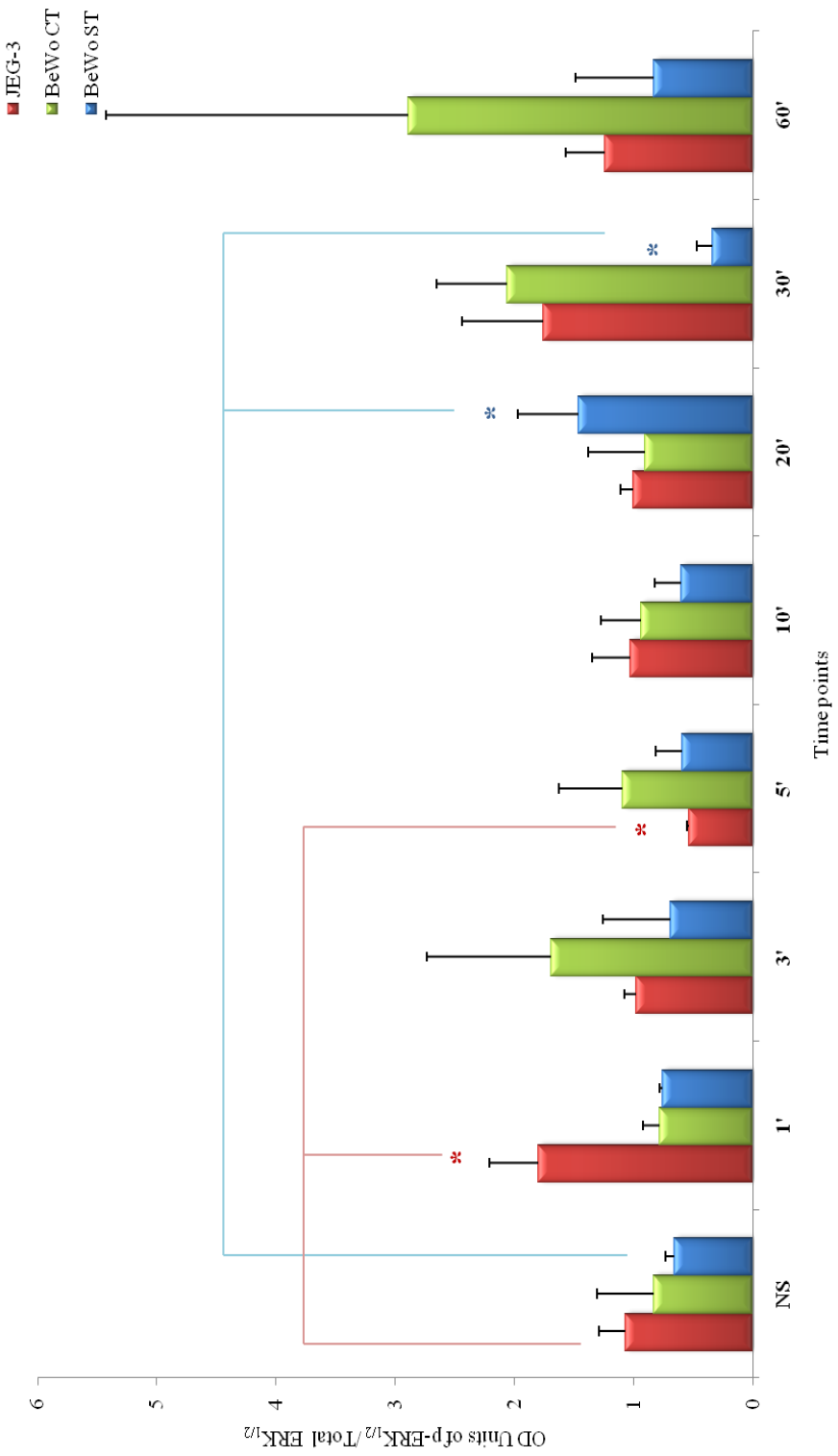


Figure 3.24: Densitometric analysis of phosphorylated $ERK_{1/2}$ protein normalized to total $ERK_{1/2}$ protein and reported as optical density (OD) units ± SEM. Statistically significant differences from non supplement were determined by paired t-test, $p < 0.05$*. JEG-3, BeWo CT and BeWo ST cells were treated with 100 nM of P4.

OD 02-0, a specific mPR ligand was used to treat JEG-3, BeWo CT and BeWo ST cells at a concentration of 100 nM for 1, 3, 5, 10, 20, 30 and 60 minutes and the phosphorylation of $ERK_{1/2}$ was measured. After exposing JEG-3 cells to 100 nM of OD 02-0 no significant differences in the activation of $ERK_{1/2}$ were detected (Fig.3.25). The mean $ERK_{1/2}$ phosphorylation was significantly decreased 4.8 fold over NS after 1 minute of treatment with 100 nM of OD 02-0 in BeWo CT cells. Also a significant decrease (3.2 fold) over NS was detected after 3 minutes of treatment, after 20 minutes a significant 3.6 fold decrease was observed and after 30 minutes of treatment a significant 5 fold decrease over NS was detected (Fig.3.25). Treatment of BeWo ST cells with 100 nM of OD 02-0 resulted in a significant increase in $ERK_{1/2}$ activity after 20 and 30 minutes where the mean $ERK_{1/2}$ phosphorylation was increased 12 fold and 19 fold over NS respectively.

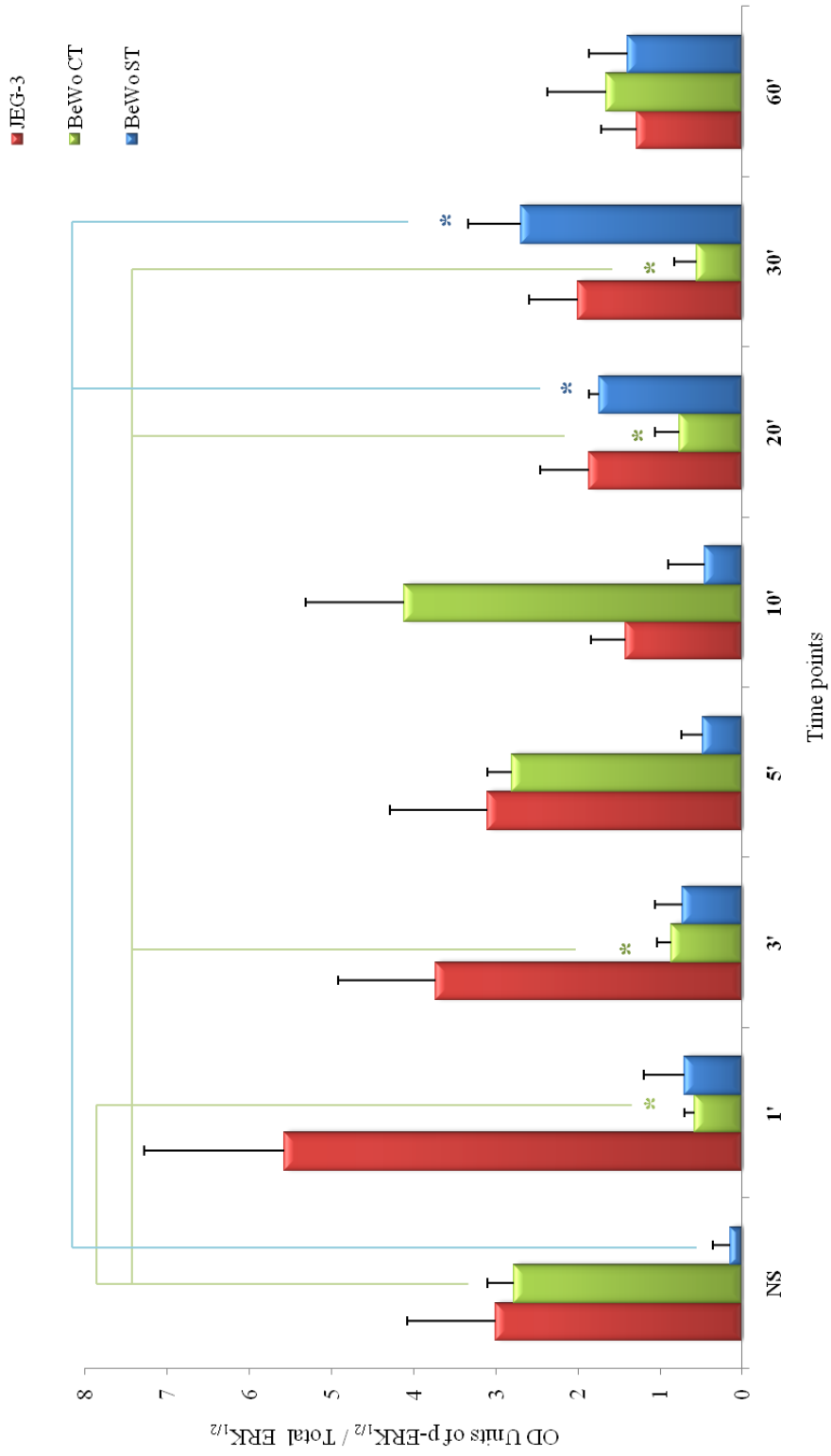


Figure 3.25:Densitometric analysis of phosphorylated $ERK_{1/2}$ protein normalized to total $ERK_{1/2}$ protein and reported as optical density (OD) units ±SEM. Statistically significant differences from non supplement were determined by paired t-test, $p < 0.05$*. JEG-3, BeWo CT and BeWo ST cells were treated with 100 nM of OD 02-0.

Next JEG-3, BeWo CT and BeWo ST cells were treated with 30 nM of R5020 a selective nuclear PR agonist for 1-60 minutes as previously and the activation of $ERK_{1/2}$ was measured. Treatment of JEG-3 cells with 30 nM of R5020 resulted in a significant increase of $ERK_{1/2}$ phosphorylation after 1 and 20 minutes of treatment (Fig.3.26). The mean $ERK_{1/2}$ activation was increased 1.7 fold and 1.6 fold over NS respectively. After treatment of BeWo CT and BeWo ST cells with 30 nM of R5020 no significant changes in the activity of $ERK_{1/2}$ were detected (Fig.3.26).

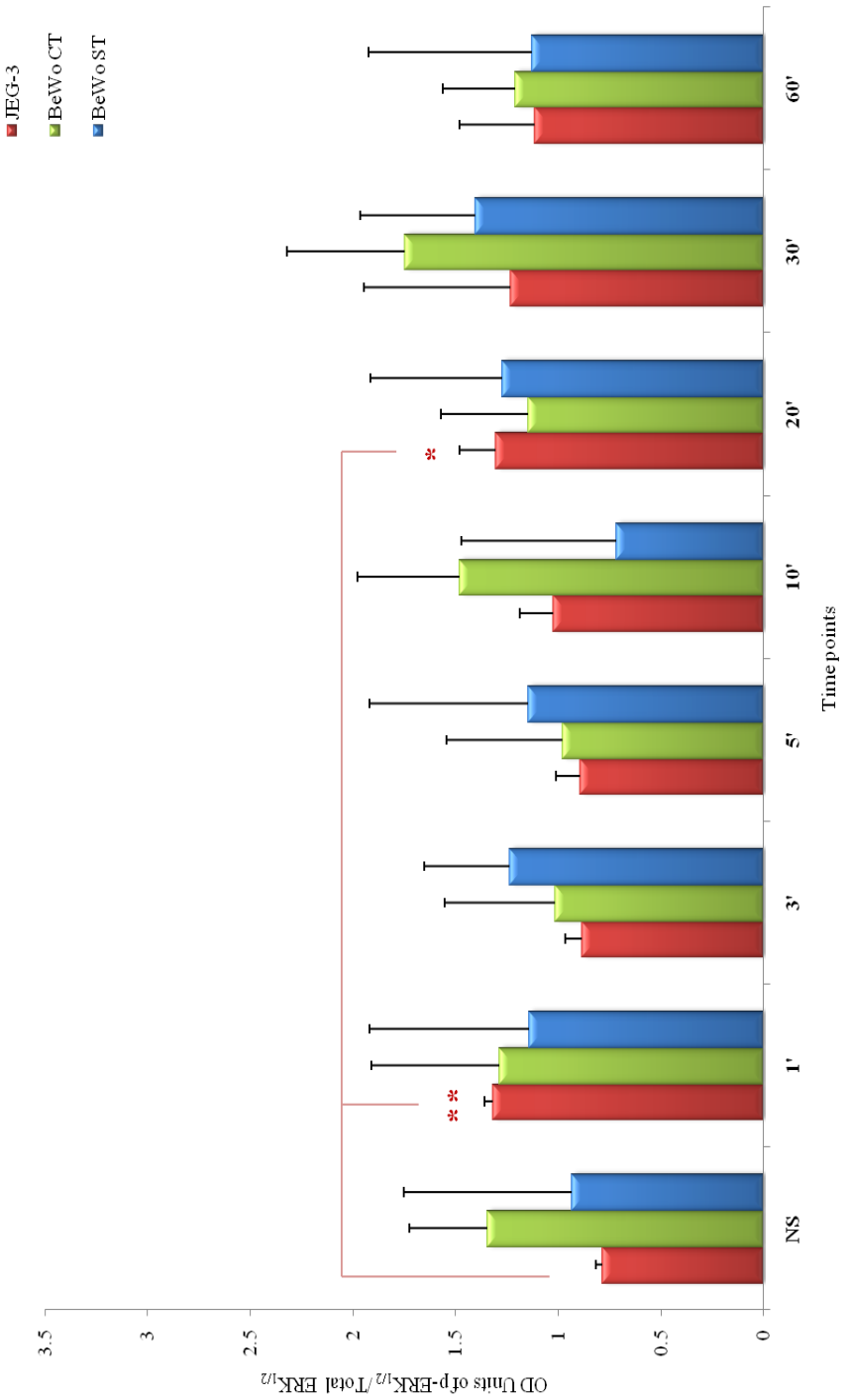


Figure 3.26: Densitometric analysis of phosphorylated $ERK_{1/2}$ protein normalized to total $ERK_{1/2}$ protein and reported as optical density (OD) units ±SEM. Statistically significant differences from non supplement were determined by paired t-test, $p < 0.05$*, $p < 0.001$**. JEG-3, BeWo CT and BeWo ST cells were treated with 30 nM of R5020.

A selective MEK $_{1/2}$ inhibitor, UO126 was used to inhibit the activity of $MEK_{1/2}$. JEG-3, BeWo CT and BeWo ST cells were incubated with 1µM of UO126 and the activity of $ERK_{1/2}$ was measured. The basal $ERK_{1/2}$ activity after $MEK_{1/2}$ inhibition is shown in graph in Fig 3.27. Even though $MEK_{1/2}$ was inhibited, phosphorylation of $ERK_{1/2}$ was detected in our cells (Fig.3.27).

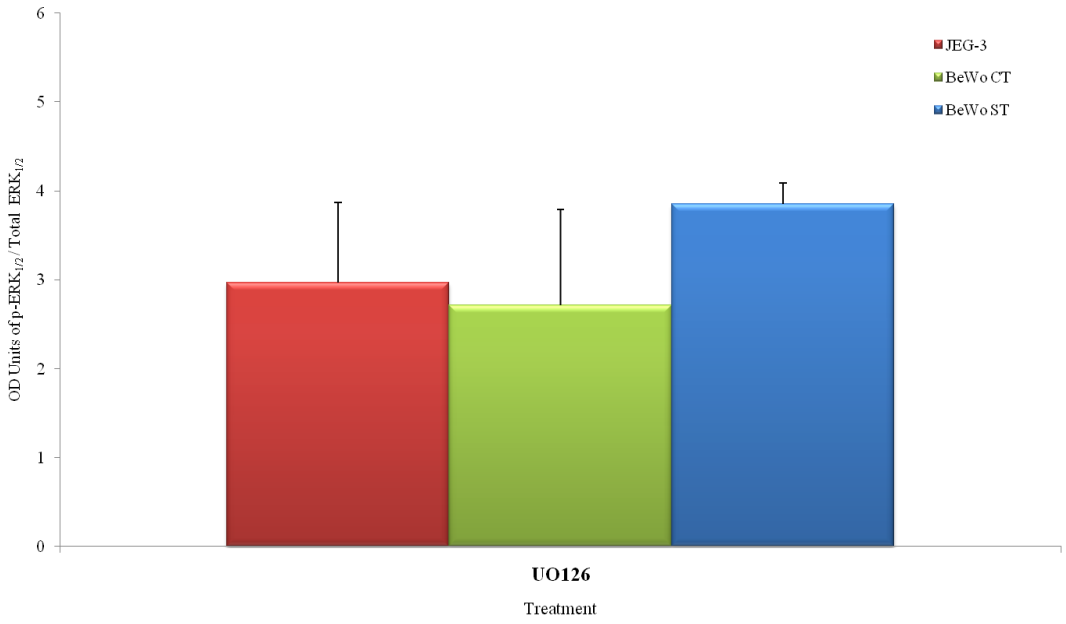


Figure 3.27: Densitometric analysis of phosphorylated $ERK_{1/2}$ protein normalized to total $ERK_{1/2}$ protein and reported as optical density (OD) units ±SEM. JEG-3, BeWo CT and BeWo ST cells were treated with 1µM of UO126.

Following, JEG-3, BeWo CT and BeWo ST cells were pre-incubated with UO126 before being treated with 100 nM of P4 for 1- 60 minutes and the activity of $ERK_{1/2}$ was assessed. Co-treatment of JEG-3 cells with these two compounds resulted in a significant increase in the phosporylation levels of $ERK_{1/2}$ after 1 minute of treatment compared to NS whereas at

60 minutes of P4 treatment the phorpshorylation of $ERK_{1/2}$ in JEG-3 cells decreased significantly compared to the NS (Fig 3.28). Co-treated BeWo CT cells showed no significant changes in the phosphorylation levels of $ERK_{1/2}$ (Fig 3.28). Exposure of BeWo ST cells to these two compounds significantly decreased the activity of $ERK_{1/2}$ after 1, 5, 30 and 60 minutes of treatment (Fig 3.28). The mean activity of $ERK_{1/2}$ was decreased by 1.1 fold, 1.7 fold, 1.8 fold, 1.9 fold and 1.8 fold over NS respectively.

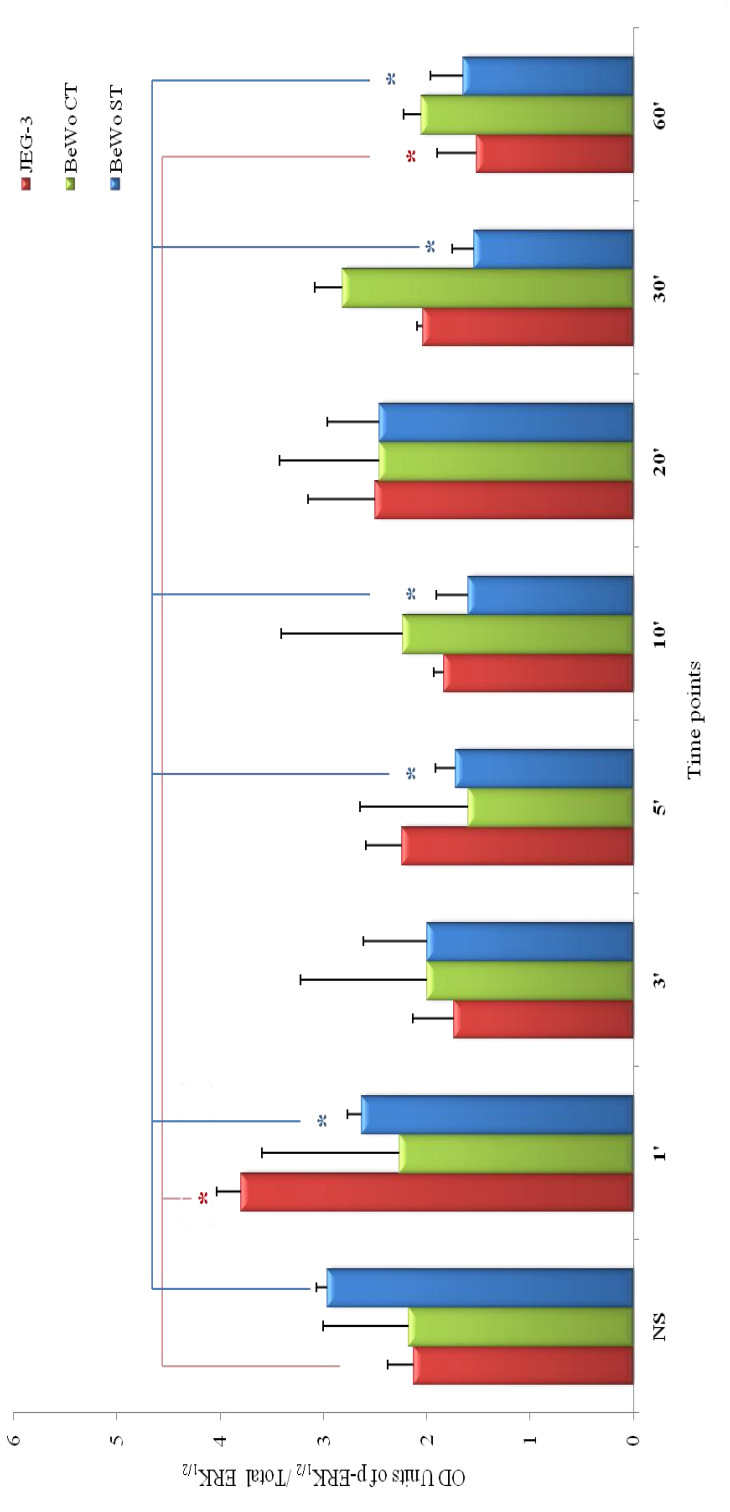


Figure 3.28: Densitometric analysis of phosphorylated $ERK_{1/2}$ protein normalized to total $ERK_{1/2}$ protein and reported as optical density (OD) units ±SEM. Statistically significant differences from non supplement were determined by paired t-test, $p < 0.05$*. JEG-3, BeWo CT and BeWo ST cells were pre-incubated with 1μM of UO126 and treated with 100 nM of P4.

3.6. Discussion

3.6.1. Evidence for non-genomic signalling at placental level

The MAP kinase family controls a wide range of cellular events such as the process of embryogenesis, cell differentiation, cell proliferation, and cell death as well as regulating short term process required for homeostasis and hormonal responses (Lewis *et al.,* 1998).

More specifically, the expression of total $ERK_{1/2}$ was shown by Kita *et al.* (2003) to be expressed in the human villous cytotrophoblast cells throughout gestation. Total $ERK_{1/2}$ was exclusively localised in the cytotrophoblast as no expression was detected in the syncytiotrophoblasts (Kita *et al.,* 2003). Further to this, a role of $ERK_{1/2}$ in the proliferation of human villous cytotrophoblast was suggested as the phosphorylated $ERK_{1/2}$ was detected in the cytotrophoblast cells up to 12 weeks of the gestation period (Kita *et al.,* 2003). The role of $ERK_{1/2}$ in the early differentiation process was supported by experiments performed showing that the suppression of $ERK_{1/2}$ activity early in the differentiation process leads to an impaired trophoblast differentiation process as the levels of hCG secretion were dramatically reduced compared to non-experimental culture conditions (Daoud *et al*., 2005).

Even though both cells lines used in these experiments were of choriocarcinoma origin and have known fusigenic differences, interestingly further cell specific differences were evident from our results. Similarly, findings from a recent study have indicated that approximately 20% of genes represented on a microarray analysis were differentially expressed between JEG-3 and BeWo choriocarcinoma cell line (Burleigh *et al*., 2007).

Rapid activation of $ERK_{1/2}$ was detected in JEG-3 cells upon stimulation with progesterone and the nuclear progesterone receptor agonist R5020 as early as 1 minute of treatment. Rapid stimulation of ERK activity has also been previously reported, upon 2 minutes of treatment with R5020 in T47D cells (Migliaccio *et al*., 1998). Evidence has been provided for the

association of the nuclear progesterone receptor with p42 MAPK in the xenopus oocyte (Migliaccio *et al*., 1998) and by ERK activation in mammalian cells mediated by progesterone (Bagowski *et al*., 2001; Boonyaratanakornkit *et al*., 2001) as well as activation of $ERK_{1/2}$ through mPR mediated signalling (Zhu *et al*., 2003[A]). This JEG-3 specific rapid activation of $ERK_{1/2}$ can be attributed as the result of up-regulation of genes involved in the MAPK cascade in JEG-3 cells in comparison to BeWo cells. Genes that have been shown to be up-regulated include the mitogen-activated protein kinase kinase kinase 7 interacting protein 2 (MAP3K7IP2) mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1), mitogen-activated protein kinase 1 (MAPK1), mitogen-activated protein kinase 14 (MAPK14), mitogen-activated protein kinase 3 (MAPK3), GRB2-associated binding protein 1 (GAB1) and Son of sevenless homolog 1 (SOS1) (Burleigh *et al*., 2007). Thus, the progestin induced rapid $ERK_{1/2}$ phosphorylation in JEG-3 compared to BeWo can be a direct result of an overactive MAPK signalling pathway.

Heat shock protein 70 and heat shock protein 90 (HSPs) are responsible for maintaining the progesterone receptor in an inactive state when not bound to a ligand (Rowan & O'Malley, 2000). The genes encoding for these two HSPs were shown to be up-regulated in BeWo cells compared to the expression detected in JEG-3 cells (Burleigh *et al*., 2007). HSP gene overexpression in BeWo's may compromise the signalling capacity of the nuclear progesterone receptor in BeWo cells as no significant activation of $ERK_{1/2}$ was observed when stimulating these cells with progesterone or R5020. For examples a recent study performed in transgenic mice overepxressing HSP70 showed that glucocorticoid receptor expression was suppressed despite the high levels of corticosterone being detected. Also, in this mouse model a reduced ligand binding of this steroid receptor has been detected (Vanhooren *et al*., 2008) which can be attributed to the overexpression of HSP70.

Activation of $ERK_{1/2}$ was also examined in the pre differentiated and differentiated state of BeWo cells. Minimal $ERK_{1/2}$ phosphorylation was detected in BeWo CT cells. In BeWo ST cells, significant $ERK_{1/2}$ phosphorylation was induced by stimulating the cells with progesterone (at 20 minutes post treatment) or the membrane progesterone agonist OD 02-0 (at 20 and 30 minutes post treatment). The phosphorylation status of $ERK_{1/2}$ remained unaltered upon stimulation with the nuclear receptor agonist, R5020. As $ERK_{1/2}$ was activated at 20 minutes post treatment with progesterone and at 20 and 30 minutes post treatment with OD 02-0 it is attractive to speculate that this rapid $ERK_{1/2}$ phosphorylation is mediated via the mPRs and not the nuclear progesterone receptor. More specifically the proposed receptor for mediating this response is mPRβ as the expression of this receptor was significantly higher than the expression of the other mPRs in BeWo ST. The involvement of PGRMC-1 should not be ruled out as this gene was shown to be highly up-regulated in BeWo ST compared to BeWo CT but the expression was lower compared to that of mPRβ.

3.7. Human placenta and the expression of progesterone receptors

3.7.1. Expression of mPRs at gene level in the human placenta

Human placental tissue was used to map in detail the expression of mPRs in an attempt to gain a better insight of their role *in vivo*. This was performed as these newly described membrane receptors still have not had a sole role attributed in the female reproductive system and specifically in the placenta. The placental samples were collected from women without any pregnancy complications or known pathologies and were grouped according to the time of delivery (term or preterm) and according to the contractile state (labour or non-labour). Totally forty one (n=41) placental samples were collected and available for further analysis (Table 3.3).

Placental samples			
Term	Pre - Term	Labour	Non- Labour
22	19	27	14

Table 3.3: Human placenta tissue sample distribution.

Following extraction of RNA and cDNA synthesis, the expression of the three genes of interest – mPRα, mPRβ and mPRγ - was assessed in the samples using Q-PCR analysis. Considerable variation in the expression of the mPRs between individuals was observed (Fig. 3.29-3.31).

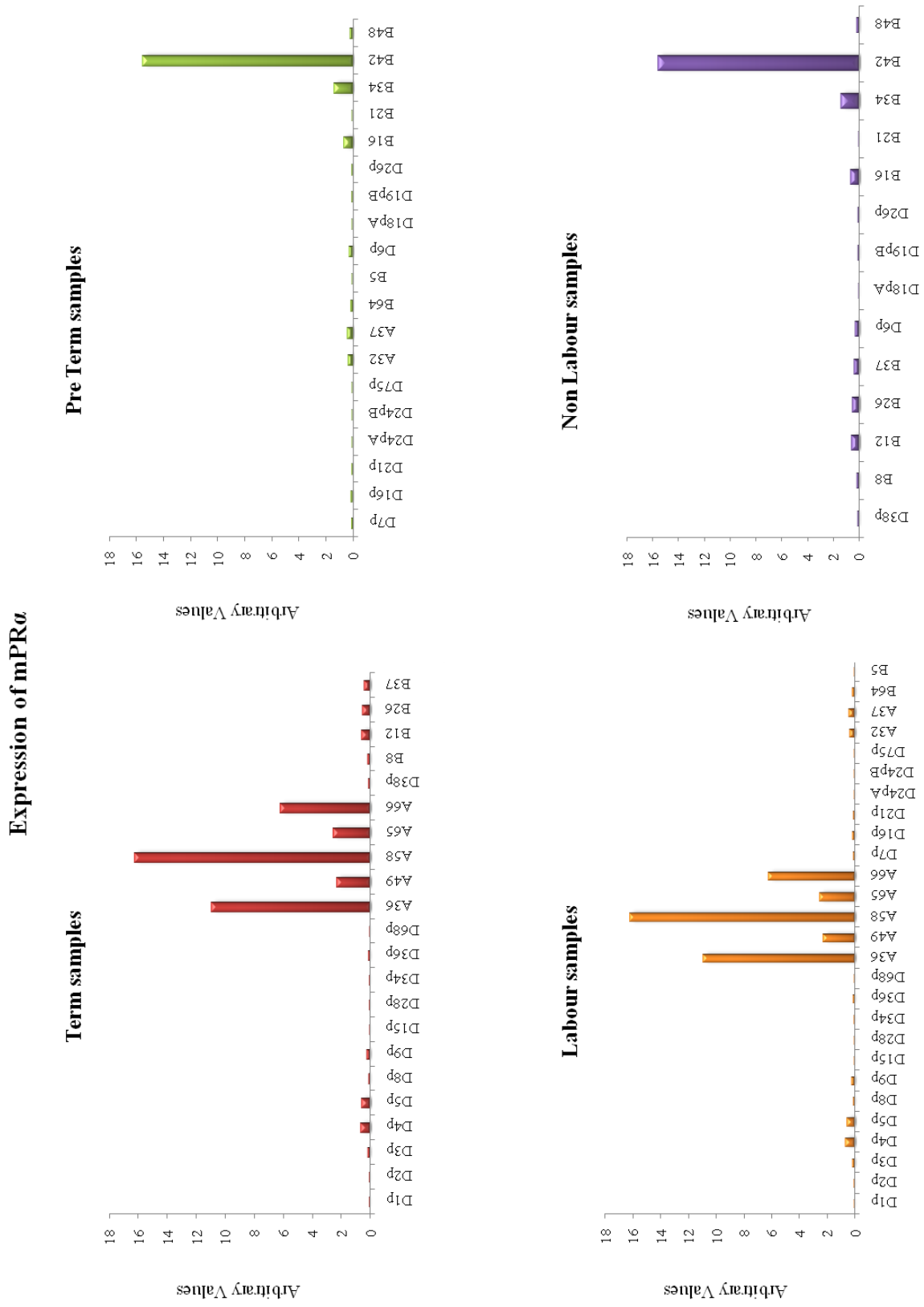


Figure 3.29: Expression of mPRα in human placental samples (experimental triplictaes per sample), presented according to the contractile state and the time of delivery. The data are presented in arbitrary values ($2^{-\Delta Ct}$) which is the result of the correction of the gene of interest over β-actin house keeping gene of each individual placental sample.

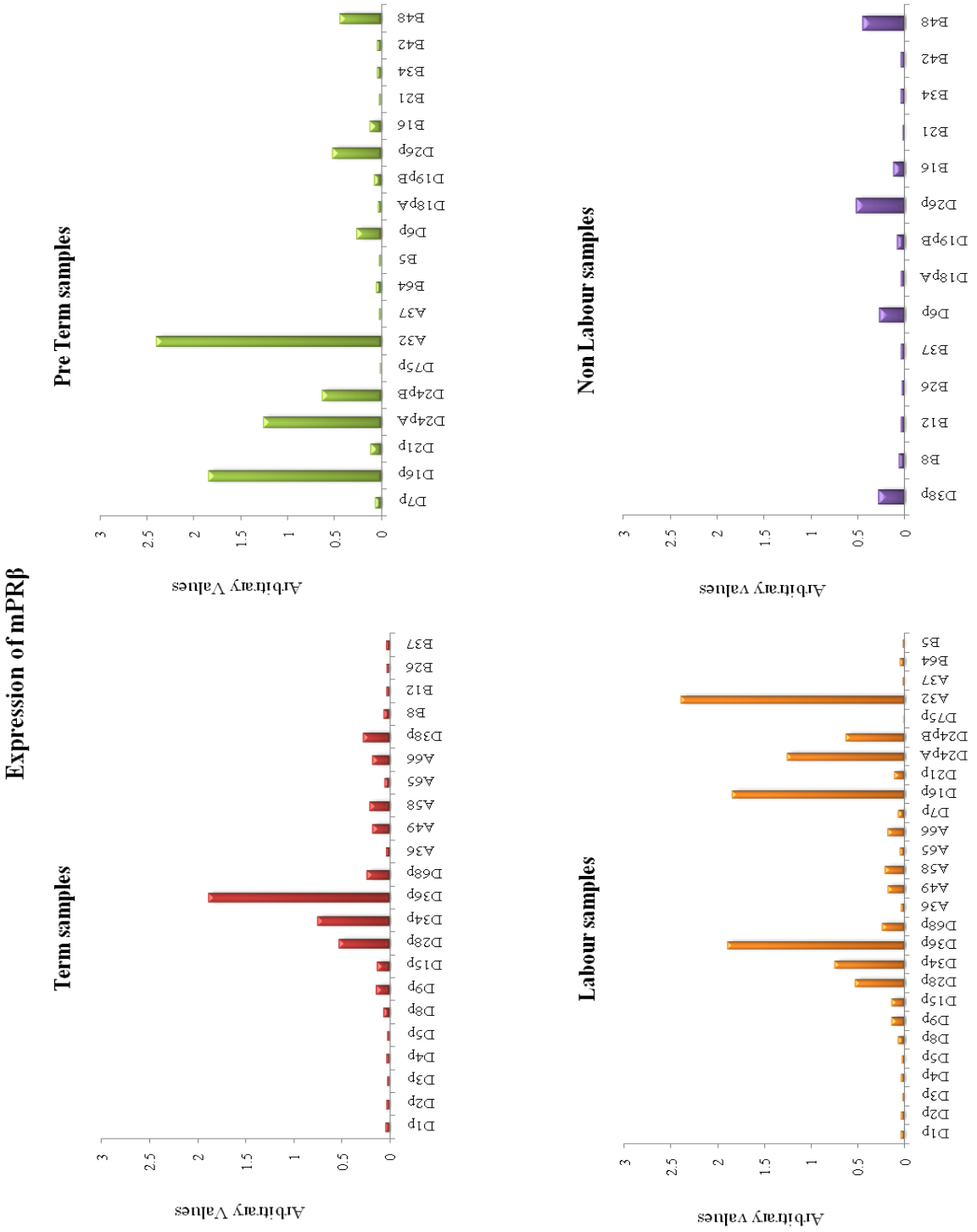


Figure 3.30: Expression of mPRβ in human placental samples (experimental triplictaes per sample), presented according to the contractile state and the time of delivery. The data are presented in arbitrary values ($2^{-\Delta Ct}$) which is the result of the correction of the gene of interest over β-actin house keeping gene of each individual placental sample.

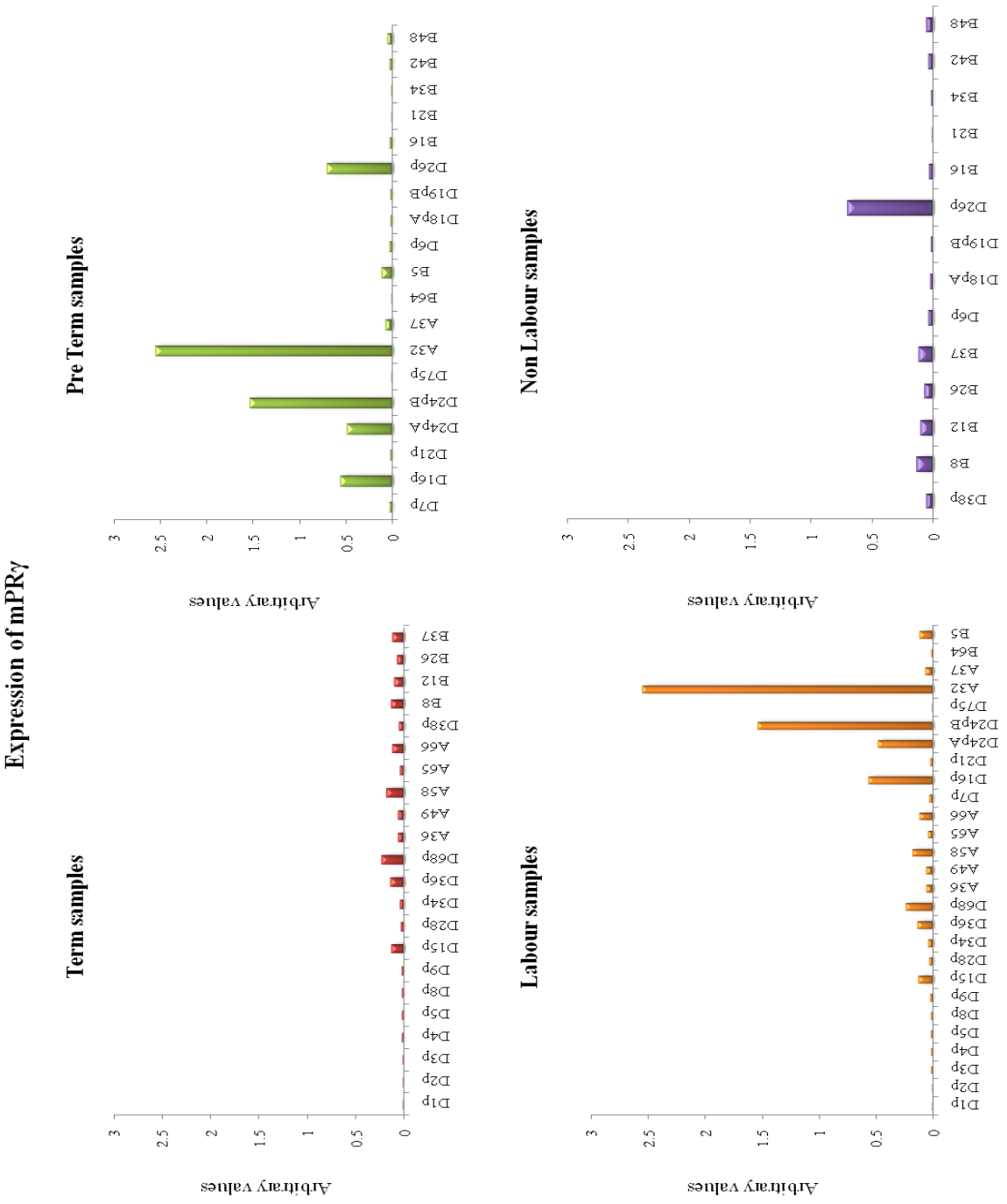
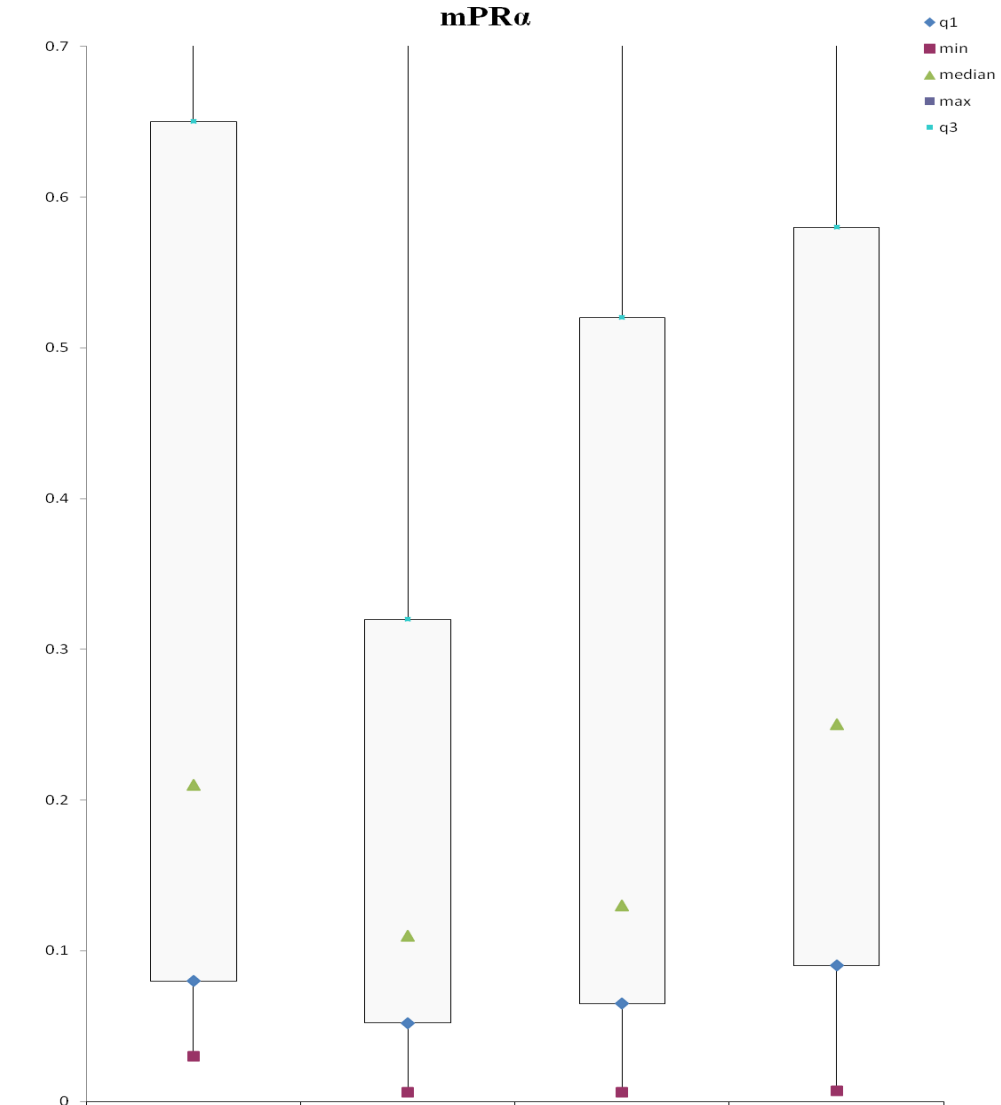


Figure 3.31: Expression of mPRγ in human placental samples (experimental triplictaes per sample), presented according to the contractile state and the time of delivery. The data are presented in arbitrary values ($2^{-\Delta Ct}$) which is the result of the correction of the gene of interest over β-actin house keeping gene of each individual placental sample.

The data illustrated in Fig. 3.32-3.34 shows three box plots of median variation with associated 25^{th}, 50^{th} and 75^{th} percentile ranges, respectively of the results collected from the analysis of mPRα, mPRβ and mPRγ. The boundaries of the box indicate the 25th percentile and the 75th percentile. The maximum values are indicated in the box below.

Variability can be determined from the length of the box where the spread of the mpRα data is greater in the term and non- labour category and a less great spread is observed in the pre-term category. Higher expression is seen in the term category as the 75^{th} percentile indicates where the maximum values of all four categories are shown in the table below. The lowest minimum expression values are detected in the pre term and labour category.

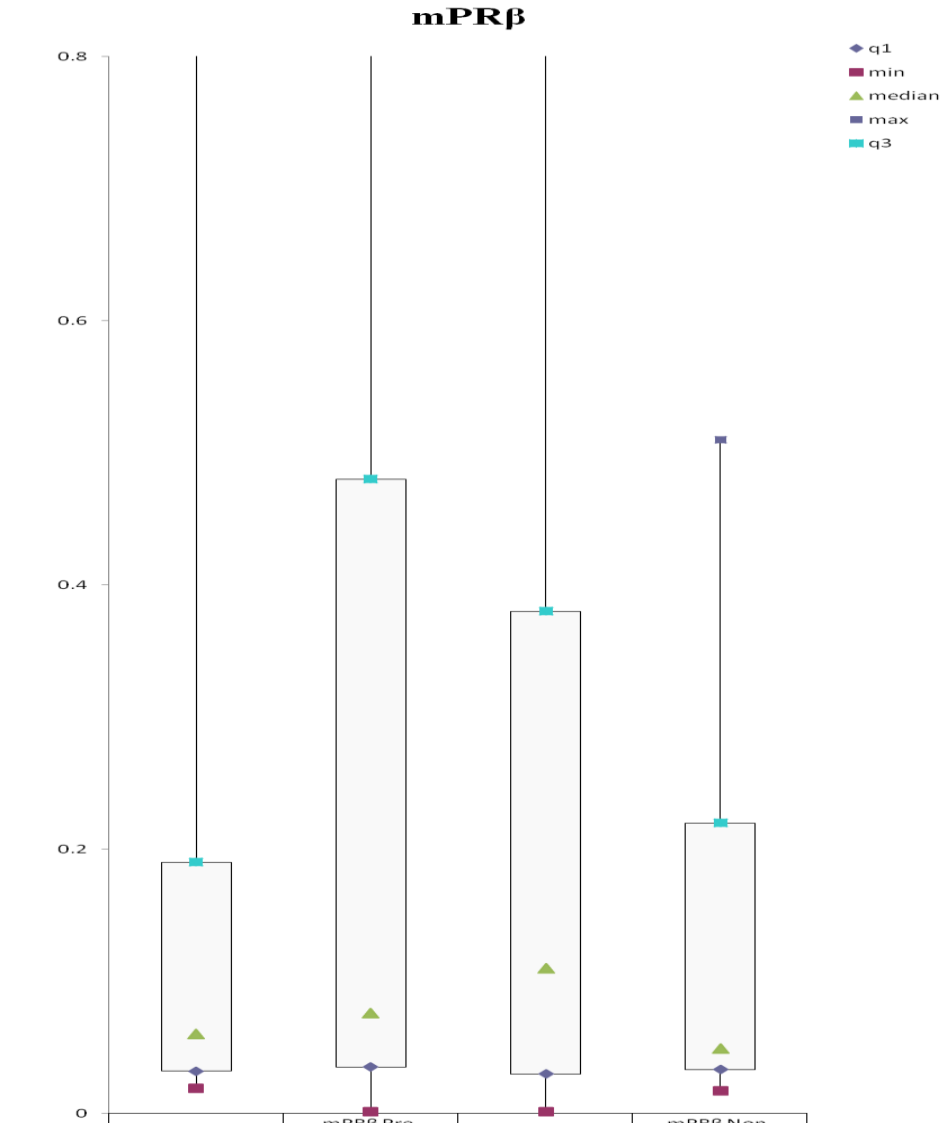


	mPRα Term	mPRα Pre-Term	mPRα Labour	mPRα Non-Labour
q1	0.08	0.052	0.065	0.09
min	0.03	0.006	0.006	0.007
median	0.21	0.11	0.13	0.25
max	16.22	15.54	16.22	15.54
q3	0.65	0.32	0.52	0.58

Figure 3.32 : Box plot obtained from placental tissue data after performing analysis of the mPRα gene. Q1= 1st quartile/ 25th percentile; Min= minimum value; Median= 2nd quartile / 50th percentile; Max= maximum value; Q3= 3rd quartile / 75th percentile

The category that shows the greater variability from the mPRβ data is the pre term and to a lesser degree the labour. Higher expression is seen in the pre term category as the 75th percentile indicates and the maximum values of all four categories are shown in the table

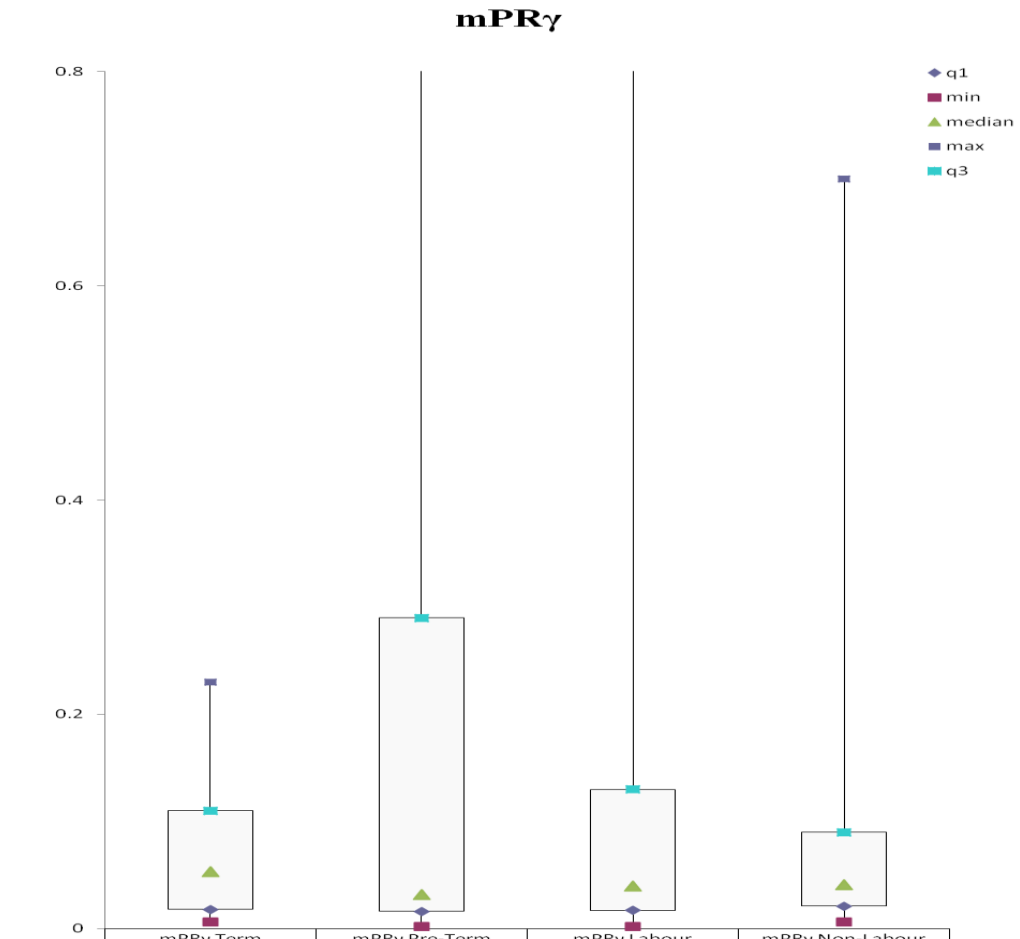
below. The lowest minimum expression values are detected in the term and non labour category.



	mPRβ Term	mPRβ Pre-Term	mPRβ Labour	mPRβ Non-Labour
q1	0.032	0.035	0.03	0.033
min	0.019	0.001	0.001	0.017
median	0.06	0.076	0.11	0.049
max	1.89	2.39	2.39	0.51
q3	0.19	0.48	0.38	0.22

Figure 3.33: Box plot obtained from placental tissue data after performing analysis of the mPRβ gene. Q1= 1st quartile/ 25th percentile; Min= minimum value; Median= 2nd quartile / 50th percentile ; Max= maximum value; Q3= 3rd quartile/ 75th percentile.

The pre term category as indicatated by the length of its box shows the greater variability from the mPRγ data where the other three catgories demonstate less variability in the expression. Higher range of expression is seen in the pre term category as the 75th percentile indicates and the maximum values of all four categories are shown in the table below. The lowest minimum expression values are detected in the term and non labour category.



	mPRγ Term	mPRγ Pre-Term	mPRγ Labour	mPRγ Non-Labour
q1	0.018	0.016	0.017	0.021
min	0.006	0.002	0.002	0.006
median	0.053	0.032	0.04	0.041
max	0.23	2.55	2.55	0.7
q3	0.11	0.29	0.13	0.09

Figure 3.34 : Box plot obtained from placental tissue data after performing analysis of the mPRγ gene. Q1= 1st quartile/ 25th percentile; Min= minimum value; Median= 2nd quartile / 50th percentile ; Max= maximum value; Q3= 3rd quartile/ 75th percentile.

The primary transcript therefore can be concluded to be the mPRα, as the expression of this gene as seen from the 75th percentile data in all four categories is higher than the values obtained from mPRβ and mPRγ. Also it should be noted that the mPRα transcript is the one with the higher maximum values compared to the other two genes of interest although this is detected on a sporadic basis.

3.7.2. Cellular localisation and distribution of mPRs in the human placenta.

The localisation of mPR proteins was assessed by immunofluorescence. From the representative images shown in Fig. 3.35 on the outer site of the chorionic villi, the syncytia layer can be seen. Directly beneath, the mononuclear cytotrophoblast cells can be visualised. mPRα was seen intensively in the syncytia layer in the cytoplasm and on the plasma membrane around the fused syntytiotrophoblast cells (Fig.3.35). The staining of mPRα in the cytotrophoblast cells showed to be mainly on the plasma membrane and the intensity is lower than in the syncytium. mPRβ showed cytoplasmic and plasma membrane staining in the syncytium and in the cytotrophoblas cells, staining of the syncytium was stronger. mPRγ protein was also localised in the syncytium and the cytotrophoblast cells with both cytoplasmic and plasma membrane staining (Zachariades *et al*., 2011).

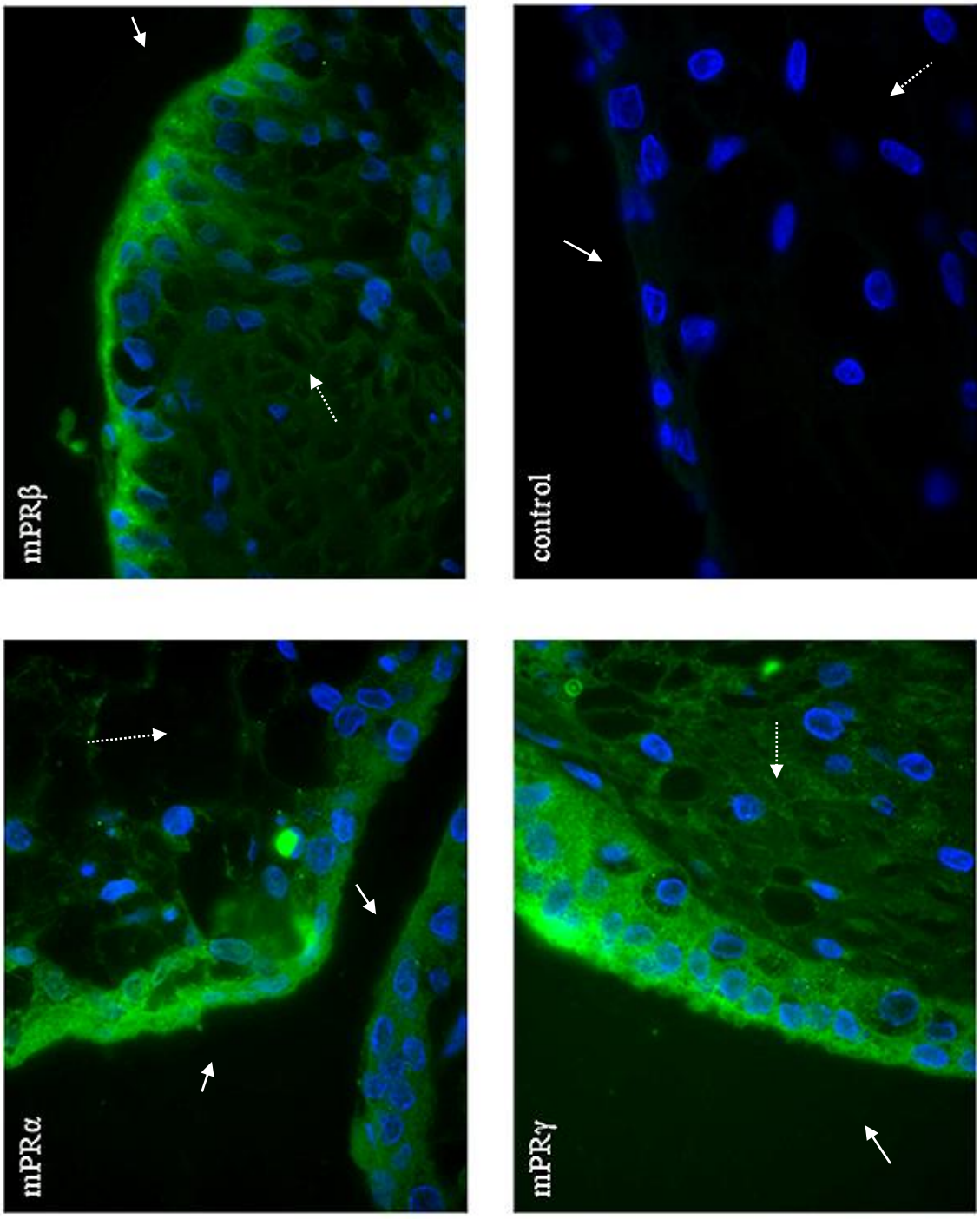


Figure 3.35: Immunofluorescence analysis for mPRα, mPRβ and mPRγ in human placental tissue sections. White arrows show the syncytia layer and the white dotted arrows the cytotrophoblast cells that lie beneath the syncytia layer.

3.7.3. Expression of PGRMC-1 at gene level in the human placenta

The expression of PGRMC-1 mRNA was examined in the human placental samples obtained and the graphs are presented in the panel of Fig.3.36.

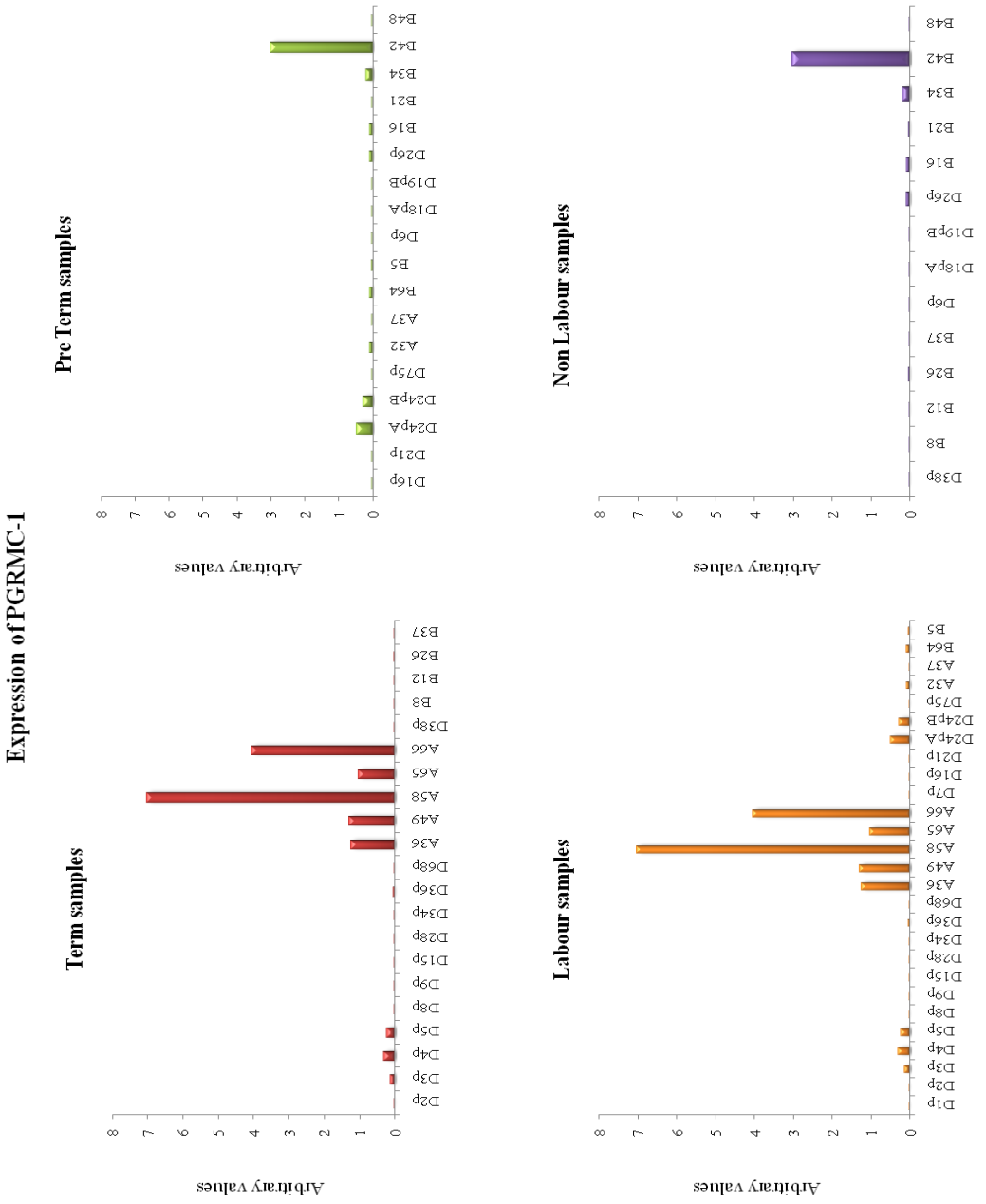
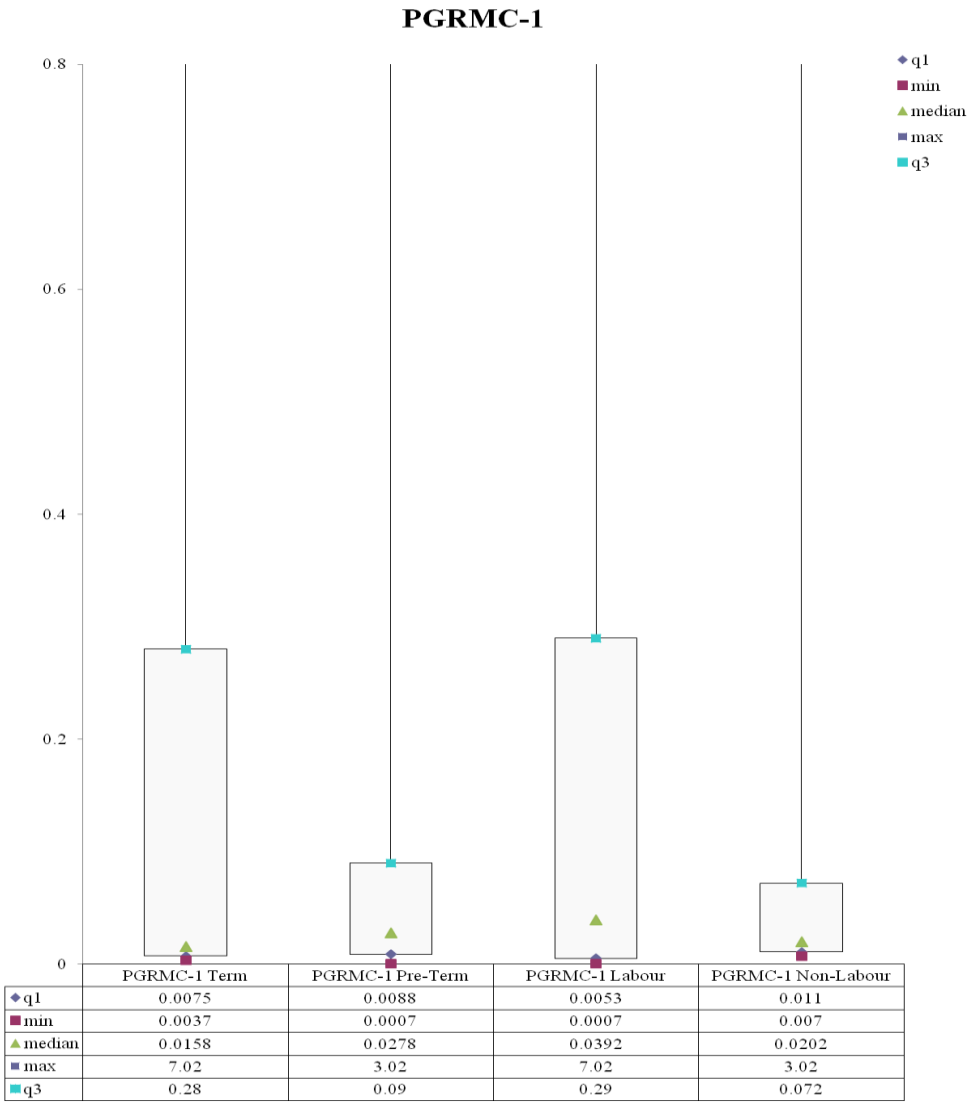


Figure 3.36: Expression of PGRMC-1 in human placental samples (experimental triplictaes per sample) and are presented according to the contractile state and the time of delivery. The data are presented in arbitrary values ($2^{-\Delta Ct}$) which is the result of the correction of the gene of interest over β-actin house keeping gene of each individual placental sample.

Below a box plot graph (Fig.3.37) depicting the mean arbitrary values of the four categories is presented. The term and the labour categories are the ones demonstating the greater spread in the expression values as indicated by the length of the boxes where the other thwo catgories demonstate less variability in their expression. Higher range of expression is again seen in the term and labour category as the 75th percentile indicates and the maximum values of all four categories are shown in the table below. The lowest minimum expression values are detected in the pre term and labour category.



	PGRMC-1 Term	PGRMC-1 Pre-Term	PGRMC-1 Labour	PGRMC-1 Non-Labour
q1	0.0075	0.0088	0.0053	0.011
min	0.0037	0.0007	0.0007	0.007
median	0.0158	0.0278	0.0392	0.0202
max	7.02	3.02	7.02	3.02
q3	0.28	0.09	0.29	0.072

Figure 3.37: Box plot obtained from placental tissue data after performing analysis of the PGRMC-1 gene. Q1= 1st quartile/ 25th percentile; Min= minimum value; Median= 2nd quartile / 50th percentile ; Max= maximum value; Q3= 3rd quartile/ 75th percentile.

3.7.4. Cellular localisation and distribution of PGRMC-1 in the human placenta.

The PGRMC-1 protein in the human placenta was found predominantly in the syncytium as seen in the image of the panel below (Fg.3.38). The staining was seen in both cytoplasm and plasma membrane. A distinctive plasma membrane staining was seen at a lesser degree in the cytotrophoblast cells.

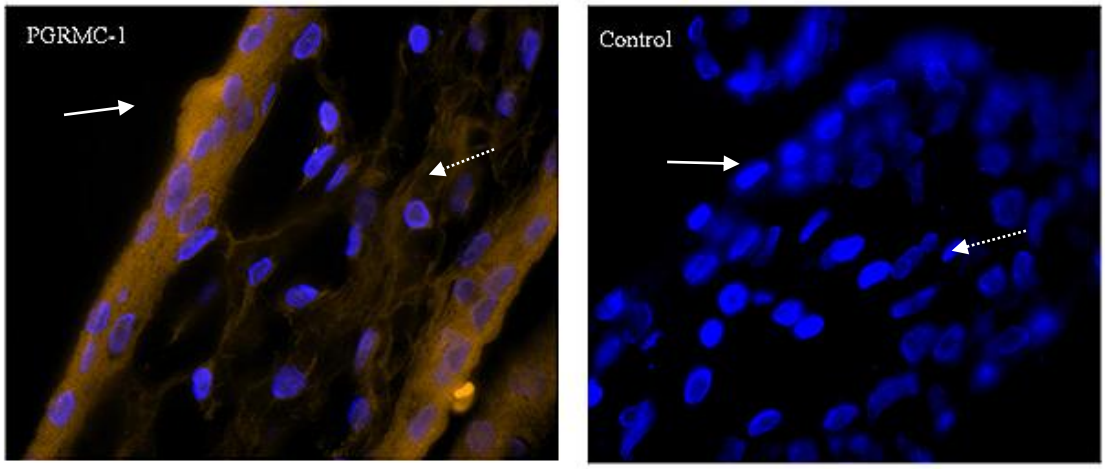


Figure 3.38: Immunofluorescence analysis for PGRMC-1 in human placental tissue section. White arrows show the syncytia layer and the white dotted arrows the cytotrophoblast cells that lie beneath the syncytia layer.

3.7.5. Expression of PR-B in the human placenta.

The expression of PR-B mRNA was examined in the human placental samples obtained and the graphs are presented in the panel of Fig.3.39.

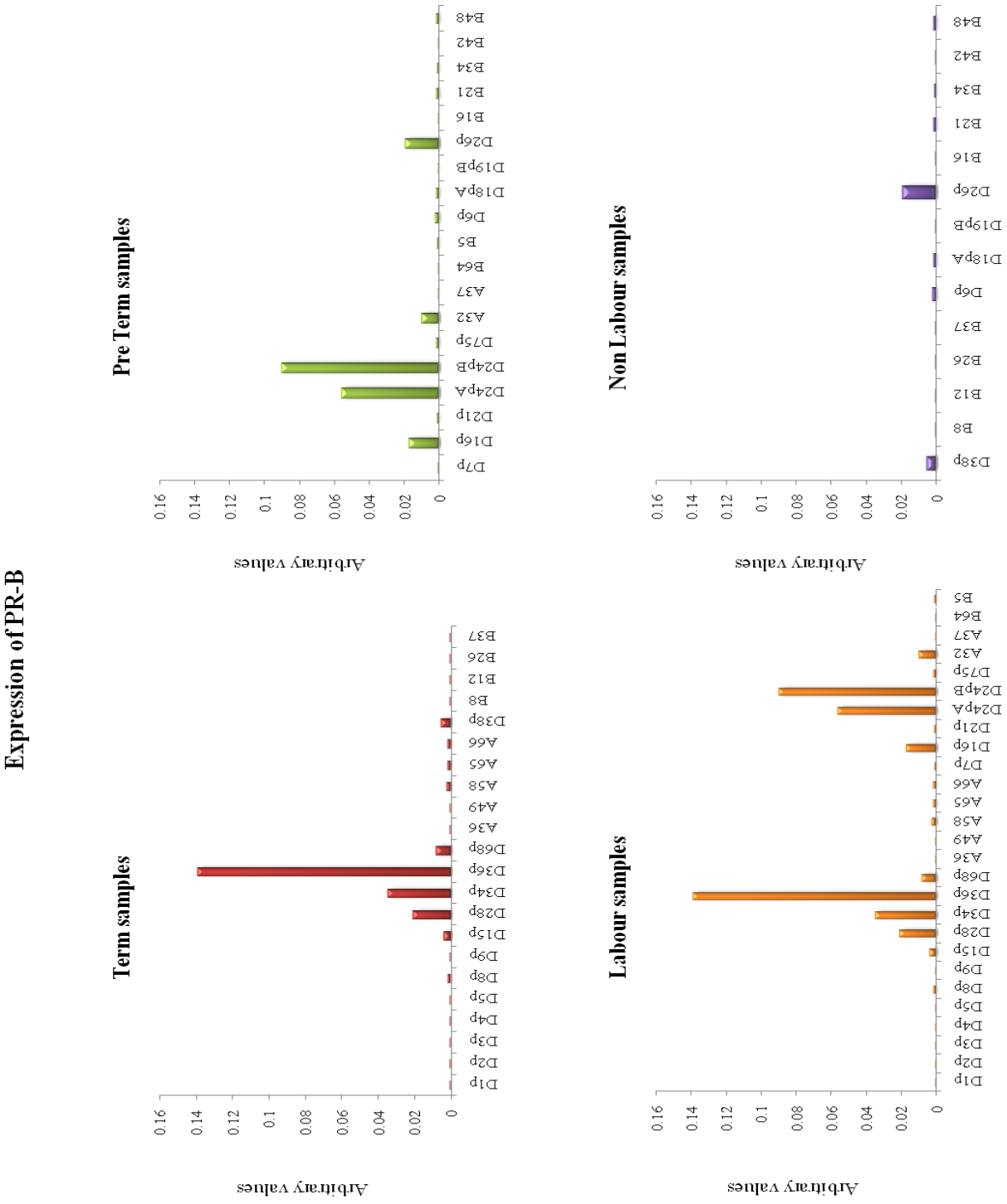
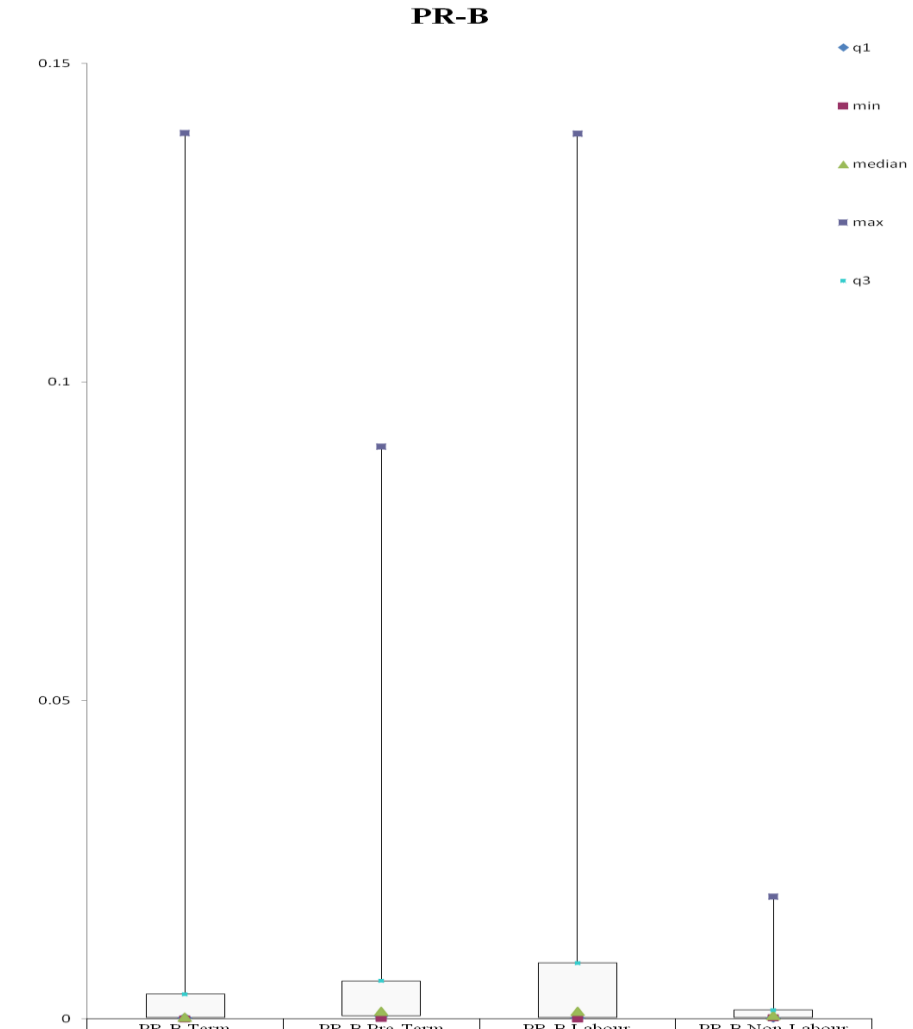


Figure 3.39: Expression of PR-B in human placental samples (experimental triplictaes per sample) and are presented according to the contractile state and the time of delivery. The data are presented in arbitrary values ($2^{-\Delta Ct}$) which is the result of the correction of the gene of interest over β-actin house keeping gene of each individual placental sample.

A box plot graph illustrated below (Fig.3.40) depicts the mean arbitrary values of the four categories. A very low expression is observed in all four categories, though a greater variability is detected in the labour category where the less variability is setected in the non labour category. Higher expression values are observed in the term and labour category although these values remail below 0.5.



	PR-B Term	PR-B Pre-Term	PR-B Labour	PR-B Non-Labour
q1	0.0002	0.0005	0.0002	0.00024
min	0.00003	0.00008	0.00003	0.00019
median	0.00034	0.00123	0.0012	0.00057
max	0.13912	0.0899	0.139	0.01919
q3	0.00388	0.00599	0.0088	0.0014

Figure 3.40: Box plot obtained from placental tissue data after performing analysis of the PR-B gene. Q1= 1st quartile/ 25th percentile; Min= minimum value; Median= 2nd quartile / 50th percentile ; Max= maximum value; Q3= 3rd quartile/ 75th percentile.

3.7.6. Statistical Correlations

The correlation between the expressions of the genes of interest was calculated using SPSS statistical software. As the ΔCt values obtained from the Q-PCR analysis of the placental samples were not normally distributed, a log transformation action was performed in order to normalise the data.

The expression values of each gene of interest were correlated with the expression values of the rest of the genes of interest to detect if a statistical relationship occurred and if so, the significance of the correlation reported as Pearson correlation coefficient. The tables below (Table 3.4 – 3.7) indicate the levels of the correlation coefficient (r) and the red asterisks indicate the levels of significance (* <0.05, ** <0.001). The correlations were performed in the four categories separately – term, pre term, labour and non labour.

		mPRα TERM	mPRβ TERM	mPRγ TERM	PGRMC-1 TERM	PR-B TERM
mPRα TERM	Pearson Correlation	1	-0.102	0.316	0.890**	-0.134
	Sig. (2-tailed)		0.653	0.151	0.000	0.551
mPRβ TERM	Pearson Correlation	-0.102	1	0.239	-.060	0.980**
	Sig. (2-tailed)	0.653		0.284	0.792	0.000
mPRγ TERM	Pearson Correlation	0.316	0.239	1	0.375	0.220
	Sig. (2-tailed)	0.151	0.284		0.086	.324
PGRMC-1 TERM	Pearson Correlation	0.890**	-0.060	0.375	1	-0.117
	Sig. (2-tailed)	0.000	0.792	0.086		0.604
PR-B TERM	Pearson Correlation	-0.134	0.980**	0.220	-0.117	1
	Sig. (2-tailed)	0.551	0.000	0.324	0.604	

Table 3.4: Levels of correlation between log values (n=22) of mPRs, PGRMC-1 and PR-B. Red asterisks indicate significance of the correlation (** Significant at the 0.01 level - 2-tailed).

For the variables where a positive correlation was seen the ΔCt values of the respective genes were plotted against each other. The two variables from the term category, as seen from the graph in Fig.3.41 were mPRα and PGRMC-1 showing a positive correlation (*r = 0.890***, *p =0.000, n=22*).

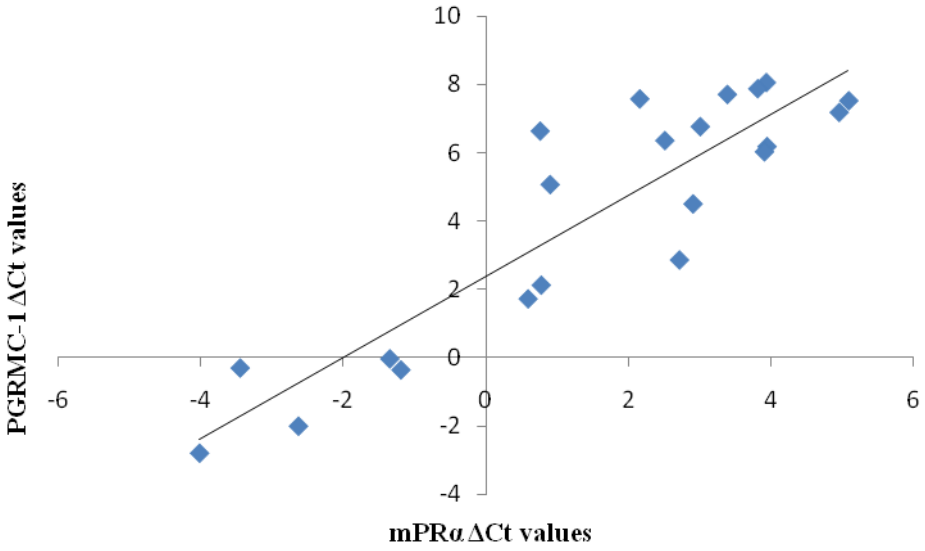


Figure 3.41: Correlation graph between mPRα and PGRMC-1 in term placentae (n=22, *r = 0.890, p =0.000).*

The variable mPRβ showed a positive correlation (*r = 0.980**, p =0.000, n=22*) with PR-B variable. This correlation is seen in graph in Fig.3.42.

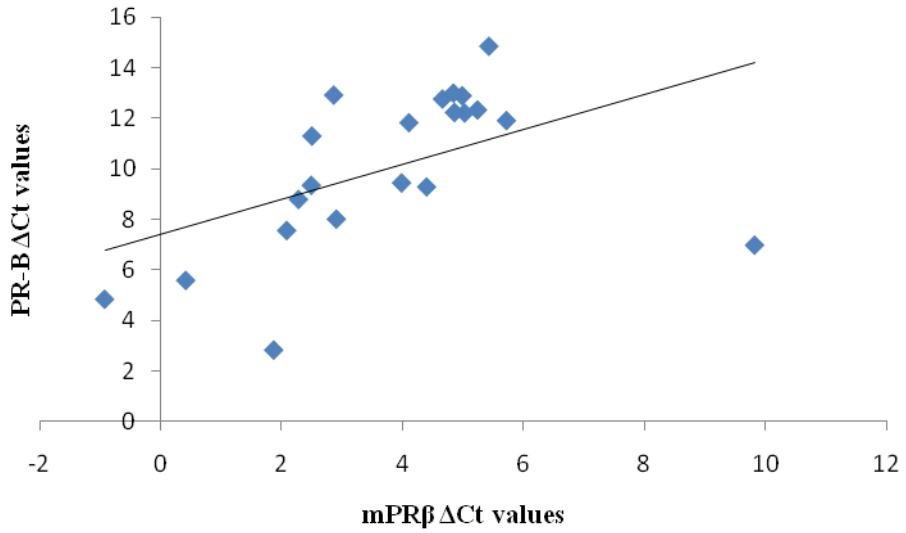


Figure 3.42: Correlation graph between mPRβ and PR-B in term placentae (n=22, *r = 0.980**, p =0.000)*

The next table has been constructed once the correlation of the expression values of the genes of interest was examined in the pre term placental samples. The levels of correlation coefficient (r) are reported and the red asterisks indicate the levels of significance (* <0.05, ** <0.001).

		mPRα PT	mPRβ PT	mPRγ PT	PGRMC-1 PT	PR-B PT
mPRα PT	Pearson Correlation	1	-0.139	-0.114	0.982**	-0.127
	Sig. (2-tailed)		0.570	0.642	0.000	0.603
mPRβ PT	Pearson Correlation	-0.139	1	0.802**	-0.081	0.376
	Sig. (2-tailed)	0.570		.000	0.743	.112
mPRγ PT	Pearson Correlation	-0.114	0.802**	1	-0.052	0.527*
	Sig. (2-tailed)	0.642	0.000		0.832	0.020
PGRMC-1 PT	Pearson Correlation	0.982**	-0.081	-0.052	1	0.031
	Sig. (2-tailed)	0.000	0.743	0.832		0.901
PR-B PT	Pearson Correlation	-0.127	0.376	0.527*	0.031	1
	Sig. (2-tailed)	0.603	0.112	0.020	0.901	

Table 3.5: Levels of correlation between log values (n=19) of mPRs, PGRMC-1 and PR-B. Red asterisks indicate significance of the correlation (** Significant at the 0.01 level, * significant at the 0.05 level).

The ΔCt values of the respective genes were plotted against each other for the variables where a correlation was seen. The first two variables from the pre term category, as seen from the graph in Fig.3.43 were mPRα and PGRMC-1 showing a positive correlation (*r = 0.982**, p =0.000, n=19*).

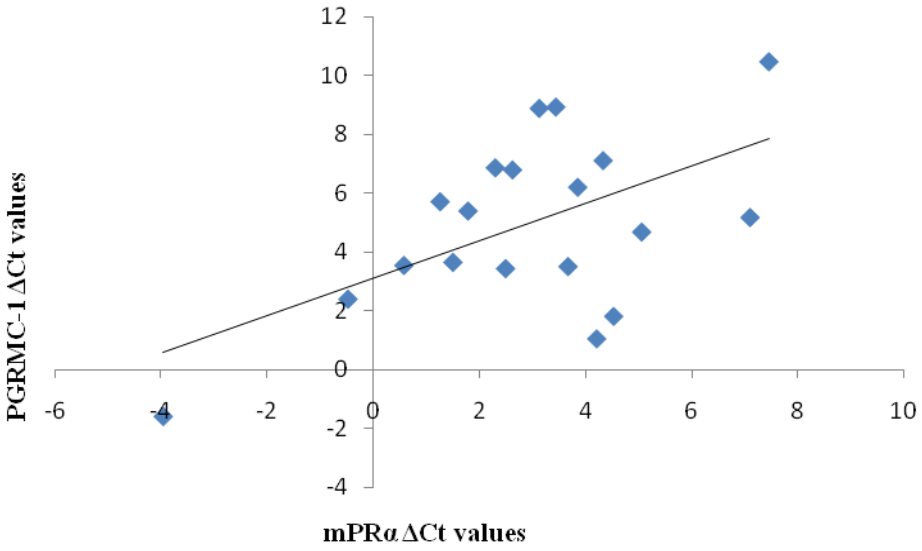


Figure 3.43: Correlation graph between mPRα and PGRMC-1 in pre term placentae (n=19, *r = 0.982**, p =0.000).*

The variable mPRβ showed a positive correlation (*r = 0.802**, p =0.000, n=19*) with mPRγ variable in the pre term category and this correlation is seen in graph in Fig.3.44.

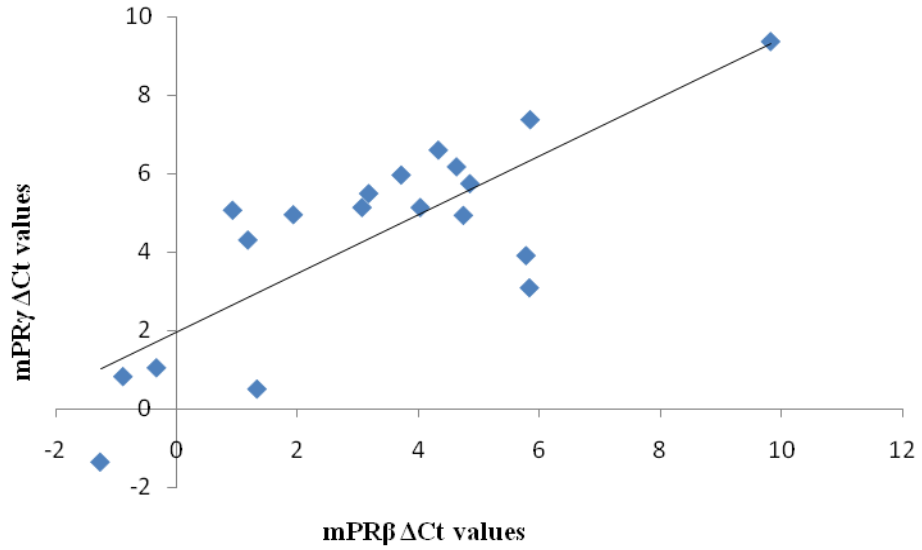


Figure 3.44: Correlation graph between mPRβ and mPRγ in pre term placentae (n=19, *r = 0.802**, p =0.000).*

The variable mPRγ showed a positive correlation (*r = 0.527*, p =0.020, n=19*) with PR-B variable in the pre term category and this correlation is seen in graph in Fig.3.45.

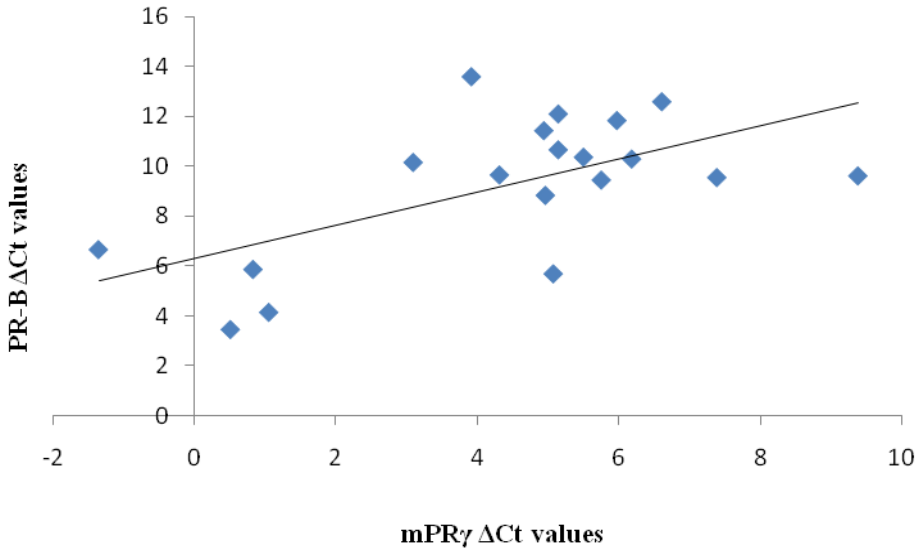


Figure 3.45: Correlation graph between mPRγ and PR-B in pre term placentae (n=19, *r = 0.527*, p =0.020).*

From the next category, placentas from the labouring cohort, a table (Table 3.6) has been constructed once the correlations of the expression values of the genes of interest were examined in this category. The levels of correlation coefficient (r) are reported and the red asterisks indicate the levels of significance (** <0.001).

		mPRα L	mPRβ L	mPRγ L	PGRMC-1 L	PR-B L
mPRα L	Pearson Correlation	1	-0.152	-0.081	0.891**	-0.164
	Sig. (2-tailed)		0.449	0.688	0.000	0.415
mPRβ L	Pearson Correlation	-0.152	1	0.687**	-0.115	0.590**
	Sig. (2-tailed)	0.449		0.000	0.566	0.001
mPRγ L	Pearson Correlation	-0.081	0.687**	1	-0.052	0.279
	Sig. (2-tailed)	0.688	0.000		0.797	0.159
PGRMC-1 L	Pearson Correlation	0.891**	-0.115	-0.052	1	-0.119
	Sig. (2-tailed)	0.000	0.566	0.797		0.553
PR-B L	Pearson Correlation	-0.164	0.590**	0.279	-0.119	1
	Sig. (2-tailed)	0.415	0.001	0.159	0.553	

Table 3.6: Levels of correlation between values (n=27) of mPRs, PGRMC-1 and PR-B. Red asterisks indicate significance of the correlation (** Significant at the 0.01 level).

The first correlation observed in this category, were the variables mPRα and PGRMC-1, where a positive correlation was seen (*r = 0.891**, p =0.000, n=27*) in Fig.3.46.

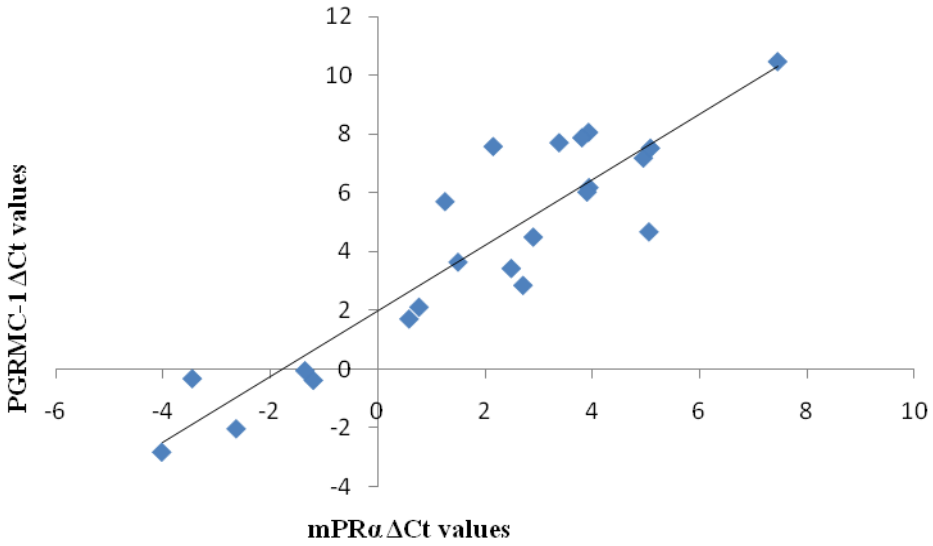


Figure 3.46: Correlation graph between mPRα and PGRMC-1 in labouring placentae (n=27, *r = 0.891**, p =0.000).*

Following, a correlation between the expression of mPRβ and mPRγ (Fig.3.47) was detected with a correlation coefficient (r) value of 0.687** and sifnificance (0.000).

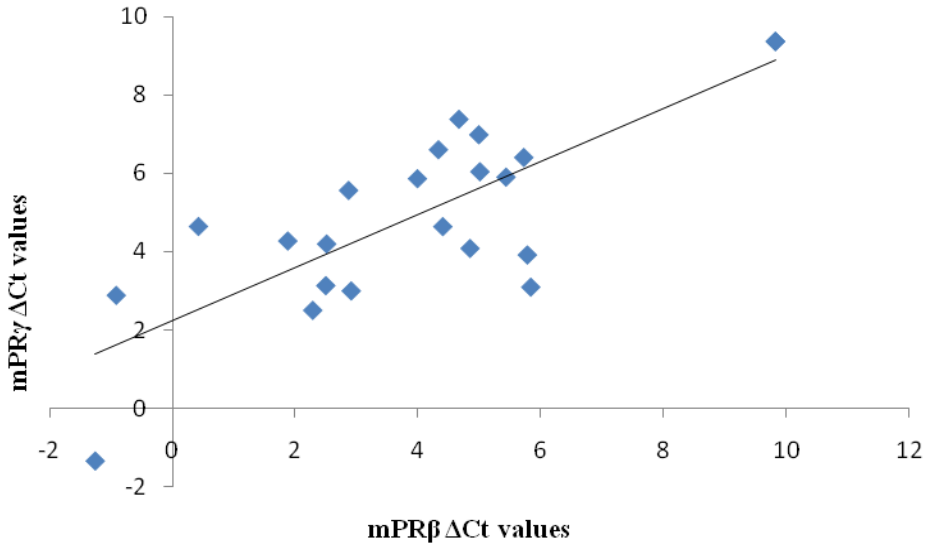


Figure 3.47: Correlation graph between mPRβ and mPR in labouring placentae (n=27, *r = 0.687**, p =0.000).*

Again in the labouring samples, variable mPRβ showed a positive correlation (*r = 0.590**, p =0.001, n=27*) with PR-B variable and this correlation is seen in graph in Fig.3.48.

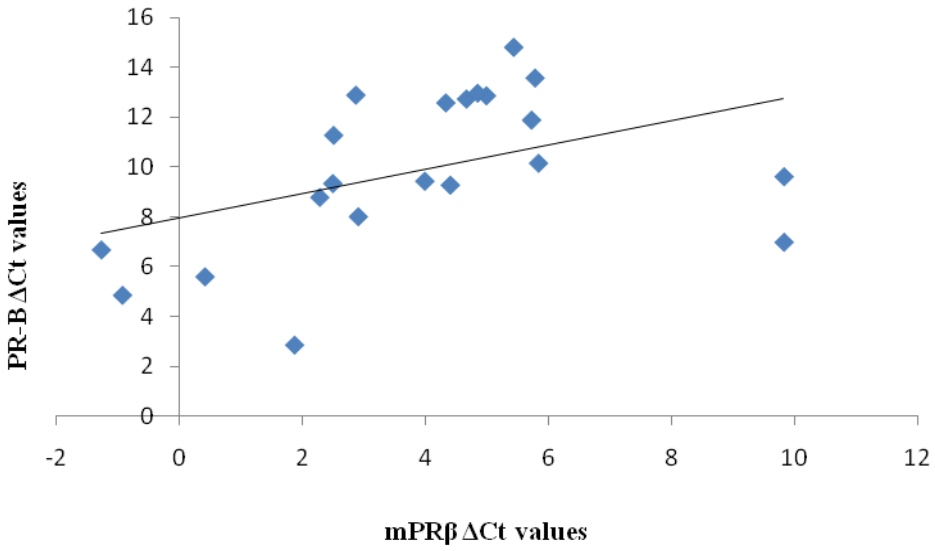


Figure 3.48: Correlation graph between mPRβ and PR-B in labouring placentae (n=27, *r = 0.590**, p =0.001).*

A table (Table 3.7) has been constructed from the values obtained in the non labouring placental samlples once the correlations of the expression values of the genes of interest were examined in this category.

		mPRα NL	mPRβ NL	mPRγ NL	PGRMC-1 NL	PR-B NL
mPRα NL	Pearson Correlation	1	-0.205	-0.124	0.998**	-0.140
	Sig. (2-tailed)		0.482	0.674	0.000	0.633
mPRβ NL	Pearson Correlation	-0.205	1	0.620*	-0.177	0.736**
	Sig. (2-tailed)	0.482		.018	0.546	0.003
mPRγ NL	Pearson Correlation	-0.124	0.620*	1	-0.092	0.927**
	Sig. (2-tailed)	0.674	0.018		0.754	0.000
PGRMC-1 NL	Pearson Correlation	0.998**	-0.177	-0.092	1	-0.101
	Sig. (2-tailed)	0.000	0.546	0.754		0.731
PR-B NL	Pearson Correlation	-0.140	0.736**	0.927**	-0.101	1
	Sig. (2-tailed)	0.633	0.003	0.000	0.731	

Table 3.7: Levels of correlation between log values (n=27) of mPRs, PGRMC-1 and PR-B. Red asterisks indicate significance of the correlation (** Significant at the 0.01 level, * significant at the 0.05 level).

The ΔCt values of the respective genes were plotted against each other for the variables where a correlation was seen in the non labouring placenta. The first two variables as seen from the graph in Fig.3.49 were mPRα and PGRMC-1 showing a positive correlation (*r = 0.998**, p =0.000, n=14*).

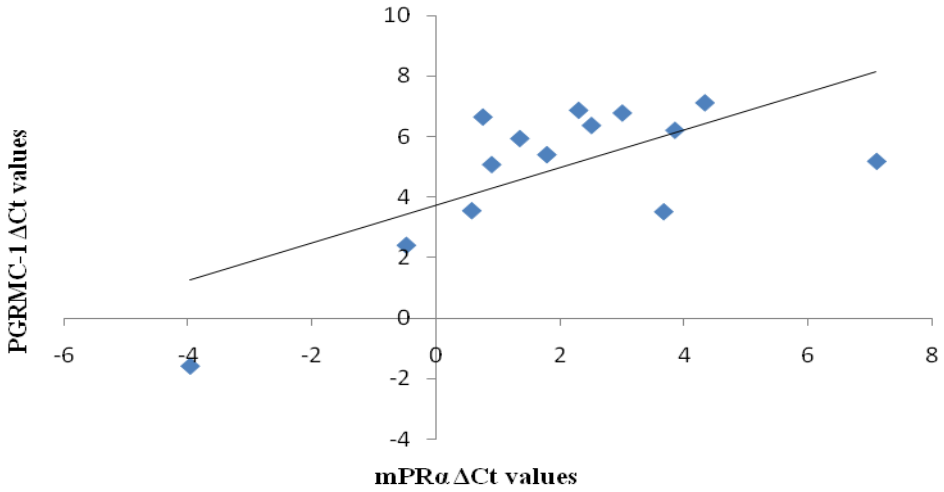


Figure 3.49: Correlation graph between mPRα and PGRMC-1 in non labouring placentae (n=14, *r = 0.998**, p =0.00).*

Variable mPRβ showed a positive correlation (*r = 0.736*, p =0.003, n=14*) with PR-B variable in the non labouring category and this correlation is seen in graph in Fig.3.50.

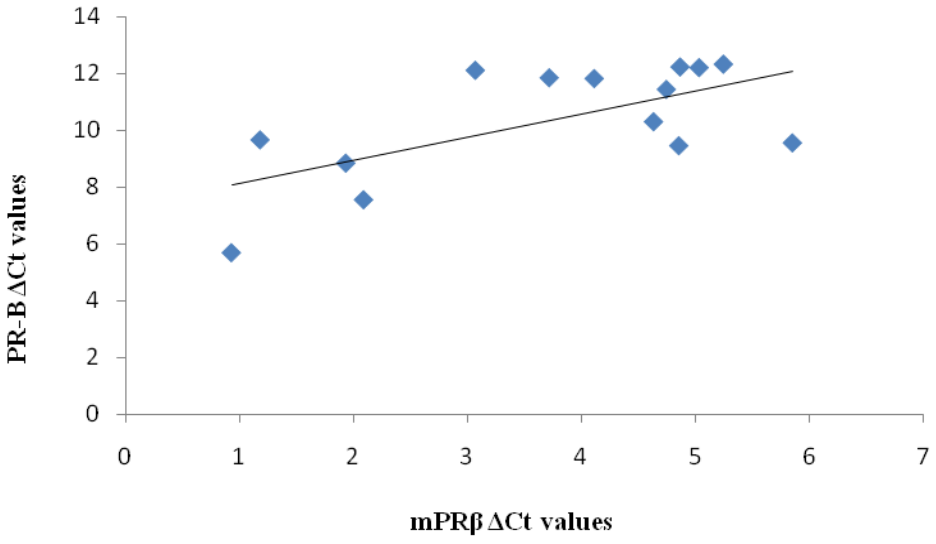


Figure 3.50: Correlation graph between mPRβ and PR-B in labouring placentae (n=14, *r = 0.736**, p =0.003).*

Lastly, a correlation was detected between the variable mPRγ showed a positive correlation (*r = 0.927**, p =0.000, n=14*) and PR-B variable in the non labouring category and this correlation is seen in graph in Fig.3.51.

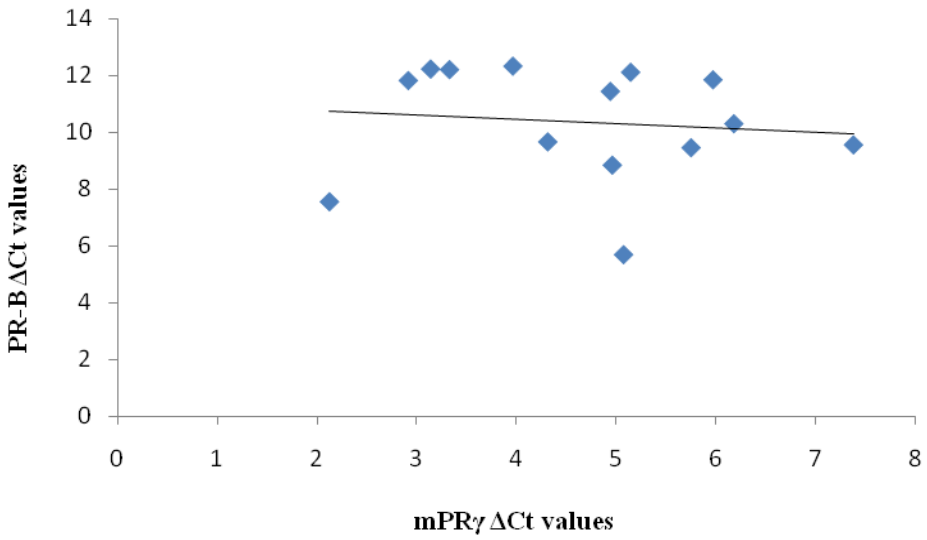


Figure 3.51: Correlation graph between mPRγ and PR-B in non labouring placentae (n=14, *r = 0.927**, p =0.000).*

Information on placental weight was obtained from only 23 patients participating in the study, as the sample number was relatively small the samples were treated as one group instead of four separate categories. The information was used to correlate the weight of the placenta with the expression of PRs and establish significance. No correlation was found for placental weight and mPRα, mPRβ, mPRγ and PGRMC-1 although the expression of mPRα and mPRγ in correlation with placental weight showed a trend, albeit non-significant. A

negative correlation (*r= -0.462, p= 0.040, n=23*) was detected between PR-B and the weight of the placentae (Table 3.8).

Correlation of PR with placental weight		
Log_mPRα	Pearson Correlation	0.419
	Significance	0.066
Log_mPRβ	Pearson Correlation	-0.284
	Significance	0.225
Log_mPRγ	Pearson Correlation	-0.433
	Significance	0.057
Log_PGRMC-1	Pearson Correlation	-0.264
	Significance	0.261
Log-PR-B	Pearson Correlation	-0.462
	Significance	0.040*

Table 3.8: Correlation of placental weight of 23 samples with the expression of mPRs, PGRMC-1 and PR-B.

3.8. Discussion

3.8.1. Human placenta and the expression of progesterone receptors

In order to gain a better insight and to correlate our *in vitro* findings with clinical samples a large human placenta tissue bank was set up comprising of samples at different contractile states (labour or non-labour) and times of delivery (term or pre-term). All mPR isoforms were expressed in our placental tissues, with mPRα and PGRMC-1 genes showing the highest expression in the term and labour group whereas mPRβ and mPRγ were shown to have the highest expression in the pre term group and the labour group. Variation in the expression of these genes was observed among the individual patients. Our study was drawn entirely from Cretan patients, a genetically homogenous Caucasian population. Caution must be taken when comparing these results with studies where a high ethnical diversity is observed in the population.

The presence of mPRα transcript was shown previously in the human placenta by the Thomas group and at the time it had been suggested that as this was the major transcript it would also be the major subtype mediating the rapid, non-genomic actions of progesterone in the placenta (Zhu *et al*., 2003[B]). Our study though shows the presence of all three mPR subtypes as well as PGRMC-1 in the human placenta and when category analysis was performed, mPRα was found to be the major transcript in two categories (labour and term) and also exhibited a high 75th percentile in the non labour category thus leading to the conclusion that the expression of mPRα depend on the time and mode of delivery.

In the human placenta mPRα was localised in the synsytia layer demonstrating a higher intensity signal compared to that detected in the cytotrophoblast cells. This is in agreement with a study by Fernandes *et al*, demonstrating that mPRα expression is restricted to the syncytiotrophoblast at the periphery of term chorionic villi (Fernandes *et al*., 2005). mPRβ

was localised in the syncytia layer and the cytotrophoblast cells beneath the multinucleated layer, where a higher signal intensity was detected. mPRγ was also localised in both cell types of the human placenta, this time the signal intensity observed in the cytotrophoblasts was almost the same as the signal intensity in the syncytia layer. Apart from mPRα, which its presence was reported in term placentae, mPRβ and mPRγ have not been localised in the human placenta before.

We have localised the PGRMC-1 protein in the syncytium, where it is present in the cytoplasm as well as in the plasma membrane and also in the cytotrophoblast cells exhibiting mainly membrane staining. These findings agree and also extend the results described by Zhang *et al*., where they only detected this protein in the syncytiotrophoblast cells (Zhang *et al*., 2008).

Earlier studies demonstrated expression of PR mRNA in human term placental samples (Shanker & Rao, 1998). At protein level, they demonstrated only the presence of PR-A but not PR-B in their samples (Shanker & Rao, 1998). At gene level, we identified the presence of PR-B in our placental tissue bank with the highest expression of PR-B being detected in the term and labour category.

Interestingly, the tissue expression of the mPRα transcript was correlated with the expression of PGRMC-1 in all four categories where this protein – protein interaction is supported by the STRING analysis. Expression of mPRβ was significantly correlated in a positive manner to the expression of mPRγ and PR-B. No predicted interactions with either protein were detected by the STRING database. The placental expression of mPRγ transcript was significantly correlated to the expression the nuclear PR-B in the pre term and the non labour caegories, an interaction indicated also by the STRING database (See Appendix I).

It is evident that the nuclear PR-B is the only transcript that shows positive and negative correlations with all mPR gene transcripts in the human placenta suggesting a differential regulatory pathway for these receptors in the human placenta as these receptors have a different expression pattern distinguished by time and mode of delivery. A plausible explanation for the correlation between mPRs and PR-B may be the transactivation ability of PR-B by mPRs which has been reported to exist in the human myometrium (Karteris *et al*., 2006).

The placental weight in our cohort showed a negative correlation with the expression of the nuclear PR-B indicating a possible inverse relationship between the growth ability of this organ and the progesterone receptor. A positive trend, albeit non-significant, between the expression of mPRα and mPRγ was also detected indicating a possible differential regulation of these genes arising from the actions of progesterone mediated by these receptors. The growth of the human placenta may indeed be regulated in a positive manner by the membrane progesterone receptors and in a negative manner by the nuclear receptors. PRs may stimulate the production and secretion of progesterone at the beginning of the pregnancy where the need of progesterone to support the pregnancy is more demanding and as a steady rise is seen during the gestation period this may create an internal feedback loop in this transient reproductive organ.

3.9. Functionality of membrane Progesterone Receptors – mPRs & PGRMC-1

The functionality of progesterone receptors (mPRs & PGRMC-1) was investigated once the detailed expression of these receptors in two placental cell lines and in human placental samples was accomplished. IL-1β, a pro-inflammatory cytokine was used to treat BeWo CT and JEG-3 cells for 24 hours. This cytokine has been shown to be able to modulate the proliferation of trophoblast cells and also it has been associated with trophoblast differentiation. In abnormal pregnancy pathophysiologies such as pre-eclpampsia, IUGR and preterm labour the levels of IL-1β cytokine are elevated (Rossi *et al*., 2005).

Once BeWo CT and JEG-3 cells were grown to confluence (~70%) combination treatments at different concentrations were performed and trypan blue assay was used to investigate the effect of the treatments on trophoblast cell death.

The following compounds and concentrations were used during the treatments:

Compound	Stock Concentration	Final concentration
Progesterone - a known activator of mPRs, PGRMC-1 and PRs	10^{-1} M	1nM, 10nM, 100nM, 1000 nM ± U0126 (1μM)
10-ethenyl-19-norprogesterone (OD 02-0) – a selective agonist of mPRα	10^{-3} M	1nM, 10nM, 100nM, 1000 nM ± U0126 (1μM)
Promegestone (R5020) - a specific agonist of nPR	10^{-2} M	30nM ± U0126
U0126 – a selective $MEK_{1/2}$ inhibitor	10^{-3} M	1μM
IL-1β		10ng/μl

Fig. 3.52 depicts the percentage of dead cells following a 24 hour treatment with P4 and OD 02-0. Controls (NS) cells were not treated and established the "baseline" of cell death in BeWo CT cells. Exposure of BeWo CT cells to 10 nM, 100 nM and 1000 nM of P4 significantly decreased the percentage of dead cells by 5.5 fold, 1.8 fold and 6.7 fold respectively over the untreated BeWo CT cells. When the BeWo CT cells were incubated with OD 02-0 no significant changes in the percentage of dead cells were observed, although there was a general trend towards a reduction in cell death.

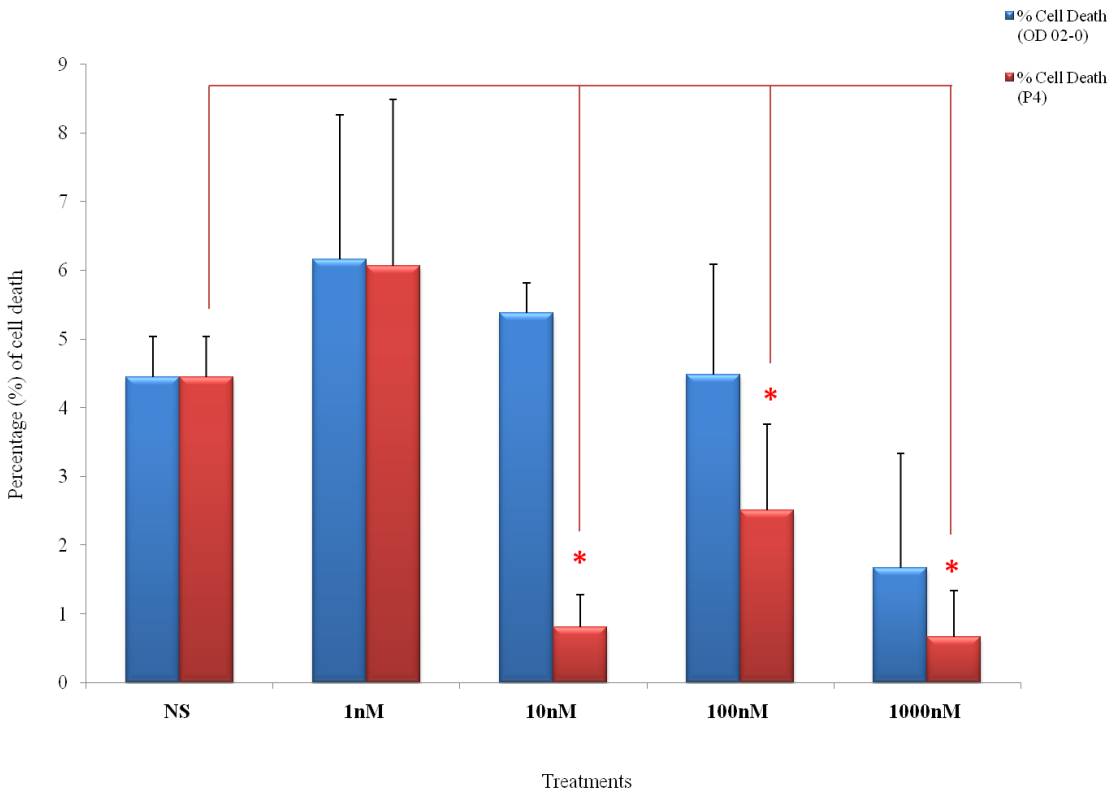


Figure 3.52: Percentage of dead BeWo CT cells following treatment with 1nM, 10nM, 100nM, 1000nM of Progesterone (red) and OD 02-0 (blue) following a 24hr incubtation. Each histogram represents the mean ± SEM of three individual experiments. Red and blue lines indicates significance of treated cells with NS ($p < 0.05$*).

BeWo CT cells were co-incubated with 10ng/µl of the cytokine IL-1β and 1 nM –1000 nM P4 or OD 02-0 before assessing cell death by trypan blue exclusion assay. Fig. 3.53 shows that the percentage of dead BeWo CT cells after treatment with IL-1β significantly increased 3 fold over the NS BeWo CT cells.When BeWo CT cells were treated with 100 nM of P4 in the presence of 10ng/µl IL-1β it significantly reduced 5.5 fold the percentage of dead cells compared to the IL-1β treated BeWo CT cells. A significant 3.3 fold reduction in the percentage of dead BeWo CT cells was detected when the cells were treated with 1000nM of P4 in parallel with 10ng/µl IL-1β compared to the IL-1β treated BeWo CT cells. When the cells were incubated with OD 02-0 in parallel to 10ng/µl IL-1β, a significant decrease in the percentage of dead cells was detected, specifically with 10 nM a 6.4 fold decrease, with 100 nM a 7.6 fold decrease and with 1000nM a 2.6 fold decrease compared to the BeWo CT cells treated with 10ng/µl IL-1β alone (Fig 3.53).

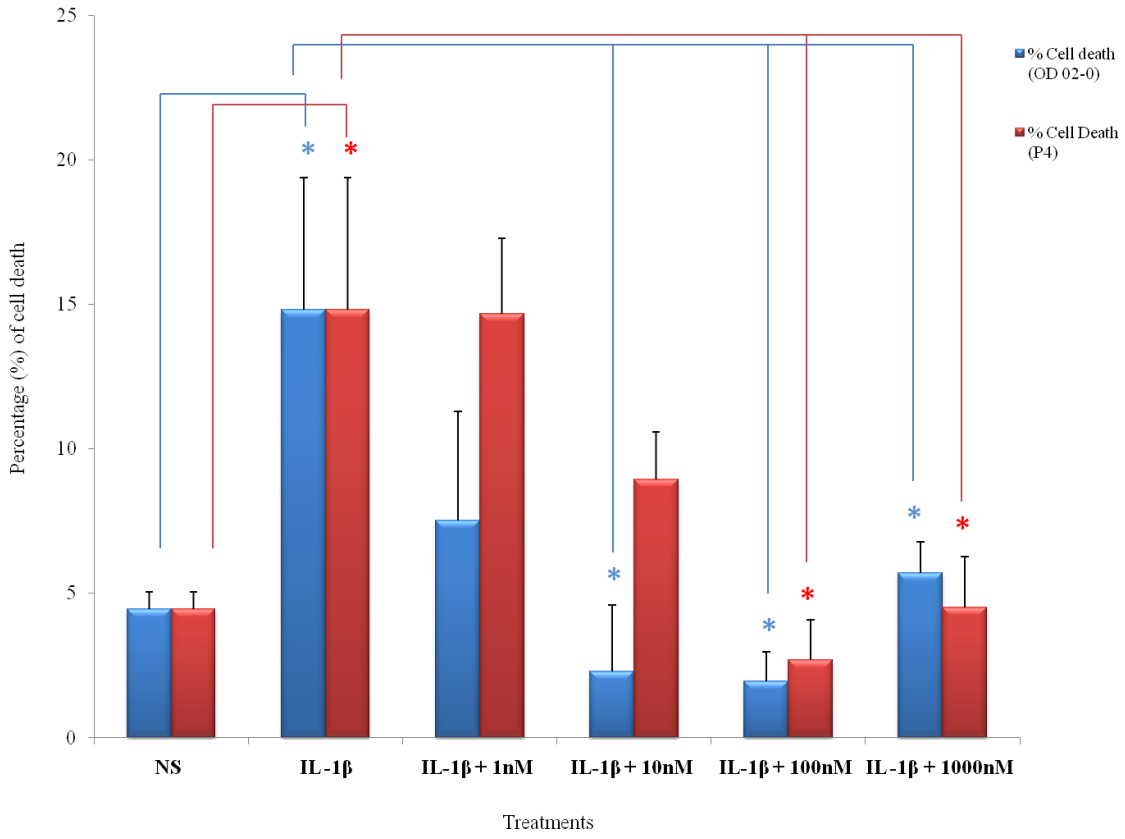


Figure 3.53: Percentage of dead BeWo CT cells following combination treatment with IL-1β (10ng/µl) and various concentrations of progesterone (red) or OD 02-0 (blue) (1-1000nM) following a 24hr incubtation. Each histogram represents the mean ± SEM of three individual experiments. Red and blue lines indicate significance of NS with IL-1β alone and treated cells with IL-1β alone ($p < 0.05$*).

BeWo CT cells were pre-incubated with the $MEK_{1/2}$ inhibitor, UO126 prior to treating with P4 or OD 02-0 at a range of concentrations (1 nM – 1000 nM) in the presence of 10ng/µl of IL-1β. The graph in Fig.3.54 illustrates the percentage of dead BeWo cells obtained after performing a trypan blue exclusion assay. Cells exposed to 1000 nM of P4 in the presence of IL-1β and having $MEK_{1/2}$ activity inhibited by UO126, showed a significant 5.5 fold decrease in the percentage of BeWo CT dead cells compared to the percentage of dead cells detected

after exposure to IL-1β alone. The percentage of dead cells was significantly higher when cells were treated with IL-1β compared to the non-treated cells. When OD 02-0 was used to treat the UO126 pre-incubated cells in the presence of IL-1β a significant lower percentage of dead cells was observed when using 10 and 100 nM of OD 02-0. The percentage of dead cells was decreased by 3.2 fold and 5.4 fold respectively in comparison to the percentage of dead cells detected when treating the cells with IL-1β only (Fig 3.54).

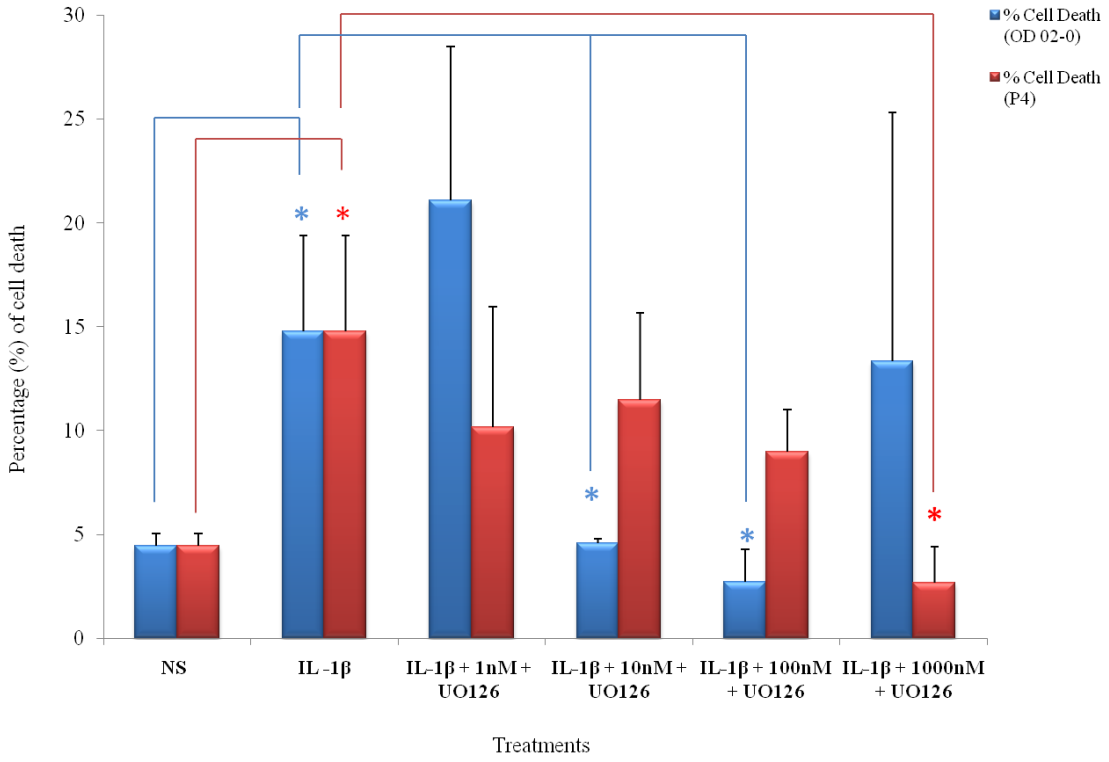


Figure 3.54: Percentage of dead BeWo CT cells after pre-incubating cells with UO126 (1μM) and performing combination treatment with IL-1β (10ng/μl) and various concentrations of progesterone (red) or OD 02-0 (blue) (1-1000nM) following a 24hr incubtation. Each histogram represents the mean percentage ± SEM of three individual experiments ($p < 0.05$*). Red and blue lines indicate significance of NS with IL-1β alone and IL-1β alone with treated cells.

A specific nPR agonist, R5020 was used to treat BeWo CT cells at a concentration of 30 nM. This compound was used alone to treat cells, in the presence of IL-1β (10ng/µl) and repeating the combination treatment but pre-incubating the cells with UO126 for 30 minutes prior to adding the 24 hour treatment. Graphical representation in Fig.3.55 shows the percentage of dead BeWo CT cells following the treatments and assessing cell death by trypan blue exclusion assay. BeWo CT cells treated with IL-1β showed significant increase in the percentage of cell death when compared to NS and R5020 treated cells. In comparison to IL-1β only treated cells, the percentage increased significanlty (2 fold) when the cells were pre-incubated with UO126 and co-treated with R5020 and IL-1β (Fig 3.55).

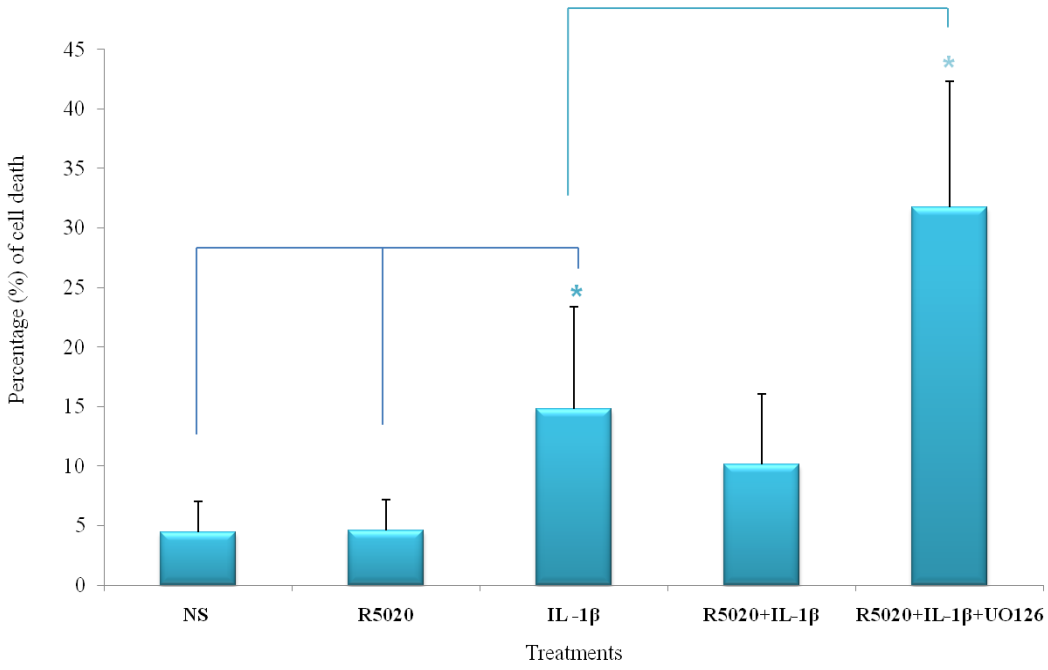


Figure 3.55: Percentage of dead BeWo cells after treating cells with R5020, R5020 + IL-1β (10ng/µl) and pre-incubating cells with UO126 and combination treatment with IL-1β (10ng/µl) + R5020 following a 24hr incubtation. Each histogram represents the mean ± SEM of three individual experiments ($p < 0.05$*). Blue line indicates significance of the percentage of dead cells obtained after treating cells with IL-1β alone compared to NS and R5020 and upon comparison of pre-incubating cells with UO126 and treating with R5020 + IL-1β with IL-1β treated cells.

The same sets of treatments were performed in the second *in vitro* model the JEG-3 cell line. The percentage of dead cells was assessed by trypan blue exclusion assay after the period of 24 hour. The first set of results is presented in Fig.3.56 where the percentage of dead JEG-3 cells was identified following treatment with P4 and OD 02-0 at different concentrations (1nM, 10nM, 100nM, 1000nM). Exposure of JEG-3 cells to 100 and 1000 nM of P4 significantly increased the percentage of dead cells 2.4 fold and 3.3 fold respectively over NS. Exposure of JEG-3 cells to OD 02-0 resulted in a significant increase of the percentage of dead cells detected. At 1 nM of OD 02-0 the increase over NS was 3.8 fold, at 10 nM of OD 02-0 was 5 fold, at 100 nM of OD 02-0 was 6.7 fold and at 1000 nM the increase was 2.3 fold over NS (Fig 3.56).

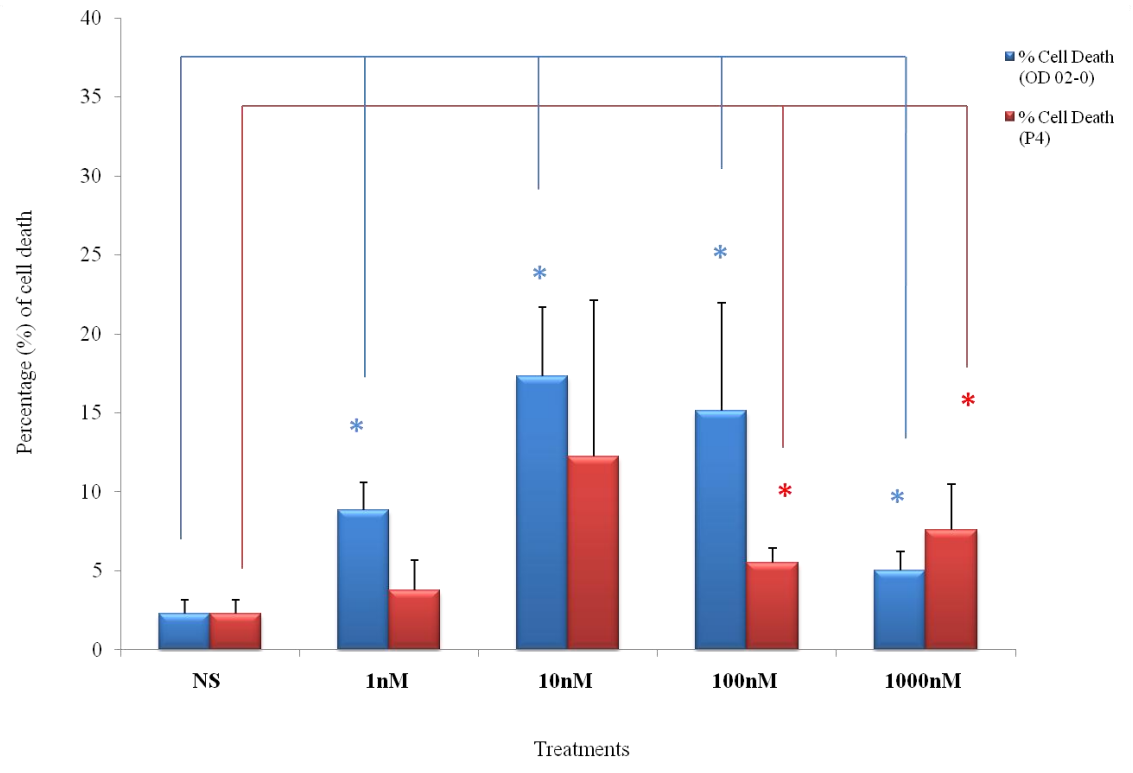


Figure 3.56: Percentage of dead JEG-3 cells following treatment with 1nM, 10nM, 100nM, 1000nM of Progesterone (red) and OD 02-0 (blue) following a 24hr incubtation. Each histogram represents the mean ±SEM of three individual experiments ($p < 0.05$*). Red and blue lines indicate significance of treated cells with NS.

JEG-3 cells were then treated with the cytokine IL-1β (10ng/μl) alone and in the presence of P4 or OD 02-0 at the same concentration range as before. By staining with trypan blue the number of dead JEG-3 cells was calculated and the percentage of dead cells is depicted in Fig.3.57. Incubating JEG-3 with 10ng/μl of IL-1β resulted in an 18 fold increase of the percentage of dead cells over the percentage of dead cells of the non treated cells. When JEG-3 cells were co-incubated with 10ng/μl of IL-1β and 1-1000 nM of P4 the high incidence of cell death was reduced significantly. 1 nM of P4 reduced the percentage of dead JEG-3 cells by 8 fold, 10 nM of P4 reduced the rate of cell death by 7.5 fold, 100 nM of P4 by 8.3 fold wehereas 1000 nM of P4 significanly reduced the rate of cell death by 208 fold compared to the percentage of dead cells detected when treating the cells with IL-1β alone (Fig 3.57) . When OD 02-0 was used to treat cells in parallel to IL-1β, significant reduction in the percentage of dead cells recorderd was achieved when 1 nM, 100 nM and 1000 nM where applied. The mean percentage of dead cells was significanlty reduced by 11 fold, 3 fold and 24 fold respectively compared to the percentage of dead cells detected when treating the cells with IL-1β alone (Fig 3.57).

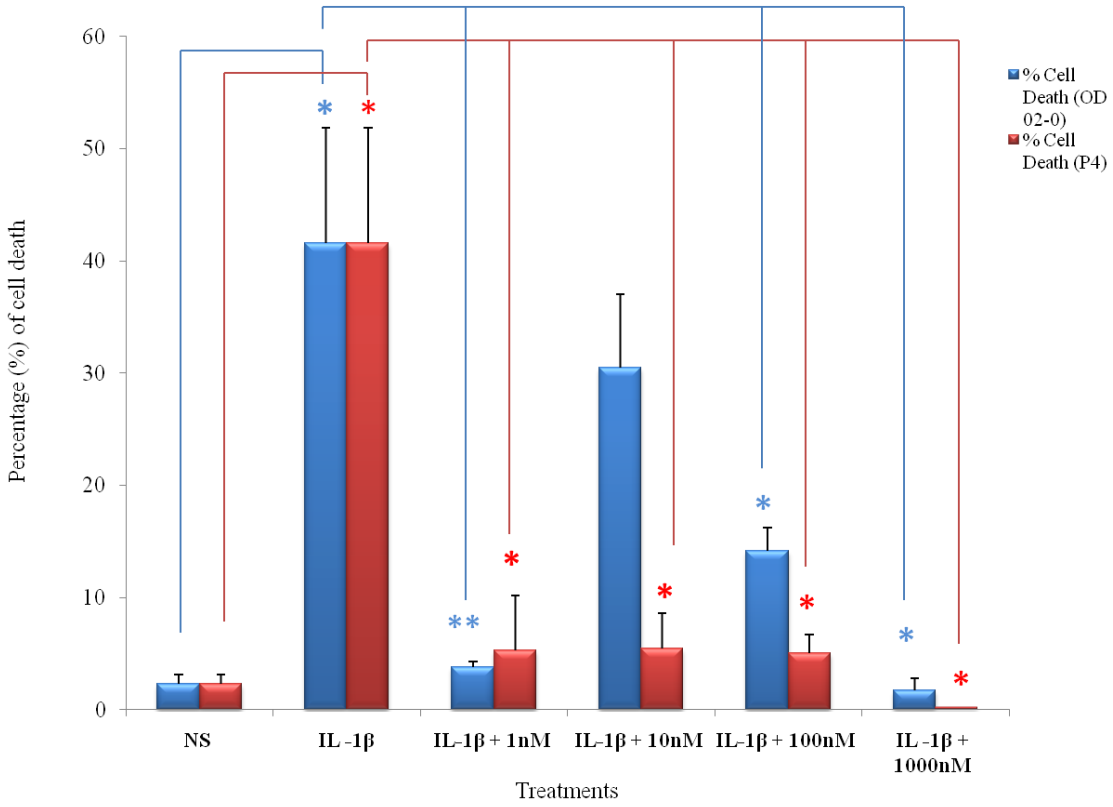


Figure 3.57: Percentage of dead JEG-3 cells following treatment with IL-1β (10ng/µl) and various concentrations of progesterone (red) and OD 02-0 (blue) (1-1000nM) following a 24hr incubtation. Each histogram represents the mean ±SEM of three individual experiments. Red and blue lines indicate significance of NS with IL-1β alone and treated cells with IL-1β alone ($p < 0.05$*, $p < 0.001$ **).

For the next set of treatments, before treating JEG-3 cells with IL-1β (10ng/µl) and either of the two compounds P4 and OD 02-0 (1 nM – 1000 nM), the cells were pre-incubated with UO126 (1 µM) for 30 minutes. The results obtained from the trypan blue exclusion assay are illustrated below in Fig.3.58.

The 18 fold increase in the percentage of JEG-3 dead cells treated with IL-1β over the non-treated cells was significanlty reduced when the UO126 pre- treated cells were co-incubated with IL-1β and 1, 100 and 1000 nM of either P4 or OD 02-0. JEG-3 cells pre-treated with UO126 and then co-incubated with 10ng/µl of IL-1β and 1nM of P4 showed a significant

reduction in the percentage of dead cells recorded by 2.8 fold compared to the IL-1β only treated cells. Whereas when 100nM of P4 was used it reduced the cell death by 3.7 fold and a 4.5 fold reduction was recorded when 1000nM of P4 was used in this set of co-treatments. When the second compound (OD 02-0)was used to treat the pre-treated with UO126 JEG-3 cells in the presecne of 10ng/µl of IL-1β, a subtle reduction (1.7 fold) in the percentage of dead cells was detected when 1nM of OD 02-0 was used compared to the percentage of dead cells recorded once cells were treated with 10ng/µl of IL-1β alone. When 100nM of OD 02-0 were used to co-incubate cells with, a 2.5 fold decrease in the percentage of dead cells was observed and the same fold decrease was also detected when 1000nM of OD 02-0 were administrated, all compared to the IL-1β only treated JEG-3 cells.

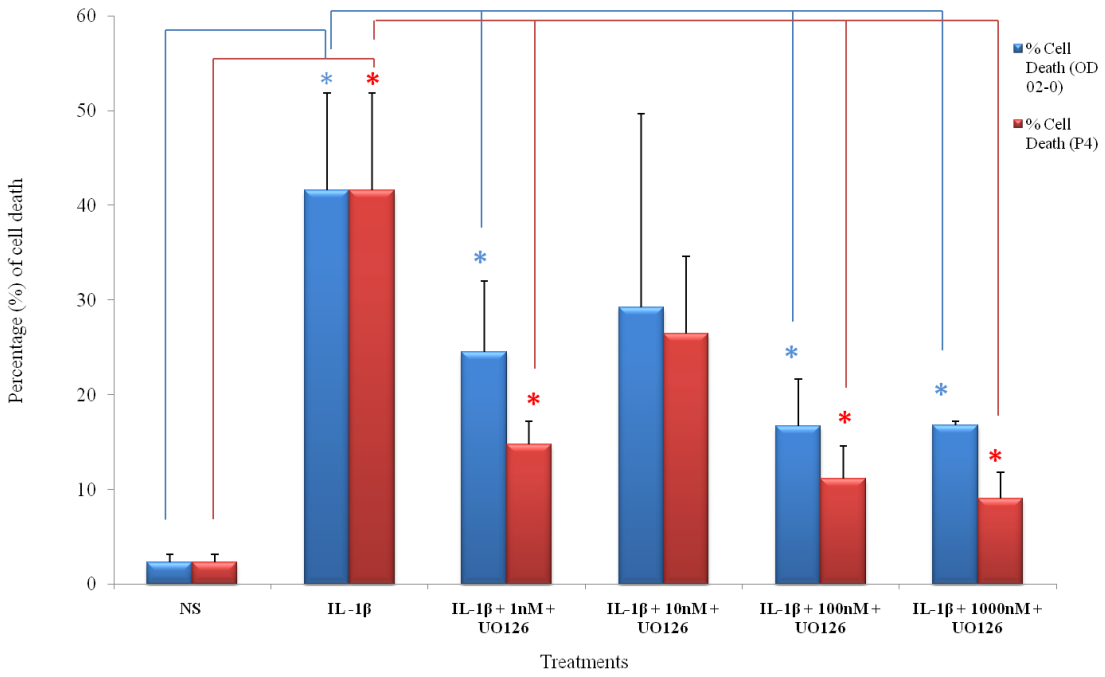


Figure 3.58: Percentage of dead JEG-3 cells after pre-incubating cells with UO126 and combination treatment with IL-1β (10ng/µl) and various concentrations of progesterone (red) and OD 02-0 (blue) following a 24hr incubtation. Each histogram represents the mean ± SEM of three individual experiments ($p < 0.05$*). Red and blue lines indicate significance of NS with IL-1β alone and treated cells with IL-1β alone.

Lastly, the nPR specific activator R5020 (30 nM) was administrated to the JEG-3 cells either alone, in parallel with 10ng/µl IL-1β and repeating the combination treatment but pre-incubating the cells with with UO126 for 30 minutes prior to adding the 24 hour treatment. Fig. 3.59 depicts the percentage of JEG-3 dead cells recorded after performing the treatments and assessing cell death by trypan blue exclusion assay.

Exposure of JEG-3 to IL-1β significanlty increased the percentage of dead cells by 18 fold and 6.6 fold over NS and R5020 respectively. When cells were co-incubated with R5020 and IL-1β a significant 20 fold decrease was detected compared to the IL-1β only treated cells. When the same co-incubation was performed on UO126 pre-treated cells a significant 10 fold reduction was detected compared to the IL-1β only treated JEG-3 cells (Fig. 3.59).

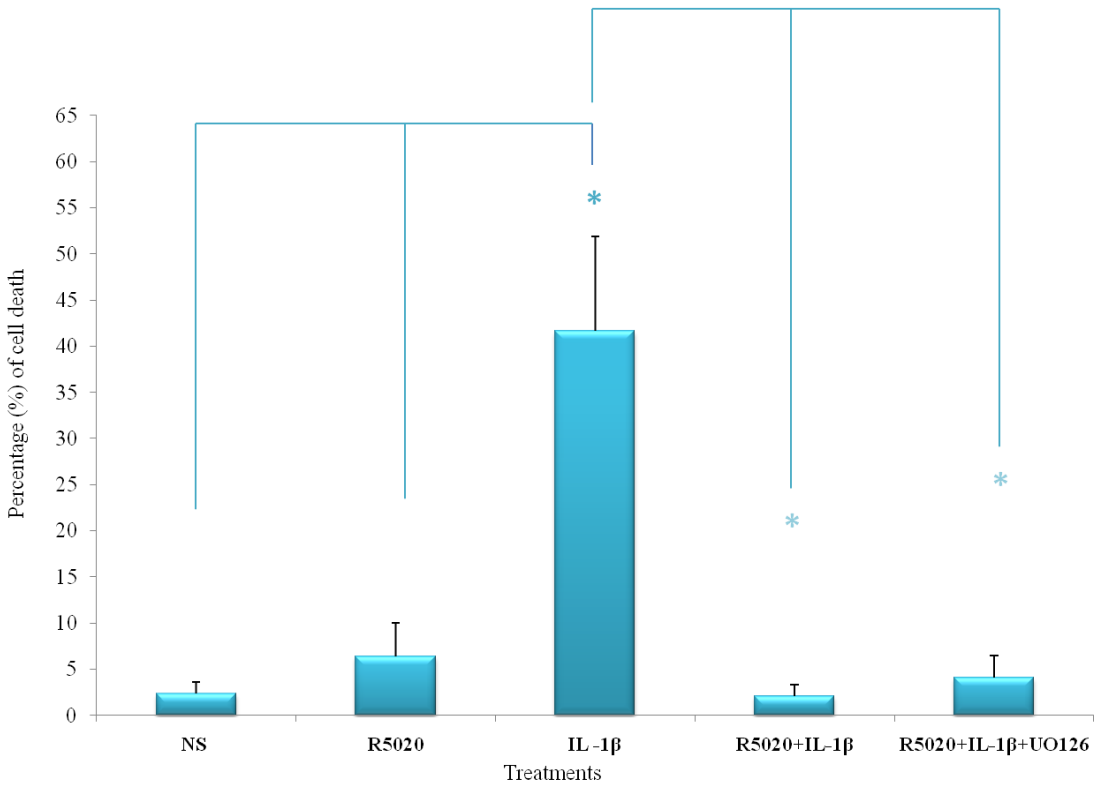


Figure 3.59: Percentage of dead JEG-3 cells after treating cells with R5020, R5020 + IL-1β (10ng/µl) and pre-incubating cells with UO126 and combination treatment with IL-1β (10ng/µl) + R5020 following a 24hr incubtation. Each histogram represents the mean ± SEM of three individual experiments ($p < 0.05$*). Blue lines indicate significance of the increase in percentage of dead cells after treating cells with IL-1β compared to NS and R5020. Significant decrease obtained after pre-incubating cells with UO126 and treating cells with R5020 + IL-1β, and by treating cell with R5020 + IL-1β compared to IL-1β only treated cells.

3.9.1. Effect of IL-1β combinational treatments on the expression of membrane Progesterone Receptors (mPRs and PGRMC-1).

As the objective was to examine the functionality of the membrane progesterone receptors in our placental cell lines, BeWo CT and JEG-3 cells were treated with IL-1β (10ng/μl), P4 (100nM), OD 02-0 (100nM) and combination treatments of IL-1β (10ng/μl) + P4 (100nM) and IL-1β (10ng/μl) + OD 02-0 (100nM) for 24 hours. The expression of membrane progesterone receptors (mPRs and PGRMC-1) was assessed by Q-PCR analysis to establish the relative quantity values (RQ).

The expression of mPRα in both cell lines following the 24 hour treatments was obtained and the RQ values are depicted in Fig.3.60. In BeWo CT cells the expression of mPRα was significantly down-regulated 6.8 fold after treating the cells with P4 in the presence of IL-1β. Even though an altered expression in comparison to non-treated cells was seen for mPRα in BeWo CT cells following different treatments, the results were not significant. After treating JEG-3 cells with IL-1β the expression of mPRα was detected to be significantly 7.1 fold down regulated compared to NS (Fig. 3.60). Also significant down-regulation of this gene was detected following the next set of treatments. With P4 a 5.2 fold decrease was detected, with OD 02-0 a 7 fold decrease, P4 and IL-1β a 9.9 fold decrease and OD 02-0 and IL-1β a 5.5 decrease in mPRα gene expression compared to NS.

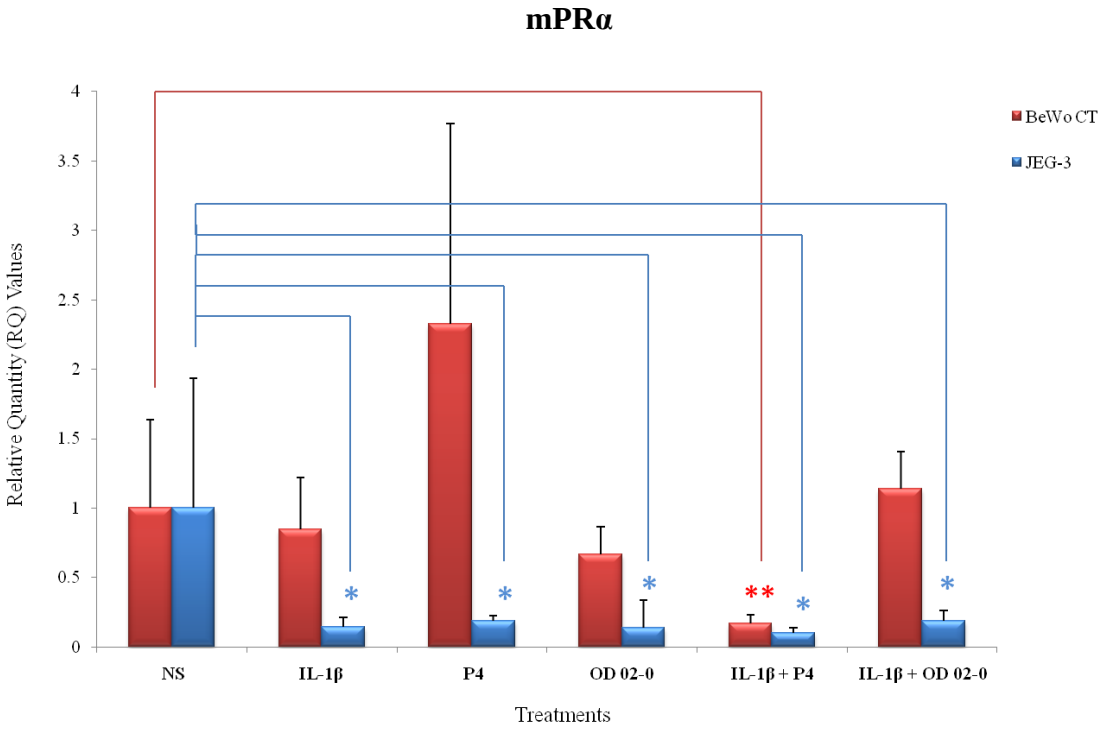


Figure 3.60: RQ values of the expression of mPRα in BeWo and JEG-3 cells after treating with IL-1β, P4, OD 02-0, IL-1β + P4, IL-1β + OD 02-0. Each histogram represents the mean ± $[2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments. Statistical significant differences are shown by blue and red lines and were determined using t-test ($p < 0.05$*, $p < 0.001$**). Red line indicates significant decrease in the expression of mPRα in BeWo CT cells treated with IL-1β + P4 compared to the NS. Blue lines indicates significant decrease in the expression of mPRα in JEG-3 cells treated with IL-1β, P4, OD 02-0, IL-1β + P4, IL-1β + OD 02-0 compared to the NS.

The expression levels of mPRβ were evaluated in BeWo CT and JEG-3 cells after treating the cells with the compounds stated above. The RQ values are indicated in the graph in Fig.3.61. In BeWo CTcells, the expression of mPRβ was significanlty 7.1 fold down-regulated after treating cells with P4, it was 15.4 fold downregulated when cells where treated with P4 in the presence of IL-1β compared to the expression in the non-treated cells. Treating BeWo CT cells with OD 02-0 in the presence of IL-1β resulted in a 3 fold increase in the expression levels of mPRβ over the expression levels detected in NS (Fig. 3.61).

When JEG-3 cells were treated with IL-1β a significant 7.1 fold decrease in the expression of mPRβ was detected compared to the expression in the non-treated cells. Treatment of JEG-3 cells with P4 in the presence of IL-1β resulted in a significant 8.6 increase in the expression levels of this gene compared to NS, whereas co-treatment with OD 02-0 in the presence of IL-1β indicated a significant 8.7 increase in the expression levels of mPRβ compared to the non treated cells (Fig. 3.61).

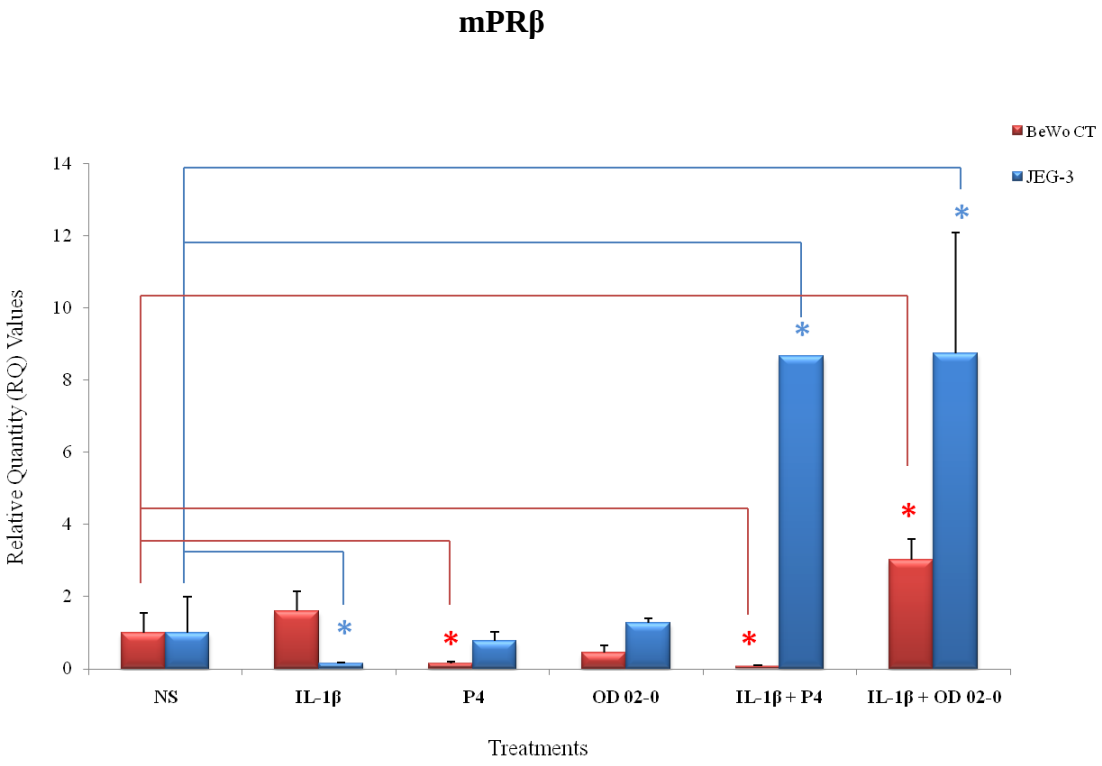


Figure 3.61: RQ values of the expression of mPRβ in BeWo and JEG-3 cells after treating with IL-1β, P4, O2, IL-1β + P4, IL-1β + O2. Each histogram represents the ± $[2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments. Statistical significant differences are shown by blue and red lines and were determined using t-test ($p < 0.05$*). Red lines indicate significant changes in the expression of mPRβ in BeWo CT cells treated with P4, IL-1β + P4 and IL-1β + OD 02-0. Blue lines indicate significanct changes in the expression of mPRβ in JEG-3 cells treated with IL-1β, IL-1β + P4, IL-1β + OD 02-0.

After treating the trophoblast cell lines as previously the expression of mPRγ was calculated following Q-PCR analysis of the samples. The results are indicated below in Fig.3.62. Co-

treatment with P4 with IL-1β decreased significantly by 9.1 fold the expression of mPRγ in BeWo CT cells when compared to the non-treated samples. In JEG-3 cells the expression of mPRγ was 11 fold down-regulated following treatment with IL-1β.

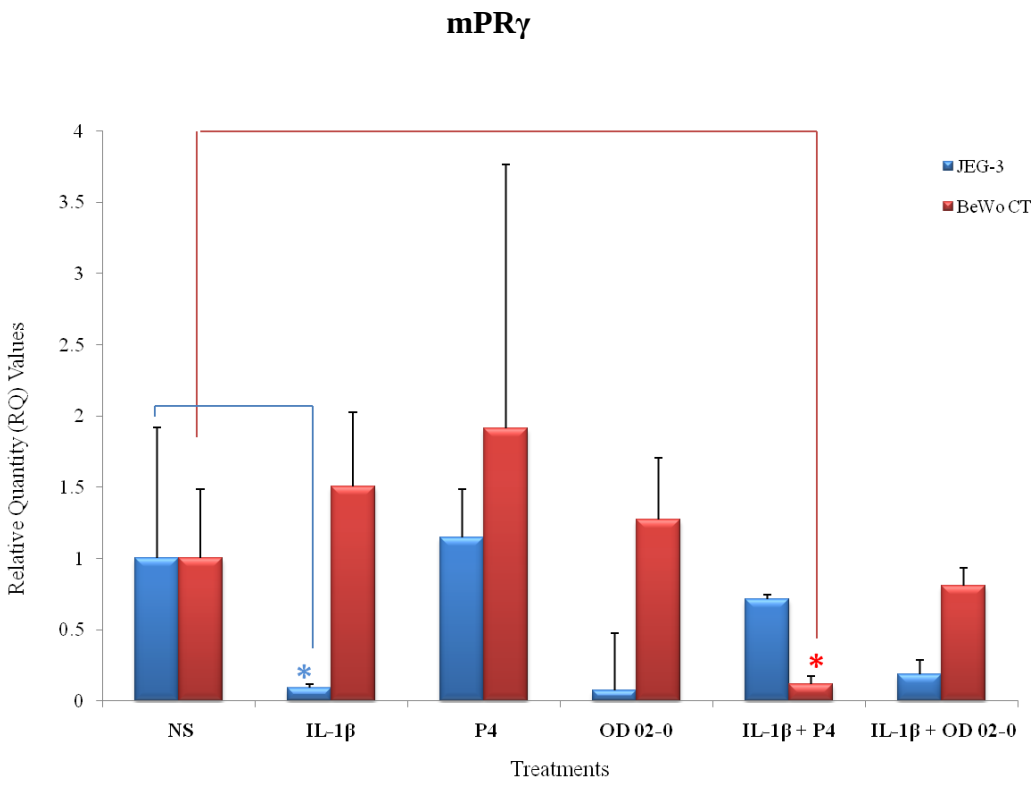


Figure 3.62: RQ values of the expression of mPRγ in BeWo and JEG-3 cells after treating with IL-1β, P4, OD 02-0, IL-1β + P4, IL-1β + OD 02-0. Each histogram represents the mean ± $[2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments. Statistical significant differences are shown by blue and red lines and were determined using t-test ($p < 0.05$*). Red line indicates significanct changes in the expression of mPRγ in BeWo cells treated with IL-1β + P4. Blue line indicates significanct changes in the expression of mPRγ in JEG-3 cells treated with IL-1β.

Lastly, the expression of PGRMC-1 was examined in our placental cell lines that were treated with the same compounds as previously. The RQ values obtained are indicated in Fig.3.63. The expression of PGRMC-1 was significanly down-regulated by 10.1 fold as a result of

treating the BeWo CT cells with P4 in the presence of IL-1β compared to the NS. In JEG-3 cells significant increase was detected in the expression of this gene following treatment with P4, OD 02-0, and repeating the same treatments in the presence of IL-1β. The fold increase over NS respectively was 6.5, 7.6, 4.4 and 6.4.

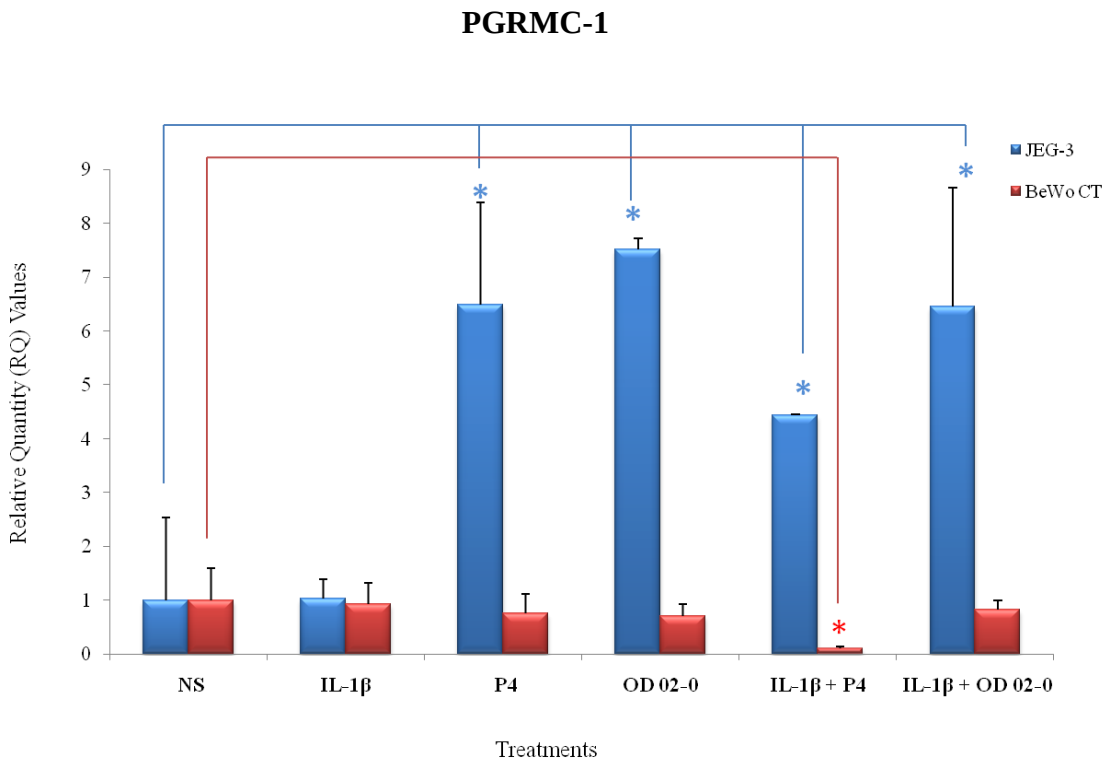


Figure 3.63: RQ values of the expression of PGRMC-1 in BeWo and JEG-3 cells after treating with IL-1β, P4, O2, IL-1β + P4, IL-1β + O2. Each histogram represents the mean ± [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments. Statistical significant differences are shown by blue and red lines and were determined using t-test ($p < 0.05$*). Red line indicates significance in the expression of PGRMC-1 in BeWo cells treated with IL-1β + P4. Blue lines indicate significant changes in the expression of PGRMC-1 in JEG-3 cells treated with P4, OD 02-0, IL-1β+P4, IL-1β + OD 02-0.

3.10. Discussion

3.10.1. Effects of progestins on cell proliferation

IL-1β is a proinflammatory cytokine which at the early stages of pregnancy is produced and secreted by the maternal decidua (Romero *et al*., 1989). There have been studies suggesting a role for this cytokine during pregnancy. Specifically, this cytokine may potentially be an inducer of placental trophoblast invasion by stimulating the release of metalloproteinase (MMP-9) by human placental cytotrophoblast (Librach *et al*., 1994). Also it has been associated with trophoblast cell differentiation by increasing the production of hCG hormone in isolated first trimester villous trophoblast (Yagel *et al*., 1989) as well as in JEG-3 human choriocarcinoma cell line (Seki *et al.*, 1997). The action of this cytokine on the stimulation of proliferation of human first trimester extravillous trophoblast cell lines (Nilkaeo *et al*., 2000[A]) was shown by inducing growth factors such as IL-6 and leukemia inhibitory factor (LIF) (Nilkaeo *et al*., 2000[B]; Nilkaeo *et al*., 2000[C]).

Cytokines participate in complex networks and under balanced production and function, a successful human pregnancy can be achieved (Orsi, 2008). hCG - induced release by IL-1β have been shown in first trimester placentae indicating a direct role of IL-1β in pre-eclampsia (Masuhiro *et al*., 1991), as increased concentrations of hCG have been associated with this pahtophysiology (Hsu *et al*., 1994). Recently, a study has shown that in women with pre-eclapmpsia significantly increased levels of IL-1β and hCG were found compared to controls individuals (Kalinderis *et al*., 2011)

IL-1β mediated apoptosis in placental cells has been shown in a recent study (Nilkaeo & Bhuvanath, 2006); also the involvement of this cytokine in pregnancy pathophysiologies such as pre-eclampsia (Wenstrom *et al*., 1994) and preterm labour (Romero *et al*., 2005) has been documented. Apoptosis has been induced in cytotrophoblast and syncytiotrophoblast in

culture by addition of TNF-α and IFN-γ cytokines and the apoptotic action of these cytokines has been rescued by EGF (Garcia-Lloret *et al*., 1996). Further research indicated that additional "survival" factors existed able to inhibit these apoptotic actions. Growth factors, such as bFGF, IGF-1, and PDGF-AA were shown to significantly inhibit the TNF-α and IFN-γ mediated apoptosis in placental cells (Smith *et al*., 2002). Others have shown anti-apoptotic effects of the steroid hormone progesterone in granulosa cells of the ovary, where this effect was PR-specific and was reversed when RU486, an antiprogestin was used (Peluso & Pappalardo, 1998). Also progesterone - induced suppression of apoptosis was recently demonstrated in the rat mammary gland during lactation (Berg *et al*., 2002).

As evidence exist of progesterone mediated anti-apoptotic effects in organs of the female reproductive system (Berg *et al*., 2002; Peluso & Pappalardo, 1998), we tested the hypothesis that progesterone may have anti apoptotic effect on placental cells reversing the adverse effect of treatment with the proinflammatory cytokine IL-1β.

Our results show increased percentage of BeWo CT cell death upon 24 hour treatment with IL-1β compared to the non-treated cells. These findings corroborate a previous study performed on JAR choriocarcinoma cells where a dosage of IL-1β induced a 1.8 fold increase in cell death (Nilkaeo & Bhuvanath, 2006).

In accordance with previous studies (Berg *et al*., 2002), our results illustrate that progesterone reduces the percentage of BeWo CT dead cells compared to the basal levels at different dosages over a period of 24 hours, a result not seen when using a selective mPRα agonist. These findings indicate a level of complexity of the involved progesterone receptors, where synergistic actions between all different PRs promote a rescuing effect.

Co-incubation of BeWo CT cells with IL-1β and different dosages of progesterone or OD 02-0, an mPRα selective agonist rescued cells from death. A more sensitive response is seen when

treating cells with OD 02-0, as being a selective agonist the rescuing effect is detected with a lower dose than progesterone. Treating cells with OD 02-0, inhibition of cell death was firstly observed at 10nM, and subsequently at 100nM and 1000nM whereas with progesterone the inhibition of cell death was obvious at 100nM and 1000nM.

As the signalling capacity of nuclear PR can be compromised due to overexpression of the chaperone protein hsp 70 and hsp 90 in BeWo cells keeping the receptor in an inactive state, this may explain the "inability" to dissociate and act upon co-treatment with the cytokine and the nuclear progesterone receptor agonist, R5020.This observation is further strengthened as the percentage of cell death was found to be the same when treating the cells with R5020 as with the non-treated cells. Pre-incubating cells with a MEK inhibitor, results in an increased percentage of cell death which is higher than the percentage recorded when treating cells with IL-1β alone. This may be an indicator of the involvement of the MAPK signalling pathway in the rescuing effect upon activation of the nuclear PR, where "switching off" this pathway renders cells incapable of reversing the effect when the R5020 mediated responses are blocked. In contrast, JEG-3 cells that do not overexpress these chaperone proteins, the phenomenon of cell death is rescued upon co-incubation of the cytokine with R5020.

Compared to the BeWo cells the JEG-3 cells upon treatment with the cytokine showed a higher percentage of dead cells. This may be the result of an upreglation of interleukin 1 receptor, which according to an extensive microaaray analysis in both cell lines, was shown to be upregulated in the JEG-3 cells accounting for the sensitivity shown to this cytokine (Burleigh *et al*., 2007). Upon co-treatment of JEG-3 cells with IL-1β and progesterone a reduction in the percentage of cell death is seen in comparison to the cytokine only treated cells. A dramatic reduction is seen when 1000nM of P4 was used. It should be taken into consideration the fact that since treatment was for 24 hours it is plausible that P4 could activate multiple PRs in parallel. Beyond the anti apoptotic effects of progesterone, OD 02-0

was shown to be able to mediate inhibitory effects as well. Blockage of MEK activity prior to treating the cells with progestins and IL-1β was shown to rescue cells from the effect of the cytokine. Whereas this rescuing effect is exacerbated when the MEK activity is not blocked thus indicating a partial block of the MEK activity was achieved. The rescuing effect seen upon treatment with progestins is assumed to be via all receptors as no further distinction can be made. Research has shown that MAPK signalling pathway mediates the increase proliferation and survival of the extravillous trophoblast cells (Nilkaeo *et al*., 2001).

The complex nature of progesterone's actions are further corroborated by recent studies that show P4 induced inhibition of cisplatin-mediated apoptosis in ovarian cancer cells both *in vitro* and *in vivo* (Wehling & Losel, 2006). The ability of progesterone to either induce or inhibit apoptosis has been a reason why ovarian cancer clinical trials have failed to present a beneficial effect of progesterone on the progression of this cancer (Cahill, 2007). The anti-apoptotic effects of progesterone cannot be attributed to be solely mediated by PRs, as other putative mediators of the action of progesterone have been shown to be expressed, such as the mPRs (Zhu *et al*., 2003[B]) and PGRMC-1 (Meyer *et al*., 1996).

It is evident from our study that a PR synergy could be in place leading to the "rescuing" effect observed. A ligand dependent PR-B transactivation by mPRs has been described previously in the human myometrium (Karteris *et al*., 2006), a scenario that cannot be ruled out in this study where progesterone or OD 02-0 activation of mPR signalling may potentiate the nuclear PR, thus having an inhibitory effect on cell apoptosis. Indeed, we speculate on the potential cross talk or complex formations leading to a synergistic effect between mPRs and PGRMC-1 enhancing the anti apoptotic effects, as PGRMC-1 has received attention as being an anti apoptotic mediator of progesterone. Evidence has been presented from a recent study strengthening the significance of PGRMC-1 as the receptor mediating the progesterone induced anti apoptotic effects in human granulosa/luteal cells of the ovary. When activated, it

potentially triggers the activation of signal transduction pathways that prevent the apoptosis of these cells (Engmann *et al*., 2006).

3.10.2. Hormonal regulation of membrane PRs

The expression of the progesterone receptor gene has been previously reported to be regulated by sex steroid hormones including progesterone in the rabbit hypothalamus (Camacho-Arroyo *et al*., 1996) and therefore we examined the effects of progesterone, a progestin compound and a cytokine on the expression of mPRs and PGRMC-1 transcripts in choriocarcinoma cell lines. The regulation of progesterone receptor genes forms a critical aspect of the responsiveness to progesterone hormone in the human placenta during gestation.

For example nPRs can be modulated at placental level. By treating the placental cells with prostaglandins, the expression of PR-B was down -regulated altering the ratio in the placenta in favour of the PR-A/PR-B ratio thus altering the pregnancy status (Ziyan *et al*., 2010). After the 37^{th} week which is the hallmark of normal human gestation period, the levels of circulating progesterone rise exponentially and this could have an affect on PR expression in an autocrine manner. These findings indicate that prostaglandins can regulate the expression of PRs in the human placenta and determining any responses of this organ during gestation.

The gene expression of mPRα, mPRβ, mPRγ and PGRMC-1 was significantly downregulated in BeWo cells compared to the non-treated cells upon co-incubation with the cytokine IL-1β and progesterone. The expression of mPRβ was significantly downregulated upon treatment with progesterone as well as co-incubation with IL-1β leading to the conclusion that progesterone has a negative effect on the expression of this gene. The expression of this gene was significantly up - regulated upon treatment with the progestin compound OD 02-0 in combination with IL-1β thus allowing us to conclude that a synergistic effect results in the upregulation of this receptor on BeWo cells. A plausible synergistic effect is also seen the

expression of mPRβ in JEG-3 cells upon con-incubation with progesterone and IL-1β as well as with OD 02-0 and IL-1β.

IL-1β significantly downregulated the expression of all mPRs but did not exert a significant effect on the expression of PGRMC-1 in JEG-3 cells. Nonetheless, a differential regulation of mPRα is observed upon treatment with progesterone or OD 02-0 and co-incubation of these progestagens with IL-1β resulted in significant down - regulation of this transcript in JEG-3 cells. Possibly, a negative effect is exerted by progestins on the regulation of the expression of this gene.

Our results lead to the conclusions that the progestin compounds when used alone or in combination with the cytokine lead to differential regulation of the progesterone receptor genes. Our findings are in agreement with earlier studies that have shown differential expression of the mPR genes in the endometrium during the different stages of the menstrual cycle where the levels of progesterone vary (Fernandes *et al*., 2005). Expression of PGRMC-1 gene has also been shown to be regulated by endocrine factors. Dynamic patterns of expression were seen under the physiological conditions such as the estrous cycle and pregnancy and expression of this gene was shown to be upregulated by P4 in an ovariectomised mouse mode (Zhang *et al*., 2008).

4

CHAPTER 4

4. GENERAL DISCUSSION

4.1. Concluding remarks

Preterm labour (PTL) and in particularly before the 32nd weeks of gestation, is associated with high fetal morbidity and mortality and thus is the subject of intense research with the ultimate goal to devise preventative or therapeutic strategies. Approximately two-thirds of preterm deliveries are due to spontaneous onset of preterm labour or to preterm premature rupture of membranes (Tattersall *et al*., 2008).

The interventions to improve the prognosis of preterm labour are very few (Farine *et al*., 2008). Many years ago, Csapo et al., was the first to promote the progesterone see-saw theory, stating that high progesterone levels are able to prevent contractions of the uterine muscles whereas low levels facilitate such contractions (Caspo, 1956). It emerges that the hormonal control of the contractions and labour in humans is more complex in comparison to other animals and that the role of progesterone may indeed be more limited than in animal models (Norwitz *et al*., 1999). Many studies have examined the use of progesterone for prevention of preterm labour. Mackenzie et al reported that from 735 such existing studies only three were found appropriate for inclusion in their meta-analysis on preterm therapy during the second trimester, which had showed that the use of progestins by women at risk for preterm labour reduced its occurrence by 43% (Mackenzie *et al*., 2006).

At term, the human placenta produces about 250mg of progesterone per day and in order to exert its effects it must bind and activate progesterone receptors (Tuckey, 2005). In this study we have mapped in detail the expression of all known progesterone receptors. We have shown that the canonical nuclear PR-B is barely expressed in the human placenta both at term and preterm. However, a multitude of alternative progesterone receptors appear to be expressed, including mPRα, mPRβ, mPRγ and PGRMC-1. Of those, mPRβ and mPRγ were upregulated in preterm placentas with the latter reaching significance. We then assessed the relative abundance of mPRs transcripts relative to mPRα. The ratio of mPRα/mPRβ was upregulated in the non labour group when compared to the labour cohort and again uperegulated in the term category when compared to the preterm. Similar results were found in the ratio of mPRα/mPRγ whereas the ratio of mPRα/PGRMC-1 was upregulated in the non labour category and the preterm category compared to labour and term respectively.

In addition, downregulation of the ratio of mPRα/PR-B in preterm samples when compared to term was also evident. However, given that the expression of PR-B was remarkably low across all samples tested, that makes its role in mediation of placental progesterone responses questionable.

This is the first study that mapped in detail all three classes of progesterone receptors in term and preterm placental tissues. Previous studies have shown changes in the expression of mPRs in other intrauterine tissues related to gestational age. Fernandes et al have shown that myometrial mPRα but not mPRβ levels also declined upon preterm labour, although the differences were not statistically significant. In contrast, term or preterm was not associated with statistically significant changes in mPRγ in the human myometrium (Fernandes *et al*., 2005). In a recent study, PGRMC-1 had a lower expression in myometrium collected from women at term either not in labour or in labour compared with tissues from women in preterm non-labour (Wu *et al*., 2011). In terms of their localisation mPRα was localised

around syncytiotrophoblasts with a cytoplasmic localization, whereas mPRβ was expressed both in the cytoplasm as well as on the plasma membrane *(*also around syncytiotrophoblasts), and mPRγ was expressed in both syncytiotrophoblasts and cytotrophoblasts. The expression was stronger around syncytiotrophoblasts, where mPRγ appeared to be expressed mainly on the plasma membrane. This is in agreement with a study by Fernandes et al., demonstrating that mPRα expression is restricted to the syncytiotrophoblast at the periphery of term chorionic villi (Fernandes *et al*., 2005). With regards to PGRMC-1, it has been previously shown that it is expressed in smooth muscle of the placental vasculature, villous capillaries and the syncytiotrophoblast (Zhang *et al*., 2008). However, regulation of mPRs at the cell surface is still unclear, since there is still a debate about the cellular distribution of these receptors (Krietsch *et al*., 2006). Membrane localisation of mPRs has been demonstrated by several research groups in a variety of other cell types (Tubbs & Thomas, 2009; Dosiou *et al*., 2008; Josefsberg *et al*., 2007; Nutu *et al*., 2007; Thomas *et al*., 2007; Hanna *et al*., 2006; Zhu *et al*., 2003). Other researchers have reported that mPRs are localized intracellularlly in the endoplasmic reticulum (Lemale *et al*., 2008; Ashley *et al*., 2006; Krietsch *et al*., 2006). An answer to these discrepancies could be the trafficking of these receptors. An important characteristic of 7TMRs is that they are rapidly internalised upon activation in a ligand-dependent manner, an event critical for their proper functioning (Moore *et al*., 2007). Data from our laboratory revealed that mPRα can undergo internalisation upon exposure to high levels of progesterone in the human myometrium (Foster *et al*., 2010). It is possible therefore, that in an adjacent intrauterine tissue like the placenta, progesterone acts in an autocrine manner to drive mPR internalisation, thus leading to their cytoplasmic appearance. Moreover, treatment of JEG-3 cells with P4 led to a significant decrease of mPRα and mPRβ, a phenomenon indicative of homologous desensitisation. Downregulation of a 7TMR at mRNA level is another feature of agonist-promoted regulation that takes place during

homologous desensitisation. For example, in human myometrial cells treated with oxytocin, a decrease in oxytocin receptor mRNA expression was accompanied with receptor desensitisation (Phaneuf *et al*., 1998), and as a result subsequent internalisation like the GLP-1 receptor (Widmann *et al*., 1997). Therefore, internalisation provides another explanation of why several 7TMRs are found in the cytoplasm (Thompson *et al*., 2005) and may also explain why some investigators have observed mPRs intracellularly.

In kidney tissue mPRα was found to be restricted to the endoplasmic reticulum (ER) due to a C-terminal ER retention motif (Lemale *et al*., 2008). This is not unusual for 7TMRs, since many require accessory proteins to ensure transport to the plasma membrane (Cooray *et al.*, 2008[A]; Cooray *et al*., 2008[B]). An example is the melanocortin 2 receptor, which is localised within the ER and unable to be transported to the plasma membrane unless it is co-expressed with the melanocortin 2 receptor accessory protein (Cooray *et al.*, 2008[A]). It is possible therefore that mPRα might require an accessory protein that is expressed in tissue- or cell-specific manner, thus affecting its transport to the membrane. A recent study has suggested such role for PGRMC-1 that can affect epidermal growth factor receptor (EGFR) signalling, by binding EGFR and stabilizing plasma membrane pools of the receptor (Ahmed *et al*., 2010). Interestingly, data from our placental tissues points towards a consistent (i.e. irrespective of any pathologies) and significant positive correlation between mPRα and PGRMC-1. These strong correlations were not influenced by gestational age or labouring status. Moreover, we moved to an *in silico* analysis using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING 8) which revealed a potential complex formation of those two receptors. It is attractive to speculate that PGRMC-1 can act as a potential accessory protein and –like EGFR- stabilizes mPRα at the plasma membrane by masking its ER retention motif. This might also explain the controversy that surrounds the cellular localization of mPRs.

There is also a 1.6 fold increase in the mPRα/PGRMC-1 ratio in preterm placentas when compared with the term ones. This might result in a compromised function of mPRα in these tissues as fewer receptors will be directed to the plasma membrane. However, there is a higher order of complexity in preterm labour and other cytokines/hormones acting in an autocrine or paracrine manner might also influence progesterone-mediated signalling. One of these cytokines –that shows certain cross-talk with progesterone- is interleukin-1β (IL-1β). It has been suggested that IL-1β could be a possible predictor of preterm delivery since its levels in amniotic fluid are elevated in mid-trimester and positively associated with preterm birth (Puchner *et al*., 2011) as well as microbial invasion of the amniotic cavity (Marconi *et al*., 2011). Dubicke et al have also shown that the actual mRNA levels of IL-1β are elevated in the human cervix during preterm labour (Dubicke *et al*., 2010). However, we have chosen not to investigate the expression of IL-1β receptor in our clinical samples, as it has already been documented that protein expression of placental IL-1β receptor was neither dependent on term or preterm labour, nor on the presence of intrauterine infection (Steinborn et al., 1998).

In this study we also provide a novel insight of the role of IL-1β and progesterone within the placental microenvironment. In both *in vitro* models tested (BeWo and JEG-3) IL-1β appears to increase cell death, whereas progesterone has an opposite "rescuing" effect. We have dissected this response further using specific agonists for mPRs as well as the nuclear PR. When both cell lines were treated with OD 02-0 (a specific mPR agonist) a similar inhibition of IL-1β's effects was noted as that of progesterone, indicating that there is involvement of mPRs in the rescuing from cell death. When both cell lines were treated with the nuclear progesterone receptor agonist, R5020 at a concentration that will only activate the nuclear PRs, there was no apparent effect on BeWo cells whereas in JEG-3 it completely abolished the IL-1β's effects. These data indicate a certain amount of synergy amongst multiple PRs at

placental level. This is not surprising as previous findings point towards a potential transactivation of the nuclear PR by mPRα and mPRβ. In human myometrial cells activation of mPRs via coupling to Gi α-subunits serves to potentiate hormone-activated nuclear PR-B (Karteris *et al*., 2006). The expression of the nuclear progesterone receptor (PR)-responsive genes is decreased in the uterus of the primate at term , suggesting that functional progestin withdrawal involves the repression of the PR transcriptional activity. One potential intermediary in this withdrawal is the truncated isoform of PR, PR-A, which is up-regulated in laboring myometria and can function as a dominant negative to repress the transcriptional activity of PR-B (Karteris *et al*., 2006).

However, cautiousness should be exercised extrapolating *in vitro* findings as it is evident, R5020 only exerted a significant response in JEG-3 but not in BeWo cells were as both cell lines are of a choriocarcinoma origin a similar response would be anticipated. As previously discussed (chapter 3.6) this could be due to inherent differences in the expression of certain components in these immortalised cell lines (Burleigh *et al*., 2007).

Given the protective effects of progestins in these placental models, we dissected this response further by assessing the capacity of the compounds to alter the phosphorylation status of $ERK_{1/2.}$ We have carefully chosen this particular MAPK, since $ERK_{1/2}$ appears to play a key role in promoting cytotrophoblast cell differentiation and proliferation. Moreover, activation of mPRs can also rapidly phosphorylate $ERK_{1/2}$ *in vitro*. This has been demonstrated by several studies where mPRα and mPRβ were stably expressed in the nuclear progesterone receptor – deficient mammalian breast cancer cells MDA-MB-23, following progestin treatment a significant activation of MAPK was detected within 5 minutes (Thomas *et al*., 2002; Zhu *et al*., 2003; Hanna *et al*., 2006). A recent report has also documented the activation of $ERK_{1/2}$ upon progestin treatment in the granulosa and theca cells isolated from the ovaries of the Atlantic croaker (Dressing *et al*., 2010). Moreover, R5020 has been shown

to exert a similar effect in T47D breast cancer cells expressing PR-B (Kariagina *et al*., 2010). Of interest, progesterone as well as R5020 also altered the phosphorylation status of this MAPK in our cellular preparations as previously discussed. Pre-treatment with the selective MEK1/2 inhibitor U0126 inhibited substantially the cell rescuing effect of progesterone in both cell lines. In contrast, U0126 significantly reversed the effects of OD 02-0 and R5020 in JEG-3 cells. This dichotomy appears to be consistent with our previous observations, where a more pronounced effect was evident in JEG-3 cells. Collectively, these data point towards an involvement of a MAPK pathway following activation of multiple activated progesterone receptors. This is not the first time that interplays between IL-1β and progesterone have been documented. In breast cancer cells, progesterone appears to inhibit IL-1β effects (Hardy *et al*., 2006). Subsequent studies from the same group indicated that PR plays an important anti-inflammatory role in breast cancer cells via ligand-dependent and ligand-independent mechanisms (Hardy *et al*., 2008). Moreover, in poorly differentiated endometrial cancer cells, treatment of progesterone downregulated gene expression of IL-1β (Davies *et al*., 2004). Therefore, we also investigated the combined effects of progesterone and IL-1β in an attempt to mimic the hormonal-cytokine cross-talk in a tissue microenvironment. When BeWo cells were treated with progesterone and IL-1β, all mPRs and PGRMC-1 were downregulated. However, this effect did not reach significance. In JEG-3 cells the combinatorial treatment downregulated mPRα and had no apparent effect on mPRγ but surprisingly, it induced an upregulation of mPRβ and PGRMC-1.

Over the past 20 years many research groups have shown that steroids also act at the cell surface of many target tissues and cell types to initiate rapid responses, via binding to membrane receptors (Falkenstein *et al.*, 2000). However, the lack of comprehensive information on the identity and molecular structure of any membrane steroid receptor has prevented understanding of critical molecular aspects of steroid actions via this alternative

mechanism and a more widespread appreciation of its significance (Watson, 2003). The major scientific contribution from this study was the detailed mapping of progesterone receptors in clinical samples (term/preterm and labour/non labour), the elucidation of their cellular distribution and their potential cross-talk with the inflammatory cytokine IL-1β. The functionality of the various PRs was assessed by using specific ligands for the nuclear and membrane PRs. Collectively, our results provide a first insight into a complex regulation of PRs in the human placenta. The PR-induced rescuing effect from cell death could provide a platform for future therapeutic interventions.

4.2. Study limitations

We would also like to acknowledge that our study has a number of limitations. The clinical samples come from a relatively homogeneous population of the Greek island of Crete. Indeed, it would have been interesting to examine the expression of mPRs in an expanded population sample that would provide a better insight and also uncover any mutations that may be related to ethnic backgrounds. However, the relative uniformity of our sample could also have certain advantages - we do not need to control for the effects of other factors/variables such as lifestyle, diet and ethnicity, as these may have been rather similar among participants. Another limitation of our studies was the access to clinical samples of the first and second trimester, due to ethical considerations. The placental samples could have been higher, providing a better insight into the expression patterns of these genes throughout pregnancy. Indeed, when we have used a placental synsytialisation model to represent the third trimester, significant changes in the expression of all mPRs were observed. As we have also demonstrated there are differences in the response of progesterone and IL-1β in the two *in vitro* models we have used. No direct conclusions can be drawn regarding activation of certain intracellular signalling pathways unless these studies are repeated in primary cell cultures or using placental explants as an *ex vivo* model. Moreover, the changes seen in the

clinical samples using quantitative PCR should be confirmed at protein level. We were also unable to study further protein expression and cellular distribution of mPRδ and mPRε due to lack of commercially-available antibodies. Finally the rescuing effect of progesterone –in terms of induced cell death- should be further evaluated.

4.3. Future studies

With regards to future studies, they should concentrate addressing some of the limitations of the current study as well as dissecting further the progesterone signalling at placental level.

1. **Which G proteins can the mPRs activate in the human placenta?** This is an important step, as emerging evidence suggest that mPRs can couple to numerous G-proteins (Karteris *et al.,* 2006). This coupling "promiscuity" of 7TMRs is a common phenomenon, usually dictated by tissue-specificity and disease state. This first objective will be studied using photoaffinity labelling in placental tissues acquired from normal and pathological complicated pregnancies. With the use of specific G-protein α-subunit antibodies (i.e. Gs, Gi, Gq and Go) and radiolabelled GTP-azidoanalide (GTP-AA), we will be able to gain a better insight if there is a switch in G protein coupling of mPRs in a disease state, i.e. IUGR, pre-eclampsia, or preterm labour.

2. **What are the subsequent downstream signalling pathways affected by mPR activation?**

We propose to use commercially-available radioimmunoassays in order to study the effects of progestins on second messenger systems such as cAMP, IP_3 and cGMP *in vitro*. Downstream effectors will also be studied in detail, by expanding on the repertoire of phosphorylation targets, including the remaining MAPKs p38, SAPK/JNK and other related targets such as

mTOR, Akt and S6K. We would also like to alter the ratios of mPRs *in vitro*, in an attempt to mimic the changes detected in clinical samples and subsequently assess their signalling capacity, by measuring changes in the phosphorylation status of the MAPK cascade.

Furthermore, as it is evident from this study, it appears that there is a potential interplay between mPR and activated nuclear PR. Therefore, we would like to study further the transactivation of PR-B by all three different mPRs in placental cell lines as previously described (Karteris *et al*., 2006) as well as investigate the progesterone-induced phosphorylation of PR in the absence of mPRs. We propose to dissect these responses using specific agonists, Pertussis toxin as inhibitor of Gi as well as specific siRNAs for all mPRs and PGRMC-1.

3. How is the progesterone "rescuing" effect mediated?

The progesterone effect on IL-1β induced cell death should be further investigated, as it the pathways of necrosis or apoptosis should be determined. Caspases are central regulators of apoptosis. Here we propose to use a wide repertoire of caspases: Cleaved Caspase-3 (Asp175), Cleaved Caspase-6 (Asp162), Cleaved Caspase-7 (Asp198), and Cleaved Caspase-9 (Asp315) to assess the effects of IL-1β in the presence of specific progestin ligands. We also propose to use the colorimetric TUNEL system so we can determine nuclear DNA fragmentation, an important biochemical indicator of apoptosis.

4. Is PGRMC-1 involved in trafficking of placental mPRs?

Internalisation and recycling of 7TMRs is a critical regulatory event for their signalling. The mechanism(s) by which mPR number is regulated on the cell surface is unclear. A key finding from the correlation studies was the highly significant correlation between mPRα and PGRMC-1 irrespective of pathologies. Given that PGRMC-1 can act as an adaptor protein we

would like to elucidate the role of PGMRC-1 on mPR trafficking *in vitro*. For this objective, two different placental cell lines can be co-transfected with PGMRC-1 and different mPRs. PGRMC-1 expression will be modulated using a Tet-On and Tet-Off system, and subsequent cellular distribution of mPRs will be determined using immunofluorescent analysis, ImageStream and TEM.

5. Should the clinical cohort number be increased?

Important conclusions arising from the clinical cohort we have used regarding differential expression of progesterone receptors in preterm labour for example. However, emerging studies emphasise that there is ethnic diversity in expression of certain genes, as well as certain polymorphisms. Our samples were obtained from a genetically homogeneous population, from the island of Crete. A larger clinical cohort involving tissues from other parts of Europe would provide a better insight not only regarding the expression at mRNA/protein level but also provide us with an opportunity to assess any correlations of PR and IL-1β polymorphisms with certain gestational pathologies.

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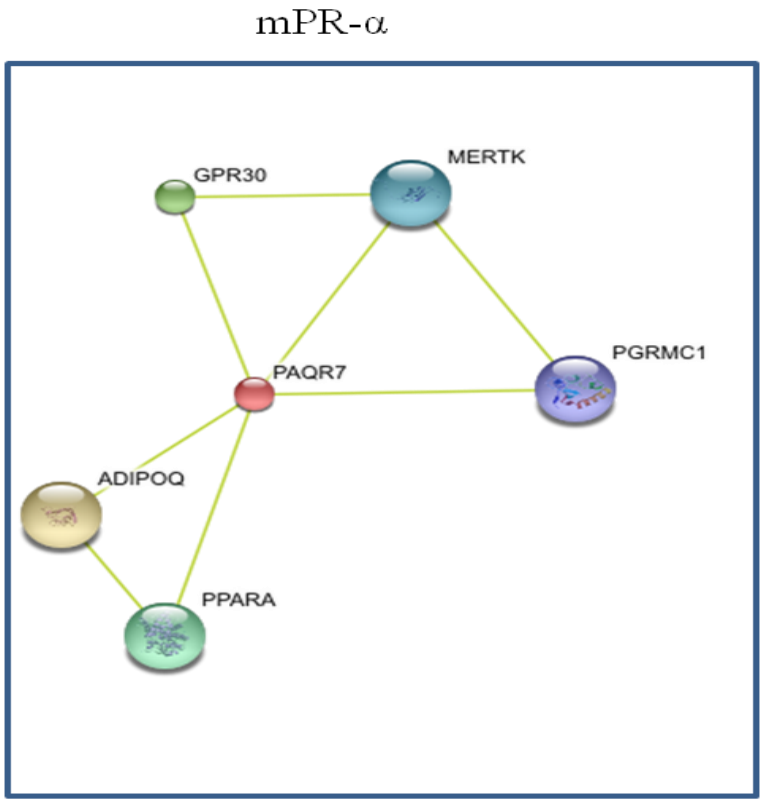
Appendix I

STRING for mPRα:



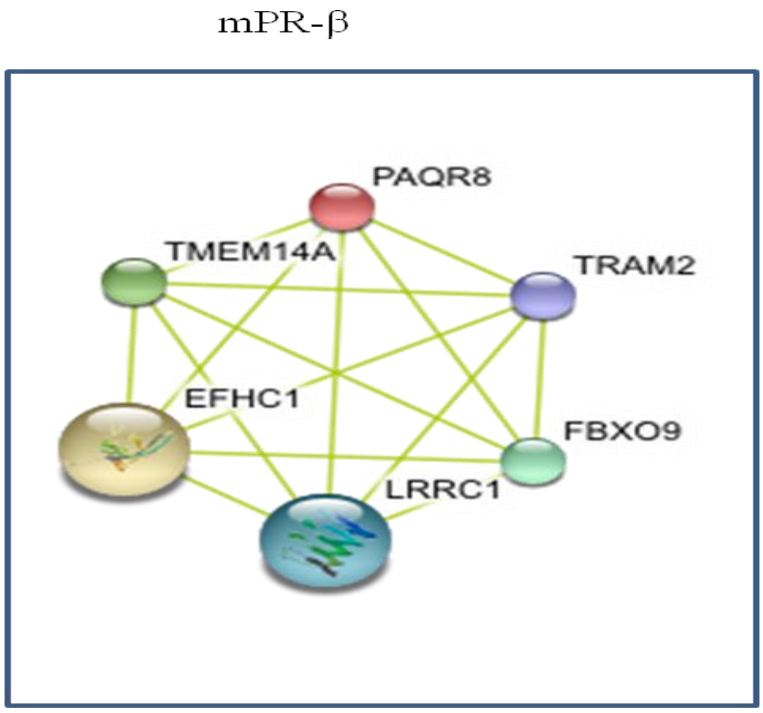
GPR30 : Adiponectin precursor (Adipocyte, C1q and collagen domain- containing protein)

PPARA: Chemokine receptor- like 2 (G-protein coupled receptor 30)

MERTK: Proto-oncogene tyrosine-protein kinase MER precursor

PGRMC-1: Membrane associated progesterone component 1- Receptor for progesterone

STRING for mPRβ:



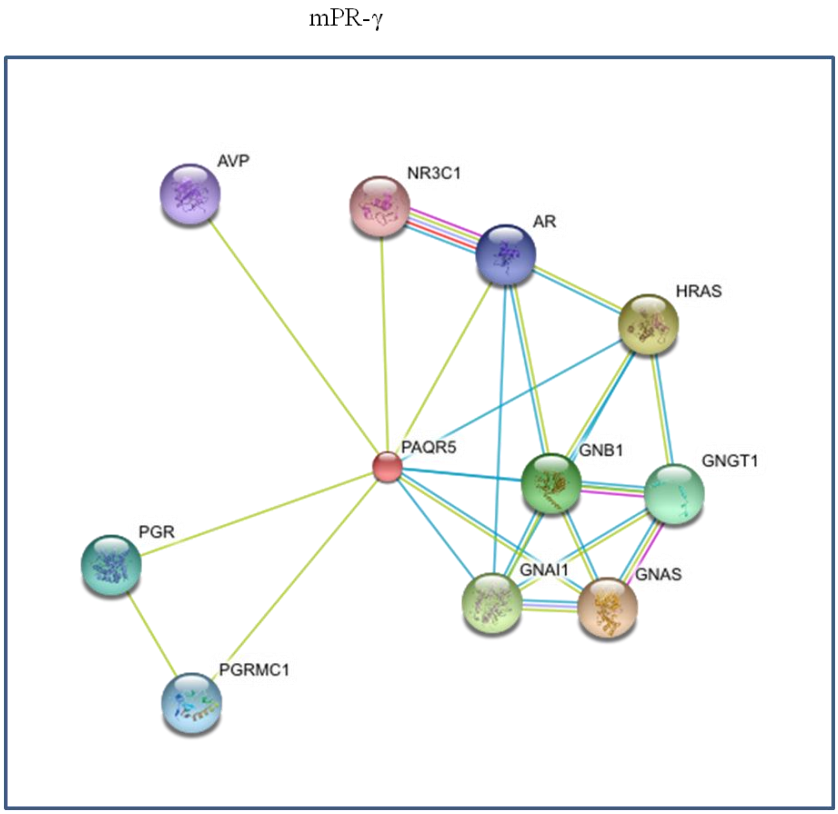
TMEM14A: EF- hand domain-containing protein 1

FBXO9: Transmembrane protein 14A

LRRC1: F-box only protein 9

TRAM2: Translocation- associated membrane protein 2

STRING for mPRγ:



HRAS: Neuroendocrine secretory protein 55

GNAI1: GTPase HRas precursor

GNB1: Guanine nucleotide- binding protein subunit beta 1

GNGT 1: Guanine nucleotide-binding protein gamma-T1 subunit precursor

PGR: Progesterone Receptor (PR)

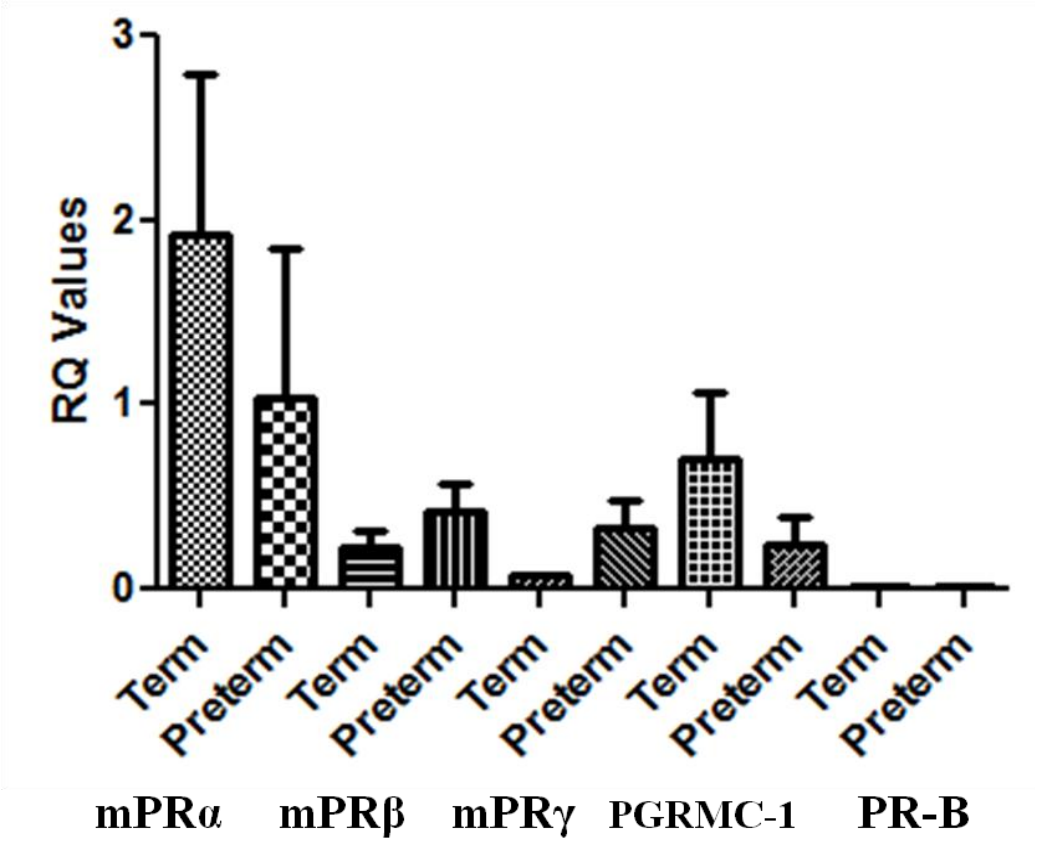
PGRMC-1: Membrane associated progesterone component 1- Receptor for progesterone

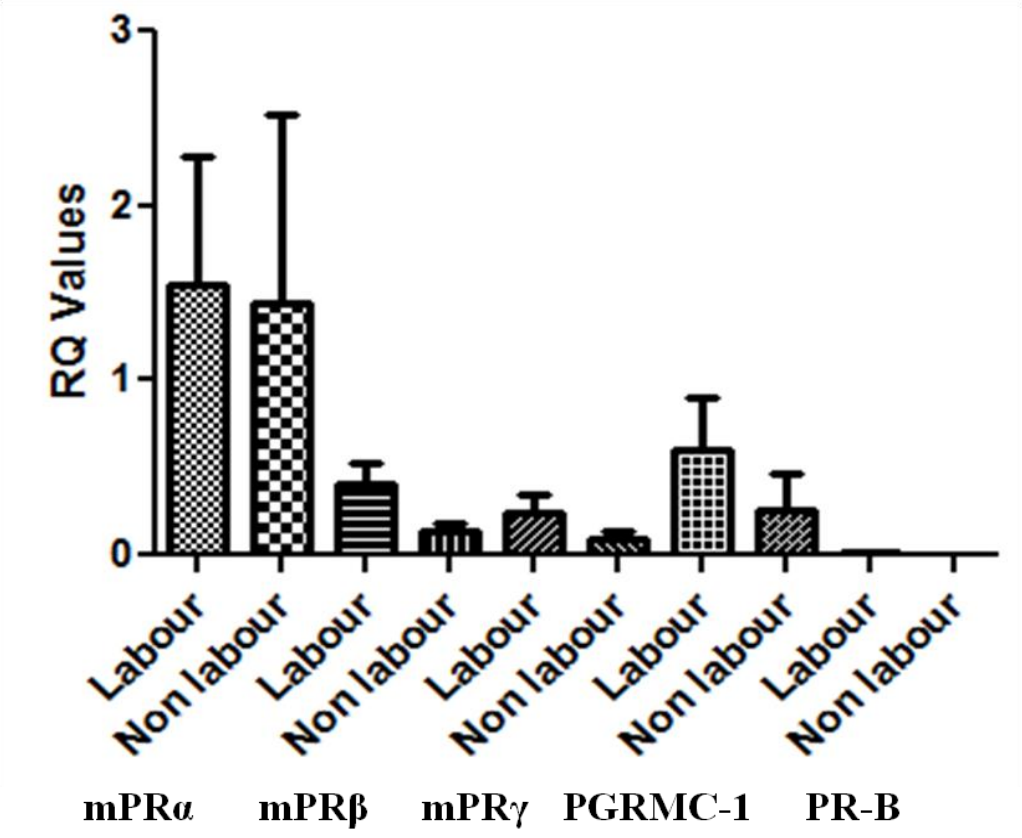
AR: Androgen Receptor

AVP: Vasopressin- neurophysin 2- copeptin precursor

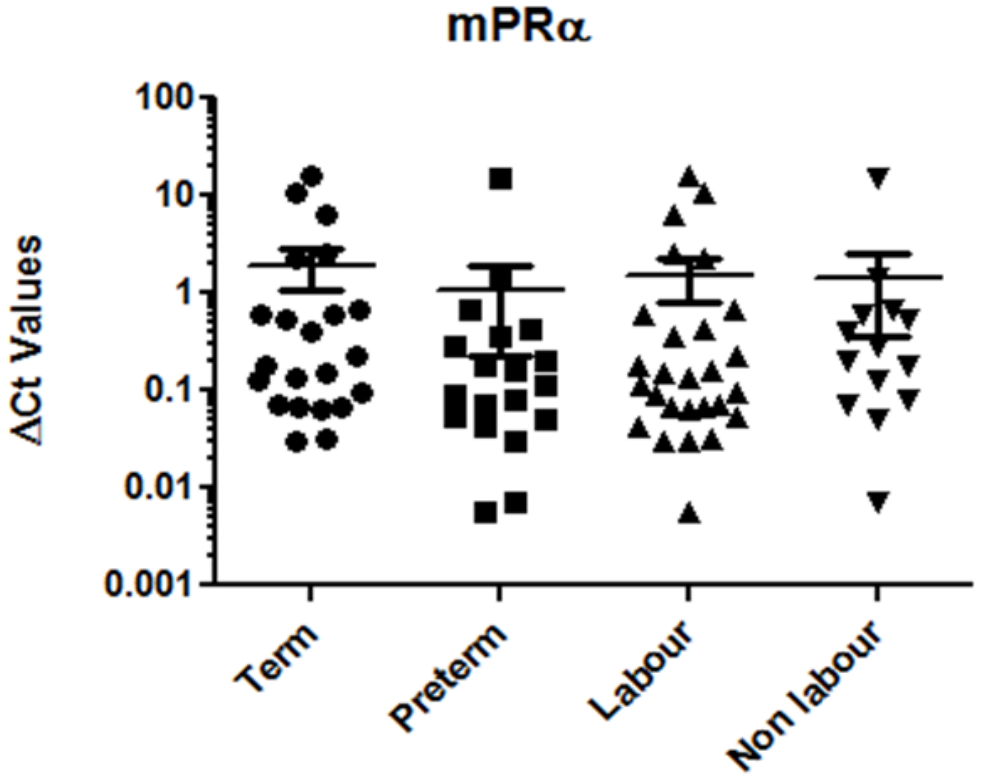
NR3C1: Glucocorticoid Receptor (GR)

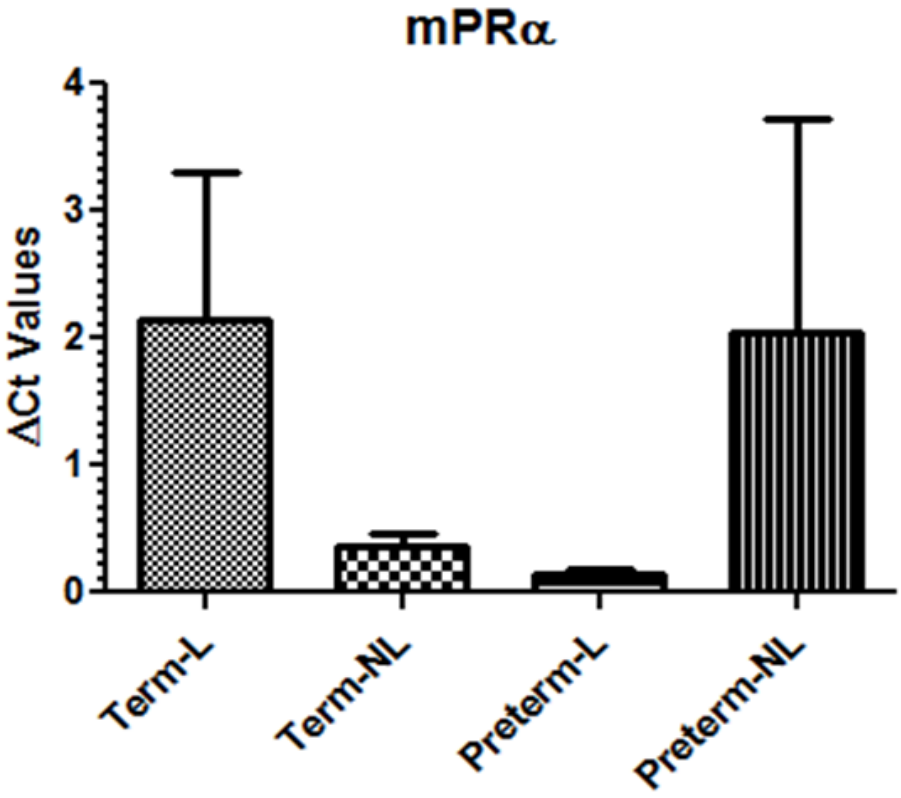
Appendix II

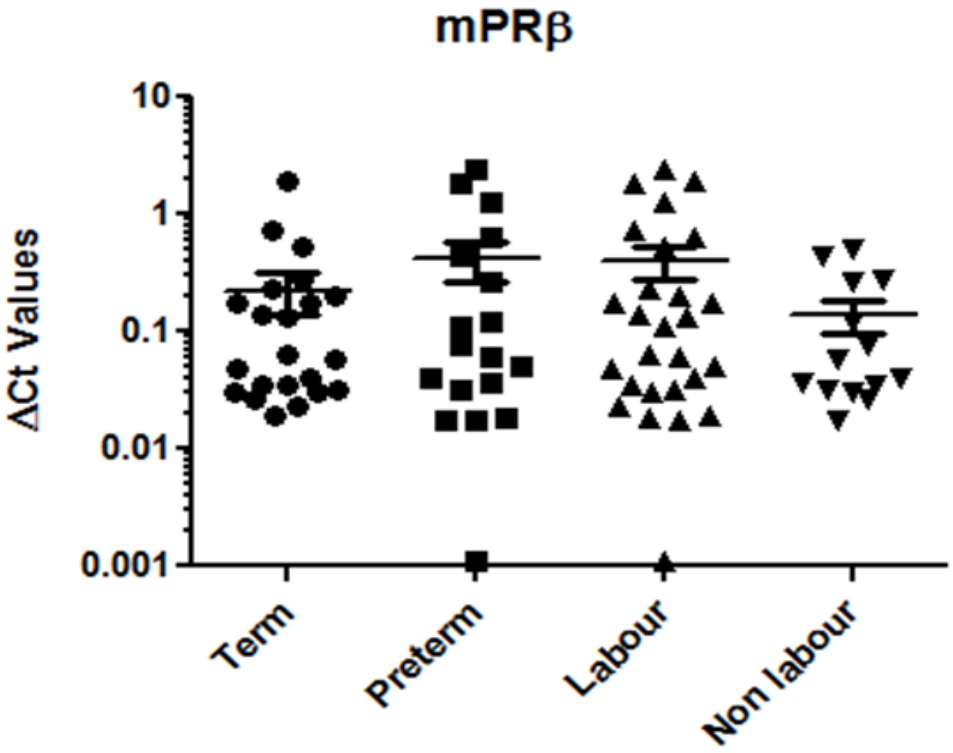

RQ Values
3
2
1
0
Term
Preterm
mPRα
mPRβ
mPRγ
PGRMC-1
PR-B

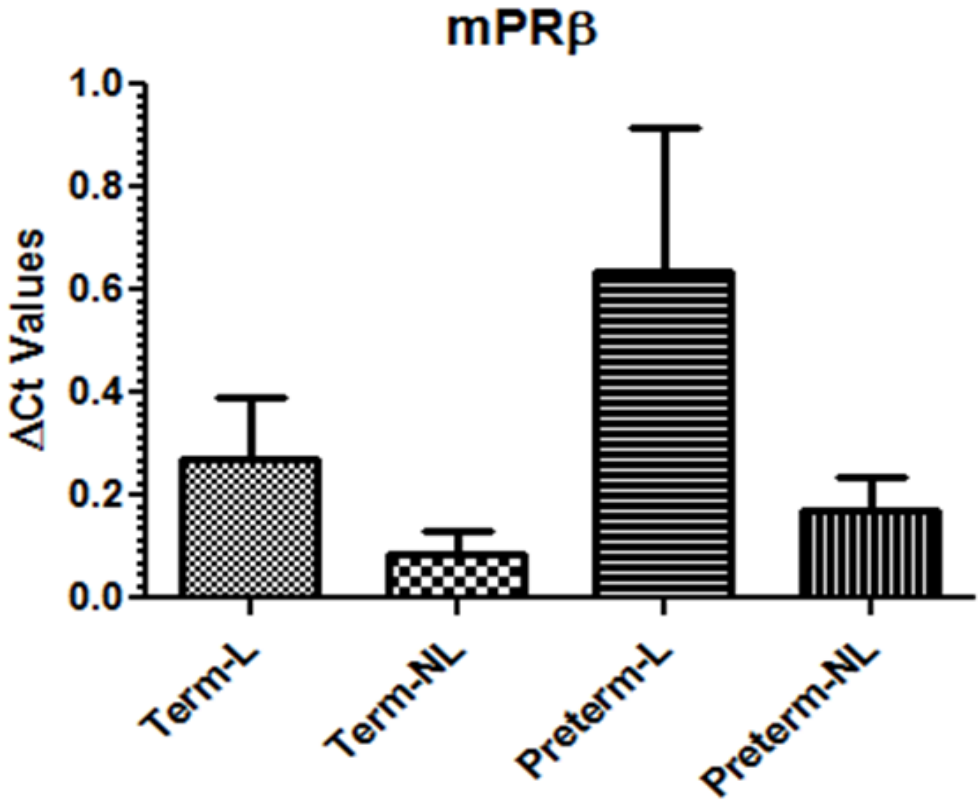

RQ Values
3
2
1
0
Labour
Non labour
mPRα
mPRβ
mPRγ
PGRMC-1
PR-B

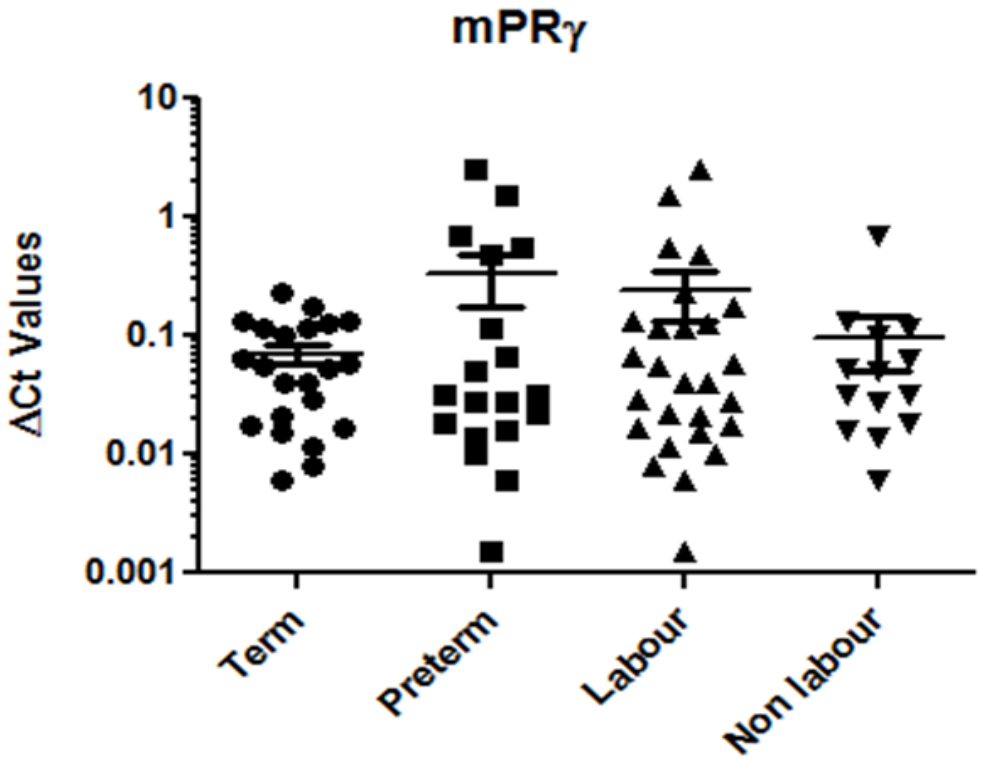
Appendix III

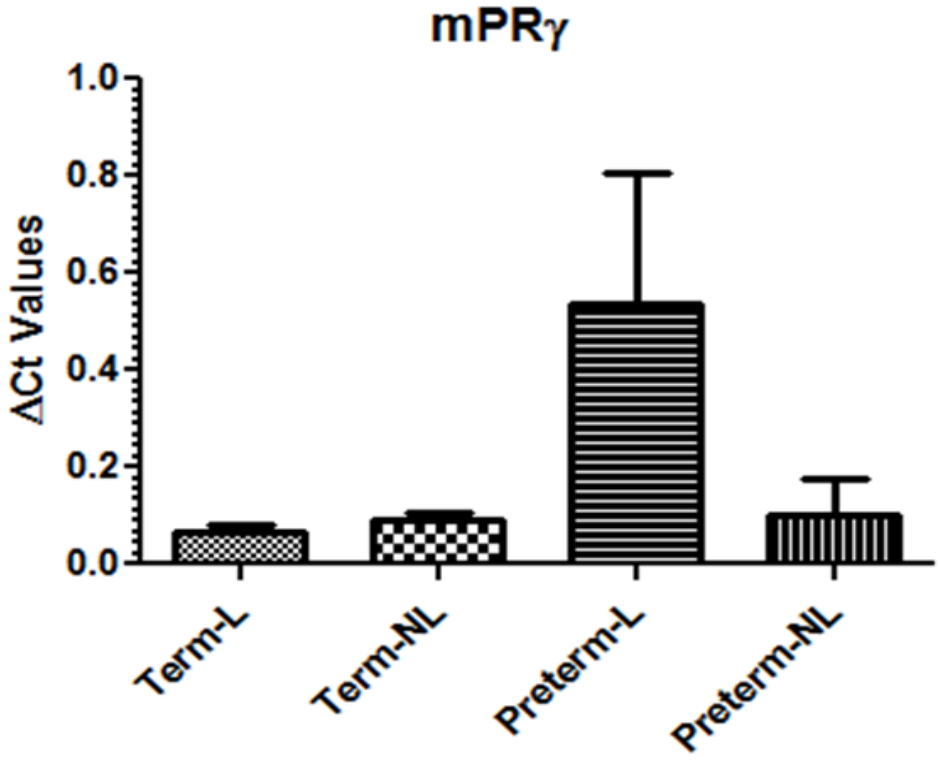

mPRα
ΔCt Values
100
10
1
0.1
0.01
0.001
Term
Preterm
Labour
Non labour

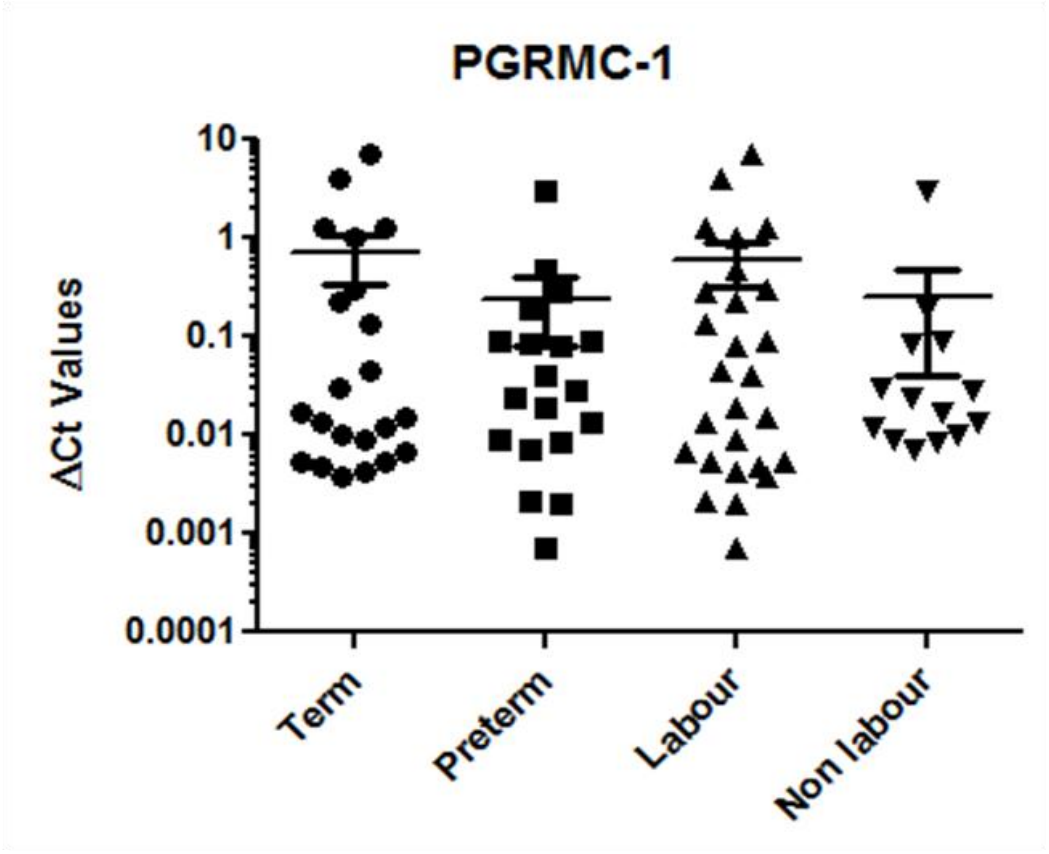

mPRα
ΔCt Values
4
3
2
1
0
Term-L
Term-NL
Preterm-L
Preterm-NL

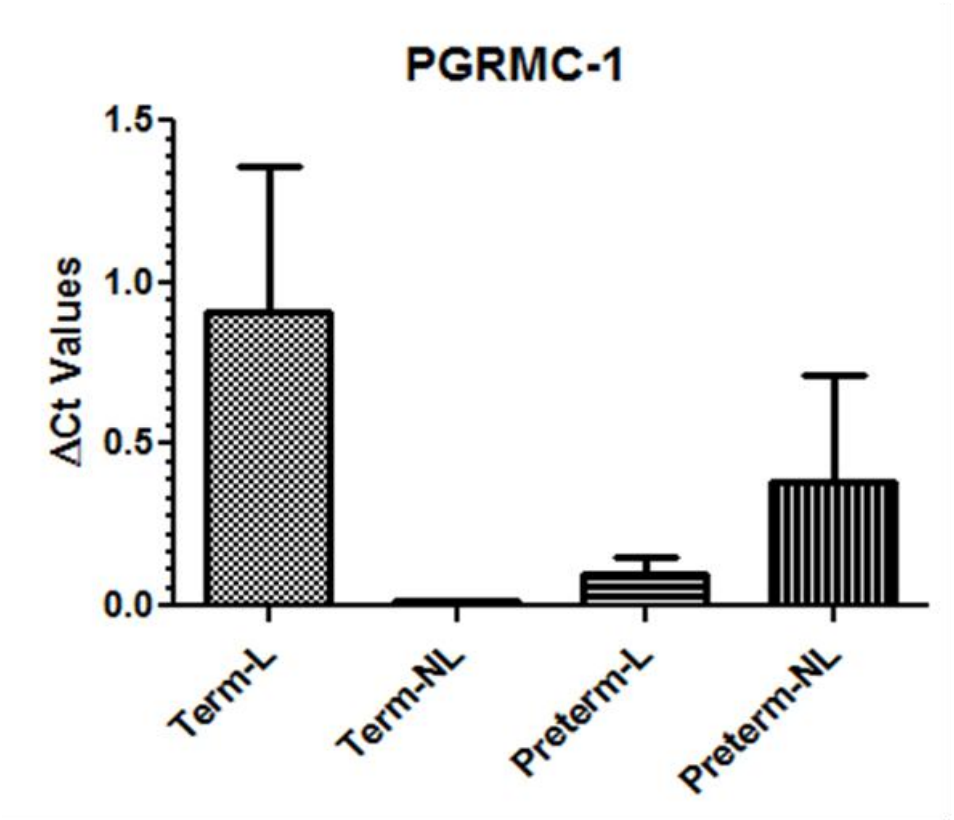

mPRβ
10
1
0.1
0.01
0.001
ΔCt Values
Term
Preterm
Labour
Non labour

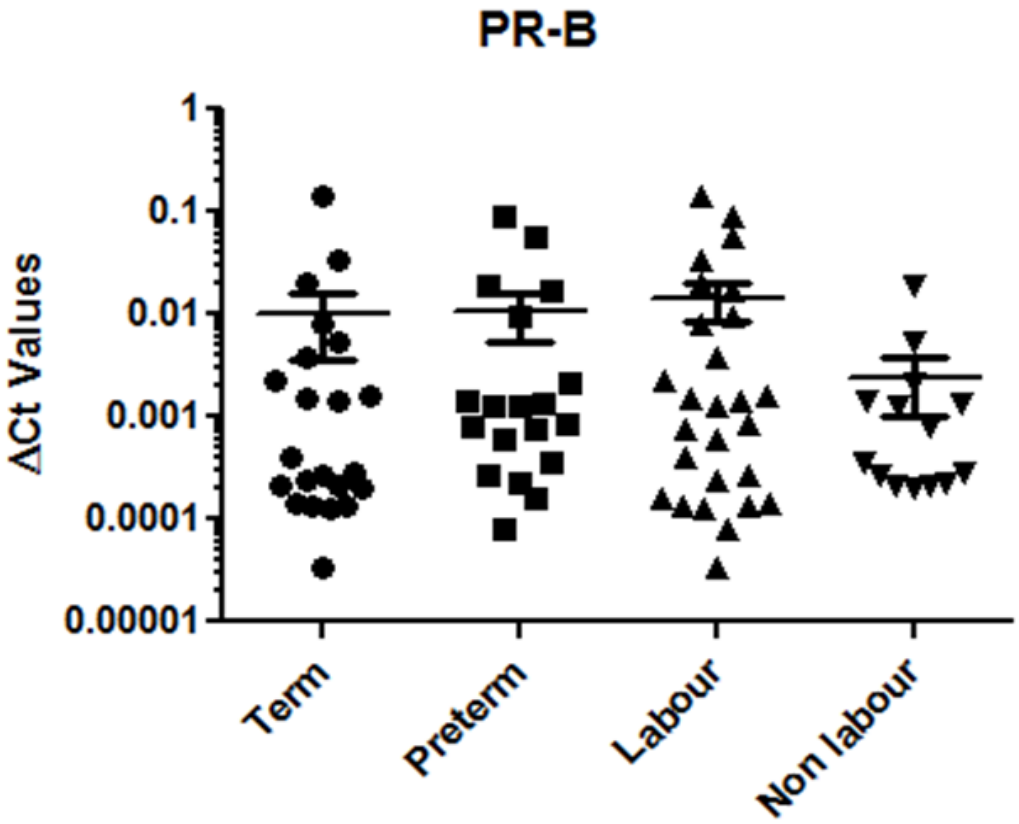

mPRβ
1.0
0.8
0.6
0.4
0.2
0.0
ΔCt Values
Term-L
Term-NL
Preterm-L
Preterm-NL

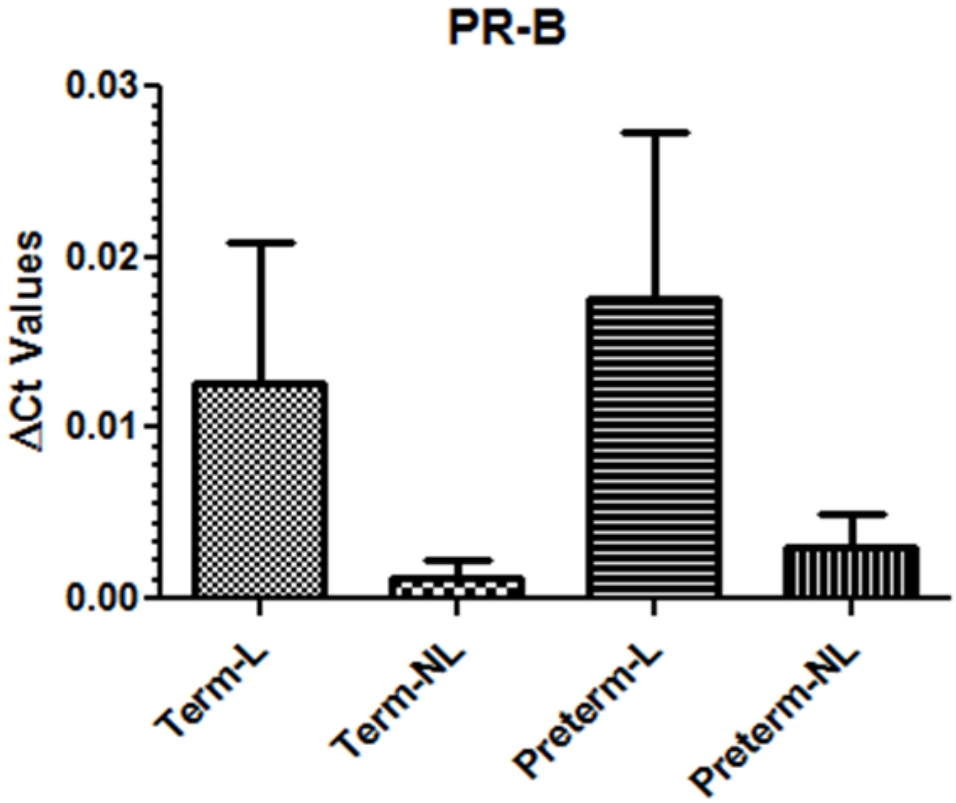

mPRγ
ΔCt Values
10
1
0.1
0.01
0.001
Term
Preterm
Labour
Non labour


mPRγ
ΔCt Values
1.0
0.8
0.6
0.4
0.2
0.0
Term-L
Term-NL
Preterm-L
Preterm-NL


PGRMC-1
ΔCt Values
10
1
0.1
0.01
0.001
0.0001
Term
Preterm
Labour
Non labour


PGRMC-1
ΔCt Values
1.5
1.0
0.5
0.0
Term-L
Term-NL
Preterm-L
Preterm-NL


PR-B
ΔCt Values
1
0.1
0.01
0.001
0.0001
0.00001
Term
Preterm
Labour
Non labour


PR-B
ΔCt Values
0.03
0.02
0.01
0.00
Term-L
Term-NL
Preterm-L
Preterm-NL

Appendix IV

Population	Count	%Gated
Double positive & Focus cells & Single Cell	**4480**	**100**

Double positive & Focus cells & Single Cell	Count	%
Nuclear localisation	**3808**	**85**
Cytoplasm localisation	**1072**	**15**

Expression of PR-B in BeWo cells using ImageStream analysis

Population	Count	%Gated
Double positive & Focus cells & Single Cell	**1320**	**100**

Double positive & Focus cells & Single Cell	Count	%
Nuclear localisation	**0**	**0**
Cytoplasm localisation	**118**	**9**
Membrane localisation	**954**	**91**

Binding study using P4-BSA-FITC in BeWo cells using ImageStream analysis