THE ROLE OF SOLUBLE FACTORS AFFECTING THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I MOLECULES IN AN *IN VITRO* MODEL OF THE FETOMATERNAL INTERFACE

A thesis submitted for the degree of Doctor of Philosophy

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This thesis is dedicated to all the mothers in the world, for putting their bodies through tremendous changes to give us life. For supporting us, through thick and thin and, for their unconditional love. This thesis is especially dedicated to my mother who never stopped giving herself to us in countless ways. For her constant encouragement, limitless giving, love and sacrifice, made this thesis possible.

Je t'aime mama

Abstract

Human main histocompatibility complex is encoded in the short arm of chromosome 6 and can be divided into three main regions based on the proteins that it encodes. We have class I, class III and class II. Class I encodes for proteins that are expressed on the cell surface of almost all somatic cells and is related to the presentation of self-antigens. Class I can be sub-divided into two groups known as classical class I (Ia) and non-classical class I (Ib), and expressed on the cell surface as human leukocyte antigen (HLA). HLA-G is one of the members of HLA-Ib and, together with HLA-E and HLA-F, is thought to play a key role in maternal tolerance to the semi-allogenic embryo, one haplotype comes from the mother, and is shared with her, while the other comes from the father. At the trophoblast stage, embryos do not express HLA-II and only express HLA-C (Ia member). Based on these facts, important questions about the role of these antigens during pregnancy have arisen. Our premise is that PreImplantation Factor (PIF), a 15 amino acid peptide secreted only by viable embryos, seems to plays a key role in this regulation. In this study we have used the JEG-3 cell line as a human trophoblastic model to study the effect of PIF on HLA-I expression. JEG-3 cells were incubated at different concentrations and time points of PIF. Using a wide variety of techniques, we could detect that PIF significantly induced HLA-I expression, mainly HLA-G and -E, increased their invasion, proliferation *in vitro*. Also, PIF modified protein profile, detected by 2D electrophoresis. Compared with the untreated cells, 14 proteins were over-expressed and 8 were under-expressed. Our study suggests that PIF, has a regulatory effect on HLA-I in this cellular model and the fact that not only HLA-G was over-expressed can suggest new regulations pathways under the control of HLA-E.

Papers and conference papers

Article (abstract): A. Jabeen, S. M. Hakam, J. M. Miranda-Sayago and N. Fernandez (2015). Regulation of the major histocompatibility complex class I molecules on trophoblast cells: role of the preimplantation factor (PIF*). "Poster Abstracts." American Journal of Reproductive Immunology 73: 23-49.

Article (abstract): A. Jabeen A., B. Obara, S. Hayrabedyan, S. M. Hakam, J. Pallavi, P. S. Spencer, P. P. Laissue and N. Fernandez (2014). "Effect of infection on heterotypic associations of immune receptors expressed on trophoblast-derived cells." Journal of Reproductive Immunology 101: 30-31.

Article (abstract): S. M. Hakam, J. H. Pallavi, T. Sachiyo, E. R. Barnea and N. Fernandez (2014). "Preimplantation factor (PIF*) regulates HLA-G expression at the fetomaternal interface." Journal of Reproductive Immunology 101–102: 51.

Article: P. S. Spencer, S. M. Hakam, P. P. Laissue, A. Jabeen, P. Jain, S. Hayrabedyan, K. Todorova, A. Blanch, J. M. McElhinney, N. Muhandiram, S. Alkhatib, G. B. Dealtry, J. M. Miranda-Sayago and N. Fernandez (2012). "Key cellular components and interactive histocompatibility molecules regulating tolerance to the fetal allograft." Am J Reprod Immunol 68(2): 95-99.

Article (abstract): P. Jain, A. Jabeen, A. Blanch, S. M. Hakam and P. P. Laissue. (2012). The interaction of the cytoskeleton with histocompatibility molecules on trophoblast cells: Relevance for foeto-maternal tolerance. "15th Biennial Meeting of the European Society for Immunodeficiency." Journal of Clinical Immunology 32(1): 1-379.

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Abbreviations

2-DE	2 dimensional electrophoresis gel
5-HT	Serotonin
β2M	β2-microglobulin
ABC	Antigen binding capacity
AM	Acetomethylester moiety
BAE	Background antibody equivalent
BCA	Bradford protein assay
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesuflate
COX-2	Cyclooxygenase-2
DM	Decidual macrophages
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNK	Decidual natural killer cells
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assays
EVCT	Extravillous cytotrophoblast
FACS	Flow analysis cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GH	Growth hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
hCG	Human chorionic gonadotropin
hCS	Human chorionic somatomammotropin
HhCG	Cyperglycosylated hCG
HLA	Human leukocyte antigen
hPL	Human placental lactogen
hPR	Human progesterone receptor
IEF	Isoelectric focusing
IFN	Interferon

IGF-2	Insulin growth factor 2
IL	Interleukin
ILT	Immunoglobulin-like transcript
IPG	Immobilized pH gradient
IVF	*In vitro* fertilization
KIR	Killer immunoglobulin like receptor
LIF	Leukaemia inhibitory factor
LILRB	Leukocyte immunoglobulin like receptor family B
МΦ	Macrophages
mAb	Monoclonal antibody
MALDI-TOF-MS	Matrix assisted laser desorption ionization time of flight mass spectrometry
M.F.I.	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMP	Metalloproteinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MS	Mass spectrometry
Mw	Molecular weight
NK	Natural killer
P4	Progesterone
PAGE	Polyacrylamide gel electrophoresis
pAPCs	Professional antigen presenting cells
PBS	Phosphate buffered saline
PBL	Peripheral blood lymphocytes
PD	Population doubling
PE	Pre-eclampsia
PES	Phenazine ethosulfate
PFA	Paraformaldehyde
pI	Isoelectric point
PIBF	Progesterone-induced blocking factor
PIF	Preimplantation factor
PMF	Peptide mass fingerprinting
pNK	Peripheral blood natural killer cell
PR	Progesterone receptor
PRE	Progesterone receptor element

PRL	Prolactin
PVDF	Polyvinyldine fluoride
RIA	Radioactive immunosorbent assay
RLX	Relaxin
RO	Reverse osmosis
RT	Room temperature
SABC	Specific antibody-binding capacity
SDS	Sodium dodecyl sulphate
sPIF*	Synthetic scrambled PIF
sPIF	Synthetic PIF
ST	Syncytiotrophoblast
SSC	Side scatter
TEMED	Nnnn-tetramethylethylediamine
TGF	Tumour growth factor
Th	T helper lymphocyte
TNF	Tumour necrosis factor
Treg	Regulatory T lymphocyte
uNK	Uterine natural killer
VCT	Villous cytotrophoblast
VEGF	Vascular endothelial growth factor
Wb	Western blot

Note: Not all the abbreviations that can be found in the text are in this list as they are international units that should be known by a person with a degree or higher (i.e. h for hours, min for minutes, kDa for kilodalton, M for Mole, etc.)

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CHAPTER I

INTRODUCTION

1.1 Background

Evolution is a conservative process that relies on established mechanisms of change, these being mutation, migration, genetic drift, and most importantly natural selection. Ancestral mammals, like the modern day duck billed platypus were oviparous, and immunity as we know it, was in place. Only about 100 million years ago did placental reproduction and thus longer gestation periods begin to emerge (Ji *et al.*, 2002; Weil, 2002). The change from external to internal fertilisation and gestation, allowed for the conceptus to be better protected from external hazardous environmental factors. It also meant that a higher proportion of the young were live-born (Hunt *et al.*, 2007). However, this presented the pregnant female with a considerable immunological challenge as this destined the reproductive and immune systems to work in close partnership with one another. This relationship between these two systems would in theory hinder the survival of the human species, and in effect all mammals, as the two have contrasting functions (Sacks *et al.*, 1999). While the primary role of the immune system is to destroy any non-self-structures or damaged tissues, the role of the reproductive system is to nourish and maintain a foetus, which in essence is a semi-foreign body to the maternal system. However, on closer inspection, the immune and reproductive systems are in fact similar, as Parham suggested in 2004; whilst the immune system processes are driven by microbial factors, those of the reproductive system are mediated by hormones (Parham, 2004). Based on these principles, mammalian (human) reproduction thus poses a paradox. Peter B. Medawar was one of the first researcher that tried to address this paradox (Medawar, 1953).

1.2 The immunological paradox as first addressed by Medawar

As an embryo is the result of two different non-histocompatible individuals in an outbred population mating, it is thus likened to a semi-allogenic graft (Thellin *et al.*, 2000; Thellin and Heinen, 2003). Throughout pregnancy, the foetus requires nutrients and gas exchange from the mother, which means that both share an intimate contact with one another. However, this close relationship between the foetus and the mother has one main disadvantage for the foetus as it is a semi-allogenic graft. The

expression of molecules from the genetic father's side that can be recognized as non-self-antigens, from the mother's immune system, and trigger an immune response and an abortion. This however, does not happen. Several hypotheses have been proposed to explain foetal tolerance, the most notable of which are those suggested by Peter B. Medawar in 1953 (Billington, 2003; Chaouat, 2015; Ribatti, 2015).

When Medawar first addressed this phenomenon he termed it as being an immunological paradox, because any mechanism which is used by the foetus to avoid rejection by the mother will, somehow, be inhibited by the need of both of these organisms to be able to ward off any invading organisms. Medawar thus put forth a couple of hypothesis as to how the foetus is able to escape the usual providence of transplants. How was the foetus able to thrive in the mother's body during gestation, whilst allowing her to maintain her immunity so as to fight off any harmful pathogens? The hypotheses suggested by Medawar at the time were "that the anatomical separation of the foetus or inertness of the mother" (Medawar, 1953; Billington, 2003; Veenstra van Nieuwenhoven *et al.*, 2003). These hypotheses have provided the theoretical starting platform that has enabled the scientific research to proceed for the better half of a century.

Through countless research, it has been observed that the foetus does in fact possess immunogenic properties, that the mother's immune system is not suppressed and that the "uterus does not uniquely protect the conceptus as an immune privileged site" (Thellin *et al.*, 2000; Gronvik *et al.*, 1987). Years of research have disproved two of the three hypothesis, that of immature foetal antigens, and maternal immunosuppression. Today the majority of research is reinforcing that it is in fact the anatomical separation between mother and foetus that allows for such a paradox to exist, and to be considered as natural (Moffett and Loke, 2004).

1.3 The miracle that is pregnancy

1.3.1 Menstrual cycle

The menstrual cycle is a complex and coordinated system, which can however be easily disturbed by environmental factors (stress, eating disorders, etc.) and genetic influences (fragile X pre-mutations, point mutations, etc.) (Popat *et al.*, 2008). Menstruation is a recurring bodily function, in which the uterine lining sloughs off as a result of the interactions that take place between the hormones that are produced by the ovaries, pituitary and the hypothalamus (Hawkins and Matzuk, 2008). This process is purely an opportunity by which to re-establish reproductive competence in an effort to prepare the uterus for the next cycle and, maybe, the annealing of a fertilized ovule (Figure 1.1).

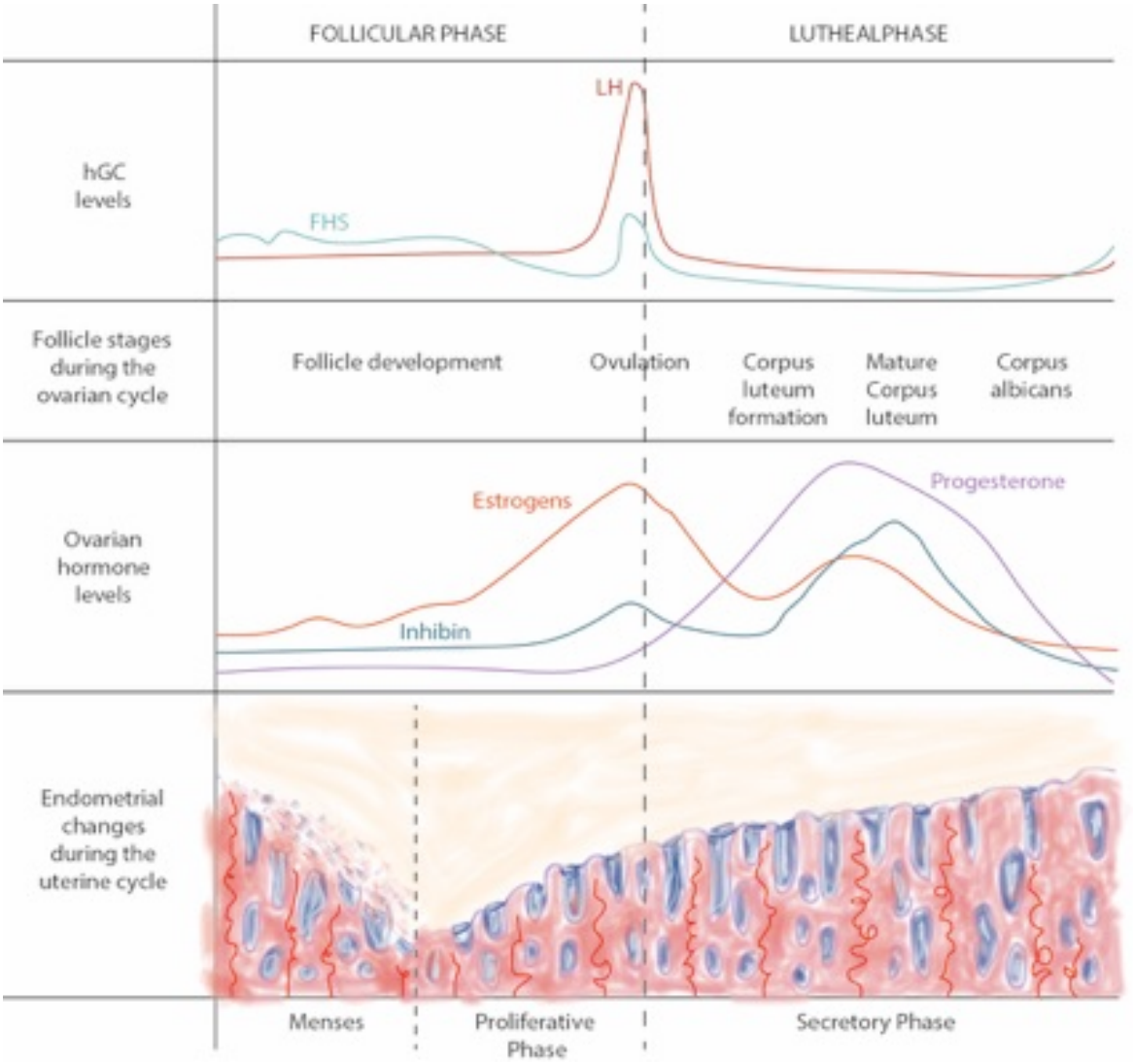

Figure 1.1. Human females' menstrual cycle.
This cycle consists of 28 days during which the woman's body undergoes different hormonal changes directly related to the success of pregnancy. In the case that the ovule is not fertilized by a spermatozoon, the ovule and the endometrium then start to decompose. This is the well-known process of menstruation.

In human females, the normal ovarian-uterine cycle can be divided into three stages based on the events that take place in each. These are: the follicular-proliferative phase, the secretory phase, and the luteal phase. During the proliferative phase (day 1-9), the endometrium thickens and becomes abundantly vascularised and infiltrated with small agranular natural killer (NK) cells. In the secretory phase (day 10-23), which is marked by ovulation, the NK cells begin to proliferate and differentiate, preparing the uterus for a possible embryo implantation. In the absence of an embryo, the NK cells die, and the outer endometrium sloughs off, marking the start of menstruation. This occurs in the absence of different

hormones like the human chorionic gonadotropin (hCG). If during the secretory phase, an embryo adheres and subsequently implants itself to the endometrium (essentially becoming a blastocyst and an allograft), pregnancy ensues this bringing menstruation to a halt for the coming nine months. If the embryo adheres to the endometrium it stars secrete hCG which, in turn, stimulate the cells of the corpus luteum to secrete estrogen and progesterone (P4) so that they decidualize the endometrium while supporting the embryo until the placenta is mature enough function independently and nurture the embryo.

1.3.2 Embryo implantation

Though placental mammals are very diverse, in their early stages, they all follow a similar progression (Hardy *et al.*, 1989). Proceeding fertilization, the zygote which is enclosed in the zona pellucida undertakes several cleavage divisions, compacts to create a morula, after which at the blastocyst stage it collects fluid in the central blastocoel cavity (Cunningham *et al.*, 2009). This blastocyst expands after which it hatches from the zona pellucida and implants itself in the uterine wall (Hardy *et al.*, 1989). After fertilization, the implantation process takes place anywhere from six to seven days ensuing fertilisation (Staun-Ram and Shalev, 2005). The process of implantation can be divided into three stages: apposition, stable adhesion, and lastly invasion. Apposition, which is an unstable, is where the pinopodes interlock with the microvilli on the apical syncytiotrophoblast surface of the blastocysts, thus establishing the primary adhesion of the blastocyst to the uterine wall (Vigano *et al.*, 2003; Norwitz *et al.*, 2001). In the next stage, there is an amplified physical contact between the uterine epithelium and the blastocyst, this denoting the second stage of implantation, the stable adhesion (Staun-Ram and Shalev, 2005). Lastly, trophoblast cells, more specifically the syncytiotrophoblast cells, penetrate the uterine epithelium and the mononuclear cytotrophoblasts invade the entire endometrium. By proliferating outwards to form cell columns, the villous cytotrophoblast cells are able to migrate into the decidua and, hence, invade the maternal spiral arteries, allowing the trophoblast to be in direct with the maternal blood, thus establishing the utero-placental circulation (Loke and King, 2000).

1.3.3 Establishing a pregnancy

The predecessor cells of the placenta are the trophoblast cells, which appear four days following fertilization in the form of the outer cell mass of the blastocyst, which is also composed of an inner cell mass which later develops into the foetus. The early blastocyst trophoblast cells differentiate to yield all of the cell subtypes that comprise the human placenta (Loke and King, 2000). Within the first week of gestation, a separation between the maternal tissues and the foetal cells is already established by a layer of cytotrophoblast and syncytiotrophoblast cells. This parting is essential for nutrient exchange, but most importantly to protect the developing foetus from any form of immunologic attack that may be triggered by the mother (Thellin and Heinen, 2003). As of four weeks of gestation, the basic structure of the mature placenta is already in place though the foetus is less than two centimetres in diameter. At this stage, foetal circulation that ends in capillary loops within the chorionic villi penetrates the maternal blood-filled intervillous space, which is supplied by spiral arteries and drained by uterine veins. The chorionic villi which are closest to the maternal blood supply continue to develop and expand into a corpus of chorionic tissue, better known as the placenta (Godfrey, 2002).

The foetal circulation enters the placenta via the umbilical arteries which are embedded in the umbilical cord, where they then branch out into cotyledons. The finest of these branches are composed of capillary loops within the chorionic villi (Cunningham *et al.*, 2009). Once the foetus has absorbed the nutrients carried to it by the blood and released its waste products, the foetal blood collects in the umbilical vein where it is returned to the umbilical cord. On the other hand, the maternal blood uses the spiral arteries of the uterus to enter the placenta where, the maternal blood is poured into the intervillous uterine veins (Cunningham *et al.*, 2009). At term, up to 35% of the maternal blood flow passes through the intervillous space in order to sustain the quickly developing foetus. In the placenta, the chorionic villi are the main functional units and are responsible for eliminating waste, mediating the absorption of nutrients, and producing the majority of hormones that are produced by the placenta during the course of pregnancy (Godfrey, 2002). When fully developed, the placenta is able to produce a physical separation between the mother and the developing foetus, while harbouring and feeding the foetus (Loke and King, 2000).

Simultaneous with the development of the placenta is the differentiation of the cytotrophoblast (original trophoblast) into three distinct subtypes (Vicovac *et al.*, 1995; King *et al.*, 1997). The trophoblast differentiates based on their subsequent role in pregnancy. The undifferentiated cytotrophoblast can differentiate into either a:

1. Hormonally active syncytiotrophoblast (ST).
2. Extravillous cytotrophoblast (EVCT).
3. Villous trophoblasts (VCT).

During all times of pregnancy, within the villi of the human placenta, there always is an undifferentiated population of cytotrophoblast cells, which are available for differentiation into any of these sub types, if necessary (Hemberger *et al.*, 2001; Hemberger and Cross, 2001).

The human placenta is considered to be an extension of the foetus into the mother, and acts as a barrier known as the fetomaternal barrier or interface (PrabhuDas *et al.*, 2015; Erlebacher, 2013a). The fetomaternal interface is the tissue that is most involved in pregnancy and is a transitional organ composed of both maternal and foetal cells (Cunningham *et al.*, 2009). The placenta is composed of three layers of cells, each playing a specific role in a successful pregnancy: the invasive extravillous cytotrophoblast cell layer which mediates implantation and invasion into the uterus, the cytotrophoblast cell layer which anchors the villi of the placenta, and lastly the chorionic villi or the syncytiotrophoblast layer, which is that which lies in direct contact with maternal blood (Cunningham *et al.*, 2009; Hemberger *et al.*, 2001; Hemberger and Cross, 2001).

In addition, to offering tolerance at the fetomaternal interface, the placenta is responsible for releasing a wide array of hormones and enzymes into the maternal bloodstream as well as regulating the transport of foetal nutrients and metabolic products, namely oxygen and carbon dioxide (Malassine *et al.*, 2003). During pregnancy, foetal and maternal components do no come into direct contact with one another, with the exception of the placenta, where these two are in direct contact, hence establishing the fetomaternal interface.

1.3.4 The trophoblast cell

Trophoblast cells are the major cell type involved in pregnancy and are the only foetal cells that ever come into direct contact with the maternal tissue in the host uterus (Ober, 1998; Hunt *et al.*, 2006). The trophoblast is an extraembryonic cell, originating from the trophectoderm which, in the embryo, is the first lineage to differentiate (Cunningham *et al.*, 2009).

The trophoblast can be subdivided into several populations depending on its type of differentiation, position in the foetus, and role in stimulating tolerance to the foetus (Hunt *et al.*, 2006). As previously mentioned, there are three subpopulations of trophoblast present, the ST, the EVCT and the VCT (Kurman *et al.*, 1984). Both the ST and EVCT are found within the placental villi. The ST is formed by the fusing of the cytotrophoblasts and is responsible for producing the majority of placental hormones critical to pregnancy, for the bi-directional transport of nutrients from mother to foetus, and of waste products from foetus to mother (Kurman *et al.*, 1984). The forefathers of all differentiated trophoblast subpopulations, the cytotrophoblast have a more structural role. These cells form the anchoring cell columns that are seen at the junction of the placenta and the endometrium throughout pregnancy. In other words, they fuse with the syncytium, so as to provide more placental space for the developing foetus. Also arising from the cytotrophoblast are the EVCT cells, which travel through the intersitium to replace the endothelial cells, removing control on vasodilation and vasoconstriction, and essentially allowing the mother's blood to flow over the placenta. Trophoblast cells play other pivotal roles apart from preparing and remodelling the endometrium, to allowing for the implantation of the foetus and subsequently modulating the immune response of the mother against the semi-allogenic graft that is the foetus (Aplin and Kimber, 2004).

The trophoblasts and the placenta are not only essential in establishing the fetomaternal interface, but also in producing essential hormones. In pregnancy, there is an overall hormonal change, as shown in Figure 1.2.

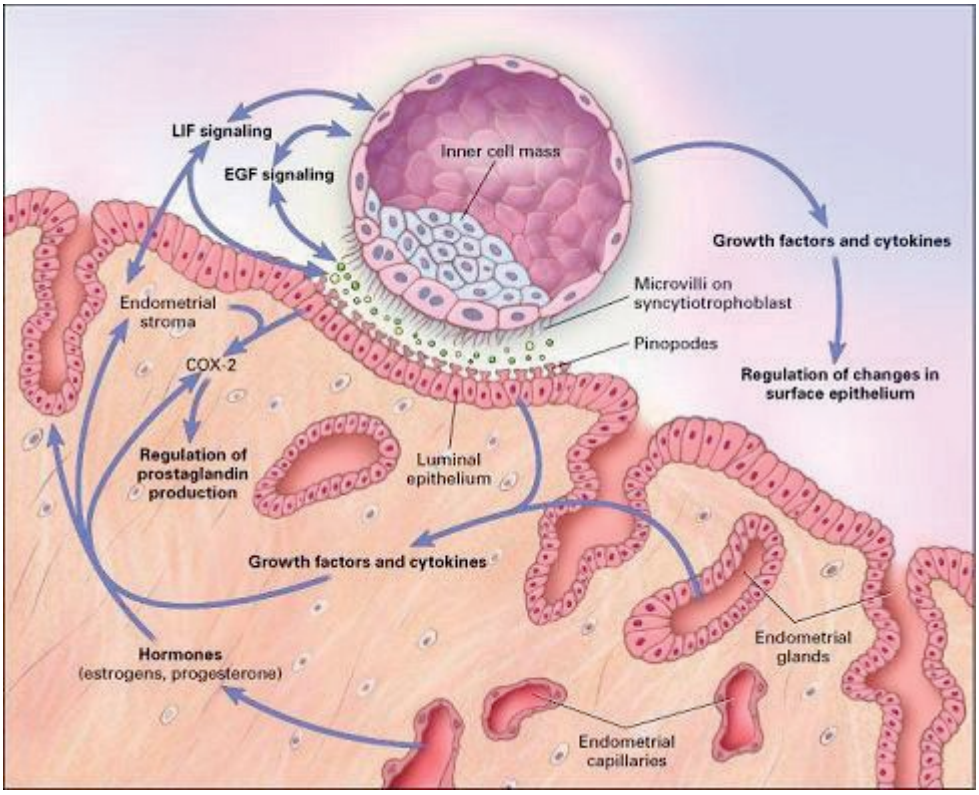

Figure 1.2. The human fetomaternal microenvironment.
The pregnant microenvironment is one which involves a complex cocktail of cytokines, hormones, immune cells and various other soluble factors, all of which work together so as to ensure the well-being of the mother whilst ensuring a successful pregnancy, and the survival of the embryo. (Figure is courtesy of Norwitz et al., (Norwitz et al., 2001)).

1.4 Immune cells involved in pregnancy

Pregnancy is important for the conservation of the species, and thus, all the means by which to protect the mother and the offspring are reinforced. During this time, there is a modulation of the immune system, which leads to different responses depending on the stages of pregnancy. The decidua is populated with a variety of cells, these include stromal cells, mesenchymal cells, and leukocytes. Throughout pregnancy, contrary to popular belief, a reduction or absence in the number of immune cells has harmful consequences on placental development, implantation, and decidual formation (Le Bouteiller and Piccinni, 2008; Hanna *et al.*, 2006). As a result, this means that immune cells at the site of implantation are a means by which to facilitate and protect pregnancy. Thus the immune system in the uterus is not suppressed, but is carefully regulated, we can say it is dynamically regulated (Mor *et al.*, 2011). There are several subpopulations of immunocompetent cells present in this environment, the primary three are: macrophages (MΦ), uNK cells, and T lymphocytes. In the endometrium, B lymphocytes are virtually absent (Mor *et al.*, 2011; Shimada *et al.*, 2006).

1.4.1 Natural killer cells (NK cells)

Even before pregnancy, the endometrium is populated with NK cells, whose levels fluctuate during the menstrual cycle and throughout the course of pregnancy. Moreover, as NK cells are present even before conception, this suggests that the foetus does not have an effect on the distribution of these cells (Bulmer, 1989; King and Loke, 1991).

NK cells make up to 70% of the decidual leukocyte population in early pregnancy. However, as gestation progresses, the number steadily declines to become essentially absent at term (Koopman *et al.*, 2003; Ashkar and Croy, 1999). In comparison to the NK cells found in peripheral blood, the major subset of NK cells in the uterus are the uNK, also referred to as the large granular lymphocytes ($CD56^{bright}$ $CD16^{-}$ $CD3^{-}$). This unique population of cells is found both in the pregnant and the non-pregnant endometrium (King and Loke, 1991). Though these cells are insignificantly cytotoxic, in contrast to their peripheral blood counterparts, they are nevertheless highly proliferative, and are known to express a wide variety of adhesion molecules, cytokines, and NK receptors for interleukin (IL)-2, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and leukaemia inhibitory factor (LIF) (Figure 1.3) (Cooper and Broxmeyer, 2001). Uterine NK cells express a wide range of inhibitory receptors that are known to interact with human leukocyte antigen (HLA) class I molecules, delivering inhibitory signals to these cells once they recognize HLAs bearing target cells. Of these inhibitory receptors, the most well-known are the killer cell immunoglobulin like receptors (KIRs), CD94/NKG2 and immunoglobulin like transcript (ILT) (Menier *et al.*, 2008; Borrego *et al.*, 2005). These are reported to bind to some of HLA class Ib molecules, i.e. HLA-G on the trophoblast cell. The exact role of uNK cells is still not yet well established. However, it has been reported that these cells regulate placental development, by mediating the foetal extraembryonic trophoblast invasion (Moffett-King, 2002). In addition, as the number of the uNK cells decreases from the start to the end of pregnancy, it has been hypothesized that the primary role of these cells is carried out during the first trimester (Moffett-King, 2002).

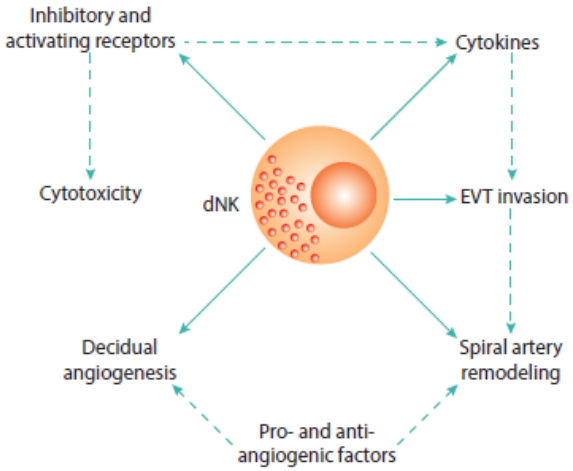

Figure 1.3. Role of natural killer cells at the fetomaternal environment.
At the fetomaternal interface, the most abundant immune cell present in the pregnant uterus is that of the decidual natural killer cells (dNK), also referred to as the uNK cells. These cells, which are part of the innate immune system, produce a number of angiogenic factors, and cytokines, that aid the remodelling of the spiral arteries and in the development of the placenta, both of which are pivotal events if a term pregnancy is to ensue (figure is adapted from St. George's University of London, http:// www.sgul.ac.uk/).

1.4.2 Macrophages (MΦ)

Macrophages, are multipurpose cells that clear debris in the body, but are also one of the major professional antigen presenting cells (pAPCs) (Faas *et al.*, 2014). In the uterus, where these are also referred to as decidual macrophages (DM), these cells represent 20-30% of the population of the decidual cells. Moreover, the concentration at which these cells are found remains high throughout the course of pregnancy, unlike uNK cells. During pregnancy, these cells are proposed to also play a role in implantation, placental growth, fetomaternal immune tolerance, in addition to protecting the mother from infections. This last hypothesis is supported by findings that have showed heavy MΦ infiltration at the fetomaternal interface (Hunt and Robertson, 1996). In addition, DM support the remodelling of the uterus that takes place as a result of the expansion of the extra-embryonic tissue (Mor *et al.*, 2006). In the decidua, MΦ are known to both synthesize and secrete growth factors and cytokines. However, a change in the distribution of DM, can lead to pre-eclampsia (PE). In PE, the MΦ are located around the spiral arteries, instead of near them, creating a barrier between the invading trophoblast and the spiral arteries. In normal pregnancies, MΦ facilitates the invasion of the trophoblast through the placental bed (Mor and Abrahams, 2003). However, in PE, or other pregnancy related pathological conditions, the MΦ act as a barrier by inducing trophoblast apoptosis, and ultimately inhibiting the modification of the spiral arteries.

Nevertheless, these cells remain true to their nature and carry out their duty of clearing debris even in this environment. At the fetomaternal interface, a high concentration of the MΦ are found next to apoptotic cells (Abrahams *et al.*, 2004).

The DM have a similar phenotype to other MΦ present in other tissues. However, these MΦ show increased activation, expression of a higher density of HLA-DR and CD11c (more than the peripheral blood monocytes), and low levels of co- stimulatory molecules CD80 and CD86. Low levels of co-stimulatory molecules suggest that DM can induce tolerance of maternal T lymphocytes by denying them sufficient levels of co-stimulatory signal (Heikkinen *et al.*, 2003). DM are also known to produce elevated levels of IL-10, a cytokine known to hinder expression of HLA-DR on pAPCs, and to modulate dendritic and T lymphocytes. Moreover, DM are known to produce lower levels of pro-inflammatory IL-1, this could be important as in the case of an intrauterine infection this would result in less inflammation (Abrahams *et al.*, 2004; Heikkinen *et al.*, 2003). MΦ are able to stimulate cell growth, whilst hindering harmful inflammatory immune reactions by establishing a favourable environment through their cytokine profile.

1.4.3 T lymphocytes

During pregnancy, the decidua is mainly dominated by the cells of the innate immune system. However, T lymphocytes make up 10% of the endometrial stromal leukocyte population (Williams *et al.*, 2009). Throughout, both the menstrual cycle and pregnancy, the decidual T lymphocytes experience changes. At the beginning of pregnancy the T lymphocytes steadily decrease to only experience an increase becoming the most abundant leukocyte population by the end of pregnancy (T lymphocytes are higher in non-pregnant uteri than they are in pregnant uteri during the first trimester). The increase in T lymphocytes at the end of gestation and their expression of activation markers such as CD25 and HLA-DR at this point, infers that these cells are important in the maintenance of pregnancy. A subpopulation of the T lymphocytes, are the regulatory T lymphocytes (Treg), which are able to control immune responsiveness to both self and non-self-antigens, and are important in transplantation tolerance and in suppressing autoimmunity. Another subset of T lymphocytes are the T helper (Th) lymphocytes, of which there are two subsets: Th1 cells, which are involved in cellular immunity, and Th2 cells, which are

involved in humoral immunity (Mosmann *et al.*, 1986; Saito, 2010). These two subsets, once comprised the Th1/Th2 paradigm, which recently has been changed and is now known as the Th1/Th2/Th17 and the Treg cell paradigm (Saito *et al.*, 2010). The Th17 cells, which are part of the paradigm, are known to play an important role in inflammation and produce the pro-inflammatory cytokine IL-17 (Saito *et al.*, 2010). The Th cells, are reported to be regulated by the Treg cells. The formers capacity to produce cytokines is also seen to be suppressed by immunoregulatory cytokines such as the transforming growth factor beta (TGF-β) and IL-10, or by cell-cell interaction (Huber *et al.*, 2011). In addition, it has also been proposed that Treg cells may be responsible for controlling the Th1 activity, which is characteristic of a healthy normal pregnancy (Figure 1.4) (Shima *et al.*, 2010; Mjosberg *et al.*, 2010).

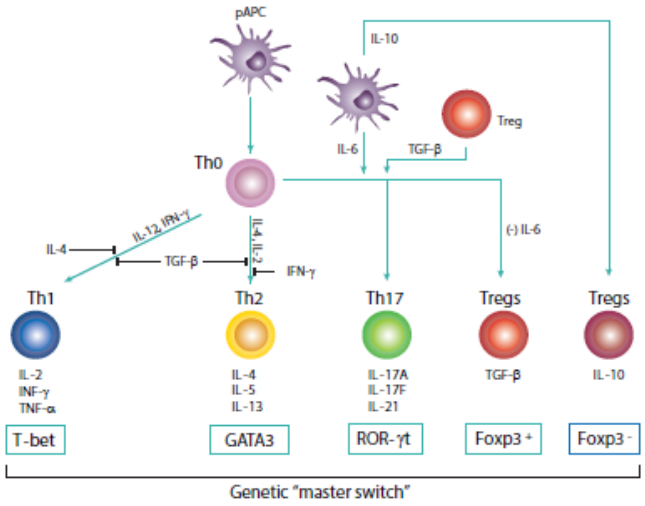

Figure 1.4. The T cells at the fetomaternal environment.
T cells play an important role in both immunoregulation and immunostimulation. T cells give rise to several different T cell subsets such the T helper cells (Th), which are identified as Th1, Th2, and Th17 and the T regulatory cells (Treg). Each subset of T cells carries out a specific function, all of which are important in ensuring an adequate environment for pregnancy. (Figure is a courtesy of Peterson, 2013).

1.5 Soluble factors at the fetomaternal interface

1.5.1 Cytokines

Cytokines, a family of proteins synthetized intracellularly, are produced by various immune cells that are involved in both the generation, and the maintenance of the immune response (Beigi *et al.*, 2007). Cytokines are released under certain signals, i.e. TCR-HLA interaction (T cell receptor), and hold an important role in the immunological processes concerning placental growth and the maintenance of pregnancy. A wide range of cytokines, are known to be produced by the uterine and decidual cells and by the placenta (Weetman, 1999). Cytokines are primarily produced by NK and T cells, and are categorized into two groups based on their paracrine activity (Sanjabi *et al.*, 2009a). The first group, termed as pro-inflammatory Th1 type cytokines produces IL-1, IL-2, IL-12, IL-15, IL-18, TNF-α and IFN-γ. These cytokines interact with the phagocytes, assisting them in destroying intracellular pathogens. On the other hand, anti-inflammatory Th2 type group encompasses cytokines such as IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and GM-CSF. Inside Th2 cytokines, IL-6 has ubiquitous properties as it can be a pro- and an anti-inflammatory cytokine (Brandt and Pedersen, 2010). Th2 cells and cytokines interact with B

lymphocytes, aiding them in their differentiation process and in the immunoglobulin class switching (Akdis *et al.*, 1998). Leukocytes use cytokines in order to carry out their functions, and can influence the activation status or/and the cytokine profile of other leukocyte subtypes. In addition, through cytokines, these cells are also able to effect the differentiation or/and the cellular behaviour of non-immune competent cells (von Rango, 2008).

1.5.2 Pregnancy as a Th1 or Th2 state

Over the years, pregnancy has been repeatedly described as being a Th2/anti-inflammatory state. However, recent studies have postulated that pregnancy is not a single state event, but an event that has three individual and distinct phases. The first stage of pregnancy, which was identified as the first and very early second trimester, are identified as being a pro-inflammatory phase, as it is during this time where the blastocyst implants itself into the uterus, essentially damaging the endometrial tissue. The process of implantation and securing a viable placental-foetal blood supply make for high numbers of invading, dying, and repairing cells. Therefore, it is essential to have a pro-inflammatory environment in order to ensure the repair of the uterine epithelium, and the clearing of cellular debris (Bowen *et al.*, 2002). The second phase of pregnancy, which is termed as an anti-inflammatory phase, as the principal immunological feature is the initiation of an anti-inflammatory state, dominates the late-early through to the end of the second trimester of pregnancy. This, is often referred to as the optimal time for the mother as it is when she no longer suffers from the symptoms of pregnancy (Mor *et al.*, 2011). During the anti-inflammatory phase of pregnancy, the foetus experiences rapid growth and development and the foetus, placenta, and the mother have a synergetic relationship. During the third phase (third trimester of pregnancy), the immunological feature goes back to being a pro-inflammatory state (Keelan *et al.*, 2003). During this trimester, the foetus has completed its growth and all its organs are ready. As the foetus needs to be delivered, an inflammatory state is required (Gomez-Lopez *et al.*, 2014). Delivery of the foetus is represented by an invasion of immune cells into the myometrium so as to stimulate an inflammation (Rusterholz *et al.*, 2007). This inflammation encourages the uterus to contract, which leads to the delivery of the baby and the placenta (Mor *et al.*, 2011; Romero *et al.*, 2007). Therefore, pregnancy depending on the stage/trimester, is both an anti- and a pro-inflammatory state. Each state and the cytokines associated

with it, serve a purpose whether it's to help in the implantation process, in the growth of the foetus or in the labour (Mor, 2008; Xu *et al.*, 2010).

In the end, a successful pregnancy is characterized by low levels of Th1 cytokines, and ultimately a shift in the cytokine profile from Th1 to Th2. This shift, referred to as the Th1/Th2 paradigm of reproduction, thus highlights that pregnancy is not just a Th2 state, but that the Th1 state is also important. In addition, it also suggests that the cytokine profile needs to be carefully controlled, as though Th1 cytokines are essential in establishing pregnancy as rejection of the foetus is primarily mediated by the Th1 cytokines while, maintenance and acceptance of the foetus and its growth is mediated by Th2 cytokines (Du *et al.*, 2014). An additional source of Th1 and Th2 cytokines during pregnancy, are the EVCT and ST cells, the chorion, amnion and Hofbauer cells (von Rango, 2008).

1.5.3 Importance of cytokines in pregnancy

During the luteal stage of the menstrual cycle, the cells of the endometrium show an increase in Th2 cytokines, namely IL-4 and IL-6, as compared to the Th1 cytokines. Secreted by the endometrium infiltrating lymphocytes IL-4 and IL-6 are reported to stimulate the production of LIF in the endometrium. LIF is very important in the implantation process as it not only facilitates trophoblast invasion, but also endometrial decidualization, and it aids in the regulation of interactions between decidual lymphocytes and trophoblast cells (Nachtigall *et al.*, 1996). On the other hand, LIF secretion is inhibited by Th1 cytokines. Moreover, low concentrations of LIF, IL-4, IL-6, and IL-10 in the endometrium and the decidua are associated with implantation failure and recurrent abortions (Wilczynski, 2005). During the implantation process, the embryo itself has the ability to actively control interactions with the decidua, generally by decreasing secretion of Th1 cytokines, namely IL-2 and TNF-α, and by increasing the secretion of GM-CSF (Sanjabi *et al.*, 2009a). The embryo is also able to secrete IL-10, which decreases the invasiveness of the trophoblast cells, and the secretion of TNF-α by the maternal tissue. *In vitro* and *in vivo* studies have both shown the that peripheral blood lymphocytes (PBL) of pregnant women secrete more Th2 cytokines and less Th1 cytokines than PBLs isolated from non-pregnant women and, in pregnant women, the number of IL-4 secreting PBLs increases gradually from the start to the end of pregnancy. The source of Th2 cytokines are the trophoblast, decidua, chorionic and

the amniotic membranes (Mor, 2008). These cytokines help to create the ideal environment for the secretion of other Th2 cytokines, while simultaneously decreasing the inflammatory effects of IL-1 and TNF-α. Such a mechanism allows for the initiation of premature labour to be evaded (Hunt and Robertson, 1996; Gomez-Lopez *et al.*, 2014).

The early implantation phase, premature, and at term labour are all situations associated with Th1 activity, and are ones where the Th1 profile dominates over the Th2. Th1 cytokines are very important in the process of implantation. MΦ are the primary source of IL-1 and TNF-α. A Th1 dominating phase is important in promoting a change in the number of endometrial leucocytes, in activating growth factors, rebuilding the extracellular matrix and in preparing the maternal uterus for embryo implantation. Additionally, TNF-α and IL-2 fuel the cytolytic activity of the uNK cells and lymphokine-activated T lymphocytes, which are able to inhibit excessive trophoblast invasion and propagation which, if are not controlled, could be detrimental to the process of a successful pregnancy. Moreover, during the implantation process IL-1, which is present in the uterine environment as an outcome of the inflammation that is taking place in response to the paternal components, stimulates trophoblastic metalloproteinase nine (MMP-9), thus increasing their invasive properties. IL-1, along with TNF-α, is also known to stimulate the production of LIF, which is known to have a positive influence on the growth and differentiation of the trophoblast cells. Embryonic and uterine cells secrete IL-2, a cytokine that stimulates uNK cells, which along with MΦ, who secrete IL-12 and TNF-α, have the potential to control trophoblast infiltration, as these cytokines are known to have the potential to trigger trophoblast apoptosis (Guenther *et al.*, 2012). At term, pro-inflammatory cytokines stimulate the decidual prostaglandins, hence triggering uterine contractions. These pro-inflammatory cytokines, in addition to mechanical stimuli induce IL-8 production, whose high levels in the cervical mucus are responsible for cervical ripening and opening (Keelan *et al.*, 2003; Ekman-Ordeberg and Dubicke, 2012).

1.5.4 Hormones

1.5.4.1 Serotonin (5-HT)

Serotonin (5-HT), which can acts as a neurotransmitter or as a hormone, is involved in a wide range of physiological functions including regulation of the cardiovascular system, control of

gastrointestinal motility and secretion, appetite, sexual behaviour, etc. (Ahern, 2011; Gellynck *et al.*, 2013; Hoyer *et al.*, 2002). Increasing number of studies have also demonstrated that before acting as a neurotransmitter, 5-HT plays a role in development. In adults, 5-HT regulates the differentiation of T lymphocytes, and the concentration of 5-HT is closely related to the regulation of Th cells, NK cells, MΦ and dendritic cells function (Ahern, 2011; Cloez-Tayarani and Changeux, 2007; Shajib and Khan, 2015). 5-HT shows these functions through specific receptors expressed on immunocompetent cells (Cloez-Tayarani and Changeux, 2007). 5-HT is able to regulate reproductive processes by having a direct effect on the reproductive organs, and not only through the hypothalamo-hypophyseal system (Dube and Amireault, 2007; Sirotkin and Schaeffer, 1997; Jorgensen, 2007).

5-HT has also been detected in very early embryos of various kinds of organisms, and considered to play an important role in 'pre-nervous' development (Malm, 2012; Bonnin and Levitt, 2011). 5-HT receptors are also known to be expressed at very early stage in foetus development (Dube and Amireault, 2007; Kyle, 2006). 5-HT is one of the essential factors for early embryo growth, as it controls the immune system development of the foetus and modulates the differentiation of various foetal tissues (Basu *et al.*, 2008). A lack of 5-HT during the prenatal period, can result in significant changes of the immune system both in the T- and B- response (Latendresse *et al.*, 2013). The morphogenetic effect of 5-HT deficiency is related to the T lymphocytes differentiation of $CD4^+$ cells (Th) in the thymus, which are going to play an essential roll with B lymphocytes in the postnatal period (Afanas'eva *et al.*, 2009).

In the maternal system, 5-HT is detected in the tubal fluid of the oviduct, cervix and uterus (Basu *et al.*, 2008; Amenta *et al.*, 1992). Maternal 5-HT is considered as being crucial in pregnancy as it is involved in the control of morphogenesis during embryo development (Cote *et al.*, 2007).

1.5.4.2 Relaxin (RLX)

Relaxin (RLX) has a molecular weight of 6 kDa and is a member of the insulin like growth factor family. This hormone has a wide range of functions in the female reproductive tract. It has been found in female circulation during the luteal stage of the menstrual cycle, and during pregnancy, where the source is the ovarian corpus luteum (Goldsmith and Weiss, 2009; Hsu *et al.*, 2002). In addition, recent studies have reported that RLX is also synthesized by the endometrium (Goldsmith and Weiss, 2009). Some of

the known functions of RLX include the regulation of growth and development of the mammary gland, growth and dilation of the cervix, relaxation of the pubic symphysis, and inhibition of the uterine contractions (Goldsmith and Weiss, 2009; Min and Sherwood, 1996). As a hormone, RLX is seen to have several effects on the endometrium, which are critical in establishing a successful pregnancy. This is further supported by the finding that RLX stimulates new blood vessel formation and increases cytokine-containing lymphocyte numbers while maintaining endometrial connective tissue integrity, decreasing endometrial levels of MMP-1 and MMP-3, and binding with high affinity to the endometrial cells, particularly in the epithelium of the endometrial glands and uterine lumen (Goldsmith *et al.*, 2004; Einspanier *et al.*, 2001; Bond *et al.*, 2005; Osheroff and King, 1995). However, though this hormone is important in rearranging the endometrium, high circulating levels of maternal RLX are harmful and have been linked with preterm birth (Goldsmith and Weiss, 2009; Weiss *et al.*, 1993). Elevated levels of RLX can be detrimental during pregnancy, as can be the absence of circulating RLX. Elevated circulating maternal RLX concentrations can be associated with premature labour, as RLX can regulate MMP-1 and MMP-3 levels. Moreover, the absence of circulating RLX during pregnancy in women may have negative consequences upon glucose metabolism. Therefore, during pregnancy the effect of RLX may be dependent on the concentration of the hormone that is circulating, just as it would on the local endocrinological milieu (Goldsmith and Weiss, 2009). As pregnancy can ensue without any circulating RLX, this suggests that RLX must be synthesized locally if it plays a functional role in human pregnancy. Recent data has shown that RLX is synthesised by the endometrial cells (Palejwala *et al.*, 2002).

RLX is also known to selectively increase the level of neutrophils, uNK, and MΦ ($CD68^+$) in the endometrium. Such a regulation of these cells is important as uNK cells are crucial in spiral artery remodelling, cytokines secretion, angiogenic factors release, etc. which are important for both implantation and the maintenance of pregnancy (Croy *et al.*, 2002). RLX is also known to have the ability to regulate the levels of other hormones. RLX is known to inhibit protein levels of progesterone receptor A, B, and ERα in the endometrium. This is important as the drop in the levels of these hormones in the secretory phase of the menstrual cycle, correlates with the rising circulating levels of RLX. Therefore

RLX has distinct effects on the functions of the endometrium, making it an important player in early pregnancy, both in establishing and maintaining it (Goldsmith and Weiss, 2009).

1.5.4.3 Human placental lactogen (hPL)

Human placental lactogen (hPL), also known as human chorionic somatomammotropin (hCS), is a hormone of 191 amino acids with two intramolecular disulphide bridges, of the lactogen/growth hormone (GH)/prolactin (PRL) protein family (Rygaard *et al.*, 1998). This glycoprotein is produced by the syncytiotrophoblast cells, and can be detected in embryo as early as 5-10 days after implantation, and at six weeks of pregnancy in the maternal blood circulation. hPL has the highest concentration in maternal serum compared with any of the other hormones. Its levels increase throughout the course of gestation, with levels peaking at the third trimester (Josimovich and Archer, 1977).

Another source of hPL are the invasive trophoblasts, which produce this hormone during the first trimester of pregnancy. During pregnancy, this hormone regulates the mother's carbohydrate and lipid metabolism. At the fetomaternal interface, hPL is known to inhibit the lymphocyte reactivity so as to protect the foetus (Siiteri and Stites, 1982). This hormone, has also been associated with the cytokines that are involved in fetomaternal tolerance while, Th2 cytokines are reported to contribute in the secretion of hPL, as well as hCG from the trophoblasts (Licht *et al.*, 2001a).

1.5.4.4 Human chorionic gonadotropin (hCG)

The ST is also known to produce one of the most studied hormones, the hCG (Malassine *et al.*, 2003). This hormone is crucial in pregnancy as it prevents the corpus luteum from degeneration, ultimately sustaining the release of P4 by the ovarian granulosa cells, and facilitating cell growth and differentiation (Staun-Ram and Shalev, 2005). In addition, hCG, which is one of the earliest secreted hormones in pregnancy, is also reported to reach its highest concentration levels between the eight and twelfth week of gestation, after which falls to a lower level and stays there until term. The early form of the hCG, the hyperglycosylated hCG (HhCG), is a variant of the hCG that has extra-large O-linked oligosaccharides and is produced by the invasive trophoblast cells. This form of the hCG is mainly expressed in the early stages of pregnancy, where an "aggressive" invasion on the part of the trophoblast is needed, and as gestation advances its levels decrease (Cole *et al.*, 2003). During the early stages, the

receptor for this hormone is expressed in an inactive and truncated form by the trophoblast. Starting from the ninth week of gestation, the full-length receptor of the hCG is expressed, hence allowing for the hormone to serve out its autocrine regulation role, among which is the differentiation of the trophoblast cells (Licht *et al.*, 1993; Licht *et al.*, 2001b).

Additionally, hCG has also been reported to have a stimulatory effect of trophoblast invasiveness. MMP-9, a key-element in trophoblast invasion, is also seen to be increased by the hCG (Licht *et al.*, 2001b; Klein *et al.*, 1995). Trophoblast migration is also stimulated by hCG through the effect of the insulin-like growth factor 2 (IGF-2) (Zygmunt *et al.*, 2005). In addition, to assisting the trophoblast in its early stages, the hCG is also reported to influence uterine factors by increasing LIF, vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2), an enzyme renowned for being involved in prostaglandin biosynthesis. It has also been reported that hCG is involved in endometrial vascularisation.

1.5.4.5 Progesterone (P4)

P4 is a steroid hormone belonging to the class of hormones called progestogens. This hormone supports ovulation and uterine and mammary gland development. During the follicular phase of the menstrual cycle, P4 levels are low, ranging from 1-2 nmol/l, while in the early-mid-late luteal phases, its concentrations increase to 15-20, 35-50, and 20-40 nmol/l, respectively (Arck, 2004). P4 is primarily secreted by the corpus luteum during the second half of the menstrual cycle. It is also important in maintaining pregnancy, where it is secreted by the placenta, starting at 12^{th} weeks of pregnancy (Arck, 2004).

P4 is important in preparing the uterus and ensuring a successful pregnancy. It stimulates the growth of the maternal breast tissue, prevent lactation, and strengthen the pelvic wall muscles in preparation for labour (Challis *et al.*, 2000). Owing to both, its endocrine and immunological effects in pregnancy, this hormone is considered as being crucial in the process of gestation from implantation through to delivery. P4 is believed to effect invasiveness by down-regulating MMP-9 expression, and is also reported to inhibit the invasion of the trophoblast into the endometrium (Staun-Ram and Shalev, 2005; Shah and Nagarajan, 2013). It is also responsible for triggering suppressor T lymphocytes

generation, inhibiting cytotoxic T cells activity, and reducing NK cells activity in the uterus (Yie *et al.*, 2006a; Brierley and Clark, 1987).

The immunological activity of P4, is believed to be modulated by the interaction with progesterone receptor (PR). Human progesterone receptor (hPR), is encoded by the *PGR* gene. Using separate promoters and translational start sites, *PGR* can be translated into two isoforms called PR-A and PR-B, where PR-B is longer than PR-A. Both isoforms are transcription factors, so they work as ligand-activated modulators of gene expression (Zakar and Mesiano, 2011). Upon immunological recognition of pregnancy, there is an up-regulation of PR on dNK cells and on the placental lymphocytes (Druckmann and Druckmann, 2005). In addition, in the presence of P4, activated lymphocytes and dNK cells produce progesterone-induced blocking factor (PIBF), which is a 34 kDa immunomodulatory protein that has several anti-abortive effects *in vivo* (Druckmann and Druckmann, 2005; Roussev *et al.*, 1993). The protective system of PIBF prompts an increase in asymmetric "blocking" antibodies, a Th2 biased immune response, and a reduction in NK cells activity. PIBF stimulates secretion of Th2 cytokines from activated lymphocytes, which are associated with B cell antibody production, whilst reducing the cytotoxic and pro-inflammatory Th1 type cytokines (Druckmann and Druckmann, 2005; Faust *et al.*, 1999). The Th2 cytokines are essential for the secretion of other pregnancy related hormones such as hPL and hCG from the trophoblast cells. This is important as the released hCG inhibits apoptosis of the corpus luteum, as well as induces the synthesis of P4 by the corpus luteum (Saito, 2000). Moreover, Th2 cytokines are also able to reduce the secretion of Th1 cytokines. This bias towards a Th2 profile leads to a favourable environment for a successful pregnancy (Dealtry *et al.*, 1998). Although the mechanisms by which P4 promotes immune tolerance have not been fully elucidated, P4 is also known to modulate HLA expression on trophoblast cells, which is one of the key players to protect the foetus from the maternal immune system. The Yie group reported that P4 enhances the expression of HLA-G both in protein and gene level using as a model the human trophoblasts cell line JEG-3 (Yie *et al.*, 2006b). HLA-G has been known to play a vital role in fetomaternal immunological tolerance with protecting the fetomaternal unit from NK cell lysis and T cell cytotoxic activity (Deniz *et al.*, 1994; Kapasi *et al.*, 2000). HLA-G inhibits

NK cell activity by binding NK receptors' ILT2 and ILT4 (Colonna, 1998). P4 also induces fetomaternal immune tolerance indirectly through promoting HLA-G expression.

1.5.4.6 Preimplantation factor (PIF)

Preimplantation factor (PIF) is a 15 amino acid peptide (MVRIKPGSANKPSDD) (Paidas *et al.*, 2010). This peptide is only secreted by viable mammalian embryos and is done so as early on as the two-cell stage and remains present throughout the course of a viable pregnancy (Stamatkin *et al.*, 2011a). Though PIF is present during the course of pregnancy, there is a progressive increase in circulating concentrations in the first and second trimester, while in the third trimester, prior to delivery, there is a notable decrease (Ramu *et al.*, 2013; Stamatkin *et al.*, 2011b). As it is secreted by the preimplantation embryo and later on by the placenta, this peptide is specific to pregnancy and plays a primordial role in implantation and the overall wellbeing of the embryo by acting directly on the decidua, hence modulating local immunity, enhancing embryo-decidual adhesion, and controlling apoptosis (Barnea *et al.*, 2012a). The Barnea group, have also reported that PIF has the capacity to modulate and orchestrate maternal immunity, without triggering any adverse immune outcomes, all while preparing the local uterine environment for implantation, the first step of pregnancy (Barnea *et al.*, 2012b). This peptide also has the ability to create a complimentary environment for early pregnancy as PIF is also believed to be the primary message of viability and acceptance that is secreted by the embryo to the mother, to promote maternal tolerance without triggering any form of suppression (Azar *et al.*, 2013; Barnea, 2007). This peptide has also been described as being universally mammalian, due to its cross species effect, non-toxic, as it is embryo derived, and is able to act in low doses in the range of nanomolar (nm). Its synthetic counterpart, sPIF also a 15 amino acid peptide is reported to mimic native PIF's features, and like PIF, inhibits NK cell cytotoxicity at low concentrations. However, scrambled PIF (sPIF*) has no effect on NK cell cytotoxicity and in no way aids the embryo during the implantation process. This clearly demonstrates the specificity of sPIF and PIF (Roussev *et al.*, 2013).

Figure 1.5 summarizes the main factors that have previously been enumerated as having an important impact or the ability to induce changes during the course of human pregnancy.

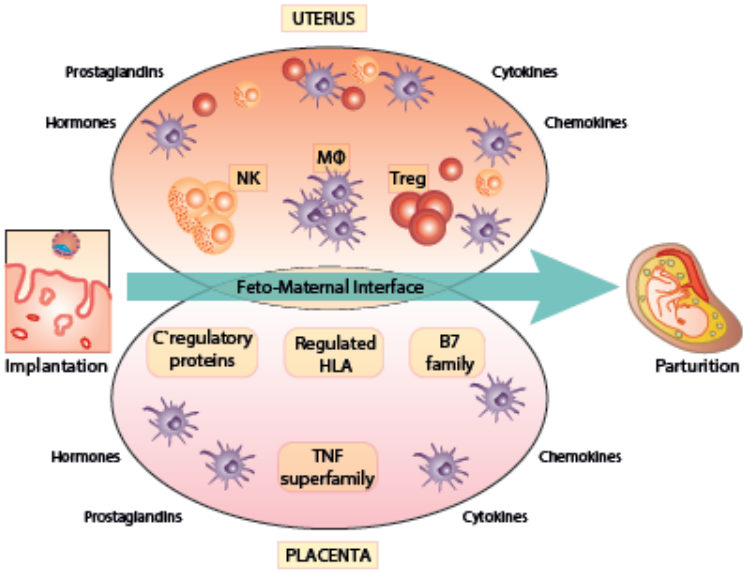

Figure 1.5. Role of soluble factors at the fetomaternal interface.
At the fetomaternal interface, several hormones, cytokines, HLAs, chemokines, and prostaglandins, among many other soluble factors are involved in order to establish a pregnancy. These soluble factors are important from implantation to parturition, and an imbalance in this tightly regulated environment can have deleterious effects.

1.6 The Major histocompatibility complex

The human major histocompatibility complex (MHC) is a set of molecules encoded by approximately 130 functional genes, which span the length of 4 Mbp on the short arm of chromosome 6 (Chow *et al.*, 2005; Salter, 2005) (Figure 1.6). The term "MHC genes" generally refers to the highly polymorphic "classical" loci that encode for class I and II antigen binding molecules (Chow *et al.*, 2005; Salter, 2005). These genes control lymphocyte recognition, antigen presentation, and immune response regulation; in humans these are referred to as HLA.

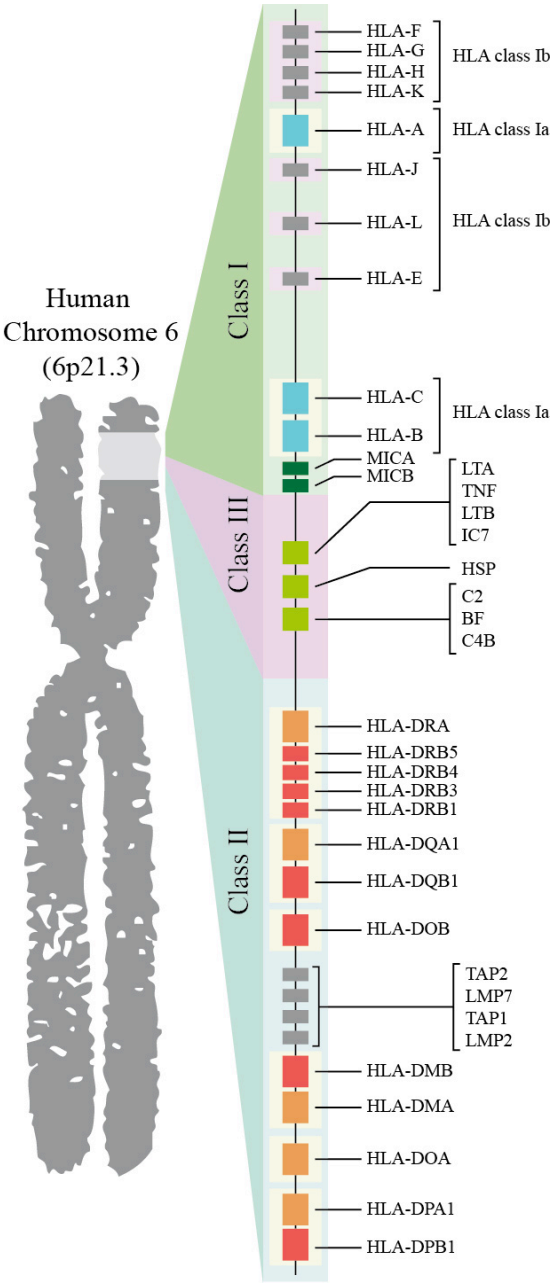

Figure 1.6. Human chromosome 6 and MHC region.
The MHC is codified in the chromosome 6, short arm, band 21.3. It can be divided in three main sub-regions that codified for different proteins: class I molecules (classical (Ia) and non-classical (Ib)), class II (mainly DR, DP and DQ) and class III (TNF, complement factors or LTA).

The *MHC* is categorized into three major classes: class I and II (which are expressed on tissues and cells), and class III (which is mainly present in serum and body fluids). These classes are further subdivided. The class I gene complex comprises of three major loci: A, B, and C, each of which encode for an alpha-chain polypeptide that associates to a soluble protein called β2-microglobulin (B2M) that is encoded on chromosome 15 (Fruh and Yang, 1999; Maenaka and Jones, 1999). HLA class I, are used by any somatic cell to present 8-11 amino acid intracellularly derived peptides to both, NK cells and $CD8^+$ T cytotoxic cells (Salter, 2005). Likewise, the class II gene complex also contains three loci, each of which encode for one alpha and one beta chain polypeptide which associate together to form the class II antigens. These loci are: DP, DQ and DR. Both of these types of receptors have a groove in their membrane distal domain (either α1α2 or α1β1) which binds to the peptides and displays them to different immunocompetent cells. *MHC* class III genes are more conserved than the class I and II genes, and are known to encode a range of secreted proteins, a few of which are involved in inflammatory responses, antigen processing and in the complement cascade (Singh *et al.*, 2007). In humans, *MHC* class II genes are renowned for their polymorphism. These genes mainly express their protein products on pAPCs such as dendritic cells, MΦ, and B cells. HLAs class II present 13-18 exogenously derived antigenic peptides

to $CD4^+$ T helper cells. These genes are also important in the preparing and maintaining of the humoral immune response (Maenaka and Jones, 1999).

As has been described, each HLA class I and II has a different structure and this structure is related to the type of antigen determinant (epitope) that they can load inside their groove or pocket. Basically, we can say that HLA-I display on the cell surface small epitopes because its pocket is closed, while the epitope that can be loaded inside the pocket of HLA-II is bigger as the pocket sides open. Another difference between both molecules is the type of molecules that are loaded inside the groove. Though they both load proteins, their origins are different. While HLA-I mainly displays epitopes from proteins synthetized inside the cell and processed by the endogenous pathway, HLA-IIs load proteins that have been internalized from outside the cell and processed by the exogenous pathways (Hviid, 2006; 1999). This is the reason as to why only pAPCs express HLA-II, as they are the only ones that can carry out phagocytosis. While the rest of the somatic cells also present antigens, they cannot carry out the process of phagocytosis.

HLA class I has developed into two distinct though structurally related subgroups termed classical or Ia, and nonclassical or Ib. Whilst the Ia molecules have evolved into highly polymorphic, omnipresently expressed membrane bound molecules, making them an important factor in establishing immune response diversity; the Ib molecules, on the other hand, have maintained a selective and limited polymorphism with restricted expression (regulated and tissue restricted) (Kedzierska and Turowski, 2001). The Ib molecules, which include HLA-G, -E and –F, are believed to have a more specialised function in antigen presentation than their Ia counterparts. The *MHC* class I genes polymorphism is concentrated in their epitope binding region and this polymorphism is generated by mutations that introduces small changes in the amino acid sequence that can be conserved or not (balancing selection). HLA class I have been identified as being expressed during pregnancy by the trophoblast, along with the interactions that take place between the different HLA molecules, and between HLA molecules and components of the immune system during pregnancy. In the human body, trophoblast cells are one of just of handful of physiological cellular populations that don't have the typical HLA profile, as they don't express HLA class Ia -A and -B or class II.

1.6.1 Human leukocyte antigen G (HLA-G)

HLA-G was the first HLA-Ib to be identified and still holds great interest (Hunt *et al.*, 2006; Moreau *et al.*, 2002). Due to its features HLA-G can be considered as a very unique molecule. The gene that encoded HLA-G (*HLA-G)* is comprised of 8 exons, which are arranged in a similar sequence as that of the other class I genes. These 8 exons can suffer from alternative splicing that can produce seven transcripts, i.e. in exon 6, a premature stop codon during the translation can cause a HLA-G with a truncated cytoplasmic tail which can explain why this isoform has a slower turnover rate, and a prolonged cell surface expression (Hunt *et al.*, 2005).

1.6.1.1 HLA-G isoforms

In addition, *HLAG* gene is also known to be alternatively spliced to give rise to seven transcripts; four membrane bound and three soluble (McCormick *et al.*, 2009; Kovats *et al.*, 1990). HLA-G1, apart from its truncated cytoplasmic tail is similar in structure to its class I genes. HLA-G2 isoform differs from the primary transcript in that it lacks exon 3, and is known to form a class II like structure through homodimerization (Ishitani and Geraghty, 1992). HLA-G3 isoform is the result of spliced exons 3 and 4, while HLA-G4 transcript has a spliced fourth exon. HLA-G5 and HLA-G6 transcripts are the same as HLA-G1 and HLA-G2 transcripts, with the inclusion of intron 4 sequences, and the fact that they are expressed as soluble isoforms (McCormick *et al.*, 2009; Hunt and Langat, 2009). Exon 2 and intron 2 are both part of HLA-G7, though in this transcript, intron 4 is spliced. To date, little is known about HLA-G4 and HLA-G7 transcripts, though it has been reported that in the placenta, their mRNAs are not present in abundance (Hunt *et al.*, 2005; Hunt and Langat, 2009).

1.6.1.2 HLA-G ligands

Studies by the Hunt group and Apps group have shown that the leukocyte immunoglobulin-like receptors (LILR) are the major HLA-G receptors on leukocytes (Hunt *et al.*, 2005; Apps *et al.*, 2007). The LILR group has 13 loci, is expressed by NK cells, mononuclear phagocytes, T and B lymphocytes and is believed to interfere with activating signals (Hunt and Langat, 2009; Apps *et al.*, 2007). From the LILR family, two inhibitory receptors, LILRB1 and LILRB2 distinguish all of HLA-I (Borges and Cosman, 2000). For lymphocytes, it is stipulated that LILRB1 is the main binding receptor, while

LILRB2 is believed to be the primary receptor on MΦ and DCs for HLA-G (Hunt and Langat, 2009). In a study carried out by the Apps group, it was shown that all HLA-DR^+ cells in the decidua expressed LILRB1 and LILRB2 (Apps *et al.*, 2007). In addition, various experiments from other groups have demonstrated that the modulation of myelomonocytic cells function is the direct result of HLA-G binding with LILRB, and that such an interaction has an impact on antigen presentation (Apps *et al.*, 2007; Ristich *et al.*, 2005).

1.6.1.3 HLA-G in pregnancy

HLA-G is a molecule of interest as it may hold the key to human successful reproduction. Researchers consider that a pregnancy specific signal is delivered to the local maternal leukocytes by HLA-G trophoblast cells as they infiltrate the uterine mucosa, thereby changing the function of these cells so as to hold the placenta and foetus (Apps *et al.*, 2007).

1.6.2 Human leukocyte antigen E (HLA-E)

Like HLA-G, HLA-E is a molecule with low polymorphism compared to its classical class I counterparts and is one which is highly conserved (Shaikly *et al.*, 2010). Widely expressed, "cell-surface expression of HLA-E heavy chain requires the formation of a non-covalent complex with β2M and a leader peptide antigen, derived from the N'-terminal sequence of other HLA class I molecules such HLA-G or HLA-C (Shaikly *et al.*, 2010; Rodgers and Cook, 2005). Protection to the foetus from the maternal system may be the result of the "affiliated" cell surface expression of HLA-G and HLA-E, as the cell surface expression of the latter is dependent on that of the former (Shaikly *et al.*, 2010). In 2004, Trundley and Moffet reported that trophoblast cells were protected from NK cytotoxicity due to the binding of HLA-E with CD94/NKG2A expressed on the surface of uNK cells (Trundley and Moffett, 2004). The Shaikly group, further hypothesized that this interaction may also be responsible for controlling other "functions" at the site of implantation (Shaikly *et al.*, 2010). The Ishitani group, reported "overlapping expression" at the trophoblast cell surface of HLA-G and HLA-E (Ishitani *et al.*, 2003). This results were reconfirmed in 2010 by the Shaikly group who also showed that HLA-G and HLA-E are co-localized on the cell surface in clusters of four molecules (tetramers) or other higher order clusters (Shaikly *et al.*, 2010). These clusters are a sign that, at the cell surface, HLA-E and HLA-G work

together to unite to receptors. The juxtaposition of these clusters on the implanting blastocyst is important at the fetomaternal interface as they are now believed to be responsible for the modulation of the immune responses of the leukocyte cells present at this site (Shaikly *et al.*, 2010).

1.6.3 Human leukocyte antigen C (HLA-C)

Of HLA-Ia, only HLA-C is expressed by the trophoblast cells at the fetomaternal interface. This molecule is highly polymorphic, in comparison to HLA-Ib expressed by the trophoblast, and has the ability to elicit an allogenic T cell response (Heemskerk *et al.*, 2005). Due to its relative polymorphism, HLA-C could thus elicit a maternal anti-foetal acquired immunity, if the paternal alleles differed from those of the mother (Hunt *et al.*, 2005). HLA-C is reported to interact with KIR2D receptors, which are expressed by the uNK cells and, is also believed, that this molecule also has the ability to control the cytokine profile of the uNK cells. HLA-C is expressed by the invasive or extravillous trophoblast cells. HLA-C has two main haplotypes called C2 and C1, which may be recognized by KIR2DL1/KIR2DS1 and KIR2DL2/KIR2DL3/KIR2DS2, respectively. C2 binds and interacts with KIR with more affinity than C1 (Boyington *et al.*, 2001; Nowak *et al.*, 2011a; Nowak *et al.*, 2011b). Different aggrupation and interaction of maternal uNK cell KIRs and trophoblast HLA-C will have different effects on the trophoblast cells, i.e. a combination of HLA-C1 and $KIRB^{+}$ uNK cells will promote trophoblast invasion, however HLA-C2 and $KIR2^{+}$ uNK cells will decrease trophoblast invasion. This combination is one which dominates in woman suffering from pre-eclampsia (Hiby *et al.*, 2004; Sargent *et al.*, 2006). This thus demonstrates that HLA-C KIR epitope and maternal KIR incompatibility (ultimately, a foetal mismatch) results in an increase in activated maternal T cells in the decidua, ultimately leading to pregnancy complications and spontaneous abortions (Tilburgs *et al.*, 2009). Though all HLA class Ib are expressed by the trophoblast cells, they are done so at different levels, and they serve different functions.

1.6.4 Human leukocyte antigen F (HLA-F)

HLA-F was the last class Ib HLA to be discovered. This molecule, expressed as a β2M-associated protein of 40-41 kDa, was first identified by the Geraghty group in 1990 (Geraghty *et al.*, 1990). Like the other class Ib molecules, HLA-F displays very low allelic polymorphism and is highly conserved. However, compared to the other HLA class I, HLA-F is shorter. In the mature HLA-F mRNA exon 7 is

missing, hence yielding an HLA-F protein that has a shortened cytoplasmic domain in comparison to the other HLA molecules (Lee *et al.*, 2010). HLA-F expression has been identified in the cytoplasm of peripheral blood B cells, some tissue cells, but most importantly expression of HLA-F was seen on extravillous trophoblast normal term placenta cells (Ishitani *et al.*, 2003; Shobu *et al.*, 2006). Expression of HLA-F by the trophoblast cells suggests that this molecule plays an important role in the fetomaternal interaction. Though countless studies, HLA-F expression have been detected in the cytoplasm of the thymus, tonsil, and spleen cells but not on their surfaces, while their function has yet to be elucidated (Wainwright *et al.*, 2000). The Shobu group had reported HLA-F expression in the cytoplasm of decidual extravillous trophoblasts during early gestation, and on the surface of these cells from mid-gestation to term, while the expression of this molecules was weak in the cytoplasm of the villous cytotrophoblasts and syncytiotrophoblasts (Shobu *et al.*, 2006). Tetramers of HLA-F molecule have been identified to bind to the ILT2 and ILT4 NK receptors however, it still needs to be identified whether or not HLA-F has an NK inhibitory function (Shobu *et al.*, 2006; Lepin *et al.*, 2000). In addition, HLA-F, like HLA-E expression, is seen to increase towards the later stages of pregnancy. Extravillous trophoblast expression of HLA-F is reported to be increased, and to move towards the cell surface during the later stages of pregnancy. This, hence suggesting this molecule plays an important role in the maintenance of pregnancy. Though several studies have been carried out on HLA-F and its expression, to date little is known about its function.

1.6.5 HLA in pregnancy and medicine

The MHC, since it was discovered in 1967, has significantly advanced the field of not only immunology, and reproductive immunology, but also the fields of organ and tissue transplantation (Bernard, 1967). However, to date, due to the very complex immunobiology of transplantation and due to the involvement of several components, namely antibodies, antigen presenting cells, cytotoxic T cell subsets, immune cells cytokines, etc. which all play an important role in innate and adaptive immunity, the mechanism with which a graft can avoid rejection has not been elucidated. The foetus, which is considered to be a semi-allograft, however, is able to survive in the maternal body for nine months, but more importantly, it is protected, and nourished, without causing any deleterious effects to the mother.

The way in which the foetus is able to avoid maternal rejection, is still not well understood. However, if such an event is understood, this may give the field of medicine a breakthrough where the organ transplantation is concerned.

1.7 Project aims

HLA class I have been reported to be expressed at the fetomaternal interface by the trophoblast cell. The expression of these HLAs is pivotal if the foetus is to avoid maternal rejection. However, these proteins are not the only factors used by the semi-allograft to avoid rejection, as the fetomaternal interface is a site of complex interactions, where several factors work together to not only protect the foetus, but also ensure the well-being of the mother. In the present study, the effects of soluble factors on the human-derived-trophoblast cell line JEG-3 and ACH-3P were studied, as a means by which to determine what soluble factors are involved at the fetomaternal interface.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell lines and cell culture techniques

2.1.1 Cell lines

Cell lines used in this study included the human first trimester choriocarcinoma trophoblastic cell lines JEG-3 and ACH-3P. The JEG-3 cell line was kindly provided by Professor I. L. Sargent of the Nuffield Department of Obstetrics and Gynecology at the University of Oxford, and its characterization and establishment is described in previous studies (Kohler and Bridson, 1971; Kohler *et al.*, 1971; Fogh *et al.*, 1977). ACH-3P, a novel trophoblast hybrid cell line, was kindly provided by Dr. G. Desoye of the Department of Obstetrics and Gynecology at the Medical University of Graz, Austria. Establishment and characterization of the ACH-3P cell line, is described in a study by the Hiden group (Hiden *et al.*, 2007). The cell lines used were routinely maintained in our laboratory in small aliquots stored in liquid nitrogen. A description of all cell lines used in this study is given in Table 2.1. Both cell lines were not DNA fingerprinted as there is no consensus in the scientific community about it. The ATCC has a document with the title "Recommendations (see appendix)", here, you can read the recommendations that the ATCC suggests. Clearly, the ATCC shows that there is no established protocol by which to characterise a cell line, but does suggest some techniques. We used morphology, growth ratio, mycoplasm detection (our group technician Julie Ardvison did it for us) and finally, we did flow cytometry for specific antigens for human trophoblast-derived populations. We thought that it was enough, even when there is not a standard protocol. Another fact that has to be taken into account is the cost/effectiveness of DNA fingerprinting: this is quite an expensive technique that needs to be checked with another technique like Western blot, as not all mRNAs are translated to proteins. It is obvious that cell lines are not going to have the same behaviour as real tissue but sometimes, in research, it is important to understand that access to human tissue is not always easy and facilities are not always up to a standard which is required when working with human tissue. So, in very early research stages it is better to start and establish a cellular model using cell lines and then extrapolate it to isolated human tissue. One the advantages that cell lines have is that they are supposed to be homogeneous so we can avoid the bias introduced by the characteristic of each human donor.

Table 2.1. List of human-derived-trophoblast cell lines used

Cell line	Description	Source	Reference
JEG-3	Human placental choriocarcinoma cell line. Cell line comprised of one main population; extravillous cytrophoblast.	Professor I. L. Sargent; Nuffield Department of Obstetrics and Gynecology at the University of Oxford.	(Kohler and Bridson, 1971)
ACH-3P	First trimester human trophoblast choriocarcinoma cell line. Comprised of two main subpopulations extravillous cytotrophoblast and the villous cytotrophoblast.	Dr. G. Desoye; Department of Obstetrics and Gynecology at the Medical University of Graz, Austria.	(Hiden *et al.*, 2007)
ACH-3P HLA-G^+ subpopulation	First trimester human trophoblast choriocarcinoma cell line. Comprised of one extravillous cytotrophoblast population.	Specific HLA-G cell sorting	
ACH-3P HLA-G^- subpopulation	First trimester human trophoblast choriocarcinoma cell line. Comprised of one villous cytotrophoblast population.	Specific HLA-G cell sorting	

* All cell lines were grown in either 25 cm^3 or 75 cm^3 tissue culture flasks (Nunc) or in tissue culture grade Petri dishes (Gibco). Both HLA-G^+ and HLA-G^- ACH-3P subpopulations were generated through an HLA-G cell specific cell sorting using a BD FACS Aria I.

2.1.2 Media and cell culture growth conditions.

The JEG-3 cell line used in this study was cultured in Dulbecco Modified Eagle Medium/Ham's F-12 (DMEM-F12) (PAA-GE Healthcare) supplemented with 10% of foetal calf serum (FCS) (Hyclone). ACH-3P was maintained in Ham's F-12 with stable glutamine medium (PAA-GE Healthcare), supplemented with 10% of FCS. The ACH-3P HLA-G^+ and HLA-G^- sorted subpopulations were both maintained in the same medium as the unsorted cell line.

Cells were maintained in a cellular incubator with a full humidified atmosphere at 37 ºC and 5% CO_2. Contamination controls and incubators maintenance and linear checking were done with the help of our laboratory technician, Julie Ardvison. Once per week, the sensors for temperature and CO_2 concentration were checked with external probes to avoid bias in the culture conditions.

2.1.3 Cell culture techniques

2.1.3.1 Passaging

All the cell lines used in this study grow as an adherent monolayer, and require passaging when they are approximately 70-80% confluent. When passaging the cells, the culture media was removed, and cells washed with 3 ml of pre-warmed (37 ºC) phosphate-buffered saline (PBS). Cells were then detached by adding 1 ml of pre-warmed Accutase™ (PAA-GE Healthcare) and incubating the flask in a dry chamber at 37 ºC for 2-8 min. After examining the cells under the microscope, to ensure their detachment, the Accutase™ was diluted ten times with the appropriate warm complete media. The media was then gently pipetted to help cells detach from the flask and to avoid cell clump formation. The cell suspension was then transferred to a tube and centrifuged at 1237 *g* for 5 min. The supernatant was aspirated off, and the cell pellet was re-suspended in fresh complete media. Cells were either transferred to new flasks for future culture or used for experiments. The maximum culture time that the cells could be maintained in culture for was up to one month, after which they had to be replaced with a fresh stock.

2.1.3.2 Counting and seeding of cells

To count the number of cells, 10 µl of the final cell suspension was obtained as described above and the cell number determined through the use of a hemocytometer. The average number of cells present in four corner squares was determined and multiplied by 10^4 giving us the concentration of cells per ml. Upon determination of a cell count, cells were diluted to the required concentration which was normally between $1.0x10^5$ - $1.5x10^5$ cells/ml using appropriate media, and were seeded in six wells plates, microscopy chambers or flasks, as required per the experimental set up.

2.1.3.3 Freezing and defrosting cell stocks

Cells were passaged as described in section 2.1.3.1, a cell count was carried out and 0.8 ml of the cell suspension (approximately $1x10^6$ cells/ml) was diluted with 0.8-1.0 ml of freezing media (10% Dimethyl Sulfoxide (DMSO) (Sigma) and 90% FCS). The cell mix was transferred to cryotubes (Nunc) and stored at -80 ºC freezer wrapped with insulating material in order to prevent rapid cooling, that will avoid ice crystal formation that could damage the cells. After 3-4 days, these cells were then moved to a liquid nitrogen container for longer storage.

To thaw the cells, the cryogenic tubes were removed from liquid nitrogen using dry ice to transport them from the cryogenic facility to the cell culture room. Tubes were then placed in a dry chamber, to avoid possible contamination, and pre-warmed at 37 ºC so as to defrost them quickly. The thawed cell suspension was centrifuged at 1237 *g* for 5 min and the supernatant was removed, as at room temperature (RT) DMSO is toxic and can induces changes in the behaviour of the cells (Preisler and Giladi, 1975; Galvao *et al.*, 2014). The cell pellet was then pipetted into a small culture flask containing 10 ml of pre-warmed culture medium and maintained at 37 ºC and 5% CO_2, until the cells reached 70-80% confluency

2.1.4 Cell stimulations

2.1.4.1 Progesterone stimulations

Progesterone (Sigma-Aldrich) was diluted to a final concentration of 1 mg/ml using absolute ethanol, as was suggested by the manufacturer. This was further diluted in DMEM-F12 medium to a concentration of 1 µg/ml.

To assess the effect of P4 on JEG-3 cells HLA profile and cytokine expression, cells were passaged and cultured at a density of $1x10^6$ cells/ml in complete medium. After 24 h, the cells were serum starved by replacing the medium with DMEM-F12 supplemented with 0.1% FCS (starving medium). Cells were incubated in this medium for 6 h after which it was refreshed and supplemented with P4 at a final concentration of 1 µg/ml. Cells were cultured in starving medium to decrease HLA surface expression as its turnover is around 3 hours (Mandelboim *et al.*, 1997; Davis *et al.*, 1997).

For dose dependent experiments, cells were incubated with concentrations of P4 ranging from 1-1000 ng/ml for a period of 24 h. For time dependent experiments, cells were incubated with P4 at a dose of 1000 ng/ml for time periods ranging from 0-72 h.

2.1.4.2 Preimplantation factor stimulations

Preimplantation factor, which was kindly supplied by Dr. Eytan Barnea (Society for the Investigation of Early Pregnancy and BioIncept, LLC, Cherry Hill, NJ, USA) and was reconstituted in double distilled water to a final concentration of 20 µM.

JEG-3 cells were cultured in complete medium at a density of $1x10^6$ cells/ml. After 24 h, the medium was changed for fresh starving medium. Cells were incubated in this medium for 6 h after which the starving medium was refreshed and supplemented with PIF. For dose dependent experiments, cells were incubated with concentrations of PIF ranging from 0-1000 nM for a period of 24 h. For time dependent experiments, cells were incubated with PIF at a dose of 200 nM for time periods ranging from 0-72 h.

2.1.4.3 IL-17 stimulations

IL-17RA (Life technologies) was reconstituted in sterile double distilled water to a concentration of 200 µg/ml. This was further diluted to a final concentration of 100 ng/ml using culture medium.

To assess the effect of IL-17 on the secretion of P4 by JEG-3 cells, cells were passaged after which they were re-suspended at a density of $1x10^6$ cells/ml in complete medium. After 24 h, the medium was removed and fresh starving medium was added. Cells were incubated in this medium for 6 h after which the medium was changed with fresh starving medium and supplemented with IL-17RA at final concentration of 1, 10 and 100 ng/ml, for 24 h. This was the range of concentration of IL-17 used, as it is in this range that IL-17 can exert activity in a wide variety of *in vitro* applications (Pongcharoen and Supalap, 2009). Controls for this experiment were JEG-3 cells incubated in medium without IL-17RA stimulation. After 24 h, the supernatant was harvested and kept at -20 ºC until determination of P4 secretion.

2.1.4.4 Enzyme Linked Immunosorbent Assay for cytokine determination

An enzyme linked immunosorbent assay (ELISA), is a biochemical technique that is widely used in the laboratory (Berg *et al.*, 2002). This technique combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antigens or antibodies coupled to an easily-assayed enzyme, by which to detect the presence of these, and provide a measurement of their concentration in a wide variety of biological samples was first described by Perlmann and Engvall in 1971 (Engvall and Perlmann, 1971). We can consider two among the several types of ELISA. The indirect ELISA is used to detect the presence of antibody and is one of the techniques used to test for HIV infection. In that test, viral core proteins (the antigen) are absorbed to the bottom of a well. Antibodies from a patient are then

added and allowed to bind to the antigen. Finally, enzyme-linked antibodies against human antibodies are added and allowed to react. Unbound antibodies are removed by washing. Substrate is then applied. A coloured well implies that the patient had antibodies to the viral antigen. The sandwich ELISA allows both the detection and the quantification of antigens. Antibody to a particular antigen is first absorbed to the bottom of a well. Next, the sample containing the antigen is added to the well and allowed to interact. Finally, a second, different antibody to the antigen is added. This antibody is enzyme linked and is processed as described for indirect ELISA. In this case, the extent of reaction is directly proportional to the amount of antigen present. Consequently, it permits the measurement of small quantities of antigen. One variation that we can use is to change the linked-enzyme for a fluorescent-linked antibody. In addition, we can also link an isotope, this technique is called RIA (radioactive immunosorbent assay) (Crowther, 1995).

To carry out the ELISA, supernatants from stimulated and unstimulated cellular cultures were collected. The concentrations TNF-α, IL-1, IL-8, IL-10, IFN-γ, TGF-β1, GM-CSF, and P4 were measured through the use of commercially available ELISA kits (eBioscience), following the manufacturer's instructions. In summary, the protocol required dilution of the capture antibody, which is a monoclonal antibody specific for the respective human biomolecule, to a concentration of 3 µg/ml in coating buffer. Using a 96 well plate, 100 µl of this antibody was then applied to the appropriate wells, after which the plate was covered, and incubated in 4 overnight. The plate was then washed five times with PBS and blocked adding 200 µl of blocking buffer. The plate was then incubated at room temperature (RT) for 2 h. 100 µl of the protein standards or samples was then added into the wells, after which the plate was again incubated for 2 h at RT. The plate was washed three times with PBS to remove any unbound antigen. Then, 100 µl of biotin conjugated antibodies specific for the appropriate cytokine were added to each of the wells, and the plate was incubated at RT for 1 h. Plate was washed five times using PBS and 100 µl of substrate added. The reactions resulted in the wells turning blue, this indicated a positive reaction. The stop solution was then added, resulting in a yellow colour. The absorbance of each well was measured through the use of an ELISA reader at 450 nm, and a standard curve used to determine the concentration of each cytokine was obtained. The samples were each assayed as triplicates.

2.1.5 Cell proliferation assay

A way in which to measure cell proliferation is through the metabolic activity of the cellular population. One such assay is the CellTiter96 AQueous One Solution Cell Proliferation Assay (Promega), which is a colorimetric method based on the use of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) as well as an electron coupling reagent (phenazine ethosulfate; PES). As PES has a solid chemical stability, it is combined with MTS so as to form a stable solution. The principle is centred on the reduction of the MTS tetrazolium compound by cells into a coloured formazan product that is soluble in cell culture medium. This change is attributed to NADH, which is produced by the dehydrogenase enzymes found in metabolically active cells. Briefly, this assay is carried out by adding a small amount of CellTiter96 AQueous One Solution Cell Reagent directly into cell cultures and incubating these for a period of 1-4 h. Then, the absorbance is measured using a 96-well plate reader at 490 nm (1992). In this assay, the quantity of formazan produced is directly proportional to the number of live cells in culture.

Cell proliferation was measured using the MTS CellTiter96 AQueous One Solution assay following the manufacturer's instructions. To do this, cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate in 100 µl of fresh complete medium. Following 24 h, cells were then serum starved for a period of 6 h, after which they were treated with fresh starving medium or with fresh starving medium containing the appropriate concentrations of P4, PIF or hCG (1000 ng/ml, 200 nM or 1000 ng/·ml, respectively). Camptothecin (Sigma-Aldrich), a well-known cell apoptosis inducing agent, was used as negative control at a concentration of 4 mM in starving medium. Cells were then incubated at 37 ºC and 5% CO_2 for time different time points (0-72 h). Following incubation, 20 µl of cell titter solution reagent was added to each well. Absorbance was then measured at 490 nm using a microplate reader.

2.1.6 Invasion assay

An invasion assay provides means by which to study the invasion capacities of cells in an *in vitro* environment. This assay makes use of a modified Boyden chamber-like design consisting of two chambers that are separated by a filter coated with extracellular matrix components (Albini *et al.*, 1987a; Albini *et al.*, 1987b). Cells are then placed in the top chamber, and incubated in the presence of a specific

medium that contains certain chemoattractants in the bottom chamber. Cells will migrate from the top chamber through the coated filter pores to the bottom filter. Cell invasion is then detected and quantified using Calcein acetomethylester moiety (AM). The migrated cells are then dissociated from the filter through the use of Calcein AM solution, which is internalized by the cells, and causing the intracellular esterases to cleave the AM. As the free Calcein fluoresces brightly, it is thus used to quantify the number of cells that have migrated.

To assess JEG-3 cells invasion capacity, Matrigel-coated (Corning) porous filters (8.0 μm pore size) were used as a barrier in a Boyden-like chamber. The cells were seeded in a multi-well plate in starving medium at a concentration of $5x10^4$ cells/well and incubated under standard culture conditions. Different soluble factors were added to the medium (PIF at a concentration of 200 nM and P4 at a concentration of 1000 ng/ml). Five wells were used for each stimulation. After incubation and fixation with 70% ethanol, the non-invasive cells were removed from the upper surface of the membrane through the use of a cotton swab, and stained with haematoxylin and eosin. The filters were then examined under the microscope for cells on the underside of the membrane. The number of cells in eight fields was then counted and the mean determined. The invasion was expressed as the number of cells invading through the Matrigel relative to the cells migrating through the control membrane.

2.1.7 Methods for total protein extraction and analysis

The Western blot (Wb) technique is used to separate and identify proteins. Using this technique, proteins are separated based on their molecular weight, through the use of gel electrophoresis. The proteins are then transferred to a membrane, which is then incubated with antibodies specific to the protein of interest.

2.1.7.1 Preparation of samples for protein analysis by SDS-PAGE

2.1.7.1.1 Cell lysates

Treated and untreated cells, as per the experimental set up, were detached, counted, and pelleted as described in section 2.1.3.1. They were immediately lysed using sodium dodecyl sulphate (SDS)-lysis buffer at a ratio of $1x10^5$ cells per 20 μl of buffer. The cell pellet was then vortexed, and heated at 95 ºC

for 5 min after which it was stored at -20 ºC until required for protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.1.7.1.2 Protein concentration

A Bradford protein assay (BCA) is used as a tool to measure the total protein concentration of a sample. This assay is based on the principle that when the proteins bind to the Coomassie dye under acidic condition, a shift in absorption occurs from 465-595 nm resulting in a colour change from brown to blue. This assay was carried out using the BCA, as previously described (Bradford and Richards, 1976). We used bovine serum albumin (BSA) at a concentration of 4 mg/ml as a calibration standard. Five microliters of the standard was diluted sequentially in a microplate with PBS to produce a standard curve. Five microliters of each protein sample was then aliquoted into a microplate, using 5 µl of PBS as a control. Two hundred and fifty microliters of Bradford reagent (Sigma-Aldrich) were then added to each well. The microplate was incubated at RT for 5 min, and absorbance was read using a plate reader at 595 nm. Protein concentrations for each sample was determined using the standard curved using the absorbance of the standards.

2.1.7.1.3 SDS-PAGE and Western blot analysis.

SDS-PAGE is one of the most commonly used analytical method to separate components of a protein mixture based on their molecular weights under the effect of an electric charge. For this technique, a discontinuous polyacrylamide gel is used as a support medium. SDS, is also used to denature the protein, so that all the samples acquire a uniform charge, so that the separation (electrophoretic mobility) will only depend on the molecular weight (Mw) of the protein. SDS is also used as it will give the proteins a negative charge, so that when loaded onto a gel, and placed in an electric field, they will migrate towards the anode, and will be separated by a molecular sieving effect that is based on size: the smaller molecules will migrate faster than the larger ones, which are restrained by the polyacrylamide gel matrix (Chrambach and Rodbard, 1971).

In this research, we used a modified SDS-PAGE system (Bio-Rad), which is nevertheless based on the original technique (Laemmli, 1970; Laemmli *et al.*, 1970). Cell lysates were prepared as described in 1.7.1.1. Samples were diluted five times in SDS-sample buffer and heated for 5 min at 95 ºC. Samples

were then loaded onto the casted polyacrylamide gel (stacking gel at 4%) and resolved in the resolving gel at 12% for 30 min (table 2.3) at 30 V after which, it was increased to 100 V until the gel finished running (approximately 1 h - 2 h). A pre-stained standard protein marker (Fermentas), containing proteins of known Mw, was also loaded onto the gel and run parallel to the protein samples. After the separation using SDS-PAGE, protein samples can be visualized using a Coomassie blue staining (Meyer and Lamberts, 1965). However, as we said, in this study we were more interested in looking for predetermined proteins of known sizes, SDS-PAGE was thus followed by a technique referred to as Wb or immunoblotting. All the buffers and gel compositions are summarized in Tables 2.2 and 2.3.

2.1.7.1.4 Western blotting

Western blotting is a technique used in the detection and analysis of proteins based on their ability to bind to specific antibodies. In this technique, the proteins resolved by SDS-PAGE, are transferred to a polyvinylidene difluoride (PVDF) membrane, which is then incubated with a specific antibody against a target protein (Towbin *et al.*, 1979). The transferred protein is further detected using a secondary antibody that is either conjugated to an enzyme that produces a chemiluminescence signal in the presence of an appropriate substrate, or that is labelled with a fluorescent tag. When the unbound antibody is washed off, only the antibody that is bounded to the protein of interest is left. Development of the signal will confirm the presence of the protein of interest in the total cell lysate analysed. The thickness of the band produced by the antibody corresponds to the amount of protein, hence this is why this technique is often referred to as semi-quantitative: using a standard can help to calculate the amount of protein present.

Our proteins resolved using SDS-PAGE, were transferred onto a PVDF membrane (Immobilion P Millipore Inc.), through the use of a semi-dry electro blotting apparatus Mini Trans- Blot Cell (Bio Rad) using the following method. Before transfer, the SDS-PAGE gel was incubated in gel running buffer for 15 min. The PVDF membrane was dehydrated in absolute methanol for 10 sec and immediately washed with reverse osmosis (RO) water. A stack/sandwich consisting of sponge, three Whatman papers soaked in transfer buffer (Table 2), followed by the PVDF membrane, the SDS-PAGE gel, three Whatmann papers, and sponge was then made. To ensure there were no air bubbles between the gel and the PVDF

membrane, a glass rod was gently rolled over the stack. This stack was placed on the semi-dry electro blotter, which was transferred to the blotting apparatus. Ice cold transfer buffer was added to the apparatus, a water cooling core added, the electrodes placed, and the apparatus set to transfer for 1 h at 110 mA/40 V. After transfer, the stack was disassembled, and the membrane washed with RO water. The membrane was then incubated in blocking buffer (Table 2.2) for 1 h at RT or overnight at 4 ºC. After blocking, the membrane was washed with washing buffer PBS-T (Table 2.2). This was then probed with the primary antibody diluted in washing buffer (against the protein of interest) for 1 hr at RT or overnight at 4 ºC. The membrane was then washed three times with washing buffer (10 min each wash), after which the membrane was incubated with secondary antibody IRDye 800CW© Donkey anti-Mouse IgG (Li-Cor Biosciences) prepared in washing buffer for 1 h at RT. Signals were detected using the OSDSSEY© infrared imaging system (Li-Cor Biosciences).

It is very important to be aware that the data produced with a Wb is typically considered to be semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of quantity. There are two reasons for this. First, there are variations in loading and transfer rates between the samples in separate lanes, which are different on separate blots. These differences will need to be standardized before a more precise comparison can be made. Second, the signal generated by detection is not linear across the concentration range of samples. Thus, since the signal produced is not linear, it should not be used to model the concentration.

Table 2.2. Western blot buffer composition

Buffer	**Composition**
SDS-lysis buffer	1% SDS (0.1 g SDS + 8 ml deionized water: adjust water accordingly so final volume is 10 ml), 100 mM Tris-HCl pH 7
PBS	2 mM KH2PO4, 10 mM Na2PO4, 137 mM NaCl, and 2.7mM KCl (pH 7.4)
Blocking buffer	0.1% Tween, 3% dried skimmed milk and PBS
Washing buffer	0.1% Tween and PBS
Tris-glycine running buffer	25 mM Tris/HCl, 250 nm glycine, and 0.1% SDS
Transfer buffer	20 mM Na_2PO_4, 20% Methanol, and 0.05% SDS

Table 2.3. Western blot gels composition

Resolving gel composition (12%)	
1.5 M Tris pH8.8	3.8 ml
30% acrylamide	6 ml
RO water	4.9 ml
20% SDS (20 g SDS + 80 ml deionized water: adjust water accordingly so final volume is 100 ml)	100 µl
10% APS	150 µl
TEMED	6 µ

Stacking gel composition (4%)	
0.5 M Tris pH 6.8	2.5 ml
30% acrylamide	1.33 ml
RO water	6.1 ml
20% SDS (20 g SDS + 80 ml deionized water: adjust water accordingly so final volume is 100 ml)	25 µl
10% APS	50 µl
TEMED	10 µl

2.1.8 Flow cytometry

Flow cytometry is a technique that measures the optical and fluorescence characteristics of single cells or any other particles. Using this technique, cells are introduced into a sheath of buffer fluid and are passed by a laser beam one at a time. When the labelled cells pass the light source, the fluorescent molecules that are conjugated to the antibody are excited to a higher energy state, and when these return to their resting state, the fluorochromes emit light energy at higher wavelengths. As the particles pass through the laser beam, several parameters can be measured amongst which are the forward scatter (FSC), side scatter (SSC) and the fluorescence intensity, which are determined using the three main systems of the flow cytometer, these being optics, fluidics and electronics (Figure 2.1).

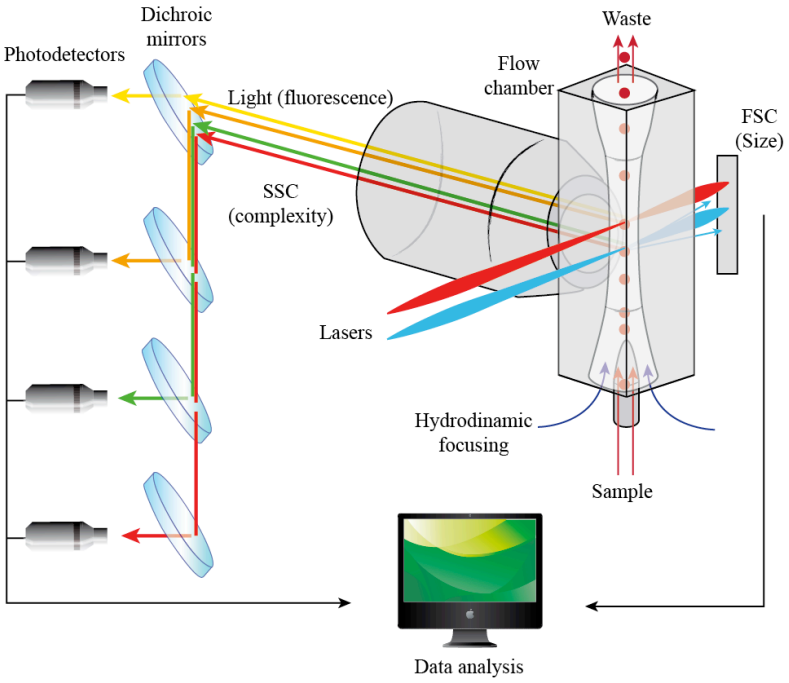

Figure 2.1. Summarized flow cytometry system.
The hydrodynamic focusing allows the analysis of single events one by one. The events interact with the lasers and the first two parameters are measured, size (FSC) and complexity (SSC). If the samples is stained with an immunofluorescence tagged antibody, the florescence will be detected by a photodetector. The dichroic mirrors are polarized mirrors that can allow or reflex certain light wave length. Finally, all the data are acquired, recorded and analysed using a computer.

2.1.8.1 Indirect cell staining for surface markers

Cells were detached using Accutase™ and washed with PBS, as previously described. They were then counted and $1x10^6$ cells were used per sample. Samples were incubated with 0.1% BSA in PBS for 30 min at RT, to block unspecific antibody binding sites. Monoclonal antibodies (mAbs) MEM-G/9, MEM-E/7, and MEM-12 (Exbio), 3D11 (kindly provided by Dr. Daniel Geragthy, CEO of Scisco Genetics Inc., Seattle), and L31 (Media Pharma) were employed in the indirect immunofluorescence staining of HLA-G, HLA-E, HLA-F, HLA-C, and HLA-DR, respectively. Cells were pre-incubated with saturating concentrations of primary antibodies, followed by washing and labelling with FITC conjugated goat anti mouse IgG (Santa Cruz Biotechnology). Cells were then re-suspended in 500 µl of PBS and analysed using a BD FACS Aria I (BD Biosciences).

2.1.8.2 Indirect staining for intracellular markers

For intracellular staining, after detaching and washing the cells, the pellet was fixed with 4% paraformaldehyde (PFA) on ice. Cells were then washed with 0.1% saponin-BSA in PBS, and permeabilised for 10 min at RT using 0.3% saponin in PBS. For all subsequent steps, 0.1% saponin in

PBS was used. The staining procedure was the same as that for the cell surface staining. After secondary antibody staining, cells were washed with PBS, re-suspended in 500 µl of PBS, and analysed using the BD FACS Aria I (BD Biosciences).

2.1.8.3 Surface antigens quantification

For surface antigen quantification, the method previously described by the Shaikly group was used (Shaikly *et al.*, 2010). Briefly, we used the Qifikit beads kit from Dako. The cells were prepared in the same manner as described in section 2.1.8.1 up to the primary antibody staining stage. To detect the antibody, cells, set up beads, and calibration beads were all stained with FITC conjugated secondary antibody which was supplied with the kit, and steps were carried out following the manufacturer's instructions. For both, the JEG-3 and the ACH-3P cell lines, the relative number of HLA-G, HLA-E, HLA-F, and HLA-C were quantified, using a QIFI assay (quantitative immunofluorescent indirect assay), a technique based on the linear relationship that exists between the mean fluorescence intensity, and the number of binding sites of the mAb per cell. Using Qifikit beads, cell surface antigen quantification was performed by means of indirect staining. The system used, a BD FACS Aria I, was calibrated using both the cell isotype and the set up beads. Using these same parameters, both cell samples and calibration bead readings were acquired. Using the readings, a calibration curve was constructed for the mean fluorescence intensity, and the beads populations, as is seen through Figure 2.2, after which the antigen binding capacity of the cells was calculated by interpolations using the calibration curve.

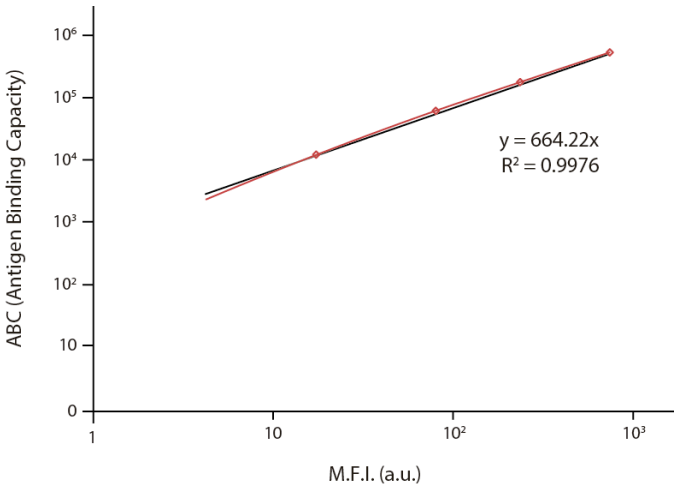

Figure 2.2. Bead population calibration curve tabulated mean fluorescence intensity (M.F.I) vs. antigen binding capacity (ABC) (log-log scale) for the calibration bead populations.
The M.F.I of each population of calibration beads was determined, and plotted vs. the ABC on a double logarithmic graph in order to determine the line of best fit. For the relevant samples, the M.F.I of each cell population was calculated, after which the ABC was obtained by interpolation using the calibration curve. Through this, we were able to calculate the antigen density of each relevant protein.

To calculate the cell surface mean antigen density of the JEG-3 and ACH-3P cells, the following formula was used:

$$SABC = ABC - BAE$$

Where:

- SABC Specific Antibody-Binding Capacity.
- ABC Antibody Binding Capacity
- BAE Background Antibody Equivalent.

2.1.8.4 Antibodies used in immunological techniques

The antibodies used in each immunological technique are summarised in Table 2.4 and 2.5.

Table 2.4. Antibodies used for proteomic techniques.

Antibody	Isotype	Specificity	Technique	Manufacturer
MEM-G/09	IgG1	Reacts with the native form of human HLA-G1 on the cell surface and with the soluble HLA-G5 isoform in its β2-microglobulin associated form.	FC	Exbio
MEM-G/01	IgG1	Reacts with the denatured HLA-G α1 domain (HLA-G1-G7) in its β2-microglobulin associated form.	Wb	Exbio
MEM-E/07	IgG1	Recognizes the native surface expressed HLA-E but not with the denatured heavy chain of HLA-E.	FC	Exbio
MEM-E/02	IgG1	Reacts with the denatured heavy chain of HLA-E.	Wb	Exbio
MEM-12	IgG1	The antibody MEM-12 recognizes common epitope on human HLA-DR which is dependent on the association of alpha and beta chains.	Fc	Exbio
3D11	IgG1	This antibody recognizes native and denatured forms of HLA-F and does not cross react with any other HLA type	FC Wb	Dr. Daniel Geraghty
L31	IgG1	Reacts with an epitope present on all HLA-C alleles (CW1 through CW8) and is known to cross react with HLA-B alleles	FC	Media Pharma
D-9	IgG1		Wb	Biolegend

FC: Flow Cytometry; Wb: Western blot.

Table 2.5. Secondary antibodies used in proteomic techniques.

Antibody	**Manufacturer**
FITC-conjugated anti-mouse immunoglobulins	Dako
IRDye 800CW Donkey anti-Mouse IgG	Li-Cor Bioscience

2.1.8.5 Two dimensional Electrophoresis

Two dimensional electrophoresis (2-DE) is a useful and widely used method for the analysis of protein mixtures extracted from tissues, cells, or any other biological samples that was first introduced by O'Farrell in 1975 (O'Farrell, 1975). This technique, separates proteins based on two independent properties; their isoelectric point (pI) and their Mw. The first step, or as is commonly referred to, the first dimension of this technique is identified as isoelectric focusing or IEF, while the second dimension is the SDS-PAGE. Following the second dimension, the gels are then stained, and analysed using a computer software.

For this technique, cell lysates were prepared as previously described (section 2.1.7.1.1), and their protein concentration assessed using a BCA (section 2.1.7.1.2). The frozen cell pellets were dissolved in hot lysis buffer and sonicated using a bioruptor. Five percent of 2-mercaptoethanol was then added. Samples were dissolved in solubilisation buffer and left to solubilize at RT for 1 h. The samples were then centrifuged at 12000 x g for 30 min. To concentrate the protein samples acetone precipitation was used. To do this, four parts of ice-cold acetone were added to every one part protein solution. The mixture was incubate overnight and then centrifuged at 12000 x g at 4 ºC for 30 min. The supernatant was then discarded and the protein pellet was washed with a mixture of ice-cold methanol-chloroform and re-suspended in IEF buffer. Finally, samples were quantified using a Bradford assay. All buffers used are summarised in Table 2.6.

2.1.8.5.1 First dimensional isoelectric focusing

For first dimensional IEF, immobilized pH Gradient (IPG) dry strip gel pH 3-10NL, 11 cm (Bio-Rad), were rehydrated with a mixture containing 55 µg of protein lysate dissolved in IEF buffer

containing 1 µl IPG buffer and 2.4 µl destreak reagent, all make up to a total volume of 202 µl. Strips were allowed to rehydrate for 24 h at RT. The IPG strips were then focused using an IPGphor system. Rehydrated filter wicks were placed between the electrodes and the IPG strip. The separation of the protein mixture in the first dimension was carried out at 20 ºC for 24 h, following the manufacturer's instructions. After IEF, strips were rinsed in deionized water and stored at -80 ºC to be used at a later time.

2.1.8.5.2 Second dimensional SDS-PAGE

IPG strips were incubated in equilibrium buffer 1 for 15 min at RT, and in equilibrium buffer 2 for 15 min at RT. Strips were then rinsed in electrophoresis buffer and second dimensional analysis was carried out using a Criterion gel system (Bio-Rad). Each strip was placed into the well of a ready-to-use 12% SDS-PAGE gels (Bio-Rad) and sealed with agarose solution. Electrophoresis was carried out at 50 V for 30 min, after which it was increased to 150 V for 4-5 h. This was all carried out at RT. The gel was then fixed overnight in fixing solution and stained using silver staining.

2.1.8.6 Silver staining.

The gels were stained using the silver staining protocol as described by the Shevchenko group (Shevchenko *et al.*, 1996). Gels were stained with 2.5% silver nitrate solution for 20 min after which, they were washed with water for 1 min. Gels were then developed for 15 mins using developing solution and the reaction was stopped by washing the gels with stop solution for 10 min. The gels were washed three times with water and stored in 2% acetic acid at 4 ºC. Each sample was prepared and run as a triplicate. Both solutions composition can be consulted in Table 2.7.

2.1.8.7 Spot analysis

The gels were scanned and analysed using SameSpots analysis software (Nonlinear dynamics Ltd.). Digitized images from 12 silver stained gels were analysed for spot detection and quantification. Image analysis included spot detection, editing, background subtraction, and spot matching. A master image was created and all spots in the other gels were matched to this master image, both manually and digitally. The size of each protein was expressed as the volume of the spot, which was defined as the sum of the intensity of all the pixels that made up to spot was also calculated using the software. The spots,

which were identified as being differentially present, were excised, and analysed using mass spectrometry (MS) analysis.

Table 2.6. 2-DE buffers.

Buffer	**Composition**
Hot lysis buffer	1% SDS, 100 mM Tris-HCl pH 7
Solubilisation buffer	8 M urea, 2.5 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesuflate (CHAPS), 50 mM DTT, 24 mM spermine tetrachloride
Isoelectric focusing (IEF) buffer	8 M urea, 2.5 M thiourea, 4% CHAPS
Equilibrium buffer 1	6 M/l urea, 20% w/v glycerol, 4% w/v SDS, 0.375 M/l Tris-HCl (pH 8.8), 5% 2-mercaptoethanol
Equilibrium buffer 2	6 M/l urea, 20% w/v glycerol, 4% w/v SDS, 0.375 M/l Tris-HCl (pH 8.8), 2.5% iodoacetamide
Agarose sealing solution	125 g agarose, 25 ml electrophoresis buffer
Electrophoresis buffer	60.57 g Tris base, 288.27 g glycine, 20 g SDS, double distilled water up to 20 L

Table 2.7. Silver staining solutions.

Fixing solution	50% methanol, 5% acetic acid and 45% water
Washing solution	50% methanol and 50% water
Rinsing solution	double distilled water
Sensitizing solution	0.02% sodium thiosulfate and 99.98% water
Staining solution	0.05g silver nitrate in 50 ml water
Developing solution	0.04% formalin (35% formaldehyde), 2% sodium carbonate and 97.96% water
Stop solution	5% acetic acid and 95% water

2.1.8.6 Mass spectrometry and bioinformatics

2.1.8.6.1 Spot preparation for MALDI-TOF analysis

Protein spots that were identified as being differentially expressed between the samples were manually excised from the 2-DE gels using a disposable sterile scalpel spot cutter (Swann- Morton). Each spot was washed in filter-sterile water (HPLC grade; Sigma-Aldrich). Each spot was then subjected to in-gel-digestion alkylation, along with tryptic digestion so as to yield peptide fragments for mass spectrometry analysis following the protocol as previously described by the Hassan group (Hassan *et al.*, 2008).

2.1.8.6.2 MALDI-TOF MS analysis of peptides

Peptide digests were subjected to MS analysis at the sub-picomole level through the use of a matrix assisted laser desorption ionisation-time of flight (MALDI-TOF MS) so as to generate peptide mass fingerprinting (PMF). This technique consists of three components: an ionization source, a mass analyser, and a detector. The MALDI-TOF analysis was carried out using a Bruker Daltonics Reflex IV instrument using a linear mode with a laser power attenuation setting at 30, as described by Padliya and Wood (Padliya and Wood, 2008). In linear mode, the ions travel down a linear flight path and their mass/charge (m/z) ratio determined by the length of time it took the ions to reach the detector. The instrument is called a time of flight (TOF) instrument. Once the data was examined and calibrated using the program M/Z, the results were transferred to a search engine MASCOT software from Matrix Science Limited for protein identification.

2.1.9 Statistical analysis

The statistical analysis was performed using the software SigmaPlot version 12.5 (Systat Software Inc.). The descriptive statistics and normality test were done as a first step. If the population followed a normal distribution (Gauss' Bell) Student's t-test was used to compare the different groups (treated *vs.* non-treated, surface *vs.* intracellular antigen expression, etc.). If the population did not follow a normal distribution Mann-Whitney U test was used. This test allows the comparison of groups, samples or data that do not follow a normal distribution pattern. The multiparametric comparison was performed by ANOVA. A statistically significant value of $P \leq 0.05$ was chosen *a priori*.

CHAPTER III

RESULTS

JEG-3 AND ACH-3P CELL LINES CHARATERIZATION AND SELECTION

3.1 Introduction

The aim of the present study was to investigate how soluble factors affect HLA expression by trophoblast cells. In this research, to study the role of HLAs, the choriocarcinoma cell lines JEG-3 and ACH-3P were used. Both cell lines are good surrogates for primary trophoblast cells (Orendi *et al.*, 2011). JEG cell line, was established by the Kohler group in 1971 (Kohler and Bridson, 1971; Kohler *et al.*, 1971). It is a hypertriploid high-proliferating human cell line with a long lifespan, in comparison with other chorocarcinoma cell lines like BeWo or Jar (Soares and Hunt, 2006a). From the parent cell line, which was isolated from the Woods strain of Erwin-Turner tumour, six different clonal cell lines have been established: JEG-1, JEG-2, JEG-3, JEG-4, JEG-7 and JEG-8. Though all the cell lines display different morphologies, none are reported to form a syncytium (Kohler and Bridson, 1971; Kohler *et al.*, 1971). Of these clonally derived cell lines, JEG-3 is that which was used through the course of our investigation. ACH-3P is a new hybrid, immortal cell line that was established by the Hiden group (Hiden *et al.*, 2007). Immortalization of this cell line was attained through fusion of primary first trimester trophoblast cells with the choriocarcinoma cell line AC1-1 (Hiden *et al.*, 2007). The ACH-3P cell line was established in an effort to overcome limitations posed by the already existing cell lines such as a lack of subpopulations and divergent genetic background.

HLAs expressed by the trophoblast cells are reported to exhibit both surface and intracellular expression. To map the expression of these molecules in different cell lines, a comparative study of both JEG-3 and ACH-3P cell lines was carried out, in an attempt to identify which of these cell lines would be best suited for ensuing studies.

3.2 Results

3.2.1 Trophoblast-derived cell lines culture

Cell culture plays an important role in immunology, as it allows for a constant or critical number of cells that can be studied biologically and immunologically to be obtained. Various methods to study cell viability and proliferation in culture are available. To determine the proliferation of both cell lines, the

total number of cells was determined by an MTS assay for a period of 9 days. Cell viability was examined using trypan blue and a hemocytometer. The cells were counted and their growth curves determined, as shown in Figure 3.1.

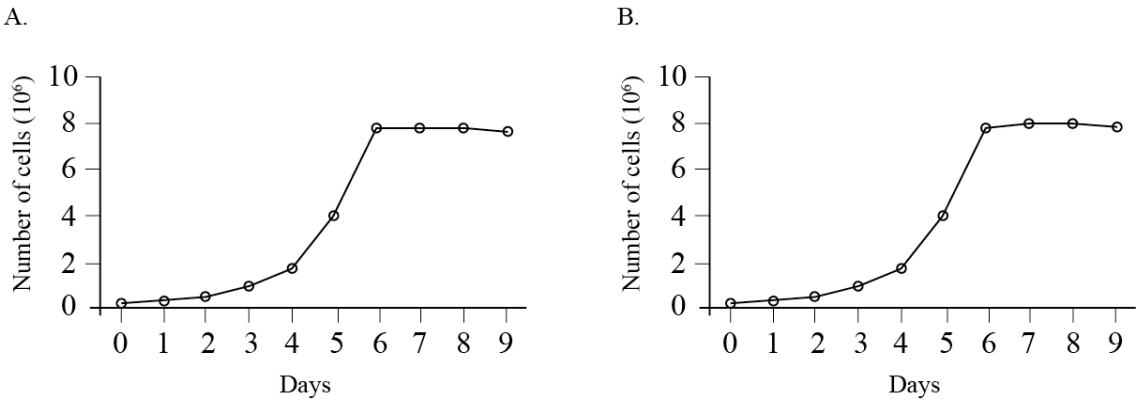

Figure 3.1. JEG-3 and ACH-3P growth curves.
Cells were seeded at a density of 30,000 cells/well in a 6 well plate in medium containing 0.1% FCS for 9 days. Cells were continuously grown in serum-free medium, which was replaced every 2 days, Cell numbers were determined through the use of an MTS assay. The JEG-3 (A) and the ACH-3P (B) cell line followed a similar growth curve. Both cell lines entered the exponential growth phase on day 3 following seeding. The cells continued to grow exponentially, and entered a stationary phase on around day 7.

The population doubling time (PD) of these two cells lines was also recorded, in addition to their growth mode, preferred growing medium, and morphology (Table 3.1).

Table 3.1. PD time, morphology, and growth mode of trophoblast JEG-3 and ACH-3P cell lines.

	PD	**Morphology**	**Growth mode**	**Culture medium**
JEG-3	~24 h	Epithelial-like	Adherent, monolayer	DMEM-F12
ACH-3P	~24 h	Epithelial-like	Adherent, monolayer	Ham's F12

All trophoblast cell lines are reported to strongly express cytokeratin-7 but are void of vimentin and the class II molecule HLA-DR. In an effort to check if the cell lines JEG-3 and ACH-3P were true trophoblast-derived cell lines, both were tested for these markers. Both cell lines were cytokeratin-7^+, vimentin$^-$ and HLA-DR$^-$, so both met the appropriate criterion of a trophoblast-derived cell line, as shown in Figure 3.2.

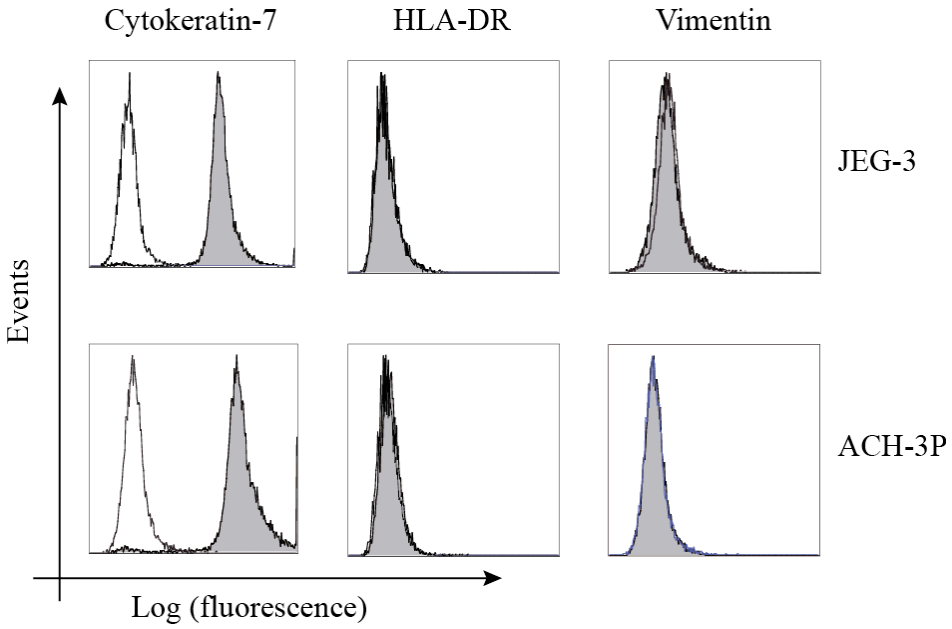

Figure 3.2. Results of the phenotype analysis studied by flow cytometry in a representative culture of JEG-3 and ACH-3P.
Expression pattern of typical trophoblast surface markers. The corresponding isotype control is shown as blank and the marker studied is shaded.

3.2.2 Immunoblot analysis of HLA-I in the JEG-3 and ACH-3P cells

HLA class I total protein expression in both cell lines was explored using Wb and mAbs that were specific for their respective soluble and membrane-bound HLA isoforms. The same amount of total protein in each cell lysate (40 µg) was loaded and separated using a 12% SDS-PAGE and blotted to PVDF membranes using the technique as described in the Materials and Methods section.

The results (Figure 3.3), afford a semi-quantitative estimate of protein amount, in that the thickness and brightness of protein bands is an indicator, but not an absolute measure, of protein quantity. HLA-G was seen to be expressed in both the JEG-3 and the ACH-3P cell lines, and was detected at a molecular weight of 39 kDa. When the JEG-3 cell sample was compared to the ACH-3P sample, the HLA-G band produced both a thicker and a brighter band, demonstrating a higher amount of HLA-G in JEG-3 cells. JEG-3 cells also yielded a thicker and brighter HLA-E band, detected at 42 kDa, compared to ACH-3P cells. This indicated that JEG-3 cells expressed higher level of HLA-E, compared with the ACH-3P cell line. For HLA-F, both cell lines displayed a considerably fainter protein band, reflecting the low expression levels of this HLA. Of both cell lines, JEG-3 cells showed a more visible band, and thus higher HLA-F expression, compared to ACH-3P cells. Both cell lines expressed a band for HLA-C,

which was similar in brightness and thickness, showing that both cell lines expressed similar levels of this molecule. In this experiment, β-actin was used as a loading control to check the integrity of the protein lysate. Both cell lines expressed this protein at 42 kDa, indicating that the protein samples were on par.

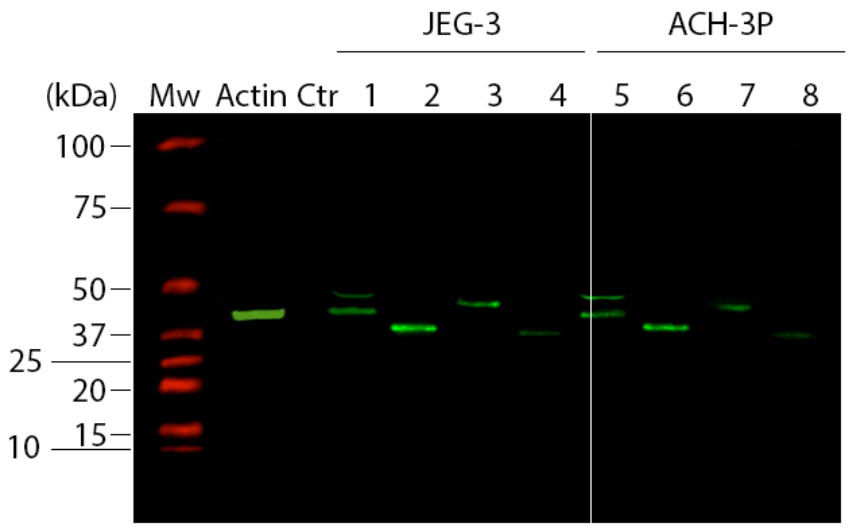

Figure 3.3. Western blot analysis of lysates from both JEG-3 and ACH-3P cell cultures.
Western blot analysis for denatured soluble and membrane bound protein heavy chains of HLA molecules. Lane 1, 2, 3 and 4 represent HLA-G, HLA-E, HLA-C and HLA-F protein respectively in the JEG-3 cell line. In the ACH-3P cell line, HLA-G, HLA-E, HLA-C and HLA-F are represented by lane 5, 6, 7 and 8 respectively. Ctrl is the negative control and Actin is the loading control HLA-G, HLA-E, HLA-F, and HLA-C were detected at different levels, in both cell lines at 39 kDa, 42 kDa, 39 kDa and 42 kDa, respectively.

To study the expression and the distribution of HLA class I intracellular and cell surface expression, in JEG-3 and ACH-3P cell lines, flow cytometry analysis was carried out. Experiments were carried out using stained permeabilazed cells. The results, summarised in Figure 3.4, showed that HLA class I molecules -G, -E, -F, and -C, were expressed intracellularly in both cell lines. Statistical analysis of HLA expression patterns between both cell lines showed significant results, with differences in HLA-G, -E and -C expression between JEG-3 and ACH-3P ($P \leq 0.01$), and that for HLA-F ($P \leq 0.05$), as seen in Figure 3.4. Also, JEG-3 displayed higher HLA levels compared with ACH-3P cells.

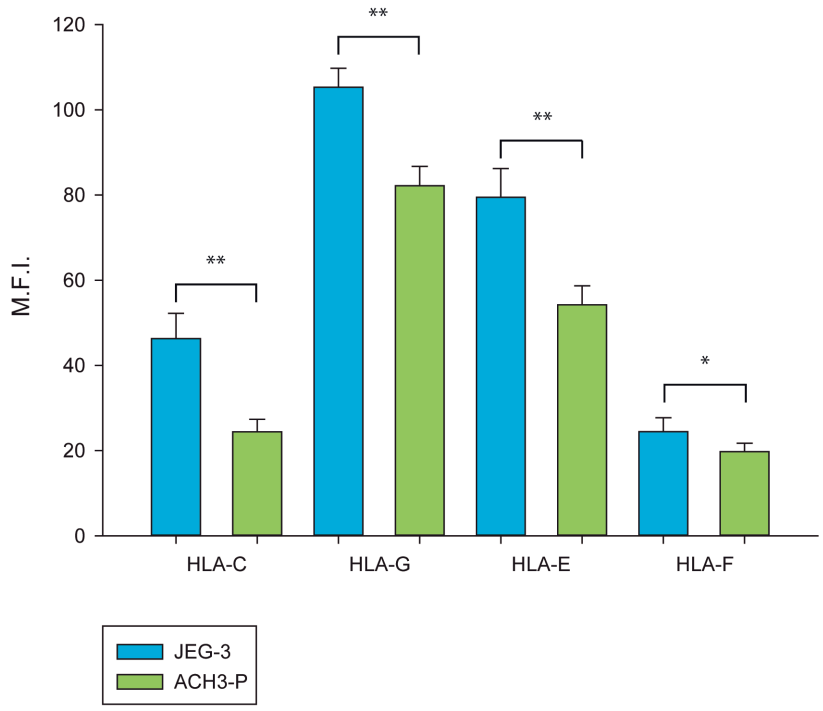

Figure 3.4. Comparative study of intracellular HLA class I expression on JEG-3 and ACH-3P cell lines.
JEG-3 (blue) and ACH-3P (green) cell lines were cultured in serum free medium, permeabilised, and analysed for intracellular expression of HLA class I molecules using FACS. HLA expression in the two cell lines was measured as the mean fluorescence intensity (M.F.I), and the difference for each HLA in both cell lines compared. Values are a mean of six independent experiments ± SD. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.In the Y-axis we have represented the mean fluorescent intensity (M.F.I.) while, in the X-axis we have represented each of the measured HLAs.

In both JEG-3 and ACH-3P cells, HLA-G, HLA-E and HLA-C were detectable at the cell surface (Figure 3.5). HLA-F was not detectable at the cell surface in either cell line. Expression of HLA-C and HLA-G between the two cell lines was statistically significant with a $P \leq 0.05$. At the cell surface, there were no statistical differences between HLA-E expressions in either cell line. As with the intracellular HLA expression, the cell surface expression of the molecules was higher in the JEG-3 cell line than in the ACH-3P cell line.

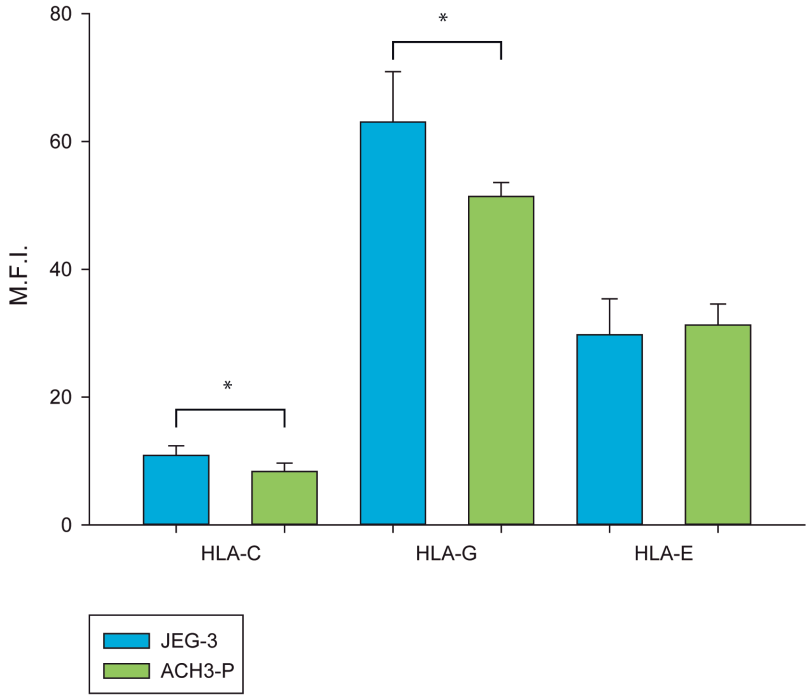

Figure 3.5. Comparative study of surface HLA class I expression of model trophoblastic cell lines.
JEG-3 and ACH-3P cell lines were cultured in serum free medium, and analysed for surface expression of HLA class I using FACS. HLA expression in both cell lines was measured as the M.F.I, and the difference for each HLA in the cell lines compared. Values are a mean of 6 independent experiments ± SD. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

3.2.3 Surface antigen quantification

The relative number of HLA Ia and Ib in JEG-3 and ACH-3P cells was quantified using a quantitative immunofluorescence assay. Table 3.2 summarize the number of each antigen on the cell surface of each studied cell line. As we observed in the western blot analysis and in the flow cytometry, JEG-3 showed a higher expression of all the studied HLAs, with the exception of HLA-F that was not detected in either cell line.

Table 3.2. Antigen quantification of surface HLAs as expressed by the JEG-3 and ACH-3P cell lines.

HLA isotype	JEG-3	ACH-3P
HLA-G	44,947.16 ± 2,391.47	19,191.83 ± 872.21
HLA-E	8,692.50 ± 625.44	6,879.5 ± 224.68
HLA-F	N.D.	N.D.
HLA-C	4,980.00 ± 243.71	3,292.00 ± 265.07

N.D.: not detectable.

Statistical analysis revealed a highly significant difference in quantifiable HLA-E and HLA-C cell-surface expression between JEG-3 and ACH-3P cells ($P \leq 0.01$) (Figure 3.6). The expression of HLA-G was also significant ($P \leq 0.05$). Compared to the ACH-3P cell line, the JEG-3 cell line displayed higher levels of HLA expression on the cell surface. HLA-F was not present on the surface of either cell line, and was thus not quantified.

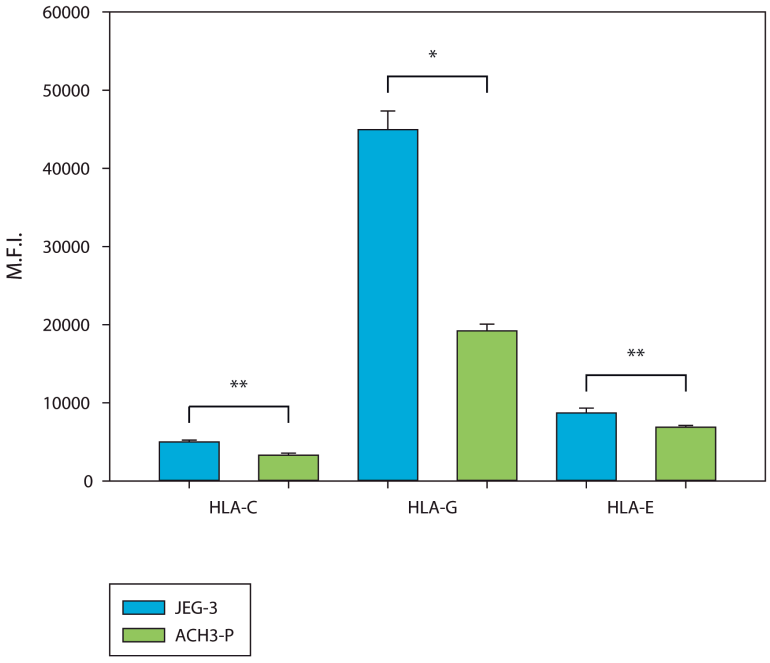

Figure 3.6. Comparative study of surface antigen quantification of HLA class I molecules in model trophoblastic cell lines.
The statistical analysis showed that the difference between the JEG-3 and ACH-3P HLA-E and HLA-C receptors was statistically highly significant ($P \leq 0.01$). The difference in HLA-G surface receptors was statistically significant ($P \leq 0.05$). Values are a mean of 6 independent experiments ± SD. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

3.2.4 Trophoblast cytokine profile

To analyse which cytokines were expressed in our experimental model, the cytokine profile of the JEG-3 and ACH-3P cell lines was investigated through the use of an ELISA. The cytokines that were investigated were those that have been previously reported to be expressed by the placenta and at the fetomaternal interface. In this study, the cytokines assayed were IL-1β, IL-8, IL-10, TNF-α, IFN-γ, GM-SCF, and TGF-β1. The ACH-3P and JEG-3 cell lines analysed did not secrete IFN-γ. Both showed a secretion level under the threshold level of detectability, as shown in Table 3.3. Both cell lines secreted appreciable quantities of IL- 1β, IL-8, IL-10, GM-SCF and TGF-β1, with the JEG-3 cell line secreting almost twice as much as the ACH-3P cell line.

Table 3.3. Cytokine secretion by JEG-3 and ACH-3P cell lines.

	IL-1β	**IL-8**	**IL-10**	**GM-SCF**	**TNF-α**	**IFN-γ**	**TGF-β1**
JEG-3	309 ± 36.9	631.5 ± 31.4	186 ± 11.2	256.2 ± 15.4	101.4±8.6	< 10	321±23.9
ACH-3P	144 ± 22.7	329.9 ± 25.6	134 ± 17.5	208.6 ± 13.3	89.2 ± 6.9	< 10	188±48.7

All results are given in pg/ml. All results shown are mean values ± SD from six experiments.

3.2.5 HLA-G-specific cell sort of the ACH-3P cell line

The behaviour of the JEG-3 and ACH-3P cell lines was examined under *in vitro* culture conditions. The ACH-3P cell line, a polyclonal cell line, was established by the Hiden group in 2007 to allow more detailed studies involving the paracrine and autocrine interplay between the different trophoblast populations (Hiden *et al.*, 2007). Our initial studies used both the JEG-3 and the ACH-3P cell lines to map HLA and cytokines profile in both cell lines. However, during our first experiments we thought that maybe the use of the ACH-3P cell line could be more advantageous than the more established JEG-3 cell line, since the ACH-3P cell line is comprised of two trophoblast subpopulations, one HLA-G^+ and another one HLA-G^-.

The two populations were isolated based on their HLA-G surface expression (Figure 3.7). After the cell sorting, the isolated HLA-G^+ subpopulation was much less in number than the HLA-G^-, as was confirmed through the use of a counting chamber.

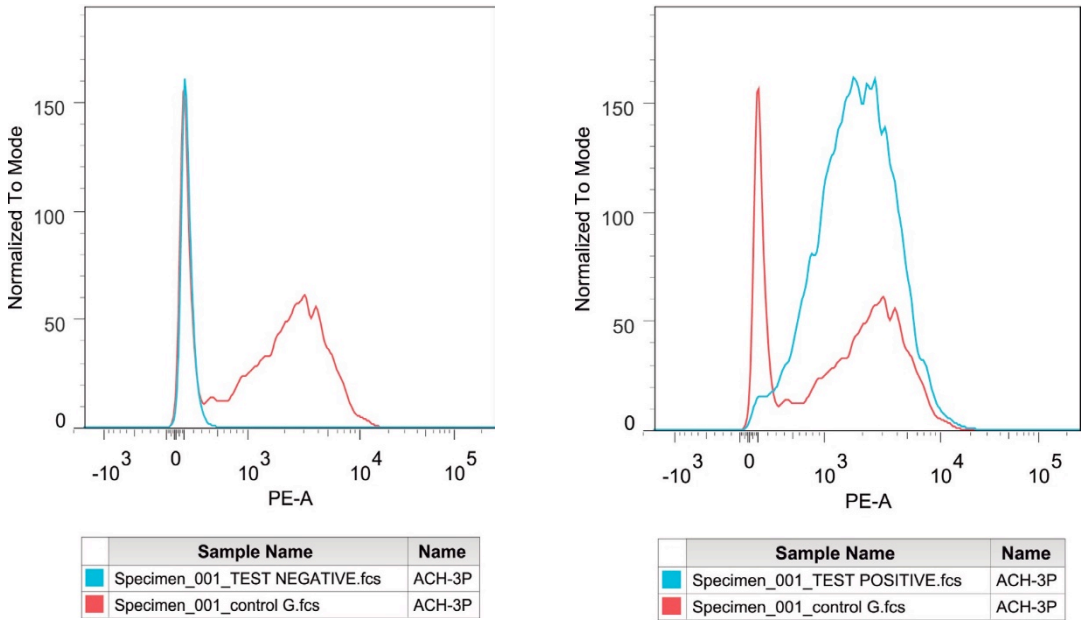

Figure 3.7. ACH-3P cell line specific sort using HLA-G as a marker.
The ACH-3P cell line was cultured in serum free medium, and their surface HLA-G expression analysed using FACS. The ACH-3P subpopulations were sorted based on their HLA-G expression. Left and right plots show the results of the cell sort using the MEM-G/9 antibody. In the left plot, the red histogram is representative of the original ACH-3P cell line tagged with the MEM-G/9. In the right blot, the red histogram is the original ACH-3P cell line, while the blue histogram is representative of the EVCT subpopulation; the small overlap between the two histograms is believed to be due to the low proportion of other subpopulations that could be present in this polyclonal cell line.

Once the two subpopulations of the ACH-3P cell line were sorted, they were cultured separately as previously described (see Materials and Methods). Cell viability and growth curves were carried out to determine the number of live cells after the cell sort and their growing patterns. This was done to know the lifespan of each subpopulation under *in vitro* culture conditions (Table 3.4).

Table 3.4. ACH-3P subpopulation characteristics.

	PD	Morphology	Growth mode	Growth medium
ACH-3P	~ 20 h	Epithelial-like	Adherent, monolayer	Ham's-F12 10% FCS
ACH-3P HLA-G^+	N.D.	Epithelial-like	Adherent, monolayer	Ham's-F12 10% FCS 1% Pen/Strep 1% Ampho B
ACH-3P HLA-G^-	~ 96 h	Epithelial-like	Adherent, monolayer	Ham's-F12 10% FCS

N.D.: Not detectable.

The separation of HLA-G positive and negative subpopulations resulted in two morphologically different subpopulations. HLA-G^+ subpopulation resembled the parent ACH-3P cell line in that it had a higher number of pseudopodia and a similar morphological shape than HLA-G^- subpopulation The morphology of each subpopulation started to change after subsequent passaging, resembling that of the parent cell line. In addition, a cell viability and growth assay showed that while HLA-G^- subpopulation was able to proliferate, as was shown by an increasing number of cells most of which were viable, HLA-G^+ subpopulation did not fare as well. Through the viability assay and growth curve carried out for HLA-G^+ subpopulation, it was determined that the cell number of this subpopulation only just increased, and most of the cells were dead (Figure 3.8).

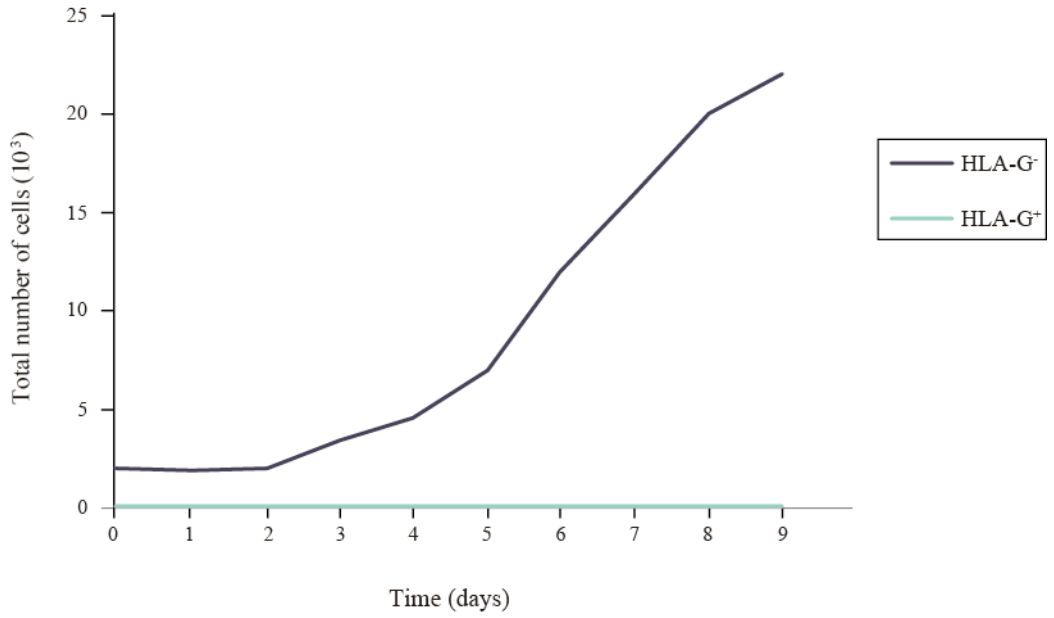

Figure 3.8. Growth curve for HLA-G$^+$ and HLA-G$^-$ ACH-3P subpopulations.
Cells were seeded at a density of 30,000 cells/well in a 12 well plate in starving medium for 9 days. Cells were continuously grown in serum-free medium, which was replaced every 2 days, Cell numbers were determined through the use of an MTT assay. HLA-G$^+$ subpopulation followed a growth pattern similar to that of a healthy ACH- 3P cell line. HLA$^-$ subpopulation did not grow, and its initial numbers quickly deteriorated.

Once both subpopulations were isolated, HLA-G$^-$ subpopulation was stimulated using P4 and INF-γ in an attempt to induce HLA-G expression. Stimulation was carried out as outlined in Materials and Methods after which, HLA-G expression by this subpopulation was analysed through flow cytometry. Neither treatment induced a significant increase in HLA-G expression. Results showed that even with stimulation, the HLA-G$^-$ subpopulation showed M.F.I values that were similar to those of the isotype control (Figure 3.9).

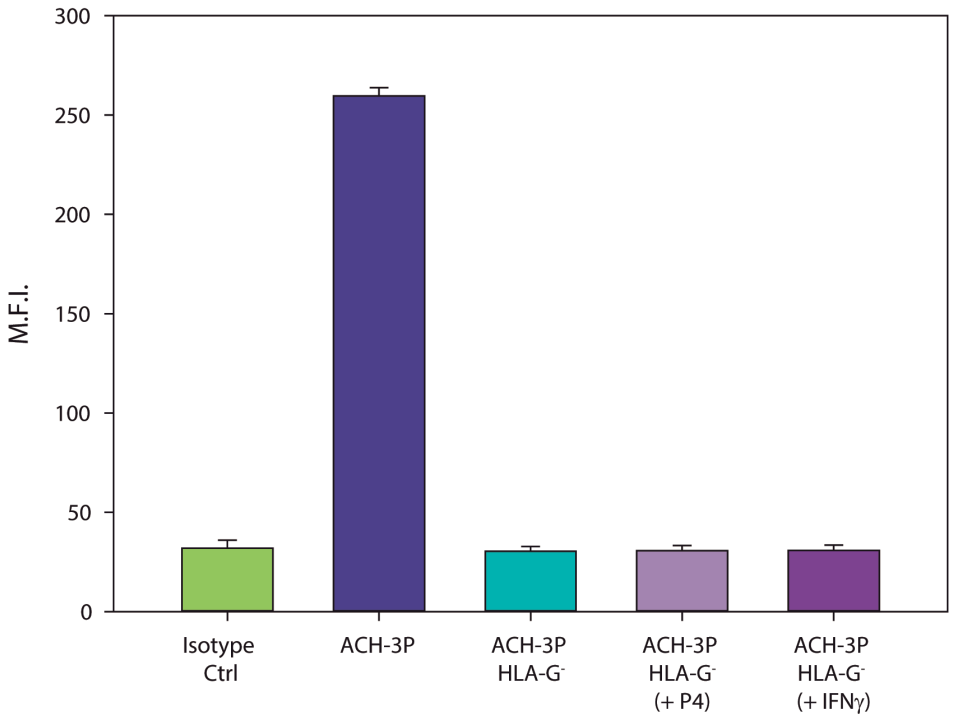

Figure 3.9. Expression of HLA-G in ACH-3P HLA-G$^-$ subpopulation upon stimulation with P4 and IFN-γ.
HLA-G$^-$ subpopulation was cultured in serum free medium, stimulated with either P4 or IFN-γ for a period of 24 h, and their surface HLA-G expression analysed using FACS. The ACH-3P cell line was also cultured in serum free medium and its surface HLA-G expression analysed. Isotype control was performed by staining ACH-3P cells with an isotype matched control antibody. ACH-3P displayed significantly higher levels of surface HLA-G, while the unstimulated and the stimulated HLA-G$^-$ subpopulations, displayed a surface HLA-G expression similar to that of the isotype control. Values are a mean of 3 independent experiments ± SD.

3.3 Discussion

HLAs are the keystone of the antigen presentation system in humans and their expression, or lack thereof, is important in pregnancy (Davies, 2007). As a means by which to study pregnancy, several trophoblast cell lines have been well established and characterized, all of which have features that resemble those of a distinct trophoblast subpopulation. Of these cell lines, JEG-3 is one which has been extensively used in studies about the fetomaternal interface. Though these cell lines have proved essential in studying pregnancy, they present some limitations. *In vitro* culture methods used to increase the lifespan may alter the regulation of cell division, affecting the gene expression and differentiated functions (Sutton *et al.*, 2003; Roobrouck *et al.*, 2011). Moreover, these cell lines sometimes have characteristics that are related to their malignant origins (see appendix). Nevertheless, these cell lines have been proved to be essential in studies addressing the interactions that take place at the fetomaternal interface when there is no access to human, or even rat/mouse, trophoblasts or placental tissue.

Upon checking the origin of the JEG-3 and ACH-3P cell lines, their growth pattern was characterised to distinguish when they are in the exponential phase, so as to ensure their viability and phenotypic stability. Here, we want to clarify that there is no consensus about how to characterise cell lines. The ATCC suggests some tests, which have been mentioned in the appendix. We performed three of the five suggested methods (which we consider as being sufficient). Through the use of a cell viability test and a generated growth curve, we determined that the ACH-3P and JEG-3 cell lines enter the log exponential phase three days after seeding. In this phase, cells will continue to proliferate until they run out of nutrients or their growing conditions change. The PD time was approximately 24 h. This was important for later experiments in which the cells needed to be stable and in a proliferative phase prior to their stimulation. This will help to avoid quiescent or senescent cells or cells in the G_0 phase of the cell cycle which decreases metabolism hence interfering with any response to stimulus. The results acquired suggest that the two cell lines were behaviourally similar to an immortalised cell line, i.e. that they evade normal cellular senescence. The JEG-3 cells were isolated from a choriocarcinoma while the ACH-3P cell line was immortalized (Hiden *et al.*, 2007). Such a feature is important as it means that unlike

primary cells cultured *in vitro*, which have a limited proliferative lifespan, a choriocarcinomic cell line will grow readily in culture providing an abundant supply of cells that will display a stable phenotype over the course of many passages (Soares and Hunt, 2006a).

By studying the growth pattern of the JEG-3 and ACH-3P cell lines, it was possible to identify the optimal time point to passage them into subsequent cultures, or to use them for experiments. The two cell lines were positive for a full range of HLAs that we enumerated previously (check Materials and Methods or points 3.2.1 to 3.2.4 in this section). Cells used for the phenotyping of HLAs on JEG-3 and ACH-3P were usually in the later stage of their exponential phase.

It has recently been recognized that trophoblast cells are not "blank" cells and, in addition to HLAs, also secrete a wide range of cytokines through the three trimesters of pregnancy (Blaschitz *et al.*, 2001; Hutter *et al.*, 1996; Juch *et al.*, 2012). Stringent regulation of these cytokines is required if a successful pregnancy is to happen. The first trimester of pregnancy, often described as a Th1-dominant phase, is one where several pro-inflammatory cytokines are produced. Through the ELISA based studies, it was seen that in both cases the JEG-3 and ACH-3P cell lines secreted constitutively the pro-inflammatory cytokines IL-1β, IL-8, IL-10, GM-SCF, and TGF-β1 at relatively abundant levels. High levels of TNF-α were also observed. These findings are in agreement with the theory that the first trimester is Th1 dominated (Saito *et al.*, 2010; Wilczynski, 2005). In addition, the results showed that the JEG-3 cell line secreted a higher level of cytokines, namely IL-1β, when compared to the ACH-3P cell line. We believe that such a result was because this cytokine is primarily found in EVCTs and not in VCTs, which are the second major subpopulation in the ACH-3P cell line. This may be due to the different trophoblast subpopulations between both cell lines. The trophoblast is reported to differentiate into functionally different subpopulations (Naruse *et al.*, 2010). Thus, it is possible that during the first trimester the EVCT population provides a rich source of cytokines, which ensure proper invasion and implantation of the foetus. This observation is supported by the Bischof group, who reported that the EVCT was the only trophoblast population with IL-1 receptors (Bischof *et al.*, 2000a; Bischof *et al.*, 2000b).

HLA-G is especially important in pregnancy as it has the ability to extenuate the immunological function of several immunocompetent cells and, after implantation and development of the placenta, it shifts the uterus' status to that of an immune privileged site (Hunt *et al.*, 2007). The presence of soluble HLA-G in maternal circulation, in addition to expression of HLA-G by embryos, is also reported to result in higher rates of successful pregnancy (Lynge Nilsson *et al.*, 2014). Through our results, gathered through proteomic techniques, namely flow cytometry and Wb, we were able to establish that JEG-3 and ACH-3P cells express HLA-G, both on the cell surface and intracellularly. However, the JEG-3 cell line, which is comprised of only EVCT cells, expressed a higher level of HLA-G, both on the surface and intracellularly, than did the ACH-3P cell line. This difference in the expression of HLA-G between the two cell lines can be attributed to the fact that while JEG-3 is formed of one population while, the ACH-3P cell line, based on its origin, is formed from two main populations, where the extravillous cells only make up 40% of this cell population (Hiden *et al.*, 2007). Thus, the number of HLA-G^+ cells is lower than in the JEG-3 cell line.

Our results also showed that after HLA-G, both cell lines most readily express HLA-E. It has been reported that HLA-E expression is dependent on that of HLA-G. Specifically, cell surface expression of HLA-E has been viewed as dependent, or facilitated, by the co-expression of HLA-G (Shaikly *et al.*, 2010; Guleria and Sayegh, 2007). However, our findings suggested that not all HLA-G expressed by the JEG-3 and ACH-3P cells, was associated with HLA-E. We observed HLA-G and HLA-E values that differed from one another. This could suggest that not all HLA-G and HLA-E expressed by the cells are functionally associated. This is in agreement with results from previous research that reported that HLA-G is found to be expressed independently of HLA-E (Ishitani *et al.*, 2003; Jabeen *et al.*, 2013).

In a previous study, it was reported that HLA-C is a ligand for KIRs, which are expressed on NK cells who are well-known for their cytotoxic properties and ability to elicit an immune response (Blais *et al.*, 2011). Our results showed that significant levels of HLA-C were present in both JEG-3 and ACH-3P cells, although compared to the other HLAs, its levels were lower. HLA-C plays a crucial role since if an infection were to occur, it allows the mother to protect herself without adversely affecting the foetus. Additionally, in the case of pregnancy complications, HLA-C will trigger a response that could induce a

preterm labour, protecting the mother. Although HLA-C expression levels in trophoblast cells are low compared to that of HLA-G and HLA-E, it nevertheless remains important in pregnancy, being a ligand for the different KIRs expressed by the uNK cells. The Hunt group have reported that HLA-C should not be expressed by the trophoblast, due to its moderate polymorphism and ability to stimulate maternal anti-foetal acquired immunity, if the paternal alleles differed greatly from those of the maternal host (Hunt *et al.*, 2005). It is here advocated that this molecule is important, because if HLA alleles are similar, this will decrease the variability in the population and will lead to an abortion.

HLA-F is a molecule of little known function. It has only recently become of interest in human pregnancy. It has been suggested that HLA-F is expressed on the trophoblast cells surface. However, this remains an area of controversy and has only been reported by a few groups (Shobu *et al.*, 2006; Ishitani *et al.*, 2006). Our results showed intracellular HLA-F expression in our experimental models. This is in agreement with the findings of the Jabeen group, who reported that HLA- F's expression was restricted to the cell cytoplasm (Jabeen *et al.*, 2013). On the other hand, the Shobu group have reported evidence that trophoblast cells express HLA-F on their surface during late gestation stages (Shobu *et al.*, 2006). However, these discrepancies between our results and those published by the Shobu group can be explained by the fact that whole the Shobu group used cells from mid to term gestation, we used first trimester isolated cells. Therefore, it is possible that at the beginning of pregnancy, cells have the ability to regulate the expression of autoantigens on the surface by an interference mechanism with other HLA molecules or with molecules involved in the antigen groove loading mechanism, while at the later stages of pregnancy this ability is lost.

Our study also appraised if the use of the new choriocarcinoma cell line, ACH-3P, which consists of two distinct subpopulations, has advantages over the JEG-3 cell line. Through a specific cell sort, two subpopulations were isolated based on their HLA-G expression, HLA-G^+ and HLA-G^- subpopulation. HLA-G^+ subpopulation, made up of the extravillous cells, was fewer in number than HLA-G^- subpopulation. This result is in agreement with those as reported by the Hiden group, who observed only 40% of the ACH-3P population to be extravillous and thus, HLA-G^+ (Hiden *et al.*, 2007). In addition, when both isolated subpopulations were cultured, we found that, unlike HLA-G^- subpopulation, HLA-G^+

extravillous subpopulation was unable to grow and the number of viable cells quickly decreased. Such a decline in this population may be in part due to the low levels of viable cells that remained after sorting, or due to the morphological dissimilarities of the two cell lines: HLA-G^+ subpopulation displayed a higher number of pseudopodia than did HLA-G^- subpopulation (Hiden *et al.*, 2007). To date, no data have been published that could explain why HLA-G^+ and HLA-G^- subpopulations of ACH-3P cells are unable to grow. Even, the Hiden group reported that once separated, the subpopulations of the ACH-3P cell line are only stable up to the 5^{th} passage after which, it is necessary to carry out another sort to isolate a fresh pool of ACH-3P subpopulations (Hiden *et al.*, 2007). Because the cytotrophoblast population displayed a more appropriate growth curve, and was the one with the more viable cells, an attempt was made to induce HLA-G expression in the HLA-G^- subpopulation. However, expression of HLA-G by this subpopulation did not occur, even when cells were treated with P4 and IFN-γ, two soluble factors which are reported to induce the expression of HLA-G in other cells isolated from human placenta (Carosella *et al.*, 2001; Lefebvre *et al.*, 2000). This is possibly because HLA expression, especially HLA-G expression, is restricted to the EVCT cell population (Hunt *et al.*, 2005).

The use of primary cell cultures from human organs for research purposes is a coveted opportunity, and even when this is the case, several limitations, such as restricted quantities and diseased tissue, are posed to the investigator (Petroff *et al.*, 2006). Using cell lines to study the fetomaternal relationship greatly limits out ability to infer conclusions to an *in vivo* condition. Nonetheless, as cell lines are readily available, have excellent viability, and there is limited ethical controversy surrounding their use, they remain an essential tool in the field of reproductive immunology. Over the last three decades several cell lines, have been established as a tool, by which to investigate, the fetomaternal interface. Of these, BeWo, JEG, and Jar cell lines are those which have been adapted to standard cell culture conditions, and those which have proven to be the most valuable for investigating human trophoblast differentiation and function (Soares and Hunt, 2006b). In our study, the reliability and usefulness of JEG-3 and the ACH-3P cell lines was appraised by mapping differences and similarities of HLA and cytokines that these cell lines express. Subsequent research is likely to focus on the effect of soluble factors on HLA expression and cytokine secretion. This will require a cell line that has consistent HLA expression. Through our

research, we found that the JEG-3 and the ACH-3P cell lines showed positive expression of the proteins of interest while, JEG-3 cells consistently expressed higher levels of HLA molecules.

Trophoblast cells express a unique HLA profile, with different subpopulations expressing different cytokines and HLAs. This study has allowed us to choose the cell line that is best suited to proceeding investigations. It has also provided a better understanding of the JEG-3 and ACH-3P cell lines through mapping of the differences in their HLA and cytokine expression profiles. The observed difference in HLA profiles in the JEG-3 and ACH-3P lines is probably the result of the distinct trophoblast subpopulations that constitute them. Whilst JEG-3 is comprised of extravillous cells, ACH-3P is comprised of two subpopulations, extravillous and villous cytotrophoblasts.

CHAPTER IV

RESULTS

AFFECTS OF PROGESTERONE ON JEG-3 CELLS

4.1 Introduction

In the previous chapter we have showed the results that we got after studying two models of human-derived-trophoblast cell lines. We studied the JEG-3 cell line, a well-known cell line, and the relatively newer ACH-3P cell line. The aim of this study was to analyse the effects of soluble factors over the behaviour of trophoblast cells. As we did not have access to human primary trophoblast cells, we decided to use cell lines. The previous results showed that JEG-3 cells displayed a better behaviour under our experimental conditions. This cell line expressed higher concentrations of the studied HLAs, both intra- and extra-cellularly, and secreted higher amounts of interleukins than ACH-3P. Also, the JEG-3 has the benefit of being composed for just one clonal population, while the ACH-3P is a mixture of, at least, two subpopulations with different HLA-G expression.

As we have seen in Chapter I, in the fetomaternal interface there is a convergence of different components (cellular, biochemical, physical, etc.). One of these components are hormones, one of the most important of which is P4. So, once we had chosen our cell line model (JEG-3) the next logical step was to analyse the effect this hormone has on the biological behaviour of JEG-3. We have to remember that P4 is considered as the keystone of pregnancy (Druckmann and Druckmann, 2005; Szekeres-Bartho *et al.*, 2009).

4.2 Results

4.2.1 Time and dose progesterone induction experiments

To study if P4 would have any effect on the JEG-3 HLA-G expression *in vitro*, a series of time and dose experiments were carried out. Cells were incubated in medium containing increasing concentrations of P4 from 0 (control) to 1000 ng/ml for a period of 4 h. The Yie group reported that at this time point the strongest reaction after treatment with P4 is generated (Yie *et al.*, 2006b). Through these dose and time dependent experiments, the dose that caused the highest increase in JEG-3 HLA-G surface expression was that of 1000 ng/ml (Figure 4.1).

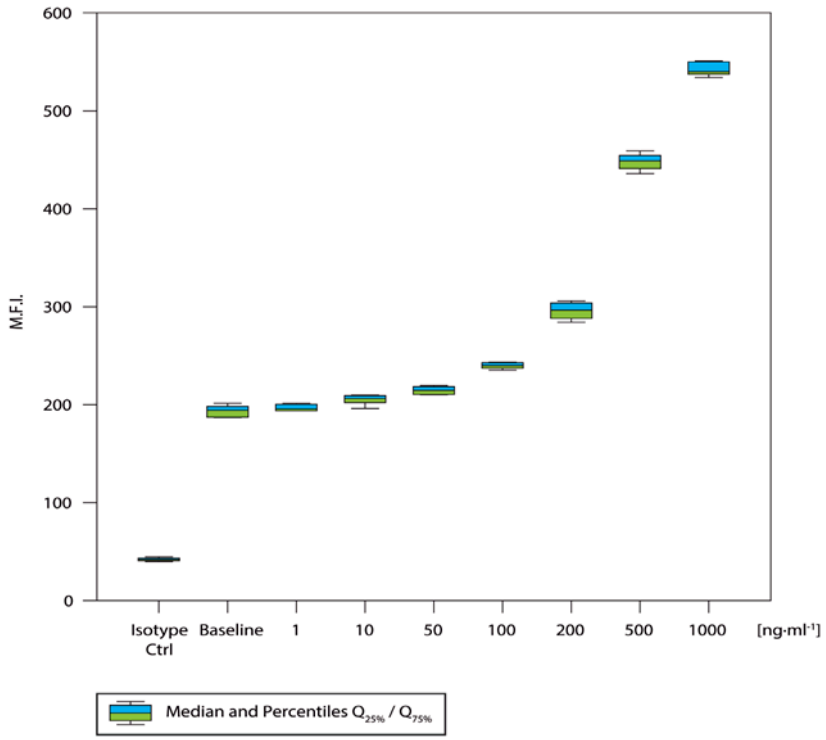

Figure 4.1. Evaluation of progesterone dose effect on HLA-G surface expression by cultured JEG-3 cells.
Cells were cultured in serum free medium, and stimulated with progesterone concentrations ranging from 0 to 1000 ng/ml for a period of 24 h. The surface HLA-G expression was measured as the M.F.I. Values are a mean of six independent experiments ± SD.

A time dependent experiment was then carried out. JEG-3 cells were incubated with 1000 ng/ml of P4 for different time periods ranging from 0 to 72 h, to study at this dose which time point would cause the highest increase in HLA-G surface expression (Figure 4.2). Semi- quantitative and quantitative data generated, using Wb and flow cytometry, respectively, concluded that at this dose, at 24 h we had the highest HLA-G expression (Figure 4.2, X-axis). Based on the results from the concentration and time incubation studies, we decided to use for all future experiments with P4 a concentration of 1000 ng/ml and 24 h as an incubation time.

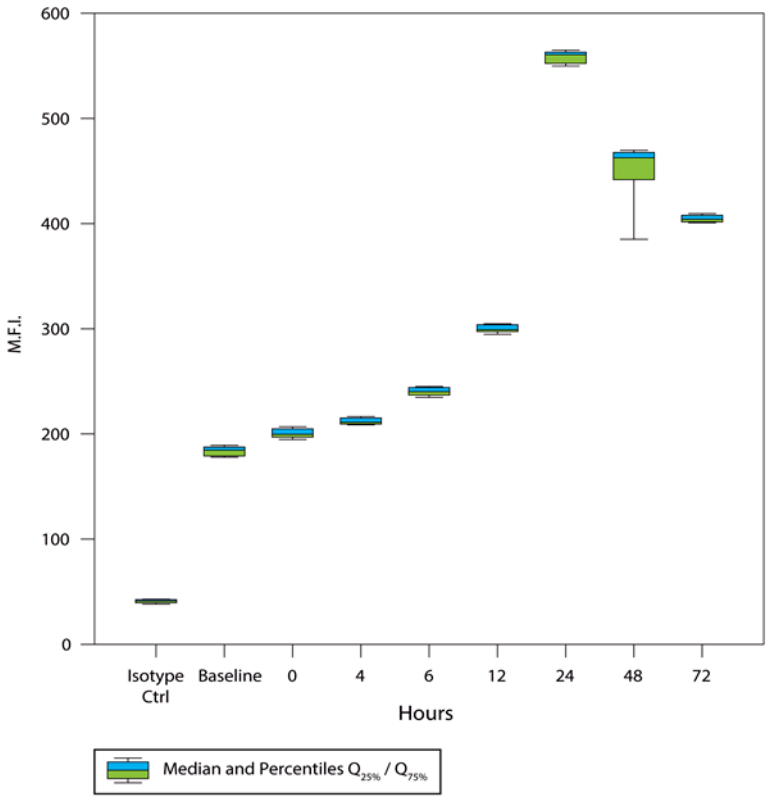

Figure 4.2. Evaluation of progesterone incubation time over HLA-G surface expression by cultured JEG-3 cells. Cells were cultured in serum free medium, and stimulated with P4 at a concentration of 1000 ng/ml for a period ranging from 0-72 h. The surface HLA-G expression was measured as the M.F.I. Values are a mean of six independent experiments ± SD.

Upon establishing a time and dose at which P4 has the highest effect on HLA-G profile of JEG-3 cells, the effect of this hormone on the other HLAs that are expressed by this cell line was then investigated. Through another set of time and dose dependent experiments (see appendix). The results showed that the ideal time point and dose to use was the same as for HLA-G.

4.2.2 Immunoblotting of the effect of P4 on HLA-I expressed in JEG-3

Using the 24 h time point and a concentration of 1000 ng/ml, a Wb experiment was carried out to determine which of HLAs expressed by the JEG-3 cells experienced the highest increase in its expression following P4 stimulation. Cells were processed as previously described in "Materials and Methods". Through the semi-quantitative data provided by the Wb, we saw that HLA-G was that which generated the thickest and brightest band after P4 stimulation, hence indicating that HLA-G was that with the highest amount of protein. However, this is not an absolute measure by which we can measure protein quantity.

In addition, we also observed that all the JEG-3 cell samples stimulated with P4 resulted in a thicker and brighter HLA bands compared with the unstimulated control JEG-3 cells (Figure 4.3).

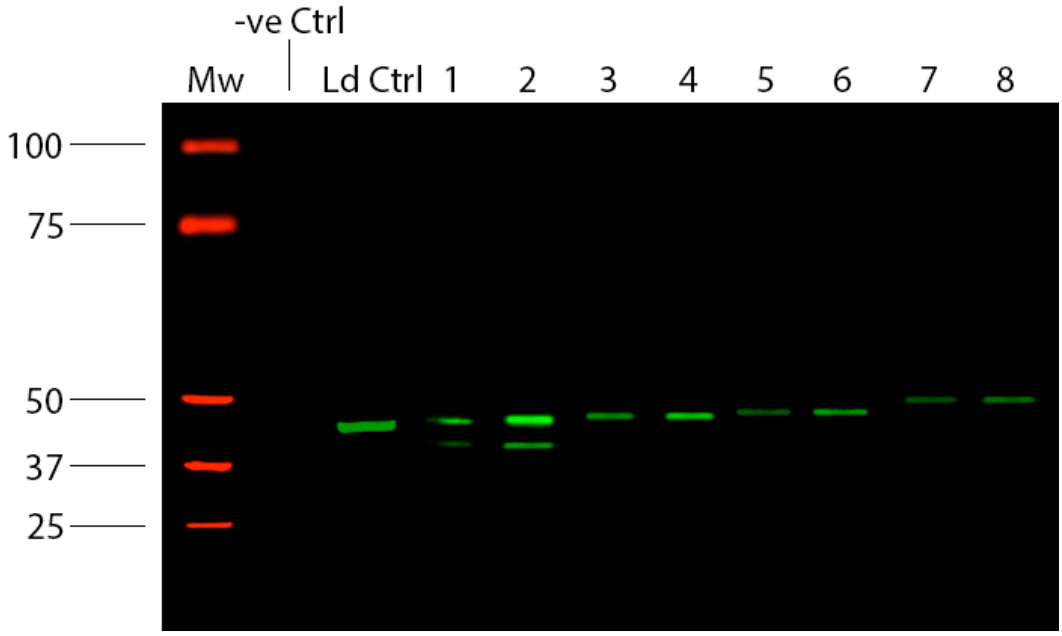

Figure 4.3. Western blot analysis of P4 stimulated JEG-3 supernatants.
Cells were incubated in serum free medium and stimulated with P4. Lane 1, 3, 5 and 7 represent HLA-G, HLA-E, HLA-C and HLA-F, respectively. Lanes 2, 4, 6 and 8 represent HLA-G, HLA-E, HLA-C, and HLA-F after P4 stimulation. -ve Ctrl is the control lane, and was loaded with water. The Ld Ctrl is tubulin (loading control), which is detected at a molecular weight of 42 kDa. HLA-G, HLA-E, HLA-F, and HLA-C were detected at different levels, in both unstimulated and P4 stimulated JEG-3 supernatants at 39 kDa, 42 kDa, 39 kDa and 42 kDa, respectively.

4.2.3 Effect of P4 over the phenotype of HLA-I in JEG-3

To support this data, a flow cytometry experiment using the same treatment conditions was then carried out. Cells were cultured with or without P4, permeabilazed, stained with the appropriate mAb, and results acquired through the use of a flow cytometer BD FACS Aria I. Results obtained reinforced the semi-quantitative data obtained using the Wb technique. Intracellular flow cytometry results showed that the HLA that experienced the highest increase in its expression, in comparison to the control, was HLA-G and this increase was statistically highly significant, and the same happened to HLA-C and -F ($P \leq 0.01$). We also observed that HLA-E also experienced an increased expression, as was previously seen through the Wb data. The detected increase in this protein was statistically significant ($P \leq 0.05$) (Figure 4.4).

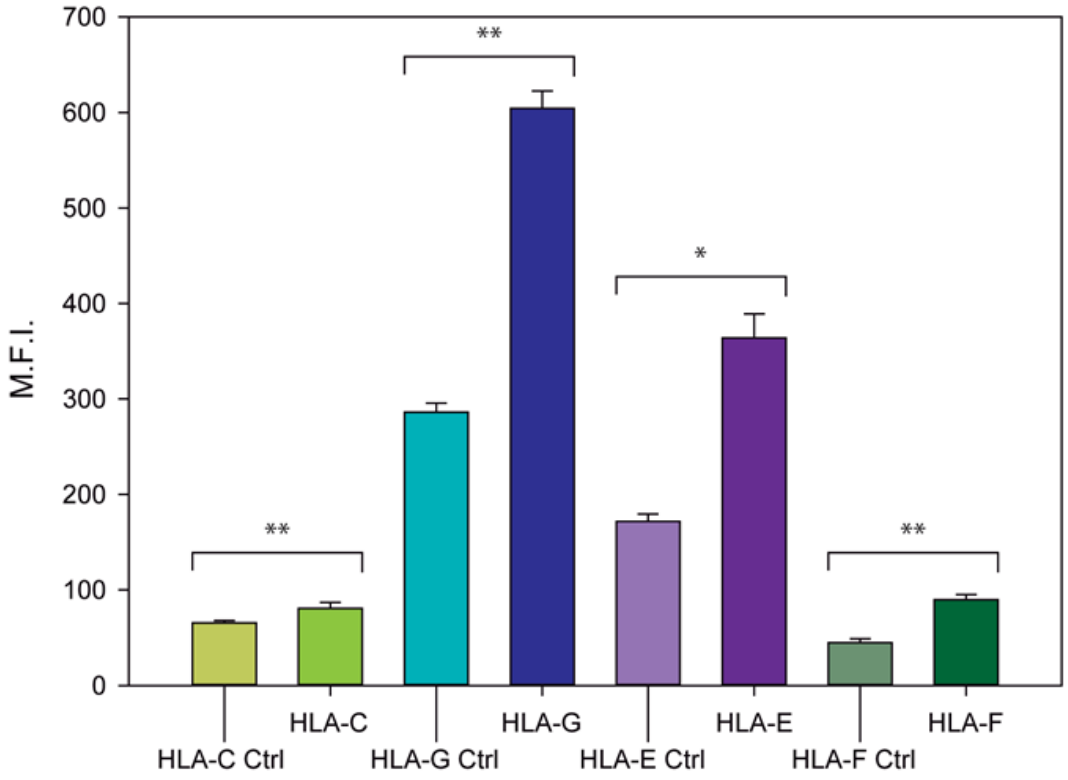

Figure 4.4. Effect of P4 on intracellular HLA expression on JEG-3 cells.
Cells were cultured for 24 h in serum free medium containing no P4 (HLA control) or P4. Data are a mean of six independent flow cytometry experiments ± SD for each antigen. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

Surface flow cytometry analyses were then carried out to see if HLAs surface expression followed a trend similar to that as their cytoplasmic counterparts. Through the surface expression of the molecules, it was observed that stimulation with P4 did not induce HLA-F surface expression. In addition, the increase in surface HLA-C and HLA-E expression, following P4 stimulation, was statistically highly significant, as was observed at the intracellular level ($P \leq 0.01$). HLA-G following stimulation had the highest M.F.I value, the increase of its expression level was statistically significant ($P \leq 0.05$), as can be seen in Figure 4.5, like at the intracellular level, as was seen through Wb and flow cytometry, where the increase was statistically highly significant (see previous subheading).

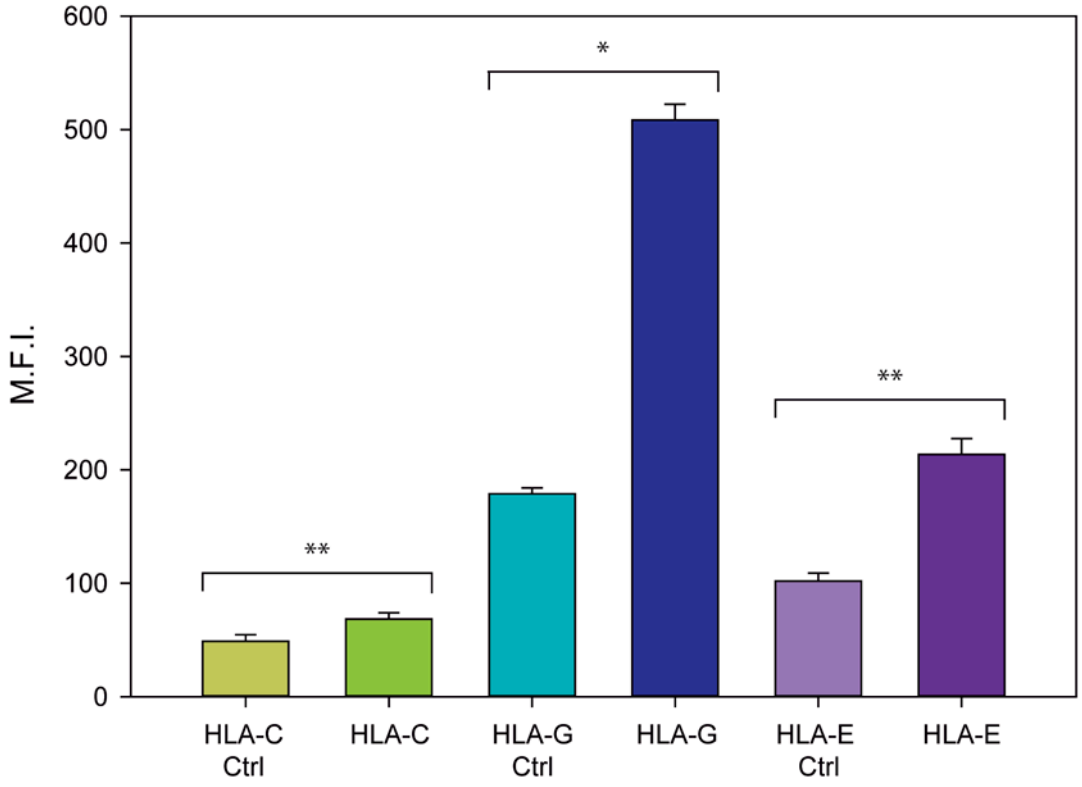

Figure 4.5. Effect of P4 on HLA surface antigen expression on JEG-3 cell line.
Data are a mean of six independent flow cytometry experiments ± SD for each antigen (**) $P \leq 0.01$ and (*) $P \leq 0.05$. Ctrl are the basal expression of each HLA (JEG-3 cultured without P4).

Results from both the intracellular and the surface antigen expression experiments were then compared in order to determine whether P4 caused a significant difference in the increased expression between HLAs at these levels. Through statistical analysis, it was seen that the difference between HLA-G surface and intracellular increase was highly significant ($P \leq 0.01$). In addition, it was also observed that intracellular HLA-G experienced the higher increase in expression following P4 treatment. The difference in increase between surface and intracellular levels of both HLA-E and HLA-C was statistically significant ($P \leq 0.05$), though the change between surface and intracellular M.F.I of HLA-C was not as obvious as that of HLA-E (Figure 4.6).

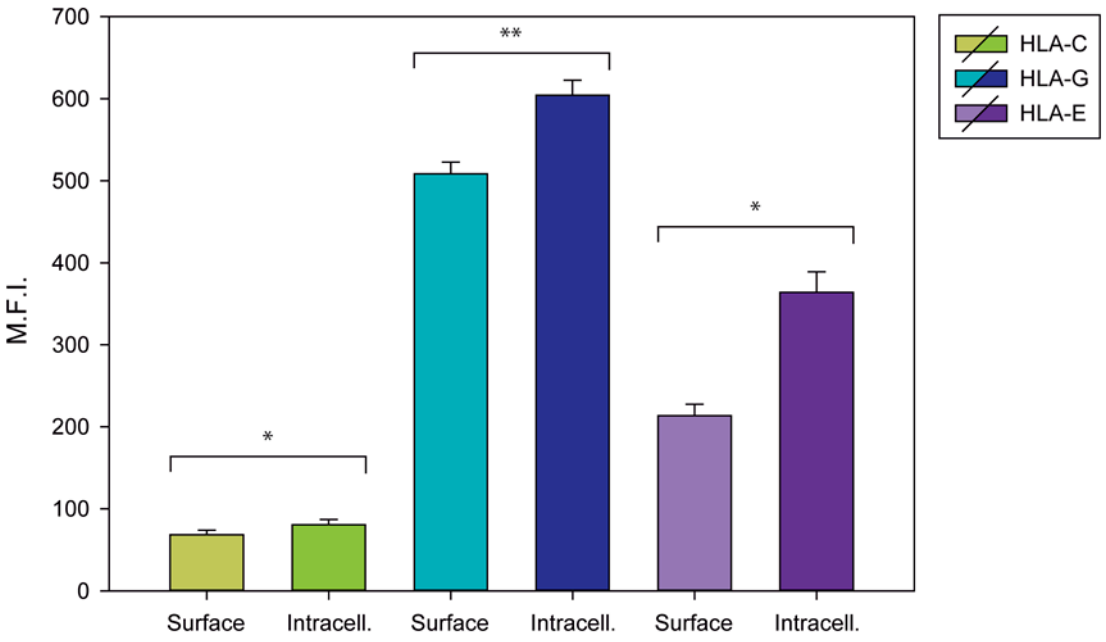

Figure 4.6. Surface vs. intracellular HLA expression of the JEG-3 trophoblastic cell line upon P4 stimulation. Readings of the surface and intracellular HLA expression was taken for each of them, and statistical analysis carried out. The difference between the surface and intracellular expression of all HLAs was statistically significant. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

4.2.4 Progesterone's effect on JEG-3 cells migration

One of the most important events that takes place in early pregnancy is the invasion of the uterine wall by the trophoblast cells. Such an episode is controlled by many factors such as hormones, proteases, components of extracellular matrix, etc. To analyse the role that P4 and hCG could have on JEG-3's invasion and migration behaviour, *in vitro* cultured cells were stimulated with P4 (1-1000 ng/ml) and hCG (1-1000 ng/m1). Treatment of JEG-3 cells with these hormones resulted in an increase in the number of cells that invaded through the Matrigel coated porous filter. In addition, the increase in cell invasion capacity was seen to be dose dependent. For both hormones, the concentration that produced the highest number of invading cells was 1000 ng/ml. The comparison between hCG and P4's effect on cell invasion showed that though hCG does increase JEG-3's invasion capacity, the increase in invasion produced by stimulation with P4 was much greater, with P4 resulting in a 3 fold change compared to unstimulated cells and hCG resulting in an 2.5 fold change when compared to unstimulated cells (Figure 4.7).

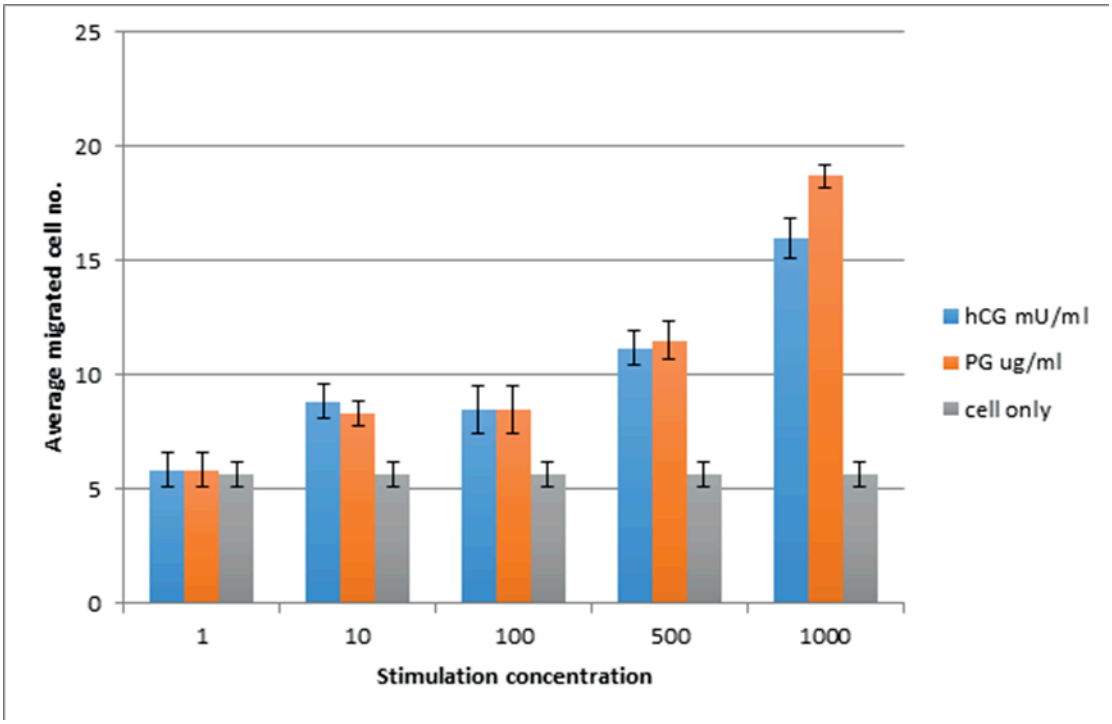

Figure 4.7. Representative invasive response of JEG-3 cells treated with or without hCG and P4.
JEG-3 cells were treated with different concentrations of hCG and P4 (1-1000 ng/ml) for 24 h. Treatment of JEG-3 cells with different concentrations of hCG and P4 resulted in a dose dependent increase in the number of cells that invaded through the matrigel coated porous filter. Each bar represents the number of JEG-3 cells on the underside of an insert filter. Results are presented as cell mean number of three independent experiments ± SD. For significance levels refer to appendix.

4.2.5 Progesterone's effect on JEG-3 cells proliferation

The proliferation of cells is a biological process that is tightly regulated by a number of different factors; such soluble factors include cytokines and hormones. To assess the affect that P4 has on the proliferation capacity of the JEG-3 cell line, a colorimetric assay for the quantification of viable cells in proliferation was carried out. Camptothecin, a known cell apoptosis inducing agent, and hCG, a hormone which has been reported to increase invasion *in vitro*, were both used as controls. JEG-3 cells were cultured and stimulated with different concentrations of P4 ranging from 0 to 1000 ng/ml at different time points (0 to 72 h). Cells were also stimulated with camptothecin and hCG (1000 ng/ml) at the same time points as P4. Camptothecin was used as negative control and hCG as positive control. Through this experiment, it was observed that hCG increased cell proliferation in comparison to unstimulated JEG-3 cells. When measured against P4, the proliferation of JEG-3 cells was either on par with or higher than that of those cells stimulated with hCG. The concentrations of P4 that resulted in the highest number of proliferating cells were 500 ng/ml and 1000 ng/ml. The 1000 ng/ml concentration of P4 was that which

induced the highest number of proliferating cells, especially after 24 h (Figure 4.8). This is in line with the data showed in the previous subsections, where the concentration of 1000 ng/ml of P4 was found to be that which caused the greatest increase of HLA expression.

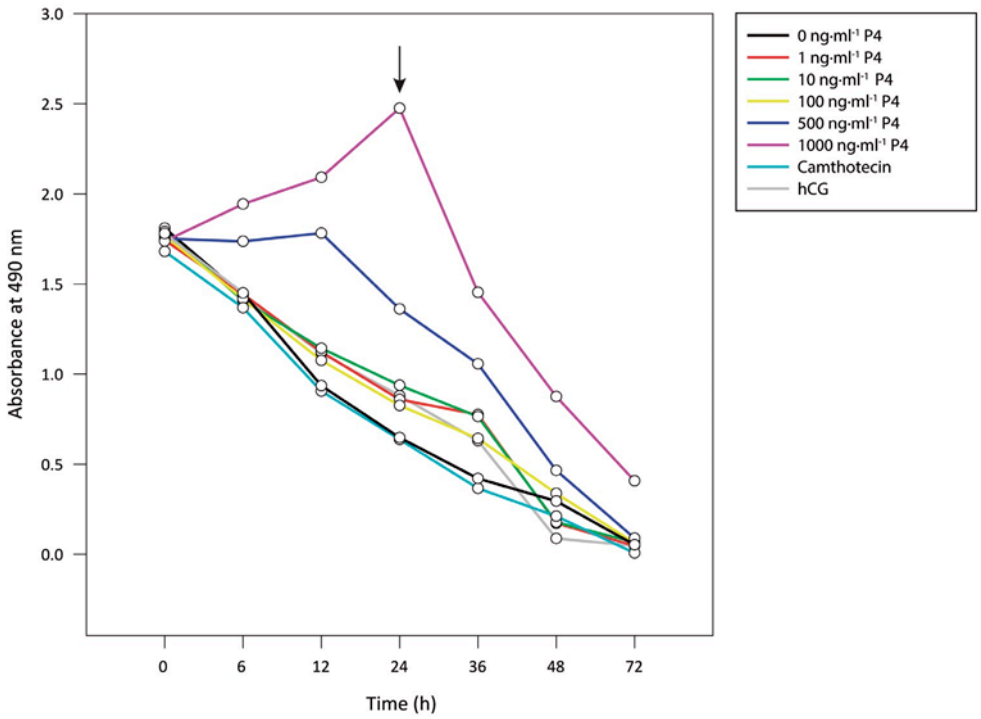

Figure 4.8. Effect of P4, hCG and campthotecin on the proliferation of the JEG-3 cells.
Cells were incubated in starving medium and stimulated with different concentrations of P4 at different time points (0-72 h). Cell proliferation was measured. Results showed that JEG-3 proliferation is time and dose dependent. hCG did not cause a change in proliferation when compared to the unstimulated cell. Campthotecin blocked cell proliferation. Results are presented as a mean of six independent experiments ± SD. For significance levels refer to appendix.

4.2.6 JEG-3 progesterone secretion in response to IL-17

Through current results, it was observed that P4 is an important inducer or regulator in HLA-G expression so we decided to study the role of other soluble factors over the effects of P4. IL-17 has a role in trophoblast invasion, in addition to having the ability to regulate P4 secretion by trophoblast cells *in vitro*. Cells were stimulated using different concentrations of IL-17 (1, 10 and 100 ng/ml) for five time points (6 to 72 h). JEG-3 supernatants were then analysed by ELISA for P4 concentration. It was observed that the secretion of P4, in comparison to the control, was increased in response to IL-17 stimulation. The difference in P4 secretion between treated and untreated cells was significant at all time points (see appendix), though this difference was more noticeable in the earlier time points (6 to 48 h).

The concentration of IL-17 that caused the greatest increase of P4 secretion at all time points, notably at the 24 h time, point was 10 ng/ml (Figure 4.9).

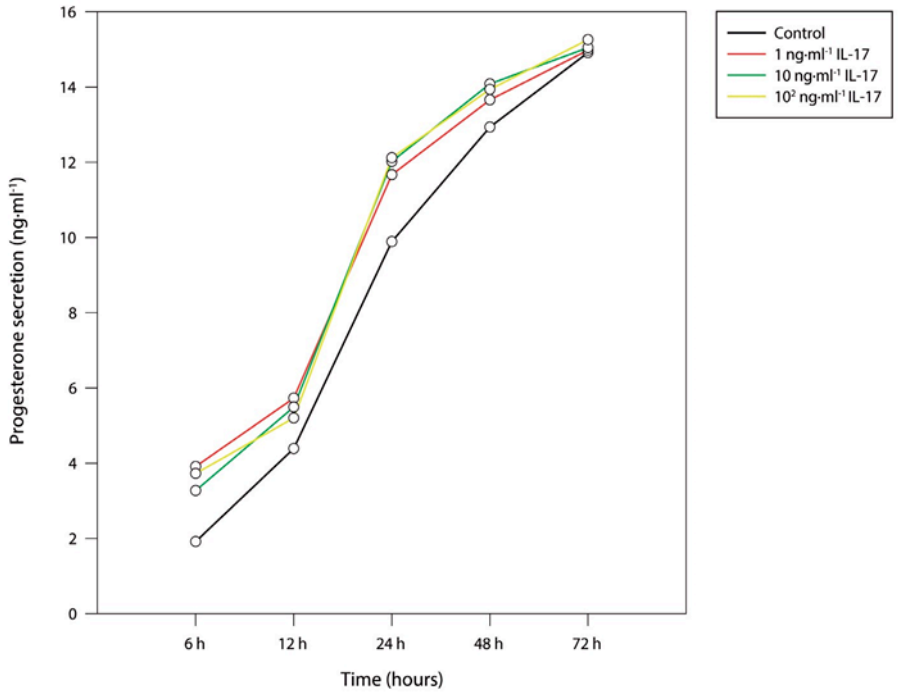

Figure 4.9. Representative P4 secretion by JEG-3 cells in response to IL-17.
Each line represents the mean concentration of P4 from three independent experiments. For significance levels refer to appendix.

4.2.7 Effect of IL-17 on HLA-G JEG-3 surface expression

Once we observed that IL-17 increases P4 secretion, it was logical to analyse if this interleukin had any effect on HLA-G expression. JEG-3 cells were treated for 24 h with 10 ng/ml of IL-17 or 1000 ng/ml of P4. We also had the respective controls of untreated cells (Ctrl IL-17 and Ctrl P4). Through the results, it was seen that IL-17 alone (JEG-3 IL-17) did not have any effect on the expression of HLA-G, as shown in Figure 4.10. However, P4 as previously showed had a statistically significant increase on the expression of HLA-G. The difference in HLA-G expression between IL-17 stimulated and P4 stimulated cells (last two bars) was statistically significant with $P \leq 0.05$.

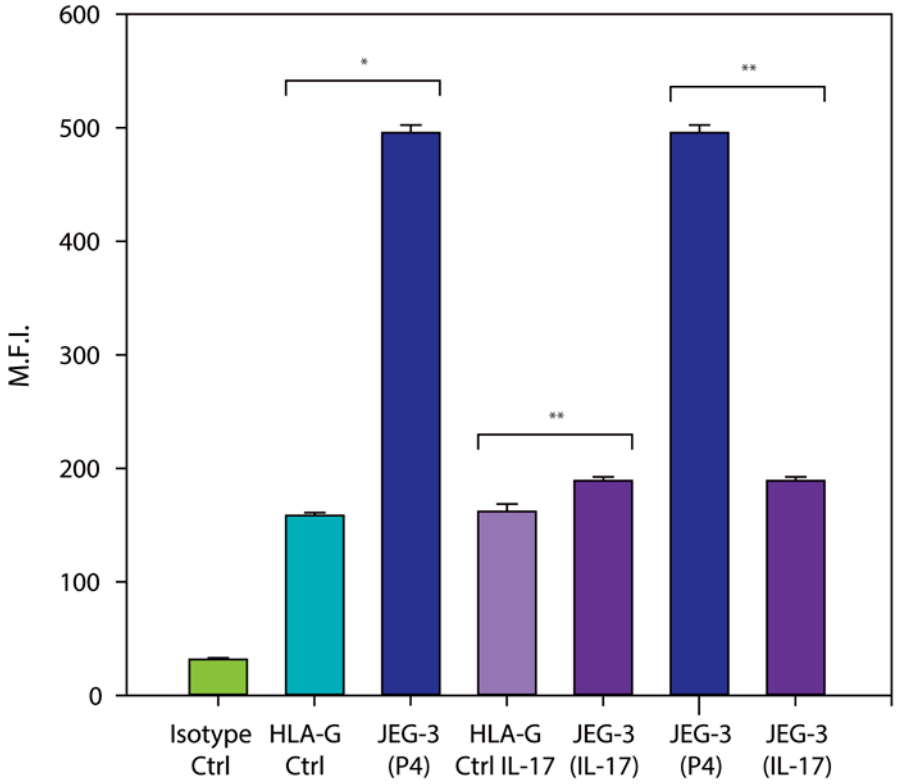

Figure 4.10. The effect of IL-17 on HLA-G expression of JEG-3 cells.
Results are presented as a mean of six independent experiments ± SD. (**) P ≤ 0.01 and (*) P ≤ 0.05. Ctrl means control (HLA-G basal expression of untreated cells). Between brackets we have the respective treatment

4.3 Discussion

Progesterone is present even before pregnancy, being secreted by the corpus luteum during the luteal phase of the menstrual cycle. Upon signals of pregnancy, the main source of P4 becomes the trophoblast cells (Norwitz *et al.*, 2001). The roles attributed to P4 in pregnancy are extensive, ranging from preparing the endometrium for embryo invasion, to inducing stromal cells to proliferate and differentiate, etc. (Halasz and Szekeres-Bartho, 2013). It is secreted in large amounts at the fetomaternal interface and is believed to play an important role in trophoblast invasion (Bischof *et al.*, 2000a). In addition, it has been reported that the P4, that is produced either by the placenta and/or ovaries, is essential in maintaining mammalian pregnancy due to its ability to inhibit the T lymphocyte cell mediated responses that are involved in tissue rejection (Szekeres-Bartho, 2009). Moreover, in 1977 the Siitteri group also reported that the local suppression of the maternal cellular immune response by P4 may be one of the ways that the foetus avoids rejection (Siiteri *et al.*, 1977). Due to its dominant presence in pregnancy, for many years this has been used in the field of *in vitro* fertilisation (IVF) and reproductive immunology, in an effort to avoid adverse outcomes in pregnancy such as preterm labour or miscarriages (Szekeres-Bartho and Balasch, 2008; Szekeres-Bartho *et al.*, 2008).

As we have reported in the previous chapter, HLA-G is an important molecule in pregnancy and its immunomodulatory properties are believed to be crucial for a successful pregnancy. Like HLA-G, P4 is also reported to induce tolerance, though the exact mechanisms by which this is achieved are still not fully understood (Yie *et al.*, 2006b). As these mechanisms to date remain undefined, the Yie group hypothesized that one possible pathway may be through the modulation of the *MHC-G* gene expression in the placenta (Yie *et al.*, 2006a). Through their study, this group reported that P4 had an up-regulatory effect on the expression of *MHC-G* gene in the cytotrophoblast and in the JEG-3 trophoblastic cell line. The Yie group also reported that after 4 h of incubation, HLA-G transcription reached its maximum, insinuating that, at least, part of progesterone's actions are mediated through the regulation of transcription (Yie *et al.*, 2006a). The increase witnessed by the Yie group was investigated to see if it would translate into one at the protein level, as an increase at the gene levels is not always related to one

at the protein level. Through our study, it was seen that P4 increased HLA-G expression, in addition to the other studied HLAs expressed by the EVCT cells, a finding that to date has still not be reported. Through P4 induction experiments, it was established that the time point at which this hormone caused the greatest increase in HLA-G and the other HLAs expression was 24 h. The difference in time points between our research and the Yie group's report is in large due to the fact that while they measured the regulation of HLA-G by P4 at the mRNA level, our study addressed the effect that P4 had on the expression of HLA-G at the protein level, at the surface and intracellularly. It is quicker to detect mRNA than a protein expression, because to have the protein first we need to process the mRNA (post-transcriptional modifications), translate it, and process once again the protein (post-translational modifications), and finally express it on the cell surface or intracellularly, this accounting for the time difference observed between our results and Yie's.

We found that the P4 concentration that was found to cause the greatest increase in HLA-G surface expression was at 1000 ng/ml. Such a finding is in agreement with the Yie group, who reported that maximal enhancement of steady state mRNA was seen at a P4 concentration of 100 ng/ml or higher (Yie *et al.*, 2006b). In addition, these findings (our and Yie's), are further supported by the Szerkeres-Bartho group. This group have reported that during pregnancy, while circulating P4 concentrations are found to be at 200 ng/ml, the concentration of P4 in the placenta, at the fetomaternal interface, can reach concentrations of up to 1720-1243 ng/g of tissue (Feinshtein *et al.*, 2010). Some of the immunomodulatory effects that are displayed by P4 during pregnancy may be mediated indirectly through modulation of *MHC-G* gene transcription. Our induction experiments revealed that P4 increased the expression of HLA-G in both a time and dose dependent manner, which is in agreement with the results observed by the Yie group for regulation of the *MHC-G* gene (Yie *et al.*, 2006a). Our time and dose dependent induction experiments of HLA-E, and -C also identified the 24 h time point and the 1000 ng/ml concentration, to be those which caused P4 to have a maximal effect on the expression of both HLAs. As HLA-F is a molecule that is not expressed on the surface of the JEG-3 cell line, the induction experiments for this HLA were focused in the intracellular expression. The optimal dose and time was identical to that identified for the other expressed HLAs.

Upon successful identification of an appropriate time and dose, the surface and intracellular expression of HLA molecules in response to P4 stimulation, and how these differed from one another was analysed. Through our investigation, it was observed that of all HLAs, HLA-G was the one on which P4 had the maximum effect, in addition to it undergoing the most important difference in expression between the surface and intracellular level. Such findings *in vitro* are important and relevant as they suggest that P4 may play a crucial role in the regulation of HLAs *in vivo*.

It has been well established that for a successful pregnancy, a number of immune modulatory factors from both the mother and the foetus are required. The effects of P4 on the immune system during pregnancy are well reported, ranging from its ability to induce lymphocyte-blocking proteins to triggering suppressor cell generation (Brierley and Clark, 1987; Mannel *et al.*, 1990). Previous studies from other groups have attributed to HLA-G functions similar to those of P4, as both are reported to reduce NK cell cytotoxicity and block cytotoxic T cell activity, among other things (Kapasi *et al.*, 2000; Chumbley *et al.*, 1994). In our study we have showed that HLA-G was that on which P4 had a maximal effect, though the other HLAs also experienced an increase in their expression. Such is important granting they are not identified as the leading HLA in parturition their presence is, nevertheless, still crucial if a favourable outcome is to be met.

HLA-C, though a class Ia protein was still observed to undergo an increase both at the surface and intracellular level when stimulated with P4. This increase is equally as important as that experienced by HLA-G, as HLA-C is also able to present antigenic peptides and can be recognized by T cells (Hutter *et al.*, 1996). Additionally, HLA-C is also important in pregnancy as it is believed to have the ability to regulate placentation through its interaction with KIRs. It can thus be proposed that HLA-C has a role in the invasion of the trophoblast, as the expression of KIR that binds HLA-C on uNK cells is higher in the decidua, in comparison to those found in the blood (Trowsdale and Moffett, 2008).

HLA-E is also reported as being essential in pregnancy as through CD94/NKG2A, a receptor expressed by $CD56^{bright}$ uNK cells, it is able to inhibit cytolysis carried out by NK cells (Dahl and Hviid, 2012). Following P4 stimulation, HLA-E also experienced a significant increase in both its surface and intracellular expression. This increase on the part of HLA-E upon P4 stimulation was expected because

surface expression of this protein is dependent on a nonamer sequence that is generally provided by the other HLAs. Hence, it was hypothesized that because the other HLAs experienced an increase, a greater number of molecules would thus be available to provide a nonamer signalling sequence, which would translate into a higher number of HLA-E molecules (Dahl and Hviid, 2012; Lee *et al.*, 1998).

An increase in HLA-F molecule was also observed at the intracellular level. Though expression is low in the trophoblast cells to begin with, a significant increase of HLA-F in response to P4 was witnessed. In 2010 the Lee group hypothesized that, in pregnancy, HLA-F may have a role in regulating the immune response by interacting with Treg cells to induce a suppressive signalling (Lee *et al.*, 2010). However, as data on HLA-F still remains contradictory, whether or not HLA-F holds a central role in developing tolerance during pregnancy can still not be established.

As the hormone of pregnancy, P4 is critical if a pregnancy is to be established and maintained. Invasion and proliferation are one of the earliest crucial events that take place during pregnancy, without a successful invasion pregnancy would not ensue. The process of invasion is an active biochemical process and not just a process that occurs due to passive growth pressure (Bischof *et al.*, 2000a). *In vivo*, trophoblast cells are only invasive during the first trimester and, even then, their invasion capacity is limited to the endometrium and to the promixal myometrium hence, they are only transiently invasive (Bischof *et al.*, 2000a; Pijnenborg *et al.*, 1980). Two the most important regulators of trophoblast invasion are the hCG and the P4 hormones. Through our study, we have showed that both hormones increase trophoblast invasion in a dose dependent manner: maximum invasion, was witnessed at a concentration of 1000 ng/ml for both the hormones, though P4 caused a higher number of cells to invade at this concentration. In 1998 the Shimonovitz group reported that P4 down-regulates the production of MMP-9, a matrix metaloproteinase, which is believed to mediate invasion of cells *in vitro* (Shimonovitz *et al.*, 1998). Where the hCG hormone is concerned, previous studies have reported that it could play an autocrine role in its control of trophoblast growth and invasion, inhibits trophoblast invasion in a dose-dependent manner, and increases trophoblast invasion (Shimonovitz *et al.*, 1998; Zygmunt *et al.*, 1998; Yagel *et al.*, 1993). Our results are in agreement with these previously published data, in that we also saw an increase in trophoblast invasion. However, to date the effect of hCG on trophoblast invasion is still not

clear and further studies are required. Proliferation is another important aspect in early pregnancy. Our investigation has found that while hCG increases trophoblast invasion, it has no effect on the proliferation of JEG-3 cells. However, P4 has an important effect on the JEG-3 cell proliferation, not only did it increase cell number, but it seems to do so in a dose-dependent manner, as the higher doses of P4 caused a greater increase in the cell number.

In pregnancy, P4 not only effects the uterus by regulating tissue repair and regeneration among other things, but it also creates a favourable immunological environment for the foetus by mediating interactions between the immune and endocrine systems (Szekeres-Bartho, 2009). Previously, cytokines have been reported to play an important role in the production of hormones by the trophoblast, one of these cytokines is IL-17 which was previously described to be expressed by the human placenta (Wu *et al.*, 2014). In our study we have observed that IL-17 significantly increases the secretion of the P4. The increase in P4 secretion upon IL-17 stimulation was seen during the earlier time points (6-48 h), and at all studied concentrations, though the concentration of IL-17 that had the highest stimulatory effect was 100 ng/ml. As of the 72 h time point, P4 secretion of IL-17 stimulated cells was similar to that of the unstimulated cells. Such an observation is in agreement with that of Pongcharoen and Supalap (Pongcharoen and Supalap, 2009). These researches reported that IL-17, has a stimulatory effect on P4 secretion during the earlier time points (6-24 h). This could be explained because IL-17 may only have a stimulatory effect on the secretion of P4 during the earlier time points, as after a certain time, there may not be any more IL-17 (no longer sufficient receptor occupancy). Another reason why an increase in P4 secretion was only seen at the earlier time points may be because, after 48 h, our cells have almost reached 100% confluency hence, their response to IL-17 may decrease (this may be due to a chance in their response to a higher concentration of P4). Nevertheless, the fact that IL-17 increases P4 secretion *in vitro* is important as it may allow an alternative way in which to control HLA-G expression.

Increase of P4 secretion by IL-17, prompted the question of whether this interleukin would be able to directly affect the expression of HLA-G, as it was previously reported that P4 has an up-regulatory effect on HLA-G, thereby IL-17 should have some effect on the expression of this protein. However, we observed that IL-17 has no direct effect on the expression of HLA-G while, P4 stimulation increased

HLA-G expression. The stimulation of JEG-3 cells with IL-17 had no observable effect on HLA-G expression. It was therefore hypothesized that the effect of P4 on the expression of HLAs is mediated through PR activation, which binds to a unique progesterone response element (PRE) sequence, which is located in the promoter region (Yie *et al.*, 2006a).

P4 is an important regulator in the establishment, and throughout the course of pregnancy. P4 is able to effect the regulation of the cell cycle, the differentiation, proliferation, and invasion of the trophoblast cells, all of which are important if the endometrium is to be ready to host the embryo. Progesterone's regulatory effects on HLA-G, were first reported by the Yie group and our study, further supported this in addition, to also showing that P4 also exerts its regulatory effects on the other HLAs expressed at the fetomaternal interface. Such findings are important as P4 is a well-established modulator of the immune system and it can be hypothesized that one of the ways in which this hormone carries out its functions, may be through the regulation of HLA-G expression.

CHAPTER V

RESULTS

EFFECTS OF PREIMPLANTATION FACTOR ON JEG-3 CELLS

5.1 Introduction

In the previous chapter, we have characterised and established our model of study. After that, we have studied how some soluble factors affect the biology of JEG-3 cells. We have analysed the role of P4 and IL-17 over the expression of HLA-C, -E, -F, and -G. However, to date, the specific endogenous factors involved in regulating HLA expression in pregnancy are not fully characterized. PIF, which is reported to regulate global systemic immunity and trophoblast invasion was used in conjunction with the JEG-3 trophoblastic cell line to analyse the effect of PIF on HLA-I expression (-C, -E, -F, and -G) in a controlled environment, in comparison to other soluble factors, mainly P4.

5.2 Results

5.2.1 Effect of PIF on HLA expression

To observe the possible effects of PIF on HLA expression patterns *in vitro,* JEG-3 cells were incubated without PIF (control) or with a range of different concentrations of PIF (50-1000 nM). HLA-G expression was measured, by flow cytometry, before and after treatment. Results showed that the exogenous PIF increases HLA-G levels in JEG-3 cells in a dose dependent manner in comparison to the untreated samples (Figure 5.1).

The detected increase was statistically significant ($P \leq 0.01$) when the cells were treated with a dose of 1 nM of PIF or higher. The concentration of PIF that resulted in the greatest increase of HLA-G was 200 nM. However, with concentrations of PIF higher than 200 nM we observed a decrease in HLA-G expression levels. This can be related to the saturation of the mechanism or receptor involved in regulating HLA-G expression. The mechanism that is involved in the regulation of HLA-G expression by PIF is still not well understood. One of our hypotheses is that PIF acts as a "protection system" that the foetus has before P4 reaches optimal concentrations. PIF is not going to regulate just HLA-G expression other mechanisms will also be invariably regulated by PIF. For this reason, we decided to also analyse the proteasome by 2-DE (Chapter VI. Results 6.2), to detect possible proteins which expression could be significantly increased or decreased after PIF stimulation.

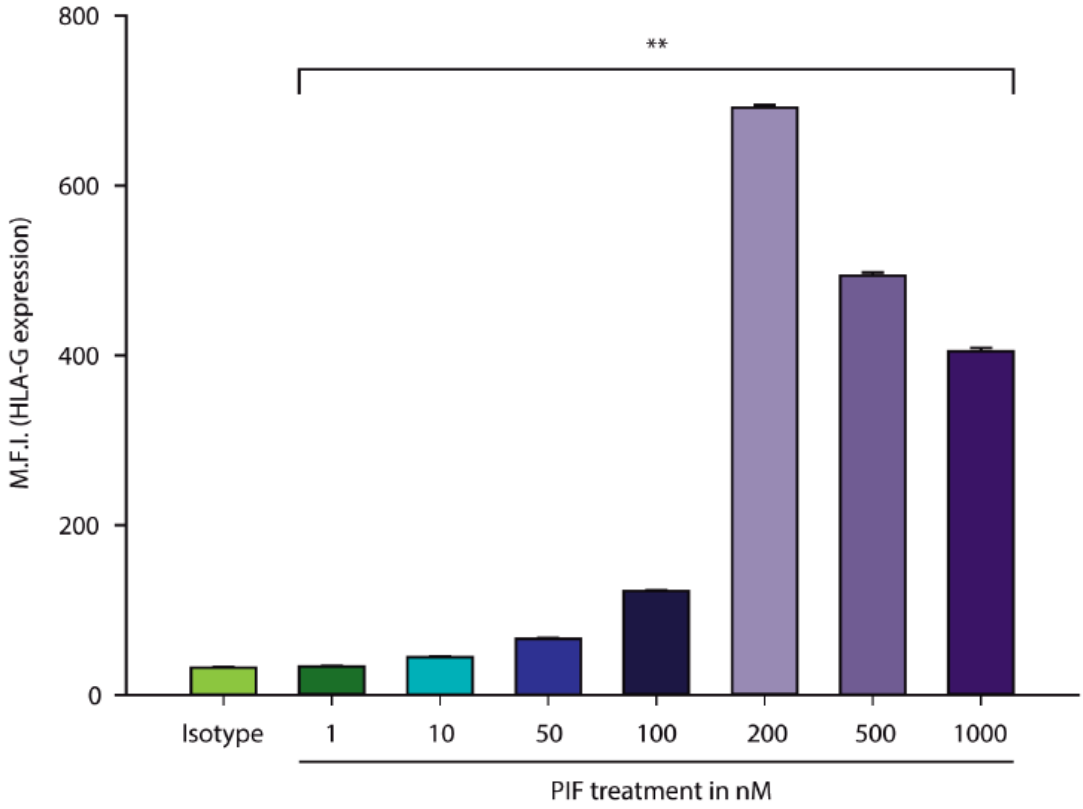

Figure 5.1. Evaluation of PIF dose effect on HLA-G surface expression.
JEG-3 cells were cultured in serum free medium and treated with PIF [0-1000 nM] for of 24 h. Their surface HLA-G expression was measured as the M.F.I using flow cytometry. Values are a mean of six independent experiments ± SD. (**) $P \leq 0.01$.

Upon determination of an appropriate dose, the time course of this effect on the cells was investigated. Using the optimal PIF concentration of 200 nM, time points between 6-72 h were analysed to determine at which point the most significant increase in HLA-G expression would be detected. The results showed that after 24 h of incubation, HLA-G reached its maximum surface expression (Figure 5.2). However, all the times points equal or over 6 h were statistically significant ($P \leq 0.05$).

Our experimental conditions were set up for the rest of the experiments, with an incubation time of 24 h and using a concentration of PIF of 200 nM. Experiments designed to detect the role of PIF on the other HLAs expressed by the JEG-3 cells, both at the surface and intracellular level were then carried out.

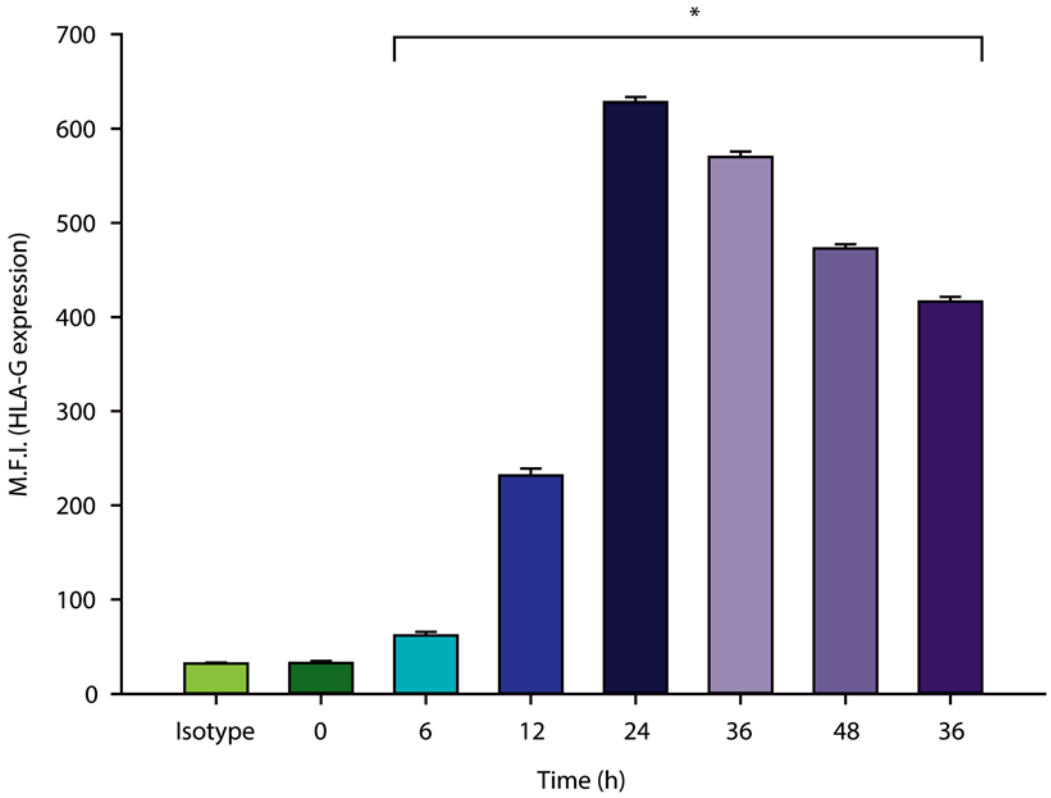

Figure 5.2. Evaluation of PIF time effect on HLA-G surface expression of JEG-3 cells.
The cells were cultured in serum free medium and stimulated with PIF at a concentration of 200 nM for a period ranging from 0-72 h. The surface HLA-G expression was measured as the M.F.I using a flow cytometer. Values are a mean of six independent experiments ± SD. (*) $P \leq 0.05$.

Surface expression of HLA-C, -E, -F, and -G was analysed using flow cytometry. We observed that, in comparison to the untreated cells, PIF treated cells had a statistically highly significant ($P \leq 0.01$) effect on the expression of HLA-G and HLA-E while, HLA-C showed significant changes ($P \leq 0.05$), as we can observe in Figure 5.3. HLA-F was not detected at the cell surface.

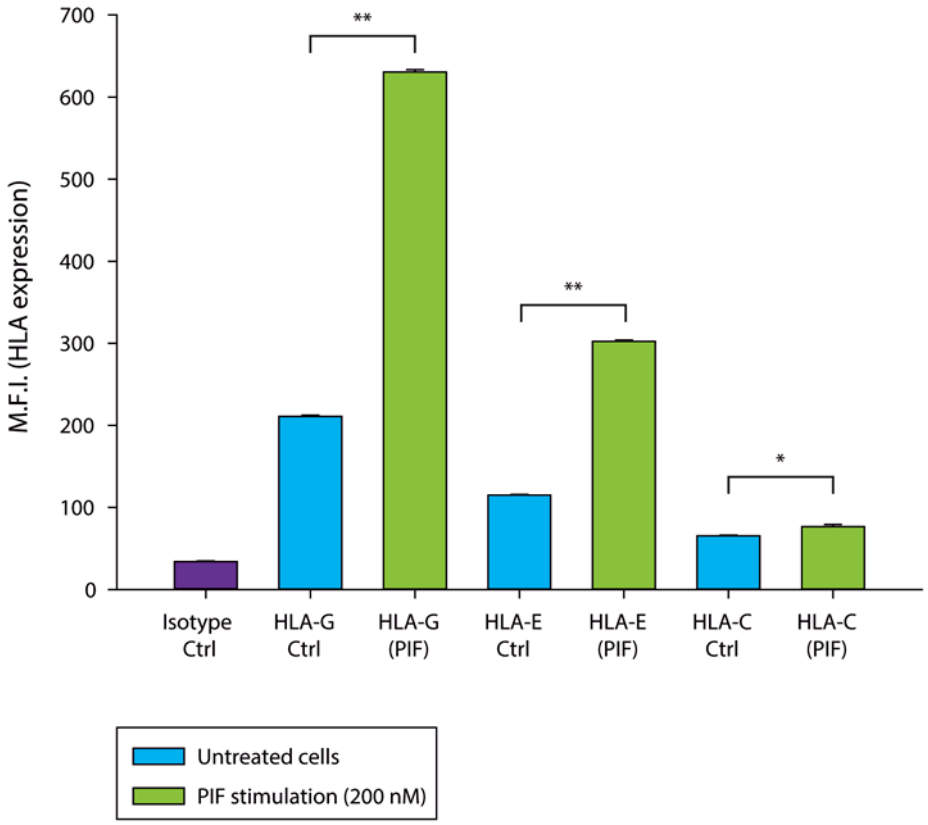

Figure 5.3. Effect of PIF on HLA surface antigen expression on JEG-3 cell line.
HLA-G and HLA-E were HLAs that showed higher changes compared with the control group. HLA-C also changed its expression significantly but not as dramatically as HLA-G or HLA-E. Data are a mean of six independent experiments ± SD for each antigen (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

All HLAs experienced an increase in their expression, though those with the highest levels of expression were HLA-G and HLA-E. To see if there was any correlation with an increase of their expression at the intracellular level we permeabilazed the cells, as described in Chapter II. The increased expression detected at the cell surface followed a similar pattern intracellularly (Figure 5.4). HLA-G and HLA-E were the antigens, which showed the highest increase, though HLA-C and HLA-F also experienced an increase. We observed a significant change in HLA-F expression this was not reflected in a surface expression, as we have previously showed. Contrary to what we observed at the cell surface, HLA-C showed a greater statistical difference between the control cells and those cells treated with PIF, at the intracellular level. This could be related to the role that this HLA plays at the fetomaternal interface, compared to that of HLA-Ib. While HLA-Ib, are mostly related to immunomodulation, HLA-C still plays a role in the activation of the immune system at the fetomaternal interface, and that could mean

that its expression has to be more controlled, hence why there is a higher expression at the intracellular level.

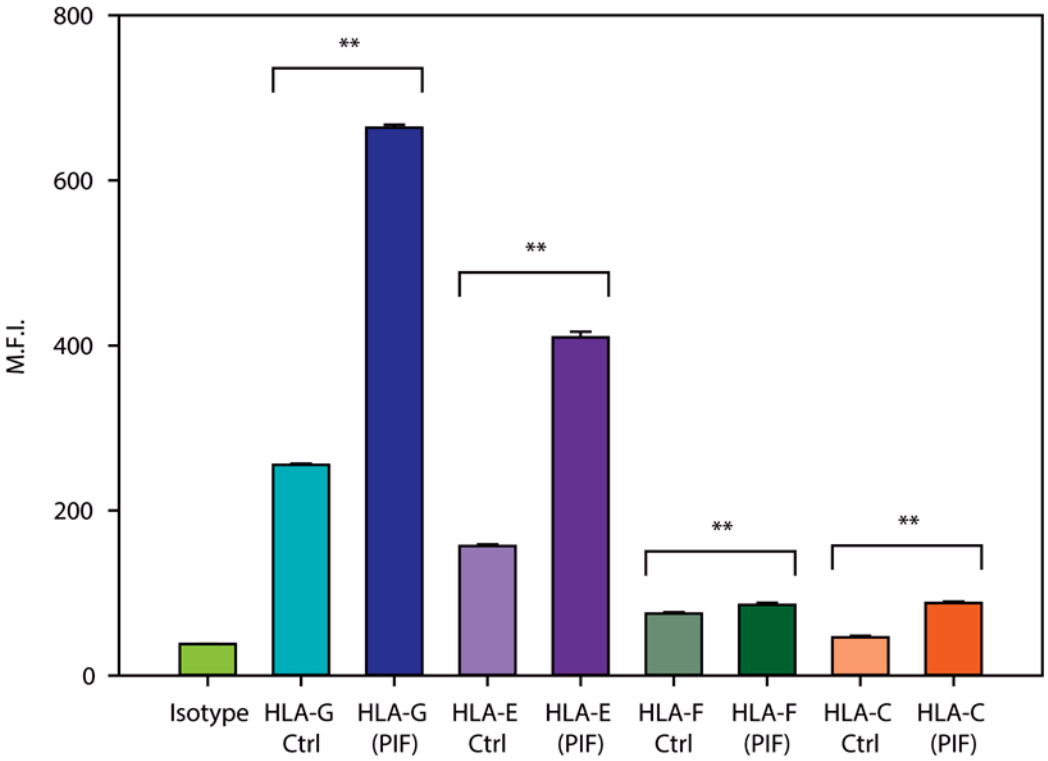

Figure 5.4. Effect of PIF on intracellular HLA expression of the JEG-3 cell line.
Cells were cultured for 24 h with or without PIF and their intracellular HLA expression analysed using a flow cytometer. HLA-G and -E showed the highest expression increase compared with -F and -C. However, both HLA-F and HLA-C also showed an increased expression. Data are a mean of six independent experiments ± SD for each antigen. (**) $P \leq 0.01$.

5.2.2 Analysis of the effect of P4 and PIF on HLA- I in JEG-3 cells

A comparison between the effects of PIF and P4 on the intracellular expression of HLAs was carried out using both qualitative and semi-quantitative techniques. JEG-3 cells were incubated using either PIF or P4, after which they were processed, as described in Chapter II, for Wb or flow cytometry. The Wb experiments showed that upon stimulation with PIF, HLA that showed the higher response in comparison to its P4 treated counterpart was HLA-G, as can be seen in Figure 5.5. We also observed that after PIF stimulation the increase in HLA-E was higher than that generated through P4 treatment. The Wb also showed that HLA-C and HLA-F expression did not differ between either of the stimulations.

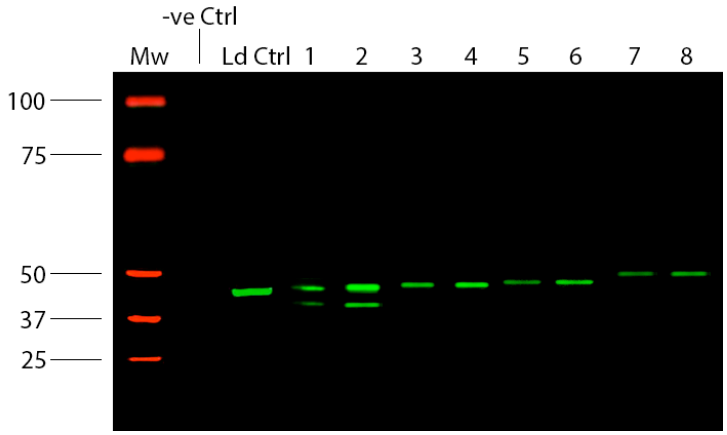

Figure 5.5. Western blot analysis of P4 and PIF stimulated JEG-3 supernatants.
JEG-3 cells were stimulated with or without PIF or P4 for 24 h. Lane 1, 3, 5 and 7 represent HLA-G, HLA-E, HLA-C and HLA-F, respectively, in the P4 stimulated samples. Lanes 2, 4, 6 and 8 represent HLA-G, HLA-E, HLA-C, and HLA-F in the PIF treated samples. The negative control (-ve Ctrl) was loaded with water, and the loading control (Ld Ctrl) is tubulin, which has a Mw of 42 kDa. HLA-G, HLA-E, HLA-F, and HLA-C were detected at different levels, in both P4 stimulated and PIF stimulated cells at a Mw of 39 kDa, 42 kDa, 39 kDa and 42 kDa, respectively.

We have previously shown that P4 can induce an increase in the expression of HLAs in our experimental model. PIF results showed that like P4, PIF also increases the expression of HLAs. Both soluble factors were thus examined to determine which of the two has a greater increasing potential.

The effect of PIF and P4 over HLA intracellular expression was examined using flow cytometry. Cells were incubated with or without PIF or P4 for 24h. Cells were permeabilazed and results acquired using the BD FACS Aria I. Our results showed that PIF induced a greater increase in intracellular HLA expression than P4 (Figure 5.6). Both HLA-E and HLA-F, though not those molecules with the highest readings experienced a statistically significant increase in their expression when stimulated with PIF than P4 ($P \leq 0.01$). Compared to the increase experienced after P4 treatment, the change in HLA-G and HLA-C intracellular expression in response to PIF treatment was also significant ($P \leq 0.05$). However, the molecules that showed the highest increase in their expression after PIF and P4 treatment were HLA-G and HLA-E (these were the molecules with the highest M.F.I values), as shown in Figure 5.6.

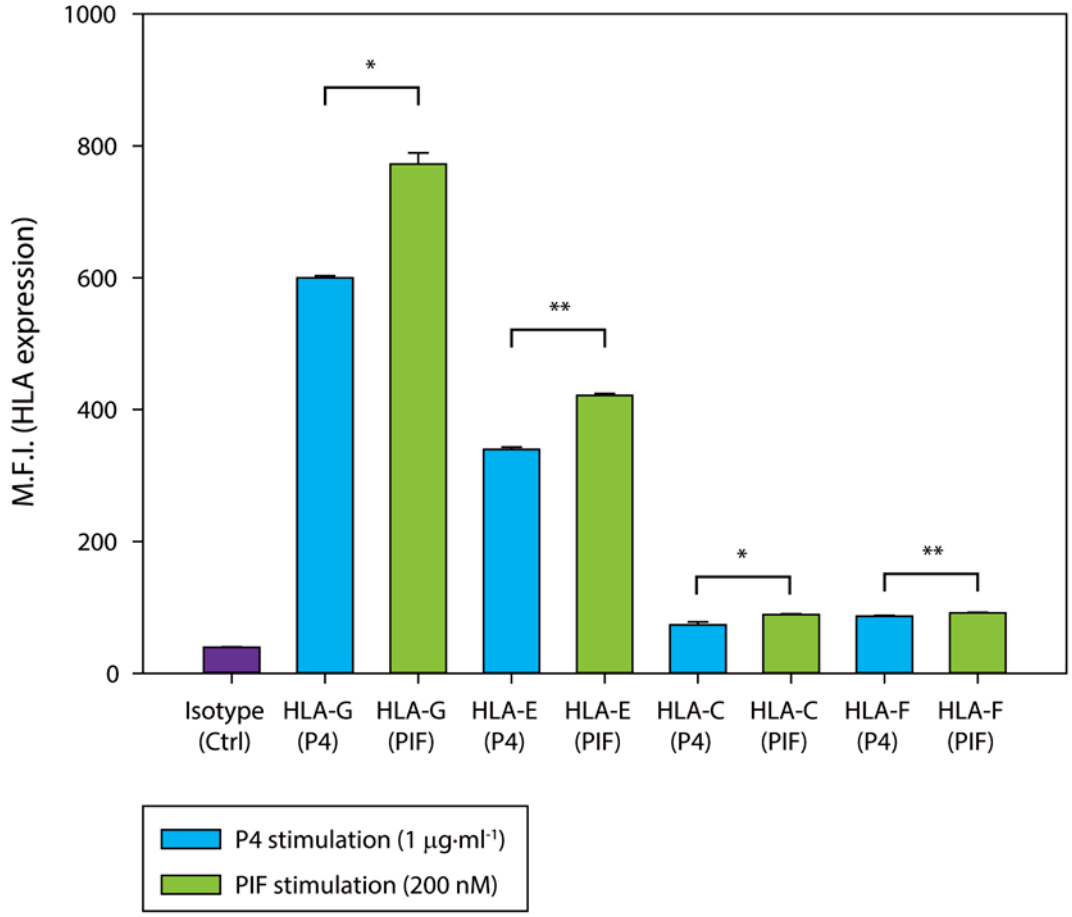

Figure 5.6. Comparative study of effects of P4 and PIF on intracellular HLAs.
JEG-3 cells were treated with P4 or PIF for 24h and processed for flow cytometric analysis. HLA-G and HLA-E were the molecules that showed higher increase in their expression after P4 of PIF treatment. However the statistical differences were different them. HLA-E and HLA-F showed highly significant changes while, HLA-G and -C showed significant changes. Data are a mean of six independent experiments ± SD for each antigen. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

A comparison of the effect of the two soluble factors on HLA surface expression was then carried out to see if the increase that was observed intracellularly would be reflected at the cell surface. To do this, we studied both the M.F.I. levels and the number of surface antigens on the cell surface. Cells were incubated with either P4 or with PIF, and analysed using flow cytometry based on their M.F.I. Then, using beads, the number of quantifiable antigens on the surface was measured. In comparison to the P4 treated samples, all the PIF treated samples experienced a significant increase in their HLAs ($P \leq 0.01$, for HLA-E and HLA-C, and $P \leq 0.05$ for HLA-G), as shown in Figure 5.7.

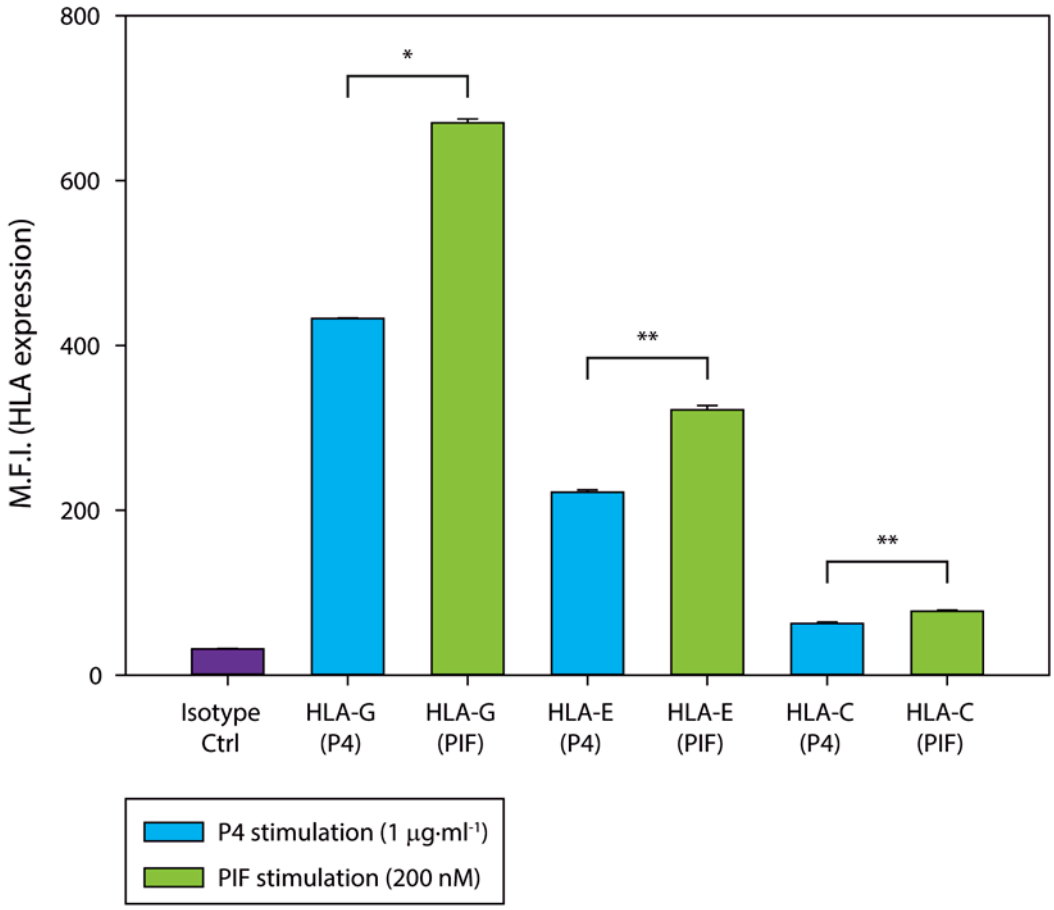

Figure 5.7. Comparative study of effect of P4 and PIF on the surface expression of HLAs by JEG-3.
Cells were treated with P4 or PIF for 24h. The comparison between P4 treatment and PIF treatment showed that PIF can induces higher surface expression than P4. Data are a mean of six independent experiments ± SD for each antigen. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

A quantitative comparison between the surface antigens after P4 or PIF treatment was also carried out (Figure 5.8). All HLAs showed higher expression levels on the cell surface after PIF treatment, in comparison to P4 treatment. The difference between the number of HLA-G and HLA-E receptors after PIF vs P4 treatment was statistically significant ($P \leq 0.01$). Through both sets of data (Figure 5.7 and 5.8), we observed that the increase at the intracellular level was translated to an increase in HLAs at the surface level, with PIF treated cells displaying both a higher M.F.I. (Figure 5.7) and surface antigen number (Figure 5.8), in comparison to cells treated with P4.

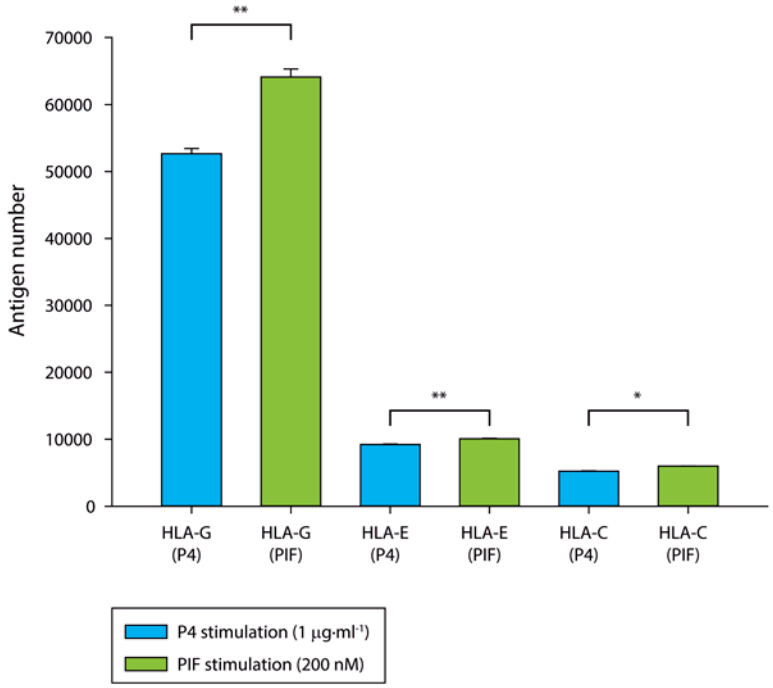

Figure 5.8. Comparative surface HLAs quantification after P4 or PIF treatment.
Cells were cultured and stimulated with P4 or PIF for 24 h. Their surface HLAs were quantified. The statistical study showed that the difference between the P4 and PIF HLA-E and HLA-G receptors was statistically highly significant (**). The difference in HLA-C surface receptors was statistically significant (*). Values are a mean of six independent experiments ± SD. (**) $P \leq 0.01$ and (*) $P \leq 0.05$.

5.2.3 Cell proliferation

Implantation generally takes place six or seven days after fertilisation and, once the blastocyst is adhered to the uterine epithelium, invasion begins. The processes of cell proliferation and invasion are important in pregnancy for normal tissue homeostasis, embryo implantation, and development. Hence a tight regulation of the two is required.

To study the proliferation of the trophoblast cell in response to PIF, cells were incubated at different time points (0-72 h) and PIF concentrations (0-1000 nM). Cells were also incubated with camptothecin and P4, as negative and positive controls respectively. The highest number of proliferative cells was observed after 24 h (Figure 5.9). All the PIF studied concentrations induced an increase in the number of proliferative cells, except 1 nM and 1000 nM. The concentration of PIF that induced the highest number of proliferative cells was 200 nM (Figure 5.9), the same value that we previously observed to induces highest changes in HLAs expression.

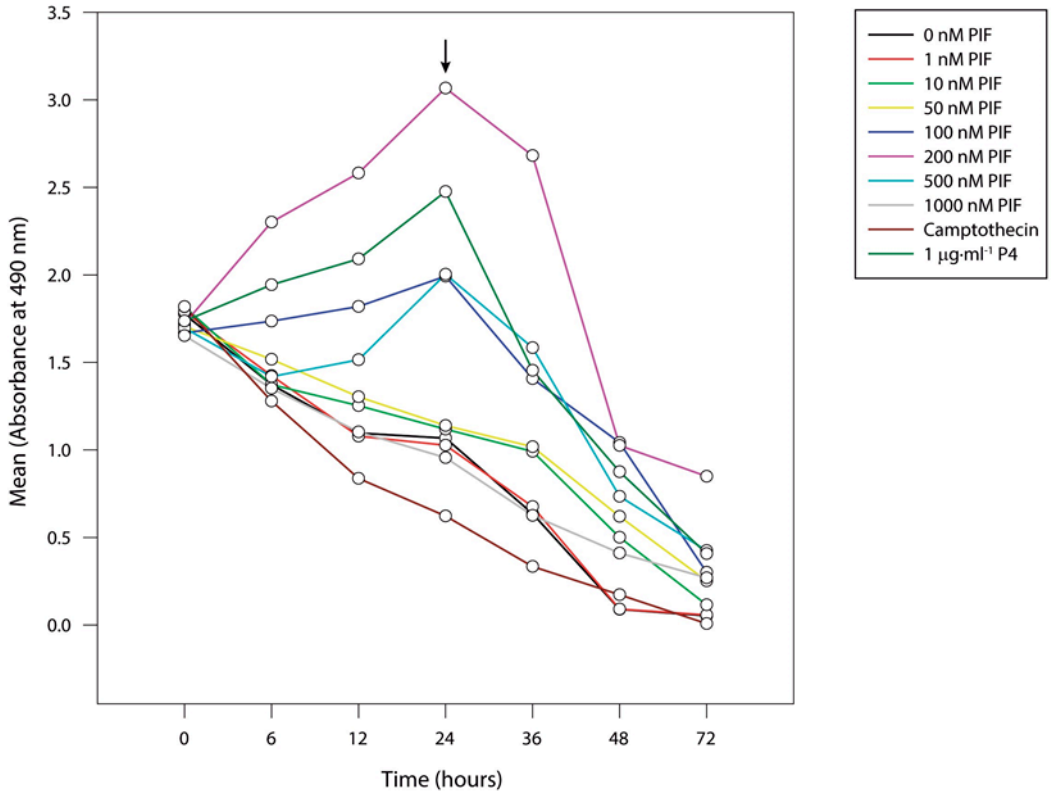

Figure 5.9. Effect of campthotecin, P4, and PIF on the proliferation of JEG-3 cells.
Results showed that JEG-3 proliferation is time and dose dependent. The arrow head indicates the most significant value for time and dose. P4 induces an increase in cell proliferation (positive control). Campthotecin impedes cell proliferation (negative control). Results are presented as a mean of six independent experiments ± SD.

5.2.4 JEG-3 cell invasion in response to P4 and PIF

As a means by which to understand the role of PIF on JEG-3 invasion, a Matrigel invasion assay was performed. Cells were grown over a Matrigel and subsequently challenged with hCG (1000 mU/ml), P4 (1000 ng/ml) or PIF (200 nM) for 24 h. Upon analysis, we saw that all three soluble factors showed a significant increase in cell invasion when they were compared to untreated cells, as seen in Figure 5.10. In comparison to P4 and hCG, treatment with PIF showed a difference of 1.43 change and 1.7 change, respectively, in invasiveness.

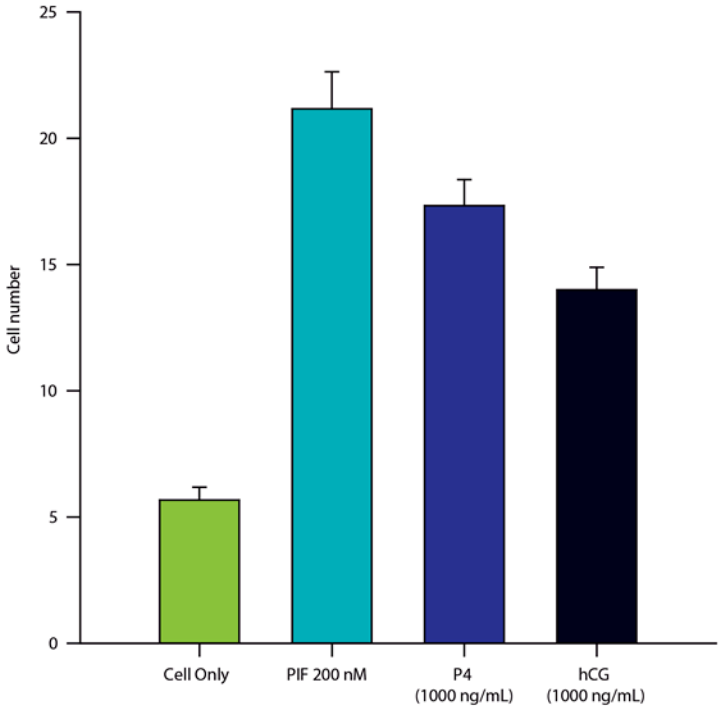

Figure 5.10. Representative invasive response of JEG-3 cells to PIF, P4, and hCG.
Cells were treated with or without PIF, hCG, and P4 for 24 h. Each bar represents the number of JEG-3 cells on the underside of an insert filter. When compared to cell only, all three treatments were statistically highly significant ($P \leq 0.01$). Results are presented as a cell mean number of three independent experiments ± SD.

5.2.5 Effect of PIF and IL-17 on P4 secretion on JEG-3 cells

We have previously observed that IL-17 affects the secretion of P4 by the JEG-3 cell. As a means by which to investigate if PIF would also be able to modulate the secretion of P4 by this trophoblastic cell line, JEG-3 cells were cultured and treated with either IL-17 or PIF. The incubations time points ranged from 6 to 72 h. We selected these time points as the secretion of P4 is regulated and needs gene transcription, mRNA translation and protein processing. P4 secretion was quantified using ELISA. We observed that both IL-17 and PIF caused a statistically significant increase in the secretion of P4 in comparison to the untreated JEG-3 cells (Figure 5.11). The statistical analysis and results are in the appendix and are not represented in Figure 5.11 as the number of them was so high that it will have made the figure very difficult to understand.

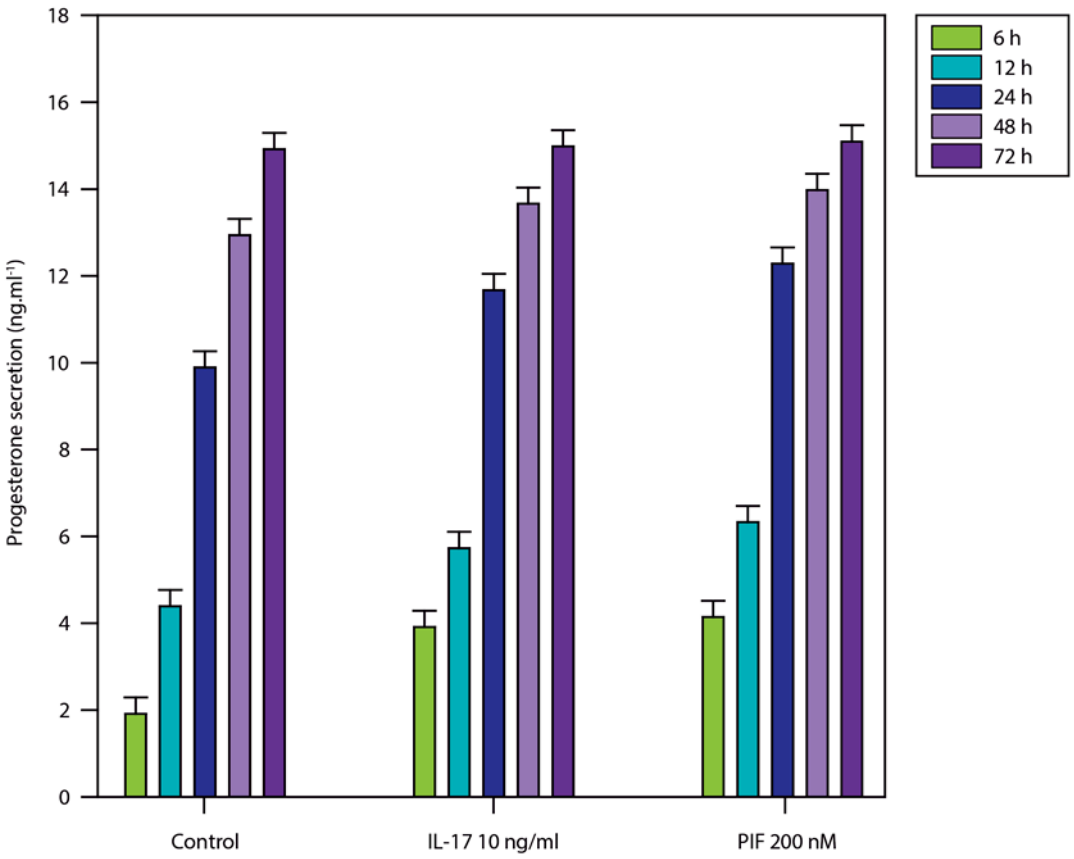

Figure 5.11. Representative P4 secretion by JEG-3 cells in response to of IL-17 and PIF at varying time points. JEG-3 cells were treated with IL-17 and PIF for 6, 12, 24, 48 and 72 h. Each bar represents the mean concentration of progesterone from three independent experiments. For significance levels refer to appendix.

5.2.6 Cytokine profile of JEG-3 in response to soluble factors

One of the aims of this study was to investigate how certain soluble factors, such as P4 and PIF, affect the cytokine profile in the JEG-3 trophoblastic cell line. To achieve this, cells were grown and stimulated with either P4 or PIF for 24 h. The change in cytokine production was detected using an ELISA. The experiment showed that P4 and PIF stimulation both increased the production of pro-inflammatory cytokines in comparison to the unstimulated JEG-3 cells. In addition, stimulation of the JEG-3 cells with these two soluble factors, also induced secretion of other factors like TNF-α (Table 5.1). Compared to P4, PIF induced a greater increase in the production and secretion of the studied cytokines. This increase was significant.

Table 5.1. Effect of P4 and PIF on the secretion of cytokines by JEG-3 cells.

	IL-1β	**IL-8**	**IL-10**	**GM-SCF**	**TNF-α**	**IFN-γ**	**TGF-β**
JEG-3 cells	303 ± 33.5	601.5 ± 43.7	183 ± 11.2	226.2 ± 23.7	112.2±13.7	<10	313 ± 29.1
JEG-3 + P4	394 ± 27.7	685 ± 58.2	243 ± 15.6	310.1 ± 36.3	198 ± 21.3	10 ± 1.2	398 ± 57.4
JEG-3 + PIF	436 ± 46.3	754 ± 50.7	301 ± 23.1	398 ± 32.4	137 ± 16.4	14 ± 2.7	462 ± 39.2

5.3 Discussion

Since the exact moment of implantation until the moment of giving birth, the developing foetus and the mother are in continuous regulated communication where different soluble factors play an essential role to ensure a successful pregnancy, which is inextricably linked with placental growth and differentiation (Albrecht and Pepe, 1999; Barnea, 2001). However, without a successful implantation into the uterine wall, a pregnancy cannot progress further than the blastocyst stage (Aplin and Kimber, 2004). During this stage, several soluble factors have been reported to interact with one another, but more so to complement each other's functions so as to promote a healthy foetus. During the onset of pregnancy, many hormones are present, amongst which are hCG and P4. Though present during the first trimester of pregnancy, neither hCG nor P4 are present for the whole nine months (Malassine *et al.*, 2003). PIF, a peptide secreted by embryos as early as the two-cell stage, is however present from the onset of pregnancy and remains so up until birth (Paidas *et al.*, 2010). In this study we have studied one of the roles that this peptide can have in a pregnant woman is the regulation of HLAs expression at the surface of trophoblastic-derived cells. In addition, as PIF is a soluble factor that is released before P4, we also analysed the possible impact that PIF can have over the cytokine profile of the trophoblast-derived cells. The effects PIF has on HLAs expression and cytokines secretion regulation were compared with the effects induced by P4.

In this study, evidence of the effect of PIF on the expression of HLA by the JEG- 3 cell line has been acquired through the use of techniques such as Wb and flow cytometry. Results showed that PIF can induce an increase in the expression of the studied HLAs in a dose and time dependent manner, both at the intracellular and at the surface level. Our *in vitro* dose-time experiment showed that PIF had a significant effect on HLAs expression at doses over 1 nM and as soon as 6 h of incubation. The concentration and time at which PIF induces its maximal effects on JEG-3 cells measured as HLA-G expression, was at a dose of 200 nM and after 24 h of incubation. The Barnea group have hypothesized that PIF's ability to act at low doses is in part due to its early secretion and its small size (Barnea *et al.*, 2012a). We believe that, based on our results, this could be possible because a small peptide of only 15 amino acids can diffuse the membrane freely and mimics the role of other bigger molecules and its

synthesis would not take as long as that of a more complex molecule. An early secretion, at the two-cell-stage, can mean that it will act in a very low dose, basically autocrinely, while in the meantime the concentration of PIF continues to increase, and its effects will also become paracrine, i.e. at the uterus during or after the annealing. Results from the Than group have also eluded to this by showing that this peptide is only 15 amino acids, inherently nontoxic, universally mammalian and able to act a low doses (Than *et al.*, 2007). PIF can be found in the maternal circulation at a concentration ranging between 30-150 nM (Duzyj *et al.*, 2010). As it has been previously published, the concentration of PIF in the peripheral maternal blood has a wide range, which is in line with our findings, which found that a PIF concentration of 200 nM was that which had the highest expression increase effect. We have to be careful with this data, as the concentration that can be found in the peripheral blood is different than that which is found at the fetomaternal interface. The difference between the two concentrations may be because only a small amount of PIF can diffuse to the maternal blood stream, hence foetal concentrations are higher. Our results focused on the effect of PIF over a trophoblast cell line (which is made up of foetal cells), not over cells isolated from the maternal side. This could be another reason for such discrepancy in the concentrations of PIF. In addition, the time point of 24 h that was observed is similar to that which it takes embryos to reach the two-cell stage when they start to secrete PIF (Barnea, 2001). Our induction experiments were successful in identifying an appropriate time and dose which are similar to those conditions observed *in vivo*. This reinforces and partially validates the present experimental model as being a suitable system by which to study the role and impact of PIF at the fetomaternal interface. Physiological and *in vitro* culture conditions are never are going to be equal as there are so many different factors that can induce changes in the results, that is why our model is partially validated (this is something completely different from invalidated).

Through our experiments, it was also seen that PIF has an increasing effect on all HLAs as expressed by our cellular model, at the surface and intracellular level, as does P4 (Yie *et al.*, 2006b). It was observed that at the intracellular level, in comparison to untreated cells, PIF treated cells underwent a significant change in their HLA profiles. At the cell surface level this change was translated for all HLAs, notably HLA-G and HLA-E, and to a lesser degree HLA-C, with the exception of HLA-F which is not

expressed at this level. This study is the first to show a regulation of HLAs by PIF and is important as it shows that several soluble factors, using different pathways, are involved in the regulation of HLAs during pregnancy, which is important as it helps to ensure survival of the foetus.

Previous research has shown that P4 and hCG are important in regulating the process of pregnancy (Miyaura and Iwata, 2002; Okabe *et al.*, 2014). Our investigation furthered those results by showing that in addition to increasing HLA-G expression, P4 also increases other HLAs expressed on the trophoblast. Once we observed this, we compared the effects of PIF with those of P4. Our experimental results indicated that PIF and P4 both exhibit increasing effects on HLAs expression. We can hypothesize that PIF causes a higher increase in HLAs expression than does P4, as PIF is secreted soon after fertilization and before secretion or production of P4 by the corpus luteum or trophoblast, respectively (Maccarrone *et al.*, 2001). This is important because without an adequate uterine environment or implantation of the embryo, which is believed, to be partially mediated by PIF a healthy pregnancy will not occur. This hypothesis is in line with other groups that have reported that in pregnancy, problems regularly occur during the peri- and pre-implantation period (Aplin and Kimber, 2004; Sargent *et al.*, 2006).

After PIF and P4 exerted their regulatory affects, it was observed that HLA-G remained that which was the highest expressed, followed by HLA-E, -C and -F that was only detected intracellularly. The dominant expression of HLA-G over the other HLAs expressed by our cellular model, even after treatment with PIF, consistently points to this molecule as being pivotal in pregnancy. The Apps group reported that HLA-G was the first of HLA-Ib molecules to be discovered in the placental trophoblast cells, due to its elevated levels of expression at this site in comparison to the other HLAs (Apps *et al.*, 2007). In addition, an observation made by the Hutter group who stated that at the site of the human placenta, the expression of HLA-G is that which favoured further backs our hypothesis (Hutter *et al.*, 1996).

It was also hypothesized that after PIF stimulation, HLA-E and HLA-F would experience an increase similar to that experienced after P4 stimulation, while HLA-C would experience either a decrease in its expression or remain unchanged. Our results are not in line with this hypothesis as our results showed that even when HLA-E and HLA-F experienced an increase after PIF treatment, this was

significantly higher than that resulting from stimulation with P4. Our results also showed that HLA-G, HLA-E, and HLA-F underwent a significant increase in their expression in response to PIF, as they are essential in maintaining a healthy pregnancy. We hypothesized that the foetus ensures its own survival by secreting PIF, which acts on these HLAs in a positive manner. Our results also showed that HLA-C experienced an increase in its expression after PIF treatment and, even when the change was not as high as with HLA-G or -E, it was statistically significant compared with P4 treatment. We believe that the increase that we detected in HLA-C expression was related to its role in the regulation of the uNK cells. However, as HLA-C is a member of HLA-Ia, it is still a polymorphic molecule that is more related to antigen presentation. If a viral infection was to occur during pregnancy, or the foetus were to start to produce truncated or abnormal proteins, HLA-C will be the molecule that will trigger the mother's immune response. This response maybe will induce a preterm labour but surely will save the mother (Chazara *et al.*, 2011). In the end, the immune response is finely regulated where the net balance between activating and tolerogenic signals will decide if the immune system is activated or not (Thellin and Heinen, 2003; Tadokoro, 2012).

Through our investigation we also observed that PIF greatly affects the invasion and proliferation of JEG-3 cells *in vitro*. PIF enhanced JEG-3 invasion to a greater extent than hCG or P4. Our results, regarding hCG and P4, were in agreement with a previous work that described that P4 enhanced JEG-3 invasion to a greater degree than hCG (Zygmunt *et al.*, 1998). We also detected that cell proliferation was also increased after PIF stimulation, notably using a concentration of 200 nM and an incubation period of 24 h. Based on our data, we can assert that PIF's effects over cell invasiveness and proliferation is statistically significant compared with the results measured after hCG or P4 stimulation. This moves the classical paradigm of P4 as the "hormone of pregnancy" to something more complex to include others actors that can may be just as important as P4 in pregnancy. One of these new actors could be PIF as not only is it secreted before either of these hormones, but it's also assumed that it is an important factor in modulating the maternal immune system and preparing the uterine environment so as to promote receptivity. Our findings support previous groups' hypothesis and findings of PIF's multi-targeted effects

and ability to prepare and adapt the maternal body to pregnancy (Stamatkin *et al.*, 2011a; Stamatkin *et al.*, 2011b; Duzyj *et al.*, 2010; Bose *et al.*, 1989).

PIFs early detection in pregnancy drove us to investigate its effect on the secretion of P4. We have been able to detect that in comparison to IL-17, PIF induced a significant increase in the P4 secretion levels of JEG-3 cells. We believe that PIF's modulation of the secretion of P4 is the embryo's way of ensuring that there is a convenient level of P4, as well as warranting its renewal. In addition, we also believe that PIF induces higher secretion levels of P4 by signalling to the maternal host that it is viable and needs to be maintained, hence the latter needs to continually replenish its P4 levels. We postulate that only a properly implanted embryo would be able to control the secretion of P4, as only viable embryos synthetize and secrete PIF (Bose *et al.*, 1989; Roussev *et al.*, 1996; Adinolfi, 1988).

In pregnancy, cytokines have been shown to act in an autocrine and paracrine manner, so as to regulate the implantation process and the development of the embryo (Sanjabi *et al.*, 2009a; Sanjabi *et al.*, 2009b). As at the fetomaternal interface, there is a complex network of cytokines and hormones that are at play, the interactions between these two players are inevitable. This study was successful in showing that P4 and PIF both have an increasing effect on the synthesis and releasing of pro-inflammatory cytokines produced by the JEG-3 cells, in addition to inducing the expression of some anti-inflammatory cytokines. More so, it was also observed that in their regulation of cytokines, PIF had a more prominent effect on the regulation of the cytokines than P4. Our results are in line with those published by the Chaouat group, who reported that different hormones were able to influence the production of local soluble factors secreted by the endometrium, i.e. cytokines (Chaouat *et al.*, 2005). The regulation of cytokine production by PIF and P4 is important, as it highlights their roles in modulating the process of implantation and embryo development. In addition, PIF's dominant regulation of the cytokines further highlights its important role in embryo implantation and its role as an autocrine regulator of embryo development. By regulating the cytokine production, PIF is able to ensure that the necessary levels of cytokines needed for a successful implantation and maintenance of the embryo are thus met. This conclusion is alongside that of the Stamatkin group, who reported that PIF has multi-targeted effects, one of which was its autocrine supporting effect on the development of the foetus (Stamatkin *et*

al., 2011a; Stamatkin *et al.*, 2011b). The modulation of the local immune system by PIF is important as it points to its importance in the foetus' wellbeing.

The main source of P4 in the earliest stages of pregnancy is the corpus luteum and it is only when the placenta has been established that this becomes the primary source of P4. On the other hand, the embryo under development, as early as the two-cell stage, is the first source of PIF secretion. This study is the first to present evidence showing PIF's ability to regulate other HLAs expressed by a cellular trophoblast-derived model JEG-3, in an *in vitro* environment. In addition, it also showed that PIF has an effect on the proliferative and invasive behaviour of the JEG-3 cells, as well as the capacity to change or modulate JEG-3 cells' P4 secreting abilities and cytokine profile. PIF's modulation of HLAs, and the local environment, and its increasing effect of P4 secretion are important in that it highlight its importance, not only, in the earliest stages of pregnancy, but also point to the embryo as being a "selfish" entity as it produces PIF for its own benefit, in that it ensures its requirements are met. Pregnancy is an event, which involves multiple soluble factors, which have to act in a harmonious way in order to ensure a favourable outcome. Though the mother ensures the embryo's survival by providing a receptive endometrium and favourable local immunity, the embryo nevertheless guarantees its own survival by producing PIF, a peptide renowned for its multi-targeted effects.

CHAPTER VI

RESULTS

PROTEOME AND 2-DE ANALYSIS

6.1 Introduction

As we have shown in the previous chapters both, PIF and P4, play an important role in the regulation of several critical factors clearly involved in human pregnancy. Maybe the main role is the regulation of HLA-G expression, but not only, as both soluble factors also regulate the expression of HLA-E, HLA-F, and HLA-C. However, as had been highlighted in this research and in the bibliography, the mechanisms by which these soluble factors carry out their roles are still not well understood (Ramu *et al.*, 2013; Hakam *et al.*, 2014; al-Sebai *et al.*, 1995; Arkaravichien and Kendle, 1990; Barrera *et al.*, 2007; Coulam *et al.*, 1995; Moindjie *et al.*, 2014).

In this study we have observed that the effects induced by PIF are pretty much similar to those induced by P4. We also know that the expression of any protein is more complex, and involves many mechanisms that do more than just ensure its secretion, especially if this protein, is constitutively secreted by the cell. Protein secretion or expression requires the regulation of multiple factors such as enzymes, nuclear factors or enhancers, surface or intracellular receptors, etc. Based on these premises, we decided to investigate how PIF induces these changes that we have measured in the previous chapters, in order to better understand the role of PIF. To carry out this investigation, we decided to use 2-DE (two dimensional polyacrylamide gel electrophoresis), which is a powerful tool used to separate and fraction complex protein mixtures from cells, tissue or any biological sample.

As 2-DE analysis is not only cheaper than a microarray, but also easier to perform, and the bioinformatics analysis is simpler to carry out and not as time-consumable as the data-mining needed for a microarray it was the next logical step that was carried out in this investigation. In addition, 2-DE analysis offered us another advantage over a microarray study in that the expression of false positives is lower than those that occur in a microarray as when a protein is detected. However, with a microarray the information about all the mRNAs is provided, meaning both those that will be translated and those that will not. Hence with a microarray, for the data to be validated, a second technique, such as a Quantitative Real Time PCR (qRT-PCR) or newer and more expensive techniques, such as RNASeq or whole

transcriptome sequencing, will have to be carried out. Therefore, for this investigation, we thought that the 2-DE technique was the best way by which to investigate how a protein induces changes in both the phenotype and the genotype of a cell

6.2 Results

6.2.1 Protein profiles analysis from JEG-3 cells, treated and untreated

The protein expression of untreated JEG-3 and PIF treated and P4 treated cells was analyzed through the use of 2-DE. Four gels from each of the three groups (untreated, PIF treated and P4 treated) were analyzed. Figure 6.1 shows a representative gel of each treatment. The overall protein expression profiles from each sample were similar: spots were distributed throughout the gels, ranging from p/ 3 to 10 and with a Mw ranging between 10-150 kDa.

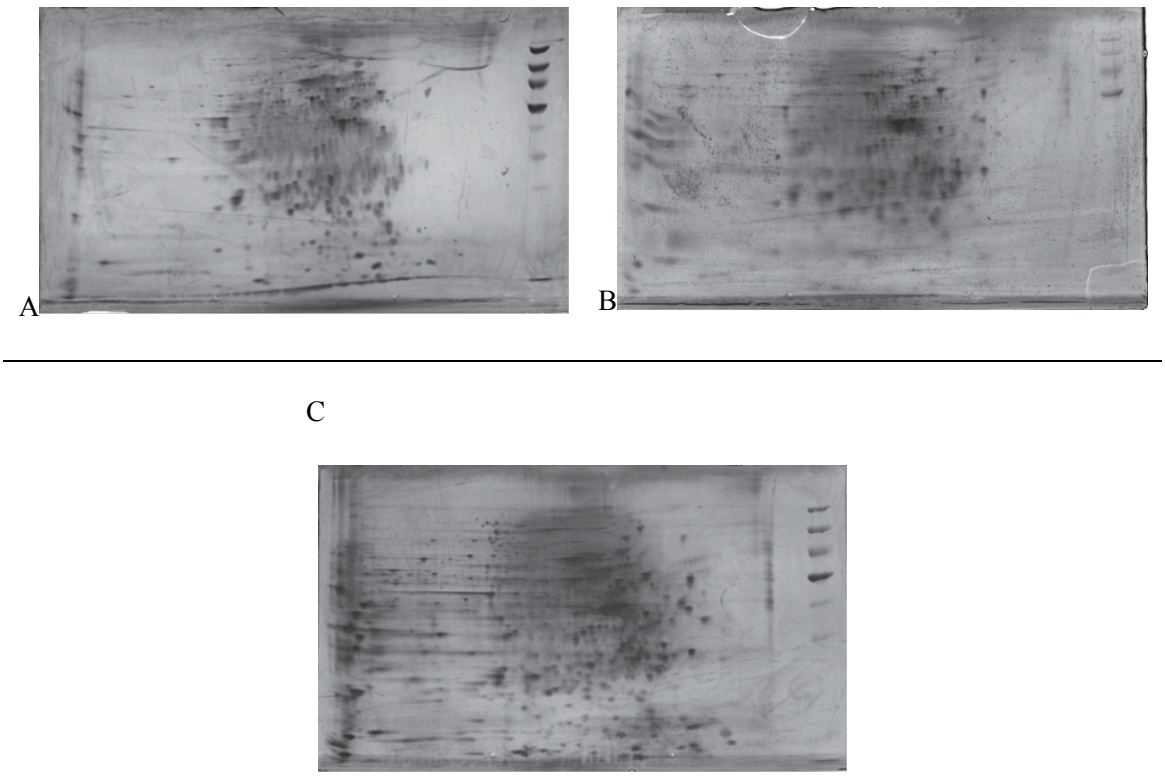

Figure 6.1. Representative 2-DE.
(A) Untreated cells. (B) Progesterone treatment. (C) PIF treatment.

Proteins were first separated on the basis of their pI, which ranged from 3-10 in the first dimension, after which they were separated based on their size by SDS-PAGE in the second dimension. To identify the changes that took place in protein expression between groups, a single master gel image representing all 12 gels was prepared using SameSpots Progenesis software (Figure 6.2). Through this, 22 spots were

found to be present and clearly focused among the samples and all showed a statistical significance. In Figure 6.2 spots are highlighted in blue in the gel. These spots were all subjected to MS analysis.

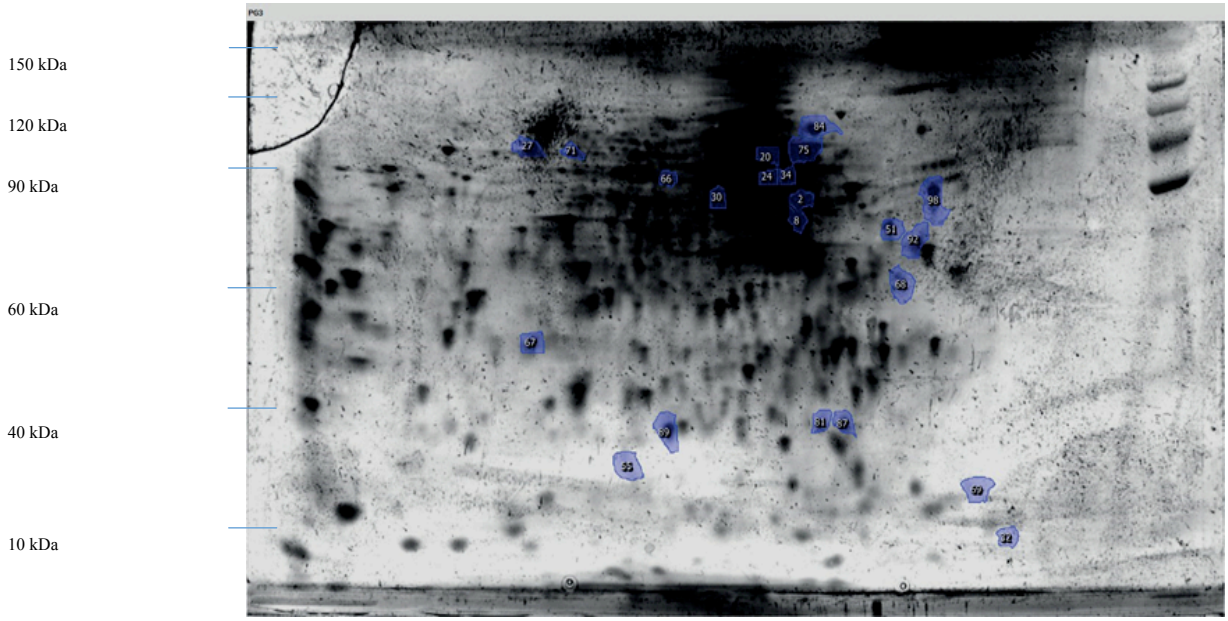

Figure 6.2. A master 2-DE gel representing twelve gels.
Twenty two spots common to all gels (identified by the circles) were identified. 14 proteins, were found to be significantly decreased between the three groups ($P \leq 0.05$) and 8 were found to be significantly increased ($P \leq 0.05$). Mw marking ranging from 10-150 kDa is displayed on the right side of the gel.

A comparison of the average spot volume (mean ± SD, 4 samples/group) of the 22 common proteins was then carried out (Figure 6.3). Between groups, the spot volumes were analyzed statistically, there was a significant difference between untreated JEG-3 cells and PIF treated JEG-3 cells: 14 polypeptide spots from the PIF treated group were shown to be significantly decreased ($P \leq 0.05$) compared to the untreated cells group, whereas eight protein spots from the same group were shown to be significantly increased ($P \leq 0.05$) compared to the untreated cells group. This is summarized in Table 6.1. The difference between untreated JEG-3 cell and P4 treated cells, as well as that between P4 treated *vs.* PIF treated cell was also statistically significant with $P \leq 0.05$. All of these proteins were excised from the gels, subjected to trypsin digestion, and prepared for identification by MS.

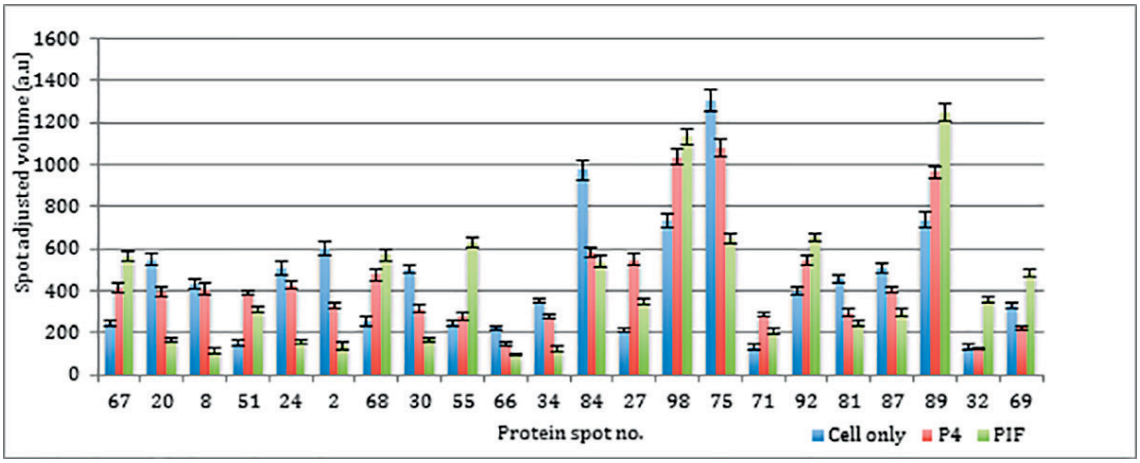

Figure 6.3. Histograms of protein spot adjusted volumes (mean ± SD) of the 22 spots identified to be differentially expressed between the groups.

6.2.2 Identification and study: differentially regulated protein in JEG-3 cells

The trypticaly digested spots were then analyzed using MALDI-TOF MS. Through this, it was possible to obtain a resolved mass fingerprint of all of the 22 proteins. The MS data was then submitted to the ProFound search facility, where all 22 of the proteins were identified. All the search results obtained were then evaluated on the basis of accepted standards so as to take into account the number of peptides that were matched to the candidate protein. In instances where multiple proteins were identified from a single spot, peptide counts representing each protein were then used to ensure the most significant protein was used. All of the proteins were identified with ProFound probability scores ranging from 0.99 to 1.00. All protein identifications made agreed with the expected Mw and pI range based on their positions on the gel. As for some proteins, it was not possible to obtain confident identifications, these proteins are thus identified as unknown (Table 6.1).

Table 6.1. Differentially expressed spots as identified by MS

Spot	ANOVA (*P*)	Fold	Protein Description	Average Normalised Volumes		
				Untreated cells	P4	PIF
67	> 0.001	▲2.3	DCBD2	243.571	413.865	566.578
20	> 0.001	▼3.4	Unknown	548.379	395.572	161.874
8	> 0.001	▼3.9	HSPA4L	431.610	407.206	111.996
51	0.001	▼2.6	CD59	150.222	388.079	310.118
24	0.001	▼3.3	ERp60	508.002	425.280	155.610
2	0.002	▼4.4	HSP70	601.820	328.658	136.959
68	0.002	▲2.2	Unknown	255.434	476.075	571.194
30	0.002	▼3.0	EGFR	498.008	313.949	166.079
55	0.002	▲2.5	PGR	246.889	277.710	625.968
66	0.003	▼2.3	PRDX2	220.649	144.399	94.620
34	0.004	▼2.9	LDP1	349.317	276.712	121.548
84	0.005	▼1.8	eEF2	971.456	578.216	535.644
27	0.007	▼3.1	Unknown	208.919	647.790	351.062
98	0.007	▲1.5	FOXP3	732.057	1035.457	1129.905
75	0.015	▼2.0	KPYM	1299.172	1078.025	649.914
71	0.015	▼2.1	ATPS	132.881	283.644	207.615
92	0.016	▲1.7	HBB	394.897	544.421	653.066
81	0.016	▼1.9	EPCAM	455.831	294.987	245.081
87	0.018	▼1.7	GAPD	510.311	399.647	294.760
89	0.019	▲1.7	HLAG	733.573	961.078	1248.309
32	0.023	▲2.9	ZNF38 ZFP36	132.122	123.015	359.905
69	0.027	▼2.2	CLIC3	326.428	220.958	484.635

From the 22 spots, three of them were undetectable or not clearly characterized and were removed from further studies. The ontology and pathway analysis was done over 19 spots that represented 19 different proteins, nine of them overexpressed and ten under-expressed. In Table 6.2 the proteins have been grouped as over- or under- expressed.

Table 6.2. Statistically significant spots classified as overexpressed and under-expressed

	Spot	ANOVA (*P*)	Fold	Protein Accesion Number (UniProt)	Protein name	Average Normalised Volumes		
						Untreated control	*P4*	*PIF*
Overexpressed	67	> 0.001	2.3	Q96PD2	DCBD2	243.571	413.865	566.578
	51	0.001	2.6	P13987	CD59	150.222	388.079	310.118
	55	0.002	2.5	P06401	PRGR	246.889	277.710	625.968
	98	0.007	1.5	Q9BZS1	FOXP3	732.057	1035.457	1129.905
	71	0.015	2.1	P06576	ATP5B	132.881	283.644	207.615
	92	0.016	1.7	P68871	HBB	394.897	544.421	653.066
	89	0.019	1.7	P17693	HLAG	733.573	961.078	1248.309
	32	0.023	2.9	Q9Y5A6 P26651	ZSC21 TTP	132.122	123.015	359.905
	69	0.027	2.2	O95833	CLIC3	326.428	220.958	484.635
Under-expressed	8	> 0.001	3.9	O95757	HS74L	431.610	407.206	111.996
	24	0.001	3.3	P30101	PDIA3	508.002	425.280	155.610
	2	0.002	4.4	P08107	HSP71	601.820	328.658	136.959
	30	0.002	3.0	P00533	EGFR	498.008	313.949	166.079
	66	0.003	2.3	P32119	PRDX2	220.649	144.399	94.620
	34	0.004	2.9	P02545	LMNA	349.317	276.712	121.548
	84	0.005	1.8	P13639	EF2	971.456	578.216	535.644
	75	0.015	2.0	P14618	KPYM	1299.172	1078.025	649.914
	81	0.016	1.9	P16422	EPCAM	455.831	294.987	245.081
	87	0.018	1.7	P04406	G3P	510.311	399.647	294.760

All the proteins identified are associated with a wide variety of cellular functions, such as signal transduction, cytoskeletal, and antioxidation functions, and related to different cell subtypes, tissues, and have the ability to interact with other proteins as described at the website database http://www.ncbi.nlm.nih.gov/gene/ from PubMed. In brief, the characteristic of each protein is as follows:

- **DCBD2**, which is encoded by the *DCBLD2* gene, is highly expressed in testis, heart, skeletal muscle and also in cultured vascular smooth muscle cells. This protein is found to be significantly increased in the case of lung cancer, specifically during the process of tumour progression, wound healing, and can act as a negative regulator of cell growth. In addition, this protein also has the ability to interact with different proteins, i.e. EGFR,

CRKL, SEMA4B and TRAF6 (the two latter ones are strongly associated with the immune system).

- **CD59**, is encoded by the *CD59* gene. This protein, which is also referred to as a membrane attack complex (MAC)-inhibitory protein (MAC-IP), is responsible for the regulation of complement-mediated cell lysis, as well as being involved in the process of lymphocyte signal transduction. This protein is a potent inhibitor of the MAC, whereby it binds C8 and/or C9, thereby inhibiting the incorporation of multiple copies of C9 into the complex, which is necessary for osmolytic pore formation. This protein also plays a role in signal transduction pathways namely in the activation of T cells. CD59 interacts with several different proteins, such as the envelope surface glycoprotein gp120 and gp41, which are present in HIV-1, or with EGFR complement elements C8 and C9 and their derivate and SMAD4.
- **PRGR**, encoded by the *PGR* gene, is a progesterone receptor of the steroid receptor superfamily. PRGR, mediates the physiological effects of the progesterone hormone, which plays a central role in reproductive events associated with both the establishment and the maintenance of pregnancy. PRGR also interacts with different proteins such as ESR1 and SP1 and with the histones HIST3H3 and HIST4H4.
- **FOXP3**, is encoded by the *FOXP3* gene. The FOXP3 protein is a member of the forkhead/winged-helix family of transcriptional regulators. It has the ability with which to interact with Tat protein from the HIV-1, the nuclear factors AT (NFAT5, NFATC1 and 2) and HDCA proteins. FOXP3 is known to be one of "master switches" during the Treg differentiation.
- **ATPB**, encoded by the gene *ATP5B*, is a subunit of mitochondrial ATP synthase. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. In addition, it has also been reported that ATPB can inhibit HIV-1 replications in HeLa cells by interacting with the viral proteins Tat, Vpr and Vpu. ATPB can also interact with other proteins such as other ATP5 or ASB proteins.

- **HBB** protein is encoded by the *HBB* gene, is related to human adult haemoglobin, which is a tetramer consisting of two alpha and two beta chains. The central role of haemoglobin is the transport of oxygen and it has the potential to interact with proteins like ORC2, HBA2 or SELT.
- **HLA-G**, which encoded by the *HLAG* gene, is one of the prime members of the non-classical HLA class I molecules (HLA-Ib). HLA-G is involved in antigen presentation and in the regulation of immune responses. HLA-G is expressed by certain types of tumour cells, as a way with which to avoid cellular cytotoxicity. HLA-G can interact with a variety of different viral proteins such as the HIV-1 like gp120, gp41 or gp160. In the human body HLA-G can also interact with KIR2-DEL4, LILRB1 or TAP1 and 2.
- **ZSC21**, encoded by the *ZSCAN21* gene, is a member of the family of zinc-finger proteins that binds to DNA and regulates its transcription. ZSC21 can also interact with other ZNF proteins.
- **TTP**, encoded by the *ZFP36* gene, is a member of the family of zinc ring finger proteins, can interact not only with the DNA but also with COX2 mRNA. At the proteomic level, this protein can also interact with several proteins like YWHA proteins, PTGS2 or APP.
- **CLIC3**, is encoded by the *CLIC3* gene. Is a member of the chloride channels, which are a diverse group of proteins that regulate fundamental cellular processes including stabilization of cell membrane, potential trans-epithelial transport, maintenance of intracellular pH, and regulation of cell volume. The chloride intracellular channel 3, is a member of the p64 family, and is predominantly localized in the nucleus. It is renowned for its stimulation of chloride ion channel activity. This protein is also reported to participate in the process of cellular growth control, based on its observed association with a member of the MAP kinase family, ERK7.
- **HS74L**, is encoded by the *HSPA4L* gene. This protein, which is a member of the heat shock protein 70 family showed chaperone activity *in vitro*, where it is seen to inhibit

aggregation of citrate synthase. The expression of this protein is also renowned to be induced by both osmotic imbalance and heat shock.

- **PDIA3** is encoded by the *PDIA3* gene. This protein can be detected in the endoplasmic reticulum where is reported to interact with the lectin chaperones, calreticulin and calnexin, so as to modulate the folding of newly synthesized glycoproteins. It has recently been demonstrated that this protein has disulphide isomerase activity, and not a phospholipase as was once believed. The complexes of lectins and this protein mediate protein folding through the promotion of the formation of disulphide bonds in their glycoprotein substrates.
- **HSP71**, is encoded by the *HSPA8* gene. Is a member of the heat shock protein 70 family, HSP71 belongs to the basic expressed members group, which are more commonly referred to as heat-shock cognate proteins. This protein functions as a chaperone, in addition to binding to nascent polypeptides as a mean by which to expedite correct folding. Moreover, this protein also functions as an ATPase in the disassembly of clathrin-coated vesicles during the process of the transportation of membrane components through the cell.
- **EGFR**, encoded by the *EGFR* gene. This protein, is a transmembrane glycoprotein member of the protein kinase superfamily. Moreover, it also acts as a cell surface receptor for members of the epidermal growth factor of extracellular protein ligands. Induction of receptor dimerization and tyrosine autophosphorylation, which ultimately leads to cell proliferation is triggered by the binding of the protein to a ligand. Mutations arising from this protein are highly related to lung cancer.
- **PRDX2**, encoded by the *PRDX2* gene, is a member of the peroxiredoxin family of antioxidant enzymes. This protein is known to reduce hydrogen peroxide and alkyl hydroperoxides, in addition to playing an antioxidant protective role in cells. This protein may also contribute to the antiviral activity of $CD8^+$ T cells. This protein is also seen to prevent haemolytic anaemia as a result of oxidative stress through the stabilization of haemoglobin. In addition, PRDX2 is also reported as having a proliferative effect, and may play a role in both the development and progression of cancer.

- **LMNA** is encoded by the *LMNA* gene, and is also known as LAMIN A/C. The lamin family of proteins have been highly conserved through evolution. Lamins are components of the nuclear lamina, which is a fibrous layer on the nucleoplasmic side of the inner nuclear membrane. During the process of mitosis, as the lamin proteins are phosphorylated, the lamina matrix is simultaneously reversibly disassembled. Moreover, the lamin proteins are believed to be involved in the processes of chromatin structure, gene expression, and nuclear stability. The proteins are also known to be able to interact with other lamin line proteins, such as LMNB, CASP1 and 6 or EGF.
- **EF2**, encoded by the *EEF2* gene, is a translation elongation factor with five domains. This G-protein, is a member of the GTP-binding translation elongation factor family. EF2 is also an essential factor for protein synthesis, and promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome. This protein is also known to interact with CDK proteins, TP53 or CASP7.
- Encoded by the *KPM* gene, the **KPYM** protein is a serine/threonine protein kinase belonging to the LATS tumour suppressor family, and is also involved in glycolysis. This protein has the role of o localize the centrosomes during the interphase, and early and late metaphase stages of mitosis. KPYM also interacts with the centrosomal proteins aurora-A and ajuba, and is essential for the accumulation of gamma tubulin and spindle formation at the beginning of the mitosis process. As well as interacting with a negative regulatory of p53, this protein also functions as a positive feedback loop with p53, responding to cytoskeletal damage. Lastly, the KPYM protein acts as a co-repressor of androgen-responsive gene expression.
- **EPCAM**, encoded by the *EPCAM* gene, is an epithelial cell adhesion molecule. EPCAM is part of a family that includes at least two type I membrane proteins, in addition to being a carcinoma-associated antigen. This protein functions as a transmembrane glycoprotein mediating Ca^{2+} independent homotypic cell-cell adhesion in the epithelia.
- **G3P**, also known as triose phosphate, is encoded by the *GAPDH* gene. G3P, which participates in the Calvin cycle, has both glyceraldehyde-3-phosphate dehydrogenase and

nitrosylase activities, thereby playing a role in glycolysis and nuclear functions, respectively. This protein is also known to hold a role in nuclear events, such as those of transcription, DNA replication, apoptosis, and RNA transport. It is most probably involved in nuclear functions due to its nitrosylase activity, which mediates cysteine S-nitrosylation of nuclear target proteins such as SIRT1, HDAC2 and PRKDC. This protein is also involved in modulating the assembly and organization of the cytoskeleton, in addition to facilitating the CHP1-dependent microtubule and membrane associations through its ability to stimulate the binding of CHP1 to microtubules. Is a component of the GAIT (gamma interferon-activated inhibitor of translation) complex and is involved in interferon-gamma-induced transcript-selective translation inhibition in inflammation processes. G3P, when is treated with interferon-gamma assembles into the GAIT complex, which then binds to stem loop-containing GAIT elements in the 3'-UTR of diverse inflammatory mRNAs, and as a result, suppresses their translation.

The selected significant spots were then analysed for their ontogeny using the web tools provided by PIR (Protein Information Resource, a UniProt consorcium member), hosted by the University of Georgetown at http://pir.georgetown.edu/pirwww/index.shtml.

The main focus of this study addressed the gene ontology (GO) of the 19 differentially expressed proteins. The analysis that was performed dealt with these proteins biological functions, as is summarized in Table 6.3. The analysis supplied data on the role of PIF during the first stages of pregnancy, and how it differs from the effects of P4 during this stage. Through the analysis of the 19 differentially expressed proteins, it was possible to detect 59 different ontological terms, as is summarized in the next table.

Table 6.3. GO analysis of biological functions.

GO ID number	Term	Proteins		Frequency
GO:0050896	Response to stimulus	▼HS74L	▲HLA-G	16
		▲CLIC3	▲TTP	
		▼EGFR	▼PDIA3	
		▼LMNA	▼PRDX2	
		▼G3P	▲HBB	
		▲PRGR	▲DCBD2	
		▼HSP71	▲FOXP3	
		▲CD59	▼EPCAM	
GO:0050789	Regulation of biological process	▲CLIC3	▲CD59	16
		▼EGFR	▼EPCAM	
		▼LMNA	▲HLA-G	
		▼G3P	▲TTP	
		▲PRGR	▼PDIA3	
		▼HSP71	▼PRDX2	
		▼EF2	▲DCBD2	
		▲FOXP3	▲ZSC21	
GO:0032501	Multicellular organismal process	▼EGFR	▼EPCAM	10
		▼LMNA	▲HLA-G	
		▲PRGR	▲TTP	
		▲ATPB	▲HBB	
		▼EF2	▲FOXP3	
GO:0007154	Cell communication	▲CLIC3	▲CD59	10
		▼EGFR	▼EPCAM	
		▼LMNA	▲HLA-G	
		▲PRGR	▼PDIA3	
		▼HSP71	▲DCBD2	
GO:0032502	Developmental process	▼EGFR	▼EPCAM	9
		▼LMNA	▲HLA-G	
		▲PRGR	▲TTP	
		▲ATPB	▲FOXP3	
		▼EF2		
GO:0008283	Cell proliferation	▼EGFR	▼EPCAM	7
		▲PRGR	▲HLA-G	
		▼HSP71	▲FOXP3	
		▲CD59		
GO:0002376	Immune system process	▼G3P	▲TTP	7
		▼EF2	▼PDIA3	
		▲CD59	▲FOXP3	
		▲HLA-G		
GO:0016265	Death	▼LMNA	▼KPYM	6
		▼HSP71	▼PDIA3	
		▲CD59	▼PRDX2	
GO:0065008	Regulation of biological quality	▼EGFR	▼PDIA3	6
		▲ATPB	▲HBB	
		▲TTP	▲FOXP3	
GO:0006351	Transcription, DNA-templated	▲PRGR	▲FOXP3	5
		▼EPCAM	▲ZSC21	
		▲TTP		

GO ID number	Term	Proteins		Frequency
GO:0008152	Metabolic process	▼G3P ▲ATPB ▼KPYM	▼PRDX2 ▲HBB	5
GO:0019538	Protein metabolic process	▼LMNA ▼EF2	▲CD59 ▼PDIA3	4
GO:0007155	Cell adhesion	▲CD59 ▼EPCAM	▲HLA-G ▲FOXP3	4
GO:0006464	Cellular protein modification process	▼G3P ▼EF2	▼PDIA3 ▲FOXP3	4
GO:0005975	Carbohydrate metabolic process	▼G3P ▼KPYM ▼PDIA3		3
GO:0006810	Transport	▲CLIC3 ▲ATPB ▲HBB		3
GO:0006412	Translation	▼G3P ▼EF2 ▲TTP		3
GO:0051641	Cellular localization	▼EGFR ▲ATPB ▼PDIA3		3
GO:0040011	Locomotion	▼EGFR ▼LMNA ▼HSP71		3
GO:0051704	Multi-organism process	▲PRGR ▼EF2 ▼KPYM		3
GO:0043170	Macromolecule metabolic process	▼EF2 ▼EPCAM ▲TTP		3
GO:0006091	Generation of precursor metabolites and energy	▼G3P ▲ATPB ▼KPYM		3
GO:0001775	Cell activation	▲CD59 ▲HLA-G ▲FOXP3		3
GO:0016070	RNA metabolic process	▼HSP71 ▲TTP		2
GO:0006457	Protein folding	▼HS74L ▼PDIA3		2
GO:0050817	Coagulation	▲CD59 ▲HBB		2
GO:0015031	Protein transport	▼EGFR ▼PDIA3		2
GO:0051179	Localization	▼EGFR ▼LMNA		2
GO:0031647	Regulation of protein stability	▼G3P ▼HSP71		2

GO ID number	Term	Proteins	Frequency
GO:0009057	Macromolecule catabolic process	▼HSP71 ▲TTP	2
GO:0006793	Phosphorus metabolic process	▼EGFR ▼KPYM	2
GO:0006811	Ion transport	▲CLIC3 ▲ATPB	2
GO:0006928	Cellular component movement	▼EGFR ▼LMNA	2
GO:0006082	Organic acid metabolic process	▼G3P ▼KPYM	2
GO:0050878	Regulation of body fluid levels	▲CD59 ▲HBB	2
GO:0055114	Oxidation-reduction process	▼PRDX2	1
GO:0022411	Cellular component disassembly	▼LMNA	1
GO:0051276	Chromosome organization	▲FOXP3	1
GO:0006996	Organelle organization	▼G3P	1
GO:0061024	Membrane organization	▼LMNA	1
GO:0016458	Gene silencing	▲TTP	1
GO:0009059	Macromolecule biosynthetic process	▼PDIA3	1
GO:0007049	Cell cycle	▼LMNA	1
GO:0001816	Cytokine production	▲TTP	1
GO:0000003	Reproduction	▲PRGR	1
GO:0048511	Rhythmic process	▲PRGR	1
GO:0065009	Regulation of molecular function	▲FOXP3	1
GO:0006997	Nucleus organization	▼LMNA	1
GO:0006818	Hydrogen transport	▲ATPB	1
GO:0016192	Vesicle-mediated transport	▲HBB	1
GO:0016032	Viral process	▼HSP71	1
GO:0009117	Nucleotide metabolic process	▲ATPB	1
GO:0007005	Mitochondrion organization	▼LMNA	1
GO:0048469	Cell maturation	▲PRGR	1
GO:0016049	Cell growth	▲DCBD2	1
GO:0065003	Macromolecular complex assembly	▲HBB	1
GO:0008015	Blood circulation	▲HBB	1
GO:0009116	Nucleoside metabolic process	▲ATPB	1
GO:0016043	Cellular component organization	▲DCBD2	1

6.3 Discussion

The purpose of the 2-DE analysis was to identify the partial changes in the proteome of the human choriocarcinoma cell line JEG-3 in response to PIF and P4 treatments. JEG-3 cellular proteins were displayed by 2-DE, after their treatment with or without P4 or PIF. We found that 42 proteins were common to all the gels (untreated, P4 treated and PIF treated). Of these proteins, only 22 were seen to be differentially expressed; 14 were consistently under-expressed and 8 were overexpressed. MALDI-TOF MS was successful in identifying 19 of these 22 proteins.

Through the gene ontology of the biological functions of the identified proteins, 59 GO terms were associated to the significant proteins. Of the 59 terms, 10 of them were believed to be pivotal in pregnancy, and were closely looked at and examined. In addition, as being a hierarchical system of classification, gene ontology does not require that all the terms identified be defined as they are interrelated to one another.

Of the proteins identified, 16 fall under the GO identification GO:0050896, also known as response to stimulus. Of the 16 proteins, eight were under-expressed, and the other eight were overexpressed. Among the overexpressed proteins we had CLIC3, which acts as an ion channel, PRGR, which is responsible for the mediation of P4 signalling as well as cell-cell signalling. In addition, CD59 was also among the overexpressed proteins. This protein is associated with the tyrosine kinase complex, which is responsible for inducing the start of different intracellular signalling cascades, which have the ability to activate different nuclear factors such as NFκB or NFAT, both of which are related to FOXP3. TTP, is a negative regulator of the translation of micro RNA (miRNA) which has the ability to silence a wide range of different genes expression interfering with their mRNA (Bartel, 2009; Brennecke *et al.*, 2005; Lim *et al.*, 2005). Other proteins, such as HBB, which is involved in the transportation of volatile messengers i.e. NO and oxygen species, and HLA-G, which is related to the response and interaction of different immune competent cells were also overexpressed after stimulation. The last overexpressed protein was DCBD2, which is reported to positively regulate the extracellular matrix. On the under-expressed proteins side, we had HS74L and HSP71, both of which are involved in protein folding, as they act as chaperones. EGFR

was another under-expressed protein; this has the ability to regulate the response to estradiol which plays a crucial role during the course of pregnancy, where EGFR increases uterine blood flow, breast size, the number of oxytocin receptors, and prepares the cervix for parturition. LMNA is involved in the stability of the nucleus, as well as in the regulation of the condensed state of chromatin and in gene expression. G3P, is notorious for its involvement in the modulation of the assembly state of the cytoskeleton, while both PDIA3 and PRDX2 play a central role in the cellular redox homeostasis. The last under-expressed protein was EPCAM, which is widely involved in cell-to-cell interactions.

Regulation of biological process, a GO term also identified as GO:0050789, also encompassed 16 significant proteins, eight of which were overexpressed, and eight of which were under-expressed. From Table 6.3, it can be observed that the proteins that fall under this GO term, are almost identical to those which fall under the GO term response to stimulus. Such can be explained, as because ontology is a hierarchical system of classification, thus numerous proteins will thus be involved in a variety of biological functions. In other words, the regulation of the biological process may be associated with the regulation at the genetic level either by an intracellular phosphorylation cascade, which would involve CD59 or EGFR, it may also be related with the activation of nuclear factors, which involves FOX3P, or may directly interact with the DNA or translation processes which involve LMNA or TTP. Though 14 out of the 16 proteins involved in the regulation of biological processes are also involved in response to stimulus, there were two proteins, which differ between these two groups. These two proteins were ZCS21 and EF2. The overexpressed ZSC21, is a strong transcriptional activator, while the under-expressed EF2 is observed to be essential for the synthesis of proteins at the ribosomal level, in addition to being able to interact with CDK molecules, p53 (which are termed as the "gatekeeper" of the genome) and caspases such as CASP7.

The GO:0007154 term identifies the process of cell communication. Such can be considered to be a result of response to stimulus, as cells either use chemical or electrical signals to communicate between themselves. A response to stimuli or a means of communication may involve a chemical messenger such as P4, an interleukin, or even a change in the membrane's electric potential (i.e. internalisation or release

of ions from or to the extracellular environment). Due to this, the proteins involved in this term, are all ones, which are involved in other functions, and have been previously described.

The function of developmental process is identified by the GO term GO:0032502. The processes of cell-cell signalling also referred to as communication between cells, and the response to stimulus, which can induce change in a group of cells, as well as maturity, division, etc., can all trigger mechanisms which are involved in the developmental processes of the foetus. Under this category nine proteins were observed, four of which were under-expressed and five overexpressed. All the proteins belonging to this group, have previously been detected and described in other groups, with the exception of one overexpressed protein, ATPB. This protein constitutes part of the ATP complex synthase in the mitochondria. Any process that involves a developmental process, requires energy, and the energetic coin in cells is ATP. As the biosynthesis of cells depends on the ATP synthase, it is obvious why we observed an increase in its expression levels; the foetus under development will need energy to synthetize proteins, gene translation, transduction, etc.

Identified by the GO term GO:0008283, the biological function of cell proliferation had seven of the 20 differentially expressed proteins. Of these, were the overexpressed CD59, which is related with intracellular signalling, and PRGR and FOXP3, which are related with genetic regulation. The three under-expressed proteins included EGFR, which is related with chromatin binding, HSP71, a protein used as a means by which to control both protein expression and cell adhesion, and also EPCAM, which is used as a mechanism by which to keep cells tied together.

The biological immune system process, also identified by the GO number 0002376, is a somewhat confusing term, as one must remember that the cells used in this investigation are in fact not immunocompetent cells. The immune system does not “produce” proteins to carry out its normal functions, but instead uses proteins that are already involved in such processes. In this case, it was observed that four overexpressed proteins, CD59, HLA-G, TTP, and FOXP3, and three under-expressed proteins G3P, EF2, and PDIA3, fall under this category. Under this classification, it can be said that HLA-G holds the most crucial role, being described as an immune regulator of the immune system. In

addition, during pregnancy, this protein is reported to interact with KIR receptors on the surface of uNK cells.

The biological process of cell death (GO:0016265), at the ontological level, differs from apoptosis and necrosis. In the ontological classification, cell death is at the upper end of the hierarchy ladder, in comparison to apoptosis or necrosis. Where this process is concerned, one of the overexpressed proteins was CD59, which is known to protect cells against the complement system, through inhibition of MAC, ultimately avoiding the polymerization of the factor C9, which produces pores on the surface of cells. We identified five under-expressed proteins among which was LMNA, which is related with nucleus stability; HSP71, which is involved in the depolymerisation of clathrin coated intracellular vesicules; KPYM, which plays a general role in caspase independent cell death; PDIA3, which is responsible for avoiding cellular cytotoxicity by decreasing antigen presentation through the regulation of folding, assembly and peptide loading of class I molecules. Lastly, PRDX2 is known for its promotion of apoptosis. Under the biological function of cell death, all six proteins are related with cell survival, hence there is a decrease in those proteins, which are renowned for their involvement in apoptosis so as to not promote cell death.

The GO:0008152 identifies the biological function of metabolic process. Under this term, two overexpressed and three under-expressed proteins were reported. ATPB, is related with ATP production, and HBB is known for its ability to protect the cell from radical species of oxygen and nitrogen. Both molecules are clearly related with metabolism: ATPB as the "producer" of energy and HBB as "a shield" against the free radicals that can be produced during the process.

The GO:0007155 identifies the biological function of cell adhesion. The overexpressed proteins under this term were CD59, HLA-G and FOXP3, all of which are related with soft cell adhesion and cell-cell interaction. EPCAM, a protein related to tight joints, which hinders cell growth due to them being in too close proximity with each other, was found to be under-expressed.

Cell activation, is identified by the GO term GO:0001775. Under this biological process, the overexpression of three proteins was observed of which was that of CD59, which is related with the

activation by a tyrosin kinase complex; HLA-G, which can either induce or inhibit the activation of immunocompetent cells; and FOXP3, which has the ability to regulate the activation of numerous cells.

The data obtained in this investigation represents the first proteomic profiling of the effect of PIF on JEG-3 cells. The proteins identified through this study are key components in determining cell proliferation, signalling, immunomodulation, etc. All the proteins identified to be either overexpressed, or under-expressed as a result of PIF are consistent with the profile of pregnancy. In addition, this study establishes and validates proteomic analysis as a useful tool by which to profile the effect of soluble factors, such as PIF or P4, on protein expression.

CHAPTER VII

FINAL DISCUSSION

7.1 Introduction

Reproductive immunology is a field of medicine that addresses the interactions that take place between the immune system and the reproductive system. Studies of the maternal immune system and the foetal-placental unit have been the major focus of reproductive immunology, for the reason that many have sought to address an apparent paradox: the foetus, which in essence is an allograft (semi-allogenic), not only avoids immune recognition, but grows and develops in the maternal uterus without adverse effects. Medawar was the first to recognize the unique immunology that exists at the fetomaternal interface in his paper from 1953 (Medawar, 1953). It was in this paper where the "foetal allograft" analogy was first described, whereby the foetus was compared to a semi-allogenic graft, which in part is comprised of paternal genes, hence is foreign to the maternal immune system, yet the foetus is able to evade maternal rejection. Medawar theorized that the unknown mechanism used by the foetus to avoid rejection by the maternal body could be due to three things: "(1) the antigenic immaturity of the foetus, (2) the immunological inertness of the mother, and (3) the anatomical separation of the foetus from the mother" (Moffett and Loke, 2004; Mor and Abrahams, 2003). Adding on the Medawar's original hypothesis, a fourth hypothesis to try and explain how the foetus avoids rejection was proposed in 1964 by Billingham who hypothesized that the uterus may be an immune privileged site, where the foetus shifts the maternal host's immune response to a protective and tolerant one: the concept of immune privileged sites was by no account new as it was recognized by immunologists for nearly 135 years when Billingham proposed it (Billingham, 1964a; Billingham, 1964b; Billingham, 1964c). Research about how the foetus avoids the mother's immune system and is able to grow and develop, including the study that you have between your hands, has shown that the foetus does not present any antigenic immaturity, and the maternal system is not inert, as can be seen through the wide array of cytokines, HLAs, growth factors, hormones, and many other soluble factors that exist and act at the fetomaternal interface (Gomez-Lopez *et al.*, 2014; Guleria and Sayegh, 2007; Erlebacher, 2013b; Girardi *et al.*, 2006; Spencer *et al.*, 2012). Such findings in fact support Medawar's theory of the existence of a physical separation between the mother and the foetus, as though both mother and foetus display 'active' immune systems, they are able to tolerate one another, and the foetus is able to thrive in the host's body.

Humans possess a form of hemotrophic viviparity that is distinguished by the presence of a placenta that acts as a physical separation between the mother and foetus (Colucci *et al.*, 2014). This physical separation that develops between the mother and the foetus is biologically significant as are the cells of the placenta, more specifically the trophoblast cells, which exert the immunomodulatory functions that allow the survival of the foetus. The trophoblast cells also downregulate the expression of HLAs, as well as express regulators of the complement system (Girardi *et al.*, 2006). The human placenta, a haemochorial transitional organ, is essential for the development of the embryo (Dancis, 1965). The placenta acts to facilitate the bi-directional exchange of soluble factors between the mother and the foetus. This is important for the development of the embryo, maintenance of pregnancy, and induction of labour (Sacks *et al.*, 1999; Schneider, 1991; Sacks *et al.*, 2000). Such soluble factors, which include biologically active molecules, locally produced cytokines, a wide array of hormones, and the MHC proteins are important in controlling the immune response at the pregnancy level, in addition to supporting the growth of the foetus.

Whilst the role of MHC proteins during fertilization remains controversial, they are believed to be implicated in several different stages of reproduction, most importantly blastocyst and trophoblast formation, embryo implantation, and the development and overall survival of the foetus (Rodgers and Cook, 2005; Blaschitz *et al.*, 2001; Juch *et al.*, 2012; Choudhury and Knapp, 2001). In a quest to help and explain the enigma of the foetal allograft, this study has focused on the expression of MHC molecules and their potential effect during pregnancy, as well as addressing the effect that soluble factors have on the expression of MHC molecules at the fetomaternal interface. To study such interactions that take place at the level of pregnancy, well established choriocarcinomic cells lines and proteomic techniques were used.

7.2 Trophoblast cell lines, a means by which to study human pregnancy

The physical and molecular interactions that take place between maternal and foetal tissues are often complex, and studying them is a difficult task. Implantation of the human blastocyst into the maternal endometrium, and all the interactions that take place during this process, cannot be studied *in*

vivo and neither can they be easily studied *ex vivo*. In addition, there are countless ethical restrictions and technical limitations that exist in trying to perform studies in pregnant women. Worldwide, only a handful of laboratories have the privilege to have access to human embryos that have been donated for research purposes, and though endometrial curettage material is more available, it nevertheless remains very hard to come by (Hannan *et al.*, 2010). Therefore, the majority of researchers turn to an alternative, yet established method, of using *in vitro* cell lines as a means by which to examine the interactions that take place at the fetomaternal interface. Each cell line has a different phenotype and, while one may be useful for studying a certain response or function of pregnancy it may not be the best to study another function (Bazer and Salamonsen, 2008). Therefore, it is very important to choose those, which are best fitted to each individual investigation.

In the present study, cell lines were selected based on three criteria: (1) HLA expression, (2) cytokines secretion, such as the IL-1β, GM-CSF, IL-12, IL-10, all of which are considered to be important in the first trimester of pregnancy, and (3) their ability to migrate through basement membrane-like Matrigel barriers. Four candidate cell lines, JAR, HTR-8/SVneo, JEG-3 and ACH-3P, were identified through a literature search. The JAR cell line does not express HLA-G, while the HTR-8/SVneo cell line, when grown on a Matrigel, only expresses HLA-G. The JEG-3 and ACH-3P cell lines, however, actively express HLA-G, in addition to several other HLAs, hence displaying similar characteristics to *in vivo* trophoblast cells (Kovats *et al.*, 1990; Kilburn *et al.*, 2000; Frank *et al.*, 2000). In addition, the JEG-3 cell line is suited for invasion and migration assays, while on the other hand the ACH-3P cell line is a new cell line and an instructive one to study. Hence both the JEG-3 and ACH-3P cell lines were selected to be tested and validated (Hannan *et al.*, 2010). Through several experiments, it was determined if the JEG-3 or ACH-3P cell line would offer the ability to study all the *a priori* selected biological characteristics, and would thus be better suited to this specific investigation. By studying phenotypic characteristics and HLA and cytokine expression patterns of the JEG-3 and ACH-3P cell lines (an aspect which remains unpublished), it was found that both express HLA-G, HLA-E, HLA-C and HLA-F, with the JEG-3 cell line expressing a significantly higher level of these HLAs. In addition, the JEG-3 cell line was that with the higher cytokine expression profile, in that it expressed higher levels of

the investigated cytokines. The difference in the relative expression of HLAs between JEG-3 and ACH-3P cells can be explained by the presence of two trophoblast subpopulations, ST and EVCT, in the ACH-3P cell line, as reported by the Hiden group (Hiden *et al.*, 2007). The JEG-3 cell line was chosen for this investigation, since unlike the ACH-3P cell line, it had a stable and higher basal HLA protein and cytokine expression profile. The JEG-3 cell line also had a similar HLA profile as its EVCT counterpart *in vivo*. This cell line is also reported to have similar biological and biochemical characteristics to EVCT (Table 7.1) (Wadsack *et al.*, 2003). Similarities between the JEG-3 and EVCT *in vivo* were essential for the results to be conclusive.

Table 7.1: JEG-3 cell line vs primary EVCT

	JEG-3	**Primary EVCT**
Origin	Human	Human
Tissue	Placenta	Placenta
Morphology	Epithelial	Epithelial
Culture properties	Adherent	Adherent
Cytokeratin-7	Yes	Yes
CD9	Yes	Yes
hCG	Yes	Yes
Progesterone	Yes	Yes
Vitamin A (retinol)	Yes	Yes
TGF-β	Yes	Yes
TNF-α	Yes	Yes
MMPs	Yes	Yes
IL-1	Yes	Yes
IL-10	Yes	Yes
IFN-	No	Yes
IFN-	Yes	Yes
IL-6	Yes	Yes
EGFR	Yes	Yes
Estradiol	Yes	Yes
HLA-G	Yes	Yes

Experiments, entailing the careful selection of the cell line(s) to be used for the study, were an essential starting point. Selecting appropriate cell lines is important to ensure the generation of relevant, but also extrapolatable results. In addition, these experiments also permitted the characterization of the cellular phenotype, showing that both, cell line and cells of interest *in vivo*, shares similar characteristics, i.e. HLA expression pattern. The selection criteria included whether the cell line being appraised expressed a similar receptor profile, and if it had a similar response to soluble factors as human primary trophoblast cells, and if the changes in the expression profile of the cells can be related to those that take place in the body (Bazer and Salamonsen, 2008). Although cell lines might yield information on mechanisms of cellular action, there is often the problem of over or under expression of certain factors. In an *in vivo* milieu there is a complex balance involving many more factors that can regulate over or under expression, than can be studied *in vitro*. Within this context, these experiments remain important, as only through them can it be determined if the results obtained are indicative of the processes that occur *in vivo*.

7.3 HLA molecules and their role in human pregnancy

The present investigation has confirmed that HLA-G, HLA-E, HLA-F and HLA-C are all expressed by the JEG-3 trophoblast, and indirectly the resting EVCT. It also established that there is a difference between cell-surface and intracellular MHC expression levels, whereby a significantly higher level of HLA-G, -E and -C is expressed intracellularly than at the surface. HLA-F, was however observed to be entirely intracellularly expressed, in contrast to the findings of the Shobu group who reported surface expression of HLA-F on EVCTs, through the use of cell lines (Shobu *et al.*, 2006). Another study by the Nagamatsu group et al., (2006) reported HLA-F to be restricted to the intracellular level in cultured EVCTs (Nagamatsu *et al.*, 2006). Our results are in line with those from the Nagamatsu group however, they observed that HLA-F expression increased with time in culture while our results did not show that, as it was not the aim of this study. We have focused all our efforts in the detection of phenotypical changes that take place after treatment with P4 and PIF, and whether any of them were able to induce a surface expression of HLA-F. Both treatments induced a faint, but significant increase in the intracellular expression of HLA-F, however none induced a surface expression. We believe that this discrepancy in surface and intracellular HLA levels is due to the fact that the molecules are retained

inside the trophoblast cell until they are required at the cell surface. This may be in part due to a limited availability of peptides that are able to bind the respective peptide groove, or to the presence of a regulation point that exists at the intracellular level, or, potentially, at the cell surface. Since HLA-F is not expressed at the surface level, it may not have any peptides with which to bind, or its function is one that does not require its expression at the cell surface, like HLA-DM. Our Western blot and flow cytometric analysis confirmed the translation of these molecules from mRNA to proteins in the studied cell lines.

In the maternal body, prior to implantation, there is an overall downregulation of the majority of MHC class Ia and II molecules, whose polymorphism and cell surface expression, is neither limited nor low and restricted like that of the class Ib molecules (Derre *et al.*, 2006). This under-expression of the MHC proteins should permit the trophoblast to avoid recognition by CTLs. Conversely, the trophoblast would become vulnerable to uNK cells, which are programmed to identify and destroy any HLA null cells. However, during implantation and throughout the course of pregnancy, a unique profile of HLAs (HLA-G, HLA-E, HLA-Fand HLA-C) is expressed, and such an adverse outcome is avoided (Guleria and Sayegh, 2007). In addition, uNK cells, instead of attacking the trophoblast, are thought to play an important role in the acceptance of the foetus through their interaction with HLA (Guleria and Sayegh, 2007; Clark *et al.*, 1996). Both the presence, and lack thereof, of these HLAs at the fetomaternal interface alludes to their importance in maintaining pregnancy, and might represent evolutionary tuning of the immune function between foetal and maternal tissues to avoid recurrent pregnancy losses.

HLA-G, which was here confirmed to be expressed at highest abundance by our trophoblastic cellular model, has had several immunomodulatory functions ascribed to it, amongst which are its inhibition of CTL responses and NK cell functions (Hunt *et al.*, 2006; Hunt *et al.*, 2005; Hunt and Langat, 2009) (Kapasi *et al.*, 2000). HLA-G interaction with its KIR2DL4 receptor, which is located on the uNK cell's surface is important, as this triggers a secretion of inflammatory factors, which are important in promoting the infiltration and vascularisation of the decidua (Long *et al.*, 2013). This interaction is also reported to protect the trophoblast from cell lysis, through the interaction of HLA-G homodimers with KIR2DL4 and B2 (ILT2 and ILT4) (Jabeen *et al.*, 2013). Moreover, as this protein preferentially binds nonamer peptides, and may play a role in presenting viral antigens to T cells, it is also believed to play a

role in immunosurveillance during the course of pregnancy (Hunt *et al.*, 2006; Hunt *et al.*, 2005; Rebmann *et al.*, 2014). Since trophoblast cells express high levels of HLA-G, it was hypothesized that HLA-G may be the highest expressed HLA in the fetoplacental unit. HLA-G differs from other HLA proteins in its function. It is the only class I molecule that forms a β2M-associated homodimer, and thus has a higher avidity to LILRB than to the other 'conventional' class I monomeric molecules. It is believed that such interactions give the trophoblast and the placenta the ability to modulate the local immune response in the uterus so as to prevent foetal death (Trowsdale and Moffett, 2008). In addition, it is supposed that HLA-G has a potential role as an immune-regulator. This is related with the expression of HLA-E. The intracellular form of HLA-G has previously been reported to bind with that of HLA-E, which leads to an increased level of cell-surface expression of both molecules. HLA-G/HLA-E cell-surface complexes might have the ability to interact with other inhibitory receptors of NK cells as a means by which to prevent lysis (Ishitani *et al.*, 2006). In humans, HLA-G is also thought to facilitate the expression of HLA-E (Allan *et al.*, 2002; Hunt, 2006).

Our investigation had found HLA-E was greatly expressed by the JEG-3 cell line and that high expression, like that of HLA-G, reflects its importance in pregnancy. The functions of HLA-E at the fetomaternal interface are thought to be closely related to those of trophoblast-specific HLA-G. A study by the Shaikly group reported that HLA-E was co-expressed with HLA-G at the surface of the trophectorderm of preimplantation embryos, suggesting the importance of both these molecules in the process of implantation (Shaikly *et al.*, 2010). In addition, the co-expression of these molecules means that without HLA-G, HLA-E would be unsuccessful in producing a successful implantation. It is thus postulated that HLA-G regulates HLA-E's expression, hence making it the second highest expressed HLA by the trophoblast. HLA-E can only bind nonamer peptides derived from the signal sequence of other HLA class I molecule, notably HLA-G, which seem to both stabilize and increase HLA-E. Therefore, cell-surface expression of HLA-E will only happen if such a binding occurs, and is thus dependent upon the availability of a leader sequence (Lee *et al.*, 1998; Llano *et al.*, 1998). Thus a high HLA-G expression will invariably affect the level of HLA-E expression, and this will result in an inhibitory effect on the activity of uNK cells. Like HLA-G, HLA-E also interacts with NK cells as it is a

ligand for CD94/NKG2 family of receptors that are expressed by these cells, and it is theorized that HLA-E inhibits cell lysis through this interaction (Jabeen *et al.*, 2013). In addition, it is also postulated that HLA-G and HLA-E work together to inhibit NK cytotoxicity by interacting with KIR2DL4 and CD94/NKG2 receptor family respectively, as previous results have reported the two HLAs to be co-localized (Jabeen *et al.*, 2013).

HLA-F is potentially significant for fetomaternal tolerance, but this molecule has only recently been well studied. The expression of cell-surface HLA-F has been the subject to debate. The Ishitani group have reported its surface expression on EVCT *in vivo* (Ishitani *et al.*, 2003). However, others have found these cells to be devoid of any HLA-F surface expression (Lepin *et al.*, 2000; Nagamatsu *et al.*, 2006). The present study found that HLA-F was significantly expressed at the intracellular level, but without detectable surface expression. This may indicate that HLA-F lacks any immunological function at the cell surface, at least during the first trimester of pregnancy, and exists during this phase as an empty heterodimer free of any peptides. The Shobu group have also reported HLA-F cell-surface expression on extravillous cells during the second trimester, with a noticeable increase in the levels of expression with the progression of pregnancy (Shobu *et al.*, 2006). A study by the Wainwright group reported HLA-F to mainly be present in the cytoplasm of peripheral blood cells and cell lines, and not on the surface, which led them to the conclusion that this protein does not bind any peptides (Wainwright *et al.*, 2000). However, the Lepin group have contended that HLA-F forms a complex with transporter associated with antigen processing TAP, thus indicating that this molecule does have the potential to bind to peptides (Lepin *et al.*, 2000). To date it is still uncertain whether or not HLA-F binds any peptides but is believed to not be involved in antigen presentation (Ishitani *et al.*, 2006; Djurisic and Hviid, 2014). The techniques used for the present study did not detect the presence of surface HLA-F during the first trimester; if this molecule is expressed at the surface its expression level is very low, and undetectable by the methods used. The study by the Shobu group has however reported surface expression of this molecule during the second trimester and thereafter (Shobu *et al.*, 2006). This may mean that the translation of HLA-F from cytoplasm to cell surface is triggered at the second trimester, possibly due to the dominant Th2 cytokine profile, hence suggesting that surface HLA-F expression is characteristic of the second trimester. It has

also been observed that though IFN-γ has the ability to induce the translation of HLA-F, this will not necessarily result in its cell surface expression (Wainwright *et al.*, 2000).

HLA-C was observed to be expressed at high levels in JEG-3, and ACH-3P cells, and was present both intracellularly and at the cell surface. The presence of HLA-C is important during pregnancy as it interacts with KIR, and may serve as a mediator of uNK cell control of the invasion of the trophoblast (Hiby *et al.*, 2004). Uterine NK cells could affect the ability of the trophoblast to invade and transform the spiral arteries, by regulating the blood flow to the intervillous space, rather than by acting directly on the spiral arteries of the uterus. Through the remodelling of the uterus, the placenta is formed and supported with an adequate blood supply (Moffett and Loke, 2006). HLA-C/KIR interactions that take place at the fetomaternal interface, are known to be important in the process of placentation, although it is still not clear why this is (Chazara *et al.*, 2011). One study has demonstrated a higher KIR expression on uNK cells than on peripheral blood natural killer (pNK) cells from the same woman, which highlights the importance of KIR/HLA-C interactions in pregnancy (Sharkey *et al.*, 2008). There is also a higher KIR expression in the earlier stages of pregnancy, and this is seen to decrease as the first trimester progresses (Verma *et al.*, 1997). Since both KIR and HLA-C are polymorphic, individual pregnancies are distinguished by different maternal KIR and foetal, HLA-C variant combinations. Certain combinations of maternal KIR and HLA-C variants, together with foetal HLA-C groups, when over presented, can lead to defective trophoblast invasion, or an incomplete transformation of arteries, which ultimately leads to major pregnancy disorders such as pre-eclampsia, or recurrent miscarriages (Hiby *et al.*, 2004; Hiby *et al.*, 2010). This suggests that HLA-C has a very important role in the process of uterine remodelling and implantation. HLA-C's presence at the fetomaternal interface remains a point of controversy, as compared to the other HLAs expressed here, which are almost invariant, whilst HLA-C is polymorphic (Trowsdale and Moffett, 2008). Nevertheless, though its presence at the fetomaternal interface remains a point of great controversy, in pregnancy HLA-C holds an important role in placentation, which if unsuccessful or incomplete would hinder all ensuing processes that take place in foetal development (Chazara *et al.*, 2011; Parham *et al.*, 2012).

Studies investigating the cytokine profile of EVCT *in vitro* during the first trimester have showed a clear predominance of the different cytokines, namely IL-1β, TGF-β, IL-8, IL-10, IL-12, and GM-SCF. In our study, we observed that TNF-α was detected above the threshold level in untreated JEG-3 cells. We believe that the values that we detected for TNF-α were related with the reality as previous results showed that JEG-3 cells could secrete TNF-α. All the repetitions showed the same results. This observation is in line with previous studies that have reported the first trimester of pregnancy as being a Th1 dominated period (Saito *et al.*, 2010; Wilczynski, 2005; Saito, 2000).

In pregnancy, cytokines are important as modulators of the maternal immune system. They also act as autocrine factors and aid in orchestrating the events of early pregnancy. During pregnancy, HLAs expressed at the fetomaternal interface are affected by and affect the cytokines present in this environment. A tightly regulated cytokine balance during pregnancy, especially during the earliest stages, is believed to be essential in promoting survival of the foetus (Saito *et al.*, 2010; Wilczynski, 2005). A dominant Th1 profile is required during the first trimester, especially during the earlier period, as this is when the crucial events of placentation and implantation take place.

In this study, IL-10 was found to be significantly elevated in the JEG-3 cell line. Although this cytokine is often referred to as a Th2 cytokine, it can be postulated that its high expression levels in first trimester cultured trophoblast cells suggests otherwise: IL-10 may be secreted by both Th1 and Th2 cells, and depending on the stage of pregnancy at which it is secreted, it is believed to have different functions (Sanjabi *et al.*, 2009a). Previous studies have suggested that IL-10 is a key contributor in establishing a fine balance between pro- and anti-inflammatory signals, which are responsible for a successful pregnancy (Simpson *et al.*, 1998; Thaxton and Sharma, 2010). In addition, the presence of IL-10 is important as it is reported to enhance HLA-G transcription in human first trimester cultured trophoblast, thereby regulating the expression of HLA-G levels (Moreau *et al.*, 1999; Rizzo *et al.*, 2005; Roth *et al.*, 1996). This may be important in that if HLA-G levels are low, this cytokine can be administered as a way of increasing them, hence providing a more favourable environment for the foetus through regulation of HLA-G.

The pro-inflammatory cytokines IL-1β, TGF-β1, IL-8, IFN-γ, and GM-SCF, were also found to be significantly expressed in the JEG-3 cell line. Detection of significantly high IL-1β levels is in agreement with earlier studies that have confirmed the presence of IL-1β in the human placenta (Jokhi *et al.*, 1997). This suggesting that this cytokine, is produced by both the placental and the uterine cells. The detection of this cytokine indicates that this synthetic ability is not lost with the malignant transformation of the trophoblast cell, once more reinforcing the use of the JEG-3 cell line as a good model by which to study the interactions that take place at the fetomaternal interface. TGF-β1 was also detected at significantly high levels, showing that this cytokine is secreted by JEG-3 cells, and presumably by primary trophoblast cells. The presence of this cytokine in the first trimester is important, since TGF-β1 is reported to inhibit the proliferation and invasion of the trophoblast by up-regulating integrin expression. Regulation of these two processes is essential if the blastocyst is to successfully implant itself in the uterus. IFN-γ, a potent cytokine, is an inductor of Th1 responses. We believe its presence at the fetomaternal interface is important, as it will help in the process of embryo implantation, but more importantly it can modulate the expression of HLAs at this site. This theory is supported by previous studies that established that IFN-γ has the ability to induce the formation of a nuclear-binding protein, which in turn binds to class I regulatory sequences, and hence decreases the expression of MHC class II whilst increasing that of MHC class I antigens (Choudhury and Knapp, 2001; Brown *et al.*, 1993). TNF-α, a prominent inflammatory cytokine, was tested for in this study, but no detectable levels were found. It is believed that this is due to the effects of this cytokine, which are up-regulation of T cell proliferation and NK cell activity (Gordon and Wofsy, 1990; Zingoni *et al.*, 2005; Melero *et al.*, 2013). For pregnancy to proceed normally, both effects require to be limited during the first trimester.

Just as cytokines can influence HLA expression by trophoblast cells, the interactions that take place between HLA proteins can also effect the secretion of cytokines. In addition, HLAs may also hold a pivotal role in regulating the interactions that take place between factors such as cytokines and NK cells. One such consequence of HLA-E and HLA-G association that takes place at the fetomaternal interface is a surge in the release of cytokines. Such an effect on the cytokine profile is important as it may regulate the process of placentation (Baergen *et al.*, 1994; Szukiewicz *et al.*, 2014).

7.4 Soluble factors at the fetomaternal interface

The fetomaternal interface is a site of simultaneous interactions that all work in unison to ensure a successful outcome of pregnancy. At this site, both the mother and the foetus release factors not only to ensure their own survival, but also to promote the well-being of the other party. A prime example of such a scenario involves the progesterone hormone and the PIF peptide. Progesterone, which is secreted by the mother and the placenta, and PIF, which is secreted by the embryo, in this report, have both been observed to regulate the uterine environment and the placenta so as to ensure a healthy and problem-free pregnancy through their regulation of HLAs and cytokines.

The results obtained in this study demonstrate that, upon stimulation of JEG-3 cells with either progesterone or PIF, a change in HLAs and cytokine profiles takes place. Progesterone caused an overexpression of HLAs both at the intracellular and the cell surface level of the JEG-3 cells, as did PIF stimulation. As was previously seen with the unstimulated JEG-3 cell HLA profile, even after stimulation, HLA-G remained that which was the highest expressed. Therefore, although progesterone and PIF have the ability to solicit an overexpression in HLAs profile, they do not change the relative proportion of each HLA subtype. This regulation of the trophoblast HLA profile by soluble factors is important, since it highlights their significance in both the establishment and maintenance of pregnancy. In addition, the fact that an increase in HLAs, but not the sequence (meaning HLA-G was still that most expressed by the JEG-3 cell followed by HLA-E, -C and -F), resulted from progesterone or PIF treatment, also highlights that the effect of either soluble factor does not change the importance of each individual HLA in pregnancy. This is indicated in that HLA-G endures as the most important HLA.

Progesterone and PIF have a similar effect on soluble factors associated with normal pregnancy. Both cause the overexpression of HLA molecules expressed by the trophoblast and an increase in cytokine secretion, as shown by the investigations herein. It can be said that having both PIF and progesterone carry out the same functions is redundant. The reason why PIF fulfils the similar functions as progesterone might be explained by the fact that PIF is secreted as early as the two-cell stage (Barnea *et al.*, 2012a; Barnea *et al.*, 2012b; Roussev *et al.*, 2013), whereas progesterone is only secreted by the placenta at around 12 weeks of pregnancy; before that progesterone is secreted by the corpus luteum

(Szekeres-Bartho and Balasch, 2008; Szekeres-Bartho *et al.*, 2008; Okabe *et al.*, 2014; Szekeres-Bartho, 2002). In the earlier weeks of pregnancy, progesterone is secreted by the corpus luteum at relatively low levels, at 32.5 ng/ml (Shah and Nagarajan, 2013). Therefore, for progesterone to fulfil its role as an immunomodulator, and to carry out its role in pregnancy, its concentration has to be higher. It is proposed that this is achieved through PIF, which increases the expression of progesterone receptors, as was observed during the 2-DE study. Increasing the cell's sensitivity to progesterone will result in an increase in its uptake and assimilation. Pregnancy cannot be achieved without the presence of the progesterone (Szekeres-Bartho and Balasch, 2008; Szekeres-Bartho *et al.*, 2008; Okabe *et al.*, 2014). This point raises the question of why its levels are not higher from the onset of pregnancy. The delayed secretion/production of progesterone by the placenta can be taken to indicate that PIF functions as a complementary factor to progesterone. PIF is secreted before implantation and throughout pregnancy, and displays multi-targeted effects (Paidas *et al.*, 2010). Some of these effects are: its role in embryo invasion and implantation, the remodelling of the uterus and blocking of peripheral mononuclear blood cells activation (Stamatkin *et al.*, 2011a; Roussev *et al.*, 2013; Moindjie *et al.*, 2014; Barnea *et al.*, 2015; Barnea *et al.*, 2014). This study, in addition, has shown that PIF can regulate HLA profile. It can be proposed that, firstly, PIF is able to carry out the same functions as progesterone, allowing time for this hormone to take effect and to be produced in concentrations that are high enough to maintain a normal pregnancy. In addition, it can also be speculated that the reason why PIF is secreted as early as the two-cell stage, is that at that time this peptide can prepare the uterus for the process of pregnancy (Stamatkin *et al.*, 2011a; Barnea, 2001; Roussev *et al.*, 1996; Moindjie *et al.*, 2014). PIF is secreted from the time prior implantation (Roussev *et al.*, 1996). It can thus be said that this is the embryo's first line of defence against the hostile maternal environment, since PIF will guarantee proper invasion, implantation, and establishment of a blood supply to the embryo.

It can be argued that PIF and progesterone do not carry out the same functions, but that one of them, PIF, supplements the effects of progesterone. The effect of PIF, a 15 amino acid (Duzyj *et al.*, 2010), on the maternal immune system, is presumably more expidative than that of progesterone, which is substantially larger in size. In addition, due to the size of progesterone, the process of transcribing the

gene, processing the primary RNA, translating it and folding it into a protein, transporting it to the Golgi where the hydrocarbons are added, and then sending it to the membrane for secretion into the intracellular space or the blood stream, will take a considerable amount of time than it will for PIF. Furthermore, since PIF, is produced by the foetus, it does not need to be transported to the foetus to carry out its function.

The process of trophoblast invasion shares several biochemical mechanisms with tumour invasion (Karmakar *et al.*, 2004). However, unlike the latter, trophoblast invasion is a tightly regulated process, involving factors such as cytokines and growth factors in the peri-implantation uterine milieu (Bischof *et al.*, 2000b; Halasz and Szekeres-Bartho, 2013; Murphy *et al.*, 2006).

Cytokine presence is important at the fetomaternal interface, and as observed through this study, can be regulated by soluble factors, namely progesterone and PIF. Both progesterone and PIF were seen to increase the secretion of the pro-inflammatory cytokines, and even induce the expression of certain pro-inflammatory cytokines, reported to be secreted by trophoblast. By increasing the pro-inflammatory cytokine IL-1, whilst keeping its increase tightly controlled, PIF and progesterone are able to ensure proper invasion and implantation of the embryo. In addition, since IL-1 stimulates trophoblast synthesis of hCG hormone, the increase in this interleukin will have a positive effect on the state of pregnancy (Jokhi *et al.*, 1997). Regulation of the cytokine profile in trophoblasts by progesterone and PIF accentuates their role in regulating cytokines to ensure survival of the foetus. Progesterone has been reported to block IL-12 production, so as to inhibit NK activation (Szekeres-Bartho, 2008; Szekeres-Bartho *et al.*, 1996). In addition, by doing so, progesterone is able to ensure proper cell invasion and motility, since IL-12 reduces the level of both (Karmakar *et al.*, 2004). Furthermore, it can be said that the fetomaternal relationship is not just one in which the maternal system has to tolerate a foreign body, but is actually a succession of cytokine interactions that serve to guide selective immune regulation. The implicated cytokines also mediate apposition of the blastocyst to the uterus and vascularisation of the placenta. Progesterone and PIF were found to induce the secretion anti-inflammatory cytokines by the trophoblast. This is important, because these cytokines could suppress cell-mediated immunity, guarding against maternal rejection of the embryo. Increased secretion of Th2 cytokines may be a mechanism by which progesterone and PIF suppress cell-mediated immunity in the endometrium, and instead favour the

state of pregnancy and enhance embryonic implantation. Trophoblastic invasion is somewhat dependent on the local milieu's cytokine and growth factor profile, which control this process (Zourbas *et al.*, 2001). The enhanced cytokine profile induced by progesterone and PIF thus promotes it. It was observed that both soluble factors increased invasion onto the Matrigel, which was used as a representative of the endometrium, by JEG-3 cells, with PIF causing a more significant increase in invasion than progesterone. PIF enhancement of invasion over progesterone may possibly be due to its earlier expression, or its increased concentration in comparison to progesterone during the first few weeks of pregnancy. PIF was found to be secreted at concentrations ranging from 25-100 nM and 150-250 nM (Duzyj *et al.*, 2010). Progesterone, which at this stage is still secreted by the corpus luteum, is only present at a concentration of 32.5 ng/ml or 103.5 nM/l. Invasion, and subsequently uterine spiral artery remodelling, are important processes in pregnancy, permitting the embryo to implant itself and lowering maternal vascular resistance. They also increase the uteroplacental blood flow (Cui *et al.*, 2012). Without proper invasion, or remodelling of the uterus, major complications, such as pre-eclampsia will eventually arise, this being a leading cause of maternal death (Lyall *et al.*, 2001).

It was observed that PIF, and other factors, including IL-17, can induce the secretion of progesterone by the placenta. Regulation of progesterone secretion by IL-17 is implemented by means of a receptor, which is present in the JEG-3 cells. The IL-17 receptor has been characterised in trophoblast cells (Pongcharoen and Supalap, 2009). Though an increase of progesterone secretion, as a result of PIF, has been observed, the process by which this occurs has not been characterized. However, given the above facts, it can be hypothesized that the trophoblast cell is equipped with a PIF receptor.

Progesterone and PIF have been demonstrated to have the capacity to modulate the cell proteome, as has been observed through this study. This study also strengthens a previous study from the Paidas group in 2010 where they studied, using microarrays, the genomic changes induced by PIF, but without any further analysis (Paidas *et al.*, 2010). Through our 2-DE studies, it was observed that both progesterone and PIF caused 22 of the identified proteins to be differentially expressed. Both caused a significant change in the proteins in comparison to the resting JEG-3 proteome. Further comparison between the JEG-3 proteome and the PIF-stimulated cell proteome showed that PIF has the ability to

orchestrate/coordinate the maternal immune system without any detrimental effects. The effects of PIF on trophoblasts, observed through stringent proteome analysis, showed that this peptide has interdependent effects on the immune, apoptotic, adhesion, proliferative, biological, response to stimulus and communication processes in the trophoblastic cell. The biological components of some of the differentially expressed proteins identified in this study are from the same family, or are related to those that have been previously identified by the Paidas group (Paidas *et al.*, 2010). These proteins include different chaperones (BAG2, BAG), heat shock proteins (HSPB1 *vs.* HSP70 and HSP4L), proteins/genes that are related to ribosomes (RPS23 40S, RPL26 60S) and cytoskeletal proteins (LMNA, G3P). While the Paidas group addressed, namely the genomic profile of human endometrial cells, which released factors that work on the embryo in a paracrine manner, the current study addressed the proteome of trophoblasts, in which soluble factors work in an autocrine manner. These work on the trophoblast itself. The proteomic data derived from this study significantly advances the understanding of PIF's multi-targeted effects in regulating immunity.

This investigation is important in that it furthers the medical field of reproductive immunology. PIF is secreted as early as the two-cell stage, and only by viable embryos (Stamatkin *et al.*, 2011a; Stamatkin *et al.*, 2011b). Thus, it could serve as an important marker that could be employed to select the best embryos to implant in the IVF context. In addition, progesterone and PIF have the capacity to modulate the immune system, and thus could be used to sustain pregnancy in the event of a pregnancy complication. Both have been observed to have the ability to increase the cytokine and HLA profile, and at the start of a pregnancy, both assist proper invasion and implantation of the embryo.

Pregnancy cogently portrays the interactions between two different and genetically unique tissues, those that have a foetal origin and those with a uterine origin. This study sought to advance understanding of the function of soluble factors at the fetomaternal interface, focussing on that of progesterone and PIF. It was observed that PIF and progesterone are pivotal in:

1. Regulating local uterine immunity.
2. Promoting trophoblast invasion and proliferation.

3. Regulating adaptive processes related to the pre-implantation stage and the whole of pregnancy.

CHAPTER VIII

FUTURE WORK

8.1 Introduction

The immune tolerance that is offered to the foetus by the maternal host is indispensable if a healthy pregnancy is to happen. Nowhere else in the body is this immune tolerance of the foetus by the mother more important than at the site of the fetomaternal interface, and the interactions that take place at this site are ones of extreme importance. Several key components have been identified as being crucial in supplying this tolerance. The major histocompatibility complex, one of the key players in establishing this tolerance, and the soluble factors that affect these proteins expression were studied in this investigation, and different mechanisms by which tolerance is offered were proposed.

It is believed that the results generated in this study, and new data, which will be obtained through future work, will provide novel insights into the fetomaternal relationship and to how tolerance is established at this site. In addition, this data may also aid in preventing spontaneous abortions, as soluble factors that aid in maintaining a healthy pregnancy can be monitored and corrected if unsuitable.

The results that have been presented here showed the effectiveness of the JEG-3 cell line in studying the role of soluble factors affecting the MHC class I molecules and other processes (cell proliferation, invasion, etc), at the fetomaternal interface, this study could be furthered in a number of ways.

8.2 Using a primary trophoblast cell line

The use of a primary cell line would further this study as primary trophoblasts would allow us to further confirm the results that we have collected using the JEG-3 cell line. In addition, the use of primary trophoblasts would also be advantageous as the cells would not have any choriocarcinomic characteristics, hence the responses seen would be closer to what is seen *in vivo*. To obtain primary trophoblast cells, we would have to isolate them from human embryos, which would require a specific protocol and authorisation, and work conditions, in addition to ethical approval which would have to be obtained from both the University of Essex ethical committee and the Human Fertilisation and Embryology Authority. Once this is done, we would then have to work with special culture conditions,

and to confirm the results reported in this thesis, we would carry out the same experiments (under the same conditions) using primary trophoblast cells to see if the same responses would be seen.

8.3 2-DE validation studies

The proteins that were identified through the 2-DE experiments should be validated using specific antibodies and techniques such as that of the Western blot, so as to investigate the importance of each of the proteins.

CHAPTER IX

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