

# **HUMAN ENDOGENOUS RETROVIRUSES AND IMMUNE TOLERANCE IN PREGNANCY**

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## Abstract

The human placenta expresses endogenous retroviral envelope proteins which have been postulated to play an important role in the physiology of pregnancy. Of these, syncytin-1 and syncytin-2 are highly expressed in the syncytiotrophoblast and cytotrophoblast respectively and are thought to be key factors in the regulation of syncytialisation due to their fusigenic properties. In addition to their role in cell fusion, it has also been speculated that syncytin-1 and syncytin-2 may have a role in maternal immune tolerance due to the presence of the highly conserved immunosuppressive domain (ISD) within its sequence. However, no studies are yet to confirm this putative role. Another factor which has been speculated to have a role in maternal immune tolerance is Corticotropin Releasing Hormone (CRH) which has been shown to promote implantation and the maintenance of early pregnancy via the regulation of FasL. Interestingly, both syncytin-1 and FasL have been identified in immunosuppressive placental exosomes. Since CRH stimulates cyclic AMP (cAMP) production and syncytin-1, syncytin-2 and FasL are all stimulated by the cAMP second messenger pathway, it was hypothesised that syncytin-1 and syncytin-2 may be regulated by CRH. Further, it was hypothesised that syncytin-1 may contribute to the modulation of the maternal immune environment during pregnancy. To examine the regulation of syncytin-1 and syncytin-2 by CRH, a combined nucleic acid and protein extraction procedure was developed using column based nucleic acid extraction kits. Using 2D buffer, proteins extracted using this method were shown to have a comparable protein profile to conventionally extracted proteins. This method was then used to examine RNA and protein levels in CRH treated BeWo cells. Following CRH treatment of BeWo cells, a significant upregulation of syncytin-1, syncytin-2 and FasL mRNA was observed. CRH also increased the production of the syncytin-1 precursor in an exosomal fraction. To examine the immunosuppressive properties of syncytin-1, the recombinant ectodomains of human and mouse syncytins were produced and purified using a combination of affinity chromatography and gel filtration. The immunosuppressive properties of the syncytin-1 recombinant ectodomain were then tested using a whole blood culture model stimulated with LPS or PHA.

Syncytin-1 recombinant ectodomain at a concentration of 1 $\mu$ M inhibited the production of TNF- $\alpha$  by 50% and CXCL10 by 65% in whole blood cultures following maximal stimulation with LPS. Syncytin-1 recombinant ectodomain also inhibited the production of IFN- $\gamma$  by 30% in PHA stimulated PBMC. These studies demonstrate for the first time that syncytin-1 has immunosuppressive properties. Further, these studies show that CRH has a role in the stimulation of syncytin-1 and its subsequent sorting into exosomes. Circulating placental exosomes containing syncytin-1 and other immunosuppressive factors including FasL may interact with maternal immune cells to prevent an immune response against the fetal-placental unit. This is a novel mechanism that may contribute to our understanding of how a genetically different fetus can be tolerated by the mother during pregnancy.

## **Statement of Originality**

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John Even Schjenken

July, 2011

## Declaration

The work presented in chapter 2 was part of collaborations between John Schjenken and Jorge Tolosa. This work was also presented in the thesis of Jorge Tolosa as an appendice and has been published in the journal *Biotechniques* (1).

<http://dx.doi.org/10.2144/000112594>

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## Acknowledgements

I would like to take this opportunity to sincerely thank my two supervisors, Professor Roger Smith and Associate Professor Vicki Clifton for giving me the opportunity to be involved with the Mothers and Babies research centre and to present my work at both national and international conferences. You have both been incredibly supportive of me during my time with the group and have always been there whenever I needed help or advice. Most importantly, you both have been patient with my writing. I will forever be thankful for the opportunity you have provided.

I would also like to thank all of the staff and students (past and present) of the Mothers and Babies research centre. I have enjoyed the past 5 years within the group and feel that all of you have contributed in some way, whether it be chatting with me when something didn't work, or giving me scientific support, to the completion of this work. In particular, I would like to thank Dr Kaushik Maiti, Professor Phil Lowry, Ms Julia Smith and Mr Jonathan Tuke who provided me with advice and their expertise with cloning, recombinant protein purification and statistics respectively. I would also like to thank Professor Hungwen Chen and Dr Benoit Barbeau for their generous donation of syncytin antibodies.

Of all of my colleagues I would like to make particular mention of Dr Jorge Tolosa. Jorge, you have taught me so much over the past 5 years, not only in science but also in life. From day one, you took me in and encouraged me to be the best that I can be. This thesis would not have happened without you and I hope that this is just the beginning of our future scientific endeavours.

These studies were carried out with financial support from the Kiriwina Investment Group through the Hunter Medical Research Institute. Travel support was provided by the Endocrine Society of Australia and the Juvenile Diabetes Research Foundation.

I would also like to thank my family and friends for putting up with me for the past 5 years. To my friends, there has always been someone there to take my mind off work when things were not going well and you all have supported me in one way or another. Mum and Dad, you have always supported whatever I have wanted to do and this was no exception. For the past 5 years you have allowed me to continue living at home without board, given me a car to drive and given me every opportunity possible to focus on my work. I couldn't possibly have better parents.

Finally to Ang, when we met I told you that I wasn't far from finishing and that was 4 years ago. While this has taken longer than we would have liked, you were always patient and supported me in my work. You listened to all my complaints when things were not working and put up with some stressful days towards the end. Now we can look forward to the rest of our lives together. I love you very much and am very lucky to have you.

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## Abbreviations

CHAPS -	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
ACTH -	Adrenocorticotrophic hormone
AGRF -	Australian Genome Research Facility
bp -	Base pair
cDNA -	Complementary DNA
CRH -	Corticotropin Releasing Hormone
CRH-BP -	CRH binding protein
CRH-R -	CRH receptor
cAMP -	cyclic AMP
DISC -	Death Inducing Signalling Complex
DAF -	Decay Accelerating Factor
DTH -	Delayed type hypersensitivity
dH <sub>2</sub> O -	Distilled water
ERV -	Endogenous retroviral/retrovirus
ERVs -	Endogenous retroviruses
env gene -	Envelope gene
ERK -	Extracellular signal-regulated kinase
EVT -	Extra villous trophoblast
FADD -	Fas-associated death domain
FasL -	Fas Ligand
FPLC -	Fast Protein Liquid Chromatography

FeLV -	Feline Leukaemia Virus
Gcma -	Glial cells missing a
GM-CSF -	Granulocyte macrophage colony stimulating factor
HBSS -	Hanks buffered salt solution
HPLC –	High Performance Liquid Chromatography
Hitrap IMAC -	Hitrap Immobilised Metal Affinity Chromatography
HRP -	Horse radish peroxidase
HERV -	Human Endogenous Retrovirus
HERVs -	Human Endogenous Retroviruses
HIV -	Human Immunodeficiency Virus
HLA -	Human Leukocyte Antigen
Ig -	Immunoglobulin
ISD -	Immunosuppressive domain
IDO -	Indoleamine 2,3,-dioxygenase
IFN -	Interferon
IL -	Interleukin
JAK3 -	Janus Kinase 3
IPTG -	Isopropyl $\beta$ -D-1-thiogalactopyranoside
LDH -	Lactate Dehydrogenase
LAL -	Limulus Amebocyte Lysate
LPS -	Lipopolysaccharide
LCMS -	Liquid Chromatography mass spectrometry
LB –	Luria broth

MHC -	Major Histocompatibility Complex
MPMV -	Mason Phizer Monkey Virus \
MAC -	Membrane Attack Complex
MCP -	Membrane Co-factor Protein
mRNA -	Messenger RNA
μg -	Microgram
μM -	Micromolar
mg -	Milligram
mM -	Millimolar
MEK –	mitogen-activated protein kinase/ERK kinase
M -	Molar
MMuLV -	Moloney Murine Leukaemia Virus
ng -	Nanogram
nm -	Nanometres
nM -	Nanomolar
NK cells -	Natural Killer cells
OD -	Optical density
1D -	One dimensional
PBMC -	Peripheral Blood Mononuclear Cells
PMA -	phorbol 12-myristate 13-acetate
PBS -	Phosphate buffered saline
Pi3-K -	phosphoinositide-3 kinase
PHA -	Phytohaemagglutinin



PCR -	Polymerase Chain Reaction
PIBF -	progesterone induced blocking factor
PD1 -	Programmed Death 1
PDL1 or PDL2 -	Programmed death Ligand 1 / 2
PKA -	Protein kinase A
RT-PCR -	Real-time Polymerase Chain Reaction
RCA -	Regulators of Complement Activation
T-reg cells -	Regulatory T cells
rpm -	Revolutions per minute
RT -	Room temperature
SDS-PAGE -	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
ASCT2 -	Solute Carrier Family 1 (neutral amino acid transporter),
member 5	
SEB -	Staphylococcal Enterotoxin B
SOB -	Super Optimal Broth
SOCS -	Suppressors of cytokine signaling
TB -	Transformation buffer
TGF -	Transforming Growth Factor
TAE buffer -	Tris-acetate-EDTA buffer
TBST -	Tris-buffered saline Tween 20
TE buffer -	Tris-EDTA buffer
TNF -	Tumour Necrosis Factor
2D -	Two dimensional

2D buffer -	Two dimensional electrophoresis lysis buffer
Th1 -	Type 1 helper T cells
Th2 -	Type 2 helper T cells
UV -	Ultra-violet

## **Chapter 1 - General Introduction**

During mammalian pregnancy, there is an intimate relationship between maternal and fetal tissues creating an immunological problem (2, 3). As the maternal immune system is exposed to a conceptus whose genome is half paternal and half maternal, the developing embryo should be recognised resulting in immune attack on fetal tissues, however, this does not happen. Whilst the immune system retains the ability to respond to foreign antigens, there are multiple mechanisms which are present to prevent inappropriate immune responses against the developing fetus (4-6).

In the following paragraphs, the elements of the maternal-fetal interface are discussed followed by a description of established mechanisms which are known to contribute to maternal immune tolerance. In particular, this introduction will discuss recent evidence that suggests that placental retroviral envelope proteins and CRH may be novel factors which contribute to immune tolerance during pregnancy.

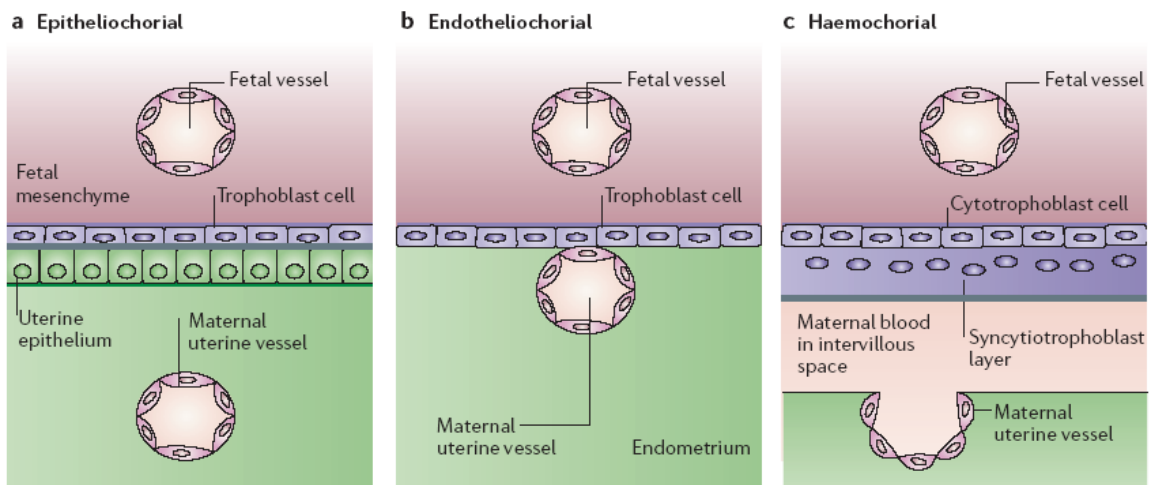
### **1.1 Brief Overview of Placental Morphology**

Viviparity, or the bearing of live young has evolved independently in many groups of vertebrates, including fish (7), reptiles (8) and mammals. Viviparity can extend from the mother simply holding yolky eggs in her body until they hatch to the complex development of the placenta that transfers nutrients from the mother to the fetus (9).

The development of the placenta is a common feature of viviparity in mammals (10, 11). The placenta is formed when fetal membranes become closely attached to the uterine wall facilitating the maternal-fetal exchange of gases, nutrients and waste during intrauterine life (9, 12). Despite the common role of the placenta, different placental species have markedly different placental morphology. However, in all placental species, the barrier between the mother and fetus is maintained by extraembryonic fetal tissue (10).

The various types of placentation seen in mammals can be classified into three groups based on the level of fetal-based trophoblast cell invasion into maternal tissues together

with the number of intervening cell layers between the fetal and maternal circulation (Figure 1.1). In all forms of placentation, trophoblast cells are the outmost layer of fetal cells that overlie the inner core of mesenchyme and fetal capillaries. In epitheliochorial placentation (e.g. whales and dolphins), the trophoblast cells can attach to the surface epithelium of the uterus but there is no trophoblast cell invasion. In endotheliochorial placentation (e.g. carnivora and elephants), the uterine epithelium is breached and trophoblast cells can migrate to maternal blood vessels while in haemochorial placentation (e.g. rodents and higher primates including humans), there is extensive trophoblast invasion where the trophoblast cells come into direct contact with the maternal blood (9, 13).



**Figure 1.1. Schematic representation of the three main types of placentation, showing the relationship between the fetal trophoblast cells and maternal blood.** *a) Epitheliochorial. Trophoblast cells of the placenta are in direct apposition with the surface epithelial cells of the uterus but there is no trophoblast-cell invasion beyond this layer. b) Endotheliochorial. The uterine epithelium is breached and trophoblast cells are in direct contact with endothelial cells of maternal uterine blood vessels. c) Haemochorial. Maternal uterine blood vessels are infiltrated by trophoblast cells causing rupture and release of blood into the intervillous space. The outer layer of the chorionic villi (syncytiotrophoblast) is now bathed in blood. Adapted from (9).*

Humans have a haemochorial placenta characterised by a high level of infiltration by extravillous trophoblast (EVT) cells and release of maternal blood into the intervillous space (Figure 1.2). This type of placentation is also typical of other primates and of rodents. In this form of placentation the mother and the fetus are separated by a villous

trophoblast layer which consists of an outer syncytiotrophoblast layer and an inner cytotrophoblast layer (Figure 1.2). The syncytiotrophoblast is formed by the fusion of the underlying mononuclear cytotrophoblast layer to form a multinucleated syncytium (14).

**Figure 1.2. Cytotrophoblast differentiation.** *Cytotrophoblasts grow into the maternal decidua from a basement membrane and differentiate into several cell types. Some form the anchoring villus that attaches the placenta to the lining of the uterus, others invade through decidual tissue until they reach the maternal spiral arteries where they transform into an endothelial phenotype, and another group fuse to form the syncytiotrophoblast layer that lines the villus space (11).*

The observation that haemochorial placentas are found in diverse species suggests the presence of strong selective pressures that favour this form of placentation. As detailed above, the placenta is the site of gas and nutrient exchange between the mother and fetus and allows the transport of hormones and peptides. The transfer of substances between the maternal and fetal circulations occurs by active transport or passive diffusion. In regards to haemochorial placentation the transfer of gases and nutrients is more efficient than the other forms of placentation due to the close proximity of the maternal and fetal circulations. Further, due to the close proximity of the maternal and fetal circulations in haemochorial placentation, placental hormones can easily be secreted into the maternal circulation to promote the adaption of maternal physiology (9-11, 15).

However, there is a disadvantage with haemochorial placentation and this is due to the direct contact that occurs between the maternal and fetal systems. As a result of the release of maternal blood into the intervillous space, the mother and fetus are no longer separated by an intact layer of epithelial cells, and this allows the fetal derived trophoblast cells to be exposed to the maternal immune system (14). This intimate association between mother and the embryo creates the potential problem of two genetically distinct individuals having to coexist for the duration of pregnancy. This requires the immune system of the mother to tolerate the presence of paternal antigens from the placenta/fetus without altering the immune response against potential pathogens (15). The mechanisms behind maternal immune tolerance will be discussed in the following section.

## **1.2 Fetal Immune Evasion Mechanisms**

Despite the presence of paternal antigens, the maternal immune system does not mount an immune response against the semi-allograft fetus. According to the classical rules of transplant immunology, paternal antigens should be recognised resulting in a maternal immune attack on fetal tissues, however, this does not happen. While the maternal

immune system continues to respond to other foreign antigens (such as from a bacterial infection) in pregnancy, the mother exhibits tolerance for fetal antigens. Without this tolerance, the growing fetus would be rejected (4-6). The mechanisms behind this immune suppression have been studied for years. Sir Peter Medawar in 1953 proposed three mechanisms which may confer immune protection to the fetus:

- That the fetal and maternal circulations are segregated;
- That fetal tissue is immunologically immature from an antigenic point of view; and
- That the maternal immune system during pregnancy has a degree of inertness (16).

In regards to the first hypothesis, Billingham in 1964 (17) drew attention to the growing evidence for bi-directional transfer of cellular elements, particularly blood cells, between the mother and fetus showing that the placenta was not an absolute barrier. It is now known that in haemochorial placentation the mother and the fetus are not separated by an intact layer of epithelial cells and that the fetal trophoblast cells are exposed to the maternal circulation. The second hypothesis proposed by Medawar, that the conceptus lacks immunogenicity has also been disproven. Hoskin and Murgita (18) showed an immune reaction against fetal cells in the mouse. In this study, splenic T cells with a helper phenotype obtained from primiparous CBA/J mice pregnant by syngeneic matings were found to proliferate in response to co-culture with fetal thymus cells. In this study, maternal anti-fetal lymphoproliferative responses were also shown to be blocked by the addition of antibodies against major histocompatibility complex (MHC) molecules (18). More recently, studies have shown that fetal tissue expresses MHC molecules which are required for immune recognition and activation (15). All together, these data showed that the conceptus had immunogenic properties.

In reference to Medawar's third hypothesis, there is ample evidence that pregnancy is recognised by the immune system. Antibodies against fetal antigens have been detected



in the sera of pregnant women showing that maternal recognition of fetal antigens does occur (19). Women undergoing normal pregnancy preferentially release type 2 helper T cell (Th2) cytokines, whereas women who have recurrent spontaneous abortions induce the production of type 1 helper T cell (Th1) cytokines (20). Thus the maternal immune system not only recognises pregnancy but responds in a way which can result in either success or failure of pregnancy maintenance.

Following on from Medawar's original hypotheses, a fourth conceptual proposal was advanced by Billingham in 1964 (17). In this publication, Billingham proposed that the uterus may be an immune privileged site. Other tissues such as testes, brain and eye are also capable of immune privilege by setting up anatomical and physiological barriers, which prevent access to cells of the immune system (3). Experimental studies have since shown that transplant immunity can be both elicited and expressed in a normal manner in the uterus. This was shown initially by Poppa *et al.*, in 1964, who demonstrated the rejection of intrauterine parathyroid allografts in pseudopregnant parathyroidectomised rats (21). Also, other studies have shown that extrauterine pregnancies are possible, suggesting that the uterus does not uniquely protect the fetus as an immune privileged site (22).

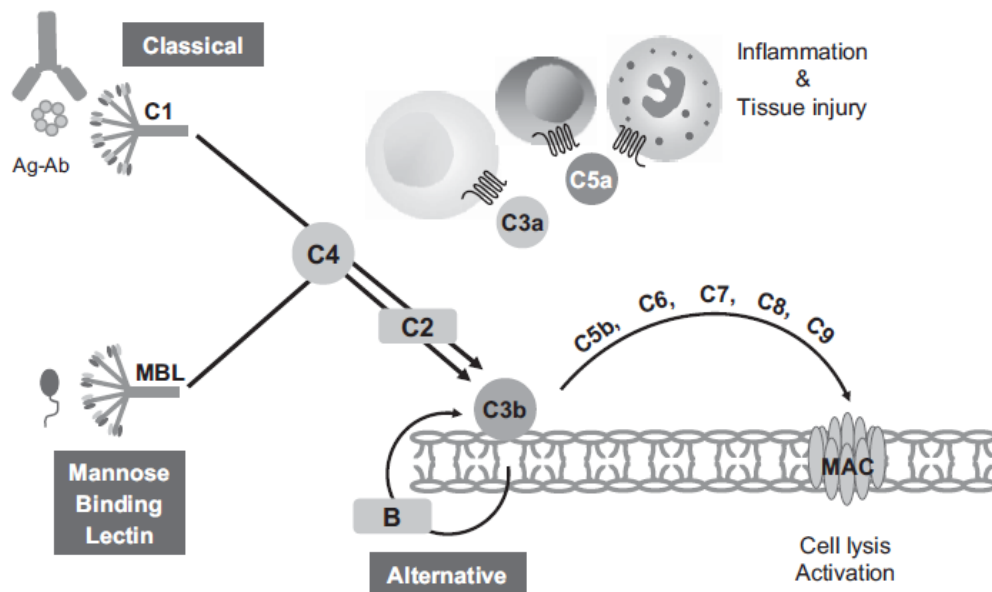
So the question is how does the placenta recognise and respond to fetal antigens in a way that promotes successful pregnancy? We know from extensive studies in transplantation that a semi-allogeneic graft will be promptly rejected without adequate pharmacological control of the recipients immune system, whereas the semiallogeneic fetus which expresses antigens derived from both the mother and the father can survive throughout normal pregnancy without immunological rejection (22). This suggests that the developing fetus has mechanisms which prevent a maternal immune response against paternal/fetal antigens. Indeed, studies have shown that during pregnancy, paternal alloantigens can avoid maternal immune rejection. Tafuri *et al.*, showed that female mice would accept an allogeneic tumour graft while pregnant with a conceptus from a father matching the allograft but if the tumour cells were transplanted from a third party allogeneic donor, they would be rejected. Further, after the delivery of the

mice, paternal tumours would be rejected showing that this tolerance only occurred during pregnancy (23).

To understand the physiological mechanisms of maternal immune tolerance to the semiallogenic fetus will not only improve our understanding of reproductive biology but also is clinically relevant for immune-mediated diseases (e.g. arthritis), cancer and transplantation. While the precise cellular factors and mechanisms involved in maternal tolerance to the semiallogenic fetus are not yet completely understood, the identity of some of the factors and mechanisms that may be critical have been uncovered and are detailed below.

#### 1.2.1 Complement System

The innate immune system comprises the cells and mechanisms that defend the host from foreign organisms in a non-specific manner. Central to innate immune responses is the complement system (24). Over 30 proteins are involved in complement activation and they act in concert to protect the host against invading organisms (24-26).



**Figure 1.3. Schematic diagram of the three complement activation pathways.** *Complement can be activated by the classical, mannose binding lectin or alternative pathways. These pathways cause the cleavage of C3 into C3a or C3b ultimately resulting in inflammation, tissue injury or cell lysis activation (adapted from (24)).*

The complement cascade (Figure 1.3) can be activated by three different pathways: the classical, mannose binding lectin, and the alternative pathway. These pathways are activated as a result of different stimuli. The classical pathway is triggered by the presence of antibody bound to a target antigen, the lectin pathway is initiated by carbohydrates on microbial surfaces and the alternative pathway is spontaneously and constantly activated on biological surfaces in plasma and in most other body fluids allowing for rapid complement activation in response to foreign antigens (27). These three activation pathways lead to the cleavage of the C3 component and the generation of C3a and C3b fragments. C3a causes the activation and release of inflammatory mediators such as histamine while C3b activates additional members of the complement cascade until the binding of C5b initiates the recruitment of the membrane attack complex (MAC). The MAC is a pore forming lipophilic complex activates cell lysis which results in permeabilisation of the cell membrane and ultimately, cell death (24, 27).

In transplantation, the activation of complement has been shown to be involved in acute graft rejections. The use of monoclonal antibodies to complement proteins has demonstrated that complement is activated and deposited on the vascular endothelium of the transplanted tissue in a significant number of acute rejections (28). However, complement can be regulated and the process of regulation can be controlled by the stimulation or inhibition of complement activation. As pregnancy is similar to transplantation, it has been suggested that appropriate complement inhibition is a requirement for successful pregnancy (24).

During pregnancy, complement may be one of the components in the maternal blood which may damage trophoblast cells after its activation by antibodies. In order to avoid complement activation, the trophoblast cells have evolved protective mechanisms which allow pregnancy to progress until term. The expression by the placenta of 3 regulators of complement activation (RCA), decay-accelerating factor (DAF), membrane co-factor protein (MCP), and CD59, support this concept (29). These regulatory proteins inhibit complement at different stages of the activation sequence. DAF and MCP both act early in the complement cascade to control C3 activation whilst CD59 is an inhibitor of the MAC (30).

The role of RCA in trophoblast complement inhibition is further supported by the absence of C3 and the terminal components of complement activation on trophoblast cells even in clinical conditions such as preeclampsia (31). Tedesco *et al.*, 1993 (29) tested the role of the RCA in the vulnerability of human trophoblast to complement activation. In these studies the inhibition of MCP and CD59 resulted in an increased susceptibility of trophoblast cells to complement mediated immunological activation suggesting the inhibition of complement by MCP and CD50 is required for pregnancy to proceed as normal. The protective function of DAF was unable to be tested due to its reduced expression on isolated trophoblasts compared to normal placental tissue.

In rare cases, individuals deficient in DAF or CD59 have been reported, but no MCP deficiencies have been found. Individuals lacking DAF or CD50 survive pregnancy

apparently unscathed making it unlikely that either regulator is essential for fetal survival but it is tempting to speculate that since there are no reported cases of individuals deficient in MCP, this protein may have an essential role in complement inhibition in pregnancy. However, it is possible that the reason that this deficiency has not been identified is because it is without consequence (30).

The most convincing evidence of complement inhibition in pregnancy comes from studies in mice and rats. These species express the three complement regulators detailed above but they also carry another regulator not found in other species called Crry (3). Xu *et al.*, 2000 generated mice that were deficient in Crry (Crry<sup>(-/-)</sup>) and showed that survival of Crry<sup>(-/-)</sup> embryos was compromised due to complement deposition and placental inflammation. This study demonstrated that complement was responsible for the fetal loss as breeding to C3<sup>(-/-)</sup> rescued Crry<sup>(-/-)</sup> from lethality (32).

### 1.2.2 Phosphocholination

Phosphocholination is a post-translational modification which involves the addition of the small haptenic molecule phosphocholine to the polysaccharide moiety of certain secretory glycoproteins (33, 34). This modification is characteristic of a wide variety of prokaryotic organisms (e.g. bacteria) as well as eukaryotic organisms (e.g. parasites). In parasites it has been suggested that phosphocholine containing proteins are secreted into host organisms and have an effect on immune cells leading to immunosuppression of the host immune system (33-35). In particular, phosphocholinated proteins in filarial nematodes have been shown to inhibit normal proliferative responses of both T and B lymphocytes *in vitro*. These effects have been attributed to the phosphocholine moiety as phosphocholine coupled to bovine serum albumin has the same inhibitory effects on T and B lymphocytes as phosphocholinated filarial nematode proteins (36, 37).

Recent studies have shown that phosphocholinated proteins can be produced by mammalian species. Foulds *et al.*, (38) identified lyso-glycerophosphocholine as an

endogenous immunosuppressive agent in bovine and rat gonadal fluids. In these studies, the immunosuppressive fractions of rat testicular interstitial fluid and bovine ovarian follicular fluid were assayed by sequential reverse phase high performance liquid chromatography (HPLC) and sequenced using capillary electrophoresis electrospray ionisation mass spectrometry. The active molecules were identified as 1-palmitoyl-sn-glycero-3-phosphocholine, 1-oleoyl-sn-glycero-3-phosphocholine, a 18:2a/lyso-GPC (putatively, 1-linoleoyl-sn-glycero-3-phosphocholine), and a 20:4a/lyso-GPC (putatively, 1-arachidonyl-snglycero-3-phosphocholine). These molecules were shown to inhibit T cell proliferation and induce apoptosis of T cells in a time and dose dependent manner (38).

In the placenta, Lovell et al., (34) showed that a large number of placental polypeptides and proteins carry phosphocholine as a tissue specific post translational modification and suggested the presence of phosphocholine groups on placental secretory proteins and peptides may play a major role in maternal immune tolerance during pregnancy. In these studies, placental neurokinin B and the precursors of corticotropin releasing hormone (CRH), adrenocorticotropin, hemokinin, activin and follistatin were shown to be post-translationally modified by the addition of phosphocholine using a combination of HPLC and two site immunometric analyses. In the case of neurokinin B, it has recently been suggested that 1-O-alkenyl-sn-glyceryl-3-phosphorylcholine may be the specific phosphocholine modification used by the placenta (39). Lowry (40) suggested that the addition of phosphocholine moieties in placental peptides and proteins may be the rule rather than the exception and that this post translational modification may play an important role in maternal immune tolerance during pregnancy.

### 1.2.3 Programmed Death Ligand 1

The activation of T lymphocytes requires two signals, one of which is delivered by the T cell receptor complex after antigen recognition and one which requires the engagement of costimulatory receptors. The second signal can be either positive, which leads to full

T cell activation, or negative, which can downregulate immune responses (41). In regards to negative T cell signalling, the inhibitory costimulatory receptor molecule programmed death 1 (PD1) and its ligands, PDL1 and PDL2 have been shown to play a role in regulating immune responses *in vivo* (42).

PD1 is a 55kDa type 1 transmembrane receptor that was initially identified in a murine T-cell hybridoma undergoing activation induced cell death (43) and is a member of the CD28 IgG superfamily (41). It has been shown to be constitutively expressed by double negative thymocytes (hematopoietic progenitor cells present in the thymus which are negative for CD4 and CD8) and natural killer (NK) cells, and its expression can be induced on activated CD4 and CD8 T cells, B cells and macrophages. The ligands for PD1, PDL1/2, are expressed on antigen presenting cells following cellular activation or exposure to interferon gamma (IFN- $\gamma$ ). PDL-1 has also been found on a subpopulation of activated T cells. In addition, the ligands can be constitutively expressed or induced by a variety of parenchymal or endothelial cells, including heart, kidney, pancreas and placenta (44).

Binding of either ligand to PD1 inhibits antigen stimulated T cell activation and cytokine production *in vitro*. *In vivo* studies have shown the critical importance of PD1 in maintaining immunological self tolerance. PD1 knockout mice have fatal autoimmune disease and in humans, polymorphisms in PD1 are associated with several autoimmune diseases (45, 46) suggesting an important role in negative T cell signalling.

As a result of their established role in negative T cell signalling, it was hypothesised that PD1/PDL(1/2) signalling may play an important role in maternal immune tolerance. During pregnancy, PDL2 is expressed on the syncytiotrophoblast in early pregnancy, while PDL1 is expressed on all trophoblast populations throughout pregnancy (47). Expression of PDL1 is low in first trimester placenta and increases throughout gestation whereas PDL2 is prominent in the syncytiotrophoblast of early placenta and decreases throughout gestation (48, 49).

Experimental studies on mice using blocking antibodies against PDL1 and PDL2 showed a dramatic loss of allogeneic fetuses in animals treated with the PDL1 but not PDL2 blocking antibody. No effect of the inhibition of PDL1 signalling was observed in syngeneic fetuses suggesting that PDL1 is participating in maternal immune tolerance (42). In addition, antibody mediated inhibition of PDL1 has been shown to result in T cell dependent fetal rejection in mice as well as complement deposition in the fetal compartment (32, 50). Interestingly, in the mouse the source of PDL1 is the maternal decidua and not the trophoblast suggesting that the decidua participates in the suppression of alloantigen specific T cells. Since, in humans, the trophoblast is the main source of PDL1, it is tempting to speculate that the humans may utilise PDL1 in a similar role as has been shown in the mouse, however, this is yet to be tested.

#### 1.2.4 Major Histocompatibility Complex (MHC) Molecules on the Trophoblast

All mammalian species studies to date possess a tightly linked cluster of genes, the MHC complex, which are involved in intercellular immunological recognition and antigen presentation to T lymphocytes. The MHC, which is referred to as the Human Leukocyte Antigen (HLA) complex in humans, is organised into three regions based on the types of molecules that are produced. Class I molecules encode glycoproteins which are expressed on the surface of nearly all nucleated cells, class II molecules encode glycoproteins primarily expressed on antigen presented cells and class III molecules encode secreted proteins associated with the immune process (e.g. soluble serum proteins) (51).

In organ transplantation, allelic differences between MHC class II and class I molecules form the primary basis for transplant rejection (52). Therefore it is relevant to note that trophoblast cells of the placenta are unique because they are one of the few mammalian cell types that do not express classical MHC class II or class Ia antigens, either constitutively or after exposure to IFN- $\gamma$  (52). This is arguably one of the most important immune evasion strategies during pregnancy.



Initially it was thought that trophoblast cells did not display HLA antigens and that this phenomenon could completely account for immunological protection of the fetus (53-55). It was later discovered that trophoblast cells exhibited the classical HLA class Ia molecule HLA-C as well as the non-classical HLA class Ib antigens HLA-E, -F and -G at high levels at the maternal fetal interface (56). Of these, HLA-G was the first trophoblast HLA molecule to be identified and is of great interest due to the strong evidence which suggests that this class Ib molecule may be important in preventing maternal immune attack against the fetus during pregnancy (57).

HLA-G can be expressed as seven isoforms, of which four are membrane bound (HLA-G1 to G4) and three are soluble (HLA-G5 to -G7) (58). In contrast to classical HLA alleles, HLA-G has a very low level of polymorphism with only 8 protein variants. Most of the polymorphisms that are encountered in the HLA-G gene are not predicted to alter the amino acid sequence, or, if they do, will not change the secondary structure of the molecule. Due to the low level of polymorphisms, paternal HLA-G expressed on the surface of trophoblast cells will be almost identical to maternal HLA-G. This minimises the risk of immunorejection.

Early studies identified HLA class I antigen expression as being specific to EVT populations, with the proteins being prominent in cells adjacent to the decidua throughout gestation (56). However, the antibody used to identify these antigens required MHC light chain ( $\beta$  2-microglobulin) and MHC heavy chain associations which are not present on all HLA-G isoforms. More recent studies using different antibodies which are capable of detecting previously undetectable HLA-G alleles showed that HLA-G isoforms are present throughout the placenta and within the chorion membrane, decidua and maternal blood (56, 59).

Since HLA-G is produced at high levels at the maternal interface it has been suggested to have a role in maternal tolerance induction. The effects of HLA-G in different tissues include impacts on NK cell killing, migration and cell viability, proliferation and IFN- $\gamma$

production, regulation of cytokine production, suppression of cytotoxic T lymphocyte killing and viability, inhibition of proliferation and induction of a suppressive phenotype in T helper cells, and alteration of dendritic cell stimulatory capacity and maturation of this lineage (2).

There are currently no reported cases of pregnancy from women in which all the forms of the HLA-G are absent. However, mutations in HLA-G alleles have been identified. For example, Ober *et al.*, (60) identified a single base pair deletion at position 1597 of exon 3 of HLA-G which is present on 7.4% of African American and 2.9% Hispanic chromosomes. This deletion causes a frameshift mutation and results in amino acid substitutions in all of the residues of the second half of exon 3. Individuals with this mutation, have no detectable HLA-G1 protein but are still able to go through normal pregnancy suggesting that certain HLA-G alleles are not essential for pregnancy. Other studies have shown that certain polymorphisms such as a 14 base pair insertion in exon 8 of the 3'-UTR may be associated with certain pregnancy complications such as preeclampsia and recurrent spontaneous abortion (61). However, these data are not well supported in the literature and different studies show different effects of HLA-G polymorphisms.

Despite the evidence which suggests that HLA-G may have an important immunosuppressive function during pregnancy, mice do not possess a clear homologue of HLA-G. However, they do express Qa-2 which has some of the structural characteristics of HLA-G and is also strongly expressed in placentas. Further, in non-human primates, placental cells produce HLA-G like proteins. Thus, although the above studies strongly suggest that HLA-G serves a key role in maternal immune tolerance in humans, definitive evidence is lacking.

### 1.2.5 Tryptophan Catabolism

The essential amino acid, L-tryptophan is required for the biosynthesis of proteins and is an important substrate for the generation of serotonin and the formation of kynurenine derivatives and nicotine adenine dinucleotides. The latter pathway, which is called the kynurenine pathway is initiated by the enzymes tryptophan pyrolase and indoleamine 2,3,-dioxygenase (IDO) (62).

IDO was initially described in 1967 by Yamamoto and Hayaishi in the rabbit intestine as an enzyme that could oxidise both L- and D-tryptophan (63) and was later shown to have an important role in microbial resistance by allowing cells to deplete tryptophan from intracellular pools or the local microenvironment (64). When an infectious agent invades a tissue, leukocytes and lymphocytes accumulate at the site of infection and secrete IFNs into the inflammatory milieu. The presence of IFN triggers IDO production and subsequent tryptophan catabolism which inhibits the growth of the infectious agent (65).

As tryptophan is an indispensable requirement for cell growth, it was proposed that IDO may have other important roles. For example, enhanced tryptophan catabolism by macrophages has been shown to inhibit T cell proliferation and it appears that the expression of IDO by cells allows them to suppress unwanted T cell responses (3, 64, 66). There are intriguing associations between altered tryptophan metabolism and cell-mediated immune responses. Studies have shown that patients receiving tryptophan for a variety of disorders experience a high frequency of autoimmune disease and although this is associative it does suggest that IDO may play an important role in mediating immunological responses (64).

IDO expression is not detected in most tissues of healthy mammals but can be increased by infection and inflammation due to the production of IFNs and other factors (67). The only tissues where IDO is expressed at constitutively high levels is the proximal male epididymis and at the maternal-fetal interface during mammalian pregnancy (67). Munn

*et al.*, (68) proposed that in the mouse the expression of IDO by the placenta is crucial in the prevention of T cell responses against the fetus. In this study Munn *et al.*, demonstrated that tryptophan is catabolised by the placenta during pregnancy and that this process suppresses T cell activity and defends the fetus against rejection. Rapid T cell-induced rejection of all allogeneic but no syngeneic fetuses occurred when pregnant mice were treated with the IDO inhibitor, 1-methyl-tryptophan (68).

In humans, Kudo *et al.*, showed that IDO activity and messenger RNA (mRNA) expression can be positively regulated by cytokines such as IFN- $\gamma$  and that tryptophan transport into the trophoblast is the rate limiting step for IDO mediated tryptophan degradation (69, 70). Knowing that IDO had similar actions in humans as in mice, Kudo *et al.*, examined the potential role of IDO in the human placenta. In these studies, Kudo *et al.*, showed that tryptophan degradation by IDO inhibited lymphocyte proliferation in placental tissues. Media conditioned by placental villi in the presence of IFN- $\gamma$  (inducer of IDO) was more depleted of tryptophan than control media and the proliferation of mononuclear cells, specifically CD4<sup>+</sup> T lymphocytes was markedly inhibited by tryptophan degradation. This inhibition could be reversed by the addition of 1-methyl tryptophan (71).

Despite all of these reports which support a role for IDO in maternal immune tolerance, a number of studies have shown that IDO is not essential for pregnancy. Baban *et al.*, developed an IDO knockout mouse and showed that in allogeneic matings, the mice were capable of producing litters of normal sizes and rates compared to control mice suggesting that redundant mechanisms may protect allogeneic fetuses in IDO knockout mice (67). Also, in humans it has been reported that IDO expression does not differ between proven fertile women and women with a history of miscarriages. Thus, there may be, as in mice, other mechanisms to establish fetal tolerance (72).

### 1.2.6 Progesterone

Since the 1970s, evidence detailing the production of various hormones and cytokines by the placenta has expanded our knowledge on mechanisms by which uterine tissue functions as well as the putative roles of these hormones and cytokines in pregnancy (73). The presence and alteration of hormones during pregnancy is essential and many important processes, such as the transport of sperm and oocytes, implantation and labour are controlled by alterations in the hormonal environment (6). Placental steroid hormones have also been considered as possible mediators of immunosuppression during pregnancy because of the immunosuppressive properties of natural and synthetic glucocorticoids (74).

Among the hormones present in maternal serum, progesterone appears to play a major role in reducing the maternal response to the fetal allograft (75). Progesterone has been shown to be essential in the maintenance of pregnancy in a number of mammalian species. In humans, progesterone is produced autonomously by the placenta at high levels (up to 250 mg a day) (6). Shortly after delivery the concentration of progesterone in maternal blood falls precipitously.

Early studies showed that high concentrations of progesterone can prolong the survival of xenogeneic and allogeneic grafts (76, 77). For example, Hansen *et al.*, (77) studied the effect of progesterone on skin transplants placed in the uterine lumen of ovariectomised ewes. Allografts placed in the uterine lumen of progesterone treated ewes were present 30 days after engraftment while allografts placed in control animals were completely resorbed. Other studies have shown that progesterone can affect various phases of the immune response *in vivo*. Siiteri *et al.*, (74) showed that in rats, progesterone can effectively block cellular immune responses both *in vivo* and *in vitro* using progesterone concentrations which mimic the high intrauterine concentrations of progesterone during pregnancy. Stites *et al.*, (78) using human blood cultures showed that progesterone was capable of inhibiting monocyte dependent T cell activation. It was

apparent in both of these studies that the immunosuppressive actions of progesterone appear to be mediated through its selective blocking of T cell activation (74, 78).

The immunosuppressive effects of progesterone are determined on the one hand by its concentration during pregnancy and also by the progesterone-binding capacity of lymphocytes. Lymphocytes carried by the maternal blood during pregnancy are extremely sensitive to progesterone which suggests receptor mediated action of progesterone on the lymphocytes. Szekeres-Batho *et al.*, (79) showed that CD8<sup>+</sup> lymphocytes produced peripherally during pregnancy were reactive to progesterone receptor monoclonal antibodies and that the level of progesterone receptor positive lymphocytes increased throughout gestation.

The biological effects of progesterone during pregnancy are manifested by a 34kDa protein, called the progesterone induced blocking factor (PIBF), which is released by lymphocytes of pregnant women following binding of progesterone to its receptor (6, 80). In pregnant women, the PIBF concentration gradually increases until the 37<sup>th</sup> week of gestation, followed by a slow decrease until term. PIBF signals through the JAK/STAT pathway and has been shown to alter the cytokine balance resulting in a preferential production of Th2 type cytokines in mice, inhibit NK cell activity through mediation by cytokines, and regulate anti-abortion effects (6, 81-83). In peripheral blood of healthy pregnant women, the percentage of PIBF positive lymphocytes is significantly higher in all trimesters of pregnancy than in women at risk for premature pregnancy termination. Further, in peripheral blood of patients undergoing spontaneous pregnancy termination at the time of samples the percentage of PIBF-positive cells was significantly decreased (84).

Based on the above data, Szekeres-Batho *et al.*, (6) proposed a mechanism for the protective effects of progesterone during pregnancy. Following the recognition of fetal antigens, progesterone receptor positive T cell receptor positive cells become activated and interact with progesterone to produce PIBF which induces a Th2 type immune response, allowing pregnancy to proceed to term.

### 1.2.7 Th1-Th2 cytokine balance in pregnancy

The best studied peripheral immune cells in pregnancy are T-lymphocytes. Within this population there are two main subsets which are defined as helper T lymphocytes and cytotoxic T lymphocytes. Helper T lymphocytes are particularly important in the context of pregnancy as they affect the function of other immune cells by producing cytokines (85). Helper T lymphocytes can be further separated into type 1 and type 2 based on their profile of cytokine production. Th1 produce tumour necrosis factor (TNF)- $\alpha$ , IFN- $\gamma$  and interleukin (IL)-2 which promote cellular immune responses while Th2 produce IL-4, IL-5, IL-9, IL-10 and IL-13 which promote humoral responses (85, 86).

It has been shown that T cells mediate many of their effects through the secretion of cytokines and in pregnancy it has been hypothesised that a correctly regulated maternal immune response determines the growth and survival of the fetoplacental unit. Studies by Chaouat *et al.*, (87, 88) examined fetal survival following the injection of various cytokines in mice and showed that granulocyte macrophage colony stimulating factor (GM-CSF), IL-3 and IL-10 enhanced fetal survival and promoted intrauterine growth while TNF- $\alpha$ , IFN- $\gamma$  and IL-2 had deleterious effects which led to fetal death. These studies, and the studies of others led to the Th1/Th2 paradigm proposed by Wegmann *et al.*, in 1993 (89) which hypothesised that Th2 cytokines inhibit Th1 responses, improving fetal survival and impairing responses against some pathogens.

Indeed, pregnancy is characterised by an increase in Th2 responses. Studies in humans and in mice have shown that the maternal immune response is biased toward a Th2 humoral response and away from cell-mediated immunity which could be harmful to the fetus (20, 89-91). In women who suffer from recurrent spontaneous abortions their peripheral blood mononuclear cells (PBMCs) respond *in vitro* to trophoblast antigens by producing high levels of the Th1 cytokine IFN- $\gamma$  and TNF- $\alpha$  (92). Conversely, in PBMC from women who are not prone to recurrent spontaneous abortions a preferential production of the Th2 cytokine IL-10 is observed (92). It is also interesting to note that humoral associated autoimmune diseases such as lupus tend to flare up during

pregnancy while cell-mediated ones such as rheumatoid polyarthritis, often enter remission (93).

Several different cells and soluble factors have been proposed as potential regulators of the altered Th1/Th2 ratio characteristic of pregnancy. The syncytiotrophoblast and cytotrophoblast are known to produce cytokines and these cells can influence cytokine production by acting on the Th1/Th2 balance (94). Decidual cells and cells of the uterine draining lymph node have also been shown to suppress immune responses *in vitro*. Factors such as progesterone, PIBF and IDO have been proposed to alter the Th1/Th2 balance (20, 85, 95) and cytokines themselves may play extremely important roles in modifying the immune system to favour a Th2 environment. Both transforming growth factor (TGF)- $\beta$  and IL-10 appear to assist in maintaining the Th1/Th2 balance. IL-10 plays an important role in preventing spontaneous pregnancy failure in mice. The injection of IL-10 alone into pregnant mice has been shown to lead to the prevention of fetal resorption. This effect can be reversed by the addition of anti-IL-10 antibodies (88). TGF- $\beta$  has been correlated with the immunosuppressive activity of decidual supernatants, appears to be reduced or absent in mice undergoing fetal resorptions and also appears to have an essential role in priming the immune system to tolerate seminal antigens (20, 96).

However, Th2 dominant immunity has also been observed in recurrent abortion cases and Th2 knockout mice can proceed normally through a pregnancy suggesting that in addition to the Th1/Th2 phenomena, there are other mechanisms which may contribute to maternal immune tolerance (97). With this in mind, the Th1/Th2 paradigm has been expanded into the Th1/Th2/Th17/T-reg paradigm. In this paradigm, Th17 cells, which produce IL-17 which are reported to be expressed at high levels in spontaneous abortion, are mediators of inflammation along with Th1 cells, while T-reg cells which are potent suppressors of inflammatory immune responses and are essential requirements to prevent autoimmunity (98, 99) may be important in the induction of antigen specific tolerance.



### 1.2.8 Regulatory T Cells

One emerging focus is the role of specialised populations of T lymphocytes termed regulatory T-cells (T-reg cells). The term T-reg refers to a subpopulation of T-lymphocytes which are required for antigen specific T-cell tolerance. Three main subsets of T-reg cells with distinctive suppressive mechanisms have been identified and can be distinguished by their phenotype, cytokine secretion and tissue origin. These are type 1 T-reg cells, T-helper 3 cells and CD4<sup>+</sup> CD25<sup>+</sup> T-reg cells. Each of these subsets has the capacity to inhibit the proliferation and effector function of other T cells. T-reg cells have two main physiological roles: control T-cell reaction with self antigens that have escaped negative selection by the thymus, and limit the extent and duration of responses exerted by T-cells reactive with alloantigens and other exogenous antigens (100).

Studies into the biological action of T-reg cells have shown that they are potent suppressors of inflammatory immune responses and are an essential requirement to prevent autoimmunity and to promote the tolerance of allogeneic organ grafts (98, 99). These unique properties of T cells have led to speculation that they may have an important role in the events of reproduction and pregnancy (100).

Recent studies by Somerset *et al.*, (101) showed that circulating human CD4<sup>+</sup>CD25<sup>+</sup>T-reg increase throughout gestation with a peak during the second trimester and then a subsequent decline postpartum. Isolated human CD4<sup>+</sup>CD25<sup>+</sup> T-reg were further shown to suppress proliferative responses of autologous CD4<sup>+</sup>CD25<sup>-</sup> T-cells to allogeneic dendritic cells. The importance of T-reg cells in pregnancy is further supported by studies of individuals who had spontaneous abortions. In these studies, the levels of CD4<sup>+</sup>CD25<sup>+</sup> T-reg were significantly lower in patients who had a spontaneous abortion compared to samples from induced abortions (102).

The role of T-reg cells in maternal immune tolerance is further supported in studies which show that paternal antigen stimulation of T-reg cells is required for optimal

protection of fetuses from rejection. Mjosberg *et al.*, showed that T-reg cells from peripheral blood of pregnant and non-pregnant women can suppress alloantigen responses *in vitro*, with increased capacity to suppress anti-paternal as opposed to irrelevant alloantigens (103).

As with humans, CD4<sup>+</sup>CD25<sup>+</sup> T-reg cells from mice have been shown to increase throughout gestation and are able to suppress both autoimmune responses and allogeneic responses directed against the fetus. The physiological significance of CD4<sup>+</sup>CD25<sup>+</sup> T-reg cells for pregnancy was further demonstrated in studies using an adoptive transfer model, where complete T-cell populations or populations depleted of CD4<sup>+</sup>CD25<sup>+</sup> T-reg cells were transferred into pregnant T-cell deficient mice. In the absence of CD4<sup>+</sup>CD25<sup>+</sup> T-reg cells, allogeneic fetuses were promptly rejected, whereas syngeneic fetuses were unaffected (104).

Collectively, these results suggest that T-reg cell may have an important role in maternal immune tolerance.

### 1.2.9 Conclusions

Despite over twenty years of research into the mechanisms behind maternal immune tolerance, the mechanisms are yet to be completely understood due to the complexity of the system. The mechanisms that are detailed above are all capable of inducing immune tolerance, however, are not necessarily required for pregnancy to be successful (67, 97). There appear to be many redundant mechanisms that exist to provide robustness to the system that is essential for mammalian pregnancy.

More recently, new factors have been identified which may play a pivotal role in maternal immune tolerance. In particular, the discovery of the presence of retroviral proteins in the human genome and the high expression levels of the peptide hormone CRH have been hypothesised to play a role in the modulation of the maternal immune

system throughout pregnancy (105, 106). Endogenous retroviral (ERV) envelope proteins are expressed at high levels in the placenta and this expression has led to speculation that they may play a physiological role in this environment (106). ERV envelope proteins expressed in the placenta of humans (syncytin-1 and syncytin-2) and mice (syncytin-a and syncytin-b) have a demonstrated role in the fusion of the cytotrophoblast to form the syncytiotrophoblast. Sequence analysis of these ERV proteins show that they have a putative immunosuppressive domain (ISD) that is characteristic of retroviral envelope proteins (107-109). The interactions of these retroviral proteins with CRH may also contribute to the maternal immune environment. In pregnancy, CRH has previously been hypothesised to have a role in maternal immune tolerance through its regulation of the pro-apoptotic cytokine, Fas Ligand (FasL) (105).

The mechanisms of CRH and retroviral mediated immune suppression are discussed below:

### **1.3 Corticotropin Releasing Hormone (CRH) and its regulation of FasL expression**

CRH is a 41 amino acid peptide hormone which was first identified by Wylie Vale in the hypothalamus of the sheep where it was shown to act as the main neurotransmitter orchestrating the stress response through the secretion of adrenocorticotrophic hormone (ACTH) or corticotropin from corticotropes of the anterior pituitary (110, 111). In addition to its function in the hypothalamic-pituitary-adrenal axis, expression of CRH has been recognised at several different sites, including regions of the central nervous system (particularly those responsible for the neuropsychological response to stress), and peripheral tissues including the placenta (111).

Immunohistochemical analysis of the human placenta shows that CRH expression can be found in the syncytiotrophoblast and intermediate trophoblasts but not the cytotrophoblasts. In the fetal membranes, CRH is found in the epithelial and subepithelial cells of the amnion, in some cells of the reticular and cellular layers of the

chorion and in some stromal cells and invasive trophoblast cells of the decidua (112). It is interesting to note that placentally derived CRH is identical to hypothalamic CRH in structure, function and bioactivity. However, the production of placental CRH only occurs in humans and other higher primates (113). There is a considerable body of evidence which indicates a role for placental CRH in the physiology of human pregnancy.

In the early stages of pregnancy, CRH can not be detected in placental tissues at significant levels but by 21 weeks it is detectable. In the maternal plasma, circulating placental CRH becomes detectable around 16-20 weeks of gestation and plasma CRH concentrations increase exponentially peaking at labour (112, 114). Plasma CRH can be bound to a high-affinity binding protein called CRH binding protein (CRH-BP). The physiological role of CRH-BP is to control the levels of free CRH. Until the final few weeks of pregnancy, CRH-BP is present at higher concentrations than CRH in the maternal plasma meaning the circulating CRH is biologically inactive (115). However, in the final weeks of pregnancy there is a rapid rise in CRH. This rise in CRH is coupled with a decrease in plasma CRH-BP concentrations thereby making circulating CRH biologically active (111, 114). Interestingly, the levels of CRH throughout pregnancy are related to the determination of gestation length (114, 116, 117) and the onset of labour (118, 119).

The increase in placental CRH that is observed throughout gestation is due to the specific upregulation of CRH gene expression (120). *In vitro* studies have shown that CRH can be stimulated by various neurotransmitters, cytokines, polypeptides, or steroid hormones (121, 122). In particular, prostaglandins E<sub>2</sub> and F<sub>2α</sub>, noradrenalin, IL-1 and oxytocin all increase CRH in cultures of syncytiotrophoblasts (123, 124). Progesterone and nitric oxide donors such as sodium nitroprusside have been shown to inhibit placental CRH secretion *in vitro* (124, 125). Further, in contrast to the hypothalamic CRH system, glucocorticoids can stimulate placental CRH. At the hypothalamic level, glucocorticoid mediated inhibition of CRH release prevents overactivation of the stress axis which can disturb homeostatic mechanisms and lead to the development of various

psychiatric, neuroendocrine and neurological disorders. The positive feed forward system is another unique feature of placenta CRH and further indicates a specific role in human pregnancy (126).

The biological effects of CRH in the placenta can be regulated through interactions with the CRH receptors. CRH receptors are plasma membrane receptors which are coupled to distinct G protein  $\alpha$  subunits (115, 126). The CRH receptors belong to the class II receptor superfamily and three have been identified: CRH-R1, -R2, and -R3, although CRH-R3 has only been described in fish species (115, 126). The primary RNA transcripts of CRH-R1 and -R2 are subjected to significant levels of alternative splicing resulting in a large number of splice variants in each gene. The CRH-R1 variants are generated by a number of partial or complete exon insertions or deletions while the CRH-R2 variants differ in their N-terminal extracellular domains (126). This variation in splicing makes the CRH receptors extremely versatile signal transducers with the potential to influence multiple cellular and biological functions (115, 126).

CRH receptors have a wide distribution and are found in the central and peripheral nervous system, adrenals, retina, spleen, heart, skeletal muscles and skin. In reproductive tissues, CRH receptors can be found in the ovaries, placenta, foetal membranes, decidua, endometrium and myometrium (115, 126). In the human placenta, both CRH-R1 and -R2 subtypes are expressed.

In most tissues, such as the pituitary, myometrium, heart and brain, the CRH receptors couple to G protein  $\alpha$  subunits resulting in the production of adenylate cyclase and cyclic AMP (cAMP) (127). Indeed, in studies of cultured myometrial cells, CRH has been shown to stimulate the production of cAMP in a dose dependent manner (128). However, in the placenta and fetal membranes, studies have shown that CRH can exert its effects by coupling to the G protein q  $\alpha$  subunit resulting in increased phospholipase C activity (127). Karteris *et al.*, showed an increase in phospholipase C activity following stimulation of placental and fetal membranes with 100nm CRH. Further, the CRH antagonist  $\alpha$ -helical blocked this effect, showing that placental and fetal membrane

CRH receptors can couple with G protein q  $\alpha$  subunits (127). The increase in phospholipase C activity following CRH administration is also observed in rat Leydig cells (129). However, other studies have shown that the main class of G proteins in the placenta and fetal membranes are G protein S  $\alpha$  subunits (127, 130) suggesting that the activation of adenylate cyclase and the production of cAMP may be a signalling pathway used following CRH stimulation in the placenta and fetal membranes.

Despite the knowledge of the regulation of CRH and its localisation in reproductive tissue, the precise biological role/s for CRH in feto-maternal tissues is yet to be elucidated. In the placenta and fetal membranes CRH appears to have a wide variety of functions, including the regulation of trophoblast cell growth and invasion, tissue remodelling through the secretion of the matrix degrading protease matrix metalloproteinase 9, control of placental vascular tone through the activation of the nitric oxide pathway, direct modulation of endocrine function, especially prostaglandin generation and bioavailability and immunological effects (105, 114, 115, 126, 131, 132).

### 1.3.1 CRH and its role in modulating the immune system

CRH has previously been shown to have a role in modulating the immune system. Traditionally hypothalamic CRH has been considered to act indirectly in an anti-inflammatory fashion as the end product of the hypothalamic-pituitary-adrenal axis is cortisol which is a well known anti-inflammatory compound. Studies have shown that CRH deficiency in mice can cause a disruption in endogenous glucocorticoid production which can result in enhanced allergen-induced airway inflammation and lung mechanical dysfunction (133). A similar pathway has been observed for central nervous system CRH where the activation of glucocorticoid and catecholamine secretion by CRH in response to stress has also been related to immune or inflammatory suppression (134). High plasma levels of cortisol, CRH and ACTH can be found in centenarians and these levels indicate an activation of the entire stress axis which is likely to be counteracting the systemic inflammatory process that occurs with age (135).

However CRH produced at peripheral inflammatory sites has been shown to possess potent proinflammatory properties that can influence both innate and acquired immune processes (115). Karalis *et al.*, showed that immunoreactive CRH can be produced at peripheral inflammatory sites where it can act as an autocrine or paracrine inflammatory cytokine (134). Theoharides *et al.*, (136) showed that one of the early effects of “immune” CRH is the degranulation of mast cells and the release of histamines and inflammatory cytokines such as TNF- $\alpha$  and IL-6.

In the reproductive system, intrauterine and ovarian CRH can have proinflammatory properties. For intrauterine CRH, various studies have shown that it may participate in the acute aseptic inflammatory response that is characteristic of embryo implantation (105). Ovarian CRH can also participate in the inflammatory processes of ovulation and luteolysis (115, 137). However, following implantation, the embryo suppresses the inflammatory response and prevents immune rejection. It has been hypothesised that the anti-inflammatory role of CRH may play a role in this process through interactions with the proapoptotic cytokine, FasL (105).

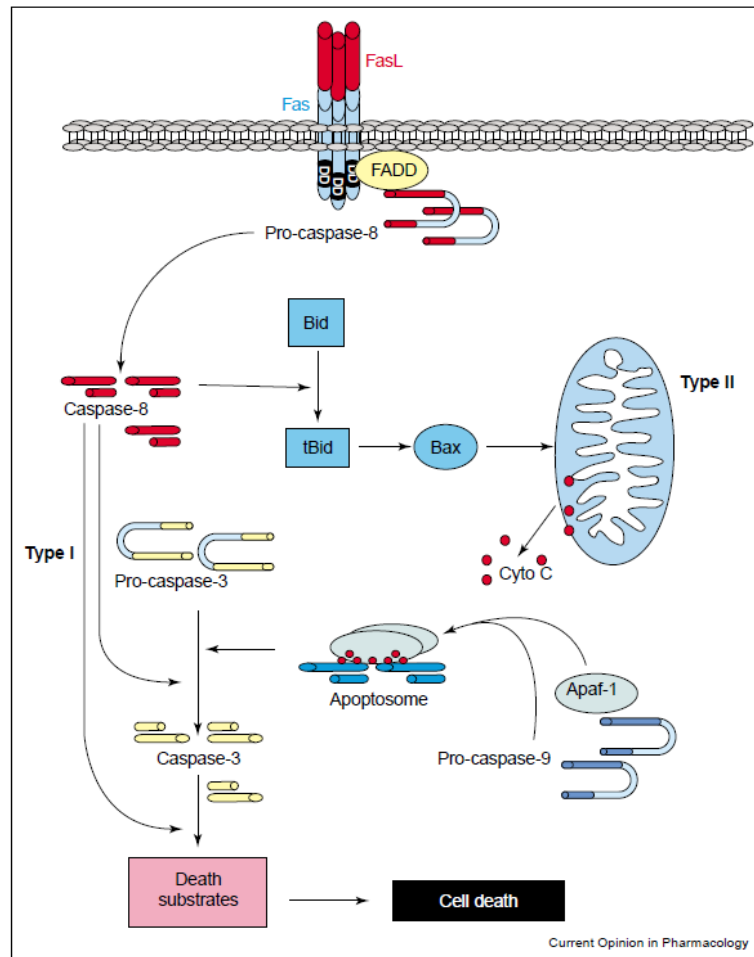
### 1.3.2 FasL

FasL is a type II membrane protein of approximately 280 amino acids that belongs to the TNF superfamily. FasL has a high level of conservation amongst species and is highly expressed on several immune cells including activated T and B lymphocytes, NK cells, monocytes and macrophages. In addition to this expression, FasL is also found on non-immune cells such as tumour cells, sertoli cells of the testis, corneal epithelial cells and endothelial cells of the eye and the placenta (115, 138).

The major function of FasL is to induce apoptosis in cells which express its receptor, Fas (138). Fas, a membrane protein that belongs to the TNF and nerve growth factor receptor family, is expressed at high levels in several immune cells, including activated

B and T lymphocytes, NK cells, monocytes, and macrophages. Binding of FasL to Fas causes a higher order aggregation of the receptor molecules and recruitment of the adaptor molecule Fas-associated death domain (FADD) (139, 140). FADD also has another domain which is called the death effector domain which recruits pro-caspase 8 and/or pro-caspase 10 to the receptor. The resulting complex is called the death inducing signalling complex (DISC) (139). At the site of DISC formation, caspase molecules are activated initiating a cascade of increasing caspase activity through the cleavage and activation of effector caspases (such as caspase 3) which in turn, cleave a set of target proteins and are ultimately responsible for the death of the cell by apoptosis (Figure 1.4). However, in some cells DISC formation following Fas/FasL interactions can be strongly reduced and a mitochondrial pathway is required for apoptosis signalling. This pathway ultimately results in the release of proapoptotic molecules such as cytochrome c which interacts with caspase molecules resulting in cell death (Figure 1.5) (139).





**Figure 1.4. Apoptosis signalling through Fas/FasL interactions.** The binding of FasL to Fas induces the recruitment of the FADD and caspases to the receptor complex to form the DISC. The DISC causes activation of caspase-3 through two pathways (death substrate or mitochondrial) which ultimately result in cell death by apoptosis. Adapted from (139)).

Due to the expression of Fas at high levels in several immune cells, it has been hypothesised that Fas/FasL interactions may be important in immunological tolerance. Griffith *et al.*, showed the importance of FasL as a mechanism of immune privilege. In these studies, the anterior chamber of the eye of mice was infected with herpes simplex type 1 virus resulting in the production of neutrophils and lymphocytes. In these mice, Fas/FasL interactions were responsible for the apoptosis of the inflammatory cells as *gld* mice, which lack functional FasL, had increased levels of inflammation and invasion of ocular tissue without apoptosis (141). Other studies have shown the importance of FasL

in tumour immune privilege. Bennett, *et al.*, studied the role of Fas/FasL interactions in oesophageal cancer and showed that in the presence of FasL the number of tumour infiltrating lymphocytes was reduced due to apoptosis (142).

### 1.3.3 CRH regulation of FasL expression

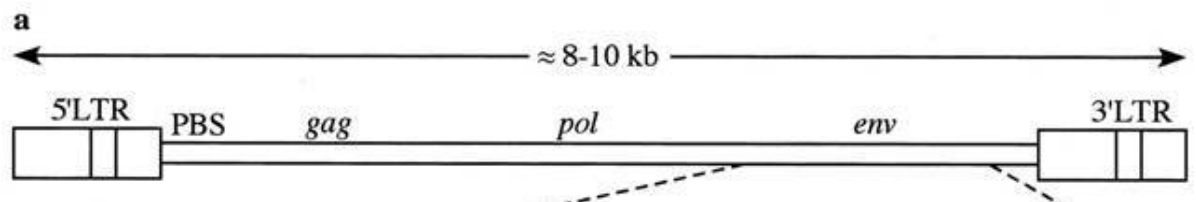
During human pregnancy, FasL is expressed on the trophoblast and decidualised endometrial cells (132). As a result of this expression profile, FasL has been speculated to have a role in embryo implantation and maternal immune tolerance. Makrigiannakis *et al.*, (105) examined the role of FasL in implantation and early pregnancy and showed the importance of CRH in this process. In these studies CRH was shown to increase FasL expression in human EVT cells which in turn increased the levels of activated T cell apoptosis in PBMC isolated from newborn children. Neutralising antibodies to FasL were shown to inhibit CRH-induced apoptosis suggesting that this effect was mediated by Fas/FasL interactions.

To determine the relevance of these *in vitro* findings, Makrigiannakis used an *in vivo* rat model to study embryo implantation. These studies showed that administration of the CRH-R1 antagonist, antalarmin, to female rats resulted in a marked decrease in FasL expression, implantation sites and live embryos. As with the *in vitro* studies, these studies showed that this was a T cell dependent process as T cell deficient rats treated with antalarmin had no difference in the number of implantation sites compared to control animals (105). As the induction of FasL by CRH requires CRH-R1 it is likely that this stimulation is a cAMP mediated effect. This is shown in studies by Xiong *et al.* and Nabhan *et al.*, where CRH-R1 in stably transfected cells was shown to couple strongly to G protein s subunits but weakly to G protein q subunits (143, 144).

However, as with IDO, studies have shown that Fas/FasL interactions are not obligatory for successful pregnancy. Hunt *et al.*, (145) examined whether the absence of FasL affected pregnancy using *gld* mice (mice unable to express a functional FasL). In these

mice, extensive leukocytic infiltrates and necrosis at the decidual-placental interface were observed which resulted in increased embryo resorption and a decrease in litter size. Interestingly, the lack of FasL in this mutant strain did not abrogate fertility suggesting that there may be other mechanisms which can control the maternal immune response in the absence of FasL.

#### **1.4 Retroviruses**



**Figure 1.5. Genomic structure of a retrovirus.** *Retroviruses consist of two long terminal repeats at the 5' and 3' ends and three genes, gag, pol, and env. Adapted from (146).*

Retroviruses can be defined as a class of enveloped viruses that have their genetic material in the form of RNA and use the enzyme reverse transcriptase to translate their RNA into DNA in a host cell (146). Retroviruses are broadly divided into two categories, simple or complex, based upon the structure of their genomes. All retroviruses contain information coding for three main domains: *gag*, *pol* and *env* (Figure 1.5), which perform the following functions:

- *gag* – directs the synthesis of internal virion proteins that form the capsid and nucleoprotein structure.
- *pol* – contains the information for the reverse transcriptase and integrase enzymes
- *env* – contains the surface and transmembrane subunits of the viron envelope protein. These are involved in cell fusion, immunosuppression and receptor recognition (147).

There is also an additional smaller coding domain called *pro* which is present in retroviruses and encodes the virion protease (147). The envelope protein has immunomodulatory functions in many viruses.

#### 1.4.1 A Brief History in Retrovirology

The retrovirus was first identified at the turn of the century in two investigations examining neoplastic disease in chickens. In 1908, Ellermann and Bang (148) showed that chicken leukosis, a form of leukaemia and lymphoma, was caused by a virus. In 1911, Dr Peyton Rous (149) studied the growth of what he described as “a transmissible sarcoma of the chicken” which was transferred in cell-free conditions. The agent discovered by Ellermann and Bang is now recognised as avian leukosis virus while the agent discovered by Rous bears the name Rous Sarcoma Virus. Both of these are members of the avian c-type virus genus which are referred to as avian sarcoma/leukosis viruses. These pioneering studies represent the beginning of what we recognise today as retrovirology.

Forty years after the initial experiments in chickens, Gross discovered that an agent in cell free extracts (now recognised as a retroviral particle) of the Rous Sarcoma Virus could induce leukaemia in infant mice (150). Over the following decades, many viruses that cause neoplastic disease in mice, cats, cattle and primates were identified (151).

Early studies of avian and murine retroviruses showed that the intracellular state of these RNA viruses was an integrated DNA which was called the proviral intermediate (152). The provirus hypothesis, proposed by the late Howard Temin stated that an integrated copy of viral DNA from a RNA based virus maintained the genetic stability of a retroviral infection (153). This hypothesis was first presented at a meeting in 1964 and was virtually ignored for the following 6 years as prior to the discovery of reverse transcriptase, it was difficult to determine how an RNA virus could take the form of a

DNA genome. This enigma was resolved following the discovery of the enzyme reverse transcriptase in 1970 (154, 155).

In the human, the first retrovirus identified was the human T-cell lymphotropic virus and was isolated from cultured leukemic cells obtained from a patient with cutaneous T-cell lymphoma (152). The infamous Human Immunodeficiency Virus (HIV) was first identified in 1983 by Montagnier and colleagues. With the discovery of human retroviruses, a new dawn in the age of retroviral research began. Future research, however, would show the potential importance of retroviral proteins in human evolution.

#### 1.4.2 Retroviral Life Cycle

Retroviruses have two markedly different life strategies. Exogenous retroviruses such as HIV reproduce within somatic tissues of infected individuals and spread through the population via transmission of fluids. Endogenous retroviruses (ERVs) use germ-line transmission. In germ-line transmission, ERVs have invaded cells of every vertebrate and replicate as an integral part of sexual reproduction in the host to be present in the genome of future generations (146).

##### 1.4.2.1 Endogenous Retroviruses (ERVs)

In rare cases, exogenous retroviruses infect germline cells or cells of the early embryo. If the proviral genome then integrates into germline DNA, an endogenous retrovirus (ERV) is formed. Providing no mutations affect the proviral genome, this newly created ERV will be able to produce infectious particles which can be transmitted horizontally. However, the great majority of human ERVs are defective by way of mutations in the gag, pol, or env genes and rely on vertical transmission, where the integrated retrovirus is passed from generation to generation in a Mendelian fashion, resulting in their presence in all human cells (146, 156).

ERVs have the same fundamental structure as exogenous retroviruses. For example, full length ERVs have their characteristic LTR-gag-pol-env-LTR structure (see Figure 1.3), reinforcing the concept that most ERVs are endogenised exogenous retroviruses which have integrated into our genomes at some point during evolution (146). In the human approximately 8% of our genome consists of retroviral elements. If the search for endogenous retroelements is extended to retroviral fragments then approximately half of our genome has retroviral origins (146).

While exogenous retroviruses are commonly associated with disease, some endogenous retroviral elements have been suggested to play an important role in physiology. It is intriguing to consider that ERVs may have played an important role in the evolution of humans (157).

### **1.5 The Human Placenta and ERVs**

The most abundant expression of different human endogenous retroviruses (HERVs) is seen in the placenta and embryonic tissues, and other reproductive tissues or cells, such as the testis and oocyte. From as early as the 1970s repeated electron microscope observations have been made of the presence of C-type ERVs within both human and animal placental tissue (158). The presence of human endogenous retrovirus (HERV) particles and proteins in these tissues suggests a normal function for these proteins in this environment (159, 160).

One of the first expressed HERVs with tissue-specific expression in the syncytiotrophoblast identified was ERV3 (161, 162). Venable *et al.*, 1995 suggested that ERV3 was likely to have a biological function as it was abundantly expressed as a full length unprocessed envelope protein in the syncytiotrophoblast of the placenta. Taking into account the known roles of retroviruses it was postulated that this function may relate to either immunosuppression or cell-fusion of the cytotrophoblasts. However, de Parseval and Heidmann in 1998 (163) showed that the ERV3 envelope protein has a number of mutations, one of which introduces a stop codon resulting in a physiological

knockout of the ERV3 envelope in 1% of the Caucasian population. Despite the original hypothesis that ERV3 had an essential role in pregnancy being incorrect, these studies showed the potential importance for ERV envelope proteins in placental development.

More recently, *in silico* genomewide systematic screening through the human genome sequence has identified 18 coding envelope (*env*) genes, whose proteins may have a physiological function (108, 164). Of these 18 *env* genes, at least 10 have been reported to be expressed in the placenta and only three have been found to cause cell-cell fusion *in vitro* (syncytin-1 (*envW*), syncytin-2 (*envFRD*) and *envP(b)*). As syncytin-1 and syncytin-2 are highly and specifically expressed in the placenta it was suggested that they have a physiological role in the fusion of the cytotrophoblast cells to form the syncytiotrophoblast layer (107, 108, 165). A similar role has been suggested for syncytin-A and -B which are envelope proteins of ERV expressed in the mouse. These envelope proteins will be discussed in greater detail in the following paragraphs.

**Table 1.1. The fusogenic potential of the 18 coding *env* genes in the human genome.** *Cell-fusion assays were performed using the following cell-types: NIH3T3, CHO, G355-5, Cos-7, TE671, 293T, and HeLa. ‘-’ negative result in all cell types, ‘+’ positive result in at least one cell type (108).*

Gene Name	Chromosome localisation	Fusion Assay
envH1	2q24.3	-
envH2	3q26	-
envH3	2q24.1	-
envK1	12q14.1	-
envK2	7p22.1	-
envK3	19q12	-
envK4	6q14.1	-
envK5	19p13.11	-
envK6	8p23.1	-
envT	19p13.11	-
envW	7q21.2	+
envFRD	6p24.1	+
envR	7q11.21	-
envR(b)	3p24.3	-
envF( c ) 2	7q36.2	-
envF( c ) 1	Xq21.33	-
envV	19q13.41	-
envP(b)	14q31.12	+

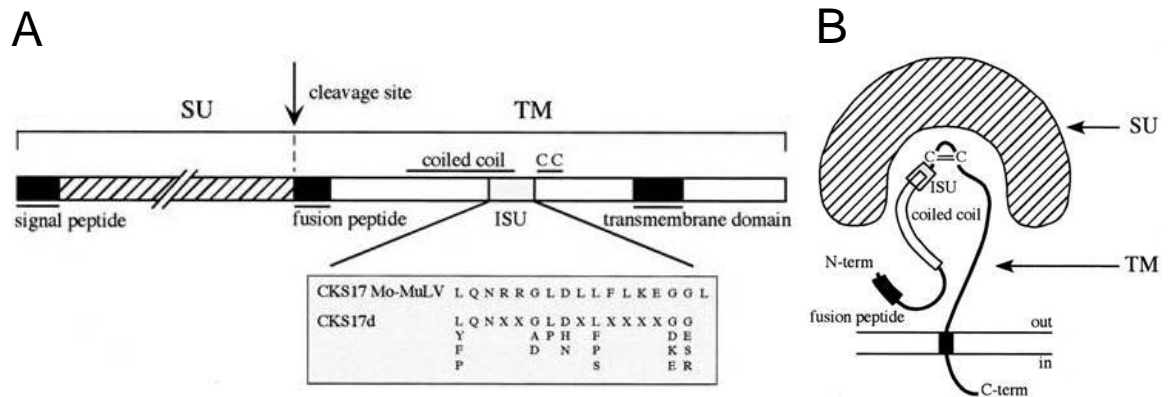
### 1.5.1 Syncytin-1 (HERV-W)

Syncytin-1 is the envelope protein of a member of the HERV-W family which was endogenised into the human germ line 25-40 million years ago (166). It was discovered by Mi *et al.*, (2000), in studies of a human testis library in a project to identify novel secreted proteins using the yeast signal sequence trap (107). DNA searches of the genome revealed the existence of several closely related sequences on chromosomes 4, 6, 7, 16, 17, 21, 22 and X. However, only the chromosome 7 sequence contained an intact open reading frame and possessed 100% similarity to the syncytin-1 DNA (107).



Flanking sequences of this region revealed the presence of the defective provirus, HERV-W (107).

The structure of syncytin-1 is similar to that of other endogenous retroviral envelope proteins. As detailed in Figure 1.6, the envelope protein consists of two subunits: the surface subunit which is involved in receptor recognition and the transmembrane subunit, which is involved in anchoring the protein to the membrane, cell-cell fusion and also putative immunosuppression (167).



**Figure 1.6 Envelope protein structure.** The envelope protein of retroviruses is divided into 2 subunits. The surface subunit (SU) is involved in receptor signalling while the transmembrane subunit is involved in anchoring the envelope to the membrane, fusion of cells and putative immunosuppression (adapted from (167)).

Studies on the newly identified syncytin-1 showed that the major site of expression is the syncytiotrophoblast cells of the placenta (107). The specific expression of syncytin-1 in the syncytiotrophoblasts may be critical to human placentation. The fusion of the cytotrophoblast to form the syncytiotrophoblast had previously been hypothesised to be a ERV envelope protein mediated event (162) and it was shown that syncytin-1 may be an important mediator of syncytialisation (107, 165, 168, 169). The receptor for syncytin-1, solute carrier family 1 (neutral amino acid transporter), member 5 (ASCT2) has been implicated in the syncytialisation process and is specifically expressed in villous cytotrophoblast (169, 170). The syncytialisation process is important in

implantation and placentation as it forms a barrier between the foetal part of the placenta and the maternal blood (171). The syncytium is responsible for many functions performed by the placenta such as the transport of oxygen and nutrients.

Syncytin-1 has also been documented in the testis using northern blot analysis (107). The presence of syncytin-1 in both the testis and the placenta suggests that this protein may play an important role during reproduction. Interestingly, the blood testis barrier modulates the immune reaction to the haploid sperm preventing anti-sperm antibodies from being formed while the placenta also regulates the maternal immune system through mechanisms which are, as yet, undefined (172, 173). The immunosuppressive properties of syncytin-1 will be detailed later.

#### 1.5.2 Syncytin-2 (HERV-FRD)

Syncytin-2 was identified in 2003 through genomewide screening for fusogenic HERV envelope proteins (108). Syncytin-2 is an envelope protein of a HERV-FRD family member which has been conserved through primate evolution for over 40 million years. During this time the syncytin-2 gene has been maintained in a functional state as a cellular gene with a low mutation rate compared to traditional retroviral genomes and syncytin-2 is present in all simians from new world monkeys through to humans (108). Studies on the crystal structure of syncytin-2 have shown remarkable structural similarity to corresponding domains of present day infective retroviruses in spite of highly divergent sequences (174).

Northern blot analysis of a series of tissue samples showed that syncytin-2 is specifically expressed in the placenta (108). Malassine *et al.*, 2007 (175) investigated the protein expression of syncytin-2 in the placenta. Using a monoclonal antibody raised against syncytin-2, immunolocalisation was detected most frequently at the membrane level of the interface between the cytotrophoblast and syncytiotrophoblast cells and only observed in the villous cytotrophoblasts (175). The receptor for syncytin-2, Major

Facilitator Superfamily Domain Containing 2 has also been demonstrated to have specific expression in the syncytiotrophoblast of the placenta and BeWo trophoblast cells suggesting that syncytin-2 may play a similar role in cell fusion to syncytin-1 (176).

Syncytin-1 had been thought to have a predominant role in cell-cell fusion but recent evidence has suggested that syncytin-2 may play an important role in this process. Vargas *et al.*, 2009 (177) showed a direct correlation between mRNA and protein levels of syncytin-2 and cell fusion. In addition, following the administration of siRNA targeting syncytin-2 a marked repression of cell fusion events was observed in BeWo and primary placental trophoblast cells.

Like syncytin-1, syncytin-2 also has a putative immunosuppressive role which will be discussed later.

### 1.5.3 Syncytin-A and Syncytin-B

Following the identification of syncytin-1 and syncytin-2 in the human placenta it was hypothesised that other species in which placental trophoblast fusion takes place would express ERV with a similar physiological role. Following the near completion of the mouse genome project, Dupressoir *et al.*, 2005 (109) reported the discovery of two previously uncharacterised murine ERV *env* genes which are phylogenetically unrelated to human syncytin-1 and syncytin-2. These genes were named syncytin-A and -B and are specifically expressed within the placenta in the syncytiotrophoblast containing labyrinthine zona and can trigger cell fusion in *ex vivo* transfection assays (109, 178). The expression of syncytin-A and -B has been shown to be specific to all species of muridae studied suggesting that the genes have retained fully coding status for over 20 million years of evolution (109).

Dupressoir *et al.*, 2009 (179) performed a landmark study on the role of envelope proteins of ERVs in the placenta. In this study, syncytin-A knockout mice were developed and its role in pregnancy examined. Homozygous syncytin-A null mouse embryos were unable to survive past 13.5 days of gestation demonstrating an important role in embryonic development. The placentas of these mice had specific disruption of syncytiotrophoblast containing labyrinth architecture, with trophoblast cells failing to fuse. The observed lack of fusion was also associated with: cell overexpansion at the level of fetal blood vessel space, cell apoptosis, decreased vascularisation, and inhibition of placental transport and fetal growth retardation (179). As yet, no such study has been performed on syncytin-B, however, such a study will provide further insight into the role of placental envelope proteins of ERVs.

As with human syncytins, syncytin-A and -B have putative immunosuppressive roles which will be discussed later.

#### 1.5.4 Regulation of Placental Syncytin Expression

As detailed above, placental syncytins are actively transcribed, placental specific ERV envelope proteins (180). In most tissues syncytin-1 and -2 expression is suppressed as ectopic expression can be dangerous for tissue organisation and integrity. For example, in studies of breast and endometrial cancers, syncytin-1 and -2 expression is turned on and this ectopic expression results in cancer cell fusions (181-183). Aberrant syncytin expression has also been related to neuroinflammatory pathologies such as multiple sclerosis (184). Outside of the placenta, syncytin-1 expression is regulated by CpG methylation of the 5' long terminal repeat. Hypomethylation of the syncytin-1 5' long terminal repeat is observed in the placenta and BeWo cells but in primary cells and cell lines that do not express syncytin-1, the sequence is heavily methylated (185).

The expression of syncytin-1 and 2 is regulated by the glial cells missing a (GCMa) transcription factor (186, 187). One of the mechanisms postulated to drive expression of

Syncytin-1 and 2 in the placenta is through the regulation of GCMA by a cAMP activated protein kinase A (PKA) signalling pathway (187). Syncytin-1 and -2 transcript and protein levels in BeWo cells can be stimulated by the addition of cAMP (107, 176) and Knerr *et al.*, (187) showed that the PKA signalling pathway acts upstream of GCMA. Stimulation of the human syncytin transcription factor GCMA through the cAMP/PKA pathway has been shown to be attributable to CBP-mediated acetylation of the GCMA protein which results in transcriptional activation (186).

Progesterone stimulates syncytin-1 expression. Noorali *et al.*, (188) used a HTR-8/SVneo cell line which was primarily isolated from chorionic villi. Real-time polymerase chain reaction (RT-PCR) analysis of these cells showed that treatment with progesterone upregulated the expression of syncytin-1 by 600-fold. Other hormones such as human chorionic gonadotropin, prolactin, estradiol, estriol, and human placental lactogen all elevated the expression of syncytin-1 when compared with the controls, but the level of induction was significantly less compared to progesterone.

CRH has also been shown to activate the cAMP/PKA pathway which is known to regulate syncytin-1 and -2 expression (189). As detailed above, CRH has been hypothesised to be involved in maternal immune tolerance through the regulation of FasL and may be even more important than previously envisioned through a possible regulation of syncytin-1 and -2 (105). The potential importance of placental syncytins and FasL in maternal immune tolerance is further supported in studies showing the co-localisation of these proteins in immunosuppressive human placental exosomes (190, 191).

## **1.6 Retroviruses and Immunosuppression**

Retroviral infections often cause severe immunosuppression in many species, which can lead to infection and death (192). The first evidence of retroviral induced

immunosuppression was published by Old *et al.*, in 1959 where he reported an inhibition of foreign (sheep) red blood cell agglutination in mice following infection with the Friend Leukaemia virus (193). These early observations were supported by data from Peterson *et al.*, 1963 which showed in detail that the infection of mice with Gross passage A leukaemia virus decreased humoral- and cell mediated-immunity (194).

The immunosuppressive effects of exogenous and ERVs are related to the transmembrane subunit of the envelope protein of the virus as initially shown by Mathes *et al.*, (195) in studies on Feline Leukaemia Virus (FeLV). Within the transmembrane subunit, a 26 amino acid sequence has a high level of conservation amongst murine, feline, bovine, simian and human retroviruses. This sequence is also found in HERVs (192).

#### 1.6.1 CKS-17

Cianciolo in 1985, demonstrated using a synthetic peptide (CKS-17 - LQNRRGLDLLFLKEGGL) homologous to a conserved region of retroviral transmembrane envelope proteins, that the conserved region possessed immunosuppressive properties (196). The CKS-17 peptide was shown to have an effect on lymphocyte proliferation by inhibiting the growth of an IL-2 dependent murine cytotoxic T cell line. The peptide also inhibited alloantigen-stimulated proliferation of murine and human lymphocytes. This immunosuppression was specific to this peptide, as other peptides representing different viral regions had no effect (196). Since this first paper, CKS-17 has been shown to inhibit:

- respiratory burst of human monocytes (197),
- human NK cell activity (198),
- monocyte-mediated tumour cell killing (199),
- IgG production (200),
- *in vitro* production of IFN- $\gamma$  and TNF- $\alpha$  by human PBMCs (201, 202),

- IL-1-mediated responses by interfering with signal transduction through a protein kinase C pathway (203, 204),
- cell-mediated immunity (delayed-type hypersensitivity (DTH) reactions *in vivo* in mice (205),
- lipopolysaccharide (LPS) -induced mortality of mice (206),
- and murine cytotoxic T lymphocyte activity (207).

These studies are detailed in the following paragraphs.

#### 1.6.1.1 Respiratory Burst of Human Monocytes

Harrell *et al.*, (197) examined the monocyte respiratory burst following treatment of human monocytes with wheat germ agglutinin, phorbol 12-myristate 13-acetate (PMA) and cytochalasin E in the presence or absence of CKS-17. When monocytes were incubated with CKS-17, they exhibited a decreased ability to stimulate the respiratory burst as shown by a decrease in oxygen consumption, O<sub>2</sub>- release and H<sub>2</sub>O<sub>2</sub> release. Further studies on other monocyte mediated functions showed that CKS-17 had no affect on chemotaxis or morphological polarisation, lysosomal enzyme secretion or phagocytosis of opsonised sheep erythrocytes. Suppression of the monocyte respiratory burst was suggested to be of biological importance because of the important role of oxygen radicals in monocyte killing of tumour cells, bacteria, protozoa, and fungi (197).

#### 1.6.1.2 Human Natural Killer (NK) Cell Activity

Harris *et al.*, (198) examined the effects of CKS-17 on human NK cell activity and showed an inhibition of NK cell function caused by interference of the lytic phase of NK cytotoxicity following treatment with CKS-17. Enriched human NK cells were assayed against K562 tumour target cells using a radioactive cytotoxicity assay. Prior incubation of the CKS-17 peptide with both freshly isolated and IFN- $\alpha$  activated human NK cells markedly and reproducibly suppressed NK cell lytic activity. However, this process was

reversible, as the continuous presence of this peptide was required for long term NK cell suppression.

#### 1.6.1.3 Monocyte-Mediated Tumour Cell Killing

Kleinerman *et al.*, (199) examined the effects of CKS-17 on monocyte mediated tumour cell killing and showed an inhibition of tumour cell lysis by monocytes following treatment with CKS-17. A375 tumour cells were incubated with human blood monocytes in the presence or absence of CKS-17. Treatment with 10 $\mu$ M CKS-17 resulted in a decrease in monocyte mediated tumour cell lysis by 80%. This inhibition was sequence specific as another peptide which had partial homology to CKS-17 failed to block monocyte mediated tumour cell killing. The mechanism of inhibition appeared to involve the inactivation of IL-1 as the direct cytotoxic action of recombinant IL-1 on A375 tumour cells was blocked by CKS-17. Interestingly, CKS-17 did not block secretion of IL-1 by LPS or IFN- $\gamma$  activated monocytes. CKS-17 also blocked IL-1 induced proliferation of murine thymocytes, the D10 T cell line and an IL-1 responsive astrocytoma cell line. These data suggests that CKS-17 is a potent inhibitor of IL-1.

#### 1.6.1.4 IgG Production

Mitani *et al.*, (200) studied the effects of purified FeLV, ultraviolet (UV) inactivated FeLV and CKS-17 on IgG production. Using a reverse haemolytic plaque assay, Mitani showed that viable and UV-inactivated virus but not CKS-17 at low concentrations were capable of activating B lymphocytes to secrete IgG. This study also demonstrated biphasic effects of FeLV as it was capable of inhibiting high levels of IgG secretion following induction of feline lymphocytes with staphylococcal protein A. The same inhibition was also seen for CKS-17 suggesting the immunosuppressive effects on immunoglobulin (Ig) synthesis are mediated by the highly conserved ISD present in retroviruses.



#### 1.6.1.5 In Vitro Production of IFN- $\gamma$ and TNF- $\alpha$ by Human Peripheral Blood Mononuclear Cells (PBMCs)

Haraguchi *et al.*, (202) examined the mRNA expression of IFN- $\gamma$  and TNF- $\alpha$  in PBMCs following treatment with CKS-17. Using a PBMC culture system, cytokines were induced by the addition of staphylococcal enterotoxin B (SEB) in the presence or absence of CKS-17 and measured by northern blot and slot blot analysis. CKS-17 was shown to be highly suppressive for SEB induced production of IFN- $\gamma$  and TNF- $\alpha$  at the mRNA level. RIA analysis of PBMC stimulated with IFN- $\gamma$  inducers and treated with CKS-17 showed that this suppression is also present at the level of protein (201, 208).

#### 1.6.1.6 Interleukin-1 (IL-1) Mediated Responses by Interfering with Signal Transduction Through a Protein Kinase C Pathway

Kleinerman *et al.*, (199) demonstrated that CKS-17 was a potent inhibitor of IL-1. Gottlieb *et al.*, (203, 204) examined the mechanisms for CKS-17 mediated IL-1 suppression using a protein kinase C assay. In this study, it was shown that CKS-17 did not interact directly with IL-1 but did interfere with protein kinase C activity which resulted in the inhibition of IL-1 mediated responses. It was hypothesised from these studies that CKS-17 is internalised and processed intracellularly generating active fragments that can interact with cytosolic or membrane-associated protein kinase C causing an inhibition of IL-1 mediated responses (203).

#### 1.6.1.7 Cell-Mediated Immunity (DTH) Reactions In Vivo in Mice

In previous studies, a relationship had been determined between CKS-17 and monocyte mediated tumour cell killing (199) and because of this, the effects of CKS-17 on cell mediated immunity delayed type hypersensitivity (DTH) on tumour cells *in vivo* were examined in mice (205). DTH had previously been used to assess cell mediated immune responses. Conjugates of CKS-17 inhibited DTH reactions to sheep erythrocytes in the feet of mice in a dose dependent manner. Further, immunisation of CKS-17 conjugates

rendered mice resistant to DTH reactions caused by CKS-17 conjugates and also by the products of cultured tumour cells. Cellular inflammatory reactions induced in mouse footpads by concanavalin A were also depressed by the addition of CKS-17 conjugates.

#### 1.6.1.8 Lipopolysaccharide (Endotoxin)-Induced Mortality of Mice

In an abstract presented to a clinical research meeting in 1990, Fuchs *et al.*, (206) showed that CKS-17 has anti-inflammatory properties in the mouse model. The inflammatory stimulator, LPS was administered to mice at doses which would normally cause mortality. In the presence of CKS-17, an inhibition of granulocytic functions (inflammation response) was observed which resulted in a reduction of the mortality levels in the mice.

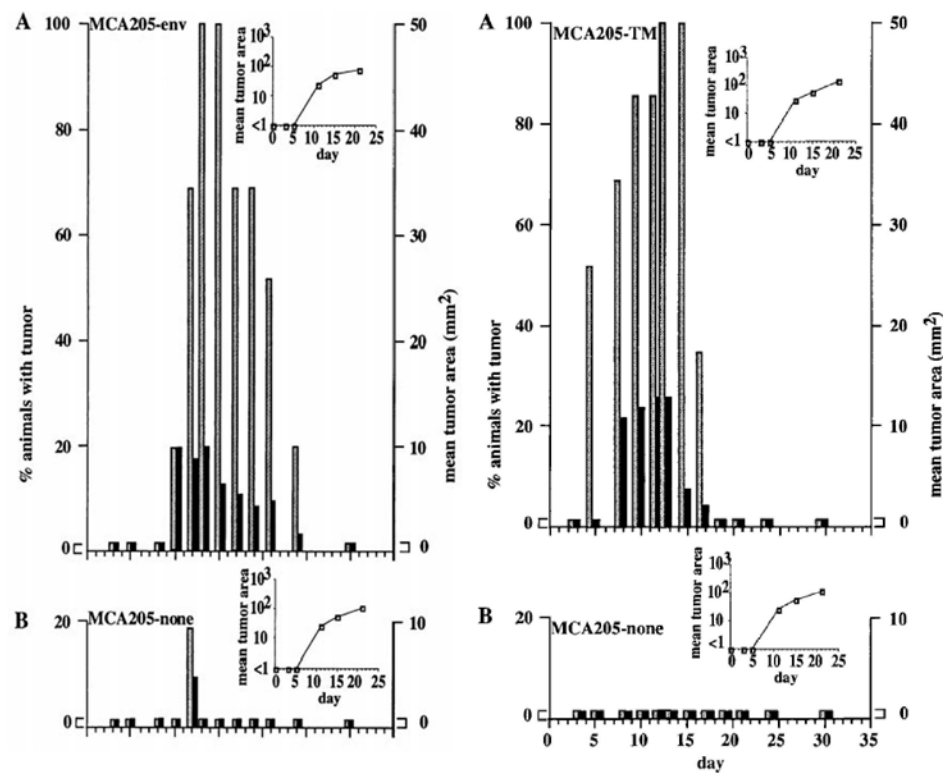
#### 1.6.1.9 Murine Cytotoxic T Lymphocyte Activity

Ogasawara *et al.*, (207) studied the effects of CKS-17 on the production of murine alloantigen-specific cytotoxic T lymphocytes *in vitro*. Spleen cells from B6, C3H and Balb/C mice were used as responders and stimulators. Responder cells were cultured with UV-irradiated stimulator cells in the presence or absence of CKS-17. A suppressive activity of CKS-17 was observed when the peptide was introduced into the immunologically stimulating culture concomitant with, or up to 48 hours after the initiation of culture. CKS-17 acted in these experiments by reducing the number of precursor T cells. An abrogation of this suppressive activity was observed following the addition of the lymphokines IL-2 and IL-4 suggesting that the immunosuppressive actions of retroviruses can be modulated by cytokines under certain conditions.

#### 1.6.2 In Vivo Studies of Retroviral Envelope Protein Mediated Immune Suppression

Mangeney and Heidmann in 1998 demonstrated by expressing the Moloney Murine leukaemia virus (MMuLV) in tumour cells, that infectious retroviruses have

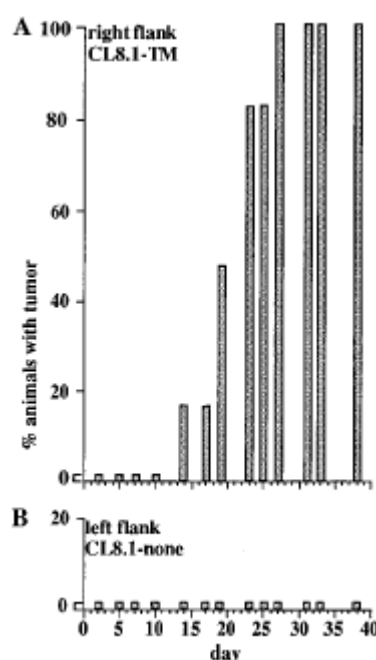
immunosuppressive properties. They examined the immunosuppressive properties of the entire envelope protein of MMuLV as well as the transmembrane subunit (Fig 1.7). Their results showed that tumour cells expressing both the entire envelope as well as the transmembrane subunit were able to proliferate without immune rejection (Figure 1.7A). Cells not expressing any of the retrovirus were immediately removed by the immune system (Figure 1.7B). This demonstrated that MMuLV transmembrane subunit has immunosuppressive properties (209).



**Figure 1.7 Tumour cell growth in mice expressing fragments of the envelope protein of MMuLV.** Induction of tumour growth by retroviral envelope expression only requires the TM subunit as demonstrated by the figure on the right. Mice were injected with tumour cells either expressing the envelope or transmembrane subunit (A) or a control (B). The percentage of animals with tumours is represented by grey bars while the black bars represent mean tumour area (mm<sup>2</sup>) (209).

To prove that the introduction of the transmembrane subunit was not causing suppression of the entire immune system, Mangeney analysed the effect on non-transduced tumour cells transplanted at a different site (Figure 1.8). To achieve this,

transmembrane subunit expressing tumour cells were injected subcutaneously into the right flank while control tumour cells were injected on the contralateral side. As detailed in Figure 1.8, tumour cells expressing the transmembrane subunit proliferated (Figure 1.8A) while control cells were removed by the immune system (Figure 1.8B). These results suggested that the immune suppression was local and specific to cells that expressed the transmembrane subunit (209).

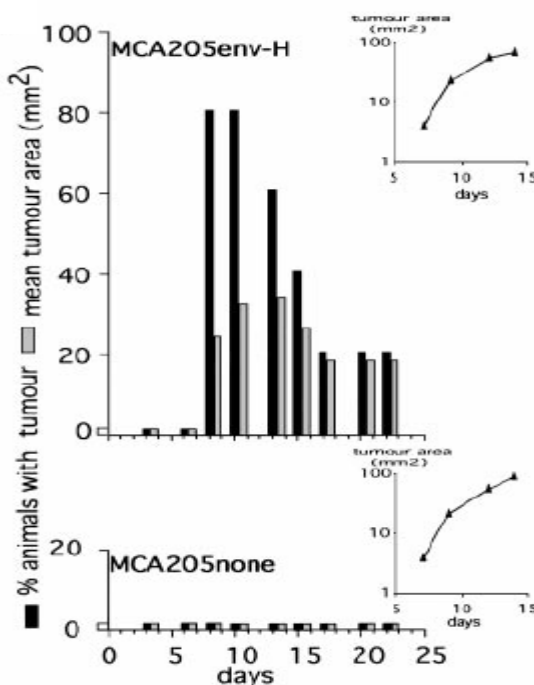


**Figure 1.8. The effects of retroviral-mediated immune suppression on distant sites of the body.**

*Transmembrane subunit expression does not affect the rejection of non-transduced cells at a distant site. Immune regulation was observed in tumour cells expressing the transmembrane subunit (A) while control tumour cells were rejected by the immune system (B) (209).*

MMuLV is an infectious exogenous retrovirus and may possess different properties to ERV proteins. However, ERV proteins are associated with immunosuppression. *In vivo* studies by Mangeney *et al.*, 2001 (210) showed that ERV proteins can have immunosuppressive properties. In this experiment, tumour cells expressing the full-length envelope of HERV-H were injected subcutaneously into mice and their survival was measured. Cells expressing the HERV-H envelope protein were able to proliferate while control cells were not, suggesting that HERV-H down-regulated the hosts immune

system (Figure 1.9) (210). These data showed that ERV envelope proteins can have immunosuppressive properties.

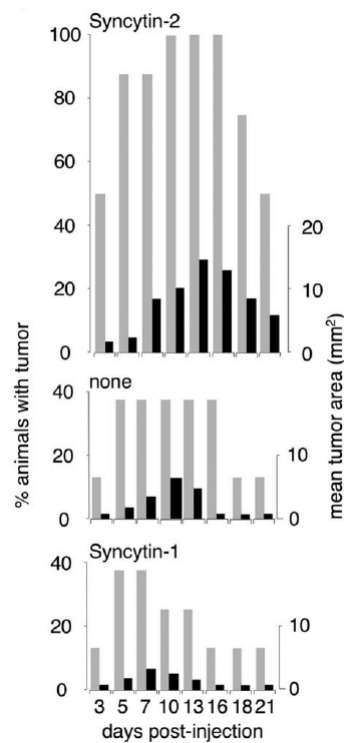


**Figure 1.9. Immunoppressive properties regulated by the HERV-H envelope protein.** Tumour cells expressing the HERV-H envelope protein and control tumour cells were injected subcutaneously into mice. The percentage of animals with the tumour was examined to determine the immunosuppressive properties. MCA205env-H represents tumour cells containing the HERV-H envelope while MCA205none are control cells. Black bars represent the percentage of animals with the tumour while grey bars represent the mean tumour area (mm<sup>2</sup>) (210).

### 1.6.3 Immunoppressive Role of Placental ERV Envelope Proteins

In line with the hypothesised immunosuppressive role of HERVs in placentation, Villarreal (211) in 1997 presented the metavirus hypothesis, whereby “retroviruses made by the embryo are proposed to specifically and transiently repress the local development of maternal immune recognition of the developing embryo”. However, until recently, the immunosuppressive properties of these proteins had not been studied.





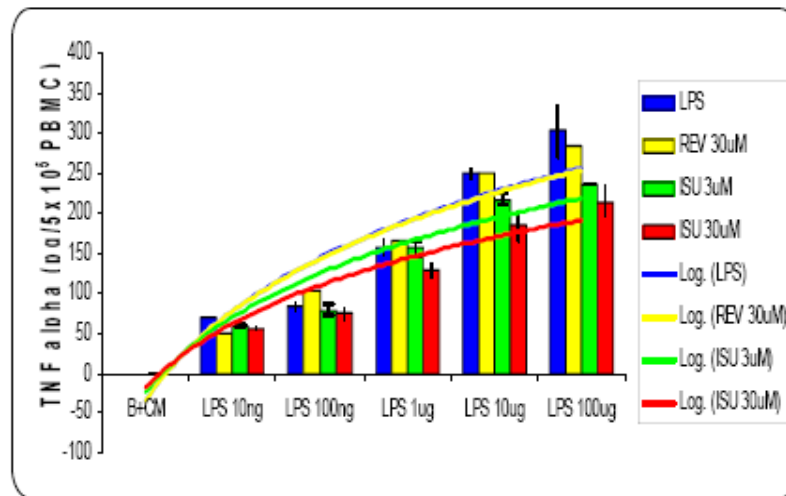
**Figure 1.10. Immunosuppressive properties of placental syncytins.** Tumour cells expressing syncytin-1 and syncytin-2 and control tumour cells were injected sub cutaneously into mice. The percentage of animals with the tumour was examined to determine the immunosuppressive properties. Grey bars represent the percentage of animals with the tumour while black bars represent the mean tumour area (mm<sup>2</sup>) (213).

Mangeney *et al.*, 2007 (213) provided the first evidence for an immunosuppressive function for placental syncytins. Human syncytin-1 and -2, and mouse syncytin-A and -B were transfected into an allogeneic tumour cell line and transplanted subcutaneously into mice. Tumour cell growth was used as a measure of immunosuppression. Using this model, Mangeney had previously shown that cells expressing ERV envelope proteins could transiently escape immune rejection (209, 210) (detailed in figure 1.9). This study showed that in both humans and mice, one of the two syncytin proteins (syncytin-2 in humans, syncytin-B in mice) was immunosuppressive, while the other is not (syncytin-1 in humans, syncytin-A in mice) (syncytin-1 and syncytin-2 data shown in figure 1.10). They further showed that an amino acid substitution at position 14 of the ISD appeared to regulate the immunosuppressive function.

To further characterise placental syncytins immunosuppressive properties, Mangeney analysed the humoral immune response of syncytin-1 and -2 using recombinant syncytin ectodomains which are 63- or 64-residue-long fragments of the TM subunit, including the ISD. These ectodomains are large enough to adopt the proper physiological conformation (174, 213, 214). An assay was designed based on the production of antibodies by mice injected with the purified ectodomain. The trimeric human syncytin proteins were injected twice at a 1 week interval and sera were collected 4 days after the last injection for analysis of IgG titres. The results showed that a humoral response was only mounted against the “non-immunosuppressive” syncytin-1 suggesting that syncytin-2 had an inhibitory effect on the antibody response (213). However, Mangeney did not study the cytokine profile following administration of these peptides.

Jorge Tolosa, during his PhD at the Mothers and Babies Research Centre, studied the immunosuppressive properties of a synthetic peptide homologous to the syncytin-1 ISD. The data produced by these experiments was in direct contrast to that produced by Mangeney *et al.*, 2007 (213), where syncytin-1 was not shown to be immunosuppressive. In these experiments, 30µM syncytin-1 synthetic ISD was able to inhibit TNF-α production in human blood by 30% following treatment of whole blood with 10 µg/ml LPS (190) (Figure 1.11). Further, Noorali *et al.*, 2009 (188) suggested that due to its localisation in the syncytiotrophoblast, syncytin-1 should be a prime candidate as an immunosuppressive ERV envelope protein in the placenta.





**Figure 1.11. Representative experiment showing the effect of Syncytin-1 ISD peptide on TNF- $\alpha$  production by human blood cells after stimulation with different concentrations of LPS. A 30% inhibition in TNF- $\alpha$  production was observed with 30uM Syncytin-1ISD when blood was stimulated with 10ug or 100ug of LPS. No effect was observed with the Syncytin-1ISD Reverse sequence at any concentration. A logarithmic curve fitting is shown. Bar graphs show mean  $\pm$  SD. Results are representative of three experiments (190).**

To our knowledge, no further studies have been completed on the immunosuppressive properties of syncytin-2, -A and -B so further analysis of these proteins are required. In regards to syncytin-1, it is clear that the current knowledge on the involvement of this protein in maternal immunotolerance requires further characterisation.

### **1.7 The Trojan Exosome Hypothesis and Immunosuppressive Placental Exosomes**

Stephen J Gould. 2003 (215) first presented the Trojan exosome hypothesis. This hypothesis states that retroviruses can exploit a cell-encoded pathway of intracellular vesicular traffic, exosome exchange, for both the biogenesis of retroviral particles and a low efficiency mode of infection. This hypothesis can explain the transmission between cells by retroviruses in the absence of a retroviral envelope protein.

Exosomes are small membrane vesicles, classically defined as having a diameter of 30-100nm (216), though larger exosomes have also been described (215, 217-219). These vesicles are released from cells upon fusion of internal multivesicular bodies with the plasma membrane (215-218). The biological function of exosomes is unclear. They were discovered over 25 years ago and thought to be involved in the removal of cell surface molecules in reticulocytes (220). Since then, exosome production has been demonstrated from a number of different cells, including dendritic cells (221), B cells (222), T cells (223), mast cells (224), epithelial cells (225), and tumour cells (226). Within all exosomes are common subsets of proteins which are essential for their biogenesis, structure and cell trafficking. Exosomes also have cell-specific components which can determine a specific function (227). For example, exosomes have been shown to modulate immune responses (reviewed in (227)).

The human placenta produces and secretes exosomes which have immunosuppressive properties (191, 228-231). Frangmyr *et al.*, 2004 showed that placental exosomes carry FasL and hypothesised that the secretion of the exosome-associated form of FasL may be a mechanism by which the placenta promotes immune privilege (229). Taylor *et al.*, 2006 identified FasL and an unidentified '24' kDa protein as being the exosomal components associated with immunosuppression. Interestingly, the 24kDa syncytin-1 transmembrane subunit has recently been demonstrated to be carried by human placental exosomes and it has been postulated that syncytin-1 could have a role in immunosuppression mediated by exosomes (190). Overall, the evidence presented suggests an immunosuppressive role of placental exosomes during pregnancy which may involve the expression of immunosuppressive retroviral envelope proteins including syncytin-1.

## **1.8 Thesis Hypothesis**

In light of the information presented in this literature, the general thesis hypothesis is

*That the endogenous retroviral envelope protein syncytin-1, which is expressed by the syncytiotrophoblast of the human placenta and carried on human placental exosomes can contribute to the immunological tolerance toward the developing fetus by inhibiting the production of Th1 cytokines. Further, the expression of syncytin-1 can be regulated by the hormone CRH in order to exert its immunosuppressive role.*

When this study began, there were no known studies examining the immunosuppressive properties of human and mouse syncytins. If the biological pathways involved in the modulation of the immune system during pregnancy are fully characterised, they have tremendous implications in medicine. Most importantly, in organ transplantation procedures, the pathways could be used so that the transplant recipient will no longer require the severe immunosuppressive drug regime that is currently utilised.

### **1.8.1 Thesis Aims**

The objectives of the present study are to:

- Set up a method to simultaneously extract DNA, RNA and proteins from a small experimental sample,
- Using the above method, assess the role of CRH in the induction of syncytin-1, syncytin-2 and FasL in the BeWo cell model and to examine an exosomal fraction following CRH treatment, and
- To produce and purify recombinant syncytin-1 ectodomain
- Assess the immunosuppressive capacity of the syncytin-1 recombinant ectodomain protein using a biologically relevant PBMC culture system.

## **Chapter 2 - A Column Based Method to Simultaneously Extract DNA, RNA and Protein from the Same Sample**

## **2.1 Abstract**

A procedure for the simultaneous extraction of proteins and nucleic acids from the same experimental sample is described allowing for direct correlations between genetic, genomic and proteomic data. This approach, using commercially available column-based nucleic acid extraction kits which are designed to efficiently extract DNA and RNA, requires no hazardous chemicals and is a quick, reliable and consistent method for concomitant protein extraction. Buffer choice is critical to completely solubilise all proteins in the sample. RIPA buffer was not suitable for this protocol as column-based methods extracted less proteins compared to conventional methods using the same buffer at the one dimensional (1D) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) level. Proteins extracted from the columns and solubilised in two dimensional (2D) electrophoresis lysis buffer (2D buffer) had a similar profile to conventionally 2D buffer extracted proteins when analyzed at both the 1D level and the 2D level. Proteins extracted using these methods were also compatible with Western Blotting analysis. This technique provides a simple and effective way to analyze protein and nucleic acids simultaneously from the same sample without affecting yield and quality.

## **2.2 Introduction**

Biological complexity emerges from different organisational levels in a highly regulated space-time coordination of processes that involves the participation and orchestrated interaction of DNA, RNA and proteins between each other and their environment. Fully understanding normal biological processes such as cell differentiation, development and ageing, and pathological conditions requires integrated genomic, transcriptional and proteomic studies (232-234), which demand the simultaneous isolation of DNA, RNA and proteins from the same sample.

Quick and reliable methods that perform simultaneous extraction of DNA, RNA and protein from a single sample are ideal for the generation of genomic and proteomic data. These procedures can save time and money and allow for the efficient use of small and precious biological samples. Researchers are increasingly turning away from classic RNA and protein extraction techniques such as phenol/chloroform separation (235) or time consuming cesium chloride gradient centrifugation because of the hazardous chemicals that are required and also because these methods are generally unsuited for routine use in the laboratory. Spin column technology is a simple and quick approach to extracting nucleic acids from small biological samples. Furthermore, most column based procedures do not require hazardous chemicals such as phenol and chloroform that are used in traditional nucleic acid extraction procedures (236).

Recently Morse *et al.* (236) discussed the combined extraction of RNA and proteins using RNA spin column-based technology and Hummon *et al.* (237) showed an improved method for isolation and solubilisation of proteins after Trizol extraction of RNA and DNA from the same sample. However, none of these authors performed a complete analysis of the proteins obtained at the level of 2D electrophoresis to compare the protein profile obtained with the new technology against that obtained with conventional proteomic methods. Here, a methodology is presented to simultaneously

extract RNA and proteins and/or DNA, RNA and proteins from the same sample using commercially available column-based nucleic acid extraction kits. In this study, the profile of proteins from these kits is further compared to the profile obtained with some of the dedicated protein extraction methods using 2D-electrophoresis and it is shown that buffer choice is critical in the efficient extraction of proteins from these kits.

## **2.3 Methods**

### **2.3.1 Tissue Preparation**

All experimental procedures involving human placenta were approved by the University of Newcastle Human Ethics Committee and the Hunter New England Health Human Ethics Committee.

Normal term placentas were collected at John Hunter Hospital and placental tissues were snap frozen in liquid nitrogen prior to use. Before protein or nucleic acid extraction, samples were pulverised in liquid nitrogen before homogenisation.

### **2.3.2 Spin Column Extractions**

Pulverised placental tissue was homogenised in Buffer RLT (RNeasy mini kit – 30mg) or Buffer QRL1 (DNA/RNA kit – 10mg) respectively, using the Ultra-Turrax T25 homogenizer (Janke and Kunkel IKA-Labortechnik). Both buffers were supplemented with  $\beta$ -mercaptoethanol as detailed in the manufacturer's instructions.

#### **2.3.2.1 Purification of Total RNA and Protein using the RNeasy Mini Kit (QIAGEN)**

RNA was extracted using the RNeasy kit following the manufacturer's instructions (<http://www1.qiagen.com/literature/handbooks>). Briefly, samples were passed through the column facilitating the binding of RNA to the column. The columns were then washed to remove contaminating proteins and eluted in RNase free water. The flow through from each of the steps was pooled and from this, proteins were left to precipitate overnight at -20°C.



#### 2.3.2.2 Purification of DNA, RNA and Protein using the DNA/RNA kit (QIAGEN)

DNA, RNA and proteins were extracted using the DNA/RNA kit following the manufacturer's instructions (<http://www1.qiagen.com/literature/handbooks>). Briefly, tissue samples were homogenised in buffer QRL1 (Qiagen) and the majority of the protein was precipitated out of the homogenate following the addition of buffer QRV1 (Qiagen). The supernatant (containing RNA and DNA) was passed through the column facilitating the binding of RNA and DNA. The columns were washed and pure RNA was eluted in a high salt buffer (buffer QRU) which prevents non-specific DNA elution. DNA was eluted following the addition of buffer QF. The flow through from all of these steps was pooled and added to the initial protein precipitate before the addition of an equal volume of ethanol (100%) and the proteins were precipitated overnight at -20°C.

Following extractions, the absorbances at 260 and 280nm were measured and the 260:280 ratios were calculated to determine quality and quantity of the purified nucleic acids using a Nano-drop® ND-1000 spectrophotometer (Biolab). Nucleic acid quality was further examined by agarose gel electrophoresis.

#### 2.3.3 Protein extraction

##### 2.3.3.1 Conventional

Tissues were homogenised on ice using an Ultra-Turrax homogeniser in either conventional RIPA protein extraction buffer or 2D buffer. 1ml of buffer was added per 100mg of tissue. The homogenate was then centrifuged at 12 000 x g for 15 minutes at 4°C and the supernatant was collected.

#### 2.3.3.2 Column based proteins

Flow through from the column was precipitated overnight at -20°C. Following precipitation, the flow was spun at 10,000 x g to pellet the proteins. Protein pellets were then washed three times in acetone by incubating at -20°C for 30 minutes and centrifuging the samples at 10,000 x g for 30 minutes. Washed protein pellets were resuspended in the appropriate buffer (RIPA or 2D). To completely resuspend the protein pellet, samples were stored overnight at 4°C.

#### 2.3.4 Protein Determination

##### 2.3.4.1 BCA kit

Proteins extracted using RIPA buffer were quantified using the BCA Protein Assay Kit (Pierce) following the manufacturer's instructions (<http://www.piercenet.com/files/1296dh4.pdf>). Briefly, the working reagents were mixed together and added to samples and standards. Prepared samples were incubated at 37°C, read at 570nm on a spectrophotometer and compared to known standards.

##### 2.3.4.2 2D Quant kit

Proteins extracted in 2D buffer were quantified using the 2D Quant Kit (GE Healthcare) following the manufacturer's instructions ([http://www5.gelifesciences.com/APTRIX/upp00919.nsf/content/06BAEBA0622B6A43C125748800812E19?OpenDocument&Path=Catalog&Hometitle=Catalog&entry=5&newrel&LinkParent=C1256FC4003AED40-9FF83ACAC523805DC1257019004908D6\\_RelatedLinksNew-C821BEC677D8448BC1256EAE002E3030&newrel&hidesearchbox=yes&moduleid=undefined](http://www5.gelifesciences.com/APTRIX/upp00919.nsf/content/06BAEBA0622B6A43C125748800812E19?OpenDocument&Path=Catalog&Hometitle=Catalog&entry=5&newrel&LinkParent=C1256FC4003AED40-9FF83ACAC523805DC1257019004908D6_RelatedLinksNew-C821BEC677D8448BC1256EAE002E3030&newrel&hidesearchbox=yes&moduleid=undefined)).

### 2.3.5 Protein analysis

#### 2.3.5.1 SDS-PAGE

Placental proteins were resolved using NuPAGE<sup>®</sup> Novex 10% Bis-Tris gels with MOPS Running Buffer at 200V for 50 minutes. Gels for silver staining were then fixed in silver stain fixative (40% methanol and 10% glacial acetic acid) overnight. Gels for western blotting were transferred to nitrocellulose membranes for later use.

#### 2.3.5.2 Silver stain

Gels fixed in silver stain fixative were silver stained using SilverQuest<sup>™</sup> (Invitrogen) to visualize the protein profile at the one dimensional level. Protocols were followed as detailed by the manufacturer ([http://www.invitrogen.com/content/sfs/manuals/silverquest\\_man.pdf](http://www.invitrogen.com/content/sfs/manuals/silverquest_man.pdf)). To determine differences between extraction techniques, a densitometric scan was undertaken using Multi Gauge software (version 2.3, Fujifilm).

#### 2.3.5.3 Western Blotting

Proteins resolved by gel electrophoresis were transferred to a nitrocellulose membrane at 25mA for 60 minutes using NUPAGE<sup>®</sup> transfer buffer. The loading and transfer of proteins was checked using FasGreen prior to western blotting. After extensive washes in tris buffered saline tween 20 (TBST), membranes were blocked with 5% skim milk in TBST. Membranes were then washed in TBST and immunoblotted with alpha-actin A4700 (Sigma-Aldrich – 1:1000) in 5% skim milk rocking overnight at 4°C. Unbound antibody was washed in TBST and incubated with an anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnologies – 1:2000) for 1 hour rotating at room temperature (RT). Following a final wash in TBST,

alpha-actin was detected on the membranes using the enhanced chemiluminescence (ECL) reagent (GE healthcare) and the LAS-3000 (Fujifilm) imaging system.

#### 2.3.5.4 2D Gel Electrophoresis

2D gel electrophoresis was undertaken using the ZOOM<sup>®</sup> IPGRunner™ System (Invitrogen) following the manufacturer's instructions ([http://www.invitrogen.com/content/sfs/manuals/zoomipgrunner\\_man.pdf](http://www.invitrogen.com/content/sfs/manuals/zoomipgrunner_man.pdf)). Briefly, proteins were electrofocused in the first dimension by isoelectric focusing using pH 3-10 immobilised pH gradient strips. Proteins were then separated based upon their molecular weight to achieve a second dimension separation using a 4-12% Bis-Tris gel. Following separation, proteins were visualised by Sypro<sup>®</sup> Ruby Protein stain (Invitrogen) following the manufacturer's protocol (<http://probes.invitrogen.com/media/pis/mp12000.pdf>) and photographed using the LAS-3000 (Fujifilm).

## **2.4 Results**

### **2.4.1 Nucleic acid quantification and quality**

Nucleic acids extracted from the RNeasy and DNA/RNA kits were quantified using a nano-drop spectrophotometer and the 260:280 ratios were calculated. Nucleic acid integrity was checked using agarose gel electrophoresis. RNA ratios were shown to be between 1.8-2.1, while DNA ratios were between 1.7 and 2. Both DNA and RNA extracted using these techniques were of high quality as expected from kits designed to extract nucleic acids (data not shown).

### **2.4.2 One-dimensional protein analysis**

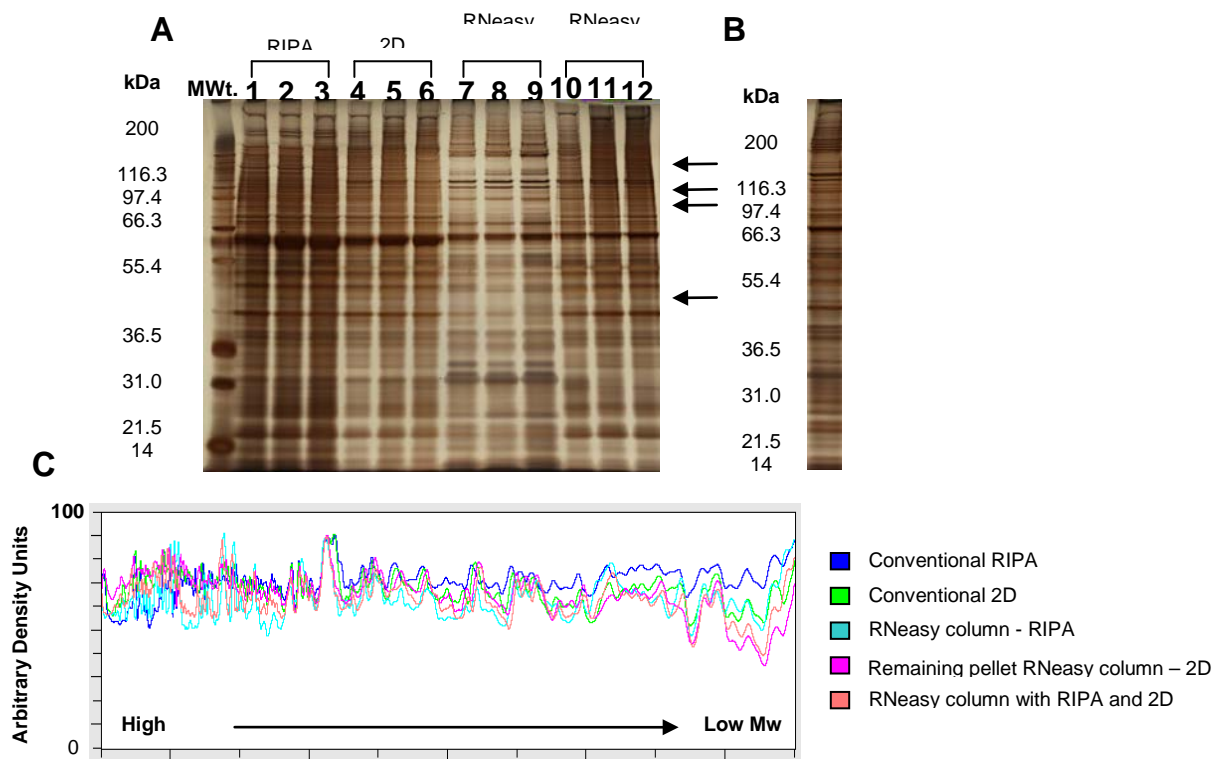
These studies were designed to confirm Morse's observations (236) by analysing the profile of the proteins extracted with the RNeasy columns using silver staining, followed by quantitative analysis using densitometry.

The initial observation was that the proteins extracted using the RNeasy column were unable to be completely resuspended in RIPA buffer, even with heat treatment and/or overnight resuspension at 4°C. This was not the case for proteins resuspended in 2D buffer as proteins purified from the column and resuspended in 2D buffer were easily solubilised. Protein determination of these samples showed that the RIPA buffer was inefficient in extracting proteins compared to 2D buffer (data not shown).

The profile of the proteins was then analyzed by SDS-PAGE followed by silver staining and densitometry. As observed in Figure 2.1, conventional extraction of placental proteins using RIPA and 2D buffer yielded a wide spectrum of proteins displaying a similar profile with some small differences, notably in the high molecular weight range (Figure 2.1a, lanes 1-3 and 4-6). Proteins that were extracted using the RNeasy column

and resuspended in either of the two buffers also showed a wide spectrum of proteins, however, the proteins resuspended with the RIPA buffer clearly had missing bands at certain molecular weights (Figure 2.1a - arrows) when compared with the conventional extraction method using RIPA or 2D buffer (Figure 2.1a, lanes 7, 8 and 9). Proteins from the RNeasy column resuspended in 2D buffer (Figure 2.1a, lanes 10, 11 and 12) had a highly comparable protein profile to the conventionally extracted proteins using the same buffer (Figure 2.1a, lanes 4, 5 and 6). These observations were further confirmed using densitometrical analysis (Figure 2.1c).

As detailed above, proteins extracted using the RNeasy column were unable to be completely resuspended in the RIPA buffer. However, if the remaining pellet after resuspension in RIPA buffer was further resuspended in 2D buffer and these samples were pooled together, a representative profile of the entire protein population could be observed. This method contained proteins highly abundant in both RIPA and 2D buffers (Figure 2.1b).



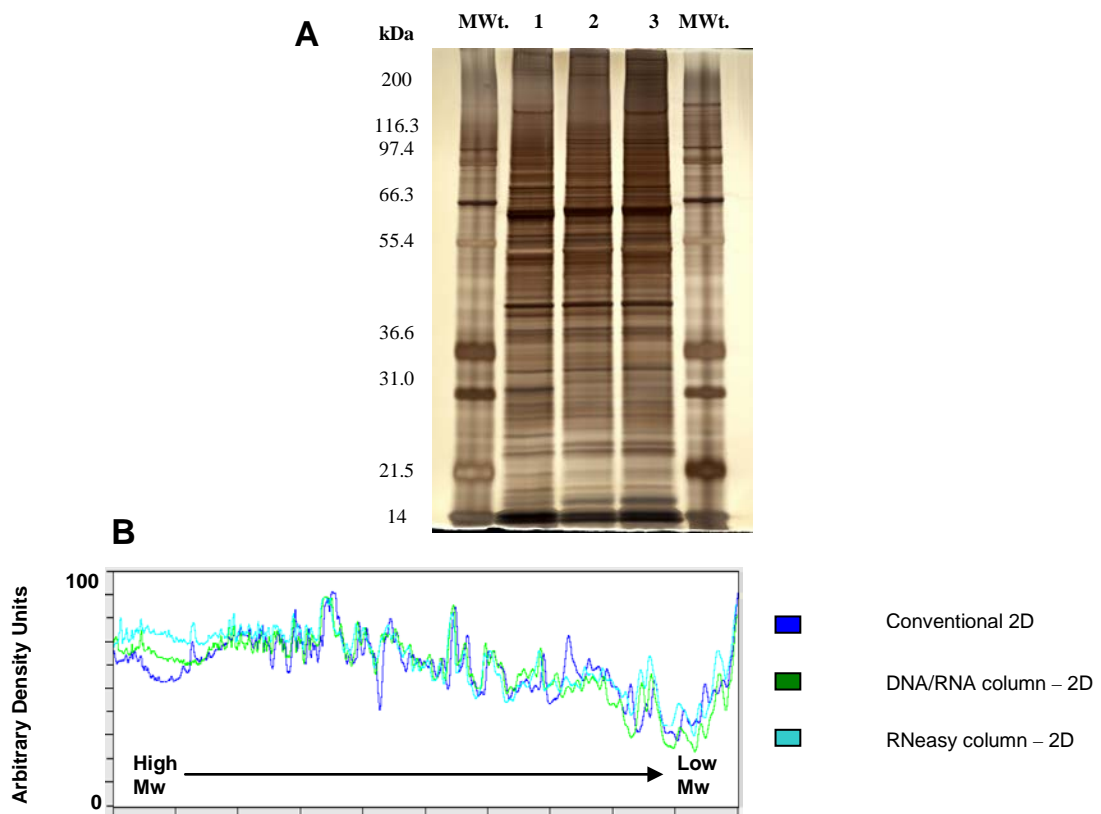
**Figure 2.1. Representative silver stain and densitometry analysis of placental proteins extracted using conventional methods and the RNeasy kit (Qiagen).** A) SDS-PAGE silver stained placental proteins: Lanes 1-3 Proteins conventionally extracted using RIPA buffer; Lanes 4-6 Proteins conventionally extracted using 2D buffer; Lanes 7-9 Proteins extracted using RNeasy column and resuspended in RIPA buffer; Lanes 10-12 Proteins extracted using RNeasy column and resuspended in 2D buffer. Molecular weight standard (Mark12 Invitrogen). B) Representative silver stain of pooled placental proteins initially resuspended in RIPA buffer and the remaining pellet resuspended in 2D buffer. C) Densitometrical analysis of pooled placental proteins extracted using conventional methods and the RNeasy kit (Qiagen). Fugi-film software was used to analyze the density of each band and a densitometrical plot was obtained. Blue = Proteins conventionally extracted using RIPA buffer, Green = Proteins conventionally extracted using 2D buffer, Aqua = Proteins extracted using the RNeasy column and resuspended in RIPA buffer, Pink = Remaining pellet from proteins extracted with RNeasy using RIPA, resuspended in 2D buffer, Salmon = Combined proteins extracted with RNeasy using RIPA and 2D buffer.

#### 2.4.3 Comparative analysis of protein profiles obtained using the RNeasy column, DNA/RNA column and conventional 2D protein extraction buffer

The initial studies confirmed Morse's observations that the RNeasy column was capable of extracting proteins following a few simple additional steps. However, these studies had further shown that a conventional extraction buffer such as RIPA is not compatible with this technique. Further to the Morse study, these studies aimed to determine whether other column based kits were capable of combined nucleic acids and protein extractions. Using the 2D buffer which was shown to be highly compatible with column based extractions, the capacity of the DNA/RNA column (Qiagen) to co-extract DNA, RNA and proteins from the same sample was analysed.

Placental proteins were extracted using the conventional method with 2D buffer, the RNeasy column method and the DNA/RNA column method. Using silver stain and densitometry the profile of the proteins at the one dimensional level was compared (Figure 2.2 a and b). A wide range of proteins were observed and the protein profiles were comparable between all extraction techniques when analysed by silver staining (Figure 2.2a) and densitometrical analysis (Figure 2.2b).

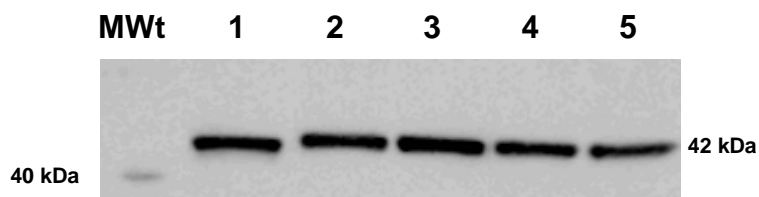




**Figure 2.2. Representative silver stain and densitometry analysis of placental proteins conventionally extracted using 2D buffer or RNeasy column and DNA/RNA column and resuspended in 2D buffer.** A) Representative silver stain of pooled placental proteins conventionally extracted using 2D buffer and column based methods. Molecular weight standard (Mark12). Lane 1 Pooled proteins conventionally extracted using 2D buffer; Lane 2, Pooled proteins extracted using the DNA/RNA column and resuspended in 2D buffer; Lane 3 Pooled proteins extracted using the RNeasy column and resuspended in 2D buffer. B) Densitometric analysis of pooled placental proteins using Fugifilm densitometrical analysis software. Blue = Pooled proteins conventionally extracted with 2D buffer, Green = Pooled proteins extracted with DNA/RNA columns and resuspended in 2D buffer, Aqua = Pooled proteins extracted with the RNeasy columns and resuspended in 2D buffer.

#### 2.4.4 Western Blotting

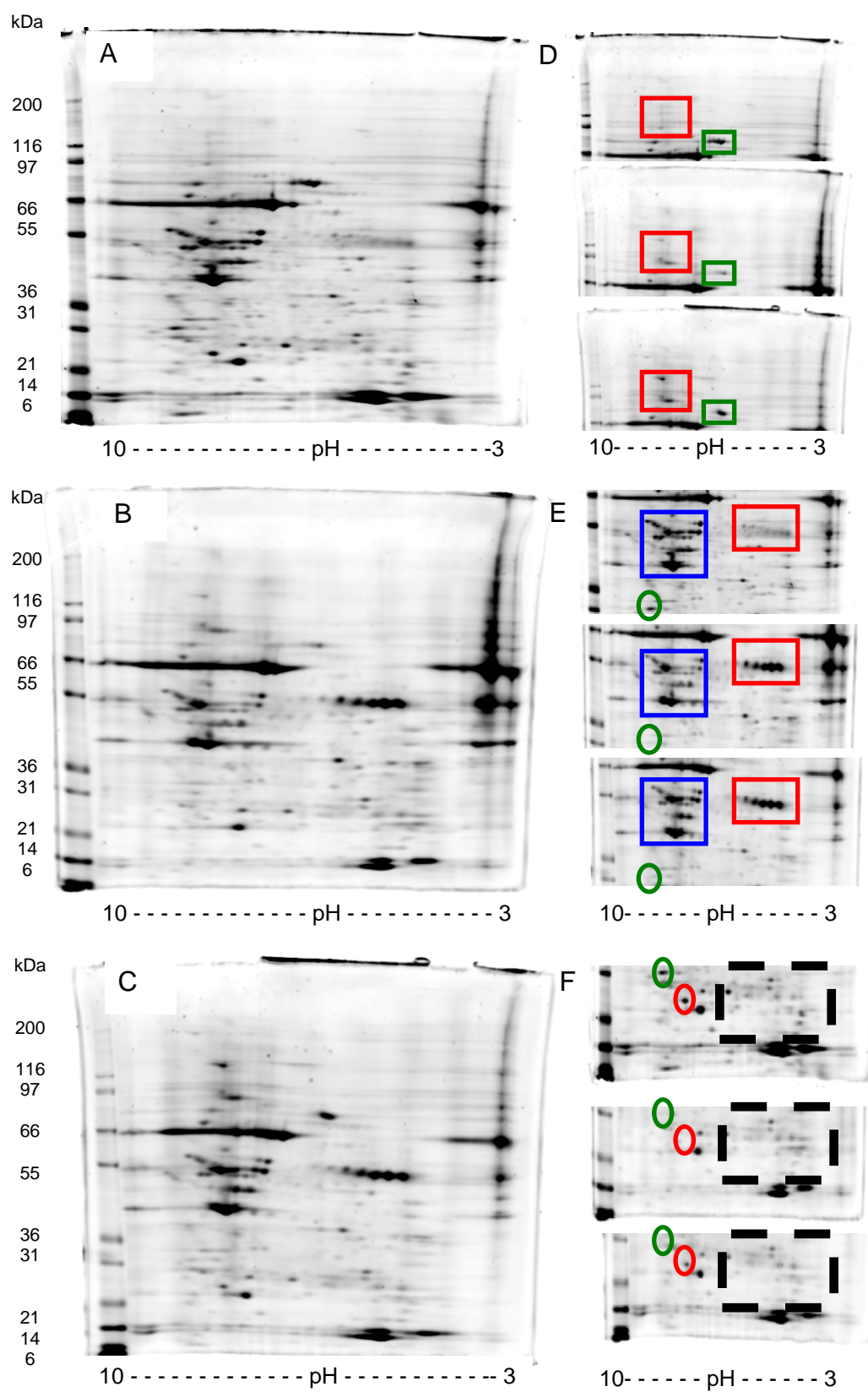
To confirm the compatibility of the RNeasy and DNA/RNA column based protein extraction methods with Western Blotting, a commercial actin antibody was tested. A positive reaction was observed at 42kDa, corresponding to the expected size for actin showing that these extraction methods are compatible with common downstream analytical applications (Figure 2.3).



**Figure 2.3. Western blot of placental proteins extracted using the conventional method and the column based methods.** *Proteins were checked for their compatibility with Western Blotting using an antibody against alpha actin (42kDa). Lane 1 Proteins conventionally extracted using 2D buffer, Lane 2 & 3 Proteins extracted using the DNA/RNA columns and resuspended in 2D buffer, Lane 4 & 5 Proteins extracted using the RNeasy column and resuspended in 2D buffer.*

#### 2.4.5 2-Dimensional analysis of proteins extracted using conventional and column based methods

Following the analysis of proteins simultaneously extracted with nucleic acids at the 1D level the profile of proteins at the 2D level was examined. The overall analysis of these gels showed a highly comparable pattern of proteins with some differences. The most notable differences between the extraction methods were the relative abundance of some proteins preferentially extracted with one method in comparison with another. The column based extraction methods in some cases enriched the protein profile compared to conventional extraction methods (Figure 2.4 (red box)). However, in some cases the conventional extraction method appeared to enrich the proteins present in certain areas of the gel, particularly at low molecular weights (Figure 2.4). However, when the images of each 2D gel protein extraction method were matched by overlapping, the overall picture of the proteins showed a highly similar pattern.



**Figure 2.4. 2D gels detailing conventional 2D buffer extraction, RNeasy and DNA/RNA proteins resuspended in 2D buffer.** *Sypro Ruby* was used to detect the protein bands. A) Proteins conventionally extracted using 2D buffer B) Proteins extracted using DNA/RNA columns and resuspended in 2D buffer C) Proteins extracted using RNeasy columns and resuspended in 2D buffer. Whole gels were then separated into regions for better analysis (D to F). Proteins were extracted using the conventional (top), DNA/RNA (middle) and RNeasy (bottom) (D to F). Dashed boxes are used to define regions where similarities can be observed. Other boxes and circles are used to define regions where differences can be observed: D) 66-200 kDa, E) 31-61 kDa, and F) 6-31 kDa.

## **2.5 Discussion**

Current methodologies limit the ability of the researcher to analyse nucleic acids and proteins from the same biological sample. To our knowledge there are only a few simultaneous DNA, RNA and protein extraction techniques (237-240) and most of these are based in variations of the Chomczynski method (238). Hummon *et al.*, (237) recently published a methodology for the extraction of nucleic acids and protein using the Trizol® reagent based upon the Chomczynski method. Using this technique, Hummon *et al.* (237) claimed that 98% of the total protein was recovered and the protein was not degraded following long term storage. However, Hummon's modified technique is time consuming and requires lengthy dialysis to completely solubilise the protein. Although these techniques allow for the simultaneous extraction of nucleic acids and proteins, new column based extraction methods are less time consuming and require the use of less hazardous chemicals.

Recently, Morse *et al.*, (236) demonstrated using a column designed to extract RNA (Qiagen's RNeasy column) that it is also possible to extract proteins, following some simple additional steps. In Morse's protocol, following the initial centrifugation step outlined in the manufacturer's protocol, the flow through was retained and stored on ice. All subsequent wash steps were pooled with the original flow-through and the samples were precipitated overnight at -20°C. However, Morse *et al.*, (236) did not use a conventional in gel staining method to show the profile of the proteins obtained and hence his studies were replicated to determine the range and efficiency of proteins extracted with this method.

The initial observations following resuspension of the proteins were that the RIPA buffer was unable to completely resuspend the protein pellet. This may be due to the fact that the wash steps were unable to completely remove the guanidine salts present from the extraction which are incompatible with SDS-based buffers, as previously reported

(241). Subsequent analysis of the proteins extracted from these methods showed that RNeasy proteins resuspended in RIPA buffer had a relatively poor profile compared to those proteins extracted using the conventional method with RIPA or 2D buffer and also RNeasy proteins resuspended in the 2D buffer (Figure 2.1). Morse *et al.*, (236) suggested that the proteins could be resuspended in a buffer of choice, however, this study has shown that buffer choice is integral in the extraction of proteins.

Because of the inherent difficulties of protein solubilisation using column based extraction procedures, it is important to optimise the protein extraction buffer prior to using this method. Churchward *et al.*, showed the importance of optimising the protein extraction buffer detergent mix in a 2D electrophoresis study of membrane proteins. In this study, proteins were solubilised in different buffers containing varying compositions of a variety of non-ionic and zwitterionic detergents and detergent-like phospholipids and it was shown that certain buffers were incompatible with different extraction methods (242). For example, in brain tissue, the most effective solvent for the extraction of proteins was 2% Diethylamine whilst 2% CHAPS was found to be the least effective (240, 242). In the studies presented in this chapter, CHAPS was found to be quite effective in extracting proteins from the human placenta using the column based methods. However, other buffers may optimally extract the protein of interest depending on the tissue.

The studies presented in this chapter also aimed to determine whether the DNA/RNA kit (Qiagen) was capable of a simultaneous extraction of nucleic acids and proteins. Using this kit, all flow through wash steps were pooled along with the initial pellet from the buffer QRV1 centrifugation step. Following an overnight incubation, the precipitated protein was centrifuged and the same protocol as the RNeasy columns was followed. Using proteins extracted with the DNA/RNA column and resuspended in 2D buffer, a highly comparable protein profile was observed between both the conventional and RNeasy extraction methods using 2D buffer at the 1D and 2D level (Figures 2 and 4). Although some differences were observed at the 2D level the most important point was

that all spots were present but at different intensities. Proteins extracted using these techniques were also capable of being analysed by Western Blotting (Figure 3).

In the case of the RNeasy column, no additional reagents are required to achieve protein precipitation. However, the DNA/RNA column flow through requires the addition of an equal volume of ethanol prior to precipitation due to the presence of low levels of ethanol in the extraction buffers. The subsequent protein pellet was washed three times in acetone following the Morse method before air drying the pellet and resuspending in our buffers. It is strongly recommend not using Trichloroacetic acid to precipitate the proteins due to the formation of highly reactive compounds when combining guanidine salts and acidic solutions (see <http://www1.qiagen.com/literature/protocols>).

There are other techniques which utilize column based technology to simultaneously extract nucleic acids and proteins, however these techniques generally have disadvantages. Commercially, kits such as Sigma's Tri Reagent and Ambion's PARIS kit allow for the dual isolation of total RNA and protein, however these methodologies are not as simple as those described by Morse *et. al.* (236). The Sigma Tri Reagent uses hazardous chemicals and requires 3 separate procedures to obtain DNA, RNA and protein while the PARIS kit requires the sample to be separated, therefore decreasing the maximal yield of nucleic acids and proteins.

Following the publication of the protocol detailed in this chapter, Qiagen released a new series of combined RNA and protein and DNA, RNA and Protein extraction kits under the commercial name of ALLPREP. These kits are now available for ready use but come at a significantly increased price compared to the conventional RNeasy extraction kit or the DNA/RNA kit. Despite the information published in this paper, Qiagen provides an SDS-based buffer for protein solubilisation. This buffer is claimed to provide adequate solubilisation for use in protein techniques such as western blotting. Despite these claims, it would still be highly recommended for the user to strongly consider the protein extraction buffer prior to using these combined DNA/RNA/Protein extraction methods.



One added bonus of using simultaneous extraction techniques is that contaminating DNA and RNA are removed from the proteins. This is particularly relevant in 2D analysis where contaminating nucleic acids appear as streaks on the gels. The removal of these nucleic acids increases the clarity of the 2D gels and improves protein yield and spot resolution (243).

Overall the modification of methods presented here provides a simple, safe and effective way to simultaneously extract nucleic acids and proteins from the same sample. The technique described here was then used in the following chapter: A new mechanism for CRH induced immunosuppression during pregnancy in which combined RNA and protein extraction using RNeasy columns was used to analyse the affect of CRH on BeWo cells. While there was no limitation in the availability of sample for this study, the technique was applied due to the reliability and simplicity of the protocol. In addition, this technique was used to minimise the use of reagents such as CRH and allow for direct comparisons between RNA, total cell protein and exosomal protein from the same BeWo cells.

### **Chapter 3 - A new mechanism for CRH induced immunosuppression during pregnancy**

### **3.1 Abstract**

During pregnancy the mother does not reject the fetal allograft, however the mechanisms through which this tolerance is achieved remain uncertain. Separate publications have indicated that placental exosomes, CRH, syncytin-1, syncytin-2 and FasL may act as immunomodulators during pregnancy. CRH, syncytin-1, syncytin-2 and FasL all interact with the cAMP second messenger pathway. Exosomes exhibit FasL and an unknown 24kDa membrane protein that we have proposed to be syncytin-1. We therefore hypothesised that CRH upregulates the production of the immunosuppressive factors FasL, syncytin-1 and syncytin-2 in the placenta and stimulates the sorting of syncytin-1 into placental exosomes. Following CRH (10-200nM) treatment of the BeWo human placental cell line for 24h, RT-PCR analysis identified a significant upregulation of syncytin-1 (50nM,  $p<0.01$ ), syncytin-2 (50nM,  $p<0.01$ ) and FasL (50nM,  $p<0.01$ ) mRNA in a dose dependent fashion. CRH also increased exosomal protein production. Syncytin-1 precursor protein in BeWo exosomes was increased in a dose dependent pattern with a maximum increase of 3.2 fold following treatment with 50nM CRH. These data indicate that CRH stimulates placental production of immunosuppressive factors which likely contribute to the modulation of the maternal immune system during pregnancy.

### **3.2 Introduction**

During pregnancy the growing fetus is a semi-allograft, expressing antigens derived from the maternal and paternal genomes (5, 16). The maternal immune system tolerates the presence of these paternal antigens (5). However, while the maternal immune system is tolerant of fetal tissues, it still retains the ability to respond to other foreign antigens (5).

The mechanisms that lead to the survival of the fetal allograft remain unclear. Common hypothesised sources of immune privilege during pregnancy include: regulation of complement activation against the placenta (26), Leukaemia Inhibitory Factor production (244), Programmed Death Ligand synthesis (42), absence of MHC class II molecules (245), and increased tryptophan catabolism (68). The expression of immunosuppressive retroviral envelope proteins in the placenta and the secretion of immunosuppressive exosomes are additional potential modifiers of the immune environment of pregnancy (191, 211).

Exosomes are small membrane vesicles, classically defined as having a diameter of 30-100nm (216), though larger exosomes have also been described (215, 217-219). These vesicles are released from cells upon fusion of internal multivesicular bodies with the plasma membrane (215). The biological function of exosomes is unclear. They were discovered over 25 years ago and thought to be involved in the removal of cell surface molecules in reticulocytes (220). Since then, exosome production has been demonstrated from a number of different cells, including: dendritic cells (221), B cells (222), T cells (223), mast cells (224), epithelial cells (225), and tumour cells (226). Within all exosomes is a common subset of proteins which are essential for their biogenesis, structure and cell trafficking. Exosomes also have cell-specific components which can determine function (227); for example, exosomes derived from dendritic cells, tumour

cells and placental cells have been shown to modulate immune responses (reviewed in (227)).

Taylor *et al.*, 2006 demonstrated that exosomes produced by placental cells are immunosuppressive (191). Placental exosomes incubated with a T cell line, Jurkat cells, modulated the JAK-3 immune signalling pathway by suppressing the proteins CD3- $\zeta$  and Janus Kinase 3 (JAK-3) while amplifying suppressors of cytokine signalling 2 (SOCS-2) (191, 230). Using a continuously eluting electrophoresis system, the immunosuppressive components of placental exosomes were found to be FasL and an unidentified 24kDa protein (191). Our group hypothesised that the unknown immunosuppressive 24kDa protein observed by Taylor is an endogenous retroviral envelope protein (246) and have recently demonstrated the presence of the 24kDa transmembrane subunit of the ERV envelope protein syncytin-1 in human placental exosomes (190).

Retroviruses are a class of enveloped RNA viruses (146). ERVs are the remnants of ancient germ-line infections by exogenous retroviruses which have been incorporated into the genome of the host (247). As a result of the endogenisation process, approximately 8% of the human genome consists of HERV sequences (146). ERV sequences are mostly transcriptionally inactive, however, certain integrants still retain expression and replication capabilities (157).

The Trojan exosome hypothesis, first proposed by Stephen J Gould *et al.*, 2003, suggests that retroviruses may use the exosomal pathway for both biogenesis of retroviral particles and for transmission of infection across cells (215). This pathway may be used by endogenous or exogenous retroviruses. While exogenous retroviruses are commonly associated with disease, Sverdlov hypothesised that ERVs may play an important role in the evolution and divergence of humans (157). Preserved ERV elements have also been proposed to play an important role in physiology. In particular HERV envelope proteins could play an important role in several processes, including;

protection against retroviral infection through receptor interference, cell-cell fusion and immunosuppression (108).

In the human genome, there are 18 coding HERV *env* sequences (108, 164). Of these 18 *env* genes, at least 10 have been reported to be expressed in the placenta and only three have been found to cause cell-cell fusion *in vitro* (syncytin-1, syncytin-2 and *envP(b)*). In particular, syncytin-1 and syncytin-2 are thought to be mediators of cell-cell fusion of the placental cytotrophoblasts to form the syncytiotrophoblast (107, 108). The expression of these ERV envelope proteins in the placenta may also have a role in generating maternal immune tolerance of fetal tissues.

Retroviruses are thought to modify immune function through the presence of a highly conserved ISD within the transmembrane subunit of the envelope protein (248). Syncytin-1 (107) and syncytin-2 (108) are expressed at high levels in the placenta and each possesses this putative immunosuppressive region. Villarreal therefore proposed the metavirus hypothesis in which retroviruses made by the embryo may specifically and transiently repress the local development of maternal immune recognition of the developing embryo (211).

Syncytin-1 and syncytin-2 expression can be stimulated by increased cAMP formation (107, 249). CRH also stimulates cAMP synthesis via the type 1 CRH receptor (250). CRH is a 41-amino acid neuropeptide which is expressed at high levels by the human placenta and is proposed to elicit maternal tolerance to the embryo through its ability to stimulate FasL. The expression of FasL can induce apoptosis of surrounding maternal T lymphocytes expressing the Fas membrane protein. This effect appears to be specifically mediated by the CRH type 1 receptor (105).

Based on these previous studies, the hypothesis of this study is that CRH induces the expression of syncytin-1, syncytin-2 and FasL which have an immunosuppressive role during pregnancy. Furthermore, it is hypothesised that CRH induces the sorting of

syncytin-1 into placental exosomes. These hypotheses were tested using a human placental cell line.

### **3.3 Materials and Methods**

#### **3.3.1 Cell culture**

BeWo cells (a human placental cell line) were maintained at 37°C in HAM-F12 medium (Sigma-Aldrich) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were subcultured and grown for 1 day to the stage of 50% confluence. The medium was then changed to one containing CRH (10, 25, 50, 100 or 200nM) or vehicle and incubated for 24 hours. At the end of the incubation, media was taken for exosome extraction and cells were taken for concurrent RNA and protein extraction.

#### **3.3.2 RNA and protein extraction**

RNA and protein were extracted from BeWo cells using the RNeasy kit (Qiagen) following the methods stated in Tolosa *et al.*, 2007 (1). Briefly, BeWo cells were homogenised in buffer RLT and run through the RNeasy column following the manufacturer's instructions ([http://www1.qiagen.com/HB/RNeasyMinEluteCleanupKit\\_EN](http://www1.qiagen.com/HB/RNeasyMinEluteCleanupKit_EN)). All flow-through from the RNeasy column was collected, pooled and stored at -20°C overnight to precipitate protein. Protein eluates were centrifuged at 13,000 x g and washed in acetone to remove contaminating salts. This step was repeated 3 times prior to suspension of proteins in 2D buffer.

Following extraction, all nucleic acids were quantified using a Nano-drop® ND-1000 spectrophotometer (Biolab).



#### 3.3.2.1 RNA sample purification

Contaminating DNA was removed by RNase free DNase treatment (Qiagen). Up to 30µg of RNA was treated with 13.6 Kunitz Units of RNase free DNase for 25 minutes at 25°C. RNA was then cleaned following the RNA clean up procedure detailed by Qiagen for the RNeasy kit ([http://www1.qiagen.com/KB/RNeasyMinEluteCleanupKit\\_EN](http://www1.qiagen.com/KB/RNeasyMinEluteCleanupKit_EN)). RNA purity and quality were confirmed using agarose gel electrophoresis.

#### 3.3.2.2 Reverse Transcription

A total of 2µg of RNA was reverse transcribed using the Superscript III kit (Invitrogen) following the manufacturer's instructions ([http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand\\_pps.pdf](http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand_pps.pdf)) using Oligo d(T) primers. Complementary DNA (cDNA) was diluted to an equivalent of 20ng/µl starting RNA with MilliQ water.

#### 3.3.2.3 Real-Time PCR

RT-PCR was used to quantify the mRNA levels of syncytin-1, syncytin-2, FasL and 18s rRNA in BeWo cells following CRH treatment. mRNA levels of these genes were measured using the SYBR green method. Reactions contained 50ng of cDNA, 200nM primers and 1x SYBR green master mix (Applied Biosystems) to a final volume of 25µl. The protocol for amplification was 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The machine used was the ABI PRISM 7700 (Applied Biosystems). Amplicons were sequenced by the Australian Genome Research Facilities (St Lucia, Australia) to confirm specific gene amplification.

**Table 3.1. Primer Sequences for RT-PCR**

Gene	Forward	Reverse	GenBank
<b>Syncytin-1</b>	GAAGGCCCTTCATAACCAATGA	GATATTTGGCTAAGGAGGTGATGTC	NM_014590
<b>Syncytin-2</b>	CCAGAGAATGGACAAGCATAGAGG	GGCTATTCCCATTAGGTTG	NM_207582
<b>Fas Ligand</b>	GCAGCCCTTCAATTACCCAT	CAGAGGTTGGACAGGGAAGAA	NM_000639
<b>18s rRNA</b>	CGAGCCGCCTGGATACC	CCTCAGTTCCGAAAACCAACAA	NR_003286

#### 3.3.4 Protein determination – 2D quant kit

Proteins extracted in 2D buffer were quantified using the 2D Quant Kit (GE Healthcare) following the manufacturer's instructions

([http://www5.gelifesciences.com/APTRIX/upp00919.nsf/content/06BAEBA0622B6A43C125748800812E19?OpenDocument&Path=Catalog&Hometitle=Catalog&entry=5&newrel&LinkParent=C1256FC4003AED40-9FF83ACAC523805DC1257019004908D6\\_RelatedLinksNew-C821BEC677D8448BC1256EAE002E3030&newrel&hidesearchbox=yes&moduleid=undefined](http://www5.gelifesciences.com/APTRIX/upp00919.nsf/content/06BAEBA0622B6A43C125748800812E19?OpenDocument&Path=Catalog&Hometitle=Catalog&entry=5&newrel&LinkParent=C1256FC4003AED40-9FF83ACAC523805DC1257019004908D6_RelatedLinksNew-C821BEC677D8448BC1256EAE002E3030&newrel&hidesearchbox=yes&moduleid=undefined)).

#### 3.3.5 Exosome extraction

Exosomes were extracted from the BeWo cell culture medium following 24 hours of CRH treatment. All centrifugation steps in the extraction of exosomes were performed at 4°C.

Exosomes were extracted using differential centrifugation and sucrose cushion methods. Briefly, media was spun twice at 2,000 x g for 10 minutes to pellet cellular debris. This was followed by 1 spin at 10,000 x g for 1 hour to remove insoluble proteins and large vesicles. The clarified exosomal supernatant was then filtered using a 0.22µm filter and stored at 4°C following the addition of complete protease inhibitor cocktail tablets (Roche). Exosomes were separated from contaminants by layering the fraction of interest on a 30% sucrose cushion. This fraction was spun at 100,000 x g for 1 hour and 10 minutes. The sucrose cushion, which contained the exosomal fraction was taken and diluted in phosphate buffered saline (PBS) containing protease inhibitor cocktail tablets and washed at 100,000 x g for 2 hours. The resulting pellet was resuspended in 50µl of NuPAGE LDS sample buffer (1x) (Invitrogen) for protein analysis or electron microscopy buffer for electron microscopy analysis.

### 3.3.6 SDS-PAGE

BeWo cell and BeWo exosomal proteins were resolved using NuPAGE® Novex 10% Bis-Tris gels in 1 x MOPS running buffer (Invitrogen) at 200V for 55 minutes. Gels for silver staining were fixed in silver stain fixative overnight. Gels for western blotting were transferred to nitrocellulose membranes for later use.

#### 3.3.6.1 Silver stain

Proteins resolved by gel electrophoresis were silver stained using SilverQuest™ (Invitrogen) to visualize the protein profile at the 1 dimensional level following the manufacturer's instructions

([http://www.invitrogen.com/content/sfs/manuals/silverquest\\_man.pdf](http://www.invitrogen.com/content/sfs/manuals/silverquest_man.pdf)).

#### 3.3.6.2 Western Blotting

Proteins resolved by gel electrophoresis were transferred to nitrocellulose membranes at 25V, 160mA, 17 Watts using NUPAGE® transfer buffer. Membranes were blocked by rocking in skim milk (5% skim milk diluted in TBST) for 1 hour at RT. Membranes were immunoblotted with antibodies targeting either: syncytin-1 (1:1000 – generated in rabbits using synthetic syncytin-1 ISD peptide), syncytin-2 (1:1000 – obtained from Academia Sinica, Taiwan), FasL (1:1000 – Cell Signalling Technology) or actin ab8227 (1:2000 – Sapphire), in skim milk rocking overnight at 4°C. Unbound antibody was washed in TBST prior to incubation with a HRP conjugated secondary antibody (syncytin-1 and FasL: Goat-anti-rabbit (Millipore), 1:2000; syncytin-2: Rabbit-anti-Guinea Pig (Invitrogen), 1:2000; actin: Rabbit-anti-mouse (Abcam), 1:2000) in skim milk (5%) for 1 hour rocking at RT. Following a final wash in TBST, syncytin-1, syncytin-2 or FasL was detected on the membranes using the ECL reagent (GE Healthcare) and images of the developed membranes were taken using the LAS-3000

(Fujifilm). To determine differences between treatment groups, a densitometric scan was undertaken using Multi Gauge software (version 2.3, Fujifilm).

### 3.3.7 Electron microscopy

Exosomes purified by differential centrifugation and sucrose cushion methods for use in transmission electron microscopy were fixed in 2% paraformaldehyde, 1% glutaraldehyde in PBS. Fixed exosomes were loaded onto formvar coated slot grids and allowed to air dry. The grids were then stained in 2% uranyl acetate solution in distilled water (dH<sub>2</sub>O) for 2 mins and then washed in dH<sub>2</sub>O and allowed to air dry. Exosomes were visualised using a JEOL 1010 electron microscope 100 kV at the University of Queensland electron microscope facility. Digital images were imported into adobe photoshop for minor adjustments to brightness and contrast.

### 3.3.8 Statistics

All experiments detailed in this study were performed a minimum of 3 times. Stata 9.2 software (StataCorp, College Station, Texas) was used for statistical analysis. Multiple linear regression was used to model the relative mRNA expression for syncytin-1, syncytin-2 and FasL against the CRH concentration (as a categorical variable, using no treatment as the reference) while adjusting for experiment. A two-tailed significance level of 5% was used throughout. The assumptions of linear regression were checked. Results are reported with coefficients, 95% confidence intervals (CI) and p values. Graphics show means and standard error bars.

Wilcoxon rank-sum tests (non-parametric tests of group medians) were used to test differences between no treatment and treatment groups and also no treatment and vehicle groups in a CRH treated protein expression analysis of total cellular protein (syncytin-1, syncytin-2 and FasL) and exosomal protein (syncytin-1) following Western

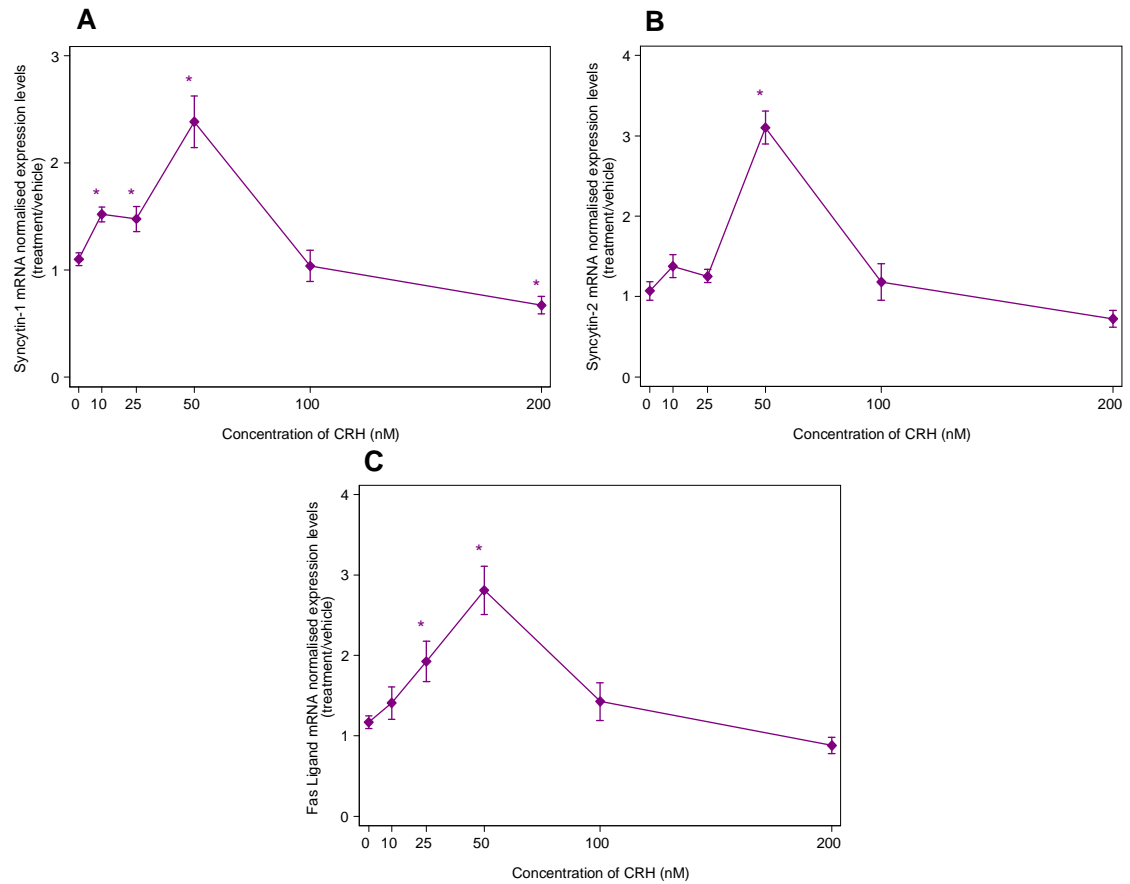
Blotting. A two-tailed significance level of 5% was used throughout.

### **3.4 Results**

#### **3.4.1 Syncytin-1, Syncytin-2 and FasL Expression Profile Following CRH Treatment**

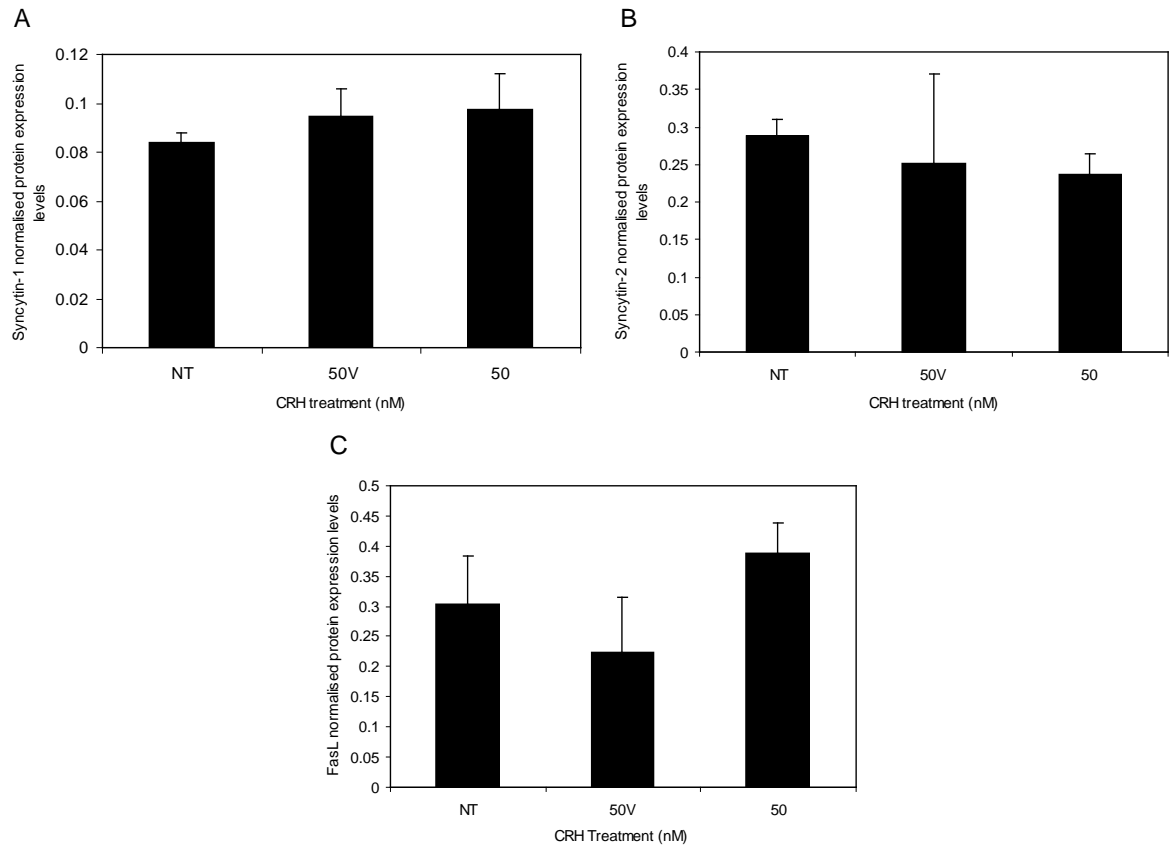
mRNA expression levels in BeWo cells of syncytin-1, syncytin-2 and FasL were measured following CRH treatment (10-200nM) for 24 hours (Figure 3.1). The relative amount of mRNA for all genes significantly increased with a maximum response at 50nM CRH treatment (syncytin-1,  $p<0.01$ , syncytin-2,  $p<0.01$  and FasL,  $p<0.01$ ). Syncytin-1 mRNA expression at this concentration increased 2.38 fold (Figure 3.1a) while syncytin-2 was increased by 3.1 fold (Figure 1b) and FasL was increased 2.81 fold (Figure 3.1c). No response was observed using higher concentrations of CRH (100nM and 200nM).

Protein expression of syncytin-1, syncytin-2 and FasL was not significantly increased in the BeWo cells following 50nM CRH treatment (Figure 3.2).



**Figure 3.1. RT-PCR analysis of syncytin-1 (A), syncytin-2 (B) and FasL (C) in BeWo cells treated with different concentrations of CRH.** *18srRNA* was used as an internal control and the values were calculated using the delta *C(t)* method. Expression levels were normalised by dividing treated sample readings by the equivalent vehicle. \* indicates  $p < 0.05$  when compared to the no treatment control (0). *P* values; syncytin-1; 10( $p=0.028$ ), 25( $p=0.048$ ), 50( $p<0.001$ ), 200( $p0.024$ ); syncytin-2; 50( $p<0.001$ ); FasL; 25( $p=0.013$ ), 50( $p<0.001$ ).  $n = 3$ .



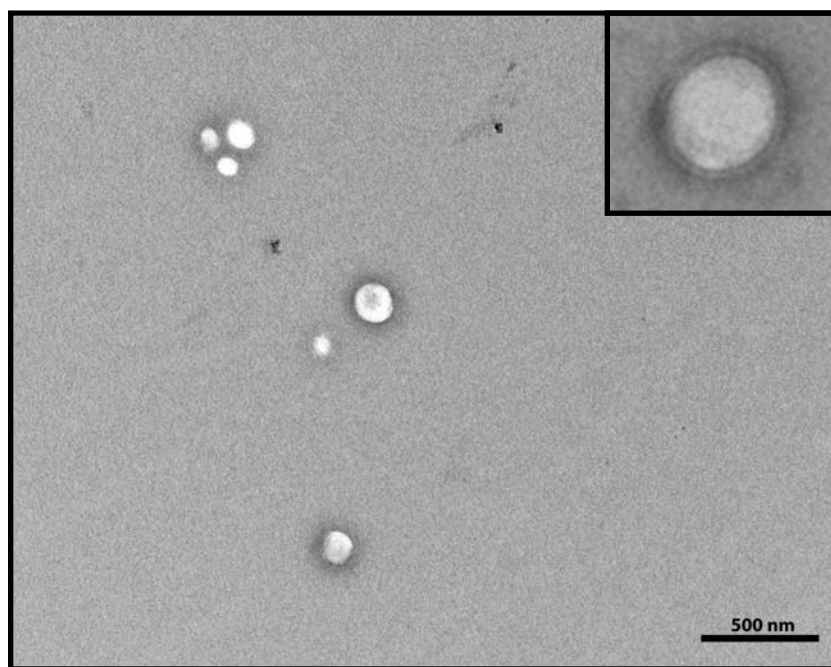


**Figure 3.2. Densitometric analysis of syncytin-1 (A), syncytin-2 (B) and FasL (C) protein expression determined by western blot in BeWo cells treated with 50nM CRH.** *Actin was used as the internal standard and the values were calculated using Fujifilm densitometry software. Expression levels were normalised by dividing specific protein values (syncytin-1, syncytin-2 or FasL) by actin and graphed. No significant differences were detected between any of the treatment groups of syncytin-1 (A), syncytin-2 (B) and FasL (C). Molecular weight standard; Magic mark (Invitrogen), NT; No treatment. 50V; 50nm CRH vehicle. 50; 50nm CRH.*

### 3.4.2 Exosomes increase in BeWo cell media following CRH treatment

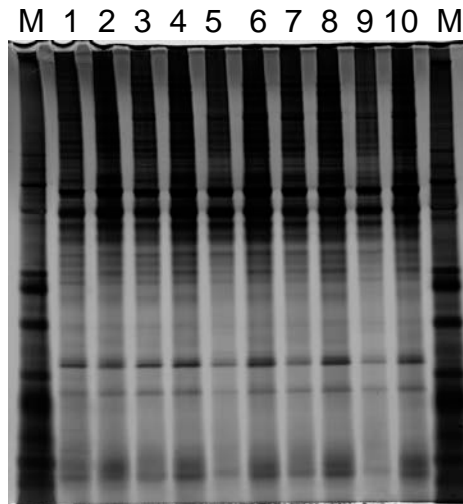
To examine the level of total exosomal protein secreted from BeWo cells following CRH treatment, SDS-PAGE silver stain, western blotting and electron microscopy analysis were performed on treated exosomal fractions. The presence of exosomes was initially confirmed by transmission electron microscopy (Figure 3.3).

Following stimulation with CRH, the total protein in the exosomal fraction was observed to increase (Figure 3.4a). To examine the level of syncytin-1 in the exosomal fraction, Western Blotting was performed. Syncytin-1 precursor protein was increased in a dose dependent pattern (Figure 3.4b) with a significant increase of 3.2 fold ( $p < 0.05$ ) following treatment with 50nM CRH.

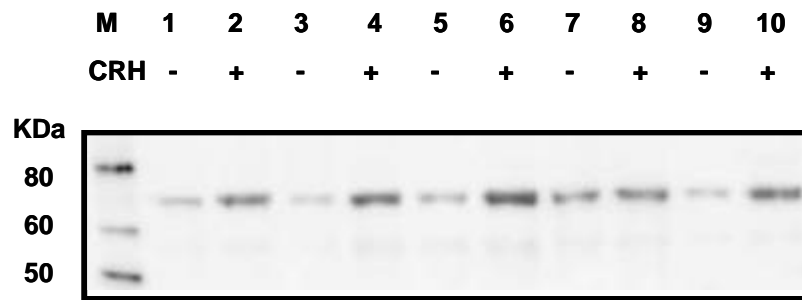


**Figure 3.3. Transmission electron micrographs of exosomes collected from BeWo cells using differential centrifugation followed by sucrose cushion.** *Exosomes are membrane nanovesicles approximately 100nm in diameter with a density ranged from 1.120 to 1.160 g/cm<sup>3</sup>. Inset – close up image of BeWo exosome.*

A



B



**Figure 3.4 Representative silver stain and syncytin-1 western blot of exosomal proteins extracted from CRH treated BeWo cells.** A) Representative silver stain of CRH treated exosomal protein. B) Representative western blot of syncytin-1 protein expression in exosomes produced by BeWo cells after CRH stimulation. In these experiments, an equal number of BeWo cells in an equal volume of culture media were treated by CRH. At the end of the treatment, the same volume of supernatant was taken from each well and exosomes were extracted using differential centrifugation and sucrose cushion methods in parallel. Observe the dose dependent increase in syncytin-1 protein in exosomes, reaching a significant peak at 50nM CRH treatment (lane 6). Results are representative of 3 experiments. M - Molecular Weight standard; Mark12 (Invitrogen); 1 - Vehicle for 10nM CRH; 2 - 10nM CRH; 3 - Vehicle for 25nM CRH; 4 - 25nM CRH; 5 - Vehicle for 50nM CRH; 6 - 50nM CRH; 7 - Vehicle for 100nM CRH; 8 - 100nM CRH; 9 - Vehicle for 200nM CRH; 10 - 200nM CRH.

### **3.5 Discussion**

This study has demonstrated for the first time that CRH stimulates gene transcription of syncytin-1, syncytin-2 and FasL in a placental cell line. CRH also stimulated the production of exosomes which contain immunosuppressive factors including syncytin-1. These data suggest that CRH upregulates the production of the immunosuppressive factors FasL, syncytin-1 and syncytin-2 in the placenta and stimulates the exosomal fraction through either promoting the sorting of syncytin-1 into placental exosomes or promoting the production of placental exosomes containing syncytin-1.

CRH is associated with early fetal immune tolerance through its modulation of FasL expression. The first evidence for such effects of CRH was presented by Makrigiannakas *et al.*, who demonstrated using EVT cells and the JEG3 cell line that CRH can promote implantation and early pregnancy by increasing FasL expression which led to the killing of activated T cells (105). This effect was mediated by CRH-R1, as the presence of antalarmin, a type 1 receptor antagonist, reduced T-cell apoptosis to control levels (105). In our study, CRH stimulated FasL transcription in a placentally derived BeWo cell line (Figure 3.1 c) but did not influence the total FasL protein concentrations.

While data has shown an immunosuppressive effect mediated by FasL on EVT cells, it should be noted that mice with a missense or inactivating mutations of the FasL gene (*gld*) can reproduce, which suggests that trophoblast FasL expression is not obligatory for maternal immune tolerance in mice. Therefore other mechanisms are also involved in mediating immuntolerance during pregnancy in mice which may include the production of endogenous retroviral envelope proteins (105).

Retroviral envelope proteins in the human placenta such as syncytin-1 and 2 are commonly associated with cell-cell fusion but they also have a potential

immunosuppressive role (157). These proteins have been shown to be upregulated following cAMP treatment and this is generally correlated with cell fusion events (107, 108, 249). Mi *et al.*, demonstrated that BeWo cell syncytin-1 transcription increased 5-fold following forskolin (cAMP) stimulation. This stimulation correlated with increasing BeWo cell fusion (107). Chen *et. al.*, demonstrated that BeWo cell syncytin-2 transcription significantly increased in a time dependent manner following forskolin (cAMP) stimulation (249) and syncytin-2 has also recently been hypothesised to play a major role in trophoblast cell fusion (177). In our study, CRH stimulation (up to a concentration of 50nM) of BeWo cells led to increased messenger RNA levels of syncytin-1 and syncytin-2 (Figure 3.1 a and b) but was not associated with a change in protein concentrations (Figure 3.2 a and b). In support of these findings, Chen *et al.*, (251) showed that CRH can promote syncytialisation in the BeWo cell line.

In BeWo cells, the CRH concentration has a threshold of maximal stimulation. Higher concentrations than 50nM inhibited the increase in messenger levels and could potentially be explained by CRH receptor saturation and subsequent receptor internalisation. Receptor internalisation following treatment with high concentrations of CRH (10nM or greater) has previously been reported in myometrial tissue (252). As the levels of CRH progressively increase during pregnancy, it is likely that the internalisation of CRH receptor may provide an important mechanism for regulating CRH responsiveness and the induction of immunosuppressive proteins such as syncytin-1, syncytin-2 and FasL.

Expression of syncytin-1 and 2 is regulated by the GCMA transcription factor (186, 187). One of the mechanisms postulated to drive expression of syncytin-1 and 2 in the placenta is through the regulation of GCMA by a cAMP mediated PKA signalling pathway (187). Knerr *et al.*, (187) showed that the PKA signalling pathway acts upstream of GCMA. Following transient transfection of BeWo cells with PKA an upregulation of syncytin-1 transcripts was observed (187). CRH has also been shown to activate the cAMP/PKA pathway (189). Stimulation of GCMA transcriptional activity

through the cAMP/PKA pathway has been shown to be attributable to CBP-mediated acetylation of GCMA which results in transcriptional activation (186).

While FasL is an established immunosuppressive molecule, there is more limited evidence that syncytin-1 and syncytin-2 have immunosuppressive properties. Mangeney *et al.*, 2007 (213) examined the immunosuppressive properties of syncytin-1 and 2 using an mouse allogenic tumour cell model. Interestingly, Mangeney *et al.*, 2007 showed that only syncytin-2 had immunosuppressive properties.

The question that arises in relation to our data is whether the stimulation of syncytin-1 by CRH is a mechanism for modulating the immune system? The success of a pregnancy has been correlated with a shift from Th1 to Th2 type responses. Th1 cytokines which are deleterious for pregnancy such as TNF- $\alpha$  and IFN- $\gamma$  are down regulated whereas Th2 cytokines such as IL-10 which are beneficial for pregnancy are produced at higher levels (20, 90, 253). The highly conserved consensus immunosuppressive peptide (CKS-17) of retroviruses has previously been shown to downregulate Th1 responses and induces the production of Th2 cytokines (254). Sequence analysis of the highly conserved consensus immunosuppressive peptide (CKS-17) to human syncytins shows a high level of homology with the CKS-17 suggesting that syncytin-1 may be immunosuppressive (213). Tolosa recently presented data showing that the synthetic syncytin-1 ISD peptide can inhibit the production of the Th1 cytokines TNF- $\alpha$  (190). These data are shown in figure 1.11 of chapter 1.

Knowing that CRH stimulates syncytin-1, syncytin-2 and FasL and that these factors may be important in maternal immune tolerance, we considered potential mechanisms that these factors may use to interact with the maternal immune system. Previous studies had shown that FasL can be expressed in cytoplasmic granules (exosomes) and the secretion of these exosomes containing FasL may be a mechanism the placenta uses to promote a state of immune privilege (229). Taylor *et al.*, had also demonstrated that the immunosuppressive components of these exosomes were FasL and an unidentified 24kDa protein. We have previously hypothesised that this unidentified protein is the

envelope protein of a HERV expressed in the placenta, syncytin-1 (246) and have recently shown that syncytin-1 is carried by human placental exosomes (190).

Exosomes from other models have been shown to have immunomodulatory properties and have been studied in much greater detail (227). Peche *et al.*, 2003 (255) elegantly demonstrated a prolongation of allograft survival using donor-specific dendritic-cell exosomes. In this study, exosomes from donor bone marrow dendritic cells were administered prior to transplantation to modulate heart allograft rejection. Following transplantation, allografts from exosome treated rats displayed a significant decrease in graft infiltrating leukocytes and expression of IFN- $\gamma$  compared to untreated animals. A decrease in anti-donor T cell responses was also observed. This effect was donor specific because syngeneic exosomes had no effect.

Since CRH stimulated placental immunosuppressive factors and since exosomes could have an important immunosuppressive role during pregnancy, we examined the production of exosomes following treatment of the placentally derived BeWo cells with CRH. Exosomes treated with CRH exhibited an increase in their protein content (Figure 4). Specific analysis of syncytin-1 precursor protein in BeWo exosomes revealed a dose dependent increase following treatment with CRH (up to 3.2 fold increase with 50nM CRH). CRH regulation of the exosomal population could be important in the maintenance of a tolerogenic state throughout pregnancy.

Although the BeWo cell model has been extensively used as a model for investigating functions of the trophoblast including the role of syncytin-1 (for examples see (6-11)), it does have its limitations. In particular, it is important to note that this model was generated from a choriocarcinoma (12). While many properties of the trophoblast have been compared with those of cancer cells (12), care must be taken when drawing conclusions from these data. Previous studies of syncytin-1 function in BeWo cells have been validated using primary cytotrophoblast cells (for examples see (6, 11)) suggesting that syncytin-1 biology in BeWo cells may closely resemble that observed in vivo.

Future experiments that could be undertaken to further validate the role of CRH in the stimulation of syncytin-1 and -2 and the sorting of syncytin-1 into exosomes include ex vivo studies using placental primary explants or primary cytotrophoblast cultures. Further, the western blotting data of total cell protein could be confirmed using immunohistochemistry to show specific localisation of syncytin-1, syncytin-2 and FasL following treatment with CRH.

This study demonstrates for the first time that CRH initiates gene transcription of syncytin-1, syncytin-2 and FasL in BeWo cells. Both syncytin-2 and FasL have documented immunosuppressive functions and we have recently shown that syncytin-1 may also modulate the immune system. Surprisingly, Western Blot analysis of the total cell proteins revealed no increase in syncytin-1, -2 or FasL at any time points. In contrast, proteins from the exosomal fraction increased following CRH treatment. These data suggest that CRH upregulates the production of the immunosuppressive factors FasL, syncytin-1 and syncytin-2 in the placenta and stimulates the exosomal fraction through either promoting the sorting of syncytin-1 into placental exosomes or promoting the production of placental exosomes containing syncytin-1.



## **Chapter 4 - The Generation and Characterisation of Recombinant Syncytin Ectodomain Proteins**

#### **4.1 Abstract**

In this chapter, a methodological approach for the generation of the recombinant ectodomains of syncytin is described. Using restriction enzyme cloning methods, the recombinant ectodomains of syncytin-1 and syncytin-2 from humans, and syncytin-A and syncytin-B from mice were cloned into the pET-28b bacterial expression plasmid. These proteins were then overexpressed using the *lac* operon system and purified using a combination of immobilised metal affinity chromatography and gel filtration chromatography. The resulting proteins were oligomeric. The expression of syncytin recombinant proteins was confirmed by SDS-PAGE, western blotting and liquid chromatography mass spectrometry (LCMS). The use of affinity chromatography followed by gel filtration provides an effective way to produce and purify bioactive, oligomeric, syncytin recombinant ectodomains.

## **4.2 Introduction**

It has been known for more than 30 years that ERVs are expressed within the placenta of humans and other mammals, yet the significance of this observation is still not fully understood and is the subject of extensive speculation (106, 158).

In particular, the envelope proteins of ERVs are expressed in the placenta at high levels and it has been speculated that their presence may be important in placentation (106, 107, 211, 260-266). Various roles have been speculated for these proteins: protection of the fetus against exogenous retroviral infections through receptor interference (260, 261), cell-cell fusion of the cytotrophoblast to form the syncytiotrophoblast (262, 263), and protection of the fetus from the maternal immune response through local immunosuppression (106, 107, 211, 264-266).

It is well known that retroviral proteins can exert immunosuppressive effects during an infection (192-194, 209, 267) however it is not known if ERV expressed in the placenta have an immunosuppressive effect on the maternal system during pregnancy. It is known, however, that the source of many retroviral mediated immunosuppressive effects is a highly conserved sequence within the envelope protein of retroviruses named the ISD (209, 210, 248, 267-272). Furthermore, a sequence with high homology to the consensus sequence of the retroviral ISD is present in syncytin-1 and syncytin-2; two ERV envelope proteins expressed in the human placenta. These proteins have an established role in cytotrophoblast cell fusion (107, 108), and have also been hypothesised to play a role in maternal immune tolerance due to the presence of this domain. In addition, it has been hypothesised that ERV envelope proteins that are expressed in the placentas of other mammals (i.e. syncytin-A and syncytin-B in the mouse) may be immunosuppressive (109). However, the role of these proteins *in vivo* in maternal immune tolerance remains speculative.

Mangeney *et al.*, (213) performed the only published study examining the immunosuppressive role of placental syncytins. Using a previously described tumour cell model to study cell-mediated immunity and recombinant proteins to study humoral immunity (209, 210, 267), human syncytin-2 and mouse syncytin-B were shown to have immunosuppressive properties whereas human syncytin-1 and mouse syncytin-B did not have immunosuppressive properties. However, the immunosuppressive functions described above were studied using an ectopic expression tumour cell model to study syncytin-1 immunosuppression. Tumour cell expression of syncytin-1 may not have the same physiological function as placental syncytin expression. Therefore the immunosuppressive properties of human and mouse syncytin proteins still require further characterisation. .

Mangeney *et al.*, described two potential ways to examine the immunosuppressive properties of these proteins. Firstly, the complete envelope protein can be stably transfected into a cell line and the cell line can then be transplanted into an allogeneic host animal. Following transplantation, the immunosuppressive index can be determined by cell survival or blood can be collected from the host and the cytokine profile be determined by ELISA. However, this model has its disadvantages as it is difficult to stably transfect syncytin proteins (S Drewlo personal communication) and ectopically transfected syncytin proteins are yet to be shown to be processed correctly or expressed at the level of the membrane (273). Another way to examine the immunosuppressive properties of these proteins is through the generation of soluble recombinant ectodomain proteins (see Mangeney *et al.*, (213)). The ectodomain of placental syncytins corresponds to a soluble part of the extracellular domain of the TM subunit (containing the ISD) and has boundaries close to those of previously crystallised and highly structured domains of retroviral envelope proteins (174, 213, 214). Recombinant ectodomains are large enough to adopt the physiological oligomeric conformation and, as such are an excellent model for examining immunological processes *in vitro* mediated by the ISD of retroviral envelope proteins (174, 214, 274).

This chapter describes how the recombinant ectodomain proteins of human and mouse syncytins were developed in order to address the hypothesis “That placental syncytin proteins may modulate the maternal immune system to promote immunological tolerance toward the developing fetus”. To achieve these aims, the ectodomain proteins were amplified from placental tissue by Polymerase Chain Reaction (PCR) and cloned into the pET 28-b plasmid. Cloned ectodomains were then overexpressed in BL21-CodonPlus competent cells and purified using a combination of affinity chromatography and gel filtration.

## **4.3 Methods**

### **4.3.1 cDNA Preparation**

#### **4.3.1.1 RNA Extraction**

RNA was extracted from term placental tissue from elective caesarean sections using RNeasy columns (Qiagen) following the manufacturer's instructions (<http://www1.qiagen.com/literature/render.aspx?id=352>). Extracted nucleic acids were quantified using the Nano-drop® ND-1000 spectrophotometer (Biolab).

#### **4.3.1.2 RNase-Free DNase Treatment**

To remove contaminating DNA's, RNA samples were treated with RNase free-DNase from the RNase free DNase kit (Qiagen) following the manufacturer's instructions (<http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000291>) with the following exceptions; RNase free DNase was used at a final concentration of 5%. Treated samples were left at 25°C for 25 minutes to remove contaminating DNA. Following treatment, RNA samples were cleaned up using the RNeasy mini kit RNA clean-up method (<http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000291>).

#### **4.3.1.3 RNA Analysis**

RNA purity was determined by analysis of the 260:280 absorbance ratios.

RNA integrity was checked by agarose gel electrophoresis. These procedures were carried out using 1% agarose gels with a final concentration of 50µg/ml ethidium

bromide in 1 x tris-acetate-EDTA (TAE) buffer. 500ng of RNA was electrophoresed in agarose gels and run at 80Volts for 40 minutes. Agarose gels were examined under UV light conditions using the Biodoc-It™ Imaging System (Pathtech).

All samples were required to have good quality RNA shown by clear 28S and 18S RNA bands and a 260:280nm ratio between 1.8 and 2.1.

#### 4.3.1.4 Reverse Transcription

RNA was reverse transcribed using Superscript III kit (Invitrogen) following the manufacturer's instructions ([http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand\\_pps.pdf](http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand_pps.pdf)). As a negative control, a reverse transcription reaction was prepared where reverse transcriptase was not added. A total of 2µg RNA was used. cDNA was diluted to an equivalent of 20ng/µl starting RNA with MilliQ water.

#### 4.3.2 Polymerase Chain Reaction (PCR) Amplification of Syncytin Recombinant Ectodomain Sequences

##### 4.3.2.1 Primer Design

PCR primers were based upon those used in Mangeney *et. al.*, 2007 (213) and designed to specifically target syncytin-1, syncytin-2, syncytin-A and syncytin-B soluble ectodomain sequences (Table 4.1). Sequence specificity of the primers was confirmed by Basic Local Alignment Search Tool (BLAST) analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primers were purchased as lyophilised nucleotides. Primers were reconstituted to a final concentration of 100µM in MilliQ-water before being diluted to a 10µM final stock before use in PCR reactions.

**Table 4.1. PCR and sequencing primers for syncytin ectodomains.** PCR primers are based upon those used in Mangeney *et. al.*, 2007 (213). T7 promoter primer was used as the sequencing primer Syncytin-1 and syncytin-2 primers are direct copies of Mangeney *et al.*, 2007 primers while syncytin-A and syncytin-B were altered slightly to include *NcoI* and *XhoI* restriction enzyme sites. **Bold** = Spacer, *Italics* = Enzyme cut site, Normal = Sequence.

Sequence	Accession Number	Forward	Reverse
<b>Syncytin-1 Ectodomain</b>	Designed from Mangeney <i>et. al.</i> , 2007	<b>ATACA</b> <i>tcatga</i> GTCAAGAACTAAATGGGGACATGGAACG	<b>TGTAT</b> <i>ctcgag</i> TTTCTCAGTGACGATTCCGGATTGA
<b>Syncytin-2 ectodomain</b>	Designed from Mangeney <i>et. al.</i> , 2007	<b>ATACA</b> <i>tcatga</i> CAAAGGAAATAGC	<b>TATGT</b> <i>ctcgag</i> GTTGTCTTGTACTT
<b>Syncytin-A Ectodomain</b>	Designed from Mangeney <i>et. al.</i> , 2007 using <i>NcoI</i> and <i>XhoI</i> RE sites	<b>GGATAT</b> <i>tcatga</i> AGGCTCTCTCGGACAGCCTAG	<b>GATATAT</b> <i>ctcgag</i> TGCCGCGTGCCGGACTAC
<b>Syncytin-B ectodomain</b>	Designed from Mangeney <i>et. al.</i> , 2007 using <i>NcoI</i> and <i>XhoI</i> RE sites	<b>GGATAT</b> <i>tcatga</i> AGGTTCTTTCCGAAACCTTGGA	<b>TATATA</b> <i>ctcgag</i> TGCCGCGTCCCGGACTA
<b>T7 promoter primer</b>	Recommended by Novagen for pET 28-b sequencing	TAATACGACTCACTATAGGG	



#### 4.3.2.2 PCR

PCRs were performed in 50µl reactions containing 50ng of placental cDNA. As controls, a cDNA negative control and water were used. To each reaction, 1x Pfx amplification buffer, 0.3mM dNTP mixture, 1mM MgSO<sub>4</sub>, 400nM primer pairs, 1 unit of Platinum Pfx polymerase and Milli Q water were added sequentially. The protocol for amplification was 94°C for 5 minutes and 25 cycles of 94°C for 30 seconds, x°C (x = 58°C for syncytin-1 and syncytin-2, 55°C for syncytin-A and 63.4°C for syncytin-B). PCR reactions were performed in a thermal cycler (ABI Prism Thermal Cycler).

#### 4.3.2.3 Agarose Gel Electrophoresis

PCR products were separated by size using agarose gel electrophoresis (see RNA analysis for preparation of agarose gel). The TrackIt™ 100 base pair (bp) DNA ladder (Invitrogen) was used as a marker. Gels were electrophoresed at 100V for 1 hour. Following electrophoresis, gels were visualised and photographed under UV light conditions using Biodoc-IT™ Imaging System.

#### 4.3.2.4 DNA Fragment Purification

DNA fragments were excised from agarose gels under UV light and were purified using the Wizard® SV gel and PCR clean up system following the manufacturer's instructions ([http://www.promega.com/applications/pcr/featuresandbenefits/Wizard\\_SV\\_Gel\\_PCR\\_Clean-Up\\_System.htm](http://www.promega.com/applications/pcr/featuresandbenefits/Wizard_SV_Gel_PCR_Clean-Up_System.htm)). Purified DNA was eluted in a final volume of 15µl MilliQ H<sub>2</sub>O.

### 4.3.3 Cloning of Syncytin Recombinant Ectodomains

#### 4.3.3.1 Restriction Enzyme Digestion

pET 28-b was digested with 2.5 Units of NcoI, 10 Units of XhoI, 1 x buffer 2 (New England Biolabs) and 1 x BSA for 4 hours at 37°C in a heated water bath. Following digestion, 0.5 Units of calf intestinal alkaline phosphatase (New England Biolabs) was added for 30 minutes at 37°C to prevent self-ligation. To stop the reaction, digested pET 28-b was heated at 65°C for 10 minutes. Digested plasmids were stored at -20°C until use.

Syncytin ectodomain sequences were digested with 1 Unit of BspHI, 1 Unit XhoI, 1x buffer 2 (New England Biolabs) and 1 x BSA for 16 hours at 37°C. Digested ectodomains were stored at -20°C until use.

Digested pET 28-b and syncytin ectodomain sequences were run on a 1% agarose gel (see RNA analysis for preparation of agarose gel – 4.3.1.3) in 1 x TAE buffer at 100V for 1 hour and 30 minutes. Digested samples were excised and purified using the Wizard SV gel and PCR purification kit (see DNA fragment purification).

#### 4.3.3.2 Ligation Reaction (pET 28-b)

Ectodomain targets were ligated into the pET-28b bacterial expression plasmid (Novagen) using a 5:1 molar ratio. Plasmid and insert in a 5:1 molar ratio were mixed with 400 cohesive end units of T4 DNA ligase and 1 x T4 ligase buffer (New England Biolabs) for 1 hour at 37°C. Ligated plasmids were immediately transformed into *E. coli*.

#### 4.3.3.3 Competent Cell Preparation

Home-made competent cells were prepared from glycerol stocks of One Shot Omni Max™ 2 T1 Phage-Resistant Cells (Invitrogen) using a modified version of methods published by Inoue *et. al.*, 1990 (275). Stocks of One Shot Omni Max™ 2 T1 Phage-Resistant cells were initially cultured for 8 hours at 37°C in Luria broth (LB) (Sigma). Cultured cells (0.02 volumes of Super Optimal Broth (SOB) medium) were then inoculated with SOB medium and cultured at 18°C in a shaking water bath till the Optical Density (OD)<sub>600</sub> reached 0.6. Cells were placed on ice for 10 minutes prior to centrifugation at 2,500 x g for 10 minutes at 4°C. The pellet was resuspended in ice-cold transformation buffer (TB), incubated on ice for 10 minutes and spun down as detailed above. The resulting pellet was gently resuspended in ice-cold TB buffer and dimethyl sulfoxide was added with gently swirling to a final concentration of 6.5%. Competent cells were checked for contamination by growing on LB Agar containing 50µg/ml ampicillin and LB Agar containing 30µg/ml kanamycin overnight at 37°C. The competent cells were found to have an efficiency of  $5.6 \times 10^6$  colonies/µg pET 28-b.

#### 4.3.3.4 pET 28-b Transformation

Transformation of ligated pET 28-b plasmids was performed using a 1:50 (ligation reaction:competent cell) ratio. The ligation reaction:competent cell mixture was incubated for 30 minutes on ice before being heat-shocked at 42°C for 30 seconds and placed on ice for 2 minutes. LB (1ml) containing no antibiotics was added and the mixture was incubated for 1 hour at 37°C. Transformed bacteria were centrifuged at 5,000 x g for 5 minutes at RT and the resulting pellet was resuspended in 200µl of LB media. The transformation mixture was then spread onto a LB agar plate containing 30µg/ml kanamycin and incubated overnight at 37°C. Colonies were incubated in a volume of LB media containing 30µg/ml kanamycin shaking overnight at 37°C and glycerol stocks (15% glycerol) were made and stored at -80°C.

#### 4.3.3.5 Plasmid Mini-Prep

To quickly check for the presence of a correct sized insert, an in-house plasmid purification procedure was used. Briefly, an aliquot from the glycerol stocks was incubated in a volume of LB containing 30µg/ml kanamycin shaking overnight at 37°C. An aliquot of each culture was transferred to a microcentrifuge tube and the bacteria were pelleted for 30 seconds at 13,000 x g. The media was removed by aspiration and the bacterial pellet was resuspended in 200µl cold alkaline lysis solution I. To lyse the cells, 200µl of alkaline lysis solution II was added to each bacterial solution and the tube was inverted several times and incubated at RT for 5 minutes. This reaction was stopped by the addition of 200µl of cold alkaline lysis solution III followed by inversion of the tube several times. The bacterial lysate was centrifuged at 13,000 x g for 20 minutes at 4°C to pellet non-specific genomic DNA. Plasmid DNA was precipitated by the addition of 2 volumes 100% ethanol and the samples were stored at -20°C for 10 minutes. Precipitated nucleic acids were collected by centrifugation at 13,000 x g for 20 minutes at 4°C. This precipitation step was repeated before the plasmid pellet was allowed to air-dry. Purified plasmids were resuspended in 30µl tris-EDTA (TE) buffer. The presence of a correct sized insert was confirmed by PCR (refer to PCR methodology – 4.3.2.1) using ectodomain specific primers (Table 4.1). As a positive control, placental cDNA was used. As a negative control, water was used. Plasmids that had the correct sized insert as determined by PCR were purified using the Gen-Elute plasmid mini-prep kit (Sigma) following the manufacturer's instructions (<http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/pln350bul.pdf>).

#### 4.3.3.6 Sequencing

Plasmids purified by the Gen-Elute plasmid mini-prep kit were sent to the Australian Genome Research Facility (AGRF) for sequencing. Plasmid samples were prepared by combining 400-1000ng of purified plasmid with 6.4pmol T7 promoter primer (Table 4.1) in an eppendorf tube.

#### 4.3.4 Syncytin Recombinant Ectodomain Protein Induction

##### 4.3.4.1 BL21-CodonPlus® Competent Cell Transformation

Syncytin ectodomain clones were transformed into chemically competent BL21-CodonPlus® competent cells (Stratagene) following the manufacturer's instructions (<http://www.stratagene.com/manuals/230240.pdf>). Briefly, competent cells were mixed with 1µl of a 1:10 XL10-gold 2-mercaptoethanol solution. The competent cells were incubated with the 2-mercaptoethanol solution for 10 minutes with regular gentle shaking. Plasmid was added to the prepared competent cell mixture in a 1:50 plasmid:competent cell ratio. This mixture was incubated for 30 minutes on ice before being heat-shocked at 42°C for 20 seconds and placed on ice for 2 minutes. LB (0.9ml) containing no antibiotics was added and the mixture was incubated shaking for 1 hour at 37°C. A small aliquot of the transformation mixture was then spread onto a LB agar plate containing 50µg/ml ampicillin and incubated overnight at 37°C. 10 colonies from each plate were selected and glycerol stocks (15% glycerol) were made. The glycerol stocks were stored at -80°C until use.

##### 4.3.4.2 Optimisation of Recombinant Ectodomain Protein Expression

Clones were tested for the expression of syncytin ectodomains by stimulation with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Glycerol stocks of BL21-CodonPlus® syncytin ectodomain clones were grown in LB media containing 30µg/ml kanamycin and 50µg/ml chloramphenicol overnight at 37°C (250rpm). A 0.05 volume aliquot of this culture was then incubated in LB media (without antibiotics) for 2 hours at 37°C (250rpm). Following this incubation, 0.1 volumes of bacterial suspension was taken to serve as a non-induced negative control. To the rest of the culture IPTG was added to a final concentration of 1mM. This culture was incubated for 2 hours at 37°C (250rpm). An aliquot of this culture was then taken and resuspended in 1 x NuPAGE® LDS

sample buffer for analysis using SDS-PAGE Coomassie stain (for protein analysis protocol see 4.3.6). Clones with high expression levels were used for subsequent work.

#### 4.3.4.3 Recombinant Ectodomain Protein Induction Protocol (Syncytin-1, 2 and B)

Syncytin ectodomain clones were stimulated with 1mM IPTG following the methods stated in optimisation of recombinant ectodomain protein expression. Instead of resuspending the culture in 1 x NuPAGE® LDS sample buffer, the bacterial suspension was centrifuged at 10,000 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in Bugbuster master mix (Novagen) (5ml/g wet cell paste) and left shaking at 200rpm for 20 minutes to digest nucleases. The Bugbuster protein mixture was then spun at 40,000 x g for 30 minutes at 4°C to pellet insoluble proteins. The soluble fraction was removed by aspiration and was immediately processed using affinity chromatography.

#### 4.3.4.4 Recombinant Ectodomain Protein Induction Protocol (Syncytin-A)

Syncytin-A ectodomain pET 28-b plasmids were stimulated with 1mM IPTG and prepared following the methods stated in recombinant ectodomain protein induction (syncytin-1, 2 and B). However, syncytin-A recombinant ectodomain protein was expressed in inclusion bodies in the insoluble fraction so a slightly different protein purification procedure was used. The insoluble pellet from the Bugbuster master mix treatment (as detailed in the above paragraph) was completely resuspended in the same volume of 1x Bugbuster master mix that was used to resuspend the original cell pellet. To this mixture, 6 volumes of 1:10 diluted (in deionised water) Bugbuster master mix was added and the samples were vortexed for 1 minute. The suspension was centrifuged at 5,000 x g for 15 minutes at 4°C to collect the inclusion bodies. Inclusion bodies were then washed in 0.5 volumes of 1:10 diluted Bugbuster, mixed by vortexing, and collected by centrifugation as described above. The inclusion bodies were resuspended

once more and then centrifuged at 16,000 x g for 15 minutes at 4°C. For purification, the inclusion bodies were resuspended in denaturing resuspension buffer.

#### 4.3.5 Purification of Syncytin Recombinant Ectodomain Proteins

A two step procedure was used for the purification of syncytin recombinant ectodomain proteins. Initially, affinity chromatography (Hitrap IMAC) was used followed by gel filtration chromatography using the Superdex 75 10/300GL column.

##### 4.3.5.1 Hitrap Immobilised Metal Affinity Chromatography (Hitrap IMAC)

The maximum flow rate used in Hitrap IMAC was 5ml/min. Columns were coated in 100mM NiSO<sub>4</sub> and washed thoroughly in dH<sub>2</sub>O. Recombinant protein samples were supplemented with 75mM imidazole and applied to a Hitrap column which had been equilibrated in binding buffer. Weakly bound proteins were removed in a series of washes using a pH gradient of binding buffer (pH7.4, 6.8, 6 and 5). Bound proteins were eluted using a competitive elution gradient system in elution buffer (100-500mM Imidazole in 100mM increments) and collected in 5ml fractions. Fractions 1-4 from the elution buffer containing 200mM imidazole were taken and diluted to a final imidazole concentration of 75mM. The Hitrap IMAC procedure was then repeated and the proteins were eluted in an elution buffer containing 500mM imidazole. Fractions were quantified using A280nm absorbance readings and the protein profile of specific fractions was determined by SDS-PAGE gel analysis.

##### 4.3.5.2 Gel Filtration – Superdex 75 10/300GL

Gel filtration using Superdex 75 10/300GL was performed with an ÄKTA™ Fast Protein Liquid Chromatography (FPLC™) (GE healthcare) using Unicorn™ software version 5.1.1 (GE Healthcare). All buffers were de-gassed and filtered prior to use. To

determine the expected elution time for syncytin recombinant ectodomain proteins, 2.5mg Ribonuclease A(13.7kDa) and 1.25mg Ovalbumin (43kDa) were run through the Superdex 75 10/300GL at 0.15ml/min in FPLC buffer in triplicate.

Fractions from the Hitrap IMAC containing syncytin recombinant ectodomain proteins were further purified by gel filtration using a Superdex 75 10/300GL column. Syncytin recombinant ectodomain proteins were dialysed using the slide-a-lyser dialysis cassette, 3,500 MWCO (Thermo Scientific) into dialysis buffer overnight at 4°C. The dialysed fraction was concentrated using a speed-vacuum system to a final volume of 500µl. Samples were added to the Superdex 75 10/300GL column and run through at 0.15ml/min in FPLC buffer. Fractions were collected and size was estimated by retention time. Purity was determined by SDS-PAGE.

#### 4.3.6 Confirmation of Syncytin Recombinant Ectodomain Protein Expression

##### 4.3.6.1 SDS-PAGE

Syncytin recombinant ectodomain proteins were resolved using NuPAGE® Novex 12% Bis-Tris gels (Invitrogen) in MES Running Buffer (Invitrogen) for 55 minutes at 200V. Gels for Coomassie staining were fixed in gel fixative for 1 hour. Gels for silver staining were fixed in gel fixative overnight. Gels for Sypro ruby protein analysis were fixed for 1 hour in Sypro ruby fixative. Gels for western blotting were transferred to nitrocellulose membranes for later use.

##### 4.3.6.2 Coomassie Gel Staining

Recombinant ectodomain proteins resolved by SDS-PAGE were stained with Coomassie brilliant blue solution to visualise the protein profile at the one dimensional level. Gels were fixed for one hour in gel fixative before being stained with Coomassie



brilliant blue solution overnight at RT on a rocking platform. Gels were destained for at least 24 hours in Coomassie destain solution with regular changes of the destain solution.

#### 4.3.6.3 Silver Stain

Recombinant ectodomain proteins resolved by SDS-PAGE were silver stained using SilverQuest™ (Invitrogen) to visualize the protein profile at the one dimensional level. Protocols were followed as detailed by the manufacturer ([http://www.invitrogen.com/content/sfs/manuals/silverquest\\_man.pdf](http://www.invitrogen.com/content/sfs/manuals/silverquest_man.pdf)).

#### 4.3.6.4 Sypro Ruby

Recombinant ectodomain proteins resolved by SDS-PAGE were stained with Sypro Ruby gel stain to visualise the protein profile for LCMS sequencing. All steps in this procedure were performed in a dark box and on a rocking platform at RT. Gels were fixed for one hour in Sypro Ruby fixative. Sypro Ruby gel stain (60ml) (Invitrogen) was added and gels were stained overnight. The following morning, gels were washed in Sypro Ruby wash solution for 30 minutes and visualised under UV light. Bands were excised for LCMS sequencing.

#### 4.3.6.5 Western Blotting

Proteins resolved by gel electrophoresis were transferred to a nitrocellulose membrane at 25V, 160mA, 17 Watts for 60 minutes using NUPAGE® transfer buffer. After extensive washes in TBST, membranes were blocked with skim milk (5%) in TBST. Membranes were then immunoblotted with primary antibodies (Table 4.2 – 1:1000 dilution) in 5% skim milk rocking overnight at 4°C. Unbound antibody was washed off in 5 x 5 minute washes in TBST and the membrane was incubated with a HRP conjugated secondary antibody (Table 4.2 – 1:2000 dilution) for 1 hour rocking at RT.

Following a final set of washes in TBST, specific binding was detected on the membranes using the ECL reagent (GE healthcare) and these membranes were photographed using LAS-3000 (Fujifilm).

**Table 4.2. Antibodies used for Western Blotting.** *The primary antibody is detailed in the left column and the corresponding secondary antibody is detailed in the right column.*

Primary Antibody	Secondary antibody
Syncytin-1 ISD Ab-L (J. Tolosa)	Goat-anti-rabbit (Millipore)
Syncytin-1 ISD Ab-L Pre-Immune (J. Tolosa)	Goat-anti-rabbit (Millipore)
His-tag (Merck)	Rabbit-anti-mouse (Abcam)

#### 4.3.7 Protein Sequencing

Syncytin recombinant ectodomain proteins were prepared for LCMS sequencing through two separate methods. Gel plugs were used to sequence specific proteins from total bacterial proteins and for highly purified fractions, aliquots of samples were trypsin digested and run directly through the LCMS. At all stages during these protocols, Fluka water was used to dilute buffers unless otherwise specified.

##### 4.3.7.1 Gel-Plug Preparation for LCMS Sequencing

Excised monomeric recombinant ectodomain gel plugs from Sypro Ruby stained gels were washed 5 times for 30 minutes in LCMS wash solution. Gel plugs were dehydrated by leaving the plate uncovered at RT overnight. Following dehydration, gel plugs were digested with a volume of trypsin solution overnight at 37°C. Digested proteins were extracted from the gel plug with 6 washes in LCMS gel extraction buffer. The supernatant, which contained digested peptides was speed vacuumed prior to LCMS sequencing.

#### 4.3.7.2 In Solution Preparation for LCMS Sequencing

An aliquot of purified syncytin recombinant ectodomain proteins was taken and made to a final volume of 400µl in fluka water (Fluka). To this sample, an equal volume of methanol and 0.5 volumes of chloroform were added sequentially with vortexing following each addition. The mixture was centrifuged at 10,000 x g for 3 minutes at 4°C. The resulting aqueous phase was discarded and 300µl of methanol was added. Following mixing, the samples were spun at 10,000 x g for 15 minutes at 4°C. The supernatant was aspirated and the protein pellet allowed to air dry before resuspension in 25mM ammonium bicarbonate. Trypsin solution was added (1µg trypsin/50 µg protein) and the sample was incubated at 37°C rotating overnight. Digested peptides were speed vacuumed prior to LCMS sequencing.

#### 4.3.7.3 LCMS Sequencing

Separation of tryptic peptide mixtures was achieved by nanoscale reverse-phase HPLC, in combination with online ESIMS. For the HPLC separation, a nano-multidimensional LC (MDLC) system (Ettan MDLC, GE Healthcare) was used, employing a linear gradient of 0–80% buffer B over 60 min.

The column system consisted of a trap (300 µm id 5mm length, c18) purchased from Agilent Technologies (Palo Alto, CA) and a separation column (75 µm id, 150 mm, c18) packed in house. While column and trap 1 were running, column and trap 2 were equilibrated with buffer A to allow continuous running of the sample through two columns. Mass spectrometric analysis was performed on a LTQ linear Ion Trap system (ThermoFinnigan, San Jose, CA). For online coupling, a nano-ion spray source was used, equipped with a New Objective ESI needle (30 µm tip) with a voltage of 1.6 kV in positive ion mode. The scan cycle consisted of a MS survey scan (scanning a mass range of 500–2000 atomic mass units (amu)) followed by MS/MS of the six most intense

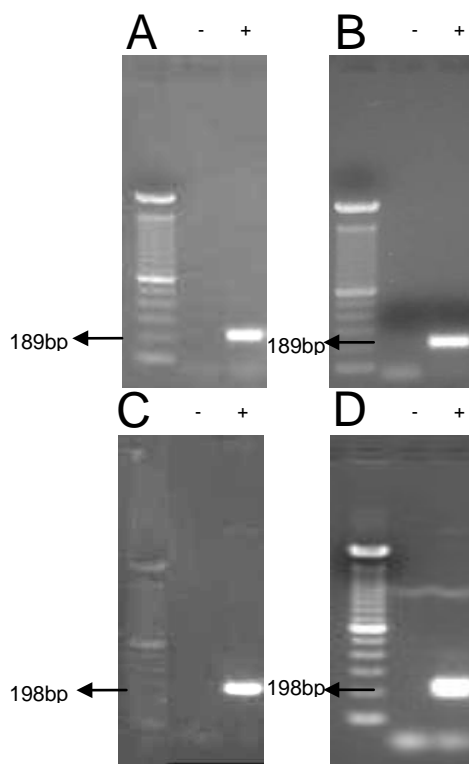
signals observed in the survey scan. An exclusion list for ion signals was set to 25 seconds after one occurrence.

The derived mass spectrometric datasets were converted to generic format (\*.dta) files using the Bioworks Browser (3.3.1) (ThermoFinnigan, San Jose, CA) and searched against the Human and Mouse IPI database (fasta version 3.33), containing the sequence of human and mouse proteins using the Bioworks (v3.31) search algorithm, Sequest™ (v3.3.1). The number of allowed miscleavages was set to 2, oxidation of methionine (M+15.99492) and phosphorylation (STY + 79.96633) of serine, threonine and tyrosine were selected as differential amino acid modifications. Precursor ion tolerance was set to 1.4 Da and ion intensity threshold was set to 100. The peptide ion selection was set to 1.4 Da, with fragment ion set to 0.7 Da. The following filters were set for every peptide: (i) minimum cross correlation score (Xcorr) by Sequest value of 2.9 for peptides of charges 1 and 2 and 3.2 for peptides with a charge of 3 and all peptides passing this filter had to have a SP (preliminary raw score) >500 and a Rsp (Rank of preliminary score) < 5.

All passing peptides were then manually validated.

## **4.4 Results**

### **4.4.1 Cloning of Syncytin Recombinant Ectodomains**

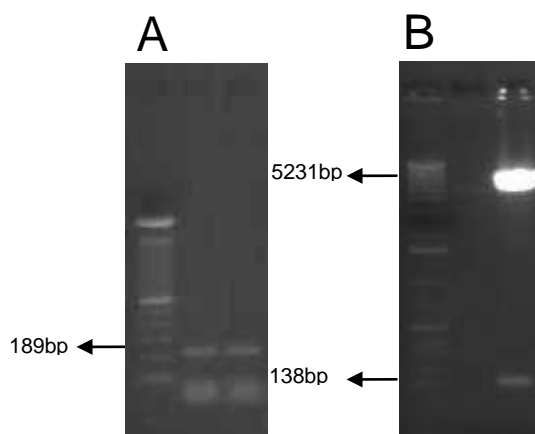


**Figure 4.1. Syncytin recombinant ectodomain amplification.** *Syncytin ectodomain gene sequences were amplified by PCR using gene specific primers and resolved using agarose gel electrophoresis. Amplicons were excised and purified for use in molecular cloning. DNA ladder = 100bp ladder (Invitrogen), ‘-’ = reverse transcription negative control, ‘+’ = Amplified ectodomain sequence. A) Syncytin-1 (189bp), B) Syncytin-2 (189bp), C) Syncytin-A (198bp), D) Syncytin-B (198bp).*

Syncytin ectodomain gene sequences were amplified using PCR. Amplicons corresponding to the expected size were observed for all primers (Figure 4.1 – ‘+’). No amplification was observed in the negative control (Figure 4.1 – ‘-’). Amplicons were excised and purified by the Wizard SV gel and PCR purification kit.

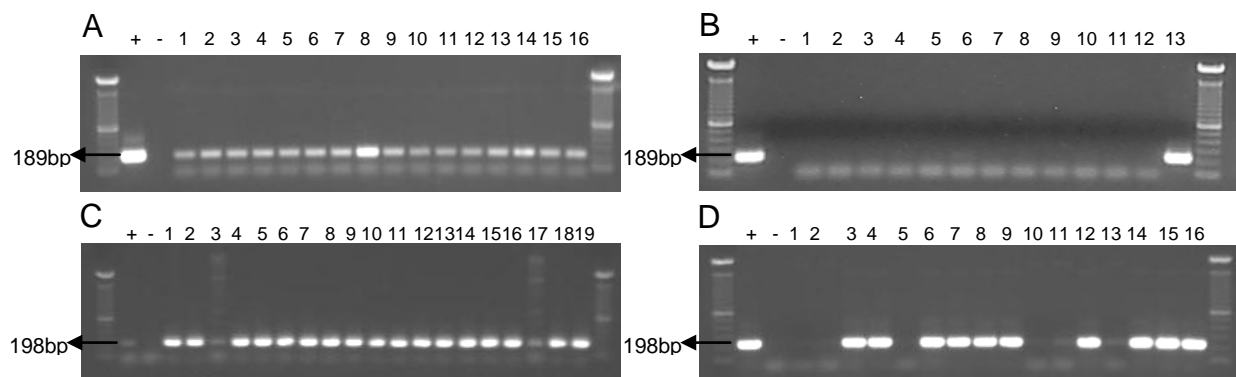
Purified syncytin ectodomain gene sequences were digested with BspH1 and Xho1. Digested ectodomains were purified using agarose gel electrophoresis (for example see

syncytin-1 digestion in figure 4.2a). The digested ectodomain ran as a 189 base pair (bp) oligomer. Digested syncytin ectodomains were excised and purified prior to cloning.



**Figure 4.2. Restriction enzyme digestion of syncytin ectodomain gene sequences and the pET 28-b plasmid.** Agarose gel electrophoresis was used to resolve digested plasmid or insert prior to purification using Wizard SV gel PCR purification kit (Promega). A) Ectodomain inserts were digested with 1Unit of *Bsp*HI and 1Unit of *Xho*I at 37°C for 16hours. Ladder; 100bp ladder (Invitrogen). The example shown in A) is digested syncytin-1 ectodomain B) 3µg pET-28b was digested with 2.5 Units *Nco*I and 10 Units *Xho*I at 37°C for 4 hours. Ladder; 1kb ladder (Invitrogen).

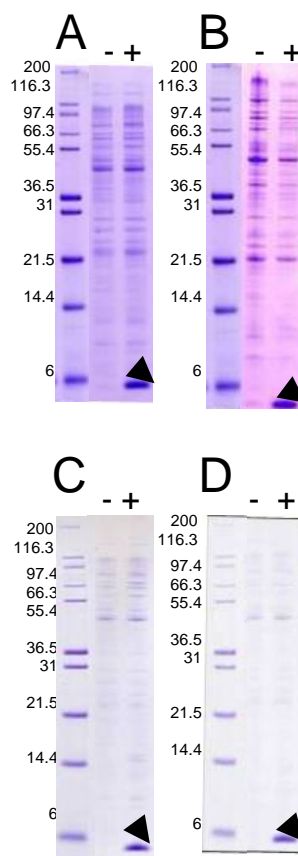
pET 28-b (5369bp) was prepared for cloning by digestion with *Nco*I and *Xho*I. Digested pET 28-b plasmid was separated from other non-digested fragments by agarose gel electrophoresis (Figure 4.2b). The digested plasmid ran as a high molecular weight oligomer of 5231bp. The low molecular weight band of 138bp corresponds to the fragment created by the digestion process and was not used for further work. Digested pET 28-b was excised and purified prior to cloning.



**Figure 4.3. Syncytin ectodomain pET28-b clones.** *Syncytin ectodomain gene sequences cloned into pET 28-b were amplified by PCR using gene-specific primers and resolved using agarose gel electrophoresis. Correct sized clones (depicted by an amplicon of similar intensity to the positive control – placental tissue) were purified using the Gen-Elute Plasmid mini-prep kit (Sigma) and sent to AGRF for DNA sequencing. Ladder = 100bp ladder (Invitrogen), ‘+’ = positive control, ‘-’ = negative control, ‘#1-16’ = syncytin ectodomain pET 28-b plasmid number. A) syncytin-1 (189bp), B) syncytin-2 (189bp), C) syncytin-A (198bp), D) syncytin-B (198bp).*

Digested syncytin ectodomain gene sequences and pET 28-b plasmids were ligated using an optimised 5:1 molar ratio. Ligated plasmids were transformed into chemically competent *E. coli* and grown overnight prior to isolation of purified plasmid DNA. The presence of a correct sized syncytin ectodomain insert in the pET 28-b plasmid was confirmed by PCR using gene specific primers (Table 4.1) (Figure 4.3). Plasmids that tested positive to the specific ectodomain are demonstrated by an amplicon of similar or higher intensity to the placental cDNA positive control (see Figure 4.3 A) syncytin-1: #8, B) syncytin-2: #13, C) syncytin-A: #1, 2, 4-16, 18, 19, D) syncytin-B: #3, 4, 6-9, 12, 14-16) and were sent for sequencing to AGRF using the T7 sequencing primer (Table 4.1). In some cases, a weak amplification could be observed in a number of plasmids. These plasmids were not able to be correctly sequenced and may be partially ligated syncytin ectodomain pET 28-b plasmids (Figure 4.3).

#### 4.4.2 Optimisation and Characterisation of Syncytin Recombinant Ectodomains Proteins from Total Bacterial Lysates

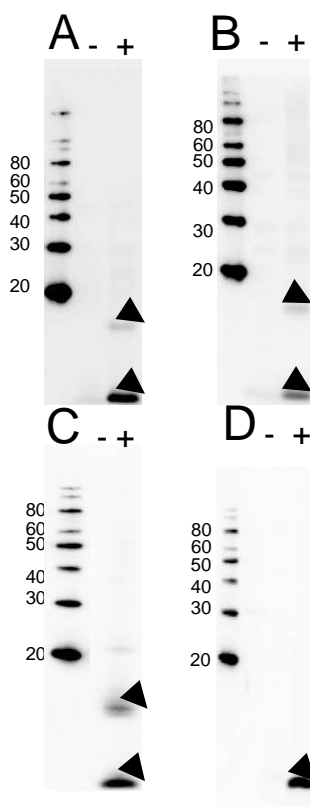


**Figure 4.4. Optimisation of syncytin recombinant ectodomain protein induction.** *Syncytin recombinant ectodomain pET 28-b clones were grown in BL21–Codon Plus® competent cells and induced with 1mM IPTG for 2 hours. Total bacterial proteins were run on a 12% Bis-Tris SDS-PAGE gel with MES running buffer and the protein profile was determined by staining with Coomassie brilliant blue gel stain. Ladder = Mark12 (Invitrogen), UI = uninduced sample, I = induced sample. A) syncytin-1, B) syncytin-2, C) syncytin-A, D) syncytin-B. Arrows indicate the induced recombinant protein.*

Chemically competent BL21 codon plus competent cells were transformed with the syncytin ectodomain pET28-b plasmids. Clones were tested for production of recombinant ectodomain proteins by stimulation with 1mM IPTG for 2hrs. The production of syncytin recombinant ectodomain proteins were observed for all samples, characterised by a 5-7kDa protein being expressed in induced, but not un-induced



samples (arrows - Figure 4.4 A-D). The induced protein corresponded to the expected size for syncytin recombinant ectodomain monomeric protein. The specificity of the induced protein was confirmed with Western Blotting using an anti-His-tag antibody (Figure 4.5). Binding to the 5-7kDa protein was observed for all ectodomains in induced but not in un-induced samples (Figure 4.5). With the exception of syncytin-B (Figure 4.5D), binding could also be observed for a 14kDa protein which corresponded to the expected molecular weight of the dimer of syncytin ectodomain proteins. A very faint band corresponding to the trimeric ectodomain protein could be observed for syncytin-1 (Figure 4.5A) and syncytin-2 (Figure 4.5B). LCMS sequencing of the 5-7 kDa induced monomeric protein confirmed the expression of each syncytin recombinant ectodomain protein.

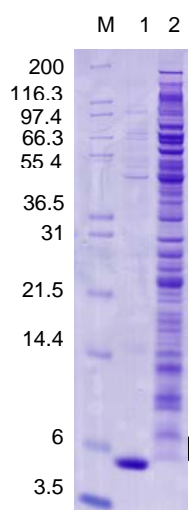


**Figure 4.5. Western Blotting analysis of syncytin recombinant ectodomains using His-tag antibody.** Syncytin recombinant ectodomain pET 28-b plasmids were grown in BL21 – Codon Plus® competent cells and induced with 1mM IPTG for 2 hours. Total bacterial proteins were run on a 12% Bis-Tris SDS-PAGE gel with MES running buffer and transferred to nitrocellulose membranes. Membranes were probed with anti-His-tag antibody (1:1000 dilution) to confirm the specificity of the protein to syncytin recombinant pET 28-b plasmid. Ladder = Magic mark (Invitrogen), - = uninduced sample, + = induced sample. A) Syncytin-1, B) Syncytin-2, C) Syncytin-A, D) Syncytin-B. Arrows indicate the induced recombinant proteins.

#### 4.4.3 Purification and Characterisation of Syncytin Recombinant Ectodomain Proteins

A two step procedure was used to purify syncytin recombinant ectodomain proteins. With the exception of syncytin-A, soluble proteins were extracted from a total *E. coli* protein fraction. For syncytin-A, an inclusion body extraction procedure was used (as described in 4.3.4.4) as syncytin-A preferentially was expressed in inclusion bodies (Figure 4.6). The Hitrap IMAC column (Amersham) was used to bind histidine-tagged

proteins from a suspension of soluble or inclusion body bacterial proteins. Using a standard elution protocol involving competitive elution with high concentrations of imidazole, a peak of proteins was observed for all syncytin recombinant ectodomain samples at elution number 6 by measurement of the absorbance at 280nm and SDS-PAGE Coomassie gel staining (Figure 4.8-4.11 A). It was observed that the fraction following Hitrap IMAC purification was only partially purified and contained high molecular weight contaminating proteins. The main proteins (monomer-6kDa, dimer-14kDa, trimer-21kDa) corresponded to the expected size for our recombinant ectodomain proteins (Figure 4.8-4.11 B).



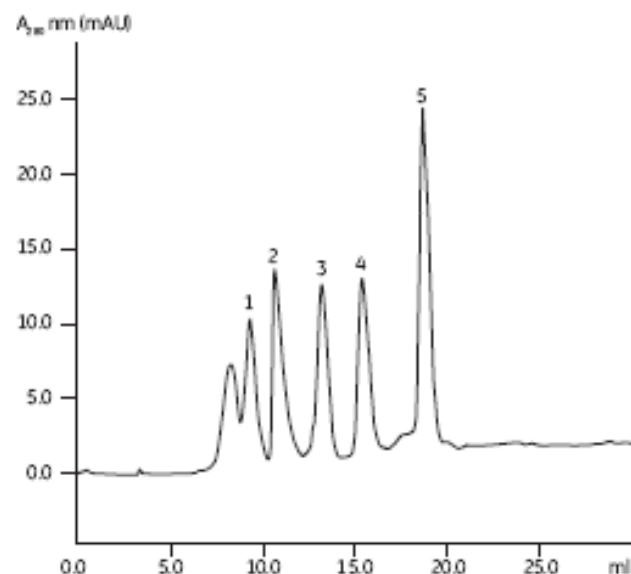
**Figure 4.6. Syncytin-A is expressed in inclusion bodies.** Syncytin-A recombinant ectodomain plasmids were grown in BL21 – Codon Plus® competent cells and induced with 1mM IPTG for 2 hours. Total bacterial proteins were separated into soluble and insoluble inclusion body fractions and were run on a 12% Bis-Tris SDS-PAGE gel with MES running buffer. The protein profile was determined by staining the gel with Coomassie brilliant blue gel stain. Ladder = Mark12 (Invitrogen), 1 = Insoluble inclusion body fraction, 2 = Soluble protein fraction. An arrow indicates the induced recombinant proteins which appear only in the insoluble inclusion body fraction.

A

Column Superdex 75 10/300 GL

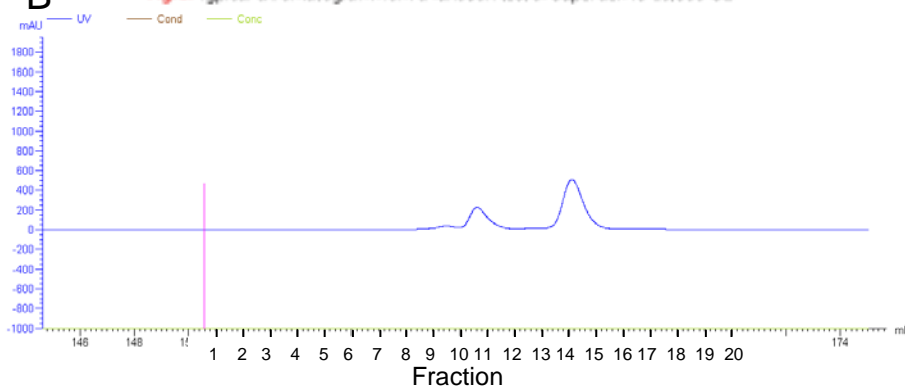
Sample: 1. BSA (M, 67 000) 8 mg/ml  
2. Ovalbumin (M, 43 000) 2.5 mg/ml  
3. Ribonuclease A (M, 13 700) 5 mg/ml  
4. Apotinin (M, 6 512) 2 mg/ml  
5. Vitamin B12 (M, 1355) 0.1 mg/ml

Sample volume: 500 µl  
Eluent: 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.0  
Flow rate: 0.4 ml/min, room temperature  
Detection: 280 nm



B

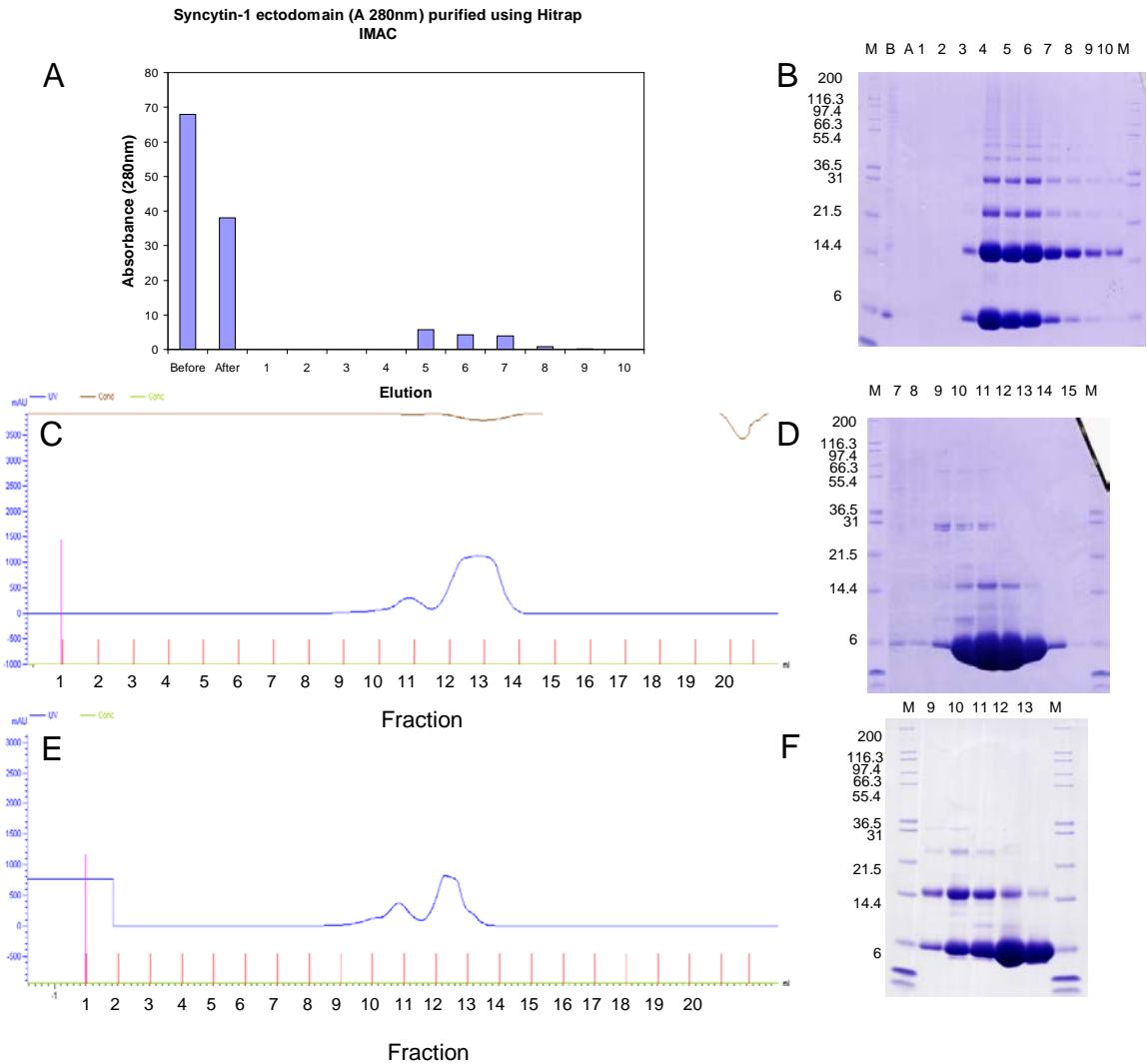
Fig. 2. Typical chromatogram from a function test of Superdex 75 10/300 GL.



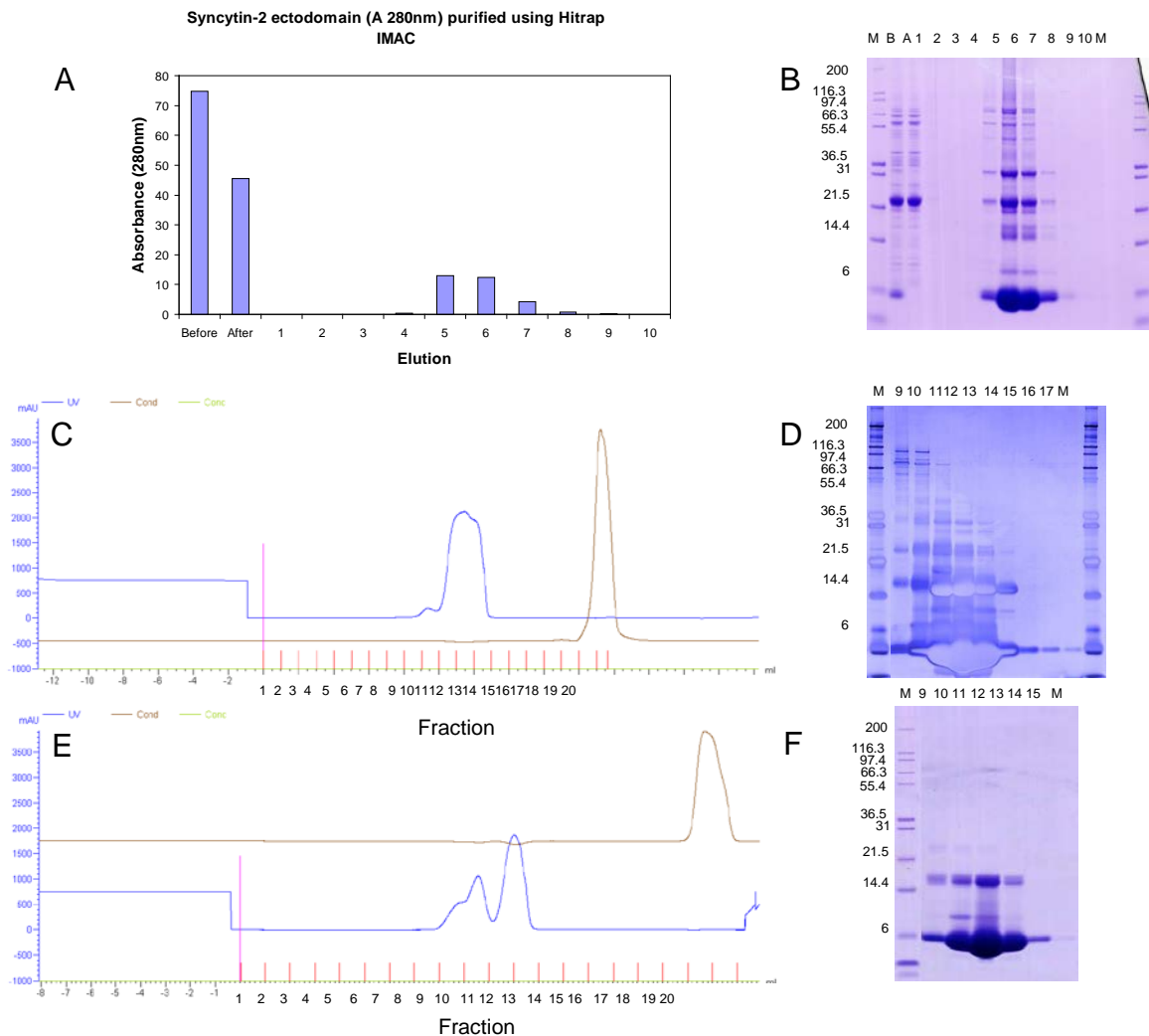
**Figure 4.7. Chromatogram of Ovalbumin and Ribonuclease A standards run using Superdex 75 10/300 GL column (GE healthcare) on ÄKTA™ FPLC™ (GE healthcare).** A) GE healthcare elution profile for 500 µl standard (BSA (1) – 67kDa - 8mg/ml, Ovalbumin (2) – 43kDa – 2.5mg/ml, Ribonuclease A (3) – 13.7kDa – 5mg/ml, Apotinin (4) – 6.5kDa – 2mg/ml, Vitamin B12 (5) – 1.355kDa – 1mg/ml) after sample was run on Superdex 75 10/300 GL in 50mM phosphate buffer, 150mM NaCl, pH7 at 0.4ml/min. B) 500 µl of 2.5mg/ml Ovalbumin (43kDa) and 5mg/ml Ribonuclease A (13.7kDa) was run

*through Superdex 75 10/300 GL in 25mM HEPES, 150mM NaCl, pH 8.5 at 0.15ml/min. Ovalbumin eluted in fraction 10l while Ribonuclease A eluted at 14ml.*

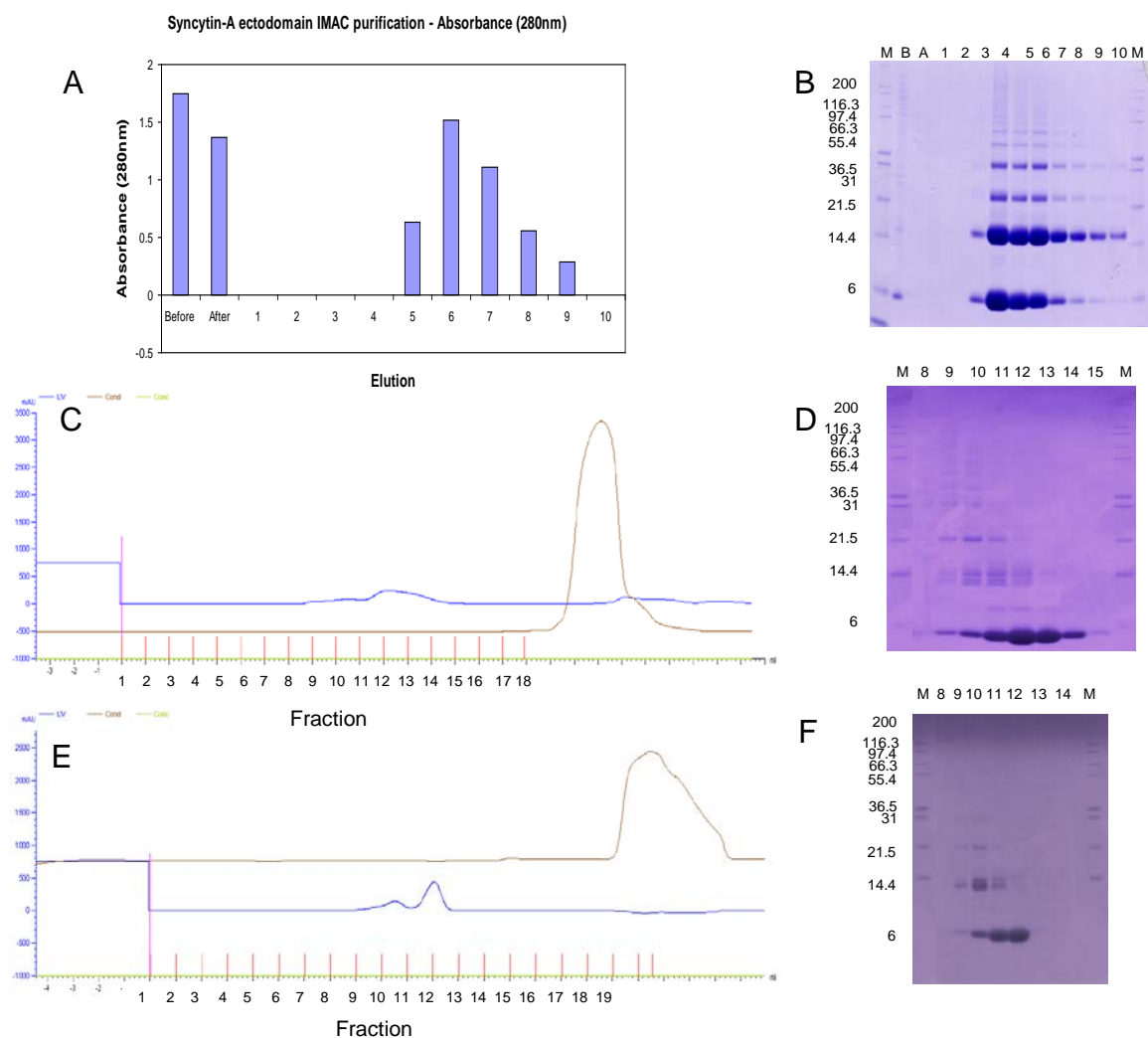
Superdex 75 10/300 GL gel filtration columns were used in the final step to yield purified oligomeric recombinant ectodomain proteins. Initially, the expected elution profiles of our oligomeric proteins were determined by running recommended molecular weight standards through the Superdex 75 10/300 column. The globular proteins Ovalbumin (43kDa) and Ribonuclease A (13.7kDa) were used to represent the molecular weight range where the oligomeric recombinant protein was expected to elute. According to GE healthcare elution profiles, Ovalbumin elutes at 11ml while Ribonuclease A elutes at 13-14ml using their running conditions (Figure 4.7A). To determine our experimental elution patterns, Ovalbumin and Ribonuclease A were run on the Superdex 75 10/300 GL column using the recombinant ectodomain experimental conditions in FPLC buffer at 0.15ml/min. The elution profile showed that Ovalbumin eluted at 10ml and Ribonuclease A eluted between 13-14 ml (Figure 4.7 B).



**Figure 4.8. Syncytin-1 ectodomain purification.** Syncytin-1 ectodomain was induced in 1mM IPTG and soluble protein was isolated using centrifugation methods. Syncytin-1 ectodomain was then purified by affinity chromatography using Hitrap IMAC HP methods (A, B). Complete purification was achieved through two runs through the Superdex 75 10/300 gel filtration column in 25mM HEPES, 150mM NaCl pH7.4 (C-F). A, C and E show representative graph/chromatograms of syncytin-1 ectodomain following Hitrap IMAC HP methods (A) or Superdex 75 10/300GL purification (C and E – blue line). B, D and F show a representative Coomassie stained SDS-PAGE gel of syncytin-1 ectodomain eluted from either Hitrap IMAC (B) or Superdex 75 10/300 GL (D or F) columns. Protein ladder = Mark12 (Invitrogen); Before (B) = Sample before application to Hitrap column; After = Sample after application to Hitrap column; #1-20 = Sample elution from column.

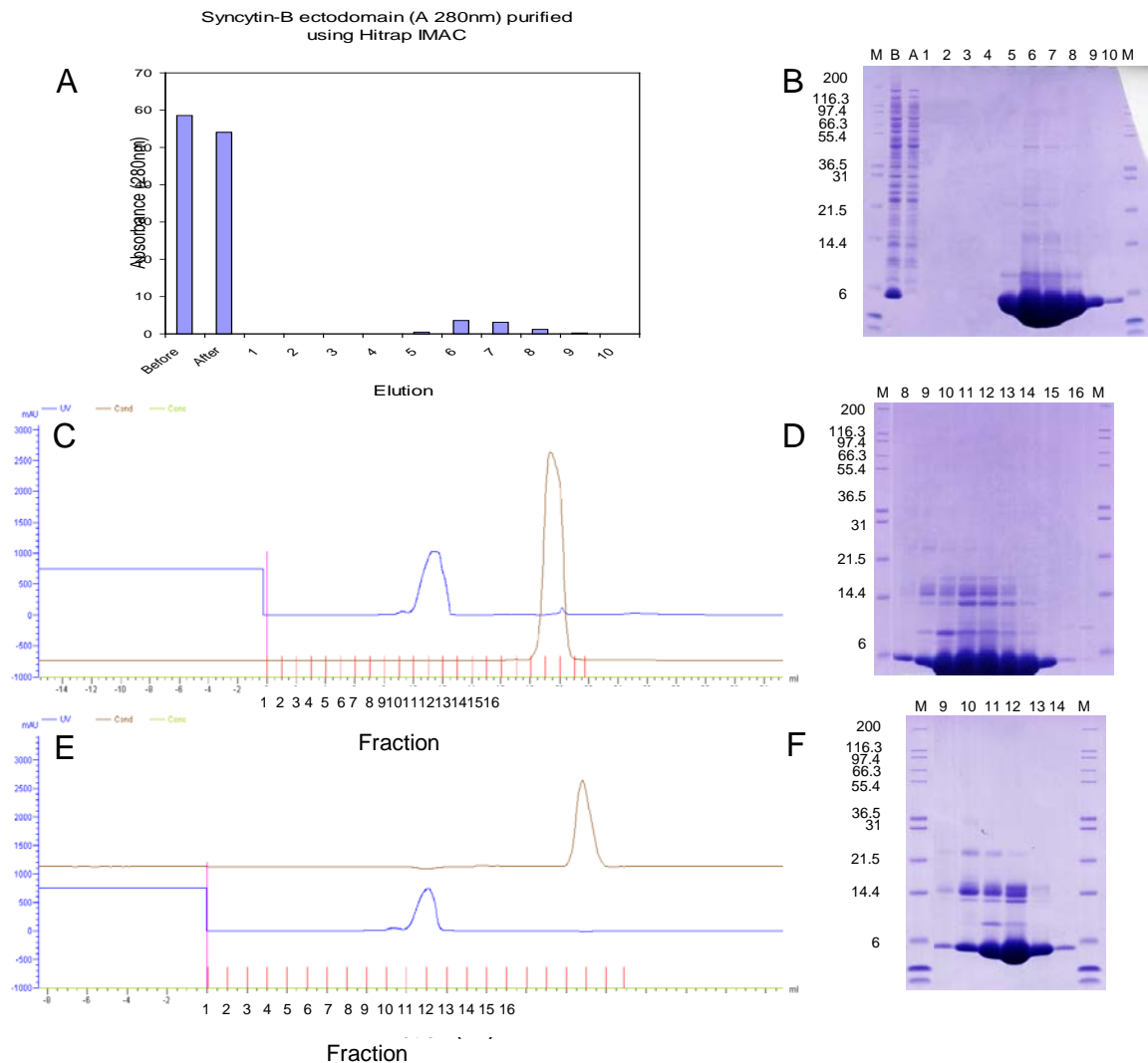


**Figure 4.9. Syncytin-2 ectodomain purification.** Syncytin-2 ectodomain was induced in 1mM IPTG and soluble protein was isolated using centrifugation methods. Syncytin-2 ectodomain was then purified by affinity chromatography using Hitrap IMAC HP methods (A, B). Complete purification was achieved through two runs through the Superdex 75 10/300 gel filtration column in 25mM HEPES, 150mM NaCl pH7.4 (C-F). A, C and E show a representative graph/chromatogram of syncytin-2 ectodomain following Hitrap IMAC HP methods (A) or Superdex 75 10/300GL purification (C and E – blue line). B, D and F show a representative Coomassie stained SDS-PAGE gel of syncytin-2 ectodomain eluted from either Hitrap IMAC (B) or Superdex 75 10/300 GL (D or F) columns. Protein ladder = Mark12 (Invitrogen); Before (B) = Sample before application to Hitrap column; After = Sample after application to Hitrap column; #1-17 = Sample elution from column.



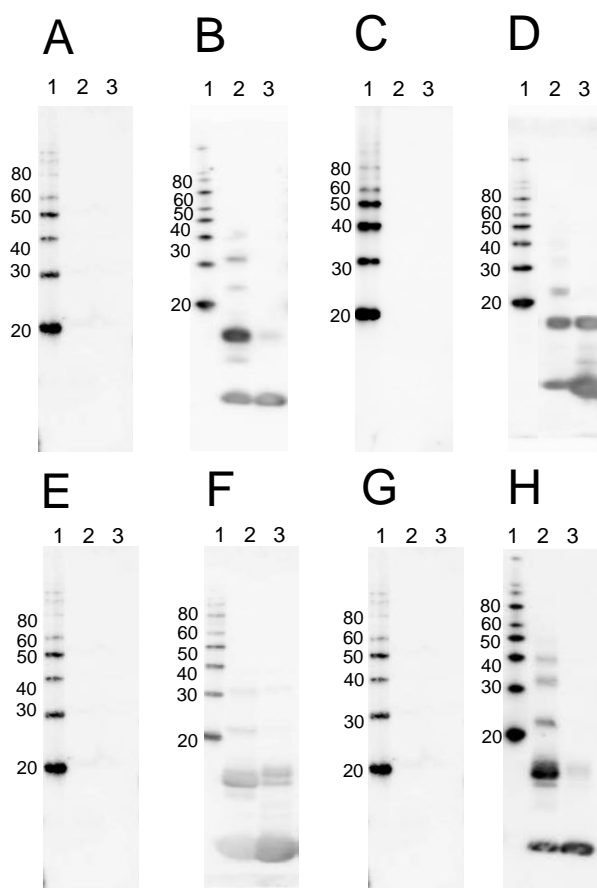
**Figure 4.10. Syncytin-A ectodomain purification.** Syncytin-A ectodomain was induced in 1mM IPTG and the insoluble inclusion body fraction was isolated using centrifugation methods. Syncytin-A ectodomain was initially purified by affinity chromatography using Hitrap IMAC HP methods (A, B). Complete purification was achieved through two runs through the Superdex 75 10/300 gel filtration column in 25mM HEPES, 150mM NaCl pH7.4 (C-F). A, C and E show a representative graph/chromatogram of syncytin-A ectodomain following Hitrap IMAC HP methods (A) or Superdex 75 10/300GL purification (C and E – blue line). B, D and F show a representative Coomassie stained SDS-PAGE gel of syncytin-A ectodomain eluted from either Hitrap IMAC (B) or Superdex 75 10/300 GL (D or F) columns. Protein ladder = Mark12 (Invitrogen); Before (B) = Sample before application to Hitrap column; After = Sample after application to Hitrap column; #1-19 = Sample elution from column.





**Figure 4.11. Syncytin-B ectodomain purification.** Syncytin-B ectodomain was induced in 1mM IPTG and soluble bacterial protein was isolated using centrifugation methods. Syncytin-B ectodomain was then purified by affinity chromatography using Hitrap IMAC HP methods (A, B). Complete purification was achieved through two runs through the Superdex 75 10/300 gel filtration column in 25mM HEPES, 150mM NaCl pH7.4 (C-F). A, C and E show a representative graph/chromatogram of syncytin-B ectodomain following Hitrap IMAC HP methods (A) or Superdex 75 10/300GL purification (C and E – blue line). B, D and F show a representative Coomassie stained SDS-PAGE gel of syncytin-B ectodomain eluted from either Hitrap IMAC (B) or Superdex 75 10/300 GL (D or F) columns. Protein ladder = Mark12 (Invitrogen); Before (B) = Sample before application to Hitrap column; After = Sample after application to Hitrap column; #1-15 = Sample elution from column.

Following the determination of the expected elution profile for our recombinant ectodomain oligomeric proteins, the second step of purification was completed using gel filtration. Partially purified recombinant ectodomains from Hitrap IMAC were dialysed into dialysis buffer and concentrated to a final volume of 500 $\mu$ l, filtered through a 0.44 $\mu$ M filter, and run through the Superdex 75 10/300 GL column. Generally, non-specific protein contamination was observed following the first Superdex run, however the main peak eluted in fraction 11-12 (with the majority of protein in fraction 12 as determined by the BCA protein assay) with a smaller peak eluting in fraction 10 (Figure 4.8-4.11 C and D). These peaks were subsequently dialysed into 5mM HEPES, 15mM NaCl, pH7.4 and were concentrated prior to a second run (Figure 4.8-4.11 E and F). Following the second run, the main peak observed eluted in fraction 11-12 and contained a highly purified recombinant ectodomain protein fraction as observed by non-reducing SDS-PAGE Coomassie stain however more oligomeric protein could be observed in proteins from the peak eluting in fraction 10 (Figure 4.8-4.11 F). Studies of the major peak were performed using fractions 12 as fraction 11 also contained carryover protein from the peak at fraction 10 (Figure 4.8-4.11E – peak from fraction 10 never completely reaches baseline). Purified protein was subjected to western blotting using an antibody targeting the highly conserved immunosuppressive peptide of syncytin-1. Analysis of Western Blots showed that purified recombinant ectodomain proteins had affinity to syncytin-1 ISU AbL for the monomer, dimer and higher order oligomer proteins (Figure 4.12 B, D, F and H). No binding was observed using syncytin pre-immune serum (Figure 4.12 A, C, E and G). Finally, LCMS sequencing confirmed the expression of specific purified recombinant retroviral ectodomain proteins.



**Figure 4.12. Representative Western blot of purified syncytin recombinant ectodomains.**

*Representative Western Blot of purified recombinant ectodomains of human syncytin-1 and syncytin-2 (A-B and C-D); and mouse syncytin-A and syncytin-B (E-F and G-H) generated in E. coli. 2µg of purified protein was used. Layout of membranes are as follows; A) and B) 1 - Magic mark (Invitrogen), 2 – syncytin-1 ectodomain fraction 10, 3 – syncytin-1 ectodomain fraction 12; C) and D) 1 - Magic mark (Invitrogen), 2 – syncytin-2 ectodomain fraction 10, 3 – syncytin-2 ectodomain fraction 12; E) and F) 1 - Magic mark (Invitrogen), 2 – syncytin-A ectodomain fraction 10, 3 – syncytin-A ectodomain fraction 12; G and H 1 - Magic mark (Invitrogen), 2 – syncytin-B ectodomain fraction 10, 3 – syncytin-B ectodomain fraction 12. A), C), E) and G) syncytin AbL Pre-Immune Sera (1:1000), B), D), F) and H) syncytin AbL Immune Sera (1:1000).*

Purified proteins were then quantified and used for immunotesting as detailed in Chapter 5.

## **4.5 Discussion**

The results shown in this chapter detail the generation of human and mice syncytin recombinant ectodomain proteins using a bacterial expression system. These recombinant proteins have previously been shown to be biologically active when they fold into a multimeric state (213, 276, 277).

The recombinant protein purification protocol described by Mangeney (213) was used as the basis for the protocol detailed in this chapter however, the results from the purification in the Mangeney study were not shown and as such, our findings are unable to be compared. (213).

In the protocol detailed in this chapter, placental syncytin ectodomain sequences were cloned into the pET-28b plasmid and transformed into BL21 Codon Plus competent *E. coli* which is designed for high level expression of recombinant proteins. Recombinant proteins were induced with 1mM IPTG and proteins were extracted using centrifugation methods. The purification of syncytin-1 (Figure 4.8), -2 (Figure 4.9) and -B (Figure 4.11) recombinant ectodomain proteins were completed using soluble protein extraction methods (detailed in 4.3.4.3) while syncytin-A (Figure 4.10) was purified from an inclusion body fraction (Figure 4.6 – protocol detailed in 4.3.4.4). Recombinant proteins were then purified by Hitrap IMAC methods with a pH gradient (pH7.4-5 binding buffer) wash step. His-tagged protein was eluted using competitive binding with imidazole, dialysed into 5mM HEPES, 25mM NaCl, pH7.4 and then completely purified in 25mM HEPES, 150mM NaCl using a Superdex 75 10/300GL column gel filtration column connected to the ÄKTA™ FPLC™.

Analysis of the Superdex 75 10/300 chromatograms of all purified recombinant ectodomains showed the major peak eluting in fraction 11-12 with a smaller protein peak eluting in fraction 10 (Figure 4.8-4.11E – blue line). The protein profiles of the syncytin recombinant proteins were observed using Coomassie staining and the majority

of the proteins present in these fractions corresponded to the expected size for the monomer, dimer or higher order oligomers. In particular, in fraction 10, trimeric and tetrameric proteins were observed using SDS-PAGE Coomassie staining. The identity of the proteins in these fractions was confirmed by western blotting using the syncytin-1 ISD antibody and LCMS sequencing. A small amount of protein which did not correspond to the expected size for the recombinant proteins was also observed in these fractions and was most likely contamination from bacterial proteins.

SDS-PAGE gel analysis of the purified syncytin recombinant ectodomain proteins always resulted in the dissociation of the recombinant proteins into monomer, dimer, trimer or tetramer and a stable oligomeric protein was never observed. This dissociation is due to the stability of these proteins. In its natural environment, stability is achieved through interactions between the envelope protein and the cell membrane through the transmembrane domain of the transmembrane subunit. This membrane interaction assists in stability through the refolding of the protein onto itself forming a very stable oligomer of hairpin like proteins which are generated in the endoplasmic reticulum of an infected cell (278, 279). However, the recombinant ectodomain is a soluble protein that does not carry the transmembrane domain and is stabilised by weak non-covalent interactions (280). It has even been stated in the literature that due to the presence of these weak interactions, it would be difficult to generate stable soluble ectodomains which are suitable for biological analysis (280). The presence of these non-covalent interactions for the syncytin recombinant ectodomains would decrease their stability under non-reducing SDS-PAGE gel conditions and result in the oligomeric pattern that is observed (Figure 4.8-4.11F fractions 10 and 11-12)).

In an effort to minimize the stability issue associated with these proteins, approaches have been designed to stabilise soluble oligomers. These approaches include; addition of GCN4 trimeric helices, disruption of the cleavage site and introduction of cysteine residues into the coiled coil region to form intersubunit disulfide bonds (281, 282). In the protocol described in this chapter, changes were not introduced to stabilise the

proteins as the effects of any of these substitutions on a potential immunosuppressive function have not been documented.

The majority of studies on the structure of the ectodomain of retroviral envelope protein are on HIV or other exogenous retroviruses such as MMuLV. These studies have shown that the retroviral ectodomain exists in its native form as an  $\alpha$ -helical oligomeric complex. (276, 277, 283-285). Chemical cross linking studies of retroviral ectodomain proteins have shown the presence of high order oligomers such as trimers and tetramers which have been shown to dramatically increase biological activity in comparison with the monomeric version of the protein (276, 277, 283-287). In regards to placental syncytins, Renard *et al.*, examined the crystal structure of the syncytin-2 ectodomain using the hanging-drop method and showed that syncytin-2 forms a trimeric molecule (174). The structure of syncytin-2 was remarkably similar to that of other retroviral ectodomains such as MuLV and HTLV-1.

Based upon the elution patterns of the molecular weight standards (Figure 4.7), it was predicted that the biologically active recombinant ectodomain molecule would approximately elute in fraction 11-12. Analysis of fraction 11-12 using SDS-PAGE and western blotting showed only monomeric and dimeric molecules and did not appear to be the biologically active multimeric fraction. It should be noted that the elution pattern of the protein is not the only important feature in determining size in gel-filtration. Another feature which can be very important is the hydrodynamic volume, which is the radius of the folded molecule. For example, globular proteins such as those described in the standards may appear elute later than proteins which are in an  $\alpha$ -helical or random-coil form (288).

In fraction 10, the elution profile shows higher order oligomeric proteins which are folded into more complex structures than the trimeric structure that has been published for syncytin-2. However, as detailed above, retroviral proteins can be active in multiple oligomeric states and do not necessarily have to form one particular conformation for optimal biological activity (277, 280, 286, 289). The SDS-PAGE profile observed in

this chapter (Figure 4.8-4.12) corresponds to the profile of other biologically active retroviral ectodomain proteins (277, 280, 289) suggesting this may be the biologically active fraction.

Unlike the other recombinant ectodomain proteins, syncytin-A was unable to be purified using a soluble protein extraction procedure. Using this method a very low yield of protein was obtained. Syncytin-A recombinant protein had previously been identified in a total bacterial protein fraction (Figure 4.4C and Figure 4.5C) at similar levels to other syncytin recombinant proteins, so these results suggested that syncytin-A was not binding to the column or was not being expressed in the soluble protein fraction. We tested these possibilities and found that syncytin-A recombinant ectodomain was being expressed in the insoluble inclusion body fraction (Figure 4.6).

Inclusion bodies in *E. coli* are densely packed denatured protein molecules which are formed as particles (290). Proteins from inclusions bodies are devoid of biological activity and generally require complicated solubilisation, refolding and purification procedures to recover a functionally active product (290). The intracellular expression of recombinant proteins is common even when an expression system with secretion signals is used. The expression from strong promoters such as T7 has been observed to increase the tendency for intracellular aggregation (291). The pET 28-b vector system uses the T7 promoter. Despite the problems associated with the formation of inclusion bodies they can be advantageous as the expression of protein in inclusion bodies increases the homogeneity of the protein of interest (290).

There is no universal method for the refolding of proteins from inclusion bodies and the usual course is to test out a number of previously published refolding protocols. The protocol used for syncytin-A ectodomain was an in-house protocol based upon the general methods published in “Purifying Challenging Proteins” (292). Inclusion bodies were extracted in a highly denaturing buffer containing 6M Guanidine Hydrochloride to dissociate all proteins. Proteins were then purified using Hitrap IMAC HP methods and eluted in standard elution buffer following wash steps (Figure 4.9 A-B). These steps

removed the highly denaturing buffer from the sample and put the protein in an environment which encouraged re-folding. Syncytin-A recombinant protein was then purified using the Superdex column using 25mM HEPES, 150mM NaCl pH7.4 (Figure 4.9 C-F). This buffer was used as it had been shown to encourage correct folding in the production of syncytin-1, -2 and -B recombinant ectodomains.

Using these methods, syncytin-A recombinant ectodomain was purified as an oligomeric protein which eluted with the same pattern as other placental syncytins on the Superdex 75 10/300 GL size exclusion column (Figure 9 E and F). Using this purification procedure, the proteins exhibited a tendency to aggregate even after solubilisation as shown by the tendency of the fraction to precipitate following vacuum centrifugation. This aggregation prevented the purification of high quantities of syncytin-A ectodomain in these experimental procedures.

The results shown in this chapter detail the generation of oligomeric syncytin recombinant ectodomain proteins from *E. coli*. A standard soluble protein extraction procedure was used for syncytin-1, -2 and -B. Syncytin-A was expressed in inclusion bodies. These recombinant proteins were purified using Hitrap-IMAC affinity chromatography followed by gel filtration using the Superdex 75 10/300 GL column. Purified proteins were shown by an elution peak at approximately 11ml and also by a smaller peak at 10ml. While fraction 11-12 corresponds to the expected elution profile for globular proteins, fraction 10 peak has the characteristic oligomeric protein profile of retroviral recombinant proteins as observed by SDS-PAGE. These data, coupled with LCMS sequencing data have demonstrated that the experimental procedure described in this chapter produces purified recombinant syncytin ectodomain proteins. Biological activity of these proteins will be described in the following chapter.



## **Chapter 5 - Immunosuppressive Properties of the Placental Endogenous Retroviral Envelope Protein Syncytin-1**

## **5.1 Abstract**

Retroviral envelope proteins have previously been shown to inhibit Th1 immune responses and induce a shift towards Th2 immune responses. The human placenta expresses ERV envelope proteins, including syncytin-1. Syncytin-1 is highly expressed in the syncytiotrophoblast and is thought to be a key factor in regulating syncytialisation due to its fusogenic properties. Syncytin-1 also carries a sequence homologous to the consensus ISD of retroviral envelope proteins. In light of this information, we hypothesised that syncytin-1 has, in addition to its known fusogenic function, immunosuppressive properties and therefore may play a role in maternal immune tolerance towards the fetus. Syncytin-1 recombinant ectodomain, carrying the ISD, inhibited the production of TNF- $\alpha$  and CXCL10 from whole blood cultures following maximal stimulation with LPS. A maximum of 50% TNF- $\alpha$  and 65% CXCL10 inhibition was observed following 1 $\mu$ M syncytin-1 treatment. Syncytin-1 recombinant ectodomain also inhibited the production of IFN- $\gamma$  by 30% in phytohaemmagglutinin (PHA) stimulated PBMC. These data suggest that syncytin-1 may mediate the shift from Th1 to Th2 cytokines observed during pregnancy.

## **5.2 Introduction**

The mechanisms behind maternal immune tolerance for the fetus have been speculated upon since the hypotheses of Sir Peter Medawar in 1953 (16). More recently, novel factors have been identified which may play an integral role in maternal immune tolerance. In particular, the discovery of the ERV envelope protein, syncytin-1, which is expressed at high levels in the placenta (293) has led to speculation that syncytin-1 may have an important role in maternal immune tolerance during pregnancy (162).

From as early as the 1970s, electron microscopy observations have identified retroviral-like particles within both human and animal placental tissue (158). Retroviral sequences have been identified in the human genome and the expression of ERV mRNA and protein has been demonstrated in the placenta (107, 108, 161, 265, 294). The high abundance of ERV in placental and embryonic tissues has led to the suggestion that ERV proteins may have a physiological function in this environment (159, 160).

In 1999, a placental specific ERV envelope protein belonging to the HERV-W family was identified (212) and in 2000 this protein was denoted syncytin (syncytin-1) (107). Syncytin-1 was found to be expressed in syncytiotrophoblast cells and shown to have a role in the cell-cell fusion of the cytotrophoblast to form the syncytiotrophoblast. Later on, in a genome wide study of fusigenic ERV envelope genes, the cytotrophoblast specific syncytin-2 was identified (108). We now know that human (107, 108), mouse, rat, gerbil, vole, hamster (109) and most recently rabbit (295) all specifically express in their placentas endogenous retroviral envelope proteins which appear to play an important biological role.

In addition to their role in cell-cell fusion, it has been hypothesised that placental ERV envelope proteins may have a role in maternal immune tolerance (211). Retroviral infections are commonly associated with immunosuppression in many species which

can result in susceptibility to other infections (192). The majority of studies on the immunosuppressive effects of retroviruses have focused on exogenous retroviruses (194, 209, 267) however there is growing evidence that ERVs may play an immunosuppressive role at the maternal-fetal interface (210, 211). The source of this immunosuppression is a highly conserved amino acid sequence called the ISD present in the transmembrane subunit of the envelope protein of most retroviruses (248).

A synthetic peptide with the 17 amino acid consensus sequence of the ISD called CKS-17, was originally produced by Cianciolo *et al.*, (248) and has been extensively studied (197-201, 204-207, 296). Initially, CKS-17 was shown to inhibit the proliferation of an IL-2 dependent murine cytotoxic T cell line as well as alloantigen stimulated proliferation of murine and human lymphocytes (248). Since these initial studies by Cianciolo, CKS-17 has been shown to have important immunological effects ultimately resulting in an inhibition of cell-mediated immunity (Th1 type responses) and a shift towards humoral immunity (Th2 type responses) (197-201, 204-207, 296). The maintenance of pregnancy has also been correlated with a shift from Th1 to Th2 type responses (89).

The immunosuppressive properties of the retroviral ISD have been further explored *in vivo*. Exogenous retroviruses such as MMuLV (209) and Mason Pfizer Monkey Virus (MPMV) (267) as well as the ERV, HERV-H (210) have been shown to have immunosuppressive properties using an *in vivo* mouse tumour model. Allogeneic tumour cells that would normally be rejected by the mice were transfected with the envelope protein of the retroviruses and tumour cell growth and proliferation was examined. Retroviral envelope expression was able to block immune-mediated elimination of the tumour cells (209, 210, 267). Interestingly, in studies on MPMV, this immunosuppression was shown to be specific to tumour cells expressing the envelope protein, as tumour cells not expressing the envelope protein injected simultaneously into the same mouse were rejected (209).

From these studies it is clear that ERV envelope proteins can possess immunosuppressive properties and that some aspects of the change in maternal immune function during pregnancy may be explained by the expression of the ERV envelope protein syncytin-1, in the syncytiotrophoblast cells of the placenta. However, while syncytin-1 has been extensively studied in regards to its role in cell-cell fusion, its potential role in maternal immune function is yet to be characterized. The study presented in this chapter aimed to assess the immunosuppressive properties of the syncytin-1 recombinant ectodomain *in vitro*, using a biologically relevant whole blood culture system. This system was used as it allows for the assessment of basic immune mechanisms without preparation and cultivation artifacts. Further this system is biologically relevant because blood cells are employed in their physiological proportions and environment. The hypothesis for this study is:

*“That the endogenous retroviral envelope protein syncytin-1, which is expressed by syncytiotrophoblast cells of the human placenta, can inhibit the production of Th1 cytokines”*

In order to address this hypothesis, the immunosuppressive properties of the recombinant ectodomain of syncytin-1 were examined *in vitro*, using a biologically relevant whole blood culture system. This system was used as it allows for the assessment of basic immune mechanisms without preparation and cultivation artifacts. Further, this system is biologically relevant because blood cells are employed in their physiological proportions and environment. Using this model, the Th1 cytokines examined (TNF- $\alpha$  and IFN- $\gamma$ ) were chosen as previous studies on the immunosuppressive properties of retroviral envelope proteins have shown an inhibition of TNF- $\alpha$  and IFN- $\gamma$  (202). In addition, as chemokines are suggested to play an important role in determining the outcome of pregnancy (297-301), CXCL10 was studied. This chemokine is known to be induced by Th1 cytokines, including TNF- $\alpha$  and IFN- $\gamma$  (302-304) and therefore would be expected to be inhibited by syncytin-1.

## **5.3 Methods**

### **5.3.1 Recombinant Proteins**

#### **5.3.1.1 Production and Purification**

The recombinant proteins used in this study were generated following the detailed protocols described in chapter 4 “Characterisation of recombinant syncytin ectodomain proteins”. In brief, syncytin-1 recombinant ectodomain was cloned into the pET-28b vector (Novagen) using BspH1, Nco1 and Xho1 restriction enzymes. The proteins were expressed in *E. coli* BL21 (DE3) cells (Stratagene) as soluble cytoplasmic material, purified twice on a nickel coated Hitrap IMAC HP column, and then twice on a superdex 75 10\_300 GL column using FPLC buffer. Two fractions were collected: fraction 10 and fraction 12. As a control, pET-28b plasmid without insert was expressed in *E. coli* BL21 (DE3) cells and purified using the same protocol.

#### **5.3.1.2 Quantification of Recombinant Protein Concentration**

The concentration of syncytin-1 recombinant protein was calculated using the BCA protein assay kit (Pierce) following the manufacturer’s instructions (<http://www.piercenetcom/files/1296as8.pdf>). Basically, syncytin-1 recombinant protein was diluted 1:3 in milliQ water and 25µl of each sample was added to a 96-well plate. Working reagent (200µl of 50 parts working reagent A: 1 part of working reagent B was added to each well and the plate was mixed for 30 seconds on a plate shaker before being covered and incubated at 37°C for 30 minutes. The plate was cooled to RT before measuring the absorbance at 570nm using the Thermo Labsystem Original Multiskan EX plate reader. The sensitivity of the assay was 2mg/ml - 25µg/ml.

#### 5.3.1.3 Endotoxin Calculation

The endotoxin concentration in the purified recombinant proteins was calculated using the Limulus Amebocyte Lysate (LAL) QCL-1000® endotoxin assay (Lonza) following the manufacturer's instructions

([http://www.lonza.com/group/en/products\\_services/products/catalog\\_new.ParSys.0007.File0.tmp?path=eshop/Instructions/Endotoxin\\_Detection/802589-LONZA\\_INSERT\\_20PG.pdf](http://www.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop/Instructions/Endotoxin_Detection/802589-LONZA_INSERT_20PG.pdf)). Basically, a 96-well plate was pre-equilibrated at 37°C in a heating block prior to beginning the assay. Throughout the assay the plate was kept at this temperature. Samples were diluted 1:50 to a final volume of 50µl in LAL reagent water, and added to the plate. In blank wells, 50µl of LAL reagent water was added. An equal volume of LAL was added to each well sequentially followed by gentle mixing. At 10 minutes, 100µl of substrate solution (prewarmed to 37°C) was added followed by mixing. The reaction was stopped by the addition of stop solution (25% acetic acid in dH<sub>2</sub>O) at 16 minutes. The absorbance at 410nm was detected using the Thermo Labsystem Original Multiskan EX plate reader. The sensitivity of the assay was 0.1 endotoxin units (EU)/ml which is the equivalent of 10pg endotoxin/ml.

#### 5.3.2 Blood Collection

Venous blood was collected from healthy female adult volunteer donors with no known history of pregnancy and no clinical evidence of infection or immunological compromise. The age range of participants was 22 to 29 years with an average of 26 years.

##### 5.3.2.1 Whole Blood Culture

A whole blood culture system was chosen to assess the immunosuppressive properties of the syncytin-1 recombinant ectodomain proteins (fractions 10 and 12) using LPS as a stimulus. Collected venous blood samples were diluted 1:5 in blood culture buffer and

loaded into 96 well-plates containing different concentrations of the syncytin-1 recombinant protein (diluted in blood culture buffer). Recombinant proteins/diluted blood were thoroughly mixed and incubated for 2 hours at 37°C, 5% CO<sub>2</sub> prior to the addition of 10µg/ml (maximum stimulating dose) LPS. In the LPS dose response experiments, diluted blood was incubated with different concentrations of LPS (1ng-50µg/ml). Whole blood was also incubated without LPS stimulus and/or recombinant protein as controls for TNF-α release.

Following incubation for 24 hours at 37°C, 5% CO<sub>2</sub>, the supernatant was collected and stored at -80°C until further use.

#### 5.3.2.2 Peripheral Blood Mononuclear Cell (PBMC) Culture

A human PBMC culture system was chosen for analysis of the immunosuppressive properties of syncytin-1 recombinant protein following stimulation with PHA. The samples of blood were diluted 1:1 in Hanks Balanced Salt solution (HBSS) (Gibco) and layered on a Ficoll gradient using a 3:1 ratio of blood to Ficoll-Paque PLUS (GE Healthcare). The Ficoll gradient was centrifuged at 400 x g for 40 minutes without the centrifuge brake on. The lymphocyte layer, which was present at the interface was transferred to a falcon tube containing at least 3 volumes of HBSS and gently resuspended using a pasteur pipette. This fraction was centrifuged at 500 x g for 10 minutes to pellet the PBMC. The supernatant was removed and the lymphocytes were resuspended in an equal volume of HBSS before repeating the centrifugation step. The final PBMC pellet was resuspended in RPMI 1640 media. An aliquot of these cells was taken for PBMC cell counting using a haemocytometer. Cell viability was determined using the trypan blue exclusion assay. A total of 500 000 cells/well were loaded into 24 well plates containing different concentrations of the syncytin-1 recombinant protein (diluted in blood culture buffer) and incubated at 37°C, 5% CO<sub>2</sub> for 48 hours in the presence of 2µg/ml PHA. Following incubation for 48 hours, the PBMC supernatant was collected and stored at -80°C until further use.



### 5.3.3 Cell Viability

#### 5.3.3.1 Lactate Dehydrogenase (LDH) Activity Assay

The Cytotox 96® Non-radioactive Cytotoxicity Assay (Promega) was used to measure cell cytotoxicity following LPS treatment of human blood containing syncytin-1 recombinant proteins. This method was used following the manufacturer's instructions (<http://www.promega.com/tbs/tb163/tb163.pdf>).

Briefly, 50µl of each whole blood sample treated with LPS and syncytin recombinant proteins was added to wells in a 96 well plate. To these wells, 50µl of substrate mix was added. The samples were slowly mixed and incubated for 30 minutes in the dark at RT. To stop the reaction, 50µl of LDH stop solution was added and the absorbance read at 490nm using the Thermo Labsystem Original Multiskan EX plate reader. As a positive control, lysed blood was used. As a negative control, supernatant from non-treated blood was used. Blood without substrate mix was also used as a control to measure the background absorbance.

### 5.3.4 Cytokine Production

#### 5.3.4.1 ELISA

The supernatants from whole blood cell cultures were assayed for TNF- $\alpha$  using a commercially available ELISA kit (BD Pharmingen ELISA kit). The supernatants from PBMC cultures were assayed for IFN- $\gamma$  using a commercially available ELISA kit (BD Pharmingen ELISA kit). All ELISAs were performed using matched antibody pairs according to the manufacturer's instructions. All dilutions performed in this assay were lot specific.

ELISA plates were coated overnight at 4°C with 100µL per well capture antibody diluted in coating buffer. Coated plates were washed three times with PBS/0.05% Tween-20 and blocked with 200µl/well assay diluent for 1h at RT. The plates were again washed three times with PBS/0.05% Tween-20 and 100µl/well of standards and samples were added and incubated for 2 hours at RT. After 5 wash cycles, the plates were incubated for 1h with working detector solution (detection antibody plus streptavidin-peroxidase in assay diluent buffer). After 7 washes with 30 seconds soaking in between, 100µl/well tetramethylbenzidine and hydrogen peroxide liquid substrate solution (BD Pharmingen) was added and the plates were incubated at RT for 30 min in the dark. After addition of 50µl/well stop solution, the absorption was measured at 450- and 570-nm with the Thermo Labsystem Original Multiskan EX plate reader and concentrations of TNF- $\alpha$  or IFN- $\gamma$  were calculated. The sensitivity of the assays was 7.8pg/ml for TNF- $\alpha$  and 4.7pg/ml for IFN- $\gamma$ . Wavelength correction was performed by subtracting the absorbance at 570nm from the absorbance at 450nm.

#### 5.3.4.2 Bead Array

The supernatants from whole blood stimulated with 10µg/ml LPS and 1µM syncytin-1 fraction 10 were assayed for IFN- $\gamma$ , IL-10, IL-13 and CXCL-10 (IP-10) using the BD CBA Human Soluble Protein Flex Set (BD Pharmingen). The supernatants for all of the samples were diluted with an equal volume of assay diluent to give a 1:2 dilution factor.

Standards were prepared for CXCL10 to be analysed. For the assay, a lyophilised vial of CXCL10 was pooled into a 15ml tube and reconstituted with 4ml assay diluent. The standard was allowed to equilibrate prior to making dilutions. While the standard was equilibrating, capture beads were prepared by dilution in capture bead diluent (1µl of each capture bead to a final volume of 50µl per well). Then, 50µl of sample/standard followed by 50µl capture beads were added to each tube and they were mixed gently and incubated for 1 hour at RT in the dark. Following the incubation, 50µl mixed PE detection reagent (BD Pharmingen) detection reagent was added to each tube and they

were mixed gently and incubated for 2 hours at RT in the dark. After the addition of 1ml wash buffer, the tubes were centrifuged for 5 minutes at 200 x g. The supernatant was aspirated from each assay tube and the beads were resuspended in 300µl wash buffer by a brief vortex. The samples were then transferred to a 96 well flat bottomed ELISA plate (NUNC). Cytokine levels were determined on the BD FACS Canto®II using BD FACS diva software. The sensitivity of the assay was 10pg/ml for all cytokines examined.

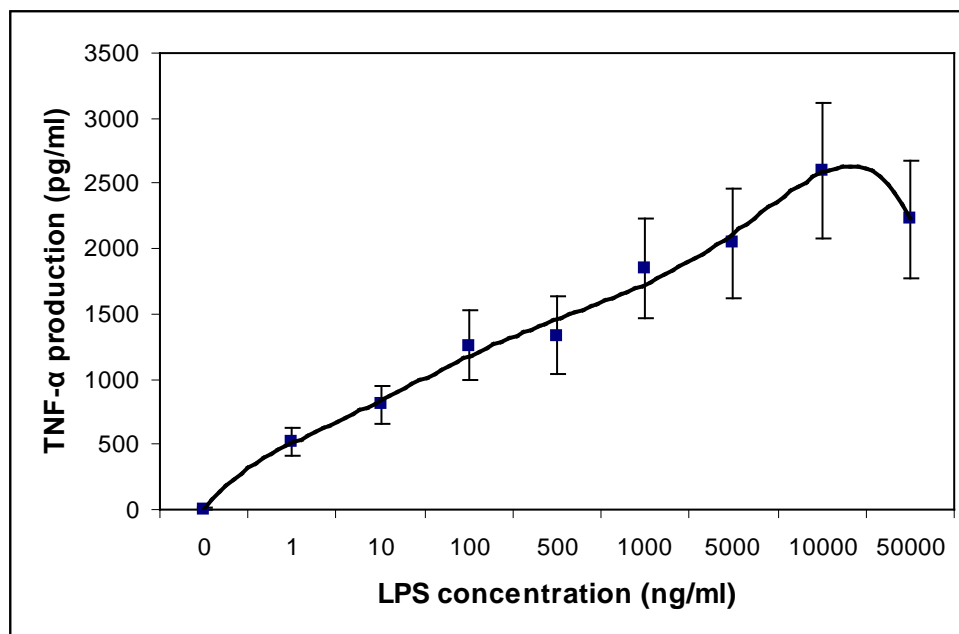
#### 5.3.5 Statistics

R software (305) was used for statistical analysis. A Welch two sample t-test was used to model the relative IFN- $\gamma$  and CXCL10 expression following syncytin-1 treatment. As the data did not meet the assumptions of normal distribution and equal variance, Wilcoxon rank-sum tests (non-parametric tests of group medians) were then used to test differences between the groups. Graphics show means and standard error bars.

A one way analysis of variance (ANOVA) was used to model the differences between no treatment and treatment groups in the TNF- $\alpha$  assays of syncytin-1 recombinant protein treated whole blood. As the data did not meet the assumptions of normal distribution and equal variance, a Kruskal Wallis rank-sum test (non-parametric equivalent of one way ANOVA) was performed. Wilcoxon rank-sum tests (non-parametric tests of group medians) were then used to test differences between each individual treatment group. Graphics show means and standard error bars.

## 5.4 Results

### 5.4.1 TNF- $\alpha$ Response to LPS stimulation

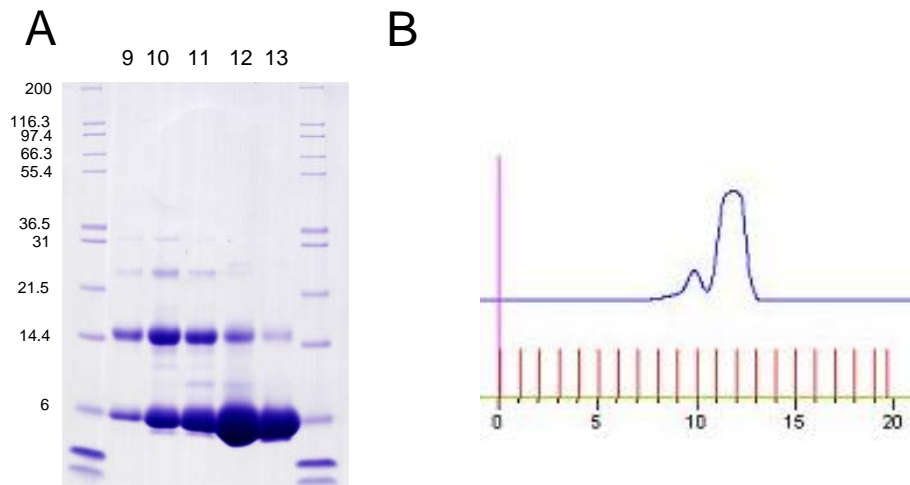


**Figure 5.1.** TNF- $\alpha$  secretion in whole blood cells in response to different concentrations of LPS. LPS dose response experiment showing stimulation of TNF- $\alpha$  production by whole human blood.. Whole human blood was diluted in 25mM HEPES, 150mM NaCl, pH7.4 to give a final concentration of 500 000 cells/well and incubated for 24 hours at 37°C with different concentrations of LPS. The amount of TNF- $\alpha$  secreted by culture media alone was at the detection limit of our assay. The maximum stimulating dose of LPS was 10000ng/ml (10 $\mu$ g/ml). The data are from 4 independent experiments and the error bars refer to standard error.

To validate the experimental model, the production of TNF- $\alpha$  was evaluated by ELISA in response to different concentrations of LPS. The TNF- $\alpha$  response to LPS was measured from four donors. A high level of individual difference in levels of TNF- $\alpha$  was observed between donors but the overall dose dependent pattern was comparable. As detailed in Figure 5.1, the maximum stimulating dose of LPS was 10 $\mu$ g. These data are consistent with the literature and the maximum stimulating dose of LPS was used for subsequent experiments to test the immunosuppressive properties of syncytin recombinant ectodomain proteins.

#### 5.4.2 Characterisation of the Immunological Properties of Syncytin-1 Recombinant Ectodomain

Recombinant proteins were produced in *E. coli* and initially purified using Hitrap IMAC affinity chromatography. Proteins were further purified using gel filtration in 25mM HEPES, 150mM NaCl, pH 7.4. Using this methodology, 2 main peaks were observed to elute from the column; a peak at 10ml and a peak at 12 ml (Figure 5.2). Of these fractions, fraction 10 carried the typical protein profile of a multimeric retroviral ectodomain protein which is required for immunosuppression (277, 280, 287, 289).

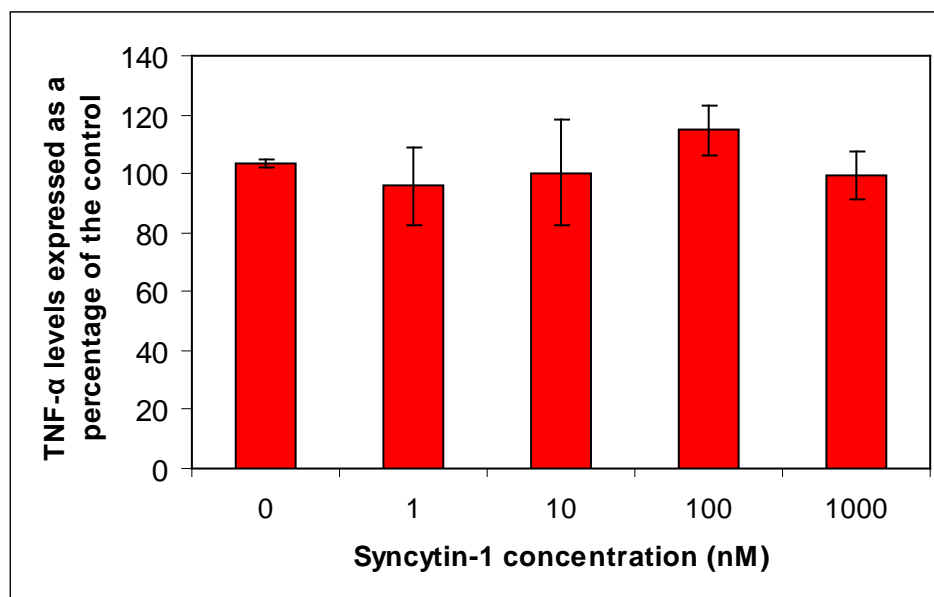


**Figure 5.2. Syncytin-1 recombinant ectodomain elution profile.** A) SDS-PAGE of purified syncytin-1 ectodomain. B) FPLC chromatogram of purified syncytin-1 ectodomain. Syncytin-1 recombinant ectodomain was produced in *E.coli* as a soluble His-tagged protein and purified using affinity chromatography (Hitrap) and gel filtration chromatography (Superdex 75 10/300GL). The recombinant protein eluted as two peaks; a smaller 10ml peak (Fraction 10) and a 12ml peak (Fraction 12) which corresponded to the majority of protein in the sample.

#### 5.4.2.1 Immunosuppressive Properties of Syncytin-1 Recombinant Ectodomain

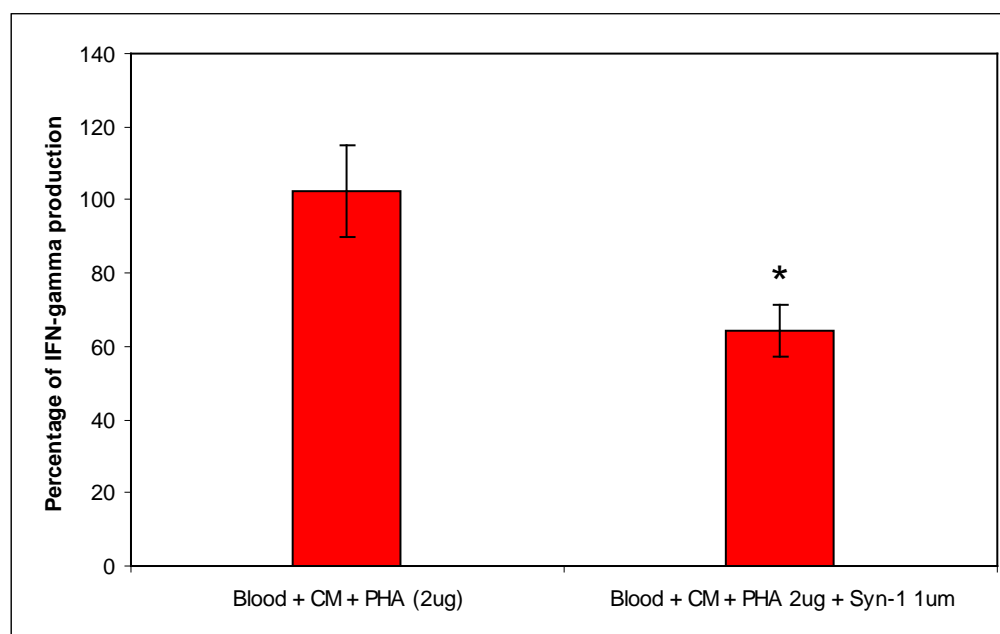
To examine the effect of syncytin-1 recombinant proteins on the TNF- $\alpha$  response, whole blood was incubated in the presence of maximal stimulating doses of LPS at 37°C for 24 hours with different concentrations of the syncytin-1 recombinant protein.

Initial studies characterised the immunosuppressive properties of the different fractions eluted from the FLPC by testing the inhibition of the production of TNF- $\alpha$  in whole blood cultures. As expected, fraction 12, which had the protein profile of an intermediately folded retroviral ectodomain (Figure 5.2), was unable to inhibit the production of TNF- $\alpha$  at any concentration studied (Figure 5.3). Further, the addition of fraction 12 to whole blood did not increase cellular toxicity as assessed by the LDH activity assay (Figure 5.5).

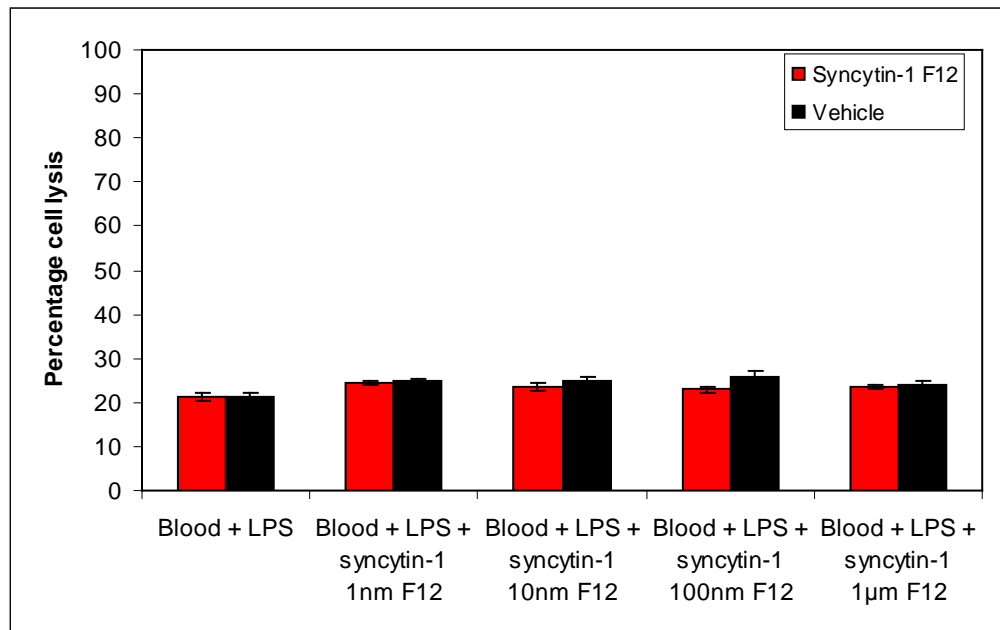


**Figure 5.3. Inhibition of TNF- $\alpha$  production using syncytin-1 recombinant protein from fraction 12.** No decrease in TNF- $\alpha$  production from whole blood was observed following maximum stimulating doses of LPS (10ug/ml) and treatment with different concentrations of syncytin-1 recombinant protein from fraction 12. Data are the mean from 4 individual experiments and the error bars refer to standard error.

As IFN- $\gamma$  is an important Th1 cytokine which induces cytotoxic and inflammatory reactions and can terminate normal pregnancy when injected into pregnant mice (20, 90, 253) the production of IFN- $\gamma$  was examined in a separate experiment. In order to examine the production of IFN- $\gamma$ , PBMC were isolated from whole blood and treated with 2 $\mu$ g/ml PHA at 37°C for 48 hours with 1 $\mu$ M syncytin-1 recombinant protein from fraction 12. The levels of IFN- $\gamma$  were readily detected in this assay. A 35% inhibition of IFN- $\gamma$  was observed following treatment with 1 $\mu$ M syncytin-1 recombinant protein (Figure 5.4).



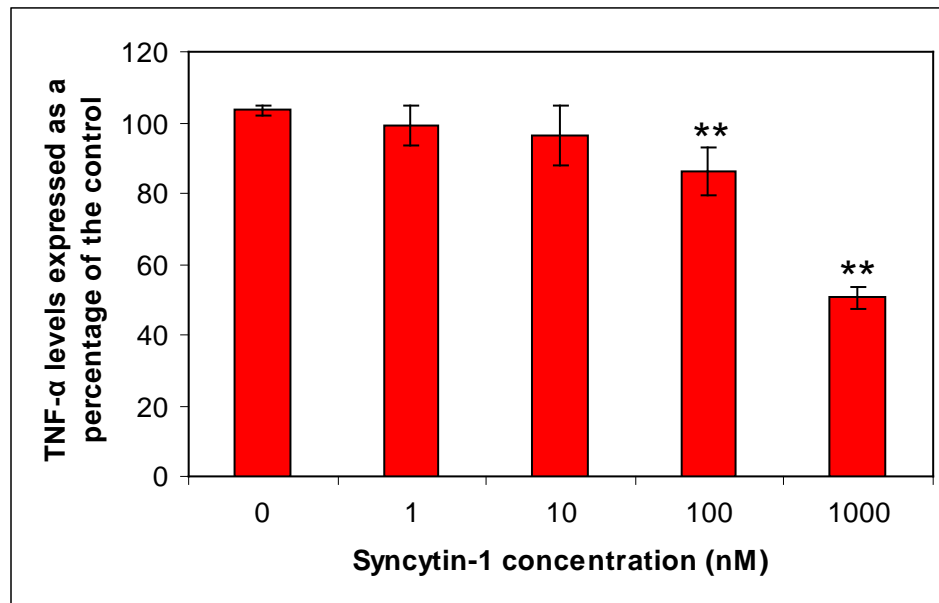
**Figure 5.4. Inhibition of IFN-gamma production in PHA stimulated whole blood using syncytin-1 recombinant protein.** A 30% inhibition of IFN-gamma compared to the vehicle control was observed using 1 $\mu$ M syncytin-1 recombinant protein following treatment of PBMC with PHA. \* -  $P < 0.05$  compared to Blood + CM + PHA (2 $\mu$ g). Data are the mean from one donor PBMC sample analysed in triplicate. The error bars refer to standard error.



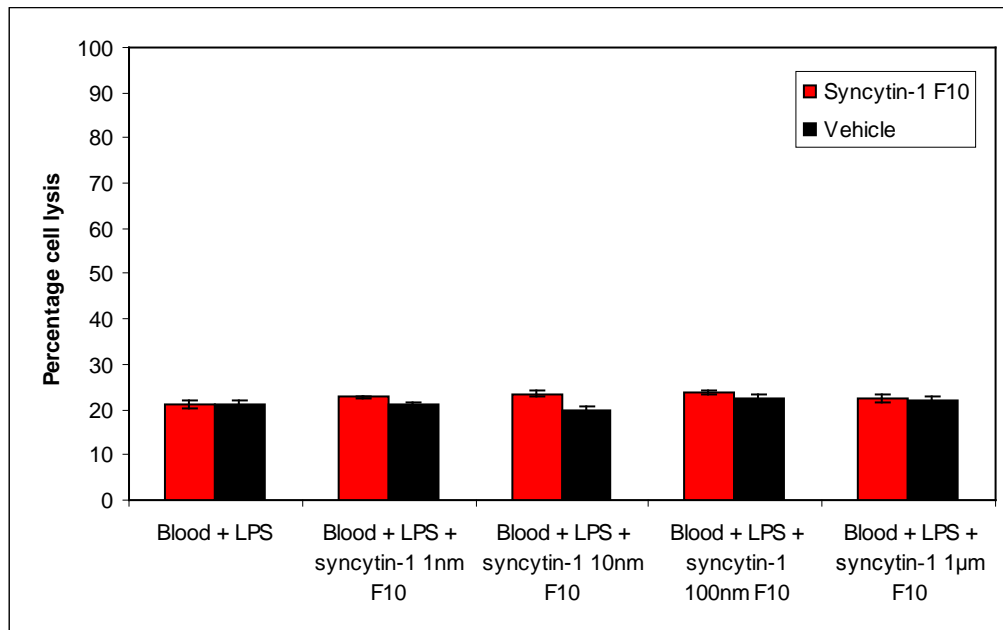
**Figure 5.5. Cytotoxicity of whole blood cells stimulated with LPS and treated with syncytin-1 recombinant protein from fraction 12.** Cytotoxicity of whole human blood cells treated with maximum stimulating doses of LPS and syncytin-1 recombinant protein was measured by the LDH assay. No significant difference in LDH was observed between treatments and vehicles at any concentration of syncytin-1 recombinant protein. Data are the mean from 3 individual experiments and the error bars refer to standard error.

In contrast to fraction 12, fraction 10 was able to inhibit the production of TNF- $\alpha$  in a dose dependent manner. Figure 5.6 shows a dose responsive inhibition in the production of TNF-alpha by syncytin-1 recombinant protein from fraction 10 in whole blood stimulated with LPS. A maximum inhibition of TNF- $\alpha$  (50%) was observed using 1 $\mu$ M syncytin-1 recombinant protein (Figure 5.6). Following the combination of fraction 10 and 12 a similar response was observed with more variability in the lower concentrations (Figure 5.8). The inhibition of TNF- $\alpha$  in both of these fractions was not purely a secondary effect due to nonspecific toxicity of the recombinant syncytin-1 ectodomain to blood cells as assessed by the LDH activity assay (Figure 5.7 (F10) and 5.9 (F10-12)).

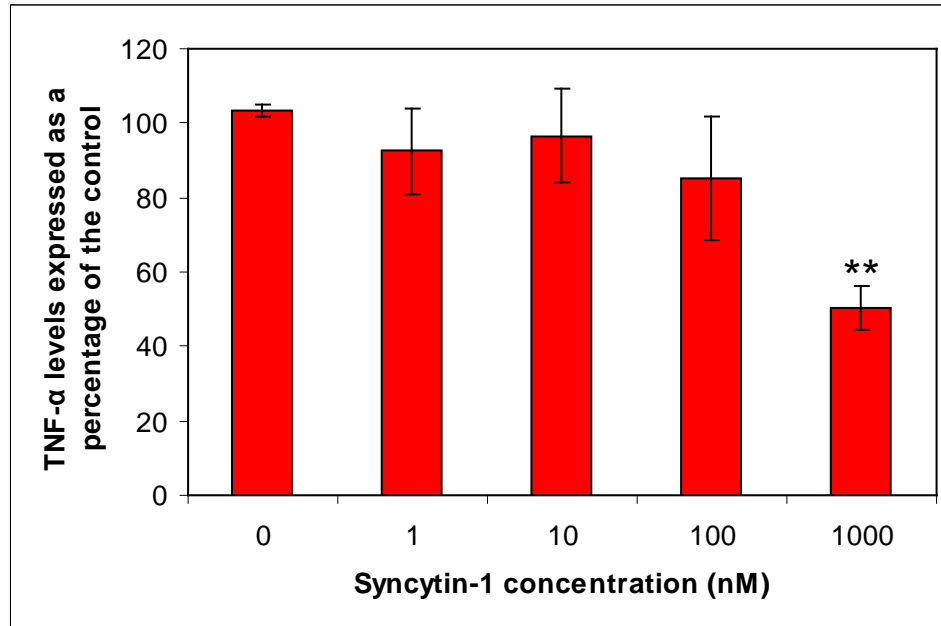




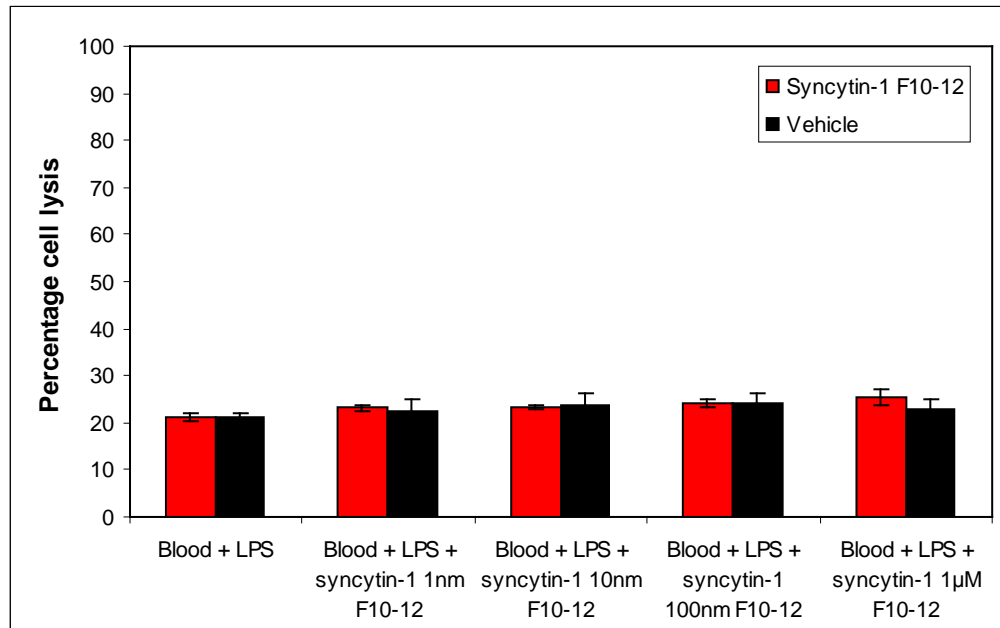
**Figure 5.6. Inhibition of TNF-alpha production using syncytin-1 recombinant protein from fraction 10.** A dose dependent decrease in TNF-alpha production from whole blood was observed following maximum stimulating doses of LPS (10ug/ml) and treatment with different concentrations of syncytin-1 recombinant protein fraction 10. An inhibition of 50% TNF-alpha compared to the vehicle control was observed using 1uM syncytin-1 recombinant protein. Data are the mean from 5 individual experiments and the error bars refer to standard error. \*\* -  $P < 0.01$  compared to the no treatment control.



**Figure 5.7. Cytotoxicity of whole blood cells stimulated with LPS and treated with syncytin-1 recombinant protein from fraction 10.** Cytotoxicity of whole human blood cells treated with maximum stimulating doses of LPS and syncytin-1 recombinant protein was measured by the LDH assay. No significant difference in LDH was observed between treatments and vehicles at any concentration of syncytin-1 recombinant protein. Data are the mean from 3 individual experiments and the error bars refer to standard error.

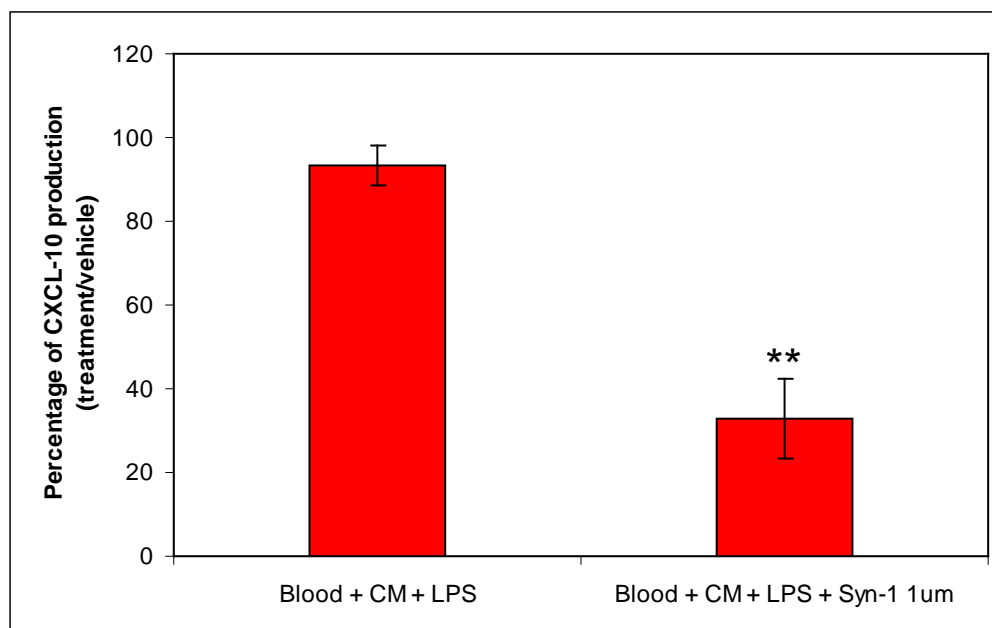


**Figure 5.8. Inhibition of TNF-alpha production in whole blood stimulated with LPS using syncytin-1 recombinant protein from fraction 10-12.** A dose dependent decrease in TNF-alpha production was observed following maximum stimulating doses of LPS (10ug/ml) and treatment with different concentrations of syncytin-1 recombinant protein. An inhibition of 45% TNF-alpha compared to the vehicle control was observed using 1uM syncytin-1 recombinant protein. Data are the mean from 5 individual experiments and the error bars refer to standard error. \*\* -  $p < 0.01$  compared to the no treatment control.



**Figure 5.9. Cytotoxicity of whole blood cells stimulated with LPS and treated with syncytin-1 recombinant protein from fraction 10-12.** Cytotoxicity of whole human blood cells treated with maximum stimulating doses of LPS and syncytin-1 recombinant protein was measured by the LDH assay. No significant difference in LDH was observed between treatments and vehicles at any concentration of syncytin-1 recombinant protein. Data are the mean from 3 individual experiments and the error bars refer to standard error.

To examine the effects of syncytin-1 recombinant protein on the production of the Th1 inducing chemokine CXCL10, whole blood was stimulated with maximal stimulating doses of LPS at 37°C for 24 hours with 1µM syncytin-1 F10 proteins and CXCL10 levels were assayed by bead array. At a concentration of 1µM syncytin-1 recombinant protein, a 67% inhibition of CXCL10 was observed compared to the vehicle control (Figure 5.10). Using bead array, IFN-γ production was also measured from these samples. At the time point used in this study, IFN-γ levels were below the limit of detection for most samples (data not shown). However, there was a tendency for detectable readings in the samples treated with LPS alone suggesting an inhibition of IFN-γ by syncytin-1.



**Figure 5.10. Inhibition of CXCL10 production in LPS stimulated whole blood using syncytin-1 recombinant protein fraction 10.** An inhibition of 65% CXCL10 compared to the vehicle control was observed using 1µM syncytin-1 recombinant protein following treatment of whole blood with maximal stimulating doses of LPS (10ug/ml). Data are the mean of 5 individual experiments and the error bars refer to standard error. \*\* -  $p < 0.01$  compared to Blood + CM + LPS.

## **5.5 Discussion**

It is well established that retroviral infections with both exogenous and endogenous retroviruses can induce severe immunosuppression in humans and animals. This immunosuppression has been observed both *in vitro* and *in vivo* and has been related to the highly conserved retroviral ISD sequence which is present in the transmembrane subunit of the envelope protein of most retroviruses. Studies using the consensus ISD peptide (CKS-17), UV-inactivated FeLV and HIV protein preparations have shown immunosuppressive effects on lymphocytes, monocytes, macrophages, NK and T killer cells (192, 196, 198-201, 204-207, 254, 296, 306-311). This highly conserved retroviral ISD sequence is found in syncytin-1 leading to the suggestion that syncytin-1 may have immunosuppressive properties.

In support of a role for syncytin-1 in immunosuppression, recent studies have shown that both syncytin-1 and CKS-17 can activate similar signalling pathways. The immunosuppressive effects of CKS-17 have been shown to be mediated by a variety of intracellular signaling molecules and treatment with CKS-17 can elevate cAMP levels and induce phosphorylation of extracellular signal-regulated kinase (ERK 1 and 2), mitogen-activated protein kinase/ERK kinase (MEK), Raf1, phospholipase C gamma and phosphoinositide-3 kinase (PI3-K) (reviewed in (312)). The syncytin-1 ISD peptide has been shown to activate the phosphorylation of ERK2 (MAPK1), Akt1, Akt2, Akt pan and GSK-3 $\alpha/\beta$  in preliminary studies (190). The activation of PI3-K and ERK2 by CKS-17 and the activation of Akt, which is an important downstream factor in the PI3-K pathway (313), and ERK2 by syncytin-1 suggests that these peptides may activate similar signalling pathways and may have similar immunosuppressive functions.

For the assessment of the immunosuppressive properties of syncytin-1 in the study presented in this chapter, the recombinant ectodomain protein, which contains the highly conserved retroviral ISD was used. The recombinant protein was tested *in vitro* using a

biologically relevant whole blood system stimulated with the polyclonal activators LPS and PHA. These stimuli are commonly used *in vitro* to stimulate the innate or adaptive immune response and have previously been used to stimulate the cytokines examined in this study (190, 206, 314-323). Further, these stimuli have previously been used as an immune stimulating reagent in studies of the immunology of pregnancy as well as the immunosuppressive effects of ERVs (190, 206, 319-323). To stimulate whole blood cultures, the maximal stimulating dose of LPS (10µg/ml) was used and while this dose can potentially mask the extent of the inhibition of cytokine production, graft rejection (such as fetal rejection in pregnancy) is characterised by a massive influx of cytokines (300, 324, 325) and therefore molecules which are involved in the inhibition of cytokine production during pregnancy should be capable of responding to high levels of stimulus.

In the study presented in this chapter, the inhibition of the Th1 cytokines, TNF- $\alpha$  and IFN- $\gamma$ , as well as the Th1 chemokine CXCL10 was observed following treatment with the syncytin-1 recombinant protein. As expected, the multimeric protein (fraction 10) was required for maximal immunosuppression with the partially folded fraction 12 being unable to inhibit TNF- $\alpha$ . However, fraction 12 was able to partially inhibit IFN- $\gamma$  suggesting that this fraction possesses some immunosuppressive properties. These observations are supported by numerous studies which have shown that multimeric retroviral ectodomains are required for maximal immunosuppression (277, 280, 287, 289). For example, Ruegg *et al.*, (287) showed that higher concentrations of monomeric Rauscher murine leukaemia virus p15E are required to inhibit IL-2 dependent proliferation of the murine T cell line CTLL-2 compared to multimeric preparations.

The observation in this study that syncytin-1 is able to inhibit Th1 responses is particularly relevant for pregnancy given the shift from Th1 to Th2 cytokine responses that is observed in pregnancy (20, 89). Studies on the modulation of Th1 and Th2 responses by CKS-17 have shown an inhibition of the Th1 cytokines TNF- $\alpha$ , IFN- $\gamma$  and IL-2 coupled with an induction of the Th2 cytokine IL-10 (202, 254, 326). In pregnancy, Th1 cytokines which are deleterious for pregnancy such as TNF- $\alpha$ , IFN- $\gamma$  and IL-2 are downregulated (20) while Th2 cytokines such as IL-4, IL-5 and IL-10 are upregulated

(20, 90, 327). Studies have shown that the presence of high levels of Th1 cytokines may lead to adverse effects for the fetus. For example, high levels of TNF- $\alpha$  and IFN- $\gamma$  result in the termination of pregnancy as well as the inhibition of embryonic and fetal development (87). Further, a greater bias toward a Th1 cytokine profile is observed in women with unexplained recurrent abortions or miscarriage (20). In contrast, the presence of Th2 cytokines appears to control Th1 dependent reactivity and promote fetal development (20, 88, 89, 328). While the study presented in this chapter did not examine the production of Th2 cytokines following treatment with syncytin-1, the fact that retroviral ISD proteins have previously been shown to induce the production of the Th2 cytokine IL-10 (254, 329) allows us to speculate that syncytin-1 will fulfil the parameters described for retroviral mediated modulation of Th2 responses and may potentially play an important role in the shift from Th1 to Th2 responses during pregnancy.

In addition to the regulation of cytokines during pregnancy, chemokine regulation has been suggested to play an important role in determining the outcome of pregnancy (297-301). Chemokines are known to promote the response, development and homeostasis of the immune system and an expression of chemokines have been observed in many pathological processes including preeclampsia (301, 330, 331). While there are no studies which detail the role of CXCL10 in the inhibition of chemokines, it has been shown that CXCL10 may play a role in the suppression of chemoattractant induced polarization of monocytes (332). Studies of HIV-1 have also shown that anti-HIV activity in LPS stimulated PBMCs is strongly associated with the production of beta-chemokines (333). The chemokine studied in this chapter was examined as it is known to be regulated by cytokines which are inhibited by CXCL10. CXCL10 can be induced by the Th1 cytokines TNF- $\alpha$  and IFN- $\gamma$  (302-304) and can be suppressed by the Th2 cytokine, IL-10 (314). High levels of CXCL10 are also associated with increased levels of inflammation in adverse pregnancy outcomes as well as in graft rejection (330, 331). Further, high levels of this chemokine have been implicated in states characterised by prominent Th1 responses such as multiple sclerosis, herpetic encephalitis in mice, experimental autoimmune encephalomyelitis, inflammatory bowel/colon disease and



type 1 diabetes (330). The observation that syncytin-1 can inhibit CXCL10 is the first study to our knowledge showing an inhibition of CXCL10 by a retroviral ISD. As CXCL10 is involved in Th1 responses, this observation further supports the evidence suggesting that syncytin-1 may inhibit Th1 responses during pregnancy.

All of this evidence suggests that the ERV envelope protein, syncytin-1, which is expressed at high levels in the placenta, may have immunosuppressive properties and participate in maternal immuntolerance. However, its role in the inhibition of immune responses remains controversial (188, 190, 213, 266).

In contrast to the data presented in this chapter, Mangeney *et al.*, (213) showed that syncytin-1 was not immunosuppressive using a mouse tumour cell model and recombinant ectodomain model. To evaluate the inhibition of the cellular immune response, Mangeney *et al.*, (213) injected MCA205 tumour cells transfected with the syncytin-1 or syncytin-2 retroviral envelope proteins into allogeneic BALB/c mice. Syncytin-2 expressing MCA205 cells were able to grow leading to the development of larger tumours that persisted for the length of the study. However, syncytin-1 expressing MCA205 cells, like control cells, were rapidly rejected.

Using a recombinant ectodomain approach, Mangeney examined humoural immunity in the context of Ig production and showed that syncytin-1 induced IgG titres 10-30 fold higher than syncytin-2. According to Mangeney, these data further illustrated that syncytin-1 did not have an immunosuppressive effect. However, an increase in IgG production is not necessarily an indicator of a lack of immunosuppressive function. Mitani *et al.*, (200) studied purified FeLV and showed that viable and UV-inactivated virus at low concentrations were capable of activating B lymphocytes to secrete IgG.

The findings of Mangeney *et al.*, (213) are rather puzzling as the localisation of the immunosuppressive syncytin-2 is within the villous cytotrophoblast cells where it is not directly exposed to the maternal circulation while syncytin-1 is localised in the maternal blood bathed syncytiotrophoblast (334, 335). Also, while syncytin-1 levels have been

shown to increase throughout pregnancy, syncytin-2 has a decreasing pattern of expression (336). Transcriptional levels of syncytin-2 are also about 10-fold lower than syncytin-1 in the first trimester and 40-fold lower at term.

While the data presented by Mangeney *et al.*, are contrary to the data presented in this chapter, it is important to note that the experimental conditions used to examine the immunosuppressive properties of syncytin-1 in the two studies are different. To examine cell-mediated immunity, Mangeney *et al.*, (213) overexpressed the whole syncytin-1 protein ectopically in an *in vivo* mouse tumour cell model. This tumour cell model has previously been used successfully to examine the immunosuppressive properties of retroviral proteins (210, 213, 267). However, it is important to note that the expression, folding and localisation of syncytin-1 within these tumour cells may not necessarily mimic that of syncytin-1 in the syncytiotrophoblast, bearing in mind that this study uses human proteins in a mouse tumour cell model. Studies have shown that correct maturation and processing of syncytin-1 is an essential requirement for syncytin-1 to possess fusogenic properties (166). Ectopic expression of syncytin-1 has been shown to reduce the amount of proteolytically cleaved mature protein in HeLa cells and alter the glycosylation status in CHO and HEK293 cells in comparison to BeWo cells (165, 273). Mangeney *et al.*, even stated that the amino acids present in the syncytin-1 ISD appeared to alter the expression, folding or trafficking of syncytin-1 in the tumour cells (213). Differential expression and cell sorting of syncytin-1 in various cell types may potentially explain the lack of immunosuppression observed in this model. If this is the case, then it is integral that future studies of syncytin-1 use a physiologically relevant model to examine its immunosuppressive properties.

The model used in this study used a physiologically relevant human whole blood system *in vitro*. Using this model, the potency with which the syncytin-1 recombinant protein inhibited cytokine secretion was consistently observed at micromolar concentrations, yet it is not expected that the native syncytin-1 protein would be found at these concentrations *in vivo*. While the purification of recombinant syncytin-1 allows us to generate proteins that may more closely resemble the native syncytin-1 structure, the

presence of the full length syncytin-1 may facilitate interaction between the protein and its cellular target. Indeed, it has been observed that the potency of a synthetic peptide containing the retroviral ISD increased 10-fold when present as a recombinant protein monomer and as much as 1000 fold when presented as the multimeric complex of the native protein (42).

While this study shows that syncytin-1 has immunosuppressive properties, the cytokines that are studied in this chapter are more relevant toward retroviral mediated immunosuppression than maternal immune tolerance. Due to time constraints, a complete analysis of Th1 and Th2 cytokines was not completed. Also, while the LDH assay showed us that this protein had no cellular toxicity, this assay has its limitations. Most notable, is the fact that LDH release can be slow relative to the deterioration of cell morphology (337). For example, cells undergoing apoptosis may not necessarily show an increase in LDH activity. Although syncytin-1 has been shown to be anti-apoptotic (338, 339), it is important that future studies further examine cell survival following treatment with the recombinant proteins. For example, cell proliferation assays will show the number of cells dividing in culture and are a good measure of cell viability.

In conclusion, this study shows for the first time, using recombinant proteins, that syncytin-1 can inhibit the production of the Th1 cytokines TNF- $\alpha$  and IFN- $\gamma$  as well as the Th1 inducing chemokine CXCL10. Previously syncytin-1 has been regarded primarily as a fusogenic protein which is involved in cytotrophoblast fusion. This study opens new perspectives for the role of syncytin-1 during pregnancy. If syncytin-1 is able to mediate the induction to Th2 responses as expected, it will be tempting to speculate that many of the immunological changes observed during pregnancy, such as the shift from a Th1 to a Th2 dominated immunoresponse (340, 341), could be explained by the actions of this endogenous retroviral envelope protein.

## **Chapter 6 - General Discussion**

## **6.1 General Discussion**

It has been hypothesised that the expression of ERV envelope proteins in the placentas of humans and other mammals play a major role in the physiology of pregnancy. In particular, syncytin-1 and syncytin-2, which are the envelope proteins of members of the HERV-W and -FRD families have been extensively studied and shown to play an important role in human cytotrophoblast cell fusion (165, 177, 249). In addition to cell fusion, it has also been speculated that these proteins may have a role in maternal immune tolerance (107, 108), however, at the beginning of this thesis, no studies had been published which examined this potential function. This project examined the role and regulation of syncytin-1 in the context of maternal immune tolerance.

In order to examine this, two models were developed. Firstly, the BeWo cell model was used to examine the regulation of syncytin-1, syncytin-2 and FasL by the placental peptide hormone CRH. In this study, a combined DNA/RNA/protein extraction method was used to examine mRNA and protein expression while an exosome protein extraction procedure was used to examine syncytin-1 levels in exosomes from CRH treated BeWo cells. Secondly, recombinant retroviral ectodomain proteins were produced and the immunosuppressive properties of the syncytin-1 ectodomain were examined by studying the inhibition of Th1 cytokines using a whole blood culture model.

To investigate the regulation of placental syncytins during pregnancy a combined DNA/RNA/Protein extraction technique was initially developed (Chapter 2). This approach, which is detailed in chapter 2, used commercially available column-based nucleic acid extraction kits which are designed to efficiently extract DNA and RNA but not protein. Using a simple modification (collecting the column flow-through) this method also allowed us to efficiently extract proteins from the columns in using a CHAPS based buffer. This method required no hazardous chemicals and was shown to be a quick, reliable and consistent method for simultaneous nucleic acid and protein extraction in comparison to previous simultaneous extraction methods. Since the

publication of this paper, a number of commercial simultaneous nucleic acid and protein extraction kits have been released, however, these kits are significantly more expensive than the traditional spin column extraction kits and do not take into account the importance of extraction buffers. The method developed in this chapter was subsequently used to test whether CRH could contribute to maternal immune tolerance through the regulation of placental syncytin expression.

The only studies examining the role of CRH in maternal immune tolerance have used the extravillous trophoblast cell line JEG3. In these cells, FasL expression increased in response to CRH treatment (105). However, the stimulation of placental syncytins is best represented using a villous trophoblastic cell line such as BeWo cells. BeWo cells have been shown to constitutively express syncytin-1 and syncytin-2 as well as their receptors. Using this model, studies have shown that the induction of syncytin-1 and syncytin-2 expression by cAMP results in an increase in cell fusion (107, 171, 177). With this in mind the BeWo cell line was used to examine the regulation of placental syncytins as well as FasL by the placental peptide hormone CRH (Chapter 3).

In this study, a significant upregulation of syncytin-1, syncytin-2 and FasL mRNA was observed following CRH treatment in a dose dependent fashion. It was postulated that the stimulation of these mRNAs by CRH was a cAMP mediated event as CRH has been shown to stimulate cAMP which signals through the cAMP/PKA signalling pathway (189). This pathway has also been shown to be involved in syncytin-1 and syncytin-2 production (107, 249), while FasL mRNA has been shown to respond to cAMP in BeWo cells (251). However, the increase in mRNA that was observed in this study was not observed at the level of total cell protein. Initially this result was rather puzzling as Makrigiannakis had shown an increase in FasL protein following treatment with CRH in the JEG3 cell line (105). However, BeWo cells represent villous cytotrophoblast while JEG3 are invasive trophoblast cells suggesting that these cell lines may respond to CRH differently.

One characteristic of syncytin-1 that may alter its protein expression on syncytiotrophoblast cells is its presence on placental exosomes. These vesicles are secreted by syncytiotrophoblast cells and, in addition to syncytin-1 have previously been shown to carry FasL (229). Further, the cAMP/PKA pathway, which is proposed to regulate syncytin-1 expression in BeWo cells, has been shown to promote the sorting of the TNF receptor into exosome like vesicles (342). As exosomal expression of syncytin-1 could explain the lack of total cell protein stimulation in BeWo cells, the effects of CRH on the exosomal fraction were examined. In these studies following CRH treatment, increased levels of the syncytin-1 precursor in BeWo exosomes was observed suggesting that CRH promotes the sorting of syncytin-1 into exosomes.

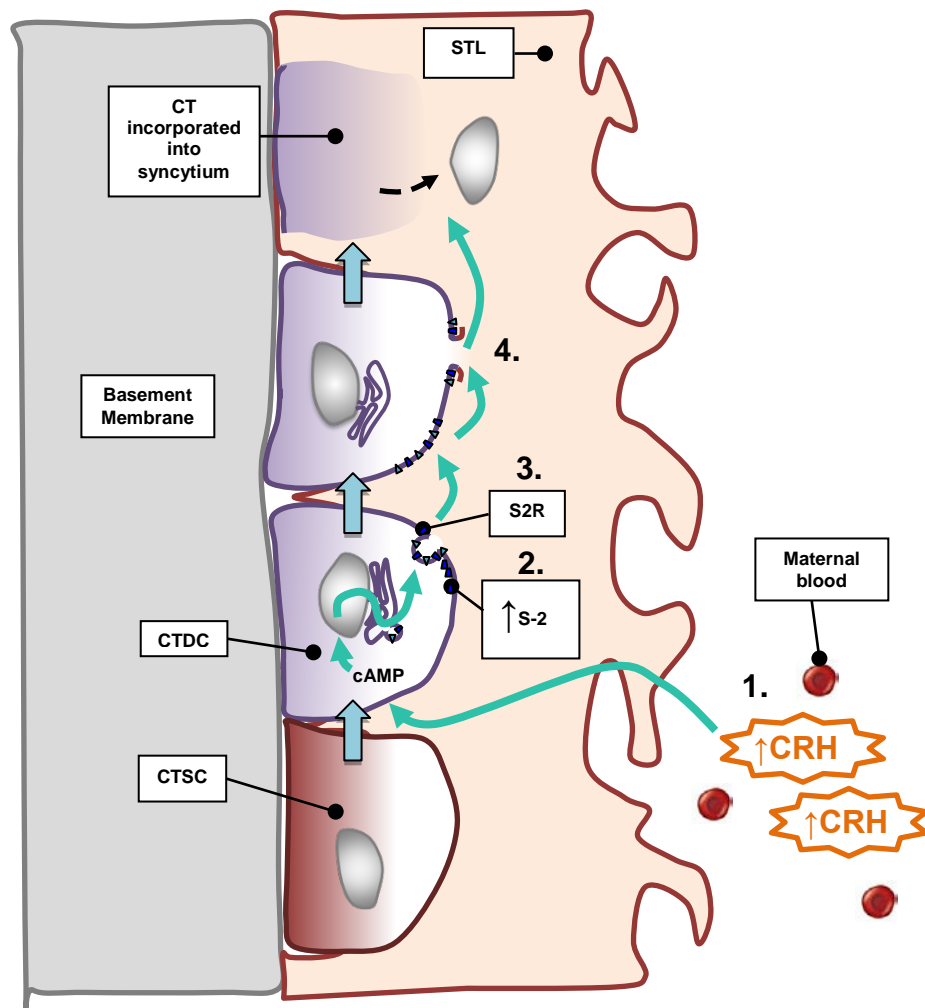
Taking into account the postulated roles of retroviral envelope proteins in pregnancy, CRH stimulation of retroviral envelope expression and its sorting into exosomes may have functions beyond that of cell fusion. It is already well established that the envelope proteins of retroviral proteins, including syncytin-1 carry an ISD which has been hypothesised to contribute to maternal immune tolerance (107, 108) and recent studies have speculated that placental exosomes may play an important role in maternal immune tolerance (191, 229, 231, 343). CRH has also been associated with maternal immune tolerance where it can promote implantation and early pregnancy by increasing FasL expression, leading to the killing of activated T cells (105).

In addition to CRH, other maternal hormones have been shown to have an effect on syncytin-1, most notably progesterone (188). Progesterone, like CRH, has a known role in maternal immune tolerance and has been shown to block cellular immune responses in rats and also inhibit monocyte dependent T cell activation in humans through its capacity to selectively block the activation of T cells (74, 78). As maternal hormones such as CRH and progesterone are considered mediators of immunosuppression during pregnancy, it could be speculated that they stimulate the sorting of retroviral proteins into exosomes to contribute to maternal immune tolerance. However, evidence that placental syncytin proteins are immunosuppressive is required to support this hypothesis.

The immunosuppressive properties of placental syncytins were first studied by Mangeney *et al.*, (213). In this study, an established tumour cell model, which has been detailed in numerous papers (209, 210, 213, 267) and throughout this thesis, was used. The data generated in this study suggested that the cytotrophoblast specific syncytin-2 (175) has immunosuppressive properties, while the syncytiotrophoblast specific syncytin-1 does not (107, 344). However, Mangeney did not provide a physiological explanation for the role of syncytin-2 in maternal immune tolerance, given that it is not in contact with the maternal circulation. Considering the localisation of syncytin-2, it could be speculated that the predominant function of this protein may be in cell fusion.

Recent studies have suggested that syncytin-2 may play a more important role in cell fusion than previously envisioned. RT-PCR analysis of differentiating trophoblast cells showed a direct correlation between mRNA and protein levels of syncytin-2 and cell fusion while an inverse correlation was observed for syncytin-1 (177). Further, syncytin-2 siRNA transfected BeWo and primary trophoblast cells resulted in reduced cell fusion while syncytin-1 siRNA had a more modest effect (177). Although this isn't the focus of this thesis, the major role of syncytin-2 in pregnancy may be in cytotrophoblast cell fusion to form syncytiotrophoblast and CRH stimulation of syncytin-2 may potentially play an important role in regulating syncytin-2 levels and cell fusion events (Figure 6.1).





**Figure 6.1. Schematic of the possible role of syncytin-2 during pregnancy.** *Recent studies have suggested that syncytin-2 has immunosuppressive properties, however, the authors have not attempted to put this immunosuppressive role in context considering that syncytin-2 is expressed in the cytotrophoblast and is not in direct contact with the maternal circulation. This thesis proposes that the increasing CRH levels during gestation (1) stimulate the production of syncytin-2 (2) in the cytotrophoblast which interacts with the syncytin-2 receptor (3) on the syncytiotrophoblast resulting in cytotrophoblast cell fusion (4). STL = Syncytiotrophoblast layer, CT = Cytotrophoblast, S2R = Syncytin-2 receptor, S-2 = Syncytin-2, CTDC = Cytotrophoblast Daughter Cell, CTSC = Cytotrophoblast Stem Cell.*

If syncytin-2 plays a major role in cell fusion as is suggested by Vargas *et al.*, (177), then syncytin-1 which is expressed on the syncytiotrophoblast and has an increasing pattern of expression throughout gestation (344), is positioned as a candidate retroviral immunosuppressive molecule. While the data presented by Mangeney (213) suggests that syncytin-1 is not immunosuppressive, it is important to note that differential expression of syncytin-1 in the tumour cells may potentially explain the lack of immunosuppression in this model. Therefore, further studies on the immunosuppressive properties of syncytin-1 were required.

In order to investigate the role of syncytin-1 in maternal immuntolerance, a recombinant retroviral ectodomain protein model was established (see chapter 4). This model was based on the publication by Mangeney *et. al.*, (213) which had used recombinant ectodomain proteins to examine humoural immunity (antibody production) in the mouse model. The advantage of this model is that it is large enough to adopt the proper, multimeric conformation of retroviral envelope proteins (276, 277, 283-285) and therefore closely mimics the native structure in comparison to synthetic peptides.

However, one of the problems associated with the generation of retroviral recombinant proteins is their stability in solution. These proteins are stabilised by weak non-covalent interactions (280) and it has been suggested that these interactions make it difficult to generate stable, soluble ectodomains suitable for biological analysis. Due to these problems, approaches have been developed to stabilise these proteins such as the introduction of cysteine residues to form intersubunit disulfide bonds or the addition of GCN4 trimeric helices (281, 282), however the effects of any of these substitutions on potential immunosuppressive functions are not documented. As such, the methods developed in this thesis did not utilise any of these approaches.

To generate these proteins the recombinant ectodomains of human syncytin-1 and syncytin-2 as well as mouse syncytin-A and syncytin-B were cloned into the pET-28b bacterial expression plasmid. These proteins were then overexpressed using the *lac* operon system and purified using a combination of IMAC and gel filtration

chromatography resulting in the protein of oligomeric proteins. Using this method, the predominant fraction eluting from the gel filtration column consisted of monomeric and dimeric proteins and did not exhibit the expected protein profile of the biologically active multimeric fraction. However the second, smaller fraction, folded into more complex structures and the protein profile corresponded to the profile of other biologically active retroviral envelope proteins (277, 280, 289). The specificity of these fractions was confirmed using both western blotting and LCMS sequencing prior to the examination of the immunosuppressive properties of syncytin-1.

To test the hypothesis that syncytin-1 can contribute to maternal immune tolerance through the inhibition of Th1 cytokines, the syncytin-1 recombinant protein was tested *in vitro* using a biologically relevant whole blood system stimulated with the polyclonal activators LPS and PHA (Chapter 5). These stimuli had previously been used as immune stimulating agents in studies of the immunology of pregnancy as well as the immunosuppressive properties of ERVs (190, 206, 319-323). In the studies presented in chapter 5, the multimeric syncytin-1 recombinant ectodomain protein was shown to inhibit the production of the Th1 cytokines, TNF- $\alpha$  and IFN- $\gamma$  as well as the Th1 inducing chemokine CXCL10 suggesting that syncytin-1 may contribute to the maternal immune environment during pregnancy by inhibiting the production of Th1 cytokines.

The success of a pregnancy has been correlated with an inhibition of Th1 cytokines and a shift from the production of Th1 to Th2 type responses (89). While the study presented in this thesis did not examine the production of Th2 cytokines following treatment with syncytin-1, retroviral ISD proteins have previously been shown to induce the production of the Th2 cytokine IL-10 (254) allowing us to speculate that syncytin-1 will fulfil the parameters described for retroviral mediated modulation of Th2 responses and may potentially play an important role in the shift from Th1 to Th2 responses that is observed during pregnancy.

However, Th2 dominant immunity has also been observed in recurrent abortion cases and Th2 knockout mice can proceed normally through a pregnancy suggesting that in

addition to the Th1/Th2 phenomena, there are other mechanisms such as T-reg cells, which may play an important role in maternal immune tolerance (98, 99).

In pregnancy, T-reg cells seem to play an important role in mediating maternal immune tolerance to the fetus. The presence of T-reg cells has been shown to rescue pregnancy in abortion prone mice and in normal pregnancy there is a systemic expansion of the T-reg cell pool. The absence of T-reg cells also leads to gestational failure due to immunological rejection of the fetus (101, 104, 345). One of the mechanisms suggested for the regulation of T-reg cell development is the presence of TGF- $\beta$ , which has been shown to be a key regulator of the signalling pathways that initiate and maintain T-reg suppressive function (346).

Interestingly, it has been suggested that syncytin-1 may have TGF- $\beta$  agonist properties. Studies have shown that a longer version of the CKS-17 peptide, the CKS-25 peptide contains a functional TGF- $\beta$  motif (RGLD) which has partial TGF- $\beta$  agonist activity (347). In relation to this observation, it is interesting to note that the syncytin-1 ISD has some homology to the CKS-17 peptide. Syncytin-1 also contains this TGF- $\beta$  motif (RGLD) and it has recently been suggested that interactions between syncytin-1 and TGF- $\beta$  may contribute to the aetiology of endometrial carcinoma progression (348). If syncytin-1 has TGF- $\beta$  agonist properties then it may have an important role in promoting the proliferation of T-reg cells throughout pregnancy.

In addition to this observation, the CKS-17 peptide has previously been demonstrated to contribute to numerous immunosuppressive effects both *in vitro* and *in vivo* on lymphocytes, monocytes, macrophages, natural killer cells and T killer cells including the inhibition of the respiratory burst of human monocytes (197), human natural killer (NK) cell activity (198), monocyte-mediated tumour cell killing (199), IgG production (200), IL-1-mediated responses by interfering with signal transduction through a protein kinase C pathway (203, 204), cell-mediated immunity (delayed-type hypersensitivity, DTH) reactions *in vivo* in mice (205), lipopolysaccharide (endotoxin)-induced mortality of mice (206), and murine cytotoxic T lymphocyte activity (207). If syncytin-1 has

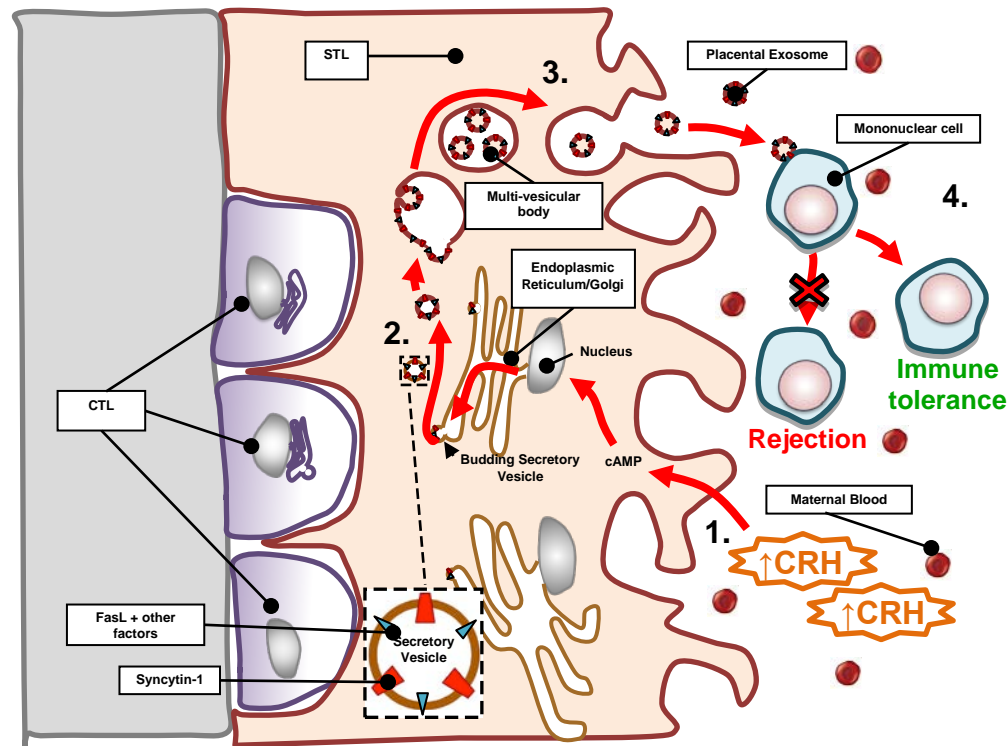
similar effects to CKS-17 on these cells, then the role of syncytin-1 in maternal immune tolerance may be even more important than the mechanisms detailed in this thesis.

If syncytin-1 is to contribute to maternal immune tolerance, it requires an expression pattern which allows it to interact with maternal immune cells. As detailed previously, syncytin-1 is expressed at the level of the maternal-fetal interface on syncytiotrophoblast cells however, its specific localisation within this tissue remains controversial. Within the syncytiotrophoblast compartment, studies have shown that syncytin-1 is expressed in the basal membrane, apical membrane, and uniformly distributed throughout the syncytiotrophoblast (169, 188, 349, 350). Recent studies by our group using the syncytin-1 ISD peptide have shown that syncytin-1 expression is predominantly localised in the cytoplasm of syncytiotrophoblast cells (190), suggesting that on the syncytiotrophoblast, syncytin-1 does not come into contact with the maternal immune system. Therefore, the recent discovery that syncytin-1 is carried by placental exosomes and our finding that CRH promotes the production of placental exosomes which contain syncytin-1, may be essential to explain a role for syncytin-1 in maternal immune tolerance.

Placental exosomes have recently emerged as novel regulators of the maternal immune system during pregnancy and it has been postulated that these vesicles may educate the immune system to maintain tolerance towards fetal antigens (190). These nanovesicles have been shown to be critical in modulating T-cell activation and suppressing effector T cells by enhancing lymphocyte apoptosis and CD3-zeta loss (191, 230, 351) as well as downregulating natural killer receptor ligands that may inhibit natural killer cell activity (231) although it is speculated that they may have additional immunosuppressive functions in addition to those already described (190, 343). In addition to syncytin-1, studies have attributed the immunosuppressive effects of placental exosomes to the presence of FasL (191), PDL-1 (230) and NKG2D ligands (231). Further, Luo *et al.*, (352) also identified the presence of miRNA's in placental exosomes and preliminary studies by our group has demonstrated the presence of miRNAs which may be involved in the regulation of immune signalling (Jorge Tolosa, unpublished data). While the

research on the immunosuppressive properties of placental exosomes is still in the early stages, the data that has been presented so far suggests that the presence of syncytin-1 and numerous other immunosuppressive factors carried by placental exosomes may have a major role in regulating maternal immune tolerance in pregnancy.

Taking into account the findings of this thesis, it could be hypothesised that the stimulation of syncytin-1 by CRH and its subsequent sorting into placental exosomes may be important in generating maternal immune tolerance (Figure 6.2). The presence of syncytin-1 on exosomes could be more beneficial to the modulation of the immune system compared to syncytiotrophoblast specific expression as exosomes may serve as a more advantageous carrier of signal delivery, favouring stable conformational conditions, increased bioactivity, and improved bio-distribution in comparison to syncytiotrophoblast specific expression of syncytin-1.



**Figure 6.2. Schematic of the possible immunosuppressive role of syncytin-1 throughout gestation.**

*During pregnancy, the semi-allogenic fetus expresses antigens derived from both the mother and the father without immunological rejection. This thesis proposes that the increasing CRH levels during gestation (1) stimulate the production of syncytin-1 and FasL which are sorted into exosomes (2) along with a number of other immunosuppressive factors and released into the maternal circulation (3) impairing the ability of circulating immune cells to mount an immune response against the semi-allograft fetus (4) resulting in maternal immune tolerance. STL = Syncytiotrophoblast layer, CTL = Cytotrophoblast layer.*

Normal pregnancy is not the sole scenario which can benefit from the study of the immunosuppressive properties of syncytin-1. Understanding how the fetal allograft is tolerated may have implications in the pathogenesis of pregnancy disturbances such as preeclampsia and other related diseases as well as implications outside of pregnancy, especially in cancer, where immune tolerance mechanisms are exploited by cancer malignancies, and transplantation, where improvements on current immunosuppressive regimes are required. In the following paragraphs, this discussion will also examine the

potential role of the immunosuppressive syncytin-1 in pregnancy complications, cancer as well as in future transplantation strategies.

#### 6.1.1 Preeclampsia

It has been suggested that an inadequate or perturbed tolerance response may contribute to adverse pregnancy outcomes such as in preeclampsia and HELLP syndrome (353). Although much is known about the diagnosis of these conditions, the molecular aetiology of preeclampsia and HELLP remains unknown. Studies have hypothesised that these syndromes are characterised by an increased placental inflammatory response (354, 355). It is therefore interesting to note that in preeclampsia and HELLP syndrome, a decreased pattern of syncytin-1 expression is observed (356, 357)

The observed decrease in syncytin-1 expression in preeclampsia may contribute to the aetiology of this condition through placenta-specific deregulation of cytotrophoblast cell fusion leading to increased apoptosis (356-358). However, this study suggests that the decreased expression of syncytin-1 in preeclamptic and HELLP syndrome patients could contribute to the aetiology of these conditions through its capacity to inhibit Th1 cytokine responses. This suggestion is supported in studies which show that hypoxia associated preeclampsia triggers the degradation of the Gcm1 transcription factor (syncytin-1 transcription factor) by suppressing the p13K-Akt signalling pathway (359). This signalling pathway is used by CKS-17 and preliminary studies have suggested that it may be also used by syncytin-1 in immunosuppression (190).

#### 6.1.2 Cancer

Despite the fact that both trophoblast and cancer cells are immunogenic they are able to avoid immunological rejection. While the precise mechanisms involved in these processes are yet to be completely understood, it appears that they may be surprisingly



similar. In relation to this thesis, increasing levels of hormones (360), a shift toward Th2 cytokines (361) and the presence of immunosuppressive exosomes (362) have all been postulated to contribute to immunosuppression during cancer. As these sources of immunosuppression may be related to the expression of retroviral envelope proteins in cancer cells, it may be speculated that the presence of retroviral proteins may contribute to the development of cancer.

Interestingly, it has already been hypothesised that syncytin-1 may have a role in cancer. Bjerregaard *et al.*, (181) identified syncytin-1 expression in breast cancer cells and showed that the presence of this protein increased breast cancer cell fusions. These observations may be extended to other cancers as syncytin-1 has been shown to be expressed in endometrial, ovarian, colonic and rectal cancers (182, 183, 363, 364) and the levels of syncytin-1 have been shown to be significantly upregulated in all benign and malignant tissues in endometrial carcinoma samples with higher levels of expression than placental syncytin-1 (348). Although syncytin-1 has been implicated in cancer cell fusions, the immunosuppressive properties described in this thesis for syncytin-1 suggest that this protein may be used by cancer cells to modulate the immune system.

### 6.1.3 Transplantation

A better understanding of the regulation of immune responses in pregnancy will not only help to understand the pathogenesis of disease but can also achieve new therapeutic approaches in terms of inducing immunological tolerance. In transplantation, the induction of donor-antigen-specific immunological tolerance still remains the ultimate aim of organ transplantation and it has previously been speculated that factors involved in maternal immune tolerance may be used in transplantation strategies (365, 366). In regards to the studies presented in this thesis, it is conceivable that syncytin-1 in the future may be a candidate protein in transplantation studies. However, the abnormal expression pattern of syncytin-1 in ectopically transfected cells (165, 166, 213, 273) and

the possibility of viral transcomplementation may prevent this protein from being used in clinical transplantation studies.

#### 6.1.4 Future studies

It is reasonable to say that the findings of this thesis have opened a new avenue of research into potential mechanisms of maternal immune tolerance during pregnancy. However, a greater understanding of the immunosuppressive properties of syncytin-1 is still required. Future studies will determine the extent of the inhibition of Th1 cytokines by syncytin-1 recombinant protein in whole blood by utilising multiplex ELISA technology. These studies will not only characterise the inhibition of Th1 cytokines by syncytin-1 but also examine the changes in the Th2 cytokine profile as it is expected that syncytin-1, like other retroviral proteins will induce the production of Th2 cytokines. Further, knowing that CKS-17 has numerous immunosuppressive effects extending beyond the shift from Th1 to Th2 cytokine responses, the effects of syncytin-1 on the inhibition of monocytes, macrophages, NK cells and potentially even T-reg cells should also be studied. These studies could be repeated using the recombinant ectodomains of syncytin-2, syncytin-A and syncytin-B (see chapter 4) to gain an increased understanding of the role of retroviral proteins in maternal immune tolerance during human and murine pregnancy. Finally, syncytin-A and syncytin-B knockout mice could be generated to study *in vivo*, the role of mouse placental syncytins on pregnancy outcome. The syncytin-A knockout mouse has already been studied and embryos from these mice have been shown to die *in utero* between days 11.5 and 13.5 as a result of defective development of the placenta (179). However, whether this *in utero* death is also a result of a heightened immune response remains to be studied.

In addition to examining the immunosuppressive properties of syncytin-1, it will be important to examine the role of placental exosomes in maternal immune tolerance. Currently, our understanding of the immunosuppressive properties of placental exosomes is lacking and a complete characterisation of the exosome proteome and

genome is required. These studies are currently being completed in our laboratory. Further, using purified placental exosomes, similar experiments as the ones detailed above for syncytin-1 recombinant ectodomain could be performed to determine the role of placental exosomes in maternal immune tolerance. Presuming that exosomes also play an important role in murine pregnancy, *in vivo* models using the TSAP/Steap-3 null mice (in which, exosome secretion is severely compromised) could be used where TSAP/Steap-3 null mice pregnancy success is compared to wild type controls.

## **6.2 Final conclusions**

The knowledge gained from this research has lead to a greater understanding of the processes vital to the establishment and maintenance of maternal immune tolerance, including the mechanisms by which syncytin-1 and placental exosomes may generate tolerance toward fetal antigens during pregnancy. These findings can not only contribute to developing therapeutic strategies to treat pregnancy related disorders such as preeclampsia or infertility but also to develop interventions to address malignancies in cancer and also to improve transplantation success rates.

## **Chapter 7 – References**

## **7.1 References**

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## **Appendix 1**

### **A.1 Buffers**

2D buffer	30mM Tris 7M Urea 2M Thiourea 4% CHAPS
30% sucrose cushion	30% sucrose dissolved in 20mM HEPES pH 7.4
Alkaline lysis solution 1	50mM Tris 10mM EDTA pH8
Alkaline lysis solution 2	0.2N NaOH 1% SDS
Alkaline lysis solution 3	3M KOAc pH5.5
Assay diluent	PBS supplememented with 10% FBS pH7
Blood culture buffer	25mM HEPES 150mM NaCl pH7.4
Buffer B	84% Acetonitrile 0.1% Formic acid
Coating buffer	0.1M Carbonate buffer pH 9.5
Coomassie Brilliant Blue Solution	0.2% Coomassie Blue 7.5% Glacial Acetic Acid 50% Ethanol
Coomassie Destain Solution	7.5% Glacial Acetic Acid
Denaturing resuspension buffer	6M Guanidine Chloride 20mM Tris-HCl 0.5M NaCl 50mM Imidazole 1mM 2-mercaptoethanol pH8



Dialysis buffer	5mM HEPES 25mM NaCl pH7.4
Electron microscopy buffer	2% Paraformaldehyde 1% Gluteraldehyde pH 7.4
Elution buffer	20mM NaPO4 100-500mM Imidazole 500mM NaCl pH7.4
FasGreen	50% Methanol 10% Glacial acetic acid 0.1% FasGreen powder
FPLC buffer	25mM HEPES 150mM NaCl pH7.4
Gel fixative	40% Methanol 10% Glacial acetic acid
HAM-F12 medium	1 bottle HAM-F12 powder (Sigma) 25mM HEPES 14.05mM Sodium Bicarbonate pH 7.4
Hi-trap binding buffer	20mM NaPO4 50mM Imidazole 500mM NaCl pH7.4, pH6.8, pH6 and pH5
LCMS gel extraction buffer	50% Acetonitrile 0.1% Formic acid
LCMS wash solution	25mM Ammonium Bicarbonate 50% Methanol
LDH stop solution	1M glacial acetic acid
Luria Agar	25g/L Luria broth 15g/L Agar
Luria Broth	25g/L Luria broth
MOPS running buffer	1x MOPS running buffer 20% Methanol in distilled water

MES running buffer	1 x MES running buffer 20% Methanol in distilled water
NuPAGE LDS sample buffer	141mM Tris base 2% LDS 10% Glycerol 0.51mM EDTA 0.22mM SERVA blue G250 0.175mM Phenol Red pH 8.5
Phosphate buffered saline	137mM NaCl 2.7mM KCl 4.3mM Na <sub>2</sub> HPO <sub>4</sub> 1.47mM KH <sub>2</sub> PO <sub>4</sub>
RIPA protein extraction buffer	50mM Tris-HCl 150mM NaCl 0.1% SDS 0.5% sodium deoxycholate 1% triton X-100 2mM EDTA 1mM DTT Complete protease inhibitor cocktail tablet ( 1 tablet/50ml pH 7.4
RPMI 1640 media	RPMI 1640 media 2mM L-glutamine 25mM HEPES 40µg/ml Gentamicin
Silver stain fixative	40% Methanol 10% Glacial acetic acid
SOB medium	2% Peptone 0.5% Bacto Yeast extract 10mM NaCl 2.5mM KCl 10mM MgCl <sub>2</sub> 10mM MgSO <sub>4</sub>
Stop solution	2N H <sub>2</sub> SO <sub>4</sub>
Sypro Ruby fixative	50% Methanol 7% Glacial Acetic acid

Sypro Ruby wash solution	10% Methanol 7% Glacial Acetic acid
TAE buffer	40mM Tris Acetate 1mM EDTA pH 8
TBST	10mM Tris (base) 150mM NaCl 0.1% Tween 20 pH 7.4
TE buffer	10mM Tris 1mM EDTA pH7.5
Transformation buffer	10mM PIPES 15mM CaCl <sub>2</sub> 250mM KCl 55mM MnCl <sub>2</sub>
Trypsin solution	1µg Trypsin/50µg protein prepared in 25mM Ammonium Bicarbonate
Working reagent A	containing Sodium Carbonate Sodium Bicarbonate Bicinchoninic acid Sodium Tartrate Dissolved in 0.1M Sodium Hydroxide
Working reagent B	4% Cupric Sulfate