

# **An Investigation of the Regulation of Abnormal Trophoblast Cell Turnover in Pre-eclampsia**

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## Abstract

An Investigation of the Regulation of Abnormal Trophoblast Cell Turnover in Pre-eclampsia  
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Pre-eclampsia affects between 3-5% of pregnancies in the developed world; it is responsible for 13% of maternal deaths and 17% of stillbirths in the UK. Pre-eclampsia is thought to originate from the placenta; several studies report increased apoptosis, a form of programmed cell death, in the placenta of pregnancies complicated by pre-eclampsia. The origin of this excessive cell-death and the role it plays in the pathophysiology of pre-eclampsia is not clear. It is hypothesised that increased apoptosis may originate from altered delivery of oxygen to placental villi, resulting in hypoxia or accumulation of reactive oxygen species (ROS). Cell turnover is tightly regulated; the p53 pathway holds a critical role in the control of apoptosis in response to cell damage. This study investigated the expression of the constituents of the p53 pathway in placental tissue in normal pregnancy and those complicated by pre-eclampsia. In addition, the effects of hypoxia and ROS on trophoblast apoptosis were assessed.

Expression of several pro-apoptotic members of the p53 pathway including: p53, p21, and Bax is increased in pre-eclampsia. Conversely, the expression of anti-apoptotic Mdm2 was decreased. The balance between p53 and Mdm2 is essential for normal embryonic development and cell survival. In human trophoblast, a reduction in Mdm2 following treatment with siRNA was associated with increased apoptosis, which was reduced to control levels by concomitant p53 knockdown, suggesting that the balance of p53 and Mdm2 is critical in determining trophoblast survival, and that the imbalance between these proteins observed in pre-eclampsia can induce trophoblast apoptosis *in vitro*.

Apoptosis was induced in the trophoblast-derived cell-line BeWo and placental villous explants following culture in hypoxic culture conditions and in the presence of ROS. In BeWo cells, exposure to ROS increased p53 and decreased Mdm2 expression. Culture of placental explants in hypoxic conditions was associated with an increase in p53 and p21, although no change in Mdm2 expression was observed. In placental explants, the increased apoptosis and reduced proliferation induced following exposure to reactive oxygen species were reduced to control levels following treatment with epidermal growth factor. These findings imply that the increased apoptosis may not be an inevitable response to cell damage, but may be the output following a combination of exposure to pro-apoptotic and pro-survival signals. In conclusion, the findings of this study imply that the p53-pathway is involved in the excessive apoptosis observed in pre-eclampsia. Taken together with the findings that culture in hypoxia or with ROS induced apoptosis within trophoblast, these data imply that apoptosis observed in pre-eclampsia results from cell damage originating from oxidative stress. A greater understanding of the mechanism underlying these events provides opportunities for novel strategies to reduce trophoblast apoptosis, including growth factors and p53-pathway antagonists.

## **Declaration**

Part of the work (20%) presented in Chapter 8 has been presented in support of an application for the degree of Master of Research awarded by the University of Manchester by Dr Sarah Moll, a student under my direct supervision. I devised and supervised these experiments and analysed the data. All of the text presented in Chapter 8 is original.

No other part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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## Abbreviations

ADP	Adenine diphosphate
AIF	Apoptosis inducing factor
APAF-1	Apoptotic protease-activating factor-1
APES	Aminopropyltriethoxysaline
ASPP	Apoptosis stimulating proteins of p53
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BIR	Baculovirus inhibitor of apoptosis repeat
BSA	Bovine serum albumin
CBP	Creb binding protein
CDK	Cyclin dependent kinase
CHM	Complete hydatidiform mole
cIAP	cellular inhibitor of apoptosis protein
DAB	3',3'-diaminobenzidine
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal related kinase
FCS	Fetal calf serum

GTD	Gestational trophoblast disease
hCG	Human chorionic gonadotrophin
HIAP	Human inhibitor of apoptosis protein
HIF	Hypoxia inducible factor
IAP	Inhibitor of apoptosis protein
IGF	Insulin-like growth factor
IUGR	Intrauterine growth restriction
JNK	Jun N-terminal Kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinase
mdm2	murine double minute-2 (murine form)
Mdm2	murine double minute-2(human form)
MLC	Myosin light chain
MMP	Matrix metalloproteinase
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NES	Nuclear export signal
NET	Neutrophil extracellular traps
NGS	Normal goat serum
NIAP	Neuronal inhibitor of apoptosis protein
NLS	Nuclear localisation signal
NRS	Normal rabbit serum
pRb	Retinoblastoma protein

PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PES	Polyethersulfone
PHM	Partial hydatidiform mole
PIC	Protease inhibitor cocktail
PVDF	Polyvinylidene difluoride
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
SOD	Superoxide dismutase
STBM	Syncytiotrophoblast microparticles
TBS	Tris buffered saline
TNF- $\alpha$	Tumour necrosis factor alpha
TUNEL	Terminal dUTP nick-end labelling
VDAC	Voltage dependent anion channel
XIAP	X-linked inhibitor of apoptosis protein



## Peer-Reviewed Publications Arising from this Thesis

**Heazell AEP**, Taylor NNJ, Baker PN, Crocker IP. Hypoxia and Reactive Oxygen Species Alter Cell Turnover of BeWo Choriocarcinoma Cells *Reproductive Biomedicine Online* (In Press)

**Heazell AEP**, Buttle HR, Baker PN, Crocker IP. Altered Expression of Regulators of Caspase Activity within Trophoblast of Normal and Pre-eclamptic Pregnancies. *Reproductive Sciences* (In Press)

**Heazell AEP**, Lacey HA, Jones CJP, Huppertz B, Baker PN, Crocker IP. Effects of oxygen on cell turnover and expression of regulators of cell turnover in human placental trophoblast. *Placenta* 2008;29(2):175-186.

Moll SJ, Jones CJP, Crocker IP, Baker PN, **Heazell AEP**. Epidermal Growth Factor Rescues Trophoblast Apoptosis Induced by Reactive Oxygen Species *Apoptosis* 2007;12:1611-1622.

**Heazell AEP**, Moll SJ, Jones CJP, Baker PN, Crocker IP. Formation of syncytial knots is increased by hyperoxia, hypoxia and reactive oxygen species. *Placenta* 2007;28(Suppl A):S33-40.

**Heazell AEP**, Harris L, Forbes K, Crocker IP. Placental cell turnover in health and disease. *Reviews in Gynecological and Perinatal Practice* 2006;6:80-86.

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Taylor N, Crocker I, Baker P, **Heazell AEP**. A model of altered trophoblast turnover following exposure to hypoxia or reactive oxygen species. *Placenta* 2007;28(8-9):A47.

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**Heazell AEP**, Buttle HR, Baker PN, Crocker IP. Altered expression of regulators of caspase activity within trophoblast of normal and pre-eclamptic pregnancies. *Reproductive Sciences* 2007;14(1):219-220A.

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**Heazell AEP**, Huppertz B, Jones CJP, Baker PN, Crocker IP. Evidence for Aponecrosis within Trophoblast exposed to Hypoxia In Vitro. *Journal of the Society for Gynecologic Investigation* 2006;13(2):114-115A.

**Heazell AEP**, Forbes K, Jones CJP, Baker PN, Westwood M, Crocker IP, Aplin JD. In Vitro Proliferation of First and Third Trimester Cytotrophoblasts is increased by Exposure to Hyperoxia. *Journal of the Society for Gynecologic Investigation* 2006;13(2):114A.

**Heazell AEP**, Brown LM, Baker PN, Crocker IP. The influence of oxygen on the expression of oncoproteins p53 and Mdm2 within cultured placental villous explants. *Placenta* 2005;26:A33.

**Heazell AEP**, Brown LM, Baker PN, Crocker IP. Expression of Oncoproteins p53, BAK and p21 in normal pregnancies and those complicated by pre-eclampsia. *Placenta* 2005;26:A59.

**Heazell AEP**, Brown LM, Baker PN, Crocker IP. The altered expression of oncoproteins in trophoblast of normal and pre-eclamptic pregnancies. *British Journal of Obstetrics and Gynaecology*, 2005;112(10):1446.

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## Lay Summary

Pre-eclampsia affects approximately 3-5% of pregnancies in the UK. Currently, there is no effective treatment for this disorder other than delivery of the baby. In severe cases, the baby will often be premature. Premature delivery due to pre-eclampsia is responsible for 1 in 6 babies requiring neonatal care cots. This increase in premature delivery is linked to medical problems in the neonatal period and through to adult life.

Although the exact cause of pre-eclampsia is unknown, it is associated with abnormalities of the placenta. The placenta is responsible for transferring all the oxygen and nutrients to the growing baby. Pre-eclampsia is associated with increased cell death in the placenta which disrupts the normal functions of the placenta. The exact cause of the increased cell death in the placenta is unknown. Cell death can result from many causes including damage from low or fluctuating oxygen levels or a lack of survival factors. I investigated whether these factors may have a role in placental cell death in pre-eclampsia.

Exposure of placental tissue to low oxygen or reactive oxygen species (created when tissue is exposed to fluctuating levels of oxygen) can induce cell death. This increased cell death affects placental functions such as metabolism and hormone synthesis. The cell death resulting from low oxygen or reactive oxygen species activates a specific cell death pathway, involving a protein called p53. p53 is essential in the regulation of cell death and collects signals from cell damage or survival factors to produce a final message to enable the cell to live or die. The increased placental cell death and p53 protein after damage with low oxygen or reactive oxygen species are similar to that seen in placentas in pre-eclampsia. This suggests that the increased cell death occurring in pre-eclampsia may result from low or fluctuating oxygen levels.

Although cell death is increased by cell damage, it can be reduced by survival factors. Survival factors are also called growth factors. These experiments showed that treatment with EGF reversed placental cell death following damage from reactive oxygen species. This suggests that survival of placental cells is determined by the balance between cell damage and growth factors. If this is the case, the increased placental cell death in pre-eclampsia could be rescued by treatment with growth factors.

Ultimately reducing placental cell death in pre-eclampsia may allow this common condition to be treated. This will prolong pregnancies and decrease premature deliveries with their associated long-term complications.

We balance probabilities and choose the most likely. It is the scientific use of the imagination

Sherlock Holmes in *The Hound of the Baskervilles*

In memory of Jack.

## Chapter 1 - Introduction

### 1.1 Pre-eclampsia – An Overview

Pre-eclampsia is a serious multi-system disorder of human pregnancy; the World Health Organisation estimates that 70,000 women die worldwide from pre-eclampsia per annum (Myers and Brockelsby 2004). Although infrequent in the UK, pre-eclampsia accounts for 14% of maternal deaths (Confidential Enquiry into Maternal and Child Health 2004). The Confidential Enquiry into Stillbirths and Deaths in Infancy estimates that 1 in 6 stillbirths in the UK occur in pregnancies complicated by pre-eclampsia (Bailey and Lee 2001). The reported incidence of pre-eclampsia lies between 2.9-5.8% in developed countries (Campbell et al. 1985; Xiong et al. 2002). Risk factors for the development of pre-eclampsia include nulliparity, obesity, maternal renal disease, pre-existing hypertension, a family history of pre-eclampsia, diabetes mellitus and twin pregnancy (Myers and Brockelsby 2004). Pre-eclampsia is defined as new onset hypertension of greater than 140/90 mmHg and the presence of proteinuria greater than 0.3g / 24hr after 20 weeks of gestation (Davey and MacGillivray 1988).

Current theories regarding the pathophysiology of pre-eclampsia highlight widespread maternal endothelial activation leading to end-organ dysfunction including: eclamptic seizures, renal and hepatic impairment and inappropriate activation of the coagulation cascade. This endothelial dysfunction occurs as a result of circulating factors within maternal plasma which are thought to arise from the placenta, as delivery of the placenta leads to an improvement in the maternal condition (Redman and Sargent 2005). Placental damage in pre-eclampsia may be evident macroscopically by the presence of infarction (Benirschke and Kaufmann 2005). At the level of the placental villus, there is a reduction in the area of syncytiotrophoblast – the covering layer of villi (Daayana et al. 2004), increased apoptosis - a form of programmed cell death, and an increase in aggregations of syncytiotrophoblast nuclei with apoptotic appearances (Allaire et al. 2000; Leung et al. 2001; Ishihara et al. 2002; Benirschke and Kaufmann 2005). It is suggested that these changes represent premature ageing of the syncytiotrophoblast and syncytial fragments are subsequently lost into the maternal circulation resulting in the widespread syndrome. In support of this proposition, an increase in syncytiotrophoblast fragments within the maternal circulation has been noted in women with pre-eclampsia (Johansen et al. 1999; Sargent et al. 2003) and trophoblast debris can induce

endothelial cell dysfunction in vitro (Gupta et al. 2005). The precise origin of these placental changes is unknown, although hypoxia and oxidative stress have been implicated as reduced trophoblast invasion of the spiral arteries has been reported in pre-eclampsia, preventing the normal myometrial arterial wall remodelling, thereby altering the maternal blood flow and oxygen delivery to the placenta (Pijnenborg et al. 1991; Meekins et al. 1994).

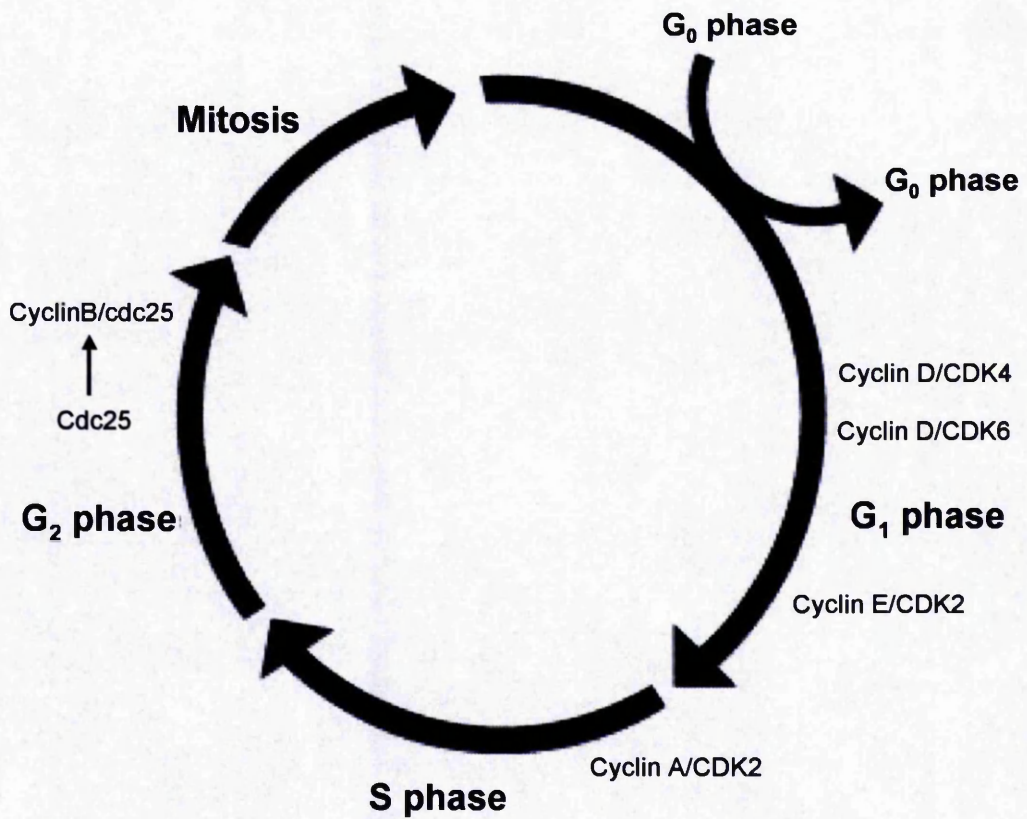
In the present model of the pathophysiology of pre-eclampsia, trophoblast has a central role. Initially, in the failure of invasion and conversion of the maternal spiral arteries which ultimately leads to damage of the villous trophoblast (Huppertz and Kingdom 2004). The response of the villous trophoblast to noxious stimuli leading to cell death and aberrant cell turnover may be an important step in the development of pre-eclampsia. However, the regulation of villous trophoblast cell turnover in pre-eclampsia is not well understood. Improved understanding of the control of cell turnover, particularly cell death, in villous trophoblast may provide insights into the pathogenesis of pre-eclampsia. This was the primary purpose of the work described herein.

## **1.2 Cell Turnover and the Cell-Cycle**

The mammalian cell cycle is divided into distinct phases, termed  $G_0$ ,  $G_1$ , S,  $G_2$  and M (Figure 1.1) (Nasmyth 1996). Quiescent (non-dividing) cells exist in the  $G_0$  phase and enter the cell cycle at  $G_1$ . They then pass through the S phase where their nuclear DNA is replicated and progress through the  $G_2$  phase before entering mitosis (M phase). The  $G_1$  and  $G_2$  phases exist to provide time for additional cell growth between cycles of DNA replication and cell division.

The cell cycle is strictly controlled and transition between each phase is regulated by the activity of cyclin/cyclin-dependent kinase (CDK) complexes, which are predominantly localised to the nucleus. While levels of CDKs remain unchanged throughout the cell cycle (Lees 1995), cyclins have a short half-life and undergo a cycle of synthesis and degradation during each round of replication (Pardee 1989). Damaged cells halt cell cycle progression by either inhibiting the activity of the cyclin/CDK complexes or reducing the production of cyclins (Sherr and Roberts 1995).





**Figure 1.1-** The mammalian cell cycle. Quiescent cells are maintained in the G<sub>0</sub> phase of the cell cycle and re-enter the cell cycle in the G<sub>1</sub> phase. DNA replication occurs during S-phase, and in the G<sub>2</sub> phase cells are prepared for mitosis. Cell cycle progression is controlled by cyclins and cyclin dependent kinases.

## **1.3 Cell Death and Apoptosis**

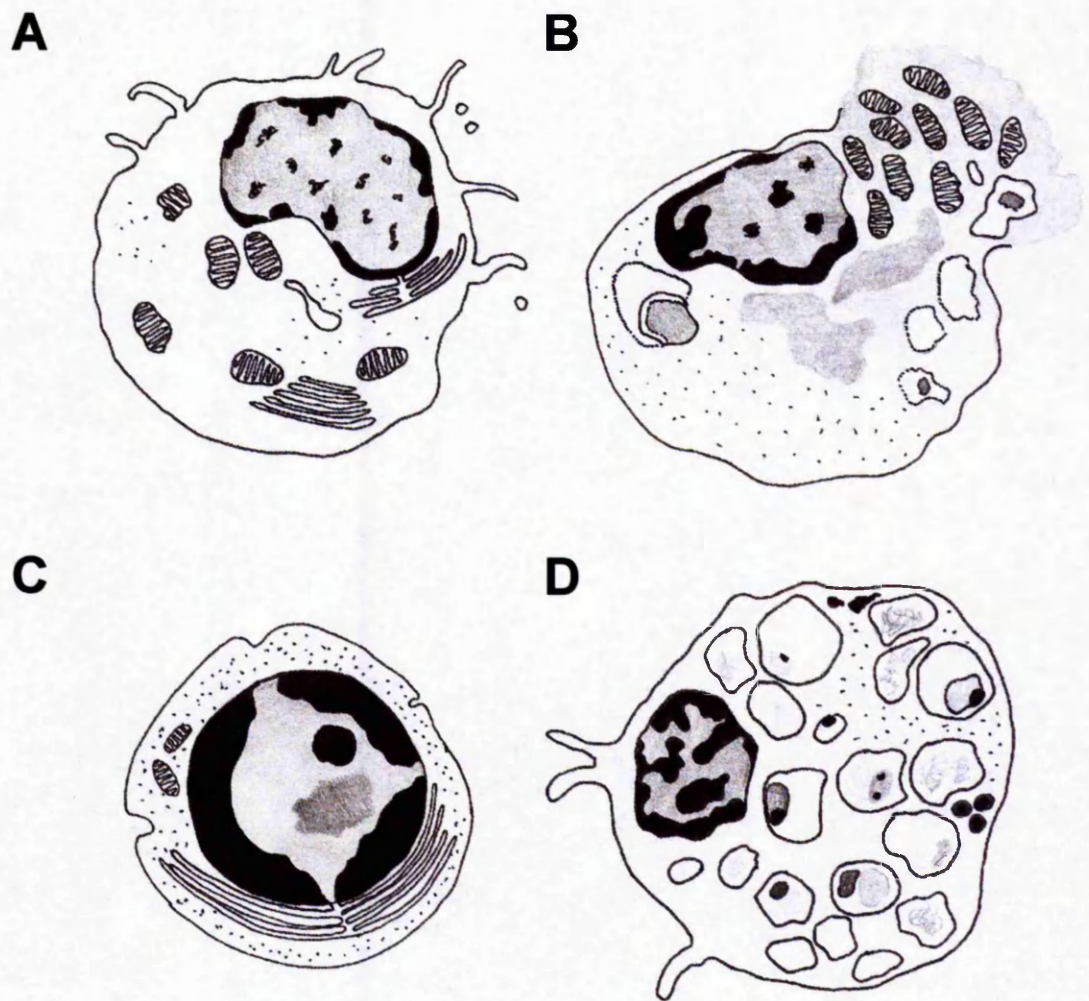
### **1.3.1 Types of Cell Death**

In a mature tissue undergoing proliferation cells must also die or be lost to maintain a constant number. Cell death may be divided by histological observations into necrosis or programmed cell death.

Necrosis is the oldest form of cell death observed microscopically, and is derived from the Greek "nekros" (dead body). Necrosis is always a pathological process; in coagulative necrosis, the most common form, the outer plasma membrane structure remains initially, although the cytoplasm and organelles are lost into the extracellular matrix from where they can induce an immune response (Figure 1.2). Necrosis does not require cellular energy production and may occur in response to severe hypoxia (Goepel 1996).

Programmed cell death may be divided into two forms, apoptosis and autophagy. Apoptosis was first described in 1972, when pathologists described a novel pattern of cell death in which the organelles fragment, condense, and are packaged with the cell membrane to form dense bodies which are phagocytosed by neighbouring cells (Kerr et al. 1972). This process is termed apoptosis, from the Greek resembling "curling up and dropping off" of flowers or leaves from trees. Unlike necrosis, apoptosis has been described in both physiological and pathological conditions (Kerr et al. 1972). Autophagy was described more recently than apoptosis, and has elements of both apoptosis and necrosis.

Apoptosis characteristically affects single cells and occurs in two stages, the formation of apoptotic bodies, and their subsequent phagocytosis by neighbouring cells or macrophages. The formation of apoptotic bodies occurs by condensation of the nucleus, organelles and cytoplasm secondary to the extrusion of water and ions (Figure 1.2). Although the nuclear membrane remains intact, the nuclear lamina - structural proteins which anchor chromatin are broken down, allowing nuclear DNA to be cleaved in a rapid non-sequence specific manner into 200 base pair fragments which can be seen using gel electrophoresis (Arends and Wyllie 1991; Lazebnik et al. 1993). To assist in phagocytosis by adjacent cells, the plasma membrane undergoes specific changes, most notably the externalisation of phosphatidylserine (Fadok et al. 1992). Following phagocytosis, the small membrane bound fragments are digested by lysosomal enzymes. Importantly, no material remains in the extra cellular space, thereby



**Figure 1.2** – Schematic representation of morphological features of normal, necrotic, apoptotic and autophagic cells based on electron micrographs presented in Edinger and Thompson. *Curr Opin Cell Biol* 2004;16:664. (A) Normal cell. (B) Necrotic cell demonstrating rupture of plasma membrane and release of intracellular contents. (C) Apoptotic cell death, in which there is a decrease in cell volume due to cytoplasmic condensation, and chromatin becomes deposited at the nuclear periphery. There is no loss of cellular material into the extracellular environment. (D) Autophagic cell death, in which the cell forms vacuoles which fuse with lysosomes to make autophagosomes, which digest cell contents, allowing them to be recycled. Cytoplasmic vacuolation is seen in necrosis and autophagy, but not normal or apoptotic cells.

avoiding an immune response (Kerr et al. 1972). Due to the nature of the processes involved, apoptosis is energy dependent.

An important difference between apoptosis and necrosis is that apoptosis occurs in tissue under physiological conditions, being part of normal cell turnover preventing hyperplasia and neoplasia by counterbalancing cell proliferation. Perhaps the most important physiological role of apoptosis is to enable normal cells to respond to damaging stimuli, especially those which alter DNA (Arends and Wyllie 1991). These include ultra-violet and ionising radiation, hypoxia and reactive oxygen species. Apoptosis also has an important role in embryonic development of all major organ systems including the development of lumina within tubular structures such as bowel, formation of digits from limb buds or involution of vestigial structures (Glucksmann 1951; Saunders 1966). In addition to embryonic development, apoptosis is a normal event in the human placenta (Smith et al. 1997b). In adults, apoptosis is important in the development of the immune system, preventing autoimmunity by the destruction of auto-reactive T-cells in the thymus (MacDonald et al. 1988).

Aberrant apoptosis may also be involved in the development of pathological conditions; inhibition of apoptosis leads to the development of neoplasia, such as in individuals with absent p53 – a protein essential for regulating apoptosis, who are unable to respond adequately to DNA damage (Bursch et al. 1984; Malkin et al. 1990). Aberrations involving the delay of apoptosis are also noted in viral infection, as the virus prevents cell death until viral nucleic acid and protein replication has taken place, such as the delayed death of CD4 cells following human immunodeficiency virus infection (MacDonald et al. 1988; Osborne 1995). Suspension of apoptosis secondary to viral infection may subsequently lead to the development of neoplasia (Henderson et al. 1993). Increased levels of apoptosis during development may also lead to embryonic malformations resulting from teratogens causing apoptosis at their site of action (Crawford et al. 1972; Kerr et al. 1972).

It has been suggested that the presence or absence of ATP determines whether a cell undergoes apoptosis or necrosis. By inhibition of oxidative phosphorylation, a combination of apoptosis and necrosis has been described. This has been termed 'aponecrosis' as it is initiated in a manner similar to apoptosis, but is completed with features suggestive of necrosis (Formigli et al. 2000). As this phenomenon has only been demonstrated in cultured fibroblast

cells, its biological significance is unknown, although it provides support for the hypothesis that the decision to undergo apoptosis or necrosis is dependent on the presence of ATP.

Autophagy, is derived from the Greek, phage – to eat, and auto – oneself. In plants, unicellular and simple multicellular organisms autophagy may represent a survival mechanism in which cell proteins and organelles are digested to recycle amino acids and lipids via a purpose-built lysosome fused with a vacuole, termed the autophagosome (Levine and Klionsky 2004) (Figure 1.2D) However, autophagy can continue with cells digesting themselves, in an alternative form of programmed cell death. Evidence for this is derived from observations that cell death induced by pro-apoptotic stimuli can occur even when caspases - enzymes critical for apoptosis - are inhibited, with features more typical of necrosis than apoptosis (Edinger and Thompson 2004). It is proposed that activation of poly-ADP ribose polymerase (PARP) leads to rapid depletion of nuclear and cytoplasmic nicotinamide adenine dinucleotide (NAD) inhibiting glycolysis, leading to energy depletion and death by necrosis. In fact, apoptosis may inhibit autophagy as PARP, an enzyme capable of DNA repair is cleaved by caspase-3 (Zong et al. 2004). However, studies in other animals, most notably *manduca sexta* sp, has demonstrated co-existence of apoptosis and autophagy (Jochova et al. 1997). It is currently thought that autophagy may play a role in normal development, as deletion of genes involved in autophagy lead to embryonic lethality in mice (Yue et al. 2003). Notwithstanding these findings, few studies exist in human cells or tissues and the role of autophagic cell death in physiological and pathological processes is yet to be elucidated.

The identification and knowledge of these forms of cell death has increased markedly over the past three decades, particularly the existence of programmed cell death. Of the forms of programmed cell death described apoptosis is the most studied, and has been identified in human tissues in physiological and pathological conditions and a significant amount of data exists regarding the regulation of apoptosis in human cells. Therefore, this investigation focuses on the initiation and execution of apoptosis, as at present, it appears to be the most relevant form of programmed cell-death in the regulation of cell turnover in the human placenta.

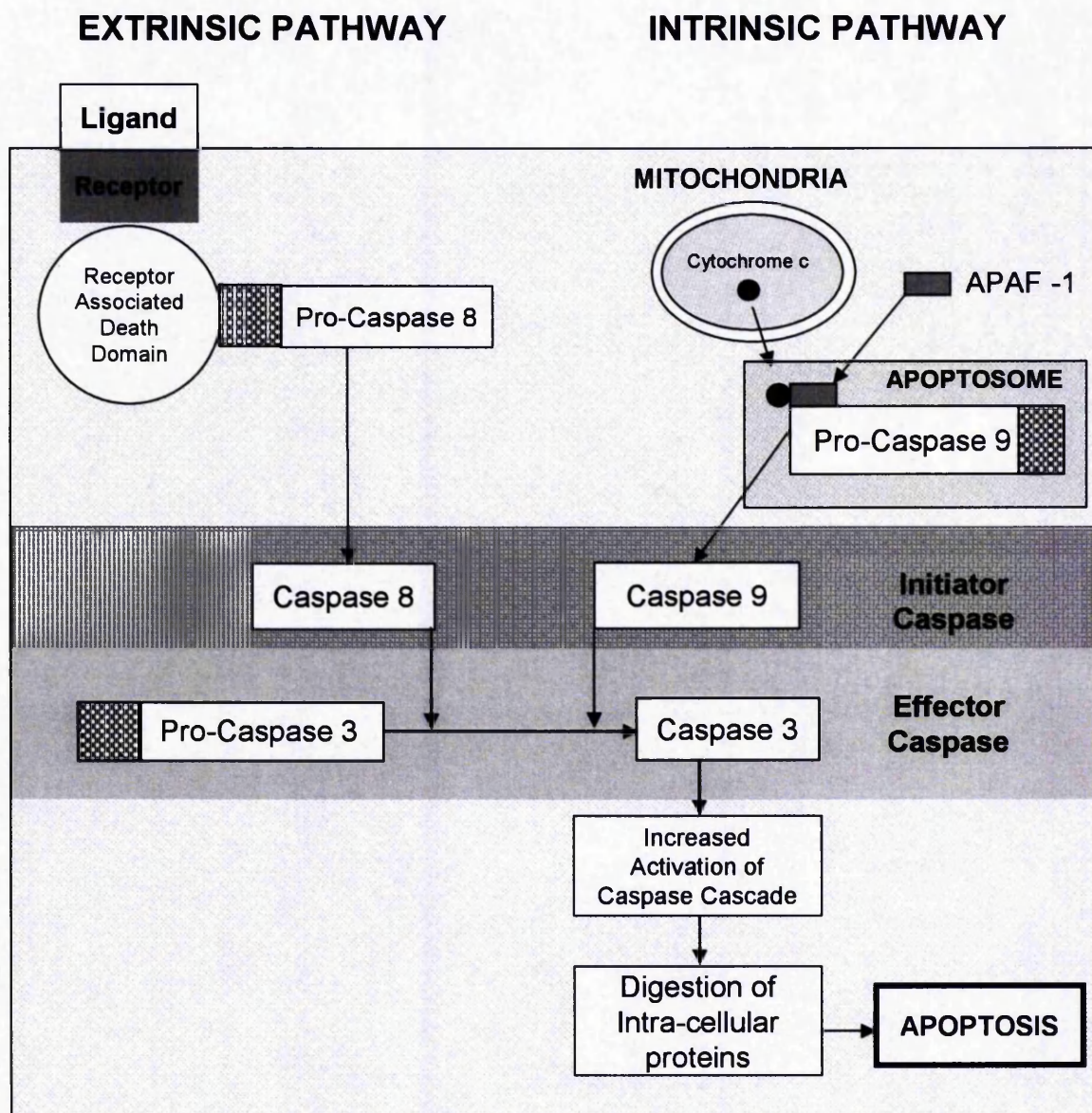


### 1.3.2 Apoptotic Pathways

The similar appearance of apoptotic cells between different species and individual tissues of an organism suggests a well preserved control mechanism. Apoptosis may result from a pathway which is initiated from within the cell (intrinsic) or one which originates from an external signal (extrinsic). However, these are not distinct pathways and cross-activation can occur (Fadeel and Orrenius 2005). Both pathways culminate in the activation of aspartate-specific cysteine proteases, known as caspases, which digest intracellular proteins thereby mediating apoptosis (Figure 1.3).

The caspases are a family of 14 proteolytic enzymes, which can be divided into those involved in apoptosis and those which regulate cytokine production (Nunez et al. 1998). Caspases 2,3,6,7,8,9 and 10 are associated with apoptosis; these can be further divided into initiator (2,8,9,10) and effector caspases (3,6,7), dependent on their place and function in the caspase cascade (Nunez et al. 1998). Caspases share a similar morphology, from the N-terminal end there is a pro-domain, a large subunit, small subunit, and linker domains. All caspases have a cysteine residue within their active site and cleave proteins after aspartate amino acid residues, an uncommon substrate for proteolytic enzymes, allowing caspases to be highly specific. In addition, pro-caspases contain aspartate residues at cleavage points in the molecule allowing activation by caspases upstream in the enzymatic cascade. Initiator caspases contain a long pro-domain which facilitates protein-protein interactions, particularly with the death receptor effector domains of the tumour necrosis factor (TNF) family and the apoptosome, such as in the interaction between procaspase-8 and the TNF receptor associated death domain (TRADD) (Muzio et al. 1996). The upstream to downstream flow of caspase activation has been demonstrated by the inactivation of initiator caspase-9, which removes the effects of caspase-3 (Kuida et al. 1998).

Effector caspases, the most well described being caspase-3, activate enzymes related to DNA and structural protein degradation, such as caspase-activated deoxyribonuclease and Rho-associated kinase 1 (ROCK-1) (Enari et al. 1998; Coleman et al. 2001). In addition, effector caspases can potentiate the apoptotic signal, for example, caspase-3 increases caspase-9 levels via a positive feedback loop, and inactivates X-linked inhibitor of apoptosis protein (XIAP) - a potent caspase-3 inhibitor (Slee et al. 1999; Slee et al. 2001). Furthermore, caspases can link the extrinsic and intrinsic apoptotic pathways. For example, caspase-8



**Figure 1.3** – Schematic diagram of apoptotic pathways within the cell showing convergent systems for caspase activation. The extrinsic pathway involving receptor-ligand interaction is shown on the left, the intrinsic pathway resulting from altered mitochondrial membrane permeability on the right.

cleaves Bid, a protein regulating mitochondrial membrane permeability, which is central to the intrinsic apoptotic pathway, to tBid (its activated form), further augmenting the apoptotic cascade (Luo et al. 1998). Therefore, the caspase cascade produces a signal that grows in magnitude producing the classical morphological appearances of apoptosis.

The intrinsic apoptotic pathway may be programmed by the cell, i.e. at a certain stage of development an individual cell is programmed to undergo apoptosis. Alternatively, a cell may use the intrinsic pathway of apoptosis to respond to cellular damage. The intrinsic pathway involves alteration of mitochondrial membrane permeability, which results in cytochrome c release (Figure 1.3). This combines with apoptotic protease-activating factor-1 (APAF-1) present in the cytoplasm to form a heptamer, termed the apoptosome, which is able to combine with pro-caspase-9 cleaving it to caspase-9, which then activates effector caspases-3, -6 and -7 (Nicholson and Thornberry 2003).

The extrinsic pathway utilizes receptors which bind either pro-survival or pro-apoptotic ligands. One example, the TNF receptor family, has been studied in depth, these receptors bind to a death signalling complex. In the case of TNF Receptor-1 (TNFR1) this is the TNFR associated death domain (TRADD) which, following binding of TNF $\alpha$ , associates with procaspase-8, cleaving it to caspase-8 which then activates the caspase cascade (Dhein et al. 1995; Hsu et al. 1995; Zheng et al. 1995; Kischkel et al. 2000). Although these mechanisms were initially reported in regulation of lymphocytes, binding of TNF $\alpha$  to TNFR1 produces a potent stimulus for apoptosis within the trophoblast in vitro (Yui et al. 1994b).

In common with all cells trophoblast has two potential mechanisms for activation of the apoptotic pathway, a reaction to cellular or DNA insult (intrinsic pathway) and receptor-ligand interactions (extrinsic pathway). In addition to the intrinsic and extrinsic death signals leading to apoptosis, the absence of survival signals may also induce apoptosis. The administration of platelet derived growth factor to rat optic nerve cells results in an increased cell number without an increase in cell proliferation, inferring a reduction in cell death. However, there is no evidence that an absence of growth factor decreases the number of neurones (Raff 1992). It is hypothesised that the cell integrates both pro-apoptotic and pro-survival signals when forming a response. This has been demonstrated in placental cytotrophoblasts, in which epidermal growth factor reduced apoptosis in response to treatment with TNF- $\alpha$  (Garcia-Lloret et al. 1996).



Due to the widespread and permanent changes affecting a cell undergoing apoptosis, activation of the apoptotic pathway must be tightly regulated to prevent unwanted cell death which may compromise tissue and organ function. This regulation is controlled by several complex pathways within the cell, using proteins to convey the signals. These proteins were initially termed oncoproteins, as they have been largely studied in cells which have undergone malignant transformation, and were initially thought to have a causative role in the development of cancer.

#### **1.4 Regulation of Apoptosis by Intracellular Proteins**

In response to sublethal cell damage a cell may respond in one of three ways: it may undergo apoptosis, it may stop replicating and attempt repair, or it may survive. The reaction will depend on the type of cell, the nature and magnitude of the noxious stimulus and the presence of survival signals. Regulation of the intrinsic pathway of apoptosis may be considered as consisting of four component parts: (i) the recognition of cellular damage / death signal, (ii) altered transcription of downstream components of the apoptotic cascade, (iii) altered mitochondrial membrane permeability and (iv) the modification of caspase activity. For ease of description these components may be regarded as temporally and spatially distinct, whereas, in reality there is much overlap between the elements of the apoptotic cascade. The regulation of transcription of downstream proteins occurs in the nucleus, the recognition of cell damage occurs predominantly in the cytoplasm, as does the modification of mitochondrial membrane integrity and regulation of caspase activity. Therefore, these proteins may be classified as to their predominant site of action as well as their role.

The recognition of cellular damage occurs through many proteins capable of detecting changes in the cellular environment, such as hypoxia inducible factors (HIF), mitogen activated protein kinase (MAPK) and jun N-terminal kinase (JNK). Many of these proteins are either transcription factors - directly promoting transcription of downstream proteins or kinases - capable of modifying proteins, altering their structure and function. One such downstream target is p53, a transcription factor capable of promoting cell-cycle arrest and/or apoptosis (Haupt et al. 2003). The effects of p53 are regulated by Mdm2; this interaction will be described in detail in Section 1.4.1 (Wu et al. 1993). Due to its predominant function as a transcription factor the majority of the effects of p53 occur within the nucleus. Therefore, p53

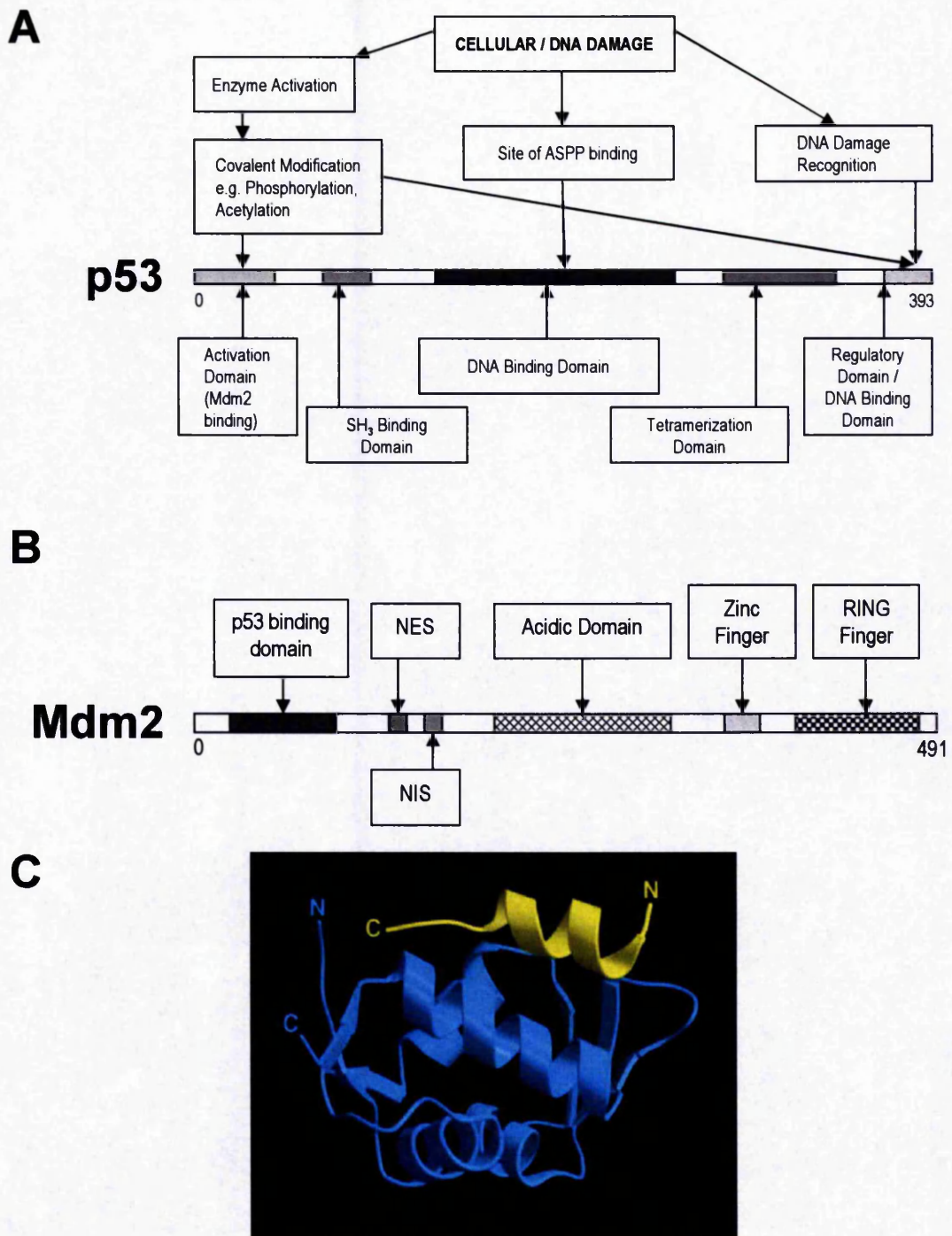
and Mdm2 may be regarded as regulating the intrinsic apoptotic pathway at the nuclear level. The control of mitochondrial membrane permeability is governed by another family of proteins, termed the Bcl-2 family, which contains both pro- and anti-apoptotic members. Finally, caspase activity is regulated in the cytoplasm by proteins released from the mitochondria and those already present in the cytosol.

Therefore, proteins responsible for the regulation of apoptosis can be grouped according to their roles, and may be further classified into those which are pro-apoptotic / cell cycle arrest or pro-survival in their nature, although it should be noted that the response to different proteins has been found to be tissue specific (Bouvard et al. 2000). Due to their position near to the beginning of the intrinsic apoptotic pathway, p53 and Mdm2 have an essential role in regulation of the apoptotic response and will be discussed in greater depth.

#### **1.4.1 The role of p53 in cell cycle control**

The p53 protein is one of the most important oncoproteins in the co-ordination of the cellular response to damage. Elevated levels of p53 have been shown to induce apoptosis (Yonish-Rouach et al. 1991). Abnormalities of p53 are amongst the most common molecular abnormalities in human carcinogenesis, and are present in approximately 50% of cancers. It is hypothesised that the loss of apoptotic control prevents the removal of cells with damaged DNA, allowing them to continue proliferating eventually leading to carcinogenesis. This is supported by evidence that humans with a non-functional p53 mutation or deletion develop malignancies throughout childhood and adult life (Malkin et al. 1990). p53 is a 393 amino acid residue, 53kD protein which is encoded on the short arm of chromosome 17 (17p13.1). In common with other transcription factors p53 is a modular protein, having several different functional domains (Arrowsmith and Morin 1996; Soussi and May 1996; 2002). Five important domains have been identified, namely the activation, SH<sub>3</sub> binding, sequence specific DNA binding, tetramerization and basic domains, shown in Figure 1.4 (Prives and Hall 1999)). p53 is most effective as a transcription factor in a tetramer due to conformational changes in the DNA binding region (Jeffrey et al. 1995).

p53 is present in the normal cell at low levels, but in response to a variety of noxious stimuli including: genotoxic agents, heat, hypoxia, hyperoxia, cytokines, growth factors, metabolic changes, cell-cell interactions and activated oncogenes, p53 levels increase rapidly with an



**Figure 1.4** – Diagrammatic representations of the domain structure of p53 and Mdm2. (A) p53 possesses domains which undergo post-translational modification which regulate the ability of p53 to interact with Mdm2 (activation domain), p53 also has a tetramerization domain, facilitating the formation of a p53 tetramer, which promotes DNA binding. (B) Mdm2 has a p53 binding domain and nuclear import (NIS) and export signals (NES) enabling the regulation of p53 ubiquitination. The RING finger domain is a common feature with other E3-ubiquitin ligases. (C) Mdm2 interacts with a p53 via binding with the transactivation domain at the n-terminus of p53. This is demonstrated by this computer generated image showing Mdm2 (blue) and the transactivation domain of p53 (yellow) Used with permission from the Sloan-Kettering Institute, NY, USA. <http://www.mskcc.org/mskcc/html/10861.ctm>.

increase in half life from around 30 minutes to up to 24 hours (Oren et al. 1981; Maltzman and Czyzyk 1984). This has led to the hypothesis that p53 is at the centre of the cellular response to noxious stimuli – indeed, p53 has been described as the 'guardian of the genome' (Lane 1992).

As a transcription factor, p53 is active within the nucleus of a cell, which is confirmed by the finding that p53 expression is strongest in the nucleus of both normal and transformed cells (Yewdell et al. 1986). p53 possesses a nuclear localization signal which, via an active transport mechanism, causes p53 to move from the cytosol to the nucleus. This process is rapid, taking only 3 minutes (Dang and Lee 1989; Addison et al. 1990; Shaulsky et al. 1990; Middeler et al. 1997). The nuclear export of p53 is also rapid, energy dependent and carefully controlled. Nuclear export involves another specific protein sequence, the nuclear export signal (NES), found in the tetramerization domain of p53 (Figure 1.4) (Stommel et al. 1999). p53 is rapidly broken down through binding with Mdm2, which firstly inactivates p53 and leads to nuclear export followed by ubiquitination, a process whereby a ubiquitin residue is attached to the protein, which then targets the protein for destruction by the proteasome, a complex structure which enzymatically degrades proteins (Haupt et al. 1997; Roth et al. 1998; Tao and Levine 1999a; Lu et al. 2000). This interaction between p53 and Mdm2 gives an essential negative feedback loop, as p53 is a transcription factor for Mdm2. This balance between p53 and Mdm2 leads to the constant low basal levels of p53 preventing inappropriate cell cycle arrest / apoptosis (Barak et al. 1993; Wu et al. 1993).

To enable controlled activation and nuclear localisation in response to cell damage, p53 undergoes many post-translational changes. In response to cellular damage these are largely confined to the activation and regulatory domain at the N-terminal and C-terminal ends respectively (Giaccia and Kastan 1998; Prives and Hall 1999; Balint and Vousden 2001) (Figure 1.4). Many stress signals including DNA damaging agents activate kinases within the cytosol such as Casein kinase 1 and 2 (Adler et al. 1997), ataxia telangiectasia mutated (ATM) kinase (Banin et al. 1998; Canman et al. 1998), JNK (Milne et al. 1994; Adler et al. 1997) and DNA-activated protein kinase (DNA-PK) (Woo et al. 1998). These enzymes phosphorylate residues within the N-terminal domain. Phosphorylation of this domain *in vitro* has been shown to inhibit binding of Mdm2. It is hypothesised that a reduction in Mdm2 binding leads to stabilisation of p53 by decreasing ubiquitination (Shieh et al. 1997).

The C-terminal domain of p53 undergoes a variety of modifications including phosphorylation, dephosphorylation (Adler et al. 1997), acetylation (Gu and Roeder 1997) and glycosylation (Shaw et al. 1996). This may result from binding of transcriptional co-activators such as myc or p300/Creb Binding Protein (CBP) at the N-terminal end or as the result of specific enzymatic interactions. Modifications of the C-terminal region have been shown to modify p53-mediated protein transcription. Although the actual mechanism is unknown at present, it is hypothesised that covalent changes to the C-terminal end may cause conformational changes to the DNA binding region or alter the ability of p53 to form a tetramer, both of which will modify transcription.

Other proteins, such as the apoptosis stimulating proteins of p53 (ASPP) 1 and 2, interact with p53 forming complexes which alter specific DNA binding without producing a change in the molecular structure of the protein. This promotes the binding of p53 to pro-apoptotic genes rather than those which cause cell cycle arrest (Samuels-Lev et al. 2001). In addition, p53 may be stabilised by Hypoxia-Inducible Factor 1 $\alpha$  (HIF-1 $\alpha$ ) in response to cellular hypoxia with acidosis in the absence of DNA damage (An et al. 1998; Pan et al. 2004). Ultimately, the molecular decision for apoptosis or cell cycle arrest lies within the individual gene transcription promoted by p53, which is regulated by covalent and non-covalent modifications of the p53 protein described above. Therefore, p53 can be regarded as a central signalling protein integrating a variety of inputs and then promoting the appropriate effect, such as apoptosis or cell-cycle arrest.

p53 has transcription dependent and independent functions with the former being of greater importance. As a transcription factor p53 binds DNA at specific sites, causing unwinding of the DNA enabling the RNA polymerase to attach to the site, leading to the transcription of mRNA. p53 usually binds to a sequence consisting of two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs (el-Deiry et al. 1992). p53 can also induce genes via binding to a microsatellite sequence (Contente et al. 2002) or a palindromic sequence (Yin et al. 2003). p53 is responsible for promoting transcription of many genes, including those which control the cell cycle directly and those which are involved with apoptosis both in the extrinsic and intrinsic pathways.

In cells, p53 induces transcription of p21 (Also known as Waf/Cip-1) which plays a critical role in producing cell-cycle arrest following DNA damage (el-Deiry et al. 1993; Brugarolas et al.

1995). The N-terminal end of p21 is a CDK inhibitor preventing progression through G<sub>1</sub> and G<sub>2</sub> checkpoints (Zhang et al. 1993; Harper et al. 1995). The C-terminal region of p21 interacts with proliferating nuclear cell antigen (PCNA), cyclin and CDK, preventing DNA synthesis by DNA polymerase  $\delta$  (Flores-Rozas et al. 1994; Waga et al. 1994; Gibbs et al. 1997).

Promotion of the intrinsic apoptotic cascade occurs through multiple downstream pathways. The transcription of APAF-1, a key constituent of the apoptosome, is promoted by p53 through a response element in the APAF-1 promoter sequence (Kannan et al. 2001; Moroni et al. 2001). p53 promotes the transcription of pro-apoptotic mitochondrial membrane proteins including Bax, Puma and Noxa (Miyashita and Reed 1995; Oda et al. 2000; Nakano and Vousden 2001). As well as up-regulation of constituents of the intrinsic apoptotic pathway, p53 can directly regulate caspase-6 expression, providing another point of activation of the intrinsic apoptotic pathway (MacLachlan and El-Deiry 2002). p53 may also promote apoptosis through mechanisms other than those which modify the intrinsic pathway. p53 is able to cause cleavage of PARP, a marker of caspase 3 activity in cells with DNA damage, in the absence of Bax, a mitochondrial pore protein present in the intrinsic pathway of apoptosis (See Section 1.4.3). It is hypothesised that p53 directly alters transcription of caspase 3 or may cause caspase activation upstream of the apoptosome (Ding et al. 1998).

The extrinsic apoptotic pathway is also promoted by increased p53 levels. The 'death' signal receptors Fas and DR5 are upregulated in the presence of p53 (Owen-Schaub et al. 1995; Wu et al. 1997). Whilst the transcription of DR5 is promoted directly, Fas levels are increased as a result of increased transport by the Golgi apparatus. Therefore, an external 'death signal' in the presence of a cell with elevated p53 is increased in magnitude (Bennett et al. 1998; Takimoto and El-Deiry 2000). In addition to transcription dependent functions, p53 has non-transcription dependent activities involving the C-terminal end which recognise areas of DNA damage including single stranded DNA (Bakalkin et al. 1995), mismatches (Lee et al. 1995), Holliday junctions (Lee et al. 1997) and irradiated DNA (Reed et al. 1995). Following binding to damaged DNA, p53 is able to bind DNA repair proteins such as Transcription Factor IIH (TFIIH) and DNA Topoisomerase directly repairing DNA whilst arresting the cell cycle until repair is complete (Leveillard et al. 1996).

In addition to the promotion of apoptotic genes, p53 increases the transcription of Mdm2 by binding its P2 promoter sequence. This forms a tightly regulated negative feedback loop to

ensure that a cell is never exposed to unopposed levels of p53. Mdm2 also inhibits the interaction with co-factors which may increase the activity of p53, such as p300/CBP-associated factor (PCAF) and p300/CBP (Wadgaonkar and Collins 1999; Kobet et al. 2000; Jin et al. 2002).

p53 also interacts with two related proteins, p63 and p73. These proteins are structurally homologous with p53, having a similar domain structure. However, p63 and p73 have an additional sterile alpha domain which is thought to mediate specific protein-protein interactions between p73, p63 and p53, altering the DNA binding properties of p53 (Chi et al. 1999). p53 has a greater pro-apoptotic effect in the presence of p63 and p73 due to the promotion of pro-apoptotic members of the Bcl-2 family (see Section 1.4.3), including Bax (Miyashita and Reed 1995; Thornborrow et al. 2002), Noxa (Oda et al. 2000) and Bid (Nakano and Vousden 2001). These proteins are key effectors in the internal pathway of apoptosis, altering mitochondrial membrane potential, allowing cytochrome c to escape, forming the apoptosome (Bossy-Wetzel and Green 1999). Therefore, the combined action of p53 and related proteins is to promote apoptosis by both the intrinsic and extrinsic pathways.

#### **1.4.2 The Role of Mdm2 in Cell Cycle Control**

mdm2 (murine double minute gene) was originally identified as one of three genes found on extrachromosomal nuclear bodies (termed double minutes) in the 3T3 mouse fibroblast cell line (Cahilly-Snyder et al. 1987). The human form of the protein is termed Mdm2 or human double minute 2 (Hdm2). Mdm2 is an essential regulatory oncoprotein, the main function of which is to regulate p53 levels via ubiquitination as described above. Mdm2 is essential for normal development; mice without mdm2 (*mdm2*<sup>-/-</sup>) die early in gestation with very little embryonic material present, although this is not the case if p53 is also absent (Jones et al. 1995). Mdm2 is essential for normal human cell turnover as mutations have been found in a number of cancers (Cordon-Cardo et al. 1994; Lu et al. 2002), and overexpression of mdm2 in mice leads to spontaneous tumour formation (Jones et al. 1998). In addition to the regulation of p53, Mdm2 also interacts with other proteins involved within the cell cycle (Ganguli and Wasylyk 2003).

The human Mdm2 gene is located on the 12q14 locus and has 12 exons. Due to alternative splicing, several different protein variants can be made. In humans, there are two proteins

produced in response to the p53 response element: the complete 90kD and a 76kD isoform which lacks part of the p53 binding domain, acting as a dominant negative form of Mdm2 (Iwakuma and Lozano 2003; Peri et al. 2003). The structure of the 90kD Mdm2 variant is shown in Figure 1.4. In common with p53, Mdm2 has a nuclear localisation and export signal, controlling the cellular location of Mdm2. Mdm2 also undergoes ubiquitination via interaction with other Mdm2 molecules, and is disposed of via the proteasome. Mdm2 is more short-lived than p53, having a half-life of approximately 20 minutes (Olson et al. 1993; Keleti et al. 1996). However, when cells are damaged Mdm2 breakdown is accelerated, reducing its half-life to 5 minutes in vitro (Stommel and Wahl 2004).

The functions of Mdm2 can be divided into those which occur with proteins upstream of its interaction with p53, directly modifying that interface, and those which occur downstream of Mdm2. The interaction between p53 is understood to be the most important function of Mdm2. As described earlier, Mdm2 leads to nuclear export, ubiquitination and disposal of p53, thereby reducing the strength of the quiescence / apoptotic signal (Haupt et al. 1997; Roth et al. 1998; Tao and Levine 1999a; Lu et al. 2000). The importance of Mdm2 in the regulation of p53 is underlined by the fact that Mdm2 is a key target for transcriptional activation, mediated by p53 (Barak et al. 1993). Adaptation of the interaction between p53 and Mdm2 occurs by two processes, covalent modification of either p53 or Mdm2 to alter the efficacy of binding, or co-factor modification leading to altered ubiquitination of p53 or Mdm2.

An important signal from DNA damage which leads to disruption of the p53-Mdm2 interaction is p14<sup>ARF</sup>, one product of the INK4a locus (ARF stands for alternative reading frame) (Lowe and Sherr 2003). p14<sup>ARF</sup> binds directly to Mdm2, blocking nuclear export, causing accumulation in the nucleolus (Tao and Levine 1999b). In addition, p14<sup>ARF</sup> inhibits Mdm2's E3 ubiquitin ligase activity, reducing turnover of p53, thereby delivering a pro-apoptotic/quiescence signal (Honda and Yasuda 1999). Conversely, p300/CBP increases the degradation of p53. In the absence of p300/CBP a single ubiquitin residue is added to p53, which is insufficient to cause degradation, as at least four residues are required (Thrower et al. 2000; Lai et al. 2001); in the presence of p300/CBP p53 is polyubiquitinated leading to degradation, producing an anti-apoptotic signal (Iwakuma and Lozano 2003). In addition to the effects on p53, Mdm2 also interacts with proteins including Retinoblastoma (pRb), another



tumour suppressor protein. The binding between Mdm2 and pRb disrupts cell cycle arrest, increasing proliferation (Xiao et al. 1995)

Like p53, Mdm2 can undergo post-translational phosphorylation at multiple sites, producing changes in function. In some cases, enzymes involved with p53 phosphorylation are also responsible for Mdm2 phosphorylation. For example, the p53 binding domain of Mdm2 undergoes phosphorylation by DNA-PK at the Ser-17 residue, which decreases the Mdm2-p53 interaction *in vitro* (Mayo et al. 1997). Other stress signals dephosphorylate Mdm2, inactivating ubiquitination (Blattner et al. 2002). The net result of both these changes is to increase p53 and induce apoptosis. Conversely, phosphorylation of the Ser-166 and 186 residues which lie near the NLS and NES may facilitate nuclear transport and interaction with p300/CBP and p53 providing a pro-survival stimulus (Mayo and Donner 2001; Zhou et al. 2001).

In addition to the effects on p53, Mdm2 interacts with p63 and p73, although the effects of this are unknown (Kaelin 1999; Calabro et al. 2002). The action of Mdm2 is to confer cell survival, largely by opposing the effects of p53. Therefore, the interaction between Mdm2 and p53 can be viewed as integrating cell death and survival signals to produce a downstream signal which may be anti- or pro-apoptotic.

#### **1.4.3 Bcl-2 Family Proteins**

The most important family of proteins downstream of the p53:Mdm2 interaction is the Bcl-2 family, this contains proteins which are both anti- and pro- apoptotic (Table 1.1). The majority of the Bcl-2 family of proteins consist of a combination of four domains, BH-1, BH-2, BH-3, and BH-4. The anti-apoptotic members of the family consist of all four domains (with the exception of Bcl-XL), the pro-apoptotic members Bax and Bak, lack a BH-4 and BH-3 domain respectively. Some such as Bid, Puma and Noxa consist of a BH-3 domain alone (Tsujimoto and Croce 1986). Different members of the Bcl-2 family express some or all of these domains and are able to form homo- and hetero-dimers using individual BH domains. For example Bcl-2 can bind to Bax, Bak, and Bid rendering them inactive (Huang et al. 1998).

Anti-Apoptotic	Pro-Apoptotic BH3-Domain Only	Pro-Apoptotic multi-domain
Bcl-2	Bid	Bax
Mcl-1	Bad	Bak
Bcl-xL	Noxa	Bok
Bcl-w	Puma	Bcl-xs

**Table 1.1** – Function of Bcl-2 family members with respect to apoptosis (Sorenson 2004).

Members of the Bcl-2 family elicit effects on mitochondrial membrane permeability – the mitochondria contains many proteins, such as cytochrome c, smac, HtrA2/omi and apoptosis inducing factor (AIF), the release of which contributes to the intrinsic pathway of apoptosis. The nature of the interaction of the Bcl-2 family with the mitochondrial membrane is unclear (Donovan and Cotter 2004). One model proposes that the Bcl-2 family acts as membrane pores, either allowing or preventing proteins such as cytochrome c from entering the cell cytoplasm. In support of this, members of the Bcl-2 family form pores in artificial membranes, although these are not large enough to permit the release of proteins such as AIF (57kD) (Schendel et al. 1997). Another model proposes that the Bcl-2 family regulates pre-existing channels such as the permeability transition pore (PTP), which contains a voltage dependent anion channel (VDAC) within the outer mitochondrial membrane; members of the Bcl-2 family interact with VDAC in vitro, preventing cytochrome c release (Shimizu et al. 1999). In addition, Bcl-2 antagonises apoptosis by preventing oligomerization and activation of caspases (Nunez et al. 1998) and nuclear import of p53 by the formation of a complex with nuclear pores (Beham et al. 1997). Of primary importance is the fact that the Bcl-2 family add another layer of regulation to the apoptotic pathway, independent of caspases (Donovan and Cotter 2004).

Members of the Bcl-2 family are important in development, a loss of Bcl-2 in mice leads to renal hypoplasia, involution of the spleen and thymus and impaired neuronal development in the brain (Sorenson 2004). A reduction in pro-apoptotic Bax leads to lymphoid hyperplasia and defective spermatogenesis leading to infertility. Loss of Bak alone does not result in

developmental abnormalities. Loss of Bak and Bax leads to multiple neurological and haematological abnormalities and the retention of interdigital skin webs, which are normally lost by apoptosis during embryogenesis (Sorenson 2004).

#### **1.4.4 Regulators of Caspase Activity**

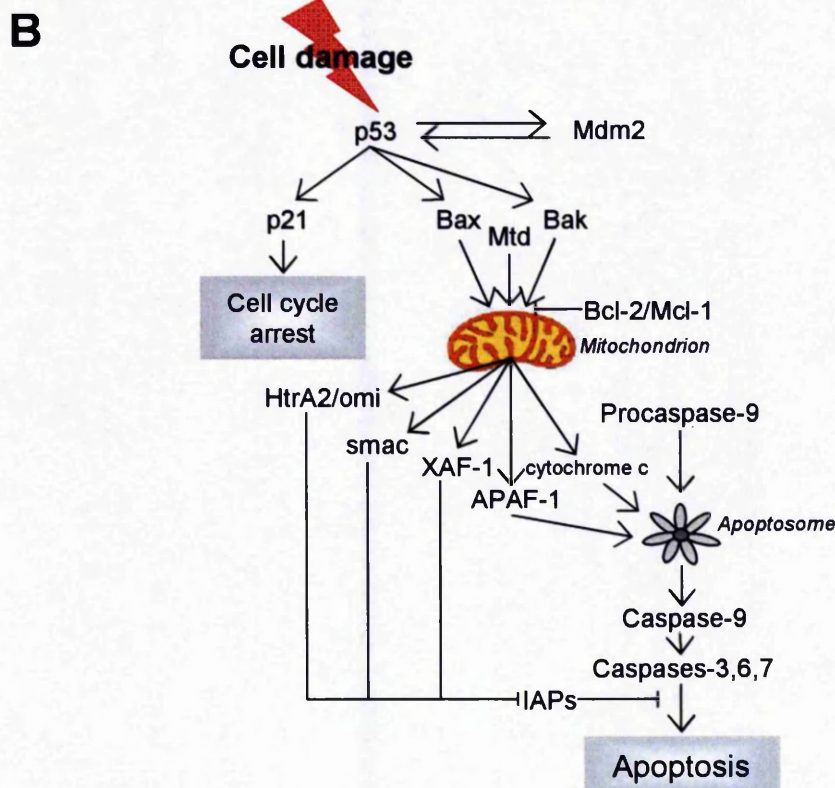
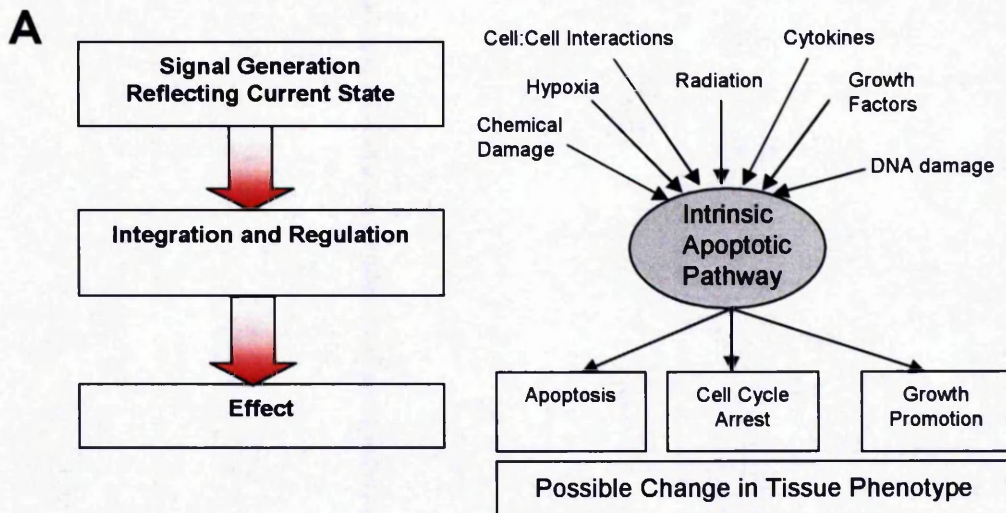
Caspase activity is regulated in the cytosol by inhibitors of apoptosis proteins (IAPs) such as XIAP, cellular IAP (cIAP), neuronal IAP (NIAP), survivin and livin. XIAP is the most extensively characterized IAP, acting as an inhibitor of caspases-3, -7 and -9 via three baculoviral inhibitor of apoptosis repeat (BIR) regions (Holcik et al. 2001). Survivin possesses one BIR region, which is analogous to one of those in XIAP, and can also interact with caspases-3 and -7, although the nature of this interaction is less clear than XIAP (Wheatley and McNeish 2005). In addition to its anti-apoptotic role, survivin associates with the microtubule spindle complex during mitosis, suggesting a role in cell replication. The mode of action of other IAPs, such as NIAP, are less clear (Davoodi et al. 2004).

The activity of IAPs is modulated by proteins released from the mitochondria; the most extensively characterized examples are smac and HtrA2/Omi. smac binds to XIAP, cIAP and survivin preventing their inhibitory action of active caspase-3 (Verhagen et al. 2000). XAF-1 another pro-apoptotic mitochondrial protein interacts with XIAP antagonising its activity against caspase 3 (Straszewski-Chavez et al. 2007). Following release from the mitochondria HtrA2/Omi acts by competitive antagonism of the binding of IAPs, particularly XIAP to caspase-3, 7 and 9 (Figure 1.5B) (Saelens et al. 2004) By reducing the activity of IAPs, smac and HtrA2/Omi are able to amplify the apoptotic signal transmitted by the intrinsic apoptotic pathway.

#### **1.4.5 An Integrated Pathway**

Due to the importance of apoptosis in the survival of multi-cellular organisms, there is a highly developed and complex system capable of responding to both pro-survival and pro-apoptotic signals. For the purposes of this study, these pathways may be reduced to three key stages, as illustrated in Figure 1.5A (i) An initiator stage, in which the cell generates signals to reflect its current state (within a tissue) such as hypoxia, damage by  $\gamma$ -radiation, or stimulation by growth signals, (ii) integration of these signals via a central regulatory process involving (iii)

effector stage in which the response is transmitted via transcription and non-transcription independent mechanisms. The inputs to this process are many and varied, and the effect they have is dependent on cell state and the tissue in which the cell is located (Bouvard et al. 2000). Together the proteins of the intrinsic pathway form a complex system (Figure 1.5B). In the healthy cell much emphasis is placed on the balance between pro- and anti-apoptotic protein expression in the determination of cell survival, and this may be regulated by the balance between p53-Mdm2, Bax/Bak-Bcl-2, and smac/HtrA2/omi-IAPs. An imbalance at any point in the pathway appears to be sufficient to induce apoptosis (de Rozières et al. 2000; Du et al. 2000; Sorenson 2004). The relationship between p53 and Mdm2 potentially has the greatest effect as it is at the beginning of the intrinsic apoptotic pathway, which may explain the devastating effects of an imbalance in p53 and mdm2 on embryonic viability (Jones et al. 1995). Control of apoptosis during normal embryonic development is important, as deletion of several of the proteins described leads to developmental abnormalities (Donehower et al. 1992; Sorenson 2004).



**Figure 1.5 – (A)** Illustration of the role of the intrinsic apoptotic pathway in the control of cell turnover leading to either apoptosis, cell cycle arrest or proliferation. Proteins regulating the intrinsic pathway of apoptosis. **(B)** The interaction between p53 and Mdm2 is modified in response to cell damage, allowing p53 to promote the transcription of p21 and Bax, and have transcription-independent interactions with other proteins such as Bak. Bax, Bak and other pro-apoptotic members of the Bcl-2 family act on the mitochondrial membrane to increase permeability; these effects are antagonised by Bcl-2 and Mcl-1. APAF-1 and cytochrome c combine with procaspase-9 to form the apoptosome producing active caspase-9, which then activates the effector caspases-3,6 and 7. Caspase activity is regulated by the actions of IAPs, which are themselves inhibited by the action of proteins released from the mitochondrion such as XAF-1, smac and HtrA2/omi.

## **1.5 Apoptosis and Oncoprotein Expression within the Placenta**

### **1.5.1 Placental Structure**

The placenta is a fetal organ responsible for the transfer of oxygen (O<sub>2</sub>), nutrients and waste products between maternal and fetal circulations, as well as the synthesis of hormones required during pregnancy. In common with some other primates, the human placenta is haemomonochorial, whereby the placenta and maternal blood are in direct contact (Page 1993). Placental structure, particularly that of the villi, is well adapted to optimise function, such that there are profound changes to placental structure as gestation progresses.

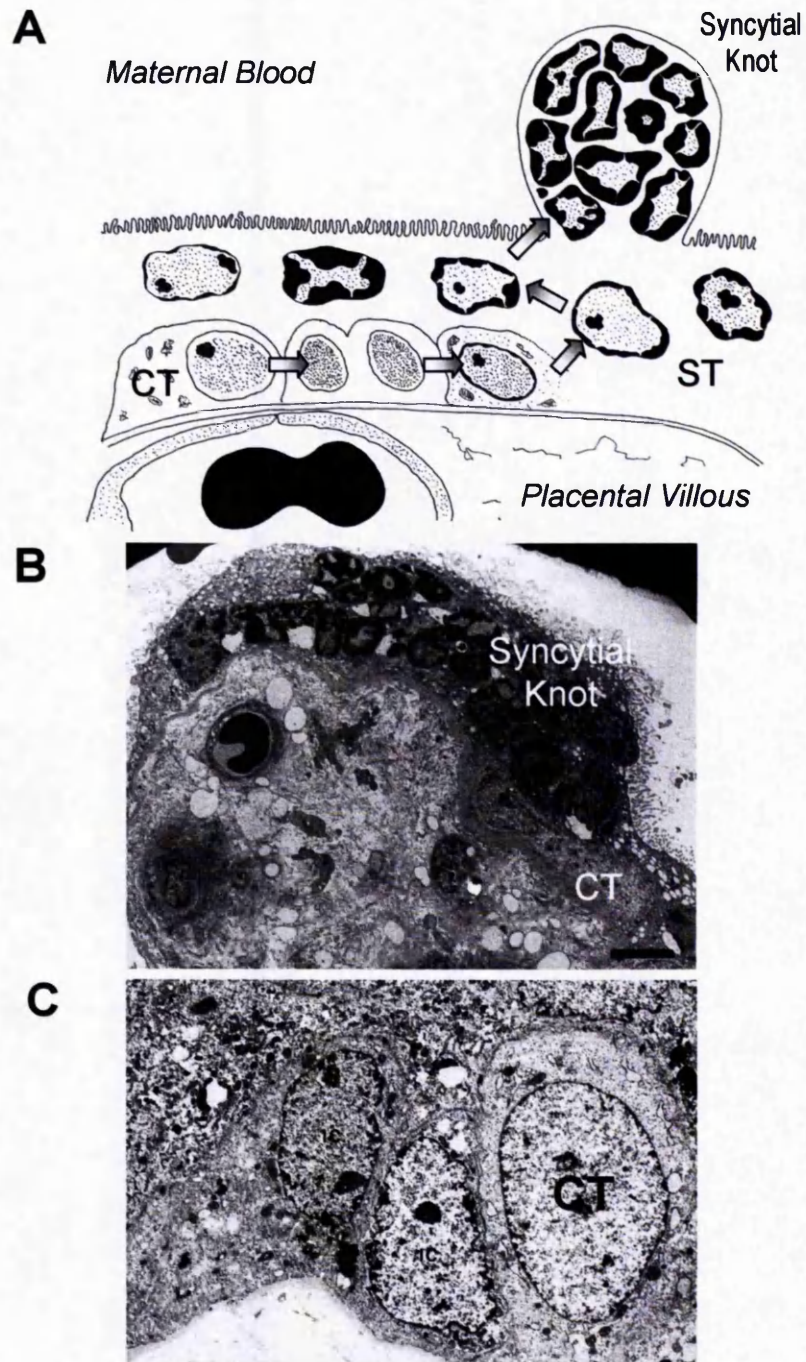
The initial development of the placenta occurs following attachment of the blastocyst to the endometrial epithelium, when the trophoblast covering the embryo invades the maternal decidua. These invading cytotrophoblast cells fuse to form a syncytium, termed the syncytiotrophoblast. This invading tissue is termed the primary chorionic villi. As pregnancy continues, the primary chorionic villi are invaded by mesenchymal cells, creating a fibrous core. This structure is now termed a secondary chorionic villus. The tips of the secondary chorionic villi contain many cytotrophoblast cells covered with a thin layer of syncytium and as gestation progresses the cytotrophoblasts break through the syncytium forming columns of invasive extravillous cytotrophoblast. These invasive extravillous cells penetrate maternal uterine vessels, leading to their conversion into the wide bore vessels which provide adequate blood flow to the placenta. These changes commence before 10 weeks of gestation, prior to the onset of maternal blood flow to the placental unit (Jauniaux et al. 2000). Even so, arterial remodelling by the extravillous trophoblast is thought to continue until 20 weeks gestation.

The placental villi further differentiate to form tertiary chorionic villi, which contain a mesodermal core with fibroblasts, collagen and fetal blood vessels. These tertiary villi are covered in a layer of cytotrophoblast cells which fuse to give rise to an overlying syncytium (Figure 1.6). As pregnancy progresses, the fetus has increased requirements for nutrients and the structure of the placental villus develops to meet this need. First trimester tertiary villi contain many cytotrophoblast cells; these become less densely packed across gestation (Figure 1.7). In the second trimester, the density of cytotrophoblasts is reduced compared to the first trimester, although cytotrophoblasts are still interposed between fetal capillaries and

the overlying syncytium (Figure 1.7). The normal third trimester placenta villus has a thin syncytium, with nuclei gathered into clusters, termed syncytial knots. Underlying this syncytium are fetal vessels, forming a structure called the vasculosyncytial membrane (Figure 1.7). The reduction in cytotrophoblast and gathering of syncytial nuclei together are hypothesised to remove unnecessary barriers to diffusion and thus optimise the efficiency of nutrient and gas transport across the vasculosyncytial membrane.

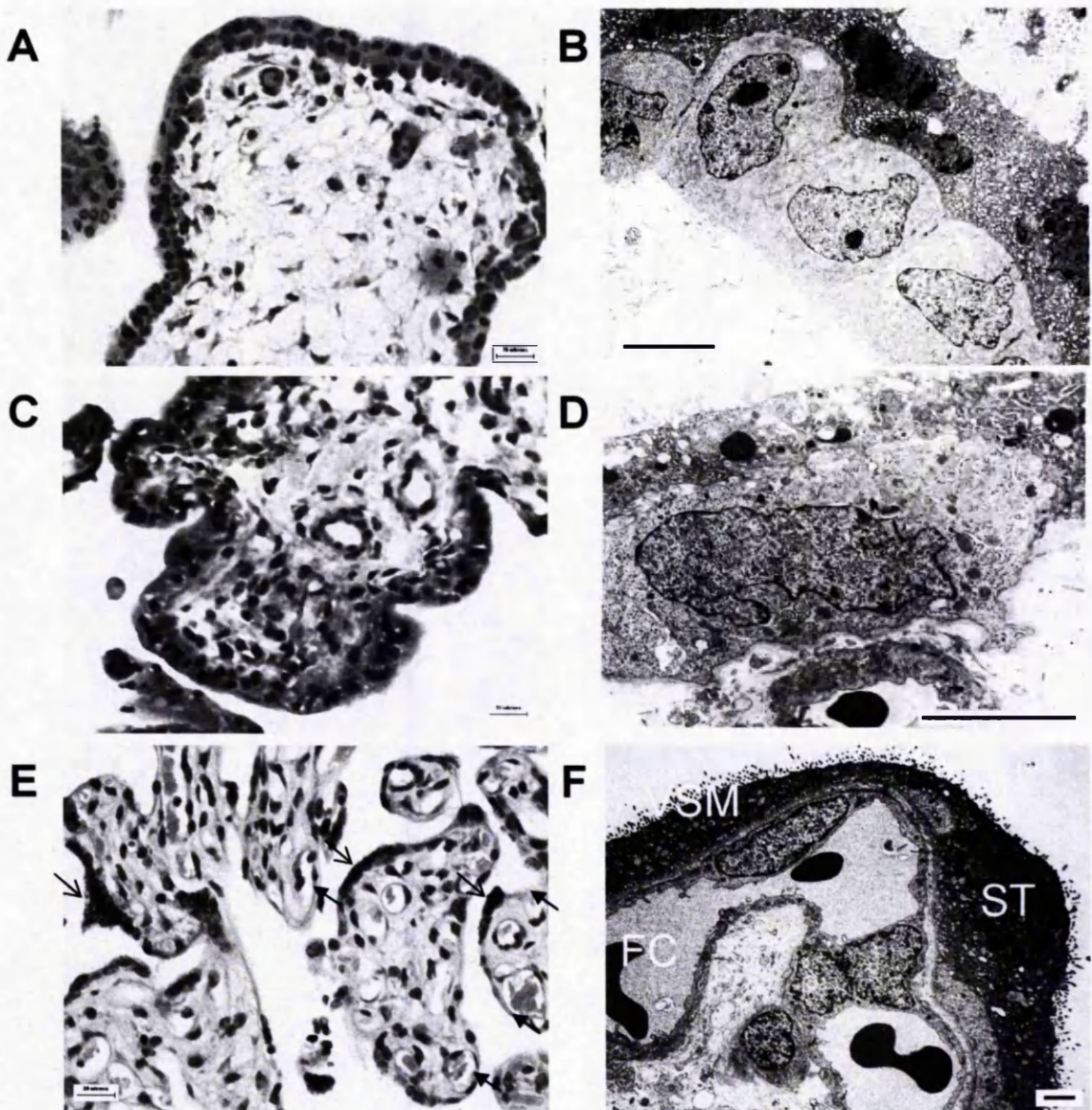
At term, terminal villi consist of an outer layer of trophoblast which encapsulates a matrix of connective tissue and blood vessels. This trophoblast layer consists of cytotrophoblast cells which proliferate and then fuse to give rise to a multinucleate syncytiotrophoblast. As the syncytiotrophoblast is formed from the cytotrophoblast it lacks many of the pathways of a normal cell, and is therefore unable to regenerate or transcribe mRNA (Hoshina et al. 1982; Huppertz et al. 1999). Nuclei within the syncytiotrophoblast show signs of chromatin condensation and a proportion of nuclei aggregate in 'syncytial knots' (Jones and Fox 1991) which may then be lost into the maternal circulation (Johansen et al. 1999; Huppertz and Kingdom 2004). The formation of the syncytiotrophoblast shows similarities with apoptosis, namely externalisation of phosphatidylserine, caspase 8 activation and DNase activity (Huppertz et al. 1999; Black et al. 2004). However, caspase activation occurs prior to anabolic activity of the syncytiotrophoblast and subsequently decreases (Yusuf et al. 2002). Activation of caspases may also have a role in degeneration of the syncytiotrophoblast, especially surrounding syncytial knots (Huppertz et al. 1999). Furthermore, the effects of caspase 3 may be identified in the syncytiotrophoblast by cleavage of cytokeratin-18, to form the M30 neo-epitope (Kadyrov et al. 2001).





**Figure 1.6** – Cell turnover in normal placental tissue. (A) Schematic representation of cell turnover in normal villous trophoblast demonstrating proliferation and fusion of the underlying cytotrophoblast (CT) in the maintenance of the syncytiotrophoblast (ST) layer (shown by arrows). As nuclei undergo fusion they show signs of degeneration, particularly peripheral chromatin condensation followed by a decrease in volume. Some degenerate nuclei are gathered together in syncytial knots, which are then lost into the maternal circulation. (B) Electron micrograph of terminal villus demonstrating some of the features outlined above. (C) Prior to fusion some peripheral chromatin condensation is apparent in CT prior to fusion, marked IC (intermediate CT as described by Jones and Fox, 1991). Electron micrographs used by kind permission of Dr Carolyn Jones, University of Manchester.





**Figure 1.7** – Morphological changes of placental villi as gestation advances. (A) In the first trimester there are many cytotrophoblasts underlying the syncytiotrophoblast, and fetal capillaries are inconspicuous (Marker = 20µm). (B) Electron microscopy shows the cytotrophoblasts abut each other, forming an almost continuous layer underneath the syncytiotrophoblast (marker = 5µm). (C) In the early second trimester, there is evidence of fetal capillaries (FC), although they are not yet adjacent to the trophoblast, there are still many cytotrophoblasts underlying the syncytiotrophoblast (Marker = 20µm). (D) In the second trimester, the cytotrophoblasts are interposed between syncytiotrophoblast and the fetal capillary (marker = 5µm). (E) In the term villous, there are few cytotrophoblasts, and syncytial nuclei are gathered together (marked with open arrows), some in syncytial knots, to minimise cellular material between the syncytiotrophoblast and the fetal capillary. Where the syncytiotrophoblast and the capillary are adjacent is termed the vasculosyncytial membrane (marked with closed arrows). Marker = 20µm. (F) The vasculosyncytial membrane (VSM), and aggregation of nuclei within the syncytiotrophoblast is demonstrated on this electron micrograph (marker = 5µm). Electron micrographs used by kind permission of Dr Carolyn Jones, University of Manchester.

### 1.5.2 Placental Apoptosis in Normal and Abnormal Pregnancy

For clarity, where investigations have localised changes to a specific cell type, these are described as localising to either syncytiotrophoblast or cytotrophoblast; where the localisation of these changes is unclear the term 'villous trophoblast' is used. Apoptosis appears to occur in placental villi under normal conditions, with the location of apoptotic nuclei predominantly localised to the trophoblast layer (Smith et al. 1997b). In addition to caspase activation during cytotrophoblast fusion, nuclei within the syncytiotrophoblast demonstrate morphological features consistent with apoptosis such as peripheral chromatin condensation. The initiation of nuclear degeneration appears to be coincident with cytotrophoblast fusion (Figure 1.6C) (Jones and Fox 1991). Additional morphological features consistent with apoptosis are present within syncytiotrophoblast, including pyknotic nuclei, cytoplasmic condensation and membrane blebbing (Jones and Fox 1977; Jones and Fox 1980; Smith et al. 1997b). Apoptosis has been identified in normal placental tissue from both first and third trimesters of pregnancy, using staining for cytokeratin M30 neoepitope and terminal dUTP nick-end labelling (Smith et al. 1997b; Kadyrov et al. 2001). The amount of apoptosis within tertiary villi increases with gestation, and appears to be greatest in pregnancies over 40 weeks gestation (Smith and Baker 1999; Athapathu et al. 2003). In addition to a role in villous trophoblast cell turnover, apoptosis may also be involved in specific components of placental development, such as the regression of the cells in the chorion laeve – the villi away from the site of implantation – which occurs during the first trimester in response to the onset of maternal blood flow (Jauniaux et al. 2003).

The amount of apoptosis is altered in several clinical conditions associated with abnormal placental structure or function. Apoptosis is increased in all forms of gestational trophoblast disease (GTD), including partial hydatidiform mole (PHM), complete hydatidiform mole (CHM) and choriocarcinoma (Wong et al. 1999; Chiu et al. 2001). In PHM, CHM and choriocarcinoma the increase in apoptosis is accompanied by an increase in trophoblast proliferation implying a global increase in cell turnover (Kale et al. 2001). Apoptosis is also increased in pregnancies complicated by missed miscarriage in the first trimester (Hempstock et al. 2003). In addition, apoptosis is increased in clinical conditions which present later in pregnancy, such as intrauterine growth restriction (IUGR) and pre-eclampsia (Smith et al. 1997a; Allaire et al. 2000; Leung et al. 2001; Ishihara et al. 2002; Levy et al. 2002). In pre-eclampsia the increased

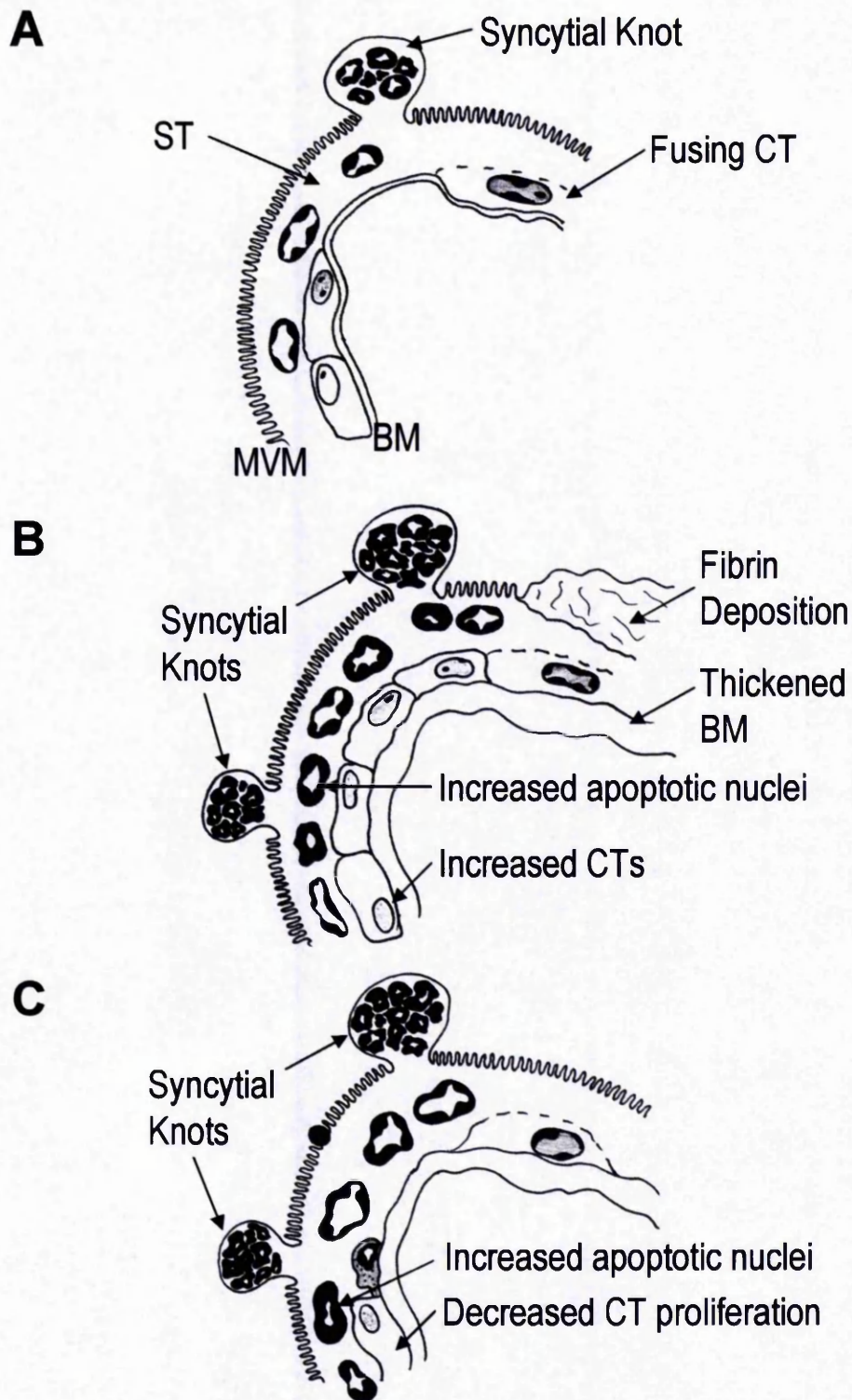
apoptosis occurs largely within the syncytiotrophoblast, and is associated with a reduction in syncytiotrophoblast area (Crocker et al. 2004a) (Figure 1.8). Interestingly, despite being a continuous cell layer, apoptosis appears to occur in discrete areas of the syncytiotrophoblast, particularly in response to cell damage, as indicated by deposition of fibrin-type fibrinoid (Ratts et al. 2000). In addition to increased trophoblast apoptosis in pre-eclampsia, there are increased numbers of syncytial knots, with 10-50% of tertiary villi possessing them in normal term pregnancy to almost all terminal villi possessing them in pre-eclampsia (Tenney and Parker 1940) (Figure 1.8).

As well as being present in clinical conditions known to be associated with placental pathology, apoptosis is increased in villous trophoblast in maternal diabetes (Sgarbosa et al. 2006), a condition which predisposes women to pre-eclampsia and IUGR (Myers and Brockelsby 2004). Furthermore, preliminary data suggests that apoptosis of invasive extravillous trophoblast is increased in first trimester pregnancies with abnormal blood flow in the uterine artery (Whitley et al. 2007), mirroring similar changes seen in patients with evident pre-eclampsia (DiFederico et al. 1999). In IUGR, the increase in apoptosis is associated with a decrease in cytotrophoblast proliferation (Chen et al. 2002). Further to the changes in villous trophoblast turnover observed in vivo, placental villous explants taken from pregnancies complicated by pre-eclampsia and IUGR have an increased apoptotic response when exposed to TNF- $\alpha$  or hypoxia, as compared to normal pregnancies. This suggests an underlying susceptibility to apoptosis in pregnancies complicated by pre-eclampsia or IUGR (Crocker et al. 2003; Crocker et al. 2004b). In summary, there is growing evidence that aberrant cell turnover, particularly increased apoptosis, is present in a number of placental disorders, and may be important in their pathogenesis.

### **1.5.3 Regulation of Placental Apoptosis in Normal and Abnormal Pregnancy**

It is surprising that despite the importance of regulated cell turnover in normal placental villi, data regarding the expression of proteins regulating villous trophoblast cell turnover are incomplete. However, valuable insights into the regulation of trophoblast cell turnover have been gained from past studies of normal and abnormal villous trophoblast. From these it is clear that trophoblast expresses many constituents of both the extrinsic and intrinsic apoptotic pathways. The elements of the extrinsic apoptotic pathway present in both cytotrophoblast and





**Figure 1.8** – Cell turnover in villous trophoblast in normal third trimester pregnancy and pregnancies complicated by pre-eclampsia and IUGR. (A) In the third trimester of pregnancy, the syncytiotrophoblast (ST) is maintained by proliferation and fusion of underlying cytotrophoblast (CT). (B) In pre-eclampsia, there is evidence of damage as shown by fibrin deposition on the microvillus membrane (MVM) and a loss of syncytiotrophoblast. There is increased apoptosis and number of syncytial knots. There is thickening of the basement membrane (BM) and increased numbers of cytotrophoblasts. (C) In IUGR, there is increased apoptosis, syncytial knots and a decrease in cytotrophoblast proliferation.

syncytiotrophoblast, include: TNF receptors, Fas, Fas-ligand (Fas-L) and caspase-8 (De Falco et al. 2004; Pongcharoen et al. 2004; Kharfi et al. 2006). Some of these proteins are altered in pre-eclampsia, but these proteins, particularly Fas/Fas-L are thought to mediate immune interactions of the trophoblast, as they induce immune cell apoptosis (Abrahams et al. 2004) and are present most strongly in invasive trophoblast. The presence of Fas/Fas-L and TNF receptors are not related to the amount of apoptosis within trophoblast (Pongcharoen et al. 2004; Kharfi et al. 2006), which is consistent with the hypothesis that the increased trophoblast apoptosis observed in pre-eclampsia results from cell damage, with transmission of the apoptotic signal via the intrinsic pathway. As the expression of elements of the intrinsic apoptotic pathway may have more significance to pregnancy complications of late gestation, they have been previously investigated in depth. Due to the amount of data described by these studies, only the key findings have been reported in the text, more detail regarding specific studies is contained in Appendix A, Tables A1-A17.

The most extensively studied protein in placental cell turnover is p53, probably as a result of its central role in apoptosis and oncogenesis. The majority of studies have investigated the expression of p53 in early pregnancy and in cases of GTD (summarised in Appendix A – Table A1). In normal pregnancy, p53 is predominantly expressed in cytotrophoblast nuclei, with expression appearing to be up-regulated in proliferative cells (Roncalli et al. 1994; Marzusch et al. 1995). In common with this observation, p53 is expressed more strongly in cytotrophoblast cells in the first trimester compared to the third (Roncalli et al. 1994; Sakuragi et al. 1994) and is rarely expressed in syncytiotrophoblast nuclei (Haidacher et al. 1995; Quenby et al. 1998; Endo et al. 2005). In GTD, which is associated with increased apoptosis and proliferation, there is increased p53 expression (Fulop et al. 1998; Qiao et al. 1998; Cheung et al. 1999). In accordance with this observation, p53 is strongly expressed in choriocarcinoma-derived cell lines (Sakuragi et al. 1994; Haidacher et al. 1995; Bae et al. 2007).

IUGR, which is associated with increased apoptosis and decreased proliferation, is also associated with increased p53 in villous trophoblast (Levy et al. 2002), although when investigated by semi-quantification of immunohistochemical staining an increase in p53 expression is not always apparent (Endo et al. 2005; Jeschke et al. 2006). Preliminary data suggest that p53 in villous trophoblast is wild-type, as no mutations of the p53 gene have been identified in normal tissue or choriocarcinoma (Shi et al. 1996; Cheung et al. 1999).

Mdm2, the negative regulator of p53, has been predominantly investigated in first trimester tissue, although preliminary data suggest that it is present in isolated term trophoblast (summarised in Table A2). In contrast to p53, Mdm2 is expressed in the cytoplasm of cytotrophoblast and syncytiotrophoblast of first trimester villous tissue (Fulop et al. 1998). The expression of Mdm2 is increased in GTD, which may represent a downstream effect of p53 (Fulop et al. 1998; Cheung et al. 1999).

Further evidence of transcription-dependent effects of p53 is also seen in the expression of p21 (summarised in Appendix A, Table A3). In first trimester villous tissue, p21 is strongly expressed, especially in proliferative cell types such as villous cytotrophoblasts and invasive trophoblast cell columns (Quenby et al. 1998). This positive correlation with proliferation is borne out by the increased expression of p21 in GTD (Fulop et al. 1998) and the presence of p21 in BeWo cells (Bae et al. 2007). Differentiated cells such as syncytiotrophoblast showed variable immunostaining, with one study reporting patchy staining for p21 in the syncytiotrophoblast (Quenby et al. 1998), and another reporting strong immunoreactivity in the syncytiotrophoblast of first trimester tissue (Toki et al. 1999). p21 is present in third trimester villous trophoblast, but expression is reduced in third trimester villous trophoblast compared to first trimester tissue, with some staining in the syncytiotrophoblast nuclei and cytoplasm and weak immunostaining of cytotrophoblast (De Falco et al. 2007). pRb, another regulator of cell-proliferation was strongly present in cytotrophoblasts but reduced or absent in syncytiotrophoblast in both first trimester and term villous tissue (summarised in Appendix A, Table A4) (Fulop et al. 1998; Quenby et al. 1998). pRb was also up-regulated on differentiation of BeWo choriocarcinoma cells, consistent with its role promoting terminal differentiation (Nampoothiri et al. 2007). The expression of p53 and related regulators of cell turnover and apoptosis appear to be primarily expressed in cell types with greater proliferative activity.

Members of the Bcl-2 family, responsible for regulation of mitochondrial membrane permeability are also present within villous trophoblast. The most extensively investigated is Bcl-2 (summarised in Appendix A Table A5) which was first described in trophoblast in 1994, and is present in the syncytiotrophoblast cytoplasm throughout gestation and in choriocarcinoma-derived cell lines (Sakuragi et al. 1994; Bae et al. 2007). The majority of studies have described that in terminal villi, Bcl-2 is only expressed in trophoblast, being absent from mesenchymal and endothelial cells (Toki et al. 1999; Yamada et al. 2001; Danihel

et al. 2002). Bcl-2 expression in cytotrophoblast cells appears weaker than in syncytiotrophoblast, with some studies reporting absence of immunoreactivity in cytotrophoblasts (Ratts et al. 2000; Axt-Fliedner et al. 2001; Danihel et al. 2002). Several studies have demonstrated that the presence of Bcl-2 is inversely proportional to the amount of apoptosis, and is reduced in the region of syncytial knots (Huppertz et al. 1998; Ishihara et al. 2002). The expression of Bcl-2 is not increased in GTD (Qiao et al. 1998). A single report using semi-quantified analysis of Bcl-2 expression described a reduction in Bcl-2 in pre-eclampsia and IUGR (Ishihara et al. 2002), although other studies report no difference in IUGR or maternal diabetes (Levy et al. 2002; Sgarbosa et al. 2006). Another anti-apoptotic member of the Bcl-2 family, Mcl-1, is also present in first and third trimester trophoblast (Hu et al. 2006a). In a similar manner to Bcl-2, Mcl-1 expression is localised to the syncytiotrophoblast cytoplasm and was reduced in the region of syncytial knots (Huppertz et al. 1998) (summarised in Appendix A Table A6). Bcl-xL, another anti-apoptotic member of the Bcl-2 family, has been identified in villous tissue lysate at the protein and mRNA level, although there are no reports of cellular localisation (Charles et al. 2005; Hu et al. 2006a) (summarised in table A7).

Pro-apoptotic members of the Bcl-2 family are also present in trophoblast, Bax is present in first and third trimester villous trophoblast and BeWo choriocarcinoma cells (Bae et al. 2007) (summarised in table A8). In first trimester villous trophoblast, Bax is localised to the cytoplasm of cytotrophoblast (Qiao et al. 1998), whereas in the third trimester, Bax is expressed in discrete areas of syncytiotrophoblast cytoplasm, especially in areas associated with damage or degeneration, such as those with fibrin deposition or syncytial knots (Ratts et al. 2000; Yamada et al. 2001). In the third trimester, Bak has similar localisation to Bax (summarised in Appendix A Table A9). No alteration of Bax or Bak expression has been reported in GTD, IUGR or pre-eclampsia (Allaire et al. 2000; Levy et al. 2002; Endo et al. 2005).

Another pro-apoptotic member of the Bcl-2 family, Mtd (also known as Bok) has a novel placental isoform (Soleymanlou et al. 2005b). The expression of all Mtd isoforms are localised to the cytotrophoblast in the first trimester. However, Mtd isoforms are only weakly expressed in the syncytiotrophoblast cytoplasm in the third trimester (summarised in Appendix A Table A10). Mtd expression is increased in severe early-onset pre-eclampsia and localised to syncytial knots (Soleymanlou et al. 2005b). In cultured explants, elevated Mtd expression in

response to hypoxia is associated with increased mitochondrial membrane permeability and caspase-3 activity, demonstrating that stimulation of the intrinsic pathway of apoptosis leads to expected downstream effects in villous trophoblast (Soleymanlou et al. 2005b).

In contrast to the multi-domain members of the Bcl-2 family, little is known about the BH-3 domain-only proteins. A single investigation has demonstrated pro-apoptotic Nix and BNip3 in villous trophoblast, with both being strongly expressed in cytotrophoblast cytoplasm. In the syncytiotrophoblast, BNip3 is weakly expressed, but Nix is absent (Stepan et al. 2005) (summarised in table A11). Contrary to expectations the expression of these pro-apoptotic proteins was decreased in pregnancies complicated by IUGR and pre-eclampsia.

Trophoblast expresses many IAPs including: livin, survivin, human IAP-1 (HIAP-1), human IAP-2 (HIAP-2), NIAP and XIAP (Gruslin et al. 2001; Ka and Hunt 2003; Shiozaki et al. 2003). The majority of these anti-apoptotic proteins are expressed within the syncytiotrophoblast cytoplasm. For example, XIAP is present in this location throughout pregnancy, although expression is stronger in the first trimester than the third (Ka and Hunt 2003) (summarised in Appendix A Table A12). However, the majority of XIAP present in the third trimester is thought to be an inactive 30kDa isoform (Straszewski-Chavez et al. 2007). Survivin is also present in villous trophoblast throughout pregnancy, and has also been described in Jeg-3, JAr and BeWo choriocarcinoma-cell lines (Ka and Hunt 2003; Shiozaki et al. 2003). Survivin is expressed in nuclei and cytoplasm of cytotrophoblast and syncytiotrophoblast cytoplasm (Ka and Hunt 2003) (summarised in table A13). Survivin appears to be important in ensuring trophoblast survival as reduction of survivin expression using anti-sense oligonucleotides was associated with a decrease in survival of isolated cytotrophoblast (Shiozaki et al. 2003). hIAP-1, hIAP-2 and NIAP are also expressed within villous trophoblast (summarised in table A14), hIAP-1 and -2 do not show any change in their expression throughout gestation, whereas NIAP is strongly expressed by many cell types in the third trimester, compared to weaker expression in cytotrophoblast and syncytiotrophoblast in the first trimester (Ka and Hunt 2003). XIAP-associated factor-1 (XAF-1) a mitochondrial protein and positive regulator of caspase activity, is present in nuclei and cytosol of syncytiotrophoblast of normal first and third trimester villous tissue (Straszewski-Chavez et al. 2007) (summarised in Appendix A Table A15). There are no reports of other mitochondrial regulators of caspase activity such as smac and HtrA2/omi in villous trophoblast.



Many caspase enzymes are present in villous trophoblast. Due to its central role in cell digestion associated with apoptosis, caspase-3 has been the subject of significant investigation (studies summarised in Appendix A Table A16). The presence of pro-caspase-3 has been described at the mRNA and protein level, localising to cytotrophoblasts (Huppertz et al. 1998; Huppertz et al. 1999). Active caspase-3 has been demonstrated by Western blotting, immunohistochemistry and specific activity assay (Hung et al. 2002; Huppertz et al. 2003; Crocker et al. 2004c; De Falco et al. 2004). In contrast to the expression of pro-caspase-3, active caspase-3 is only weakly expressed in cytotrophoblast and predominantly localises to the syncytiotrophoblast cytoplasm (Huppertz et al. 1998; De Falco et al. 2004). Active caspase-3 is increased in pregnancies complicated by IUGR (Endo et al. 2005).

Caspases-6 and -7 show similar patterns of expression to caspase-3 for the pro- and active forms, with the activation only occurring in the syncytiotrophoblast (Huppertz et al. 1999) (summarised in Appendix A Table A17). In contrast, both the pro- and active forms of caspase-8 are present in cytotrophoblast, and caspase-8 activity is an important component of cytotrophoblast fusion with overlying syncytiotrophoblast (Black et al. 2004). Caspase-9 shows weak punctate staining in the both trophoblast compartments in the first trimester, but strong immunostaining in the third trimester (De Falco et al. 2004). Pro-caspase-10 is expressed in cytotrophoblast, although at present the active form has not been described in villous trophoblast (Huppertz et al. 1999). Similarly, pro-caspase-14 has been shown in both cytotrophoblast and syncytiotrophoblast, but the active form has not been identified; pro-caspase-14 is more strongly expressed in the first trimester compared to term placental tissue (Kam et al. 2005).

To summarise, many factors capable of regulating apoptosis are present in villous trophoblast, although their expression patterns change as pregnancy progresses (Table 1.2). In the third trimester, the syncytiotrophoblast is well protected against unwanted apoptosis, expressing Bcl-2, Mcl-1 and several different IAPs. Owing to the low rate of cell proliferation of villous cytotrophoblasts in the third trimester, there is low expression of cell-cycle regulators such as p53 and p21. Prior to cytotrophoblast fusion, cytotrophoblast express pro-forms of caspase enzymes, although the majority of these only become active in the syncytiotrophoblast. The exception is caspase-8, which has a role in cytotrophoblast fusion. Despite the dysregulation of trophoblast cell turnover described in GTD, IUGR and pre-eclampsia, comparatively few data

exist describing alterations in the expression of regulators of cell turnover and apoptosis (Table 1.2). Nevertheless, in placental pathologies changes in expression of regulators of cell turnover have been described at all levels of the apoptotic pathway, including proteins predominantly active in the nucleus, such as p53, and those regulating mitochondrial membrane permeability, such as Bcl-2 and Mtd, culminating in increased caspase activity.

Protein	Tissue Type	Study	Method	Results
<b>p53</b>	Normal first trimester, CHM, PHM and CCA	Shi et al. 1996	DNA Sequencing	No mutations in p53 gene
	Normal first trimester, CHM, PHM and CCA	Qiao et al. 1998	Immunohistochemistry	Expression increased in CTs in CHM.
	Normal first trimester, CHM, PHM and CCA	Fulop et al. 1998	Immunohistochemistry DNA Sequencing	No mutations in p53 in CHM, PHM or CCA. p53 increased in PHM, CHM and CCA.
	Normal first trimester, CHM, PHM and CCA	Cheung et al. 1999	Immunohistochemistry DNA Sequencing	No mutations identified in CHM, PHM or CCA. p53 increased in PHM, CHM and CCA.
	Normal third trimester and IUGR pregnancies	Levy et al. 2002	Immunohistochemistry Western Blotting	p53 expression increased in IUGR pregnancies in CT nuclei.
	Normal third trimester and IUGR pregnancies	Endo et al. 2005	Immunohistochemistry	Not altered in IUGR
	Normal third trimester, pre-eclampsia, HELLP and IUGR pregnancies	Jeschke et al. 2006	Immunohistochemistry	Increased in HELLP syndrome, unchanged in pre-eclampsia, and decreased in IUGR.
<b>Mdm2</b>	Normal first trimester, CHM, PHM and CCA	Fulop et al. 1998	Immunohistochemistry	Increased in CHM, PHM and CCA
	Normal first trimester, CHM, PHM and CCA	Cheung et al. 1999	Immunohistochemistry	Increased expression in PHM, CHM and CCA.
<b>p21</b>	Normal first trimester, CHM, PHM and CCA	Fulop et al. 1998	Immunohistochemistry	Increased in CHM, PHM and CCA
<b>pRb</b>	Normal first trimester, CHM, PHM and CCA	Fulop et al. 1998	Immunohistochemistry	Increased in CHM, PHM and CCA
<b>Bcl-2</b>	Normal third trimester, pre-eclampsia and IUGR pregnancies	Isihara et al. 2002	Immunohistochemistry	Reduced in severe pre-eclampsia / IUGR.
	Normal third trimester and IUGR pregnancies	Levy et al. 2002	Western Blotting	Not altered in IUGR.
	Normal third trimester pregnancies and pre-eclampsia	Allaire et al. 2000	Immunohistochemistry	Not altered in pre-eclampsia
<b>Bak</b>	Normal third trimester and IUGR pregnancies	Levy et al. 2002	Western Blotting	Not altered in IUGR.
<b>Bax</b>	Term villous tissue from normal pregnancies and pre-eclampsia	Allaire et al. 2000	Immunohistochemistry	Not altered in pre-eclampsia.
	Normal third trimester and IUGR pregnancies	Levy et al. 2002	Western Blotting	Not altered in IUGR.
<b>Mtd</b>	Gestational-age matched pregnancies and pre-eclampsia	Soleymanlou et al. 2003	Immunohistochemistry Western blotting real-time PCR	Increased in pre-eclampsia.
<b>BNip3</b>	Normal third trimester and HELLP, pre-eclampsia and IUGR	Stepan et al. 2005	Immunohistochemistry	Reduced in HELLP, pre-eclampsia and IUGR.
<b>Nix</b>	Normal third trimester and HELLP, pre-eclampsia and IUGR	Stepan et al. 2005	Immunohistochemistry	Reduced in HELLP, pre-eclampsia and IUGR.

**Table 1.2** – Reports of the expression of apoptotic regulatory proteins in placental pathology including GTD, IUGR and pre-eclampsia. CCA = Choriocarcinoma, CHM = Complete hydatidiform mole, CT = cytotrophoblast, HELLP = Haemolysis Elevated Liver Enzymes and Low Platelets, PHM = Partial hydatidiform mole, ST = syncytiotrophoblast.

#### **1.5.4 Potential Role of Apoptosis in the Development of Placental Pathology**

The precise role of apoptosis in the development of placental pathology and clinical conditions such as pre-eclampsia and IUGR is yet to be determined. Undoubtedly, apoptosis in pre-eclampsia and IUGR will disrupt normal cytotrophoblast cell turnover in the villus, preventing the normal replenishment of mRNA for the syncytiotrophoblast, which may further decrease placental function and may explain the relationship between pre-eclampsia and IUGR. There is evidence that stimulation of both intrinsic and extrinsic pathways induces apoptosis in trophoblast in vitro (Yui et al. 1994b; Crocker et al. 2004c). More specifically, challenges implicated in the development of pre-eclampsia and IUGR, such as hypoxia and hypoxia-reoxygenation injury, can induce apoptosis in cultured villous trophoblast (Levy et al. 2000; Hung et al. 2002). Some experiments also describe an association between exposure of trophoblast to hypoxia and subsequent increased expression of p53 and Mtd-1 or decrease in Bcl-2 expression (Levy et al. 2000; Soleymanlou et al. 2005b; Hu et al. 2006b); similar changes have been observed in placentas from pregnancies complicated by pre-eclampsia and IUGR (Ishihara et al. 2002; Levy et al. 2002; Soleymanlou et al. 2005b). These experiments suggest that the increased apoptosis observed in villous trophoblast in vivo occurs as a result of exposure to a noxious stimulus such as hypoxia or oxidative stress.

The presence of a placenta is essential for the development of pre-eclampsia. Furthermore, the presence of increased placental mass such as a twin pregnancy and in advanced cases of GTD is associated with increased incidence of pre-eclampsia (Acosta-Sison 1956; Myers and Brockelsby 2004). Pre-eclampsia, but not IUGR, is associated with increased presence of syncytiotrophoblast microparticles (STBM) - fragments of syncytiotrophoblast which exhibit features of apoptosis, in the maternal circulation (Knight et al. 1998; Johansen et al. 1999; Goswami et al. 2006). In addition, cell-free DNA of fetal origin is increased in the maternal circulation in pre-eclampsia, but not IUGR (Zhong et al. 2002). It is hypothesised that both STBMs and cell-free fetal DNA may be released as a result of apoptotic cell death (Abumaree et al. 2006b). This is supported by the observation that the STBMs and cell-free DNA were increased in trophoblast cultured in hypoxia and hypoxia-reoxygenation (Orozco et al. 2006; Tjoa et al. 2006). Recently, STBM particles have been shown to alter behaviour of macrophages, promoting immune tolerance (Abumaree et al. 2006a). It is hypothesised that excessive amounts of STBM or cell-free fetal DNA may provoke an excessive maternal

inflammatory response (Sargent et al. 2003). Consistent with this hypothesis is the observation that STBM increases the formation of neutrophil extracellular traps (NET), indicating activation of neutrophils (Gupta et al. 2006). Interestingly, the occurrence of NETs are increased in placentas of pregnancies complicated by pre-eclampsia (Gupta et al. 2006). STBMs also disrupt endothelial cells in vitro, reducing proliferation (Gupta et al. 2005; Hoegh et al. 2006). This disruption is also associated with a reduction in endothelial dependent relaxation (Cockell et al. 1997). These preliminary data suggest a potential link between apoptotic cell death within trophoblast and changes to the maternal immune system, unifying trophoblast cell death with the presence of systemic changes in the mother.

In opposition to this hypothesis is the argument that apoptosis is an epiphenomenon, occurring during exposure of the placenta to noxious environments. Some noxious stimuli may induce the release of soluble factors such as soluble VEGF receptor 1 (sVEGFR1 - also known as sFlt) and soluble endoglin, as exposure of villous explants to hypoxia induces expression of sVEGFR1 mRNA (Nevo et al. 2006). These findings indicate that hypoxia may promote apoptosis of villous trophoblast and promote the expression of vasoactive proteins. As both sVEGFR1 and soluble endoglin are implicated in the endothelial dysfunction of pre-eclampsia (Levine et al. 2006), this hypothesis proposes that the production of these proteins is a key event in the placenta dysfunction, rather than apoptosis or cell death. Nevertheless, the release of sVEGFR1 may itself be related to syncytiotrophoblast degeneration, as sVEGFR1 expression is strongest in syncytial knots and STBM (Guller et al. 2007), both of which are elevated in pre-eclampsia. Therefore, the increased apoptosis, formation of syncytial knots, generation of STBMs, release of sVEGFR1 (also known as sFlt-1) and soluble endoglin may all be markers of syncytiotrophoblast degeneration.

### **1.5.5 Evidence for Altered Regulation of Cell Turnover Following Pregnancies Complicated by Pre-eclampsia**

In addition to the regulation of apoptosis in placental tissues, some epidemiological studies have demonstrated that women who have pre-eclampsia have an increased risk of developing cancer in later life when compared to women who had normal pregnancies, IUGR or pregnancy induced hypertension alone (Paltiel et al. 2004). This effect is particularly pronounced in women who develop severe early onset pre-eclampsia (Paltiel et al. 2004). As

the placenta is a fetal organ, one would also expect the infant to have an increased risk of developing cancer, and it has been reported that children born to mothers suffering from pre-eclampsia in that pregnancy have a 1.7x risk of developing Non-Hodgkins Lymphoma (NHL) during childhood, compared to those from normal pregnancies (Roman et al. 2005). These observations suggest that there may be shared aetiology between pre-eclampsia and other disorders of cellular regulation, as p53 mutations are noted in both solid tumours and NHL.

### **1.6 Interaction between the Placenta and Maternal Immune System in Pre-eclampsia**

Although significant evidence demonstrates abnormal placental cell turnover in pregnancies complicated with pre-eclampsia, these events alone are not responsible for the clinical syndrome of pre-eclampsia. The relationship between the placenta and maternal immune system is critical to the development of pre-eclampsia. There are both maternal and paternal genetic components to pre-eclampsia, but the maternal contribution is greater than the fathers (Esplin et al. 2001). It is proposed that the maternal genetic contribution may involve the maternal immune system.

The involvement of the immune system can be divided into interactions early in pregnancy between the invasive extravillous trophoblast and the maternal decidua and interaction later in pregnancy between villous trophoblast and the maternal blood (Sargent et al. 2007). To interact with immune cells, somatic cells express specific antigens, the most common group of which are the major histocompatibility complex (MHC). Extravillous trophoblast do not express major T-cell ligands (HLA-A and HLA-B), instead they express HLA-G, HLA-E and HLA-C. Villous trophoblast appears not to express HLA complexes (Moffett-King 2002).

The earlier interaction occurs between extravillous trophoblast and decidual natural killer (NK) cells. HLA-C is the dominant ligand on extra-villous trophoblast for decidual NK cells; HLA-C interacts with killer-immunoglobulin like receptors (KIRs). Different combinations of HLA-C and KIRs can be either activating or inhibitory, altering the secretion of cytokines (Moffett and Hiby 2007). In the mouse, decidual NK cells are critical in the remodelling of spiral arteries (Croy et al. 1997) and human decidual NK cells can secrete angiogenic factors such as VEGF (Lash et al. 2006). Therefore, an alteration in the interaction between the extravillous trophoblast and decidual NK cells might result in reduced conversion of spiral arteries in humans. Notably, some combinations of HLA-C and KIRs are more common in women who develop pre-

eclampsia (Hiby et al. 2004), adding support to the hypothesis that an interaction between extravillous trophoblast and decidual NK cells might alter spiral artery remodelling in humans, resulting in reduced spiral artery conversion and reduced blood supply to the placenta, culminating in damage to villous trophoblast. In addition, one study has found that HLA-G is decreased in extra-villous trophoblasts in pre-eclampsia (Goldman-Wohl et al. 2000), indicating that other HLA types may also be dysregulated in pre-eclampsia.

The latter immune interaction involves the villous trophoblast and immune cells in the maternal circulation. In normal pregnancy, there was proposed to be a change from Th1 immunity to Th2 immunity, this was proposed to result in a decreased inflammatory response (Wegmann et al. 1993). This is supported by evidence that the placenta secretes interleukin-4 and interleukin-10, both of which stimulate a Th2-like response (Saito and Sakai 2003). Monocytes from peripheral blood secrete hormones consistent with a Th2-like response in normal pregnancy which appears to change to a Th1-type response in women with recurrent miscarriage or pre-eclampsia (Saito and Sakai 2003). More recently, this simplistic view of Th-1 and Th-2 responses in pregnancy has been challenged and a greater role for circulating NK-cells rather than T-helper lymphocytes has been proposed (Sargent et al. 2007). The material shed from the syncytiotrophoblast, STBMs, interact with immune cells (Redman and Sargent 2007). It is hypothesised that an increase in STBM and trophoblast debris leads to an enhanced pro-inflammatory response; some preliminary data suggests that these changes may be most pronounced in monocytes and dendritic cells, which can then lead to activation of other components of the maternal immune system (Sargent et al. 2007).

Placental dysfunction and changes in cell turnover are intimately related to the interaction between the placenta and the maternal host, initially leading to decreased conversion of maternal spiral arteries leading to reduced blood delivery to the placenta leading to oxidative stress. Such stress, may lead to increased apoptosis and increased release of debris from villous trophoblast which interacts with the maternal immune and cardiovascular systems to produce a systemic maternal response.

## **1.7 Summary**

Despite significant advances in the last decade, the precise cause of placental pathology leading to pre-eclampsia and IUGR remains unclear. For pre-eclampsia, the current favoured

pathophysiological model (summarised by Redman and Sargent, 2005) highlights abnormal placentation in early pregnancy, leading to decreased invasion and conversion of spiral arteries, leading to placental damage. In later pregnancy, the release of vasoactive factors combined with shedding of syncytiotrophoblast material leads to the powerful combination of maternal systemic inflammatory response and endothelial activation, which culminates in the clinical syndrome of pre-eclampsia. There is less agreement regarding the placental pathology associated with idiopathic IUGR, probably due to the heterogeneity of this condition and multiple causative factors. Nevertheless, the clinical syndrome of IUGR is frequently associated with signs of placental dysfunction, including increased placental vascular resistance, as measured by umbilical artery Doppler waveform and abnormal villous trophoblast function (Sibley et al. 2005). In addition, the co-existence of IUGR and pre-eclampsia in some pregnancies provides evidence of a common aetiology.

The increasing body of evidence highlighting apoptosis in villous trophoblast in pregnancies complicated by pre-eclampsia and IUGR suggests that this form of cell death may have a role in the development of these complex disorders. The observation that trophoblast isolated from pregnancies complicated by pre-eclampsia and IUGR have a greater susceptibility to apoptosis (Crocker et al. 2004b) has led to the hypothesis that trophoblast in these conditions may have an imbalance in the regulators of apoptosis, making cell-death more likely when challenged with a noxious stimulus. In eukaryotes, apoptosis is regulated by well conserved signalling pathways, the constituents of which appear to be present in trophoblast. Although several studies have described increased apoptosis in the villous trophoblast in pre-eclampsia, there have been few studies investigating changes in the expression of proteins regulating apoptosis (See Table 1.2).

The expression of proteins involved in the regulation of apoptosis in villous trophoblast in pre-eclampsia merits closer examination for several reasons. Firstly, it is important to identify whether the increased rate of apoptosis observed in pre-eclampsia results from activation of the extrinsic or intrinsic apoptotic pathway, as this may provide insight into the origins of the increased syncytiotrophoblast apoptosis observed. Secondly, since activation of the apoptotic pathway, causes alteration in downstream effector proteins and specific post-translational modifications, evaluation of the patterns of oncoprotein expression may identify specific candidates capable of inducing apoptosis in pre-eclampsia, such as placental hypoxia, reactive



oxygen species or a reduction of growth factors. Finally, understanding the pathways involved in regulating cell turnover may reveal novel molecular targets to antagonise apoptosis in syncytiotrophoblast, thereby providing a potential therapeutic approach for pre-eclampsia.

## **1.8 Hypotheses and Aims**

### **1.8.1 Hypotheses**

The central thesis of this study is that the increased apoptosis in placentas of pregnancies complicated by pre-eclampsia is associated with an imbalance in the expression of pro- and anti-apoptotic proteins in villous trophoblast. In addition, it is hypothesised that exposure of placental villous tissue to challenges implicated in the development of pre-eclampsia, such as hypoxia and oxidative stress, will induce alterations in cell turnover and changes in the expression of regulators of apoptosis in villous trophoblast.

### **1.8.2 Aims**

The first aim of this project is to comprehensively characterise the expression of regulators of the intrinsic pathway of apoptosis in villous tissue of normal placentas and those from pregnancies complicated by pre-eclampsia. Depending upon the findings of the primary investigation, the second aim is to assess the ability of an alteration in the expression of apoptotic regulators to alter cell turnover in trophoblast. Thirdly, this project aims to describe changes in cell turnover and expression of regulators of apoptosis following exposure of trophoblast to environments implicated in the development of pre-eclampsia, specifically hypoxia and reactive oxygen species. The final aim is to investigate whether survival factors can reduce apoptosis in trophoblast.

## **2 Materials and Methods**

The main methods used in the experiments in this thesis are described in this chapter. Methods specific to individual experiments are detailed in the relevant chapter. Unless specifically stated all chemicals are from Sigma-Aldrich Chemical Company, Poole, Dorset, UK.

### **2.1 Preparation of Culture Medium**

For culture of placental villous explants, CMRL-1066 culture medium (ICN-Biomedicals Irvine, CA, USA) was supplemented with 0.03M Sodium Bicarbonate ( $\text{NaHCO}_3$ ), 250 units/l penicillin, 50mg/l streptomycin, 500  $\mu\text{l/l}$  gentamicin, 100 $\mu\text{g/l}$  hydrocortisone, 1mg/l of insulin, 100 $\mu\text{g/l}$  retinol acetate and 10% (v/v) heat-inactivated fetal calf serum (FCS). The pH of the solution was corrected to 7.2 using 1M sodium hydroxide (NaOH). The finished solution was then filtered through a 22 $\mu\text{m}$  polyethersulfone (PES) membrane (Corning Incorporated, Corning, NY, USA) to remove contaminants. The media was stored at 4°C.

For culture of floating placental villous explants: Dulbecco's Modified Eagles Medium (DMEM) was combined with Hams F-12 in a 1:1 (v/v) ratio. This mixture was supplemented with 1% (v/v) insulin, transferrin and sodium selenite (ITS). The media was stored at 4°C.

For culture of BeWo cells: a 1:1 (v/v) mixture of DMEM (Cambrex, Verviers, Belgium) and Hams-F12 (Cambrex) was supplemented with 10% heat-inactivated FCS, 250 units/l penicillin, 50mg/l streptomycin, 50 $\mu\text{g/l}$  gentamicin and 146 $\mu\text{g/l}$  glutamine. The resulting culture medium was filtered through a 22 $\mu\text{m}$  PES membrane (Corning Incorporated) and stored at 4°C.

## **2.2 Placental Explant Culture**

### **2.2.1 Placental Explant Preparation**

#### **2.2.1.1 Supported Placental Villous Explants**

Placentas were obtained within 30 minutes of delivery of live infants by normal vaginal delivery or Caesarean section. Informed consent was sought from the mother following Local Research Ethics Committee approval (Manchester, UK). After transfer to laboratory the placenta was placed on a tray with the maternal side facing upwards. A sampling frame made from an acetate sheet with intersecting lines at 10cm intervals was placed over the surface of the placenta. Three full thickness samples approximately 2cm<sup>3</sup> were taken from the placenta at the location of the intersecting lines. This tissue was placed in warmed sterile phosphate buffered saline (PBS) at 37°C and then transferred to a biological safety cabinet to ensure aseptic conditions for further tissue preparation.

Each placental sample was washed three times with warmed PBS (37°C) to remove blood and tissue debris. The placental sample was then transferred to a sterile disposable Petri dish (Bibby-Sterilin, Stone, UK) with the maternal side of the sample facing upwards. Using sterile scissors and forceps the first 5mm of the maternal surface of the placenta was removed to prevent sampling of the maternal decidua. 3mm<sup>3</sup> pieces (approx 5mg) of placental villous tissue were dissected using sterile scissors; from this point onwards these will be termed "explants". The explants were washed in a sterile Petri dish containing warmed sterile PBS (37°C) to remove further blood and tissue debris, and then placed in a sterile Petri dish containing warmed culture medium prior to culture.

#### **2.2.1.2 Floating Villous Explants**

Placentas were obtained within 30 minutes of delivery of live term infants by normal vaginal delivery or Caesarean section with ethical approval of the institutional board of Mount Sinai Hospital, Toronto, Canada. The placentas were transferred to the laboratory within 30 minutes of delivery. Three areas of villous tissue were identified using the method described in section 2.2.1.1. After the placental tissue had been washed three times in sterile warmed PBS, villous tissue was dissected to yield single placental villi, approximately 3mm in length, containing a

stem villus with distal terminal villi. The stem villous was then implanted in a sterile polystyrene cube and then placed on a dish of culture media (prepared according to section 2.1), allowing the villous explant to float in the culture media.

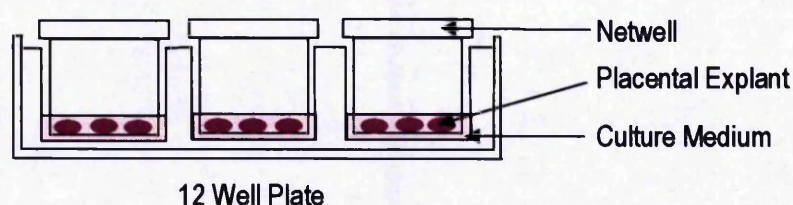
## **2.2.2 Preparation and Storage of Fresh Placental Tissue/Explants (Day 0)**

Following preparation of placental explants according to the methods described, additional samples were taken to allow analysis of placental cell turnover, protein and mRNA expression and morphology of fresh placental tissue. Depending on the experiment these explants were processed for protein analysis, RNA quantification, immunohistochemistry or electron microscopy according to the methods outlined in sections 2.3.1-2.3.3.

## **2.2.3 Incubation of Placental Explants and Change of Culture Medium**

### **2.2.3.1 Supported Placental Explants**

The placental explants were transferred to 12 well culture plates (Corning Incorporated). The Netwells (Corning Incorporated) had been sterilised in 2% Virkon (Antec International, Sudbury, UK) and washed in double distilled water prior to use. Each Netwell was placed in 1.5ml of warmed explant culture medium (37°C). Three placental explants were carefully transferred to each netwell support using sterile forceps. Care was taken to ensure explants were not touching each other or the sides of the netwell (Figure 2.1).



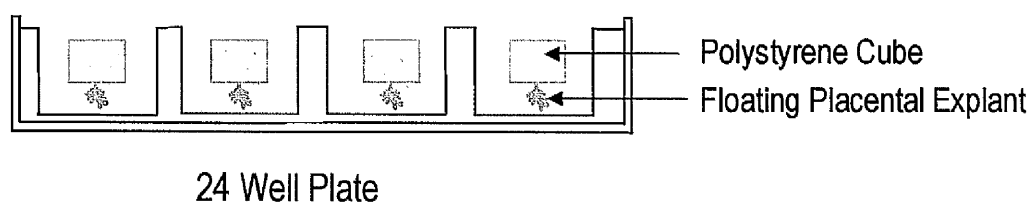
**Figure 2.1** Diagrammatic representation of placental explants in Netwells resting at the gas-liquid interface of the culture medium.

For each culture environment, 25ml of culture medium was placed in a ventilated container for 24 hours in the relevant atmospheric O<sub>2</sub> tension, to allow the culture medium to equilibrate to the ambient O<sub>2</sub> tension. In all culture environments, carbon dioxide (CO<sub>2</sub>) was maintained at

5% and temperature at 37°C. After 48 hours incubation, the culture medium was replaced with medium equilibrated to the correct O<sub>2</sub> tension, thus preventing exposure to atmospheric O<sub>2</sub> which may produce oxidative stress (Newby et al. 2005). In addition, culture medium was changed in the same atmospheric O<sub>2</sub> tension as the culture conditions. After 96 hours of incubation, the explants were removed from the incubator and transferred to the biological safety cabinet for processing of tissue under aseptic conditions.

### 2.2.3.2 Floating Villous Explants

After preparation, floating placental villous explants attached to polystyrene cubes were transferred to a 24-well plate (Corning Incorporated) containing 1ml of warmed explant culture media per well (37°C). The explants were not handled directly due to their fragile nature; the polystyrene block was manipulated to ensure that the explant was suspended in culture media (Figure 2.2).



**Figure 2.2** Diagrammatic representation of floating placental explants showing villous tissue floating in culture medium.

The explants were cultured in 8% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. 25ml of culture medium was equilibrated in 8% O<sub>2</sub> for 24 hours prior to the change of medium. After 24 hours the culture medium was replaced and cultures continued for a further 48 hours.

### 2.2.4 Culture of Placental Villous Explants in Different Atmospheric Oxygen Tension

Following preparation of placental villous explants, each 12-well plate was transferred to an incubator with an atmospheric O<sub>2</sub> concentration of either 20% O<sub>2</sub> (Galaxy B, RS Biotech, UK), 6% O<sub>2</sub> (Sanyo Biomedical, Loughborough, UK) or 1% O<sub>2</sub> (Coy Laboratory Products, Grass-Lake, MI, USA). All incubators maintained a CO<sub>2</sub> level of 5%. The O<sub>2</sub> tension was measured in the culture medium at 37°C using a digital meter (Strathkelvin Instruments, Glasgow, UK). The

O<sub>2</sub> tension in the culture medium was found to be  $150.1 \pm 5.0$  mmHg in 20% atmospheric O<sub>2</sub>,  $70.5 \pm 7.5$  mmHg in 6% atmospheric O<sub>2</sub>, and  $20.2 \pm 8.6$  mmHg in 1% atmospheric O<sub>2</sub>.

The O<sub>2</sub> tensions used in these experiments were selected according to the tissue or cell being cultured. Culture of placental explants was undertaken in 2 settings, Manchester and Toronto. Normoxic culture conditions were regarded as 6% O<sub>2</sub> and 8% O<sub>2</sub> respectively. This reflects international consensus on normoxic conditions for third trimester placenta (Miller et al. 2005), which results from measurements of O<sub>2</sub> tension in the uterine vein (Schaaps et al. 2005) and O<sub>2</sub> tension of 66 mmHg in the intervillous space at term (8.6%) (Fujikura and Yoshida 1996). The decision to use 20% O<sub>2</sub> and 1% O<sub>2</sub> to represent hyperoxic and hypoxic conditions respectively was taken as differences in similar O<sub>2</sub> tensions had already been demonstrated to alter trophoblast cell-turnover (Crocker et al. 2004b; Crocker et al. 2004c).

For BeWo cell culture, 20% O<sub>2</sub> was regarded as normoxic and 6% O<sub>2</sub> was regarded as hypoxic conditions. This is not representative of O<sub>2</sub> conditions in the first trimester or term placenta. However, following isolation from a metastatic choriocarcinoma, BeWo cells were cultured in 20% O<sub>2</sub> environment (Pattillo and Gey 1968) which may have resulted in this level of oxygenation being adopted as normoxic conditions for this cell-line. The use of these O<sub>2</sub> tensions is in accordance with other studies of the effects of altered O<sub>2</sub> tension on BeWo cells (Kudo et al. 2003a; Kudo et al. 2003b; Baumann et al. 2007).

## **2.3 Processing of Placental Tissue**

### **2.3.1 Processing of Tissue for Protein Analysis**

A 1% (v/v) solution of Protease inhibitor cocktail (PIC) (P8340, Sigma) was made in sucrose lysis buffer (10mM HEPES, 250mM sucrose, 1mM EDTA). Each placental sample was homogenised on ice in 250µl of PIC/sucrose lysis buffer mixture. The resultant suspension was centrifuged at 10,000 rpm for 3 minutes (9503g). The supernatant was collected and stored at -80°C. The pellet was resuspended in a further buffer (0.5mM DTT, 10mM HEPES 3.3mM magnesium chloride, 10mM potassium chloride, 0.5M sucrose) containing 1% PIC and subjected to further centrifugation and re-suspension as previously described to isolate nuclear material (Lontay et al. 2005).

### **2.3.2 Processing of Tissue for RNA Analysis**

Explants from each placental sample were placed in RNA later (Ambion, Austin, TX, USA) and stored at -80°C.

### **2.3.3 Fixation of Tissue**

#### **2.3.3.1 Tissue Fixation and Processing for Immunohistochemistry**

Formol Saline was prepared from 9g Sodium Chloride (NaCl) and 10ml 40% Formaldehyde (H<sub>2</sub>CO) added to 90ml of distilled water, giving a final concentration of 4% formaldehyde. Explants were fixed in 5ml of formol saline for 24 hours at 4°C. After this period, explants were washed 3 times in sterile PBS and stored at 4°C prior to wax embedding. Explants were dehydrated by immersion in an ascending series of alcohols, a series of washes in xylene each for 30 minutes. Explants were then wax embedded.

#### **2.3.3.2 Tissue Fixation and Processing for Electron Microscopy**

Tissue was fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 hours. The glutaraldehyde was then removed with several washes in 0.1M sodium cacodylate buffer with 3mM calcium chloride (pH 7.3). Post-fixation was carried out in 1% osmium tetroxide (Agar Scientific Ltd, UK) in 0.05M sodium cacodylate buffer (pH 7.3) for 1 hour at 4°C followed by a rinse in 0.05M sodium cacodylate buffer. The tissue was then dehydrated in an ascending alcohol series, treated twice with propylene oxide for 15 minutes each, then left in a 1:1 mix of propylene oxide and Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK) for 1 hour at room temperature. The tissue was then left overnight on a rotator at 4°C in a mixture of 1:3 propylene oxide and epoxy resin and the following day given three changes of fresh resin at 45°C for 1 hour each before being embedded in gelatin capsules and polymerised for 72 hours at 60°C.

### **2.3.4 Collection of Tissue-Conditioned Culture Medium**

After all the placental explants had been prepared in stages 2.5-2.7, the conditioned culture medium was collected and stored at -80°C.



## **2.4 Culture of BeWo Cells**

### **2.4.1 Preparation of BeWo Cells**

BeWo cells were obtained from either the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) or as a kind gift from Mrs Dora Baczyk (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto) and stored in liquid nitrogen. For experimentation, cells were rapidly thawed using culture medium warmed to 37°C, transferred to a 75cm<sup>3</sup> flask and grown to confluence. When confluent, cells were washed with warmed PBS, and incubated with 5ml 0.05% trypsin-EDTA for 2 minutes, after which the excess solution was removed. 10ml of warmed BeWo culture medium (as described in section 2.1) was then added and allowed to stand for 2 minutes. The flask was then tapped sharply to release adherent cells. The cells were then counted using a haemocytometer (Marienfeld, Germany). Cells were then seeded into either 6-well plates or 96 well-plates for subsequent culture. Remaining cells were replaced in the 75cm<sup>3</sup> flask (Corning Incorporated) in 10ml warmed culture medium and grown to confluence for future passaging. All experiments were conducted between passages 7 and 25.

### **2.4.2 Seeding and Culture of BeWo Cells**

Cells were seeded at a density of  $1.5 \times 10^6$ /well in 6 well plates, and between  $1 \times 10^5$ - $5 \times 10^5$ /well in 96 well plates depending on the experiment to be performed. In addition, sterile 16mm glass cover-slips (Fisher Scientific, Loughborough UK) were placed in 6 well plates, to enable cell to form monolayers on the coverslips. The culture plates were then transferred to an incubator at 37°C in 20% O<sub>2</sub> (RS Biotech), 8% O<sub>2</sub> (Sanyo Biomedical) or 6% O<sub>2</sub> (Sanyo Biomedical); CO<sub>2</sub> was maintained at 5%.

## **2.5 Processing of BeWo Cells**

### **2.5.1 Collection of Cell-Conditioned Culture Medium**

After culture, the conditioned cell culture medium was removed using sterile pipette and stored at -80°C.

### **2.5.2 Processing of Cells for Protein Analysis**

After removal of the culture medium, cells were washed with warmed PBS (37°C), 250µl of ice cold PBS was then added to each well and the plate placed on ice for 2 minutes. Cells were then removed with a cell scraper and the resultant suspension aspirated through a 20 gauge needle to aid cell fragmentation. The cell lysate was then stored at -80°C.

### **2.5.3 Collection of Cell RNA**

Following removal of the culture media, cells were washed twice in warmed PBS (37°C) for 2 minutes each. 200µl of RLT buffer (Qiagen, Mississauga, Canada) with 0.1% β-mercaptoethanol was added to each well and the cells detached with a cell scraper. The resultant suspension was then stored at -80°C.

### **2.5.4 Fixation of Cells Adherent to Cover Slips**

Glass cover slips coated in BeWo cells were removed from the culture media, washed in warmed PBS (37°C) twice for 5 minutes each. The cover slips were then covered with cold methanol (VWR, Lutterworth, UK, 4°C) and transferred to -20°C for 20 minutes. Following removal of the methanol, the cells were washed 3 times in PBS for 5 minutes each. They were then stored in PBS at 4°C.

### **2.5.5 Fixation of cells for Flow Cytometry**

Following removal of culture medium, BeWo cells were washed with warmed PBS (37°C). Cells were incubated with 200µl trypsin-EDTA per well for 5 minutes at 37°C, 200µl of culture medium was then added to buffer the trypsin-EDTA and the resulting cell suspension centrifuged at 631 rpm (80g) for 5 minutes at 4°C (Eppendorf, Mississauga, Canada). The supernatant was then removed, and cells resuspended in 5ml warmed PBS (37°C), the cell suspension was centrifuged at 631 rpm (80g) for 5 minutes at 4°C. The supernatant was then removed and the cell pellet fixed in 2ml ethanol. The cells were kept on ice for one hour and then stored at -20°C prior to analysis.

## **2.6 Protein Assay**

The amount of protein in the supernatant and protein pellet samples obtained in sections 2.3.1 and 2.5.2 was determined using a commercially available protein assay based on the Lowry method (Biorad, Hercules CA, USA). This compares the absorbance of a sample of unknown protein concentration against a series of solutions with known protein concentration.

A 10 mg/ml solution of Bovine Serum Albumin (BSA) solution was made, and a standard curve made by serial dilutions in the relevant lysis buffer, giving a range of 0 to 10µg/µl. The protein samples were defrosted on ice, and reagents added as per the manufacturers' instructions. The absorbance of the resulting solution was measured at a wavelength of 750nm by a spectrophotometer (Versamax, Molecular Devices, Wokingham, UK). Each sample was analysed in triplicate to allow an average reading of the final analysis. The mean intra-assay variability for this method was 7.2%. This data were then entered into Graphpad Prism software version 4.0 (Graphpad Software, San Diego, USA) and the protein content of the samples determined using linear regression.

## **2.7 Protein Analysis by Western Blotting**

### **2.7.1 Protein Preparation for Western Blotting**

The protein was removed from the -80°C freezer and defrosted on ice. Each sample was combined with commercially available Laemmli buffer containing 2-mercaptoethanol, sodium dodecyl sulphate (SDS) and bromophenol blue in a 1:1 ratio. This mixture was boiled at 95°C for 5 minutes to denature the protein. After boiling, the samples were transferred immediately to ice. If the samples were not to be used immediately they were returned to -80°C.

### **2.7.2 Placental Fibroblast Preparation for Western Blotting**

Confluent cultured placental fibroblasts were obtained from Dr Adrian Miller for use as a positive control. The culture media was removed and the base of the flask was gently washed with sterile PBS. 300µl of Laemmli Buffer was boiled at 100°C for 5 minutes and then run over the base of the cell culture flask. This solution was agitated using a pipette. The flask was viewed under the microscope to ensure that the majority of cells had been removed from the base of the flask. The Laemmli buffer containing the cell suspension was boiled for a further 5 minutes. The resultant cell lysate was stored at -80°C.

### 2.7.3 Western Blotting

Western blotting was carried out using discontinuous SDS poly-acrylamide gel electrophoresis (SDS-PAGE). This method uses two separate (discontinuous) gels of differing density to separate proteins with respect to their size / electrical charge ratio. All SDS-PAGE experiments described used a stacking gel of 3.5% acrylamide with a resolving gel of 10% or 12% acrylamide. Prior to the production of the SDS-Polyacrylamide gel stock solutions of 1.5M Tris-HCl, 0.5M Tris-HCl, 10% sodium dodecyl sulphate (SDS), 10% ammonium persulphate, running buffer, transfer buffer and tris-buffered saline (TBS) were made (See Appendix B).

The gels were assembled using the Biorad SDS-PAGE system (Biorad). All equipment was cleaned with methanol before use to remove any residual protein. The resolving gel (10% or 12% acrylamide) (See Appendix B) was prepared first and allowed to set. The stacking gel (3.5% acrylamide) (See Appendix B) was then made and placed above the resolving gel and a comb inserted to allow placement of the protein samples. After the both gels had set, the SDS-polyacrylamide gel was placed in the electrode assembly and transferred into the electrophoresis tank. The tank was then filled to the top of the assembly with running buffer and protein sample added to each lane. Pre-stained SDS-PAGE markers (Biorad) were used to enable identification of proteins by molecular weight, these were boiled for 1 minute prior to use.

The protein samples were subjected to a current of 75mA while the samples were in the stacking gel, when the protein samples had moved to the more dense resolving gel the current was increased to 125 mA. The proteins were then allowed to move through the SDS-Polyacrylamide gel until the 10kD marker had reached the base of the gel. The gel was then removed from the electrophoresis cell, the stacking gel removed and the resolving gel placed in transfer buffer for 15 minutes at room temperature. The proteins were then transferred to a Polyvinylidene Difluoride (PVDF) membrane (Schleicher and Schuell Bioscience, Dassel, Germany) using a wet transfer system (Biorad) with a current of 400mA. The duration of transfer was dependent on the molecular weight of the protein of interest, ranging from 40 to 90 minutes. After transfer, the membranes were placed on blotting paper to dry for 15 minutes in air to fix the proteins to the membrane.

The membranes were blocked for 1 hour at room temperature in a solution of 3% milk in 0.05% TBS-Tween solution. The PVDF membrane was then exposed to primary antibody overnight at

4°C. The primary antibodies, sources, clone (where applicable) and concentrations are shown in Table 2.1. The PVDF membrane was divided at an appropriate place for the proteins of interest and constitutively expressed proteins to ensure that expression of the protein of interest could be standardised. In most cases the PVDF membrane was divided at approximately 40kD. A negative control, by omission of primary antibody, was performed to ensure antibody specificity. The choice of constitutively expressed protein was made according to the molecular weight of the protein of interest. For example, myosin light chain (21kD) was used to standardise p53 (53kD), as  $\beta$ -actin (44kD) was too close to accurately divide the membrane.

After incubation with primary antibody the membranes were washed in 0.05% TBS-Tween for 10 minutes, this process was repeated three times. The membrane was then exposed to the appropriate secondary antibody in 1% milk in 0.05% TBS-Tween: either goat anti-mouse (P0447, Dako-Cytomation, Ely, UK) or anti-rabbit (P0448, Dako-Cytomation) conjugated to horseradish peroxidase at a dilution of 1:1000 for 1 hour at room temperature. Following incubation with secondary antibody, the PVDF membrane was washed 3 times in 0.05% TBS-Tween for 10 minutes. The presence of secondary antibody revealed using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) as per the manufacturers' instructions; the reagent mixture was applied to each membrane and allowed to react for 3 minutes. The membrane was then removed from the solution placed with the protein-antibody complex side uppermost and placed between two sheets of clear acetate in a film cassette (Amersham Biosciences, Amersham, UK). Photosensitive film (Amersham Biosciences) was placed in the cassette for periods of 1, 5, 10, 60, or 120 minutes up to 24 hours depending on the strength of the signal from the protein of interest. The film was then placed into developing solution (Eastman-Kodak, Rochester, NY, USA) for 5-10 seconds, washed in water and then placed in fixative solution (Eastman-Kodak) for a further 5-10 seconds. The presence of the protein of interest was assessed by densitometric analysis of the bands on the developed film using Molecular Analyst software (Biorad).

Antibody Type	Target	Clone	Supplier	Concentration
Monoclonal Mouse	anti-p53	DO-1	Merck Biosciences, Nottingham, UK	1µg/ml
Monoclonal Mouse	anti-Mdm2	2A10	Merck Biosciences.	2µg/ml
Monoclonal Mouse	anti-p21	EA10	Abcam, Cambridge, UK	1µg/ml
Polyclonal Rabbit	anti-Bax	-	Abcam	1µg/ml
Monoclonal Mouse	anti-Bcl-2	100/D5	Abcam	1µg/ml
Monoclonal Mouse	anti-Bak	TC102	Merck Biosciences	1µg/ml
Polyclonal Rabbit	anti-smac	-	Abcam	0.5µg/ml
Monoclonal Rabbit	anti-HtrA2/omi	E55	Abcam	2.0µg/ml
Monoclonal Mouse	Anti-XIAP	48	BD Biosciences, San Jose, USA	10µg/ml
Polyclonal Rabbit	Anti-survivin	-	R & D Systems, Minneapolis, USA	0.5µg/ml
Monoclonal Mouse	anti-Myosin Light Chain	MY21	Abcam	0.1µg/ml
Monoclonal Mouse	anti-β actin	AC15	Sigma Aldrich	Dilution 1:10,000

**Table 2.1** – Type, target, and supplier of primary antibodies used for Western Blotting.

#### **2.7.4 Stripping PVDF Membranes**

If the molecular weight of the protein of interest was too close to that of β-actin or myosin light chain, equal protein loading was confirmed by stripping the PVDF membrane of primary antibody and re-probing with mouse anti β-actin antibody. Membranes were stripped by soaking in methanol for 10 seconds, then transferred to a solution of 0.5M Tris-HCl, 2% SDS (v/v), 2% β-mercaptoethanol (v/v), which was placed in a water bath at 60°C for 30 minutes. The membranes were then removed from the stripping buffer and washed 5 times in distilled

water for 10 minutes. The membranes were then exposed to primary antibodies against  $\beta$ -actin as outlined in section 2.7.3.

## **2.8 Immunohistochemistry and Immunocytochemistry**

### **2.8.1 Preparation of Slides and Tissue Sections**

Prior to cutting tissue sections, microscope slides (BDH, Loughborough, UK) were coated with 3-aminopropyltriethoxysilane (APES) to enhance adhesion between the tissue section and slide as previously described (Maddox and Jenkins 1987) (See Appendix C for detailed method).

5 $\mu$ m tissue sections were cut using a microtome (Reichert-Jung, Vienna, Austria), floated on a water bath and placed on glass slides. The finished slides were then incubated at 37°C for 48 hours to ensure adhesion between the tissue section and the slide.

### **2.8.2 Immunoperoxidase Staining**

Immunohistochemistry was conducted using a microwave antigen retrieval method followed by a peroxidase staining technique. This technique utilised a secondary antibody conjugated to a biotin residue. Subsequent application of avidin peroxidase conjugate enables the identification of bound antibody complex with the substrate 3',3'-diaminobenzidine (DAB) which forms a characteristic brown precipitate. The protocol used has been modified from that described in a textbook of immunohistochemical techniques (Miller 1996).

Tissue sections were warmed to 56°C for 10 minutes, and then de-paraffinised in 3 washes of xylene, each for 4 minutes. The sections were rehydrated in descending grades of ethanol (100% to 70%). Endogenous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol (VWR) for 40 minutes at room temperature. The sections were then washed in distilled water for 3 minutes and then subjected to microwave antigen retrieval (800W) in 300ml 0.01M tri-sodium citrate solution (pH 6) for between 5 and 12 minutes; after the first 5 minutes, the volume of tri-sodium citrate was restored to 300ml. The sections were allowed to cool in the tri-sodium citrate solution for 20 minutes.

A reservoir for liquid was made around each section and the slides placed in a humidified chamber. Non-specific antibody binding was prevented by application of a protein block containing either 10% normal rabbit serum (NRS), 10% normal goat serum (NGS) or 10% NGS

with 1% BSA for 20 minutes at room temperature. Tissue sections were then exposed to primary antibody solutions diluted in the appropriate blocking solution overnight at 4°C. The primary antibodies, sources, clone (where applicable) and concentrations are shown in Table 2.2. Negative controls were used for each immunostaining procedure. For mouse monoclonal antibodies, isotype specific mouse IgG was used at an appropriate dilution, for rabbit primary antibodies, blocking solution alone was applied.

After incubation with primary antibody the tissue sections were washed 3 times in PBS each for 5 minutes. The sections were incubated with either biotinylated polyclonal goat anti-mouse (E0354, Dako-Cytomation) or biotinylated polyclonal goat anti-rabbit (E0432, Dako-Cytomation) antibodies at a dilution of 1:200 in PBS for 1 hour at room temperature. Following this, the slides were washed in PBS 3 times each for 5 minutes. The sections were then incubated with avidin peroxidase (5µg/ml) in 0.125M TBS for 30 minutes at room temperature. The slides were then washed in PBS as previously described. The tissue sections were exposed to concentrated DAB generated using a kit (Sigmafast) for 3 minutes at room temperature; the reaction was stopped by placing the sections in running water.

The slides were counterstained with either methyl green or haematoxylin. If haematoxylin was used, tissue sections were blued in warm running tap water and differentiated using acid-alcohol (1N HCl in 70% ethanol). If methyl green was used, excess staining was removed by washing in cold tap water. The slides were then dehydrated in an ascending alcohol series (70% to 100%), then immersed in xylene for 3 washes each for 2 minutes. The tissue sections were then mounted in DPX mountant (BDH).



Antibody Type	Target	Clone	Supplier	Concentration	Microwave Time for Antigen Retrieval
Monoclonal Mouse	anti-p53	DO-1	Merck Biosciences	7.8µg/ml	10 minutes
Monoclonal Mouse	anti-Mdm2	2A10	Merck Biosciences	2µg/ml	5 minutes
Monoclonal Mouse	anti-p21	EA10	Abcam	4µg/ml	12 minutes
Polyclonal Rabbit	anti-Bax	-	Abcam	2µg/ml	5 minutes
Monoclonal Mouse	anti-Bcl-2	100/D5	Abcam	2µg/ml	5 minutes
Monoclonal Mouse	anti-Bak	TC102	Merck Ltd	2µg/ml	5 minutes
Polyclonal Rabbit	anti-smac	-	Abcam	5µg/ml	5 minutes
Monoclonal Rabbit	anti-HtrA2/omi	E55	Abcam	10µg/ml	10 minutes
Monoclonal Mouse	Anti-XIAP	48	BD Biosciences	10µg/ml	5 minutes
Polyclonal Rabbit	Anti-survivin	-	R & D Systems	0.5µg/ml	5 minutes
Monoclonal Mouse	Anti phosphorylated AKT	587F11	Cell Signalling Technology, Danvers, USA	2µg/ml	5 minutes
Polyclonal Rabbit	Anti phosphorylated PI3-Kinase	-	Cell Signalling Technology	0.5µg/ml	5 minutes
Monoclonal Mouse	anti-Ki67	MIB-1	Dako-Cytomation	1.6µg/ml	12 minutes

**Table 2.2** – Type, target, and supplier of primary antibodies used for immunoperoxidase staining.

### **2.8.3 Immunocytochemistry**

BeWo cells were cultured on coverslips and fixed as described in section 2.5.4. The cells were washed twice in PBS to remove any debris. Non-specific antibody binding was reduced by blocking the coverslips in 1% BSA with 0.05% Tween for 20 minutes at room temperature. The coverslips were then incubated with primary antibody solution of mouse monoclonal anti-desmoplakin (Clone ZK31, Sigma-Aldrich, 19µg/ml) or anti-cytokeratin 7 (Clone OV-TL, Dako, dilution 1:200) for 1 hour at room temperature. A negative control was made by the omission of primary antibody. The coverslips were then washed 3 times in PBS, each for 5 minutes, before being exposed to a fluorescein conjugated rabbit anti-mouse antibody at a dilution of 1:200. The coverslips were then washed a further three times in PBS, and then mounted in medium containing propidium iodide (Vector, Burlingame, USA). The slides were examined using a laser-scanning confocal microscope (Olympus IX70 Microscope and Biorad Laser) and 10 representative images taken per experimental condition.

## **2.9 Real-time Quantitative Polymerase Chain Reaction**

### **2.9.1 Extraction and Quantification of Total RNA**

#### **2.9.1.1 Method Used for BeWo cells**

Total RNA was extracted using an RNEasy kit (Qiagen, Mississauga, Canada) according to the manufacturers' instructions. This included treatment with DNase I to remove any contaminant genomic DNA. The concentration of RNA was determined using the absorbance at 260nm.

#### **2.9.1.2 Method Used for Placental Villous Explants**

Total RNA was extracted using Absolutely RNA (Stratagene, La Jolla, USA) according to the manufacturers' instructions. All samples were treated with DNase I to eliminate genomic DNA contamination. Total RNA was quantified using Ribogreen (Molecular Probes, Invitrogen, UK) and the MX4000 analyser (Stratagene, La Jolla, USA) according to the manufacturer's instructions. Integrity of total RNA was confirmed by visualization of ribosomal band integrity by gel electrophoresis.

### 2.9.2 Synthesis of cDNA

A constant amount of total placental RNA (100 ng) was used for each batch of reverse transcription (RT) reactions. To allow adjustment for run-to-run variation, a calibrator sample (100ng) of quantitative reference RNA (Stratagene) was included in the RT reaction. This calibrator sample was used in all RT reactions in parallel to unknown placental samples. In addition, cDNA synthesis reactions included a no RT enzyme control to exclude the presence of genomic DNA. First strand cDNA synthesis was performed using random primers (300 ng) (Stratascript First Strand, Stratagene) and cDNA was stored at -20°C.

### 2.9.3 Primer design

Sequences for all genes were derived from GenBank (Table 2.3). All primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Int., Palo Alto, CA, USA) with reference to structural and Basic Local Alignment Search Tool (BLAST) assessment. Primer and amplicon sequences were subjected to a BLAST search to ensure gene specificity and no amplification from related or other gene sequences. Primers were synthesised by either Invitrogen (Paisley, UK – for Manchester experiments) or Qiagen (for Toronto experiments).

Primer	Forward Sequence	Reverse Sequence
p53	CTCCTCAGCATCTTATCCGAGTG	GTGGTACAGTCAGAGCCAACC
Mdm2	GTGAAGGAAACTGGGGAGTCTT	AGGTACAGACATTTTGGTATTGCA
p21	AGGTGGACCTGGAGACTCTCA	CGGCGTTTGGAGTGGTAGAAA
Bax	GCTGTTGGGCTGGATCCAAG	TCAGCCCATCTTCCAGA
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG

**Table 2.3** Primer sequences used for real time PCR

### 2.9.4 Real-Time Quantitative Polymerase Chain Reaction (rt-QPCR)

p53, Mdm2, Bax, p21, TBP, and SDHA mRNA expression in placenta was quantified using an MX4000 using SYBR Green I and melt curve analysis to ensure amplification specificity. All

reactions were run in triplicate and 5-carboxy-x-rhodamine (ROX) was always included as passive reference dye. QPCR reactions were performed in 25  $\mu$ l volumes, containing 1  $\mu$ l cDNA using Stratagene Brilliant QPCR mastermix. All samples were run in triplicate for 40 cycles of PCR under the following conditions: initial enzyme activation and template denaturation for 10 minutes at 95°C followed by 30 seconds at 95°C, 1 minute annealing at 55-58°C (depending on primer pairing) and extension phase for 30 seconds at 72°C. For each gene transcript an amplification plot was created for each sample plotting dRn (Rn is fluorescence emission intensity of the reporter dye normalized to ROX and dRn is the Rn of an unreacted sample minus the Rn value of the reaction) vs the product cycle threshold (Ct). To quantify expression sample, Ct values were used to calculate amounts against a standard curve constructed from cDNA generated from human reference RNA (1  $\mu$ g/ $\mu$ l; Stratagene, La Jolla, USA) ranging from 7.8 ng to 5  $\mu$ g for determination of assay efficiency and sample quantification (Figures 2.3-2.6). All assays were validated on this serial dilution and only used for subsequent analysis if the reaction efficiency was determined to be between 93 and 105%. Examples of rt-PCR amplification plots, dissociation curves and products are shown in Figures 2.3-2.6.

## **2.10 Assessment of Apoptosis**

### **2.10.1 Terminal d-UTP Nick-End Labelling**

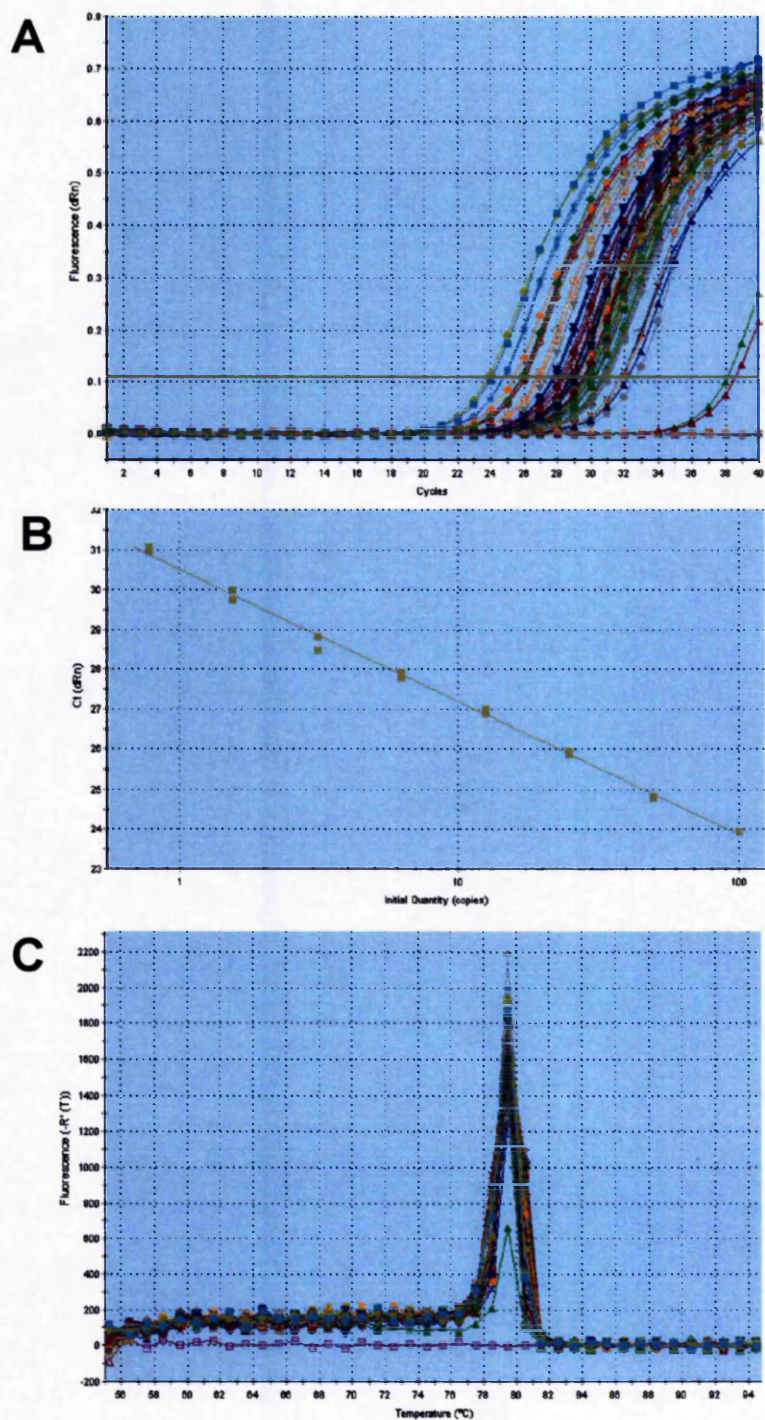
Terminal d-UTP Nick-End Labelling (TUNEL) is used to identify apoptotic cells by labelling fragmented DNA occurring as a result of nuclear DNA breakdown (Gavrieli et al. 1992). A commercially available TUNEL kit was used (Roche Applied Science, Lewes, Sussex, UK) with modifications from the manufacturers' instructions. The key components of the kit were terminal deoxynucleotidyl transferase enzyme solution and a fluorescein conjugated nucleotide solution. In some cases a peroxidase-conjugated anti-fluorescein antibody was used to reveal TUNEL staining.

For both fluorescein and peroxidase methods, 5 $\mu$ m tissue sections were prepared from wax-embedded tissue as described in section 2.8.1 and were subjected to the same microwave antigen retrieval method as described for immunohistochemistry in section 2.8.2. If the TUNEL technique included the use of a peroxidase conjugated antibody, endogenous peroxidase activity was quenched using 3% H<sub>2</sub>O<sub>2</sub> (v/v) in methanol for 40 minutes at room temperature

followed by rinsing in distilled water. If TUNEL was imaged using fluorescein alone tissue sections were transferred to 70% ethanol for 2 minutes, and then distilled water for 2 minutes. For both methods, tissue sections were then subjected to microwave antigen retrieval (800W) in 300ml 0.01M tri-sodium citrate solution (pH 6) for 5 minutes. The trisodium citrate was then replenished to 300ml and sections heated for a further 5 minutes at 800W. The sections were allowed to cool in the tri-sodium citrate solution for 20 minutes.

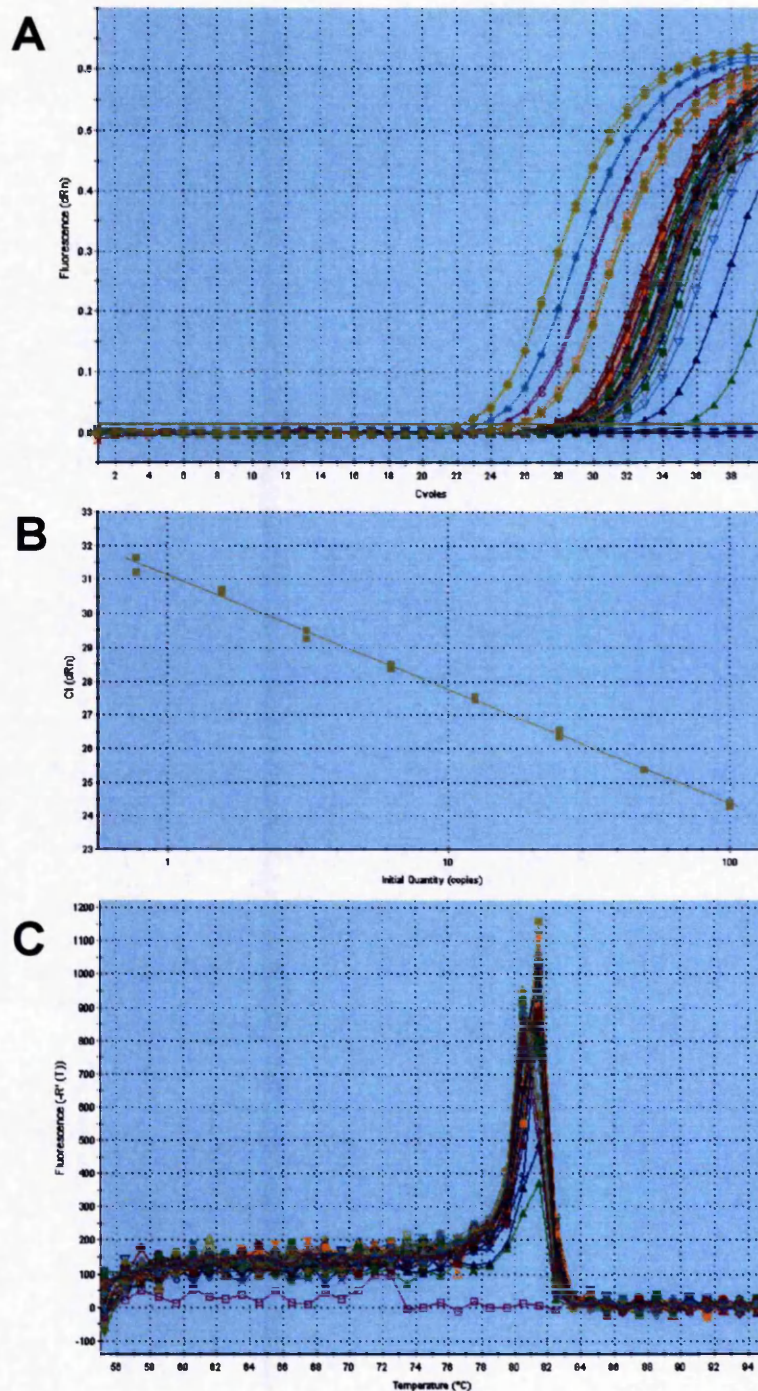
A reservoir was made around the tissue section and the slides were placed in a humidified chamber. A tissue section was incubated with DNase I (3000 u/ml in PBS) for 15 minutes at 37°C as a positive control. The deoxynucleotidyl transferase enzyme solution supplied with the kit was diluted 1 in 5 in PBS. This dilute enzyme solution was added to the labelling compound in a 1:9 ratio and the combined solution added to each slide. A negative control slide was incubated with label solution alone. All slides were incubated for 1 hour at 37°C, followed by washing twice in PBS each for 5 minutes.

If the fluorescein label was to be visualised directly, tissue sections were mounted in medium containing propidium iodide (Vector), the slide was then sealed by application of varnish around the edge of the cover slip. If the TUNEL was to be revealed using immunoperoxidase, unwanted antibody binding was blocked using 10% NRS with 1% BSA (w/v) for 30 minutes at room temperature. Slides were then incubated with peroxidase conjugated sheep anti-fluorescein antibody for 30 minutes at 37°C. The tissue sections were then washed 3 times in PBS each for 5 minutes. The sections were then exposed to concentrated DAB for 3 minutes at room temperature, after which the reaction was stopped by placing the sections in cold running water. The slides were then counterstained with Haematoxylin for 2 minutes, which was blued by rinsing in warm water for 5 minutes, followed by differentiating the sections in acid-alcohol (1N HCl in 70% ethanol). The sections were dehydrated using an ascending alcohol series (70%-100%) for 2 minutes each. The slides were placed in xylene for 3 washes, each for 2 minutes. The tissue sections were mounted using DPX mountant (BDH).

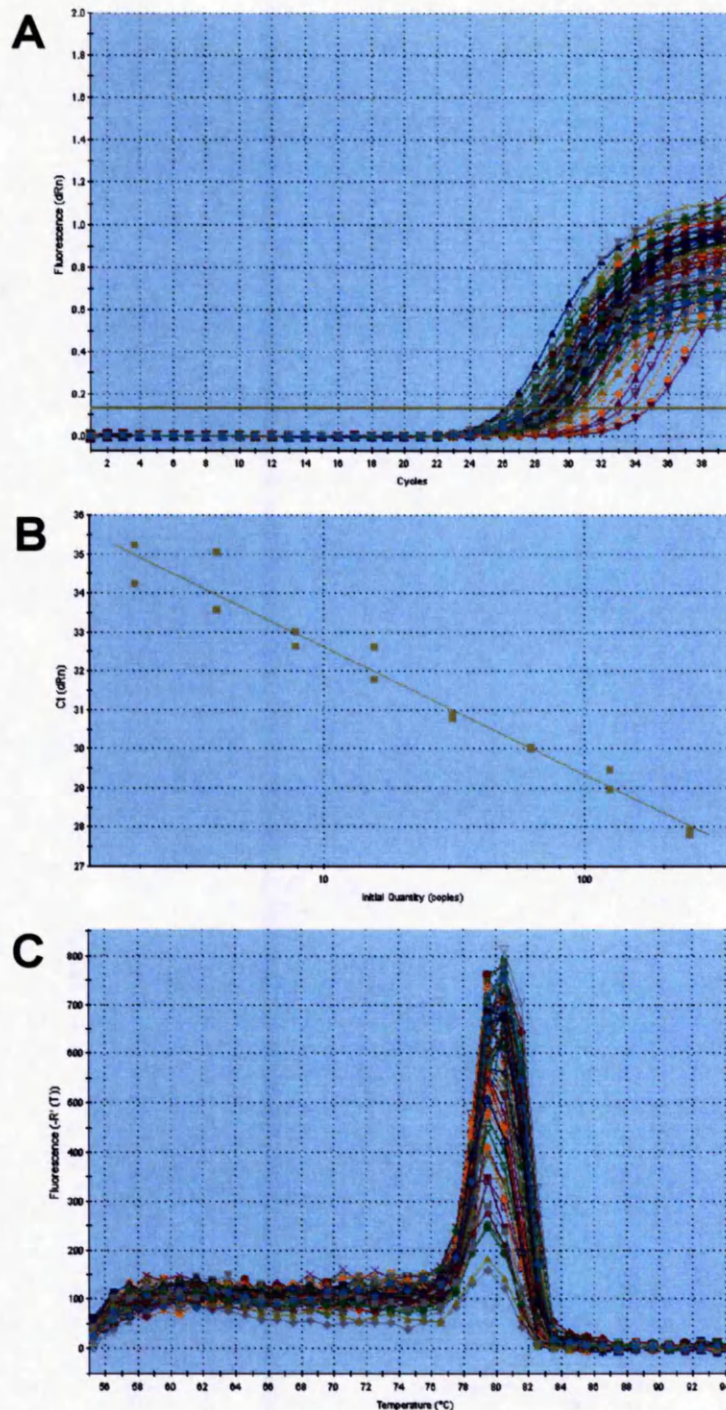


**Figure 2.3** – Real-time PCR plots for p53 mRNA. A) Amplification plot demonstrating that samples were amplified within the standard curve and that sample amplification was parallel to that of the standard curve. B) Standard curve for p53 siRNA, the reaction efficiency was 100%. C) Dissociation curve for p53 mRNA showing a single peak.



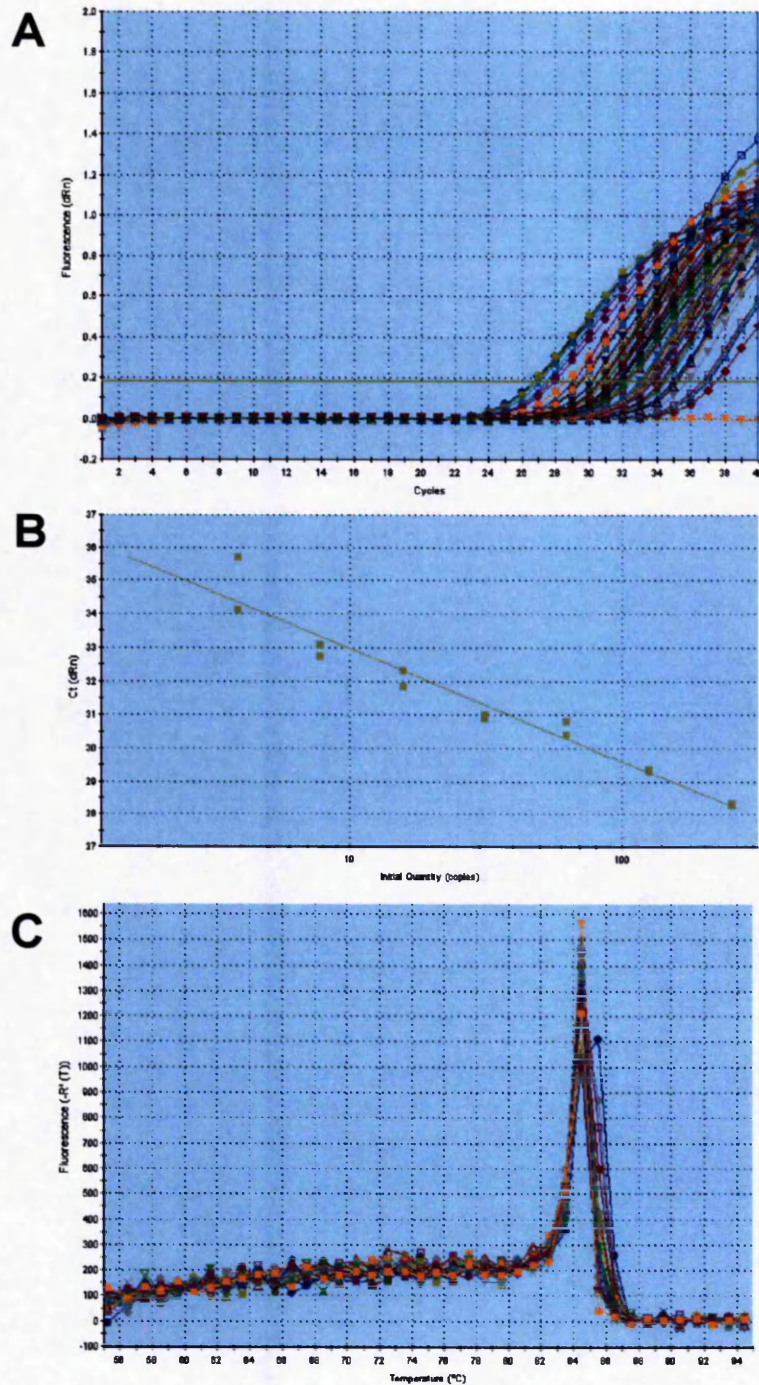


**Figure 2.4** – Real-time PCR plots for Mdm2 mRNA. A) Amplification plot demonstrating that samples were amplified within the standard curve and that sample amplification was parallel to that of the standard curve. B) Standard curve for Mdm2 siRNA, the reaction efficiency was 97.5%. C) Dissociation curve for Mdm2 mRNA showing a single peak.



**Figure 2.5** – Real-time PCR plots for p21 mRNA. A) Amplification plot demonstrating that samples were amplified within the standard curve and that sample amplification was parallel to that of the standard curve. B) Standard curve for p21 siRNA, the reaction efficiency was 101.7%. C) Dissociation curve for p21 mRNA showing a single peak.





**Figure 2.6** – Real-time PCR plots for Bax mRNA. A) Amplification plot demonstrating that samples were amplified within the standard curve and that sample amplification was parallel to that of the standard curve. B) Standard curve for Bax siRNA, the reaction efficiency was 98%. C) Dissociation curve for Bax mRNA showing a single peak.

TUNEL staining was quantified using light and confocal microscopy of immunoperoxidase and fluorescein labelled sections, respectively. The light microscopy technique used a Leitz microscope at 400x magnification and Image Pro-Plus software (MediaCybernetics, Wokingham, UK). Ten fields of view were assessed for each tissue section from each experimental condition. To avoid bias the microscope was taken out of focus between frames. The number of TUNEL positive nuclei were counted manually and the total number of nuclei were measured by sequential colour thresholding as previously described (Crocker et al. 2004a). From these values a ratio was derived, representing the TUNEL positive index (% of nuclei TUNEL positive).

### **2.10.2 Immunohistochemistry for Cytokeratin-18 M30 Neoepitope**

Cytokeratin-18, a cytoskeletal protein present in cells of epithelial origin, is cleaved by caspase-3 during apoptosis, creating a new epitope, termed M30. This neo-epitope has been used to detect apoptosis in trophoblast (Kadyrov et al. 2001). 5µm tissue sections were deparaffinised and subjected to microwave antigen retrieval as for immunohistochemistry (Section 2.8.2). Sections were blocked with 10% NRS for 60 minutes at room temperature and then exposed to mouse monoclonal anti-M30 antibody (Roche Applied Science, 1:200) overnight at 4°C. An isotype specific mouse immunoglobulin was used as a negative control. Tissue sections were then washed in PBS and exposed to secondary antibody, avidin peroxidase and DAB as defined in the immunohistochemistry protocol (Section 2.8.2). 10 areas of each tissue section were viewed at x400 magnification using a light microscope (Leitz) and representative images taken (Image Pro-Plus, MediaCybernetics).

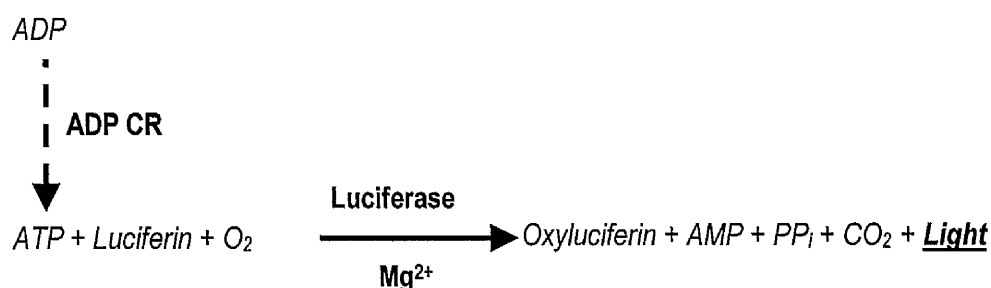
### **2.10.3 Apopercantage Assay**

In BeWo cells apoptosis was assessed using three methods. The first, the Apopercantage assay (Biocolor, Newtonabbey, UK) identified apoptotic cells by absorption of a dye during the externalisation of phosphatidylserine from the inner aspect of the plasma membrane to the outer surface. The accumulation of dye reflects the amount of apoptosis. The assay was carried out according to the manufacturers' instructions. In summary, BeWo cells were cultured in a 96-well plate at a density of  $1 \times 10^5$ /well. After culture, the original culture medium was

replaced with culture media containing 5% (v/v) Apopercut dye. Cells were then returned to their culture environment for 30 minutes. After this, cells were washed twice with warmed PBS (37°C) to remove unbound dye. Images were then taken using an inverted microscope (Leica). Following this cells were lysed and the dye released into solution, the absorbance of this solution was then measured at 550nm using a spectrophotometer (Molecular Devices).

#### 2.10.4 Adenylate Nucleotide Ratio Assay

Cell death from either necrosis or apoptosis can be assessed by determining the release of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) from cells (Bradbury et al. 2000). ATP can be measured by a bioluminescent method, which comes in a kit (Apoglow, Cambrex) previously used in trophoblast (Crocker et al. 2003). This method utilises luciferase which generates light in the presence of ATP and luciferin (Figure 2.7). The emitted light intensity is proportional to the amount of ATP. Subsequently, ADP can be measured following enzymatic conversion to ATP.



**Figure 2.7** Conversion of ATP and luciferin by luciferase to oxyluciferin and adenosine monophosphate (AMP) results in the emission of light. The Apoglow kit uses an ADP converting reagent (ADP CR) to measure ADP, by conversion to ATP.

BeWo cells were cultured at a density of  $2 \times 10^5$ /well in white-walled 96-well culture plates in order that adjacent wells would not interfere with the measured luminescence of adjacent wells. After culture, culture medium was removed from the well, and the cells incubated for 15 minutes at room temperature with 100µl of 'nucleotide releasing reagent' to liberate nucleotides from cells. The luciferin-containing 'nuclear monitoring reagent' was then added, and a reading taken immediately using a luminometer (Gemini XS, Molecular Devices)

(Reading A), reflecting the amount of ATP released from the cells. The culture plate was incubated for 20 minutes in the dark at room temperature to allow this activity to decay and reach stable levels, at which point the luminescence was recorded (Reading B). Then 20µl of 'ADP converting reagent' was added to convert any ADP present to ATP. After 1 minute the luminescence was measured again (Reading C). The ADP:ATP ratio was calculated by the formula:

$$ADP : ATP \text{ Ratio} = \frac{\text{Reading C} - \text{Reading B (The amount of ADP)}}{\text{Reading A (The amount of ATP)}}$$

### 2.10.5 Assessment of BeWo Cell Morphology by Flow Cytometry

BeWo cells were fixed as described in section 2.5.5. The cell suspensions were taken from the -20°C freezer, centrifuged at 6000 rpm (8300g) for 2 minutes and the supernatant removed. Cells were then suspended in 500µl PBS with 0.12% Triton X (v/v) and 0.12% EDTA (v/v) and centrifuged at 6000rpm (8300g) for a further 2 minutes. The supernatant was removed and cells resuspended in the PBS-Triton-EDTA buffer with 0.05% DNase free RNase, agitated and then incubated at 37°C for 30 minutes. After this incubation, 2.5µl propidium iodide was added per ml of buffer solution and the suspension gently agitated.

The resultant suspension of BeWo cells was analysed by FACSCalibur (BD Biosciences) using absorbance at 585nm +/- 21nm. Cells were analysed by side scatter, size and the amount of propidium iodide (PI) per cell. Data were analysed using FlowJo v6.3.4 (FlowJo, Ashland, OR, USA). The presence of cell debris was excluded by gating. The proportion of cells with less than diploid complement (<2n) and the proportion of cells in S, G1 and G2 stages of the cell cycle were identified using assessing the amount of PI in cells. The number of cells <2n was taken as an indicator of apoptosis, as this has previously been used in isolated primary trophoblast cells (Yui et al. 1994b).

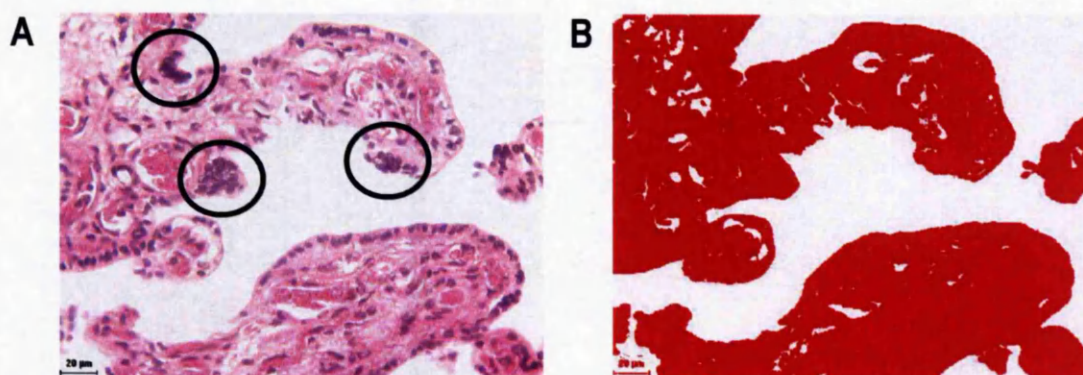
### 2.11 Quantification of Syncytial Knots

Syncytial knots are defined as accumulations of degenerating nuclei which may protrude from the villous surface and are thought to reflect the ageing process and/or degeneration of the syncytiotrophoblast. 5µm sections were deparaffinised and rehydrated as for



immunohistochemistry (section 2.8.2). The tissue sections were stained with Harris' haematoxylin for 5 minutes, blued in warm running tap water, and differentiated using acid-alcohol (1N HCl in 70% ethanol). The tissue sections were rinsed in tap water for a further two minutes, and then counterstained in eosin for five minutes; excess eosin staining was removed by rinsing in cold tap water. The slides were dehydrated in an ascending alcohol series, (70% to 100%) and then immersed in xylene for 3 washes of 2 minutes each. The tissue sections were then mounted in DPX mountant (BDH).

Using a 40x objective, the number of syncytial knots was counted in 10 fields of view for each experimental condition. To avoid bias the microscope was taken out of focus between frames. A syncytial knot was defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface, not in direct contact with adjacent villi (Cantle et al. 1987). The number of syncytial knots was counted manually. The trophoblast area was measured by sequential colour thresholding following spatial calibration of the image analysis software (Image ProPlus, Mediacybemetics) as previously described (Daayana et al. 2004). This technique enables selection of all tissue stained with haematoxylin and eosin (Figure 2.8). Data were normalised to give a measure of the number of syncytial knots per mm<sup>2</sup> of villus.



**Figure 2.8** Determination of the number of syncytial knots A) 5µm section of placental villous tissue stained with haematoxylin and eosin. Syncytial knots are highlighted by black circles. B) Sequential colour thresholding can be used to select total villous area which can then be measured by image analysis software.

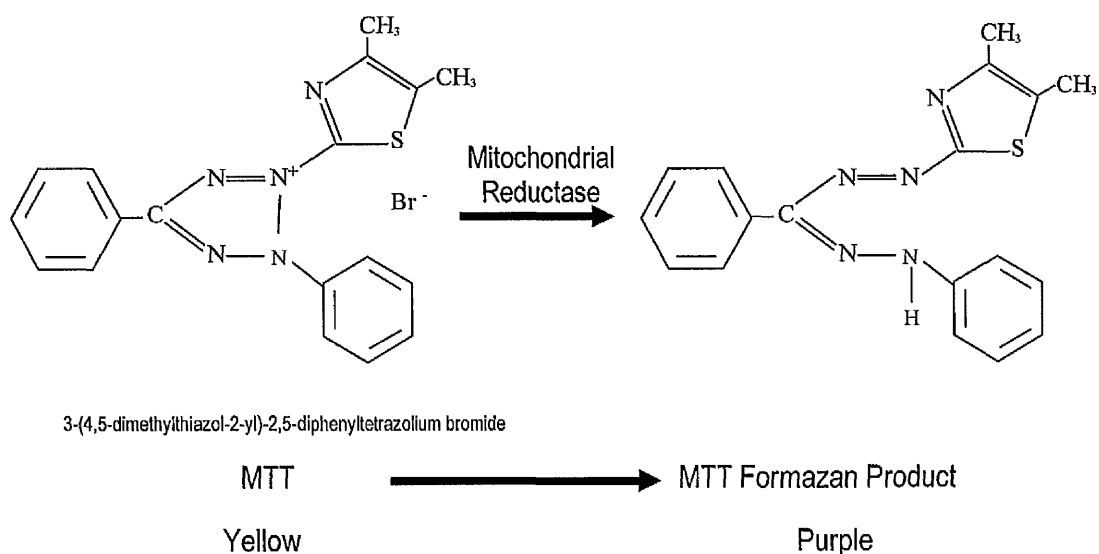
## **2.12 Assessment of Proliferation**

### **2.12.1 Immunoperoxidase staining for Ki67 antigen**

The Ki67 antigen is expressed in nuclei undergoing mitosis, and has been previously used to assess proliferation in trophoblast (Cheung et al. 1994a). Immunoperoxidase staining was carried out as described in section 2.8.2 using a primary mouse-monoclonal anti-Ki67 antibody (Dako-Cytomation, Clone MIB-1) at a concentration of 1.6µg/ml. Ki67 immunostaining was assessed using light microscopy with a Leitz microscope at 400x magnification and Image Pro-Plus software (MediaCybernetics, Wokingham, UK). Five fields of view were assessed for each experimental condition, to avoid bias the microscope was taken out of focus between frames. The number of Ki67 positive nuclei were counted manually. The total number of nuclei was then measured by sequential colour thresholding as previously described. (Crocker et al. 2004a) From these values a ratio was derived; the proliferative index (% of total nuclei Ki67 positive)

### **2.12.2 MTT Assay**

Proliferation of BeWo cells was assessed by the MTT assay, which measures conversion of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to purple formazan (Figure 2.9). This process is catalysed by mitochondrial enzymes, the amount of which is thought proportional to the number of viable cells (Mosmann 1983). This has previously been used in BeWo cells, and was proportional to the expression of Ki67 (Al-Nasiry et al. 2006). BeWo cells were seeded onto 96 well plates at a density of  $10 \times 10^5$ /well. Following culture, 10µl of culture medium was removed from each well and replaced by 0.01M MTT solution, giving a final concentration of 1mM MTT per well. The cells were then returned to their original culture environment for 3 hours. The cells were then gently washed twice with warmed PBS for 2 minutes each. The purple formazan dye was then dissolved using 100µl dimethylsulphoxide (DMSO), the absorbance of the resulting solution was assessed using a spectrophotometer (Molecular Devices) at a wavelength of 550nm.



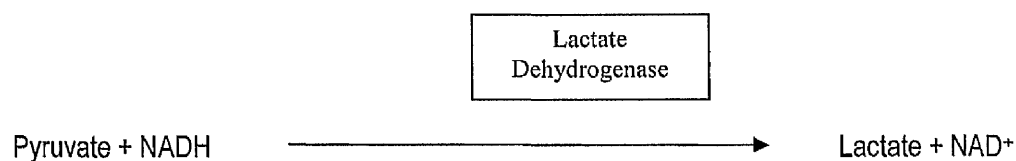
**Figure 2.9** MTT Assay - MTT is reduced by mitochondrial reductase to form an MTT formazan product resulting in a colour change from a yellow solution to a purple precipitate.

## 2.13 Assessment of Secreted Proteins in Conditioned Culture Medium

### 2.13.1 Lactate Dehydrogenase Assay

#### 2.13.1.1 Conditioned Culture Medium from Placental Villous Explants

This assay relies on the ability of lactate dehydrogenase (LDH) to transform pyruvate to lactate in the presence of the reduced form of nicotinamide dinucleotide (NADH). In this reaction NADH is oxidised to nicotinamide dinucleotide (NAD<sup>+</sup>), therefore the decrease in absorbance of NADH is directly related to LDH activity.



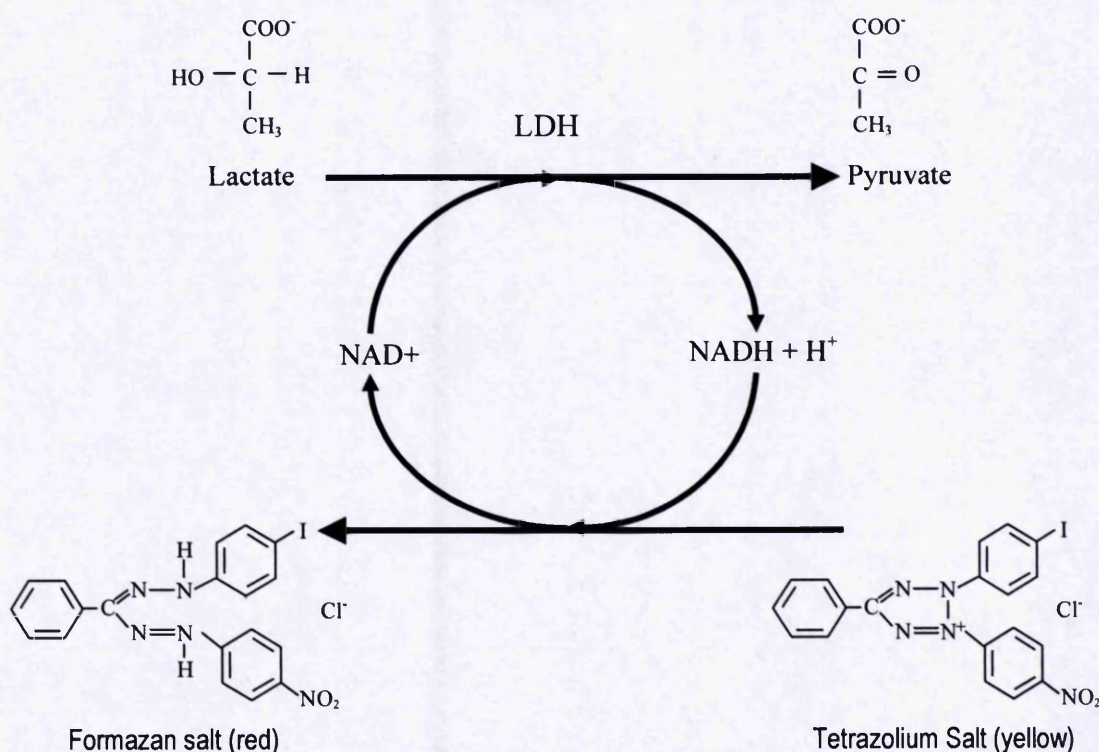
A 0.4mM solution of NADH (Roche Applied Science, Lewes, UK) and a solution of 0.35mM pyruvate were prepared in 80 mmol/L Tris-HCl, pH 7.5 (See Appendix B). The conditioned culture medium was defrosted on ice and agitated for 5 seconds. 100μl of culture media was

placed in a cuvette (1cm wide) and combined with 1ml of NADH solution and 1ml of pyruvate solution. The cuvette was then placed immediately in the spectrophotometer (Genova Jenway, Dunmow, UK), and the reaction allowed to continue for 3 minutes after which time the decrease in absorbance at 340nm was recorded. Each sample was repeated in triplicate. The amount of LDH activity was calculated using a formula containing the concentration of NAD and pyruvate, and the extinction co-efficient of NADH at 340nm.

#### **2.13.1.2Conditioned Culture Medium from BeWo Cells**

In conditioned culture media from BeWo cells, LDH activity was assessed using a commercially available kit (Roche), measuring the conversion of a tetrazolium salt (yellow) to a formazan salt (red). This requires the presence of NADH, which is generated by the conversion of lactate to pyruvate by LDH (Figure 2.10). Conditioned culture medium was defrosted on ice, and agitated for 5 seconds. Unconditioned culture media was used as a negative control. BeWo cells lysed with 2% Triton were used as a positive control. 50 $\mu$ l of culture media was placed in each well of a 96-well plate, this was combined with the kit reagents then incubated for 20 minutes at room temperature. The reaction was then halted by addition of 100 $\mu$ l 1M HCl stop solution. The absorbance of each well was then read at a wavelength of 490nm (Molecular Devices). For this assay the intraassay variability was 6.0%.





**Figure 2.10** – LDH assay. The conversion of lactate to pyruvate by LDH results in the generation of NADH. This is able to reduce the tetrazolium salt to a formazan salt, resulting in a colour change from yellow to red.

### 2.13.2 Human Chorionic Gonadotrophin Assay

Human Chorionic Gonadotrophin (hCG) is produced by the syncytiotrophoblast and fused BeWo cells. The quantity of hCG in the conditioned culture medium was assessed using a commercially available radioimmunoassay kit (MP Biomedicals, Orangeburg, USA). This kit uses test-tubes coated with an antibody against the  $\beta$ -subunit of hCG, which binds the hCG, then a radio-labelled ( $^{125}\text{I}$ ) secondary antibody is added. The amount of gamma emissions from the  $^{125}\text{I}$  can then be measured.

This experiment was conducted in accordance with the local rules for the handling and use of radioactive substances. The culture media was defrosted on ice and agitated for 5 seconds. 50 $\mu\text{l}$  of each prepared standard (0, 2.5, 5, 10, 25, 100, 250, 500 and 1000 iu/l) and 50 $\mu\text{l}$  of each culture media, was added to individual marked test tubes. Each sample was performed in

triplicate to allow calculation of an average reading to be used for the final analysis. The tubes were incubated for 30 minutes at room temperature on a shaker. After each tube was rinsed twice with wash solution, 50µl of radio-labelled antibody was added and this was incubated and agitated for 30 minutes at room temperature. The tubes were then washed twice with wash solution and transferred to a gamma-counter (Cobra II Auto Gamma, Packard Instrument Company, Meriden, USA). Each tube was read for 10 minutes. The gamma count of samples was converted to a linear scale by logarithmic transformation and an hCG content for each unknown sample calculated using linear regression (GraphPad). The intra-assay variability was 10.5%.

#### **2.13.3 Assessment of Human Placental Lactogen in Conditioned Culture Medium**

Human Placental Lactogen (hPL) was measured using a commercially available ELISA kit (Bioserv Diagnostics, Rostock, Germany) according to the manufacturers' instructions. The culture media was defrosted on ice and agitated for 5 seconds. 50µl of each prepared standard (0, 1.25, 5, 10, and 20 mg/l) and 50µl of conditioned culture media was added to each well of a 96-well plate. To reduce intra-assay error samples were analysed in triplicate. The samples were incubated for 30 minutes at room temperature and then washed 5 times with PBS. An enzyme conjugate solution was then added and incubated for 30 minutes at room temperature, then washed 5 times with PBS. Then 100µl substrate was added and incubated for 20 minutes, after which time the reaction was stopped using 1M HCl. The absorbance at 450nm was then measured using a spectrophotometer (Molecular Devices). The content of unknown samples was calculated by linear regression using GraphPad software. The intra-assay variability was 12.2%.

#### **2.13.4 Assessment of Alkaline Phosphatase in Conditioned Culture Medium**

Alkaline phosphatase was measured in conditioned culture medium by Ms Naseem Sheikh in the Department of Clinical Biochemistry, Manchester Royal Infirmary.

#### **2.13.5 Assessment of Actin and Placental Protein 13 in Conditioned Culture Medium**

These experiments were conducted in collaboration with Professor Berthold Huppertz, Medical University of Graz, Austria. The presence of alpha actin in tissue conditioned culture medium was assessed using an ELISA technique that makes use of a monoclonal anti-actin antibody

(A9718, Sigma, 1µg/µl) and a polyclonal coating anti-actin antibody (sc1616, Santa-Cruz, 1:50). Absorbance was measured at a wavelength of 450nm. Optical densities were transformed to ng/ml (actin). The intra-assay and inter-assay variabilities were 6.85% and 4.97%, respectively.

Placental protein 13 (PP-13), which is synthesized by the syncytiotrophoblast and may be either actively or passively released, was also quantified using an ELISA technique (a generous gift of Dr H. Meiri, Diagnostic Technologies, Haifa, Israel) and performed as previously described (Burger et al. 2004). Optical densities were transformed to pg/ml (PP-13). Absorbance was measured at a wavelength of 450nm. For PP-13, intra-assay and inter-assay variabilities were 2.26% and 14.8% respectively.

#### **2.14 – Assessment of Villous Morphology by Electron Microscopy**

Following tissue fixation in 2% glutaraldehyde (section 2.3.3.2) villous explants were incubated in 1% osmium tetroxide (Agar Scientific Ltd) in 0.05M sodium cacodylate buffer pH 7.3 for 1 hour at 4°C followed by a rinse in buffer. The tissue was then dehydrated in an ascending alcohol series, treated twice with propylene oxide (15 minutes each), then left in a 1:1 mix of propylene oxide and Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK) for one hour at room temperature. It was then left overnight on a rotator at 4°C in a mixture of 1:3 propylene oxide and epoxy resin and the following day given three changes of fresh resin at 45°C for one hour each before being embedded in gelatin capsules and polymerised for 72 hours at 60°C.

Semithin sections 0.5µm thick were cut on a Reichert Ultracut microtome, stained with 1% toluidine blue in 1% borax. After inspection to identify areas of interest, ultrathin sections were prepared with a diamond knife, mounted on copper grids and stained with uranyl acetate and lead citrate. These were examined in a Philips CM10 electron microscope at an accelerating voltage of 80kV and appropriate areas photographed.

## **Chapter 3 - Is The Aberrant Cell Turnover of Villous Trophoblast in Pre-eclampsia Associated with Altered Expression of Constituents of the p53 Pathway?**

### **3.1 Introduction**

Pre-eclampsia, defined as new onset hypertension and proteinuria after 20 weeks of pregnancy, is a severe multi-system disorder which affects 2.9-5.8% of all human pregnancies (Campbell et al. 1985; Xiong et al. 2002). As discussed in section 1.5 the precise origins of pre-eclampsia are unclear, but symptoms may manifest from mid-gestation and are regarded as signs of inappropriate utero-placental interactions. Currently, a two-stage hypothesis is favoured in which (i) placental damage is associated with the release of single or multiple agents which potentially account for the (ii) maternal endothelial and/or leukocyte activations which characterise this disorder (Myers and Baker 2002; Redman and Sargent 2005). The responsible factors remain undefined, but a potential candidate is material liberated from the syncytiotrophoblast (Redman and Sargent 2005). The trigger for excessive syncytiotrophoblast release and suggested premature aging of the syncytiotrophoblast in pre-eclampsia is unknown, although early and exaggerated cytotrophoblast cell turnover is implicated (Huppertz and Kingdom 2004).

In addition to mitosis, apoptosis is essential for appropriate cell turnover. In the human placenta, apoptosis increases with advancing gestation (Mayhew et al. 1999; Smith and Baker 1999; Athapathu et al. 2003) whilst proliferation declines (Mayhew et al. 1999). Alterations in apoptosis are associated with complications and failures of human pregnancy, such as pre-eclampsia (Allaire et al. 2000; Leung et al. 2001; Ishihara et al. 2002). Throughout normal placental development mononucleate cytotrophoblasts are in equilibrium with the overlying syncytiotrophoblast. Recruitment of cytotrophoblasts to the syncytium occurs via differentiation and fusion and is offset by apoptosis and subsequent egress of syncytial fragments into the maternal circulation (Huppertz et al. 1999). In pre-eclampsia the equilibrium and renewal of syncytiotrophoblast is disrupted, resulting in a decrease in syncytiotrophoblast area (Crocker et al. 2004a; Huppertz and Kingdom 2004). Apoptosis is enhanced in pre-eclampsia and both villous tissue fragments and extracted cytotrophoblast cells show signs of increased apoptotic

susceptibility to intrinsic and extrinsic factors, such as tumour necrosis factor alpha (TNF $\alpha$ ) and hypoxia (Allaire et al. 2000; Ishihara et al. 2002; Crocker et al. 2004b). The increased presence of aggregates of syncytial nuclei in terminal villi from pregnancies complicated by pre-eclampsia have been noted in for many years, and have been eponymously termed 'Tenney-Parker' changes (Tenney and Parker 1940); closer inspection of these structures identifies nuclei with features of advanced apoptosis including a high euchromatin:heterochromatin ratio and the presence of annulate lamellae (Jones and Fox 1977; Mayhew et al. 1999).

The intracellular mechanisms leading to the increased apoptosis observed within villous trophoblast in pre-eclampsia are unknown. In other cell types this process may be activated by extrinsic stimuli such as receptor-ligand interactions, or by intrinsic cell damage, typically oxidative stress, hypoxia and UV radiation. In pre-eclampsia, the increased apoptosis is thought to result from cell damage rather than a response to an extrinsic signal. This hypothesis is based on two important observations in pregnancies complicated by pre-eclampsia. The first is the reduced conversion of maternal 'spiral' arteries within the decidua and myometrium which prevents the formation of flaccid conduits facilitating the delivery of large volumes of maternal blood to the placenta and fetus, potentially exposing the placenta to periods of ischaemia (Meekins et al. 1994). The second observation is that the placenta shows features of damage in pregnancies complicated by pre-eclampsia including areas of infarction and fibrin deposition (Benirschke and Kaufmann 2005).

If the increased apoptosis described in placentas from pregnancies complicated by pre-eclampsia occurs in response to cell damage, it would be expected to occur via activation of the signalling proteins in the intrinsic apoptotic pathway. Preliminary data supporting this hypothesis is equivocal, studies reporting semi-quantitative assessment of immunohistochemical staining of sections from pre-eclamptic placentas have described no change in either p53 (Jeschke et al. 2006) or Bax (Allaire et al. 2000) and either a decrease or no change in the expression of Bcl-2 (Allaire et al. 2000; Ishihara et al. 2002). In contrast, pre-eclampsia is associated with an increase in the presence and activity of caspase-3, 6 and 8 in placental tissue lysate, indicating that there is activation of the intrinsic apoptotic pathway in the placental villus (Crocker et al. 2005). If this hypothesis is correct, then isolated elevation of

a single protein would be unexpected, instead, a continuum culminating in activation of caspase enzymes would be consistent with activation of the intrinsic apoptotic pathway.

The constituents of the intrinsic apoptotic pathway, specifically: p53, p21, Bax, Bak, Bcl-2, IAPs, caspases 3,6 and 9 have been described in human term villous trophoblast (see Section 1.5.3 and Appendix A for references). In these experiments, we investigated whether increased apoptosis in pre-eclampsia was associated with altered expression of the constituents of the intrinsic apoptotic pathway. To achieve this we firstly considered the expression levels and localizations of constituents of the pathway including: p53, Mdm2, p21, Bax, Bak, Bcl-2, smac, HtrA2/omi, XIAP and survivin.

### **3.2 Hypothesis**

The increased apoptosis of villous trophoblast observed in pre-eclampsia is associated with an alteration in the expression of proteins in the intrinsic apoptotic pathway.

### **3.3 Methods**

#### **3.3.1 Tissue Procurement**

Ethical approval for this project was given by Central Manchester local research ethics committee and all participants provided written informed consent. Placentas were obtained from women with pre-eclampsia (n=6-8) and with uncomplicated pregnancies (n=6-8). Pre-eclampsia was defined as a blood pressure of >140/90 mmHg on two or more occasions after the 20th week of pregnancy in a previously normotensive woman in the presence of significant proteinuria (either >300 mg/L in a 24hour period or >2+ on a urine dipstick in the absence of urinary tract infection) (Davey and MacGillivray 1988). Women with essential hypertension, renal disease, diabetes or had evidence of intra-uterine growth restriction were excluded.

#### **3.3.2 Protein Preparation and Immunoblotting**

Protein was extracted from placental samples as described in section 2.3.1, and protein content was determined using a standardised commercial assay (Biorad). Tissue lysate was subjected to SDS-PAGE, transferred to a PVDF membrane, blocked and exposed to antibodies to the protein of interest (section 2.7.3). Negative controls were achieved by omission of primary antibody. Resulting bands were visualised on photo-sensitive film

(Amersham Biosciences Ltd, Chalfont St. Giles, UK) using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). Densitometry was performed on the Bio-Rad 700 system and bands of interest standardized against constitutively expressed proteins ( $\beta$ -actin, Myosin Light Chain). The choice of  $\beta$ -actin or Myosin Light Chain was dependent on the predicted molecular weight of the protein of interest.

### **3.3.3 Immunostaining**

Sections of placental villous tissue were washed and fixed for 24 hours in 4% formal saline (section 2.3.3.1). Tissue sections were deparaffinised and exposed to microwave pre-treatment as described in section 2.8.2. Endogenous tissue peroxidase activity was quenched and non-specific binding was blocked by exposure to 10% (v/v) non-immune serum as described. Tissue sections were exposed to antibodies against the protein of interest then probed with biotin conjugated goat anti-mouse or anti-rabbit antibodies followed by incubation with avidin-peroxidase. Immunostaining was revealed by exposure to concentrated 3,3-DAB for 3 minutes. Negative control sections were exposed to isotype specific mouse immunoglobulins or blocking solution alone if rabbit antibodies were used. Slides were counterstained with methyl green or Harris' Haematoxylin and sections viewed using a Leitz microscope with ImageProPlus 3.0 imaging software (Media Cybernetics Inc). To determine protein localisation, 10 randomly selected areas of each tissue sample were inspected by 2 observers and a qualitative assessment was made.

### **3.3.4 Quantitative PCR**

Total RNA was extracted from fresh placental tissue as described in section 2.9.1. cDNA synthesis was performed using a reverse transcriptase reaction kit (section 2.9.2). mRNA expression was quantified with specific primers for p53, Mdm2, Bax and p21 using SYBR Green I as detailed in section 2.9.4. Due to the wide range of expression of housekeeping genes in whole placental tissue Ct values were used to calculate a standard curve constructed from the cDNA of the human reference RNA and samples normalized to the calibrator sample as previously described (Lacey et al. 2005). All assays were between 93 and 105% efficiency.

### **3.3.5 Statistical Analysis**

Statistical significance was tested using the Mann-Whitney U-Test for non-parametric data and results are presented as median and interquartile range. Graphical data is presented as box and whisker plots, with the boxes representing the interquartile range and whiskers the whole data range. p-values of less than 0.05 were considered to be statistically significant.

## **3.4 Results**

### **3.4.1 Study Participants**

The median values and ranges of participant demographics are given in Table 3.1. No differences in maternal age, gravidity, parity and mode of delivery were identified between normal pregnancies and those with pre-eclampsia. Both systolic and diastolic blood pressures were significantly elevated at sampling in pre-eclampsia and a significant reduction in gestational age at delivery was noted. There was no difference in the mode of delivery between patients with normal pregnancies and those complicated by pre-eclampsia.



	Normal n=8	Pre-eclampsia (n=8)	Statistical Significance
Age	26 (20 – 35)	31 (25-38)	NS
BMI	25 (19-31)	27 (21-33)	NS
Gravidity	2 (1-3)	2 (1-4)	NS
Parity	1 (0-2)	1 (0-2)	NS
MABP in First Trimester (mmHg)	82 (73-87)	88 (80-110)	NS
Maximum MABP (mmHg)	87 (81-95)	125 (113-141)	p < 0.01
Gestation at delivery (weeks)	39 <sup>+3</sup> (37 <sup>+0</sup> -.41 <sup>+5</sup> )	37 <sup>+0</sup> (28 <sup>+3</sup> -.41 <sup>+3</sup> )	NS
Individualised Birthweight Ratio	47 (17-74)	4 (2-46)	p<0.05
Mode of delivery	4 VD, 4 CS	4 VD, 4 CS	NS

**Table 3.1** - Demographic data for women with normal pregnancies and those with pregnancies complicated by pre-eclampsia. BMI = Body Mass Index. MABP = Mean Arterial Blood Pressure, mmHg = millimetres of mercury. VD = Vaginal Delivery, CS = Caesarean section.

### 3.4.2 Expression of p53 and Transcriptionally-Regulated Proteins of the Intrinsic Apoptotic Pathway

#### 3.4.2.1 p53

Western blotting for p53 demonstrated a single band at approximately 53kD corresponding to the expected molecular weight. The expression of p53 was significantly elevated in placentas from pregnancies complicated by pre-eclampsia (Figure 3.1A). This was not accompanied by a corresponding increase in p53 mRNA (Figure 3.1B); although a wide variation in p53 mRNA expression was evident in both normal and pre-eclamptic placental tissue. Differences in the localisation of p53 in the villous trophoblast of normal term and pre-eclamptic pregnancies were discernable, particularly in focal areas. In normal pregnancies, p53 was evident in

occasional cytotrophoblast and syncytiotrophoblast nuclei and very weakly expressed in cytotrophoblast and syncytiotrophoblast cytoplasm (Figure 3.1C). No increased staining was identified in syncytial knots. The exaggerated p53 in pre-eclampsia was localised to syncytiotrophoblast nuclei (Figure 3.1C). In addition, moderate staining was also noted in discrete areas of the syncytiotrophoblast cytoplasm.

#### **3.4.2.2 Mdm2**

There was a no statistically significant change in Mdm2 mRNA expression (Figure 3.2A). In contrast to the increase in p53, protein expression of the anti-apoptotic 90kD isoform of Mdm2 was significantly reduced in pre-eclampsia (Figure 3.2B). In normal pregnancies, Mdm2 was expressed throughout the syncytiotrophoblast cytoplasm and in the cytoplasm of some cytotrophoblasts (Figure 3.2C). On inspection of 10 areas of 8 tissue sections, Mdm2 immunostaining appeared to be decreased in the syncytiotrophoblast cytoplasm in samples from pregnancies complicated by pre-eclampsia compared to normal pregnancy (Figure 3.2C).

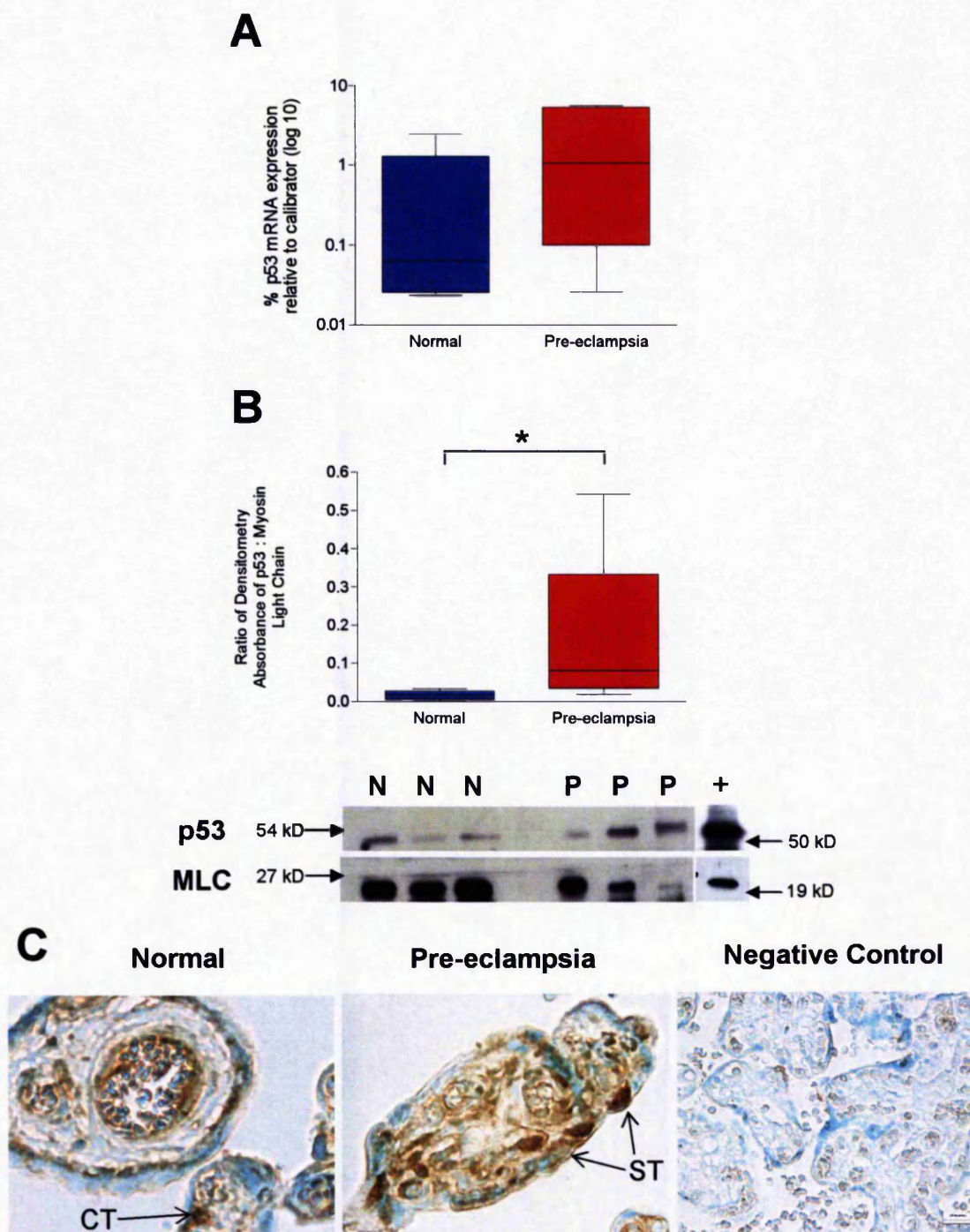
#### **3.4.2.3 p21**

The increased levels of p53 were associated with increased expression of downstream transcriptionally-regulated elements of the intrinsic apoptotic pathway. Western blotting for p21 protein demonstrated two bands, one at approximately 21kD and the other approximately 16kD. Both 21kD protein and mRNA levels were significantly increased in pre-eclampsia ( $p<0.05$ ) (Figure 3.3A and B). A 16kD band was present in 3 out of 6 samples taken from pregnancies complicated by pre-eclampsia (Figure 3.3B). In normal pregnancy, p21 expression was confined to occasional cytotrophoblast and syncytiotrophoblast nuclei, with no cytoplasmic expression in either cell type (Figure 3.3C). In pre-eclampsia, the increase in protein expression appeared to localise to syncytiotrophoblast nuclei (Figure 3.3C).

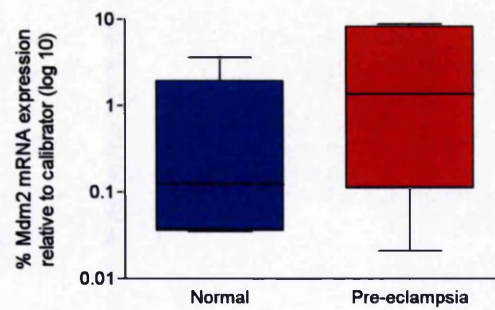
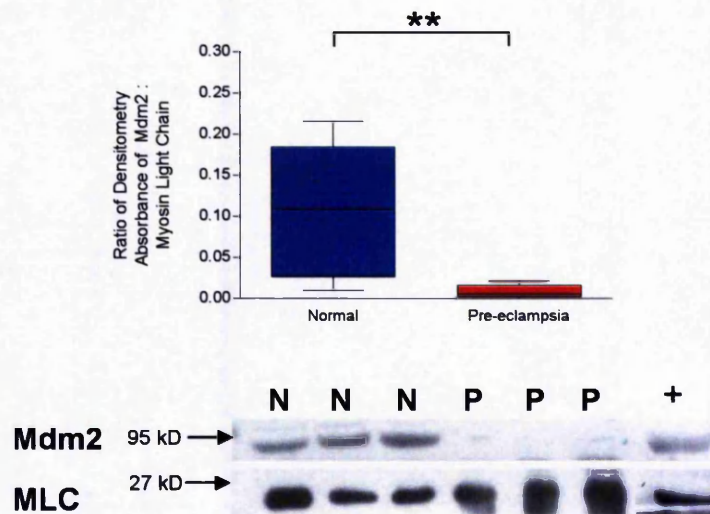
#### **3.4.2.4 Bax**

Although Bax mRNA was not significantly elevated (Figure 3.4A), Western blotting for Bax protein demonstrated a single band at approximately 24kD, which was significantly increased in the pre-eclamptic cases ( $p<0.05$ ) (Figure 3.4B). In a similar manner, Bax was moderately expressed throughout the syncytiotrophoblast, cytotrophoblast and occasional stromal



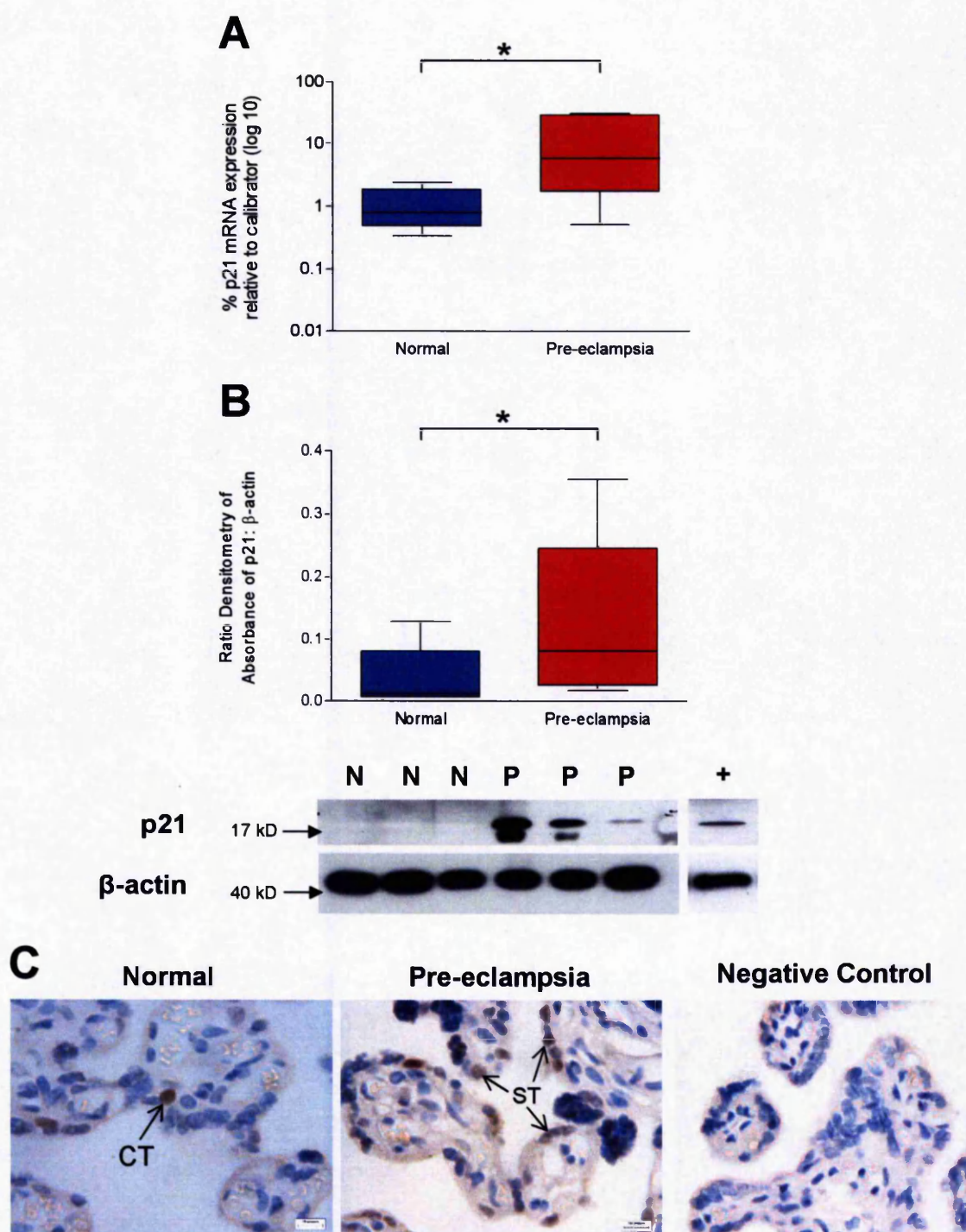


**Figure 3.1 – Expression and Localisation of p53.** (A) There was no significant difference in p53 mRNA expression between normal pregnancies and pre-eclampsia. (B) Western blotting of samples and positive control (A431 cell lysate) demonstrates a single band at 53kD. Densitometry standardised to myosin light chain (MLC) showed p53 protein expression was significantly increased in pregnancies complicated by pre-eclampsia (\* =  $p < 0.05$ , Mann-Whitney u-test,  $n=6$ ). (C) Representative images of immunoperoxidase staining for p53. In normal villous tissue p53 was present in few cytotrophoblast (CT) or syncytiotrophoblast (ST) nuclei. In pre-eclampsia p53 expression was seen in ST nuclei and in discrete areas of ST cytoplasm, images counterstained with methyl green.

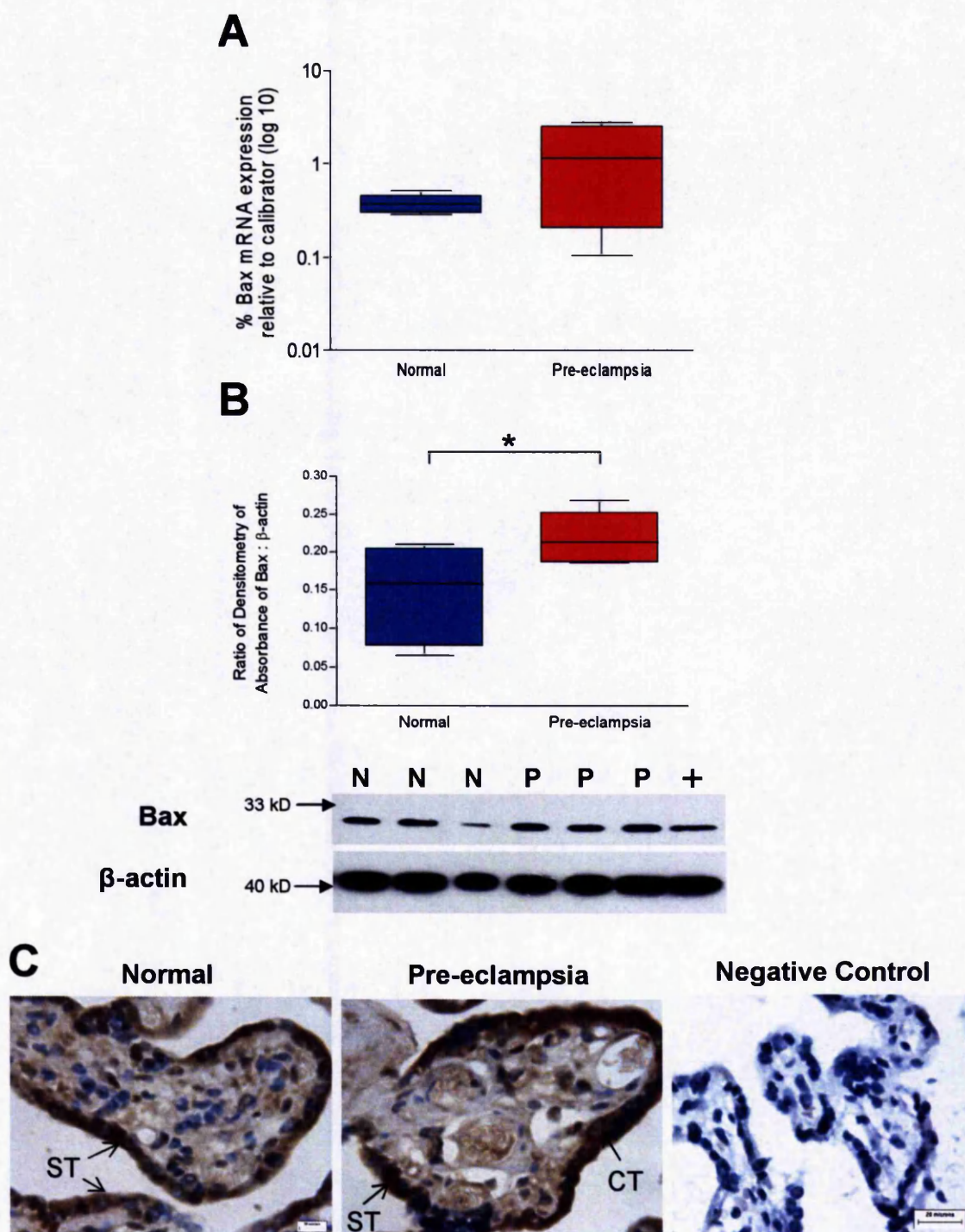
**A****B****C**

**Figure 3.2 – Expression and Localisation of Mdm2.** (A) There was no significant difference in Mdm2 mRNA expression between normal pregnancies and pre-eclampsia. (B) Samples and positive control (A549 cell lysate) show a band at 90kD. The protein expression of the 90kD Mdm2 protein was significantly decreased in pre-eclampsia (\*\*  $p < 0.01$ , Mann-Whitney u-test,  $n=6$ ). (C) Representative images of immunoperoxidase staining for Mdm2. In villous tissue Mdm2 was present in the cytoplasm of syncytiotrophoblast (ST) and more weakly in stromal cells.





**Figure 3.3 – Expression and Localisation of p21.** (A) p21 mRNA is increased in pre-eclampsia compared to normal pregnancies (\*  $p < 0.05$ , Mann-Whitney u-test,  $n=6$ ). (B) Samples and positive control (BeWo cell Lysate) show a band at 21kD. The protein expression of the 21kD p21 protein was significantly increased in pre-eclampsia (\*\*  $p < 0.01$ , Mann-Whitney u-test,  $n=6$ ). An additional band was present at 16kD in some samples from pre-eclampsia. (C) Representative images of immunoperoxidase staining for p21. (C) In normal villous tissue, p21 was present in occasional CT and ST nuclei. In PE, p21 predominantly localised to ST nuclei.



**Figure 3.4 – Expression and Localisation of Bax.** (A) There was no difference in Bax mRNA between normal pregnancies and pre-eclampsia. (B) Samples and positive control (Fibroblast Lysate) show a single band at approximately 30kD. The protein expression of the 30kD Bax protein was significantly increased in pre-eclampsia (\*  $p < 0.05$ , Mann-Whitney u-test,  $n=6$ ). (C) Representative images of immunoperoxidase staining for Bax. In villous tissue Bax was present in discrete areas of syncytiotrophoblast (ST) cytoplasm, cytotrophoblast (CT) cytoplasm and some stromal cells.

cytoplasm in normal placental villous tissue, but appeared to be enhanced in pre-eclampsia in both syncytiotrophoblast and cytotrophoblasts (Figure 3.4C).

### **3.4.3 Expression of Regulators of Mitochondrial Membrane Permeability**

#### **3.4.3.1 Bak**

The protein expression of regulators of mitochondrial membrane permeability which are not transcriptionally-regulated by p53 were further investigated using Western blotting for Bak, which demonstrated a single band at approximately 24kD, the predicted molecular weight of Bak (Figure 3.5A). In placentas from normal pregnancies and those with pre-eclampsia, Bak localised to the syncytiotrophoblast cytoplasm with staining also apparent in some stromal cells (Figure 3.5B).

#### **3.4.3.2 Bcl-2**

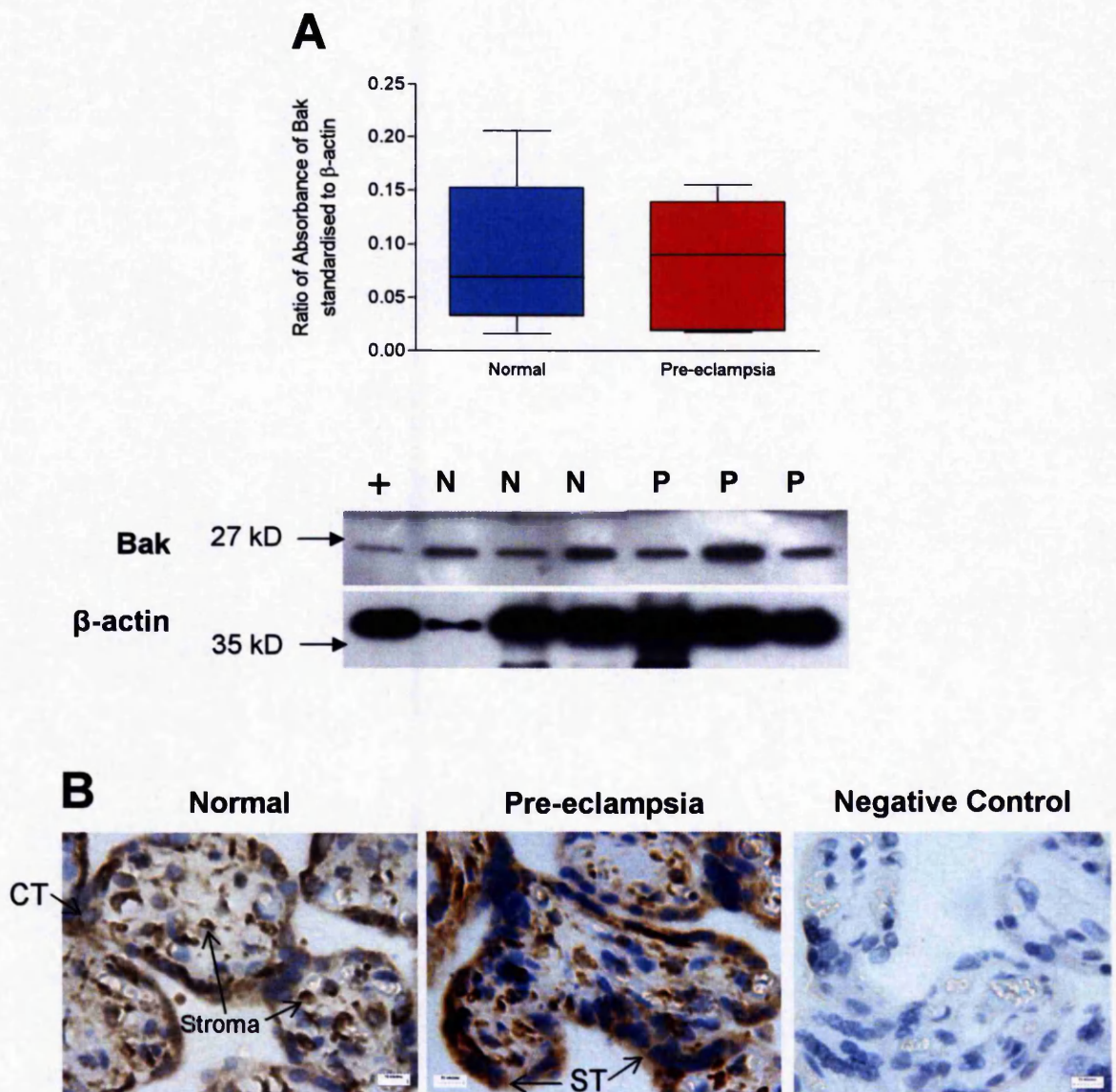
Western blotting for Bcl-2 showed a single band at approximately 27kD, which is consistent with the predicted molecular weight (Figure 3.6A). The protein expression of Bcl-2 was not altered in pregnancies complicated by pre-eclampsia. In cases from both normal and pre-eclampsia, Bcl-2 localised strongly to the cytoplasm of syncytiotrophoblast (Figure 3.6B). Bcl-2 was weakly expressed in the cytoplasm of cytotrophoblasts.

### **3.4.4 Expression of Regulators of Caspase Activity**

#### **3.4.4.1 Smac**

Western blotting for smac demonstrated a band at 27kD, corresponding to the expected molecular weight (Figure 3.7A); an additional band was identified at approximately 35kD in mouse heart lysate (positive control). In addition, an unidentified band was visible at approximately 50kD in some samples from pre-eclampsia which was not visible on the negative control lane. The expression of 27kD smac was significantly increased in pregnancies complicated by pre-eclampsia ( $p < 0.05$ ). In normal pregnancy, smac predominantly localized to the syncytiotrophoblast cytoplasm, with weak immunostaining in some cytotrophoblasts and endothelial cells. On inspection of 10 areas of 8 tissue sections, immunostaining of the

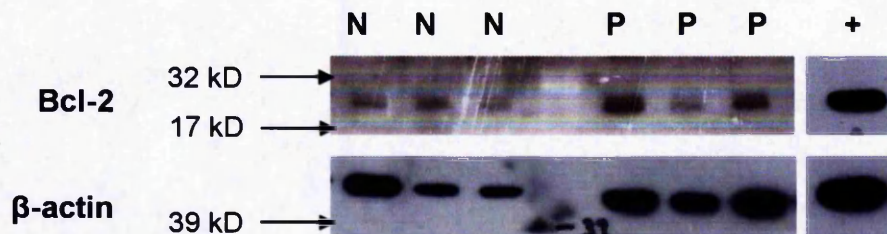
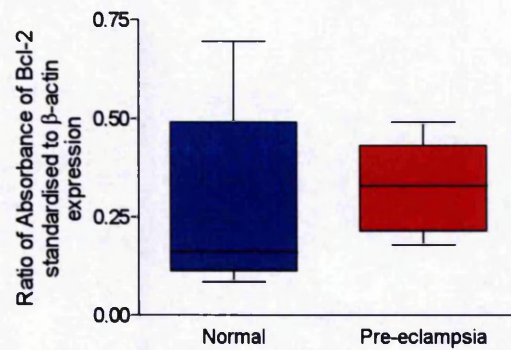




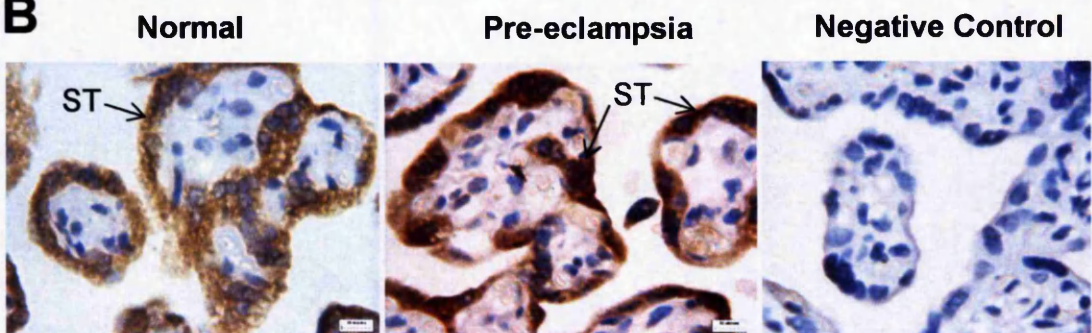
**Figure 3.5 – Expression and Localisation of Bak.** (A) Samples and positive control (Fibroblast Lysate) show a single band at approximately 24kD. The protein expression of Bak was not significantly different in pregnancies complicated by PE (n=6). (C) Representative images of immunoperoxidase staining for Bak. In normal villous tissue Bak was present in the syncytiotrophoblast (ST) cytoplasm, cytotrophoblast (CT) and stromal cytoplasm. In pre-eclampsia, Bak has a similar localisation to normal villous trophoblast.



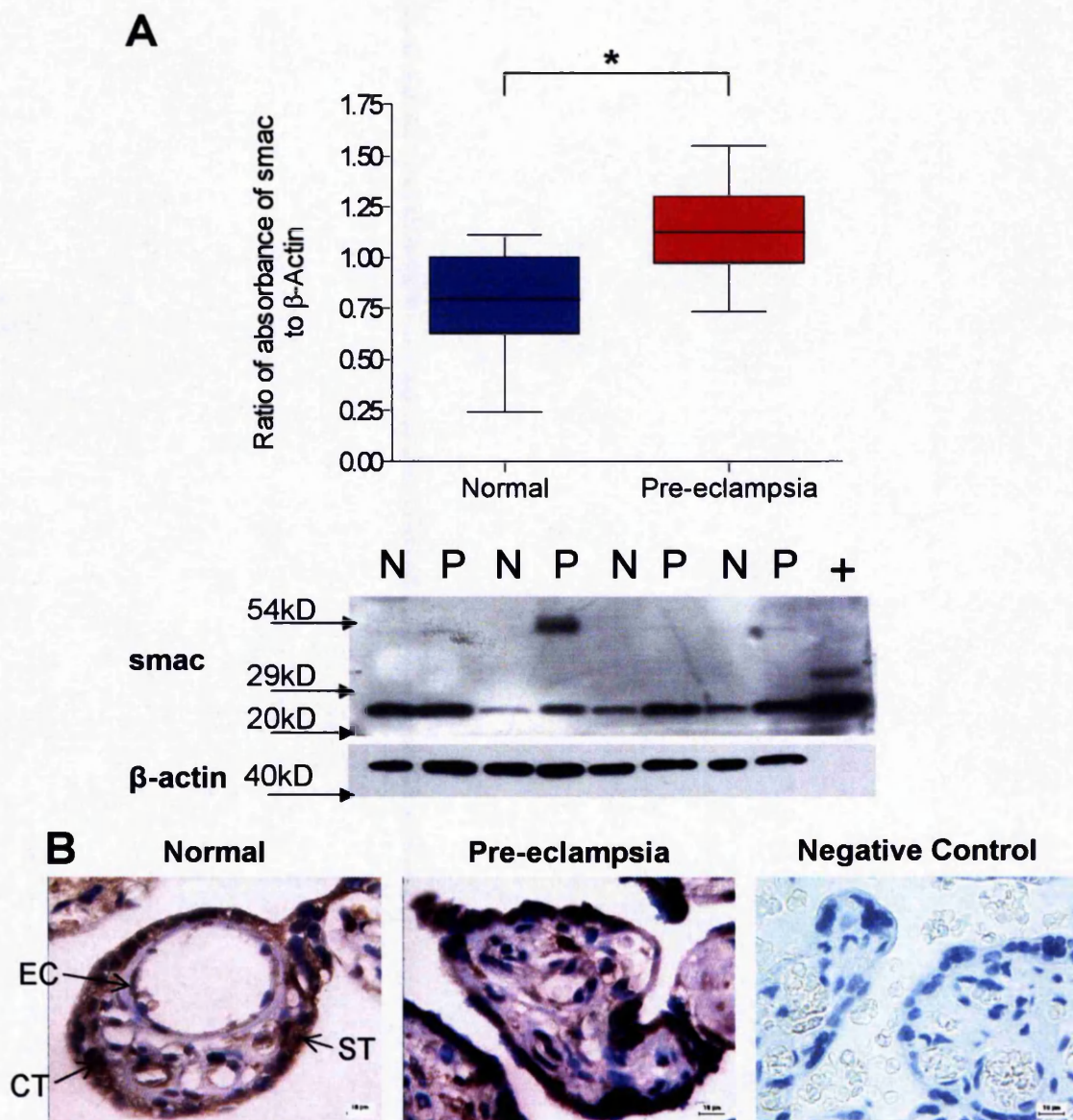
**A**



**B**



**Figure 3.6** – Expression and localisation of Bcl-2. (A) Samples and positive control (Fibroblast Lysate) show a single band at approximately 27kD. The protein expression of Bcl-2 was not significantly different in pregnancies complicated by pre-eclampsia (n=6). (C) Representative images of immunoperoxidase staining for Bcl-2. Immunostaining for Bcl-2 in normal villous tissue and pre-eclampsia demonstrating strong reactivity in the ST cytoplasm.



**Figure 3.7** – Protein expression and localisation of smac. (A) The protein expression of smac was significantly increased in pregnancies complicated by PE (\* $p < 0.05$ , Mann-Whitney u-test,  $n = 8$ ). Western blot showing the presence of two bands at approximately 27 and 35kD in the mouse heart positive control (+). There is no signal for  $\beta$ -actin in the positive control, as this specifically detects human  $\beta$ -actin. (B) Representative image of immunoperoxidase staining for smac. In normal villous tissue, smac was present in the syncytiotrophoblast (ST) cytoplasm, cytotrophoblast (CT) cytoplasm and endothelial cells (EC). In pre-eclampsia, smac has a similar localisation to normal villous trophoblast.

syncytiotrophoblast cytoplasm appeared denser in pregnancies affected by pre-eclampsia, and was also present in the region of syncytial knots (Figure 3.7B).

#### **3.4.4.2 HtrA2/Omi**

While a band of 35 kD corresponding to the expected molecular weight of HtrA2/omi was seen in the positive control tissue (mouse heart lysate) there was no detectable signal in the whole placental lysate, indicating that the presence of HtrA2/omi was beneath the level of detection despite loading 80µg of protein (Figure 3.8A). HtrA2/omi had similar cellular localization to smac, as weak immunostaining of the syncytiotrophoblast cytoplasm was identified in both normal and pre-eclamptic tissue (Figure 3.8B). On inspection of 10 areas of 8 placentas there did not appear to be any difference in the immunostaining between normal and pre-eclamptic tissue.

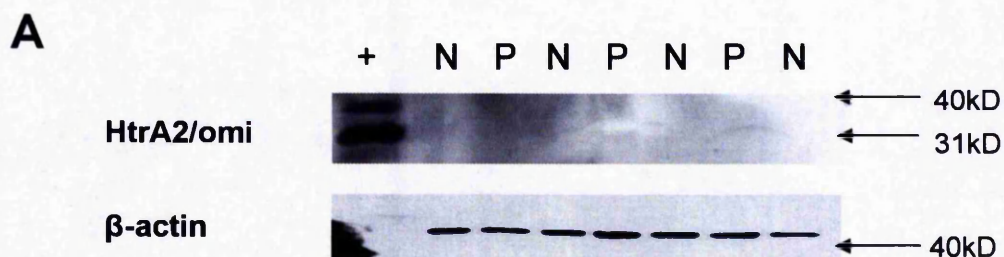
#### **3.4.4.3 XIAP**

Western blotting for XIAP demonstrated a single band at 57kD in the positive control (Jurkat cells); no reaction was evident in the negative control. In whole placental lysate, XIAP appeared as a doublet band. There was no significant difference in the expression of total XIAP (both bands) between placentas of normal pregnancies and those complicated by pre-eclampsia (Figure 3.9A). Similarly, XIAP localized to the syncytiotrophoblast cytoplasm in normal pregnancies and pre-eclampsia, with weak staining in some cytotrophoblast cytoplasm and no immunostaining in the endothelium (Figure 3.9B).

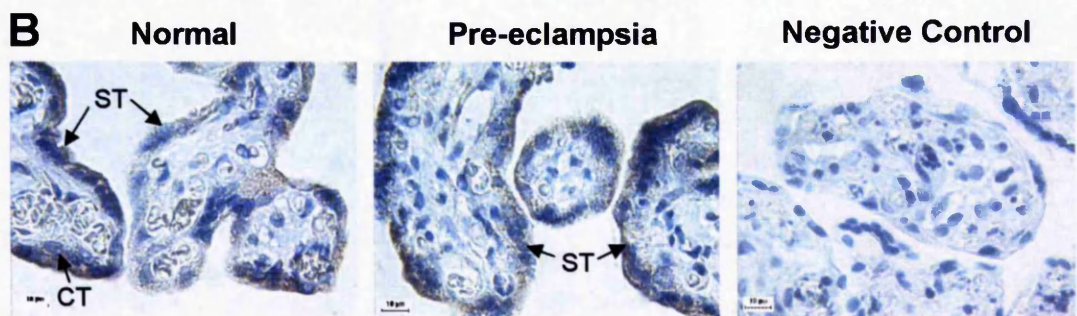
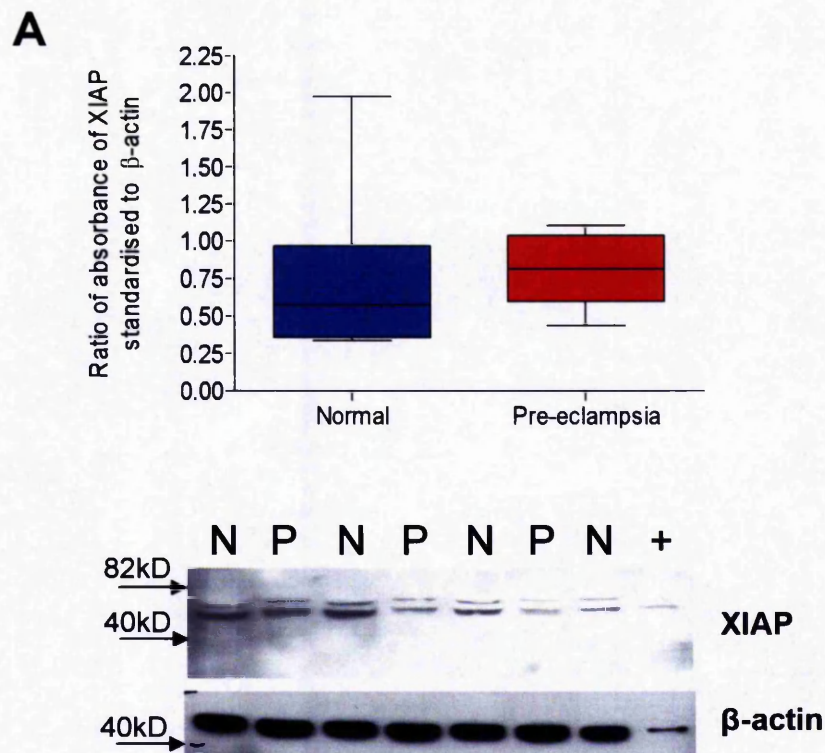
#### **3.4.4.4 Survivin**

Western blotting for survivin demonstrated a single band at approximately 17kD (Figure 3.10A). There was no difference in the expression of survivin between normal pregnancies and those complicated by pre-eclampsia. In contrast to XIAP, survivin localised to the syncytiotrophoblast, cytotrophoblast, occasional stromal cells and the endothelium (Figure 3.10B). In placental villi, survivin was rarely expressed in nuclei, although staining was sometimes evident in adjacent nuclei. On inspection, no alteration in the localization of survivin between normal and pre-eclamptic tissue was identified (Figure 3.10B).





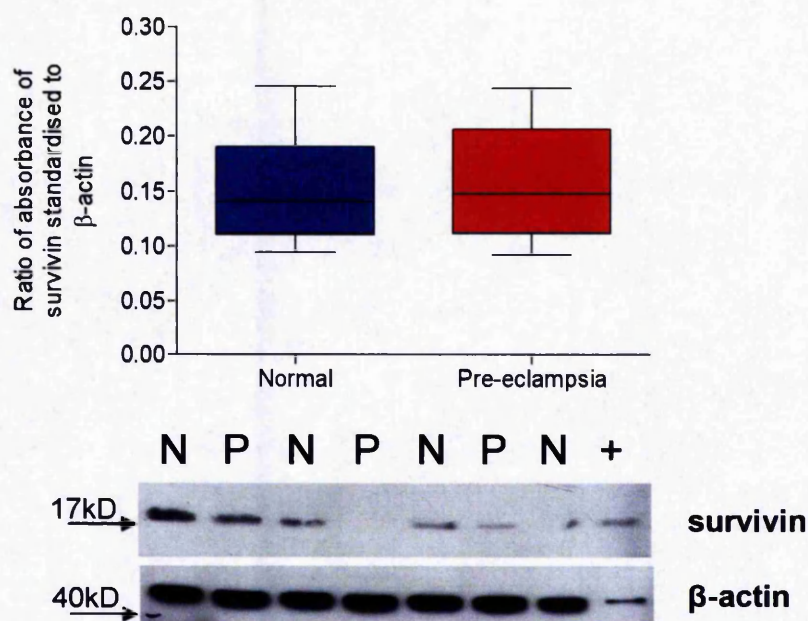
**Figure 3.8** – Protein expression and localisation of HtrA2/Omi. (A) Western blot showing the presence of two bands at approximately 31 and 38 kDa in the mouse heart positive control (+). There was no signal in whole placental lysate (80 μg/lane). There is no signal for β-actin in the positive control, as this specifically detects human β-actin. (B) Representative image of immunoperoxidase staining for HtrA2/Omi in normal villous tissue demonstrating weak reactivity in the syncytiotrophoblast (ST) cytoplasm which had similar localisation and magnitude of staining in pre-eclampsia.



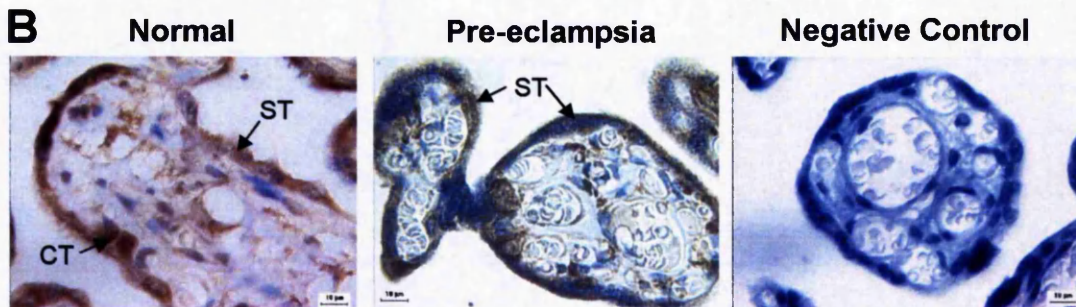
**Figure 3.9** – Protein expression and localisation of XIAP. (A) The protein expression of XIAP was not significantly different between normal pregnancies and those complicated by pre-eclampsia ( $n=8$ ). In Jurkat cells, XIAP appeared as a single band. In placental villous lysate, XIAP appeared as a doublet band at approximately 57kD. (B) Representative images of immunoperoxidase staining for XIAP. In normal villous tissue and PE, XIAP was present in the syncytiotrophoblast (ST) cytoplasm and more weakly in cytotrophoblast (CT) cytoplasm.



**A**



**B**



**Figure 3.10** – Protein expression and localisation of survivin. (A) In Jurkat cells and placental lysate survivin appeared as a single band at 17kD. The protein expression of survivin was not significantly different in placentas from normal pregnancies and those complicated by pre-eclampsia (n=8). (B) Representative images of immunoperoxidase staining for survivin. In normal villous tissue survivin was present in the syncytiotrophoblast (ST) and cytotrophoblast (CT) cytoplasm. Nuclear localization in two adjacent CT nuclei is shown. Survivin is also present in endothelial and some stromal cells.

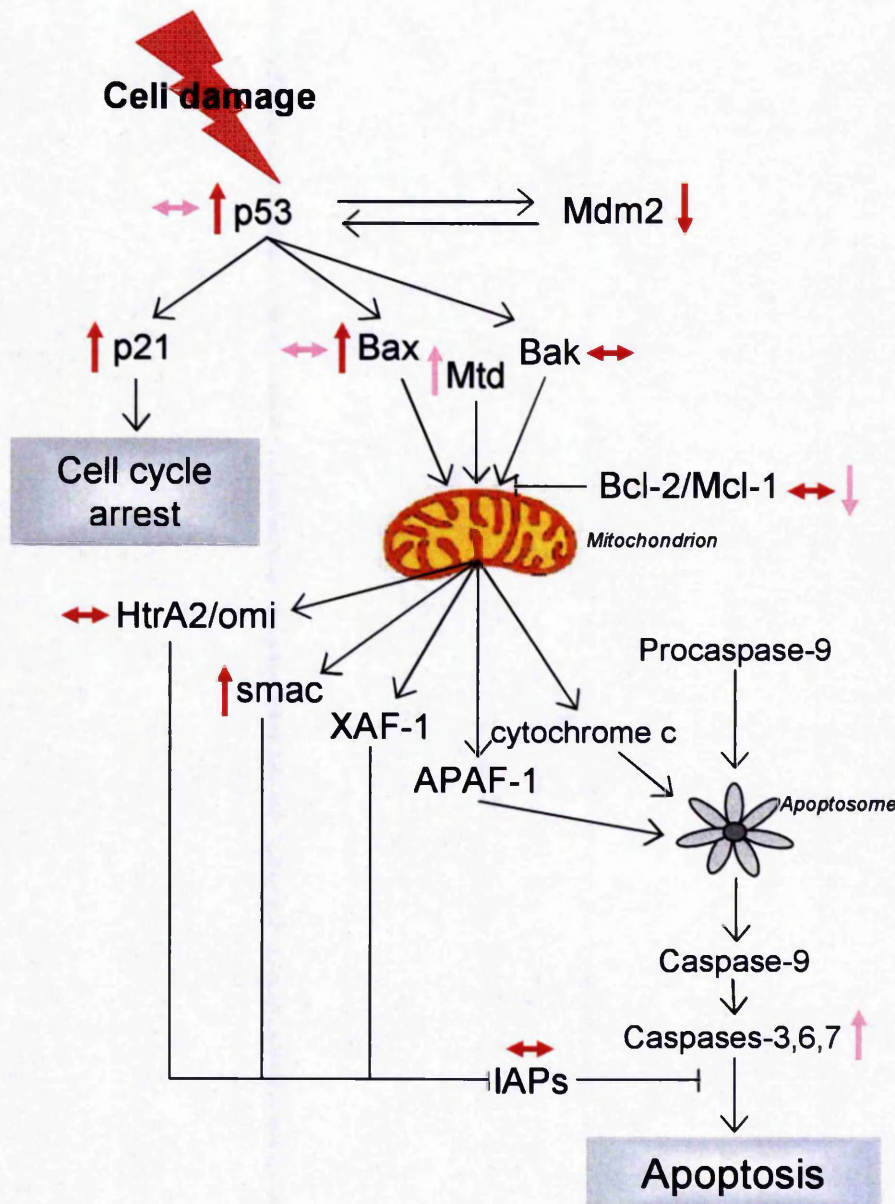
<b>Protein</b>	<b>Change in pre-eclampsia</b>	<b>Localisation</b>
Bak	Protein Unaltered	Normal and Pre-eclampsia - syncytiotrophoblast cytoplasm with some staining in stromal cells
Bax	mRNA unaltered Protein increased	Normal – cytoplasm of syncytiotrophoblast, cytotrophoblast and occasional stromal cells. Pre-eclampsia - staining appeared enhanced in syncytiotrophoblast and cytotrophoblasts
Bcl-2	Protein unaltered	Normal and Pre-eclampsia – strongly in syncytiotrophoblast cytoplasm and weakly in cytotrophoblast cytoplasm
HtrA2/Omi	Protein unaltered	Normal and Pre-eclampsia - weak immunostaining of syncytiotrophoblast cytoplasm
Mdm2	mRNA unaltered Protein decreased	Normal – widespread expression in cytoplasm of syncytiotrophoblast, cytotrophoblasts and some stromal cells. Pre-eclampsia - decreased in the syncytiotrophoblast cytoplasm.
p21	mRNA increased Protein increased	Normal – occasional cytotrophoblast and syncytiotrophoblast nuclei, with no cytoplasmic expression. Pre-eclampsia – increased expression in syncytiotrophoblast nuclei
p53	mRNA unaltered Protein increased	Normal – Occasional cytotrophoblast and syncytiotrophoblast nuclei. Pre-eclampsia – Staining in multiple syncytiotrophoblast nuclei and discrete areas of syncytiotrophoblast cytoplasm
smac	Protein increased	Normal - syncytiotrophoblast cytoplasm, with weak immunostaining in some cytotrophoblasts and endothelial cells. Pre-eclampsia - syncytiotrophoblast cytoplasm, no change in the degree of staining in cytotrophoblast and endothelial cells.
Survivin	Protein unaltered	Normal and Pre-eclampsia - syncytiotrophoblast, cytotrophoblast, occasional stromal cells and endothelium
XIAP	Protein unaltered	Normal and Pre-eclampsia – XIAP present in syncytiotrophoblast cytoplasm with weak staining in some cytotrophoblast cytoplasm

**Table 3.2** – Summary of expression of proteins of the intrinsic apoptotic pathway in placental tissue from normal pregnancies and those complicated by pre-eclampsia.

### 3.5 Discussion

These studies have demonstrated that proteins essential in the control of cell turnover are present in the placental villous trophoblast (Summarised in Table 3.2). These investigations also highlighted changes in some of these proteins in villous trophoblast from pregnancies complicated by pre-eclampsia (Figure 3.11). Our findings are in agreement with the majority of descriptions of localisation and expression of these proteins in normal term villous tissue already published; p53 is expressed in few cytotrophoblast nuclei and rarely in syncytiotrophoblast (Haidacher et al. 1995; Marzusch et al. 1995). There have been no studies of Mdm2 localisation in term villous trophoblast, but the presence of Mdm2 in the cytoplasm of syncytiotrophoblast and cytotrophoblast differed from descriptions in choriocarcinoma, in which Mdm2 localised predominantly to the cytotrophoblast nuclei (Cheung et al. 1999). However, both the 75kD and 90kD isoforms of Mdm2 have been identified in isolated trophoblast from term placentas by Western blotting (Hu et al. 2006a). In normal villous trophoblast, the expression of p21 was confined to few cytotrophoblast nuclei, consistent with previous reports of localisation and a decrease in p21 expression from the first to the third trimester of pregnancy (Quenby et al. 1998; De Falco et al. 2007). The localisation of pro-apoptotic members of the Bcl-2 family, Bax and Bak to the syncytiotrophoblast cytoplasm was more widespread in this study than previously reported, as Bax and Bak were previously described in discrete regions of the syncytiotrophoblast, especially in the region of syncytial knots or fibrin deposition (Ratts et al. 2000; Yamada et al. 2001). However, our findings are in agreement with another description of Bax being more widespread in the trophoblast (De Falco et al. 2001). In contrast, the localisation of Bcl-2 in these studies is concordant with the almost universal agreement that Bcl-2 is expressed throughout the syncytiotrophoblast cytoplasm in term villous tissue (Axt-Flidner et al. 2001; De Falco et al. 2001; Yamada et al. 2001; Danihel et al. 2002; Ishihara et al. 2002; Levy et al. 2002); although some reports have suggested that Bcl-2 is reduced in the region of syncytial knots (Huppertz et al. 1998; Sgarbosa et al. 2006). In agreement with previous data, we localised the expression of XIAP to the syncytiotrophoblast cytoplasm in our study (Gruslin et al. 2001; Ka and Hunt 2003). Although we were unable to detect the 30kD isoform identified previously (Straszewski-Chavez et al. 2007). The distribution of survivin was more widespread than that of XIAP, consistent with previous reports, being





**Figure 3.11** – Schematic representation of altered expression of proteins in the intrinsic apoptotic pathway in villous trophoblast of pregnancies complicated by pre-eclampsia. Alterations in expression described in this study are indicated by red arrows, and by other studies are shown by pink arrows (↑ = increased, ↓ = decreased, ↔ = unchanged). In pre-eclampsia the negative feedback loop between p53 and Mdm2 is lost due to a reduction in Mdm2 allowing p53 to promote the transcription of p21 and Bax. Bax and Mtd act on the mitochondrial membrane to increase permeability; these effects are antagonised by Bcl-2. The increased mitochondrial membrane permeability is associated with increased presence of smac in the cytoplasm, where it can regulate the IAPs such as XIAP and survivin. Inhibition of the actions of IAPs will further promote the activity of effector caspases.

noted in some cytotrophoblast nuclei and cytoplasm (Ka and Hunt 2003; Shiozaki et al. 2003). We also detected survivin in stromal and endothelial cells. To date there have been no reports of the expression of smac or HtrA2/omi in normal villous trophoblast to enable comparison of our data, although XAF-1, another protein of mitochondrial origin has been described in syncytiotrophoblast cytoplasm (Straszewski-Chavez et al. 2007). Several important trends in protein expression in the normal placental villus can be inferred from our data combined with data from previous studies. Firstly, the majority of proteins responsible for the regulation of apoptosis were detected within the trophoblast; with lower levels of expression in the villous stroma or endothelium. This may reflect greater rates of cell turnover, proliferation, cell division and apoptosis in the trophoblast compartments compared to the mesenchymal core. Secondly, under normal circumstances the syncytiotrophoblast is well protected against unwanted apoptosis, expressing Mdm2, Bcl-2, Mcl-1, XIAP and survivin (Huppertz et al. 1998; Ka and Hunt 2003) antagonizing the effects of p53, Bax, Bak and caspase-3 respectively.

This investigation of the expression of several components of the intrinsic apoptotic pathway in villous trophoblast in pregnancies complicated by pre-eclampsia is consistent with the hypothesis that there is activation of the intrinsic pathway of apoptosis from p53 downstream to the activation of caspase-3 (Crocker et al. 2005) (Figure 3.11). These changes predominantly localise to the syncytiotrophoblast which coincides with previous observations of increased apoptosis localizing to the syncytiotrophoblast and reduced syncytiotrophoblast cover in these placentas (Ishihara et al. 2002; Crocker et al. 2004a). Furthermore, our data suggest that the increase in p53 protein expression is associated with expected downstream changes in mRNA and protein expression, such as that of p21 and Bax. The expression of oncoproteins such as Bak and Bcl-2, the transcription of which is not promoted by p53, were not altered in pre-eclampsia, which provides further evidence that the downstream effects of p53 are similar to those described in other cell types. An increase in the ratio of Bax:Bcl-2 would be expected to increase mitochondrial membrane permeability, promoting the release of proteins such as smac and HtrA2/omi into the cytoplasm. In the case of villous trophoblast, the expression of smac is increased in pre-eclampsia, which can potentiate the actions of caspase-3, even in the presence of normal levels of XIAP (Verhagen et al. 2000).

There have been few other reports of differences in the expression of proteins involved in the intrinsic apoptotic pathway in pre-eclampsia (shown in Table 3.3). As highlighted previously,

three studies have used semi-quantitative analysis of immunohistochemical staining, to describe p53, Bax and Bcl-2 expression in villous trophoblast; these studies described no difference in the expression of p53, Bax or Bcl-2 (Allaire et al. 2000; Jeschke et al. 2006). In the only semi-quantitative study to identify a difference between normal pregnancy and pre-eclampsia, Ishihara et al. described a reduction in Bcl-2 in cases of severe disease (Ishihara et al. 2002). In contrast, the expression of Mtd (also known as Bok), a pro-apoptotic member of the Bcl-2 family is increased in pregnancies complicated by pre-eclampsia and when over-expressed in trophoblast increases apoptosis (Soleymanlou et al. 2005b). In contrast to reports of increased expression of proteins favouring apoptosis, a single report identified a reduction in pro-apoptotic Nix and BNip3 in pregnancies complicated by pre-eclampsia or IUGR (Stepan et al. 2005).

<b>Bak</b>	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	Western Blotting	Present in villous tissue, not increased in IUGR.
<b>Bax</b>	Term villous tissue from normal and PE pregnancies	Allaire et al. 2000	Immunohistochemistry	Localisation not stated, no difference in H-score. between normal and PE
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	Western Blotting	Present in villous tissue, not increased in IUGR.
	Term villous tissue from normal and IUGR pregnancies	Endo et al. 2005	Immunohistochemistry	Expressed in some areas of trophoblast Not altered in IUGR
<b>Bcl-2</b>	Term villous tissue from normal and PE pregnancies	Allaire et al. 2000	Immunohistochemistry	Localisation not stated, no difference in H-score. between normal and PE
	Villous tissue from normal term placenta and PE/IUGR	Isihara et al. 2002	Immunohistochemistry	Present throughout ST. Reduced in severe PE/IUGR.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	Western Blotting	Present in villous tissue, not increased in IUGR.
<b>BNip3</b>	Villous tissue from normal tissue, HELLP, PE, IUGR	Stepan et al. 2005	Immunohistochemistry	Strongly present in CT, weakly expressed in ST. Reduced in CT and ST in HELLP, PE and IUGR.
<b>Mtd</b>	Villous tissue from first trimester, normal term pregnancies and PE	Soleymanlou et al. 2003	Immunohistochemistry Western blotting mRNA	Protein expressed in ST cytoplasm. Increased in pre-eclampsia.
<b>Nix</b>	Villous tissue from normal tissue, HELLP, PE, IUGR	Stepan et al. 2005	Immunohistochemistry	Strongly present in CT, not detectable in ST. Reduced in CT in HELLP, PE and IUGR.
<b>p53</b>	Term villous tissue from normal, pre-eclampsia (PE), HELLP and IUGR pregnancies	Jeschke et al. 2006	Immunohistochemistry	Expressed in CTs in normal tissue. Increased in HELLP syndrome, unchanged in PE, and decreased in IUGR.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	Immunohistochemistry Western Blotting	Present in CT nuclei. p53 expression increased in IUGR pregnancies

**Table 3.3** – Proteins in the intrinsic apoptotic pathway reported in placentas from normal pregnancies and those complicated by pre-eclampsia, IUGR or HELLP syndrome.

The increase in p53 in villous trophoblast in cases of pre-eclampsia is supported by similar findings in extravillous trophoblast, suggesting that this invasive form of trophoblast may also up-regulate p53 and undergo apoptosis following exposure to damaging stimuli present in pre-eclampsia (DiFederico et al. 1999). In addition, p53 is also elevated in villous trophoblast of pregnancies complicated by IUGR, a condition related to pre-eclampsia, in which there is also increased trophoblast apoptosis (Smith et al. 1997a; Levy et al. 2002; Endo et al. 2005).

Likewise, p53 expression is increased in hydatidiform mole and choriocarcinoma which are both associated with increased trophoblast cell turnover and apoptosis (Fulop et al. 1998; Cheung et al. 1999; Wong et al. 1999; Chiu et al. 2001). Therefore, p53 expression appears to be increased in conditions associated with increased trophoblast apoptosis, suggesting a potential role for p53 in the regulation of this form of cell death or in forming a response to cell damage in trophoblast.

Undoubtedly, the complexity of p53 signalling raises several issues with regard to the control of trophoblast cell turnover. This is particularly evident in the syncytiotrophoblast, thought not to synthesize DNA or RNA; an idea derived originally by Hoshina et al. who demonstrated that hCG is transcribed in the cytotrophoblasts, but released by the syncytiotrophoblast (Hoshina et al. 1982), and extended by assessment of radiolabelled cytidine and uridine uptake in normal first trimester placental villous explants, showing intense staining only in cytotrophoblast, indicative of active nucleic acid synthesis (Huppertz et al. 1999). In pre-eclampsia, the increased expression of p53 was greatest in the syncytiotrophoblast, and the expected downstream transcription-dependent effects of p53, specifically the promotion of p21 and Bax were also observed in the syncytiotrophoblast. As the promotion of p21 and Bax requires mRNA transcription, this change would be expected to occur in cytotrophoblasts, at a time prior to the point of fusion. Alternatively, it may be hypothesised that this results from observations that transcription in the first trimester is in variance with that of the third, i.e. the syncytiotrophoblast can transcribe mRNA in the third trimester or that in pre-eclampsia the syncytiotrophoblast retains its capacity for transcription as a result of accelerated fusion events and the early incorporation of 'immature' cytotrophoblasts into the syncytiotrophoblast layer.

The hypothesis that an imbalance in the relationship between pro- and anti-apoptotic proteins may lead to activation of the intrinsic apoptotic pathway in pre-eclampsia, is supported by evidence that either an isolated increase in Mtd or a reduction of survivin is capable of increasing apoptosis in studies of trophoblast cell lines in vitro (Shiozaki et al. 2003; Soleymanlou et al. 2005b). At present there are no data relating to the role of p53 and Mdm2 in normal human trophoblast, therefore the consequences of an imbalance in these proteins in trophoblast is unknown. However, a reduction in mdm2 expression induces apoptosis in a p53-dependent manner in studies of mouse fibroblasts (de Rozières et al. 2000). Furthermore, a reduction in mdm2 is embryonic lethal in mice at day 5.5 post coitum, a phenotype rescued by

concomitant knockout of p53 (Jones et al. 1995), supporting a balance between p53 and mdm2 in the normal embryonic development in the mouse. A similar effect on embryonic viability is noted in survivin knockout mice (Wheatley and McNeish 2005), but not in XIAP or Bcl-2 knockouts (Harlin et al. 2001; Sorenson 2004), suggesting that the presence of some anti-apoptotic proteins are more essential to embryonic survival than others.

In the case of villous trophoblast in pre-eclampsia, these investigations identified an increase in the expression of pro-apoptotic p53 and decreased expression of anti-apoptotic Mdm2, leading to a number of downstream changes, including those observed in p21, Bax and smac. This therefore raises the hypothesis that the increased apoptosis observed in pre-eclampsia is associated with the imbalance between p53 and Mdm2. To investigate this hypothesis further, a study of the effects of such an imbalance in p53 and Mdm2 in villous trophoblast was undertaken.

## 4 – Is an Imbalance Between p53 and Mdm2 Capable of Inducing Apoptosis in Villous Trophoblast?

### 4.1 Introduction

Apoptosis may be increased following cell damage. A key component of this response is p53, which following exposure to noxious stimuli such as DNA-damage or oxidative stress, promotes the downstream transcription of elements involved in apoptosis and cell-cycle arrest, including p21 (el-Deiry et al. 1993; Gartel and Tyner 1999), APAF-1 (Kannan et al. 2001) and Bax (Miyashita and Reed 1995; Kannan et al. 2001). Under normal circumstances, p53 is present at low concentrations and levels are maintained within the cell by Mdm2 (Haupt et al. 1997) which functionally inhibits p53 activity, causes nuclear export and ultimately targets it for destruction by the proteasome (Meek 2004). Under normal circumstances the gene encoding Mdm2 is transcriptionally regulated by p53, providing an important feedback loop, preventing exposure of cells to excessive p53 thereby inducing apoptosis (de Rozieres et al. 2000). The balance between p53 and Mdm2 is essential for normal cell turnover and embryogenesis. Using a mutant mouse model Jones et al. engineered mice heterozygous for a functionally inactive mutant allele of *mdm2*; crossing these mice produced *mdm2* mutant/mutant (*mt/mt*) offspring. No viable homozygous *mdm2<sup>mt/mt</sup>* embryos were detected at day 7.5, 8.5 or 9.5 of gestation, and approximately 25% of embryos had reabsorbed by this stage (Jones et al. 1995). Embryos were then examined at day 6.5 of gestation, and those embryos found to be homozygous for *mdm2<sup>mt/mt</sup>* had fewer embryonic ectoderm cells, lacking in normal architecture and smaller in size than both wild-type homo- and heterozygote embryos. In-situ hybridization demonstrated that p53 and *mdm2* are expressed within the murine embryo at day 6.5, although the presence of *mdm2* precedes p53, as *mdm2* appears ubiquitously expressed from day 5.5 onwards. Subsequently, *mdm2<sup>mt/mt</sup>* mice were crossed with p53 null/- mice; *mdm2<sup>mt/mt</sup>* p53 -/- mice were viable. These studies concluded that absence of p53 rescues embryonic lethality of *mdm2* deficient mice (Jones et al. 1995).

These findings are augmented by studies of mouse embryonic fibroblasts from p53/- and *mdm2*/- mice. Transfection with p53 led to expression of p53 similar to wild-type cells. In cells without *mdm2* this led to apoptosis, whereas, in cells expressing wild-type *mdm2* there was no

increase in cell death (de Rozieres et al. 2000). These initial findings propagated further studies, most notably within cancer biology, in which the p53-pathway is often functionally inactive and cells overexpress Mdm2. Studies of cell-lines developed from malignant neoplasia retaining wild-type p53 demonstrate that a reduction in Mdm2 leads to increased apoptosis (Tortora et al. 2000; Zhang et al. 2004).

The apparent sensitisation of tumour cells to apoptosis has led to studies of Mdm2 inhibition as a potential agent to increase the efficacy of chemotherapy. However, recent data suggest that systemic inhibition of Mdm2 function induces apoptosis in many normal cell types. An elegant experiment using Mdm2<sup>-/-</sup> mice crossed with mice expressing a form of p53 only active in the presence of 4-hydroxytamoxifen demonstrated effects of unopposed p53 on cell-cycle arrest and apoptosis in bone marrow, thymus, spleen and small intestine (Ringshausen et al. 2006). These studies did not address any effects on reproductive organs or embryogenesis. When viewed collectively, these data suggest that the balance between p53 and Mdm2 is one of the key determinants of apoptosis or cell survival.

The data presented and literature reviewed in chapter 3 describes p53 in human and other animal placental villous trophoblast. Interestingly, p53 expression in human villous trophoblast is similar to findings in the rat and rhesus monkey, which identified p53 at the maternal-fetal interface (Yamauchi et al. 2004; Wei et al. 2005). As reviewed in chapter 3, p53 is mainly expressed in proliferating cell-types such as villous cytotrophoblasts and cell columns (Quenby et al. 1998). p53 is present in nuclear and cytoplasmic compartments and in the nucleus, p53 is a tetramer - its active DNA binding form (Cohen et al. 2007). Sequencing experiments demonstrated that p53 in trophoblast is wild-type (Cheung et al. 1994b; Shi et al. 1996), and in these experiments seems to elicit expected downstream effects such as increased expression of p21 and Bax. Aside from the data presented in chapter 3, Mdm2 has not been localised in third trimester villous trophoblast. Studies in first trimester tissue have described Mdm2 predominantly in cytotrophoblast nuclei. Interestingly, in common with other malignancies, both p53 and Mdm2 are increased in choriocarcinoma (Fulop et al. 1998; Cheung et al. 1999).

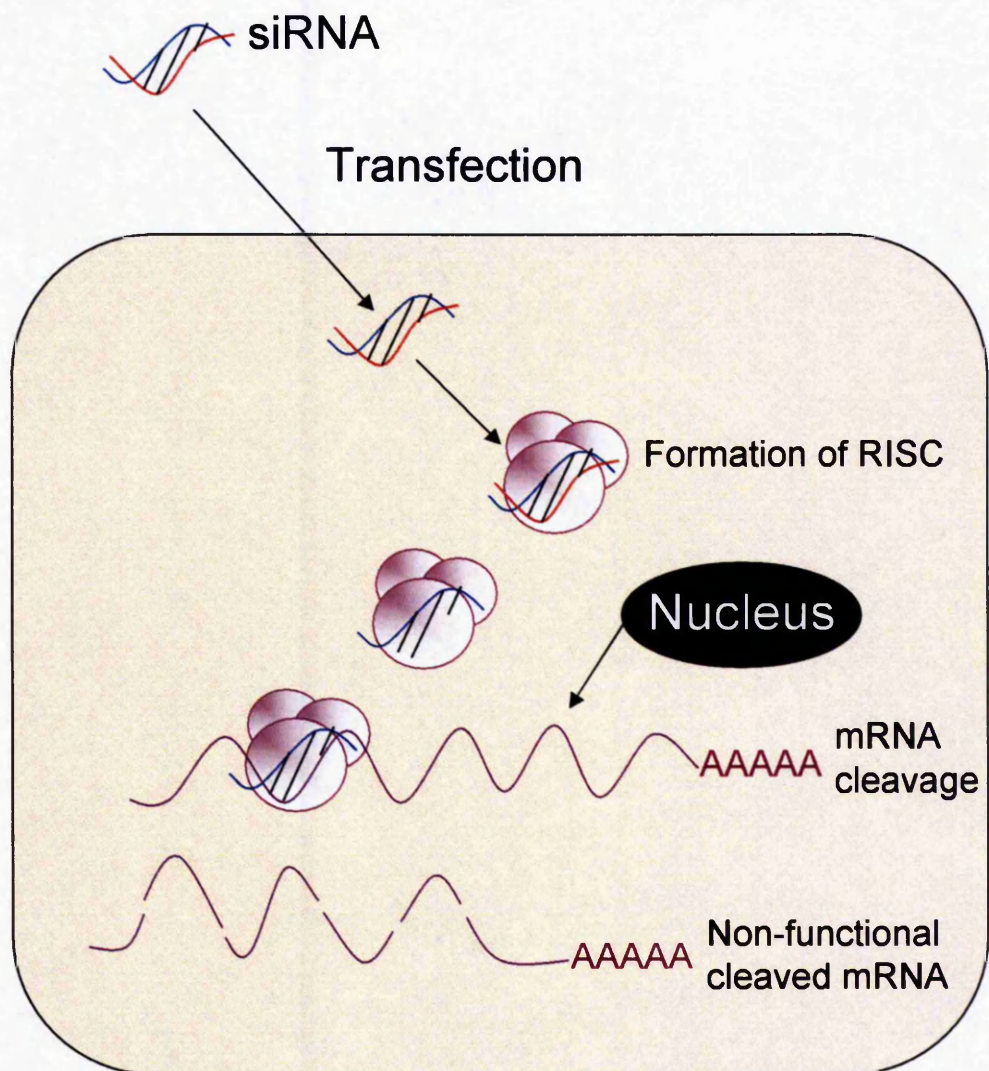
The conservation of p53 expression in the maternal-fetal interface of several species suggests that p53, and Mdm2 by association, may play an important regulatory role in the control of trophoblast cell turnover. Some authors have proposed that p53 may regulate proliferation, as it most strongly expressed in nuclei of proliferating trophoblast, and expression falls as



gestation progresses, in common with a similar reduction in proliferation. Other authors have proposed that in cytotrophoblast cell columns p53 may regulate invasion by enzymes such as matrix-metalloproteinases (MMPs) (Cohen et al. 2007). However, there are currently no data regarding the function of p53 and Mdm2 in trophoblast.

An imbalance between p53 and Mdm2 in association with increased apoptosis in villous trophoblast could provide a potential mechanism for the increased apoptosis observed in pre-eclampsia. This hypothesis is supported by data indicating an increase in p53-dependent apoptosis in the absence of mdm2 (de Rozieres et al. 2000). As the roles of p53 and Mdm2 are not fully understood, the possibility that an imbalance of these proteins could induce trophoblast apoptosis merits further investigation. Studies to assess the function of specific proteins in trophoblast have utilised anti-sense oligonucleotides or small-interfering RNA (siRNA) to specifically reduce the expression of specific proteins in trophoblast (Black et al. 2004).

RNA interference technology using oligonucleotides, morpholinos or siRNA allows investigation of the effects of individual proteins in individual cell types. Each utilises a slightly different approach to prevent the translation of protein from mRNA. siRNA was first described in 2001, and has proved a widely used and useful technique, resulting in the award of the Nobel Prize for Medicine to Andrew Fire and Craig Mello in 2006. siRNA uses 21-mer RNA duplexes homologous to the gene to be silenced (Elbashir et al. 2001; Timmons et al. 2003). Once taken up by cells, these duplexes then attach to a RNA-induced silencing complex (RISC) (Figure 4.1). The siRNA in the RISC complex unwinds, exposing the sequence which then binds to the mRNA of the gene to be knocked-down. This mRNA is then cleaved by the RISC, reducing mRNA from the gene of interest (Hammond et al. 2000). Although proteins of the RISC have not been specifically identified in trophoblast, their presence may be inferred from efficacy of other studies utilising siRNA in trophoblast (Kudo and Boyd 2004; Chen et al. 2006; Meade et al. 2007).



**Figure 4.1** – Schematic representation of the mechanism of action of short-interfering RNA (siRNA). Following introduction of siRNA into the cell by transfection the double stranded siRNA forms a complex with the RNA-induced silencing complex (RISC). This then becomes single-stranded RNA which binds to a complementary sequence on the mRNA to the protein of interest. The mRNA is then cleaved by enzymes contained within the RISC resulting in non-functional fragments of mRNA.

## **4.2 Hypothesis and Aim**

It was hypothesised that a reduction in Mdm2 in trophoblast would be associated with an increase in apoptosis which would be attenuated by contemporaneous knockdown of p53. In experiments to test this hypothesis siRNA methodology was utilised to reduce p53 and Mdm2 expression within a choriocarcinoma cell-line and normal term villous trophoblast. These experiments aimed to produce effective knockdown of p53 and Mdm2 and observe any related changes in cell turnover.

## **4.3 Methods**

These experiments were carried out at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto under the supervision of Professor John Kingdom, whose laboratory were one of the first groups to successfully describe the use of siRNA to knock-down transcription of a protein involved in regulation of cell turnover (Baczyk et al. 2006). The techniques described here make use of techniques optimized by Mrs Dora Baczyk, particularly the concentrations of Lipofectamine and siRNA used.

### **4.3.1 Tissue Procurement**

The research ethics board of Mount Sinai Hospital (Toronto, ON, Canada) gave approval for this study; all participants provided written informed consent. Placentas were obtained from women with uncomplicated pregnancies. Women with pre-existing medical conditions, gestational diabetes, pre-eclampsia or had evidence of intra-uterine growth restriction were excluded.

### **4.3.2 Culture and Transfection of BeWo Choriocarcinoma Cells**

BeWo cells were donated by Mrs Dora Baczyk (originally obtained from the American Tissue and Cell Collection - CCL-98). The BeWo choriocarcinoma cell line was chosen as these cells undergo proliferation and spontaneous fusion, mirroring normal cell turnover within the trophoblast. BeWo cells were maintained in DMEM with F12K (DMEM-F12K) containing 10% FCS, penicillin, streptomycin at 37°C in a 5% CO<sub>2</sub>/air atmosphere. BeWo cells were plated at 1x10<sup>6</sup> cells per ml and cultured for 24 hours to allow cell attachment, giving a confluence between 40-50%. The cells were then washed with Optimem medium (Invitrogen, CA) and

transfected with a mixture of Lipofectamine (Invitrogen) and siRNA for 5 hours. We used previously published siRNA oligonucleotides to p53 (Qiagen, Mississauga, ON) and custom-made sequences for Mdm2 (Qiagen) (Table 4.1) (Martinez et al. 2002; Uchida et al. 2005). Sequence homology was confirmed using BLAST search. We employed control experiments using Lipofectamine alone and a fluorescent labelled scrambled siRNA sequence (Qiagen) which was checked against BLAST searches to verify a lack of homology to other genes. After 5 hours transfection, the mixture was supplemented with DMEM-F12K with 20% FCS. The cells were then cultured for a further 48 hours in a 5% CO<sub>2</sub>/air atmosphere. After 48 hours culture images were taken using phase contrast microscopy and conditioned culture medium was collected from each well (section 2.5.1). Cells were then either lysed in ice-cold PBS (section 2.5.2) or in RLT buffer with 0.1%  $\beta$ -mercaptoethanol prior to RNA extraction (section 2.5.3) or fixed for immunocytochemistry (section 2.5.4) or flow cytometry (section 2.5.5).

Sequence	Sense	Antisense
p53	GCAUGAACCGGAGGCCCA	AUGGGCCUCCGGUUCAUG
Mdm2	AAGGAAUAAGCCCUGCCC	UGGGCAGGGCUUAUUCU
Scrambled	UUCUCCGAACGUGUCACG	ACGUGACACGUUCGGAGA

**Table 4.1** – siRNA sequences used to knockdown p53 and Mdm2, scrambled siRNA was used as a non-silencing control.

### 4.3.3 Culture and Transfection of Floating Villous Explants

Placental tissue for culture experiments using siRNA was obtained from uncomplicated term pregnancies (n=6). Three areas of each placenta were randomly sampled; villous tissue was dissected into single villous explants as described (section 2.2.3.2). These were mounted in polystyrene cubes and were allowed to float in phenol-red free DMEM supplemented with 1% (v/v) insulin, transferrin and selenium (ITS) at 37°C in 8% O<sub>2</sub>; CO<sub>2</sub> was maintained at 5%. After 24 hours the culture medium was changed and replaced with culture medium containing the relevant siRNA sequence. The explants were cultured for a further 48 hours, after which the villous tissue was collected for analysis of protein and RNA (sections 2.3.1-2.3.2) or fixed prior

to wax embedding or electron microscopy (section 2.3.3.1-2). Conditioned culture media was collected from each well (section 2.3.4).

#### **4.3.4 Protein Preparation and Western Blotting**

Protein content was determined using a standardised commercial assay (Biorad). 40µg of tissue lysate was subjected to 10% discontinuous SDS-PAGE, transferred to a PVDF membrane, blocked and then probed with antibodies to the protein of interest (section 2.7.3). Following exposure to a suitable secondary antibody conjugated to horseradish peroxidase, resulting bands were visualised on photo-sensitive film (Amersham Biosciences Ltd) using enhanced chemiluminescence reagents (Pierce). Densitometry was performed on the Bio-Rad 700 system and bands of interest standardized against constitutively expressed proteins ( $\beta$ -actin).

#### **4.3.5 Immunohistochemistry and Immunocytochemistry**

Tissue sections were deparaffinised and exposed to microwave pre-treatment as described in section 2.8.2. Endogenous peroxidase activity was quenched and non-specific binding was blocked as described (section 2.8.2). Tissue sections were exposed to antibodies against the protein of interest, then probed with biotin conjugated goat anti-mouse or anti-rabbit antibodies followed by incubation with avidin peroxidase. Each tissue was incubated with a matching concentration of either isotype specific non-immune mouse immunoglobulin or 10% NRS with 1% BSA (w/v) to serve as negative controls for mouse and rabbit primary antibodies respectively. Immunostaining was revealed by exposure to concentrated 3,3-DAB for 3 minutes. Slides were counterstained with Harris' Haematoxylin and sections viewed using a Leitz microscope with ImageProPlus 3.0 imaging software (Media Cybernetics Inc, Silver Spring, MD, USA). All samples for comparison were stained in the same batch.

For assessment of BeWo morphology cells adherent to cover slips were fixed as described in section 2.5.4. These were then washed and non-specific antibody binding was reduced by blocking the coverslips in 1% BSA with 0.05% Tween for 20 minutes at room temperature. The coverslips were then incubated with primary anti-cytokeratin 8 (Dako) antibody for 1 hour at room temperature. The coverslips were then washed PBS and then exposed to a fluorescein conjugated rabbit anti-mouse antibody at a dilution of 1:200 (Dako). A negative control was

achieved by the omission of primary antibody. The coverslips were washed and mounted in medium containing propidium iodide as described in section 2.8.3.

#### **4.3.6 Quantitative PCR**

Total RNA was extracted from placental villous explants and BeWo cell lysate as described in sections 2.3.2 and 2.5.3 respectively. cDNA was synthesised using a reverse transcriptase kit (section 2.9.2). mRNA expression was quantified using specific primers for p53, Mdm2, Bax, p21, TBP and SDHA using SYBR Green I as detailed in section 2.9.4. Due to the wide range of expression of housekeeping genes in whole placental tissue Ct values were used to calculate a standard curve constructed from the cDNA of the human reference RNA and samples normalized to the calibrator sample (Lacey et al. 2005). However, in BeWo cells expression was standardized to the geometric mean of expression of the housekeeping genes TBP and SDHA. All assays were between 93 and 105% efficiency.

#### **4.3.7 Assessment of Apoptosis**

##### **4.3.7.1 Assessment of Apoptosis in BeWo cells**

In BeWo cells apoptosis was assessed by flow cytometry. Cells were fixed and prepared for flow-cytometry as described in sections 2.5.5 and 2.10.5. The resulting suspensions of BeWo cells stained with PI ( $n=3$ ) were analysed by FACSCalibur (BD Biosciences) using absorbance at  $585\pm 21\text{nm}$ , cell debris was excluded by gating. Cell morphology was assessed, and apoptosis determined by the amount of PI per cell. Cells with less than diploid content were determined to be apoptotic. Likewise, the relationship between cell size and PI was used to determine whether cells were in G<sub>1</sub>, S or G<sub>2</sub> phase of the cell cycle.

##### **4.3.7.2 Assessment of Apoptosis and Syncytial Knots in Placental Villous Explants**

In tissue sections apoptosis was assessed using a commercially available TUNEL kit (Roche Applied Diagnostics) with adaptations to the manufacturers' instructions as described in section 2.10.1. TUNEL staining was visualised by light microscopy of 10 randomly selected fields of view for each experimental condition. The number of TUNEL positive nuclei was counted manually and total number of nuclei quantified using a sequential colour thresholding technique. A ratio was derived from these values; the TUNEL positive index. TUNEL staining

was confirmed by immunohistochemistry using a monoclonal mouse antibody against cytokeratin-18 M30 neopeptide as described in section 2.10.2.

For assessment of syncytial knots, 5µm tissue sections were stained with haematoxylin and eosin as described in section 2.11. The number of syncytial knots defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface not in direct contact with adjacent villi (Cantle et al. 1987) was counted manually in 10 fields of view for each experimental condition. The trophoblast area was measured by sequential colour thresholding (Image ProPlus, Mediacybernetics) and data normalised to give a measure of the number of syncytial knots per mm<sup>2</sup> of villus.

#### **4.3.8 Assessment of Cell Proliferation**

Cell proliferation within placental explants was assessed using Ki-67 (Mib-1) immunostaining as described in section 2.12.1. 10 areas of each section were assessed using light microscopy, Ki67 positive nuclei were counted manually and total number of nuclei assessed using a sequential colour thresholding technique, deriving a ratio of proliferative nuclei:total nuclei termed the proliferative index.

#### **4.3.9 Electron Microscopy**

Explants from each experimental condition were fixed and treated for electron microscopy as described in section 2.14. Ultra-thin sections were examined using a Philips CM10 electron microscope at an accelerating voltage of 80kV and representative areas photographed.

#### **4.3.10 Analysis of Collected Culture Medium**

The amount of cell necrosis was assessed by the presence of lactate dehydrogenase (LDH) in conditioned culture media using commercially available assay (Roche Applied Bioscience) (sections 2.13.1.1-2). Biochemical evidence of trophoblast differentiation was assessed by the measurement of human chorionic gonadotrophin (hCG) within conditioned culture medium using a commercially available radio-immunoassay in accordance with the manufacturers instructions (ICN Pharmaceuticals, Basingstoke, UK) (Section 2.13.2).

#### **4.3.11 Statistical Analysis**

Statistical significance was tested using the Friedman test for matched non-parametric data or Kruskal-Wallis test for non-matched non-parametric data. Results are presented as median and range and plotted as box and whisker plots (Box = interquartile range, Whiskers = total range). p-values of less than or equal to 0.05 were considered statistically significant.

### **4.4 Results**

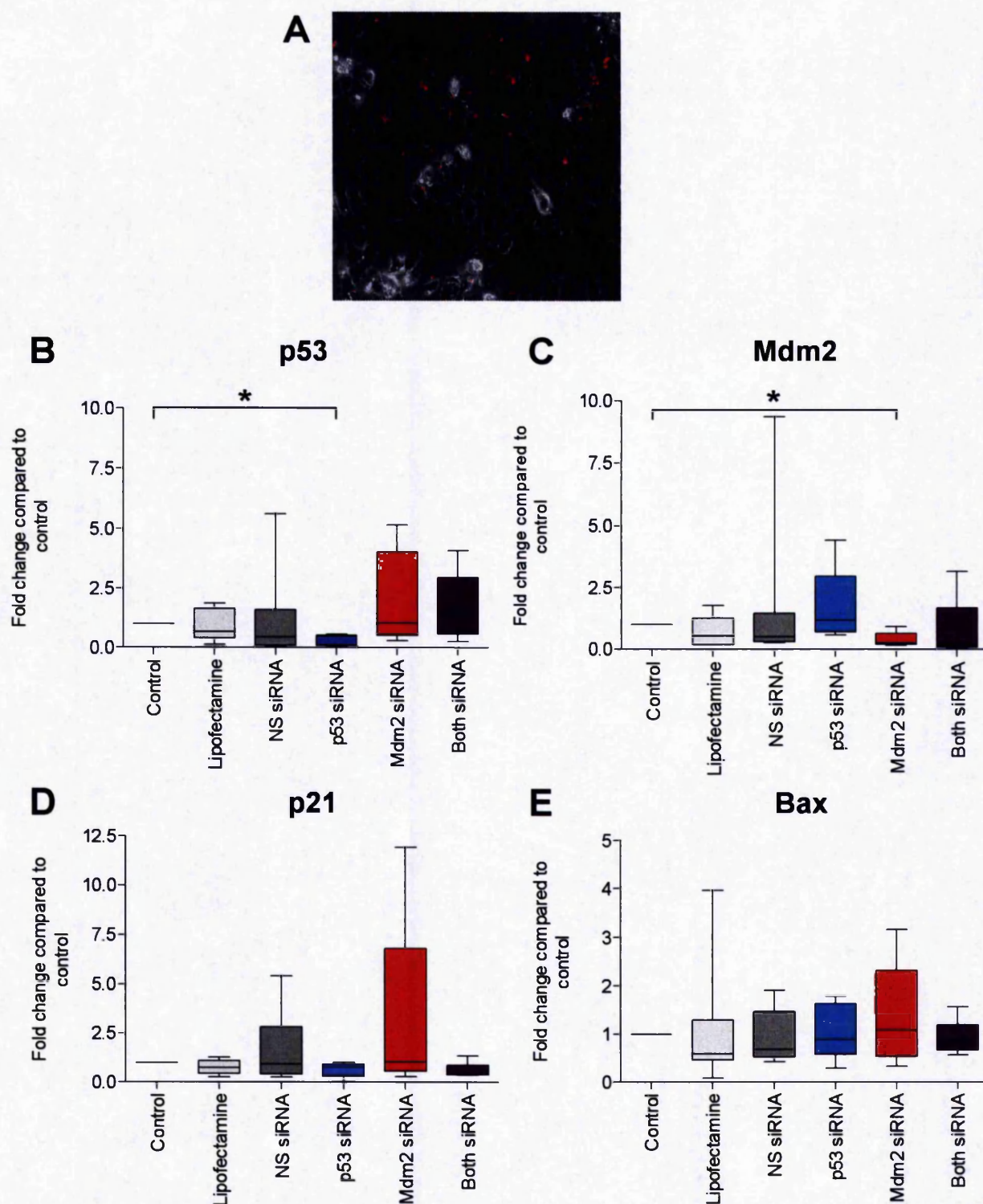
#### **4.4.1 Effects of BeWo Cell Transfection on p53, Mdm2, p21 and Bax mRNA expression**

Transfection of fluorescent labelled non-silencing siRNA indicated that approximately 50% of BeWo cells were successfully transfected with siRNA (Figure 4.2A). There was no significant effect of control conditions on the expression of p53, Mdm2, p21 or Bax mRNA (Figures 4.2B-E). BeWo cells exposed to p53 siRNA showed a median reduction of p53 siRNA of 54% compared to control (Figure 4.2B) and treatment with Mdm2 siRNA showed a 52% decrease in Mdm2 mRNA (Figure 4.2C). There was no significant difference in the expression of p21 or Bax mRNA following treatment with any siRNA sequence (Figure 4.2B-E). Western blotting was carried out on a single passage of cells to assess protein expression; this demonstrated that although there was a significant reduction in p53 mRNA following transfection with p53 siRNA, this was not associated with a decrease in p53 protein expression (Figure 4.3A). Mdm2 knockdown appeared to be associated with increased p53 protein expression.

#### **4.4.2 Effects of BeWo Cell Transfection on Cell Morphology**

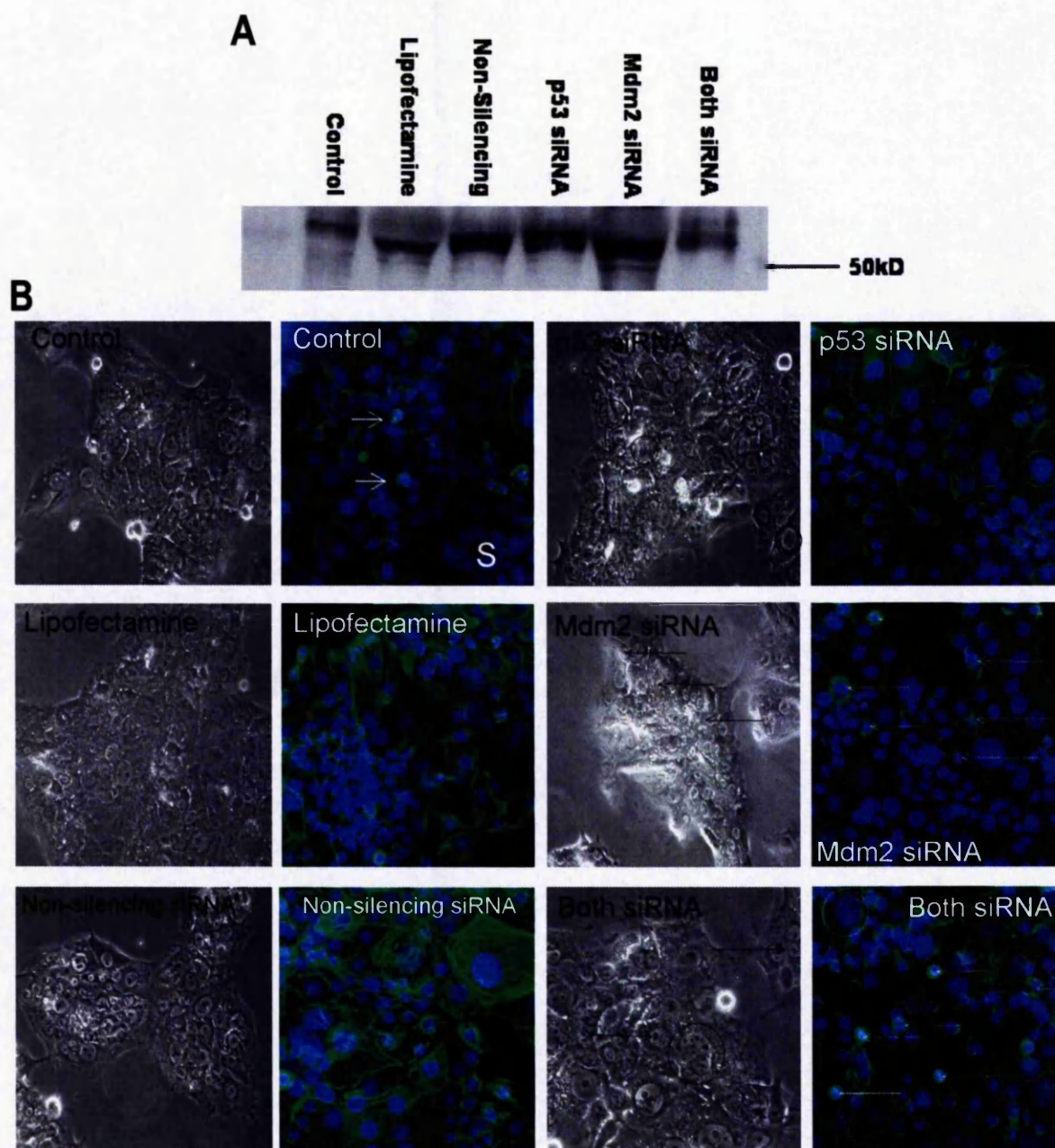
Inspection of images of BeWo cells by two independent observers blinded to culture conditions identified several changes in morphology in response to treatment with p53 and Mdm2 siRNA. In control conditions occasional pyknotic cells and some morphological evidence of cell fusion were seen (Figure 4.3B). BeWo cells exposed to p53 siRNA showed increased numbers of nuclei and nucleoli, without cell fusion. Some BeWo cells treated with Mdm2 siRNA exhibited morphological appearances of apoptosis, with increased nuclear:cytoplasmic ratio and pyknotic nuclei. Cells exposed to both p53 and Mdm2 siRNA showed mixed morphology, some cells had the appearances of apoptosis while a proportion were multinucleate with prominent nucleoli.





**Figure 4.2** – The effect of transfection of BeWo cells with p53 and Mdm2 siRNA (A) Fluorescent labelled siRNA was present in 50% of cells. (B) p53 mRNA expression was significantly reduced by treatment with p53 siRNA compared to control, \*  $p < 0.05$  Wilcoxon signed rank test. (C) Mdm2 mRNA was significantly reduced by treatment with Mdm2 siRNA compared to control. (D) p21 mRNA was not significantly altered by treatment with p53 or Mdm2 siRNA. (E) Bax mRNA was unaffected by exposure to p53 or Mdm2 siRNA. Lipofectamine or non-silencing siRNA (NS siRNA) did not alter the expression of p53, Mdm2, p21 or Bax siRNA. Both siRNA = p53 + Mdm2 siRNA. (n=5-7).





**Figure 4.3** – Effects of transfection of BeWo cells with siRNA on p53 protein expression and cell morphology. (A) Western blot for p53 demonstrating an increase in p53 expression in BeWo cells treated with Mdm2 siRNA. Cells treated with p53 siRNA did not show a reduced level of p53 protein expression. (B) BeWo cell morphology was altered in the presence of p53 and Mdm2 siRNA. In control samples cells appeared mononucleate with areas of multinucleate syncytium (marked S). Occasional cells had cytoplasmic shrinkage, increased nuclear:cytoplasmic ratio (marked with open arrows). Exposure to lipofectamine and non-silencing siRNA did not alter the phenotype. Treatment with p53 siRNA led to the appearance of cells with multiple nuclei and nucleoli without syncytia formation (shown in circles). Conversely cells treated with Mdm2 siRNA showed an increase in apoptotic appearances such as increased cell density and reduced nuclear:cytoplasmic ratio. Exposure to both p53 and Mdm2 showed mixed appearances with evidence of apoptotic and endoreduplicating cells. Phase contrast micrographs original magnification x250, Fluorescent micrographs original magnification x400.

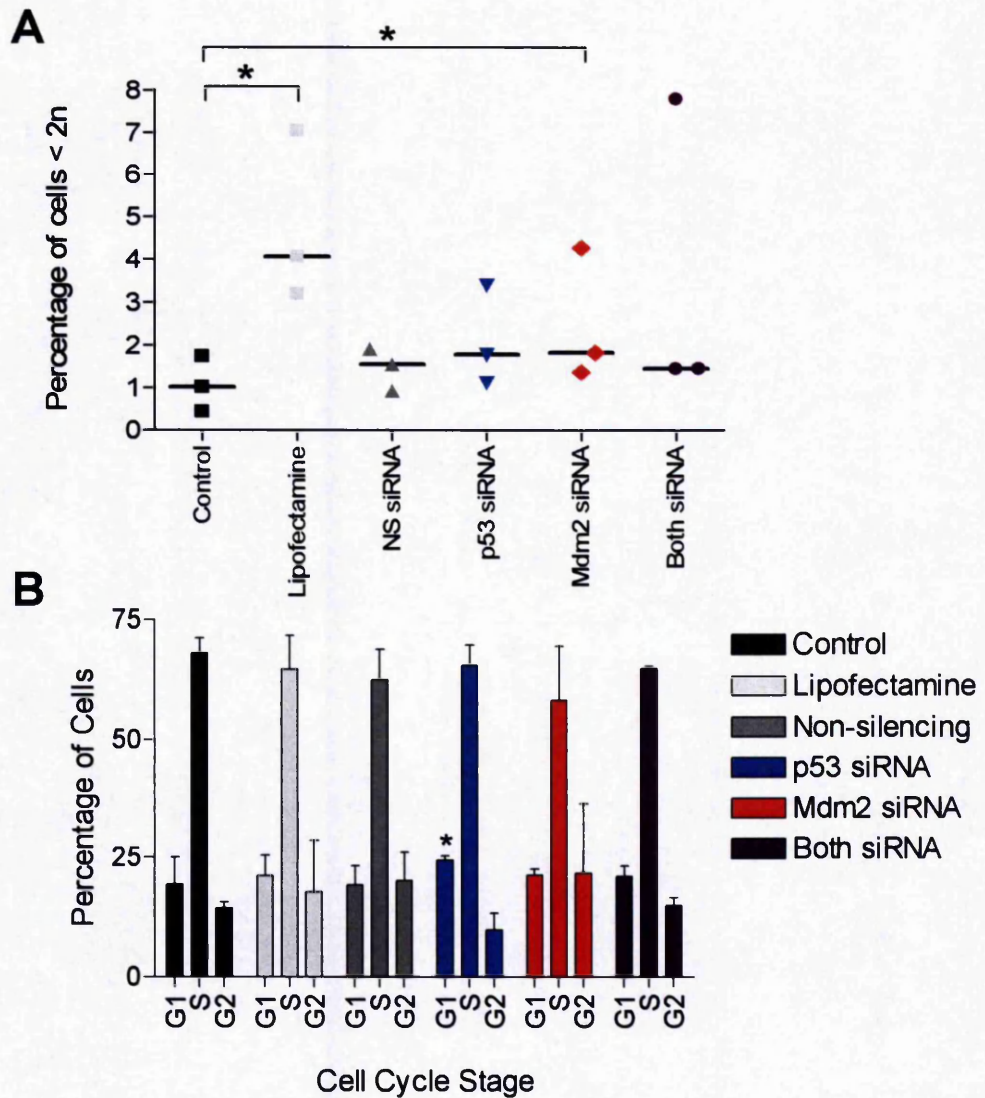
The qualitative differences in BeWo cell morphology were further investigated by flow cytometry (n=3). The percentage of cells having less than a diploid complement of DNA was increased following treatment with Mdm2 siRNA, although a more pronounced elevation was noted in cells treated with Lipofectamine alone which had no apparent change in morphology ( $p<0.05$ , Figure 4.4A). Cells treated with p53 siRNA had a significant increase in the percentage of cells in G<sub>1</sub>-phase of the cell-cycle compared to with other treatments ( $p<0.05$ , Figure 4.4B). Despite these changes in morphology, apoptosis and cell turnover, there were no statistically significant increases in LDH activity or hCG secretion of the BeWo cells in response to exposure to control or siRNA treatment (Figure 4.5 A and B).

#### **4.4.3 Effects of Transfection of Term Placental Villous Explants on p53 and Mdm2 mRNA and Protein Expression.**

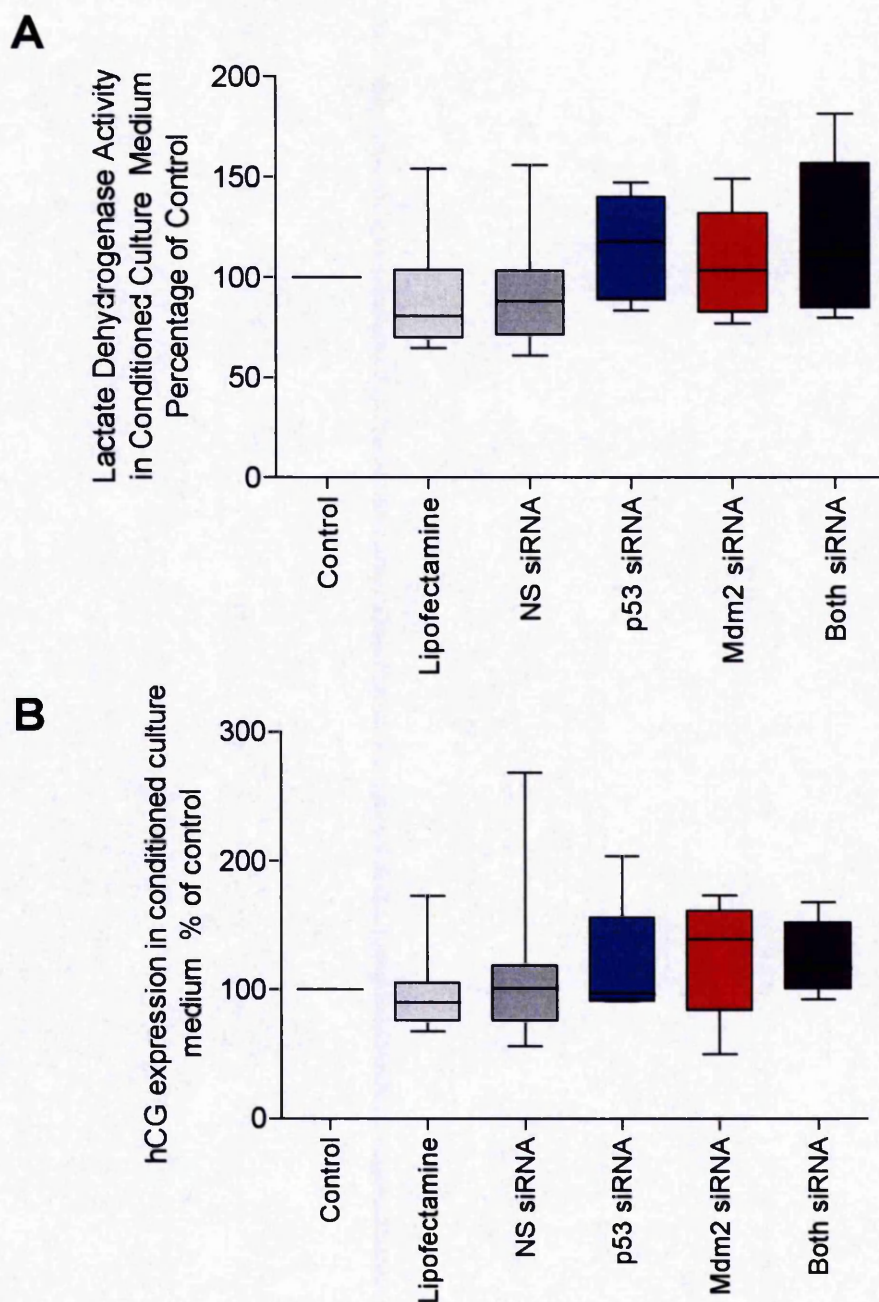
Fluorescent labelled non-silencing siRNA was evident in the syncytiotrophoblast and cytotrophoblast cytoplasm of villous explants exposed to siRNA for 48 hours (Figure 4.6A). There was no significant effect of non-silencing siRNA on the expression of p53 or Mdm2 mRNA. Culture with p53 siRNA alone reduced the expression of p53 mRNA to 50% (median) of control levels ( $p<0.05$ , Kruskal-Wallis test) (Figure 4.6B), p53 mRNA was reduced to a median of 60% of control levels by transfection with both siRNAs (A mixture of 50% p53 and 50% Mdm2 siRNA). Transfection with Mdm2 siRNA was associated with a median 80% decrease in Mdm2 mRNA expression compared to control ( $p<0.01$ ). Transfection with both siRNAs resulted in a median 50% decrease in mRNA expression compared to control ( $p<0.05$ ) (Figure 4.6C). In addition to effects on p53 mRNA, treatment with p53 siRNA resulted in a significant reduction in p21 mRNA ( $p<0.05$ ) (Figure 4.6D). The expression of Bax mRNA was not affected by treatment with non-silencing, p53- or Mdm2 siRNA (Figure 4.5E).

In common with observations in BeWo cells, treatment with p53 siRNA did not reduce p53 protein expression in placental villous lysate (Figure 4.7A). However, exposure to Mdm2 siRNA appeared to increased p53 expression (Figure 4.7A). Mdm2 protein expression was decreased in placental villous lysate following treatment with Mdm2 siRNA (Figure 4.7B).



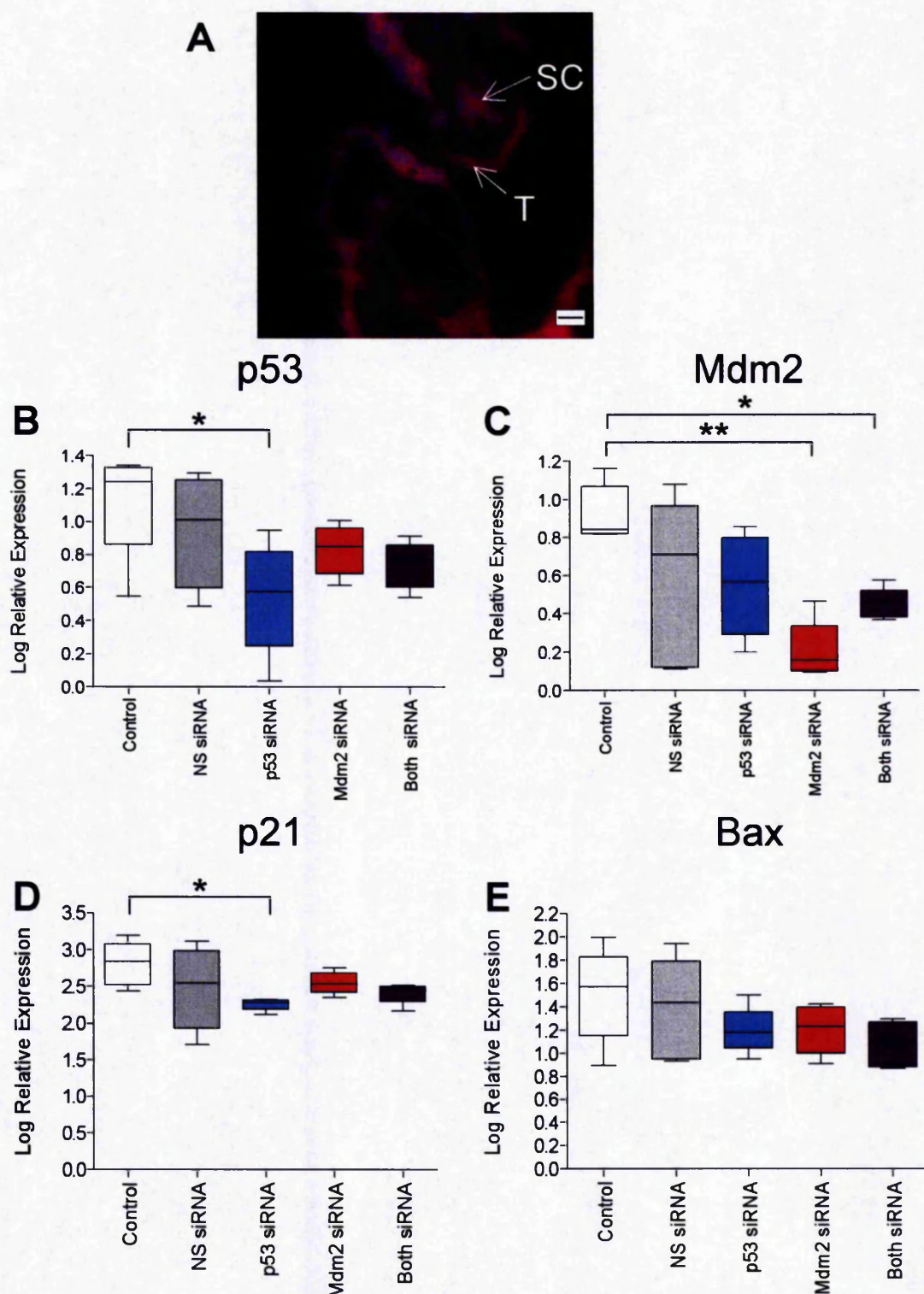


**Figure 4.4** - Assessment of apoptosis and stage of cell-cycle of BeWo cells using flow cytometry. Percentage of cells with DNA complement <2n demonstrating that cells exposed to lipofectamine alone and Mdm2 siRNA, had more cells <2n than control, (\*  $p < 0.05$ , Kruskal-Wallis test). The solid black line indicates the median value ( $n=3$ ). (B) Graph showing median percentage of cells designated as 2n in cell cycle stage G1, S or G2. BeWo cells exposed to p53 siRNA had significantly more cells in G1 than control or other culture conditions (\*  $p < 0.05$ , Kruskal-Wallis test). Error bars indicate the upper range of data ( $n=3$ ).

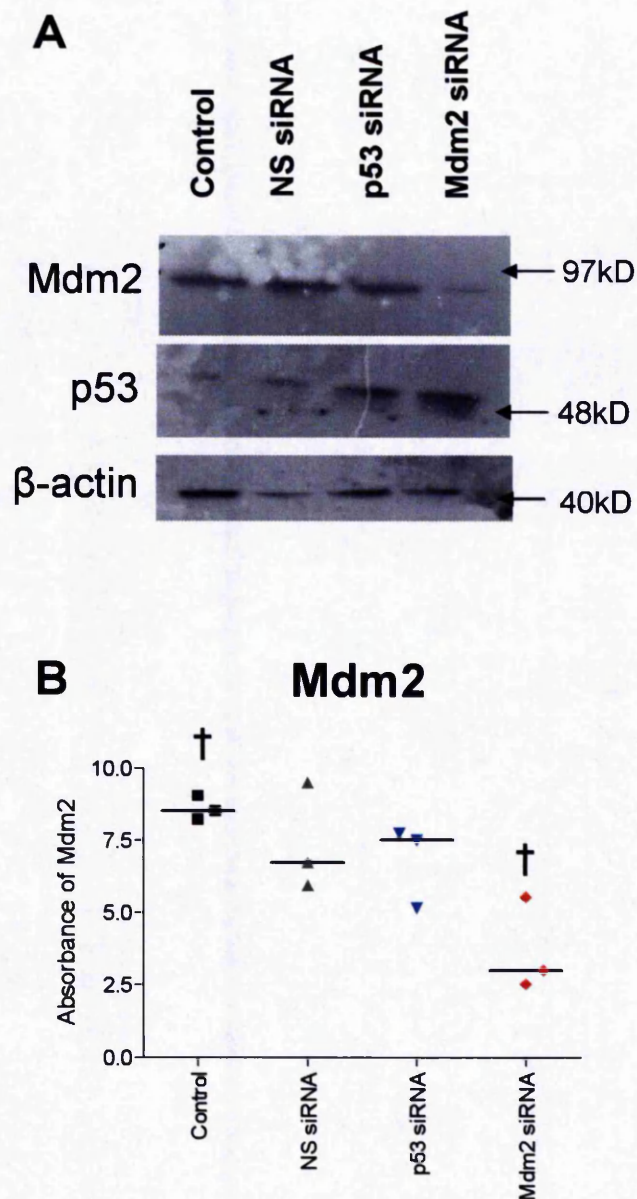


**Figure 4.5** – Effects of transfection with siRNA on necrosis and hCG production of BeWo cells. (A) LDH expression was not increased by culture in the presence of lipofectamine or any siRNA (n=6). (B) hCG expression is not increased by culture in the presence of lipofectamine or any siRNA (n=6). NS siRNA = Non-silencing siRNA. Both siRNA = p53 + Mdm2 siRNA.





**Figure 4.6** – Effect of transfection with siRNA on expression of p53, Mdm2, p21 and Bax in placental villous explants. (A) Transfection with fluorescently labelled non-silencing (NS) siRNA demonstrates uptake by trophoblast (T) and stromal cells (SC), Scale bar 10µm. (B) Transfection with p53 siRNA leads to a significant reduction in p53 mRNA (\*  $p < 0.05$ ), Kruskal-Wallis Test. (C) Transfection with Mdm2 siRNA leads to a significant reduction in Mdm2 siRNA. (D) Transfection with p53 siRNA significantly reduced the expression of p21 mRNA. (E) The amount of Bax mRNA was not altered by transfection with p53 or Mdm2 siRNA (n=4-6).



**Figure 4.7** – Effect of transfection with siRNA on protein expression of Mdm2. (A) Representative Western blots showing an apparent reduction in Mdm2 and an increase in p53 protein expression in explants transfected with Mdm2 siRNA. (B) Densitometry of Western blotting (n=3) demonstrated a trend towards reduced Mdm2 protein following transfection with Mdm2 siRNA († p=0.05, Friedman test).



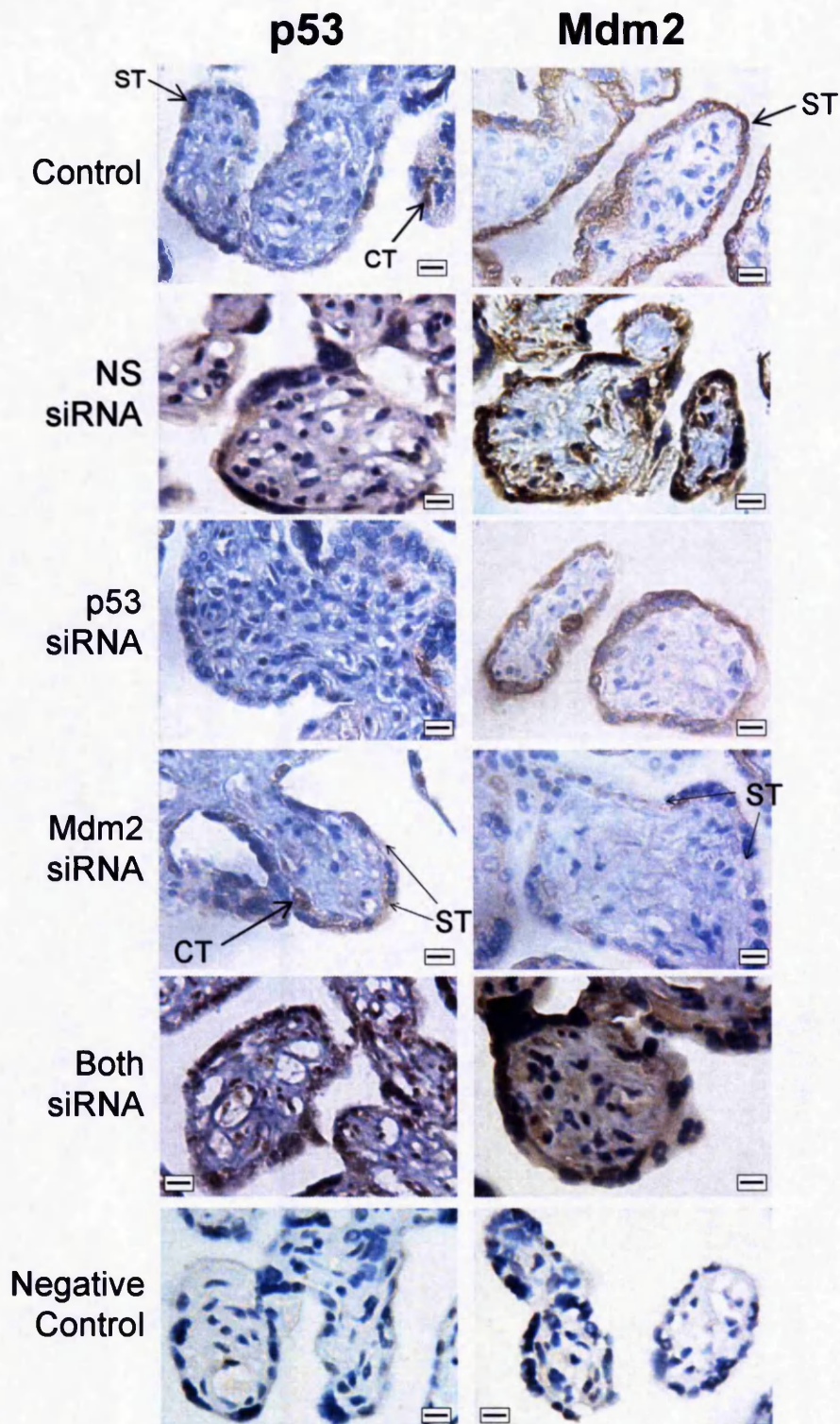
The changes in p53 and Mdm2 expression appeared to predominantly localise to the syncytiotrophoblast. In control conditions, p53 was expressed in few cytotrophoblast nuclei and discrete areas of the syncytium, which was not altered by culture in the presence of non-silencing or p53 siRNA (Figure 4.8). p53 expression appeared stronger in both syncytiotrophoblast and cytotrophoblast compartments in villous explants treated with Mdm2 siRNA, although this was not observed in explants treated concurrently with both p53 and Mdm2 siRNA. Mdm2 protein levels in the syncytiotrophoblast cytoplasm appeared to be reduced by treatment with Mdm2 siRNA compared to villous explants exposed to control conditions, non-silencing, p53 siRNA alone or both p53 and Mdm2 siRNA (Figure 4.8). The localisation of p21, Bax and Bcl-2 was also similar to that previously described in fresh villous trophoblast (Chapter 3) and was not altered by treatment with non-silencing, p53- or Mdm2-siRNA (Figure 4.9).

#### **4.4.4 Effects of p53 and Mdm2 siRNA on Cell Turnover of Term Placental Villous Explants**

The rate of apoptosis in villous explants was significantly increased in the presence of Mdm2 siRNA ( $p < 0.05$ , Kruskal-Wallis test), but was unaffected by transfection with non-silencing siRNA, p53 siRNA or concurrent p53 and Mdm2 siRNA ( $p < 0.05$ , Figure 4.10A). Explants treated with Mdm2 siRNA demonstrated increased TUNEL staining in trophoblast and stromal cells (Figure 4.10B-E). This distribution of apoptotic cells was confirmed by cytokeratin-M30 immunostaining (Figure 4.11).

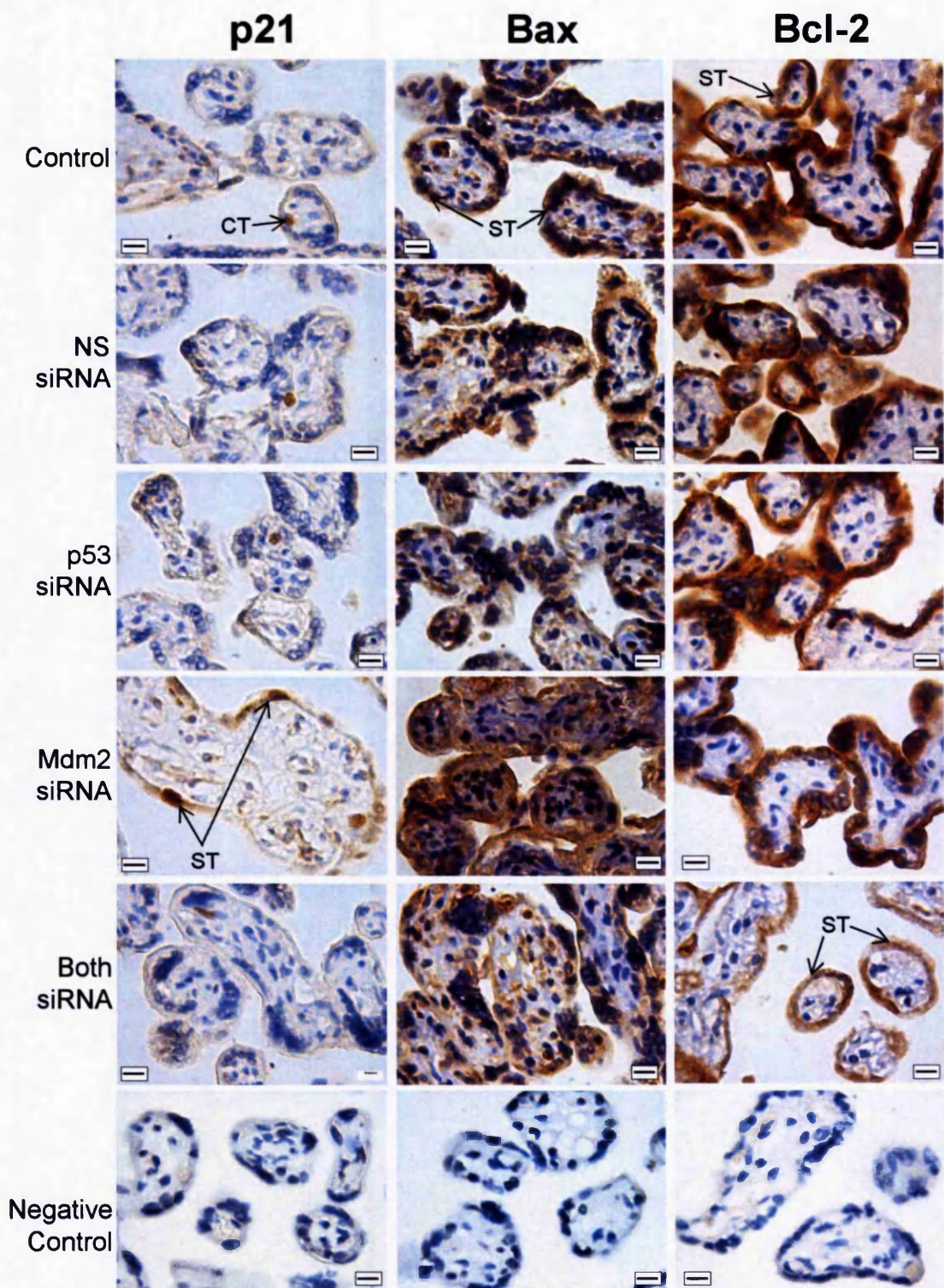
In cultured placental villous explants, Ki67 immunostaining was confined to cytotrophoblast nuclei. Cytotrophoblast proliferation was decreased in explants cultured in the presence of Mdm2 siRNA ( $p < 0.05$ , Figure 4.12A). The changes in proliferation in response to treatment with Mdm2 siRNA were reduced to baseline levels in explants exposed to both Mdm2 and p53 siRNA.

The presence of syncytial knots was increased in explants cultured in the presence of Mdm2 siRNA compared to control ( $p < 0.05$ , Figure 4.13A). Culture in the presence of siRNA was not associated with increased presence of LDH within the culture media compared to control conditions (Figure 4.14A). In addition, there were no significant effects of siRNA on the expression of hCG within conditioned culture media (Figure 4.14B).



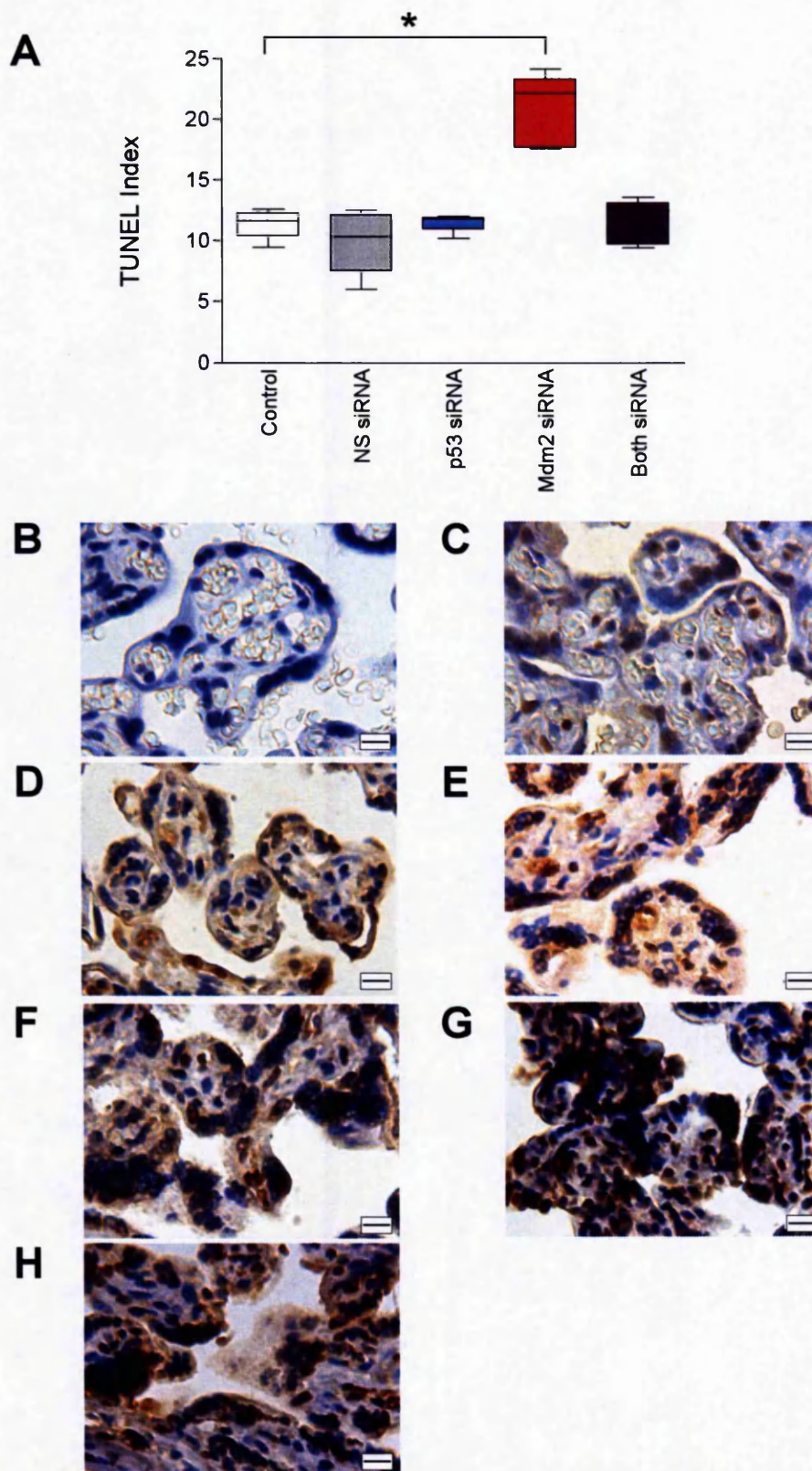
**Figure 4.8** – Assessment of protein expression of p53 and Mdm2 in villous explants following treatment with siRNA. Representative images of immunoperoxidase staining. In these studies, p53 and Mdm2 were predominantly expressed by trophoblast, with little evidence of staining in the stromal cells. In control explants, p53 was expressed in occasional cytotrophoblast (CT) and syncytiotrophoblast (ST) nuclei and small areas of the ST cytoplasm. Mdm2 was predominantly expressed in ST cytoplasm. Treatment with p53 siRNA did not appear to alter the expression of p53 or Mdm2. In the presence of Mdm2 siRNA, Mdm2 expression in the ST cytoplasm was reduced. In the same explants p53 expression was increased, especially in the trophoblast layer. There was no evidence of immunostaining in the negative control. Bar = 10 $\mu$ m.



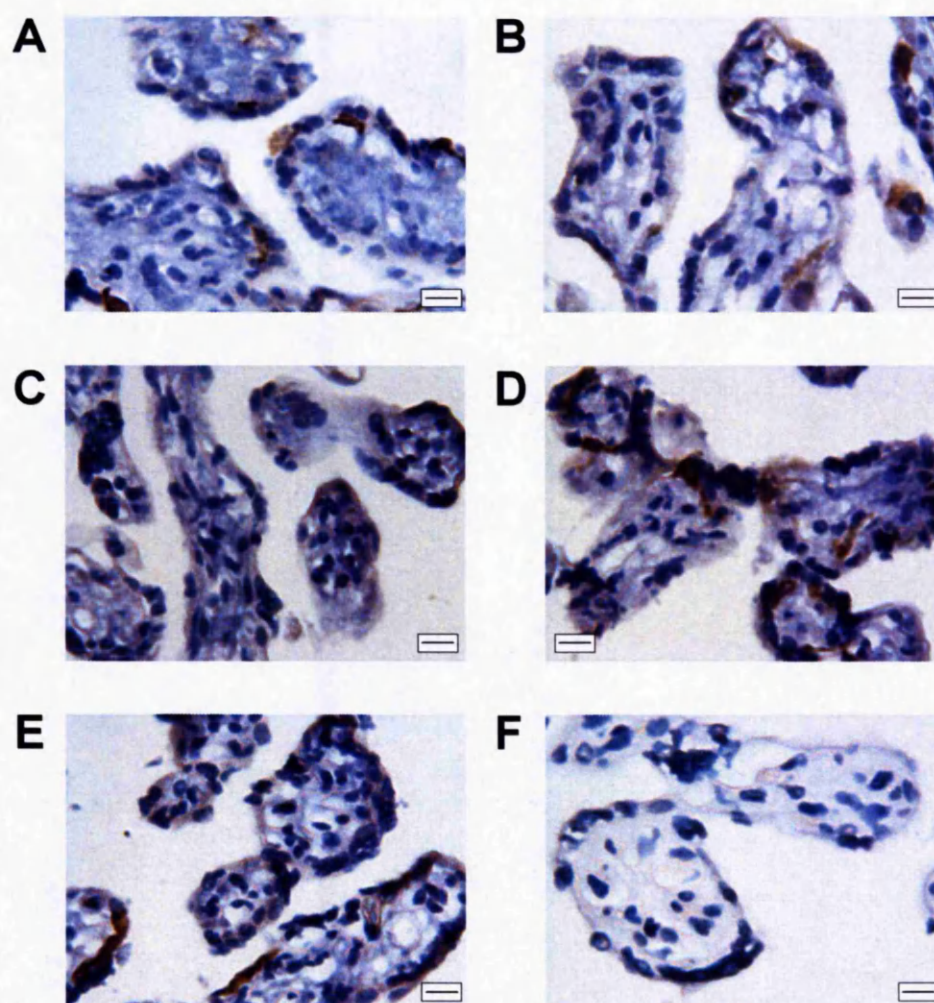


**Figure 4.9** – Assessment of protein expression of p21, Bax and Bcl-2 in villous explants following treatment with siRNA. Representative images of immunoperoxidase staining. In these studies, p21, Bax and Bcl-2 were predominantly expressed by trophoblast. In control explants, p21 was expressed in occasional cytotrophoblast (CT) nuclei. Bax was expressed in syncytiotrophoblast (ST) and stromal cell cytoplasm. Bcl-2 was present in the ST cytoplasm. Treatment with p53 siRNA did not appear to alter the expression of p21, Bax or Bcl-2. In the presence of Mdm2 siRNA, p21 expression appeared to be increased in CT and ST layers; Bax and Bcl-2 appeared to be unaltered. There was no evidence of immunostaining in the negative control.



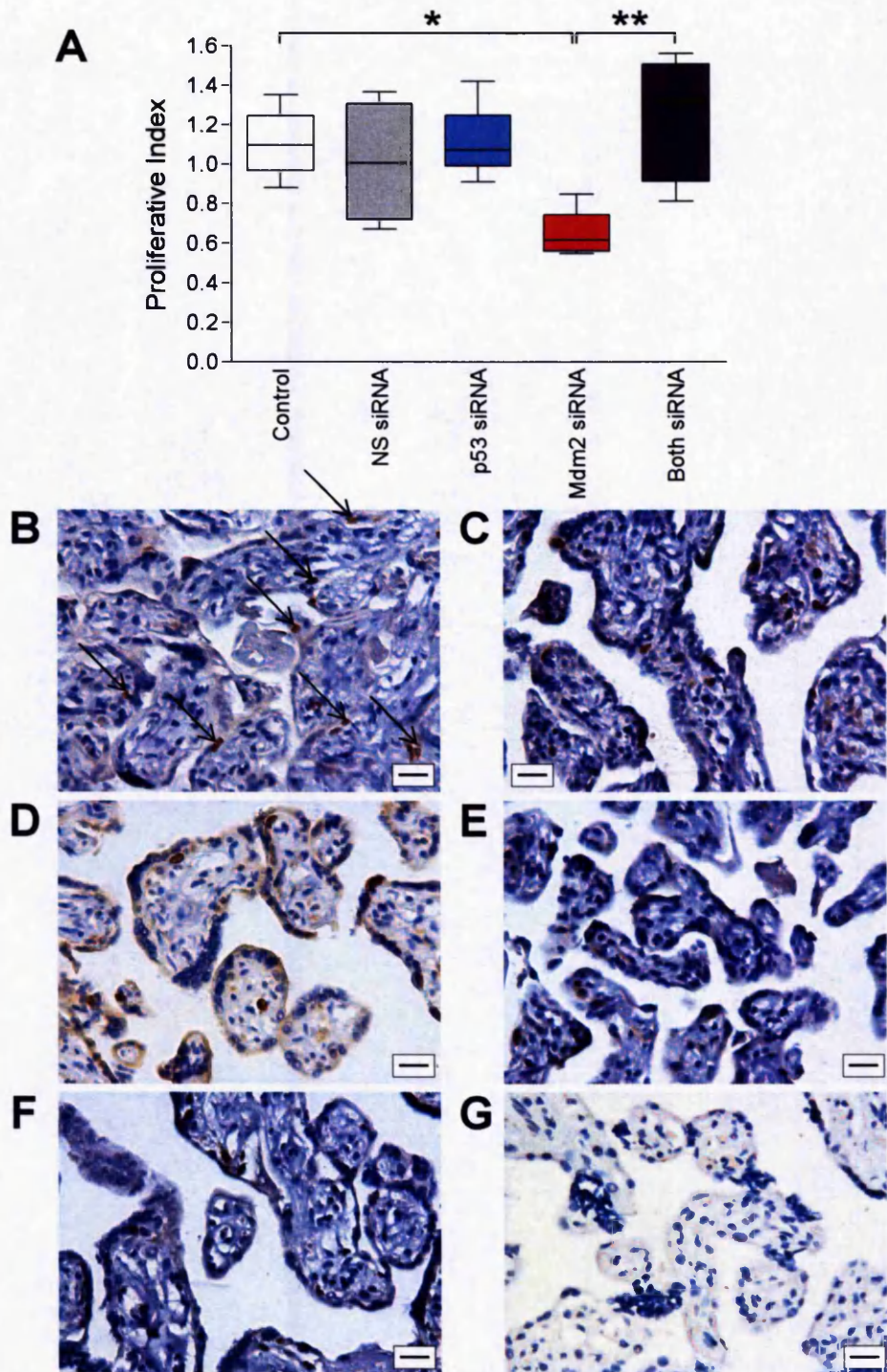


**Figure 4.10** – Effect of transfection with siRNA on apoptosis in placental explants. (A) Transfection with NS or p53 siRNA did not alter the TUNEL index. Transfection with Mdm2 siRNA increased TUNEL index compared to control. \* =  $p < 0.05$  (Kruskal-Wallis Test). (n=4-6). B) Negative control C) TUNEL positive control. Representative images of TUNEL staining in D) control conditions, E) NS siRNA, F) p53 siRNA, G) Mdm2 siRNA and H) Both p53 and Mdm2 siRNA. Line = 10µm.



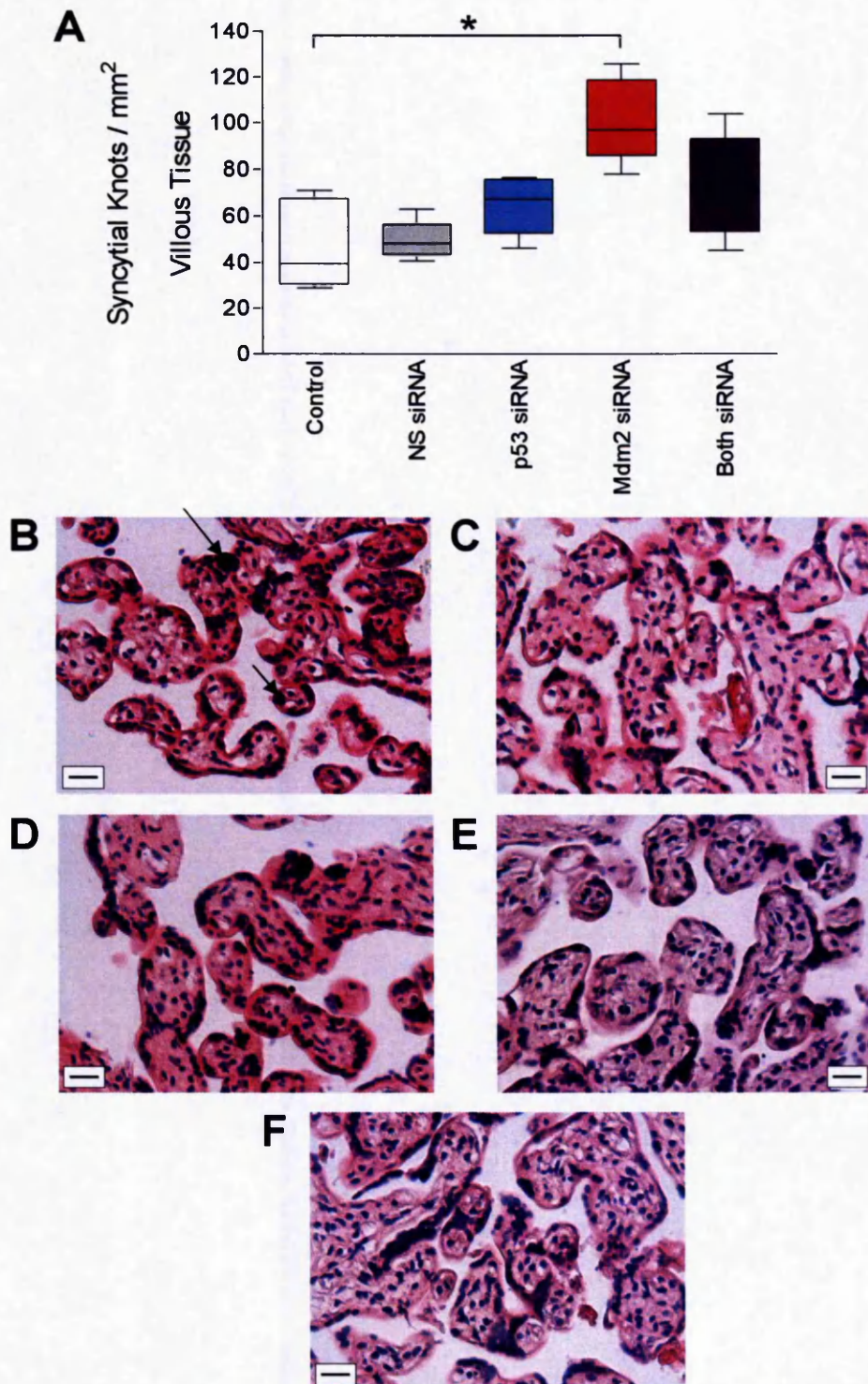
**Figure 4.11** – Effect of transfection with siRNA on apoptosis in placental explants as assessed by cytokeratin-M30 immunostaining. Representative immunostaining in A) control conditions, (B) NS siRNA, (C) p53 siRNA, (D) Mdm2 siRNA and (E) Both p53 and Mdm2 siRNA. (F) Negative control. Line = 10µm.



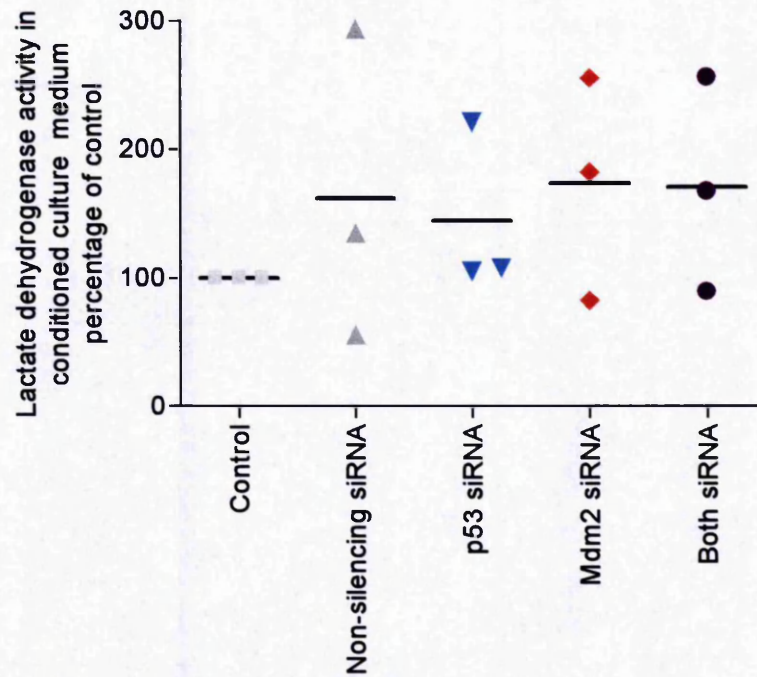
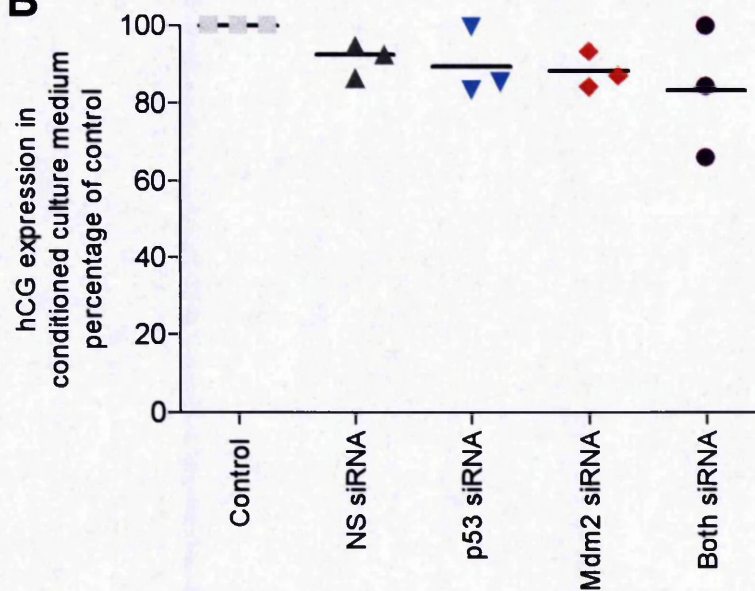


**Figure 4.12** – Effect of transfection with siRNA on proliferative index in placental explants. (A) Transfection with non-silencing (NS) siRNA or p53 siRNA did not alter the proliferative index. The proliferative index was significantly reduced by treatment with Mdm2 siRNA, \*  $p < 0.05$  (Kruskal-Wallis Test). The level of proliferation returned to levels of control with co-administration of p53 and Mdm2 siRNA, \*\*  $p < 0.01$ . (n=4–6). Representative images of Ki67 staining in (B) control conditions (nuclei marked with open arrows), (C) NS siRNA, (D) p53 siRNA, (E) Mdm2 siRNA and (F) Both p53 and Mdm2 siRNA. (G) Negative control. Line = 20 $\mu$ m.





**Figure 4.13** – Effect of transfection with siRNA on number of syncytial knots in placental explants. (A) Transfection with non-silencing (NS) siRNA or p53 siRNA did not alter the proliferative index. The number of syncytial knots (marked with closed arrows) was significantly increased by treatment with Mdm2 siRNA, \*  $p < 0.05$  (Kruskal-Wallis Test).. Representative images of syncytial knots in B) control conditions, C) NS siRNA, D) p53 siRNA, E) Mdm2 siRNA and F) Both p53 and Mdm2 siRNA. Line = 20 $\mu$ m.

**A****B**

**Figure 4.14** – Assessment of necrosis and biochemical differentiation in placental villous explants transfected with siRNA. Data was normalised to control conditions for each placenta. (A) The presence of lactate dehydrogenase (LDH) in conditioned culture medium was not significantly increased by treatment with any specific siRNA sequence (n=3). (B) Expression of hCG was not significantly altered by treatment with any specific siRNA sequence, but treatment with siRNA did produce a trend towards reduced presence of hCG in conditioned culture medium (n=3).

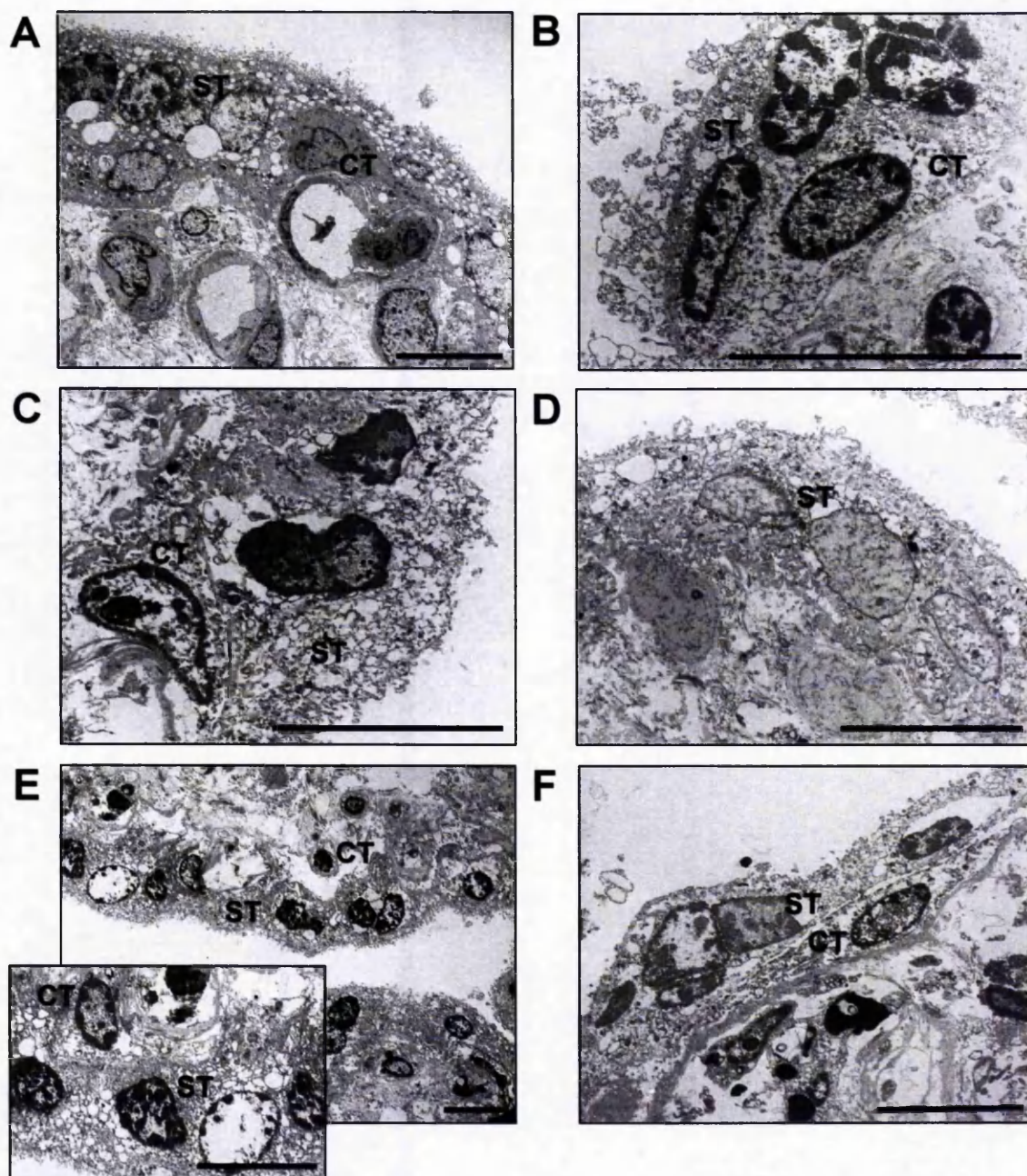
The changes in cell turnover were confirmed by electron microscopy. Cultured placental villous explants were inspected by two observers not blinded to the culture conditions. Explants cultured in control conditions had evidence of normal nuclear ageing, with syncytiotrophoblast nuclei showing evidence of peripheral chromatin condensation with underlying cytotrophoblast beginning to show similar evidence of degeneration (Figure 4.15B). Explants cultured with non-silencing siRNA demonstrated similar nuclear changes of circular aggregates in the nuclear periphery, although these were not typical of apoptotic appearances (Figure 4.15C). However, in the presence of p53 siRNA the nuclei do not show evidence of ageing (Figure 4.15D). In contrast, explants exposed to Mdm2 siRNA showed evidence of advanced apoptosis, including nuclear condensation of syncytiotrophoblast and condensation of cytotrophoblast nuclei and cytoplasm (Figure 4.15E). Few changes of apoptosis were evident following treatment with both p53 and Mdm2 siRNA, with cytotrophoblasts retaining healthy appearances (Figure 4.15F).

#### **4.5 Discussion**

These experiments demonstrate that both BeWo choriocarcinoma cells and term placental villous explants can be successfully transfected with siRNA. Exposure to scrambled non-silencing siRNA did not alter any specific mRNA tested here. In this study, siRNA sequences from published studies demonstrating significant knockdown in other cell types were used (Martinez et al. 2002; Uchida et al. 2005). Ideally, several different siRNA sequences directed at different targets of the mRNA would be tested. Due to time constraints replicate experiments were not undertaken here. However, a number of preliminary studies were compared and the most efficacious siRNA sequences were utilised in these investigations. The reductions in p53 and Mdm2 siRNA observed in this study were comparable to those reported in other studies using the same siRNA sequences (Martinez et al. 2002; Uchida et al. 2005). Treatment with siRNA did not lead to a significant alteration in LDH release from BeWo cells or placental villous explants into culture medium indicating that there was no increase in necrotic cell death. Likewise, there was no alteration in hCG expression in culture media, suggesting that treatment with siRNA had no adverse effects on trophoblast viability.

The reduction in Mdm2 mRNA was associated with a decrease in Mdm2 protein expression in placental villous explants. In both BeWo cells and explants Mdm2 knockdown was associated





**Figure 4.15** – Assessment of villous morphology in placental villous explants transfected with siRNA. Representative electron micrographs of (A) fresh placental tissue and explants cultured in (B) control conditions demonstrating an intact syncytiotrophoblast (ST) with underlying cytotrophoblast cells (CT). (C) Explants treated with non-silencing siRNA show some nuclear changes with peripheral aggregates appearing in nuclei. (D) These nuclear aggregates were not present in explants treated with p53 siRNA. (E) Treatment with Mdm2 siRNA was associated with appearance of many pyknotic nuclei with dense peripheral chromatin consistent with exaggerated apoptosis (highlighted in inset). (F) Explants treated with a combination of p53 and Mdm2 siRNA had similar appearance of CT and ST to explants cultured in control conditions. Scale bar = 5µm.

with an increase in p53 expression, but treatment with p53 siRNA did not reduce p53 protein despite a significant decrease in p53 mRNA. These data may result from differences in the turnover of p53 protein, whose half-life may increase to 24 hours following activation, compared to approximately 30 minutes for Mdm2 (Oren et al. 1981; Pan et al. 2004; Stommel and Wahl 2004). Presently, there are no data regarding the half-life of p53 and Mdm2 protein in trophoblast to support or refute this proposal. It is notable that p53 knockdown did appear to attenuate the increased p53 protein expression in the presence of Mdm2 siRNA and may therefore prevent translation of further p53, but cannot modify p53 protein which has been activated and stabilised.

Treatment with Mdm2 siRNA altered BeWo cell morphology, increasing the proportion of cells  $<2n$  and increased evidence of pyknotic cells with high nuclear:cytoplasmic ratio. Further investigation using different analyses of apoptosis and cell viability are required to confirm these preliminary data. Concurrent exposure of BeWo cells to p53 and Mdm2 siRNA did not reverse the phenotype associated with Mdm2 siRNA, instead cells adopted appearances of either apoptosis or replication. Several explanations may be postulated for this inconsistency, including (i) the stage of the cell cycle at which transfection occurred, (ii) BeWo cells may respond to the predominant knockdown within an individual cell or (iii) there maybe an imbalance between the potency of p53 and Mdm2 siRNA. This question could be answered by the use of novel siRNA technologies (developed after these experiments were performed), which have the ability to transfect several siRNA sequences in the same duplex enabling matched delivery of p53 and Mdm2 siRNA to each cell.

In placental villous explants, transfection with Mdm2 siRNA led to a reduction of Mdm2 mRNA and protein and an increase in p53 protein expression which predominantly localised to the syncytiotrophoblast, which was consistent with the distribution of siRNA. Interestingly, it is similar to the altered expression of these proteins seen in pregnancies complicated by pre-eclampsia. This imbalance between Mdm2 and p53 was associated with changes in trophoblast cell turnover, most notably, a two-fold increase in apoptosis as assessed by TUNEL, confirmed by cytokeratin-M30 immunostaining and electron microscopy. In these experiments, the increased apoptosis was combined with an increased formation of syncytial knots. This increase in apoptotic cell death and degeneration was allied to a decrease in proliferation; both changes consistent with a cellular response to unopposed p53. In the case

of cytotrophoblast proliferation, the changes associated with Mdm2 siRNA were reduced to levels of control by concurrent administration of p53 and Mdm2. These data may be attributable to lower doses of individual siRNA used for these experiments (33nM compared to 66nM). Examination of the effects of 33nM Mdm2 siRNA alone would provide insight whether the reduction in apoptosis observed here was due to a lesser knockdown of Mdm2. In addition, co-transfection with p53 and Mdm2 siRNA in a single sequence could address whether the observed reduction in apoptosis and increase in proliferation resulted from concurrent administration of p53 and Mdm2 siRNA, thereby preventing a rise in p53 transcription.

Although Mdm2 knockdown was associated with increased p53 expression there was no complementary increase in downstream p53 targets p21 and Bax at either the mRNA or protein level. p53 has many downstream pro-apoptotic targets including Puma and Noxa, which were not investigated here. It is possible that Puma and Noxa may be active in promoting the increased apoptosis observed following Mdm2 knockdown (Oda et al. 2000; Nakano and Vousden 2001). These experiments do not exclude the possibility that a reduction in Mdm2 may have other cytotoxic effects other than an increase in p53 expression. This possibility has been investigated in a variety of cell types using Nutlin-3a - a specific pharmacological antagonist of the interaction between p53 and Mdm2. In the presence of this agent, Mdm2 cannot bind p53, thereby increasing p53 stability by preventing its nuclear export and destruction (Vassilev et al. 2004). In the presence of Nutlin-3a, any effects are due to increased presence of p53 rather than a loss of Mdm2 per se. Treatment with Nutlin-3a increases apoptosis and decreases proliferation in cancer-cell lines (Vassilev et al. 2004; Logan et al. 2007). Exposing trophoblast to Nutlin-3a would indicate whether the excessive cell death observed in these experiments occurred solely as a result of increased p53 expression, rather than due to a reduction of other functions of Mdm2.

In the absence of Mdm2, regulation of p53 activity may be provided by MdmX (also known as Mdm4), an analogue of Mdm2 (Jackson and Berberich 2000). The susceptibility of cells to Mdm2 knockdown is thought to be determined by the expression of MdmX (Wade et al. 2006). There are no data regarding the expression of MdmX in trophoblast, and current reports have only demonstrated the presence of MdmX in malignancies of mesenchymal or endodermal origin including: retinoblastoma, soft tissue sarcomas and glioma (Marine et al. 2007). The presence of MdmX in villous trophoblast may reduce the importance of an imbalance between



p53 and Mdm2, such as that observed in pre-eclampsia. However, these data imply that significant expression of MdmX in normal term trophoblast is unlikely as Mdm2 knockdown led to a significant increase in apoptosis making the presence of a surrogate protein unlikely.

In conclusion, the findings of these experiments are in agreement with previous data in other cell types demonstrating that a reduction in Mdm2 is associated with increased apoptosis, and that this is related to increased expression of p53. Therefore, the increase in villous trophoblast apoptosis observed in pre-eclampsia may be related to an imbalance between Mdm2 and p53 as described in Chapter 3. In pre-eclampsia the reduction in Mdm2 protein was not associated with a decrease in Mdm2 mRNA. Therefore, it is hypothesised that in pre-eclampsia either Mdm2 translation is reduced or consumption of the protein may be increased. In support of the latter hypothesis, Mdm2 protein expression may be reduced by exposure to stressful stimuli such as DNA damage (Stommel and Wahl 2004).

In pre-eclampsia, many factors may be present in the placental milieu which could activate the intrinsic pathways of apoptosis and explain the stimulation of p53, i.e. hypoxia, reactive oxygen species (ROS) and nitric oxide (Polyak et al. 1997; Hammond and Giaccia 2005). No factor yet identified can fully explain the reduction in protective Mdm2. Mdm2 protein stability is decreased following activation of DNA-damage-activated protein kinases (PK) such as ATM-PK and DNA-PK (Stommel and Wahl 2004). This observation is consistent with a decrease in Mdm2 protein but not mRNA. There are few reports regarding direct effects of hypoxia or ROS on expression of Mdm2. In tumour cell-lines Mdm2 interacts with hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) (Zhang and Hill 2004; Nieminen et al. 2005), an important intracellular O<sub>2</sub> sensor. However, hypoxia has different effects on different cell types leading to an increase in Mdm2 in KHT (sarcoma) cells (Zhang and Hill 2004), but not in human colorectal carcinoma cell-line HCT116 (Nieminen et al. 2005). There are no studies of the effects of ROS on Mdm2 expression. However, Mdm2 stability is proportionally regulated by Daxx, a death-domain associated protein (Tang et al. 2006) which is increased in HeLa cells following exposure to oxidative stress (Kim et al. 2005), inferring that Mdm2 expression may be increased as a result of oxidative stress. However, this inference is questioned by the increase in apoptosis following exposure to oxidative stress. Recent data suggests that elevation of N-myc down-regulated gene (NDRG1) which may occur in response to hypoxia decreases the dominant

negative p60 form of Mdm2 in trophoblast indicating that exposure of placental tissue to noxious stimuli may alter the expression of Mdm2 (Chen et al. 2006).

It is hypothesised that the increased apoptosis in villous trophoblast observed in pre-eclampsia results from exposure to hypoxia or following the generation of ROS. This hypothesis originates from the observation of reduced conversion of spiral arteries (Meekins et al. 1994), which may reduce blood flow to the intervillous space leading to periods of hypoxia or generate reactive oxygen species from intermittent perfusion of the intervillous space. This hypothesis is supported by the finding that HIF1 $\alpha$  and 2 $\alpha$  are increased in villous trophoblast of pregnancies complicated by pre-eclampsia (Rajakumar et al. 2001; Rajakumar et al. 2003; Rajakumar et al. 2004). HIF1 $\alpha$  can be induced in trophoblast following exposure to hypoxia or reactive oxygen species (Caniggia et al. 2000a; Cindrova-Davies et al. 2005). Furthermore, microarray studies have demonstrated that culture of first trimester trophoblast to 3% O<sub>2</sub> produces a similar pattern of mRNA transcription compared to pre-eclampsia (Soleymanlou et al. 2005a). In addition, hypoxia alters villous trophoblast cell turnover in vitro, increasing apoptosis and reducing differentiation (Levy et al. 2000; Crocker et al. 2004c). Therefore, exposure to hypoxia or oxidative stress may be responsible for alterations in trophoblast cell turnover. In the same way, it may be hypothesised that these noxious stimuli may alter the balance of expression of p53 and Mdm2 in villous trophoblast.

## **Chapter 5 – Do Hypoxia and Reactive Oxygen Species Alter Cell Turnover of BeWo Choriocarcinoma Cells?**

### **5.1 Introduction**

Cell turnover is a tightly regulated event, when a tissue or cell colony is in a state of equilibrium a balance must be maintained between cell proliferation and cells lost by damage or death. In villous trophoblast proliferation and fusion of mononucleate cytotrophoblast cells is balanced by degeneration of the syncytiotrophoblast, with changes similar to apoptosis, and formation of syncytial knots, some of which are lost into the maternal circulation (Huppertz and Kingdom 2004). The equilibrium between maintenance and loss of syncytiotrophoblast is disturbed in pre-eclampsia which is associated with increased apoptosis (Allaire et al. 2000; Leung et al. 2001), increased formation of syncytial knots (Tenney and Parker 1940) and increased numbers of cytotrophoblasts (Jones and Fox 1980; Soma et al. 1982). In pre-eclampsia, increased apoptosis and syncytial degeneration is associated with increased expression of pro-apoptotic p53, p21, Bax and Caspase-3 and a reduction in anti-apoptotic Mdm2 (as reviewed Chapter 3). Furthermore, inducing an imbalance between p53 and Mdm2 induces apoptosis in term placental villous explants (Chapter 4).

Presently, the origin of the pro-apoptotic shift in p53, Mdm2, p21 and Bax expression in pre-eclampsia is not known. The aberrant turnover of villous trophoblast in pre-eclampsia is thought to result from exposure to hypoxia and/or oxidative stress, as severe pre-eclampsia is associated with a failure of conversion of spiral uterine arteries to wide flaccid conduits required for the delivery of maternal blood to the placenta (Meekins et al. 1994). It is hypothesised that this reduction in blood flow results in placental hypoxia or hypoxia-reperfusion injury (Hung and Burton 2006). This is supported by evidence that placentas from pregnancies complicated by pre-eclampsia show similar gene expression to placental villous explants cultured in hypoxic conditions (Soleymanlou et al. 2005a), and apoptosis may be induced in isolated term cytotrophoblast following exposure to hypoxia (Levy et al. 2000) or hypoxia-reperfusion injury (Hung et al. 2002). In addition, the increased apoptosis induced following exposure to hypoxia is associated with an increase in p53 expression (Levy et al. 2000). Previous studies (reviewed in Section 1.5.3) have demonstrated the presence of

constituents of the p53 pathway in villous trophoblast at the mRNA and protein level, indicating that they may have a role in regulating cell turnover (Fulop et al. 1998; Qiao et al. 1998; Allaire et al. 2000; Ratts et al. 2000).

Various in vitro models have been used to investigate the effects of hypoxia and ROS on trophoblast cell turnover including: culture of whole tissue, primary trophoblast and choriocarcinoma-derived cell lines. The BeWo choriocarcinoma cell line has been used to investigate aspects of trophoblast cell turnover, including: fusion and formation of syncytia, apoptosis and proliferation (Kudo et al. 2003b; Al-Nasiry et al. 2006; Bae et al. 2007; Hu et al. 2007). The use of BeWo cells for preliminary assessment of the effects of altered oxygen tension or oxidative stress is advantageous for several reasons. Firstly, BeWo cells have been well-characterized and represent a pure trophoblast cell line which may be cultured simply. Secondly, altered fusion of BeWo cells following O<sub>2</sub> restriction has been described allowing comparisons to be made with published data (Kudo et al. 2003a; Kudo et al. 2003b). Thirdly, BeWo cells increase human chorionic gonadotrophin (hCG) secretion following fusion, enabling a biochemical measure of trophoblast differentiation (Kudo et al. 2003a). Finally, BeWo cells undergo proliferation, which may not be the case in primary trophoblast cell cultures (Yui et al. 1994a). In addition, previous studies have demonstrated the presence of p53, Mdm2, p21 and Bax in BeWo cells (Haidacher et al. 1995; Bae et al. 2007). Therefore, BeWo cells may provide a useful in vitro model to study the effects of hypoxia and oxidative stress on trophoblast apoptosis, proliferation, fusion and the expression of p53 and Mdm2.

## **5.2 Hypothesis**

It was hypothesised that exposure of BeWo cells to reduced oxygen tension or ROS in the form of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) would induce apoptosis and alter proliferation and fusion. In addition, it was hypothesised that any observed changes in cell turnover would be associated with altered expression of proteins in the p53 pathway.

## **5.3 Methods**

### **5.3.1 BeWo Cell Culture**

BeWo cells were thawed and cultured to confluence in DMEM-F12 supplemented with 10% FCS, 30mg/l penicillin, 50mg/l streptomycin and 146µg/l glutamine. When confluent, cells were

washed, and treated with 5ml 0.05% trypsin-EDTA solution. Cells were then seeded into either 6-well plates at a density of  $1.5 \times 10^6$ /well or 96 well-plates at a density of  $10\text{--}50 \times 10^5$ /well for subsequent culture. Cells were cultured for 48 hours in 20% atmospheric O<sub>2</sub> as control, as this is the most common culture condition described in previous studies. To assess the effects of low O<sub>2</sub>, cells were cultured for 48 hours in 6% O<sub>2</sub> or in 20% O<sub>2</sub> for 24 hours followed by 6% O<sub>2</sub> for 24 hours. The effect of ROS was investigated by culture in 20% O<sub>2</sub> for 24 hours and 5µM, 10µM or 50µM H<sub>2</sub>O<sub>2</sub> added for a further 24 hours (Figure 5.1).

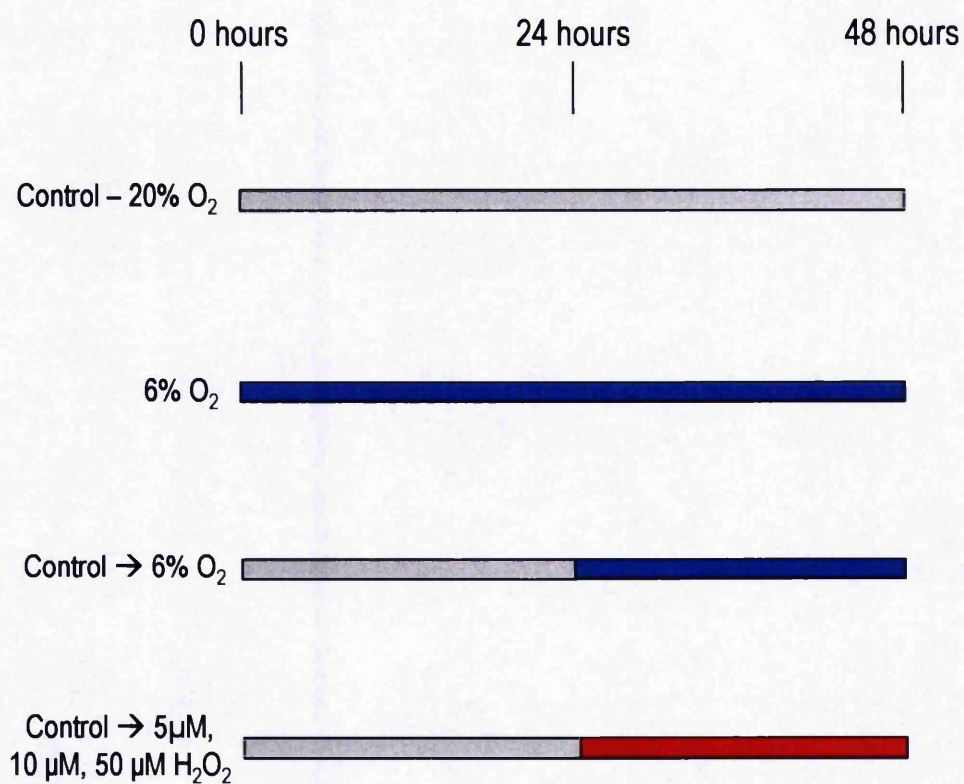
### **5.3.2 Assessment of Apoptosis**

As assessment of cell morphology by flow cytometry was unavailable, apoptosis of BeWo cells was assessed in these experiments using two different methods. Firstly, by the Apopercantage kit (Biocolor), for which cells were cultured on 96-well plates at a density of  $5 \times 10^5$ /well; 8 wells were cultured per experimental condition for 5 separate passages (n=5). After culture, cells were exposed to dye as described in section 2.10.3. After the cells were washed, representative photographs were taken using an inverted microscope (Leica). Cells were then treated with dye release reagent and absorbance measured at 550nm.

Apoptosis was also assessed using a commercially available Adenosine Diphosphate:Adenosine Triphosphate (ADP:ATP) ratio kit, which has been previously used on primary trophoblast culture (Crocker et al. 2003). BeWo cells were cultured in a white walled 96-well plate at a density of  $2 \times 10^5$ /well; 8 wells were cultured per experimental condition for 5 separate passages (n=5). Following culture cells were exposed to reagents to release adenylate nucleotides from cells as described in section 2.10.4. The presence of ADP and ATP were measured and a ratio of ADP:ATP calculated.

### **5.3.3 Assessment of Lactate Dehydrogenase in Conditioned Culture Medium**

Conditioned culture media was collected after 48 hours culture (section 2.5.1). Necrotic cell death was quantified using a commercially available lactate dehydrogenase (LDH) kit according to the manufacturers' instructions (section 2.13.1.2). LDH activity was normalised to protein content as determined by the Lowry method (section 2.6).



**Figure 5.1** - Schematic diagram to illustrate the experimental protocol, indicating duration of exposure of BeWo cells to either 20 or 6% ambient O<sub>2</sub> and treatment with H<sub>2</sub>O<sub>2</sub>. Culture continued for a total of 48 hours.



#### **5.3.4 Assessment of Cell Viability**

The conversion of MTT to a formazan salt was used as a measure of BeWo cell viability as previously described (Al-Nasiry et al. 2006). Cells were seeded onto 96 well plates at densities of  $1 \times 10^5$ /well; 8 wells were cultured per experimental condition for between 6-8 separate passages. Following culture, the conversion of MTT was assessed as described in section 2.12.2.

#### **5.3.5 Assessment of hCG in Conditioned Culture Medium**

The secretion of hCG by BeWo cells was measured in conditioned culture medium using a commercially available radio-immunoassay (ICN Pharmaceuticals) as described in section 2.13.2. hCG expression was then normalised to cell protein measured using the Lowry method (section 2.6).

#### **5.3.6 Assessment of Cell Morphology and Fusion**

BeWo cells were cultured on sterile glass cover slips placed in 6-well plates seeded at a density of  $1.5 \times 10^6$  cells/well. Following culture, cells were fixed and immunocytochemistry performed as described in sections 2.5.3 and 2.8.3, respectively. Cell membranes were identified by immunostaining with monoclonal mouse anti-desmoplakin (Sigma-Aldrich, Clone ZK31, 19 $\mu$ g/ml). Immunostaining was visualised using confocal laser scanning microscopy (Biorad) and 10 images per field were taken. BeWo cell fusion, as defined by the presence of three or more nuclei contained within a single cell membrane, was quantified by two independent observers blinded to experimental conditions.

#### **5.3.7 Western Blotting**

20 $\mu$ g of cell lysate was subjected to 10% discontinuous SDS-PAGE and transferred to a PVDF membrane as described in section 2.7.3. After blocking, membranes were probed with mouse monoclonal antibodies to p53, Mdm2 or rabbit polyclonal antibody to Bax. Negative controls were achieved by omission of primary antibody. After incubation with an appropriate horseradish peroxidase conjugated secondary antibody resulting bands were visualised on photo-sensitive film (Amersham Biosciences Ltd) using enhanced chemiluminescence reagents (Pierce). Membranes were subsequently stripped and reprobed with mouse-monoclonal anti- $\beta$ -

actin (section 2.7.4). Densitometry was performed on a Bio-Rad 700 (BioRad) and bands of interest standardized against  $\beta$ -actin, which has been used previously to standardise protein expression in similar experiments (Bae et al. 2007).

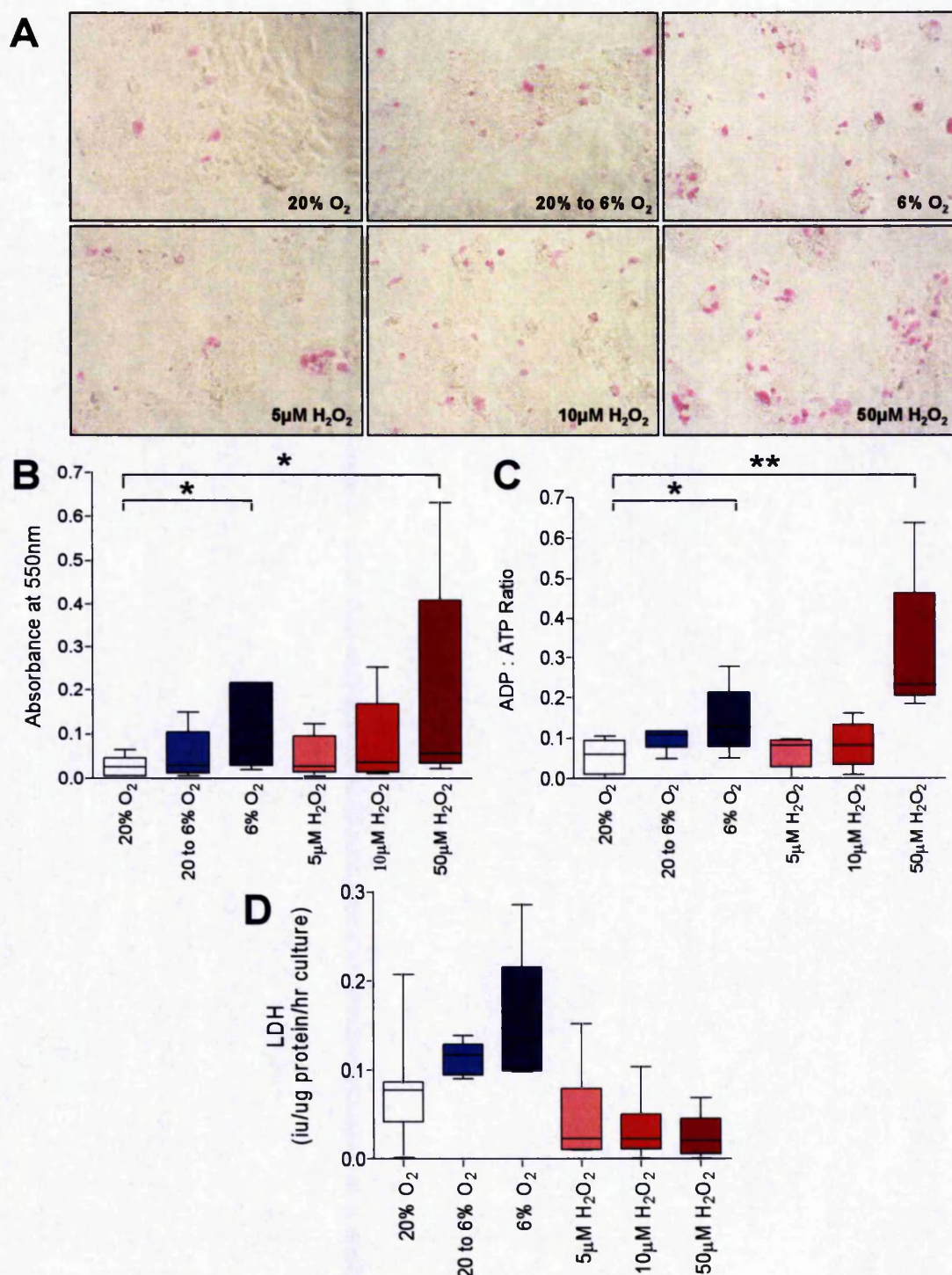
### **5.3.8 Statistical analysis**

As the number of experiments undertaken numbered between 5 and 9 passages, statistical significance was tested using either the Kruskal-Wallis test for unmatched non-parametric data or Friedman test for matched non-parametric data as normal distribution cannot be assumed. Friedman test was used where data for all experimental cultures were obtained from the same cell passage. Dunn's post-hoc test was performed as appropriate. Results are presented as median and range and plotted as box and whisker plots.

## **5.4 Results**

### **5.4.1 Effects of Culture in 6% O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> on Apoptosis in BeWo cells**

Dissolved O<sub>2</sub> tension measured in conditioned culture medium at 37°C after 24 hours incubation was  $149 \pm 3.2$  mmHg in 20% atmospheric O<sub>2</sub> and  $42.7 \pm 3.7$  mmHg in medium from 6% atmospheric O<sub>2</sub>. Images obtained following treatment with the Apopercantage method demonstrated that cells stained with the dye are condensed and rounded-up (Figure 5.2A). There appeared to be a greater proportion of positive stained cells in BeWo cells cultured in 6% O<sub>2</sub> and in the presence of 50  $\mu$ M H<sub>2</sub>O<sub>2</sub>. After cell lysis, there was increased absorbance in cells cultured in 6% O<sub>2</sub> and 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> indicating increased apoptosis (Figure 5.2B). This finding was confirmed by the ADP:ATP ratio (Figure 5.2C). Exposure to 6% O<sub>2</sub> for 24 hours or smaller doses of H<sub>2</sub>O<sub>2</sub> did not significantly increase the rate of apoptosis. Assessment of necrotic cell death by the measurement of LDH in conditioned culture medium demonstrated a wide range of LDH activity within conditioned culture medium from control conditions (Figure 5.2D). As a result, there was no statistically significant increase in LDH activity in conditioned culture medium from BeWo cells exposed to 6% O<sub>2</sub> or 5-50  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared to control.



**Figure 5.2** – Effect of culture in 6% O<sub>2</sub> and treatment with H<sub>2</sub>O<sub>2</sub> on apoptosis and necrosis of BeWo cells. (A) Apoptosis was increased in BeWo cells; there appeared to be more cells stained with pink Apopercantage™ dye following culture in 6% O<sub>2</sub> and 50μM H<sub>2</sub>O<sub>2</sub> compared to control. Cells stained with dye are rounded up - a characteristic feature of apoptosis. (B) Quantification of apopercantage staining confirmed increased staining in BeWo cells exposed to 6% O<sub>2</sub> and 50μM H<sub>2</sub>O<sub>2</sub>. (C) Increased apoptosis was confirmed by measurement of ADP:ATP ratio (\*\* p<0.01, \* p<0.05, Friedman test, n=5). (D) There was a large range of LDH secretion by cells under control conditions (20%O<sub>2</sub>), there was no significant increase in LDH release under any experimental conditions (n=5-11).

#### **5.4.2 Effect of Culture in 6%O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> on Viability, Differentiation and Fusion of BeWo Cells**

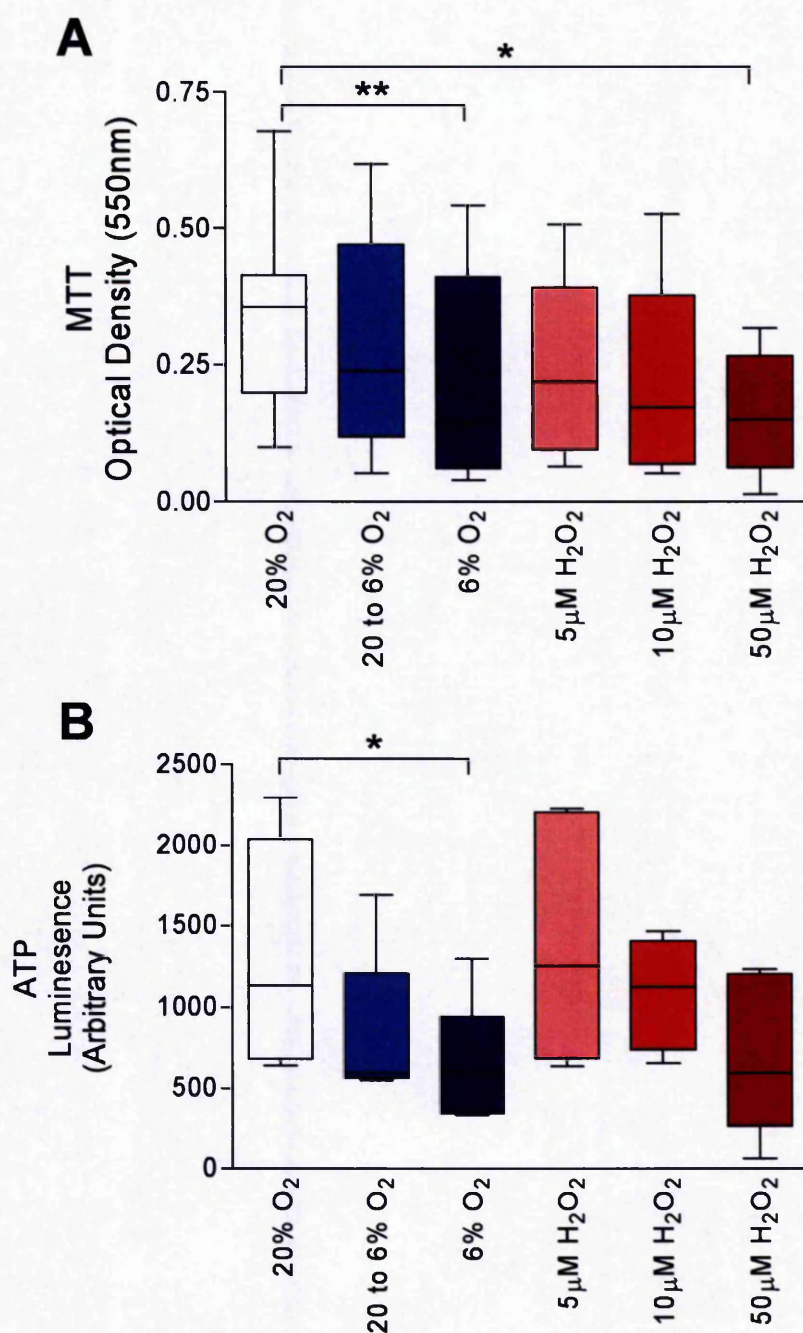
MTT conversion was reduced in cells exposed to 6% O<sub>2</sub> and 50µM H<sub>2</sub>O<sub>2</sub> (Figure 5.3A). A similar pattern was noted in the presence of ATP extracted from BeWo cells (Figure 5.3B). There was no effect of culturing cells in 5 or 10µM H<sub>2</sub>O<sub>2</sub> on either MTT or ATP assays. Transferring cells from 20% to 6% O<sub>2</sub> had no significant effect on reduction of MTT or ATP generation.

Culture in 6% O<sub>2</sub> and 50µM H<sub>2</sub>O<sub>2</sub> reduced the expression of hCG in conditioned culture media (Figure 5.4A). However, only culture in the presence of 5 or 50µM H<sub>2</sub>O<sub>2</sub> resulted in a significant decrease in formation of multinucleate BeWo cells, although there was a trend towards reduced multi-nucleation in BeWo cells cultured in 6% O<sub>2</sub> ( $p = 0.09$ ) (Figures 5.4B and 5.5). When all culture conditions were analysed together, hCG secretion correlated with the percentage of nuclei contained within multinucleate BeWo cells ( $r^2 = 0.33$ ,  $p < 0.0041$ ) (Figure 5.4C).

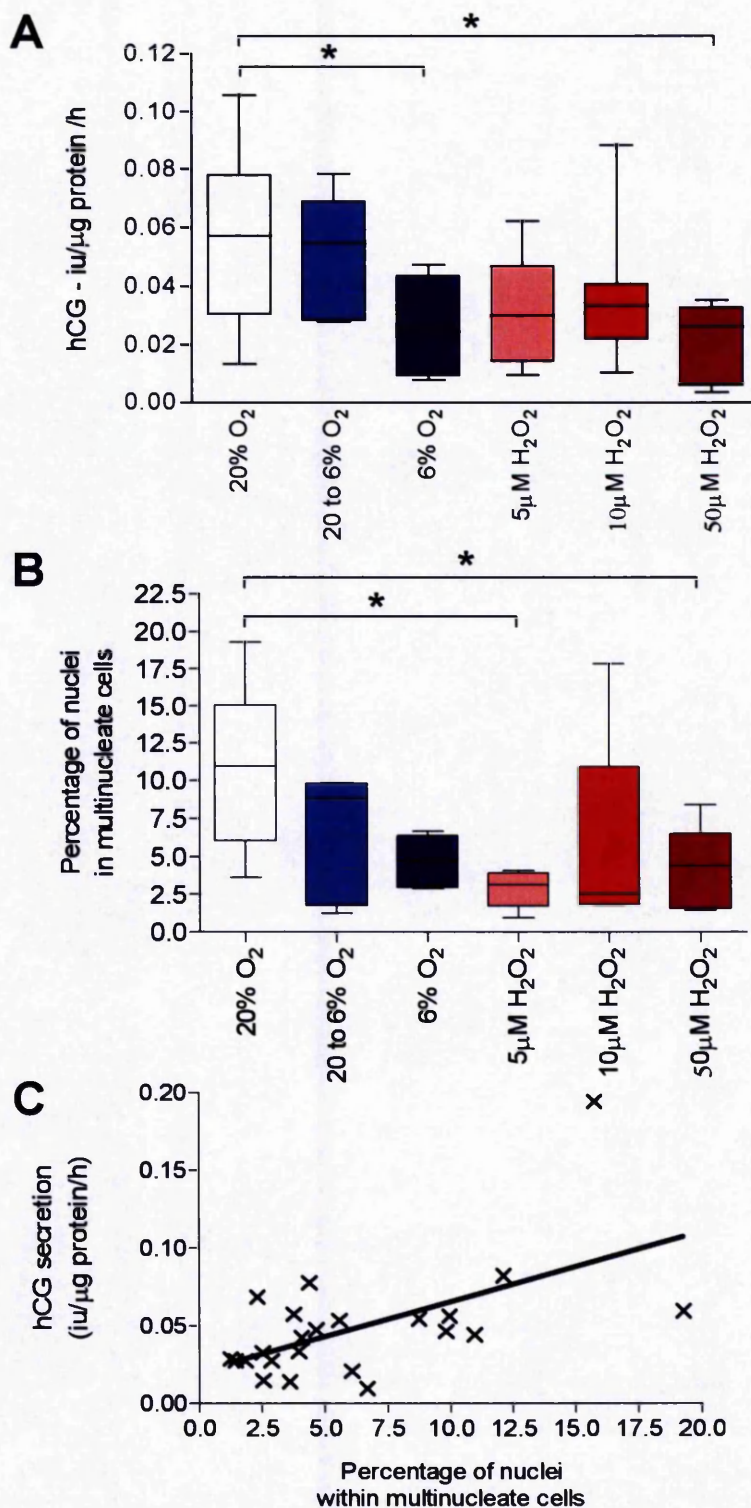
#### **5.4.3 Effect of Culture in 6%O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> on Protein Expression of p53 and Mdm2**

In BeWo cell lysate p53 expression appeared as a single band at approximately 53kD (Figure 5.6A). Densitometry demonstrated that exposure to 50µM H<sub>2</sub>O<sub>2</sub> was associated with increased expression of p53 (Figure 5.6B). This was associated with a decrease in Mdm2 expression (Figure 5.6A and C). Bax expression was not altered by treatment with 50µM H<sub>2</sub>O<sub>2</sub> (5.6A and D). The expression of p53, Mdm2 and Bax was not altered by culture in 6% O<sub>2</sub>.



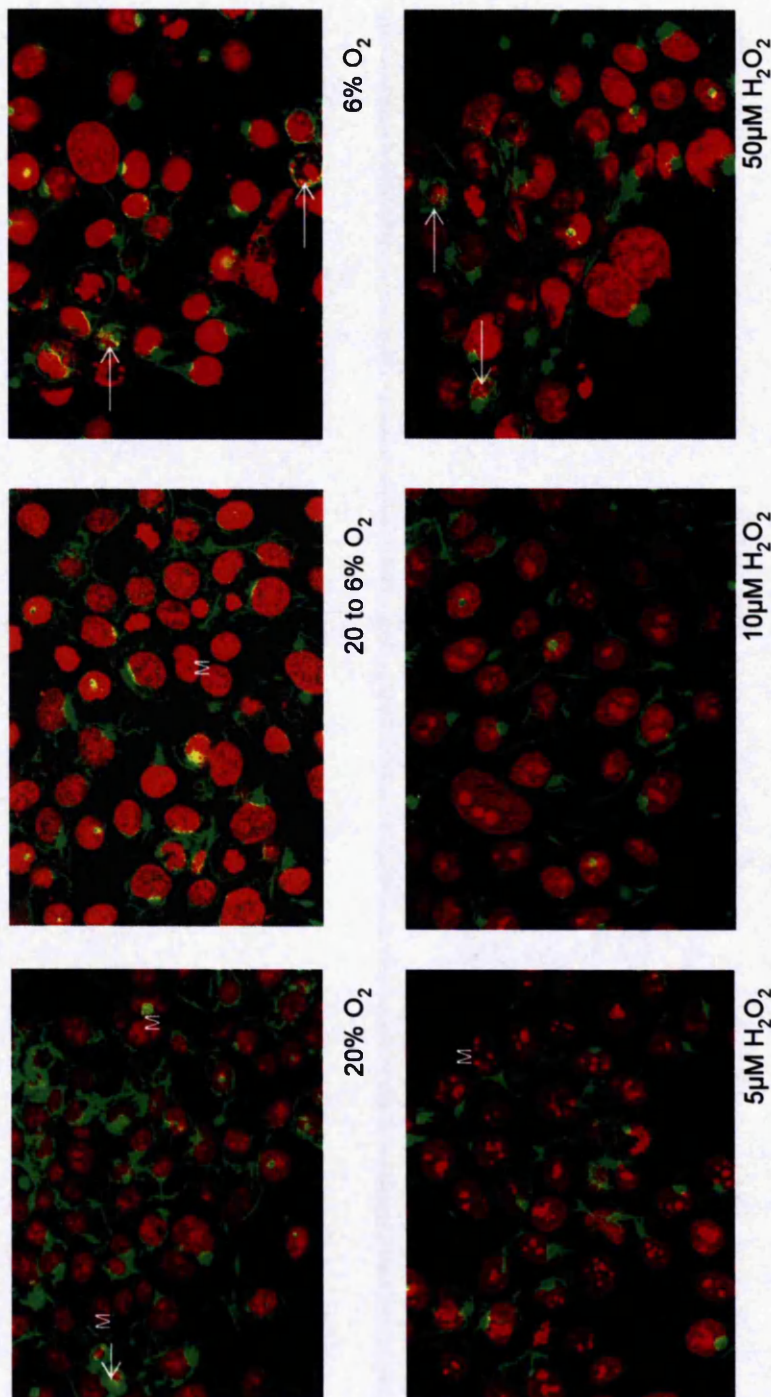


**Figure 5.3** – Effect of culture in 6% O<sub>2</sub> and treatment with H<sub>2</sub>O<sub>2</sub> on cell viability. (A) Absorbance at 550nm indicating reduction of MTT, demonstrating reduced viability of cells cultured in 6% O<sub>2</sub> and 50μM H<sub>2</sub>O<sub>2</sub> compared to control (20% O<sub>2</sub>) (\*\* p<0.01, \*p<0.05, Friedman test, n=6). (B) ATP as measured by luminescence was significantly reduced in cells cultured in 6% O<sub>2</sub> (\* p<0.05, Friedman test, n=5).

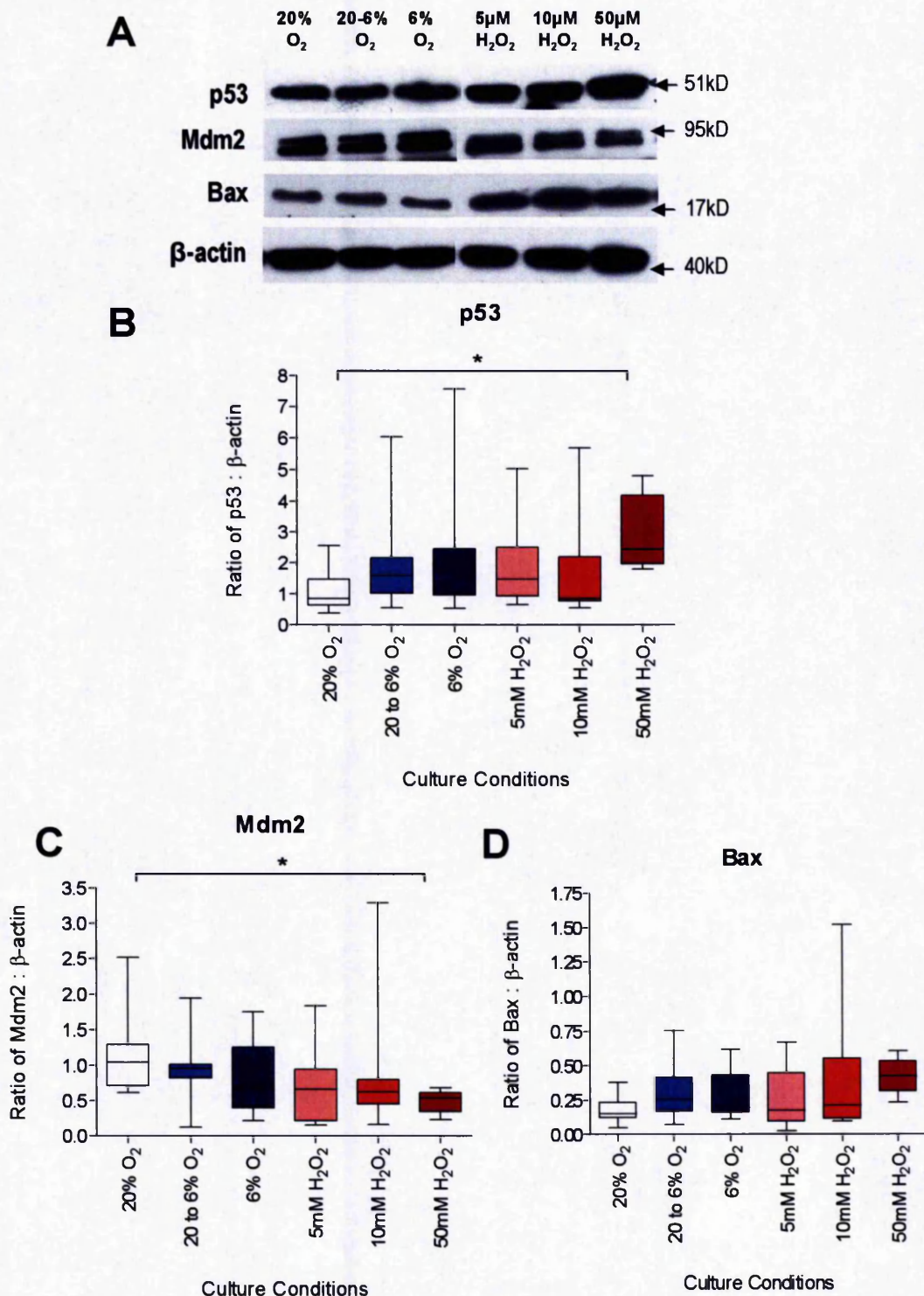


**Figure 5.4** – Effect of culture in 6% O<sub>2</sub> and treatment with H<sub>2</sub>O<sub>2</sub> on differentiation and fusion of BeWo cells. (A) The presence of hCG was reduced in conditioned culture media taken from BeWo cells cultured in 6% O<sub>2</sub> and exposed to 50μM H<sub>2</sub>O<sub>2</sub> (\* p<0.05, Kruskal Wallis test, n=5-10). (B) The percentage of nuclei contained in multinucleate cells, defined as 3 or more nuclei enveloped by a single membrane, was significantly reduced by culture in 6% O<sub>2</sub> and 50μM H<sub>2</sub>O<sub>2</sub> (\* p<0.05, Kruskal Wallis test, n=5-9). (C) There was a weak correlation between hCG secretion and the proportion of nuclei in multinucleate cells in all culture environments investigated.





**Figure 5.5** – BeWo cell morphology assessed by immunocytochemistry using anti-desmoplakin antibody. BeWo cells cultured in control conditions show evidence of multinucleation (marked M). A small proportion of cells cultured in control conditions (20% O<sub>2</sub>), and a greater number of cells cultured in 6% O<sub>2</sub> and 50μM H<sub>2</sub>O<sub>2</sub> demonstrate some morphological features of apoptosis such as increased nuclear : cytoplasmic ratio (marked with open arrows).



**Figure 5.6** – Protein expression of p53, Mdm2 and Bax in BeWo cells cultured in 6% O<sub>2</sub> and treated with H<sub>2</sub>O<sub>2</sub>. (A) Representative Western blots for p53, Mdm2 and Bax. β-actin expression was not altered, indicating equal protein loading. Western Blotting for p53 showed the presence of single band at 53kD. (B) p53 was increased in cells treated with 50μM H<sub>2</sub>O<sub>2</sub> (\*p<0.05, Kruskal-Wallis test, n=5-9). (C) The 90kD isoform of Mdm2 appeared as a doublet band and was reduced in cells exposed to 50μM H<sub>2</sub>O<sub>2</sub> (\*p<0.05, Kruskal-Wallis test, n=5-9). (D) Bax expression was not altered by 6% O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> (n=5-9).

## 5.5 Discussion

These experimental data demonstrate that BeWo cells undergo apoptosis following culture in 6% O<sub>2</sub> and ROS in the form of 50µM H<sub>2</sub>O<sub>2</sub>. These data are strengthened by two different assessments of apoptotic cell death, specifically, the disruption of the phospholipid membrane and the ADP:ATP ratio. In addition, the findings of these assays were in agreement with the observations of cell morphology using phase contrast and confocal microscopy. The increase in apoptosis did not appear to be related to increased necrosis. Although there was a trend towards increased LDH expression in culture medium from BeWo cells cultured in 6% O<sub>2</sub>, these changes are moderate in comparison to those observed in widespread cell necrosis, in which up to a 3000% increase may be observed (S.L.Greenwood, personal communication). Therefore, rather than necrosis, this observation may reflect increase in LDH transcription, which is promoted in choriocarcinoma cell-lines by hypoxic culture conditions (Kay et al. 2007). The increase in apoptosis following culture in 6% O<sub>2</sub> demonstrates that a comparatively small reduction in oxygen tension can induce apoptosis. Other studies of BeWo cell turnover have used lower oxygen tensions between 1%-5% O<sub>2</sub> (Kudo et al. 2003b; Meissner et al. 2003; Hayashi et al. 2005). Interestingly, HIF-1α expression is increased in BeWo cells cultured for 2h in 5% O<sub>2</sub>, indicating mild hypoxia can induce changes in O<sub>2</sub> sensing proteins which may elicit downstream effects (Hayashi et al. 2005). Differences in cell turnover described in other studies may result from the density at which cells are plated; MDA-MB-231 (A breast cancer cell-line) cells show resistance to apoptosis at higher densities, with confluent cells being more resistant than sparsely plated cells (Fang et al. 2007). These differences relate to the basal expression of HIF-1α, with confluent cells again having the greatest expression, which in this cell line was protective against apoptosis.

It appears that despite having origins in first trimester trophoblast, normally exposed to <20mmHg (<2.6% O<sub>2</sub> assuming 1atm = 760 mmHg), BeWo cells have adjusted to regard 20% O<sub>2</sub> (152 mmHg) as normoxic conditions. This may result from their original isolation from a choriocarcinoma metastasis which occurred in 21% O<sub>2</sub> (Pattillo and Gey 1968). Indeed, 20% O<sub>2</sub> is used as control culture conditions in the majority of published data (Kudo et al. 2003b; Meissner et al. 2003; Hayashi et al. 2005; Bae et al. 2007; Nampoothiri et al. 2007). Therefore,

in common with native trophoblast, BeWo choriocarcinoma cells undergo apoptosis in response to decreased O<sub>2</sub> tension (Levy et al. 2000; Kilani et al. 2003).

In agreement with previous data these experiments demonstrate that despite being derived from choriocarcinoma, BeWo cells retain the ability to undergo apoptosis in response to stimuli capable of inducing apoptosis in other cell-types such as zinc-citrate compound (Bae et al. 2007), TNF $\alpha$  (Al-Nasiry et al. 2006), and galectin-1 (Wiest et al. 2005).

The increase in apoptosis following culture in 6% O<sub>2</sub> and in the presence of 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with a decrease in the conversion of MTT. The MTT assay was developed as a measure of proliferation in homogenous cell populations, and the reduction of MTT by the mitochondria is thought to reflect cell number. In these experiments, similar patterns in MTT assay and ATP production were noted, indicating that both may represent mitochondrial function. This finding questions whether MTT conversion may relate solely to mitochondrial activity which will be affected by periods of anaerobic respiration or oxidative stress. In BeWo cells, fusion may affect the MTT assay. These concerns have been addressed by investigation of BeWo fusion stimulated by forskolin or epidermal growth factor (EGF); in these studies, fusion was associated with a decrease in MTT conversion and in Mib-1, a marker of proliferation, suggesting that MTT was proportional to proliferative activity (Al-Nasiry et al. 2006). However, a decrease in proliferation may either result from a decrease in cell viability or terminal differentiation. As relatively low levels of fusion were observed in the experiments presented here, it may be concluded that MTT conversion probably provides a reflection of cell number and thereby proliferation.

The altered BeWo cell turnover was also reflected in cell fusion and differentiation. Previous studies have utilised BeWo cells as a model of syncytium formation, which is markedly increased by the addition of forskolin (Kudo et al. 2003b; Hu et al. 2007; Nampoothiri et al. 2007). Following stimulation with forskolin, BeWo cell differentiation, as measured by cell fusion and hCG secretion, was reduced by hypoxia (Kudo et al. 2003a; Kudo et al. 2003b; Hu et al. 2007). In this experiment hCG secretion was reduced by culture in 6% O<sub>2</sub> compared to 20% O<sub>2</sub>, although there was not a concurrent fall in multi-nucleation, which suggests that morphological and biochemical differentiation may be separated by culture in 6% O<sub>2</sub>. However, in agreement with previous data an association between hCG secretion and the percentage of nuclei contained within multinucleate cells was observed. The inconsistencies between these



data and those reported in the literature may reflect the low percentage of multinucleate cells, approximately 10%, compared to studies using forskolin which report up to 80% of nuclei within multinucleate syncytia (Nampoothiri et al. 2007).

In BeWo cells treated with 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> the induction of apoptosis does not appear to be an isolated event and is associated with decreased proliferation and ultimately the formation of a multinucleate syncytium. Therefore, it may be speculated that apoptosis may not only be a phenomenon reflecting widespread damage to the villous trophoblast, but may be an essential component of the aberrant cell turnover observed in pre-eclampsia. In support of this hypothesis, apoptosis and proliferation are inversely related in placental villi from normal first and third trimester pregnancies suggesting these events are linked in vivo (Chan et al. 1999; Yamada et al. 2001).

It is interesting that the response of BeWo cells to decreased O<sub>2</sub> tension and H<sub>2</sub>O<sub>2</sub> are similar to findings in other models of trophoblast cell turnover. For example, the fall in hCG secretion at high doses of H<sub>2</sub>O<sub>2</sub> concurs with data from isolated trophoblast, although in that model lower doses of H<sub>2</sub>O<sub>2</sub> promoted hCG secretion which was not observed in these experiments (Kharfi Aris et al. 2007). Furthermore, the reduction in hCG secretion in response to lowering O<sub>2</sub> from 20% and 6% O<sub>2</sub> is similar to that reported in normal term placental villous explants (Crocker et al. 2004c). In addition, oxidative stress in the form of hypoxia and hypoxia-reoxygenation also induces apoptosis in primary trophoblast cells and placental villous explants (Levy et al. 2000; Hung et al. 2002). Therefore, despite arising from a choriocarcinoma, BeWo cells share some characteristics with native trophoblast, and may provide a useful model for investigating the response of trophoblast to noxious stimuli.

In agreement with previous data we found that p53 was expressed by BeWo cells (Haidacher et al. 1995). Apoptosis induced by exposure to 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with an increase in expression of p53 and Bax and a decrease in Mdm2 protein expression. However, this was not the case for the increased apoptosis observed following culture in 6% O<sub>2</sub>. Extrapolating data from BeWo cells treated with Mdm2 siRNA (Chapter 4), it is speculated that the observed increase in p53 protein may result from reduced degradation in the presence of a reduction in Mdm2. The increased apoptosis observed following culture in 6% O<sub>2</sub> may be induced via a p53-independent mechanism. Alternatively, p53 and Mdm2 and their ability to regulate apoptosis may be impaired in BeWo cells, as both p53 and Mdm2 expression is increased in



choriocarcinoma compared to normal first trimester villous tissue (Fulop et al. 1998; Qiao et al. 1998). However, p53 has been sequenced in molar pregnancies and choriocarcinoma and found to be of wild-type (Cheung et al. 1994b; Shi et al. 1996; Fulop et al. 1998). In support of a p53-independent response to lowered O<sub>2</sub> BeWo cells express many other regulators of apoptosis including Bcl-2, p21 and pRb, indicating that many components of the intrinsic apoptotic pathway are present in this cell type (Bae et al. 2007; Nampoothiri et al. 2007).

Several studies have concluded that BeWo cells are a useful model to study trophoblast cell turnover. Using this model we have demonstrated that ROS and hypoxic stimuli, both of which are implicated in the development of pre-eclampsia, can induce apoptosis *in vitro*. In the case of ROS, these changes are observed at a concentration present in maternal serum (50µM) (Kharfi et al. 2005). Furthermore, these changes are associated with an increase in p53 expression similar to that described in villous trophoblast from pregnancies complicated by pre-eclampsia as described in chapter 3. Although findings of studies in choriocarcinoma cell-lines must be extrapolated with caution, these experiments provide preliminary data that oxidative stress, such as that implicated in the pathogenesis of pre-eclampsia can induce alterations in trophoblast cell turnover that if present in villous trophoblast could be deleterious to placental function.

In the third trimester, tertiary placental villi contain many different cell types including trophoblast, endothelial cells, macrophages, and fibroblasts. When cytotrophoblasts are cultured in isolation they are deprived of cell-cell interactions which may alter cell phenotype and/or the response to damaging stimuli. Culture of placental villous fragments or explants, in which trophoblast retains cell-cell contacts, has been used to investigate the effects of hypoxia, hyperoxia and hypoxia-reoxygenation on cell-turnover of villous trophoblast (Siman et al. 2001; Hung et al. 2002; Crocker et al. 2004c). Therefore, this model has been utilised for further investigation of the effects of altered oxygen tension or ROS on trophoblast turnover and expression of constituents of the p53 pathway.

## **Chapter 6 – Does Altered Oxygen Tension Alter Cell Turnover and Expression of Regulators of Apoptosis in Human Term Placental Villous Explants?**

### **6.1 Introduction**

As already described, the syncytiotrophoblast is maintained by the continuous proliferation and subsequent fusion of underlying cytotrophoblasts (Huppertz and Kingdom 2004). Following fusion, the nuclei progressively degenerate, and gather together into syncytial knots, some of which are subsequently lost into the maternal circulation (Mayhew et al. 1999). Aberrant cell turnover, particularly apoptosis, is thought to have a role in the origins of pre-eclampsia (Allaire et al. 2000; Leung et al. 2001; Ishihara et al. 2002). Increased apoptosis is thought to result from exposure to hypoxia or ROS resulting from a failure of conversion of maternal spiral arteries into low resistance high flow vessels (Meekins et al. 1994), hypothetically reducing the delivery of O<sub>2</sub> to the intervillous space.

In normal pregnancy the villous trophoblast is exposed to wide variations in O<sub>2</sub> tension during placental development, ranging from a partial pressure of O<sub>2</sub> (pO<sub>2</sub>) of <20 mmHg (<2.6% assuming 1 atm = 760 mmHg) in the intervillous space at 8 weeks gestation, to 50mmHg (6.5%) at week 12 (Jauniaux et al. 2000) and 66 mmHg at term (8.6%) (Fujikura and Yoshida 1996; Schaaps et al. 2005). Due to its potential regulatory role in normal placental development and pathogenesis of pre-eclampsia, the effects of O<sub>2</sub> on villous trophoblast structure and function have been investigated for many years. When appraising studies of the effects of O<sub>2</sub> on trophoblast it is essential to evaluate three features (i) the type and (ii) the gestational age of trophoblast used and (iii) the pO<sub>2</sub> employed in the experiments. The type of trophoblast alters the response to O<sub>2</sub>, for example, invasive extravillous trophoblast undergoes proliferation in response to hypoxia (15.2 mmHg, 2%) and differentiates when exposed to higher O<sub>2</sub> concentrations (152 mmHg, 20%) (Genbacev et al. 1997). Whereas, BeWo cells and term villous explants exhibit decreased proliferation and formation of syncytium in 15.2 mmHg (2%) compared to 152 mmHg O<sub>2</sub> (20%) (Kudo et al. 2003b; Crocker et al. 2004c). The ability of hypoxia to induce changes in cell turnover also alters with gestation, villous explants from first trimester placentas cultured in a pO<sub>2</sub> of 22mmHg (3%) had less apoptosis than those cultured

in 152 mmHg (20%) (Huppertz et al. 2003). However, severe hypoxia <15 mmHg (< 2%) induced apoptosis in isolated villous cytotrophoblasts from term placentas (Levy et al. 2000; Kilani et al. 2003). In addition to the type of trophoblast and gestational age, the pO<sub>2</sub> used in experiments must be evaluated. Early studies undertaken prior to the measurement of pO<sub>2</sub> in the IVS often used supra-physiological O<sub>2</sub> conditions from 197.6 to 722 mmHg (26-95%), regarding culture in 45.6 mmHg (6%) as hypoxic (Fox 1970; Amaladoss and Burton 1985). Evaluation of the effects of O<sub>2</sub> tension is particularly important in studies of term trophoblast turnover, as apoptosis is induced by culture in supraphysiological O<sub>2</sub> tension (Reti et al. 2007). In multi-cellular tissues, such as placental villi, cell turnover is tightly regulated by many proteins. Cell damage such as insults from hypoxia or oxidative stress may lead to increased apoptosis and decreased proliferation, these changes may be associated with expression of p53, such as that observed in BeWo cells exposed to ROS (Chapter 5) or in primary term cytotrophoblasts cultured in 15.2 mmHg O<sub>2</sub> (Levy et al. 2002). Culture of isolated cytotrophoblast cells and villous explants in hypoxic conditions is associated with increased expression of HIF1 $\alpha$  (Hayashi et al. 2005) which can exert downstream effects on p53 and Mdm2 expression (An et al. 1998; Nieminen et al. 2005). NDRG-1, another hypoxia-responsive gene also increases p53 in isolated term trophoblasts (Chen et al. 2006). This suggests that p53 may be up-regulated in trophoblast by more than one mechanism following culture in low O<sub>2</sub>. Downstream effects of the p53 pathway have also been described in isolated first trimester trophoblast cultured in 14mmHg (1.8%) O<sub>2</sub>, leading to an increase in pro-apoptotic Bax and a reduction in Bcl-2 (Hu et al. 2006b). Therefore, culture of trophoblast in hypoxic conditions is frequently associated with an increase in apoptosis and an imbalance in regulatory proteins of the intrinsic pathway. However, apoptotic cell death may not be the only form of cell death in trophoblast following culture in low O<sub>2</sub>. Culture in 15.2mmHg (2%) O<sub>2</sub> was associated with increased necrotic cell death of the syncytiotrophoblast (Huppertz et al. 2003).

In spite of a large volume of research investigating the role of O<sub>2</sub> in regulating trophoblast cell turnover, few studies have investigated effects of O<sub>2</sub> tension on third trimester villous explants, and those that have been conducted have not investigated the expression of constituents of the p53-pathway. As p53 expression is altered in pre-eclampsia, its regulation merits further investigation. Since altered oxygenation is hypothesised to play an important role in the development of abnormal cell turnover of villous trophoblast in pre-eclampsia, these conditions

may also alter p53 expression. To investigate the expression of components of the p53-pathway in response to altered oxygenation these studies have employed a tissue culture model previously used to investigate the reaction of placental tissue to an altered culture environment.

This culture model was developed from initial observations that after degeneration of the original syncytiotrophoblast after 24-48 hours, a new functional syncytial layer is reformed from 72 hours onwards (Siman et al. 2001). Unlike isolated trophoblasts, this model allows the study of trophoblast cell turnover in the presence of cell-cell interactions present in vivo, and has previously been used to study proliferation and differentiation of cytotrophoblasts over a 7 day culture period (Siman et al. 2001; Crocker et al. 2004b). In these investigations a 96 hour culture period was employed, as previous studies using this in vitro model have reported differences in necrosis, proliferation and differentiation in response to altered O<sub>2</sub> tension at this time (Siman et al. 2001; Crocker et al. 2004b; Crocker et al. 2004c).

## **6.2 Hypothesis and Aims**

It is hypothesised that alterations in atmospheric O<sub>2</sub> tension will result in changes of cell turnover in term placental villous explants. In addition, it is hypothesised that changes in cell turnover will be associated with altered expression of proteins in the p53 pathway.

This investigation aims to comprehensively assess the effects of altered O<sub>2</sub> tension on cell turnover of term placental tissue including: proliferation, differentiation, apoptosis, necrosis and formation of syncytial knots as well as expression and localisation of regulators of apoptosis.

## **6.3 Methods**

### **6.3.1 Preparation of Placental Explants**

Ethical approval was granted by Central Manchester local research ethics committee and all participants provided informed written consent. Placentas from uncomplicated term pregnancies (n=9) were collected within 20 minutes of delivery. Placental villous explants (each 5mg approx) were prepared from 3 randomly selected areas of placenta as described in section 2.2.1.1. Fresh villous tissue was processed for protein and mRNA expression (sections 2.3.1 and 2.3.2 respectively) as well as being fixed for immunohistochemistry (section 2.3.3.1)

and electron microscopy (section 2.3.3.2) to enable comparisons between fresh and cultured tissue.

3 explants were cultured on each Netwell support for 96 hours in CMRL-1066 culture medium supplemented with antibiotics, insulin (1mg/l), hydrocortisone (0.1mg/l), retinol acetate (0.1mg/l) and 10% FCS. For each condition, 3 Netwells were used to culture explants for later immunohistochemistry (9 explants), 3 for RNA extraction (9 explants) and 6 for homogenisation prior to Western blotting (18 explants). To maximise exposure to atmospheric O<sub>2</sub> tension explants were suspended at the gas-liquid interface as previously described at 37°C; CO<sub>2</sub> was maintained at 5% (Crocker et al. 2004b; Crocker et al. 2004c) (section 2.2.3.1). The culture medium was changed after 48h and prior to this the medium was equilibrated in the appropriate O<sub>2</sub> conditions for 24 hours (Newby et al. 2005). Explants cultured in 1% O<sub>2</sub> remained within this environment whilst cultured medium was changed. O<sub>2</sub> tension was measured in situ at 37°C using a digital meter (Strathkelvin, Glasgow, UK).

After 96 hours, tissue was processed for protein and mRNA expression, fixed for immunohistochemistry and electron microscopy (sections 2.3.3.1 and 2.3.3.2 respectively) and conditioned culture medium collected (section 2.3.4).

### **6.3.2 Western Blotting**

Western blotting was carried out according the method described in section 2.7.3; in these experiments separate blots were used for each protein of interest. Briefly, 40µg of tissue lysate was subjected to 10% discontinuous SDS-PAGE and transferred to a PVDF membrane. After membranes were blocked they were probed with antibodies to either: p53, Mdm2, Bax, Bcl-2, Myosin Light Chain, β-actin, or HP1α. Membranes were then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase. Resulting bands were visualised on photo-sensitive film (Amersham Biosciences Ltd) using enhanced chemiluminescence reagents (Pierce). Densitometry was performed on a Bio-Rad 700 (BioRad) and bands of interest standardized against constitutively expressed proteins (β-actin, MLC, HP1α) which were unchanged in response to oxygen in these studies. β-actin has also been used previously to standardise protein expression in similar experiments (Levy et al. 2000) and does not alter with O<sub>2</sub> tension (Alsat et al. 1996).



### **6.3.3 Immunohistochemistry**

Tissue sections were deparaffinised and exposed to microwave pre-treatment as described in section 2.8.2. Endogenous peroxidase activity was quenched and non-specific binding was blocked as described. Where mouse monoclonal antibodies were used, tissue sections were exposed to antibodies against p53, Mdm2, p21, Bax or Bcl-2. Each tissue was incubated with a matching concentration of non-immune mouse IgG to serve as negative controls. Sections were then probed with appropriate biotin conjugated secondary antibodies, followed by incubation with avidin-peroxidase. Immunostaining was revealed by exposure to concentrated DAB for 3-5 minutes. Slides were counterstained with methyl green or haematoxylin and sections viewed using a Leitz microscope with ImageProPlus 4.5 (Media Cybernetics Inc). All samples for comparison were stained in the same batch.

### **6.3.4 Real-Time Quantitative PCR**

Total RNA was extracted and described in section 2.9.1.2. cDNA synthesis was performed as described in section 2.9.2. mRNA expression was quantified using specific primers for p53, Mdm2, p21 and Bax and SYBR Green I with melt curve analysis to ensure amplification specificity as detailed in section 2.9.4. To quantify expression, Ct values were used to calculate a standard curve constructed from cDNA derived from human reference RNA and samples normalized to the calibrator sample as previously described (Lacey et al. 2005). All assays were between 95 and 104% efficiency.

### **6.3.5 Assessment of Apoptosis**

Apoptosis was assessed using a commercially available TUNEL kit (Roche Applied Diagnostics) with modifications to the manufacturer's instructions as detailed in section 2.10.1. TUNEL staining was quantified by assessing 10 random fields of terminal villi per experimental condition. The number of TUNEL positive nuclei were counted manually, and the total number of nuclei measured using sequential colour thresholding as previously described (Crocker et al. 2004a). A ratio was derived from these values; the TUNEL index (% of total nuclei TUNEL positive). The presence of apoptosis was confirmed by immunostaining for cytokeratin-18 M30 neo-epitope as described in section 2.10.2.

### **6.3.6 Assessment of Syncytial Knots**

5µm tissue sections were stained with haematoxylin and eosin as described in section 2.11. The number of syncytial knots defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface (Cantle et al. 1987) was counted manually in 10 fields of view for each experimental condition. The trophoblast area was measured by sequential colour thresholding using image analysis software (Image ProPlus, Mediacybernetics) and data normalised to give a measure of the number of syncytial knots per mm<sup>2</sup> of villus.

### **6.3.7 Assessment of Proliferation**

Cell proliferation was assessed using a mouse monoclonal anti-Ki-67 antibody (Clone Mib-1) as described in section 2.12.1. Ki67 staining was quantified in 10 randomly selected fields of terminal villi per experiment. The number of Ki67 positive nuclei were counted manually and the total number of nuclei measured by sequential colour thresholding. A ratio was derived; the proliferative index (% of total nuclei Ki67 positive).

### **6.3.8 Electron Microscopy**

Randomly selected explants from each experimental condition were fixed and treated for electron microscopy as described in section 2.14. Semi-thin sections, 0.5µm thick, were inspected to identify areas of interest. Multiple areas of ultrathin sections were examined by two observers using a Philips CM10 electron microscope at an accelerating voltage of 80 kV. Representative areas were then photographed.

### **6.3.9 Analysis of Conditioned Culture Media**

Necrosis was assessed by the presence of LDH and actin in conditioned culture medium using an enzyme-linked assay as described in section 2.13.1.1. The intra-assay variabilities for these assays were 12.67% and 6.85%, respectively. Trophoblast differentiation was assessed by the measurement of hCG in the culture medium, using a commercial quantitative immunoradiometric assay in accordance with the manufacturers' instructions (ICN Pharmaceuticals; Basingstoke, UK) as detailed in section 2.13.2, the intra-assay variability was 9.0%. Further evidence of trophoblast differentiation was assessed by the presence of human

placental lactogen (hPL) and alkaline phosphatase, both of which are secreted by the syncytiotrophoblast (sections 2.13.3 and 4). Both proteins were assessed by commercially available ELISA techniques according to manufacturers' instructions, the intra-assay variabilities were 12.3% and 4.65% respectively. Placental protein 13 (PP-13) is synthesized by the syncytiotrophoblast and may be either actively or passively released. PP13 was also quantified using an ELISA technique as described in section 2.13.5. For this assay intra-assay variability was 2.26%.

#### **6.3.10 Statistical analysis**

As tissue from each placenta was cultured at 20%, 6% or 1% O<sub>2</sub>, statistical significance was tested using the Friedman test for matched non-parametric data. Dunn's post-hoc test was performed as appropriate. Results are presented as median and interquartile range, and plotted as box and whisker plots (Box = interquartile range, Whiskers = total range). A p-value of 0.05 was regarded as statistically significant.

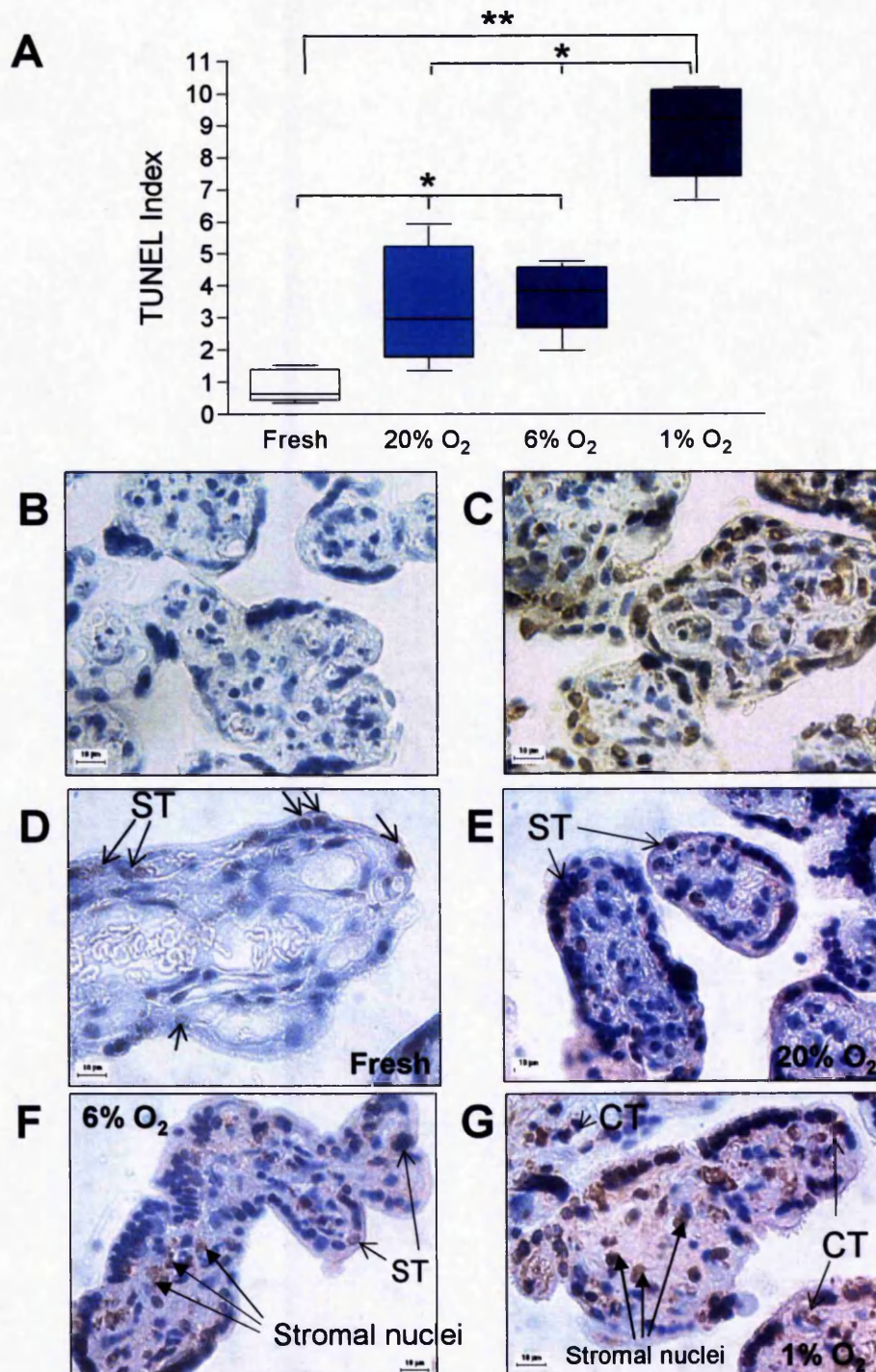
### **6.4 Results**

#### **6.4.1 Effect of Culture in 20%, 6% and 1% atmospheric O<sub>2</sub> on O<sub>2</sub> tension in Culture Media.**

O<sub>2</sub> tension within culture media after 96 hours culture in 20% atmospheric O<sub>2</sub> was 150.1 mmHg  $\pm$  5.0 mmHg (Mean  $\pm$  SD), in 6% atmospheric O<sub>2</sub> was 70.5 mmHg  $\pm$  7.5 mmHg, and in 1% atmospheric O<sub>2</sub> was 20.2 mmHg  $\pm$  8.6 mmHg.

#### **6.4.2 Effects of O<sub>2</sub> Tension on Apoptosis**

The TUNEL index was increased in all cultured explant tissues after 96 hours incubation compared to fresh tissue (Figure 6.1A). Apoptosis was increased by culture in 1% O<sub>2</sub> compared to 20% and 6% O<sub>2</sub> (Figure 6.1A). TUNEL was specific for detection of DNA strand breaks as no staining was seen in the negative control (Figure 6.1B) and the positive control showed strong staining in many component nuclei (Figure 6.1C). In fresh tissue and explants cultured in 20% and 6% O<sub>2</sub>, the majority of TUNEL positive nuclei were seen within the syncytiotrophoblast and occasionally in the stroma (Figures 6.1D-G). In explants cultured in 1% O<sub>2</sub> there was additional TUNEL staining in cytotrophoblast and stromal cells (Figure 6.1G).



**Figure 6.1** – Assessment of apoptosis in placental explants exposed to altered O<sub>2</sub> conditions. (A) Histogram showing apoptotic cell death, as assessed by TUNEL, was increased in explants cultured in 20% and 6% O<sub>2</sub>, compared to fresh tissue (\* p<0.05). Explants cultured in 1% O<sub>2</sub> had significantly greater apoptosis than fresh tissue or explants cultured in 20% or 6% O<sub>2</sub> (\* p<0.05, \*\* p<0.01, Friedman test). (B) Negative control for TUNEL demonstrating no immunostaining. (C) TUNEL positive control incubated with DNase I showing positive staining in many component nuclei. (D) TUNEL staining in fresh tissue is localised to occasional syncytial nuclei. (E) TUNEL staining in explants cultured in 20% O<sub>2</sub> and (F) 6% O<sub>2</sub> demonstrate increased apoptosis in the syncytiotrophoblast (ST). (G) Explants cultured in 1% O<sub>2</sub> showed TUNEL positive nuclei in ST, cytotrophoblast (CT) and stromal cells.

This distribution of apoptotic cells was confirmed by the presence of cytokeratin M30, which was present in discrete areas of syncytiotrophoblast of fresh tissue (Figure 6.2A) and was increased in the syncytiotrophoblast of explants cultured at 20% and 6% O<sub>2</sub> (Figures 6.2B and C). Explants cultured in 1% O<sub>2</sub> had M30 staining in cytotrophoblasts and stromal cells but minimal staining in the syncytiotrophoblast (Figure 6.2D).

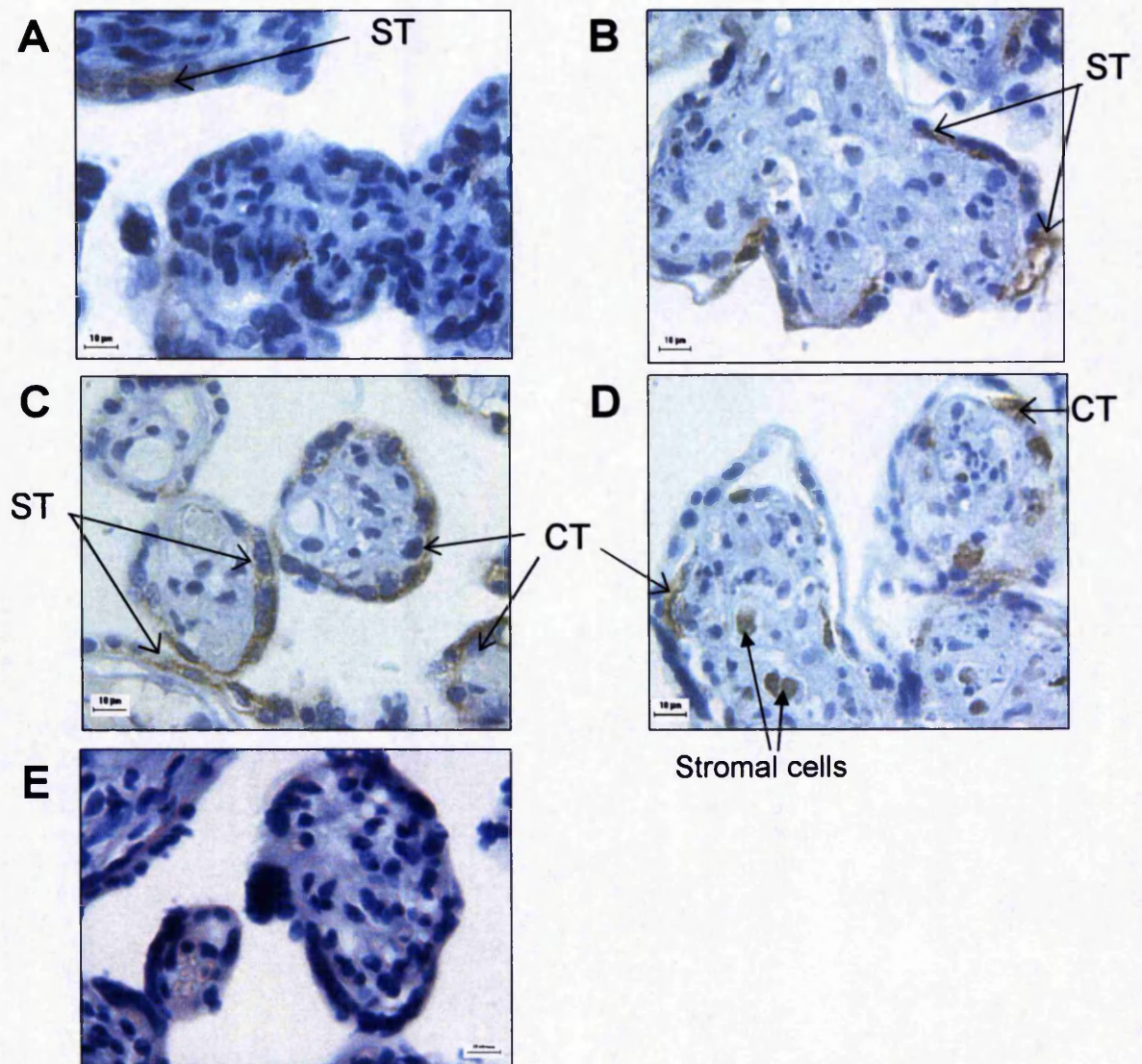
In contrast to changes in apoptosis, the estimated numerical density of syncytial knots was similar in explants cultured in 6% O<sub>2</sub> and fresh tissue (Figure 6.3A). However, the density of syncytial knots was significantly increased in explants exposed to 1% and 20% O<sub>2</sub>. Following culture, syncytial knots appeared to contain more densely packed pyknotic nuclei compared to those in fresh tissue (Figure 6.3 B-D).

#### **6.4.3 Effects of O<sub>2</sub> tension on Proliferation and Differentiation**

In all tissue, Ki-67 staining was almost exclusively localised to cytotrophoblasts, identified as morphologically large mononuclear cells with a large nucleus lying immediately underneath the syncytiotrophoblast. Placental explants cultured in 6% O<sub>2</sub> had a similar proliferative index to fresh tissue (Figure 6.4A). The proliferative index was increased in explants cultured in 20% O<sub>2</sub> compared to 6% O<sub>2</sub>. In contrast, the proliferative index was decreased in placental explants cultured in 1% O<sub>2</sub>, with very little Ki67 staining visible in villous nuclei (Figures 6.4B-E).

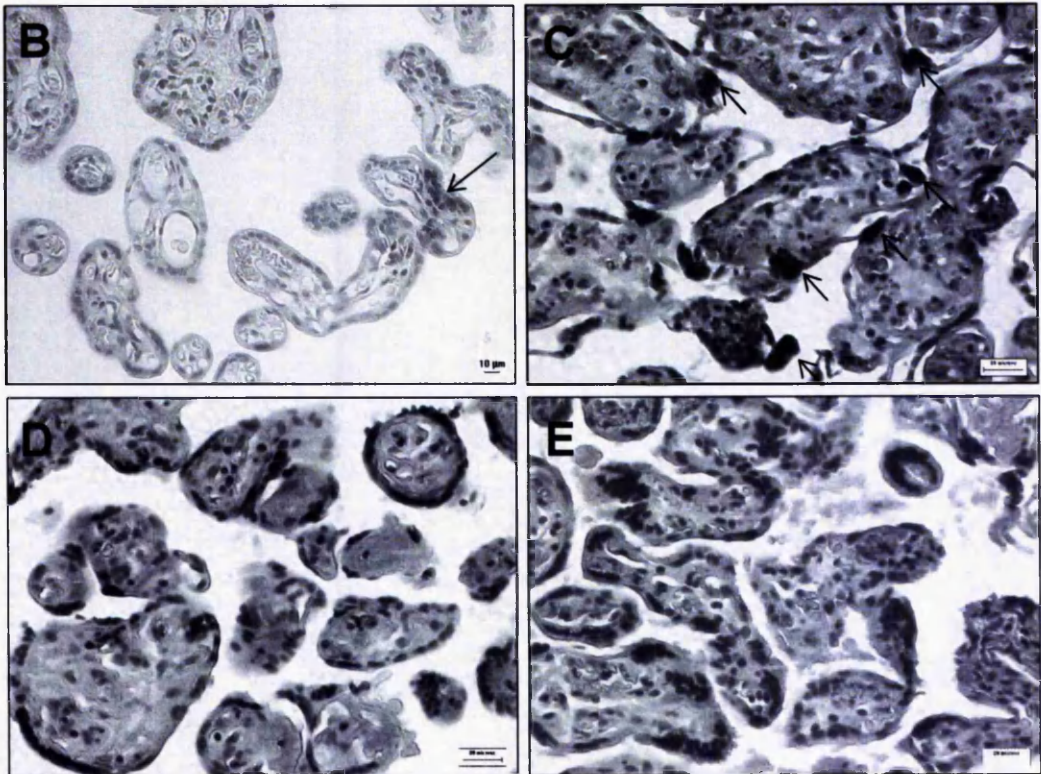
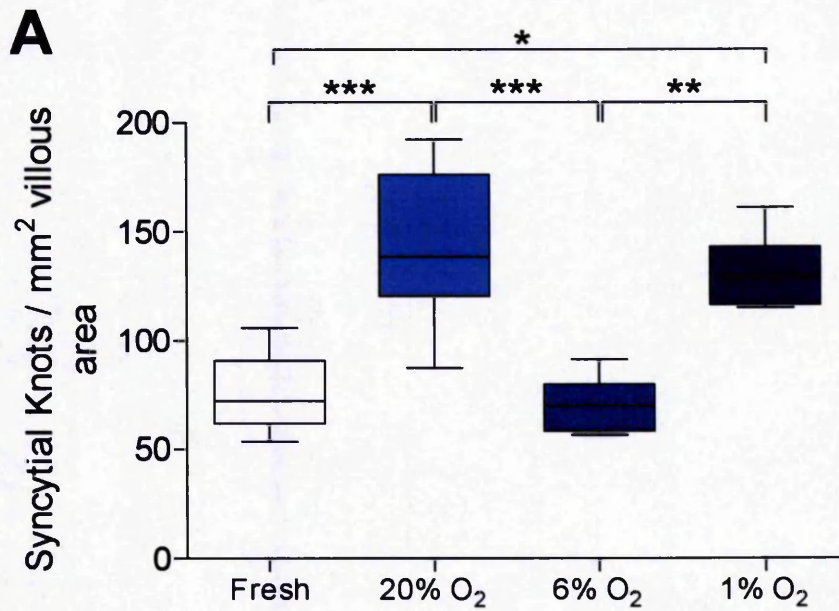
The increased proliferation in explants cultured in 20% O<sub>2</sub> was also associated with increased expression of hCG in culture medium compared to explants cultured in 6% and 1% O<sub>2</sub> (Table 6.1). The quantity of hCG in conditioned culture media was not different between explants cultured in 6% and 1% O<sub>2</sub> (Table 6.1). Other biochemical measures of trophoblast differentiation such as hPL and alkaline phosphatase were not altered by culture in different O<sub>2</sub> tensions (Table 6.1).





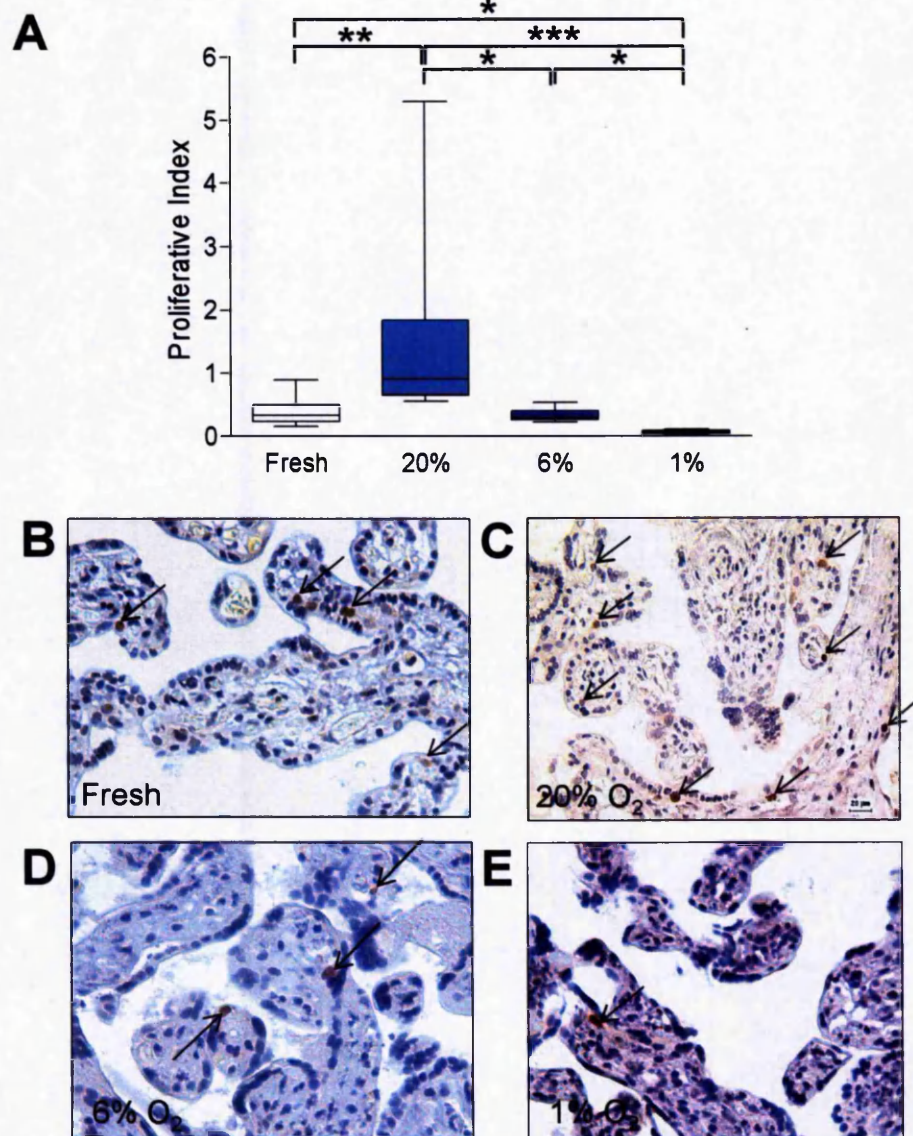
**Figure 6.2** – Localisation of increased apoptosis in explants exposed to 1% O<sub>2</sub> using cytokeratin-18 neo-epitope M30. (A) Immunostaining for cytokeratin M30 neoepitope in fresh villous tissue shows discrete areas of expression in syncytiotrophoblast (ST). (B) M30 immunostaining in explants cultured in 20% O<sub>2</sub> demonstrating immunoreactivity in isolated areas of ST. (C) M30 immunostaining in explants cultured in 6% O<sub>2</sub> is more widespread in the ST layer. CT and stromal cells do not show evidence of M30 staining. (D) In explants cultured in 1% O<sub>2</sub>, M30 immunostaining was weak in ST, but strongly expressed in CTs and some stromal cell. (E) Negative control section demonstrating no immuno-reactivity. Bar = 10µm.





**Figure 6.3** – Assessment of syncytial knots in placental explants exposed to altered O<sub>2</sub> tension. (A) The number of syncytial knots is increased in villous explants cultured in 20% and 1% O<sub>2</sub> conditions compared to fresh tissue and that cultured in 6% O<sub>2</sub>. (\*= $p < 0.05$ , \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$ , Friedman test). (B) Micrograph of terminal villi from normal term placenta showing a single syncytial knot marked by open arrow. (C) Terminal villi from an explant cultured in 20% O<sub>2</sub> show increased numbers of syncytial knots consisting of tightly aggregated nuclei (open arrows). (D) Explants cultured at 6% O<sub>2</sub> have comparable numbers of syncytial knots to fresh placental tissue. (E) Explants cultured in 1% O<sub>2</sub> have increased syncytial knots.





**Figure 6.4** – Effects of altered O<sub>2</sub> on proliferation in normal term placental villous explants. (A) Proliferative index as assessed by Ki67 immunostaining, is similar in explants cultured in 6% O<sub>2</sub> and fresh tissue. Proliferation is increased in 20% O<sub>2</sub> compared to 6% O<sub>2</sub>. Conversely, proliferation is decreased by culture in 1% O<sub>2</sub> as compared to 6% O<sub>2</sub> and fresh tissue (\* p < 0.05, Friedman test). (B) Ki67 immunostaining in fresh tissue was predominantly seen in cytotrophoblasts (CT), as marked with arrows. (C) Explants cultured in 20% O<sub>2</sub> had a significantly greater number of Ki-67 positive CTs than those cultured at (D) 6% O<sub>2</sub>. (E) Explants cultured in 1% O<sub>2</sub> had fewer Ki67 positive nuclei.

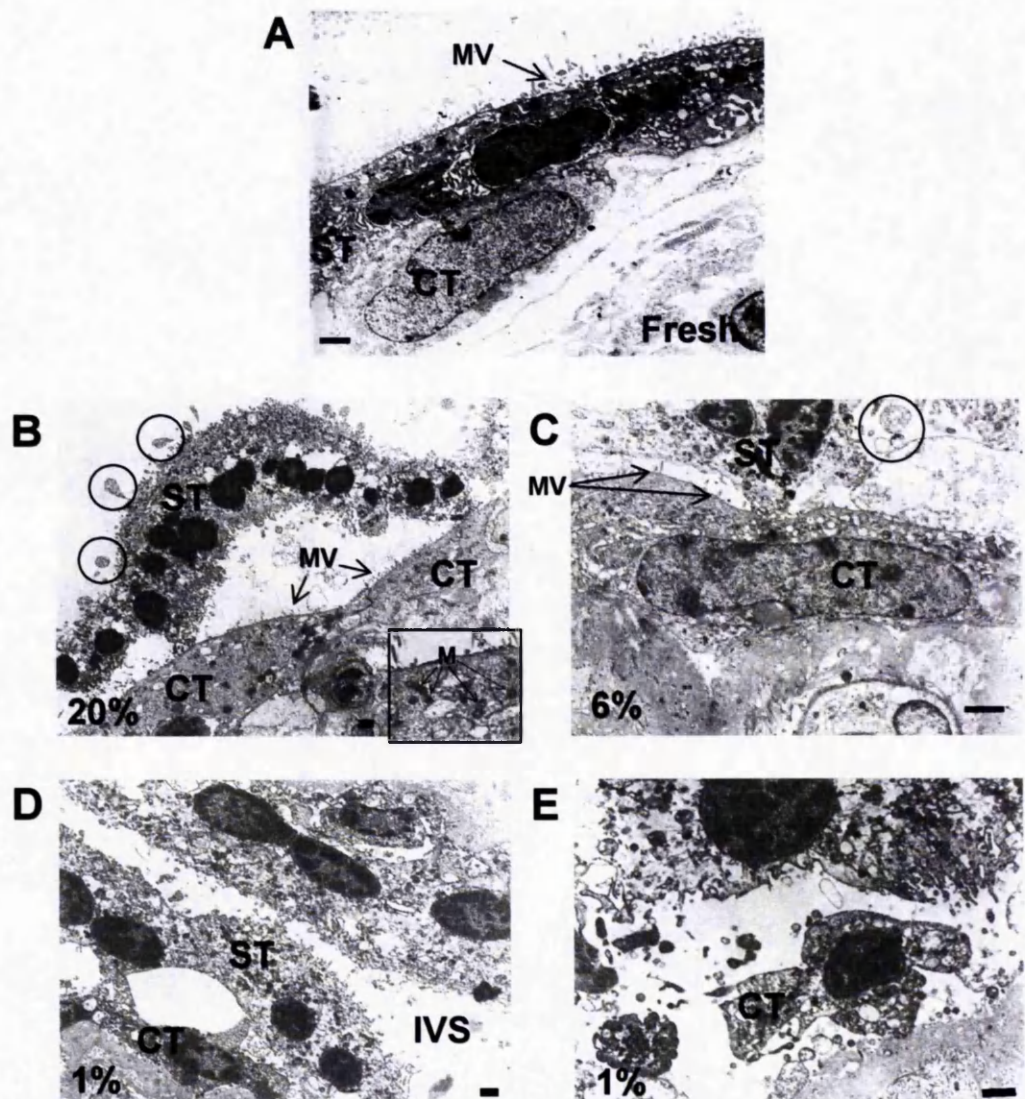
Analyte	Culture Conditions		
	20% O <sub>2</sub>	6% O <sub>2</sub>	1% O <sub>2</sub>
hCG (iu/L)	156.7 (89.4-256.5)*	50.2 (31.3-58.8)*	31.5 (15.5-76.7)*
hPL (mg/L)	11.3 (8.8-14.7)	11.4 (7.8-14.4)	13.0 (11.6-15.9)
Alkaline Phosphatase (iu/L)	23 (20.5-41.5)	24 (19.5-56)	37.5 (21-47)

**Table 6.1** – Concentrations of hCG, hPL and alkaline phosphatase in conditioned culture medium from placental explants cultured in different oxygen tension (n=9). Results are median with interquartile range shown in parentheses. \* p<0.05, Friedman test.

#### 6.4.4 Effects of Altered O<sub>2</sub> tension on Trophoblast Morphology and Syncytiotrophoblast Necrosis

The syncytiotrophoblast showed signs of degeneration at 96 hours of culture in all explants irrespective of O<sub>2</sub> tension when compared to fresh tissue (Figure 6.5A). Indeed, characteristic changes of apoptosis such as peripheral chromatin condensation could be seen in the majority of syncytiotrophoblast nuclei. In fresh and cultured tissue, cytotrophoblasts were identified as large mononucleate cells immediately underneath the syncytiotrophoblast. In explants cultured in 20% and 6% O<sub>2</sub>, rounded vesicles were present at the apical surface of the syncytiotrophoblast; these measured approximately 3-5µm in diameter, in comparison to microvilli, which are approximately 0.2-0.5µm (Figure 6.5B and C). These rounded structures were not present in explants incubated at 1% O<sub>2</sub>, where irregular fragments were present on the apical surface of syncytiotrophoblast (Figure 6.5D). In 20% O<sub>2</sub>, the cytotrophoblasts had many surface microvilli (approximately 0.8µm long and 0.2-0.3 µm wide) and prominent mitochondria (Figure 6.5B). Cytotrophoblasts had similar appearances in 6% O<sub>2</sub>, although fewer microvilli were present, mitochondria were seen and multiple nucleoli were present (Figure 6.5C). In contrast, placental explants cultured in 1% O<sub>2</sub> contained cytotrophoblasts with apoptotic features including membrane blebbing and peripheral nuclear chromatin condensation (Figure 6.5E). An increase in necrosis, particularly degeneration of the





**Figure 6.5** – Effects of altered  $O_2$  on villous morphology after 96 hours culture. (A) In fresh tissue, syncytiotrophoblast (ST) is in contact with the underlying cytotrophoblast (CT), ST nuclei have features of degeneration such as peripheral chromatin condensation. The CTs show evidence of metabolic activity with numerous mitochondria and a prominent nucleolus. (B) Explants cultured in 20%  $O_2$  show ST degeneration and the loss of membrane bound fragments shown in circles (~3-5 $\mu$ m diameter) The underlying CT show regeneration of microvilli (MV) (~0.2-0.5 $\mu$ m diameter), and possess several mitochondria (Inset). (C) Explants cultured in 6%  $O_2$ , also show ST degeneration in bound fragments, the underlying CTs show evidence of microvilli on the apical border and have mitochondria and organelles. The nucleus appears to contain nucleoli. (D) Explants cultured in 1%  $O_2$ , show ST degeneration, and ST nuclei have peripheral chromatin condensation, these appearances are consistent with advanced apoptosis. The syncytial debris lost into intervillous space (IVS) is not contained in membrane bound vesicles, but appears as irregular small fragments. (E) Explants cultured in 1%  $O_2$  show evidence of CT apoptosis, specifically, peripheral nuclear chromatin condensation and cytoplasmic shrinkage. Scale bar = 1 $\mu$ m in all images.

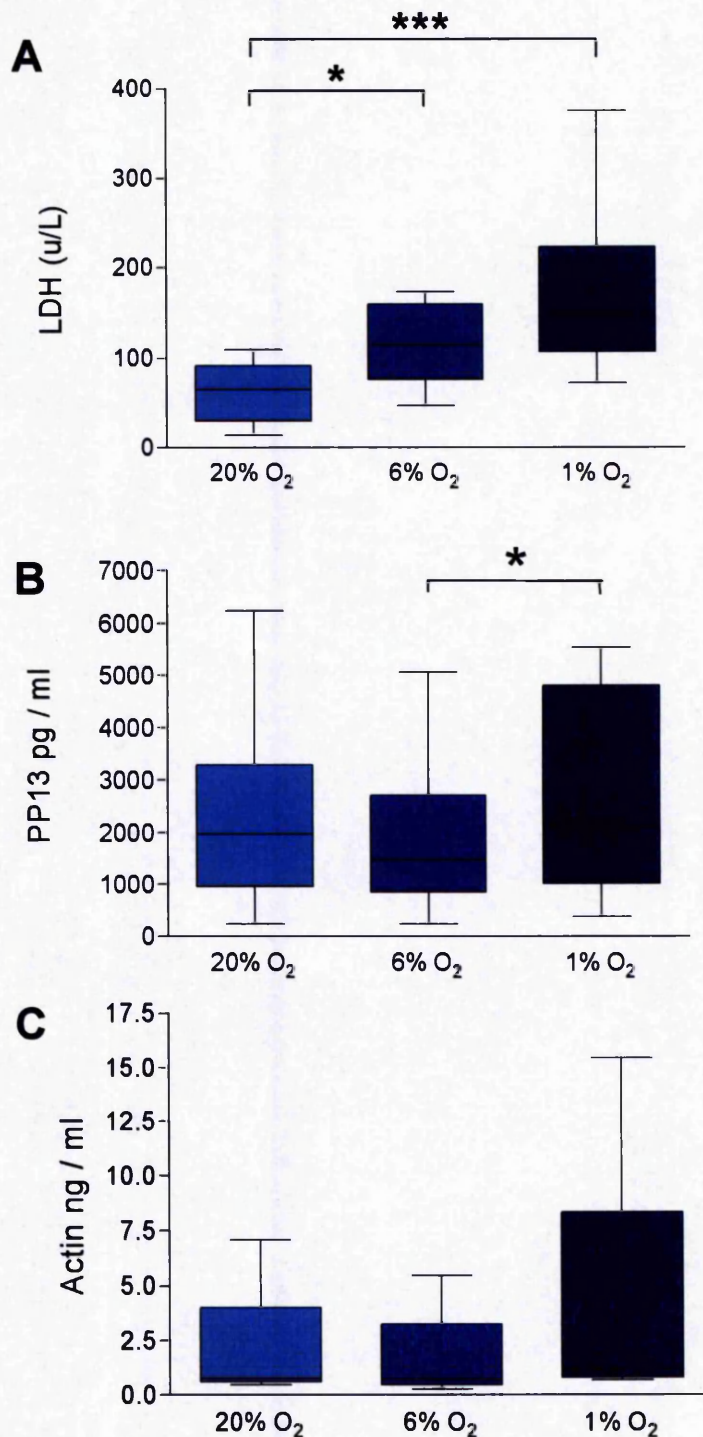
syncytiotrophoblast, was also evident at 1% O<sub>2</sub> by the increased release of LDH and PP13 into the tissue culture medium (Figures 6.6A and 6.6B respectively). However, there was no significant increase in liberated actin (Figure 6.6C).

#### **6.4.5 Effects of Altered O<sub>2</sub> Tension on Expression of p53, Mdm2, p21, Bax and Bcl-2**

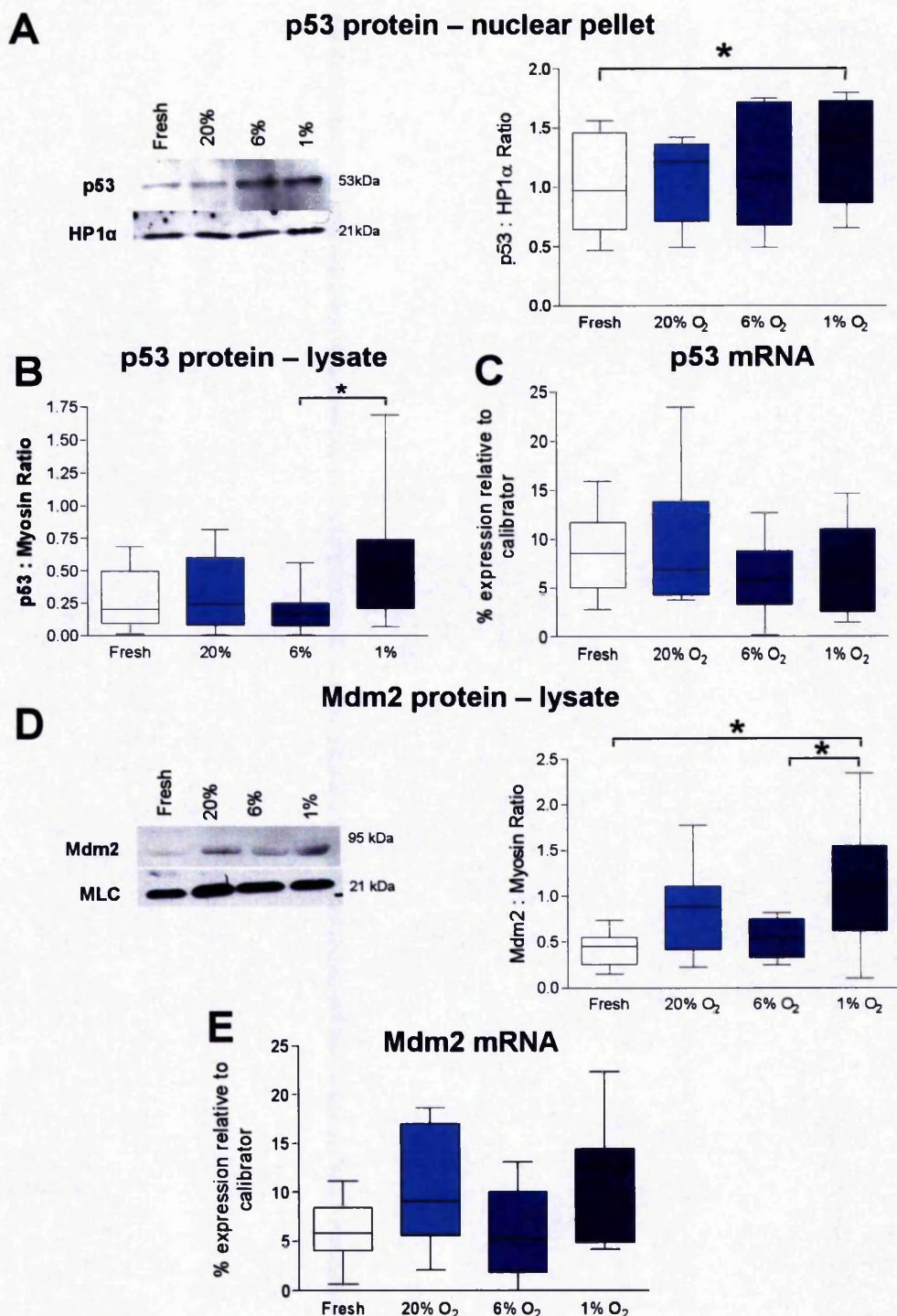
p53 expression was significantly elevated in the nuclear pellet of explants cultured in 1% O<sub>2</sub> compared to fresh tissue (Figure 6.7A). p53 expression was increased in the whole tissue lysate of explants cultured in 1% O<sub>2</sub> compared to that of 6% O<sub>2</sub> (Figure 6.7B). There was no change in p53 mRNA expression under any of the oxygen conditions tested (Figure 6.7C). Mdm2 expression was greater in explants cultured in 20% and 1% O<sub>2</sub>, compared to fresh tissue and 6% O<sub>2</sub> (Figure 6.7D). There was no significant change in Mdm2 mRNA in response to altered O<sub>2</sub> tension, although there was a trend towards an increase in explants cultured in 1% O<sub>2</sub> ( $p=0.08$ ) (Figure 6.7E). No significant variations were evident in Bax protein or mRNA between fresh tissue and villous explant cultures irrespective of O<sub>2</sub> tension (Figures 6.8A and B). Similarly, Bcl-2 protein expression was not altered in response to variations in O<sub>2</sub> tension (Figure 6.8C). p21 mRNA was increased in explants cultured at 1% O<sub>2</sub> compared to fresh tissue and 6% O<sub>2</sub> (Figure 6.7D).

In fresh placental tissue, p53 localised to some cytotrophoblasts, with very little expression in syncytiotrophoblast or stromal cells (Figure 6.9A). In all cultured tissue, there was some immunostaining within the stroma and cytotrophoblasts, which was increased in 1% O<sub>2</sub> (Figure 6.9A). Mdm2 was expressed in the cytoplasm of syncytiotrophoblast and cytotrophoblasts of fresh tissue. In cultured tissue, Mdm2 additionally localised to the cytoplasm of stromal cells, although there was no apparent change in localisation in response to different O<sub>2</sub> tensions (Figure 6.9B). In fresh tissue, p21 localised to a limited number of cytotrophoblast nuclei. Culture for 96 hours was associated with an increase in p21 expression in both stromal and cytotrophoblast cells; this change was most prominent in explants cultured in 1% O<sub>2</sub> (Figure 6.9C). Bax localised to the cytoplasm of cytotrophoblast, syncytiotrophoblast and stromal cells of the fresh tissue. The localisation of Bax did not alter with variations in O<sub>2</sub> (Figure 6.10A). Bcl-2 localised to the cytoplasm of cytotrophoblast and syncytiotrophoblast, with the latter being most prominent. The expression or localisation of Bcl-2 was not affected by alterations in O<sub>2</sub> tension (Figure 6.10B).



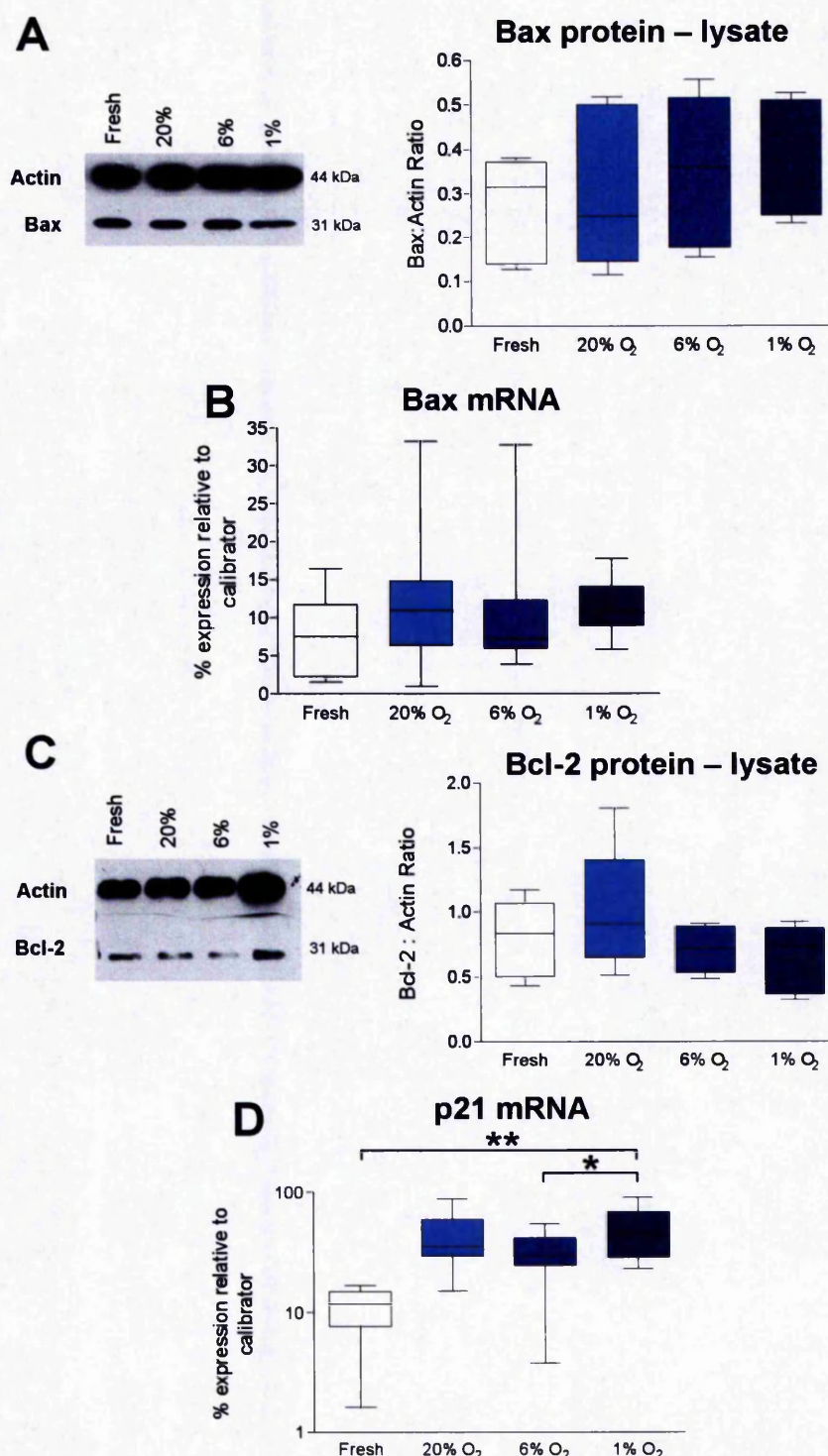


**Figure 6.6** – Assessment of necrotic cell death by release of intracellular contents into conditioned culture medium. (A) Explants cultured in 1% O<sub>2</sub> have increased liberation of lactate dehydrogenase (LDH) into conditioned culture media compared to explants cultured in 20% O<sub>2</sub>. Conditioned culture media taken from explants in 20% O<sub>2</sub> have less LDH activity than those at 6% oxygen (\* p<0.05, \*\*\* p<0.001, Friedman test, n=9). (B) Placental-protein -13 (PP13) was increased in conditioned media from explants cultured in 1% O<sub>2</sub> as compared to those at 6% O<sub>2</sub> (\* p<0.05, Friedman test) (n=6). (C) The presence of actin in conditioned culture medium was not significantly altered by O<sub>2</sub> tension (n=5).



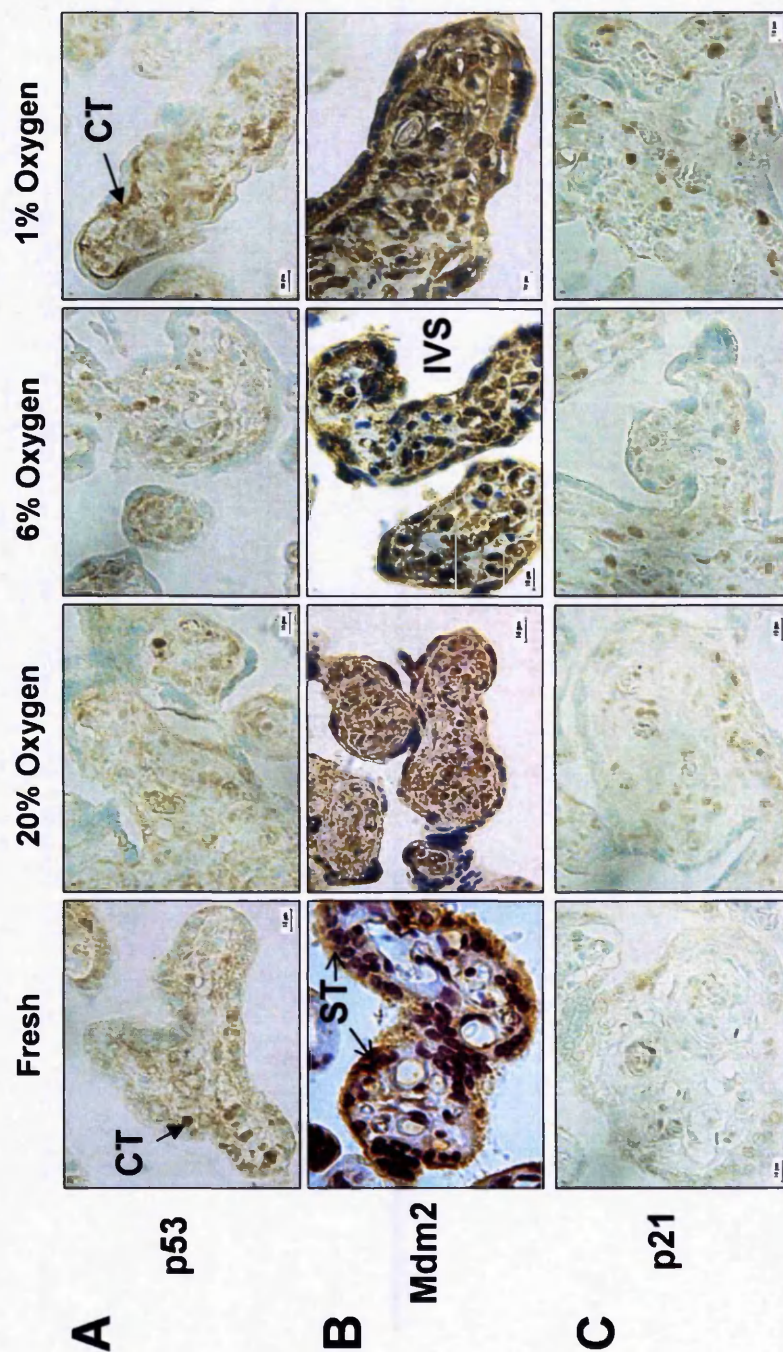
**Figure 6.7** – Protein and mRNA expression in fresh explants and those cultured in 20%, 6% and 1% O<sub>2</sub>. (A) Representative western blot and densitometry of p53 protein standardised to HP1α in the nuclear pellet. p53 protein expression is increased in explants cultured in 1% O<sub>2</sub> compared to fresh tissue (\*  $p < 0.05$ , Friedman test). (B) p53 protein standardised to MLC indicating p53 expression is increased in cell lysate following culture in 1% O<sub>2</sub> compared to 6% O<sub>2</sub>. (C) p53 mRNA expression assessed by qPCR standardised to the calibrator sample. (D) Representative western blot and densitometry of Mdm2 protein standardised to myosin light chain in whole villous lysate demonstrating increased expression of Mdm2 in explants cultured in 1% O<sub>2</sub> compared to fresh tissue and 6% O<sub>2</sub> (\*  $p < 0.05$  Friedman test). (E) Mdm2 mRNA expression assessed by qPCR standardised to the calibrator sample. All experiments  $n=9$ .





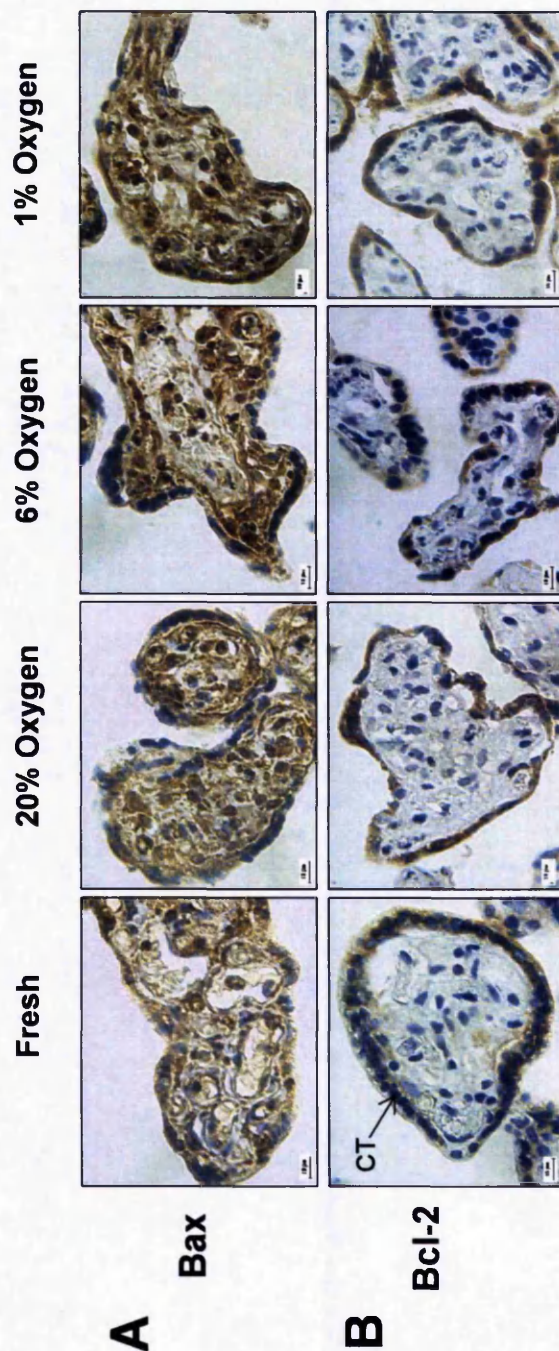
**Figure 6.8** – Protein and mRNA expression in fresh explants and those cultured in 20%, 6% and 1% O<sub>2</sub>. (A) Representative western blot and densitometry of Bax protein standardised to  $\beta$ -actin in whole villous lysate, showing no difference in expression in response to altered O<sub>2</sub>. (B) Bax mRNA expression assessed by qPCR standardised to the calibrator sample. (C) Representative western blot and densitometry of Bcl-2 protein standardised to  $\beta$ -actin in whole villous lysate showing no significant difference in expression in response to altered O<sub>2</sub>. (D) p21 mRNA expression as assessed by qPCR standardised to calibrator sample, showing increased expression in explants cultured in 1% O<sub>2</sub> (\*  $p < 0.05$ , \*\*  $p < 0.01$ , Friedman test). All experiments  $n=9$ .





**Figure 6.9** – Immunohistochemistry for p53, Mdm2 and p21 in fresh tissue and explants cultured in 20%, 6% and 1% oxygen. (A) p53 protein localised to occasional CT nuclei in fresh tissue and explants cultured in 20% and 6% oxygen. In explants cultured in 1% oxygen, p53 localised to many CT and stroma. (B) Mdm2 protein localised to the ST cytoplasm in fresh tissue. In cultured explants Mdm2 protein not only localised to the ST cytoplasm, but was also expressed in the CT and stromal cells. (C) p21 localised to occasional CT nuclei in fresh tissue. In tissue cultured in 20%, 6% and 1% , p21 localised to some CT and stromal nuclei, with the expression in 1% oxygen most prominent.





**Figure 6.10** – Immunohistochemistry for Bax and Bcl-2 in fresh tissue and explants cultured in 20%, 6% and 1% oxygen. (A) In fresh tissue Bax immunostaining was present in the ST, CT and endothelial cell cytoplasm. In cultured tissue, Bax was present in the ST, CT and stromal cell cytoplasm. The expression or localisation of Bax did not appear to be altered by oxygen tension. (B) In fresh tissue, Bcl-2 localised strongly to the ST cytoplasm, and was weakly expressed in CT cytoplasm. The localisation and expression of Bcl-2 was not altered by culture in 20%, 6% or 1% oxygen.



## 6.5 Discussion

This study demonstrates that atmospheric O<sub>2</sub> has profound effects on cell turnover in villous explants from term placentas. Culture in 20%, 6% and 1% atmospheric O<sub>2</sub> produced levels in culture media respectively considered to be hyperoxic, normoxic and hypoxic for term placental villous trophoblast (Miller et al. 2005). Ongoing viability of placental explants was demonstrated by the secretion of hCG and evidence of proliferation by staining for Ki67 antigen, providing evidence for the use of explants for a 96 hour culture period which has previously been questioned (Di Santo et al. 2003).

The higher TUNEL index of cultured tissue compared to fresh samples may reflect syncytiotrophoblast degeneration previously observed in this culture model (Siman et al. 2001; Hung et al. 2002; Crocker et al. 2004c). Culture in 1% O<sub>2</sub> was associated with an additional increase in TUNEL index which predominantly localised to cytotrophoblasts and stromal cells. Owing to variations in the degree of staining and evidence of staining in necrotic tissue, the specificity of TUNEL as a marker of apoptosis in trophoblast has been questioned (Smith et al. 1997b; Burton et al. 2003). In this study, the specificity of TUNEL staining was confirmed by recognition of cytokeratin M30 and electron microscopy, which highlighted the increased apoptosis of cytotrophoblasts in explants cultured in 1% O<sub>2</sub>. However, TUNEL identified a greater number of nuclei than M30-immunostaining. This is likely to result from the specificity of the M30 neopeptide, which only identifies apoptotic cells which express cytokeratin-18 and will not detect other cell types such as endothelial cells or fibroblasts which may also undergo apoptosis in 1% O<sub>2</sub>. Some stromal cells were identified by M30-immunostaining; cytokeratin-18 positive cells have been reported in the stroma, although their significance to this investigation is unclear (Haigh et al. 1999). The confirmation of TUNEL staining by electron microscopy, the gold standard tool for identification of apoptosis, demonstrating morphological features of apoptosis in cytotrophoblast and syncytiotrophoblast suggests that TUNEL appears to be an acceptable marker of apoptosis in these trophoblast investigations. These different experimental approaches provide a significant body of evidence indicating that apoptosis is increased in explants cultured in 1% O<sub>2</sub>. This finding of increased apoptosis following culture in 1% O<sub>2</sub> (~20mmHg) is supported by investigations of isolated term trophoblasts which describe increased apoptosis in an O<sub>2</sub> tension of 15mmHg (Levy et al. 2000; Kilani et al. 2003).

In common with previous studies of term placental explants, culture in 1% O<sub>2</sub> induced features of necrosis (Huppertz et al. 2003). A combination of apoptosis and necrosis, termed 'aponecrosis', has been described in cells exposed to a pro-apoptotic stimulus and subsequently deprived of oxygen (Formigli et al. 2000). Oxygen deprivation reduces energy generation and as apoptosis is energy-dependent, cell death is completed by necrosis (Huppertz et al. 2003). In contrast to apoptosis, aponecrosis results in release of unbound cytoplasmic contents from the degenerating trophoblast, a feature identified by increase LDH and PP13 release from explants cultured in 1% O<sub>2</sub>. Aponecrotic release of unbound syncytial material has been proposed as a potential mechanism in the development of pre-eclampsia (Huppertz and Kingdom 2004) and may lead to the release of cell-free DNA in pre-eclampsia and following culture of placental villous explants subjected to hypoxia-reperfusion injury (Zhong et al. 2002; Tjoa et al. 2006).

Using Ki67 immunostaining, we demonstrated similar levels of proliferation to that reported in fresh placental tissue from term pregnancies (Yamada et al. 2001). As staining localised almost exclusively to cytotrophoblasts, the proliferative index reflects trophoblast proliferation. In addition to increased cell death, explants cultured in 1% O<sub>2</sub> demonstrated reduced proliferation. These data are consistent with the finding of reduced proliferation in BeWo cells cultured in low O<sub>2</sub> tension described in Chapter 5. However, a reduction in proliferation has not been described in earlier studies of placental villous explants (Fox 1970; Crocker et al. 2004c), which may be due to the magnitude of hypoxia used, as in previous studies the effect of 3% atmospheric O<sub>2</sub> was considered for a shorter culture period. In the experiments presented here explants cultured in 20% O<sub>2</sub> exhibited increased proliferation. This observation may represent differences in the response of cytotrophoblasts to syncytiotrophoblast ageing, as syncytial knot formation is increased following culture in 20% O<sub>2</sub>. Using our current model of villous trophoblast cell turnover (Chapter 1), the degenerating syncytium would be expected to be replaced by cytotrophoblast proliferation with subsequent differentiation. It is hypothesised that this process is intact in tissue cultured in 20% O<sub>2</sub>, hence an association between syncytial knots and cytotrophoblast proliferation. This may not occur in 1% O<sub>2</sub> due to loss of cytotrophoblast viability due to apoptosis. A similar relationship between proliferation and apoptosis was seen in BeWo cells cultured in low O<sub>2</sub> (Chapter 5).

The altered proliferation of cytotrophoblasts in response to culture in different O<sub>2</sub> tension was accompanied by changes in hCG expression in conditioned culture medium. In addition to increased proliferation, culture in 20% O<sub>2</sub> increased hCG expression compared to 6% and 1% O<sub>2</sub> as previously reported (Alsat et al. 1996; Crocker et al. 2004c). This suggests that cytotrophoblasts in explants cultured in 20% O<sub>2</sub> may either be undergoing accelerated differentiation or that expression of hCG is uncoupled from morphological features of differentiation, such as that observed in isolated trophoblast treated with 8-bromo-cAMP (Kao et al. 1988). In support of the former hypothesis, cytotrophoblasts cultured in 20% O<sub>2</sub> exhibit more microvilli and mitochondria, suggesting a greater degree of differentiation compared to explants cultured in 6% O<sub>2</sub>. hCG may be secreted earlier in the differentiation of cytotrophoblasts and precedes elevations in hPL (Kato and Braunstein 1989; Dodeur et al. 1990), which may explain why the increase in hCG secretion was an isolated finding in these studies.

The culture of placental villous explants allows the study of cell turnover in the presence of cell-cell interactions between stroma, cytotrophoblast and syncytiotrophoblast. Microarray data from villous explants from the first trimester of pregnancy cultured in low O<sub>2</sub> was similar to that seen from high altitude suggesting that an explant culture model responds to altered O<sub>2</sub> in a similar manner to trophoblast in vivo (Soleymanlou et al. 2005a). The changes in expression of Bax, p21 and Mdm2 within stromal cells as well as in the cytotrophoblasts, demonstrates that changes in O<sub>2</sub> tension affect different cell types within the villus and raises the possibility that different cell types are able to transmit apoptotic or survival signals.

The control of cell turnover in placental tissue in response to changes in O<sub>2</sub> is complex. The constituent proteins of the intrinsic apoptotic pathway are present in many cell types within the placental villus, although all are expressed in villous trophoblast as reviewed in Chapter 1 and further investigated in Chapter 3. The localisation of p53, Mdm2, p21, Bcl-2, and Bax in fresh villous trophoblast was in agreement with previous studies and experiments described in chapter 3, confirming their potential role in regulating cell survival in this cell layer (Fulop et al. 1998; Qiao et al. 1998; Ratts et al. 2000). p53, Mdm2 and p21 are increased in term villous explants cultured in 1% O<sub>2</sub>. The observation that p53 is increased by exposure to low O<sub>2</sub> agrees with previous data from isolated term trophoblast and also microarray studies of first trimester villous explants exposed to 3% O<sub>2</sub> (Levy et al. 2000; Soleymanlou et al. 2005a).

The increase in p53 protein in the absence of increased mRNA suggests that p53 protein may be stabilised in 1% O<sub>2</sub> rather than an increase in p53 transcription. This is consistent with regulation of p53 following other sources of cell damage, which induce post-translational modifications of the p53 protein preventing its degradation, or facilitating downstream interactions (Prives and Hall 1999). One downstream interaction of p53, the promotion of p21 transcription, was increased following culture in 1% O<sub>2</sub>. This increase in p21 may relate to the decreased proliferation seen in explants cultured in 1% O<sub>2</sub>, as p21 is a potent cell-cycle inhibitor (Waga et al. 1994). Despite this association with downstream effects of p53, there was no significant increase in Mdm2 or Bax mRNA in explants cultured in 1% O<sub>2</sub>. This may result from alternative pro-apoptotic signalling of p53; in which the transcription of anti-apoptotic genes (e.g. XIAP, survivin) is repressed (Hammond and Giaccia 2005), this idea is not refuted by this study. Mdm2 may be independently regulated by hypoxia, which may account for the increased Mdm2 observed in 1% O<sub>2</sub> (Zhang and Hill 2004). These observations suggest that some proteins of the intrinsic apoptotic pathway may be independently regulated by oxygenation, making the regulation of cell-death with villous trophoblast extremely complex. Given this complexity, further studies are required to determine whether the observed alterations in cell turnover are p53-dependent and the potential mechanisms of p53 stabilisation and/or activation.

Although altered O<sub>2</sub> tension alters trophoblast cell turnover, only one study thus far has investigated the effects on p53 expression, describing an association between increased apoptosis and p53 protein expression in isolated term cytotrophoblasts (Levy et al. 2000). Much of the previously published work on hypoxia in trophoblast has focused on HIF1 $\alpha$  as a central mediator of downstream effects of hypoxia. HIF1 $\alpha$  has been described in villous trophoblast and is increased in pre-eclampsia and explants subjected to hypoxic conditions (Caniggia et al. 2000a; Caniggia et al. 2000b; Rajakumar et al. 2003). HIF1 $\alpha$  has been demonstrated to have direct actions on the promotion of VEGF and sflt-1 expression, two factors known to be altered in pre-eclampsia (Nevo et al. 2006). O<sub>2</sub> sensing proteins such as HIF1 $\alpha$  can stabilise p53, thereby increasing cellular levels promoting cell-cycle arrest and apoptosis (An et al. 1998; Hammond and Giaccia 2005). This suggests that HIF1 $\alpha$  may have a dual role promoting VEGF/sflt1 expression and stabilisation of p53.

In conclusion, the culture of placental explants in altered O<sub>2</sub> tension is able to reproduce some of the aberrant trophoblast cell turnover described in pre-eclampsia. Culture in 1% O<sub>2</sub> produced hypoxic conditions in culture which was associated with increased apoptosis, necrosis and an increase in p53 protein. Increased p53 expression is in agreement with the data from villous trophoblast from pregnancies complicated by pre-eclampsia (Chapter 3) and IUGR (Levy et al. 2002). In combination, the increased apoptosis, formation of syncytial knots and necrosis in response to hypoxia may lead to increased syncytial shedding which has been implicated in the pathogenesis of pre-eclampsia (Sargent et al. 2003). Conversely, hyperoxic conditions for term villous trophoblast were not associated with a significant increase in apoptosis, but did show increased proliferation and hCG secretion into culture media compared to normoxia. Culture in hyperoxic conditions for 96h did not reproduce the morphological features of placental apoptosis seen in pre-eclampsia. Further investigation is needed to determine the role of O<sub>2</sub>, hypoxia and oxidative stress in the development of pre-eclampsia and the cell signalling pathways involved. Other origins of oxidative stress, such as the generation of ROS by hypoxia-reoxygenation, are more able to reproduce the villous trophoblast phenotype seen in pre-eclampsia (Hung et al. 2002). However, the effects of hypoxia-reoxygenation on proliferation and syncytial knot formation of villous trophoblast have not been described.



## **Chapter 7 – Does Exposure to Reactive Oxygen Species Alter Cell Turnover In Term Placental Villous Explants?**

### **7.1 Introduction**

As previously stated, the altered cell turnover in pre-eclampsia is hypothesised to result from failure to convert uteroplacental arteries from tortuous spiral vessels to flaccid wide-bore conduits. Some authors have proposed that rather than leading to constant chronic hypoxia, preservation of arterial smooth muscle in unconverted placental bed vessels leads to retention of vasoconstriction, which may subject the placenta to periods of ischaemia followed by reperfusion (Hung and Burton 2006). Such an ischaemia-reperfusion injury generates reactive oxygen species (ROS). In normal pregnancies, placental villi are able to cope with periods of oxidative stress as trophoblast expresses enzymes such as copper/zinc superoxide dismutase (Cu/Zn SOD) (Ali Akbar et al. 1998), manganese superoxide dismutase (Mn SOD), xanthine oxidase, glutathione peroxidase, catalase and anti-oxidant molecules such as glutathione (Wang and Walsh 1996; Telfer et al. 1997; Watson et al. 1998). However, these critical enzymes are only weakly expressed within the syncytiotrophoblast, with Cu/Zn SOD also being strongly expressed in stromal cells and Mn SOD predominantly expressed in fetal endothelium (Myatt et al. 1997). In pre-eclampsia the antioxidant activity of Cu/Zn SOD and glutathione peroxidase in villous trophoblast is reduced, while the pro-oxidative xanthine oxidase is increased (Many et al. 2000; Wang and Walsh 2001). These changes result in increased ROS as demonstrated by an increase in the presence of nitrotyrosine, a product of peroxynitrite generation by ROS in trophoblast sampled from pregnancies complicated by pre-eclampsia (Many et al. 2000).

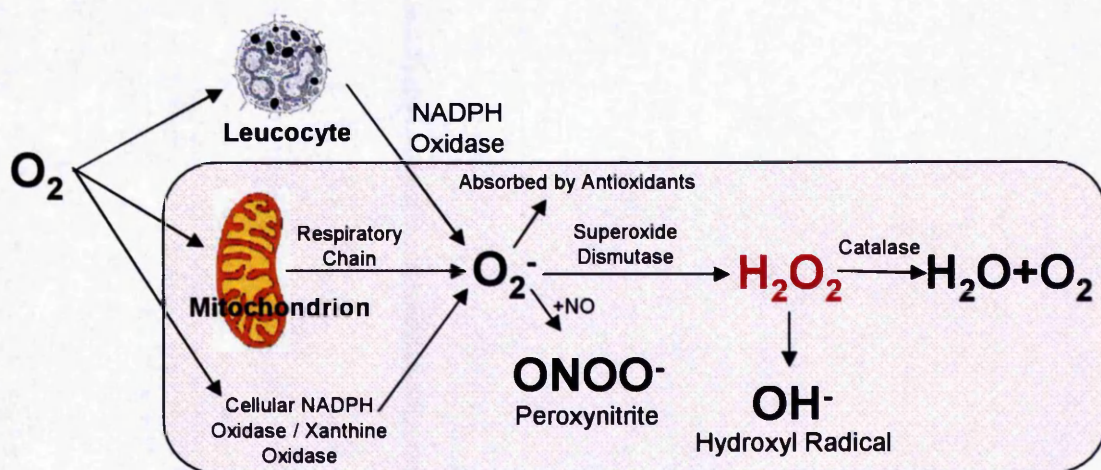
While there is evidence of placental oxidative stress, evidence of oxidative stress in maternal plasma in pre-eclampsia is equivocal. The concentration of ROS such as  $H_2O_2$  is raised in maternal plasma of pregnancies complicated by pre-eclampsia (Kharfi et al. 2005). Likewise, products of oxidation such as malondialdehyde and lipid peroxides are also increased in pre-eclampsia (Kumar and Das 2000; Aydin et al. 2004). However, other studies have found no difference in urinary isoprostane (Regan et al. 2001), plasma prostaglandin  $F_{2\alpha}$ , lipid peroxide or malondialdehyde between women with normal pregnancies and those with pre-eclampsia

(Ishihara et al. 2004). Interpretation of the differences described by these studies is complicated, as they describe results for a small number of patients which may result in selection bias. In addition, ROS are unstable and differences in sample collection may account for some of the variation described. Currently, there are no conclusive data to link placental oxidative stress to systemic oxidative stress and both may arise through separate processes. Therefore, equivocal evidence of systemic oxidative stress does not preclude placental oxidative stress.

Mimicking placental ischaemic-reperfusion injury in vitro by short (1h) cycles of hypoxia-reoxygenation is associated with increased apoptosis and secondary necrosis of syncytiotrophoblast (Hung et al. 2002). This increase in cell-death is likely to be related to the generation of ROS as hypoxia-reoxygenation is associated with increased evidence of nitrotyrosine residues (Hung et al. 2002) and treatment with potent anti-oxidants such as glyceryl trinitrate or carbon monoxide (CO) attenuates this increase in apoptosis (Bainbridge et al. 2006; Belkacemi et al. 2007). Nitrotyrosine residues are generated by the presence of the peroxynitrite species (ONOO<sup>-</sup>) generated by the combination of nitric oxide with O<sub>2</sub><sup>-</sup> ion (Figure 7.1). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), generated by action of SOD can also give rise to the reactive hydroxyl (OH<sup>-</sup>) species, which can denature DNA, producing strand breaks which may then induce apoptosis (Collins and Horvathova 2001). Investigation of this reaction in *Escherichia coli* sp lead to the proposal that H<sub>2</sub>O<sub>2</sub> is reduced in the presence of a divalent metal ion such as iron (Fe<sup>2+</sup>) via a Fenton reaction (Imlay and Linn 1988). This may explain why desferrioxamine, an iron-chelating agent, provides protection from hypoxia-reoxygenation injury in trophoblast (Hung et al. 2002).

In addition to inducing apoptosis in villous explants, H<sub>2</sub>O<sub>2</sub> can alter trophoblast cell turnover as indicated by altered hCG secretion in isolated trophoblast, promoting hCG secretion at lower doses and reducing secretion at higher doses (Kharfi Aris et al. 2007). In BeWo cells treatment with H<sub>2</sub>O<sub>2</sub> decreased proliferation and reduced hCG secretion (Chapter 5). Therefore, treatment with H<sub>2</sub>O<sub>2</sub> can alter trophoblast cell turnover and may provide a means by which a defined quantity of ROS can be administered and alterations in cell turnover assessed.

As oxidative stress and the generation of ROS are implicated in the pathogenesis of pre-eclampsia it is important to investigate the effects of a measured dose of ROS on apoptosis and the expression of components of the p53 pathway. As similar changes in cell turnover in



**Figure 7.1** – Generation of reactive-oxygen species (ROS). ROS in the form of  $O_2^-$  can be generated by the action of NADPH Oxidase or by the electron transport chain. These are then either absorbed by intracellular anti-oxidants such as glutathione or denatured by enzymes such as Superoxide Dismutase (SOD). Hydrogen Peroxide ( $H_2O_2$ ) is then degraded by catalase into water and oxygen. However, in the presence of divalent metal ions such as Iron ( $Fe^{2+}$ ),  $H_2O_2$  can generate hydroxyl radicals ( $OH^\cdot$ ) which lead to DNA strand breaks.

response to culture in 1%O<sub>2</sub> were associated with altered expression of p53 in term placental villous explants (Chapter 6), this in vitro culture model was also utilised to investigate cell turnover in response to treatment with ROS. The use of this culture model also allows comparison between published studies using hypoxia-reoxygenation which also induces ROS formation in vitro. Once again, a 96 hour culture period was used in these experiments as differences in necrosis, proliferation and differentiation have been described after this duration of culture (Siman et al. 2001; Crocker et al. 2004b; Crocker et al. 2004c).

## **7.2 Hypothesis**

It was hypothesised that exposure of term placental villous explants to ROS in the form of H<sub>2</sub>O<sub>2</sub> would alter cell turnover and changes in cell turnover would be associated with altered expression of proteins in the p53 pathway. This investigation aimed to characterise the effects of ROS on cell turnover including: proliferation, differentiation, apoptosis, necrosis and formation of syncytial knots. The expression of regulators of apoptosis were localised by immunohistochemistry.

## **7.3 Methods**

### **7.3.1 Preparation and Culture of Term Placental Villous Explants**

Following ethical approval, term placentas (n=6) were obtained following elective Caesarean section of uncomplicated pregnancies at 37-40 weeks gestation. Placentas were used following Caesarean sections as labour has been shown to alter the transcription of genes related to oxidative stress (Cindrova-Davies et al. 2007).

3 areas of each placenta were randomly sampled within 20 minutes of delivery as previously described (Crocker et al. 2004a) and placental villous explants (each 5mg approx) as described in section 2.2.1.1. Fresh villous tissue was fixed for immunohistochemistry (section 2.3.3.1) and electron microscopy (section 2.3.3.2) to enable comparisons between fresh and cultured tissue.

Explants were cultured for 96 hours in CMRL-1066 culture medium supplemented with antibiotics, insulin (1mg/l), hydrocortisone (0.1mg/l), retinol acetate (0.1mg/l) and 10% FCS. The explants were supported at the gas-liquid interface as previously described in 6%O<sub>2</sub>/ 5% CO<sub>2</sub>/ 89%N<sub>2</sub> at 37°C (Crocker et al. 2004b) (section 2.2.3.1). Explants were initially cultured

for a 48 hour period to allow syncytial shedding and regeneration (Crocker et al. 2004b; Crocker et al. 2004c). After 48 hours, the culture medium was replaced with medium containing either 0, 5, 10, 50, 100, or 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> (VWR International) and cultured for a further 6, 12, 24 or 48 hours. Following tissue culture, explants from each well were weighed on a fine balance before being fixed for wax embedding or electron microscopy (sections 2.3.3.1 and 2.3.3.2 respectively). Conditioned culture medium was collected as described in section 2.3.4.

### **7.3.2 Immunohistochemistry**

Tissue sections were deparaffinised, exposed to microwave pre-treatment, quenched for endogenous peroxidase activity and blocked for non-specific binding, as described in section 2.8.2. Tissue sections were exposed to antibodies against p53, Mdm2, p21, Bax or Bcl-2. Where mouse antibodies were used, tissue was incubated with a matching concentration of non-immune mouse IgG to serve as a negative control. Sections were then probed with an appropriate biotin conjugated secondary antibody followed by incubation with avidin peroxidase. Immunostaining was revealed by exposure to concentrated DAB for 3 minutes. Slides were counterstained with haematoxylin and sections viewed using a Leitz microscope with ImageProPlus 4.5 (Media Cybernetics Inc). All samples for comparison were stained in the same batch.

### **7.3.3 Assessment of Apoptosis**

Apoptosis was assessed using a commercially available TUNEL kit (Roche Applied Diagnostics) with modifications to the manufacturers' instructions as detailed in section 2.10.1. In these experiments TUNEL staining was performed using a fluorescent label and images quantified by confocal microscopy of 6 random fields of terminal villi per experimental condition. The number of TUNEL positive nuclei were counted manually and the total number of nuclei per cross-section of tissue measured using sequential colour thresholding as previously described (Crocker et al. 2004a). A ratio was derived from these values; the TUNEL index (% of total nuclei TUNEL positive). TUNEL staining was confirmed by immunostaining for cytokeratin-18 M30 neo-epitope as described in section 2.10.2.



### **7.3.4 Assessment of Syncytial Knots**

5µm tissue sections were stained with haematoxylin and eosin as described in section 2.11. The number of syncytial knots, defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface not in direct contact with adjacent villi (Cantle et al. 1987), was counted manually in 10 fields of view for each experimental condition. The trophoblast area was measured using image analysis software (Image ProPlus, Mediacybernetics) (Daayana et al. 2004). Data were normalised to give a measure of the number of syncytial knots per mm<sup>2</sup> of villus.

### **7.3.5 Assessment of Proliferation**

Cell proliferation was assessed using a mouse monoclonal anti-Ki-67 antibody (Clone Mib-1) as described in section 2.12.1. Ki67 staining was quantified using 10 random fields of terminal villi per experiment. The number of Ki67-positive nuclei were counted manually and the total number of nuclei per cross-section of villous tissue measured by sequential colour thresholding. A ratio was derived; the proliferative index (% of total nuclei Ki67 positive).

### **7.3.6 Electron Microscopy**

Explants from each experimental condition were fixed and treated for electron microscopy as described in section 2.14. Semi-thin sections, 0.5µm thick, were inspected to identify areas of interest. Multiple areas of ultrathin sections were examined by two investigators using a Philips CM10 electron microscope at an accelerating voltage of 80 kV and representative areas photographed.

### **7.3.7 Analysis of Conditioned Culture Medium**

Necrosis was assessed by the presence of LDH in conditioned culture medium using an enzyme-linked assay as described in section 2.13.1.1. Trophoblast differentiation was assessed by the measurement of hCG in the culture medium, using a commercial quantitative immunoradiometric assay in accordance with the manufacturers instructions (ICN Pharmaceuticals; Basingstoke, UK) as detailed in section 2.13.2.

### 7.3.8 Statistical Analysis

As tissue from each placenta was cultured in 0, 5, 10, 50, 100 and 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> statistical significance was tested using the Friedman test for matched non-parametric data. Dunn's post-hoc test was performed as appropriate. Results are presented as median and interquartile range, and plotted as box and whisker plots. A p-value of <0.05 was regarded as significant.

## 7.4 Results

### 7.4.1 Effects of Treatment with H<sub>2</sub>O<sub>2</sub> on Apoptosis and Syncytial Knots

Preliminary experiments (n=3) were carried out to determine the concentration of H<sub>2</sub>O<sub>2</sub> and the culture period that induced apoptosis in placental villous explants. The median apoptotic index for all culture conditions and time periods studied is shown in Table 7.1. These data indicate that exposure to H<sub>2</sub>O<sub>2</sub> increased the proportion of TUNEL nuclei in a dose dependent manner (Explants cultured for 48 hours, Spearman correlation coefficient 0.98, p<0.001). There was no effect of additional duration of culture. Nevertheless, the greatest effects on TUNEL index were observed in placental explants cultured in either 100 or 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> for 48 hours. Therefore, remaining experiments were carried out using 100 or 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> for a 48 hour culture period (n=6).

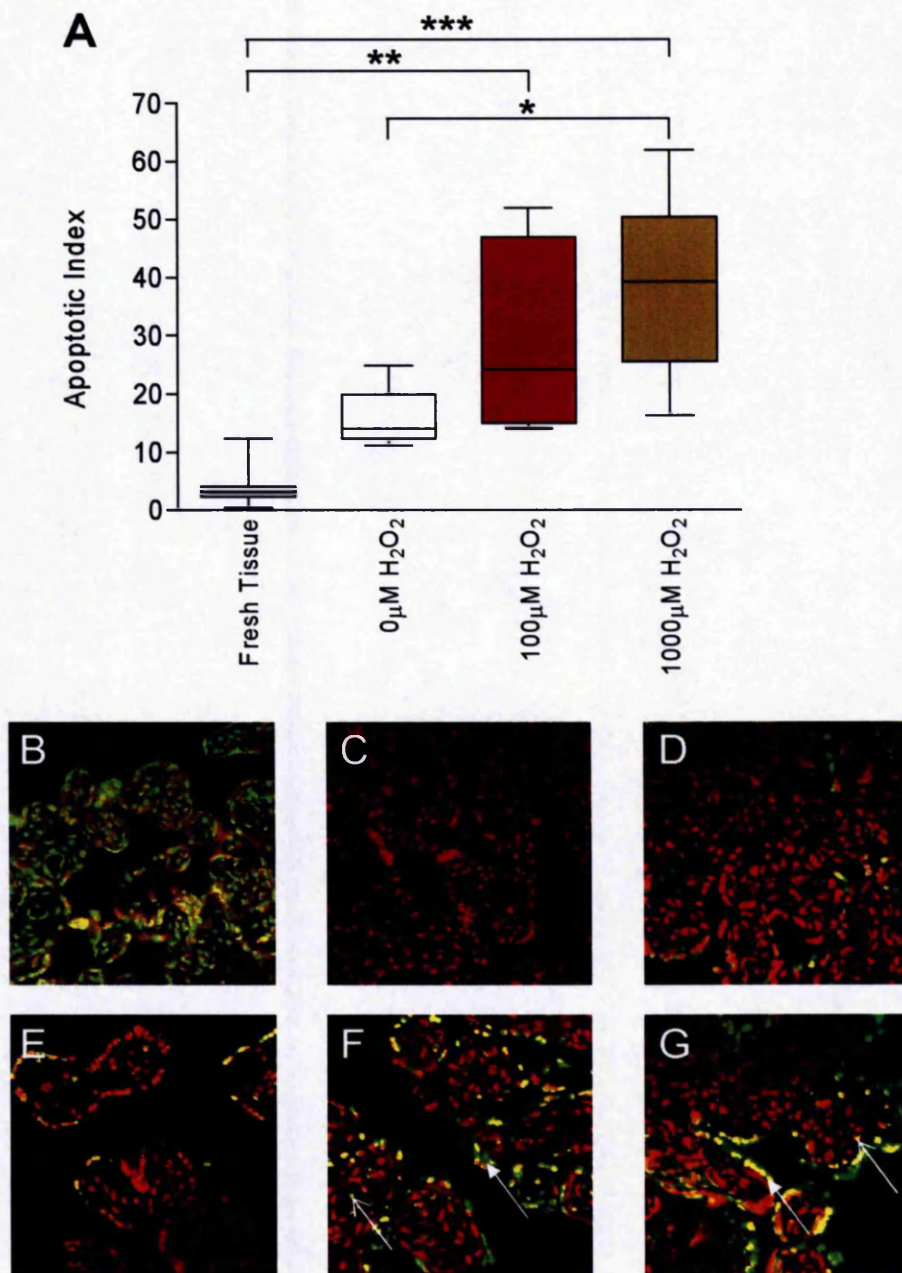
Culture Period (hours)	Concentration of H <sub>2</sub> O <sub>2</sub> ( $\mu$ M)					
	0	5	10	50	100	1000
6	11 (17-19)	25 (24-28)	37 (34-37)	35 (30-44)	52 (24-55)	52 (28-57)
12	8 (5-25)	29 (16-31)	23 (23-25)	46 (44-50)	54 (38-54)	51 (41-58)
24	13 (9-16)	30 (25-37)	37 (32-50)	42 (27-56)	58 (25-60)	62 (62-85)
48	14 (14-19)	29 (29-47)	29 (27-51)	30 (13-73)	48 (46-52)	60 (41-62)

**Table 7.1** – Median apoptotic index (figures in parentheses indicate range) in placental villous explants exposed to increasing doses of H<sub>2</sub>O<sub>2</sub> for culture periods ranging from 6-48h (n=3).

The TUNEL index was significantly increased in all cultured placental explants compared to fresh tissue (Figure 7.2A). Following culture with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> for 48 hours, TUNEL index was increased compared to tissue cultured in control conditions (Figure 7.2A). Positive control tissue treated with DNase, demonstrated TUNEL staining in many nuclei (Figure 7.2B). There was no TUNEL staining in the negative control sections. In fresh tissue, there were few TUNEL positive nuclei, all of which were localised to the syncytiotrophoblast (Figure 7.2D). Following tissue culture, there were increased numbers of TUNEL positive nuclei in the syncytiotrophoblast compared to fresh tissue (Figure 7.2A and E). Following exposure to 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> TUNEL positive nuclei were detected throughout the syncytiotrophoblast (Figure 7.2F). Following treatment with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> TUNEL positive nuclei were seen in the syncytiotrophoblast, underlying cytotrophoblast and some stromal nuclei (Figure 7.2A and G). The distribution of apoptotic cells was confirmed by cytokeratin-M30 immunostaining, demonstrating strong staining in the syncytiotrophoblast of explants cultured in the presence of 1000 H<sub>2</sub>O<sub>2</sub> (Figure 7.3). In addition to inducing apoptosis in the syncytiotrophoblast, H<sub>2</sub>O<sub>2</sub> increased the formation of syncytial knots compared to fresh tissue and tissue cultured in control conditions (Figure 7.4A). Unlike the TUNEL index, the presence of syncytial knots was significantly increased by culture in both 100 $\mu$ M and 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> compared to control conditions.

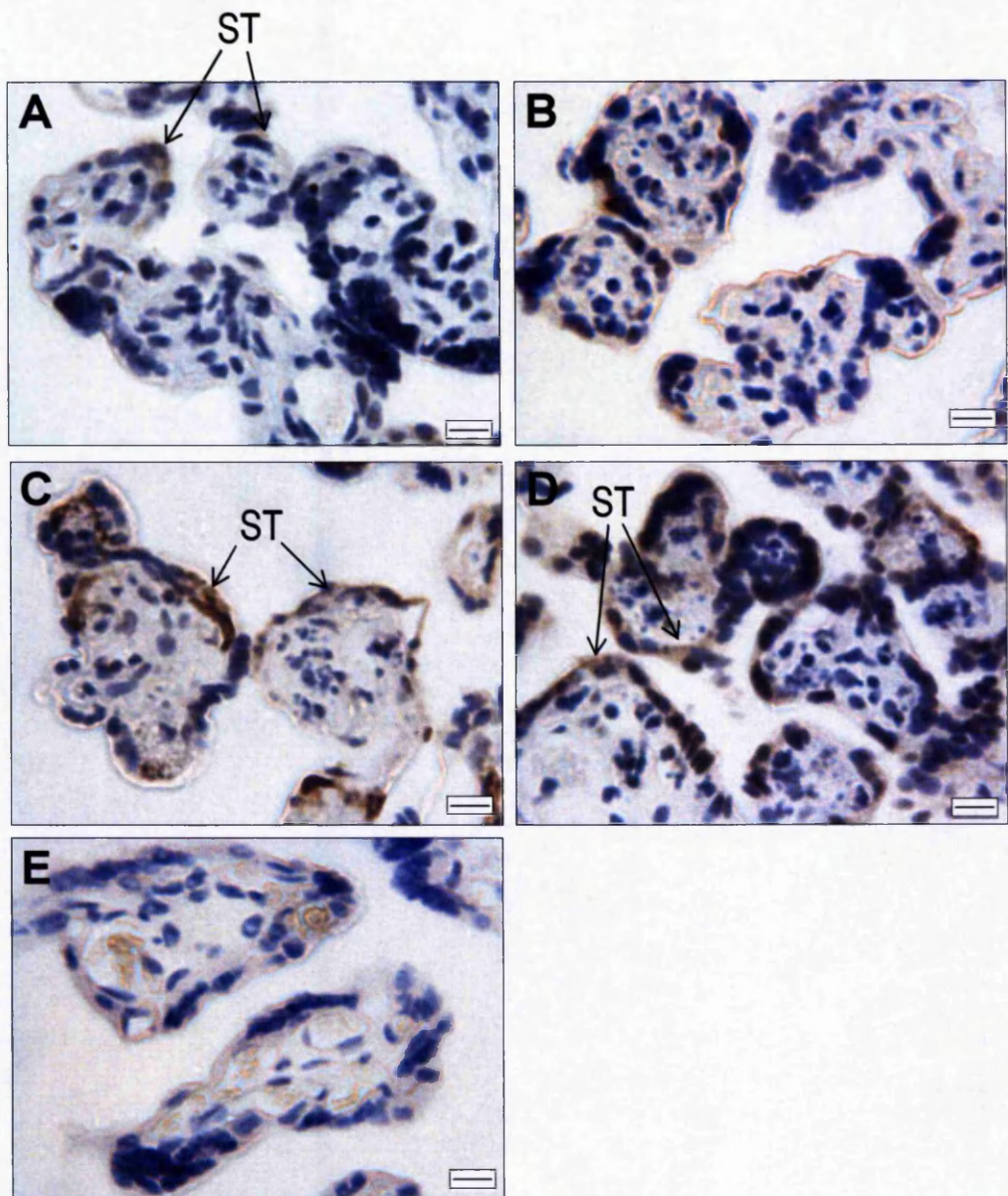
#### **7.4.2 Effects of Treatment with H<sub>2</sub>O<sub>2</sub> on Proliferation and hCG Expression**

In all tissue sections examined, Ki67 immunostaining was localised to cytotrophoblast nuclei. In these studies, cytotrophoblasts were identified morphologically as large mononuclear cells underlying the syncytiotrophoblast. The proliferative index was not significantly altered by culture in control conditions compared to fresh tissue (Figure 7.5A). Exposure to 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> significantly reduced the proliferative index (Figure 7.5A). Despite effects on apoptosis and proliferation, preliminary data suggests that exposure to H<sub>2</sub>O<sub>2</sub> did not alter hCG expression in conditioned culture medium; mean hCG expression in control conditions = 0.27 iu/mg tissue/hr, 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> = 0.24 iu/mg tissue/hr and 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> 0.25 iu/mg tissue/hr (N=2, samples pooled from n=6).



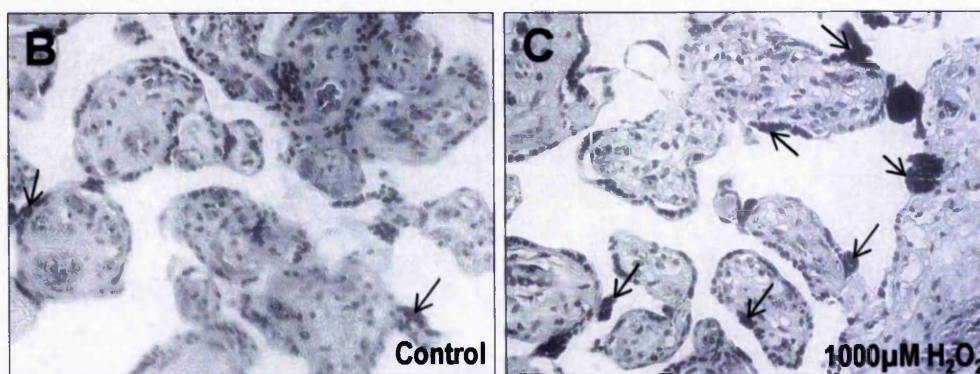
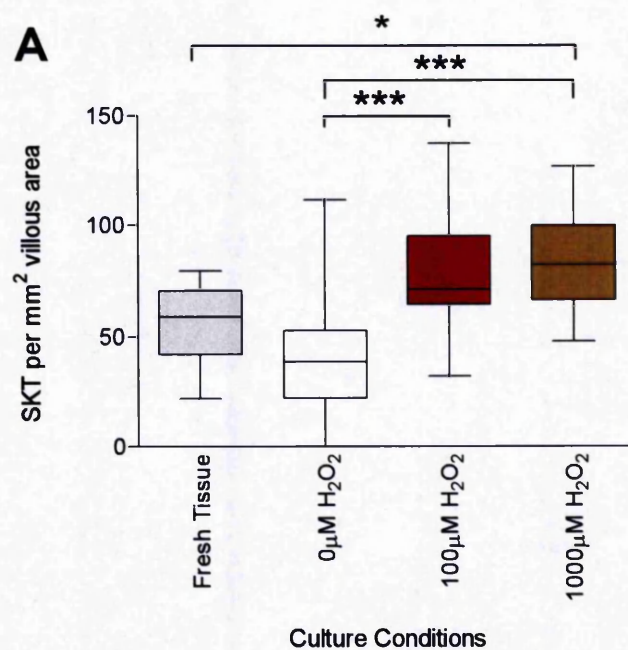
**Figure 7.2** – Assessment of apoptosis in placental villous explants exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 48 hours. (A) Apoptosis is significantly increased in placental villous explants cultured in 1000µM H<sub>2</sub>O<sub>2</sub> compared to control (\* p<0.05, \*\* p<0.01, \*\*\* p <0.001, Friedman test, n=6). Images obtained by confocal microscopy demonstrating TUNEL staining (green) counterstained with propidium iodide (red). (B) Positive control – incubated with DNase. (C) Negative control. (D) In fresh tissue occasional TUNEL positive nuclei are seen in the syncytiotrophoblast (ST). (E) Tissue cultured in control conditions shows TUNEL positive nuclei more diffusely in the ST. (F) After culture with 100µM H<sub>2</sub>O<sub>2</sub>, in addition to nuclei in ST (closed arrows), condensed TUNEL positive nuclei are visible in stromal cells (open arrows). (G) Explants cultured in 1000µM H<sub>2</sub>O<sub>2</sub> have widespread TUNEL staining in the ST (closed arrows) and cells underlying the syncytium (open arrows). Original Image Magnification x400.



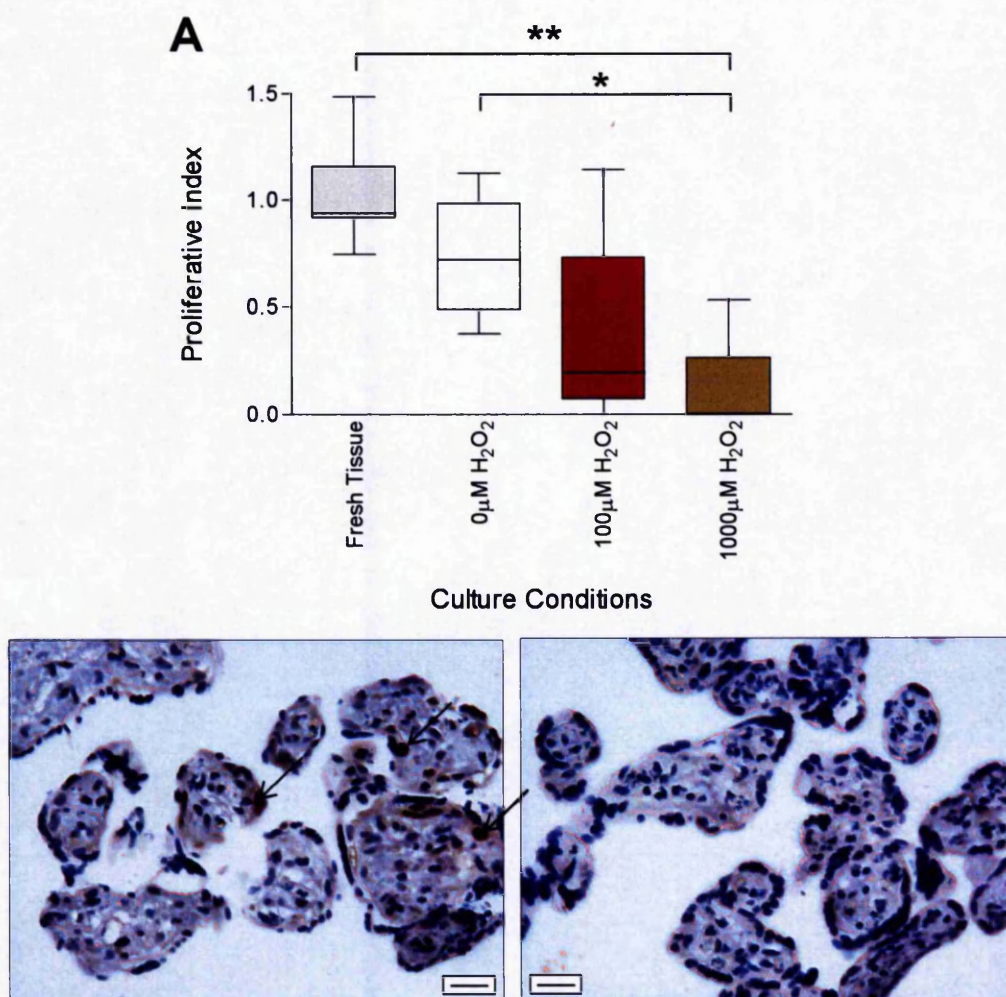


**Figure 7.3** - Localisation of increased apoptosis in explants exposed to  $\text{H}_2\text{O}_2$  using cytokeratin-18 neo-epitope M30. (A) Immunostaining for cytokeratin M30 neoepitope in fresh villous tissue shows discrete areas of expression in syncytiotrophoblast (ST). (B) M30 immunostaining in explants cultured in control conditions demonstrating immunoreactivity in isolated areas of ST. (C) Explants cultured in  $100\mu\text{M}$   $\text{H}_2\text{O}_2$  have increased M30 staining in ST. (D) Explants cultured in  $1000\mu\text{M}$   $\text{H}_2\text{O}_2$  have widespread M30 staining in the ST. (E) Negative control section demonstrating no immunoreactivity. Bar =  $10\mu\text{m}$ .





**Figure 7.4** – Assessment of syncytial knots in placental villous explants exposed to H<sub>2</sub>O<sub>2</sub> for 48 hours. (A) The formation of syncytial knots was increased following culture in the presence of 100 and 1000µM H<sub>2</sub>O<sub>2</sub> (\*  $p < 0.05$ , \*\*\*  $p < 0.001$ , Friedman test,  $n = 6$ ). (B) Low power micrograph showing occasional syncytial knots in tissue cultured in control conditions (open arrows). (C) Low power micrograph of tissue cultured in 1000µM H<sub>2</sub>O<sub>2</sub> showing increased numbers of syncytial knots (open arrows). Original magnification x250.



**Figure 7.5** – Assessment of proliferative index in explants exposed to H<sub>2</sub>O<sub>2</sub>. (A) Proliferative index was reduced following treatment with 1000µM H<sub>2</sub>O<sub>2</sub> (\*\* p<0.01, \* p<0.05, Friedman test, n=6). (B) Ki67 immunostaining in fresh tissue localised to cytotrophoblasts, as marked with open arrows. (C) Explants cultured in 1000µM H<sub>2</sub>O<sub>2</sub> showing reduced numbers of nuclei positive for Ki67 immunostaining. Bar = 20µm

#### **7.4.3 Effects of Treatment with H<sub>2</sub>O<sub>2</sub> on LDH activity in Conditioned Culture Medium**

There was no significant increase in necrosis following treatment with 100 $\mu$ M and 1000 $\mu$ M as assessed by LDH activity in conditioned culture media; median LDH activity in control = 8.2 iu/mg protein (Range 2.8-51.4), 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> = 14.4 iu/mg protein (Range 1.9-49.7) and 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> = 17.58 iu/mg protein (Range 3.5-26.1).

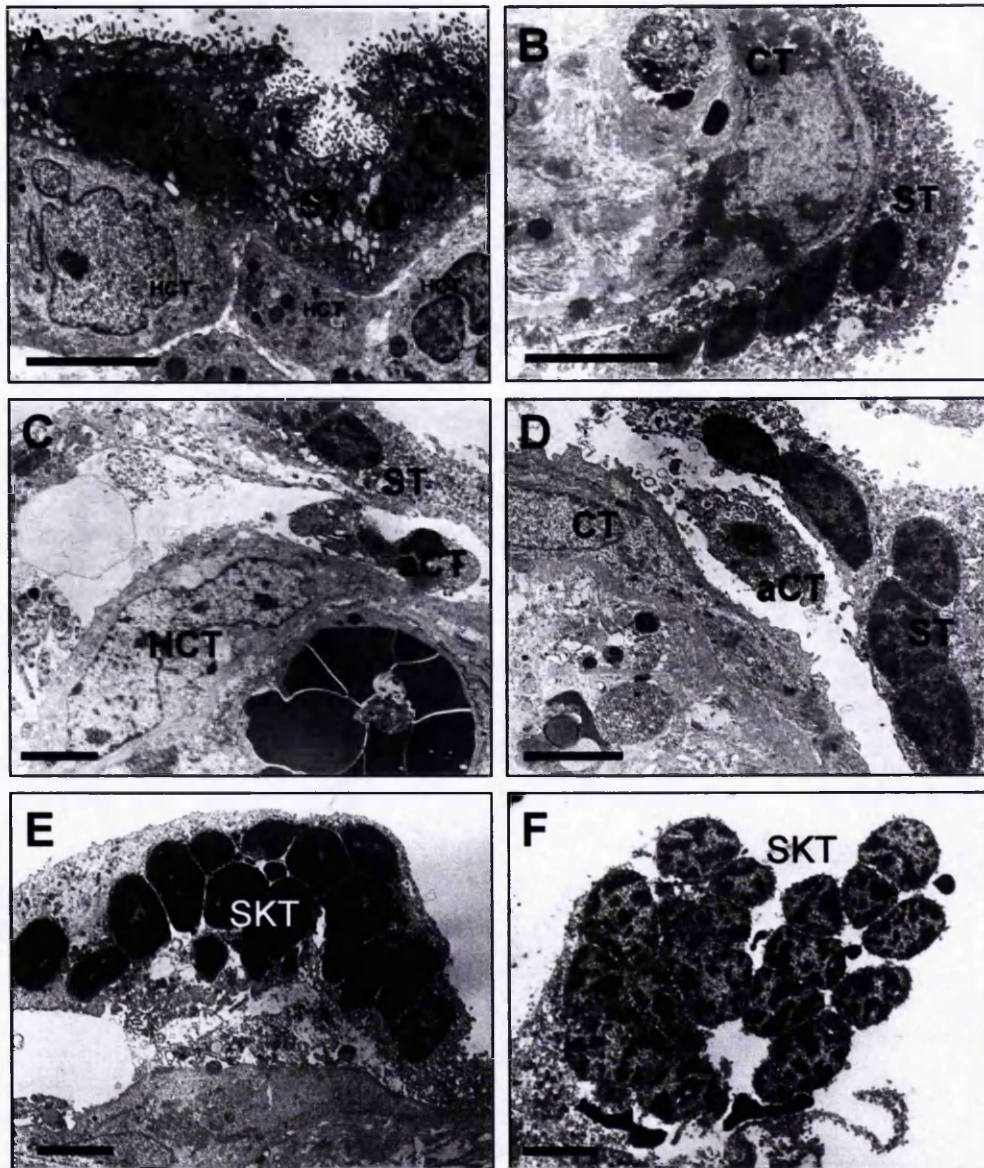
#### **7.4.4 Effects of Treatment with H<sub>2</sub>O<sub>2</sub> on Villous Morphology**

The changes observed in cell turnover were associated with altered villous morphology. In fresh tissue the syncytiotrophoblast was intact with healthy underlying cytotrophoblasts (Figure 7.6A). After culture in control conditions, the syncytiotrophoblast showed signs of degeneration including: vacuolation of cytoplasm; and dense clumps of peripheral chromatin in the syncytial nuclei (Figure 7.6B). There was little change in the appearances of cytotrophoblasts (Figure 7.6B). Culture in the presence of 100 $\mu$ M and 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with increased vacuolation of syncytiotrophoblast cytoplasm and peripheral chromatin condensation in syncytiotrophoblast nuclei. Following culture with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> the majority of cytotrophoblasts appeared intact (Figure 7.6C). However, exposure to 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with nuclear and cytoplasmic condensation of cytotrophoblasts, although some healthy cells remain (Figure 7.6D). Treatment with H<sub>2</sub>O<sub>2</sub> was associated with the formation of syncytial knots, aggregates of degenerate nuclei with much dense euchromatin (Figure 7.6E). Rarely syncytial degeneration was so marked following treatment with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> that syncytial integrity was compromised releasing syncytiotrophoblast nuclei into the intervillous space (Figure 7.6F).

#### **7.4.5 Effects of treatment with H<sub>2</sub>O<sub>2</sub> on Localisation of p53, Mdm2, p21, Bax and Bcl-2**

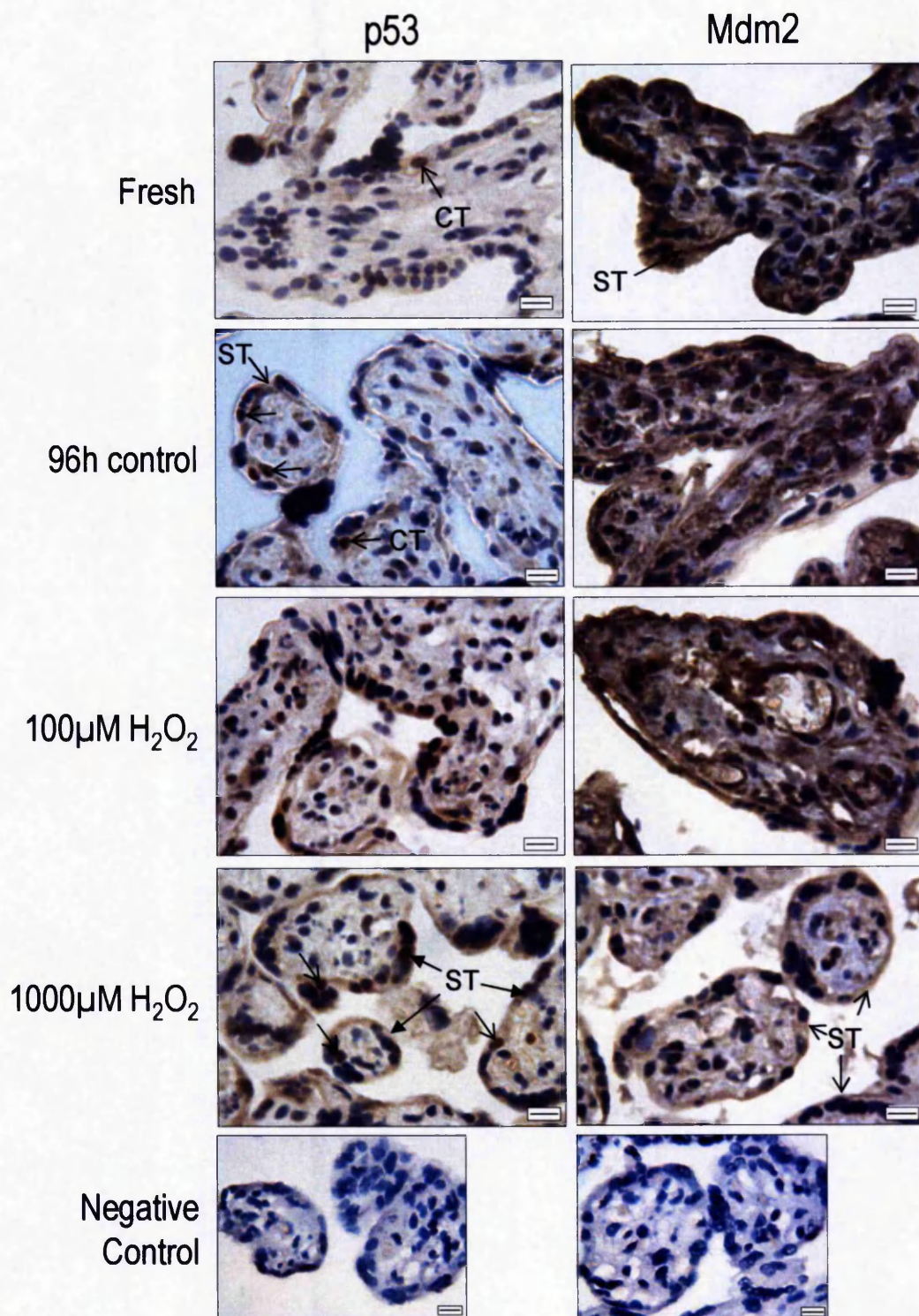
In fresh tissue, p53 protein localised to occasional syncytiotrophoblast and cytotrophoblast nuclei with very little cytoplasmic expression (Figure 7.7). In tissue cultured under control conditions p53 expression was increased in nuclei and cytoplasm of stromal cells and was present in some cytotrophoblast nuclei and rarely in syncytiotrophoblast nuclei. A similar pattern of p53 localisation was noted in explants treated with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub>, although weak staining was present in discrete areas of syncytiotrophoblast cytoplasm (Figure 7.7). In





**Figure 7.6** – Assessment of morphology of placental villous explants treated with  $H_2O_2$  by electron microscopy. (A) In fresh villous tissue there is a layer of healthy cytotrophoblast (HCT) cells underlying syncytiotrophoblast (ST). The ST has a healthy microvillous membrane and nuclei show typical appearances of peripheral chromatin condensation. (B) Placental villous explant cultured for 4 days, in this area the ST is still in contact with the underlying cytotrophoblast (CT), although the ST nuclei show more advanced features of apoptosis, with increased euchromatin:heterochromatin ratio compared to fresh tissue. The CT contains evidence of homogenous deposits, which may represent fat droplets. (C) Following 48 hours culture in the presence of  $100\mu M H_2O_2$  there is some evidence of apoptotic CT (aCT) with pyknotic nuclei and cytoplasmic shrinkage. However, other CTs appear healthy. (D) Explants cultured in  $1000\mu M H_2O_2$  show a similar mixture of apoptotic and healthy CTs. ST nuclei show increased evidence of degeneration compared to control. (E) Syncytial knots (SKT) – aggregations of apoptotic nuclei were evident in explants cultured in  $100\mu M H_2O_2$  and  $1000\mu M H_2O_2$ . (F) In some areas degeneration had progressed to rupture of the SKT releasing nuclei into the intervillous space. Scale bars =  $5\mu m$ .





**Figure 7.7** – Representative images of immunoperoxidase staining for p53 and Mdm2 in explants treated with H<sub>2</sub>O<sub>2</sub>. In fresh term villous tissue p53 was present in occasional nuclei (marked with arrow). After 96 culture in control conditions, p53 was present in multiple nuclei and discrete areas of syncytiotrophoblast (ST) cytoplasm. Treatment with 1000µM H<sub>2</sub>O<sub>2</sub> was associated with p53 expression in ST cytoplasm (closed arrows) and nuclei in the ST and underlying CT (open arrows). In fresh tissue, Mdm2 was strongly expressed in the cytoplasm of ST and some stromal cells. Culture in control conditions or treatment with 100µM H<sub>2</sub>O<sub>2</sub> was not associated with any alteration in the localisation of Mdm2. Treatment with 1000µM H<sub>2</sub>O<sub>2</sub> was not associated with any changes in the localisation of Mdm2. However, the density of staining appeared to be reduced in the ST. There was no immunostaining evident in negative control tissue sections. Bar = 10µm.



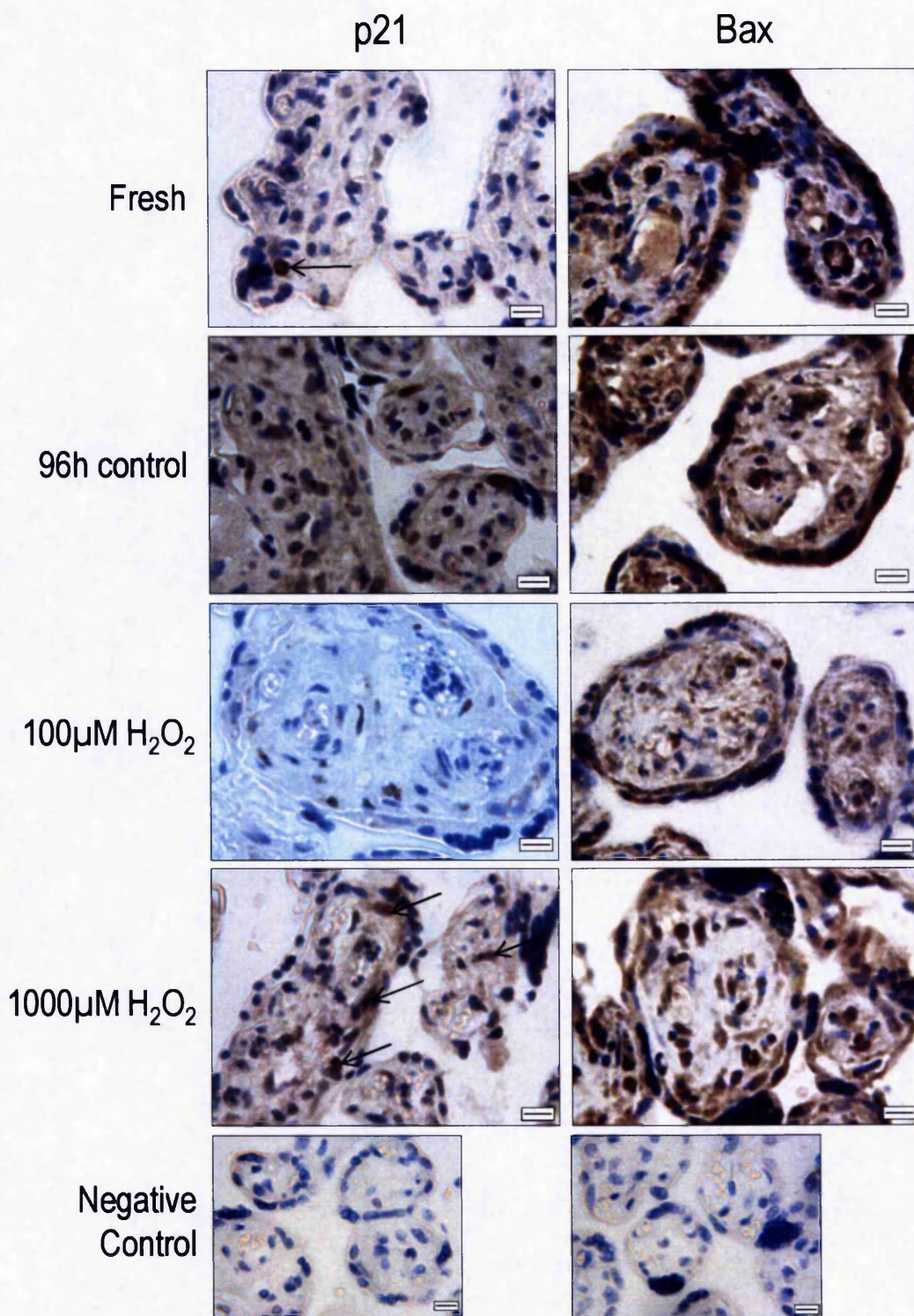
explants treated with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub>, p53 expression localised to nuclei of both trophoblast compartments and syncytiotrophoblast cytoplasm. Even though p53 appeared to be increased in the syncytiotrophoblast, p53 was not present in the region of syncytial knots (Figure 7.7).

In fresh tissue Mdm2 strongly localised to syncytiotrophoblast cytoplasm and more weakly to the cytoplasm of some stromal cells (Figure 7.7). There was little variation in the presence of Mdm2 within syncytiotrophoblast and there was no loss of Mdm2 in syncytial knots. Culture in control conditions was associated with increased immunostaining for Mdm2 in stromal cells, although Mdm2 was still present in syncytiotrophoblast cytoplasm (Figure 7.7). In explants treated with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> Mdm2 localisation was not altered and was present in syncytiotrophoblast even when separating from the villus (Figure 7.7). In contrast to culture in control conditions, exposure to 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> appeared to be associated with a decrease in Mdm2 staining in the cytoplasm of both syncytiotrophoblast and stromal cells (Figure 7.7).

p21 was confined to occasional cytotrophoblast nuclei in fresh villous tissue; there was no expression in stromal cells or syncytiotrophoblast (Figure 7.8). In tissue cultured in control conditions p21 was present in few nuclei of cytotrophoblast, syncytiotrophoblast and stromal cells. Culture in the presence of 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> did not alter the localisation of p21 compared to control conditions (Figure 7.8). Treatment with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with p21 expression in cytotrophoblast and stromal nuclei, but this was not accompanied by an increase in p21 expression in syncytiotrophoblast nuclei (Figure 7.8).

In fresh tissue, Bax was present in the cytoplasm of syncytiotrophoblast and some cells of the stromal villous core including endothelial cells (Figure 7.8). In cultured explants, the expression of Bax in the syncytiotrophoblast appeared to be weaker than in fresh tissue. Conversely, Bax expression was increased in the cytoplasm of stromal cells compared to fresh tissue. Similarly, Bax was strongly expressed in syncytiotrophoblast cytoplasm and stromal tissue of explants exposed to 100 $\mu$ M or 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub>. (Figure 7.8).

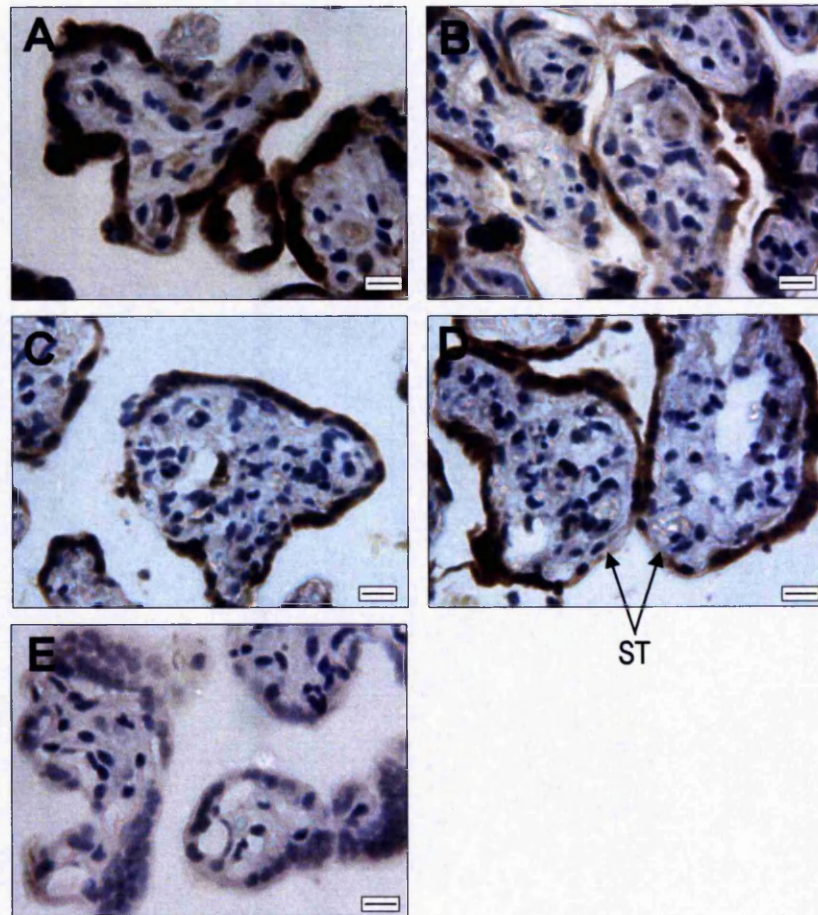
Anti-apoptotic Bcl-2 was strongly expressed in the syncytiotrophoblast cytoplasm of fresh tissue, and neither the localisation nor degree of staining was altered following culture in control conditions (Figure 7.9). Bcl-2 immunostaining was not present throughout the syncytiotrophoblast after treatment with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub>, but was not reduced in the region of syncytial knots (Figure 7.9). Bcl-2 expression appeared to be reduced in discrete areas of explants cultured in the presence of 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub>. This may reflect loss of the syncytio-



**Figure 7.8** – Representative images of immunoperoxidase staining for p21 and Bax in explants treated with H<sub>2</sub>O<sub>2</sub>. In fresh term villous tissue p21 was present in occasional nuclei (marked with arrow). After 96 culture in control conditions, p21 was present in multiple nuclei, including those in stromal tissue. Treatment with 1000μM H<sub>2</sub>O<sub>2</sub> was associated with p21 expression in ST and CT nuclei (open arrows). In fresh tissue, Bax was strongly expressed in the cytoplasm of ST and some stromal cells. Culture in control conditions or treatment with either dose of H<sub>2</sub>O<sub>2</sub> was not associated with any alteration in the localisation or degree of staining of Bax. There was no immunostaining evident in negative control tissue sections. Bar = 10μm.



## Bcl-2



**Figure 7.9** – Representative images of immunoperoxidase staining for Bcl-2 in explants treated with  $\text{H}_2\text{O}_2$ . (A) In fresh tissue, Bcl-2 was strongly expressed in syncytiotrophoblast (ST) cytoplasm and some stromal cells. (B) Culture in control conditions was not associated with any alteration in the localisation or degree of staining of Bcl-2. (C) Following treatment with  $100\mu\text{MH}_2\text{O}_2$  Bcl-2 was not altered, remaining strongly expressed in ST cytoplasm. (D) After treatment with  $1000\mu\text{MH}_2\text{O}_2$  Bcl-2 was decreased in discrete areas of the ST cytoplasm, marked with arrows. (E) There was no immunostaining evident in negative control tissue sections. Bar =  $10\mu\text{m}$ .

trophoblast layer, although areas of intact syncytiotrophoblast were seen with absent Bcl-2 immunostaining (Figure 7.9)

## 7.5 Discussion

This study demonstrates that ROS in the form of  $H_2O_2$  can induce apoptosis in term placental villous explants. As described in Chapter 6, TUNEL appeared to be a specific marker for DNA-strand breaks as treatment with DNase showed positive staining in almost all nuclei. The increase in TUNEL-labelled nuclei predominantly localised to the syncytiotrophoblast, with TUNEL staining only evident in underlying cells treated with  $1000\mu M$   $H_2O_2$ . In these experiments there appeared to be a dose-dependent relationship between TUNEL index and  $H_2O_2$ . However, the TUNEL index was not affected by duration of culture suggesting that DNA fragmentation occurred within 6 hours after treatment with  $H_2O_2$ . Similarly rapid changes in TUNEL staining were observed following hypoxia-reoxygenation injury indicating that exposure to ROS can rapidly induce apoptosis (Hung et al. 2002). As  $H_2O_2$  can cause DNA damage directly, apoptotic cell death was confirmed by immunostaining for the cytokeratin-M30 neo-epitope; this confirmed that the increased apoptosis following exposure to  $H_2O_2$  was predominantly localised to the syncytiotrophoblast. The increased apoptosis in the syncytiotrophoblast was associated with an increase in the number of syncytial knots compared to fresh tissue and explants cultured in control conditions, suggesting that ROS may lead to a combination of apoptosis and syncytiotrophoblast ageing. However, unlike placental villous explants cultured in 1%  $O_2$  (Chapter 6), the increase in apoptotic cell death and syncytial knots was not accompanied by an increase in necrosis, indicating that despite a profound insult the trophoblast could still generate sufficient energy to complete cell death by apoptosis.

The increased apoptosis following exposure to ROS is supported by evidence that exposure to hypoxia-reperfusion injury also induces apoptosis in a similar in vitro model (Hung et al. 2002), which found comparable localisation of apoptotic nuclei to that described here. The relative sparing of cytotrophoblast and stromal cells may result from a lack of penetrance of ROS to deeper tissues, as they may be absorbed in the syncytiotrophoblast by antioxidants such as glutathione or are degraded by the activity of SOD (Wang and Walsh 1996; Telfer et al. 1997). Alternatively, the stronger expression of Cu/Zn SOD, MnSOD and catalase in cytotrophoblasts

may provide protection for this cell type, which may finally be overwhelmed *in vitro* when exposed to 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> (Myatt et al. 1997; Watson et al. 1998).

In addition to inducing apoptosis, culture in the presence of 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> was associated with a decrease in proliferative index, a situation comparable with the culture in 1% O<sub>2</sub> (Chapter 6), providing evidence that apoptosis and proliferation may be inversely related in trophoblast *in vitro*, a relationship previously identified *in vivo* (Yamada et al. 2001). Although proliferation was decreased following treatment with 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub>, there was no corresponding fall in hCG secretion. This finding agrees with data presented in Chapter 6 demonstrating no reduction in hCG in low O<sub>2</sub> concentration compared to control (6% O<sub>2</sub>). Interestingly, hCG is increased in maternal serum in pregnancies complicated by pre-eclampsia (Bartha et al. 2003; Gurbuz et al. 2004) and is increased in explants taken from pregnancies complicated by pre-eclampsia. These observations suggest that apoptosis is not necessarily related to a reduction in hCG secretion in placental tissue. Nevertheless, the combination of increased apoptosis and syncytial knot formation and decreased proliferation seen following culture in low O<sub>2</sub> and exposure to ROS will disrupt the renewal of syncytiotrophoblast (Figure 1.5). It may be speculated that this might have an impact on other aspects of villous function such as nutrient transport or gas transfer. If this were the case, the increased apoptosis observed in pre-eclampsia and IUGR *in vivo* may play an important role in the pathophysiology of these disorders.

H<sub>2</sub>O<sub>2</sub> can induce apoptosis in many different cell types including cells derived from gastric carcinoma cells (Mao et al. 2006), glial cells (Bonini et al. 2004), cardiomyocytes (von Harsdorf et al. 1999) and fibroblasts (Chen et al. 2000). Furthermore, intracellular H<sub>2</sub>O<sub>2</sub> may act as a secondary mediator of apoptosis induced by treatment with agents such as Doxorubicin (Tsang et al. 2003). In other cell types H<sub>2</sub>O<sub>2</sub> is associated with altered expression of constituents of the intrinsic apoptotic pathway, particularly p53, which may be activated by phosphorylation (Chen et al. 2000; Mao et al. 2006). Following oxidative damage, p53 identifies DNA damage and initiates the appropriate base repair machinery (Achanta and Huang 2004). However, if DNA or cell damage is too great then the cell will undergo apoptosis. This dual role of p53 may explain the elevation in proteins promoting both cell-cycle arrest (e.g. p21) and apoptosis (e.g. Bax) observed in placental explants following treatment with H<sub>2</sub>O<sub>2</sub> (Yin et al. 1999). A similar pattern of protein expression was described in melanoma cells, in



which exposure to 0.5mM H<sub>2</sub>O<sub>2</sub> induced p53, p21 and Bax protein expression (Gomez Sarosi et al. 2003). Conversely, studies of glial cells identified that the increase in p53 following treatment with H<sub>2</sub>O<sub>2</sub> predominantly localised to the mitochondrial membrane (Bonini et al. 2004). In this cell type the elevated apoptosis was abolished by addition of the caspase inhibitor zVAD-fmk but not pifithrin-1α – an inhibitor of p53-dependent transcription; the authors propose that p53 may elicit pro-apoptotic effects by direct action on mitochondria (Bonini et al. 2004). Interestingly, this study also described a caspase-dependent degradation of Mdm2 following treatment with H<sub>2</sub>O<sub>2</sub>, in this instance accelerated degradation of Mdm2 was associated with accumulation of p53 in nuclei (Bonini et al. 2004). In trophoblast, p53 appears to have a number of transcriptional effects potentially including increased expression of pro-apoptotic Bid, Bad and Bcl-Xs mRNA (Hu et al. 2006a) and Bax protein expression (Chapter 3).

An imbalance in the regulators of mitochondrial membrane permeability is frequently described following treatment with H<sub>2</sub>O<sub>2</sub> (Gomez Sarosi et al. 2003; Tsang et al. 2003; Bonini et al. 2004; Mao et al. 2006). There is an intricate balance between regulators of mitochondrial membrane permeability and H<sub>2</sub>O<sub>2</sub>. In cardiomyocytes, following oxidative stress, mitochondria may generate excessive H<sub>2</sub>O<sub>2</sub>, the release of which is controlled by anti-apoptotic Bcl-2 and Mcl-1. This provides chronic low-grade exposure to ROS which protects these cells against apoptosis induced by acute oxidation (Kowaltowski et al. 2004). Furthermore, there was no change in expression of Bcl-2 and Bax, although Bax translocated to the mitochondrial membrane promoting cytochrome c release and apoptosis (von Harsdorf et al. 1999). This indicates that subtle changes to the mitochondrial environment are sufficient to induce apoptosis, and more detailed investigation is required to determine whether these changes are present in trophoblast.

Culture of placental explants or BeWo cells in the presence of H<sub>2</sub>O<sub>2</sub> produced similar effects on cell turnover, increasing apoptosis and decreasing proliferation (Chapters 5 and 7). Interestingly, in both models of villous trophoblast cell-turnover, exposure to H<sub>2</sub>O<sub>2</sub> produced a greater increase in apoptotic index than hypoxia. In addition, treatment with H<sub>2</sub>O<sub>2</sub> was not associated with an increase in necrosis. In explants, the increased apoptosis predominantly localised to the syncytiotrophoblast, thereby more accurately mimicking the increased apoptosis observed in pre-eclampsia or IUGR (Ishihara et al. 2002). Furthermore, explants

cultured in the presence of 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> demonstrate similar alterations in expression of regulators of apoptosis to those described in pre-eclampsia. A role for ROS rather than chronic hypoxia in the origin of trophoblast apoptosis is also supported by evidence that chronic hypoxia has no effects on placental infarction or villous trophoblast apoptosis, and appears to lessen oxidative stress (Zamudio et al. 2007).

The ability of antioxidants, such as carbon monoxide or vitamins C and E, to attenuate apoptosis induced by oxidative stress, has led to the proposal that antioxidant therapy may reduce the incidence of pre-eclampsia (Cindrova-Davies et al. 2005; Bainbridge et al. 2006). However, the first large-scale randomized-controlled trial of such an intervention reported that there was no reduction in the incidence of pre-eclampsia in women given vitamin C and E compared to placebo (Poston et al. 2006). This finding has raised a number of questions regarding the role of both systemic and placental oxidative stress in pre-eclampsia. Currently, there are no reports regarding placental tissue from women who received vitamins C and E during their pregnancies. A single study of villous trophoblast found that apoptosis was decreased in women with a higher prenatal vitamin C level. Given the large body of evidence demonstrating a reduction in antioxidant defences in villous trophoblast in pre-eclampsia and the ability of oxidative stress to reproduce trophoblast apoptosis, the role of oxidative stress in promoting the placental phenotype observed in pre-eclampsia cannot be discounted.

## **Chapter 8 – Can Epidermal Growth Factor Attenuate Apoptosis Induced by Oxidative Stress in Term Placental Villous Explants?**

### **8.1 Introduction**

In previous chapters emphasis has been placed on cell damage as a key initiator of apoptosis. However, the precise cellular response represents an integration of apoptotic and survival signals. Pro-apoptotic impulses may come from the external environment such as TNF- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ) (Fadeel and Orrenius 2005) or the internal milieu via the p53 pathway. Survival signals, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) or insulin-like growth factor 1 and 2 (IGF1 and 2) may be received from the extracellular surroundings. The constituents of both intrinsic and extrinsic apoptotic pathways are present and functional within villous trophoblast (Levy and Nelson 2000), as apoptosis has been induced in trophoblast *in vitro* by treatment with TNF- $\alpha$  and IFN- $\gamma$  (Yui et al. 1994b; Crocker et al. 2004c) and hypoxia, ROS or hypoxia-reoxygenation (Levy et al. 2000; Hung et al. 2002). Likewise, survival factors alter trophoblast turnover; EGF promotes differentiation of villous trophoblast and regulates invasion of extravillous trophoblast (Morrish et al. 1987; Anteby et al. 2004). EGF receptors are expressed in both extravillous and villous cytotrophoblasts and syncytiotrophoblast of first trimester placenta (Bulmer et al. 1989; Ladines-Llave et al. 1991; Hofmann et al. 1992; Muhlhauser et al. 1993). Therefore, in common with the majority of other cell-types it is likely that villous trophoblast integrates apoptotic and survival signals to determine the response to cell damage.

Growth factors can promote survival by directly antagonising apoptosis. Barres et al. demonstrated that apoptosis induced in rat optical nerve glial cells by serum deprivation (Barres et al. 1992) was attenuated by concurrent administration of growth factors, such as platelet-derived growth factor (PDGF) (Barres et al. 1992). As this attenuation of cell loss was not associated with an increase in cell proliferation it is proposed that administration of growth factors antagonises apoptosis. These findings have been extended, and it has been suggested that cell survival is dependent upon a plentiful supply of growth factors (Raff 1992). Certainly, growth factors are able to inhibit apoptosis of primary term cytotrophoblasts induced by exposure to TNF $\alpha$  and IFN- $\gamma$  ceramide or hypoxia (Garcia-Lloret et al. 1996; Payne et al.

1999; Levy et al. 2000; Smith et al. 2002). The effect of growth factors on trophoblast apoptosis has been previously studied with the greatest reduction in apoptosis noted in response to EGF, with partial efficacy of bFGF, IGF-1, and PDGF-AA also recorded (Smith et al. 2002). It is noteworthy that the receptors of these growth factors are all direct protein kinases, suggesting a similar mode of action (Smith et al. 2002).

As mentioned previously, EGF promotes differentiation of cytotrophoblasts and regulates invasion of extravillous trophoblast (Morrish et al. 1987; Anteby et al. 2004). EGF receptors are decreased when apoptosis is induced via the extrinsic pathway (Tavare and Holmes 1989; Filla et al. 1993; Toth et al. 1997). Perhaps of greatest relevance to clinical pathologies, a reduction in EGF is associated with abnormal pregnancy outcome. The concentration of EGF is reduced in urine of women with pregnancies complicated by IUGR (Lindqvist et al. 1999) and a reduction in maternal EGF in mice is associated with a reduced birthweight (Kamei et al. 1999). However, there are presently no data regarding concentrations of EGF in pre-eclampsia.

Thus far, studies of the role of EGF as a survival factor in the placenta have used either primary cytotrophoblasts or first trimester explant cultures (Maruo et al. 1992; Garcia-Lloret et al. 1996; Payne et al. 1999; Levy et al. 2000; Smith et al. 2002). A tissue culture technique may be advantageous as normal tissue architecture, cell-cell interactions and paracrine effects, the absence of which may potentiate apoptosis, are preserved. In addition, culture of third trimester tissue may be more appropriate to investigate the effects of EGF with respect to apoptosis in pre-eclampsia because this is when the syndrome becomes clinically manifest.

## **8.2 Hypothesis**

It is hypothesised that treatment with EGF will reduce apoptosis and alter cell turnover in term placental villous explants. Due to its putative survival effects, it is anticipated that EGF will attenuate apoptosis induced by exposure to ROS in the form of H<sub>2</sub>O<sub>2</sub>. As EGF acts via a protein-kinase, it is hypothesised that treatment with EGF will increase phosphorylation of signalling molecules such as phospho-inositol 3-kinase (PI3-kinase) and AKT.

### **8.3 Methods**

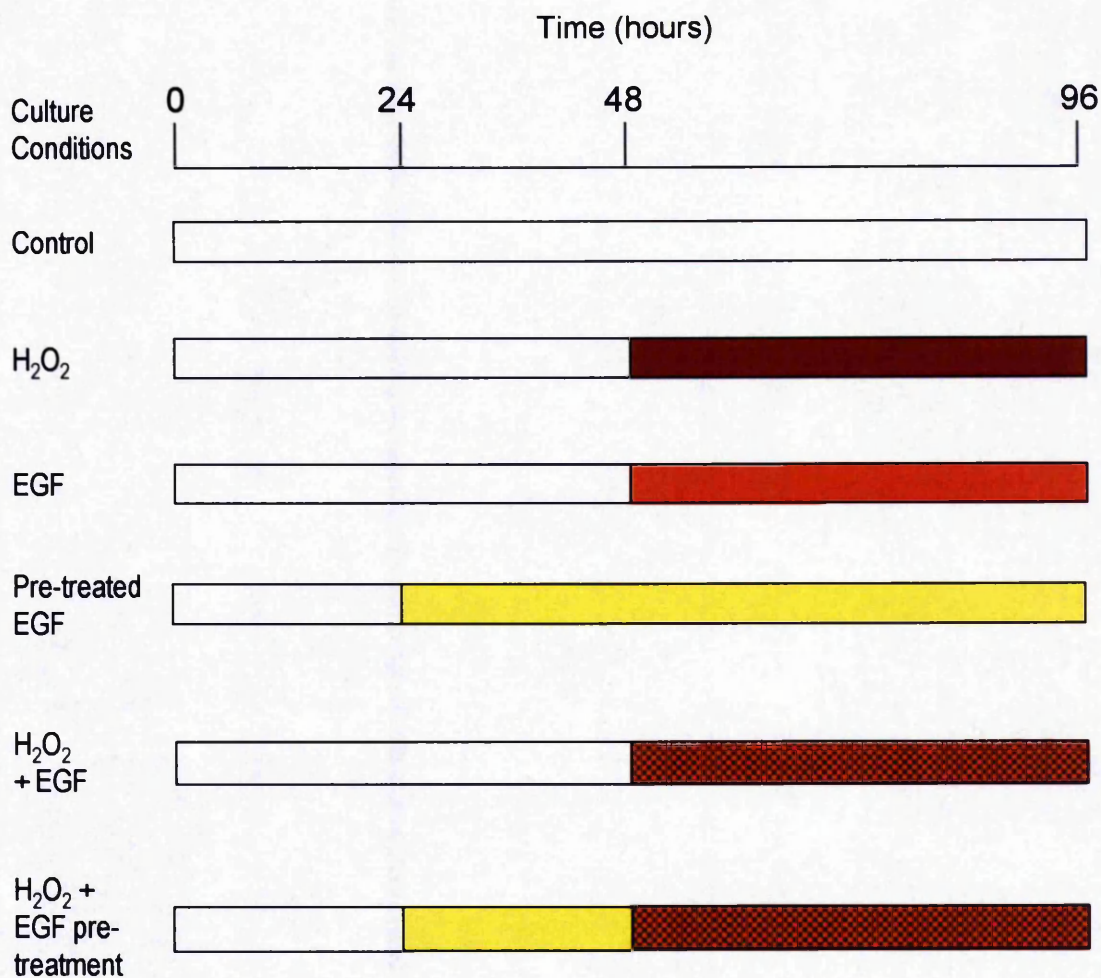
#### **8.3.1 Preparation and Culture of Term Placental Villous Explants**

Human term placentas (n=6) were obtained following elective Caesarean section of uncomplicated pregnancies at 37-40 weeks gestation. Women were normotensive throughout pregnancy and had no pre-existing medical conditions. Ethical approval was granted by the Central Manchester Local Research Ethics Committee.

3 areas of each placenta were randomly sampled within 20 minutes of delivery as previously described (Crocker et al. 2004a); Placental villous explants (5mg approx) were prepared from three randomly selected areas of placenta as described in section 2.2.1.1. Fresh villous tissue was fixed for immunohistochemistry (section 2.3.3.1) and electron microscopy (section 2.3.3.2) to enable comparisons between fresh and cultured tissue. Explants were cultured for 96 hours in CMRL-1066 culture medium supplemented as described in section 2.1. Explants were suspended at the gas-liquid interface as previously described (Crocker et al. 2004b; Crocker et al. 2004c) in 6% O<sub>2</sub>, 5% CO<sub>2</sub>, and 89% N<sub>2</sub> at 37°C (section 2.2.3.1). Explants were initially cultured for 48 hours to allow syncytial shedding and regeneration (Crocker et al. 2004b). Subsequently the culture medium was replaced with media containing either H<sub>2</sub>O<sub>2</sub> alone or in combination with EGF as shown in Figure 8.1. To assess the effects of EGF alone, unaltered culture medium was used as control. To investigate whether EGF could attenuate the apoptotic effects of H<sub>2</sub>O<sub>2</sub>, explants were cultured with 1000µM H<sub>2</sub>O<sub>2</sub> alone or in combination with 10 or 100 ng/ml EGF. The explants were then cultured for a further 48 hours. In addition, a further group of explants were exposed to EGF (10 or 100 ng/ml) for 24 hours before addition of H<sub>2</sub>O<sub>2</sub> and EGF to determine whether prolonged exposure to EGF was able to confer additional survival benefit.

Following culture, explants from each well were weighed before fixation prior to embedding in wax or resin (sections 2.3.3.1 and 2.3.3.2 respectively). Conditioned culture medium was collected as described in section 2.3.4.





**Figure 8.1** - Schematic diagram to illustrate placental explant culture protocol indicating duration of exposure to epidermal growth factor (EGF) and H<sub>2</sub>O<sub>2</sub>. Clear bars indicate control conditions, brown bars indicate culture in the presence of hydrogen peroxide, orange bars indicate culture in the presence of EGF and chequered bars indicate culture in the presence of hydrogen peroxide and EGF. Pre-treatment with EGF is shown by the yellow bars. Culture continued for 48 hours after the change of culture medium.

### **8.3.2 Immunohistochemistry**

Tissue sections were deparaffinised and exposed to microwave pre-treatment as described in section 2.8.2. Endogenous peroxidase activity was quenched and non-specific binding was blocked as described. Tissue sections were exposed to antibodies against phosphorylated-AKT (p-AKT) or phosphorylated PI3K (p-PI3K) (concentrations and sources shown in section 2.8.2). Each tissue was incubated with either a matching concentration of non-immune mouse IgG or blocking solution alone to serve as a negative control for mouse and rabbit primary antibodies respectively. Sections were then probed with appropriate biotin conjugated secondary antibodies, followed by incubation with avidin-peroxidase. Immunostaining was revealed by exposure to a concentrated DAB kit for 3 minutes. Slides were counterstained with haematoxylin and sections viewed using a Leitz microscope with ImageProPlus 4.5 (Media Cybernetics Inc). All samples for comparison were stained in the same batch. There was no immunoreactivity in negative control sections. Qualitative assessment of immunostaining was made of ten images of each experimental condition by two independent observers.

### **8.3.3 Assessment of Apoptosis**

Apoptosis was assessed using a commercially available TUNEL kit (Roche Applied Diagnostics) with modifications to the manufacturers' instructions as detailed in section 2.10.1. For these experiments TUNEL staining was quantified using images generated by confocal microscopy, assessing 6 randomly selected fields of terminal villi per experimental condition. The number of TUNEL positive nuclei were counted manually, and the total number of nuclei per cross-section of tissue measured using sequential colour thresholding as previously described (Crocker et al. 2004a). A ratio was derived from these values; the TUNEL index (% of total nuclei TUNEL positive).

### **8.3.4 Assessment of Syncytial Knots**

5µm tissue sections were stained with haematoxylin and eosin as described in section 2.11. The number of syncytial knots as defined by Cantle et al. was counted manually in 10 randomly selected fields of view for each experimental condition (Cantle et al. 1987). The trophoblast area was measured as described in section 2.11 and data normalised to give a measure of the number of syncytial knots per mm<sup>2</sup> of villus.

### **8.3.5 Assessment of Proliferation**

Cell proliferation was assessed using immunostaining for mouse anti-Ki-67 antibody (Clone Mib-1) as described in section 2.12.1. Ki67 staining was quantified using 10 random fields of terminal villi per experiment. The number of Ki67 positive nuclei were counted manually and the total number of nuclei per cross-section of villous tissue measured by sequential colour thresholding. A ratio was derived; the proliferative index (% of total nuclei Ki67 positive).

### **8.3.6 Electron Microscopy**

Explants from each experimental condition were fixed and treated for electron microscopy as described in section 2.14. Semi-thin sections, 0.5µm thick, were inspected to identify areas of interest. Ultrathin sections were examined by two observers using a Philips CM10 electron microscope, at an accelerating voltage of 80 kV, and representative areas of tissue photographed.

### **8.3.7 Analysis of Conditioned Culture Medium**

Necrotic cell death was assessed by the presence of LDH in the conditioned culture medium using an enzyme-linked assay as described in section 2.13.1.1. The intra-assay variability was 9.79%. The presence of hCG in culture medium was quantified using a commercial quantitative immunoradiometric assay in accordance with the manufacturers instructions (ICN Pharmaceuticals; Basingstoke, UK), detailed in section 2.13.2. The intra-assay variability using this technique was 7.05%. Samples (n=6) were pooled for quantification of hCG to give N=2.

### **8.3.8 Statistical Analysis**

As tissue from each placenta was exposed to EGF and H<sub>2</sub>O<sub>2</sub>, statistical significance was tested using the Friedman test for matched non-parametric data. Dunn's post-hoc test was performed as appropriate. Results are presented as median and interquartile range (IQR), and plotted as box and whisker plots (Box = IQR, Whiskers = total range).

## 8.4 Results

### 8.4.1 Effects of EGF alone on Apoptosis, Syncytial Knots and Necrosis

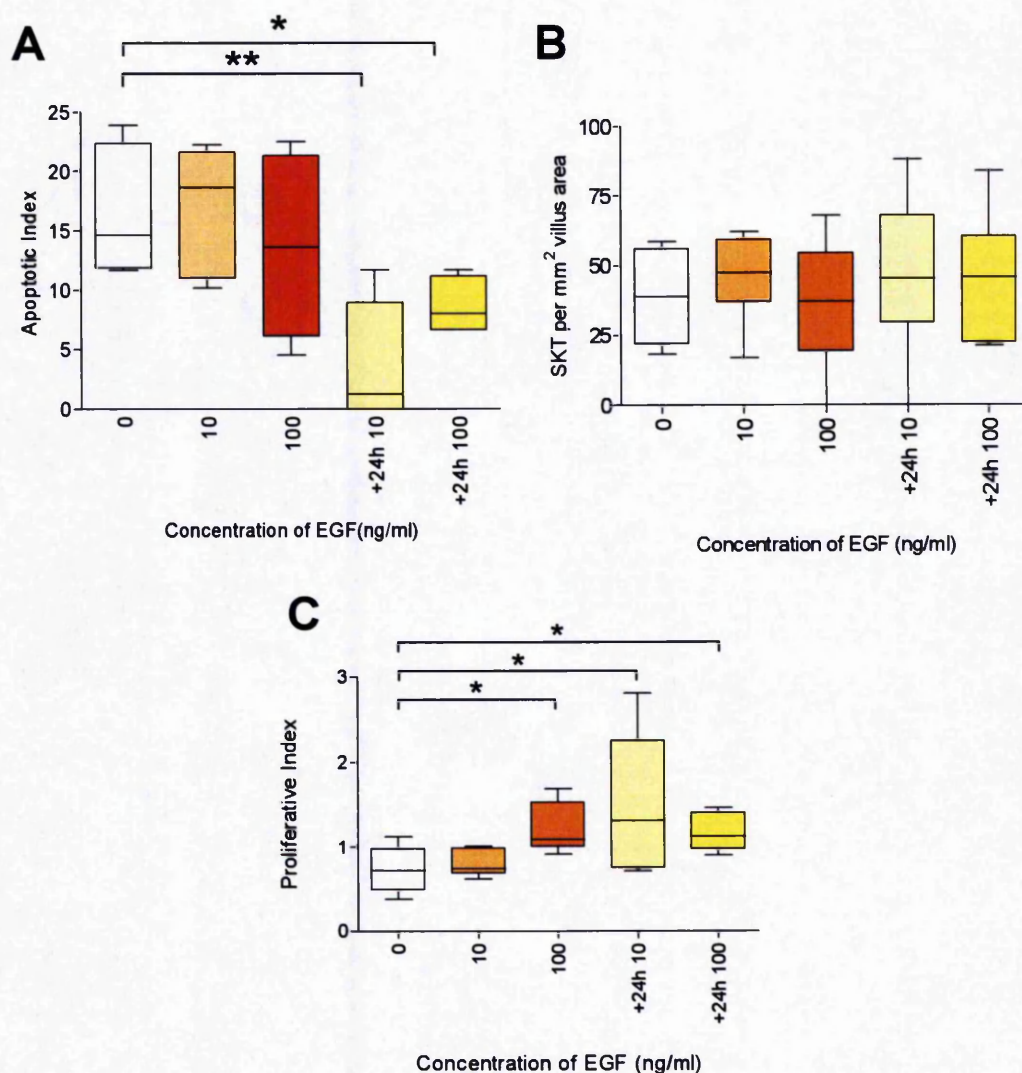
The apoptotic index was increased in tissue cultured for 96 hours in control conditions compared to fresh tissue, the median apoptotic index of cultured tissue 14% (IQR 11.9-22.3) vs 3.17% (IQR 2.3-4.1) for fresh tissue. Treatment with EGF at 10 or 100ng/ml for 48 hours did not alter the apoptotic index. However, treatment with either 10 or 100ng/ml for a total of 72 hours (24 hours pre-treatment + 48 hours standard treatment) significantly reduced the apoptotic index compared to control (Figure 8.2A). The density of syncytial knots was not altered by treatment with 10 or 100ng/ml EGF irrespective of the duration of exposure (Figure 8.2B). The alterations in apoptotic index were not associated with an increase in necrotic cell death as assessed by LDH activity in conditioned culture medium (Table 8.1).

Culture Conditions	Control	10ng/ml EGF 48h	100ng/ml EGF 48h	10ng/ml EGF 72h	100ng/ml EGF 72h
LDH activity % of Control	100	209.3 (95.3-314.7)	209.9 (51.7-338.1)	139.4 (77.4-161.3)	126 (42.2-247.4)

**Table 8.1** - LDH activity in conditioned culture medium expressed as a percentage of control. Figures shown are medians, with ranges shown in parentheses.

### 8.4.2 Effects of EGF alone on Proliferation and hCG secretion

Proliferative index in control explants was comparable to that in fresh tissue, the median proliferative index of cultured tissue was 0.72% (IQR 0.48-0.98) vs 0.94% fresh tissue (IQR 0.91-1.16). The proliferative index was significantly increased in explants treated with 100ng/ml EGF for 48 hours and 10 or 100ng/ml EGF for 72 hours (24 hours pre-treatment + 48 hours standard treatment) (Figure 8.2C). This increase in proliferation did not appear to be accompanied by an increase of hCG in the conditioned culture medium (Table 8.2).



**Figure 8.2** – Assessment of cell turnover in placental villous explants treated with EGF. (A) Exposure to EGF for 48 hours did not alter the apoptotic index, although exposure to either 10 or 100ng/ml of EGF for an additional 24 hours (72 hours in total) significantly reduced the number of TUNEL positive nuclei. (B) Exposure to EGF for either 48 or 72 hours did not significantly alter the number of syncytial knots (n=6). (C) Culture in the presence of 10 ng/ml EGF for 72 hours significantly increased the proliferative index compared to control conditions (\*  $p < 0.05$ , \*\*  $p < 0.01$ , Friedman test, n=6).



Culture Conditions	Control	10ng/ml 48h	100ng/ml 48h	10ng/ml 72h	100ng/ml 72h
hCG expression (iu/mg of tissue /hr)	0.27	0.25	0.35	0.32	0.31

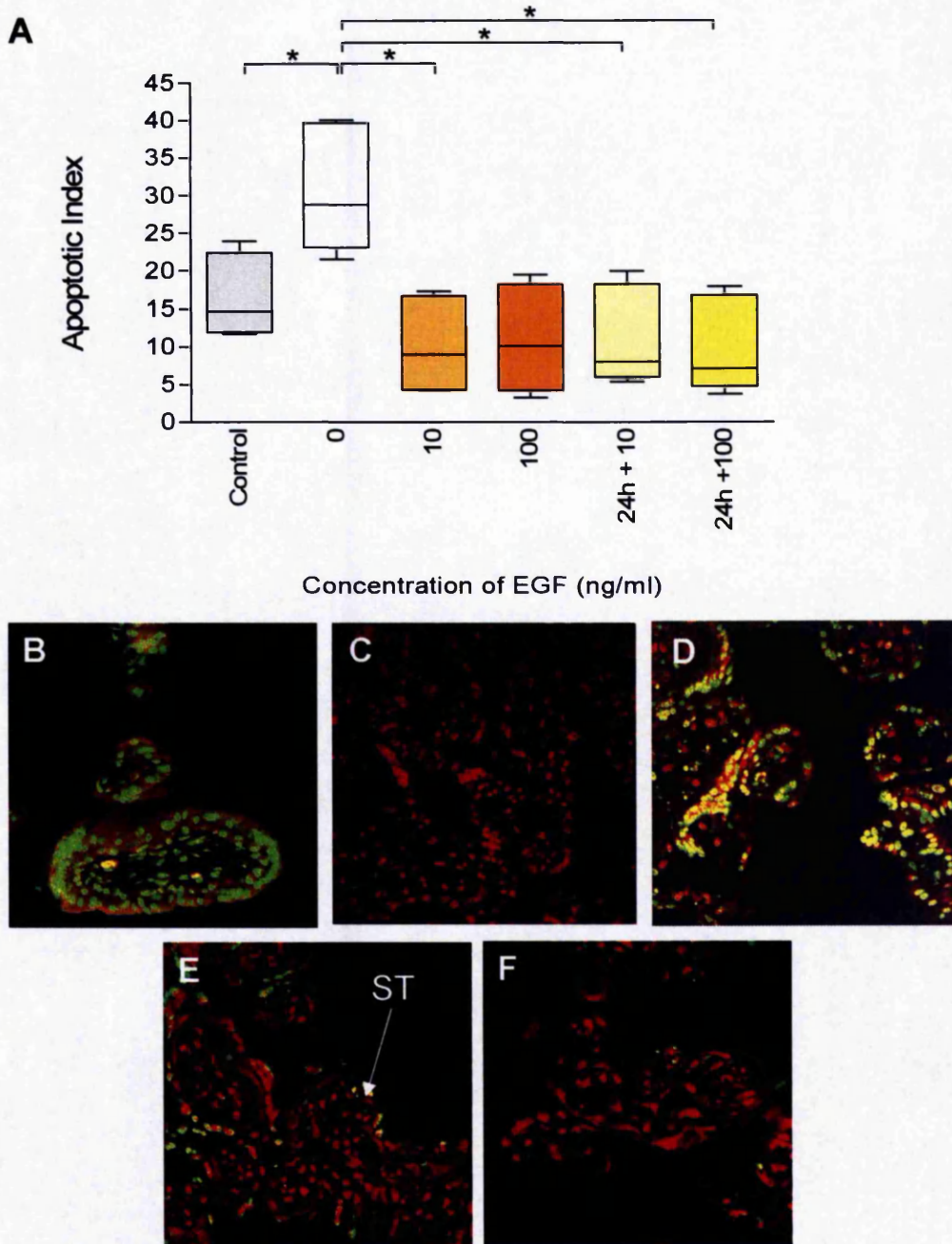
**Table 8.2** – hCG expression in conditioned culture medium with increasing duration and doses of EGF. Figures shown are mean (N=2, n=6).

#### **8.4.3 Effects of EGF on Apoptosis and Syncytial Knots in Explants exposed to H<sub>2</sub>O<sub>2</sub>**

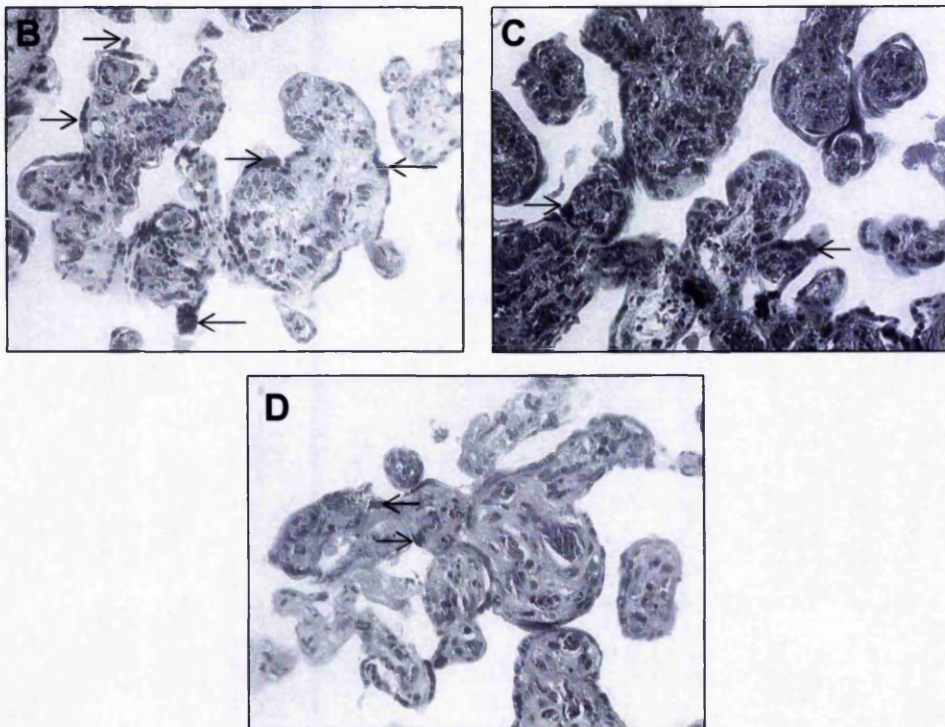
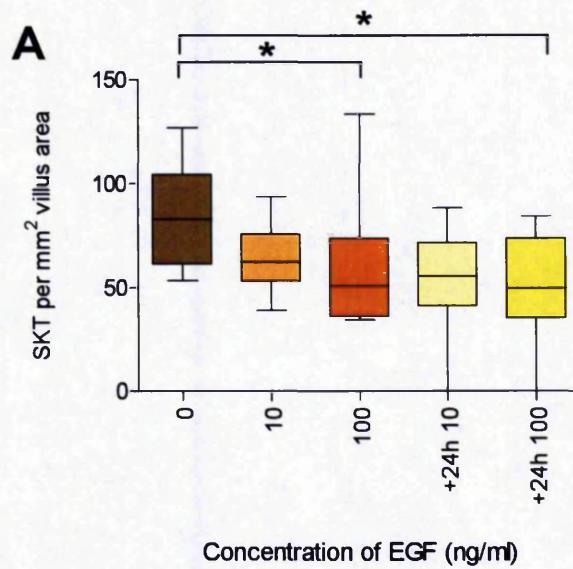
Following exposure to 1000µM H<sub>2</sub>O<sub>2</sub>, treatment with 10 or 100ng/ml EGF reduced the apoptotic index to control levels (Figure 8.3A). There was no additional effect of pre-treatment with EGF for 24 hours compared to that treated for 48 hours alone. Following exposure to 1000µM H<sub>2</sub>O<sub>2</sub>, TUNEL positive nuclei were predominantly localised to the syncytiotrophoblast (Figure 8.3D) with some staining evident in the underlying cells, perhaps representing apoptotic cytotrophoblast or stromal cells. Following treatment with EGF, few TUNEL positive nuclei were detected in the villi (Figure 8.3E and F). However, occasional TUNEL staining was seen in the syncytiotrophoblast in a similar pattern to that of control tissue (Figure 7.2D). Treatment with EGF also reduced the number of syncytial knots induced following treatment with 1000µM H<sub>2</sub>O<sub>2</sub> (Figure 8.4A). There was some evidence of a dose-dependent effect as only 100ng/ml attenuated the formation of syncytial knots and there was no evidence of a time-dependent effect as pre-treatment for an additional 24 hours did not produce an additional decrease in the number of syncytial knots.

#### **8.4.4 Effects of EGF on Proliferation and hCG release in Explants exposed to H<sub>2</sub>O<sub>2</sub>**

After exposure to 1000µM H<sub>2</sub>O<sub>2</sub> treatment with either 10 or 100ng/ml restored the proliferative index to levels comparable with control conditions (median = 0.74%) (Figure 8.5A). In common with the effects observed on apoptotic index or syncytial knot formation there was no evidence of dose- or time-dependent nature of this effect. Treatment with EGF in the presence of H<sub>2</sub>O<sub>2</sub> did not appear to alter the presence of hCG in culture medium (Table 8.3).

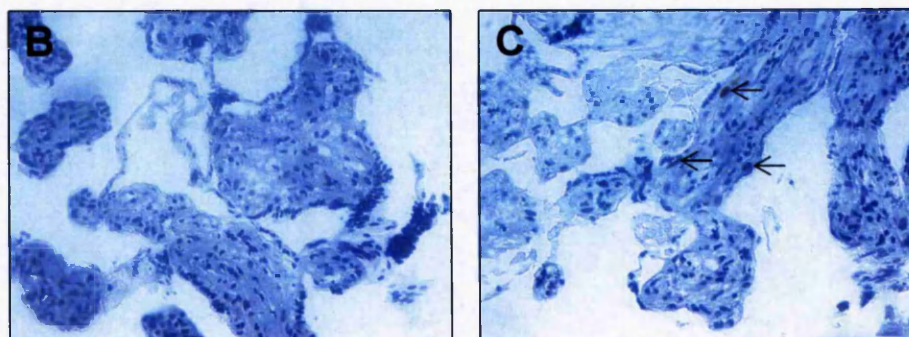
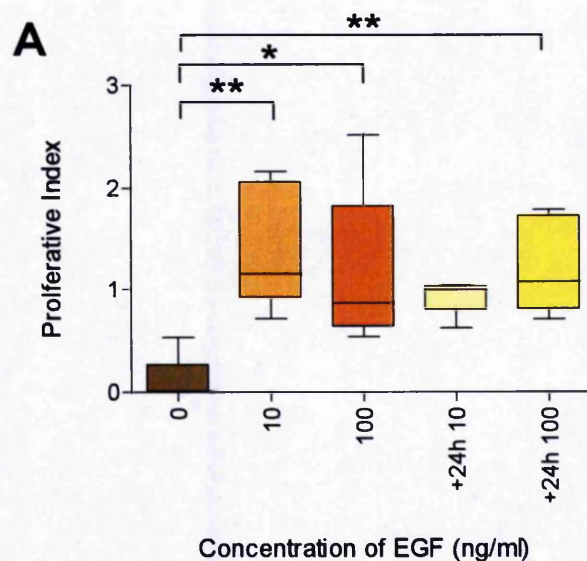


**Figure 8.3** – Assessment of apoptosis in placental villous explants exposed to  $H_2O_2$  and EGF for 48 and 72 hours. (A) In explants cultured in the presence of  $1000\mu M H_2O_2$  TUNEL index is reduced by treatment with 10 or 100 ng/ml EGF (\*  $p < 0.05$ , Friedman test,  $n=6$ ). Images obtained by confocal microscopy demonstrating TUNEL staining (green) counterstained with propidium iodide (red). (B) Positive control – incubated with DNase. (C) Negative control. (D) Explants cultured in  $1000\mu M H_2O_2$  have widespread TUNEL staining in the ST and cells underlying the syncytium. (E) Explants cultured in  $1000\mu M H_2O_2$  and 10ng/ml EGF have fewer TUNEL positive nuclei than in the absence of EGF especially in the stroma. (F) Explants cultured in  $1000\mu M H_2O_2$  and 100ng/ml EGF demonstrating few TUNEL positive nuclei in the ST or stromal compartments. Original Image Magnification x400.



**Figure 8.4** – Assessment of syncytial knots (SKTs) in placental villous explants exposed to  $H_2O_2$  and EGF for 48 and 72 hours. (A) In explants cultured in the presence of  $1000\mu M H_2O_2$  the number of SKTs is reduced by treatment with  $100\text{ ng/ml}$  EGF for either 24 or 72 hours (\*  $p < 0.05$ , Friedman test,  $n=6$ ). (B) Explants cultured in  $1000\mu M H_2O_2$  have increased numbers of SKTs marked by open arrows. (C) Explants cultured in  $1000\mu M H_2O_2$  and  $100\text{ng/ml}$  EGF for 24 hours have fewer SKTs. (D) Explants cultured in  $1000\mu M H_2O_2$  and  $100\text{ng/ml}$  EGF for 72 hours few SKTs and an relatively intact ST layer.





**Figure 8.5** –Assessment of proliferation in placental villous explants exposed to  $H_2O_2$  and EGF for 48 and 72 hours. (A) In explants cultured in the presence of  $1000\mu M H_2O_2$  the proliferative index is increased by treatment with 10 or 100 ng/ml EGF for 24 hours (\*  $p < 0.05$ , \*\* $p < 0.01$ , Friedman test,  $n=6$ ). (B) Explants cultured in  $1000\mu M H_2O_2$  have little or no Ki67 staining. (C) Explants cultured in  $1000\mu M H_2O_2$  and 100ng/ml EGF for 24 hours have increased Ki67 immunostaining, marked by open arrows.

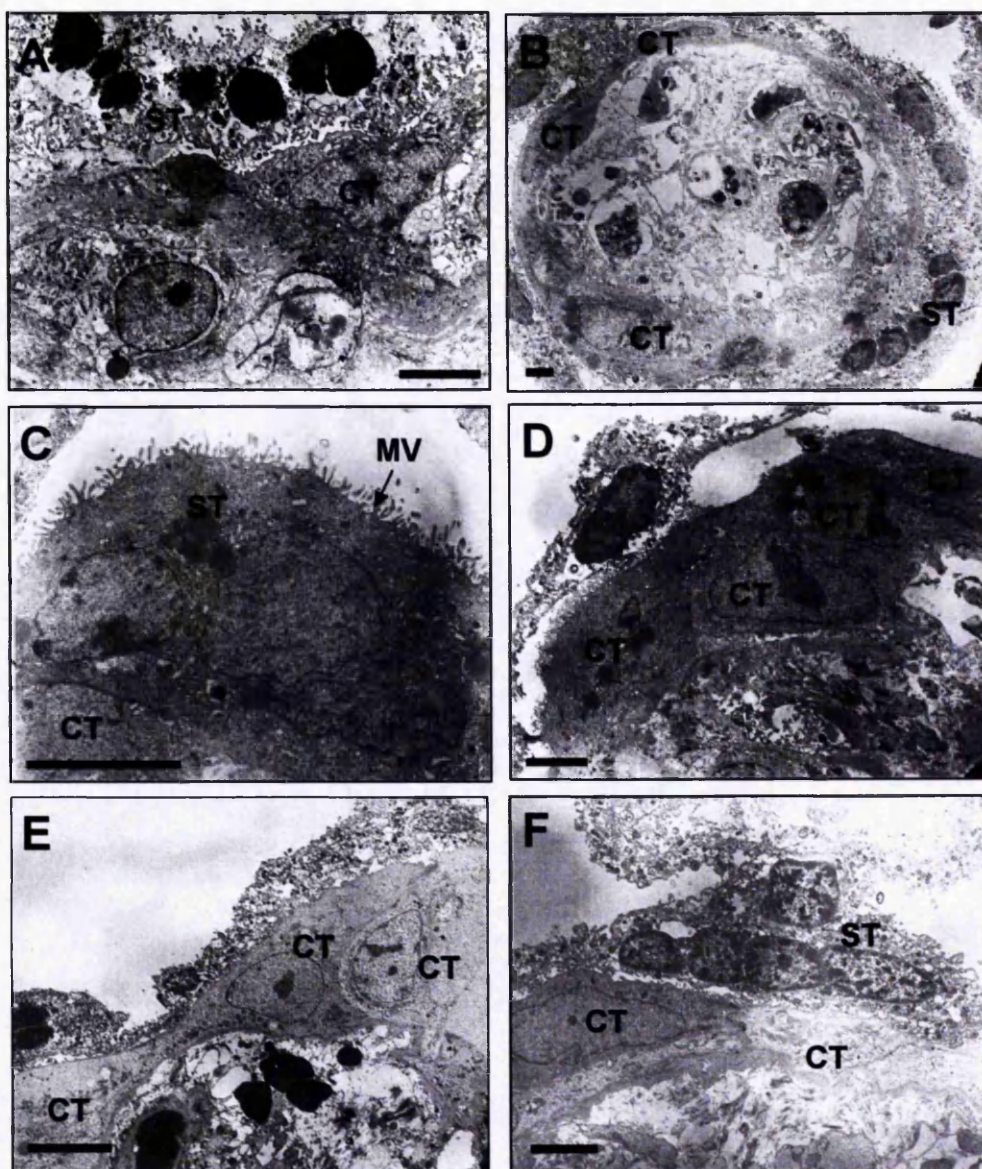
Culture Conditions	1000 $\mu$ M H <sub>2</sub> O <sub>2</sub>	1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> + 10ng/ml EGF 48h	1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> + 100ng/ml EGF 48h	1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> + 10ng/ml EGF 72h	1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> + 100ng/ml EGF 72h
hCG expression (iu/mg of tissue /hr)	0.24	0.28	0.35	0.21	0.20

**Table 8.3** – hCG expression in conditioned culture medium following treatment with H<sub>2</sub>O<sub>2</sub> and increasing doses of EGF. Figures shown are mean (N=2, n=6).

#### 8.4.5 Effects of EGF on Villous Morphology of Explants exposed to H<sub>2</sub>O<sub>2</sub>

As observed in previous experiments the syncytiotrophoblast showed signs of degeneration after 96 hours of culture compared to fresh tissue, including evidence of cytoplasmic vacuolation and dense clumps of peripheral chromatin in the syncytial nuclei (Figure 8.6A). Few changes were observed in the appearance of cytotrophoblasts (Figure 8.6A). Treatment with 100ng/ml EGF alone did not reduce the degeneration of the syncytiotrophoblast cytoplasm, but nuclei did not have the dense euchromatin observed in control conditions (Figure 8.6B). The underlying cytotrophoblasts appeared healthy, forming an almost complete layer around the villus rarely seen in normal term placental tissue (Figure 8.6B) suggesting regeneration of a new syncytiotrophoblast layer. Following treatment with 100ng/ml EGF for an additional 24 hours cytotrophoblasts fusion is visible and features of differentiation are present, including many apical microvilli (Figure 8.6C). When explants were treated with H<sub>2</sub>O<sub>2</sub> and EGF degradation was always seen. But when explants were treated with either 10 or 100ng/ml EGF underlying cytotrophoblasts had a healthy appearance (Figures 8.6D and E respectively). Explants exposed to 1000 $\mu$ M hydrogen peroxide following pre-treatment with EGF for 24 hours also exhibited syncytial degradation, although the ratio of euchromatin:heterochromatin in syncytial nuclei was lower than in explants treated with H<sub>2</sub>O<sub>2</sub> alone (Figure 8.6F). Underlying cytotrophoblasts appeared healthy, with no classical features of apoptosis (Figure 8.6F).





**Figure 8.6** – Assessment of morphology of placental villous explants exposed to EGF and  $H_2O_2$  by electron microscopy. (A) Following culture in control conditions for 96 hours the ST undergoes degeneration, but underlying CT are intact. (B) A cross sectional view of a villus following treatment with 100ng/ml EGF for 48 hours demonstrating reduced degeneration of ST compared to control, and complete CT layer surrounding the basement membrane. (C) Higher power view of villus treated with 100ng/ml EGF showing localised regeneration of the ST with microvilli. (D and E) Explants cultured in the presence of 100 $\mu$ M  $H_2O_2$  and 100ng/ml EGF showing apoptotic ST nuclei and with underlying multiple healthy CTs. (E and F) Explants treated with 1000 $\mu$ M  $H_2O_2$  and 100ng/ml EGF showing ST nuclei showing early signs of apoptosis within an intact cytoplasm. Underlying the ST are healthy CTs with no apoptotic appearances. All scale bars = 5 $\mu$ m.

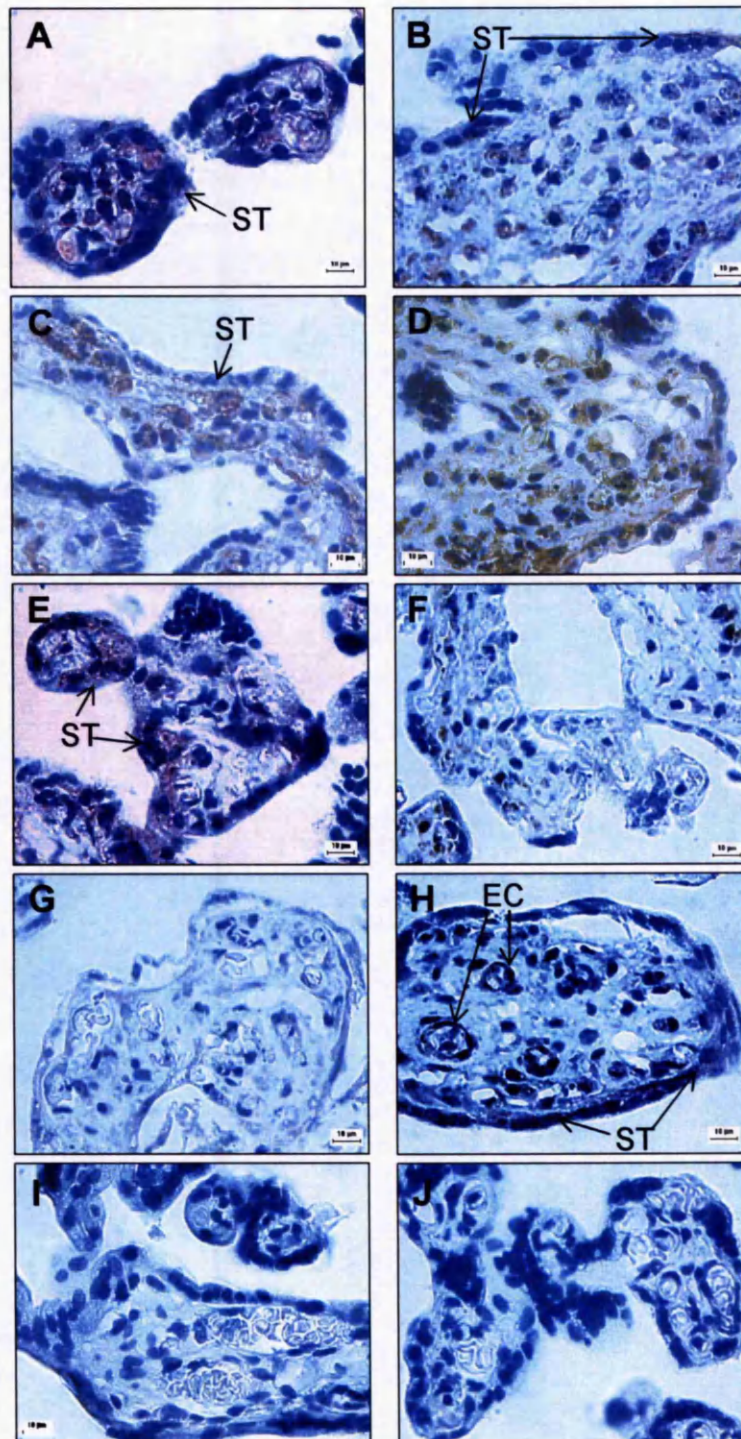
#### **8.4.5 Effects of EGF and H<sub>2</sub>O<sub>2</sub> on Expression and Localisation of p-AKT and p-PI3K**

In fresh villous tissue p-AKT was expressed in stromal cells, macrophages, endothelial cells and discrete areas of syncytiotrophoblast (Figure 8.7A). Following culture in control conditions, p-AKT was expressed in discrete areas of the syncytiotrophoblast cytoplasm and stromal cells; exposure to 1000µM H<sub>2</sub>O<sub>2</sub> was associated with increased p-AKT immunostaining in cytotrophoblast and stromal cells (Figure 8.7B). Treatment with EGF increased the degree of p-AKT staining within stroma and trophoblast compartments (Figure 8.7C and D). In fresh placental tissue, p-PI3K localised to discrete areas of the syncytiotrophoblast in fresh placental tissue (Figure 8.7E). Following culture in control conditions p-PI3K staining was evident in stromal cells and weakly present in trophoblast (Figure 8.7F). The expression of p-PI3K was decreased by culture in the presence of H<sub>2</sub>O<sub>2</sub> with very little staining observed in trophoblast (Figure 8.7G). However, treatment with EGF increased p-PI3K staining, especially in syncytiotrophoblast and endothelial cells (Figure 8.7H).

#### **8.5 Discussion**

The data presented suggest that EGF is able to alter trophoblast cell turnover of term placental villous explants, a finding in concordance with studies of isolated cytotrophoblasts and first trimester villous explants (Maruo et al. 1992; Garcia-Lloret et al. 1996; Levy et al. 2000; Smith et al. 2002). Following prolonged exposure, EGF has both anti-apoptotic and pro-proliferative effects. Although morphological evidence suggested increased renewal of syncytiotrophoblast, no increase in hCG secretion was demonstrated in these studies. Furthermore, these pro-survival effects of EGF were able to attenuate the negative effects of H<sub>2</sub>O<sub>2</sub> on cell turnover, reducing apoptosis and the formation of syncytial knots and restoring cytotrophoblast proliferation to levels of control explants. Neither treatment with H<sub>2</sub>O<sub>2</sub> nor EGF altered LDH activity within conditioned culture media, indicating no alteration in tissue viability. In agreement with other investigations, these results suggest that EGF can act as a placental survival factor (Jinno et al. 1988; Smith et al. 2002). The pro-survival effects of EGF were associated with morphological changes in the villous, especially the increased numbers of cytotrophoblasts with microvilli present on the apical surface, which is highly suggestive of





**Figure 8.7** – Representative micrographs of immunoperoxidase staining for p-AKT and p-PI3K in villous explants. (A) In fresh villous tissue p-AKT is present in ST and stromal cells (B) Under control conditions p-AKT is present in discrete regions of ST and many stromal cells. (C) Following culture with 1000µM H<sub>2</sub>O<sub>2</sub> p-AKT is strongly expressed in stromal cells but not ST. (D) Treatment with 1000µM H<sub>2</sub>O<sub>2</sub> and 100ng/ml EGF was associated with strong p-AKT immunostaining in stromal cells and trophoblast. (E) p-PI3-kinase in fresh villous tissue is present in ST. (F) Following 48 hours culture in control conditions p-PI3K was present in some stromal cells but weakly expressed in trophoblast. (G) Culture in the presence of 1000µM H<sub>2</sub>O<sub>2</sub> was associated with weak expression of p-PI3K. (H) Treatment with 1000µM H<sub>2</sub>O<sub>2</sub> and 100ng/ml EGF increased p-PI3K staining especially in ST and endothelial cells (EC). (I) Negative control for pAKT (J) Negative control for pPI3K.

accelerated syncytiotrophoblast regeneration.

This investigation did not show any dose- or time-dependent effects of EGF on apoptosis, this suggests that (i) 10ng/ml EGF is sufficient to antagonise apoptotic effects of 1000 $\mu$ M H<sub>2</sub>O<sub>2</sub> and (ii) that the maximal pro-survival effects of EGF occurs within 48 hours. Interestingly, while treatment with EGF reduced apoptosis in villous explants, there was no reduction in the number of syncytial knots. This may result from syncytiotrophoblast degeneration, a degree of which is inevitable in an in vitro culture system (Di Santo et al. 2003).

This study is the first reported investigation of the effects of EGF on cell turnover in term placental villous explants and has shown that EGF can also attenuate apoptosis resulting from reactive oxygen species in the form of H<sub>2</sub>O<sub>2</sub>, a potent inducer of apoptosis in term trophoblast explants (Hung et al. 2002). Similar anti-apoptotic effects of EGF to those described here have already been demonstrated in isolated cytotrophoblasts (Garcia-Lloret et al. 1996; Levy et al. 2000; Smith et al. 2002). Smith et al examined the extrinsic pathway of apoptosis in primary cytotrophoblast initiated by TNF $\alpha$  and IFN $\gamma$ , whilst Levy et al. demonstrated that the activation of the intrinsic apoptotic pathway by hypoxia could be antagonised by EGF (Levy et al. 2000). Combining the findings of this and previous investigations suggests that EGF can inhibit apoptosis initiated by both intrinsic and extrinsic apoptotic pathways (Levy et al. 2000; Smith et al. 2002). More recently, Nelson et al. have shown that this may occur via phosphorylation of Bad, decreasing mitochondrial membrane permeability, antagonising the effects of Bak and Bax (Nelson et al. 2007).

Whilst there is a growing body of evidence to support the anti-apoptotic role of EGF, its ability to promote proliferation and differentiation of cytotrophoblasts is far less clear. In the experiments described, EGF restored proliferation and promoted morphological features of differentiation. In support of the increase in proliferation, Maruo et al. (1992) found that EGF stimulated proliferation in early placental explants from 4-5 weeks gestation, although this was not seen in explants taken from 6-12 week placentas. The ability of EGF to increase differentiation has been noted in cultured primary term trophoblast (Yui et al. 1994a), although the ability of EGF to promote trophoblast proliferation is unclear. This may result from the use of primary term cytotrophoblast which showed no increase in the number of nuclei during culture, concluding that cytotrophoblasts underwent differentiation but did not proliferate (Kliman et al. 1986). However, subsequent investigation suggests that isolated term

trophoblast may undergo increased proliferation, as detected by incorporation of bromodeoxyuridine, following stimulation with EGF (Johnstone et al. 2005).

The most significant reduction in TUNEL positive nuclei following treatment with EGF alone was localised to the syncytiotrophoblast; there are several possible explanations for this phenomenon. Firstly, the increased proliferation and subsequent differentiation of cytotrophoblasts may decrease apoptosis as syncytiotrophoblast is more resistant to apoptotic stimuli *in vitro* (Garcia-Lloret et al. 1996; Crocker et al. 2001). This decreased apoptotic susceptibility may result from relatively high (compared to cytotrophoblast) levels of expression of anti-apoptotic proteins such as: Bcl-2, inhibitors of apoptosis proteins (IAPs) and Mdm2 (Sakuragi et al. 1994; Fulop et al. 1998; Toki et al. 1999; Ka and Hunt 2003). The second possibility is that EGF may directly up-regulate the expression of anti-apoptotic proteins. The only study investigating this possibility described no effect of EGF on the protein expression of Bcl-2 in isolated term trophoblasts (Ho et al. 1999). Currently, there are no reports of effects of EGF on isolated syncytiotrophoblast fragments; further study is therefore required to determine whether EGF up-regulates the expression of anti-apoptotic proteins in this cell layer. Lastly, EGF may also up-regulate anti-oxidant enzymes such as Cu/Zn SOD which would protect trophoblast from damage resulting from ROS. Indirect evidence for such an effect is suggested by alteration in Cu/Zn SOD mRNA between mononucleate and fused trophoblasts, when fusion has been stimulated by EGF (Morrish et al. 1996).

EGF acts via a complex array of pro-survival signals including: MAPK, JNK, PI3K, extracellular signal related kinase (ERK) and Akt, all of which may be activated by phosphorylation. PI3K and JNK are activated following EGF binding to its receptor suggesting that the survival mechanisms of EGF may act via pathways activated by other growth factors (Kawamata et al. 2003; Qiu et al. 2004). Immunostaining for p-PI3K, but not p-AKT is increased in placental villous explants following treatment with EGF in the presence of hydrogen peroxide. These data concur with evidence that EGF leads to activation of PI3K, thereby promoting trophoblast survival (Datta et al. 1997). Other activities of EGF, such as promotion of trophoblast differentiation, occurs via MAPK 11/14, which is not involved in the anti-apoptotic effects of EGF (Kawamata et al. 2003; Johnstone et al. 2005). It is likely that EGF acts through multiple anti-apoptotic pathways, as inhibition of the PI3K pathway with LY294002 does not reduce the pro-survival effect of EGF (Perkins et al. 2002).



As treatment with EGF antagonises apoptosis induced by both extrinsic and intrinsic stimuli, it is hypothesised that EGF affects common elements of both pathways, such as members of the Bcl-2 or IAP families. The anti-apoptotic effects of EGF do not appear to be mediated through altered expression of Bcl-2 in primary cytotrophoblast (Ho et al. 1999), although this may reflect the relatively low expression of Bcl-2 in cytotrophoblast compared to differentiated syncytiotrophoblast (Sakuragi et al. 1994). Recent data suggest that Bad, a pro-apoptotic mitochondrial pore protein is altered following culture of trophoblast in the presence of EGF, providing a potential mechanism of action (Nelson et al. 2007). Further research is required to ascertain the mode of action of EGF with regard to cell survival, including the receptor signalling pathway and whether the expression of downstream are altered, particularly those in the p53-pathway, as these are altered in pre-eclampsia and in placental explants exposed to low O<sub>2</sub> tension. A greater understanding of the anti-apoptotic mechanisms of EGF may be used to develop EGF as a therapeutic strategy to attenuate excessive trophoblast apoptosis in vivo.

The potential role of EGF as a survival factor for villous trophoblast is supported by observations that EGF levels are reduced in the presence of abnormal pregnancies. EGF levels are significantly reduced in pregnancies complicated by Chagas' disease, an infection with the parasite *Trypanosoma cruzi* (Lin et al. 2004); women with Chagas' disease have a higher incidence of miscarriage and IUGR, which is associated with severe placental pathology including placental infarction and necrosis. Furthermore, women with IUGR have less EGF, as measured in urine, than women with normal pregnancies, indicating that circulating EGF may be lower in IUGR (Lindqvist et al. 1999). These observations have been extended in animal models. In mice the predominant source of EGF is from the salivary glands. Following sialadenectomy, mice have lower levels of EGF in serum, and show evidence of IUGR (Kamei et al. 1999). It may be concluded that EGF has important endocrine actions on placental structure and function, which may be impaired in placental pathology such as pre-eclampsia and IUGR. Further research is required to develop these findings, with particular respect to defining the mechanism of action of EGF in the maintenance of normal placental structure and function, but also the understanding of pre-eclampsia and IUGR. This study also suggests that EGF or an analogous agent may be useful to antagonise the increased placental apoptosis observed in pre-eclampsia or IUGR. Further research is required to determine whether this

might prove a useful therapeutic strategy to either prevent or treat pre-eclampsia or IUGR.

## Chapter 9 - Discussion

### 9.1 Pre-eclampsia, the Placenta and Apoptosis

Despite significant research there has been little progress in the development of therapeutic or preventative strategies for pre-eclampsia. Although maternal blood pressure can be controlled by anti-hypertensive agents, delivery of the infant remains the only curative treatment. The lack of progress in clinical interventions may be partially attributed to a paucity of knowledge regarding the pathophysiology of this complex condition. Undoubtedly, abnormal placentation is central to the development of pre-eclampsia, commencing with abnormal trophoblast invasion of the decidua (Brosens et al. 2002) and a reduction in the conversion of utero-placental arteries in the first trimester continuing to produce evidence of placental damage at the macroscopic and microscopic level in the third trimester (Benirschke and Kaufmann 2005). This hypothesis is supported by evidence that it is delivery of the placenta that brings about resolution of pre-eclampsia. However, the link between abnormal trophoblast invasion, placental damage, decreased placental function and maternal endothelial dysfunction remains unclear. Due to the widespread nature of the endothelial dysfunction, it has been attributed to circulating factors within the maternal circulation; suggestions of such circulating factors include fragments of syncytiotrophoblast (Redman and Sargent 2005), free fetal DNA (Zhong et al. 2002) or proteins released from degenerating syncytiotrophoblast (Maynard et al. 2003). It is hypothesised that apoptosis may be involved in the generation of these putative circulating factors. Syncytiotrophoblast microparticles have features of apoptosis and can be isolated following culture of villous explants (Abumaree et al. 2006b). Apoptosis could also be associated with the release of free-fetal DNA from the placenta (Tjoa et al. 2006). In vitro, apoptosis is not an isolated event, it is associated with altered cytotrophoblast proliferation, differentiation and generation of syncytial knots. A similar inverse relationship is seen between apoptosis and proliferation in placental tissue in vivo (Yamada et al. 2001). If these changes in cell turnover are present in vivo this may disrupt the renewal of syncytiotrophoblast, impacting on placental function, particularly nutrient transport and hormone synthesis. Therefore, apoptosis may lead to production of circulating factors and impair placental function, ultimately impacting on the outcome of pregnancy.

Due to its potential role in the pathogenesis of pre-eclampsia or intra-uterine growth restriction, apoptosis of villous trophoblast has been the subject of a number of investigations and review articles since its identification in 1997. Although, the presence of an increased proportion of apoptotic nuclei in pre-eclampsia is a consistent finding, the origin of this increased apoptosis and the cellular signals involved are less clear. Nevertheless, investigation of apoptosis in villous trophoblast may increase the knowledge of the pathophysiology of pre-eclampsia by identifying the origin of cell damage. In addition, an understanding of the cell-signalling events involved in the promotion of apoptosis may identify target molecules which may be used to antagonise the apoptotic signal, thereby reducing apoptosis in pre-eclampsia. The potential benefits of investigating the regulation of apoptosis in trophoblast have resulted in a rapid expansion of this field, illustrated by the consistent growth in published research on human placental apoptosis rising from 23 papers in 1997 to 523 by the end of 2007. As a result of this growth, knowledge regarding the regulation of apoptosis in villous trophoblast has developed since work on this thesis commenced. Therefore, it is essential to contextualise the findings of the investigations presented in this thesis within the advances made in the wider field of trophoblast cell turnover.

The primary aim of this thesis was to characterise the expression of proteins capable of regulating apoptosis in villous tissue from normal third trimester pregnancies and those complicated by pre-eclampsia. Secondly, this thesis aimed to describe changes in cell turnover of villous trophoblast in response to exposure to adverse placental environments purported to be involved in the development of pre-eclampsia. Lastly, this thesis addressed whether apoptosis was an inevitable event following cell damage or whether survival can be increased by administration of an exogenous growth factor. Each of these aims addresses the central hypothesis that the increased apoptosis observed in pre-eclampsia occurs as a result of cell damage, initiating a series of signalling events which alter villous trophoblast turnover.

## **9.2 Limitations of the Experimental Data Presented in this Thesis**

### **9.2.1 Limitations when Studying Placental Tissue in Pregnancies Complicated by Pre-eclampsia**

As in all studies, the experiments presented in this thesis have limitations. These include limitations of both experimental concepts and methods. Investigation of the placenta in pregnancies complicated by pre-eclampsia is restricted as placental tissue cannot easily be obtained until delivery, a point at which the disease is clinically evident and in some cases advanced; the data presented in Chapter 3 was acquired from placentas following delivery. Therefore, the role of apoptosis and the p53 pathway in the development of pre-eclampsia cannot be precisely determined.

This problem has been partially addressed by studies of first trimester placental tissue with evidence of high-resistance maternal circulation which demonstrates altered trophoblast cell-turnover and reduced invasion. However, these specimens are taken from termination of pregnancy so their potential to develop pre-eclampsia cannot be fully assessed. To address this question, placental tissue would need to be obtained from a sufficient number of pregnancies expected to continue to the third trimester; differences in apoptosis and expression of the p53 pathway could then be investigated.

The study of samples collected from pregnancies complicated by pre-eclampsia is also problematic due to the heterogenous nature of the disorder, which varies in severity, gestation at onset, its association with IUGR, the end-organs affected and its progression. This study focused on pre-eclampsia occurring after 28 weeks gestation which was not associated with IUGR; a complete study of placental dysfunction in pre-eclampsia would be sufficiently powered to detect differences between normal pregnancy, pre-eclampsia occurring after 37 weeks, between 28-37 weeks gestation and before 28 weeks gestation, with and without evidence of IUGR. Such a study would be complex and would probably require collaboration between multiple centres to achieve adequate sample numbers and was outwith the scope of the experiments presented in this thesis. Nevertheless, to confirm the role of placental apoptosis and investigate the role of p53 in the pathogenesis of pre-eclampsia such a study should be regarded as essential.



### **9.2.2 Limitations of in vitro Culture Models**

The origins of the increased expression of p53 in villous trophoblast were explored in chapters 4-7. These experiments used two in vitro culture models of normal villous trophoblast cell turnover, BeWo cell culture and the culture of placental explants. Inevitably, in vitro culture models lack many features of the in vivo environment, including maternal and fetal blood flow. Pre-eclampsia results from a complex interaction between the placenta and the maternal immune system, which is absent from the explant culture system employed here. In explant culture, the syncytiotrophoblast degenerates and sloughs off, eventually being replaced by a regenerated layer originating from cytotrophoblasts; this marked degeneration of the syncytium is not thought to reflect the in vivo situation. This has led some authors to culture explants for shorter periods (~6h). However, a 96h duration of culture was employed in these experiments as this time period exhibits maximal secretion of hCG, indicating the greatest renewal of syncytiotrophoblast in culture. Ultimately, no culture model is without flaws, but findings must be carefully interpreted in the context of previous studies using a specific culture model.

The use of BeWo cell culture is not only complicated by the absence of a maternal environment, but utilises a transformed cell-line. BeWo cells have been used by several research groups for a number of years to investigate aspects of trophoblast cell turnover, most notably cell fusion. As BeWo cells were derived from choriocarcinoma cells, the initiation and regulation of apoptosis and proliferation in these cells may be altered compared to native trophoblast. Certainly, BeWo cells undergo apoptosis following exposure to a number of noxious environments which elicit similar effects in native trophoblast. However, there are many pathways capable of inducing apoptosis, more rigorous study is required to investigate whether these are similar in BeWo and native trophoblasts, and whether p53 and Mdm2 have the same roles in this cell line. Until this time, investigations utilising choriocarcinoma cell lines must compare their findings with normal trophoblast.

### **9.2.3 Limitations in Studying an Evolving Pathway**

The intrinsic apoptotic pathway in trophoblast is incompletely understood. Primary studies reporting the identification of apoptotic regulatory proteins rarely use placental tissue despite using many other human and animal tissue types. Due to its importance in cancer biology,

studies of the nature and role of proteins regulating apoptosis are frequently determined in transformed cell-lines derived from cancer. Therefore, the studies undertaken in this thesis make an assumption that these proteins have a similar function in trophoblast to that described in other cell types.

Studies of the intrinsic apoptotic pathway are further limited by the evolving understanding of this pathway. Since these studies began, novel proteins have been identified that are likely to interact with the p53-pathway to modulate apoptosis in trophoblast. In addition, knowledge of proteins regulated by p53 has also expanded; for example, Puma and Noxa are currently thought to have a greater role than Bax in the regulation of mitochondrial membrane permeability, and therefore apoptosis, following activation of p53. This evolution complicates studies of the regulation of apoptosis in trophoblast and necessitates study of the expression of further proteins in trophoblast of pregnancies complicated by pre-eclampsia.

In addition to identification of novel proteins and expansion of the roles of known proteins knowledge of the role of p53 in reproduction has increased. Initial studies predicted that although p53 was present in a number of cell types in the reproductive system, its role was uncertain. Recent studies have demonstrated that p53 is important for successful implantation - an unexpected role for "the guardian of the genome" (Stewart 2007). While knowledge of the role of p53 in normal reproduction is incomplete, interpretation of the data presented in this thesis will need to be re-evaluated in the light of new findings. As understanding of the role of p53 in healthy reproduction grows, the role of p53 in the development of pregnancy complications such as pre-eclampsia and IUGR will increase.

### **9.3 Regulation of Apoptosis in Normal Villous Trophoblast**

It is apparent from this research and findings of other studies that trophoblast express many regulators of apoptosis. Although these regulators were originally described in other cell types, for purpose of this investigation it has been assumed that these proteins have the same function in trophoblast. Certainly, trophoblast expresses regulators of apoptosis which can modulate the apoptotic signal at the level of the nucleus, mitochondrion and the cytoplasm. This study has focused on regulators of apoptosis involved in the cellular response to damage via the intrinsic apoptotic pathway, particularly those relating to p53. The expression of the majority of regulators of apoptosis in normal term villous trophoblast appears to be spatially

distinct, with separation between cytotrophoblast and syncytiotrophoblast compartments. The expression of p53 and p21 localised to a proportion of cytotrophoblast nuclei. In comparison, the non-proliferative syncytiotrophoblast rarely expressed either p53 or p21, suggesting that these proteins may play a role in regulating cytotrophoblast proliferation. However, both trophoblast compartments strongly expressed anti-apoptotic Mdm2. Expression of both pro- and anti-apoptotic members of the Bcl-2 family predominantly localised to the syncytiotrophoblast, and a similar distribution was also observed in regulators of caspase activity including: smac, XIAP and survivin. Investigations of effector caspase activity have also demonstrated differences in spatial localisation, with active caspase-3 localising to the syncytiotrophoblast, whereas the pro-form is more strongly present in cytotrophoblast (Huppertz et al. 1998). The concentration of regulators of apoptosis in the syncytiotrophoblast strongly suggests that apoptosis plays a role in syncytiotrophoblast degeneration, and that a constant balance between pro- and anti-apoptotic factors must be maintained to preserve integrity of this essential cellular layer. Therefore, dysregulation of apoptosis may result in excessive apoptosis and loss of this layer, such as that seen in pre-eclampsia.

#### **9.4 Regulation of Villous Trophoblast Apoptosis in Pre-eclampsia**

This thesis has shown that increased apoptosis in placentas of pregnancies complicated by pre-eclampsia is associated with altered expression of constituents of the intrinsic apoptotic pathway (Chapter 3). These include factors which are predominantly active at the nuclear level (p53, Mdm2, p21), at the mitochondrial membrane (Bax, Bak, Bcl-2) and in the cytoplasm (smac, XIAP). When combined with the findings of other studies (Ishihara et al. 2002; Soleymanlou et al. 2005b) these data provide evidence that rather than a change in a single pro- or anti-apoptotic factor, there is an imbalance in a number of pro- and anti-apoptotic factors favouring apoptosis. Importantly, these changes are predominantly localised to the syncytiotrophoblast, the site of increased apoptosis in pre-eclampsia. The most striking finding of these investigations was an imbalance between p53 and Mdm2, which due to its position high in the intrinsic apoptotic pathway can have profound effects on cell survival; p53 unopposed by Mdm2 results in apoptosis, which abolishes normal murine embryonic development (Jones et al. 1995; de Rozieres et al. 2000). By reducing Mdm2 using siRNA these studies have shown increased apoptosis of both trophoblast and stromal cells in

placental villous explants, this change was reduced to control levels by concomitant administration of p53 siRNA. Therefore, the negative feedback loop between pro-apoptotic p53 and anti-apoptotic Mdm2 seems to be an important regulator of apoptosis in villous trophoblast. This reduction in Mdm2 may allow p53 to promote the transcription of downstream genes such as p21 and Bax predisposing the syncytiotrophoblast to apoptosis. Apoptosis may also be induced in trophoblast by an imbalance between pro-apoptotic Mtd-1 and anti-apoptotic Mcl-1 (Soleymanlou et al. 2005b), both of which are also dysregulated in the syncytiotrophoblast in pre-eclampsia. As there are changes in expression of proteins throughout the intrinsic apoptotic pathway, it is probable that a number of imbalances may be present in villous trophoblast favouring apoptosis. As regulation by p53-Mdm2 occurs upstream of imbalances in Mcl-1/Mtd-1 and smac/XIAP, exaggerated expression of p53 may promote or repress the expression of some of these downstream proteins; for example, the expression of XIAP following exposure to hypoxia. Therefore, the regulation of p53 expression may be central to the initiation and regulation of the excessive apoptosis described in villous trophoblast in pre-eclampsia.

### **9.5 The Role of Oxidative Stress in Villous Trophoblast Apoptosis**

There have been few investigations to identify the origin of the imbalance between pro- and anti-apoptotic proteins in pre-eclampsia, where investigations have been carried out they tend to focus on a specific protein of interest rather than constituents of a pathway. In experiments presented in Chapter 6, apoptosis induced by culture of term placental villous explants in low atmospheric O<sub>2</sub> was associated with an increase in p53 expression. Similarly, apoptosis is increased in isolated term cytotrophoblasts by culture in low O<sub>2</sub> tension and by prolonged culture (Levy et al. 2002; Hu et al. 2006a). In both experiments the increased apoptosis was associated with elevation in p53, although neither of these environments report any change of the 90kD isoform of Mdm2. These experiments highlight two important observations, firstly the potential role of altered placental oxygenation in the induction of apoptosis, and secondly the association of increased p53 expression with increased apoptosis of trophoblast. An increase in p53 in response to reduced oxygenation is not an isolated finding; an imbalance in Mcl-1/Mtd-1 can also be induced in placental villous explants cultured in low atmospheric O<sub>2</sub> tension (Soleymanlou et al. 2005b). However, these experiments were carried out using first

trimester placental tissue which is exposed to low oxygenation *in vivo*, and may not reflect the response of term placental tissue to low oxygenation. Nevertheless, as the relationship between Mtd-1 and Mcl-1 is downstream of p53, the possibility that p53 may exert downstream effects on these proteins merits further investigation.

Rather than inducing placental hypoxia, some investigators have proposed that the reduced conversion of the utero-placental vasculature results in the generation of ROS leading to oxidative stress. Certainly, in the experiments presented in this thesis, H<sub>2</sub>O<sub>2</sub>, a potent oxidant, induces apoptosis in BeWo cells and placental villous explants. It is interesting that increased apoptosis induced by treatment with H<sub>2</sub>O<sub>2</sub> was associated with an increase in p53 and decrease in Mdm2 expression in BeWo cells (Chapter 5). Treatment with H<sub>2</sub>O<sub>2</sub> induced apoptosis in placental villous explants (Chapter 7) in a similar pattern to that described following hypoxia-reoxygenation injury (Hung et al. 2002). Unlike culture in reduced O<sub>2</sub> tension exposure to H<sub>2</sub>O<sub>2</sub> was not associated with any increase in necrosis, indicating that oxidative stress induces apoptosis, rather than aponecrosis.

It is evident from these and other investigations that exposure of placental tissue, isolated cytotrophoblasts or choriocarcinoma cell-lines to reduced oxygenation or oxidative stress exerts profound effects on cell turnover (Levy et al. 2000; Hung et al. 2002; Kilani et al. 2003; Crocker et al. 2004c; Al-Nasiry et al. 2006). Both noxious environments increase apoptosis, decrease proliferation and reduced differentiation. These similar *in vitro* effects may result from activation of a common pathway. This may result from a similar biochemical insult resulting from two distinct stimuli. For example, if cellular oxygenation were reduced to such an extent that the electron transport chain is interrupted, then superoxide anions (O<sub>2</sub><sup>-</sup>) will be released into the mitochondria, initiating the release of mitochondrial contents into the cytosol (Guzy et al. 2005). A similar release of superoxide may also occur after administration of H<sub>2</sub>O<sub>2</sub>. Alternatively, the intrinsic pathway of apoptosis may represent a common mechanism, with both reduced oxygenation and ROS increasing the expression of p53, ultimately leading to altered cell turnover. If the intrinsic pathway of apoptosis acts as a final common pathway for the induction of apoptosis in placental tissue in pre-eclampsia, the precise origin of placental damage such as hypoxia, hypoxia-reperfusion, nitrate stress etc. may not be important, providing that the stimulus could induce pro-apoptotic proteins such as p53, Bax, Mtd-1 or smac.



If the intrinsic apoptotic pathway represents a common path for induction of trophoblast apoptosis in pre-eclampsia, the altered expression of the constituents of the intrinsic apoptotic pathway is likely to regulate these critical events. The balance between proteins such as p53/Mdm2, Bax/Bcl-2, Mcl-1/Mtd, and smac/XIAP act as a series of rheostats, adjusting the response to cell injury. It is hypothesised that modulation of the balance between these proteins will alter downstream effects on cell turnover, potentially antagonising apoptosis.

## **9.6 Antagonising Apoptosis in Villous Trophoblast**

As previously stated, apoptosis is a carefully controlled event in villous trophoblast. The increased apoptosis in villous trophoblast in pre-eclampsia is associated with activation of the intrinsic apoptotic pathway, most likely following exposure to a damaging environment. However, apoptosis is not an inevitable result of exposure to noxious stimuli; apoptosis induced in placental tissue by culture in the presence of H<sub>2</sub>O<sub>2</sub> was reduced to levels of control by treatment with EGF. This is supported by other studies of isolated trophoblast demonstrating that a variety of growth factors can prevent the induction of apoptosis in trophoblast from either intrinsic or extrinsic stimuli (Garcia-Lloret et al. 1996; Levy et al. 2000; Smith et al. 2002). These growth factors may act by several different mechanisms and may promote the expression of anti-apoptotic proteins or decrease the potency of pro-apoptotic proteins including p53. As growth factors are reduced in some placental pathologies, it is hypothesised that the balance between pro-apoptotic and pro-survival factors is important in determining the extent of placental cell death in vivo. Thus, growth factors may provide a mechanism by which villous trophoblast apoptosis could be prevented in vivo.

## **9.7 Modulation of Villous Trophoblast Apoptosis In Vivo – A Potential Therapeutic Strategy?**

This thesis is based on the basic observation that the presence of a placenta is central to the development of pre-eclampsia. In pre-eclampsia, several changes have been observed in the villous trophoblast, some of which may play a direct role in the pathogenesis of this complex disorder. At the most simplistic level, pre-eclampsia originates from the placenta. Therefore prevention of placental pathology may arrest the development of pre-eclampsia, which would

be more effective than attempting to control the hypertension and endothelial dysfunction when the clinical disorder becomes manifest.

Until the role of apoptosis in the pathophysiology of pre-eclampsia has been elucidated the potential therapeutic implications for factors which can reduce villous trophoblast apoptosis are restricted to speculation. Assuming that apoptosis is an integral component of the pathophysiology of pre-eclampsia and that normalisation of trophoblast cell turnover could improve placental function in pre-eclampsia, two potential strategies exist to attenuate apoptosis 1) administer growth factors 2) directly antagonise the effects of pro-apoptotic proteins.

As the administration of growth factors decreases villous trophoblast apoptosis *in vitro* and the availability of growth factors in pre-eclampsia may be reduced, treatment with exogenous growth factors is an attractive therapeutic strategy. However, the mode of action of growth factors is not fully understood nor which would be the most efficacious agent to use, although EGF appears to have the greatest anti-apoptotic effect *in vitro*. The use of growth factors may also have potential systemic effects; growth factors may promote cell proliferation, potentially leading to the development of neoplasia. Such unwanted effects may be overcome by delivery of growth factors directly to the placental villi; this could be achieved by administration of growth factor in microbubbles which are burst when in contact with ultrasound (Dijkmans et al. 2004). While the ultrasound and materials technology exist to undertake such a therapeutic intervention, the safety of this approach in pregnancy has not been determined. Rather than using growth factors, other agents with anti-apoptotic effects could be used to attenuate apoptosis *in vivo*. One such agent is low molecular weight heparin (LMWH), which decreases apoptosis in first trimester villous cytotrophoblasts (Bose et al. 2005). Preliminary reports suggest that treatment with LMWH decreases the incidence of pre-eclampsia (Sergio et al. 2006). In addition to antagonising apoptosis, there could be several reasons for this effect, including correction of thrombophilia or prevention of placental thrombosis. Nevertheless, it is exciting that a pharmacological agent which is used and appears to be safe in pregnancy could decrease apoptosis of villous trophoblast.

Recent developments, particularly in the field of oncology, have suggested that direct modulation of the intrinsic apoptotic pathway could be used as a therapeutic strategy. Anti-apoptotic proteins such as Mdm2, Bcl-2 or XIAP are frequently overexpressed in neoplastic

tissue. The development of siRNA has led some authors to speculate that this method may be used to inhibit the expression of anti-apoptotic proteins, to sensitise cancer cells to chemotherapy. In addition, pharmacological agents have been developed to specifically antagonise the effects of Mdm2, the Nutlin family. Nutlin-3a, the most active agent, antagonises binding between Mdm2 and p53, stabilising p53 and inducing apoptosis (Vassilev et al. 2004). Conversely, agents have been developed to antagonise the downstream effects of p53, preventing transcription of proteins which promote apoptosis or cell cycle arrest; these are pifithrin- $\alpha$  and pifithrin- $\mu$  (Komarov et al. 1999; Strom et al. 2006). As the exaggerated apoptosis of villous trophoblast in pre-eclampsia is associated with an increase in p53 expression, treatment with pifithrin may prevent downstream activation of the apoptotic cascade in response to cell injury (Gudkov and Komarova 2005). Alternatively, p53 expression could be reduced by treatment with siRNA. One advantage of this strategy is that disruption of the p53-pathway is unlikely to have any detrimental effects on fetal development as p53  $-/-$  mice and humans with Li Fraumeni syndrome (absent p53) develop normally in utero, and do not have an excess of miscarriage (Hartley et al. 1994). However, such strategies are untested in human pregnancy and would need extensive investigation in tissue culture and animal models before any justification could be made for their use in vivo.

### **9.8 Future Work – The Regulation of Apoptosis in Villous Trophoblast**

In order to develop interventions to arrest the development of pre-eclampsia a greater understanding of the pathophysiology is required, particularly with respect to apoptosis in villous trophoblast. The data presented and discussed in this thesis raise a number of avenues meriting further investigation: 1) The role of apoptosis in placental dysfunction, 2) The origins of apoptosis in villous trophoblast, 3) The expression of other constituents in the p53 pathway, 4) The mechanism and effects of attenuating apoptosis.

Firstly, the role of apoptosis in the pathogenesis of pre-eclampsia and associated intra-uterine growth restriction is not clear. It is essential to determine whether apoptosis of villous trophoblast is an epiphenomenon or a central event in the development of pre-eclampsia. In vitro, apoptosis is accompanied by a reduction in proliferation of underlying cytotrophoblasts which may prevent renewal of the syncytiotrophoblast, reducing nutrient transport and hormone synthesis. While increased apoptosis is sometimes associated with a decrease in

release of hCG in vitro, there are no data to associate increased apoptosis with altered placental transport. Further research is also required to link apoptosis with the generation of circulating factors, be they syncytiotrophoblast microparticles, cell-free fetal DNA or angiogenic factors. These questions could be answered using the tissue and cell-culture models used in this thesis, as they have also been used to investigate placental transport and hormone synthesis.

Apoptosis can be induced in villous trophoblast by exposure to various forms of oxidative stress. Investigation of the origin of increased apoptosis will provide insight into the placental insult which may be amenable to therapeutic intervention, such as directed anti-oxidant therapy. Animal models, which develop a clinical syndrome similar to pre-eclampsia could be utilised to determine the placental phenotype and describe changes to the environment e.g. measure placental oxygenation.

The balance between p53 and Mdm2 appears to be important in the regulation of apoptosis in villous trophoblast. This allows p53 to act unopposed, ultimately leading to activation of p53. There were many factors not investigated in these studies such as Puma and Noxa, which may mediate downstream effects of p53. Investigation of these factors would provide a more complete picture of the activation of the intrinsic pathway in villous trophoblast. Furthermore, detailed study of the C- and N-terminal domains of p53 itself would provide an insight into the origins of p53 stabilisation and activation following placental damage.

Lastly, if experimental data support a direct role of apoptosis in the pathogenesis of pre-eclampsia then mechanisms to attenuate apoptosis require detailed examination. Given the strong anti-apoptotic effects of growth factors, particularly EGF, it would seem logical to commence with these agents. In addition, to restoring normal trophoblast cell turnover, the effects of growth factors on placental transport and secretion of microparticles should be investigated.

## **9.9 Conclusions**

The placenta is a remarkable organ; it is able to sustain life, prevent immune recognition of a semi-allograft and develops throughout pregnancy to fulfil its functions. In common, with all organs, the placenta can fail when placed under adverse conditions. Uniquely, in the case of pre-eclampsia, this affects two lives rather than one. These adverse conditions lead to a loss

of the syncytiotrophoblast as a result of excessive apoptosis, which is mediated by a series of tightly regulated proteins. In pre-eclampsia the balance of these proteins is altered favouring apoptosis, in particular an imbalance between pro-apoptotic p53 and anti-apoptotic Mdm2. p53 appears to be an important regulator of apoptosis in villous trophoblast, being increased in environments which induce apoptosis in villous trophoblast, such as reduced oxygenation. Further work is required to investigate the role of p53 and Mdm2 in the complex life-cycle of villous trophoblast. It is hoped that modulation of trophoblast apoptosis could be used to promote cell survival thereby improving placental function in vivo. Ultimately, this might provide a therapeutic strategy for a disorder, for which there is currently no treatment.



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## Appendices

### Appendix A

Protein	Tissue / Cell Type	Author	Method of Study	Main findings
p53	Jeg-3 choriocarcinoma cell line, first trimester and normal term villous tissue	Sakuragi et al. 1994	WB Northern blotting	Protein abundant in Jeg-3 cells. Differentiation of JEG-3 cells associated with decrease in p53 mRNA. p53 has stronger expression in first trimester compared to term tissue lysate.
	Normal first trimester and term villous tissue	Roncalli et al. 1994	IHC	In first trimester villous tissue, present in CT, very weak staining in ST In third trimester villous tissue almost no staining in ST.
	Normal first trimester and term villous tissue, Jeg-3, JAr and BeWo CCA cell line	Haidacher et al. 1995.	IHC WB	All CCAI lines stained with 3 different monoclonal antibodies to p53. In first trimester villous tissue, present in 6-8% of CTs. In third trimester tissue, present in 1-2% of CTs and 1% of ST nuclei.
	First trimester, Second trimester and Term villous tissue	Marzusch et al. 1995	IHC	<5% of nuclei stained for p53, Some villous CT positive, rare positive nuclei in ST in all gestations studied.
	Normal first trimester villous tissue, CHM, PHM and CCA	Shi et al. 1996	DNA Sequencing	No mutations in p53 gene
	Normal first trimester villous tissue	Quenby et al. 1998	IHC	Present in occasional CTs. Absent in ST
	Normal first trimester villous tissue and CHM	Qiao et al. 1998	IHC	In normal first trimester tissue, seen in CT, some intermediate tissue and rarely in ST. Expression increased in CTs in CHM.
	Normal first trimester villous tissue, CHM, PHM, CCA	Fulop et al. 1998	IHC DNA Sequencing	No mutations in p53 in CHM, PHM or CCA. p53 present in CT nuclei. p53 increased in PHM, CHM and CCA compared to normal first trimester villous trophoblast.
	First trimester tissue, CHM, PHM, CCA. Normal term tissue.	Cheung et al. 1999	IHC DNA Sequencing	No mutations identified in p53 sequence in HM or CCA. In first trimester, present in CT, weakly present in ST nuclei. In term tissue, present in CT, but reduced compared to first trimester tissue.

<b>p53</b>	First trimester villous tissue, CHM, PHM, miscarriage	Kale et al. 2001	IHC	Present in CT. Increased in CHM > PHM > Non-molar pregnancies.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	IHC WB	Present in CT nuclei. p53 expression increased in IUGR pregnancies
	Term villous tissue from normal and IUGR pregnancies	Endo et al. 2005	IHC	Present in some CT, rarely in ST nuclei, no increase in IUGR pregnancies
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast
	Term villous tissue from normal, PE, HELLP and IUGR pregnancies	Jeschke et al. 2006	IHC	Expressed in CTs in normal tissue. Increased in HELLP syndrome, unchanged in PE, and decreased in IUGR.
	BeWo choriocarcinoma cell line	Bae et al. 2007	WB	Present in BeWo cell lysate

**Table A1** – Studies reporting expression of p53 in trophoblast-derived choriocarcinoma cell lines and villous trophoblast at different stages of pregnancy and in partial hydatidiform mole (PHM), complete hydatidiform mole (CHM), choriocarcinoma (CCA), intra-uterine growth restriction (IUGR), HELLP syndrome and pre-eclampsia (PE). IHC = IHC, WB = Western Blotting.

<b>Mdm2</b>	Normal first trimester villous tissue, CHM, PHM, CCA	Fulop et al. 1998	IHC	Present in CT and ST of normal villus trophoblast. Immunostaining stronger in CHM and PHM than in normal villous tissue.
	First trimester tissue, CHM, PHM, CCA. Normal term tissue.	Cheung et al. 1999	IHC	In first trimester tissue, present in CT, and ST. Increased expression in PHM, CHM and CCA. Decreased in term compared to first trimester tissue.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated tissue in 2 isoforms

**Table A2** – Studies reporting expression of Mdm2 in villous trophoblast at different stages of pregnancy and in PHM, CHM and CCA.

<b>p21</b>	Normal first trimester villous tissue	Quenby et al. 1998	IHC	Strongly expressed by villous CT, Moderately expressed by intermediate tissue (IT). Patchy staining in ST.
	Normal first trimester villous tissue, CHM, PHM, CCA.	Fulop et al. 1998	IHC	Expressed in villous CT and ST nuclei. Immunostaining stronger in CHM and PHM than in normal villous tissue.
	Normal first trimester villous tissue	Toki et al. 1999	IHC WB	Positive staining mainly in ST nuclei, focal areas of CT positive for p21. Weakly positive on Western blot.
	BeWo choriocarcinoma cell line	Bae et al. 2007	WB	Present in BeWo cell lysate
	Normal first trimester and term villous tissue	De Falco et al. 2007	IHC	In first trimester, strongly present in ST nuclei and some CT nuclei. In third trimester, localized to some ST nuclei and cytoplasm, weak CT staining.

**Table A3** – Studies reporting expression of p21 in trophoblast-derived choriocarcinoma cell lines, villous trophoblast at different stages of pregnancy and in pregnancies complicated by PHM, CHM, and CCA.

<b>pRb</b>	Normal first trimester and term villous tissue	Roncalli et al. 1994	IHC	In first trimester villous tissue, strongly present in CT, no staining in ST. In third trimester villous tissue, present in CT, no staining in ST.
	Normal first trimester villous tissue	Quenby et al. 1998	IHC	Moderately expressed by CT, IT and ST
	Normal first trimester villous tissue, CHM, PHM, CCA	Fulop et al. 1998	IHC	Present in CT, small amount of staining in ST. Immunostaining stronger in CCA and CHM than in normal villous tissue and PHM.

**Table A4** – Studies reporting expression of pRb in villous trophoblast at different stages of pregnancy and in pregnancies complicated by PHM, CHM, and CCA.



<b>Bcl-2</b>	Jeg3 choriocarcinoma cell line, first trimester and normal villous tissue	Sakuragi et al. 1994	WB Northern Blotting	Protein weakly expressed in Jeg-3 cells. Present in ST of villous tissue, weakly expressed in CTs.
	Normal term villous tissue	Huppertz et al. 1998	IHC	Present in ST, absent in the region of syncytial sprouts.
	Normal first trimester villous tissue	Quenby et al. 1998	IHC	Present in ST and in the region of syncytial knots.
	Normal first trimester villous tissue and CHM	Qiao et al. 1998	IHC	In normal first trimester tissue, present in ST, no staining in CT. Unchanged in CHM.
	Normal first trimester villous tissue	Toki et al. 1999	IHC WB	Positive staining throughout ST. Present on Western blot.
	Normal term villous tissue	Ratts et al. 2000	IHC	Present throughout ST cytoplasm, weakly expressed in CT.
	Term villous tissue from normal and PE pregnancies	Allaire et al. 2000	IHC	Localisation not stated, no difference in H-score. between normal and PE
	Normal term villous tissue	Axt-Fliedner 2001	IHC	Present throughout ST cytoplasm, reduced in areas of syncytial sprouts. Absent from CTs.
	Normal term villous tissue	Yamada et al. 2001	IHC	Present throughout ST cytoplasm.
	Normal first trimester and term villous tissue	De Falco et al. 2001	IHC	Present throughout ST cytoplasm.
	Normal first trimester and term villous tissue	Daniel et al. 2002	IHC	In first trimester tissue, and third trimester tissue present in ST. Absent in CT and mesenchymal cells.
	Villous tissue from normal term placenta and PE/IUGR	Isihara et al. 2002	IHC	Present throughout ST. Reduced in severe PE/IUGR.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	WB	Present in villous tissue, not increased in IUGR.
	Normal term villous tissue, villous tissue from diabetic pregnancies	Sgarbosa et al. 2006	IHC	Present throughout ST cytoplasm and in the region of some syncytial knots.
	Isolated first trimester trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.
	Villous tissue from growth-discordant twins	Kim S-K et al. 2006	WB	Present in villous tissue.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.
	BeWo choriocarcinoma cell line	Bae et al. 2007	WB	Present in BeWo cell lysate.

**Table A5** – Studies reporting expression of Bcl-2 in cell lines, first trimester trophoblast, normal term villous trophoblast and in pregnancies complicated by diabetes, IUGR, HELLP and PE.

<b>Mcl-1</b>	Normal term villous tissue	Huppertz et al. 1998	IHC	Present in ST, absent in the region of syncytial sprouts.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.

**Table A6** – Studies reporting expression of Mcl-1 in normal term villous trophoblast.

<b>Bcl-XL</b>	Normal first trimester villous tissue	Charles et al. 2005	real time RT-PCR	mRNA present in villous tissue.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.

**Table A7** – Studies reporting expression of Nix in normal term villous trophoblast.

<b>Bak</b>	Normal term villous tissue	Ratts et al. 2000	IHC	Present in isolated areas of ST cytoplasm, associated with fibrin deposits
	Normal term villous tissue	Yamada et al. 2001	IHC	Present in isolated areas of ST cytoplasm in the region of syncytial knots.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	WB	Present in villous tissue, not increased in IUGR.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast

**Table A8** – Studies reporting expression of Bak in normal term villous trophoblast and in pregnancies complicated by IUGR.

<b>Bax</b>	Normal first trimester villous tissue and CHM	Qiao et al. 1998	IHC	In normal first trimester tissue, seen in CT, no staining in ST.
	Normal term villous tissue	Ratts et al. 2000	IHC	Present in isolated areas of ST cytoplasm, associated with fibrin deposits
	Term villous tissue from normal and PE pregnancies	Allaire et al. 2000	IHC	Localisation not stated, no difference in H-score. between normal and PE
	Normal first trimester and term villous tissue	De Falco et al. 2001	IHC	Present in discrete areas of ST cytoplasm.
	Normal term villous tissue	Yamada et al. 2001	IHC	Present in isolated areas of ST cytoplasm in the region of syncytial knots.
	Term villous tissue from normal and IUGR pregnancies	Levy et al. 2002	WB	Present in villous tissue, not increased in IUGR.
	Term villous tissue from normal and IUGR pregnancies	Endo et al. 2005	IHC	Expressed in some areas of trophoblast and trophoblast surrounding
	Normal term villous tissue	Charles et al. 2005	real time RT-PCR	mRNA present in villous tissue.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.
	Villous tissue from growth-discordant twins	Kim S-K et al. 2006	WB	Present in villous tissue.
	Isolated first trimester trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.
	BeWo choriocarcinoma cell line	Bae et al. 2007	WB	Present in BeWo cell lysate.

**Table A9** – Studies reporting expression of Bax in normal first trimester and term villous trophoblast and in pregnancies complicated by IUGR and PE.

<b>Mtd</b>	Villous tissue from first trimester, normal term pregnancies and PE	Soleymanlou et al. 2003	IHC WB mRNA	Protein expressed in ST cytoplasm. Increased in PE
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**Table A10** – Studies reporting expression of Mtd / Bok in normal first trimester and term villous trophoblast and in pregnancies PE.

<b>BNip3</b>	Villous tissue from normal tissue, HELLP, PE, IUGR	Stepan et al. 2005	IHC	Strongly present in CT, weakly expressed in ST. Reduced in CT and ST in HELLP, PE and IUGR.
<b>Nix</b>				Strongly present in CT, not detectable in ST. Reduced in CT in HELLP, PE and IUGR.

**Table A11** – Study reporting expression of BNip3 and Nix in normal term villous trophoblast and in pregnancies complicated by IUGR, HELLP and PE.

<b>XIAP</b>	Normal first trimester and term villous tissue	Gruslin et al. 2001.	IHC WB	Present throughout gestation. Stronger expression in first trimester compared to term. Present in ST.
	Normal first trimester and term villous tissue	Ka and Hunt. 2003.	IHC WB RT-PCR	Present throughout gestation, expression stronger in third trimester compared to first trimester. In first trimester, XIAP localised to CT cytoplasm. In third trimester XIAP localised to CT and ST cytoplasm.
	Normal first trimester and term villous tissue	Straszewski-Chavez et al. 2007	IHC WB	Expressed in first trimester and in third trimester tissue in the ST cytoplasm. In 3rd trimester, majority is inactive 30kDa form.

**Table A12** – Studies reporting expression of XIAP in choriocarcinoma cell lines, in first trimester trophoblast and normal term villous trophoblast.

<b>Survivin</b>	Normal term villous tissue, Jeg-3 and BeWo choriocarcinoma cell lines	Shiozaki et al. 2003	IHC RT-PCR WB	mRNA present in placental tissue, Jeg-3 and BeWo cells, weakly expressed by villous tissue. Protein present in first trimester tissue CT, and weakly in ST. In third trimester tissue CT staining in nucleus and cytoplasm.
	Normal first trimester and term villous tissue	Ka and Hunt. 2003	IHC WB RT-PCR	mRNA present in villous tissue and in Jeg-3 and JAr cell lines. Expression greater in first trimester than term villous tissue. In first trimester, survivin present in villous CT cytoplasm. In term patchy staining of CT and ST cytoplasm.

**Table A13** – Studies reporting expression of XIAP in choriocarcinoma cell lines, in first trimester trophoblast and normal term villous trophoblast.

<b>NIAP</b>	Normal first trimester and term villous tissue, Jeg-3 and JAr choriocarcinoma cell lines	Ka and Hunt. 2003	IHC RT-PCR WB	mRNA present in villous tissue and in Jeg-3 and JAr cell lines. Strongly expressed by trophoblast. Expression greater in third trimester than first trimester, NIAP present in CT, ST and endothelial cells.
<b>HIAP-1</b>				mRNA present in villous tissue and in Jeg-3 and JAr cell lines. Expression and localisation did not change with gestation. HIAP1 was present in cytoplasm of CT, ST and mesenchymal cells.
<b>HIAP-2</b>				mRNA present in villous tissue and in Jeg-3 and JAr cell lines. Expression and localisation did not change with gestation. HIAP1 was present in cytoplasm of CT and ST.

**Table A14** – The presence of other IAPs in choriocarcinoma cell lines, in first trimester trophoblast and normal term villous trophoblast



<b>XAF-1</b>	Normal first trimester and term villous tissue	Straszewski-Chavez et al. 2007	IHC WB	Not expressed in first trimester tissue. In third trimester expressed in ST nuclei and cytoplasm.
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**Table A15** – Studies reporting expression of XAF-1 in first trimester trophoblast and normal term villous trophoblast.

<b>Caspase 3</b>	Normal first trimester and term villous tissue	Huppertz et al. 1998	IHC	In first trimester, procaspase-3 predominantly in CT cytoplasm, active caspase-3 in focal areas of ST cytoplasm. In third trimester, throughout ST cytoplasm.
	Normal term villous tissue	Huppertz et al. 1999	IHC Caspase activity assay	Procaspase 3 expressed in CTs, active caspase 3 only present in ST. Need to check paper.
	Normal term villous tissue	Hung et al. 2002	IHC	Active caspase-3 present in syncytiotrophoblast.
	Normal term villous tissue	Huppertz et al. 2003	IHC	Active caspase-3 present in syncytiotrophoblast
	Normal first trimester and term villous tissue	De Falco et al. 2004	IHC	In first trimester caspase-3 present in perinuclear region of CT. In third trimester, present in CT and ST in cytoplasm and perinuclear areas.
	Normal term villous tissue	Crocker et al. 2004	Caspase activity assay	Caspase 3 activity present in villous tissue.
	Normal term villous tissue	Charles et al. 2005	real time RT-PCR WB Caspase activity assay	Procaspase 3 mRNA and protein present and caspase 3 active in villous tissue.
	Term villous tissue from normal and IUGR pregnancies	Endo et al. 2005	IHC	Active caspase 3 present in villous tissue. Increased in IUGR pregnancies.
	Isolated term trophoblast	Hu et al. 2006	WB	Present in isolated trophoblast.
	BeWo choriocarcinoma cell line	Bae et al. 2007	WB Caspase activity assay	Present and active in BeWo cell lysate.

**Table A16** – The presence of Caspase-3 (pro- and active forms) in choriocarcinoma cell lines, in first trimester trophoblast, normal term villous trophoblast and pregnancies complicated by IUGR.

<b>Caspase 6</b>				Procaspase 6 expressed in CTs, active caspase 6 only present in ST.
<b>Caspase 7</b>	Normal term villous tissue	Huppertz et al. 1999	IHC Caspase activity assay	Procaspase 7 expressed in CTs, active caspase 7 only present in ST.
<b>Caspase 8</b>	Normal first trimester and term villous tissue	De Falco et al. 2004	IHC	Procaspase 8 expressed in CTs, active caspase 8 present in CT.
<b>Caspase 9</b>	Normal first trimester and term villous tissue	De Falco et al. 2004	IHC	Expressed in CT cytoplasm in first trimester. In third trimester, caspase-8 present in ST cytoplasm.
<b>Caspase 10</b>	Normal first trimester and term villous tissue	Huppertz et al. 1999	IHC Caspase activity assay	In first trimester weak punctate staining in cytoplasm of ST and CT. In third trimester, strong expression in cytoplasm of CT and ST.
<b>Caspase 14</b>	Normal first trimester and term villous tissue	Kam et al. 2005	IHC WB Caspase activity assay	Procaspase 14 present in first and third trimester villous CT and ST. No evidence of cleaved caspase 14 in the placenta. More procaspase 14 in first trimester compared to third trimester.

**Table A17** – The presence of other caspases (pro- and active forms) in first trimester trophoblast and normal term villous trophoblast.

## Appendix B

The following section describes the constituents of solutions used in Western blotting (section 2.7), Immunoperoxidase staining (2.8), and lactate dehydrogenase assay (2.13.1).

### Solutions used in Western Blotting Protocol

1.5M Tris-Hydrochloride (Tris HCl) was made by dissolving 18.5g of Trizma base ( $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$ ) in 100ml water whilst stirring continuously, when the Trizma base had dissolved completely the pH was reduced to 8.8 by the addition of concentrated hydrochloric acid (HCl). The solution was stored at room temperature.

0.5M Tris-HCl was produced by adding 6g of Trizma base in 100ml water whilst stirring continuously. When all the Trizma base has dissolved the pH was adjusted to 6.8 by the addition of concentrated HCl. The solution was stored at room temperature.

10% (w/v) Sodium Dodecyl Sulphate ( $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$ ) (SDS). 10 mg of SDS was weighed out on the ventilated balance due to the toxic nature of this substance. This was then added to 100ml of water and stirred vigorously whilst being heated to  $60^\circ$ . The solution was stored at room temperature.

10% Ammonium Persulphate (APS) ( $(\text{NH}_4)_2\text{S}_2\text{O}_8$ ). 1g of APS was dissolved in 10ml of distilled water and stirred gently. Following this the solution was divided into aliquots of 500 $\mu\text{l}$  and frozen at  $-20^\circ\text{C}$  until required.

10x Running buffer was made by dissolving 30.25g of Trizma base and 144g of glycine in 850ml while stirring. Once all the solid material has dissolved the pH was adjusted to 8.3 using concentrated HCl. To this solution 100ml of 10% SDS (produced by the method shown above) was added, and the final solution made up to 1000ml by the addition of distilled water. The 1x running buffer used in Western blotting was 0.025M Tris-HCl, 0.05M Glycine, 1% SDS.

Blotting buffer was made by adding 3.03g of Trizma Base (0.025M) and 14.4g of glycine (0.05M) to 800ml of distilled water whilst stirring, once all in solution the pH was adjusted to 8.3 using 1M HCl, to this solution 200ml of Methanol was added. This solution was then stored at 4°C.

10x Tris Buffered Saline (TBS) was made from 121.1g of Trizma base and 88g of NaCl was added to 800ml of distilled water in a clean bottle. After all the powder had dissolved the pH was decreased to 7.5 using concentrated HCl. The volume was then increased to 1000ml using distilled water. The finished solution was stored at room temperature. A solution of 1x TBS was made using distilled water and 10x TBS in a ratio of 9:1 giving a final concentration of 0.1M TBS. To this Tween 20 was added to give 0.05% Tween, therefore 0.25ml of Tween 20 was used in 500ml of 1xTBS. This solution was then stored at room temperature.

#### SDS-Polyacrylamide Gels for Electrophoresis

The resolving gel (10%) was made from 5.0 ml of 1.5M Tris-HCl, 8.1 ml of distilled water, 200µl of SDS, 100µl of APS and 6.6ml of 30% Acrylamide (29:1 Acrylamide:Bis-acrylamide) (Biorad, Hercules, CA, USA), this mixture was then agitated. 15µl of N,N,N',N'-Tetramethylethylene-diamine (T-MED) was added to the acrylamide solution, this was then

agitated for a further 10 seconds. This solution was then gently placed into the assembled mould using a 1ml pipette. To prevent any bubbles forming in the gel, distilled water was gently placed above the SDS-Polyacrylamide mixture immediately, so as not to disturb the setting of the gel. The remaining gel mixture was left to set in the specimen pot, when this had set the mould was inverted and the water allowed to drain out.

The separating gel (3.5%) was made from 2.5ml of 0.5M Tris-HCl, 6.1ml of distilled water, 100µl of SDS, 50µl of APS and 1.33ml of 30% Acrylamide (29:1 Acrylamide:Bis-acrylamide) (Biorad, Hercules, CA, USA). This mixture was agitated for 10 seconds. 15µl of T-MED was added to this solution and agitated again for 10 seconds. This solution was then placed on top of the set 10% resolving gel until the liquid reached the top of the mould, at this point the plastic comb was gently introduced at a 45° angle to prevent bubbles forming at the base of the wells which hold the protein samples. The remaining solution is once again left in the specimen pot until it sets. When the separating gel has set the moulds were removed from the stand and placed in the electrode assembly.

#### **Solutions used in Immunoperoxidase Staining Protocol**

Tri-sodium citrate solution (0.01M) was made using 1.47g Tri-sodium citrate dissolved in 400ml of distilled water. Once all the material was in solution the pH was adjusted to 6.0 using 1M HCl. This was then made up to 500ml using distilled water.

#### Coating slides in 3-aminopropyltriethoxysilane (APES)

A solution of 9ml APES in 300ml acetone was made. The slides were cleaned in 100% ethanol and agitated in a bath containing the APES-Acetone solution for 30 seconds. The slides were



then transferred to a bath of 100% acetone and agitated for 5 seconds. Finally the slides were transferred to a bath of distilled water and agitated for a further 5 seconds. The slides were then shaken dry and transferred to a 60°C oven for 24 hours.

#### **Solutions used in Lactate Dehydrogenase Assay Protocol**

80mM Tris-HCl solution was made using 1.938g of Tris-base and 3.376g of Sodium Chloride (BDH, Leicestershire, UK) in 200ml of distilled water. The pH of this solution was adjusted to 7.5 using concentrated hydrochloric acid.

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