

University of Alberta

*TRAIL-induced apoptosis of vascular endothelial cells occurs using both the intrinsic and extrinsic pathways and is modulated by phosphoinositide 3-kinase through c-FLIP.*

by

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## LIST OF ABBREVIATIONS AND UNITS

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The following abbreviations, definitions and units have been used throughout this thesis.

°C	degrees Celsius
ANOVA	analysis of variance
Apaf1	apoptotic protease-activating factor 1
BAD	Bcl-2/ Bcl-X <sub>L</sub> associated death promoter
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
Bcl-X <sub>L</sub>	Long form of Bcl-X
Bid	Bcl-2 inhibitory BH3 domain-containing protein
Bim	Bcl-2 interacting protein
bp	base pair
Ca <sup>2+</sup>	calcium ion
CaCl <sub>2</sub>	calcium chloride
cAMP	cyclic adenosine monophosphate
caspase	cysteinyl aspartic acid-protease
CDK	cyclin-dependent kinase

c-FLIP<sub>L</sub>.....cellular FADD-like, IL-1 $\beta$ -converting enzyme inhibitory protein  
long form

c-FLIP<sub>S</sub>.....cellular FADD-like, IL-1 $\beta$ -converting enzyme inhibitory protein  
short form

cGMP .....cyclic guanine monophosphate

cIAP1 .....cellular inhibitor of apoptosis protein 1

cIAP2 .....cellular inhibitor of apoptosis protein 2

CO<sub>2</sub>.....carbon dioxide

CREB .....cyclic AMP (cAMP)-response element binding protein

CREs .....cAMP response elements

C-terminal .....carboxy terminal

Cys .....cysteine (amino acid)

Cyto c .....cytochrome *c*

DR.....death receptor

DcR .....decoy receptor

DD.....death domain

DED .....death effector domain

DFF .....DNA fragmentation factor

DISC .....death inducing signalling complex

DPBS.....Dulbecco Phosphate Buffered Saline

ECGS .....endothelial cell growth supplement

ECM.....extracellular matrix



EDTA.....ethylenediaminetetraacetic acid di-sodium salt

*et al.*.....*et alii* (Latin, 'and others')

FADD.....Fas-associated death domain

FBS .....fetal bovine serum

*g*.....acceleration due to Earth's gravity ( $9.8 \text{ m}\cdot\text{s}^{-2}$ )

*g*.....gram(s)

GEFs .....guanine-nucleotide-exchange factors

GSK3.....glycogen synthase kinase 3

Gly.....glycine (amino acid)

*h*.....hour(s)

H<sub>2</sub>O .....water

HCl.....hydrochloric acid

HUVECs .....human umbilical vein endothelial cells

IAP .....inhibitor of apoptosis proteins

*i.e.*.....*id est* (Latin, 'that is')

IgG .....Immunoglobulin G

I $\kappa$ B .....inhibitory subunit for NF- $\kappa$ B

IKK.....inhibitors of  $\kappa$ B kinase

KCl.....potassium chloride

K<sub>d</sub>.....kinetic dissociation constant

kD.....kilodalton

l .....litre(s)

m .....meter(s)

M.....moles·l<sup>-1</sup>

M199 .....medium for endothelial cells

mAbs .....monoclonal antibodies

MgCl<sub>2</sub>.....magnesium chloride

min .....minute(s)

mRNA .....messenger ribonucleic acid

n.....number of samples

N/A.....not applicable

NaCl .....sodium chloride

NaN<sub>3</sub>.....sodium azide

NAIP .....neuronal apoptosis inhibitory protein

N/D.....not determined

NF-κB .....nuclear factor kappa B

NK cells .....natural killer cells

N-terminal .....amino-terminal

O<sub>2</sub> .....molecular oxygen

OD .....optical density

OPG.....osteoprotegerin

p-Akt .....phosphorylated Akt

PARP .....poly(ADP-ribose)polymerase

PBLs.....peripheral blood lymphocytes

PBS .....phosphate buffered saline

PDK-1 .....phosphoinositide-dependent kinase-1

PE.....phycoerythrin

PH .....pleckstrin homology domains

pH.....logarithmic unit measuring acidity

PI.....propidium iodide

PI3K .....phosphoinositide 3-kinase

PKA.....protein kinase A/cyclic-AMP-dependent protein kinase

PKB.....protein kinase B otherwise known as Akt

PKC.....protein kinase C

PLAD .....pre-ligand-binding assembly domain

PtdIns .....1,2-Diacyl-*sn*-glycero(3)phospho(1)-D-*myo*-inositol;  
phosphoinositide

PTEN.....phosphoinositide-lipid 3-phosphatase

p value .....probability (of incorrectly rejecting the null hypothesis)

RB .....running buffer

Rb.....retinoblastoma

RNA .....ribonucleic acid

RNAi .....RNA interference

RIP .....receptor interacting protein

s.....second(s)

SD .....standard deviation of the mean

SDS .....sodium dodecyl sulphate

siRNA .....small interfering RNA

SEM .....standard error of the mean

Ser .....serine (amino acid)

SHIP.....SH2-containing inositol (poly)phosphate 5- phosphatase

Smac/DIABLO.....second mitochondria-derived activator of caspase/direct inhibitor  
of apoptosis binding protein [IAP] with low pI

tBid.....truncated Bid

TBST .....Tris buffered saline with Tween-20

Thr.....threonine (amino acid)

TNF $\alpha$ .....Tumor Necrosis Factor alpha

TNFR .....TNF receptor

TRAIL.....TNF-related apoptosis-inducing ligand

TRAIL-R.....TRAIL receptor

Tris-HCl .....tris[hydroxymethyl]-amino methane hydrochloride

XIAP .....X-linked inhibitor of apoptosis proteins

Zn .....zinc

Mathematical prefixes

k.....kilo ( $10^3$ )

c.....centi ( $10^{-1}$ )

m .....milli ( $10^{-3}$ )

$\mu$  .....micro ( $10^{-6}$ )

n.....nano ( $10^{-9}$ )

## **CHAPTER 1. RATIONALE, HYPOTHESES AND LITERATURE REVIEW.**

---

### ***1.1 RATIONALE***

TRAIL and its receptors are widely expressed in normal tissues, unlike TNF or FasL, whose expression is restricted to a limited set of immune effector cells. This suggests that TRAIL exerts multiple functions in a wide variety of tissues, including the cardiovascular system. TRAIL apoptosis has been widely studied in cancer but not in normal cells. In endothelial cells, TRAIL may play a role in endothelial apoptosis, a process that is important in both atherosclerosis and in angiogenesis. The PI3K pathway may be downregulated in these states to promote endothelial apoptosis in response to various ligands including FasL and TRAIL. We wished to explore the role of the PI3K pathway in regulating the susceptibility of vascular endothelial cells to TRAIL-induced apoptosis. This may have implications in understanding the role of TRAIL in situations where endothelial apoptosis is promoted, such as atherosclerosis and angiogenesis.

## 1.2 HYPOTHESES

*Hypothesis 1:* Vascular endothelial cells are resistant to the apoptotic effects of non-tagged TRAIL and require inhibition of the PI3K/Akt pathway in order to become sensitive to TRAIL-induced apoptosis. *Experimental approach:* HUVECs were subjected to TRAIL treatment alone or in combination with PI3K inhibitor LY294002 and resulting cell numbers assessed by acid phosphatase assay.

*Hypothesis 2:* TRAIL activates both the intrinsic and extrinsic pathway in order to induce apoptosis in endothelial cells where the PI3K pathway is inhibited. *Experimental approach:* HUVECs were subjected to TRAIL treatment alone or in combination with the PI3K inhibitor LY294002. The resulting cell lysates were assessed by Western blot analysis for extrinsic and intrinsic pathway activation.

*Hypothesis 3:* The PI3K/Akt pathway modulates endothelial cell sensitivity by regulating anti-apoptotic protein. *Experimental approach:* HUVECs were subjected to PI3K inhibitor LY294002 treatment and resulting cell lysates were assessed by Western blot analysis for expression of pro- and anti-apoptotic regulatory proteins. In addition, the role of PI3K in modulating TRAIL sensitivity was confirmed by siRNA gene silencing.

### ***1.3 OVERVIEW***

The regulation of cell growth and death balances a fine line, where uncontrolled growth in the absence of death disturbs the homeostasis and can result in diseases such as cancer, autoimmunity, and degenerative disorders [1]. Cells have the intrinsic ability to self-destruct or commit suicide, a process called apoptosis [1]. Mutations or instabilities in genes that take part in apoptosis or those that induce proliferation can result in deregulation of the two processes contributing to malignancy [2]. In recent years, research into treating cancer has been focused on targeting apoptotic mechanisms. Two main signaling pathways exist that control the initiation of apoptosis: the intrinsic pathway involving mitochondria [3] and the extrinsic pathway involving surface death receptors [4]. Consequently, the TNF-superfamily of death ligands and death receptors became of considerable interest for therapeutic development. The superfamily includes the ligand/receptor pairs: TNF $\alpha$ /TNFR1, FasL (CD95L)/Fas (CD95), Apo3L/DR3, and TNF-related apoptosis-inducing ligand (TRAIL)/DR4 and DR5 [4]. Both TNF $\alpha$  and FasL have been found to induce apoptosis in solid tumors. However, the potential clinical usage of these two death ligands was abandoned due to their severe toxicity to normal cells and tissues [5].

TRAIL, the newest member of the TNF superfamily [6, 7], has recently generated much interest and excitement in research circles because of its ability to selectively kill cancer



cells while leaving normal cells intact [8-10]. The tumor suppressor activity of TRAIL was first found in mice where a subset of natural killer (NK) cells found in mouse livers expressed TRAIL on their surface [11, 12] which contributed in protecting their livers from tumor metastasis [12]. Although not all murine NK cells express TRAIL on their surface, expression can be induced on lung and spleen NK cells resulting in the killing of tumor cells *in vivo* through upregulated TRAIL expression [11]. These studies support the role of TRAIL as a tumor surveillance mechanism in the innate immune system.

Experiments systemically administering recombinant non-tagged TRAIL in mice and non-human primates observed inhibition of tumor growth with little or no toxicity to the animals [8, 13]. In contrast, several other versions of TRAIL, namely those that are epitope-tagged with histidine tags or leucine zippers or antibody crosslinked at the amino-terminus, have generated controversy due to their toxicity to normal cells and tissues [13-16]. Not all transformed cells and primary tumors are sensitive to TRAIL-induced apoptosis raising questions to the plausibility of using TRAIL in potential cancer therapies [17].

The normal physiological roles of TRAIL as well as its ability to induce apoptosis in endothelium, which can be part of normal processes such as angiogenesis are not well studied. In a recent study, it was reported that tagged-TRAIL activates the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, which modulates TRAIL-induced

apoptosis in human umbilical vein endothelial cells HUVECs [18]. This pathway is of central importance to the vascular endothelium during angiogenesis, where it regulates endothelial cell survival, differentiation and migration [19]. Therefore, further study of this pathway, and its potential to modulate TRAIL-induced apoptosis in vascular endothelial cells is warranted.

#### **1.4 THE TRAIL RECEPTOR FAMILY**

Five receptors have been identified to bind TRAIL. Two receptors DR4 [20] and DR5 [21], are type I transmembrane proteins and are implicated in pro-apoptotic signaling. They both contain a conserved death domain (DD) motif in their cytoplasmic tails and upon binding to TRAIL are responsible for transducing the death signal to the intracellular machinery. Overexpression of the two death receptors has been found to induce apoptosis independent of ligand binding due to oligomerization of the DDs, whereas deletion of the death domains on both receptors blocks TRAIL death signal [20, 22]. Decoy receptor 1 (DcR1) [21] and decoy receptor 2 (DcR2) [23, 24] possess extracellular domains that have close sequence homology to the extracellular domains of DR4 and DR5. However, these receptors are unable to transduce a death signal upon binding to TRAIL and their overexpression is believed to inhibit TRAIL-induced apoptosis. Transformed cell lines lacking DcR1 were sensitive to TRAIL-induced apoptosis until transfected and overexpressed with the decoy receptor at which point they became resistant to the actions of TRAIL [22]. In another study, cells resistant to TRAIL and expressing DcR1 such as human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HUMECs) became sensitive to TRAIL-induced apoptosis upon treatment with PI-PLC or removal of DcR1 from the cell surface [21]. DcR2 contains a truncated death domain motif and DcR1 is a GPI-anchored protein that lacks a cytoplasmic tail altogether [21-24]. As a result, both receptors lack a functioning death domain motif in order to communicate a death signal into the inside of the cell.

High expression levels of mRNA for DcR1 and DcR2 have been found in normal cells including PBLs, spleen, heart, lung, kidney, liver, bone marrow, and placenta [21, 22, 25]. In contrast, low levels of both decoy receptors have been detected in transformed cells lines and their overexpression in sensitive cell lines confers resistance to TRAIL induced apoptosis [21-24]. Hence, it is hypothesized that DcR1 and DcR2 compete with DR4 and DR5 for the binding of TRAIL and thereby protect normal cells from TRAIL-induced apoptosis. The decoy receptors have also been implicated in signaling by activation of NF- $\kappa$ B [24].

In addition, a soluble receptor for TRAIL, osteoprotegerin (OPG) [26, 27] has also been identified but its physiologic role as a TRAIL receptor has not firmly been established because of its low affinity for TRAIL at 37°C ( $K_d$ = 400 nM) [28]. Initial experiments have outlined the role of OPG in regulating bone resorption and bone mass through a decrease in osteoclast differentiation [26]. However, a recent study showed that OPG may play an important role in survival of hormone-resistant prostate cancer cells against TRAIL-induced apoptosis [29].

Discovery of a conserved pre-ligand-binding assembly domain (PLAD) on the surface of TNFRs [30] as well as pre-formed Fas trimers [31] suggest that the TRAIL receptors also exist in pre-associated trimer complexes on the surface of cells and that the preformed receptor complexes may be required for ligand signaling [31].

### ***1.5 TRAIL: GENE AND PROTEIN***

TRAIL normally exists as a 281 amino acid containing, type II transmembrane protein [7]. Although most TRAIL exists in a vesicle-associated or membrane bound form, proteolytic cleavage of the extracellular domain results in a soluble version of TRAIL that has also been detected in small quantities [32, 33]. Expression of TRAIL mRNA has been found in most human tissues such as the lungs, spleen, and prostate but not in the brain, liver, testes and HUVECs [7, 34]. Expression of the protein ligand itself has been detected mostly on cells of the immune system such as natural killer cells of the innate immune system [11] as well as on activated monocytes [35] and dendritic cells [36]. However, recently TRAIL protein expression was also detected on the surface of vascular smooth muscle [34]. Similar to FasL and TNF $\alpha$  in both sequence homology and signaling characteristics, TRAIL also forms homotrimers that binds three receptor molecules [37, 38]. This results in a receptor/ligand hexagonal complex where the ligand binds each receptor between two of its subunits thereby initiating a signaling cascade [37, 38].

Optimal biological activity of TRAIL is dependent on the stability of the trimeric ligand and on the final tertiary configuration of the ligand complex itself [39]. In both recombinant soluble forms as well as cell-associated forms of TRAIL, the presence of Cys230 is critical for maintaining activity of the ligand as it can either bind another Cys

to form a disulfide bridge or can chelate a Zn atom per one TRAIL trimer unit [39]. In the case where the Cys of interest forms a disulfide bond, the resulting TRAIL was found to be poorly active as it reduced the affinity for TRAIL for DR5 [39]. Alternately, when a Zn atom was coordinated to one TRAIL trimeric unit the resulting ligand retained its biological activity suggesting a role for Zn in stabilizing the ligand configuration for receptor binding [39]. Because of this specific interaction, the amount of Zn in forms of recombinant soluble TRAIL for research and clinical use, may be important in determining the final selectivity and cytotoxicity of the ligand [40].

Toxicity of different versions of recombinant TRAIL to hepatocytes may be due to the preparation of the ligand. Hepatocyte toxicity [16, 41] was reported in groups that used TRAIL prepared in the absence of Zn, which has been suggested to reduce solubility of the ligand product and cause aggregation [40]. This is contrasted by forms of recombinant soluble, non-tagged versions of TRAIL produced in the presence of stoichiometric Zn, which had no adverse cytotoxic effects on human and non-human primate hepatocytes [15, 41] as well as keratinocytes [14]. In addition, certain forms of aggregated, poly-histidine tagged, and antibody crosslinked TRAIL can also trigger apoptosis in normal cell types such as astrocytes [13, 15], keratinocytes [14], hepatocytes [16] and neurons [15] indicating that although some cells are resistant to the trimeric form of the ligand they can be quite sensitive to other higher order oligomerized forms of TRAIL [42].

## 1.6 APOPTOSIS

Apoptosis is a form of programmed cell death that allows multicellular organisms to remove old or damaged cells in a process that is distinct from necrosis [1]. Necrosis, a form of pathological or accidental cell death, is instigated upon acute injury to a cell resulting in irreversible swelling of the cytoplasm and organelles followed by the eventual lysing and release of its contents to its surroundings [43]. The release in cellular components affects neighbouring cells and can potentially cause a damaging inflammatory response. The process of necrosis typically occurs when cells are exposed to traumatic conditions such as hypoxia, ischemia, hyperthermia, complement attack from the innate immune system, metabolic toxins as well as direct cell trauma [44].

Unlike necrosis, apoptosis is morphologically characterized by chromatin condensation and fragmentation, cell shrinkage and membrane blebbing [1, 45, 46]. Changes in the plasma membrane composition, during apoptosis, provide signals for phagocytes to absorb the apoptotic cell without provoking a generalized inflammatory response [45, 46]. This process not only prevents cellular contents from being released into the surrounding and initiating an inflammatory reaction, but also allows for recycling of the organic components of the dying cell [44]. Apoptosis is an important physiological event that is required for normal cell development as well as controlling pathological processes [1, 44]. Because of its importance, apoptosis is a tightly regulated process, hence any

abrogation from the normal apoptotic regulation can lead to a number of diseases (reviewed in [43]). For example, accumulation of unwanted cells because of insufficient apoptosis can lead to cancer, and cell loss due to excess apoptosis results in stroke, neurodegeneration and heart failure [43]. In angiogenesis, apoptosis plays an important role in removing excess and/or redundant endothelial cells generated during the process of new blood vessel formation [19]. In atherosclerosis, apoptosis removes endothelial cells creating a prothrombotic surface on atherosclerotic plaques.

The execution of apoptosis is carried out by a set of intracellular proteins called caspases, which are responsible for inducing the morphological and biochemical changes that are associated with apoptosis [47, 48]. Caspases are aspartic-specific cysteine proteases that exist in a single chain, inactive zymogen form and therefore require proteolytic cleavage into their active forms [47, 49]. Since their discovery in *C. elegans*, thirteen caspases have been identified in mammalian cells [47, 49]. They can be subdivided into two groups, initiator and effector caspases, based on their structure and function [47, 49]. ‘Initiator’ caspases or caspases -8, -9, or -10, have long prodomains, which allow them to interact with protein ‘adaptor’ molecules [47, 49]. In the event of apoptotic stimuli, recruitment of these caspases through clustering of adaptor molecules results in their close proximity to each other and leads to activation of their intrinsic protease activity ending in their cleavage and activation [50]. When activated, these ‘initiator’ caspases can go on, either directly or indirectly, to activate caspases with shorter prodomains, termed ‘effector’ caspases that include caspases -3, -6, and -7 [47, 49, 51, 52]. Once these



'effector' caspases are activated, they can further go on to cleave substrates such as structural and regulatory proteins leading eventually to apoptosis. This caspase cascade is selective since caspases recognize a specific protein motif (e.g. DEVD, IETD, VDVAD, etc) in their substrates, including on other caspases, resulting in cleavage after aspartate residues within the specific protein motif [49]. End substrates cleaved by 'effector' caspases include PARP, DFF as well as lamins, actin, cytokeratins and Bcl-2 and Bcl-X<sub>L</sub>, these substrates result in the morphological and biochemical changes associated with apoptosis [49].

Although most caspases are known to exist in the cytoplasm, a few have been found sequestered to other organelles and are thought to facilitate organelle specific responses to stress [49]. For instance, caspase-2 has been found in the nucleus and Golgi apparatus and caspase-12 associated with the outer membrane of the endoplasmic reticulum (ER) in order to respond to nuclear, Golgi and ER stress respectively [49].

## **1.7 TRAIL SIGNALING EVENTS**

### ***1.7.1 TRAIL-Induced DISC/Extrinsic Pathway of Apoptosis***

TRAIL induces apoptosis through the extrinsic and intrinsic pathways (See **Figure 1.1**). The extrinsic pathway is initiated by binding of TRAIL to its cognate death receptors, DR4 and DR5 on the cell surface [20-22, 53]. This results in the recruitment of specific cytoplasmic proteins to the intracellular death domains of the receptor forming a death-inducing signaling complex (DISC) [54]. One of the proteins recruited first to the DISC is the adaptor protein, Fas-associated death domain (FADD) [53-56]. Early dominant negative studies where FADD was overexpressed saw the recruitment of FADD to both the DR4 and DR5 DISC upon coimmunoprecipitation [57, 58]. However, FADD failed to coimmunoprecipitate in the DISC of DR4 but was observed in the DISC of DR5 by a later group in experiments using receptor overexpression [25]. Subsequent experiments coimmunoprecipitating the native TRAIL DISC without gene transfection or overexpression of FADD, DR4 and DR5 showed FADD to be present in both the DR4 and DR5 DISC, with caspase-8, just after TRAIL addition to the cells [55, 59, 60]. FADD is composed of two protein interaction domains: a carboxy-terminal death domain (DD) and an amino-terminal death effector domain (DED) [61, 62]. It interacts with the receptor to initiate formation of the DISC with homophilic interactions between the DD

domains on the receptors and itself and subsequently recruits procaspase-8 through homophilic interactions between DED domains on itself and caspase-8 [54, 63].

In the DISC, close proximity of procaspase-8 to other procaspase-8 precursors leads to their activation through trans- and autocatalytic cleavage [64], to form activated caspase subunits in a two-step mechanism [65]. In the first step, precursor or procaspase forms, p55 and p53, are cleaved into p43 and p12 fragments [65]. The p12 unit undergoes further processing to generate an active p10 fragment [65]. Receptor-bound p43 is also cleaved into a smaller p26 fragment which subsequently produces an active p18 fragment [65, 66]. Both active subunits of caspase-8, p10 and p18, are released into the cytosol where they can activate downstream effector caspases [63, 66] such as caspase-3. Caspase-3 is activated from its proenzyme p32 precursor through cleavage by activated caspase-8, into p20, p17 and p10 fragments [67]. Once activated, caspase-3 can then induce and execute programmed cell death through the cleavage and activation of substrates that inhibit cell survival such as DNA fragmentation factor [68].

Caspase-10, another initiator caspase closely related to caspase-8, was recently confirmed to be recruited to the DR4, DR5 and Fas DISC through FADD [69, 70] and induce subsequent downstream activation of caspase-3 in caspase-8 deficient cells [71, 72]. However, caspase-3 activation was attenuated suggesting that caspase-10 may play a role different from caspase-8 [71, 72].

### ***1.7.2 TRAIL-Induced Activation of the Intrinsic Mitochondrial Pathway***

In certain cells, induction of apoptosis by effector caspase pathways requires amplification of the DISC signals by activation of the cell-intrinsic pathway, which utilizes factors released by mitochondria [40]. This pathway is normally activated to induce apoptosis in response to DNA damage when cells are exposed to DNA-damaging agents such as chemotherapeutic agents and radiation [40]. Activation of the intrinsic pathway can also be achieved by caspase-8, which therefore activates both the extrinsic and intrinsic pathways (**See Figure 1.1**). Active caspase-8 subunits cleave pro-apoptotic Bcl-2 inhibitory BH3-domain containing protein (Bid) to form truncated Bid (tBid) [73]. Upon cleavage tBid translocates to the mitochondrial membrane and induces the mitochondrial release of apoptotic factors Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis binding protein [IAP] with low pI) [74, 75] and cytochrome *c* [76] into the cytosol. Cytochrome *c* together with the cytosolic proteins Apaf1, and procaspase-9 as well as ATP form a protein complex called an apoptosome resulting in the activation of caspase-9 [77]. Caspase-9 is activated from its precursor p47 form to activated p37 and p35 subunits [77]. These active subunits can then go on to activate caspase-3, contributing to apoptosis through the intrinsic pathway [77]. In the cytosol, Smac/DIABLO acts by interfering with XIAP (X-linked inhibitor of apoptosis protein) which inhibits caspase 9 [78] and caspase-3 [79]; when Smac interacts with XIAP it displaces it, and therefore releases its inhibition of caspase-9 and caspase-3,

thereby promoting further activation and amplification of the death ligand signal through the intrinsic pathway [78, 79].

The activation of the intrinsic pathway is regulated by Bcl-2 family members: Bcl-2, Bcl-X<sub>L</sub>, Bax, Bak and Bid (**See Figure 1.1**). Translocation of tBid to the mitochondrial membrane results in the loss of mitochondrial membrane potential and release of apoptotic factors, Smac/DIABLO and cytochrome *c* into the cytosol where they activate caspases-9, and -3 in order to induce apoptosis [73]. The release of Smac/DIABLO and cytochrome *c* is facilitated by pro-apoptotic proteins, Bak and Bax [80]. While Bak is normally present on the surface of the mitochondrial membrane, Bax is recruited to the mitochondrion by interacting with tBid which results in all three proteins oligomerizing, leading to the loss of mitochondrial membrane potential [81] and triggering the release of Smac/DIABLO and cytochrome *c* [82]. In contrast, anti-apoptotic members Bcl-2 and Bcl-X<sub>L</sub>, which reside on the outer mitochondrial membrane, block mitochondrial release of these apoptotic factors by interacting with Bak and Bax thus blocking their actions on perturbing the mitochondrial membrane potential [80].

## ***1.8 MODULATION OF TRAIL SENSITIVITY***

### ***1.8.1 Modulation of the TRAIL DISC***

A protein thought to modulate sensitivity of cells to TRAIL-induced apoptosis by being recruited to the DISC is referred to as “cellular FADD-like, IL-1 $\beta$ -converting enzyme inhibitory protein” (c-FLIP) [83]. Like other proteins recruited to the DISC through homologous DD or DED domain interactions, c-FLIP is recruited to the DISC through DED domain interactions with FADD where it modulates caspase-8 recruitment and activation [71].

The gene encoding c-FLIP consists of 13 exons and is found between the genes encoding caspase-8 and caspase-10 on chromosome 2 [84, 85]. Four mRNA splice variants are transcribed from the c-FLIP gene, but only two forms are translated into proteins that are found in the cell [83, 86, 87]. The short form of c-FLIP (c-FLIP<sub>S</sub>) has a molecular weight of 28 kDa and contains two DED domains [83]. In contrast, the long form of c-FLIP (c-FLIP<sub>L</sub>) has a molecular weight of 55 kDa and contains two DED domains in addition to a caspase-like domain [83]. This caspase-like domain of c-FLIP<sub>L</sub>, however, lacks catalytic activity as a critical active site tyrosine residue is substituted with a cysteine thereby abolishing its caspase-activity [83]. Both forms of c-FLIP have been found to be expressed in many different tissues with the short form being predominantly found in

lymphatic tissue [83]. Three isoforms of the long form of c-FLIP are known to exist [42]. In addition, the c-FLIP expression has been found to be regulated by CaMKII [88], mitogen-activated protein kinase kinase [89] and phosphatidylinositide 3-kinase (PI3K) [90].

TRAIL treatment results in the recruitment of both the long and short forms of c-FLIP to the DISC, where cleavage of c-FLIP<sub>L</sub> occurs to form an intermediate p43 fragment [71]. Both this intermediate form of c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> when bound in the DISC prevent the second step cleavage and activation of caspase-8 [71]. There is also some evidence that c-FLIP might also activate NF- $\kappa$ B to promote cell survival, as was seen in gene transfection and overexpression experiments with c-FLIP<sub>L</sub> [91, 92]. However, more research is required to elucidate the signaling events that link the DISC to downstream NF- $\kappa$ B activation.

### ***1.8.2 Inhibitor of Apoptosis Proteins (IAPs)***

Inhibitor of apoptosis proteins (IAPs) are a family of proteins that inhibit apoptosis by binding to caspases and preventing their activation. Five IAPs have been identified in humans and they include: NAIP (neuronal apoptotic inhibitory protein), c-IAP1, c-IAP2, XIAP and survivin [49]. The proteins c-IAP1, c-IAP2 and XIAP prevent the activation of procaspase-9 and can also directly bind and inhibit effector caspases -3, and -7 thus

inhibiting both the extrinsic and intrinsic apoptotic pathways [93]. In addition, some IAPs can also ubiquitinate either themselves or caspase substrates thereby mediating their removal from the cell [94]. The role of IAPs in regulating caspase activity appears to be important as the molecule Smac/DIABLO, normally sequestered in mitochondria, is released during apoptotic signalling in order to promote caspase activation by inhibiting the activity of XIAP [49].



### ***1.9 ACTIVATION OF NF- $\kappa$ B BY TRAIL***

The TRAIL DISC has also been found to contain a protein called receptor-interacting protein (RIP) [95]. RIP is an adaptor protein, which contains a serine/threonine kinase domain, an intermediate domain, and like FADD, a death domain, which allows it to be recruited to DR4/DR5 during TRAIL signaling [57, 95]. In the DISC, RIP interacts with inhibitors of  $\kappa$ B kinase (IKK) complex [96]. This acts by inducing degradation of I $\kappa$ B and ultimately leads to the TRAIL-induced activation of NF- $\kappa$ B (outlined in Section 1.10.1) [97]. Activated NF- $\kappa$ B transcribes genes with anti-apoptotic functions and can also induce transcription of genes that induce cell proliferation [98]. RIP mediated NF- $\kappa$ B activation is thought to be responsible for the TRAIL-induced proliferation seen in cancer cell lines that resist TRAIL-induced apoptosis [99].

## **1.10 PHOSPHOINOSITIDE 3-KINASE SIGNALING**

### ***1.10.1 Phosphoinositide 3-kinases***

Phosphoinositide 3-kinases (PI3Ks) are responsible for phosphorylating the 3'-OH position of inositol rings contained in inositol phospholipids or phosphoinositides (PtdIns). Its kinase activity produces three lipid products that include: PtdIns(3)P, PtdIns(3,4)P<sub>2</sub>, and PtdIns(3,4,5)P<sub>3</sub>. The phosphoinositides can go on to induce intracellular signaling events by binding proteins containing pleckstrin homology (PH) domains, and controlling as well as localizing their activity [100].

Multiple isoforms of PI3K exist in mammalian cells, which can be divided into three classes [101]. Class I PI3Ks are heterodimers composed of an adaptor/regulatory subunit in addition to a p110 catalytic subunit (~110 kDa) [102]. Class II PI3Ks are 170 – 210 kDa proteins with catalytic domains that are 45-50% homologous to their class I counterparts [103]. However, class II PI3Ks are defined by their C-terminal C2 domains which can bind phospholipids in a Ca<sup>2+</sup>-independent manner, unlike other C2 domain containing proteins such as isoforms of protein kinase C [104, 105]. Class II PI3Ks are known to phosphorylate PtdIns and PtdIns(4)P *in vitro* but their *in vivo* targets have yet to be determined [103]. Class III PI3Ks are analogous to the yeast vesicular-protein-sorting protein Vps34p and are thought to regulate intracellular vesicle trafficking processes in

the cell [102, 103]. They are only known to phosphorylate PtdIns to produce PtdIns(3)P and are responsible for generating most of the PtdIns(3)P present in cells [102].

Most research on PI3Ks has focused on the class I isoforms, as their activity is coupled to extracellular signals and stimuli [103]. Once activated, they phosphorylate their predominant substrate PtdIns(4,5)P<sub>2</sub> to produce PtdIns(3,4,5)P<sub>3</sub> (**See Figure 1.2**) [106]. This lipid product can be further modified to other phosphoinositide forms or it can itself bind PH domains of proteins to modify their activity or localize the protein to specific areas of the plasma membrane [107]. Various extracellular stimuli, such as growth factors and hormones, activate class I PI3Ks which are believed to influence a wide range of cellular processes including cell growth, cell cycle progression, cell adhesion, cell motility and cell survival (**See Figure 1.2**) [100, 103, 108].

The class I family of PI3Ks can be additionally divided into two subclasses: class IA and class IB [102]. Class IA enzymes contain three isoforms of the p110 catalytic subunit: p110 $\alpha$ , p110 $\beta$  and p110 $\delta$  as well as seven adaptor proteins derived from splice variants of regulatory subunits p85 $\alpha$ , p85 $\beta$  and p55 $\gamma$  [102]. The different splice variants can combine with the three catalytic isoforms to form functional PI3K complexes [101]. Tissue distribution of p110 $\alpha$  and p110 $\beta$  is widespread in mammals, however, p110 $\delta$  distribution is more restricted and is primarily found in leukocytes [109]. Class IB PI3Ks contain a

p110 $\gamma$  catalytic subunit complexed with a 101 kDa (p101) regulatory protein [102]. The distribution of class IB PI3K is restricted to white blood cells in mammals [102].

At least one PI3K class IA isoform is expressed in all mammalian cells [102]. Activation of this class of PI3K is seen with stimulation of almost every receptor that induces tyrosine kinase activity including receptors that contain intrinsic tyrosine kinase activity as well as non-receptor tyrosine kinases such as JAK kinases [102]. The activated tyrosine receptor complex recruits p110 subunits by its p85 adaptor subunit, where it can phosphorylate its main substrate PtdIns(4,5)P<sub>2</sub> to generate PtdIns(3,4,5)P<sub>3</sub> [103]. The non-receptor tyrosine kinases including the src-family kinases or JAK kinases have been implicated in activating class IA PI3Ks by B-cell and T-cell receptors, cytokine receptors, co-stimulatory molecules, cell-cell and cell-matrix adhesion [110, 111].

The p110 $\gamma$ /p101 heterodimers, making up the class IB PI3Ks, are activated by the G $\beta\gamma$  subunits of heterotrimeric G (guanine-nucleotide-binding) proteins which are controlled by serpentine receptors such as chemokine receptors [112]. Studies also show that the p110 $\gamma$  can be allosterically regulated through the binding of Ras which results in the stimulation of the p101 lipid kinase activity [113-115].

### *1.10.2 Inactivation of Class I PI3Ks*

Two major pathways have been identified for inactivation of class I PI3K product PtdIns(3,4,5)P<sub>3</sub>. These two pathways are: 1. dephosphorylation of the product by phosphoinositide-lipid 3-phosphatase PTEN [116, 117] and 2. dephosphorylation by SH2-containing inositol (poly)phosphate 5- phosphatase (SHIP) (**See Figure 1.2**) [118].

PTEN, a tumor suppressor has been found to be deleted or mutated in a variety of human tumors [103]. Cells which lack a functioning PTEN enzyme, have constitively activated PI3K signaling pathways mediated through the Tec family of kinases [119], Akt/PKB [116, 117] and Rac/Rho GTPases [120].

The second phosphatase, SHIP, which also negatively regulates PI3K activation has been observed to be important for signaling in lymphocytes [103]. Loss of a wild type SHIP has been observed to result in an unbalanced immune response as well as a disruption of the homeostasis of the immune system resulting in the development of autoimmunity [121-123].

### ***1.10.3 Inhibition of PI3Ks***

Two low molecular weight, cell permeable inhibitors are known to inhibit PI3K activity: wortmannin and LY294002 [102]. These inhibitors have been widely used to elucidate and study the function of PI3Ks.

Wortmannin, a fungal metabolite, acts as a non-competitive inhibitor of PI3K by covalently binding to and modifying residues in the active site of PI3K preventing the binding of its substrate [124]. It is the more potent inhibitor of the two with an inhibitory concentration ( $IC_{50}$ ) of 5 nM [102] and it displays a similar toxicity for class I, II and III PI3Ks *in vitro* [102, 125]. However, because of its relatively short half life [126, 127] and its ability to also inhibit other PI3K-related enzymes such as mTOR, DNA-PK [128-131], wortmannin is not as widely used as LY294002.

LY294002, also known as 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4one [132], is a flavonoid-based compound [102] which, unlike wortmannin, acts as a competitive inhibitor as it can compete with ATP for binding and activating PI3Ks [132]. It also displays a similar inhibitory effect on class I, II and III PI3Ks as wortmannin and has an  $IC_{50}$  of 1  $\mu$ M [102]. Recent evidence indicates that LY294002 can also inhibit the widely expressed protein casein kinase-2 (CK-2) [133].

## **1.11 DOWNSTREAM TARGETS OF PHOSPHOINOSITIDE 3-KINASES**

### ***1.11.1 Guanine –nucleotide-binding proteins Rac and Rho***

GTPases, Rac and Rho, are known to organize and coordinate actin cytoskeletal rearrangements as well as assembly of integrin structures [103]. RhoA is responsible for regulating the formation of actin stress fibers and focal adhesions whereas Rac1 is responsible for controlling lamellipodia formation [103]. Although it is not clear how exactly PI3K regulate their activation, recent groups have found that the class I PI3K product PtdIns(3,4,5)P<sub>3</sub>, potentiates activation of different Rac and Rho kinases through tyrosine phosphorylation on guanine-nucleotide-exchange factors (GEFs), which contain a N-terminal PH domain that binds to PtdIns(3,4,5)P<sub>3</sub> allowing them to be recruited to the plasma membrane and be phosphorylated by Src kinases [103, 134, 135]. The GEF can then go on to activate Rac or Rho [103]. Constitutive activation of PI3K has been found to induce Rac- and Rho- mediated lamellipodia formation and actin stress fibers during chemotaxis [136, 137]. In addition to controlling cytoskeletal rearrangements, Rac and Rho are also known to regulate stress activated MAP kinases p38 and JNKs as well as activate various transcription factors [103, 137]. Their activation by PI3Ks however, does not stimulate these regulatory roles [137]. It is not fully understood how PI3K restrict Rac and Rho activity to those involving motility, but it is hypothesized that architectural organization of the proteins ensures that only a subpopulation of the GTPases are activated near the plasma membrane instead of the whole [103]. Rac and Rho have also

been implicated in the regulation of endothelial vascular permeability through effects on the cytoskeleton [138].

### *1.11.2 Activation of Tec family of tyrosine kinases*

The TEC family of kinases which include ITK, TEC, and BTK, are activated by the PI3K product, PtdIns(3,4,5)P<sub>3</sub> and in turn activate downstream PLC signaling for development and function of cells of the immune system [103]. These kinases are activated by phosphorylation of their activation loops by Src kinases [139, 140]. In order for activation by Src kinases, ITK, TEC and BTK bind to PtdIns(3,4,5)P<sub>3</sub> by their N-terminal PH domain [141]. This results in the association and recruitment of these kinases to the plasma membrane and with Src kinases also located in the plasma membrane [103, 119, 141]. Mice lacking a functioning regulatory p85 $\alpha$  subunit show profound defects in B-cell function [142], which is consistent with mice deficient in BTK indicating essential roles of PI3K in B-cell development and function [140].



### ***1.11.3 Serine/threonine kinase Akt/PKB***

The serine/threonine kinase protein kinase B (PKB), also known as Akt, mediates PI3K activity [143, 144]. Upon production of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, Akt is recruited to the plasma membrane by the binding of the PI3K products to its PH domain [103]. Akt colocalizes with phosphoinositide-dependent kinase-1 (PDK1) which is also recruited to the plasma membrane due to the binding of PtdIns(3,4,5)P<sub>3</sub> with its C-terminal PH domain [103, 145, 146]. At the membrane, Akt is phosphorylated at Thr308 and Ser273 [147, 148]. The phosphorylation of Thr308 is essential and is mediated by PDK1 due to growth factor stimulation [149, 150]. PDK1 is also known to regulate PKC [151, 152] and the ribosomal kinases S6K and S6K1 [153, 154]. The kinase responsible for phosphorylating Ser 273 on Akt has yet to be established, although possible candidates include among others PDK1, PDK2 as well as Akt itself [155].

Presently, three isoforms of Akt have been found to exist: Akt1 (PKB $\alpha$ ) [156], Akt2 (PKB $\beta$ ) [157] and Akt3 (PKB $\gamma$ ). Most of the work discussed involves Akt1 as it is the best studied. Once activated Akt can influence a variety of cellular processes including cell survival, gene expression, and cellular proliferation.

## ***1.12 DOWNSTREAM TARGETS OF AKT/PKB***

### ***1.12.1 Pro-survival and Anti-apoptotic mechanisms***

One major role of Akt has been its critical involvement in cell survival from stimuli such as growth factors and the extracellular matrix (**See Figure 1.3**) [158]. Although this involvement of Akt in cell survival has been known for a number of years, the mechanisms by which it regulates cell survival have only recently emerged [159].

A mechanism by which Akt regulates cell survival is through the phosphorylation of transcription factors resulting in either their activation in order to mediate transcription of pro-survival genes or inhibition of transcription of pro-apoptotic genes [159]. The forkhead family of transcription factors which include FKHR, FKHL1, and AFX are an example of a set of transcription factors phosphorylated and negatively regulated by Akt [160-164]. Normally the forkheads reside in the nucleus where they promote transcription of pro-apoptotic Fas-L, IGFBP-1, and Bim [161, 165]. However, phosphorylation of the forkheads by Akt leads to their export out of the nucleus [160] and into the cytosol where they are sequestered by 14-3-3 [161]. The localization of the forkheads in the cytosol prevents the transcription factors from re-entering the nucleus and transcribing the pro-apoptotic products (**See Figure 1.3**) [159, 166].

In addition, Akt can also positively regulate the activity of transcription factors NF- $\kappa$ B [167, 168] and CREB [169] (See **Figure 1.3**). NF- $\kappa$ B is known to be involved in regulating cell apoptosis, cell proliferation and survival in response to growth factors and cytokines [159]. It is regulated by being associated with an inhibitory cofactor, I- $\kappa$ B. When bound to NF- $\kappa$ B, I- $\kappa$ B retains the transcription factor in the cytoplasm and thereby inhibits its activity. However, phosphorylation of the cofactor by upstream kinases, called IKKs, targets I- $\kappa$ B for degradation. As a result, NF- $\kappa$ B is released and can be translocated into the nucleus where it can induce gene expression. One IKK, called IKK $\alpha$ , has been shown to interact and be activated through phosphorylation by Akt [167]. In addition, some evidence also suggests that Akt is essential for the destruction of I- $\kappa$ B, mediated through IKKs, and activation of NF- $\kappa$ B [167, 168]. NF- $\kappa$ B is known to activate transcription of the prosurvival genes c-IAP1, c-IAP2, XIAP, c-FLIP, Bcl-X<sub>L</sub> and Bcl-2 [98]. Akt is also known to increase the activity of the cyclic AMP (cAMP)-response element binding protein (CREB) transcription factor. Phosphorylation of CREB by Akt [169] results in its increased association with accessory proteins resulting in the transcription of genes containing cAMP response elements (CREs). Such target proteins include the anti-apoptotic protein Bcl-2 [170, 171] as well as increased expression of the mcl-1 gene [172].

Akt can also influence cell survival and apoptosis through the direct activation or inactivation of key regulatory proteins (**See Figure 1.3**) [159]. An example of this would be through BAD, a member of the Bcl-2 family of proteins. This family of proteins promotes apoptosis by binding to anti-apoptotic family members Bcl-2 and Bcl-X<sub>L</sub> thereby preventing their anti-apoptotic functions at the mitochondrial membrane and so promoting apoptosis. Phosphorylation of BAD by Akt results in its binding and sequestering to 14-3-3 and as a result BAD can no longer bind to Bcl-2 and Bcl-X<sub>L</sub> which can continue with their anti-apoptotic functions [173]. Although this mechanism of cell survival has been studied, it is not considered a universal mechanism by which Akt promotes cell survival as BAD is not present in all cell types [174]. In addition, some studies also link Akt in the regulation of apoptosis through procaspase-9 [175]. The initiator caspase was found to be phosphorylated by Akt, which blocked its intrinsic protease activity and hence blocked activation of the intrinsic mitochondrial apoptotic pathway [175]. However, this study was later refuted by another group who reported that procaspase-9 was not a direct substrate for Akt but that its caspase activity could be blocked by an unknown factor regulated by Akt [176].

### ***1.12.2 Cell Cycle Progression***

Each cell undergoes a sequence of events in which it replicates its DNA and then divides into two cells. This sequence of events is called the cell cycle, which is divided into 4

phases. DNA replication occurs in S phase, which followed by gap 2 (G2), mitosis and cell division occur in M phase, which is finally followed by another gap phase called G1 or gap 1 where the cell prepares to undergo the entire cell cycle over again [177]. The two gap phases are time delays that allow cells to grow and monitor internal and external conditions in order for cell cycle progression. The point at which the cell commits itself to division is referred to as the restriction point, which occurs in late G1 [177].

Akt/PKB has also been found to be involved in the regulation of cell cycle progression from G1 into S phase (**See Figure 1.4**) [159]. Progression of cells through the restriction point or the G1/S checkpoint is regulated by a protein called retinoblastoma (Rb). Rb is a major anti-proliferation protein and acts as one of the main brakes in cell cycle progression as it represses the expression of genes required to traverse the checkpoint. Phosphorylation of Rb by cyclin-dependent kinases (CDKs) inactivates its inhibitory role and allows cells to move into S-phase. CDKs require cyclins for activation as their names imply. Inactivation of Rb is achieved by kinase activity of Cdk4 and D-type cyclins, which are crucial in progression of the cycle in late G1. CDKs are themselves regulated by a family of protein kinase inhibitors of which p21<sup>CIP1</sup> and p27<sup>KIP1</sup> are important in this discussion. Akt has been found to regulate cell cycle progression by controlling the expression and activity of cyclin D1, p21<sup>CIP1</sup> and p27<sup>KIP1</sup> and therefore modulating activity of Rb phosphorylation [178].

Cyclin D1 expression can be controlled by Akt at the levels of gene transcription, mRNA translation and protein stability (See **Figure 1.4**) [159, 179-181]. Activation of Akt results in the direct downstream phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3) [182, 183]. GSK-3 has been known to play a major role in differentiation and proliferation by phosphorylating cyclin D1 and targeting it for degradation by the proteasome pathway [184]. Inactivation of GSK-3 by Akt, therefore, results in the stabilization of cyclin D1 levels in the cell and promotes cell cycle progression into S-phase. In addition, active GSK-3 also targets  $\beta$ -catenin, a protein that mediates transcription of D-cyclins, for degradation [185]. Consequently, activation of Akt promotes transcription of D-cyclins by inactivation of GSK-3, thus, preventing the degradation of  $\beta$ -catenin. Akt has also been found to phosphorylate and activate the protein kinase mTOR/FRAP [181], which upregulates protein synthesis of D-cyclins by controlling mRNA translation [186].

p21<sup>CIP1</sup>, an inhibitor of CDKs, prevents progression of the cell cycle from G1 to S-phase by binding to the CDK complexes in the nucleus and preventing them from inhibiting Rb. There are a number of mechanisms that reduce p21<sup>CIP1</sup> levels during late G1 allowing cells to move into S-phase including the Ras-ERK pathway. p21<sup>CIP1</sup> has been shown to be a direct target for phosphorylation by Akt resulting in the localization of p21<sup>CIP1</sup> into the cytosol where it can no longer inhibit CDKs [187]. Studies have also showed that

phosphorylation of p21<sup>CIP1</sup> by Akt decreases the affinity of the kinase inhibitor for the CDK complexes allowing cell cycle progression (See **Figure 1.4**) [188].

Akt also regulates p27<sup>KIP1</sup>, another kinase inhibitor, which like p21<sup>CIP1</sup> also negatively regulates CDK activity in the nucleus (See **Figure 1.4**). One way in which Akt regulates p27<sup>KIP1</sup> levels is by inactivation of the transcription factors responsible for its expression [189, 190]. These transcription factors, namely AFX, are part of the forkhead family of transcription factors, which are inactivated when phosphorylated by Akt and sequestered in the cytoplasm (discussed in Section 1.10.1) [166]. As a result, activation of the Akt pathway results in decreased levels of p27<sup>KIP1</sup> allowing G1/S transition. There are also reports that Akt can directly phosphorylate p27<sup>KIP1</sup> leading to its localization in the cytosol where it cannot inhibit CDK activity in the nucleus [191].

### **1.13 VASCULAR ENDOTHELIAL CELLS**

The cardiovascular system is made up of a branching network of blood vessels with diverse roles, including transport functions through the conveyance of blood such as the supply and removal of tissue metabolites, and transport of hormones and cells between tissues, as well as the regulation of blood pressure and fluid homeostasis [192]. The vascular endothelium fulfils a central role in these critical processes. It is made up of a single layer of cells that lines the luminal surface of all blood vessels, and therefore comes into direct contact with the blood [193]. Vascular endothelial cells have multiple functions that relate to its critical position at the interface between blood and tissue. The endothelium acts as a barrier to the passage of cells and molecules between blood and tissue [194]. It acts as an anticoagulant surface, preventing blood and platelets from clotting under normal physiological conditions. Endothelial cells also secrete multiple factors that regulate the activity of surrounding cells [195-198]. For example, endothelial cells maintain vascular tone and contribute to the mechanics of blood flow by secreting vasoactive substances that can contract or relax underlying vascular smooth muscle [195-198]. This role is critical to the regulation of blood pressure [195-198]. The endothelium has also been centrally implicated in disease processes of the cardiovascular system [194, 199]. In atherosclerosis, a disease in which the vessel wall is infiltrated by a plaque containing lipids, inflammatory cells and calcium, the endothelium regulates the accumulation of these plaque components and breaches in the endothelium or active loss of endothelial cells through apoptosis, lead to clot formation and vessel occlusion, a



major cause of cardiac and cerebral ischemia [200]. In wounds and adjacent to tumors, the process of new vessel formation, called angiogenesis, includes the processes of endothelial proliferation and regression through apoptosis [19, 201]. Formation of new blood vessels require generation of new endothelial cells by proliferation, their migration through the extracellular matrix and the eventual elimination of excessive endothelium through apoptosis [19]. These processes find their counterpart in the *de novo* generation of blood vessels during embryonic development, when a similar proliferation and paring of new vessels occurs [19]. There has been considerable interest in recent years, in the processes that regulate the balance of proliferation and apoptosis in endothelial cells under these conditions.

### ***1.13.1 PI3K pathways and the relevance to endothelial cells***

The PI3K/Akt pathway has been found to be important in controlling endothelial cell viability and survival and a variety of factors have been found to activate the PI3K/Akt pathway to promote endothelial cell survival during angiogenesis, where this survival promotes the formation of new blood vessels (**See Figure 1.5**) [159, 202]. Stimuli that promote angiogenesis such as angiopoietin-1 (Ang-1) [203, 204] and vascular endothelial growth factor (VEGF) [205] have been found to activate the PI3K/Akt pathway preventing apoptosis of endothelial cells [206]. In addition, endothelial nitric oxide synthase (eNOS) is activated through phosphorylation by Akt [207-209]. This leads to

the release of endothelial nitric oxide (NO) leading to capillary formation and protecting endothelial cells from apoptosis [210, 211]. The PI3K/Akt pathway has also been found to stabilize the transcription factor hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), via the protein kinase mTOR/FRAP [212-214]. HIF-1 $\alpha$  regulates cellular responses to hypoxia by inducing transcription of genes, such as VEGF and glucose transporters, that are involved in angiogenesis [215]. Protein stability is activated by hypoxic conditions [215], as well as insulin [212, 216] and various growth factors [212]. The PI3K/Akt pathway has also been found to be activated by factors including: insulin-like growth factor-I (IGF-I) [209], sphingosine-1-phosphate (SIP) [217, 218], hepatocyte growth factor [219], fluid shear stress [220], estrogen [221], reactive oxygen species [222] and corticosteroids [223]. By implication, factors that inhibit the PI3K/Akt pathway can promote endothelial apoptosis. Examples include members of the TNF receptor superfamily, including FasL [224], and most recently TRAIL itself [225].

### ***1.13.2 The roles of TRAIL in endothelial cells***

TRAIL has been shown to induce apoptosis in malignant cells without affecting normal cells [8, 13, 41] supporting its potential as a cancer therapy. Much less is known about the role of TRAIL in normal cell physiology, especially vascular endothelial cell biology. TRAIL itself has been found to be expressed by vascular smooth muscle [34]. In addition, expression of TRAIL receptors has also been reported on the surface of human

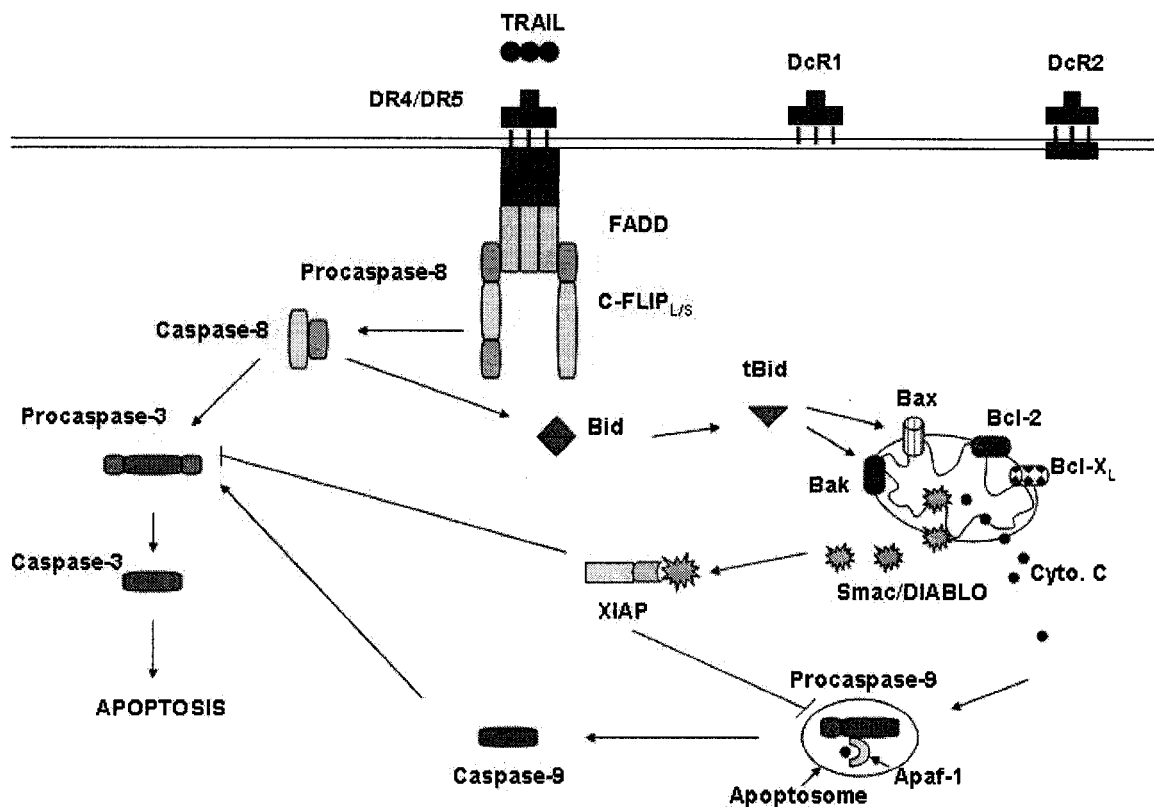
umbilical vein cells (HUVECs) including DR4 [18, 226], DR5 [18, 226, 227], DcR1 [18, 226] and DcR2 [18].

HUVECs appear to be resistant to TRAIL-induced apoptosis [8, 18, 226, 228, 229], however the findings of these groups was recently contradicted in a report by Li *et al* [227]. As mentioned earlier, the form of TRAIL and the method in which it is prepared plays an important role in the outcomes of apoptosis experiments. Numerous studies have shown that non-tagged versions of TRAIL produced in the presence of stoichiometric Zn are not toxic to normal cells including hepatocytes, keratinocytes and HUVECs. However a non-tagged form of TRAIL was found to directly induced apoptosis in HUVECs as well as human dermal microvascular endothelial cells (HDMECs) [227]. Activation of the NF- $\kappa$ B pathway was detected and TRAIL was found to induce inflammatory gene expression including that of E-selectin, ICAM-1 and IL-8 expression and leukocyte adhesion [227]. However, there are recent indications that the particular form of non-tagged TRAIL used in this study and supplied by R&D systems has a tendency to form aggregates, and effectively behaves as though it were tagged (unpublished data). In addition, aggregated and tagged forms of TRAIL have also been found to induce apoptosis in normal cells. This is contrasted by a recent study that did not observe TRAIL-induced apoptosis using a polyhistidine-tagged version of TRAIL [18]. However, this group did observe activation of the PI3K/Akt pathway by TRAIL as well as inactivation of this pathway by trophic withdrawal [18]. When PI3K was inhibited with the inhibitor LY294002 this rendered the endothelial cells sensitive to

TRAIL-induced apoptosis [18]. Additionally, proliferation of TRAIL-treated endothelial cells, in the absence of inhibitor, was also observed which was attributed to activation of the ERK1/2 pathway but not p38 or the JNK pathways [18]. As opposed to the results published by Li *et al*, this group did not see activation of the NF- $\kappa$ B pathway or an increase in expression of inflammatory genes such as E-selectin, ICAM-1, or VCAM-1 in TRAIL stimulated cells [18]. TRAIL was also found to phosphorylate eNOS resulting in activation of the enzyme and increased NO synthesis [230]. NO plays an important role in blood vessel dilation as it mediates underlying vascular smooth muscle relaxation through cGMP levels. In addition, TRAIL was also found to increase prostacyclin production although activation of the NF- $\kappa$ B nor cyclooxygenase 2 (COX-2) pathways was not detected [230].

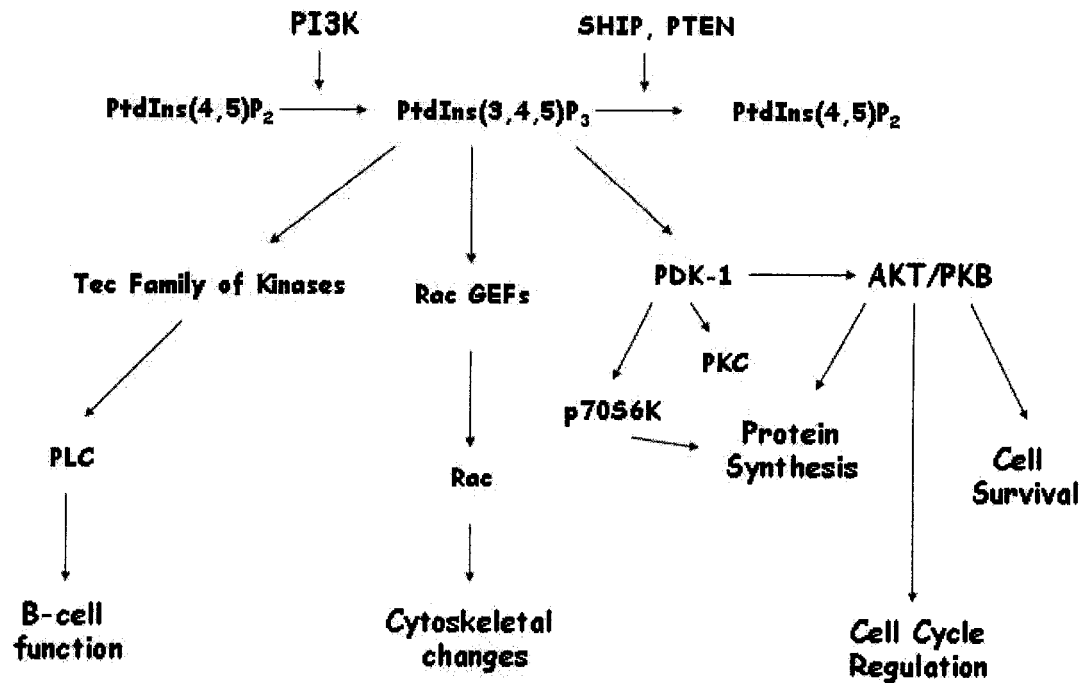
### ***1.14 SUMMARY***

TRAIL has been shown to induce apoptosis in cancer cells without harming normal cells supporting its potential as a cancer therapy. However, the normal physiological roles of TRAIL on the human vascular endothelium are not well studied. Endothelial apoptosis plays important roles in both physiological and pathological processes including atherosclerosis and angiogenesis. The phosphatidylinositide-3 kinase (PI3K)/Akt pathway is of central importance to the vascular endothelium during angiogenesis, where it regulates endothelial cell survival, differentiation and migration. Therefore, further study of this pathway, and its potential to modulate TRAIL-induced apoptosis in vascular endothelial cells is warranted.



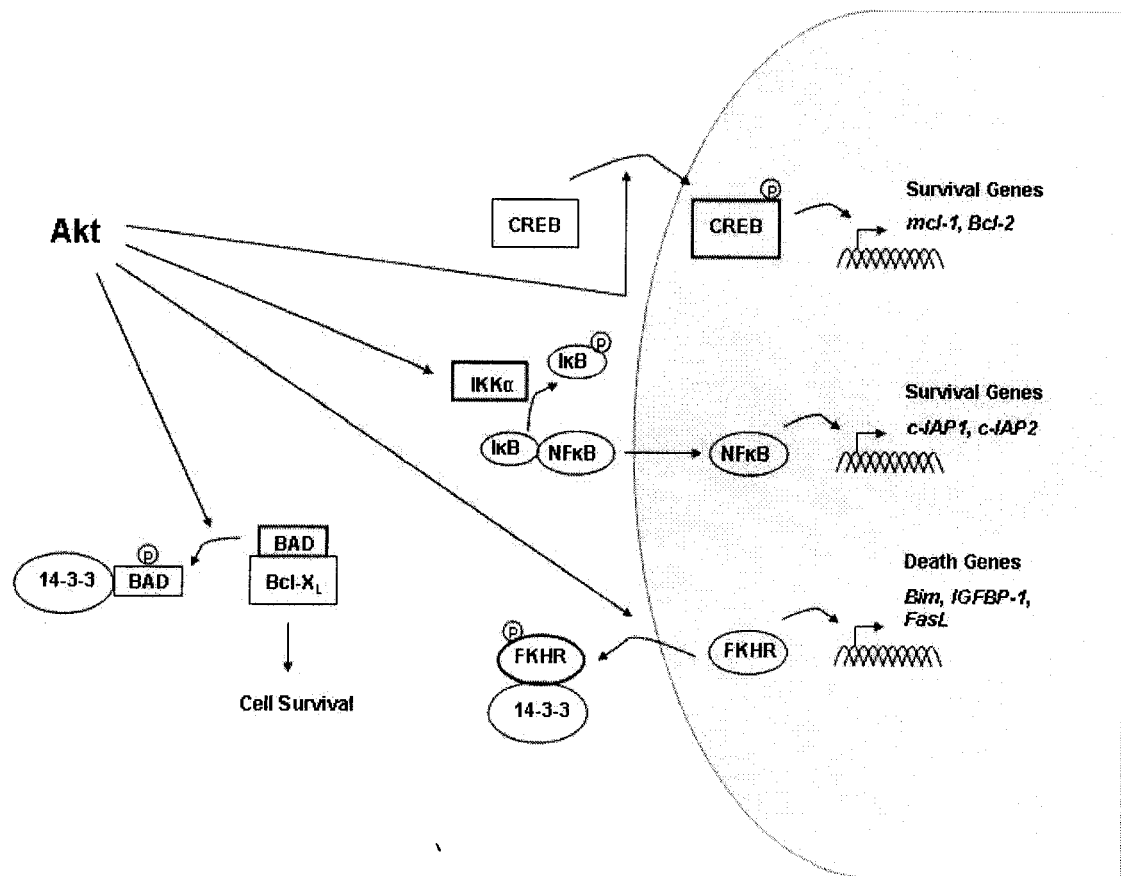
**Figure 1.1 TRAIL-induced apoptosis through the extrinsic and intrinsic pathways.**

The diagram illustrates the signal transduction events following TRAIL-induced apoptosis through the death receptor (DR4/DR5)-mediated extrinsic pathway and the mitochondrial intrinsic pathway. Adapted from Hao *et al*, 2004.



**Figure 1.2 Downstream events of the PI3-kinase pathway.**

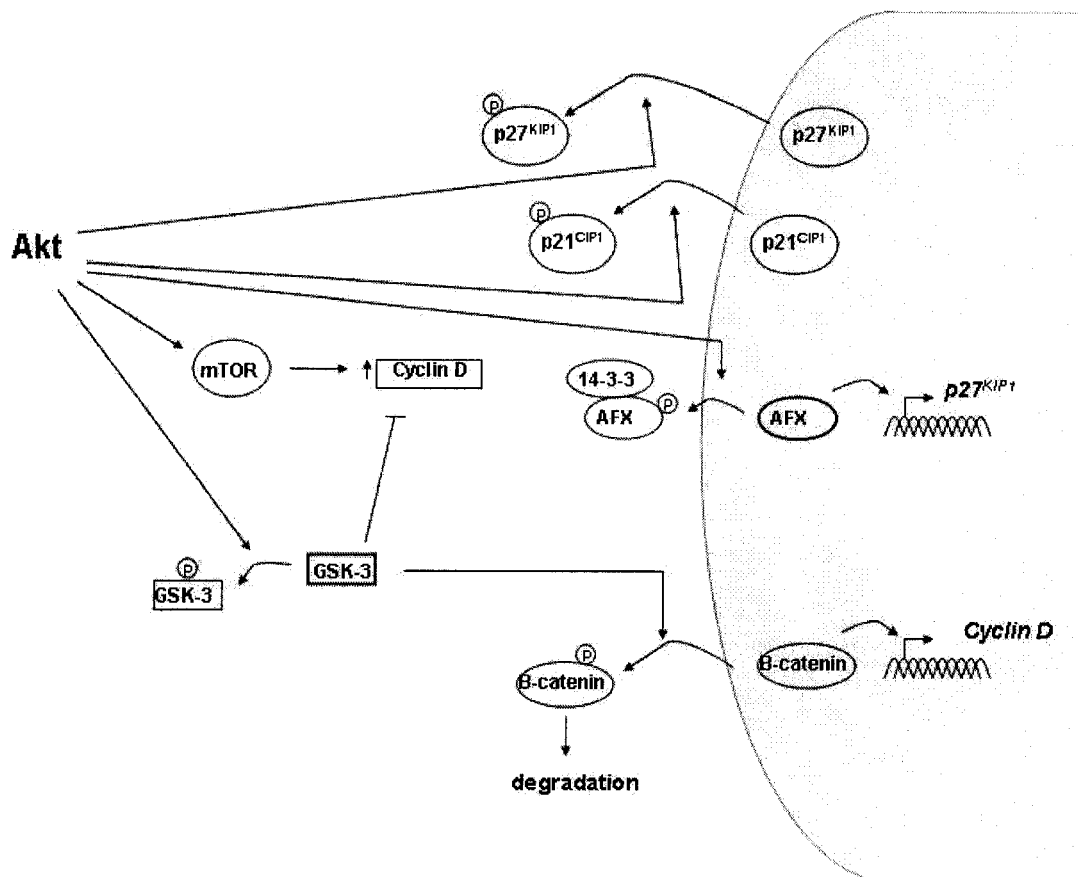
Activation of PI3K pathway induces downstream signaling events that affect cell survival, cell growth and cell movement. PI3K can be activated by a variety of stimuli such as growth factors, integrin-dependent cell adhesion and by G-protein couple receptors. Lipid product of PI3K activation, phosphatidylinositol -3,4,5-triphosphate PtdIns(3,4,5)P<sub>3</sub> recruits proteins with pleckstrin homology (PH) domains to the plasma membrane where they are activated. Ultimately, these proteins can go on to influence actin polymerization, protein synthesis, cell growth and survival and cell cycle entry. Figure adapted from Cantrell *et al*, 2001.



**Figure 1.3 Downstream Akt targets involved in pro-survival.**

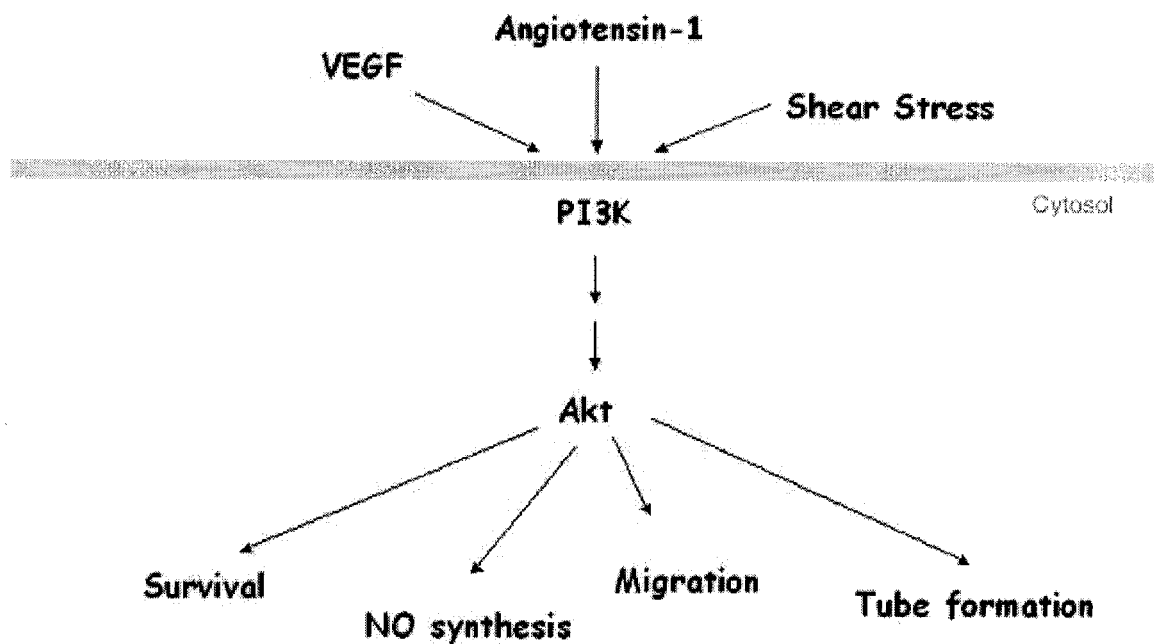
Upon activation by PI3K, Akt/PKB regulates cell survival through three mechanisms. Direct phosphorylation of BAD by Akt inhibits BAD from binding to Bcl-X<sub>L</sub> and allows it to inhibit activation of the intrinsic pathway. Activation of CREB, via phosphorylation, stimulates transcription of pro-survival genes for Bcl-2 and Mcl-1. Phosphorylation of the forkhead family of transcription factors, inhibits their translocation into the nucleus due to cytoplasmic sequestering by 14-3-3 and thus prevents transcription of pro-apoptotic genes for Fas-L, Bim and IGFBP-1. Akt can also activate transcription factor NF-κB, by phosphorylating IKKα which relieves inhibition on NF-κB in order to transcribe pro-survival genes. Adapted from Nicholson *et al*, 2002.





**Figure 1.4** Role of Akt on cell cycle entry.

Activated Akt regulates cell cycle progression by increasing cyclin D levels and decreasing activity of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>. Cyclin D expression is increased by transcription through inactivation of GSK-3 and activation of cyclin D translation through phosphorylation and activation of mTOR. p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are directly phosphorylated by Akt resulting in their localization to the cytosol in order to prevent Cdk inhibition in the nucleus. Inactivation of forkhead family transcription factor AFX, via phosphorylation by Akt, also inhibits transcription of p27<sup>Kip1</sup>. Adapted from Nicholson *et al*, 2002.



**Figure 1.5** Activation of PI3K and Akt pathway in endothelial cells.

The PI3K/Akt pathway is responsible for activating a number of pathways in endothelial cells in order to stimulate angiogenesis. Angiogenic stimuli activate the PI3K/Akt pathway and activate endothelial cell survival, migration, tube formation and NO production required for angiogenesis. Adapted from Shiojima *et al*, 2002.

## **CHAPTER 2. MATERIALS AND METHODS.**

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### **2.1 ISOLATION AND CULTURING OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECS)**

Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated as described previously [231]. Fresh umbilical cords were obtained from patients at the Royal Alexandria Hospital (Edmonton, Alberta) and were used within the first two days of delivery. The umbilical vein was slowly perfused (flow rate of 1 drop per second) with 10 - 15 ml of Dulbecco Phosphate Buffered Saline (DPBS) (Invitrogen, Burlington, ON 14190-144) before being treated with 0.1% collagenase solution (Worthington Biochemical Corporation, Lakewood, NJ, LS4197, Lot S2C5490) for 13 min at 37°C. The collagenase digest was collected by rinsing the umbilical vein with 10 ml of DPBS into 5 ml of fetal bovine serum (FBS) (Invitrogen 16000-044). Cells were separated from the digest solution by centrifugation (1000 x g) and were cultured on plastic tissue culture T25 and T75 cm<sup>3</sup> flasks (Corning, NY) coated with 0.1% gelatin (Sigma G1890) and grown in M199 medium (Invitrogen 11100-059) (supplemented with 20% fetal bovine serum (FBS), 2mM L-glutamine (Invitrogen 25030-081) and 1% penicillin/streptomycin (Invitrogen 15070-063), 100 µg/ml heparin (Sigma H3149) and 100 µg/ml endothelial cell growth supplement (Becton Dickinson CACB356006). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After the initial isolation (P<sub>0</sub>), once

the cells had attached they were rinsed three times with Hanks Balanced Salt Solution (HBSS) (Invitrogen 14170-112) (within the first 24 hours of isolation) in order to remove contaminating red blood cells that were collected during the isolating procedure. For harvesting cells, 0.25% Trypsin/EDTA (Invitrogen 25200-056 and diluted with HBSS) was added for 5 minutes or until cells detached in sufficient numbers. This harvesting procedure was always used except when cells were used for flow cytometry in order to maintain cell surface membrane integrity (see Section 2.2.1). HUVECs were also plated on 0.1% gelatin coated 96-well plates with  $1.5 \times 10^4$  viable cells/well for the acid phosphatase assay and on coated 6-well plates with a  $1.0 \times 10^5$  viable cells/well for siRNA gene silencing experiments. During experiments, HUVECs were transferred to a reduced or low serum medium made of M199 with 1% FBS, 2mM L-glutamine, and 1% antibiotics and treated with TRAIL and LY294002 either alone or in combination or left untreated upon immediate transfer of cells into the reduced serum medium.

## **2.2 ANTIBODIES AND REAGENTS**

Human recombinant, non-tagged soluble TRAIL (amino acids 114-281) was purchased from PeproTech (Rocky Hill, NJ). The PI3K inhibitor LY 294002 (Cell Signalling 9901) was prepared as a 50  $\mu$ M stock in DMSO (Sigma). Protein quantification reagents for Bradford Assay were purchased from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). The following mouse anti-human monoclonal antibodies were used: FADD (610399), cytochrome *c* (556433), XIAP (610762) (Transduction Laboratories, Lexington, KY), caspase-8 (M032-3) (MBL, Watertown, MA, USA) and c-FLIP NF6 (ALX-804-428) (Alexis Biochemicals, San Diego, CA). Rabbit anti-human polyclonal antibodies used were: caspase-3, ERK1/2 (Stressgen, Victoria, BC), caspase-9 (9502), Akt (9272), Phospho-Akt (9271) (Cell Signalling), Bax (sc-493), Bcl-2 (sc-492) (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-X<sub>L</sub> (556361) (Transduction Laboratories), Bak (06-536) (Upstate Biotechnology, Lake Placid, NY) and Bid (44-433) (Biosource, Camarillo, CA). Flow cytometry antibodies were phycoerythrin (PE)-conjugated anti-human TRAIL-R1/DR4, -R2/DR5 (BD Pharmingen, San Diego, CA), -R3/DcR1 (12-6238) and -R4/DcR2 (12-6239) (eBioscience, San Diego, CA), and IgG<sub>1</sub> isotype control (555749) (BD Pharmingen). HRP-conjugated goat anti-rabbit antibody (Jackson, West Grove, PA) and HRP-conjugated goat anti-mouse IgG<sub>2b</sub> antibody (Southern Biotechnology, Birmingham, AL) were used as secondary antibodies.

### **2.3 FLOW CYTOMETRY**

Flow cytometry measures the light intensity of a cell or particle. This light can either be the laser light being scattered as it shines through cells or it can be fluorescent light emitted from a fluorochrome [232]. Forward and side light scatter from a laser allows one to determine the size and granularity of cells passing through the laser and therefore gives information about the population of cells such as granularity, size and viability [232]. In most cases, however, flow cytometry is used to measure and analyze cellular components and function that can be detected with fluorescent compounds [232]. For the purposes of this study flow cytometry was used to study cell surface receptor expression and cell cycle progression (See Appendix).

Surface receptor expression can be measured by obtaining antibodies against the receptors of interest conjugated to fluorochromes such as phycoerythrin (PE) [232]. Cells that express the receptor of interest on their surface will bind the PE-conjugated antibody which can be detected by the cytometer as the cells pass through. The fluorescence can be quantitated and compared to the fluorescence obtained from the negative control in which a non-specific, isotype matched PE-conjugated antibody is used.

Surface expression of TRAIL-R1/DR4, R2/DR5, R3/DcR1 and R4/DcR2 was analyzed by flow cytometry with antibodies specific for surface epitopes of the receptors. Cells

were grown to 90% confluency before being transferred to fresh growth medium or the reduced serum medium for 6 hours. After incubation the cells were harvested in 10 ml of PBS containing 0.04% EDTA and then divided into tubes containing  $10^6$  cells per 1 ml of PBS. The usual method of harvesting HUVECs using 0.25% Trypsin/EDTA was not used in order to maintain the native state of the surface receptor.

Phycoerythrin (PE) -conjugated anti-human antibody (0.1  $\mu\text{g/ml}$ ) for each receptor was added to  $10^6$  cells in 200  $\mu\text{l}$  of immunofluorescence (IF) buffer (phosphate buffered saline (PBS), 2% FBS and 0.2% sodium azide) and incubated in the dark at  $4^\circ\text{C}$  for 1 hour. In addition, a non-specific isotype-matched negative control PE-conjugated antibody was also added to  $10^6$  cells at a concentration of 0.1  $\mu\text{g/ml}$  and incubated in the conditions stated previously. After three washes with fresh IF buffer in order to remove any non-specifically bound antibody, the cells were resuspended in 500  $\mu\text{l}$  of PBS and 10,000 cells were analyzed using BD FACScan and the resulting data processed using Cell Quest software (Becton Dickinson, Mountain View, CA). Healthy cells were determined from side and forward scattered histograms and gated before being subsequently analyzed for fluorescent intensity using FLH-2 detector for PE. Fluorescent activity of each antibody was compared to that of the negative control and were plotted against each other on contour plots.

## **2.4 ASSESSMENT OF CELL NUMBER UTILIZING ACID PHOSPHATASE ASSAY**

Endothelial cell numbers were assessed by the acid phosphatase assay as described previously [233]. This assay utilizes the cells' own lysosomal acid phosphatase enzyme to assess cell number by relating enzymatic activity to cell numbers [234]. The native enzyme is used to remove phosphate esters from compounds and release them into cells to be used as building blocks for new molecules. However, because the enzyme can also catalyze the hydrolysis of phosphate esters on many different substrates the use of artificial phosphate esters, which produce chromagenic products when cleaved by the enzyme, are commonly used in laboratory techniques that measure enzyme activity [234]. In this case, the substrate for the reaction is p-nitrophenylphosphate (pNPP) which after ester hydrolysis by the enzyme releases p-nitrophenol in addition to an inorganic phosphate [234]. The reaction is stopped by the addition of NaOH at a given time, which reacts with the p-nitrophenol product by removing the phenolic proton to produce p-nitrophenolate, a yellow colored product that absorbs at 405 nm and therefore can be quantitated [234].

In performing the assay,  $1.5 \times 10^4$  cells/well were seeded on 96-well plates overnight, as mentioned previously, and treated the following day (at about 50% confluency) with 100 $\mu$ l of reduced serum medium containing TRAIL and LY294002 as indicated in the Results. After treatment, the medium was removed and the cells were washed once with



200  $\mu$ l of PBS and then 100  $\mu$ l of buffer solution containing 0.1 M Na-acetate [pH 5.5], 0.1% (v/v) Triton X-100 and 10 mM *p*-nitrophenyl phosphate (104 phosphatase substrate pNPP) (all from Sigma) in order to make the cells permeable to the pNPP substrate solution. Following incubation for 1 hour at 37°C, 10  $\mu$ l of 1 M NaOH was added to each well to stop the reaction and the developed absorbance measured with a microplate reader (Bio-Rad, Mississauga, ON) at 405 nm. During calibration, a linear relationship was obtained between cell numbers and enzyme activity over a range of  $10^3$ - $10^5$  cells ( $R^2= 0.996$ ) and the optimum plating density of cells was determined to be  $1.5 \times 10^4$  cells/well for maximum sensitivity of the assay. All data is expressed as cell numbers normalized to the control obtained with untreated cells, and expressed as a %.

## 2.5 STATISTICS

For acid phosphatase experiments, results are presented as means  $\pm$  SEM of eight separate experiments. The differences between mean values was tested by one-way analysis of variance (ANOVA) followed by the Bonferroni *t*-test (SigmaStat, SPSS, Chicago, IL).  $P < 0.05$  was taken as significant.

## 2.6 CELL LYSATES

### 2.6.1 *Subcellular fractionation*

Subcellular organs such as the endoplasmic reticulum (ER), Golgi body and mitochondrion can be separated from the rest of the cellular components based on their size, shape and density by a technique called differential centrifugation. Differential centrifugation involves centrifuging cell homogenates at progressively higher speeds to fractionate cell lysates into their components on the basis that larger heavy organelles or components will sediment faster than small light organelles [235].

Sub-confluent HUVEC (about 50%) were treated as indicated in the Results with TRAIL and LY294002, harvested with 0.25% Trypsin/EDTA, and the resulting pellet suspended in 5 volumes of isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.5), 1mM Na- EDTA) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (PIC) mixture (all from Sigma). After incubation on ice for 15 minutes, the cells were passed 15 times through a 22-gauge needle and then centrifuged at 800g for 10 minutes at 4°C in order to pellet the nuclear fraction, which contains nuclei, some mitochondria, plasma membrane fragments as well as any unbroken cells and debris. The resulting supernatant was collected and centrifuged at 13,000g for 10 minutes at 4°C in order to pellet the heavy fraction

containing predominately mitochondria. The resulting supernatant contained the soluble portion of the cellular cytoplasm and was designated the cytosolic fraction. After determining the protein concentration of the cytosolic fractions using the Bradford Assay (see Section 2.6), the fractions were subjected to Western blot (see Section 2.7) analysis to determine the release of mitochondrial proteins, Smac/DIABLO and cytochrome *c* into the cytosol.

### ***2.6.2 Whole-cell lysates***

Sub-confluent HUVEC (about 50%) were treated as indicated in the Results with TRAIL and LY294002, harvested with 0.25% Trypsin/EDTA and lysed through vigorous pipetting in ice cold lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with 1mmol/L PMSF and 0.2% PIC (all from Sigma). Lysates were centrifuged at 18,000g for 15 minutes at 4°C in order to remove components of the plasma membranes as well as any unbroken cells and debris. The resulting supernatants of whole cells lysates were used in Western blot analysis (see Section 2.7) after determination of protein concentration using the Bradford Assay (see Section 2.6).

## **2.7 PROTEIN QUANTIFICATION USING THE BRADFORD ASSAY**

Protein concentrations of the lysates were determined using Bradford Assay purchased from Bio-Rad. The Bradford Assay consists of a protein binding dye, Coomassie Brilliant Blue G-250, which has a shift in absorbance maximum from 465 nm to 595 nm upon binding protein [236-239]. The anionic form of the dye binds proteins primarily at arginine as well as at tryptophan, tyrosine, histidine and phenylalanine residues through Van der Waals forces and hydrophobic interactions [237]. Upon binding protein, the absorbance of the dye shifts to 595 nm and therefore the amount of protein can be quantitated by measuring the absorbance of the resulting solution at 595 nm and determining the protein amount using a standard curve. A standard curve is obtained by measuring the absorbance of known amounts of a standard protein.

In performing the protein assay, standards were set up containing 5, 10 and 20  $\mu\text{g}$  of BSA in 1 ml of dye reagent to obtain the standard curve. Duplicates of the lysates were also set up where 2  $\mu\text{l}$  of the lysate was added to 1 ml of dye reagent. The protein concentration of the lysates were obtained by reading off the protein amounts from the standard curve.

## 2.8 WESTERN BLOT ANALYSIS

SDS-polyacrylamide gel electrophoresis is a method used to separate proteins based on their molecular weight. Cell lysates are loaded to a support matrix of polyacrylamide and the proteins are separated based upon their size as they move through the porous matrix in an applied electric field. The anionic detergent, SDS, is used in order to denature proteins as well as uniformly coat them with negative charges so that proteins are able to move through the electric field and be separated based on their molecular weight not on their native charge. Differences of pH in the polyacrylamide gel allow proteins to be resolved into a single band in the top stacking portion of the gel, and then be separated based on their molecular weight in the bottom separating gel [240].

The proteins separated by gel electrophoresis can be detected using Western blot analysis. This technique involves transferring the proteins separated on the polyacrylamide gel onto a nitrocellulose membrane using an electric field. The proteins, which are now immobilized, can be detected by using a double-antibody, enzyme linked system that can be detected using a chemiluminescent substrate [241].

Equal amounts of protein from cells lysates (50 or 100  $\mu\text{g}$ ) and cytosolic fractions (20 $\mu\text{g}$ ) were separated by SDS-polyacrylamide gel electrophoresis using Bio-Rad's Mini-PROTEAN 3 system with 15% separating gels and 3.5% stacking gels in 1X Running

Buffer for 1.5 hours at 150 V. The separated proteins were then transferred to nitrocellulose membranes using Mini Trans-Blot Cell from Bio-Rad at 30V for 960 min. After blocking unoccupied regions of the membrane in 5% milk in Tris buffered saline (TBS) with 0.05% Tween-20 (TBST) (Sigma), the membranes were blotted overnight with various primary antibodies as indicated in the Results. Following three successive washes (10, 10, 15 min), the membranes were incubated for 1 hour with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies. After incubation with the secondary antibody, the membranes were again subjected to three successive washes (15, 15, 25 min) and developed by ECL Western Blotting Detection Reagents (Amersham Biosciences) on Kodak BioMax MR Film.

## **2.9 GENE SILENCING USING SMALL INTERFERING RNA (siRNA)**

RNA interference is a recently developed method of gene silencing that introduces double stranded RNA (dsRNA) into cells resulting in post-transcriptional gene silencing. The small interfering RNAs (siRNA) consist of 21-23 nucleotide (nt) double stranded RNA with symmetric 2 nt 3' overhangs [242]. Upon transfection into mammalian cells, they are incorporated into the RNA-induced silencing complex (RISC), a nuclease complex that cleaves nucleic acid, which targets mRNA complementary to the siRNA for cleavage leading to silencing of that gene [242-245].

Four different synthetic siRNAs (PI3-K1, PI3-K2, PI3K-3, and PI3K-4) for sequences corresponding to the p110 $\beta$  subunit of class IA PI3-kinases (GeneBank number 006219) were purchased from Qiagen (Mississauga, ON). Single transfections were carried out according to manufacturer's protocol, using TransMessenger Reagent (Qiagen). Initially, each lyophilized siRNA product was resuspended in siRNA Suspension Buffer to give a working stock concentration of 1  $\mu\text{g}/\mu\text{L}$ . This was followed by heating the tubes to 90  $^{\circ}\text{C}$  for 1 min and then incubating them at 37  $^{\circ}\text{C}$  for 60 min in order to disrupt any aggregates to ensure optimal transfection efficiency.

The day before transfection,  $1.0 \times 10^5$  viable cells were plated per well on 0.1% gelatin coated 6-well plates and were allowed to grow in normal growth medium for 24 hours



before being treated with siRNA at a confluency of 30%. siRNA complex formation was achieved by adding 2 µg of each siRNA to 4 µL of Enhancer-R in 92 µl of Buffer EC-R and 8µl of TransMessenger Transfection Reagent (all provided by the manufacturer). The complex mixture was mixed with 100 µl of medium without serum or antibiotics and applied dropwise to subconfluent cells (30-50%) contained in 1.1 ml serum-free and antibiotic-free medium. The siRNA complex was incubated for 3 hours at 37°C with the siRNA complex, the cells were returned to normal serum medium for 24 hours, followed by treatments in reduced serum medium (1% FBS), as indicated in the Results.

The synthetic siRNA sequences used are:

PI3-K1      5'-r(GCUCAUCGUAGCUGUUCAU)d(TT)-3' sense

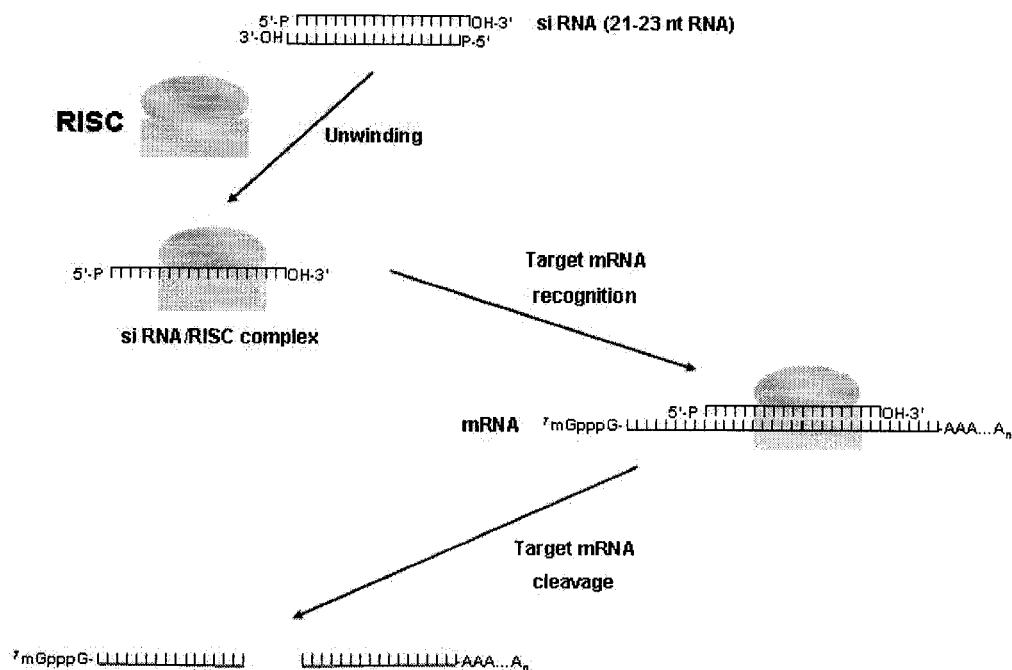
5'-r(AUGAACAGCUACGAUGAGC)d(TT)-3' anti-sense

PI3-K2      5'-r(GCAGUGAUAGUGCUGAAUGU)d(TT)-3' sense

5'-r(ACAUUAGCACUAUCACUGC)d(TT)-3' anti-sense

PI3-K3      5'-r(CUGAAUGCCGUGAAGUUAA)d(TT)-3' sense  
                 5'-r(UUAACUUCACGGCAUUCAG)d(TT)-3' anti-sense

PI3-K4      5'-r(AGGGAGCGAGUGCCUUUUA)d(TT)-3' sense  
                 5'-r(UAAAAGGCACUCGCUCCCU)d(TT)-3' anti-sense



**Figure 2.1 Model of siRNA gene silencing mechanism.**

Small interfering RNAs (siRNAs) are transfected into cells, where they interact with the nuclease complex, RNA-induced silencing complex (RISC). In the RISC, siRNAs are unwound by the helicase activity of the complex and target complementary mRNA, through base pairing, for cleavage and degradation by the RISC complex. Adapted from Wall *et al*, 2003 [246]

## **CHAPTER 3. RESULTS.**

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### ***3.1 TRAIL RECEPTOR EXPRESSION ON THE SURFACE OF HUVECS***

TRAIL receptor expression was examined by flow cytometry in the low serum conditions. HUVECs were found to express TRAIL-R2/DR5 and TRAIL-R3/DcR1 as indicated by an increase in fluorescent intensity when the receptor-specific labelling was compared to isotope matched control antibody. In contrast, minimal expression of TRAIL-R1/DR4 or TRAIL-R4/DcR2 was seen under low serum conditions. **(See Figure 3.1)**. Therefore HUVECs were found to express the functional death receptor DR5 and the putative decoy receptor DcR1, but did not demonstrate surface expression of DR4 or DcR2.

## 3.2 TRAIL-INDUCED APOPTOSIS

### *3.2.1 HUVECs increase in cell number with TRAIL treatment*

Since the functional TRAIL death receptor TRAIL-R1 was identified on the surface of HUVEC, the cells were examined for their responses to exogenously applied TRAIL. Treatment with TRAIL for 24 hours (3, 30, 100 and 300 ng/ml) which commenced immediately following transfer of cells into reduced serum medium (1% FBS), significantly increased cell numbers to a maximum of 140 % compared to untreated controls (See **Figure 3.2A**). This suggests that TRAIL may stimulate endothelial growth and also confirmed that the recombinant soluble version of TRAIL used did not induce apoptosis in the endothelial cells. The same treatments with TRAIL were carried out in 20% serum conditions for 24 hours, with no increase in cell number compared to untreated cells (See **Figure 3.2B**).

### ***3.2.2 Endothelial cell numbers decrease with LY294002 and TRAIL***

To study how we could sensitize endothelial cells to TRAIL induced apoptosis and determine the mechanisms of apoptosis, we employed LY294002, an inhibitor of the phosphoinositide 3-kinase pathway, previously shown to sensitize endothelial cells to FasL and TRAIL induced apoptosis [18, 247]. The phosphoinositide 3-kinase (PI3K) pathway is an important pathway that stimulates cell growth, cell cycle entry, cell migration and cell survival in response to extracellular stimuli [100].

Initially, these experiments were carried out in flasks so that any apoptosis could easily be seen with the naked eye under phase contrast microscopy. Cells treated with the combination of LY294002 and TRAIL did undergo apoptosis when cells were treated with increasing concentrations, 5  $\mu$ M and 20  $\mu$ M, of LY294002 and TRAIL (100 ng/ml) upon immediate transfer into the low serum containing medium. Apoptotic cells are identified from rounding and surface blebbing when viewed by phase contrast microscopy. The combination treatment did not induce apoptosis in cells that were treated in normal serum containing medium. There was a significant increase in apoptotic cells compared to controls, when cells were pre-treated with 1% serum medium for 6 hours before applying TRAIL/LY294002 for a further 6 hours. However if cells were pre-treated for 18 hours followed by 6 hours combined treatment, both treated and control cells showed indistinguishably high levels of apoptosis. This indicated that serum

deprivation itself induces apoptosis and thus remaining experiments were limited to a maximum 6 hours of treatment with reagents applied upon immediate transfer into the low serum medium.

Because cell death was seen when the cells were treated with LY294002 and TRAIL in combination, an acid phosphatase assay was conducted in order to quantify the effect in terms of cell numbers.

### ***3.2.3 Acid phosphatase assay to determine cell death with LY 294002 and TRAIL.***

Using the same conditions as in the previous experiments (See Section 3.2.2), HUVECs were then treated with TRAIL (100 ng/ml) together with increasing concentrations of the PI3K inhibitor LY294002 [132] (5, 20  $\mu$ M) for 6 hours in reduced serum medium in 96-well plates (See Figure 3.3). Control wells were left untreated but exposed to reduced serum medium alone for 6 hours. The combination treatment of LY294002 (5 and 20  $\mu$ M) and TRAIL for 6 hours resulted in a significant ( $P < 0.001$ ) decrease in cell number of almost 60% with the higher dose (20  $\mu$ M) of LY294002. There were no significant differences in cell number between untreated controls and wells treated with the two doses of LY294002 (5 and 20  $\mu$ M) alone indicating that the decrease in cell number was not attributable to the inhibitor. These results indicate that the combination treatment of

LY294002 and TRAIL significantly reduced cell numbers suggesting that PI3K inhibition sensitizes HUVECs to TRAIL killing.



### **3.3 TRAIL-INDUCES APOPTOSIS THROUGH BOTH THE EXTRINSIC AND INTRINSIC PATHWAYS**

#### ***3.3.1 Activation of the extrinsic pathway***

To determine whether the reduction in cell numbers caused by TRAIL in combination with LY294002 reflected the induction of apoptosis, investigations were carried out to detect activation of apoptosis by both the extrinsic and intrinsic pathways.

The extrinsic pathway (discussed in more detail in Section 1.6.1) is activated by trimerization of the death receptors upon binding TRAIL resulting in the recruitment of the adaptor protein, FADD and caspase-8 to form the death-inducing signaling complex (DISC). In the DISC, caspase-8 is activated from its procaspase form and can go on to activate caspase-3, an effector caspase, to propagate apoptosis through the extrinsic pathway.

The extrinsic pathway was examined by detecting cleavage of procaspase-8 and procaspase-3 into their active forms. Caspase-8 cleavage in the DISC occurs in 2 consecutive steps. The first cleaves p55/p53 precursors into p43, p41 and p12 subunits. The p12 subunit is processed to an active p10 fragment. Second step cleavage of p43/p41

releases an active p18 fragment [65]. Caspase-3 p32 precursor is cleaved into p20, p17 and p10 active subunits which execute apoptosis [67]. Cells were treated in combination with 100 ng/ml TRAIL and 20  $\mu$ M LY 294002, in reduced serum medium for 1, 3 and 6 hours, and whole cell lysates examined for caspase -8 and -3 cleavage products on Western blots (**See Figure 3.4**). Caspase-8 p18 cleavage subunits were first detected at 3 hours, and to a greater extent at 6 hours. Caspase-3 p32 precursor was cleaved into active p20 and p17 subunits, detectable at 3 hours and increasing at 6 hours.

### ***3.3.2 Activation of the intrinsic pathway***

The intrinsic pathway (discussed in detail in Section 1.6.2) can be independently activated through intracellular mechanisms that detect DNA damage and can also be activated by the extrinsic pathway in order to amplify apoptotic signals from the DISC. Activated caspase-8 can cleave Bid to form truncated Bid (tBid) which can move to the mitochondrion and together with pro-apoptotic proteins, Bax and Bak, result in the release of cytochrome *c* and Smac/DIABLO into the cytosol. The two factors can then go on to activate caspase-9 from its procaspase form. Once activated, caspase-9 can go on to activate caspase-3 reinforcing apoptosis through the intrinsic pathway.

The intrinsic mitochondrial pathway was examined by detecting release of cytochrome *c* and Smac/DIABLO from mitochondria into the cytosol, with downstream cleavage of

caspase-9 (**See Figure 3.5**). Smac/DIABLO and cytochrome *c* release were detected in the cytosolic fraction, which was obtained through subcellular fractionation, at 3 and 6 hours. Downstream activated caspase-9 p35 and p37 cleavage products were detected at 3 and 6 hours. Caspase-9 cleavage products and release of Smac/DIABLO and cytochrome *c* were not detected in cells treated for 6 hours with 100ng/ml TRAIL or 20  $\mu$ M LY 294002 alone indicating there was no activation of the intrinsic pathway by either the inhibitor or TRAIL treatment alone.

Additionally, caspase-3 cleavage and activation was not detected in cells treated in combination with 100ng/ml TRAIL and 20 $\mu$ mol/L LY294002 in normal serum (20% FBS, **See Figure 3.6**). These data confirm that TRAIL alone does not induce apoptosis in normal or reduced serum conditions, and extend this observation to show activation of the extrinsic and intrinsic pathways with inhibition of PI3-kinase in reduced serum but not normal serum medium.

### **3.4 EFFECTS OF LY294002 ON REGULATORY PROTEIN EXPRESSION**

#### ***3.4.1 Regulatory Proteins in the DISC***

Regulation of TRAIL-induced apoptosis occurs at the levels of both the DISC and the mitochondrion. In the DISC, TRAIL-induced apoptosis can be modulated by changes in levels of proteins necessary to initiate the signaling cascade. These proteins can include the adaptor protein FADD, caspase-8 (discussed in Section 1.6.1) and the inhibitor of apoptosis, c-FLIP (discussed in more detail in Section 1.7.1). c-FLIP inhibits apoptosis at the level of the DISC by competing with caspase-8 for binding to FADD as well as inhibiting the protease activity required for caspase-8 activation in order to initiate apoptosis.

To determine how LY294002 predisposes HUVECs to TRAIL killing, proteins that regulate apoptosis at the level of the DISC were analyzed by Western blotting, in cells left untreated or treated with 20  $\mu$ M LY294002 for 1, 3 and 6 hours (See **Figure 3.7**). Levels of FADD and caspase-8, which initiate apoptosis in the DISC, were unchanged (See **Figure 3.7**). In contrast, levels of both the long (c-FLIP<sub>L</sub>) and the short (c-FLIP<sub>S</sub>) forms of c-FLIP proteins were decreased with LY294002 treatment in a time-dependent manner. The level of c-FLIP<sub>L</sub> decreased at 6 hours, while c-FLIP<sub>S</sub> was undetectable at 3

and 6 hours (See **Figure 3.7**). This indicates that LY 294002 sensitizes HUVECs to TRAIL-induced apoptosis by downregulating c-FLIP.

### ***3.4.2 Pro- and anti-apoptotic regulatory proteins of the mitochondrial pathway***

Regulation of the intrinsic pathway occurs from the balance of pro- and anti-apoptotic factors of the Bcl-2 family of proteins (discussed in Section 1.6.2). When cleaved by caspase-8 in response to death ligand, tBid moves to the mitochondria and together with pro-apoptotic members Bak and Bax polarize the mitochondrial membrane potential to induce the release of apoptotic factors cytochrome *c* and Smac/DIABLO in order to activate apoptosis through the intrinsic pathway. The release of Smac/DIABLO and cytochrome *c* is inhibited by anti-apoptotic members Bcl-2 and Bcl-X<sub>L</sub>. In addition, the anti-apoptotic protein, XIAP, also regulates apoptosis at the post-mitochondrial level (discussed in Section 1.6.2). It binds to caspases –3 and –9 in their procaspase forms and prevents their activation. This inhibition by XIAP is relieved by the binding of Smac/DIABLO when it is released from the mitochondria in response to apoptotic stimuli. XIAP can also be cleaved to from a p57 precursor into p25 subunits to promote apoptosis [17].

Members of the Bcl-2 family of proteins, that regulate the intrinsic pathway, were also examined. The levels of the pro-apoptotic members, Bax, Bak and Bid were unaltered by treatment with LY294002. Levels of the anti-apoptotic proteins Bcl-2 and Bcl-x<sub>L</sub> were similarly unaffected (**See Figure 3.8**). We found no change in the expression of XIAP or its cleavage on treatment with LY294002 (**See Figure 3.8**).

### ***3.4.3 Akt is a downstream target of PI3K.***

A major downstream target of PI3K is Akt, which mediates cell survival and anti-apoptotic signals. Key sites of phosphorylation within the Akt1 isoform include threonine 308 and serine 473[158]. Total levels of Akt were unchanged by treatment with LY 294002, but levels of serine 473 phosphorylated Akt were reduced following treatment, becoming undetectable at 6 hours (**See Figure 3.9**).

### 3.5 SMALL INTERFERING RNA (siRNA) AGAINST THE PI3K p110 $\beta$ SUBUNIT

To reinforce the data obtained with LY294002, we used siRNA oligonucleotides directed against the  $\beta$  isoform of the p110 catalytic subunit of PI3K. This treatment was previously shown to reduce phosphorylated Akt levels and to inhibit cell growth in HeLa cells [248]. Also LY294002, although considered a specific inhibitor of PI3K [132], can exert other cellular effects that include altered calcium currents, inhibition of estrogen receptors, casein kinase 2 and other protein kinases of the PI3K superfamily such as mTOR [102, 249-251]. It was, therefore, important to attribute the apoptosis seen solely to inhibition of the PI3K pathway when combined with TRAIL treatment.

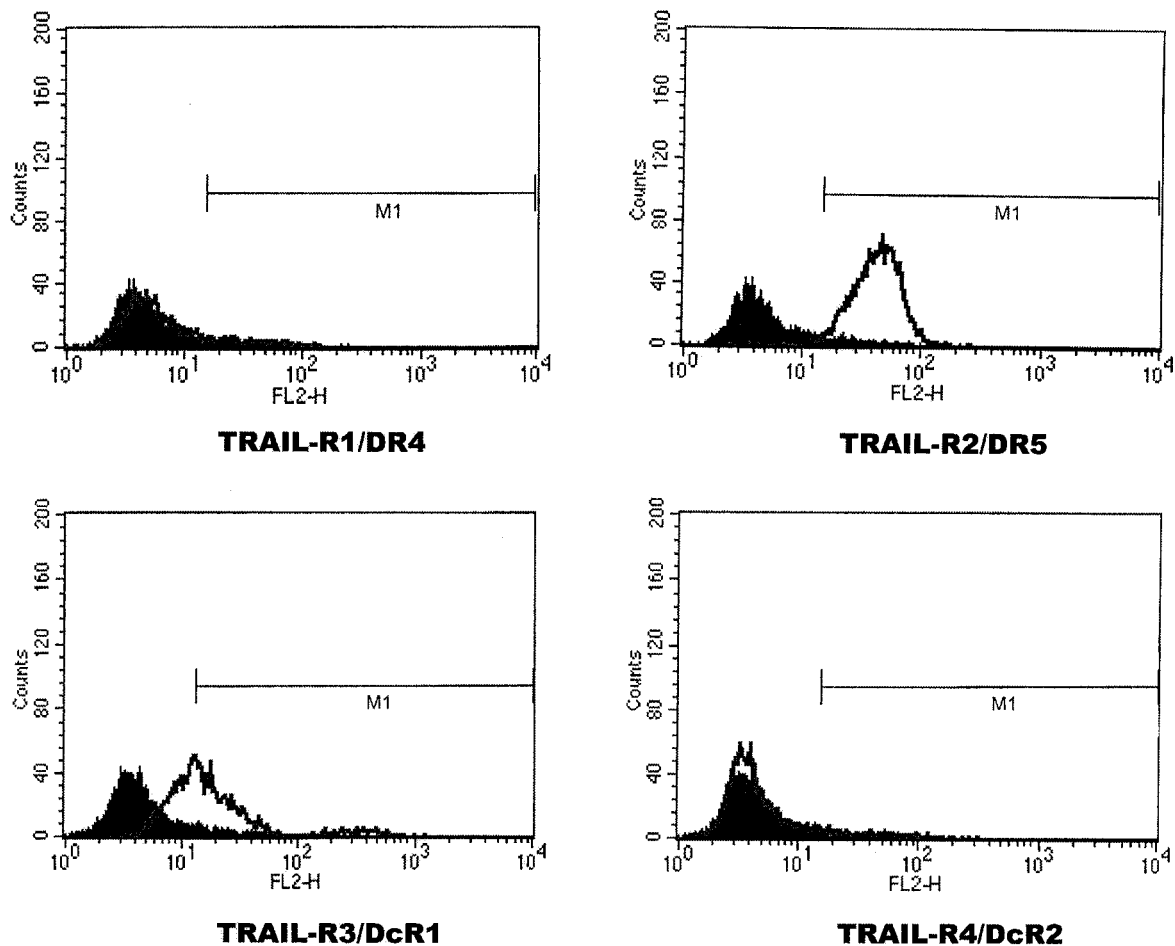
Four siRNA molecules of different sequences (sequences detailed in Section 2.8) were purchased to target the p110 $\beta$  subunit of class 1A PI3Ks (discussed in detail in Section 1.8.1). Each sequence was transfected into HUVECs and followed by TRAIL treatment in reduced serum conditions in order to observe which sequence was the most efficient at silencing PI3K activity. By visualizing cell death under the microscope, PI3-K2 was determined to be the most effective and was used in further transfection experiments (data not shown).

Figure 3.10 compares mock transfected cells (exposed to the transfection protocol without siRNA) with siRNA transfected cells treated for 6 hours in reduced serum

medium with or without 100ng/ml TRAIL. Indicators of apoptosis, such as cell rounding and membrane blebbing, were noted by phase contrast microscopy only in siRNA transfected cells treated with TRAIL (See **Figure 3.10**).

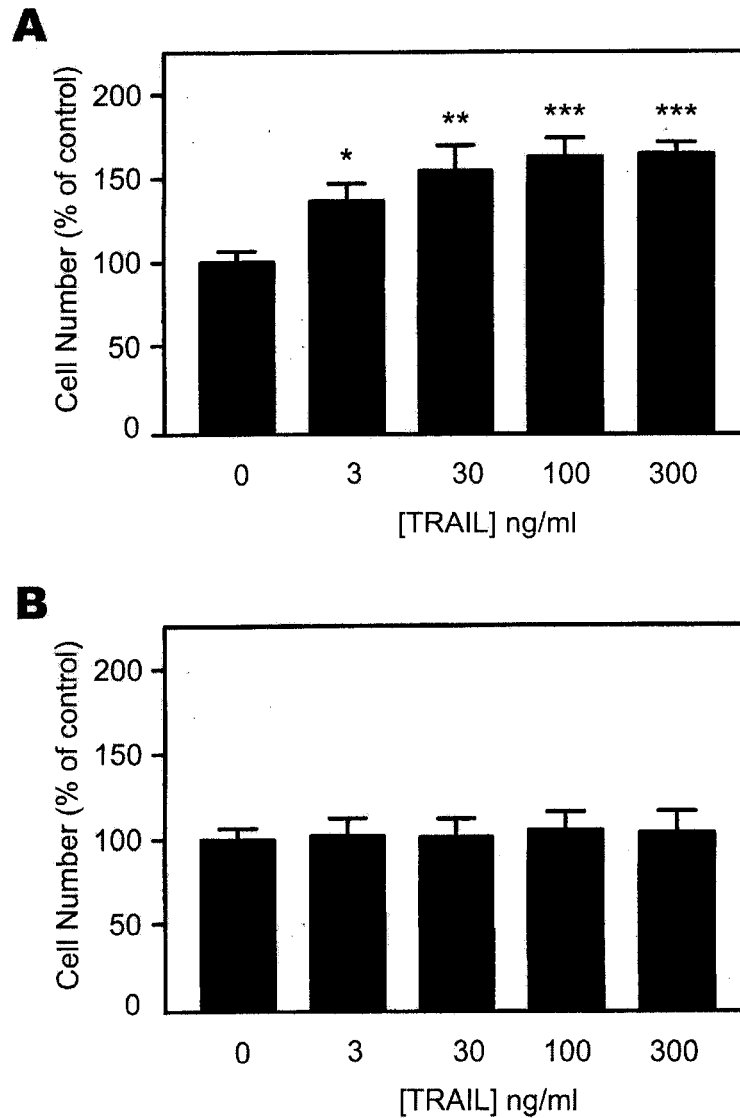
In addition, cleavage of caspase 3 from the p32 precursor into p17 active subunits was detected only in TRAIL/siRNA treated cells (See **Figure 3.11**). Compared to mock transfected cells, cells treated with siRNA showed a clear reduction in the level of c-FLIP<sub>S</sub> with a modest reduction in c-FLIP<sub>L</sub> (See **Figure 3.11**). There was no clear change in the levels of either Bcl-2 or Bcl-X<sub>L</sub> (See **Figure 3.11**). In cells treated with TRAIL, the subsequent cleavage of c-FLIP<sub>L</sub> into an intermediate p43 form was seen in both mock and siRNA transfected cells. Also there was a general reduction in protein expression levels in cells undergoing apoptosis. This results from protease activation during cellular breakdown.





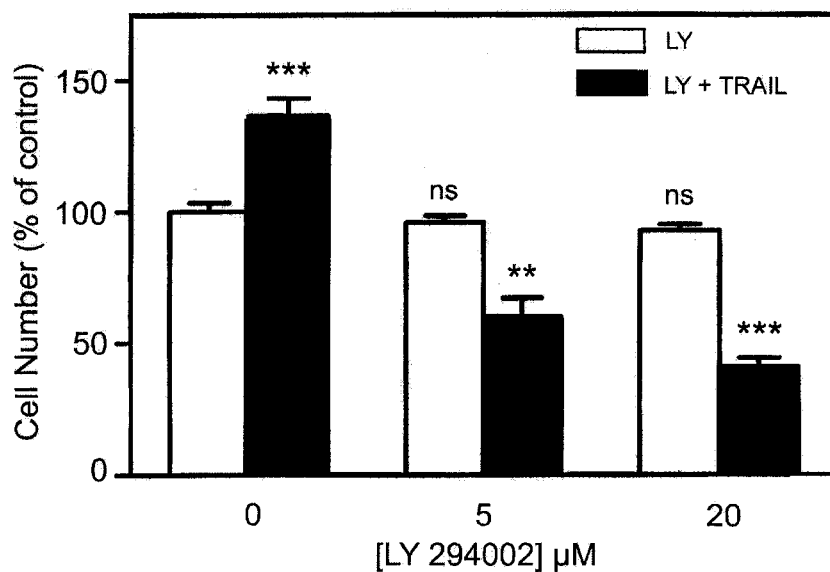
**Figure 3.1** Flow cytometry plots of surface expression of TRAIL receptors DR4, DR5, DcR1, DcR2 on HUVECs.

Surface expression of TRAIL receptors DR4, DR5, DcR1, and DcR2 on the surface of HUVECs using flow cytometry. HUVECs were incubated to PE-conjugated antibody against specific TRAIL receptors and fluorescence measure using a BD FACScan cytometer. The panels are representative blots of samples from one experiment (n=3) carried out using HUVECs from one umbilical cord.



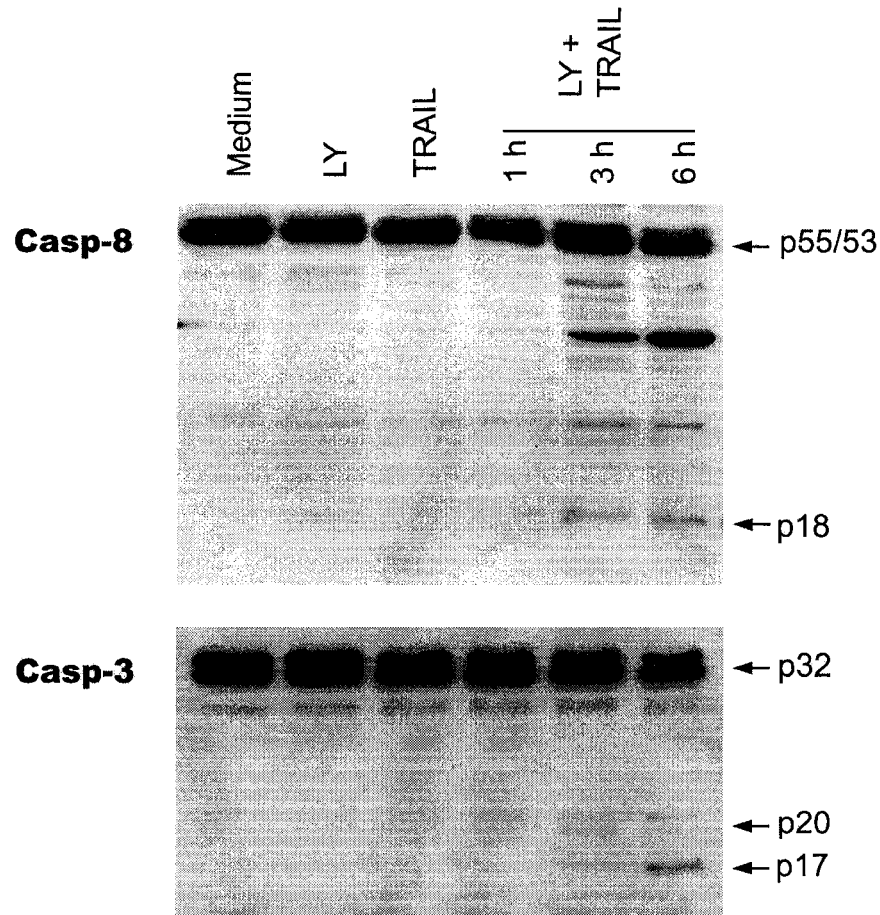
**Figure 3.2** Effects of TRAIL on HUVECs cell numbers using acid phosphatase assay.

**A.** Cells were treated with indicated doses of TRAIL for 24 hours in reduced serum medium. **B.** HUVECs were treated with indicated doses of TRAIL for 24 hours in 20% serum containing medium. Data is mean  $\pm$  standard error mean (n=8). Significance is compared to the untreated control (ns = not significant, \* = P<0.05, \*\* = P<0.01, \*\*\* = P<0.001).



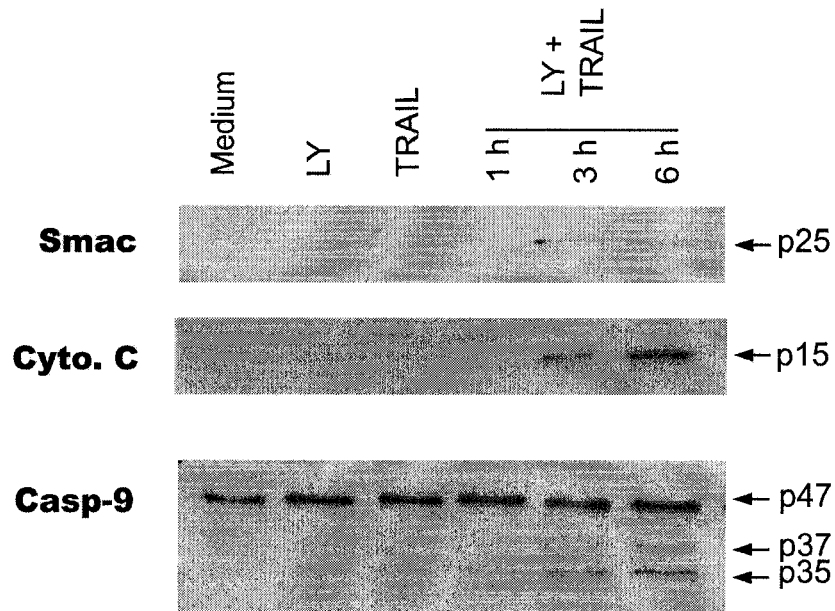
**Figure 3.3** Effects of TRAIL and LY294002 on HUVECs cell numbers using acid phosphatase assay.

Cells were treated with indicated doses of LY 294002 either alone (open bars, LY) or combined with 100ng/ml TRAIL (filled bars, LY + TRAIL) for 6 hours in reduced serum medium. Data is mean  $\pm$  standard error mean (n=8). Significance is compared to the untreated control (ns = not significant, \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ ).



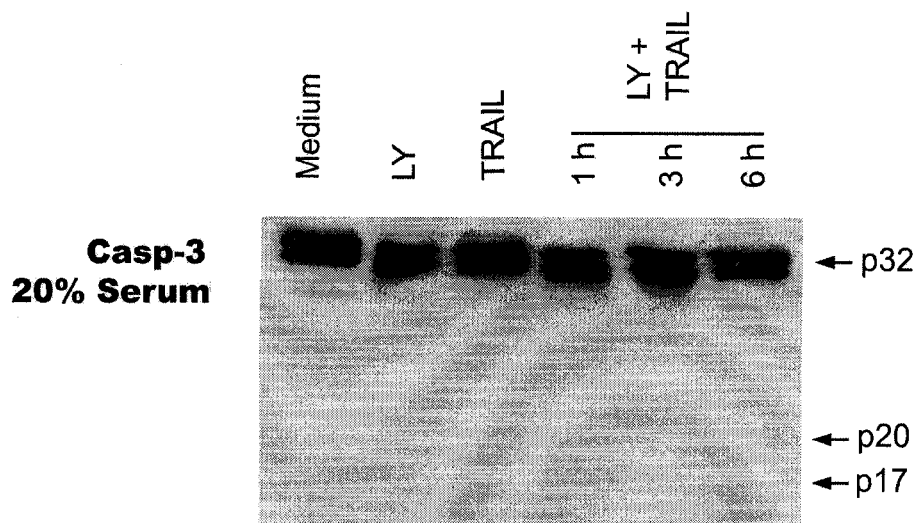
**Figure 3.4 TRAIL induces apoptosis using the extrinsic pathway in HUVECs when PI3K activity is inhibited.**

Detection of activation of the extrinsic pathway of TRAIL induced apoptosis in HUVECs in cells treated with TRAIL (100 ng/ml) and LY 294002 (20  $\mu$ M) alone for 6 hours or in combination for 1, 3 and 6 hours in 1% serum conditions. Detection of TRAIL-induced cleavage of caspases -8 and -3 in the extrinsic pathway. Whole cell lysates were subjected to Western blot analysis (100  $\mu$ g of protein per lane) as described in the Methods section. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.



**Figure 3.5 TRAIL induces apoptosis using the intrinsic pathway in HUVECs when PI3K activity is inhibited.**

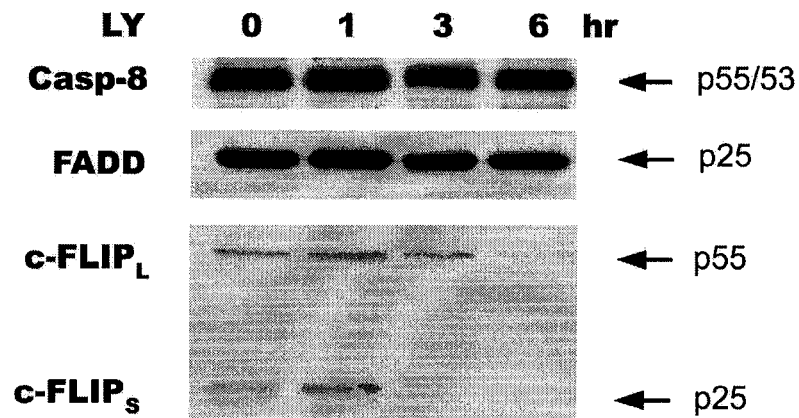
Detection of activation of the intrinsic pathway of TRAIL induced apoptosis in HUVECs in cells treated with TRAIL (100 ng/ml) and LY 294002 (20  $\mu$ M) alone for 6 hours or in combination for 1, 3 and 6 hours in 1% serum conditions. Detection of mitochondrial release of cytochrome *c* and Smac/DIABLO into the cytosol (from subcellular fractions) as well as caspase-9 cleavage. Whole cell lysates were subjected to Western blot analysis (100  $\mu$ g of protein per lane) as described in the Methods section except in the case of subcellular fractionation where 25  $\mu$ g of lysate was loaded for Western blot analysis. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.



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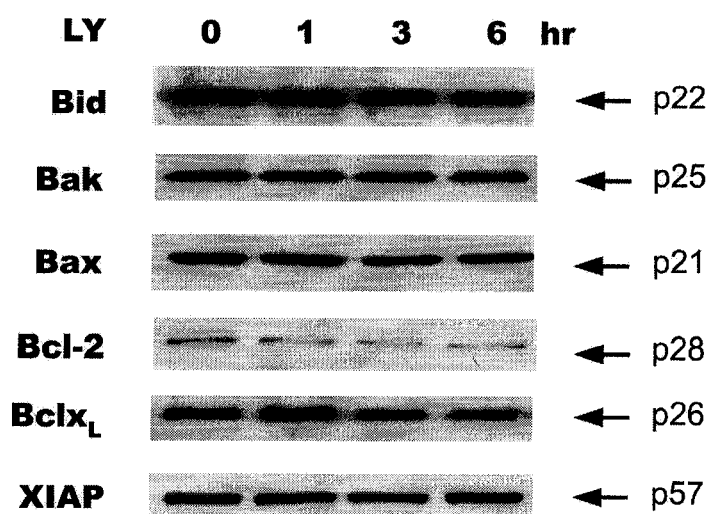
**Figure 3.6 TRAIL does not induce apoptosis in HUVECs with PI3K inhibition in 20% serum containing medium.**

Detection of activation of caspase-3 in HUVECs in cells treated with TRAIL (100 ng/ml) and LY 294002 (20  $\mu$ M) alone for 6 hours or in combination for 1, 3 and 6 hours in 20% serum conditions. Whole cell lysates were subjected to Western blot analysis (100  $\mu$ g of protein per lane) as described in the Methods section. The panels are representative blots of samples from one experiment carried out using cells from one umbilical cord.



**Figure 3.7** Effects of PI3K inhibition on regulatory proteins of the extrinsic pathway in the TRAIL DISC.

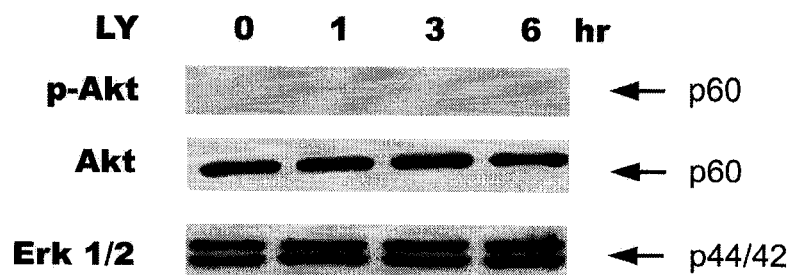
Expression levels of regulatory proteins of the extrinsic pathway in the when the PI3K pathway is inhibited by LY294002 (20  $\mu$ M) for 1, 3 and 6 hours in 1% serum containing medium. Detection of expression levels of FADD, caspase-8 and c-FLIP (short and long form) in response to PI3K inhibition. Whole cell lysates were subjected to Western blot analysis (50  $\mu$ g of protein per lane) as described in the Methods section. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.



**Figure 3.8** Effects of PI3K inhibition on regulatory proteins of the intrinsic pathway at the mitochondria .

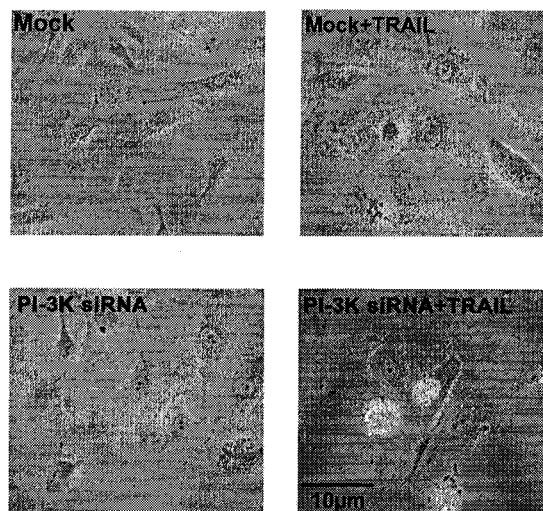
Expression levels of regulatory proteins of the intrinsic pathway at the mitochondria when the PI3K pathway is inhibited by LY294002 (20  $\mu$ M) for 1, 3 and 6 hours in 1% serum containing medium. Levels of expression of pro-apoptotic Bid, Bak and Bax as well as anti-apoptotic Bcl-2, Bcl-X<sub>L</sub>, and XIAP in response to PI3K inhibition. Whole cell lysates were subjected to Western blot analysis (50  $\mu$ g of protein per lane) as described in the Methods section. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.





**Figure 3.9** Effects of PI3K inhibition on downstream target Akt.

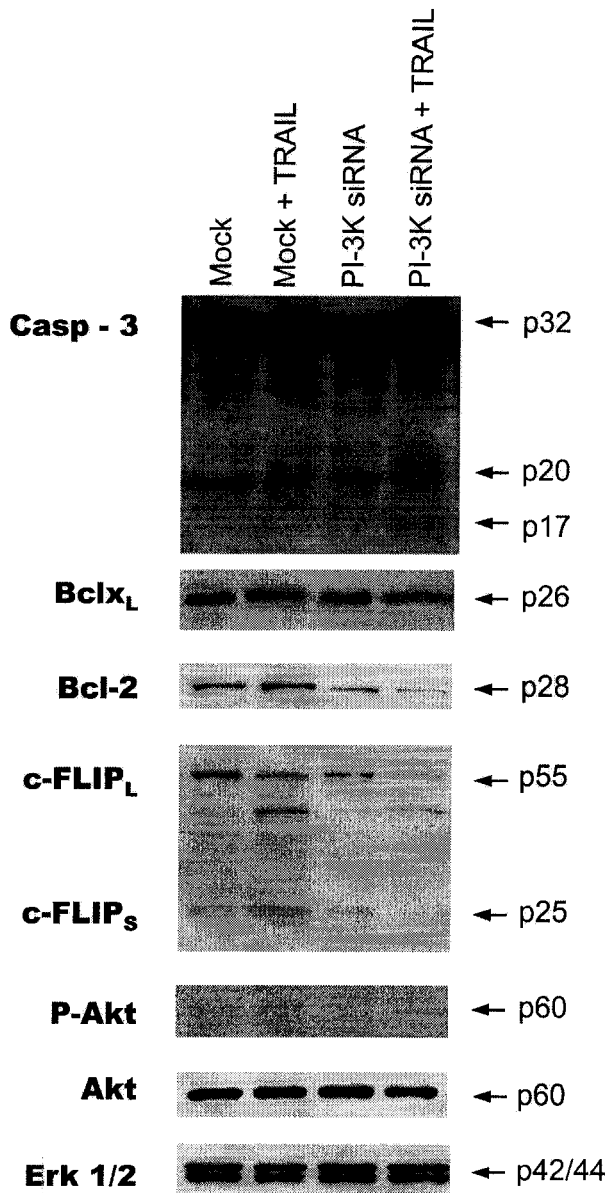
Expression levels of downstream mediators of the PI3K pathway, Akt and Phospho-Akt when the PI3K pathway is inhibited by LY294002 (20  $\mu$ M) for 1, 3 and 6 hours in 1% serum containing medium. Detection of phosphorylation of downstream target of PI3K, Akt in response to PI3K inhibition. Whole cell lysates were subjected to Western blot analysis (50  $\mu$ g of protein per lane) as described in the Methods section for. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.



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**Figure 3.10** Effects of PI3K gene silencing using siRNA on TRAIL-induced apoptosis of HUVECs.

Detection of cellular apoptosis, using phase contrast microscopy, in response to TRAIL treatment (100 ng/ml) and PI3K gene silencing of HUVECs in 1% serum containing medium. HUVECs were treated with TRAIL or left untreated when either transfected with PI3K siRNA or mock transfected and apoptotic bodies were identified through morphological changes such as cell rounding and shrinkage and membrane blebbing. The photos (X400 magnification) are representative of samples from one experiment (n=3) carried out using cells from one umbilical cord.



**Figure 3.11 Effects of PI3K gene silencing using siRNA on TRAIL-induced apoptosis and regulatory protein expression in HUVECs.**

Effects of regulatory protein expression in response to TRAIL treatment (100 ng/ml) and PI3K gene silencing of HUVECs in 1% serum containing medium. Detection of caspase-3 cleavage and expression of anti-apoptotic proteins c-FLIP, Bcl-2 and Bcl-X<sub>L</sub> in HUVECs transfected or mock transfected with PI3K siRNA and then treated with TRAIL or left untreated. Phosphorylation of downstream target of PI3K, Akt in response to PI3K gene silencing with siRNA was also detected in order to confirm silencing of catalytic activity of PI3K. Whole cell lysates were subjected to Western blot analysis (50 µg of protein per lane) as described in the Methods section. The panels are representative blots of samples from one experiment (n=3) carried out using cells from one umbilical cord.

## **CHAPTER 4. GENERAL DISCUSSION.**

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### **4.1 SUMMARY**

We demonstrate in this study that endothelial cells are resistant to TRAIL-induced apoptosis, but that inhibition of the PI3K pathway sensitizes them to TRAIL-induced apoptosis, through both the extrinsic and intrinsic pathways, by removing molecular inhibition of c-FLIP at the TRAIL death-inducing signalling complex (DISC).

TRAIL may be involved in regulating apoptosis of normal tissues because it is so widely expressed. Clearly it does not do this normally, but under particular circumstances this function may come into operation. In endothelial cells, apoptosis plays central roles in both normal and pathological processes. Endothelial cell apoptosis is thought to contribute to atherosclerosis, a disease that results in the formation of vascular lesions. In addition, the removal of excess endothelial cells through apoptosis is important during angiogenesis, a process of blood vessel formation that occurs during embryonic development as well as in wound healing and tumor formation. The phosphoinositide 3-kinase (PI3K) pathway has been shown to act as a gatekeeper for apoptosis in endothelial cells in response to both FasL and TRAIL. This study explores in greater detail the molecular mechanisms by which this important pathway modulates the sensitivity of endothelial cells to TRAIL. To this end, we decided to explore the roles of non-tagged

TRAIL on the human vascular endothelium, using HUVECs as a source, in order to investigate the signal transduction events induced by TRAIL on endothelial cell apoptosis as well as to study the molecular mechanisms that modulate and control TRAIL-induced apoptosis of endothelial cells. The PI3K pathway is an important pathway that stimulates cell growth, cell cycle entry, cell migration and cell survival in response to extracellular stimuli [100]. In endothelial cells, the PI3K pathway is of particular interest as it is activated by angiogenic factors and regulates downstream targets involved in endothelial cell survival, migration and angiogenesis [202].

#### 4.2 TRAIL DOES NOT INDUCE APOPTOSIS IN HUVECS

Studying TRAIL receptor expression on the surface of HUVECs, we found that they mainly expressed DR5 with only minimal levels of the second death receptor DR4 detected. With regards to decoy receptor expression, DcR2 was found on the surface of HUVECs, however similar to DR4, only a minimal level of DcR1 was expressed (**See Figure 3.1**). HUVEC TRAIL receptor expression has been published by several other groups in the past with all groups detecting the presence of DR5 [18, 226, 227, 230] and DcR1 expression [18, 21, 226, 227, 230]. In contrast to the data in this study, previous studies have found DR4 [18, 226, 227, 230] and DcR2 [18, 21, 226, 227, 230] on the surface of HUVECs. Perhaps TRAIL receptor expression varies from one HUVEC culture to another, which may explain the variability in receptor expression between different groups. In addition, due conflicting reports about the validity of antibodies used in flow cytometry, various techniques have been used in these studies to detect for receptor expression including flow cytometry, Western blot analysis and detection of mRNA expression. Hence, the different techniques used to study surface receptor expression may yield conflicting results since both Western and Northern blot analysis give information on intracellular expression levels of the protein and mRNA, but give no information about expression of the receptors on the cell surface.

We report that non-tagged TRAIL does not induce apoptosis in human vascular endothelium, consistent with previous reports that did not observe toxicity to normal cells

and tissues [8, 18, 226, 228, 229]. Studies of TRAIL on HUVECs have resulted in conflicting results. A histidine-tagged version of TRAIL was not found to induce apoptosis in HUVECs [18], however unlike this study, a non-tagged version of TRAIL was found to induce apoptosis in HUVECs [227]. This emphasizes that the method of ligand preparation and ligand structure affects toxic properties of the ligand. TRAIL treatment of HUVECs, in this study, instead resulted in an increase of cell numbers when cells were treated with increasing concentrations of TRAIL. This observation was also reported by Secchiero *et al* [18] who characterized this as a proliferative response to TRAIL by HUVECs using an ERK-dependent mechanism.

### **4.3 TRAIL-INDUCED APOPTOSIS REQUIRES PI3K INHIBITION AND UTILIZES BOTH THE EXTRINSIC AND INTRINSIC PATHWAYS**

TRAIL-induced apoptosis was observed in HUVECs upon inhibition of the PI3K pathway in the 1% serum conditions. These findings concur with those by Secchiero *et al* [18], who also observed apoptosis in HUVECs but only after inhibition of the PI3K pathway. Interestingly, the version of TRAIL that was used in experiments by Secchiero *et al* was histidine tagged [18]. Contrary to past reports that observed TRAIL-induced apoptosis of normal cells when using the poly-histidine tagged version of TRAIL [16], Secchiero *et al* were only able to induce apoptosis of HUVECs upon inhibition of the PI3K pathway in the serum deprived conditions [18] indicating that the toxicity of recombinant soluble TRAIL in HUVECs is also dependent on the method of preparation of the ligand. This study further characterizes the signaling events that modulate TRAIL-induced apoptosis when PI3K is inhibited. It is shown that when PI3K is inhibited, TRAIL induces apoptosis through both the extrinsic and intrinsic pathways. We went on to further characterize the signaling events involved in TRAIL-induced apoptosis and show, for the first time, that TRAIL stimulates both the extrinsic and intrinsic pathways in the presence of PI3K inhibition to induce apoptosis in HUVECs.

Activation of the extrinsic pathway involves the binding of TRAIL to its two death receptors DR4 and DR5 at the plasma membrane [4, 20-22, 58]. Once bound to TRAIL, DR4 and DR5 are able to transduce the death signal into the cytosol using their death



domain (DD) motifs to attract and recruit the adaptor protein FADD through its own DD motif at the carboxy-terminal [55, 60]. FADD also contains a death effector domain (DED) on its amino terminus, and in turn recruits caspase-8 through homophilic interactions between DEDs forming a death-inducing signaling complex (DISC) [54, 66]. In the DISC, caspase-8 is activated in a two-step cleavage process releasing activated caspase-8 subunits into the cytosol [64]. Activated caspase-8 subunits are then able to go on to cleave downstream caspases such as caspase-3, inducing apoptosis through the extrinsic pathway [71].

Activation of the extrinsic pathway in HUVECs in response to TRAIL was demonstrated by measuring cleavage products of caspases -8 and -3 using Western blot analysis. In HUVECs treated with TRAIL and the PI3K inhibitor, LY294002, activated caspase-8 subunit p18 was seen within 3 hours of the combined treatment. Similarly, activated caspase-3 subunits p20 and p17 were also seen, beginning at 3 hours, with stronger activation occurring with 6 hours of combination treatment (**See Figure 3.4**). This activation of the extrinsic pathway was most likely due to TRAIL binding to DR5 and initiating DISC formation as it was the predominant death receptor detected on the surface of HUVECs in this study.

TRAIL has also been reported to activate the intrinsic pathway as a result of activation of the extrinsic pathway [17]. Activated caspase-8 can either directly activate caspase-3 to

induce apoptosis through the extrinsic pathway or also cleave Bid in order to activate the intrinsic pathway [252]. Cleaved Bid or truncated Bid (tBid) can move to the mitochondrion and together with the pro-apoptotic proteins, Bak and Bax, trigger the depolarization of the mitochondrial membrane potential and release of Smac/DIABLO and cytochrome *c* [80-82]. Smac/DIABLO is able to bind to XIAP and relieve its inhibition on caspase-9 and caspase-3 allowing them to be activated in response to apoptotic stimuli [78, 85]. Cytochrome *c*, together with Apaf1, ATP and caspase-9 form an apoptosome in the cytosol resulting in the activation of caspase-9 [77, 253, 254]. Activated caspase-9 can stimulate apoptosis through the intrinsic pathway by further activating and cleaving caspase-3 [77].

Activation of the intrinsic pathway in response to TRAIL was studied in HUVECs by determining release of Smac/DIABLO and cytochrome *c* into the cytosol and looking for caspase-9 cleavage using Western blot analysis. Smac/DIABLO and cytochrome *c* release into the cytosolic fraction was observed in cells treated with TRAIL and LY294002 beginning at 3 hours. In addition, this was supported by the appearance of active caspase-9 cleavage products p37 and p35 at 3 hours and more pronounced with 6 hours of the combination treatment (**See Figure 3.5**).

HUVECs treated with the inhibitor alone or TRAIL alone did not show activation of the intrinsic (no release of cytochrome *c* and Smac/DIABLO or activation of caspase-9) or extrinsic pathway (no activation of caspases-8 and -3) in the serum deprived conditions.

This data exhibits that TRAIL-induced apoptosis in HUVECs utilizes both the intrinsic and extrinsic pathways when the PI3K pathway is inhibited in reduced serum medium.

#### **4.4 TRAIL-INDUCED APOPTOSIS ONLY OCCURS IN REDUCED SERUM CONDITIONS**

The ability of TRAIL to induce apoptosis with inhibition of the PI3K pathway was observed only culture conditions containing 1% serum. This was confirmed by Western blot analysis of HUVECs treated with LY294002 and TRAIL in 20% serum conditions where no activation of caspase-3, the chief effector caspase of apoptosis, was noted.

The low serum conditions required in order to observe TRAIL-induced apoptosis of HUVECs in the presence of PI3K inhibition has been observed previously [18], but the specific contribution that low serum conditions play is poorly understood. The role of serum on endothelial cell survival has been well documented with serum deprivation initiating HUVEC apoptosis [255, 256]. This was observed in this study in those experiments where cells were left in reduced serum medium for over 10 hours (**See Section 3.2.2**). Studies have shown that serum starvation causes cells to become quiescent with an arrest in cell cycle progression where cells do not enter S-phase and as a result stop in G1 [257]. Also, multiple studies in endothelial cells are only able to induce apoptosis when cells are placed in reduced serum medium [18, 258-261]. The data suggests that reduced serum medium produces synergistic effects to those of PI3K inhibition to result in apoptosis, but the nature of these effects is obscure. It has been hypothesized that serum albumin plays a major role in inhibiting endothelial cell apoptosis because cells cultured in serum free media are protected from apoptosis by

adding supplemental serum albumin [262, 263]. As a result, perhaps the low serum culture conditions predispose HUVECs to apoptosis induced by TRAIL because the low concentrations of serum albumin decreases the expression of anti-apoptotic factors making cells more sensitive to death signals. Albumin is thought to signal to endothelial cells with regards to vessel perfusion, where poorly perfused vessels are lost due to endothelial cell apoptosis as a result of low albumin concentrations [262, 263].

#### **4.5 ROLE OF PI3K IN MODULATING TRAIL-INDUCED APOPTOSIS**

The PI3K pathway plays central roles in cellular growth, survival, cell cycle entry and migration in response to various extracellular stimuli such as growth factors and hormones [100]. Activation of PI3Ks results in the activation of a number of pathways that are responsible for inducing downstream effects, most notably the serine/threonine kinase Akt/PKB pathway [143]. When activated, Akt is capable of stimulating the transcription of pro-survival genes and inhibiting the transcription of pro-apoptotic genes (discussed in 1.10.1) as well as stimulating cell cycle progression into S-phase (discussed in 1.10.2) [159]. As a result, we went on to further elucidate the role that PI3K plays in modulating the TRAIL-induced apoptosis seen in HUVECs.

We studied the effects of PI3K inhibition on levels of regulatory proteins involved in the extrinsic pathway at the DISC and in the intrinsic pathway at the mitochondria in 1% serum conditions. Proteins recruited to the TRAIL-induced DISC include the adaptor FADD and caspase-8 as well as c-FLIP, which is recruited to the DISC by DED interactions with FADD [71]. Two isoforms of c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> exist which are recruited to the TRAIL DISC where they bind FADD and compete for binding with caspase-8 as well as prevent its second step cleavage and activation [71]. These actions combine to inhibit TRAIL-induced apoptosis. There is data to implicate c-FLIP as an inhibitor of TRAIL-induced apoptosis in cancer cells as well as normal cells [83, 89, 264] but no data yet to implicate c-FLIP in modulation of TRAIL apoptosis in vascular

endothelial cells. However there are studies showing that c-FLIP modulates sensitivity of endothelial cells to TNF [265] and FasL [259] as well as other factors such as oxidized LDL [266]. Previous studies have also suggested that inhibition of PI3K results in downregulation of c-FLIP to sensitize endothelial cells to FasL [259]. The role of c-FLIP is still controversial, with some transfectant studies showing that it promotes rather than inhibits apoptosis [71, 88].

Western blot analysis of proteins recruited to the DISC in HUVECs treated with the PI3K inhibitor LY294002, revealed that there were no differences in expression levels of FADD or caspase-8. However, expression of both the short and long forms of c-FLIP were found to decrease with inhibitor treatment for 1, 3 and 6 hours with expression of the short form disappearing by 6 hours. This work therefore suggests, for the first time, that c-FLIP plays a role in regulating the susceptibility of vascular endothelial cells to TRAIL-induced apoptosis.

As mentioned earlier, the intrinsic pathway is regulated by a balance between pro- and anti- apoptotic proteins of the Bcl-2 family of proteins [267]. Upon cleavage by activated caspase-8, Bid can move to the mitochondrion where together with the pro-apoptotic proteins, Bax and Bak, it results in the mitochondrial release of apoptotic factors cytochrome *c* and Smac/DIABLO that activate the intrinsic pathway for apoptosis [74-76, 81, 268, 269]. The release of these factors from the mitochondria is inhibited by anti-

apoptotic family members, Bcl-2 and Bcl-X<sub>L</sub> [17, 267]. In addition, the inhibitor of apoptosis protein, XIAP also inhibits apoptosis by preventing the activation of caspases-3 and -9 [78]. The binding of Smac/DIABLO to XIAP relieves this inhibition and allows apoptosis to continue through the intrinsic pathway [78]. XIAP may also be directly cleaved to release its inhibitory effect [17].

HUVECs treated with the PI3K inhibitor LY294002 were subjected to Western blot analysis in order to determine if there were any changes in protein expression levels of those proteins involved in regulating the mitochondrial pathway. No changes in levels were detected for the pro-apoptotic proteins Bid, Bax, or Bak nor were there any changes in levels of the anti-apoptotic proteins Bcl-2, Bcl-X<sub>L</sub>, or XIAP.

The results obtained from PI3K inhibitor experiments were reinforced by siRNA gene silencing in order to confirm that modulation of TRAIL-induced apoptosis was due to inhibition of PI3K activity and not due to any other cellular processes that the inhibitor, LY294002 might affect. Interfering RNAs were chosen to target the catalytic p110 $\beta$  subunit of class 1A PI3Ks. As mentioned in section 1.8.1, of the three classes of PI3Ks that exist, class 1A is responsible for signal transduction events in response to extracellular stimuli such as growth factors and hormones [103]. In addition, a previous study showed that siRNA targeted against the p110 $\beta$  subunit abolished PI3K activity in



HeLa cells [248]. Hence, siRNAs were directed against the same subunit in HUVECs in order to abolish PI3K activity and observe the effects on TRAIL-induced apoptosis.

Cells that were transfected with the siRNA for PI3K became sensitive to TRAIL-induced apoptosis in 1% serum conditions. This is in contrast to cells that were mock transfected and remained resistant to TRAIL treatment (**See Figure 3.10 and 3.11**). When protein levels of anti-apoptotic proteins, c-FLIP, Bcl-2 and Bcl-X<sub>L</sub> were checked in response to PI3K siRNA transfection, no changes were detected in the levels of Bcl-2 and Bcl-X<sub>L</sub>. However, levels of both the long and short forms of c-FLIP decreased with PI3K gene silencing. In addition, in cells treated with TRAIL the intermediate p43 form of c-FLIP<sub>L</sub> was observed. This indicated that TRAIL was successful in recruiting the long form of c-FLIP to the DISC where it is processed into its intermediate form and remains bound with c-FLIP<sub>S</sub> in order to inhibit second step cleavage of caspase-8 and as a result prevent TRAIL-induced apoptosis [71]. These results confirmed the results with LY204002 that c-FLIP was downregulated in response to PI3K inhibition.

Our results imply that resistance of HUVECs to TRAIL is conferred by the PI3K/Akt pathway acting through the downstream regulator c-FLIP. Previous studies have suggested that c-FLIP is either not present in HUVECs [226], or that only the long isoform is present [266]. However we found both long and short isoforms of c-FLIP and suggest that c-FLIP<sub>S</sub> may be more important than c-FLIP<sub>L</sub> in regulating TRAIL-induced

apoptosis, since its level becomes undetectable upon inhibition of PI3K with LY294002. In a previous study [270], inhibition of PI3K with the inhibitor LY294002 had no effect on levels of c-FLIP in HUVECs. However, unlike the conditions of this study, which were carried out in 1% serum, this latter study was carried out in 20% serum. Other work has implicated the PI3K pathway in the reduction in c-FLIP in endothelial cells [259], however this is the first study to show a correlation between this reduction in c-FLIP and endothelial sensitivity to TRAIL-induced apoptosis.

We did not see changes in expression levels of XIAP, Bcl-2, and Bcl-X<sub>L</sub> proteins known to be regulated by PI3K/Akt through NF- $\kappa$ B. In addition, no changes in expression levels of Bax, Bak and Bid as well as caspase-8 and FADD were detected indicating that the primary site of regulation of apoptosis in HUVECs by PI3K occurs through c-FLIP at the beginning of the extrinsic pathway in the DISC.

The mechanism by which c-FLIP is downregulated as a result of PI3K inhibition has been attributed to the downstream mediator of PI3K activation, Akt/PKB in cancer cell lines as well as normal cells [71, 90, 271, 272]. Akt is known to activate the transcription factor NF- $\kappa$ B [167, 168]. Recent studies have found that regulation of c-FLIP expression can be controlled by NF- $\kappa$ B in human aortic endothelial cells [265], and cancer cell lines [271, 273]. The regulation of c-FLIP levels observed in transformed cancer cell lines in response to PI3K inhibition led to the hypothesis that PI3K is the predominant regulator

of c-FLIP in tumor cells [90]. This would support our data where we observed a decrease in c-FLIP levels due to PI3K inhibition in endothelial cells. The PI3K/Akt pathway also acts through c-FLIP to modulate vascular endothelial apoptosis in response to Fas ligand [259], which is closely related to TRAIL, suggesting that a common mechanism regulates endothelial apoptosis through both death receptors. In this study we also found that inhibition of PI3K resulted in a reduction in Akt phosphorylation, implying that PI3K acts via Akt. However, we have not ruled out the possibility that PI3K acts by effects on other downstream molecular mediators.

#### 4.6 FUTURE WORK

To specifically attribute sensitization of HUVECs to TRAIL-induced apoptosis in the presence of PI3K inhibition due to the downregulation of c-FLIP expression, future experiments studying the effects of c-FLIP silencing either through siRNA gene silencing, dominant negative and anti-sense gene transfections would be required. In addition, this study did not elaborate on the signal transduction events that lead to the decrease in expression of c-FLIP due to PI3K inhibition. Hence, experiments investigating the role that Akt may play in downregulating c-FLIP expression, either through inhibition or knockout studies, are warranted in order to elucidate the causative role of the PI3K/Akt pathway in regulating TRAIL-induced apoptosis.

It is important to note that the role of TRAIL and PI3K was not directly related to angiogenesis and atherogenesis in this study. However, endothelial cell survival in response to angiogenic stimuli such as VEGF [205, 206], Ang-1 [203, 204], corticosteroids [223], estrogen [221] and reactive oxygen species [222] have all been observed to occur through activation of the PI3K pathway. Inhibition of the PI3K pathway has been shown to prevent endothelial cell survival in the presence of VEGF and has been implicated in mediating vessel regression [206]. As a result, observing the roles of TRAIL and PI3K inhibition in angiogenic model systems *in vitro* and *in vivo* would be

examples of future experiments in order to directly characterize the effects of TRAIL on angiogenesis and vessel regression.

## **CHAPTER 5.    WORKS CITED.**

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## **APPENDIX: CELL CYCLE ANALYSIS OF HUVECS TREATED WITH TRAIL**

Cell cycle analysis was used to determine the effects that TRAIL has on HUVEC cell cycle. As mentioned in Section 3.2.1, HUVECs treated with TRAIL (various doses) for 24 hours, increased in cell number as determined by the acid phosphatase assay when treatment occurred in 1% serum containing medium. This was also observed by Secchiero *et al* when HUVECs were pretreated with reduced serum medium for 18 hours prior to TRAIL treatment [18]. In addition, TRAIL has been shown to induce proliferation of cancer cell lines resistant to TRAIL-induced apoptosis [99]. Consequently, the increases in cell number was analysed for cell cycle progression in order to determine if TRAIL was elucidating a proliferative response in HUVECs

### **CELL CYCLE ANALYSIS USING PROPIDIUM IODIDE STAINING**

Propidium iodide (PI) is used in flow cytometry to analyze cell cycle progression [274]. It is a stain that stoichiometrically binds DNA and as a result, gives information about the DNA content of a cell. Because at different points of the cell cycle cells are either replicating, growing or dividing PI staining can then be used to determine which phase of



the cell cycle G1, S, G2 or M the cell is in [274]. Cells that are actively proliferating spend much of their time in S-phase replicating their DNA. This is in contrast to cells that are non-cycling or not proliferating and spend most of their time in G1. Therefore, proliferating cells exhibit an increased content of DNA, and therefore increased PI staining, compared with non-proliferating cells.

Cell cycle analysis using PI staining was determined by flow cytometry. Cells were grown to 60% confluency, treated with TRAIL (100 ng/ml) in both 20% and 1% serum containing medium for 24 hours and then harvested in 0.25% Trypsin/EDTA. After washing the cells in PBS they were divided into tubes containing  $10^6$  cells per 1 ml of fresh PBS. To each tube of PBS, 5 ml of 70% ethanol was added in order to fix the cells at 4°C overnight.

The next day, the tubes were centrifuged (1000 x g) and the ethanol removed followed by three successive washes with PBS in order to remove any excess ethanol. Next, the cells were incubated in 0.25 ml of PI Buffer (1 µg/ml propidium iodide (Sigma), 100 µg/ml Rnase A (Qiagen)) for 25 min in the dark. 20,000 cells were then analyzed using BD FACScan and the resulting data processed using ModFit LT 2.0 (Becton Dickinson, Mountain View, CA). Healthy cells were determined from side and forward scattered histograms and gated before being subsequently analyzed for fluorescent intensity using the ModFit LT 2.0 program.

### **CELL CYCLE ANALYSIS OF HUVECs TREATED WITH TRAIL**

In order to determine if the increase in cell number that was observed with TRAIL treatment was due to TRAIL-induced endothelial cell proliferation, we looked at cell cycle progression of HUVECs treated with TRAIL (100 ng/mL) at 6, 12 and 24 hours in both the 1% and 20% serum containing media using PI staining (**See Figure A1**).

In cells cultured in the 20% serum containing medium and treated with TRAIL (filled bars), there was no difference in the proportion of cells in S-phase at 6, 12 and 24 hours when compared to untreated cells in the same conditions for 6, 12 and 24 hours (**See Figure A.1a**). Although the proportion of cells in S-phase increased with time from 41% at 6 hours to 73% at 24 hours, the TRAIL treated and untreated cultures did not show any differences at the respective times. This would indicate that TRAIL does not stimulate proliferation of HUVECs in 20% serum containing medium.

Similarly, cells cultured in the 1% serum containing media and treated with TRAIL (filled bars) for 6, 12 and 24 hours also showed no differences in proportions of cells in S-phase compared to their untreated control counterparts (**See Figure A.1b**). There was an increase in cells in S-phase through the time course, but there were no differences

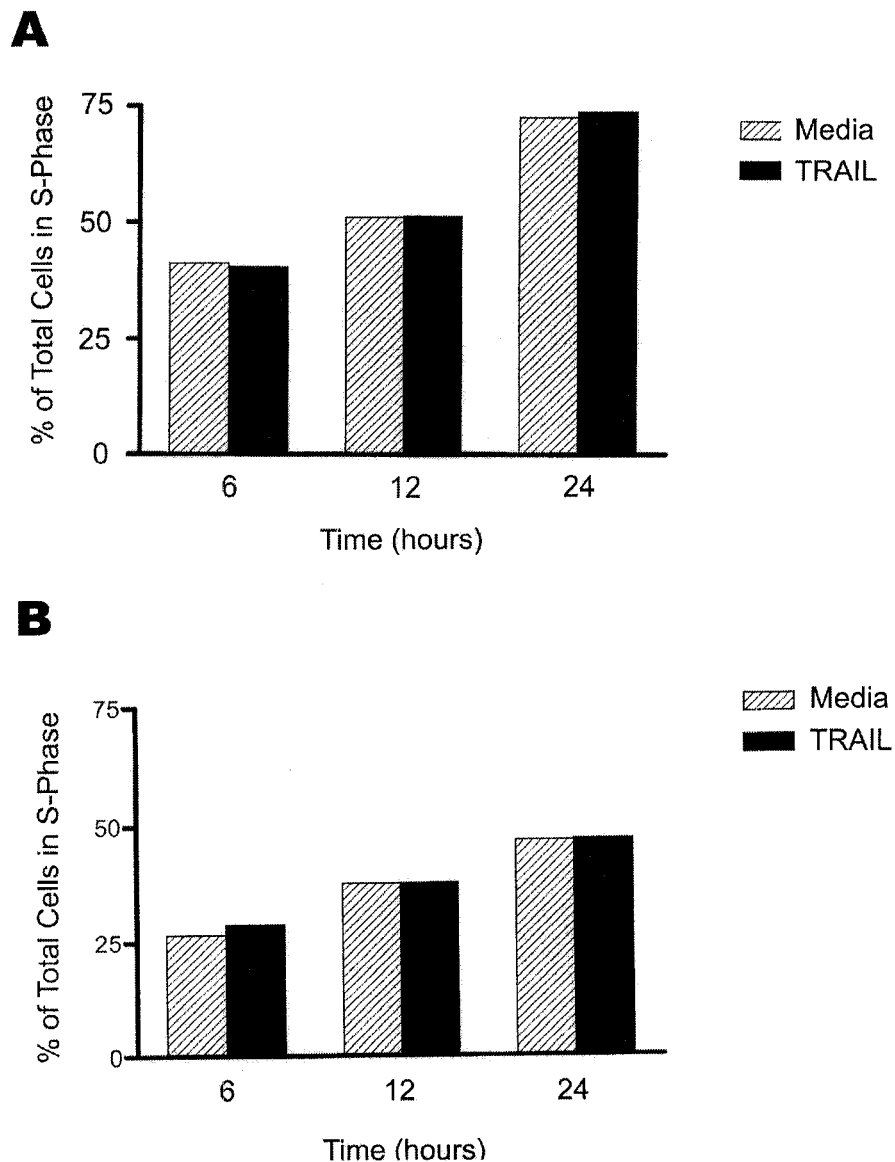
between cells left in the low serum medium or those treated with TRAIL. This would also indicate the TRAIL does not induce proliferation in HUVECs in the 1% serum conditions.

The increase in the proportion of cells in S-phase over time observed in both the 20% and 1% serum containing medium can be attributed to cell growth and proliferation occurring in both conditions. As expected, cells cultured in the 20% serum conditions have a higher proportion of cells in S-phase when compared to cells propagated in the 1% serum containing medium. This is due to the fact that the high serum containing environment in the 20% serum containing medium, promotes growth and proliferation unlike that of the 1% serum containing medium, which maintains the metabolic activity of cells at a basal rate [275].

### **TRAIL DOES NOT INDUCE PROLIFERATION IN HUVECS**

As shown in this study, TRAIL does not induce HUVEC proliferation. No differences were detected in numbers of cells in S phase between TRAIL treated and untreated cells. This conflicts with the results by Secchiero *et al*, who characterize the increase in HUVEC cell number with TRAIL treatment to TRAIL-induced proliferation in an ERK-dependent mechanism in the low serum conditions [18]. The increase in cell number was characterized as proliferation using a [<sup>3</sup>H]thymidine uptake assay which essentially

measures an increase in DNA [18]. The proliferation observed with resistant cancer lines was reported to occur in an NF- $\kappa$ B dependent mechanism [99]. Interestingly, Secchiero *et al* reported that no activated NF- $\kappa$ B was observed in TRAIL treated HUVECs [18].



**Figure A.1 Cell cycle analysis of HUVECs treated with TRAIL in 1% serum conditions and 20% serum conditions.**

Cell cycle analysis and detection of HUVECs in S-phase in response to TRAIL treatment (100 ng/ml) for times indicated in 20% serum containing medium (**A**) and in 1% serum containing medium (**B**). **A.** Total proportion of cells in S-phase when cultured in 20% serum conditions and treated with TRAIL (filled bars) or left untreated (hatched bars). **B.** Total proportion of cells in S-phase when cultured in 1% serum conditions and treated with TRAIL (filled bars) and left untreated (hatched bars). Data is representative of one experiment (n=2) carried out using cells from one umbilical cord.