Mechanisms of BNIP3 Transcriptional Regulation under Hypoxia

By

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A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
for the degree of

Master of Science

Department of Biochemistry & Medical Genetics
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In memory of my father

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LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor-1
ARNT	aryl hydrocarbon nuclear translocator
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
BH1-4	Bcl-2 homology doamin 1-4
BHLH	basic helix loop helix
Bid	BH3-interacting domain death agonist
BNIP3	Bcl-2,nineteen kD-interacting protein-3
Ca	calcium
C.*elegans*	*Caenorhabditis elegans*
dATP/ATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ETC	electron transport chain
FADD	Fas-asociated protein with death domain
H_2O_2	hydrogen peroxide
HEK	human embryonic kidney
HDAC	histone deacetylase

HIF	hypoxia inducible factor
HRE	hypoxia response element
IAP	inhibitor of apoptosis
K	potassium
Na	sodium
NLS	nuclear localization signal
NIX	BNIP3-like protein X
O_2^-	superoxide anion radical
ODDD	oxygen-dependant degradation domain
PBS	phosphate buffer saline
PEST	prolyl, glutamic acid, serine , threonine rich domain
PHD	prolyl hydroxylase
PT	permeability transition
PVHL	Von Hippel Lindau protein
ROS	Reactive oxygen species
SDS	sodium dodecyl sulfate
TAD	transactivation domain
TBST	tris buffer saline
TBID	truncated BID
TM	transmembrane
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
$\Delta\Psi m$	mitochondrial membrane potential

ABSTRACT

Hypoxia is responsible for the pathological aspects of many diseases such as cancer, cardiovascular and cerebrovascular disorders. Hypoxic regions within solid tumors are often resistant to chemotherapy and radiation. During coronary ischemia and stroke, hypoxia induces cell death that leads to tissue injury ultimately resulting in heart and brain failure. BNIP3 (Bcl-2/E1B 19kDa interacting protein) is a pro-apoptotic member of the Bcl-2 family that is expressed in hypoxic regions of tumors. During hypoxia, BNIP3 expression is increased in many cell types and upon forced over expression BNIP3 induces cell death. Herein, we have demonstrated that blockage of hypoxia induced BNIP3 expression using antisense oligonucleotides against BNIP3 or blockage of BNIP3 function through expression of a dominant negative form of BNIP3 inhibits hypoxic induced cell death in HEK293 cells. Hypoxia mediated BNIP3 expression is directly regulated by the transcription factor, Hypoxia Inducible Factor 1α (HIF-1α) in human epithelial cell lines. In cells over expressing HIF-1α, BNIP3 expression was increased and cells defective in HIF-1α activation failed to increase human BNIP3 promoter activity. Furthermore, HIF-1α binds to a consensus HIF-1α responsive element (1) in the human BNIP3 promoter that upon mutation of this HRE site eliminates the hypoxic responsiveness of the promoter.

I. INTRODUCTION

1. Hypoxia

Oxygen homeostasis is essential for the survival of all vertebrate and invertebrate organisms. In humans, complex cardiovascular, hematopoetic and respiratory systems assure optimal oxygen delivery to all cells of the body. Oxygen from the atmosphere enters the respiratory system and diffuses from the pulmonary alveoli through the capillaries into the arterial blood. In this system, the heart plays a crucial role in pumping the blood through the vasculature, thus ensuring optimal oxygen delivery to all parts of the body. Impairment in any component of this elaborate physiological system can result in an imbalance between oxygen supply and demand. Such imbalance is characterized by regions of low oxygenation or hypoxia.

There are four types of hypoxia. Anemic hypoxia refers to the reduced oxygen carrying capacity of the blood. Systemic hypoxia is characterized by adequate perfusion with a reduction in partial pressure of oxygen in the arterial blood. Histologic hypoxia refers to the inability of the cells to utilize the available oxygen in the presence of sufficient blood flow. Ischemic hypoxia is due to an insufficient oxygen delivery caused by insufficient blood flow (2).

Oxygen is essential for the survival of human cells. Molecular oxygen plays a major role in energy generation in the form of ATP through aerobic metabolism. Although human cells have the ability to carry anaerobic metabolism, this process is not efficient and is used in few circumstances only. To assure oxygen homeostasis, all cells within the human body are capable of sensing oxygen concentration and responding to hypoxia. Depending on the duration and severity of the hypoxic condition, cells initiate a

variety of responses to survive at low oxygen concentration and to restore oxygen homeostasis. These biological changes comprise physiological changes such as increased heart rate, increase in blood pressure, and development of new blood vasculature. The molecular mediators responsible for these changes will be discussed later.

While initially, low oxygen levels induce cell survival responses in cells, sustained hypoxia generally results in cell death (3). There are two major types of cell death during hypoxia: apoptosis and necrosis. Apoptosis or programmed cell death is an evolutionary conserved and genetically regulated process characterized by chromatin condensation, DNA fragmentation, plasma membrane blebbing and formation of apoptotic bodies (4). Apoptosis is a cell suicide program where highly regulated and conserved pathways assure that the cell destruction occurs in a systematic and structured manner avoiding inflammatory responses. Apoptosis is essential for the development and homeostasis in multicellular organisms and is triggered by a variety of extrinsic and intrinsic signals including growth factors withdrawal, TNFα, Fas ligand, loss of matrix attachment, heat shock, viral infection, chemotherapeutic drugs, radiation, DNA damage, and hypoxia (5).

Since the discovery of apoptosis, two major cellular pathways leading to cell suicide have been characterized. Both pathways involve common molecular players such as members of the Bcl-2 (B-cell lymphoma/leukemia) family, Apaf-1 like adapter proteins, caspases, and inhibitor of apoptosis (IAPs). The initial apoptotic trigger distinguishes the extrinsic and intrinsic apoptotic pathways. Indeed, while extra-cellular triggers initiate the extrinsic pathway, intracellular signals are responsible for the activation of the intrinsic pathway. The extrinsinc apoptotic pathway is initiated by the

activation of death receptors at the cell membrane. Activated death receptors then recruit initiator procaspases-8. Caspases are a family of cysteine proteases that are constitutively present as inactive proenzymes. Active caspases are able to cleave either other caspases or key target molecules in the cell resulting in a caspase cascade and cell disassembly (6). The agglomeration of initiator caspases by activated death receptors results in the formation of protein complexes where the procaspases-8 are in close proximity to each other. The caspases autocatalyse and activate themselves. Once activated, caspase-8 cleaves Bid a Bcl-2 family member (7). The truncated Bid or t-Bid then translocates to the mitochondria membrane. Once at the mitochondria, t-Bid further activates proapoptotic Bcl-2 family members such as Bax (8). This results in a series of changes at the mitochondria that culminates in the release of cytochrome-c (a component of the electron transport chain) into the cytoplasm. In the presence of ATP, cytochrome-c complexes with Apaf-1 and procaspase-9. This process activates caspase-9 which in turn activates caspase-3 resulting in cell death (9). The intrinsic apoptotic pathway is intiated by intracellular death triggers such as tumor suppressor protein p53 that is activated by DNA damage and hypoxia. p53 has been shown to limit cellular proliferation by inducing cell cycle arrest and apoptosis. One of the well characterized function of p53 is as a regulator of transcription. It has been shown that stress signals such as DNA damage or hypoxia stabilize and activate p53 that in turn upregulates the transcription of proapoptotic Bcl-2 family members such as Bax, Bak, and Noxa (10). Transactivation of Bax results in its translocation to the mitochondria membrane. Once at the mitochondria, Bax induces the same series of molecular changes as described earlier resulting in cell death. The description of both the extrinsic and intrinsic apoptotic pathways has been

simplified and thus omits the involvement of many other molecular players. The apoptotic pathways are far more complex than outlined here.

Unlike apoptosis, necrosis is considered a passive form of cell death in response to toxicant and physical injury that involves inflammatory responses (11). Morphological features of necrosis include extensive mitochondrial swelling, cytoplasmic vacuolation and early plasma membrane permeability. Molecular markers of necrosis are less well known. However caspase- independent cell death involving Bcl-2 family members have been reported. For instance, Bax expressing thymocytes, treated with caspase inhibitors die with necrotic like morphologies.

During hypoxia, the prevalence of one form over the other depends on the severity of the hypoxic condition and the type of cells and tissues affected.

Endogenous cellular responses to hypoxia are critical to ensure oxygen homeostasis. However these same mechanisms can be responsible for a major aspect of the pathology of many diseases. Hypoxia indeed plays a major role in diseases such as cancer, heart and cerebrovascular disorders.

2. Hypoxia and diseases

2.1. Hypoxia and cancer

2.1.1. Occurrence of hypoxic regions in solid tumors

Solid tumors masses are the result of alterated cell physiology characterized by self sufficiency in growth signals, insensitivity to inhibitory growth signals, evasion of cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. These changes promote dysregulated cell proliferation and as a consequence

abnormal morphogenesis. As a result, solid tumors have a structurally abnormal blood supply that results in a microenvironment characterized by heterogeneous pH, oxygen tension distribution and nutrient delivery. Limited oxygen supply develops in severe regional hypoxia in tumors (12).

Hypoxic regions have been observed in neoplasms that are only microscopic in size. In fact, prior to vascularization tumors are maintained at a volume of several mm^3 because the rate of cell death is equal to the rate of cell division. However tumors adapt to hypoxia and thus acquire the ability to overcome hypoxic stress (13).

Hypoxia occurs in well-differentiated, slowly growing, monmetastatic tumors as well as in rapidly growing, anaplastic, aggressive malignancies. Hypoxic regions correlate with poor prognosis. Cells in hypoxic regions constitute a problem for treatment because they are more resistant to the effects of radiotherapy and many chemotherapeutic agents. Moreover hypoxia has also been shown to increase mutation rate, resulting in the selection of cells that are more resistant to apoptosis and less responsive to therapies (14).

2.1.2. Hypoxia and drug resistance

Chemotherapeutic drugs are injected into the blood. However since most solid tumors have a limited blood supply and as a consequence regions of hypoxia drug delivery is thus a challenge.

Hypoxia has a protective effect during radiation. To achieve the same biological effect in hypoxic tissues as in normal cells, 3-fold higher doses of radiation have to be used. It has been suggested that during radiotherapy, the high-energy radiation affects protein and nucleic acid structure. One of the mechanisms by which radiation kills cancer

cells is mediated through molecular oxygen. High-energy radiation reacts with oxygen to form unstable reactive oxygen radicals that damage DNA and thus lead to cell death. Hence in the absence of oxygen, the killing power of radiation diminishes (15,16).

Hypoxic conditions also affect the efficiency of a number of chemotherapeutic drugs, as they need oxygen to be maximally cytotoxic. One such drug is bleomycin, a family of antibiotics that have demonstrated antitumor effect. Once inside the cell, bleomycins form DNA-bleomycin-ferrous ion dioxygen complexes, which upon oxidation reduce molecular oxygen to highly reactive species. These radicals then produce single and double-strand breaks in DNA and ultimately induce cell death. Thus oxygen availability plays a crucial role in the killing effect of bleomycin (17-19).

Another example of chemotherapeutic drugs that need oxygen is etoposide. Etoposide is a podophyllotoxin derivative that has been shown to cause both single stranded and double stranded DNA breaks and DNA protein cross-links in mammalian cells. Etoposide has been shown to mediate these effects through an interaction with topoisomerase II. The direct involvement of oxygen in the cytotoxicity of etoposide is unknown. However, it has been shown that free radical scavengers, dehydrogenase inhibitors and dehydrogenase substrates prevent the formation of DNA breaks during etoposide treatment. Moreover, etoposide shows a selective cytotoxicity to normally oxygenated cells in vitro, and when combined with an oxygen carrying perfluorochemical, the antitumor activity of etoposide is enhanced (19-21).

Following the discovery that tumor growth is dependant upon vacularization, angiogenesis targeted therapies were designed. Angiogenesis targeted therapies inhibit the expression of genes necessary for vascularization and thus ultimately represses

vaculature formation. The resulting limited blood supply starves the growing tumor from nutrients and oxygen necessary for survival, thus killing cells by inducing hypoxia. However some cells survive hypoxia and thus become more resistant to death triggers. Hence, angiogenesis targeted therapy promotes hypoxia resistant cell growth and thus have failed to become a successful strategy for cancer treatment (22).

2.1.3. Hypoxia and genetic instability

As cancers progress they often become more aggressive. This phenomenon has been associated with genomic instability that is seen by an accumulation of genetic aberrations. Two possible contributors of genetic instability are increased mutation rate and decreased DNA repair activity. Large numbers of mutations found in malignant cells can not be accounted for by the low rate of mutations observed in normal cells. Studies have suggested that tumor microenvironment and in particular hypoxia contribute directly to genomic instability. It has been shown that cells exposed to hypoxia have a diminished DNA repair activity. The exact mechanism of this DNA repair impairment is unknown. However it is believed that changes in the oxidation state that accompany hypoxia affect enzyme activity.

Hypoxic conditions also favor immortalization. In normal cells, telomeres are believed to shorten with time. This progressive reduction of telomeres limits replicative ability of cells and telomerases (enzymes that assure telomere length) are repressed. However, under hypoxic conditions, telomerase activity has been shown to increase thus favoring immortalization (26).

2.1.4. Hypoxia and treatments

As described earlier, hypoxia promotes cancer progression and maligancy. Moreover hypoxia has also been shown to favor drug resistance. However hypoxia is one characteristic that differentiates cancer cells from normal cells. Cancer therapies are designed to specifically target cancer cells without affecting normal cells. Thus hypoxia can be an invaluable tool in designing cancer therapies. This concept has been exploited in developing therapeutic drugs that would only be active under hypoxia.

Mytomycin C is one of such bioreductive alkylating agent available for clinical use. In vitro mytomycin C has shown to be more toxic in hypoxic cells than towards normally oxygenated tumor cells. A clinical trial of radiation therapy in combination with mytomycin C in patient with head and neck cancer has shown a significant improvement in patient outcome (27,28).

Tirapazamine (SR 4233) is another hypoxia selective cytotoxin. Under hypoxic conditions tirapazamine is reduced to a radical that leads to DNA double stranded breaks, single stranded breaks and base damage. Tirapazamine has shown to enhance the cell killing and delay tumor growth of several murine tumor when injected in combination with radiation treatment. Importantly, tirapazamine is selectively toxic to hypoxic cells. In the presence of oxygen tirapazamine is present in a harmless form. Therefore tirapazamine is an attractive cytoxin for the treatment of solid tumors (29-31).

It has been shown that hypoxia leads to increased histone deacetylase (HDAC) activity. Histone deacetylation has been implicated in alteration of chromatin structure and transcription silencing. Studies have directly linked the hypoxia induced HDAC activity to angiogenic stimulation and have suggested the potential therapeutic value of HDAC inhibitors. Indeed, one of such HDAC inhibitor, FK228 is currently in a phase I

clinical trial for cancer therapy and has shown promising results as a potent angiogenic inhibitor (23,24).

2.2. Hypoxia and the heart

One of the most common cardiac disorder is coronary insufficiency. In order to function, the heart requires a constant supply of oxygenated blood. The coronary arteries assure this transport. Fatty matter, calcium and proteins can build up within the arteries and form plaques that restrict blood flow. The area of the heart muscle supplied by the blocked artery becomes starved with oxygen and nutrients. Within a short period of time the cardiomyocytes die causing permanent damage of the heart muscle or myocardial infarction. Each coronary artery supplies blood to different regions of the heart muscle. The severity of the damage depends on the size of the area supplied by the blocked artery and the time between injury and treatment.

There is a wide range in the ability of cells to survive anoxic, hypoxic or ischemic stress. However cardiac muscle cells are highly oxygen-dependant and thus highly vulnerable to hypoxia. One of the major pathological aspects of cardiac infarction is cell death. Thus investigation in the cell death pathways induced by hypoxia have been undertaken in order to understand these mechanisms and ultimately to minimize cell death following ischemia.

It was traditionally believed that restoration of blood flow would be sufficient in rescuing cardiomyocytes from cell death. However, the restoration of blood flow or reperfusion may subject cells to further damage through oxidative stress In fact, free radicals are thought to be a major cause of ischemia/reperfusion injury. The electron transport chain (ETC) carriers in cardiomyocyte mitochondria are capable of generating

oxidants. Under normal conditions, oxidants generation is minimal, and well controlled by the cellular antioxidants. However if the ETC becomes reduced as it occurs during ischemia and early reperfusion, the electron carriers transfer electrons to any available oxygen. This results in the production of free radicals. During reperfusion, the burst in oxygen in the reduced environment increases the production of free radicals. The mitochondrial ETC inhibition can generate enough ROS to cause loss of contractile function and accelerated cell death (32,33).

Free radicals are highly reactive atoms or molecules with an unpaired electron in their outer most orbits. The production of free radicals occurs either by the addition or removal of an electron in oxidation/reduction reaction. Oxygen has 2 electrons in its outer most shell and requires 4 electrons to be completely reduced to water. The addition of one electron at a time results in the formation of reactive oxygen species. The addition of one electron to molecular oxygen results in the formation of superoxide anion radical (O_2^-). The addition of one electron to O_2^- forms hydrogen peroxide (H_2O_2). H_2O_2 is not a radical by itself but is capable of causing cell damage by interacting with metals such as iron. A single electron reduction of H_2O_2 results in the formation of hydroxyl radical (26). OH^- is a highly reactive molecule with a very short half-life, and therefore has a very limited diffusion capacity (32,34).

Free radicals injure cells in various ways. For instance, free radicals induced lipid peroxidation has been suggested to affect membrane fluidity, thus altering membrane structure and function. There is also evidence that free radicals modify protein structure and function. In the myocardium, oxygen radicals have been shown to affect Na^+/Ca^{2+}

exchanger, Na^+-K^+ ATPase and Ca^{2+} ATPase activities. These changes can lead to cell death and loss of the contractile function of the heart (35,36).

While oxidative stress accounts for the majority of tissue damage, other factors such as collateral damage from necrosis and infiltrating macrophages have also been shown to contribute to the overall tissue loss.

2.3. Hypoxia and the brain

Hypoxia affects the brain in a way similar to the heart. A stroke occurs when the blood supply to a part of the brain is suddenly interrupted or when a blood vessel in the brain bursts. Similarly to cardiomyocytes, brain cells die when they no longer receive oxygen and nutrient from the blood or when they are damaged by sudden bleeding into or around the brain. These damaged cells can linger in a compromised state for several hours. Similarly to the co-lateral damage in the heart, some brain damage that results from stroke may be secondary to the initial death of cells caused by the lack of blood flow. This brain damage is a result of a toxic reaction to the primary damage (37).

Although stroke is a disease of the brain, it can affect the entire body. Some of the disabilities that can result from stroke include paralysis, cognitive deficits, and speech problems.

3. Molecular mechanisms of hypoxia responsiveness

Physiological changes in response to hypoxia, described earlier, have a molecular basis. In fact, upon detection of hypoxia, cells trigger a variety of signal transduction pathways that result in the expression and accumulation of proteins. These proteins

initiate biological changes to ensure at first cell survival. However, if hypoxic conditions are sustained, death inducing pathways are also triggered.

One major regulator of hypoxic responses is transcription factor Hypoxia Inducible Factor 1 (HIF-1). HIF-1 is activated by low oxygen conditions and induces changes to match oxygen supply and demand.

3.1 HIF-1 structure

HIF-1 is a heterodimeric transcription factor consisting of HIF-1α and HIF-1 β (also known as aryl hydrocarbon nuclear translocator or ARNT) subunits. The amino terminal of HIF-1β contains a basic helix-loop-helix (bHLH) domain and a PER-ARNT-SIM (PAS) homology domain. The bHLH domain and PAS domain are characteristic of a family of eukaryotic transcription. The HIF-1α subunit also contains a bHLH (aa.17-71). The PAS domain of HIF-1α is divided into two subdomains, PAS-A (aa.85-158) and PAS-B (aa.228-298) that are required for dimerization and binding to HIF-1β and the HRE (hypoxia response element) DNA core recognition sequence (5'RCGTG-3'). While the N-terminal half of the 826-amino acids HIF-1α protein is required for dimerization and binding the C-terminal half is essential for transactivation. The transactivation domains (TADs) are localized to aa. 531-575 (N-terminal TAD) and aa. 786-826 (C-terminal TAD), which are separated by an inhibitory domain. Nuclear localization signal (NLS) is localized at C-terminal (aa.718-721) and plays an important role in mediating nuclear transport. Moreover the C-terminal also contains two sequences rich in proline (P), glutamic acid (E), serine (S), and threonine (T). These PEST like motifs are involved in the intracellular degradation of the protein. HIF-1α is a very unstable protein with a half-life of less than 5 minutes under nomoxic conditions. It has now been shown that a

oxygen dependant degradation domain (ODDD) which contains the two PEST like motifs controls HIF-1α degradation by the ubiquitin-proteosome pathway (32,38,39).

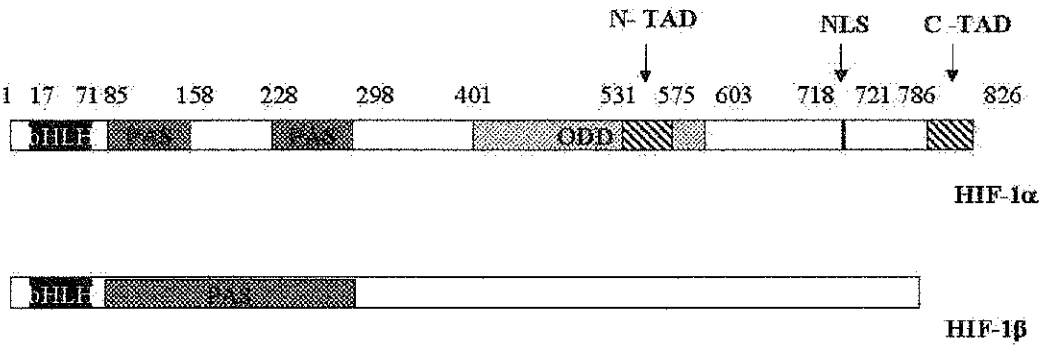

Figure 1: Schematic diagram of HIF-1 alpha and beta subunits. HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits. The black domains represent the homologous basic helix-loop-helix (bHLH) domain and dark gray area the PER-ARNT-SIM homology (PAS) domains. Carboxy terminal domains control gene transcription (C-TAD: transcription activation domains). Oxygen dependant domain (ODD) regulate the oxygen dependant degradation. Nuclear localization domains are indicated by NLS. (Adapted from Semenza et. Al.)

3.2. Oxygen-dependant regulation of HIF-1α

The biological activity of transcription factor HIF-1 is determined by the expression and activity of the HIF-1α subunit. While the ARNT subunit is constitutively expressed, the HIF-1α subunit is expressed but rapidly degraded under normoxic conditions such that almost no HIF-1α protein accumulates. Degradation of the HIF-1α subunit under normal conditions is triggered by post-translational hydroxylation of conserved proline residues (402 and 564) within the oxygen dependant degradation domain (ODDD). These proline residues are embedded within the amino acids motif LXXLAP, which is conserved in the HIF-1α protein of different species and HIF-2α.

The hydroxylation reaction is executed by a family of oxygen dependant iron (II) prolyl hydroxylase enzymes (Semenza, 2001 78 /id) (40). These enzymes use molecular oxygen as a substrate and ferrous iron as a cofactor to hydroxylate the proline residues and generate carbon dioxide and succinate as by-products. The motifs containing hydroxylated proline residues are than recognized by the Von Hippel-Lindau protein (pVHL), a componenet of the E3 ubiquitin ligase protein complex that tags the HIF-1α subunit for degradation by the proteasome. Since the activity of PHDs depend on the availability of oxygen, hypoxic conditions limit the hydroxylation of prolines residues thereby preventing binding of pVHL to HIF-1α, hence stabilizing the protein. HIF-1α subunit can then accumulate within the nucleus. Upon binding to its ARNT partner, the homodimeric transcription factor can now bind to hypoxia responsive elements (Fisher, 1994 2 /id) within the promoters of hypoxia responsive genes. After the HIF transcription factor binds to HRE sequences on target genes, P300/CBP transcriptional co-activators must be recruited to initiate transcription. Under normal conditions, an asparaginyl hydroxylase hydroxylates a conserved asparagine residue. Similarly to PHDs, the asparagyl hydroxylase also uses molecular oxygen as a substrate. Hydroxylation of the asparagine residue blocks interaction of the C-TAD with the p300/CBP transcriptional coactivators thus preventing transcription. Under hypoxic conditions, asparagine hydroxylation is inhibited, thereby facilitating the recruitment of coactivators by HIF-1α, and promoting transcription. It has been shown that both prolyl and asparagyl hydroxylase reactions should be prevented for full induction of HIF-1. It has been suggested that regulation involving two independent checkpoints, ensures a tight control of the hypoxic response pathways (41).

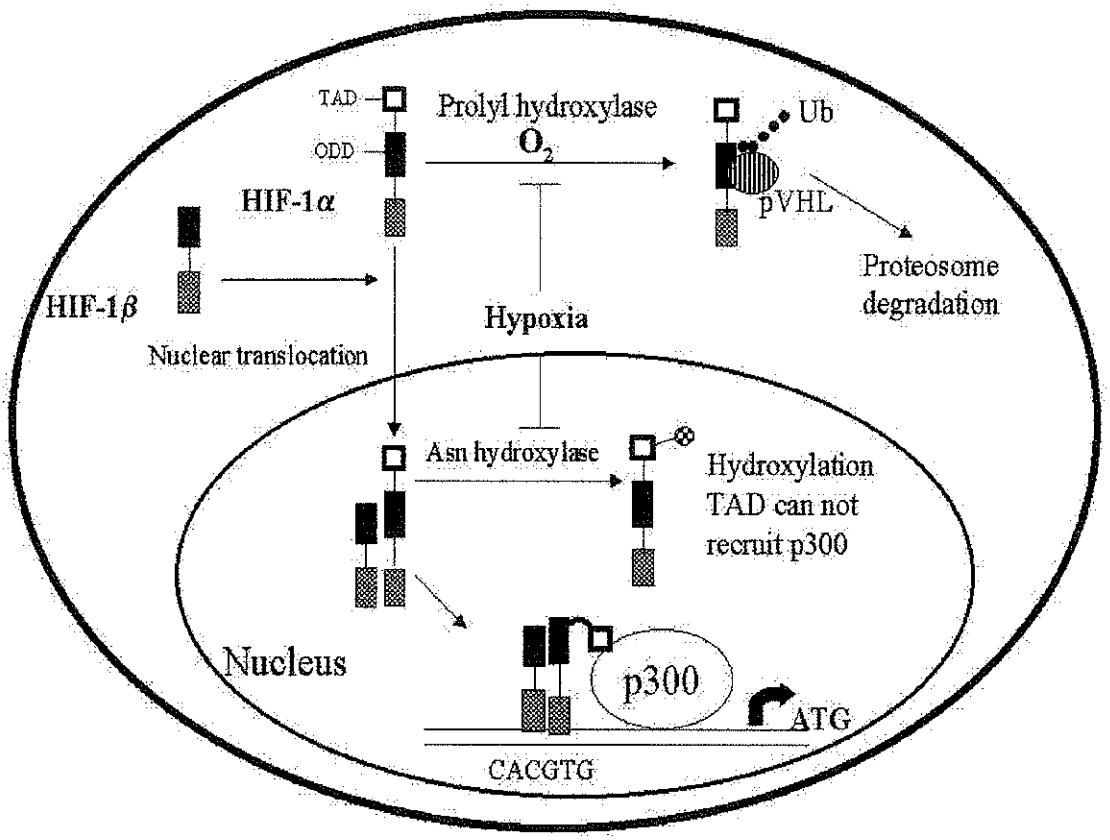

Figure 2. Schematic diagram of oxygen dependant regulation of HIF-1 transcrition factor.

Hif-1α and Hif-1β are constitutively expressed. Under normal conditions, Hif-1 α's oxygen dependant degradation domain (ODD) is hydroxylated in a oxygen dependant manner by prolyl hydroxylase. PVHL recognizes the hydroxylated Hif-1 α and recruits other ubiquitination enzymes. Once ubiquitinated, Hif-1α is degraded via the proteasome. Under hypoxic conditions however, prolyl hydroxylase is not functional, thus leading to the accumulation of HIF-1α. Upon binding to Hif-1β, the Hif-1α, Hif-1β heterodimer translocates in the nucleus. In the presence of oxygen, Asn hydroxylase hydroxylates Hif-1α at the transactivation domain (42). Hydroxylated TAD can not recruit transcription activator p300. In the absence of oxygen, Asn hydrozylase is inactive, thus allowing the recruitement of p300 and the transcription of target genes. Adapted from (43)

3.3 HIF-1α responsive genes

Once activated by hypoxia, HIF-1 translocates to the nucleus and initiates the transcription of target genes that provide cells with coping mechanisms against hypoxic stress. The activation of such genes allows cells to undergo a variety of biological changes and to adapt to low oxygen levels. In multicellular organisms, oxygen plays a fundamental role in energy metabolism. Under hypoxic condition, HIF-1 activates pathways to first adapt cells to low oxygen conditions to ensure the survival of cells. However under chronic hypoxic conditions, death inducing pathways are activated. HIF-1 activated genes can be classified into three major categories that reflect their function: energy metabolism, angiogenesis, and cell death.

3.3.1 HIF-1 target genes involved in energy metabolism

Under normal conditions, cells mainly harvest energy in the form of ATP through the oxygen dependant cellular respiration. This process is the most efficient catabolic pathway. In fact, aerobic respiration yields 38 ATP molecules per molecule of glucose. The metabolic reactions of cellular respiration occur in the mitochondrion and are catalysed by enzymes. Oxygen acts as the final acceptor of electrons that are passed from carrier to carrier of the electron transport chain. Therefore under hypoxic conditions, energy can not be generated through aerobic respiration and cells switch their method of glucose metabolism to glycolysis. In contrast to cellular respiration, glycolysis is an inefficient process that provides only two ATP molecules per molecule of glucose. Therefore under hypoxia, optimization in energy metabolism is crucial for survival. Indeed, HIF-1 has been shown to upregulate the expression of all the enzymes of the

glycolytic pathway (table 1) as well as glucose transporters (GLUT1 and GLUT3) (44-46).

Table 1. Glycolytic enzymes that have been shown to be upregulated by HIF

Glycolytic Enzymes upregulated by HIF-1	Reference
Hexokinase1 and 2	(44)
Phosphofructokinase L	(44)
Aldolase A and C	(45)
Glyceraldehyde phosphate dehydrogenase	(44)
Phosphoglycerate kinase 1	(46)
Pyruvate kinase	(44)
Enolase 1	(44)

Genes encoding for glycolytic proteins ensure an alternative form of energy production. However due to the lack of efficiency, the amount of energy produced during glycolysis is not adequate for long-term survival. Alternative mechanisms destined to increase oxygen delivery are critical for cell survival are initiated by HIF-1.

HIF-1 regulates the expression of several other genes that are involved in oxygen transport and iron metabolism. These genes include ceruloplasmin, erythropoietin, ferritin light chain, heme-oxygenase-1, transferrin, and transferrin receptor. When expressed these genes increase oxygen delivery and thus could restore aerobic respiration (47,48).

3.3.2 HIF-1 regulated genes involved in angiogenesis

The genes described above can increase oxygen delivery, only if blood vessels are present and intact. A second wave of HIF-1 regulated genes contributes to the development of new blood vessel formation or angiogenesis. This set of genes protects cells against a local obstruction in oxygen delivery and provides a long-term alternative to oxygen delivery. These include genes such as vascular endothelial growth factor (VEGF)-A, VEGF receptor-1, adrenomedullin, angiopoeitin, endothelin-1 and-2, fibroblast growth factor-3, hepatocyte growth factor, nitric oxide synthase and osteopoetin (47,48).

Angiogenesis plays a critical role in tumor progression. Studies have demonstrated that tumors can not grow beyond a diameter of 0.2 to 2 mm unless they have recruited surrounding blood vessels. This process requires an increase in angiogenic factors. VEGF is one of such angiogenic factors that is essential and has been studied extensively with respect to HIF-1. Hypoxia has been shown to induce VEGF transcription in a HIF-1 dependent manner. Hypoxia conditions have also been shown to stabilize VEGF mRNA (49).

3.3.3. HIF-1 regulated genes involved in cell death

The regulation of HIF-1 induced genes involved in glycolysis, erythropoesis and angiogenesis have been extensively studied in the last few years. However the mechanisms by which HIF-1 induces cell death are less well known.

A role for HIF-1 in cell death was first suggested by the ability of hypoxia to increase apoptosis in wild type HIF-1 +/+ embryonic stem cells but not in HIF-1 -/- embryonic stem cells. It was later shown that HIF-1 directly interacts and stabilizes p53

(50-52). p53 is a tumor suppressor gene. HIF-1 has been shown to promote p53 dependant apoptosis that is mediated by APAF-1 and caspase-9. Under hypoxic conditions, it has been shown that p53 binds to dephosphorylated HIF-1 and blocks transcriptional activity of HIF-1. Phosphorylated form of HIF-1 binds to ARNT and initiates the transcription of HIF-1 target genes. Hence it is thought that by binding and blocking HIF-1 transcriptional activity, p53 might prevent HIF-1 from transcribing survival genes. Furthermore binding of p53 to HIF-1 prevents its degradation through MDM2. Following modification by phosphorylation, p53 initiates the transcription of pro-apoptotic Bcl-2 family member such as BAX and thus induces cell death (52-55).

Recently, HIF-1 has also shown to directly induce the transcription of two related pro-apoptotic Bcl-2 family members: BNIP3 and NIX. Expression of both of these genes has been associated with induction of cell death (49,56,57).

Bcl-2 family of proteins constitutes one of the most biologically relevant family of cell death regulatory proteins. Originally, the Bcl-2 (B cell lymphoma/leukemia-2) gene was identified as the first proto-oncogene that contributed to neoplasia by cell survival rather than cell proliferation. Since the discovery of Bcl-2, the family of related mammalian proteins has expanded significantly. The Bcl-2 family is comprised of two functional groups: those that inhibit cell death and those that promote cell death. The death inducing category of Bcl-2 related proteins has been further divided into subclasses depending on the distribution of the four Bcl-2 homology domains (BH1, BH2, BH3, and BH4). Class I of pro-apoptotic proteins contain BH1, BH2 and BH3 domains and feature proteins such as BAX, BAK, and BOK. Class II proteins contain only BH3 and BH4. Bcl-Xs is one member of such protein. Class III proteins contain only the BH3 domain

and are therefore also named " BH3-only" proteins. This class features protein such as Bim and Bad. The BNIP3 subfamily is part of this class of proteins.

Bcl-2 family members regulate cell death by several mechanisms. Briefly, many of the Bcl-2 family members sub-localize to the mitochondria where pro-apoptotic members destabilize the membrane, resulting in a change in the mitochondrial membrane potential and promote the release of death inducing agents such as cytochrome c. On the other hand, anti-apoptotic proteins, stabilize the mitochondrial membrane by sequestring pro-apoptotic proteins from the mitochondria and thus prevent the release of death inducing agents. Furthermore domains of anti-apoptotic members Bcl-2 and Bcl-XL can bind to Apaf-1 thus inhibiting the association of caspase-9 with Apaf-1 and by consequence the activation of caspase-9(58).

3.4. HIF-1 and diseases

3.4.1. HIF-1 and cancer

HIF-1 α is overexpressed in over 90% of all colon, lung, ovary, prostate and stomach cancer. HIF-1α is also overexpressed in the majority of breast and colon cancer metastasis. HIF-1 α is not detectable in normal tissue from any of these organs(59-61).

HIF-1α expression in tumor cells may be increased by both physiologic induction (hypoxia) and genetic alterations. In fact mutations that activate oncogene v-src or inactivate tumor suppressor VHL are associated with increased HIF-1α protein expression. Autocrine growth factor stimulation can also activate HIF-1α expression. In fact insulin like growth factor 2(IGF 2) induces expression of HIF-1α (62).

HIF-1 α may play a role in the major processes of tumor progression. In fact, when injected into nude mice, tumor cells lacking HIF-1 α expression have an impaired

growth pattern and vascularization than their HIF-1 α expressing counterpart. It has been suggested that HIF-1 contributes to tumor progression through the following mechanisms: 1) HIF-1 mediates hypoxia mediated angiogenesis via activation of VEGF gene transcription which is necessary for both the establishment of the primary tumor and generation of metastases. 2) HIF-1 mediates adaptation to hypoxia via increases glycolysis and glucose transport. Adaptation to hypoxia is essential for tumor progression. 3) HIF-1 induced cell death may select for genes resistant to both hypoxia and cell death.

The involvement of HIF-1 in tumor progression may offer a therapeutic window to treat cancer. Thus strategies designed to inhibit HIF-1 expression or activity may represent a novel approach to treat cancer (63-65).

3.4.2. HIF-1 and cerebral and myocardial ischemia

As discussed earlier, HIF-1 possesses the ability to induce both survival and cell death in response to hypoxia. The implication of such paradoxically dual role of HIF-1 in ischemia is not very well understood. Some studies have suggested that HIF-1-dependant cell death might be a contributing factor to tissue injury. While other studies have suggested that HIF-1 might be involved in activating survival pathways thus rescuing cells from hypoxia induced cell death (66-68).

4. The BNIP3 Subfamily

4.1. BNIP3 and NIX

BNIP3 (Bcl-2/nineteen kD-interacting protein-1; formerly NIP3) forms a Bcl-2 subfamily of death-inducing mitochondrial proteins that includes the mammalian

homologue NIX (BNIP3-like protein X; also called BNIP3α/BNIP3L/B5)(7,69,70). BNIP3 was originally identified in a yeast two-hybrid screen using the adenovirus E1B 19 Kda protein as bait(71) . The E1B 19 Kda is a viral product that protects against cell death induced by certain stimuli including viral infection. BNIP3 was subsequently found to interact with the antiapoptotic protein Bcl-2(72). Search for homologues to the human BNIP3, which is located on chromosome 14q11.2-q12 led to the discovery of NIX. NIX shares 56% identity with BNIP3 and maps to chromosome 8q21(73-75).

4.2. BNIP3 and NIX structures

BNIP3 family members share similar structure. They contain a NH_2-terminal PEST sequence, a BH3 domain and a C-terminal TM domain. The PEST domain is rich in proline (P), glutamate (E), serine (S), and threonine (T). PEST sequences are usually present in proteins that are degraded by the proteosome. It has been shown that cells that are transfected by BNIP3 or NIX show decreasing amount of the transfected protein over time suggesting a mechanism involved in their degradation. Treatment with lactacystin, a proteosome inhibitor, resulted in an inhibition of the degradation.

BNIP3 family members also contain a region homologous to the BH3 domain, hence their classification as BH3-only members of the Bcl-2 family. The BH3 domain is an eight amino acid core sequence with conserved leucine and aspartate at position 1 and 6. These two residues are thought to be involved in heterodimerization with Bcl-2 protein. Typically the BH3 domain of pro-apoptotic Bcl-2 proteins mediates heterodimerization with Bcl-2/Bcl-X_L, however BNIP3 and ceBNIP3 bind Bcl-2/Bcl-X_L independently of their BH3 domains(74,76). Instead, the NH_2-terminus (residues 1-49) and TM domain of BNIP3 are required for binding Bcl-2, and either region is sufficient

for heterodimerization with Bcl-X_L. BH3 independant homo- and heterodimerization has also been observed for NIX. ceBNIP3 has also been shown to heterodimerize with CED-3 and CED-4(76), although the homologous interactions for BNIP3 and NIX have not yet been reported. Functionally, deletion of the BH3 domain has no significant effect on the killing activity of BNIP3. However, substitution of the BNIP3 BH3 domain for the corresponding sequences in Bax functionally restores Bax pro-apoptotic activity and heterodimerization with Bcl-X_L. Thus, the BH3 domain of BNIP3 appears to be functionally equivalent to the Bax BH3 domain, but only in the context of the Bax peptide, which may be due to conformational changes that differ from BNIP3. This suggests that BNIP3 and NIX induce cell death in a unique fashion unseen in other BH3-only proteins.

BNIP3 also contains a carboxyl-terminal TM domain at residues 164 to 184 that is essential for mitochondrial localization, homodimerization, and cell killing activity. Deletion of the TM domain abolishes the killing activity of both proteins. Although the TM domain is required for mitochondrial localization, chimeric BNIP3 proteins that are targeted to nonmitochondrial sites by TM domain swapping are nearly as efficient at inducing cell death as the wild type provided that the protein is anchored to an intracellular membrane.

Expression of BNIP3 lacking the TM domain protects cells against BNIP3 induced cell death. It was suggested that this dominant negative form of BNIP3 might interact with the endogenous BNIP3 and prevent its translocation to the mitochondria thus preventing cell death. A truncated isoform of NIX termed sNIX that did not contain

the TM domain was shown to heterodimerize with NIX and protect against NIX induced cell death.

Like Bax, endogenous BNIP3 is loosely associated with the mitochondria, but is integrated into the outer membrane when overexpressed. This would suggest that active BNIP3 is integrated into the outer mitochondrial membrane in response to an apoptotic stimulus, although the mechanism remains unknown (73-76).

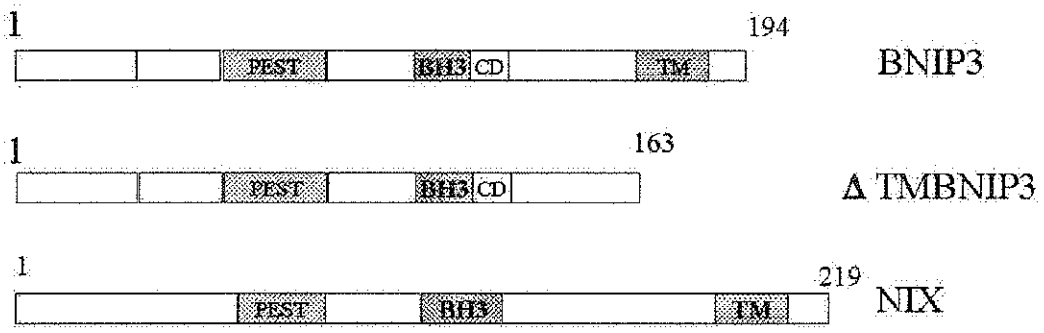

Figure 3 : Schematic diagram of the BNIP3 , Δ TMBNIP3 and NIX proteins. BNIP3 and NIX are members of the BH3 only family of Bcl-2 proteins. They both contain proline (P), glutamate (E), serine (S), and threonine (T) PEST domain that is involved in protein degradation. They also contain the BH3 domain and the mitochondria targetting transmembrane domain (TM). Schematic diagram of the BNIP3 dominant negative form lacking the TM domain (Δ TM BNIP3) is also shown.

4.3. Model of BNIP3 induced cell death

Overexpression of BNIP3 and NIX induces cell death in a variety of mammalian cell lines(77,78). Although Bcl-2 and Bcl-X_L can heterodimerize with these BNIP3

family members, overexpression of Bcl-2 and Bcl-X_L only delays the onset of cell death, and can completely surppress it only when expressed at very high levels (79).

Vande Velde et al. (2000) described a necrotic-like cell death in BNIP3-expressing cells as opposed to apoptotic cell death. Overexpression of BNIP3 induced cell death with early plasma membrane permeability, mitochondrial damage and mininal nuclear damage that are characteristic of necrosis. Furthermore, BNIP3-induced cell death was independent of cytochrome *c* release, Apaf-1, caspase-9, and caspase-3. Treatment with pan-caspase inhibitor z-VAD-fmk did not suppress BNIP3 induced cell death. BNIP3 induces cell death that is caspases, Apaf-1, and cytochrome c release independent (80).

A rapid opening of the mitochondrial permeability transition (PT) pore, $\Delta\Psi m$ suppression, and increased ROS production were also observed. The PT pore inhibitors cyclosporin A and bongkrekic acid efficiently blocked BNIP3-induced cell death, indicating that mitochondrial dysfunction is the primary cause of death. In addition, electron microscopy of BNIP3-transfected cells revealed extensive cytoplasmic vacuolation, autophagosomes, and mitochondrial deformation in which the internal cisternae were destroyed. This study showed that BNIP3 induces a necrotic like cell death in human epithelial cells (81).

The literature is however divided with respect to the characteristics of BNIP3 induced cell death. In ventricular myocytes, treatment with z-VAD-fmk suppressed BNIP3 induced cell death in a dose dependant manner suggesting the involvement of caspase (82). Furthermore the expression of BNIP3 in ventricular myocytes also induces mitochondria cytochrome release.

Yet another study showed that under hypoxia and acidosis, cardiomyocytes were dying with intact plasma membrane and above 70% ATP levels until late time points. Since necrotic cell death typically involves a rapid loss of plasma membrane integrity and ATP levels, the authors concluded that under hypoxia/acidosis, cardiomyocytes were dying of a cell death similar to apoptosis. However upon treatment with broad range caspase inhibitors, BNIP3 induced cell death was not inhibited in these cardiomyocytes. These results suggest that under hypoxia and acidosis, BNIP3 induces a caspase independent cell death with apoptotic morphology in cardiomyocytes (Guo, 2001 24 /id).

The differences in BNIP3 induced cell death characteristics may be due to the differences in cell types and hypoxic conditions studied. While BNIP3 may induce a necrotic like cell death in epithelial cells, in ventricular myocytes BNIP3 might induce alternative pathways that are caspase dependant or independent, with apoptotic morphology.

Recently, it has been shown that after T cell stimulation via CD47, BNIP3 translocates to the mitochondria to induce cell death. CD47 is a surface receptor which induced either coactivation or cell death in lymphocytes. The CD47 induced cell death pathway is independent of caspase activation and cytochrome c release and is characterized by suppression of mitochondrial membrane potential. Immunoprecipitation analysis and yeast two-hybrid system using CD47 as bait showed that BNIP3 and CD47 interact with each other. Futhermore, CD47 induced cell death was inhibited by reduced BNIP3 expression mediated via BNIP3 antisense oligonucleotide treatment. This study has shown that BNIP3 induced cell death can be induced by surface receptors (83,84).

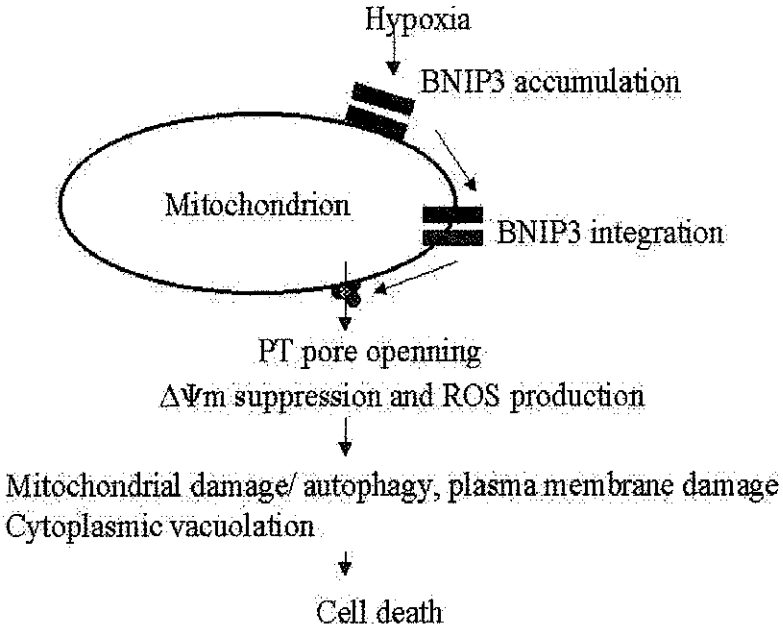

Figure 3: Model of BNIP3 induced cell death under hypoxia. Overexpression under hypoxia permits integration of BNIP3 into the outer mitochondrial membrane through its TM domain. BNIP3 than initiate permeability transition pore opening and $\Delta\psi m$ suppression with increased ROS production in an undefined sequence, leading to cell death. Adapted from Vande velde et al. 2000.

4.4. BNIP3 and HIF-1

BNIP3 protein and mRNA levels have been shown to increase under hypoxic conditions in both epithelial cells and cardiomyocytes. Similarly, Nix mRNA also increased when cells were incubated under hypoxia (85,86). No BNIP3 protein was detected in cells incubated under normoxic conditions even in the presence of proteosome inhibitor. This suggests that expression of endogenous protein is weak under normal oxygen levels.

Few mechanisms have been found to induce transcription of target genes under hypoxia. Hypoxic conditions have shown to activate both transcription regulators HIF-1α and p53. It has also been reported that the expression of p53 is increased upon hypoxia through a HIF-1 dependant pathway. Overexpression of HIF-1α but not p53 induced the expression of BNIP3 thus suggesting that HIF-1α directly upregulates BNIP3 expression, via a p53-independent pathway. In fact, renal cancer cell line (RCC4), lacking functional pVHL, has high levels of BNIP3. As described earlier, pVHL is involved in the degradation of transcription factor HIF-1α. Reintroduction of functional pVHL in these cells restored HIF-1 degradation and reduced BNIP3 levels. Furthermore in a Chinese Hamster Ovary (CHO) cell line that contains defective HIF-1α, BNIP3 protein levels failed to increase upon hypoxic exposure.

The rodent BNIP3 promoter has been characterized for hypoxia responsiveness. A functional hypoxia response element that matched consensus HIF-1 binding sequence was identified in the rodent BNIP3 promoter. Mutation of this HRE site in the rodent BNIP3 promoter lead to a complete loss of hypoxia induced transcriptional activation (87). It is however unknown whether the human BNIP3 promoter is similarly hypoxia regulated.

4.5. BNIP3, Nix and Cancer.

Hypoxic treatment induces BNIP3 expression in bladder, endothelial, colon, and prostate human carcinoma cell lines. Moreover Northern blot analysis of RNA extracted from tumor and distant normal breast obtained from mastectomy samples has shown that Nix and BNIP3 are highly expressed in tumors samples compared to normal tissue. BNIP3 mRNA was also detected in 5/9 tumors consisting of 1 /2 SSC head and neck

carcinomas, 1 pancreas carcinoma, 1 /2 ovarian carcinomas, and 2 breast carcinomas. BNIP3 expression was not detected in 1 lung carcinoma, 1 lymphoma, and in two cases of normal breast tissue. Using serial analysis of gene expression (SAGE) it was also revealed upregulation of NIX in a glioblastoma cell line under hypoxia. In situ hybridization showed that BNIP3 protein is expressed in necrotic regions of tumors (56).

The implication of BNIP3 and Nix overexpression in human tumors is not understood at the present time. It is however speculated that BNIP3 and NIX expression is in cells resistant to hypoxia induced cell death. On the other hand, BNIP3 and NIX both induce cell death upon overexpression. Thus additional information on the BNIP3/ NIX induced cell death pathways might be helpful in understanding their implication in cancer and design novel therapies.

4.6. BNIP3, NIX and cardiomyocytes

Understanding of the mechanisms underlying hypoxia induced cardiomyocyte cell death is under active investigation. As seen earlier, hypoxia induces massive cell death during both cardiac ischemia and stroke. It was shown that both BNIP3 and NIX are overexpressed in cardiomyocytes exposed to hypoxia. Futhermore, BNIP3 overexpression in cardiomyocytes leads to cell death. The implication of BNIP3 induced cell death is ischemia is under investigation. It was shown that reduction in BNIP3 protein expression upon treatment with antisense oligonucleotides against BNIP3 results in a reduced cardiomyocyte death. Furthermore, cell death was also reduced in ventricular myocytes expressing the dominant negative form of BNIP3 (ΔTM BNIP3)(88).

II. Rational and Hypothesis

HIF-1 transcription factor is activated under hypoxia. BNIP3 has been shown to be upregulated by hypoxia. Forced BNIP3 expression induces cell death. The mechanism of rodent BNIP3 upregulation by HIF-1 has been elucidated. However the transcriptional upregulation of human BNIP3 promoter is unknown.

We hypothesized that the human BNIP3 gene is upregulated by transcription factor HIF-1 during hypoxia and is involved in hypoxia induced cell death.

III. Objectives

1. To determine the contribution of BNIP3 during hypoxia induced cell death in human epithelial cells.
2. To determine the role of HIF-1 in the transcriptional upregulation of human BNIP3 during hypoxia.
3. To determine regions within the human BNIP3 promoter critical to its hypoxia responsiveness

IV. Materials and methods

1. Reagents

All chemicals were purchased from Sigma-Aldrich Chemical co. (St Louis,MO) unless otherwise stated.

2. Media

2.1 Bacterial culture media

All bacterial cultures were grown in standard Luria Bertani broth and agar. Pre-mixed powders for LB broth and LB agar from Difco (89) were dissolved in the required amount of distilled deionized water (ddH2O) and autoclaved at 120°C for 20 minutes. The LB broth was stored at 4°C until required and was supplemented with ampicillin at a final concentration of 50 μg /mL prior to use. The LB agar was cooled to 55°C, then supplemented with 50 μg /mL ampicillin and poured into 100 mm petri plates (Fisher Scientific Canada, Nepean, ON, Canada). The agar was allowed to solidify and dried at room temperature, then stored at 4°C until needed. The hBNIP3 BAC bacterial culture was grown in chloramphenicol (60 μg /ml) supplemented LB broth and agar plates. For bacterial transformation, commercially available S.O.C medium (Invitrogen) was used.

2.2. Cell culture media

Dulbeco's Modified Eagle Media (DMEM, GIBCO BRL), α-minimal essential medium (α-MEM, GIBCO BRL), and F12 media were made according to manufacturers' instructions. The pH was adjusted to between 7.0 to 7.2. All media were filter sterilized

and stored at 4°C. Prior to use, the media were supplemented with 10% v/v fetal bovine serum (FBS) (GIBCO BRL), 5% penicillin/streptomycin and pre-warmed to 37°C.

3. Antibodies

Mouse monoclonal antibody against human BNIP3, amino 112-124 (Sowter, 2001 40 /id) (1/20000) and mouse monoclonal antibody that recognizes human HIF-1 α (Transduction laboratories) (1/5000) was used for detection on Western blots. Goat anti-mouse horseradish peroxidase (Bio-Rad laboratories Canada Ltd, Mississauga, ON) (1/10000) and enhanced chemiluminescence developing reagents (Perkin Elmer Life Science) were used to detect the immunoreactive bands.

4. Bacterial Strains

Plasmid DNA was transformed into commercially available competent *Escherichia coli* cells (INVF', competent cells, Invitrogen). All transformed bacterial stocks were maintained at –80 °C in 20 % glycerol. Transformants were recovered by streaking on to LB plates containing the appropriate antibiotic and incubating at 37°C overnight.

5. Cell Lines

Human embryonic kidney 293T epithelial cells were grown in DMEM (GIBCO BRL) supplemented with 10% v/v FBS. MCF-7 breast carcinoma cells were grown in α-MEM supplemented with 10 % v/v FBS, 5% L-Glutamine (200 mM, GIBCO BRL), 5% MEM Sodium pyruvate (100 mM, GIBCO BRL) and 5 % HEPES Buffer solution (1M GIBCO BRL). Chinese Hamster Ovary (CHO) parental cell line and CHO Hypoxia Inducible

Factor-1 alpha (HIF-1α) defective cell line (CHO KA13) (gift from Dr. A. Harris, Institute of Molecular Medicine, UK) were cultured in F:12 medium supplemented with 10% FBS, 1% L-glutamine (GIBCO, BRL) and antibiotics. All cell lines were grown in humidified incubators in the presence of 5% CO_2 at 37°C. When indicated, cells were maintained under hypoxic conditions at 37°C within a hypoxic chamber (Forma Scientific, Marietta. Ohio) filled with 5% CO_2 balanced with N_2. The hypoxic chamber thus creates an atmosphere that contains less than 2% oxygen.

6. Construction of deletion and base pair BNIP3 promoter mutants.

BAC clone H_NH0045A17 containing the human BNIP3 genomic sequence was used as template with appropriate primers to amplify 1.2 kb of the BNIP3 promoter. Primers were designed to incorporate Bgl II and Mlu I restriction enzyme sites at the 5' and the 3' ends respectively. To generate of the deletion mutant constructs, primers containing the same Bgl2 and MluI sites were designed to systematically delete approximately 200 bp at a time (table 2). To generate base pair mutants, primers were designed to incorporate the mutation at the HRE1 and HRE2 sites. Following restriction digestion, the inserts were ligated upstream of a luciferase gene into the pGL3 basic (Promega) reporter vector. The Hif-1α CDNA and dominant negative plasmids were kindly provided by Dr. Lorrie Kirshenbaum.

Table 2: Primers and their corresponding sequence used in generating delation and base pair BNIP3 promoter mutants.

Primer	Sequence
Deletion mutant 1 (90) primer	**Gggacgcgt**tcctctgaaaagtgcctccca
Deletion mutant 2 (D2) primer	**Gggacgcgt**ctctttcgactctgctcgagc
Deletion mutant 3 (44) primer	**Gggacgcgt**gagacgctcagctccggccc
Deletion mutant 4 (D4) primer	**Gggacgcgt**gtggtagccagtgcccagaga
Deletion mutant 5 (D5) primer	**Gggacgcgt**accggaggcctctgcccctc
Deletion mutant 6 (D6) primer	**Gggacgcgt**gccctgccctgtgagttcctc
Deletion mutant 8 (D8) primer	**Gggacgcgt**ctccgacctccgctttc
BNIP3 promoter 5' primer	**Gggacgcgt**ctttcccgcaagaccagacac
BNIP3 promoter 3' primer	**Gggagatct**caactgcggcgatcggagtc
	Gggacgcgt: MluI restriction site **Gggagatct: BglII restriction site**

7. Western blot analysis

Western blot analysis by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to identify proteins in cell lysates using specific antibodies. Cell were lysed using a buffer containing 50 mM Tris HCl (pH 7.4), 250 mM NaCl, 0.5% NP40, 50 mM NaF, 15 mM sodium pyrophosphate, 1mM glycerophosphate, 1mM Na3VO4, 500 nM okadeic acid, 20 μg aprotinin/mL, 0.7μg pepstatin/mL, 5μg leupeptin/mL, and 1mM PMSF. Each sample (50μg to 100 μg) of cell lysate was separated by electrophoresis at 80 V through a 5 mm 10% SDS-polyacrylamide gel (reference) in running buffer. To transfer the proteins to a nitrocellulose membrane (Bio-

Rad laboratories Canada Ltd, Mississauga, ON), the gel, the membrane and filter papers were soaked in transfer buffer, layered in a transfer apparatus and set at 1 Amp for 45 minutes. The membrane was then incubated in blocking buffer (2% skim milk in 0.1 M TBS supplemented with 0.2 % V/V Triton X) for 1 hour at room temperature. The membrane was incubated overnight at 4°C with the appropriate antibody diluted in blocking buffer. After removal of the primary antibody solution, the membrane was washed for 40 min in 0.1 M TBST with agitation changing the buffer every 5 minutes. The membranes were then incubated in blocking buffer containing the secondary antibody for 1 hour. The blots were washed again for 40 min in 0.1 M TBST with agitation changing the buffer every 5 minutes. The blots were then developed using enhanced chemiluminescence as described by manufacturer (ECL, Amersham Pharmacia Biotech). Blots were exposed to X-ray film for desired length of time and processed in a tabletop automated developer.

8. Transient transfection: lipofectAMINE method

In preparation for transient transfection, cells were plated 24 to 48 hours in advance at a density of $1x10^5$ cells per 6 well plates or 6mm plates. Cells were transfected using the polycationic lipid LipofectAMINE reagent (GIBCO BRL). For each transfection 0.8 μg of the desired plasmid and 0.2 μg of the plasmid monitoring for transfection efficiency (β-galactosidase in pcDNA3) were diluted in 100 μl of serum free medium (OPTI-MEM 1 Reduced Serum Medium, GIBCO BRL) in a 15 ml conical tube. In another tube, 7 μl LipofectAMINE reagent was diluted in 100 μl of serum free medium. The diluted DNA was added drop-wise to the LipofectAMINE reagent. The

DNA-liposome complexes were allowed to form for 30 minutes at room temperature. An additional 0.8 ml serum free medium was added to each transfection mix. Cells were washed once with serum free media and overlaid with the 1ml of transfection mix. Following 4 to 5 hours incubation at 37°C, the lipofectamine mixture was removed and normal growth medium was added. The cells were then cultured in either hypoxic or normal conditions as required and then analyzed for either luciferase activity, cell viability, or protein expression.

9. B-galactosidase cell death assay

HEK 293 T ($1x10^5$) cells were seeded in 6 well plates. The next day, using lipofectamine reagent (Invitrogen) cells were transfected with 0.2 μg of pcDNA3-β-galactosidase (pcDNA3-β-gal) plasmid plus 1 μg of pcDNA3-BNIP3ΔTM. After 4 hours transfection, cells were incubated either in DMEM under normal or hypoxic conditions for 48 hours. Following incubation, cells were fixed in 0.2 % glutaraldehyde and washed three times with 0.1M phosphate buffer saline (PBS) and stained in Xgal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 3mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6 \bullet 3H_2O$, 1mM $MgCl_2$ in 0.1 M PBS) to detect for β-galactosidase expression as described previously (Miura et al.1993). The percentage of dead cells was calculated by assessing the number of round, condensed, blue cells in the total population of flat blue cells. A total number of at least 200 cells were counted for each experiment.

10. Electrophoretic Mobility Shift Assay

Oligonucleotides analyzed in this experiment are as follows: control (W18) (5'-

AGCTTGCCCT**ACGTG**CCTGTCTCAG-3'), HRE1 (5'GCTTCCCTG**CACGTC**CTC ACGC-3'), HRE2 (5-CGTGTGG**CACGTG**CGGCGCGT-3') and HRE2 mutant (5'-CGTGTGG**GTGGTG**CGGCGCGT-3'). Probes (control, HRE1, HRE2 and HRE2 mutant) were radioactively 5' end-labeled with [γ-^{32}P] ATP (Perkin Elmer) and T4 polynucleotide kinase (BioLabs). Cell incubated under normoxic and hypoxic conditions were lysed in a buffer containing 50 mM Tris HCl (pH 7.4), 250 mM NaCl, 0.5% NP40, 50 mM NaF, 15 mM sodium pyrophosphate, 1mM glycerophosphate, 1mM Na_3VO_4, 500 nM okadaic acid, 20 μg aprotinin/mL, 0.7μg pepstatin/mL, 5μg leupeptin/mL, and 1mM PMSF. Binding reactions were carried in a total volume of 20 μl containing 5 μg of protein extract and 0.1μg of denatured herring sperm DNA (Sigma) in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50mM NaCl, 1mM $MgCl_2$, 1 mM EDTA, 5mM DTT, and 5% glycerol. After preincubation of the protein mixture for 5 minutes at room temperature, probes were added to the binding reactions and the incubation was continued for an additional 30 minutes. The reaction mixtures were then loaded onto a 5% nondenaturing polyacrylamide gel. Electrophoresis was performed at 180V in a 0.3X TBE (1X TBE is 89 mM Tris-HCl, 89 mM boric acid, and 5mM EDTA) at 4 °C. Gels were vacuum dried and exposed to X-ray films at –80 °C for 18 to 24 hours. The films were processed in a tabletop automated developer.

11. Antisense Experiment

Antisense oligonucleotide (5'-GCGCTCCGTTCTGCGACATG-3') (Sigma Genesys, Oakville, ON) and sense oligonucleotide (5'-GTACAGCGTCTTGCCTCGCG-3') were complimentary to bases –1 to +19 of the human Bnip3 gene. Random sequence

oligonucleotide (5'-GCAGTCAGCGACGTCGAAGC-3') contained the same bases in a scrambled sequence. Oligonucleotides were phosphorothioate modified to prevent degradation. Oligonucleotides were diluted in Opti-MEM (Gibco BRL) were mixed with oligofectamine (Invitrogen) and incubated with HEK 293 cells for 4 hours then hypoxic medium was added and cells were further incubated under hypoxia for 24 hours to 48 hours. At indicated times, cell death was determined via Trypan blue exclusion assay, or cells were lysed and protein extracts were analyzed for BNIP3 expression as described in Western blot analysis.

12. Trypan blue cell death assay.

Trypan blue exclusion assays were performed to identify compromised plasma membranes. Culture media were removed and replaced with 0.4% Trypan blue in PBS for 5 minutes at 37°C. The percentage of dead cells was calculated by counting the number of blue cells in a population of clear cells. A total number of at least 200 cells were counted for each assay.

V. RESULTS

1. Treatment with antisense oligonucleotide against BNIP3 blocks hypoxia induced cell death.

It has been shown previously that when overexpressed BNIP3 induces cell death. It has also been shown that in rodent BNIP3 protein levels increase under hypoxic condition. Blockage of BNIP3 induction in mouse cardiomyocytes reduces ischemia induced cell death. It is however unknown if BNIP3 is involved in hypoxia induced cell death in human epithelial derived cells. Hence, using antisense oligonucleotides against BNIP3 to reduce BNIP3 expression, we determined if hypoxia induced cell death was affected in HEK 293 cells. For 48 hours, 293 cells were treated with random, sense and antisense oligonucleotide against human BNIP3, exposed to hypoxic treatment and analyzed for cell death by trypan blue exclusion assays. In order to determine if antisense treatment was specific against BNIP3, BNIP3 protein levels were first analyzed by Western blotting. As shown in Fig.5A, only treatment with antisense oligonucleotides resulted in decreased expression of BNIP3 protein as compared to non-treated cells or cells treated with random or sense oligonucleotides under hypoxic conditions. Oligonucleotide treatments had no significant effect on β-actin levels. The lower expression of BNIP3 protein in cells treated with antisense oligonucleotides correlated with a reduced cell death at 48 hours under hypoxic conditions. As shown in Fig.2B, 48 hours hypoxic treatment resulted in 29%, 28 % and 27 % in non-transfected, random oligonucleotide transfected and sense oligonucleotide transfected cells respectively. In contrast, cells transfected with the antisense oligonucleotide against BNIP3 had a 12%

cell death. Therefore, treatment with antisense oligonucleotide against BNIP3 protects against hypoxia induced cell death.

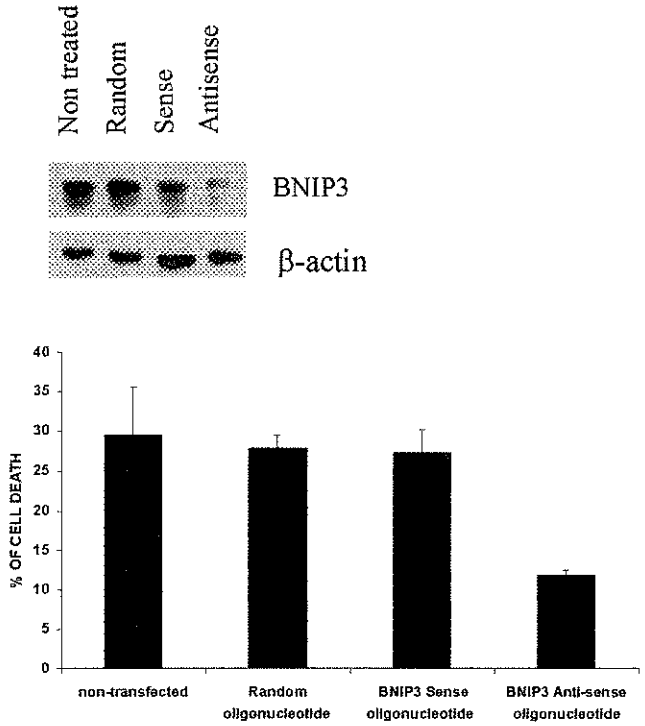

Figure 5 :Treatment with antisense oligonucleotides against BNIP3 reduces hypoxia induced cell death. A)HEK 293 cells were incubated with random, sense and anti-sense oligonucleotides against BNIP3. Cells were subjected to hypoxia for 48 hours. Proteins were isolated and Western blotted with monoclonal anti-BNIP3 antibody. The blots were stripped and reprobed for β-actin to determine equal loading. B) Cells were treated with oligonucleotides as in A. After a 48 hours hypoxic incubation, cell death was measured by trypan blue exclusion assay. Differences between cells treated with random oligonucleotide and antisense oligonucleotide were statistically significant with a p value of 0.002. The error bars represent standard deviation between three independent experiments.

2. Expression of human BNIP3 protein lacking the transmembrane domain reduces hypoxia induced cell death.

Deletion of the transmembrane domain of BNIP3 (BNIP3ΔTM) (Fig.6A) abolishes the death promoting activity of BNIP3 upon overexpression. Furthermore when expressed in cardiomyocytes, BNIP3ΔTM acts as a dominant negative protein blocking endogenous BNIP3 from translocating to the mitochondria and inducing cell death. In order to confirm that BNIP3 activity is required in HEK 293 cells to induce cell death under hypoxic conditions, BNIP3ΔTM was transiently expressed and cells were exposed to hypoxic conditions for 48 hours. As shown in Fig.6B, hypoxia induced cell death was reduced from 30% in cells transfected with vector alone as negative control to 18 % in cells expressing BNIP3ΔTM. These results suggest that blockage of endogenous BNIP3 activity results in reduced hypoxia induced cell death suggesting that endogenous BNIP3 plays a role in hypoxia induced cell death.

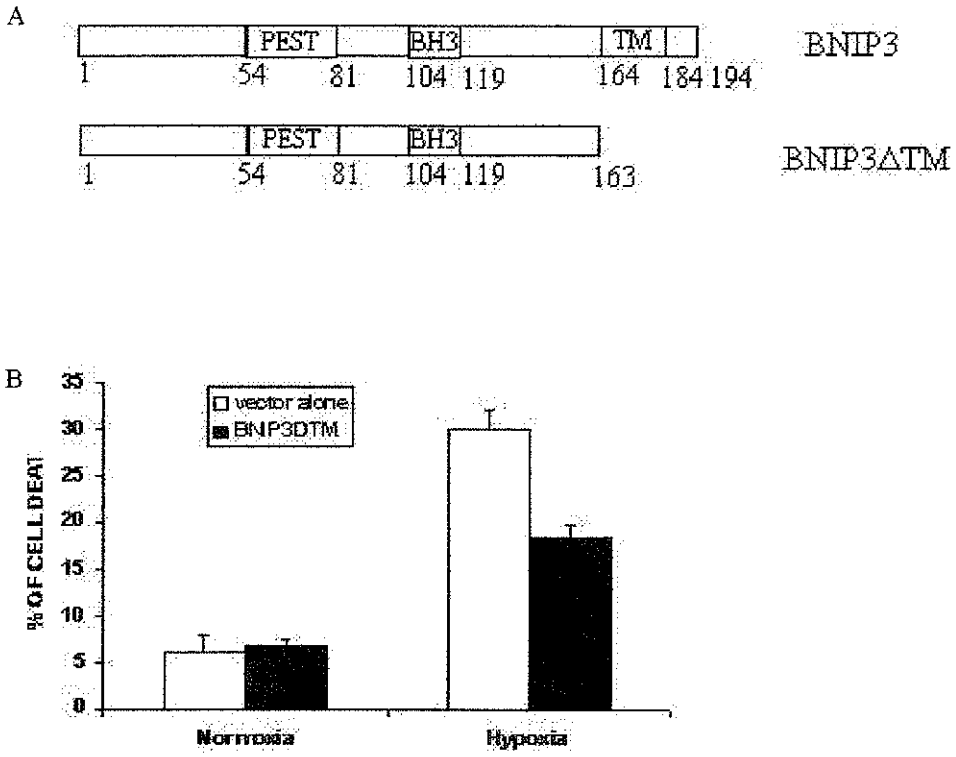

Figure 6: Expression of dominant negative BNIP3 reduces hypoxia induced cell death A) Schematic diagram of the human BNIP3 and ΔTm BNIP3 protein B)HEK 293 cells were transfected with the dominant negative BNIP3ΔTM form of BNIP3 in a pcDNA3 expression vector or vector alone in combination with β-galactosidase cDNA in pcDNA3. Cells were incubated under hypoxia for 48 hours, and cell death was measured as changes in the morphology in β-galactosidase positive cells. Differences between cells transfected with BNIP3ΔTM and vector alone were statistically significant with a p value of 0.05. The error bars represent standard deviation between three independent experiments.

3. Hypoxia and HIF-1α overexpression increase BNIP3 protein expression.

One of the major mediators of hypoxia responsiveness is the transcription factor HIF-1α. In rodent it has been shown that BNIP3 upregulation is mediated by HIF-1α. Hypoxic regulation of the human BNIP3 protein is unknown. In order to determine if in human epithelial derived cells hypoxic exposure increases HIF-1 α protein, MCF-7 cells were incubated under hypoxia or normoxia for 24, 48 and 72 hours and lysates were analyzed by Western blot method. As shown in Fig.7A, hypoxic exposure results in increased levels of both BNIP3 and HIF-1α proteins in MCF-7 cells. Furthermore increased HIF-1α expression is detected at 24 hours while BNIP3 protein increases at 48 hours. Neither BNIP3 nor HIF-1α was detectable in cells cultured under normal conditions. To determine if BNIP3 upregulation was HIF-1α dependant, MCF-7 cells were transiently tranfected with HIF-1α and incubated under normal conditions. Cell lysates were analyzed for BNIP3 expression. Transient overexpression of HIF-1 α resulted in increased expression of BNIP3 in MCF-7 cells under normal conditions at 24 hours as shown in Fig 7B. Only 30 % of the cells were transfected with HIF-1α (data not shown). Therefore the expression of BNIP3 protein was lower in cells transiently transfected with HIF-1α than in cells incubated under hypoxic condition. These results show that both hypoxia and HIF-1α overexpression upregulate BNIP3 expression.

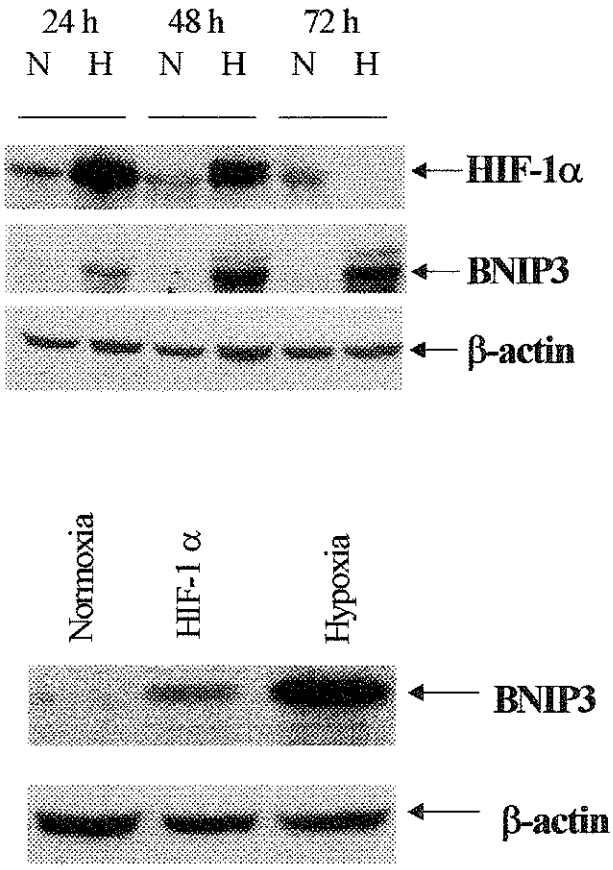

Figure 7: Hypoxia and HIF-1α overexpression increase BNIP3 protein levels A)MCF-7 cells were incubated under hypoxic condition over a time course of 72 hours. At indicated times, cells were lysed and protein lysates were Western blotted with monoclonal anti-HIF-1α antibody. The blots were than stripped and reprobed for BNIP3 with monoclonal anti-BNIP3 antibody. The blots were also analyzed for β-actin expression to ensure equal loading. Results are representative of three independent experiments.B) HEK 293T cells were transiently transfected with HIF-1α cDNA in pCEP expression vector. Cells were incubated under hypoxic or normoxic conditions for 24 hours. Protein lysates were isolated and Western blotted with anti-BNIP3 antibody. The blots were than stripped and reprobed for β-actin. Results are representative of three independent experiments.

4. Hypoxia and HIF-1α expression activate the human BNIP3 promoter.

Previously, the rodent promoter was characterized and HIF-1α was determined to control its activity. The regulation of human BNIP3 promoter is however unknown. We have isolated a human 1.2 kb BNIP3 promoter. This promoter was placed upstream of a luciferase reporter gene and used to monitor promoter activity in MCF-7 cells. As shown in Fig 8. , luciferase expression was induced 15-fold when MCF-7 cells were incubated for 20 hours under hypoxic conditions or when cells were co-transfected with HIF-1α. Luciferase activity in cells incubated under normal conditions showed no increase. Furthermore expression of a dominant negative form of HIF-1α resulted in complete loss of luciferase activity from the BNIP3 promoter in cells exposed to hypoxia. These results suggest that HIF-1 α is essential for BNIP3 promoter activation under hypoxic conditions.

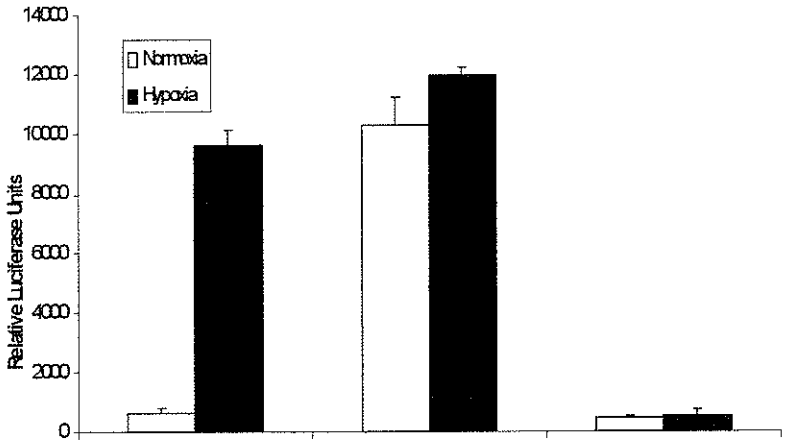

Figure 8: BNIP3 promoter is activated by transcription factor HIF-1α under hypoxia. MCF-7 cells were transfected with a luciferase reporter vector (pGL3-basic) regulated by the BNIP3 promoter in combination with a β-galactosidase vector in pcDNA3. When indicated cells were also co-transfected with HIF-1α cDNA, HIF-1α dominant negative (DN) or pCEP expression vector alone. Cells were incubated under either hypoxia or normoxia for 20 hours. Cells were than lysed and luciferase and β-galactosidase activities measured as described in *materials and methods*. Results represent average relative luciferase activity of three independent experiments and error bars represent standard deviation.

5. HIF-1α is essential for BNIP3 promoter activation under hypoxia.

To confirm if HIF-1α is essential to BNIP3 promoter activation under hypoxia CHO parental and CHO HIF-1α defective cells, kindly provided by Dr. Adrian Harris, were transfected with BNIP3 promoter luciferase construct. In CHO parental cells, a 4-fold induction in luciferase activity was observed upon a 20 hours hypoxic treatment as shown in Fig. 9. Parental and HIF-1α defective cells incubated under normal conditions did not show any induction in luciferase activity. The CHO HIF-1α defective cells incubated under hypoxia did not show an increase in luciferase activity either. Thus, absence of functional HIF-1α lead to a complete loss of hypoxia mediated responsiveness of the BNIP3 promoter. To confirm that such loss of hypoxia responsiveness was due to the absence of a functional HIF-1α, HIF-1α was transfected in the CHO Hif-1α defective cells. The restoration of functional HIF-1α by transient transfection resulted in a 4-fold induction of luciferase activity from the BNIP3 promoter at 24 hours post transfection under normal and hypoxic conditions. This induction was similar to the upregulation in luciferase activity in CHO parental cells exposed to hypoxia. These results indicate that DNA sequences located immediately upstream of the BNIP3 gene confer transcriptional induction in response to hypoxia. This effect is mediated through transcription factor HIF-1α.

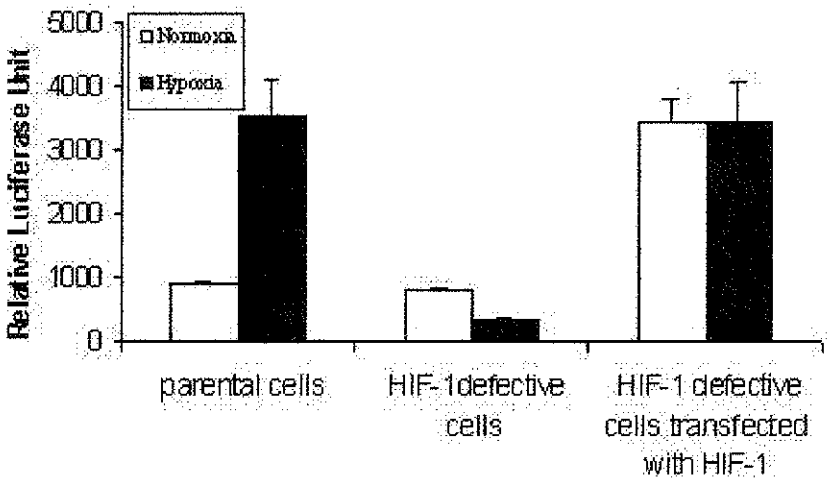

Figure 9: Transcription factor HIF-1α is critical for BNIP3 promoter activation under hypoxia. CHO parental and CHO HIF-1α defective cells were transfected with a luciferase reporter construct containing the BNIP3 promoter in combination with β-galactosidase cDNA in a pcDNA3 expression vector. When indicated cells were also transfected with HIF-1α cDNA in a pCEP expression vector. After incubation under hypoxia or normoxia for 20 hours, luciferase and β-galactosidase activities were determined. Results represent average relative luciferase activity of three independent experiments and error bars represents standard deviation.

6. Deletion mutant study of the BNIP3 promoter.

Analysis of the BNIP3 promoter using the TFSEARCH database showed two possible HRE sites (HRE1: 5'-CACGTC-3' located at –206 bp and HRE2: 5'CACGTG-3' located at –609 bp) that are almost identical to the consensus sequence 5'ACGTG 3'. In order to identify elements within the human BNIP3 promoter that are essential for transcriptional activation under hypoxia, 6 BNIP3 promoter deletion mutant constructs were made. Each of the mutated inserts were ligated upstream of a luciferase gene in the pGL3-basic vector. Each construct was transiently transfected in HEK 293T cells and promoter activity monitored. Results show that increased deletion of the promoter results in decreased promoter activity (Fig.10). Upon hypoxic incubation, deletion mutant D1, D2, D3 did not show much decrease in luciferase induction as compared to the wild type promoter. Indeed the wild type, D1, D2 and D3 promoter constructs showed a 7-fold increase in luciferase activity upon hypoxic exposure compared to the luciferase activity under normal conditions. The induction of luciferase activity upon hypoxic exposure was decreased from 7-fold in the wild type promoter to 4-fold in the deletion mutants D3, D4 and D5 deletion mutant. Interestingly, deletion mutant 6 (D6) that did not contain any of the two potential HRE showed the least induction upon incubation under hypoxia. The level of induction of D6 was comparable to the background luciferase activity as observed in cells transfected with the wild type promoter and incubated under normal conditions. Only the difference is luciferase induction between the wild type and deletion mutant D6 was statistically significant ($p < 0.05$) as analysed by the student t-test.

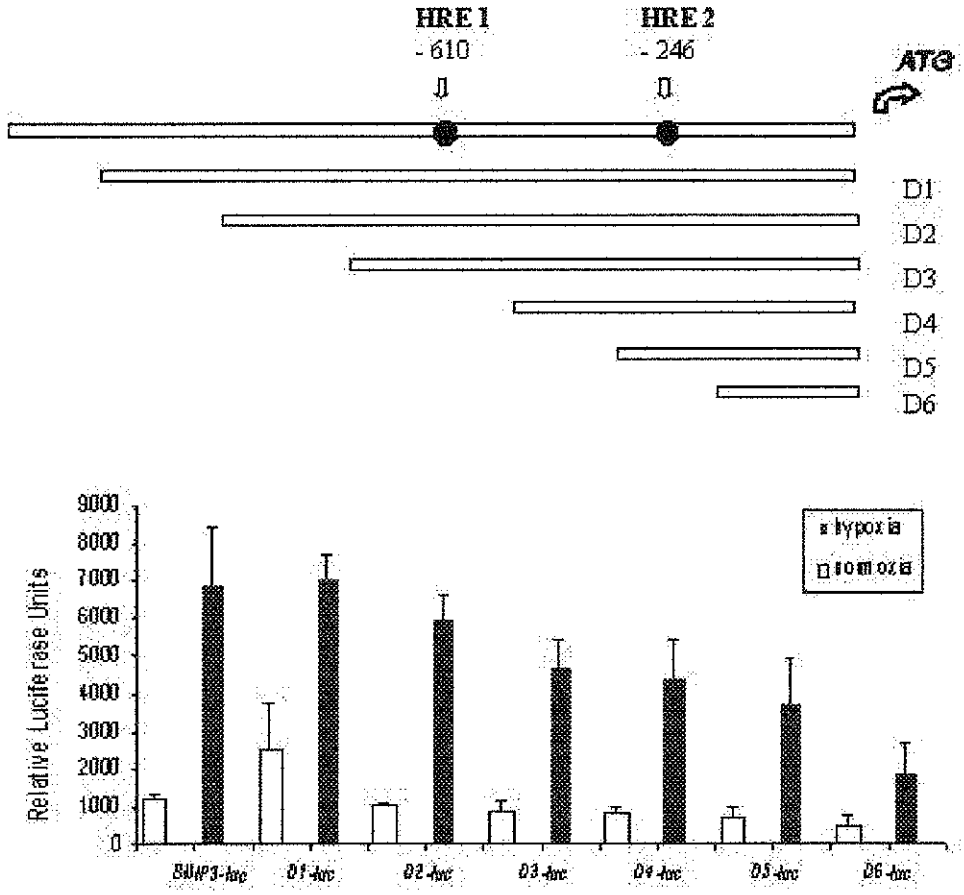

Figure 10: Deletion of the BNIP3 promoter leads to decrease in promoter activation under hypoxia. A) Schematic diagram of the wild type and deletion mutants BNIP3 promoter B)Luciferase reporter constructs containing the BNIP3 wild type promoter(BNIP3-luc) or the BNIP3 promoter deletion mutants were transfected in 293T cells in combination with β-galactosidase in a pcDNA3 expression vector. Cells were incubated under hypoxia or normoxia for 20 hours. Cells were lysed and luciferase and β-galactosidase activities determined. Results represent average relative luciferase activity of three independent experiments and error bars indicate standard deviation.

7 Human BNIP3 promoter has a hypoxia response element (1) at –204 bp that is essential for transcriptional activation.

HIF-1α binds to a consensus 5'-RCGTG-3'site on target gene promoters to activate their transcriptional activity. On the 0.5 kb rodent BNIP3 promoter, a HRE site was found to be essential for transcriptional activation by HIF-1α. Analysis of the human BNIP3 promoter using the TFSEARCH database showed two possible HRE sites (HRE1: 5'-CACGTC-3' located at –610 bp and HRE2: 5'CACGTG-3' located at –246 bp) that are almost identical to the consensus sequence. Comparison between the rodent and human BNIP3 promoters using BLAST 2 sequences showed that a 18 bp sequence containing the functional rodent HRE and the human HRE2 site was conserved between the two species. The human BNIP3 promoter containing both of HRE sites was cloned upstream from a luciferase reporter gene. As shown in Fig.11, mutation in the HRE located at -246 bp lead to a complete loss of hypoxia responsiveness. Cells transfected with the wild type BNIP3 promoter and exposed to normal conditions had a background luciferase activity of 1000 RLU. This activity was almost identical to the activity of cells transfected with the mutated HRE2 and exposed for 19 hours under hypoxia (700 RLU). In contrast, luciferase activity of cells transfected with a mutated HRE 1 located at -610 bp and exposed to hypoxic conditions was 6200 RLU. This was comparable to the activity of cells transfected with the wild type BNIP3 promoter and exposed to hypoxic conditions (8000 RLU).

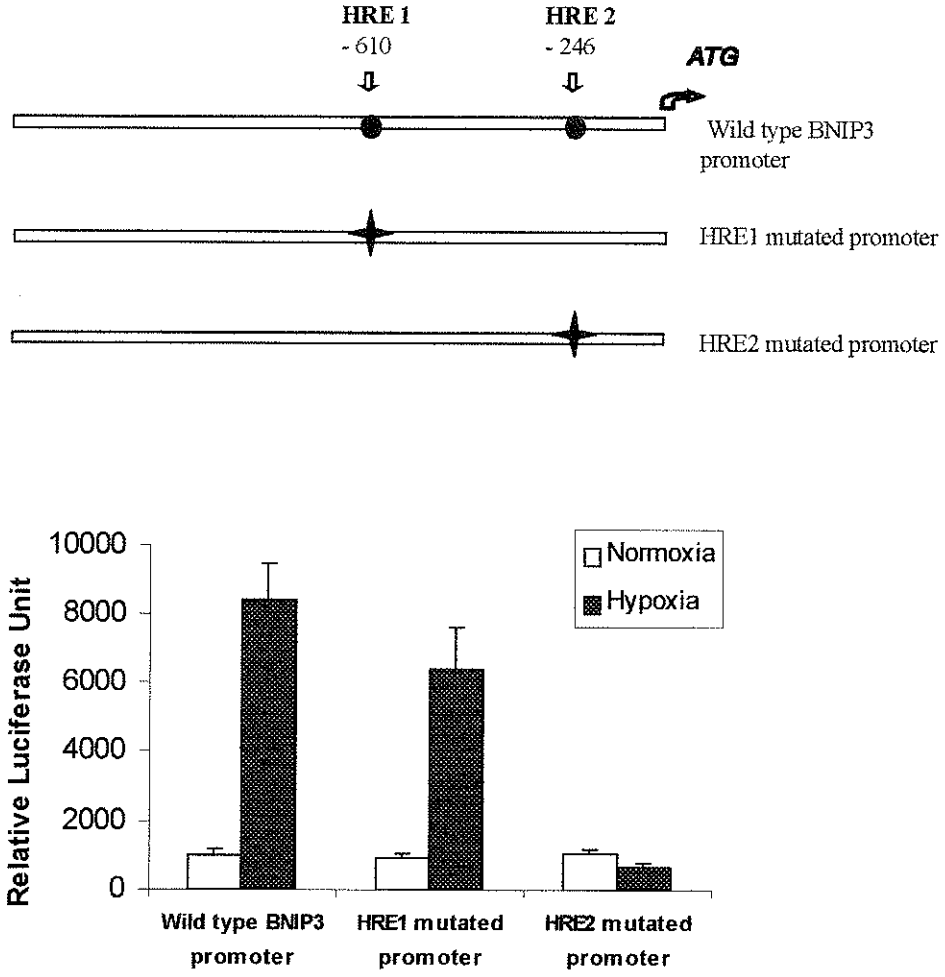

Figure 11: Hypoxia response element located at –246 bp is critical for BNIP3 promoter activation under hypoxia. A) Schematic diagram of the wild type, HRE1 and HRE2 mutated BNIP3 promoter B)Luciferase reporter constructs containing the BNIP3 wild type promoter or the BNIP3 promoter containing a mutation at HRE1 or HRE2 were transfected in 293T cells in combination with β-galactosidase in a pcDNA3 expression vector. Cells were incubated under hypoxia or normoxia for 20 hours. Cells were lysed and luciferase and β-galactosidase activities determined. Results represent average relative luciferase activity of three independent experiments and error bars indicate standard deviation.

8. HRE2 site on human BNIP3 promoter binds to transcription factor HIF-1α and controls its hypoxic induction.

To determine if HIF-1α directly binds to the BNIP3 promoter at the HRE2 site, oligonucleotides containing the HRE2 site and a mutated HRE2 site were analyzed for HIF-1 α binding by electromobility shift assay. A probe (W18) containing a consensus HIF-1α responsive element that binds to HIF-1α was used as positive control. All probes were radioactively labeled and mixed with lysates from cells incubated under either hypoxic or normal conditions. Three different shifts in probe migration were observed on the blots. The highest intensity signal is free probe that was not bound to any protein. In addition, a nonspecific binding activity that was seen for all samples. The shifted band was hypoxia dependant and was only seen with probes W18 and HRE2 upon incubation with lysates from hypoxic cells. When the W18 and HRE2 probes were incubated with lysates from cells exposed to normal conditions, no binding could be detected. Furthermore mutation in the HRE2 site resulted in the loss of binding suggesting a sequence specificity essential for binding to occur. Incubation of the HRE1 probe with lysates from hypoxic cells or normoxic cells did not show any binding for HIF-1. In order to confirm that the band that was observed for probes W18 and HRE2 when incubated with hypoxic cell lysates was HIF-1 specific, anti-HIF-1α antibody was incubated with the protein lysates prior to incubation with the probes. As see on Fig.11, upon incubation with anti HIF-1 α antibody, the hypoxia dependant shift observed for HRE2 disappeared.

This suggests that the HRE located –246 bp is essential for binding of transcription factor HIF-1α and hypoxia mediated activation of BNIP3 promoter.

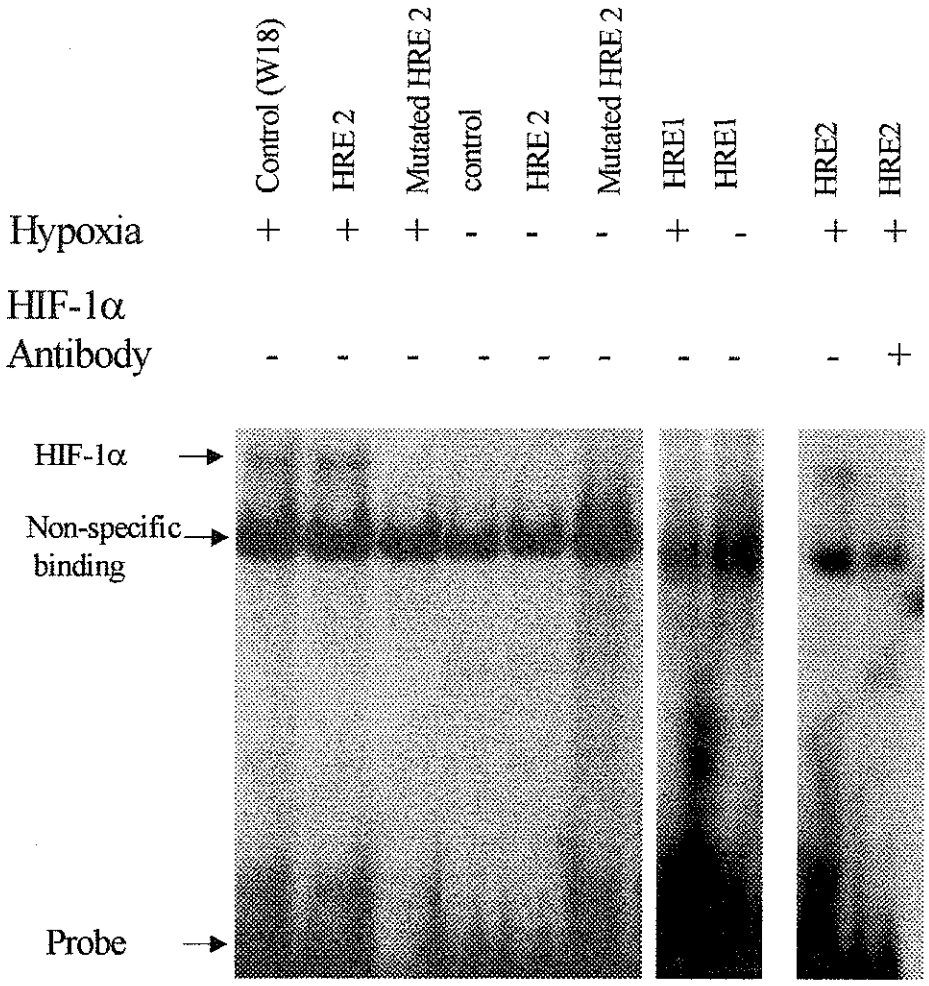

Figure 12: HIF-1α binds to the HRE2 site of the BNIP3 promoter. MCF-7 cells were incubated under either hypoxic or normoxic conditions for 24 hours prior to protein extract preparation. Extracts were mixed with probes containing the human HRE2 site, the mutated HRE2 site, the HRE1 site and a probe containing a consensus HIF-1α binding site (control) as indicated on top of the blot. To identify HIF-1 α, protein extract were mixed with anti- HIF-1 α antibody prior to incubation with HRE 2 probes. Binding reactions were analyzed by EMSA.

VI. DISCUSSION

Hypoxia has pathogenic features during cancer progression and ischemic injury of the heart and brain. Extensive studies have thus been undertaken to understand the implication of hypoxia in these diseases. Indeed, the relevance of hypoxia in epithelial derived tumor is becoming increasingly important. In solid tumors, hypoxia appears to be a two edged sword. On one hand, hypoxia is a micro-environmental condition that differentiates tumor cells from normal cells. This property is being manipulated to design therapeutic agents that are active under low oxygen levels and thus would selectively target tumor cells. On the other hand, it has been shown that cancer cells can survive hypoxic stress and consequently become more aggressive. Indeed, hypoxic tumor cells can survive and repopulate the tumor after antiangiogenic therapy (91). Hypoxic tumor cells are also more resistant to chemotherapy primarily due to a lack of an efficient way to deliver drugs to these cells (92). Furthermore, radiation therapy that relies on the formation of reactive oxygen species to induce apoptosis is also ineffective in hypoxic cells due to the low rate of oxidative phosphorylation (93). In addition, hypoxia induces genomic instability that promotes the generation of mutated cells more resistant to death stimuli(91). While cell death is the target of cancer therapies, cell survival is the focus of treatments for ischemic injury of the heart and brain. During stroke and cardiac infarction, interruption in blood supply leads to hypoxia in the heart or brain. Sustained hypoxic conditions translate into cell loss and consequently tissue injury. Thus understanding of hypoxia mediated cell death machinery such as BNIP3 is important for treatment of cancer and ischemic injury of the heart and brain.

BNIP3 is one of the most hypoxic responsive genes with its mRNA and protein levels dramatically increasing following hypoxia (94). Increased BNIP3 expression induces cell death through mitochondrial dysfunction in many cell types including cancer derived epithelial cells (74,76,95) and cardiomyocytes (88). In mouse cardiomyocytes, ischemia induced cell death is inhibited by blockage of BNIP3 expression. In the present study, we have determined that blockage of BNIP3 expression, by using antisense oligonucleotide against BNIP3, results in over 50% reduction in hypoxia-induced cell death in human epithelial cells.

Characterization of the BNIP3 protein structure has revealed that deletion of the transmembrane (TM) domain fails to induce cell death upon overexpression. Furthermore BNIP3 ΔTM acts as a dominant negative molecule blocking the endogenous BNIP3 from integrating to the mitochondria (73). To confirm results obtained through antisense technology, we transiently transfected the dominant negative form of BNIP3 (BNIP3 ΔTM) to block endogenous BNIP3 function and subsequently monitor hypoxia induced cell death. Hypoxia-induced cell death was reduced from 30 % for cells transfected with vector alone as a control to 18% for cells expressing BNIP3 ΔTM. It was not surprising that results obtained in this study were similar to those observed in cardiomyocytes transfected with BNIP3 ΔTM (88) and incubated under ischemic conditions.

Interestingly, during both antisense experiment and transient expression of ΔTM BNIP3, hypoxia induced cell death is not completely blocked. Hence, it is likely and not surprising that BNIP3 does not solely mediate hypoxia induced cell death. Expression of

other hypoxia responsive death inducing molecules may also contribute to the overall response. Indeed, NIX expression has been shown to be upregulated during hypoxia (56). As discussed earlier BNIP3 and NIX share sequence homology. Furthermore, insight into NIX function has revealed its similarity to BNIP3 (74). Indeed, similarly to BNIP3, Nix has also been shown to induce cell death upon over-expression. Furthermore, similarly to BNIP3ΔTM, a truncated NIX isoform, termed sNIX was shown to heterodimerize with NIX and protect against NIX induced cell death also by preventing its localization to the mitochondria. Although, BNIP3 is clearly not the only player in hypoxia involved cell death, we have nonetheless determined that BNIP3 function is necessary for the full hypoxia induced cell death response in epithelial derived cells.

Weak to undetectable levels of BNIP3 in cell lines under normal conditions suggest that BNIP3 expression is tightly regulated. We thus hypothesized that BNIP3 accumulation is the result of transcriptional upregulation .

Analysis of the 1.2 kb sequence of the BNIP3 promoter using the TFSEARCH data base identified potential transcription factors binding sites for transcription factors Sp1, Sp3, HIF-1. All three transcription factors have been shown to be hypoxia inducible. We hence looked at the possibility of the involvement of each of these transcription factors in the upregulation of BNIP3 under hypoxia. Interestingly, only the deletion mutant that omitted the HIF-1α response element site (HRE 2) closest to the starting codon showed significant decrease in luciferase activity upon hypoxic treatment. Although promoter deletion mutants are routinely being used to characterize promoters, our deletion mutant study did not lead to any conclusive results. Differences in luciferase activities between the wild type and the mutants were not significant. We believe that

deletion of DNA regions changes the overall promoter structure which is important for transcriptional regulation.

HIF-1 is a transcription factor that binds on specific DNA sequences on target promoters known as hypoxia response elements. It has been previously shown that rodent BNIP3 promoter is activated by HIF-1 through a HRE that is critical for its transcriptional upregulation (87). Comparison between the 1.2 kb fragment of the human BNIP3 promoter directly upstream of ATG and the 0.5 kb rodent BNIP3 promoter using blast 2 sequences program revealed a 24 bp sequence conservation. Interestingly the functional hypoxia response element involved in the rodent promoter is located within this region thus suggesting the importance of this site the transcriptional regulation of human BNIP3 and the importance of HIF-1 in BNIP3 activation under hypoxia.

In the present study we have determined that transcription factor HIF-1 is essential for BNIP3 transcriptional activation under hypoxia. Indeed, transient transfection of HIF in cells incubated under normal conditions showed upregulation in BNIP3 promoter activity. Furthermore, defect in HIF-1 function resulted in complete loss of hypoxia responsiveness of the BNIP3 promoter in cells incubated under hypoxia. Finally, the mutation of the HRE2 site on the BNIP3 promoter also resulted in complete loss of hypoxia responsiveness. Finally, EMSA showed that there is indeed a physical interaction between that HRE2 DNA motif and HIF-1α. This HRE2 sequence is indeed the one conserved between the human and rodent BNIP3 promoters. We have thus shown that during hypoxia transcription factor HIF-1 alpha upregulates the BNIP3 transcription by binding to the HRE located at –246 bp of the BNIP3 promoter.

This study has shown that under hypoxia transcription factor HIF-1 is essential for the upregulation of the human BNIP3 promoter. It has been also shown that the transcription factor PLAGL-2 (pleomorphic adenoma gene-like 2) induces rodent BNIP3 promoter activation under hypoxia. However HIF-1 is still required for the full activation of the promoter. The implication of PLAGL-2 in regulating human BNIP3 promoter remains to be elucidated. PLAGL-2 is a transcription factor that is expressed in response to hypoxia or iron deficiency. While, mouse PLAGL-2 is ubiquitously expressed, human PLAGL-2 is only highly expressed in fetal tissues (96). Thus taken together, HIF-1 is the major regulator of BNIP3 expression under hypoxia.

Transcription factor HIF –1 is one of the key regulator of genes that are activated under low oxygen levels. Genes upregulated by HIF-1 encode proteins involved in glucose uptake, glucose metabolism angiogenesis and erytropoesis. Expression of these genes contributes to the adaptation against oxygen deficiency by optimizing oxygen delivery and energy generation. This, thus suggests that the initial hypoxic insult results in the activation of genes that promote cell survival. HIF-1 however also upregulates genes involved in cell death, BNIP3 and NIX. HIF-1 is thus involved in both promoting or preventing cell death. The regulatory mechanisms that would control the switch between two opposite actions are still unknown. It has been however been speculated that the differences in transcriptional activation kinetics of HIF-1 target genes might be involved in this regulation. For instance, a hypoxic insult might initially trigger a survival response. However, sustained hypoxic conditions would allow the accumulation of HIF-1 target death inducing genes and thus initiate cell death. Indeed, we have shown that hypoxia induced BNIP3 expression was detectable 24 hours after the initial upregulation

of HIF-1 and peaked after 72 hours. Meanwhile, VEGF expression has been shown to increase after only 12 hours of hypoxia, suggesting that BNIP3 expression occurs later in the hypoxic response than other genes such as angiogenic factor VEGF. Thus HIF-1 activates both survival and cell death genes in which the balance between these genes would likely determine the cell's fate under hypoxia.

In conclusion, the evidence presented here demonstrated that Bcl-2 family member BNIP3 is involved in hypoxia induced cell death, that transcription factor HIF-1 is essential for BNIP3 transcriptional activation under hypoxia. This upregualtion is mediated via a conserved HRE located in the BNIP3 promoter. A model for hypoxia induced cell death involving BNIP3 could be as follows: hypoxic conditions block the degradation of HIF-1α, HIF-1α then translocates to the nucleus and binds to the HRE site on the BNIP3 promoter, activation of BNIP3 transcription then leads to increase in BNIP3 protein expression, BNIP3 protein migrate to and integrate in the mitochondria outer membrane, BNIP3 then initiate PT pore opening, which results in mitochondrial dysfunction and consequently cell death.

VII. FUTURE DIRECTION

1) Other factors beside hypoxia have been reported to modulate BNIP3 expression. Indeed it has been reported that nitric oxide (NO) downregulates BNIP3 mRNA expression in hepatocytes (97). Another study has shown that BNIP3 expression is upregulated in interleukin-3 deprived cells. Molecular mechanisms underlying either of these two conditions are unknown. Determination of the molecular mechanisms leading to upregulation in BNIP3 expression during interleukin-3 deprivation or downregulation in BNIP3 expression during NO signaling thus need to be examined. Furthermore the implication of these conditions on cell death and diseases also need to be addressed.

2) We have examined the effect of hypoxia on the activation of the 1.2 kb BNIP3 promoter. Thus, analysis of the promoter sequence upstream of 1.2 kb in search of other conserved transcription binding sites might result in a further understanding of BNIP3 transcriptional regulation.

3) BNIP3 expression is essential but might not be sufficient in initiating cell death. Indeed it has been shown that BNIP3 is expressed in hypoxic regions of tumors but fails to induce cell death due to growth factor signalling. Epidermal growth factors EGF and insulin-like growth factor IGF have been shown to protect against BNIP3 induced cell death. Thus, elucidation of the molecular mechanisms involved during BNIP3 activation will be crucial in our understanding of BNIP3 implication in cancer, and ischemic injury of the heart and brain.

Reference List

1. **Hofmann, K.** 1999. The modular nature of apoptotic signaling proteins. Cell Mol.Life Sci. **55**:1113-1128.

2. **Semenza, G. L.** 2000. Hypoxia, HIF-1, and the pathophysiology of common human diseases.

3. **Brunelle, J. K. and N. S. Chandel**. 2002. Oxygen deprivation induced cell death: an update. Apoptosis. 7:475-482.

4. **Formigli, L., L. Papucci, A. Tani, N. Schiavone, A. Tempestini, G. E. Orlandini, S. Capaccioli, and S. Z. Orlandini**. 2000. Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J.Cell Physiol **182**:41-49.

5. **Cory, S. and J. M. Adams**. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat.Rev.Cancer **2**:647-656.

6. **Daniel, P. T.** 2000. Dissecting the pathways to death. Leukemia **14**:2035-2044.

7. **Kruidering, M. and G. I. Evan**. 2000. Caspase-8 in apoptosis: the beginning of "the end"? IUBMB.Life **50**:85-90.

8. **Esposti, M. D.** 2002. The roles of Bid. Apoptosis. 7:433-440.

9. **Adams, J. M. and S. Cory**. 2002. Apoptosomes: engines for caspase activation. Curr.Opin.Cell Biol. **14**:715-720.

10. **Schuler, M. and D. R. Green**. 2001. Mechanisms of p53-dependent apoptosis. Biochem.Soc.Trans. **29**:684-688.

11. **Proskuryakov, S. Y., A. G. Konoplyannikov, and V. L. Gabai**. 2003. Necrosis: a specific form of programmed cell death? Exp.Cell Res. **283**:1-16.

12. **Tomida, A. and T. Tsuruo**. 1999. Drug resistance mediated by cellular stress response to the microenvironment of solid tumors. Anticancer Drug Des **14**:169-177.

13. **Vaupel, P., D. K. Kelleher, and O. Thews**. 1998. Modulation of tumor oxygenation. Int.J.Radiat.Oncol.Biol.Phys. **42**:843-848.

14. **Tomes, L., E. Emberley, Y. Niu, S. Troup, J. Pastorek, K. Strange, A. Harris, and P. H. Watson**. 2003. Necrosis and hypoxia in invasive breast carcinoma. Breast Cancer Res.Treat. **81**:61-69.

15. **Harrison, L. B., M. Chadha, R. J. Hill, K. Hu, and D. Shasha**. 2002. Impact of tumor hypoxia and anemia on radiation therapy outcomes. Oncologist. 7:492-508.

16. **Overgaard, J. and M. R. Horsman**. 1996. Modification of Hypoxia-Induced Radioresistance in Tumors by the Use of Oxygen and Sensitizers. Semin.Radiat.Oncol. **6**:10-21.

17. **Dorr, R. T.** 1992. Bleomycin pharmacology: mechanism of action and resistance, and clinical pharmacokinetics. Semin.Oncol. **19**:3-8.

18. **Solaiman, D., E. A. Rao, D. H. Petering, R. C. Sealy, and W. E. Antholine**. 1979. Chemical, biochemical, and cellular properties of copper and iron bleomycins. Int.J.Radiat.Oncol.Biol.Phys. **5**:1519-1521.

19. **Yamauchi, T., T. A. Raffin, P. Yang, and B. I. Sikic**. 1987. Differential protective effects of varying degrees of hypoxia on the cytotoxicities of etoposide and bleomycin. Cancer Chemother.Pharmacol. **19**:282-286.

20. **Grau, C. and J. Overgaard**. 1992. Effect of etoposide, carmustine, vincristine, 5-fluorouracil, or methotrexate on radiobiologically oxic and hypoxic cells in a C3H mouse mammary carcinoma in situ. Cancer Chemother.Pharmacol. **30**:277-280.

21. **Tomida, A. and T. Tsuruo**. 1999. Drug resistance mediated by cellular stress response to the microenvironment of solid tumors. Anticancer Drug Des **14**:169-177.

22. **Longo, R., R. Sarmiento, M. Fanelli, B. Capaccetti, D. Gattuso, and G. Gasparini**. 2002. Anti-angiogenic therapy: rationale, challenges and clinical studies. Angiogenesis. **5**:237-256.

23. **Kwon, H. J., M. S. Kim, M. J. Kim, H. Nakajima, and K. W. Kim**. 2002. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int.J.Cancer **97**:290-296.

24. **Mie, L. Y., S. H. Kim, H. S. Kim, S. M. Jin, H. Nakajima, K. H. Jeong, and K. W. Kim**. 2003. Inhibition of hypoxia-induced angiogenesis by FK228, a specific histone deacetylase inhibitor, via suppression of HIF-1alpha activity. Biochem.Biophys.Res.Commun. **300**:241-246.

25. **Williams, R. J.** 2001. Trichostatin A, an inhibitor of histone deacetylase, inhibits hypoxia-induced angiogenesis. Expert.Opin.Investig.Drugs **10**:1571-1573.

26. **Seimiya, H., M. Tanji, T. Oh-hara, A. Tomida, I. Naasani, and T. Tsuruo**. 1999. Hypoxia up-regulates telomerase activity via mitogen-activated protein kinase signaling in human solid tumor cells. Biochem.Biophys.Res.Commun. **260**:365-370.

27. **Smid, L., H. Lesnicar, B. Zakotnik, E. Soba, M. Budihna, L. Furlan, M. Zargi, and Z. Rudolf**. 1995. Radiotherapy, combined with simultaneous chemotherapy with mitomycin C and bleomycin for inoperable head and neck cancer--preliminary report. Int.J.Radiat.Oncol.Biol.Phys. **32**:769-775.

28. **Gatzemeier, U.** 2002. Hypoxic active drugs. Suppl Tumori **1**:S15-S16.

29. **Peters, K. B. and J. M. Brown**. 2002. Tirapazamine: a hypoxia-activated topoisomerase II poison. Cancer Res. **62**:5248-5253.

30. **Gandara, D. R., P. N. Lara, Jr., Z. Goldberg, Q. T. Le, P. C. Mack, D. H. Lau, and P. H. Gumerlock**. 2002. Tirapazamine: prototype for a novel class of therapeutic agents targeting tumor hypoxia. Semin.Oncol. **29**:102-109.

31. **Peters, L. J.** 2001. Targeting hypoxia in head and neck cancer. Acta Oncol. **40**:937-940.

32. **Kehrer, J. P.** 1989. Concepts related to the study of reactive oxygen and cardiac reperfusion injury. Free Radic.Res.Commun. **5**:305-314.

33. **Singh, N., A. K. Dhalla, C. Seneviratne, and P. K. Singal**. 1995. Oxidative stress and heart failure. Mol.Cell Biochem. **147**:77-81.

34. **Wolin, M. S., T. H. Hintze, W. Shen, H. Mohazzab, and Y. W. Xie**. 1997. Involvement of reactive oxygen and nitrogen species in signalling mechanisms that control tissue respiration in muscle. Biochem.Soc.Trans. **25**:934-939.

35. **Piper, H. M.** 1989. Energy deficiency, calcium overload or oxidative stress: possible causes of irreversible ischemic myocardial injury. Klin.Wochenschr. **67**:465-476.

36. **Hess, M. L. and N. H. Manson**. 1984. Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. J.Mol.Cell Cardiol. **16**:969-985.

37. **Biagas, K.** 1999. Hypoxic-ischemic brain injury: advancements in the understanding of mechanisms and potential avenues for therapy. Curr.Opin.Pediatr. **11**:223-228.

38. **Iyer, N. V., S. W. Leung, and G. L. Semenza**. 1998. The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. Genomics **52**:159-165.

39. **Semenza, G. L., F. Agani, G. Booth, J. Forsythe, N. Iyer, B. H. Jiang, S. Leung, R. Roe, C. Wiener, and A. Yu**. 1997. Structural and functional analysis of hypoxia-inducible factor 1. Kidney Int. **51**:553-555.

40. **Semenza, G. L.** 2001. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus.

41. **Semenza, G. L.** 2001. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus.

42. **Lai, J., J. Flanagan, W. A. Phillips, G. Chenevix-Trench, and J. Arnold**. 2003. Analysis of the candidate 8p21 tumour suppressor, BNIP3L, in breast and ovarian cancer. Br.J.Cancer **88**:270-276.

43. **Semenza, G. L.** 2001. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus.

44. **Iyer, N. V., L. E. Kotch, F. Agani, S. W. Leung, E. Laughner, R. H. Wenger, M. Gassmann, J. D. Gearhart, A. M. Lawler, A. Y. Yu, and G. L. Semenza**. 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. **12**:149-162.

45. **Ryan, H. E., J. Lo, and R. S. Johnson**. 1998. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. **17**:3005-3015.

46. **Carmeliet, P., Y. Dor, J. M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C. J. Koch, P. Ratcliffe, L. Moons, R. K. Jain, D. Collen, E. Keshert, and E. Keshet**. 1998. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature **394**:485-490.

47. **Bracken, C. P., M. L. Whitelaw, and D. J. Peet**. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol.Life Sci. **60**:1376-1393.

48. **Semenza, G. L.** 1999. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu.Rev.Cell Dev.Biol. **15**:551-578.

49. **Mu, D., X. Jiang, R. A. Sheldon, C. K. Fox, S. E. Hamrick, Z. S. Vexler, and D. M. Ferriero**. 2003. Regulation of hypoxia-inducible factor 1alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model. Neurobiol.Dis. **14**:524-534.

50. **Achison, M. and T. R. Hupp**. 2003. Hypoxia attenuates the p53 response to cellular damage. Oncogene **22**:3431-3440.

51. **Hansson, L. O., A. Friedler, S. Freund, S. Rudiger, and A. R. Fersht**. 2002. Two sequence motifs from HIF-1alpha bind to the DNA-binding site of p53. Proc.Natl.Acad.Sci.U.S.A **99**:10305-10309.

52. **An, W. G., M. Kanekal, M. C. Simon, E. Maltepe, M. V. Blagosklonny, and L. M. Neckers**. 1998. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature **392**:405-408.

53. **Achison, M. and T. R. Hupp**. 2003. Hypoxia attenuates the p53 response to cellular damage. Oncogene **22**:3431-3440.

54. **Hansson, L. O., A. Friedler, S. Freund, S. Rudiger, and A. R. Fersht**. 2002. Two sequence motifs from HIF-1alpha bind to the DNA-binding site of p53. Proc.Natl.Acad.Sci.U.S.A **99**:10305-10309.

55. **Ravi, R., B. Mookerjee, Z. M. Bhujwalla, C. H. Sutter, D. Artemov, Q. Zeng, L. E. Dillehay, A. Madan, G. L. Semenza, and A. Bedi**. 2000. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. **14**:34-44.

56. **Sowter, H. M., M. Ferguson, C. Pym, P. Watson, S. B. Fox, C. Han, and A. L. Harris**. 2003. Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J.Pathol. **201**:573-580.

57. **Kothari, S., J. Cizeau, E. McMillan-Ward, S. J. Israels, M. Bailes, K. Ens, L. A. Kirshenbaum, and S. B. Gibson**. 2003. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene **22**:4734-4744.

58. **Cory, S. and J. M. Adams**. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat.Rev.Cancer **2**:647-656.

59. **Semenza, G. L.** 2001. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol.Med. 7:345-350.

60. **Semenza, G. L.** 2001. Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. Pediatr.Res. **49**:614-617.

61. **Semenza, G. L.** 2000. HIF-1 and human disease: one highly involved factor. Genes Dev. **14**:1983-1991.

62. **Feldser, D., F. Agani, N. V. Iyer, B. Pak, G. Ferreira, and G. L. Semenza**. 1999. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res. **59**:3915-3918.

63. **Giaccia, A., B. G. Siim, and R. S. Johnson**. 2003. HIF-1 as a target for drug development. Nat.Rev.Drug Discov. **2**:803-811.

64. **Semenza, G. L.** 2003. Targeting HIF-1 for cancer therapy. Nat.Rev.Cancer **3**:721-732.

65. **Maxwell, P. H.** 2002. Oxygen homeostasis and cancer: insights from a rare disease. Clin.Med. **2**:356-362.

66. **Giaccia, A., B. G. Siim, and R. S. Johnson**. 2003. HIF-1 as a target for drug development. Nat.Rev.Drug Discov. **2**:803-811.

67. **Stroka, D. M., T. Burkhardt, I. Desbaillets, R. H. Wenger, D. A. Neil, C. Bauer, M. Gassmann, and D. Candinas**. 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J. **15**:2445-2453.

68. **Semenza, G. L., F. Agani, D. Feldser, N. Iyer, L. Kotch, E. Laughner, and A. Yu**. 2000. Hypoxia, HIF-1, and the pathophysiology of common human diseases. Adv.Exp.Med.Biol. **475**:123-130.

69. **Chen, G.** 1999. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins.

70. **Ray, R.** 2000. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites.

71. **Boyd, J. M.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins.

72. **Yasuda, M.** 1998. Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence.

73. **Ray, R., G. Chen, V. C. Vande, J. Cizeau, J. H. Park, J. C. Reed, R. D. Gietz, and A. H. Greenberg**. 2000. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. J.Biol.Chem. **275**:1439-1448.

74. **Chen, G., J. Cizeau, V. C. Vande, J. H. Park, G. Bozek, J. Bolton, L. Shi, D. Dubik, and A. Greenberg**. 1999. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. J.Biol.Chem. **274**:7-10.

75. **Chen, G., R. Ray, D. Dubik, L. Shi, J. Cizeau, R. C. Bleackley, S. Saxena, R. D. Gietz, and A. H. Greenberg**. 1997. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. J.Exp.Med. **186**:1975-1983.

76. **Cizeau, J., R. Ray, G. Chen, R. D. Gietz, and A. H. Greenberg**. 2000. The C. elegans orthologue ceBNIP3 interacts with CED-9 and CED-3 but kills through a BH3- and caspase-independent mechanism. Oncogene **19**:5453-5463.

77. **Vande, V. C.** 2000. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore.

78. **Chen, G.** 1999. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins.

79. **Ray, R.** 2000. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites.

80. **Vande, V. C.** 2000. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore.

81. **Vande, V. C.** 2000. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore.

82. **Regula, K. M.** 2002. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes.

83. **Crow, M. T.** 2002. Hypoxia, BNip3 proteins, and the mitochondrial death pathway in cardiomyocytes.

84. **Lamy, L.** 2003. CD47 and the 19 kDa interacting protein-3 (BNIP3) in T cell apoptosis.

85. **Crow, M. T.** 2002. Hypoxia, BNip3 proteins, and the mitochondrial death pathway in cardiomyocytes.

86. **Sowter, H. M.** 2001. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors.

87. **Bruick, R. K.** 2000. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc.Natl.Acad.Sci.U.S.A **97**:9082-9087.

88. **Regula, K. M., K. Ens, and L. A. Kirshenbaum**. 2002. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. Circ.Res. **91**:226-231.

89. **Ravi, R., B. Mookerjee, Z. M. Bhujwalla, C. H. Sutter, D. Artemov, Q. Zeng, L. E. Dillehay, A. Madan, G. L. Semenza, and A. Bedi**. 2000. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. **14**:34-44.

90. **Achison, M. and T. R. Hupp**. 2003. Hypoxia attenuates the p53 response to cellular damage. Oncogene **22**:3431-3440.

91. **Yu, J. L., B. L. Coomber, and R. S. Kerbel**. 2002. A paradigm for therapy-induced microenvironmental changes in solid tumors leading to drug resistance. Differentiation **70**:599-609.

92. **Harris, J. M., N. E. Martin, and M. Modi**. 2001. Pegylation: a novel process for modifying pharmacokinetics. Clin.Pharmacokinet. **40**:539-551.

93. **Koukourakis, M. I.** 2001. Tumour angiogenesis and response to radiotherapy. Anticancer Res. **21**:4285-4300.

94. **Guo, K., G. Searfoss, D. Krolikowski, M. Pagnoni, C. Franks, K. Clark, K. T. Yu, M. Jaye, and Y. Ivashchenko**. 2001. Hypoxia induces the expression of the pro-apoptotic gene BNIP3. Cell Death.Differ. **8**:367-376.

95. **Vande, V. C., J. Cizeau, D. Dubik, J. Alimonti, T. Brown, S. Israels, R. Hakem, and A. H. Greenberg**. 2000. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. Mol.Cell Biol. **20**:5454-5468.

96. **Mizutani, A., T. Furukawa, Y. Adachi, S. Ikehara, and S. Taketani**. 2002. A zinc-finger protein, PLAGL2, induces the expression of a proapoptotic protein Nip3, leading to cellular apoptosis. J.Biol.Chem. **277**:15851-15858.

97. **Mizutani, A.** 2002. A zinc-finger protein, PLAGL2, induces the expression of a proapoptotic protein Nip3, leading to cellular apoptosis.