STUDIES ON THE MOLECULAR MECHANISMS OF HYDROGEN PEROXIDE-MEDIATED REGULATION OF CELL PROLIFERATION

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Abstract

Mammalian cells have the ability to endogenously generate and metabolize hydrogen peroxide (H_2O_2). This H_2O_2 can interact with protein and lipid components of intracellular signal transduction pathways to regulate cell behaviour. It has been demonstrated in our laboratory and by others that many types of cells, including transformed cells, require H_2O_2 for efficient growth in culture. Excess H_2O_2 has long been identified as a source of oxidative stress that can mediate cell growth arrest and death. However, the addition of the H_2O_2 scavenger catalase to culture media or the overexpression of intracellular catalase can block proliferation. This effect is reversed by inactivation of the enzyme or upon co-incubation of cells with H_2O_2 and H_2O_2-generating sources such as glucose oxidase. Thus, at non-toxic levels, H_2O_2 appears to act as a cellular growth factor. The purpose of this work is to help elucidate molecular mechanisms underlying this H_2O_2-regulated aspect of cell physiology. The effects of extracellular H_2O_2 level manipulation upon the activities of the HER-2/Neu receptor tyrosine kinase, mitogen-activated protein kinases (MAPKs), and stress-activated protein kinases (SAPKs) are discussed.

The c-Jun-NH_2 terminal kinase 1 (JNK1), a member of the SAPK family, is involved in several diverse aspects of cellular functioning including apoptosis and transformation. The JNK1 signal has also been implicated as a cell sensor of redox (reducing/oxidizing) stress. We have observed that the growth-inhibitory effect of both high level H_2O_2 treatment and H_2O_2-scavenging catalase treatment is accompanied by a transient increase in JNK1 activity. To determine the importance of this response in

growth regulation, the JNK1 signal was stably altered in SK-OV-3 human ovarian adenocarcinoma cells by the expression of ectopic JNK1 (HA-JNK1). Levels of HA-JNK1 protein expression correlated with increases in basal c-Jun phosphorylation in a dose-dependent manner. Transient expression of HA-JNK1 potentiated cell growth arrest by catalase activity, however with stable expression a degree of resistance to this response was observed. Resistance was accompanied by a lowered endogenous production of H_2O_2. Transient HA-JNK1 expression also reduced H_2O_2 generation, and this effect was reversed by the JNK inhibitor SP600125. These results indicate that the JNK1 stress response contributes to the inhibition of proliferation by catalase treatment, possibly via additional reductions in environmental H_2O_2 caused by a lowered endogenous production. Stable amplification of the JNK1 pathway leads to cellular adaptation to its signal, resulting in a diminished reliance upon basal H_2O_2 levels for efficient growth. These data contribute to the understanding of the mechanisms involved with the overexpression and/or hyperactivity of JNKs observed in certain cancers.

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List of Abbreviations

AP-1	Activator Protein 1
APAF-1	Apoptotic Protease-activating Factor 1
ARE	Antioxidant/Electrophile Responsive Element
ATP	Adenosine triphosphate
BrdU	5-bromo-2'-deoxyuridine
Cdk	Cyclin-dependent Kinase
CKI	Cyclin-dependent Kinase inhibitor
CMV	Cytomegalovirus (promoter)
CO_2	Carbon dioxide
CoQ	Coenzyme Q
COX	Cyclooxygenase
CREB	Cyclic AMP-responsive Element Binding Protein
Cu,ZnSOD	Copper/Zinc-containing Superoxide Dismutase
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Erk	Extracellular signal-regulated kinase
ETC	Electron Transport Chain
G	Heterotrimeric G Protein
GEF	Guanine nucleotide exchange factor
GF	Growth Factor
GPx	Glutathione Peroxidase
GSH	Glutathione (reduced)
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HA	Haemagluttinin
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HIF	Hypoxia-inducible Factor
HRP	Horseradish Peroxidase

Hsp	Heat Shock Protein
HTLV	Human T cell Leukemia Virus
IκB	Inhibitory Factor kappa B
IMM	Inner Mitochondrial Membrane
JAK	Janus kinase
JNK	c-Jun-NH_2-terminal Kinase
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated Protein Kinase
MAPKK/MEK	Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase
MAPKKK/MEKK	MAPKK/MEK Kinase
MBP	MAPK-binding Protein Phosphatase
Mdm-2	mouse double minute-2
ME	Mercaptoethanol
MEF	Mouse Embryo Fibroblast
MLK	Mixed-lineage Protein Kinase
MMP	Matrix Metalloprotease
MMR	Mismatch Repair (DNA)
MnSOD	Manganese-containing Superoxide Dismutase
MPF	Mitosis-promoting Factor
MPT	Mitochondrial Permeability Transition
MT	Metallothionein
mtDNA	Mitochondrial DNA
NAC	*N*-acetyl cysteine
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NFκB	Nuclear Factor kappa B
NO	Nitrogen oxide
Nrf	NF-E2-related Factor
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PAK	p21-activated Kinase
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PG	Prostaglandin
PHS	Prostaglandin H Synthase
PI	Propidium iodide
PI3-K	Phosphatidyl inositol 3'-OH Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PM	Plasma Membrane
PMOR	Plasma Membrane Oxidoreductase Complex
PP	Protein Phosphatase

PPAR	Peroxisome Proliferator-activated Receptor
Prx	Peroxiredoxin
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RSOH	Sulphenic acid group
RSO_2H	Sulphinic acid group
RSO_3H	Sulphonic acid group
RTK	Receptor Tyrosine Kinase
SAPK	Stress-activated Protein Kinase
SDS	Sodium dodecyl sulfate
SEK	Stress-activated Protein Kinase/Extracellular Signal-regulated Protein Kinase Kinase
SOD	Superoxide Dismutase
TCF	Ternary Complex Factor
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
Trx	Thioredoxin
XOR	Xanthine Oxidoreductase

Table of Contents

List of Figures

List of Tables

1.0 Introduction

1.1 Molecular Regulation of Cell Proliferation

The ability of mammalian cells to proliferate, differentiate or die is crucial to the proper development and functioning of the organism as a whole. All of these cellular functions are known to be responses to a variety of intracellular and extracellular stimuli; the focus of this thesis is upon the regulation of proliferation by the hydrogen peroxide (H_2O_2) signal. The coordination of specific cells to divide or remain in a state of non-proliferation, or quiescence, is required for efficient maintenance of body tissues and organs, and deregulation of such coordination may lead to disease and death. For example, inappropriate cell proliferation is a marker of tumourigenesis. Many cell types in an adult organism are quiescent, but most of these have the capacity to return to a state of proliferation, also referred to as the cell cycle (Norbury and Nurse 1992; Malumbres and Barbacid 2001). The cell cycle consists of four distinct phases: the G1 phase (or pre-synthesis), S phase (DNA synthesis), G2 phase (preparation for mitosis), and mitosis; if a cell is removed from the cycle it may be referred to as being in G0 phase (Lloyd *et al.* 1982). One way that cancer cells are altered from normal cells is that they retain the ability to proliferate with little or no mitogenic stimulation and are more resistant to signals that induce growth arrest (Cohen and Ellwein 1990; Ortega *et al.* 2002).

In order for a cell to complete a cell cycle with proper DNA replication, chromosome segregation, and organellar and macromolecule redistribution, defined cycle checkpoints must be passed through successfully (Hartwell and Weinert 1989; Walworth 2000). The term "restriction point" (or "R") was first used to describe the transition

point during G1 that determines cellular removal from the cell cycle or advancement to late G1 and S phases (Pardee 1974). After a given cell has initiated G1, removal of mitogenic stimuli before R allows a return to G0, however after this point there occurs a switch to mitogen-independent progression through G1 (Planas-Silva and Weinberg 1997). An extensive amount of research has implicated members of the retinoblastoma (Rb) protein family (pRb, p107, p130) as regulators of the passage through R (Sherr 1994; Weinberg 1996). This appears to be accomplished by Rb family-mediated binding of a number of transcription factors required for coordinated expression of cell cycle machinery. Such binding inactivates these factors by sequestering them from DNA binding sites (for review see Sherr 1996). The Rb proteins are considered to be tumour suppressors, due to their role in cell proliferative regulation and their loss of expression or function in certain cancers (Harbour and Dean 2000).

An initial event in early G1 phase is the synthesis of the D-type cyclins (primarily cyclin D1); the cyclins are a family of proteins critical to cell cycle progression (Sherr 1996; Ortega *et al.* 2002). D-type cyclins bind to and activate cyclin-dependent kinases 4 and/or 6 (Cdk4/6), and these kinase complexes then can act to inhibit Rb family activity through direct phosphorylation (Adams 2001). Cdk4/6 are themselves tightly regulated by the INK4 family of cell cycle inhibitors (cyclin-dependent kinase inhibitors, or CKIs), which are also considered to be tumour suppressors (for review see Sherr and Roberts 1999; Malumbres and Barbacid 2001). Basically, the levels of cyclin expression and activities of Cdks and CKIs, as determined by cellular mitogenic or antimitogenic signals, are responsible for the cell's ability to "stop" or "go" at a given cycle checkpoint. The

major regulatory events proposed to occur at the R point of the G1/S transition are displayed in figure 1.

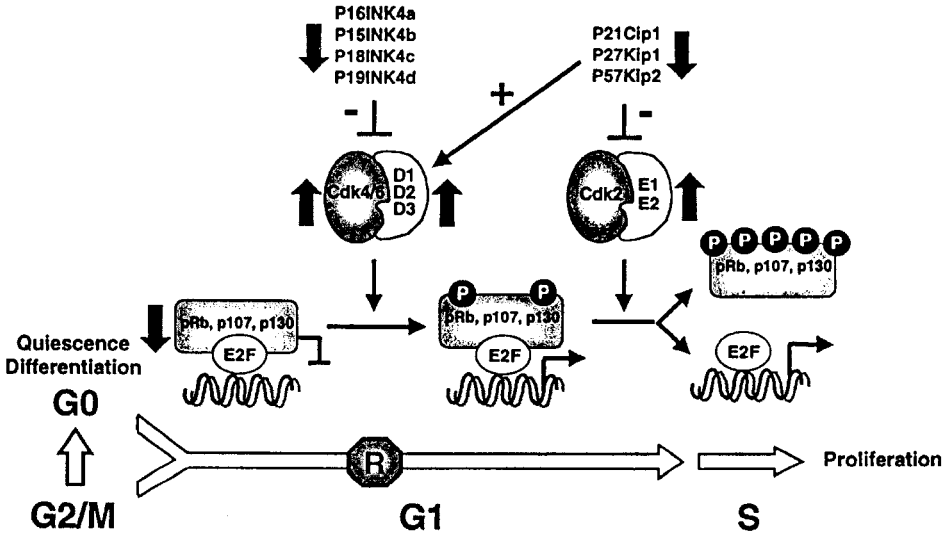

Figure 1. Regulation of the Restriction Point during G1 Phase
(Reprinted from Ortega *et al.* 2002)
Rb proteins are non-phosphorylated in G0, and can bind to transcription factors such as the E2F family. Phosphorylation of Rb proteins leads to their inactivation, and to increased cyclin/Cdk activity. A lowered H_2O_2 stimulus can also decrease cyclin D expression. Arrows signify mutations often found in human cancer that cause downregulation (downward arrows) or upregulation (upward arrows) of the activity of the indicated enzymes.

The progression from G2 phase to mitosis (G2/M transition) is another point of cell cycle regulation, however here the checkpoint stimulus appears to be DNA-damaging stress rather than mitogen withdrawal. Mitosis is thought to be initiated through the activity of the Cdk1 (also referred to as Cdc2) and B-type cyclin (primarily cyclin B1) complex; accordingly, this complex is also known as mitosis promoting factor (MPF) (for

review see Lew and Kornbluth 1996; Ohi and Gould 1999; Takizawa and Morgan 2000). During G2, Cdk1 is maintained in a phosphorylated and inactive state via Wee1 and Myt1 kinase regulation, until its dephosphorylation by the phosphatase Cdc25 (Hoffman *et al.* 1993; Kumagi and Dunphy 1996; Blasina *et al.* 1999). MPF activity then allows for the transition to mitosis. During mitosis, most cyclin B/Cdk1 complexes translocate to the nucleus to participate in nuclear envelope breakdown; the remaining cytosolic complexes are thought to aid in Golgi apparatus breakdown and centrosome separation (Bailly *et al.* 1992; Lowe *et al.* 1998; Hagting *et al.* 1999). At later stages in M, proper chromosome alignment and separation to "daughter" cells are controlled by complex (and not completely understood) interactions with cellular microtubule systems (for review see Khodjakor *et al.* 1999; Kapoor and Compton 2002). The molecular mechanisms leading to cyclin B/Cdk1 inactivation and mammalian cell cycle arrest at the G2/M checkpoint are linked to DNA damage-induced activity of the ATM/ATR and Chk1/Chk2 groups of kinases (Matsuoka *et al.* 1998; Hirao *et al.* 2000; Walworth 2000). These kinases participate in DNA mismatch repair (MMR) signalling that occurs after S phase, and therefore represent one bridge between DNA repair systems and the G2/M cycle arrest required for their efficient operation (Modrich and Lahue 1996; Jeggo *et al.* 1998). Failure of the G2/M regulatory systems is linked to certain human cancers, as observed with deregulation of the G1/S checkpoint. The ATM/ATR and Chk1/Chk2 kinases have also been observed to stabilize the tumour suppressor protein p53; this stabilization allows for such p53-mediated effects as cell cycle arrest and apoptosis in response to DNA damage and other stresses (for review see Ko and Prives 1996; Oren 1999).

p53 is a DNA-binding protein with a variety of transcriptional upregulatory and downregulatory activities (for review see Donehower and Bradley 1993). Under normal conditions, cytoplasmic p53 is bound to a regulatory factor, the ubiquitin ligase mouse double mutant-2 (Mdm-2) and targeted for proteosome-mediated destruction, however upon cell stress the amino terminus of p53 is phosphorylated, allowing for Mdm-2 dissociation (Siliciano *et al.* 1997; Oren 1999). Stress-induced nuclear stabilization of p53 is not only an important component of G2/M cycle arrest, but can also signal arrest at the G1/S checkpoint. This function of p53 is dependent upon its transcriptional activation of the *p21/WAF1/Cip1* gene (Harper *et al.* 1993; El-Diery *et al.* 1993). The p21 protein binds with cyclin/Cdk complexes (particularly cyclin D/Cdk4/6) to form a quatrenary complex; at high p21 concentration this association blocks Cdk activity and promotes G1 arrest (Xiong *et al.* 1993; Ko and Prives 1996). It follows that functionally-inactivating p53 mutations are observed at high frequency in tumour cell lines and in the majority of human cancers (Hollstein *et al.* 1994). As p53 participates in cell growth arrest and programmed cell death pathways, it is one, but not the only, signal that must be blocked in order for cellular immortalization and tumour progression to occur.

Explanted mammalian cells, or "primary" cells, when cultured are capable of a defined number of cell divisions but then permanently arrest in G1, a state referred to as senescence (Lundberg *et al.* 2000). One intrinsic mechanism of senescence is telomere shortening. Telomeres, the ends of mammalian chromosomes, can not be completely replicated with each round of cell division, and thus become shortened to a degree where critical DNA sequences are vulnerable to damage (Olovnikov 1973; Greider and

Blackburn 1996). Therefore telomeres appear to act as cellular "alarm clocks" *in vivo*, participating in the mechanisms in most somatic cells that allow for only a finite amount of divisions. Induced activity of telomerase, the enzyme that maintains telomere integrity by mediating polynucleotide addition onto telomere ends, can impede senescence, however other growth arrest signals must also be overcome for immortalization to occur *in vitro* (Kiyono *et al.* 1998). Oncogenic stimulation of proliferative signals can also act as a cell stress and induce senescence (Lin *et al.* 1998). Presumably, an innate cellular regulatory mechanism exists that responds to inappropriate cell proliferation and induces growth arrest; currently it is believed that such arrest is ultimately mediated via CKI activity (Lin *et al.* 1998; Lundberg *et al.* 2000). Therefore, a number of regulatory systems must be dysfunctional (for example loss of the senescence pathway) in order for neoplasia to occur. The major known mechanisms of senescence are illustrated in figure 2.

Interestingly, oncogene-stimulated cell senescence may occur via the induction of endogenous cellular ROS production, which acts as a stress stimulus (Lee *et al.* 1999). Here we see an example of peptide growth factor signalling mediated by ROS such as H_2O_2. The same appears to hold true for other cellular responses such as proliferation and differentiation. An important question is: how can a single stimulus result in completely different cellular responses such as senescence and proliferation? The answer appears to be the context in which a signal is received. The myriad of other cues encountered by the cell and the unique way that these are processed (via altered

signalling pathways; changes in gene expression, mutation etc.) will ultimately define a particular phenotypic outcome. This thesis focuses upon the specific role of non-toxic

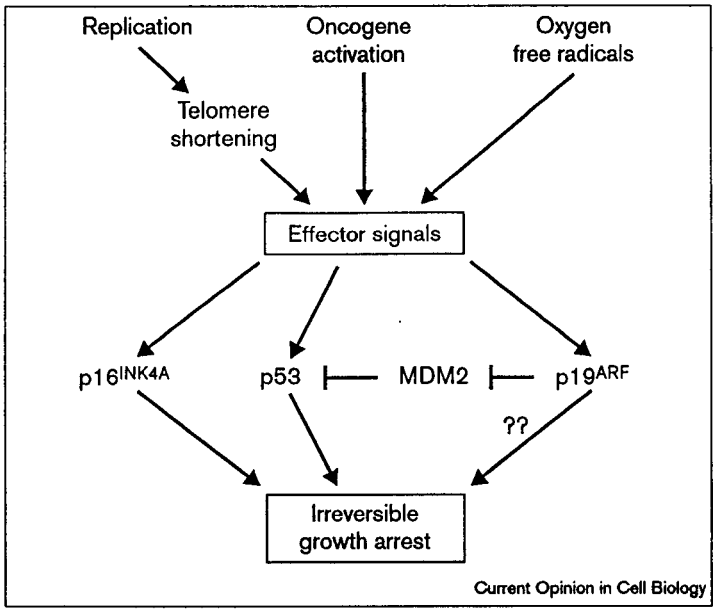

Figure 2. Stimuli and Effector Pathways of Senescence
(Reprinted from Lundberg *et al.* 2000)

levels of H_2O_2 as an inducer of cell proliferation. Appropriate mitogenic signals, when not processed as a cell stressor, are critical in activating the cell cycle machinery for progresion through cell division. For example, growth factors and growth factor-responsive pathways are observed to rapidly induce D-type cyclin expression, stability, and assembly with Cdks (Malumbres and Pellicer 1998; Diehl *et al.* 1998). It follows that ROS may regulate specific signalling pathways to exert similar effects, and could induce cell proliferation. Evidence for this function of H_2O_2 is described next. In the current and following sections, the term cell growth is used along with proliferation, with growth referring to an increase in tissue culture population or increased tumour burden.

It is important to note that *in vivo* increased cell number can result from either an upregulation in mitosis and/or downregulation of programmed cell death or necrosis, the effects of H_2O_2 regulation discussed here relate to changes in mitotic potential only.

1.2 The Cellular Production of H_2O_2

Mitochondrial Generation-

Mitochondria make an integral contribution to the regulation of several aspects of cell biology such as energy production, molecular metabolism, redox status, calcium signalling and programmed cell death (apoptosis). In accordance with an endosymbiotic origin, mitochondria rely upon the nucleus for synthesis and function. These organelles are approximately 1μm diameter and variable length, comprised of up to 15% of total cellular protein, varying in accordance with the energetic demands of the cell they supply (Schatz 1995). They are surrounded by an outer membrane, an intermembrane space and a relatively impermeable inner membrane that is responsible for inhibiting passage of polar molecules lacking specific transporters. The inner mitochondrial membrane (IMM) is folded into numerous cristae that increase surface area to accommodate thousands of copies of the enzyme complexes comprising the electron transport chain (ETC), required for the generation of adenosine triphosphate (ATP).

Permeabilization of the IMM to large solutes, referred to as the mitochondrial permeability transition (MPT), is mediated by a proteinaceous pore complex (for review see Gunter and Pfeiffer 1990; Richter and Kass 1991; Zoratti and Szabo 1995). Strong activation of the MPT causes mitochondrial swelling and release of cytochrome C (an ETC component) from the IMM into the cytosol; here it is thought to interact with

apoptotic protease-activating factor 1 (APAF-1) and procaspase-9 to induce caspase-mediated apoptosis (for review see Cai *et al.* 1998). Enclosed by the IMM is the mitochondrial matrix containing mitochondrial DNA (mtDNA), ribosomes and enzymes used for the citric acid cycle and fatty acid oxidation (Alberts *et al.* 1998). Of the hundreds of proteins found in the mitochondrion, only 13 are encoded on mtDNA and synthesized within the organelle. mtDNA consists of a double-stranded, circular, 16.6-kb genome, almost entirely made of coding sequences (Anderson *et al.* 1981). Because all of the proteins encoded by the mtDNA are vital to the assembly and function of the ETC, both replication and transcription of these genes are dependent on nuclear-encoded DNA and RNA polymerases (Grivell 1995). ETC dysfunction can thus be the result of nuclear DNA mutations, as this genome coordinates the synthesis and translocation of the majority of the oxidative phosphorylation machinery (Grivell 1995).

ETC inefficiency causes accumulation of electrons at NADH:ubiquinone oxidoreductase (complex I that catalyzes the exchange of electrons from NAD^+/NADH and ubiquinol/ubiquinone) and cytochrome bc_1 (complex III that transfers electrons from ubiquinone to cytochrome C) which can then react with molecular oxygen to form $\mathbf{O_2^{\bullet -}}$ (a one electron reduction of molecular oxygen) (for review see Wallace 1999; Saraste 1999; Lenaz *et al.* 2002). A large proportion of cellular hydrogen peroxide is derived from this superoxide production after the catalytic conversion of superoxide radical by manganese-containing superoxide dismutase (MnSOD) present in mitochondria (Boveris *et al.* 1972; Chance *et al.* 1979; Hunt *et al.* 1998). The SOD net reaction: $\mathbf{2O_2^- + 2H^+}$ **to** $\mathbf{H_2O_2 + O_2}$. Inside the cell, H_2O_2 can react with macromolecules, or can react with positively charged

transition metals (e.g. Fe, Cu) to produce the highly reactive and unstable hydroxyl radical; a process known as the Fenton reaction (net reaction: $\mathbf{H_2O_2 + Me^{n+}}$ **to** $\mathbf{OH^- + OH^{\cdot} + Me^{(n+1)+}}$) (Croft *et al.* 1992). Our laboratory has observed that extracellular generation of H_2O_2 by human cancer cell lines is significantly reduced by treatment with the electron transport chain inhibitor rotenone (unpublished data). These effects could be reflected in the growth kinetics of the tumour *in vivo*. For example, quiescent cells within the inner region of tumours exhibit lower respiration rates by downregulating mitochondrial function (Freyer 1998).

As a major source of ROS, it is thought that mitochondria make a large contribution to the redox status of the cell. High levels of cellular ROS are toxic, therefore to maintain viability it is believed that the cell controls its redox environment by balancing antioxidant enzyme levels with oxidative species production, particularly at the mitochondria (Dorward *et al.* 1997). The sensitivity of the mitochondrial genome to mutagens (discussed further in the *oxidative stress and cancer* section) suggests that mtDNA mutations may represent an initiating step in carcinogenesis. These changes can affect ETC components, resulting in changes to the cellular redox status. Therefore it is thought that non-toxic levels of ROS from mitochondrial alterations can act in tumour promotion, however too much mtDNA instability and ROS levels are cytotoxic. A significant amount of toxicity resulting from the administration of current anti-neoplastic agents has been traced to mtDNA damage (Singh 1989; Singh *et al.* 1992). It is proposed that protection from mitochondrial-mediated induction of apoptotic enzyme (caspase)

activity and/or production of toxic levels of ROS may result in cancer cell resistance to these therapies.

Generation from Other Cellular Compartments-

There are other cellular sources of ROS production than the ETC of mitochondria. These consist of a wide variety of redox enzymes, capable of transferring electrons to a wide variety of chemical substrates including molecular oxygen (Boveris *et al.* 1972; Thannickal and Fanburg 1995; Lander 1997). ROS from these systems are produced in several cellular compartments including the cytoplasm, endoplasmic reticulum, peroxisomes, and the plasma membrane. Specific examples of ROS production in each of these compartments are discussed.

Many of the substrates of ROS-producing enzymes are exogenous chemicals that become oxidized as part of cellular catabolic pathways. A variety of aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs; one example is the carcinogen benzo[*a*]pyrene), and aromatic compounds with oxygen-containing side chains such as phenols, hydroquoinones, quinones and catechols can be converted in the cell to epoxides by the activities of oxygenase and peroxidase enzymes (Gelboin 1980; Monks *et al.* 1992). Such aromatic compounds are present in natural or engineered (referred to as "xenobiotics") biochemicals; quinones are a general term for their derivatives that can undergo cycling with radical isoforms to generate superoxide radical anion (Bolton *et al.* 2000). The cytoplasmic xanthine oxidoreductase (XOR) complex, composed of xanthine oxidase and reductase, performs the rate-limiting step in purine catabolism but can also metabolize several xenobiotics (for review see Pritsos 2000).

This oxidase activity can generate both nitrogen oxide (NO) and superoxide. Various investigators have attempted to determine if the ROS produced by XOR activity plays a role in cancer development or cancer resistance to drug therapy. However, conflicting data showing increased or decreased XOR activities in cell, animal and clinical investigations have failed to define a precise mechanism.

Cyclooxygenase (COX), also known as prostaglandin H synthase (PHS), is another prominent enzyme in the transformation of xenobiotics into ROS-producing intermediates (Parman *et al.* 1998). COX also functions as the rate-limiting step of arachidonic acid metabolism to prostaglandins: initial oxygenase activity converts arachidonic acid into prostaglandin G_2 (PGG_2) and then the hydroperoxidase component of COX modifies PGG_2 into PGH_2; a process that can generate superoxide (Parman *et al.* 1998; Cao and Prescott 2002). Activity of the inducible form of COX, COX-2 (or PHS-2), is thought to promote carcinogenesis and other disease states such as atherosclerosis and arthritis. The association with cancer arises from observations of COX-2 as both an inducer of cell proliferation and inhibitor of apoptosis (Coffey *et al.* 1997; Cao and Prescott 2002). COX-2-induced proliferation may indeed be a result of ROS production. However, the antiapoptotic attributes of COX-2 activity appear to be mediated by a decrease in cellular arachidonic acid levels that are linked to toxic ceramide levels, and by the production of PGE_2 from PGH_2, that can act as an autocrine and/or paracrine signal to increase the expression of the apoptosis inhibitor Bcl-2 (upregulated in B-cell lymphoma-2) (Chan *et al.* 1998; Sheng *et al.* 1998). In addition, PGE_2 is capable of stimulating another cell response which appears to induce cell growth and

transformation: activation of peroxisome proliferator-activated receptors (PPARs) (for review see Hihi *et al.* 2002).

PPARs are steroid/thyroid nuclear hormone receptors that are themselves activated by polyusaturated fatty acids, prostaglandins and leukotrienes, and act at specific DNA sites as transcriptional regulators (Wahli and Martinez 1991; Hihi *et al.* 2002). Induction of PPAR-mediated gene expression leads to an increased cellular peroxisome content, and increases in expression and activity of peroxisomal β-oxidation system components and some microsomal cytochrome P450 isoforms (Yeldandi *et al.* 2000). Peroxisomal fatty acyl-CoA oxidase performs the initial step of the β-oxidation of cellular lipids; this reaction is another generator of ROS (Hashimoto 2000). As well, the activity of various mitochondrial- and endoplasmic reticulum-localized cytochrome P450 oxidases/reductases has been demonstrated to generate ROS during xenobiotic metabolism, much in the same way as proposed for COX (Cross and Jones 1991). Again we see evidence for reactive oxygen species (particularly H_2O_2) participating in proliferative signals. Indeed, PPAR signalling stimulates both increases in cellular H_2O_2 levels and cell transformation in models of hepatocellular carcinoma (HCC), and this specific induction of H_2O_2 is transforming in other cell types (Lake *et al.* 1987; Ashby *et al.* 1994; Chu *et al.* 1995).

An increasing number of studies have identified a plasma membrane-localized multimolecular β-nicotinamide adenine dinucleotide (NADH)-oxidase complex (also referred to as the plasma membrane oxidoreductase (PMOR) complex; Mox1 or Nox1) in mammalian nonphagocytic cells such as hepatocytes and fibroblasts (Thannickal and

Fanburg 1995; Arnold *et al.* 2001). This system is thought to be distinct from the NADPH oxidase of neutrophils, and is activated in response to a number of extracellular stimuli such as hormones, growth factors (Brightman *et al.* 1992) and the cytokines interleukin-1 (IL-1), tumour necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) (Thannickal and Fanburg 1995). Interestingly, the small guanosine triphosphate (GTP)-binding protein Rac1 is a component of the plasma membrane NADH oxidase complex, and in rabbit fibroblasts Rac1 activation resulted in the generation of reactive oxygen species (Kheradmand *et al.* 1998).

H_2O_2 generated from the NAD(P)H oxidase complex (as the dismutase product of superoxide radical anion) has been proposed to mediate cell growth, transformation, and tumourigenesis in mice (Arnold *et al.* 2001; Arbiser *et al.* 2002). It is also reported that the Rac1 GTPase can regulate superoxide production and mitogenesis by this system (Joneson and Bar-Sagi 1998). The c-Jun NH_2-terminal kinases (JNKs) are one of the downstream targets of Rac1 activity; Shin and colleagues (Shin *et al.* 1999) have demonstrated an arachidonic acid-mediated positive feedback loop of Rac1 and JNK activities which correlate with H_2O_2 generation. Rac1 is critical for the growth factor-induced membrane ruffling and lamellipodia alterations observed in cancer cell motility and invasion (Michiels *et al.* 1995; Keely *et al.* 1997; Anand-Apte *et al.* 1997). It is therefore possible that certain levels of H_2O_2 may not only be required for the efficient transmission of growth signals, but may also participate in the anchorage-independent and migratory qualities indicative of the tumour cell at later stages of cancer progression. At this time, such associations have not been well characterized. The known significant

contributors to the endogenous cellular production and metabolism of ROS are displayed in figure 3.

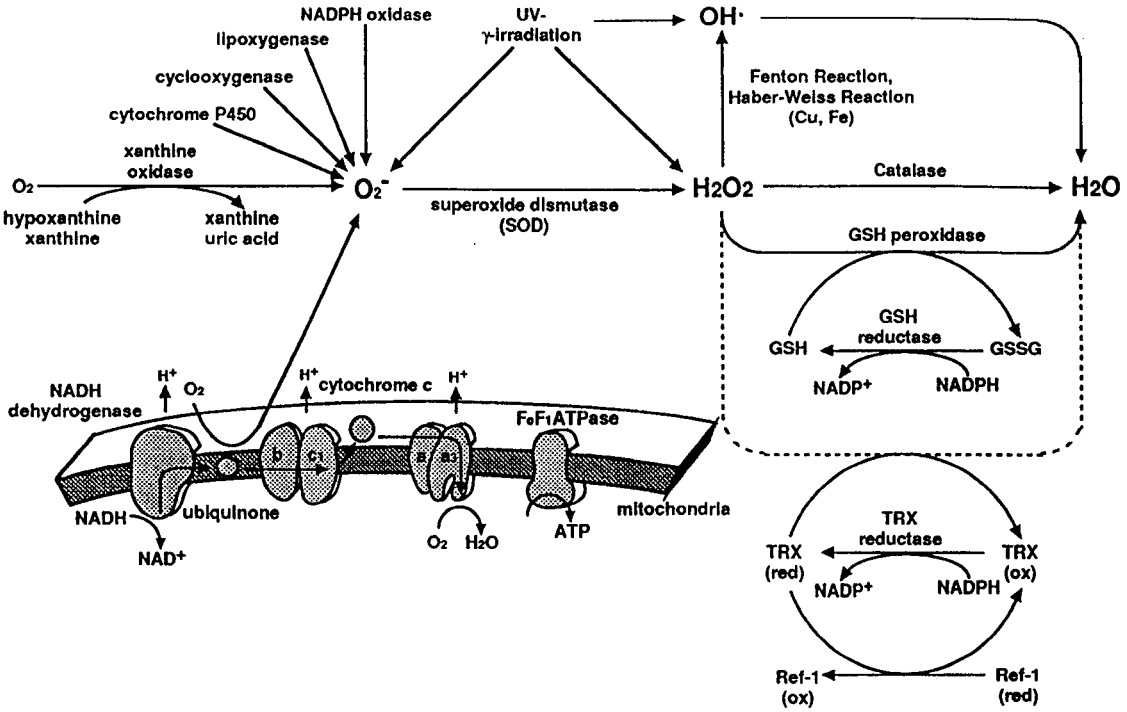

Figure 3. Major Systems of Cellular ROS Metabolism
(Reprinted from Kamata and Hirata 1999)
Major sources of superoxide anion radical (O_2^-) are displayed. H_2O_2 is mostly degraded to H_2O by glutathione (GSH) peroxidases, catalase and thioredoxins (TRX). Ref-1 represents refolding of oxidized proteins.

1.3 Antioxidants and Cell Redox Balance

In addition to the well-characterized cytotoxic effects of reactive oxygen species (e.g., superoxide ($O_2^{\bullet}$-), hydrogen peroxide, hydroxyl radical (•OH)) generation, lower levels may participate in the regulation of other cell responses. There appears to exist a window of ROS levels needed for efficient cell growth in culture (Preston *et al.* 2001); above this window cell death is activated, and below it proliferation is blocked. As cells have evolved within an oxidative environment, their ability to utilize ROS for a variety of processes has been balanced with antioxidant mechanisms for the protection of cellular

components. Reaction kinetics and physical barriers within the cell may help to avoid unwanted reactions between many oxidants and antioxidants. The term antioxidant, as used here, refers to all cellular enzymes that actively metabolize ROS into less- or non-reactive molecules, thiol-containing proteins that can be used to reduce ROS, and any other compounds with reducing potential. Upon reaction with ROS, the sulfhydryl group (thiol) on cysteine residues can be oxidized to form a disulfide bond with another thiol, or to form sulphenic (RSOH)/sulphinic (RSO_2H)/sulphonic (RSO_3H) acids (Kamata and Hirata 1999).

The primary endogenous antioxidant systems that metabolize ROS are the protein substrate glutathione (GSH; reduced form of γ-glutamylcysteine-glycine) and associated enzymes (GSH peroxidases, reductases, S-transferases and efflux pumps), the protein substrate thioredoxin (Trx) and associated enzymes (Trx reductases, peroxidases (also known as "peroxiredoxins")), and the enzymes catalase and superoxide dismutases (SODs) (Oberley and Oberley 1997; Kamata and Hirata 1999; Powis and Montfort 2001). Non-enzymatic antioxidants other than GSH and Trx are naturally produced, and can be supplied to human cells by self-production or diet. These include vitamins A (retinoids), C and E, carotenoids and plant flavonoids (Sies and Krinsky 1995; Krinsky 1998; Middleton Jr. *et al.* 2000). Most are low molecular weight compounds based upon an aromatic ring structure (Middleton Jr. *et al.* 2000). Cellular antioxidant enzyme systems are described in further detail here.

Superoxide dismutase activity converts superoxide radical anion into the less-reactive hydrogen peroxide and water (Boveris *et al.* 1972). Higher eukaryotes possess

three individual *SOD* genes encoding cytosolic copper/zinc-containing SOD (Cu,ZnSOD/SOD1), mitochondrial-localized manganese-containing SOD (MnSOD/SOD2) and secreted "extracellular" SOD (SOD3) (McCord 2002). Decreased cellular SOD activity leads to increased ROS levels, however increased SOD activity, for example by forced overexpression in cells, can result in excess lipid peroxidation from H_2O_2 (Epstein *et al.* 1987). Thus it appears that a balance between ROS production and SOD activity regulates peroxidation in the cell, and a shift in balance on either side can result in redox deregulation and toxicity (Nelson *et al.* 1994; McCord 2002). This problem is addressed by other enzyme systems that act to further metabolize H_2O_2 into water.

Glutathione is the most prominent cellular thiol-containing molecule and can scavenge free radicals, or reduce H_2O_2 or organic hydroperoxides in conjunction with glutathione peroxidases (Gpxs) or glutathione-S-transferases (GSTs), respectively (Meister 1988; Hayes and McLellan 1999). There are four known mammalian selenium-containing Gpxs (Gpx1-4), of which Gpx1 is the major form observed in several cellular compartments (for review see Arthur 2000). Cell culture and animal studies have shown that Gpx1 overexpression can effectively protect cells against oxidative stress, however Gpx1 knockout mouse models do not demonstrate significant increases in cellular ROS, presumably as a result of functional redundancy of other Gpx isoforms (Taylor *et al.* 1993; Yoshida *et al.* 1996; Cheng *et al.* 1997; Ho *et al.* 1997). Unlike the Gpxs that appear to be constitutively expressed, the expression of mammalian GSTs can be highly induced by cell treatment with prooxidant chemicals or ROS (Marrs 1996; Hayes and

McLellan 1999). This response is shown to be caused by antioxidant responsive element (ARE) enhancer-mediated transcriptional upregulation (Rushmore and Morton 1991; Nguyen *et al.* 1994). A critical cofactor in glutathione/Gpx/GST reactions is NADPH which maintains glutathione in reduced form (GSH); thus another enzyme of importance in GSH-mediated antioxidant activity is glucose-6-phosphate dehydrogenase (G6PD), as it catalyzes NADPH/NADH cycling thereby maintaining a GSH pool (Mehta 1994; Nicol *et al.* 2000).

H_2O_2 metabolism is also mediated by Trxs: small thiol-containing proteins (approx. 12 kDa) that act with Trx-associated antioxidant enzymes to primarily function as reducers of oxidized cysteine groups on other proteins (for review see Powis and Montfort 2001). Two mammalian Trx proteins are known; Trx1 is found in the cytosol and nucleus and Trx2 is localized to mitochondria (Gasdaska *et al.* 1994; Spyrou *et al.* 1997). Surprisingly, as opposed to other antioxidants, Trx activity is observed to promote cell proliferation and transformation. These functions are proposed to result from Trx1-mediated induction of transcription factor activity in the nucleus (DNA binding) and cell protection from peroxide-induced apoptosis (Hayashi *et al.* 1993; Powis and Montfort 2001). The last well-characterized H_2O_2 dismutase enzyme to be briefly described is catalase. Typical catalases (the most predominantly expressed; e. g. human Catalase-1) exist in the cell as homotetrameric haem proteins (Zámocký and Koller 1999). The majority of eukaryotic catalases are located in peroxisomes, and are directed to these organelles by a carboxy-terminal tripeptide peroxisomal targetting sequence (PTS) (Subramani 1993; Trelease *et al.* 1996). Catalases act to protect cell structures from

harmful oxidation at these sites, as well as to a lower degree in the cytosol (Zámocký and Koller 1999). As with the glutathione antioxidant system, catalase requires the cofactor NADPH in order to be maintained in active catalytic form (Kirkman *et al.* 1999). Although not a true physiological representation of normal catalase function, in our experiments the addition of catalase to the cell culture medium or forced cytosolic expression of Catalase-1 was utilized as a specific means of scavenging environmental H_2O_2 (Preston *et al.* 2003).

Additional mechanisms for the reduction of intracellular H_2O_2 concentration continue to be identified. The metallothioneins (MTs) represent a family of small (60 to 7 kDa) thiol-rich proteins that sequester and distribute copper and zinc ions within the cell, due to their high affinity for binding metal atoms (for review see Nath *et al.* 2000; Romero-Isart and Vašák, 2002). In response to cell stress (high Cu or Zn, oxidative and chemical stresses) or injury, increased metallothionein levels are induced by direct transcriptional upregulation of MT genes (Dalton *et al.* 1994). It is proposed that the cysteine residues of MTs are also utilized for the scavenging of ROS, and that this is a mechanism of cell protection (Hussain *et al.* 1996; Nath *et al.* 2000). The removal of free copper and other metals from cellular compartments may itself be one mechanism of MT-mediated protection from oxidative damage, via the removal of metals that can participate in the hydroxyl radical-producing Fenton reaction. Indeed, intracellular copper has been demonstrated to enhance ROS stress, and this stress can be reduced by antioxidant activity (Austin *et al.* 1998; Cai *et al.* 2001).

Another stress protein family that, like the MTs, is activated in response to elevated ROS levels is the heat shock proteins (Hsp) (Del Razo *et al.* 2001). There are approximately two dozen Hsps currently known, and these are categorized by their molecular weight: Hsp10, small Hsps, Hsp 40, Hsp60, Hsp70, Hsp90 and Hsp110 families (Xu 2002). Hsps are part of a class of enzymes known as molecular chaperones, defined as proteins that mediate the correct folding of other polypeptides but do not take part in the final assembly of new protein structures (Welch 1993). In addition to heat stress, hormonal signals, metabolite imbalances, chemical insults and ROS can induce Hsp activity both at the transcriptional and post-translational levels (Outinen *et al.* 1999; Xu 2002). In response to stress, endoplasmic reticulum-localized Hsps can also activate gene expression and protein kinase pathways that participate in apoptosis (Ferri and Kroemer 2001). A recent study suggests that the transcription of Hsp33 is regulated by heat shock, however post-translational regulation of its activity is specifically controlled by oxidative stress (Graf and Jakob 2002). It is intriguing that MT activity can also be regulated by post-translational protein modification (Zalups and Koropatnick 2000); therefore cellular defence systems that were previously believed to respond to specific and diverse stimuli may in fact all be coordinated in a similar manner upon the condition of oxidative stress.

To protect mitochondrial membranes, proteins and DNA from harmful reaction with ROS, a number of mitochondria-specific detoxifying mechanisms exist. As discussed, protection from superoxide is provided by MnSOD (Boveris *et al.* 1972; Wenk *et al.* 1999). Additionally, the peroxiredoxin group III (PrxIII) proteins that utilize Trx as an

electron donor for the reduction of H_2O_2 are localized to the mitochondria (Kang *et al.* 1998). A member of this group, the SP-22 protein, was recently shown to be a critical anti-oxidative stress defense for cardiovascular system mitochondria *in vitro* and in a rat model (Araki *et al.* 1999). Gpx is another characterized mediator of mitochondrial protection from ROS. Gpx1, the most abundant Gpx present both in the mitochondria and cytosol has been demonstrated to allow for the maintenance of mitochondria function and cell viability in mice using targeted gene (*gpx1*) knockout and disruption strategies (de Haan *et al.* 1998; Esposito *et al.* 1999). Coenzyme Q (CoQ; CoQ_{10} in human cells), a component of the ubiquinol or "Q" cycle of the mitochondrial ETC that can generate ROS, can act as an antioxidant at this site and at the plasma membrane in association with quinone reductases (Villalba *et al.* 1995; Crane and Navas 1997; Lenaz *et al.* 2002). The effectiveness of CoQ_{10} as a protector of mitochondrial function *in vivo* is not yet determined.

Such detoxifying enzymes may be particularly important to redox homeostasis in mitochondria as ROS themselves can further inhibit the ETC, resulting in a cycle of oxidative stress amplification. For example, Esposito *et al* (Esposito *et al.* 1999) disrupted mitochondrial OXPHOS in mouse heart/muscle by the removal of functional adenine nucleotide translocator (Ant1), causing increased ROS levels and mtDNA mutation rate; effects that were averted by a cellular response to upregulate MnSOD and Gpx1 expression. It is apparent that ETC dysfunction, whether resulting from genomic or mitochondrial DNA mutation, can result in additional damage to mitochondria and other cell components via oxidative stress. Research investigating the biology of

apoptosis-related disorders, in particular neurodegenerative disorders, has increasingly focused upon this process as an underlying mechanism of these pathologies (de Haan *et al.* 1998; Wallace 1999). In the case of cancer, high ROS generation from mitochondria coupled with increased resistance to toxicity from oxidation (as observed in tumour resistance to conventional chemotherapy and radiotherapy treatments) may be a key mediator of the transformed phenotype.

The manipulation of mitochondrial-generated oxidative stress by the control of specific antioxidant enzyme levels and activities at the mitochondria has been studied extensively using cell culture models. Overexpression of MnSOD is known to inhibit cell growth, decrease mitochondria membrane potential and is proposed to represent a mechanism of tumour suppression (Li *et al.* 1998a; Li *et al.* 1998b). Researchers (Wenk *et al.* 1999) point out, however, that such effects are subject to the particular responses (i. e., alterations in the expression of other cellular antioxidants) of each given cell type. Human epidermal fibroblasts overexpressing MnSOD were shown to produce more H_2O_2, which caused the AP-1-mediated upregulation of matrix-degrading metalloprotease-1 (MMP-1) expression and cellular invasive potential (Wenk *et al.* 1999). These seemingly contradictory results may be explained after closer study of the exact responses to altered SOD activity in certain cells. For instance, the balance of $O_2^{\bullet}$- to H_2O_2 may be critical to the outcome of MnSOD overexpression, with high [$O_2^{\bullet}$-] stimulating cell stress and death and high [H_2O_2] enhancing cancer-related properties like proliferation and metastasis. Thus, in cells exhibiting efficient H_2O_2 catabolism (from glutathione peroxidases, catalase, thiol "sinks"), high SOD activity could cause lowered

amounts of all ROS forms and inhibit cell growth, but if H_2O_2 levels remain high other pro-cancer responses may be promoted.

1.4 Oxidative Stress and Cancer

The free radical theory of ageing is founded upon the idea that cells are chronically exposed to ROS, and this exposure damages critical cellular components over time, resulting in physical deficiencies (Harman 1956; Harman 1988). This theory has been proposed as one of the factors contributing to tumourigenesis. However, the full implications for the contributions of ROS to cancer cell functioning *in vivo* have yet to be determined. Oxidative stress refers to a deregulation of cellular ROS levels caused by increased ROS of environmental or biochemical origin, and/or a decreased ability of cellular antioxidant defences to remove these ROS. Certainly, animal studies have shown that damaging oxidative stress can kill cells and decrease life span. A report by Migliaccio and colleagues (Migliaccio *et al.* 1999) suggests that, in mice, such a response may be primarily regulated by the stress-responsive $p66^{Shc}$ adaptor protein. Because of their ability to induce genomic mutation, ROS are also considered to be carcinogens. In theory, a cell which survives increased oxidation may become neoplastic, and further exposure to ROS could in fact promote tumour progression. Treatment of malignant mouse keratinocytes with carcinogens (ionizing radiation or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine) indicated that the ability of these agents to increase cell growth *in vitro* and *in vivo* depended upon elevated ROS concentrations (Gupta *et al.* 1999). It is critical to note that ROS are required in other physiological processes (both normal and pathological), such as wound healing (particularly via angiogenic effects) and rheumatoid

arthritis (Sen *et al.* 2002). Reactive nitrogen species such as nitric oxide (NO) are also thought to contribute to these processes and others via the regulation of intracellular signaling cascades and cytokine production, and ROS may function in a similar fashion (Schwentker *et al.* 2002). Only the ROS relationship with cancer will be further discussed.

The pattern of elevate ROS concentration and carcinogenesis is also observed in humans, although this research is to date preliminary. Human renal, lung and prostate cancers often display decreased levels of antioxidant enzymes (particularly catalase and glutathione peroxidases) and increased oxidative stress as compared to non-transformed tissues (Coursin *et al.* 1996; Oberley and Oberley 1997). One clinical study suggests that women who are homozygous for a variant of the *MnSOD* gene, that encodes a mutant MnSOD incapable of transport to the mitochondria, have a significantly higher risk of breast cancer (Ambrosone *et al.* 1999). Therefore, H_2O_2 levels could be contributing to this putative risk. Interestingly, catalase overexpression in the cytosol and especially in the mitochondria of HepG2 cells has been demonstrated to raise sensitivity to TNFα-mediated caspase activation and apoptosis (Bai and Cederbaum 2000). These results correlate with our investigations on the importance of the H_2O_2 stimulus in cell signalling (Preston *et al.* 2001; Preston *et al.* 2003). Such observations suggest that the ROS stimulus may contribute to cancer initiation, maintenance and progression *in vivo*. It is critical to point out that there exists no conclusive evidence for a direst correlation between decreased antioxidant activities and cancer, however. In theory, the response to ROS may be shifted from one of stress to growth in cells having constitutive activation of

growth pathways and defects in the apoptotic program. Therefore, antioxidant therapy could act as an anti-cancer treatment, at least in conjunction with other conventional agents.

Certainly, the hypothesis that changes to mitochondrial OXPHOS, linked to ROS production from the electron transport chain, might be associated with cancer has been studied for some time (Warburg 1954). Homoplasmic (present in every mitochondrial genome of a cell) mtDNA mutations have been observed in many human colorectal tumours and tumour cell lines studied, and it is postulated that these mutations confer a cell growth advantage by inhibiting the ETC and causing higher levels of ROS production (Polyak *et al.* 1998). Despite findings like these, the significance of mitochondria-derived ROS in human cancer progression remains to be fully understood. Mutations within the cellular genome including those that have been connected to neoplasia can drastically affect the functioning of mitochondria. The nucleus supplies the polymerase enzymes and other factors responsible for the replication and expression of the mtDNA, and also encodes many of the proteins critical for such mitochondrial processes as OXPHOS (Grivell 1995). However, genes specific to the mitochondria themselves have a profound influence upon cell physiology, through participation in metabolic pathways including lipid, haem, and pyridine nucleotide synthesis, transfer and ribosomal RNA production, and of course energy provision in the form of ATP production (Sweet *et al.* 1998).

Through these critical processes as well as cellular redox regulation, the mitochondria have the ability to influence tumour cell phenotype by mechanisms which

are both dependent and independent of the nucleus. As genomic mutation, chromosomal instability and aneuploidy have been linked with many cancers (Cohen and Ellwein 1990; White 1996), the mitochondrial dysfunction resulting from these nuclear events as well as mtDNA damage may play an important role during the progression of such diseases. mtDNA itself is highly sensitive to mutation, much more so than nuclear DNA, due to lack of protective histones, the high error rate during replication by DNA polymerase γ, and proximity to reactive oxygen species generated from O_2 respiration (Kunkel and Loeb 1981; Sweet *et al.* 1998). The alteration of mitochondrial genes can have substantial effects upon other compartments within the cell.

One approach used to study the ability of mitochondrial signals to affect cell behaviour is to deplete mtDNA by serial cell passages in the presence of ethidium bromide or by inhibition of mRNA translation by mitochondrial ribosomes using chloramphenicol (Wang and Morais 1997). After EtBr treatment, "ghost" mitochondria remain intact, with maintenance of a membrane potential ($\Delta\Psi m$) and ATP metabolism (primarily via the glycolytic pathway), however there is no replication or expression of mtDNA. In avian cells transformed by the MC29 strain of avian leukosis virus (containing v-*myc*), loss of mtDNA blocked the anchorage-independent phenotype, which was rescued after reintroduction of wild type mitochondria by cybrid fusion (Zinkewich-Péotti *et al.* 1990). In the same model, lowered mtDNA expression resulted in the increased transcription of several genes including viral-encoded v-*myc* (Wang and Morais 1997), and in a reciprocal manner the levels of mitogen-activated protein kinase kinase 2 (MEK2) were increased after treatment (Wang *et al.* 1997). So, by affecting the

mitochondria, nuclear gene expression can be manipulated, and tumour physiology may be altered. It should be noted that conflicting results from experimentation with human cells in cybrid studies has stimulated questions regarding the effects of cell species, tissue type and state of differentiation on the penetrance of mitochondria-derived signals.

It is this mitochondrial and ROS deregulation that may be an important aspect of tumour biology: a decrease in electron transport chain efficiency may result in greater production of ROS, which could manipulate a host of cell signalling cascades, while cytotoxicity is resisted. If the mitochondria have any significant contribution to this difference, it could be realized by changes in the electron transport chain and/or local antioxidant proteins such as superoxide dismutase, glutathione/glutathione peroxidase, and catalase. It is these same enzymes which are upregulated in fibroblasts after chronic exposure to oxidative stress (Spitz *et al.* 1993; Hunt *et al.* 1998), therefore cancer cells which are resistant to the toxicity inherent with increased cellular oxidation have presumably acquired this characteristic by some other defense mechanism(s).

Oberley *et al* have studied the relationship between the level of expression of MnSOD and neoplastic transformation extensively. In immortalized as well as malignant cell lines, overexpression of MnSOD resulted in growth inhibition (Oberley *et al.* 1995; Li *et al.* 1998a; Li *et al.* 1998b). This increase in MnSOD has also been demonstrated to suppress the malignant phenotype of human glioma, oral squamous carcinoma and breast cancer cells (Li *et al.* 1995; Liu *et al.* 1997; Zhong *et al.* 1997). More recently, H_2O_2 generated from the mitochondrial compartment has been linked to activation of mitogen-activated protein kinases (MAPKs) and increased matrix metalloprotease (MMP)

expression (Ranganathan *et al.* 2001). A decrease in overall MnSOD level should translate into a higher concentration of its catalytic substrates, oxygen radicals, and lower concentration of product, H_2O_2. However, the interplay between SODs and other antioxidant enzymes at the mitochondria and elsewhere among various cell types is an important consideration in this process. The investigators hypothesize that MnSOD may function as a tumour suppressor and possible marker of cell differentiation. If so, the endogenous regulation of the MnSOD gene may be affected by the changes to characterized tumour suppressors, such as p53, seen in a cancerous state. Collectively, these research findings suggest that increased oxidation may confer a selective advantage to cancer cells *in vivo*, and the regulation of such an effect with therapies directed against cellular sources of ROS or aimed towards activation of scavenging enzymes might help control tumour growth.

1.5 H_2O_2 Regulation of Cellular Signal Transduction Pathways

The interactions of ROS with lipid species and thiol-containing proteins are considered important in multiple aspects of normal cell physiology including growth and differentiation, as well as altered states such as transformation (Szatrowski and Nathan 1991; Burdon 1995; Lander 1997). Of the work directed at studying cellular redox effects upon cell functioning, there is a growing focus on the ability of H_2O_2 to act as a specific mediator of both stress and proliferative signalling. The role of H_2O_2 in association with cellular toxicity during physiological processes such as phagocytic attack has long been characterized. Depending upon cell type, the concentrations of H_2O_2 sufficient to induce cell death in culture conditions range from 10^{-6} to 10^{-3} M,

however low levels of endogenous cellular H_2O_2 production (10^{-10} to 10^{-7} M) are generally non-toxic and in fact appear critical for efficient culture growth (Szatrowski and Nathan 1991; D'Souza *et al.* 1993; Tokoyuni *et al.* 1995; Lander 1997). It is yet undetermined if this H_2O_2 dependency is intrinsic to cell functioning or an artifact of the tissue culture environment. However, reports implicating H_2O_2 as a promoter of several well-characterized proliferation pathways, as well as the previously discussed evidence of lowered H_2O_2 scavenger enzyme levels in rapidly proliferating cells and tumours suggest that the effects are real (Sundaresan *et al.* 1995; Rao 1996; Coursin *et al.* 1996).

There exists a large amount of evidence for the participation of H_2O_2 in a number of signal transduction pathways associated with human cancers (Lander 1997). Most of the information collected comes from tissue culture models in which exogenous levels of H_2O_2 in the micromolar range are added to cells with subsequent investigation of cellular signalling, or the reciprocal study of H_2O_2 production responses to the introduction of activated oncogenes. All mammalian tissues in culture, whether transformed or not, generate variable levels of H_2O_2, however lines exhibiting growth pathway upregulation such as those derived from human tumours have been shown to produce higher peroxide levels and also display increased resistance to cytotoxicity from oxidative stress (Szatrowski and Nathan 1991; Yang and de Bono 1997). One report postulates that in the human breast carcinoma cells, increases in reactive oxygen species generated by tumor necrosis factor α (TNFα) in turn induce resistance to stress by modulating heat shock protein 27 (Hsp27) activity (Mehlen *et al.* 1995). Thus, in the cancer cell where growth

pathways can be inappropriately activated and apoptosis avoided, oxidative stress may confer a selective growth advantage.

One question concerning H_2O_2-directed research is whether the presence of elevated H_2O_2 levels in growth-stimulated cells is a critical component of this phenotype, or simply a downstream effect of increased metabolism. Indeed, cellular H_2O_2 levels have been observed to rise quickly in response to stimulation by growth factors (Chen *et al.* 1995; Bae *et al.* 1997) although such observations may be representative of a positive feedback-type mechanism for the maintenance of signal pathway activation. Data from our laboratory (Preston *et al.* 2001; Preston *et al.* 2003) which indicates that transformed cells in culture undergo growth arrest upon scavenging of endogenous H_2O_2 is in support of such a model. This scavenging is extracellular, which raises an important question: how can this affect intracellular signalling? The loss of extracellular H_2O_2 may act as a "sink" in to which cytosolic peroxides are lost, or the critical effects may be occuring at the plasma membrane. In some mammalian tissues such as smooth muscle, H_2O_2-catabolizing enzymes such as catalase appear to be able to cross the plasma membrane, and if so a major site of function could be within the cell (Sundaresan *et al.* 1995).

Another point of controversy concerns how, specifically, reactive compounds like H_2O_2 function to assist in the transduction of signals in the cell. Cellular redox potential has been demonstrated to regulate cellular enzymatic activity (Dorward *et al.* 1997; Flohé *et al.* 1997). A number of investigators suggest that protein kinase activity may be maintained indirectly by H_2O_2 as a result of negative regulation of cytoplasmic protein phosphatase functioning (reviewed by Meng *et al.* 2002). Apparently, ROS-mediated

oxidation of critical cysteine residues at the active sites of such phosphatases inhibits their specific activities (Meng *et al.* 2002). Candidate enzyme targets for this inhibition by H_2O_2 which are important in the regulation of growth signals include protein tyrosine phosphatases (PTPs) such as PTP1C (targeting RTKs and the Src kinase family) and protein serine/threonine phosphatases (PSPs) including PP1 (targets Raf and Src), PP2A (targets mitogen-activated protein kinases (MAPKs) and MAPKKs) and the MAPK-binding protein phosphatases (MBPs) (Sun *et al.* 1993; Hunter 1995; Misra-Press *et al.* 1995; Muda *et al.* 1996). In mouse fibroblasts expressing human EGFR, addition of H_2O_2 decreases overall protein tyrosine phosphatase activity (Sullivan *et al.* 1994). By either direct kinase activation and/or phosphatase inhibition, it is observed that several protein kinase pathways are affected by H_2O_2 levels, as modelled in figure 4.

Regulation of Mitogenic Signals-

Survival and proliferative signals associated with tumour promotion and maintenance appear to require ROS for their efficient transmission to the nucleus. For example, studies on H_2O_2 effects upon cells have demonstrated activation of receptor tyrosine kinases (RTKs) (Sundaresan *et al.* 1995; Kamata and Hirata 1999), the Ras/MAPK signal transduction pathway (Rao 1996; Guyton *et al.* 1996), protein kinase C (PKC) signalling (Gopalakrishna and Jaken 2000), as well as phospholipase Cγ (PLCγ) and phosphatidylinositol 3'-kinase (PI3K) pathways (Kamata and Hirata 1999). It is likely that ROS interactions resulting in kinase activation, protein phosphatase inhibition or both are responsible for such observations.

A specific oncogenic signalling cascade of importance in many cancers is mediated by epidermal growth factor (EGF) stimulation (van der Geer and Hunter 1994). EGF binds and activates members of the EGF receptor (EGFR) family at the plasma membrane, which then transmit signals to several cytoplasmic transducer molecules resulting in altered gene expression, usually directed towards cell growth promotion. Signal messenger proteins in this pathway which have been shown to be critical for its propagation are members of a family of small guanine nucleotide exchange factors including p21 Ras, Rho and Rac (Lowy 1993; van der Geer and Hunter 1994). The small GTPase p21 Ras is located on the inner side of the plasma membrane and acts as a transducer of cell signals from tyrosine kinases such as receptors and the Src kinase family onto cytoplasmic serine/threonine kinases including Raf1 and the MAPKs Erk1 and Erk2 (extracellular signal-regulated kinase 1 and 2) and JNK (Khosravi-Far and Der 1994; Marshall 1995). Upregulation of Ras activity transforms mammalian cells in culture through stimulation of growth responses, cell cycle manipulation and cytoskeletal changes (Mansour *et al.* 1994; Peeper and Bernards 1997) and activating Ras mutations have been detected in approximately one third of all human cancers (Khosravi-Far and Der 1994). Because of their importance in tumour cell biology, many investigators have focused on the responses of specific EGF and Ras pathway components to H_2O_2 treatment in order to elucidate the molecular mechanisms which underlie the cell proliferative effects observed: addition of H_2O_2 (micromolar range) to a variety of cell types including cultured rodent and human fibroblasts, human carcinoma cells and vascular smooth muscle has been demonstrated to cause an increase in tyrosine

phosphorylation of RTKs such as the epidermal growth factor receptor (EGFR), as well as an increase in Ras effector complex localization to these sites (Gamou and Shimizu 1995; Rao 1996). As would be expected, hydrogen peroxide and other peroxide compounds also promote MAPK family activation and translocation to the nucleus (Stevenson *et al.* 1994; Guyton *et al.* 1996).

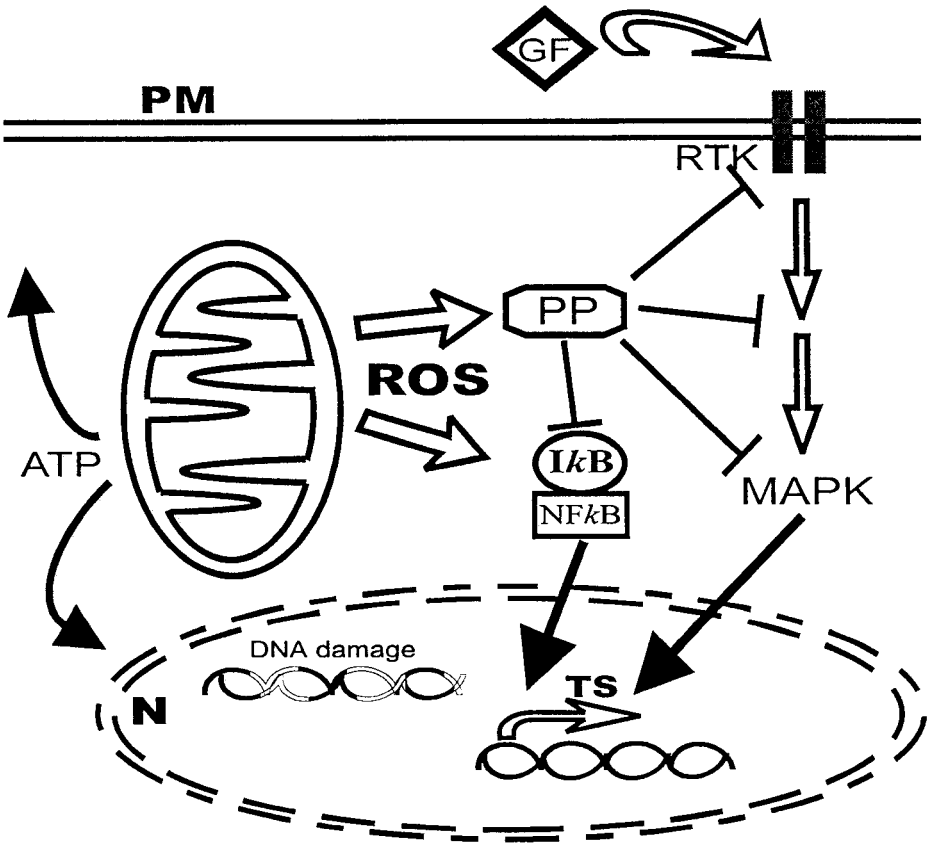

Figure 4. Well-characterized Models of ROS-mediated Signalling Regulation
(Reprinted from Preston and Singh 2001)
ROS generated by the mitochondria are given as an example. ROS can promote nuclear (N) and mtDNA damage, and can also regulate signal transduction pathways such as those originating from growth factor (GF) stimulation of receptor tyrosine kinases (RTK). One proposed mechanism for the induction of mitogenic signals by ROS is via the inhibition of cytosolic protein phosphatases (PP). Affected signalling molecules shown are the mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NFκB). These proteins can then translocate to the nucleus to regulate gene transcription (TS). IκB: inhibitory factor kappa B; PM: plasma membrane.

Interactions between H_2O_2 and peptide growth factor signalling are further demonstrated by reciprocal experiments to those using exogenous H_2O_2 addition, where the activation of specific growth pathway components by peptide growth factor addition or genetic mutation then causes an increase in H_2O_2 generation within the cell. For example, stimulation of starved carcinoma cells with EGF or vascular smooth muscle cells with platelet-derived growth factor (PDGF) induces a rapid response of cellular H_2O_2 generation (Sundaresan *et al.* 1995; Bae *et al.* 1997), and the expression of activated v-Ras or v-Rac1 in non-transformed lines increases cytoplasmic levels of ROS (Irani *et al.* 1997). Some progress has been made in analyzing these effects from the opposite direction, by blocking the H_2O_2 signal using the expression of inhibitory mutants of Src, Ras and Raf1 (Aikawa *et al.* 1997). Interestingly, H_2O_2 appears to be required for the maintenance of these and other cell signals, and scavenging of H_2O_2 by antioxidants or antioxidant enzymes can in turn downregulate growth pathway activation. Pretreatment of a variety of cell types with the glutathione precursor *N*-acetyl-cysteine (NAC) has been shown to block the H_2O_2-dependent activation of MAPK (Guyton *et al.* 1998). As well, cytosolic catalase activity is inversely correlated with the stimulation of MAPK through ROS generation in the cell response to lysophosphatidic acid (LPA) (Chen *et al.* 1995). One report also indicates a role for the recently characterized peroxiredoxin (Prx) enzymes, peroxidases which use thioredoxin as a source of reducing equivalent, as regulators of EGF, PDGF and TNF-α signaling in mammalian cells (Kang *et al.* 1998). Peroxiredoxin III (Prx III) is exclusively localized to the mitochondria, and

may serve as a useful point of study in order to evaluate the contribution of mitochondrial-generated H_2O_2 in the propagation of the cell growth response.

More recently, research has been conducted to try and elucidate the specific molecular targets of H_2O_2 stimulation that initiate mitogenic pathway activations. p66 Shc is an adaptor protein (mediating kinase complex formation) that functions inside the plasma membrane to recruit the p21 Ras-activating guanine nucleotide exchange factor (GEF) Grb2/Sos complex for interaction with activated receptor tyrosine kinases (Aronheim *et al.* 1994). As previously discussed, the p66 Shc protein is implicated as a molecular link between cellular oxidative stress and decreased life span in mice (Migliaccio *et al.* 1999). Additional information regarding the signalling mechanisms underlying this process has been presented, and implicates the downregulation of pro-survival pathways as a critical factor. Specifically, in mammalian cell culture, Shc-induced ROS production was observed to abolish the activity of forkhead transcription factors, which act as downstream effectors of the PI3-K/protein kinase B (PKB/Akt) survival cascade (Nemoto and Finkel 2002).

Other investigation has indicated the involvement of another plasma membrane-localized target of H_2O_2-induced mitogenesis: the Src kinase family. Stimulation of Erk1/2 MAPK activity by H_2O_2 is proposed to require signalling from both the Src kinsases (Src, Fyn, Lck) and Ras (Irani *et al.* 1997). A putative mechanism of this pathway synergism is the Src family (Src or Fyn, depending on the cell type studied)-dependent phosphorylation of JAK2 kinase, which activates the Shc/GEF/Ras pathway (Han *et al.* 1996; Abe and Berk 1999). Interstingly, a similar yet distinct pathway is

proposed for H_2O_2 stimulation of the stress induced JNK signal; here, H_2O_2-induced Src activity signals to the JNK cascade via the Cas adaptor protein (Yoshizumi *et al.* 2000). In haematopoeitic cells, ROS signalling to Erk1/2 appears also to initiate at the plasma membrane, however by a Src-independent mechanism of heterotrimeric G-protein activation (Nishida *et al.* 2000; Nishida *et al.* 2002). Therefore, the critical site of cellular initial environmental redox "sensing" may occur at the plasma membrane. As H_2O_2 level alterations may occur from both intra- and extracellular sources, this would be an ideal compartment for this function. Effects upon various signal transduction pathways downstream of these sensors could then determine the cellular response to redox changes.

From the information gathered so far, it becomes difficult to determine whether non-toxic ROS levels act to help initiate the activation of signals associated with cell proliferation and transformation or if they are required in an autocrine and/or paracrine manner for the maintenance of these signals after initiation. The latter model seems to be a more accurate description of these effects. For instance, *c-myc* expression in hepatoma cells starved from growth factors efficiently induces apoptosis, and this induction appears to be dependent upon oxidative stress by elevated intracellular H_2O_2 levels (Xu *et al.* 1997). Without prior growth and survival pathway activation, the H_2O_2 response becomes one of stress.

Regulation of Stress Signals-

ROS production by the mitochondria and other sources can be regulated by a number of stress stimuli associated with cytotoxicity. Increases in ROS are observed in

response to a number of mitochondrial "poisoning" agents, during hypoxia/reperfusion injury, and after stimulation of the inflammatory TNFα pathway. In these situations, the oxygen radicals and peroxides can directly damage the mitochondrial membranes and other sites within the cell, and also cause oxidation of mitochondrial-generated pyridine nucleotides which then stimulates Ca^{2+} release (Richter *et al.* 1995). This Ca^{2+} from mitochondrial channels contributes to overall Ca^{2+} cycling kinetics of the cell and is another mechanism by which damage occurs (Gunter *et al.* 1994). The TNFα pathway is of particular interest when studying cancer, as TNFα is an important immune system-regulating cytokine involved in the targeted destruction of tumour cells through the induction of apoptosis. Upon binding to the subtypes of its receptor (TNFR), activation of the phosholipase A_2 and protein kinase C pathways occurs as well as generation of ceramide, a sphingolipid thought to directly damage mitochondria by interference with complex III of the electron transport chain (Garcia-Ruiz *et al.* 1997). This process may be responsible for the large increases in ROS observed with TNF-mediated toxicity. Other evidence also implicates mitochondria as downstream targets of the TNF signal from the plasma membrane, for example the 55 kDa subtype of TNFR can induce mitochondrial clustering in fibrosarcoma lines via specific interaction with microtubules (De Vos *et al.* 1998).

One way that mitochondrial integrity may become resistant to TNFα stimulation is by increased protection against the damaging effects of reactive oxygen species. Mitochondrial glutathione, sequestered from the cytoplasmic pool by a specific transporter, can protect from oxidative stress, as depletion of this GSH results in cell

sensitivity to the prooxidant effects of signals such as TNFα (Fernandez-Checa *et al.* 1997). Increased levels of this ROS scavenger at the mitochondria may be an important factor promoting cell survival. Many tumour cells also display an altered mechanism for glucose metabolism typified by an increase in aerobic glycolysis (Brand 1997). In theory, a reduction in mitochondrial oxygen consumption could lower the ROS produced as byproducts from the electron transport chain, thereby decreasing the effects of upstream signals which target this site to induce cell death. Products of the glycolytic pathway such as pyruvate may also contribute to this effect themselves by acting as scavengers of ROS (Brand 1997).

In addition to stress pathway mediation of ROS production, the activation of stress pathways by oxidative stimuli has also been documented. Thus a positive feedback loop of stress signaling and ROS concentration appears to exist. Initially, stress responses were believed to solely mediate apoptosis, however more recent investigations have revealed that some stress signals may have additional involvement in cell protection from oxidative stress. Another event observed upon TNFR stimulation or upon increases in ROS is the nuclear translocation of the transcription factor nuclear factor kappa B (NFκB), which is involved with the modulating the expression of a variety of inflammatory factors in lymphoid cells including TNFα itself (Siebenlist *et al.* 1994; Mercurio and Manning 1999). The activation of NFκB after TNFα treatment of cells and along with cytoplasmic redox changes is well characterized and has been attributed to the function of NFκB as a downstream effector of the TNFα pathway resulting in toxicity (Siebenlist *et al.* 1994). However, it seems as though this view of NFκB may be too

simplistic. Several studies have determined that the response of NFκB to these cell stresses may actually represent an antagonistic signal utilized by the cell as a protective mechanism against TNFα. Fibroblasts and macrophages derived from knockout mice lacking both copies of the *rel A* gene, which encodes the p65 subunit of NFκB, display increased sensitivity to the TNFα death signal (Beg and Baltimore 1996). This phenotype has also been shown to be manifested in primary and neoplastic human cell lines that express a dominant inhibitory form of IκB (inhibitory factor of κB), the regulator which binds NFκB in the cytosol to sequester it from the nucleus (Van Antwerp *et al.* 1996), and is also observed in response to other apoptotic activators such as ionizing radiation and cancer chemotherapeutics (Wang *et al.* 1996). These *in vitro* and animal studies could be reflected in the clinic. Activating mutations and overexpression of NFκB are prevalent in breast and ovarian cancers, and inhibition of IκB is observed in ovarian carcinoma and ataxia telangiectasia (Luque and Gélinas 1997).

In an attempt to elucidate how NFκB might be priming the cell for survival, research has, as would be expected, focused on NFκB-mediated changes in genomic expression. Published reports so far indicate that some promoter regions targeted by NFκB are within anti-apoptotic genes: transcription of TRAF (TNFR-associated factor) and c-IAP (inhibitor of apoptosis) genes is induced by NFκB, and the protein products of this expression appear to be required for suppression of the TNF-induced apoptosis by an unknown mechanism (Wang *et al.* 1998). An immediate early response gene, *IEX*-1L, has also been demonstrated to be activated by NFκB in a variety of transformed human

cell lines after treatment with TNFα, and may be critical for increased resistance to cell death (Wu *et al.* 1998). In addition to promoting survival, the NFκB pathway seems to be a contributing factor in tumour cell growth. The stimulation of NFκB activity during mitogenesis is well documented, and this activity can act synergistically with other growth-supporting transcription factors such as c-Jun and c-Fos (Siebenlist *et al.* 1994; Luque and Gélinas 1997).

Some of the investigation as to how these effects might be related to cancer has been via the study of the Rel members of the NFκB family, which have similarity to the v-Rel oncogene product of the avian Reticuloendotheliosis Virus (REV-T) (Bose 1992). v-Rel efficiently transforms avian lymphoid cells upon infection by the REV-T retrovirus, but does not produce the same effect when expressed in mammalian cell lines (Bose 1992; Siebenlist 1997). In spite of this apparent species specificity, alterations of the c-*rel* locus in human lymphomas (Siebenlist 1997) suggest the existence of similar mechanisms of oncogenesis in mammalian cells. It was previously thought that these properties of v-Rel were a result of interference with normal NFκB signaling, however another model in which the activation of cellular genes by v-Rel is critical for its functioning has gained acceptance. The activation of the c-*jun* promoter by v-Rel through binding at a κB site and promoter region has been observed in HeLa cells, and in chicken fibroblasts the AP-1 complex of c-Jun and c-Fos are required for v-Rel-induced transformation (Fujii *et al.* 1996; Kravola *et al.* 1998). Another retroviral oncogene product, the Tax protein of the Human T cell Leukemia Virus-1 (HTLV-1), has recently been shown to transactivate the expression of cellular genes required for lymphocyte proliferation by acting on both AP-1

and NFκB-like sites on DNA (Mori *et al.* 1998). Thus, NFκB family members may participate in similar signaling pathways to those associated with AP-1 transcription factors, and this cooperation may be necessary for cell growth.

Mitochondria may act as critical regulators of NFκB signaling through their contribution to the local cytoplasmic redox environment. The increase in cellular H_2O_2 observed in response to stimulation by growth factors including TNFα is known to activate NFκB, but the molecular events leading to this activation have yet to be determined (Siebenlist *et al.* 1994; Lander 1997). It is assumed that any NFκB signal inducer acts to promote the phosphorylation of IκB on amino-terminal serine residues 32 and 36, leading to its disassociation from the regulatory complex, ubiquitination, and degradation by the 26S proteosome (Flohé *et al.* 1997). A number of serine/threonine kinases linked to mitogenesis including protein kinase C (PKC), Ras, Raf, and MAPK kinases (MAPKKs or MEKs) have been proposed to directly phosphorylate Ser32 and Ser36 of IκB (Remacle *et al.* 1995; Flohé *et al.* 1997; Siebenlist 1997). As already mentioned in the preceding section, the activity of these enzymes can also be controlled by reactive oxygen species.

The NFκB pathway is not the only stress-induced cell survival response implicated as a target of the H_2O_2 stimulus. Rapidly growing tumours are capable of expanding beyond the perfusion capacity of the local vasculature, causing much of the cancerous tissue to be deprived of oxygen (hypoxia) and nutrients. The response of cancer cells to this environment is critical for their survival until new blood vessels are produced (angiogenesis). One way that hypoxia-mediated death is avoided is by inhibition of the

senescence- and apoptosis-inducing p53 tumour suppressor pathway, accomplished by lowered p53 expression, altered subcellular localization and stability, or mutation (Ashcroft *et al.* 2000). Another adaptive mechanism is activation of hypoxia-inducible factor 1 (HIF-1), a transcription factor that promotes the expression of genes involved in glucose transport, glycolysis, angiogenesis, erythropoiesis and cell proliferation (for review see Semenza 2000). HIF-1 functions as a dimer of HIF-1α and HIF-1β subunits; studies on the effects of HIF-1α upon the cancer cell response to hypoxia have demonstrated that it is required for tumour survival and growth in such conditions (Carmeliet *et al.* 1998; Ryan *et al.* 2000).

The possible mechanisms by which the cell senses hypoxia for the upregulation of signals like HIF-1 are of great interest. An emerging hypothesis is that mitochondria act as this "sensor", altering their functions to regulate energy consumption and induce survival responses. Cell culture experiments demonstrate a reduced oxygen consumption rate and $\Delta\Psi m$ at mitochondria after exposure to hypoxia, and it is proposed that this may result from specific effects of low $[O_2]$ upon the catalytic function of cytochrome oxidase (Chandel *et al.* 1997; Freyer 1998). As well, Chandel *et al* suggest that this change in cytochrome oxidase activity alters ETC function, causing increased H_2O_2 generation which is responsible for HIF-1 pathway induction (Chandel *et al.* 1998; Chandel *et al.* 2000). In these studies, H_2O_2 from complex III of the ETC stimulated accumulation of HIF-1α and expression of HIF-1 target genes; depletion of mtDNA and treatment of cells with catalase both blocked this signal. However, other researchers have made the contradictory observation (Canbolat *et al.* 1998) of decreased H_2O_2 levels being required

for HIF-1 pathway activity. These inconsistent results may be related to differences in experimental procedures (nature of hypoxia treatment, type of ROS assay), target genes studied, and cell type. Certainly, it appears that changes in environmental [O_2] affect intracellular ROS levels, and this can affect the HIF-1 pathway. It may be that H_2O_2 can inhibit HIF-1 activity directly in certain conditions, but the overall effect observed is HIF-1 pathway induction due to ROS-mediated MAPK stimulation (Minet *et al.* 2000). Effects upon mitogenic signals like the MAPK cascade are more unclear, however, as MAPK inhibition has also been observed upon cell hypoxia (Laderoute *et al.* 1999). As would be expected, hypoxia also activates other cellular stress pathways such as the JNK signal (Lee and Corry 1999). Cancer cells often exhibit an increased oxidative state and elevation in ROS-responsive stress pathways, as previously discussed. And this may promote resistance to oxidation- and drug-induced apoptosis. Accordingly, inhibiting specific survival-associated stress pathways and/or ROS production by drug therapy may predispose the tumour cell to other anti-cancer drugs. Thus, an increase in ROS-mediated signalling caused by hypoxic (or other) stress may be a critical mediator of tumour progression *in vivo*, as shown in figure 5.

Activation of the JNK members of the stress-activated protein kinase (SAPK) family is also regulated by redox changes. JNK activity is known to be induced by ROS (Tibbles and Woodgett 1999). As well, antioxidants have been shown to regulate this activity; for instance under normal conditions glutathione S-transferase pi (GSTp) binds and inhibits JNK activity, but with oxidative treatments (H_2O_2) GSTp dissociates and the JNK signal is upregulated (Adler *et al.* 1999). As with both NFκB and HIF-1α, JNKs are

postulated to be involved in the promotion of cell death and regulation of cell proliferation. There is also evidence linking JNKs with survival and oncogenic signalling. It is clear that the roles these enzymes play in cell physiology should not be subject to strict interpretation, as the interplay between these signals and others most likely acts to determine any phenotypic outcomes.

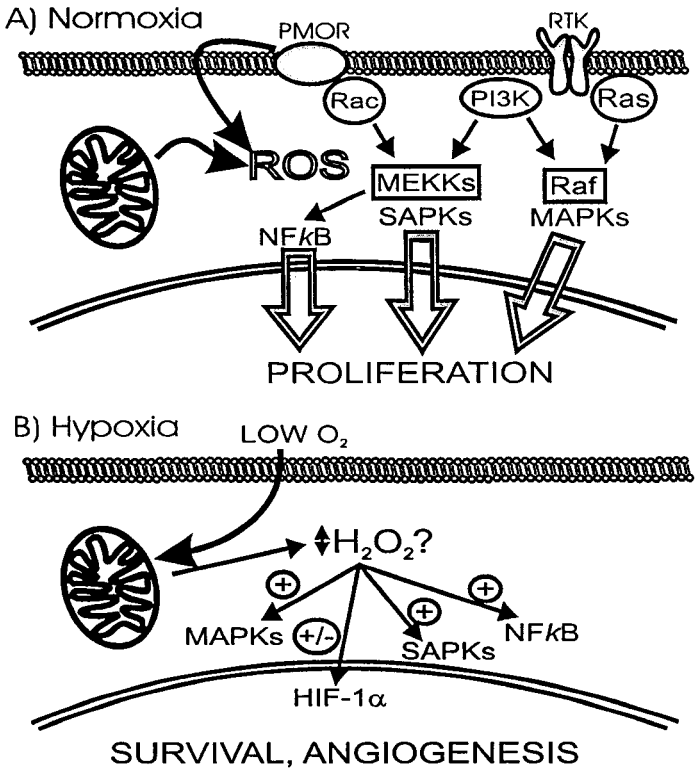

Figure 5. Growth and Stress Signal Responsiveness to Hydrogen Peroxide
(Reprinted from Preston *et al.* 2001b)
Non-toxic levels of ROS from electron "leakiness" at the ETC of mitochondria and/or other sources such as the plasma membrane NAD(P)H oxidoreductase (PMOR) complex can act as a growth stimulus (A). In conditions of hypoxia (B), cytochrome components of the ETC may act as sensors of low [O_2] and respond by altering ROS production. Changes in [H_2O_2] are proposed to affect stress responses such as the HIF-1 pathway.

JNK1 (p46 SAPKγ) is a member of the stress-activated protein kinase (SAPK) family (Dérijard *et al.* 1994). Upon dual phosphorylation of a tripeptide motif (Thr-Pro-Tyr), JNKs translocate to the nucleus and activate transcription factors involved in activator protein-1 (AP-1) complex-mediated gene transcription such as c-Jun, JunB, ATF-2 and Elk1 (for review see Tibbles and Woodgett 1999; Davis 2000). Upstream signals including the Ras, Rac and Rho GTPase pathways and PI3K/protein kinase B (PKB/Akt) pathway can activate JNK1 via phosphorylation of MAPKKKs (MEKK1-4) and mixed-lineage protein kinases (MLKs) (Tang *et al.* 1999; Bar-Sagi and Hall 2000). These kinases in turn phosphorylate MAPKK4 and 7 (MKK4,7/SEK1,2) which are direct upstream activators of JNK1 (Yan *et al.* 1994; Tournier *et al.* 1997). Upstream JNK effectors are illustrated in Figure 39 of the discussion section. JNKs can induce apoptosis in response to a variety of stresses; the proposed mechanisms of this effect are the induction of new gene expression as well as transcription-independent influences upon mitochondrial and caspase signalling (Bossy-Wetzel *et al.* 1997; Tournier *et al.* 2000). However, the JNK/AP-1 pathway has also been associated with cell growth and transformation (Davis 2000; Xiao and Lang 2000). This may be the result of a JNK-initiated homeostatic pathway. For example, in response to oxidative stress Jun proteins associate with NF-E2-related factors 1 and 2 (Nrf1/2) transcription factors to induce the protective response of antioxidant/electrophile response element (ARE)-directed expression of detoxifying and antioxidant genes (Venogupal and Jaiswal 1998). The influences of cell type and cellular environment certainly play a role in the outcome of JNK signaling. It follows that the JNK induction observed in certain cancer cells may

contribute to the transformed phenotype only following the deregulation of other cellular mitogenic and/or apoptotic pathways. Thus, adaptation to the growth- and survival-inhibitory effects of JNK expression and activity may represent one mechanism of tumour progression, much as with the adaptation to changes in cell redox status.

1.6 The Requirement of H_2O_2 for Cell Proliferation *in vitro*: Mechanistic Studies

As our laboratory and others have previously demonstrated the requirement of "basal" H_2O_2 levels for efficient eukaryotic cell proliferation in culture, the purpose of the research presented in this thesis was to determine what molecular signal(s) may be critical mediators of this effect. Knowledge of key molecular targets of the H_2O_2 stimulus would allow for manipulation of the cellular responses to redox changes, and this could help to control the behaviour of cells in which these responses have become dysfunctional, as is observed in some cancers. Much research has studied the induction of cell proliferation and mitogenic signal transduction cascades upon the addition of exogenous H_2O_2 to cell culture. This work investigates the opposite context of H_2O_2-mediated growth regulation, where the removal of physiological levels (referring to endogenous production by immortalized cell culture) of H_2O_2 results in a proliferative block. Initially, the requirement of low levels of H_2O_2 for the proliferation of Rat-1 fibroblast was investigated.

Rat-1 clones exhibiting differential growth and transformation properties resulting from specific alterations of the HER-2/Neu receptor tyrosine kinase were utilized as a model for the responses to removal of the H_2O_2 stimulus (Siegel and Muller 1996; Dankort *et al.* 1997). HER-2/ErbB-2 (Neu refers to the murine homologue) is a member

of the ErbB/epidermal growth factor receptor (EGFR) family (Reese and Slamon 1997). Activation of HER-2 by the EGF ligand causes heterodimerization with other EGFR members, and this induces cell growth- and survival-promoting signals such as MAPKs and PI3K (Wada *et al.* 1990). As H_2O_2 levels are proposed to regulate receptor activity via effects upon disulfide bond formation and receptor dimerization, we wished to utilize this HER-2/Neu model to determine if such receptor-specific effects are critical for the cell proliferative block caused by catalase-mediated scavenging of H_2O_2. Multiple signals downstream of Neu are implicated in oncogenesis, and HER-2 overexpression and/or activating mutation is observed in 25 to 30 percent of human breast and ovarian cancers (Reese and Slamon 1997; Ménard *et al.* 2000). The activities of the Neu receptor, extracellular signal-regulated kinase 1 and 2 (Erk1/2) MAPK and JNK1 effector pathways were studied after the addition of the H_2O_2 scavenger catalase to cell cultures. All Rat-1 clones displayed comparable sensitivities to growth inhibition by this treatment regardless of the level of Neu activity. It was found that scavenging of H_2O_2 caused stable inhibition of the Erk1/2 MAPK signal, transient induction of the JNK1 pathway, and no effect upon p38 MAPK activity. Constitutive Neu activation could rescue the catalase-mediated block of Erk1/2 activity while only slightly increasing resistance to growth inhibition. Therefore, these results indicated that removal of extracellular H_2O_2 can both downregulate growth signals and activate stress-associated signals, however the stress response appears to play a larger role in the anti-proliferative effects observed.

Due to these findings, research became directed at a further study of the involvement of JNK1 in this cell response. The JNK1 signal was manipulated in a

human ovarian carcinoma cell model by transient and stable transfection of an ectopic JNK1-expressing plasmid construct. From this work it was observed that alteration of this signal is capable of affecting the cellular response to both extracellular catalase treatments and catalase overexpression. Transient induction in JNK1 levels contributed to growth inhibition by catalase treatments and decreased endogenous cellular generation of H_2O_2. Cell lines that presumably adapted to stable induction of JNK1 levels and c-Jun phosphorylation also displayed a lowered endogenous production of H_2O_2, however an increased resistance to the catalase-mediated block of proliferation was observed. After further experimentation, it is presumed that these effects are related to JNK control over cellular ROS production, through JNK-directed gene expression or by direct effects upon ROS-generating systems. Certainly, this JNK-H_2O_2 relationship represents only one part of the mechanisms underlying redox regulation of cell proliferation. However, our results are the first to implicate the induction of a specific cellular stress pathway upon "reducing" stress (lowering of basal H_2O_2 levels), and the ability of this pathway to itself alter cellular H_2O_2 production. We propose that JNK signalling may be an important effector of the H_2O_2 stimulus, and that deregulation of this pathway may play a role in the altered proliferative capacity of cancer cells.

1.7 Hypothesis

The autocrine and/or paracrine generation of non-toxic levels of H_2O_2 acts upon a variety of intracellular signalling pathways to promote cell proliferation. Removal of this stimulus can act to induce growth arrest. Both growth- and stress-associated signals can be affected by the alteration of H_2O_2 concentrations, and the balance of such signalling

may determine the response of a given cell to these changes. The manipulation of molecular targets of the H_2O_2 signal can alter the ability H_2O_2 to act as a cell mitogen, and may provide a strategy for drug therapy of diseases related to proliferative deregulation such as cancer. Additionally, if removal of H_2O_2 (for example by antioxidant activity) can not block inappropriate proliferation, it may decrease cancer cell resistance to antineoplastic agents.

1.8 Objectives

1. To observe the effects of the scavenging of extracellular H_2O_2 upon mitogenic and stress signalling pathways, from the receptor tyrosine kinase level to the transcription factor level.
2. To determine critical protein kinase targets of the H_2O_2 stimulus from such observations.
3. To alter the functioning of the H_2O_2 target(s) by genetic and pharmacologic manipulation, in order to gain understanding of the molecular mechanisms of H_2O_2-mediated cell proliferation.

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3.0 RESULTS

3.1 Scavenging of Extracellular H_2O_2 by Catalase Inhibits Cell Proliferation

H_2O_2 is Required for Efficient Proliferation of Rat-1 Fibroblasts in Culture-

Addition of catalase preparations to the culture media has been shown by our laboratory to block the proliferation of a variety of immortalized cell types in a dose-dependent and reversible manner (unpublished observations). Extracellular catalase treatments have also been utilized to inhibit the effects of exogenous H_2O_2 in cell culture (Dalton *et al.* 1994; Hansen *et al.* 1999) and forced overexpression of catalase by cells *in vitro* or in transgenic mouse models can inhibit DNA synthesis and cell growth (Nilakantan *et al.* 1998; Brown *et al.* 1999). To determine if exogenous catalase activity could produce a similar response in a wild type and HER-2/Neu-transformed Rat-1 fibroblast model, 1000 cells/well were seeded into 96-well plates, cells allowed to adhere then treated with doses of catalase (0 to 2000 U/ml) or heat-inactivated preparation, and total cell number after 4 days culture was observed (Fig. 6). The growth of all Rat-1 lines was blocked after catalase addition; removal of this treatment during incubation or heat-inactivation of the enzyme reversed this effect. Catalase treatment (up to 48 hours) caused growth arrest without significant induction of the apoptotic program, as observed by cell morphology and trypan blue exclusion (not shown).

To confirm that catalase treatment of the cell culture media was indeed lowering H_2O_2 concentrations, Rat-1NeuN and SK-OV-3 human ovarian adenocarcinoma cells were plated, media treated with varying amounts (0 to 2,000 U/ml) *Aspergillus niger* catalase preparation, and extracellular H_2O_2 production quantitated (Fig. 7). Addition of

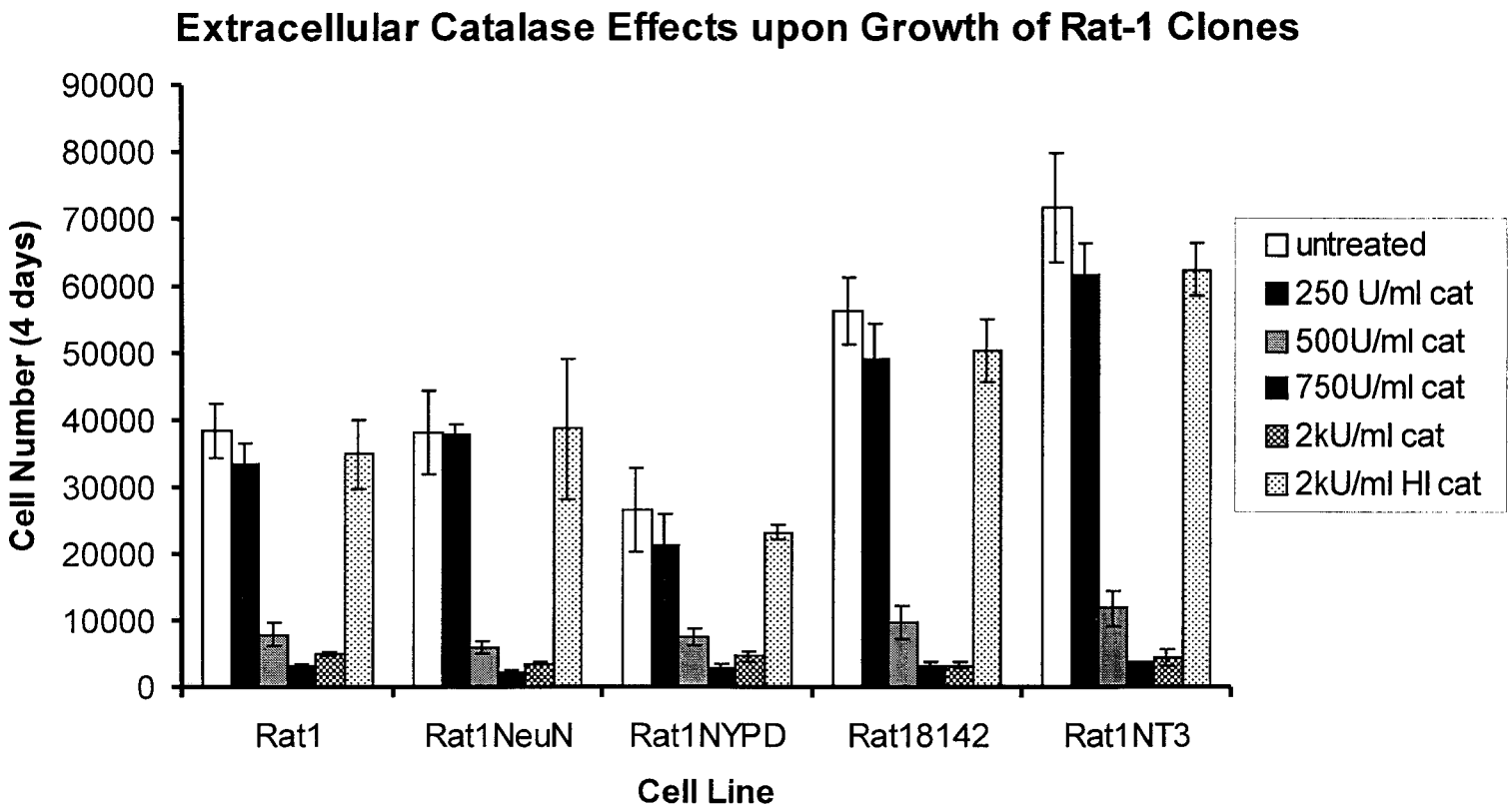


Figure 6: Growth Inhibitory Effects of Extracellular Catalase Treatment
Cells were plated at a density of 1,000 cells/well (96-well plate), allowed to adhere, and then subjected to the indicated treatments. Cat=bovine liver catalase preparation (0-2,000 U/ml); HI cat= heat-inactivated catalase (2,000 U/ml). After four days of incubation, cell numbers in wells were calculated. Dose-dependent growth inhibition by catalase was observed at comparable levels in all Rat-1 cell lines, regardless of level of transformation, and this effect was not observed upon heat-inactivation (boiling 10 minutes) of the enzyme.

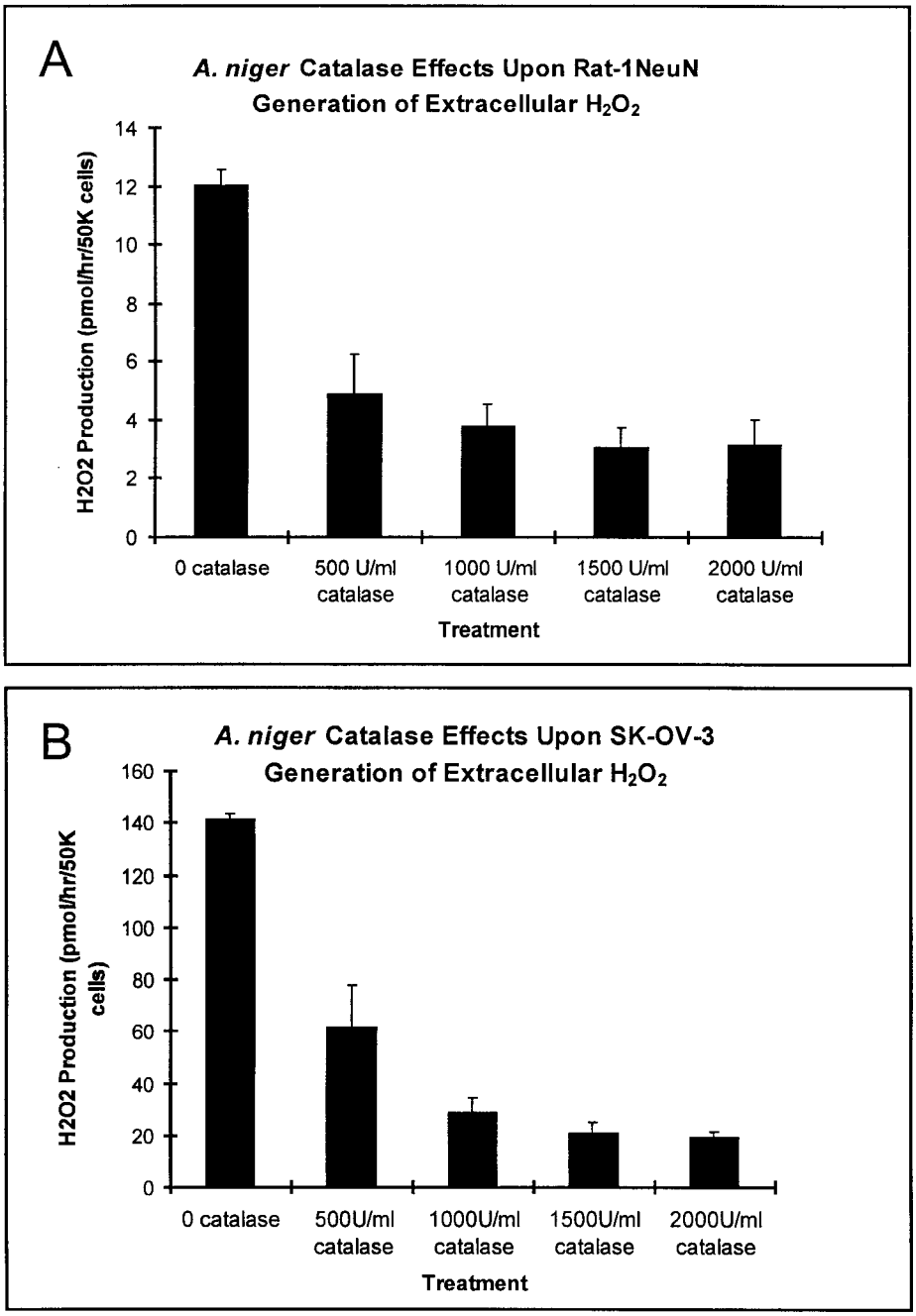
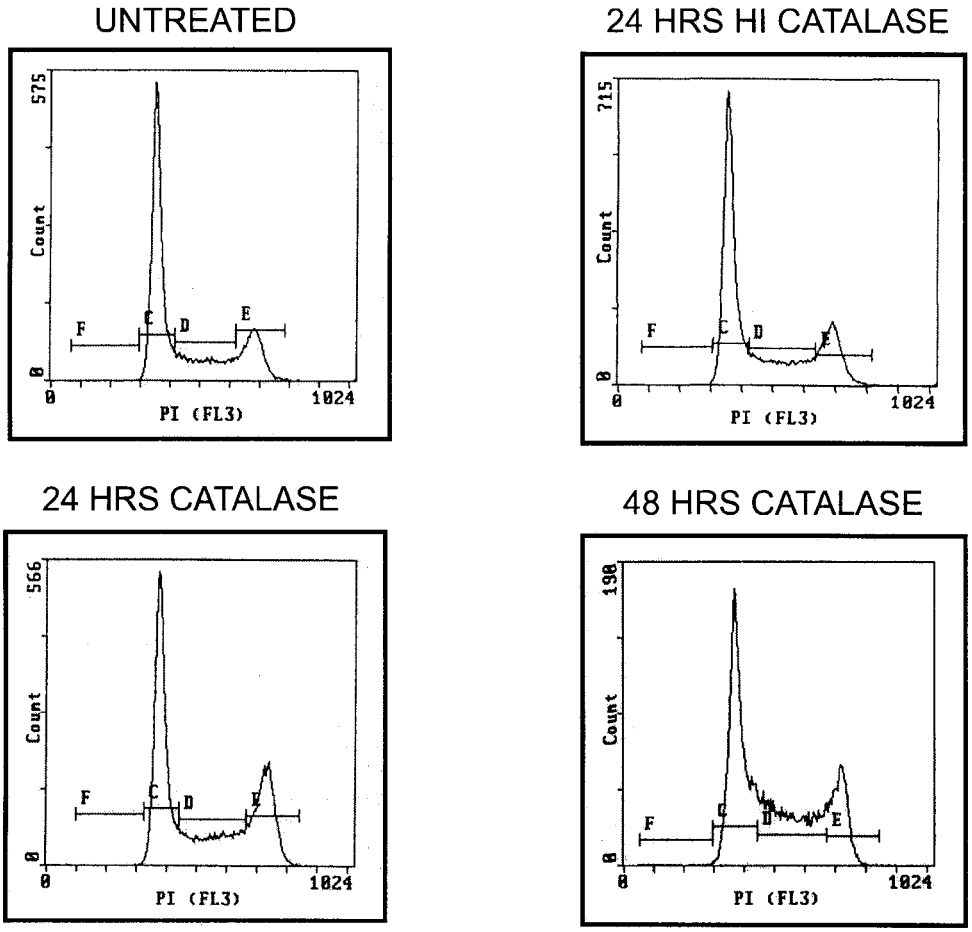


Figure 7: Scavenging of Extracellular H_2O_2 by *Aspergillus niger* Catalase
A, 50,000 Rat-1NeuN cells were seeded into multiple wells and analyzed for extracellular hydrogen peroxide production. The indicated amounts of catalase preparation were added into the culture solution prior to measurement of H_2O_2 levels. Addition of catalase effectively scavenged extracellular H_2O_2. *B,* the identical experiment using SK-OV-3 cells. Note the large amount of H_2O_2 generation by this cell line, which was also reduced after the indicated catalase treatments.

Aspergillus niger catalase preparation to the media lowered extracellular H_2O_2 levels by 2- to 3-fold as compared to untreated culture. H_2O_2 levels were effectively diminished in a dose-dependent manner both from the Rat-1 clone that produces relatively low amounts of H_2O_2, and the SK-OV-3 cell line that generates much higher levels. The bovine catalase preparation used in growth experiments was not tested, as some component of this preparation caused an increased background absorbance during the H_2O_2 assay. As the same effects on cell proliferation were observed with both *A. niger* or bovine catalase preparation, it is assumed that both have comparable scavenging activities.

Catalase Treatment Blocks Rat-1 Proliferation at the S and G2/M Phases of the Cell Cycle, but Does Not Inhibit Cell Size Progression-

As catalase activity was shown to block cell proliferation in our cell model, the specific effects upon cell cycle progression were assessed in order to gain more insight into possible mechanisms of the growth inhibition. We investigated the cell cycle response of Rat-1NeuN cells to bovine catalase treatment of increasing duration (6 to 48 hours), and compared this response to growth arrest from serum starvation or to heat-inactivated catalase control treatment (Fig. 8). The cell cycle profile of the fibroblasts was assessed by propidium iodide staining of total DNA content and flow cytometry analysis. In our model system a discreet block of the cell cycle at the G1/S or G2/M checkpoints was not observed, however an overall accumulation of cell populations in S and G2 phases suggests a prolongued S phase and inhibition of entry into mitosis. These findings correlate with those obtained upon overexpression of catalase in cell cultures (personal communication from Dr. A. I. Cederbaum).



UNTREATED			24 HR HI CATALASE			24 HR NO SERUM		
%G_1	%S	%G_2	%G_1	%S	%G_2	%G_1	%S	%G_2
51.2	33.3	15.4	48.2	35.4	16.5	74.1	17.3	8.7
Chi sq. 10.7			Chi Sq. 16.5			Chi Sq. 14.7		
12 HR CATALASE			**24 HR CATALASE**			**48 HR CATALASE**		
%G_1	%S	%G_2	%G_1	%S	%G_2	%G_1	%S	%G_2
45.4	36.2	18.4	40.5	35.6	23.9	33.2	52.2	14.6
Chi Sq. 14.2			Chi Sq. 19.0			Chi Sq. 8.0		

Figure 8: Catalase Effects Upon Rat1NeuN Cell Cycle
Cells were treated for the indicated times with 1000U/ml catalase or heat-inactivated (HI) catalase preparations. Cell cycle profiles were then determined by examining total cell DNA content (propidium iodide (PI) staining) using flow cytometry. The table represents multicycle (mcycle) analysis of the results, as percentages of cell populations in a given stage of the cell cycle. Catalase treatment increased the percentage of Rat1NeuN cells in the S and G_2 phases. F=sub-G_1 population, C=G_1 population, D=S phase population, E=G_2 population.

To determine if cell cycle-inhibitory effects of catalase also applied to cycle-dependent increases in cell size, cultures underwent identical catalase treatments and cell size changes were observed (Fig. 9) from the forward scatter of an argon laser during flow cytometry. In contrast to effects upon S and G2/M phase progression, increasing durations of bovine catalase did not hinder increases in cell size, resulting in progressively larger cells unable to proliferate. Treatment with heat-inactivated preparation did not hinder cell cycle progression, and thus cells retained their regulated size (as compared to untreated control). These findings indicate the cell cycle machinery was inhibited by treatment, but other cellular gene expression may proceed. Therefore, scavenging of H_2O_2 may specifically induce mitotic arrest.

Co-incubation of cells with an H_2O_2-generating glucose oxidase preparation in addition to catalase also rescued the proliferation block (Fig. 10), providing evidence that catalase-mediated effects are indeed a result of changes in H_2O_2 levels. Treatment with glucose oxidase alone at activities greater than 4×10^{-3} U/ml was toxic to cells, suggesting that a defined window of extracellular H_2O_2 concentration is required for cell survival and growth. Apparently cells need a defined level of environmental H_2O_2 as a mitogenic stimulus, however excess H_2O_2 acts as a toxic stress. Glucose oxidase catalyzes the production of both H_2O_2 and D-galactone from glucose (supplied in the media), and previous work in our lab has shown that D-galactone alone does not affect catalase-mediated growth inhibition (not shown).

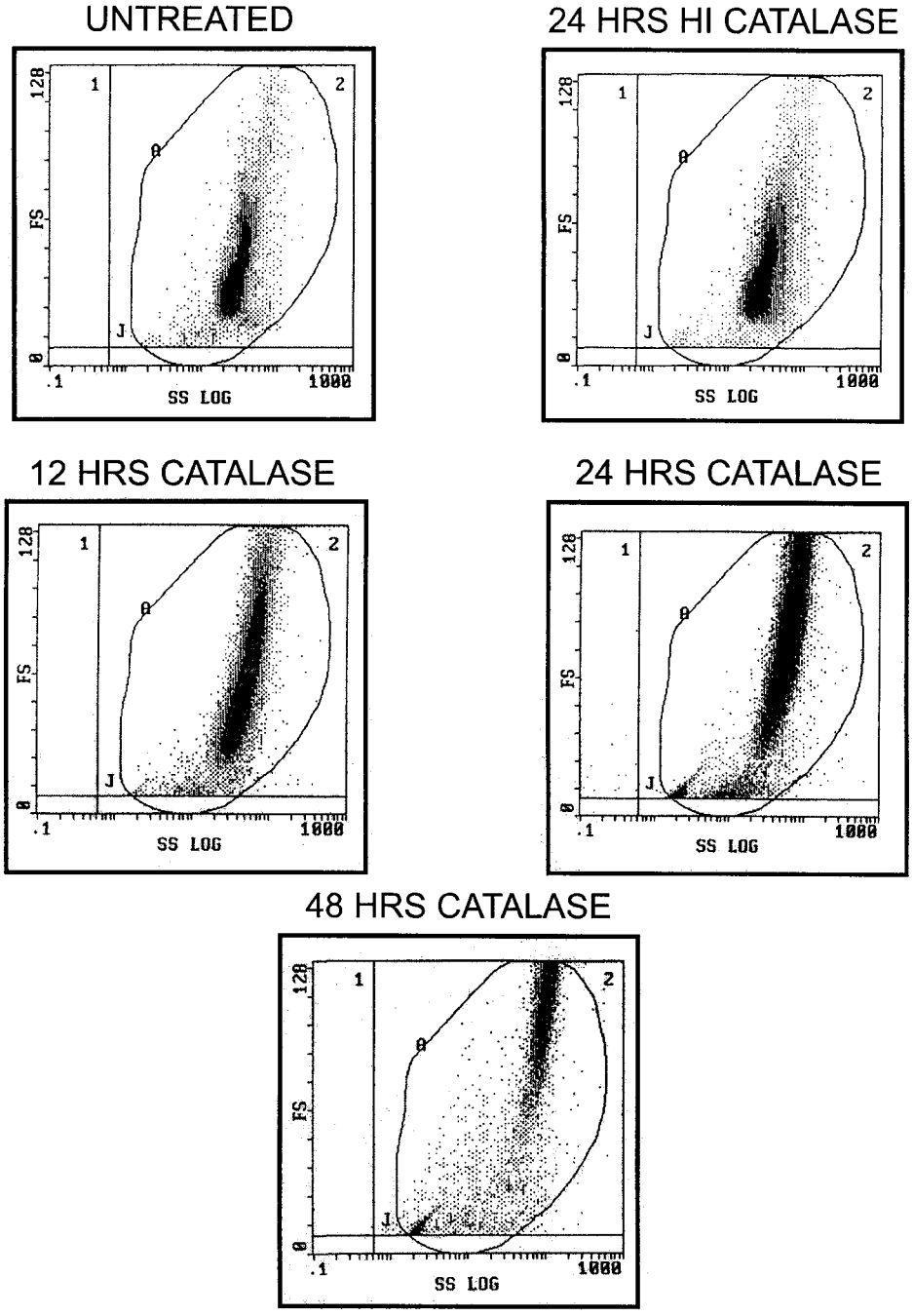


Figure 9: Catalase Effects upon Rat1NeuN Cell Size
Cells were treated for the indicated times with 1000 U/ml catalase or heat-inactivated (HI) catalase preparations. Cell size changes were determined using flow cytometry; forward scatter (FS) of the argon laser beam represents cell size, and side scatter (SS) of the beam represents cell granularity. Cell size increased with continued catalase treatment, but was unaffected by treatment with heat-inactivated enzyme.

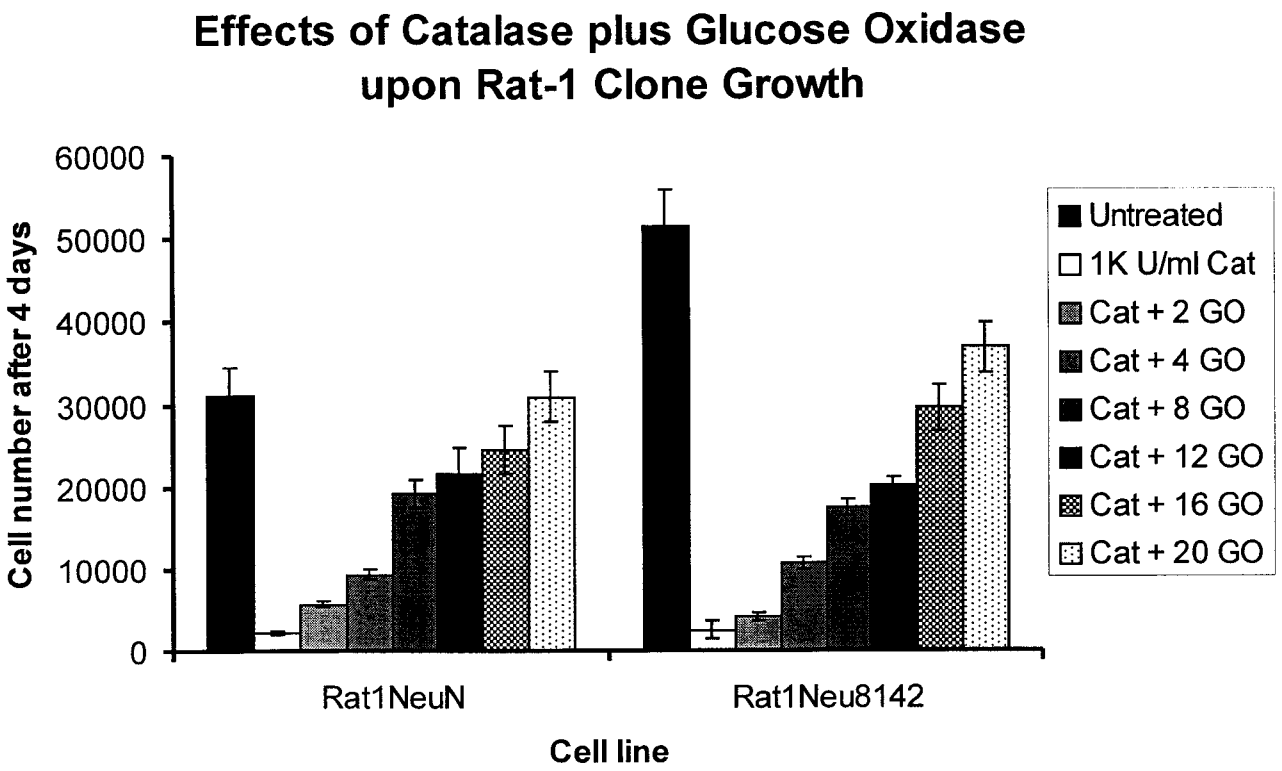


Figure 10: Glucose Oxidase Activity can Rescue Cell Growth Inhibition by Catalase Treatment
Rat-1NeuN and Rat-1Neu8142 cells were seeded 1,000 cells/well (96-well plate), allowed to adhere and then treated with 0 (untreated) or 1,000 U/ml catalase preparation (Cat). Some samples were co-incubated with increasing concentrations ($2X10^{-3}$ U/ml; shown as 2 GO, to $2X10^{-2}$ U/ml; shown as 20 GO) of a *Aspergillus niger* glucose oxidase (GO) preparation. After four days incubation, cell numbers in wells were calculated. Catalase treatment efficiently blocked cell proliferation, however this effect was reversed in a dose-dependent manner upon co-treatment of cells with increasing amounts of H_2O_2-generating glucose oxidase. Levels of glucose oxidase activity over $4X10^{-3}$ U/ml were themselves toxic to all Rat-1 clones tested (not shown).

Catalase Treatment Lowers Active DNA Synthesis-

Various mammalian cell types have been shown to secrete H_2O_2 into the surrounding culture medium (Szatrowski and Nathan 1991; Patterson *et al.* 1999). This generation is thought to regulate culture growth, as manipulation of intracellular or plasma membrane-specific H_2O_2 production results in changes to both the amount of extracellular H_2O_2 and to levels of active DNA synthesis and proliferation (Li *et al.* 1995; Li *et al.* 1998; Brown *et al.* 1999; Wenk *et al.* 1999). DNA synthesis in Rat-1 clones was examined in response to manipulation of H_2O_2 levels in the media. Consistent with the observations of lower cell number and a prolongued S phase and entry into mitosis, active DNA synthesis was decreased 2- to 4-fold by 2000 U/ml catalase as observed by BrdU or [^{3}H-methyl]-thymidine incorporation into cellular DNA (Figs. 11 and 12). As well, the addition of 200 μM H_2O_2 increased DNA synthesis in all lines tested, in accordance with the well-documented growth promoting effects of low level H_2O_2 treatment (although not to statistically significant levels in all experiments).

Rat-1 Clone Proliferative Rates are Proportional to Rates of Extracellular H_2O_2 Production-

To further evaluate the correlation between extracellular hydrogen peroxide levels and cell growth efficiency, the endogenous generation of H_2O_2 by Rat-1 clones was examined (Fig. 13). The amounts of H_2O_2 generation from Rat-1 clones corresponded with their rates of growth in culture. These levels (approximately 5 to 15 pmol/hr/50,000 cells) are comparable to that observed in other non-phagocytic mammalian lines. Our findings suggest a reciprocal regulation between endogenous cellular H_2O_2 production

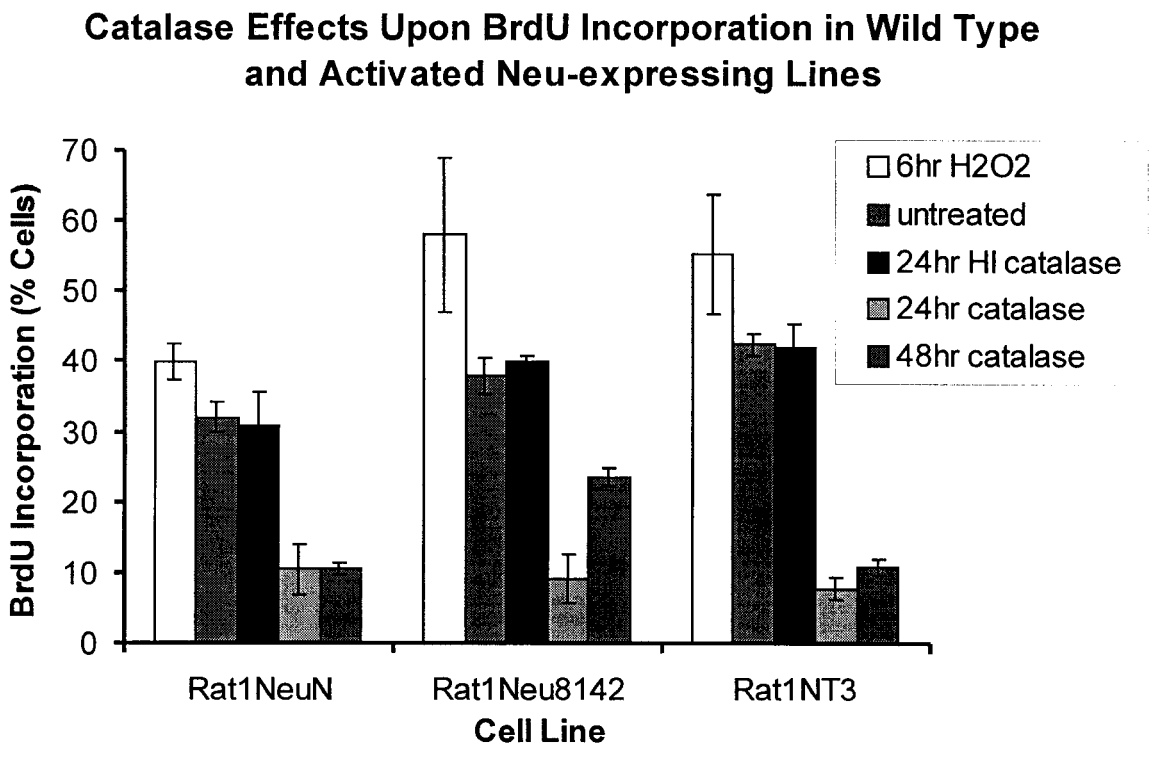


Figure 11: Inhibition of Active DNA Synthesis by Catalase Treatment
Equal numbers of cells were seeded onto 100 mm plates and treated for the specified amounts of time (200 μM H_2O_2; 2,000 U/ml catalase or heat-inactivated (HI) catalase). Samples were then dosed with BrdU and labelled with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody and propridium iodide, then analyzed by flow cytometry. Active DNA synthesis was stimulated after the addition of non-toxic levels of H_2O_2, whereas scavenging of endogenous H_2O_2 by catalase decreased this synthesis in all clones tested.

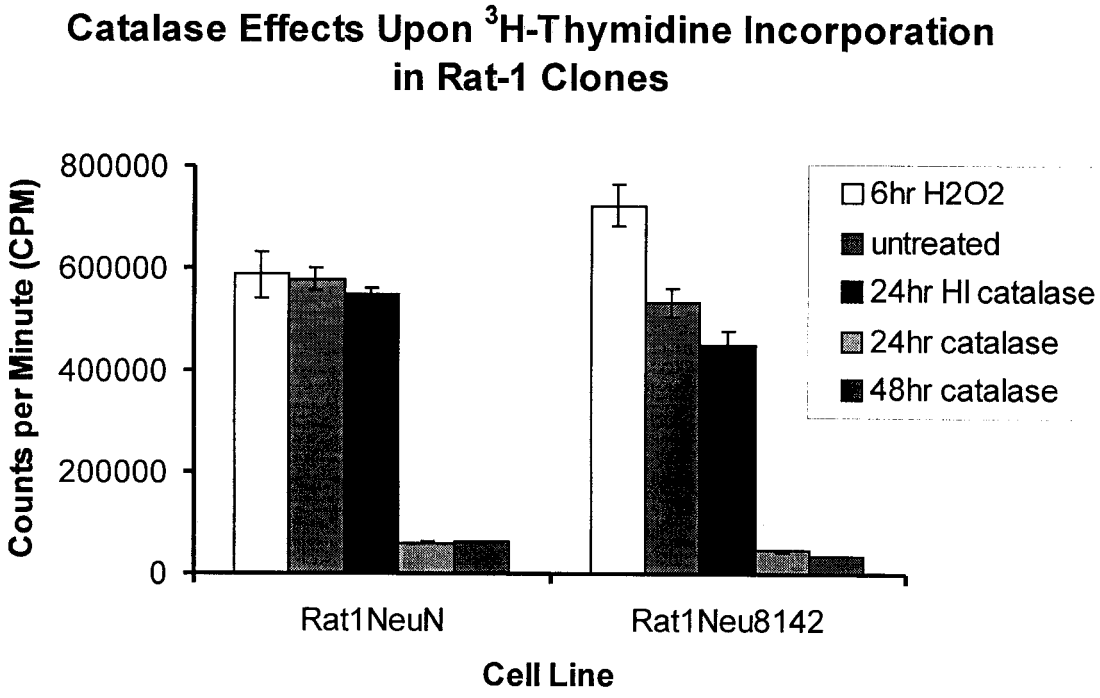


Figure 12: Inhibition of Active DNA Synthesis by Catalase Treatment (II) [*methyl*-^{3}H] thymidine incorporation was utilized as a measure of active DNA synthesis. 50,000 cells/plate were seeded (60 mm plates) and then exposed to the indicated treatments (200 μM H_2O_2; 2,000 U/ml catalase or heat-inactivated (HI) catalase preparations). Cultures were labelled with 1 μCi/ml isotope for 4 hours. Cells were incorporated onto glass microfibre filters, added to scintillation cocktail, and assessed for total radioactivity (measured as counts per minute). As observed in BrdU experiments, H_2O_2 induced thymidine incorporation, however it's removal by catalase blocked incorporation.

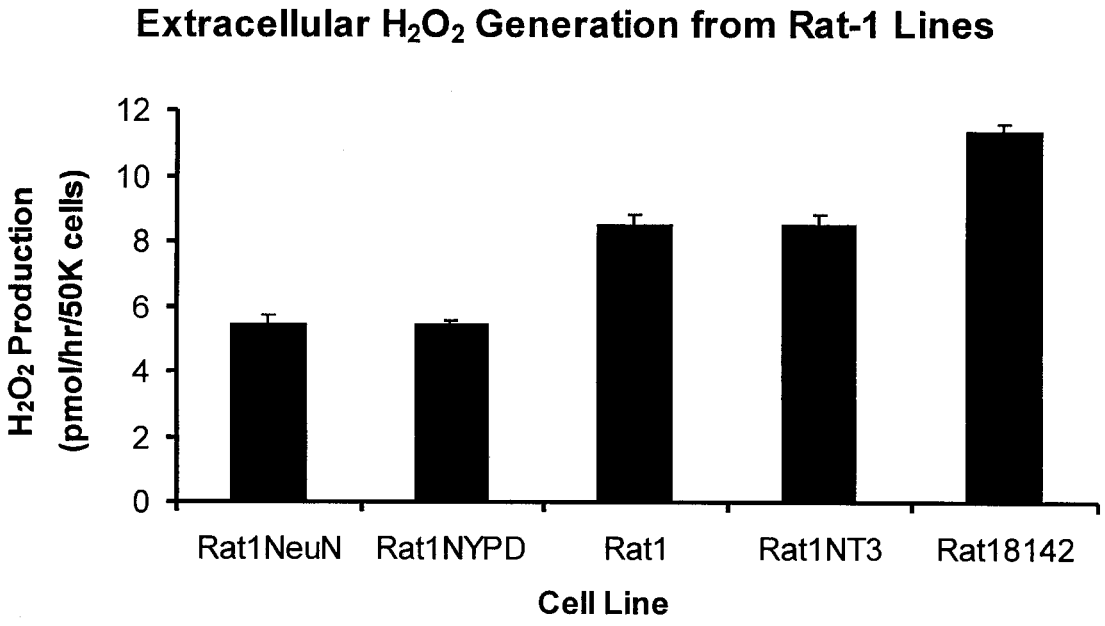


Figure 13: Rat-1 Fibroblast Clones Generate Extracellular H_2O_2
50,000 cells/well of each Rat-1 clone were seeded into multiple wells of a 96-well plate and incubated until adherent. Media was removed and replaced with a buffer containing the "Amplex Red" reagent for the colourimetric detection of endogenous cellular production of H_2O_2 into the extracellular compartment. The amount of H_2O_2 produced corresponded with the growth rates of each Rat-1 clone; the highly transformed clones (NT3, Neu8142) exhibited both the most rapid proliferation and largest levels of H_2O_2 secretion. Slower growing clones (NeuN, NeuNT-NYPD) generated less H_2O_2. Rat-1NeuN and Rat-1Neu8142 clones were compared in subsequent experiments due to their differential status of transformation, HER-2/Neu activty, and extracellular H_2O_2 production.

and mitogenesis: the non-toxic H_2O_2 stimulus promotes proliferation, and higher rates of proliferation (and therefore also cell metabolism) result in increases in ROS levels. The constitutively dimerized Neu-expressing Rat-1Neu8142 line consistently demonstrated a 2-fold or higher increase in H_2O_2 production compared to the Rat-1NeuN line expressing wild type Neu. Because of the significant differences in HER-2/Neu activity, extracellular H_2O_2 generation and level of transformation exhibited by these two clones, they were compared directly in subsequent experiments focusing on molecular responses to catalase treatment.

3.2 Responses of the HER-2/Neu Receptor to Extracellular Catalase Treatment

Scavenging of H_2O_2 Does Not Significantly Affect Neu Receptor Dimerization-

An important target of an oxidizing environment at the plasma membrane may be the regulation of membrane receptor dimerization and signalling via the stabilization of disulfide bonds or induction of autokinase activity (for review see Burdon 1995; Chakraborti and Chakraborti 1998; Kamata and Hirata 1999). Thus, in our model the direct manipulation of HER-2/Neu activity by changes in "pericellular" (close proximity to the plasma membrane and associated enzymes) redox status could be a key factor in the growth responses observed. To investigate catalase effects upon receptor dimerization kinetics, Neu was immunoprecipitated from total cellular protein lysates and subjected to SDS-PAGE under reducing and non-reducing conditions, in order to observe the proportion of Neu in dimer form (Figs. 14 and 15). The addition of 200 μM H_2O_2 to cell cultures, a concentration capable of increasing the rate of DNA synthesis, did not

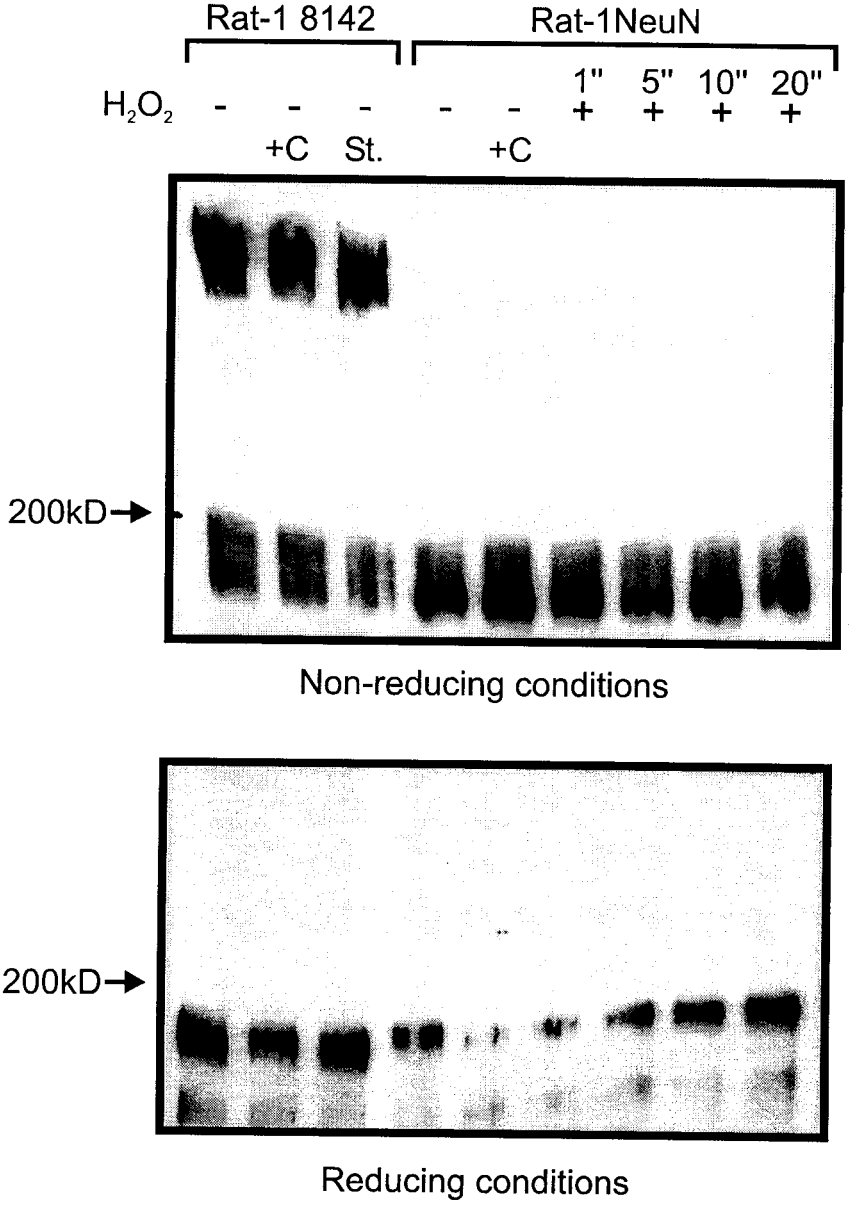


Figure 14: Addition of H_2O_2 Does Not Induce Neu Receptor Dimerization
Neu was immunoprecipitated from Rat-1Neu8142 and Rat-1NeuN cell lysates in the presence of iodoacetamide and the resulting samples included in SDS-PAGE under reducing (0.8 M β-mercaptoethanol) and non-reducing conditions. Upper bands (approx. 370 kDa) in non-reducing conditions indicate receptor dimerization. +C= 24 hour treatment with 2,000 U/ml catalase; St.=24 hour serum starvation; H_2O_2= 200 μM H_2O_2 treatment for the designated times (1 to 20 minutes). The mutant 8142 receptor was constitutively dimerized. Addition of exogenous H_2O_2 to the wild type Neu-overexpressing clones (Rat-1NeuN) did not induce disulfide bond formation.

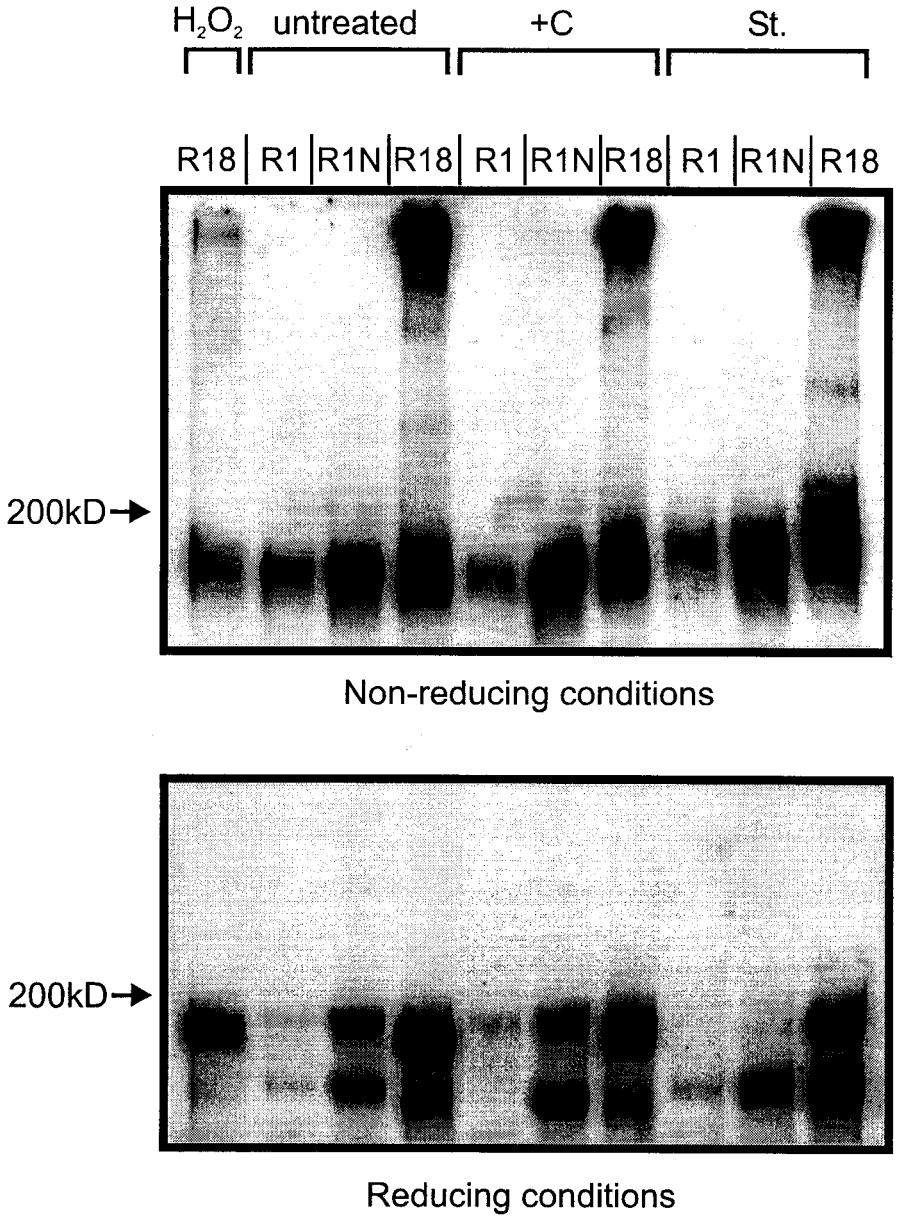


Figure 15: Scavenging of H_2O_2 Does Not Regulate HER-2/Neu Dimerization
Neu receptor was immunoprecipitated from Rat-1 (R1), Rat-1NeuN (R1N) and Rat-1Neu8142 (R18) cell lysates and analyzed for dimerization as described in the preceding figure. H_2O_2=200 μM H_2O_2 treatment for 24 hours; +C=2,000 U/ml catalase treatment for 24 hours; St.=24 hour serum starvation. Neu dimers were only observed from Rat-1Neu8142 cell lysates (constitutively dimerized Neu). Removal of extracellular H_2O_2 or peptide growth factors resulted in cell growth inhibition, but did not inhibit Neu8142 receptor dimerization.

induce the dimerization of wild type Neu in Rat-1NeuN cells after treatments from 1 to 20 minutes (and up to 24 hours; not shown) as seen in Fig. 14.

The constitutively dimerized mutant Neu in Rat-1Neu8142 cells was always observed in this form, regardless of catalase addition or serum starvation (Figs. 14 and 15). It is not expected that subtle redox manipulation would have an appreciable effect upon the Neu8142 receptor owing to the 12 a.a. deletion in its extracellular region. This mutant receptor should remain in a dimerized state during any environmental changes, although receptor phosphorylation and downstream signalling may be affected. However, the fact that Rat-1Neu8142 cells remain very much susceptible to growth inhibition by catalase indicates that both Neu dimerization kinetics and kinase activity are not a component of the catalase-induced response.

Scavenging of H_2O_2 Does Not Elicit Prolongued Effects Upon Neu Phosphorylation-

Effects upon the status of Neu phosphorylation and kinase activity after manipulation of H_2O_2 levels were evaluated by immunoprecipitation of the receptor and Western blotting for phosphorylated tyrosine residues (Fig. 16), and by *in vivo* Neu kinase assay (Fig. 17), respectively. Again, Rat-1Neu8142 cells were examined for their receptor characteristics following a variety of treatments. Phosphotyrosine analysis showed that the mutant Neu receptor activity was relatively unchanged after the addition of catalase (2,000 U/ml for 24 hrs.), H_2O_2 (200 μM for 10 min.), or both to cells (Fig. 16). The level of phosphorylation was compared to Rat-1NeuN (low activity) and Rat-1NeuNT(3) (high activity) lysates. Thus, phosphorylation responses of HER-2/Neu in the Rat-1 clones correlated with the results of dimerization experiments.

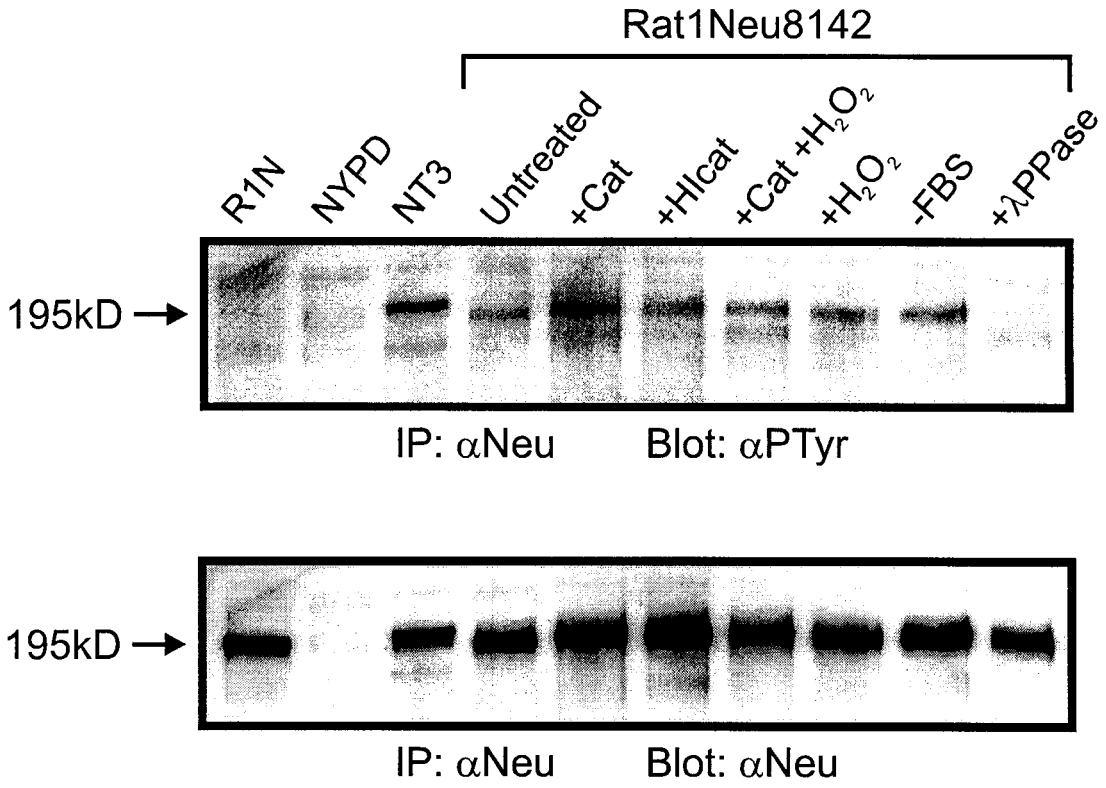


Figure 16: Alteration of Extracellular H_2O_2 Levels Does Not Significantly Effect HER-2/Neu Receptor Tyrosine Phosphorylation

HER-2/Neu was immunoprecipitated from 200 μg of total cell lysate, included in SDS-PAGE and Western blotted. Tyrosine-phosphorylated receptor was observed by labelling of the blot with PY20 anti-P-Tyr Ab. Membrane was then stripped of Ab and reprobed for total Neu content by labelling with anti-Neu Ab. Rat-1NT3 (NT3) lysate was used as a positive control of phosphorylation, Rat-1NeuNT-NYPD (NYPD) lysate was used as an indicator of low phosphorylation, and lambda protein phosphatase (+λPPase; 500 U, 2 hours)-treated Rat-1Neu8142 lysate was included as a negative control. R1N=Rat-1NeuN; Cat=2,000 U/ml catalase (24 hours); HIcat=2,000 U/ml heat-inactivated catalase (24 hours); +H_2O_2=200 μM H_2O_2 (10 minutes); -FBS=24 hours serum starvation. 24 hours of catalase treatment did not lower Neu phosphorylation, and H_2O_2 treatment did not increase phosphorylation. These treatments were capable of influencing Rat-1Neu8142 growth, however.

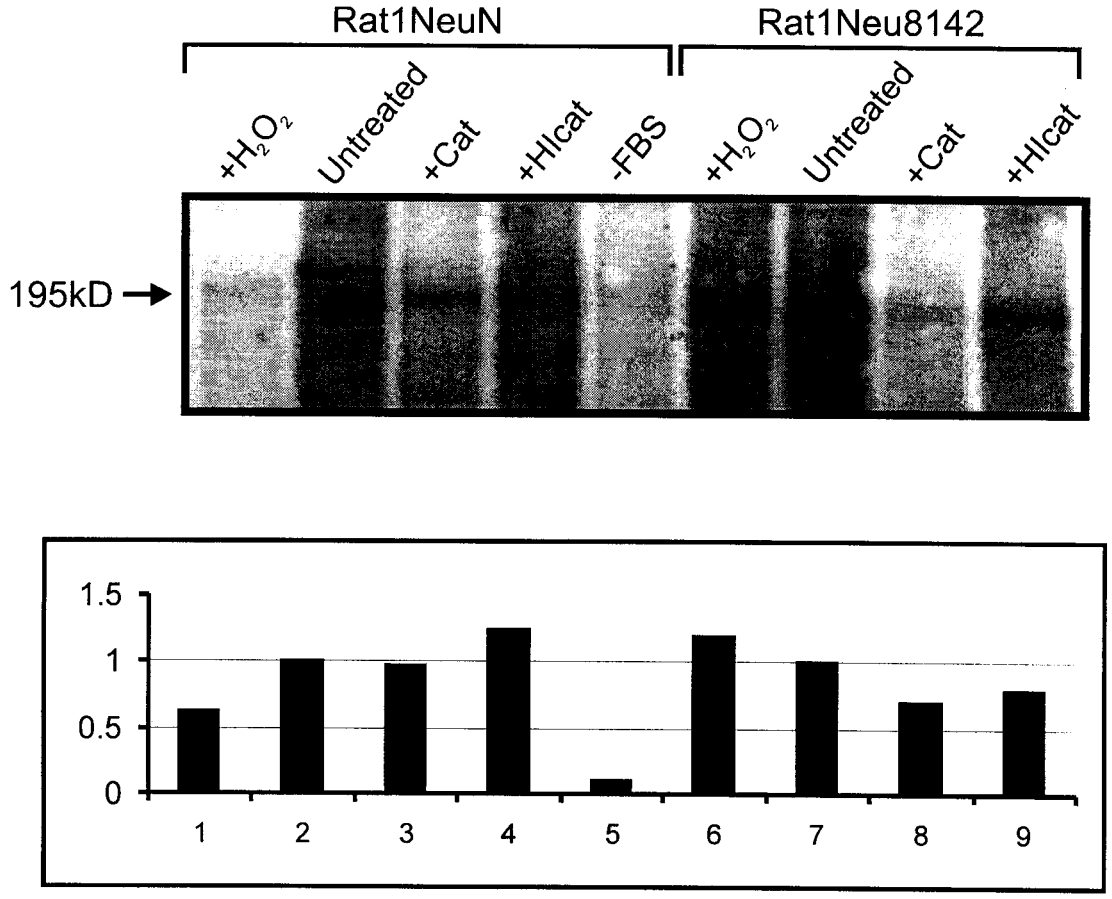


Figure 17: HER-2/Neu Kinase Activity Does Not Participate in Growth Regulation from Alteration of Extracellular H_2O_2 Levels

An *in vivo* Neu kinase assay for additional analysis of **relative** Neu activity responses to exogenous H_2O_2 and catalase treatments. $5X10^5$ cells (Rat-1NeuN or Rat-1 Neu8142) were plated for each sample, and phosphate starved. Cultures were treated as indicated (+H_2O_2=200 μM H_2O_2 for 30 minutes; +Cat=2,000 U/ml catalase for 24 hours; +HIcat=2,000 U/ml heat-inactivated catalase for 24 hours; -FBS=24 hour serum starvation) and then incubated with 0.5 mCi/plate of ^{32}P-labelled orthophosphoric acid for 5 hours. Cells were then lysed for protein, total radioactivity of 1 μl from each supernatant compared by TCA precipitation and scintillation counting, and the appropriate normalized sample volumes used for immunoprecipitation (IP) of Neu. Neu samples were included in SDS-PAGE and transferred onto a PVDF membrane. The above image was generated by phosphorimager analysis of the membrane, and the **histogram** represents the relative intensity of the ^{32}P signal from each lane at 195 kD (untreated=1.0 intensity). Separate background measurements were used for each lane. Only serum starvation had a pronounced effect upon Neu kinase activity.

Similarly, overall kinase activity of Neu in Rat-1NeuN and Rat-1Neu8142 lines did not appear to be significantly altered with catalase treatment. H_2O_2 did increase kinase activity somewhat in certain clones (see Rat-1Neu8142; Fig. 17), which complements previous findings of H_2O_2-induced receptor activity as seen by *in vitro* kinase assay (personal communication from Dr. W. J. Muller, McMaster University). Regardless of this effect, in no experimental model could growth inhibitory concentrations of catalase substantially downregulate Neu tyrosine kinase activity (as compared to serum starvation of wild type receptor; Fig. 17). These experimental results suggest that the hydrogen peroxide stimulus functions downstream of the receptor tyrosine kinase level in our Rat-1 fibroblast model.

3.3 Response of Mitogen- and Stress-activated Protein Kinases to Extracellular Catalase Treatment

p44/42 MAPK (Erk1/2) Phosphorylation is Blocked by Catalase Treatment and this Effect is Rescued by Constitutively Activated Neu-

The Ras-MAPK signaling cascade is an important mediator of cell survival and growth regulation and also has been shown to be activated by H_2O_2 (Khosravi-Far and Der 1994; Guyton *et al.* 1996; Rao 1996). We wished to determine if the removal of H_2O_2 would produce the opposite effect of Erk1/2 MAPK downregulation, as further evidence for the redox sensitivity of this pathway (Figs. 18 and 19). Protein lysates were run on SDS-PAGE and Western blotted; Thr202/Tyr204-phosphorylated Erk 1 and 2 were detected using phospho-MAPK-specific primary antibodies. Levels of phosphorylated Erks were compared to total Erk1/2 levels. Rat-1NeuN cells treated with

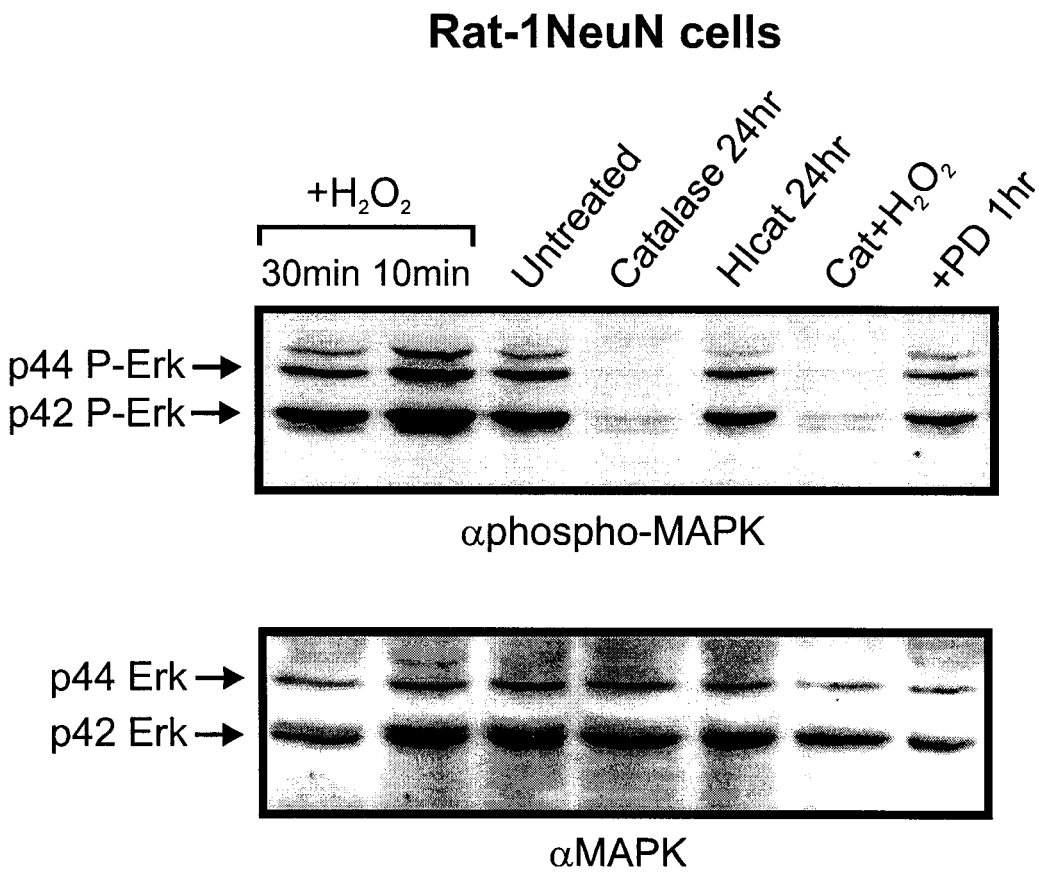


Figure 18: P44/P42 MAPK (ERK1/2) Activity is Blocked by Catalase Treatment in Wild Type Neu-Expressing Fibroblasts

Rat-1NeuN cells were treated (2,000 U/ml catalase or heat-inactivated catalase (HIcat) preparation; 200 μM H_2O_2; +PD=30 μM PD98059) and protein lysates analyzed by SDS-PAGE and Western blotting. The membrane was labelled with phospho-specific anti-ERK (Thr202/Tyr204) Ab to observe levels of activated p44/p42 MAPKs, then stripped of Ab and reprobed with anti-ERK Ab to observe overall levels of expression. H_2O_2 treatment transiently induced ERK phophorylation, and catalase treatment abolished this phosphorylation. H_2O_2 treatment did not rescue inactivation by catalase after 30 min. treatment (not tested at longer treatment durations) Heat-inactivated catalase treatment produced no change in ERK activation. The MEK1 inhibitor PD98059 did not significantly reduce phophorylation (increased reduction was observed with longer treatment period; not shown).

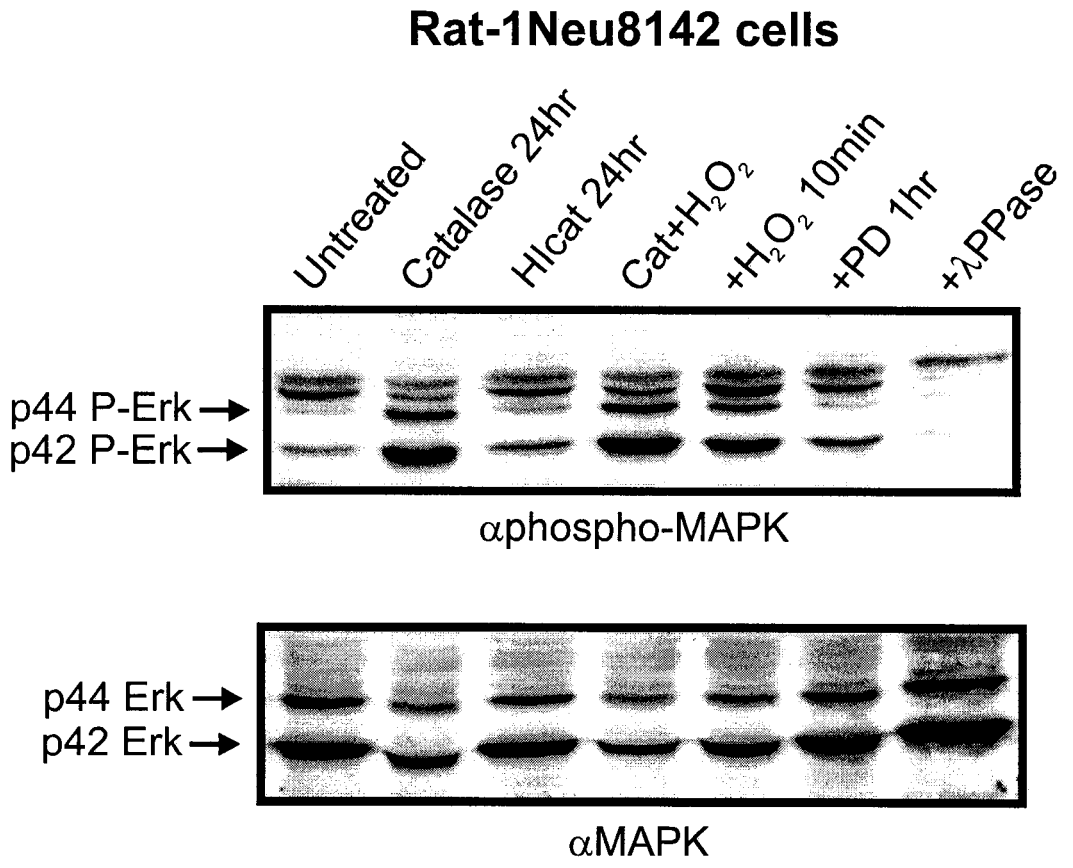


Figure 19: P44/P42 MAPK (ERK1/2) Activity is Increased by Catalase Treatment in Dominant Active Neu-Expressing Fibroblasts
Rat-1Neu8142 cells were treated (2,000 U/ml catalase or heat-inactivated catalase (HIcat) preparation; 200 μM H_2O_2; +PD=30 μM PD98059) and protein lysates analyzed by SDS-PAGE and Western blotting. +λPPase=treatment of lysate with 500 U/ml lambda protein phosphatase for 2 hours (negative control). The membrane was labelled with phospho-specific anti-ERK (Thr202/Tyr204) Ab to observe levels of activated p44/p42 MAPKs, then stripped of Ab and reprobed with anti-ERK Ab to observe overall levels of expression. H_2O_2 treatment transiently induced ERK phosphorylation, and catalase treatment stably induced phosphorylation. This is the opposite effect to that observed in Rat-1NeuN cells. Heat-inactivated catalase treatment produced no change in ERK activation. The MEK1 inhibitor PD98059 moderately reduced phosphorylation, and treatment of lysate with λPPase abolished phosphorylation.

200 μM H_2O_2 (10 min.) displayed an increase in p44/42 MAPK phosphorylation, while the addition of 2,000 U/ml catalase for 24 hours (with or without 10 minutes of exposure to H_2O_2) abolished MAPK activity (Fig. 18). Heat-inactivated catalase did not produce such an effect. Therefore, the proliferation-stimulating Erk1/2 signal is a target of catalase-mediated removal of the H_2O_2 stimulus in Rat-1NeuN cells.

In contrast, this decrease in Erk1/2 phosphorylation was not observed after the identical treatment of Rat-1Neu8142 cells, with the constitutively active form of HER-2/Neu. Indeed, both catalase and H_2O_2 appeared to stimulate Erk activation (Fig. 19). 30 μM of the MEK1 inhibitor PD98059 (Dudley *et al.* 1995; Cohen 1997) was added to cells for 1 hour as a control for Erk1/2 downregulation. These observations demonstrate that the alteration of Neu receptor activity can modulate the response of the MAPK pathway to a decrease in H_2O_2 levels, and that prior hyperstimulation of Erk1/2 induces insensitivity to the regulatory effects of other stimuli. The response of Rat-1Neu8142 cells to catalase treatments differed slightly (but significantly) from that of Rat-1NeuN cells (see Fig. 20, *A* + *B*), with the Rat-1Neu8142 clone displaying an increased resistance to growth inhibition. Thus, the MAPK pathway appears to represent one of the critical mediators of H_2O_2 signalling. However, as the catalase-induced proliferation block observed appears largely independent of effects upon both the HER-2/Neu receptor and p44/42 MAPK signalling pathways, there must be other signal transduction responses responsible for these cellular effects.

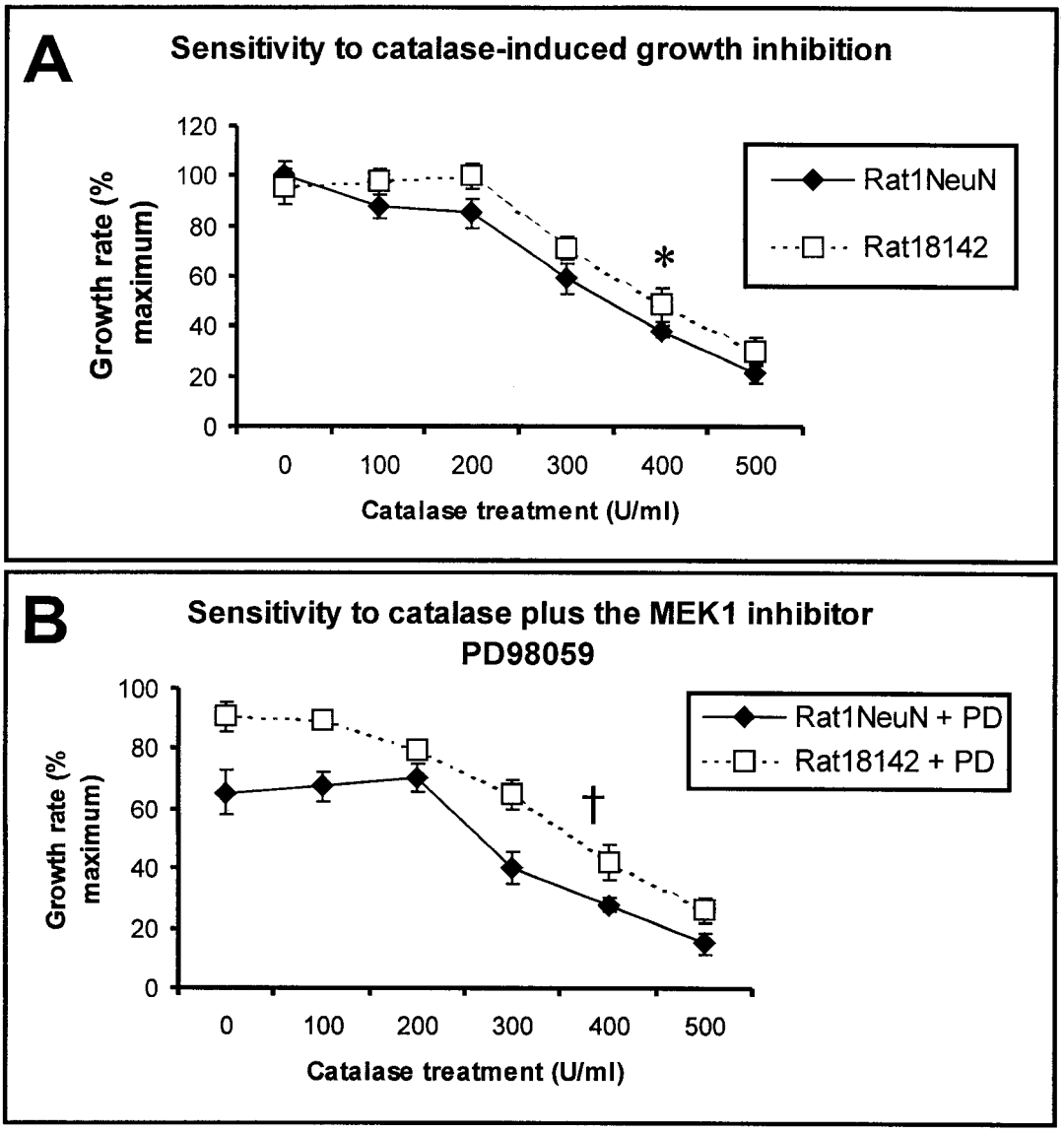


Figure 20: Sensitivity of Rat-1NeuN Cells vs. Rat-1Neu8142 Cells to Catalase Treatment or Catalase Treatment plus the MEK1 inhibitor PD98059
A, 1,000 cells/well of each clone were seeded into multiple wells of a 96-well plate, incubated until adherent, and then treated with increasing doses of catalase (0 to 500 U/ml) for four days. Total cell number in wells was quantitated by Hoechst staining to observe total DNA in wells. No catalase treatment was considered 100% maximum growth. *B,* the same clones were treated as in *A,* however 5 μM of the MEK1 inhibitor PD98059 was added to wells along with catalase treatments. The Rat-1Neu8142 clone displayed a slightly increased resistance to growth inhibition from catalase treatment, as compared to Rat-1NeuN. A significant increased resistance to Erk inhibition was observed in Rat-1Neu8142 cells. (*,†) indicate a significant difference in IC_{50} of catalase or catalase plus PD98059 treatments between clones ($p<0.05$).

Erk1/2 Activity Partially Protects Rat-1 Cells From the Growth-inhibitory Effects of Catalase Treatment-

In order to further characterize the differences in the sensitivities of Rat-1NeuN and Rat-1Neu8142 lines to H_2O_2 scavenging, the response of these cells to a range (0 to 500 U/ml) of catalase activities was compared (Fig. 20A). Percentage of optimal cell growth was determined by a DNA-specific fluorimetric assay as previously described. Rat-1Neu8142 cells displayed a slightly higher resistance to the effects of catalase than Rat-1NeuN; this may result from differences in MAPK pathway activity or in the amount of endogenous H_2O_2 production observed between the two clones. To test for the latter possibility, SK-OV-3 cells were also examined for resistance to catalase (not shown). These cells generate extracellular H_2O_2 at a level approximately 6-fold higher than the Rat-1Neu8142 line. This increased H_2O_2 did not correlate to an increase in resistance to growth inhibition, indicating that the altered cell responses observed are at least in part due to differences in Erk1/2 activity.

To further analyze how changes in Erk1/2 pathway activation may alter the cell proliferation response to changes in H_2O_2 levels, the sensitivity of cells to removal of H_2O_2 in the presence of the MEK1 inhibitor PD98059 was investigated (Fig. 20B). This concurrent treatment blocked the growth of Rat-1NeuN cells to a greater extent than catalase treatment alone, while the proliferation of Rat-1Neu8142 cells which exhibited increased p44/42 MAPK phosphorylation upon catalase treatment was less affected by co-incubation with the MEK1 inhibitor. It should be noted that it is assumed that the Neu8142 clone would demonstrate a higher degree of insensitivity to the effects of

PD98059, due to hyperstimulation of MAPKs. These data provide additional evidence that the Erk1/2 pathway is responsible for the small differences in growth inhibition by catalase observed. Increased MAPK activity in the Neu8142 clone did not rescue cell growth to a substantial degree, however. Thus it appears that although the H_2O_2 signal can regulate Erk1/2 activities, there are also other intracellular signals affected by changes in H_2O_2 levels which are responsible for blocking proliferation.

p38 MAPK Phosphorylation is Induced by H_2O_2 but not by Catalase Treatment-

As the effects of H_2O_2 scavenging upon Rat-1 fibroblasts could not be fully attributed to the downregulation of mitogenic MAPK signals, the possible contribution of an upregulation of stress-activated MAPK signalling was investigated. The stress-induced p38 MAPK is known to be responsive to a variety of cell stimuli including hyperosmolarity and ROS, and its signal has been associated with cell growth inhibition and death (Han *et al.* 1994; Chuang *et al.* 2000). p38 MAPK was phosphorylated upon addition of exogenous H_2O_2 in all cells tested (Figs. 21 and 22). This response to oxidative stress was sustained over time, and no p38 activation was observed after treatment of cells with catalase or heat-inactivated catalase preparations. Levels of induction of p38 phosphorylation in both Rat-1NeuN (Fig. 21) and Rat-1Neu8142 (Fig. 22) lines by 2 mM H_2O_2 was comparable to that observed under hyperosmotic conditions (0.2M NaCl for 30 minutes). The phosphorylation of one p38 MAPK substrate, the transcription factor ATF2, was also investigated under the same conditions using an *in vitro* kinase assay (Figs. 21 and 22). No significant changes in phosphorylation of GST-ATF2 peptide by p38 were observed after catalase or H_2O_2 treatments. It is

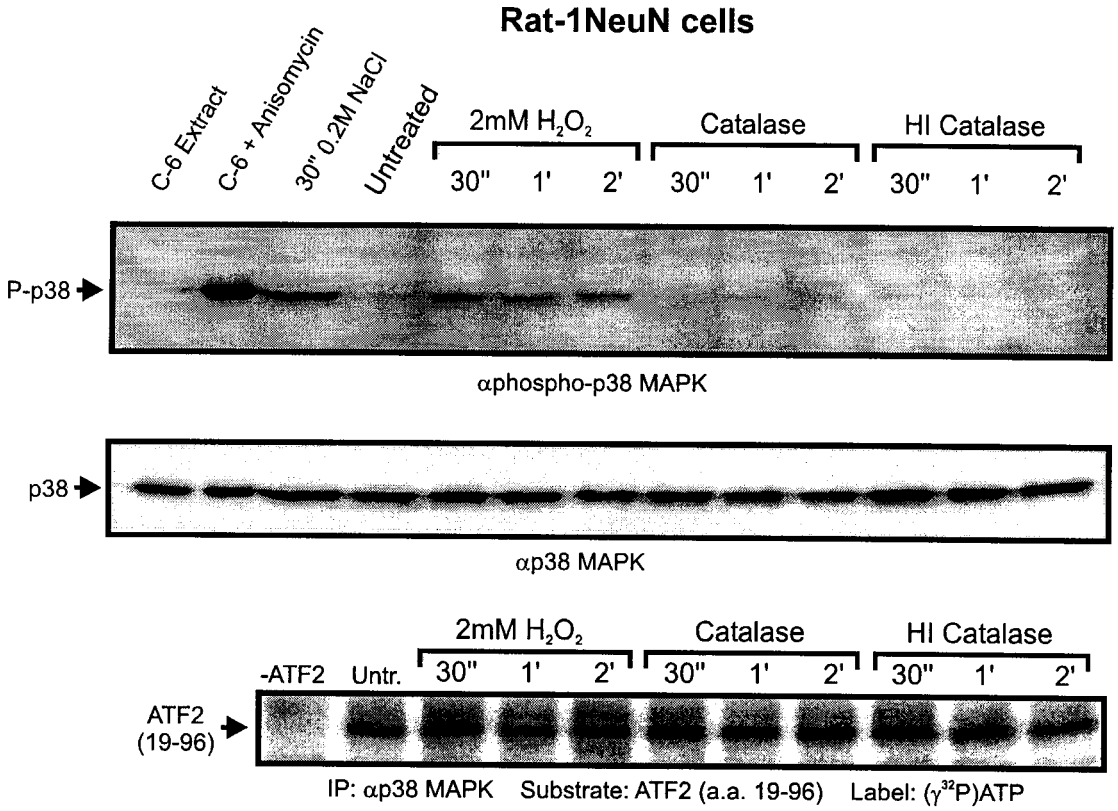


Figure 21: p38 MAPK Phosphorylation is Stably Induced by H_2O_2 Stress but is Unaffected by Catalase Treatment in Rat-1NeuN Cells

Cultures were treated as indicated (catalase/heat-inactivated (HI) catalase=500 U/ml; treatment times 30 minutes to 2 hours (30"-2')) and protein lysates analyzed by Western blotting. C-6 cell extract was used as a negative control of p38 activation, and both extract from C-6 cells treated with anisomycin as well as treatment with 0.2M NaCl were used as a positive control. The membrane was labelled with phospho-specific anti-p38 (Thr180/Tyr182) Ab to observe levels of activated p38 MAPK, then stripped of Ab and reprobed with anti-p38 MAPK Ab to observe overall level of p38 expression. p38 MAPK phosphorylation was induced by H_2O_2 treatment but not by catalase treatment. p38 MAPK was also immunoprecipitated from cell lysates for use in a kinase reaction with a p38 substrate (ATF-2; amino acids 19-96) and label ([γ-^{32}P]ATP). -ATF2=no substrate included; Untr.=no treatment. No significant change to the phosphorylation of the ATF-2 substrate was observed.

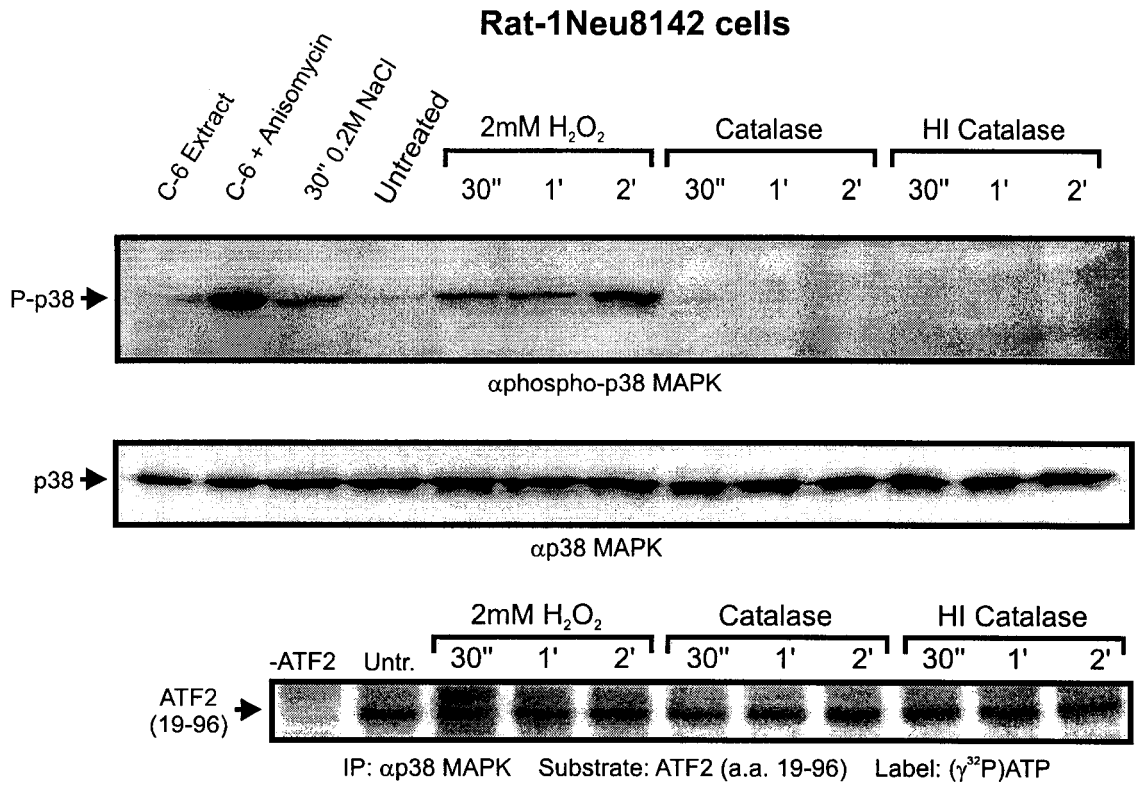


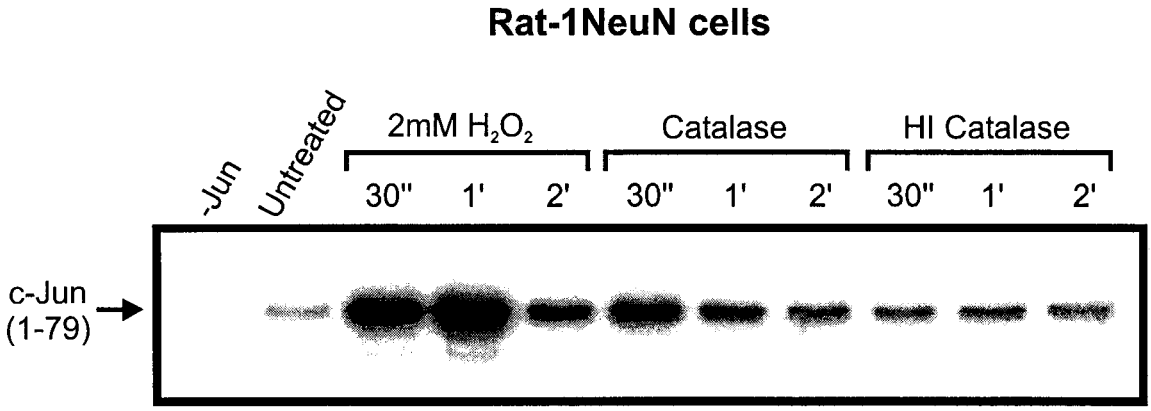
Figure 22: p38 MAPK Phosphorylation is Stably Induced by H_2O_2 Stress but is Unaffected by Catalase Treatment in Rat-1Neu8142 Cells

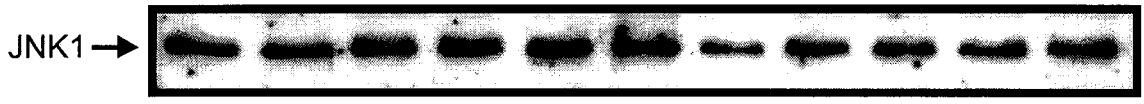
Cultures were treated as indicated (catalase/heat-inactivated (HI) catalase=500 U/ml; treatment times 30 minutes to 2 hours (30"-2')) and protein lysates analyzed by Western blotting. C-6 cell extract was used as a negative control of p38 activation, and both extract from anisomycin-treated C-6 cells and treatment with 0.2M NaCl were used as positive controls. The membrane was labelled with phospho-specific anti-p38 MAPK (Thr180/Tyr182) Ab to observe levels of activated p38 MAPK, then stripped of Ab and reprobed with anti-p38 MAPK Ab to observe overall level of p38 expression. p38 MAPK phosphorylation was induced by H_2O_2 treatment but not by catalase treatment. p38 MAPK was also immunoprecipitated from cell lysates for use in a kinase reaction with a p38 substrate (ATF-2; amino acids 19-96) and label ([γ-^{32}P]ATP). -ATF2=no substrate included; Untr.=no treatment. No significant change to the phosphorylation of the ATF-2 substrate was observed.

undetermined if this finding is a result of a functional impairment of p38 in the cells tested, or the inability of the substrate utilized to respond to the p38 signal. In either case, the absence of any direct p38 activation by catalase at levels observed to affect Erk1/2 and JNK1 suggests that the block in Rat-1 fibroblast proliferation is independent of this signal.

Extracellular Catalase Treatment Transiently Activates the JNK1 Pathway-

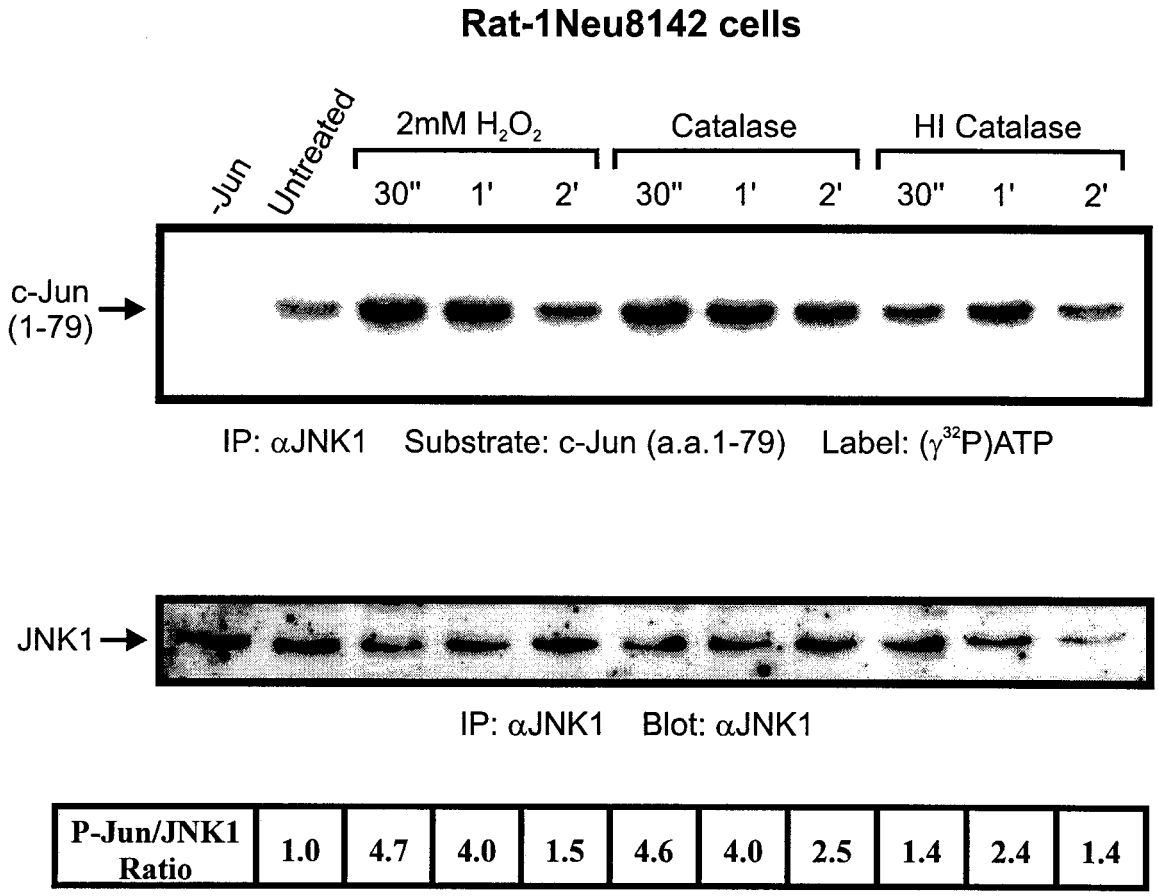
As the molecular effects of catalase treatment did not include p38 MAPK activation, we examined the response of another prominent stress-activated protein kinase (or SAPK) signal; the JNK1 pathway. The phosphorylation of c-Jun at amino-terminal serines 63 and 73 by JNK1 (p46 JNK) is a well-characterized cell response to stress, including oxidative stress (Pulverer *et al.* 1991; Smeal *et al.* 1992; Dérijard *et al.* 1994; Wisdom *et al.* 1999). JNK1 activity in response to changes in H_2O_2 levels was examined by an *in vitro* JNK kinase assay (Figs. 23 and 24). All treatments induced a transient increase in JNK1 kinase activity as quantitated by level of substrate (GST fusion peptide of c-Jun amino acids 1-79) phosphorylation. In Rat-1NeuN cells (Fig. 23) a toxic level of H_2O_2 (2 mM) activated JNK1 up to approximately 28-fold over basal levels, with peak activity occurring 1 hour after treatment. It is critical to note that non-toxic levels of H_2O_2 could not increase JNK1 activity (not shown). 500 U/ml catalase ($\sim IC_{75}$) induced the JNK1 signal approximately 7-fold with peak activity after 30 minutes. Heat-inactivated catalase (500 U/ml) could also activate JNK1 to a lesser extent (approximately 2-fold over basal levels) indicating that the addition of this protein preparation itself has subtle effects upon JNK signalling, possibly by some remaining





P-Jun/JNK1 Ratio	1.0	17.0	28.8	4.2	7.3	3.9	2.4	1.8	1.9	1.8

Figure 23: Both Cytotoxic Levels of H_2O_2 and Growth Inhibitory Levels of Extracellular Catalase Activate JNK1 in Rat-1NeuN Cells
JNK1 was immunoprecipitated from Rat-1NeuN protein lysates after the indicated treatments (catalase/heat-inactivated (HI) catalase=500 U/ml; treatment times from 30 minutes to 2 hours (30"-2')). Immunoprecipitates were then included in an *in vitro* kinase reaction with a JNK1 substrate (c-Jun; amino acids 1-79) and label ([γ^{32}P]ATP). -Jun=no c-Jun peptide substrate included. ^{32}P phosphorescence was quantitated after protein transfer onto PVDF membrane. 2mM H_2O_2 treatment markedly and transiently induced JNK1-mediated phosphorylation of c-Jun peptide. JNK1 activity was also induced transiently and to a lesser extent after catalase treatment, and to a lesser extent still after heat-inactivated catalase treatment. To compare c-Jun phosphorylation with total JNK1 protein level in each sample, the membrane was then incubated with anti-JNK1 Ab. The table displays densitometry analysis of the c-Jun phosphorylation/JNK1 level ratio for each lane (untreated ratio considered 1).



P-Jun/JNK1 Ratio	1.0	4.7	4.0	1.5	4.6	4.0	2.5	1.4	2.4	1.4

Figure 24: Both Cytotoxic Levels of H_2O_2 and Growth Inhibitory Levels of Extracellular Catalase Activate JNK1 in Rat-1Neu8142 Cells
JNK1 was immunoprecipitated from Rat-1Neu8142 protein lysates after the indicated treatments (catalase/heat-inactivated (HI) catalase=500 U/ml; treatment times from 30 minutes to 2 hours (30"-2')). Immunoprecipitates were then included in an *in vitro* kinase reaction with a JNK1 substrate (c-Jun; amino acids 1-79) and label ([γ^{32}P]ATP). -Jun=no c-Jun peptide substrate included. ^{32}P phosphorescence was quantitated after protein transfer onto PVDF membrane. 2mM H_2O_2 treatment transiently induced JNK1-mediated phosphorylation of c-Jun peptide. JNK1 activity was also induced transiently after catalase treatment. A small increase in activity was observed after heat-inactivated catalase treatment. To compare c-Jun phosphorylation with total JNK1 protein level in each sample, the membrane was then incubated with anti-JNK1 Ab. The table displays densitometry analysis of the c-Jun phosphorylation/JNK1 level ratio for each lane (untreated ratio considered 1).

catalase activity or other factor(s) present in the preparation. However, these effects do not result in any change in cell viability and growth as discussed. The response of Rat-1Neu8142 cells (Fig. 24) was similar but less marked. Here, 2 mM H_2O_2 increased phosphorylation of the c-Jun substrate in an identical manner as catalase treatment (approximately 4- to 5-fold increase, peak at 30 min.). The Rat-1Neu8142 clone therefore appears more resistant (at least in JNK1 response) to high levels of H_2O_2, however the mechanism of this property is not determined.

Our observations demonstrate that opposing directions of H_2O_2 imbalance, namely the addition of high concentrations of H_2O_2 or removal of endogenous H_2O_2 levels by the scavenger enzyme catalase, can both stimulate the JNK1 stress-activated pathway in a similar fashion. As well, the activation of this SAPK by catalase treatment is unaffected by differential HER-2/Neu receptor activity, suggesting that it may be a critical component of the growth inhibition observed with all Rat-1 fibroblasts studied. It is not clear if these JNK1-mediated effects are sufficient for the induction of a proliferative arrest. These data indicate that the upregulation of stress associated signalling pathway(s) is a common response to removal of extracellular H_2O_2 in our Rat-1 fibroblast model.

3.4 JNK1 Activity Contributes to Catalase-mediated Inhibition of Cell Proliferation by Lowering Endogenous H_2O_2 Levels

Transient HA-JNK1 Expression Potentiates Catalase-mediated Cell Growth Inhibition-

Our observations demonstrate that scavenging of H_2O_2 by the addition of catalase to the culture media can block the proliferation of cells as well as mediate a rapid and

transient induction of c-Jun phosphorylation (Preston *et al.* 2001). As discussed, JNK1 exhibits a high affinity for binding and phosphorylation of c-Jun, and is also involved in growth arrest and apoptosis signalling (Derijard *et al.* 1994; Tournier *et al.* 1997; Zou *et al.* 2001). To determine if p46 JNK1 could itself actively participate in the cellular growth arrest response to catalase treatment, Cos-1 cells (SV40-transformed monkey kidney fibroblasts) were transiently transfected with increasing doses of HA-JNK1/pcDNA3 expression vector, and vector dose-dependent expression of HA-JNK1 protein was observed by Western blotting (Fig. 25A).

The effects of this ectopic expression upon the response to catalase treatment were then observed (Fig. 25B). The growth of Cos-1 cells was inhibited after catalase addition (1,000 U/ml); removal of this treatment during cell incubation or heat inactivation of the enzyme reversed this effect (not shown). Expression of the HA-JNK1 protein potentiated growth inhibition in a dose-dependent manner, however this effect was not observed upon transfection with the pcDNA3 control vector (Fig. 25B). As Cos-1 cells express SV40 viral proteins, it is expected that transfection of these cells with plasmids containing an SV40 origin of replication site (ori), such as pcDNA3 and HA-JNK1/pcDNA3, results in in vivo plasmid replication (Gluzman 1981). This replication itself can result in cell toxicity, which could be interpreted as inhibition of cell growth in the assay used. However, toxicity from plasmid replication alone should be equal for both vectors used. Therefore, these results indicate that ectopic HA-JNK1 expression, like catalase treatment, can antagonize cell proliferation. However, it is not determined

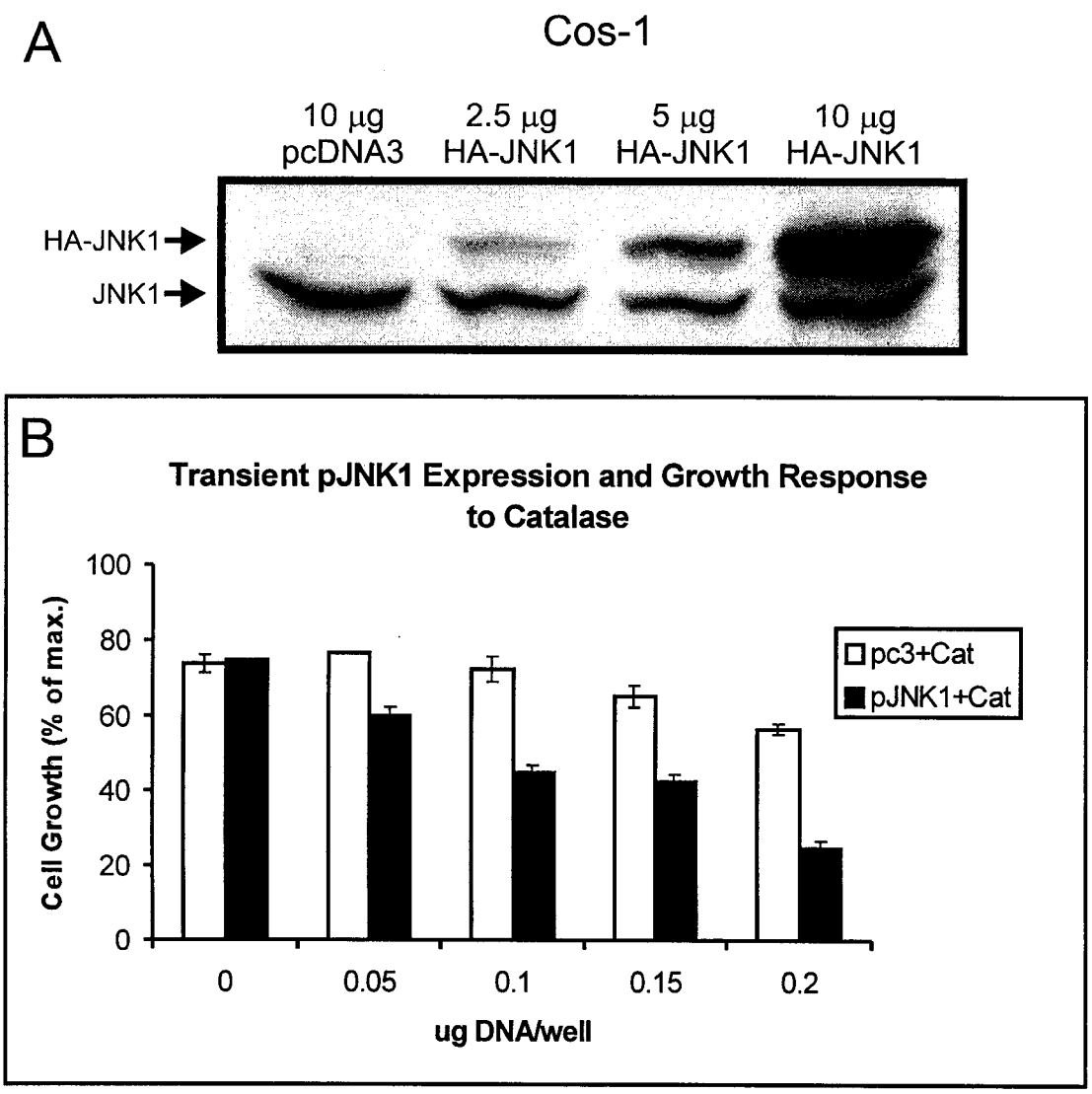


Figure 25: Growth Inhibitory Effects of Catalase Treatment and Transient Expression of HA-JNK1 Upon Cos-1 Fibroblasts

A, Cos-1 cells transfected with the designated amounts (transfections in 100 mm plates) of HA-JNK1/pcDNA3 plasmid or pcDNA3 control plasmid, and total cell protein lysates collected. JNK1 protein expression was analyzed by Western blotting; membrane was probed with anti-JNK1 Ab. Both endogenous JNK1 and ectopic HA-JNK1 proteins were detected. *B,* 1,000 cells/well (96-well plate) were incubated until adherent and then treated with transfection reagent alone (0 DNA) or transfected with increasing amounts of HA-JNK1/pcDNA3 (pJNK1) plasmid or pcDNA3 (pc3) control plasmid. Four hours post-transfection, cells were treated with 1,000 U/ml heat-inactivated catalase preparation (considered maximum (max.) growth) or 1,000 U/ml catalase preparation for four days. Cell number after treatment was determined by Hoechst staining to observe total DNA content in wells. Both catalase treatment and HA-JNK1 expression inhibited cell proliferation in a dose-dependent manner.

whether the removal of extracellular H_2O_2 triggers growth arrest through JNK1 or other independent signals.

Stable Expression of HA-JNK1 in Ovarian Tumour Cells Effectively Increases Basal Levels of c-Jun Phosphorylation-

To directly manipulate the JNK1 signal, SK-OV-3 cells were transfected with HA-JNK1/pcDNA3, selected for G418 sulfate resistance, and clones screened for stable HA-JNK1 protein expression. These cells were utilized as they produce a very large amount of extracellular H_2O_2 that contributes to their proliferation in culture. Three positive clones were obtained that express varying levels of HA-JNK1 (clones JNK1 2a, JNK1 2d, JNK1 3c) as compared to a pcDNA3-transfected clone (pCDNA3 1c) which expresses only endogenous JNK1, as determined by anti-JNK1 and anti-HA tag Western blots (Fig. 26). To assess the functional significance of the stable changes in JNK1 levels, SK-OV-3 clones were treated with toxic levels of H_2O_2 (1 mM), growth-inhibitory levels of catalase (500 U/ml) or heat-inactivated preparation (500 U/ml) and cell lysates were used in *in vitro* JNK1 kinase reactions (Fig. 27). As expected, a pronounced and stable c-Jun peptide phosphorylation by JNK1 was induced (from approximately 2.5- to 10-fold induction) by H_2O_2 treatment. Catalase treatment produced a small and transient increase in phospho-c-Jun peptide levels in all clones, but these increases were not significant when compared to the effects of heat-inactivated catalase preparation. Here we observe the differences between the JNK1 responses of murine Rat-1 fibroblasts andhuman SK-OV-3 epithelial cells, as the epithelial cell line appears more insensitive to small changes in environmental H_2O_2 levels. However, the overall levels of c-Jun

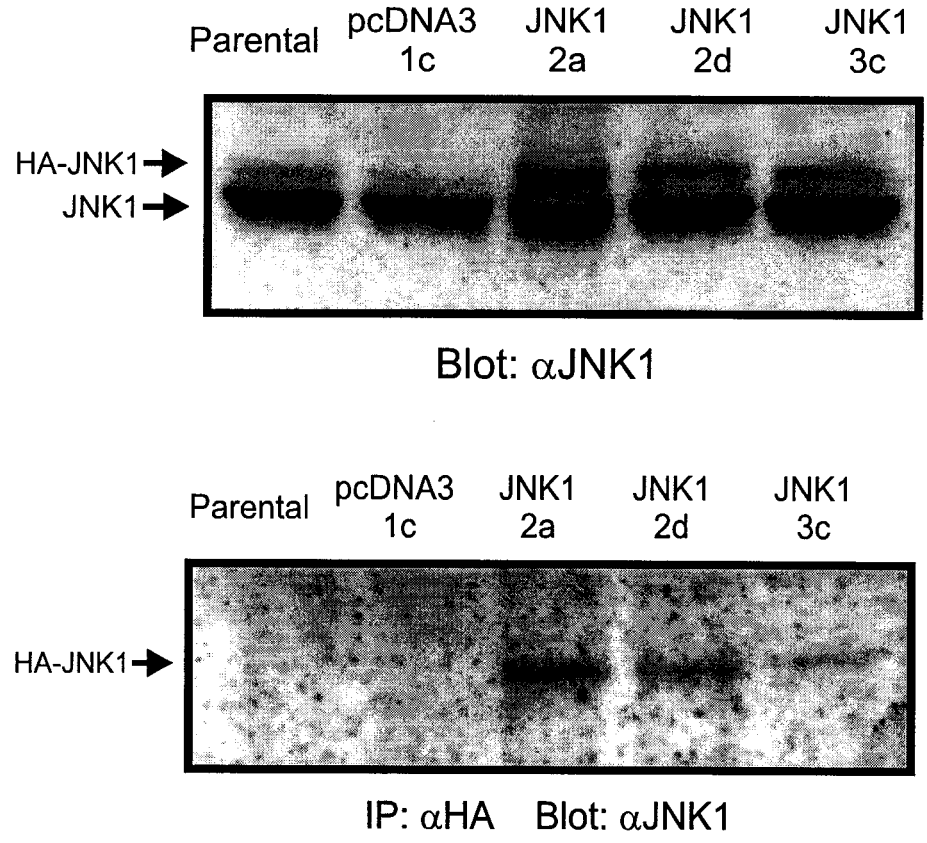


Figure 26: Stable Expression of Ectopic HA-JNK1 in Three SK-OV-3 Ovarian Carcinoma Clones

SK-OV-3 cells were transfected with HA-JNK1/pcDNA3 plasmid or pcDNA3 control plasmid, selected for resistance to G418 sulfate, and clones screened for HA-JNK1 protein expression. In the top image, total protein lysates from clones were analyzed by Western blotting; membrane was probed with anti-JNK1 Ab. Both endogenous and HA-JNK1 were detected in three clones (SK-OV-3 JNK1 2a, 2d, 3c). Parental cells and control clone SK-OV-3 pcDNA3 1c also expressed a protein that corresponded in size with HA-JNK1, however this may represent phosphorylated JNK1. To confirm the presence of HA-JNK1 in the three positive clones and its absence in control cells, HA-tagged protein was immunoprecipitated from lysates using anti-HA Ab (bottom image). Immunoprecipitates were analyzed by Western blotting, and membrane probed with anti-JNK1 Ab. HA-JNK1 expression was only observed in the clones 2a, 2d and 3c. Clone JNK1 2a expressed the highest levels of HA-JNK1, however expression in all clones was relatively low.

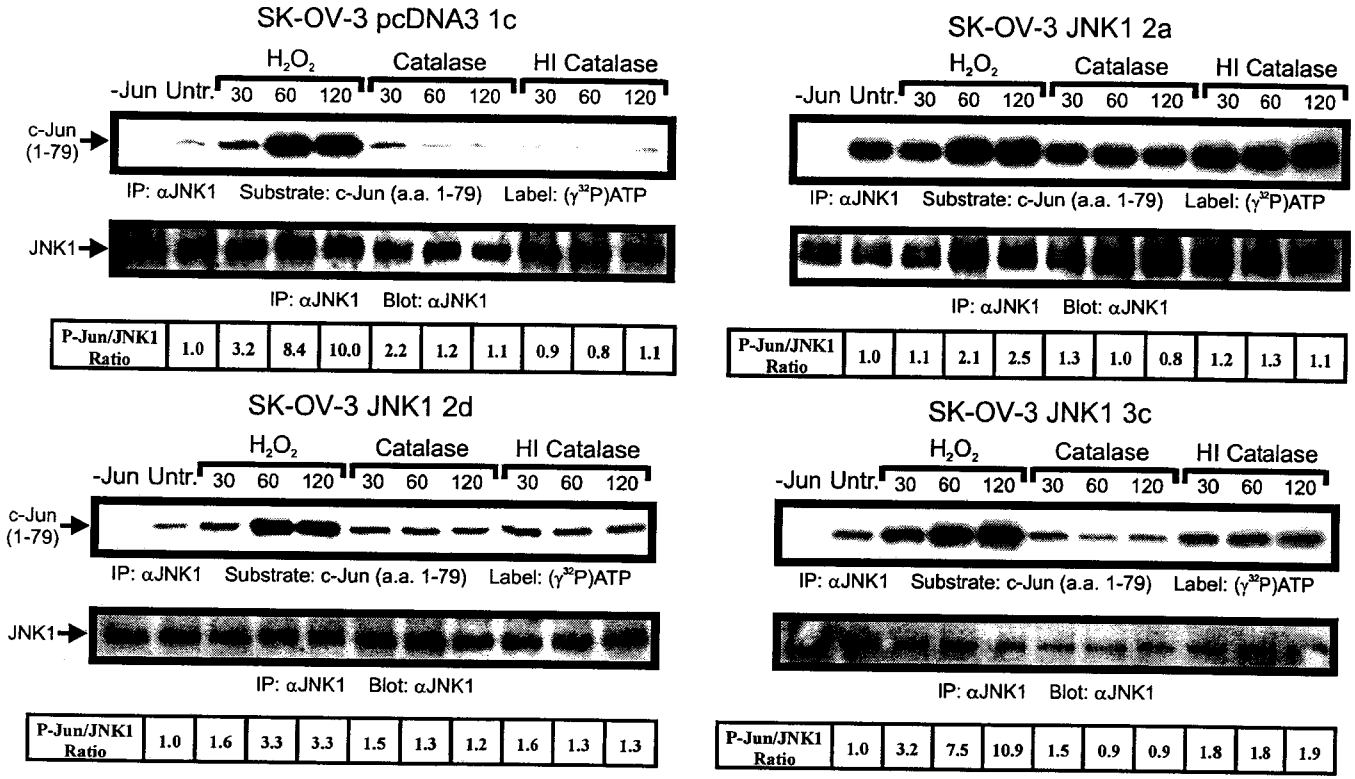


Figure 27: Stable Expression of HA-JNK1 in SK-OV-3 Cells Increases Basal Levels of c-Jun Phosphorylation JNK1 was immunoprecipitated from protein lysates after the indicated treatments. Untr.=no treatment; H_2O_2=1 mM H_2O_2; Catalase/Heat-inactivated (HI) Catalase=500 U/ml; treatment times 30 minutes to 2 hours (30-120). Immunoprecipitates were included in an *in vitro* kinase reaction with a JNK1 substrate (c-Jun peptide, amino acids 1-79) and label ([^{32}P]ATP). Samples were then run on SDS-PAGE and Western blotted. ^{32}P phosphorescence representing c-Jun phopshorylation by JNK1 was quantified using phosphorimager analysis. c-Jun phosphorescence was compared to total JNK1 protein levels. H_2O_2 treatment caused a sustained induction of JNK1 activity. Basal c-Jun phosphorylation by JNK1 was increased in all three HA-JNK1-expressing clones as compared to the control clone. The level of basal phosphorylation increases correlated to the level of ectopic HA-JNK1 expression. Tables display densitometry analysis of the c-Jun phosphorylation/ JNK1 level ratio for each lane (untreated ratio considered 1).

phosphorylation varied consistently between clones regardless of treatment, and corresponded to the levels of stable HA-JNK1 expression. Therefore, a range of permanent amplifications of the basal JNK1 signal was obtained in the SK-OV-3 JNK1 clones.

Stable Amplification of the JNK1 Signal Lowers Endogenous Cellular H_2O_2 Production and Increases Resistance to Catalase Treatment-

A number of studies have demonstrated the regulation of JNK1 pathway components by changes in intracellular H_2O_2 levels (Adler *et al.* 1999; Nemoto *et al.* 2000; Chen *et al.* 2001; Preston *et al.* 2001). To determine if alteration of JNK1 activity could affect cell-generated H_2O_2 levels in a reciprocal fashion, we analyzed the extracellular H_2O_2 production of HA-JNK1-expressing SK-OV-3 clones and pcDNA3 1c control clone (Fig. 28). Interestingly, H_2O_2 production was decreased 2- to 3-fold in all HA-JNK1-expressing clones. This alteration in H_2O_2 generation profile did not significantly affect the growth rates of selected clones compared to SK-OV-3 parental cells (not shown). These data suggest a decreased requirement of H_2O_2 for efficient growth in culture.

To address this hypothesis, we investigated how these changes in the JNK1 clones affected their growth response to H_2O_2 removal by catalase treatment (Fig. 29). Clones JNK1 2d and JNK1 3c displayed an approximate 2-fold resistance to growth inhibition by catalase (0-1,000 U/ml). The growth response of clone JNK1 2a, with the highest HA-JNK1 expression, compared to that of the pcDNA3 1c control clone, with slight resistance observed at large catalase doses. Although the level of resistance to catalase

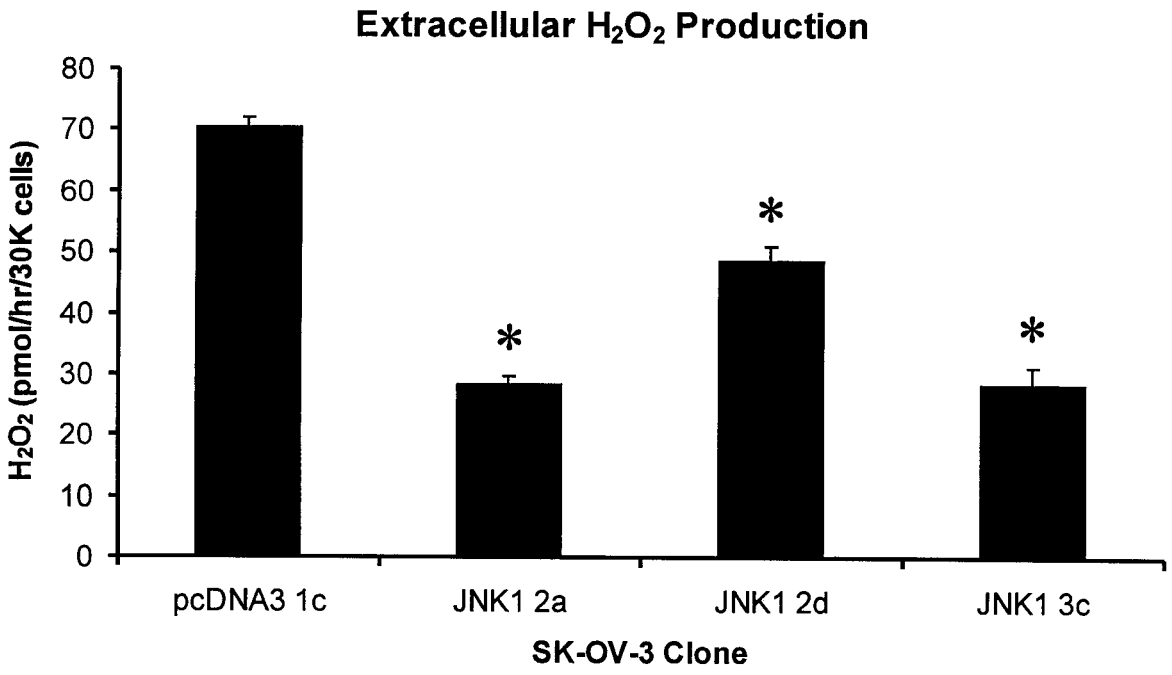


Figure 28: Stable HA-JNK1 Expression in SK-OV-3 Clones Results in Lowered Endogenous H_2O_2 Generation

30,000 cells/well of each clone were seeded into multiple wells of a 96-well plate and incubated until adherent. Media was removed from wells and replaced with an assay solution containing Amplex Red reagent in order to detect the extracellular accumulation of H_2O_2. Fluorescence in each well was recorded over three hours to determine H_2O_2 production over time (pmol/hr). HA-JNK1-expressing clones exhibit a 2- to 3-fold decrease in extracellular H_2O_2 generation as compared to the pcDNA3 1c control clone. Values were obtained from multiple experiments. (*) indicates a significant decrease in H_2O_2 production as compared to clone pcDNA3 1c ($p<0.05$).

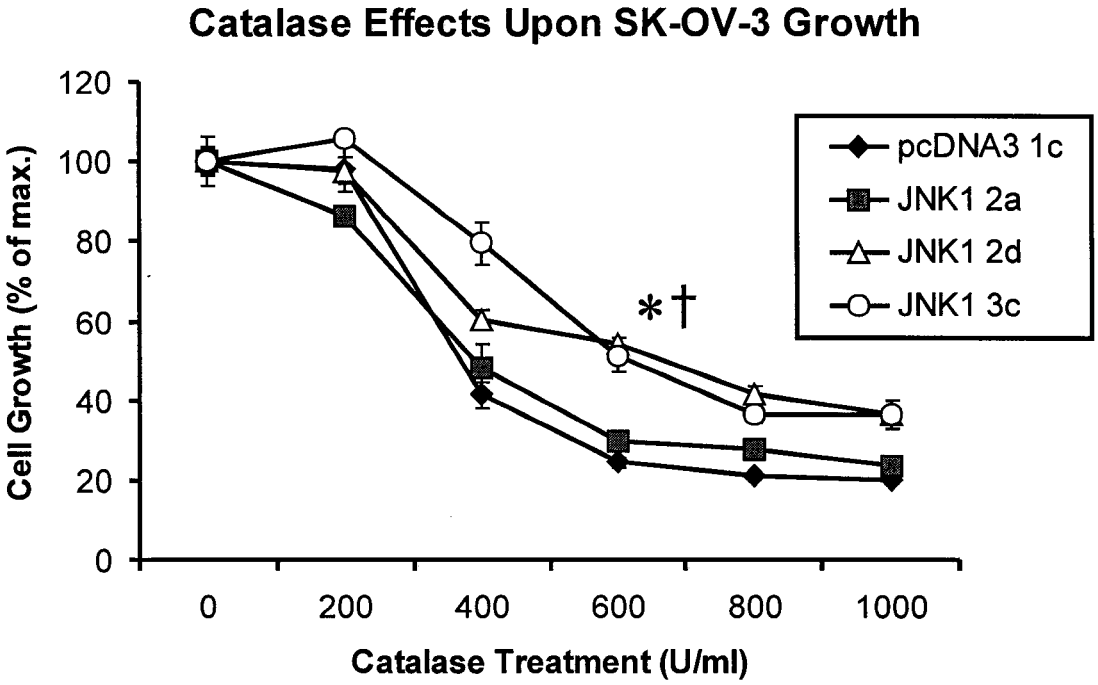


Figure 29: Stable HA-JNK1 Expression in SK-OV-3 Cells Results in Increased Resistance to Growth Arrest from Scavenging of Extracellular H_2O_2
1,000 cells/well of each clone were seeded into multiple wells of a 96-well plate, incubated until adherent, and then treated with increasing doses of catalase (0 to 1,000 U/ml) for four days. Total cell number in wells was quantitated by Hoechst staining to observe total DNA in wells. No catalase treatment was considered 100% maximum (max.) growth. The JNK1 2a clone displayed a slight increase and clones JNK1 2d and 3c displayed an approximate 1.5- to 2-fold increase in resistance to the growth-inhibitory effects of catalase treatment. (*,†) indicate a significant difference in IC_{50} of catalase treatment between JNK1 clones 2d and 3c, and the control clone pcDNA3 1c ($p<0.05$).

treatment does not directly correlate with the level of ectopic HA-JNK1 expression in the three SK-OV-3 clones, all clones display a stable increase in JNK1 activity, lowered H_2O_2 generation and decreased requirement for basal levels of environmental H_2O_2. These findings provide further evidence for a JNK1-mediated adaptation which provides a lowered requirement of the H_2O_2 stimulus for efficient proliferation.

In order to determine if the increase in resistance to the growth-inhibitory effects of extracellular catalase-mediated scavenging of H_2O_2 observed in the JNK1 clones (only clones 2d and 3c significantly) could also be applied to an increased intracellular catalase activity, a human *catalase-1* gene expression vector was constructed. The use of ectopic catalase expression in our experiments also avoids any of the concerns raised by the use of catalase preparation treatment of the culture media, such as the effects of non-enzymatic preparation components. The cloning strategy for this vector construction is illustrated in figure 30. Upstream HindIII and downstream XbaI restriction endonuclease target sequences were incorporated onto the ends of the *catalase-1* open reading frame using designed primers and PCR. After HindIII/XbaI digestion, the gene was incorporated into the pFLAG-CMV-2 transient expression vector. Confirmation of correct vector components was obtained by HindIII/XbaI digest and analysis of gene insert and vector backbone DNA fragment migration in ethidium bromide-stained agarose gels (Fig. 31A). Successful component ligation was confirmed by vector size analysis after EcoRI/XhoI digest (Fig. 31B) and amplification of the *catalase-1* gene from the ligation product using specific primers and PCR (Fig. 31C). Correct frame

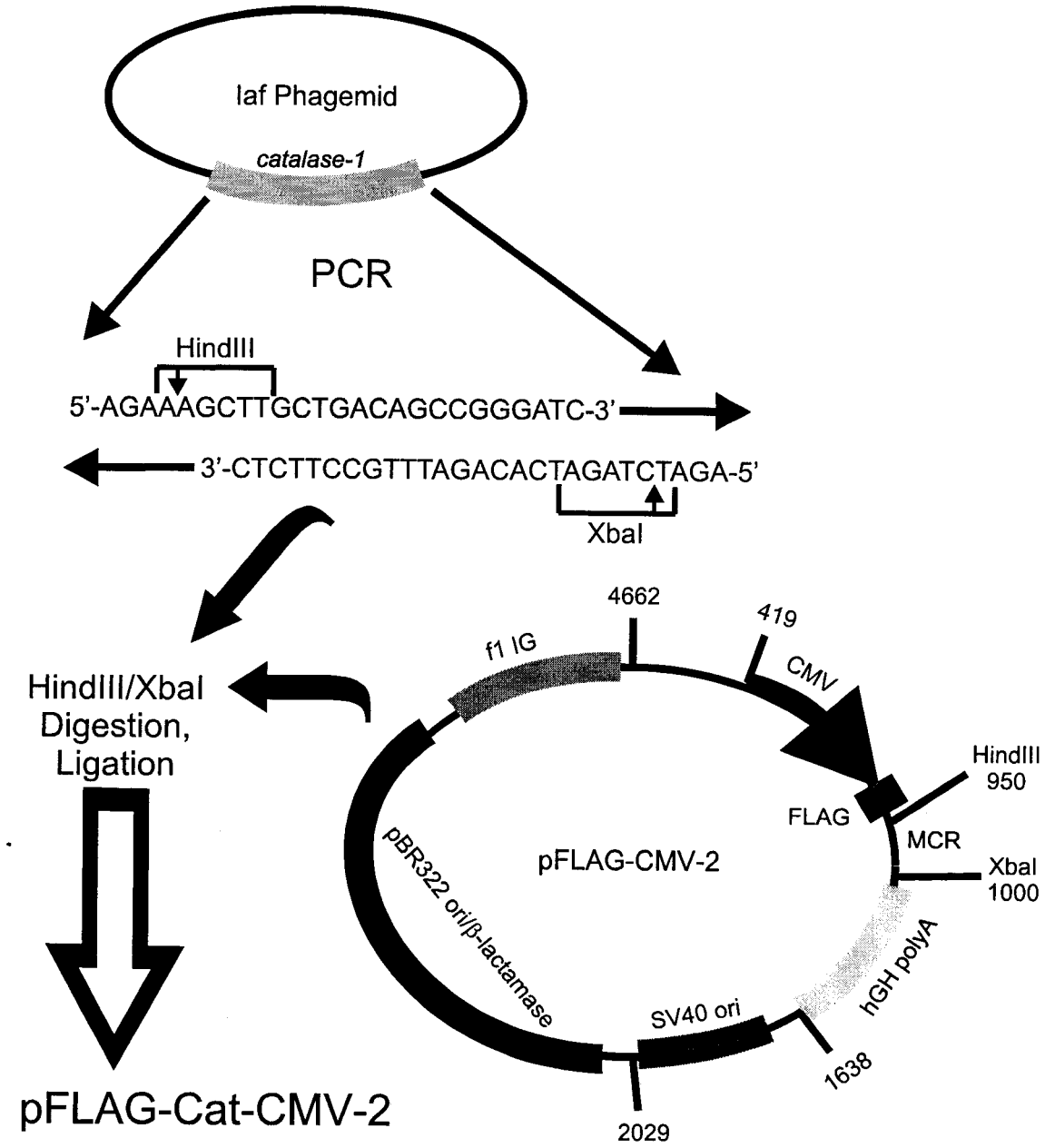


Figure 30: Cloning Strategy for the pFLAG-Cat-CMV-2 Transient Expression Vector

The human *catalase-1* gene was obtained from ATCC. An upstream HindIII and downstream XbaI target sequences were added using specific primers and PCR. The parental plasmid and the *catalase-1* PCR product were digested with the appropriate restriction endonucleases and then ligated together to form pFLAG-Cat-CMV-2.

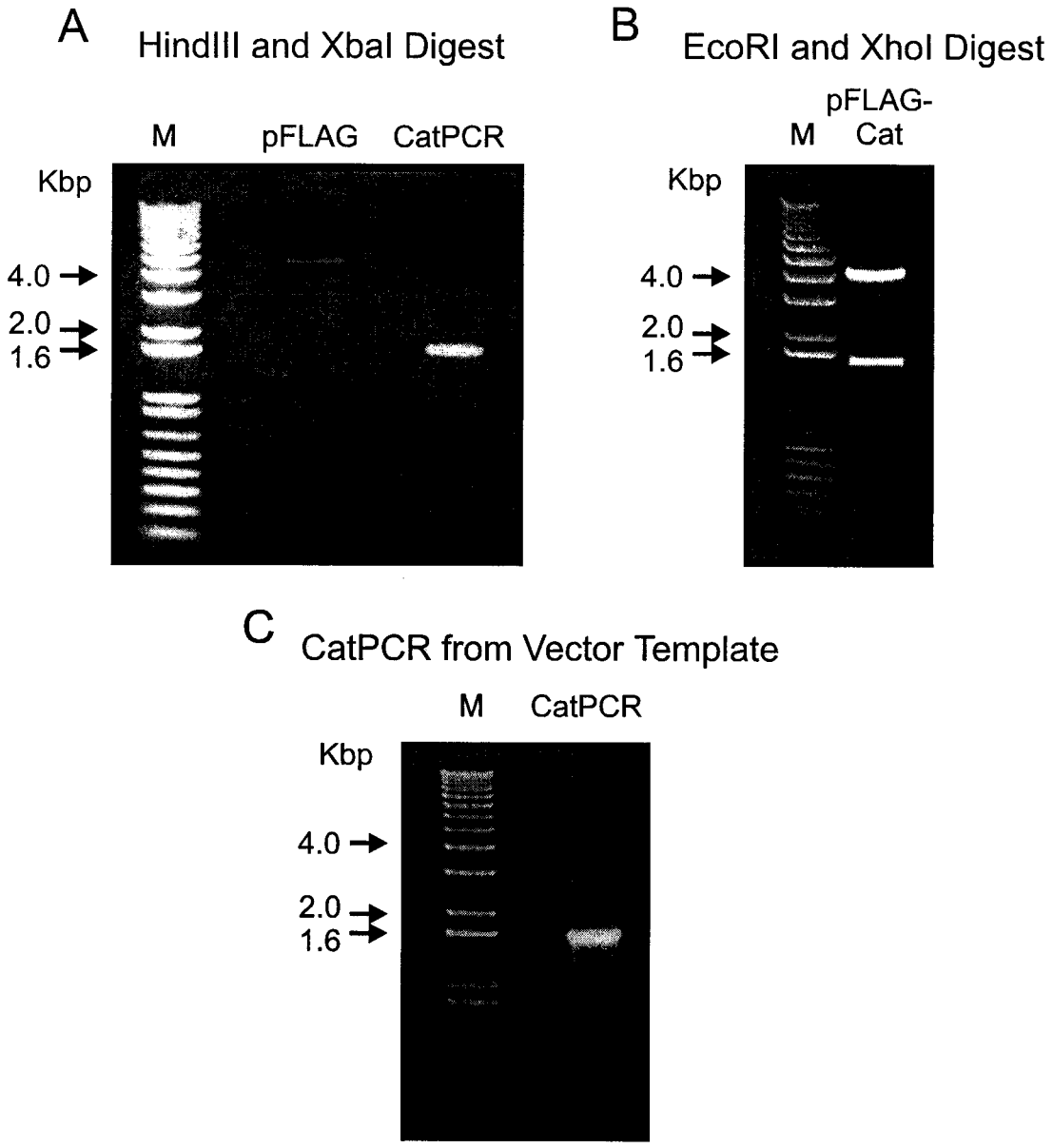


Figure 31: Cloning of the pFLAG-Cat-CMV-2 Plasmid
A, pFLAG-CMV-2 plasmid and the human *catalase 1* ORF were both digested with HindIII and XbaI prior to ligation reaction. Expected band sizes are approximately 4.62 Kbp and 1.59 Kbp. *B,* after ligation, competent cells were transformed, pFLAG-Cat-CMV-2 plasmid amplified from resistant clones, and plasmid digested with EcoRI and XhoI. Expected band sizes are approximately 4Kbp and 2.2 Kbp; bands of 4Kbp, 1.4 Kbp and 1 Kbp were observed due to EcoRI star activity. *C,* for further confirmation of catalase gene insertion, *catalase 1* was amplified using the pFLAG-Cat-CMV-2 plasmid as a template. M=markers (0.1-12 Kbp).

insertion and proper unmutated sequence of the insert gene were further confirmed upon sequencing analysis (not shown). The vector was designated pFLAG-Cat-CMV-2.

The effects of intracellular expression of ectopic catalase upon SK-OV-3 clones were then examined. pFLAG-Cat-CMV-2 was introduced into SK-OV-3 cells by transient transfection. FLAG-catalase protein levels were then assessed by Western blotting (Fig. 32A). As the ectopic *catalase*-1 cDNA did not contain a peroxisome-specific leader sequence, it is assumed that the protein product was located in the cytosol. SK-OV-3 cells express endogenous catalase at a high level as compared to other cell lines tested (Rat-1 fibroblast clones are shown for comparison). The presence of ectopic FLAG-catalase was confirmed upon labeling with anti-FLAG antibody (Fig. 32A). An intracellular increase in H_2O_2 scavenging after transfection with 10 μg of pFLAG-Cat-CMV-2 plasmid was observed after native polyacrylamide gel electrophoresis of cell lysates and H_2O_2-linked gel staining (Fig. 32B). H_2O_2 removal was increased approximately 2-fold in total cell lysates after pFLAG-Cat-CMV-2 transfection as compared to pcDNA3 (10 μg) or pFLAG-CMV-2 (10 μg) transfection. As with experiments using the addition of catalase preparations to the cell culture media, the growth response of SK-OV-3 clones to ectopic FLAG-catalase activity was studied (Fig. 32C). Clones were exposed to transfection reagent alone, transfected with 0.2 μg of pFLAG-Cat-CMV-2 plasmid or transfected with 0.2 μg of pcDNA3 control plasmid per well. Transfection efficiency was tested independently using a grenn fluorescence protein (GFP)-expressing plasmid; optimal efficiency in SK-OV-3 culture was approximately 35% (measured using flow cytometry). As was observed with

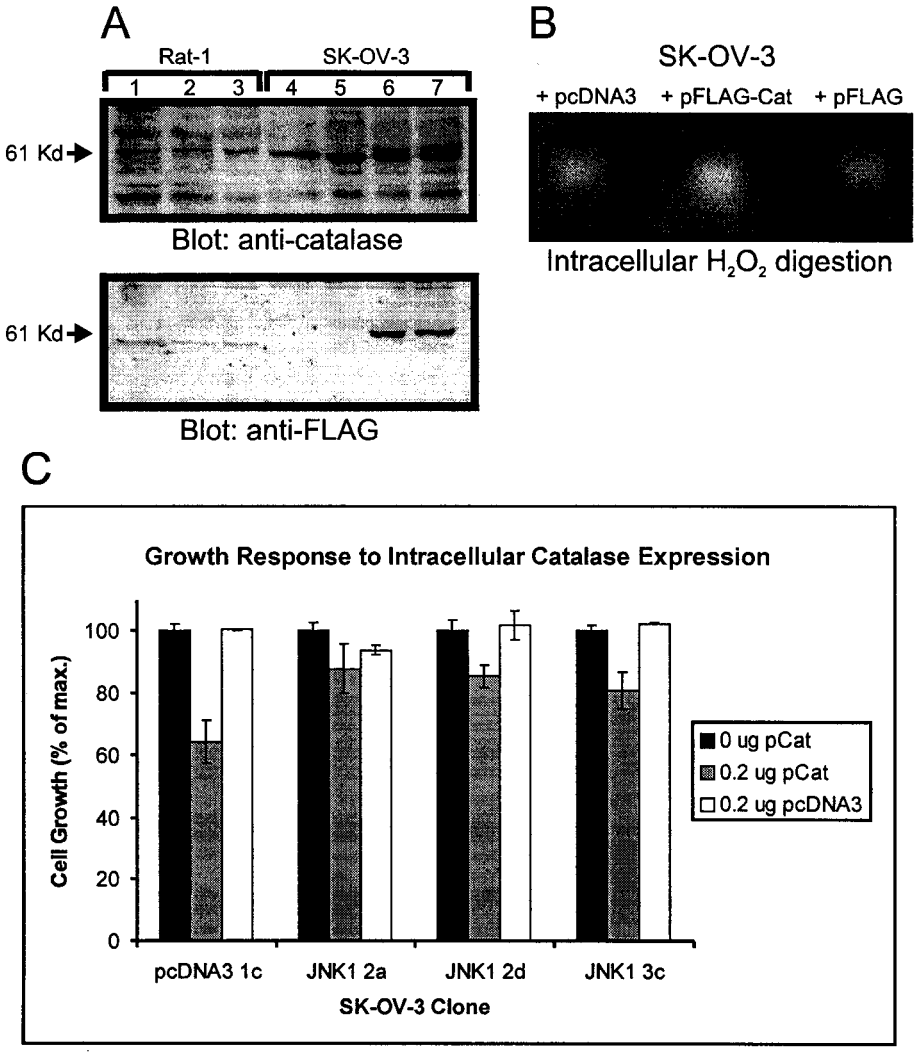


Figure 32: Stable HA-JNK1 Expression in SK-OV-3 Cells Causes Increased Resistance to Growth Inhibition from Intracellular Expression of Ectopic Catalase
A, FLAG-catalase expression after transfection with pFLAG-Cat-CMV-2 plasmid was detected by Western blotting and probing with anti-catalase Ab. SK-OV-3 cells express high levels of endogenous catalase (compare to Rat-1 lysates). The expression of ectopic FLAG-catalase was confirmed by reprobing with anti-FLAG Ab. Lanes 1-3: Rat-1 clone lysates; Lane 4: SK-OV-3 lysate; Lane 5: SK-OV-3 transfected with 10 μg pFLAG-CMV-2 control; Lanes 6+7: SK-OV-3 transfected with 10 μg pFLAG-Cat-CMV-2. *B,* SK-OV-3 cells were transfected with the indicated plasmids, and intracellular catalase activity assessed by native PAGE and H_2O_2-linked gel staining. FLAG-catalase expression increased catalase activity approximately 2-fold. *C,* 2,000 cells/well of each clone were plated and then transfected with no DNA (0 μg pCat), 0.2 μg pFLAG-Cat-CMV-2 or 0.2 μg pcDNA3. Cells were incubated for three days; all HA-JNK1-expressing clones displayed resistance to growth inhibition from catalase expression as compared to the pcDNA3 1c control clone.

extracellular catalase treatment, the HA-JNK1-expressing clones displayed partial resistance to growth inhibition from increased intracellular catalase activity. Growth inhibition was not observed after control plasmid transfection. Because H_2O_2 can diffuse freely across the plasma membrane, it is expected that its removal from the cellular environment or from the cytosol would have similar effects upon cell behaviour. These results indicate that stable expression of HA-JNK1 results in partial resistance to the anti-proliferative effects of both extracellular and intracellular catalase activity.

Inhibition of Kinase Activity Upstream of JNK1 via Transient Expression of Dominant-negative MKK4 or MKK7 Does Not Induce Resistance to Catalase Treatment-

MKK4 (SEK1) and MKK7 (SEK2) directly activate JNK1 by phosphorylation of Threonine 183 and Tyrosine 185 (Yan *et al.* 1994; Tournier *et al.* 1997). To investigate the possibility that a mechanism of catalase-induced inhibition of cell growth is through the targeting of factor(s) upstream of the JNK1 signal, dominant-negative mutants of MKK4 (HA-MKK4-AL) and MKK7 (MKK7-AL) were transiently expressed in Cos-1 cells (Figs. 33A and 33B). Presumably, blocking the activity of one or both of these kinases may suppress the lowered H_2O_2 signal to JNK1, thereby restoring cell proliferative capacity to optimal levels. This was tested by cell proliferation assay, after treatment of Cos-1 cells with increasing catalase dose (Fig. 33C). Cell growth efficiency was not enhanced with either expression of HA-MKK4 or MKK7-AL. Indeed, a slight decrease in growth rate was observed after transfection with these vectors as compared to pcDNA3 control plasmid, particularly after transfection with pMKK7-AL. This suggests that the previously observed transient induction of JNK1 activity by catalase (Preston *et*

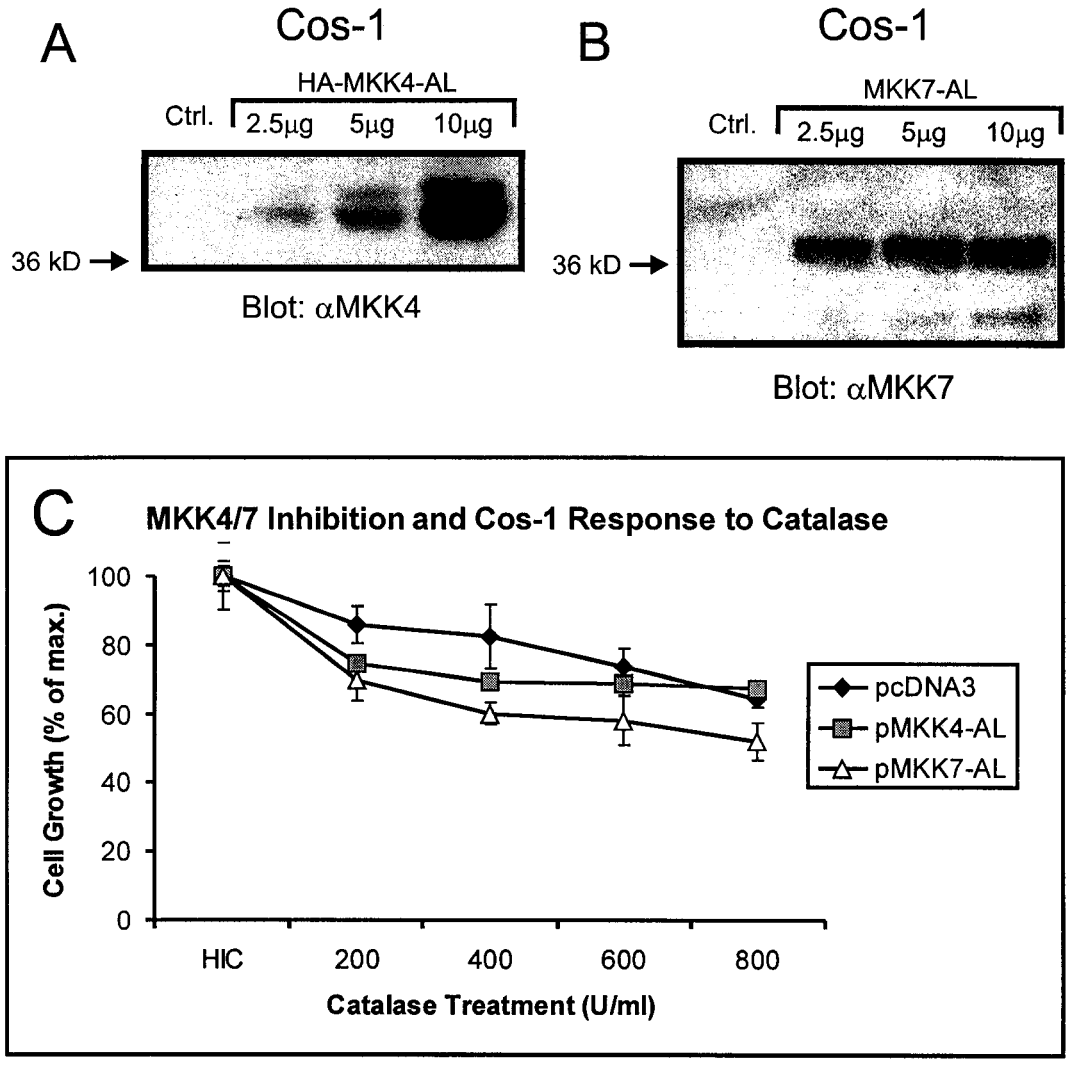


Figure 33: Inhibition of MKK4 (SEK1) or MKK7 (SEK2) Activity in Cos-1 Cells Does Not Confer Resistance to Catalase Treatment

A+B, cells were transfected with the indicated amounts of HA-MKK4-AL/pcDNA3.1 (*A*) or MKK7-AL/pcDNA3.1 (*B*) plasmids or 10 μg pcDNA3 control plasmid (Ctrl.). Cell lysates were collected and HA-MKK4-AL or MKK7-AL protein expression detected by Western blotting and probing of membranes with anti-MKK4 or anti-MKK7 Abs. *C,* 1,000 cells/well were seeded into multiple wells of a 96-well plate and allowed to adhere. Cultures were transfected with 0.1 μg control (pcDNA3) or MKK-AL expression vectors (pMKK4-AL, pMKK7-AL). Four hours post-transfection, cells were treated with 800 U/ml heat-inactivated catalase (HIC) or 200-800 U/ml catalase for four days. Cell growth was not restored upon expression of either dominant-negative enzyme; MKK-7-AL expression slightly potentiated growth inhibition.

al. 2001) may mediate a protective response that maintains cell cycle progression. However, a larger and sustained increase in JNK1 activity may produce the opposite effect of growth inhibition, as observed in figure 25. Further evidence against a role for the MKK4 signal in catalase-mediated proliferation effects was obtained using a cell growth assay after treatment of MKK4 +/+ and -/- mouse embryo fibroblasts (MEFs) (Fig. 34). No difference in growth responses to catalase was observed between the wild type and MKK4 null fibroblasts. These results indicate that the ability of JNK1 activity to potentiate catalase treatment effects may be dependent upon other upstream signals, or upon direct effects of altered H_2O_2 levels on JNK1 itself.

Inhibition of JNK Activity Does Not Reverse the Resistance of HA-JNK1-expressing SK-OV-3 Clones to the Catalase-mediated Proliferative Block-

In order to determine if the resistance to H_2O_2 scavenging observed in SK-OV-3 JNK1 clones is due directly to an increase in basal JNK1 activity or due to an adapted unresponsiveness to the JNK1 signal, this activity was inhibited by co-treatment with the specific JNK inhibitor SP600125 (Bennett *et al.* 2001). The inhibitory effect of SP600125 upon JNK Thr183/Tyr185 phosphorylation was confirmed by Western blotting (Fig. 35A). JNK phosphorylation in SK-OV-3 clones was induced to a high degree with anisomycin treatment (250 ng/ml, 30 min.) and to a lesser degree with catalase treatment (1000 U/ml, 30 min.). Therefore, we see that even in the SK-OV-3 cells, extracellular catalase treatment is capable of transiently inducing JNK activity, albeit to a lesser degree than previously observed in Rat-1 fibroblasts. Pre-treatment with SP600125 (50 μM, 1 hr.) inhibited JNK phosphorylation after anisomycin or catalase treatments. These

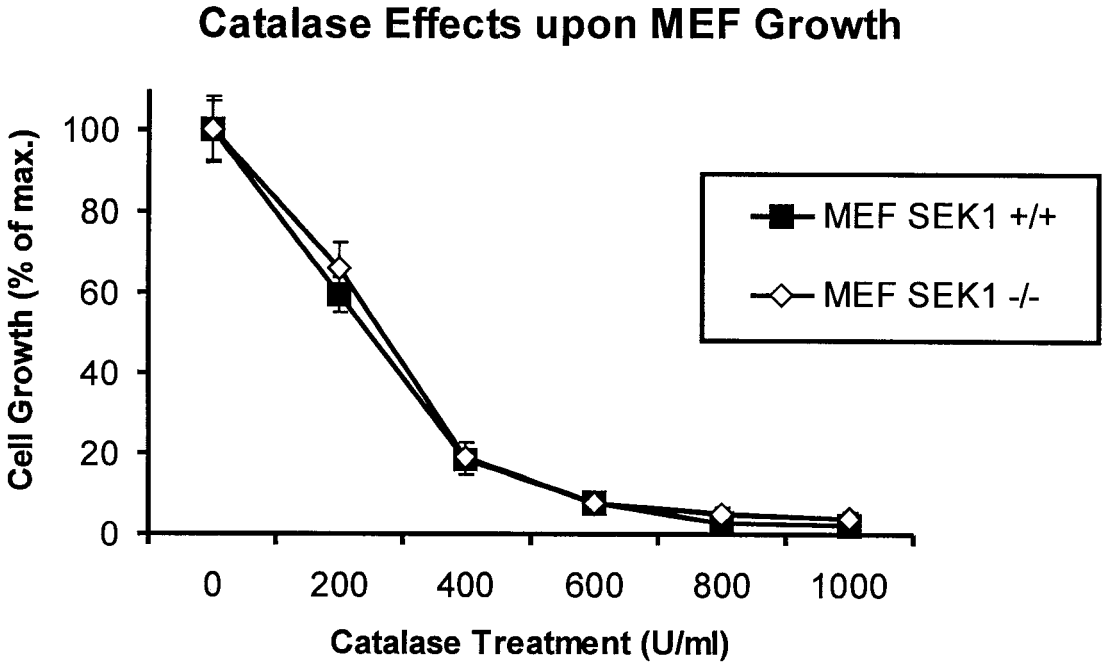


Figure 34: The Absence of MKK4 (SEK1) Does Not Alter the Cell Growth Response to Catalase Treatment
Wild-type (SEK1 +/+) or SEK1 knockout (SEK1 -/-) mouse embryo fibroblasts (MEFs) were plated at 1,000 cells/well into multiple wells (96-well plate) and incubated until adherent. 0 to 1,000 U/ml catalase (0 catalase considered 100% maximum (max.) growth) was then added to wells and cultures incubated for four days. Cell number in wells was quantitated using Hoechst staining for total cellular DNA. All MEFs were highly sensitive to growth arrest from catalase treatment, regardless of *SEK1* status.

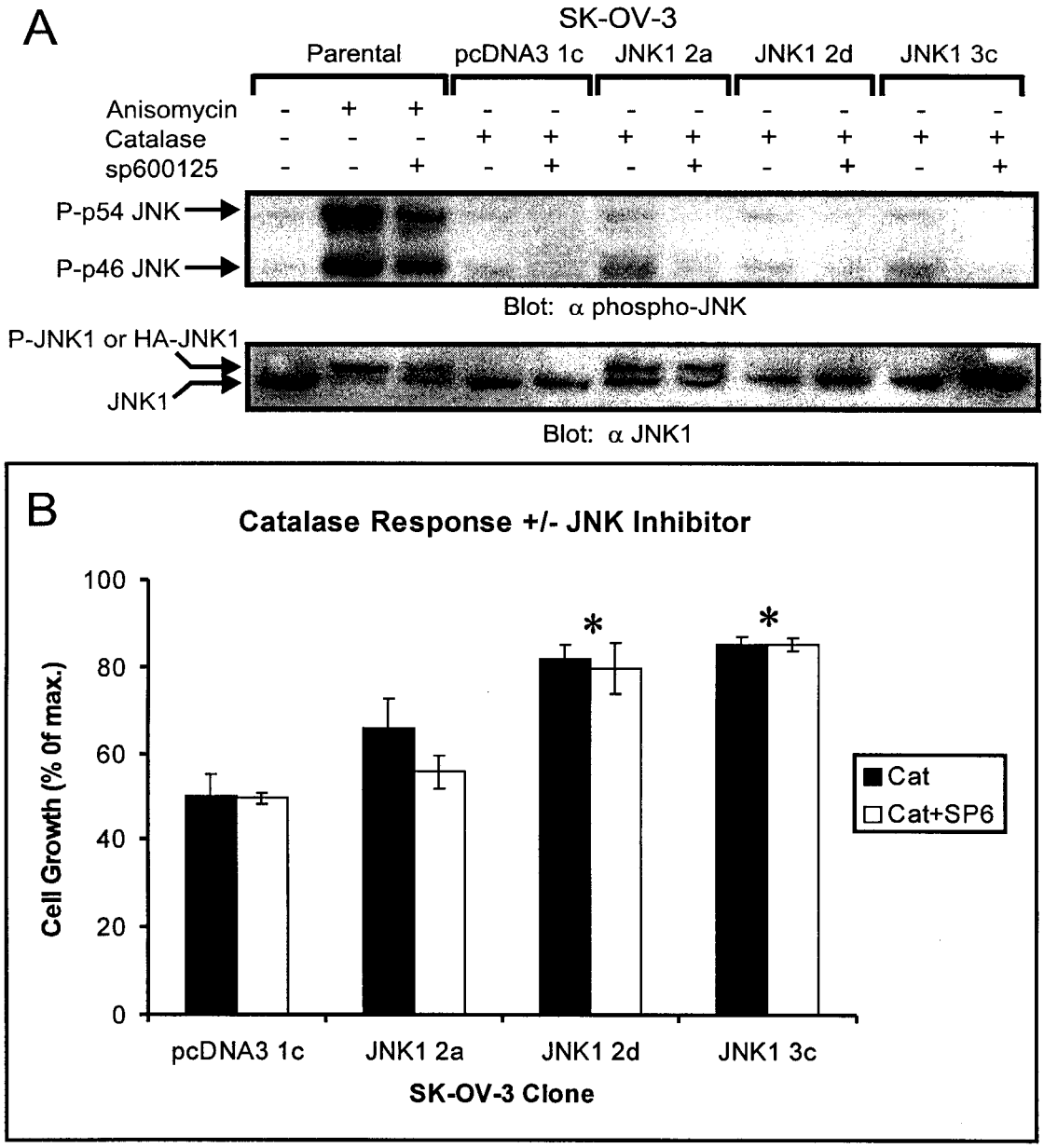


Figure 35: Inhibition of JNK Activity Does Not Reverse SK-OV-3 JNK1 Clone Resistance to Growth Arrest from Catalase Treatment

A, JNK phosphorylation in SK-OV-3 clones after treatments was assessed by labelling with anti-phospho JNK Ab. Anisomycin=250 ng/ml, 30 min.; Catalase=1 kU/ml, 30 min.; SP600125=50 μM, 1 hr. Anisomycin treatment caused a high degree, and catalase treatment caused a low degree, of JNK phosphorylation. This was inhibited by the JNK inhibitor SP600125. *B,* 1,000 cells/well were treated (Cat=500 U/ml catalase; Cat+SP6=500 U/ml catalase + 20 μM SP600125) for four days. Growth inhibition by catalase was not significantly affected by Sp600125 treatment. (*) indicates a significant increase in resistance to cell growth inhibition as compared to clone pcDNA3 1c, for both Cat and Cat+SP6 treatments ($p<0.05$).

phosphorylation changes were compared to total JNK1 levels (Fig. 35A). SP600125 was then used in conjunction with catalase treatment of HA-JNK1-expressing SK-OV-3 clones and the pcDNA3 1c control clone to determine if lowering basal JNK activity could reverse the partial resistance of the HA-JNK1 expressors to growth inhibition (Fig. 35B). 1,000 cells/well were incubated until adherent, and then left untreated (considered 100% growth), treated with 500 U/ml catalase preparation, or treated with catalase plus 20 μM SP600125 for four days. As previously determined, the HA-JNK1-expressing clones remained partially resistant to the effects of catalase treatment as compared to the control. Co-incubation with SP600125 did not significantly alter growth responses of these clones. Therefore, the stable HA-JNK1-expressing clones display a lowered requirement of the H_2O_2 stimulus for growth, as well as a decreased responsiveness to alteration of the JNK1 signal. It is this insensitivity to JNK1 that may be responsible for the resistance to catalase treatment observed.

Increased JNK1 Activity after Transient Expression of HA-JNK1 in Cos-1 Cells Inhibits Cellular H_2O_2 Production-

To investigate if the decreased H_2O_2 generation seen in the stable HA-JNK1-expressing SK-OV-3 clones was a direct result of JNK1 activity induction or other mechanism(s), the extracellular H_2O_2 production profile of cells after transient manipulation of JNK1 activity was observed (Fig. 36). Inhibition of basal JNK1 activity (already low) by treatment with the JNK1 inhibitor SP600125 (100 μM for 16 hours) or transfection with pcDNA3 control plasmid (0.4 μg/well, 16 hours) did not affect the generation of H_2O_2. However, transfection with the pHA-JNK1/pcDNA3 expression vector (0.4 μg/well, 16

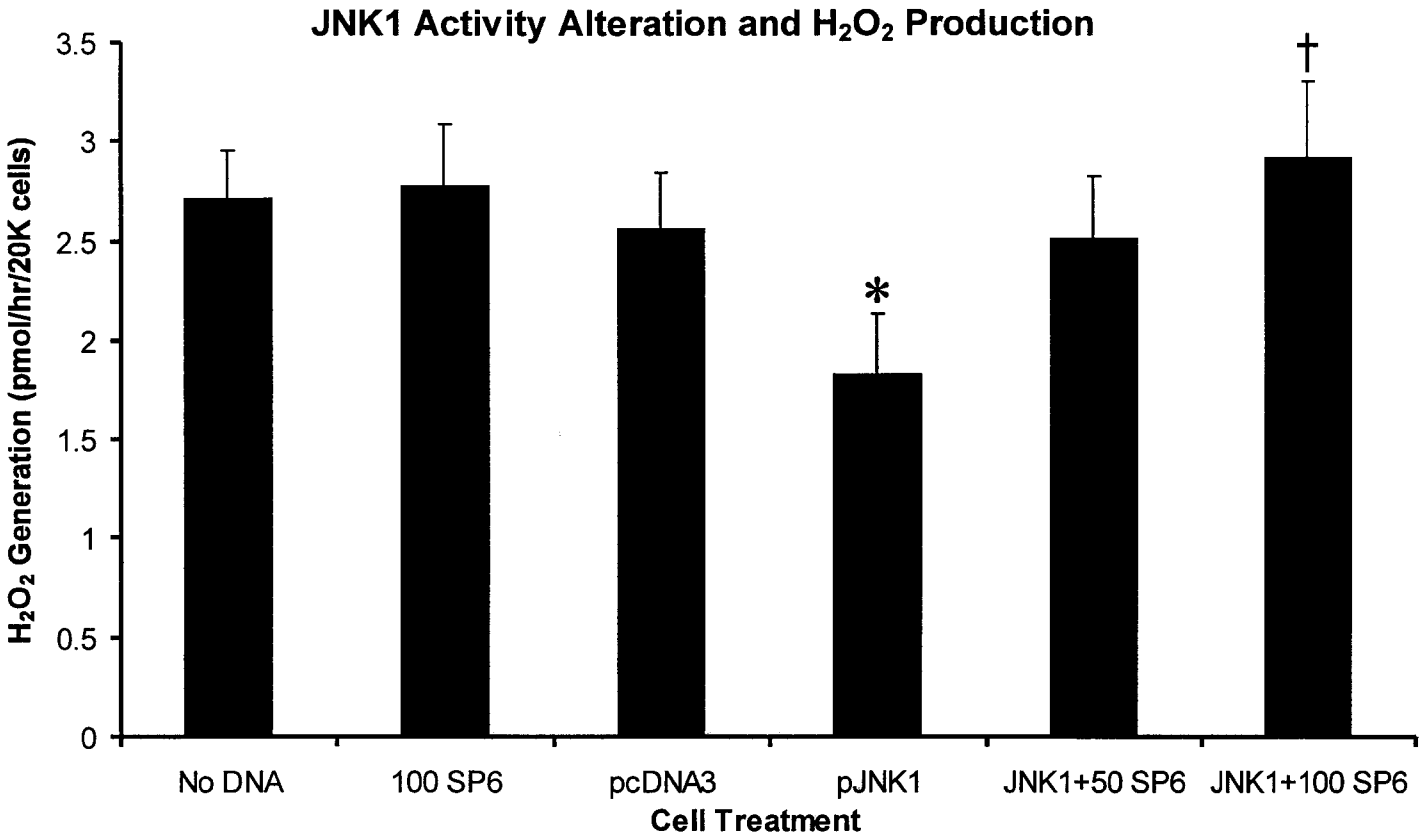


Figure 36: JNK1 Activity Lowers Endogenous Generation of H_2O_2 by Cos-1 Cells
20,000 cells/well were seeded and incubated until adherent. Cultures were then transfected and treated as indicated. No DNA=transfection reagent without plasmid; 100 SP6=transfection reagent without plasmid plus 100 μM of the JNK inhibitor SP600125; pcDNA3=transfection with 0.4 μg pcDNA3/well; pJNK1=transfection with 0.4 μg HA-JNK1/pcDNA3/well; 50 SP6=treatment with 50 μM SP600125. Four hours post-treatment, media was replaced with an Amplex Red assay solution to detect the extracellular accumulation of H_2O_2. Fluorescence in each well represents H_2O_2 production over time (pmol/hr). HA-JNK1 expression lowered H_2O_2 production, and this effect was reversed upon addition of SP600125. (*) indicates a significant decrease in H_2O_2 production as compared to transfection with no DNA ($p<0.05$). (†) indicates a significant increase in H_2O_2 production as compared to transfection with pJNK1 ($p<0.05$).

hours) did reduce H_2O_2 levels significantly. Co-treatment with SP600125 (50 and 100 μM, 16 hours) reversed this effect. These observations provide further evidence that JNK activity inhibits cellular H_2O_2 production.

Reduced H_2O_2 Generation from Stable Expression of HA-JNK1 Is Not Accompanied by an Increase in the Activity of Prominent Cellular H_2O_2-scavenging Enzymes-

Due to the observation that ectopic expression of HA-JNK1 lowers endogenous generation of H_2O_2 in both transient and stable transfection models, the putative mechanism(s) underlying this effect was investigated. As Jun proteins are known to heterodimerize with Nrf2 or Nrf1 to activate ARE-mediated expression of genes involved with xenobiotic detoxification and ROS removal (Venugopal and Jaiswal 1998; Jeyogupal and Jaiswal 2000; Satoh *et al.* 2002), JNK1 could lower cellular H_2O_2 levels via induced transcription of H_2O_2 scavenging enzymes. To address this possibility, the status of major H_2O_2 metabolizing systems (catalase, glutathione peroxidase (GPx)/reduced glutahtione (GSH)) in the stable HA-JNK1-expressing clones was examined. The level of catalase expression in SK-OV-3 clones was determined by Western blotting (Fig. 37A). All three HA-JNK1-expressing clones displayed a reduced expression of catalase as compared to the pcDNA3 1c control clone; it is likely that this downregulation may be an adaptive response of these clones to attempt to increase H_2O_2 levels. Alternatively, owing to a lowered level of endogenous H_2O_2 generation, there is simply a reduced requirement for catalase expression in the SK-OV-3 JNK1 clones.

Total GPx activity in these clones was also examined using a native polyacrylamide gel enzymatic activity assay (Fig. 37B). A slight increase in the H_2O_2

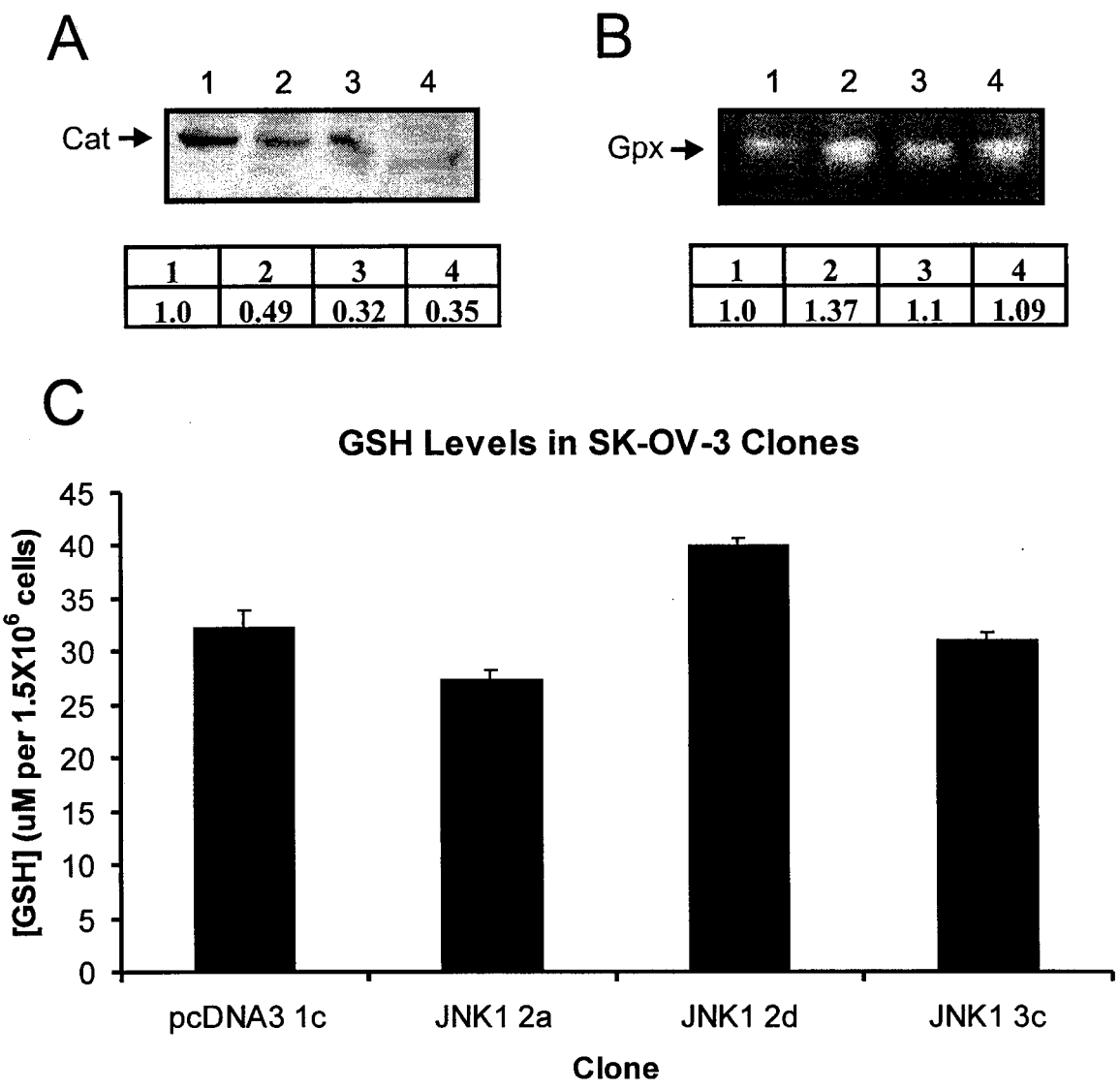


Figure 37: H_2O_2-scavenging Profiles of HA-JNK1-expressing SK-OV-3 Clones
A, the level of total catalase (*Cat*) expression in SK-OV-3 clones (*1*=pcDNA3 1c control clone; *2*=clone JNK1 2a; *3*=clone JNK1 2d; *4*=clone JNK1 3c) was determined by Western blotting. All HA-JNK1-expressing clones displayed lower levels of catalase protein. *B,* total glutathione peroxidase (*GPx*) activity in SK-OV-3 clones (numbers representing clones as in *A*) was quantitated using a native gel activity assay. Achromatic bands represent GPx-mediated scavenging of H_2O_2. All JNK1 clones displayed a slightly increased level of GPx activity as compared to the control clone. Tables display band densitometry analysis (pcDNA3 1c clone considered density 1.0) *C,* total glutathione (*GSH*) levels in SK-OV-3 clones was measured by an *in vitro* colourimetric plate assay. GSH levels were similar in all clones; HA-JNK1-expressing clones displayed variable changes total levels as compared to control pcDNA3 1c clone.

scavenging activity of GPx was observed in the stable HA-JNK1-expressing clones, however not at levels high enough to be wholly responsible for the decreased cellular H_2O_2 concentrations observed. In addition, total cell GSH levels were comparable between all SK-OV-3 clones, as observed by a colourimetric assay for GSH concentration (Fig. 37C). These observations suggest that the JNK1-mediated decrease in cellular H_2O_2 production may result from the induction of other signalling pathways downstream of the JNK1 stimulus or by direct regulation of cellular ROS generating systems. Collectively, these observations are suggestive of a putative model of JNK1 involvement in the regulation of endogenous cellular H_2O_2 generation and in the growth inhibitory response to catalase activity (Fig. 38).

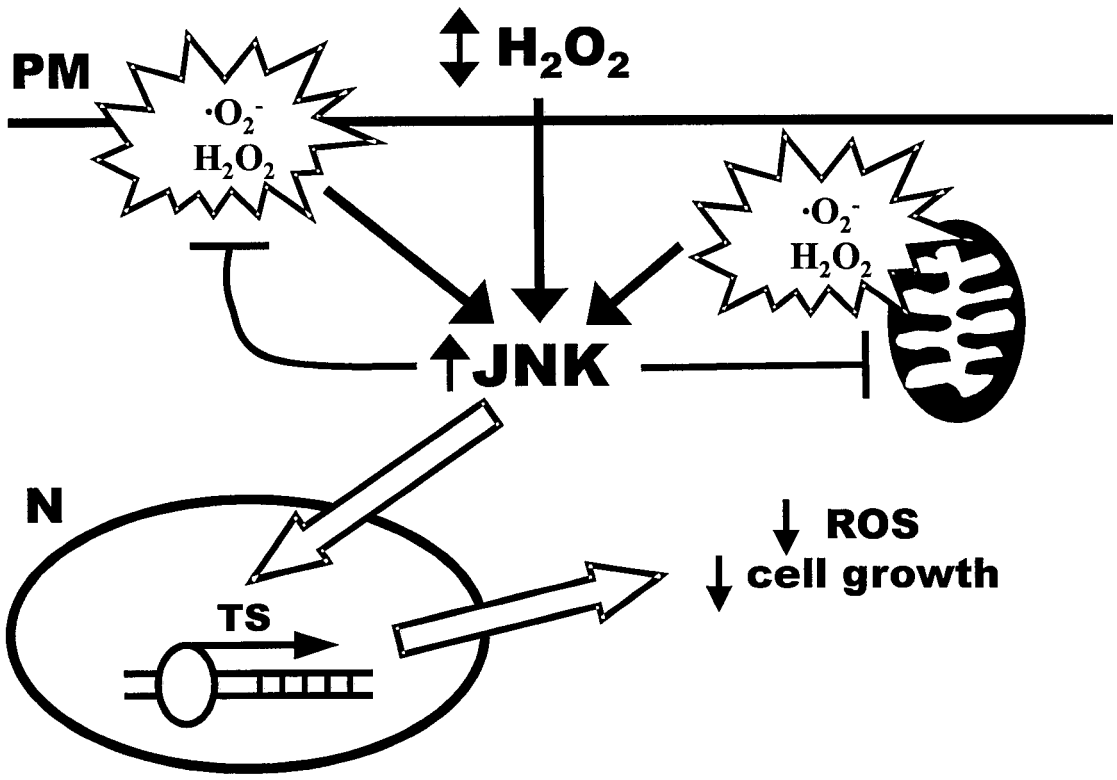


Figure 38: Model for JNK as a Both a Sensor and Regulator of Cellular H_2O_2 Levels

JNK activity can be induced **(in)directly** by both increases and decreases in cellular H_2O_2 levels, that can occur as a result of altered endogenous production (mitochondrial and plasma membrane sources displayed) or experimental manipulation. This response may be due to direct activation of JNK and/or by inhibition of JNK phosphatases. Activation of JNK causes a reduction in H_2O_2 generation, which may represent a mechanism of proliferation block. It is undetermined precisely how JNK1 activity can lower H_2O_2 levels; however we have determined in SK-OV-3 cells that it is not via a reduction in catalase or Gpx enzyme expression or activity. Changes in other JNK1-targetted gene expression or direct effects upon cellular sites of H_2O_2 generation remain possible alternative mechanisms. These effects may be responsible for reductions in cell growth observed upon both catalase-mediated scavenging of H_2O_2 and increased JNK1 activity. PM=plasma membrane; N=nucleus; TS=transcription. Arrows signify positive regulation and blocked lines signify negative regulation.

4.0 Discussion

4.1 H_2O_2 as a Growth Factor

The participation of cellular signal transduction pathways to and from the nucleus in cancer biology has been studied extensively. From this work, key oncogenic stimuli present in various cellular compartments ranging from secreted factors to nuclear transcription factors have been characterized. Many oncogenic pathways are observed to induce cell proliferation. Known molecular "triggers" for this process include peptide and steroid growth hormones, reactive lipid species, and cell/cell or cell/extracellular matrix attachment. It is becoming apparent that all of these signals act together to determine cell phenotype; all environmental cues are processed by the cell, and deregulation of this communication may lead to pathalogical states. The idea of reactive oxygen species as an additional level of environmental regulation of cell behaviour is not new, however the role of this stimulus in the promotion of cell transformation and tumourigenesis has more recently garnered much attention. One mechanism by which ROS can act as regulators of intracellular protein signalling by oxidizing thiol groups on cysteine residues, thereby altering enzymatic activity (Schmid *et al.* 1999; Meng *et al.* 2002). This can have multiple effects upon cell behaviour, including the induction of proliferation. Uncontrolled proliferation may be an advantage for the survival of populations of unicellular organisms, but is dangerous to multicellular organisms as entire organ systems can be destroyed by this process. The work presented in this thesis substantiates, and contributes a molecular mechanism to, an established concept that non-toxic levels of hydrogen peroxide are required for cell proliferation. Additionally, it

offers insight into the signal transduction pathways critical for this process, and their involvement in tumour progression.

This research describes the effects of the removal of endogenously produced H_2O_2 upon specific mitogen- and stress-responsive signalling pathways. In order to remove H_2O_2 from the cellular environment, a catalase preparation was directly added to the culture media. This strategy was adopted to delineate the molecular signalling responses to changes in H_2O_2 status at the plasma membrane. The issue of enzyme purity raises concerns that the effects observed could result from contaminants. To address this issue, we compared the effects of catalase to heat-inactivated catalase in all experiments. Heat-inactivated catalase did not affect cell viability and growth, nor did it affect Neu receptor or Erk activities. JNK1 activity did increase slightly upon the addition of heat-inactivated catalase, but at a lower level than that observed after treatment with active catalase. Direct scavenging of H_2O_2 in cell culture media by bovine liver catalase could not be directly measured due to fluorescence interference, however a comparable *Aspergillus niger* catalase preparation markedly reduced extracellular H_2O_2 levels, while heat-inactivation of this treatment blocked the effect. In addition, catalase-induced growth inhibition was rescued upon co-treatment of cells with H_2O_2 generating glucose oxidase. The levels of glucose oxidase activity required for this rescue were cytotoxic if added alone, indicating that the two enzymes are inversely affecting H_2O_2 levels. In SK-OV-3 cells, human Catalase-1 levels and activity were increased using a transient expression vector, and this produced a similar impairment of proliferation as observed

with catalase addition to the media. These results provide evidence that the responses to the catalase preparation used specifically reflect its H_2O_2 scavenging activity.

With extracellular catalase treatments, all of the cell cultures tested exhibited a decrease in proliferation without induction of apoptosis. Cells were capable of reentering the cell cycle upon removal of catalase after up to 2 days of treatment; with longer incubations cells lost the ability to proliferate, and eventually died. As cells remain trapped in the late stages of the cell cycle, apoptosis may then be triggered by a "mitotic crisis". As well, although proliferation was inhibited by catalase, cell size increase did not appear to be affected. A key process for cell size progression is the accumulation of enzymes required for RNA translation machinery; signalled primarily by the ribosomal S6 kinase pathway (Thomas 2000). It appears that reduction of H_2O_2 in cell culture can act to block the activities of cell cycle machinery without effects upon other gene expression, however this phenomenon was not studied further. Increases in ROS levels that can damage macromolecules such as DNA are known to induce cell cycle arrest at the G1/S and G2/M phase transitions, by p53-dependent and –independent mechanisms that are not fully understood (Shackelford *et al.* 2000; Kastan 2001). It is intriguing that the reduction of "basal" levels of ROS can produce similar effects. Burdon (Burdon 1995) has proposed that H_2O_2 acts as a paracrine signal in cell culture to allow for proliferation, and removal of this stimulus has the same effect on culture growth as lowering cell density. Indeed, in our Rat-1 clones the rate of proliferation correlated with the amount of H_2O_2 production. However, we observed the highest levels of H_2O_2 generation by SK-OV-3 cells, which proliferate relatively slowly compared to the

fibroblast lines tested. Thus, inherent differences between cell lines appear to regulate H_2O_2-mediated responses.

Observation of mammalian cell culture responses to redox changes are not a true reflection of what may happen *in vivo*, as these cells are exposed to constant hyperoxia as compared to their natural tissue environment. In fact, it is proposed that dysfunction of the senescence program in immortalized cell lines may be a reflection of cell tolerance to increased oxidative stress (Lundberg *et al.* 2000). Interestingly, inappropriate growth factor signalling and activation of mitogenic oncogenes can also induce cell senescence (Serrano *et al.* 1997). In this manner ROS have the same effects as growth factors. If a cell gains resistance to the growth arrest and/or apoptotic effects of these stresses, then the outcome of these signals may be proliferation. Indeed, increased ROS production has been associated with tumour cell growth, migration and resistance to cytotoxic agents *in vitro* and in clinical studies (Oberley and Oberley 1997; Liu *et al.* 2001; Brown and Bicknell 2001). Therefore the resistance to chronic oxidative stress attributed to many immortalized cell lines in culture may in fact mimic *in vivo* tumour progression, where an increased H_2O_2 stimulus could become a selective advantage for clonal survival and growth.

It is likely that changes in the concentration of extracellular H_2O_2 will in turn affect intracellular redox status, as H_2O_2 can freely diffuse across cell membranes. Therefore, the effects of the catalase treatment used here are best described as a response to the alteration of the "pericellular" redox environment, that is both outside and inside of the plasma membrane. Certainly the fact that Neu receptor activity in Rat-1 cells was not

significantly altered after the addition of catalase while MAPK activity was (described below) indicates the direct manipulation of intracellular signalling molecules. These findings support a number of studies implicating cytosolic, plasma membrane-localized kinases and adaptor proteins in the initial molecular response to environmental H_2O_2 changes (Irani *et al.* 1997; Abe and Berk 1999; Migliaccio *et al.* 1999; Nishida *et al.* 2000; Nemoto and Finkel 2002). Subsequent work was not directed at further study of the initial effects of catalase treatment at the plasma membrane, but at the downstream responses of MAPK and SAPK activity.

4.2 The JNK Pathway as a Sensor of Cell Redox Balance

Our observation of the inhibition of Erk1/2 MAPK phosphorylation upon catalase addition (and upregulation observed with exogenous H_2O_2) to Rat-1NeuN cells agrees with the previous findings that demonstrate the responsiveness of the MAPK cascade to H_2O_2 (Stevenson *et al.* 1994; Guyton *et al.* 1996; Rao 1996). Constitutive activation of the Neu receptor in Rat-1 Neu8142 cells induced basal Erk1/2 activity and protected it from catalase-induced inhibition. Neu interacts with several signaling cascades including the Ras/Raf/MEK pathway, important in Erk1/2 regulation and mitogenesis (Reese and Slamon 1997). The rescue of Erk phosphorylation was accompanied by only a slight increase in resistance of fibroblasts to growth inhibition by catalase. This result indicates that while contributing to the cellular responses observed, Erk1/2 signal downregulation is not critical for them.

Unlike the Erk response, the stress-activated JNK1 signal was rapidly and transiently induced by treatments that both increased or decreased H_2O_2 in Rat-1NeuN

and Rat-1Neu8142 lines. However, the signalling mechanism(s) promoting this JNK activation in response to catalase have yet to be elucidated. JNK1 is known to be responsive to oxidative stress (Tibbles and Woodgett 1999; Yoshizumi *et al.* 2000). H_2O_2 is observed to induce JNK activity in a number of ways: through direct activation (via cysteine residue oxidation), activation of the MKK4- and MKK7-signalling Apoptosis Signal-regulating Kinase 1 (ASK1), inhibition of the JNK phosphatase M3/6, or by the Src pathway (Nemoto *et al.* 2000; Chen *et al.* 2001; Chen *et al.* 2001b; Zou *et al.* 2001). Our findings suggest an additional role for this signal in response to an antioxidant enzyme. The suppression of JNK activation by endogenous antioxidants such as glutathione-S-transferases (Adler *et al.* 1999; Cho *et al.* 2001) and thioredoxin (Saitoh *et al.* 1998) is documented, but we also report a novel response of transient JNK1 activation following catalase-mediated scavenging of H_2O_2. The JNK1 activation observed was transient; perhaps reduced ROS may initially induce JNK signalling but ultimately result in pathway downregulation.

Because of our findings with Rat-1 fibroblasts, we then tested this response in another cell type: SK-OV-3 epithelial cells, which produce a high level of H_2O_2 in culture that is required for their proliferation. Similar effects from catalase treatment were observed, however the response in this cell line differed somewhat from that seen in the Rat-1 model. High levels of exogenous H_2O_2 produced a more marked and stable JNK1 activation, but activation by catalase treatment was significantly reduced. Again, this provides evidence of cell-specific responses to redox changes. However, regardless of the lower JNK1 response in SK-OV-3 cells, upregulation of this signal did result in a

small but significant resistance to growth inhibition to reduction of H_2O_2 levels, and affected cell redox status (as discussed in the following section). These results suggest that although different cell types exhibit altered JNK pathway responses, this signal may be a general mechanism of the response to changes in ROS. It is not known whether catalase treatment can affect JNK1 activity directly (via H_2O_2 removal) , or perhaps via MKK7 upregulation. A block of the MKK4 signal to JNK1 by transient expression of dominant-negative HA-MKK4 in Cos-1 cells or by the use of MKK4-deficient mouse embryo fibroblasts did not significantly affect the cell growth arrest response to catalase. A slight potentiation of growth inhibition was observed after catalase treatment of Cos-1 cells expressing dominant-negative MKK7-AL. This suggests that the transient JNK1 activation observed upon H_2O_2 removal (and that may participate in growth arrest induction) may result from an upstream signal to MKK7. Further experiments are required in order to gain a conclusive understanding of the mechanisms for this JNK1 induction, however.

The association of JNK signalling with the regulation of endogenous cellular H_2O_2-generating systems is also reported. Rac1 and Cdc42, members of the Rho family of small GTPases involved in actin cytoskeleton regulation and cell transformation, have been shown to activate the JNKs through interaction with p21-activated kinase (PAK) (Coso *et al.* 1995; Minden *et al.* 1995; Tang *et al.* 1999). In addition, Rac1 is a cytoplasmic component of the plasma membrane-localized NAD(P)H oxidase complex that directs the extracellular generation of $O_2^{\cdot-}$ and its dismutase product, H_2O_2 (Quinn *et al.* 1993). Interestingly, the functioning of this complex appears to be essential not only

for ROS generation but also for cell survival and growth (Patterson *et al.* 1999; Shin *et al.* 1999). It follows that Rac may stimulate the JNK pathway through PAK as well as directly via H_2O_2 production.

Since our data demonstrate MAPK inhibition and SAPK activation after catalase treatment, it could be that loss of the H_2O_2 stimulus blocks the Ras pathway while inducing other JNK-dependent signals like PI3K and Rac. The PI3K and Rac1 signals are observed to be synergistic, however other evidence suggests that PI3K can also inhibit the Rac/JNK signal under certain circumstances: the survival-promoting PI3K target Akt kinase (protein kinase B) was shown to phosphorylate Rac1 at serine 71 and abolish its GTPase activity (Kwon *et al.* 2000). Treatment of our Rat-1 fibroblast lines with a 5 μM concentration of the PI3K inhibitor LY294002 inhibited cell proliferation to a comparable extent regardless of HER-2/Neu activity levels (not shown). Thus, changes to the PI3K/Akt signal may also contribute to the catalase-induced effects upon JNK1 observed, however this remains to be determined. It is the balance of mitogen- and stress-activated kinase pathway activities, as determined by genetic and environmental regulation, that determine the physiological outcome of a given cell response. Known mechanisms of communication between these pathways is illustrated in figure 39. As JNK is a regulator of cell proliferation, it may represent an important component of the growth-inhibitory response to "reducing" stress. It is clear that many signalling pathways in addition to the JNK signal can be affected by redox changes that may also regulate this response.

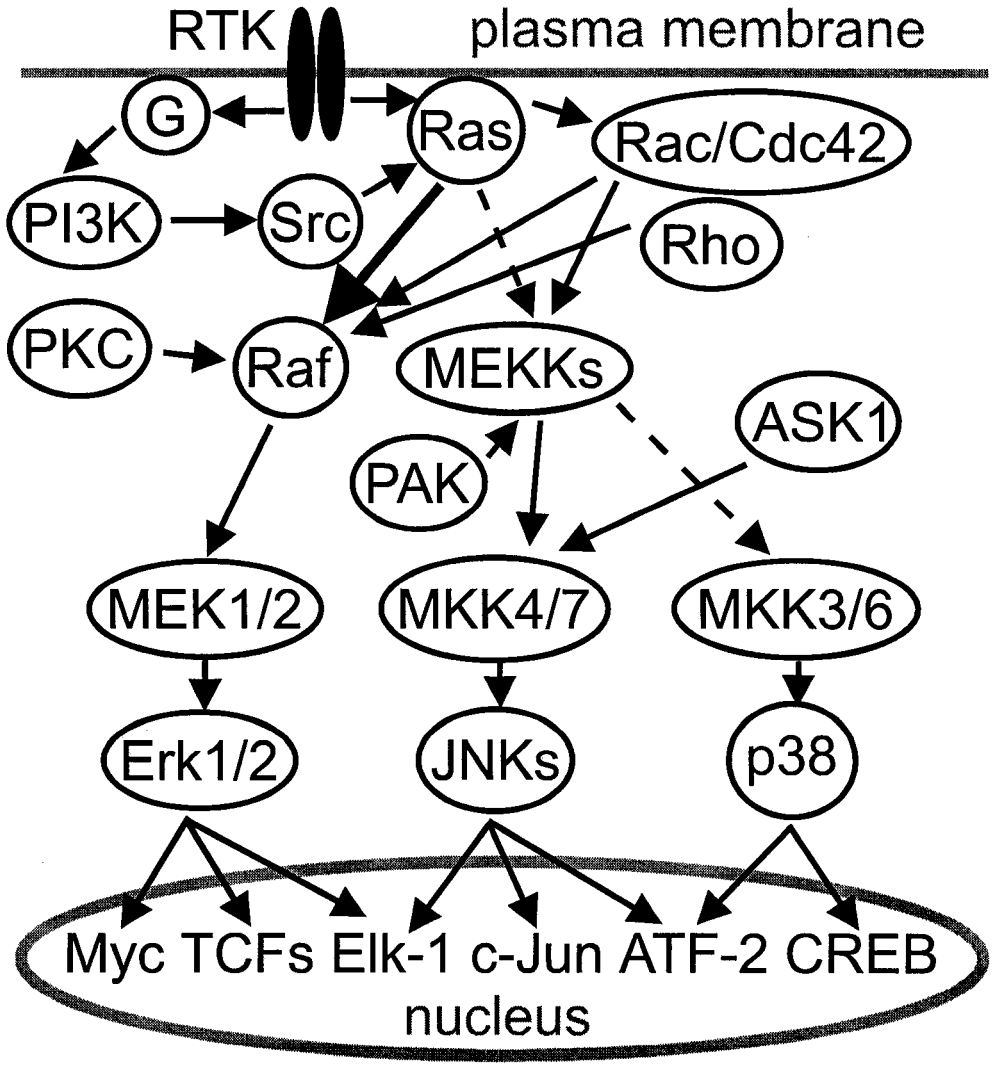


Figure 39: Mitogen- and Stress-activated Protein Kinase Signal Transduction Pathways Involved in Cell Proliferation.
Growth factors activate receptor tyrosine kinases (RTK), and these primarily transduce signals to mitogen-activated protein kinases (MAPKs; Erk1/2) and to a lesser extent the stress-activated protein kinases (SAPKs; JNK, p38 MAPK). Cell stressors are more potent inducers of SAPK activity. Upon activation by phosphorylation, MAPKs/SAPKs translocate to the nucleus and phosphorylate a number of transcription factors. Many of these are required for AP-1-mediated regulation of gene transcription (TCFs such as Elk-1, c-Jun, ATF-2). Myc activity can induce cell proliferation and apoptosis, and CREB can inhibit these processes. These transcriptional responses determine the cellular response to a given stimulus. Kinase/kinase interactions are simplified, and all known cross-talk between other signalling molecules is not shown. Hatched arrows represent weak or disputed interactions. G=heterotrimeric G proteins; TCF=ternary complex factor; CREB=cyclic AMP-responsive element binding protein.

4.3 JNK1 Signalling and Cellular ROS Production: Implications in Cell Proliferation and Cancer

Observation of the activation of JNK1 by the catalase-mediated scavenging of physiological concentrations of H_2O_2 led us to investigate if alteration of the JNK1 signal could affect the anti-mitotic response to this treatment. Using a SK-OV-3 human ovarian carcinoma cell line model, we found that JNK1 activity can partially influence resistance to catalase activity, and can also itself downregulate cellular H_2O_2 generation. It is this function of JNK that may participate in its well-characterized ability to mediate cell growth inhibition. Initial experiments of transient ectopic JNK1 expression in Cos-1 cells showed that increasing JNK1 levels potentiated the catalase-induced proliferative block, supporting the hypothesis that JNK1 may be one of the molecular mediators of this response. To our surprise, however, stable expression of ectopic JNK1 in SK-OV-3 cells actually caused a partial increase in resistance to the effects of catalase treatments. Subsequent experimentation indicates that this results from an acquired insensitivity to the JNK1 signal itself. Due to the toxic effects of JNK pathway activation this could be expected, as stable JNK1-expressing clones may be able to survive only if other cell alterations have allowed them to adapt to constitutive amplification of this signal.

The precise role of JNK in cell cycle regulation remains to be elucidated. JNK activity is known to induce p53-mediated signalling in certain cell models, which is capable of causing cycle arrest in both G1/S and G2/M, or apoptosis (Leppä and Bohmann 1999; Tibbles and Woodgett 1999; Davis 2000). c-Jun, the major downstream target of JNK, contributes to a number of contrasting biological processes such as cell

growth regulation as part of the AP-1 transcriptional complex (Leppä and Bohmann 1999). Because JNK activity is involved in a number of cell responses, the outcome of this activity is likely dependent upon cross-talk with other intracellular signals. For example, the degree of SAPK activity balanced with other AP-1-inducing signals such as the Erks could determine progression through the cell cycle. In our Rat-1 fibroblast model, Erk inhibition and JNK activation were observed with cell growth arrest.

There is a large amount of evidence implicating the stimulation of mitogenic pathways as the mechanism of H_2O_2-induced cell growth (for review see Burdon 1995; Lander 1997; Kamata and Hirata 1999; Rhee 1999; Gopalakrishna and Jaken 2000). We have proposed that maintenance of basal H_2O_2 levels may also allow for mitogenesis via the repression of growth-inhibitory stress signals such as JNK (Preston *et al.* 2001). Some stress-induced signalling pathways have also been implicated as cell survival responses, and there is also evidence for the JNK signal as a pro-survival factor. As NF-κB activation is a target of the JNK pathway, particularly during cell responses to redox changes, this may mediate resistance to oxidative stress (Lee *et al.* 1997; Das 2001). Increased JNK, c-Jun and AP-1 activities are proposed to induce the resistance of prostate and breast tumours to the toxic effects of anticancer drugs such as staurosporine, vincristine and tamoxifen (Schiff *et al.* 2000; Chang 2001; Lafarge *et al.* 2001). In certain cancer cells, it is proposed that this acquisition of chemoresistance is accomplished by JNK pathway-mediated induction of the expression of multidrug resistance protein efflux pumps (Cripe *et al.* 2002). Conflicting data regarding JNK signalling and cell survival is also reported. For example, one study (Qu *et al.* 2002)

indicates that the process of malignant transformation *in vitro* requires resistance to apoptosis via JNK1/2 downregulation. It may be that in non-transformed cells, JNK activity induces growth arrest and death, but upon transformation, resistance to JNK upregulation by alteration of the activity of downstream stress and apoptosis signalling molecules causes resistance to certain chemotoxic agents. An alternate relationship appears to exist in the context of pro-apoptotic JNK signalling, with the survival-associated NF-κB and protein kinase B (PKB/Akt) pathways cooperating to downregulate JNK activity (Madrid *et al.* 2000; De Smaele *et al.* 2001; Park *et al.* 2002). Therefore, it is apparent that JNK1 regulates cell responses to stress in conjunction with other signals like NF-κB and Akt.

Studies by Wisdom *et al.* (Wisdom *et al.* 1999) indicate that JNK-mediated phosphorylation of c-Jun is specific for stress responses only, while c-Jun involvement in the stimulation of cell proliferation is triggered by other factors. Interestingly, other researchers have demonstrated that the JNK signal can prolongue the cell cycle at S and G2/M phases in a p53-independent manner (Yamamoto *et al.* 1999). In one study JNK1 activity was found to be required for S phase arrest caused by the protease inhibitor *N*-acetyl-leucinyl-leucinyl-norleucinal which acts to prevent Cyclin B degradation (in addition to other effects) (Tchou *et al.* 1999). Therefore one mechanism of JNK1-induced growth arrest may be via the regulation of Cyclin B/Cdk complex stability. Indeed, we observe an increase of the proportion of Rat-1 fibroblast clones in S and G2 phases after incubation with catalase. Although this cannot be solely attributed to the

transient induction of JNK1 activity seen upon this treatment, JNK1 was later observed to influence this antiproliferative response Cos-1 and SK-OV-3 cells.

A discrete range of JNK activation may act as a cell sensor of oxidative and reducing stresses, leading to a response of growth arrest and/or death. We propose that one mechanism by which JNK1 inhibits cell proliferation is via the downregulation of the endogenous H_2O_2 stimulus. As previously discussed, Jun family targets of JNK activity have been demonstrated to participate with Nrf2 in ARE-mediated transcriptional responses to oxidative stress. Although the transcriptional regulation of genes encoding H_2O_2 scavenging enzymes like catalase and glutathione peroxidase is not shown to be mediated by ARE elements, they are responsive to altered ROS conditions. It is also possible that JNK1 may stimulate the expression of these enzymes by AP-1-mediated transcription, or indirectly via the upregulation of NF-κB activity (Das 2001; Zhou *et al.* 2001). However, ectopic HA-JNK1 expression and increased c-Jun activation in our SK-OV-3 model did not correlate with increases in H_2O_2-scavenging antioxidant enzyme expression. Indeed, catalase expression was diminished under these circumstances, most likely as a cellular adaptation to rescue basal H_2O_2 levels. Increased synthesis of glutathione is reported to be caused by ARE-mediated gene expression (Jeyogupal and Jaiswal 2000), but in the SK-OV-3 JNK1 clones no increased GSH levels were observed. Therefore it may be that the H_2O_2 downregulation function of JNK1 is mediated by other mechanisms than the activation of AP-1-, Nrf2/Jun- or NF-κB-induced transcription of antioxidant genes. The expression levels and activities of Hsps and MTs in the SK-OV-3 JNK1 clones was not investigated; it could be that alteration of the JNK signal induced

other diverse stress-responsive factors such as these, resulting in a decreased H_2O_2 generation.

Our present findings not only support a model for JNK regulation by both stressful increases in, or the removal of basal levels of, H_2O_2, but they also indicate that JNK1 can itself alter cellular H_2O_2 production. Therefore the JNK pathway may possibly function as a mechanism to either maintain redox homeostasis upon toxic increases in environmental H_2O_2, with the side effect of growth inhibition. Alternatively, upon reduction of the basal levels of H_2O_2 necessary for cell proliferation, JNK may further reduce this stimulus, thereby signalling the cell to arrest and die. A block of the SAPK/JNK pathway is known to suppress cell growth arrest and death induced by high levels of H_2O_2 (Kim *et al.* 2001), and we have observed a similar effect in the alternate context of H_2O_2 removal. Unresponsiveness to the JNK1 signal, as displayed by the stable HA-JNK1-expressing SK-OV-3 clones, may therefore induce resistance to the effects of environmental redox changes. This has also been observed with the JNK response to other cell stresses such as cisplatin and tumor necrosis factor treatments, where sustained JNK pathway activation results in a resistant phenotype (Hayakawa *et al.* 1999; Tobiume *et al.* 2001). As JNK1 activity lowers the cellular production of H_2O_2, it follows that cells which have adapted to grow efficiently with an increased JNK1 signal should display a lowered requirement of the H_2O_2 stimulus for proliferation. This may account for the upregulation of JNK1 expression and activity observed in certain cancers.

4.4 Future Studies

One critical focus of further study is to elucidate the mechanism(s) by which JNK activity can inhibit cellular H_2O_2 generation. Investigation of gene transcriptional changes induced after the scavenging of extracellular or intracellular H_2O_2, or after JNK pathway manipulation (expression of ectopic JNK1, pharmacological activation or inhibition of JNK) can be undertaken in order to gain a better understanding of the relationship between these signals. Our laboratory has experience using reagents and scanning equipment from the Ontario Cancer Institute (OCI) microarray facility, and from a number of commercial sources. It is hoped that these experiments would allow for insight into JNK1-mediated trends in gene transcription that could account for a reduction of H_2O_2 levels. If the expression of HA-JNK1 produces no significant effects upon the transcription of genes associated with cell redox regulation, this would suggest a model of JNK1 regulation of ROS production through direct interaction with ROS-generating complexes. One way that JNK is thought to regulate apoptosis is by direct interaction with mitochondria (Davis 2000). Perhaps by another unknown mechanism, JNK may also lower ROS generation at this site. Mitochondrial electron transport chain inhibitors such as antimycin A and rotenone can be utilized to study the contribution of mitochondrial-generated ROS in the response to JNK1 activity. Additionally, the small GTP-binding proteins Rac1 and Cdc42 have been demonstrated to regulate JNK1 activity (Coso *et al.* 1995). As described, Rac1 is a component of the plasma membrane-localized NAD(P)H oxidoreductase complex, and can affect cell proliferation via the regulation of ROS generation from this site (Joneson and Bar-Sagi 1998; Arnold *et al.*

2001; Arbiser *et al.* 2002). Any putative role of this ROS-generating system in the regulation of H_2O_2 production by JNK1 can be assessed upon inhibition of its activity with drugs such as capcaisin and silymarin. To investigate the specific function of Rac1 in this process, we can control its activity by the use of dominant-active and dominant-negative mutant Rac1 expression vectors (Upstate Biotechnology).

Although JNK1 appears to modulate the effects of decreased H_2O_2, our findings indicate that there must be other signals controlling this response. The involvement of other components of SAPK signalling as well as other stress-induced pathways is probable. It would be interesting to study cell survival signals from Akt (protein kinase B) and NF-κB in relation to the H_2O_2 stimulus. The serine/threonine protein kinase Akt responds to survival signals (growth factors, nutrients, normoxia) while the NF-κB transcription factor responds to stress signals, and both have been shown to negatively regulate JNK1 (Madrid *et al.* 2000; De Smaele *et al.* 2001; Park *et al.* 2002). Interestingly, NF-κB itself is activated by JNKs in response to cellular redox changes (Lee *et al.* 1997; Das 2001). The contribution of these signalling pathways to JNK1, H_2O_2 and mitogenic regulation can be assessed by molecular manipulation, such as using wild type and mutant Akt1, and Inhibitor of NF-κB (IκB) expression vectors (Upstate Biotechnology). This research may lead to a better understanding of how the deregulation of these pathways contributes to cell transformation.

The specific locations of endogenous H_2O_2 generation that are most critical for cell growth regulation have previously been studied in our laboratory. It was found in an ovarian carcinoma cell line that mitochondria were responsible for approximately 40% of

H_2O_2 production, and that inhibition of this production reduced cell proliferation (unpublished observations). Of course the most prominent sources of mitogenic ROS generation may vary in a cell type-specific manner. It may be that specific molecular responses, like the JNK1 response, to modulation of H_2O_2 levels may be differentially affected depending upon the compartment(s) from which this signal arises. The relative importance of mitochondrial-generated H_2O_2 in H_2O_2-mediated signalling versus other sites of production remains unclear. In our experiments, scavenging of extracellular H_2O_2 affects cells profoundly. However, as H_2O_2 can diffuse freely across the plasma membrane, this catalase treatment may also be acting as a "sink", thereby decreasing intracellular H_2O_2 levels as well. To address this question, we can target catalase expression to cytosolic, mitochondrial, and extracellular compartments, and compare cell growth responses. We have constructed a FLAG-tagged catalase expression vector (pFLAG-Cat-CMV-2) and achieved expression of functional protein in the cytosol. This expression can also inhibit cell proliferation. Additionally, a proopiomelanocortin (POMC) signal sequence that targets protein products for extracellular secretion has been obtained from Dr. A. K. Grover. By comparison of induced expression of catalase in the cytosol or outside the cell, we can observe the crucial locations of H_2O_2 activity. The addition of a mitochondrial-directed signal sequence such as the manganese SOD leader sequence can also be utilized for further investigation of a specific mitochondrial contribution to ROS signalling.

It is not known how increased catalase activity can be correlated with tumour progression *in vivo*; there is evidence for decreased antioxidant levels in cancer (see

introduction), however this is not always observed, and the antioxidant systems involved may vary. Transgenic mice with tissue-specific (e.g. liver, myocardium) expression of ectopic catalase have been generated, and a similar model could be used to investigate a putative relationship between catalase activity and *in vivo* tumourigenesis. No catalase knockout mice exist at this time, however mouse lines with JNK pathway deficiencies are available and may be utilized in order to observe both changes in tissue H_2O_2 production and cancer cell proliferation.

5.0 References

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6.0 Appendix

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