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CHARACTERIZATION OF THE AP-1 AND NF-KAPPA B TRANSCRIPTION FACTORS IN THE U-87 MG ASTROCYTOMA CELL LINE

by

Denise L. Smith

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Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Biological Sciences
Advisor: Bruce Bejcek, Ph.D.

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Denise L Smith

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CHAPTER I

INTRODUCTION

Cancer

Historically, cancer incidence increases with modernization and wealth, as well as with increasing age of the population. Little is known prior to recorded history, but a skeleton of *Australopithecus* or *Homo erectus* with cancer has been found suggesting that cancer was problematic even 500,000 to 1,000,000 years ago. The advanced medical practices as well as written records of the ancient Egyptians have left a large amount of information as well as mummies with cancer. The earliest documentation came from the ancient Egyptian Eber and Smith medical papyri which described non-treatable breast, stomach and uterine cancer as tumors against the gods (Nunn, 1996).

The rise of the ancient Greek civilization 1100 years later saw further advancement in cancer knowledge. Hippocrates described the crab leg-like veins found in breast tumors with the Greek word for crab, karkinoma, as well as distinguished between benign and malignant tumors (Bishop & Weinberg, 1996). By the 4th century AD, Galen and other contemporary physicians believed that cancer was an infectious disease caused by an imbalance of the body's four vital fluids; blood, phlegm, yellow and black bile, and hospitals were established to isolate patients (Varmus & Weinberg, 1993). Yet, it would take another 1500 years before

researchers would begin to unravel the complicated disease that is cancer.

The 18th century saw the first link to a cancer cause hypothesized. In 1761, Londoner John Hill believed that inhalation of snuff might cause nasal cancer and Percival Pott in 1775 realized that men who were London chimney sweeps as boys were prone to cancer of the scrotum later in life (Varmus & Weinberg, 1993). Thus, the beginning of cause and effect was established. By the 19th century, the publication of works by Mendel and Darwin opened the door to a genetic approach to cancer. In 1866, Broca described the first genetic link to cancer when he described a family containing several members with breast or liver cancer and Warthin in 1895 described familial cancer as inherited in a Mendelian autosomal dominant manner (Bast et al., 2000).

The 20th century was pivotal, showing significant strides in the cancer knowledge base with laboratory experimentation, the cornerstone to understanding cancer, gaining momentum during this century. Prior to this, most of the knowledge about cancer was developed from observation with little experimentation. A 1907 epidemiological study showed that the incidence of cancer was higher in meat eating Germans, Irish and Scandinavians when compared to pasta eating Italians and rice eating Chinese living in Chicago (Varmus & Weinberg, 1993) suggesting a lifestyle component to cancer.

The study of carcinogenesis and mutagenesis started in the previous century continued to develop during the 20th century. Experimentation on rabbits and mice revealed that skin cancer was caused by x-rays and coal tar placed on the ears and

further work by H. J. Muller revealed that x-rays were mutagenic as well as carcinogenic in *Drosophila*. As further work developed suggesting a link between carcinogens and mutagens, the theory that all external carcinogens might be mutagens was established. With the development of this theory, the identification of mutagens became a major research focus and a quick test to identify mutagens was developed by Bruce Ames (Bishop & Weinberg, 1996). The Ames test had many negative results for suspected mutagens which further experimentation revealed metabolic conversion of the mutagen was required for activity (Bishop & Weinberg, 1996).

The principle of initiators and promoters in carcinogenesis was established by Isaac Berenblum (Varmus & Weinberg, 1993). He found that cancer was a multi-step process and that two types of insults to the cell had to occur, neither of which had the ability to cause cancer alone. The first step, initiation, consisted of a single exposure to a mutagenic agent, usually a polycyclic aromatic hydrocarbon. The second step, promotion, required numerous exposures to another agent that promoted the growth of the tumor or the conversion from a benign to a cancerous tumor. He believed that the promoter from the second step stimulated the growth of the initiated cells.

The rapid advancements in cellular biology during the 21st century led to a greater understanding of cancer and the study of cytogenetics revealed a link between genetic anomalies and cancer. In 1914 Theodor Boveri stated that cancer was caused by abnormal loss or gain of chromosomes (Bishop & Weinberg, 1996), and other genetic anomalies such as the translocation seen in the Philadelphia chromosome were found.

Viral experimentation that developed during the 21st century revealed a link between viruses and cancer which lead to the study of tumor viruses. In 1911 Peyton Rous described a virus which caused sarcomas in chickens (Varmus & Weinberg, 1993), and in 1960 the simian vacuolating virus 40 (SV40), which is an important cancer model and research tool was discovered. The use of cell culture became more common and it was shown by Hlozanek and others that normal cells could be transformed by several tumor viruses such as Rous sarcoma virus (RSV; 1969). Work since this time has focused on the molecular players in cancer (Table 1).

Table 1

Major Gene Classifications Involved in Cancer and Examples (Aggarwal, 2006).

Gene Type	*Description*	*Examples*
Oncogenes	Promote abnormal cell growth/division	PDGF, Ras, c-myc, c-Rel, c-Fos, c-Jun
Tumor suppressor: Caretaker	Control the maintenance of genetic information	PTEN, BRCA1, BRCA2
Tumor suppressor: Gatekeeper	Directly regulate tumor growth	pRb, p53, NF1
Tumor suppressor: Landscaper	Have an indirect effect on pre-cancerous cells	SMAD4
Angiogenesis	Promote new blood vessel formation	VEGF, PDGF
Invasion & Metastasis	Allow the cancer cell to invade and colonize new tissue	PI3K/Akt/IKK pathway

All cancer cells have several characteristics in common. They are self-sufficient in growth factors, insensitive to anti-growth signals, able to evade apoptosis, able to repeatedly replicate, able to promote and sustain angiogenesis, and able to invade and metastasis (Hanahan & Weinberg, 2000). All of these characteristics have been linked to platelet-derived growth factor.

Platelet-derived Growth Factor

Platelet-derived growth factor (PDGF) is a family of molecules that bind with two membrane bound receptor tyrosine kinases (RTKs). These RTKs phosphorylate proteins which cause functional changes of these proteins such as location, enzyme activity levels, and/or association with other proteins. It is this kinase ability that makes PDGF and its receptors so powerful, providing the starting location of many signaling pathways. PDGF was first identified in whole blood serum (Kohler, 1974; Ross et al., 1974; Westermark & Wasteson, 1976) and is the major growth factor in human serum (Fenstermaker et al., 1993). It has been implicated in numerous cancers that the PDGF subunits B, PDGFRα, and PDGFRβ are oncogenes.

PDGF Structure and Processing

Active PDGF exists as a disulfide-bonded dimer of five different isoforms, the homodimers AA, BB, CC, DD and the heterodimer AB. Each of the four chain (A, B, C and D) mRNAs varies in structure and fall into two groups; A- and B-chains in one group, and C- and D-chains in the other. The A- and B-chain mRNAs have seven exons with exons 4 and 5 containing the mature protein sequences while exon 6 contains a C-terminal sequence that is proteolytically processed for removal except in the A-chain where it can remain to create two splice variants (Johnsson et al., 1984; Bonthron et al., 1988; Rorsman et al., 1988). The C-chain has six exons and the D-chain has seven with exons 2 and 3 containing the CUB (Complement subcomponents Clr/Cls Urchin EGF-like protein and Bone morphogenic protein-1) domain while exons 5 and 6 in the C-chain or exons 6 and 7 in the D-chain encode

the mature growth factor protein.

Each chain is processed differently to produce the mature protein. The A-chain is cleaved by furin at RRKR86 (Siegfried et al., 2003; Reigstad et al., 2005) to create the 30 kDa mature AA homodimer which is then exported from the cell. Processing of the B-chain cleaves the protein at RGRR81 by an unknown enzyme which is probably convertase (Robbins et al., 1983; Beckmann et al., 1988; Bywater et al., 1988; Ostman et al., 1988; Fredriksson et al., 2004a) creating a secreted 30 kDa protein. There is also a 24 kDa B-chain made up of proteolytic N-terminal fragments that is retained within the cell. The addition of C-terminal extensions creates a longer A- and B-chain isoforms. The C- and D-chain mRNAs have a two-domain organization consisting of an N-terminal CUB domain and a C-terminal growth factor domain. The C- and D-chains are excreted by the cell in an inactive form and later processed to remove the CUB domain extracellularly to activate the 31 and 50 kDa proteins, respectively. Cleavage of the CUB domain in the C-chain occurs at RKSR234 by plasmin (Li et al., 2000a; Gilbertson et al., 2001) or tissue plasminogen activator (Fredriksson et al., 2004b) depending on cell type, while the D-chain is cleaved at RKSK257 by plasmin (Bergsten et al., 2001) or RGRS250 by urokinase plasminogen activator (Reigstad et al., 2005; Ustach, 2005). It is unclear of the specific role of the CUB domain but is believed to be responsible for protein-protein and protein-glycosaminoglycan interactions.

The two members of the dimer exist in anti-parallel orientation and are joined by three disulfide bonds, which allow the dimer to fold into the highly conserved

cystine knot structure which is characteristic of this family of growth factors. Each mature chain member ranges between 196-370 amino acids in length with 60% sequence similarity, of which the greatest conservation of sequence occurs within the cystine knot region (Heldin et al., 1998; Heldin et al., 1999a) suggesting the importance of the cysteine knot structure.

For proper functioning, the chains have regions of specific activity. There is a C-terminal basic region in all chains except D which interacts with extracellular matrix molecules such as heparin (Lustig et al., 1996) and collagen (Lustig et al., 1996; Somasundaram & Schuppan, 1996) allowing the producing cell to retain the chain. Unlike the other chains, the D-chain has no such sequence. There is a cationic region in chains A and B which is required for receptor binding and mitogenic activity, while the C- and D-chains require the removal of the CUB domain for receptor binding and mitogenic activity to occur.

PDGF Receptor Structure

The PDGF α and β receptors contain five immunoglobulin-like extracellular domains and a split kinase domain in the cytoplasmic region making them class III receptor tyrosine kinases. The two receptors are transcribed into approximately 120 and 125 kDa proteins, respectively and are glycosylated to form the mature 170-180 and 170-185 kDa proteins, respectively. In the un-stimulated cell, the receptor exists as a monomer which non-covalently dimerizes upon ligand binding and both members of the dimer become activated in the case of homodimers and in some heterodimeric situations. The various binding affinities of the PDGF isoforms allow

for novel properties to emerge.

The receptor is divided into four domains, signal peptide, cytoplasmic, transmembrane, and extracellular (Figure 1).

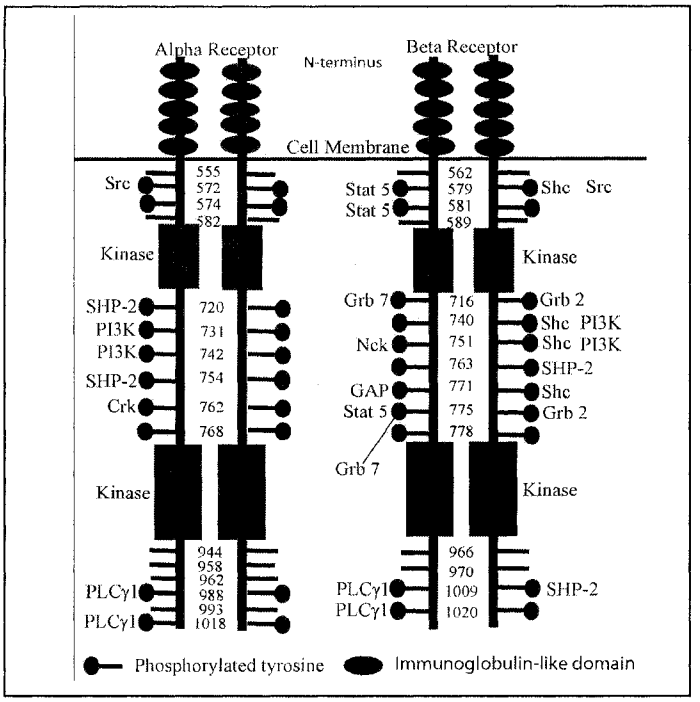


Figure 1. PDGF Receptor with Binding Locations for Signaling Molecules. Numbers indicate tyrosine residue positions.

The extracellular binding segment of the receptor consists of five immunoglobulin-like domains with each domain playing a specific role in binding, dimerization and signal transduction. The three N-terminal domains are disulfide-bonded creating loops that are involved in ligand binding, two and three are required for ligand binding (Heidaran et al, 1990) with the second domain the most important (Mahadevan et al., 1995; Lokker et al., 1997; Miyazawa et al., 1998). Domain four is

involved in receptor-receptor dimerization which helps to stabilize the ligand-receptor complex (Lokker et al., 1997; Shulman, et al., 1997; Omura et al., 1997) and domain five is required for signal transduction not ligand binding (Lokker et al., 1997; Omura et al., 1997). The ligand binding domains have varying affinities for the different PDGF chain dimers. Domains one through three determines high affinity binding of the ligand and it has been shown that the AA homodimer binds to domains two and three with higher affinity than the BB homodimer (Heidaran et al., 1990) while the BB homodimer binds to domains one and two with higher affinity than the AA homodimer (Miyazawa et al., 1998). Extracellular domain one is required for proper orientation of the AA homodimer (Mahadevan et al., 1995). The PDGF AA homodimer only binds with the $\alpha\alpha$ receptor, the AB heterodimer binds with the $\alpha\alpha$ and the $\alpha\beta$ receptors, the BB homodimer binds with the $\alpha\alpha$, $\alpha\beta$ and the $\beta\beta$ receptors, the CC homodimer binds with the $\alpha\alpha$ and the $\alpha\beta$ receptors, and the DD homodimer binds with the $\alpha\beta$ and the $\beta\beta$ receptors.

The transmembrane region consists of approximately 25 hydrophobic amino acids. The intracellular portion of the receptor consists of a split tyrosine kinase domain separated by approximately 100 hydrophobic amino acids (Claesson-Welsh, 1994) and throughout the intracellular portion are tyrosine residues that when phosphorylated become docking locations for various signal transduction molecules.

Consequences of Ligand Binding

Upon ligand binding, the monomeric receptors dimerize causing activation of

the receptor which is one of the earliest results of ligand binding. Once the receptors dimerize, autophosphorylation of tyrosine residues occurs in *trans*, providing locations for signal transduction molecules containing Src homology 2 domains (SH2) to bind. In the receptor, up to eleven tyrosines can become phosphorylated which provide up to ten locations for signaling molecules to bind. Activation of the kinase also occurs during the autophosphorylation of Tyr 849 in the α receptor and Tyr 857 in the β receptor (Fantl et al., 1989; Kazlauskas & Cooper, 1989). Interestingly, at high receptor concentrations autophosphorylation is not dependent upon ligand binding to the receptor leading to an autocrine signaling pathway (Herren et al., 1993).

Expression of PDGF and Receptors

PDGF is a ubiquitous molecule found to stimulate or be secreted via a paracrine signaling pathway by many cell types. It has been shown to be secreted by activated macrophages (Shimokado et al., 1985), endothelial cells (Harlan et al., 1986), smooth muscle cells (Ross et al., 1974; Walker et al., 1986), glial cells (Westermark & Wasteson, 1976), fibroblasts, (Kohler, 1974; Paulsson et al., 1987), and the cellular fragmented platelets (Shimokado et al., 1985; Bryckaert et al., 1989), while glial cells, fibroblasts, and smooth muscle cells are the major targets (Fretto et al., 1993).

Generally cells only express certain PDGF chains. For example the AB heterodimer is the major form found in human platelets (Hammacher et al., 1988),

while the CC homodimers is predominately found in angiogenic tissues (Cao et al., 2002). PDGF-A have been found in neurons (Yeh et al, 1991) while PDGF-A and PDGF–B have been found in glial cells (Shih & Holland, 2003). PDGF has also been found to be restricted to various stages of development. PDGF-A has been found in embryonic lung tissue mesenchyme (Bostrom et al., 1996) and PDGF-C has been found in epidermal openings of embryonic mice (Ding et al., 2000, Hamada et al., 2000, Li et al., 2000b, Aase et al., 2002).

Receptor isoform types also vary by cell type. The α-receptor only is found in platelets, astrocytes, glial precursors (Yeh et al., 1993), and postnatal neurons (Oumesmar et al., 1997). The β-receptor has been found in macrophages, T-lymphocytes, myeloid lineage hematopoietic cells, mammary epithelium, capillary endothelial cells (George, 2003), neurons and Schwann cells (Davis & Stroobant, 1990; Eccleston et al., 1990). Fibroblasts and smooth muscle cells have been shown to express both receptor types but have a higher number of β-receptors (Raines et al., 1990). These variations in PDGF chain and receptor composition determine the vast array of cellular responses attributed to PDGF. Interestingly in glioma cells, PDGF-A, PDGF-B, PDGFRα, and PDGFRβ are all present enabling PDGF to use autocrine signaling (Takeuchi et al., 2004).

PDGF in Normal Physiology

The physiological responses to PDGF are as varied as the cells that are the targets. One of the primary functions is wound healing and PDGF is released during blood clotting. PDGF has been found to be secreted by platelets upon adhesion to the

capillary endothelium and may be part of a feedback loop that controls platelet aggregation. Other important cellular roles for PDGF are proliferation (Seifert et al., 1989; Funa et al., 1990), angiogenesis (Dvorak, 1986; Plate et al., 1992), chemotaxis (Seifert et al., 1989; Claesson-Welsh, 1994; Khachigian et al., 1994), inhibition of apoptosis (Nikkhah et al., 1993; Simm et al., 1994; Bennett et al., 1995; Yao, 1995), increased metabolic rates (Seifert et al., 1989; Lokker et al., 1997), changes in cellular morphology (Lokker et al., 1997), and regulation of DNA synthesis (Ross et al., 1986; Aaronson, 1991; Yu et al., 1995). Many of the previous processes are involved in wound healing.

PDGF has also been found to be important during embryogenesis and cellular differentiation. It has been shown to play a role in the differentiation of glial cells into astrocytes and oligodendrocytes by stimulating the glial progenitor cells to divide until they are no longer responsive to PDGF which allows them to differentiate (Hart et al., 1989).

Individual isoforms of PDGF and receptor have been shown to have specific roles. PDGF-A stimulates the expansion of neuronal progenitors from neural stem cells as well as the partial differentiation of these cells (Heldin, 1999b; Demoulin et al., 2006) and it mediates chemotaxis, and anchorage independent growth (Beckmann et al., 1988; Matsui et al., 1989; Heidaran et al., 1991; Heidaran et al., 1993; Yu et al., 1995), while the B-chain has roles in proliferation (Shah & Vincent, 2005), chemotaxis, and angiogenesis (Reigstad et al., 2005). Knockout of the A- and B-chains causes embryonic death and illustrates the importance of these chains during

development.

The A- and B-chains have been studied for many years and a great deal has been discovered about these two chains, while the C- and D-chain discoveries are relatively recent and much less is currently known. The C-chain has been shown to play a major role in wound healing through inflammation, proliferation and remodeling (Li et al., 2000b; Grazul-Bilska et al., 2003; Fang et al., 2004; Jinnin et al., 2005; Li et al., 2005), while both chains have been found to play a role in angiogenesis (LaRochelle et al., 2002; Li et al., 2003a; Uutela et al., 2004).

Since the α-receptor binds with all isoforms except the D-D homodimer, the roles attributed to the α-receptor should show some overlap with the roles for the various chain isoforms. The α-receptor is responsible for brain development (Soriano, 1997; Clarke & Dirks, 2003), differentiation of oligodendrocyte precursor cells (Noble et al., 1988), and anchorage independent growth (Yu et al., 1995). The α–receptor has been found to have a role in early development and mice deficient in the α-receptor die during embryogenesis because of defects to the oligodendrocytes and alveolar smooth muscle cells. This suggests that the primary importance of the α-receptor is developmental. Again, some overlap of activities should be seen between the β-receptor and the B-B and D-D homodimers. The β-receptor has been shown to be responsible for angiogenesis (Risau et al., 1992; Claesson-Welsh, 1994), pericyte recruitment to capillaries, smooth muscle development in coronary vessels (Pietras et al., 2003), cellular proliferation, migration, survival, (Ross, 1999; Sano et al., 2001; Bergers et al., 2003) and cell cycle progression (Twamley-Stein et al., 1993; Barone

& Courtneidge, 1995). These processes reveal a much more diverse role for the β-receptor than the α-receptor.

Cell Signaling by PDGF

Various signal transduction pathways are activated through PDGF and because of extensive cross-talk it is difficult to sort out the different pathways. Known molecules that interact with phosphorylated tyrosine residues on both PDGF receptors include phospholipase C-γ (PLC-γ), tyrosine kinases Src and PI-3-K, the tyrosine phosphatase SHP-2. The adaptor Crk only binds to the α receptor, while adaptors Grb 2 and 7, Shc, and Nck, the tryrosine kinase guanosine triphosphase-activating protein (Ras-GAP), and the transcription factor family STAT only bind to the β receptor (Meisenhelder et al., 1989; Molloy et al., 1989; Cantley et al., 1991; Li et al., 1992; Feng et al., 1993). These various molecules provide starting points for the signaling pathways stimulated by PDGF.

Regulation of PDGF and Receptors

Regulation of PDGF can occur at many different levels. Regulation of PDGF chains can occur through expression levels varying over time, mRNA copy number, mRNA availability, translation time, processing time, and turnover time. It can also be regulated by varying splice form expression which can change binding affinity and the presence of specific proteases can determine which isoforms and/or splice variants of PDGF are available. Other cellular conditions can cause binding pattern changes

which allow some chains to bind while preventing others. Lastly, the expression of some receptors and not others determine which chains are allowed to bind. Regulation can also occur at the receptor level. PDGF receptor numbers do not remain constant and can vary from 10,000 to 1,000,000 present on the cell surface and in concentrations within caveolae (Anderson et al., 1992; Anderson, 1993; Sargiacomo et al., 1993; Chang et al., 1994; Shenoy-Scaria et al., 1994; Bishop & Weinberg, 1996; Liu et al., 1996a). Regulation of the activated receptor can occur via internalization of the receptor-ligand into endosomes which fuse with lysosomes for degradation or dissociation of the ligand from the receptor and return of the receptor to the cell membrane. Regulation can also occur via cytoplasmic degradation in proteasomes after ubiquitination. The process of internalization is dependent upon the activity level of the kinase in the receptor (Sorkin et al., 1991) and the interaction of the receptor with PI3-kinase (Joly et al., 1994). Negative regulation occurs through the phosphatase SHP-2 which de-phosphorylates the tyrosine residues of PDGF (Klinghoffer, 1995).

Specific regulation of the A- and B-chain occurs through the zinc finger transcription factors Egr1 and Sp1 (Khachigian et al., 1995) as well as the transcription factor Kruppel-like factor 5 (Aizawa et al., 2004). Additional regulation of the B-chain is a result of both positive and negative signals as well as the ratio of PDGF isoforms and the receptors present. Regulation occurs within the gene by an approximately 1kb long 5' un-translated region in the B-chain mRNA that acts as a translational inhibitor (Heldin et al., 1999a), while the promoter can be regulated by

activators of protein kinase C (Khachigian et al., 1994). External sources such as the tumor suppressor gene $p19^{ARF}$ have also been shown to negatively regulate PDGF (Silva et al., 2005).

Transcription factor binding helps to regulate both α- and β-receptors. The α-receptor promoter has a binding site for the transcription factor GATA-4 (Wang & Stiles, 1994; Afink et al., 1995; Kawagishi et al., 1995) and the β-receptor promoter's binding site is for transcription factor nuclear factor Y (Ballagi et al., 1995; Ishisaki et al., 1997). Lastly, a few chemicals have been shown to have regulatory effects on PDGF. It was shown that the anti-oxidant epigallocatechin-3-gallate (EGCG) interferes with PDGF signaling (Chen, 2003) and ellagic acid inhibits PDGF-induced phosphorylation of the receptors in smooth muscle cells (Labrecque et al., 2005).

PDGF, Its Receptors, and Disease

PDGF has been implicated in a number of disease processes other than cancer (Table 2). These diseases appear to be caused by over-expression, constitutive activation and/or autocrine or paracrine loops of PDGF and/or the receptors. The abnormalities in PDGF have also been shown to play a role in the transformation of normal cells into malignant ones. PDGF transformation of cells requires either phospholipase Cγ or PI-3K (DeMali et al., 1997) and utilizes the ras/MAPK pathway (Rosenmuller et al., 2001; Reigstad et al., 2005). Numerous cancers have been found to have PDGF abnormalities (Table 3).

Specific roles in cancer have been linked to specific chains and/or receptors. The A-

chain has been found in numerous transformed cell lines and cancers (Table 4), but only plays a minor role in cancer and transformation. The simian sarcoma virus encodes the v-sis gene product that has been implicated in cellular transformation. The v-*sis* gene is the homolog of the c-*sis* proto-oncogene encoding the PDGF B-chain (Devare et al., 1983; Doolittle et al., 1983; Clarke et al., 1984; Bejcek et al., 1992; Varmus & Weinberg, 1993).

Table 2

Diseases Other than Cancer with a PDGF Role.

Disease	*Reference*
Atherosclerotic plaques	Ross et al., 1986; Barrett & Benditt, 1987; Libby et al., 1988; Rubin et al., 1988; Wilcox et al., 1988; Libby et al., 1989; Ross et al., 1990; Fenstermaker et al., 1993; Ferns et al., 1991; Fretto et al., 1993; Herren et al., 1993; Raines & Ross, 1993; Ostman, 2001; He et al., 2003
Post angioplasty restenosis	Johnson et al., 1990; Majesky et al., 1990; Raines & Ross, 1993; Ross, 1993; Kanzaki et al., 1994; Khachigian et al., 1994; Yu et al., 1995; Uchida et al., 1996; Lokker et al., 1997; Kaetzel et al., 1998; Liu et al., 2001; He et al., 2003; Fang et al., 2004
Rheumatoid arthritis	Rubin et al., 1988; Herren et al., 1993; Kaiser et al., 1998; Liu et al., 2001
Lung fibrosis	Martinet et al., 1987; Wangoo et al., 1993; Liu et al., 2001
Liver fibrosis	Pinzani, 1995
Kidney fibrosis	Iida et al., 1991
Inflammatory response	Yu et al., 1995
Bronchiolitis	Aubert et al., 1997; Bergmann et al., 1998; Liu et al., 2001
Glomerulomephritis	Gesualdo et al., 1991; Iida et al., 1991; Johnson et al., 1992; Fretto et al., 1993; Lokker et al., 1997

In sis transformed cells, PDGF binds to immature receptors in the endoplasmic reticulum and causes autophosphorylation allowing for upregulation of the β-receptor. It has been found that amino acids 105-144 in the PDGF B-chain is responsible for the transforming activity seen (Giese et al., 1990; LaRochelle et al.,

1990; Fenstermaker et al., 1993) and the B-chain has been found to be up to 100 times more efficient at transformation than the A-chain (Beckmann et al., 1988; Kim et al., 1994; Uren et al., 1996) but is insufficient for full transformation. B-chain has implicated tumorigenic and metastatic effects and many cancers have been shown to have aberrant B-chain activity (Table 5).

Table 3

Involvement of PDGF in Cancer.

Cancer type	*References*
Medulloblastomas	Black et al., 1996; Andrae et al., 2002
Fibrosarcoma	Pantazis et al., 1985; Liu et al., 2001
Melanoma	Barnhill et al., 1996; Liu et al., 2001; Loizos et al., 2005
Breast carcinoma	Bronzert et al., 1987; deJong et al., 1998; Liu et al., 2001; Loizos et al., 2005
Lung carcinoma	Antoniades et al., 1991; Liu et al., 2001; Schiffer, 2001; Uehara et al., 2003; Zhang et al., 2003; Takeuchi et al., 2004; Loizos et al., 2005
Esophageal carcinoma	Liu et al., 1996a; Liu et al., 2001
Osteosarcoma	Sulzbacher et al., 2000; Loizos et al., 2005
Stomach cancer	Schiffer, 2001; Uehara et al., 2003
Pancreatic cancer	Schiffer, 2001; Uehara et al., 2003
Prostate cancer	Fudge et al., 1994; Schiffer, 2001; Uehara et al., 2003; Loizos et al., 2005
Kaposi's sarcoma	Ralf et al., 1996; Liu et al., 2001
Ovarian cancer	Henriksen et al., 1993; Matei et al., 2001; Loizos et al., 2005; Wilczynski et al., 2005; Kumar et al., 2006
Basal cell carcinoma	Xie et al., 2001; Kumar et al., 2006
Gastrointestinal stromal tumors	Heinrich et al., 2003; Kumar et al., 2006
Ependymoma	Black et al., 1996; Heldin, 1999b
Meningioma	Nakamura et al., 1993; Heldin, 1999b
Pituitary adenomas	Leon et al., 1994; Heldin, 1999b
Capillary Hemangioblastoma	Heldin, 1999b
Mesothelioma	Heldin, 1999b; Filiberti et al., 2005
Testicular germ cell tumors	Heldin, 1999b
Midgut carcinoid	Heldin, 1999b
Gliomas	Takeuchi et al., 2004

Table 4

Cancers Where PDGF A-chain has a Role.

Cancer type	*Reference*
Osteosarcomas	Kaetzel et al. 1994; Heldin et al., 1999a; Bast et al., 2000
Medulloblastoma	Huber et al., 2001; Black et al., 1996; Heldin et al., 1999a
Ependymoma	Black et al., 1996; Heldin et al., 1999a
Meningioma	Heldin et al., 1999a; Yang & Xu, 2001b
Pituitary adenomas	Leon et al., 1994; Heldin et al., 1999a
Mesothelioma	Heldin et al., 1999a; Metheny-Barlow et al., 2001; Filiberti et al., 2005
Ovarian carcinoma	Heldin et al., 1999a; Apte et al., 2004
Breast cancer	Ginsburg & Vonderhaar, 1991; Heldin et al., 1999a
Prostate carcinoma	Kim et al., 1997; Heldin et al., 1999a
Chronic myelogenous leukemia	Kimura et al., 1995; Heldin et al., 1999a
Malignant melanomas	Barnhill et al., 1996; Heldin et al., 1999a; Bast et al., 2000; Lazar-Molnar et al., 2000
Glioblastomas	Heldin et al., 1999a; Bast et al., 2000; Clarke & Dirks, 2003
Non-small cell lung cancer	Shikada et al., 2005
Choriocarcinoma	Pedigo et al., 2005
Colorectal carcinoma	Mantur et al., 2008

PDGF C-chain has also been found in many malignancies and acts like the heterodimer AB which is the most mitogenic of all dimer isoforms. The C-chain has been found in medulloblastomas and Ewing sarcomas (Reigstad et al., 2005), as well as gliomas and glioblastomas (Lokker et al., 2002). The D-chain has also been found in gliomas and glioblastomas (Uutela et al., 2001; LaRochelle et al., 2002; Lokker et al., 2002; Li et al., 2003a) while the levels are low in normal cells. PDGF D-chain transformed cells exhibit increased proliferation, anchorage independent growth and angiogenesis via an autocrine or paracrine loop (Li et al., 2003a).

Table 5

Cancers Associated with PDGF B-chain.

Cancer type	*References*
Fibrosarcoma	Pech et al., 1989; Liu et al., 2001; Fthernou et al., 2006
Vascular connective tissue stroma	Forsberg et al., 1993; Liu et al., 2001
Ependymoma	Black et al., 1996; Heldin et al., 1999a
Meningioma	Nakamura et al., 1993; Heldin et al., 1999a
Pituitary adenomas	Leon et al., 1994; Heldin et al., 1999a
Kaposi's sarcoma	Pistritto et al., 1994; Koster et al., 1996; Heldin et al., 1999a
Mesothelioma	Heldin et al., 1999a; Filiberti et al., 2005
Malignant melanoma	Barnhill et al., 1996; Heldin et al., 1999a
Choriocarcinoma	Holmgren et al., 1993; Miller et al., 1998; Heldin et al., 1999a
Ovarian carcinoma	Heldin et al., 1999a; Apte et al., 2004; Matei et al., 2007
Breast cancer	Ginsberg & Vonderhaar, 1991; Heldin et al., 1999a; Bos et al., 2005
Colorectal carcinoma	Heldin et al., 1999a; Mantur et al., 2008
Chronic myelogenous leukemia	Kimura et al., 1995; Heldin et al., 1999a
Osteosarcoma	Weich et al., 1986; Liang et al., 1996
Astrocytoma	Nakamura et al., 1993
Glioblastoma	Nakamura et al., 1993
Oligodendroglioma	Nakamura et al., 1993
Gliomas	Mauro et al., 1991

As before there should be some overlap between the activities of the receptors and the individual chains. The α-receptor has been shown to have a lesser role in cancer and transformation than the β-receptor, but has been found to have mutations leading to autocrine or paracrine loops, over-expression, and/or gene amplification in malignancies (Table 6).

The α-receptor has been implicated in processes such as focus formation chemotaxis and anchorage independent growth. PDGFRβ has been implicated in

many malignancies (Table 7). It has been shown that amino acids 142-1027 of the β-receptor in the second domain of the kinase was responsible for the transforming activity and regions outside of the kinase domain were responsible for focus formation (Uren et al., 1996). Experimentation found that transformation of fibroblasts occurred with PDGF-BB but not PDGF-AA implying that transformation occurred only through the β-receptor (Heldin, 1999b; Yu et al., 2000; Li et al., 2003b).

Table 6

Cancers Associated with PDGFRα.

Cancer type	*Reference*
Liver carcinoma	Stock et al., 2007
Prostate carcinoma	Dolloff et al., 2007
Ovarian carcinoma	Apte et al., 2004; Matei et al., 2006; Vrekoussis et al., 2007
Breast carcinoma	Crowley et al., 2005
Ependymomas	Black et al., 1996
Malignant melanoma	Barnhill et al., 1996
Glioblastoma	Clarke & Dirks, 2003

PDGF and Gliomas

Aberrent PDGF signaling seems to be a hallmark of gliomas. Gliomas in general and specifically astrocytomas have been shown to express all four PDGF chains and both receptors, and over-expression has been found in poorly differentiated gliomas (Nister et al., 1988; Fleming et al., 1992; Kumabe et al., 1992; Guha et al., 1995; Westermark et al., 1995; Kirsch et al., 1997; Clarke & Dirks,

2003). It is believed that the formation of the autocrine loop is a rate limiting step in the formation of gliomas and it was found that the autocrine loop involving PDGF-A and PDGFRα is enough to establish gliomas in mice (Uhrborn et al., 1998; Takeuchi et al., 2004). Individually, the B-chain has been shown to cause and maintain an undifferentiated progenitor cell phenotype in glial cells (Markert et al., 2005) and placement of the B-chain downstream of the nestin promoter causes gliomas in mice (Dai et al., 2001; Ding et al., 2000). This implies that creation and/or maintenance of the neurospheres is in part because of PDGF B-chain.

Table 7

Cancers Associated with PDGFRβ.

Cancer type	*Reference*
Glioblastoma	Mauro et al., 1991; Heldin et al., 1999a
Medulloblastoma	Black et al., 1996; Heldin et al., 1999a
Ependymoma	Black et al., 1996; Heldin et al., 1999a; Fakhrai et al., 2004
Meningioma	Heldin et al., 1999a; Yang & Xu, 2001b
Pituitary adenomas	Leon et al., 1994; Heldin et al., 1999a
Kaposi's sarcoma	Pistritto et al., 1994; Heldin et al., 1999a
Choriocarcinoma	Holmgren et al., 1993; Heldin et al., 1999a
Breast cancer	Heldin et al,, 1999a; Vrekoussis et al., 2007; Cristofanilli et al., 2008
Prostate carcinoma	Kim et al., 1997; Heldin et al., 1999a
Ovarian carcinoma	Apte et al., 2004

The over-expression of PDGF has been shown to lead to transformation of NIH-3T3 mouse fibroblasts in cell culture (Clarke et al., 1984; Verbeek et al., 1999; Yu et al., 2000; Rosenmuller et al., 2001). Does PDGF play similar roles in human cancer? Numerous commercially available glioma cell lines over-express PDGF making them valuable to study the role of PDGF in human cancer.

Gliomas

Gliomas, a deadly glial derived brain cancer, play a small role in the disease that is cancer. Even though they only represent 2.4% (Markert et al., 2005) of all cancer, the impact of gliomas is substantial. Generally the target population is children and adolescents, causing the largest number of solid tumors for these groups. It is also the third leading cause of death for young adults (Alemany et al., 1999). With the 2008 diagnosis of Senator Ted Kennedy's glioma, the general population has been introduced to this devastating disease.

Treated glioma patients have a five year survival rate of less than 3% (Markert, 2003; Markert et al., 2005). There are several reasons for the low patient survival including the cell type the tumor arose from (ependymal, astrocytes, oligodendrocytes, or mixed cell type), the resistance of the tumor to ionizing radiation and an inability of therapeutic agents to cross the blood-brain barrier. The only currently accepted treatment is surgical intervention, but because of the highly invasive nature of gliomas there is little success with surgery and patients are subject to frequent relapse.

Gliomas fall into four categories based on glial cell type involved,

astrocytomas, oligodendrogliomas, ependymomas, and mixed gliomas (containing more than one cell type). Astrocytomas are the most common and are further classified into four groups; grade I (pilocytic astrocytoma), grade II (low-grade astrocytoma), grade III (anaplastic astrocytoma) and grade IV, (glioblastoma multiforme, GBM). GBM is the most aggressive and lethal of all gliomas, and accounts for 82% of all astrocytomas in the United States (Markert et al., 2005). Through the alterations of the proto-oncogenes and tumor suppressor genes GBM can develop in one of two ways, de novo or progressively through the four grades.

It is now accepted that tumor cells are morphologically diverse in nature especially in their ability to proliferate and differentiate and these cells express unique markers. One important population of cells found in most gliomas represents a de-differentiated cell referred to as a neural stem cell-like (NSC-like) cell. These NSC-like cells are believed to provide the source of tumor growth, have self-renewal ability (Lapidot et al., 1994; Larochelle et al., 1996; Bonnet, 1997; Al-Hajj et al., 2003; Dick, 2003; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004; Kapoor et al., 2004; Kondo et al., 2004; Singh et al., 2004; Yuan et al., 2004) and are found within neurospheres (Yuan et al., 2004). Generally, the higher the grade of astrocytoma, the more likely there are neurospheres.

The various cells within the neurosphere display different surface markers. The intermediate filament nestin is a CNS stem cell marker (Hockfield, 1985; Lendahl et al., 1990; Singh et al., 2004; Nagato et al., 2005), while glial fibrillary acid protein (GFAP) is expressed in differentiated astrocytes (Markert et al., 2005). The

glycoprotein CD133 is expressed in glial stem cells (Singh et al., 2003; Singh et al., 2004), and the neural RNA binding protein Musashil is a selective neural stem cell marker (Sakakibara et al., 1996; Sakakibara, 1997; Kancko et al., 2000; Nagato et al., 2005). It is believed that NSC-like cells represent a de-differentiated cell that has mutated growth factors, cytokines, hormones, and cell cycle regulators.

Our lab has previously evaluated a panel of eight transcription factors for increased activity as a result of the over-expression of PDGF. This revealed that the over-expression of PDGF in the astrocytoma cell line U-87 MG led to the over-expression of only two of these eight transcription factors, activator protein-1 and nuclear factor-kappa B.

Nuclear Factor-kappa B

The transcription factor nuclear factor-κB (NF-κB) was first isolated in 1986 by David Baltimore's lab and found to be a requirement for immunoglobulin kappa light chain transcription in B cells (Sen & Baltimore, 1986; Ghosh et al., 1998; Rayet & Gélinas, 1999; Chen & Ghosh, 1999). Since that time, NF-κB has been found as a requirement for transcription of numerous genes especially those requiring rapid induction since it is already synthesized and kept inactive in the cytoplasm. To activate NF-κB, bound regulator proteins called inhibitor κB (IκB) dissociate from NF-κB and phosphorylation of the NF-κB proteins must occur. Once activated, NF-κB quickly translocates to the nucleus and binds to promoters and enhancers. The initiation of gene expression through direct DNA contact was believed to be the only role of NF-κB, but it has been found to also play a role in elongation (West et al.,

2001; Dreikhausen et al., 2005) as well as interaction with other DNA-bound transcription factors (Heckman et al., 2002; Gilmore et al., 2004). NF-κB response has been described as "waves" of activity and non-activity lasting from 15-45 minutes (Gilmore, 1999).

NF-κB has been found in organisms as diverse as humans (Ghosh et al., 1998), *Drosophila* (Steward, 1987; Ip et al., 1993; Dushay et al., 1996; Ghosh et al., 1998), viruses (Chen et al., 1983), *Xenopus* (Kucharezak et al., 2003), plants (Whitham et al., 1994; Ghosh et al., 1998), and oysters (Montagnani et al., 2004), but has yet to be found in yeast and *C. elegans* (Gilmore, 1999). In mammals there are five NF-κB proteins, p50/p105, p52/p100, p65 (RelA), c-Rel, and RelB, while a sixth, NFAT, has been accepted by some. Active NF-κB consists of homo- or heterodimers of the five proteins, but not all fifteen different combinations are found in all cells or in the same quantity. The most common dimer found in cells is p50/p65 and is generally referred to as NF-κB. Knockout mice for each of the five proteins have shown that the function of each protein is generally unique with some redundancy.

The five proteins fall into two classes: one class contains p50/p105 and p52/p100, while the other class contains p65, c-Rel and RelB. Near the N-terminus, each protein contains a highly conserved 300 amino acid region termed the Rel homology domain (RHD) and within the RHD there are two immunoglobulin-like repeats responsible for sequence specificity, dimerization of the proteins, interaction with IκBs, as well as DNA-binding, and the location for the nuclear localization sequence (Figure 2).

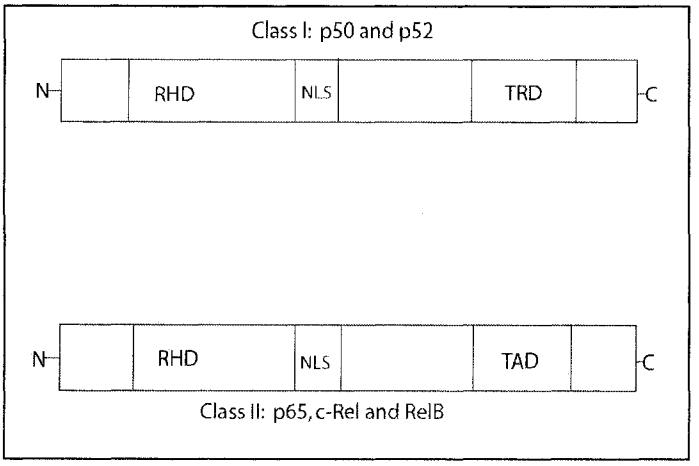


Figure 2. Structure of NF-κB. Rel homology domain (RHD) is responsible for DNA binding, IκB binding and dimerization. Transactivation domain (TAD) is responsible for transcription facilitation. Transrepression domain (TRD) contains ankyrin repeats. Nuclear localization sequence (NLS) allows translocation to the nucleus.

p65, c-Rel and RelB contain a transactivating domain (TAD) near the C-terminus while p50/p105 and p52/p100 do not. The TAD facilitates transcription by recruiting activators and displacing repressors (Gilmore, 1999; Li & Verma, 2002; Hayden & Ghosh, 2004). Without the TAD, there is little or no transcription. In contrast, the C-terminus of p105 and p100 contain numerous copies of ankyrin repeats which act as inhibitors and require removal of the C-terminus to create the p50 and p52 proteins.

NF-κB binds to specific 9- or 10-bp sites on the DNA termed κB sites which vary slightly causing asymmetry of the binding site. This asymmetric arrangement accounts for the differential binding affinities of the various dimer combinations and it is believed that the κB site is recognized as half sites by the individual proteins of the dimer. The NF-κB dimer straddles the DNA and makes contact especially with the major groove via ten flexible loops within the immunoglobulin-like domains

(Chen & Ghosh, 1999).

Generally, NF-κB stimulates gene expression but has been found to repress it as well. NF-κB has been found to play a role in activating many genes (Table 8) and has been found in most if not all cell types.

Table 8

Genes Regulated by NF-κB.

Process	*References*
Inflammation	Sha et al., 1995; Baeuerle & Baltimore, 1996; Barnes & Karin, 1997; Franzoso et al., 1997; Ghosh et al., 1998; Epinat & Gilmore, 1999; Karin, 1999; Alcamo et al., 2001; Karin et al., 2002a; Karin & Lin, 2002b; Li & Verma, 2002; Shaulian & Karin, 2002; Fujioka et al., 2004; Hayden & Ghosh, 2004; Ravi, 2004; Marienfeld et al., 2006; Mauro et al., 2006
Embryonic development	Epinat & Gilmore, 1999; Li et al., 1999; Shaulian & Karin, 2002; Fujioka et al., 2004; Hayden & Ghosh, 2004; Marienfeld et al., 2006
Lymphoid differentiation	Li et al., 1999; Shaulian & Karin, 2002; Fujioka et al., 2004
Oncogenesis	Epinat & Gilmore, 1999; Li et al., 1999; Shaulian & Karin, 2002; Fujioka et al., 2004
Pro-apoptosis	Barkett & Gilmore, 1999; Li et al., 1999; Pahl, 1999; Karin & Lin, 2002b; Shaulian & Karin, 2002; Fujioka et al., 2004; Hayden & Ghosh, 2004; Marienfeld et al., 2006; Mauro et al., 2006
Anti-apoptosis	Barkett & Gilmore, 1999; Li et al., 1999; Pahl, 1999; Karin & Lin, 2002b; Shaulian & Karin, 2002; Fujioka et al., 2004; Hayden & Ghosh, 2004; Marienfeld et al., 2006; Mauro et al., 2006
Immune responses	Sha et al., 1995; Baeuerle & Baltimore, 1996; Barnes & Karin, 1997; Franzoso et al., 1997; Ghosh et al., 1998; Epinat & Gilmore, 1999; Karin, 1999; Alcamo et al., 2001; Karin et al., 2002a; Karin & Lin, 2002b; Li & Verma, 2002; Bonizzi & Karin, 2004; Hayden & Ghosh, 2004; Ravi, 2004; Marienfeld et al., 2006; Mauro et al., 2006
Cell growth	Heldin et al., 1998; Epinat & Gilmore, 1999
Viral gene expression	Epinat & Gilmore, 1999
Dorsal-ventral polarity in insects	Baeuerle & Baltimore, 1996; Karin, 1999
Cellular proliferation	Inta et al., 2006

Table 8-Continued

Process	*References*
Directed cellular movement	Heldin et al., 1998
Stress response	Sha et al., 1995; Barnes & Karin, 1997; Franzoso et al., 1997; Ghosh et al., 1998; Alcamo et al., 2001; Karin et al., 2002a; Karin & Lin, 2002b; Ravi, 2004; Mauro et al., 2006
Cell cycle progression	Ravi, 2004
Invasion	Ravi, 2004
Angiogenesis	Ravi, 2004
Transcription factor activation	Pahl, 1999
Growth factor activation	Chen & Ghosh, 1999
Cytokine activation	Chen & Ghosh, 1999
Cell adhesion molecule activation	Bond et al., 1999; Takeshita et al., 1999; Wang et al., 1999; Ravi, 2004
Matrix metalloproteinase activation	Bond et al., 1999; Takeshita et al., 1999; Wang et al., 1999; Ravi, 2004
IkappaB family activation	Pahl, 1999
Actin reorganization	Heldin et al., 1998

The Role of NF-κB

NF-κB has many target genes and most of these target genes can be linked to onset and/or progression of inflammation (Hayden & Ghosh, 2004; Stuhlmeier & Pollaschek, 2005; Adli & Baldwin, 2006), cell survival, proliferation, and immune responses (Hayden & Ghosh, 2004; Adli & Baldwin, 2006).

NF-κB and Disease

NF-κB has been found aberrantly activated in numerous disease processes such as inflammation, tumor progression, metastasis, and angiogenesis, as well as cellular transformation. It has been shown that the transformation of NIH3T3 cells by the over-expression of PDGF is dependent upon NF-κB (Shimamura et al., 2002). Aberrations of NF-κB have also been implicated in specific cancers (Table 9). NF-

κB has been found to play a role in many diseases or disease processes other than cancer (Table 10). Not all increases in NF-κB levels will lead to disease, many normal processes as well as cellular insults can lead to increases (Table 11).

Table 9

Cancers Found to have NF-κB Involvement.

Cancer	*References*
Colorectal	Lind et al., 2001; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Thyroid	Visconti et al., 1997; Gilmore et al., 2003
Breast	Nakshatri et al., 1997; Sovak et al., 1997; Newton et al., 1999; Patel et al., 2000; Bhat-Nakshatri et al., 2002; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Lung	Madrid & Baldwin, 2003; Ravi, 2004
Pancreas	Wang et al., 1999; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Head & neck	Ondrey et al., 1999; Gilmore et al., 2003
Oral	Nakayama et al., 2001; Gilmore et al., 2003
Prostate	Palayoor et al., 1999; Huang et al., 2001; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Glioblastoma	Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Melanoma	Yang & Richmond, 2001a; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Multiple myeloma	Berenson et al., 2001; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004
Lymphoma	Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi, 2004; Xiao et al., 2004
Ovary	Dejardin et al., 1999; Huang et al., 2000; Gilmore et al., 2003
Kidney	Oya et al., 2001; Gilmore et al., 2003
Liver	Tai et al., 2000; Gilmore et al., 2003
Kaposi's sarcoma	Brinkman, 2003
Cervical	Prusty et al., 2005
Leukemia	Kordes et al., 2000; Guzman et al., 2001; Gilmore et al., 2003; Madrid & Baldwin, 2003; Ravi. 2004; Xiao et al., 2004

p50

The p50 protein was first isolated in 1991 and the structure is now well known (Meyer et al., 1991). The *nfkb1* gene produces the p105 protein which is

proteolytically processed to the N-terminal p50 protein. The affinity of dimer formation indicates that the heterodimer p50-p65 forms preferentially before the homodimer p50 which forms preferentially before the p65 homodimer (Kunsch et al., 1992; Ganchi et al., 1993; Chen & Ghosh, 1999). The p50 homodimer lacks the TAD and therefore often represses transcription except when paired with p65, c-Rel or RelB which have the TAD to activate transcription (Kang et al., 1992). p50 has been found in virtually all cell types especially in B cells (Horwitz et al., 1997; Pohl et al., 2002), T cells (Sundstedt et al., 1996), and dendritic cells (Artis et al., 2005).

Table 10

NF-κB's Involvement in Diseases, Infections, and Processes Other than Cancer. All have been found to activate NF-κB except the last two which block NF-κB activity.

Disease/Infection/Process	*References*
Sepsis & Septic shock	Liu, 2006
Hypertension	Rodriguez-Iturbe et al., 2005
Rheumatoid arthritis	Cheon et al., 2004
Hypercholesterolemia	Zhou et al., 2005
Inflammatory bowel	Li & Verma, 2002
Multiple sclerosis	Li & Verma, 2002
Asthma	Li & Verma, 2002
Infarct development	Brown et al., 2005
Autoimmune disease	Fracchiolla et al., 1993; Mackay et al., 1999; Maeda et al., 2005; He et al., 2006
HIV	Kovacs et al., 1995; Roulston et al., 1995; Pahl, 1999; Hiscott et al., 2001; Cook et al., 2003; Gedey et al., 2006
Epstein-Barr	Luque & Gélinas, 1997; Dudziak et al., 2003
Respiratory syncytial virus	Choudhany et al., 2005
Influenza A	Bernascon et al., 2006
Hepatitis B	Diao et al., 2001
Herpes	Pati et al., 2001
Orthopox	Oie & Pickup, 2001; Shisler & Jin, 2004; Gedey et al., 2006
Hepatitis C	Joo et al., 2005

Table 11

Ways to Increase NF-κB Activity.

Regulator	*References*
Exercise	Ji et al., 2004; Ho et al., 2005
Increased calcium levels	Hughes et al. 1998; Hughes et al., 2001; Ho et al, 2005
Reactive oxygen species	Schreck et al., 1991; Muller et al., 1997; Oka et al., 2000; Kamata et al., 2002; Ho et al., 2005
Cell irradiation	Brach et al., 1991; Campbell et al., 2004; Hayden & Ghosh, 2004
DNA damage	Brach et al., 1991; Campbell et al., 2004; Hayden & Ghosh, 2004
Brain injury	Gapuzan et al., 2002
Spinal cord injury	Gapuzan et al., 2002
Cytokines	Beg et al., 1995; Sha et al., 1995; Beg & Baltimore, 1996; Barnes & Karin, 1997; Thomas et al., 1998; Hilliard et al., 2002
Growth factors	Beg et al., 1995; Sha et al., 1995; Beg & Baltimore, 1996; Barnes & Karin, 1997; Thomas et al., 1998; Hilliard et al., 2002
Stress	Beg et al., 1995; Sha et al., 1995; Beg & Baltimore, 1996; Barnes & Karin, 1997; Thomas et al., 1998; Hilliard et al., 2002
Infection	Beg et al., 1995; Sha et al., 1995; Beg & Baltimore, 1996; Barnes & Karin, 1997; Thomas et al., 1998; Hilliard et al., 2002

p50 is an important regulator of development. It has been found that p50 is not required for embryogenesis or hemopoiesis, yet embryonic tissue cells all express p50. p50 paired with p65 has been shown to cooperatively activate PDGF-A chain through protein-protein interactions (Aizawa et al., 2004; Benitah et al., 2003), creating a positive feedback loop. p50 paired with other NF-κB proteins lead to novel properties not seen with the p50 homodimer. Knockout studies have revealed that the absence of p50 and p52 leads to growth retardation, craniofacial abnormalities, and blocked B-cell development (Franzoso et al., 1997; Iotsova et al., 1997; Gerondakis et al., 1999), while the absence of p50 and p65 leads to embryonic death at around E13

(Horwitz et al., 1997; Gerondakis et al., 1999).

The involvement of p50 in cancer is cell specific. Thus far, in hematologic malignancies no genetic alterations in *nfkb1* have been found. p50 over-expression has been found in non-small cell lung cancer and rearrangements have been found in leukemia (Luque & Gélinas, 1997).

p52

The p52 protein was isolated in 1991 the structure has been determined by numerous labs (Neri et al., 1991). The *nfkb2* gene produces the p100 protein which is proteolytically processed to the N-terminal p52 protein. p52 has been shown to have a death domain (DD) which participates in caspase activation (Wang et al., 2002; Turco et al., 2004) and lacks a transactivation domain.

p52 has been found to have limited expression, but is important for the immune system. Expression was believed to be limited to stomach epithelium, thymic medulla, and the marginal zone of the spleen (Attar et al., 1997; Gerondakis et al., 1999), but has since been found in dendritic cells (Lind et al., 2008) and neurons (Cao et al., 2008). Knockouts of *nfkb2* show disruption of splenic and lymph node architecture (Franzoso et al., 1997; Caamano et al., 1998; Gerondakis et al., 1999) and the inability to mount a normal T-cell antibody response (Gerondakis et al., 1999). It has been found that p52 is required for the immune response and B-cell activation.

p52 has been implicated in numerous cancers usually involving the regulatory regions of p100/p52. The *nfkb2* gene was the first NF-κB gene found altered in malignancies and rearrangements removing the DD have been found in lymphoid

tumors (Turco et al., 2004). Other genetic alterations removing the C-terminal IκB property have also been shown in some malignancies (Hacker & Karin, 2002; Wang et al., 2002; Turco et al., 2004). p52 over-expression has been found in breast and colon carcinoma and rearrangements have been found in lymphoma (Luque & Gélinas, 1997).

p65

p65 was isolated in 1991 (Ruben et al., 1991) and has been shown to have a role in development since knockout of the gene leads to embryonic death. The p65 protein has been found in virtually all cells including some astrocytes, neuron precursors, and glial cells (Denis-Donini et al., 2005), and has been shown to have redundant functions in differentiation, regulation, and survival in multiple hemopoietic cells (Grossman et al., 1999), as well as epidermal development (Gugasyan et al., 2004).

Dysregulation of p65 have been observed in many diseases with most leading to over-expression. Over-expression of p65 inhibits apoptosis (Beg & Baltimore, 1996; Liu et al., 1996b; Van Antwerp et al., 1996; Wang et al., 1996; Wu et al., 1996; Ghosh et al., 1998; Karin et al., 2002a; Turco et al., 2004), increases DNA binding (Mosialos & Gilmore, 1993; Neumann et al., 1995; Hayden & Ghosh, 2004), and mediates anchorage-independent growth (Zahir et al., 2003).

RelB

RelB was isolated in 1992 (Ruben et al., 1992) and has a very limited distribution and functionality. *relb* is an immediate early response gene which cannot

bind κB sites on its own but only as a heterodimer with p50. Normal RelB is confined to dendritic cells and immune cells and is up-regulated during generation of dendritic cells and macrophages (Neumann et al., 2000), but small amounts have been found in neuron precursors (Denis-Donini et al., 2005). Absence of RelB as well as p50 leads to organ inflammation and death within 3-4 weeks of birth in mice (Weih et al., 1997; Gerondakis et al., 1999). RelB alterations have not been identified in hematologic malignancies.

c-Rel

As a proto-oncogene, c-Rel has been studied extensively and was isolated in 1986 (Brownell et al., 1986). It is an intermediate early gene with mRNA expression within 30 minutes of stimulation. It generally forms homodimers, or heterodimers with p50 or p65. c-Rel has been found expressed in numerous cell types. Little c-Rel protein has been found in fibroblasts, muscle cells or brain tissue, but is widely expressed in embryonic and adult central nervous system. c-Rel has also been detected in embryonic lymphoid tissues.

Regulation of c-Rel can be variable. It can enhance its own expression since it has κB sites. DNA binding (Neumann et al., 1992; Glineur et al., 2000; Gilmore et al., 2004) of c-Rel and C-terminal transactivation activity (Martin & Fresno, 2000; Martin et al., 2001; Gilmore et al., 2004) can be affected by phosphorylation.

c-Rel has been implicated in numerous cellular processes (Table 12). The heterodimer c-Rel and p50 have been shown to play a role in cell growth and proliferation, while c-Rel and p65 in survival. The homodimer plays a role in survival

and proliferation. c-Rel has been shown to compensate somewhat for missing p65 (Grossmann et al., 1999).

Table 12

Cellular Processes with c-Rel Involvement. Research has conflicting data regarding c-Rel involvement in embryonic development.

Cellular Process	*References*
Proliferation	Gilmore et al., 1996; Baichwal, 1997; Gilmore, 1999; Karin, 1999; Liss, 2002
Cell cycle progression	Liss, 2002
T helper cell differentiation	Hilliard et al., 2002; Pai, 2002
Autoimmune inflammation	Hilliard et al., 2002; Pai, 2002
B-cell proliferation	Gilmore et al., 2004
B-cell survival	Gilmore et al., 2004
Apoptosis	Gilmore et al., 1996; Baichwal, 1997; Gerondakis et al., 1999; Gilmore, 1999; Karin, 1999
Epidermal growth	Gugasyan et al., 2004
B- & T-cell activation	Kontgen et al., 1995; Gerondakis et al., 1996; Grigoriadis et al., 1996; Grumont et al., 1998; Gerondakis et al., 1999; Grossmann et al., 2000; Starczynowski et al., 2003
Embryonic development	Kontgen et al., 1995; Tumang et al., 1998; Gerondakis et al., 1999; Grossman et al., 1999; Gilmore et al., 2004

A large amount of data regarding the role of c-Rel in cancer has been generated. *c-rel* is the homologue of the viral oncogene *v-rel* of the avian reticulendotheliosis (REV-T) retrovirus isolated in 1958 and is responsible for leukemias and lymphomas in mice and chickens. To transform cells, c-Rel must activate transcription, but interestingly, deletions in the transactivation region increase the transforming activity (Starczynowski et al., 2003). Genetic alterations of *c-rel* in cancer are numerous (Table 13).

Table 13

Alterations of c-Rel Implicated in Cancer.

Alteration	*References*
Amplifications	Brownell et al., 1988; Houldsworth et al., 1996; Joos et al., 1996; Barth et al., 1998; Rao et al., 1998; Rayet & Gelinas, 1999; Furman et al., 2000; Bernal et al., 2001; Davis et al., 2001; Joos et al., 2002; Barth et al., 2003; Cheng et al., 2003; Gilmore et al., 2003; Turco et al., 2004
Rearrangements	Gilmore et al., 2003; Gilmore et al., 2004
Deletions	Gilmore et al., 2004
Over-expression	Houldsworth et al., 1996; Joos et al., 1996; Rao et al., 1998; Rayet & Gelinas, 1999; Furman et al., 2000; Bernal et al., 2001; Davis et al, 2001; Joos et al., 2002; Barth et al., 2003; Cheng et al., 2003; Gilmore et al., 2003

c-Rel alterations in cancer have been studied extensively (Table 14). The *c-rel* gene has been found altered in many hematologic malignancies, endometrial carcinoma, and non-small cell lung carcinoma.

Regulation

Regulation of NF-κB is a complex process and can occur in many ways. Dimerization of NF-κB regulates whether or not NF-κB is active. Differing affinities of the various NF-κB dimers for individual κB sites regulate what is available to bind. Dimerization affinity occurs with amino acids Asp-254 and Tyr-267 in p50 and the amino acid analogs in other family members (Chen & Ghosh, 1999). Inhibitory κBs (IκBs) and their inhibitory IκB kinases (IKKs) can regulate whether NF-κB is available or not. Degradation of IκBs by potent regulators means more NF-κB.

Table 14

Specific Cancer Alterations.

Genetic alteration	*Phenotype*	*Cancer/Cell type*	*References*
Over-expression	Cell cycle arrest	HeLa cells	Pahl, 1999
Over-expression	Protection from apoptosis	All	Beg & Baltimore, 1996; Liu et al., 1996b; Van Antwerp et al., 1996; Wang et al., 1996; Wu et al., 1996; Ghosh et al., 1998; Pahl, 1999; Zong et al., 1999; Karin & Lin, 2002b; Turco et al, 2004
Over-expression		B-cell leukemia & lymphoma	Houldsworth et al., 1996; Joos et al., 1996; Rao et al., 1998; Rayet & Gelinas, 1999; Furman et al., 2000; Bernal et al.. 2001; Davis et al., 2001; Joos et al., 2002; Barth et al., 2003; Cheng et al., 2003
Amplification		B-cell leukemia & lymphoma	Lu et al., 1991; Houldsworth et al., 1996; Joos et al., 1996; Rao et al., 1998; Goff et al., 2000; Nagy et al., 2000; Neat et al., 2001; Palanisamy et al., 2002; Barth et al., 2003; Starezynowski et al., 2003; Ravi, 2004
Over-expression	Transformation	Chicken spleen cells	Gilmore et al., 1996; Gilmore, 1999; Gilmore et al., 2003; Cheng et al., 2003; Fan et al., 2004
Over-expression	Apoptosis	Cell type specific	Bernard et al., 2002
Rearrangements		Leukemia & lymphoma	Luque & Gelinas, 1997
Amplification		Leukemia & lymphoma	Luque & Gelinas, 1997
Over-expression		Gliomas	Conti et al., 2005; Raychaudhuri et al., 2007

The ability to bind to DNA can regulate whether or not a gene is activated. Individual dimer compositions have varying recognition and affinity for DNA binding sites. Phosphorylation of p50 increases the affinity for DNA binding (Li et al., 2004; Chen & Ghosh, 1999; Kushner & Ricciardi, 1999) as well as increase the transcriptional activity of p65 (Chen & Ghosh, 1999). Some promoters/enhancers

require multiple transcription factors to bind prior to transcription thus adding another layer of regulation. Covalent modifications have been shown to alter the ability of NF-κB to bind to DNA, recruit co-activators and interact with IκBs.

Not all cells respond to a stimulus at the same level causing further regulation. Specific regulators include cell cycle progression by p53 through NF-κB (Rocha et al., 2003), decreased potassium levels up-regulate DNA binding of NF-κB in apoptotic neurons (Tao et al., 2006), and inhibition of NF-κB by anti-oxidants (Epinat & Gilmore, 1999; Li et al., 1999; Pahl, 1999).

In an unstimulated cell, NF-κB is bound to IκBs and remains inactive (Figure 3). There are seven members that have different binding affinities for the different NF-κB dimers, IκBα, IκBβ, BCL-3, IκBε, IκBγ and the NF-κB precursor proteins p105 and p100. These proteins contain five to seven ankyrin repeats which bind to the individual NF-κB dimer RHDs and cover the nuclear localization sequence, an N-terminal regulatory domain and a C-terminal PEST motif domain. The seven IκB family members can form hetero- and homodimers.

IκBα and IκBβ are two of the best documented IκBs. They are serine/threonine kinases with IκBβ activated when the cell is stimulated by proinflammatory cytokines while IκBα is much more diverse in what stimulates it. In IκBα and IκBβ, the kinase domain is located N-terminally and the C-terminal region has a helix-loop-helix motif and a leucine zipper domain with the leucine zipper required for kinase activity. IκBα has been shown to shuttle NF-κB in and out of the

nucleus (Gilmore, 1999; Aguilera et al., 2006) in unstimulated cells as well as stimulated cells (Johnson et al., 1999; Huang et al., 2000a; Aguilera et al., 2006), whereas IκBβ has been shown to sequester NF-κB within the cytoplasm (Liou, 2003).

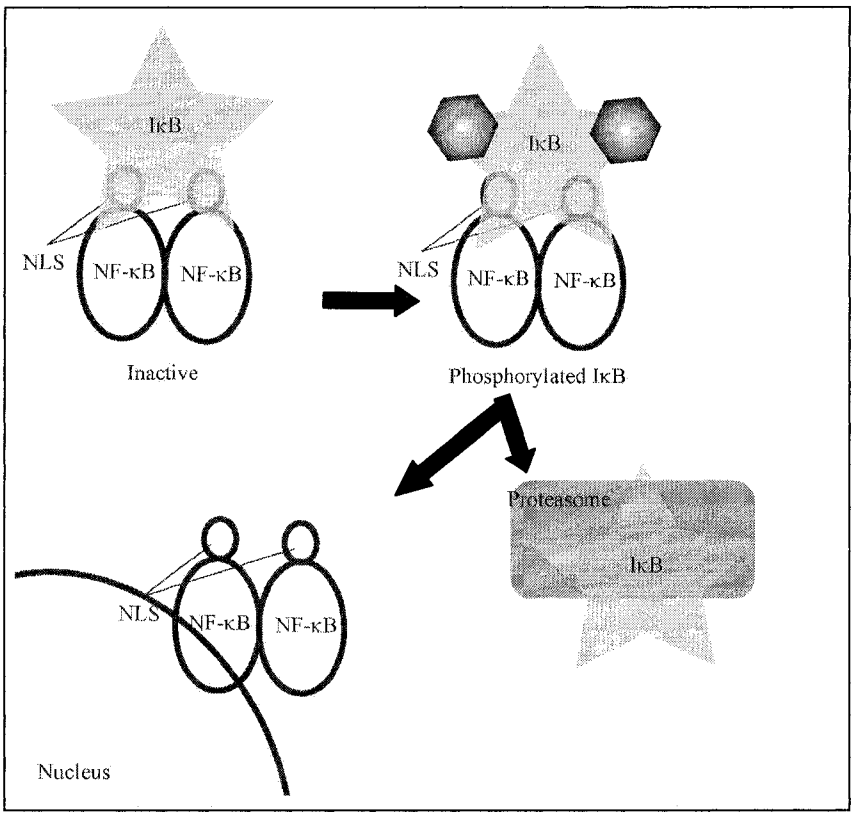


Figure 3. Regulation of NF-κB by IκBs. Nuclear localization sequence is covered by IκB and remains inactive in the cytoplasm. Upon phosphorylation of IκB, NF-κB translocates to the nucleus and binds to DNA, while IκB is degraded by a proteasome.

Upon stimulation of the cell NF-κB is activated in one of two ways, IκBs are phosphorylated, ubiquitinated and degraded via the proteasomal pathway thus allowing NF-κB to translocate to the nucleus and bind to κB sites or by phosphorylation of the IκB-like portion of p105 and p100 which results in proteolytic

processing and release of p50 and p52 respectively. Interestingly, NF-κB has been found to regulate IκBα synthesis by presence of a κB site (Latchman, 1990; Ting & Endy, 2002; Hayden & Ghosh, 2004).

The regulation of IκBs is provided by an IκB kinase (IKK) complex composed of catalytic subunits IKKα and IKKβ in homo- or heterodimers and the regulatory subunit IKKγ or NEMO. IKKα and IKKβ are responsible for the phosphorylation and subsequent degradation of IκBs, while NEMO is responsible for ubiquitination. Two different pathways of regulation utilize different IKKs. The noncanonical pathway which has been shown to be stimulated by CD40 and lymphotoxin-beta receptor and others (Pomerantz & Baltimore, 2002) is dependent upon IKKα and NEMO (Claudio et al., 2002; Dejardin et al., 2002; Hayden & Ghosh, 2004), activates p105 and p100 processing (Senftleben et al., 2001; Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Mortmuller et al., 2003; Muller & Siebenlist, 2003; Hayden & Ghosh, 2004; Ramakrishnan et al., 2004; Xiao et al., 2004; He et al., 2006; Marienfeld et al., 2006), and requires NF-κB-inducing kinase (NIK) for phosphorylation and activation of IKKα (Senftleben et al., 2001; Xiao et al., 2001; Hayden & Ghosh, 2004; He et al., 2006) while the canonical pathway which has been shown to be stimulated by lymphotoxin-beta receptor and others (Pomerantz & Baltimore, 2002) is dependent upon IKKβ and NEMO, phosphorylates and activates the remaining NF-κB proteins.

Various upstream and extracellular molecules can activate the NF-κB pathway

(Table 15), while other molecules have been shown to inhibit NF-κB. Downregulation of tumor necrosis factor receptor-associated factor 2 (TRAF2) by E2F has been shown to inhibit NF-κB and E2F has also been shown to inhibit NF-κB by deactivating the DNA binding sites (Seville et al., 2005). Curcumin has been shown to inhibit NF-κB expression through Akt (Aggarwal et al., 2006). *Yersinia* virulence factor YopJ inhibits NF-κB activation (Zhou et al., 2005).

Table 15

Agents Implicated in Activating NF-κB.

Activating agent	*References*
Pathogens	Brown et al., 1995; DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Mauro et al., 2006
Cytokines	Brown et al., 1995; DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Mauro et al., 2006
dsRNA	Karin, 1999; Pahl, 1999
Ionizing radiation	Karin, 1999; Pahl, 1999
Phorbol ester	Latchman, 1990; Karin, 1999
Tumor necrosis factor α	Latchman, 1990; Karin, 1999
IL-1	Latchman, 1990; Karin, 1999; Cook et al., 2003
Bacterial lipopolysaccharide	Latchman, 1990; Karin, 1999
Cigarette smoke	Yang et al., 2006
PDGF	Klinghoffer, 1995; Heldin et al., 1998; Abraham, 2003; Chow et al., 2005; Liu, 2006
Ras	Madrid et al, 2000; Madrid et al., 2001; West et al., 2002; Ravi, 2004
TRAF3	He et al., 2006
T-cell receptor	Rossman et al., 2006
Receptor tyrosine kinases	Wang et al., 2007; Ringseis et al., 2008; Yi et al., 2008

Activator Protein-1

Activator protein-1 (AP-1) transcription factor was first identified bound to

promoter DNA of genes stimulated by phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Angel et al., 1987). Active AP-1 can form 18 hetero- and homodimers of the Jun (JunB, c-Jun, and JunD) and Fos (FosB, c-Fos, Fra-1, and Fra-2) family members. Jun family members form low-affinity homodimers and high-affinity heterodimers with Fos members, while Fos members do not form homodimers.

Dimerization is essential for AP-1 activity and specificity. The AP-1 dimer binds to TPA response elements (TRE) or cAMP response element binding (CREB) sites located in the promoter region of the gene. DNA binding and the binding specificities of the different dimers are similar but their transactivation abilities are different yielding a variety of response among the various dimers.

The AP-1 structure varies between the Jun and Fos families (Figure 4). It contains an evolutionarily conserved basic leucine zipper (bZIP) motif. The highly charged basic region is required for DNA binding while the amphipathic leucine zipper is required for dimerization and specificity of dimer composition. The N-terminal region contains a highly variable transactivation domain in both Jun and Fos family members while the Fos family also contains a second transactivation domain in the C-terminal region.

AP-1 has been found in nearly all vertebrate cell types and has been found to regulate numerous genes. It has been shown to be involved in a vast array of cellular processes (Table 16). AP-1 family members have been shown to regulate both basal and induced expression in more than 129 genes (Chanda et al., 2003) and have

overlapping as well as unique functions.

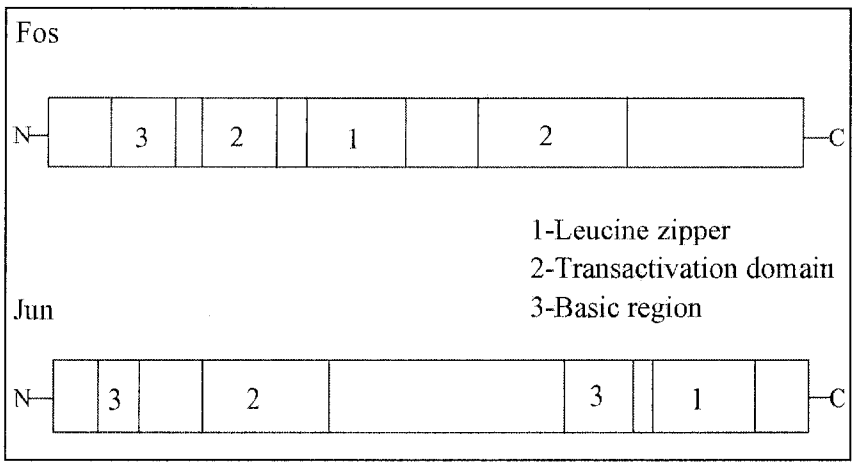


Figure 4. Structure of AP-1 Family Members. The leucine zipper facilitates dimerization and the basic region allows for DNA binding.

Regulation

AP-1 activity can be regulated at the level of expression as well as post-translational modification. Post-translational modifications are generally phosphorylation events (Bishop & Weinberg, 1996; Yazgan & Pfarr, 2002). Regulation of expression can occur through increases or decreases in the transcription of the genes for the AP-1 family members, mRNA stability, turnover of AP-1 family members, and association with other proteins. Numerous extracellular stimuli (Table 17) have been found to activate intracellular pathways (Table 18) that terminate with AP-1 activation.

Table 16

AP-1 Involvement in Cellular Processes.

Process	*References*
Cell growth	Hirai et al., 1989; Angel & Karin, 1991; Karin, 1995; Bejer et al., 1999; Wisdom, 1999; Bejer et al., 2000; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Shaulian & Karin, 2002; Short & Pfarr, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Hess et al., 2003; Pramanik et al., 2003; Hess et al., 2004; Kenner et al., 2004; Xie et al., 2005
Proliferation	Hirai et al., 1989; Angel & Karin, 1991; Karin, 1995; Bejer et al., 1999; Wisdom, 1999; Bejer et al., 2000; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Shaulian & Karin, 2002; Short & Pfarr, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Hess et al., 2003; Pramanik et al., 2003; Hess et al., 2004; Kenner et al., 2004; Xie et al., 2005
Transformation	Hirai et al., 1989; Angel & Karin, 1991; Karin, 1995; Shaulian & Karin, 2001; Short & Pfarr, 2002; Yazgan & Pfarr, 2002; Pramanik et al., 2003; Fujioka et al., 2004; Ameyar-Zazoua et al., 2005
Cell stress	Angel & Karin, 1991; Wisdom, 1999; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Short & Pfarr, 2002
Hypoxia	Angel & Karin, 1991; Wisdom, 1999; Shaulian & Karin, 2001; Short & Pfarr, 2002
Cytokines	Angel & Karin, 1991; Wisdom, 1999; Shaulian & Karin, 2001; Short & Pfarr, 2002
Differentiation	Angel & Karin, 1991; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Fujioka et al., 2004; Hess et al., 2004; Xie et al., 2005
Apoptosis	Angel & Karin, 1991; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Shaulian & Karin, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Fujioka et al., 2004; Hess et al., 2004; Xie et al., 2005
Survival	Shaulian & Karin, 2002; Hess et al., 2004; Xie et al., 2005
Immune responses	Angel & Karin, 1991; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001
Angiogenesis	Eferl & Wagner, 2003a; Xie et al., 2005
Invasion	Eferl & Wagner, 2003a; Xie et al., 2005
Inflammation	Shaulian & Karin, 2001; Fujioka et al., 2004
Embryonic development	Fujioka et al., 2004
Wound healing	Shaulian & Karin, 2002
Skeletal development & maintenance	Wagner, 2002; Naito et al., 2005

Diseases

Many diseases other than cancer have been shown to stimulate AP-1 activity. The connection between viruses and AP-1 has been well established. AP-1 activity increases with many viral infections and has been shown to be activated via JNK in

promonocytes and macrophages by the Vpr (Varin et al., 2005) and Nef (Varin et al., 2003) proteins of the HIV virus. Rotavirus has been shown to activate AP-1 through JNK in intestinal cells (Holloway & Coulson, 2006) and retroviral homologs of some of the AP-1 family members exist. Viruses are not the only pathogens that activate AP-1. Bacteria such as *Salmonella enterica* activate AP-1 through MAPK (Galdiero et al., 2002). Recently, a connection between AP-1 and Alzheimer's disease has been established. Apoptosis of endothelial cells of the brain during Alzheimer's disease has been shown to be mediated through AP-1 (Yin et al., 2002; Hess et al., 2004) causing a decrease in blood flow to the areas of the brain involved.

Table 17

Stimuli that Activate AP-1.

Stimuli	*References*
Cytokines	Karin, 1995; Orlandini et al., 1996; Wisdom, 1999; Shaulian & Karin, 2002; Hess et al., 2004
Growth factors	Bravo et al., 1985; Curran et al., 1985; Buscher et al., 1988; Quantin & Breathnach, 1988; Ryder & Nathans, 1988; Wu et al., 1989; Karin, 1995; Orlandini et al., 1996; Lee et al., 1998; Schreiber et al., 1999; Wisdom, 1999; Shaulian & Karin, 2002; Mathas et al., 2002; Ventura et al., 2002; Fujioka et al., 2004; Hess et al., 2004; Monje et al., 2005
PDGF	Wisdom; 1999; Monje, 2003
Stress signals	Bravo et al., 1985; Curran et al., 1985; Buscher et al., 1988; Karin, 1995; Orlandini et al., 1996; Lee et al., 1998; Schreiber et al., 1999; Wisdom, 1999; Mathas et al., 2002; Shaulian & Karin, 2002; Hess et al., 2004
Infection	Shaulian & Karin, 2002; Hess et al., 2004
T-cell activators	Karin, 1995; Orlandini et al., 1996; Wisdom, 1999
Neurotransmitters	Shaulian & Karin, 2002
Hormones	Shaulian & Karin, 2002
Serum	Lamph et al., 1988; Ryder & Nathans, 1988; Shaulian & Karin, 2001
Oncogenic signals	Angel & Karin, 1991; Shaulian & Karin, 2001; Eferl & Wagner, 2003a; Hess et al., 2004

Table 18

Intracellular Pathways that Activate AP-1.

Pathways activated	*References*
MAPK	Treisman, 1994; Karin, 1995; Whitmarsh & Davis, 1996; Cohen, 1997; Hunter, 1997; Karin et al., 1997; Ono & Han, 2000; Suh et al., 2000; Shaulian & Karin, 2001; Eferl & Wagner, 2003a; Pramanik et al., 2003; Monje et al., 2005
ERK	Wisdom, 1999; Fujioka et al., 2004; Monje et al., 2005
JNK	Smeal et al., 1991; Franklin et al., 1992; Smeal et al., 1992; Hibi et al., 1993; Kallunki et al., 1996; Wisdom, 1999; Bast et al., 2000; Davis, 2000; Chang et al., 1994; Mathas et al., 2002; Yazgan & Pfarr, 2002; Hess et al., 2004; Shahabi et al., 2006
PI3K/Akt	Li et al, 2004
MEKK1	Kim et al., 1999
Jak-Stat	Symes et al., 1997
PKC	Li et al., 2000c; Arnott et al., 2002
Src	Okada et al., 2000; Granet et al., 2002

AP-1 and Cancer

AP-1 has been linked to cancer by extensive research. v-Fos and v-Jun, the retroviral homologs of c-Fos and c-Jun were first identified in Finkel-Biskis-Jinkins osteosarcoma virus and avian sarcoma virus 17, respectively (Vogt, 2002; Eferl & Wagner, 2003a), and later found to be members of the AP-1 family. AP-1 has also been found to be induced by tumor promoters further suggesting a link between AP-1 and transformation. During transformation, the transactivation domain plays a significant role and those proteins lacking the domain have either a weak or nonexistent transforming ability (Eferl & Wagner, 2003a). AP-1 has been linked with cancers such as fibrosarcoma, squamous cell carcinoma, hepatocarcinoma, chronic myeloid leukemia, Hodgkin lymphoma, rhabdosarcoma, osteosarcoma (Hess et al., 2004), and gliomas (Debinski & Gibo, 2005), as well as many cancer physiological

processes (Table 19).

Table 19

Cancer Processes Activated by AP-1.

Process	*References*
Angiogenesis	Crowe et al., 1999; Westermarck & Kahari, 1999; Leaner et al., 2005; Xie et al., 2005
Growth	Leaner et al., 2005
Proliferation	Leaner et al., 2005
Apoptosis	Leaner et al., 2005
Invasion	Leaner et al., 2005; Xie et al., 2005
Metastasis	Reichmann et al., 1992; Hennigan et al., 1994; Miao & Curran, 1994; Lamb et al., 1997a; Lamb et al., 1997b; Malliri et al., 1998; Johnston et al., 2000; Ozanne et al., 2000; Spence et al., 2000; Stapleton et al., 2002; Eferl & Wagner, 2003a; Zhai et al., 2003; Bahassi et al., 2004; McGarry et al., 2004; Scott et al., 2004; Leaner et al., 2005; Spence et al., 2006

c-Jun

c-*jun*, an immediate early gene that encodes a 39 kDa protein that maps to an area of chromosome 1 that has frequent translocations and deletions involved in cancer, and was first identified as a homolog of the retroviral oncogene v-*jun*. c-Jun is considered a strong transactivator and regulation occurs through phosphorylation by JNK which increases its half-life through decreases in ubiquitination and degradation (Treier et al., 1994; Bishop & Weinberg, 1996; Fuchs et al., 1996; Musti et al., 1997; Kayahara et al., 2005; Monje et al., 2005), and through gene transcription control (Angel et al., 1988; Kayahara et al., 2005) which is partly through AP-1 sites in the c-*jun* promoter.

c-Jun has been shown to play a role in many physiological processes (Table 20) as well as embryogenesis. Knockout studies have shown that lacking c-Jun is

lethal at embryonic day 12.5 (Short & Pfarr, 2002; Eferl et al., 2003b; Naito et al., 2005) and that embryonic fibroblasts proliferate slower than normal cells.

Table 20

Physiological Processes Activated by c-Jun.

Process	*References*
Proliferation	Herdegen et al., 1997; Ham et al., 2000; Szabowski et al., 2000; Jochum et al., 2001; Mechta-Grigoriou et al., 2001; Behrens et al., 2002; Qi et al., 2002; Short & Pfarr, 2002; Vogt, 2002; Eferl et al., 2003b; Li et al., 2003b; Zenz et al., 2003; Hess et al, 2004; Besirli et al., 2005; Sun et al., 2005
Growth	Varmus & Weinberg, 1993
Differentiation	Szabowski et al., 2000; Hess et al., 2004
Survival	Jochum et al., 2001; Qi et al., 2002; Vogt, 2002
Hepatogenesis	Hilberg et al., 1993; Eferl et al., 1999; Szabowski et al., 2000; Behrens et al., 2002; Eferl et al., 2003b; Hess et al., 2004; Stepniak et al., 2006
Heart development	Hilberg et al., 1993; Eferl et al., 1999; Szabowski et al., 2000
Apoptosis	Herdegen et al., 1997; Ham et al., 2000; Szabowski et al., 2000; Jochum et al., 2001; Mechta-Grigoriou et al., 2001; Behrens et al., 2002; Qi et al., 2002; Short & Pfarr, 2002; Vogt, 2002; Eferl et al., 2003b; Li et al., 2003b; Zenz et al., 2003; Hess et al., 2004; Besirli et al., 2005; Sun et al., 2005
Transformation	Jochum et al., 2001; Qi et al., 2002; Vogt, 2002

Regulation of c-Jun can occur by different agents and on different levels. Stress and growth factors such as PDGF can stimulate intracellular pathways such as MAPK and JNK via PI3K/Akt to activate c-Jun. Heterodimerization with c-Fos increases stability (Halazonetis et al., 1988; Kouzarides & Ziff, 1988; Shaulian & Karin, 2001), epigallocatechin (EGCG) down regulates c-Jun, and the bacterium *Helicobacter pylori* induces c-*jun* transcription (Meyer-ter-Vehn et al., 2000).

Specific proteins and protein dimers have been found to have specific effects such as c-Jun/Fra2 promotes growth (Bakiri et al., 2002; Eferl & Wagner, 2003a), while c-Jun/c-Fos causes anchorage independence (van Dam & Castellazzi, 2001;

Shaulian & Karin, 2002; Eferl & Wagner, 2003a). c-Jun has been shown to interact with NF-κB protein p65 to increase binding efficiency to NF-κB sites as well as AP-1 sites (Stein et al., 1993; Smith et al., 1999) and RelB can be induced by c-Jun/Fra2 heterodimer in response to cytomegalovirus IE1 (Wang et al., 1995).

As a proto-oncogene, c-Jun has been implicated in numerous cancers such as fibrosarcoma, squamous cell carcinoma, and hepatocarcinoma, and cancer processes such as invasion, metastasis, and anchorage-independence. *ras* induced transformation occurs via c-Jun. Over-expression enhances osteosarcoma (Wang et al., 1995; Mechta-Grigoriou et al., 2001; Su et al., 2005) as well as glioma (Akbasak & Sunar-Akbasak, 1992) formation, and causes an invasive and hormone resistant breast cancer (Smith et al., 1999). Hodgkin lymphoma expresses aberrant c-Jun which causes cellular proliferation which increases in conjunction with NF-κB (Mathas et al., 2002). c-*jun* is required early in hepatic tumor development (Eferl et al., 2003b). Invasion of squamous cell carcinoma is dependent upon c-Jun (Malliri et al., 1998; Spence et al., 2006). c-Jun has been shown to be needed in androgen-independent prostate cancer (Edwards et al., 2004). Interestingly, in some cell types, differentiation states, and/or stages of the tumor can cause c-Jun induced tumor suppression (Eferl & Wagner, 2003a).

JunB

JunB, a proto-oncogene located on chromosome 19 is an immediate early gene that exhibits low protein levels until exposed to serum which activates transcription within 15 minutes. v-src has also been shown to increase *jun*B transcription (Apel et

al., 1992) and it has been shown to have anti-proliferative effects in the cell (Eferl & Wagner, 2003a).

The role of JunB in cellular processes and development suggest a role in pregnancy and embryogenesis. Knockout of *jun*B showed no alterations in cell growth in culture, but mice were born with osteopenia and a chronic myeloid leukemia-like disease (Kenner et al., 2004), while other studies reported that the lack of JunB is lethal to the embryo (Eferl & Wagner, 2003a; Naito et al., 2005). JunB is a negative regulator of proliferation, but in the absence of c-Jun, it can promote proliferation, suggesting the anti-proliferative effect requires a c-Jun/JunB heterodimer (Hess et al., 2004). JunB has role in placentation (Schorpp-Kistner et al., 1999; Szabowski et al., 2000; Kenner et al., 2004), pro- and anti-differentiation that is cell specific (Hartenstein et al., 2002; Hess et al., 2004; Meixner et al., 2004), and transcriptional regulator of myelopoiesis (Passegue et al., 2004).

It has been shown that c-Jun and JunB have antagonizing effects. These effects have been shown for cell cycle progression, transformation, differentiation, and AP-1-dependent target gene expression, but both stimulate angiogenesis.

JunB has been shown to have positive as well as negative effects on cancer that is specific to the cancer. It has been shown to be involved in chronic myeloid leukemia and Hodgkin lymphoma, but has also been shown to have tumor suppressor activity in some cell types. Hypermethylation of *jun*B impairs expression in chronic myeloid leukemia (Yang et al., 2003; Hess et al., 2004). JunB is a negative regulator of Ras-mediated transformation (Mechta-Grigoriou et al., 2001). Thus far, there has

been no role shown for JunB in gliomas.

JunD

*jun*D, the third member of the Jun family is located on chromosome 19. High levels of JunD are found in quiescent cells while the lack of JunD causes altered growth, yet JunD switches from growth promotion to growth suppression in the presence of the tumor suppressor menin (Agarwal et al., 1999; Knapp et al., 2000; Agarwal et al., 2003; Eferl & Wagner, 2003a; Hess et al., 2004).

JunD has been shown to have both positive and negative roles in many cellular processes. JunD plays a role in differentiation of T-helper cells and suppresses differentiation of osteoblasts. It also plays a role in both pro- and anti-apoptosis depending upon cell type. Lymphocytes have been shown to have increased proliferation when JunD is absent. Knockout of JunD exhibits male sterility (Eferl, 2003a) reduced proliferation and smaller cell size (Behrens et al., 2002; Thepot et al., 2000; Weitzman et al., 2000; Shaulian & Karin, 2001; Shaulian & Karin, 2002).

Changes in JunD expression has been shown to be a factor in cancer. Over-expression of JunD exhibits slowed cell growth and antagonizes *ras*-induced transformation (Schutte et al., 1989; Castellazzi et al., 1991; Pfarr et al., 1994; Mechta-Grigoriou et al., 2001; Short & Pfarr, 2002; Meixner et al., 2004), while constitutive activity of NF-κB p50/p65 along with JunD/Fra-1 are responsible for the deregulated interleukin 6 in prostate cancer (Szabowski et al., 2000). No role for JunD in gliomas has been found.

c-Fos

c-Fos is a 55 kDa protein first isolated in 1983 (Van Beveren et al., 1983). c-Fos exists at low levels in resting cells, but upon stimulation by growth factors such as PDGF, c-Fos levels increase within minutes making c-Fos an immediate early gene. c-Fos is implicated in numerous cellular processes (Table 21).

Regulation of c-Fos comes from many different sources. It is sensitive to growth factors and requires PDGF pathway members SHP-2 and Shc. Regulation of c-Fos through PDGF via ERK causes an increase in c-Fos (Chen et al., 1993; Treisman, 1994; Whitmarsh et al., 1995; Murphy et al., 2002; Monje et al., 2003; Monje et al., 2005). c-Fos expression is induced by *Helicobacter pylori* via MAPK (Meyer-ter-Vehn et al., 2000) and Fra-1 has been shown to repress c-*fos* (Kessler et al., 1999).

c-Fos is implicated in numerous cancers and cancer processes. Some cancers associated with c-Fos are rhabdosarcoma, osteosarcoma, and squamous cell carcinoma. c-Fos enhances the transforming ability of c-Jun and creates a more stable heterodimer. c-Fos has been implicated in the invasion of breast cancer (Milde-Langosch et al., 2004) and constitutive expression in transformation of cervical cancer (van Riggelen et al., 2005). Over-expression in fibroblasts causes transformation (Miller et al., 1984; Wang et al., 1991; Grigoriadis et al., 1995; Orlandini et al., 1996; Matsuo et al., 1999; Shaulian & Karin, 2001), and osteosarcomas have been seen to develop (Grigoriadis et al., 1993; Naito et al., 2005), yet it has the ability to suppress tumor formation and anchorage-independent growth in some cell types (Hess et al.,

2004). Interestingly, in glioblastoma cells c-Fos is amplified and over-expressed (Wen et al., 1989), yet the protein levels are low (Debinski et al., 2001).

Table 21

Cellular Processes in which c-Fos Plays a Role.

Process	*References*
Gametogenesis	Wang et al., 1991; Johnson et al., 1992; Orlandini et al., 1996
Bone cell formation	Wang et al., 1991; Johnson et al., 1992; Grigoriadis et al., 1993; Grigoriadis et al., 1994; Grigoriadis et al., 1995; Orlandini et al., 1996; Matsuo et al., 1999; Naito et al., 2005
Loss of cell polarity	Miao & Curran, 1994; Orlandini et al., 1996
Transformation	Miller et al., 1984; Wang et al., 1991; Grigoriadis et al., 1995; Orlandini et al., 1996; Matsuo et al., 1999; Shaulian & Karin, 2001
Apoptosis	Mikula et al., 2003; Hess et al., 2004
Lymphocyte proliferation	Wang et al., 1991; Johnson et al., 1992; Orlandini et al., 1996
Development	Grigoriadis et al., 1993; Naito et al., 2005
Phospholipid biosynthesis	Borioli et al., 2002
Scaffold in transcriptional complexes	Kaneyama et al., 2002
Bone homeostasis	Takayanagi et al., 2002
Cell cycle progression	Hess et al. 2004
Proliferation	Orlandini et al., 1996
Tumor development	Orlandini et al., 1996
Tumor progression	Orlandini et al., 1996
Angiogenesis	Marconcini et al., 1999; Eferl & Wagner 2003a
Invasion	Reichmann et al., 1992; Fialka et al., 1996; Eferl & Wagner, 2003a; Milde-Langosch et al., 2004
Metastasis	Reichmann et al., 1992; Fialka et al., 1996; Eferl & Wagner, 2003a
Differentiation	Grigoriadis et al., 1995; Orlandini et al., 1996; Matsuo et al., 1999; Hess et al., 2004

FosB

FosB, the FBJ murine osteosarcoma viral oncogene homolog B located on chromosome 19 exists in two isoforms, FosB and ΔFosB. In the resting cell, FosB levels are low but upon stimulation levels increase rapidly.

FosB has generally been shown to play a role in many cellular processes in

immune and skeletal cells. FosB regulates gene expression of anti-Ig-mediated B-cell activation (Dickinson et al, 1995), cell cycle progression in T cells (Hess et al., 2004), differentiation in bone cells (Hess et al., 2004), and mechanical stress-induced signaling that activates osteoblasts (Inoue et al., 2004). In addition to its role in immune and skeletal cell processes, FosB has been implicated in cancer processes of angiogenesis, and invasion, and ΔFosB has been implicated as a molecular switch in addiction (Nestler et al., 2001).

Changes in FosB levels cause minor changes within the cell compared to other members of AP-1. Over-expression does not transform cells but causes increased bone formation and development of osteosclerosis and knockout of the gene show normal proliferation, but have nuturing defects, but knockout of FosB as well as c-Fos have a reduced proliferative capacity and are smaller (Brusselbach et al., 1995; Brown et al., 1998; Shaulian & Karin, 2002).

Fra-1

Fra-1 was isolated in 1988 (Cohen & Curran, 1988; Ito et al., 1990) and the gene is located on chromosome 11. Fra-1 lacks a transactivation domain and does not induce tumors.

Fra-1 has been shown to play a role in bone and eye cell regulation as well as development. Fra-1 is important in osteoclast lineage and is a bone matrix activator. Fra-1 potentiates osteoclastic differentiation and differentiation of bone cells. Fra-1 has been shown to be involved in apoptosis of photoreceptor cells (Hess et al., 2004). Knockout of Fra-1 leads to embryonic lethality at E9.5 caused by placental defects

and osteosclerosis (Eferl & Wagner, 2003a; Eferl et al., 2004).

Many AP-1 proteins have been shown to regulate Fra-1. Fra-1 is activated through the PI3K and Akt pathway and can be transcriptionally activated by the c-Jun/c-Fos heterodimer. Constitutive expression of c-Fos, FosB, Fra-1 or c-Jun up-regulates *fra*-1 (Bergers et al., 1995).

Fra-1 is implicated in numerous cancers and stimulates invasion and is up-regulated in invasive tumors. In glioma cells, Fra-1 is constitutively over-expressed which leads to proliferative inhibition, apoptosis and reduced tumorigenicity (Shirsat & Shaikh, 2003) and is implicated in their maintenance and progression (Debinski & Gibo, 2005). Fra-1/c-Jun heterodimer has been found to be up-regulated in glioblastoma (Debinski et al., 2001). Over-expression of Fra-1 has been detected in colorectal adenocarcinomas and increased ERK-MAPK protects Fra-1 from proteasomal degradation. High levels of Fra-1 are required for negative AP-1 regulation by estradiol in breast cancer (Philips et al., 1998) and are associated with a more malignant phenotype in breast cancer (Belguise et al., 2005). Over-expression of Fra-1 leads to lung tumors (Jochum et al., 2000; Eferl & Wagner, 2003a). In mesotheliomas, Fra-1 is linked to anchorage-independent growth (Ramos-Nino et al., 2002).

Fra-2

The Fra-2 proto-oncogene located on chromosome 2 produces a 46 kDa protein that was isolated in 1990 (Ito et al., 1990; Matsui et al., 1990; Nishina et al., 1990). It has been found to be present in fibroblasts at low levels and increase

slightly upon serum addition. Fra-2 has been shown to be required during development by knockout studies that show missing Fra-2 is lethal at birth and is partly regulated by c-Fos and c-Jun.

Fra-2 has been shown to be involved in cancer as well as addiction. Cocaine up-regulates Fra-2 (Liu et al., 2005). Over-expression of Fra-2 has been shown to lead to epithelial tumors (Jochum et al., 2000; Eferl & Wagner, 2003a) and Fra-2 transforms cells when over-expressed by avian retrovirus (Nishina et al., 1990). Fra-2 lacks a transactivating domain and cannot transform cells.

NF-κB and AP-1

NF-κB and AP-1 are activated by many of the same stimuli, yet they are generally regulated by different pathways. AP-1 response is enhanced by the presence of NF-κB (Fujioka et al., 2004) and NF-κB can induce expression of c-*fos* and *fos*B via ERK (Fujioka et al., 2004). c-Jun/Fra-2 has been shown to inhibit the transactivating effects of NF-κB (Li, 2000c; Udalova & Kwiatkowski, 2001) as well as AP-1 and suppresses anchorage-independent growth (Li et al., 2000c).

The Hypothesis

Our lab has previously shown that the Grade III anaplastic astrocytoma cell line U-87 MG (ATCC) over-expresses PDGF which directly causes over-expression of the transcription factors NF-κB and AP-1 which lead to the following research hypothesis:

The different subunits of NF-kB and AP-1 play different roles in cell growth,

survival and differentiation in the U-87 MG astrocytoma cell line.

CHAPTER II

MATERIALS AND METHODS

Cell Cultures

The established cell line U-87 MG grade III astrocytoma cells (ATTC) were used in the transfection of the various shRNA recombinant plasmid DNAs, as well as the four stable cell lines created. These cells were grown in Minimum essential medium (MEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) and 0.5% penicillin/streptomycin antibiotic (Invitrogen). The cells received fresh MEM on Monday, Wednesday and Friday and were sub-cultured when they were approximately 80% confluent. The cells were washed with 1ml 1x trypsin-EDTA (Invitrogen; 0.025% trypsin, 0.01% EDTA, pH 7.4) followed by a five minute incubation of 1ml 1x trypsin-EDTA. The cells were then removed from the plate via repeated pipetting. The cells were counted with a hemocytometer and added to new plates according to plate size. 100mm tissue culture plates (Falcon) received 1,000,000 cells/ml, 60mm tissue culture plates (Falcon) 250,000 cells/ml, 8-well tissue culture plates (Nunc) 50,000 cells/ml, and 96 well plates (Corning) 5,000 cells/ml. All work was done within a SterilGard II laminar flow hood. Plates were incubated at 37^{0}C and 5% CO_2 in a Fisher Scientific incubator.

The initial culture came from lab stock stored in liquid nitrogen (-195^{0}C). This was placed into a 100mm tissue culture dish (Falcon) with MEM (Invitrogen), 10% FBS (Invitrogen) and 0.5% penicillin/streptomycin antibiotic (Invitrogen). For

all cell lines, at first sub-culture, half of the cells were returned to liquid nitrogen storage. This was done by placing 500µl into a 1.7ml tube and centrifuged (Eppendorf 5415C) at 455 x g for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in 1ml 95% Fetal Bovine Serum (FBS; Invitrogen) and 5% DMSO (Sigma). The cells were then transferred to a 2ml cryovial (Corning). This was placed into a benchtop microcentrifuge cooler and placed into a -80^{0}C freezer for 24 hours. The cryovial was then transferred to liquid nitrogen.

Small Hairpin RNA (shRNA) Plasmid Construction

Small hairpin RNA (shRNA) plasmids were generated for all AP-1 and NFκB transcription factor proteins as well as scrambled controls for a total of 48 plasmid constructs. These were used to suppress protein expression of the various subunits of AP-1 and NF-κB. Sequence location was 63-190 nucleotides from the start codon, began with an adenine dimer, had a guanine/cytosine content ranging from 47-80%, and was 19 nucleotides in length (Elbashir et al., 2001a; Elbashir et al., 2001b). The 19 nucleotide DNA sequences that were chosen were also scrambled to create controls. Once the sequence and its control were found they were compared to other DNA sequences using the BLAST search at the National Center for Biotechnology Information (NCBI) website to verify that each sequence did not target other mammalian genes. These sequences were then inserted into a DNA template (Figure 5) and four separate oligonucleotides varying in length from 26-38 nucleotides were synthetically produced (Figure 6; Invitrogen). Upon transcription a hairpin loop allowed the creation of double stranded RNA (Figure 7 Paddison et al., 2002).

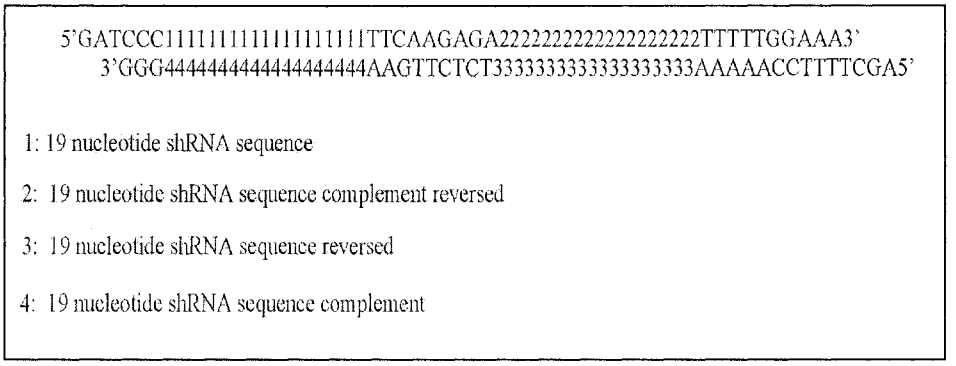


Figure 5. DNA Template to Create shRNA. The 5' and 3' ends contain restriction enzyme recognition sequences for BglII and HindIII respectively.

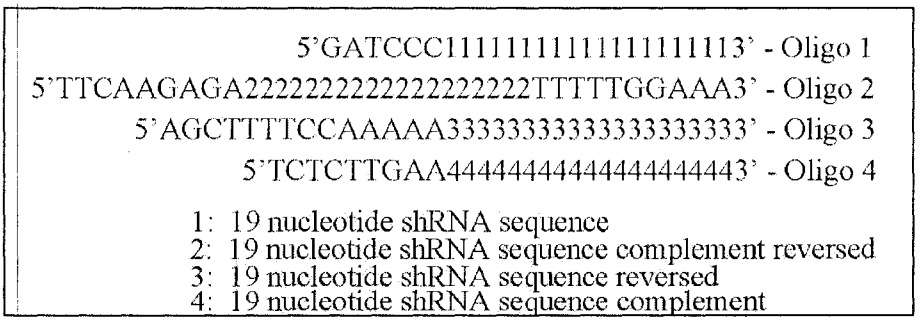


Figure 6. Oligonucleotides Used to Create shRNA.

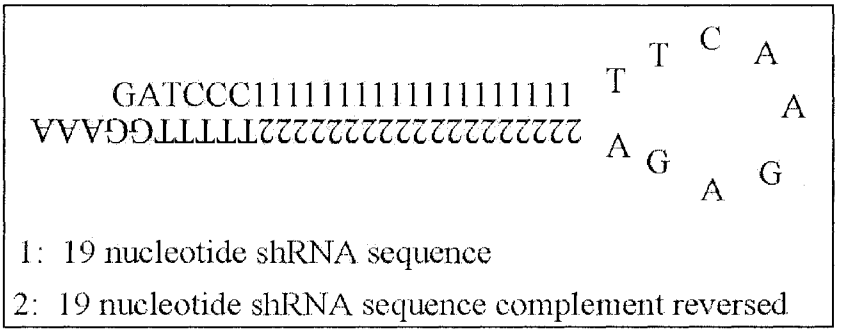


Figure 7. shRNA Hairpin. The only base pairing occurs between 1 and 2, creating the dsRNA.

The four lyophilized oligonucleotides (oligos) were reconstituted to a

concentration of 0.5μg/μl DNA in distilled-deionized water via vortexing (Fisher Vortex Genie 2). The oligos (2μg) were then phosphorylated in a solution of 25μl of T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5; New England Biolabs) and 10 units of T4 DNA polynucleotide kinase (PNK; New England Biolabs) in four separate 0.65ml tubes for 1 hour at 37°C in a Thermolyne 17600 dry heat block. The oligos were then allowed to hybridize by combining 0.5μg of oligo 1 and 0.5μg of its compliment oligo 4 in one tube and 0.5μg of oligo 2 and 0.5μg of its compliment oligo 3 in a second tube with 2μl of 10X annealing buffer (100mM Tris-HCl pH 7.5, 1M NaCl, 10mM EDTA) and 21μl DEPC treated distilled-deionized water in each tube thereby creating 2 double-stranded oligos. These were incubated at 65°C for 10 minutes in a dry heat block (Thermolyne 17600) and allowed to cool at room temperature for 1 hour.

The pBlueScript plasmid vector (2μg; Stratgene), with the H1 RNA promoter inserted into the polycloning site (pBlueScript+H1) was digested with 5 units *Hind*III restriction endonuclease (New England Biolabs), 2μl buffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9), and distilled-deionized water to 20μl. This was incubated at 37°C for 1 hour in a dry heat block (Thermolyne 17600) followed by digestion with 5 units *Bgl*II (New England Biolabs) at 37°C for 1 hour in a dry heat block.

The hybridized oligos (0.04μg from each tube) were combined with the digested plasmid vector (0.2μg) and distilled-deionized water for ligation. This was

incubated at 45°C for 5 minutes in a water bath and cooled 3 minutes on ice. Following incubation, 2µl DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5) and 400 units T4 DNA ligase (New England Biolabs) were added to the tube for a final reaction volume of 20µl and incubated at 16°C for 1 hour in a water bath.

The recombinant plasmid DNA was then transformed into DH5α *Escherichia coli* (*E. coli*) cells. This was done by adding the ligation mixture from above to 200µl chemically competent DH5α cells and incubated on ice for 30 minutes. The cells were heat shocked at 45°C for 90 seconds in a water bath and then placed on ice for 3 minutes. The transformed cells were allowed to recover in 800µl of Luria Bertani broth (LB; 8mg tryptone, 4mg yeast extract, 8mg NaCl in 800µl distilled-deionized water, pH 7.5) without ampicillin at 37°C in a shaking incubator (New Brunswick Scientific). 200µl of the transformed cells were spread on an LB (40 mg tryptone, 16mg yeast extract, 40mg NaCl, 60mg agar in 4ml distilled-deionized water, pH 7.5) with ampicillin (60µg/ml) agar plate and incubated at 37°C overnight. Individual bacterial colonies were transfered from the plate grown overnight in 3ml liquid LB (30mg tryptone, 12mg yeast extract, 30mg NaCl in 3ml distilled-deionized water, pH 7.5) with ampicillin (60µg/ml) in a shaking incubator (New Brunswick Scientific) at 37°C.

To isolate the recombinant plasmid DNA, 1.5ml of the overnight culture was centrifuged (Eppendorf 5415C) in a 1.7ml tube at 14,000 x g for 10 minutes. The 1.5ml volume of the culture remaining was refrigerated. Following centrifugation,

the supernatant was removed and the pellet resuspended in 350µl STET buffer (100mM NaCl, 10mM Tris-HCl pH 8, 1mM EDTA pH 8, and 5% Triton X-100). 10µl of lysozyme (50mg/ml) was added to the tube, briefly vortexed (Fisher Vortex Genie 2), and placed in boiling water for 45 seconds. The tube was then centrifuged (Eppendorf 5415C) for 15 minutes at 14,000 x g. After centrifugation, the cell debris pellet was removed. 35µl of 3M Sodium Acetate pH 5.2 and 350µl of Isopropanol was added to the tube, briefly vortexed (Fisher Vortex Genie 2), and centrifuged (Eppendorf 5414C) for 5 minutes at 14,000 x g. The supernatant was removed and the DNA pellet was resuspended in 200µl of distilled-deionized water, 4 µl of 5M NaCl and 500µl of ice cold ethanol, briefly vortexed (Fisher Vortex Genie 2), and centrifuged (Eppendorf 5415C) at 14,000 x g for 5 minutes with the supernatant removed at the end.

The isolated recombinant plasmid DNA was analyzed by restriction endonuclease digestion. The pellet was allowed to air dry briefly and resuspended in 2µl Buffer 3 (New England Biolabs; 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9), 5 units of *Bgl*II restriction endonuclease (New England Biolabs), and distilled-deionized water for a 20µl reaction volume, briefly vortexed (Fisher Vortex Genie 2) and incubated at 37°C for 1 hour in a dry heat block (Thermolyne 17600). The digested DNA along with digested and undigested pBlueScript+H1 (controls) were electrophoresed, after 2µl of loading dye was added to the sample, for 2 hours at 50 V in a 1% agarose gel (Invitrogen) with 2µl ethidium

bromide (BioRad; 10mg/ml) using a Mini Sub Cell GT electrophoresis apparatus (BioRad) and a Power Pac 300 power supply (BioRad). The gel was visualized via a UV light box (Fotodyne).

When a digested DNA sample failed to be cleaved with the restriction endonuclease, the 1.5ml refrigerated bacterial culture was again grown in 3ml of liquid LB with ampicillin as previously described. The plasmid from this culture was isolated using a Mini-prep kit (Qiagen) to enable DNA sequencing at a later time. The 1.5ml culture was centrifuged (Eppendorf 5415C) for 10 minutes at 14,000 x g, the supernatant removed, and the plasmid DNA was isolated as follows. The remaining 1.5ml culture was refrigerated. The pellet was resuspended in 300µl Buffer P1 (50mM Tris-HCl, pH 8; 10mM EDTA; 100µg/ml RNase A) by vortexing (Fisher Vortex Genie 2). Buffer P2 (300µl; 200mM NaOH; 1% SDS) was added; the tube inverted five times to mix, and incubated at room temperature for five minutes. Ice-cold buffer P3 (300µl; 3M potassium acetate, pH 5.5) was added; the tube inverted five times to mix, and incubated on ice for five minutes. The tube was centrifuged (Eppendorf 5415C) for 10 minutes at 14,000 x g. During centrifugation, a Qiagen 20 gravity flow column was equilibrated with 1ml buffer QBT (750mM NaCl; 50mM MOPS, pH 7; 15% isopropanol; 0.15% Triton X-100) which was allowed to flow through the column. The supernatant was loaded onto the column. After the supernatant passed through, the column was washed with 2ml buffer QC (1M NaCl; 50mM MOPS, pH 7; 15% isopropanol) x two. The DNA was eluted into a 1.7ml tube using 800µl buffer QF (1.25M NaCl; 50mM Tris-Cl, pH 8.5; 15% isopropanol).

The DNA was precipitated by addition of 560µl room temperature isopropanol, mixed via vortexing (Fisher Vortex Genie 2) and centrifuged (Eppendorf 5415C) for 30 minutes at room temperature at 14,000 x g. The supernatant was removed; the pellet was allowed to air dry and was resuspended in 25µl of distilled-deionized water by vortexing (Fisher Vortex Genie 2). The isolated DNA (10µl) was digested with *Bgl*II and analyzed by electrophoresis as above. Any DNA bands on the electrophoresis gel which migrated through the gel like the undigested plasmid control had DNA sequencing performed by Dr. Todd Barkman's lab (Western Michigan University). This was repeated for each AP-1 and NF-κB subunit and scrambled control (see appendix). For many of the subunits and controls a second set of shRNA was generated to show specificity of the shRNA and provide a further control.

Each sequenced DNA was verified by comparison with original shRNA sequence and correct sequences had the 1.5ml refrigerated culture grown overnight in 100ml of liquid LB (1gm tryptone, 0.4gm yeast extract; 1gm NaCl in 100ml distilled-deionized water, pH 7.5) with ampicillin (60µg/ml) at 37°C in a shaking incubator (New Brunswick Scientific). The recombinant plasmid DNA was isolated using a plasmid midi-prep kit (Qiagen). The culture was centrifuged (Sorvall) at 36,000 x g and 4°C for 15 minutes and the supernatant removed. The pellet was resuspended in 4ml P1 buffer (50mM Tris-HCl, pH 8; 10mM EDTA; 100µg/ml RNase A) by vortexing (Fisher Vortex Genie 2). P2 buffer (4ml; 300µl; 200mM NaOH; 1% SDS) was added to the tube; the tube inverted five times to mix, and incubated at room

temperature for five minutes. Ice-cold P3 buffer (4ml; 300µl; 3M potassium acetate, pH 5.5) was added to the tube; the tube inverted five times to mix, and incubated on ice for fifteen minutes. The tube was centrifuged (Sorvall) for 30 minutes at 65,000 x g at 4°C. During centrifugation, a Qiagen 100 gravity flow column was equilibrated with 4ml buffer QBT (750mM NaCl; 50mM MOPS, pH 7; 15% isopropanol; 0.15% Triton X-100) which was allowed to flow through the column. The supernatant was loaded onto the column. After the supernatant passed through, the column was washed with 10ml buffer QC (1M NaCl; 50mM MOPS, pH 7; 15% isopropanol) x 2. The DNA was then eluted into a 50ml centrifuge tube using 5ml buffer QF (1.25M NaCl; 50mM Tris-Cl, pH 8.5; 15% isopropanol). The DNA was precipitated by addition of 3.5ml room temperature isopropanol (Sigma), briefly vortexed (Fisher Vortex Genie 2), and centrifuged (Sorvall) for 30 minutes at 4°C and 56,000 x g. The supernatant was removed and the pellet was allowed to air dry and resuspended in 500µl of distilled-deionized water by vortexing (Fisher Vortex Genie 2).

A stock culture of each recombinant plasmid DNA in DH5α cells was kept at -80°C in freezing solution (65% glycerol, 100mM $MgSO_4$ and 25mM Tris-HCl pH 8). The isolated recombinant plasmid DNA was kept at -20°C.

Transfection of shRNA Recombinant Plasmid DNA

U-87 MG grade III astrocytoma cells (ATCC) were transfected individually with all AP-1 and NFκB transcription factor subunit shRNA plasmid DNAs as well as scrambled controls (see p.59). Cells were sub-cultured from stock plates into 100mm

tissue culture dishes (Falcon), 60mm tissue culture dishes (Falcon), 8-well tissue culture plates (Nunc) or 96 well tissue culture plates at concentrations described above (p. 58). A transfection reaction was set up 24 hours later. Into sterile 0.65ml tubes, 100µl of Minimum Essential Medium (MEM) without Fetal Bovine Serum (FBS), 6µl of FuGene 6 (Roche) transfection reagent and 2µg of shRNA plasmid were combined. This mixture was allowed to incubate for 30 minutes at room temperature. At the end of the incubation, the MEM with 10% FBS was removed and the cells were washed with MEM without FBS to remove any residual FBS. MEM (4ml) without FBS was added to the cells as was the contents of the 0.65ml tube and then gently swirled to mix. The plate was placed in the incubator at 37°C, 5% CO_2 for four hours. At the end of the four hours, MEM with 10% FBS was added to the cells and allowed to incubate 18 hours at 37°C and 5% CO_2.

Creation of pTet-On+Hygromycin Plasmid DNA

Inducible stable cell lines expressing the shRNA for c-Jun, c-Fos, p65, and c-Rel were created using the pTet-On plasmid (Clontech) and the pSuper plasmid (OligoEngine). Both the pTet-On and the pSuperior plasmids had the neomycin resistance gene to allow selection in the U-87 MG cells. With both plasmids expressing neomycin resistence there would be no way to determine which plasmid was in the cells. Therefore, the pTet-On plasmid had the neomycin resistence gene removed and hygromycin resistence gene inserted (Figure 8).

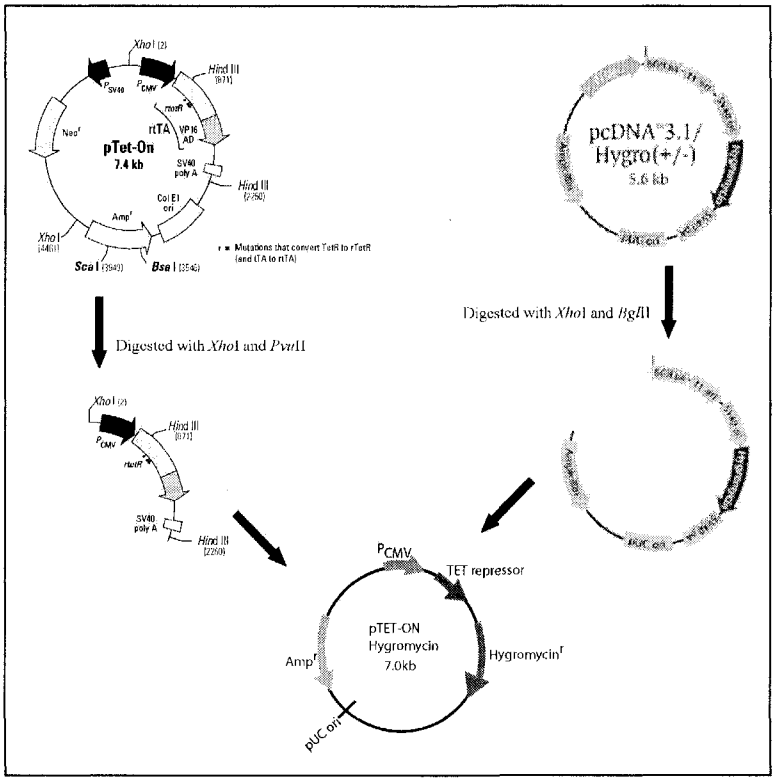


Figure 8. Creation of the pTet-On+Hygromycin Plasmid (pTet-On, Clontech; pcDNA 3.1+, Invitrogen).

pTet-On plasmid was digested with *Xho*I restriction endonuclease (New England Biolabs) in a 20µl reaction with 5 units *Xho*I, 2µl Buffer 2 (New England Biolabs; 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9), and distilled-deionized water at 37°C for 1 hour in a dry heat block (Thermolyne 17600) followed by digestion with 5 units *Pvu*II restriction endonuclease (New England Biolabs) for 1 hour at 37°C in a dry heat block (Thermolyne 17600). The plasmid pcDNA 3.1+ was digested with *Bgl*II restriction endonuclease (New England Biolabs) in a 20µl reaction with 5 units *Bgl*II, 2µl Buffer 2 (New England Biolabs; 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9), and

distilled-deionized water at 37°C for 1 hour in a dry heat block (Thermolyne 17600) followed by digestion with 5 units *Xho*I (New England Biolabs) for 1 hour at 37°C in a dry heat block (Thermolyne 17600).

Loading dye (2µl) was added to the digested pTet-On and pcDNA 3.1+ plasmids as well as *Hind*III cut λDNA standard (New England Biolabs) and these were electrophoresed for 2 hours at 50 V in 1% agarose gel (Invitrogen) with 2µl ethidium bromide (BioRad; 10mg/ml) using a Mini Sub Cell GT electrophoresis apparatus (BioRad) and a Power Pac 300 power supply (BioRad). The gel was visualized via a UV light box (Fotodyne). The 2398bp DNA *Xho*I-*Pvu*II fragment from pTet-On (containing the cytomegalovirus (CMV) promoter and the tetracycline repressor gene) was removed from the gel as was the 4487bp DNA *Xho*I-*Bgl*II fragment from pcDNA3.1+ (which had the CMV promoter removed) and placed into separate 0.65ml tubes which were weighed (138mg and 321mg, respectively) to facilitate the extraction of the DNA from the agarose gel.

DNA was extracted from the gel using the gel extraction kit (Qiagen). Three volumes Buffer QG (proprietary formula) was added to the gel slices (414µl and 963µl, respectively). The tubes were incubated at 50°C for 10 minutes in a dry heat block (Thermolyne 17600). One gel volume of room temperature isopropanol was added to the tubes (138µl and 321µl, respectively) and vortexed (Fisher Vortex Genie 2). Two QIAquick spin columns were placed into 2ml tubes and placed into the centrifuge (Eppendorf 5415C). The contents of the tubes were placed into the columns and centrifuged for 1 minute at 14,000 x g. The supernatant was discarded;

500µl of buffer QG (proprietary formula) was added to the column and centrifuged (Eppendorf 5415C) for 1 minute at 14, 000 x g. The supernatant was discarded; 750µl of buffer PE (proprietary formula) was added to the column and centrifuged (Eppendorf 5415C) for 1 minute at 14,000 x g. The supernatant was discarded and the column was centrifuged (Eppendorf 5415C) for 1 minute at 14, 000 x g. The column was placed into a new 1.7ml tube and the DNA was eluted with 20µl distilled-deionized water by centrifugation (Eppendorf 5415C) for 1 minute at 14,000 x g.

The 2398bp DNA *Xho*I-*Pvu*II fragment from pTet-On and the 4487bp DNA *Xho*I-*Bgl*II fragment from pcDNA3.1+ (2µg each) were placed into a 0.65ml tube and then incubated at 45°C for 5 minutes in a water bath and cooled for 3 minutes on ice. To the tube was added 2µl DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5) and 400 units T4 DNA ligase (New England Biolabs) in a 20µl reaction volume with distilled-deionized water and incubated at 16°C for 1 hour in a water bath. Two µl of 3M Sodium Acetate (pH 5.2) and 20µl of room temperature isopropanol was added to the tube and centrifuged (Eppendorf 5414C) for 5 minutes at room temperature and 14,000 x g. The supernatant was removed and the pellet was resuspended in 200µl of distilled-deionized water, 4µl of 5M NaCl and 500µl of ice-cold ethanol and centrifuged (Eppendorf 5415C) at room temperature for 5 minutes at 14,000 x g with the supernatant removed at the end. The pellet was resuspended in 10µl of water. The above procedure ligated the *Xho*I ends of 2398bp DNA *Xho*I-*Pvu*II fragment from

pTet-On and the 4487bp DNA *Xho*I-*Bgl*II fragment from pcDNA3.1+.

The *Bgl*II end of pcDNA 3.1+ had a 5' overhang which the *Pvu*II end of pTet-On did not have. This required the removal of the 5' overhang on the *Bgl*II end to allow ligation to the *Pvu*II blunt end and was done using the T4 DNA polymerase. To the tube containing the 10µl ligated *Xho*I ends of the 2398bp DNA *Xho*I-*Pvu*II fragment from pTet-On and the 4487bp DNA *Xho*I-*Bgl*II fragment from pcDNA3.1+.was added 2µl Buffer 2 (New England Biolabs; 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9), 20µM dNTPs, and 1 unit T4 DNA polymerase (New England Biolabs) in a 20µl reaction volume with distilled-deionized water. This was incubated for 30 minutes at 15°C in a water bath. The tube was then heated to 75°C for 10 minutes to inactivate the polymerase. 2µl of 3M Sodium Acetate (pH 5.2) and 20µl of room temperature isopropanol was added to the tube and centrifuged (Eppendorf 5414C) for 5 minutes at room temperature and 14,000 x g. The supernatant was removed and the pellet was resuspended in 200µl of distilled-deionized water, 4 µl of 5M NaCl and 500µl of ice cold ethanol and centrifuged (Eppendorf 5415C) for 5 minutes at room temperature and 14,000 x g with the supernatant removed at the end. The pellet was resuspended in 10µl of water. The tube was incubated at 45°C for 5 minutes in a water bath followed by cooling for 3 minutes on ice. To the tube was added 2µl DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5) and 400 units T4 DNA ligase (New England Biolabs) in a 20µl reaction volume with distilled-deionized water and incubated at 16°C for 1 hour in a water bath. The above

procedure ligated the blunt *Bgl*II and *Pvu*II ends creating the pTet-On+hygromycin plasmid.

The pTet-On+hygromycin plasmid was then transformed into DH5α cells as previously described. This was followed by isolation of the plasmid DNA as previously described. Electrophoresis was performed as previously stated except digested and undigested pcDNA 3.1+ was used for the comparison to verify correct plasmid size. Any DNA showing an undigested pattern had the 1.5ml refrigerated colony grown in 100ml LB broth (1gm tryptone, 0.4gm yeast extract; 1gm NaCl in 100ml distilled-deionized water, pH 7.5) with ampicillin (60μg.ml) overnight and the DNA was isolated using a plasmid midi-prep kit (Qiagen) as previously reported (p. 65). A stock culture of pTet-On+hygromycin plasmid DNA in DH5α cells were kept at -80°C in freezing solution (65% glycerol, 100mM $MgSO_4$ and 25mM Tris-HCl pH 8). The isolated pTet-On+hygromycin plasmid DNA was kept at -20°C.

Stable Cell Line Creation

The reconstituted oligos (2μg) c-Jun, c-Fos, c-Rel and p65 from earlier (p. 61) were phosphorylated in a solution of 25μl of T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5; New England Biolabs) and 10 units of T4 DNA PNK (New England Biolabs) in four separate 0.65ml tubes for 1 hour at 37°C in a dry heat block (Thermolyne 17600). The oligos were then allowed to hybridize by combining 0.5μg of oligo 1 and 0.5μg of its compliment oligo 4 in one tube and 0.5μg of oligo 2 and 0.5μg of its compliment oligo 3 in a second

tube with 2μl of 10X annealing buffer (100mM Tris-HCl pH 7.5, 1M NaCl, 10mM EDTA) and 21μl DEPC treated distilled-deionized water in each tube thereby creating 2 double-stranded oligos. These were incubated at 65°C for 10 minutes in a dry heat block (Thermolyne 17600) and allowed to cool at room temperature for 1 hour.

The pSuperior plasmid vector (2μg; OligoEngine) was digested with 5 units *Hind*III, 2μl Buffer 2 (New England Biolabs; 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, & 1 mM Dithiothreitol, pH 7.9), and distilled-deionized water in a 20μl reaction volume at 37°C for 1 hour in a dry heat block (Thermolyne 17600) followed by digestion with 5 units *Bgl*II restriction endonuclease (New England Biolabs). The hybridized oligos for c-Jun, c-Fos, c-Rel, and p65 (0.04μg each) were combined with the digested pSuperior plasmid vector (0.2μg) and distilled-deionized water for ligation in individual 0.65ml tubes. These were incubated at 45°C for 5 minutes in a water bath and then cooled for 3 minutes on ice. To the tubes were added 2μl DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM Dithiothreitol, pH 7.5), 400U T4 DNA ligase (New England Biolabs) for a 20μl reaction and was incubated at 16°C for 1 hour in a water bath. This created the pSuperior+c-Jun, pSuperior+c-Fos, pSuperior+c-Rel, and pSuperior+p65 plasmids

The four plasmids (pSuperior+c-Jun, pSuperior+c-Fos, pSuperior+c-Rel, and pSuperior+p65) were then transformed into DH5α cells and isolation of the plasmid was performed as previously reported (p. 63). The isolated plasmid DNA was analyzed by restriction endonuclease digestion as previously stated except digested and undigested pSuperior was used for comparison (p. 63). When a sample failed to

be cut with the restriction endonuclease, the remaining culture was again grown in 3ml of liquid LB with ampicillin and the DNA was isolated using a plasmid mini-prep kit (Qiagen) and DNA sequencing was performed by Dr. Todd Barkman's lab (Western Michigan University) as previously reported (p. 64). The sequences were verified by comparison with original shRNA sequences and the refrigerated bacterial colonies from correct sequences were grown in 100ml of liquid LB with ampicillin overnight and DNA was isolated using a plasmid midi-prep kit (Qiagen) as previously stated (p 65). A stock culture of pSuperior+c-Jun, pSuperior+c-Fos, pSuperior+c-Rel, and pSuperior+p65 plasmids in DH5α cells were kept at -80^{0}C in freezing solution (65% glycerol, 100mM $MgSO_4$ and 25mM Tris-HCl pH 8). The isolated plasmids were kept at -20^{0}C.

pTet-On+hygromycin was transfected into U-87 MG cells. Cells were sub-cultured from stock plates into a 60mm tissue culture dish (Falcon) and a transfection using 2μg of pTet-On+hygromycin was performed as previously stated (p. 67). The next morning fresh Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS) was added to the dish and the cells were allowed to grow for 48 hours. At this time the MEM with 10% FBS was changed and hygromycin (100μg/ml) was added to the medium. At each medium change, the hygromycin was added for 14 days at which time the hygromycin was removed from the medium. All cells remaining on the dish were assumed to be hygromycin resistant, suggesting the presence of the pTet-On+hygromycin plasmid. This created the U-87 MG+pTet-On+hygromycin cell line. A stock cell line of U-87 MG+pTet-On+hygromycin was

placed into liquid nitrogen storage.

The pSuperior+c-Jun, pSuperior+c-Fos, pSuperior+c-Rel, and pSuperior+p65 plasmids (p. 73) were individually transfected into the U-87 MG+pTet-On+hygromycin cell line. Cells were sub-cultured from U-87 MG+pTet-On+hygromycin cell line stock plates into four 60mm tissue culture dishes (Falcon) and transfection reactions were performed as previously reported (p. 67). Fresh MEM with 10% FBS was added 24 hours after transfection to the cells which were allowed to grow for 48 hours at 37^0C and 5% CO_2. At this time the MEM with 10% FBS was changed and neomycin (500μg/ml; Sigma) was added to the medium. At each medium change, the neomycin was added for 21 days at which time the neomycin was removed from the medium. All cells remaining on the dish were assumed to be neomycin resistant, suggesting the presence of the pSuper+c-Jun, pSuper+c-Fos, pSuper+c-Rel, or pSuper+p65 plasmids. This created the U-87 MG+pTet-On+hygromycin+pSuper+c-Jun, U-87 MG+pTet-On+hygromycin+pSuper+c-Fos, U-87 MG+pTet-On+hygromycin+pSuper+c-Rel, and U-87 MG+pTet-On+hygromycin+pSuper+p65 cell lines. These stable cell lines were created from a population of cells growing on the plate rather than a single clone. A culture of each cell line was placed into liquid nitrogen storage.

Proliferation Assay

This assay utilizes the dehydrogenase activity in the cell to cleave the tetrazolium salt WST-8 substrate to create the colored formazan dye product. The four stable cell lines were grown in 96 well plates (Corning) using six columns and

four rows for the induced cells and six columns and four rows for the non-induced cells. Upon introduction of cells to the plate, 2μg/ml of doxycycline was added to the medium of the induced wells to induce the plasmid and replenished with each medium change. Four hours after the introduction to the 96 well plates, a 1:10 dilution of Cell Counting Kit-8 (CCK8; Dojindo Laboratories) solution was put into the eight wells of the first column. This was incubated at 37^0 and 5% CO_2 for 60 minutes. A microplate spectrophotometer at a wavelength of 450nm was used to quantify the color change in the wells containing CCK8. The CCK8 was placed into all wells of the second column on day two, the third column on day four, and the fifth column on day six. On day four and six, columns four and six respectively had the doxycycline inducer removed by medium change to show that any changes in growth were not caused by the inducer. All were incubated for 60 minutes and read using the microplate reader. The optical densities were plotted for each date and growth curves for each stable cell line induced, non-induced, and inducer removal were generated. A student's t-test, standard deviation and standard error were performed comparing the induced to the non-induced results to determine whether the differences seen were statistically significant. This assay was repeated in triplicate.

SDS-Page and Western Blotting

U-87 MG cells were transiently transfected with 3μg of plasmid DNA in 60mm dishes with one dish for the plasmid containing the shRNA plasmid and one dish for the control plasmid. The four stable cell lines, pSuperior+pTet-On+hygromycin+c-Jun, pSuperior+pTet-On+hygromycin+c-Fos, pSuperior+pTet-

On+hygromycin+c-Rel, and pSuperior+pTet-On+hygromycin+p65 were grown in two plates, one for the induced with doxycycline and one for the non-induced. Upon introduction of stable cell lines to the dishes, 2 μg/ml of doxycycline was added to the medium of the induced plate and replenished with each medium change. Each set (transient and stable cell lines) was done in triplicate.

24 hours after transfection for the shRNAs and 48 hours after transfection for the stable cell lines, the cells were harvested by removing the MEM with 10% FBS and washing the dish with 2ml of cold PBS (without Mg^{+2} or Ca^{+2}; Invitrogen) twice. 200μl of lysis buffer (Cell Signaling Technologies; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) was placed onto the plate and incubated on ice for 5 minutes. The cells were scraped off the plate and placed into a 1.7ml microcentrifuge tube and centrifuged for 5 minutes at 4^0C. The supernatant was removed and placed into a new 1.7ml tube.

A protein concentration assay was performed using a BCA protein assay kit (Pierce). BSA standards (0, 0.1, 0.25, 0.5, 1, 1.5, 2, and 4mg/ml) and harvested protein supernatant were placed in triplicate into a 96 well plate. 200μl of assay buffer (50μl Assay Reagent A:1μl Assay Reagent B dilution) was placed into each well, covered, and incubated 30 minutes at 37^0C. The plate was read using a microplate spectrophotometer at 562nm wavelength. Using linear regression a standard curve was generated using the protein standards, and the unknown sample protein concentrations were determined.

Electrophoresis of the protein samples was done using an X Cell SureLock electrophoresis chamber (Novex) and a Power Pac 3000 (Bio-Rad). 20μg of total protein, from the transient shRNA or induced shRNA, scrambled or non-induced controls, and untreated U-87 MG cells were placed into individual 0.65ml tubes and 1/3 volume of Nupage LDS 2x sample buffer (Invitrogen) was added to each tube. The samples were incubated in a boiling water bath for 5 minutes and then loaded onto a 10% polyacrylamide gel (Invitrogen) with 1x Nupage MOPS SDS running buffer (Invitrogen) with 0.0025% Nupage antioxidants (Invitrogen).

The gel was electrophoresed for 75 minutes at 200 volts in an ice bath. During electrophoresis, an Immobulon nylon membrane (Millipore) was hydrated in methanol for ½ hour and then Tris-glycine (84mM Tris, 1M glycine, 10% methanol) transfer buffer for the remaining time. The gel was removed from the cassette and placed into an electric transfer apparatus with the Immobulon membrane (Millipore) and Tris-glycine transfer buffer (84mM Tris, 1M glycine, 10% methanol). The protein was transferred to the Immobulon membrane for 2 hours, 6 volts, at 4^0C. The membrane was then blocked using 0.2% I-Block (Tropix) solution (1x TBS, 0.2% I-Block, 0.0001% Tween) for 60 minutes at room temperature with shaking. The blocking reagent was removed and the membrane was incubated 18 hours in 0.2% I-Block solution (1x TBS, 0.2% I-Block, 0.0001% Tween) and the appropriate primary antibody for the shRNA used (0.0001%; Table 21) with shaking at 4^0C.

18 hours after the primary antibody was introduced, the membrane was washed x 3 with 1X TBS 0.01% Tween for 5 minutes, 10 minutes and 20 minutes.

The membrane was then incubated in 0.2% I-Block solution (1X TBS, 0.2% I-Block, 0.0001% Tween) and the secondary antibody either mouse or rabbit depending upon what organism the primary antibody came from (1:2500 dilution; Amersham) for 60 minutes with shaking at room temperature. The membrane was again washed x 3 with TBS-Tween 0.01% for 5 minutes, 10 minutes, and 20 minutes.

Table 22

Antibodies Used in Western Blots.

Antibody	*Manufacturer*	*Grown In*
c-Jun	Upstate	Rabbit (polyclonal)
JunB	Santa Cruz	Mouse (monoclonal)
JunD	Santa Cruz	Rabbit (polyclonal)
c-Fos	Upstate	Rabbit (polyclonal)
FosB	Santa Cruz	Mouse (monoclonal)
Fra-1	Santa Cruz	Rabbit (polyclonal)
Fra-2	Santa Cruz	Rabbit (polyclonal)
c-Rel	Upstate	Rabbit (polyclonal)
p50	Upstate	Rabbit (polyclonal)
p52	Upstate	Rabbit (polyclonal)
p65	Upstate	Rabbit (polyclonal)
RelB	Santa Cruz	Mouse (monoclonal)
Cleaved PARP	Cell Signaling Technologies	Rabbit (polyclonal)
Nestin	Chemicon	Mouse (monoclonal)

The membrane was then taken to the darkroom where the wash buffer was removed and 1ml of each of the detection reagents 1 and 2 from the ECL Western Blot kit (Amersham) were diluted in 1ml of water then added to the membrane and incubated in the light for 1 minute. The lights were extinguished and the membrane was exposed to Kodak OMax film from 15 seconds to 1 hour and then developed for 3 minutes in Kodak developer, washed for 1 minute in water, 3 minutes in Kodak

fixer and then a final 1 minute wash. The image was scanned using an Epson Perfection 1200S scanner. Each transient shRNA and its scrambled control, as well as each induced stable cell line and its non-induced control experiment was performed in triplicate.

Transcription Factor Activity Assay

U-87 MG cells were transfected with 2μg of shRNA plasmid DNA and 2μg of reporter plasmid DNA (either pNF-κB SEAP, pAP-1 SEAP, or pTAL; Clontech) or the four stable cell lines were transfected with 2μg of reporter plasmid DNA (either pNF-κB SEAP, pAP-1 SEAP, or pTAL; Clontech) in 8-well plates (6 wells with either NF-κB or AP-1 and 2 wells with pTAL; one-half the wells with shRNA or induction and the other half with scrambled shRNA or non-induced). Upon introduction of the stable cell lines to the dishes, 2 μg/ml of doxycycline was added to the medium of the induced wells and replenished with each medium change. For the shRNA, 24 hours after transfection and for the stable cell lines, 48 hours after transfection the medium was removed and centrifuged for 5 minutes at room temperature and 14,000 x g (Eppendorf centrifuge 5415 C). To 200μl of recovered supernatant was added 200μl 1X dilution buffer (Great Escape kit; Clontech) in a 0.65ml tubes and vortexed (Fisher Vortex Genie 2). Tubes were placed into a thermocycler (Eppendorf) at 65°C for 30 minutes to create a constant temperature. 100μl of the above solution was placed into an opaque white 96-well plate in triplicate (Costar). 99μl of assay buffer (1M diethanolamine, 0.05M $MgCl_2$, 0.1M

homoarginine) and 1μl of 4-methylumbelliferyl phosphate (Clontech) was added to each well. The plate was covered with foil to prevent light contamination and incubated at room temperature for 60 minutes, and read in a fluorometer (Perkin Elmer LS-50B) with excitation at 360nm wavelength and emission at 449nm wavelength. The triplicate wells were averaged and a graph was generated from the data. A Student's t-test, standard deviation and standard error were performed to compare the transient shRNA results with the scrambled control shRNA results and the induced stable shRNA results with the non-induced shRNA results to determine if any changes seen were statistically significant. This assay was repeated in triplicate.

Cell Staining for Neurosphere Determination

Every other day for six days, two wells, one induced (2μg/ml doxycycline) and one non-induced, for each stable cell line were sub-cultured into 6-well plates (Falcon) at a concentration of 25, 000 cells/ml per well to allow growth for six days. At the end of the sixth day the medium was removed, plates were placed on ice and the wells were washed with ice-cold 1X PBS (Invitrogen). Cells were fixed on ice using 1ml of ice-cold methanol per well for 10 minutes and removed. The cells were then stained with 1ml per well 0.5% crystal violet solution (in methanol) at room temperature for 10 minutes and removed. Plates were gently rinsed with water until clear. Plates were allowed to dry at room temperature. Photos were taken using a Canon Rebel camera with a Sigma 105mm DG Macro lens. The settings were f stop of 22, aperture priority, autofocus, and ¼ sec exposure. This assay was performed at three separate times on three different different populations of cells..

Caspase Assay

This assay utilizes a non-fluorescent Z-DEVD-R110 substrate which is cleaved by caspases 3 and 7 to create the rhodamine 110 fluorescent product. The activity of caspase 3 and 7 was assayed using the Apo-ONE assay kit (Promega). The four stable cell lines (pSuperior+pTet-On+hygromycin+c-Jun, pSuperior+pTet-On+hygromycin+c-Fos, pSuperior+pTet-On+hygromycin+c-Rel, pSuperior+pTet-On+hygromycin+p65) were sub-cultured into a 96-well white plate (Costar) with one column of eight per cell line, and half the column induced (2μg/ml doxycycline) and half the column non-induced. One additional column contained Minimum Essential Medium (MEM) for all eight wells and and four wells also containing doxycycline (2μg/ml) but no U-87 MG cells. Induction (2μg/ml doxycycline) occurred at the time of sub-culturing. 24 hours after induction, 100μl caspase reagent was added to each well and the plate was covered and allowed to incubate for 18 hours at room temperature. The plate was read on a fluorometer (Perkin Elmer LS-50B) with a wavelength of 485nm for excitation and 530nm for emission. A Student's t-test, standard deviation and standard error were performed to determine if the differences seen were statistically significant. This assay was performed in triplicate.

Photomicrographs

Photomicrographs were taken of cells growing in culture at 24 hours post-transfection or 48 hours post induction (2μg/ml doxycycline) using a Canon S40 digital camera with autofocus and autoprogram settings.

CHAPTER III

RESULTS

Transient shRNA Assays

Protein Levels

AP-1

Protein levels of the individual Ap-1 subunits were determined using a western blot in cells transiently transfected with shRNA or scrambled shRNA using 20μg total cellular protein 24 hours post transfection to evaluate whether the protein was present in the cell as well as the ability to block protein expression. Protein levels were quantified by pixel count. Protein levels were found to be decreased 55% in the transient c-Jun shRNA cells when compared with the scrambled shRNA controls (Figure 9A). Protein levels were decreased 26% in transient JunB shRNA when compared to scrambled JunB shRNA controls (Figure 9B). Protein levels when JunD shRNA was transiently transfected into U-87 MG cells showed a decrease of 43% when compared to the scrambled shRNA controls (Figure 9C). Cells transiently transfected with c-Fos shRNA showed a 45% decrease in protein levels when compared with scrambled (Figure 9D). FosB shRNA transiently transfected showed a 55% decrease in protein levels when compared to the scrambled shRNA controls (Figure 9E). Protein levels were shown to be reduced 28% in the Fra-1 transfected cells when compared to the scrambled shRNA controls (Figure 9F). Protein levels were decreased 80% in cells transfected with Fra-2 shRNA when compared with

scrambled shRNA controls (Figure 9G).

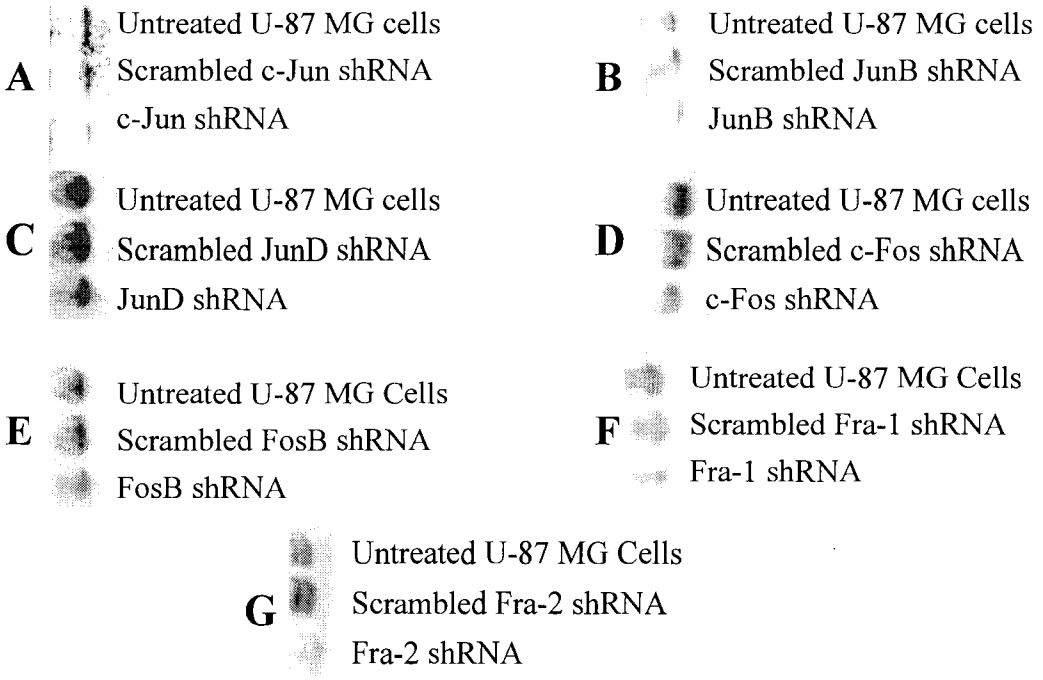


Figure 9. Protein Levels for Transiently Transfected AP-1 Subunits. SDS-Page and western blots of 20μg total protein were performed for all AP-1 subunits transiently tranfected with the shRNA 24 hours post transfection and protein levels were quantified by pixel count. c-Jun levels were decreased 55% (A), JunB levels were decreased 26% (B), JunD levels were decreased 43% (C), c-Fos levels were decreased 45% (D), FosB levels were decreased 55% (E), Fra-1 levels were decreased 28% (F), and Fra-2 levels were decreased 80% (G) when compared with their own scrambled shRNA control.

NF-κB

Protein levels of the individual NF-κB subunits were determined using a western blot in cells transiently transfected with shRNA or scrambled shRNA using 20μg total cellular protein 24 hours post transfection to evaluate whether the protein was present in the cell as well as the ability to block protein expression. Protein levels were quantified by pixel count. c-Rel transiently transfected into cells was

reduced 27% when compared with scrambled c-Rel shRNA controls (Figure 10A). Protein levels were decreased 32% in p50 shRNA when compared with scrambled controls (Figure 10B). Protein levels were decreased 34% in cells containing p52 shRNA when compared with the scrambled shRNA controls (Figure 10C). Protein levels of transient p65 shRNA were decreased 58% when compared with scrambled shRNA controls (Figure 10D). Protein levels were found to be decreased 44% in RelB shRNA cells when compared with scrambled controls (Figure 10E).

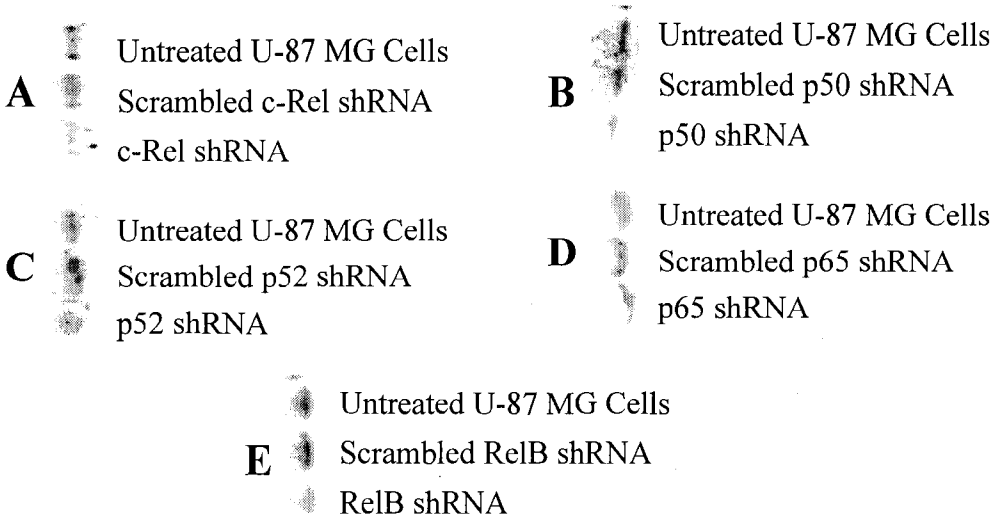


Figure 10. Protein Levels for Transiently Transfected NF-κB Subunits. SDS-Page and western blots of 20μg total protein were performed 24 hours post transfection for all NF-κB subunits transiently tranfected with the shRNA and protein levels were quantified by pixel count. c-Rel levels were decreased 27% (A), p50 levels were decreased 32% (B), p52 levels were decreased 34% (C), p65 levels were decreased 58% (D), RelB levels were decreased 44% (E) when compared with their own scrambled shRNA control.

Transcription Factor Activity

AP-1

The reporter gene assay for AP-1 transcription factor activity at 24 hours post transfection quantified the secreted alkaline phospatase produced as a result of AP-1 binding to the promoter. U-87 MG cells transiently transfected with c-Jun shRNA had a reduction in AP-1 activity of 95% when compared to the scrambled control levels (Figure 11). AP-1 activity was only reduced 14% in JunB shRNA transfected cells when compared to the scrambled shRNA controls (Figure 11). AP-1 activity was decreased 55% in cells transiently transfected with JunD shRNA when compared with cells transiently transfected with scrambled JunD shRNA controls (Figure 11). When c-Fos shRNA was transiently transfected into U-87 MG cells, AP-1 transcription factor activity was decreased 84% when compared to the scrambled shRNA controls (Figure 11), AP-1 activity was found to be 60% reduced in cells transiently transfected with FosB shRNA when compared to scrambled shRNA controls (Figure 11). AP-1 activity was found to be 63% reduced in cells transfected with Fra-1 shRNA when compared with scrambled shRNA controls (Figure 11). AP-1 activity was decreased 76% in cells transfected with Fra-2 shRNA when compared with scrambled shRNA controls (Figure 11).

NF-κB

The reporter gene assay for NF-κB transcription factor activity quantified the secreted alkaline phospatase produced at 24 hours post transfection as a result of NF-κB binding to the promoter. U-87 MG cells transiently transfected with c-Rel shRNA

had a reduction in NF-κB activity of 98% when compared to the scrambled control levels (Figure 12). NF-κB activity was reduced 60% in RelB shRNA transfected cells when compared to the scrambled shRNA controls (Figure 12). NF-κB activity was decreased 97% in cells transiently transfected with p50 shRNA when compared with cells transiently transfected with scrambled JunD shRNA controls (Figure 12). When p52 shRNA was transiently transfected into U-87 MG cells, NF-κB transcription factor activity was decreased 72% when compared to the scrambled shRNA controls (Figure 12), NF-κB activity was found to be 94% reduced in cells transiently transfected with p65 shRNA when compared to scrambled shRNA controls (Figure 12).

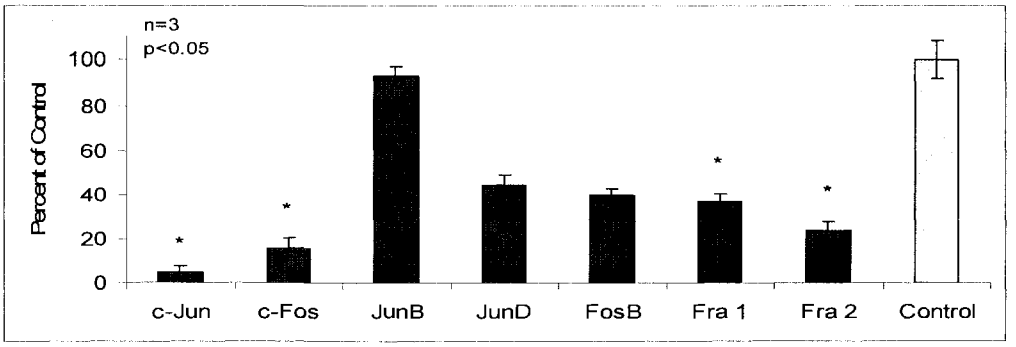


Figure 11. AP-1 Transcription Factor Activity 24 Hours Post Transfection when shRNA is Transiently Transfected into U-87 MG Cells. Secreted alkaline phosphatase production was quantified as a result of AP-1 transcription factor binding for each transiently transfected shRNA. Transcription factor activity was reduced 95% for c-Jun, 84% for c-Fos, 14% for JunB, 55% for JunD, 60% for FosB, 63% for Fra-1, and 76% for Fra-2 when compared to their own scrambled controls. The reduction in activity was statistically significant for c-Jun, c-Fos, Fra-1, and Fra-2. ($n=3$; $p \leq 0.05$)

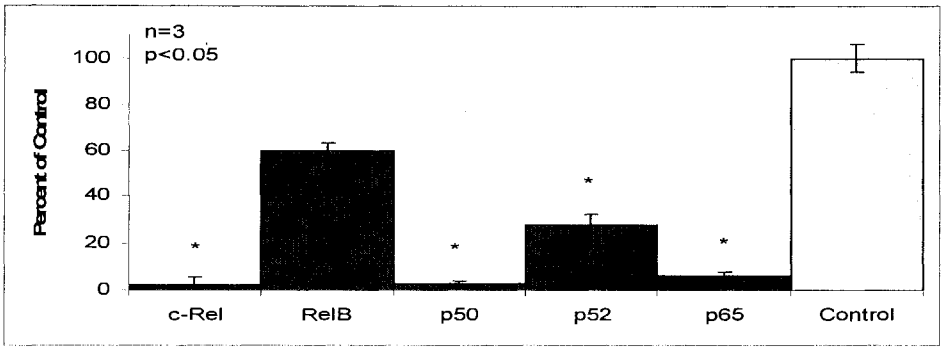


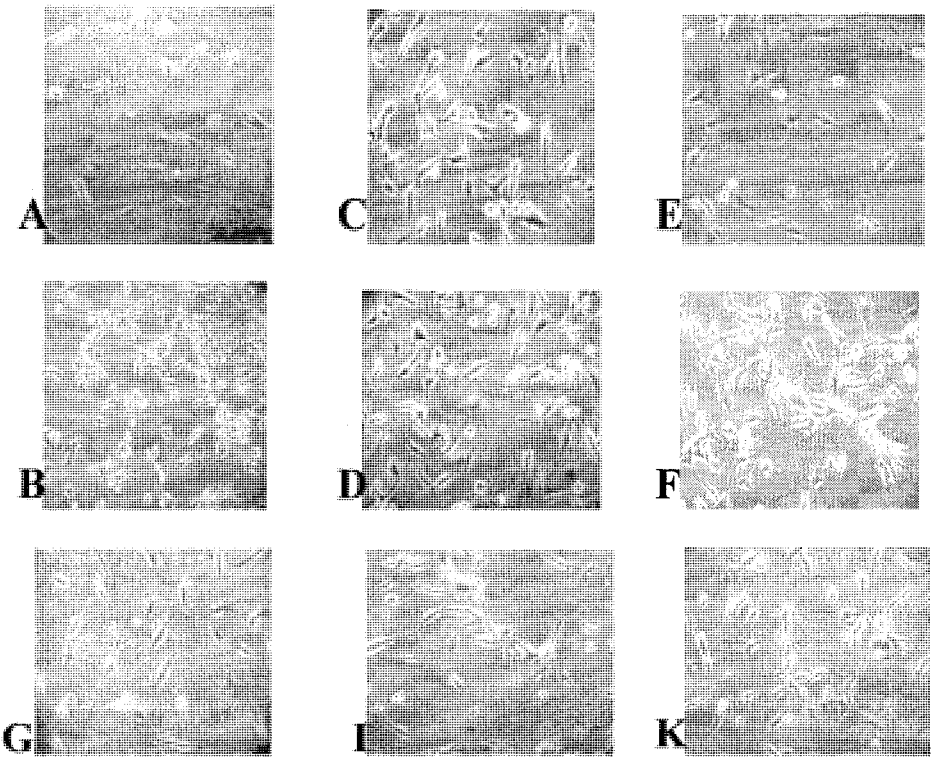
Figure 12. NF-κB Transcription Factor Activity 24 Hours Post Transfection when shRNA is Transiently Ttransfected into U-87 MG Cells. Secreted alkaline phosphatase production was quantified as a result of NF-κB transcription factor binding for each transiently transfected shRNA. Transcription factor activity was reduced 98% for c-Rel, 60% for RelB, 97% for p50, 72% for p52, and 94% for p65 when compared to their own scrambled controls. The reduction in activity was statistically significant for all but RelB. (n=3; $p \leq 0.05$)

Photomicrographs

AP-1

Photomicrographs were taken to document changes in cell numbers or morphology between transient transfections and their scrambled controls. At 24 hours post transfection there was a reduction in cell number in plates transiently transfected with c-Jun (Figure 13A) when compared to plates transfected with the scrambled control (Figure 13B). Photomicrographs of JunB shRNA transiently transfected cells showed no difference in cell growth (Figure 13C) when compared to the scrambled shRNA (Figure 13D). Photomicrographs of transiently transfected JunD shRNA and scrambled shRNA controls.showed a decrease in cell numbers (Figure 13E) when compared with the scrambled shRNA (Figure 13F). Photomicrographs of cells transiently transfected with c-Fos shRNA (Figure 13G)

showed decreased cell numbers when compared with scrambled shRNA (Figure 13H). Photomicrographs of cells transiently transfected with FosB shRNA showed no changes in cell number (Figure 13I) when compared to scrambled shRNA (Figure 13J). Photomicrographs of cells transiently transfected with Fra-1 shRNA were slightly decreased (Figure 13K) when compared to scrambled shRNA controls (Figure 13L). Photomicrographs of cells transiently transfected with Fra-2 shRNA showed decreased cell numbers (Figure 13M) when compared to scrambled shRNA control plates (Figure 13N). In all cases there were no neurospheres present in plates containing the shRNA, but were present in all plates with the scrambled controls.



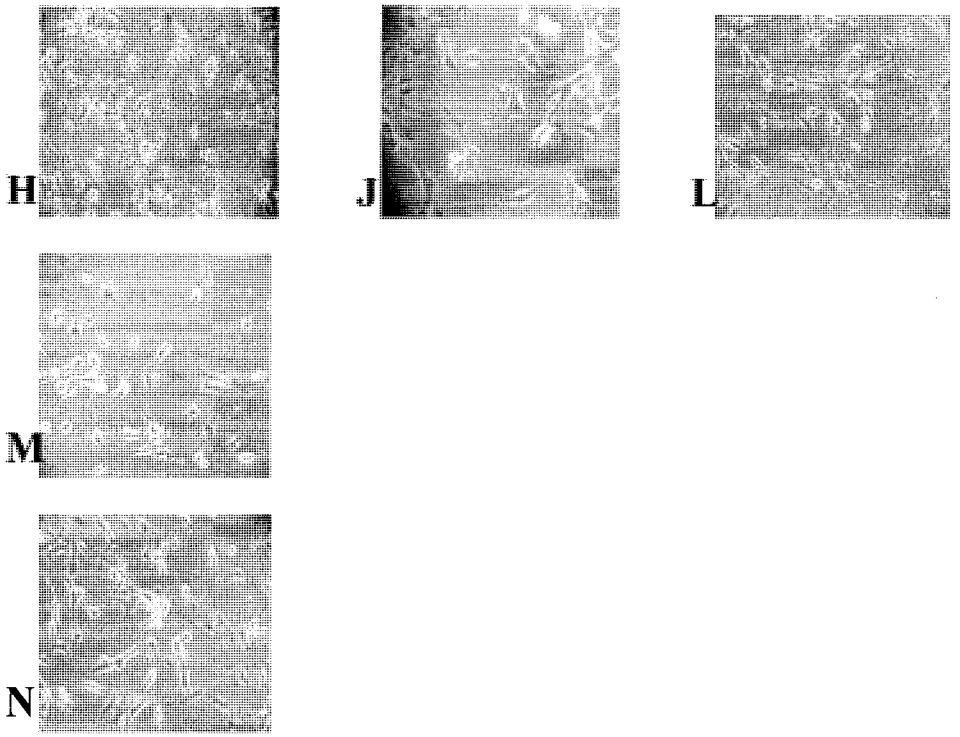
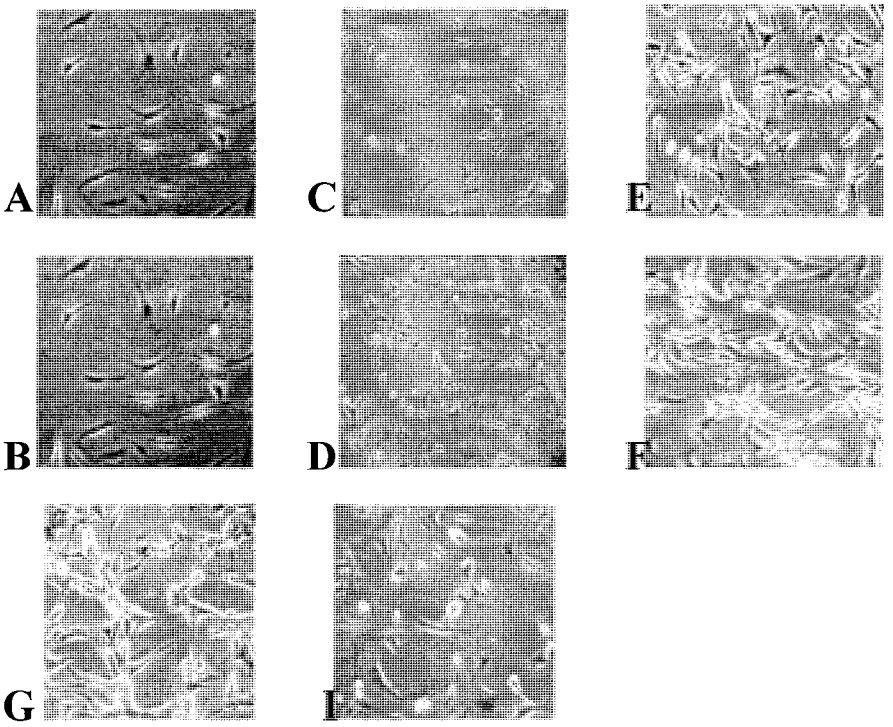


Figure 13. Photomicrographs Taken 24 Hours After Transient Transfection of the AP-1 Subunits. Transiently transfected c-Jun had reduced cell numbers on the plate (A) when compared with the scrambled control (B). Transiently transfected JunB showed no change in cell numbers (C) when compared with the scrambled controls (D). Transiently transfected JunD had reduced cell numbers on the plate (E) when compared with the scrambled control (F). Transiently transfected c-Fos had reduced numbers on the plate (G) when compared with the scrambled control (H). Transiently transfected FosB showed no change in cell numbers (I) when compared with the scrambled control (J). Transiently transfected Fra-1 showed no change in cell numbers (K) when compared with the scrambled control (L). Transiently transfected Fra-2 had reduced cell numbers on the plate (M) when compared with the scrambled control (N). In all transiently transfected shRNA plates there were no neurospheres present but neurospheres were present on the plates with the scrambled controls.

NF-κB

Photomicrographs were taken to document changes in cell numbers or morphology between transient transfections and their scrambled controls. At 24 hours after transfection there was a reduction in cell number in plates transiently transfected with c-Rel (Figure 14A) when compared to plates transfected with the

scrambled control (Figure 14B). Photomicrographs of RelB shRNA transiently transfected cells showed a difference in cell growth (Figure 14C) when compared to the scrambled shRNA (Figure 14D). Photomicrographs of transiently transfected p50 shRNA and scrambled shRNA controls.showed no decrease in cell numbers (Figure 14E) when compared with the scrambled shRNA (Figure 14F). Photomicrographs of cells transiently transfected with p52 shRNA (Figure 14G) showed no decrease in cell numbers when compared with scrambled shRNA (Figure 14H). Photomicrographs of cells transiently transfected with p65 shRNA showed decreased cell numbers (Figure 14I) when compared to scrambled shRNA (Figure 14J). In all cases there were no neurospheres present in plates containing the shRNA, but were present in all plates with the scrambled controls.



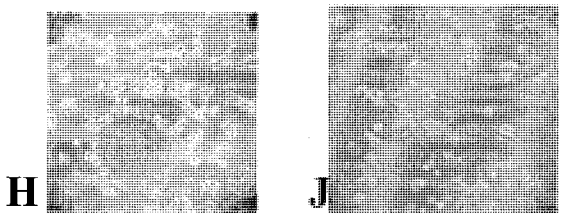


Figure 14. Photomicrographs Taken 24 Hours After Transient Transfection of the NF-κB Subunits. Transiently transfected c-Rel had reduced cell numbers on the plate (A) when compared with the scrambled control (B). Transiently transfected RelB had reduced cell numbers on the plate (C) when compared with the scrambled controls (D). Transiently transfected p50 showed no cell number differences on the plate (E) when compared with the scrambled control (F). Transiently transfected p52 had no cell number differences on the plate (G) when compared with the scrambled control (H). Transiently transfected p65 had reduced cell numbers (I) when compared with the scrambled control (J). In all transiently transfected shRNA plates there were no neurospheres present but neurospheres were present on the plates with the scrambled controls.

The AP-1 (Table 23) and NF-κB (Table 24) summaries follow.

Table 23

Summary of Results for AP-1.

	Protein Level Decrease	Transcription Factor Decrease	Cell Numbers
c-Jun	55%* ± 4	95%* ± 13	Reduced
c-Fos	45% ± 10	84%* ± 7	Reduced
JunB	26% ± 4	14% ± 6	No Change
JunD	43% ± 6	55% ± 20	Reduced
FosB	55%* ± 5	60% ± 7	No Change
Fra-1	28% ± 4	63%* ± 2	No Change
Fra-2	80%* ± 15	76%* ± 10	Reduced

Table 24

Summary of Results for NF-κB.

	Protein Level Decrease	Transcription Factor Decrease	Cell Numbers
c-Rel	27% ± 5	98%* ± 4	Reduced
RelB	44% ± 10	60% ± 11	Reduced
p50	32% ± 4	97%* ± 8	No Change
p52	34% ± 5	72%* ± 10	No Change
p65	58%* ± 6	94%* ± 4	Reduced

Stable shRNA Assays

Protein Levels

Protein levels were determined using a western blot on 20μg total cellular protein at 48 hours post induction (2μg doxycycline) in induced and non-induced stable shRNA cells to evaluate whether the protein level decrease was similar to that seen in the transient assays. Protein levels were quantified by pixel count. Protein levels were found to be decreased 59% in induced stable c-Jun shRNA cells when compared with non-induced shRNA controls cells (Figure 15A). Protein levels were found to be decreased 65% in induced stable c-Fos shRNA cells when compared with non-induced shRNA control cells (Figure 15B). Protein levels were found to be decreased 60% in induced stable c-Rel shRNA cells when compared with non-induced shRNA control cells (Figure 15C). Protein levels were decreased 57% in induced p65 shRNA cells when compared with non-induced p65 shRNA cells (Figure 15D).

Transcription Factor Activity

Stable U-87 MG cells expressing one of the four shRNAs (c-Jun, c-Fos, c-Rel,

or p65) were evaluated for transcription factor activity at 48 hours post induction (2μg doxycycline). The reporter gene assay for transcription factor activity quantified the secreted alkaline phospatase produced as a result of transcription factor binding to the promoter. Induced c-Jun shRNA had a reduction in AP-1 activity of 91% when compared to the non-induced control levels (Figure 16). AP-1 activity was reduced 98% in induced c-Fos shRNA cells when compared to the non-induced shRNA controls (Figure 16). NF-κB activity was decreased 96% in induced cells with c-Rel shRNA when compared with non-induced c-Rel shRNA controls (Figure 16). NF-κB activity was found to be 87% reduced in induced cells with p65 shRNA when compared to non-induced shRNA controls (Figure 16).

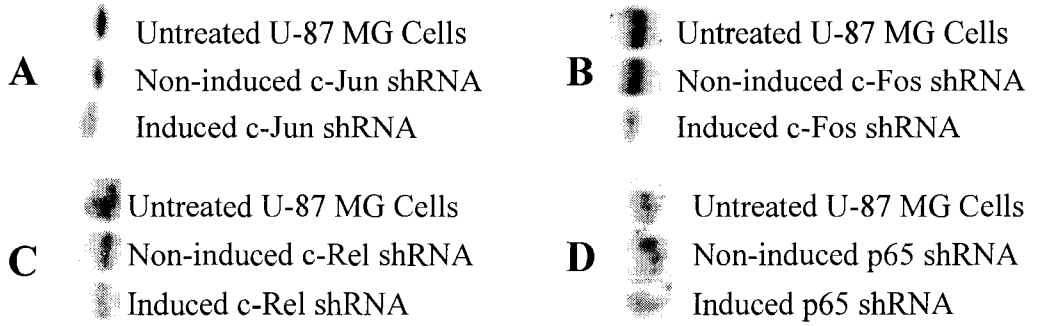


Figure 15. Protein Levels for Induced and Non-induced Stable Cell Line Subunits. SDS-Page and western blots of 20mg total protein were performed at 48 hours post induction (2μg doxycycline) for the four stable subunits tranfected with the shRNA and protein levels were quantified by pixel count. c-Jun levels were decreased 59% (A), c-Fos levels were decreased 65% (B), c-Rel levels were decreased 60% (C), p65 levels were decreased 57% (D) when compared with their own scrambled shRNA control.

Photomicrographs

Photomicrographs were taken to document changes in cell numbers or

morphology between transient transfections and their scrambled controls. At 48 hours post induction (2μg doxycycline) there was a reduction in cell number in plates transiently transfected with c-Jun (Figure 17A) when compared to plates transfected with the scrambled control (Figure 17B). Photomicrographs of c-Fos shRNA transiently transfected cells showed a decrease in cell growth (Figure 17C) when compared to the scrambled shRNA (Figure 17D). Photomicrographs of transiently transfected c-Rel shRNA showed a reduction in cell numbers (Figure 17E) when compared with the scrambled shRNA (Figure 17F). Photomicrographs of cells transiently transfected with p65 shRNA showed decreased cell numbers (Figure 17G) when compared to scrambled shRNA (Figure 17H). In all cases there were no neurospheres present in plates containing the shRNA, but were present in all plates with the scrambled controls.

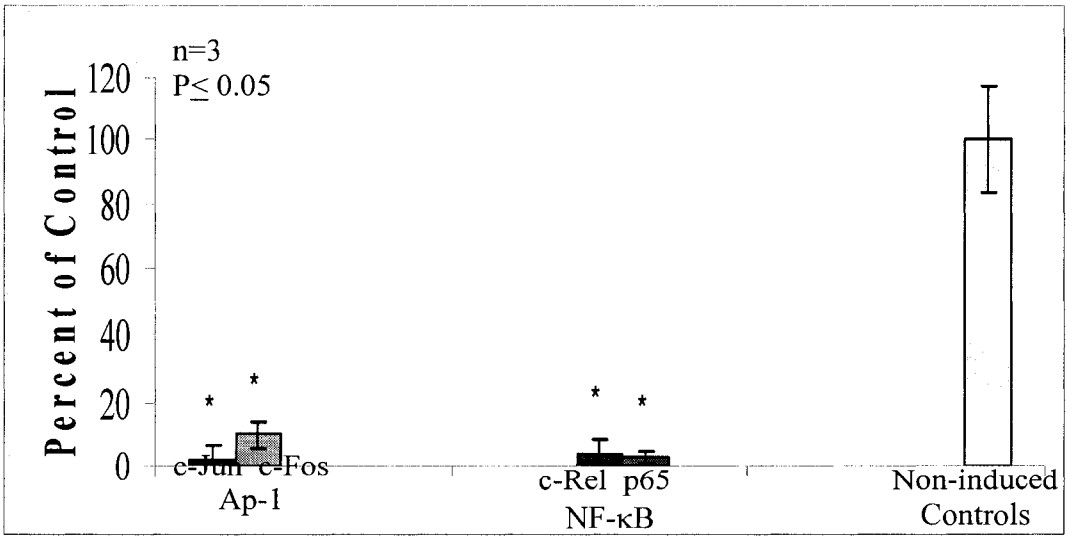


Figure 16. Transcription Factor Activity in U-87 MG Cells Expressing Stable shRNA (c-Jun, c-Fos, c-Rel, p65) 48 Hours Post Induction (2μg Doxycycline). Secreted alkaline phosphatase production was quantified as a result of NF-κB or AP-1

transcription factor binding for each stable shRNA (c-Jun, c-Fos, c-Rel, or p65). Transcription factor activity was reduced 91% for c-Jun, 98% for c-Fos, 96% for c-Rel, and 87% for p65 when compared to their own non-induced controls. The reduction in activity was statistically significant for all. ($n=3$; $p \leq 0.05$)

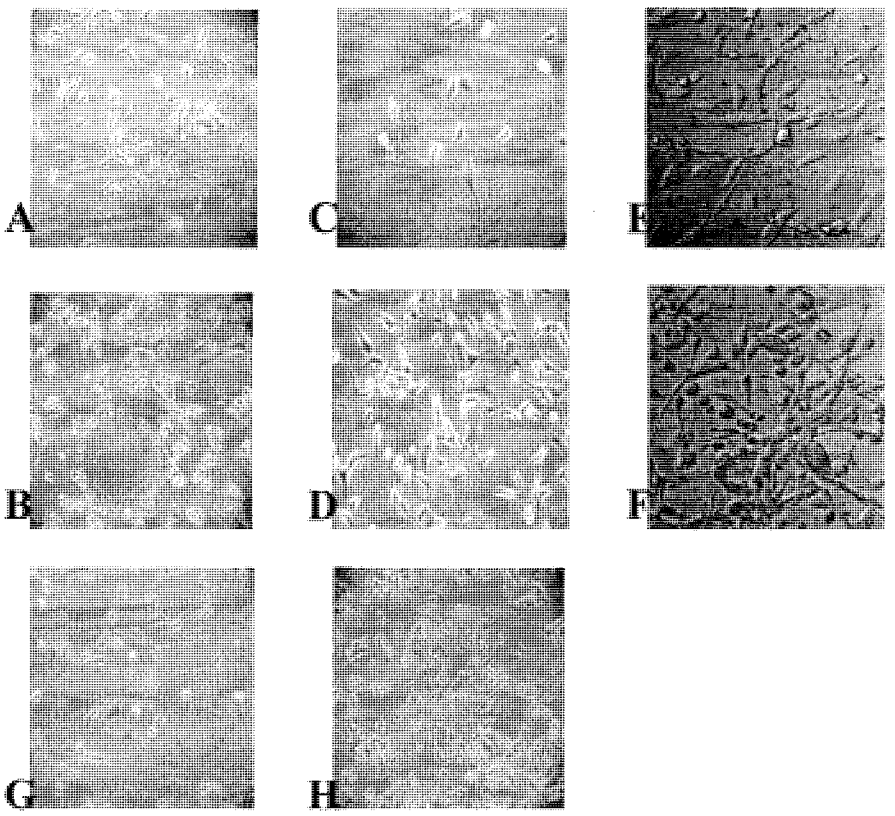


Figure 17. Photomicrographs Taken 48 Hours After Induction of the Four NF-κB and Ap-1 Subunits (c-Jun, c-Fos, c-Rel, p65). Induction (2μg doxycycline) of c-Jun showed reduced cell numbers on the plate (A) when compared with the non-induced (B). Induction of c-Fos showed reduced cell numbers on the plate (C) when compared with the non-induced (D). Induction of c-Rel showed reduced cell numbers on the plate (E) when compared with the non-induced (F). Induction of p65 showed reduced cell numbers on the plate (G) when compared with the non-induced (H). In all induced shRNA plates there were no neurospheres present but neurospheres were present on the plates with the non-induced controls.

Differentiation

Nestin, a protein expressed during development of glial cells is used as a

marker for un-differentiation in glioma cells. Protein levels were quantified by pixel count. A nestin western blot was performed 48 hours post induction using 20μg total cellular protein on induced (2μg doxycycline) and non-induced c-Jun U-87 MG cells and nestin protein levels were found to be decreased 74% on induction of the shRNA when compared with the non-induced cells (Figure 18A). A nestin western blot was performed on induced and non-induced c-Fos U-87 MG cells and nestin protein levels were found to be decreased 70% on induction of the shRNA when compared with the non-induced cells (Figure 18B). A nestin western blot was performed on induced and non-induced c-Rel U-87 MG cells and nestin protein levels were found to be decreased 72% on induction of the shRNA when compared with the non-induced cells (Figure 18C). A nestin western blot was performed on induced and non-induced p65 U-87 MG cells and nestin protein levels were found to be decreased 54% on induction of the shRNA when compared with the non-induced cells (Figure 18D).

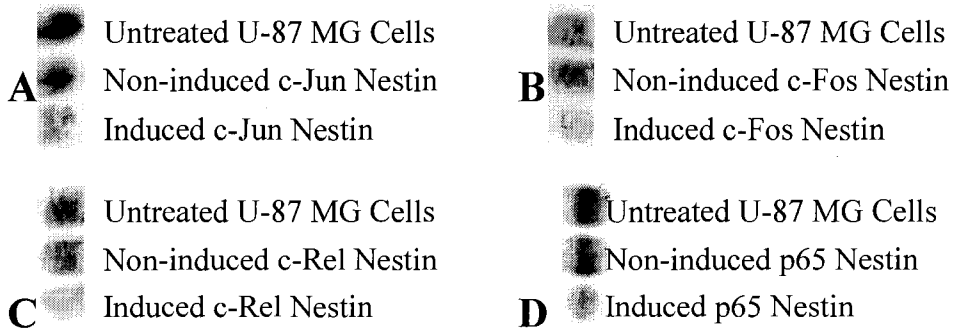
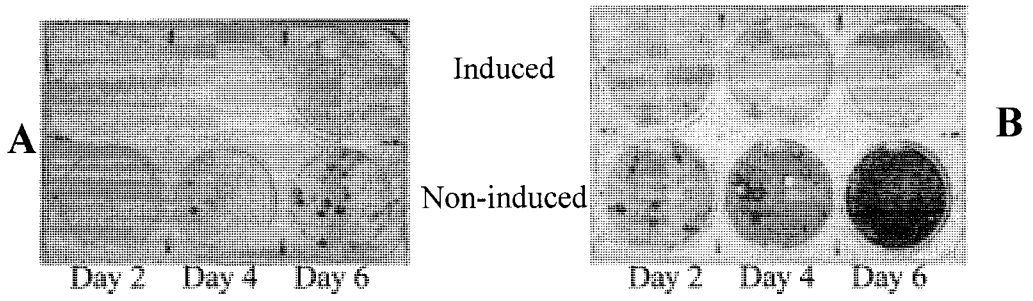


Figure 18. Differentiation Assay Comparing Nestin Protein Levels in Induced shRNA Cells (c-Jun, c-Fos, c-Rel, p65) and Non-induced shRNA Cells. A western blot of 20μg total protein was performed at 48 hours to compare the induced cells nestin levels with the non-induced nestin levels. Protein levels were quantified by pixel count. The nestin protein levels were decreased 74% in induced c-Jun cells when compared with non-induced. The nestin protein levels were decreased 70% in induced c-Fos cells when compared with non-induced. The nestin protein levels were

decreased 72% in induced c-Rel cells when compared with non-induced. The nestin protein levels were decreased 54% in induced p65 cells when compared with non-induced.

Neurosphere Staining

The impact of the shRNA on neurospheres was addressed by growing cells over a six day period comparing induced (2μg doxycycline) stable shRNA U-87 MG cells with non-induced cells in neurosphere size, numbers, and date of appearance. Induced c-Jun shRNA resulted in neurospheres which were smaller, were fewer in number, and onset was delayed (Figure 19A) when compared with the non-induced shRNA cells. Induced c-Fos shRNA resulted in neurospheres which were smaller, were fewer in number, and onset was delayed (Figure 19B) when compared with the non-induced shRNA cells. Induced c-Rel shRNA resulted in neurospheres which were smaller, were fewer in number, and onset was delayed (Figure 19C) when compared with the non-induced shRNA cells. Induced p65 shRNA resulted in neurospheres which were smaller, were fewer in number, and onset was delayed (Figure 19D) when compared with the non-induced shRNA cells.



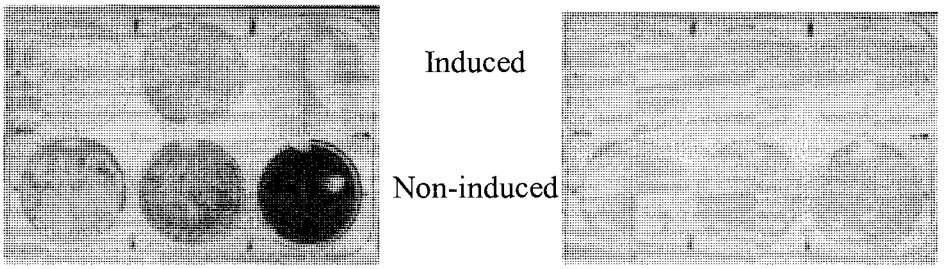
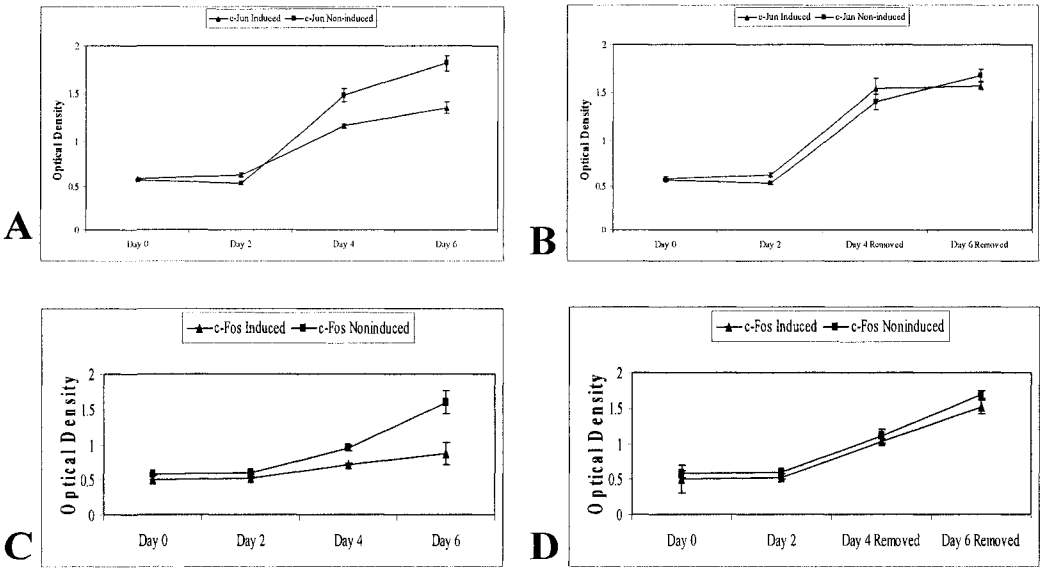


Figure 19. Staining of U-87 MG Cells that have Induced shRNA were Compared with Non-induced shRNA. Induced (2μg doxycycline) and non-induced cells (c-Jun, c-Fos, c-Rel, p65) were grown over six day and then stained to look at the neurospheres. Cells that contained induced c-Jun have fewer neurosphere that are smaller in size and have a delayed onset when compared with non-induced cells (A). Cells that contained induced c-Fos have fewer neurosphere that are smaller in size and have a delayed onset when compared with non-induced cells (B). Cells that contained induced c-Rel have fewer neurosphere that are smaller in size and have a delayed onset when compared with non-induced cells (C). Cells that contained induced p65 have fewer neurosphere that are smaller in size and have a delayed onset when compared with non-induced cells (D).

Cellular Proliferation Assay

Cell proliferation of induced and non-induced stable shRNA U-87 MG cells was evaluated. Cells were grown over six days and cells were quantified at day two, four, and six. The inducer (2μg doxycycline) in two populations was removed at day four and six to assess whether the changes seen was because of the inducer. Induced c-Jun cells showed that there was 26% less cells when compared to non-induced cells (Figure 20A). The induced cells did continue to proliferate, but not as fast as the non-induced cells. When the inducer was removed at day four, the cells began to grow again indicating that the reduction in growth was not caused by the presence of the inducer (Figure 20B). Induced c-Fos cells showed that there was 45% less cells when compared to non-induced cells (Figure 20C). The induced cells appear to stop proliferating. When the inducer was removed at day four, the cells began to grow

again indicating that the reduction in growth was not caused by the presence of the inducer (Figure 20D). Induced c-Rel cells showed that growth was reduced by 49% when compared to non-induced cells (Figure 20E). The induced cells appear stop proliferating. When the inducer was removed at day four, the cells began to grow again indicating that the reduction in growth was not caused by the presence of the inducer (Figure 20F). Induced p65 cells showed that growth was reduced by 52% when compared to non-induced cells (Figure 20G). The induced cell number decrease appears to be because of the failure to proliferate and/or because of apoptosis. When the inducer was removed at day four, the cells began to grow again indicating that the reduction in growth was not caused by the presence of the inducer (Figure 20H).



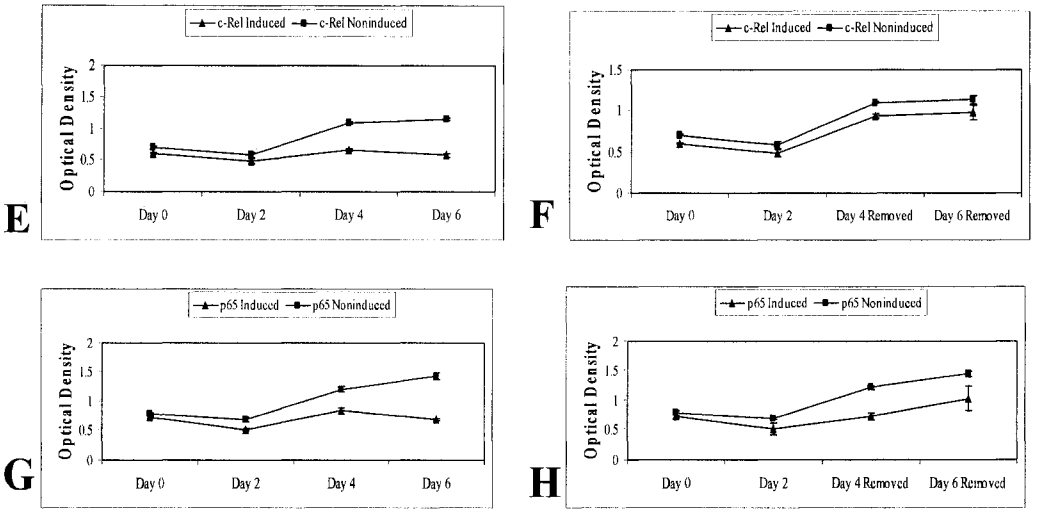


Figure 20. Proliferation Assay Comparing the Induced shRNA Cells with the Non-induced shRNA Cells. Colormetric assay to compare induced shRNA (2μg doxycycline) with the non-induced shRNA cells over six days in the four stable cell lines (c-Jun, c-Fos, c-Rel, p65). Induced c-Jun cells were decreased 26% when compared with non-induced cells (A). With the removal of the inducer at day four allowed the cells to recover and grow (B). Induced c-Fos cells were decreased 45% when compared with non-induced cells (A). With the removal of the inducer at day four allowed the cells to recover and grow (B). Induced c-Rel cells were decreased 49% when compared with non-induced cells (A). With the removal of the inducer at day four allowed the cells to recover and grow (B). Induced p65 cells were decreased 52% when compared with non-induced cells (A). With the removal of the inducer at day four allowed the cells to recover and grow (B). All decreases in induced cell numbers were statistically significant when compared with non-induced controls. (n-3; $p \leq 0.05$).

Caspase 3/7 Activity

The activity of executioner caspases 3 and 7 were evaluated in the induced (2μg doxycycline) and non-induced c-Jun U-87 MG cells at 48 hours post induction using the cleavage of a non-fluorescent molecule by the caspases to produce a fluorescent product. This assay revealed that the level of caspases 3 and 7 increased by 98% when c-Jun shRNA was induced (Figure 21). This assay revealed that the

level of caspases 3 and 7 increased by 97% when c-Fos shRNA was induced (Figure 21). This assay revealed that the level of caspases 3 and 7 increased by 98% when c-Rel shRNA was induced (Figure 21). This assay revealed that the level of caspases 3 and 7 increased by 98% when p65 shRNA was induced (Figure 21).

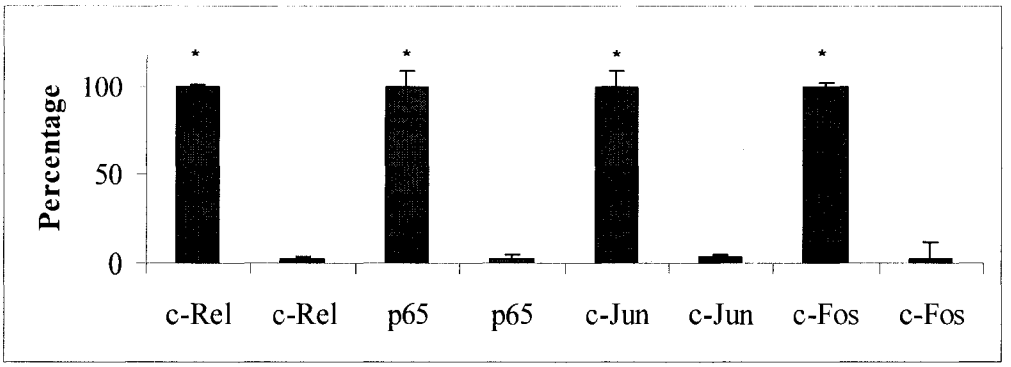


Figure 21. Caspase 3/7 Activity in Induced and Non-induced Cells. The production of a fluorescent product by caspase 3/7 activity was evaluated in the stable cell lines (c-Jun, c-Fos, c-Rel, p65) at 48 hours post induction (2μg doxycycline). The caspase activity increased 98% when c-Jun was induced, 97% when c-Fos was induced, 98% when c-Rel was induced, and 98% when p65 was induced. The first column is induced and the second column is non-induced for each subunit. All were statistically significant. ($n=3$; $p \leq 0.05$)

Cleaved PARP Assay

PARP (poly ADP-ribose), a cleavage target of caspase 3, is an indicator of death by apoptosis. Induced and non-induced stable shRNA U-87 MG cells were evaluated at 48 hours post induction (2μg doxycycline) for cleaved PARP protein levels. A western blot was performed using 20μg total cellular protein to evaluate the cleaved PARP levels and all proteins levels were quantified by pixel count. Cleaved PARP increased 53% when c-Jun shRNA was induced when compared with the non-

induced controls (Figure 22A). Cleaved PARP increased 61% when c-Fos shRNA was induced when compared with the non-induced controls (Figure 22B). Cleaved PARP increased 71% when c-Rel shRNA was induced when compared with the non-induced controls (Figure 22C). Cleaved PARP increased 28% when p65 shRNA was induced when compared with the non-induced controls (Figure 22D).

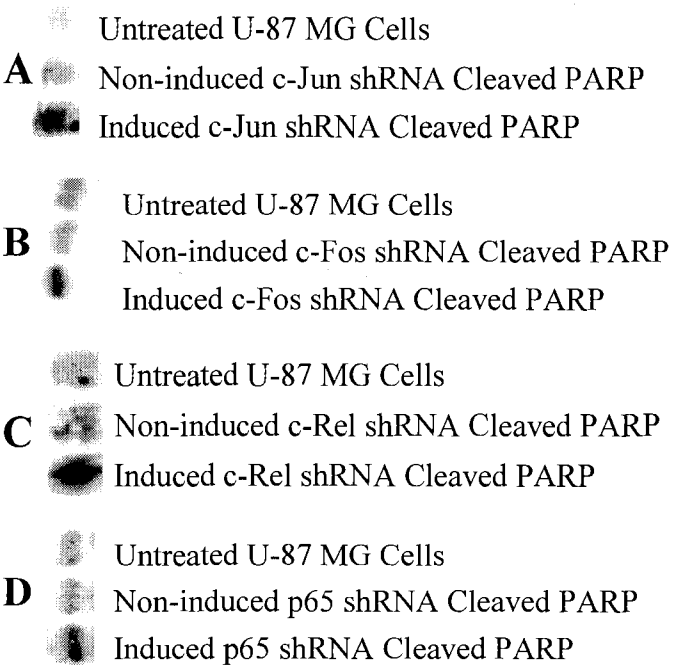


Figure 22. Cleaved PARP Western Blot for Induced and Non-induced shRNA. A western blot of 48 hour induced (2μg doxycycline) and non-induced (c-Jun, c-Fos, c-Rel, p65) shRNA of 20μg total protein. Protein levels were quantified by pixel count. The western blot of induced c-Jun showed that cleaved PARP protein levels increased 53% when compared with the non-induced control (A). The western blot of induced c-Fos showed that cleaved PARP protein levels increased 61% when compared with the non-induced control (B). The western blot of induced c-Rel showed that cleaved PARP protein levels increased 71% when compared with the non-induced control (C). The western blot of induced p65 showed that cleaved PARP protein levels increased 28% when compared with the non-induced control (D).

The summary of results for the four stable cell lines (c-Jun, c-Fos, c-

Rel, p65) follows (Table 25).

Table 25

Summary of Results for the Stable Cell Lines.

	Protein Level Decrease	Transcription Factor Decrease	Proliferation Assay	Caspase 3/7 Activity	Cleaved PARP Increase	Nestin Decrease
c-Jun	59%* ± 9	91%* ± 14	26%* ± 5	97%* ± 19	53%* ± 8	74%* ± 16
c-Fos	65%* ± 18	98%* ± 9	45%* ± 6	98%* ± 10	61%* ± 12	70%* ± 19
c-Rel	60%* ± 12	96%* ± 22	49%* ± 4	98%* ± 8	71%* ± 13	72%* ± 10
p65	57%* ± 8	87%* ± 10	52%* ± 6	98%* ± 22	28% ± 4	54%* ± 8

CHAPTER IV

DISCUSSION

This research has revealed many new and interesting facts, especially in the composition of the two transcription factors, AP-1 and NF-κB, and the role they play in the transformation and/or maintenance of the malignant phenotype of the U-87 MG cells. This research has also supported many of the previous reported research. The ability to induce apoptosis and differentiation when many of these proteins are decreased suggests a possible therapeutic treatment for this malignancy.

AP-1 and NF-κB

The seven AP-1 and five NF-κB proteins were all present in the U-87 MG cells and were found to be decreased when the specific shRNA was either transiently transfected into the cell or the stable cell line was induced indicating that the shRNA was functional. The varying degrees of protein blockage could indicate that the target location on the gene was not the optimal location.

AP-1 and NF-κB activity was found to be decreased when any of the individual protein levels were decreased except for JunB, suggesting that the remaining six AP-1 and the five NF-κB proteins can all form AP-1 and NF-κB dimers in the U-87 MG cells. Interestingly, the reduction of AP-1 or NF-κB in the cell did not always lead to changes that were assayed in this project, except for neurosphere formation. In each of the transiently transfected shRNAs and the induced stable cell lines no neurospheres were noted at 24 and 48 hours, respectively. This suggested

that each protein in the AP-1 dimer played a role in the development and/or maintenance of the neurospheres. But whether these roles are unique or redundant remains to be established. Nestin was found to be reduced in varying amounts in induced stable cell lines suggesting that differentiation was occurring which supports the decrease in neurosphere formation, delayed onset and smaller size. AP-1 has previously been shown to play a role in differentiation (Angel & Karin, 1991; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Fujioka et al., 2004; Hess et al., 2004; Xie et al., 2005).

The difference between the photomicrographs and the growth curves indicate that another growth assay might prove useful since it appears that the dehydrogenase activity in the U-87 MG cells may be changing with the introduction of the some of the shRNAs. The current assay did reveal differences in growth between the induced and non-induced plates, but not necessarily the correct amount of difference. The decreases do suggest that both AP-1 and NF-κB play a role in cellular proliferation (Hirai et al., 1989; Angel & Karin, 1991; Karin, 1995; Bejer et al., 1999; Wisdom, 1999; Bejer et al., 2000; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Shaulian & Karin, 2002; Short & Pfarr, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Hess et al., 2003; Pramanik et al., 2003; Hess et al., 2004; Kenner et al., 2004; Xie et al., 2005; Inta et al., 2006) and/or anti-apoptosis (Angel & Karin, 1991; Barkett & Gilmore, 1999; Li et al., 1999; Pahl, 1999; Mechta-Grigoriou et al., 2001; Shaulian & Karin, 2001; Karin & Lin, 2002b; Shaulian & Karin, 2002; Yazgan & Pfarr, 2002; Eferl & Wagner, 2003a; Fujioka et al., 2004; Hayden & Ghosh, 2004;

Hess et al., 2004; Xie et al., 2005; Marienfeld et al., 2006; Mauro et al., 2006) which has been previously found. The suggestion of a role in anti-apoptosis is further supported by the caspase and cleaved PARP assays which showed that the cells were dying by apoptosis. Since evasion of apoptosis is a hallmark of cancer (Hanahan & Weinberg, 2000), these results reveal one of the more important findings of this research.

AP-1

c-Jun

The decrease of c-Jun protein in both transient shRNA as well as induced stable shRNA revealed many changes in the U-87 MG cells. The protein levels were decreased 55% (Figure 9A) and in the transient shRNA experiments when compared with the scrambled controls and 59% (Figure 15A) in the induced stable cell lines when compared with the non-induced shRNA cells. The AP-1 transcription factor levels were decreased 95% in the transient assay (Figure 11) and 98% in the induced stable assay (Figure 16) suggesting that c-Jun is part of AP-1 in this cell type which is in agreement with previous reports of AP-1 dimer being c-Jun/c-Fos.

c-Jun has been shown previously to be involved in cellular proliferation (Herdegen et al., 1997; Ham et al., 2000; Szabowski et al., 2000; Jochum et al., 2001; Mechta-Grigoriou et al., 2001; Behrens et al., 2002; Qi et al., 2002; Short & Pfarr, 2002; Vogt, 2002; Eferl & Wagner, 2003b; Li et al., 2003b; Zenz et al., 2003; Hess et al., 2004; Besirli et al., 2005; Sun et al., 2005), growth (Varmus & Weinberg, 1993) and survival (Jochum et al., 2001; Qi et al., 2002; Vogt, 2002). The cell numbers in

both the transient assay (Figure 13A) and the induced stable assay (Figure 17A) were decreased in the photomicrographs as well as in the proliferation assay (Figure 20A), but the photomicrograph cell number decreases looked far greater than the 26% that the growth curves showed. The proliferation assay uses dehydrogenase activity which could be influenced by the decreased c-Jun levels, yielding an artificially high cell count.

One of the characteristics of cancer cells is the evasion of apoptosis (Hanahan & Weinberg, 2000). When c-Jun protein levels were decreased, caspases 3 and 7 were elevated by 98% (Figure 21) and cleaved PARP was increased 53% (Figure 22A) as well, indicating that cells were dying by apoptosis suggesting a role for c-Jun in evasion of apoptosis.

Tumor growth occurs from neurospheres (Lapidot et al., 1994; Larochelle et al., 1996; Bonnet, 1997; Al-Hajj et al., 2003; Dick, 2003; Hemmati et al., 2003; Singh et al., 2003; Galli et al., 2004; Kapoor et al., 2004; Kondo et al., 2004; Singh et al., 2004; Yuan et al., 2004), and the photomicrographs showed that when c-Jun levels were decreased, the neurospheres were absent (Figure 13A & 17A). To address whether the neurospheres developed later than 48 hours, cell staining of neurospheres at later time points was performed. When c-Jun was decreased neurosphere formation was delayed from day 2 to day 6, the neurospheres were smaller and decreased in number, suggesting that c-Jun has a role in neurosphere formation and/or maintenance (Figure 19A). This was further supported by the fact that nestin levels were decreased by 74% (Figure 18A) in cells with decreased c-Jun indicating that

some of the dedifferentiated cells within the neurosphere were becoming differentiating.

JunB

Transiently transfected JunB showed no changes in AP-1 levels (Figure 11) or in the photomicrographs (Figure 13C) when compared with the scrambled controls, yet the protein was decreased in the western blot by 26% (Figure 9B). This suggests that the lack of JunB in the U-87 MG cells is not a part of AP-1 and essential for cell growth, proliferation or apoptosis in these cells. This lack of change in growth is supported by other research (Shaulian & Karin, 2001). This is not surprising when most research indicates that JunB is important for bone and immune cells, as well as embryogenesis. It has been shown to have some role in differentiation (Hartenstein et al., 2002; Hess et al., 2004; Meixner et al., 2004), but that was not explored in this research. The question of why it is expressed in these cells remains to be determined.

JunD

With the reduction of JunD protein of 43% (Figure 9C), the cells had reduced AP-1 levels of 55% (Figure 11) and reduced cell numbers in the photos (Figure 13E) when compared with the scrambled controls. This suggests that JunD is part of the AP-1 dimer in this cell type and has a role in cell growth and/or anti-apoptosis. This is in agreement with others who found that lack of JunD caused altered cell growth and decreased proliferation (Behrens et al., 1999; Thepot et al., 2000; Weitzman et al., 2000; Shaulian & Karin, 2001; Shaulian & Karin, 2002) as well as decreased apoptosis (Pfarr et al., 1994; Passegue et al., 2000; Weitzman et al., 2000; Mechta-

Grigoriou et al., 2001; Shaulian & Karin, 2001; Bakiri et al., 2002; Eferl & Wagner, 2003a; Hess et al., 2004; Meizner et al., 2004). Interestingly, it was found that in the absence of the tumor suppressor menin, JunD promotes growth, suggesting that menin might be missing in this cell line (Agarwal et al., 1999; Knapp et al., 2000; Agarwal et al., 2003; Eferl & Wagner, 2003a; Hess et al., 2004).

c-Fos

Decreased c-Fos protein in both the transient 45%; Figure 9D) and stable cells (65%; Figure 15B) revealed a significant decrease in AP-1 levels of 84% (Figure 11 & 16) for both the transient and induced stable cells when compared with their controls. This suggests that c-Fos is another protein making up the AP-1 dimer in the U-87 MG cells which is in agreement with the general consensus that Ap-1 dimer is c-Jun/c/Fos.

The decreased protein levels in cells resulted in decreased cell numbers in both the photomicrographs (Figure 13G) and proliferation assay (Figure 20C). This suggests that c-Fos plays a role in cell growth and/or proliferation. This is in agreement with other research in the area (Orlandini et al., 1996). The decrease in cell numbers could also be caused by increased apoptosis which is supported by the increase in caspases 3 and 7 of 98% (Figure 21) as well as the increase of 61% in cleaved PARP (Figure 22B). This also is in agreement with other research (Mikula et al., 2003; Hess et al., 2004) suggesting a role in evasion of apoptosis.

c-Fos has been shown to have a role in differentiation (Grigoriadis et al., 1995; Orlandini et al., 1996; Matsuo et al., 1999; Hess et al., 2004) and this research

supports these findings. Nestin levels were decreased by70% (Figure 18B) and neurospheres were reduced in size and number which suggests a role for c-Fos in dedifferentiation and neurosphere formation and maintenance (Figure 19B).

FosB

Decreased FosB protein levels of 55% (Figure 9E) led to decreased levels of AP-1 transcription factor of 60% (Figure 11) suggesting that FosB also is part of AP-1 in this cell type. Morphologically there were no changes seen in the photos (Figure 13I) except for the lack of neurospheres when FosB was decreased suggesting a role in neurosphere development or maintenance. Cell numbers remained normal when FosB was decreased which supports previous findings (Eferl & Wagner, 2003a).

Fra-1

Fra-1 was shown to have decreased protein levels of 28% (Figure 9F) when transfected with shRNA. This led to a decrease of 63% (Figure 11) in AP-1 transcription factor levels as well suggesting a role in the AP-1 dimer. There was no change in cell numbers seen in the photos (Figure 13K). This partially supports earlier findings that showed Fra-1 caused proliferative inhibition and apoptosis (Shirsat & Shaikh, 2003). There were also no neurospheres present which suggests a role in neurosphere development and/or maintenance.

Fra-2

The presence of the shRNA led to an 80% (Figure 9G) decrease in protein levels of Fra-2 which led to a 76% (Figure 11) decrease in AP-1 transcription factor

levels suggesting a role in the AP-1 dimer. There were also decreased cell numbers in the photos (Figure 13M) which suggests a role for Fra-2 in proliferation and/or evasion of apoptosis. There were also no neurospheres present which suggests a role in neurosphere development and/or maintenance.

NF-κB

p50

There was a decrease in p50 protein levels of 32% (Figure 10B) in the U-87 MG cells that contained p50 shRNA transiently transfected into them which led to a 97% (Figure 12) decrease in NF-κB levels suggesting a major role of p50 in the NF-κB dimer. This is in agreement with the published literature that p50 is one of the major proteins in the NF-κB dimer. With this decrease in NF-κB levels there was a decrease in cell numbers on the plate (Figure 14C) which suggests a role for p50 in proliferation and/or evasion of apoptosis, a role that p50 has been shown to have in other cell types (Young et al., 2006). The lack of neurospheres suggests a role in differentiation which has been found in other cell types (Hilliard et al., 2002). The decrease in cell numbers and the lack of neurospheres is interesting since p50 has no transactivation domain, leading to the question of how p50 exerts its effects.

p52

The 34% decrease in p52 protein (Figure 10C) in the presence of p52 shRNA led to a 72% decrease in NF-κB transcription factor levels (Figure 12), suggesting a role in the NF-kB dimer. There was no noticeable change in the number of cells

growing on the plate with the shRNA when compared with the scrambled shRNA plates (Figure 14E).

p65

Protein levels were decreased 58% (Figure 10D) in the transiently transfected as well as the 57% reduction in induced stable shRNA U-87 MG cells (Figure 15D) when compared with their controls. This protein decrease led to decrease of NF-κB transcription factor of 94% in transiently transfected shRNA (Figure 12) and 87% in induced stable shRNA (Figure 16) when compared with their controls. This supports the fact that the NF-κB dimer is made up of p50/p65.

The lack of NF-κB in the cells revealed a decrease in cell numbers in both the proliferation assay of 52% (Figure 20D) and in the photomicrographs (Figure 14G & 17G), suggesting a role in proliferation and/or evasion of apoptosis. The caspase decrease of 98% (Figure 21) and decrease of 28% in cleaved PARP (Figure 22D) assays revealed that the cell death seen was caused by apoptosis which supports earlier findings that showed that over-expression of p65 inhibited apoptosis (Liu et al., 1996b; Pahl, 1999; Zong et al., 1999).

There was a 54% decrease in nestin (Figure 18D) and a decrease in neurosphere formation, with smaller neurospheres and later onset which suggests that p65 has a role in neurosphere formation and/or maintenance. The decrease in nestin suggests a role in differention which has been previously shown (Grossman et al., 1999).

RelB

The 44% decrease in protein (Figure 10E) led to a 60% decrease in NF-κB levels (Figure 12) as well as a decrease in cell numbers (Figure 14I). This suggests that p50/p65 is not the only dimer combination in the U-87 MG cells and that RelB is part of the NF-κB dimer in these cells. The decrease in cell numbers suggests a role in proliferation and/or evasion of apoptosis. Previous work showed that RelB was confined to dendritic and immune cells. This suggests that the transformed U-87 MG cells have activated RelB.

c-Rel

The 27% decrease in transiently transfected c-Rel shRNA (Figure 10A) and 60% decrease in induced stable shRNA (Figure 15C) protein led to a 98% and 96% decrease in NF-κB transcription factor activity in transiently transfected shRNA (Figure 12) and induced stable shRNA (Figure 16) respectively when compared with their scrambled controls. This suggests a role for c-Rel in dimer composition that is as important in the U-87 MG cells as p50 and p65. The decrease in NF-κB levels led to a 49% decrease in cell numbers shown in the proliferation assay (Figure 20E) as well as photomicrographs (Figure 14A & 17E) suggesting a role for c-Rel in proliferation and/or evasion of apoptosis. The 98% decrease in caspase activity (Figure 21) and 71% increase in cleaved PARP (Figure 22C) further supports the suggestion that c-Rel plays a role in evasion of apoptosis. This is in agreement with previous research that showed a role for c-Rel in proliferation (Gilmore et al., 1996; Baichwal, 1997; Gilmore, 1999; Karin, 1999; Liss, 2002), apoptosis (Gilmore et al.,

1996; Baichwal, 1997; Gerondakis et al., 1999; Gilmore, 1999; Karin, 1999), and cell cycle progression (Liss, 2002).

The 72% decrease in nestin (Figure 18C) and the decrease in neurosphere formation, size, and onset (Figure 19C) suggest a role in differentiation as well as neurosphere formation and/or maintenance. Previous work has shown that c-Rel plays a role in T helper cell differentiation (Hilliard et al., 2002; Pai, 2002).

This research has shown the involvement of these two transcription factors in proliferation and/or evasion of apoptosis, differentiation, and neurosphere maintenance and/or development yet much remains to be determined. It remains to be determined what role JunB is playing in these cells and whether any of the subunits play a role in cellular growth. Stable cell lines need to be created for the remaining eight subunits (JunB, JunD, FosB, Fra-1, Fra-2, p50, p52, and RelB) to determine further their role in these cells. Lastly, the determination of the actual transcription factor dimer composition should be done. The most promising of the subunits appears to be c-Rel which should be further evaluated.

APPENDIX

pc-Jun-1 siRNA 68%GC 84nt from start

AACCTGGCCGACCCACTGG

GAT CCC AAC CTG GCC GAC CCA CTG G,cjun-1,
TTC AAG AGA CCA GTG GGT CGG CCA GGT TTT TTT GGA AA,cjun-2
AGC TTT TCC AAA AAA ACC TGG CCG ACC CAC TGG,cjun-3
TCT CTT GAA CCA GTG GGT CGG CCA GGT TGG G,cjun-4

GATCCCAACCTGGCCGACCCACTGGTTCAAGAGACCAGTGGGTCGGCCAGGTTTTTTTGGAAA
GGGTTGGACCGGCTGGGTGACCAAGTTCTCTGGTCACCCAGCCGGTCCAAAAAAACCTTTTCGA

pc-JunC-1 siRNA 68%GC

AAGACTACGCGCTCCGCGC

GAT CCC CAA GAC TAC GCG CTC CGC GC,cjunC-1,
TTC AAG AGA GCG CGG AGC GCG TAG TCT TTT TTT GGA AA,cjunC-2,
AGC TTT TCC AAA AAA AGA CTA CGC GCT CCG CGC,cjunC-3,
TCT CTT GAA GCG CGG AGC GCG TAG TCT TGG G,cjunC-4,

GATCCCCAAGACTACGCGCTCCGCGCTTCAAGAGAGCGCGGAGCGCGTAGTCTTTTTTTGGAAA
GGGTTCTGATGCGCGAGGCGCGAAGTTCTCTCGCGCCTCGCGCATCAGAAAAAAACCTTTTCGA

pc-Jun-2 siRNA 53% GC 347nt from start

AACTGCACAGCCAGAACAC

GAT CCC CAA CTG CAC AGC CAG AAC AC,cjun2-1,
TTC AAG AGA GTG TTC TGG CTG TGC AGT TTT TTT GGA AA,cjun2-2,
AGC TTT TCC AAA AAA ACT GCA CAG CCA GAA CAC,cjun2-3,
TCT CTT GAA GTG TTC TGG CTG TGC AGT TGG G,cjun2-4,

GATCCCCAACTGCACAGCCAGAACACTTCAAGAGAGTGTTCTGGCTGTGCAGTTTTTTTGGAAA
GGGTTGACGTGTCGGTCTTGTGAAGTTCTCTCACAAGACCGACACGTCAAAAAAACCTTTTCGA

pc-JunC-2 siRNA 53%GC

TAAGACCGACCGACCAACA

GAT CCC CTA AGA CCG ACC GAC CAA CA,cjunC2-1,
TTC AAG AGA TGT TGG TCG GTC GGT CTT ATT TTT GGA AA,cjunC2-2,

AGC TTT TCC AAA AAT AAG ACC GAC CGA CCA ACA,cjunC2-3,
TCT CTT GAA TGT TGG TCG GTC GGT CTT AGG G,cjunC2-4,

GATCCCCTAAGACCGACCGACCAACATTCAAGAGATGTTGGTCGGTCGGTCTTATTTTTGGAAA
GGGATTCTGGCTGGCTGGTTGTAAGTTCTCTACAACCAGCCAGCCAGAATAAAAACCTTTTCGA

pJunD-1 siRNA 84%GC 183nt from start

AAGCCTGCGGCCGCGCCGC

GAT CCC CAA GCC TGC GGC CGC GCC GC,JunD-1,
TTC AAG AGA GCG GCG CGG CCG CAG GCT TTT TTT GGA AA,JunD-2,
AGC TTT TCC AAA AAA AGC CTG CGG CCG CGC CGC,JunD-3,
TCT CTT GAA GCG GCG CGG CCG CAG GCT TGG G,JunD-4,

GATCCCCAAGCCTGCGGCCGCGCCGCTTCAAGAGAGCGGCGCGGCCGCAGGCTTTTTTTGGAAA
GGGTTCGGACGCCGGCGCGGCGAAGTTCTCTCGCCGCGCCGGCGTCCGAAAAAAACCTTTTCGA

pJunDC-1 siRNA 84%GC

CGCCGCGCCGGCGTCCGAA

GAT CCC CCG CCG CGC CGG CGT CCG AA,JunDC-1,
TTC AAG AGA TTC GGA CGC CGG CGC GGC GTT TTT GGA AA,JunDC-2,
AGC TTT TCC AAA AAC GCC GCG CCG GCG TCC GAA,JunDC-3,
TCT CTT GAA TTC GGA CGC CGG CGC GGC GGG G,JunDC-4,

GATCCCCCGCCGCGCCGGCGTCCGAATTCAAGAGATTCGGACGCCGGCGCGGCGTTTTTGGAAA
GGGGCGGCGCGGCCGCAGGCTTAAGTTCTCTAAGCCTGCGGCCGCGCCGCAAAAACCTTTTCGA

pJunD-2 siRNA 84%GC 1255nt from start

AAGGGGCGGGCTCGCGGGG

GAT CCC CAA GGG GCG GGC TCG CGG GG,JunD2-1,
TTC AAG AGA CCC CGC GAG CCC GCC CCT TTT TTT GGA AA,JunD2-2,
AGC TTT TCC AAA AAA AGG GGC GGG CTC GCG GGG,JunD2-3,
TCT CTT GAA CCC CGC GAG CCC GCC CCT TGG G,JunD2-4,

GATCCCCAAGGGGCGGGCTCGCGGGGTTCAAGAGACCCCGCGAGCCCGCCCCTTTTTTTGGAAA
GGGTTCCCCGCCCGAGCGCCCCAAGTTCTCTGGGGCGCTCGGGCGGGGAAAAAAACCTTTTCGA

pJunDC-2 siRNA 84%GC

GGGGGGGGGGGGACCACTC

GAT CCC CGG GGG GGG GGG GAC CAC TC,JunDC2-1,
TTT AAG AGA GAG TGG TCC CCC CCC CCC CTT TTT GGA AA,JunDC2-2,
AGC TTT TCC AAA AAG GGG GGG GGG GGA CCA CTC,JunDC2-3,
TCT CTT GAA GAG TGG TCC CCC CCC CCC CGG G,JunDC2-4

GATCCCCGGGGGGGGGGGGACCACTCTTTAAGAGAGAGTGGTCCCCCCCCCCCCTTTTTGGAAA
GGGCCCCCCCCCCCCCTGGTGAGAAGTTCTCTCTCACCAGGGGGGGGGGGGAAAAACCTTTTCGA

pJunB-1 siRNA 80%GC 148nt from start

AAGCGCCTGGGGCTCGCGG

GAT CCC CAA GCG CCT GGG GCT CGC GG,JunB-1,
TTC AAG AGA CCG CGA GCC CCA GGC GCT TTT TTT GGA AA,JunB-2,
AGC TTT TCC AAA AAA AGC GCC TGG GGC TCG CGG,JunB-3,
TCT CTT GAA CCG CGA GCC CCA GGC GCT TGG G,JunB-4,

GATCCCCAAGCGCCTGGGGCTCGCGGTTCAAGAGACCGCGAGCCCCAGGCGCTTTTTTTGGAAA
GGGTTCGCGGACCCCGAGCGCCAAGTTCTCTGGCGCTCGGGGTCCGCGAAAAAAACCTTTTCGA

pJunBC-1 siRNA 80%GC

GCGGCGGAGGCATCCGTCG

GAT CCC CGC GGC GGA GGC ATC CGT CG,JunBC-1,
TTC AAG AGA CGA CGG ATG CCT CCG CCG CTT TTT GGA AA,JunBC-2,
AGC TTT TCC AAA AAG CGG CGG AGG CAT CCG TCG,JunBC-3,
TCT CTT GAA CGA CGG ATG CCT CCG CCG CGG G,JunBC-4,

GATCCCCGCGGCGGAGGCATCCGTCGTTCAAGAGACGACGGATGCCTCCGCCGCTTTTTGGAAA
GGGCGCCGCCTCCGTAGGCAGCAAGTTCTCTGCTGCCTACGGAGGCGGCGAAAAACCTTTTCGA

pJunB-2 siRNA 52%GC 259nt from start

AACGCCTGATTGTCCCCAA

GAT CCC CAA CGC CTG ATT GTC CCC AA,JunB2-1,
TTC AAG AGA TTG GGG ACA ATC AGG CGT TTT TTT GGA AA,JunB2-2,
AGC TTT TCC AAA AAA ACG CCT GAT TGT CCC CAA,JunB2-3,
TCT CTT GAA TTG GGG ACA ATC AGG CGT TGG G,JunB2-4,

GATCCCCAACGCCTGATTGTCCCCAATTCAAGAGATTGGGGACAATCAGGCGTTTTTTTTGGAAA
GGGTTGCGGACTAACAGGGGTTAAGTTCTCTAACCCCTGTTAGTCCGCAAAAAAACCTTTTCGA

pJunBC-2 siRNA 52%GC

CACCATGATCACGATCGTC

GAT CCC CCA CCA TGA TCA CGA TCG TC,JunBC2-1
TTC AAG AGA GAC GAT CGT GAT CAT GGT GTT TTT GGA AA,JunBC2-2,
AGC TTT TCC AAA AAC ACC ATG ATC ACG ATC GTC,JunBC2-3,
TCT CTT GAA GAC GAT CGT GAT CAT GGT GGG G,JunBC2-4,

GATCCCCCACCATGATCACGATCGTCTTCAAGAGAGACGATCGTGATCATGGTGTTTTTTGGAAA
GGGGTGGTACTAGTGCTAGCAGAAGTTCTCTCTGCTAGCACTAGTACCACAAAAACCTTTTCGA

pFra2-1 siRNA 63%GC 151nt from start

AACGCCATCACGACCAGCC

GAT CCC CAA CGC CAT CAC GAC CAG CC,Fra2-1,
TTC AAG AGA GGC TGG TCG TGA TGG CGT TTT TTT GGA AA,Fra2-2,
AGC TTT TCC AAA AAA ACG CCA TCA CGA CCA GCC,Fra2-3,
TCT CTT GAA GGC TGG TCG TGA TGG CGT TGG G,Fra2-4,

GATCCCCAACGCCATCACGACCAGCCTTCAAGAGAGGCTGGTCGTGATGGCGTTTTTTTGGAAA
GGGTTGCGGTAGTGCTGGTCGGAAGTTCTCTCCGACCAGCACTACCGCAAAAAAACCTTTTCGA

pFra2C-1 siRNA 63%GC

CCGACCAGCACTACCGCAA

GAT CCC CCC GAC CAG CAC TAC CGC AA,Fra2C-1,
TTC AAG AGA TTG CGG TAG TGC TGG TCG GTT TTT GGA AA,Fra2C-2,
AGC TTT TCC AAA AAC CGA CCA GCA CTA CCG CAA,Fra2C-3,
TCT CTT GAA TTG CGG TAG TGC TGG TCG GGG G,Fra2,

GATCCCCCCGACCAGCACTACCGCAATTCAAGAGATTGCGGTAGTGCTGGTCGGTTTTTGGAAA
GGGGGCTGGTCGTGATGGCGTTAAGTTCTCTAACGCCATCACGACCAGCCAAAAACCTTTTCGA

pFra2-2 siRNA 63%GC 223nt from start

AACCCATACCCTCGCTCGC

GAT CCC CAA CCC ATA CCC TCG CTC GC,Fra22-1,
TTC AAG AGA GCG AGC GAG GGT ATG GGT TTT TTT GGA AA,Fra22-2,
AGC TTT TCC AAA AAA ACC CAT ACC CTC GCT CGC,Fra22-3,
TCT CTT GAA GCG AGC GAG GGT ATG GGT TGG G,Fra22-4,

GATCCCCAACCCATACCCTCGCTCGCTTCAAGAGAGCGAGCGAGGGTATGGGTTTTTTTGGAAA
GGGTTGGGTATGGGAGCGAGCGAAGTTCTCTCGCTCGCTCCCATACCCAAAAAAACCTTTTCGA

pFra2C-2 siRNA 63%GC

CACACATACCTCGCGCCTC

GAT CCC CCA CAC ATA CCT CGC GCC TC,Fra2C2-1,
TTC AAG AGA GAG GCG CGA GGT ATG TGT GTT TTT GGA AA,Fra2C2-2
AGC TTT TCC AAA AAC ACA CAT ACC TCG CGC CTC,Fra2C-3,
TCT CTT GAA GAG GCG CGA GGT ATG TGT GGG G,Fra2C-4,

GATCCCCCACACATACCTCGCGCCTCTTCAAGAGAGAGGCGCGAGGTATGTGTGTTTTTGGAAA
GGGGTGTGTATGGAGCGCGGAGAAGTTCTCTCTCCGCGCTCCATACACACAAAAACCTTTTCGA

pFra1-1 siRNA 53%GC 95nt from start

AAGTTCCACCTGGTGCCAA

GAT CCC CAA GTT CCA CCT GGT GCC AA,Fra1-1,
TTC AAG AGA TTG GCA CCA GGT GGA ACT TTT TTT GGA AA,Fra1-2,
AGC TTT TCC AAA AAA AGT TCC ACC TGG TGC CAA,Fra1-3,
TCT CTT GAA TTG GCA CCA GGT GGA ACT TGG G,Fra1-4,

GATCCCCAAGTTCCACCTGGTGCCAATTCAAGAGATTGGCACCAGGTGGAACTTTTTTTGGAAA
GGGTTCAAGGTGGACCACGGTTAAGTTCTCTAACCGTGGTCCACCTTGAAAAAAACCTTTTCGA

pFra1C-1 siRNA 53%GC

TCATCAGTCATCAGGCCCA

GAT CCC CTC ATC AGT CAT CAG GCC CA,Fra1C-1,
TTC AAG AGA TGG GCC TGA TGA CTG ATG ATT TTT GGA AA,Fra1C-2,
AGC TTT TCC AAA AAT CAT CAG TCA TCA GGC CCA,Fra1C-3,
TCT CTT GAA TGG GCC TGA TGA CTG ATG AGG G,Fra1C-4,

GATCCCCTCATCAGTCATCAGGCCCATTCAAGAGATGGGCCTGATGACTGATGATTTTTGGAAA
GGGAGTAGTCAGTAGTCCGGGTAAGTTCTCTACCCGGACTACTGACTACTAAAAACCTTTTCGA

pFra1-2 siRNA 63%GC 226nt from start

AACCCCGGCCAGGAGTCAT

GAT CCC CAA CCC CGG CCA GGA GTC AT,Fra12-1,
TTC AAG AGA ATG ACT CCT GGC CGG GGT TTT TTT GGA AA,Fra12-2,
AGC TTT TCC AAA AAA ACC CCG GCC AGG AGT CAT,Fra12-3,
TCT CTT GAA ATG ACT CCT GGC CGG GGT TGG G,Fra12-4.

GATCCCCAACCCCGGCCAGGAGTCATTTCAAGAGAATGACTCCTGGCCGGGGTTTTTTTGGAAA
GGGTTGGGGCCGGTCCTCAGTAAAGTTCTCTTACTGAGGACCGGCCCCAAAAAAACCTTTTCGA

pFra1C-2 siRNA 63%GC

CGCCACGACGACTAGTAGC

GAT CCC CCG CCA CGA CGA CTA GTA GC,Fra1C2-1,
TTC AAG AGA GCT ACT AGT CGT CGT GGC GTT TTT GGA AA,Fra1C2-2,
AGC TTT TCC AAA AAC GCC ACG ACG ACT AGT AGC,Fra1C2-3,
TCT CTT GAA GCT ACT AGT CGT CGT GGC GGG G,Fra1C2-4,

GATCCCCCGCCACGACGACTAGTAGCTTCAAGAGAGCTACTAGTCGTCGTGGCGTTTTTGGAAA

GGGGCGGTGCTGCTGATCATCGAAGTTCTCTCGATGATCAGCAGCACCGCAAAAACCTTTTCGA

pc-Fos-1 siRNA 68%GC 139nt from start

AAGGCTGGCTTCCCGTCGC

GAT CCC CAA GGC TGG CTT CCC GTC GC,c-fos-1,
TTC AAG AGA GCG ACG GGA AGC CAG CCT TTT TTT GGA AA,c-fos-2,
AGC TTT TCC AAA AAA AGG CTG GCT TCC CGT CGC,c-fos-3,
TCT CTT GAA GCG ACG GGA AGC CAG CCT TGG G,c-fos-4,

GATCCCCAAGGCTGGCTTCCCGTCGCTTCAAGAGAGCGACGGGAAGCCAGCCTTTTTTTGGAAA
GGGTTCCGACCGAAGGGCAGCGAAGTTCTCTCGCTGCCCTTCGGTCGGAAAAAAACCTTTTCGA

pc-FosC-1 siRNA 68%GC

GATCAGGTCGCCTCTGCCG

GAT CCC CGA TCA GGT CGC CTC TGC CG,cfosC-1,
TTC AAG AGA CGG CAG AGG CGA CCT GAT CTT TTT GGA AA,cfosC-2,
AGC TTT TCC AAA AAG ATC AGG TCG CCT CTG CCG,cfosC-3,
TCT CTT GAA CGG CAG AGG CGA CCT GAT CGG G,cfosC-4,

GATCCCCGATCAGGTCGCCTCTGCCGTTCAAGAGACGGCAGAGGCGACCTGATCTTTTTGGAAA
GGGCTAGTCCAGCGGAGACGGCAAGTTCTCTGCCGTCTCCGCTGGACTAGAAAAACCTTTTCGA

pc-Fos-2 siRNA 58%GC 323nt from start

AAGAATTGGTTCCCCCTTC

GAT CCC CAA GAA TTG GTT CCC CCT TC,cfos2-1,
TTC AAG AGA GAA GGG GGA ACC AAT TCT TTT TTT GGA AA,cfos2-2,
AGC TTT TCC AAA AAA AGA ATT GGT TCC CCC TTC,cfos2-3,
TCT CTT GAA GAA GGG GGA ACC AAT TCT TGG G,cfos2-4,

GATCCCCAAGAATTGGTTCCCCCTTCTTCAAGAGAGAAGGGGGAACCAATTCTTTTTTTGGAAA
GGGTTCTTAACCAAGGGGGAAGAAGTTCTCTCTTCCCCCTTGGTTAAGAAAAAAACCTTTTCGA

pc-FosC-2 siRNA 58%GC

CATTTCTTCGCAGCATCAG

GAT CCC CCA TTT CTT CGC AGC ATC AG,cfosC2-1,
TTC AAG AGA CTG ATG CTG CGA AGA AAT GTT TTT GGA AA,cfosC2-2,
AGC TTT TCC AAA AAC ATT TCT TCG CAG CAT CAG,cfosC2-3,
TCT CTT GAA CTG ATG CTG CGA AGA AAT GGG G,cfosC2-4,

GATCCCCCATTTCTTCGCAGCATCAGTTCAAGAGACTGATGCTGCGAAGAAATGTTTTTGGAAA
GGGGTAAAGAAGCGTCGTAGTCAAGTTCTCTGACTACGACGCTTCTTTACAAAAACCTTTTCGA

pFosB-1 siRNA 47%GC 167nt from start

AAGCTGATTCTGTACAGCG

GAT CCC CAA GCT GAT TCT GTA CAG CG,FosB-1,
TTC AAG AGA CGC TGT ACA GAA TCA GCT TTT TTT GGA AA,FosB-2,
AGC TTT TCC AAA AAA AGC TGA TTC TGT ACA GCG,FosB-3,
TCT CTT GAA CGC TGT ACA GAA TCA GCT TGG G,FosB-4,

GATCCCCAAGCTGATTCTGTACAGCGTTCAAGAGACGCTGTACAGAATCAGCTTTTTTTGGAAA
GGGTTCGACTAAGACATGTCGCAAGTTCTCTGCGACATGTCTTAGTCGAAAAAAACCTTTTCGA

pFosBC-1 siRNA 47%GC

TTTAGCACCTAGGAGCATG

GAT CCC CTT TAG CAC CTA GGA GCA TG,FosBC-1,
TTC AAG AGA CAT GCT CCT AGG TGC TAA ATT TTT GGA AA,FosBC-2,
AGC TTT TCC AAA AAT TTA GCA CCT AGG AGC ATG,FosBC-3,
TCT CTT GAA CAT GCT CCT AGG TGC TAA AGG G,FosBC-4,

GATCCCCTTTAGCACCTAGGAGCATGTTCAAGAGACATGCTCCTAGGTGCTAAATTTTTGGAAA
GGGAAATCGTGGATCCTCGTACAAGTTCTCTGTACGAGGATCCACGATTTAAAAAACCTTTTCGA

pFosB-2 siRNA 74%GC 209nt from start

AACCCCTCCCTCGCCCCTG

GAT CCC CAA CCC CTC CCT CGC CCC TG,FosB–1,
TTC AAG AGA CAG GGG CGA GGG AGG GGT TTT TTT GGA AA,FosB-2,
AGC TTT TCC AAA AAA ACC CCT CCC TCG CCC CTG,FosB-3,
TCT CTT GAA CAG GGG CGA GGG AGG GGT TGG G,FosB-4,

GATCCCCAACCCCTCCCTCGCCCCTGTTCAAGAGACAGGGGCGAGGGAGGGGTTTTTTTGGAAA
GGGTTGGGGAGGGAGCGGGGACAAGTTCTCTGTCCCCGCTCCCTCCCCAAAAAAACCTTTTCGA

pFosBC-2 siRNA 74%GC

CCTCCACCTCCACCGCCTG

GAT CCC CCC TCC ACC TCC ACC GCC TG,FosBC2-1,
TTC AAG AGA CAG GCG GTG GAG GTG GAG GTT TTT GGA AA,FosBC2-2,
AGC TTT TCC AAA AAC CTC CAC CTC CAC CGC CTG,FosBC2-3,
TCT CTT GAA CAG GCG GTG GAG GTG GAG GGG G,FosBC2-4,

GATCCCCCCTCCACCTCCACCGCCTGTTCAAGAGACAGGCGGTGGAGGTGGAGGTTTTTGGAAA
GGGGGAGGTGGAGGTGGCGGACAAGTTCTCTGTCCGCCACCTCCACCTCCAAAAACCTTTTCGA

p65-1 siRNA 74%GC 66nt from start

AAGTGCGAGGGGCGCTCCG

GAT CCC CAA GTG CGA GGG GCG CTC CG,p65-1,
TTC AAG AGA CGG AGC GCC CCT CGC ACT TTT TTT GGA AA,p65-2,
AGC TTT TCC AAA AAA AGT GCG AGG GGC GCT CCG,p65-3,
TCT CTT GAA CGG AGC GCC CCT CGC ACT TGG G,p65-4,

GATCCCCAAGTGCGAGGGGCGCTCCGTTCAAGAGACGGAGCGCCCCTCGCACTTTTTTTGGAAA
GGGTTCACGCTCCCCGCGAGGCAAGTTCTCTGCCTCGCGGGGAGCGTGAAAAAAACCTTTTCGA

p65C-1 siRNA 74%GC

GGAGGAGGTACGCCTGCCG

GAT CCC CGG AGG AGG TAC GCC TGC CG,P65C-1,
TTC AAG AGA CGG CAG GCG TAC CTC CTC CTT TTT GGA AA,P65C-2,
AGC TTT TCC AAA AAG GAG GAG GTA CGC CTG CCG,P65C-3,
TCT CTT GAA CGG CAG GCG TAC CTC CTC CGG G,P65C-4,

GATCCCCGGAGGAGGTACGCCTGCCGTTCAAGAGACGGCAGGCGTACCTCCTCCTTTTTGGAAA
GGGCCTCCTCCATGCGGACGGCAAGTTCTCTGCCGTCCGCATGGAGGAGGAAAAACCTTTTCGA

p65-2 siRNA 63%GC 234nt from start

AAGGACTGCCGGGATGGCT

GAT CCC CAA GGA CTG CCG GGA TGG CT,p652-1,
TTC AAG AGA AGC CAT CCC GGC AGT CCT TTT TTT GGA AA,p652-2,
AGC TTT TCC AAA AAA AGG ACT GCC GGG ATG GCT,p652-3,
TCT CTT GAA AGC CAT CCC GGC AGT CCT TGG G,p652-4,

GATCCCCAAGGACTGCCGGGATGGCTTTCAAGAGAAGCCATCCCGGCAGTCCTTTTTTTGGAAA
GGGTTCCTGACGGCCCTACCGAAAGTTCTCTTCGGTAGGGCCGTCAGGAAAAAAACCTTTTCGA

p65C-2 siRNA 63%GC

GACGATGCCGATGAGGTCG

GAT CCC CGA CGA TGC CGA TGA GGT CG,p65C2-1,
TTC AAG AGA CGA CCT CAT CGG CAT CGT CTT TTT GGA AA,p65C2-2,
AGC TTT TCC AAA AAG ACG ATG CCG ATG AGG TCG,p65C2-3,
TCT CTT GAA CGA CCT CAT CGG CAT CGT CGG G,p65C2-4,

GATCCCCGACGATGCCGATGAGGTCGTTCAAGAGACGACCTCATCGGCATCGTCTTTTTGGAAA
GGGCTGCTACGGCTACTCCAGCAAGTTCTCTGCTGGAGTAGCCGTAGCAGAAAAACCTTTTCGA

pc-Rel-1 siRNA 53%GC 63nt from start

AACTTTCCTCTCCCCCAGT

GAT CCC CAA CTT TCC TCT CCC CCA GT,cRel-1,
TTC AAG AGA ACT GGG GGA GAG GAA AGT TTT TTT GGA AA,cRel-2,
AGC TTT TCC AAA AAA ACT TTC CTC TCC CCC AGT,cRel-3,
TCT CTT GAA ACT GGG GGA GAG GAA AGT TGG G,cRel-4,

GATCCCCAACTTTCCTCTCCCCCAGTTTCAAGAGAACTGGGGGAGAGGAAAGTTTTTTTGGAAA
GGGTTGAAAGGAGAGGGGGTCAAAGTTCTCTTGACCCCCTCTCCTTTCAAAAAAACCTTTTCGA

pc-RelC-1 siRNA 53%GC

CTCCATCTCCATCACTCTG

GAT CCC CCT CCA TCT CCA TCA CTC TG,cRelC-1,
TTC AAG AGA CAG AGT GAT GGA GAT GGA GTT TTT GGA AA,cRelC-2,
AGC TTT TCC AAA AAC TCC ATC TCC ATC ACT CTG,cRelC-3,
TCT CTT GAA CAG AGT GAT GGA GAT GGA GGG G,cRelC-4,

GATCCCCCTCCATCTCCATCACTCTGTTCAAGAGACAGAGTGATGGAGATGGAGTTTTTGGAAA
GGGGAGGTAGAGGTAGTGAGACAAGTTCTCTGTCTCACTACCTCTACCTCAAAAACCTTTTCGA

pc-Rel-2 siRNA 58%GC 195nt from start

AAGGTCTTTCTTGCGGGCC

GAT CCC CAA GGT CTT TCT TGC GGG CC,c-Rel2-1,
TTC AAG AGA GGC CCG CAA GAA AGA CCT TTT TTT GGA AA,c-Rel2-2,
AGC TTT TCC AAA AAA AGG TCT TTC TTG CGG GCC,c-Rel2-3,
TCT CTT GAA GGC CCG CAA GAA AGA CCT TGG G,c-Rel2-4,

GATCCCCAAGGTCTTTCTTGCGGGCCTTCAAGAGAGGCCCGCAAGAAAGACCTTTTTTTGGAAA
GGGTTCCAGAAAGAACGCCCGGAAGTTCTCTCCGGGCGTTCTTTCTGGAAAAAAACCTTTTCGA

pc-RelC-2 siRNA 58%GC

GATCATGTTGCCGTTGCGC

GAT CCC CGA TCA TGT TGC CGT TGC GC,cRelC2-1,
TTC AAG AGA GCG CAA CGG CAA CAT GAT CTT TTT GGA AA,cRelC2-2,
AGC TTT TCC AAA AAG ATC ATG TTG CCG TTG CGC,cRelC2-3,
TCT CTT GAA GCG CAA CGG CAA CAT GAT CGG G,cRelC2-4,

GATCCCCGATCATGTTGCCGTTGCGCTTCAAGAGAGCGCAACGGCAACATGATCTTTTTGGAAA
GGGCTAGTACAACGGCAACGCGAAGTTCTCTCGCGTTGCCGTTGTACTAGAAAAACCTTTTCGA

pRelB-1 siRNA 63%GC 170nt from start

AAGGAGAACGGCTTCGGCC

GAT CCC CAA GGA GAA CGG CTT CGG CC,RelB-1,
TTC AAG AGA GGC CGA AGC CGT TCT CCT TTT TTT GGA AA,RelB-2,
AGC TTT TCC AAA AAA AGG AGA ACG GCT TCG GCC,RelB-3,
TCT CTT GAA GGC CGA AGC CGT TCT CCT TGG G,RelB-4,

GATCCCCAAGGAGAACGGCTTCGGCCTTCAAGAGAGGCCGAAGCCGTTCTCCTTTTTTTGGAAA
GGGTTCCTCTTGCCGAAGCCGGAAGTTCTCTCCGGCTTCGGCAAGAGGAAAAAAACCTTTTCGA

pRelBC-1 siRNA 63%GC

GAGAGAGTCGTCCGCGCAA

GAT CCC CGA GAG AGT CGT CCG CGC AA,RelBC-1,
TTC AAG AGA TTG CGC GGA CGA CTC TCT CTT TTT GGA AA,RelBC-2,
AGC TTT TCC AAA AAG AGA GAG TCG TCC GCG CAA,RelBC-3,
TCT CTT GAA TTG CGC GGA CGA CTC TCT CGG G,RelBC-4,

GATCCCCGAGAGAGTCGTCCGCGCAATTCAAGAGATTGCGCGGACGACTCTCTCTTTTTGGAAA
GGGCTCTCTCAGCAGGCGCGTTAAGTTCTCTAACGCGCCTGCTGAGAGAGAAAAACCTTTTCGA

pRelB-2 siRNA 68%GC 393nt from start

AAGCAGCGCGGCATGCGCT

GAT CCC CAA GCA GCG CGG CAT GCG CT,RelB2-1,
TTC AAG AGA AGC GCA TGC CGC GCT GCT TTT TTT GGA AA,RelB2-2,
AGC TTT TCC AAA AAA AGC AGC GCG GCA TGC GCT,RelB2-3,
TCT CTT GAA AGC GCA TGC CGC GCT GCT TGG G,RelB2-4,

GATCCCCAAGCAGCGCGGCATGCGCTTTCAAGAGAAGCGCATGCCGCGCTGCTTTTTTTGGAAA
GGGTTCGTCGCGCCGTACGCGAAAGTTCTCTTCGCGTACGGCGCGACGAAAAAAACCTTTTCGA

pRelBC-2 siRNA 68%GC

CATGAGACGGGCAGCTCGC

GAT CCC CCA TGA GAC GGG CAG CTC GC,RelBC2-1,
TTC AAG AGA GCG AGC TGC CCG TCT CAT GTT TTT GGA AA,RelBC2-2,
AGC TTT TCC AAA AAC ATG AGA CGG GCA GCT CGC,RelBC2-3,
TCT CTT GAA GCG AGC TGC CCG TCT CAT GGG G,RelBC2-4,

GATCCCCCATGAGACGGGCAGCTCGCTTCAAGAGAGCGAGCTGCCCGTCTCATGTTTTTGGAAA
GGGGTACTCTGCCCGTCGAGCGAAGTTCTCTCGCTCGACGGGCAGAGTACAAAAACCTTTTCGA

p52-1 siRNA 68%GC 83nt from start

AACCCAAGGAGCCAGCCCC

GAT CCC CAA CCC AAG GAG CCA GCC CC,p52-1,
TTC AAG AGA GGG GCT GGC TCC TTG GGT TTT TTT GGA AA,p52-2,
AGC TTT TCC AAA AAA ACC CAA GGA GCC AGC CCC,p52-3,
TCT CTT GAA GGG GCT GGC TCC TTG GGT TGG G,p52-4,

GATCCCCAACCCAAGGAGCCAGCCCCTTCAAGAGAGGGGCTGGCTCCTTGGGTTTTTTTGGAAA
GGGTTGGGTTCCTCGGTCGGGGAAGTTCTCTCCCCGACCGAGGAACCCAAAAAAACCTTTTCGA

p52C-1 siRNA 68%GC

CAGGACCCGACCACAGCCA

GAT CCC CCA GGA CCC GAC CAC AGC CA,p52C-1,
TTC AAG AGA TGG CTG TGG TCG GGT CCT GTT TTT GGA AA,p52C-2,
AGC TTT TCC AAA AAC AGG ACC CGA CCA CAG CCA,p52C-3,
TCT CTT GAA TGG CTG TGG TCG GGT CCT GGG G,P52c-4,

GATCCCCCAGGACCCGACCACAGCCATTCAAGAGATGGCTGTGGTCGGGTCCTGTTTTTGGAAA
GGGGTCCTGGGCTGGTGTCGGTAAGTTCTCTACCGACACCAGCCCAGGACAAAAACCTTTTCGA

p52-2 siRNA 58%GC 246nt from start

AACTACGAGGGACCAGCCA

GAT CCC CAA CTA CGA GGG ACC AGC CA,p522-1,
TTC AAG AGA TGG CTG GTC CCT CGT AGT TTT TTT GGA AA,p522-2,
AGC TTT TCC AAA AAA ACT ACG AGG GAC CAG CCA,p522-3,
TCT CTT GAA TGG CTG GTC CCT CGT AGT TGG G,p522-4,

GATCCCCAACTACGAGGGACCAGCCATTCAAGAGATGGCTGGTCCCTCGTAGTTTTTTTGGAAA
GGGTTGATGCTCCCTGGTCGGTAAGTTCTCTACCGACCAGGGAGCATCAAAAAAACCTTTTCGA

p52C-2 siRNA 58%GC

ACCAGGACGCCATGAGACA

GAT CCC CAC CAG GAC GCC ATG AGA CA,p52C2-1,
TTC AAG AGA TGT CTC ATG GCG TCC TGG TTT TTT GGA AA,p52C2-2,
AGC TTT TCC AAA AAA CCA GGA CGC CAT GAG ACA,p52C2-3,
TCT CTT GAA TGT CTC ATG GCG TCC TGG TGG G,p52C2-4,

GATCCCCACCAGGACGCCATGAGACATTCAAGAGATGTCTCATGGCGTCCTGGTTTTTTGGAAA
GGGTGGTCCTGCGGTACTCTGTAAGTTCTCTACAGAGTACCGCAGGACCAAAAAACCTTTTCGA

p50-1 siRNA 63% GC 190nt from start

AAGGCCCATCCCATGGTGG

GAT CCC CAA GGC CCA TCC CAT GGT GG,p50-1,
TTC AAG AGA CCA CCA TGG GAT GGG CCT TTT TTT GGA AA,p50-2,
AGC TTT TCC AAA AAA AGG CCC ATC CCA TGG TGG,p50-3,
TCT CTT GAA CCA CCA TGG GAT GGG CCT TGG G,p50-4,

GATCCCCAAGGCCCATCCCATGGTGGTTCAAGAGACCACCATGGGATGGGCCTTTTTTTGGAAA
GGGTTCCGGGTAGGGTACCACCAAGTTCTCTGGTGGTACCCTACCCGGAAAAAAACCTTTTCGA

p50C-1 siRNA 63%GC

GAGCACTAGTACGCGCGCT

GAT CCC CGA GCA CTA GTA CGC GCG CT,p50C-1,
TTC AAG AGA AGC GCG CGT ACT AGT GCT CTT TTT GGA AA,p50C-2,
AGC TTT TCC AAA AAG AGC ACT AGT ACG CGC GCT,p50C-3,
TCT CTT GAA AGC GCG CGT ACT AGT GCT CGG G,p50C-4,

GATCCCCGAGCACTAGTACGCGCGCTTTCAAGAGAAGCGCGCGTACTAGTGCTCTTTTTGGAAA
GGGCTCGTGATCATGCGCGCGAAAGTTCTCTTCGCGCGCATGATCACGAGAAAAAACCTTTTCGA

p50-2 siRNA 58%GC 374nt from start

AACTGCTGGACCCAAGGAC

GAT CCC CAA CTG CTG GAC CCA AGG AC,p502-1,
TTC AAG AGA GTC CTT GGG TCC AGC AGT TTT TTT GGA AA,p502-2,
AGC TTT TCC AAA AAA ACT GCT GGA CCC AAG GAC,p502-3,
TCT CTT GAA GTC CTT GGG TCC AGC AGT TGG G,p502-4,

GATCCCCAACTGCTGGACCCAAGGACTTCAAGAGAGTCCTTGGGTCCAGCAGTTTTTTTGGAAA
GGGTTGACGACCTGGGTTCCTGAAGTTCTCTCAGGAACCCAGGTCGTCAAAAAAACCTTTTCGA

p50C-2 siRNA 58%GC

GTCAGAGACCCATGAGCAC

GAT CCC CGT CAG AGA CCC ATG AGC AC,p50C2-1,
TTC AAG AGA GTG CTC ATG GGT CTC TGA CTT TTT GGA AA,p50C2-2,
AGC TTT TCC AAA AAG TCA GAG ACC CAT GAG CAC,p50C2-3,
TCT CTT GAA GTG CTC ATG GGT CTC TGA CGG G,p50C2-4,

GATCCCCGTCAGAGACCCATGAGCACTTCAAGAGAGTGCTCATGGGTCTCTGACTTTTTGGAAA
GGGCAGTCTCTGGGTACTCGTGAAGTTCTCTCACGAGTACCCAGAGACTGAAAAACCTTTTCGA

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