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Ap 1 in Glioblastomas

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1. Introduction

Although the origins of cancer can be exceptionally complex due to the range of genes that may be involved it is also apparent that there are "consensus" genes, ones that are frequently mutated. The AP-1 family of transcription factors represent a set of proteins that are commonly involved in cancers as well as other developmental and pathological conditions including heart development, apoptosis, and hepatogenesis (reviewed in (1). Although defined as a protein that can bind to a consensus sequence in a variety of promoters, AP-1 is in fact a series of heterodimmers that are created between at least 7 distinct proteins including c-jun, junB, Fos, FosB, Fra1,Fra2 and the Maf family of proteins (2). Each of these proteins is the product of distinct genes and although they are different at the amino acid level they share homology and structural similarities including a leucine zipper, transactivation domain and a basic region. Expression of these different proteins is tightly controlled and they may be expressed at different times of development and in different tissues. Control of AP-1 activity revolves around the control of these protein's transcription, translation and post-translation modification

There have been several reports that have detailed the role that some of the family members may play in several tumors. Through the use of dominant negative mutants for c-jun it has been demonstrated that this protein is necessary for tumor survival while inhibition of jun-B may enhance tumor survival (3-5). In all of these experiments it is not clear if any of the other family members that comprise AP-1 are actually present in the cell and if they might play roles as well. Instead of developing dominant negative mutants that could mask the role that the other family members play in tumorigenesis we have taken the approach of creating a series of either siRNAs or shRNAs to investigate the role that each of these family members play in a glioblastoma cell line. Our results demonstrate that in the U87-MG cell line all family members are expressed. However the necessity of each these members varies greatly for cell proliferation and survival.

2. Materials and methods

Cells and transfections: Cell lines U87-MG, U118-MG and U373-MG were obtained from the American Type Culture Collection and maintained according to their instructions. Cells transfections, selections in geneticin sulfate and secreted alkaline phosphatase assays were performed as essentially as described (6)

shRNA: siRNAs were developed by Elbashir using the methods described by (7, 8). At 63-190 nucletoides downstream of the start codon a 19 nucleotide sequence was chosen. This sequence began with an adenine dimer and had a 47-80% GC content. Controls were created from these sequences by randomizing the order of the bases. Both the selected shRNA sequence and its cognate control were compared to the nucleotide databases at the National Institute for Biotechnoilogy using the BLAST program (9). Only those sequences that were unique were used for oligonucleotides synthesis. These sequences were then used to direct the synthesis of oligonucleotides. In addition to the shRNA sequences these oligonucleoitides contained a short region that resulted in a hairpin region. Oligonucleotides were ligated into pBluescript that contained an H1 promoter (10) and pSuperiorneo (Oligoengines). Clones were verified by sequence analysis.

Cell growth assays. Cells were seeded into 96 well plates. On day 0 cells were dosed with media that contained the control solvent or media that contained 2µg/ml doxycycline . Medium was replenished every 2 days thereafter with the appropriate additions. Cell division was determined using the 10 µl per well of CCK8 (Dojindo) a formazan dye based assay. After 1 hour of incubation with the dye the amount of dye converted was measured by spectrophotometry at a wavelength of 459 nm.

Western blots. Western blots were performed essentially as described (6). Protein amounts in each sample were determined using the BCA assay (Pierce) with bovine serum albumin as a standard. Antibodies to c-jun, c-fos (Upstate Biotechnologies) JunB, JunD, FosB, Fra-1 and Fra-2 (Santa Cruz), and cleaved PARP (Cell Signalling Technologies) were purchased.

Caspase assays. Assays for activated caspase 3 and 7 were performed using the Apo-ONE kit (Promega). Lysates from induced cells were compared to cells that were not induced with 2µg/ml of doxycyclin. Assays were read on a Perkin Elmer LS-50B fluorometer with an excitation wavelength of 485nm and an emission wavelength of 530nm according to manufacturer's recommendations.

3. Results

To determine if the transcription factor AP-1 was present at elevated levels in glioblastomas we transfected a reporter plasmid, pAP-1SEAP, or a control plasmid pTal into the glioblastoma cell lines U87-MG, U118-MG and U373-MG. After 24 hour cells were harvested, the amounts of protein and β-galactosidase measured. Identical amounts of protein were then used for a secreted alkaline phosphatase assay and the assay results normalized to the specific activity of β-galactosidase. The results are presented in Fig.1 and demonstrate that the levels of AP-1 were significantly elevated in both U87-Mg and U118-MG cells. U373-MG cells did not have elevated levels of SEAP indicating that they did not have activated Ap-1 (data not shown). The AP-1 activity was sensitive to genistein indicating the possible involvement of a tyrosine kinase in activating the AP-1 pathway. We have obtained similar results with NF-κB activation (6). When compared to a control cell line, MCF-10A, which is a cell line derived from normal breast epithelium, U87-MG and U118-MG Ap-1 levels also appeared elevated (data not shown).

Although it was clear that AP-1 levels were elevated at least in the two glioblastomas cell lines it was not clear which of the family members of AP-1 might be present and the contribution that each family member was making. To determine what roles each individual
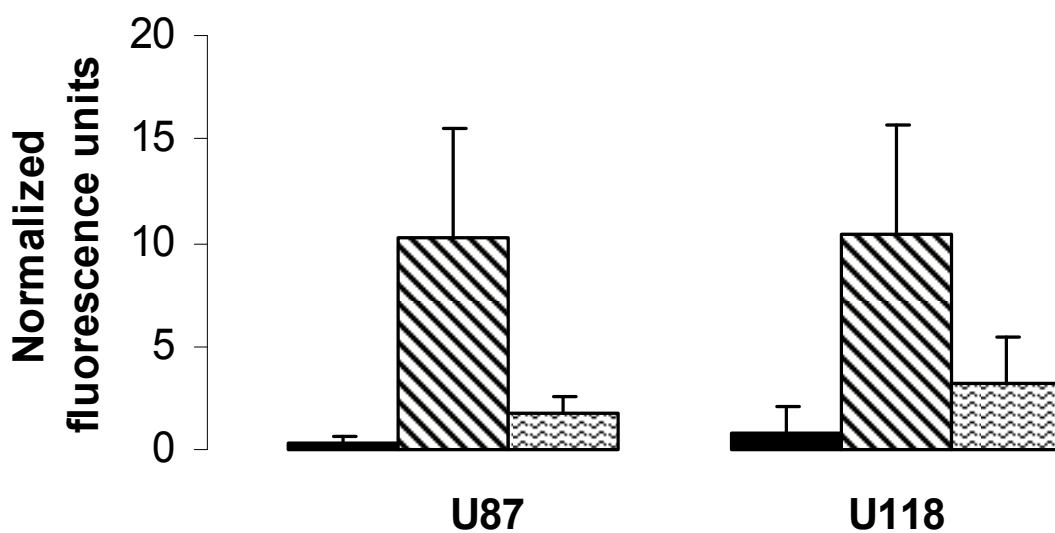
Fig. 1. AP-1 is elevated in the glioblastoma cell lines U87-MG and U118-MG. Cells were co-transfected with either the pTAL (black bars), or pAP1SEAP (diagonal striped bars), or pAP1SEAP in the presence of genistein (horizontal striped bars) and a plasmid that constitutively expressed the β-galactosidase protein. Media was assayed for the presence of secreted alkaline phosphatase and the values were normalized to the specific activity of β–galactosidase levels from cell lysates. Levels of SEAP in pAP1SEAP transfected cells were statistically significantly different from either the pTAL control or the cells treated with genistein (p0.05).

Protein	siRNA Sequence
c-jun-1	GATCCCAACCTGGCCGACCCACTGGTTCAAGAGACCAGTGGGTCGGCCAGGTTTTTTTGGAAA GGGTTGGACCGGCTGGGTGACCAAGTTCTCTGGTCACCCAGCCGGTCCAAAAAAAACCTTTTCGA
c-junC-1	GATCCCCAAGACTACGCGCTCCGCGCTTCAAGAGAGCGCGGAGCGCGTAGTCTTTTTTTGGAAA GGGTTCTGATGCGCGAGGCGCGAAGTTCTCTCGCGCCTCGCGCATCAGAAAAAAAACCTTTTCGA
JunD-1	GATCCCCAAGCCTGCGGCCGCGCCGCTTCAAGAGAGCGGCGCGGCCGCAGGCTTTTTTTGGAAA GGGTTCGGACGCCGGCGCGGCGAAGTTCTCTCGCCGCGCCGGCGTCCGAAAAAAAACCTTTTCGA
JunDC-1	GATCCCCCGCCGCGCCGGCGTCCGAATTCAAGAGATTCGGACGCCGGCGCGGCGTTTTTGGAAA GGGGCGGCGCGGCCGCAGGCTTAAGTTCTCTAAGCCTGCGGCCGCGCCGCAAAAACCTTTTCGA
JunB-1	GATCCCCAAGCGCCTGGGGCTCGCGGTTCAAGAGACCGCGAGCCCCAGGCGCTTTTTTTGGAAA GGGTTCGCGGACCCCGAGCGCCAAGTTCTCTGGCGCTCGGGGTCCGCGAAAAAAAACCTTTTCGA
JunBC-1	GATCCCCGCGGCGGAGGCATCCGTCGTTCAAGAGACGACGGATGCCTCCGCCGCTTTTTGGAAA GGGCGCCGCCTCCGTAGGCAGCAAGTTCTCTGCTGCCTACGGAGGCGGCGAAAAACCTTTTCGA
Fra2-1	GATCCCCAACGCCATCACGACCAGCCTTCAAGAGAGGCTGGTCGTGATGGCGTTTTTTTGGAAA GGGTTGCGGTAGTGCTGGTCGGAAGTTCTCTCCGACCAGCACTACCGCAAAAAAAACCTTTTCGA
Fra2C-1	GATCCCCCCGACCAGCACTACCGCAATTCAAGAGATTGCGGTAGTGCTGGTCGGTTTTTGGAAA GGGGGCTGGTCGTGATGGCGTTAAGTTCTCTAACGCCATCACGACCAGCCAAAAACCTTTTCGA
Fra1-1	GATCCCCAAGTTCCACCTGGTGCCAATTCAAGAGATTGGCACCAGGTGGAACTTTTTTTGGAAA GGGTTCAAGGTGGACCACGGTTAAGTTCTCTAACCGTGGTCCACCTTGAAAAAAAACCTTTTCGA
Fra1C-1	GATCCCCTCATCAGTCATCAGGCCCATTCAAGAGATGGGCCTGATGACTGATGATTTTTGGAAA GGGAGTAGTCAGTAGTCCGGGTAAGTTCTCTACCCGGACTACTGACTACTAAAAACCTTTTCGA
c-fos-1	GATCCCCAAGGCTGGCTTCCCGTCGCTTCAAGAGAGCGACGGGAAGCCAGCCTTTTTTTGGAAA GGGTTCCGACCGAAGGGCAGCGAAGTTCTCTCGCTGCCCTTCGGTCGGAAAAAAAACCTTTTCGA
c-fosC-1	GATCCCCGATCAGGTCGCCTCTGCCGTTCAAGAGACGGCAGAGGCGACCTGATCTTTTTGGAAA GGGCTAGTCCAGCGGAGACGGCAAGTTCTCTGCCGTCTCCGCTGGACTAGAAAAACCTTTTCGA
FosB-1	GATCCCCAAGCTGATTCTGTACAGCGTTCAAGAGACGCTGTACAGAATCAGCTTTTTTTGGAAA GGGTTCGACTAAGACATGTCGCAAGTTCTCTGCGACATGTCTTAGTCGAAAAAAAACCTTTTCGA
FosBC-1	GATCCCCTTTAGCACCTAGGAGCATGTTCAAGAGACATGCTCCTAGGTGCTAAATTTTTGGAAA GGGAAATCGTGGATCCTCGTACAAGTTCTCTGTACGAGGATCCACGATTTAAAAACCTTTTCGA

shRNA sequences. Sequences for the shRNAs are given in table 1.

Table 1. Sequences of the siRNAs that were synthesized. The designation of c indicates control oligonucleotides that are scrambled. Note that the overhanging ends are compatible with HindII and BglII sites in the vectors that were used.

family member might be playing in the increased levels of AP-1 we designed a series of shRNAs that were directed specifically to each individual family member (Table 1). We then tested these constructs in both transient expression and in stable expression assays. Western blotting demonstrated that all of the family members were present in the U87-MG cells that there were no family members that were expressed. Furthermore, levels of these proteins could be reduced with either transient or stable expression studies as compared to cognate controls (Fig. 2).

To determine what effect, if any, this reduction in protein might have on AP-1 activity we transfected into cells the pAP1-SEAP reporter plasmid or its control pTAL. From this assay it became apparent that the contributions that the different AP-1 family members had on AP-1 levels were quite variable and ranged from a low of 7% for JunB to 84% for c-Fos.

We were interested what the affect was of inhibiting these proteins would have on the cells in stable assays. Transient transfections could only allow us to assay the affects of the shRNA constructs on a potentially minor proportion of the population of cells. By creating stable transfectants we could investigate the role of some of these proteins consistently within a population. The shRNAs for c-Jun and c-Fos were cloned into a modified pSuperior neo vector that we created so that it expressed the hygromycin resistance gene in place of
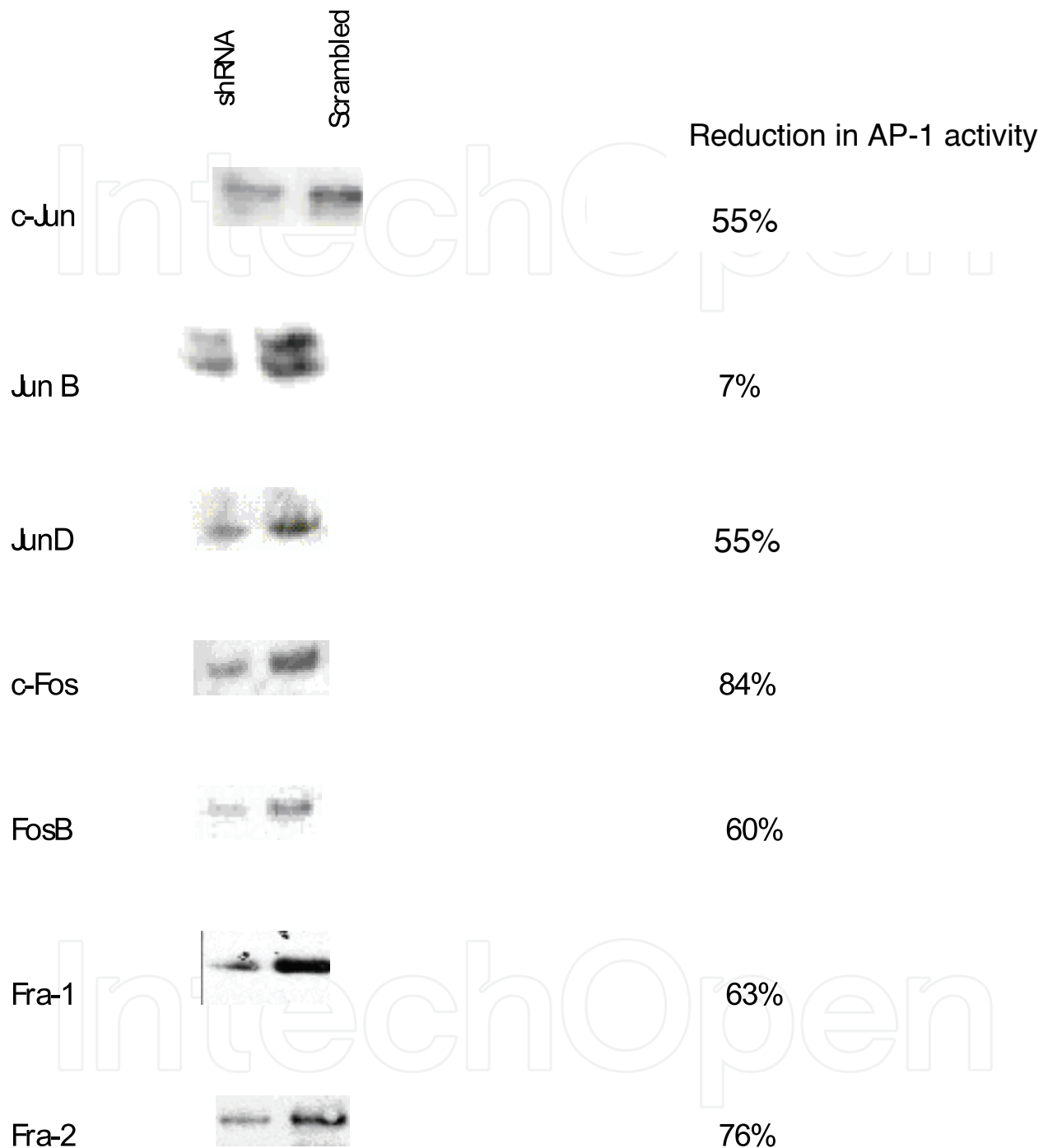
Fig. 2. Expression of AP-1 family members transiently transfected with plasmids that either expressed shRNA or the scrambled control shRNA. Cels were transfected with indicated plasmids and lysates were tested for expression of the various family members by western blot analysis of whole cell lysates. Cells were also transfected with the pAP1SEAP reporter plasmid and the media assayed for SEAP.
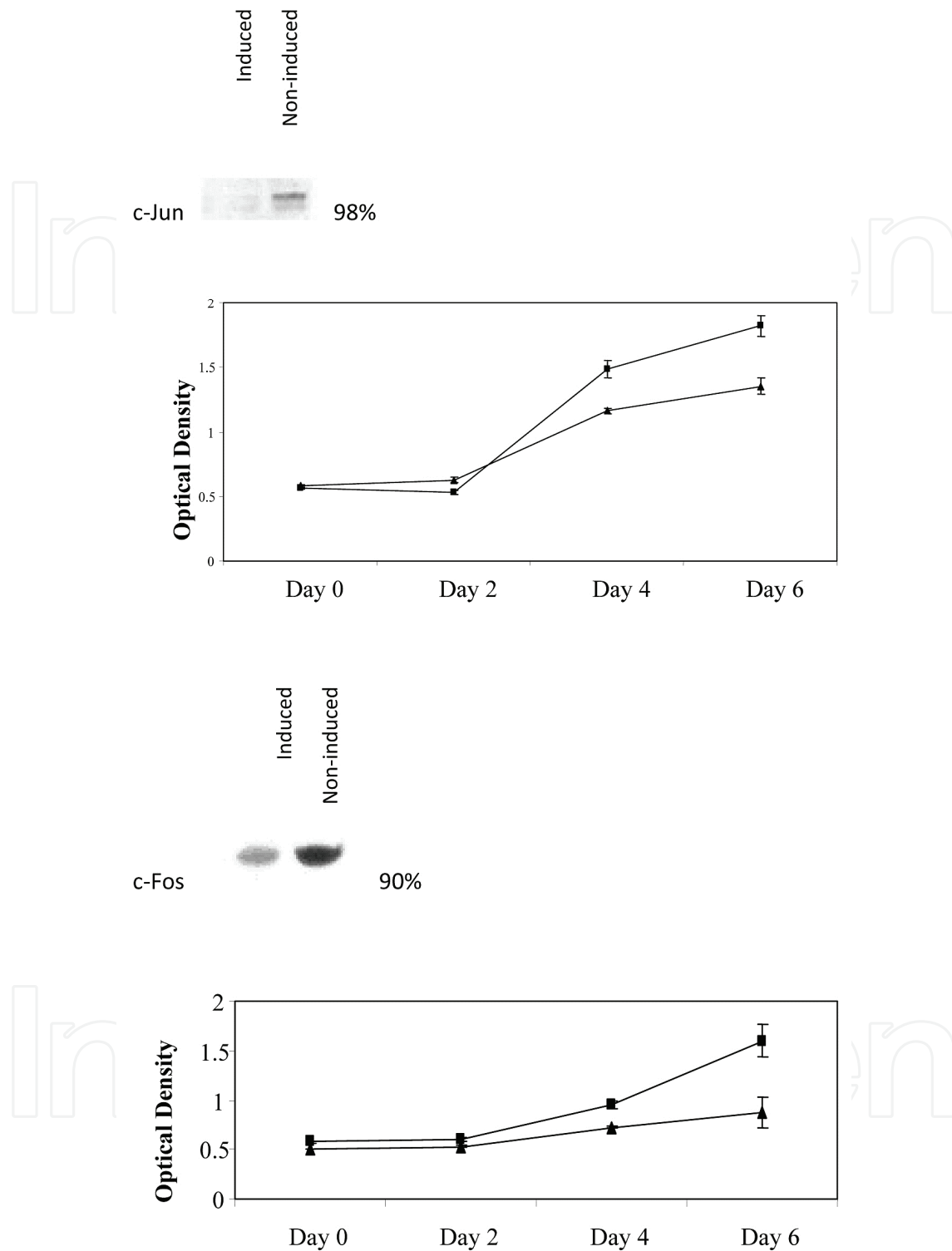
Fig. 3. Stable transfection and inhibition of cell doubling with c-Jun and c-Fos shRNAs. U87-MG cells that expressed the pTET-ON plasmid were transfected with the pSuperior constructs of the shRNAs for c-Jun and c-fos shRNAs or their cognate controls. Cells selected in hygromycin were then tested for their ability to replicate in the presence or absence of the inducer for shRNA, doxtcyclin. In both cases day 6 values are statistically significantly different between the control and shRNA expressing cells (p<0.05).

the neo resistance gene. The promoter in this vector that drives the expression of the shRNAs is an H1 promoter that has additional sequences that make is responsive to the Tet repressor. Cell lines of U87-MG were created that expressed the Tet repressor and these were transfected and selected so that they also carried either the c-Fos or c-Jun shRNAs that were used in the transient assays described above. As expected induction of the shRNA expression with doxycycline resulted in depression of c-Jun (90%) and c-Fos (98%) protein levels as compared to their uninduced controls (Fig. 3a). To determine what effect this type of reduction would have on the ability of the cells to divide cells were treated with doxycycline and their division measured over a period of several days using a formazan dye derivative. The results (Fig. 3b) demonstrate that a reduction in c-Fos and c-Jun did have a modest but statistically significant suppressive affect on cell division which was not seen in the cells that were transfected with the scrambled control oligonucleotides. If doxycycline was removed part way through the assay this effect was not seen indicating that the effect could be cytostatic as opposed to resulting in apoptosis or cell death. However, western blots of the cell lines demonstrated that they had increased levels of cleaved PARP and caspase 3,7 activity indicating that at least some of the cells were undergoing apoptosis in response to expression of the shRNAs.

4. Discussion

AP-1 is a transcription factor that is frequently up regulated in a variety of tumors. In this study we determined that AP-1 is up regulated in some glioblastoma cell lines. Transcription factors are typically defined by the sequences that they are capable of binding even though the transcription factor itself may be composed of several different but related polypeptides. The purpose of this study was to determine if the cell lines that expressed higher levels of activated AP-1 only expressed certain family members. Similar to what we previously found with the NF-κB family of transcription factors (6), most if not all of the family members of AP-1 were present in these cells and they appeared to play a role in the level of AP-1 that was found within the cell. Although inhibition of c-Jun, JunD, c-Fos, FosB, Fra-1 and 2 all had similar effects on the levels of AP-1, inhibition of JunB did not have a substantial impact on AP-1 levels indicating that certain family members were not as important for the AP-1 effect on the cells. This is in contrast to previous findings that JunB had an antagonistic effect to c-Jun on cells (4). Whether our results differ due to the fact that we used established cell lines as opposed to fresh tumor tissue is unclear.

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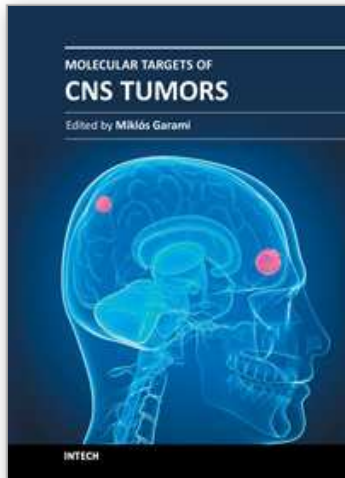
Molecular Targets of CNS Tumors

Edited by Dr. Miklos Garami

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