Abstract

Andrew R. Taylor. BCL2'S EFFECT ON GENE REGULATION IN MCF10A CELLS AS DETECTED BY QRT-PCR. (Under the direction of Thomas J. McConnell, Ph.D. and Mary A. Farwell, Ph.D.) Department of Biology. East Carolina. April, 2009.

Cells over-expressing the proto-oncogene B-cell lymphoma (BCL2), belonging to the Bcl-2 family of proteins, have been shown to slow the cell cycle and resist apoptosis. Microarray data has shown differences in a number of genes within and between several gene systems in the MCF10A cell line. We hypothesized that genes shown to be differentially expressed in MCF10A cells could be further verified through quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Using Ingenuity Pathway Analysis, twelve differentially expressed genes were chosen from the top five highest rated networks of genes from the microarray data. Through the use of the comparative C_t method ten of the genes were found to be up-regulated, while the remaining two were shown to be down-regulated. The two genes shown to be down-regulated were MCL-1 and FOXM1. The ten genes shown to be up-regulated are as follows: THRA, ITGB5, MAPIIIKII, ANXA1, PSENEN, RPS6KB1, HCLS1, PYCARD, RPS6KB2, and USP3. Of the twelve genes, seven correlated with the microarray data and are: MCL1, PSENEN, RP6KB2, HCLS1, PYCARD, and USP3. One-way analysis of variance (ANOVA), a statistical test, showed that the differential expression of ANXA1, PSENEN, HCLS1, and PYCARD were significant. We concluded that the over-expression of BCL2 does affect the expression of other genes within the MCF10A cell line, and that these expressional changes could be directly affecting the regulation of apoptosis and the cell cycle. The exact mechanism to how this regulation is occurring remains unclear, however these experiments have provided a better understanding of gene expression regulation in MCF10A cells.

BCL-2'S EFFECT ON GENE REGULATION IN MCF10A CELLS AS DETECTED BY QRT-PCR

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TABLE OF CONTENTS

List of Figures.....iii

List of Tables.....iv

List of Abbreviations.....v

Introduction.....1

Hypothesis.....7

Materials and Methods.....8

Cell Lines.....8

Cell Culture.....8

RNA Isolation and Purification.....9

cDNA Synthesis.....10

Primer Design.....11

Quantitative Reverse Transcription Polymerase Chain Reaction.....11

Results.....14

Discussion.....18

Conclusion.....23

Future Research.....25

Figures.....26

Tables.....32

References.....38

LIST OF FIGURES

1. Ingenuity Pathway Analysis (IPA) demonstration of the most highly rated network of microarray data.
2. Native gel electrophoresis of sample RNA extraction.
3. Native gel electrophoresis of DNase treated RNA.
4. Native gel electrophoresis of PCR, following reverse transcription, of the FOXM1 gene.
5. Quantitative reverse transcription PCR amplification curves.
6. Bar graphs of the fold change in expression of the four significant genes.

LIST OF TABLES

1. List of gene symbols, names, and functions.

2. List of accession numbers and primer sequences.

3. Chart of the ΔC_t values for each sample and gene, from both cell lines.

4. Chart of the $\Delta\Delta C_t$ values for each sample and gene, from both cell lines.

5. List of the average fold change and standard deviation between the two cell lines.

6. ANOVA results for each gene.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ANXA1	Annexin A1
Apaf-1	Apoptotic peptidase activating factor 1
Aph-1	Anterior pharynx-defective 1
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
BAD	BCL2-associated agonist of cell death
BAX	BCL2-associated X protein
Bcl-2	B-cell lymphoma
Bcl-xL	B-cell lymphoma extra large
BH	Bcl-2 homology
BLAST	Basic Local Alignment Search Tool
CCND1	Cyclin D1
CD95	Tumor necrosis factor receptor superfamily, member 6
CDC25A	Cell division cycle 25A
CDC42	Cell division cycle 42

CED-3	Cell death abnormality protein 3 precursor
CO_2	Carbon dioxide
C_t	Cycle threshold
DMEM/F12	Delbecco's Modified Eagle's Medium
dNTP	Deoxyribonucleotide triphosphate
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FN1	Fibronectin 1
FOXM1	Forkhead box M1
Hax-1	HCLS1 associated protein X-1
HCLS1	Hematopoietic cell-specific Lyn substrate 1
IAA	Isoamyl alcohol
IPA	Ingenuity Pathway Analysis
Ipaf	ICE-protease-activating factor
ITGB5	Integrin, beta 5
LLC-PK1	Renal epothilial cell line derived from porcine kidneys
Lyn	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog

MAPIIIKII	Mitogen-activated protein kinase kinase kinase 2
MCL1	Myeloid cell leukemia sequence 1
MMLV-RT	Moloney murine leukemia virus reverse transcriptase
NF-kappa	nuclear factor kappa
PAGE	Parametric Analysis of Gene set Enrichment
p53	Tumor protein 53
PBS	Phosphate Buffered Saline
PSENEN	Presenilin enhancer 2 homolog
Pen-2	Presenilin enhancer 2
PYCARD	Caspase recruitment domain-containing protein
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RPS6KB1	Ribosomal protein S6 kinase, 70 kDa, polypeptide 1
RPS6KB2	Ribosomal protein S6 kinase, 70 kDa, polypeptide 2
SH2	Src Homology 2
TBE	Tris-borate-ethylenediamine tetraacetic acid
TGFB3	Transforming growth factor, beta 3
THRA	Thyroid hormone receptor, alpha

U937	Human leukemic monocyte lymphoma cell line
USP3	Ubiquitin specific peptidase 3

Introduction

The B-cell lymphoma gene (BCL-2 proper) is one of a family of homologs which have been shown to have distinct effects on programmed cell death. Programmed cell death has proven to be a necessary sequence of events in the maintenance and stability of an organism's life cycle. Programmed cell death usually occurs through an evolutionarily conserved form of cell suicide termed apoptosis (Kerr *et al* 1972). Apoptosis is required for development and morphogenesis, homeostasis, and the removal of damaged or infected cells in organisms. In order for survival, each of these cellular functions must be regulated through the processes of programmed cell death.

The dysregulation of apoptosis has shown to be important in the development and progression of cancer. Apoptosis is regulated by cellular signaling that includes numerous genes within the cell. The first gene whose mutations were shown to occur in a majority of human tumors was p53, a tumor suppressor gene (Wallace-Brodeur *et al* 1999). Interference with the Fas/CD95 receptor pathway, which eliminates cells in the immune system through apoptosis, can lead to cancers (Beltinger *et al* 1998). Many proto-oncogenes as well as oncogenes, when mutated or over-expressed, help to induce cancer. BCL-2 is an oncogene, whose expression promotes cell survival and proliferation (Vaux *et al* 1988; McDonnell *et al* 1989).

BCL-2 proper was first described in 1988 as a mammalian homolog for CED-3, which is a gene found in *Caenorhabditis elegans* that is necessary for programmed cell death (Vaux *et al* 1988). Bcl-2 is also the name of a family of proteins that regulate apoptosis. The members of this family have at least one of the four Bcl-2 homology domains termed BH1, BH2, BH3, and

BH4, which contain α-helices (Adams and Cory 1998; Kelekar and Thompson 1998; Reed 1998). The number of domains conserved in each gene seems to determine the functions of each gene member. Anti-apoptotic members, for instance, conserve all four domains, while pro-apoptotic genes conserve several domains or the BH3 domain only. It is argued that the BH3 domain should be considered a crucial "death" domain in the pro-apoptotic members (Polster *et al* 2001).

The general structure of the Bcl-2 family members is a hydrophobic helix surrounded by amphipathic helices, with many of the genes containing a transmembrane domain as well. The anti-apoptotic genes encode proteins that are found initially as integral membrane proteins in the mitochondria, endoplasmic reticulum, or the nuclear membrane (Hockenbery et al. 1990; Krajewski et al. 1993). Prior to a death signal, a majority of pro-apoptotic proteins can be found within the cytosol or associated with the cytoskeleton of the cell (Hsu *et al* 1997). Once this death signal has been received, these proteins undergo a conformational change that allows them to integrate into membranes (Gross *et al* 1999). Members of this family also display the ability to form homodimers and heterodimers, which suggests a neutralizing competition between them (Gross *et al* 1999). For example, the BH1, BH2, and BH3 domains of an anti-apoptotic protein form a hydrophobic cleft to which the BH3 domain of an amphipathic alpha helix can bind (Sattler *et al* 1997).

Bcl-2 family pro and anti-apoptotic proteins are regulated on the transcriptional and post-translational level. It has been shown that the BAX gene, a pro-apoptotic member of the Bcl-2 family, is transcriptionally activated by the tumor suppressor protein p53 (Miyashita *et al* 1995). Phosphorylation of the BH3 subfamily member Bad regulates its subcellular localization as well as its protein-protein interactions (Condorelli *et al* 2001). Each Bcl-2 family protein displays its

own characteristic method of action. Bcl-2 proper has been shown to directly or indirectly prevent the release of cytochrome c from the mitochondria (Yang et al 1997). Another example of the family's method of action is that the protein Bcl-xL is able to bind to the protein Apaf-1 inhibiting the activation of caspase 9 (Hu *et al* 1998). Overall, this family has been shown to act as checkpoints upstream of caspases and mitochondrial dysfunction (Chao *et al* 1998).

Although often over-expressed in cancer cells, Bcl-2's role the progression of cancer in unclear, and our lab is interested in the role of Bcl-2 in non-cancer cells. Recently two cell lines have been produced termed MCF10A/Bcl-2 and MCF10A/Neo, the former being a breast epithelial cell line that over expresses the protein Bcl-2, by retroviral infection (Long *et al* 2008). MCF10A/Neo serves as a control cell line that is resistant to neomycin. As mentioned above, the Bcl-2 protein is a part of the Bcl-2 family of proteins and displays anti-apoptotic abilities. This ability can be seen when cells over-expressing BCL-2 are exposed to a number of various cytotoxic insults, such as gamma- and UV-irradiation, cytokine withdrawal, dexamethasone, staurosporine, and cytotoxic drugs (Chao and Korsmeyer, 1998).

In addition to preventing apoptosis, BCL-2 over-expression has been shown to slow the cell cycle (Lin *et al* 2001). The MCF10A cells over-expression of BCL-2 displayed significantly slower proliferation compared to a control line, particularly between the G1 and S phases (Long *et al* 2008). Microarray analysis, performed previously, has shown that BCL-2 over-expression has a significant effect on the expression pattern of 363 different genes (Long *et al* 2008). Several of these genes that were either up or down-regulated are known to be involved with cell cycle and proliferation; examples include CDC25A, CDC42, CCND1, FN1, and TGFB3.

The microarray data that showed differences in expression between the MCF10A/Bcl-2 and MCF10A/Neo cell lines were compared using specific data analysis tools. These included first obtaining z-scores for each gene from both the MCF10A/Bcl-2 and MCF10A/Neo cell line. The z-score normalization method normalizes the raw intensity values from the Phosphoimager 860 (Molecular Dynamics, Sunnyvale, CA) to allow for comparison between different microarray analyses (Long *et al* 2008). The z-scores for each gene in the array were then compared between the experimental and control cell lines to calculate z-ratios (Vawter *et al* 2002). The value of the z-ratio determined which genes were differentially expressed. Those genes, whose z-ratio was greater than 1.5 and a p-value less than 0.05 were measured to be significant (Long *et al* 2008). This statistical analysis gave the results of 363 significant genes, with 307 being upregulated and 56 being downregulated.

The microarray data was also subjected to two separate pathway analysis tools that provided biological information regarding how the genes interact: Parametric Analysis of Gene Set Enrichment (PAGE) and Ingenuity Pathway Analysis (IPA) (Long *et al* 2008). PAGE grouped the genes by function into gene sets that are significantly enhanced and provided a graphical representation of the microarray data (Kim & Volsky 2005). This analysis is done through the use of an aggregate of z-scores from the comparison of the fold change between the two cell lines on each of the genes in the microarray (Kim & Volsky 2005). IPA created networks of genes, whose expression was altered compared to the control line, assigning function and how they directly or indirectly interact with one another. The software used a knowledge base of published literature in order to create these networks (Calvano *et* al 2005). Networks are then rated based upon how many significant genes they contain. Finally, in order

to confirm the results of the microarray analysis, a number of genes were assessed by qRT-PCR, and the results from the microarray and qRT-PCR were found to be directly correlated.

Similar studies examining the expression levels of genes between cell lines have been performed as well. Microarray data analyzed by IPA and then confirmed to be up- or downregulated by qRT-PCR was shown in spontaneous cancer regression in melanoma pigs (Rambow *et al* 2008). These experiments were also performed to identify viral and cellular genes regulated during Pseudorabies viral infection of porcine epithelial cells (Flori *et al* 2008). Another set of experiments following similar procedures were to determine if the expression patterns of genes could be distinguished between primary and recurrent ovarian tumors (Laios *et al* 2008). Many other gene expression patterns have been analyzed through microarray analysis followed by validation through qRT-PCR.

We performed further analysis of the differential gene expression shown through microarray analysis, by analyzing twelve genes using qRT-PCR. The genes that were analyzed have been determined to be significant through statistical analysis of the microarray data, which has determined that the genes are differentially expressed between MCF10A/Bcl-2 and a control cell line MCF10A/Neo. The twelve genes used were chosen from the Ingenuity Pathway Analysis of the previous microarray data. An example of a network created by Ingenuity Pathway Analysis can be seen in Figure 1. Each of the genes was selected from the top five-functionality networks at random, and possesses their own function within the cells. The twelve genes chosen and their associated function can be seen in Table 1. Some like MCL1 are involved with apoptosis, while some such as MAPIIIKII are kinases. Quantitative RT-PCR allowed us to show differential expression levels between the MCF10A/Bcl-2 cell line and the

MCF10A/Neo cell line, while allowing for determination of correlation with previous microarray results.

Hypothesis

Bcl-2 over-expression has an effect on several gene systems in the MCF10A/Bcl-2 cell line compared to the control MCF10A/Neo cell line, as shown by microarray analysis. Quantitative real-time polymerase chain reaction (PCR) will be performed that can be compared to the previous microarray data. We expect that the twelve genes being observed through qRT-PCR will have expression levels that correlate directly to the up- or down-regulation that was observed by microarray analysis.

Materials and Methods

Cell Lines

The immortalized, non-tumorigenic human breast epithelial cell line, MCF10A, was obtained from the Karmanos Cancer Institute in Detroit MI. In the laboratory of Dr. James McCubrey at the Brody School of Medicine, Mary Kushman constructed a MCF10A cell line that stably over-expressed Bcl-2 as well as a MCF10A cell line to be used as a control (Kushman 2000). These were produced through retroviral infection of either both murine Bcl-2 and neomycin resistance genes (MCF10A/Bcl-2), or though a vector of neomycin resistance genes alone (MCF10A/Neo). The MCF10A/Bcl-2 cell line has been shown to have a significantly lower growth rate when compared to the control MCF10A/Neo cells (Long *et al* 2008).

Cell Culture

The MCF10A/Bcl-2 and MCF10A/Neo cell lines were cultured in Dulbecco's modified Eagle's Medium/Ham's F-12 media mixture (DMEM/F12), containing 15 mM HEPES and 2.5 mM L-glutamine, supplemented with 5% heat inactivated equine serum, 500 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 100 ng/ml gentamicin, and 10 ng/ml cholera toxin (hereafter referred to as DMEM/F12 complete). Both cell lines were grown under the same conditions of incubation at 37°C and in an atmosphere of 5% CO_2. Once cells were grown to confluency, they were either harvested or re-seeded. This was performed by trypisinization at 37°C with 0.1% trypsin until cells were detached, followed by centrifugation. If the cells were to be reseeded, they were resuspended in DMEM/F12 complete and aliquoted accordingly into appropriate vessels.

RNA Isolation and Purification

RNA lysates from both the MCF10A/Bcl-2 and MCF10A/Neo cell lines were prepared using Ambion's ToTALLY RNA™ Kit. Cells were grown to confluency in a 75 cm^2 flask, washed in PBS, trypsinized, and then pelletted in a 15 ml conical tube. Once pelletted, the remained liquid was aspirated from the top of the pellet. From each flask, approximately 1.6 x 10^6 cells were obtained. Next, 350 µl of Denaturing Solution was added to the pellet and vortexed to obtain a homogenous lysate. Using the organic phase of Pheonol:Chloroform:IAA (isoamyl alcohol, 25:24:1, pH 6.6/7.9), one starting volume was added to the lysate. The sample was vortexed for one min and placed on ice for five min. Next, the samples were centrifuged at 12,000 x g for five min and the upper aqueous phase of the solution was transferred to a new 1.5 ml microcentrifuge tube. The aqueous phase was measured and 1/10 volume of 30mM sodium acetate (pH 4.5) was added, and then mixed by inversion for 10 sec. One starting volume of the organic phase of Acid-Phenol:Chloroform (5:1, pH 4.5 +/-0.2) was then added to the sample. The samples were again vortexed for one min, placed on ice for five min, and centrifuged at 12,000 x g for five min. The aqueous phase was measured and transferred to a new RNase-free vessel. An equal volume of isopropanol was added to the RNA preparation and mixed well. To precipitate the RNA, the prep was then placed at -20°C for thirty min. The sample was then centrifuged for fifteen min at 12,000 x g, after which the supernatant was removed by aspiration with a pipette. Residual salts were removed by washing the pellet in 300 µl of 70% ethanol, gently vortexing for three min, and centrifuged for five min at 7,500 rpm. The ethanol supernatant was then removed by aspiration with a fine-tipped pipette. The pellets were resuspended in 100 µl of Elution Solution and stored at -80°C.

Upon completion of the RNA isolation, possible DNA contamination was treated through the use of TURBO DNA-*free*™ kit from Ambion. First, 0.1 volume of 10x TURBO DNase Buffer and 1 µl TURBO DNase (2U/µl) was added to the RNA, and mixed gently. The sample was then incubated at 37°C for 30 min. In order to inactivate the DNase, 2 µl of DNase Inactivation Reagent was added to the solution, and mixed well. The sample was then incubated at room temperature for two min, with occasional mixing. Next, the sample was centrifuged at 10,000 x g for 1.5 min and the supernatant was transferred to a new tube. The RNA was then tested for purity and quantity using two methods. Checking the absorbance ratios at A_{260}/A_{280}, measures the purity of the RNA sample. The reading should fall between 1.8 and 2.1. The quantity of RNA in the sample is determined by the absorbance at A_{260}, where an A_{260} of 1 is equal to 40 µg/ml of RNA. DNase-free RNA samples were also subjected to electrophoresis on a 1% agarose gel in TBE buffer; and observing both the 28S and 18S rRNA subunits on the gel with the 28S subunit being twice the intensity of the 18S subunit. This indicates that no RNA degradation has occurred, and that the sample is of good quality. The samples were then stored at -80°C.

cDNA Synthesis

Using Ambion's RETROscript® Kit, the first strand cDNA was prepared. To remove any possible secondary structure within the RNA, the RT method with heat denaturation was chosen. RNA (1 µg), 2 µl of random decamers (50µM), and 8 µl of nuclease free water mixed, spun briefly and heated at 82°C for three minutes. The tubes were removed to ice, spun briefly, and placed back on ice. Next, 2 µl of 10x RT Buffer, 4 µl of dNTP mix (2.5mM each), 1 µl of RNase Inhibitor (10U/µl), and 1 µl of MMLV-RT reverse transcriptase (100U/µl) were added to

the sample. The sample was mixed, spun briefly, and incubated at 44°C for one hour. The reverse transcriptase was then inactivated, by incubating the sample at 92°C for ten min. The sample was then stored for later use in qRT-PCR reactions at -20°C.

Primer Design

The computer software program, Vector NTI was used to display the mRNA sequences of target genes. This was made possible through the use of NCBI's Genebank search program for accession numbers of the genes. Several other programs were then used to select a primer sequence, optimal for qRT-PCR, for each gene in question. First a 300 bp sequence crossing an exon-exon junction was selected and entered into BLAST analysis. This analysis ensures that no other gene is homologous with the gene in question (McGinnis & Madden, 2004). From there, the 300 bp sequence was subjected to the program Mfold (Zuker 2003). This program is used to show how the mRNA would fold at 55°C and is essential in preventing primer binding within secondary structures. The reason 55°C was chosen for the program is due to the fact that this would be the annealing temperature for each of the primer sets. Finally, the sequenced was pasted into Primer3 (Rozen and Skaletsky 2000) to produce the optimal forward and reverse primers for each gene. The primers chosen were 19-23 base pairs in length and were to produce an amplicon that was within the range of 75-150 base pairs. The primer sequences selected for use can be seen in Table 2.

Quantitative Reverse Transcription Polymerase Chain Reaction

This procedure was performed in the laboratory of Dr. Baohong Zhang, located in the Biology Department at East Carolina University, on an ABI 7300 (Applied Biosystems, Foster

City, CA). Each reaction used cDNA from both the MCF10A/Bcl-2 cell line and the MCF10A/Neo cell line. The control or housekeeping gene used for establishing expression differences between the two cell lines was β-Actin. The reactions were carried out on a 96-well plate and contained 25 µl of the following: 12.5 µl of *Power* SYBR® Green PCR Master Mix (SYBR® Green 1 Dye, AmpliTaq Gold® DNA Polymerase LD, dNTPs with dUTP/dTTP blend, Passive Reference 1 and optimized buffer components) from Applied Biosystems, 50-900 nm forward primer, 50-900 nm reverse primer, 1-100 ng cDNA, and 7.5 µl nuclease free water. The cycle total was set to 40 with the conditions being 95°C for fifteen seconds to denature and 55°C for one minute to anneal and extend.

In order to calculate the differences in expression for each gene between the MCF10A/Bcl-2 and MCF10A/Neo cell lines, the comparative cycle threshold method ($2^{-\Delta\Delta Ct)}$ was used (Livak & Schmittgen, 2001). This method first requires that the ΔC_t values for each gene of the two cell lines be obtained. These are determined by subtracting the C_t value for the housekeeping gene from the C_t value of the gene in question ($|C_t$ Bcl-2 – C_t Actin $|$ or $|C_t$ Neo – C_t Actin $|$). The cycle threshold (C_t) value is the amount of fluorescence above a background level, due to Sybr Green binding onto dsDNA. The more dsDNA produced in a reaction, the lower the C_t value. Next, the ΔC_t values of each gene from the MCF10A/Neo cell line are subtracted from the ΔC_t values of each corresponding gene from the MCF10A/Bcl-2 cell line (ΔC_t Bcl-2 - ΔC_t Neo). A positive value indicates a decrease in expression levels, while a negative value indicates an increase in expression levels due to Bcl-2. The fold change in the gene expression of each gene can then be calculated as follows: $2^{-\Delta\Delta Ct}$. The average of the fold change for each gene between the replicates is then calculated for the determination of the standard deviation amongst the genes in question. The $\Delta\Delta C_t$ values were then subjected to a

statistical test (one-way ANOVA) to determine if the differences found from qRT-PCR were significant. One-way analysis of variance (ANOVA) is used to test for differences among two or more independent groups. This statistical analysis considers both fold change and variability amongst replicates to create a p-value. A significant p-value indicates that the data obtained was due to the up or down-regulation of the genes due to over-expression of Bcl-2 alone and not by random chance. The p-value chosen for significance was set at 95% confidence or 0.05. In order for the qRT-PCR results to be considered relevant, the $\Delta\Delta C_t$ values between the MCF10A/Bcl-2 and MCF10A/Neo cell lines must fall below the set p-value. Genes whose ANOVA value is <0.05 are said to be significantly different. The genes found to be significantly different were then compared to the microarray results from previous research to determine if there is a direct correlation with the qRT-PCR results.

Results

RNA Isolation and Purification

Using the ToTALLY RNA™ Kit from Ambion, samples of RNA from both the MCF10A/Bcl-2 and MCF10A/Neo cell lines were isolated. These samples were then tested for quality and purity using two analyses: spectrophotometry and native gel electrophoresis. The ratio of absorbance readings at 260nm and 280nm can give a reliable measurement as to how pure an RNA sample is. Samples of isolated RNA having a ratio within a range of 1.8 to 2.1 were selected for further testing by native gel electrophoresis with ethidium bromide. The 28S and 18S human rRNA bands should be clearly visible in order to ensure optimal quality of the sample RNA. The 28S subunit also should be about twice the intensity of the 18S subunit, indicating that no RNA degradation has taken place. An example of a native electrophoresis gel can be seen in Figure 2. A total of ten samples, five from the Bcl-2 and five from the Neo cell lines, were collected from these procedures. These samples were each from separate 75 cm^2 flasks grown to confluency under the conditions previously describe. Each sample was also originated from flasks that had been split and reseeded due to confluency over a six week period. To further ensure purity of the RNA, each sample was subjected to DNase treatment. The DNA-*free*™ kit from Ambion removed possible DNA contamination from the RNA samples. This can be seen from comparing Figure 2, untreated RNA, with Figure 3. Lane 5, in Figure 2, is a sample from the MCF10A/Bcl-2 cell line, and can be compared with Lane 3 in Figure 3. Lane 2, in Figure 2, is a sample from the MCF10A/Neo cell line, and can be compared with Lane 4 in Figure 3. Both the MCF10A/Bcl-2 and MCF10A/Neo samples show removal of DNA contamination.

Reverse Transcription followed by PCR

Ambion's RETROscript™ First Strand Synthesis Kit was used to create a cDNA complement of the ten samples of isolated RNA. Primers were designed corresponding to specific genes for analysis of the potential gene expression differences between the MCF10A/Bcl-2 and MCF10A/Neo cell lines. The cDNA was then subjected to PCR in order to determine the primers' efficiency, as well as the appropriate conditions required for quantitative PCR. Standard PCR also ensures that only the gene on interest is being amplified. Figure 4 shows a representative of four samples of cDNA from both the MCF10A/Neo cell line and the MCF10A/Bcl-2 cell line that underwent PCR using a primer set from one of the genes in question, along with control primers provided with the kit. Twelve genes were chosen in total for analysis, with each requiring their own specific primer set. All twelve genes were analyzed using PCR with their corresponding primer set for each of the ten cDNA samples. Each of the ten cDNA samples showed amplification of only one amplicon in question for each of the twelve genes, at the expected size on the gel. These primer sets were then used for further analysis by real-time, quantitative PCR.

Real-Time Quantitative Polymerase Chain Reaction

Using the primer sets mentioned above, real-time quantitative polymerase chain reaction was carried out to determine the potential differences in gene expression between the MCF10A/Bcl-2 cell line and the MCF10A/Neo cell line. Figure 5 gives an example of the outcome of the twelve genes in question as well as the housekeeping gene, Actin. The number of importance gained from this reaction is the cycle threshold (C_t) value, which is the cycle number where the fluorescence of the gene of interest is in the exponential phase and has passed

the level of background fluorescence. The background fluorescence is that of which is not caused by amplification of the genes of interest, and is established by the threshold line. A lower C_t value indicates that a higher concentration of the mRNA was present, while a higher C_t value means that a lower concentration of mRNA was expressed. Comparison of the C_t values between the two cell lines shows which expresses more of each target gene in question. The delta C_t values for each gene in question can be seen in Table 3.

The quantitative approach, termed the comparative C_t method, uses the values described above to calculate the fold increased or decreased expression of each gene between the two cell lines. Table 4 shows the delta delta C_t values for each gene found between the MCF10A/Bcl-2 cell line and the MCF10A/Neo cell line. The delta delta C_t values can then be placed into the following equation, to give the fold increase or decrease in expression: $2^{-\Delta\Delta Ct}$. A value greater that 1.00 indicates an increase in the level of expression between the MCF10A/Bcl-2 cell line and the MCF10A/Neo cell line. A value less than 1.00 indicates a decrease in the level of expression between the two cell lines. The values of the average fold change expression for each gene amongst the five qPCR replicates, along with the standard deviation within the replicates, can be seen in Table 5. Using this method, two genes were shown to be down-regulated: MCL1 and FOXM1. Ten genes were found to be up-regulated and are as follows: THRA, ITGB5, MAPIIIKII, ANXA1, PSENEN, RPS6KB1, HCLS1, PYCARD, RPS6KB2, and USP3. Of the twelve genes analyzed, seven correlated with the microarray and are as follows: MCL1, PSENEN, RP6KB2, HCLS1, PYCARD, and USP3. Statistical analysis was done in order to clarify whether or not the expression levels gathered through qRT-PCR were due to the over-expression of Bcl-2 alone.

One-way analysis of variance (ANOVA) was used to establish whether the expression levels of each gene in question was significantly different from one another between the two cell lines. The significance value also determines whether the difference in expression is due to the over-expression of Bcl-2, and not by random chance. It was found that four of the twelve genes had a value of significance to be <0.05, and can be seen in Table 6. Those genes were ANXA1, PSENEN, HCLS1, and PYCARD. Figure 6 shows the up-regulated expression of the four genes found to be significantly different. The eight other genes were confirmed to be too close to one another, between cell lines, in order for there to be distinguishable difference of expression. Of the four significant genes three correlated with the previously gained microarray data and are PSENEN, HCLS1, and PYCARD. These genes were shown to be significantly up-regulated between the MCF10A/Bcl-2 and MCF10A/Neo cell line.

Discussion

Twelve genes, chosen from the five highest rated networks of IPA, were analyzed by qRT-PCR to verify if they were being differentially expressed between the MCF10A/Bcl-2 and MCF10A/Neo cell lines. From the twelve genes, four were determined by one-way ANOVA to be significantly different at a confidence of 95%. Those genes were ANXA1, PSENEN, HCLS1, and PYCARD. Of the four genes found to be significantly different, only ANXA1 was found not to be directly correlated with the microarray results. For this section, I have focused on the four genes that were shown to have expression levels significantly different between the MCF10A/Bcl-2 and MCF10A/Neo cell lines. I have explained the results obtained from the qRT-PCR experiments, and possible reasons as to how the levels of expression may have an effect on the anti-apoptotic characteristics of the MCF10A/Bcl-2 cell line.

PSENEN

Through qRT-PCR, PSENEN was shown to be up-regulated due to over-expression of Bcl-2. Pen-2 is a subunit of the gamma secretase complex, an integral membrane protein that cleaves single-pass transmembrane proteins at residues within the transmembrane domain (Kimberly *et al* 2003). Pen-2 is a 101-amino acid protein whose N-terminus and C-terminus first face the lumen of the endoplasmic reticulum and later the extracellular environment (Francis *et al* 2002). The C-terminus has shown to be a critical factor in the formation of an active gamma secretase complex, with a conserved motif as well as required length (Hasegawa *et al* 2007). The gamma secretase complex has not been fully characterized, but is composed minimally of four subunits: presenilin, nicastrin, Aph-1, and Pen-2 (Kaether *et al* 2006). Pen-2 binds to the

transmembrane domain of presenilin for association with the complex (Wantanabe *et al* 2005). PSENEN over-expression has been shown to have an anti-apoptotic effect by lowering the p-53 dependent control of caspase-3 (Dunys *et al* 2007). Presenilins 1 and 2 can promote the degradation of FKBP38 and Bcl-2, inhibiting mitochondrial targeting of Bcl-2 (Wang *et al* 2005). Along with functioning on the late ER, gamma secretase complexes have been shown to localize within the mitochondria (Hansson *et al* 2004). This localization may play a role in anti-apoptosis of cells. It is possible that up-regulation of PSENEN increases protein expression and sequesters the amount of active p-53 within and outside of the mitochondria, thus reducing its apoptotic activity. Another possible theory is that the up-regulation of PSENEN increases presenilin 1 and 2 association, limiting their ability to promote the degradation of Bcl-2. This would in turn increase the anti-apoptotic ability of Bcl-2.

HCLS1

HCLS1 is a gene that codes for hematopoietic cell-specific Lyn substrate 1, and was found to be significantly up-regulated in the MCF10A/Bcl-2 cell line. The HCLS1 gene is expressed in hematopoietic cells and encodes the protein $p75^{HS1}$ (Yamanashi *et al*, 1993). The protein is 75-kDa; with functional properties as a major substrate of protein-tyrosine kinases regulated by B-cell antigen receptors, along with association with the SH2 domain of Lyn kinase upon receptor mediated signaling. It contains 3.5 tandem repeats, a coiled-coil region, and an SH3 domain at the C-terminus (Hao *et al* 2005). It was first thought that HCLS1 protein expression was limited only to cells of hematopoietic origin. Using sensitive expression analysis, including RT-PCR, $p75^{HS1}$ was also shown to be found in a number of human tissues (Fischer *et al* 2005). In B lymphocytes $p75^{HS1}$ is highly phosphorylated along tyrosine residues during B cell antigen receptor-mediated signaling, and low expression levels have been shown to

be insensitive to BCR-mediated apoptosis (Yamanashi *et al* 1997). Hax-1 is a Bcl-2-family-related-protein, which is associated with $p75^{HS1}$, and has been shown to have anti-apoptotic effects in lymphocytes and neurons (Chao *et al* 2008). HCLS1 upregulation may increase the amount of protein within the cell. This increase may help to stabilize the effects that Hax-1 has on anti-apoptosis within the MCF10A/Bcl-2 cell line. Due to the $p75^{HS1}$ having a known effect on signal transduction along with its finding in other cells not limited to hematopoietic origin; the up-regulation of HCLS1 may also have a possible influence on the anti-apoptotic nature of the MCF10A/Bcl-2 cell line in this manner. This may occur due to the possibility that an increase in the level of the $p75^{HS1}$ within the cells is causing the natural signal transduction pathways to be altered. This alteration thus limits the ability of the MCF10A/Bcl-2 cell line to initiate proper apoptosis.

PYCARD

PYCARD was found to be up-regulated by qRT-PCR, and is a gene that encodes an adaptor protein (ASC) which contains two protein-protein interaction domains labeled PYD and CARD (Shiohara *et al* 2002). PYD is a PYRIN domain, a novel domain found in apoptosis and inflammation proteins. CARD is a caspase-recruitment domain, originally found to be associated with apoptosis and caspase activation. CARD protein is a 195 amino acid, 22-kDa protein with the N-terminus being the PYRIN domain and the C-terminus comprising the CARD motif (Masumoto *et al,* 1999). The PYCARD protein has been shown to be an essential activating adaptor for caspases and apoptotic factors. In an Ipaf signaling pathway, ASC is a mediator of NF-kappa activation and Caspase-8-dependent apoptosis (Masumoto *et al,* 2003). ASC also is involved in mediating the formation of caspase-1-inflammasome signaling complex, acting as an adaptor protein (Srinivasula *et al* 2002). It has been reported that ASC over-

expression induced apoptosis via caspase-9 pathway, along with inhibiting growth of breast cancer cells (Conway *et al* 2000). The p53-Bax mitochondrial apoptosis pathway is affected by ASC, due to its association as a Bax adaptor protein (Ohtsuka *et al* 2004). In type II cells, cells that undergo mitochondrially-mediated apoptosis, ASC mediated apoptosis has been inhibited by Bcl-2 and/or Bcl-Xl (Hasegawa *et al* 2007). Bcl-2 over-expression causes a number of pro-apoptotic proteins such as Bad and Bax to be expressed differently within the cell as well (Fagg *et al* 2008). The cells may be upregulating the expression of PYCARD to counteract the anti-apoptotic abilities of Bcl-2. This up-regulation would eventually lead to the normalization of the cells' ability to induce proper apoptosis.

ANXA1

ANXA1 is another up-regulated gene whose expression level was found to significantly different between the MCF10A/Bcl-2 and the MCF10A/Neo cell lines. It encodes the protein Annexin 1, and belongs to a family of Ca^{2+} dependent phospholipid-binding proteins. This family of proteins consists of 13 calcium or calcium and phospholipid binding proteins with 40-60% homology between them (Raynal and Pollard 1994). Annexin 1 is a 37 kDa protein, and was discovered to be actively involved in inhibiting eicosanoid synthesis and PLA2 (Lim and Pervaiz 2007). The N-terminus contains sites for phosphorylation and proteolysis, deeming it the regulatory region of the protein (Lim and Pervaiz 2007). In A549 lung cancer cells, Annexin 1 had inhibitory effects on cell growth and proliferation (Croxtall *et al,* Mar 1993); suggesting tumor suppression functions. There have been contradicting studies in the role of Annexin 1 apoptosis; the reasons is unknown, but could be linked to cell type (Lim and Pervaiz 2007). In U937 cells, apoptosis was shown to be enhanced due to over-expression of Annexin 1 (Canadier *et al* 2000). An interesting finding is that over-expression of Bcl-2 in porcine renal LLC-PK1

cells, inhibited the localization of Annexin 1 to the nucleus during TNF-α-induced apoptosis (Ishido 2005). Decreased levels of Annexin 1 were seen in progression of prostate cancer (Xin *et al* 2003). In breast tissue the expression of Annexin 1 is often conflicting. Expression in ductal epithelial cells of human mammary tissue was found to be increased in association with tumors (Ahn *et al* 1997). On the other hand, a recent tissue microarray analysis indicated decreased expression of Annexin 1 correlated with breast cancer development and progression (Shen *et al* 2006). One possible outcome of the up-regulation of ANXA1 in MCF10A/Bcl-2 is that since Bcl-2 is able to block Annexin 1 from performing its pro-apoptotic functions through direct protein-protein interaction, the gene may be upregulated to compensate for over-expression of Bcl-2. With ANXA1 being upregulated, this counteraction to Bcl-2 over-expression may allow for proper apoptosis to take place. Although the process by which this is occurring is unknown; another possible outcome is that in this cell line, ANXA1 is having an anti-apoptotic effect within the cells.

Conclusion

In the MCF10A cells, the over-expression of Bcl-2 was shown through qRT-PCR to have a significant differential regulation pattern for four of the twelve genes analyzed. The up-regulation of these genes could potentially have a profound effect on the anti-apoptotic characteristics of these cells. Of the four genes described in the discussion the following were shown to be correlated with the microarray results: PSENEN, PYCARD, and HCLS1. I would have expected to observe a direct correlation between the two experiments amongst all the genes. The z-ratios of the microarray analysis for each of the four significant genes were found to be under 3-fold. Lower correlations are consistently found between microarray and qRT-PCR for genes showing a small degree of change. Steps were taken to carefully ensure that the biological and technical procedures were similar to that of the microarray analysis preparation. This included proper production of primer sets used to analyze each gene of the twelve genes in question. Considering the fact that no standard definition of validation exists, many factors can attribute to discrepancies between microarray and qRT-PCR results such as: RNA quality, contaminants, data normalization, as well as experimental design (Morey *et al* 2006).

Bcl-2 has been shown to associate with several proteins within the MCF10A cell line, not being limited to those whose function directly involves apoptosis. These associations can lead to a cascade or "domino-effect" of multiple interactions with other proteins, altering the expression of genes within the cell line. This would also lead to the MCF10A/Bcl-2 cell line displaying several characteristics that would be different from the MCF10A/Neo cell line. It is possible that these effects were taking place due to the over-expression of only Bcl-2, accounting for the up- or down-regulation of many different genes with different cellular functions in the cell. Exactly what influence the over-expression of Bcl-2 has on the MCF10A cell line remains unclear;

however these experiments have provided a better understanding of the genome expression of breast cells, and may eventually shed some light on the development of breast cancer.

Future Research

Many additional experiments could be performed with the intentions of providing evidence towards the effects of the over-expression of Bcl-2 in MCF10A cells. Genes with higher levels of differential expression between the MCF10A/Bcl-2 and MCF10A/Neo cells could be chosen for further analysis by qRT-PCR. This could help with the validation of the microarray experiments previously performed. As for the four genes shown to be significantly different in my research, protein and enzymatic work could be done. This could not only further confirm the qRTPCR or microarray results, but give insight into the mechanisms behind the effects of Bcl-2 over-expression. Information gathered through these experiments would not be limited to knowledge gained to the MCF10A cell lines, but could potentially describe possible ways in which the cascade of protein-protein interactions take place in other cell lines as well.

Figure 1

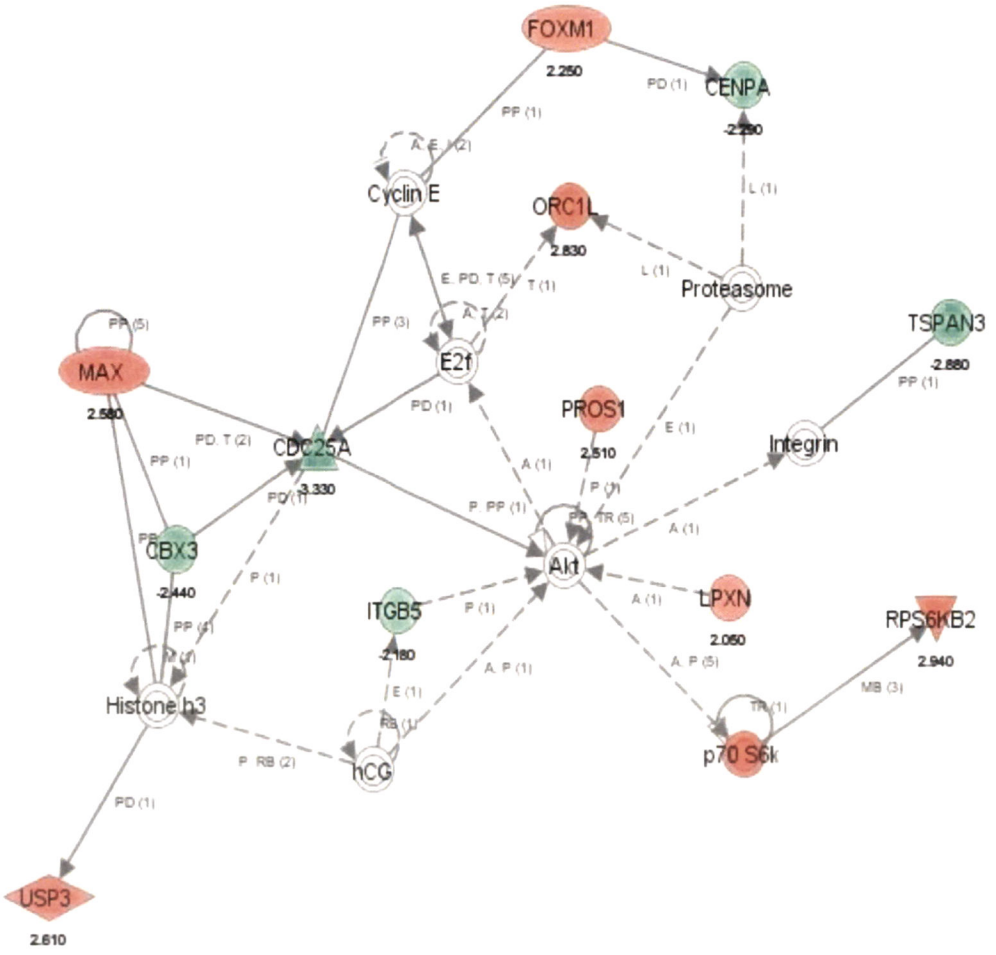

Figure 1: Ingenuity Pathway Analysis representation of the most highly rated network of the microarray data. Genes that are shaded green denote those which were deemed significantly down-regulated. Genes that are shaded red are those which are significantly up-regulated. The z-ratio below each gene shows the level to which each gene is up or down-regulated. Solid lines indicate direct interaction between each gene product, while a dotted-line indicates an indirect interaction.

Figure 2

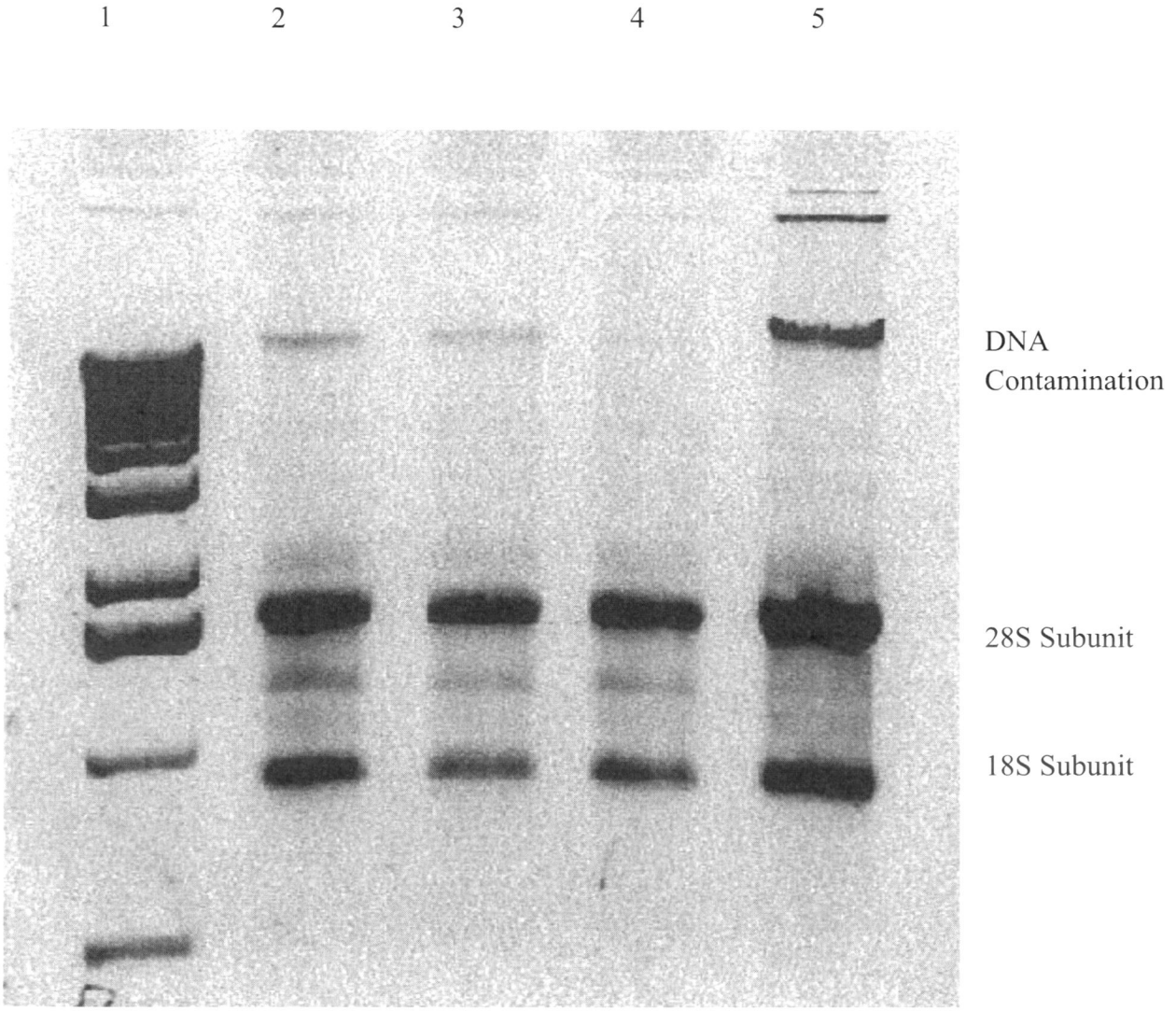

Figure 2: Native gel electrophoresis of sample RNA extraction. The gel was prepared with 1% agarose, TBE buffer, and ethidium bromide for visualization. The samples in lanes 2-5 show the 28S and 18S ribosomal subunits. Lane 1 was loaded with 1kb ladder.

Figure 3

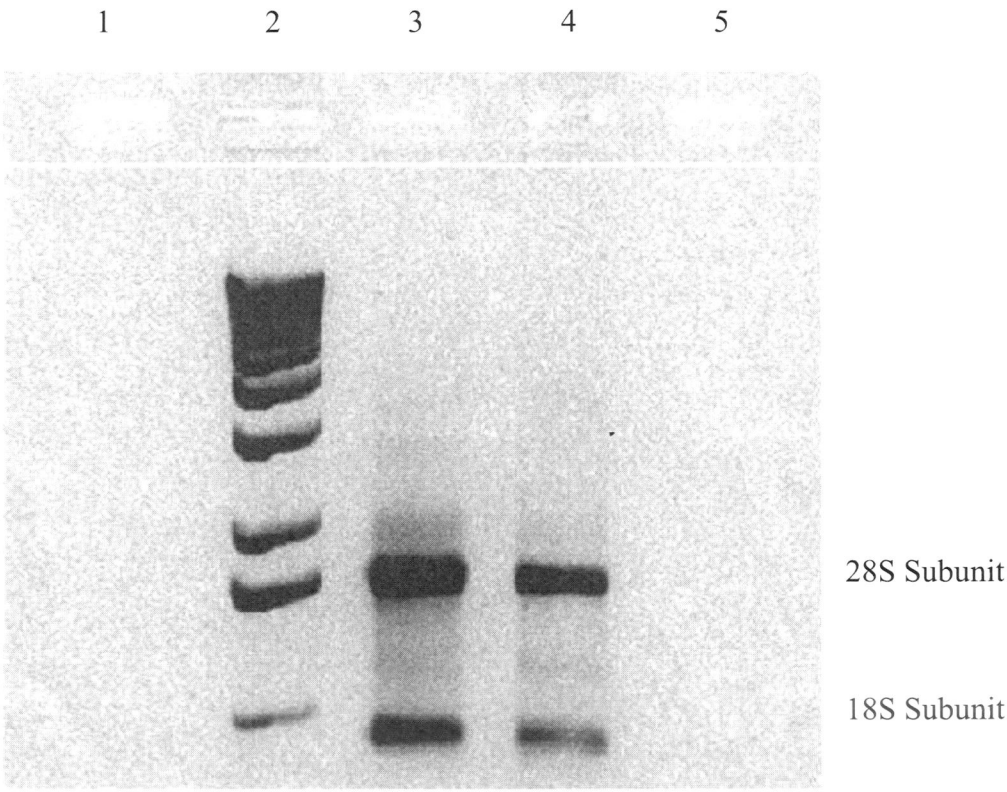

Figure 3: Native gel electrophoresis of extracted RNA that had been treated with DNase. The gel was prepared with 1% agarose, TBE buffer, and ethidium bromide for visualization. Lanes 3 and 4 were loaded with RNA, and show the 28S and 18S ribosomal subunits. Lanes 3 and 4 can be compared with lanes 5 and 2 respectively, from Figure 2. Lane 1 was loaded with 1kb ladder to be used as a reference.

Figure 4

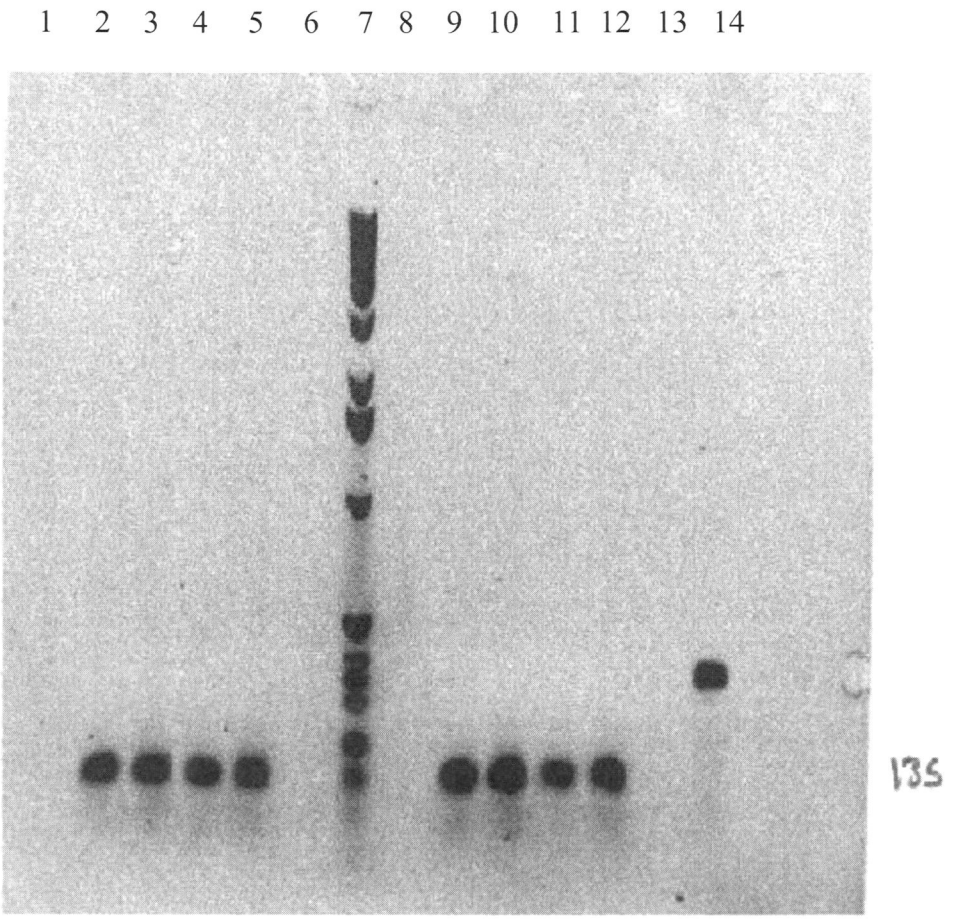

Figure 4: Native gel electrophoresis of PCR, following reverse transcription, of the FOXM1 gene. The gel was prepared using 1% agarose, TBE Buffer, and ethidium bromide for visualization. Lanes 2-5 are separate samples of cDNA from the MCF10A/Neo cell line, while Lanes 9-12 are separate samples of cDNA from the MCF10A/Bcl-2 cell line. The primers were specific for a 135bp product, and can be seen when compared to the 1kb ladder used in Lane 7. Lane 14 is a control primer set for the human rig/S15, and was used with a cDNA sample from the MCF10A/Bcl-2 cell line. The control primer set amplifies a 361bp product.

Figure 5

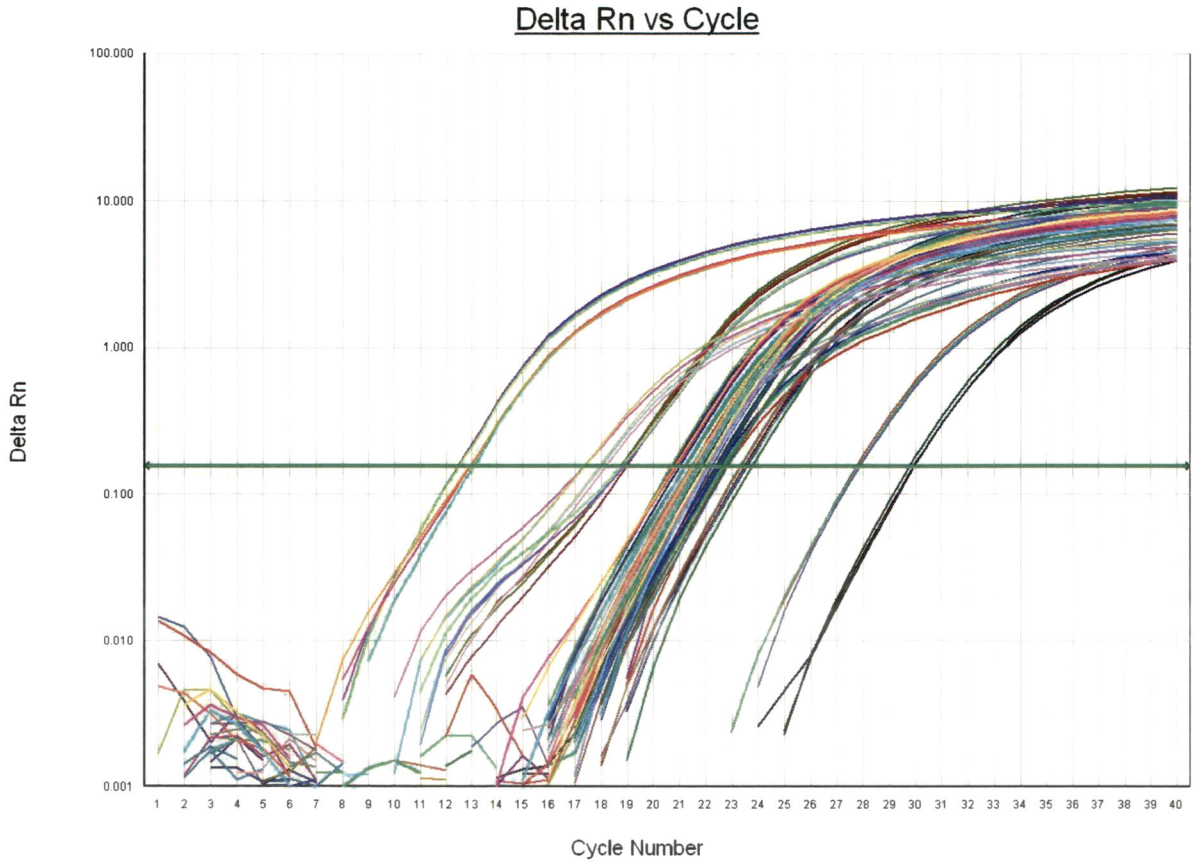

Figure 5: Quantitative RT-PCR analysis of the 12 genes, along with the housekeeping gene, chosen from the Ingenuity Pathway Analysis (IPA). The cycle number is located along the x-axis of the graph, with the amount of relative fluorescence along the y-axis. The green horizontal line indicates the cycle threshold level, which is defined as the point where the amount of fluorescence due to gene amplification is above that of background fluorescence. Background fluorescence can be seen in the lower left quadrant of the figure, and is due to unused wells on the 96-well plate.

Figure 6

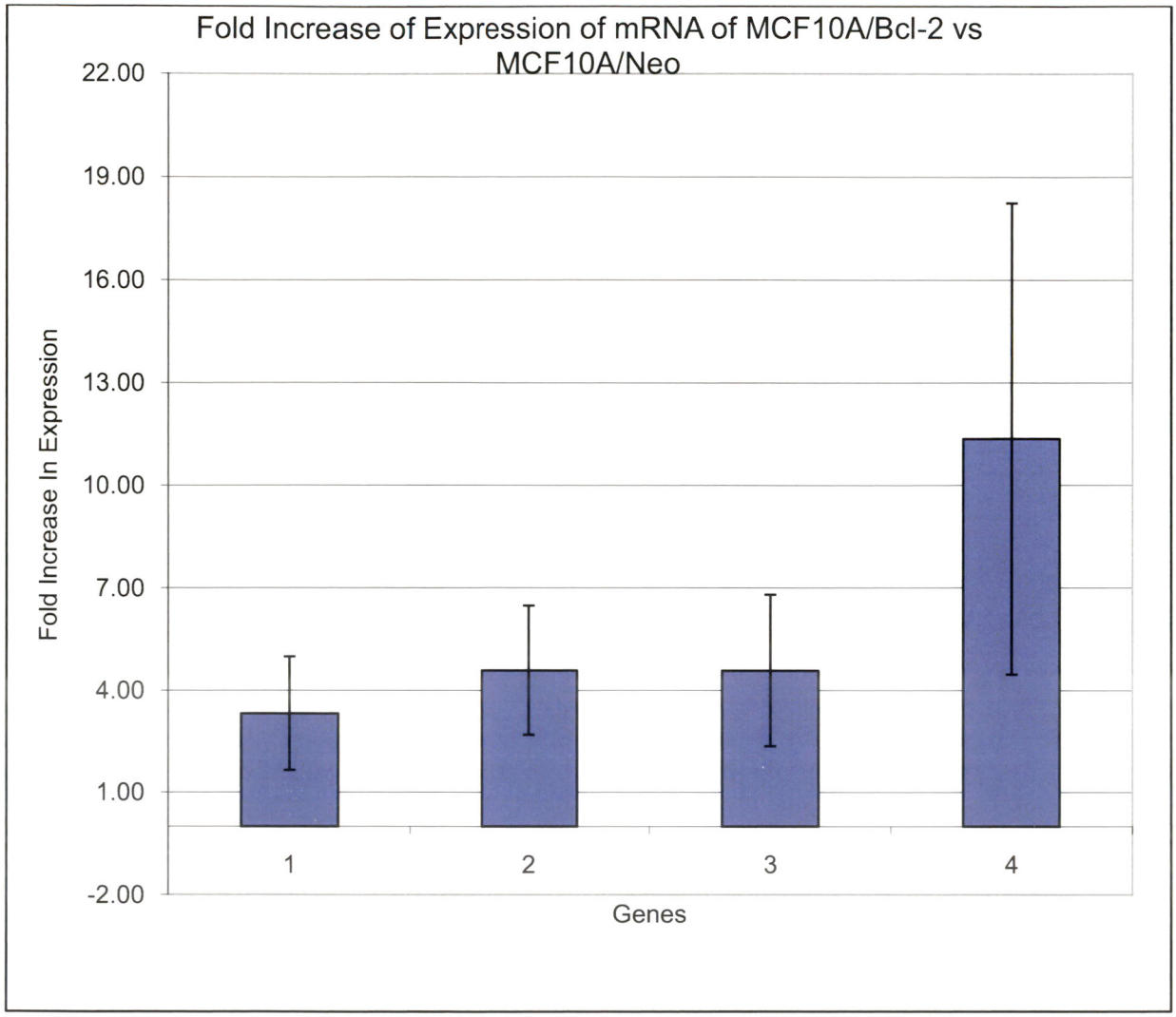

Figure 6: Increase in fold expression of mRNA in the MCF10A/Bcl-2 cell line, due to the over-expression of Bcl-2. These expression values were gathered from quantitative PCR, and the fold increase was determined by using the comparative cycle threshold method (Livack 2001). Lanes 1-4 are the corresponding genes: ANAX1, PSENEN, HCLS1, and PYCARD. The error bars are the range in expression (standard deviation) values between replicates.

Table 1

Gene Symbol	Gene Name	Gene Function
MCL1	Myeloid cell leukemia sequence 1	Anti-apoptosis
THRA	Thyroid hormone receptor, alpha	Nuclear hormone receptor
FoxM1	Forkhead box M1	Transcription factor
ITGB5	Integrin, beta 5	Receptor
MAPIIIKII	Mitogen-activated protein kinase kinase kinase 2	Kinase
ANXA1	Annexin A1	Exocytosis
PSENEN	Presenilin enhancer 2 homolog	Endoprotease Complex
RPS6KB2	Ribosomal protein S6 kinase, 70kDa, polypeptide 2	Kinase
HCLS1	Hematopoietic cell-specific Lyn substrate 1	Substrate in Signal Transduction Pathway
PYCARD	Caspase recruitment domain-containing protein	Apoptosis
RPS6KB1	Ribosomal protein S6 kinase, 70kDa, polypeptide 1	Kinase
USP3	Ubiquitin specific peptidase 3	Protease

Table 2

Gene Symbol	Accession Number	Transcript Size	Primer Sequence
MCL1	NM_021960.3	297 bp	For 5'-ATGCTTCGGAAACTGGACAT-3' Rev 5'-TCCTGATGCCACCTTCTTCTAGG-3'
THRA	NM_003250.4	137 bp	For 5'-AGTTACCTGGACAAAGACGAG-3' Rev5'-GGAATAGGTGGGATGGAG-3'
FoxM1	NM_021953.2	135 bp	For 5'-ACTTGGAATCACAGCAGAAAC-3' Rev 5'-TGGATAGGTACCAGGTATGAG-3'
ITGB5	NM_002213.3	117 bp	For 5'-AATTGGCAGAGAACAACATC-3' Rev 5'-GAGTCTCCATCTAAAATCTCCAC-3'
MAP3K2	NM_006609.3	82 bp	For 5'-CAGTATTTGGAGCAGAGAGG-3' Rev 5'-GTAACCTGGGGGAGGAGAAC-3'
ANXA1	NM_000700.1	86 bp	For 5'-AATGGTTAAAGGTGTGGATG-3' Rev 5'-TATGCTGCTTTGATCTGTTG-3'
PSENEN	NM_172341.1	91 bp	For 5'-GAGTGTCCAATGAGGAGAAATTG-3' Rev 5'-AAGATGTTGACCAACCAGAG-3'
RPS6KB2	NM_003952.2	88 bp	For 5'-GAATTGGGACGACCTTCTG-3' Rev 5'-GGGTATCAAACTGGCTCAC-3'
HCLS1	NM_005335.4	109 bp	For 5'-GACTATGAGGACGTTGAGGAG-3' Rev 5'-CCAGAGCAGAAGAAAAAGAAG-3'
PYCARD	NM_013258.3	116 bp	For 5'-TGGCTGCTGGATGCTCTGTAC-3' Rev 5'-CAGGCTGGTGTGAAACTGAAG-3'
RPS6KB1	NM_003161.2	85 bp	For 5'-CTGGAGGAAGTAAAAGCATCC-3' Rev 5'-GATACTCAAGGATGAGGTAGAG-3'
USP3	NM_006537.2	117 bp	For 5'-TTGGGTCTGTTTGACTTGTTC-3' Rev 5'-TGCTTTTCTGATTTCTTATGG-3'

Table 3

Cell line	Sample	MCL1 ΔCt	THRA ΔCt	FoxM1 ΔCt	ITGB5 ΔCt	MAP3K2 ΔCt	ANXA1 ΔCt
Bcl-2	1	7.735	6.8579	6.7216	7.0684	7.8783	3.4666
Bcl-2	2	8.986	7.4208	7.6397	7.5669	8.3863	4.399
Bcl-2	3	7.1718	8.7653	8.4588	8.4172	10.4748	4.1947
Bcl-2	4	9.042	9.5899	10.0647	9.339	10.395	5.5085
Bcl-2	5	10.4337	8.663	8.8527	8.8462	9.6997	5.1275
Neo	1	6.8872	7.9172	6.4295	7.1532	9.3981	6.0901
Neo	2	7.7984	8.7677	7.1778	8.2738	9.6571	5.3945
Neo	3	6.6542	9.6165	8.1449	9.2283	10.8298	5.975
Neo	4	7.5059	9.6384	9.2738	9.3978	11.5202	6.8766
Neo	5	8.7089	9.6793	9.208	9.1741	10.9339	6.4059

		PSENEN ΔCt	RPS6KB2 ΔCt	HCLS1 ΔCt	PYCARD ΔCt	RPS6KB1 ΔCt	USP3 ΔCt
Bcl-2	1	5.1368	7.0022	14.4191	4.8834	7.0358	7.4543
Bcl-2	2	5.5637	7.7414	15.0344	4.411	7.5381	7.9905
Bcl-2	3	7.2735	8.8085	16.2595	8.3836	8.9381	8.7892
Bcl-2	4	7.2884	9.5044	16.5543	6.8813	9.4141	9.423
Bcl-2	5	6.0935	8.8752	14.9046	5.8742	8.5065	9.1322
Neo	1	8.0637	7.2156	17.0351	7.4201	8.4127	8.0523
Neo	2	7.4446	8.2877	16.0987	8.7499	8.2045	9.8684
Neo	3	8.7394	9.1093	17.3803	10.1525	9.2739	9.8429
Neo	4	9.1655	9.9071	19.1972	10.4567	9.7012	10.2463
Neo	5	8.4715	9.0363	17.543	9.8219	9.5199	10.7826

Table 4

Sample #	MCL1 ΔΔCt	THRA ΔΔCt	FoxM1 ΔΔCt	ITGB5 ΔΔCt	MAP3K2 ΔΔCt	ANXA1 ΔΔCt
1	0.8478	-1.0593	0.2921	-0.0848	-1.5198	-2.6235
2	1.1876	-1.3469	0.4619	-0.7069	-1.2708	-0.9955
3	0.5176	-0.8512	0.3139	-0.8111	-0.355	-1.7803
4	1.5361	-0.0485	0.7909	-0.0588	-1.1252	-1.3681
5	1.7248	-1.0163	-0.3553	-0.3279	-1.2342	-1.2784
	PSENEN ΔΔCt	RPS6KB2 ΔΔCt	HCLS1 ΔΔCt	PYCARD ΔΔCt	RPS6KB1 ΔΔCt	USP3 ΔΔCt
1	-2.9269	-0.2134	-2.616	-2.5367	-1.3769	-0.598
2	-1.8809	-0.5463	-1.0643	-4.3389	-0.6664	-1.8779
3	-1.4659	-0.3008	-1.1208	-1.7689	-0.3358	-1.0537
4	-1.8771	-0.4027	-2.6429	-3.5754	-0.2871	-0.8233
5	-2.378	-0.1611	-2.6384	-3.9477	-1.0134	-1.6504

Table 5

Gene	Average Fold Change	Standard Deviation
MCL1	0.47	0.161
THRA	1.90	0.553
FOXM1	0.84	0.263
ITGB5	1.35	0.328
MAPIIIKII	2.22	0.583
ANXA1	3.32	1.673
PSENEN	4.59	1.901
RPS6KB2	1.26	0.137
HCLS1	4.58	2.229
PYCARD	11.36	6.888
RPS6KB1	1.74	0.578
USP3	2.43	0.929

Table 6

Gene Symbol	F value	Significance
MCL1	2.969	.123
THRA	2.060	.189
FoxM1	.144	.715
ITGB5	.454	.520
MAP3K2	2.746	.136
ANXA1	13.726	.006
PSENEN	15.871	.004
RPS6KB2	.260	.624
HCLS1	9.600	.015
PYCARD	12.766	.007
RPS6KB1	1.907	.205
USP3	4.185	.075

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