

**The Role of FUS (TLS) in Differentiation
in Acute Myeloid Leukaemia**

Elisabeth Jane Walsby

PhD 2004

University of Wales College of Medicine

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Publications and Presentations arising from this work

Publications

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The over-expression or inhibition of FUS (TLS) changes the response to induced differentiation which is dependent on the cytogenetic abnormality in human and murine leukaemia cell lines. 18th Annual Postgraduate Day. University of Wales College of Medicine 2003

Abbreviations

A ₅₉₅	absorbance at 595nm
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
AML1	acute myeloid leukaemia gene 1
APL	acute promyelocytic leukaemia
Ara-C	arabinoside
A/S or AS	antisense
ATRA	all-trans retinoic acid
BSA	bovine serum albumin
CBFB	core binding factor β
CBP	CREB binding protein
CBP	core binding protein
CBS	Core Biotechnology Services
cDNA	copy DNA
CML	chronic myeloid leukaemia
CTD	carboxy terminal domain
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotriphosphates
DTT	dithiothreitol
EPO	erythropoietin
ETS	E26 Transformation Specific
EWS	Ewings sarcoma protein
FAB	French American and British leukaemia classifications
FCS/FBS	foetal calf serum/foetal bovine serum
FL	FLT2/FLT3 ligand
Flt3	FMS-like tyrosine kinase 3
FUS AS	FUS antisense
G	glycine
G-CSF	granulocyte colony-stimulating factor
G-CSF-R	granulocyte colony-stimulating factor receptor
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony- stimulating factor
h	hours
HAT	histone acetyl transferases
HDAC	histone deacetylase
hnRNP	heterologous nuclear ribonuclear protein
inv	inversion
IVT	<i>in vitro</i> transcription
LB	Luria-Bertoni
LDS	lithium dodecyl sulphate
IL	interleukin
LP	long pass
LTR	long terminal repeat
MAP	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factor

MDS	myelodysplastic syndrome
MGDF	megakaryocyte growth and development,
min	minutes
MP-4	myelopeptide 4
MPD	myeloproliferative disorder
MPO	myeloperoxidase
mRNA	messenger RNA
NF- κ B	nuclear factor - κ B
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
RNAi	RNA interference
RPM	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
Ph	Philadelphia chromosome
PI3K	phospho-inositol-3-kinase
PIC	pre-initiation complex
PKA	protein kinase A
PKC	protein kinase 3
PML	promyelocytic leukaemia protein
PVDF	polyvinylidene difluoride
Q	glutamine
RAR	retinoic acid receptor
RAR α	retinoic acid receptor α
RARE	retinoic acid response element
Rb	retinoblastoma protein
RGG	arginine-arginine-glycine
RISC	RNA inducible silencing complex
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RQ-PCR	reverse quantitative-PCR
RRM	RNA recognition motif
RT-PCR	reverse transcription-PCR
RXR	retinoid X receptor
S	serine
SAPE	streptavidin conjugated phycoerythrin
SCID	severe combined immuno deficient
SCF	stem cell factor
SDS	sodium dodecyl sulphate
sec	seconds
siRNA	small interfering RNA
snRNP	small nuclear ribonuclear protein
SR	serine-arginine
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
STE buffer	salt, tris, EDTA buffer
TAF _{II} S	TBP associated factors
TBE	tris-borate-EDTA
TBP	TATA-binding protein
TE	tris-EDTA
TET	TAF _{II} 68, EWS, TLS
TLS	translocated in liposarcoma gene

TPA	12-O-tetradecanoyl phorbol-13-acetate
TPO	thrombopoietin
TRAIL	tumour necrosis factor related apoptosis inducing ligand
UC	universal container
uv	ultra violet light
WHO	World Health Organisation
Y	tyrosine

Abstract

Leukaemia is the result of molecular abnormalities which lead to a block in differentiation that is the defining characteristic of myeloid leukaemia cells. Work to identify the genes that are associated with this defective differentiation led to the identification of the involvement of the FUS gene. FUS (TLS) is a housekeeping gene that is capable of binding DNA and RNA. FUS has roles in coupling mRNA transcription and processing and in the repair of double stranded DNA breaks. Previous work within this department has shown that FUS is down-regulated in human leukaemia cell lines in response to induction of differentiation with ATRA and that FUS is up-regulated in acute myeloid leukaemia (AML) patients. The aim of this study was to identify whether this is a causative or correlative relationship and also to identify target genes associated with FUS dysregulation. Transduction of human (HL-60, NB4, NB4R2) and murine (32D, 32D AML-ETO, 32D B2A2) leukaemia cell lines with retroviral constructs expressing FUS or antisense FUS resulted in some over-expression or down-regulation of FUS in these cells. The effect of FUS modulation in the transduced cell lines was assessed through cell growth and viability. No effect on the growth and viability of transduced cells was observed as a result of FUS modulation. To determine whether FUS had a role in differentiation, the transduced cell lines were induced to differentiate using ATRA and G-CSF. Differentiation was assessed by measurement of cell growth, viability and the expression of cell surface markers by flow cytometry. The result of expression of FUS antisense in the ATRA sensitive NB4 and 32D cell lines was to generate resistance to differentiation induction using ATRA. Conversely in the ATRA resistant NB4R2 and 32D B2A2 cells, expression of FUS antisense reinstated the ability to differentiate in these cells. In response to treatment with G-CSF, the 32D cells expressing FUS antisense developed a resistance to differentiation while the previously G-CSF resistant 32D B2A2 cells became capable of differentiation when FUS antisense was expressed in these cells. Following the demonstration of an altered phenotype in response to treatment with different differentiation inducers in cells containing FUS antisense constructs, genes acting as target genes of FUS were identified using the Affymetrix gene expression system. The effect of FUS over-expression and down-regulation were studied in all the murine 32D derived cell lines and in the human NB4 cell line. In addition to this, gene expression changes resulting from FUS modulation in the NB4 cells during treatment with ATRA over 96 hours was investigated in this manner. Expression levels of genes associated with FUS dysregulation were verified using quantitative RT-PCR. Genes identified as having altered expression as a result of the expression of the FUS antisense construct included transcription factors, genes involved in apoptosis and differentiation and genes that have previously been shown to interact with, or have homology to, FUS itself. Further analysis of these candidate genes suggested that they were not likely to have a dominant effect in the altered phenotype seen in the transduced cells but were more likely to play a participatory role in the effects observed. This study has concluded that FUS may have a role in haematopoietic differentiation induced by both ATRA and G-CSF but this role appears to be context dependent making it important to study the effects of its modulation in primary AML blasts. The mechanism through which FUS affects the ability of the cells to differentiate remains unresolved.

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Chapter 1

Introduction

1.1 Haematopoiesis

Haematopoiesis is the process by which blood cells are made and this occurs in the bone marrow of the central skeleton and the proximal ends of long bone in adults. Stem cells have the ability to self replicate, proliferate and differentiate to increasingly specialized progenitor cells eventually resulting in red cells, granulocytes, monocytes, platelets and lymphocytes in a sufficient but not excessive quantity (Mehts A *et al.* 2000; Olsson *et al.* 1996; Orkin 2000). Primitive cell differentiation probably involves mainly stochastic processes, but as the cell becomes more lineage restricted, deterministic processes appear to be more relevant as the cell has to respond to immediate changes in the environment (Enver *et al.* 1998).

1.1.1 Role of cytokines

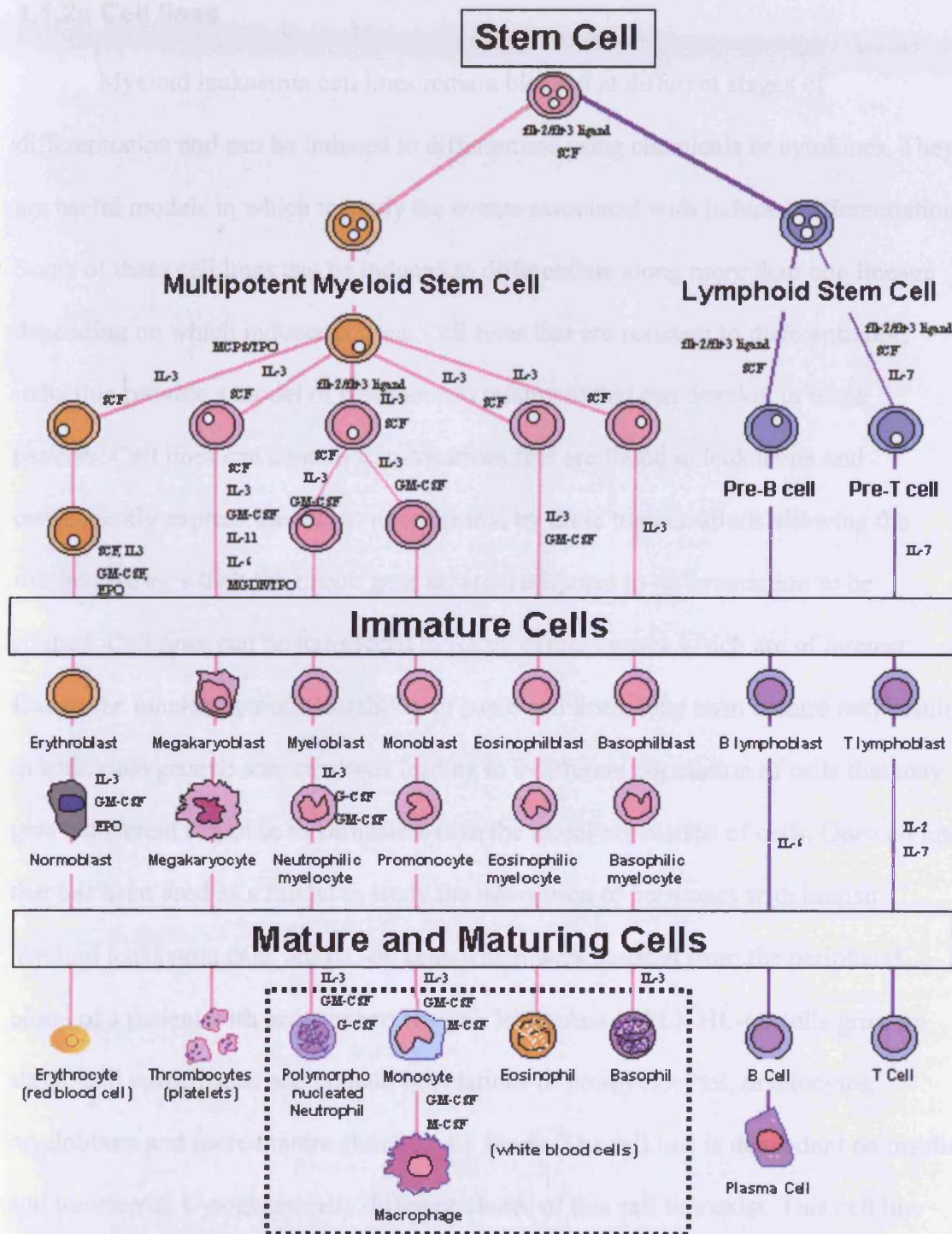
The process of haematopoiesis is controlled by a group of glycoproteins known as growth factors and cytokines and these include G-CSF, GM-CSF, IL-3, IL-4, IL-6 and IL-8. These growth factors act in synergy with each other and regulate the progression of differentiation of a pluripotent stem cell into all the restricted cell lineages found in the blood. Following commitment to a particular lineage, cells are dependent upon the supply of growth factors for continued viability. The progression of a pluripotent stem cell to myeloid restricted lineages is controlled by the cytokine IL-3 which stimulates proliferation and produces differentiated progeny of all the myeloid cell types. Following their response to IL-3, progenitor cells continue to differentiate and become responsive to GM-CSF and M-CSF, resulting in granulocyte or macrophage restricted progeny (Mehts A *et al.* 2000; Ribatti *et al.* 2001) Growth factors that act on early multipotent cells are IL-3, IL-4, IL-6 and GM-CSF. Growth factors that act on cells which have already been committed to specific lineages are G-

CSF, M-CSF, IL-5, erythropoietin and thrombopoietin, although these can also have effects on earlier cells.

Binding of a growth factor to the cell surface receptor usually results in activation of a protein kinase pathway within the cell, taking the message from the cell surface receptor to the nucleus of the cell. This in turn may result in activation or inhibition of gene transcription resulting in the cell starting the cell cycle, commencing differentiation, inhibiting apoptosis or becoming functionally active (Hibbert *et al.* 2001; Mehts A *et al.* 2000; Olsson *et al.* 1996). The transcription factors are involved in protein-protein interactions which control the lineage the cell will eventually take and the stage of differentiation that will be achieved (Fisher 2002; Olsson *et al.* 1996). Transcription factors that are restricted to expression in particular lineages establish a gene expression program that is intrinsic to differentiation along that lineage and these are capable of promoting the differentiation along one lineage while simultaneously and actively suppressing another (Enver *et al.* 1998; Orkin 2000). One example of such a transcription factor is the AML1 protein that forms one subunit of a heterodimeric complex with the CBF β protein. The dimer functions as a transcription factor with the ability to bind CBF recognition sites found in genes important for myeloid differentiation. This transcriptional regulation leads to expression of myeloid specific proteins including the myeloperoxidase (MPO) enzyme (Olsson *et al.* 1996). Growth factors can also affect the function of mature cells. The diagram in figure 1.1 shows the differentiation of the haematopoietic stem cell into the different lineages derived from it and the point at which growth factors and cytokines act within the different differentiation pathways.

1.1.2 Models for studying haematopoiesis

Fig. 1.1. Differentiation of the stem cell into different haematopoietic lineages including the point at which cytokines and growth factors act within these programs of differentiation. Diagram adapted from Chugai Pharmaceuticals Ltd.



1.1.2 Models for studying haematopoiesis

Different models exist for studying the process of haematopoiesis. These models include cell lines, primary cells and whole animals. Each of these systems has advantages and limitations in their use.

1.1.2a Cell lines

Myeloid leukaemia cell lines remain blocked at different stages of differentiation and can be induced to differentiate using chemicals or cytokines. They are useful models in which to study the events associated with induced differentiation. Some of these cell lines can be induced to differentiate along more than one lineage depending on which inducer is used. Cell lines that are resistant to differentiation induction provide a model of resistance to treatment that can develop in some patients. Cell lines can contain translocations that are found in leukaemia and consequently express the fusion gene created by these translocations allowing the mechanism by which the fusion gene creates resistance to differentiation to be studied. Cell lines can be transduced to stably express genes which are of interest. Due to the inherent genetic instability of some cell lines, long term culture may result in additional genetic abnormalities leading to a different population of cells that may give a different response to stimulants than the initial population of cells. One cell line that has been used as a model to study the interaction of hormones with human myeloid leukaemia cells are HL-60 cells which were isolated from the peripheral blood of a patient with acute promyelocytic leukaemia (APL). HL-60 cells grow as single-cell suspensions and contain populations of promyelocytes, myelocytes, myeloblasts and more mature granulocytic forms. The cell line is dependent on insulin and transferrin. Cytogenetically different clones of this cell line exist. This cell line has been used to demonstrate that colony stimulating factor (CSF) induces colony

formation *in vitro* in these cells and maturation can be induced with dimethyl sulphoxide (DMSO) and butyric acid. HL-60 cells that have been induced to mature respond to chemotaxins, develop complement receptors and produce superoxide (Collins 1987; Koeffler *et al.* 1980). This cell line has been suggested to be a good model for studying the interaction of hormones with human myeloid leukaemia cells and the synergistic effects of mediators of granulocyte, monocyte and macrophage differentiation despite not truly reflecting the differentiative potential of normal promyelocytes (Collins 1987; Koeffler *et al.* 1980). Other cell lines used in this study are discussed in detail later.

1.1.2b Primary cells

Primary cells will differentiate into any of the haematopoietic lineages with appropriate cytokines. Normal primary cells do not contain the recurrent translocations that are typically found in leukaemia cell lines and have a finite lifespan *in vitro*. Normal primary cells are good models of normal, non leukaemic differentiation but can also be used to study the influence of individual genes on a normal genetic background. It is possible to express a transgene or suppress the expression of a gene using an antisense copy of the gene to study its effect on normal differentiation of the over- or under-expression of this gene. Generally immature subsets of cells (e.g. CD34⁺) are employed for these studies which allow the entire process of differentiation to be studied.

1.1.2c Animals

Cell lines and primary cells provide good models of haematopoiesis and differentiation but are only capable of showing the reactions of cells in isolation. The use of whole animals as models in which to study the process of differentiation provides an opportunity to study the cells in the position it would be in within the

animal with all the interactions from the other systems of the body. Examples of the ways in which animal models are utilised include the generation of knockout mice from which genes are removed or inactivated in a specific tissue, domain specific, isoform specific manner or with a combination of these alterations to the genome. These models are not trivial to generate and give information about the function of genes *in vivo* (Bartel *et al.* 2000). Additionally the SCID-hu mouse model provides a small animal model for human physiology and pathophysiology and the treatment of disease. In these animals organs from the human haematolymphoid system were introduced into the mouse to become vascularised and interact with the systems in the mouse resulting in the production of suitable microenvironments for the differentiation and functioning of human haematopoietic cells of multiple lineages. The introduction of bone marrow, fetal liver and thymus have shown good results while artefacts are generated when elements of the lymphoid systems are introduced. These artefacts must be considered when the results are generated using these models as must physiological variations in the mouse. These SCID-hu mice have been used to study cytokine interactions both individually and in combination as well as irradiation and human stem cell therapy (McCune 1996).

1.2 Differentiation

1.2.1 Assessment of differentiation

Differentiation is a sequence of events by which cells undergo an orderly change from immature cells into mature cells and the mechanisms governing cell fate decisions are under tight but flexible control (Akashi *et al.* 2000; Phillips *et al.* 2000). Regulation of myeloid differentiation involves receptors and intracellular processing of external signals eventually leading to the activation of specific genetic programs of differentiation. Examples of factors influencing the pathway of development include

G-CSF which stimulates the proliferation and maturation of neutrophil progenitors but can also act to trigger the proliferation of non cycling primitive progenitor cells.

Factors which act late in lineage commitment include Epo, which regulates erythropoiesis and IL-5 which is important in the development of esinophils (Ogawa 1993). These programs of differentiation depend on the correct temporal and spatial expression of myeloid specific transcription factors that are coupled to cell cycle regulation. Tumour suppressor proteins may be centrally placed in intra-cellular pathways that mediate extra-cellular differentiation signals converging both in myeloid transcription factors and cell cycle regulation (Olsson *et al.* 1996).

1.2.2 Measurement of the effects of haematopoiesis

1.2.2a Proliferation

Once committed to differentiation along any of the haematopoietic lineages, the developing cells will only be programmed to undergo a limited number of divisions leading to the ultimate phenotype and specialised cell. The actual number of divisions is highly variable. Cell lines are capable of continued proliferation which will be reduced if they respond to treatment with an inducer. By assessing the proliferation of a population of cells under different conditions of culture it can be determined whether they have the ability to respond to an inducer of differentiation or whether their growth rate is affected by the expression of a transgene.

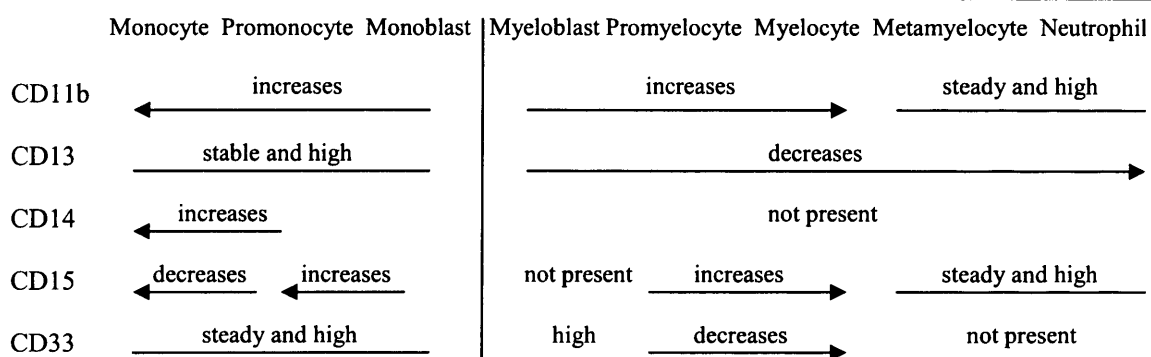
1.2.2b Colony formation

Colony formation by cells is used as a way to measure the potential of the cells to proliferate and the different types of colonies that are formed are used to identify progenitor subsets present within a population.

1.2.2c Immunophenotyping

Cells express different proteins on their surfaces at different points in their development and in response to different stimuli. Determining which of these cell surface markers are expressed on the cells while inducing differentiation or following the progression of cells through their differentiation program can give information about the differentiation state of the cells. More than one type of cell surface marker can be detected and it can be informative to study the increase in expression of one cell marker while another declines in expression. The expression of these cell markers are controlled by different processes and which of these is occurring at any particular point is informative about the mechanism by which differentiation is occurring and the stage to which differentiation has proceeded. The diagram shown in figure 1.2 shows the level of expression of different cell surface markers throughout different stages of development. Of the cell surface markers illustrated in the diagram, CD14 is predominantly expressed in the monocytic cell lineage, CD11b and CD15 are acquired in the early stages of both granulocytic and monocytic differentiation, CD33 is present throughout monocytic differentiation and is lost early in granulocytic differentiation and CD13 is present at all stages of both lineages but decreases in expression in granulocytes.

Fig 1.2 Levels of expression of selected cell surface markers in the monocytic and granulocytic lineages. This diagram was adapted from Drexler 1987.



1.2.2d Cellular morphology

The morphology of cells is altered as they differentiate. The cells become more distinctive and have different ratios of nuclear to cytoplasmic volume, some develop cytoplasmic extrusions, lobulated nuclei, become visibly more granular in their cytoplasm, or develop other characteristics. Some morphological characteristics of differentiated cells are indicated in figure 1.1. Populations of cells may be studied and the proportions of cells with different morphological phenotypes scored to indicate the stages of differentiation of the cells that are present within the population.

1.3 Leukaemia

1.3.1 Leukaemia as dysregulated haematopoiesis

It is thought that haematological malignancies arise from a single cell in the bone marrow, thymus or peripheral lymphoid system (Mehts A *et al.* 2000). The cell undergoes a genetic mutation leading to a cell with altered developmental properties. Mitotic divisions of this cell lead to a clone of cells with the same genetic mutation. Further genetic alterations may give rise to additional subclones (Manoharan 1998; Nucifora *et al.* 1995; Phillips *et al.* 2000). Leukaemia cells are arrested at an immature stage of development and the genes involved in this differentiation block are often important in myeloid differentiation (Olsson *et al.* 1996). Different classes of genes are thought to contribute to the transformation of a cell into a malignant clone.

Myelogenous leukaemia displays the hallmark of the rapid growth of immature cells that have no ability to differentiate (van der Reijden *et al.* 1996). The block in differentiation that is seen is frequently the result of chromosomal rearrangements or gene amplification and both these events result in multiple simultaneous effects.

1.3.1a Anti-oncogenes

Anti-oncogenes, like the tumour suppressor gene p53, encode proteins having a critical role in the suppression of tumour growth. Removal of these genes at one allele by chromosomal deletion results in a cell that is vulnerable to deletion or mutation of the remaining allele of the gene, which may promote transformation (Mehts A *et al.* 2000). The theory discussed by Knudsen suggests that tumourigenesis is the result of two genetic alterations happening to the cell from which the cancer is formed (Knudson 1995). The first mutation in the gene may be either inherited or acquired in the somatic cell. For example, in retinoblastoma, the presence of a mutation in the Rb gene was not sufficient to ensure that all patients with an inherited mutation developed a tumour suggesting that on its own the first mutation has no effect on the cell. In this theory the rate limiting event in tumour development is the loss of, or mutation of, both copies of the gene within one cell.

1.3.1b Oncogenes

Oncogenes are genes whose protein products can give rise to neoplastic transformation and are normally derived from normal genes (proto-oncogenes) in the cells. The proteins encoded by the proto-oncogenes are involved in signal transduction, cell cycling, resistance to apoptosis or differentiation. The inappropriate expression or activation of an oncogene may cause the transformation of a cell. Aberrant expression of oncogenes can result from amplification, point mutation or translocation from one chromosomal location to another. Translocation is the most common mechanism by which oncogenes are inappropriately expressed in haematological malignancies and examples of translocations involved in leukaemias are discussed in section 1.3.5 (Mehts A *et al.* 2000). Mutation of genes can also result in aberrant expression of the protein product. In the Flt-3 (fms-like tyrosine kinase 3)

gene which encodes a tyrosine kinase receptor protein, internal tandem duplications (ITD) are commonly observed in acute myeloid leukaemia (AML) cases. These ITD vary in their location and length but are always located in the juxtamembrane region of the protein and are in-frame. The presence of an ITD mutation leads to the constitutive activation of tyrosine kinase signalling, resulting in the activation of the signal transducer and activator of transcription 5 (STAT5) and mitogen-activated protein kinase (MAP) kinase signalling molecules among others (Mehts A *et al.* 2000). Point mutations in the AML-1 gene, which is involved in the chromosomal translocation discussed in section 1.3.5b, result in myeloid leukaemias with the M0 subtype. These point mutations in the AML1 gene which is a regulator of haematopoiesis are biallelic and total loss of the gene results in a differentiation block (Osato *et al.* 2001). Non-sense and frameshift mutations are also seen in the AML-1 gene and these lead to limited DNA binding by the protein disrupting its function. The more reduced the activity of the AML-1 gene, the higher the leukaemogenicity resulting from the mutation (Osato *et al.* 2001).

1.3.1b/ Inhibition of apoptosis

Genes whose protein products provide resistance to apoptosis are involved in haematological malignancies. Elevated expression of these genes prevents the cell from undergoing programmed cell death which normally occurs in damaged cells. An example of a gene with this function is Bcl-2 whose expression has been shown to be up-regulated in cells with some chromosomal translocations (Mehts A *et al.* 2000). Intact apoptotic pathways within the cell are necessary for the cell to be able to respond to insults. A death pathway at which several signalling pathways converge is controlled by the bcl-2 family of genes. Bcl-2 codes for a protein that blocks programmed cell death, so promoting cell survival. Over-expression of Bcl-2 is commonly found in follicular B-cell lymphoma and lymphoproliferative disease. The

ratio of expression level of the proteins in the bcl-2 family determines whether the cells will undergo apoptosis or survive as a result of dimers forming between the different members of the family. The over-expression of the bcl-2 or bcl-x_L genes results in the cell bypassing the apoptotic pathway and continuing to proliferate despite any damage that it may have acquired (Irvine *et al.* 2002). Deletion of tumour suppressor genes may also result in cell transformation (Rabbitts 1994). The p53 tumour suppressor gene is often inactivated through deletion in solid tumours and to a lesser extent in haematological malignancies. This is a tumour suppressor gene that is triggered by cellular stress including the results of DNA damage, hypoxia and oncogene activation and leads to the activation of downstream genes including WAF1, MDM2 and GADD45. The loss of p53 removes the ability of the cell to respond to the damage it has received and allows it to continue to proliferate without first repairing the damage (Drexler 1998; Padua *et al.* 2001).

1.3.2 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a clonal proliferative disorder which displays an increase in neutrophils and their precursors in the peripheral blood of the patient. The bone marrow of patients has an excess of granulocyte precursors resulting in increased cellularity. More than 95% of patients with CML have the t(9;22) Philadelphia chromosomal translocation encoding the fusion protein BCR-ABL which is also seen in acute lymphocytic leukaemia and is discussed further in section 1.3.5c (Gordon *et al.* 1996; Mehts A *et al.* 2000). CML has an insidious onset and a greater incidence in older patients (Rabbitts 1991).

1.3.3 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a rare disease in childhood but is much more frequent in adults where the prognosis is less favourable (Bene *et al.* 1999).

AML accounts for 77% of acute leukaemias (Walker *et al.* 1994) and no pre-clinical phase is generally manifested (Rabbitts 1991). AML is a heterogeneous disease caused by a variety of pathogenic mechanisms. At the morphologic level this heterogeneity is manifested by variability in the degree of commitment to and differentiation of cell lineages (Hayashi 2000). Acute leukaemias originate from immature haematopoietic cells that can undergo self-renewal (Ribatti *et al.* 2001). AML disrupts the maturation and apoptotic processes of the cell, leading to the accumulation of long-lived immature cells (Bene *et al.* 1999). The disruption of the normal expression of transcription factors, cytokine receptors and genes regulating the cell cycle may be involved in the development of AML (Cleary 1991; Olsson *et al.* 1996). Chromosomal translocations in AML often involve transcription factors which are part of the regulation of myeloid cell differentiation. These translocations result in the fusion of two genes creating a chimeric protein with transforming ability being drawn from one or both of the genes (Olsson *et al.* 1996). Examples of chromosomal translocations affected in this manner are discussed in section 1.3.5.

AML patients can have one or more chromosomal abnormality that is not detectable at complete remission but can reappear at relapse, sometimes with an additional or different abnormality (Walker *et al.* 1994).

Chromosomal abnormalities in AML often activate transcription factors that are important in differentiation rather than cell division. The expression of these transcription factors are developmentally regulated in a normal cell and encode proteins that have the potential to dimerize with other transcription factors resulting in either negative or positive transcription control (Rabbitts 1991). Chromosomal translocations seen in leukaemia and their cellular effects are discussed in section 1.3.5.

1.3.4 Classification of different types of AML

According to the French, American and British (FAB) classification (Bennett *et al.* 1976) leukaemia can be classified as lymphoid or myeloid with further sub classification within this system. The classification that is attached to a patient is dependent on the morphology of the cells of blood films taken from the patient before any cytotoxic drugs have been given. Sub-classification of lymphoid leukaemias gives classes L1, L2 and L3. Myeloid leukaemias receive a classification of M0 to M7 depending on the differentiated status of the cells. In brief the FAB classifications of AML are (Bruserud *et al.* 2000):

M0 and M1	minimal differentiation
M2	has a minor maturing granulocytic compartment, a sub group is associated with t(8;21)
M3	Acute promyelocytic leukaemia (APL)- dominating accumulation of promyelocytes. Have t(15;17)
M4 and M5	myelomonocytic differentiation.
M6	erythroid predominance
M7	megakaryoblastic leukaemia

Progress in the development of monoclonal antibodies which are specific for developmental subsets of haematopoietic cells, coupled with laser and computer technology, has led to multi-parameter flow cytometry being used with panels of antibodies to increase the precision of diagnosis and classification of AML (Weir *et al.* 2001). In 2002 the World Health Organisation (WHO) proposed a new system which uses morphologic, genetic, immunophenotypic, biologic and clinical information to define haematopoietic and lymphoid neoplasms into disease entities. These new criteria include many of the criteria used in the FAB group classifications

of leukaemia. The revised classifications for AML consist of four major groups with further classified subgroups and these are shown below as described in Vardiman (Vardiman *et al.* 2002).

- AML with recurrent genetic abnormalities
 - AML with t(8;21)(q22;q22)
 - AML with abnormal bone marrow eosinophils and inv(16)(p13q22), or t(16;16)(p13;q22)
 - APL with t(15;17)(q22;q12)
 - AML with 11q23 abnormalities
- AML with multilineage dysplasia
 - Following myelodysplastic syndrome (MDS) or MDS/myeloproliferative disorder (MPD)
 - without MDS or MDS/MPD but with dysplasia in at least 50% of cells in two or more lineages
- AML and myelodysplastic syndromes, therapy related
 - alkylating agent/radiation-related type
 - topoisomerase II inhibitor-related type
 - others
- AML, not otherwise classified
 - AML- minimally differentiated
 - AML without maturation
 - AML with maturation
 - acute myelomonocytic leukaemia
 - acute monoblastic/acute monocytic leukaemia
 - acute erythroid leukaemia
 - acute megakaryoblastic leukaemia

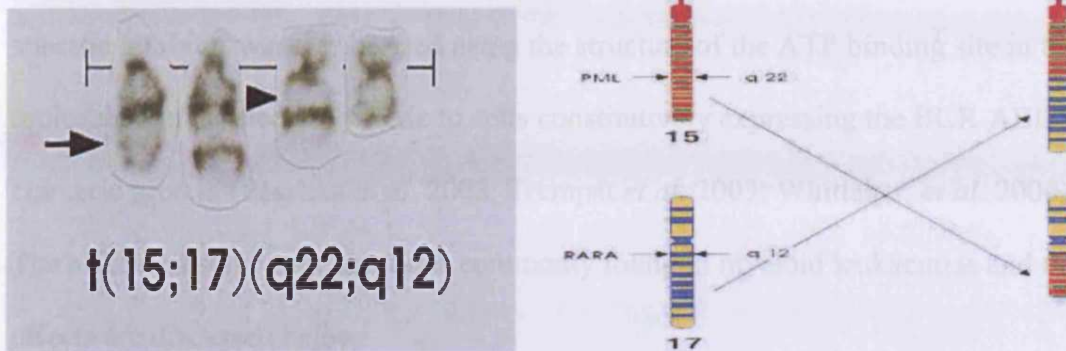
- acute basophilic leukaemia
- acute panmyelosis with myelofibrosis
- myeloid sarcoma

Patients exhibiting specific recurring cytogenetic abnormalities (t(8;21), inv(16), t(16;16)) should be classified as having AML regardless of the blast count that is measured. One potential problem with these classifications is the time required for generating the genetic information that is required for each patient. In these cases a preliminary classification can be made to allow treatment to commence and this refined once the genetic information has been determined. Within these classifications scope has been left for further refinement of the subgroups as a result of further advances in diagnostic techniques.

1.3.5 Translocations in AML

A chromosomal translocation occurs when part of one chromosome becomes detached and fuses to a different chromosome. A reciprocal translocation gives rise to two hybrid chromosomes both containing parts of the other chromosome which have been broken apart and rejoined to the other chromosome. An example of this is the t(15;17) translocation. A diagram of chromosomes 15 and 17 before translocation and the chromosomes resulting from the translocation are shown in figure 1.3.

Fig. 1.3. Chromosomal translocation between chromosomes 15 and 17. Diagram from www.meds.com/molmime/leukemia/guide/1660s16.jpg



Translocations affect only one allele of the gene meaning that the resulting fusion protein needs to have a dominant effect over the protein product of the unaffected allele in order to transform the cell (Olsson *et al.* 1996). The two consequences of translocations and inversions are that a gene comes to lie near a strong promoter and is activated by it, or that the chromosomal break occurs in one gene on each of the translocated chromosomes resulting in the joining of these two genes and the creation of a fusion protein. Transcription factors are often involved in fusion proteins, leading to altered transcription of the transcription factor's target genes (Rabbitts 1994). Chimaeric proteins created through chromosomal translocations in leukaemia are believed to act in a dominant-negative manner to block the action of critical lineage determining factors (Orkin 2000), an example of this is the $t(15;17)$ translocation which creates the PML-RAR α fusion protein which acts on the RAR α , RXR and C/EBP α receptors which are essential for granulocytic differentiation (Orkin 2000). Fusion proteins created by chromosomal translocation exert some of the effects that result in the differentiation block while others are exerted by the truncated products of alternative splicing of genes following chromosomal rearrangement (van der Reijden *et al.* 1996). Fusion proteins created by

chromosomal translocations are tumour specific antigens and have been identified as potential targets for therapy (Rabbitts 1994). An example of this is seen in CML. In patients with the t(9;22) translocation, a specific tyrosine kinase inhibitor that acts to inhibit the Abl protein tyrosine kinase has been licensed for use in adult patients. This specific inhibitor was synthesised using the structure of the ATP binding site in the molecule and is selectively toxic to cells constitutively expressing the BCR-ABL chimeric protein (Paschka *et al.* 2003; Trempat *et al.* 2003; Whittaker *et al.* 2000). The effects of some translocations commonly found in myeloid leukaemias and their effects are discussed below.

1.3.5a t(15;17)

The t(15;17) translocation fuses the retinoic acid receptor gene $RAR\alpha$ to the promyelocytic leukaemia protein gene PML (Duprez *et al.* 1992). PML is a tumour repressor gene which forms transcription-regulatory complexes with various molecules (Kawasaki *et al.* 2002). The $RAR\alpha$ gene encodes a protein that acts as a receptor for retinoic acid and is involved in the differentiation process in normal granulocytic differentiation. The retinoic acid binding domain of $RAR\alpha$ is retained in the fusion protein and functions as an aberrant retinoid receptor at physiological levels of retinoic acid. Downstream target genes are repressed by the recruitment of nuclear co-repressors, deacetylase complexes and DNA methyl transferases to the transcription factor complex, contributing to the block in differentiation (Thompson *et al.* 2003).

The PML/ $RAR\alpha$ fusion protein has been shown to augment STAT3 activity (Kawasaki *et al.* 2002). The STAT3 gene regulates cytokine dependent growth of haematopoietic cells by promoting the progression of the cells from G_1 to S phase in the cell cycle through the induction of the c-myc and cyclin D1 genes. Through its

interaction with STAT3, the PML/RAR α fusion protein results in more cells passing from G₁ to S phase without the usual intracellular controls taking place to ensure the cell is competent to proceed through the cell cycle.

1.3.5b t(8;21)

The t(8;21)(q22;q22) translocation in which the AML1 gene is fused to the ETO gene is seen in 10-15% of acute myeloid leukaemias and in 40% of M2 AML patients with normal karyotypes (Lasa *et al.* 2002; Nucifora *et al.* 1995). This translocation is the second most common recurring chromosomal abnormality seen in AML and indicates a favourable outcome following treatment (Gamerdinger *et al.* 2003; Pabst *et al.* 2001). AML1 is a strong transcriptional activator (Nucifora *et al.* 1995) as well as being associated with CBP histone acetyl transferase (Puccetti *et al.* 2002). AML1 is also known as CBF α and is the DNA binding subunit of the heterodimer core binding factor (CBF) which is a transcription factor essential for normal haematopoiesis mediating up-regulation of a number of genes including GM-CSF and IL-3 involved in the differentiation of haematopoietic stem cells (Israels *et al.* 2002). ETO is a transcriptional co-repressor protein which is recruited through DNA binding proteins but which cannot itself bind DNA (Amann *et al.* 2001). ETO is also known as CDR or MTG8. Fusion with ETO converts AML1 from a transcriptional activator to a transcriptional repressor. The fusion protein may act as an inhibitor by binding the normal AML1 binding sites where it acts as a dominant negative competitor of AML1 (Nucifora *et al.* 1995). The fusion protein aberrantly recruits histone deacetylase (HDAC) activity (which is recruited by the normal ETO protein (Puccetti *et al.* 2002) and results in a block in the vitamin D₃-induced differentiation (Puccetti *et al.* 2002)). Haematopoietic progenitor cells with t(8;21)

have impaired differentiation as well as deregulated proliferation (Puccetti *et al.* 2002).

1.3.5c t(9;22)

The t(9;22) translocation results in a structure known as the Philadelphia chromosome in which the c-ABL gene is fused to the BCR gene. c-ABL is a non-receptor tyrosine protein kinase found in the nucleus and the cytoplasm. BCR forms a complex with c-Abl in haematopoietic cells (Ling *et al.* 2003). Two naturally occurring forms of BCR/ABL exist and these differ in the extent of the BCR protein that is fused to the ABL protein (Cortez *et al.* 1995). The breakpoint in the bcr gene in the p185 fusion protein is seen within the first intron of the bcr gene while in the p210 fusion protein the breakpoint in the bcr gene is 5kb further downstream. The proportion of the ABL protein that is fused to the BCR protein is the same in both fusion proteins. Of these two forms, BCR/ABL p210 results in CML and the BCR/ABL p185 leads to ALL (Cortez *et al.* 1995; Lugo *et al.* 1990). Human CML cells and the UT-7 and BaF3 cell lines expressing BCR-ABL have constitutive tyrosine kinase activity and increased genetic instability (Deutsch *et al.* 2003). In 32Dcl3 cells reduced susceptibility to apoptosis, growth factor independent cell proliferation and differentiation arrest was observed as a result of the expression of the BCR/ABL fusion protein (Perrotti *et al.* 1998). The ability of BCR-ABL to transform cells depends on its tyrosine kinase activity which is essential to recruit and activate multiple biochemical pathways transducing oncogenic signals (Perrotti *et al.* 1998). Numerous pathways have been reported as being activated by the BCR/ABL fusion protein including PI3K, protein kinase C and the Jak/STAT pathway (Zou *et al.* 1999). The activity of transcription and post-transcription factors that modulate the expression of specific differentiation related genes is altered by BCR-ABL expression although the molecular mechanisms for this are unknown (Perrotti *et al.* 2002b). The

MZF-1 transcription factor is one example of a transcription factor whose activity in two separate complexes is regulated by the BCR/ABL protein and in addition to this, the post transcriptional activity of the FUS protein itself was shown to be regulated by the binding of the BCR/ABL fusion protein in 32D cells (Perrotti *et al.* 1998).

Expression of BCR-ABL leads to the post-transcriptional down-regulation of the BRCA1 protein which is involved in the maintenance of genomic integrity (Deutsch *et al.* 2003).

1.3.5d t(16;21)

The t(16;21) translocation is seen in all subtypes of AML except M3 but is not a commonly occurring translocation (Kong *et al.* 1997; Panagopoulos *et al.* 1994).

This translocation results in the 5' end of the FUS gene, also known as TLS, becoming fused to the 3' end of the ERG gene. FUS is located on chromosome 16p11 and is expressed in many tissues. FUS is required for active transcription and is capable of binding both RNA and DNA (Perrotti *et al.* 1998; Zinszner *et al.* 1997b).

The ERG gene is a sequence specific transcriptional activator comprising two transcriptional activation domains, one located in the amino terminal domain and the other in the carboxy terminal domain of the protein (Yi *et al.* 1997). ERG is located on chromosome 21q22 and is also seen fused to EWS in a subset of Ewings Sarcomas (Panagopoulos *et al.* 1994; Yi *et al.* 1997). The FUS domain in the fusion protein regulates the DNA binding activity of the fusion protein and acts as a transcriptional activation domain which is essential for the tumorigenic activity of the fusion protein (Kong *et al.* 1997; Panagopoulos *et al.* 1994; Yang *et al.* 2000). Alterations in DNA binding of FUS changes the protein's transcriptional activation properties and are thought to be responsible for the pathogenesis of t(16;21) AML (Hayashi 2000; Yi *et al.* 1997). The DNA binding ability of the fusion protein is lower than that of the normal ERG protein (Prasad *et al.* 1994).

FUS-ERG was the first example of two genes fused in a haematopoietic malignancy in which both genes were known to be recombined in solid tumour types (FUS in myxoid liposarcoma, ERG in Ewings sarcoma) (Panagopoulos *et al.* 1994). FUS-ERG protein inhibits apoptosis induced by calcium ionophore in NIH3T3 cells and both the FUS and ERG domains of the fusion protein are required for this (Yi *et al.* 1997). FUS-ERG expression in normal human haematopoietic cells results in the cells showing an increased capacity for self-renewal and proliferation but they do not acquire immortality (Pereira *et al.* 1998). Enhanced expression of G-CSF-R is seen when FUS-ERG is expressed, this can also be seen in AML1-ETO expressing mouse myeloid precursor L-G cells (Ichikawa *et al.* 1999). FUS-ERG inhibits the docking function of the FUS molecule which usually recruits SR splicing factors in pre-mRNA splicing (Yang *et al.* 2000). Further discussion of the FUS gene is presented in section 1.4.

1.3.6 Treatment of AML

Treatment of AML generally involves intensive chemotherapy which is administered as induction treatment aiming to achieve complete haematological remission followed by consolidation therapy which aims to eradicate residual disease and prevent relapse. Consolidation therapy and autologous stem cell transplantation are associated with a relatively high risk of relapse while allotransplantation leads to a lower risk of relapse but a higher risk of treatment related mortality (Bruserud *et al.* 2000). Specific subtypes of AML receive more specialised treatment.

In APL, differentiation therapy is attempted using retinoids which have important effects on cell development, proliferation and differentiation (Agadir *et al.* 1994; Bruserud *et al.* 2000). The presence of all-trans retinoic acid (ATRA), which is a vitamin A derivative, has been shown to increase the fraction of differentiated cells

with the functional characteristics of normal neutrophils, increase cytokine secretion, induce a mature membrane molecule phenotype, inhibit leukaemic cell proliferation and induce apoptosis (Bruserud *et al.* 2000). ATRA treatment has also been shown to decrease the level of bcl-2 expression. When bcl-2 is over-expressed cells show a poor response to chemotherapy while down regulation of bcl-2 with ATRA may increase the sensitivity of the cells to chemotherapy (Seiter *et al.* 2000). ATRA-induced responses are often short-term and therapy with ATRA can be associated with the development of ATRA resistance (Mills 2000).

Differentiation therapy applied to non APL AML blasts has shown that these cells can be induced to differentiate to several myeloid lineages. An example of differentiation therapy achieved in non APL AML patients was seen when 12-O-tetradecanoyl phorbol-13-acetate (TPA) was used to treat patients with myelocytic leukaemia. A large decrease in the number of bone marrow myeloblasts was observed and used alone or in combination with arabinoside (Ara C), complete remission could be achieved (Han *et al.* 1998). Also in non APL AML patients, ATRA and low doses of Ara C used in combination resulted in complete remission in 48% of patients as a result of the anti-leukaemic activity of the combination of ATRA and Ara C (Venditti *et al.* 1995). GM-CSF and IL-3 have both been used in clinical trials to recruit leukaemia progenitor cells into cycle, attempting to make them more sensitive to the cytotoxic effects of cycle specific agents including ara-C and topoisomerase II inhibitors although the sensitivity of patients to chemotherapy was equally increased and decreased following exposure to IL-3 (Gore *et al.* 1995). In different subsets of patients, treatment with identical cytokines will not achieve the same result as that seen in a different subset of the disease. The pathways of differentiation that are achieved are varied between patients (Bruserud *et al.* 2000). Regulation of

differentiation in AML blasts may involve several intracellular pathways and can be independent of the regulation of apoptosis (Bruserud *et al.* 2000).

Alterations in the response to apoptosis-inducing stimuli by the disruption of the apoptotic pathway or by altered DNA repair may account for resistance to chemotherapy and treatment failure. Quiescent cells and those cells with disrupted cell cycle checkpoints may also display decreased drug sensitivity. This has led to attempts to recruit cells into the cell cycle with haematopoietic growth factors to enhance their sensitivity to drug-induced apoptosis (Ketley *et al.* 2000). There are currently a large number of prototypes and second generation agents that are capable of inducing differentiation in myeloid and lymphoid cell lines. Many of these agents have been used in a clinical situation often in combination with other factors (Mills 2000). Briefly, these agents include ICRF-193 which is a potent catalytic inhibitor of DNA topoisomerase II which does not induce DNA strand breaks. This agent may act by trapping the enzyme in the form of a closed ATP-modulated protein clamp resulting in the prevention of the formation or stabilisation of cleavable complexes (Niitsu *et al.* 2002). This agent has reduced cytotoxicity which indicates it may be used for long periods of time and be suitable for chemo-differentiation therapy and has been used in conjunction with ATRA or retinoic acid receptor ligands to induce differentiation in NB4 cells (Niitsu *et al.* 2002). Recombinant tumour necrosis factor-related apoptosis inducing ligand (TRAIL) induces apoptosis in a number of transformed cell lines and in primary tumour cells while non-leukaemic cells are not affected (Plasilova *et al.* 2002). TRAIL may function as a negative regulator of normal erythroid differentiation in the later stages of erythropoiesis and is abundantly expressed on the surface of many cell types but its physiological role has not yet been determined (Plasilova *et al.* 2002). Additionally, myelo peptide 4 (MP-4) which is a bone marrow bioregulatory mediator, has been shown to be important in the control of

differentiation in normal and leukaemic myeloid cells and induces differentiation in both HL-60 and K-562 cells. A therapeutic use of MP-4 has been suggested in AML and myelodysplastic disorders (Strelkov *et al.* 2000). Another agent, Glivec, is discussed in section 1.3.5.

1.4 FUS/TLS

The FUS gene, also known as TLS (Translocated in Liposarcoma) is located in chromosome 16p11. This gene is expressed in many tissues and has been shown to be necessary for active transcription (Mills *et al.* 2000). FUS is composed of 15 exons in 11kb of genomic DNA and is a housekeeping gene (Aman *et al.* 1996; Morohoshi *et al.* 1998). FUS was first discovered as the N-terminal of the FUS-CHOP fusion protein seen in myxoid liposarcoma (Crozat *et al.* 1993; Rabbitts *et al.* 1993).

1.4.1 FUS function

FUS is capable of binding both DNA and RNA (Bertolotti *et al.* 1999), leading to the suggestion that it has multiple functions within the cell. The FUS protein is capable of shuttling between the nucleus and cytoplasm of a cell (Bertolotti *et al.* 1999), which is in keeping with proteins that bind mRNA species and have roles in their processing. Additionally, FUS shows some typical features of housekeeping genes by having binding sites for the transcription factors AP2, Sp1 and GCF in its promoter region (Aman *et al.* 1996).

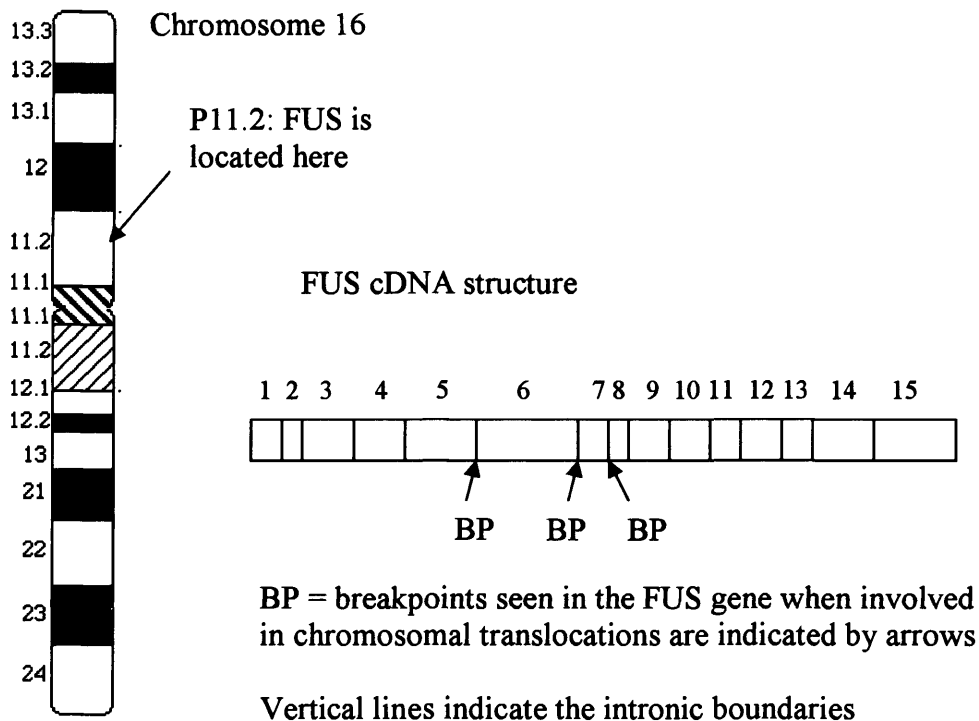
Evidence for the necessity for FUS expression in cells has been provided by the observations that FUS deficiency leads to a profound defect in spermatogenesis, a mild defect in somatic growth and an enhanced sensitivity to ionising radiation in FUS deficient mice (Kuroda *et al.* 2000). FUS is usually expressed at a high level in meiotic prophase I and FUS deficiency is associated with the absence of an activity in testicular extracts that promotes homologous pairing of DNA molecules *in vitro*.

Asynapsis of one pair of homologs in male animals leads to apoptosis of the affected spermatocyte and reduced fertility or even sterility (Kuroda *et al.* 2000). FUS has been shown to be essential for the viability of neonatal animals using homozygous knockout mice and to have an influence on lymphocyte development in a non cell-intrinsic manner as well as having an intrinsic role in the proliferative responses of B cells in specific mitogenic stimuli (Hicks *et al.* 2000). The defect in B-cell activation in FUS^{-/-} mice was similar to that shown in Abl^{-/-} mice. In the FUS^{-/-} mice 67% of the fibroblast cells examined had aneuploidy and other chromosomal abnormalities including chromosomal breakage, centromeric fusion and extra chromosomal elements. Similar events were also seen in primary B –lymphocytes. FUS was suggested to have a role in the Atm/Abl signal transduction pathway as a possible modulator or effector of gene expression in response to DNA damage and mitogenic stimuli (Hicks *et al.* 2000).

1.4.1a DNA binding

DNA strands breaks are caused by chemical damaging agents, ionising radiation and are specifically induced in meiosis. Repair of DNA double strand breaks is important for maintenance of the genomic integrity of the cell. Unrepaired, or aberrantly repaired breaks can lead to chromosomal rearrangements possibly leading in turn to tumours or cell death. Mammalian cells have two pathways of DNA repair, non-homologous end joining and homologous recombination (Baechtold *et al.* 1999). A role for FUS in the promotion of the homologous pairing of DNA has been suggested and this has been identified as having a relationship with cell proliferation (Mills *et al.* 2000).

Fig. 1.4 Diagram showing the location and structure of the FUS gene located on chromosome 16. Breakpoints seen in the FUS gene are in exon 5, the exon 6/7 boundary and in exon 8



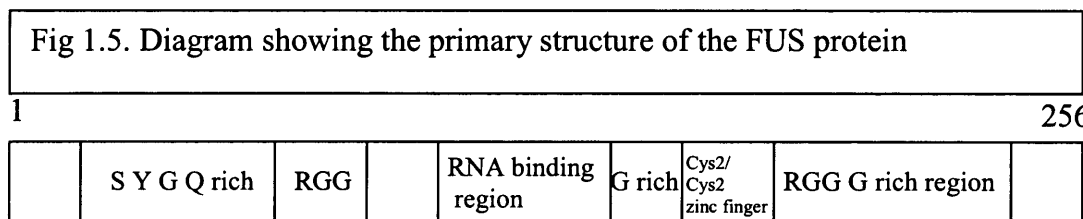
DNA homologous pairing is involved in homologous recombination which is implicated in the processes of chromosome pairing, gene inactivation, initiation of some replication processes and DNA repair (Bertrand *et al.* 1999). Incorporation of single stranded DNA into homologous duplex DNA leading to the formation of a D-loop is an essential feature of homologous recombination. FUS has a greater affinity for single stranded DNA than double stranded DNA and binds in an ATP independent manner although a divalent magnesium ion is required. D-loop formation represents the invasion of a single stranded end of one molecule into an intact homologous duplex DNA and this is an essential step in double strand breaks repair through recombination. Post-translational modification of FUS was suggested to modulate the activity of FUS in DNA homologous pairing after the ability of DNA homologous pairing to occur was measured and correlated to the level of FUS protein expressed in

HeLa cells (Bertrand *et al.* 1999). FUS itself is a target of the c-Abl protein and the phosphorylation of FUS by c-Abl is a pre-requisite for DNA homologous pairing and maybe one way in which the activity of FUS is regulated (Bertrand *et al.* 1999).

The ability of the FUS protein to bind single stranded and double stranded DNA and to promote the annealing of complementary single stranded DNA and assist the formation of D-loops in superhelical double stranded DNA suggests that it has a role in the maintenance of genomic integrity. The FUS-CHOP fusion protein is not capable of promoting DNA pairing and the loss of DNA repair in FUS-CHOP expressing cells may be one of the mechanisms by which transformation of the cell is achieved.

1.4.1b RNA processing

FUS binds non ribosomal RNA transcripts and RNA species that turn over rapidly in the cells. The RNA that is targeted by FUS contains a GGUG motif (Lerga A *et al.* 2001). This RNA is bound by FUS in the nucleus and remains bound by FUS in the cytoplasm, indicating that FUS is a heterogeneous ribonuclear protein-like chaperone of RNA (Yang *et al.* 1998).



S = serine, Y = tyrosine, G = glycine, Q = glutamine

There are three RNA binding domains in FUS and co-operation between these domains gives the protein its specificity of RNA recognition. Secondary structure is an important component of the RNA-FUS interaction and sequences as short as

GGAAG, (A/U)GGG or GGG can act respectively as either exonic or intronic splicing enhancers to improve splice site selectivity in the role FUS has in RNA splicing (Lerga A *et al.* 2001). The RNA recognition motif (RRM) of FUS is dispensable for binding RNA *in vivo*. It is this region that is the most conserved between FUS, EWS, CAZ and other members of the ETS (E26 Transformation Specific) family of RNA binding proteins and is implicated in other proteins in the sequence specific RNA binding (Zinszner *et al.* 1997b). The distinct RRM is surrounded by arginine-glycine-glycine (RGG) repeats which have also been implicated in RNA binding (Croizat *et al.* 1993; Hicks *et al.* 2000; Mills *et al.* 2000). The limited structural complexity of RGG regions found at the N-terminal side of the RNA-binding domain and at the C-terminal region suggests that they do not impart sequence specificity to RNA binding, instead serving as an auxiliary RNA-protein interaction domain (Zinszner *et al.* 1997b).

In addition to a serine-arginine-arginine rich area in the C-terminal RNA binding domain of the protein, FUS has a threonine rich area containing repeats of S-Y-S-G/Q sequences at the N-terminal region which may be important in the transactivation domain of the protein (Rabbitts *et al.* 1993), and an RNA binding domain of 90 amino acids at the central region with a highly conserved Cys₂/Cys₂-type zinc finger motif on the 3' side which is important for the protein-protein interactions of the FUS protein (Croizat *et al.* 1993; Morohoshi *et al.* 1998; Yang *et al.* 1998; Zinszner *et al.* 1997b). The hTAF_{II}68 and Ewings Sarcoma (EWS) proteins have extensive sequence similarity to FUS and the RNA binding domain is conserved between all three proteins which are all also capable of binding single stranded DNA (Bertolotti *et al.* 1999).

Heterologous nuclear ribonuclear proteins (hnRNPs) can bind a wide variety of RNA targets and are capable of binding targets that are not splicing substrates

(Calvio *et al.* 1995), additionally hnRNP's have a widespread diffuse nucleoplasmic distribution and may shuttle between the nucleus and cytoplasm in a complex with mRNA (Calvio *et al.* 1995; Zinszner *et al.* 1997a). The integrity of hnRNPs and a high level of expression are associated with an increasing proliferative capacity (Mills *et al.* 2000).

The FUS, EWS and hTAF_{II}68 proteins are part the TET (TAF_{II}68, EWS, TLS) family of proteins and are participants in the transcriptional regulation of class II (protein encoding) genes. Transcription involves several processes including promoter recognition and binding, pre-initiation complex (PIC) assembly, RNA chain initiation, RNA chain elongation and termination (Bertolotti *et al.* 1996). The FUS, EWS and hTAF_{II}68 proteins associate with the TFIID multi-protein complex which is composed of the TATA binding protein (TBP) and TBP associated factors (TAF_{II}s) (Bertolotti *et al.* 1999; Powers *et al.* 1998). In a protein coding gene, the recruitment of this complex through the binding of the TBP to the promoter in a sequence specific manner is the first step of PIC assembly (Bertolotti *et al.* 1996; Bertolotti *et al.* 1999). RNA polymerase II transcribes protein-encoding genes into mRNA. Following transcription, primary transcripts are further processed by capping, splicing, cleavage and polyadenylation of the pre m-RNA into mRNA (Bentley 1999).

During transcriptional activation, activators are thought to stimulate and/or facilitate the formation of the PIC. Activation domains of proteins can contact components of the basal transcriptional machinery directly or indirectly. These interactions serve to recruit, stabilise and induce conformational changes in the PIC. Transcriptional activation by an activator also involves modification of the chromatin. This is done by recruiting chromatin remodelling complexes to promoters which acts to de-compact the chromatin around the genes to be transcribed (Bertolotti *et al.* 1999). Activation domains can interact with histone acetyl transferases (HAT's), an

interaction which serves to open the chromatin structure around the promoter and facilitate the recruitment of the PIC to the initiation site (Bertolotti *et al.* 1999).

Artificial recruitment of one of the various components of the basal transcription machinery or HAT is sufficient to stimulate transcription (Bertolotti *et al.* 1999).

When RNA polymerase II nears the 3' end of the gene, several independent events occur. These events are the splicing of the terminal intron, cleavage at the poly adenosine site, addition of the poly adenosine tail and termination or release of the polymerase from the DNA (Bentley 1999). The carboxy terminal domain (CTD) of the RNA polymerase II large subunit is required for efficient RNA processing. The effect of the CTD on splicing may be due to recruitment of splicing factors to the pre-mRNA via protein-protein interactions with the CTD (McCracken *et al.* 1997). The presence or absence of activators of different types at initiation events results in the assembly of processive and non-processive transcription elongation processes.

Different processes can be regulated during the elongation reaction including transcriptional processing, transcript cleavage and reactivation and post-translational modifications of polymerase II (Bertolotti *et al.* 1996). Basal polymerase II transcription machinery may participate directly in the recruitment and assembly of splicing factors on the nascent pre-mRNAs and nascent pre-mRNA may contain all the information required to recruit the splicing factors required to process the transcript (Du *et al.* 1997). A phosphorylated CTD helps recruit serine-arginine (SR) splicing factors to nascent polymerase II transcripts and *in vivo* spliceosome assembly takes place progressively on pre-mRNA as it emerges from the polysome (Calvio *et al.* 1995; Du *et al.* 1997). FUS may have a role in coupling transcription and mRNA processing by entering the PIC with TF_{II}D and later associating with elongating RNA polymerase II (Bertolotti *et al.* 1999; Powers *et al.* 1998). The CTD of polymerase II has been shown to be essential for cell growth. The CTD binds DNA in a non-specific

manner that may function to remove inhibitory proteins from the promoter and may also interact with the TBP (Yuryev *et al.* 1996). FUS is associated with human nuclear RNP A1 and SF1 proteins which have splicing functions and these associations help to link FUS function to nascent pre-mRNA (Yang *et al.* 1998).

FUS shows an influence on the 5' splice site among the alternative splicing of the adenovirus E1A pre-mRNA which is governed by Spi-1. Spi-1 is an ETS protein, like FUS, which has a central role in the differentiation of macrophages and B-cells in normal haematopoiesis. The alternative selection of the pre-mRNA splicing sites may be changed by Spi-1 and this protein may modulate splicing of some pre-mRNA (Hallier *et al.* 1998).

Compartmentalisation is known to have a key role in the regulation of cellular processes (Fuchsova *et al.* 2002). When RNA polymerase II transcription or splicing is inhibited, splicing factors and small nuclear ribonuclear proteins (snRNPs) relocate from the perichromatin fibrils and coiled bodies to the interchromatin granules which act as a repository for storage and/or assembly of splicing factors. Recruitment of splicing factors from the interchromatin granules to the active site of transcription is blocked when transcription/splicing is inhibited (Thomas *et al.* 2001).

1.4.1c FUS interactions

FUS is a high affinity binding protein for the DNA binding domains of retinoid, steroid and thyroid hormone receptors and binds to them with its N-terminal domains in a ligand independent manner (Powers *et al.* 1998). This binding of FUS to the nuclear receptor may serve as a mechanism by which the transcription complexes are recruited and stabilized at the target gene although the mechanism is not yet clear (Powers *et al.* 1998). Nuclear receptor binding by FUS may serve as a primary mechanism to recruit FUS to the TFIID complex or to accelerate the subsequent processing of primary transcripts generated in response to hormone-induced

transcription (Powers *et al.* 1998). The N-terminal of FUS is a potent transcriptional activation domain when placed in the proximity of a promoter (Zinszner *et al.* 1997a; Zinszner *et al.* 1997b). The nuclear receptors for steroid hormones, vitamin D3 and retinoids mediate ligand-dependent transcriptional regulation of target genes with DNA sequences that enable receptor binding (Powers *et al.* 1998). FUS does not affect the DNA binding ability of the receptor proteins to which it binds. Structural conservation of the DNA binding domain of nuclear receptors may allow an interaction between the members of the receptor family which are diverse and a common binding protein, like FUS (Powers *et al.* 1998). Co-repressors may suppress the activity of the receptor-FUS complex and ligand-evoked dissociation of co-repressors from the ligand binding domain may relieve this repression (Powers *et al.* 1998).

FUS acts as a co-activator of nuclear factor- κ B (NF- κ B) and plays a pivotal role in the NF- κ B-mediated transactivation by acting as a co-activator of transcription when recruited to the vicinity of the promoter. NF- κ B is an inducible transcription factor which regulates a wide variety of cellular and viral genes including cytokines, cell adhesion molecules (Uranishi *et al.* 2001) as well as being a key modulator of cell survival (Saunders *et al.* 2001). Activation of NF- κ B requires multiple co-activator proteins including CREB-binding protein (CBP), CBP associated factor, and steroid receptor co-activator 1 (Uranishi *et al.* 2001). Coactivators have HAT activity and so open the chromatin and bridge to the basal transcription machinery. Retinoid receptors' interactions with NF- κ B have been shown to be involved in the inhibition of cancer cell proliferation (Na *et al.* 1999) Retinoid receptors form a transcriptionally inhibitory complex with NF- κ B. With NF- κ B transactivation, the retinoid-mediated inhibiting action appears to involved inhibition of NF- κ B-DNA interactions as well as competitive recruitment of transcription factors and co-regulators between NF- κ B and

RXR (Na *et al.* 1999). NF- κ B is usually bound to the I κ B protein in an inactive form in the cytoplasm. Treatment of cells with various inducers leads to the degradation of the I κ B proteins which releases the NF- κ B protein to translocate to the nucleus where it can activate target genes or form a transcriptionally inhibitory complex with the retinoid receptors (Na *et al.* 1999).

Other transcription factors known to interact with FUS are Spi-1/PU.1 where the association between Spi-1 and FUS impedes the translational functions of Spi-1 (Hallier *et al.* 1998; Yang *et al.* 1998). Additionally, FUS may function in the Atm/Abl signal transduction pathway, as a modulator or an effector of gene expression in response to DNA damage and mitogenic stimuli (Hicks *et al.* 2000).

1.4.2 FUS and differentiation

Cell differentiation is controlled at the gene transcription level and the level of regulation of nuclear transcription factor activity. Aberrant expression of transcription factors probably interferes with differentiation events and has a role in the pathogenesis of AML by super-activation or repression of genes regulating proliferation and differentiation or by the interference with the activity of transcription complexes for these genes (Lerga A *et al.* 2001).

The level of FUS expressed in the cells was down-regulated in the HL-60 cell line when the cells were induced to undergo granulocytic differentiation using either DMSO or ATRA. In contrast, the level of FUS expression remained fairly constant under induction towards the monocytic lineages with TPA or vitamin D₃ (Mills *et al.* 2000). ATRA resistant HL-60 cells did not show down-regulation of FUS in response to ATRA or DMSO. The cell lines that showed a limited or no response to ATRA had a higher level of FUS expression in the untreated state than the cell lines that did show a differentiation response to the treatment with ATRA. The speed at which FUS was

down-regulated indicates that it is an early response gene (Mills *et al.* 2000). The immediate cascade of gene expression after exposure to ATRA is important in initiating differentiation towards a mature phenotype. Leukaemia classifications with a more mature FAB classification (M6, M7) have a lower average expression of FUS than the immature and less committed FAB groups suggesting that FUS may be developmentally regulated (Mills *et al.* 2000).

1.4.2a FUS and BCR/ABL

The BCR/ABL protein formed as a result of the t(9:22) translocation, discussed in section 1.3.5c, requires tyrosine kinase activity for the recruitment and activation of multiple biochemical pathways to transform haematopoietic cells. FUS was detected in a complex with DNA in cells with BCR/ABL functional tyrosine kinase activity. The formation of the DNA-FUS complex was regulated by the tyrosine kinase activity of the p210 BCR/ABL oncoprotein (Perrotti *et al.* 1998). FUS activity was found to be important for the growth factor independence and reduced propensity for differentiation of BCR/ABL transformed 32D cells and the protein was readily detectable in IL-3 starved 32D cells expressing BCR/ABL. In the parental 32D cells, endogenous FUS expression was only detected when the cells were maintained in the presence of IL-3 suggesting that BCR/ABL expression circumvents the requirements for signals generated in non-transformed cells by IL-3 deprivation (Perrotti *et al.* 1998). It was also shown that FUS was phosphorylated in BCR/ABL expressing cells but not in the parental cells.

1.4.2b Degradation of FUS

The PKC family of proteins are a family of serine threonine protein kinases which are involved in the signal transduction of growth factors hormones and neurotransmitters within the cell (Krasagakis *et al.* 2002; Pinton *et al.* 2002). The

PKC pathway is involved in the regulation of FUS's binding activity and the FUS protein has multiple putative PKC phosphorylation sites on its C-terminal region. Phosphorylation of FUS at serine 256 prevents its association with c-Jun and formation of a multiprotein complex which involves c-Jun and hnRNP A1 and appears to be necessary for proteasome dependent degradation of FUS which undergoes proteasome dependent degradation without prior ubiquitination (Finn *et al.* 1990; Perrotti *et al.* 2000). Phosphorylation of FUS by PKC β II prevents the proteasome dependent proteolysis. Cells containing a mutated FUS gene where the phosphorylation site is replaced by a phosphomimetic aspartic acid show a reduced susceptibility to degradation of FUS induced by IL-3 deprivation (Perrotti *et al.* 2000).

1.4.2c Interaction of FUS with G-CSF-R

FUS may bind G-CSF-R pre-mRNA in the nucleus and interfere with its processing or export to the cytoplasm (Perrotti *et al.* 2002a). When treated with G-CSF, the level of FUS expression in 32D cells and transformed 32D cells expressing a full length FUS gene, was rapidly down-regulated. The level of FUS expression in 32D cells expressing BCR/ABL was not down-regulated upon exposure to G-CSF and these cells showed no differentiative response to G-CSF (Perrotti *et al.* 1998). 32D cells and 32D cells expressing full length FUS did differentiate in response to G-CSF stimulation while 32D cells with reduced FUS levels using an antisense construct differentiated more slowly than the parental 32D cells. 32D cells expressing both BCR/ABL and an antisense FUS construct showed an increased ability to differentiate although 32D cells expressing only the BCR/ABL transcript are resistant to differentiation induced by G-CSF (Perrotti *et al.* 1998). In BCR/ABL expressing cells, the failure to down-modulate FUS might be one mechanism by which G-CSF induced

differentiation is prevented. FUS expression is controlled in part by proteasome-mediated degradation regulated by PKC β II-dependent phosphorylation, c-Jun expression and possibly hnRNP A1 ubiquitination. Execution of these processes appears to be important for granulocytic differentiation while its suppression by BCR/ABL expression might be important in the development of BCR/ABL dependent leukaemia (Perrotti *et al.* 2000). Additionally BCR/ABL expression has been shown to lead to the up-regulation of FUS, hnRNP A1 and hnRNP E2 through post-transcriptional mechanisms leading to increased protein stability (Perrotti *et al.* 2002a).

1.4.3 Fusion partners

The ability of FUS to bind DNA and its putative role in maintaining genomic stability by the repair of DNA damage has led to the suggestion that the loss of these functions as potential mechanisms that may contribute to the oncogenic potential of FUS when the gene is translocated in human cancers (Hicks *et al.* 2000; Lerga A *et al.* 2001). Translocations involving FUS have breakpoints in exons 5, 7 and 8 of FUS (Morohoshi *et al.* 1998). Exons 1 to 5 are always in tumour associated fusion proteins involving FUS (Aman *et al.* 1996). FUS forms fusion proteins with the ERG and CHOP genes.

1.4.3a FUS-ERG

The effect of the FUS-ERG fusion generated by the t(16;21) translocation are discussed in section 1.3.5d.

1.4.3b FUS-CHOP

The t(12;16)(q13;p11) translocation results in myxoid liposarcoma and produced the fusion protein FUS-CHOP. The CHOP protein is a leucine zipper that

lacks the basic DNA binding region and the presence of the C-terminal of CHOP in the fusion protein allows hetero-dimerisation of the fusion protein with C/EBP (Rabbitts *et al.* 1993). The FUS-CHOP fusion protein is highly expressed in transformed cells whereas the CHOP protein itself is not abundantly expressed and is induced in response to stress in normal cells (Rabbitts *et al.* 1993; Thelin-Jarnum *et al.* 2002; Zinszner *et al.* 1994). The small nuclear protein CHOP is usually a dominant negative regulator of transcription but becomes a transcription activator in the fusion protein when the growth arresting properties of CHOP are removed (Ron 1997). Splicing inhibition is seen when the fusion protein is expressed in cells as the FUS domain of the fusion protein is no longer capable of recruiting splicing factors to itself in its normal manner (Rapp *et al.* 2002). The fusion protein remains capable of binding RNA and may result in the recruitment of splicing factors to CHOP target gene (Ron 1997). Different types of FUS-CHOP fusion genes have been described in which the breakpoints are different and the amount of FUS and CHOP in the fusion genes is altered although not all of these translocations result in an in-frame protein (Kanoë *et al.* 1999; Panagopoulos *et al.* 1995). Generally the portion of FUS in the FUS-CHOP and FUS-ERG fusion proteins is very similar (Perez-Losada *et al.* 2000b). The FUS domain of the fusion protein is required for transformation and the expression of the FUS-CHOP fusion protein in the cell is sufficient to produce most of the symptoms of myxoid liposarcoma without additional genetic alterations in mice (Perez-Losada *et al.* 2000a).

1.5 Summary

To summarise, FUS is a protein with the ability to bind both RNA and DNA and has suggested roles in transcriptional regulation, RNA processing and in the maintenance of genomic integrity through its ability to promote homologous pairing of DNA in the process of DNA repair following double strand breakage. The down-

regulation of FUS has been shown to enable the differentiation block of BCR/ABL expressing 32D cells to be overcome. FUS is involved in chromosomal translocations leading to the disruption of the transcription, post-transcriptional regulation of FUS targets and the altered transcriptional activation of the fusion partners of FUS in the translocations.

1.6 Project Aims

The aims of this project were to

- To assess the effect of the expression of the FUS gene in the sense and antisense orientations during differentiation induced using ATRA and G-CSF in myeloid cell lines.
- Investigate the effect of over-expressing or down-regulating FUS in myeloid cell lines with additional genetic abnormalities
- Investigate the effect of down-regulating FUS expression by antisense, and RNA interference, in myeloid cell lines with additional genetic abnormalities
- To investigate the effect of the over-expression of FUS and the down-regulation of FUS in terms of differential gene expression using gene arrays

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Composition of solutions

The compositions of all solutions that were used are given here except where the solutions were supplied with kits and solution composition was not supplied by the manufacturer.

TE buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA

Klenow reaction mix: 5mM Tris-HCl pH 7.2, 100mM MgSO₄, 1mM DTT

SOC medium: 2% tryptone, 0.5% yeast extract, 0.01M NaCl, 2.5mM KCl,

0.01M MgCl₂, 0.02M sterile glucose

LB broth: 1% bactotryptone, 0.5% yeast extract, 0.1M NaCl pH 7.5

LB agar: LB broth with 1.5% bactoagar.

Colony lift denaturing solution: 1.5M NaCl, 0.5M NaOH

Colony lift Neutralising solution: 1.5M NaCl, 0.5M Tris-HCl pH 7.4

STE (1 x): 100mM NaCl, 20mM Tris-HCl pH 7.5, 10mM EDTA

Sodium carbonate buffer for reconstitution of retronectin: 0.015M Na₂CO₃,

0.035M NaHCO₃, pH 9.6

MOPS (1 x) for Northern gels: 200mM MOPS, 0.08M Na-trihydrate. 0.01M EDTA,

0.02% formaldehyde, 0.002% 1mg/ml ethidium bromide

RNA loading buffer: 500μl formamine (undiluted), 166.4μl formaldehyde (40%),

100μl 10 x MOPS, 233.6μl DEPC treated water

RNA loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 0.2mM EDTA

pH 8.0

SSC (10 x): 1.5M NaCl, 0.15M Tri-NA-citrate, pH 7.0

RT buffer: 1.42mM dATP, 1.42mM dCTP, 1.42 dGTP, 1.42mM dTTP,

7.1mM MgCl₂, 1.4 x Buffer II

Cell lysis buffer: 50mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate,
150mM NaCl, 1mM PMSF, 1µg/ml Aprotinin, 1µg/ml leupeptin,
1µg/ml pepstatin, 1mM Na₃VO₄, 1mM NaF

Annexin V binding buffer: 10mM HEPES/NaOH, pH 7.4, 140mM NaCl,
2.5mM CaCl₂

cDNA Array probe denaturing solution: 1M NaOH, 10mM EDTA

cDNA Array probe neutralising solution: 1M NaH₂PO₄ pH 7.0

2.1.2 Primer sequences

PINCO SENSE	5'	TAGAACCTCGCTGGAAAGGA	3'
PINCO3	5'	GAACCTAGAACCTCGCTGGA	3'
PFUSRIGHT	5'	TATCCCTGGGGAGTTGACTG	3'
PASRIGHT	5'	GTGGTGGGGACAGAGGTG	3'
BCL2 LEFT	5'	AGGAGCAGGTGCCTACAAGA	3'
BCL2 RIGHT	5'	GCATTTTCCCACCACTGTCT	3'
SLAP LEFT	5'	GAAAACCCACAGGGCACTAA	3'
SLAP RIGHT	5'	GCTGAGCCCAGGTATAGGA	3'
IKB LEFT	5'	CTGTTCTAGGGCTGCTTTGG	3'
IKB RIGHT	5'	AAGGCCTGGGTTCAATTTCT	3'
IL3R LEFT	5'	CCACTCTCTGCCTGATCTCC	3'
IL3R RIGHT	5'	CCCACACTGCACATCCATAG	3'
IL15 LEFT	5'	TTGCAGTGCATCTCCTTACG	3'
IL15 RIGHT	5'	GTGCTTTGAAGAGCCAGAGG	3'
TASR LEFT	5'	CACGTCTCTGTTTCGTCAGGA	3'
TASR RIGHT	5'	CTGTGCGAACTGGATTTCAA	3'
CP450 LEFT	5'	GGCAACAGTTCTGGGTGAAT	3'

CP450 RIGHT	5'	TATTTGAGGACAATCCAACA	3'
H3H LEFT	5'	CACGAAGCAAACAGCTCGTA	3'
H3H RIGHT	5'	GTACCAGACGCTGGAATGGT	3'
EGF LEFT	5'	TGGGTGTGGTCGTTGAACAGT	3'
EGF RIGHT	5'	CTCACCTACCCGCTTCTCAG	3'
TARC LEFT	5'	AGTGGAGTGTTCCAGGGATG	3'
TARC RIGHT	5'	GTCACAGGCCGCTTTATGTT	3'
RAR LEFT	5'	CAAGAGGAGCACCCAGACCTC	3'
RAR RIGHT	5'	ACTCACTTGGAGGAGCCAGA	3'
HIP LEFT	5'	ACCCCAAAGCCATTTTAACC	3'
HIP RIGHT	5'	GTTATTGATGGCAGCCCAAT	3'
PAF LEFT	5'	TGTTTCCACTGGGTGATGAA	3'
PAF RIGHT	5'	TGCCAGTTGCAAAGTGAAG	3'
SP1 LEFT	5'	ACATGCAGTTCAGCAACTAG	3'
SP1 RIGHT	5'	CTGCCACTTTCAGGTCCATT	3'

T7 primer 5'- GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG
AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3'

Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were supplied by Oswell, Southampton, Hampshire, UK.

The T7 primer for Affymetrix was produced by MWG Biotech (UK) Ltd, Milton Keynes, UK for human samples and Genset Oligos, Proligo, Paris, France for murine samples.

2.1.3 Suppliers

All chemicals used were supplied by Sigma Aldrich UK Ltd, (Dorset, UK), Fisher Scientific UK Ltd, (Loughborough, Leicestershire, UK), Invitrogen Ltd,

(Paisley, UK), Promega, (Southampton, Hampshire, UK), BD UK Ltd, (Cowley, Oxfordshire, UK), Ambion Europe Ltd, (Huntington, Cambridgeshire, UK), BioWhittaker UK Ltd, (Wokingham, Berkshire UK), R and D Systems Europe Ltd, (Abingdon, Oxfordshire, UK) and Perbio Science UK Ltd, (Tattenhall, Cheshire, UK).

Sterile glucose, sterile water was supplied by the in-house pharmacy.

2.1.4 Radionucleotides

Radionucleotides [^{32}P]dCTP and [$\alpha^{32}\text{P}$]dATP were supplied by Amersham Pharmacia Biosciences Ltd, Little Chalfont, Buckinghamshire, UK.

2.1.5 Enzymes and PCR reagents

Enzymes were supplied with their appropriate buffers by Promega, (Southampton, Hampshire, UK), Northumbria Biologicals Ltd, (Cramlington, Northumberland, UK), Roche Diagnostics Ltd, (Lewes, East Sussex, UK), New England Biolabs (UK) Ltd, (Hitchin, Hertfordshire, UK) and Invitrogen Ltd, (Paisley, UK).

Dinucleotriphosphates (dNTPs), DNA polymerase, RNase inhibitor, MuMLV reverse transcriptase, MgCl_2 and buffers for PCR were supplied by Applied Biosystems, Roche Diagnostics Ltd, Lewes, East Sussex, UK.

DNA markers for electrophoresis were supplied by Promega, Southampton, Hampshire, UK.

2.1.6 Commercial kits

Commercially available kits used were:

QIAquick spin kit for gel extraction, QIAquick spin kit for PCR purification, QIAprep miniprep kit for purification of plasmid DNA, DyeEx spin kit, RNeasy mini kit, DNase I treatment kit, all supplied by Qiagen Ltd, Dorking, Surrey, UK

One Shot Top 10 Chemically Competent cells, XCell electrophoresis tank and XCell blot II module used with the NOVEX Western blotting system, reagents supplied by Invitrogen, MultiMark and MagicMark protein standards, WesternBreeze NOVEX Chemiluminescent Western Blot Immunodetection kit, Affymetrix reagents (5 x 1st strand buffer, 0.1M DTT, 10mM dNTP mix, Superscript II reverse transcriptase, 5 x 2nd strand buffer, *E. coli* DNA ligase, DNA polymerase I, RNase H, T4 DNA polymerase, glycogen), Herring sperm DNA, all supplied by Invitrogen Ltd, Paisley, UK.

Rediprime II random prime labelling system, Hybond N membrane, Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK

Pall Biotrade A transfer membrane, Pall Europe Ltd, Portsmouth, Hampshire, UK

Calcium Phosphate transfection kit, Sigma Aldrich UK Ltd, Dorset, UK

Strip-EZ DNA kit, Silencer siRNA labelling kit, Silencer siRNA transfection kit, Ultrahyb hybridisation fluid, Ambion Europe Ltd, Huntingdon, Cambridgeshire, UK

ABI Prism Dye Terminator Ready Reaction Kit, Applied Biosystems, Warrington, UK

Mouse cDNA Expression Arrays, Atlas Image software, Chromaspin STE 100 columns, BD UK Ltd, Cowley, Oxfordshire, UK

NucTrap probe purification columns, Push column Beta shield device, Stratagene Europe, Amsterdam Zuidoost, Netherlands:

Enzo Bioarray high yield IVT kit including HY buffer, 10 x biotin-labelled ribonucleotides, 10 x DTT, RNase inhibitor mix, T7 RNA polymerase, Affymetrix UK Ltd, High Wycombe, UK.

2.1.7 Antibodies

CD11b, CD33 and CD38 mouse anti-human antibodies and isotype-matched controls for flow cytometry were supplied by DAKO Cytomation Ltd, Ely, Cambridgeshire, UK, Ly-6G rat anti-mouse antibody was supplied by BD UK Ltd, Cowley, Oxfordshire, UK. CD11b and F4/80 rat anti mouse and isotype matched control Serotec Ltd, Kidlington, Oxford, UK

Annexin V antibody was supplied by R&D systems Europe Ltd, Abingdon, Oxfordshire, UK.

The Anti-TLS antibody used for Western blotting was supplied by BD UK Ltd.

2.1.8 siRNA

siRNA was designed, produced and supplied by Eurogentec s.a., Seraing Belgium.

2.1.9 Laboratory equipment

All additional laboratory and tissue culture consumables were supplied by Thermo Life Sciences (Basingstoke, Hampshire, UK), Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK), Abgene (Epsom, Surrey, UK), Sigma Aldrich

UK Ltd (Dorset, UK), Fahrenheit Laboratory Supplies (Milton Keynes, UK),

Bioquote Ltd, (York, UK) and BOC Gases (Guildford, Surrey, UK).

2.1.10 Cell lines

The cells lines commonly used in this study are summarised in table 2.1

Table 2.1 Cell line commonly used and their culture conditions. CM = conditioned medium

Cell line	Cell type	Known translocations	Source	Culture conditions
HL-60	Human	None	Dr Chris Bunce, Dept Medicine, University of Birmingham, UK	RPMI 1640, 10% FBS, 1% Pen/strep 37°C, 5% CO ₂
NB4	Human promyelocytic	t(15;17)	Beatson Institute for Cancer Research, Glasgow, UK	RPMI 1640, 10% FBS, 37°C, 5% CO ₂
NB4R2	Human promyelocytic	t(15;17)	Dr. Michel Lanotte, Centre G. Hayem, Hopital Saint-Louis, Paris, France	RPMI 1640, 10% FBS, 37°C, 5% CO ₂
32D	Murine myeloid	None	Beatson Institute for Cancer Research, Glasgow, UK	RPMI 1640 10% Hyclone II, 10% Wehi-3B conditioned medium 37°C, 5% CO ₂
32D B ₂ A ₂	Murine myeloid	t(9;22)	Developed in-house. (Guinn <i>et al.</i> 2000)	RPMI 1640, 10% FBS, 1% Pen/strep 37°C, 5% CO ₂
32D AML-ETO	Murine myeloid	t(8;21)	Dr. Eldad Dann, Ram Bam Medical Centre, Heifa, Israel	RPMI 1640 10% Hyclone II, 10% Wehi-3B CM 37°C, 5% CO ₂
ΦNX-Ampho	Retrovirus producing cell line	None	Asst Prof Garry Nolan, Dept Molecular Biology, Stanford University Medical School, Stanford, CA, USA	DMEM, 10% FBS, 1% Pen/Strep
Wehi-3B	Murine myelomonocytic		Dr. Brian Thomas NIMR, London	RPMI 1640, 10% FBS, 1% Pen/strep 37°C, 5% CO ₂

2.2 Vector construction

2.2.1 Excision of FUS from pBluescript

The FUS gene (bases 71-1822 of the published sequence) was supplied in pBluescript by Dr. Danilo Perrotti spotted on 3MM paper in 10 μ g aliquots. This DNA was eluted in 100 μ l of TE. The FUS gene was excised from the pBluescript plasmid by digestion with the enzymes *Spe* I and *Hind* III. The conditions for the restriction reaction were:

15 μ g pBluescript DNA (150 μ l)

15 μ l *Spe* I 10U/ μ l

15 μ l *Hind* III

20 μ l 10 x Buffer B

The reaction was incubated at 37°C overnight. The restriction fragments were separated by gel electrophoresis in a 0.8% agarose gel stained with ethidium bromide and visualised under ultraviolet light (uv) using a transilluminator. The sizes of the restriction fragments were determined by running DNA markers containing fragments of known size in the gel alongside the samples. These markers were pGEM digested with *Hin*FI, *RS*AI and *Sin*1, and 1Kb DNA ladder. These DNA markers were diluted to a concentration of 0.1 μ g/ μ l with water and 10 μ l were loaded per lane of gel. The fragment of gel containing the 1822 base pair FUS gene was excised from the gel with a disposable scalpel and the FUS DNA was extracted from the gel using the Qiagen Gel Extraction kit. This was done according to the manufacturer's instructions and involved placing the gel slice containing the DNA to be extracted into a previously weighed microcentrifuge tube, weighing the tube again to determine the weight of the gel. Three (3) volumes of buffer QG to 1 volume of gel were added to the tube (300 μ l of buffer to every 100mg of gel) and the mixture heated to 50°C for 10 min in a water

bath. The mixture was vortexed every 2-3 min of the incubation to aid the gel dissolving. One gel volume of isopropanol was added to the tube and mixed. A QIAquick spin column was placed in a 2ml collection tube, the dissolved gel mixture added and centrifuged for 1 min at 7,550 x g. The flow-through was discarded. 0.5ml buffer QG was added to the column and centrifuged for 1 min. A wash of 0.75ml buffer PE was added to the tube and centrifuged for 1 min. The flow-through was discarded and the column centrifuged for 1 min, before being placed into a clean 1.5ml microcentrifuge tube. The DNA was eluted in water by adding 30 μ l to the centre of the membrane at the base of the column and centrifuging for 1 min then repeating this elution step. Buffers supplied with the kit were QG, PE and water for elution.

Following the restriction digest of the FUS gene from pBluescript the resulting DNA fragment had an overhanging 5' end that was filled by incubating the fragment with a Klenow fragment in the following reaction:

2.7 μ g FUS DNA

4 μ l 10 x Klenow reaction mix

0.8 μ l dNTP mix (2mM each dNTP)

0.8 μ l acetylated Bovine serum albumin 1 μ g/ μ l

1 μ l DNA Polymerase I (Klenow) fragment 5U/ μ l

water to 40 μ l

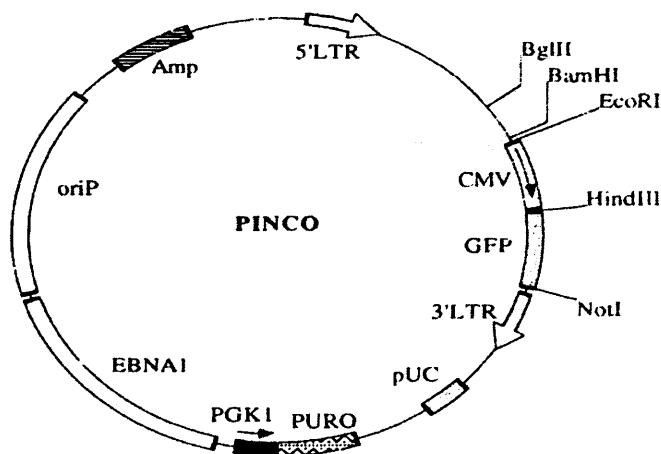
The reaction was incubated at room temperature for 10 mins, 75°C for 10 mins and cleaned to remove the unincorporated nucleotides using the QIAquick Spin kit for PCR purification from Qiagen. The protocol for the removal of unincorporated nucleotides using the kit was to add 5 volumes of buffer PB to 1 volume of the Klenow reaction and to mix the resulting mixture. The DNA was bound to a QIAquick column by placing the mixture in the column in a collection tube and

centrifuging at 10,000 x g for 1 min. The flow through was discarded and the column washed with 0.75ml buffer PE and centrifuging for 1 min at 10,000 x g. The flow through was discarded and the column centrifuged for an additional min at 10,000 x g. The column was placed in a new collection tube and the DNA eluted by the addition of 50µl water and centrifugation for 1 min at 10,000 x g for 1 min.

2.2.2 Ligation into PINCO plasmid

The PINCO plasmid (Grignani *et al.* 1998) contains the gene for the green fluorescent protein which is expressed in the cells containing the plasmid and enables the cells containing the PINCO plasmid to be detected and selected by flow cytometry.

Fig. 2.1 Diagram of PINCO plasmid showing some of the restriction sites (Grignani *et al.* 1998).



The blunt ended FUS gene was ligated into the PINCO plasmid in the sense and antisense orientations. 5µg of PINCO plasmid was prepared for ligation by digestion with 50U *Bam*HI enzyme (20U/µl) at 37°C in a 50µl reaction for 1h. 15U

DNA polymerase I (Klenow) fragment were added with 50 μ M dNTP mix (2mM each dNTP). The mixture was incubated for 30 min at 30°C then 75°C for 10 min followed by slow cooling in a DNA thermal cycler. The reaction was cleaned and the enzyme removed by using the Qiagen QIAquick Spin kit for PCR purification as described in section 2.2.1.

The blunt-ended FUS gene was ligated into the blunt ended PINCO plasmid by mixing 140ng of the FUS gene (1.8kb) with 390ng of the PINCO plasmid (13kb) in a total of 4 μ l of water. A mixture of 8.0 μ l water, 2.0 μ l 10 x ligase buffer and 6.0 μ l T4 DNA ligase (60U) enzyme was added to the DNA mixture and mixed gently in a total volume of 20 μ l. The reaction was incubated for 30 min at 16°C then used to transform competent cells.

2.2.3 Transformation of competent cells

One Shot TOP10 Chemically Competent Cells were transformed with the above ligation. This was carried out as according to the manufacturer's instruction and briefly involved centrifuging the vial containing the ligation reaction and placing on ice. One 50 μ l vial of competent cells was thawed. 5 μ l of ligation reaction was pipetted directly onto the cells and mixed by tapping gently. Remaining ligation mixture was stored at -20°C. The reaction was incubated on ice for 30 min, then for exactly 30 sec in a water bath pre-warmed to 42 °C and returned to ice. 250 μ l of SOC medium was added to the vial of cells and ligation mixture using sterile technique in a class 2 microbiological safety cabinet. The vial was then sealed, placed on its side, secured and shaken at 37°C for 1h at 225rpm in an orbital incubator. 200 μ l of the shaken mixture was spread on LB agar in a plate which was inverted and incubated overnight at 37°C. The remaining transformation mixture was stored at +4°C.

2.2.4 Colony lifts

Colonies of transformed chemically competent cells were selected by colony lifts.

2.2.4a Colony lift procedure

Colonies from the LB agar plate that the competent cells were plated out on were replicated onto a nylon filter as follows. Four pieces of 3MM Whatman paper were soaked in one of the following solutions 1) 10% SDS, 2) denaturing solution (1.5M NaCl, 0.5M NaOH), 3) Neutralising solution (1.5M NaCl, 0.5M Tris-Cl pH 7.4) and 4) 2 x SSC in separate trays. Excess liquid was poured from the paper. Pall Biodyne A transfer membrane 1.2µM cut to the size of the agar plate was labelled with a biro and placed label side down on the surface of the agar. The orientation of the membrane was marked using a flamed 18G needle in three locations. The membrane was removed from the agar with forceps and laid colony side up in 10% sodium dodecyl sulphate (SDS) for 3 min (tray 1), before being transferred to tray 2 containing denaturing solution for 5 min. The membrane was transferred to tray 3 for 5 min in neutralising solution for 5 min, then to tray 4 for 5 min soak in 2 x SSC. When transferring the membrane between the trays, the side of the tray was used to remove any excess fluid from the membrane prior to the next treatment. After the four treatments, the membrane was air dried for 30 min lying colony side up on a dry sheet of 3MM paper. Once dry, the DNA was fixed to the membrane by baking the filter at 80 °C for 2h in a Heraeus T6030 oven.

2.2.4b Probe labelling procedure

A ³²P labelled probe for the FUS gene was produced using the Rediprime II random prime labelling system. FUS DNA from the restriction digest of FUS in pBluescript was diluted to 25ng in 45µl of 10mM TE at pH8, 1mM EDTA and heated

to 100 °C in a water bath for 5 min to denature the DNA. The mixture was then rapidly cooled by placing on ice for 5 min, and added to the reaction tube containing buffered dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primer in a dried stabilised form was centrifuged to collect it at the bottom of the tube. 5µl of Redivue [³²P] dCTP was added to the tube and pipetted up and down to mix and incubated at 37 °C for 10 min. The labelling reaction was stopped by adding 5µl of 0.2M EDTA. The labelled DNA product was denatured by heating to 100 °C for 5 min then rapidly cooled on ice for 5 min, centrifuging to collect at the bottom of the tube.

2.2.4c Probe purification

The labelled, denatured probe was purified by passing it through a NucTrap Probe Purification column and Push column used with a Beta Shield Device. This was done by equilibrating the column with 80µl of 1 x STE buffer, diluting the denatured labelled probe to 80µl loading it into the push column and pushing this through slowly with the plunger, collecting it at the end of the push column.

2.2.4d Hybridisation of the probe to the filter

The probe was incubated with the nylon filter overnight at 42 °C in hybridisation tubes using 10ml of Ultrahyb hybridisation solution and allowed to prehybridise for 30 min before addition of the probe. The unhybridised probe was washed from the nylon membrane with 2 washes of 5 min in 2 x SSC, 0.1% SDS at 42 °C, followed by 2 washes of 5 min in 0.1 x SSC, 0.1% SDS at 42 °C. The level of radiation detectable on the nylon filter was monitored with a Geiger counter following every wash to ensure that the specifically bound probe was not washed off. The nylon filter was exposed to an X-OMAT AR autoradiograph film for 6h to identify the colonies positive for the FUS gene. The autoradiographs were developed using an AGFA film processor. Colonies identified as being positive for the FUS gene were

selected as discrete colonies from the LB agar plate and were inoculated into LB broth cultures and incubated overnight at 37 °C with shaking.

2.2.5 Plasmid extraction

Plasmid DNA was extracted from the LB broth cultures of the selected colonies using the Qiaprep Miniprep kit for purification of plasmid DNA according to the manufacturer's instructions which, in brief, involved harvesting 5ml of transformed bacterial cells by centrifugation at 6,000 x g then resuspending these cells in 250µl of buffer P1 with added RNase A. Adding 250µl buffer P2 and inverting 4-6 times to mix, adding 350µl buffer N3 and inverting to mix. The mixture was then centrifuged for 10 min at 16,000 x g. Supernatant from the centrifugation step was applied to the Qiaprep column sat in a collection tube and centrifuged for 30-60 sec at 16,000 x g and the flow-through discarded. The Qiaprep column was washed by centrifuging 0.5ml of buffer PB for 30-60 sec at 16,000 x g and discarding the flow through. Buffer PE was also used to wash the Qiaprep column (0.75ml) and centrifuging for 30-60 sec at 16,000 x g and the flow through discarded. An additional centrifugation step of 1 min was performed to remove any residual buffer from the Qiaprep column. Plasmid DNA was eluted from the Qiaprep column by adding 50µl of buffer EB to the column in a 1.5ml microfuge collection tube, allowing to stand for 1 min and centrifuging for 1 min at 16,000 x g.

Buffers supplied as part of the DNeasy mini DNA extraction kit were buffers P1, P2, N3, PE, EB and RNase A.

2.2.6 Restriction digests of plasmid DNA

The extracted plasmids containing FUS were digested with EcoR1. This gave an asymmetrical digest in the FUS gene and on the plasmid which enabled the orientation of FUS insertion in the PINCO plasmid to be determined by visualising

the digested plasmids on a 0.8% agarose gel as described in section 2.2.1. DNA markers pGEM and 1Kb DNA ladder were loaded into the gel to allow determination of the restriction fragment sizes. The conditions for the restriction digest were:

1µl EcoR1 12U/µl

5µl extracted plasmid DNA sample

2µl 10 x Eco R1 buffer

12µl water

The restriction reaction was incubated at 37 °C for 2h .

2.2.7 Glycerol stocks

Glycerol stocks were made of the bacterial colonies grown in LB broth from which the plasmid DNA had been extracted by mixing 50% glycerol and 50% LB broth culture of cells in a cryopreservation tube. Glycerol stocks were stored at -80 °C.

2.2.8 DNA quantitation

The amount of plasmid DNA extracted from the bacterial cells using the Qiagen Miniprep kit was determined by uv spectrophotometry using a DU 640 spectrophotometer. The absorbance of the extracted DNA was measured at 260nm and 280nm and the following formula was used to determine the purity and concentration of the DNA. A blanking control of the buffer used to elute the DNA was used to set the reference reading of the spectrophotometer.

$$\text{DNA } (\mu\text{g/ml}) = A_{260} \times 50 \times \text{dilution factor}$$

2.2.9 Sequencing by cycle sequencing PCR

To confirm the orientation of the FUS gene insertion in the PINCO plasmid cycle sequencing PCR was performed on the plasmid DNA using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. The primer used for this was

PINCO SENSE which is located before the cloning site on the PINCO plasmid. The primer (1.6pmoles) was added to 4.0 μ l of the terminator ready reaction mixture supplied in the kit, DNA at 0.2 μ g/ μ l was added (0.75-1.25 μ l) and the total reaction volume made up to 10 μ l with distilled water and overlaid with one drop of light mineral oil. Using a DNA thermal cycler an initial denaturing step of heating the mixture to 96°C for 5 min was performed, then the following temperature cycles were performed on the mixture; 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min and repeated for 25 cycles. The temperature was then decreased to 4°C and kept here until the products were cleaned to remove unincorporated nucleotides.

The DyeEx Spin Kit was used to remove unincorporated nucleotides from the products according to the manufacturer's instructions. In brief this involves resuspending the resin in the spin column by vortexing, removing the cap and bottom closure of the tube, placing in a collection tube and centrifuging at 750 x g for 3 min to compact the resin to the correct density for use. The tube was transferred to a clean collection tube and the sequencing reaction applied to the slope of the resin and centrifuged at 750 x g for 3 min. The sample was transferred to a clean small eppendorf tube and taken to Joyce Hoy in the in-house Central Biotechnology Services (CBS), Automated DNA Sequencing Service, to be run on the sequencing gel. The sequences produced by the sequencing service were aligned with the published sequence of the FUS gene in DNASTar software to check the orientation of the gene in the plasmid.

2.3 Generation of amphotrophic retrovirus using vectors based on Murine Leukaemia Virus

Retroviruses can be used as a method of introducing a gene of interest into a cell along with the promoters and regulatory elements required for its expression. The

majority of applications of retroviral vectors require that the vector is not spread from the cell into which it has been transferred. Replication incompetent retroviruses are produced by the replacement of most of the viral genome with the gene to be transferred and result in a retrovirus which can enter a cell, leading to the insertion of the target gene into the DNA of the cell while preventing the virus from further mobilising itself and spreading to any additional cells by its inability to produce the proteins required for retroviral replication. Generally, the gag, pol and env genes are removed from the retroviral genome. Replication incompetent viruses can be generated by the transfection of the recombinant retroviral construct into a packaging cell line which produces the viral proteins required to package the recombinant retroviral particles. Packaging cells are themselves incapable of generating retrovirus due to mutation of the packaging recognition site. The host range which the virus is capable of infecting is dependent on the type of the env gene expressed by the packaging cells used in the production of the retrovirus.

Amphotrophic packaging cell lines produce virus that is capable of infecting cells from man, mouse and other species. The ability of different types of cells to be infected by virus differs according to cell type, most mammalian cells are readily infectable by amphotrophic virus while the haematopoietic HL-60 cell line is infectable expression of the retroviral constructs is subsequently lost, possibly due to methylation of the retroviral sequences. The gene incorporated into the retroviral expression vector is driven by the long terminal repeat (LTR) promoter in the virus from which it is expressed. Alternative splicing may be used to express more than one gene from the same LTR promoter, or additional internal promoters can be incorporated. cDNA copies of an mRNA encoding the protein of interest are the most widely used sequences in retroviral expression constructs. These sequences contain no introns and the 5' and 3' non coding regions and poly adenylation signals can be

removed since the retroviral message itself becomes polyadenylated. There is a maximum insert size when generating a retrovirus; with packaging efficiency reducing dramatically for inserts greater than 8kb. The cDNA is inserted in the same direction as the LTR, but can be inserted in the reverse direction if wishing to generate an antisense mRNA transcript to be used for gene silencing. Selectable markers can also be incorporated into the retrovirus to allow chemical selection of transformed cells in culture or any surface protein which can be identified as the cells would not normally express this protein. Integration of the retroviral vector into the DNA of the cell does not usually result in significant rearrangement of the genome of the infected cell (Miller A.D 1992; Morgenstern J.P *et al.* 1991).

2.3.1 Transfection of Φ NX-Ampho cells

The PINCO plasmids with FUS in the sense and antisense directions were transfected into Φ NX-Ampho cells (see 2.1.10) using a calcium phosphate transfection kit according to the manufacturers instructions. Φ NX-Ampho cells are a second generation retrovirus producer cell line derived from the 293T human embryonic kidney cell line. These cells are helper virus free. The procedure for this involved plating the Φ NX-Ampho cells on the day prior to the transfection at a density of 1×10^5 cells/ml of medium in an F25 flask. Two hours prior to transfection the medium on the cells was aspirated and replaced with fresh medium. Transfection was carried out by mixing 5 μ l of DNA at 1 μ g/ μ l with 49 μ l molecular biology grade water and 6 μ l 2.5M CaCl₂. This mixture was mixed gently by pipetting in a sterile microcentrifuge tube. In a separate microcentrifuge tube 60 μ l of 2 x HEPES buffered saline (pH 7.05) was bubbled with an automatic pipette pump fitted with a 1ml serological sterile pipette and an additional sterile 1-200 μ l pipette tip fitted to the end. The calcium chloride and DNA mixture was added to the bubbling HEPES

buffered saline drop-wise with a sterile pipette tip. The precipitate that formed was allowed to sit undisturbed at room temperature for 20 min before being distributed over the cells in the flask. The flask was rocked very gently and laid flat in a 5% CO₂ cell culture incubator overnight. The medium was aspirated the following day and replaced with 5ml of fresh medium and the flask incubated at 33°C overnight. Materials supplied with the transfection kit were molecular biology water, 2.5M CaCl₂, and 2 x HEPES buffered saline (pH 7.05).

2.3.2 Harvesting the virus

The amphotropic virus produced by the ΦNX-Ampho cells was optimally harvested when the cells were between 80 and 90% confluent in the flask. The medium containing the virus was removed from the culture flask. Cellular debris was removed by centrifugation. 1ml aliquots were snap frozen in liquid nitrogen and stored at -80°C. At all times when handling virus, two pairs of latex gloves were worn and the work carried out in class II microbiological safety cabinets.

2.3.3 Retroviral infection of cell lines

Haematopoietic cell lines were infected with the retrovirus containing the FUS gene in the sense direction and the FUS gene in the antisense direction as well as a retrovirus containing the green fluorescent protein (GFP) gene alone to act as a control against which the effect of the added FUS gene and the antisense FUS gene could be compared. Transduction with retroviruses are stable and long term (Grignani *et al.* 1998).

2.3.3a Transduction of cell lines - retronectin

Cell lines that are adhesive to retronectin were transduced in retronectin coated plastic dishes. Retronectin is a biolinker which brings the target cells in culture and

the added virus into close proximity by the binding of both by its different domains and increasing the efficiency of retroviral transduction (Hanenberg *et al.* 1996; Hanenberg *et al.* 1997). Retronectin was reconstituted with 0.5ml of tissue culture water supplied by our in-house pharmacy and rested at 37°C in a dry incubator for 30 min, then diluted with 4.5ml of sodium carbonate buffer. Once diluted (100µg/ml) the retronectin solution was filtered through a 0.22µ filter, dispensed into aliquots and stored at -20°C.

The protocol used for cell transduction on retronectin coated plastic was to coat the appropriate number of wells in an uncoated 24 well tissue culture dish with 150µl of reconstituted retronectin. This was placed on a shaker at room temperature for a minimum of 2h. The retronectin was aspirated from the dish and replaced with 500µl 1x phosphate buffered saline (PBS) with 1% Bovine serum albumin (BSA) and incubated for 30 min. The PBS/BSA solution was removed and replaced with 1ml of the retroviral supernatant. The dish was sealed in a plastic bag and tucked into a plate carrier for the refrigerated centrifuge. A balance dish was prepared with water to ensure equal loading of the centrifuge. The dish was centrifuged at 33°C for 90 min at 1350 x g. Centrifugation promotes binding of virus to retronectin by an unknown mechanism. During the centrifugation, the cells to be transduced were counted and diluted to a density of 1-2 x 10⁵ cell/ml with fresh culture medium. Once the centrifugation was complete, the viral supernatant was pipetted from the dish and discarded. Immediately the viral supernatant had been removed, 500µl of the diluted target cells were placed gently in the dish before any drying of the pre-coated well could occur. The dish was then placed in a 37°C incubator, tilted gently and laid flat to ensure even coverage of the well with the cells. By this procedure both virus and cells become immobilised to the dish.

A repeat infection was performed 24h after the first infection. The cells were removed from the dish by gently jetting off with medium and maintained in a bijou flask at 37°C. Retroviral supernatant was placed into the dish and the same centrifugation steps were carried out as above. Following centrifugation, the cells were returned to the dish to undergo a further exposure to the virus and increase the efficiency of the transduction. A further 24h after the second transduction, the cells were removed from the dish and cultured under their normal conditions until selection of the transduced population by flow sorting.

2.3.3b Transduction of cell lines - spin infection

Cells that are poorly- or non-adherent to retronectin were transduced with virus containing the FUS gene in the sense and antisense orientations by spin infection. The protocol for this was to estimate the density of the target cells and treat 10^5 cells per infection with protamine sulphate at a final concentration of $10\mu\text{g/ml}$ for 60 min at 37°C in 5% CO_2 for 1h. Following the incubation with protamine sulphate, the cells were placed in a 50ml Falcon tube and centrifuged at $190 \times g$ for 3 min at 33°C. The supernatant was carefully removed without disturbing the pellet. The retroviral supernatant was thawed quickly to 37°C and 2ml placed in the Falcon tube with the pelleted cells and additional protamine sulphate was added to the 2ml to make a final concentration of $0.10\mu\text{g/ml}$. The Falcon tube was gassed with 5% CO_2 , sealed and mixed gently before being centrifuged under containment caps for 90 min at 33°C and $525 \times g$. A 24 well dish was prepared to receive the cells after centrifugation by placing 1x PBS in the outer wells of the dish to prevent excessive evaporation from the dish. The dish was maintained at 37°C to equilibriate. Once the centrifugation had finished the entire centrifuge bucket with the containment cap was removed to the class II safety cabinet and the Falcon tube removed. The supernatant

was removed from the pelleted cells using a 5ml sterile pipette with a sterile 1ml pipette tip attached to the end and discarded without dripping it into the cabinet. The pelleted cells in the Falcon tube were resuspended in 0.5ml of their specified growth medium and transferred to the prepared 24 well dish. A second transduction procedure was carried out 24h after the first to increase the efficiency of transduction on the same cells. The transduced cells were cultured under their normal growth conditions following both transduction procedures until they were present in sufficient numbers to be sorted to a population of cells positive for the GFP marker by flow cytometry.

2.3.4 Tissue culture

The haematopoietic cell lines were maintained at densities of $1 - 10 \times 10^5$ cells/ml in the appropriate medium described in table 2.1. All cell culture work was carried out in class II laminar flow cabinets where the surfaces had been sterilised with 70% ethanol prior to commencing work. All materials used in tissue culture were either purchased as sterile components or filter-sterilised before use. All contaminated waste was either soaked in pre-sept Mini Haz-Tabs for 24h before being disposed of (through a sharps bin if appropriate) or autoclaved to ensure it was not dangerous.

2.3.4a Preparation of Wehi-3B conditioned medium

The myelomonocytic cell line Wehi-3B constitutively produces IL-3. This cell line has been shown to be the most economical source for large quantities of murine IL-3 (Lee *et al.* 1982). To produce conditioned medium containing IL-3, Wehi-3B cells were grown to full confluence and the supernatant harvested by centrifugation, filtered through a 0.45μ filter to remove any residual Wehi-3B cells and stored at -20°C .

2.3.4b Cell counts

Cells were counted using an Improved Neubauer haemocytometer counting chamber. Cells were taken straight from the culture flask and placed on the haemocytometer unless dilution was required due to the numbers of cells present in the flask. When required, cells were diluted with 1x PBS. The number of cells was calculated as shown below:

$$\text{Cell count (16 squares)} \times \text{dilution factor} \times 1000 = \text{number of cells/ml.}$$

2.3.4c Cell viability

Trypan blue exclusion was used to assess the viability of the cultured cells. Cells from the culture flask were removed in 10 μ l aliquots, mixed with 2 μ l 0.4% trypan blue, placed on the haemocytometer counting chamber and analysed by light microscopy. Live cells remained capable of excluding the dye whilst dead cells and debris were stained blue. Viability was expressed as a percentage of live cells.

2.3.4d Cryopreservation of cell lines

Cultured cells were preserved for future use by storage in liquid nitrogen. 5 x 10⁶ cells were centrifuged at 300 x g for 5 min and resuspended in 1ml of IMDM medium containing 20% Foetal calf serum (FCS) and 10% di-methyl sulphoxide (DMSO). Cells were aliquoted into 1.8ml cryopreservation tubes and placed in a freezing box half filled with isopropan-1-ol. This freezing box was then placed at –80°C to ensure slow freezing. After 4h at –80°C the cryopreservation tubes were transferred to cryovats containing liquid nitrogen.

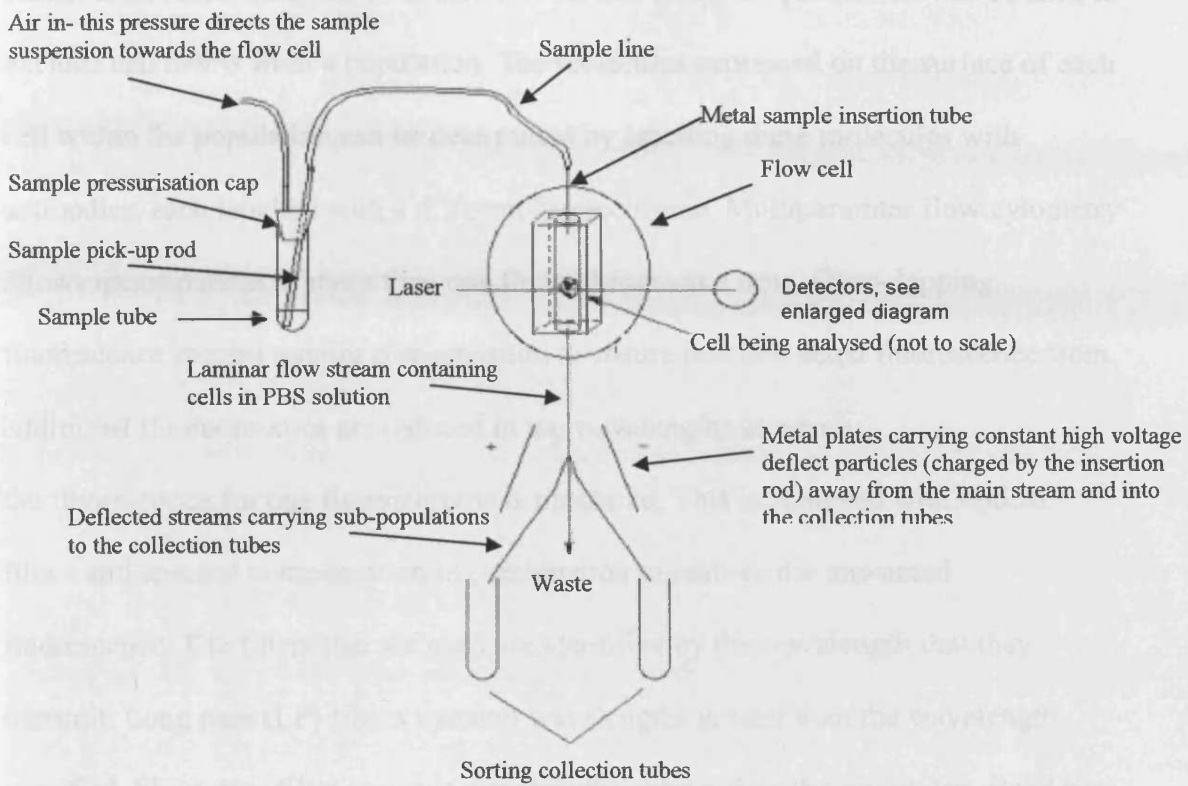
When required for culture, cells were recovered from liquid nitrogen storage by rapid thawing to 37°C followed by drop-wise dilution with 1ml of pre-warmed media to the cryopreservation tube. Cells were then transferred to a universal container (UC) and the volume made up gradually to 5ml with pre-warmed media.

Cells were harvested by centrifugation, resuspended in an appropriate volume of the appropriate culture medium and incubated at 37°C as previously described.

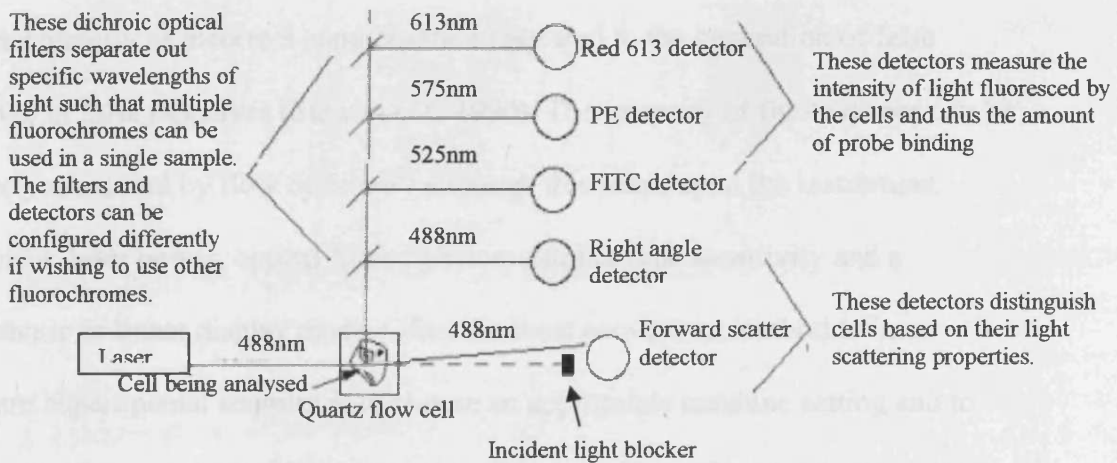
2.3.5 Cell sorting by flow cytometry

Flow cytometry can be used to define the properties of a large population of cells by collecting the information generated when each individual cell of the population is passed through a laser and the amount of light scattered by the cell and the fluorescence of the cell is collected. The principle behind the flow cytometer is to pressurize the sample, then produce a stream of cells only one cell in thickness and pass these cells one by one through a laser. The amount of side scatter, forward scatter and fluorescence generated when the cells is passed through the laser is detected by different detectors depending on the wavelengths at which the cell fluoresces. A flow cytometer that is capable of sorting sub-populations of cells with a particular phenotype works on the same principle with a decision being made as to the phenotype of the cell being made for each cell in turn while the cells are separated into separate droplets from the stream passed through the laser. Each droplet is electrically charged according the phenotype of the cell it contains and the droplets containing the cells with the phenotype required are deflected from the stream using an electrostatic field. Two separate populations can be collected simultaneously by creating positive and negatively charged droplets while leaving unwanted droplets without charge. A schematic diagram of a flow cytometer is shown in figure 2.2.

Fig.2.2 Schematic diagrams of a flow cytometer and the detectors used within them.



Detectors operating within a flow cytometer



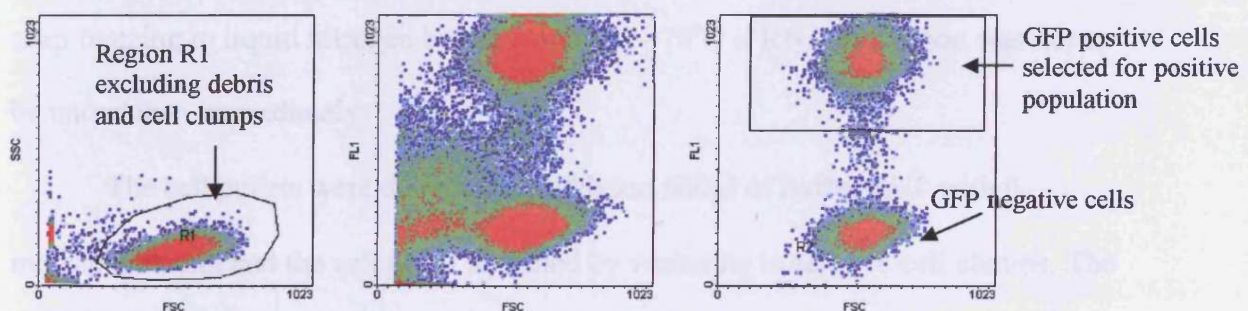
Flow cytometry diagrams adapted from The Wistar Institute

(www.wistar.upenn.edu/research_facilities/facilities/flowcytometry/assets/)

The forward scatter created from a cell is an indicator of cell size while side scatter is an indicator of the granularity of the cell and these parameters can be used to exclude cell debris from a population. The molecules expressed on the surface of each cell within the population can be determined by labelling these molecules with antibodies, each labelled with a different fluorochrome. Multiparameter flow cytometry allows measurement of more than one fluorochrome at a time. Overlapping fluorescence spectra require compensation to ensure that unwanted fluorescence from additional fluorochromes are reduced in the wavelengths at which the fluorescence for one fluorochrome is measured. This is achieved with optical filters and spectral compensation in combination to remove the unwanted fluorescence. The filters that are used are identified by the wavelength that they transmit. Long pass (LP) filters transmit wavelengths greater than the wavelength specified. Short pass filter transmit wavelengths shorter than the one stated. Band pass filters transmit wavelengths in a specific range; a 530-30 band pass filter transmits at 530nm with a band width of 30nm, so from 515nm to 545nm with maximum emission at 530nm. It is important to compensate for the overlapping fluorescence spectra correctly as incorrect compensation may lead to the generation of false positives or false negatives (Stewart C.C 1990). The intensity of fluorescence can be precisely measured by flow cytometry although this relies upon the instrument alignment, laser power, optical filters, photomultiplier tube sensitivity and a logarithmic or linear display mode. Often the most convenient method to intercompare experimental samples is to choose an appropriate machine setting and to collect data from each sample without changing the settings. This method produced data with the greatest possible resolution when small differences in sample intensity are expected (Durand R.E 1990).

Following transduction with retrovirus a pure population of cells positive for the GFP protein was produced by sorting the cells on a flow cytometer. The fluorescence emitted from GFP was detected using a 530/30nm band pass filter. Untransduced cells of each cell line were used to set a region which excluded debris and to define the baseline level of fluorescence already expressed in the cells prior to transduction. The transduced cells were collected for the GFP positive population if they expressed a higher level of fluorescence than the untransduced cells. Figure 2.3 shows how the cells in the transduced populations were selected for GFP positivity.

Fig. 2.3 The first density plot shows the region (R1) excluding debris and cell clumps, the second plot shows a population of ungated GFP positive and GFP negative cells and the third plot show the same population of GFP positive and negative cells gated on R1 indicating the GFP positive cells that were sorted into a GFP positive population of cells. Debris have been removed from the GFP positive population by applying the gate for live cells indicated in the first plot.



The populations of GFP positive cells collected were maintained and expanded in culture until there were sufficient numbers on which to perform experiments. Low passage stocks were maintained to ensure that they did not acquire any additional genetic alteration which may have affected their growth characteristics.

2.4 Validation of transgene expression/Molecular methods

Each of the GFP positive populations collected by flow cytometry for all the haematopoietic cells lines used in this study were checked to ensure that they contained the FUS gene in the sense or the antisense orientation by reverse transcription-polymerase chain reaction (RT-PCR). The function of the retroviral expression vector and its ability to produce mRNA of the correct sizes expected without any additional gene and with the FUS gene in both orientations was corroborated by evidence produced by Northern blotting. The effect of the expression of the retroviral expression vectors on the level of expression of the FUS protein within the cells was identified by Western blotting.

2.4.1 RNA extraction and quantitation

The RNeasy Mini-kit was used to produce total RNA samples from the transduced cells and untransduced cells to act as control samples. In brief, the protocol for this method of RNA extraction was to harvest 10^7 cells by centrifugation at 120 x g for 5 min. The supernatant was removed by aspiration and the cells preserved by snap freezing in liquid nitrogen before storage at -70°C if RNA extraction was not to be undertaken immediately.

The cell pellets were disrupted by addition 600 μl of buffer RLT with β -mercaptoethanol and the cell pellet loosened by vortexing to remove cell clumps. The sample was homogenised by passing the cell lysate through a 20G needle fitted to a syringe at least 6 times. A volume of 600 μl 70% ethanol was added to the homogenised sample and mixed by pipetting the sample up and down. The sample was applied to an RNeasy mini spin column in a 2ml collection tube in 700 μl aliquots and centrifuged for 15 sec at 8,000 x g. As the sample was larger than the 700 μl added to the column initially the remainder of the sample was added to the column

after the centrifugation step and the centrifugation repeated so the whole sample had been passed through the column. The flow-through from the column was discarded after each centrifugation. 700µl of buffer RW1 was added to the RNeasy column followed by centrifugation for 15 sec at 8,000 x g. The RNeasy mini spin column was transferred to a clean collection tube and 500µl of buffer RPE with ethanol was added to the column which was then centrifuged at 8,000 x g for 15 sec. The flow-through was discarded. An additional 500µl of buffer RPE was added to the column which was then centrifuged for 2 min at the centrifuge's maximum speed of 16,000 x g and the flow through discarded. The column was inserted into a clean collection tube and centrifuged for 1 min at 16,000 x g. The column was finally placed in a new 1.5ml collection tube and 30µl of RNase-free water was added directly to the membrane at the base of the column. The column was centrifuged for 1 min at 8,000 x g to elute the RNA. The elution step was repeated in the same collection tube to increase the yield of RNA eluted from the column.

The buffers supplied by Qiagen with the RNeasy mini extraction kit were buffers RLT, RW1, RPE and the RNase free water. All apparatus used in the extraction and handling of RNA was either purchased as RNase free components or treated with di-ethyl-pyrocabonate (DEPC) at 0.1% volume/volume overnight at 37°C followed by autoclaving to remove the action of the DEPC.

The amount of RNA in each of the eluates from the columns was quantitated by uv spectrophometry in the same manner as was used for DNA quantitation as described in section 2.2.8. The formula for calculating the amount of RNA per sample was:

$$\text{RNA in } \mu\text{g/ml} = \text{Ab}_{260} \times 40 \times \text{dilution factor}$$

$$\text{Total RNA } \mu\text{g} = \frac{\text{Ab}_{260} \times 40 \times 20}{1000} \times \text{volume RNA eluted in } \mu\text{l}$$

where Ab_{260} is the absorbance of the sample at 260nm.

The quantitation and quality of the extracted RNA was checked by visualising an aliquot of 2 μ g of RNA in a 1% agarose gel stained with ethidium bromide and viewed under uv light using the method described in section 2.2.1.

2.4.2 Northern blotting

Northern blotting was carried out on the extracted RNA samples made from haematopoietic cell lines to confirm that the inserted FUS gene was producing mRNA when inserted in the sense and antisense orientations and to confirm the sizes of the mRNA transcripts produced from the retroviral expression vector when transcribing the inserted FUS gene.

The protocol followed for Northern blotting was chiefly as described in Maniatis (Maniatis 1989) using formaldehyde to denature the RNA as described in (Lehrach *et al.* 1977) and a nylon filter as first described in (Reed *et al.* 1985).

2.4.2a Northern gel and sample preparation

A 1% agarose gel in 1 x MOPS solution was prepared and cast in a large electrophoresis tank in a fume hood. All bubbles were removed from the gel before it had set. The running buffer for the Northern gel was 1 x MOPS with 2 x 10⁻⁵mg/ml ethidium bromide.

20 μ g of RNA in water freeze dried in a DNA Speed Vac DNA110. The RNA was re-dissolved in 20 μ l RNA loading buffer, heated for 15 min at 55°C in a JB1 water bath and pulsed down in a microcentrifuge, 5 μ l of loading dye was added to each sample. A sample of pGEM marker was also prepared to load alongside the RNA samples to enable the sizes of the RNA transcripts to be determined.

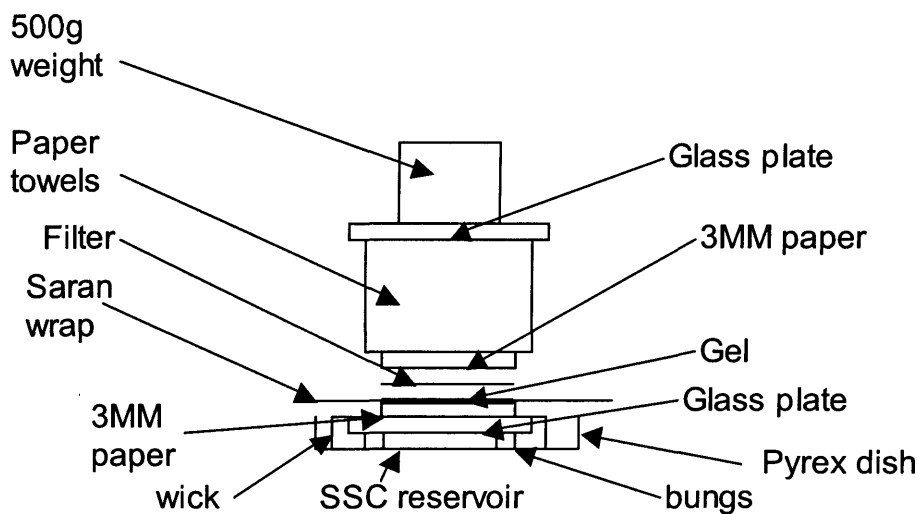
The samples were loaded into the gel and run at 60V for 1h then the current reduced to 30V and the gel run overnight. The gel was visualised and photographed

under uv light after running to ensure even RNA loading and that the ribosomal RNA have run equally in each lane.

2.4.2b Northern blotting procedure

The marker lane was removed from the gel which was transferred to a Pyrex dish and washed with DEPC water for 10 min with gentle agitation and this wash step was repeated once. The gel was equilibrated in 10 x SSC for 10 min with agitation while the blotting apparatus was prepared. Six sheets of 3MM Whatman paper, cut to the size of the gel, were soaked in 10 x SSC. The Hybond N membrane was cut to the size of the gel, soaked in DEPC water and then transferred to 10 x SSC. The blotting apparatus was assembled as shown in the diagram below:

Fig 2.4 Blotting apparatus for Northern blots



A piece of 3MM paper was placed over the glass plate on bungs and cut to the size that it hung over the sides into the 10 x SSC reservoir to act as a wick. Above the wick, three pieces of 3MM paper the size of the gel were stacked, followed by the gel, the membrane, three more sheets of 3MM paper, a stack of paper towels, a second glass plate and finally on the top a 500g weight. Saran wrap was placed up to each

side of the gel and over the top of to the SSC reservoir to prevent a short circuit from the SSC reservoir and the paper towels. The blot was left undisturbed overnight before being dismantled carefully with the orientation of the filter being marked on its reverse side. The transfer was checked under uv light and the filter was air dried for 30 min before being baked for 4h at 80°C in an oven to fix the RNA to the filter. The filter was sealed in a plastic bag and stored at 4°C until hybridisation.

2.4.3 Northern hybridisation

2.4.3a Probe production.

A ³²P labelled DNA probe was prepared using the Strip-EZ DNA kit. Probes were made to detect mRNA sequences including the FUS gene in either orientation, and probes were made to detect mRNA sequences with the GFP gene in them. The FUS probes were made from FUS DNA restricted from the pBluescript as described in section 2.2.1.

The GFP probe was made from GFP DNA restricted from the PINCO plasmid under the conditions:

12.5µl PINCO DNA 0.91ng/µl

5µl 10 x Buffer 2

2.5µl Not1 15U/µl

2.5µl *Hind*III 10U/µl

27.5µl water

The restriction mixture was incubated at 37°C overnight. The GFP DNA was separated on a 1% agarose gel, visualised with ethidium bromide under uv light. The 717 base pair band shown in the gel was cut out of the gel and then purified using the QIAquick spin kit for gel extraction as described in section 2.2.1. The amount of

DNA extracted from the gel was quantitated by uv spectrophotometry as described in section 2.2.8.

2.4.3b Probe labelling

Making the ^{32}P labelled probe with the Strip-EZ probe labelling kit was done according to the manufacturer's instruction manual, which, in brief, involves diluting 25ng of probe DNA to 9 μl with 1 x TE buffer, denaturing it at 95-100°C for 3-5 min in a heating block. The denatured DNA was snap-frozen in dry ice and ethanol, thawed, microcentrifuged and placed on ice. A primer extension mix was created by adding to the 9 μl of probe DNA 2.5 μl 10 x decamer solution, 5 μl 5 x dATP/dCTP buffer, 2.5 μl 10 x dCTP, 5 μl $\alpha^{32}\text{P}$ dATP, 1 μl exonuclease-free klenow. The contents were mixed gently and incubated at 37°C for 10 min. The reaction was stopped by adding 1 μl 0.5M EDTA and was cleaned through a NucTrap Probe Purification column as described in section 2.2.4c.

2.4.3c Hybridisation of the labelled probe to the Northern filter

The purified probe was hybridised overnight to the Northern filter using the ULTRAhyb hybridisation solution, washed twice with 2* SSC for five min at 42°C, then twice with 0.1* SSC, 0.1% SDS for 15 min at 42°C. The Northern filter developed as described in section 2.2.4d.

2.4.3d Stripping old probes from the Northern blot

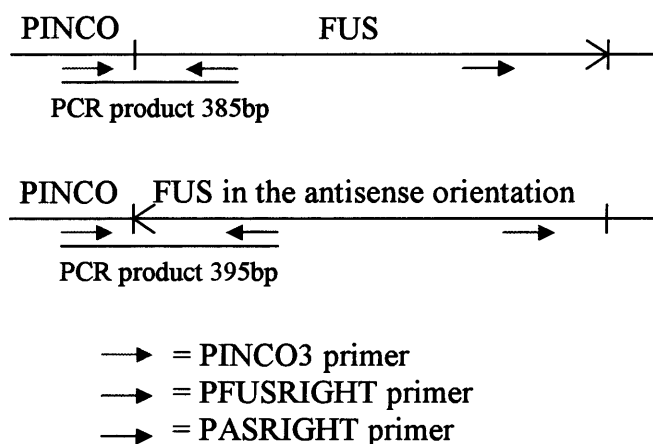
Probes were removed from the Northern blot by returning the filter to the hybridisation tube, with the RNA side facing inwards, washing with a buffer of 1x probe degradation dilution buffer and DNA probe degradation buffer made up in DEPC treated water for 10 min at 68°C. A second wash was performed with 1x blot reconstitution buffer and 0.1% SDS for 10 min at 68°C. A final wash was performed

for 10 min, at 68°C with 0.1% SDS. The buffers for stripping the filters are supplied in the Strip-EZ probe labelling kit.

2.4.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Primers were designed to produce a PCR product if the FUS gene was inserted in the sense direction or in the antisense direction only. One primer (PINCO3) was located in the retroviral expression vector upstream of the site where FUS was cloned into the plasmid. Another primer (PFUSRIGHT) was located in the FUS gene and would form a PCR product when FUS was inserted into the plasmid in the sense direction. The third primer (PASRIGHT) was also located in the FUS gene and would form a PCR product when the FUS gene was inserted in the antisense direction.

Fig 2.5 Diagram showing the location of the primers when FUS is inserted into the PINCO plasmid in the sense and antisense directions and the sizes of the resulting PCR products.



An RT-PCR was done on RNA extracted from the cell lines after 4 days treatment with all-trans retinoic acid (ATRA) at 10^{-7} M to achieve high expression of the retroviral construct, using the RNeasy RNA extraction kit described in section 2.4.1. The conditions used for the reverse transcription (RT) step were;

1 µg RNA (2.5µl total volume made up with water)

14µl RT buffer containing dNTPs

1.5µl Downstream primer 10µM (PFUSRIGHT or PASRIGHT)

1µl RNase inhibitor 20U/µl

1µl MuMLV RT enzyme 50U/µl

These reagents were mixed in a thin walled microcentrifuge tube and placed in a DNA thermal cycler. The thermal cycle for the RT reaction was one cycle of; 25°C 10 min, 42 °C 30 min, 95 °C 5 min, 4 °C soak

The reaction conditions for the PCR reaction to detect FUS in the sense orientation in the PINCO plasmid were:

5µl RT reaction using PFUSRIGHT primer

1µl 25mM MgCl₂

2µl 10 x buffer II

1µl PINCO3 primer (10µM)

1µl PFUSRIGHT (10µM)

0.25µl AmpliTaq DNA Polymerase 5U/µl

9.75µl water

The reaction conditions for the PCR reaction to detect FUS in the antisense orientation in the PINCO plasmid were:

5µl RT reaction using PASRIGHT primer

1µl 25mM MgCl₂

2µl 10 x buffer II

1µl PINCO3 primer (10µM)

1µl PASRIGHT (10µM)

0.25µl AmpliTaq DNA Polymerase 5U/µl

9.75µl water

For detection of FUS in the sense and antisense orientations the PCR reagents were mixed in a thin walled microcentrifuge tube, overlaid with light mineral oil and placed in a DNA thermal cycler. The cycling conditions for the PCR reaction were:

95°C 1 min, 65 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 64 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 63 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 62 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 61 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 60 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 59 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 58 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 57 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 56 °C 1 min, 72 °C 1 min - 2 cycles

95°C 1 min, 55 °C 1 min, 72 °C 1 min - 20 cycles

72 °C 10 min, 4°C soak

This touch-down PCR cycling program was used as it removed the need to try the PCR reaction at each of the different annealing temperatures during optimisation of the reaction.

Following the PCR reaction the samples were removed to the post-PCR room to prevent contamination of the PCR preparation area with PCR product was were visualised on a 1% agarose gel with ethidium bromide and photographed as described in section 2.2.1.

2.4.5 Real-time PCR (RQ-PCR) using a Light Cycler

The Light Cycler PCR system uses temperature cycling using air which allows faster cycling than can be achieved in a conventional thermal block cycler and produces accuracy of $\pm 0.3^{\circ}\text{C}$. The capillaries in which the reactions take place allow rapid equilibrium between the air and the reaction mixture through its high surface to volume ratio and these capillaries are suitable for use as a cuvette to measure fluorescence, which is detected at the tip of the capillary. The amount of fluorescence observed is relative to the amount of PCR product, which permits on-line monitoring of the PCR reaction. The Sybr-Green dye that was used is double strand specific and only fluoresces when incorporated as part of a double stranded DNA molecule, which means the level of fluorescence observed increases as more PCR product is produced in the reaction.

Melting curve analysis is useful for determining that the products produced in each reaction are the same as each double stranded DNA product has its own specific melting temperature at which 50% of the DNA become single stranded and at which the remaining 50% remains double stranded. This is measured at the end of the PCR reaction by increasing the temperature of the reactions and measuring the fluorescence as it decreases as the Sybr Green dye is released from the double stranded PCR products as they become single stranded with increasing temperature.

2.4.5a Reverse transcription of samples

RNA samples produced from the samples in which the level of mRNA expression was to be determined were reverse transcribed using the following reaction mixture:

25mM MgCl ₂	8 μ l
10 x PCR buffer II	4 μ l

dNTP mix	16 μ l
RNase inhibitor	2 μ l
Reverse transcriptase	2 μ l
Random hexamers	2 μ l
RNA	2ng
Water	to 40 μ l

The reaction was carried out in a thermal cycler under the following conditions: 37°C for 60 min, 75°C for 5 min, followed by a 4°C soak. The samples were stored at -20°C.

2.4.5b Primer design and PCR optimisation using a thermal cycler

Primers were designed to produce PCR products of approximately 200 base pairs from each of the genes of interest. Using these primers, which are listed in section 2.1.2, the PCR reaction was optimised in the thermal cycler. A unique product was required for genes that were to be analysed on the light Cycler. The PCR reaction mixture used to optimise the PCR was:

25mM MgCl ₂	2.5 μ l
10 X PCR buffer II	5.0 μ l
Primer 1 (10mM)	2.5 μ l
Primer 2 (10mM)	2.5 μ l
Taq	0.625 μ l
RT reaction	12.5 μ l
Water	to 50 μ l

The cycling conditions used were 95°C for 6 min, 35 cycles of 95°C 1 min, 60°C for 1 min, 72°C for 1 min, followed by a 4°C soak. Upon completion of the PCR reaction the products were visualised in an agarose gel stained with ethidium bromide

as described in section 2.2.1. If a unique product was visualised in the agarose gel, the cycling conditions were adapted for the Light Cycler. Further optimisation of the annealing temperature was carried out if more than one product was seen. For the cycling conditions given above, the programme adapted for the Light Cycler was 95°C for 10 min, 50 cycles of 95°C for 3 sec, 60°C for 5 sec, 72°C for 12 sec during which the Light Cycler acquires one fluorescence measurement per 72°C extension period to measure the accumulation of the PCR product, then the temperature of the samples was taken to 95°C, reduced to 70°C for 15 sec and raised to 95°C again. During this period, fluorescence measurements were taken continuously as this provides information about the melting point of the products produced by the PCR reaction. All temperature increases were made at a speed of 20°C/sec in the amplification and melting curve stages of the reaction. Finally the temperature was reduced to 40°C.

2.4.5c Magnesium chloride concentration optimisation

The magnesium chloride concentration to be used with each pair of primers was optimised on the Light Cycler. Concentrations of 2mM, 3mM, 4mM and 5mM magnesium chloride were used in the following reaction mixture:

MgCl ₂	2mM/3mM/4mM/5mM
Primer 1 (50ng/μl)	0.5μl
Primer 2 (50ng/μl)	0.5μl
Sybr Green reaction mixture	1.0μl
RT reaction used to optimised PCR	1.0μl
Water	to 10μl

The cycling conditions adapted from the PCR optimised from the thermal cycler were used. The samples were repeated in duplicates. The magnesium

concentration that produced the melting curve (shown in figure 2.6) that was most reproducible in the replicates and had the highest maximum was used as the optimised concentration.

2.4.5d Optimised Light Cycler PCR

The optimised magnesium concentration for each set of primers was run on the Light Cycler in the same reaction mixture as was described above and the same cycling conditions were used. The samples were repeated in duplicate and two independent sets of samples were analysed on the Light Cycler with RNA extracted from two different sets of each cell or treatment type and independently reverse transcribed. The results acquired from the Light Cycler are shown in figure 2.7. The second derivative maximum method of analysis of the data was used. The baseline value was first arithmetically determined by the Light Cycler software by subtracting the mean of the five lowest measured data points from each reading point in the sample. This method then measures the point at which the rate of change of fluorescence is fastest which usually occurs in the cycle where the sample fluorescence can first be distinguished from the background fluorescence. The value generated is denoted as the crossing point and shows the number of reaction cycles that have occurred before the level of fluorescence from the PCR product exceeds the baseline threshold. The mean of all four crossing point values generated for each sample in each gene were calculated and these were compared to the values generated for housekeeping genes. In the human samples, the housekeeping gene used was S14 while in the murine samples β -actin was used. The results for the housekeeping genes were generated in the same manner as the results for the samples.

Fig. 2.6 Melting curve analysis of PCR products

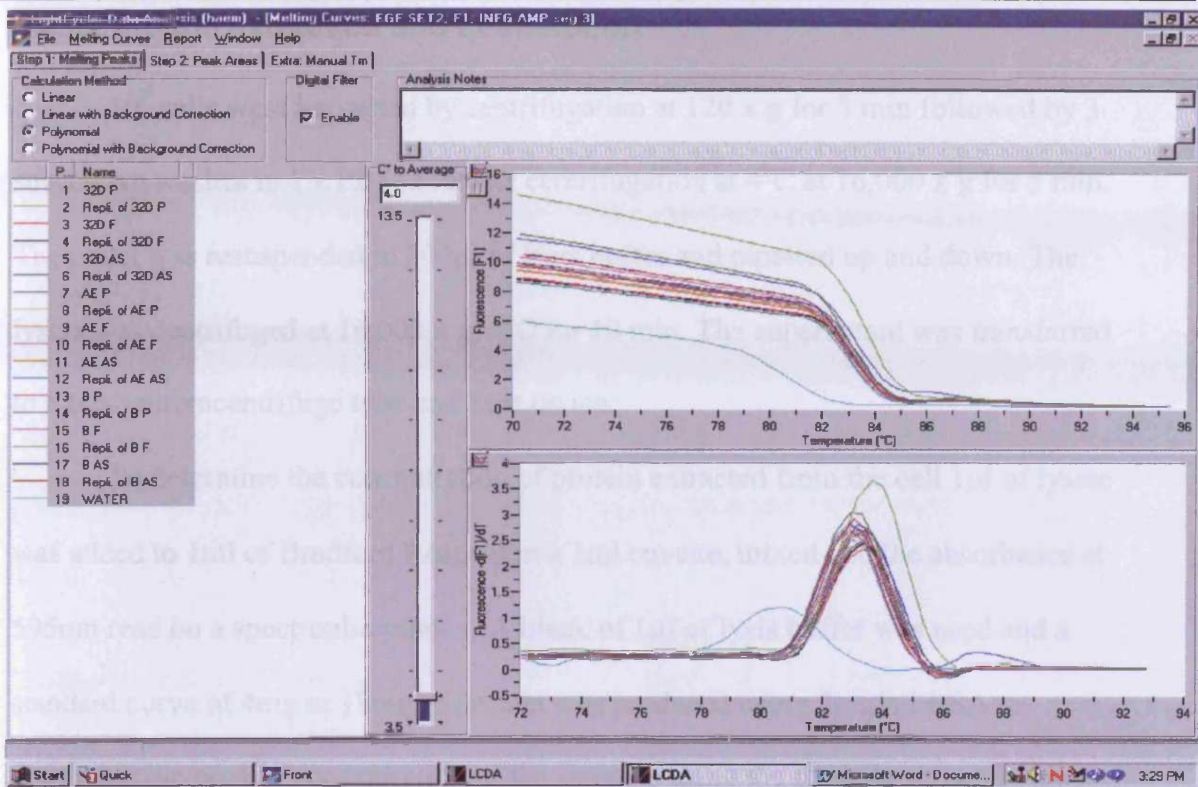
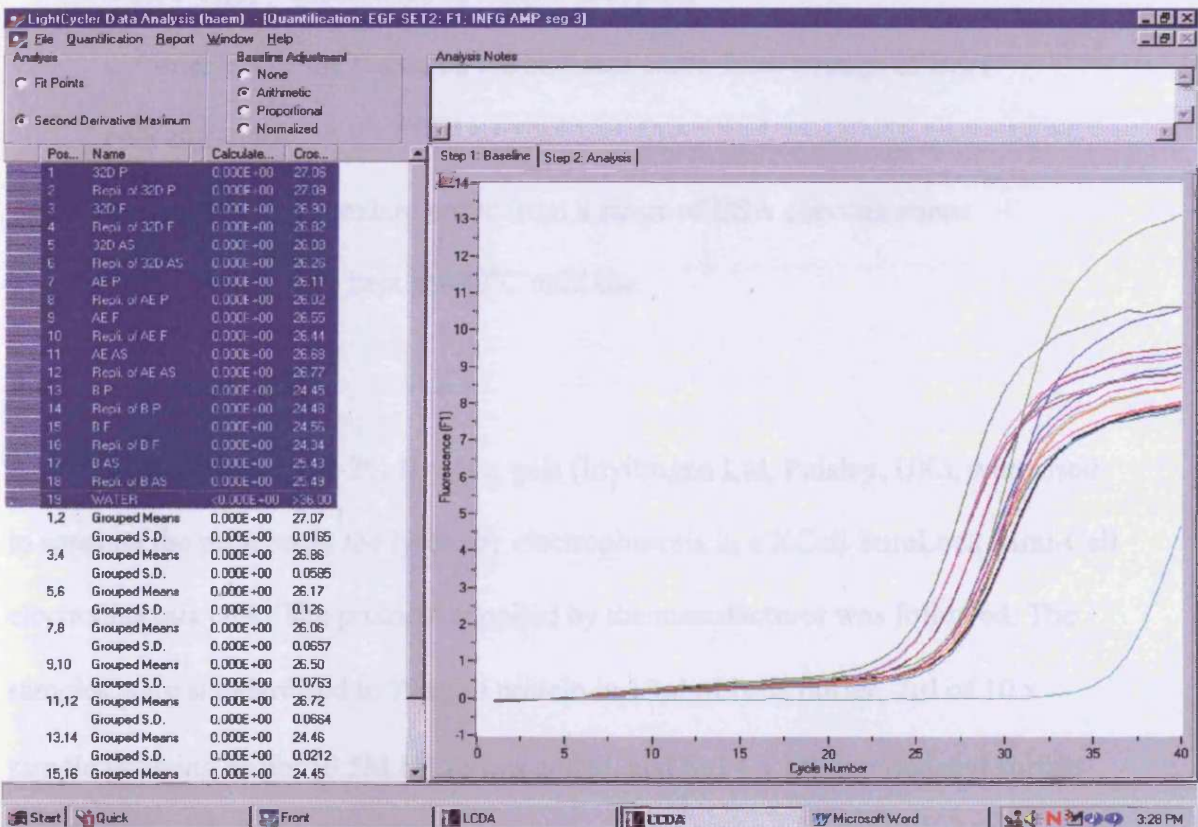


Fig. 2.7 Example of the results generated by the Light Cycler



2.4.6 Western Blotting

2.4.6a Protein extraction and quantitation

10^7 cells were harvested by centrifugation at 120 x g for 5 min followed by 3 successive washes in 1 x PBS, a further centrifugation at 4°C at 16,000 x g for 5 min. The pellet was resuspended in 200µl of lysis buffer and pipetted up and down. The lysate was centrifuged at 16,000 x g, 4°C for 10 min. The supernatant was transferred to a fresh microcentrifuge tube and kept on ice.

To determine the concentration of protein extracted from the cell 1µl of lysate was added to 1ml of Bradford Reagent in a 1ml cuvette, mixed and the absorbance at 595nm read on a spectrophotometer. A blank of 1µl of lysis buffer was used and a standard curve of 4mg to 18mg of protein was produced using 2mg/ml BSA to determine the protein concentration of the samples using the formula shown below:

$$\text{Protein } (\mu\text{g}/\mu\text{l}) = \frac{A_{595} - c}{m}$$

where A_{595} = absorbance of sample at 595nm

c = intersect of the y-axis on the standard curve from a range of BSA concentrations

m = gradient of standard curve from a range of BSA concentrations

The extracted protein was kept at -80°C until use.

2.4.6b Western gels

NuPage pre-cast 4-20% Bis-Tris gels (Invitrogen Ltd, Paisley, UK), were used to separate the proteins in the lysate by electrophoresis in a XCell SureLock Mini-Cell electrophoresis tank. The protocol supplied by the manufacturer was followed. The samples were standardised to 70µg of protein in 13µl of lysis buffer, 2µl of 10 x sample reducing buffer (0.5M DTT) was added, and 5µl 4 x lithium dodecyl sulfate

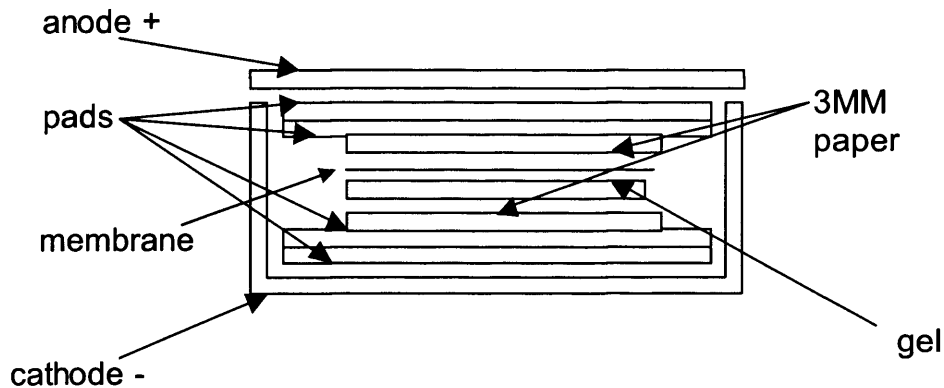
(LDS) buffer was added. The samples were heated to 70°C for 10 min. The pre-cast gel was washed with running buffer (1 x MOPS), and locked into place in the XCell SureLock Mini-Cell electrophoresis tank. 500µl of antioxidant was added to the 200ml 1 x MOPS running buffer which was then used to fill the inner chamber of the electrophoresis tank. The reduced samples were loaded into the gel after the incubation was complete along with a rainbow marker, MultiMark Multicoloured standard and a protein marker MagicMark that is detected chemiluminescently. The orientation of the samples and markers was noted. The 1 x MOPS running buffer was placed into the outer reservoir of the electrophoresis tank and the gel was run at 200V for 50 min.

2.4.6c Western Transfer

A polyvinylidene difluoride (PVDF) membrane was soaked for 30 sec in methanol, then transferred to distilled water for 5 min then soaked in transfer buffer (100ml methanol, 1ml antioxidant, 50ml 20 x transfer buffer, 849ml distilled water). The pads for the XCell II Blot Module, and 2 pieces of 3MM paper supplied with the PVDF membrane were soaked in transfer buffer. Once the gel had been run, the wells were removed from it and the blotting apparatus was assembled as shown in figure 2.8.

From the cathode (-) core of the blotting module two pads were stacked, followed by 1 piece of 3MM paper, the gel, the PVDF membrane, 1 piece of 3MM paper. Any bubbles were removed at this point. The remaining pads were placed on the top of the 3MM paper and the anode (+) core assembled. The transfer module was placed in the electrophoresis tank, covered with transfer buffer. The outer reservoir of the electrophoresis tank was filled with distilled water and transfer was carried out at 30V (380mA) for 1h.

Fig 2.8 Blotting apparatus for Western blotting.



For these procedures, buffers supplied as concentrated by Invitrogen Ltd were: reducing agent, LDS loading buffer, MOPS running buffer, antioxidant and transfer buffer. These reagents were made up to the final strength solutions as described by the manufacturer.

2.4.6d Chemiluminescent Immunodetection

The WesternBreeze Novex Chemiluminescent Western Blot Immunodetection Kit was used to detect the FUS protein on the Western membrane. The protocol used for this was supplied by the manufacturer and involved pre-wetting the membrane with methanol for 30 sec, followed by 2 washes of 5 min in water on a rotary shaker. The membrane was placed in 10ml of blocking solution (5ml water, 2ml blocker/diluent part A and 3ml of blocker/diluent part B) and incubated for 30 min on a rotary shaker at 1 revolution/sec. Two further washes of 5 min in 20ml water were carried out. 10ml of primary antibody solution was prepared (7ml water, 2ml blocker/diluent part A, 1ml blocker/diluent part B, 40 μ l anti-TLS antibody and incubated with the membrane on the rotary shaker for 1h. Four washes of 5 min with 20ml antibody wash (150ml water, 10ml antibody wash solution) were performed. The filter was incubated at room temperature for 30 min with a mouse secondary

antibody solution. Four washes of 5 min with 20ml antibody wash were performed. Three washes of the membrane with 20ml water for 2 min followed. The membrane was placed on acetate film and 2.5ml of chemiluminescent substrate was applied slowly to cover the membrane surface. The reaction was permitted to develop for 5 min before the excess substrate was blotted away with filter paper (supplied with the Western Breeze kit). The membrane was covered with an additional piece of acetate film and in a dark room exposed to Hyperfilm ECL x-ray films for exposures of different lengths from 3 sec up to 5 min. The x-ray films were developed using an AGFA film processor.

Materials supplied in the kit for chemiluminescent immunodetection were: blocker/diluent part A, blocker/diluent part B, antibody wash solution, secondary antibody, chemiluminescent substrate, filter paper.

2.5 Differentiation induction in transduced cell lines

2.5.1 All-trans retinoic acid (ATRA)

ATRA was dissolved in DMSO at 1µg ATRA /1µl DMSO giving a concentration of 3.3×10^{-3} M ATRA. 30µl of 1µg/µl ATRA solution was dissolved in 970µl RPMI + 10%FCS to give a concentration of 10^{-4} M ATRA which was stored at -20°C in the dark. The 10^{-4} M ATRA dilution was used at 10µl/1ml of cell culture to treat the culture at a concentration of 10^{-6} M ATRA. The 10^{-4} M ATRA solution was diluted to enable treatment of cell cultures at concentrations between 10^{-9} M and 10^{-6} M ATRA. The cells to be treated with ATRA were seeded at the required cell density in a new culture vessel, allowed to rest for 2h at 37°C , 5% CO_2 and then the ATRA added at the required concentration. The cells' growth, viability and expression of cell surface markers were assessed at a number of time points throughout each experiment. In all experiments where ATRA was used to treat cells, a set of the same

type of cells were left untreated to act as a control sample against which the treated cells' responses to treatment could be compared. All experiments were repeated in triplicate. All the cell lines transduced with FUS and FUS antisense were treated with ATRA at a concentration of 10^{-7} M over a period of 14 days. The ATRA was added to the normal culture medium of the cell lines and, in the case of the cell lines that were dependent on the presence of IL-3, experiments to determine the effect of ATRA at 10^{-7} M were also performed on these cell lines in the absence of IL-3 in the medium used to maintain the cell lines.

Dose response experiments were performed on the HL-60, NB4 and NB4R2 transduced cell lines. ATRA was used to treat these cell lines at 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M concentrations. These experiments were performed in triplicate and the effect of the different concentrations of ATRA were determined as described in the following section.

2.5.2 Granulocyte colony stimulating factor (G-CSF)

G-CSF at 150 μ g/ml in sterile water for injections was supplied by the in-house pharmacy and diluted to 50 μ g/ μ l in filter sterilised 1 x PBS, 1% BSA. This stock was stored at -20° C. Cells to be treated with G-CSF were seeded at the required density in a new culture vessel and allowed to rest at 37° C, 5% CO₂ for 2h before the addition of the G-CSF. The cell lines were treated with a final concentration of 100ng/ml G-CSF. As with the treatment of cells with ATRA, the effect of the G-CSF on the cells was assessed by cell growth, viability and the expression of cell surface markers at time points throughout the cells' exposure to G-CSF and relative to an untreated control set of cells and all experiments were repeated in triplicate.

The cell lines transduced with FUS and FUS antisense were treated with G-CSF at a concentration of 100ng/ml over a period of 14 days. G-CSF was added to the normal

culture medium of the cell lines and in the cases of the cells line that were dependent on the presence of IL-3 additional experiments to determine the effect of G-CSF at the same concentration were also performed on these cell lines in the absence of IL-3 in the medium used to maintain the cell lines.

2.5.3 ATRA and G-CSF in combination

The cell lines treated with ATRA and G-CSF separately were also treated with both inducers in combination. The cells were seeded, rested and treated as described in section 2.5.1 before the addition of the G-CSF and ATRA. In these experiments, G-CSF was used at a concentration of 100ng/ml and ATRA at 10^{-7} M. The effect of both inducers on the treated cells was assessed by cell growth, viability and the expression of cell surface markers at multiple time points throughout the exposure of the cells to the inducers. These experiments were repeated in triplicate.

2.5.4 Cell growth and viability measurement

Cell growth was measured by counting the number of cells/ml of culture using a haemocytometer as described in section 2.3.4b. The growth of the cells over the time course of the experiments was calculated by the formula and plotted on a graph.

$$\text{Fold expansion} = \frac{\text{cell density at start of day}}{\text{cell density at which culture left at previous time of counting}}$$

Cumulative fold expansion = fold expansion x cumulative fold expansion from previous time of counting

The cell viability was assessed and calculated as described in section 2.3.4c. The percentage viability was plotted on a graph for each of the time points in the experiments to show how the viability of the cells altered in response to treatment with the inducers.

2.6 Immunophenotyping

The expression of cell surface markers was assessed by the binding of fluorescently labelled antibodies to the cell surface markers of interest. The cells to be immunologically stained were pelleted by centrifugation at 120 x g for 3 min. The pellet was washed 3 times in 1 x PBS, 1% BSA before being resuspended in 10µl of 1 x PBS, 1% BSA. The resuspended cells were incubated at 4°C in the dark for 30 min with the labelled antibody. The binding reaction was effectively stopped by dilution with 140µl of FACSFlow. The cells were pelleted again by centrifugation and resuspended in 50µl of FACSFlow and transferred to a Luckham LP2 tube before being analysed on a FACSCalibur for the level of fluorescence shown on the cells when excited by a laser. The metal sheath acting as a droplet retrieval system was removed to allow analysis of small sample volumes. Each cell type was stained with the control antibody and the other antibodies in a separate aliquot of cells so that only the binding of a single antibody was seen on any population of cells when analysed on the FACSCalibur.

2.6.1 Markers for HL-60, NB4, NB4R2 cell lines

The antibodies used to assess the response to the inducers ATRA and G-CSF in HL-60, NB4 and NB4R2 cells were mouse anti-human CD11b, mouse anti-human CD38, and mouse anti-human CD33. The irrelevant antibody control used to show the level of background, or irrelevant binding by the antibody was mouse IgG1. All the antibodies used on these cell lines were phycoerythrin (PE) labelled. The final concentration at which these antibodies were used on a maximum of 100,000 cells was 0.01ng/µl in a total reaction volume of 25µl for the IgG1, CD11b and CD38 antibodies. The CD33 antibody was used at a concentration of 0.03ng/µl in a total reaction volume of 25µl.

2.6.2 Markers for 32D, 32D B2A2 and 32D AML-ETO cell lines

These murine cell lines were immunologically stained with the PE-labelled antibodies rat anti-mouse CD11b, rat anti-mouse F4/80, and rat anti-mouse Ly-6G. The irrelevant control antibody was rat anti-mouse IgG2b. The concentration at which these antibodies were used was 0.01ng/ μ l in a total reaction volume of 25 μ l.

2.6.3 Flow analysis

The data collected on the FACSCalibur by flow cytometry was analysed using WIN MDI version 2.8. The data was gated to exclude cell debris. From this, the level of background staining was determined using the data collected from the cells stained with the IgG control antibody. The level of antibody staining seen on the cells stained with the cell surface marker antibodies was determined by the level of fluorescence seen on these cells above the background staining level when compared to the same type of cells stained with the control antibody. The level of antibody binding was expressed as the mean fluorescence intensity seen when the results were averaged from all repetitions of the experiment.

2.6.4 Annexin V staining

Annexin V labelled with PE was used to assess whether any apoptosis occurred in the population of cells transfected with the siRNA. The protocol for this was to harvest not more than 10^4 cells by centrifugation at 120 x g for 3 min followed by 2 washes in 1 x PBS followed by re-suspension in 10 μ l of the supplied Annexin V binding buffer (10mM HEPES/NaOH (pH 7.0), 140mM NaCl, 2.5mM CaCl₂). 1 μ l of Annexin V-PE was added to the resuspended cells and incubated for 15 min at room temperature in the dark. After this incubation, 150 μ l of binding buffer was added and

the cells centrifuged at 120 x g for 3 min. The supernatant was removed and the cells resuspended in 25µl of Annexin V binding and transferred to Luckham LP2 tubes.

2.7 siRNA

2.7.1 siRNA design and production

The siRNA used was designed by Eurogentec from the sequence of FUS that was supplied to them. The sequence was

```
5' GUGACCGUGGUGGCUUCAATT 3'  
3' TTCACUGGCACCACCGAAGUU 5'
```

A scrambled siRNA control sequence bearing no significant homology to mouse, rat or human sequences supplied by Ambion Europe Ltd was also used. The sequence for the control siRNA was not supplied. The siRNA was supplied as separate strands that were lyophilised.

2.7.2 siRNA annealing

The separate siRNA strands were re-suspended in water at a concentration of 50µM. The two strands were then combined using 30µl of each and 15µl of 5 x annealing buffer. The final concentration of the siRNA was 20µM and the buffer concentration was 50nm Tris pH 7.5-8.0, 100mM NaCl in DEPC treated water. The annealing buffer was provided with the siRNA strands supplied by Eurogentec.

The separate strands were annealed by incubating the solution for 1-2 min in a water bath at 90-95°C, then allowed to cool to room temperature over 45-60 min. The annealed duplex siRNA was stored at -20°C and freeze-thawed not more than 5 times.

2.7.3 siRNA Cy3 labelling

The duplex siRNA was labelled with Cy3 using the *Silencer*TM siRNA labelling kit. The siRNA was fluorescently labelled to allow the cells containing the

siRNA following transfection to be cell sorted by flow cytometry to achieve a population of cells containing the siRNA. The protocol supplied by the manufacturer was followed for the labelling of the Cy3. In brief, this protocol was to reconstitute the labelling reagent and mix the resulting solution well by vortexing. The labelling reaction was assembled using 18.3µl nuclease free water, 5µl 10 x labelling buffer, 19.2µl duplex siRNA at 20µM and 7.5µl Cy3 labelling reagent. The mixture was mixed by vortexing and exposure to light was limited. The reaction was incubated in the dark at 37°C for 1h.

The labelled siRNA was recovered by ethanol precipitation. The protocol for this was to add 5µl 5M NaCl (0.1 volume of the labelling reaction) and 125µl (2.5 volumes) of cold 100% ethanol. The reaction was mixed well and incubated at -20°C for 30-60 min. The mixture now containing precipitated siRNA was centrifuged at 8,000 x g for 20 min. The supernatant was removed without disturbing the pellet. The pellet was washed with 175µl 70% ethanol, centrifuged for 5 min at 8,000 x g and all traces of the supernatant was removed with a pipette. The siRNA was dried at room temperature for 5-10 min then resuspended in 19.2µl nuclease free water to result in a final siRNA concentration of 20µM.

2.7.4 Transfection of siRNA

The Cy3 labelled duplex siRNA was transfected into NB4 and NB4R2 cells using the *SilencerTM* siRNA transfection kit. The protocol supplied by the manufacturer was followed for the transfection following some optimisation required for the cell lines used. The final protocol used to transfect the cells was to plate the cells at a density of 1×10^5 cells/ml in a 12 well plate the day before transfection. These cells were maintained in normal culture conditions overnight and the density of the cells adjusted to 2×10^5 cells/ml on the day of transfection. The volume of culture

was reduced to 400 μ l. The amine transfection agent (5 μ l) supplied by in the transfection kit was combined with 94 μ l of OPTI-MEM medium, vortexed to mix and incubated at room temperature for 30 min. The siRNA was added to produce final concentrations of 60nM for NB4 cells and 100nM for NB4R2 cells, mixed by flicking and incubated at room temperature for 15-20 min to allow it to complex with the amine transfection agent. Following this incubation, the amine-siRNA mixture was added dropwise to the cells in culture and incubated for 4h before 2ml of the normal cell culture medium for the cell was added.

Other experiments into the effect of siRNA on the transfected included multiple transfections of the siRNA into the original population of cells using the same protocol and performed on days 3 and 6 when the initial transfection was performed on day 0.

2.7.5 Determination of transfection efficiency

Cells positive for the Cy3 were assayed by flow cytometry to determine the proportion of the cells containing the labelled siRNA. This was done on day 1 following transfection. The cells were not sorted by flow cytometry as the entire population of cells showed an increase in fluorescence at a wavelength of 550nm indicating that all the cells had taken up siRNA during the transfection procedure. Additional measurements of the amount of Cy-3 in the cells were taken on day 3 following transfection of the siRNA into the cells.

2.7.6 Assessment of the effect of siRNA

Following transfection, the growth and viability of the siRNA positive cells were assessed as described in sections 2.3.4b and 2.3.4c. Cell counts and viability were measured and Annexin V staining, as described in section 2.6.4 were performed at multiple time points following transfection. Analysis of the annexin V staining

allowed the proportion of cells undergoing apoptosis or that were dead to be determined.

2.8 Affymetrix GeneChip Analysis

The gene expression of cells transduced with FUS and FUS antisense as well as untransduced cells were analysed using the Affymetrix system. The samples which were analysed in this manner were; NB4 control, NB4 FUS and NB4 FUS AS cells, and all these cell lines treated with ATRA at 10^{-7} M for 24h and for 96h. Additionally 32D control, 32D FUS, 32D FUS AS, 32D B2A2 control, 32D B2A2 FUS, 32D B2A2 FUS AS, 32D AML-ETO control, 32D AML-ETO FUS and 32D AML-ETO FUS AS cells were analysed using Affymetrix GeneChips.

2.8.1 Sample preparation

For each sample 10^7 cells were harvested from culture at a point when they were actively growing and not at confluence in their culture flasks.

2.8.1a RNA extraction

All equipment used in the extraction of total RNA was RNase free. Trizol Reagent was used to extract the RNA from the samples. The procedure for this was to pellet the cells by centrifugation at 300 x g for 5 min and resuspended by pipetting in 1ml Trizol reagent. 2ml tubes containing Eppendorf Phase Lock Gel-heavy were centrifuged at 1,500 x g for 30 sec to collect the gel at the bottom of the tube. The cell lysate was added to the tubes containing the Phase lock gel-heavy and the tubes were incubated for 5 min at room temperature. 0.2ml chloroform-isoamyl alcohol were added to the tubes which were then shaken vigorously for 15 sec, then centrifuged at 12,000 x g for 10 min at 2-8°C. After the centrifugation, the phasing of the tube was examined. If the clear, aqueous layer was entirely on top of the Phase lock gel it was transferred to a fresh tube. If the layers were not clearly separated, the centrifugation

was repeated. The RNA in the aqueous layer was precipitated by the addition of 0.5ml of isopropyl alcohol, which was mixed by repeated inversion of the tube and incubated at room temperature for 10 min. The precipitated RNA was centrifuged at 12,000 x g for 10 min at 2-8°C after which the RNA was visible at the bottom of the tube. The supernatant was removed following centrifugation and 1ml 75% ethanol was added. The pellet was dislodged by mixing or vortexing and centrifuged for 5 min at 7,500 x g at 2-8°C. The supernatant was removed and the RNA pellet air dried for 5-10 min to remove any residual ethanol. Once dry, the pellet was dissolved in 20µl RNase free water, incubated at 65°C for 10 min and then incubated on ice for 1h. The RNA yield was quantitated using a GeneQuant RNA/DNA Calculator and the quality assessed by analysing a sample of 500ng or less on an Agilent Bioanalyser 2100 with an RNA Pico chip.

2.8.1b cDNA synthesis

2.8.1bi First strand synthesis

7.5µg of total RNA in a volume of 11µl made up with water if necessary was mixed in a microfuge tube with 1µl T7 primer at 100pmol/µl with the sequence 5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT-3'. The primer and total RNA mixture was incubated for 10 min at 70°C in a DNA thermal cycler, then placed on ice for 5 min, then pulsed down in a centrifuge to collect the mixture at the bottom of the tube. The following reagents were added to the tube, 4µl 5 x 1st strand buffer, 2µl 0.1M DTT, 1µl 10mM dNTP mix, the reagents were mixed, pulsed down again and incubated at 42°C for 2 min. 1µl SuperScript II reverse transcriptase was added, mixed and the entire mixture incubated at 42°C for 1h.

2.8.1bii Second strand synthesis

The second cDNA strand was made by placing the first strand on ice after pulsing them down in their microcentrifuge tubes. To the first strand reaction 91µl of RNase-free water was added, 30µl 5 x second strand buffer, 3µl 10mM dNTP mix, 1µl *E.coli* DNA ligase 10U/µl, 4µl DNA Polymerase I 10U/µl and 1µl RNase H 2U/µl. The mixture was mixed and incubated for 2h at 16°C in a DNA thermal cycler. Leaving the reaction tube in the thermal cycler, 2µl T4 DNA Polymerase 5U/µl was added and the mixture incubated for a further 5 min at 16°C. The reaction was stopped by the addition of 10µl 0.5M EDTA.

2.8.1c Clean-up of cDNA

A phase-lock gel tube was pelleted by centrifuging at 12,000 x g for 30 sec. 162µl phenol:chloroform:IAA was added to the tube in which the second strand synthesis had taken place and the two solutions were mixed well. The entire mixture was then transferred to the pre-spun phase-lock gel tube and spun at 13,000 x g for 2 min. The aqueous layer above the gel following centrifugation was transferred to a clean 1.5ml tube and 85µl 7.5M ammonium acetate, 650µl 100% ethanol and 1µl 5mg/ml glycogen were added. The cDNA was allowed to precipitate overnight at –20°C. The precipitated cDNA was centrifuged for 20 min at 12,000 x g at 4°C, the supernatant removed and the pellet washed twice with 500µl 80% ethanol followed by a further spin at 12,000 x g for 5 min at 4°C. The supernatant was removed carefully as a loose pellet formed and the pellet was air-dried thoroughly to ensure any residual ethanol was removed. The pellet was resuspended in 12µl RNase-free water, heated to 65°C for 5 min and left on ice for 30-60 min to rehydrate.

2.8.1d *In Vitro* transcription (IVT) Reaction

2.8.1di IVT reaction

1µg cDNA was combined at room temperature with 11µl RNase free water, 4µl 10 x HY buffer, 4µl 10 x biotin-labelled ribonucleotides, 4µl 10 x DTT, 4µl RNase inhibitor mix, 2µl T7 RNA polymerase, mixed well and incubated in a DNA thermal cycler for 6h at 37°C mixing the reaction every 30-45 min.

2.8.1e Clean-up of cRNA

Two Chromaspin columns for each sample were prepared by repeated inversion to resuspend the gel matrix, removing the break away ends and the lid. The column was centrifuged at 700 x g for 5 min in a collection tube. The column was placed in a fresh collection tube and the IVT reaction made up to 100µl by the addition of 60µl RNase-free water. 50µl of the IVT reaction was applied to each of the two columns and centrifuged for 5 min at 700 x g. The samples of purified cRNA was collected in the collection tube during centrifugation. The addition of 50µl RNase-free water to the column and further centrifuging for 5 min at 700 x g ensured that all cRNA was recovered from the column and gave a final volume of 100µl from each column.

2.8.1ei Ethanol precipitation of cRNA

The purified cRNA samples recovered from the two chromaspin columns were pooled and precipitated together. Precipitation was carried out by the addition of 0.5 volumes (100µl) of RNase-free 7.5M ammonium acetate, 2.5 volumes (750µl) 100% ethanol and 1µl glycogen. The sample was mixed well and precipitated overnight at –20°C. The precipitated cRNA was centrifuged at 12,000 x g for 30 min at 4°C. The pellet was washed twice with 80% ethanol and centrifuged at 12,000 x g for 10 min

following each wash. The pellet was briefly air dried and resuspended in 34µl of RNase-free water, heated to 65°C for 5 min and left on ice for 30-60 min to rehydrate. The cRNA was quantitated following cleanup by measuring the optical density of the sample under uv. A 1µl aliquot of the clean cRNA was retained for analysis on an Agilent Bioanalyser 2100.

2.8.1f cRNA Fragmentation

The adjusted cRNA yield was calculated using the following formula.

$$\text{Adjusted yield} = \left(\frac{\text{amount of cRNA measured after IVT } (\mu\text{g})}{\text{amount of total RNA used in cDNA synthesis}} \right) \times \left(\frac{\text{fraction of cDNA synthesis reaction used in IVT}}{\text{fraction of cDNA synthesis reaction used in IVT}} \right)$$

An adjusted yield of ~15µg of cRNA was considered sufficient to proceed to cRNA fragmentation.

2.8.1fi cRNA Fragmentation

The fragmentation reaction was performed in a microfuge tube and consisted of 15µg (1-32µl) cRNA, 8µl 5 x fragmentation buffer, and water to 40µl final volume. This reaction was incubated at 94°C for 35 min then placed on ice. A 1µl aliquot was retained for analysis on an Agilent Bioanalyser 2100 and the remainder of the fragmented cRNA was stored at -20°C until hybridisation.

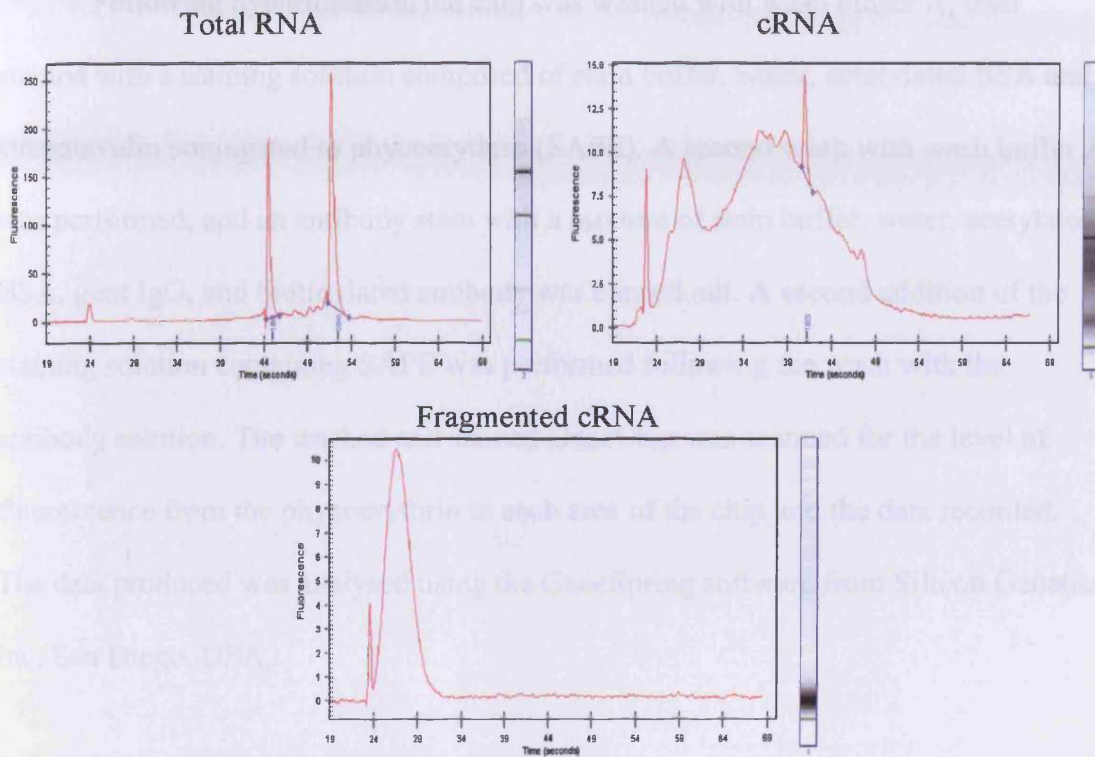
2.8.2 Agilent Bioanalyser Chip Analysis

The Agilent Bioanalyser is used to produce profiles of RNA, cDNA and cRNA which act as an indication of the quality of the product that has been produced. The bioanalyser works by mixing a very small quantity of sample with a proprietary fluorescent dye that binds specifically to DNA or RNA. The sample is then separated by size and charge following the application of an electrical current. The sample moves through a gel that fills microcapillaries in the chip in a similar manner as it

would through a standard agarose gel following the application of current to the gel. The fluorescence generated by the presence of the dye as the sample passes through a laser is detected. The level of fluorescence relates to the amount of the sample with the specific size and charge. The profiles for samples can be produced either as profiles of fluorescence against time, or as bands of different sizes as they would be seen in a conventional standard electrophoresis gel.

The 1µl aliquots of total RNA after extraction, cRNA after IVT reaction and purification, and cRNA following fragmentation were analysed on an Agilent Bioanalyser 2100 using a Agilent RNA Lab-on-a-chip. The profiles produced from this analysis are shown in figure 2.9. The profiles that are shown are those that are considered acceptable to proceed with the process of fragmented cRNA production and hybridisation to a GeneChip.

Fig 2.9 Agilent analysis profiles and representations of the agarose gels they would represent. Traces show level of fluorescence against time, which indicates the amount of sample against increasing molecular weight. Peaks equate to bands which would be seen in standard gels.



2.8.3 Chip hybridisation, Washing, Staining and Scanning of Chip

The fragmented cRNA samples were taken to Megan John in the Core Biotechnology Service who performed the hybridisation of the samples to the GeneChip followed by the washing, staining and scanning. In brief, the protocol used for this was to produce a hybridisation cocktail consisting of 15µg fragmented cRNA, a control oligo, eukaryotic hybridisation controls, herring sperm DNA, acetylated BSA, hybridisation buffer and RNase-free water in a total volume of 300µl. This cocktail was denatured by heating to 99°C for 5 min, followed by 5 min at 45°C before being collected by brief centrifugation in the preparation tube which also removes any insoluble material in the buffer. GeneChips were equilibrated to room

temperature and prehybridised for 10 min with 250µl of hybridisation buffer at 45°C while being rotated at 60rpm. Following prehybridisation the prehybridisation buffer was removed from the GeneChip and replaced with 200µl of denatured hybridisation cocktail and hybridised for 16h at 45°C rotating at 60rpm.

Following hybridisation the chip was washed with wash buffer A, then stained with a staining solution composed of stain buffer, water, acetylated BSA and streptavidin conjugated to phycoerythrin (SAPE). A second wash with wash buffer A was performed, and an antibody stain with a mixture of stain buffer, water, acetylated BSA, goat IgG, and biotinylated antibody was carried out. A second addition of the staining solution containing SAPE was performed following the wash with the antibody solution. The washed and stained GeneChip was scanned for the level of fluorescence from the phycoerythrin in each area of the chip and the data recorded. The data produced was analysed using the GeneSpring software from Silicon Genetics Inc, San Diego, USA.

Chapter 3

Transduction of cell lines with FUS and FUS antisense constructs

3.1 Introduction

Production of plasmid constructs containing the FUS gene in the sense and antisense orientations and the effective transfer of these constructs to haematopoietic cells lines is shown in this chapter. The expression of these constructs also needed to be verified at the mRNA and protein levels. In addition to the presence of the transgene within the cells, the effect of its expression in the sense and antisense orientations is shown by its effect on the ability of the cells to proliferate and on their viability in culture.

3.2 Production of PINCO constructs containing FUS and FUS

AS

Plasmid constructs containing the FUS gene (bases 71-1822 of the coding sequence) in the sense (FUS) and antisense (FUS AS) orientations were produced by the methods described in the methods sections 2.2.1 to 2.2.2. The FUS gene was excised from the pBluescript plasmid, had its overhanging 5' ends filled with a Klenow fragment, and was then ligated in a blunt ended reaction into the PINCO plasmid. The re-ligated plasmid was used to transform competent bacterial cells. Cells were identified as containing the FUS gene in either the sense or the antisense orientations by the hybridisation of a radio-labelled probe for the FUS gene to the DNA of the bacterial colonies which had been immobilised to a nylon filter. The bacterial cells identified as being positive for the FUS gene were used to extract the PINCO plasmid DNA containing FUS in the sense and antisense orientations.

3.2.1 Confirmation of PINCO plasmids containing the FUS gene in the sense and antisense orientations

The orientation of insertion of the FUS gene into these plasmids was determined in two ways, by restriction digest with the EcoR1 enzyme and by the cycle sequencing PCR reaction from a specific primer.

3.2.1.1 EcoR1 digestion

The methods for determining the sequence of FUS insertion into the PINCO plasmid are described in section 2.2.6. When cut with the EcoR1 enzyme, the PINCO plasmid is linearised and produces only one fragment of 12.748Kb while the PINCO plasmid with FUS produces fragments of 38bp, 654bp and 13,816bp and the PINCO plasmid with FUS AS gives fragments of 654bp, 1106bp and 12,742bp. This is shown graphically in figure 3.1

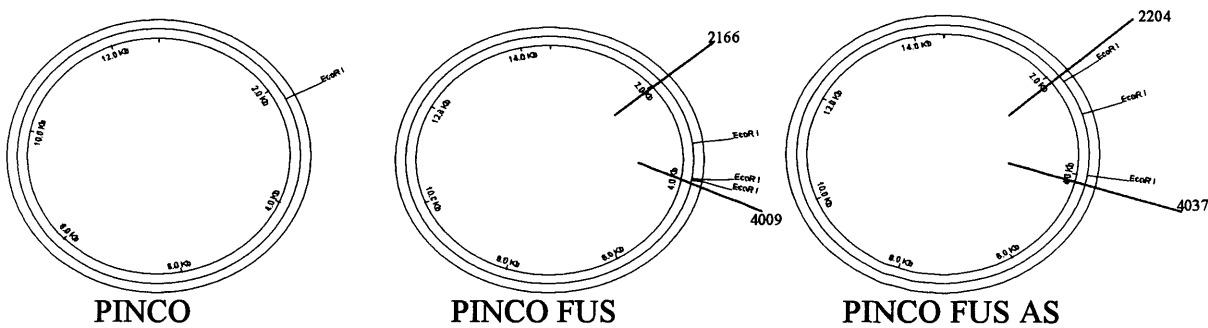


Fig. 3.1 Diagram of the EcoR1 restriction sites in the PINCO plasmid that is unaltered and with the FUS gene inserted in both directions. The blue lines indicate the sites in the plasmid at either side of the insert when the FUS or FUS AS sequences were inserted. The EcoR1 restriction sites are also indicated.

When the plasmids were digested with EcoR1 and the fragments visualised in an agarose gel, it was shown that plasmids with FUS (showing only one band of

654bp) and FUS AS (showing two bands, one of 654bp and one of 1106bp) had been generated. The restriction fragments of PINCO plasmid with FUS and FUS AS are shown in figure 3.2

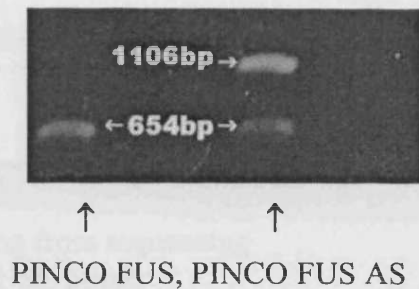
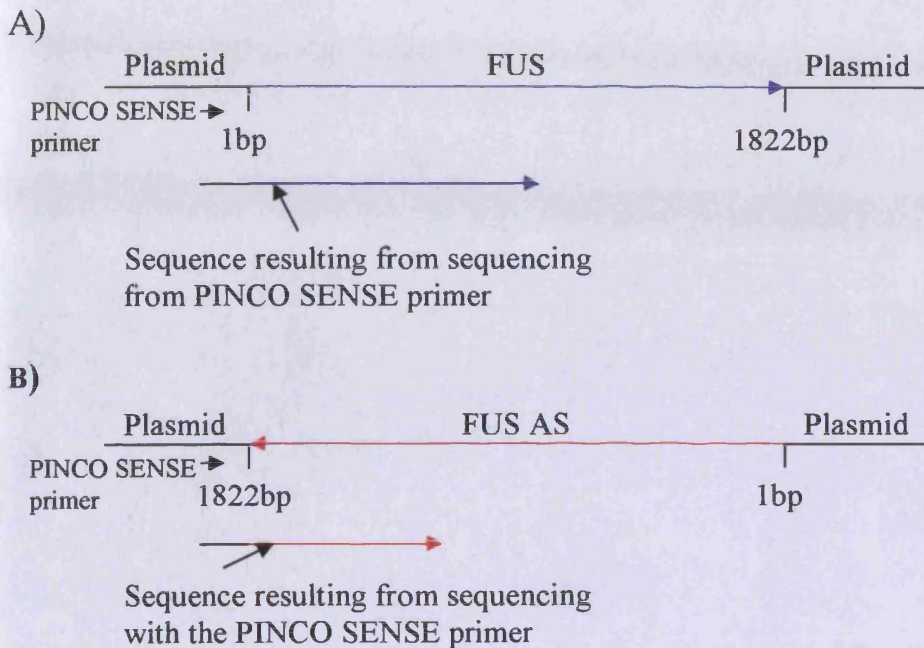


Fig. 3.2 Restriction fragments of the PINCO plasmid with FUS inserted both orientations visualised in an agarose gel.

3.2.1.2 Cycle sequencing PCR

Cycle sequencing PCR was performed on the plasmid DNA extracted from the bacterial cells using the PINCO SENSE primer which lies on the upstream side (5') of the *Hind*III cloning site into which the FUS gene was ligated. The method used to perform the cycle sequencing PCR is described in section 2.2.9. The sequences obtained from the sequencing reaction were aligned with the published FUS sequence. The result of this alignment indicated that, in agreement with the restriction digest results of the same plasmids, FUS had been inserted into the PINCO plasmid in both orientations and that the bacterial colonies containing the sense and the antisense plasmids could be identified. The results of aligning the cycle sequencing PCR reaction and the FUS sequence are shown schematically in figure 3.3.

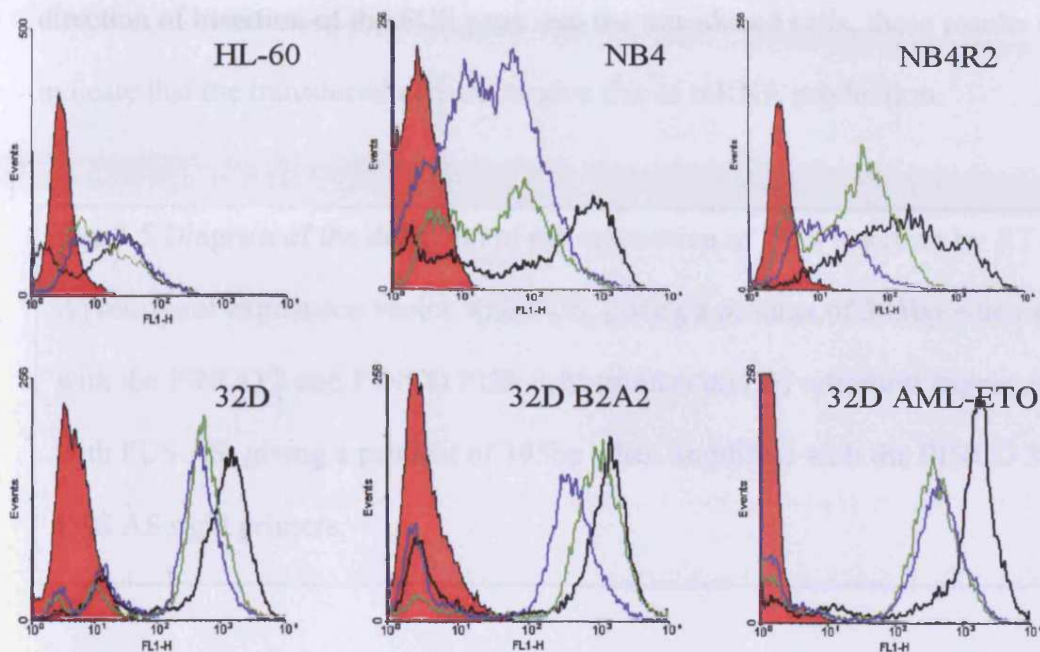
Fig. 3.3 Schematic representations of the results of the cycle sequencing PCR reaction where the sequencing results are aligned next to the FUS gene when inserted into the plasmid in A) the sense direction and B) the antisense direction.



3.3 Production of cell lines positive for FUS in the sense and antisense orientations

A population of cells positive for the GFP marker on the retroviral construct were selected from a population of cells that had been transduced using the retrovirus derived from the PINCO plasmid, or PINCO containing FUS or PINCO containing FUS AS. The methods for transduction of the cell lines with the retrovirus and sorting of the resultant population by flow cytometry are given in sections 2.3.3 and 2.3.5. Figure 3.4 shows the level of GFP expression in control untransduced cells of each cell line and the change in the level of fluorescence in the FL1 channel, where the GFP expressed within the cell is detected when the cells have been transduced with the control retroviral constructs and those containing FUS and FUS AS.

Fig 3.4 Histograms showing the fluorescence intensities of control (red) and cell lines transduced with control construct (black), FUS (green) and FUS AS (blue) constructs co-expressing GFP.



These graphs shows that all the cells lines containing the control, FUS and FUS AS constructs have a higher level of fluorescence in the FL-1 channel than the untransduced cell lines although some of the transduced cell lines fluoresce at a higher level than others containing the same construct.

3.4 Confirming the orientation of FUS insertion within transduced cell lines

To confirm the orientation of the insertion of FUS in transduced cell lines, an RT-PCR was carried out as described in section 2.4.4. In this PCR, the primers used were designed to create a product only if the FUS insert in the retroviral expression vector was in the sense orientation with one set of primers, or if the FUS gene was in

the antisense orientation with the other primer combination. This is shown diagrammatically in figure 3.5

The results of these PCRs which indicate the orientation of FUS in the integrated retroviral construct are shown in figure 3.6. In addition to confirming the direction of insertion of the FUS gene into the transduced cells, these results also indicate that the transduced constructs give rise to mRNA production.

Fig. 3.5 Diagram of the detection of the orientation of FUS insertion by RT-PCR in A) retroviral expression vector with FUS, giving a product of 385bp when amplified with the PINCO 3 and PINCO FUS right primers and B) retroviral expression vector with FUS AS, giving a product of 395bp when amplified with the PINCO 3 and PINCO FUS AS right primers.

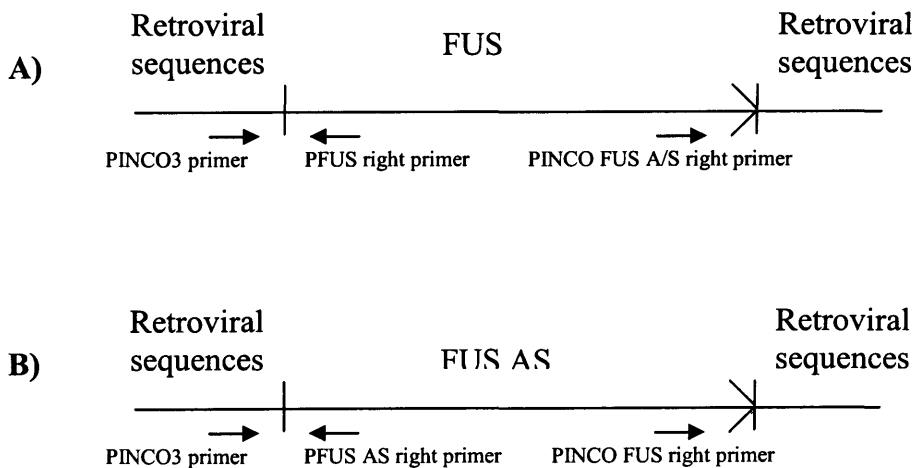
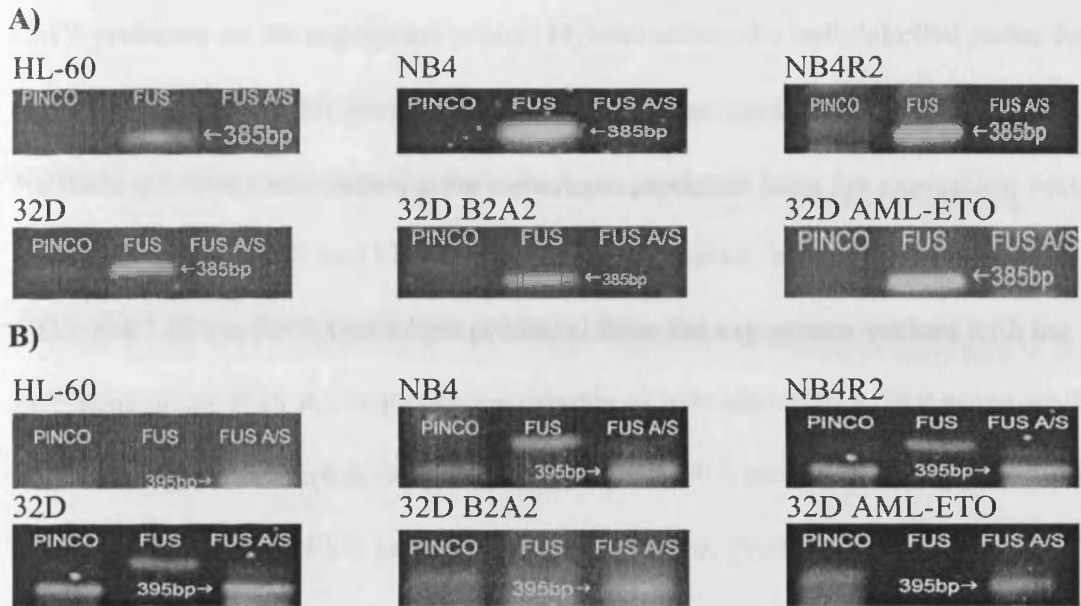


Fig. 3.6 Results of the RT-PCRs used to detect FUS orientation in transduced cell lines

A) when FUS was inserted and B) when FUS AS was inserted



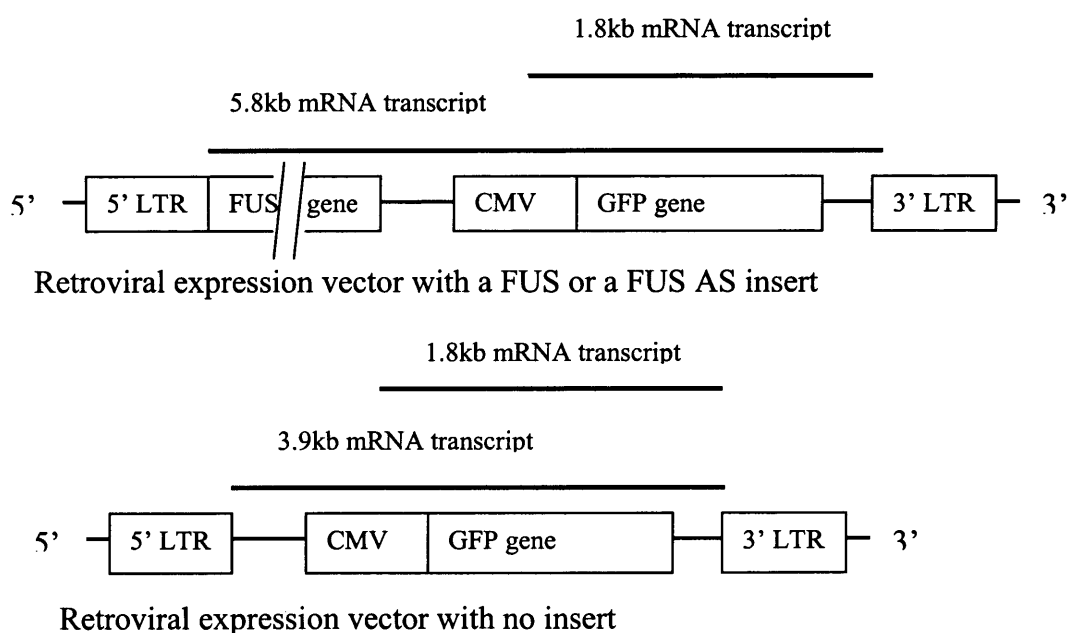
The additional band seen in the control (labelled PINCO) in the PCR reactions to detect FUS AS was not identified and it remains possible that this band contains an identical product to the FUS AS samples.

3.5 Ensuring the retroviral constructs with FUS in the sense and antisense directions are functional within the cell lines

Northern blotting was carried out on samples of the cell lines transduced with FUS and FUS AS and an unaltered retroviral expression vector to show whether the amount of mRNA produced in the cells was altered as a result of the insertion of the FUS gene and its antisense construct. The procedure used to perform the Northern blots is described in section 2.4.2. The Northern blots were hybridised with radiolabelled probes for the FUS gene. This was done to show the amount of FUS mRNA, or FUS AS mRNA that was present in the cell lines. The FUS probe was also capable of hybridising to the endogenous FUS mRNA produced in the cell lines so the blots were also hybridised to a radiolabelled probe for the GFP gene which is transcribed from the same 5' LTR promoter in the retroviral expression vector and as

a result, the GFP probe co-localises with the retroviral FUS gene on the Northern blots as well as localising to the shorter GFP transcript that is produced from the CMV promoter on the expression vector. Hybridisation of a radiolabelled probe for the housekeeping β -actin gene was carried out to show equal loading of RNA in the Northern gel. Diagrams showing the transcripts produced from the expression vector and its forms with FUS and FUS AS insertions are shown in figure 3.7. Both the 5.8kb and 1.8kb mRNA transcripts produced from the expression vectors with the FUS gene or the FUS AS sequence are capable of hybridising to a GFP probe while only the longer transcript is capable of binding to a FUS probe. The FUS probe was capable of binding the FUS and the FUS AS sequence. From the expression vector that has no inserts, the 3.9kb and 1.8kb mRNA transcripts that are produced are capable of binding to the GFP probe, while neither are capable of binding the FUS probe due to the absence of the FUS gene in either orientation from this construct.

Fig. 3.7 Diagram of the transcripts produced from the 5'LTR and CMV promoters in the retroviral expression vector.



The results of the expression of the FUS gene and FUS AS constructs within the cell lines and the effect of these on the amount of FUS mRNA detectable in the cell lines is shown in figure 3.8. These Northern blots show that mRNA produced from the inserted retroviral expression vectors is produced in the transduced cell lines. The co-localisation of the GFP probe and FUS probe indicates that the mRNA for both of these genes is being produced as one transcript of 5.7kb from the 5'LTR promoter in the constructs with the FUS or FUS AS inserts. As the empty retroviral vector has no FUS gene in either orientation, the mRNA transcript produced from it has no capability to hybridise with the FUS probe. The presence of this construct in the cells transduced with the empty vector alone is shown by the 3.9kb transcript indicated in the cells containing this vector in each cell line in the blots hybridised with a GFP probe but which is not apparent when probed for FUS mRNA. In the Northern blots that were probed for the FUS gene, a 1.8kb mRNA was also detected. This 1.8kb FUS mRNA is the endogenous FUS that is expressed by the cell lines. Overall these results are consistent with the flow cytometric analysis in figure 3.4. Expression levels are high for both the FUS and FUS AS constructs in the 32D based cell lines. In the cell lines of human origin the levels of expression are generally lower, particularly in the case of FUS expression in HL-60 cells.

The effect of the mRNA transcripts produced from the empty retroviral expression vector on the level of FUS protein detectable in the cell lines was determined by Western blotting as described in section 2.4.6. The antibody that was used to detect the expression of the FUS protein was capable of detecting the human form of the FUS protein but it was also reactive with the murine form. The results of all the western blots are shown in figure 3.9.

Fig. 3.8 Northern blots showing the expression of FUS mRNA in the transduced cell lines. 10ng of RNA was used for each sample. A) Northern blots probed with a radiolabelled probe for FUS mRNA. The level of expression of the housekeeping β -actin gene is shown below the annotated Northern blot probed with the FUS gene and B) Northern blots probed with a radiolabelled probe for GFP mRNA. The level of β -actin is the same as in the previous images as the same Northern filters were used. All samples were loaded in the order: vector control, FUS, FUS AS in all blots (left to right).

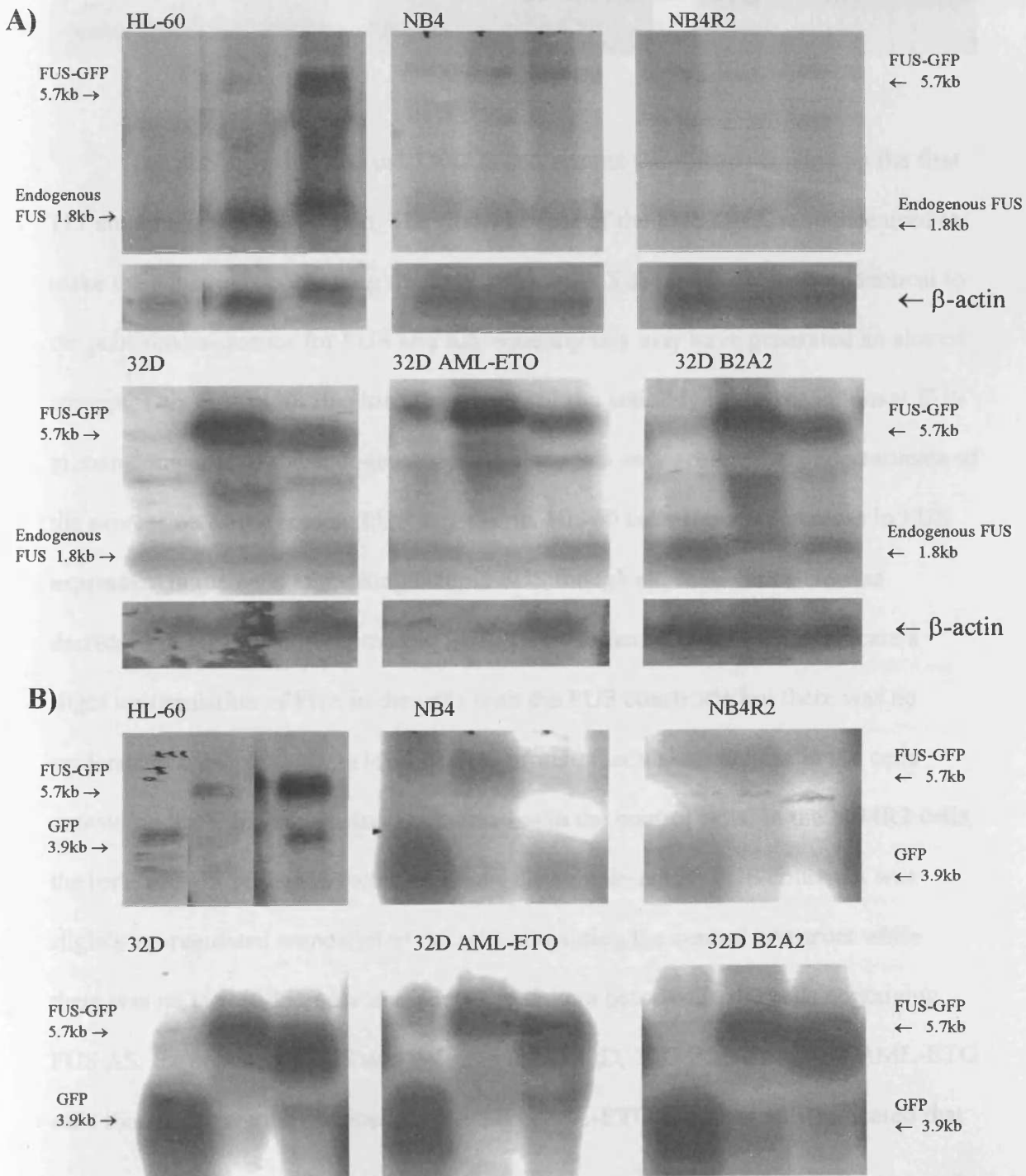
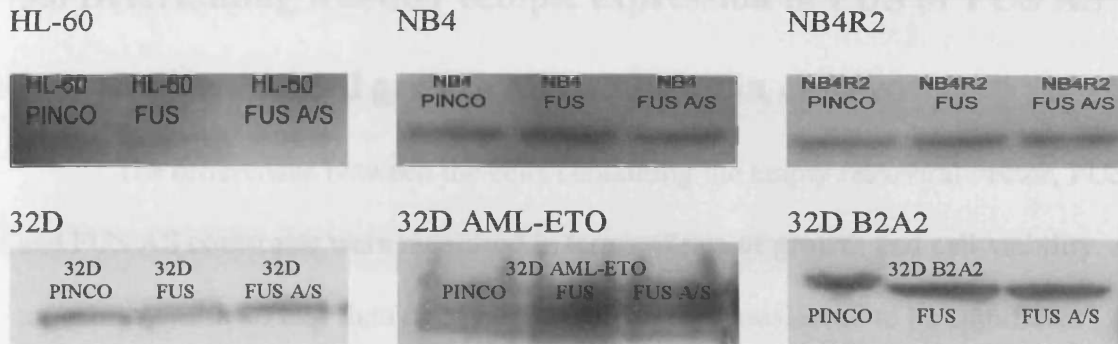


Fig 3.9 Western blots showing the level of FUS protein detectable in the different cell lines. In each blot an equal amount of protein was used from each cell type. The cells containing the empty retroviral expression vector are labelled PINCO.



The antibody that was used was raised against the epitope created by the first 117 amino acids of the protein. The first 71 bases of the FUS DNA sequence used to make the constructs containing the FUS and FUS AS sequences were not identical to the published sequence for FUS and subsequently this may have generated an altered epitope. This may result in a lower reactivity of the antibody to the recombinant FUS protein compared to the endogenous protein and this may produce an underestimate of the expression of the ectopic FUS expression. HL-60 cells show no increase in FUS expression in the cells expressing ectopic FUS though expression of FUS was decreased in HL-60 cells expressing FUS AS construct. The NB4 cells indicate a slight up-regulation of FUS in the cells with the FUS constructs but there was no evidence of a decrease in the level of FUS protein that was detectable in the cells containing the FUS AS construct compared with the control cells. In the NB4R2 cells, the level of FUS protein detectable in the cells containing the FUS construct was slightly up-regulated compared to the cells containing the control construct while there was no visible decrease in the level of protein detected in the cells containing FUS AS. Expression of FUS was detected in the 32D, 32D B2A2 and 32D AML-ETO cells though the results obtained for the 32D AML-ETO cell lines only indicated that

the presence of the FUS AS sequence did result in a decrease in the level of FUS protein detectable in the cells compared to the cells containing the FUS retroviral construct.

3.6 Determining whether ectopic expression of FUS or FUS AS had an effect on cell growth and viability in culture

The differences between the cells containing the empty retroviral vector, FUS and FUS AS constructs were measured in terms of rate of growth and cell viability. A probability value of less than or equal to $p = 0.05$ was considered to be significant when generated using Student's T-test. Table 3.1 shows the results of the statistical analysis of the cell growth and viability. These results indicate that the insertion of the FUS or FUS AS constructs into the HL-60, NB4, NB4R2, 32D, 32D AML-ETO and 32D B2A2 cells does not alter the ability of these cells to proliferate and remain viable in culture. The 32D B2A2 cell lines containing FUS and FUS AS constructs showed a lower proliferative capacity on days 3 and 4, and day 3 only respectively. The growth curves of the transduced 32D B2A2 cells only are discussed further in figure 3.10.

3.6.1 32D B2A2 cells

The proliferation rate and cell viability of 32D B2A2 cells when transduced with the control, FUS and FUS AS and maintained in normal culture conditions are shown in figure 3.10.

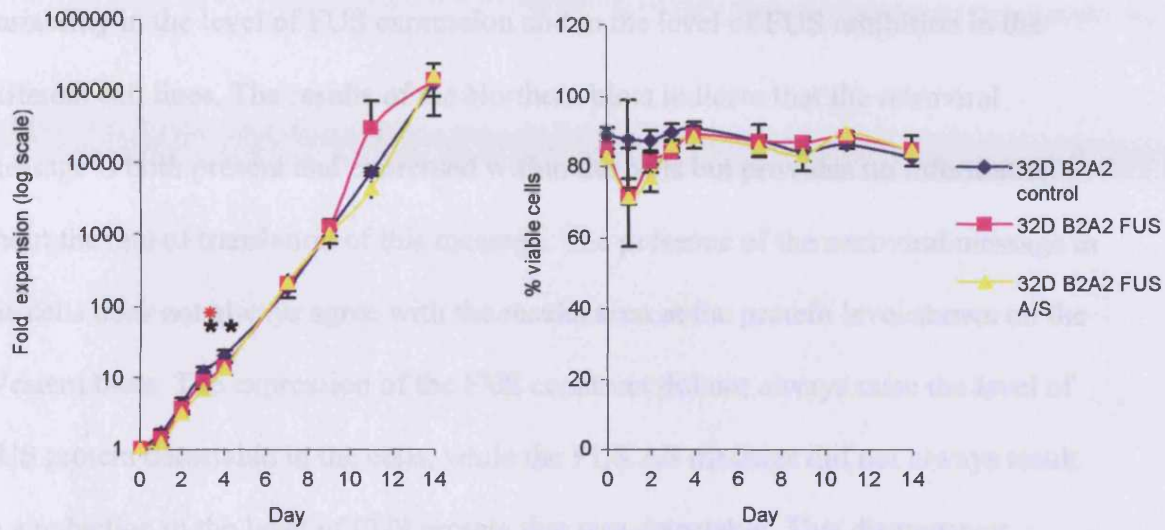
Despite the decrease seen on day 1, the viability of the 32D B2A2 cells were not significantly affected by the expression of the control, FUS and FUS AS constructs. The growth of the 32D B2A2 cells expressing FUS or FUS AS was transiently affected being significantly lower than the control cells on day 3 ($p = 0.048$) in the case of the 32D B2A2 FUS AS cells while the 32D B2A2 FUS cells

were significantly lower than the control cells on days 3 and 4 ($p = 0.043$ and 0.007 respectively).

Table 3.1 Effects of the insertion of the control, FUS and FUS AS constructs on the growth and viability on culture of the HL-60, NB4, NB4R2, 32D, 32D AML-ETO and 32D B2A2 cell lines over 14 days.

Cell line	Fold expansion at day 14 +/-SD	Probability day 14	Viability on day 14 +/- SD	Probability day 14
HL-60 control	134052 ± 11307		89.5 ± 3.00	
HL-60 FUS	140604 ± 5535	0.42	88.3 ± 2.71	0.46
HL-60 FUS AS	120370 ± 62142	0.38	84.0 ± 5.77	0.17
NB4 control	8947 ± 6307		92.19 ± 6.75	
NB4 FUS	11479 ± 1723	0.68	89.4 ± 1.05	0.579
NB4 FUS AS	9694 ± 6419	0.56	92.4 ± 6.55	0.637
NB4R2 control	3052 ± 308.4		87.1 ± 3.84	
NB4R2 FUS	3043 ± 632	0.522	89.4 ± 0.86	0.191
NB4R2 FUS AS	3098 ± 688.9	0.482	91.0 ± 1.72	0.083
32D control	262979 ± 176468		84.1 ± 9.41	
32D FUS	249083 ± 146120	0.921	86.2 ± 3.90	0.742
32D FUS AS	693186 ± 1140884	0.554	86.8 ± 2.10	0.649
32D AML-ETO control	384990 ± 436447		74.2 ± 8.50	
32D AML-ETO FUS	606583 ± 367644	0.538	85.1 ± 14.01	0.311
32D AML-ETO FUS AS	557750 ± 319961	0.609	77.6 ± 4.56	0.573
32D B2A2 control	122133 ± 40748		82.5 ± 0.20	
32D B2A2 FUS	138667 ± 42350	0.652	84.6 ± 0.489	0.927
32D B2A2 FUS AS	156000 ± 13879	0.245	84.4 ± 0.489	0.927

Fig 3.10 Graphs showing the growth and viability of 32D B2A2 cells transduced with control, FUS and FUS AS constructs. Error bars on the graph indicate \pm one standard deviation.



- * Cells containing FUS construct show significant difference
- * Cells with FUS AS construct show significant difference

3.7 Discussion of cellular response to the up- and down-regulation of FUS expression

The results presented in this chapter demonstrate that the expression constructs containing the FUS gene in both orientations were created and these were successfully transduced into the HL-60, NB4, NB4R2, 32D, 32D AML-ETO and 32D B2A2 cells lines from which populations positive for the GFP selectable marker were selected. The level of GFP expression in the selected populations was varied depending on the transduced cell lines and the construct that they contained. The presence of the FUS gene and its orientation in the transduced cell lines was determined by PCR and the presence of the mRNA for both the FUS gene and the GFP sequence was detected by Northern blotting. The PCR reactions indicated that the FUS or the FUS AS sequences were present within the cells and the Northern

blots showed that the mRNA transcripts that were produced from the retroviral expression vectors containing the FUS gene in either insertional orientation were produced within the cells and the size was as predicted.

The effect of the FUS and FUS AS sequences on the level of protein detectable within the cells was detected by Western blotting. There was apparent variability in the level of FUS expression and in the level of FUS inhibition in the different cell lines. The results of the Northern blots indicate that the retroviral message is both present and expressed within the cells but provides no information about the rate of translation of this message. The presence of the retroviral message in the cells does not always agree with the results seen at the protein level shown on the Western blots. The expression of the FUS construct did not always raise the level of FUS protein detectable in the cells, while the FUS AS message did not always result in a reduction in the level of FUS protein that was detectable. This discrepancy between the results may be due to the Northern blot results being specific for the retroviral message while the Western blot is not specific for the ectopically expressed FUS. Possible factors that may affect the expression of the retroviral construct may include de novo methylation at the 5' position of cytosine bases in CpG dinucleotides by cytosine methyltransferases (Heim *et al.* 2000; Pannell *et al.* 2001; Swindle *et al.* 2002). This methylation leads via histone 3 and 4 deacetylation by the HDAC complex to chromatin condensation and this is prohibitive to transcriptional initiation resulting in silencing of the provirus (Pannell *et al.* 2001; Swindle *et al.* 2002). The integration site of the provirus is also important as different areas of the genome have different transcriptional activity to others and insertion of the retroviral sequence into some areas of the chromosome may entirely preclude its expression (Swindle *et al.* 2002). Additionally, retroviral silencer elements may play roles in the silencing of the provirus. These are DNA binding sites for trans-acting factors that directly or

indirectly reduce transcriptional initiation at promoters. At least four of these elements are found in the LTR and adjacent primer binding site of the viral construct. These elements act in an additive manner to increase the probability of silencing. The effect of one element alone is not sufficient to induce complete silencing (Swindle *et al.* 2002). Mechanisms of silencing of murine based retroviruses may be different in the murine cell lines as mice have evolved pathways for silencing the larger number of endogenous retroviruses that are contained within their genomes (Heim *et al.* 2000).

Another factor affecting the efficacy of this approach is the high level of endogenous FUS expression in haematopoietic cell lines which has been previously reported (Mills *et al.* 2000). In this context expression of a retroviral construct containing the FUS gene may not significantly affect overall expression levels and therefore any additional increase in the level of FUS protein in these cells may not be sufficient to be detectable by Western blotting. The very high level of endogenous FUS in the cell lines may also be one reason why the antisense retroviral construct did not demonstrate silencing, or even in some cases, a reduction of the level of protein that was detectable in the cells.

The effect of expression of FUS or FUS AS sequences on the proliferation of the cells and their viability in culture did not show there to be any differences between the control cells expressing the empty retroviral construct and the cells expressing the FUS or the FUS AS sequence. Minor differences that were seen in the 32D B2A2 cells with the FUS and FUS AS sequences on single days were the results of little variation occurring within the results resulting in a very small difference being statistically significant. Previously reported work (Perrotti *et al.* 1998) in which the level of expression of the FUS gene had been modulated using ectopic FUS and FUS AS expression had shown that in 32D cells, over-expression of the FUS gene resulted in faster proliferation than the parental cells while the FUS AS expression in these

cells led to a moderate decrease in proliferation as was the case for 32D cells expressing the BCR/ABL fusion protein. These results were not seen in the cells transduced in this project and the differences seen may be due to differences in the retroviral constructs used or the fact that clonal populations were employed in the aforementioned study. This is the only report containing information on the effect of over expression or of under-expression of FUS itself as both it, and the related EWS protein, are more commonly studied as the fusion proteins they form following chromosomal translocation.

In conclusion, the presence of FUS or FUS AS constructs and their expression in the different cell lines resulted in no significant difference being observed in the growth and viability of the cells in culture. The level of FUS protein expressed in the cells is raised by the presence of the FUS construct in the NB4, NB4R2 and 32D B2A2 cell lines only and FUS AS detectably decreases the level of FUS protein in the 32D B2A2 cell line only. Nevertheless, the constructs were expressed in all the transduced cell lines and mRNA from the constructs was detectable in each transduced cell line.

Chapter 4

**Response to ATRA of haematopoietic cells
transduced with the FUS and FUS antisense
constructs**

4.1 Introduction

All-trans retinoic acid (ATRA) is the most biologically active of the retinoids and is metabolised by several cytochrome P450s, proteins that catalyse the oxidation of numerous endobiotics and xenobiotics. ATRA is metabolised into several oxidised metabolites, all having biological activity. ATRA and its metabolites can inhibit growth, induce differentiation and regulate expression of several genes involved in differentiation and embryogenesis (Idres *et al.* 2002). Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) both have three sub types, α , β and γ which belong to a family of nuclear hormone receptors acting as ligand activated transcription factors. When complexed with their ligand, ligand-receptor complexes act as inducible transcription regulators of genes by binding to specific retinoic acid response elements (RAREs). ATRA and its metabolites do not bind RXR's although they will bind RARs (Idres *et al.* 2001; Idres *et al.* 2002). 9-cis retinoic acid can bind both RARs and RXRs (Niitsu *et al.* 2002). RXR receptors form functional heterodimers with both RAR α and PML-RAR α . Mutations in the E-ligand binding region of RAR α in the PML-RAR α fusion protein have been linked to resistance to ATRA (Idres *et al.* 2001). In the HL-60 cell line, the RAR/RXR pathway was found to be more important than the RXR/RXR pathway in the differentiation and proliferation of these cells (Shiohara *et al.* 1999). A functional RAR α is not required for granulocytic differentiation and NB4 cells resistant to ATRA can differentiate using the protein kinase A (PKA)/RXR pathway as well as the RAR α -RXR pathway (Benoit *et al.* 1999).

As discussed briefly in section 1.4.2, when ATRA is used to induce differentiation in myeloid cells the expression of multiple genes is induced which leads to altered cell growth and the development of the mature myeloid cell

phenotype. Previously, differential display has been used to examine the changes in gene expression resulting from the treatment of the human promyelocytic cell line, HL-60. One hour of exposure to ATRA resulted in many genes having strongly altered expression in these cells including the oncogenes MYC and FUS. FUS has been shown to be dramatically down regulated in the process of granulocytic differentiation when induced by either ATRA or DMSO in the HL-60 cell line whereas expression of FUS is relatively stable in the same cell line when monocytic differentiation was induced using Vitamin D₃ or TPA for 24h. HL-60 cells that are less responsive to the differentiation inducing effects of ATRA did not show alteration in FUS expression. This shows changes in FUS expression is an early event in the induction of granulocytic differentiation by ATRA (Mills *et al.* 2000). Other genes which were activated immediately after exposure to ATRA included cell cycle regulators, transcription factors and regulators of cell division and apoptosis. A large number of genes relating to the mature myeloid cell function were seen to be activated within the first day of exposure to ATRA during which the cells are actively cycling. Genes activated within the first 24h may be important for the initiation of differentiation while genes showing delayed activation (over 24h after commencement of treatment) may be involved in the progression of differentiation maturation and apoptosis.

When HL-60 cells are treated with ATRA they become committed to the neutrophil lineage within 24h and this is indicated by the expression of CD38 on their surface. The induction of CD38 is an early and specific event in retinoid induced differentiation in myeloid cells. CD38 is not upregulated by other inducers that are used to generate neutrophil differentiation and is not a natural marker of neutrophil differentiation (Mills *et al.* 1999). An increased proliferative rate was observed in the HL-60 cells within 24h of commencing treatment with ATRA. Within three days of

treatment with ATRA in the HL-60 cells, the morphological, phenotypic and functional characteristics of neutrophils are evident (Mills *et al.* 1998). The cells begin to adhere to plastic (30%) and may aggregate to form clumps. More than two nuclear lobes are seen in the cells which gives them the appearance of granulocytes. The size of the cells increases and there is increased cytoplasm. The morphology of the cell becomes increasingly irregular (Hu *et al.* 1993).

HL-60 cells treated with ATRA begin to show expression of the cell surface marker CD11b before the cells have completed their current cell cycle. CD11b is expressed by both monocytes and neutrophils but not by HL-60 cells that are exponentially proliferating. The cells undergo approximately three cell cycles following treatment with ATRA and these occur at the same rate as the usual cell cycle for untreated cells. Following 96h of ATRA treatment the proliferation rate of the cells begins to decline and on subsequent days no further proliferation is observed. This effect is seen whether the cells are treated with ATRA to induce neutrophilic differentiation or vitamin D₃ to induce monocytic differentiation. It has been shown that the control of CD11b expression and the control of initiation of the maturation divisions of the cells is at least in part a separate process. Cells in the first 2h of the G₁ phase of the cell cycle are capable of responding to ATRA while cells in other phases of the cycle proceed through to their next G₁ phase before beginning to respond to ATRA. Following the ATRA sensitive period of G₁ the cells enter a period of 2 to 4h where they are insensitive to ATRA (Drayson *et al.* 2001). Similar results to those seen in HL-60 cells were observed when NB4 cells were treated with ATRA (Drayson *et al.* 2001). The processes within the cell for controlling growth arrest and cell maturation are not obligatorily linked and there might be an independent regulatory event that directly initiates maturation divisions or CD11b expression which still

affects the number of maturation divisions and the rate of maturation (Drayson *et al.* 2001; Lee *et al.* 2002).

Within 4 days, growth arrest was seen in NB4 cells treated with ATRA (Idres *et al.* 2001). The maturation resistant NB4R2 cell line was generated by mutagenization from NB4 cells (Dermime *et al.* 1993) and has a defective RAR signalling pathway which makes the ATRA metabolites inefficient at inducing differentiation in these cells. The growth rate of NB4R2 cells is unaffected by ATRA although weak phenotypic effects are seen (Dermime *et al.* 1993). This cell line will differentiate in the presence of specific RXR agonists (Idres *et al.* 2002).

The aim of this section of the study was to investigate the effect of the expression of FUS and FUS AS sequences on the differentiation responses of different haematopoietic cell lines when induced with ATRA. The effect of the expression of FUS and FUS AS sequences and the induction of ATRA was measured in the context of cell growth, viability as well as the expression of differentiation associated cell surface markers.

4.2 Experimental procedures

4.2.1 Expression of FUS transcript following treatment with ATRA

To determine whether the FUS gene was down regulated in untransduced NB4 and NB4R2 cells in response to the treatment of these cell lines with ATRA, NB4 cells and NB4R2 cells were treated with ATRA at a concentration of 10^{-7} M for 24h. Samples of these treated cells were harvested before the start of treatment and at 30 min, 1, 2, 4, 8, 16 and 24h following the start of treatment. These cells were snap frozen and the RNA extracted as described in section 2.4.1. Northern blots for FUS mRNA were performed using 10 μ g of the extracted RNA from each sample and these

Northern blots were hybridised to a radiolabelled probe as described in sections 2.4.2 and 2.4.3.

4.2.2 Effect of ATRA on transduced cells

All the cell lines transduced with control, FUS and FUS AS constructs were treated with ATRA at a concentration of 10^{-7} M for a period of 14 days. On days 0, 1, 2, 3, 4, 7, 9, 11, and 14 the growth and viability of the transduced 32D, 32D AML-ETO and 32D B2A2 cells in culture were determined as described in sections 2.5.4. The HL-60, NB4 and NB4R2 transduced cell lines were assessed on days 0, 4, 7, 11 and 14. Flow cytometric analysis was carried out on days 1, 4, 7, 11 and 14 following the start of treatment to assess the expression of cell surface markers on the cells. For the HL-60, NB4 and NB4R2 cell lines the antibodies used to identify the cell surface markers expressed by the cells were CD11b, CD38 and CD33. Expression of CD11b on granulocytes and monocytes can be considered to be a general marker used for identifying maturation of these cells (Sanchez-Madrid *et al.* 1983; Springer *et al.* 1979; Yokomori *et al.* 1998). CD38 is a membrane glycoprotein that is expressed on cells that are in the early stages of myeloid development but not in the intermediate or mature stages (Jackson *et al.* 1990; Konopleva *et al.* 1998). CD38 is specifically induced in response to treatment of the cells with ATRA (Mills *et al.* 1999). CD33 is a myeloid specific cell surface antigen that is expressed in blasts, promyelocytes and is shown to reduce in expression in cells undergoing neutrophil development but remains expressed at a high level during monocytic differentiation (Drexler 1987; Favaloro *et al.* 1987; Griffin *et al.* 1984). In the 32D, 32D AML-ETO and 32D B2A2 cell lines that are of murine origin, the antibodies used to assess the differentiation response of the treated cells were CD11b, F4/80 and Ly-6G. CD11b in these cell lines is expressed similarly as it was on the human cell lines already described. F4/80

is a membrane glycoprotein that is expressed by almost all mature mouse macrophages and circulating monocytes (Austyn *et al.* 1981; Gordon *et al.* 1992; Hume *et al.* 1984; Lee *et al.* 1985). Ly-6G is expressed on mature granulocytes. Immature monocytes in the bone marrow may also express Ly-6G but this is at a lower level than those found in the circulating population (Fleming *et al.* 1993; Lagasse *et al.* 1996). The methods used to identify the phenotype of the cells treated with ATRA by flow cytometry are described in section 2.6. All these experiments were carried out in triplicate and the mean results used in the analysis of the experiments. In all experiments treated samples were compared to untreated control cell cultures. When treating the 32D and 32D AML-ETO cell lines that are dependent on IL-3 to survive in culture, one set of experiments were performed in the presence of IL-3 in the culture while a second set of experiments were performed in the absence of IL-3. The untreated control cultures for these experiments were maintained in the presence of IL-3 as the cells would normally be maintained in culture.

4.2.3 Level of FUS protein expression in transduced cells

Western blotting was performed on the HL-60, NB4 and NB4R2 cell lines that had been transduced with control, FUS and FUS AS constructs and compared with cells treated with ATRA for 4 days. The FUS protein was detected on these Western blots using an anti-FUS antibody at a concentration of 0.1 µg/ml. The method for performing Western blots is described in section 2.4.6. The level of FUS protein expressed in the 32D, 32D AML-ETO and 32D B2A2 transduced cells lines following treatment with ATRA was not investigated since at the time of study the reactivity of this antibody with murine FUS was in doubt.

Northern blotting was also employed to determine the effect on the level of FUS mRNA expressed in the transduced cells lines following treatment with ATRA.

The Northern blots were performed as described in section 2.4.2 and the level of FUS mRNA was detected using a radio labelled FUS DNA sequence.

4.2.4 Dose response to ATRA

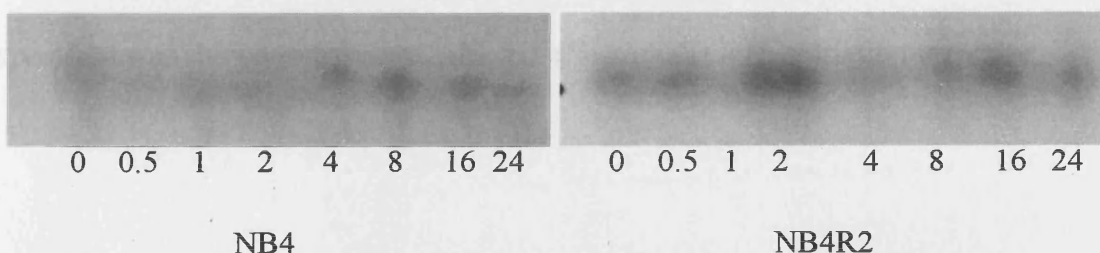
Dose response experiments using ATRA at concentration of 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M were performed and the results compared to untreated control cultures on the transduced HL-60, NB4 and NB4R2 cell lines containing control, FUS and FUS AS constructs. The effect of the different ATRA concentrations were assessed as in the previous experiments using the capacity of the cells to differentiate and expression of the CD11b, CD38 and CD33 cell surface markers on days 0, 4, 7, 11 and 14. All experiments were repeated in triplicate.

4.3 Results

4.3.1 Expression of FUS in NB4 and NB4R2 cell lines following treatment with ATRA over 24h

In HL-60 cells FUS expression had been shown to be down-regulated within 30 minutes of treatment with ATRA. To determine the effect of ATRA treatment on the expression of endogenous FUS Northern blots were performed on samples of NB4 and NB4R2 cells treated with ATRA at a concentration of 10^{-7} M at different intervals up to 24 hours.

Fig 4.1 Northern blots showing the level of FUS mRNA detectable at different time intervals in hours following commencement of treatment with ATRA at 10^{-7} M.



As with results previously published on HL-60 cells (Mills *et al.* 2000), FUS mRNA was detectable in NB4 cells before exposure to ATRA at 10^{-7} M but by 30 min of treatment the mRNA was undetectable (figure 4.1). At 8, 16 and 24h, some mRNA was detectable but at a lower level than in the untreated cells. In the NB4R2 cells that do not respond to ATRA, FUS mRNA was detectable at 30 min, 2, 4, 8, 16h and a lesser level of the mRNA was detectable at 24h following the start of treatment. The FUS mRNA sample for 1h of treatment was not detectable due to degradation of this RNA sample. These results indicate that, as in HL-60 cells, FUS is rapidly down-regulated in NB4 cells (within 30 min) upon treatment with ATRA. However, in the less responsive NB4R2 cells, the FUS gene is not down-regulated upon ATRA treatment until the much later time point of between 16 and 24h following the start of treatment. The NB4R2 cells do show a much reduced response to ATRA than the NB4 cells and this is reflected in the later reduction of FUS mRNA expression.

4.3.2 Effect of ATRA on transduced cell lines

As the FUS gene was down-regulated in response to treatment with ATRA in HL-60, NB4 and much later in NB4R2 cells the effect of treatment with ATRA of cell lines transduced with FUS and FUS AS was studied. Cell lines dependent on IL-3 for continued proliferation were treated in the presence and in the absence of IL-3 to determine whether the expression of FUS or FUS AS within these cells during treatment with ATRA abrogated the need for this growth factor. Due to the volume of results that were created by the number of cell lines that were used, the data has been collated and is represented in table 4.1. Data are shown in full where FUS or FUS AS expression was shown to have a significant effect on the differentiation response.

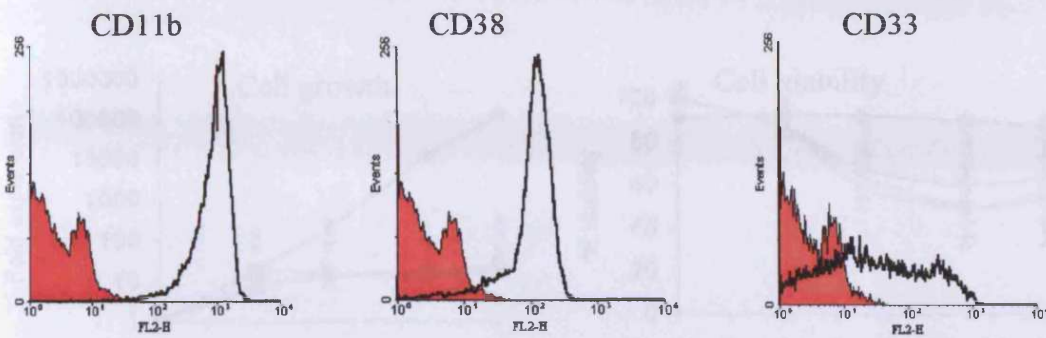
4.3.2a Overview of responses

Differences in response to ATRA treatment were seen between cells expressing FUS and FUS AS constructs in the NB4 and 32D (+IL-3) cells where the expression of FUS AS lead to some resistance to the differentiation induced by treatment with ATRA. In the case of NB4R2 and 32D B2A2 cells the presence of FUS AS leads to the differentiation of cells that otherwise show resistance to ATRA. The results represented in Appendix 3, table A3.1, indicate that there is no difference in the differentiation response to ATRA between the cells expressing FUS or FUS AS in the HL-60, 32D (-IL-3), or the 32D AML-ETO cell lines, (with or without IL-3). Example histograms indicating how the values for the expression levels of the cells surface markers were generated are shown in figure 4.2. An example of a set of results in which no difference was observed between the cells containing control, FUS and FUS AS constructs is shown in figure 4.3.

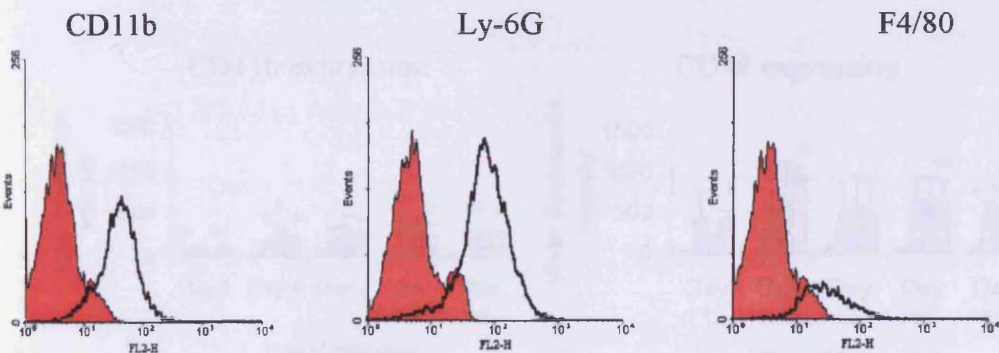
The results shown in figure 4.3 show the drop in the ability of the ATRA treated cells to proliferate compared to the untreated control cells. The small, but insignificant differences between the proliferation of the cells expressing control, FUS and FUS AS constructs when exposed to ATRA are also apparent. The drop in cell viability and the increase in expression of the cell surface markers CD11b and CD38 and decrease in the expression of CD33 in the treated cells indicates that they are undergoing differentiation into a more mature phenotype

Figure 4.2. Histograms showing how the expression levels of cells surface markers were determined. Each histogram shows the fluorescence pattern of the control IgG antibody in red and the fluorescence pattern of the other antibody over this in black.

Cell surface markers used on human cell lines

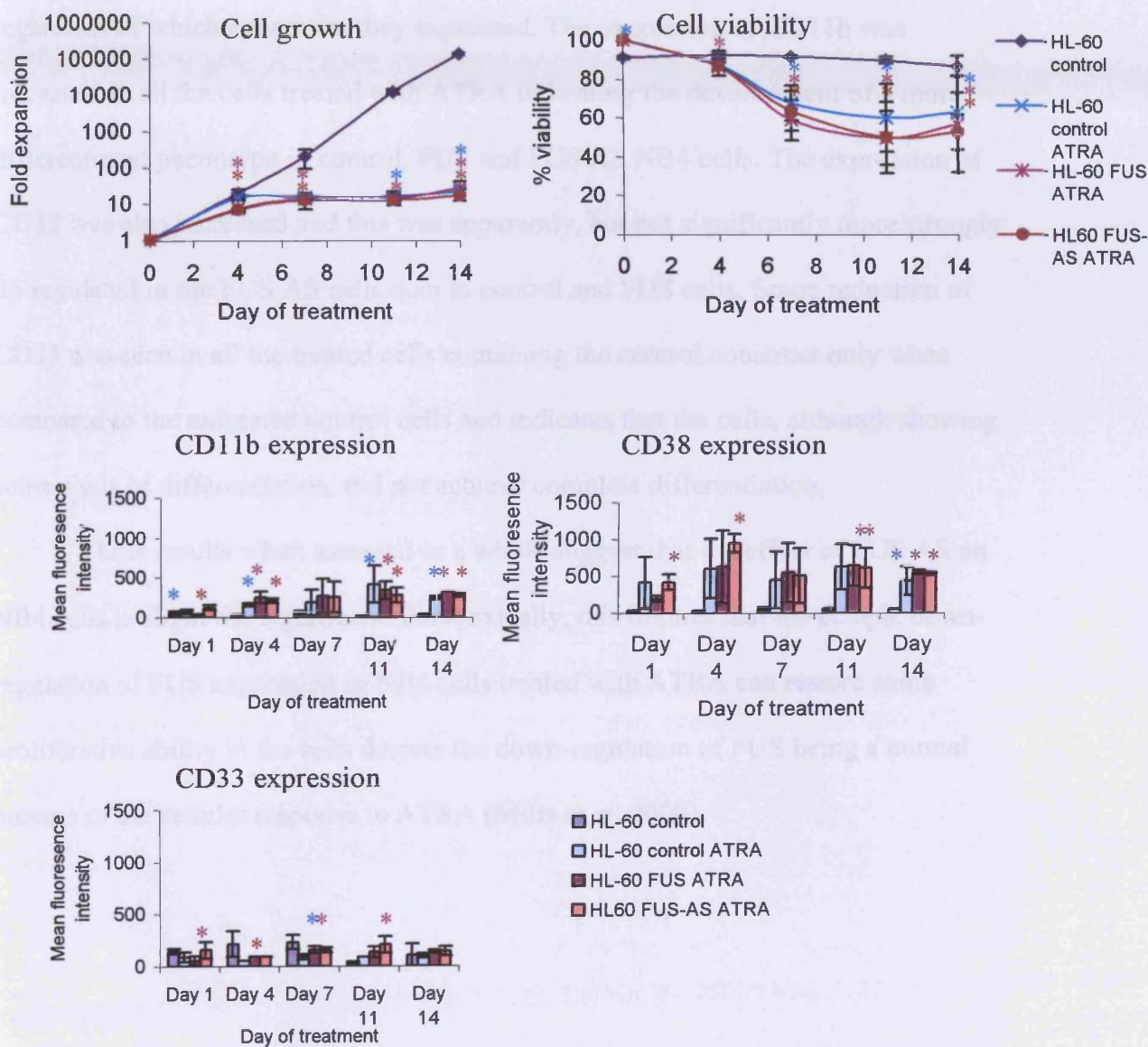


Cell surface markers used on murine cell lines



Similar patterns of response to ATRA as illustrated in figure 4.3 for the transduced HL-60 cells were also seen in the 32D cells transduced with control, FUS and FUS AS constructs and exposed to ATRA in the absence of IL-3, and the transduced 32D AML-ETO cells exposed to ATRA both in the presence and in the absence of IL-3. The most interesting results were seen in the NB4, NB4R2, 32D (in the presence of IL-3) and 32D B2A2 cells lines and these are detailed below in figures 4.4, 4.5, 4.6 and 4.7.

Fig. 4.3 Graphs showing the fold expansion, viability and expression of cell surface markers of HL-60 cells containing control, FUS and FUS AS constructs following treatment with ATRA. Error bars show +/- one standard deviation. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.

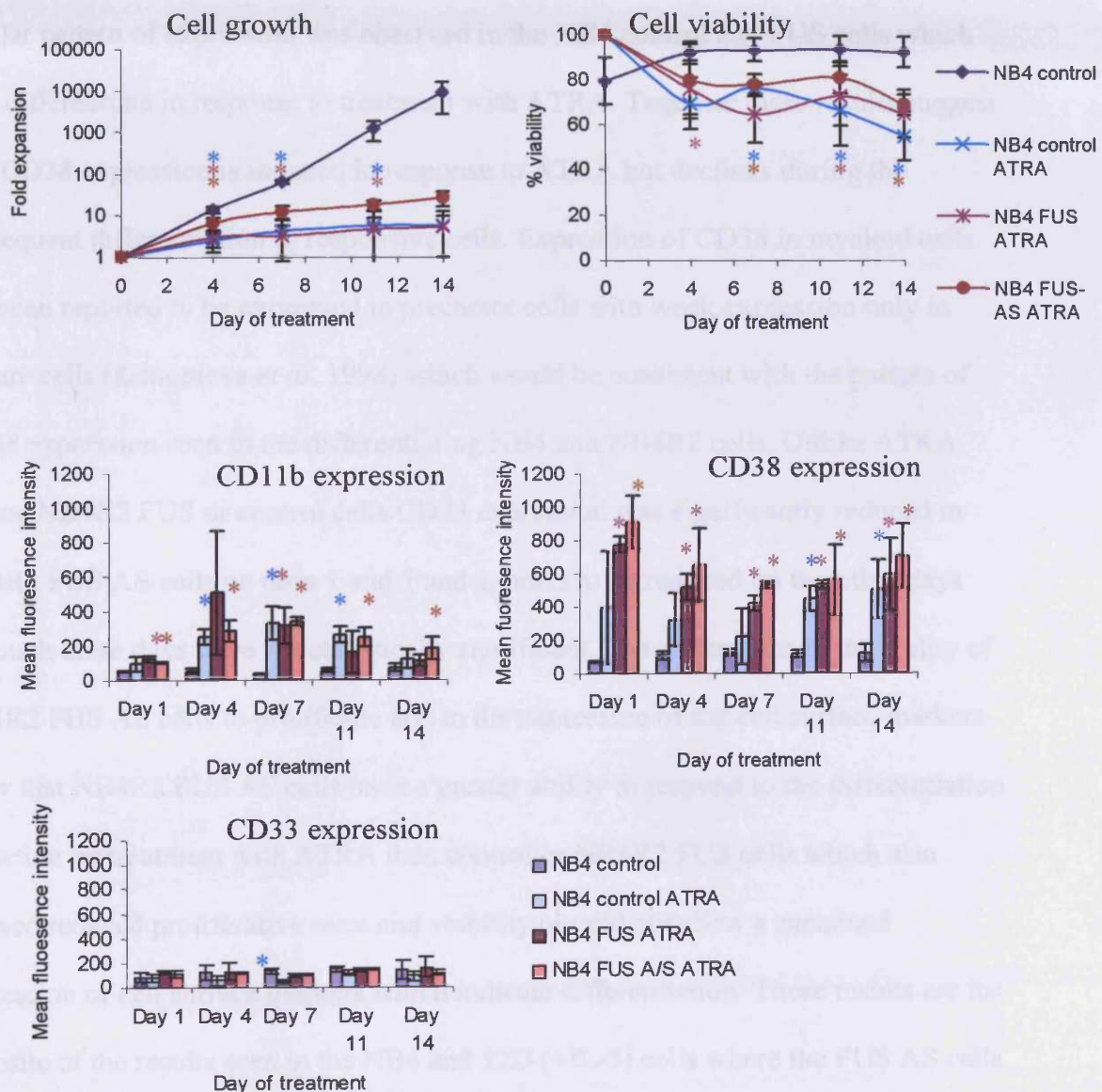


4.3.2b NB4 transduced cell lines

Figure 4.4 shows the significant decrease in proliferation in the NB4 cells expressing the control and FUS constructs when treated with ATRA. The NB4 FUS AS cells also showed a significant decrease in their ability to proliferate when treated with ATRA but remained capable of significantly more proliferation than the cells containing the other constructs. The viability of all cells treated with ATRA decreased regardless of which constructs they expressed. The expression of CD11b was increased in all the cells treated with ATRA indicating the development of a more differentiated phenotype in control, FUS and FUS AS NB4 cells. The expression of CD38 was also increased and this was apparently, but not significantly more strongly up-regulated in the FUS AS cells than in control and FUS cells. Some reduction of CD33 was seen in all the treated cells containing the control construct only when compared to the untreated control cells and indicates that the cells, although showing some signs of differentiation, did not achieve complete differentiation.

These results when assessed as a whole suggest that the effect of FUS AS on NB4 cells is slight but significant. Paradoxically, this implies that the ectopic down-regulation of FUS expression in NB4 cells treated with ATRA can restore some proliferative ability in the cells despite the down-regulation of FUS being a normal process in the cellular response to ATRA (Mills *et al.* 2000).

Fig. 4.4 Graphs showing the effect of ATRA on cell growth, viability and expression of cell surface markers on NB4 control, NB4 FUS and NB4 FUS AS (A/S) cells induced to differentiate with ATRA. Error bars show +/- one standard deviation. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.

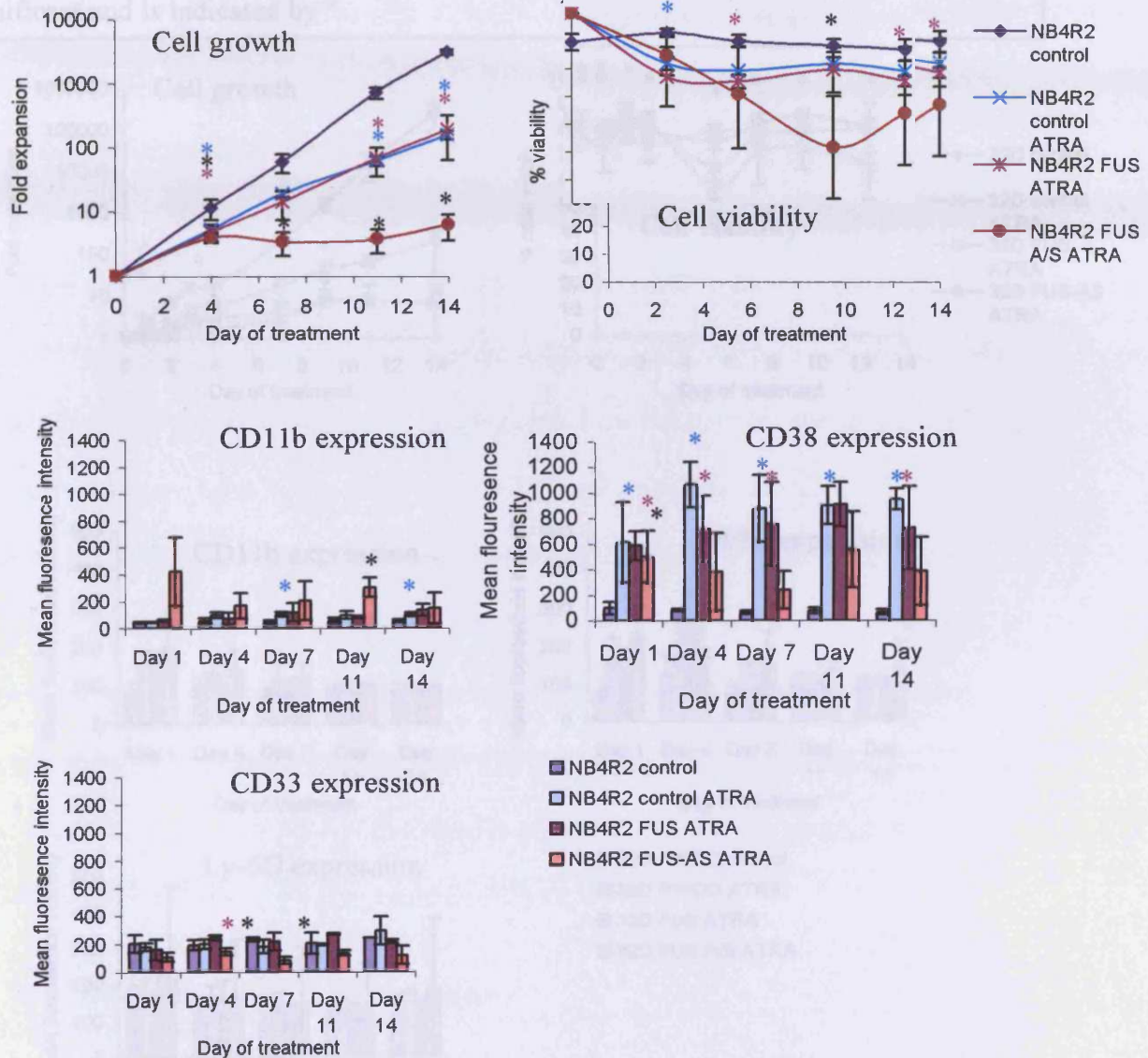


4.3.2c NB4R2 transduced cell lines

The results presented in figure 4.5 indicate that treatment with ATRA significantly affected all NB4R2 cell lines. However the proliferation and viability of

NB4R2 FUS AS cells was significantly more affected by the ATRA treatment than control or FUS NB4R2 cells. Additionally, the expression of CD11b was significantly higher in FUS AS cells (though not at all time points). This higher level of CD11b expression indicates a more differentiated phenotype in these cells than was observed in control or FUS NB4R2 cells. Expression of CD38 was shown to be up-regulated in all the cells in response to ATRA. In the NB4R2 FUS AS cells which differentiate the expression of CD38 declined more quickly than the control and NB4R2 FUS cells. A similar pattern of expression was observed in the NB4 control and FUS cells which also differentiate in response to treatment with ATRA. Together these results suggest that CD38 expression is induced in response to ATRA but declines during the subsequent differentiation in responsive cells. Expression of CD38 in myeloid cells has been reported to be expressed in precursor cells with weak expression only in mature cells (Konopleva *et al.* 1998) which would be consistent with the pattern of CD38 expression seen in the differentiating NB4 and NB4R2 cells. Unlike ATRA treated NB4R2 FUS or control cells CD33 expression was significantly reduced in NB4R2 FUS AS cells on days 1 and 7 and appears to be reduced on the other days although these days were not statistically significant. The differences in the ability of NB4R2 FUS AS cells to proliferate and in the expression of the cell surface markers show that NB4R2 FUS AS cells have a greater ability to respond to the differentiation induction by treatment with ATRA than control or NB4R2 FUS cells which also showed reduced proliferative rates and viability but did not show a sustained expression of cell surface markers which indicate differentiation. These results are the opposite of the results seen in the NB4 and 32D (+IL-3) cells where the FUS AS cells were *less* responsive to the induction of differentiation by ATRA.

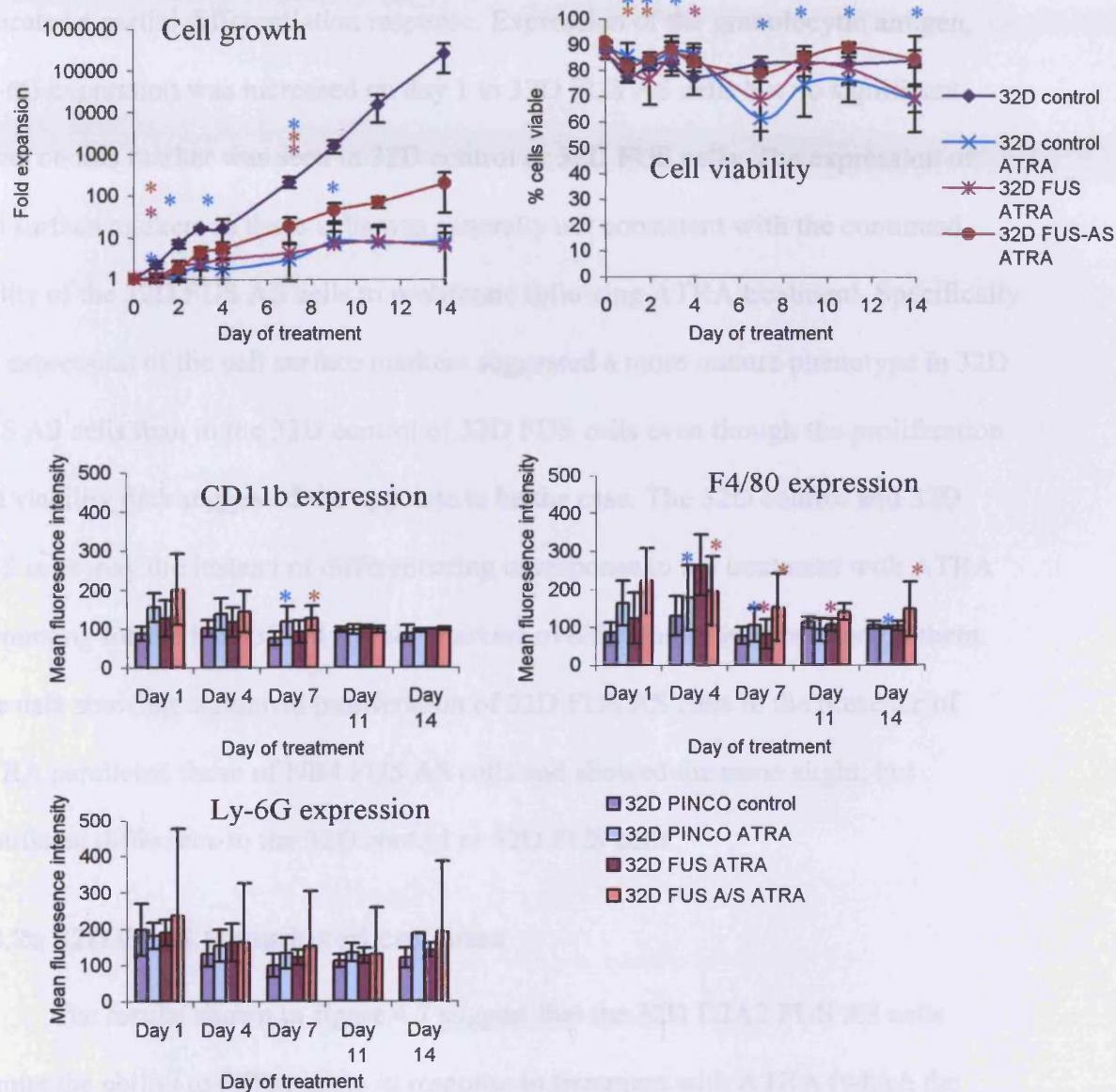
Fig. 4.5 Graphs showing the effect of ATRA on cell growth, viability and expression of cell surface markers in NB4R2 control, NB4R2 FUS and NB4R2 FUS AS cells. Error bars show +/- one standard deviation. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.



4.3.2d 32D transduced cell lines

The results in figure 4.6 show the effect of ATRA on the growth and viability of the treated 32D cells compared to an untreated control. The ability of 32D FUS AS

Fig 4.6 Graphs showing the effects of induction in 32D cells (+IL-3) expressing the control, FUS and FUS AS constructs with ATRA on cell growth, viability and expression of cell surface markers. Error bars indicate +/- one standard deviation. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.



cells to continue proliferating following treatment with ATRA is significantly increased when compared to 32D control and 32D FUS cells treated with ATRA and is also significantly lower than the untreated control cells.

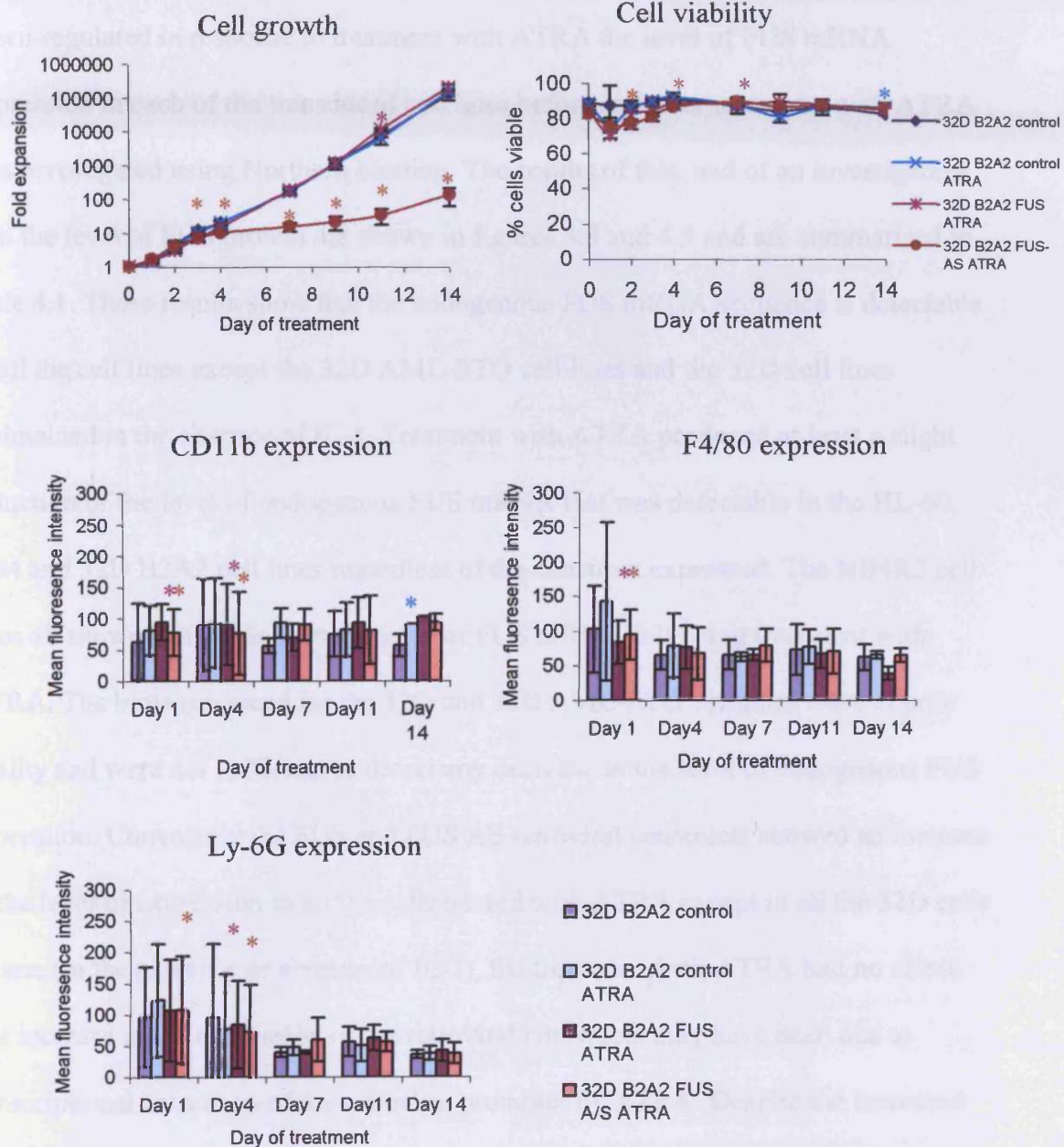
Additionally, the viability of 32D FUS AS cells was reduced to a lesser extent compared with control cells treated with ATRA. Using a t-test, expression of CD11b

in 32D FUS AS cells was only significantly increased on day 7 although the general level of expression of this cell surface marker was higher over the early time points. At no point did the 32D FUS cells show significant induction of CD11b expression. Expression of macrophage antigen, F4/80, was significantly increased in all the treated cells. Despite their continued proliferation, up-regulated F4/80 expression also indicated a partial differentiation response. Expression of the granulocytic antigen, Ly-6G expression was increased on day 1 in 32D FUS AS cells but no significant effect on this marker was seen in 32D control or 32D FUS cells. The expression of cell surface markers in these cells was generally not consistent with the continued ability of the 32D FUS AS cells to proliferate following ATRA treatment. Specifically the expression of the cell surface markers suggested a more mature phenotype in 32D FUS AS cells than in the 32D control of 32D FUS cells even though the proliferation and viability data suggested the opposite to be the case. The 32D control and 32D FUS cells may die instead of differentiating in response to the treatment with ATRA accounting for the lack of cell surface markers of differentiation expressed on them. The data showing continued proliferation of 32D FUS AS cells in the presence of ATRA paralleled those of NB4 FUS AS cells and showed the same slight, but significant difference to the 32D control or 32D FUS cells.

4.3.2e 32D B2A2 transduced cell lines

The results shown in figure 4.7 suggest that the 32D B2A2 FUS AS cells acquire the ability to differentiate in response to treatment with ATRA (which the 32D B2A2 control and 32D B2A2 FUS cells are not capable of doing). The growth of the 32D B2A2 FUS AS cells was significantly affected by the treatment with ATRA although viability was only slightly affected (significantly on days 2, 3, 4 and 14 of treatment). However when assessing cell surface marker expression in the 32D B2A2 cells treated with ATRA, there was no difference between cells expressing any of the

Fig. 4.7 Graphs showing the effects of induction with ATRA in 32D B2A2 cells expressing the control, FUS and FUS AS constructs on cell growth, viability and the expression of cell surface markers. Error bars indicate +/- one standard deviation. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.



constructs indicating that although the growth of the cells expressing FUS AS is reduced by ATRA this was not accompanied by a significant differentiation response.

4.3.3 Level of expression of FUS mRNA and protein in transduced cell lines following treatment with ATRA

As the level of endogenous FUS expressed in HL-60 cells was reported to be down-regulated in response to treatment with ATRA the level of FUS mRNA expression in each of the transduced cell lines before and after treatment with ATRA was investigated using Northern blotting. The results of this, and of an investigation into the level of FUS protein are shown in figures 4.8 and 4.9 and are summarised in table 4.1. These results show that the endogenous FUS mRNA sequence is detectable in all the cell lines except the 32D AML-ETO cell lines and the 32D cell lines maintained in the absence of IL-3. Treatment with ATRA produced at least a slight reduction of the level of endogenous FUS mRNA that was detectable in the HL-60, NB4 and 32D B2A2 cell lines regardless of the construct expressed. The NB4R2 cell lines all showed an increase in the level of FUS mRNA following treatment with ATRA. The blots produced for the 32D and 32D AML-ETO cell lines were of poor quality and were not sufficient to detect any decrease in the level of endogenous FUS expression. Conversely the FUS and FUS AS retroviral constructs showed an increase in the level of expression in all the cells treated with ATRA except in all the 32D cells where, (in the presence or absence of IL-3), the treatment with ATRA had no effect. The increase in the expression of the retroviral constructs may have been due to transcriptional activation of the retroviral promoter by ATRA. Despite the increased level of retroviral construct being expressed in the FUS AS cells, the level of endogenous FUS mRNA detectable in the cells was not decreased any further by the presence of the retroviral FUS AS sequence than it was by the action of ATRA on the

cells. This may be due to the high level of endogenous FUS expression within the haematopoietic cells as previously discussed in section 3.7. Equal loading of mRNA in the Northern electrophoresis gels was demonstrated by reprobing blots with the β -actin housekeeping gene (figure 4.8).

Fig. 4.8 Northern blots showing the level of FUS mRNA expression in cells before and after treatment with ATRA at 10^{-7} M for 4 days. All blots loaded in the order (left to right): untreated vector control, FUS, FUS AS, ATRA treated vector control, FUS, FUS AS. Representative blots from two repetitions.

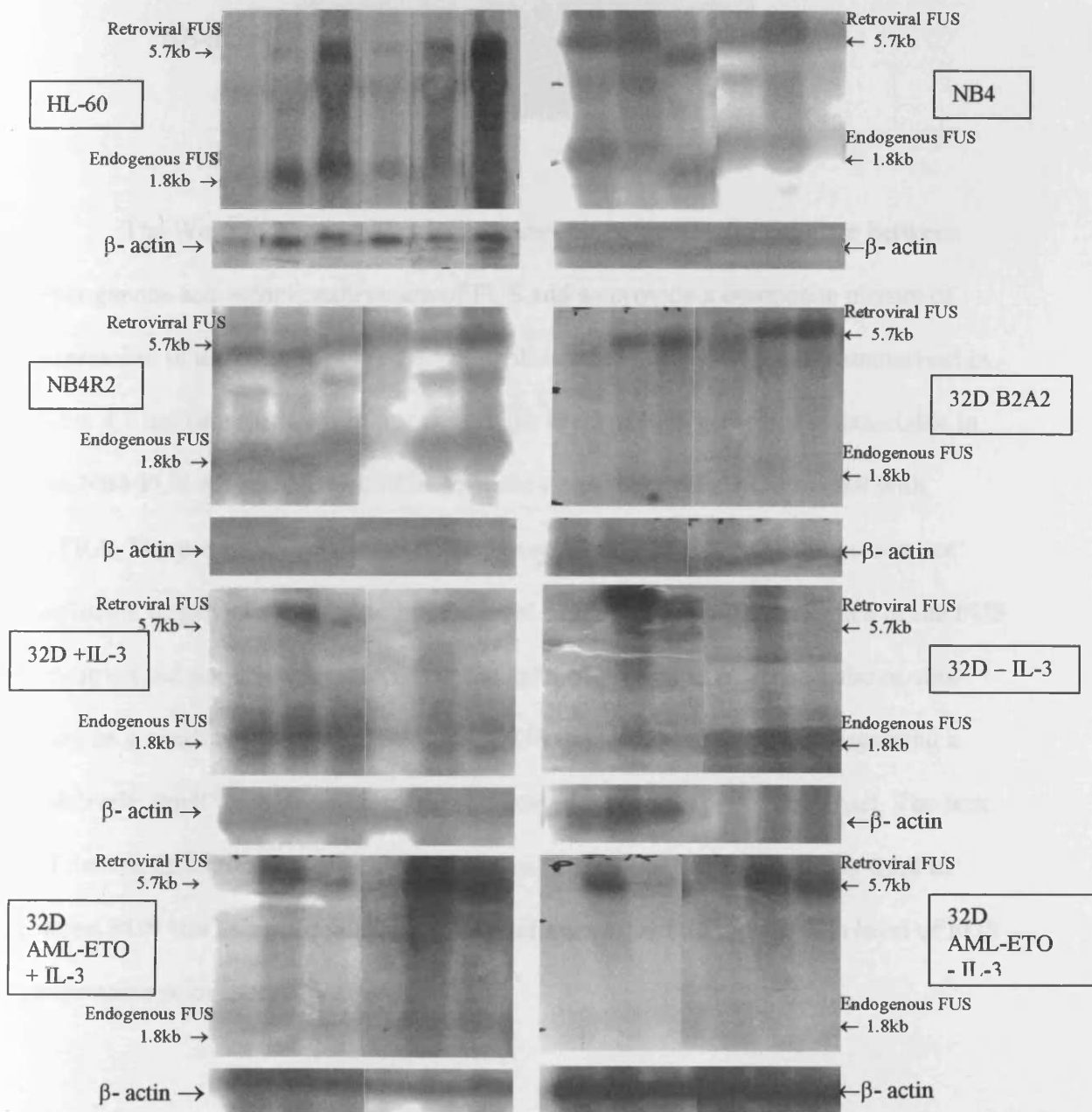
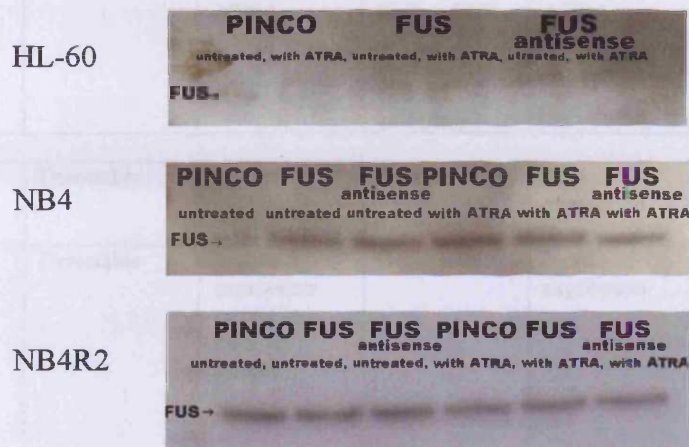


Fig. 4.9 Westerns showing the level of FUS protein expression in cells before and after treatment with ATRA at 10^{-7} M for 4 days. Control samples labelled PINCO (P), F = FUS, A/S = FUS AS. Representative blots from two repetitions.



The Western blots, unlike the Northern blots, cannot discriminate between endogenous and ectopic expression of FUS and so provide a composite picture of expression in the cells. The Western blots illustrated in figure 4.9 and summarised in table 4.1 indicate that a slight decrease in the level of FUS protein was detectable in the NB4 FUS AS and NB4R2 FUS AS cells when these cells were treated with ATRA. The presence of FUS AS in the untreated cells of these cell lines was not sufficient to generate a reduction in the level of FUS protein. The presence of the FUS construct did not create an increase in the level of protein as suggested above, this may be a result of the high expression of FUS in haematopoietic cells disguising a relatively small increase in expression generated by the retroviral construct. The lack of detectable expression of FUS in HL-60 cells suggests that the antibody used to detect FUS was of a poor quality as these cells are known to have a high level of FUS expression prior to treatment with ATRA.

Table 4.1 Results of Northern and Western blotting on transduced cells lines before and after treatment with ATRA. Results refer to cells containing the control, FUS and FUS AS constructs unless otherwise stated.

Cell line	Northern blots				Western blots	
	FUS	Effect of ATRA	Retroviral FUS or FUS AS (not in control samples)	Effect of ATRA	FUS	Effect of ATRA
HL-60	Detectable	Reduced expression in all constructs	Detectable	Increased expression		
NB4	Detectable	Reduced expression except in FUS AS sample	Detectable	Increased expression	Present and equal in all samples (Untreated control sample under-loaded)	Decreased slightly in FUS AS sample
NB4R2	Detectable	Increased expression in all constructs	Detectable	Increased expression	Present and equal in all samples	Decreased slightly in FUS AS sample
32D (+IL-3)	Detectable	Lower expression of FUS detectable	Detectable	No increase in expression	N/A	N/A
32D (-IL-3)	Not detectable	Not detectable	Detectable in FUS construct containing cells	Not detectable	N/A	N/A
32D AE (+IL-3)	Not detectable	Not detectable	Detectable	Increased expression	N/A	N/A
32D AE (-IL-3)	Detectable, low expression	Not detectable	Detectable	Increased expression	N/A	N/A
32D B2A2	Detectable	Reduced expression	Detectable	Increased expression	N/A	N/A

4.3.4 Dose response of transduced HL-60, NB4 and NB4R2 cells to ATRA

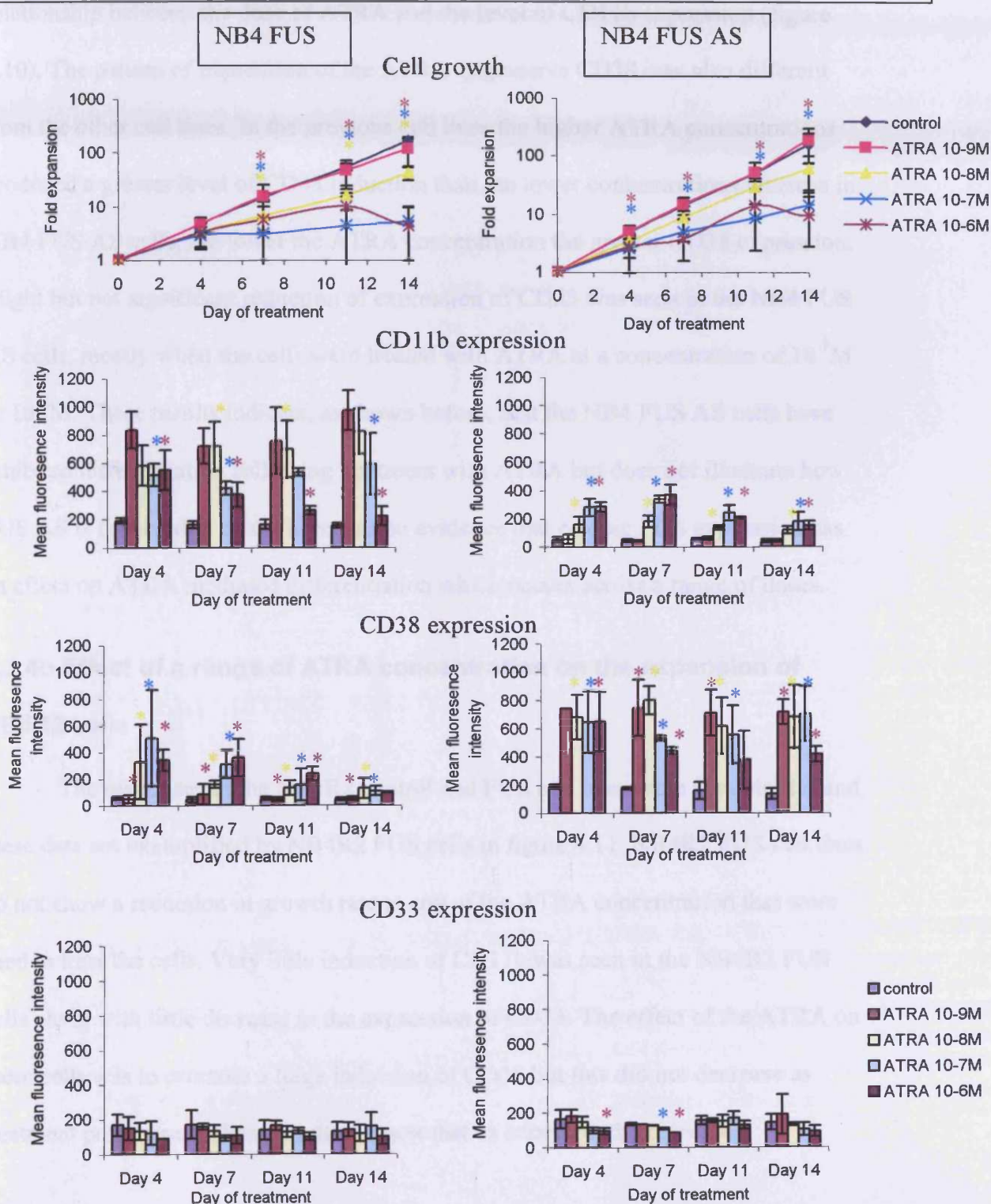
As it was thought that FUS and FUS AS may have subtle effects on differentiation, dose response experiments were carried out to show if these effects become evident by using a range of ATRA concentrations to induce differentiation. ATRA was used to induce differentiation in the transduced HL-60, NB4 and NB4R2

cells at concentrations of 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M over a period of 14 days. The results that were generated when the HL-60, NB4 and NB4R2 cells expressing the control, FUS and FUS AS constructs were treated in this way are shown in table 4.3. Comparison of the results displayed in Appendix 3, table A3.2 indicates that, with minor differences in the days at which the expression of the cell surface markers were found to be significant ectopic expression of FUS had no effect on HL-60 or NB4 cells. Likewise expression of FUS AS had no effect on HL-60, however NB4 FUS AS cells showed a different pattern of responses to the different ATRA concentrations as discussed below. The responses of NB4 FUS, NB4 FUS AS, NB4R2 FUS and NB4R2 FUS AS cells to the different ATRA concentrations are shown below.

4.3.4a Dose response effects seen in NB4 and HL-60 cells.

The same pattern of responses to different doses of ATRA were seen in the HL-60 control, HL-60 FUS, HL-60 FUS AS, NB4 control and NB4 FUS cells although some variation existed between the days at which the expression of cell surface markers were statistically significant. The responses of these cell lines are exemplified in figure 4.10 by NB4 FUS cells. These results show that, as expected, the dose of ATRA used to treat the cells was related to the magnitude of differentiation response observed. This was the case for the up-regulation of CD38 and the loss of CD33 expression. The pattern of CD11b expression which is a general marker of differentiation was interesting as the level of induced expression was inversely related to the concentration of ATRA used to treat the cells. In the NB4 FUS AS cells a much smaller induction of CD11b expression was seen compared to NB4 FUS cells and the greatest induction was observed, as might be expected in cells treated with the higher ATRA concentrations. In the responses shown by the measurements of cell growth, increase of expression of CD38 and in the decrease of CD33 on the treated cells, the affect with increasing the dosage of ATRA resulted in

Fig 4.10 Graphs showing the effect on cell growth and expression of cell surface markers when different concentrations of ATRA are used to induce differentiation in NB4 FUS and NB4 FUS AS cells over 14 days. Error bars indicate +/- one standard deviation. Experiments were repeated in triplicate. A value of less than $p = 0.05$ was considered significant and is indicated by *.

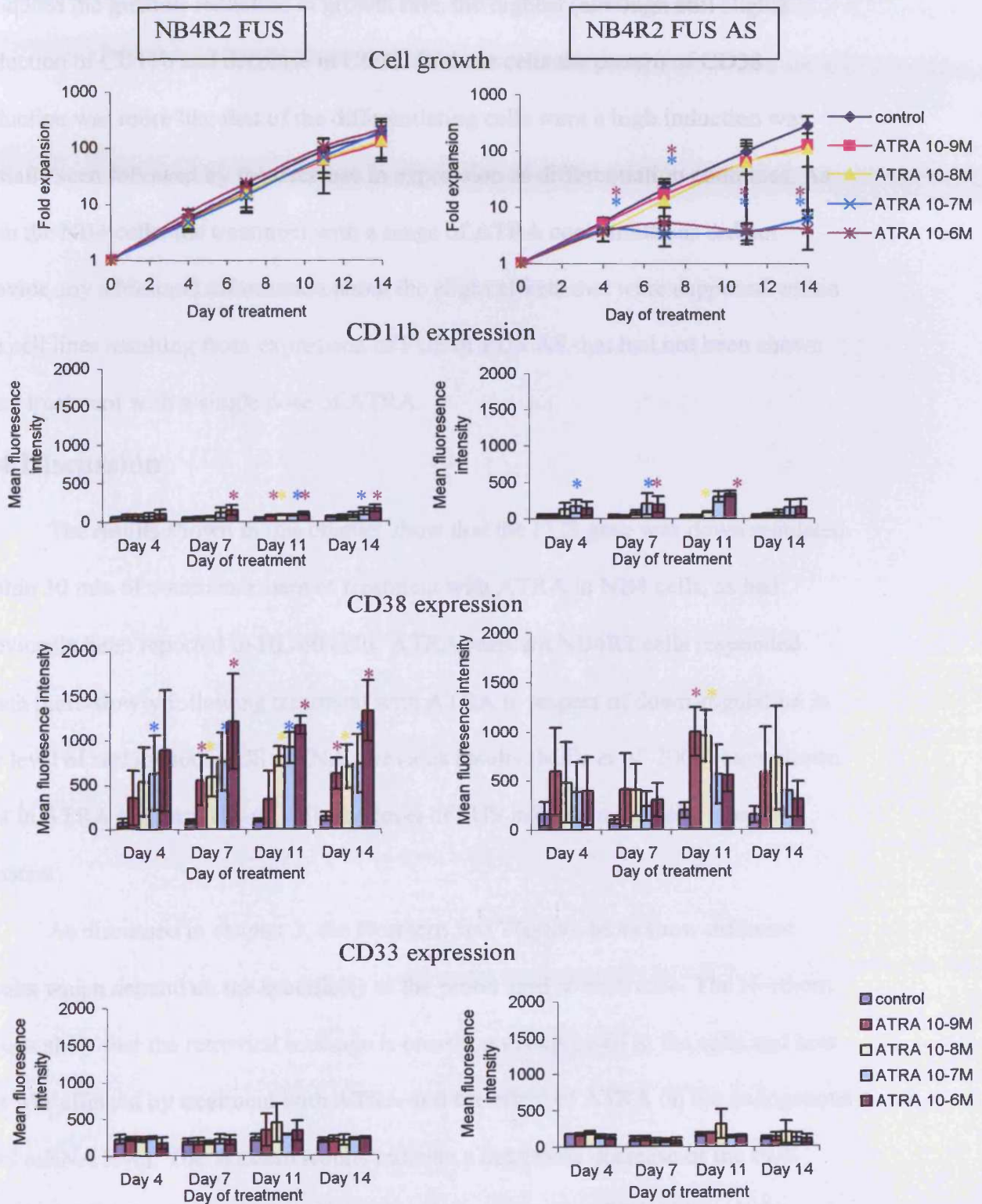


an increased response up to 10^{-7} M and then little difference was found between the responses seen between the results for 10^{-7} M and 10^{-6} M ATRA treatments. The effect seen when cells treated with ATRA at a concentration of 10^{-9} M was not significantly different from the untreated control cells. In contrast, the response of NB4 FUS AS cells differed from the NB4 FUS cells in that there was a direct rather than inverse relationship between the dose of ATRA and the level of CD11b expression (figure 4.10). The pattern of expression of the ATRA-responsive CD38 was also different from the other cell lines. In the previous cell lines the higher ATRA concentrations produced a greater level of CD38 induction than the lower concentrations whereas in NB4 FUS AS cells, the lower the ATRA concentration the greater CD38 expression. Slight but not significant reduction of expression of CD33 was seen in the NB4 FUS AS cells, mostly when the cells were treated with ATRA at a concentration of 10^{-7} M or 10^{-6} M. These results indicate, as shown before, that the NB4 FUS AS cells have inhibited differentiation following treatment with ATRA but does not illustrate how FUS AS is involved in this. There was no evidence that ectopic FUS expression has an effect on ATRA mediated differentiation which occurs across a range of doses.

4.3.4b Effect of a range of ATRA concentration on the expansion of NB4R2 cells

The responses of the NB4R2 control and FUS cell lines were very similar and these data are exemplified by NB4R2 FUS cells in figure 4.11. NB4R2 FUS cell lines do not show a reduction in growth rate at any of the ATRA concentration that were used to treat the cells. Very little induction of CD11b was seen in the NB4R2 FUS cells along with little decrease in the expression of CD33. The effect of the ATRA on these cells was to promote a large induction of CD38 but this did not decrease as treatment progressed. These results suggest that as expected, the increased

Fig 4.11 Graphs showing the effect of different ATRA concentrations on the cell growth and expression of cell surface markers on NB4R2 FUS and NB4R2 FUS AS cells. Error bars show +/- one standard deviation. Experiments were repeated in triplicate. A value of less than $p = 0.05$ was considered significant and is indicated by *.



concentrations of ATRA produced higher expression of CD11b and CD38 but did not promote differentiation in these cells.

In contrast in the NB4R2 FUS AS cells more evidence of differentiation was seen with increasing ATRA concentration. The highest ATRA concentration produced the greatest reduction in growth rate, the highest (although still slight) induction of CD11b and decrease in CD33. In these cells the pattern of CD38 induction was more like that of the differentiating cells where a high induction was initially seen followed by the decrease in expression as differentiation continues. As with the NB4 cells, the treatment with a range of ATRA concentrations did not provide any additional information about the slight effects that were supposed within the cell lines resulting from expression of FUS or FUS AS that had not been shown from treatment with a single dose of ATRA.

4.4 Discussion

The results shown in this chapter show that the FUS gene was down regulated within 30 min of commencement of treatment with ATRA in NB4 cells, as had previously been reported in HL-60 cells. ATRA resistant NB4R2 cells responded much more slowly following treatment with ATRA in respect of down-regulation in the level of endogenous FUS mRNA. Previous results (Mills *et al.* 2000) have shown that in ATRA resistant HL-60 cells the level of FUS mRNA in the cells remained constant.

As discussed in chapter 3, the Northern and Western blots show different results which depend on the specificity of the probe used in each case. The Northern results show that the retroviral message is present and expressed in the cells and how this was affected by treatment with ATRA and the effect of ATRA on the endogenous FUS mRNA level. The Western results indicate a detectable decrease of the FUS protein in NB4 FUS and NB4R2 FUS AS cells treated with ATRA although the

quality of the HL-60 Western blot was poor and did not allow any conclusions to be drawn. Treatment with ATRA resulted in increased levels of retroviral FUS mRNA that was detectable by Northern blotting in some of the cells lines. Retinoids have previously been shown to activate several viral promoters and this may be mediated by the LTR region of the viral genome (Lee *et al.* 1994). A number of studies have indicated that RARs and RXRs in the presence of their ligand modulate LTR-directed transcription (Sawaya *et al.* 1996). In addition retinoic acid mediated activation of the HIV LTR has been seen in the U38, HL-60 and HeLa cell lines (Sawaya *et al.* 1996; Towers *et al.* 1995). The Moloney murine leukaemia virus (MMLV) is under the control of a retroviral LTR which is a strong promoter in eukaryotic cells although this retroviral LTR is far less effective in promoting gene expression in haematopoietic cells (Towers *et al.* 1995). In the transduced cell lines used in this study, the transgenic FUS gene is expressed from a full length MMLV LTR in the retroviral construct and it is likely that the retinoid responsive elements in the LTR of the MMLV resulted in an up-regulation of gene expression in the presence of retinoic acid. The very high endogenous level of FUS expression in haematopoietic cells may be one reason why the retroviral constructs, even with elevated transcription in the presence of ATRA, were not capable of producing a detectable alteration of FUS expression at the protein level.

When treated with ATRA, a difference between the cells expressing the FUS and FUS AS constructs was observed in the NB4, NB4R2, 32D B2A2 and 32D cell lines. The control and FUS constructs expressed in these cells had no effect whereas in the NB4 and 32D cells the presence of FUS AS inhibited the differentiation response to ATRA. No previous evidence of this result has been reported. The lower level of FUS expression in the cells expressing FUS AS before the commencement of treatment with ATRA may be the cause of the retention of proliferative ability in

these cells. The lower level of FUS proposed to be responsible for this effect in these cells was not observed on the Northern or Western blots and may be the result of the time points at which samples for blotting were prepared being too late to observe a decrease in FUS expression in the very early time points of the experiments. Although the FUS gene is normally down-regulated in the NB4 cells following treatment with ATRA, it may be required for the very early events following treatment with ATRA and the lower expression of FUS in NB4 FUS AS cells may escape the differentiation response.

Paradoxically the NB4R2 FUS AS cell line showed an increased differentiation response to ATRA, whereas the control and FUS cells remained relatively unaffected by the treatment with ATRA. This result was also seen in 32D cells expressing the BCR-ABL fusion protein (32D B2A2 cells) and previously has been observed in 32D cells expressing the BCR-ABL fusion protein and a FUS antisense sequence in response to G-CSF (Perrotti *et al.* 1998). The differences between the previously published data and the findings reported in this chapter may be the result of differences in the antisense sequences that were transduced into the cells or the original clone of the 32D cell line that was used. This differentiation response was not seen in the control and FUS cells and the level of FUS expression in these cells was not reduced upon treatment with ATRA as is the case in ATRA-responsive cell lines (Mills *et al.* 2000).

Over expression of FUS also did not alter the sensitivity to ATRA treatment in HL-60, NB4 or NB4R2 cells. In these cell lines, the greater the dose of ATRA, the greater the reduction in proliferation, viability and CD33 expression until the optimum concentration was reached at 10^{-7} M ATRA. A higher dose of ATRA also led to a greater induction of CD38 expression in these cells. The expression pattern for CD11b was different and did not agree with previously published results (Ikezoe *et al.*

2000; Yu *et al.* 1999) which demonstrate that an increased ATRA dose results in an increased CD11b induction. The results presented in this chapter showed that a lower dose of ATRA led to a greater induction of CD11b expression which lasted for a longer period than the induction of this cell surface marker with a higher concentration of ATRA. This could be a result of the higher concentrations of ATRA resulting in a faster differentiation response in these cells producing the greatest induction of CD11b at a very early time point before CD11b was measured in this study, while lower ATRA concentrations produce a slower differentiation response which enabled the greatest induction of CD11b to be detected in the method followed in these experiments.

In NB4 cells, expression of FUS AS again led to an increased response in the cells with increasing doses of ATRA. In these cells however the increased ATRA led to increased CD11b expression in agreement with published results (Ikezoe *et al.* 2000; Yu *et al.* 1999) but a lesser induction of CD38 than at the lower concentrations that were used. The proliferative response and CD33 expression were the same as the other NB4 cell lines.

In the NB4R2 cells, FUS AS promoted the differentiation response to increasing concentrations of ATRA, enhancing its anti-proliferative effect and the induction of CD11b. The expression of CD33 was not affected by any of the ATRA concentrations while the lower concentrations of ATRA produced a higher CD38 expression.

Both the NB4 FUS AS and NB4R2 FUS AS cell lines showed that the lower concentrations of ATRA elicited a higher induction of CD38 than the higher ATRA concentrations. An increasing dose of ATRA will also increase the level of induction of expression of the retroviral FUS AS sequence within these cells as it is produced from an ATRA responsive promoter. CD38 induction is a specific event following the

treatment of the cells with ATRA and these results suggests that reduction of the level of FUS protein expression in the cells results in a reduced ability to express the CD38 cell surface marker.

The choice of cell surface markers used to determine whether differentiation is occurring is important. As in some cases the stage of differentiation that was achieved was difficult to determine due to ambiguity in the expression of the cell surface markers and growth of the cells, a larger panel of markers might be used to provide a more complete picture of the differentiation status of the cells. This larger panel of markers might additionally include CD15, HLA-DR and CD16 which can be used in conjunction with each other to determine the different stages of maturing granulocytes and CD14 which can be used alongside CD11b to distinguish different stages of monocyte maturation. In addition to these the markers CD64, CD65, CD117, CD133, CD135, MPO (myeloperoxidase) and lactoferrin have all be suggested to be useful in multi-parameter flow cytometric determination of stages of myeloid maturation (Basso *et al.* 2001; Kussick *et al.* 2003).

To summarise, the effects of ATRA on the cells expressing FUS AS constructs were found to be altered in four of the transduced cell lines only. These results are summarised in table 4.2.

Table 4.2 Summary of the effect of ATRA on cell lines containing the control, FUS and FUS AS constructs. Altered responses due to ectopic expression of FUS AS are indicated by *.

Cell line and construct expressed	Effect of ATRA
NB4 with control or FUS	Differentiation
NB4 with FUS AS *	Continued proliferation
NB4R2 with control or FUS	Continued proliferation
NB4R2 with FUS AS *	Differentiation
32D (-IL-3) with control or FUS	Differentiation
32D (-IL-3) with FUS AS *	Continued proliferation
32D B2A2 with control or FUS	Continued proliferation
32D B2A2 with FUS AS *	Differentiation

The results shown in this chapter illustrate that in some cell lines the level of the FUS protein that is expressed within the cells is important for the ability of the cells to respond to ATRA, or to remain unresponsive to its action. As FUS has been suggested to have a role in the mechanism by which G-CSF induces differentiation in murine cell lines, the effect of altering the level of FUS expression in this response was also investigated in the next chapter. Enhancement of the effect of ATRA on the transduced cell lines by combining treatment with ATRA and G-CSF will also be investigated as sensitivity to induction of differentiation by G-CSF has been reported to be facilitated by pre-treating HL-60 cells with ATRA at sub-optimal concentrations (Bunce *et al.* 1994). ATRA in combination with vitamin D3 (Bunce *et al.* 1995), medoxyprogesterone acetate (MPA), clofibrac acid (Fenton *et al.* 2003) and indomethacin (Bunce *et al.* 1994) also promote neurophillic differentiation induction in HL-60 cells. It has been suggested that differentiation inducers that act in a synergistic manner thus enabling lower, less cytotoxic concentrations to be used to induce differentiation which would be of benefit to patients (Bunce *et al.* 1994; Fenton *et al.* 2003).

Chapter 5

**Response to G-CSF of haematopoietic cell lines
transduced with the FUS and FUS AS constructs**

5.1 Introduction

Granulocyte-colony stimulating factor (G-CSF) is a major regulator of neutrophilic granulocytic differentiation and augments the proliferation, survival, maturation and functional activation of cells (Ward *et al.* 1999). The action of G-CSF is mediated through the G-CSF receptor (G-CSF-R) which is located on the cell surface. As a result of binding, G-CSF-R and G-CSF form a homo oligomeric complex. The G-CSF-R has no intrinsic tyrosine kinase activity and signals by activating cytoplasmic kinases. The kinases activated by G-CSF-R include the Janus kinases Jak1, Jak 2 and Tyk 2, the Src kinases p55^{lyn} and p56/59^{hck} and the STAT 1, 3 and 5 signal transducers and activators of transcription. Four tyrosine residues in the cytoplasmic region of G-CSF-R become phosphorylated when G-CSF is bound to the receptor and form binding sites for signalling molecules with Src homology 2 (SH2) domains or phosphotyrosine binding domains (Ward *et al.* 1999). G-CSF is the most important of the colony stimulating factors for normal granulocytic differentiation (Tkatch *et al.* 1995).

G-CSF induces granulocytic differentiation in the 32D cells line through the C/EBP ϵ pathway after 1 day of treatment and the response is maximal after 3 days. The C/EBP ϵ protein is the principle downstream target of G-CSF-R signalling and its expression alone is sufficient to support terminal granulocytic differentiation (Nakajima *et al.* 2001). C/EBP ϵ is also induced by other growth factors or differentiation inducers including retinoic acid, stem cells factor, GM-CSF and DMSO. There is some controversy as to whether G-CSF is an active differentiation inducer or just a survival factor for committed granulocyte precursors (Nakajima *et al.* 2001).

32D cells grown in the presence of IL-3 remain blast like in morphology. The

removal of IL-3 from the cells results in cell death in 1-2 days with no evidence of differentiation being seen (Ward *et al.* 1999). Addition of G-CSF leads to transient proliferation over 5 to 7 days and to terminally differentiated neutrophils in 6-10 days which are identified by increased cytoplasmic to nuclear volume, cytoplasmic granulation and lobulation of the nucleus (Ward *et al.* 1999). Up-regulation of G-CSF-R is also observed in response to treatment with G-CSF in 32D cells (Tkatch *et al.* 1995). Two myeloid leukaemic cell lines containing the BCR/ABL transcript, K562 and BV17 do not express G-CSF-R despite the normal gene structure being present within these cells. The BCR/ABL transcript may be responsible for interfering with the regulation of G-CSF-R in these cell lines (Tkatch *et al.* 1995). BCR-ABL oncoproteins regulate proliferation, differentiation and trafficking of haematopoietic cells by transcriptional and post-transcriptional mechanisms that require tyrosine kinase activity and formation of multi-protein complexes whereby signalling molecules are assembled and activated in the cytoplasm and the nucleus (Perrotti *et al.* 2002a). FUS expression is markedly increased in haematopoietic cell lines ectopically expressing BCR-ABL (Perrotti *et al.* 2002a).

Granulocytic differentiation has been shown to be accelerated in myeloid precursor cells expressing FUS AS constructs and also promotes expression of G-CSF-R. Conversely, over expression of FUS down-regulates G-CSF-R and adversely affects G-CSF dependent cells (Perrotti *et al.* 2002a). FUS expression is associated with a delay in the up-regulation of G-CSF-R in 32D cells treated with G-CSF. FUS binds a portion of the G-CSF-R mRNA and may interfere with its processing or export from the nucleus to the cytoplasm (Perrotti *et al.* 2002a). BCR-ABL induces increased FUS expression by preventing its proteasome dependent degradation. BCR-ABL expression also leads to enhanced levels of hnRNPA1 and hnRNPA2 as well as FUS. These are all RNA binding proteins and post-transcriptional mechanisms are

employed which results in the enhanced protein stability. For FUS, the phosphorylation of serine 256 (which is PKC β II dependent), disrupts the formation of the multi-protein complex that targets FUS for degradation.

NB4 cells treated with interferons show expression of PML/RAR α mRNA and two transcripts encoding RAR α . The leukocyte alkaline phosphatase (LAP) and G-CSF-R proteins are also produced and can be considered to be markers for granulocytic differentiation (Gianni *et al.* 1994; Gianni *et al.* 1996). G-CSF has no effect on the level of G-CSF-R expression in HL-60 and NB4 cells while ATRA induces an increase in the levels of both G-CSF-R mRNA and protein within these cells (Tkatch *et al.* 1995). The up-regulation of G-CSF-R in the HL-60 and NB4 cells occurs before any morphological evidence of differentiation is seen and its occurrence requires protein synthesis (Tkatch *et al.* 1995).

G-CSF acts synergistically with ATRA in NB4 and HL-60 cells. ATRA and G-CSF in combination at a concentration of 10^{-7} M ATRA and 10ng/ml G-CSF induce the maximal expression of LAP in 4 days of treatment in NB4 cells. HL-60 cells behave essentially in the same manner as the NB4 cells following induction of differentiation with the ATRA and G-CSF in combination (Gianni *et al.* 1994). These two inducers regulate the expression of CD11b and CD33 in NB4 cells. CD11b is increased by the treatment and CD33 is reduced. It has been reported that CD33 shows no decline in expression when the cells are treated with ATRA alone or G-CSF alone but a reduction in expression is seen when the two inducers are used in combination (Gianni *et al.* 1994). CD11b expression is brought about by treatment of the cells with ATRA alone but not by G-CSF alone. The two in combination also up-regulate the expression of CD11b (Gianni *et al.* 1994). Morphologically, G-CSF has no effect on NB4 cells (Gianni *et al.* 1994) and results in an incomplete differentiation where additional inducers may be required to promote the differentiation of the cells

(Gianni *et al.* 1994). G-CSF has minor effects on the amount of RAR α and β that are expressed in NB4 cells (Gianni *et al.* 1994). When treated with ATRA, one of the genes that is activated within one day of treatment and before the cells stop cycling is G-CSF-R (Drayson *et al.* 2001).

There is a weak synergistic effect between ATRA and G-CSF in CML treatment and a greater one in APL which is dependent on the PML-RAR α protein seen in NB4 cells (Gianni *et al.* 1994).

This chapter aims to show the effect of the expression of FUS and FUS AS constructs in the G-CSF induced differentiation of different haematopoietic cell lines containing different chromosomal translocations. The amount of differentiation will be measured by cell growth, viability and by the expression of cell surface markers associated with different stages of differentiation. In addition, the level of FUS protein expression in different treatment conditions will be determined by Western blotting in the cell lines of human origin. The effect of using both G-CSF and ATRA in combination will also be studied to determine whether this increases the level of differentiation achieved in the presence of FUS and FUS AS constructs.

5.2 Experimental procedures

5.2.1 Effect of G-CSF on transduced cells

The response of the transduced HL-60, NB4, NB4R2, 32D, 32D AML-ETO and 32D B2A2 cell lines to G-CSF at a concentration of 100ng/ml were studied over 14 days. The manner in which the cell lines were treated with G-CSF is described in section 2.5.2. The effect of the G-CSF was assessed in the same manner as for the ATRA treatment described in section 4.2. The same cell surface markers (CD11b, CD38 and CD33 for human cell lines, and CD11b, F4/80 and Ly-6G for murine cell lines) were used to determine the differentiation response seen in the treated cells and

were measured on days 1, 4, 7, 11 and 14 following the commencement of treatment. When treating the transduced 32D, and 32D AML-ETO cell lines the experiments were performed in the presence of IL-3 and in the absence of IL-3 to determine whether this had an effect on the ability of the cells to respond to the treatment with G-CSF. The experiments were repeated in triplicate and compared to untreated control cultures that had not been exposed to G-CSF and were also repeated in triplicate.

5.2.2 Effect of G-CSF and ATRA in combination on transduced cell lines

Using the same end points, the effect of both ATRA and G-CSF in combination was also studied. The two induction agents were combined at the same concentrations (G-CSF at 100ng/ml, ATRA at 10^{-7} M) as were used separately and the response of the cells was studied over 14 days. The method used to treat the cell lines with both differentiation inducers in combination is described in section 2.5.3. As previously, the triplicated 32D and 32D AML-ETO experiments were performed twice, once in the presence of IL-3 and once without it.

5.2.3 Level of FUS expression in transduced cells

Western blots were performed on samples of the HL-60, NB4 and NB4R2 cell lines transduced with control, FUS and FUS AS constructs treated with G-CSF for 4 days to show the level of FUS protein expressed in the cells. The method used to perform these Western blots is described in section 2.4.6. An anti-FUS antibody was used to detect the FUS protein on the Western blots. Western blots were also performed on the HL-60, NB4 and NB4R2 transduced cell lines treated with ATRA and G-CSF in combination. Western blots were not carried out on the murine cell lines due to the doubtful activity of the available antibody at the time of this study.

Northern blots were carried out to assess the level of FUS mRNA produced in each of the transduced cell lines treated with G-CSF and with G-CSF and ATRA in combination. A radio-labelled FUS DNA probe was used to detect the FUS mRNA on these Northern blots. This probe was also capable of hybridising to the FUS AS sequences. The procedures for carrying out Northern and Western blots are described in sections 2.4.2 and 2.4.6.

5.3 Results

5.3.1 Effects of treating the transduced cell lines with G-CSF

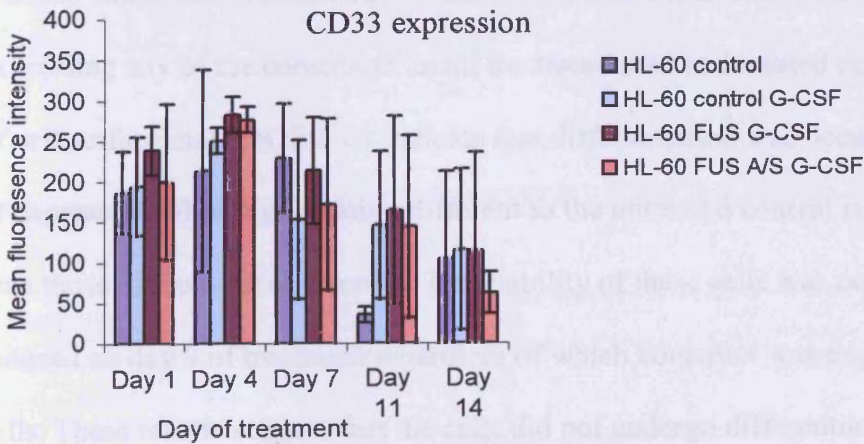
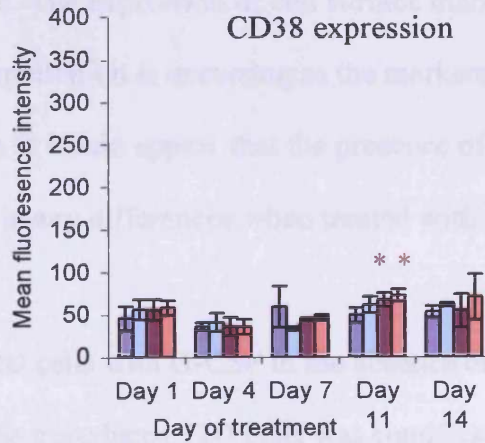
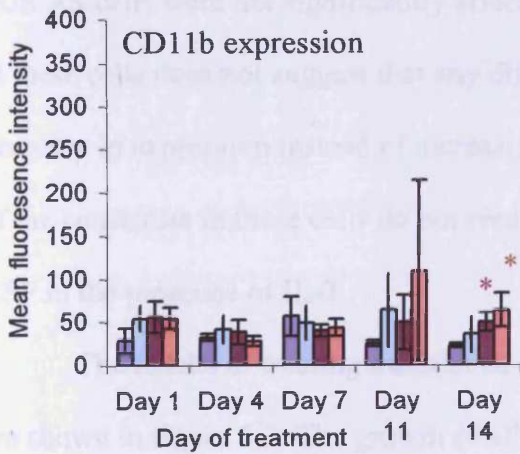
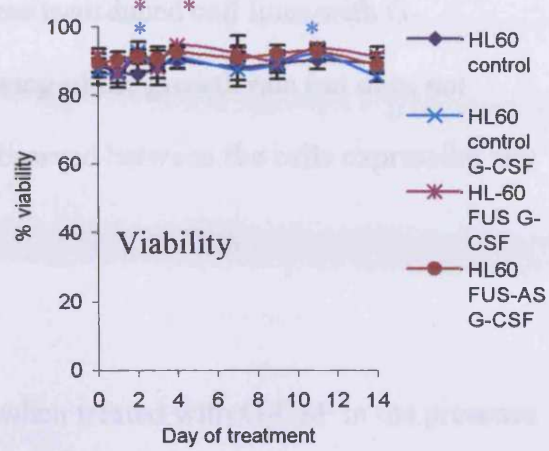
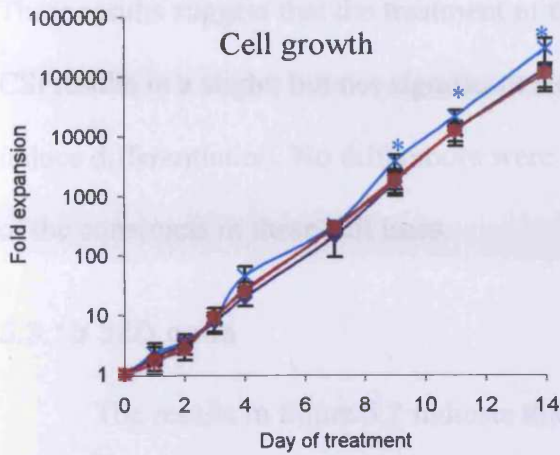
32D and 32D AML-ETO cells are known to be capable of differentiation in response to G-CSF. It has been previously reported that 32D cells expressing the BCR/ABL fusion protein become capable of differentiating in response to G-CSF when they express FUS AS. In order to determine whether the presence of FUS AS produced any sensitivity to G-CSF in other cell lines or whether ectopic expression of FUS generated an increased resistance to G-CSF, all the transduced cell lines were treated with G-CSF. The effects of treating the transduced cell lines is shown in appendix 3 table A3.2.

5.3.1a Effect of G-CSF on HL-60, NB4, NB4R2 and 32D AML-ETO (- IL-3)

The effects of treatment with G-CSF for fourteen days is shown in figure 5.1. The results shown are for HL-60 cells expressing all the constructs but the same pattern of results was observed when the transduced NB4, NB4R2 and 32D AML-ETO (in the absence of IL-3) cells were treated with G-CSF.

The results in figure 5.1 show that the growth of the transduced HL-60 cells are slightly, but not significantly slower when the cells are treated with G-CSF than when they remain untreated. Likewise cell viability was little affected. No consistent change in the level of expression of CD11b, CD38 or CD33 was seen in any of the

Fig 5.1 Effect of G-CSF treatment over 14 days on HL-60 cells measured by cell growth, viability and expression of cell surface markers. Experiments were repeated in triplicate and error bars indicate +/- one standard deviation. A probability of less than 0.05 was considered significant and is indicated by *.



human cell lines, while the expression of CD11b, F4/80 and Ly-6G was unaltered or reduced in the 32D AML-ETO cells treated with G-CSF in the absence of IL-3. Minor changes in the expression of cell surface markers were seen on the different days but these were not consistent and did not indicate that any differentiation was occurring. These results suggest that the treatment of these transduced cell lines with G-CSF results in a slight, but not significant slowing of the growth rate but does not induce differentiation. No differences were observed between the cells expressing any of the constructs in these cell lines.

5.3.1b 32D cells

The results in figure 5.2 indicate that when treated with G-CSF in the presence of IL-3, 32D control and FUS cells showed a slowing of their growth rate while 32D FUS AS cells were not significantly affected. The expression of cell surface markers in these cells does not suggest that any differentiation is occurring as the markers decrease in expression instead of increasing. It would appear that the presence of any of the constructs in these cells do not result in any differences when treated with G-CSF in the presence of IL-3.

The results of treating transduced 32D cells with G-CSF in the absence of IL-3 are shown in figure 5.3. The growth of all the transduced 32D cells was significantly reduced under these conditions. No difference was observed between the cells expressing any of the constructs. In all the transduced and treated cells the expression of cell surface markers did not indicate that differentiation was occurring as the levels of expression when significantly different to the untreated control samples were lower than those of the control samples. The viability of these cells was only significantly reduced on day 9 of treatment regardless of which construct was expressed in the cells. These results suggest that the cells did not undergo differentiation but treatment with G-CSF results in the slowing of the proliferation rate.

Fig. 5.2 Effects of treatment of transduced 32D cells with G-CSF in the presence of IL-3. Error bars indicate +/- one standard deviation about the mean. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.

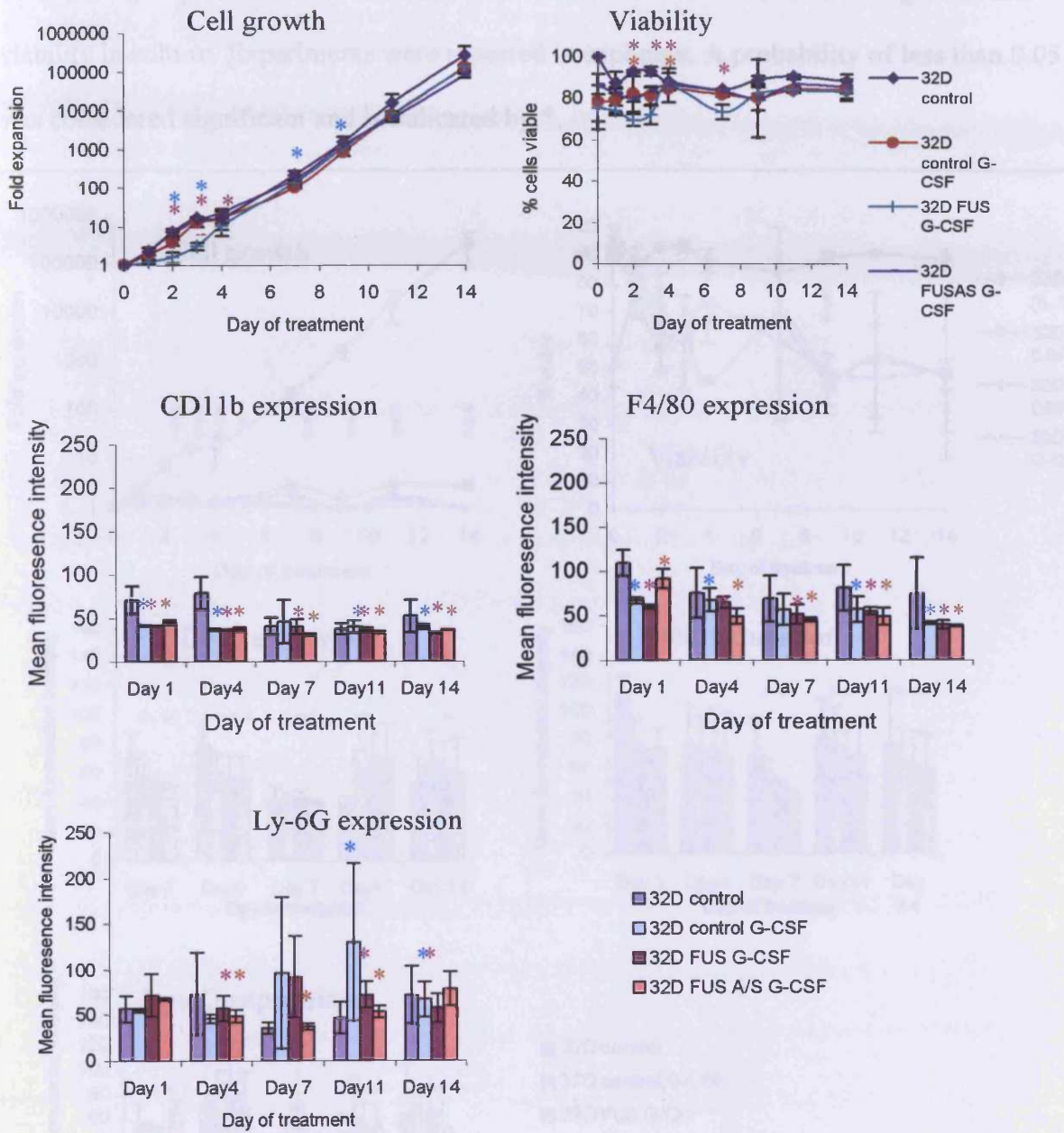
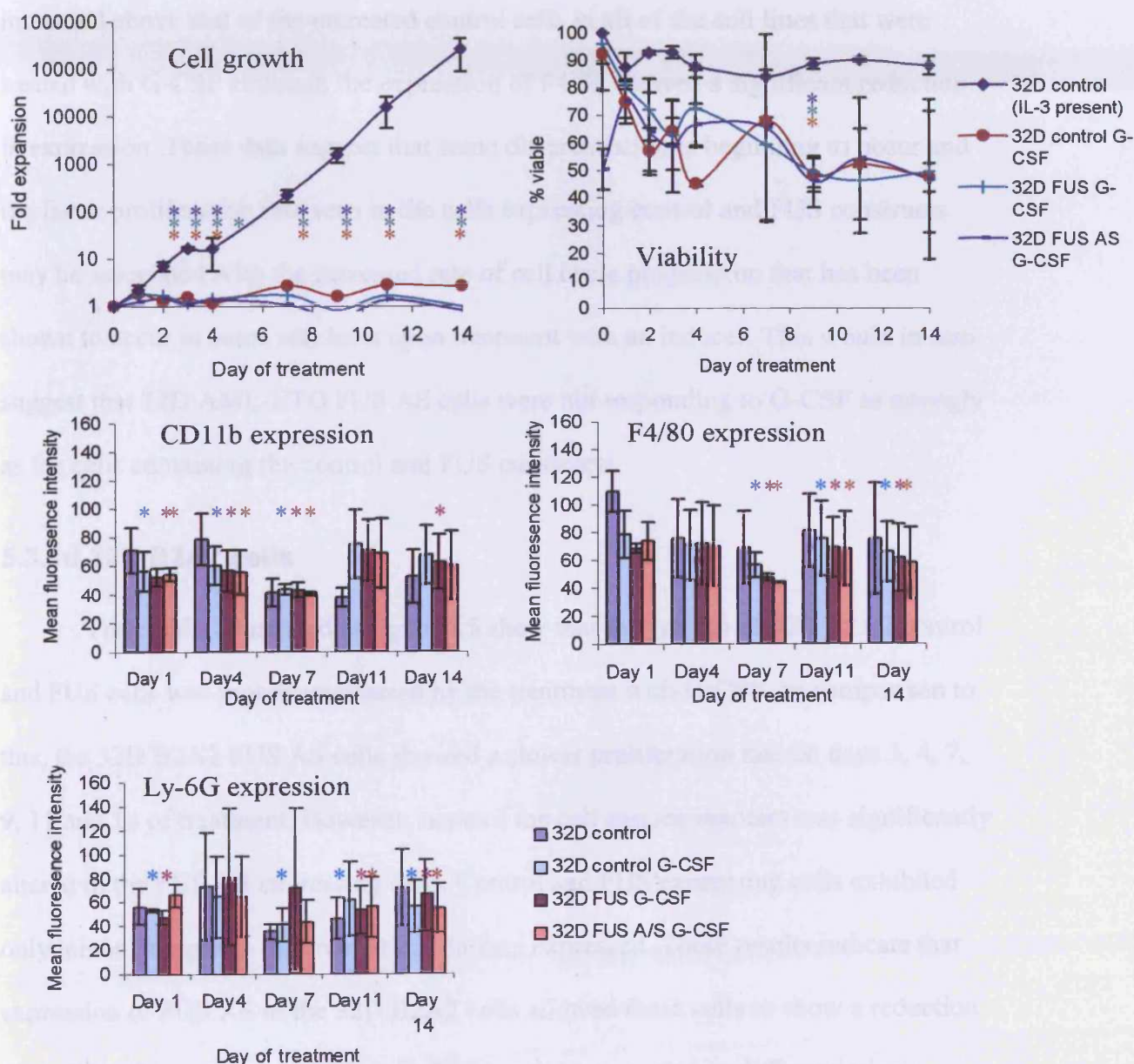


Fig. 5.3 Effect of treatment of 32D cells expressing the control, FUS and FUS AS constructs with G-CSF in the absence of IL-3. Experiments were repeated in triplicate and error bars show +/- one standard deviation of the mean. The effect of G-CSF on these cells was measured by expression of cell surface markers and the measurement of cell growth and viability in culture. Experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.



5.3.1c 32D AML-ETO cells (+IL-3)

The results illustrated in figure 5.4 show that the growth rate of the cells expressing the control and FUS constructs is significantly elevated in the early stages of treatment with G-CSF. The viability of these cells remained unaffected except in the 32D AML-ETO FUS cells which demonstrate a greater viability than the untreated cells on days 1, 2, 3 and 7 of treatment. CD11b and Ly-6G expression was increased above that of the untreated control cells in all of the cell lines that were treated with G-CSF although the expression of F4/80 showed a significant reduction in expression. These data suggest that some differentiation is beginning to occur and the faster proliferation rate seen in the cells expressing control and FUS constructs may be associated with the increased rate of cell cycle progression that has been shown to occur in some cell lines upon treatment with an inducer. This would in turn suggest that 32D AML-ETO FUS AS cells were not responding to G-CSF as strongly as the cells containing the control and FUS constructs.

5.3.1d 32D B2A2 cells

The results illustrated in figure 5.5 show that the growth of 32D B2A2 control and FUS cells was mostly unaffected by the treatment with G-CSF. In comparison to this, the 32D B2A2 FUS AS cells showed a slower proliferation rate on days 3, 4, 7, 9, 11 and 14 of treatment. However, none of the cell surface markers was significantly altered in the FUS AS expressing cells. Control and FUS expressing cells exhibited only minor changes in differentiation markers expressed. These results indicate that expression of FUS AS in the 32D B2A2 cells allowed these cells to show a reduction of proliferation rate in response to G-CSF but did not result in a differentiation response.

Fig 5.4 Effect of treatment with G-CSF in 32D AML-ETO cells expressing the control, FUS and FUS AS constructs in the presence of IL-3. Error bars indicate +/- on standard deviation either side of the mean and experiments were repeated in triplicate. A probability of less than 0.05 was considered significant and is indicated by *.

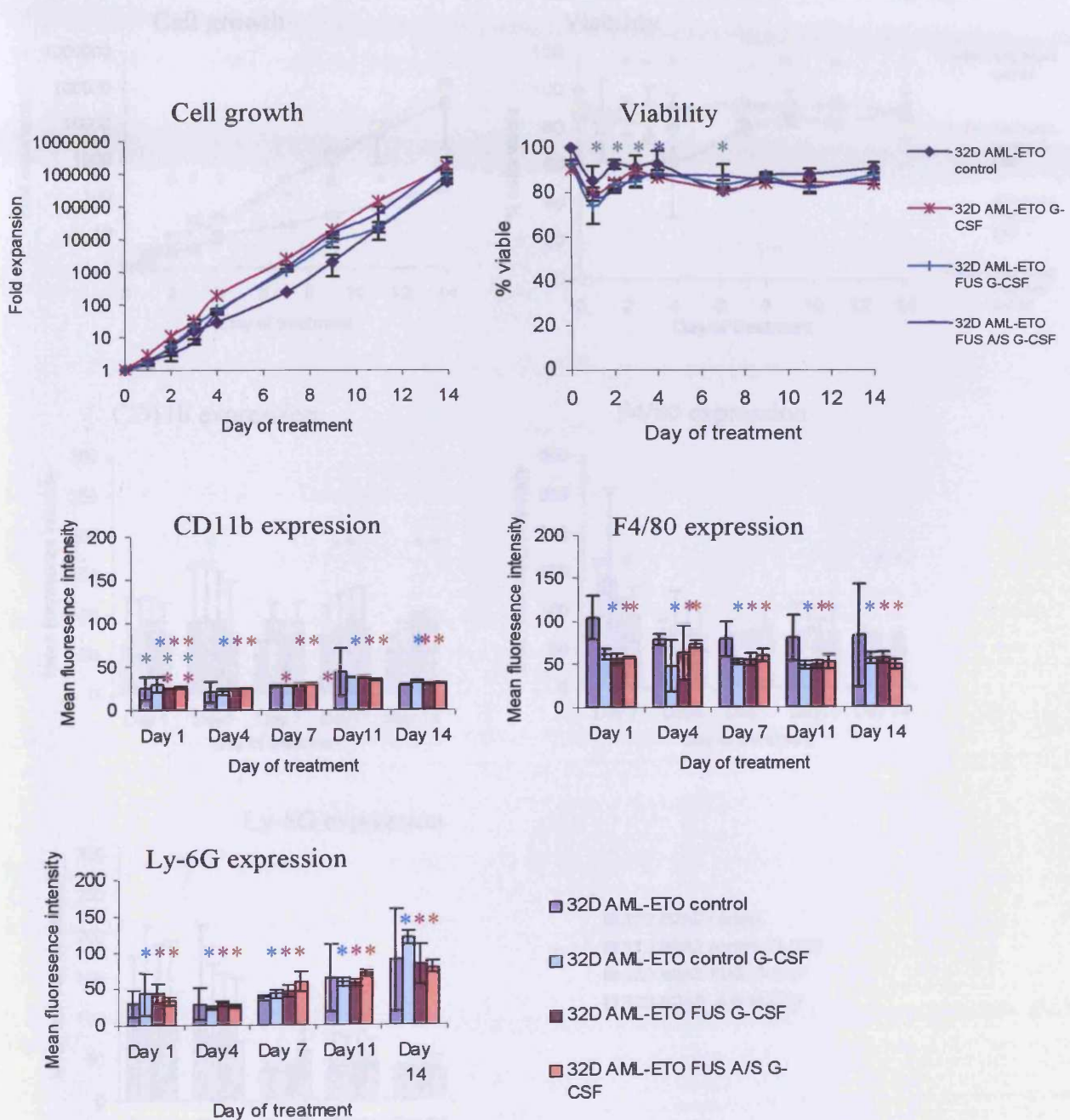
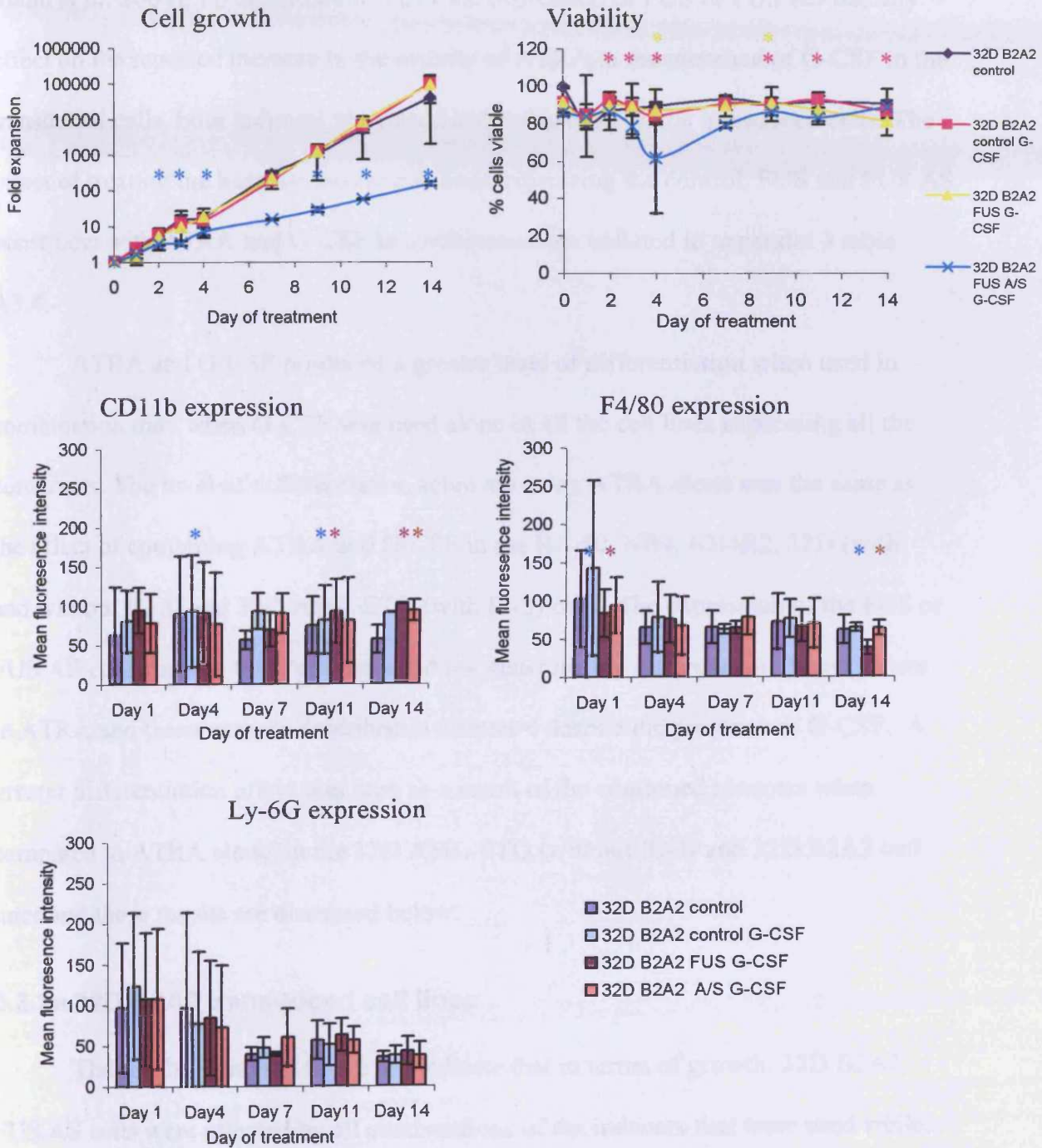


Fig. 5.5 Effect of treatment with G-CSF on 32D B2A2 cells expressing the control, FUS and FUS AS constructs over 14 days. Experiments were repeated in triplicate and error bars indicate +/- one standard deviation about the mean. A probability of less than 0.05 was considered significant and is indicated by *.



5.3.2 The combined effect of ATRA and G-CSF on haematopoietic cells containing the control, FUS and FUS AS sequences

Synergistic activity has previously been reported between ATRA and G-CSF when used in combination to induce differentiation in NB4 cells (Gianni *et al.* 1994; Maun *et al.* 2004). To determine whether the expression of FUS or FUS AS had any effect on the reported increase in the activity of ATRA in the presence of G-CSF in the transduced cells, both inducers were used in combination on the transduced cells. The effect of treating the haematopoietic cell lines expressing the control, FUS and FUS AS constructs with ATRA and G-CSF in combination are collated in appendix 3 table A3.4.

ATRA and G-CSF produced a greater level of differentiation when used in combination than when G-CSF was used alone in all the cell lines expressing all the constructs. The level of differentiation achieved using ATRA alone was the same as the effect of combining ATRA and G-CSF in the HL-60, NB4, NB4R2, 32D (with and without IL-3) and 32D AML-ETO (with IL-3) cells. The expression of the FUS or FUS AS constructs in these cell lines did not generate any difference in the responses to ATRA and these were as described in chapter 4 despite the presence of G-CSF. A greater differentiation effect was seen as a result of the combined inducers when compared to ATRA alone, in the 32D AML-ETO (without IL-3) and 32D B2A2 cell lines and these results are discussed below.

5.3.2a 32D B2A2 transduced cell lines

The results shown in figure 5.6 indicate that in terms of growth, 32D B2A2 FUS AS cells were affected by all combinations of the inducers that were used while, as with the parental cells, the 32D B2A2 FUS cells were not affected at all. Only minor differences existed between the viability of the cells treated with ATRA in

combination with G-CSF or with either separately and this was seen in cells expressing either FUS or FUS AS. The expression of the cell surface markers was seen to be increased by the use of both ATRA and G-CSF in combination compared to the levels of induction seen when either inducer was used separately. In contrast to the effect on growth, induction of different antigens was seen in both the FUS and FUS AS expressing 32D B2A2 cells suggesting that the expression of FUS AS was influencing the alteration in proliferative ability rather than differentiation.

5.3.2b 32D AML-ETO transduced cell lines

No difference was observed between the 32D AML-ETO cells expressing FUS or FUS AS in the absence of IL-3 when measured in terms of growth and viability as illustrated in figure 5.7. When induction of CD11b, F4/80 and Ly-6G were assessed it was found that combined use of ATRA and G-CSF produced a greater induction of these antigens in the 32D AML-ETO FUS AS cells than either of the inducers used alone. In contrast to this, induction of these antigens in the 32D AML-ETO FUS cells was only equal to that induced by the use of ATRA alone.

Fig 5.6 Effect of treating 32D B2A2 cells expressing FUS and FUS AS with G-CSF and ATRA in combination. Error bars indicate +/- one standard deviation about the mean and experiments were repeated in triplicate. * indicates significant difference to treatment with ATRA and G-CSF.

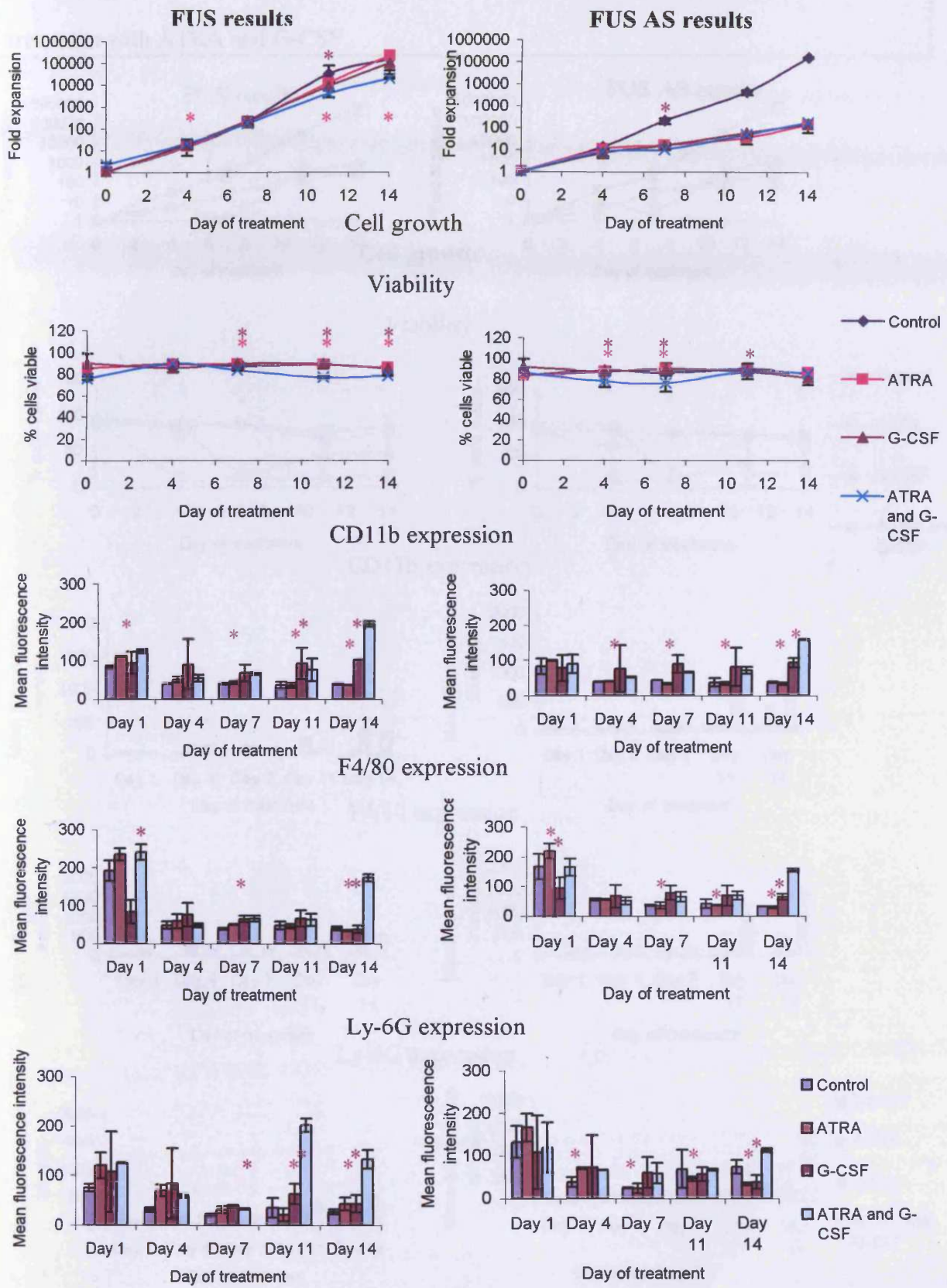
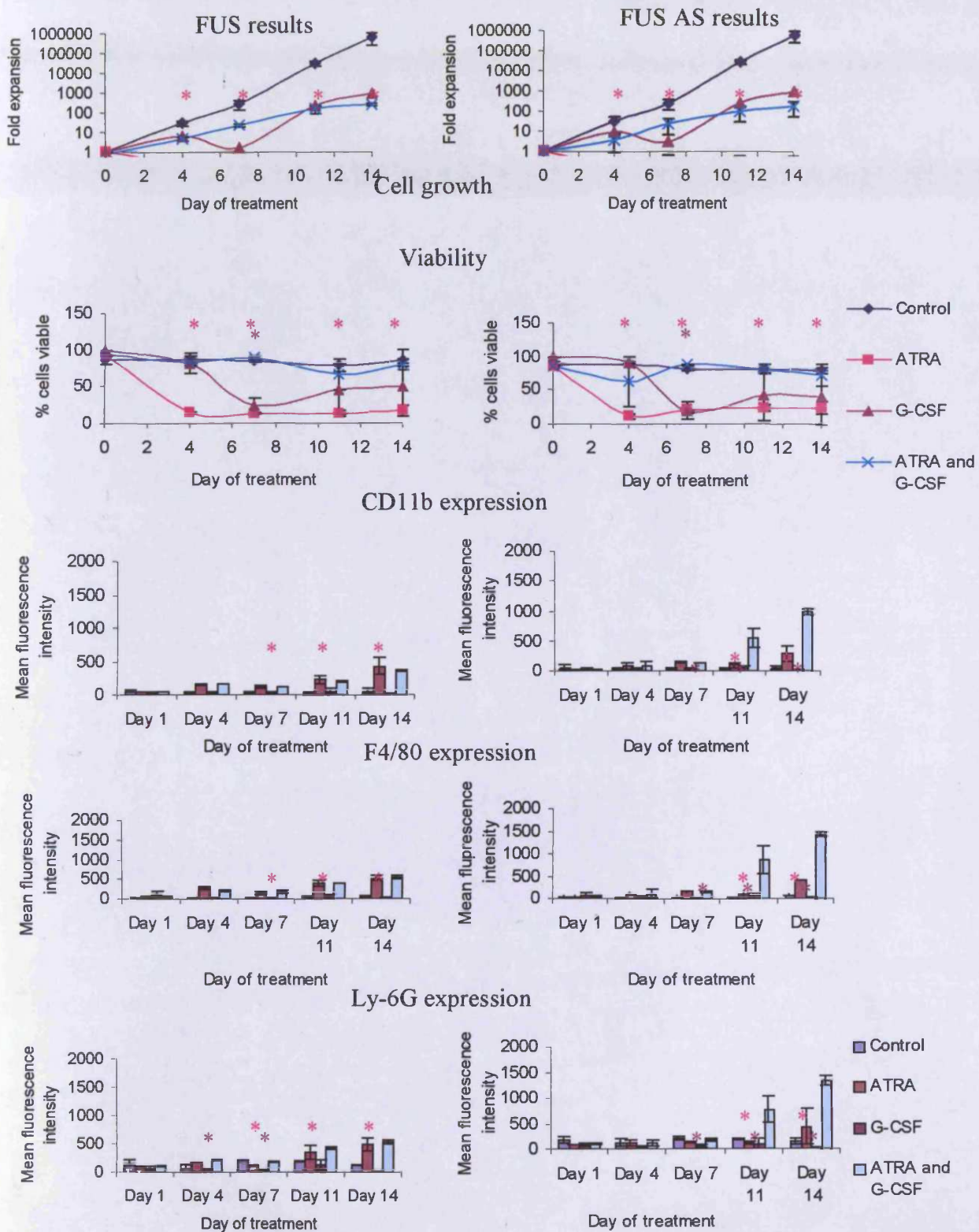


Fig. 5.7 Effect of inducing transduced 32D AML-ETO cells with ATRA and G-CSF in combination in the absence of IL-3. Error bars indicate +/- one standard deviation of the mean and experiments were repeated in triplicate. * indicates significant difference to treatment with ATRA and G-CSF.



5.3.2c Effects of G-CSF and ATRA separately and in combination on the expression of FUS mRNA and protein within the transduced cell lines

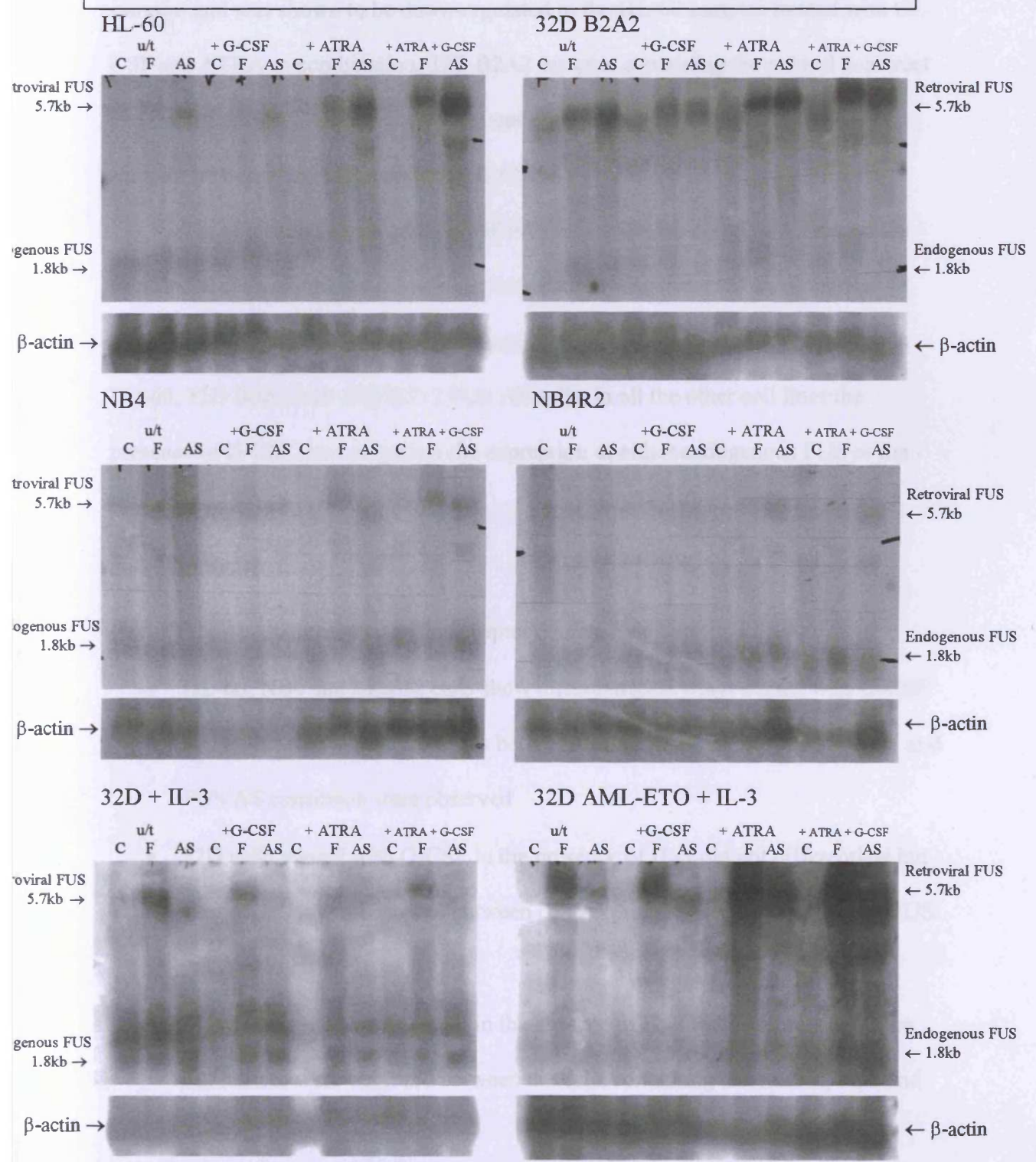
Northern blots were performed on the transduced cell lines to show whether the treatment of these cells with ATRA and G-CSF in combination produced any differences in the amount of endogenous and retroviral transcripts that were present within the cells. These Northern blots are shown in figure 5.8. Equal loading of mRNA into the Northern electrophoresis gels is shown below each filter hybridised to the β -actin housekeeping gene.

The retroviral message is up-regulated following treatment with ATRA and ATRA with G-CSF in the cells with FUS and FUS AS in the HL-60, NB4R2, 32D B2A2 and only slightly in the NB4 cells. G-CSF alone had no effect on the level of expression of the retroviral construct. In the HL-60 cells treatment with ATRA and G-CSF in combination led to a greater up-regulation of the retroviral message than was seen when the cells were treated with ATRA alone. This was not seen in the other cell lines where the up-regulation of the retroviral message was equally induced by ATRA or ATRA and G-CSF in combination.

The blots for the 32D and 32D AML-ETO cells treated in the absence of IL-3 were of poor quality due to difficulties in achieving good quality RNA as a result of the speed at which these cells die in the treatment culture conditions.

The blots for 32D and 32D AML-ETO cells treated in the presence of IL-3 show the presence of the retroviral message in the untreated cells and the cells treated

Fig 5.8 Effect of the treatment of the transduced cell lines with G-CSF, ATRA, and both inducers in combination on the expression of the endogenous FUS protein and the retroviral sequences. P = control, F = FUS, A/S = FUS AS



with G-CSF alone but the results for the cells treated with ATRA alone, or for ATRA and G-CSF used in combination again produced poor results.

The presence of the endogenous FUS gene was detected in almost all of the samples and was shown to be down-regulated in the HL-60 samples treated with G-CSF and ATRA in combination, 32D B2A2 samples containing the control construct and treated with ATRA and G-CSF in combination and the 32D B2A2 FUS AS samples treated with ATRA and with G-CSF.

These results suggest that G-CSF only augments the effect of ATRA on the transduced cells in isolated cases: these being the up-regulation of the retroviral message in HL-60 cells and the down-regulation of endogenous FUS expression in HL-60, 32D B2A2 and 32D B2A2 FUS AS cells. In all the other cell lines the presence of G-CSF does not affect the expression of either endogenous FUS or the retroviral message.

5.4 Discussion

The results presented in this chapter demonstrate that

- HL-60, NB4 and NB4R2 cells show differentiation when treated with G-CSF and in these cells no differences between cells containing the control, FUS and FUS AS constructs were observed
- 32D cells treated with G-CSF in the presence of IL-3 did not differentiate but no difference was observed between cells expressing the control, FUS or FUS AS constructs.
- 32D cells treated with G-CSF in the absence of IL-3 did differentiate but no differences were observed between the cells containing the control, FUS and FUS AS constructs.
- 32D AML-ETO cells treated with G-CSF in the presence of IL-3 did not differentiate and the cells containing the control and FUS constructs

proliferated at a faster rate than untreated cells in the early parts of the experiment. The FUS AS cells were unaffected.

- 32D AML-ETO cells treated in the absence of IL-3 did not differentiate but showed a reduction in growth rate. No difference was observed between cells expressing each of the constructs.
- 32D B2A2 FUS AS cells showed a reduced growth rate when treated with G-CSF while the 32D B2A2 control and FUS cells were unaffected.

Addressing these points one at a time, the lack of differentiation assessed by the expression of the CD11b and CD33 cell surface markers in the HL-60, NB4 and NB4R2 cells has been previously reported (Gianni *et al.* 1994). This report suggests that G-CSF alone does not generate an increase in the level of CD11b expressed on NB4 cells or a reduction in the level of CD33. Similar results were reported for HL-60 cells in the same report. The inability to detect differentiation by observing the expression of the CD11b and CD33 in the NB4 and HL-60 cells, and the lack of any morphological effect generated on these cells by G-CSF (Gianni *et al.* 1994) means that the effect of G-CSF on these cells can be observed only in the measurement of proliferation rate from the parameters that were measured in these experiments. As discussed in chapter 4, CD38 expression is an ATRA-responsive gene and this cell surface marker is not expressed in response to G-CSF. Consequently it is not possible to ask questions about the role of FUS in G-CSF mediated differentiation effects using these cells as a model.

In the 32D cell line, when treated with G-CSF in the presence of IL-3, control and FUS cells exhibited a slower growth rate than the control cells while the 32D FUS AS cells remained capable of proliferation at the same rate as the untreated control cells. The viability and expression of the CD11b, F4/80 and Ly-6G cell surface

markers remained largely unaffected or was lower in the treated cells. This was not in agreement with previously published reports that suggest that CD11b showed increased expression when 32D cells are treated with G-CSF (Panopoulos *et al.* 2002), while Ly-6G is expressed on murine granulocytes (Lagasse *et al.* 1996). It may be possible that in these cells, the effect of G-CSF, as in the NB4 and HL-60 cells is not sufficient to promote expression of the CD11b marker on the cells. If the differentiation that is achieved by treatment of these cells is incomplete it may not result in the cells expressing the F4/80 and Ly-6G markers as these are seen on mature macrophages and monocytes and on mature granulocytes and neutrophils respectively. The presence of IL-3 during treatment of these cells may also be responsible for the lack of cell surface marker expression in these cells as IL-3 is known to keep 32D cells proliferating and the signals received by the cells from the IL-3 may be more dominant than those received from the presence of the G-CSF.

When these same 32D cells were treated with G-CSF in the absence of IL-3 (to determine whether the expression of FUS or FUS AS had an effect on the dependence of the cells on IL-3 and additionally, as a response was more likely to be achieved with this cytokine being absent and therefore to gain an insight into the role of FUS in G-CSF mediated differentiation) however, the cells containing all the constructs showed a significant reduction in the growth rate compared to untreated cells and the expression of the cell surface markers that were measured were frequently reduced. The viability of these cells was only significantly reduced on day 9 of the experiments. In absence of IL-3, the effect of only reducing the growth rate of these cells but not increasing the expression of any of the cells' surface markers is not completely in agreement with previously published results (Austyn *et al.* 1981; Lagasse *et al.* 1996; Panopoulos *et al.* 2002) which suggests that increased expression of the cell's surface markers alongside the reduction in growth rate would indicate

that differentiation was occurring in these cells. These results are not consistent with previous reports (Perrotti *et al.* 2002a) which suggest that FUS AS expression in myeloid precursor cells (32D cells) resulted in an accelerated granulocytic differentiation as a result of the reduced level of FUS protein in these cells promoting an increased expression of G-CSF-R. The over-expression of FUS in 32D cells was reported to lead to a delay in the up-regulation of G-CSF-R expression following treatment with G-CSF. It is thought that FUS may exert this effect by binding a portion of the G-CSF-R mRNA and interfere with its processing or export from the nucleus (Perrotti *et al.* 2002a).

The increase in expression of CD11b in 32D AML-ETO cells treated with G-CSF in the presence of IL-3 was the only indication that the cells were responding to the treatment. G-CSF-R is expressed in response to treatment with G-CSF (Panopoulos *et al.* 2002) but this was the only parameter that indicated any sign of differentiation was occurring in these cells. Expression of F4/80 and Ly-6G markers in the 32D AML-ETO cells were reduced by the treatment with G-CSF and these would be expected to increase in expression when differentiation occurs. In the 32D AML-ETO cells treated with G-CSF in the absence of IL-3 the expression of the cell surface markers was again unaltered or showed a reduced expression. In these cells however, the growth rates and viability of the cells were reduced which indicates that in the absence of IL-3 the cells become more able to differentiate in response to G-CSF having lost the continued proliferation signals from the presence of the IL-3. This would be in line with results previously reported for the parental 32D cells line which continue to proliferate in the presence of IL-3 but will undergo G-CSF induced differentiation into mature granulocytes once IL-3 is removed (Panopoulos *et al.* 2002). No previous reports of the effect of G-CSF on 32D cells expressing the AML-ETO fusion protein were brought to light.

An interesting result was observed when 32D B2A2 cells expressing the constructs were treated with G-CSF. Though the proliferation of cells expressing the control and FUS constructs was unaffected following treatment with G-CSF, the proliferation of cells expressing FUS AS was inhibited. Despite this there was little effect on viability and expression of the cell surface markers in any of the 32D B2A2 cell lines. It has previously been reported that 32D cells expressing the BCR-ABL fusion gene regain the ability to differentiate in response to treatment with G-CSF when FUS AS is expressed (Perrotti *et al.* 1998). This report also found that suppression of FUS expression in 32D cells themselves resulted in the up-regulation of G-CSF-R expression, down-regulation of the IL-3 receptor β chain and an accelerated G-CSF stimulated differentiation. In this report the extent of differentiation was determined by morphological analysis of May-Grunwald/Giemsa stained cytopins. Of the responses to G-CSF demonstrated in this chapter, only the response of the 32D B2A2 FUS AS cells agree with previous work described by Perrotti (Perrotti *et al.* 1998) in which down-regulation of the FUS gene resulted in 32D cells expressing the BCR/ABL fusion gene regaining the ability to differentiate in response to G-CSF.

These results, like the results in chapter 4 where the FUS AS sequence expressed in different cell lines showed different effects upon the cells suggests that the effect of the FUS gene is dependent on the manner in which differentiation is induced in the cells and also upon the underlying chromosomal translocation that is present in the cells. In BCR/ABL expressing 32D cells, the mechanism by which the reduction of FUS levels by FUS AS produces an ability to differentiate in response to treatment with G-CSF could involve G-CSF-R. In 32D BCR/ABL cells the high endogenous level of FUS expression has been shown to prevent expression of G-CSF-R in response to treatment with G-CSF. This is thought to be occur by FUS binding

the G-CSF-R mRNA and mediating its rapid degradation (Perrotti *et al.* 1998). Removal of FUS may allow sufficient G-CSF-R expression for G-CSF induced differentiation to occur in 32D BCR/ABL FUS AS cells. Less work has been done on the effect of FUS expression during differentiation in cell lines with other chromosomal translocations. The endogenous level of FUS expression was shown to be down-regulated in response to ATRA treatment in HL-60 and NB4 cells (Mills *et al.* 2000) and to G-CSF treatment in 32D cells (Perrotti *et al.* 1998). These findings suggest that the presence of FUS has a role which prevents differentiation in these cells. The interaction of FUS with other proteins may be key to determining the function of FUS in differentiation. Interaction of FUS with an Ets protein, Spi-1, which itself has a central role in the differentiation of B-cells in normal haematopoiesis, was shown to impede the transcriptional function of this protein (Hallier *et al.* 1998). It was also suggested by Hallier *et al.* that FUS may suppress the transcriptional activation of Spi-1 by inhibiting its ability to bind DNA. The multifunctional roles of FUS in both activating transcription and being involved in the post-transcriptional processing of mRNA of other proteins means that it is difficult to suggest which of the proteins FUS interacts with may be responsible for the different effects expression of FUS AS has been shown to have in cell lines with a range of chromosomal translocations.

When ATRA and G-CSF were used in combination to induce differentiation, the combined treatment often created a larger effect than that produced by G-CSF alone but which was only equal to that produced to ATRA alone. This was seen in the HL-60, NB4, NB4R2, 32D (with and without IL-3) and 32D AML-ETO (with IL-3) cell lines. In the 32D AML-ETO cells treated in the absence of IL-3 and the 32D B2A2 cells the combined use of both inducers produced a greater expression of the cell surface markers than either inducer alone had managed to induce. The synergistic

effect of G-CSF and ATRA previously reported (Gianni *et al.* 1994) was not seen in these experiments as only an additive effect was seen in some of the cell lines. This may be the result of the fact that ATRA induced such strong differentiation in these cells that the added effect of G-CSF was not able to cause a significant enhancement. Previous investigations that have reported a synergistic effect between ATRA and G-CSF (Gianni *et al.* 1994; Maun *et al.* 2004) have used different end-points by which to measure the effect of induction which included phenotypic changes identified by Wright-Giemsa staining and the level of activity of the leukocyte alkaline phosphatase (LAP) enzyme which is used as a marker for the terminal differentiation of granulocytes. The murine cell surface markers however did show an increase in the level of expression when both the inducers were used to treat the cells. The results suggest that G-CSF is the least effective of the inducers and while ATRA induces more of the cell lines to differentiate, the use of both inducers in combination provides the greatest level of induction of differentiation in these haematopoietic cell lines, regardless of the construct they are expressing.

The different effects of the expression of the FUS AS construct in the different cell lines suggests that the down-regulation of the FUS gene may have several different effects depending on the type of haematopoietic cell in which it is occurring. The cells showing resistance to induction of differentiation with ATRA, (NB4R2 and 32D B2A2 cells) showed the regaining of the ability to differentiate when FUS expression was down-regulated while in other cell lines, the reduced level of FUS expression led to some previously ATRA-responsive cells (NB4 and 32D) becoming resistant to the induction when ATRA was used. When cells were induced with G-CSF, the unresponsive 32D B2A2 cells became responsive with the down-regulation of FUS expression, while, in previously responsive 32D AML-ETO cells, the lower level of FUS that was expressed appeared to afford the cells some resistance to

induction of differentiation with G-CSF (in the presence of IL-3). The effect of FUS AS on the expression of other genes that may be involved in the cell's response to inducers will be investigated further using gene expression analysis techniques. The investigation of gene expression pattern in cells containing modulated levels of FUS expression is hoped to identify further genes whose expression is altered as a result of the modulation of FUS expression and to begin to elucidate the manner in which the down-regulation of the FUS gene exerts the effect that has been observed in some of the cell lines examined.

Chapter 6

Affymetrix GeneChip analysis of the effect of modulation of FUS on gene expression in haematopoietic cell lines

6.1 Introduction

DNA arrays allow high throughput gene expression analysis and have enabled the examination of thousands of genes in various organisms in the context of different diseases, and at different stages of differentiation and progression of the disease. This has allowed diagnostic factors of diseases and indicators of risk factors to be identified in different diseases and the identification of potential targets for treatment that may be of interest to investigate. Information about the level of gene expression by the analysis of different mRNA species found within a cell or population of cells is informative about the cell's state and the activation of genes that may function in response to treatment of the cells with drugs or in response to other stresses placed upon the cells. The results presented in Appendix 1 were generated using Clontech cDNA microarrays consisting of cDNA fragments of 200 to 600 base pairs in length generated from inserts from cDNA libraries or PCR products produced from gene specific primers and which are printed onto nylon membranes containing 588 characterised genes. This work is not discussed any further in this chapter as the availability of Affymetrix macroarrays with higher analytical power provided a greater level of gene expression information.

The Affymetrix gene expression analysis system has the capacity to incorporate large numbers of genes on a single GeneChip. These genes are represented by multiple oligos that are arranged at different places across the surface of the chip. The different locations on the chip of probes for the same gene ensures that high hybridisation signals are not located to one area of the chip but are evenly distributed across it to ensure the readings of hybridisation signals are accurate and to prevent the loss of an entire gene's probesets due to damage and hybridisation artefacts. The reproducibility and specificity of the gene expression measurements generated using the GeneChips is increased above that achieved by other gene

expression analysis methods by the incorporation of multiple probes for each gene being represented in the GeneChip. Multiple control probes containing one mismatched base in the probe sequence for each gene are also incorporated into the GeneChips. These control probes provide a method of discriminating whether the binding to the actual probe for the gene is above the level of binding observed to the control oligo. Despite these obvious advantages in using this system for the investigation of gene expression levels in samples, the expense of each GeneChip and the requirement for highly specialised equipment to process and decipher the information contained in the GeneChip must be considered when making the decision to undertake the large scale gene expression analysis that is possible with the Affymetrix GeneChips.

The aims for this chapter were to

- identify genes in each of the 32D cell lines whose expression is altered by the presence of the FUS and FUS AS constructs.
- identify genes in NB4 cells whose patterns of expression is altered in response to treatment with ATRA as a result of the presence of FUS and FUS AS within the cells
- select genes of particular interest and to verify the expression patterns of these by RQ-PCR
- assess the level of FUS expression in the NB4 cells lines during treatment with ATRA

6.2 Methods

The methods detailed in chapter 2 sections 2.8.1 to 2.8.3 were used to produce results from Affymetrix GeneChips. Two types of GeneChips were used, the HU133A chip containing probesets representing 22283 human transcripts and the Mu74A v2 containing probesets representing 12488 murine transcripts. The HU133A chip was

used to produce data on the gene expression patterns of NB4 cells containing the control, FUS and FUS AS constructs before treatment with ATRA, following 24h of exposure to ATRA and following 96h of treatment with ATRA. The Mu74A v2 chip was used to generate information about the gene expression patterns in the murine 32D cells containing no insert, FUS and FUS AS, 32D AML-ETO cells without any construct and with FUS and FUS AS and 32D B2A2 cells again with no insert added and with FUS and FUS AS. The results generated were analysed using the GeneSpring software from Silicon Genetics and verification of the levels of gene expression were performed by quantitative RT-PCR (RQ-PCR) performed on a light cycler as described in section 2.4.5. In this study only one chip was used for each condition.

Normalisation of the results produced from the GeneChips was carried out using the GeneSpring software and this serves to standardise the data to enable real variations in the data and variations that are generated as a result of the measurement process to be determined. Two forms of normalisation were performed, these were a per-chip normalisation and a per-gene normalisation. A per-chip normalisation controls for experimental variations involved in the preparation of the chip. The expression values of the data points for each gene are normalised to the 50th percentile (the mean value) of all the data points. This procedure ensures that the median signal of the genes on each GeneChip stays relatively constant throughout the experiment. A per-gene normalisation accounts for the difference occurring in detection efficiency between genes and allows comparison of the relative change of gene expression levels and the display of these expression levels in a similar scale on the same graph. The expression levels are centred around a value of 1.0 and this is generated by dividing each value for a gene by the median of all the signals for that particular gene in all the chips involved in the analysis of the experiment.

The results from the murine samples were returned from the CBS who performed the final stages of hybridisation, washing and scanning of the chips, having had a TGT normalisation applied to them. A TGT is a form of normalisation that sets a target intensity of 100 for the average of the genes on the chip. The average value of the genes on the chip is calculated and a scaling factor is set which is calculated to multiply the mean intensity of the mean genes to reach 100. Each gene is then multiplied by the scaling factor to produce the normalised values. This is similar in effect to the per-chip normalisation, which was also carried out on these samples but following a TGT normalisation, had a lesser effect. TGT normalisation allows the data to be compared with other experiments and with experiments performed by other groups as opposed to only being compared within the same experiment as variability in the experiment is reduced in terms of chip batch differences and variation in the washing stages.

The data used to generate gene lists in the GeneSpring software was later selected on the basis of the absent/present calls made by Micro Array Suite 5 (MAS5) from Affymetrix. This process uses the relative levels of hybridisation to the gene probes and the mismatched gene probe to decide whether a gene is expressed above the level of non-specific binding to an oligo with one mis-matched base used as a hybridisation control. This serves to identify genes which have an apparently high hybridisation signal but which also have a high hybridisation signal on the control mis-matched oligo. This information ensures that genes denoted as present by MAS5 have actually hybridised at a greater level to the probes than to the hybridisation control oligo. A gene denoted as absent has no higher hybridisation of the probe over the hybridisation control mismatched probe. Filtering of the results was carried out using these present and absent calls in the analysis of the results.

A gene with no change in expression level was determined to be one with a level of expression of between 0.5 and 2 in all the samples under scrutiny.

Genes of particular interest were identified from their expression patterns and from the large numbers of genes which show particular expression patterns the genes to be verified by RQ-PCR were selected on criteria that included:

- the gene being known to interact with, or be homologous to FUS
- genes known to be involved in differentiation or apoptosis decisions within the cell
- genes known to have an effect on selective transcriptional activation processes
- genes known to be involved in the cellular response to ATRA
- genes coding enzymes that are chemokines or which catabolise chemokines and activation factors

Gene expression was verified using RQ-PCR as described in section 2.4.5 and the results obtained in this method were normalised to the housekeeping gene used. For murine cell lines the housekeeping gene used was β -actin and for the human cell lines S14 was used. The normalisation was carried out using the formula:

$$\text{Arbitrary units (AU)} = 2^{(s'-h')}$$

where s' = mean of the crossing point obtained for the gene and

h' = mean of the crossing points of the housekeeping gene

6.3 Results

6.3.1 Murine samples

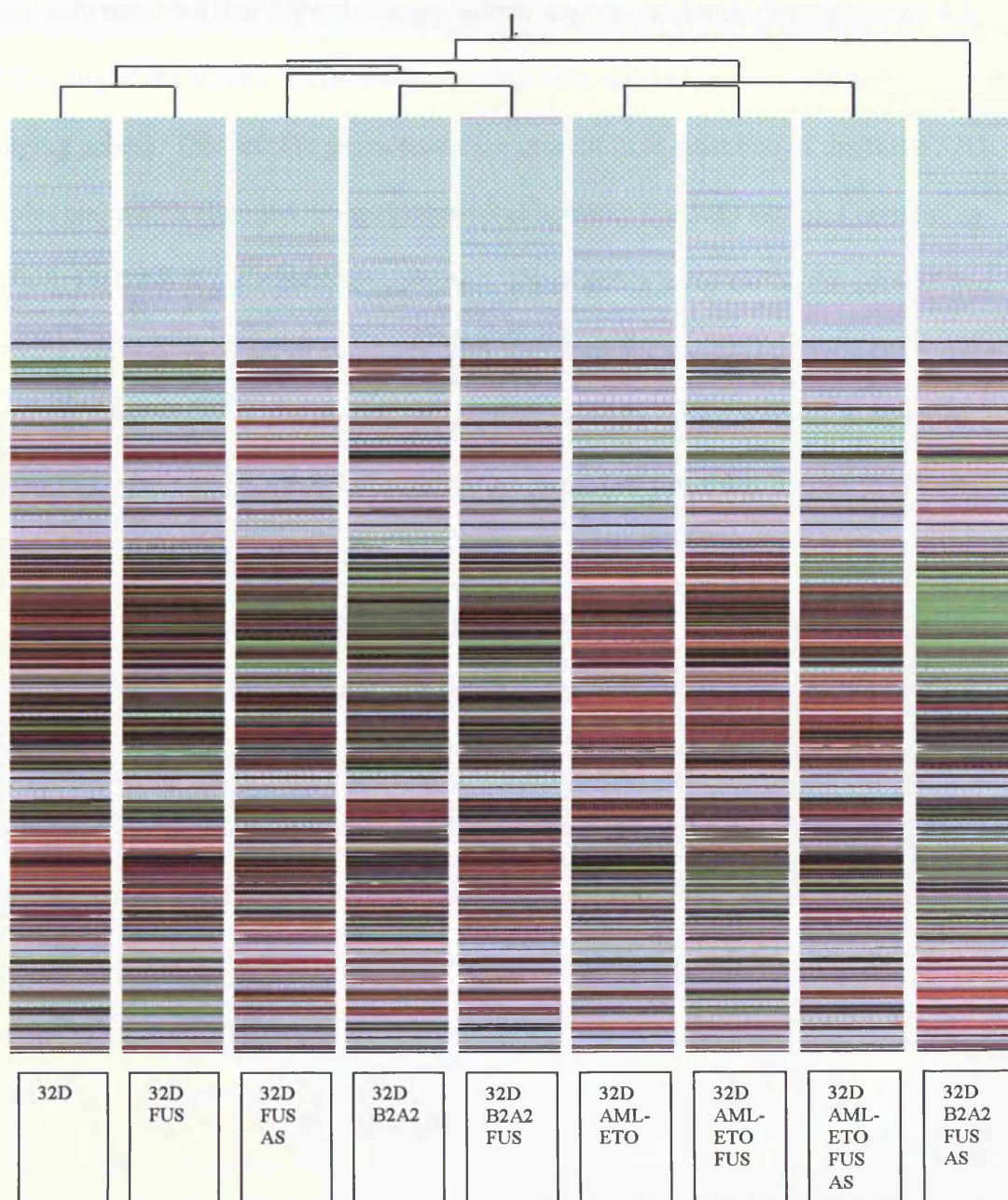
A cluster analysis was performed on the nine murine samples on which Affymetrix GeneChips had been performed. The standard correlation algorithm was used to cluster the samples and this is calculated by the formula:

$$\frac{\sum_{i=1}^n a_i b_i}{\sqrt{\left(\sum_{i=1}^n a_i^2\right)\left(\sum_{i=1}^n b_i^2\right)}}$$

The results of this cluster are shown in figure 6.1.

The results show that in terms of similarity of gene expression patterns, the 32D B2A2 FUS AS cells are the least similar sample to any of the other samples. The cluster also indicates that the addition of the FUS construct to each of the different cell types (32D, 32D AML-ETO and 32D B2A2) does not generate much alteration in the pattern of gene expression. The presence of FUS AS in the cells produces a greater alteration in the gene expression patterns. Interestingly, the 32D FUS AS cells are similar to the 32D B2A2 and 32D B2A2 FUS cells. The 32D FUS AS cells were shown in chapter 4 to have acquired an ability to continue to proliferate in response to treatment with ATRA which the 32D B2A2 cells (and the 32D B2A2 FUS cells) also exhibit. These data suggest that there may be a common underlying mechanism in the resistance of 32D B2A2 and 32D FUS AS cells to ATRA. The similarity of the 32D AML-ETO and 32D AML-ETO FUS cells, which are closely similar to the 32D AML-ETO FUS AS cells in conjunction with the lack of any difference in the responses to ATRA or G-CSF may indicate that the small differences in gene expression generated in the 32D AML-ETO cell line by modulation of FUS expression are not involved in the differentiation in these cells. Perhaps the most interesting result is that of the 32D B2A2 FUS AS cells. These cells exhibit the most altered pattern of gene expression compared with the other cell types. The expression of FUS AS in these 32D B2A2 cells appears to have resulted in a newly acquired differentiation response to both G-CSF and ATRA. The genes with altered expression in the antisense containing 32D B2A2 cells may be of particular interest.

Fig. 6.1 32D cell lines expressing the FUS and FUS AS constructs and untransduced controls clustered by sample and by gene. All genes are shown in this unsupervised cluster. The branches at the top of the cluster show the degree of similarity of the samples where the closer the branches join the samples, the more similar the samples are. Genes are indicated in red and green where a red bar indicates a high level of expression and green indicates a low level relative to the median signal for that gene across all the samples in the cluster.



6.3.1a Identification of genes that are present and changing in each of the 32D cell lines

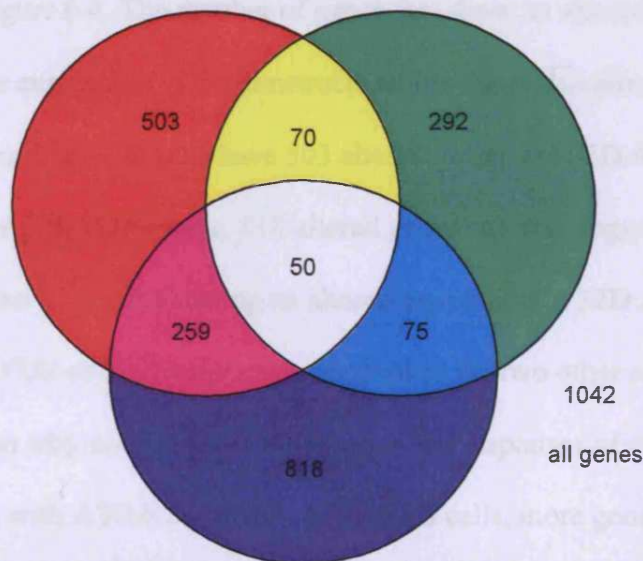
Experimental groupings for comparison were created within GeneSpring that contained only the three samples of each cell type, (for example; 32D, 32D FUS and 32D FUS AS). Using the following filtering and statistical analysis tools, genes identified as present (where the hybridisation signal exceeds that of the control oligos) in at least one of the three samples in the set were grouped in a list. From this list, genes were removed that did not change in their expression levels from between 0.5 and 2 in any of the three cells (these genes were identified as present and non-changing genes). This left the genes that were present in at least one of the three 32D samples and which changed in expression level between the 32D samples containing the FUS, FUS AS or no additional construct. These genes were called the present and changing genes in each set of cell lines. This was done for the 32D derived cells, 32D AML-ETO derived cells and the 32D B2A2 derived cells. The distribution of genes that were present and changing between the different cell lines was shown in a Venn diagram illustrated in figure 6.2.

The level of expression of the 50 genes that were present and changing in expression level in all the cell lines as a result of the expression of the FUS and FUS AS constructs have the expression profile shown in figure 6.3. The general pattern of expression of these 50 genes appear to be much the same in the 32D and 32D AML-ETO cells and a slightly altered one in the 32D B2A2 cell lines. The degree to which the expression levels are altered differs between the different cell lines but the direction of expression change remains similar as a result of the expression of FUS and FUS AS. As these genes show the similar changes in expression they are unlikely to be the genes that are involved in the changes in response to ATRA and G-CSF which were seen in the 32D FUS AS and 32D B2A2 FUS AS cells.

Fig. 6.2 Venn diagram showing the similarity of gene expression patterns of genes that were both present and changing in the 32D, 32D AML-ETO and 32D B2A2 derived cell lines

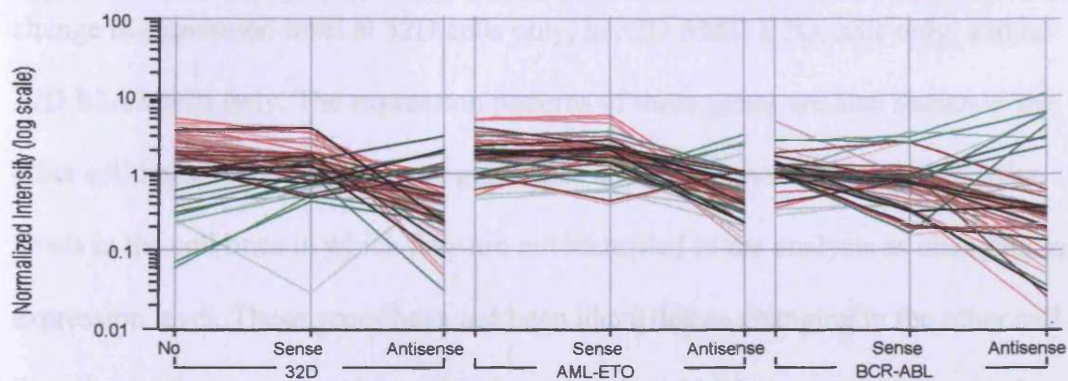
32D: genes present and changing between the untransduced cells and those containing the FUS and FUS antisense constructs

AML-ETO: genes present and changing between the untransduced cells and those containing the FUS and FUS antisense constructs



B2A2: genes present and changing between the untransduced cells and those containing the FUS and FUS antisense constructs

Fig 6.3 Expression levels of genes shown to be present and changing as a result of the expression of the FUS or FUS AS constructs in all the 32D cell lines. Samples are coloured on the basis of up- or down-regulation in the first sample analysed.

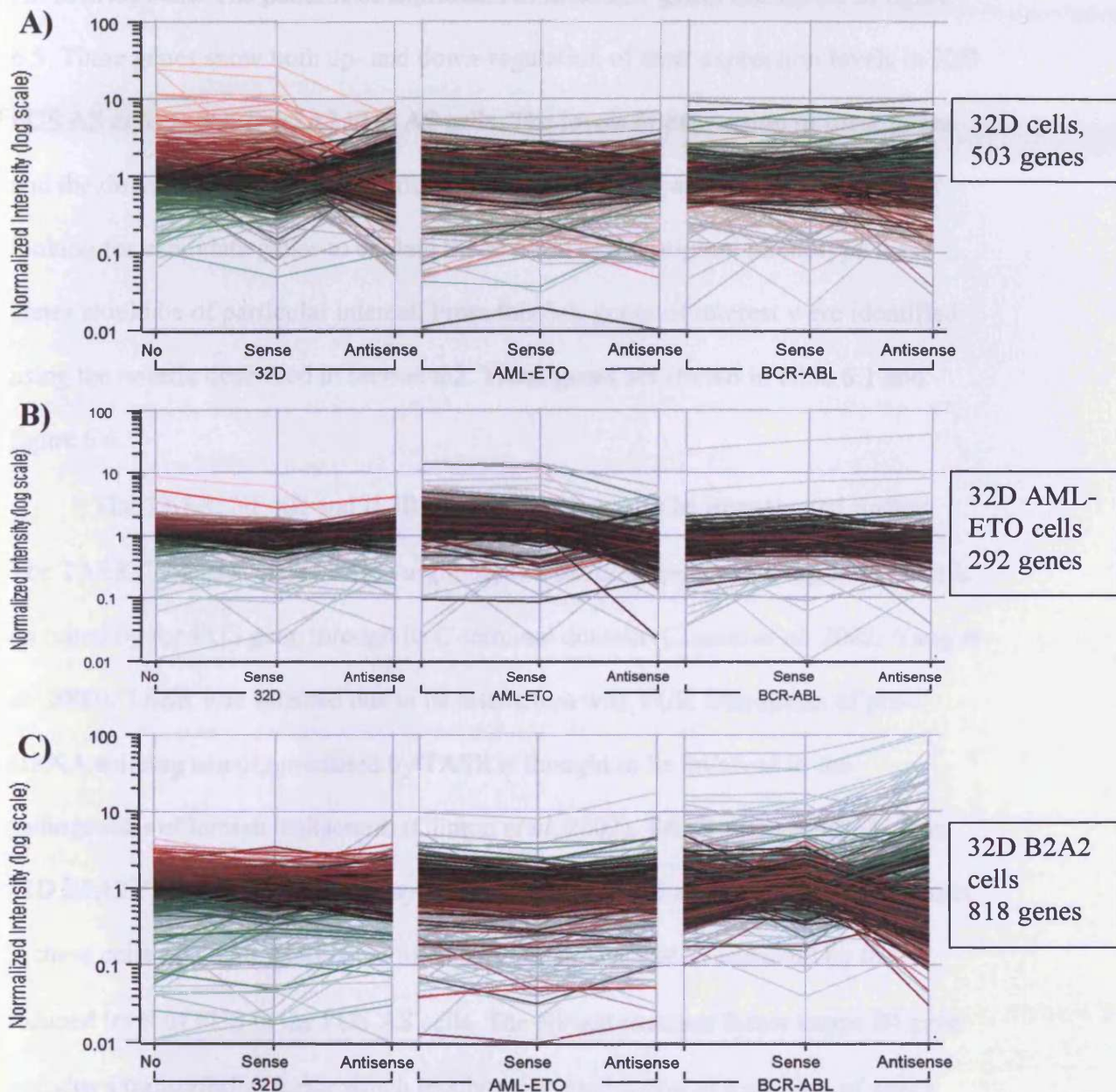


The patterns of expression of the genes which were identified as being present and changing as a result of FUS or FUS AS expression in only one cell type, for example the 32D cells only, were identified from the Venn diagram in figure 6.2 and these were studied as they may provide further information about the genes that were involved in generating the differences seen in the cells expressing FUS AS. The graphs illustrating these genes in the 32D, 32D AML-ETO and 32D B2A2 cells are shown in figure 6.4. The number of genes that show an altered expression level as a result of the expression of the constructs within the cells varies considerably between the cell lines. The 32D cells have 503 altered genes, the 32D AML-ETO cells have 292, and the 32D B2A2 cells, 818 altered genes. As was suggested previously, the lower number of genes showing an altered expression in 32D AML-ETO cells expressing FUS or FUS AS when compared to the two other cell types may also point to the reason why no difference was seen in the responses of these cells to the stimulation with ATRA or G-CSF. In the 32D cells, more genes have an altered expression but the largest number of genes with altered expression is seen in the 32D B2A2 cells which may, in part, begin to explain the more pronounced difference in the phenotype seen in the 32D B2A2 FUS AS cells when responding to induction of differentiation and compared to the phenotype seen in the 32D FUS AS containing cells.

In the graphs illustrated in figure 6.4, genes are shown which are present and change in expression level in 32D cells only, in 32D AML-ETO cells only, and in 32D B2A2 cells only. The expression patterns of these genes are also shown in the other cell lines. In each case, some genes also appear to have changing expression levels in the cell lines in which they are not identified in the analysis as changing in expression level. These genes have not been identified as changing in the other cell lines due to the present or absent decision made by MAS5. A gene is designated as

'present' if the hybridisation signals from the probes exceed those of the control probes. 'Absent' decisions relate to genes where the signals from the probes do not exceed those of the control probes. The genes were filtered on the 'presence' in at least one of the cell types from the cell lines in question and having a change in expression level between the cells expressing the control, FUS and FUS AS cells of each cell line. Where absent decisions exist in the other cell lines, these genes have not been excluded from the analysis but the apparent changes in expression levels in these cell lines are not confirmed by 'present' decision. With effects such as this, where the graph shows changing expression levels of the genes but when the filtered analysis does not, the decisions of MAS5 regarding the expression levels and the value of these results were be considered in any further analysis.

Fig 6.4 Graphs showing the expression patterns of the genes which are present and changing as a result of expression of FUS or FUS AS in A) 32D cells only, B) 32D AML-ETO cells only and C) 32D B2A2 cells only



6.3.1b Identification of genes present and changing in both the 32D and 32D B2A2 cell lines but not the 32D AML-ETO cell lines.

The subset of genes identified in figure 6.2 as having altered expression in both the 32D and 32D B2A2 cell types may also contain information about genes that are involved in the development of the phenotypes resulting from expression of FUS AS in these cells. The patterns of expression of these 259 genes are shown in figure 6.5. These genes show both up- and down-regulation of their expression levels in 32D FUS AS cells and 32D B2A2 FUS AS cells. The levels of expression of these genes and the directions of change are different between the 32D and 32D B2A2 cells. If looking for candidate genes to explain the reversal of the original phenotype these genes would be of particular interest. From this list, genes of interest were identified using the criteria described in section 6.2. These genes are shown in table 6.1 and figure 6.6.

The TASR, NF- κ B and IL3R genes were chosen to be investigated further. The TASR (TLS-associated serine-arginine) is a serine-arginine splicing factor that is recruited by the FUS gene through its C-terminal domain (Clinton *et al.* 2002; Yang *et al.* 2000). TASR was selected due to its interaction with FUS. Disruption of pre-mRNA splicing usually mediated by TASR is thought to be involved in the pathogenesis of human leukaemias (Clinton *et al.* 2002). TASR was switched off in 32D B2A2 FUS AS cells which may be consistent with the action of the AS construct in these cells as TASR itself is recruited by FUS and cannot be recruited by the reduced level of FUS in the FUS AS cells. The NF- κ B (nuclear factor kappa B) gene encodes a transcription factor which regulates the expression of a number of genes including those involved in immune function, inflammation and cellular growth control (Glavac *et al.* 1994; Le Beau *et al.* 1992; Morris *et al.* 2003). The activity of NF- κ B is regulated by I κ B which is a cellular inhibitory protein and binds NF- κ B in

an inactive form in the cytoplasm of the cell (Glavac *et al.* 1994; Morris *et al.* 2003). This gene was selected as it is a transcription factor and has previously been linked with FUS. NF- κ B was up-regulated in 32D B2A2 FUS AS and 32D FUS AS cells which may not be surprising in the light that NF- κ B is involved with the regulation of cellular growth and both these cell lines have altered growth in response to ATRA as shown in chapter 4. IL3R (interleukin 3 receptor) acts as a receptor for the potent haematopoietic growth factor IL-3 which is thought to stimulate multipotential haematopoietic stem cells and can also act as a growth factor for more committed cells. This gene was selected as the 32D B2A2 cell lines can proliferate in the absence of IL3 while the 32D and 32D AML-ETO cell lines are dependent on IL3 for continued growth (Burton *et al.* 1997; Gorman *et al.* 1990). The expression pattern for IL-3R was interesting as it was absent in the 32D B2A2 FUS AS cells which are not dependent on IL-3 for survival but was also down-regulated in 32D FUS AS cells which remain dependent on IL-3 for survival. This may suggest that the AS construct is active in both these cell types and affects the cells' requirement for IL-3 to a limited extent.

Fig 6.5 Graph showing the changes in expression level of genes identified as changing in expression in both the 32D and 32D B2A2 cell types upon insertion of the FUS AS construct.

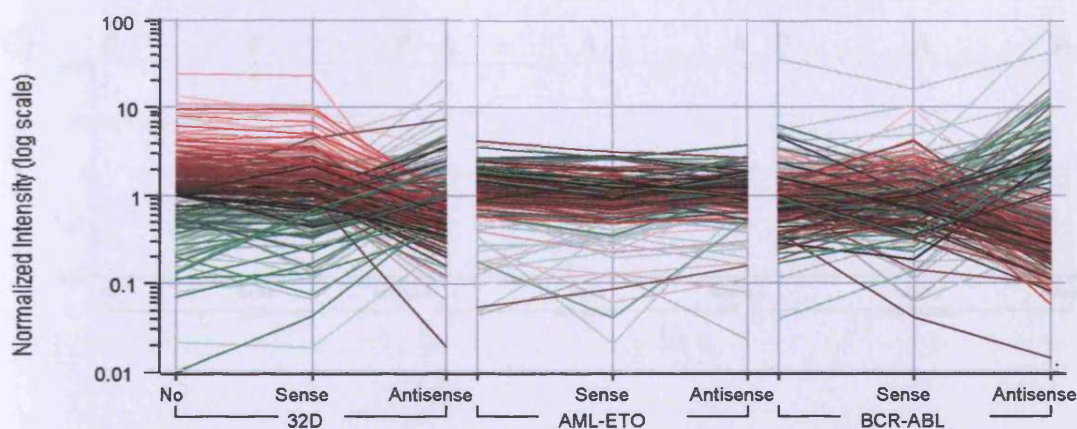
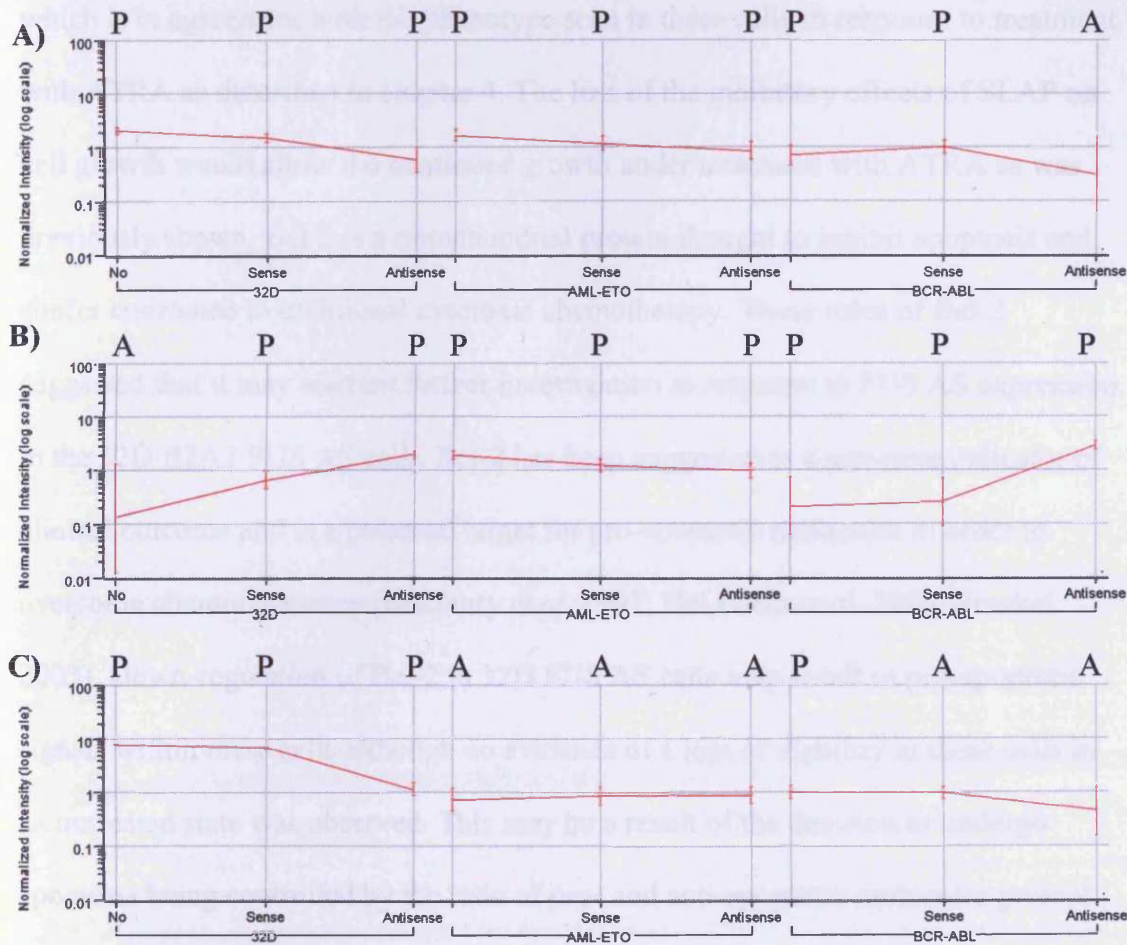


Table 6.1 Expression of genes identified as being of interest from the list of genes that had altered expression in both the 32D and 32D B2A2 cell types, P = present, A = absent as decided by MAS5. The numbers in the table indicate the normalised expression level of each gene in each cell line.

Gene	Accession number	32D			32D AML-ETO			32D B2A2											
		No insert	FUS	FUS AS	No insert	FUS	FUS AS	No insert	FUS	FUS AS									
TASR	AF060490	2.15	P	1.52	P	0.59	P	1.73	P	1.22	P	0.88	P	0.72	P	1.0	P	0.31	A
Nfkb1	U57524	0.13	A	0.67	P	2.04	P	1.91	P	1.26	P	1.0	P	0.22	P	0.26	P	2.95	P
IL3R	M34397	8.16	P	7.01	P	1.16	P	0.77	A	0.88	A	0.92	A	1.01	P	1.0	A	0.46	A

Fig 6.6 Expression of A) TASR, B) NF-κBi and C) IL3R genes in all the 32D cell lines. Error bars on the graphs indicate the level in intra experiment variation generated using GeneSpring's internal error model. P = present, A = absent.



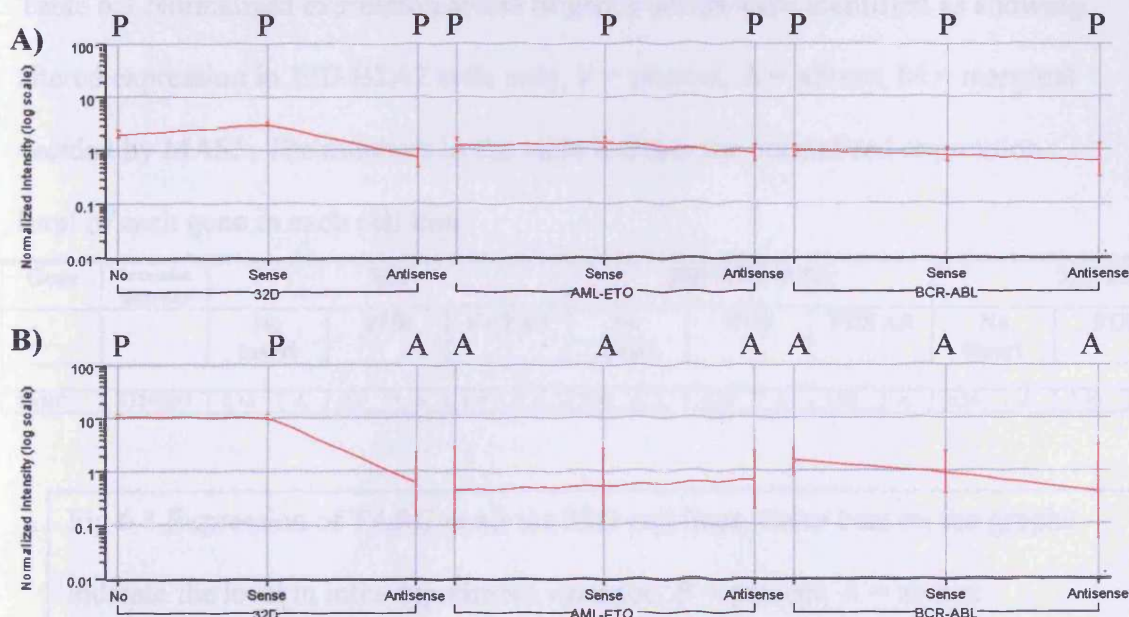
6.3.1c Identification of genes of particular interest in 32D FUS AS cells

From the 503 genes shown in figure 6.4 which were identified as being altered in expression level in the 32D cells as a result of the FUS or FUS AS construct, genes of particular interest were identified. The normalised expression levels of these genes are shown in table 6.2 and figure 6.7. The expression of selected genes, SLAP and Bcl-2, were verified using the real time PCR method. SLAP (Src-like adaptor protein) is an adaptor protein with src homology and is seen in a wide variety of cell types. SLAP acts in the Eck receptor tyrosine kinase pathway and is thought to inhibit cell growth by functioning as a negative regulator of signalling initiated by growth factors (Meijerink *et al.* 1998; Roche *et al.* 1998). The similarity of SLAP to FUS made it a candidate for further investigation. SLAP was down-regulated in 32D FUS AS cells which is in agreement with the phenotype seen in these cells in response to treatment with ATRA as described in chapter 4. The loss of the inhibitory effects of SLAP on cell growth would allow the continued growth under treatment with ATRA as was previously shown. Bcl-2 is a mitochondrial protein thought to inhibit apoptosis and confer resistance to traditional cytotoxic chemotherapy. These roles of Bcl-2 suggested that it may warrant further investigation in response to FUS AS expression in the 32D B2A2 FUS AS cells. Bcl-2 has been suggested as a sensitive indicator of clinical outcome and is a potential target for pro-apoptotic molecules in order to overcome chemoresistance (Bradbury *et al.* 1997; Del Poeta *et al.* 2003; Frankel 2003). Down-regulation of Bcl-2 in 32D FUS AS cells may result in pro-apoptotic signals within these cells although no evidence of a loss of viability in these cells in an untreated state was observed. This may be a result of the decision to undergo apoptosis being controlled by the ratio of pro- and anti-apoptotic molecules present within the cells and altering only one of these molecules may not sufficiently affect the ratio to result in apoptosis.

Table 6.2 Gene expression levels of genes identified as having altered expression levels in the 32D cells only as a result of the expression of the FUS and FUS AS in these cells, P = present, A = absent, M = marginal decided by MAS5. The numbers in the table indicate the normalised expression level of each gene in each cell line.

Gene	Accession number	32D						32D AML-ETO						32D B2A2					
		No insert		FUS		FUS AS		No insert		FUS		FUS AS		No insert		FUS		FUS AS	
Bcl-2	L31532	2.26	P	2.43	P	1.0	P	1.27	P	1.65	P	0.86	P	0.81	P	0.86	P	0.60	P
SLAP	U29056	11.51	P	11.01	P	0.62	A	0.47	A	0.57	A	0.70	A	1.60	A	0.98	A	0.43	A

Fig. 6.7 Expression of A) Bcl-2 and B) SLAP genes in all the 32D cell lines. Error bars on the graphs indicate the level of intra experiment variation. P = present, A = absent.



6.3.1d Identification of genes of interest in 32D B2A2 FUS AS cells

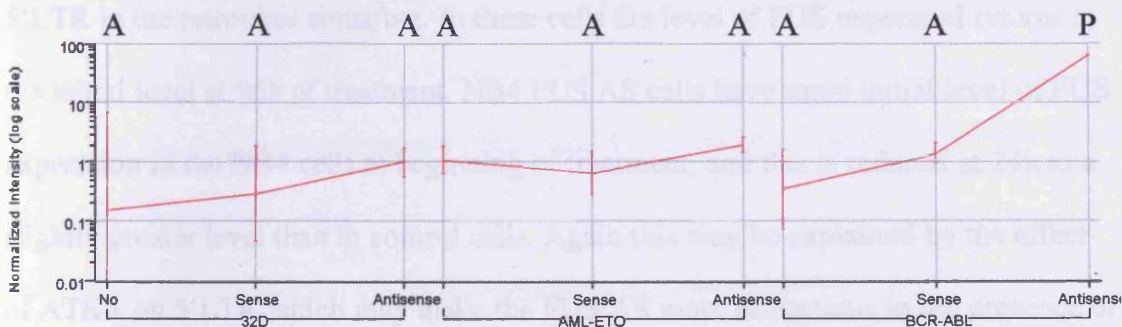
As with the 32D cells, the gene list of 818 genes used to generate the expression graphs displayed in figure 6.4 were used to further identify a set of genes that were interesting in terms of their expression levels exclusively changing in 32D B2A2 FUS AS cells. The expression levels of the gene selected for further

verification, TARC (thymus and activation regulated chemokine), are displayed in table 6.3 and figure 6.8. TARC is mainly expressed in the thymus but is also expressed in a subset of bone marrow derived dendritic cells and in human B-cells stimulated with IL-4 and TARC may play a role in the interactions between B and T-cells (Asojo *et al.* 2003; Lieberam *et al.* 1999; Lin *et al.* 2003). The actions of TARC in the development of B and T-cells made it a candidate gene for further verification. The high level of expression of TARC in 32D B2A2 FUS AS cells only suggests that it is involved in the altered response of these cells to treatment with ATRA and G-CSF but how TARC is involved in the mechanism of the response remains unclear.

Table 6.3 Normalised expression levels of genes which were identified as showing altered expression in 32D B2A2 cells only, P = present, A = absent, M = marginal decided by MAS5. The numbers in the table indicate the normalised expression level of each gene in each cell line.

Gene	Accession number	32D			32D AML-ETO			32D B2A2											
		No insert	FUS	FUS AS	No insert	FUS	FUS AS	No insert	FUS	FUS AS									
TARC	AJ242587	0.16	A	0.3	A	1.0	A	1.07	A	0.68	A	1.97	A	0.36	A	1.40	A	64.9	P

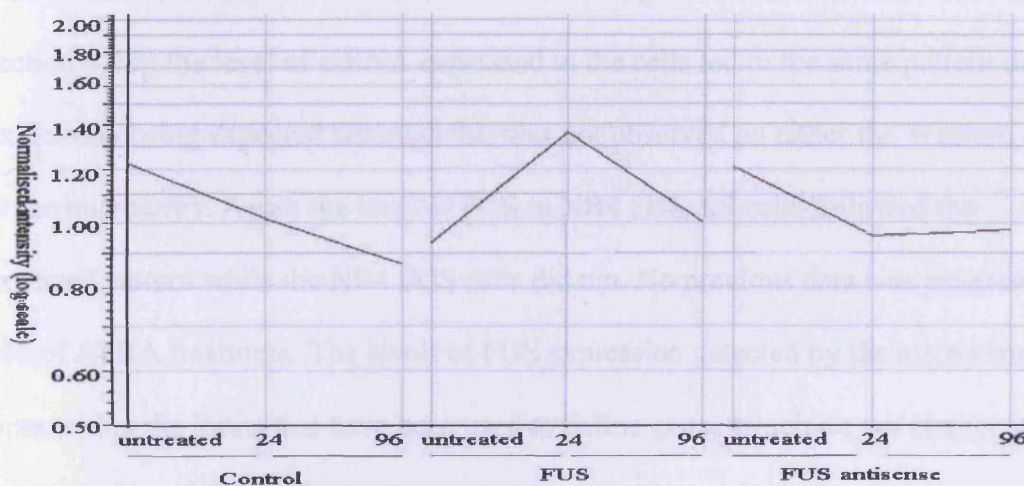
Fig 6.8 Expression of TARC in all the 32D cell lines. Error bars on the graphs indicate the level in intra experiment variation. P = present, A = absent.



6.3.2 Human cell lines treated with ATRA

The level of expression of the FUS gene detectable in the NB4 cells with the different constructs before commencement of treatment with ATRA and following 24 and 96h of exposure to ATRA are shown in figure 6.9.

Fig 6.9 Level of expression of the FUS gene before and during treatment with ATRA in the transduced NB4 cells.



The graph in figure 6.9 shows that FUS expression level is reduced in cells containing the control construct following treatment with ATRA and becomes even more reduced at 96h of ATRA exposure. In the NB4 FUS cells, the level of FUS is increased at 24h of exposure to ATRA, possibly due to the effect of the ATRA on the 5'LTR in the retroviral construct. In these cells the level of FUS expressed returns to the initial level at 96h of treatment. NB4 FUS AS cells have same initial level of FUS expression as the NB4 cells at beginning of treatment, and this is reduced at 24h to a slightly greater level than in control cells. Again this may be explained by the effect of ATRA on 5'LTR which may make the FUS AS more efficacious in the presence of ATRA. Surprisingly, the level of FUS expression decreased no further by 96h of exposure to ATRA. Microarrays can distinguish between the ectopically and

endogenously expressed FUS mRNA and the antisense FUS will not be detected.

When this data is examined in relation to data previously collected such as the Northern blots illustrated in section 3.5 and the total level of FUS expression estimated by the addition or removal of the retroviral constructs from the endogenous FUS expression, the previous results (both Northern and Western, section 3.5) show that the level of FUS in untreated NB4 FUS cells was greater than the control cells and lower than the control cells for NB4 FUS AS cells. This reduction of FUS in NB4 FUS AS cells agreed with the microarray data presented here although no increase in FUS in the NB4 FUS cells was observed. Following treatment with ATRA (96h, section 4.3.3) the level of mRNA expressed in the cells led to the same pattern of FUS expression being expected although this was not observed on either the Western blots or the microarray. Again the level of FUS in NB4 FUS AS cells followed the expected pattern while the NB4 FUS cells did not. No previous data was generated for 24h of ATRA treatment. The levels of FUS expression detected by the microarrays were within the limits that have been used to define genes which do not change in expression level. It may be that the time points used did not identify the greatest changes in FUS expression. The level of retroviral message expression determined by the Northern blots are in partial agreement with the increased FUS expression in NB4 FUS cells and NB4 FUS AS cells after 96h of treatment.

6.3.2a Gene expression patterns in NB4, NB4 FUS and NB4 FUS AS cells

NB4 and NB4 FUS cells show a differentiation response on treatment with ATRA as shown in chapter 4. NB4 FUS AS cells retain some proliferative capacity when treated under the same induction conditions. This would suggest that the patterns of gene expression in NB4 and NB4 FUS cells would be more similar than NB4 FUS AS cells. To show whether the NB4 and NB4 FUS cells showed the closest patterns of gene expression the samples were clustered according to the similarity of

the samples and the similarity of the level of genes expressed within them. The result of this clustering by both similarity of samples and by similarity of the expression of each gene is shown in figure 6.10.

The result of the clustering was to show that expression of the FUS or FUS AS constructs did alter the expression patterns of the cells but to a lesser extent than the treatment with ATRA. The untreated cell lines grouped together, as were two of the cell lines (NB4 FUS and NB4 FUS AS) at 24h and at 96h of ATRA treatment. This shows that at each time point, distinct gene expression patterns have occurred in all the cell lines as a result of the treatment with ATRA. Within these groups the presence of the constructs had smaller effects. In the untreated cells, the NB4 FUS AS cells were most dissimilar to the NB4 and NB4 FUS cells. However, surprisingly this dissimilarity was not observed when these cells were treated with ATRA. A possible explanation for this disparity is that a relatively small group of genes are altered by the expression of the FUS AS constructs but that these genes are sufficient to generate the phenotypic differences described in chapter 4.

6.3.2b Identification of genes showing changes in expression levels in response to treatment with ATRA

Genes altered as a result of ATRA treatment in all the cell lines were identified to categorize genes that were not likely to be involved in the phenotypes that were seen in the different cell lines in response to treatment. To distinguish genes with changing expression levels in all the cell lines, each cell line was first examined alone. Genes with changing expression levels were identified for each of the cell lines over the treatment time and these lists of genes were combined using a Venn diagram which allowed identification of 746 genes whose level of expression was altered in all the cell lines as a result of treatment with ATRA. These genes are shown in figure 6.11.

Fig 6.10 Cluster analysis of gene expression in all the samples. C = NB4 control cells, F = NB4 FUS cells, AS = NB4 FUS AS cells, ut = untreated cells, 24 = cells exposed to ATRA for 24h, 96 = cells exposed to ATRA for 96h. All genes are shown in this unsupervised cluster.

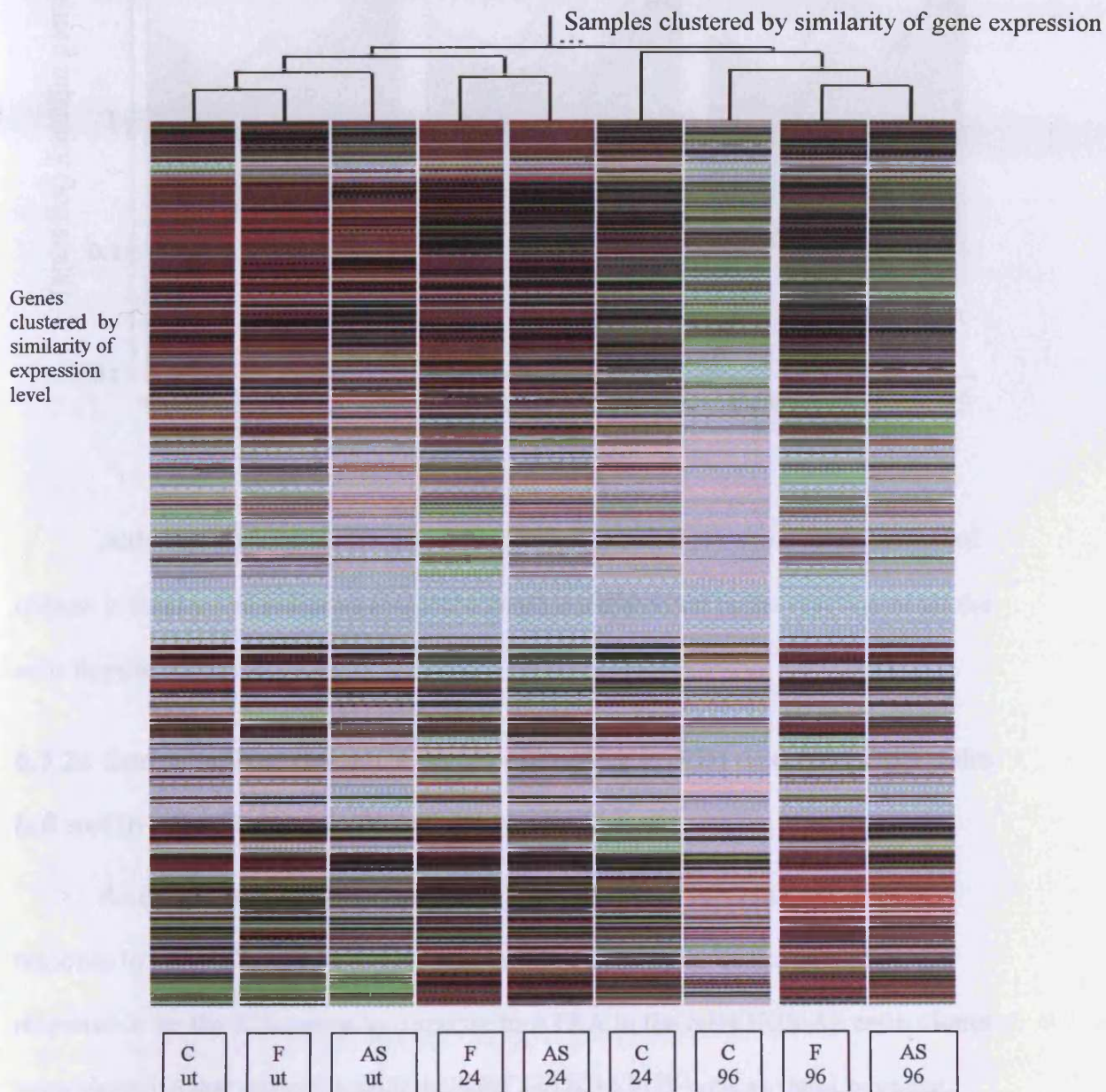
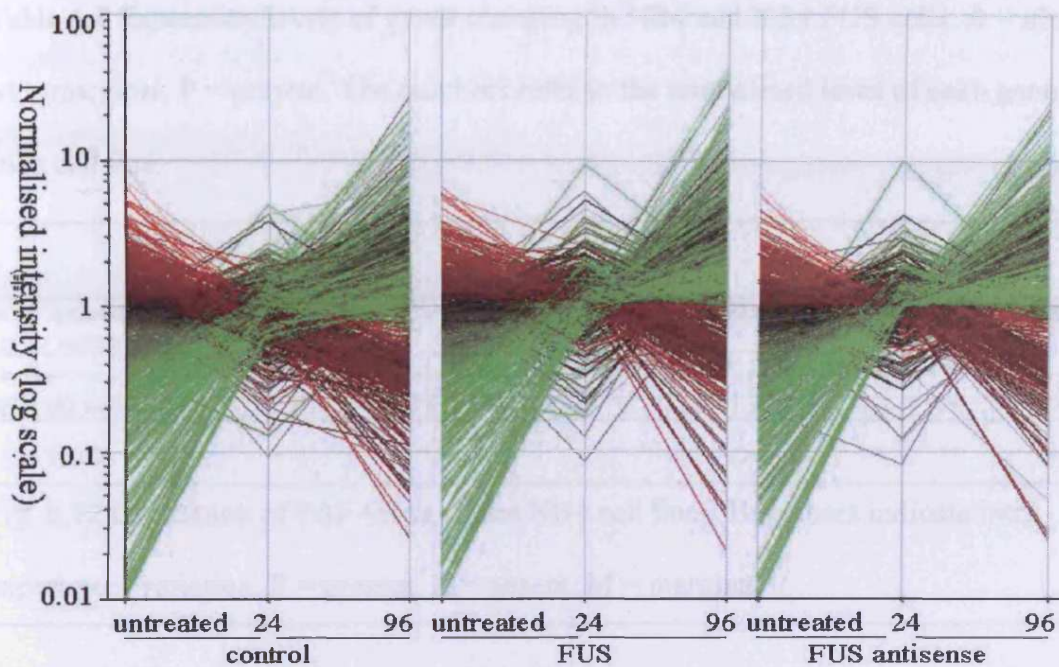


Fig. 6.11 Genes whose expression is altered in response to treatment with ATRA in all NB4 cell lines.



Although differences are present between the cell lines, the overall pattern of change is similar and indicates that these genes are important in the reaction of all the cells lines to ATRA.

6.3.2c Genes with expression levels changing in NB4 and NB4 FUS cells but not in NB4 FUS AS cells.

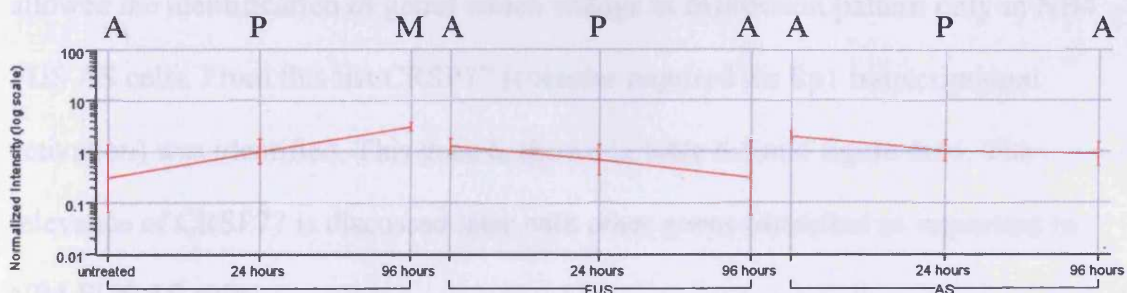
As the genes identified in section 6.3.2b are altered in all the cell lines in response to ATRA, it may be argued that they are unlikely to be the genes that are responsible for the differences in response to ATRA in the NB4 FUS AS cells. Genes were identified that change in both the NB4 and NB4 FUS cells as these have the same response to treatment with ATRA. These genes were identified from a Venn diagram of genes which change in each of the cell lines. From the resulting list of genes changing in both NB4 and NB4 FUS cell lines but not in NB4 FUS AS cells

genes of particular interest were identified. These genes are shown in table 6.4 and figure 6.12.

Table 6.4 Expression levels of genes changing in NB4 and NB4 FUS cells. A = absent, M = marginal, P = present. The numbers refer to the normalised level of each gene in each cell line.

Gene	Accession number	NB4						NB4 FUS						NB4 FUS AS					
		untreated	24 h	96 h	untreated	24 h	96 h	untreated	24 h	96 h	untreated	24 h	96 h						
PAFAH	NM_005084	0.31	A	1.0	P	3.11	M	1.28	A	0.69	P	0.30	A	2.07	P	1.07	P	0.92	A

Fig. 6.12 Expression of PAFAH in all the NB4 cell lines. Error bars indicate intra experiment variation. P = present, A = absent, M = marginal.



PAFAH (platelet-activating factor acteylhydrolase) encodes an enzyme that catabolises PAF and its expression level is increased in monocytes that are differentiated into macrophages or monocyte-derived dendritic cells (Al Darmaki *et al.* 2003). The increased expression of PAFAH during the differentiation of monocyte warranted further investigation of its expression in response to ATRA. PAFAH is present in and expressed at around 1.0 in each of the NB4 cell lines treated with ATRA at 24h. In the NB4 and NB4 FUS cells the signal was absent at 0h of treatment and absent or marginal at 96 hours of treatment so although the directions in which

the expression of this gene appears to change is different in these two cell lines, the absent decisions indicate that at these time points the gene is not expressed. The change in expression in NB4 FUS AS cells treated with ATRA fall inside the limits set as non-changing in these experiments. The equal level of expression of PAFAH at 24h of ATRA treatment in all the NB4 cell lines suggests that at this point in the treatment PAFAH is active in the same manner in each of the cell lines.

6.3.2d Genes which change in response to ATRA only in NB4 FUS AS cells

Genes changing exclusively in NB4 FUS AS cells may be genes active in generating the altered response to ATRA that was observed in the NB4 FUS AS cells and classification of these candidate genes may allow identification of genes actively involved in this altered response. A Venn diagram of genes altered in each cell line allowed the identification of genes which change in expression pattern only in NB4 FUS AS cells. From this list CRSP77 (cofactor required for Sp1 transcriptional activation) was identified. This gene is shown in table 6.5 and figure 6.14. The relevance of CRSP77 is discussed later with other genes identified as important in NB4 FUS AS cells.

Further refinement of the list generated from the Venn diagram was performed to reduce the list of candidate genes. This was done by the removal of genes expressed in the NB4 or NB4 FUS cells at any point during their treatment. This left a list of 59 candidate genes which are shown in figure 6.13. Genes of particular interest were identified from the 59 genes exclusively expressed and with expression levels that change as a result of treatment with ATRA were identified and these are shown in table 6.7 along with CRSP77. These genes are also shown in figure 6.14. As discussed previously, the genes with apparently changing expression in the NB4 and NB4 FUS cells during treatment with ATRA have absent decisions and although they appear to

change in expression level, are either not expressed or do not have changes in expression in these cell lines.

Fig 6.13 Levels of expression of the 59 genes exclusively changing in expression levels in the NB4 FUS AS cells treated with ATRA. These genes are not expressed nor do they have any alteration in their expression level in control or FUS containing cells.

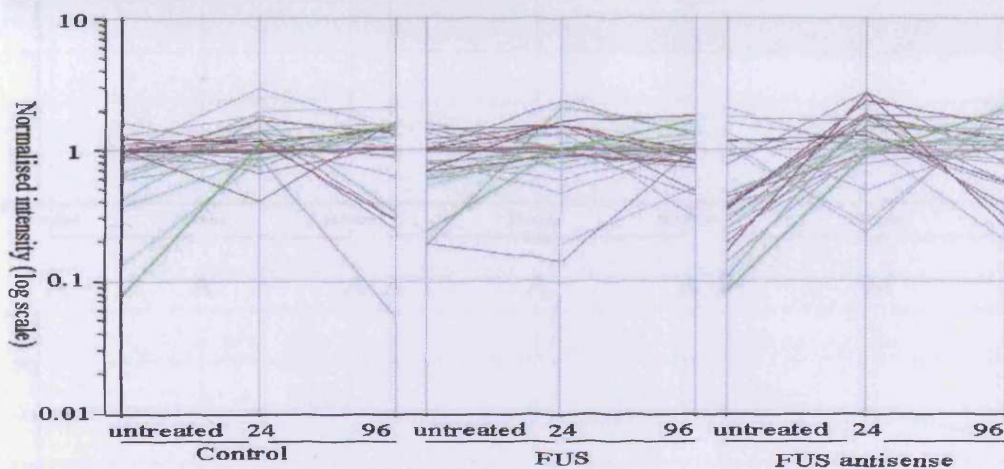
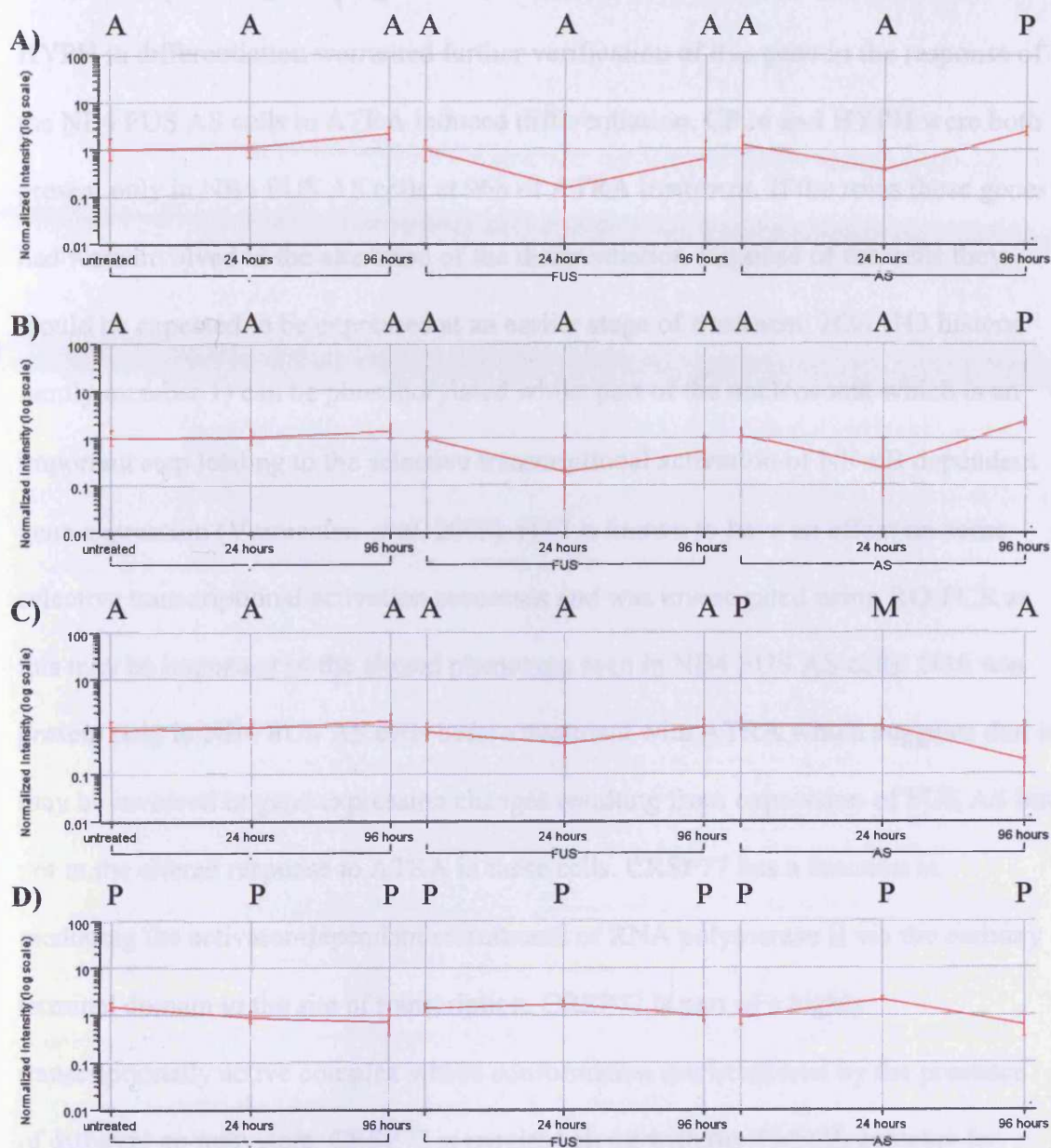


Table 6.5 Genes of interest with altered expression levels resulting from treatment with ATRA in NB4 FUS AS cells. P = present, A = absent, M = marginal. The numbers in the table refer to the normalised expression of the gene in each cell line.

Gene	Accession number	NB4						NB4 FUS						NB4 FUS AS					
		untreated		24h		96h		untreated		24h		96h		untreated		24h		96h	
CP24	NM 000782	1.0	A	1.12	A	2.17	A	0.94	A	0.15	A	0.63	A	1.25	A	0.37	A	2.50	P
HYPH	AF161412	1.0	A	1.07	A	1.37	A	0.98	A	0.19	A	0.51	A	1.14	A	0.24	A	2.27	P
H3i	NM 021018	0.87	A	1.03	A	1.29	A	0.81	A	0.46	A	1.0	A	1.18	P	1.10	M	0.20	A
CRSP77	AF105421	1.57	P	0.91	P	0.74	P	1.44	P	1.81	P	1.0	P	0.89	P	2.21	P	0.62	P

CP24 (cytochrome P450) is involved in the production of physiologically available retinoic acid which is obtained from two oxidation steps, retinol to retinal to retinoic acid. The second of these two steps is catalysed by cytochrome P450 which

Fig 6.14. Expression of A) CP24, B) HYPH, C) H3/i and D) CRSP77 in all NB4 cell lines.



can metabolise over 100 structurally diverse exogenous and endogenous molecules.

Inhibitors for cytochrome P450 are being developed for treatments (Hansen *et al.*

2000; Kawai *et al.* 2003; Liu *et al.* 2002; Tallman 1996). CP24 was selected for RQ-

PCR verification as it is known to be involved in the metabolism of retinoic acid and

so may be involved in the response of the NB4 FUS AS cells to ATRA. HYPH

(Huntingtin interacting protein H) interacts with Huntingtin which is mutated in

Huntingtons disease. A fusion between HYPH and platelet derived growth factor β results in CML. HYPH has additional roles in differentiation, proliferation and survival of spermatogenic progenitors (Rao *et al.* 2001; Rao *et al.* 2002). The role of HYPH in differentiation warranted further verification of this gene in the response of the NB4 FUS AS cells to ATRA induced differentiation. CP24 and HYPH were both present only in NB4 FUS AS cells at 96h of ATRA treatment. If the roles these genes had were involved in the alteration of the differentiation response of the cells they would be expected to be expressed at an earlier stage of treatment. H3/i (H3 histone family member 1) can be phosphorylated whilst part of the nucleosome which is an important step leading to the selective transcriptional activation of NF- κ B dependent gene expression (Vermeulen *et al.* 2003). H3/i is known to have an effect on some selective transcriptional activation processes and was investigated using RQ-PCR as this may be important in the altered phenotype seen in NB4 FUS AS cells. H3/i was present only in NB4 FUS AS cells before treatment with ATRA which suggests that it may be involved in gene expression changes resulting from expression of FUS AS but not in the altered response to ATRA in these cells. CRSP77 has a function in mediating the activator-dependent recruitment of RNA polymerase II via the carboxy terminal domain to the site of transcription. CRSP77 is part of a highly transcriptionally active complex whose conformation can be altered by the presence of different co-activators. CRSP77 is required along with the TAF(II) proteins for transcriptional activation by the Sp1 transcription factor (Naar *et al.* 2002; Ryu *et al.* 1999a; Ryu *et al.* 1999b; Taatjes *et al.* 2002). This gene was selected for further study as Sp 1 is a cofactor that interacts with the FUS protein itself. CRSP77 was slightly up-regulated in NB4 FUS AS cells at 96h of treatment with ATRA only, despite it being expressed in all the cell lines at all treatment points. The very slight up-regulation of this gene, which functions in a transcriptionally active complex to allow

transcription factor Sp1 to function suggests that the effect it mediates is only slight and therefore may not be of great importance in the altered response of NB4 FUS AS cells to ATRA.

6.3.3 Homology between murine and human GeneChips

Genes that were represented on both the human and murine GeneChips can be identified by the creation of homology tables which list the genes in the chip which are also present on the chip for the other species. This was done for the murine and human chips that have been used in this chapter.

Identification of genes represented on both the human and murine chips may allow the discovery of similar gene expression patterns in both the species which may indicate a common mechanism of response to ATRA. Analysis of the murine homology list in the human genome revealed that 4985 genes were present on both chips.

Only two genes which had been identified earlier in this chapter as being of interest due to their expression in different cell types were found to be present in the homology lists. These genes were: PAFAH which was identified as having changing expression in NB4 and NB4 FUS cells in response to ATRA; and CP24 (known as vitamin D-24-hydrolase in the murine samples) which was exclusively found to be changing in response to ATRA in NB4 FUS AS cells.

Despite these samples having apparently changing expression levels the decision made by MAS5 for each of the samples was that the signal was absent and the PAFAH gene was not expressed in any of the murine samples at a level higher than was observed for the hybridisation controls. In the human samples the level of expression in the untreated NB4 and NB4 FUS cells were also absent but it was present and at a level of 2.068 in the NB4 FUS AS cells. Following treatment with

ATRA this gene becomes expressed in all the NB4 cells. As this gene is not expressed in any of the untreated murine cells despite expression of the construct it may be expressed specifically in untreated NB4 FUS AS cells and may indicate that it is involved in the altered response to ATRA in NB4 FUS AS cells. In the other NB4 cells the altered expression of this gene may be an indication of its involvement in the response to ATRA but this observation cannot be extended to the murine cells due to the lack of information on the expression of this gene following treatment with ATRA.

As with PAFAH in murine cells, the expression values for the vitamin D-24-hydroxylase were also designated as absent in the murine samples. In the untreated NB4 cells this gene was also absent in cells containing any of the constructs. This gene was only expressed following 96h of ATRA treatment in NB4 FUS AS cells. As this gene is not registered as being expressed in any of the untreated murine or human cells its altered expression may be the result of the altered differentiation response in the NB4 FUS AS cells but further analysis of the response to ATRA in the murine cells would need to be carried out to determine if this is the case.

6.3.4 Validation of results by RT-PCR

6.3.4a Murine genes identified as changing in expression level in 32D and 32D B2A2 cells lines as a result of the expression of the FUS and FUS antisense constructs.

The graphs in figure 6.19 show the level of expression of the selected genes detected by both the microarray technique and the RQ-PCR method. Correlation was the seen in the IL3-R gene only although this may have been the result of outliers in the data. For the NF- κ B gene there appears to be two trend lines, one occurring at the lower range of the data generated by RQ-PCR and the other at the higher range.

6.3.4b Genes changing in expression level in the 32D cells with the FUS antisense constructs only.

The agreement between the result generated for the SLAP and Bcl-2 genes when assayed as part of a microarray and by RQ-PCR are shown in figure 6.20. Correlation is seen between the results generated by microarray analysis and by RQ-PCR in the case of the SLAP gene. The Bcl-2 data do not cover a wide range and this resulted in the gradient of the trend line being shallow although the general trend was for the results to increase in the same manner in both methods of measurement. The correlation in the SLAP gene is more easily observed although again this may be the result of two data points being more widely spaced than the remainder of the data.

6.3.4c Genes with altered expression in the 32D B2A2 FUS AS cells

The TARC gene up-regulated in 32D B2A2 FUS AS cells. The correlation of the expression levels of this gene when measured by microarray analysis and by RQ-PCR are shown in figure 6.21.

Most of the data points generated by both the methods used were clustered in one area of the graph but the result that was vastly different to the other results in both methods of measurement showed that the agreement between both methods of measurement in the TARC gene was good. The sample that gave the very high expression of this gene was the 32D B2A2 FUS AS sample.

Fig 6.19 Graphs of the correlation between the levels of the TASR, NF-κBi and IL3R genes determined by two different methods. Units are arbitrary.

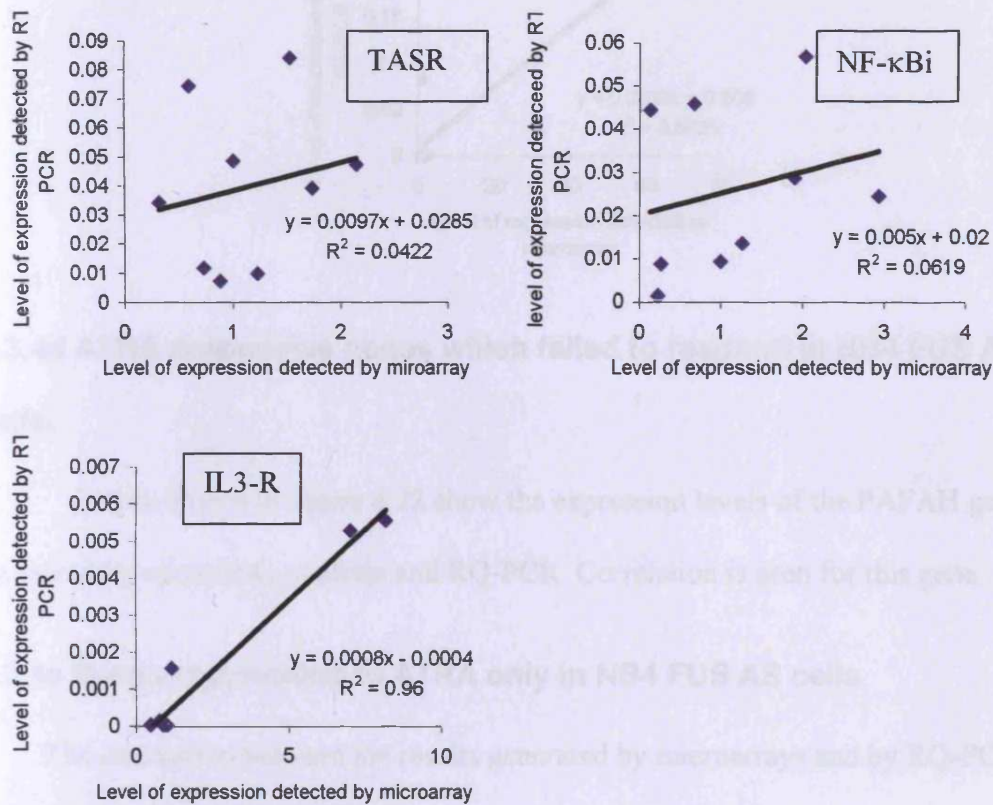


Fig 6.20 Graphs of the correlation between the levels of the SLAP and Bcl-2 genes determined by two different methods. Units are arbitrary.

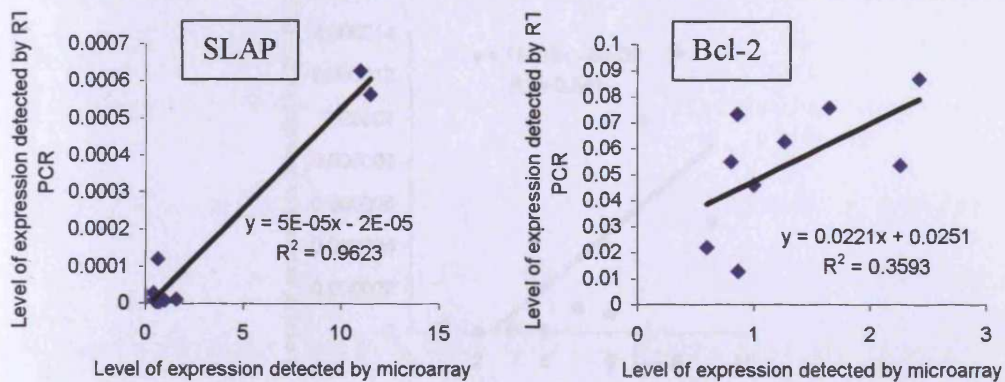
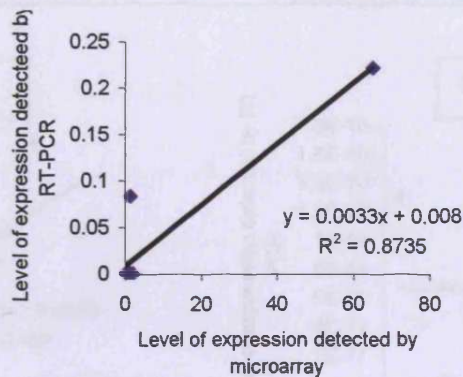


Fig 6.21 Graphs of the correlation between the levels of the TARC gene determined by two different methods. Units are arbitrary.



6.3.4d ATRA responsive genes which failed to respond in NB4 FUS AS cells.

Graphs shown in figure 6.22 show the expression levels of the PAFAH gene measured by microarray analysis and RQ-PCR. Correlation is seen for this gene.

6.3.4e Genes responding to ATRA only in NB4 FUS AS cells

The correlation between the results generated by microarrays and by RQ-PCR on the CRSP77, CP24, HYPH and H3/i genes are shown in the graphs in figure 6.23.

Fig 6.22 Graphs of the correlation between the levels of the PAFAH gene determined by two different methods. Units are arbitrary.

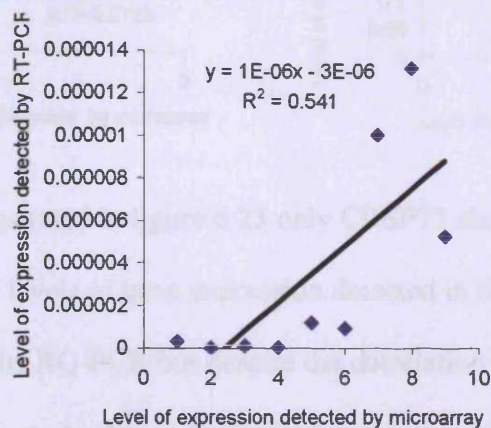
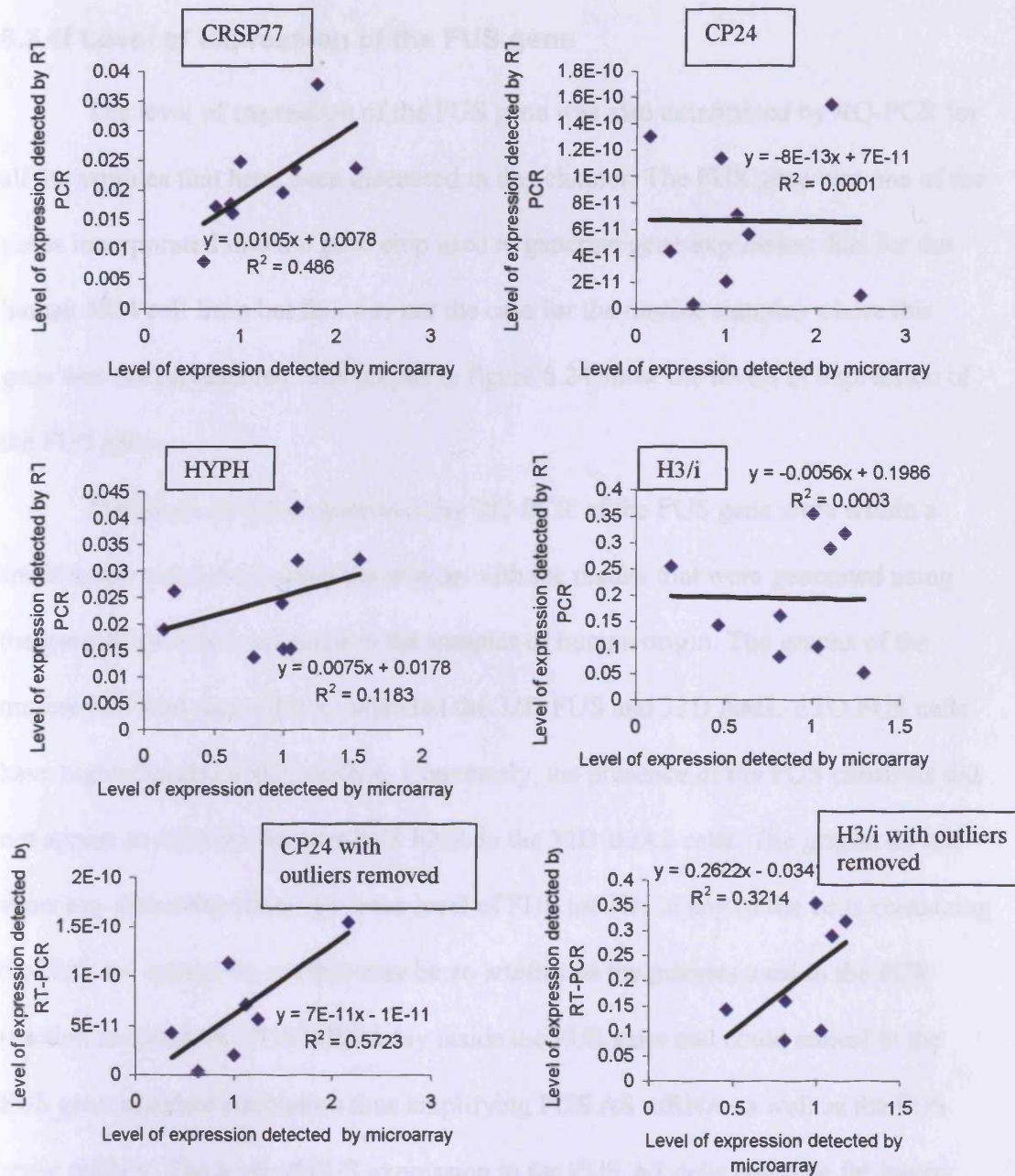


Fig 6.23 Graphs of the correlation between the levels of the CRSP77, CP24, HYPH and H3/i genes determined by two different methods. Units are arbitrary.



Of the genes illustrated in figure 6.23 only CRSP77 showed any positive correlation between the levels of gene expression detected in the microarray analysis and the levels detected by RQ-PCR but despite the correlation seen being positive it was not strong and this may be due to the limited range over which the data is arrayed. In the HYPH gene, despite the lack of correlation, some of the samples did

indicate a trend of increasing values being seen in both methods. When two outliers were removed from the CP24 or H3/i data on the basis of replicate variability, a good level of correlation was observed.

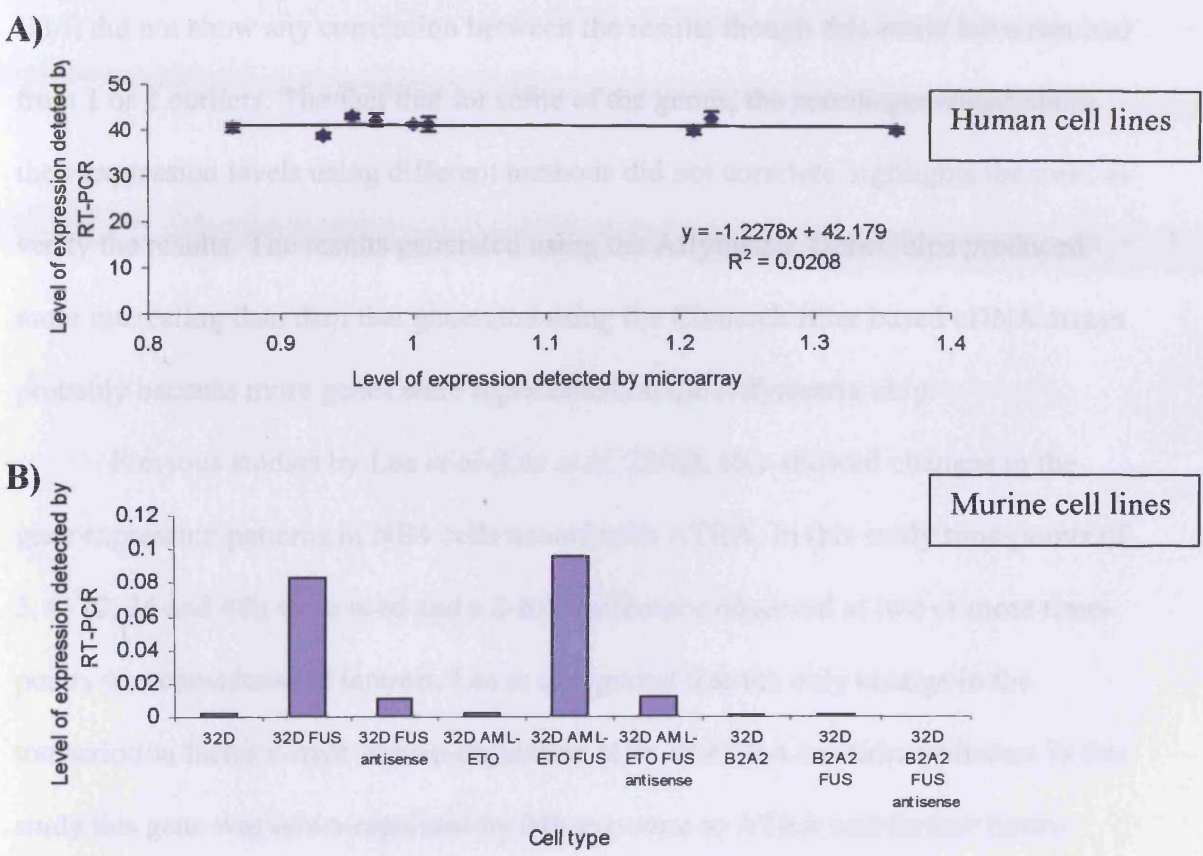
6.3.4f Level of expression of the FUS gene

The level of expression of the FUS gene was also determined by RQ-PCR for all the samples that have been discussed in this chapter. The FUS gene was one of the genes incorporated into the gene chip used to generate gene expression data for the human NB4 cell lines but this was not the case for the murine samples where this gene was not represented. The graphs in figure 6.24 show the levels of expression of the FUS gene.

The range of values generated by RQ-PCR of the FUS gene were within a small range and did not show correlation with the results that were generated using the gene chips when measured in the samples of human origin. The graphs of the murine cell lines show that as expected the 32D FUS and 32D AML-ETO FUS cells have higher levels of FUS mRNA. Conversely, the presence of the FUS construct did not appear to raise the level of FUS RNA in the 32D B2A2 cells. The graphs do not show any detectable decrease in the level of FUS mRNA in any of the cells containing the FUS AS constructs but this may be an artefact as the primers used in the PCR reaction to detect the FUS mRNA lay inside the FUS gene and could anneal to the FUS gene in either orientation thus amplifying FUS AS mRNA as well as the FUS sense mRNA. The level of FUS expression in the FUS AS cells could be far lower than is apparent in these results.

while the cells were treated with ATRA, some of the genes (IL3R, SLAP, TARC, PAPAI and CRSP77) showed a good level of correlation between the microarray

Fig 6.24 Levels of expression of the FUS gene in A) NB4 cell lines (untreated and treated with ATRA) and B) in the murine cell lines 32D cell lines. Units are arbitrary.



6.4 Discussion

The Affymetrix GeneChips have provided a very large volume of data that has allowed the identification of genes exhibiting specific expression patterns, for example, genes that show changes in their expression levels as a result of treatment with ATRA only in cells expressing the FUS AS construct, or genes which are expressed in uninduced cells that express one of the constructs but not the others. When a selection of the genes that were identified as being of interest due to their activities related to the FUS protein, other transcription factors or processes occurring

within the cells were verified using RQ-PCR some of the genes (IL3R, SLAP, TARC, PAFAH and CRSP77) showed a good level of correlation between the measurements of level of expression taken in the two different methods. Other genes (NF- κ B, Bcl-2 and HYPH) showed some agreement between the two sets of readings or a trend incorporating most of the data points and the remaining genes, (TASR, CP24 and H3/i) did not show any correlation between the results though this could have resulted from 1 or 2 outliers. The fact that for some of the genes, the results generated about their expression levels using different methods did not correlate highlights the need to verify the results. The results generated using the Affymetrix GeneChips produced more interesting data than that generated using the Clontech filter based cDNA arrays probably because more genes were represented on the Affymetrix chip.

Previous studies by Lee *et al* (Lee *et al.* 2002), also showed changes in the gene expression patterns in NB4 cells treated with ATRA. In this study time-points of 3, 8, 12, 24 and 48h were used and a 2-fold difference observed at two or more time-points was considered of interest. Lee *et al* reported that the only change in the transcription factor v-myb was up-regulation at 8h of ATRA treatment whereas in this study this gene was down-regulated by 24h exposure to ATRA and further down-regulated levels by 96h in all NB4 cell lines. This discrepancy may be a result of the different time-points that were used to measure the changes in gene expression and the more numerous and less widely spaced time-points employed by Lee *et al* may allow more resolution of early changes in gene expression.

Lee *et al* (Lee *et al.* 2002) also reported the myogenic factor 4, myogenin, and G-protein coupled receptor as being up-regulated after 8h of treatment with ATRA, these were both found to be absent from the experiments reported in this chapter. Agreement was, however, observed when the expression of C/EBP α was examined. Lee *et al* reported that this gene was up-regulated in the NB4 and also HL-60 cells 8h

after treatment with ATRA. Consistent with this I observed up-regulation C/EBP up-regulation in NB4 and NB4 FUS cells after 24h of treatment above the untreated level and a relative down-regulation by 96h indicating that C/EBP expression is involved in the intermediate term response to ATRA in these cells. In the NB4 FUS AS cells, no up-regulation of C/EBP expression was seen above untreated levels and the expression declined following treatment. This may be consistent with the altered response of these cells to ATRA.

Another report by Witcher *et al* (Witcher *et al.* 2003) also used cDNA microarrays to search for genes modulated by ATRA in NB4 cells and reported that among other genes several members of the TNF pathway were regulated by ATRA. Overall there was poor correlation with the data of Witcher *et al* with the data presented here. The reported change in Toll like receptor 3 after 96h of ATRA treatment was not observed here. These differences may be the result of differences in the preparation of samples and in performing the hybridisation to the microarrays. Different microarrays were used in each of the experiments which may result in further differences in the compatibility of the data derived from each method. The differences observed between different reports of gene expression patterns using similar methods suggest a caveat when attempting to compare data from different experiments. In addition to this, the level of fold change to be considered as significant frequently differs between research groups as are the time-points at which gene expression is measured. These issues as well as experimental design and implementation need to be taken into consideration when attempting comparison between different experiments.

Fewer reports of the result of the effects of different translocations in murine cell lines exist. One report, (Schuster *et al.* 2003), which looks at the effect of the BCR/ABL on G-CSF stimulated differentiation in 32D cells suggested that the block

in differentiation seen in these cells was accompanied by a failure to up-regulate C/EBP ϵ , while PU.1 up-regulation remained intact. In the murine samples used in this chapter C/EBP ϵ was not expressed in any of the 32D or 32D AML-ETO samples regardless of the construct they expressed while the 32D B2A2 cell lines expressed this gene (though marginally in the case of 32D B2A2 FUS AS cells). The effect of G-CSF on the expression of these genes was not assessed in this study. The PU.1 gene was expressed in all the samples measured. Comparison between the two studies remains difficult due to the differences existing in the experimental design, one aiming to look at the effect of up- and down-regulation of a single gene against a background of chromosomal translocations and the other (Schuster *et al.* 2003) assessing the effect of treatment with G-CSF and Imatinib on a single cell type.

The use of oligonucleotide and cDNA microarrays is increasingly being utilised for determining discriminatory gene-sets whose expression is altered in particular sub-classes of disease. Data recovered from these experiments may be subjected to classification, clustering, network modelling and hypothesis testing which all rely on statistical parameters to draw conclusions. To enable the conclusions to be reliable a large number of array samples need to be considered (Hwang *et al.* 2002).

cDNA microarrays were developed to reveal the expression patterns of genes which are up-regulated and down-regulated in response to biological stimuli. The development of this technology has allowed automated imaging analysis by computer and is well suited to the large scale study of genomic gene expression patterns in either tissues, cultured cells or disease samples (Riccioni *et al.* 2003). In the light of the results discussed in this chapter and in comparison to other reported findings, a more comprehensive study of the differences in gene expression may need to be undertaken. It may be of interest to analyse the changes in expression patterns

following treatment with G-CSF as well as to ATRA and to include a wider range of samples with treatment time-points that were more closely spaced than those used in this study. In addition to this, technical replicates of the same cell line at the same treatment point would allow identification of genes whose expression changes as a result of variation in sample preparation. Further verification of the expression levels of genes found to be interesting would also need to be undertaken. The work presented in this chapter has identified genes expressed in cell lines as a result of the expression of FUS or FUS AS or as a result of treatment with ATRA. The multiple roles of FUS within the cell lines means that the genes that were identified as being targets of FUS, or involved in the altered response of FUS AS containing cells to inducers are unlikely to have a dominant effect on the cells. It is more plausible to suggest that multiple genes are involved in the alteration of the responses to differentiation inducers and that the microarrays undertaken in this study are unlikely to be able to disentangle the complex pathways producing a composite effect on the different cell lines although possible targets of the FUS gene were identified in this chapter.

Chapter 7

The effect of RNA interference when used as a mechanism to down-regulate the expression of the FUS gene in human haematopoietic cell lines

7.1 Introduction

RNA interference is the process by which double stranded RNA molecules target homologous mRNA molecules and results in endonucleolytic cleavage and degradation of the target mRNA (Jiang *et al.* 2002). The system of RNA interference (RNAi) was first discovered in non-mammalian systems and has been shown to block gene expression at the post-transcriptional level and can be up to several orders of magnitude more efficient than antisense, or ribozyme treatments (Jiang *et al.* 2002). In plants, invertebrates and vertebrates small RNA molecules of less than 30 base pairs exist which silence alleles during development (Xia *et al.* 2002). In the eukaryotic cell, gene silencing at the RNA level functions in protection against viruses and mobile DNA elements (Hamilton *et al.* 2002). Insertion of foreign elements into eukaryotic cells is believed to be accompanied by the formation of double stranded RNA which is then interpreted by the cells as a signal for unwanted gene activity. Dicer RNase III endonuclease processes the double stranded RNA into smaller double stranded RNA fragment of 21-22 nucleotides in length (Jiang *et al.* 2002; Martinez *et al.* 2002). These small double stranded RNA fragments are known as small interfering RNA (siRNA) molecules and have the potential to remain stably complexed with the endonuclease and its associated proteins which are known as the RNA-induced silencing complex (RISC) and to initiate the degradation of further homologous RNA molecules (Hamilton *et al.* 2002; Jiang *et al.* 2002; Martinez *et al.* 2002). The specificity of the degradation of the target gene is mediated by base pairing (Hamilton *et al.* 2002), the siRNA duplexes acting as primers to transform the target mRNA into more double stranded siRNA molecules and so making the process of RNAi self-replicative (Jiang *et al.* 2002).

siRNA molecules of 21 nucleotides in length are sufficient to initiate the process of RNAi while longer double stranded RNA molecules induce a non-specific

interferon response that is not produced in response to the short siRNA duplexes in vertebrate animals and mammalian cells (Jiang *et al.* 2002; Martinez *et al.* 2002). The siRNA duplexes have overhangs of 2 to 3 nucleotides at each end and these contain 5' phosphate and free 3' hydroxyl termini. The 5' phosphate is required at the target complementary strand of siRNA duplexes for RISC activity (Martinez *et al.* 2002). The effectiveness of a particular siRNA molecule in silencing the target gene is influenced by the secondary structure of the RNA molecule to be silenced and the positioning of the siRNA sequence on the RNA molecule (Jiang *et al.* 2002). The growth regulatory mechanisms of mammalian cells is not adversely affected by the process of RNAi (Jiang *et al.* 2002).

Experimentally, siRNA expressed from expression constructs within a cell has advantages over synthetic siRNA molecules. siRNAs generated from expression vectors are constantly expressed within the cells and the level of siRNA in the cells is not depleted by cell division. Retroviruses may be suitable vectors for introducing siRNA molecules; giving stable expression within mammalian cells (Devroe *et al.* 2002; Xia *et al.* 2002). In retrovirally expressed vectors, the siRNA duplex is formed as a hairpin and loop structure and processed within the cell to form the 21 nucleotide duplex with overhanging ends. The positioning of the hairpin immediately adjacent to the promoter is critically important for function (Xia *et al.* 2002).

Determining the performance of the siRNA in the cell is not trivial and may in some cases give rise to a modest target gene down-regulation within a small fraction of the total cells rather than total gene silencing within all the cells of the population (Devroe *et al.* 2002). The effect triggered by siRNA is greater and sustained for longer than that produced by antisense oligonucleotides (Bertrand *et al.* 2002). The half-life of siRNA molecules has been shown to be higher in HeLa cells, cell extracts and in calf serum than that of antisense oligonucleotide (Bertrand *et al.* 2002).

Additionally the silenced gene has been shown to return to its normal level of expression between 5 and 9 days following transfection of the siRNA molecule into mammalian cells (Martinez *et al.* 2002). The effect of single stranded siRNA molecules transfected into HeLa cells was shown to silence an endogenous gene with similar efficiency to duplex siRNA (Martinez *et al.* 2002). Additionally, the simultaneous use of more than one siRNA molecule directed towards a gene may result in a greater silencing effect upon the gene (Heinonen *et al.* 2002).

The small effects that were seen in the cultured cell lines containing and expressing and antisense FUS sequence when treated with ATRA and with G-CSF and the differences observed in patterns of gene expression between the different cell lines suggested the possibility that the antisense sequence that was used to generate these cell lines was not effective at reducing the level of FUS protein expressed within the cells. This was also shown by the level of FUS protein still detectable in the human cell lines by Western blotting. To determine whether a greater reduction in the expression of the FUS protein in the different cell lines generated a more pronounced response within the cells, RNAi was used to silence the expression of the endogenous FUS gene within the NB4 and NB4R2 cells.

7.2 Methods

NB4 and NB4R2 cells were transfected with siRNA designed to silence the expression of the FUS gene using the amine transfection agent produced by Ambion Incorporated. The protocol for this is described in section 2.7.4. The efficiency of the transfection was assessed by flow cytometry on the day following the transfection as described in section 2.7.5. The effect of the siRNA on the NB4 and NB4R2 cells was measured by the effect on growth, viability and by the percentage of cells undergoing apoptosis. Cells undergoing apoptosis were identified by flow cytometry by their reactivity with annexin V while maintaining an intact cellular membrane (excluding

propidium iodide). The protocol followed for detection of apoptosis by annexin V and propidium iodide labelling is described in section 2.7.6.

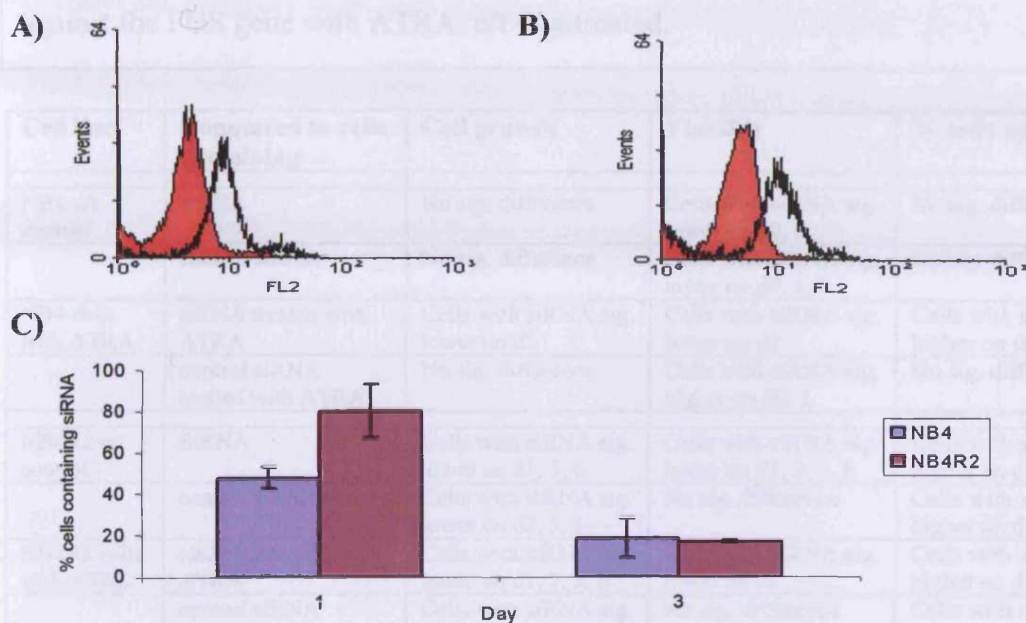
7.3 Results

7.3.1 Detection of Cy-3 labelled siRNA against the FUS gene in NB4 and NB4R2 cells on days 1 and 3 following transfection

The level of siRNA in the transfected cells was determined by flow cytometry on days 1 and 3 following transfection to determine the level of the siRNA that was present within the cells and so to determine how long the effect of the siRNA was likely to be sustained. The results of this flow cytometry are shown figure 7.1.

Both the NB4 and NB4R2 cell lines showed a significantly higher level of fluorescence than the untransfected control cells on day 1 indicating that a high level of Cy-3 labelled siRNA was present within these cells. On day 3 the level of siRNA had dropped substantially and in the NB4 cells was not significantly different to the untransfected control cells. In NB4R2 cells the level of fluorescence had dropped to a similar extent but was still shown to be significantly higher than the untransfected control cells. These results suggest that although the siRNA is transfected into the NB4 and NB4R2 cells with a high frequency of positive cells being achieved, the fast growth rate of these cells lines, (doubling time being approximately 24h), reduced the level of siRNA in each individual cell rapidly and the reduction of the concentration of siRNA in each cell. These data suggest that the effect of siRNA is likely to be insignificant beyond day 3.

Fig 7.1. Graphs showing the level of Cy-3 labelled siRNA detectable in A) NB4 and B) NB4R2 cells on day 1 where the red indicates the fluorescence pattern of untransfected cells and black indicates transfected cells C) the decline of the level Cy-3 labelled siRNA in the cells by day 3. Error bars indicate +/-one standard deviation. Experiments were repeated in triplicate.



7.3.2 Effect of ATRA on NB4 and NB4R2 cells transfected with siRNA against the FUS gene

To demonstrate the effect of FUS siRNA in NB4 and NB4R2 cells in both the presence and absence of treatment with ATRA cells were transfected on day 0.

Transfected cells were divided into two separate cultures on day 1, one culture was treated with 10⁻⁷M ATRA. The growth and viability of the cells were measured on day 0, 1, 2, 3 and 6 of the experiment and the level of apoptosis was determined on days 1, 2, 3 and 6. A control siRNA was used which was made of a sequence with no homology to any human gene. All experiments were repeated in triplicate.

The results presented in table 7.1 and figure 7.2 show the effect on NB4 cells of transfection of siRNA's without the additional treatment with ATRA. Transfection

of NB4 cells with siRNA directed against the FUS gene or a control siRNA with no homology to any genes both showed no significant effect on the growth or percentage of the cell populations undergoing apoptosis while the transfection of both siRNA sequences resulted in a lower viability compared with untransfected cells.

Table 7.1 The effects of treating NB4 and NB4R2 cells transfected with siRNA against the FUS gene with ATRA. u/t = untreated.

Cell line	Compared to cells containing	Cell growth	Viability	% cells apoptosing
NB4 u/t control	siRNA	No sig. difference	Cells with siRNA sig. lower on d2, 3	No sig. difference
	control siRNA	No sig. difference	Cells with siRNA sig. lower on d0, 1, 2	No sig. difference
NB4 cells with ATRA	siRNA treated with ATRA	Cells with siRNA sig. lower on d2	Cells with siRNA sig. lower on d2	Cells with siRNA sig. higher on d6
	control siRNA treated with ATRA	No sig. difference	Cells with siRNA sig. higher on d0, 1	No sig. difference
NB4R2 u/t control	SiRNA	Cells with siRNA sig. lower on d1, 3, 6	Cells with siRNA sig. lower on d1, 2, 3, 6	Cells with siRNA sig. higher on d3
	control siRNA	Cells with siRNA sig. lower on d2, 3, 6	No sig. difference	Cells with siRNA sig. higher on d2, 6
NB4R2 cells with ATRA	siRNA treated with ATRA	Cells with siRNA sig. lower on d1, 2, 3, 6	Cells with siRNA sig. lower on d1	Cells with siRNA sig. higher on d3
	control siRNA treated with ATRA	Cells with siRNA sig. lower on d3	No sig. difference	Cells with siRNA sig. higher on d2

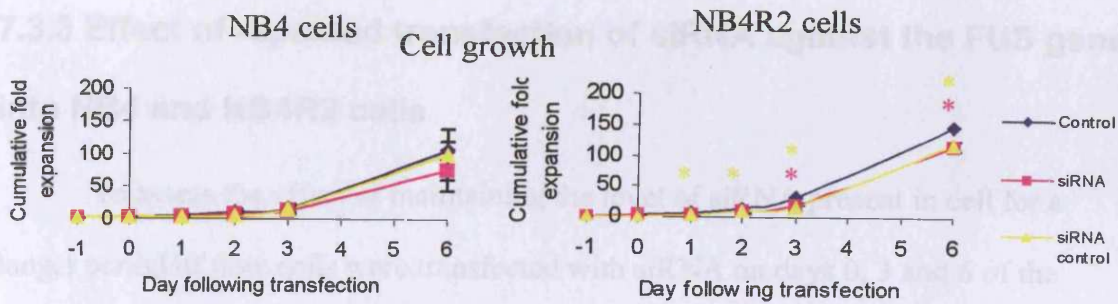
In the NB4R2 cells, transfection with control siRNA and that directed against the FUS gene both resulted in slower growth than the untransfected cells and more detectable apoptosis while only the cells with the FUS siRNA resulted in a lower viability than that in the untransfected control cultures. This suggests that the effect of transfection of the siRNA sequences in the NB4 and NB4R2 cells are different and the FUS siRNA only produced a difference to the control siRNA cells in the viability of the transfected NB4R2 cells. In turn, this suggests that the effect of the siRNA in otherwise untreated NB4 and NB4R2 cells may be too slight to be detected by the methods used in these experiments.

When treated with ATRA, the NB4 cells transfected with FUS siRNA showed a lower growth rate and viability on day 2 of treatment and more apoptosis was detectable

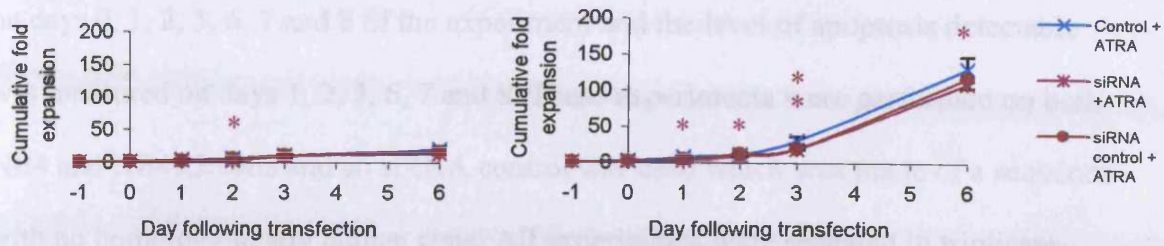
day 6 of the experiment while the cells with the control siRNA sequence were not affected in any respect. These data suggest that the siRNA sequence directed against the FUS gene was not having the same effect in the NB4 cells as the FUS AS treatment (discussed in chapter 4) which resulted in the ability to continue proliferating in response to treatment with ATRA. In the NB4R2 cells treatment with ATRA resulted in a slower growth rate and more detectable apoptosis in the cells with both the control siRNA and the anti-FUS siRNA suggesting that the FUS siRNA had an effect that was no different to the control siRNA sequence and that the effect on growth rate and apoptosis was a result of the transfection process itself. In the cells with the anti-FUS siRNA the viability of the cells was lower than NB4R2 cells which were ATRA treated but not transfected while control siRNA did not affect the viability of ATRA-treated cells. The lower growth rate and viability along with the increased level of apoptosis in the NB4R2 cells with the anti-FUS siRNA would suggest that these cells were behaving like the NB4R2 FUS AS cells which became more responsive to the effect of ATRA than the NB4R2 cells not expressing the FUS AS sequence. The similarity of the results observed when the NB4R2 cells that were transfected with the control siRNA sequence prevents this being drawn as a valid conclusion and suggests that the effect of the transfection procedure itself was responsible for these results.

Fig. 7.2 over page. Effect of treatment of NB4 and NB4R2 cells transfected with FUS siRNA with ATRA. Experiments were repeated in triplicate and error bars indicate +/- one standard deviation. Significant difference ($p < 0.05$) to control is indicated by *.

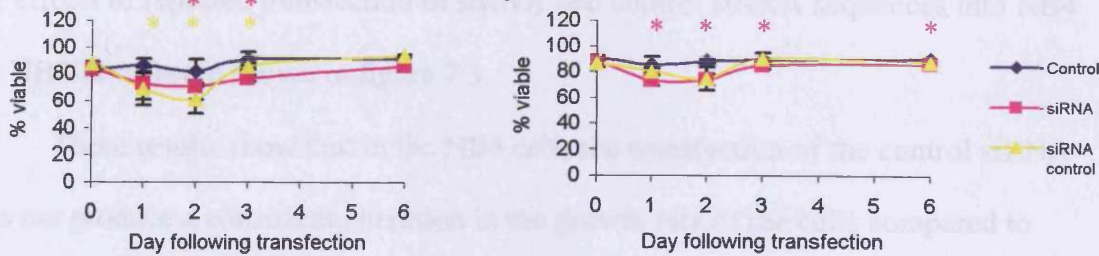
7.3.3 Effect of siRNA on cell growth of NB4 and NB4R2 cells



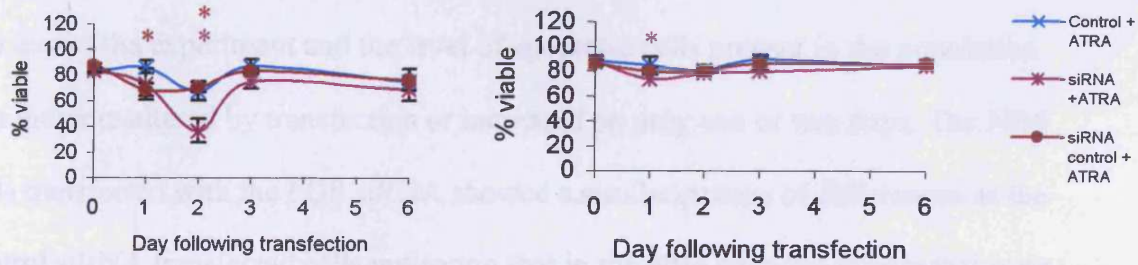
Cell growth – treated with ATRA



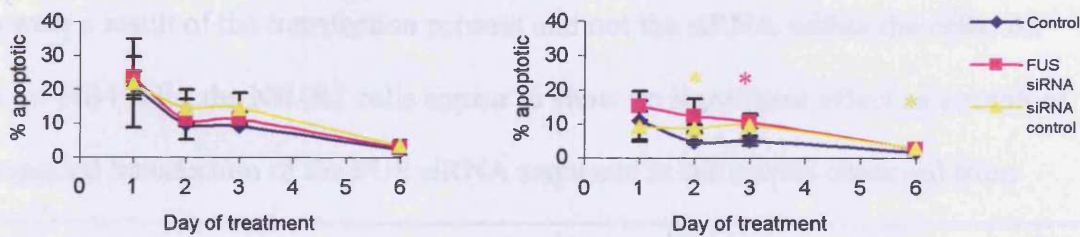
Viability



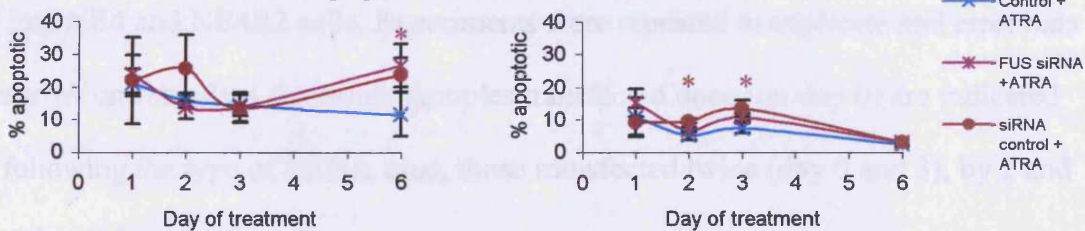
Viability – treated with ATRA



Level of apoptosis



Level of apoptosis – treated with ATRA



7.3.3 Effect of repeated transfection of siRNA against the FUS gene into NB4 and NB4R2 cells

To assess the effect of maintaining the level of siRNA present in cell for a longer period of time cells were transfected with siRNA on days 0, 3 and 6 of the experiment. In these experiments the growth and viability of the cells were measured on days 0, 1, 2, 3, 6, 7 and 8 of the experiment and the level of apoptosis detectable was measured on days 1, 2, 3, 6, 7 and 8. These experiments were performed on both NB4 and NB4R2 cells and an siRNA control was used which was made of a sequence with no homology to any human gene. All experiments were repeated in triplicate. The effects of repeated transfection of siRNA and control siRNA sequences into NB4 and NB4R2 cells are shown in figure 7.3.

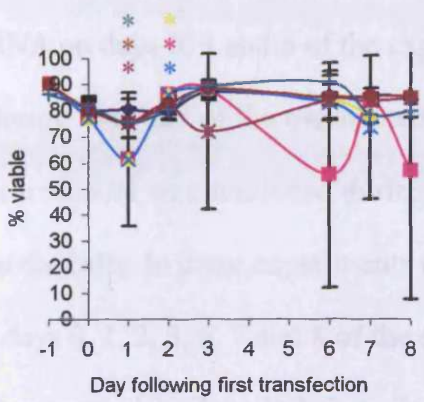
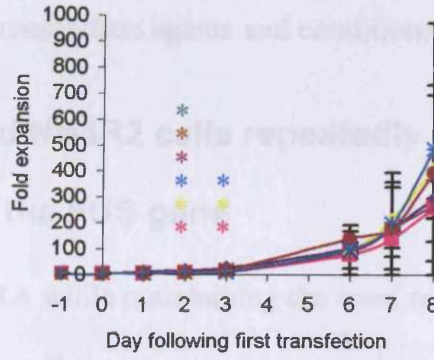
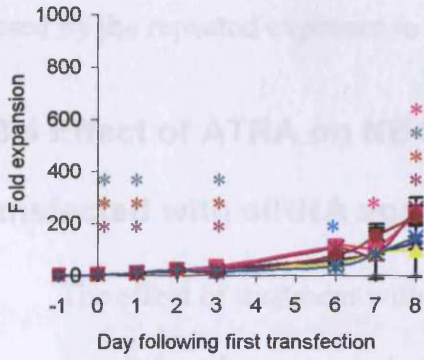
These results show that in the NB4 cells the transfection of the control siRNA does not produce a consistent alteration in the growth rate of the cells compared to untransfected control cells. The viability of the transfected cells was only affected on one day of the experiment and the level of apoptotic cells present in the population was either unaltered by transfection or increased on only one or two days. The NB4 cells transfected with the FUS siRNA showed a similar pattern of differences as the control siRNA transfected cells indicating that in the NB4 cells the effects that were seen were a result of the transfection process and not the siRNA within the cells. As with the NB4 cells, the NB4R2 cells appear to show no significant effect as a result of the repeated transfection of the FUS siRNA sequence as the results observed from

Fig. 7.3 on next page. Effect of repeated transfection of siRNA directed against the FUS gene into NB4 and NB4R2 cells. Experiments were repeated in triplicate and error bars indicate +/- one standard deviation. Samples transfected once (on day 0) are indicated by 1 following the type of siRNA used, those transfected twice (day 0 and 3), by 2 and thrice (day 0, 3 and 6) by a 3.

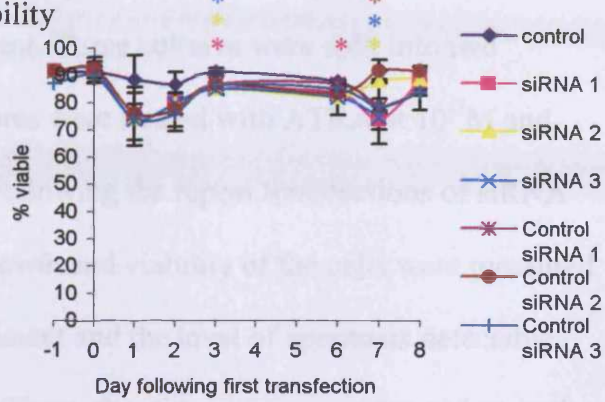
NB4 cells

Cell growth

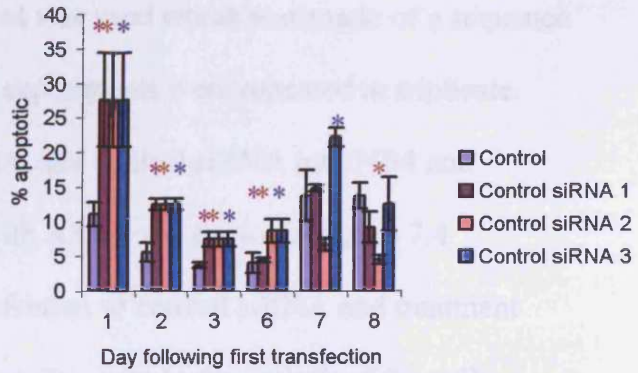
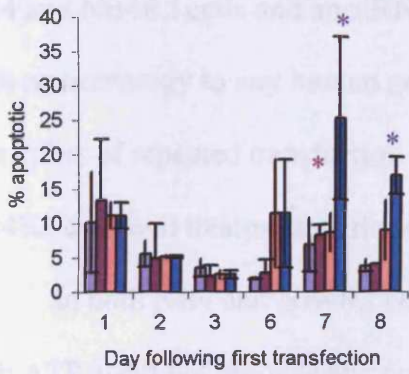
NB4R2 cells



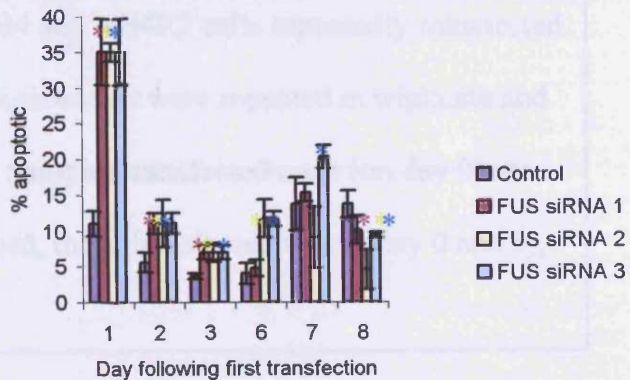
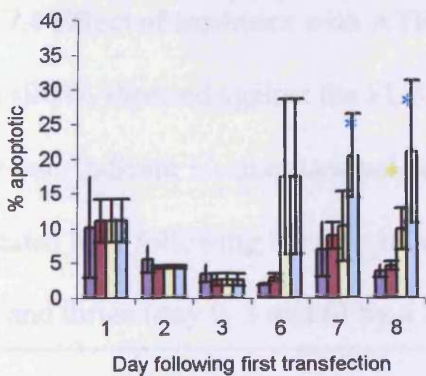
Viability



Level of apoptosis in cells with control siRNA



Level of apoptosis in cells with FUS siRNA



the FUS siRNA and the control siRNA sequence were very similar and appear to be caused by the repeated exposure to the transfection agents and conditions.

7.3.4 Effect of ATRA on NB4 and NB4R2 cells repeatedly transfected with siRNA against the FUS gene

The effect of treatment with ATRA while maintaining the level of siRNA present in cell for a longer period of time cells was assessed by transfecting cells with siRNA on days 0, 3 and 6 of the experiment. These cultures were split into two cultures. One half of the transfected cultures were treated with ATRA at 10^{-7} M and this treatment was continued during and following the repeat transfections of siRNA into the cells. In these experiments the growth and viability of the cells were measured on days 0, 1, 2, 3, 6, 7 and 8 of the experiment and the level of apoptosis detectable was measured on days 1, 2, 3, 6, 7 and 8. These experiments were performed on both NB4 and NB4R2 cells and an siRNA control was used which was made of a sequence with no homology to any human gene. All experiments were repeated in triplicate. The effect of repeated transfection of siRNA, and control siRNA into NB4 and NB4R2 cells and treatment of these cells with ATRA are shown in figure 7.4

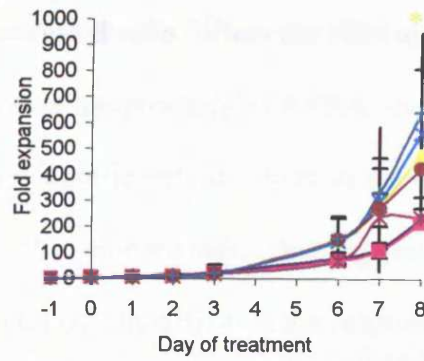
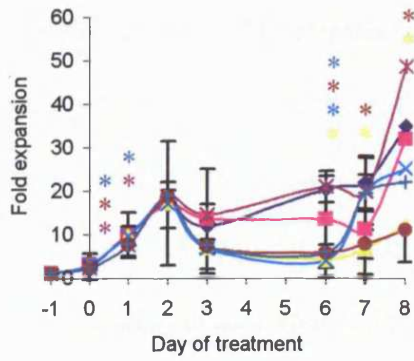
In both NB4 and NB4R2 cells transfection of control siRNA and treatment with ATRA did not produce any consistent differences in the growth of the cells although a slower growth rate was seen at some time points. Some variation was seen

Fig. 7.4 Effect of treatment with ATRA on NB4 and NB4R2 cells repeatedly transfected with siRNA directed against the FUS gene. Experiments were repeated in triplicate and error bars indicate +/- one standard deviation. Samples transfected once (on day 0) are indicated by 1 following the type of siRNA used, those transfected twice (day 0 and 3), by 2 and thrice (day 0, 3 and 6) by a 3.

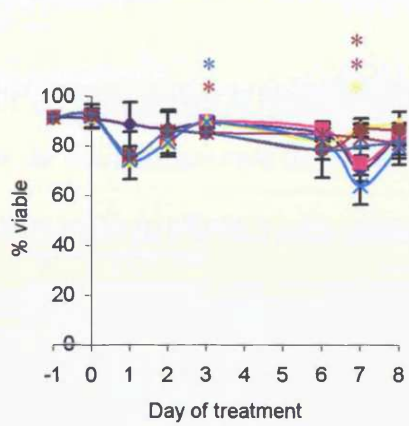
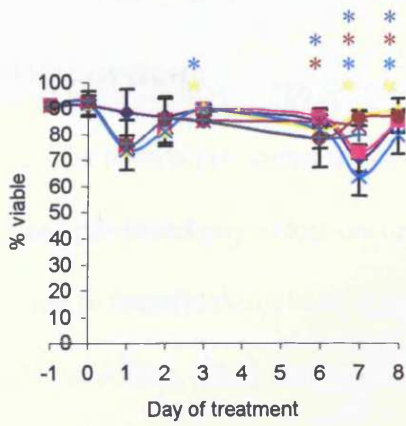
NB4 cells

NB4R2 cells

Cell growth

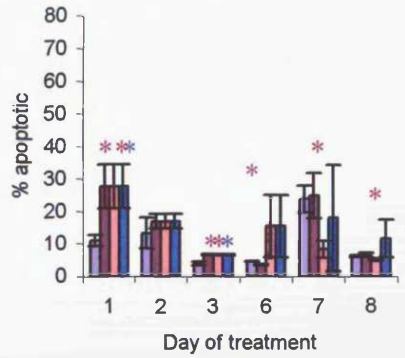
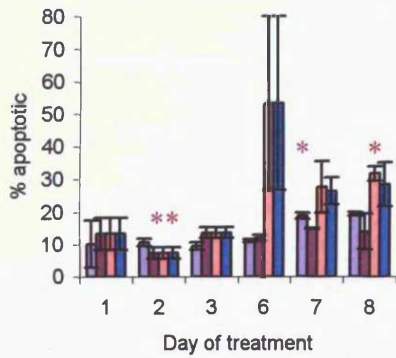


Cell viability



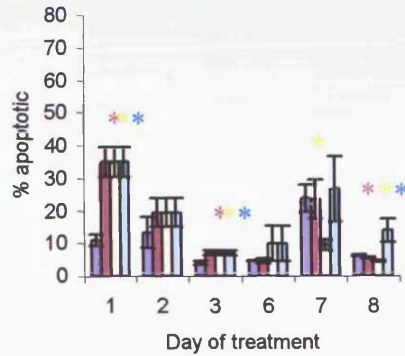
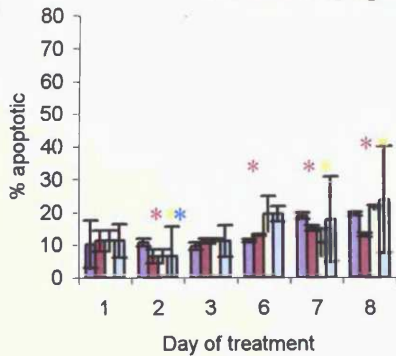
- control + ATRA
- siRNA 1 + ATRA
- ▲— siRNA 2 + ATRA
- ×— siRNA 3 + ATRA
- *— Control siRNA 1 + ATRA
- Control siRNA 2 + ATRA
- ×— Control siRNA 3 + ATRA

Level of apoptosis in cells with control siRNA



- Control with ATRA
- Control siRNA 1 with ATRA
- Control siRNA 2 with ATRA
- Control siRNA 3 with ATRA

Level of apoptosis in cells with FUS siRNA



- Control with ATRA
- FUS siRNA 1 with ATRA
- FUS siRNA 2 with ATRA
- FUS siRNA 3 with ATRA

in the viability of the transfected cells compared to the untransfected cells but in both the NB4 and NB4R2 cells this was not consistently different. The levels of apoptosis in the cells also showed no consistent differences to the untreated cells. When the NB4 and NB4R2 cells were repeatedly transfected with the FUS siRNA in the presence of ATRA, the effects on growth, viability and apoptosis in the cells were not significantly different to the cells transfected with the control siRNA sequence and treated in the same manner. This suggests that the FUS siRNA sequence used in these experiments did not have any effect on the response to ATRA in the NB4 and NB4R2 cells.

7.4 Discussion

The results presented in this chapter do not indicate that the siRNA sequence that was used produced any effect on the growth of NB4 or NB4R2 cells either when subjected to transfection alone, a repeated course of transfections, or in the response of the cells to ATRA when transfected once or up to three times in succession. It was observed that the level of siRNA in the cells was reduced drastically on the third day following the transfection procedure. Unfortunately the low frequency of transfection made determination of the level of FUS protein expressed in the transfected cells by Western blotting unfeasible as it was not possible to collect a sufficient number of cells to perform a Western blot.

In the light of other experiments where gene silencing was achieved by the use of RNAi, it may be that the siRNA sequence that was used was not the best one possible as it may have been directed to a section of the FUS mRNA sequence which had a secondary structure that made the binding of the siRNA inefficient or impossible or that the section of the mRNA that was targeted by the siRNA was not at an optimum position on the mRNA (Jiang *et al.* 2002). Later reports on siRNA design suggest that the structure of the siRNA duplex is as important as the target site on the mRNA for effectively silencing the target gene (Khvorova *et al.* 2003; Schwarz *et al.* 2003).

Particular importance has been assigned to the 5' ends of the separate siRNA strands for which of the two strands in the siRNA duplex is incorporated in the RISC complex and therefore whether this complex is targeted towards the sense or antisense RNA. The decrease in siRNA that was detectable in the cells and with it the decreasing siRNA concentration following each division of the cells may be resolved by introducing the siRNA sequence into the cell in the form of an expression vector which would result in stable expression of the siRNA within the cells (Devroe *et al.* 2002; Xia *et al.* 2002). This approach has worked well in other laboratories and may provide better experimental results if applied to the reduction of FUS gene expression. Alternatively, co-transfection of a combination of different siRNA molecules all directed to different areas of the same gene and used in conjunction with each other, has been shown to result in a greater level of silencing of the target gene and may prove to be an improved strategy to reduce the expression of the FUS gene by the use of RNAi (Heinonen *et al.* 2002). In the experiments reported in this chapter it is not possible to discriminate whether the siRNA was not showing an effect in the cells because it was sub-optimally targeted or whether it was working but at a level that was too low to have any significant effect. Use of the alternative approaches described here may allow discrimination between these possibilities.

Chapter 8

Concluding Remarks

8.1 Remarks on results generated during this study

The work carried out during this study has shown that the up- or down-regulation of expression of the FUS gene has no effect on the growth or viability of any of the cell lines into which the constructs were transduced in keeping with its role as a housekeeping gene since the activities of housekeeping proteins tend not to be regulated at the level of gene transcription. Previous work has however identified a possible role for FUS in haematopoietic differentiation in that FUS was down-regulated during differentiation and was over-expressed in leukaemia cells (which are blocked in differentiation) (Mills *et al.* 2000). One aim of this thesis was to determine whether these changes in FUS expression are causally related or merely correlate with differentiated status. When the transduced cells were treated with ATRA, some of the cell lines expressing the FUS AS construct exhibited an alteration in the response to ATRA. In the 32D FUS AS and NB4 FUS AS cells, the presence of the FUS AS construct led to the cells acquiring an ability to continue proliferating despite treatment with ATRA. Conversely, in the ATRA resistant NB4R2 and 32D B2A2 cells the presence of the FUS AS construct resulted in these cells becoming competent to differentiate in response to treatment with ATRA. Similarly, an alteration in the response to treatment with G-CSF was observed in the 32D cells (when treated in the presence of IL-3) and in the 32D B2A2 cells. When treated with G-CSF, the presence of the FUS AS construct in the 32D cells led to these cells continuing to proliferate under conditions that would usually induce them to differentiate. Again in cells expressing BCR-ABL, (32D B2A2), the presence of the FUS AS construct reinstated the ability of these cells to respond to G-CSF as well as ATRA. These data indicate that the level of FUS may influence differentiation but that the result of this influence differs markedly depending on the context in which it is expressed.

The role of the FUS gene is complicated as it appears to be involved in the mechanism of resistance to ATRA seen in the NB4R2 cells and to both ATRA and G-CSF seen in the 32D B2A2 cells. On the other hand, FUS appears to be required for the differentiation response that is seen in the NB4 cells to ATRA and the 32D cells to ATRA and G-CSF. The expression of the FUS AS construct in these cells confers resistance to the inducers.

Gene expression analysis using both cDNA filter based microarrays and GeneChip chips utilising oligos have provided a wealth of information about the changes in gene expression that have resulted from the expression of the FUS and FUS AS constructs in the 32D, 32D AML-ETO and 32D B2A2 cells lines which respectively carry no translocation, the t(8;21) and t(9;22) translocations and in the t(15;17) expressing NB4 cell line. These results have shown that the presence of the FUS AS construct in the 32D B2A2 cells has led to their gene expression pattern becoming most dissimilar to any of the other 32D based cell lines and that in the 32D cells the expression of the FUS AS construct produced an altered gene expression pattern most closely resembling the 32D B2A2 cells. Similarly the NB4 FUS AS cells were the least similar in terms of gene expression patterns of the cell types expressing the control, FUS or FUS AS constructs. Interestingly, the extent of the change in the gene expression profiles of these cells correlated with the phenotypic alteration induced by modulation of FUS (as described in section 6.3.1 (32D FUS AS and 32D B2A2 FUS AS cells) and section 6.3.2b (NB4 FUS AS cells)).

The different patterns of gene expression resulting from expression of FUS or FUS AS within the cell lines indicate that despite no effect on differentiation in some of the cell lines there was evidence of induced changes in gene expression. This does however raise the question of the authenticity of these changes or whether they could be experimental noise. This cannot be demonstrated without carrying out replicates

for each condition, however, when one of the Affymetrix samples was repeated in duplicate (this was only performed on one sample due to prohibitive cost) it clustered next to its replicate sample and further analysis showed the two samples to have very similar gene expression patterns. This suggests that the reproducibility of the Affymetrix GeneChip chips is high suggesting that the differences observed were not simply due to experimental noise. A high level of reproducibility was seen between Affymetrix GeneChip chips (Toda *et al.* 2003) and this report also proposed a theory, the FUMI theory, which uses an algorithm based on the t-test which is reported to be able to analyse data in single replicate experiments. An alternative mechanism of microarray analysis suggests that if a non-parametric method of analysis is adopted, a single repetition of each condition is sufficient to detect 2-fold changes in gene expression with high probability of accuracy (Black *et al.* 2002). However a larger number of GeneChip chips being performed would allow identification of a set of genes that were consistently changed as a result of the expression of the FUS or FUS AS constructs. Analysis by multiple methods and combination of the resultant gene lists would also help to strengthen the power of the experiment. Although only a single analysis was performed for each condition, the power of the analysis was increased by looking for common changes in gene expression induced by FUS or FUS AS between the different cell lines. This led to identification of a number of candidate genes which may be important targets for FUS. The expression patterns of these genes however, were not consistent with FUS having a dominant effect on differentiation and were thought to be among a number of participant genes in the effect of FUS AS expression rather than being directly involved in the response as was discussed in section 6.4.

In answer to the question of whether the model used was suitable to address the aims projected for this study, it would appear that the cell line models may not

have been adequate as it was not possible to effectively modulate the expression of FUS which is constitutively expressed at a high level. In addition to this, it may not have been expected that the ectopic expression of a gene that is already over expressed in the cells would generate any phenotypic change. Theoretically, the recent advances in RNAi may make this possible in principle, although achieving complete down-regulation of the FUS gene may prove to be challenging.

The conclusion, as far as this study goes, is that FUS may have a role in differentiation and in leukaemia. However, from the experiments in this study it is not possible to conclude that this is a dominant anti-differentiation effect in leukaemia cells. However, because the effect of this gene appears to be highly context dependent, it may be the case that in primary AML blasts FUS does function as a dominant anti-differentiation gene and use of RNAi in *these* cells may establish whether reduction of FUS expression is capable of inducing differentiation.

8.2 Suggestions for further study

The inability to detect a consistent down-regulation of the FUS gene at the protein level have led to the suggestion that the antisense sequence of FUS was not as effective as would have been desired. To overcome this, RNAi was employed in the NB4 and NB4R2 cells but the siRNA sequence used had no further success in identifying a phenotype resulting from down-regulation of the FUS gene. In order to further study whether the down-regulation of FUS at the protein level affects haematopoietic cells, siRNA sequences could be introduced into the cells using a retroviral vector which would stably express the siRNA sequences in the cells thus solving the problem of the rapid loss of the siRNA sequence from the cells by dilution resulting from repeated cell division. In addition to this, the use of more than one siRNA molecule directed against different areas of the FUS gene in a single cell may enhance the effect generated by the use of this method of gene silencing. As

mentioned in the previous section, the use of primary AML blasts with RNAi may allow elucidation of the context dependent effect of FUS down-regulation.

Following the gene expression analysis experiments, genes that had verified differences in expression resulting from the expression of the different constructs, (particularly the FUS AS construct) before treatment with ATRA and at different treatments during the course of treatment could be investigated further, possibly after more refinement of the array analysis. Use of additional repeats of each condition on which an array was performed may allow experimental noise to be excluded from the analysis and lead to identification of genes exerting a dominant effect on the phenotype of the cells as a result of FUS AS expression rather than genes thought to be participatory. The role of these genes in haematopoietic cells could be investigated by retroviral transduction to express additional protein or to down-regulate it either by antisense or RNAi. Additionally, the effect of removing the expression of these genes in addition to the FUS gene in cells could be investigated.

The effect of FUS on primary cells isolated from cord blood could also be of interest to study although preliminary experiments in these cells have not shown any difference between cells expressing the FUS and FUS AS constructs. The use of a more effective system to down-regulate the expression of FUS may provide more information about the relevance of FUS expression in primary cells. A system including one or more siRNA molecule reliably expressed in the cell as proposed earlier may be a better method for investigating the role of FUS in primary cells.

In the light of the deficiencies in the cell line models, an alternative model which could be used to provide further information on the role of FUS in differentiation would be the use of knockout mice. FUS^{-/-} mice have already been produced and are embryonically lethal which provides no information about the role of FUS in adult animals (Hicks *et al.* 2000). However, another report using FUS

deficient animals suggests that depletion of FUS results in defects in spermatogenesis, somatic growth and increased sensitivity to ionising radiation (Kuroda *et al.* 2000). Using a model such as this, haematopoietic cells could be studied *in vitro* to assess whether they have an ability to undergo increased differentiation or less self renewal than cells with no disruption of the FUS gene.

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