Investigating a co-operative link between TGF-β and Lyn kinase in Chronic Myeloid Leukaemia

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DEDICATION

This thesis is dedicated to my family, thank you

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ABSTRACT

Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterised by uncontrolled proliferation of haematopoietic cells driven by the fusion gene Bcr-Abl. The advent of a mechanism specific kinase inhibitor, Imatinib, targeting the constitutively active Bcr-Abl kinase has paved the way for new treatment strategies. However, resistance emerges, particularly in those in more advanced disease stages. One factor in this resistance is the presence of a quiescent subset of CML cells that are insensitive to Imatinib. CML resistance to Imatinib is linked with over-expression and activation of Lyn, a member of the Src family kinases (SFK). The main aim of the project was to investigate whether transforming growth factor-β (TGFβ) might influence drug-resistance through direct affects on Lyn kinase levels and its activation.

This research builds on previous findings of Bcr-Abl-dependent up-regulation of TGFβ signalling, implicating this cytokine in CML. Human MYL cell lines derived from a CML patient were used to show TGFβ plays a role in imatinib-resistance through direct effects on Lyn protein turnover. Stimulation of MYL cells with TGFβ produced cyclic changes in high molecular weight bands in the presence of proteasomal inhibitors. These high molecular weight bands are due to TGFβ inducing Lyn ubiquitination, and that this is mediated via TGFβ-dependent expression of the c-cbl E3 ubiquitin ligase. Cell cycle analysis in combination with poly ADP ribose polymerase (PARP) cleavage assays show TGFβ causes cell quiescence, and that the inhibition of the signalling pathway releases cells from growth arrest and enhances Imatinib-induced cell death. Collectively, data highlights a potential new dual-treatment approach using a combination of Imatinib and SB431542 to enhance imatinib-mediated cell death in

CML. Furthermore, it is speculated that hyper-activation of the TGFβ pathway in the presence of Bcr-Abl drives CML drug resistance by a co-operative mechanism involving Lyn kinase ubiquitination and activation, which is linked to the c-cbl proto-oncogene.

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LIST OF ABBREVIATIONS

Alk	activin like kinase
BMP	bone morphogenetic protein
bp	base pairs
cDNA	copy DNA
CML	chronic myeloid leukaemia
Co-Smad	common mediator smad
ddH20	double distilled water
DMEM	dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
E. Coli	*Escherichia coli*
Erk	Extracellular-signal regulated kinase
FCS	foetal calf serum
g	grams
GDF	growth differentiation factor
HECT	homologous to E6 carboxy terminus
HRP	horse radish peroxidase
I-Smad	inhibitory smad
JNK	c-Jun N-terminal kinase
Kb	kilo bases
kDa	kilo dalton
L	litre
LAP	latency-associated protein

LB	laurial broth
Luc	luciferase
M	molar
MAD	mothers against decapentaplegic
MAPK	mitogen activated protein kinase
mg	milligram
ml	millilitre
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger RNA
NEDD	Neural precursor cell expressed developmentally down-regulated
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PARP	poly ADP ribose polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
rcf	relative centrifugal force
RING	really interesting new gene
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
R-Smad	receptor regulated Smad

RT –PCR	reverse transcription-PCR
SARA	smad anchor for receptor activation
SBE	smad binding element
SDS	sodium dodecyl sulphate
siRNA	short interfering RNA
Smurf	smad ubiquitin regulatory factor
SUMO	small ubiquitin like modifier
TGF-β	Transforming Growth Factor-β
TGFβRII	TGF-β receptor I
TGFβRII	TGF-β receptor II
Ub	Ubiquitin
UBD	ubiquitin binding domain
µg	microgram
µl	microlitre
V	volts
v/v	volume/volume ratio

1. INTRODUCTION

1.1 Background

Chronic myeloid leukaemia (CML) is a devastating blood cancer typified by the occurrence of the Philadelphia chromosome translocation that leads to the combining of two genes Bcr and Abl which generates the constitutively active Bcr-Abl tyrosine kinase. The discovery of Imatinib mesylate paved the way for targeted disease treatment in the 1990's. Unfortunately, Imatinib rarely acts as a complete cure, particularly in late stage disease, and the detection of residual CML contributing to resistance has made it necessary to search for alternative treatments, and ways to overcome Imatinib resistant forms of CML.

The signalling network driving CML is complex and multifaceted. One example is the TGFβ signalling pathway which plays a role in differentiation, cell growth, apoptosis, haematopoiesis, and is well established as playing a prominent role in cancer (Meulmeester and ten Dijke, 2011). TGFβ has an inhibitory effect on haematopoietic cells, and research has suggested that de-regulation of this pathway can lead to carcinogenesis (Kim and Letterio, 2003).

This thesis has aimed to look at the involvement of TGFβ during later stages of disease progression, with the aim of establishing and validating a novel dual therapy approach that may be used to combat drug resistance. Alongside this, a more mechanistic study looked to determine the molecular mechanisms responsible for TGFβ's involvement in drug resistance in addition to highlighting novel interconnections between TGFβ and Lyn kinase activation through ubiquitination.

The remainder of this introduction reviews the current research on CML, including current treatments and proposed resistance mechanisms, together with the TGFβ signalling pathway, its involvement and connections with Lyn kinase and their association with CML drug resistance.

1.2 Leukaemia

Cancer is a consequence of uncontrolled abnormal cell growth. Under normal conditions, cells will form, mature, undertake their intended function and die, to be replaced by new cells. It is when these homeostatic cell processes are disturbed that cancer can occur.

Leukaemia is cancer of the blood, and is characterised by abnormal proliferation of the haematopoietic cells in the bone marrow. These cancer cells are immature and build up in circulating blood and organs of the body.

Leukaemia can vary depending on cell type and the mechanisms responsible for its progression, and it can fall into two main types, either an acute or chronic form. Acute forms of the disease are typified by the rapid accumulation of immature blood cells within the bone marrow preventing healthy cell production. This rapid increase in blood cells leads to spillage into the bloodstream and subsequent spread to other organs throughout the body. Of the two forms, this is the more aggressive, is often seen in childhood leukaemia and, if left untreated can lead to death within a few weeks or months. The chronic form is typified by a slow but excessive increase in blood cells, and disease progression is much slower compared to acute forms, often taking several years. Chronic leukaemia occurs more frequently in the elderly, and is often a consequence of abnormal maturation of the blood cells. If left untreated, the disease may persist for many months or even years.

Based on these classifications, there are four main disease categories for leukaemia; acute lymphoid, acute myeloid, chronic lymphoid and chronic myeloid leukaemia.

Table 1 summarises the annual incidence of leukaemia among adults in the UK (LRF 2010).

Acute Lymphoblastic Leukaemia	400
Chronic Lymphocytic Leukaemia	3300
Acute Myeloid Leukaemia	2200
Acute promyelocytic Leukaemia	200
Chronic Myeloid Leukaemia	550
Hairy Cell Leukaemia	200
T-Cell Actute Leukaemia	200
Chronic Myelomonocytic Leukaemia	300

Table 1: Annual adult incidence of Leukaemia in the UK (Adapted from Leukaemia and Lymphocytic Research Fund 2010)

1.3 Haematopoiesis

Leukaemia by definition is a disorder of the haematopoietic system. Haematopoiesis is a complex process of differentiation leading to the formation of mature blood cells from a rare population of primitive haematopoietic stem cells (HSCs). These HSCs are predominately found in the adult bone marrow where they serve to repopulate the mature cell lineages, in addition to maintaining the stem cell pool. The mature cells are short lived and a constant high turnover ensures adaptation to the surroundings. This makes the homeostatic control of this process critical to prevent haematological diseases such as leukaemia. These HSCs are identified through the expression of cell surface proteins CD34, and as such are known as $CD34^{+}$ (Collins, 1994).

There are three major cell types contained within the blood; white blood cells, red blood cells and platelets, all of which are formed from a single immature bone marrow stem cell during the process of haematopoiesis. These cells go on to develop into still immature precursor cells and subsequently into mature blood cells within the bone marrow. The first step of this maturation process is into one of two subgroups, myeloid or lymphoid. The myeloid lineage develops into the red blood cells, platelets and certain types of white blood cells. The lymphoid lineage develops into the lymphocytes. Figure 1 shows the various lineages of the blood cells and their differentiation fates.

The classification of leukaemia can be divided into categories within the affected cell type from either lymphocytic or myeloid lineages, resulting in lymphoid or myeloid leukaemia.

It is now well established that the regulation of haematopoiesis is through an intricate network of extracellular and intracellular regulatory factors (reviewed by Akala and Clarke, 2006; Blank *et al* 2008). For many years the TGFβ family has been thought to play an important role in this network. Its roles in haematopoiesis will be discussed later in this chapter.

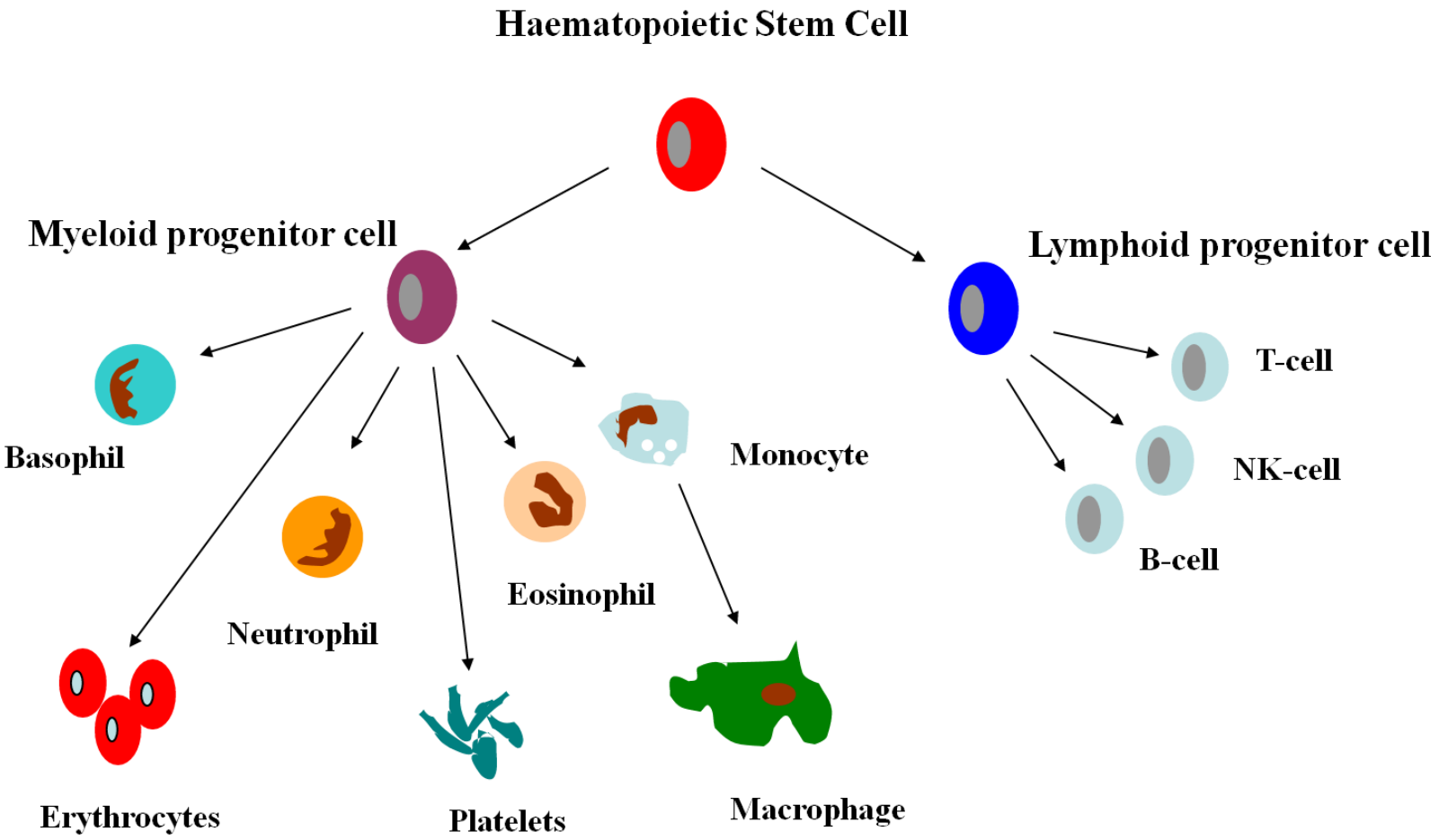


Figure 1: Cell differentiation during haematopoiesis

1.4 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) was the first malignant disease to be associated with an acquired non-random chromosomal abnormality. According to the World Health Organisation (WHO) classification, the term "myeloid" refers to all cells belonging to the granulocytic (neutrophil, eosinophil, basophil), monocyte/macrophage, erythroid, megakaryocytes and mast cell grouping. Nowell and Hungerford reported the association in 1960, along with the detection of a minute chromosome they termed the Philadelphia chromosome after the city of its discovery. This led to the finding of the reciprocal chromosomal translocation, t(9:22)(q34;q11). The consequence of which is the fusion of a number of sequences from the BCR gene at 22q11 with sequences of the c-Abl oncogene from 9q34. The resulting Bcr-Abl product is a hybrid gene found on chromosome 22. Incidence of the disease occurs at approximately 1.0 to 1.5 in every 100,000 people, and are rarely seen among children, with the average age of onset between 50-60 years. The identification of Bcr-Abl as the causative agent of CML was confirmed using a murine model in which bone marrow was transformed with Bcr-Abl and transplanted into mice that then went on to develop CML-like symptoms (Daley GQ *et al.*, 1990).

The Bcr-Abl product is believed to occur initially in a single HSC giving it a proliferative advantage over the common population, leading to the increase in cell number (Bruns *et al.*, 2009). Disease development follows a tri-phasic pattern, consisting of an initial chronic phase, which progresses to an accelerated phase and finally on to blast crisis. The chronic phase can have many years of disease evolution,

and often it is not until the presentation of worsening physical symptoms such as weight loss and sweats, along with increased blood cell production that it is diagnosed. During the accelerated stage, increased mutations occur, such as those within the kinase domain of Abl, and generation of reactive oxygen species are more commonly seen, which aid progression of the Bcr-Abl driven disease (Branford *et al.*, 2003). This second disease stage is believed to be triggered through a set of mutations which reach a "threshold" of genetic abnormalities which go on to promote disease development. In some cases the accelerated phase can be by-passed, with direct development from chronic to blast phase.

Blast crisis occurs when haematopoietic differentiation becomes arrested and immature blasts accumulate in the bone marrow and enter into the circulation (Melo and Barnes, 2007). This is driven by deleterious genetic events which occur within stem and progenitor cells of the leukaemic clone. Despite classifications, the precise definition of the three stages has been widely debated.

The progression from chronic to accelerated and blast phase has been shown to be accompanied by non-random chromosomal aberrations (Calabretta and Perrotti, 2004), the most common of which are duplication of the Philadelphia chromosome, trisomy 8, 19 (Kantarjian *et al.*, 1987). More specifically, during blast crisis, mutations in p53, Ras (Baum and Ren, 2008), and amplification of Myc have been reported.

1.5 Bcr-Abl in CML

Chronic myeloid leukaemia results from a reciprocal translocation between chromosomes 9 and 22 giving rise to a fusion gene product Bcr-Abl. This chromosomal recombination which creates a longer chromosome 9 and a shorter chromosome 22 is termed the Philadelphia chromosome. The exact location of chromosome breakage can vary. Within chromosome 22, the breakage occurs in the breakpoint cluster region (BCR) gene, for chromosome 9, it is within the c-Abl gene.

The c-Abl oncoprotein is a 145kDa non-receptor protein tyrosine kinase localised both in the cytoplasm, where it weakly associates with actin filaments, and the nucleus where it is associated with chromatin (Kipreos and Wang, 1992). It exists as an inactive monomer as a result of its tyrosine kinase domain being inhibited by its SH3 domain. The protein exists in 2 iso-forms a1 and b1, both of which are ubiquitously expressed in mammalian cells. There are differences in the N-termini of the iso-forms termed the variable domains, which are caused by alternative splicing of the first 2 exons called 1a and 1b in human genes (Ben-Neriah *et al.*, 1986). There are 3 Src homology domains (SH1-SH3) located towards the N-terminus. The SH1 domain contains the tyrosine kinase function, while the SH2 and SH3 domains allow for interaction with other proteins (Cohen *et al.*, 1995). Normal Abl function is involved in the cell cycle (Kipreos and Wang, 1992), in the response to genotoxic stress (Yuan *et al.*, 1999), and in integrin signalling (Lewis and Schwartz, 1998). However, it is important to note that the majority of this data has been obtained using ectopic expression studies in fibroblasts rather than studies of endogenously expressed proteins in hematopoietic cells.

Break-point cluster region (Bcr) is a 160kDa ubiquitously expressed protein (Laneuville, 1995). The centre of the molecule contains a *dbl*-like and pleckstrin-homology (PH) domain shown to stimulate GTP-GDP exchange (Denhardt, 1996). The C-terminus contains GTPase activity for Rac (a small GTPase of the Ras superfamily) (Diekmann *et al.*, 1991).

At the molecular level there are at least 4 different Bcr-Abl fusion genes due to the potential breakpoints in Bcr (Figure 1.1). Breakpoints within the Abl gene can occur anywhere over a large area at the 5' end upstream of the first exon b1, downstream of the second exon a1, or more frequently, between the two (Melo, 1996). This leads to the formation of 1 of 3 abnormal proteins of different molecular weight known as p210 (M-bcr). Due to alternative splicing, fusion transcripts with either b2a2 or b3a2 can be formed. Further upstream breakpoints result in p190 (m-bcr) with e1a2 mRNA, and finally the break-point downstream of exon 19 result in p230 (μ-bcr), associated with a rare Philadelphia positive chronic neutrophilia leukaemia (Pane *et al.*, 1996). Of these variants, p210 is most common among CML patients.

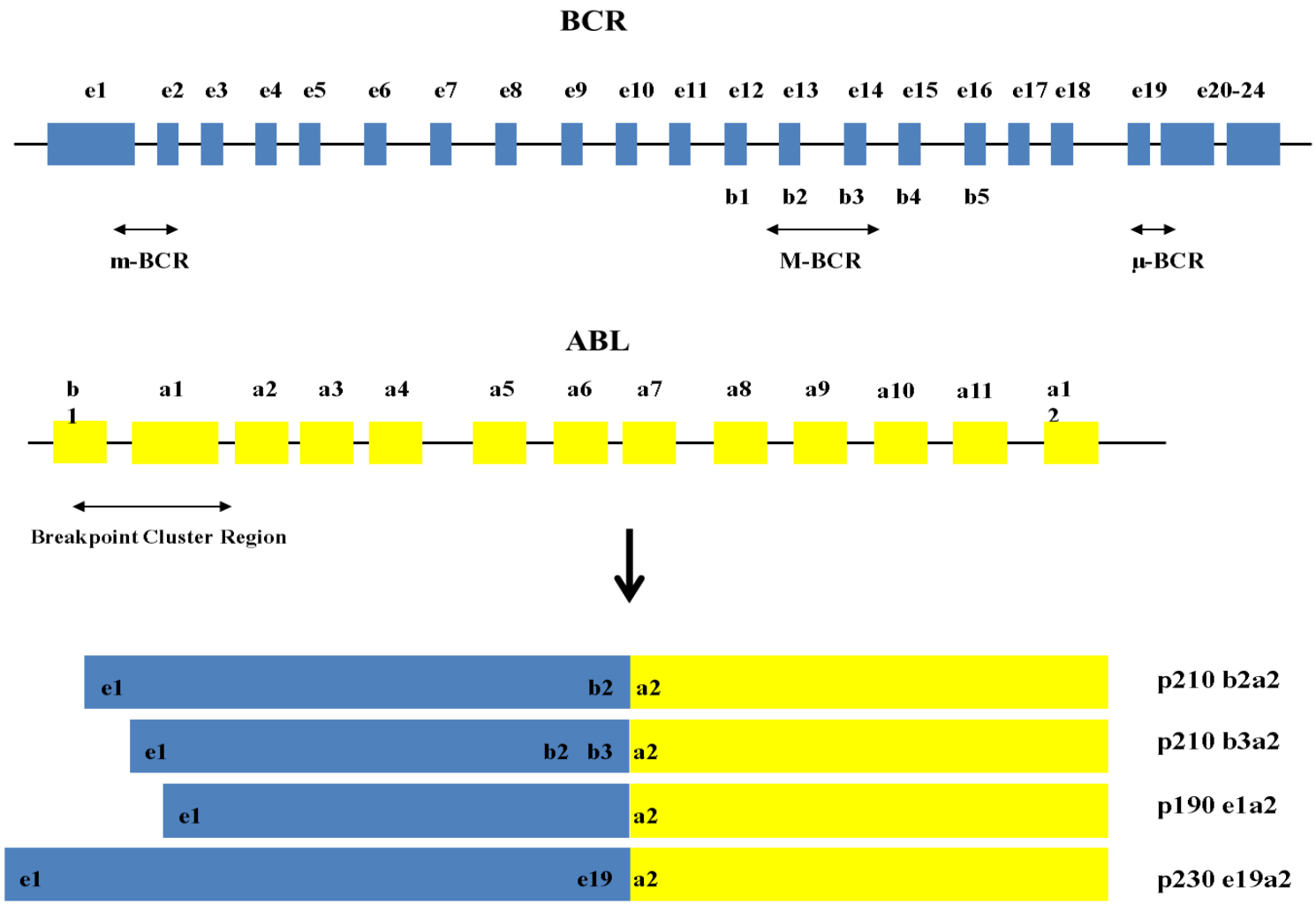


Figure 1.1 : Breakpoints in *BCR* and *ABL* genes with subsequent products: Alternative breakpoint regions result in four proteins of different weight and/or composition (Adapted from Hazelhurstet *et al* 2009)

The activation of the tyrosine kinase and transforming functions of Bcr-Abl are dependent on the fused Bcr sequences. These sequences are also responsible for altering the subcellular localisation of Abl. The Bcr-Abl proteins block nuclear translocation and activate the F-actin binding function, which is required for efficient transformation of cells (McWhirter and Wang, 1993). The cytoplasmic location of Bcr-Abl (Wetzler *et al.*, 1993) allows access to a wider range of cellular substrates than those available to the predominately nuclear Abl protein (Van Etten *et al.*, 1989). With rare exceptions, such as secondary mutations which cause constitutive activation of the STAT5 pathway resulting in maintenance of the transformed phenotype with Bcr-Abl silenced (Horita M *et al.*, 2000), Bcr-Abl expression is retained throughout the course of the disease, suggesting a selection pressure favouring continuous expression (Melo and Barnes, 2007). In support of evidence showing that epigenetic changes are reliant on constitutive Bcr-Abl activity, research shows an increase in Bcr-Abl expression levels prior to progression to advanced stage disease (Gaiger *et al.*, 1995), together with results showing altered expression of genes such as PRAME and JUN-B which may play a role in blast phase (Oehler *et al.*, 2009; Radich *et al.*, 2006).

Bcr-Abl has a wide variety of downstream substrates (Figure 1.2) which it activates through phosphorylation, and this is aided through its autophosphorylation which allow interaction with SH2 domain containing substrates such as Grb2. CrkL is a key substrate in neutrophils which is involved in the phosphoinositide 3-kinase (PI3K) pathway which is subsequently involved in processes such as protein synthesis and cell survival (Oda *et al.*, 1994).

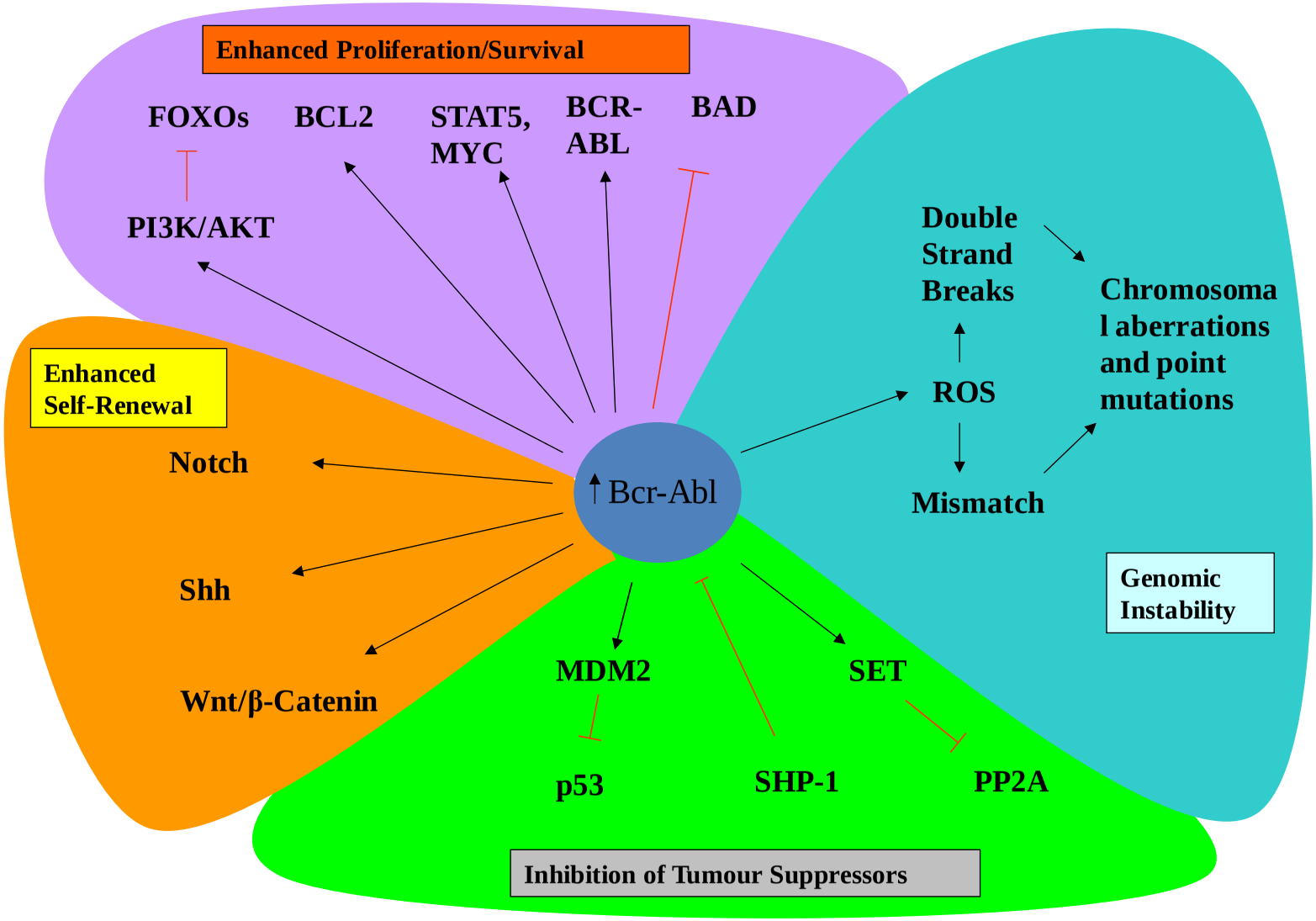


Figure 1.2: Pathways affected by Bcr-Abl during CML (Adapted from Perrotti *et al* 2010)

1.6 CML Treatment and Resistance Mechanisms

There are a range of options available to treat CML sufferers including stem cell transplantation, and a variety of chemotherapeutic drugs. To date, therapies have involved hydroxy-urea and interferon-α only as a means of prolonging life, working to slow down disease progression rather than actually offering a cure. Bone marrow transplantation is effective but is rarely an option due to donor shortages and complications with overall post-operative patient health.

During the 1990's, Imatinib mesylate (Gleevec) was specifically designed to act on the ATP binding site of the kinase domain within Bcr-Abl. Since its introduction, 5-year survival rates from CML patients have risen from 40-60% to 90% for patients with chronic phase disease (Bixby and Talpaz, 2009). Recent karyotype results show that 60-70% of patients undergo a complete cytogenetic response (CCyR), defined by the complete absence of any Philadelphia positive (Ph^+) marrow cells, and remain so 5 years after initiating Imatinib therapy (Hochhaus *et al.*, 2009). Results have also shown a decrease in the number of patients progressing to accelerated and blast phase after Imatinib therapy (Hochhaus *et al.*, 2009). The drug has since been the frontline therapy due to its effectiveness and low toxicity. Unfortunately, Imatinib resistance is a growing problem, with the detection of minimal residual disease at the molecular level through PCR (Druker *et al.*, 2006). It is currently estimated that 20-30% of patients will go on to develop resistance to Imatinib, with treatment responses seen in advanced stage CML often being slow and short-lived (Druker *et al.*, 2001).

Imatinib resistance can be either primary (known as refractory), where initial treatment is unresponsive, or secondary (known as acquired resistance) where an initial treatment response is lost (Druker *et al.*, 2006). Acquired resistance can be characterised by a loss of a major cytogenetic response and complete haematological response. Resistance can be further divided into haematological (lack of normalisation of peripheral blood counts), molecular (persistence of Bcr-Abl transcripts) and cytogenetic (persistence of the Philadelphia chromosome).

The realisation that Imatinib therapy was not the complete cure for CML in all patients prompted further investigation into the development of resistance. It is currently thought that resistance occurs through a range of factors with multiple interactions necessary for its emergence, the mechanisms can be divided in to Bcr-Abl dependent and independent.

1.6.1 Bcr-Abl Independent Mechanisms

Drug pharmacokinetics plays an important role in determining the efficacy of any treatment. Several studies have highlighted variability in Imatinib plasma levels among patients receiving 400mg orally (le Coutre *et al.*, 2004; Peng *et al.*, 2004). Imatinib is metabolised by the cytochrome p450 isoenzymes and therefore differences in concentration of these enzymes can influence Imatinib levels in the plasma. This is an important consideration as basal Imatinib plasma levels are associated with both cytogenetic and molecular responses (Picard *et al.*, 2007). In addition, the success of Imatinib as a targeted therapy has allowed its structural manipulation to develop potential treatments for other kinase related diseases such as gastrointestinal stromal tumours (Skobridis *et al.*, 2010) which often have mutations in the PDFGR or KIT receptor (Ma *et al.*, 2002).

The amount of drug that enters the target cell will depend on the balance of influx and efflux proteins. MDR-1 is a multi-drug resistance gene which encodes the p-glycoprotein involved in efflux of chemotherapeutic drugs. This gene is over-expressed in blast phase CML and implicated in Imatinib resistance (Mahon *et al.*, 2000). The mechanism of MDR-1 mediated resistance is still unclear, as over-expression in K562 cells did not confer Imatinib resistance (Ferrao *et al.*, 2003). In contrast, over-expression of MDR-1 has been observed in patients failing to induce a major cytogenetic response (Galimberti *et al.*, 2005). The adenosine triphosphate-binding cassette (ABC) transporter ABCB1 also known as MDR-1 is expressed in normal haematopoietic stem cells (Zhou *et al.*, 2001), and this may explain the apparent

inherent Imatinib insensitivity reported in CML stem cells (Chu *et al.*, 2005; Lepper *et al.*, 2005).

During Imatinib treatment, variation in cell susceptibility causes a biphasic decline in Bcr-Abl transcript levels (Komarova and Wodarz, 2007). Differentiated cells are more readily cleared than stem cells which are not affected due to their quiescence and increased ABCB1 levels. Generally, the quiescent population make up approximately 0.5% of the $CD34^+$ population and have intrinsic resistance to Imatinib (Roeder *et al.*, 2006). These cells have been shown to over-express the Bcr-Abl transcript at significantly higher levels than more mature CML cells (Copland *et al.*, 2006).

1.6.2 Bcr-Abl Dependent Mechanisms

Amplification of the Bcr-Abl gene in addition to up-regulation of the Bcr-Abl kinase in the absence of Bcr-Abl mutations has been reported in patients with blast phase CML who developed Imatinib resistance (Gorre *et al.*, 2001). Additionally, this resistance was in the absence of mutations within the kinase domain of Bcr-Abl (Mahon *et al.*, 2000). Further patient analysis has suggested that point mutations are a more common mechanism of drug resistance as only 2 of 66 patients showed gene amplification (Hochhaus *et al.*, 2002).

The development of point mutations leading to treatment resistance has long been known in CML. The occurrence of these mutations ranges from 40-90% depending on various factors such as disease phase, resistance definition, and detection method. The first discovery of Bcr-Abl mutations were found in patients who had developed Imatinib

resistance, and a significant number had the T315I mutation (Gorre *et al.*, 2001). Normally threonine 315, also known as the gatekeeper residue, will form a hydrogen bond between Abl kinase and Imatinb, inhibiting kinase activity. However, if this is replaced by an isoleucine residue, as occurs in this mutated form, the hydrogen bond interaction is disrupted, Imatinib cannot bind to the Abl kinase and the drug is ineffective (Gorre *et al.*, 2001).

Abl kinase contains an activation loop which can adopt either an open (active) or closed (inactive) conformation. Crystallographic evidence shows Imatinib can bind the closed DFG (aspartate-phenylalanine-glycine) conformation and 'locks' it closed (Schindler *et al.*, 2000), but is unable to bind the open conformation. Some mutations favour this open conformation, and include those at residues 253 and 255. They are located at the end of the P-loop of Abl kinase which is within the ATP binding domain.

Some mutations occurring at the activation loop prevent the formation of the inactive kinase, the form which Imatinib can bind. Imatinib itself has been implicated in promoting drug resistance through up-regulating the chemokine CXC receptor 4 (CXCR4) protein which increases cells movement to the bone marrow microenvironment (Jin *et al.*, 2008). This environment, which enhances survival of quiescent populations, is typically hypoxic. Strategies to overcome cell quiescence have been investigated and growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) have been used. These enhanced leukaemia initiating cell (LIC) proliferation and subsequently reduced cell number after exposure to Imatinib (Holtz *et al.*, 2007). Interestingly, LICs have been shown to have higher levels of Bcr-Abl than mature progenitor cells, which is also true in patient samples with Imatinib

resistance (Jamieson *et al.*, 2004). This suggests that these LICs may have inherent resistance to Imatinib.

Not all mutations lead to resistance, and there is variation among transforming capabilities. Analysis of the phosphotyrosine proteome has highlighted different phosphorylation signatures among the different mutants suggesting a variation in substrate specificity, leading to activation of various signalling pathways, such as in the case of the M351T kinase domain mutation which exhibits an increase in SHIP1 and consequently reduced Akt levels compared to native Bcr-Abl expressing cells (Griswold *et al.*, 2006).

1.7 Treating Imatinib Resistance

In the event of Imatinib resistance, increases in Imatinib dosage have been used with positive results. However this strategy is potentially limited as further development of disease and increased dosages will ultimately become intolerable for the patient. To combat this problem, more potent Abl tyrosine kinase inhibitors have been developed. They are divided into two main groups; ATP-competitive and ATP non-competitive inhibitors. From these, the ATP-competitive inhibitors contain two subclasses, the Src family kinase (SFK)/Abl inhibitors and 2-phenylaminopyrimidine-based compounds. Dasatinib and SKI-606 are SFK/Abl inhibitors, while Nilotinib is of the latter subclass of inhibitor (Kantarjian *et al.*, 2006). Nilotinib is structurally similar to Imatinib and has an improved topographical fit into the kinase pocket (O'Hare *et al.*, 2005; Redaelli *et al.*, 2009). Dasatinib has a completely different structure to Imatinib and Nilotinib, and is able to bind Bcr-Abl in the active conformation (Tokarski *et al.*, 2006; Vajpai *et al.*, 2008). The use of molecular modelling SKI-606 also known as Bosutinib was shown to be effective against a broad range of Bcr-Abl mutations, and to have fewer side effects, which may be due to not inhibiting KIT or PDGFR (Puttini *et al.*, 2006). Currently, the clinical trials for these next generation tyrosine kinase inhibitors are still ongoing, but initial results have shown both Dasatinib and Nilotinib to have greater efficacy in comparison to Imatinib as a front line therapy (DASSION trial 2010).

Even the advent of these new ATP-competitive inhibitors however, cannot inhibit the phosphorylation of Bcr-Abl T315I, since this mutation crucially blocks the Imatinib-binding site preventing access for the drugs. Interestingly, identical amino acid

substitutions were found at homologous positions in the kinase domain of c-Kit (T670I) and PDGFRα (T674I) kinases in Imatinib-resistant gastrointestinal stromal tumours and hypereosinophilic syndromes, respectively (Cools *et al.*, 2003). These results highlight a crucial role for this conserved amino acid in controlling the accessibility of the ATP-pocket to inhibitors. The FMS receptor has been identified as a potential therapeutic target, and research has identified predicted similarities between Dasatinib hydrogen interactions with Abl and FMS, the receptor for CSF-1 (Brownlow *et al.*, 2009).

Research into mutations of Bcr-Abl have shown there to be graded forms of resistance. Those that occur within the P-loop confer a highly resistant phenotype and poor prognosis; mutations outside of the P-Loop are associated with a less potent resistance to Imatinib. Results have shown that second generation inhibitors such as Dasatinib are able to induce or restore a complete cytogenetic response (CCyR) in 40-50% of patients who have failed initial Imatinib therapy. However there are still 20% of patients who fail to respond to either Imatinib or Dasatinib treatments (Hochhaus *et al.*, 2009).

The International Randomised Study of Interferon and Imatinib (STI571) (IRIS) results have shown that 98% of CML sufferers treated with Imatinib in early chronic phase achieve complete haematological response after 60 months, indicating that primary resistance is rare. This highlights the importance of disease treatment during early stage disease but does not address those in accelerated or blast crisis stages where resistance is a much more common occurrence.

The next step therefore was the development of the non-competitive inhibitors, where the status of the ATP-binding pocket is not relevant in their mode of inhibition. ON012380 is claimed to be the first agent capable of inhibiting both wild-type and all Imatinib-resistant kinase mutations, including T315I in vitro and in vivo (Gumireddy *et*

al., 2005). At present, ON012380 is still to undergo clinical trials to prove its safety in CML patients. Another recent approach is to target downstream proteins in the Bcr-Abl signalling pathway. Histone deactylase inhibitors LBH in combination with Vorinostat and Dasatinib have been able to lead to polyubiquitination of Bcr-Abl and downstream effectors AKT and c-Raf. In leukaemia cells, they cause apoptosis due to an increase in pro-apoptotic proteins Bax and Bim together with a reduction in anti-apoptotic Bcl-2, Bcl-XL and Survivin (Nimmanapalli *et al.*, 2003). Another novel approach being investigated is whether kinase inhibitors directed at other proteins already in clinical trials have an off target effect on the Bcr-Abl/T315I. Results indicate that the p38 MAP kinase inhibitor BIRB-796 and the aurora kinase inhibitor VX-680 are both capable of binding Bcr-Abl/T315I (Carter *et al.*, 2005; Harrington *et al.*, 2004). Alternative secondary combination treatments to prevent disease progression currently being investigated are anti-oxidants (Skorski, 2008) which would protect against cancer-causing DNA damage; farnesyl transferase inhibitors (Copland *et al.*, 2008), which prevent Ras signalling, a known substrate of Bcr-Abl; sonic hedgehog antagonists (Zhao C *et al.*, 2009) which prevent self-renewal and repair when combined with tyrosine kinase inhibitors, and protein phosphatase 2A (PP2A) (Perrotti and Neviani, 2008) activators which target Bcr-Abl and subsequent downstream substrates by remove activating phosphate groups.

Currently, none of the available treatments have significant impact on control or reversal of advanced stage disease. This makes detection and treatment paramount during chronic phase. The need for other methods of disease control could further help combat resistance. As well as targeting Bcr-Abl, it is necessary for alternative methods to be investigated, involving proteins potentially affecting disease development or resistance.

The previously mentioned compounds are effective against the majority of Bcr-Abl mutations, but not all, which paves the way for further investigation. These novel inhibitors offer targeting of Src Family kinases (SFK's) such as Lyn, and Dasatinib has been shown to reduce phosphorylated STAT5 levels in CML cell lines ((Nam *et al.*, 2007), which is a known downstream target of Lyn. Lyn kinase is a protein which has become the subject of much research interest since its association with CML drug resistance (Donato *et al.*, 2003; Wu *et al.*, 2008a; Wu *et al.*, 2008b).

1.8 Lyn Kinase

Lyn kinase is a member of the Src family of non-receptor protein tyrosine kinases of which there are eight members currently known; Lyn, Hck, Fyn, Lck, Blk, Yes, Fgr and Src. Lyn is expressed in all blood cells apart from T cells. All members of this family share similar structures (Boggon and Eck, 2004). All members have a unique N-terminal region which varies among the members, and this region contains either a myristoylation or palmitoylation site (Koegl *et al.*, 1994; Resh, 1999). This type of modification has been found to regulate protein trafficking. Lyn mono-palmitoylation causes transport from the golgi to the plasma membrane, whereas Fyn is a dually palmitoylated protein targeted directly to the plasma membrane (Izumi Sato *et al.*, 2008). The N-terminal region is adjacent to a Src homology 3 (SH3) domain which interacts with proline rich sequences via the PXXP consensus sequence (Koch *et al.*, 1991). This is followed by the Src homology 2 (SH2) domain which is involved in the interaction with phosphotyrosine motifs with the highest affinity shown to the pYEEI consensus (Koch *et al.*, 1991). The final domain is the kinase domain responsible for enzymatic activity. The proteins themselves are highly regulated through interactions between the SH2 and SH3 domains as well as other regulatory proteins.

Lyn kinase is present in B lymphocytes and myeloid cells. Lyn is expressed in two protein forms, derived from alternative splicing, and both are expressed in myeloid cells. Studies have found equal protein levels of the two isoforms in haematopoietic cells with the splicing variation resulting in a 63bp difference within the amino-terminal coding region of the gene, giving rise to 53kDa and 56kDa proteins. Kinase activity of the two

forms has been studied and they both display comparable activities (Yi *et al* 1991). Lyn kinase contains a Src homology 3 (SH3) domain which has two main functions; regulation of its kinase activity and directing binding to specific substrates, allowing association with proline rich motifs (Koch *et al.*, 1991).

1.8.1 SH2 and SH3 Domain

In addition to interacting with various proline-rich subsrates, the SH3 domain acts as a negative regulator of Lyn functional activity. Binding of the SH3 domain to the back of the kinase domain, via a proline rich linker, between the SH2 and kinase domains maintains the inactive form of the enzyme, through formation of a left-handed poly-proline type II helix (Xu *et al.*, 1999). This formation prevents closure between the N and C terminals of the kinase domain favouring the inactive form of the activation loop. A hallmark of this protein family is the possession of two tyrosine motifs with opposing regulatory functions. The C terminal tyrosine motif which is phosphorylated in the inactive state (Y508) by C-terminal kinase (Csk), while the activation loop which is located within the cleft between the N and C lobes of the kinase domain is phosphorylated to promote the active conformation (Y397). The association of the SH2 and SH3 with the kinase domain promotes an overall stable inactive protein structure, while the SH2 and SH3 interactions alone provide a low affinity interaction, not strong enough to cause a fully inactive protein. A summary of the structure is shown in Figure 1.3.

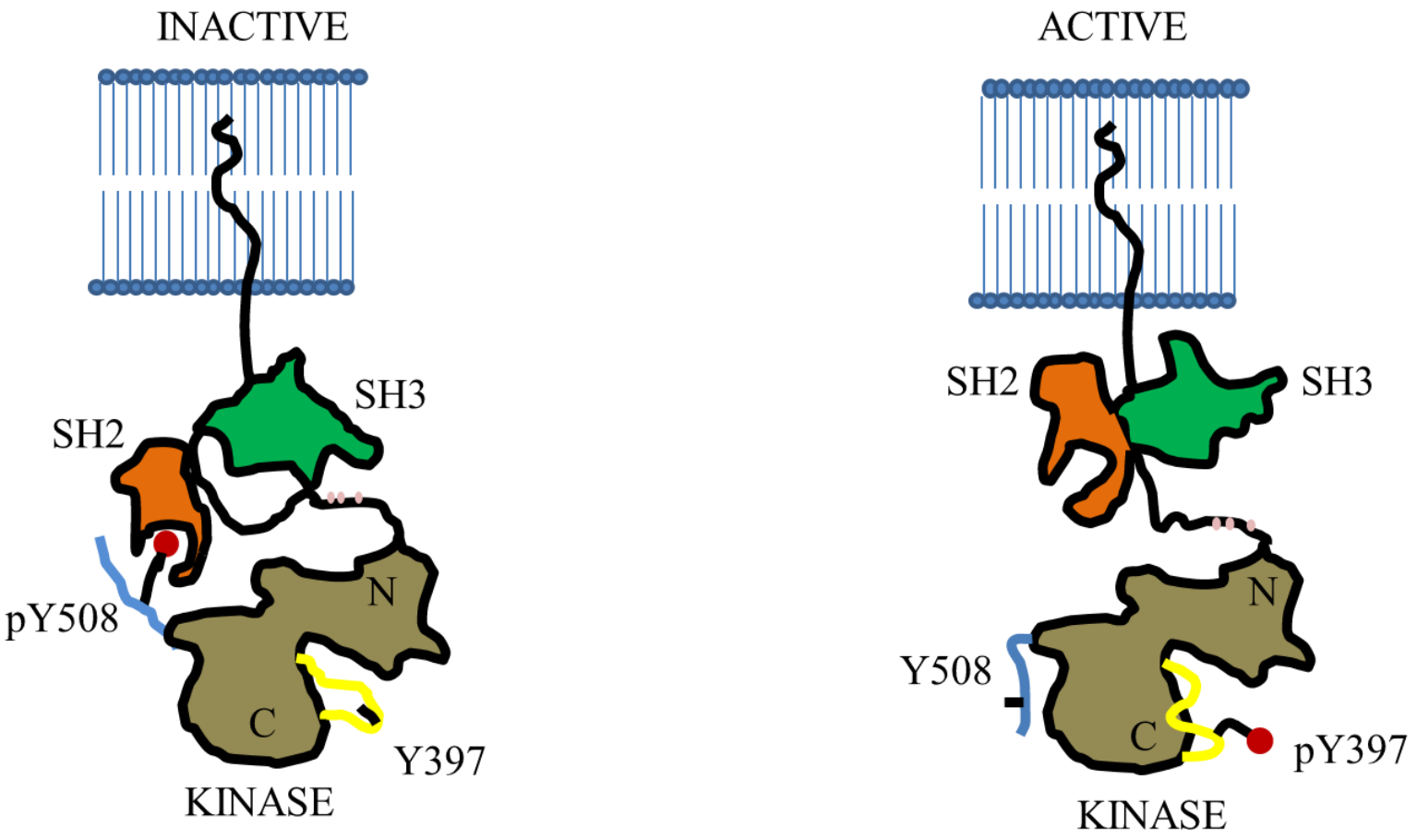


Figure 1.3: Structure of Active and inactive Src family kinase: The C-terminal tyrosine motif (Y508) is phosphorylated by Csk in in the inactivated state, binding of the SH3 domain to the kinase domain maintains the inactive form. The activation loop (Y397) is phosphorylated in the active conformation (Adapted from Ingley *et al* 2007)

1.8.2 Phosphorylation-mediated Activation

Lyn activation occurs through phosphorylation of the activation loop at residue 397. This site has also been shown to be phosphorylated through other Src family members in addition to tyrosine kinases (Sun *et al.*, 2002). Research has shown that there are graded levels of activation of Lyn protein; i) Fully inactive, which has the SH2/SH3 interactions with a phosphorylated 508 residue within the SH2 domain and an unphosphorylated activation-loop, ii) a partially active form where SH2/SH3 interactions are interrupted with the activation loop remaining unphosphorylated, or iii) a fully active form in which the 397 residue within the activation loop is phosphorylated (Chong *et al.*, 2005).

1.8.3 Extrinsic Regulation

Currently, not a great deal is known about the regulation of dephosphorylation of the two tyrosine sites via phosphatases. While not family member specific, a number of phosphatases have been implicated. Proline-enriched tyrosine phosphatase (PEP), T-cell protein tyrosine phosphatase (TCPTP), tandem SH2 domain-containing protein tyrosine phosphatase (SHP1) as well as CD45, have all been shown to dephosphorylate the 508 phosphorylated tyrosine residue (Baker *et al.*, 2000; Cloutier and Veillette, 1996; Van Vliet *et al.*, 2005). Of these, CD45 has emerged as playing a more prominent role in haematopoietic cells (Wang *et al.*, 2002). In addition to removal of the negatively

associated phospho-tyrosine, the phosphatases have also been shown to remove phosphate from the activation loop, so they are involved in activation as well inactivation of the proteins (Baker *et al.*, 2000; Van Vliet *et al.*, 2005).

The phosphorylation of the Src family kinase (SFK) proteins appears to be a multi-factorial process involving kinases in addition to adaptor/scaffold proteins. C-terminal Src kinase (Csk) and CSK-homologous kinase (CHK) are able to phosphorylate the c-terminal tyrosine residue (Chong *et al.*, 2005). Csk is usually found in the cytoplasm and relies on Csk-binding protein (Cbp) to facilitate its recruitment to the plasma membrane. This then enables Csk to interact with the SFK member. Lyn kinase has been shown to directly associate with Cbp (Ingley *et al.*, 2006). Once in its active form the SFK can bind Cbp through its SH3 domain which stabilises the active conformation (Ingley *et al.*, 2006).

Interestingly, Lyn has been shown to form a negative feedback loop with Suppressor of Cytokine Signalling 1 (SOCS1) protein which is involved in the ubiquitination of Lyn (Kyo *et al.*, 2003). SOCS1 is recruited to Lyn by the adaptor protein Cbp in the same manner as Csk (Zhang *et al.*, 1999). Increased SOCS1 showed increased levels of ubiquitinated Lyn which was significantly decreased in SOCS deficient cells (Zhang *et al.*, 1999).

1.8.4 Lyn involvement in Disease

Lyn plays an important role in B cell development and is activated through B cell receptor signalling where it acts as a negative regulator. Interestingly however, in B cell chronic lymphocytic leukaemia (B-CLL), increased levels of Lyn result in defective apoptosis (Contri *et al.*, 2005). Recently, over-expression of Lyn has been implicated in drug resistance of chronic myeloid leukaemia and shown to be activated independently of Bcr-Abl (Wu *et al.*, 2008a). Wu *et al* go on to suggest that Lyn kinase is responsible for regulating the Imatinib sensitivity in CML cells.

Recently, Lyn has been shown to act as both a negative and positive regulator in signalling responses (Lowell, 2004; Xu *et al.*, 2005). Evidence of Lyn involvement in down-regulating signalling responses came from the $lyn^{-/-}$ mice which develop auto-immune disease (Chan *et al.*, 1997; Hibbs *et al.*, 1995). In B cells, Lyn is crucial in down-regulating B cell receptor ligation-mediated activation where it is responsible for phosphorylating inhibitory receptors in the B cell signalling cascade, such as CD22 and Fc gamma IIb resulting in suppression of the active B cell responses (Lowell, 2004). as well as recruiting tyrosine phosphatase SHP-1 which turns off the activating response (DeFranco *et al.*, 1998).

Interestingly, in myeloid cells Lyn has been shown to play a positive role. In haematopoiesis Lyn is needed for stem cell factor-induced proliferation and chemotaxis (O'Laughlin-Bunner *et al.*, 2001).

Lyn is able to inhibit neutrophil apoptosis through association with the granulocyte macrophage colony-stimulating factor (GM-SCF) receptor (Wei S *et al.*, 1996).

In haematopoietic progenitor cells, Lyn $^{-/-}$ mice showed enhanced sensitivity to myeloid specific growth factors such as interleukin 3 (IL-3) and stem cell factor (SCF) (Harder *et al.*, 2001). The knockout mouse also developed significant increases in myeloid cells in the spleen, lymph nodes and bone marrow. These results suggest that Lyn plays a negative role in the modulation of myelopoiesis. It is noted however that these Lyn deficient mice do not develop leukaemia in terms of uncontrolled blast proliferation. In CML, knockdown of elevated Lyn expression by siRNA resulted in increased apoptosis and an increase in Imatinib susceptibility (Ptasznik *et al.*, 2004). Interestingly, in some studies, the development of Lyn dependent signalling has been linked to the appearance of new phosphorylation sites (Lee *et al.*, 2008).

Lyn has also been the target of the more recently developed CML drug Dasatinib which targets the Src family kinases and is aimed at patients unresponsive to Imatinib. Recently, Lyn hyper-activation has been highlighted in CML resistant populations. Acute myeloid leukaemia (AML) is also reported to have increased levels of phosphorylated Lyn kinase which could be decreased with protein phosphatase 2 (PP2A) or Lyn siRNA (Dos Santos *et al.*, 2008).

The ability of Lyn to switch from positive to negative regulator for the same agonist in different cell types suggests a much wider signalling network. With Lyn playing such a varied role among cell systems it is not surprising that tight regulation is required, and as such, deregulation causing an imbalance can lead directly to disease promotion.

Overall, it appears that in normal haematopoiesis Lyn has a role in the inhibition of myeloid cell development, yet in the context of disease it is able to act as a positive regulator of growth and survival. One study has implicated TGFβ in the regulation of Src family members suggesting it can down-regulate Lyn (Atfi *et al.*, 1994).

1.9 TGFβ Signalling

The TGFβ family play a dual role in cell signalling, influencing both normal and disease states. TGFβ is able to elicit a huge number of signalling responses through targeting a wide range of genes, giving rise to diverse cellular responses such as proliferation, apoptosis, differentiation, and epithelial-mesenchymal transition (EMT).

Effects are dependent on cell type and stimulation context (Siegel and Massague, 2003). TGFβ was initially reported to cause fibroblasts to undergo transformation (Roberts *et al.*, 1980). Often the TGFβ signalling cascade is reported to be mutated in cancers. An example of the different responses to TGFβ can be seen among epithelial cells where TGFβ stimulation causes growth arrest and apoptosis, processes associated with tumour suppression, and the induction of the epithelial to mesenchymal transition (EMT) and fibroblast activation, responses involved in the promotion of cancer metastasis and fibrotic disease (Siegel and Massague, 2003). There is growing evidence suggesting TGFβ is secreted by tumour cells which actively contributes to cancer processes such as cell growth, invasion and metastasis (Franco *et al.*, 2011; Komatsu *et al.*, 1989).

The overall ability of TGFβ to be involved in such a wide range of cellular processes would suggest a complex signalling pathway mechanism, yet the mediation of the TGFβ signal is relatively simple.

1.9.1 TGFβ Ligand and Receptors

The TGFβ superfamily can be subdivided into the TGFβ/activin/nodal family and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/Muellerian inhibiting substance (MIS) family, these classifications are based on their structural similarities and functional roles. TGFβ is the founding member of the superfamily and exists in three mammalian isoforms; TGFβ-1, TGFβ-2, and TGFβ-3. They share around 80% homology and signal through the same receptors. Research carried out in this thesis looks exclusively at TGFβ-1.

TGFβ is initially synthesised as a precursor protein, and then proteolytically processed to an inactive form bound to the amino-terminal precursor called the latency-associated peptide (LAP) (Munger *et al.*, 1997). The secreted growth factor and pro-peptide complex prevents a response being elicited prematurely. Initially synthesised as 55kDa polypeptides, TGFβ then forms dimers after production. This is then cleaved to form small latent TGFβ in the golgi apparatus (Dubois *et al.*, 1995). This complex contains the mature 25kDa protein with LAP, this usually associates with latent TGFβ binding protein (LTBP). TGFβ is activated by several mechanisms including plasmin digestion through cleavage of LAP (Grainger *et al.*, 1995), and activation of small or large latent complexes through thrombospondin (Ribeiro *et al.*, 1999). The activation of TGFβ is a multi-step process demonstrating the regulation used in the control of active TGFβ production.

There are a range of type I and II receptors which are utilised by TGFβ family members. There are seven mammalian type I receptors known as ALK 1-7 (Activin receptor-like

kinase), and five type II receptors. In the vast majority of cell types TGFβ uses ALK5 and TGFβRII. In addition, a type III receptor (TGFβRIII), also known as betaglycan, serves as a co-receptor by presenting the ligand to TGFβRII. The cytoplasmic domains of TGFβRIII and TGFβRII interact. TGFβRIII preferentially binds autophosphorylated TGFβRII, promoting the formation of a TGFβRII/TGFβRI complex (Blobe *et al.*, 2001). TGFβ family members elicit signalling through interactions with 2 receptor serine/threonine kinases known as the type I (TGFβRI) and type II (TGFβRII) receptors. Ligand binding mediates formation of a heterotetrameric complex allowing the constitutively active TGFβRII to phosphorylate the glycine-serine rich domain of TGFβRI, activating its kinase domain, causing the assembly of two type I and two type II receptors (Figure 1.4). The TGFβ family transduce their signals via the phosphorylation, and subsequent activation of receptor activated Smad proteins via the TGFβRI. Activated Smads translocate into the nucleus where they associate with other transcription factors to regulate gene expression (Massague *et al.*, 2005).

1.9.2 SMADS

Smads are the intracellular messengers of the TGFβ signalling pathway. They were first identified in Drosophila and named MAD proteins (Sekelsky *et al.*, 1995). In mammals, there are 8 known Smad proteins which are grouped according to function: receptor activated Smads (R-Smads: 1,2,3,5 and 8), the common mediator Smad (co-Smad: Smad4), and the inhibitory Smads (I-Smads: 6 and 7). TGFβ is known to signal through Smads 2 and 3, while Smads 1, 5 and 8 are activated through BMP signalling. Both R- and Co-Smads contain an N-terminal MH1 (MAD homology 1) and C-terminal MH2 domain which are connected via a non-conserved linker region.

Upon ligand binding, phosphorylation of R-SMADs by TGFβRI occurs on the C-terminal Ser-Ser-X-Ser-Ser (SSXSS) motif, also known as the MH2 domain which is highly conserved among all Smad proteins. R-Smad phosphorylation is facilitated by other proteins such as SARA (Smad anchor for receptor activation) (Tsukazaki *et al.*, 1998). SARA was originally identified as a Smad2 interacting protein which can also associate with the TGFβ receptor complex and present Smad2 to the complexes for phosphorylation and activation (Tsukazaki *et al.*, 1998). SARA contains the FYVE domain, a phospholipid binding domain, which functions as an adaptor to recruit Smads 2 and 3 to the TGFβ receptor complex. Recently, SARA has been found to bind to phosphatidylinositol 3-phoshphate (PtdIns3P) in the membrane and promote TGFβ receptor trafficking to the early endosome leading to Smad2 phosphorylation (Itoh F *et al.*, 2002). Additionally, R-SMAD phosphorylation is coupled with the internalisation of the TGFβR (Hayes *et al.*, 2002). After phosphorylation, dissociation occurs between

the SARA/receptor complex and the R-SMAD, a hetero-oligomeric complex is then formed between the R-Smad and co-Smad 4. This complex then translocates into the nucleus and interacts with Smad binding elements (SBEs) or GC rich sequences present in certain promoters (Shi *et al.*, 1998). The SBEs are often located near binding sites for various other transcription factors, consequently Smads are able to influence a wide range of gene expression mechanisms (Massague *et al.*, 2005). The complexity of signalling is regulated in part through accessory proteins in the nucleus which facilitate a specific transcriptional response. Smads have a low affinity for DNA binding, so co-activator proteins such as p300, and CREB-binding protein (CBP) and co-repressors such as Ski and SnoN aid in regulating the response.

I-Smads lack the C-terminal sites for phosphorylation by TGFβRI found in both R- and co- Smads. Smad7 inhibits the TGFβ/activin and BMP pathways whereas Smad6 exclusively inhibits the BMP pathway (Hayashi *et al.*, 1997). These two Smads are able to inhibit signalling via different mechanisms. Smad6 competes with Smad4 for binding to phosphorylated Smad1, creating a Smad1/6 complex, which is unable to regulate transcription (Hata *et al.*, 1998). Smad7 however competes with R-Smad for the interaction with TGFβRI by forming stable interactions with the receptor (Hayashi *et al.*, 1997). In addition, Smad7 is able to mediate TGFβ receptor ubiquitination and degradation by recruiting Smurf1/2 ubiquitin ligases (Kavsak *et al.*, 2000). Caveolin-1 is known to interact with TGFβRI and its overexpression leads to the promotion of receptor degradation. Smad7 and Smurf2 are located within caveolin-1 vesicles, and are thought to be involved in this receptor complex and promote proteasomal-dependent degradation (Le Roy and Wrana, 2005).

Despite the Smads being widely known as the intracellular mediators of TGFβ signalling, other pathways can be activated through TGFβ, independently of them. In primary epithelial cells and some breast cancer cell lines, activation of Ras, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK) by TGFβ has been reported (Mulder, 2000). TGFβ activated kinase-1 (TAK1) a member of the MEKK family and activator of JNK and p38 MAPK pathways (Yamaguchi *et al.*, 1995) was activated by TGFβ. Recently, the TGFβ receptor has been found to be modified through sumoylation which is a small ubiquitin-like modifier (SUMO) which enhances the TGFβ response through recruitment and activation of Smad3 (Kang *et al.*, 2008).

This evidence goes some way towards detailing the complexity surrounding the TGFβ signalling pathway and how multiple signalling transducers can have a huge range of effects within a cell.

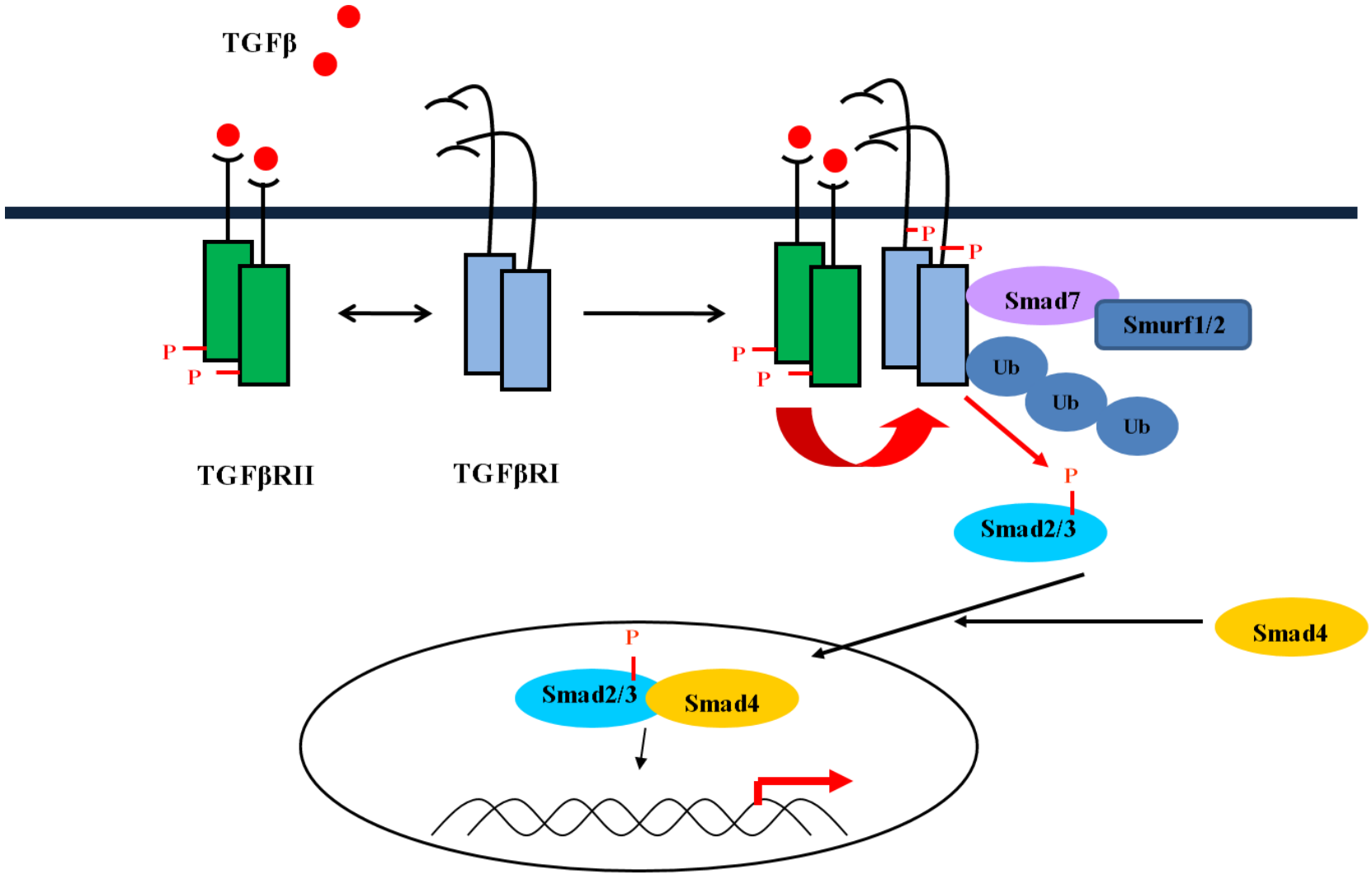


Figure 1.4: The TGFβ signalling pathway: TGFβ ligand binds the type II receptor dimer, this leads to the recruitment of the type II receptor dimer, forming a heterodimer complex catalysing the phosphorylation of the type I receptor. The activated receptor phosphorylates Smad2/3 which associate with Smad4 and translocates into the nucleus where it causes DNA transcription

1.10 TGFβ in Haematopoiesis

A number of studies have suggested TGFβ is directly involved in the inhibition of murine and human haematopoietic stem and primitive progenitor cells *in vitro* (Batard *et al.*, 2000; Keller *et al.*, 1988; Sitnicka *et al.*, 1996), whereas more differentiated cells are more resistant to these affects. In more mature cells the presence of other growth factors makes the effect on these cells more complex. Transient blockage of autocrine signalling through expression of a dominant negative TGFβRII enhances proliferation and survival of human HSCs *in vitro* (Fan *et al.*, 2002). An *in vivo* study also showed the inhibitory affects of TGFβ-1 by directly injecting it into femoral arteries of mice, demonstrating inhibition of multi-potent haematopoietic progenitors in bone marrow (Goey *et al.*, 1989). These results suggest that both autocrine TGFβ production from HSCs, and TGFβ from surrounding stromal cells are involved in the maintenance of the HSC population.

Interestingly, TGFβ-2 has been shown to have a positive affect on HSCs while TGFβ-3 only functions as an inhibitor on early haematopoietic progenitors (Jacobsen *et al.*, 1991). Another study showed that TGFβ-2 demonstrated opposing effects and this was dose dependent. In low concentrations it had a stimulatory affect, while at higher concentrations proliferation was inhibited (Langer *et al.*, 2004).

It has been observed that early progenitor $CD34^{+}CD38^{-}$ cells show a strong response to TGFβ inhibition whereas more mature $CD34^{+}CD38^{+}$ are less sensitive (Van Ranst *et al.*, 1996). In addition, research has shown a branching of the TGFβ pathway where Smad4-Smad2/3 associations promotes growth inhibition in haematopoietic cells, whereas

Smad2/3 interacting with Transcriptional Intermediary Factor 1γ (TIF1γ) mediates differentiation (Wei *et al.*, 2006).

TGFβ is believed to exert the anti-proliferative affects through two mechanisms resulting in the inhibition of cell-cycle responses. Firstly, it down-regulates c-Myc which is involved in antagonizing the TGFβ pathway through cyclin dependent kinase inhibitor (CDKI) repression, and secondly it upregulates CDKIs such as p15, p21 and p27 (Massagué *et al.*, 2000). Interestingly, $p21^{-/-}$ and $p27^{-/-}$ mice were inhibited by TGFβ, demonstrating that the anti-proliferative affect is independent of these two proteins (Cheng *et al.*, 2001). A number of studies have shown that the only CDKI to be significantly up-regulated by TGFβ is p57, and targeted siRNA has shown it to be crucial for TGFβ-mediated cell cycle arrest in haematopoietic cells (Scandura *et al.*, 2004; Yamazaki *et al.*, 2009). In addition to cell cycle proteins, TGFβ has also been shown to down-regulate haematopoietic cytokine receptors such as IL-1 and stem cell factor (SCF) (Dubois *et al.*, 1990; Jacobsen *et al.*, 1995). Smad5 has been shown to play a role in mediating the inhibitory effects of TGFβ in haematopoietic progenitor cells by using antisense oligonucleotides to knock it down, which reversed the inhibitory effects (Bruno *et al.*, 1998).

Another study looked at completely knocking out the TGFβ signalling pathway through over-expression of Smad7 in HSCs resulting in a greater ability of cells to self-renew (Blank *et al.*, 2006).

Despite the large amount of evidence presenting TGFβ as an inhibitor of haematopoietic cell growth *in vitro*, the *in vivo* studies suggest a different outcome. It is also a consideration that the high TGFβ doses used may not reflect physiological conditions. To overcome this, one study used a TGFβ receptor knockout model and concluded that

TGFβ is dispensable for regulation of HSC quiescence or self-renewal *in viv*o (Larsson *et al.*, 2003; Larsson *et al.*, 2005) It is thought that these differences may be due to the more complex *in vivo* setting, and highlights the importance the surrounding environment can have on the TGFβ-mediated effects.

1.11 TGFβ and Leukaemia

In addition to its role in cell growth inhibition and acting as a tumour suppressor, TGFβ can also promote tumour metastasis in late stage disease highlighting its involvement in both disease suppression and progression (Derynck *et al.*, 2001). The disruption of TGFβ signalling through mutations causing either inactivation or a down-regulation of expression is known to play an important role in carcinogenesis (Derynck *et al.*, 2001; Massagué *et al.*, 2000).

In acute myeloid leukaemia (AML) two mutations involving Smad4 are known, which disrupt the propagation of TGFβ transcriptional activity (Imai *et al.*, 2001). Further evidence goes on to suggest that TGFβ alone is not able to cause the onset of leukaemia but is a factor in disease progression. This is supported further by evidence that both Smad3-null and TGFβRI knockout mice independently did not go on to develop leukaemia (Datto *et al.*, 1999; Yang *et al.*, 1999), but patient data suggests loss of TGFβRI/II can lead to myeloid or lymphocytic leukaemia (Le Bousse-Kerdilès *et al.*, 1996).

A number of studies have tried to uncover the effect TGFβ has on leukaemia cell proliferation. For example, Kadin *et al* (1994) found resistance to TGFβ-mediated inhibition was associated with loss of TGFβRII expression in T-cell lymphomas. Alternatively, Cashman *et al* (1998) showed that CML cells were responsive to TGFβ growth inhibition. Alongside this, Atfi *et al* (2005) suggests Bcr-Abl positive cells inhibit the TGFβ response without adversely affecting Smad activity but by activating Akt which blocks the TGFβ effect through retention of FoxO3 in the cytoplasm. Møller

et al (2007) demonstrated that Bcr-Abl expression in TF-1 cells rendered them more sensitive to the growth inhibitory effects of TGFβ than the parental cell line. In addition to the growth inhibitory effects, TGFβ plays a vital role in maintaining the growth and differentiation balance in haematopoietic cells (Fortunel *et al.*, 2003; Kim and Letterio, 2003). More recently, growing evidence for a mixed population of tumour cells, which are able to self-renew and propagate tumour formation, has been suggested. These findings have been discovered in many organs and tissues including blood (Lapidot *et al.*, 1994), breast (Al-Hajj *et al.*, 2003) and brain (Singh *et al.*, 2004). Aberrant TGFβ signalling in adult T-cell leukaemia/lymphoma suggests that down regulation of the tumour suppressor zinc finger E-binding homeobox 1 (ZEB1) which is normally an enhancer of TGFβ signalling through Smad2/3 binding, combined with enhanced Smad7, leads to TGFβ anti-growth resistance (Nakahata *et al.*, 2010). Members of the TGFβ receptor family ALK1 and ALK5 have been studied and ALK5 has been found to be overexpressed in acute myeloid leukaemia. This correlates with a negative impact on complete remission in patients, and highlights the potential for small molecule inhibitors to target the receptor (Otten *et al.*, 2011).

A further recent development in the study of CML is the role of the stem cell niche. As mentioned previously, HSC's are thought to play a role in disease persistence and be refractory to therapy, and recently the environment harbouring these cell populations has been investigated. Matrix-metalloproteinase-9 (MMP-9), involved in matrix protein regulation, , has been reported to be up-regulated in CML (Yoon *et al.*, 2006). MMP-9 is regulated by TGFβ and this activation leads to MMP-9 induced cell migration and subsequent spread of cells away from the niche and into the circulation, promoting metastasis. It has also been shown using HSc025 (Smad inhibitor) that MMP-9 up-

regulation is Smad independent and occurs via the PI3K/Akt pathway. The PI3K/Akt pathway has previously been implicated in CML and work by Naka *et al (*2010) has shown that TGFβ critically regulates the leukaemia initiating cell (LIC) population. Inhibition of the pathway resulted in an increase in phosphorylated Akt leading to export of nuclear Foxo3a resulting in a reduction in the potency of LIC driven disease. In non-LICs, Bcr-Abl activates Akt, leading to repression of Foxo3a effects, such as apoptosis and cell cycle arrest, allowing these cells to proliferate.

One study which has tried to establish a mechanism through which leukemic cells are resistant to the effects of TGFβ looked at childhood leukaemia resulting from a *TEL-AML1* fusion gene, the most common in paediatric cancer. The study shows that *TEL-AML1* is able to bind Smad3 and suggests that this leads to a sequestering away from the nuclear target sites compromising the ability to activate negative cell cycle regulators such as $p27^{Kip1}$ in early B cell progenitors (Ford *et al.*, 2010). The cell cycle inhibitor p15 also involved in mediating TGFβ induced cytostatic effects is often silenced due to promoter hyper-methylation in AML (Krug *et al.*, 2002).

TGFβ regulation via the gene *Pml* has been found in promyelocytic leukaemia, $Pml^{-/-}$ primary cells showed impaired TGFβ induced growth inhibition. Interestingly, this was rescued by adding a cytoplasmic isoform (cPML), suggesting it is critical for TGFβ functions. This research also showed that Smad2/3 phosphorylation and nuclear translocation was reduced in $PML^{-/-}$ cells (Lin *et al.*, 2004). This followed on from work which identified retinoic acid (RA) as being able to reverse the TGFβ insensitivity in acute promyelocytic (APL) leukaemia caused by PML-RARα over-expression through degradation of the over-expressed protein (Lin *et al.*, 2004). The involvement of Smad3 in leukemogenesis has also been found in T-cell ALL where it is identified as a tumour

suppressor, with protein levels undetectable in children suffering from the disease. Finally, TGFβ can be negatively regulated, leading to leukaemia. The oncoproteins c-Ski which are overexpressed in leukaemia patients (Kronenwett *et al.*, 2005) are known to effect TGFβ signalling through interference with the R-smad/Smad4 complex in addition to the recruitment of co-repressors such as N-CoR and histone deacetylases (HDACs) (Liu *et al.*, 2001). From these examples it is clear to see that TGFβ appears to have a changing or transient role in leukaemia pathogenesis, and can act in conflicting fashion dependent on cell type.

In general, TGFβ is considered a strong inhibitor of committed progenitor cell function, and autocrine production of TGFβ maintains haematopoietic stem cells in a non-dividing quiescent state. During carcinogenesis the role of TGFβ as a tumour suppressor appears to change, emerging as an oncogenic factor. The ability of TGFβ to be involved in a range of signalling pathways, which may elicit cross-talk mechanisms, and can be either Smad dependent or independent, only highlights the vast complexity of its role in disease development. Evidence suggests that while TGFβ is important, it is not involved in leukaemia initiation, but plays a role in leukaemia progression. Combined with the observation that it is often up-regulated in leukaemia (Mori *et al.*, 2000) it can be suggested that it plays a role in disease invasion. Clearly, understanding TGFβ regulation and its downstream targets is of paramount importance in the context of CML. Protein levels within a cell can be controlled through a range of post-translational modifications, TGFβRI levels are known to be regulated through Smad7-mediated Smurf2 ubiquitination (Kavsak *et al.*, 2000), and evidence has also highlighted

TGFβ to downregulate Src kinases including Lyn (Atfi *et al.*, 1994) which may be via ubiquitination.

1.12 The Ubiquitin Proteosome System

Ubiquitination is a post-translational modification involved in the majority of cellular events, where a 76-residue ubiquitin (Ub) protein can be attached to target proteins and affect their localization, activity, structure and interaction partners through lysine residues. Proteins can become mono or polyubiquitinated. Ub is activated through an enzymatic cascade, by a ubiquitin activating enzyme the E1 enzyme, and then transferred to a ubiquitin conjugating enzyme E2. The E2 enzyme transfers the ubiquitin to an E3 ubiquitin ligase which consequently catalyzes the transfer of the ubiquitin to a lysine residue of the target protein (Figure 1.5). Substrate specificity is mainly determined by the E3 ligase (Pickart and Fushman, 2004). Ubiquitination is reversible through deubiquitinating enzymes (DUBs) which remove the Ub modification from the substrate (Komander *et al.*, 2009). In addition, proteins contain Ub binding domains (UBDs), which increase the ability for this modification to influence cellular protein activities.

1.12.1 Roles of Ubiquitination

The ubiquitin modification of a protein causes a specific functional change. Ubiquitin is able to affect its target protein based on the particular modification. The ubiquitination reaction generates an iso-peptide bond between the carboxyl terminus of Ub and the

amino group of lysine on the target substrate (Weissman, 2001), and in certain instances the terminal amine or Cysteine group can also be modified (Cadwell and Coscoy, 2006; Ciechanover and Ben-Saadon, 2004).

The substrate modification can be via different mechanisms. Monoubiquitination occurs when a single ubiquitin molecule binds a substrate, and is involved in non-proteolytic processes such as gene expression and nuclear export. Poly-monoubiquitination can occur where several lysine residues on a target protein have a single ubiquitin attached (Figure 1.6). Ub contains 7-internal lysine residues, making it possible to form chains, and therefore can be targeted for ubiquitination on these seven lysine residues serving as ubiquitin substrates. This allows a target protein to be polyubiquitinated through the addition of Ub chains (Xu *et al.*, 2009). The structural diversity of these chains, gained through the additional ubiquitination of specific lysine residues on ubiquitin, allows functionally distinct signals to be generated. Polyubiquitination through lysine 48 is known to target the substrate for proteasomal degradation, whereas ubiquitin chains linked through lysine 63 are believed to signal in a similar way to monoubiquitination (Sorrentino *et al.*, 2008).

Proteins targeted for ubiquitination are complexed with specific ubiquitin-conjugating enzymes known as E3 ligases which attach the 76-residue protein to the target substrate. Phosphorylation has been highlighted as an important factor in ubiquitination. Many parallels have been drawn between the two modifications. Phosphorylation of substrates is a well characterised way of creating binding sites for E3 ligase binding. An example of this is Cbl, a monomeric Ring E3 ligase, involved in ubiquitination of the EGFR, which binds its substrate through a phosphotyrosine binding domain, only after receptor phosphorylation (Levkowitz *et al.*, 1998).

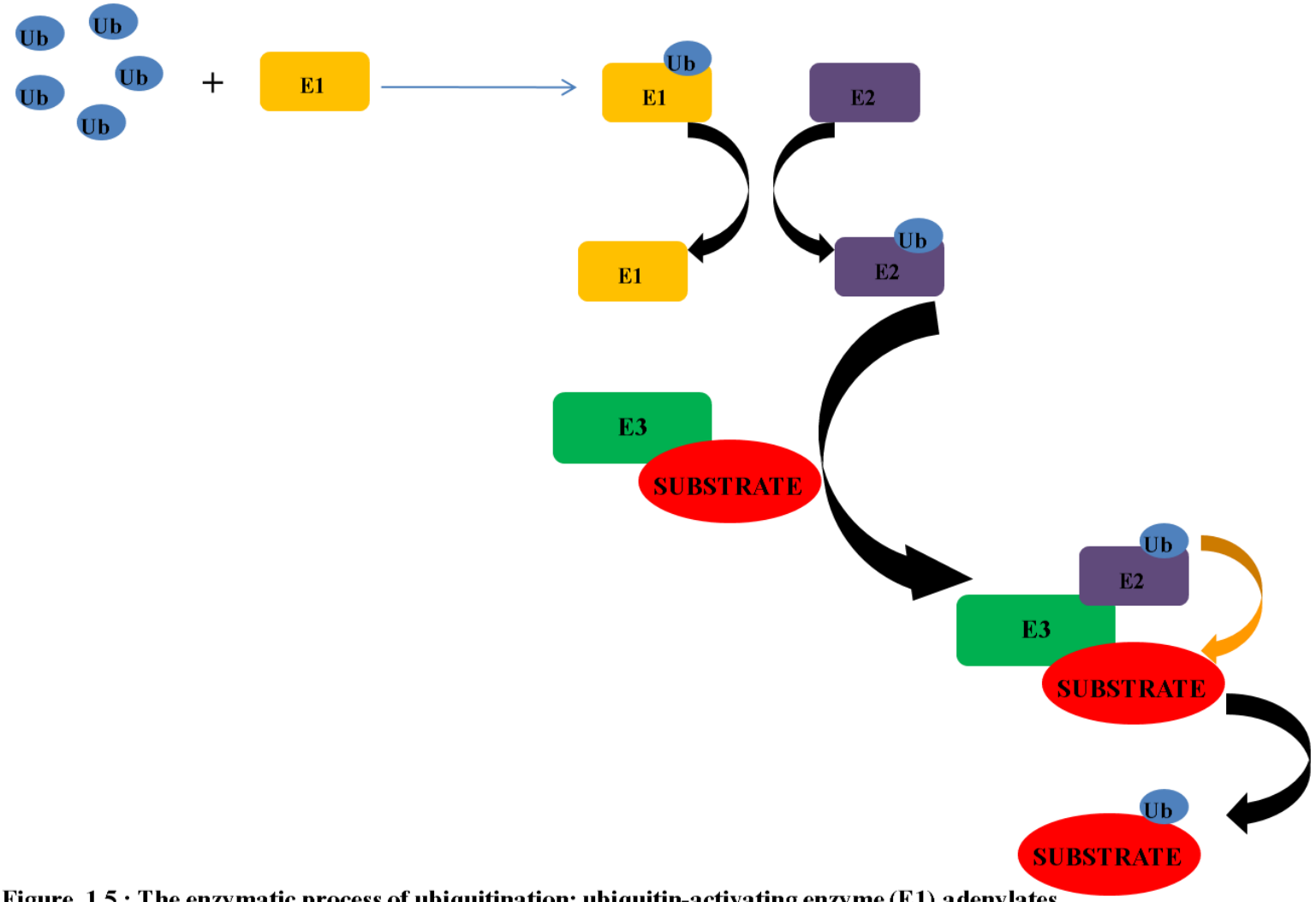


Figure 1.5 : The enzymatic process of ubiquitination: ubiquitin-activating enzyme (E1) adenylates ubiquitin and transfers it to the ubiquitin-conjugating enzyme (E2), in the final stage the ubiqutin ligase (E3) recognises the substrate and catalyses the transfer of ubiqutin from the E2 to the substrate

1.12.2 Ubiquitination in TGFβ Signalling

E3 ubiquitin ligases are essential in targeting substrate specificity, and are divided into Homologous to the E6-AP Carboxyl Terminus (HECT) domain and Really Interesting New Gene (RING)-type ligases. The RING E3s can be single-chain proteins or multi-subunit complexes and they act as scaffolds using an associated E2 ligase to transfer Ub onto target substrates. In contrast, the HECT E3s have intrinsic ubiquitin ligase activity used for direct substrate ubiquitination. Arkadia is an example of a RING finger E3 ligase, and recent data suggests it plays an important role in modulating levels of nuclear phospho-smads together with the Smad transcriptional co-repressor SnoN (Levy et al, 2007; Nagano et al, 2007). In addition, the RING E3 ligase Ectodermin/TIF1γ has been implicated in Smad4 degradation (Dupont et al, 2005). Within the HECT-domain E3 ligases are a small sub-group known as the Neural precursor cell expressed developmentally down-regulated 4 (NEDD4) family. There are nine NEDD4 family E3 ligases in human and mouse, including NEDD4-2/NEDD4L, WWP1/Tiul1, WWP2, AIP4/Itch, Smurf1, Smurf2, HecW1/NEDL1, and HecW2/NEDL2. Smurf1 (Smad-ubiquitin regulatory factor 1) is a NEDD4 family E3 ligase, and this was the first E3 ligase to be implicated in the TGFβ signalling pathway. Smurf1 binds and regulates levels of un-stimulated BMP-regulated Smad 1 and 5 (Zhu *et al*, 1999). Smurf2 binds and ubiquitinates primarily phospho-Smad 1 and 2 (Lin et al, 2000; Zhang et al, 2001; Bonni *et al*, 2001), but is also recruited to the type 1 TGFβ receptor via Smad7 (Kavsak et al., 2000; Ebisawa et al., 2001; Suzuki *et al*, 2002). Other NEDD4 family E3 ubiquitin ligases that regulate the TGFβ pathway at different levels include NEDD4-2 (Kuratomi

et al, 2005), WWP1/Tiul (Komuro *et al*, 2004; Morén *et al*, 2005; Seo *et al*, 2004), and AIP4/Itch (Bai *et al*, 2004; Lallemand *et al*, 2005), and WWP2 (Soond and Chantry, 2011).

Recently, TRAF6, a two ring domain ubiquitin ligase, was shown to be responsible for the activation of the TGFβ-activated kinase 1 (TAK1) through lysine-63 polyubiquitination, allowing the downstream activation of Smad independent JNK and p38 pathways (Sorrentino *et al*., 2008; Yamashita *et al*., 2008).

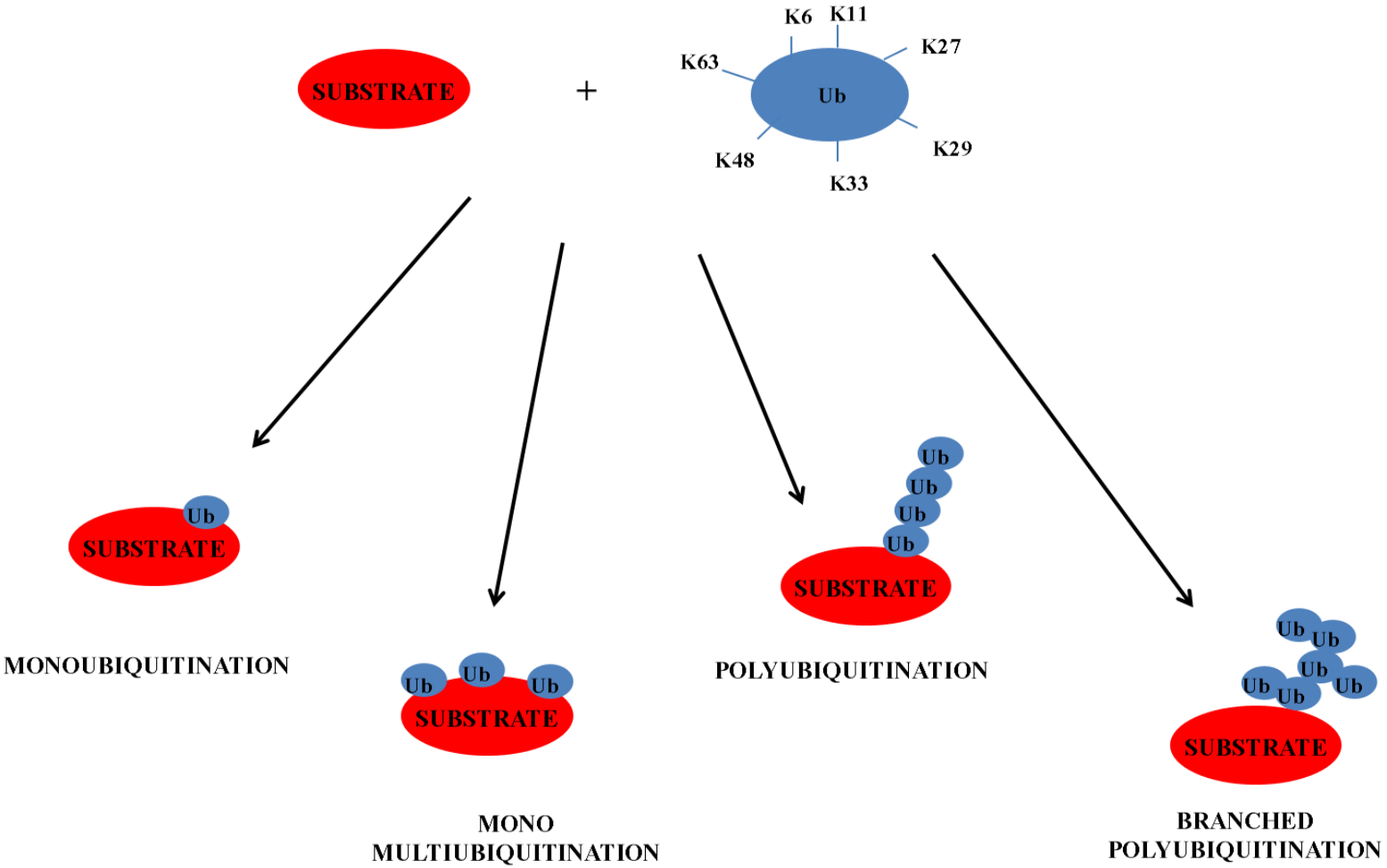


Figure 1.6: The Various types of protein ubiquitination: Monoubiquitination - a single ubiquitin attaches to the target protein, this can become Mono-multiubiquitination through addition of ubiquitin directly to the target protein. Polyubiquitination- multiubiquitination through linkage of ubiquitin protein forming chains. Branched polyubiquitination- ubiquitin forms branched chains through various lysine residue linkages

1.13 Connections between TGFβ, Lyn and E3 ubiquitin ligases

There are a number of potential E3 Ubiquitin ligases that might connect TGFβ to Lyn, and literature searches have highlighted the Ring finger E3 Ubiquitin ligase, c-cbl, as a very promising candidate (Shao *et al* 2004). C-cbl is an SH2-domain-containing E3 ubiquitin ligase that is known to interact with Lyn. In addition, other published studies highlight de-regulated TGFβ signalling in cbl -/- cells suggesting possible interactions between TGFβ and c-cbl (Wohlfert *et al* 2006).

TGFβ is known to inhibit cell growth, and this response is often lost in cancerous cells. Studies have implicated a role for TGFβ in Lyn activation. Atfi *et al* (1994) suggest TGFβ causes a decrease in cellular levels of Lyn together with a decrease in protein activity. In 1995 Pazdrak *et al* reported a TGFβ-mediated inhibition of Lyn through IL-5, suggesting an inhibitory role for TGFβ in eosinophils.

The E3 ligase Cbl has previously been linked to Lyn kinase ubiquitination (Kyo *et al.*, 2003). Importantly, they are also substrates of the SFKs (Hunter *et al.*, 1999). The Cbl proteins have largely been studied in relation to the epidermal growth factor receptor and its role in receptor recycling and degradation (Katzmann *et al.*, 2002).

1.14 Aims and Hypothesis

TGFβ has long been studied and its deregulation is well documented in many cancers. This research aims to further investigate the role of TGFβ in CML disease progression and resistance. My initial hypothesis is that up-regulation of TGFβ activity by Bcr-Abl is responsible for maintaining the small population of Imatinib-resistant stem cells in CML causing the residual persistent disease, leading to relapse. The theory was that CML could be completely eradicated only by a combination therapy; where it is necessary to administer both a Bcr-Abl inhibitor together with a TGFβ receptor inhibitor to allow the quiescent cell population to enter back into the cell cycle where Imatinib would induce apoptosis, summarised in Figure 1.7. While this was being investigated research emerged unveiling Lyn kinase as a contributor to imatinib resistance through over-expression. Futhermore, a paper by Atfi *et al (1994)* described TGFβ down-regulating Src family kinases including Lyn in prostate carcinoma cells. This provided a link between the two, so it was speculated that TGFβ may also be having an effect on Lyn kinase in CML, and indeed, on drug resistance.

Overall, the underlying hypothesis is that Bcr-Abl up-regulates TGFβ-signalling and that imatinib resistance through Lyn kinase expression is mediated by TGFβ. The aims of the project were:

1. To confirm Bcr-Abl up-regulation of TGFβ Smad-dependent signalling through luciferase reporter analysis in well established TGFβ-responsive cell lines
2. To characterise MYL and MYL-R CML derived cell lines in terms of TGFβ responsiveness, through cell proliferation and protein analysis studies.
3. Assess a dual-therapy approach to overcoming drug resistance using a TGFβ signalling inhibitor alongside Imatinib.
4. Investigate a link between TGFβ and Lyn kinase in drug resistance through over-expression and endogenous analysis in CML cells.

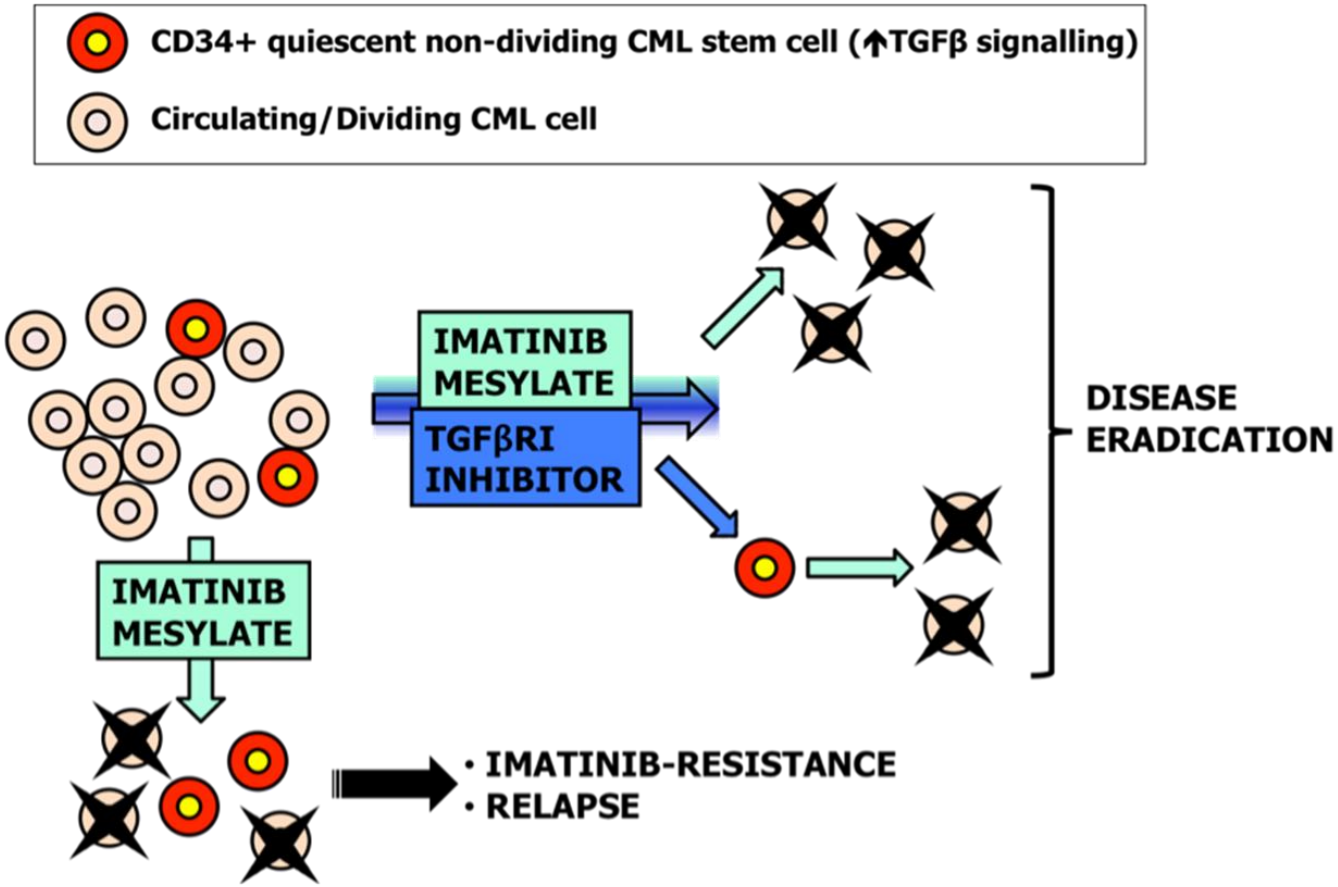


Figure 1.7: Proposed new dual therapy approach for the treatment of Chronic Myeloid Leukaemia: Circulating/Dividing CML cells are eradicated by Imatinib treatment. Quiescent non-dividing cells are resistant to Imatinib, by inhibiting the TGFβ receptor, cells are forced out of their quiescent state, begin dividing and are eradicated by Imatinib.

2. MATERIALS AND METHODS

2.1 Materials

Recombinant human TGFβ was bought from R&D Systems. SB431542 compound was bought from Sigma Aldrich. Imatinib Mesylate (STI571) was donated by Novartis.
Lyn-Flag expression vector was obtained from Hiroshi Murakami (Okayama University), and $CAGA_{12}$ from Caroline Hill (CRUK Laboratories), TRAF6 constructs were a gift from Justin McCarthy (University College Cork), Cbl constructs from Stan Lipkowitz (National Institutes of Health, Maryland), Bcr-Abl-p210 from Helen James (University of East Anglia).
Competent DH5α cells were purchased from Invitrogen.

2.2 DNA Preparation

2.2.1 LB Agar Plates/ LB Media

E. Coli DH5α cells were grown in Luria-Bertani medium (LB) which was sterilised by autoclaving. After cooling, ampicillin (100μg/ml) was added if required. If bacterial cultures were grown on solid media, agar was added to the LB prior to autoclaving. LB agar was cooled before the addition of ampicillin (100μg/ml), and then poured into 10cm dishes, left to solidify at room temperature and then stored at 4°C. The components for both are shown in Table 2.1.

	LB Media	LB Agar
Bacto-Tryptone	10g	10g
Bacto-Yeast Extract	5g	5g
Sodium Chloride	5g	5g
5M Sodium Hydroxide	200µl	200µl
Bacto-Agar	-	30g
Distilled Water	1000ml	1000ml

Table 2.1: Compositon of LB media and LB agar

2.2.2 Heat Shock Transformations

A vial containing 50µl of competent DH5α cells was thawed on ice and, 1µg of plasmid DNA was added. The same volume of water was added to a separate sample of cells to serve as a negative control. Cells were mixed gently and then incubated on ice for 30 minutes before heat-shock treatment at 42°C in a water bath for 20 seconds, before being incubated on ice for 2 minutes. 950µl of pre-warmed LB was added and incubated for 1 hour at 37°C, shaking at 225rpm. The mixture was then spun down at 6,000 rpm for 5 minutes and the supernatant was removed. The DH5α cells were re-suspended in the remaining supernatant and pipetted onto an antibiotic selective LB agar plate, and incubated at 37°C overnight.

2.2.3 DNA Purification

Competent cells were transformed as described previously. Colonies were isolated from agar plates grown up overnight using a pipette tip, and transferred to 15ml Falcon tubes each containing 2mls of LB plus a selected antibiotic. These were incubated at 37°C, 250rpm with shaking for 12-16 hours. Cells were centrifuged at 4300rpm for 5 minutes, and the supernatant was aspirated. DNA was purified from the remaining pellet using a Plasmid Spin Miniprep kit (Qiagen), and carried out in accordance with the manufacturer's guidlines. DNA concentration was analysed using Nanodrop spectrophotometer (Thermo Scientific). Samples were stored at -20°C.

2.3 RNA Techniques

2.3.1 RNA Extraction

Total RNA was extracted from cells using the Promega SV Total isolation kit (Promega) following the manufacturers guidelines. RNA was eluted in a final volume of 100µl, the RNA was quantified on a Nanodrop spectrophotometer and then stored at -80°C.

2.3.2 Reverse Transcription

Routinely, 1µg of RNA was transcribed in a 20µl reaction. The reverse-transcription was carried out using random hexamer primers, dNTP mix, 0.1M DTT, 5x First Strand buffer, RNase out (Invitrogen-RNase inhibitor) and Reverse transcriptase Superscript II (Invitrogen). The 1µg of RNA was made up to 9µl with RNase free water. To this, 2µl of random hexamers was added and the mixture incubated at 70˚C for 10 minutes, and then placed on ice and spun briefly at 4˚C. The remainder of components were added as shown in table 2.2. Particular care was taken to ensure thorough mixing as Superscript II and RNase out would sink to the bottom of the mix. The reaction mixture was incubated at 42˚C for 60 minutes, the cDNA was then stored at -20˚C.

REAGENTS	20µl Reaction
Random Primers	250ng
RNA	1µg
dNTP Mix	33mM
5x First Strand Buffer	4µl
0.1M DTT	2µl
RNase Out (40U/µ)l	1µl
Superscript II RT	1µl
H_20 up to a final volume of	20µl

Table 2.2 Reverse Transcription components

2.3.3 Semi Quantitative RT-PCR

Semi-quantitative PCR was carried out to look at expression levels of Cbl genes using GAPDH as a normalisation control. The c-Cbl primers were run at 95˚C for 5 minutes, 27 cycles of 95˚C for 30 seconds, 60˚C for 45 seconds, 72˚C for 60 seconds, one cycle of 72˚C for 10 minutes.

Cbl b primers were run at 95˚C for 5 minutes, 27 cycles of 95˚C for 30 seconds, 59˚C for 30 seconds, 72˚C for 30 seconds, one cycle of 72˚C for 7 minutes.

GAPDH primers were run at 94˚C for 4 minutes, 27 cycles of 94˚C for 1 minute, 55˚C for 1 minute, 72˚C for 1 minute, one cycle of 72˚C for 10 minutes.

Then 4 µl of 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to samples before being run out on an agarose gel.

PRIMER	LOCATION	SEQUENCE (5'-3')
C-Cbl Fwd	Exon 2	TCAGCCTAGGCGAAACCTAA
C-Cbl Rev	Exon 9	TCCCTCTAGGATCAAACGGA
Cbl-b Fwd	Exon 10	CCGGTTAAGTTGCACTCGAT
Cbl-b Rev	Exon 13	CAAAGGGGTCCACGATTATG
GAPDH Fwd	Exon 4	ACCACAGTCCATGCCATCAC
GAPDH Rev	Exon 6	TCCACCACCCTGTTGCTGTA

Table 2.3 RT-PCR primer sequences

REAGENTS	25µl Reaction
10x PCR Buffer	2.5µl
$MgCl_2$ (mM)	1.5
dNTP Mix (mM)	40
Forward Primer (10µM)	0.5µl
Reverse Primer (10µM)	0.5µl
***Taq* Polymerase**	0.2µl
cDNA	1µl
H_20 up to a final volume of	25µl

Table 2.4 Composition of RT-PCR reaction

2.3.4 Agarose Gels

To prepare 1% (w/v) and 2.5% (w/v), 1g or 2.5g of agarose were weighed out and added to a 100ml conical flask. 100ml of 1xTAE buffer (50x TAE: 242g Tris Base, 100ml 0.5M EDTA, 57.1ml acetic glacial acid) was added and the agarose was dissolved in a microwave on half power. After sufficient cooling, ethidium bromide was added at a 1/50,000 dilution, and the gel poured into the mould. An 8-well comb was inserted into the gel and left to set. After setting the comb was removed and the gel was placed into a running tank, 1xTAE solution was poured over the gel to cover. Into the first well 5µl

of Hyperladder I (Bioline) was added. Subsequent wells were loaded with 20µl of sample. Gels were then run at 100V constant for 45 minutes.

2.3.5 Real Time RT-PCR (Taqman)-18s

As a control, levels of the housekeeping gene 18s were used to normalise the gene of interest. The cDNA was diluted to 1ng/µl and a total of 5ng used per reaction. Each reaction was seeded in duplicate in a total reaction volume of 20µl. The composition of the reaction mix is shown in table 3.4. All reactions were carried out in a 96-well optical reaction plates (Applied Biosystems) and sealed with an adhesive strip. The following cycling conditions were used; Stage 1: 50˚C for 2 minutes, Stage 2: 95˚C for 10 minutes, and then 40 cycles of Step 4: 95˚C for 15 seconds and Step 5: 60˚C for 1 minute. Samples were run using an ABI 7500 thermocycler (Applied Biosystems).

2.3.6 Real Time RT-PCR (Taqman)-c-Cbl

The cDNA samples were diluted to 1ng/ µl and a total of 5ng was used in each reaction. Each reaction was carried out in duplicate in a 96-well plate using a final reaction volume of 20µl, reaction components are summarised in Table 2.5. The c-Cbl primer/probes were obtained from Applied Biosytems (Hs00231981_m1) and contained a 5' FAM reporter dye and a 3' quencher TAMRA. Reactions were carried out in 96-well optical reaction plates using an ABI 7500 thermocycler (Applied Biosystems).

REAGENTS	20µl Reaction
Primer/Probe	1µl
2x Master Mix	10µl
cDNA	5ng
H_20	5µl

Table 2.5 Composition of RealTime RT-PCR reaction

2.4 Cell Culture Techniques

2.4.1 General

Cell lines were maintained in a 5% CO_2 incubator at 37˚C. A summary of cell lines used can be found in table 2.6.

CELL LINE	TYPE	SOURCE	MORPHOLOGY	GROWTH MODE
HEK-293	Human Embryonic Kidney	ECACC	Epithelial	Adherent
HEK293T	Human Embryonic Kidney	Dr P.Powell	Epithelial	Adherent
COS-1	African Green Monkey Kidney	ECACC	Fibroblast	Adherent
MYL	Human CML Blast Crisis	Dr A.Kimura	Myeloid	Suspension
MYL-R	Human CML Blast Crisis	Dr A.Kimura	Myeloid	Suspension
K562	Human CML Blast Crisis	Prof. J.Melo	Myeloid	Suspension

Table 2.6 Cell lines

2.4.2 MYL/MYL-R Cells

These cells lines were generated and kindly donated by Dr A. Kimura (Hiroshima University). Bone marrow mononuclear cells were obtained from a 24-year old female patient in blast crisis stage of CML. Cells were initially cultured with or without a low dosage of Imatinib, cells were selected through single-cell cloning, and two populations with the greatest differential sensitivity were selected. The most sensitive was named MYL and the most resistant MYL-R. Both cell lines contained double Philadelphia chromosomes.

MYL and MYL-R cells were cultured in T75cm^2 flasks at 37°C, 5% CO_2 incubator in RPMI 1640 (Invitrogen 31870) media supplemented with 10% Foetal Bovine Serum (Autogen Bioclear), 1% penicillin streptomycin (Invitrogen), glutamine (200mM). Cells were routinely sub-cultured by transferring to a falcon tube and spinning at 1200rpm for 5 minutes. The supernatant was aspirated and the cell pellet resuspended in 10mls fresh media. The cells were then transferred into a 75cm^2 culture flask at a dilution of 1/10.

2.4.3 COS-1 / HEK 293/293T Cells

COS-1 and HEK293 cells were grown in T75cm^2 flasks at 37°C, 5% CO_2 incubator in DMEM (Invitrogen 21885) supplemented with 10% FBS (Autogen Bioclear) and 1% p/s. In order to subculture cells, once they had reached 80-90% confluency, the media was aspirated and cells were washed in 2ml PBS to remove residual media, then 1.5ml trypsin was added and cells incubated for 5 minutes at 37°C. After the cells had

trypsinised, 8mls of fresh media was added. The cells were then spun down at 1200rpm for 5 minutes in a 50ml falcon. The supernatant was aspirated and the cell pellet resuspended in 10mls of media. Cells were then added to a fresh 75cm^2 culture flask at a dilution of 1/10.

In addition to FBS and p/s, HEK293T media was supplemented with 1% Non-essential amino acids (Invitrogen) and 1% sodium pyruvate (Invitrogen).

2.4.4 Cryopreservation of Cells

Cells were prepared for long term storage to maintain sufficient cell stocks. Adherent cells were trypsinised with 2ml of trypsin for removal from the culture flask before being resuspended in 10ml of fresh media to neutralise the trypsin. Both adherent and suspension cells were spun down at 1200rcf for 5 minutes before removal of the supernatant. The cell pellet would be washed in PBS and spun to pellet the cells again. Cell pellet was then re-suspended in 1ml of freezing media, containing FCS and 10% DMSO. One ml of cell suspension was transferred to a cryopreservation vial and put on dry ice before being stored at -80°C or moved to liquid nitrogen for long term storage.

2.4.5 Raising Cells from Frozen Stocks

Vials of cryo-preserved cells were removed from either liquid nitrogen or the -80°C freezer and thawed in a water bath at 37°C. The cell suspension was added drop wise to 10ml of fresh media. Cells were spun down at 1200rcf and the supernatant aspirated.

The pellet was then re-suspended in 10mls of fresh media. The cells were transferred to a T25cm^2 flask and incubated at 37°C, 5% CO_2 incubator overnight. The following day, the media of adhesion cells was aspirated, the cells were washed in 2mls of PBS to remove debris and fresh media was added. Suspension cells were counted and media added to maintain normal growing conditions.

2.4.6 Sub-Culturing cells

Both adherent and suspension cells were maintained in T75 flasks. Fresh growth media would be added every 2-3 days. Cells were routinely split 1/10 or 1/20 depending on confluence to maintain optimal growing conditions.

2.4.7 Cell Viability and Number

Viability and cell number were analysed by mixing cell suspension with Trypan blue at a ratio of 1:1 and assessed by cell counts under a light microscope on a haemocytometer.

2.4.8 Transfection of Cells

Adherent cells were transiently transfected using either FuGene 6 (Roche) or LipoD293 (SignaGen). Transfection reagent was mixed with a range of plasmid DNA concentrations at a ratio of 3:1 and added drop-wise to sub-confluent cells and left for 24 hours at 37˚C. Cell media was replaced with 2% FBS DMEM overnight. Cells were then stimulated accordingly, and then lysed.

2.4.9 Luciferase Assays

Cells were seeded in 6 well plates and grown overnight in normal media. After 24 hours cells were transfected (see 2.3.8) with luciferase reporter plasmids. Cells were stimulated accordingly and lysed in luciferase assay cell lysis buffer (Promega). Transcriptional activity was analysed by adding 50µl of luciferase reagent to10µl of lysate in a 96-well domino plate and reading luminescence using a Wallac Victor Luminometer at 595nm.

2.4.10 MTS Assays

Into 96 well plates, $5x10^3$ cells were seeded in a volume of 100µl per well and grown overnight in 0.5% FCS-containing media. Cells were stimulated accordingly and incubated at 37°C for up to 72 hours. 10µl of the MTS solution (Promega) was added to each well. Cells were further incubated for up to 3 hours before absorbance readings were taken at 490nm using a Wallac Victor Luminometer.

2.5 Protein Techniques

2.5.1 Protein Markers

Kaleidoscope pre-stained and All Blue (Biorad) markers were used to estimate protein size when separated by SDS PAGE gels. Molecular standards ranged from 6 to 210kDa and were stained each with a separate colour for ease of identification.

2.5.2 Preparation of Cell Lysates for Suspension Cells

Cells were transferred to a fresh vial and spun down. The cell pellet was washed in ice-cold PBS and re-spun. The cell pellet was lysed for 10 minutes on ice in 50µl RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with protease and phosphatase inhibitors, sodium fluoride, and sodium vanadate, added at 1/200 and 1/100 dilutions respectively. Lysates were spun down at 13,000 rpm, at 4°C for 10 minutes. Supernatant was transferred to a fresh vial, where 2x lammelli (200mM Tris pH6.8, 10% glycerol, 6% SDS, 0.02% bromophenol blue) with 10% 0.1M DTT was added at a ratio of 1:1 with lysate. Samples were then heated to 100°C for 3 minutes, either run on SDS-PAGE gels or frozen at -20°C.

2.5.3 Co-immunoprecipitations

Cells were washed in ice cold PBS before being lysed in 250µl 1% NP40 lysis buffer (Table 2.41) and were then placed on ice for 10 minutes. Samples were spun at 13k for 10minutes at 4°C. Supernatants were transferred to new vials and 50µl would be removed and stored as whole cell lysates. The remaining sample was incubated with primary antibodies for 1 hour at room temperature on a rocker.

Next 20µl of protein A/G beads (Santa Cruz) were added to each sample before incubating at 4°C on a rocker overnight. Samples were spun down at 2000 rpm and the supernatant aspirated. Samples were washed six times in 0.1% NP40 wash buffer (Table 2.7). After the final wash approximately 5µl of wash buffer remained, to this 2x lammelli plus 10% 0.1M DTT was added to give 25µl. Samples were then run on SDS-PAGE gels.

	Cell Lysis Buffer (50ml)	**Wash Buffer (50ml)**
Tris	50mM	50mM
NP40	1%	0.1%
Glycerol	10%	10%
Sodium Chloride	0.4g	0.4g
EDTA	1mM	1mM
Sodium Vanadate 0.1M	500µl	500µl
Sodium Fluoride 1M	50µl	50µl

Table 2.7 Components of NP40 Lysis and wash buffers

2.5.4 Nickel Affinity Purification

Cells were washed in ice cold PBS, then cell samples were lysed as above (see section 2.5.3) but without the addition of EDTA in either lysis or wash buffers. After whole cell lysate removal, 40µl of nickel coated beads (Invitrogen) were added to samples and incubated at 4˚C overnight on a shaker. Samples were then washed and treated as section 2.5.3.

2.5.5 SDS-PAGE

BioRad apparatus was set up according to manufacturers guidelines using 1.5mm gel plates. All samples were run out on 10% resolving gels. The resolving gel was made up, and 8ml pipetted between the gel plates, and 70% ethanol was added on top to ensure a flat edge. After polymerisation, the ethanol was poured off and ddH_2O was added and poured off to remove residual ethanol. The stacking gel was made and added to the top of the resolving gel and a 10 well comb was inserted to form the wells. Resolving and stacking gel composition is shown in table 2.8. Once the stacking gel had polymerised, combs were removed and the gels were transferred from the casting brackets into the gel tank which was filled with 1x running buffer, the components of which can be found in table 2.9.

To lane 1 of each gel, 5µl of protein marker (Biorad) was added. In subsequent wells cell samples were added in equal volumes. Gels were run between 140V-170V for approximately 1.5hrs, varying depending on the size of the protein of interest. Gels were then removed from the tank and proteins transferred via Western Blotting.

	10% Resolving Gel	6% Stacking Gel
ddH_2O	9.5ml	7.9ml
30% Acrylamide	8ml	3ml
1.5M Tris pH8.8/0.5M Tris pH6.8	6ml	3.75ml
10% SDS	240µl	150µl
10% APS	240µl	150µl
TEMED	24µl	15µl

Table 2.8 Components of stacking and resolving gels

1 Litre	Running Buffer	Transfer Buffer
Tris	3g	3g
Glycine	15g	15g
10% SDS	12ml	-
Methanol	-	200ml

Table 2.9 Running and Transfer buffer components

2.5.6 Western blotting

Proteins were transferred to a nitrocellulose membrane using wet blotting apparatus (BioRad). Prior to transfer four pieces of Whatman filter paper were cut to the same size as the gel, along with sponges from the blotting kit which were maintained briefly in transfer buffer, the composition of which can be found in table 3.43. The 0.45µM nitrocellulose transfer membrane (BioRad) was also cut to size and hydrated briefly in 1x transfer buffer before transferring. The wet blotting apparatus was subsequently assembled according to manufacturer's instructions, with particular care taken to remove any air bubbles between layers. Proteins were transferred at 110v for 75 mintutes at

4°C. Ponceau S staining was used to confirm even protein transfer among samples. The stain was removed by several washes with ddH_20.

Membranes were blocked in 3% marvel or BSA (depending on primary antibody to be used) in 1xPBS for 60 minutes at room temperature on a shaker. Antibodies were diluted in blocking solution (Table 2.10). Membranes were then incubated with the antibodies at 4°C overnight on a rocker. Following the overnight incubation, membranes were washed for 4 x 15 minutes in washing buffer (0.1% Tween in PBS) on a shaker at room temperature. A secondary antibody was added in a 3% blocking solution in a volume of 10mls and incubated for 90 minutes at room temperature. The secondary antibodies used were horse-radish peroxidise conjugate antibodies, and were either anti-mouse (Sigma) at 1:10,000, anti-rat (Roche) 1:10,000 or anti-rabbit (Sigma) 1:40,000. Membranes were washed again in washing buffer for a further 4 x 15 minutes.

Proteins of interest were visualised using chemiluminescence (ECL reagents: 68mM p-coumaric acid in 5ml DMSO, 1.25mM luminal in 50µl 0.1M Tris pH8.5, 1.5µl 30% hydrogen peroxide) with Amersham Hyperfilm (GE Healthcare), films were developed using standard solutions.

Prior to re-probing membranes were stripped in 0.1M sodium hydroxide for 15 minutes, washed briefly in washing buffer before blocking in marvel/BSA and then incubated with the primary antibody as described previously.

Antibody	Manufacturer	Species	Dilution
Lyn Kinase	Cell Signalling	Rabbit	1:1000
p-Lyn Kinase	Cell Signalling	Rabbit	1:1000
B-Actin	Sigma	Mouse	1:1000
HA	Roche	Rat	1:500
PARP	Abcam	Rabbit	1:400
c-Cbl	Cell Signalling	Rabbit	1:1000
Flag M2	Sigma	Mouse	1:500
HIS	GE Healthcare	Rabbit	1:1000
Smad3	Zymed Laboratories	Rabbit	1:500

Table 2.10 Antibodies used on nitrocellulose membranes

2.6 Cell Sorting

2.6.1 Cell Cycle Analysis

Cell cycle analysis was carried out using flow cytometry (Accuri C6). Cells were seeded at $5x10^5$/ml in 0.5% FBS containing media and left overnight. For cells undergoing a double thymidine (0.2mM) block, it was added at this point for 16 hours before being washed out and cells returned to normal media for 9 hours, and finally thymidine added again for a further 16 hours. After this point cells were returned to normal media in 1ml aliquots in a 24-well plate and treated accordingly.

Cells were transferred to a 15ml falcon tube, spun at 1200rpm for 5 minutes, resuspended in ice-cold PBS and re-spun at 1200rpm for 5 minutes. The supernatant was aspirated and cells were fixed by adding 5ml of 70% ice-cold ethanol drop-wise to the cell pellet whilst vortexing. Cells were left for 60 minutes on ice. Cells were then spun at 1000rpm for 5 minutes and the supernatant aspirated and the pellet resuspended in 1ml PBS to remove all traces of ethanol and transferred to a 1.5ml microcentrifuge tube. This was spun at 1000rpm, the supernatant was aspirated and the cell pellet was resuspended in a final volume of 500µl of PBS. At this stage the samples were filtered, 100µg of RNAse A was added and samples were incubated at 37˚C for 30 minutes. Propidium iodide (10µg) was then added to stain the cells followed by a 15 minute incubation at room temperature before analysis using FACS.

3. ANALYSIS OF TGFβ SIGNALLING IN CML

3.1 Introduction

CML is characterized by the presence of the Philadelphia chromosome (Ph^+) and the fusion protein Bcr-Abl. The evidence of the involvement and importance of the constitutively active Bcr-Abl tyrosine kinase in CML is extensive (Deininger *et al.*, 2000; Melo, 1996). Research by Møller *et al* in 2007 looked at progenitor populations and the effects of TGFβ within $CD34^+$ cells, following an early-disease stage focus. The reciprocal translocation is believed to occur in these early progenitor haematopoietic stem cells, and findings have indicated that these cells are resistant to Imatinib (Graham *et al.*, 2002).

The aim of this first chapter was to assess the role of TGFβ signalling in CML and haematopoietic cell growth. Initially, experiments focused on signalling in an over-expression system which allowed specific effects between TGFβ and Bcr-Abl to be studied. During the project, a human CML model became available, which enabled experiments to be carried out in a more relevant cell system, allowing TGFβ analysis within haematopoietic CML cells. It also allowed the development of a novel dual therapy approach to combat drug resistance.

The research presented here aimed to look at mature stage disease. Drug resistance has become more of a recent research focus after the emergence of a reduction in Imatinib efficacy during late stage CML in addition to the reported inherent resistance of the leukemic stem cell (Jin *et al.*, 2008). In particular, my project aimed to define and characterise interconnections between Bcr-Abl and the TGFβ signalling pathway. TGFβ is well known as a growth inhibitory cytokine in haematopoietic cells (Keller *et al.*,

1990), but can have opposing effects in different cells and surroundings, such as an involvement in integrin induction during T cell migration (Kang *et al.*, 2011). It is this environmental specificity which makes the understanding of TGFβ signalling an important mechanism to uncover within all biological contexts. TGFβ is believed to play a variety of roles within cancers, both inhibiting and in driving tumourigenesis. It is the deregulation of these finely balanced signals which often results in disease. TGFβ is well known to signal through Smad intracellular messenger proteins, but recently TGFβ has been shown to signal independently of Smads, through MAPK activation via TRAF6 in Smad2/3 knockdown cells (Yamashita *et al.*, 2008). This highlights a further level of signalling complexity within the normal cell, and identifies additional potential for aberrant signalling to cause disease.

Experimentally, TGFβ signalling activity is often monitored through the activation of its intracellular messengers the Smad proteins. The activation of TGFβ responsive genes showed direct interactions between Smads 3 and 4 to CAGA sequences within the PAI-1 promoter (Dennler *et al.*, 1998). These sequences are now used artificially within luciferase-based gene reporter plasmids to analyse TGFβ signalling activity.

It is necessary to consider how different stages of CML disease can affect the treatment outcome. Research has shown in 32D cells that levels of Bcr-Abl present in the cell during various disease stages can have a direct affect on treatment outcome (Barnes *et al.*, 2005). This suggests Bcr-Abl can continue to drive disease progression throughout the course of the disease, and that resistance to Imatinib may be a hallmark of all stages. In addition to the signalling aspect of the disease, Imatinib resistance has become a growing area of research, with the need for enhanced specificity and targeted drug development. There are a number of known factors contributing to resistant phenotypes;

increased Bcr-Abl protein, increases in genomic instability and mutations in the Bcr-Abl gene itself, such as the T315I amino acid substitution. Alongside this is the population of stem cells or leukemic initiating cells (LICs) which have also been shown to confer Imatinib resistance (Graham *et al.*, 2002). In addition, the discovery of cell niches allow populations of cells to respond differently to a similar signal depending on their differentiation status. TGFβ has been found to play a critical role in the hibernation of HSCs in the bone marrow through Smad2/3 activation (Yamazaki *et al.*, 2009). Studies have shown that FOXO3a can play conflicting roles in CML. In the Ph^+ stem cell, FOXO3a mediates protection to stresses such as tyrosine kinase inhibition, whereas in the non-stem CML cell FOXO3a activation induces apoptosis (Naka *et al.*, 2010b). Another recent development in the area of resistance was the findings of Ito *et al* in 2007, showing over-expression of Lyn kinase to be involved in drug resistance. A recent review has highlighted the main factors involved in CML drug resistance within stem cells and proposes a novel therapeutic approach (Naka *et al.*, 2010a).

To develop the understanding of the TGFβ involvement in this disease, a human, CML patient-derived cell line named MYL and the acquired Imatinib resistant MYL-R were used as a model to examine the role of TGFβ in blast phase CML.

Results presented in this chapter establish the Bcr-Abl mediated up-regulation of TGFβ signalling through $CAGA_{12}$-luc induction in HEK293 and COS-1 cell lines, both are known to be TGFβ-responsive. To assess the role of Bcr-Abl on cellular growth with TGFβ stimulation in the haematopoietic system, mouse 32D and 32Dp210 cells were analysed. Results suggest a Bcr-Abl driven increase in proliferation in response to TGFβ stimulation.

Experiments in human MYL and MYL-R cells indicated variation in sensitivity to the growth inhibitory effects of TGFβ, and it is speculated that this is due to the over-expression of the Src family kinase Lyn. In addition, cell cycle analysis was performed to address the effects of TGFβ on the cycling status of CML cells. Results indicate that TGFβ induces MYL cell cycle arrest and that abrogation of TGFβ signalling using a specific TGFβ receptor kinase inhibitor reverses these effects.

Based on these findings, combination therapy experiments were performed. TGFβ signalling was inhibited through the SB431542 compound in combination with Imatinib, resulting in an increase in Imatinib-mediated cell death, analysed through MTS and PARP cleavage experiments.

Overall it is observed that blast phase CML cells retain sensitivity to TGFβ, and the outcome of the combination therapy approach highlights the TGFβ signalling pathway as a potential therapeutic target for combating CML drug resistance.

3.2 Results

3.2.1 Bcr-Abl up-regulates transcriptional activity of the $CAGA_{12}$-luc Smad-responsive reporter in a dose-dependent manner

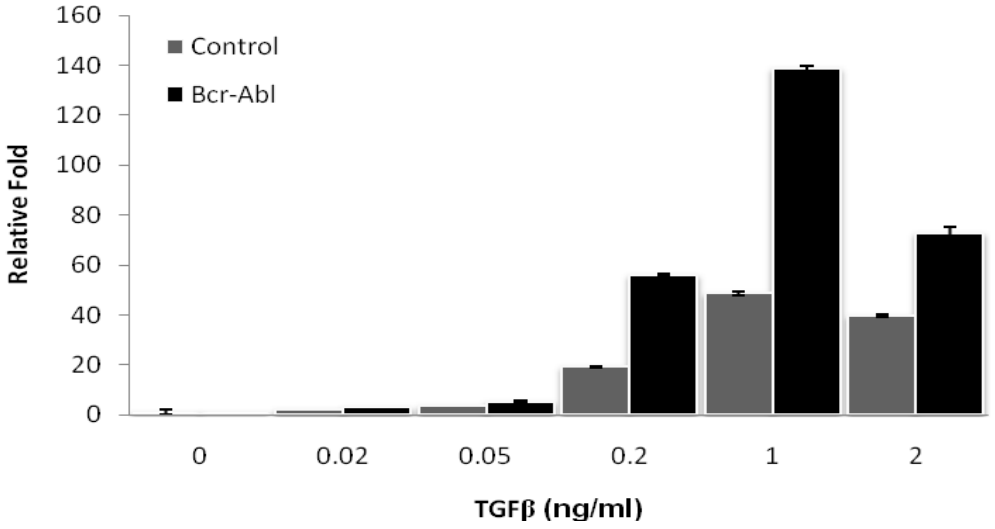
HEK293 cells were chosen to analyse the role of Bcr-Abl on the Smad3-dependent TGFβ signalling pathway. These cells originate from human epithelial kidney and have been widely used to study the effects of TGFβ signalling activity (Bhowmick *et al.*, 2003; Shukla *et al.*, 2009). The Smad3 responsive reporter plasmid $CAGA_{12}$-luc, together with a Bcr-Abl p210 plasmid were transiently co-transfected into cells. The transcriptional effects of the plasmid were monitored through measurements of luciferase activity. Cells were stimulated 48 hours post-transfection with a range of TGFβ concentrations (0-2ng/ml) for 16 hours. Results show that there is a clear TGFβ-mediated, concentration-dependent increase in Bcr-Abl transfected cells at concentrations between 0.02 and 1ng/ml (Figure 3.1a). This response appears to be reduced at 2ng/ml, possibly due to TGFβ saturation of the cells. Additionally, Bcr-Abl has minimal effect on the reporter plasmid alone.

To establish if this was a cell specific response, the experiment was repeated in the COS-1 cell line. These cells are of fibroblast origin and are well studied with respect to TGFβ responsiveness (Kretzschmar *et al.*, 1999; Zhang *et al.*, 2010). The same dose-dependent up-regulation can be seen in these cells, which does not diminish at 2ng/ml as in the HEK293 cells (Figure 3.1b).

The combined effect of Bcr-Abl and TGFβ on $CAGA_{12}$ transcriptional activity appears to be the same in both cell lines. It is apparent that there is a clear difference in

sensitivity to TGFβ between the two cell lines, with HEK293 cells giving a maximal response of 139-fold relative to the control, compared to the maximal 21-fold increase in COS-1. The variation in relative luciferase activity allows a greater degree of sensitivity to be used in future experiments. Further HEK293 experiments used 1ng/ml while for COS-1 experiments 2ng/ml was used. Data here indicate Bcr-Abl clearly up-regulating TGFβ-dependent transcriptional activity of the $CAGA_{12}$-luc reporter.

(a)



(b)

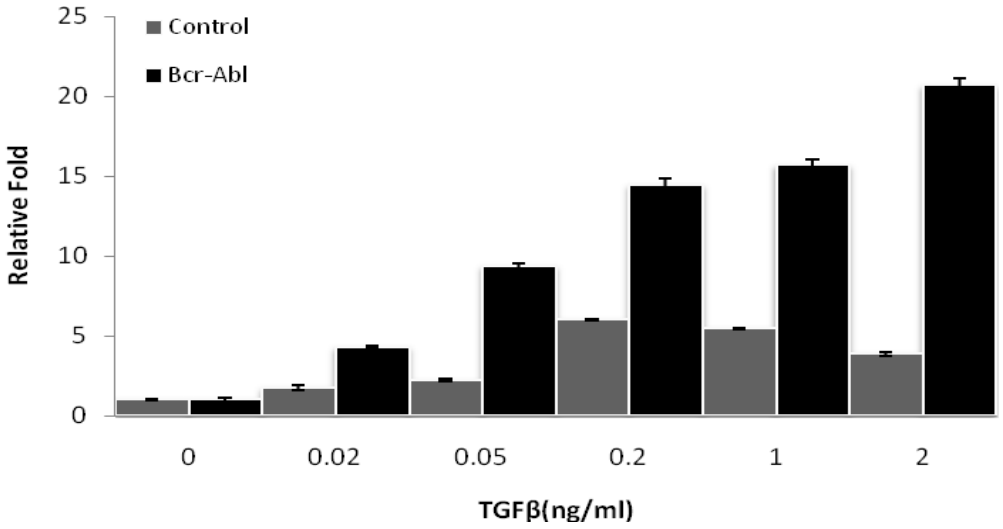


Figure 3.1 Bcr-Abl up-regulates TGFβ-dependent transcriptional activity of $CAGA_{12}$ in a dose dependent manner

Cells were co-transfected with a TGFβ-dependent Smad-responsive $CAGA_{12}$ luciferase reporter plasmid together with either the empty vector or the B3a2 Bcr-Abl variant expression plasmid. (a) HEK293 cells were stimulated for 16 hours at varying concentrations of TGFβ (0-2ng/ml). (b) COS-1 cells were stimulated for 16 hours at varying concentrations of TGFβ (0-2ng/ml). Results are analysed at 490nm on a Wallac Victor luminometer normalized to the control and standard errors calculated, based on at least three independent experiments.

3.2.2 Bcr-Abl abrogates TGFβ-induced haematopoietic cell growth arrest and enhances proliferation

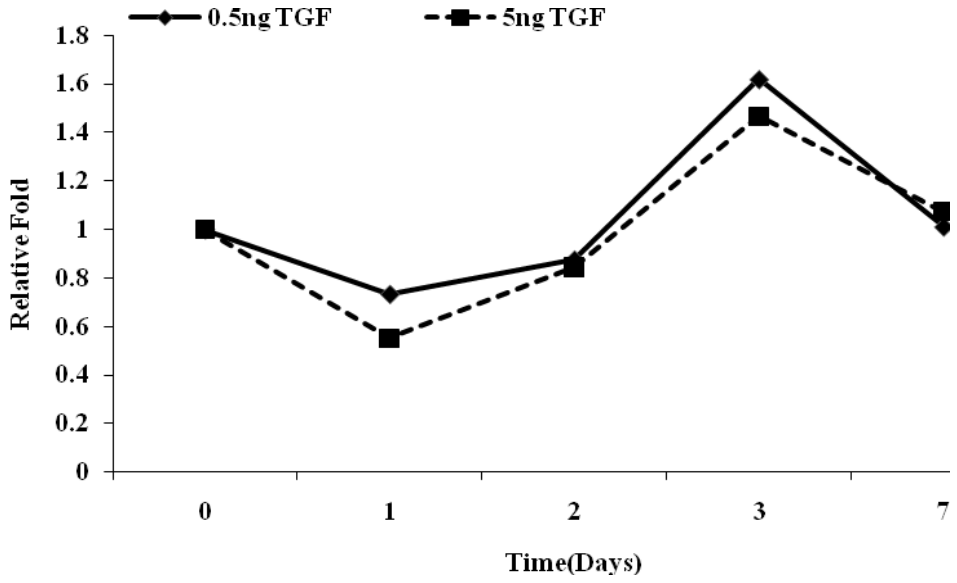
The aim of this section was to assess the proliferative effect of Bcr-Abl on cells stimulated with TGFβ in more physiologically relevant mouse haematopoietic 32D and 32Dp210 cell lines. The 32Dp210 cells are stably transfected with the p210 form of Bcr-Abl (Laneuville *et al.*, 1991). Cell proliferation was assessed daily using an MTT assay, and this experiment was run in parallel alongside a cell count assessment of growth, using trypan blue staining. The cell count method proved difficult, and culturing of cells under these conditions promoted cell clumping in the 32Dp210 cells, making accurate cell counts challenging.

To look at the effect Bcr-Abl has on cellular growth, 32D cells and the Bcr-Abl transformed 32Dp210 were treated with two different concentrations of TGFβ which was added at day 0, and no further additions or changes of media were made during the experiment. The change in TGFβ concentrations gave an insight into whether any effects seen were concentration dependent.

Results show that 32D cells are growth inhibited 24 hours post treatment so remain sensitive to TGFβ-mediated growth arrest (Figure 3.2a). This inhibition is increased with a higher concentration of TGFβ (0.5ng/ml) after 24 hours. 32D cells remain inhibited by TGFβ at 48 hours compared to the control, but at 72 hours there is a moderate increase in cell number. This is then followed by a decrease to basal levels at day 7, at which point other factors such as cell overcrowding and contact inhibition may be influencing the cellular response. The increase in cell number after 72 hours may be due

to TGFβ breakdown, removing the growth inhibitory effect observed during the first 48 hours of TGFβ stimulation. The Bcr-Abl p210 containing cells showed no growth inhibitory response to TGFβ stimulation (Figure 3.2b). After 24 hours there was no decrease in cell number compared to basal cell counts, unlike in the 32D cells. Increasing TGFβ concentration slightly enhanced proliferation after 48 hours. This is also seen after 72 hours of stimulation, where TGFβ has a proliferative affect on cell growth, suggesting it can drive cell growth in combination with Bcr-Abl. The 72 hour sample data again suggest a moderate rise in cell number, as seen in 32D cells. This may be due to the breakdown of TGFβ, however it is more likely that cells at 72 hours have reached maximal numbers, and are unable to sustain the growth, which explains the decline in cell numbers seen at day 7. The overall growth pattern in 32Dp210 cells is similar to that of 32D cells but the presence of Bcr-Abl appears to positively affect the growth responsiveness of these cells to TGFβ and prevent the TGFβ-mediated growth inhibition seen in 32D cells. Further investigation with an inducible Bcr-Abl protein alongside Bcr-Abl knockdown studies would develop this theory and allow a firmer conclusion to be reached on the affect of Bcr-Abl on the growth of these cells.

(a)



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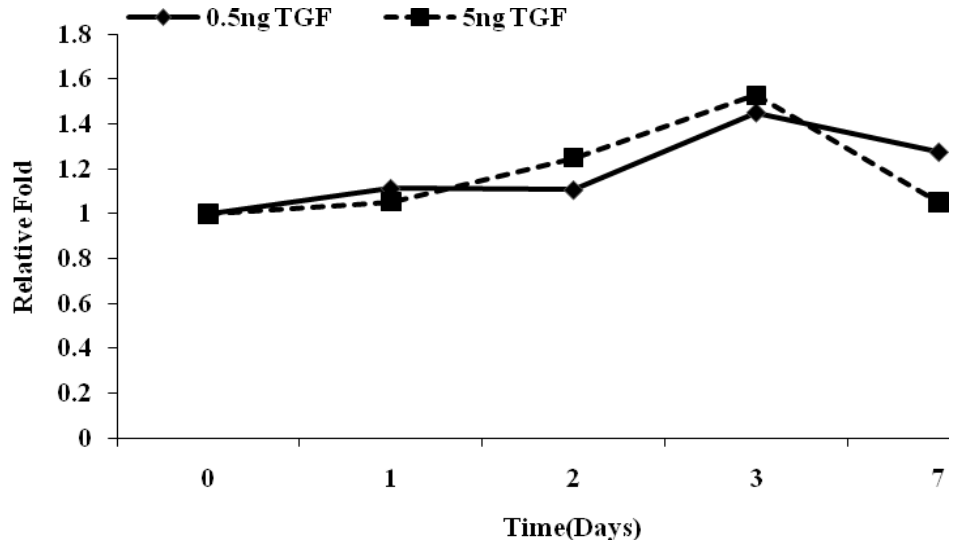


Figure 3.2 Bcr-Abl expression desensitises 32D cells to TGFβ-mediated inhibition

32D (a) and 32Dp210 (b) cells were seeded at an initial concentration of $2.5x10^5$/ml and maintained in that media throughout the experiment. TGFβ was added at day 0, at either 0.5 or 5ng/ml. Cell number was counted using a haemocytometer and cell viability assessed using trypan blue exclusion. Data presented is normalised to an untreated sample. Cells were treated in triplicate repeats, averages and standard errors were calculated.

3.2.3 MYL cells are more sensitive to Imatinib,than MYL-R cells

The emergence of Imatinib resistance with the inability to cure overall disease has led to intense research to discover the mechanisms contributing to this. A human derived CML patient cell line was used to generate resistant and sensitive cell lines. MYL cells were induced to develop resistance to Imatinib by means of cells maintained in media containing Imatinib. Successfully resistant cells were selected and cultured with increasing concentrations of the drug, leading to the generation of the Imatinib resistant MYL-R cell line. Resistant cells were negative for Bcr-Abl mutations and shown to over-express Lyn kinase (Ito *et al.*, 2007).

Initial experiments were designed to characterise the cells and establish a system to study the effects of TGFβ in CML. To test the differences in Imatinib sensitivity MTS assays were performed. Cells were seeded in 0.5% serum overnight, before 48 hours of Imatinib treatment at concentrations ranging from 0μM to 10μM. MTS solution was added and cell survival analysed through absorbance readings at 490nm.

Up to 0.1μM Imatinib the survival of both cell lines remain largely unaffected. At 0.3μM a threshold is found and MYL cell survival dropped to just over 10% (Figure 3.3). The MYL-R cells are still able to show a survival rate of 80%. MYL cells reach basal levels at 0.5μM while MYL-R cells are still at a 60% survival rate. The effect of Imatinib on MYL-R is dose dependent, with increases in concentration resulting in a fall in cell survival. These results confirm that MYL-R cells do indeed display greater resistance to Imatinib than MYL cells.

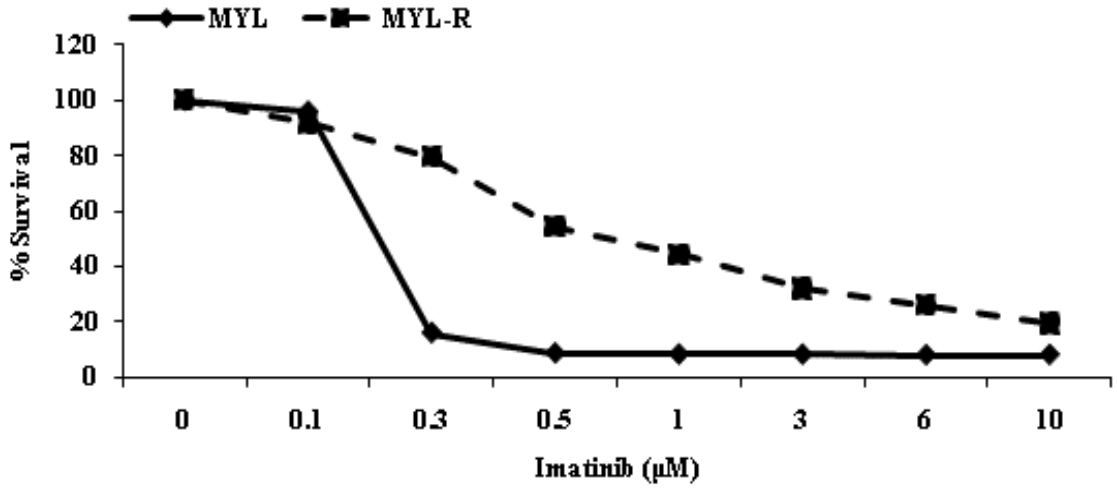


Figure 3.3 MYL-R cells are more resistant to Imatinib than MYL

MYL and MYL-R cells were seeded in 0.5% serum overnight. Imatinib was added at a range of concentrations between 0µM and 10µM for 48 hours. Cell survival was then analysed by adding MTS solution and measuring absorbance values at 490nm. Treatments were carried out in triplicate repeats, averages and standard errors calculated. Data shown is representative of at least three independent experiments.

3.2.4 MYL and MYL-R cells have increased sensitivity to TGFβ-mediated growth inhibition in low serum media

In order to investigate further the data obtained in adherent cell lines HEK293 and COS-1, together with the murine haematopoietic 32D and 32Dp210 lineages, the more biologically relevant MYL and MYL-R cell lines were used in subsequent experiments. Assesment of response to treatments was analysed with two different serum levels, either 0.5% or 10%, to give an indication of whether intrinsic growth factors in the serum were masking the effects of treatment. It would also establish a standard set of experimental conditions which would allow optimal and directly comparable data collection.

Lowering serum levels enhanced the sensitivity to the inhibitory TGFβ response, significantly so in the MYL cells (Figure 3.4a) These experiments also used the TGFβRI inihbitor SB431542 which in the MYL cells can be seen to slightly inhibit the TGFβ-mediated inhibtion at 0.5% serum levels, and at 10% serum, SB431542 is able to slightly increase proliferation levels. Furthermore, MYL-R cells appear to become sensitive to TGFβ in low serum levels which is not seen at 10% levels, the inhibitory response is less pronounced than in MYL cells. Also, the addition of SB431542 in 0.5% serum to MYL-R cells is able to show an increase in proliferation compared to the control, and this is greater than that seen in 10% samples (Figure 3.4b). Although the range seen here is not vast, and values are still in the range of those seen in previous figures of TGFβ affecting cell growth and survival, the fact that a difference is apparent suggests residual cytokines found in the serum may be affecting response to stimulation.

In addition, MYL-R cells have an increased basal growth rate compared to MYL cells, therefore a reduction in serum levels would predicatably slow down this growth and potentially re-establish TGFβ sensitivity.

As mentioned previously, the difference in range especially for MYL-R cells is within 10% so there is not a hugely significant point to draw from this other than experimental conditions are important to optimise and maintain throughout a set of experiments. From these data the conditions to be used for future experiments will be low serum at 0.5% in cell media.

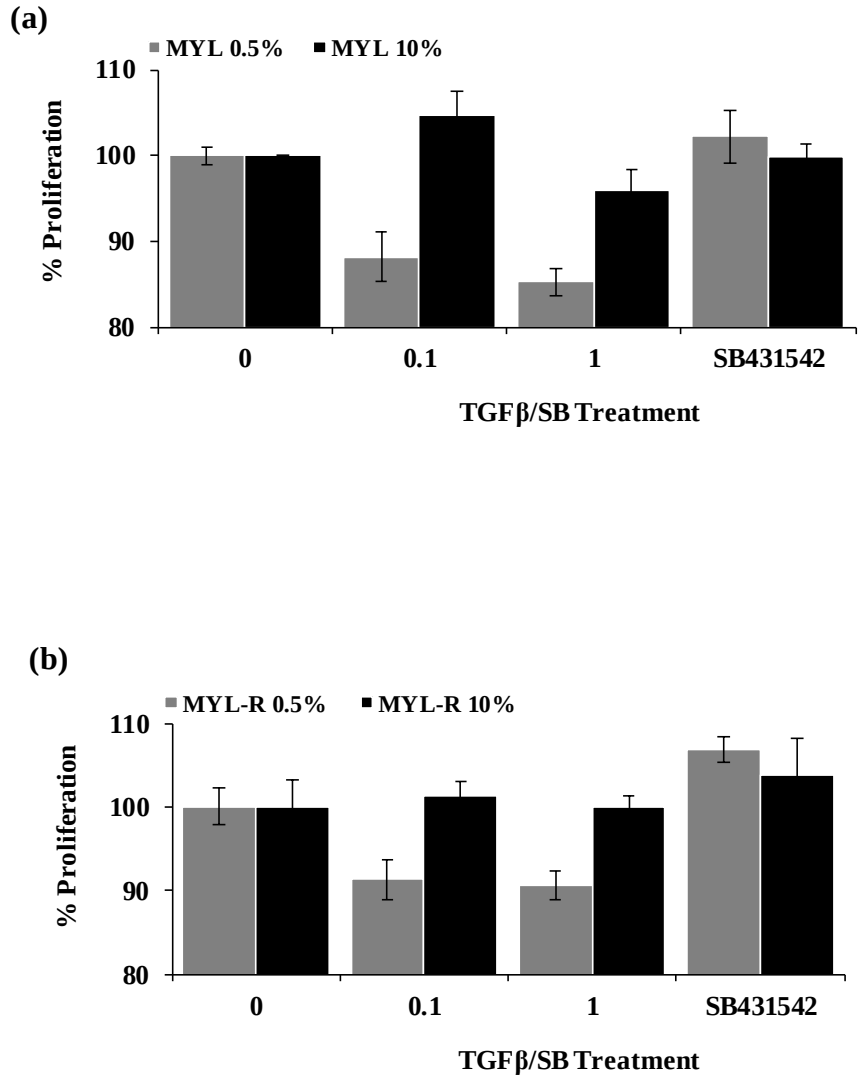


Figure 3.4 Lowering serum levels affects the sensitivity of MYL and MYL-R cells to TGFβ

MYL (a) and MYL-R (b) cells were seeded in 96-well plates at $5x10^3$ cells/well with either 0.5 or 10% FCS-containing media. SB431542 (10μM) or TGFβ (0.1 or 1ng/ml) was added to cells for 48 hours. Treatments were performed in triplicate. Data was normalised to an untreated control and standard error calculated.

After establishing an optimal set of conditions for the serum level in the experiments, the MTS protocol was then optimised and the time points of TGFβ stimulation were varied to analyse effects on cell proliferation.

MYL and MYL-R cell lines were grown overnight in 0.5% foetal calf serum containing media, and TGFβ was added at varying concentrations. The cells were seeded in a 96-well plate at $5x10^3$ per well, and treatments were carried out in triplicate repeats. At this stage the MTS assay kit was untested with regards to the optimal time to obtain absorption values. Therefore, two different time points were chosen in consultation with the manufacturers guidelines, at two and four hours after addition of the MTS solution. Results show no significant differences between the two and four hour readings, so all future readings were analysed at two hours after the addition of the MTS solution (Figure 3.5).

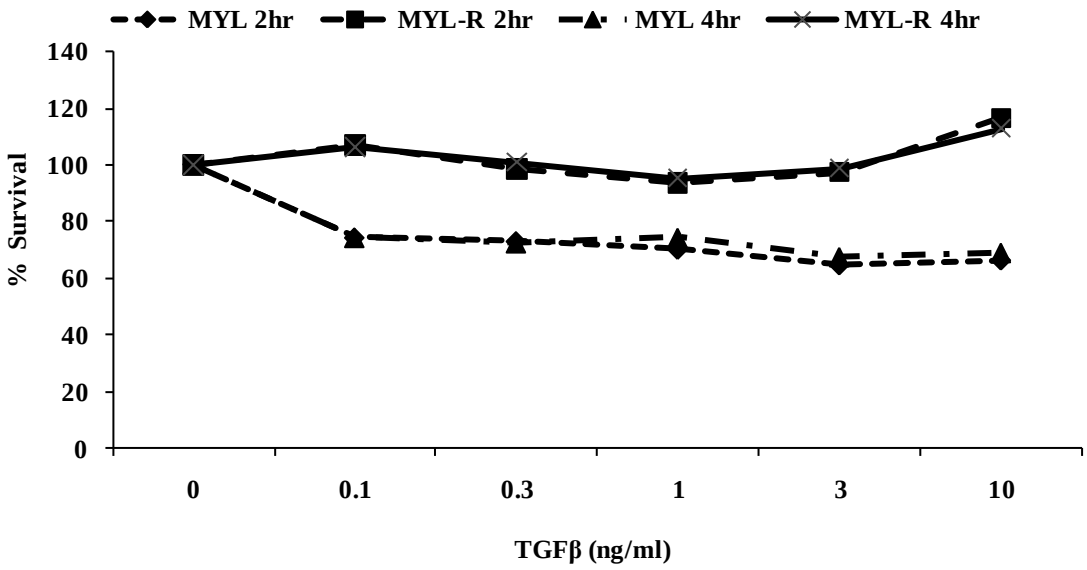


Figure 3.5 MYL and MYL-R cells show different sensitivities to TGFβ, no significant difference observed between 2 and 4 hour readings post-MTS addition

MYL and MYL-R cells were serum starved in 0.5% FCS containing media overnight. TGFβ was added at a range of concentrations between 0-10ng/ml for 16 hours to analyse the effects on cellular proliferation. MTS solution was added and cells were incubated for 2 or 4 hours and absorbance read at 490nm on a Wallac Victor luminometer. Cells were treated in triplicate repeats, averages and standard errors calculated.

3.2.5 The effects of TGFβ stimulation peaks after 48 hours in CML cells

To further characterise MYL and MYL-R cell lines, their response to TGFβ was analysed over a longer treatment period than the initial luciferase experiments. Despite the initial evidence of MYL-R unresponsiveness to TGFβ, the effects had not been monitored over longer time points so these experiments were designed to assess if there were in fact time-dependent responses.

Results show that TGFβ inhibits MYL cell survival but has minimal effect on MYL-R, which confirmed the previous findings. To assess cell survival, TGFβ was added for 24, 48 and 72 hour time points. MTS assay solution was added to each well and absorbance read at 490nm. After 24 hours the difference in response to treatment between the cell lines is seen at the lowest concentration of 0.1ng/ml (Figure 3.6 a). At this time point, increasing TGFβ concentration does not have any enhanced effects on inhibiting growth in the MYL cells, while no effect is seen throughout the treatment course in the MYL-R cells. These observed effects are more clearly demonstrated at 48 hours where at a concentration of 1ng/ml the difference in survival is over 30% between the two cell lines, showing MYL cells are growth inhibited by TGFβ and the MYL-R cells remain unaffected. The inhibition in MYL cells remains at 72 hours but is less well defined, no inhibition is seen at 0.1ng/ml and it is not until a concentration of 0.3ng/ml TGFβ that any inhibition can be seen. For this reason, future readings will be taken after 48 hours. The MYL-R cells show no significant changes in response to TGFβ throughtout the 72 hour treatment period. The largest difference is observed after 72 hours at 0.3ng/ml where TGFβ enhances proliferation.

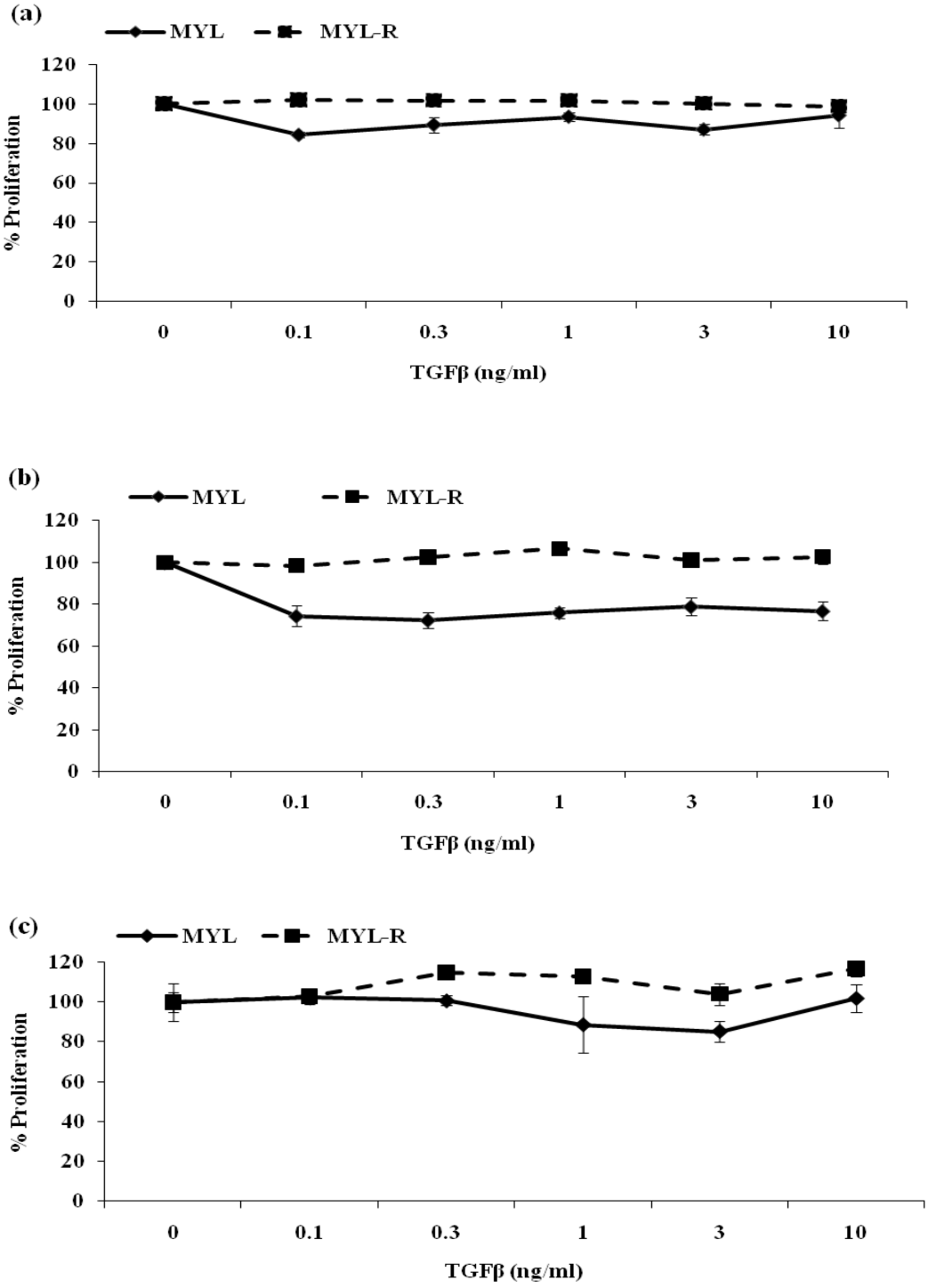


Figure 3.6 TGFβ-mediated effect is seen to be greatest between the MYL and MYL-R cells after 48 hours of treatment

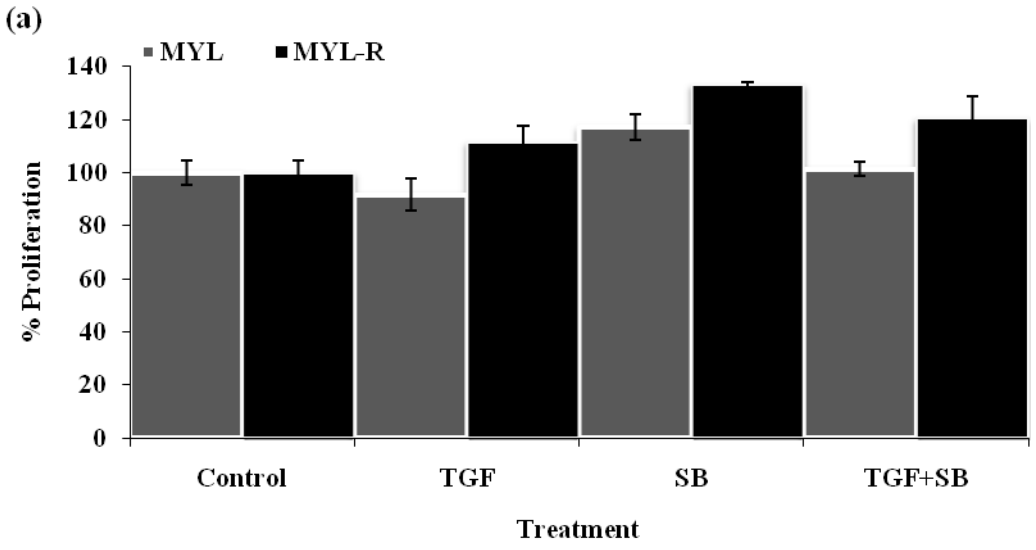
MYL and MYL-R cells were seeded at $2.5x10^5$/ml and serum starved in 0.5% serum containing media overnight. TGFβ was added at a range of concentrations between 0-10ng/ml for 24 hours (a) 48 hours (b) and 72 hours (c) to analyse the effects on cell proliferation. MTS solution was added for 2 hours and absorbance read at 490nm. Cells were treated in triplicate repeats, averages and standard errors calculated.

3.2.6 Abrogation of the TGFβ signalling pathway reverses CML growth inhibition

After establishing a TGFβ effect in MYL cells the type I TGFβ receptor kinase inhibitor was added to analyse if the observed effects could be reversed or inhibited. Again, time points of 24, 48 and 72 hours were chosen to keep in line with previous results. TGFβ inhibited growth in MYL cells as previously observed, but actually increased proliferation in MYL-R cells (Figure 3.7a). Treatment of SB431542 was able to increase survival in MYL cells, reversing the effects of TGFβ. Interestingly, SB431542 also enhanced survival in MYL-R cells to a greater extent than TGFβ addition alone. This would suggest the TGFβ pathway is still responsive in these cells. SB431542 was able to cause the same effect in MYL-R cells in combination with TGFβ but with a slightly diminished response. This could suggest that TGFβ may in part be by-passing the type I receptor to mediate effects seen here, or that additional TGFβ has no effect on MYL-R cells. It is possible that the MYL-R cells have adapted themselves to use TGFβ as a growth stimulus, yet in the absence of stimulation the default mechanism results and cells again are able to proliferate.

To compare these effects to another human-derived CML cell line, K562 cells were analysed alongside MYL and MYL-R cells (Fgure 3.7b) TGFβ is able to inhibit MYL and K562 cells which is reversed by SB431542 addition. K562 cells were more sensitive to TGFβ-mediated growth arrest with a 30% reduction in survival compared with 15% in MYL cells. Combining TGFβ and SB431542 is able to prevent inhibition in K562 cells but shows little effect in MYL which supports the previous data. MYL-R

cells show a TGFβ-mediated increase in proliferation which is not altered by SB431542 or with the combination treatments. K562 cells have a similar TGFβ response to MYL cells, which correlates with a mutual sensitivity to Imatinib.



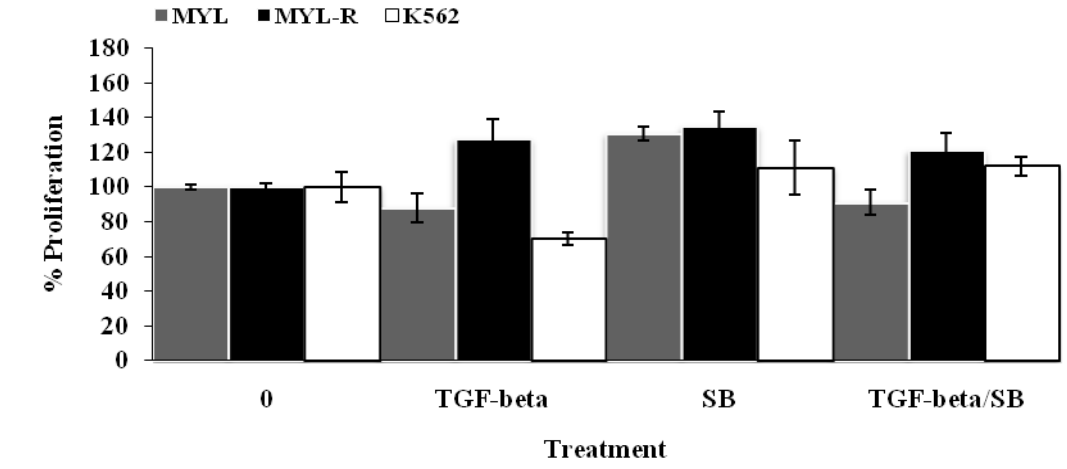


Figure 3.7 SB431542 potentiates growth in the absence of TGFβ, this is not observed in combination with TGFβ

(a) MYL and MYL-R cells were serum starved overnight in 0.5% serum-containing media before being treated in 96-well plates with either 1ng/ml TGFβ, 10μM SB431542 or a combination of the two .
(b) K562 cells were treated the same in an independent experiment.

3.2.7 MYL-R cells have enhanced SMAD3 Activation in response to TGFβ stimulation

In order to dissect the signalling pathway within these cells further, a $CAGA_{12}$ luciferase reporter construct was made which would allow TGFβ/Smad-dependent signalling to be monitored through establishing a stably transfected population of cells. The pGL3b $CAGA_{12}$-luc plasmid was modified to contain the neomycin resistance gene to allow for antibiotic selection of successfully transfected clones.

The cloned plasmid was tested by transfecting into COS-1 cells and being treated +/- TGFβ to check the Smad reporter region was still functional (Figure 3.8). MYL and MYL-R cells were transfected using Amaxa Nucleofecter protocol T016. The aim was to create stable cell lines expressing the plasmid and positive selection would be made by adding G418 to the media.

Unfortunately, cell clones were not able to be selected. It was thought this may be due to a lack of viable cells after electroporation and antibiotic selection, so an additional media was prepared which had been previously optimised to promote cell growth and viability among limited cell populations. Again this proved unsuccessful.

Instead of monitoring Smad3 activity using a luciferase reporter in these cells, endogenous Smad3 activation was looked at to establish if there were differences between sensitive and resistant cell lines, which may be underlying the variation in response to TGFβ and Imatinib treatments.

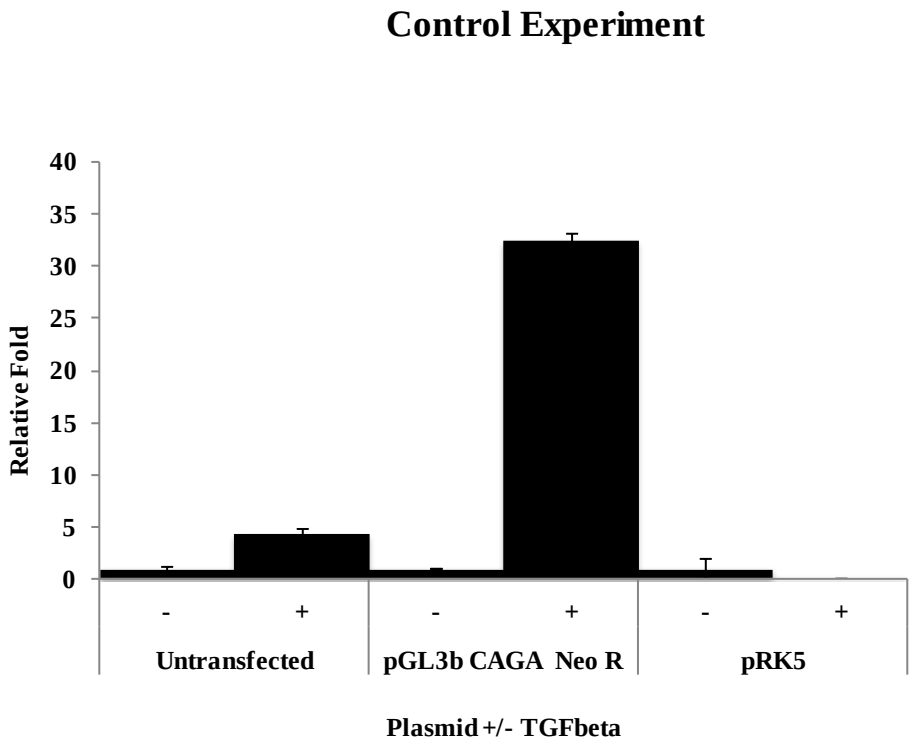


Figure 3.8 Characterisation of TGFβ luciferase reporter containing the Neomycin resistance gene

COS-1 cells were transiently transfected with the cloned vector containing $CAGA_{12}$ luciferase reporter with the neomycin resistance gene, pRK5 or un-transfected. Cells were then stimulated for 16 hours with TGFβ or an unstimulated control. Cells were lysed and analysed through luciferase activity. Treatments were carried out in triplicate repeats and averages and standard errors calculated Data presented is representative of at least three independent experiments

Cells were seeded in 0.5% FCS containing media and left overnight. A TGFβ time course was set up to look at activation of Smad3. Cells were stimulated for time points between 0-6 hours with 1ng/ml TGFβ. SB431542 treatment was added to establish if it effectively blocked the signalling pathway. Imatinib (0.5μM) and Dasatinib (0.5μM) were used to look at their effects on Smad3 activation after 60 mintutes of stimulation.

Results show that TGFβ is able to induce Smad3 phosphorylation within 10 minutes of treatment in both MYL and MYL-R cells (Figure 3.9a). Smad3 phosphorylation appears to be greater in MYL-R cells and is sustained throughtout the TGFβ treatment periods and, although MYL cells have phosphorylated Smad3 after 60 mintues, it is very much diminshed and is only just apparent above unstimulated levels. SB431542 is able to inhibit TGFβ-mediated Smad3 phosphorylation in both cell lines, and this supports the MTS data which suggests both cell lines can respond to inhibition of TGFβ signalling. Total Smad3 levels were assessed under the same experimental conditions, and no change in levels were observed in either cell line therefore increases in activated Smad3 is not due to increases in overall Smad protein. To quantify the Western blot analysis, densitometry software was used to assess changes in Smad3 activation, taking into account variation in β-actin levels (Figure 3.9b). As the blots indicated, MYL-R cells have a more sustained and intense TGFβ response, but both cell lines show the same pattern of activation over the time course, peaking at 60 minutes of stimulation. Interestingly, Dasatinib is also able to block Smad3 activation in both cell lines.

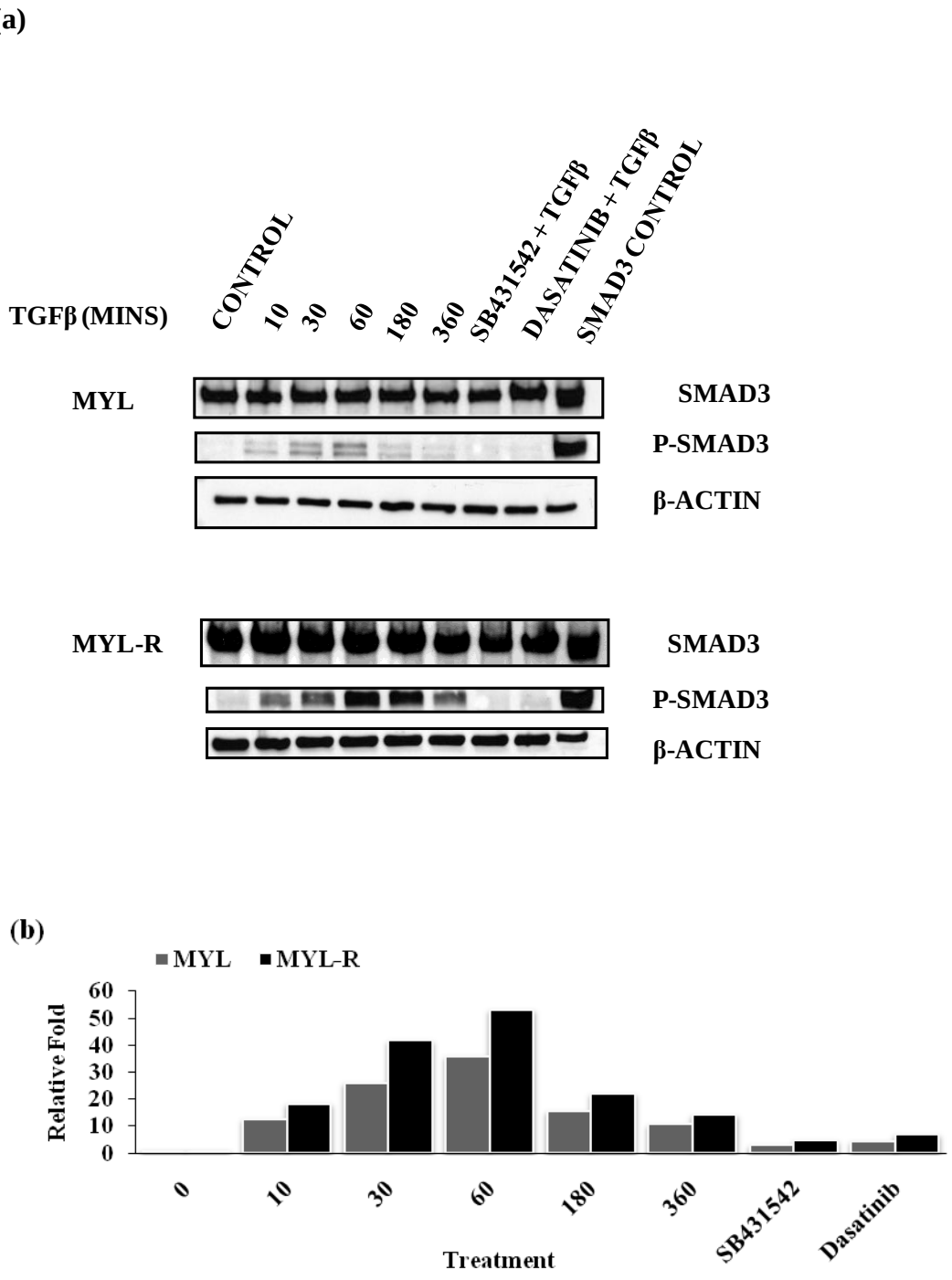


Figure 3.9 TGFβ induces Smad3 phosphorylation and is inhibited by SB431542

MYL and MYL-R cells were serum starved overnight in 0.5% serum containing media. Cells were seeded into 24-well plates at $5.0x10^5$/ml. (a)Cells were treated with 1ng/ml TGFβ for a range of time points 0-360 mins, SB431542 and Dasatinib treatments were also looked at, a Smad3 expression plasmid was transfected into HEK293 as a positive control. (b) Densitometry analysis of MYL and MYL-R western blots using ImageJ analysis

In addition to a TGFβ time course, Smad3 phosphorylation was investigated in combination with Bcr-Abl inhibitors with endogenous and exogenous addition of TGFβ. Cells were treated for 60 minutes with TGFβ in combination with SB431542, Imatinib or Dasatinib. This time point was chosen as the previous data highlighted it to be sufficient to induce maximal Smad3 phosphorylation.

Results show that Imatinib has no effect on TGFβ-mediated Smad3 phosphorylation in MYL cells, and Dasatinib reduces Smad3 activation 2-fold. This is observed in the MYL-R cells where Imatinib does not inhibit TGFβ-induced Smad3 activation but Dasatinib is able to cause inhibition (Figure 3.10b). Densitometry software was used on the Western blots to analyse the observed differences in response to these treatments more accurately, by comparing each treatment to its untreated control. Results are shown as relative fold increase using the unstimulated sample from each treatment pair. In the MYL cells, control samples show a nearly 5-fold increase in smad3 activation, SB431542 is able to completely inhibit this activation in these cells. Interstingly Imatinib is shown here to increase Smad3 activation and Dasatinib is able to reduce the activation by around 40% (Figure 3.10b).

MYL-R cells here show a much lower activation than previously observed, with TGFβ only inducing a 2-fold increase in active Smad3. SB431542 is not able to fully inhibit TGFβ activation in these cells, unlike the MYL cells. Imatinib has no affect on Smad3 activation, while Dasatinib is able to reduce activation slightly compared to the stimulated control (Figure 3.10c).

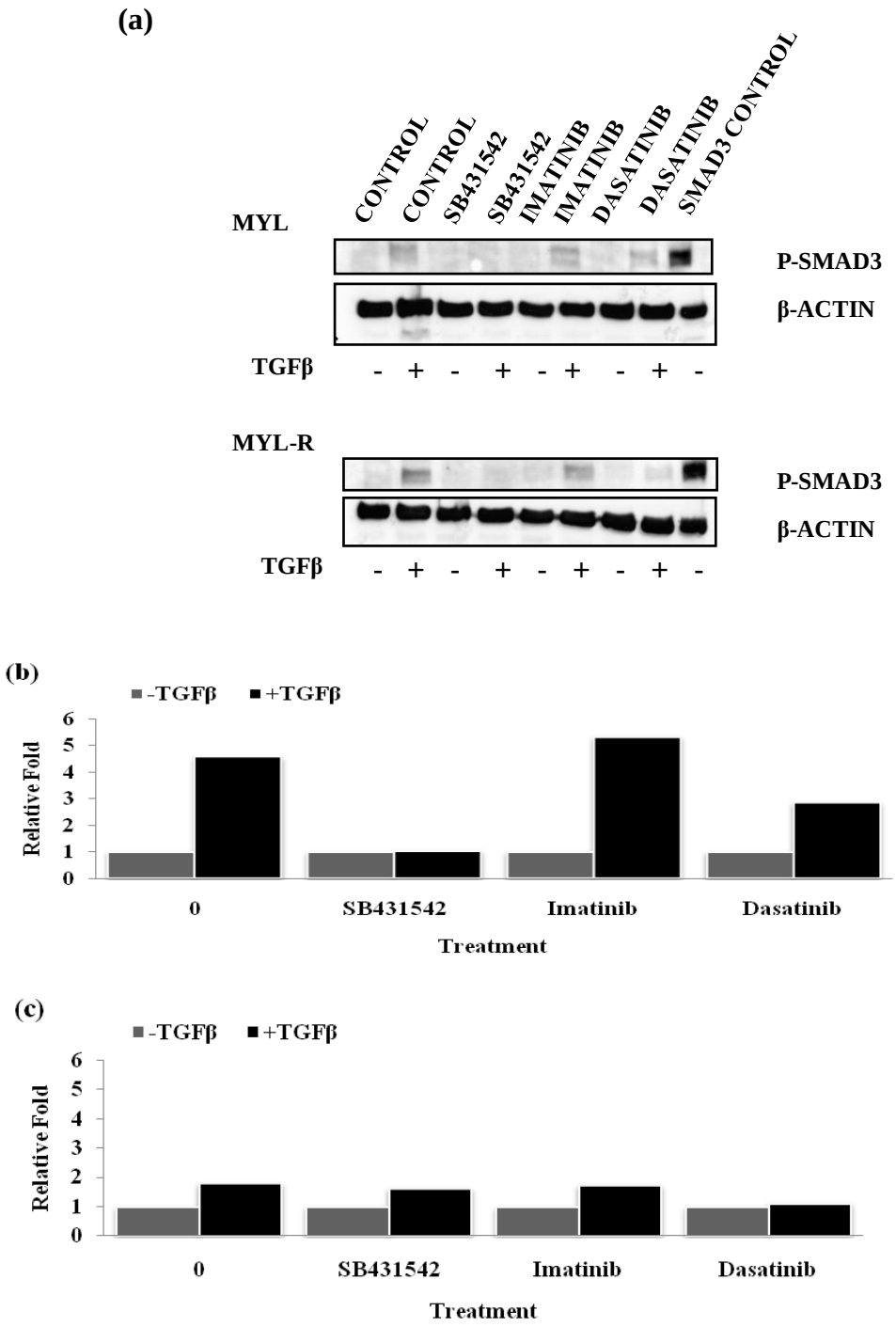


Figure 3.10 TGFβ induces Smad3 phosphorylation and is inhibited by SB431542

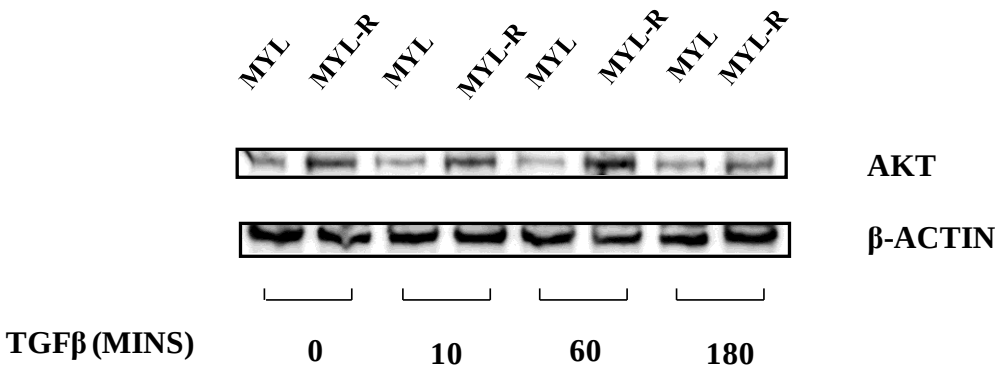
MYL and MYL-R cells were serum starved overnight in 0.5% serum containing media. Cells were seeded into 24-well plates at $5.0x10^5$/ml. (a)Cells were treated with or without 1ng/ml TGFβ for 60 minutes, in combination with SB431542, Imatinib or Dasatinib treatments. Lysates were separated through SDS-PAGE, subsequent Western blots were probed with anti-phosphorylated Smad3 antibodies, anti- β-Actin antibodies were used to check for equal protein loading. (b) Densitometery analysis of MYL phosphorylated Smad3 Western blot using ImageJ software (c) Densitometry analysis of MYL-R phosphorylated Smad3 Western blot using ImageJ software

Atfi *et al* (2005) suggest that Bcr-Abl activates Akt and suppresses TGFβ-mediated growth-inhibitory signals. A recent report suggests that the Bcr-Abl-mediated up-regulation of TGFβ signalling has been implicated in the mobilsation of CML cells into the blood through Akt/MMP-9-associated release of membrane-bound cytokines m-KitL and m-ICAM-1 (Zhu *et al.*, 2011). Also, Akt has been shown to sequester Smad3 from the nucleus (Conery *et al.*, 2004). Interestingly, Akt has been shown to regulate TGFβ signalling, via destabilsation of Ski, a negative regulator of TGFβ signalling through Smad7 (Band *et al.*, 2009).

Therefore it was decided to investigate Akt levels in MYL and MYL-R cells and analyse the effect of TGFβ on Akt over a time course.

Results show increased Akt levels in MYL-R cells compared to MYL (Figure 3.11a). After 60 minutes of treatment, Akt levels have increased in the MYL-R cells, MYL Akt levels remain constant, but have a slight increase after 180 minutes of TGFβ treatment, by which stage MYL-R levels have peaked and are in decline. The Akt increase in MYL-R cells mirrors that of Smad3 activation., which suggests the two may be linked, especially with the evidence of Akt/Smad3 associations. Densitometry analysis of the relative Akt levels from the Western blot confirms MYL-R cells to have over a 2-fold increase compared to MYL cells (Figure 3.11b).

(a)



(b)

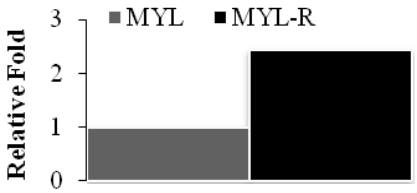


Figure 3.11 Different Akt levels in MYL and MYL-R cells

MYL and MYL-R cells were serum starved overnight in 0.5% serum containing media. Cells were seeded into 24-well plates at $5.0x10^5$/ml. (a) Cells were TGFβ treated for time points between 0-180 minutes. Lysates were separated through SDS-PAGE, subsequent Western blots were probed with anti-Akt antibodies. Anti-β-actin antibodies were used to ensure equal protein loading. (b) A comparison of Akt protein levels between MYL and MYL-R cell lines treated for 60 minutes, corrected for loading variability, analysed using Image J software.

3.2.8 Inhibiting TGFβ Signalling in Combination with Imatinib Enhances Cell Death in MYL cells

One of the theories of CML persistence is the survival of a residual population of quiescent cells which remain insensitive to the Imatinib target cell population (Copland *et al.*, 2006; Graham *et al.*, 2002). Data presented earlier in this chapter show TGFβ to have an anti-proliferative response in MYL cells, and no affect on MYL-R cells.

Experiments undertaken here aim to look at whether TGFβ may be playing a roll in Imatinib resistance. MYL and MYL-R cells were seeded in 96-well plates and treated with Imatinib at a range of concentrations between 0-0.5μM, and in combination with the TGFβ receptor I inhibitor SB431542 (10μM) to see if inhibiting this signalling pathway would have any effect on Imatinib-mediated cell death.

Results suggest that MYL-R cells are unresponsive to the control treatments of TGFβ and SB431542, and go on to show no difference in survival rates between Imatinib and Imatinib with SB431542 treatments over the entire range of Imatinib concentrations (Figure 3.12a). MYL cells show an enhanced response to inhibition of the TGFβ signalling pathway compared to the control. The combination treatments show an enhanced Imatinib-mediated cell death response at 0.03μM, where there is over a 10% reduction in cell survival. An increase in Imatinib efficiency is seen in concentrations of 0.01μM up to 0.1μM, and thereafter there appears to be no difference in cell survival compared to Imatinib treatment alone (Figure 3.12b).

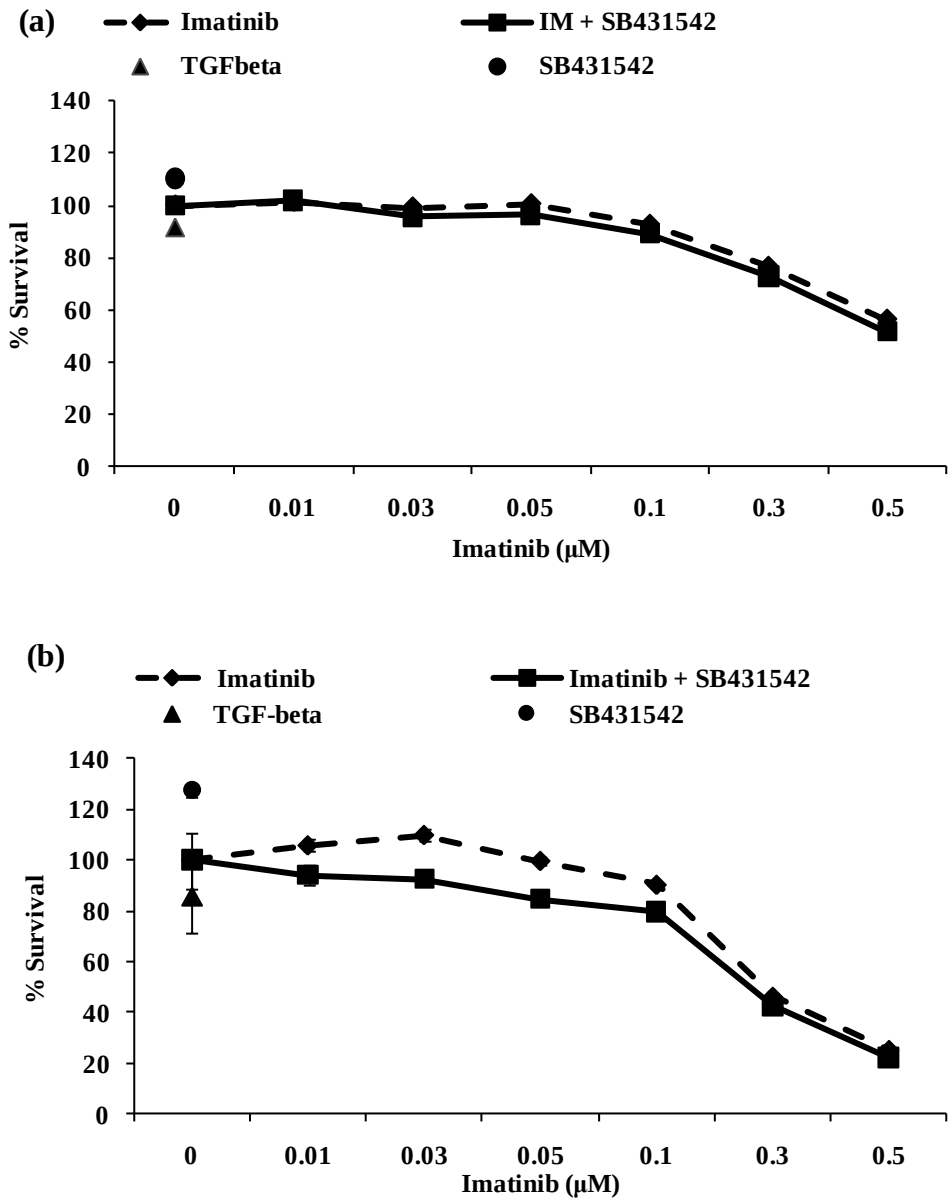


Figure 3.12 Combination treatment of Imatinib and SB431542enhance Imatinib-mediated cell death in MYL but not MYL-R cells

Cells were serum starved in 0.5% serum containing media overnight. Cells were seeded into 96-well plates and treated with either a range of Imatinib concentrations between 0-0.5µM or in combination with a TGFβ receptor inhibitor SB431542. TGFβ and SB431542 controls were also carried out. Cells were treated for 48 hours before addition of MTS solution and incubated for a further 2 hours before analysing absorbance at 490nm. (a) MYL-R cells (b) MYL cells. Cells were seeded in triplicate repeats, averages and standard error were calculated.

3.2.9 TGFβ signalling increases CML cell quiescence

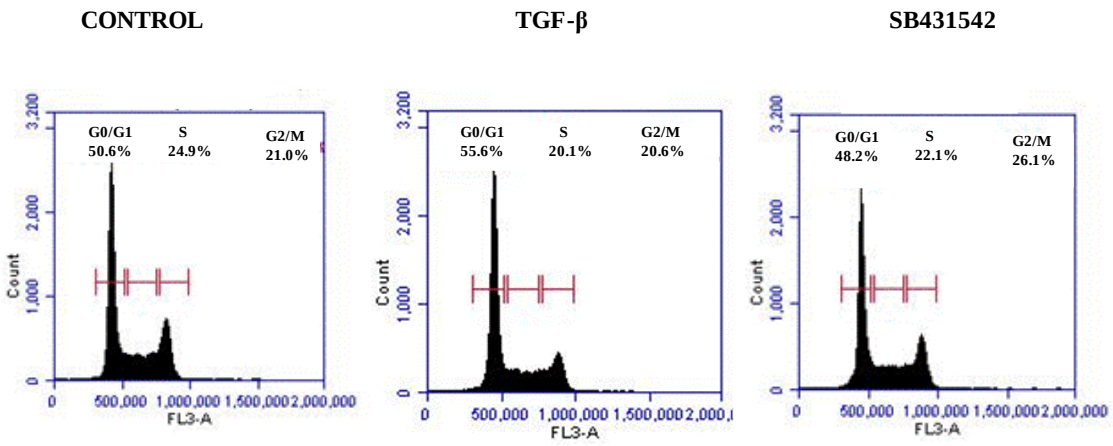
A number of studies have suggested TGFβ as having an anti-proliferative effect on cell cycle, and one of the underlying theories of residual drug resistance in CML is the quiescent population of cells, potentially mediated by TGFβ (Jonuleit *et al.*, 2000; Møller *et al.*, 2007 ; Naka *et al.*, 2010b).

Experiments were undertaken to establish the affects of TGFβ on the cell cycle in late-stage disease, using MYL cells as a model system.

MYL cells were seeded in 0.5% FCS containing media overnight and then aliquoted at $5.0x10^5$ cells/ml into 24-well plates. Cells were synchronised in G1/S phase through a double thymidine block, briefly 19 hour thymidine incubation, replace with basal media for 9 hours, then add thymidine again for an additional 16 hour incubation, TGFβ and SB431542 were then added for a period of 12 hours. Cells were fixed in 70% ethanol before staining with propidium iodide and analysed using flow cytometry.

Cell cycle histogram profiles show addition of TGFβ increases the population in G0/G1 phase by 5%. This difference is supported by the 5% reduction in S phase in TGFβ treated cells, with no difference observed in G2/M phase cells (Figure 3.13a). The TGFβ-mediated increase in G0/G1 cells can be reversed by the addition of SB431542 to inhibit endogenous TGFβ signalling. This causes a decrease in the G0/G1 cell population and an increase in those cells in the mitotic stage of the cell cycle, an increase of almost 6% compared to TGFβ treated cells. The histogram profiles have also been represented in bar chart form to show results more clearly (Figure 3.13b)

(a)



(b)

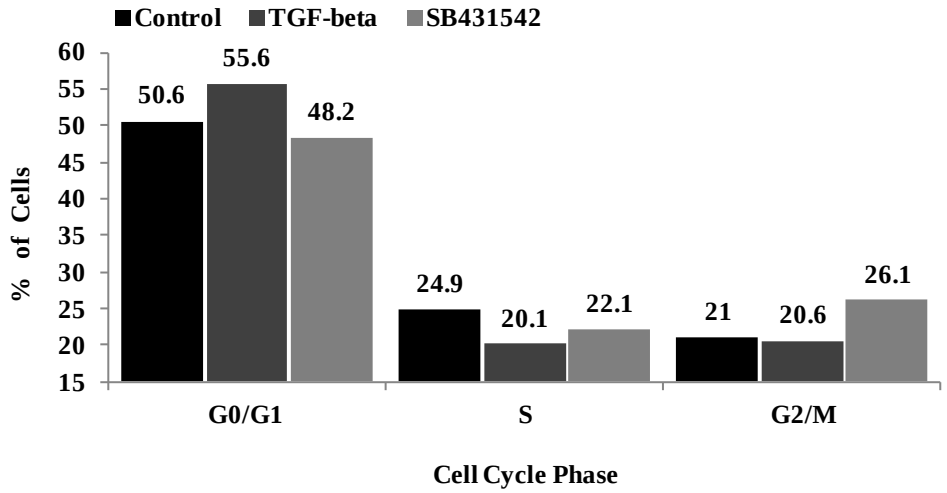


Figure 3.13 TGFβ causes cell cycle arrest in CML cells which can be reversed by SB431542 treatment

MYL cells were synchronised with a double thymidine block, then treated with either TGF β or SB431542 for 12 hours. Cells were fixed in 70% ethanol before propidium iodide was used and cell cycle status analysed on a flow cytometer. Data was then analysed using CFlow Plus software.

3.2.10 SB431542 Combined with Imatinb Increases PARP cleavage in MYL cells

To support the findings that SB431542 enhanced Imatinib-mediated cell death, PARP cleavage was examined. Poly (ADP-ribose) polymerase (PARP) is routinely used to assess cells undergoing apoptosis, and cleavage of PARP by caspase-3 activity is recognised to occur at the onset of apoptosis (Chang *et al.*, 2010; Dean *et al.*, 2010).

Initially, a preliminary experiment was set up to test the conditions to obtain a read-out of apoptotic cells using this system. MYL cells were seeded in a 24-well plate and exposed to a range of treatments to study the effect. The high molecular weight band at 113kDa can be seen strongly in the untreated lane and the 0.3μM Imatinib lane, with no bands corresponding to cleaved PARP at 29kDa. As cell treatment concentrations increase, a subsequent increase in the strength of the 29kDa band, indicating PARP cleavage, is seen. Concomitantly, a reduction in the un-cleaved band is apparent at 113kDa (Figure 3.14a).

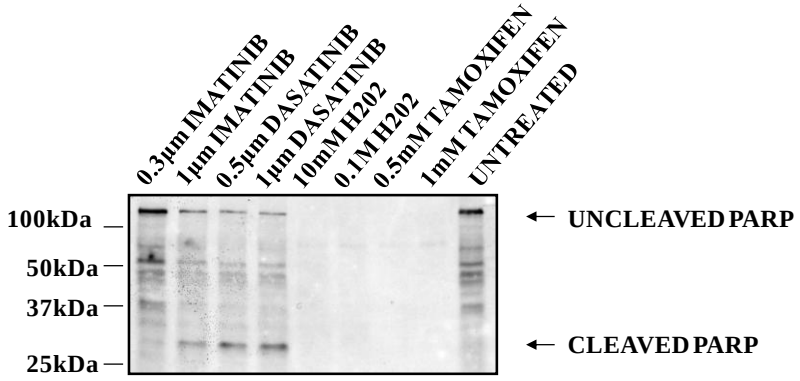
As it was unknown how these cells would respond to treatments in terms of their PARP cleavage profile, hydrogen peroxide and Tamoxifen were also added to cells as positive controls to induce apoptosis. The absence of bands for hydrogen peroxide and Tamoxifen treatments suggest the doses were too concentrated for any response to be analysed.

To look at the combination effect of TGFβ signalling inhibition and Imatinib treatment, MYL cells were seeded in 24-well plates in 0.5% FCS containing media. SB431542 was added to cells 60 minutes before Imatinib. Two separate time courses were set up for 6 and 12 hour treatment.

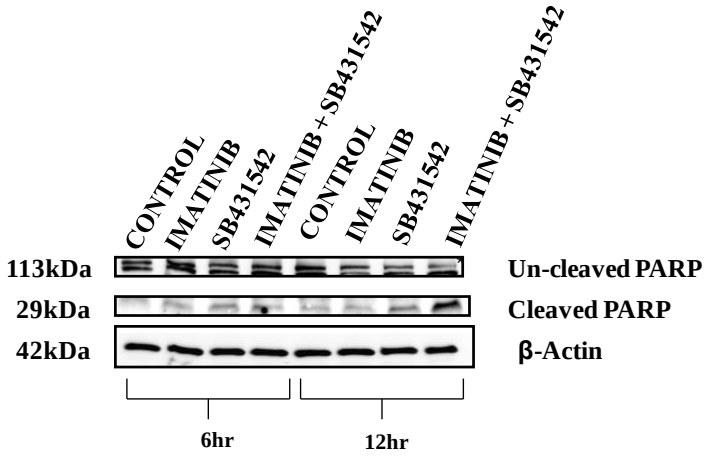
After 6 hours of treatment there appeared to be no significant changes in either the un-cleaved or cleaved PARP bands compared to the control. Samples treated for 12 hours show a reduction in un-cleaved PARP which can be seen in all three lanes in comparison to the control, but only in the SB431542 and Imatinib lane is there a clear increase in cleaved PARP at 29kDa, indicating a combination treatment is able to enhance PARP cleavage in these cells, more so than Imatinib alone (Figure 3.14b).

Densitometry analysis of Western blots shows that there is an increase in PARP cleavage after 6 hours of treatment, and that the combination of TGFβ signalling inhibition and Imatinib treatment is more efficient at inducing cleavage than the two treatments alone. The 12 hour samples reflect more strongly what is seen after 6 hours. The graph shows a clear increase in PARP cleavage correlating with a decrease in un-cleaved PARP (Figure 3.14c).

(a)



(b)



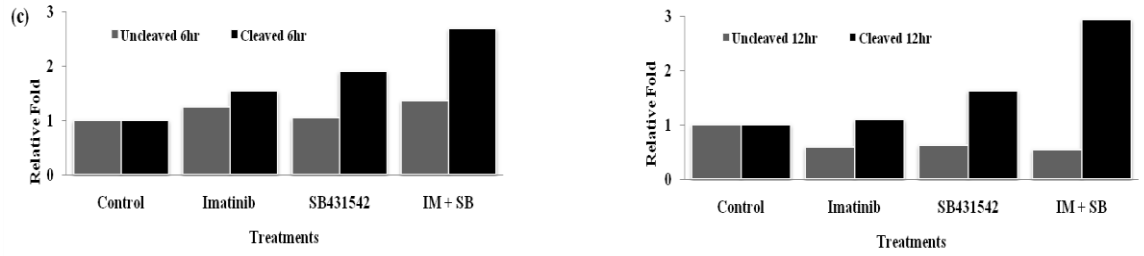


Figure 3.14 Imatinib and SB431542 combination treatments enhances PARP cleavage in chronic myeloid leukaemia cells

MYL cells were serum starved overnight in 0.5% serum containing media before treatment with (a) Imatinib, Dasatinib, hydrogen peroxide or Tamoxifen (b) either 0.3μM Imatinib, 10μM SB431542 or a combination of the two for 6 or 12 hours. Lysates were separated through SDS-PAGE, subsequent Western blots were probed with anti-PARP antibodies Expression of PARP cleavage was determined through anti-PARP western blots. Anti-β-Actin blots were probed to ensure equal protein loading (c) Densitometry analysis of PARP Western blot comparing relative fold compared to untreated controls, analysed with ImageJ software.

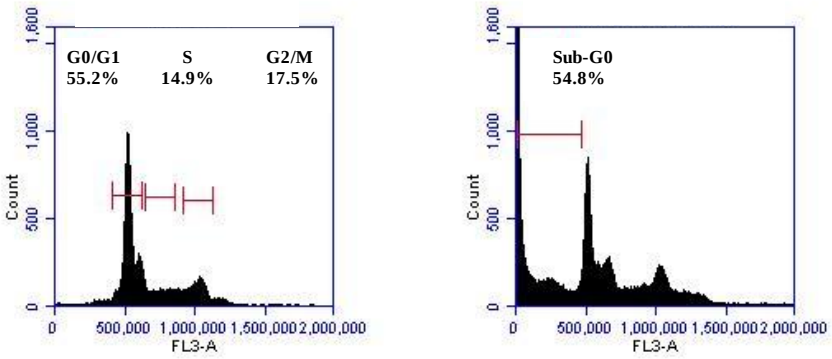
3.2.11 TGFβ inhibition combined with Imatinib increases the sub-G0 cell population

To establish if the results seen in the MTS and PARP cleavage experiments would be supported by cell cycle data, analysis to assess the sub-G0 apoptotic cell populations was carried out. MYL cells were again seeded in 0.5% FCS containing media overnight before being treated in 24-well plates at $5.0x10^5$cells/ml.

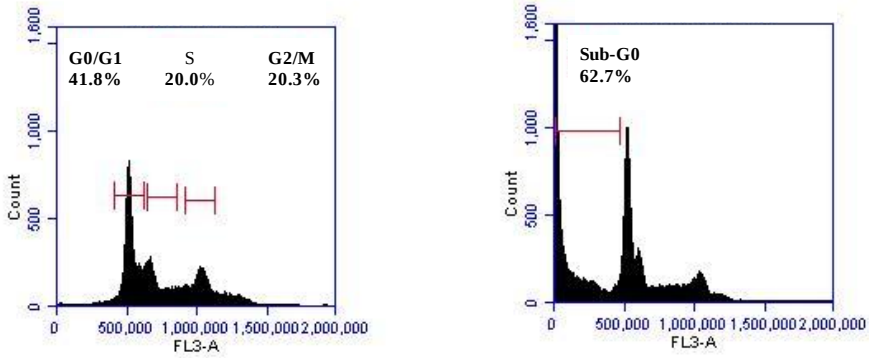
Samples were treated for 12 hours. SB431542 (10μM) was added 60 minutes before Imatinib (0.3μM) in the combination treatment. Imatinib treated samples have a G0/G1 population of 55.2% this, as previous data would suggest is reduced in the Imatinib and SB431542 treated cells by just under 14%. (Figure 3.15a) This decrease seen with combined treatment also causes an increase in Sub-G0 cells, which increases nearly 8% from 54.8% to 62.7%. The S and G2/M cell populations in the dual treated samples have a total increase of almost 8% compared to Imatinib treatment alone. Cell cycle histogram profiles are also presented in bar chart form for an easier comparison of cell populations between treatments (Figure 3.15b).

(a)

IMATINIB



IMATINIB+SB431542



(b)

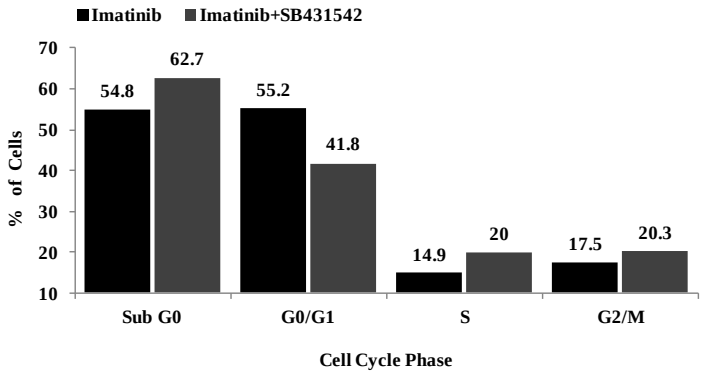


Figure 3.15 TGFβ inhibition enhances Imatinib-mediated cell death through induction of the cell cycle

MYL cells were seeded in 0.5% FCS containing media. Cells were treated with SB431542 60 minutes before Imatinib addition. After 12 hours cells were fixed in 70% ethanol before addition of propidium iodide and analysis on a cytometer.

3.3 DISCUSSION

The aim of this chapter was to analyse the effect of Bcr-Abl on TGFβ signalling, in addition to studying its effect on haematopoietic cell proliferation.

Analysing the Bcr-Abl effect on TGFβ Smad-dependent signalling showed a dose-dependent up-regulation in two independent TGFβ responsive cell lines HEK293 and COS-1. Establishing the effect of combining this cytokine and Bcr-Abl oncogene has in the 32D haematopoietic system suggested that Bcr-Abl might prevent the growth inhibitory effects of TGFβ, observed in the absence of the p210 protein, and may enhance cell proliferation in response to TGFβ, suggesting that Bcr-Abl can de-regulate the normal cytostatic effect of TGFβ (Figure 3.2). These results support the findings of Atfi *et al* (2005) who report that Bcr-Abl prevents TGFβ-mediated growth arrest in Ba/F3 cells stability transfected with a Bcr-Abl plasmid. Unfortunately, the coagulation or clumping of the mouse haematopoietic cells especially those expressing p210, made it increasingly difficult to analyse results. The use of an anti-clumping agent such as citrate dextrose may have been necessary to enable further studies in this system.

Results presented in this chapter also support the findings of Møller *et al* (2007) who show Bcr-Abl up-regulating TGFβ signalling. Interestingly, Atfi *et al* (2005) go on to suggest TGFβ signalling is not altered by the presence of Bcr-Abl in K562 cells, which is in contrast to the findings presented in this chapter.

To study and characterise the TGFβ effect in human CML-derived cell lines, MYL and MYL-R myeloid leukaemia cells were analysed to elucidate how the response might be altered within these cells. These cells are derived from a CML patient and had

previously been characterised by Ito *et al* (2007) in terms of their mutation status and Imatinib resistance, but not in terms of their responsiveness to TGFβ. Their data had highlighted an alternative mechanism of drug resistance to that proposed previously. This suggested an increase in Bcl-2 levels (Dai *et al.*, 2004), yet MYL-R cells did not have elevated levels compared to K562 or MYL cells (Ito *et al.*, 2007).

Results through MTS assays showed that MYL cells had maintained a response to TGFβ, and were growth inhibited. In contrast, the MYL-R cells did not show a significant reponse to TGFβ stimulation. Interestingly Jonuleit *et al* (2000) showed that Bcr-Abl completely blocks TGFβ-induced p27 induction, preventing normal cell cycle regulation. These observed differences in TGFβ sensitivity may be due to changes in Bcr-Abl signallling and regualtion but are independent of Bcr-Abl mutations as characterised by Ito *et al* (2007). Secretion of TGFβ has also been studied in Bcr-Abl expressing dendritic cells (DCs) and suggested this as being responsible for the reduced maturation in these cells (Mumprecht *et al.*, 2009). Further upregulation of TGFβ by c-Abl is reported in scleroderma (SSc) sufferers , and fibrotic effects can be successfully reduced with Imatininb (Bujor *et al.*, 2009). When compared to K562 cells, which have been shown to contain similar levels of Lyn to MYL cells (Ito *et al.*, 2007), a similar proliferation pattern is observed, which is not followed by MYL-R cells. The K562 cell line is a good comparison as like the MYL/MYL-R cells they also contain double philadelphia postive chromosomes. In addition, MYL-R cells have previously been shown to have enhanced activated STAT5, as a result of increased Lyn activation (Ito *et al.*, 2007). TGFβ has been reported to directly inhibit STAT5 activation (Cocolakis *et al.*, 2008). It therefore probable that TGFβ addition to MYL-R cells would antagonize the Lyn mediated activation of STAT5, but no effect on proliferation was observed.

The phosphorylation of endogenous Smad3 levels in CML cells indicate that TGFβ signalling is not inhibited in the MYL or MYL-R cell lines, as both see a TGFβ-mediated induction of Smad3 phosphorylation and an abrogation with SB431542. Interestingly both cell lines have no detectable phosphorylated Smad3 in unstimulated samples. If Bcr-Abl is up-regulating TGFβ signalling then Smad3 activation is more likely, although up-regulation could be a transient mechanism in the endogenous CML environment. Results presented here suggest that MYL-R may undergo hyperactivation in response to TGFβ compared to MYL cells, and certainly appear to have a more sustained Smad3 response, and this may explain the desensitisation of MYL-R to the TGFβ signal if Smad signalling is over-active. The activation of Smad proteins however does not guarantee they are entering the nucleus and activating transcription, and further downstream signalling pathways could be inhibiting TGFβ effects within MYL-R cells. For example, Akt has been shown to sequester activated Smad3 from the nucleus, and indeed increased Akt levels are shown to protect liver cells from TGFβ-induced apoptosis (Conery *et al.*, 2004). Within CML, the observed results could be explained by Bcr-Abl causing an up-regulation of TGFβ signalling, but the absence of Smad3 activation in untreated cells suggests if up-regulation is occurring, it is a transient process. There are studies showing that TGFβ-Smad target genes are themselves Smad interacting factors, enabling TGFβ transcriptional responses such as ATF3 (Kang *et al.*, 2003). These responses have been shown to cause sustained Smad signalling over extended periods suggesting genes such as ATF3 themselves are able to influence Smad signalling through specific binding. Research has shown that alternative pathways are able to influence Smad signalling such as the Erk/MAPK pathway which inhibits nuclear translocation of activated Smads (Derynck and Zhang, 2003). It is apparent that

signalling pathways are not exclusive in their mediators or interaction partners, and therefore it is interesting to speculate that additional proteins may be involved directly with Smad3 signalling in MYL and MYL-R cells, which may be the cause of the observed responses.

It is possible that a delay in TGFβ receptor de-activation in MYL-R cells is responsible for the potential increased levels in phosphorylated Smad3 levels. Interestingly, SNIP1 has been found to interact with Smads 1 and 2 and inhibit signalling, while a Smad3 interaction was not found. SNIP1 did inhibit the Cbp/p300 protein which is a co-activator of many genes including TGFβ-regulated genes. Furthermore, the p300 gene is located on chromosome 22 which is involved in the translocation in CML. Akt activation sequesters FoxO3 in the cytoplasm, preventing its apoptotic mechanism and leading to oncogenic transformation by Bcr-Abl (Kharas *et al.*, 2004). Akt has also been shown to sequester Smad3 outside the nucleus, preventing Smad3 regulated transcription and apoptosis (Conery *et al.*, 2004) It was therefore decided to look at the effect TGFβ had on Akt in MYL and MYL-R cells.

Results showed an increased level of Akt in MYL-R cells, this could explain the increased oncogenic capacity, through sequestering of FoxO3, leading to inhibted apoptosis. It could also be suggested that this elevated Akt level could be sequestering Smad3 levels from the nucleus in MYL-R cells which may explain why no cytostatic effect is seen despite enhanced Smad3 activation upon TGFβ stimulation. Akt is known to be constitutively active in blast crisis CML, and treatment with Imatinib has been shown to inhibit its kinase activity (Kawauchi *et al.*, 2003). Interestingly Akt has recently been shown to be involved in the relocation of p27 to the cytoplasm, resulting in an increase in CML cell proliferation (Chu *et al.*, 2010). The differences observed

here could go some way to explain the divergence in response to stimulation, and potentially highlight cross-talk between TGFβ/Akt signalling pathways which may have become more prominent in MYL-R cells and influence their enhanced growth potential in response to TGFβ, while at the same time a reduction in nuclear p27 levels would support enhanced cellular growth observed in MYL-R populations. Other studies have looked at inhibiting Akt phosphorylation in CML, and have found that Resveratrol can efficiently reduce activation, but has no effect on Bcr-Abl activation (Banerjee *et al.*, 2010).

Studies have shown that TGFβ plays an inhibitory role in early stage haematopoiesis (Park *et al.*, 2004), the point at which the initial chromosomal translocation occurs. Previous findings suggest that Bcr-Abl can reverse these inhibitory effects on cell cycle (Jonuleit *et al.*, 2000). One study suggests the effect of Bcr-Abl on $CD34^+$ cells to be more limited than previously thought, and by comparing CML and normal $CD34^+$ cells it reveals more differences between quiescent and dividing normal cells than their CML counterparts (Graham *et al.*, 2007).

In this chapter, cell cycle data shows a marked response between TGFβ stimulation and its inhibition with the SB431542 compound, suggesting MYL blast phase cells remain sensitive to TGFβ signalling (Figure 3.13). This is contrary to the findings of Atfi *et al* (2005) who suggest Bcr-Abl inhibits the TGFβ mediated cell cycle arrest, and also PARP cleavage. Their results were obtained in transfected mouse cells, while results presented in this chapter were carried out in human CML cells so the differences may be due to cell specificity. One study looking at the CML cell cycle concluded there was a defective Bcr-Abl-dependent response to growth factor deprivation (Jonuleit *et al.*, 1998). Results in this thesis support this evidence but go on to uncover specific

treatment effects. Evidence also implicates Imatinib in increasing the number of G0/G1 cells and thus increasing the quiescent population. (Jin *et al*., 2008). My results support an Imatinib-mediated increase in G0/G1 cells, but when combined with SB431542 this population decreases significantly. Interestingly, a study has shown that CML can generate leukaemic stem cells through missplicing causing aberrant β-catenin activation (Abrahamsson *et al*., 2009), which would contain Imatinib resistant cell populations. During advanced and blast crisis stages there is conflicting evidence regarding the involvement of Bcr-Abl, while some evidence suggests disease progression is met with an increased need for Bcr-Abl up-regulation (Barnes *et al*., 2005), known as Bcr-Abl addiction, whereas other reports suggest that as CML evolves it can begin to rely less heavily on oncogenic addiction in favour of other key proteins to sustain and drive the disease. So, while Bcr-Abl may still be active within cells it may not be as heavily relied on, as newly acquired oncogenic pathways emerge to drive disease progression, such as constitutively active PI3K/AKT signalling in Imatinib resistant cells (Quentmeier *et al*., 2011).

Research has shown that a K562 Imatinib-resistant cell line exhibiting a decrease in Bcr-Abl levels is more susceptible to DNA damaging agents (Ju *et al*., 2007). To overcome resistance there has been a vast range of experimental approaches with the advent of second generation tyrosine kinase inhibitors such as Dasatinib and Nilotinib.

The next experiments in this chapter focused on the theory of a sub-population of quiescent cells, being maintained in this non-dividing state through an up-regulation of TGFβ signalling, which are subsequently resistant to Imatinib. Interestingly, MYL-R cells were found to contain higher levels of CD34 than MYL cells (Ito *et al*., 2007), which is a marker for haematopoietic progenitor cells, and is known to confer inherent

drug resistance (Roeder *et al.*, 2006). By inhibiting TGFβ signalling, these cells are forced back into the cell cycle, begin to divide and become sensitive to Imatinib-mediated cell death. Cell cycle analysis of combined SB431542 and Imatinib treatment gives a significant increase in SubG0 cells which are considered to be apoptotic populations, compared to Imatinib treatment alone. The cell cycle data showing cells to be growth inhibited by TGFβ is not a dramatic effect, yet this may be explained by the fact that CML exists as mixed population of cells within the samples which respond differently to TGFβ. MYL-R cells which have induced-Imatinib resistance show no response to TGFβ, suggesting this de-sensitization is further along in the disease evolution process, as the MYL cells still retain TGFβ sensitivity.

The combination therapy approach showed that by inhibiting the TGFβ signalling pathway, cells were induced to undergo Imatinib-mediated cell death slightly more so than Imatinib treatment alone. This was confirmed by PARP cleavage analysis, which are supported by the survival assays which show an increase in CML cell death with the combined treatment. These results suggest that TGFβ does play a role in the blast phase of CML. The necessity for TGFβ signalling inhibition is supported by results indicating that Imatinib treatment has no effect on TGFβ *in vitro* (Larmonier N, 2008). Another study has shown the use of a TGFβ inhibitor together with Imatinib treatment is able to impair the colony forming ability of leukaemia initiating cells (LICs) (Naka *et al.*, 2010b). Bcr-Abl siRNA together with Imatinib has yielded promising results showing an increase in apoptosis (Koldehoff *et al.*, 2010), although this was in 32Dp210 cells, so may not be representative of blast phase disease. It is also of note that these cells carried mutations in the Bcr-Abl gene so siRNA treatment would have had more of a targeted impact than Imatinib in cells containing Bcr-Abl mutations.

CML is a slowly evolving disease which increases the likelihood for a range of mutations which could change a cell response. It is important to realise that the effects seen in these cells may be transient in terms of disease progression. So it is suggested to take these results as a snapshot of what is occurring within CML cells at their current disease stage, and that it will not necessarily apply to other types of analyses.

As the emergence of further research into drug resistance progresses, it is found that mutations in the Bcr-Abl gene are often not responsible for resistance. For this reason attention has focused on other modes of resistance development in CML. The CML cell lines being used in this research, one of which has Imatinib resistance but no Bcr-Abl mutations, suggests a Bcr-Abl independent resistance mechanism, that could be linked to elevated levels of Lyn kinase (Ito *et al.*, 2007). This leads onto the next chapter which looks at the involvement of Lyn kinase and TGFβ within the context of CML and drug resistance.

4. A ROLE FOR LYN KINASE IN TGFβ-MEDIATED DRUG RESISTANCE

4.1 Introduction

The advent of Imatinib was a huge breakthrough in the treatment of CML and saw vast improvements in survival rates. The drug is extremely effective when used during early stages of disease development where Bcr-Abl alone is driving disease progression. Over the years it has become apparent that Imatinib is not as effective on later disease stage patients (Druker *et al.*, 2001; Rousselot *et al.*, 2007). This can be due to increasing mutations, either Bcr-Abl dependent such as point mutations in the Abl kinase domain or Bcr-Abl independent mechanisms (Quintás-Cardama *et al.*, 2009). Recently there has been a much more focused drive towards understanding Bcr-Abl independent mechanisms contributing to drug resistance. Imatinib has been shown to be ineffective at curing CML completely, with persistent residual drug reported, and removal of treatment often resulting in CML re-emergence (Hughes *et al.*, 2006). It is important to consider that resistance is a multi-factorial process, often the result of many genetic alterations occurring throughout disease evolution. Clinically, Imatinib is given orally, which gives rise to a range of environmental influences such as intestinal absorption, drug efflux and influx for example, before target cells are reached, and all of these factors can play a role in affecting drug efficacy.

The advent of second generation tyrosine kinase inhibitors such as Nilotinib and Dasatinib was aimed at patients who had failed on Imatinib therapy. These new inhibitors have shown respectively a 20-30 fold and 325-fold increase in potency over Imatinib against Bcr-Abl *in vitro (Deininger et al., 2009)*. They have shown to be active against the majority of Bcr-Abl mutants, but the T315I mutant confers resistance to

Imatinib, Dasatinib and Nilotinib, (Copland *et al.*, 2006; Redaelli *et al.*, 2009), and thus highlights the need for further developments.

The previous chapter touched on dual therapy approaches to overcome drug resistance, in this next chapter experiments will aim to look at the impact of TGFβ in CML through the protein Lyn kinase which is up-regulated in an Imatinib-resistant, Bcr-Abl mutation-negative form of CML (Ito *et al.*, 2007).

Lyn kinase itself is present in myeloid cells where it is known to act as both a positive and negative modulator of cellular responses, Lyn deficient mice show hyper-proliferation of myeloid cells due to an enhanced sensitivity to colony stimulating factors (Xu *et al.*, 2005). Conversely, Lyn has been shown to act in a positive role for stem cell factor-induced proliferation and chemotaxis in primary haematopoietic cells (O'Laughlin-Bunner *et al.*, 2001).

The involvement of Lyn kinase in CML was highlighted by (Donato *et al.*, 2003; Ito *et al.*, 2007; Wu *et al.*, 2008a) who show it to be over-expressed in an Imatinib-resistant cell line. This evidence combined with research suggesting TGFβ is able to down-regulate members of the Src kinase family (Atfi *et al.*, 1994) made TGFβ and Lyn interesting subjects to look at, with a view to understanding further how they may be linked in CML.

Lyn kinase has previously been shown to be modified through ubiquitination (Kyo *et al.*, 2003; Shao *et al.*, 2004). Interestingly, Src family kinase ubiquitination and subsequent proteasomal degradation has been associated with the activity levels of these kinases. Constitutively active Lyn mutants showed increased Lyn activity, yet a decrease in protein levels (Harder *et al.*, 2001). It will therefore be important to understand what the

impact the different levels of Lyn has between resistant and sensitive cells and how this may affect its subsequent degradation.

Whilst my research was being carried out there were a number of studies that looked at Lyn and its interactions with the E3 ligase c-Cbl (Kyo *et al.*, 2003; Wu *et al.*, 2008b). Previous reports have suggested c-Cbl phosphorylation might be regulated by Bcr-Abl (Andoniou *et al.*, 1994), and silencing of Bcr-Abl has shown a reduction in c-Cbl phosphorylation (Wu *et al.*, 2008b). In addition, Lyn silencing was able to increase c-Cbl phosphorylation and protein levels (Wu *et al.*, 2008b). Research has identified c-Cbl to have negative regulatory roles in tyrosine kinase signalling. Knockout mice have been shown to develop an enlarged thymus, expanded haematopoietic progenitor pools and increased bone marrow re-population (Naramura *et al.*, 1998). It is the loss of function mutations which have given the c-Cbl protein the branding as a tumour suppressor protein. A c-Cbl knockout has been shown to accelerate blast phase CML (Sanada *et al.*, 2009). In addition, Lyn has been implicated in the substrate list of c-Cbl where it is shown to be ubiquitinated and down-regulated (Kyo *et al.*, 2003).

Here results are presented which demonstrate TGFβ to be playing a role in Lyn kinase activation and degradation through ubiquitination and it is suggested to be mediated through the E3 ubiquitin ligase c-Cbl.

The aims of this chapter are therefore to the following:

1. Investigate TGFβ-mediated effects on Lyn kinase through post-translational modification

2. Analyse these interconnections in an endogenous CML system

3. Identify the E3 ligase responsible for the modification

4.2 Results

4.2.1 TGFβ Causes Ubiquitination of Lyn kinase and Bursts of Activation

The first aim of this chapter was to analyse the effect TGFβ had on the protein Lyn kinase through Western blotting. MYL cells were seeded at $5.0x10^5$ cells/ml in 0.5% FCS-containing media. TGFβ was added for various time points between 0-3 hours, and SB431542 treatment (60 mintues) was used to look at the effect of inhibiting TGFβ signalling. The experiment was carried out in the absence and presence of the proteasomal inhibitor MG132.

Results indicate that TGFβ causes Lyn levels to fluctuate during treatment, a significant decrease in levels can be seen between 0 and 30 minutes of stimulation in the absence of the proteasomal inhibitor, and the levels then show an increase for the future time points (Figure 4.1). In contrast, the initial drop at 30 minutes in Lyn levels is not seen in the presence of MG132. Also, after 60 and 180 minute time-points, high molecular weight bands can be seen with increased intensity compared to the MG132 negative cells when probed with anti-Lyn antibodies, and bands appear at much larger molecular weights than in those without the proteasomal inhibitor. The high molecular weight band is not seen in SB431542 treated cells suggesting that the band is TGFβ dependent and can be prevented by inhibiting the signalling pathway. The absence of a drop in protein levels after 30 minutes in MG132 treated cells suggests TGFβ is involved in Lyn protein turnover.

Since these high molecular weight bands can be seen with increased intensity and abundance in the presence of proteasomal inhibitors, it is possible that these bands represent polyubiquitinated protein species.

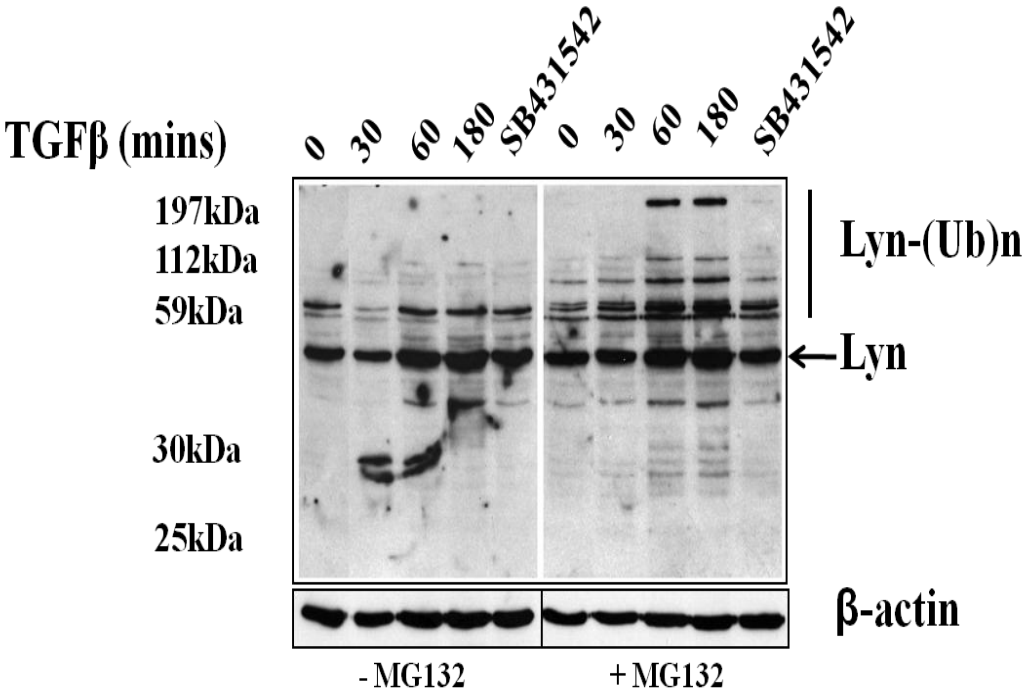


Figure 4.1 TGFβ induces Lyn ubiquitination in the prescence of the proteasomal inhibitor MG132

MYL cells were serum starved overnight in 0.5% serum-containing media before treatment with 1ng/ml TGFβ for a range of time points bewteen 0-180 minutes or 10μM SB431542 with and without the proteasomal inhibitor MG132. Cells were lysed in RIPA buffer and subsequent Western blots were probed with anti-Lyn and anti-β-Actin antibodies.

4.2.2 TGFβ mediates Lyn ubiquitination

Ubiquitination is an important post-translational modification involved in numerous biological functions such as cell growth, apoptosis, proliferation and the DNA damage response. Whilst these processes are generally considered to be carried out via proteasomal degradation (Hoeller and Dikic, 2009; Pickart, 2001), recent evidence has suggested protein trafficking and localisation as further roles for ubiquitination (Chen and Sun, 2009).

Currently, there are only a few papers suggesting links between TGFβ and Lyn (Atfi *et al.*, 1994; Pazdrak *et al.*, 1995). Evidence of Lyn ubiquitination is not published in human CML, but has been researched in rat basophilic leukaemia (Bhattacharyya, 2001; Kyo *et al.*, 2003). To look further at the effect of TGFβ on Lyn, and investigate TGFβ-mediated Lyn ubiquitination, HEK293 cells were transiently transfected with a Lyn-Flag plasmid. Cells were seeded in 2% FCS-containing media, and a range of 0-1μg of a Lyn-Flag expression plasmid was transiently transfected into cells. Using this over-expression system would potentially clarify the pattern seen in the endogenous experiment using MYL cells.

Results indicate a TGFβ mediated response. It is apparent from this experiment that HEK293 cells contain endogenous Lyn and this has been detected in the first two lanes (Figure 4.2a). TGFβ is able to cause an increase in the high molecular weight band seen previously in MYL cells. At 0.25μg of Lyn the response is less clear, possibly due to transfection efficiency, however at 0.5μg there is a high molecular weight band present in the TGFβ treated cells which is not seen in the absence of stimulation. This response

appears to be lost at the 1µg level suggesting that the induction may be Lyn concentration dependent.

In addition to this, a co-immunoprecipitation experiment was undertaken to look at the outcome of combining Lyn with an HA-Ub plasmid in the presence of TGFβ. This would look to support the evidence of high molecular weight species found in MYL cells (Figure 4.1). After lysis, anti-HA antibodies were used to precipitate out the HA-tagged Ub and any covalently bound Lyn. Western blot analysis alongside whole cell lysates shows that in this over expression system, Lyn appears to be ubiquitinated even at time 0 (Figure 4.2b). The top panel suggests that TGFβ is able to increase ubiquitination of Lyn, seen through increased smearing at 30 and 60 minute time points, SB431542 and Dasatinib treatments appear to reduce ubiquitination. These observations support evidence seen endogenously albeit not at the same time points, but these results are seen in an over expression system which differ kinetically compared to the effects seen endogenously. Also of note, this latter experiment was not carried out in the presence of any proteasomal inhibitors, yet the over-expressed proteins appear abundant enough to highlight the effect.

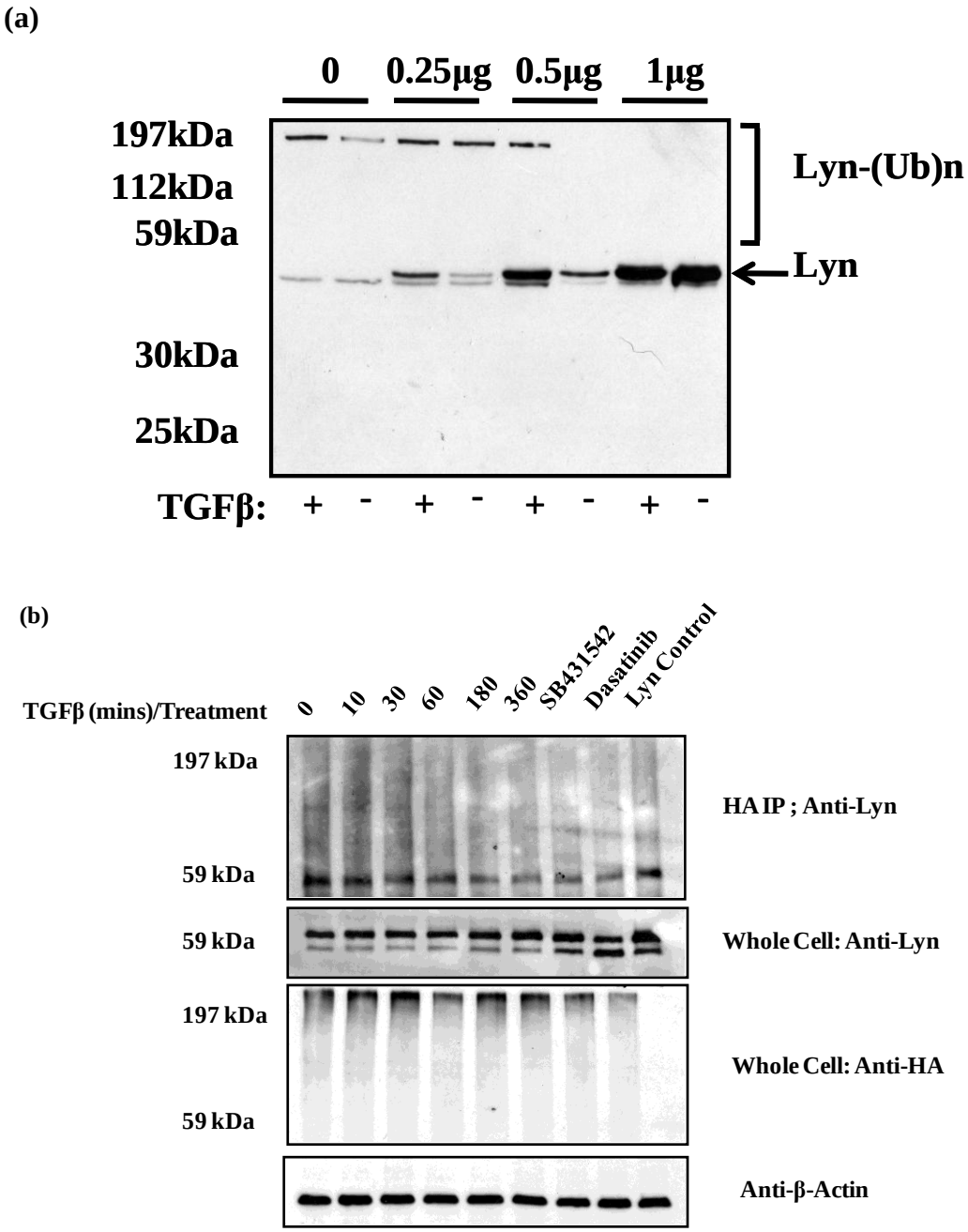


Figure 4.2 TGFβ is able to induce high molecular weight Lyn bands in HEK293 cells

(a) HEK293 cells were transfected with a range of concentrations between 0-1µg of the expression plasmid Lyn-Flag. Cells were then put into low serum 2% before being treated with 1ng/ml TGFβ for 60 minutes. Cells were then lysed in RIPA buffer and subsequent Western blots were probed with anti-Flag antibody (b) HEK293 cells were stimulated with TGFβ, SB431542(60min) or Dasatinib (60min), lysates were treated with anti-HA antibody before being analysed with Western blotting, probed with anti-Lyn, HA or β-actin.

To develop further the effect of TGFβ on Lyn ubiquitination, and clarify if this modification showed changes in Lyn protein levels, nickel affinity analysis was used. HEK293T cells were transiently co-transfected with a Lyn plasmid and a His-tagged ubiquitin plasmid. This experiment utilised the ability of Histidine to bind nickel beads after cell lysis, and therefore separate ubiquitin with any associated proteins from non-ubiquitinated proteins. The high affinity of the nickel nitrilotriacetic acid (NTA) should provide high specificity for the HIS-tagged Ub whilst reducing non-specific binding from other proteins. These proteins are then probed with specific Lyn antibodies to analyse ubiquitination levels of Lyn throughout the time course.

After 21 hours of transfection, cells were treated for a range of time points between 0-360 minutes with TGFβ or SB431542, together with the appropriate controls (Figure 4.3).

Results show a pattern of ubiquitinated Lyn seen in panel (b) which peaks at 60 minutes before declining at 180 and 360 minutes points. Whole cell Lyn levels appear to fluctuate during the treatment which may correspond to changes in ubiquitination levels. To look at ubiquitinated proteins, the whole cell extract was probed with anti-His antibody to highlight all ubiquitinated proteins. While this is not specific for Lyn protein ubiquitination, it is observed that ubiquitinated proteins are only seen in cells containing Lyn and the ubiquitin plasmid, and neither of the controls independently, suggesting that the ubiquitination seen in these lanes is indeed ubiquitinated Lyn. Additionally, the ubiquitinated species in the His-probed blot in panel (a) follows a similar pattern to that of the nickel affinity blot panel (b), and also that of the active phosphorylated Lyn blot (Figure 4.3a). This result also supports the results seen in the endogenous system with the high molecular weight band seen in the presence of MG132

after 60 minutes of TGFβ treatment (Figure 4.1). Densitometry analysis of the western blots using Image J software also support the observed changes (Figure 4.3c), panel (a) is represented as Ub, while panel (b) of the Western blot is represented as Lyn Ub. It also clarifies that SB431542 is inhibiting TGFβ ubiquitination after 60 minutes, as relative to 60 minute TGFβ stimulation, the SB431542 treated cells show lower ubiquitination levels.

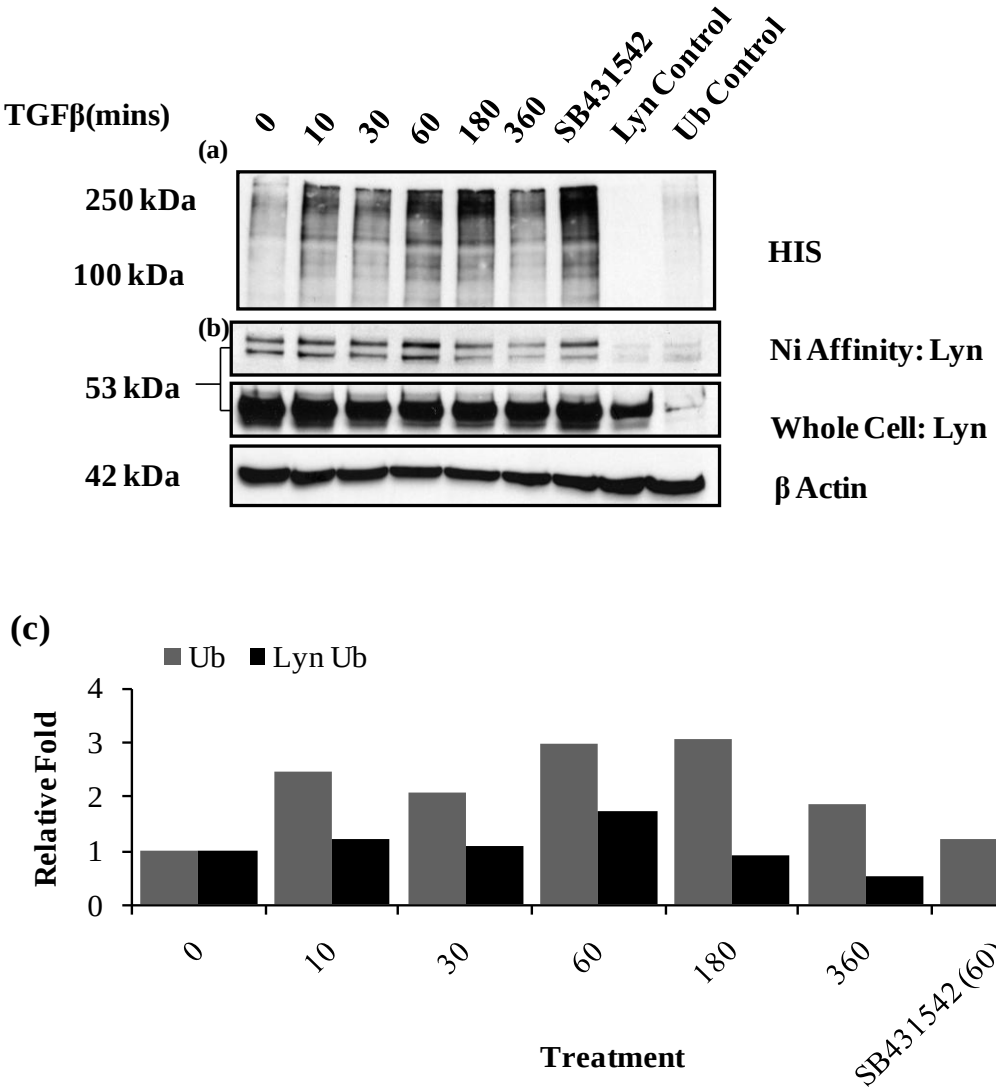


Figure 4.3 TGFβ causes ubiquitination of Lyn

HEK293T cells were co-transfected with expression plasmids for Lyn (0.5µg) and Ubiquitin-HIS (0.25µg). Cells were put into 2% FCS-containing media overnight before TGFβ treatment for a range of time between 0-360 minutes, or 10µM SB431532. Cells were then lysed in 1% NP40 lysis buffer and incubated overnight with nikel beads. Lysates were washed with 0.1%NP40 buffer, subsequent Western blots were probed with (a) anti-Ha and (b) anti-Lyn antibodies and the whole cell extracts with anti-Lyn and anti-β-Actin antibodies, (c) blot analysis showing fold changes in nickel affinity Lyn ub levels and His-ub relative to the untreated controls, images analysed using Image J software.

4.2.3 Lyn kinase is modified via Sumoylation

While looking for potential E3 ligases involved in Lyn ubiquitination, a ubiquitin like modifier was investigated to establish if TGFβ could have an effect on this type of modification.

Small ubiquitin-like modifier (SUMO) is another post-translational modification which is known to be involved in a range of cellular processes such as gene transcription, cell cycle regulation and cellular localisation. Sumoylation counteracts ubiquitination via directly competing for the same lysine residue (Bergink and Jentsch, 2009). SUMO is known to be involved in subcellular localisation, DNA binding and activation of transcription factors (Geiss-Friedlander and Melchior, 2007; Hilgarth *et al.*, 2004). Interestingly SUMO2 has the ability to form polymeric chains, whereas SUMO1 cannot (Tatham *et al.*, 2001). There has been a number of papers linking SUMO with TGFβ signalling. The TGFβ type I receptor is sumoylated which promotes activation (Kang *et al.*, 2008; Miyazono *et al.*, 2008). Smad3 has been shown to undergo sumoylation, resulting in its nuclear export (Imoto *et al.*, 2008).

Experiments again utilise HEK293T cells which are co-transfected with Lyn and SUMO2 plasmids. Cells were put into 2% FCS media post-transfection and left overnight before being treated with TGFβ (1ng/ml) or SB431542 (10μM).

Results suggest SUMO2 to have an effect on Lyn, as dark, high molecular weight bands and smearing can be seen in lanes 4 and 5 of the top anti-His panel (Figure 4.4a). There does not appear to be a significant difference between TGFβ negative and positive samples, however the SB431542 sample in lane 6 does have a marked reduction in the

smearing pattern in comparison with the previous two lanes. Also of note is the increase in Lyn protein levels in the presence of SUMO2 in the anti-Lyn panel, suggesting SUMO2 may increase Lyn protein stabilisation.

The second experiment aimed to provide evidence of Lyn sumoylation alongside that of Lyn ubiquitination, in order to provide a relative comparison of abundance. This was achieved by running samples alongside each other on the same gel (Figure 4.4b). Nickel affinity was used to pull out His-tagged SUMO2 and Ub proteins, plus any bound Lyn. Western blot analysis shows both SUMO2 and Ub are immunopreciptated with Lyn (Figure 4.7b middle panel). Ub appears to be the more prominent modification, a much larger amount of Lyn bound Ub is seen on the western compared to the SUMO2 bound Lyn. This would suggest Lyn has a greater amount of Ub modification rather than SUMO2. To compare levels of Lyn in the absence and presence of SUMO2 modification, densitometry analysis was used, and shows a 2.5-fold increase in relative protein amount (Figure 4.4c), this may implicate SUMO2 in Lyn protein stabilisation.

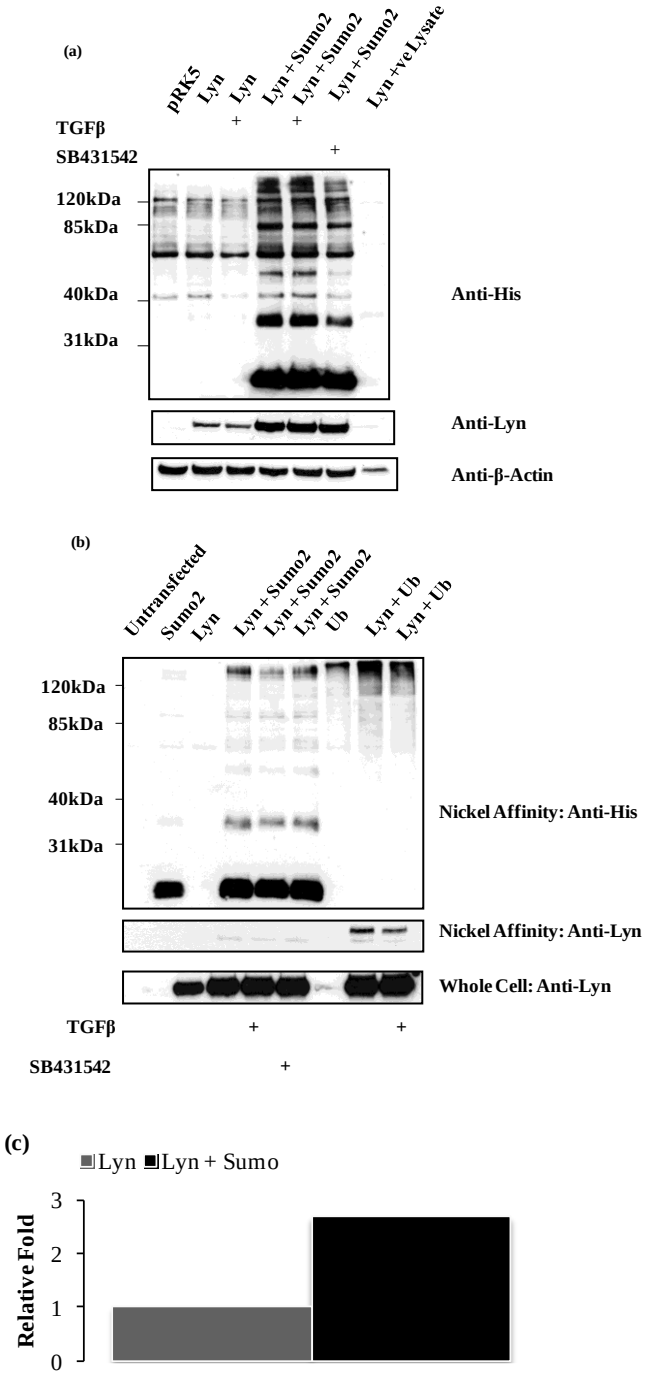


Figure 4.4 Lyn is modified by Sumoylation

(a)HEK293T cells were co-transfected with Lyn and Sumo2 plasmids. 2% FCS media was added overnight. TGFβ (1ng/ml) or SB431542 (10μM) was added to cells for 60 minutes. Blots were probed with anti-Lyn and anti-His antibodies, β-Actin was probed for to check equal loading (b) In addition to panel (a) Ub was transfected with Lyn and Nickel beads were added to lysates overnight. Anti-Lyn and Anti-His antibodies were used to detect protein levels. (c) uses ImageJ software to compare Lyn protein levels with and without SUMO2

4.2.4 TGFβ activates Lyn through turnover and enhanced activation

Protein activation is largely phosphorylation dependent, and may be linked to ubiquitination (Levkowitz *et al.*, 1999). Protein kinases can modify substrates by adding phosphate groups to them. Phosphorylation will often result in functional changes of the substrate protein. The addition of phosphate often results in changes in cellular location, enzymatic activity, and association with other proteins. Evidence exists to suggest that phosphorylation can serve as a marker for ubiquitination of kinase substrates, this has been shown for TAK1 (Bottero *et al.*, 2011). Indeed Smad proteins themselves are known to be ubiquitinated after activation through phosphorylation, Smads 2 and 3 are ubiquitinated by Smurf 2 (Lin *et al.*, 2000) and casein kinase 1 gamma 2 (Guo *et al.*, 2008), respectively. Lyn is known to be regulated through two distinct phosphorylation sites at 397 and 508 (Koch *et al.*, 1991). Recently Lyn phosphorylation and subsequent signalling has been shown to be involved in Nilotinib resistance (Gioia *et al.*, 2011). Dephosphorylation at residue 508 is necessary to induce Lyn activation, and protein tyrosine phosphatase Shp2 is known to carry out this task after granulocyte colony-stimulating factor stimulation (Futami *et al.*, 2011).

After looking at TGFβ inducing Lyn ubiquitination, the phosphorylation status of Lyn was examined to determine if there was a link between ubiquitination and phosphorylation. MYL cells were serum starved overnight in 0.5% FCS-containing media before TGFβ treatment was undertaken for a range of time-points between 0-360 minutes. SB431542 treatment was used to inhibit TGFβ signalling, and Dasatinib to prevent Lyn activation.

Results show a burst of Lyn activation with phosphorylation levels peaking at 180 minutes before diminishing at 360 minutes (Figure 4.5a). Interestingly, Lyn phosphorylation is found in untreated cells showing that active Lyn is present, possibly due to endogenous TGFβ signalling in these cells. SB431542 was added for 60 minutes and shows lower active Lyn levels compared to the 60 minute TGFβ treated cells. Dasatinib is seen to effectively prevent Lyn phosphorylation, identified by the slightly lower migrating band.

After studying Lyn activation over a time course, the difference in Lyn and phosphorylated Lyn levels were compared between MYL and MYL-R cell lines. A subsequent experiment to analyse if resistant cells responded differently to CML treatment, which may also affect their response to TGFβ, was performed. Figure 4.5b shows that there is no significant change in Lyn levels over the course of the different treatments in either MYL or MYL-R cells, although a slight reduction can be seen in MYL-R cells treated with TGFβ. The phosphorylated Lyn levels appear in much lower amounts in the MYL cells compared to MYL-R (Figure 4.5c). There is slightly elevated phosphorylated p-Lyn in TGFβ treated MYL cells compared to control cells. Imatinib has no effect on Lyn levels in either state. In both cell lines, Dasatinib is able to markedly reduce phosphorylated Lyn levels. Compared to the control, TGFβ slightly decreases phosphorylated Lyn in MYL-R cells.

Figure 4.5d-f uses Image J software to look at the differences in protein levels of Lyn, and phosphorylated Lyn through densitometry analysis of the Western blots, and also provides quantifiable data. Both MYL and MYL-R cells show very similar patterns in response to the different treatments. Dasatinib had the largest effect on phosphorylated Lyn levels, and was more effective in the MYL-R cells at reducing relative levels,

showing a 10-fold decrease, compared to 3-fold for MYL cells. TGFβ shows a greater effect in the non-resistant cell line and does appear to increase phosphorylated Lyn levels. The final figure looks at phosphorylated Lyn levels between the two cell lines and confirms that MYL-R cells have just under a 3-fold increase in levels compared to the MYL cells.

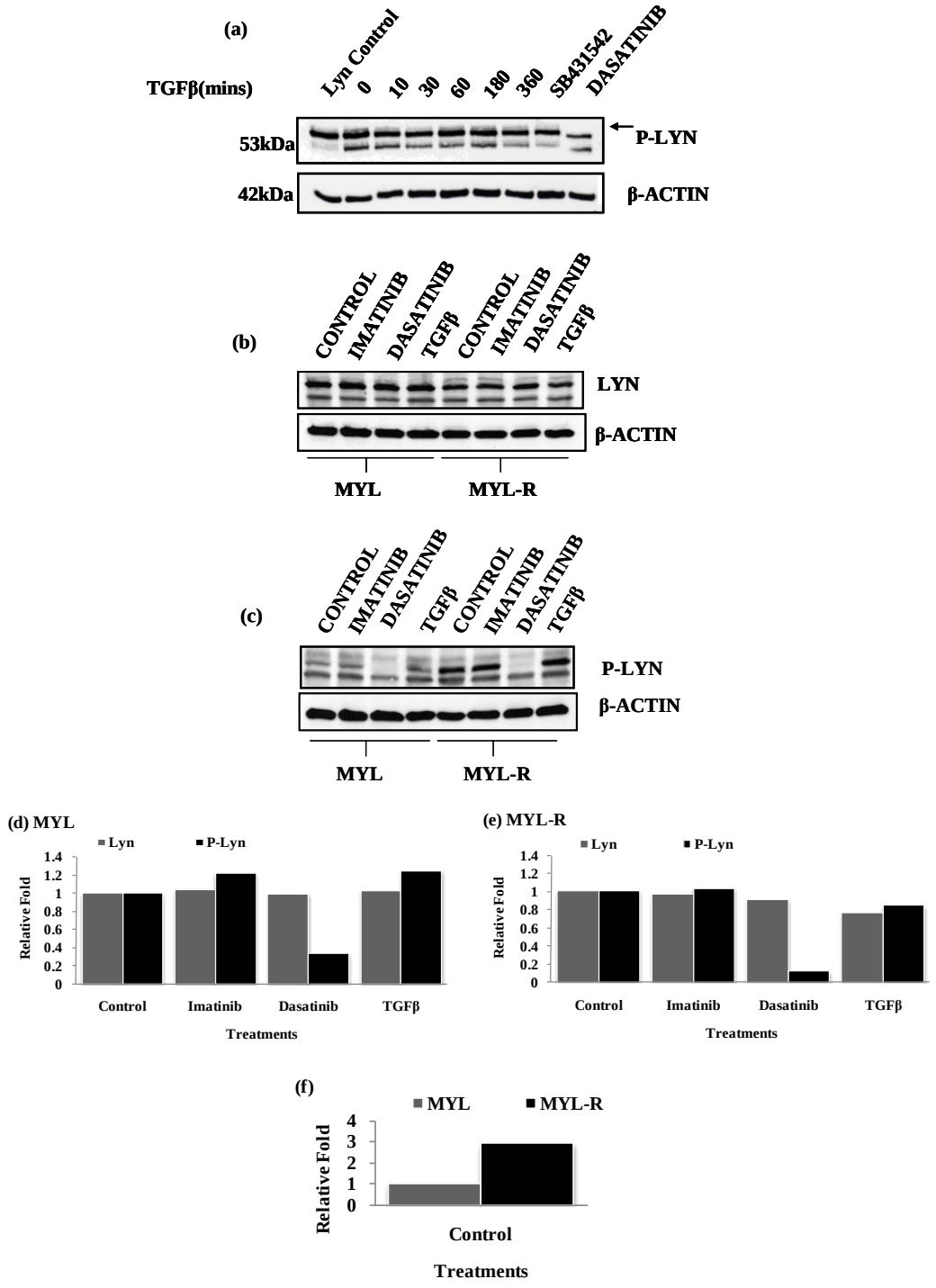


Figure 4.5 TGFβ induces f Lyn kinase activity

MYL cells were serum starved overnight in 0.5% serum containing media before treatment with (a) 1ng/ml TGFβ for a range of time points between 0-360 minutes, or with either 10μM SB431542 or 0.5μM dual Bcr-Abl/Lyn kinase inhibitor Dasatinib. Cells were treated for 60 minutes, lysed in RIPA buffer, and subsequent Western blots were probed with (a) anti-P-Lyn (b) anti-Lyn or (c) phospho-Lyn and anti- β-actin antibodies. (d-f) analysis of blot densitometery using ImageJ software.

4.3 A Candidate E3 Ligase

4.3.1 TRAF6 does not associate with Lyn kinase in the absence or presence of TGFβ

The addition of Ub to target proteins is mediated through an enzymatic cascade, resulting in an E3 ligase transferring Ub to a lysine residue of a specific target substrate. After analysing the ubiquitination of Lyn through TGFβ, the search for the E3 ligase responsible for its transfer was investigated. The E3 ligase TRAF6 was identified as a possible candidate, as it was known to associate with the activated TGFβ receptors I and II (Yamashita *et al.*, 2008). The TRAF family consist of seven members and are characterised by a C-terminal TRAF domain and a less well conserved N-terminal RING (Really Interesting New Gene) domain which is responsible for the E3 ligase activities (Twomey *et al.*, 2009). Initially TRAF6 was reported to facilitate poly lysine-63 ubiquitination, rather than lysine-48 linked chains, targeting substrates for degradation (Inoue *et al.*, 2007) TRAF6 has recently been found to be involved in the TGFβ intracellular domain-induced activation of invasive cancer promoting genes (Mu *et al.*, 2011). Therefore it was speculated it could provide the link between TGFβ and the ubiquitination of Lyn.

Four TRAF6 constructs were obtained, wild type (WT), a mutant defective in ubiquitin ligase activity (C70A), and is therefore unable to ubiquitinate its usual substrates, a mutant with a defective ubiquitin acceptor site responsible for auto-ubiquitination (K124R), auto-ubiquitination often serves as a marker of activation, and a dominant

negative mutant lacking the N-terminal RING finger and six zinc finger motifs (DN), so has no E3 ligase activity (Twomey *et al.*, 2009).

Each of the four constructs were transiently transfected into HEK293 cells and treated with or without TGFβ to look for any TGFβ-mediated effects on the protein. Analysis suggests that addition of TGFβ did not have an effect on levels of TRAF6 expression (Figure 4.6a). Whole cell extracts were also probed with β-Actin to check protein loading levels.

To establish if TRAF6 had any direct involvement with Lyn through a TGFβ-mediated interaction, HEK293 cells were transfected with WT TRAF6 and co-transfected with Lyn-Flag. Samples were co-immunoprecipitated using anti-Lyn antibodies and then probed for anti-Flag. It is important to highlight that both plasmids used were Flag-tagged. Results suggest that there is no interaction between TRAF6 and Lyn as no bands can be seen which correspond to the TRAF6 protein (Figure 4.6b)

Despite no direct interaction between Lyn and TRAF6, there was still potential for TRAF6 to be the E3 ligase responsible for Lyn ubiquitination via an intermediary protein. Therefore TRAF6 was analysed in combination with Lyn and ubiquitin. WT TRAF6, Lyn and Ub constructs were transiently transfected into HEK293 cells. Co-immunoprecipitation experiments were carried out to pull out the Flag-tagged TRAF6 and Lyn proteins and then probe with anti-HA antibodies to pick up the ubiquitinated proteins.

Unfortunately, these experiments did not yield any results at the time and are possibly something to develop again in the future. It is of note that the two proteins used for the immunoprecipitation experiments as well as both being Flag tagged are also of a similar molecular weight so would potentially be difficult to resolve through Western blotting.

I acknowledge that immunoprecipitating with Flag antibody and then probing with Lyn antibody would have been more informative in these experiments.

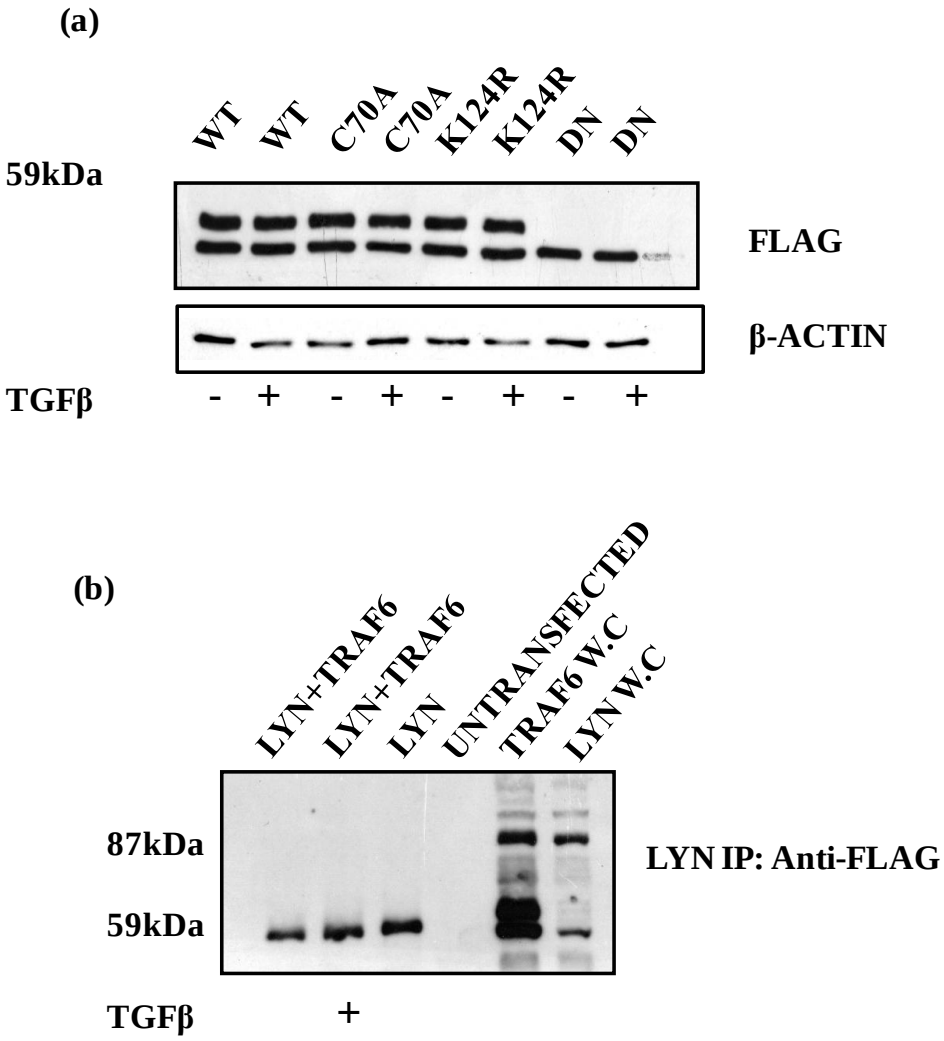


Figure 4.6 TRAF6 could be involved in Lyn activation

HEK293 cells were transfected with TRAF6 constructs, Wild Type (WT), ubiquitin ligase deficient mutant (C70A) ubiquitin acceptor site mutant (K124R). (a) Shows whole cell lysates of the WT, C70A. K124R and DN mutant constructs with and without TGFβ treatment (b) shows a co-IP of WT TRAF6 with Lyn and appropriate controls.

4.3.2 C-Cbl interacts with Lyn kinase

Alongside the TRAF6 experiments, another candidate E3 ligase was assessed to reveal if it played a role in the TGFβ mediated Lyn ubiquitination, namely the cbl E3 ligases. It was decided to investigate the involvement of these E3 ligases focusing on c-Cbl and Cbl-b. Cbl-c also exists but is mainly expressed in epithelial cells so was not considered for this analysis. C-cbl has been identified as a negative regulator of haematopoietic stem cells (Rathinam *et al.*, 2008), and therefore may play a role in the regulation of cell expansion in CML.

To look for any potential involvement, HEK293T cells were transfected with Cbl-b or c-Cbl together with Lyn to look for an association. Cells were maintained in 2% FCS media post-transfection before stimulation with TGFβ.

The HA-tag on c-Cbl plasmid was not well expressed initially, but later lysates confirmed its presence. No interaction between Lyn and cbl-b was seen in this experiment (Figure 4.7a). However, initial analysis suggested that TGFβ plays a negative role in the interaction of Lyn with c-Cbl, as addition of TGFβ appears to remove the presence of Lyn in the sample co-transfected with c-Cbl, supported by the absence of the anti-Lyn band in the TGFβ treated c-Cbl sample (Figure 4.7a).

Interestingly, after repeating the experiment and still observing no cbl-b/Lyn interaction it was decided to investigate the potential c-Cbl interaction with Lyn. In a co-immunoprecipitation experiment it appears that TGFβ increases the association between Lyn and c-Cbl, and this is seen with cells immunoprecipitated with anti-Lyn antibodies and then probed with anti-HA antibodies to pick up the associated proteins. There is a

band in the Lyn/c-Cbl TGFβ treated lane which corresponds to Lyn associated with c-Cbl (Figure 4.7b).

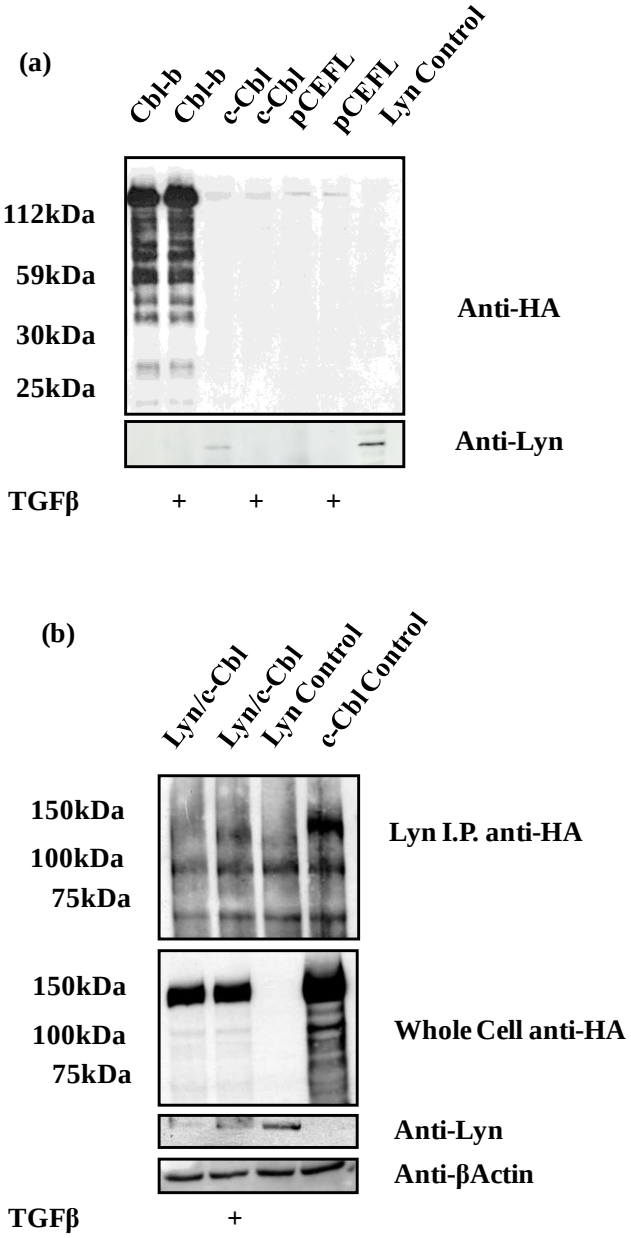


Figure 4.7 TGFβ induces Lyn/c-Cbl interaction

HEK293T cells were co-transfected with expression plasmids for Lyn and c-Cbl-HA or singly as a control. Cells were put into 2% FCS media overnight before 1ng/ml TGFβ treatment for 60 minutes. (a) Cells were lysed, and lysates mixed with anti-HA antibodies, proteins separated with SDS-PAGE, Western blot was then probed with anti-HA or Anti-Lyn antibodies (b)Cells were then lysed in 1% NP40 lysis buffer and Lyn protein immunoprecipitated overnight with anti-Lyn antibody using protein A/G agarose beads. Lysates were washed with 0.1%NP40 buffer, subsequent Western blots were probed with anti-HA, anti-Lyn and the whole cell extracts with anti-Lyn and anti-β-Actin antibodies.

Lyn is reported to be ubiquitinated by c-Cbl (Kaabeche *et al.*, 2004; Kyo *et al.*, 2003). The tyrosine kinase binding domain of c-Cbl allows it to interact with a wide range of substrates (Meng *et al.*, 1999). To assess if TGFβ was involved in c-Cbl mediated Lyn ubiquitination, different amounts of c-Cbl together with a Ub-His construct, were transfected into HEK293T cells to see if protein ubiquitination could be altered.

Cells were transfected and maintained in 2% FCS media before being stimulated with TGFβ for 60 minutes. Nickel affinity was used to pull out the Ub through its ability to bind the tagged histidine, and any bound proteins.

In control samples there is no sign of any ubiquitination occurring endogenously. After 0.25μg of c-Cbl in combination with Lyn and Ub were transfected into the cells, there is an immediate effect seen on Lyn with increased smearing, which is in the presence of TGFβ. In the 0.5μg c-Cbl sample there is further smearing seen and this is increased in the 1μg c-Cbl transfected cells in the presence of TGFβ. Interestingly, the smearing at 1μg c-Cbl can be reduced in both nickel affinity anti-Lyn and whole cell anti-Lyn panels by blocking TGFβ signalling with the SB431542 compound suggesting that TGFβ is enhancing c-Cbl mediated Lyn ubiquitination (Figure 4.8). Bcr-Abl is known to phosphorylate c-Cbl (Gotoh and Broxmeyer, 1997), which may further enhance Lyn ubiquitination in CML, but this was not looked at during this experiment.

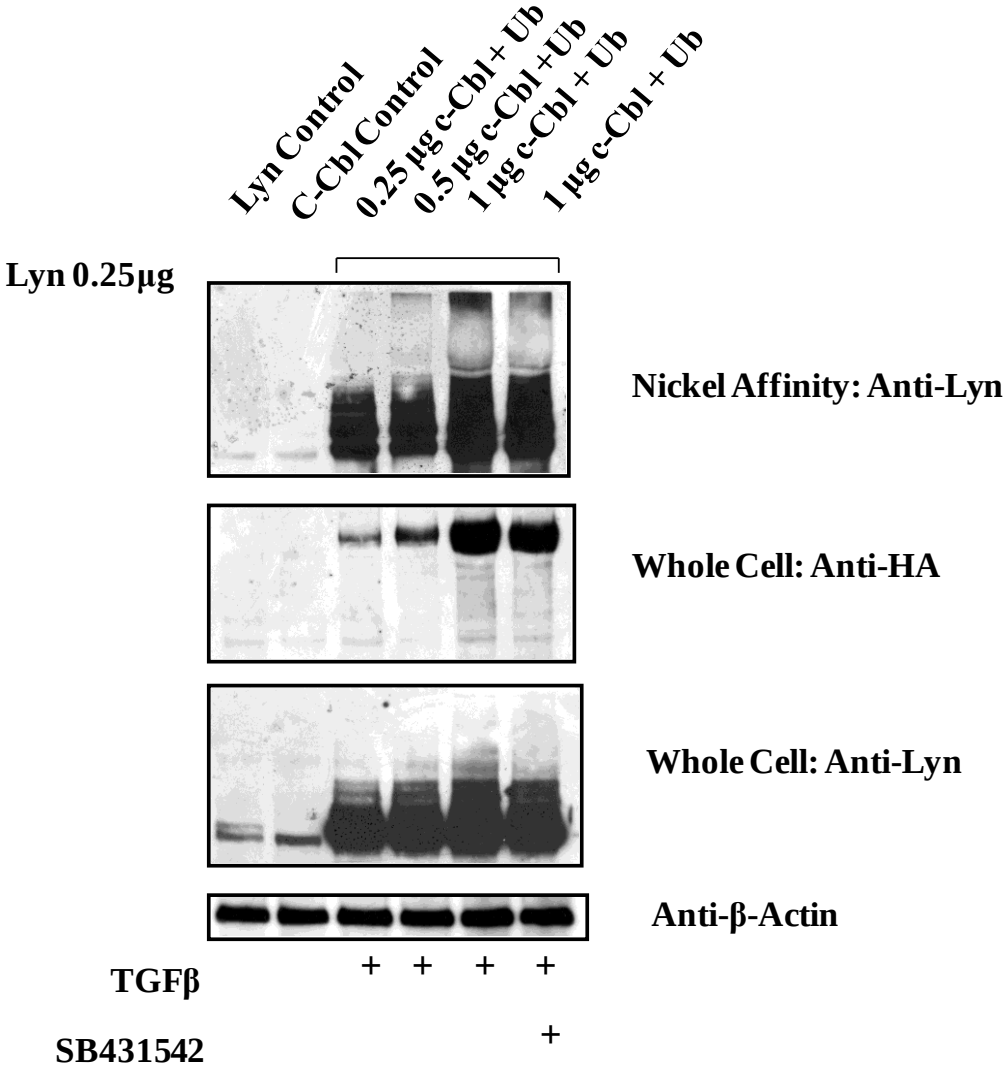


Figure 4.8 Increasing c-Cbl levels enhances Lyn ubiquitination

HEK293T cells were transfected with Lyn, c-Cbl and Ub plasmids. Cells were maintained in 2% FCS media post-transfection before TGFβ (1ng/ml) or SB431542 (10µM) stimulation for 60 minutes. Nickel beads were added to lysates overnight before blots were probed with anti-Lyn, anti-His and anti-β-Actin antibodies to analyse protein levels.

4.3.3 Lyn and c-Cbl are able to affect reciprocal protein levels

After identifying c-Cbl as a candidate E3 ligase involved in the ubiquitination of Lyn kinase, experiments were carried out in an over-expression system to see if any direct effects on protein levels were occurring. Studies have suggested that Lyn is negatively regulated by c-Cbl (Kyo *et al.*, 2003). Lyn itself has been implicated in the regulation of c-Cbl in CML cells (Wu *et al.*, 2008a).

Lyn was co-transfected with a range of c-Cbl concentrations (0.25µg-1µg). Treatments were run in duplicate with MG132 (20µM). Results show that increasing levels of c-Cbl protein cause a reduction in Lyn levels (Figure 4.9a). Over this time-point it appears that inhibition of proteasomal degradation with MG132 is not able to prevent Lyn breakdown.

Alongside this, Lyn plasmid was dosed in (0.25µg-1µg) with 0.25µg of c-Cbl. Increasing levels of Lyn are able to reduce protein levels of c-cbl (Figure 4.9b). This was done in the absence of TGFβ, inhibiting TGFβ signalling with SB431542 treatment showed no significant difference to c-Cbl levels.

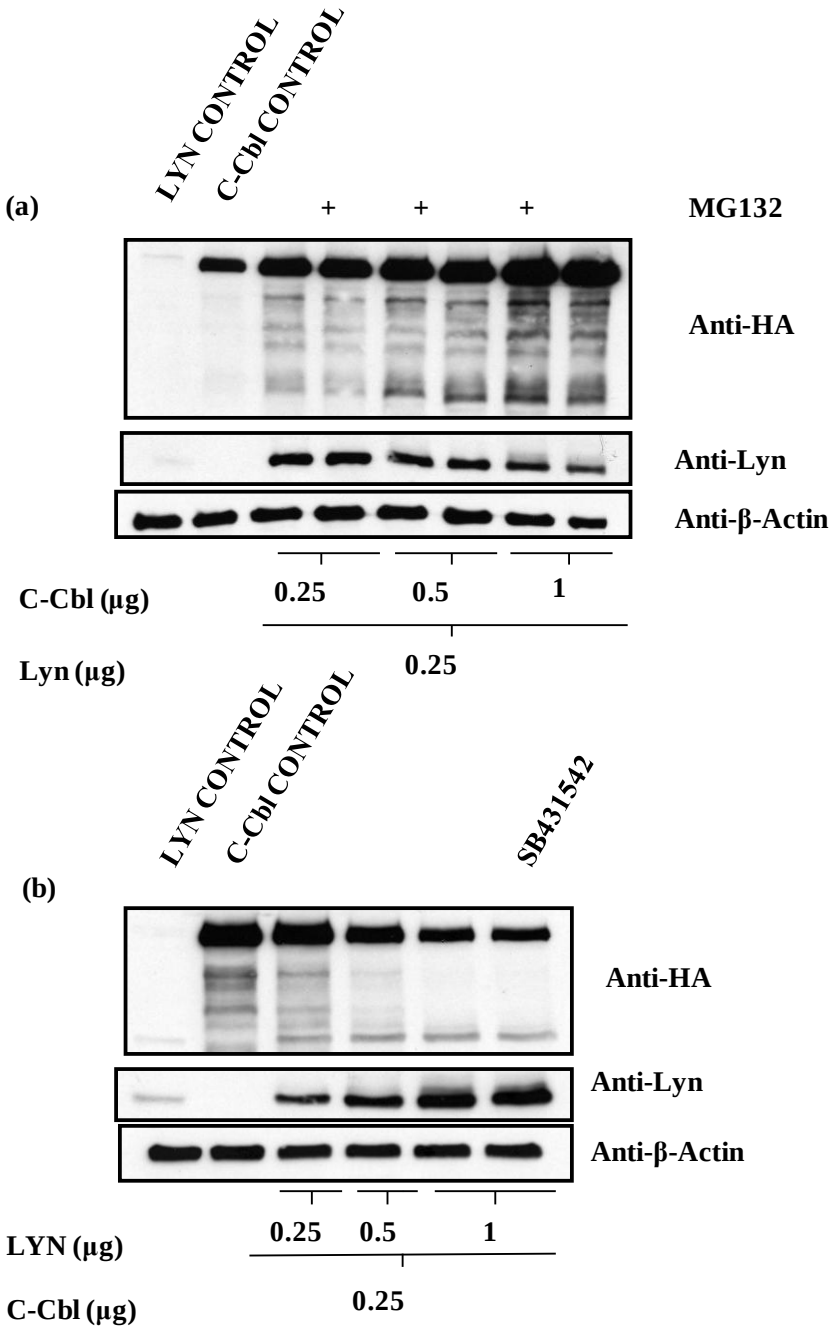


Figure 4.9 Lyn kinase and c-Cbl regulate reciprocal protein levels

HEK293T cells were transiently transfected with Lyn and c-Cbl plasmids and left for 21 hours. (a) C-Cbl was dosed in at concentrations between 0.25µg-1µg while Lyn was at 0.25µg throughout, samples were treated with and without MG132. (b) Lyn was dosed in at concentrations between 0.25µg-1µg while c-Cbl was at 0.25µg throughout.

4.4 Endogenous c-Cbl levels in MYL and MYL-R cells

c-Cbl has recently been shown to ubiquitinate Bcr-Abl, leading to its proteasomal degradation (Mao *et al.*, 2010), and c-Cbl overexpression in CML cells led to an increase in Bcr-Abl degradation (Dinulescu *et al.*, 2003). The emergence of c-Cbl mutations in a range of cancers (Caligiuri *et al.*, 2007; Klapper *et al.*, 2000; Tan *et al.*, 2010) has led to speculation of it becoming an oncogene after mutational events.

Earlier experiments have concentrated on c-Cbl in an overexpression system, next MYL and MYL-R cell lines were used to look at endogenous levels. Cells were seeded in 0.5% serum overnight, and then treated with either TGFβ (1ng/ml), SB431542 (10μM) or Imatinib (0.3μM) for 60 minutes. Cells were lysed and RNA extracted (SV total RNA isolation, Promega). RNA was then reverse transcribed to cDNA in a 20μl reaction. The cDNA from each sample was then added to a PCR mastermix before run on a thermocycler. Figure 4.10a shows the results from this experiment, in MYL cells, TGFβ causes an increase in c-Cbl levels of 30% (Figure 4.10b). SB431542 and Imatinib do not have any significant affect on c-Cbl RNA levels. MYL-R cells appear to have no changes in response to treatment, although there may be a slight increase with SB431542 treatment, but this may be explained by a slightly higher GAPDH level. To investigate the apparent TGFβ-induced increase in c-Cbl mRNA levels Taqman analysis was used to give a more sensitive and quantative readout. Existing cDNA samples of TGFβ stimulated (1ng/ml) for 60 minutes or unstimulated samples were used with Cbl Taqman primers (Applied Biosystems). The cDNA levels were normalised using 18S.

The data indicates a 8-fold increase in c-Cbl mRNA when TGFβ stimulated (Figure 4.10c).

MYL and MYL-R cells are known to have different levels of Lyn kinase (Ito *et al.*, 2007). It is proposed that these different levels are affected through TGFβ which may activate/recruit the E3 ligase c-Cbl to ubiquitinate Lyn. The following experiments were therefore designed to look at the endogenous levels of c-Cbl in the two CML cell lines.

Both cells lines were seeded at $5.0x10^5$ cells/ml and incubated overnight in 0.5% serum containing media. Cells were then stimulated with 1ng/ml TGFβ for a range of time points between 0-24 hours to look for any effects it may be having on the protein levels of the E3 ligase.

Results suggest that TGFβ does have an effect on protein levels. Initially c-Cbl was found at very low levels in the cell so an increased amount of protein was loaded onto the gel to enhance the ECL signal, consequently the β-actin signal was extremely strong. MYL cells appeared to have increased c-Cbl levels after 30 minutes of TGFβ stimulation and this appeared to follow a pattern where a reduction in levels was apparent after 60 minutes, with a subsequent rise after 180 minutes, a further reduction in levels after 360 minutes is followed by an increase after 24 hours of stimulation.

TGFβ appears to have less of an effect on MYL-R cells, although a decrease in protein levels can be seen after 30 and 60 minutes and this is increased up to the final time point of 24 hours (Figure 4.11a).

Despite the protein samples being on different blots it appeared that c-Cbl protein levels differed considerably between the cell lines, so samples from the two cell lines were run alongside each other to enable a direct comparison of protein levels. Results indicate

higher levels of c-Cbl protein in MYL cells compared to the MYL-R. Again the effects on protein levels can be seen (Figure 4.11b).

Densitometry analysis of the Western blot with Image J software confirms the increase in c-Cbl levels, after 30 minutes MYL cells have a 20% increase in levels (Figure 4.11c). This increase does not occur after 30 minutes in MYL-R cells (Figure 4.11d). Interestingly the later time points show conflicting patterns between MYL and MYL-R cells. In MYL cells c-Cbl levels appear to fluctuate, possibly representing normal protein activity and turnover. However in MYL-R cells there is an increase in c-Cbl levels from 30 minutes up to 24 hours. This may be due to the lower levels of c-Cbl in these cells, causing an increase in the time taken to see any TGFβ effect. The apparent difference in c-Cbl levels is confirmed with Image J analysis, MYL-R has 50% less c-Cbl compared to MYL cells (Figure 4.11e).

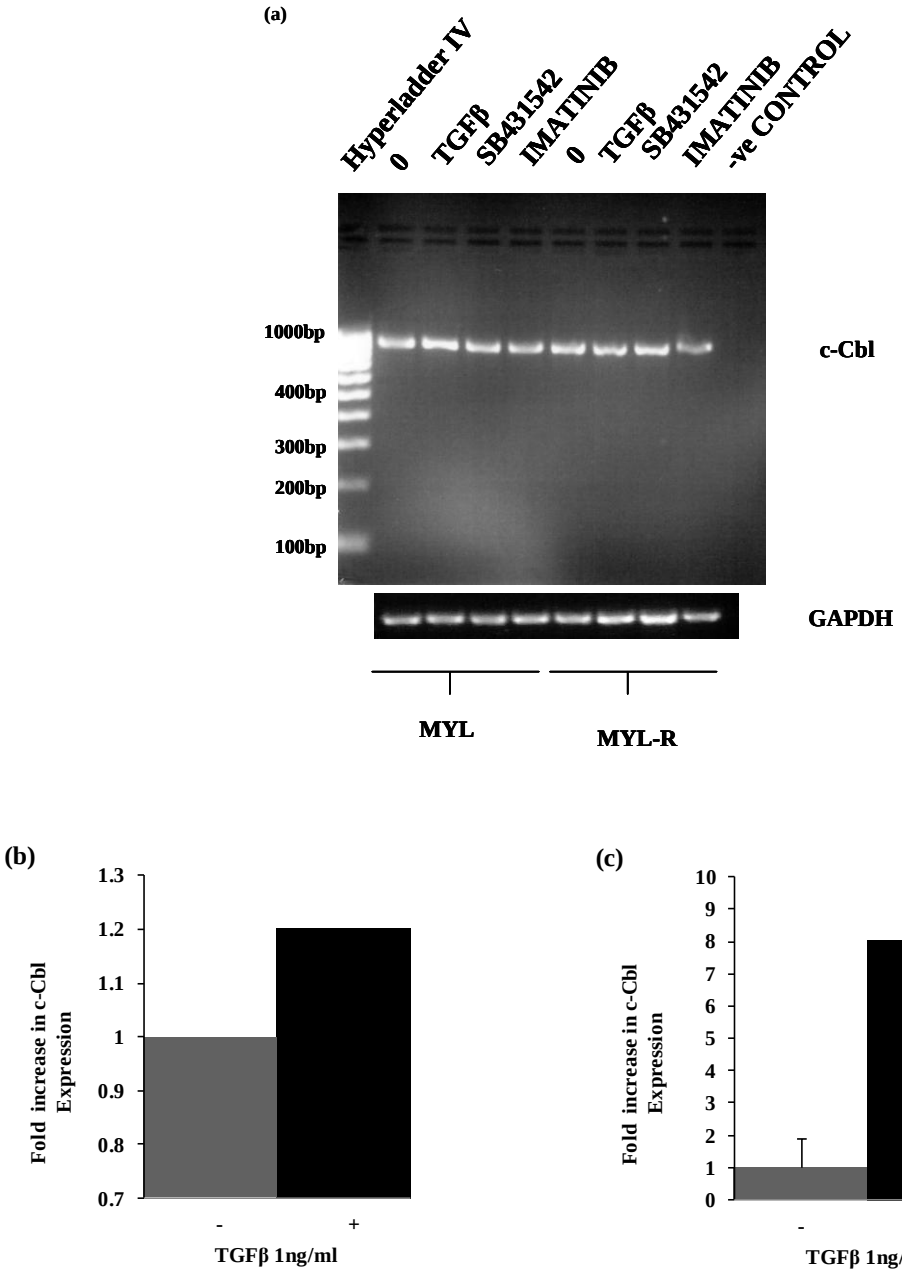


Figure 4.10 TGFβ upregulates c-Cbl mRNA levels

(a)MYL and MYL-R cells were treated with either TGFβ (1ng/ml), SB431542 (10μM), or Imatinib (0.3μM) for 60 minutes. RNA was extracted, and cDNA synthesised. Samples were then run on a thermocycler with c-Cbl primers (b) Relative levels of c-Cbl were analysed in MYL -/+ TGFβ using Image J software (c) Quantitative real time PCR data of MYL cDNA after 60minute TGFβ (1ng/ml) treatment with 5ng cDNA run with c-Cbl Taqman primers, data was normalised to an 18s control.

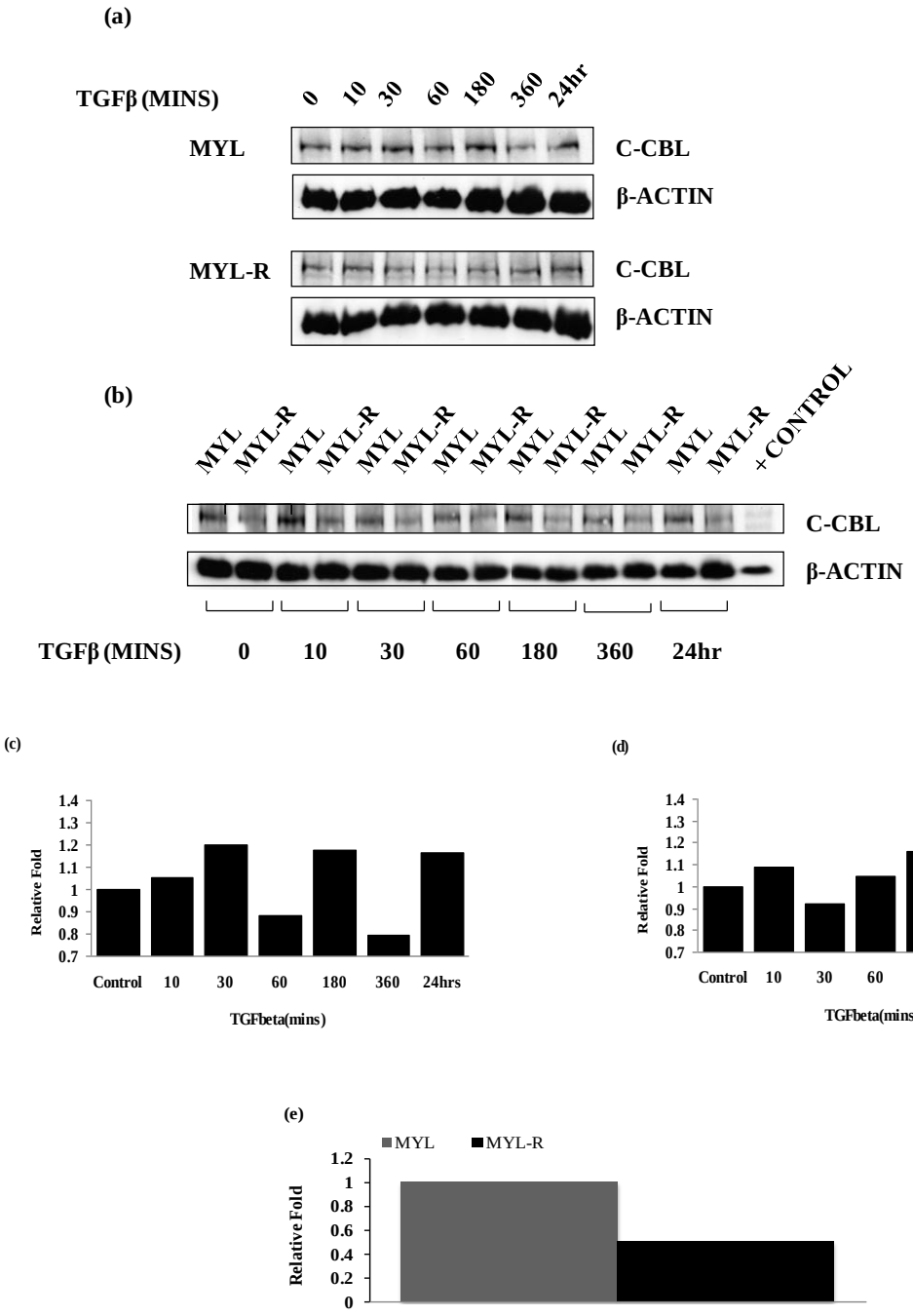


Figure 4.11 TGFβ affects c-Cbl levels in MYL and MYL-R cells

MYL and MYL-R cells were seeded in 0.5% serum-containing media overnight before being treated with TGFβ for time points ranging from 0-24 hours. (a) TGFβ time course showing C-Cbl levels in MYL and MYL-R with β-actin loading control, (b) Comparison of c-Cbl levels between MYL and MYL-R cells with β-actin loading control. (c,d) Densitometry looking at TGFβ effect on MYL and MYL-R cells (e) Densitometry analysis comparing c-Cbl levels in both cell lines, analysed using ImageJ software.

4.5 Discussion

The aim of this chapter was to analyse the effect TGFβ had on the regulation of Lyn kinase and to understand how this was mediated, and in particular assess whether the process of protein turnover mediated through Lyn ubiquitination was affected by TGFβ stimulation.

Lyn kinase has previously been shown to be up-regulated in a number of instances of Imatinib resistance in CML (Ito *et al.*, 2007; Wu *et al.*, 2008a), and TGFβ has been implicated in the regulation of Lyn (Atfi *et al.*, 1994). However, these two research areas had not previously been linked in any way. Therefore, a study to look at the effect of TGFβ on Lyn levels was undertaken. Previous research investigating Lyn ubiquitination has shown it to be mediated through a proteasome specific pathway (Bhattacharyya, 2001). Interestingly, Lyn has been shown to phosphorylate csk binding protein (Cbp) leading to the recruitment of SOCS1 which ubiquitinates Lyn (Ingley *et al.*, 2006). Lyn has also been shown to be ubiquitinated by c-Cbl in mast cells, leading to its degradation (Kyo *et al.*, 2003).

Analysing the effect of TGFβ on Lyn kinase has shown that levels of Lyn play a role in the sensitivity to TGFβ-mediated growth arrest. TGFβ is able to cause a burst of Lyn activation, measured through activating phosphorylation-specific antibody detection. Moreover, TGFβ causes Lyn ubiquitination which has been shown experimentally through endogenous and transient over-expression analyses. This ubiquitination leads to proteasomal Lyn degradation, the active Lyn levels are then replenished. This model has been shown previously in the TGFβ/BMP/Smad signalling pathway where Smad

linker region phosphorylation is necessary to cause maximal activity, yet also leads to Smad degradation through recruitment of E3 ligases (Alarcón *et al.*, 2009; Gao *et al.*, 2009). In addition, the E3 ligase c-Cbl has been identified and analysed in both endogenous and over-expression systems and is shown to be involved in the ubiquitination of Lyn.

Initial experiments highlighted TGFβ to have an effect on Lyn kinase levels in the presence of MG132 which inhibits proteasomal degradation, implicating TGFβ in protein regulation through ubiquitination (Figure 4.1). To further investigate this hypothesis, co-immunoprecipitation experiments were used to look at ubiquitination levels of Lyn over a TGFβ-stimulated time course. Whilst results were often difficult to replicate in terms of precise timings, it can be concluded that Lyn does undergo ubiquitination, seen by increased high molecular weight smearing (Figures 4.2 and 4.3).

Often closely linked with protein turnover and ubiquitination is protein activation through phosphorylation (Gao and Karin, 2005; Gao *et al.*, 2009). The EGFR requires phosphorylation before ubiquitination, with the E3 ligase binding through a phosphotyrosine binding domain (Levkowitz *et al.*, 1999), and it is possible that Lyn ubiquitination works in this way too as many parallels have been drawn between the two processes, with activated proteins often targeted for proteasomal degradation. Protein phosphorylation is also a common and well documented way of creating an E3 ligase binding site (Zhou and Snyder, 2005). CML cells were looked at in terms of their activation through activating phosphorylation-specific anti-Lyn antibodies. TGFβ appears to play a role in activation of Lyn kinase seen through bursts of activity. The increase in activated Lyn in the resistant cell line supports evidence of Lyn-mediated resistance (Ito *et al.*, 2007).

Ubiquitination analysis through nickel affinity experiments highlighted Lyn ubiquitination, which supports the activation via phosphorylation data, and shows Lyn is replenished after degradation (Figure 4.3).

In addition to ubiquitination it became apparent that this may not be the only modification occurring, with the SUMO2 protein having also been implicated in protein modification. The TGFβ receptor has been shown to be sumoylated leading to enhanced activation of the pathway (Kang *et al.*, 2008; Miyazono *et al.*, 2008). Results here suggest that Lyn is modified via sumoylation, addition of TGFβ did not appear to alter any effects of SUMO2. However, blocking the pathway with SB431542 did appear to reduce sumoylation (Figure 4.4b). Sumoylation has been highlighted to promote the stabilisation of HIF-1alpha (Carbia-Nagashima *et al.*, 2007), and SUMO3 is involved in PML nuclear stabilisation (Fu *et al.*, 2005), it is therefore likely that this modification is also involved in protein stabilisation of Lyn in CML. During experiments it was observed that Lyn protein levels were increased in the prescence of SUMO2 (Figure 4.4a and b). While the precise effects are not understood it may promote stabilisation of Lyn as it becomes more influential during the progression to a resistant phenotype and may also be involved in protein translocation, serving as a molecular chaperone, allowing Lyn to interact with downstream substrates. It would be interesting to look for direct competition for Lyn between the both Ub and sumoylation modifications, which may further contribute to the understanding of the mechanism of Lyn signalling in CML resistance.

In order to provide a link between TGFβ and Lyn ubiquitination, potential E3 ligases were investigated. A number of candidates were considered. The E3 ligase TRAF6 posed as a potential Ub donor, but co-immunoprecipitation experiments were unable to confirm a role, possibly due to the unfortunate nature of similar molecular weight and protein tags further complicating the analysis. The next candidates were the members of the Cbl family, and co-immunoprecipitation experiments show a direct interaction between Lyn and the E3 ligase c-Cbl (Figure 4.7b). Results also demonstrated direct effects of Lyn down-regulating c-Cbl and c-Cbl down-regulating Lyn protein levels (Figures 4.8 and 4.9). This down regulation of Lyn via c-Cbl has been shown previously in the rat leukaemia cell line RBL-2H3 (Kyo *et al.*, 2003), although there was no suggestion there however of Lyn negatively regulating c-Cbl. This result is supported endogenously in the two CML cell lines where c-Cbl is seen at higher levels than the MYL-R cells which are known to be over-expressing Lyn, suggesting that the higher levels of Lyn in MYL-R cells might be due to the reduction in c-Cbl levels. However, it is also possible that the higher Lyn levels in these cells may be due to a reduction in c-Cbl levels. Preliminary results of the effect of TGFβ on c-Cbl transcription through RT-PCR suggested it was able to induce c-Cbl transcription in MYL cells, although only slightly above basal levels. The more sensitive Taqman analysis of this effect showed TGFβ to have a marked effect on inducing c-Cbl transcription.

There have been many other studies which have looked at the impact of Lyn kinase in drug resistance. One study suggests Lyn activation plays a role in Imatinib resistance, but suggests no mechanism for the activation (Wu *et al.*, 2008a). Another study by the same group suggest Lyn is responsible for Bcr-Abl phosphorylation, which has been

found by numerous groups and is combined with reports of Lyn being a down-stream substrate of the Bcr-Abl signalling pathway. Bcr-Abl silencing has also been shown to reduce c-Cbl tyrosine phosphorylation (Wu *et al.*, 2008b), which may promote drug resistance. The recent introduction of Nilotinib treatment for newly diagnosed CML sufferers has increased research into the drug. Experiments to generate a Nilotinib resistant cell population have found Lyn activation to be involved in cell survival (Okabe *et al.*, 2011). Together, these results suggest a signalling network existing between Bcr-Abl and Lyn which is mediated through a range of adaptor molecules such as Gab2, c-Cbl and CrkL.

Jak2 inhibition has been shown to increase the serine/threonine phosphatase PP2A and in turn reduce levels of activated Lyn (Samanta *et al.*, 2009). Interestingly, inhibition of the Jak/Stat signalling pathway shows reduction in TGFβR (Lakner *et al.*, 2010), suggesting levels of the receptor could be playing a role in CML.

In conclusion, the data presented in this and the previous chapter suggests that TGFβ plays a role in regulating Lyn kinase protein levels with bursts of activation, mediated through its phosphorylation. This phosphorylation then targets Lyn for proteasomal degradation via c-Cbl-mediated ubiquitination.

5. FINAL DISCUSSION

5.1 Introduction

The TGFβ signalling pathway has been well studied in haematopoietic cell function and development (Blank and Karlsson, 2011; Larsson and Karlsson, 2005). Under normal conditions the TGFβ influence on these cells is to induce growth arrest through increases in p21 and p27 (Cheng *et al.*, 2001). The study into haematological diseases such as leukaemia unveiled abnormalities in this signalling pathway (Imai *et al.*, 2001; Rooke *et al.*, 1999).

In the case of CML, a disease driven by the presence of Bcr-Abl, TGFβ signalling has been found to be up-regulated (Møller *et al.*, 2007). If detected and treated early, CML can be controlled. In many cases however, the disease cannot be completely eradicated, and relapse can often occur in apparently disease free patients (La Rosée and Deininger, 2010). It is this unresolved issue which renders further research into refractory disease necessary.

In this thesis I have looked at the mechanistic role of TGFβ in late disease stage CML cells alongside over-expression cell systems in order to uncover the TGFβ involvement in drug resistance to Imatinib, paying particular attention to Lyn kinase and how this protein may be regulated in CML resistance.

5.2 Bcr-Abl up-regulates TGFβ signalling and may overcome TGFβ-mediated growth arrest

Initially, this project set out to analyse TGFβ signalling in CML. This was carried out through the utilisation of two TGFβ responsive cell lines HEK293 and COS-1. These cells were transfected with a luciferase reporter plasmid containing 12 CAGA repeat elements, which is the transcriptional binding site for Smad signalling. In addition, a Bcr-Abl plasmid was transfected into these cells along with TGFβ stimulation.

Analysis of the effect of Bcr-Abl, showed it could up-regulate TGFβ transcriptional activity in a TGFβ concentration dependent manner in both of these independent cell lines Figures 3.1a and b.

To further investigate the effect of Bcr-Abl on TGFβ signalling it was decided to see if the increase in signalling could be seen to be having an effect on cell growth. For this, a more relevant cell system was used: the mouse haematopoietic 32D and the stably expressing Bcr-Abl variant 32Dp210. These experiments highlighted Bcr-Abl in preventing the cytostatic effects of TGFβ, seen in the non-expressing cells. Although this was analysed through cell counts, which was quite a crude approach, it clearly demonstrates the selective growth advantage that Bcr-Abl presents to these haematopoietic cells, and supports the previous results of Bcr-Abl up-regulating TGFβ signalling. Evidence presented here supports that of another study which concluded that Bcr-Abl suppresses the cytostatic effects of TGFβ in CML (Atfi *et al.*, 2005).

5.3 MYL and MYL-R cells differ in their TGFβ responsiveness

TGFβ is known to have cytostatic effects on normal haematopoietic cells (Larsson and Karlsson, 2005). Human CML cell lines were obtained in the form of MYL and MYL-R cells, with MYL-R cells having increased resistance to Imatinib.

The first aim with these cells was to characterise them and establish their sensitivity to TGFβ. This was carried out using MTS assays and showed the MYL-R cells to be significantly more resistant to Imatinib than MYL cells, despite these cells themselves showing a degree of tolerance. It was found that the two cell lines exhibit different sensitivities to TGFβ. The MYL cells showed slight growth inhibition whereas MYL-R cells showed no TGFβ-mediated inhibition. Once an optimal set of conditions had been defined, the experiments were repeated with TGFβRI inhibition using SB431542 to see if this could reverse the effects seen by TGFβ stimulation. The TGFβ response remained the same, but interestingly MYL-R cells responded to the SB431542 treatment, showing a marginal increase in cell proliferation. This suggested that the MYL-R cells may have retained a degree of TGFβ sensitivity, but that this response may be masked under normal conditions in these cells. This could be due to an over-active TGFβ signalling pathway, and by inhibiting this, cell proliferation is enhanced.

5.4 Resistant CML cells have enhanced TGFβ signalling

As previously discussed, Bcr-Abl up-regulates TGFβ signalling in HEK293 and COS-1 cell lines. The aim of the next section was to study the effects of TGFβ signalling endogenously in a human CML system. The neomycin resistance gene was cloned into the previously used pGL3b-$CAGA_{12}$-luc vector, enabling successfully transfected cells to be selected through antibiotic resistance. Both MYL and MYL-R cells were electroporated to insert the plasmid, but stable cells were unable to be sustained.

Alternatively, endogenous Smad3 levels were analysed in the two cells lines. Both cell lines showed no changes of overall Smad levels for the duration of the experiment. Interestingly, MYL-R cells showed agreater activation response of Smad3 than MYL cells. This evidence correlated with the hypothesis that MYL-R cells have increased endogenous levels of TGFβ, despite the un-stimulated sample having no active Smad3, and this may be explained by transient activation of the pathway in these cells. When phosphorylated Smad3 was analysed in response to treatments in the absence and presence of TGFβ, it was interesting to find that Dasatinib was more effective in reducing phosphorylated Smad3 in the MYL-R cells than in MYL cells (Figure 3.9). Since this is specific for targeting Lyn kinase in addition to Bcr-Abl, it suggests Lyn is more actively involved in the signalling pathway in the resistant cells, while MYL cells are less dependent on Lyn activation.

TGFβ is known to activate the PI3K/Akt pathway (Horowitz *et al.*, 2004), and Akt is a known substrate of Bcr-Abl (Atfi *et al.*, 2005). Analysis of Akt levels in the two cell lines over a shorter time course indicated increased levels within MYL-R cells.

Interestingly, Akt has been implicated in the sequestering of Smad3 from the nucleus through direct physical interaction, while other reports suggest an Akt dependent suppression of Smad3 via mTOR (Song *et al.*, 2006). Therefore, this increase in Akt levels in the MYL-R cells may be responsible for attenuating the TGFβ response seen in these cells.

5.5 Does combination therapy enhance Imatinib-mediated cell death?

One of the theories of CML disease persistence is that there exists a subset of quiescent CML stem cells which is resistant to Imatinib. This is believed to occur because of signals preventing cells from entering the cell cycle. As already mentioned, TGFβ is a potent cell cycle inhibitor of normal haematopoietic cells (Batard *et al.*, 2000). It is understood that the cytostatic effects of these quiescent cells is due to the over-expression of TGFβ which is known to be driven by Bcr-Abl. Therefore in theory, if these arrested cells are able to start dividing again, they should become sensitive to the effects of Imatinib. In addition to targeting this known population of stem cells, research has found that there are a group of granulocyte macrophage progenitor cells which have the ability to require self-renewal properties during advanced stage disease (Jamieson *et al.*, 2004). This suggests that in addition to a clonal population of stem cells, disease evolution can also generate these more Imatinib resistant cell types.

In order to overcome this resistance, a combined treatment approach of TGFβ inhibition and Imatinib treatment was compared to Imatinib treatment alone. MTS results showed MYL cells to have a slight decrease in cell survival with the combined treatments, compared to Imatinib alone. No difference was seen in the MYL-R cells. This was interesting because previous SB431542 treated MYL-R cells had shown an increase in cell proliferation assays, suggesting the combination treatment would have successfully increased Imatinib-mediated apoptosis. However, the threshold at which Imatinib is able to induce cell death may be a critical difference between the two cell lines.

The unresponsiveness of MYL-R cells to this particular treatment regime meant MYL cells were used for the next analysis. To confirm the findings of increased Imatinib induced cell death, a PARP-cleavage assay was developed. Analysis confirms that after 12 hours of combination treatment there is an increase in PARP cleavage indicating apoptosis.

Finally, to support the previous data from combination treatments, cell cycle analysis was undertaken to show the effects of the treatments on the cells. Both TGFβ and SB431542 exhibited results in accordance with the previous findings of growth inhibition and increased proliferation respectively. When SB431542 treatment was combined with Imatinib a large increase in the Sub-G0 cell population was apparent which has previously been referred to in published data as apoptotic cells (Lu *et al.*, 2010). Overall, this data suggests that by inhibiting TGFβ signalling in combination with Imatinib, there is enhanced apoptosis within the CML cell population.

5.6 TGFβ causes Lyn ubiquitination

TGFβ was initially found to down-regulate Src kinases in the prostate carcinoma cell line PC3 (Atfi *et al.*, 1994). This data only analysed the effect during time-points up to 30 minutes, and indeed results presented in this thesis agree with an initial down-regulation of Lyn, mediated by TGFβ. Lyn kinase is implicated in Imatinib resistance in CML (Ito *et al.*, 2007; O'Hare *et al.*, 2008). Therefore, the potential TGFβ effect on Lyn in the CML context was investigated.

Initial experiments aimed to analyse the effects over a TGFβ time course. Interestingly, results immediately indicated a role for TGFβ in Lyn regulation within CML. Lyn co-transfected with Ub suggested an increase in Lyn ubiquitination. Alongside this, Lyn activation through phosphorylation was identified and implicated in the cascade of ubiquitination, together with evidence showing increased Lyn activation in MYL-R cells. Analysis of ubiquitination using a Ub-His tagged plasmid with its nickel affinity properties went on to further confirm a role for TGFβ-induced ubiquitination of Lyn.

Lyn was also found to undergo sumoylation via SUMO2. This may affect protein stability as well as localisation, as Lyn levels appeared to increase in the presence of SUMO2. This type of modification has been implicated in cancer, where Ubc9, the SUMO E2 enzyme has been found to be up-regulated leading to increased cancer cell growth (Moschos *et al.*, 2010). Currently there is no published work on Lyn sumoylation, so these initial findings could pave the way for a more in-depth analysis of the effects of this modification in CML resistance. The effect of SUMO2 in CML

requires much further research but could potentially pose as a therapeutic target if like other cancers it is found to be deregulated.

5.7 The E3 ligase c-Cbl is involved in Lyn ubiquitination

After implicating TGFβ in the ubiquitination of Lyn kinase, the next set of experiments aimed to determine the E3 ligase responsible.

Due to the TRAF6 involvement and known association with the TGFβRI (Sorrentino *et al.*, 2008), it was looked at as the first potential candidate. No association between Lyn and TRAF6 was found, nor did there appear to be any enhanced ubiquitination occurring.

The E3 ligase c-Cbl has now been intensively investigated (Ogawa *et al.*, 2010), yet at the time of this research was less well defined with respect to Lyn kinase in CML. Initial experiments showed a direct interaction between the two proteins which appeared to be TGFβ dependent. When increasing amounts of the E3 ligase were dosed in with Lyn and Ub plasmids, an increase in high molecular weight bands was seen.

c-Cbl had previously been implicated in Lyn regulation through ubiquitination in rat basophil leukaemia (Kyo *et al.*, 2003). Experiments in this thesis show that not only is c-Cbl able to negatively regulate Lyn, in contrast, Lyn is able to negatively regulate c-Cbl, suggesting a negative feedback loop as an additional mode of control.

Through analysing endogenous c-Cbl, it is apparent that MYL-R cells have a lower level of the E3 ligase, supporting evidence of the negative regulation by Lyn. There are many papers detailing c-Cbl as an anti-oncogenic protein, largely due to its role in targeting proteins for proteasomal degradation (Miyake *et al.*, 1998). It is believed that mutations in this protein lead it to acquire oncogenic functions (Dunbar *et al.*, 2008; Loh *et al.*, 2009).

5.8 Conclusion

In conclusion, TGFβ is shown to play a varied role within the context of CML, initially having a strong inhibitory role during normal haematopoiesis and TGFβ sensitive CML cells. It is the later stage disease, where Imatinib resistance occurs, that was the main focus of this research. A combination approach, enabling the quiescent sub-population of cells to enter back into the cell cycle and become sensitive to the Imatinib-induced cell death, shows enhanced cell death compared to Imatinib alone, paving the way for dual therapy approaches to be investigated further.

Results presented here also show TGFβ to be involved in the activation and ubiquitination of Lyn kinase which is known to be involved in drug resistance (Donato *et al.*, 2003; Ito *et al.*, 2007). The activation of Lyn is followed by its ubiquitination and subsequent degradation, and Lyn is then replenished with active forms of the protein. The ubiquitination is mediated through the E3 ligase c-Cbl, which is potentially TGFβ regulated.

Therefore, I believe that this thesis has demonstrated an important and diverse role for TGFβ in CML, highlighting a dual therapy approach to overcome drug resistance combined with an analysis of the role of TGFβ in regulating Lyn kinase ubiquitination.

At this stage I feel it is important to relate the findings of this thesis to CML disease as a whole and to try to rationalise the results. The effect of Lyn kinase is evidently involved in drug resistance in the case of MYL/MYL-R cell lines, and this is known to not exclusively be the only route of resistance. However, uncovering the network involved in Lyn-mediated resistance will develop a further understanding of the complexity

within CML. The discovery of the TGFβ involvement in Lyn-mediated resistance is a novel one and presents a further role for TGFβ in CML. The ability of TGFβ to cause activation of Lyn, and also induce the E3 ligase responsible for its degradation, suggests an extremely well regulated and balanced signalling network. Resistant cell populations have been able to reduce levels of the E3 ligase preventing degradation of Lyn, leading to persistent activation.

Overall, within this diverse signalling network it is extremely difficult to address aspects of the disease as isolated factors. TGFβ can promote cell quiescence, leading to residual disease, with suggestions of inhibiting TGFβ signalling to combat this. Yet, here it is shown that TGFβ can promote c-Cbl induction, leading to Lyn ubiquitination which could potentially reduce resistance if over-expressed. Therefore, what is needed is a tailored balance to address factors as they become apparent within CML development.

From these results I present a diagram of this signalling network detailing the interconnections between TGFβ, Bcr-Abl, Lyn kinase and c-Cbl (Figure 5).

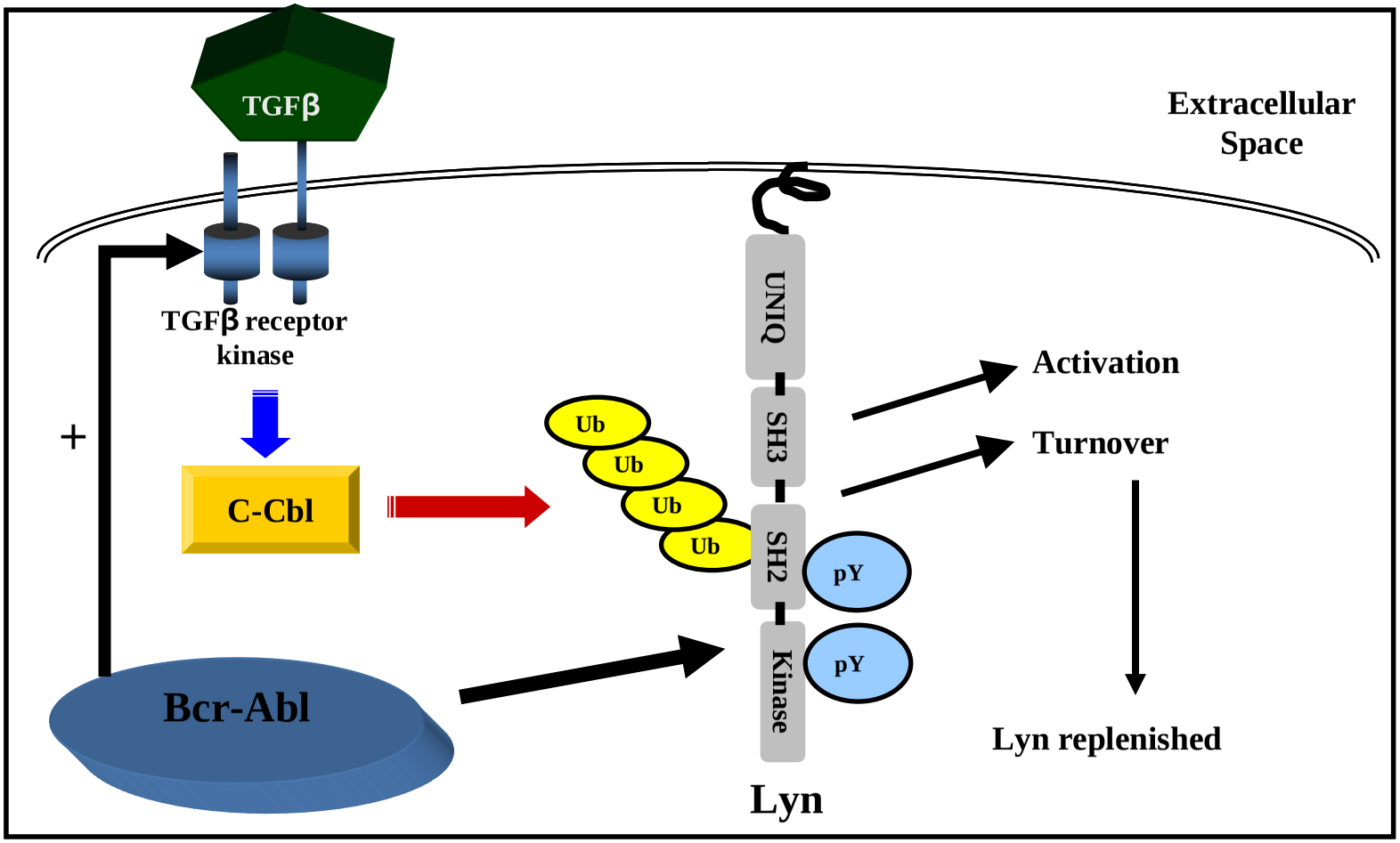


Figure 5: Proposed signalling network involving TGFβ, Bcr-Abl, Lyn and c-Cbl in CML

5.9 Future Work

The research into CML drug resistance has intensified significantly within the last few years. The involvement of TGFβ up until now has focused primarily on its effect on cell growth. Results presented in this thesis highlight its involvement in the activation and ubiquitination of Lyn kinase which has been directly linked to drug resistance.

There are always new ideas and further studies required to progress with current theories, unfortunately due to time constraints not all of these were achievable. Therefore I have proposed a number of future studies which would go on to develop the research presented here;

- Analyse further the TGFβ effect on c-Cbl. Using a haematopoetic stem cell model to look at relative levels of c-Cbl and how they are affected by the presence of TGFβ. Analysing the interconnections between Bcr-Abl, c-Cbl and Lyn with TGFβ in early CML cells would allow for a greater understanding of disease development and the signalling occurring during later disease stages. In addition, analyse the c-Cbl promoter for potential TGFβ regulatory sites. This thesis only looked at the effect on Smad3 levels, it would be informative to analyse the Smad2 signalling both endogenously and in overexpression studies using the DE4 Smad2-specific reporter plasmid.
- Using c-Cbl siRNA to assess the effect on Lyn levels and drug resistance within CML cells. A c-Cbl knockdown will allow a study into whether it develops a more resistant and aggressive phenotype.

- Obtaining primary CML cells and undertaking these experiments will provide a much clearer picture as to the significance and relevance of previous data obtained through over-expression and cell line systems.
- Developing further the dual therapy approach again using primary CML cell lines in order to ascertain the clinical significance of previous PARP and cell cycle data. Analysis using Annexin V staining of cells will give a more accurate and sensitive readout of apoptotic cells. In addition, analysing the relative levels of TGFβ sensitive regulators of the cell cycle such as p21. This may provide informative results as to the precise mechanisms involved in the cycling of MYL and MYL-R cells and indicate an explanation to the observed differences in their TGFβ response.

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