The Role of Dysregulated Transcription Factors in Drug Resistant CML

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Abstract

The kinase activity of BCR-ABL1 is central to chronic myeloid leukaemia (CML) pathology. Clinical treatment of CML with the kinase inhibitor Imatinib Mesylate (IM) is a fundamental success. However, 15-20% of CML patients develop drug resistance. For the majority of patients, the challenge is to understand how leukaemic cells can survive in the absence of BCR-ABL1 activity. To address this issue, a model of CML drug resistance was previously established using KCL22 cells. Here, the drug resistant cells recapitulate clinical findings and survive in a BCR-ABL1 kinase independent manner. Noting that the oncogene is a multi-domain complex protein, the functional relevance of the other domains of BCR-ABL1 in compensating for the loss of kinase activity was explored. In contrast to the parental KCL22 cells, depletion of the oncogene within the drug-resistant clones failed to induce apoptosis but rather the cells underwent growth arrest. A novel model is proposed for how these cells maintain a leukaemic phenotype in kinase independent CML. Here, the other domains of BCR-ABL1 regulate cell proliferation, the Src kinases inhibit apoptosis while transcription factors block cellular differentiation.

The proposed mechanism of how cells can survive in the absent of BCR-ABL1 kinase activity is based on the findings from a single cell line. To compliment the KCL22 model, a second drug resistance CML line (EM2 cells) was generated. Interestingly, while both KCL22 and EM2 models were kinase independent, they contrasted in terms of the transcription factors deregulated upon drug resistance. Lineage phenotyping of these cells identify them as erythroid and GMP respectively and prompted the 'cell of origin' hypothesis whereby the identity of the transformed progenitor dictates the nature of the transcriptome changes acquired upon drug resistance. Furthermore, clonal analysis of different EM2 single cell clones highlighted the heterogeneity of drug resistant CML and suggested that the mechanism of resistance is innately established within the cells prior to any exposure to drug.

The deregulated expression of transcription factors is a key contributing factor in the pathology of many blood cancers. Targeting these factors with the aims of restoring their normal functional activity has significant potential in the therapy of leukaemias. Notably, the expression of the SOX4 transcription factor is strongly induced upon KCL22 drug resistance. Depletion of SOX4 within drug resistant KCL22 cells failed to confer any overt phenotypic changes. However, these

studies unravel a possible redundant network among the SOXC family members (SOX4, SOX11 and SOX12).

In summary, the work herein provides a greater understanding of CML and identifies, for the first time, the potential importance of the other domains of BCR-ABL1 in establishing kinase independency. Moreover, this study suggests a personalised stratification of CML patients where future treatment of drug resistance could depend on the lineage identity of the original transformed clone.

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ABL1	Abelson murine Leukaemia viral oncogene homolog 1
AEBP1	AE Binding Protein 1
ALAS2	5'-Aminolevulinate Synthase 2
ALL	Acute Lymphoblastic Leukaemia
ALOX5	Arachinodate 5-Lipoxygenase
AML	Acute Myeloid Leukaemia
APC/Cdh1	Anaphase promoting complex/cyclome
ARRDC4	Arresting Domain Containing 4
ATF3	Activating Transcription Factor 3
AZU1	Azurocidin 1
BC-CML	Blast Crisis Chronic Myeloid Leukaemia
BCL7A	BAF Chromatin Remodeling Complex Subunit 7A
BCL11A	B-cell CLL/lymphoma 11A
BCOR	BCL6 Co-repressor
BCR	Breakpoint Cluster Region Protein
BAD	BCL2 Associated Agonist of Cell Death
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
C/EBP-α	CCAAT-Enhancer Binding Protein Alpha
CHAC1	ChaC Glutathione Specific-Gamma-Glutamylcyclotransferase 1
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukaemia
CMP	Common Myeloid Progenitor
CP-CML	Chronic Phase Chronic Myeloid Leukaemia
CREB5	cAMP Responsive Element Binding Protein 5
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9
CrkL	CRK-Like Proto-Oncogene, Adaptor Protein
Cul1	Cullin 1
DACH1	Dachshund Homolog 1
DEAF1	Deformed Epidermal Autoregulatory Factor 1 Homolog
DNMT3A	DNA-Methyltransferase 3 Alpha
EBF3	EBF Transcription Factor 3
EGF	Epidermal Growth Factor
ELF1	E74 Like ETS Transcription Factor 1
EMI1	F-Box Protein 5
EM2-P	EM2 Imatinib-sensitive parental single-cell clone
EM2-R	EM2 Imatinib-resistant single-cell clone
ETV6	ETS Variant 6
E2F	E2F Transcription Factor
FDR	False Discovery Ratio
FOXA3	Forkhead Box Protein A3
FOXL2	Forkhead Box Protein L2
GAL	Galanin and GMAP Prepropeptide
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA1	Globin Transcription Factor 1
GEO	Gene Expression Omnibus
Gfi1	Growth Factor Independent 1 Transcriptional Repressor
GFP	Green Fluorescent Protein
G-CSFRA	Granulocyte-Colony Stimulating Factor Receptor Alpha
GM-CSFRA	Granulocyte-Macrophage Colony Stimulating Factor Receptor Alpha
GMP	Granulocyte/Monocyte Myeloid Progenitor
GRB2	Growth Factor Receptor Bound Protein 2
GSEA	Gene Set Enrichment Analysis
ID2	Inhibitor of DNA Binding 2

IGF	Insulin Like Growth Factor
IM	Imatinib Mesylate
IMr	Imatinib-resistant
P	Parental cells
Hck	Hck Proto-oncogene
HEMGN	Hemogen
HGF	Hepatocyte Growth Factor
HMG	High-Mobility Group
HOXA5	Homeobox A5
HPRT	Hypoxanthine Phosphoribosyltransferase 1
HSC	Haematopoietic Stem Cell
ID2	Inhibitor of DNA-binding 2
IRES	Internal Ribosomal Entry Site
Kit	KIT Proto-Oncogene Receptor Tyrosine Kinase
LMPP	Lymphoid-Primed Multipotent Progenitor
LSC	Leukaemia Stem Cell
Lyn	Lyn Proto-oncogene
MAPK	Mitogen-activated Protein Kinase
MEF2C	Myocyte Enhancer Factor 2C
MEF2D	Myocyte Enhancer Factor 2D
M-CSFR	Monocyte Colony Stimulating Factor Receptor
MEP	Megakaryocyte/Erythrocyte Progenitor
MPP	Multipotent Progenitor
mTOR	mechanistic Target of Rapamycin
NF-E2	Nuclear Factor, Erythroid 2
NFIX	Nuclear Factor I X
NKX2-2	NK2 Homeobox1
PAX5	Paired Box 5
PCGF2	Polycomb Group Ring Finger 2
PCR	Polymerase Chain Reaction
pCrkL	Phosphorylated CrkL
PDK1	Pyruvate Dehydrogenase Kinase 1
Ph	Philadelphia Chromosome
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PITX1	Paired Like Homeodomain 1
PKLR	Pyruvate Kinase L/R
pSTAT5	Phosphorylated STAT5
PU.1	Spi-1 Proto-Oncogene
RAS	Rat Sarcoma Viral Oncogene
RB1	Retinoblastoma Transcriptional Corepressor 1
RBX1	Ring Box 1
RFP	Red Fluorescent Protein
RFX3	Regulatory Factor X3
Rho/GEF	Guanine Nucleotide Exchange Factor Rho
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RUNX1	Runt-related Transcription Factor 1
RUNX2	Runt-related Transcription Factor 2
SCL	Stem Cell Protein
SCF	SKP/Cullin/F-Box containing complex
SFK	Src Family Kinase
SH	Src-homology domain
shBCRendo	shRNA targeting the endogenous BCR mRNA
shLuc	shRNA targeting the Luciferase mRNA
shRNA	small-hairpin RNA
siBCR	Small-interference RNA targeting the BCR mRNA
SLC7A11	Solute Carrier Family 7 Member 11

siRNA	Small-interference RNA
siSOX4	Small-interference RNA targeting the SOX4 mRNA
SKP2	S-phase Kinase Associated Protein 2
SOX	SRY-related HMG-Box
SOXC	SRY Group C
SOX4	SRY-Box 4
SOX11	SRY-Box 11
SOX12	SRY-Box 12
SOS	Son of Sevenless
SRSF2	Serine and Arginine Rich Splicing Factor 2
SSX1	Synovial Sarcoma X Breakpoint 1
SSX2	Synovial Sarcoma X Breakpoint 2
STAT4	Signal Transducer and Activator of Transcription 4
STAT5	Signal Transducer and Activator of Transcription 5
tABL1	Total ABL1 protein
TAF12	TATA-Box Binding Protein Associated Factor 12
TCEA3	Transcription Elongation Factor A (SII), 3
TCEAL1	Transcription Elongation Factor A (SII)-like 1
TCEAL4	Transcription Elongation Factor A (SII)-like 4
TDRD9	Tudor Domain Containing 9
TFRC	Transferrin Receptor
TLR2	Toll-like Receptor 2
TLR4	Toll-like Receptor 4
TXNIP	Thioredoxin Interacting Protein
ZNF25	Zinc Finger Protein 25
ZNF91	Zinc Finger Protein 91
ZNF256	Zinc Finger Protein 256
ZNF302	Zinc Finger Protein 302
ZNF331	Zinc Finger Protein 331
ZNF626	Zinc Finger Protein 626
ZRSR2	Zinc Finger CCCH-Type, RNA Binding Motif and Serine/Arginine Rich 2
16P	Parental single-cell clone 16 from the KCL22 cell line
16IMr	Resistant derivative from the 16P cell line
15P	Parental single-cell clone 15 from the KCL22 cell line
15IMr	Resistant derivative from the 15P cell line

Chapter 1: Introduction

1.1 Chronic myeloid leukaemia: Clinical characteristics

Chronic myeloid leukaemia (CML) is a clonal haematopoietic stem cell (HSC) myeloproliferative disease affecting 1-2 adults per 100,000 world-wide [1, 2]. The disease is prevalent in the elderly population with a median age at diagnosis of 60-65 years and presents at a sex ratio of 1.2:1.7 female to male [3, 4]. CML is presented at a low frequency in children, being 2% of all paediatric leukaemias [5].

The pathology is associated with the reciprocal chromosomal translocation t(9;22)(q34;q11), which produces a small chromosome termed the Philadelphia chromosome. As a consequence of this translocation, the *BCR* and *ABL1* genes are fused together resulting in the generation of the *BCR-ABL1* oncogene respectively [6]. The presence of the Philadelphia chromosome is the cytogenetic, and diagnostic, hallmark of CML and the kinase activity arising from BCR-ABL1 is central for the pathogenesis of the disease [7].

Two different stages of CML have been defined which represent the natural progression of the disease: chronic phase (CP) and blast crisis (BC) [8, 9]. The early CP disease is characterised by leukaemic proliferation of neutrophils [1]. Patients are asymptomatic or possess mild symptoms that include loss of weight, fatigue and headaches. The majority of CP patients are effectively managed by drug therapy, Imatinib Mesylate (IM) [10]. Generally, CP-CML is not considered to be a fatal disease, but if untreated or develops drug resistance, the disease will transform into the more aggressive stage of BC [2].

Blast crisis CML is distinguished by the accumulation of immature myeloid blasts arising from an arrest of myeloid differentiation (acute-like) with >30% blasts in the peripheral blood [9, 11, 12]. At this stage, the leukaemia develops rapidly with patients experiencing worsening symptoms including weight loss, defects in blood coagulation, appearance of bruises and splenic inflammation. In contrast to chronic phase, drug treatment is inadequate with death occurring (systemic failure) within 12-months of diagnosis [13].

1.2 The Philadelphia chromosome

The Philadelphia chromosome is the result of a reciprocal chromosome translocation t(9;22)(q34;q11) that fuses together the 3’ end of the proto-

oncogene Abelson tyrosine kinase (ABL1) with the 5' end of the Breakpoint cluster region (BCR) gene (**Figure 1.1**). This small chromosome was first discovered in 1960 by Peter Nowell and David Hungerford at the University of Pennsylvania, Philadelphia (chromosome named after location) and details of the molecular aberration was detailed by Janet Rowley using Giemsa-staining and quinacrine banding [7, 14].

The Philadelphia chromosome is the hallmark of CML and is present in about 95% of CML cases. Moreover, this translocation exists within about 20-30% of acute lymphoblastic leukaemia (ALL) patients and treatment of this B-cell malignancy is with IM therapy [15]. Finally, some rare cases of Philadelphia-positive acute myeloid leukaemia (AML) cases have been reported [16].

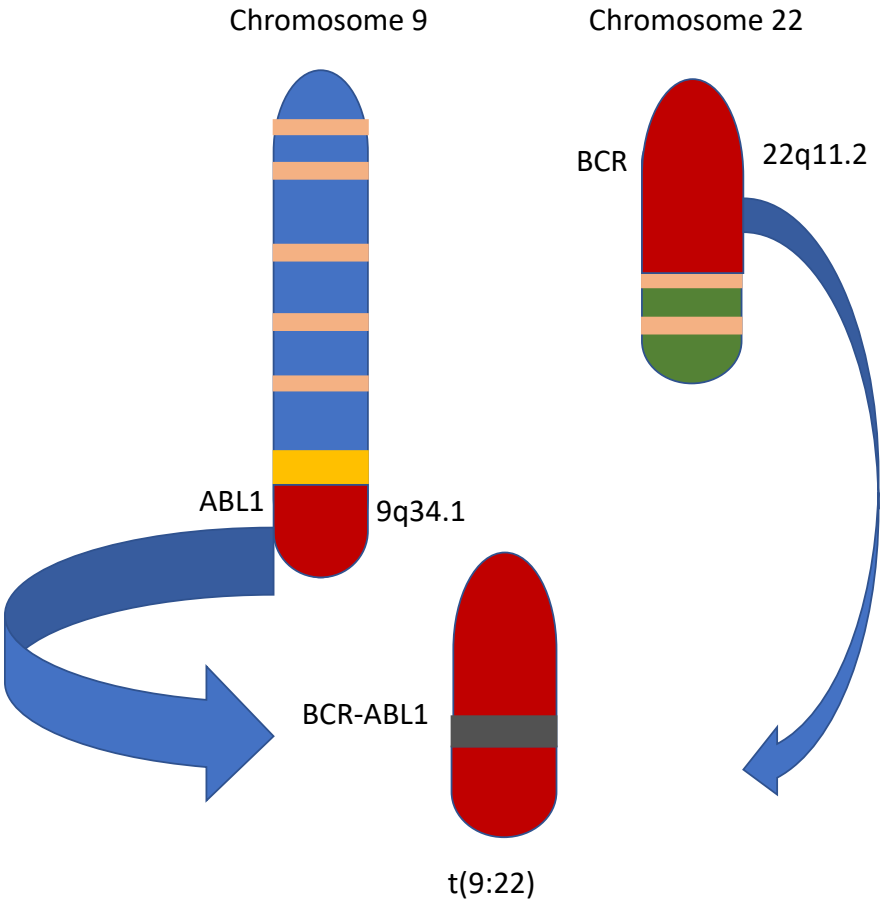


Figure 1.1: Schematic depicting the generation of the Philadelphia chromosome.

Philadelphia chromosome arises after a reciprocal translocation t(9;22)(q34;q11), which fuses the *BCR* and *ABL1* genes into a single loci.

1.3 The BCR-ABL1 fusion protein: biology and pathology

BCR is a ubiquitously expressed cytoplasmic serine/threonine kinase with GTPase-activity and regulates the Rho family of GTPases such as Rho and Rac1 [17] (**Figure 1.2a**). Despite its pattern of expression, the precise function of BCR is unclear with BCR-null mice being viable and presenting no overt phenotype; albeit the neutrophils produce more reactive oxygen metabolites following activation [18, 19]. The BCR protein contains an oligomerisation motif, located at the N-terminal site, and in the context of BCR-ABL1 pathology enables homo-dimerization and subsequent SH1-mediated trans-autophosphorylation at tyrosine residue 177. This modification subsequently recruits adapter proteins leading to the activation of various downstream signalling pathways associated with cell proliferation and differentiation such as STAT5 [20], Myc and RAS [21-23] respectively.

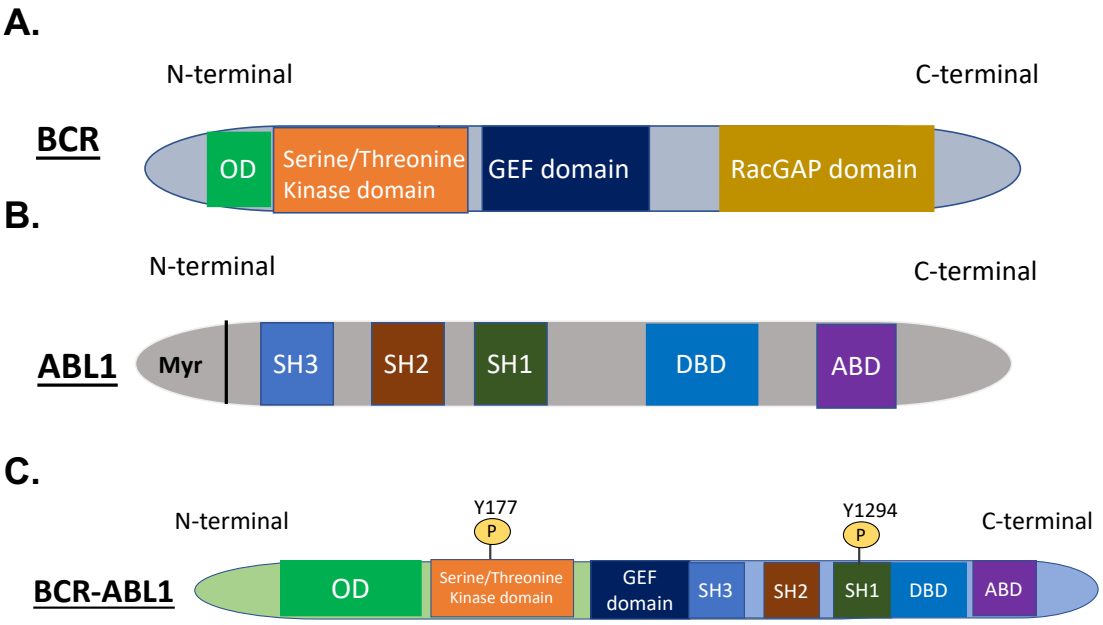


Figure 1.2: Simplified schematic of the BCR, ABL1 and BCR-ABL1 proteins.

(**A**) BCR protein contains a coiled-coil (CC) oligomerisation domain, a serine/threonine (S/T) kinase domain and a rho-specific guanine nucleotide exchange factor (Rho/GEF) domain. In this S/T domain BCR also contains binding sites for growth factor receptor bound protein 2 (GRB2) at tyrosine 177 (Y177). (**B**) ABL1 contains tandem SRC homology 3 and 2 (SH3, SH2) and the tyrosine kinase domain (SH1). The C-terminal end of ABL contains proline rich SH3 binding site (PPs), nuclear localisation signals (NLS), a DNA binding domain (DBD) and an actin-binding domain (ABD). (**C**) the BCR-ABL1 protein. Figure adapted from Ren, R. 2005 [11]).

ABL1 is a well-characterised non-receptor tyrosine kinase that is structurally similar to the Src family. It regulates a variety of functions including cellular proliferation, differentiation, adhesion, survival and response to DNA damage [24, 25]. The protein is capable of shuttling between the nucleus and cytoplasm and as a consequence of the fusion with BCR, its nucleo-cytoplasmic shuttling is inhibited and the BCR-ABL1 protein becomes anchored within the cytoplasm [17, 19, 26]. ABL1 a multi-domain protein and contains various Src-homology (SH) domains. Crucial to ABL1 (and BCR-ABL1) function, the SH1 domain has tyrosine-kinase activity [27] with *in vivo* studies clearly demonstrated that this domain is fundamental for initiating BCR-ABL1 mediated pathogenesis [28]. The SH1 kinase activity within ABL1 is tightly controlled by auto-inhibition which is deregulated upon fusion with BCR [29, 30]. The SH2 domain aides in protein-protein interactions [31] and can positively interact with the SH1 domain. Indeed, in the absence of SH2, the SH1 activity is reduced and in animal studies the CML-like leukaemia is significantly diminished [32]. Finally, the SH3 domain (60 amino acids) functions as a modular protein-protein region and binds with proline-rich specific sequences, mainly those with PxxP pattern [33]. The primary role of SH3 domain is to support the formation of large protein complexes [33]. Nevertheless, the SH3 domain can negatively influence the SH1 activity where upon its absence a more severe CML-like disease is presented within animal models [34].

Other domains of ABL1 protein includes a DNA binding domain (which is related to the SRY and LEF HMG box transcription factors [25]), a nuclear localisation signal [35] and an actin binding domain [36] (**Figure 1.2b**).

The downstream targets of BCR-ABL1 define its oncogenicity. In addition to the STAT5, Myc and RAS pathways, the SH1 domain directly phosphorylates the CrkL adaptor protein [37] which, in turn, activates the Akt-Bad pathway causing the deregulation of apoptosis [30, 34, 38, 39]. **Figure 1.3** depicts a summary of the BCR-ABL1 signaling pathways.

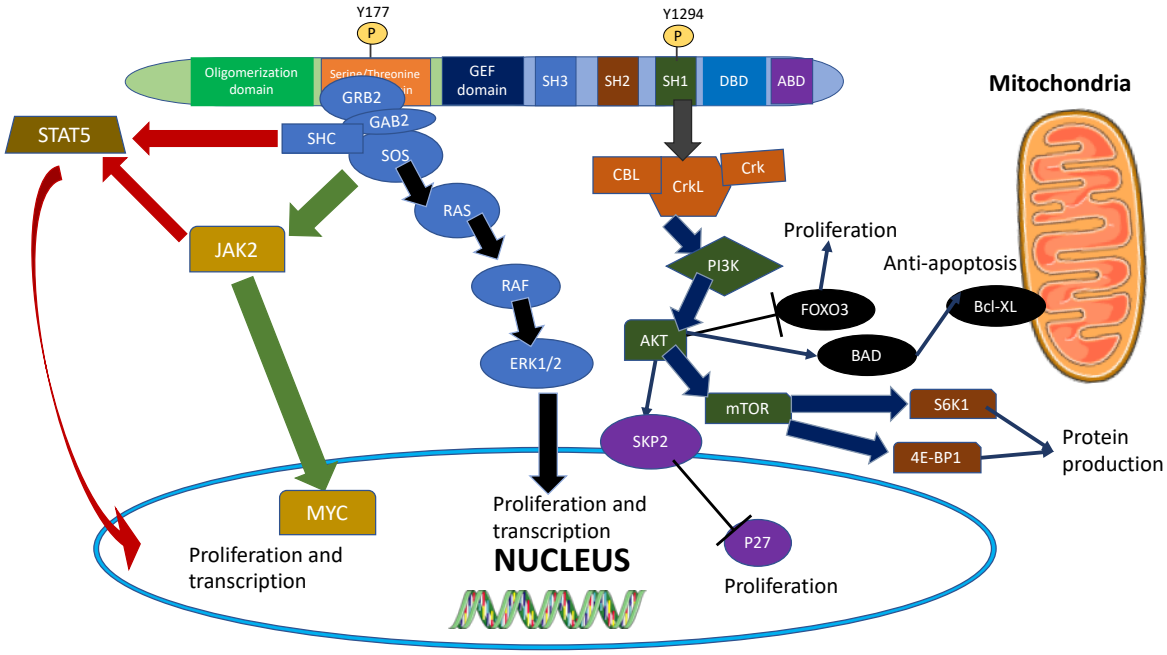


Figure 1.3: Schematic representation of the molecular pathways activated by BCR-ABL1.

BCR phosphorylation at the Y177 residue generates a high-affinity binding site for growth factor receptor-bound protein 2 (GRB2). The BCR-ABL1/GRB2 complex recruits son of sevenless (SOS) which stimulates the RAS downstream pathway that activates RAF1, MEK1/2 and MAPK proteins, driving cell proliferation. SOS also activates PI3K, which in turn activates AKT which promotes survival through regulation of apoptotic machinery. AKT also enhances cell proliferation by mTOR activation. BCR-ABL1 also leads to the activation of the JAK/STAT pathway, which is commonly activated in myeloproliferative diseases. Phosphorylation of CRKL by the SH1 tyrosine kinase domain also activates PI3K and STAT5 signaling. Adapted from: Cilloni, D. 2012 [6].

The transcriptional expression of BCR-ABL1 is regulated from the endogenous *BCR* promoter and three isoforms have been reported. Each isoform has constitutive tyrosine kinase activity which is required for cellular transformation and onset of leukaemia [40]. These isoforms differ in the amount of *BCR* that is included in the corresponding fusion gene. The p210 isoform is the most common and displays two alternate splice variants termed e13a2 (b2a2) or e14a2 (b3a2) and arises from fusion of BCR exon 13 to exon 2 of ABL1 or fusion of BCR exon 14 to ABL1 exon 2 respectively [41]. The p190 isoform (e1a2, e1a3) is primarily associated with Philadelphia chromosome-positive ALL [41, 42], while that of the p230 isoform (e19a2) is present in the rare neutrophilic CML [43].

For simplicity, from herein the BCR-ABL1 term refers to the p210 isoform.

1.4 Role of SH2 domain in cancer biology

The SH2 domain is a conserved 100-amino acid phospho-tyrosine binding site and is present within many receptor and non-receptor tyrosine kinase proteins [44, 45]. Its primary role is to augment the transduction of kinase pathways, wild-type or oncogenic, and as such their role in cancers has been well-recognized [46]. For example, small molecule inhibition of SH2 within the growth-factor receptor bound 7 protein reduces breast cancer proliferation [47] as well as pancreatic cancer migration [48]. Additionally, inhibition of SH2 within the STAT3 protein abolishes dimerization and subsequent downstream activation and within the context of pancreatic cancer results in decrease of tumour viability and survival [49].

These studies draw attention to the importance of protein-protein interactions in cancer biology and the relevance of targeting the SH2 domain in driving tumour regression. Consequently, various small molecular inhibitors and protein-like peptides (i.e. affimers and monobodies) have been generated to target the SH2 domain within oncogenic proteins in breast cancer, pancreatic cancer and melanoma respectively [50-53]. While a role for the SH2 domain in BCR-ABL1 is clearly defined in CML [32, 54] its role, if any, within drug resistance is unknown.

1.5 Imatinib as a clinical treatment against CML

Prior to 1950s, CML was treated with radiotherapy (spleenic irradiation) and was primarily used to ease the pain of patients (palliative care) rather than a valid therapeutic approach [55]. In 1953, clinical investigators at Royal Marsden Hospital in London tested a new alkylating agent, busulfan, and discovered that it was effective in controlling the leukocyte count in newly diagnosed CML patients but failed to inhibit the progression of the disease to blast crisis [56]. Almost a decade later, clinical trials demonstrated that busulfan-treated patients lived longer than those treated with radiotherapy and thereafter chemotherapy reagents became the standard treatment for CML [57]. Notwithstanding this clinical progress, chemotherapeutic treatments suffered from both severe side effects and frequent recurrence of the leukaemia.

CML therapy was revolutionised in the late-90’s by the discovery that activity from the SH1 tyrosine kinase domain was central for BCR-ABL1 oncogenesis [38, 58, 59]. This spearheaded the basis for the design of a novel inhibitor that

could target the SH1 domain of BCR-ABL1 [60]. Imatinib (IM) or Gleevec® (STI-571, Novartis), is a 2-phenylaminopyrimidine that binds with high affinity to the ATP pocket of BCR-ABL1 and inhibits the SH1-dependent phosphorylation of downstream targets [10, 61, 62]. In early trials, IM showed extremely good tolerance within patients with little side-effects and was associated with complete cytogenetic and haematological responses in 98% of patients within the first four weeks of therapy [63]. These results readily prompted the Food and Drug Administration to approve IM for clinical use in 2001 [64]. Given the clinical success of IM, second-generation tyrosine kinase inhibitors (TKI) have been generated with greater affinity towards the ATP pocket and include Nilotinib and Dasatinib respectively [65, 66].

1.6 The molecular progression from CP to BC CML

The molecular pathology of CP and BC CML are quite distinct [67, 68]. First, CP is a proliferative disorder albeit with normal development of the myeloid lineages. Yet in BC, myelopoiesis is significantly impaired with lineage differentiation blocked at an early stage resulting in the generation of proliferating immature blast cells. Secondly, drug treatment of the bulk tumour population in CP is extremely effective, yet in BC the treatment regime simply fails to be clinically effective. In this case, the leukaemia itself has transformed into a new pathology acquiring the ability to block myeloid development and can escape drug targeting.

Not surprisingly, the transition from CP to BC requires the development of additional mutations to confer the AML-like transformation [2, 69]. This includes both the activation of other oncogenes as well as loss of function of tumour suppressor genes [70]. As example, the expression of transcription factors are deregulated and contribute to both a block of cellular differentiation as well as anti-apoptosis properties and include C/EBPα, BCL-2, MYC, EVI1 and JUN-B [70]. Furthermore, increased activity of proliferation and survival pathways such as MAPK, PI3K/Akt/mTOR, Notch and Ras also contribute to the CP to BC progression [8]. Finally, genetic aberrations including mutations in the tumour suppressor genes TP53, RB1, CDKN1A and CDKN2A/B as well as the gain of new chromosomal aberrations (e.g. 8+, 1q12-21, i(17)(q10), +20, +21) [71, 72] frequently occur during this transformation.

In summary, it is clear that the transformation from CP to BC requires of additional oncogenic “hits”. Yet, what is the primary cause for acquisition of these additional oncogenic events? One key contributing factor is the unrelenting kinase activity of BCR-ABL1 itself, which promotes genome instability [73]. Such instability includes the increased production of reactive oxygen species (ROS) which, in turn, compromises DNA stability by generating double-strand breaks and diminishes the efficiency of the DNA repair mechanisms [74-76]. It is of interest to mention that ROS production reduces the activity of phosphatases *in vitro*, which could feedback and enhance the sustained activation of several kinase signalling pathways mediated by BCR-ABL1 [75, 77].

1.7 Kinase-dependent and -independent IM resistance

Despite the breakthrough and success of IM treatment, approximately 20% of CML patients develop drug resistance [78, 79]. Although a transient success, treatment with the more powerful second-generation TKIs (Nilotinib or Dasatinib) are ultimately ineffective in treating the disease [80]. Broadly speaking, two main types of resistance have been identified in CML: BCR-ABL1 kinase-dependent or kinase-independent mechanisms [78].

Kinase-dependent mechanisms involve (i) overexpression of efflux drug-pumps, (ii) gross amplification of BCR-ABL1 expression or (iii) acquisition of point mutations within the SH1 tyrosine-kinase domain. Point mutations in the SH1 domain is the most common BCR-ABL1 kinase dependent mechanism [81]. The first point-mutation (T315I) was identified by Gorre *et al.* [82] and subsequently termed the “gatekeeper” mutation. Since this initially discovery, more than 50 SH1-domain mutations have been identified (**Figure 1.4**) with 8 point-mutations directly conferring drug resistance by inhibiting the ability of IM binding to the ATP pocket via steric conformation changes [79]. While second-generation TKIs (Nilotinib) have been designed with the ability to recognise some of these mutations none are successful against the T315I gatekeeper mutation. A third-generation TKI, Ponatinib, does however target the T315I mutation yet phase-1 clinical trials demonstrate poor tolerability within patients with the advent of severe side-effects [83, 84].

M237V	E258D	E292V	Y342H	A365V	L387M	E450K
I242T	W261L	I293V	M343T	A366G	M388L	E453G
M244V	L273M	L298V	A344V	L370P	Y393C	E459K
K247R	E275K	V299L	A350V	V371A	H396P	M472I
L248V	D276G	F311L	M351T	E373K	A397P	P480L
G250E	T277A	**T315I**	E355D	**V379I**	S417F	F486S
Q252H	E279K	**F317L**	**F359V**	**A380T**	I418S	E507G
Y253F	V280A	Y320C	D363Y	F383L	A433T	
E255K	**V289A**	L324Q	L364I	L384M	S438C	

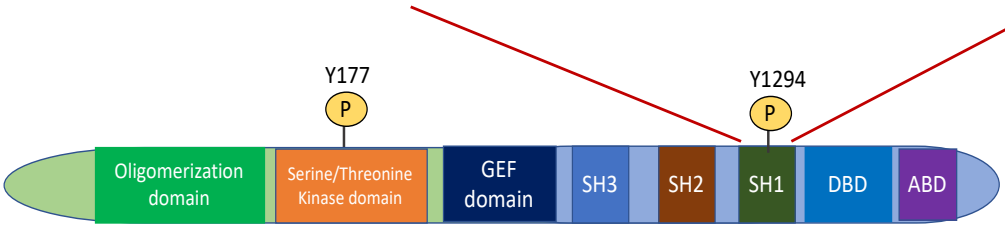


Figure 1.4: Schematic of various point mutations identified in the SH1 kinase domain of BCR-ABL1.

More than 50 point mutations have been identified at the SH1 domain have been identified in the clinic; nonetheless, only 8 (highlighted in red) have been reported to confer drug resistance.

While the point mutations within the SH1 domain is one of the most widely studied mechanism for CML drug resistance, it only accounts for approximately 40% of such cases [78, 79, 85]. Indeed, the majority of drug resistant patients harbour a wild-type *BCR-ABL1* allele (**Table 1.1**). Various international clinical studies have assessed the frequency of kinase mutations within CML drug resistant patients and these ranged from 19-63% respectively [86]. For example, the Polish MAPTEST study reported only 12% of patients harboured a SH1 domain point mutations from the 92 recruited patients. Similar frequencies were identified within the Australian study with only 19% of drug-resistant patients having a SH1-domain point mutation [87].

Clinical Study	Frequency of BCR-ABL mutations (%)	CML patients without BCR-ABL mutations (%)
GIMEMA CML WP	43% (127/297)	57%
Argentinean	23% (36/154)	77%
Chinese	58% (74/127)	42%
Korean	63% (70/111)	37%
Polish MAPTEST	12% (11/92)	88%
Indian	33% (25/76)	65%
Hungarian	36% (27/74)	64%
Australian	19% (27/144)	81%

Table 1.1: Frequency of BCR-ABL mutations in CML patients.

Data showing frequency of BCR-ABL1 mutations in patients from multinational clinical studies. Data kindly provided by G. Bheesmachar from Soverini, S. 2014 [86].

Collectively, these observations demonstrate that the majority of drug resistant CML patients carry a wild-type *BCR-ABL1* allele indicating that IM continues to bind and inhibit the SH1 domain; yet the disease has now become independent from the BCR-ABL1 signalling [86]. How do these leukaemic cells continue to survive and grow in the absence of BCR-ABL1 kinase activity?

1.8 The Src Family Kinases in drug-resistant CML

The Sarcoma virus proto-oncogenes (Src) kinase family (SFKs) are a group of non-receptor tyrosine kinases that facilitate the signal transduction of receptor tyrosine kinases [88]. The family consists of 9 tyrosine kinases: Src, Lck, Lyn, Hck, Blk, Fgr, Fyn, Yes and Yrk [89, 90]. All share a highly conserved SH1 tyrosine kinase domain, as well as two Src homology regions (SH2 and SH3) and a unique N-terminal domain [91].

The SFKs have pleiotropic activities and have been related to a variety of cellular processes such as cell growth, survival, differentiation, cell remodelling, adhesion and migration [91, 92]. Moreover, they represent the largest family of cytoplasmic kinases [93]. Importantly, the SFKs inhibit apoptosis and promote cell survival by signalling through the RAS/RAF/MEK/ERK pathway to degrade the pro-apoptotic protein Bik [94]. Within the myeloid lineage, the Fgr, Lyn and Hck proteins are the most commonly expressed [93].

The ABL1 kinase and SKFs are highly similar regarding their structure, with both enzymes possessing SH1-SH3 domains [95]. To investigate the potential role of SFKs in drug resistant CML, work by others have generated an *in vitro* model of IM-resistance [96]. In brief, the K562 cells line (established from a patient in blast

crisis CML) was cultured in sequentially increasing concentrations of IM until a resistant line (K562-R) was cloned. Notably, the K562-R cells were continuously grown in the presence of IM and in comparison, to the parental K562 cells they demonstrated a significant loss of BCR-ABL1 kinase activity. Interestingly, the Lyn kinase was overexpressed in the K562-R cells and depletion of its activity (Lyn-specific shRNA) induced cell death.

Moreover, overexpression of Lyn was observed in drug-resistant CML patients and treatment of *ex vivo* primary cells with specific Src inhibitor induced apoptosis [96]. Collectively these results suggest that acquired activation of the Lyn kinase pathway could mediate drug resistance in CML patients. Indeed, based on these observations the second-generation of tyrosine kinase inhibitors was generated (Dasatinib [97]) which has dual target specificity capable of blocking the kinase activity of BCR-ABL1, Lyn and the additional SFKs family members. Unfortunately, treatment of IM-resistant CML patients with Dasatinib provided only a transient response with patients rapidly developing resistance. As such, molecular targeting of both BCR-ABL1 kinase activity as well as the SFKs pathways failed to restore sensitivity to IM and demonstrates the complexity in how leukaemic cells of blast crisis CML have evolved to be drug resistant.

1.9 Leukaemic cancer stem cells are insensitive to IM

CML is a haematopoietic stem cell disease and the disease can originate within either the Lin- CD34+ CD38- CD90+ primitive cells (primarily seen in CP stage) or Lin- CD34+ CD38+ GMP (BC stage) populations respectively [98]. Similar to normal stem cells, these leukaemic stem cells (LSCs) have both self-renewal capacities as well as the ability to differentiate, albeit in a dysregulated manner, with the progeny populating the bulk of the tumour [99, 100]. As anticipated, key features of LSCs include their ability to serially engraft within xenograft models and can re-constitute a CML-like disorder in immune-deficient mice [100, 101].

The clinical relevance of LSCs pertains to their innate resistance to TKI therapy. Indeed, initial studies demonstrated that CD34+ bone marrow cells of chronic phase CML patients are able to survive in the presence of IM [102]. Several studies have corroborated this observation and it is now clear that LSCs, in both chronic phase and blast crisis, can indeed survive in the absence of BCR-ABL1 kinase activity [103-105]. This kinase resistance is conferred by several factors.

First, like most stem cells, the LSCs are generally non-proliferating which desensitises them from the consequences of BCR-ABL1 kinase inhibition [99]. Secondly, LSCs are innately independent from BCR-ABL1 kinase for survival, indicating that these cells possess other oncogenic pathways for proliferation and survival [106].

The failure of the LSCs to respond to clinical treatment makes CML a manageable condition, but unfortunately, not a curable disease. Simply, the withdrawal of TKIs from therapy resolves in rapid leukaemia relapse [1]. Notably, during BC the bulk leukaemic cells within the periphery also becomes insensitive to TKIs (i.e. drug resistant) [107, 108].

1.10 Drug resistance and the other protein domain(s) of BCR-ABL1

The prevalence of drug resistant LSCs has led many to question whether BCR-ABL1 kinase activity is still required for late-stage disease (i.e. blast crisis) maintenance.

Why do blast crisis LSCs give rise to drug-resistant progeny while chronic phase LSCs do not? It is likely due to the intrinsic differences between the pathology of these two populations. Recall that CP gives rise to proliferating progenitors that still generate terminally differentiated myeloid cells while blast crisis LSCs give rise to immature myeloid progenitors blocked in cellular differentiation. As such, during the transformation of the disease, the molecular pathology of the LSCs has changed in its ability to block myeloid differentiation.

How are blast crisis CML cells capable of surviving and, more importantly, blocking myeloid differentiation in the absence of BCR-ABL1 kinase activity? There are two models that could account for this observation. First, given the complexity of the BCR-ABL1 protein it is possible that other domain(s) of the oncogene can now compensate for the loss of kinase activity. Secondly, the molecular drivers of cell proliferation, survival and differentiation that are dysregulated in blast crisis CML, likely influenced by BCR-ABL1 kinase activity in early phase of the disease, can now operate autonomously. That is, the disease is now oncogene independent.

Resistance to IM demonstrates the ability of CML cells to survive in the absence of BCR-ABL1 kinase activity. However, BCR-ABL1 contains numerous protein domains and it is possible that other domains of BCR-ABL1 can compensate. Indeed, BCR-ABL1 can activate the expression of *Alox5* in the presence of IM

[109]. Furthermore, the Lyn signalling pathway continues to be activated in the presence of IM [110]. Notably, the activity of these other domains cannot induce leukaemia with initiation of the disease being strictly dependent on the SH1 tyrosine kinase. Nevertheless, these observations support the notion that other domains of BCR-ABL1 are functionally independent from its kinase activity and could therefore account as a molecular mechanism for drug resistance.

The mechanism for this BCR-ABL1 independence is unclear although in this context it is interesting to note that epigenetic modifications have been associated with the establishment of an IM-resistant phenotype. Specifically, in comparison to drug-sensitive parental cells, the IM-resistant K562 cells exhibited an upregulation of histone de-acetylaces (HDACs) with a downregulation of histone acetyltransferase [111]. Furthermore, primary cells isolated from CML patients responded well *in* vitro to a combination treatment of HDAC inhibitors and IM [112]. Collectively, these observations suggest that upon drug resistance the blast CML cells no longer require the kinase activity of BCR-ABL1 but rather have undergone epigenetic changes to maintain a leukemic phenotype.

1.11 Summary of CML and BCR-ABL1 kinase-independency

A greater understanding of the molecular mechanism(s) of how CML cells have become independent of kinase activity is of great importance. This will impact future drug therapies in directing whether efforts should target the alternate BCR-ABL1 domain(s) or focus on an ‘oncogene independent’ disease with acquired epigenetically modified gene targets. Specifically, two mechanistic models can be proposed:

Model 1: In the presence of the drug (IM), kinase activity of BCR-ABL1 is inhibited, thereby blocking the downstream pathways that lead to cell proliferation and survival. However, other domain(s) of BCR-ABL1 are still active and are responsible for maintaining the leukaemia. Therefore, although cells are not reliant on kinase domain, they are dependent on signalling from alternate domain(s) of BCR-ABL1 to sustain the leukaemic phenotype.

Model 2: Upon drug resistance, CML cells lose their dependency upon the initiating oncogene and completely become independent of BCR-ABL1 signalling. Here, the cells do not need BCR-ABL1 protein any longer for maintaining leukaemia. Other pathways, or genes, are activated in order to

compensate for the loss of kinase activity and are capable of sustaining the disease.

1.12 General overview on haematopoiesis

Haematopoiesis is a dynamic process by which the entire repertoire of blood cell lineages is generated from the haematopoietic stem cells [113]. HSCs are located in the bone marrow and are multipotent stem cells with self-renewal properties from which all differentiated blood cell types arise. Three main haematopoietic lineages have been identified: erythroid-megakaryocyte, myeloid and lymphoid [114]. The myeloid lineage gives rise to monocytes, granulocytes (including neutrophils, eosinophils and basophils), mast cells and macrophages. The lymphoid lineage gives rise to all classes of lymphocytes (B cell, T cell and natural killer).

HSCs have the ability to divide asymmetrically giving rise to one new HSC (self-renewal) and to one cell committed to differentiation to either of the three main lineages (pluripotency) [115].

1.13 Haematopoietic differentiation

Haematopoietic differentiation is represented as a cascade of cellular events that begin with the HSC and lead to the generation of mature blood cell types. The multipotent progenitors (MPPs) are the first to arise from HSC. MPPs, in turn, give rise to CLPs (common lymphoid progenitors) and CMPs (common myeloid progenitors), that result in the generation of all lymphoid or myeloid/erythroid cells respectively. CLPs give rise to all lymphoid cells but lose the ability to produce any myeloid/erythroid cell types and CMPs give rise to either MEP (megakaryocyte-erythrocyte precursors) or GMP (granulocyte-monocyte precursors). MEPs give rise to megakaryocytes or red blood cells and platelets while GMPs give rise to granulocytes, monocytes and macrophages (**Figure 1.5**) [116].

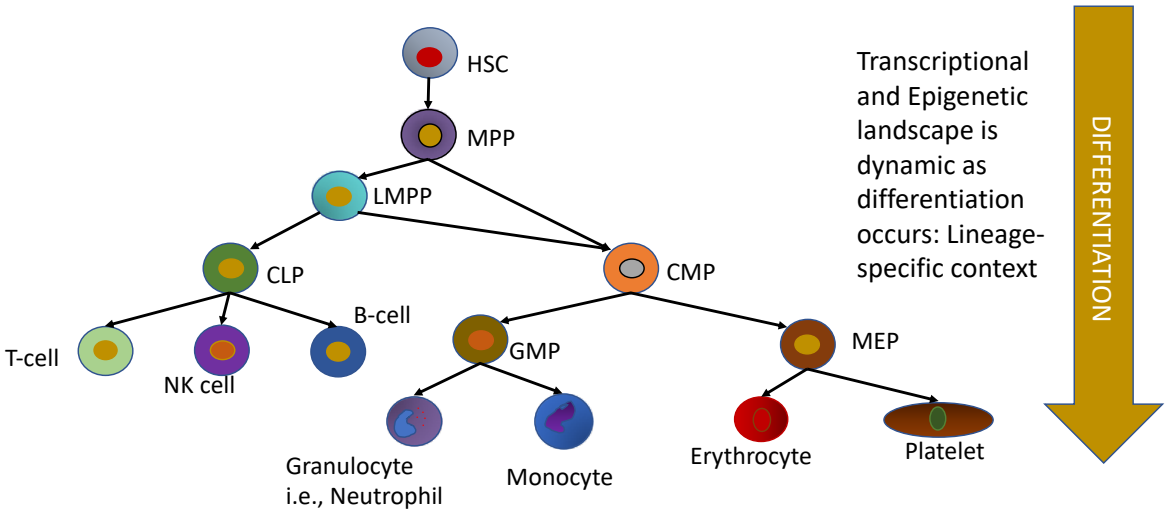


Figure 1.5: Haematopoiesis in adult bone marrow.

Schematic representation showing hierarchical model of haematopoiesis in the adult bone marrow. LT-HSC: long-term Hematopoietic stem cell, ST-HSC: short-term hematopoietic stem cell, MPP: multipotent progenitors, CLP: common lymphoid progenitor, GMP: granulocyte-monocyte progenitor, CMP: common myeloid progenitor, MEP: megakaryocyte-erythrocyte progenitor. (Figure adapted from Orkin and Zon, 2008 [113]).

The haematopoietic system represents a very good model for explaining how a multi-potential stem cell gives rise to different cell lineages. By specific targeting of signalling proteins, transcription factors and characterizing gene mutations involved in leukaemia, a large set of regulatory molecules that control generation of haematopoietic lineages has been analysed. These molecules orchestrate cell fate specification, commitment and differentiation. A general framework where the transcriptional regulatory proteins are involved in specifying distinct cell fates with in the haematopoietic system can be proposed [115]:

(1) A unique combination of transcription factors specifies each cell fate e.g., PU.1, C/EBPα, Gfi-1 and Irf8 determine the myeloid cell fate [117].

(2) The level of a transcription factors also determines the cell fate e.g., B cell fate requires low level/activity of PU.1 whereas, macrophage cell fate requires high level/activity of PU.1 [118].

(3) HSCs and multi-potential progenitors show low levels of mixed lineage patterns of gene expression [119].

(4) Cell fate specification involves the activation of lineage appropriate subsets of genes and repression of lineage inappropriate subset of genes respectively [119].

(5) Mixed lineage developmental states may be dictated by heterogeneous sets of cell fate determinants that are simultaneously active in multi-potential progenitors [120].

1.14 Transcription factors in myelopoiesis

Transcription factors have long been recognized as major regulators of myeloid development and primarily function to specify and re-enforce each cell fate decision thus ensuring the successful developmental transition from one progenitor to the next mature cell [121]. Transcription factors do not act alone but work in combination as exemplified by the functions of PU.1 and C/EBPA in regulating the expression of many myeloid-specific genes [122]. Moreover, transcription factors often cross-antagonise one another's activity to supress an alternate cell fate choice. This lineage restriction commits the progenitor along the chosen cell fate; as exemplified by PU.1 and GATA1, in the choice between the myeloid and erythroid lineages [123]. As such, transcription factors regulate a cell fate decision by controlling the expression of lineage-specific target genes as well as supressing the unwanted genetic programs of the alternate fates [124]. Transcription factors have divergent roles in different cells and at different stages of development or differentiation. They play vital roles in normal haematopoiesis and their deregulation can lead to leukaemia. A number of transcription factors including GATA-1, GATA-2, SCL, PU.1 and RUNX1 are key players in the regulation of haematopoietic progenitors. SCL/TAL1 is a member of the basic helix-loop-helix transcription factor family and is specifically required for the transition from haemangioblast to haemogenic endothelium stage. SCL/TAL1 is required for mesoderm specification and eventually for the development of megakaryocyte lineage [125]. PU.1, a member of the ETS family of transcription factors, is necessary for the development of both lymphoid and myeloid cells, and for the maintenance of the haematopoietic system. PU.1 is a master regulator of myeloid differentiation. PU.1 interacts with other regulators (C/EBPa, RUNX1 and GATA-1) and these interactions modulate the PU.1 transcriptional activity that determines the effect of PU.1 on cell fate decisions [122]. PU.1 is expressed at low levels in HSC and megakaryocyte-erythroid lineage but its expression increases during differentiation in both myeloid and lymphoid lineages [126].

1.15 Previous work in the lab

1.15.1 KCL22 cells: pre-clinical model of CML drug resistance

There is an urgent need to understand how CML cells, which were once dependent on BCR-ABL1 signalling for survival, have become kinase independent. Previous work from the research group has directly addressed this concern by establishing an *in vitro* cellular model of CML drug resistant (G. Bheeshmachar). The KCL22 cells, derived from a 32-year old BC-CML patient [127], are sensitive to IM treatment and are one of the most widely used systems to study CML [128]. Single cell clones of the KLC22 population were generated and two were randomly selected for further analysis; termed clones 15P and 16P respectively.

1.15.2 Generation of IM-resistant KCL22 cells

To establish IM-resistant (IMr) derivatives, the concentration of IM that results in >95% cell death of 15P and 16P cells respectively was determined and 1μM IM was defined as the 'drug resistant' concentration. Clones 15P and 16P were grown in the presence of IM starting at 0.1μM (10% of drug resistant concentration) with weekly increments of 0.1μM until they reached 1μM concentration; termed 15IMr and 16IMr respectively. Once established, the IMr derivatives were continuously cultured in the presence of IM with the drug replenished every 3 days.

1.15.3 Drug resistant KCL22 cells have reduced BCR-ABL1 activity

BCR-ABL1 phosphorylates itself as well as downstream target proteins including STAT5 and CrkL [129]. By western analysis, the comparison between parental against resistant cells showed a markedly decrease in BCR-ABL1 activity (**Figure 1.6**). Interestingly, the total protein expression of BCR-ABL1 was not changed after acquisition of drug resistance, suggesting that these cells have become kinase independent for survival. Notably, these drug resistant cells recapitulate the clinical observations in that activity of BCR-ABL1 is significantly diminished in the presence of IM yet the cells continue to grow and survive.

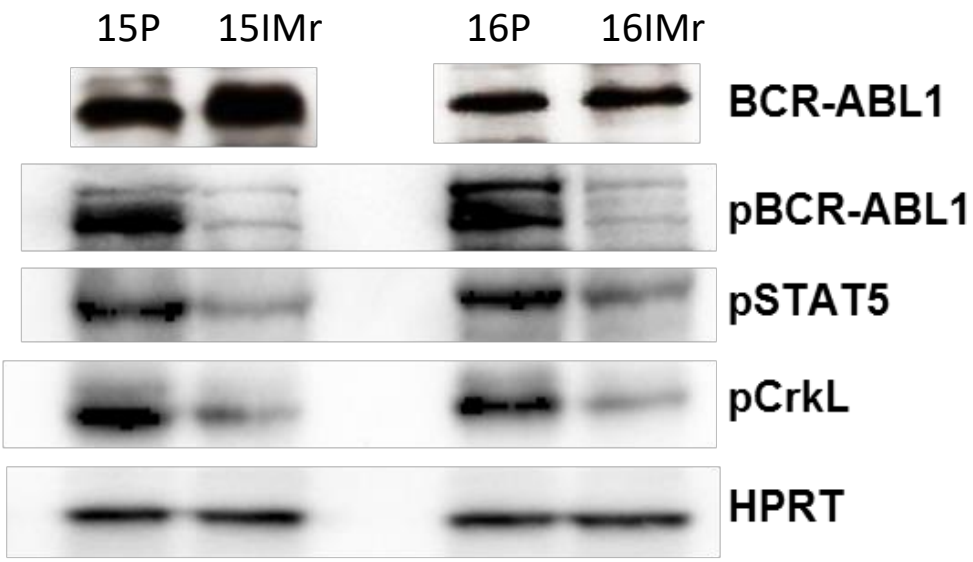


Figure 1.6: KCL22 model of BCR-ABL1 kinase-independent drug resistance.

Western blot analysis of the indicated proteins within the KCL22 model of drug resistance. HPRT was used as a loading control. Data kindly provided by G. Bheesmachar.

1.15.4 Drug resistant CML cells are oncogene independent

The consequence of BCR-ABL1 depletion on the cell viability of 15IMr and 16IMr was determined. Using siRNA molecules, a shared BCR sequence that would deplete both BCR-ABL1 and endogenous BCR proteins (siBCR) was used. The 16P cells are dependent on BCR-ABL1 for cell survival and therefore it was not surprising that depletion with siBCR resulted in significant cell death (**Figure 1.7**). This phenotype was re-iterated by the near absence of the healthy population as determined by FACS (forward- versus side-scatter plot). Of great interest was that depletion with siBCR within 16IMr failed to show any induced apoptosis. Collectively, these observations demonstrate that drug resistant CML cells are oncogene independent; at least in the preclinical KCL22 model.

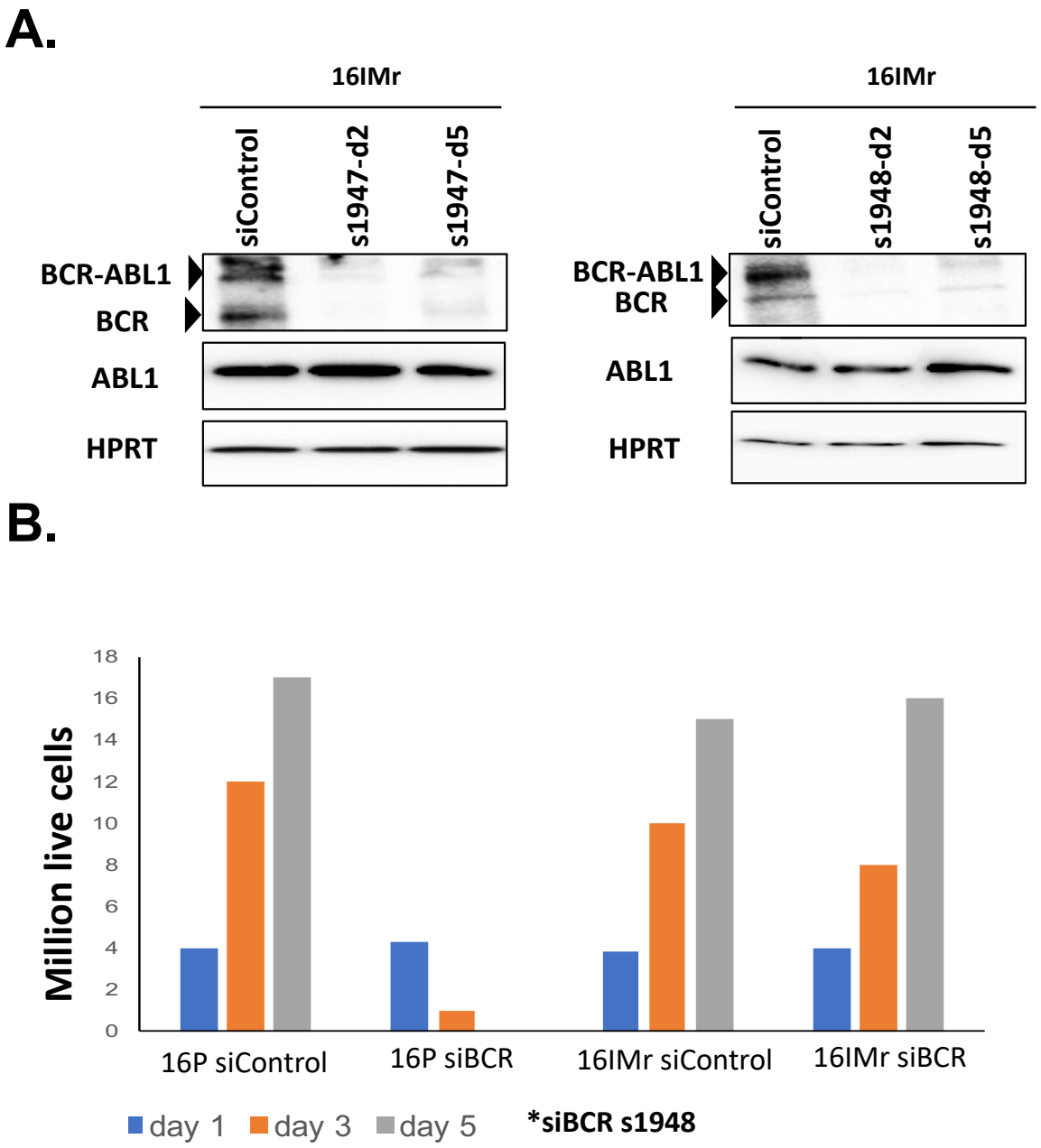


Figure 1.7: siBCR on 16IMr cells.

(**A**) The 16IMr single-cell clones were treated under two sequences of siBCR s1947, s1948 and siControl for 2 and 5 days. Western Blots show the depletion of total BCR-ABL1 molecule and BCR endogenous in 16IMr cells, tABL and HPRT are used as loading controls. (**B**) Trypan Blue exclusion of siBCR treated cells with siBCR s1947 and siControl for up to 5 days. Data kindly provided by G. Bheesmachar.

1.16 Defining a clinically relevant drug-resistant gene network

Deregulated transcription factors are commonly found in leukaemias [69, 130, 131] which makes them likely candidate(s) as new oncogenic drivers within drug-resistant KCL22 clones to compensate for the absence of BCR-ABL1 activity.

Genome-wide expression analysis was performed on parental cells (15P and 16P) and resistant cells (15IMr and 16IMr) respectively. The list of transcription factors (>2-fold regulated) was filtered based on their expression profile within a clinical cohort comprising of a publicly available microarray database of 42 CP-CML (drug sensitive) and 36 BC-CML (drug resistant) bone marrow samples respectively [132].

A candidate transcription factor from the KCL22 model was deemed clinically relevant if (i) its expression pattern mirrored that seen in the patient CP to BC transition and (ii) the adjusted p-value within the clinical samples was significant with $p<0.01$. This process narrowed the list to 30 transcription factors (23 induced and 7 repressed; (**Table 1.2**).

Gene	15IMr fold	16IMr fold	Clinical significance
AEBP1	9.23	10	1.48E-08
BCL11A	18.1	21.19	3.08E-07
CREB5	2.78	6.25	4.52E-03
DACH1	18.86	20.35	4.62E-03
DEAF1	2.57	3.09	2.93E-03
EBF3	8.04	8.14	9.67E-06
ETV6	3.55	4.45	6.51E-13
FOXA3	2.22	2.78	9.69E-20
HOXA5	3.24	4.83	9.67E-06
ID2	11.11	5.26	3.06E-14
MEF2D	3.26	4.35	1.93E-07
MEIS1	2.25	2.51	3.32E-05
NFIX	4.04	5.74	9.92E-04
NKX2-2	2.57	2.54	2.08E-05
RUNX1	5.66	6.57	6.26E-08
SOX4	5.69	6.27	8.54E-12
SSX1	14.29	20	3.18E-12
SSX2	6.25	5.56	3.06E-14
STAT4	4.62	12.78	2.81E-03
TAF12	2.44	2.22	4.11E-05
TCEA3	3.72	4.62	8.26E-04
TCEAL1	2.01	2.2	4.50E-08
TCEAL4	7.51	3.3	5.27E-09
ZNF25	2.39	2.32	2.30E-06
ZNF91	2.64	2.38	4.11E-07
ZNF256	5.06	3.13	1.37E-11
ZNF302	3.36	3.16	2.36E-20
ZNF331	2.31	2.41	4.91E-14
ZNF626	2.33	3.13	1.63E-04

Table 1.2: Clinically relevant transcription factors regulated in KCL22 cells upon drug resistance.

Table of clinically relevant transcription factors regulated upon drug resistance within KCL22 cells with expression profile within CML patient cohort in transitioning from drug-sensitive to drug-resistant ($p<0.01$). Clinical significance represents the corresponding adjusted p-value for the gene expression profile between CML CP (n=42) drug sensitive patients and BC (n=36) drug resistant patients for the dataset GSE4170 found in the GEO2R tool.

An example of this filtering process is presented displaying expression patterns of RUNX1 (~6-fold induced in IMr derivatives) and SSX1 (~15-fold supressed in IMr derivatives) from the clinical cohort (**Figure 1.8a and 1.8b** respectively). Expression of the gene network within the drug resistant KCL22 cells was confirmed accordingly via semi-quantitative PCR (16P model; **Figure 1.9**).

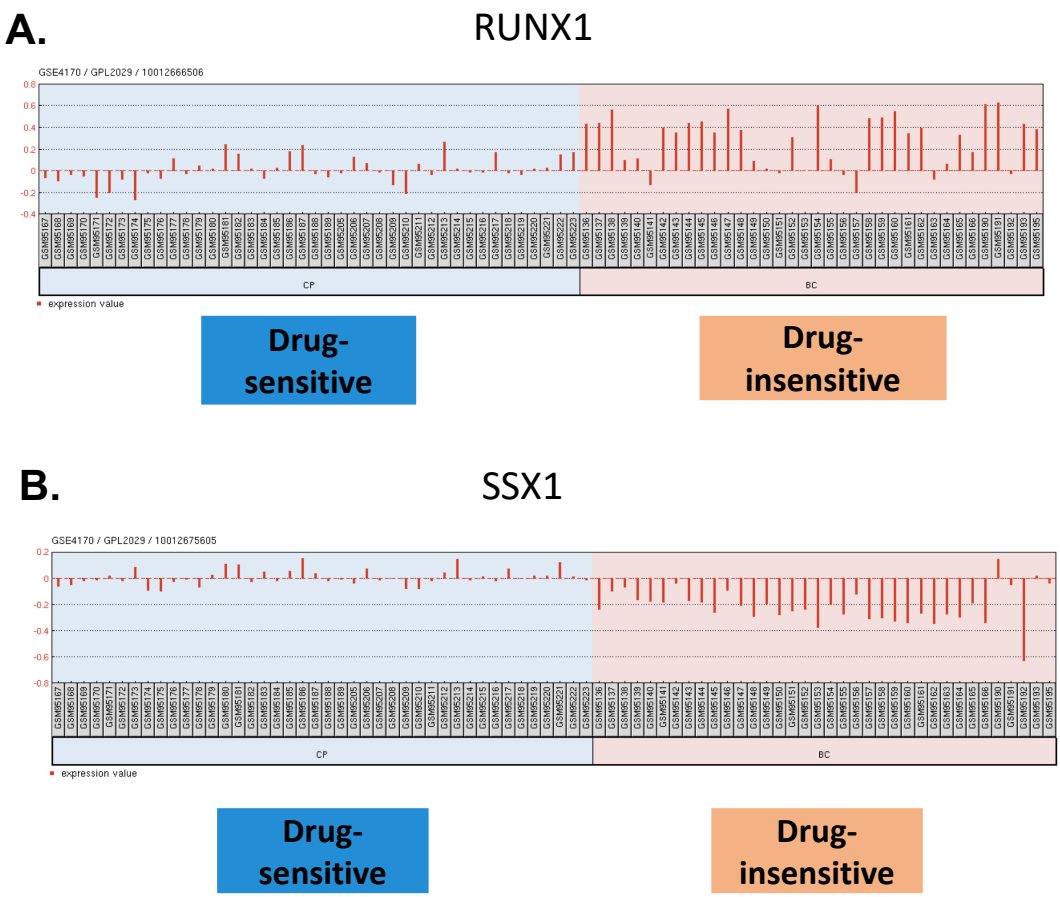


Figure 1.8: Clinical cohort database (GEO2R).

Examples of (**A)** RUNX1 upregulation and (**B**) SSX1 downregulation in BC-CML patients (drug-resistant, red panel) compared to CP-CML (drug-sensitive, blue panel).

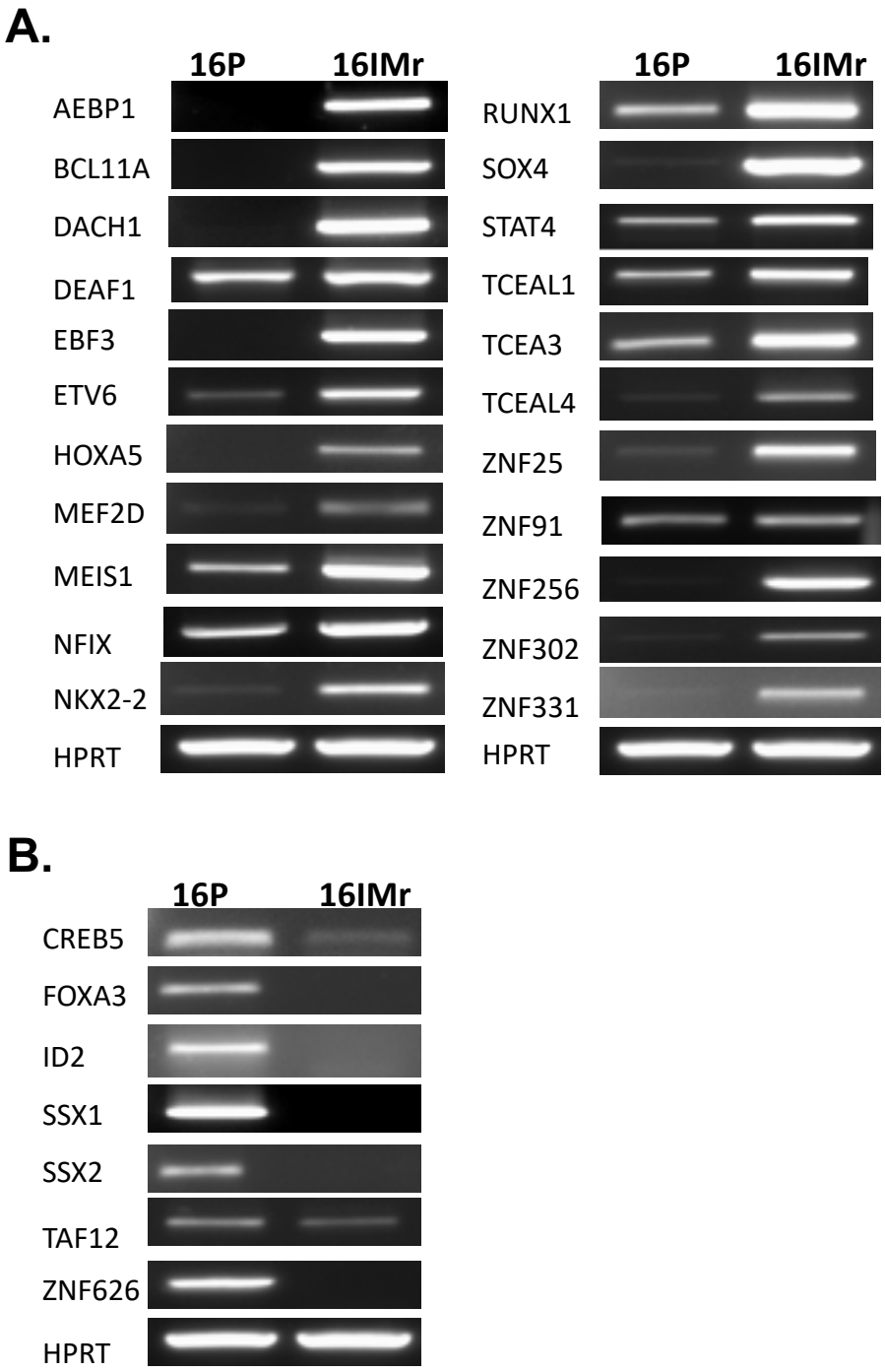


Figure 1.9: KCL22 drug resistant transcription factor gene-set.

Validation of the microarray analysis in KCL22 clone 16P and 16IMr by gene expression profile of (**A**) induced and (**B**) repressed transcription factors assessed by semi-quantitative PCR analysis. HPRT was used as a control.

1.17 SOX4 transcription factor and its role in CML

Among the list of the proposed clinically relevant transcription factors in drug resistant CML, the lab focussed on SOX4 based on (i) its expression profile in patient samples (**Figure 1.10a**), and pilot data profiling its expression in the KCL22 drug resistant model (work performed by A. Baral; **Figure 1.10b and 1.10c**).

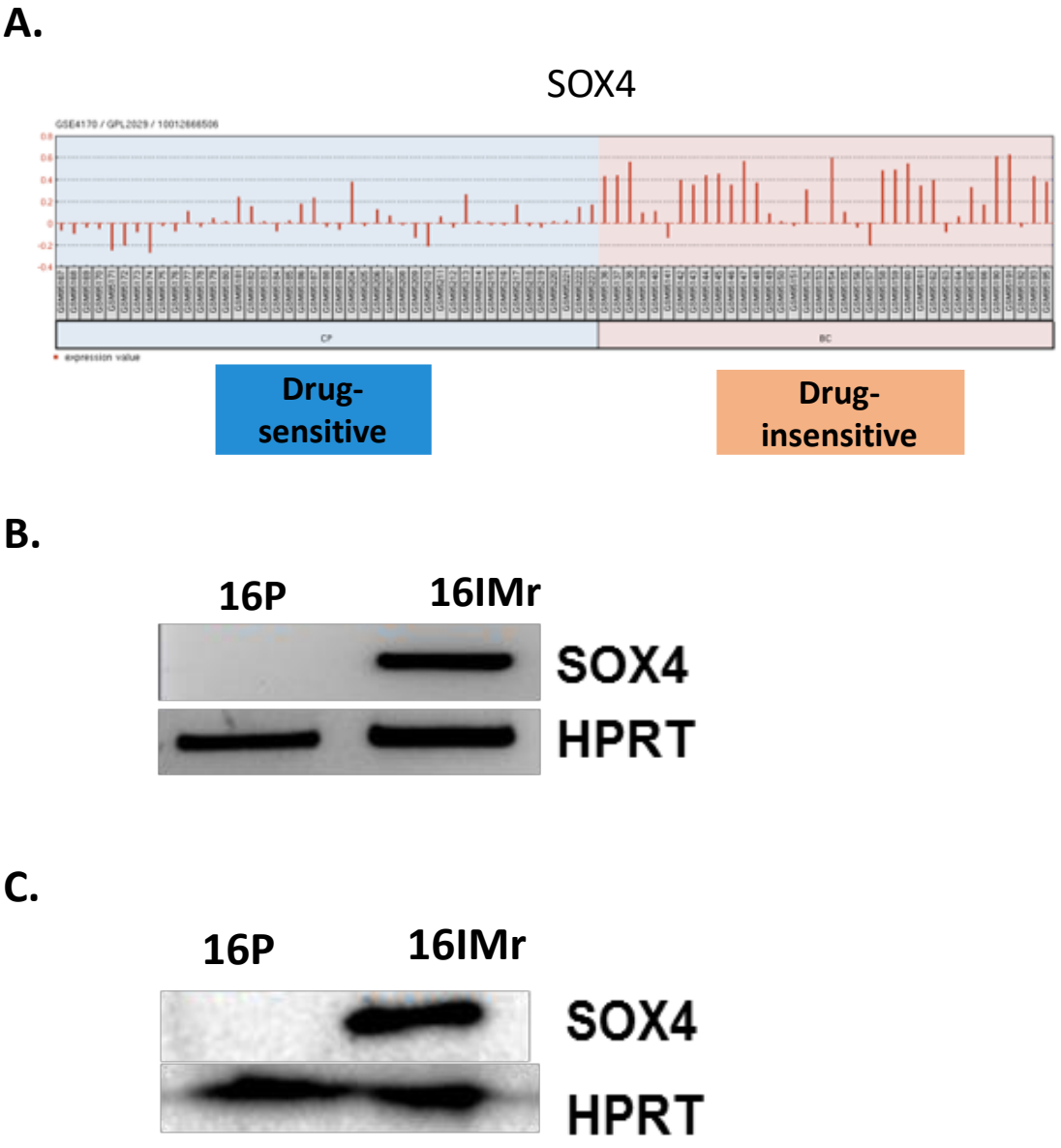


Figure 1.10: Expression of SOX4 in CML patients and the KCL22 drug resistant model.

(**A**) Representation of SOX4 expression (microarray) within drug-sensitive and -resistant respectively. Expression of SOX4 (**B**) transcripts and (**C**) protein in drug resistant KCL22 cells (16P model). HPRT was used as loading control. Data kindly provided by A. Baral.

The Sex Determining Region Y-Box 4 (SOX4) transcription factor is a member of the SRY-related High Mobility Group box family [133] and is frequently overexpressed in various cancers including breast, prostate, myeloma and hepatocellular carcinoma [134-137]. SOX4 functions as an oncogene with its increased activity promoting tumour cell proliferation, apoptotic inhibition and metastasis in variety of malignancies [138, 139].

SOX4 has a major role in haematopoiesis including the expansion of B and T progenitor cells respectively [140] as well as the development of progenitor B cells [141-143]. Although SOX4 is not required for normal myeloid differentiation it is, however, highly overexpressed within a subpopulation of AML patients that harbor a mutated or silent *C/EBPα* allele [144]. Using primary patient blast cells, as well as mouse models, it was established that targeting SOX4 within these leukaemic cells could restore normal granulocyte differentiation. As such, a primary role of SOX4 within AML blasts is to block myeloid differentiation. It is interesting to note that the driving *C/EBP*α oncogene is still active within these cells yet the leukaemic block can be overridden by the loss of SOX4. Corroborating these findings, the overexpression of SOX4 blocks granulocytic differentiation within 32D cells [145].

SOX4 is central to the leukaemic gene network of AML and these findings support the notion of a similar role in drug resistant CML. Previous work in the lab directly addressed this hypothesis (A. Baral). In brief, siRNA-mediated depletion of SOX4 within the 16IMr clone (**Figure 1.11**) reproducibly inhibited the ability of these cells to grow and expand. This pilot study clearly demonstrates that the drug resistant clone, 16IMr, is dependent upon SOX4 for cell proliferation.

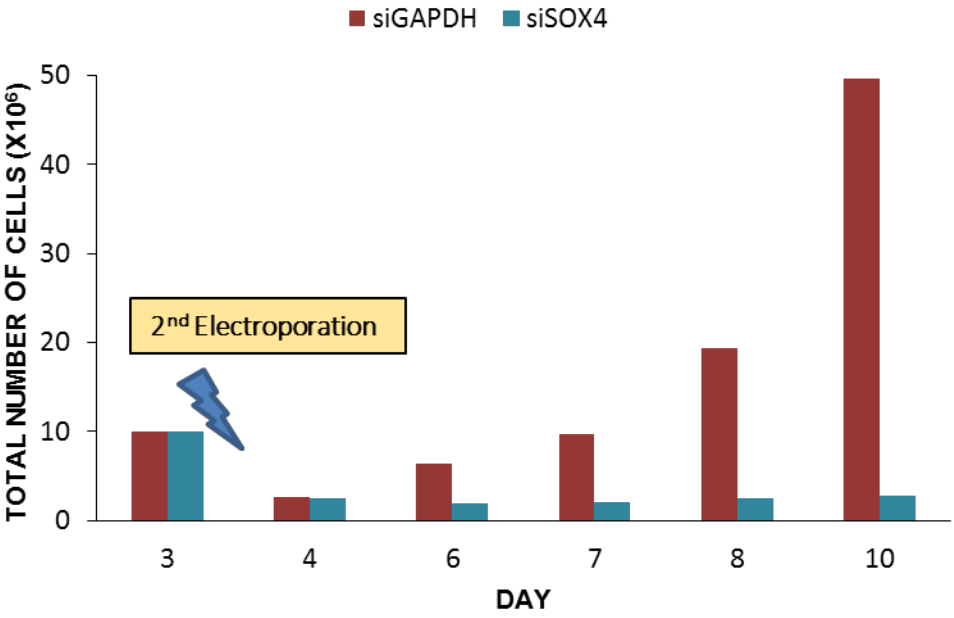


Figure 1.11: Cell growth of 16IMr cells upon depletion of SOX4.

16-IMr cells were transfected with either siSOX4 or siGAPH and left to grow for 3 days under incubation conditions. Then, a second electroporation for maintaining KD of SOX4 was performed. Tryphan blue exclusion assessing of cell viability was performed in the subsequent days, until day 10. Data kindly provided by A. Baral.

1.18 The SOXC genes: Fundamentals of their biology

The *SOX* (Sry HMG box) genes were first identified by Gubbay and co-workers in 1990 [146] and are an ancient family of transcription factors conserved across the chordate phylum. They are fundamental in embryonic development and cellular differentiation [147].

This family consists of 20 genes and all share a conserved 79-amino acid High Motility Group box (HMG) domain that allows DNA binding and transcriptional regulation. The SOX super-family is further sub-classified into nine groups based on their relative HMG box degree of similarity (70%-95% of sequence identity) [148]. In humans and mice, these groups are: SOXA, SOXB1, SOXB2, SOXC, SOXD, SOXE, SOXF, SOXG and SOXH [149]. The classification of each member into corresponding SOX group is indicated in **Table 1.3**.

Group	Members
SOXA	Sry
SOXB1	SOX1
	SOX2
	SOX3
SOXB2	SOX14
	SOX21
SOXC	SOX4
	SOX11
	SOX12
SOXD	SOX5
	SOX6
	SOX13
SOXE	SOX8
	SOX9
	SOX10
SOXF	SOX7
	SOX17
	SOX18
SOXG	SOX15
SOXH	SOX30

Table 1.3: The SOX transcription factor super-family.

The SOXC sub-group comprises of three members: SOX4, SOX11 and SOX12 [149, 150]. Each are single-exon genes and express proteins of approximately 47, 44 and 31 kDa of molecular weight, respectively. They possess two main domains: (i) HMG-box DNA binding domain and (ii) a transactivation domain (TAD) at the C-terminal region which allows for protein-protein interaction with multiple transcriptional partners [151]. The SOXC genes are highly homologous with each other bearing a HMG-box with 84% sequence identity (95% similarity) among its members and similarly for its TAD with 67% sequence identity (94% similarity) respectively [152]. Their structure is shown in **Figure 1.12**.

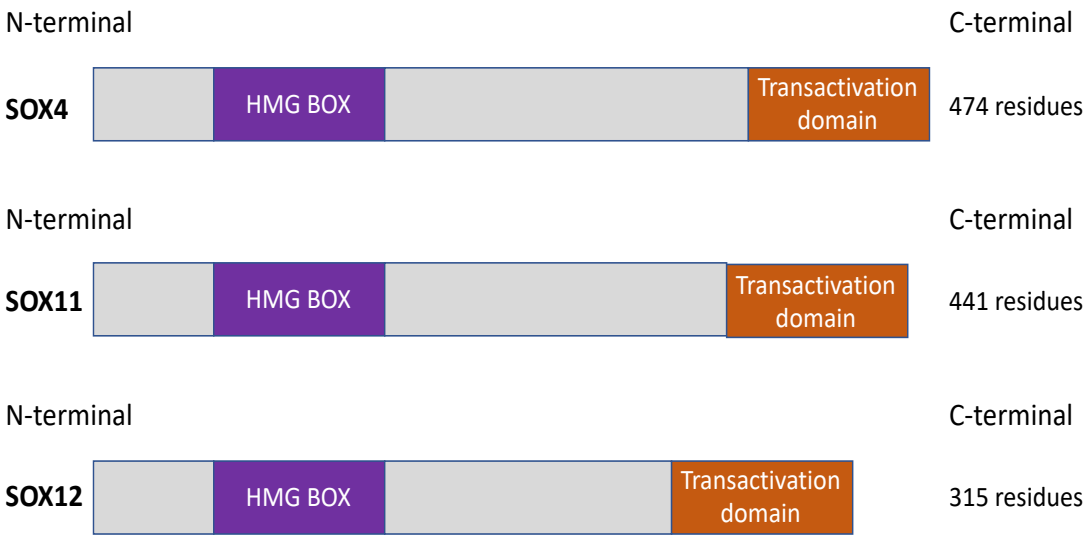


Figure 1.12: Schematic of the SOXC family of transcription factors.
The SOXC family consists of the SOX4, SOX11 and SOX12 genes sharing strong homology at the HMG box and transactivation domains respectively. Based on data by Dy *et al* 2008 [152].

The SOXC genes are frequently co-expressed in a number of different tissues, and their simultaneous expression, and function, within neuronal and mesenchymal organs is necessary for their development [152]. Indeed, the SOXC genes are required for survival of neural and mesenchymal progenitors respectively by mediating the expression and interacting with Tead2, a fundamental member of the Hippo developmental pathway [153]. Moreover, SOXC gene expression is required for skeleton morphogenesis in mouse, and this is achieved by regulating the WNT pathway [153, 154].

SOX4 was first identified in 1993 by Van de Wetering and co-workers [155], and details of this transcription factor is described earlier.

SOX11 and SOX12 were discovered by Wright et al. in 1993 [156]. SOX11 is required during mouse embryogenesis with increasing expression within the spinal cord and brain as the cells begin to migrate into what would be the peripheral nervous system; upon neuronal differentiation its expression considerably decreases [157]. The importance of SOX11 to neuronal development is furthered established by clinical-genetic findings whereby point mutations and genetic aberrations of the *SOX11* gene is correlated with the onset of Coffin-Siris syndrome, a congenital disorder characterised by intellectual impairment, congenital deficiency, facial and limb malformations as well as cardiac abnormalities [158]. Such genetic abnormalities include c.347A>G,

c.178T>C, c.305C>T and deletions at chromosome 2p25 (which involves SOX11) [159-161].

Moreover, SOX11 is essential for cardiac development with the death of null-mice shortly after birth due to arterial outflow tract malformations [162] and accompanied by skeletal and facial disruptions [163].

SOX11 is overexpressed in Mantle cell lymphoma (MCL), and is epigenetically regulated within these tumour cells with hypomethylation at the promoter in malignant lymphoma cells when compared to those located in healthy surrounding tissue [164]. In MCL, SOX11 inhibits differentiation by directly suppressing the expression of PAX5 [165]. Additionally, within MCL pathology, SOX11 expression increase PDGFA production and promotes angiogenesis and tumour growth in mouse models [166]. SOX11 is a biomarker for the diagnosis of cyclin D1-negative MCL; with a 93% correlation between this disease and high nuclear SOX11 expression [167, 168].

SOX12 is the least characterised member of the SOXC genes. Mutant mice develop normally without any noticeable alterations in their phenotype [169]. However, the loss of SOX12 within lung cancer cell lines, and patient samples, promoted cell cycle arrest and induced apoptosis [170]. Moreover, SOX12 induces mesenchymal-to-epithelial transition in hepatocellular carcinoma by regulating the expression of Twist1 and FGFBP1 which, in turn, promotes the metastatic potential of tumour cells respectively [171]. Similarly, in colorectal cancer, SOX12 overexpression has been correlated to poor prognosis and functions to promote tumour proliferation, migration and invasion *in vitro* and *in vivo* [172]. A similar role of SOX12 in tumour proliferation and invasion has been reported in breast cancer as well [173].

1.19 SOXC genes and functional redundancy

Due to their strong sequence homology, the members of the SOXC family often present overlapping functionalities and can even compete among themselves for target genes [152]. This functional redundancy is most prevalent within the limb development of mice [154]. The individual loss of *SOX4*, *SOX11* or *SOX12* fail to display any gross limb deformation. Yet, mice with the combined loss of *SOX4* and *SOX11* develop severe development of the limbs which is further exacerbated within the triple knock-out [148, 174].

1.20 Aims and Objectives

Objective 1: The KCL22 model of CML drug resistance clearly demonstrates that the cells are capable of surviving and expanding in the absence of the SH1 kinase activity. A greater understanding of the molecular mechanism(s) of how drug resistant CML cells have become independent of kinase activity is of great importance. This will impact future drug therapies in directing whether efforts should target (i) alternate BCR-ABL domains or (ii) focus on an 'oncogene independent' disease that has acquired a new drug resistant network. The first aim of this study is to investigate whether in the absence of SH1 kinase activity, do the other domains of BCR-ABL1 protein have a function in maintaining cell growth and/or survival?

Objective 2: While the KCL22 model of drug resistance mirrors clinical observations, it suffers from concerns that any novel findings are only established within a single cell line. As such, a second model of CML drug resistance would be invaluable in confirming, and validating, any hypothesis derived from the KCL22 cells. The second aim of this study is to generate and characterise an independent cell-based model of kinase-independent drug resistant CML.

Objective 3: Transcription factors are commonly deregulated in myeloid leukaemias and contribute to the malignant phenotype by impeding cellular differentiation. Upon drug resistance, the KCL22 cells differentially regulate the expression of several transcription factors. Collectively, these observations lead to the hypothesis that these deregulated transcription factors function to block differentiation within drug resistant KCL22 cells and thus maintain the leukaemic phenotype. One such transcription factor identified was SOX4. The third aim of this study is to determine the role, if any, of the SOX4 transcription factor in conferring CML drug resistance.

Chapter 2: Materials and Methods

2.1 List of reagents and equipment

Tissue culture reagents

Tissue culture reagent	Manufacturer
RPMI-1640	Sigma-Aldrich, Missouri, U.S.A.
Foetal Calf Serum (FCS)	Sigma-Aldrich, Missouri, U.S.A.
L-Glutamine	ThermoFisher Scientific, Massachusetts, U.S.A.
10% Ammonium Persulphate (APS)	Sigma-Aldrich, Missouri, U.S.A.
Penicillin-Streptomycin	ThermoFisher Scientific, Massachusetts, U.S.A.
β-mercaptoethanol	Sigma-Aldrich, Missouri, U.S.A.
Trypan Blue	Sigma-Aldrich, Missouri, U.S.A.
Imatinib (IM)	ENZO Life Sciences, Exeter, U.K.
Dasatinib (25mg)	Adoq Bioscience
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Missouri, U.S.A.
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega, Madison, Wisconsin, U.S.A.
Geniticin® (G418)	ThermoFisher Scientific, Massachusetts, U.S.A.
Hygromycin (50mg/ml)	Invitrogen, Carlsbad, California, U.S.A.
Ambion® Silencer® siRNA sequences	ThermoFisher Scientific, Massachusetts, U.S.A.

Tissue culture equipment

Tissue culture equipment	Manufacturer
Pipetman®	Gilson, Dunstable, U.K.
Neubauer Chamber (0.1mm, 1/400mm)	Hawksley, Sussex, U.K.
Electroporation cuvettes	Geneflow, Staffordshire, U.K.
BioRad® GENE PULSER II	BioRad, California, U.S.A.
Attune® Acoustic Focusing Cytometer	ThermoFisher Scientific, Massachusetts, U.S.A.
Tissue Culture Microscope Olympus CXX41	Olympus Lifesciences, Tokyo, Japan
Nikon® Eclipse E1000	Nikon, Melville, U.S.A.
Cytospin CytoCentrifuge™	ThermoFisher Scientific, Massachusetts, U.S.A.
TC20™ Automated cell counter	BioRad, California, U.S.A.
Cell Counting Slides Dual Chamber	BioRad, California, U.S.A.
Olympus U-RFLT 50	Olympus Lifesciences, Tokyo, Japan
Olympus C-7070	Olympus Lifesciences, Tokyo, Japan
Mithras® LB940 Microplate Reader	Berthold Technologies, Wildbad, Germany

Laboratory reagents

Lab bench reagents	Manufacturer
QIAzol lysis reagent	Qiagen, Hilden, Germany
Chroloform	Sigma-Aldrich, Missouri, U.S.A.
Isopropanol	Sigma-Aldrich, Missouri, U.S.A.
Ethanol	Sigma-Aldrich, Missouri, U.S.A.
Methanol	Sigma-Aldrich, Missouri, U.S.A.
Omniscript® Reverse Transcription Kit	Qiagen, Hilden, Germany
Random Nonamers	Sigma-Aldrich, Missouri, U.S.A.
Fast Gene Optima® Hot Start DNA Polymerase	Nippon Genetics, Dueren, Germany
Ultra-Pure Agarose	ThermoFisher Scientific, Massachusetts, U.S.A.
Ethidium Bromide (10mg/ml)	Sigma-Aldrich, Missouri, U.S.A.
Hyclone™ Nuclease-free water	ThermoFisher Scientific, Massachusetts, U.S.A.
Laemmli Lysis Buffer	Sigma-Aldrich, Missouri, U.S.A.
Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Missouri, U.S.A.
Acrylamide/bis-acrylamide 40%	Sigma-Aldrich, Missouri, U.S.A.
Protease Cocktail Inhibitor (PIC) 100X	Cell Signaling Technology, Danvers, Massachusetts, U.S.A.
Page Ruler™ Plus Prestained Protein Ladder	ThermoFisher Scientific, Massachusetts, U.S.A.
Dried Skimmed Milk	Marvel, Premier Foods, St. Albans, Herthfordshire, U.K.
Tween 20®	Sigma-Aldrich, Missouri, U.S.A.
Bovine Serum Albumine (BSA)	Sigma-Aldrich, Missouri, U.S.A.
SuperSignal West® Pico Chemiluminescence Kit	ThermoFisher Scientific, Massachusetts, U.S.A.
DH5α *E.coli* competent cells	New England Biolabs, Massachusetts, U.S.A.
Luria-Bertani (LB) Broth	Sigma-Aldrich, Missouri, U.S.A.
Kanamycin (50mg/ml)	ThermoFisher Scientific, Massachusetts, U.S.A.
Plasmid Maxi Kit	Qiagen, Hilden, Germany
Annexin-V (BV421)	BD Biosciences, California, U.S.A.
Propidium Iodide (PI)	Sigma-Aldrich, Missouri, U.S.A.
Binding Buffer 10X	BD Biosciences, California, U.S.A.

Laboratory equipment

Lab bench equipment	Manufacturer
Grant® Water Bath	Scientific Laboratory Supplies, Nottingham, U.K.
Heat Block	Thermolyne, Iowa, U.S.A.
Thermocycler 3000	Biometra, Göttingen, Germany
RunOne® Electrophoresis Gel	Embi Tec, San Diego, California, U.S.A.
UV transilluminator	Alpha Innotech Corporation, San Leandro, California, U.S.A.
BioRad® XR gel and protein visualiser	BioRad, California, U.S.A.
Axygen® PCR Strip tubes	Sigma-Aldrich, Missouri, U.S.A.
NanoDrop® 1000	ThermoFisher Scientific, Massachusetts, U.S.A.
MiniProtean Tetra Cell	BioRad, California, U.S.A.
PowerPac™ Basic Power Supply	BioRad, California, U.S.A.
Mini Trans-Blot® Cell	BioRad, California, U.S.A.

Homemade reagents

Homemade buffers and solutions	Composition
10X TBS Buffer	96g Tris-Base, 352g NaCl, p 7.6
10X TGS (Running Buffer)	25mM Tris, 192mM Glycine, 0.1% SDS
10X TG (Transfer Buffer)	25mM Tris, 192mM Glycine, pH 8.3
Washing Buffer	1X TBS buffer, 0.1% Tween 20
Blocking Buffer	5% Marvel milk in 1X TBS-Tween® 20
Primary Antibody Incubation Buffer	5% BSA in 1X TBS-Tween® 20
Secondary Antibody Incubation Buffer	5% Marvel milk in 1X TBS-Tween® 20
MACS Buffer	0.5% BSA, 2mM EDTA in 1X PBS
Wright-Giemsa staining Buffer	300mg powdered Wright's stain, 30g powdered Giemsa100ml absolute methanol

2.2 Cell line culture

All cell lines (KCL22, EM2, K562 and all derivatives) were cultured in RPMI-1640 media (supplemented with 10% Foetal Calf Serum, 1% L-Glutamine, 1% Penicillin-Streptomycin and 0.1% β-mercaptoethanol). Cells were split with a dilution factor of 1:3 when confluency reached approximately 80%. Drug resistant KCL22 cells were maintained in the presence of 1μM IM, while the EM2 derivatives in 0.7μM IM. K562-SD and K562-RD cells, kindly given by Dr. Patrick Auberger [175], were maintained in 2nM Dasatinib. Generation of EM2 single cell clones was carried out by serial dilution.

2.3 Cryopreservation of cells

To preserve cells in liquid nitrogen storage at -195°C, cells were spun down by centrifugation and re-suspended in FCS containing 10% DMSO. Cells were transferred to Cryovials, placed in freezing chambers and stored in a -80°C for 24 hours. For next day, cells were transferred to the LN_2 tank for long-term storage.

2.4 Trypan Blue method for cell counting

For cell proliferation assessment, cell counts were performed. From a cell suspension, 20μl volume of cells was mixed with 20μl of trypan blue and transferred to each side of the Neubauer Chamber slide. Briefly, bright cells are counted and their sum of the 4 quadrants is calculated. The average is measured and multiplied by the dilution factor and the 10^6 factor due to slide volume. Such indicates the number of cells in the dimension of million cells per ml.

2.5 MTS cell proliferation assay

Cell proliferation was measured with $0.2x10^6$ cells plated in 12-wells and incubated for 72 hrs. Next, 100μl of cells was transferred into a 96-well plate and mixed with 20μl of MTS reagent (CellTiter 96®, Promega). Plates were left to incubate for 4 hours at 37°C or until the solution presented a dark-brown colour. Plates were analysed by using a Mithras microplate reader at a 490nm wavelength for absorbance detection. Empty media was used as a blank and media with cells without MTS reagent were used as control.

2.6 Generation of drug-resistant cells

The EM2 single-cell clones P3, P4, P5, P6 and P7 were cultured in the presence of 10% of final IM with 10% weekly increments until they reached a final IM concentration. Cells were cultured at each drug concentration until a steady stable population was established. Overall, this protocol took about 8 weeks to complete. Once drug resistance was established, cells were maintained at final IM concentration. Frozen stocks were stored at -80.C.

2.7 RNA extraction and cDNA generation

Total RNA from cells was isolated using QIAzol™ reagent following the provider's instructions. RNA quality and concentration were measured using NanoDrop-1000™. Total RNA was also run on 0.8% agarose gels to determine quality. Desired values for RNA quality were 1.8-2.0 for 260/280 ratio and 2.0-2.2 for 260/230 ratio.

For reverse transcription, 2μg of RNA was used as template to generate cDNA using the Omniscript® RT (QIAGEN) kit. All components were mixed by pipetting gently and incubated at 37°C in the water bath for 2 hours. Tubes were centrifuged at high speed for 30 seconds and diluted with 180μl sterile dH2O and gently mixed by pipetting. This corresponded to 1μg/100μl cDNA concentration and was used in PCRs or frozen at for later use.

2.8 Primer design

All primers used for cloning and semi quantitative reverse transcription PCR (RT-PCR) analysis was designed using the codon regions of genes with the online Primer 3 program:

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) with following modifications to the standard settings - the length of primer was set at 350-500bp, annealing temperature of primer was set 59-60 and max self-complimentary at 5. Primers were commercially synthesized from Sigma Aldrich. All primers were suspended in nuclease free water at a concentration of 100μM and stored at -20°C.

2.9 Semi-quantitative PCR

PCR was performed using FastGene® Optima HotStart Ready Mix (Nippon Genetics Europe, Dueren, Germany) with 0.25μl of 100μM stock for a final concentration of 1μM for both reverse and forward primers. PCR reaction was performed under the following conditions: 94°C for 5 minutes followed by 94°C for 30 seconds, annealing temperature as indicated in **Tables 2, 3 and 4,** for 45 seconds and final extension at 72°C for 90 seconds. The sequences of primers and number of cycles for each gene are indicated in the corresponding tables. Amplified products were electrophoresed in 1.3% agarose 1x-TBE gel containing 10mg/ml of Ethidium Bromide (Sigma-Aldrich) and visualised in the Bio-Rad® XR gel visualizer (Bio-Rad Laboratories).

Gene	Forward primer	Reverse primer	Annealing Temp. (C°)	Cycles
AEBP1	AGAAGAACCCCTTCGTGCTG	TCCAGGCAGTTGGTATGCAG	58	30
BCL11A	CTGTGCAACTATGCCTGTGC	ATTGCCCCATACAGATCATGC	58	30
CREB5	GGGTTGGAGGCTAGACAGTTC	GCTTCCTGCAGTCACCAAATC	57	35
DACH1	ATTTCGACTTGCGGGACACT	GTGGAGATTGGGGGTTGAGG	57	32
DEAF1	AGTGTTGACATCCCTGCCTG	CTCACACGGTCACCTTCTCC	58	32
EBF3	ACCCACGAGATCATGTGCAG	GATGCACGGAGTGGCTTCT	57	32
ETV6	TCTCTCCTGCTCCTCTTCGG	TATTTGCAACTCACTGGCCCT	58	32
FOXA3	AGTGGAGCTACTACCCGGAG	ACCTTGACGAAGCAGTCGTT	60	32
HOXA5	CGGCGGGAAAAACTCCCTAA	CGGGTCAGGTAACGGTTGAA	65	32
ID2	AGGAAAAACAGCCTGTCGGA	ACCGCTTATTCAGCCACACA	65	28
MEF2D	GATCTGAACAATGCCCAGCG	CTTGATGCTGATGTGGGGGT	60	32
MEIS1	GCGCAAAGGTACGACGATCT	ATGACTCTGACGAGCAGACG	57	32
NFIX	ATGTACTCCCCGTACTGCCT	CTGAGGCGACTTGTAGAGCC	57	32
NKX2-2	CGCGTGCTTTCAAAGAAGACA	GTTGTCGCTGCTGTCGTAGA	60	32
RUNX1	TCTGACCATCACTGTCTTCA	GGTATTGGTAGGACTGATCG	60	28
SOX4	ATGATCTCGGGAGACTGGCT	TAACTCGCCTTCTTGCTGGG	58	28
SSX1	AGAGACCCAGGGATGATGCT	GGCCAGATGCTTCTGACACT	65	32
SSX2	CCCTAACCGTGGGAATCAGG	GGTACCATGAACTGCACCCA	60	35
STAT4	GCAGTTTCTGCGTGTTAGCAT	TTAGAAGCTGCCTCCCAGTC	58	35
TAF12	GGGTCAGTGTTCGAAGGACC	AATCTGCCGAGCTTTGGACT	65	32
TCEA3	ACCTCTTCCAGTGCAGCAAA	TTCTCCAATTAGGCTCCCCC	58	32
TCEAL1	TCAGGGAAGGGAATAACTGTGC	CCCTCTAAATTGCGGGCGA	58	35
TCEAL4	AGGACAGGAAAAGGAGGGGA	TTGAGGTAATGAGCCAGCCC	65	32
ZNF25	GAACAAGTTCCAGGGACCCG	TCACAGGCTTTCTCTGTGGT	65	30
ZNF91	GCTGGGAACTGTCCAATCAGG	GCTTAGAGAGAGCAATACCCAGG	60	35
ZNF256	ATGCTGGAGAACTTGACACT	GACTTCTTTCTGGTGTGAGC	58	32
ZNF302	TGAGAACCTGGTCTCTGTAGGT	TTGAAGGCTGCCCCACTTTT	65	32
ZNF331	CACAGTCCAATGAGGAGCGA	GCTTGTTCGCATTACCCCAC	58	32
ZNF626	CGTGTCCTGCAGGTATTGGG	GAGGACTTTGGCTCTCACTGT	65	32
HPRT	AGGACTGAACGTCTTGCTCG	ATCACACTCGTGGGGTC	58	28

Table 2.1: List of primers and conditions for the Transcription Factors identified in KCL22 cells. HPRT is the loading control used in RT-PCR.

Gene	Forward primer	Reverse primer	Annealing (C°)	Cycles
CD11b	TTTCTGCCCTTCTTTGCTTTGG	TAGTCGCACTGGTAGAGGCT	65	35
CD14	CAGAACCCTAGATGCCCTGC	CATCGTCCAGCTCACAAGGT	65	35
CD235a	AGCAGGCTAAGGTCAGACAC	TGAGTGTTATCTCGGTTTCCTCTT	65	35
CD71	ACCGCCGGTTAGGGG	ACGATCACAGCAATAGTCCCAT	60	32
C/EBPα	TATAGGCTGGGCTTCCCCTT	AGACGCGCACATTCACATTG	58	32
EPO-R	CATGACGTCTCACATCCGCT	GGCCTTCAAACTCGCTCTCT	65	35
GATA-1	CTACACCAGGTGAACCGGC	CCCAGAGACTTGGGTTGTCC	58	32
Gfi1	CAAGTGCCACCTGGTCTCC	GGGCACATTGACTTCTCCGA	60	35
Lactoferrin	TGGTGGCTTGTACCCCAAAG	GTAGACTTCCGCCGCTACAG	60	28
M-CSF-R	AATAATGGCCCTGCAGACCT	CTCGCCCAGGTACAGTTCAT	58	32
NF-E2	GTGACTCCACCACAGGTTTCT	CTCAGTGGCTTGGAGACTGG	58	32
PU.1	CGGCAGGCCCTTCGATAAA	GAAGTTGTTCTCGGCGAAGC	58	32
SCL (TAL1)	CAGCGAAAAAGGGGGAAAGC	GTCGCGGCCCTTTAAGTCT	58	32
TLR2	CTCGGAGTTCTCCCAGTGTTT	TGGCCGCCTTGATTCATAGA	58	32
TLR4	AGAATGCTAAGGTTGCCGCT	CGGAGTCTGAAAGCTCTGGG	58	32

Table 2.2: Table of primers and conditions for Differentiation markers.

Gene	Forward primer	Reverse primer	Annealing (C◦)	Cycles
BCR-ABL1 (b2a2 and b3a2)	ACAGCATTCCGCTGACCATCAATAAG	ATGGTCCAGAGGATCGCTCTCT	58	32

Table 2.3: Table of primers and conditions for the BCR-ABL1 gene.

2.10 Protein lysate preparation

For generating protein lysates, $10x10^6$ cells were centrifuged at 1200rpm, for 5 minutes at 4°C, washed with ice-cold PBS three times, centrifuged as aforementioned and re-suspended in 25μl per million cells in Laemmli Lysis Buffer supplemented with 100mM Protease/Phosphatase Inhibitor Cocktail. Cell lysates were placed in a water-bath at 95°C for 8 minutes. Lysates were centrifuged at $13.2x10^3$ rpm at room temperature for 1 minute and 45μl of the supernatant was aliquoted into individual tubes and stored at -80°C. For loading, the aliquot would be boiled at 95°C for 8 minutes, spun down with the previously mentioned settings and loaded into the gel.

2.11 Western Blot Analysis

Protein lysates (15μl) were loaded and run on a 10% SDS-PAGE. Protein transfer onto nitrocellulose membrane was done using the wet-transfer protocol (Bio-Rad Laboratories). Membrane was blocked in 5% skimmed milk, TBS-0.1% Tween-20 for 1hr at room temperature under gentle shaking. Primary antibodies (**Table 2.4**) were incubated in 5% BSA, TBS-0.1% Tween-20 at 4°C under 130rpm shaking, overnight. Membrane was washed 3 times in TBS-0.1% Tween-20 under shaking, followed by secondary antibodies incubations (**Table 2.5**) for 1hr at room temperature under gentle shaking. Then, membranes had a final wash round of 3 times for 5 minutes each with TBS-0.1% Tween-20. Finally, bound antibody was visualised using SuperSignal™ WestPico Chemiluminescent Substrate and using the BioRad Transilluminator.

Protein	Antibody company	Primary antibody dilution, species	Secondary antibody dilution
GAPDH	Proteintech, Illinois, U.S.A.	1:25000, mouse	1:5000, goat anti-mouse
Vinculin	Proteintech, Illinois, U.S.A.	1:3000, mouse	1:5000, goat anti-mouse
pABL1 Y1245	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit
pCRKL Y207	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit
pSTAT5 Y694	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit
tABL1	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit
SOX4	Abcam, Cambridge, U.K.	1:4000, rabbit	1:5000, goat anti-rabbit
pTyrosine	Cell Signalling, Massachusetts, U.S.A.	1:5000, mouse	1:5000, goat anti-mouse
SKP2	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit
p27	Cell Signalling, Massachusetts, U.S.A.	1:5000, rabbit	1:5000, goat anti-rabbit

Table 2.4: Primary and secondary antibodies conditions for western blot.

Secondary Antibody	Company
Peroxidase Conjugated Goat Anti-rabbit IgG	Jackson ImmunoResearch Laboratories, Pennsylvania, U.S.A.
Peroxidase Conjugated Goat Anti-mouse IgG	Jackson ImmunoResearch Laboratories, Pennsylvania, U.S.A.

Table 2.5: Secondary antibodies.

2.12 Generation of recombinant KCL22 cells

About $10x10^6$ KCL22 16P and 16IMr cells were transfected with 20μg plasmid by electroporation at 250V and 950μF. In order to generate stable lines, transfected cells were cultured with 1.25 mg/ml G418 with drug replenished every 3-4 days. As these cells were electroporated alongside a GFP vector, a blue light UV microscope was used to verify efficiency of transfection and stable generation.

2.13 Annexin-V and Propidium Iodide (PI) apoptosis analysis

For analysis of apoptosis, $5x10^5$ cells were collected and washed twice with cold 1X PBS. Then, cells were washed with 1x Binding Buffer (BD Biosciences). After this, cells were resuspended in 5% AnnexinV-1x Binding Buffer (BD Biosciences) and incubated for 15 minutes at room temperature in the dark. Next, cells were resuspended in 1x Binding buffer with 5% PI and incubated for 15 minutes at room temperature in the dark.

2.14 Genome-Wide microarray analysis

For Genome-Wide Microarray Analysis, total RNA was extracted from whole cell lysate using the QIAzol™ reagent and protocol as previously mentioned. Total RNA extracted was quantified using the Nanodrop 1000™, required ratio values

were for 260/280 and 260/230 was of 1.8 to 2.0 for both. 100ng/μl of RNA sample concentration was required.

The cells lines used were siBCR treated 15IMr and 16IMr cells (15B and 16B respectively), siControl treated 15IMr and 16IMr cells (15C and 16C, respectively) and EM2-P7 and EM2-R7 (P7 and R7, respectively), all of them in triplicates. The sample labelling, quality control values and RNA concentration obtained for each sample are shown in **Table 2.6**. Before sample submitting, total RNA was visualised in a 0.8% gel for quality assessment.

Number	Name	Concentration (ng/µl)	Volume (µl)	Total Quantity (ng)	A260/A280 Ratio	A260/A230 Ratio
1	15-B1	112.64	13	1464.32	1.77	2.29
2	15-B2	104.02	13	1352.26	1.74	2.27
3	15-B3	102.22	13	1328.86	1.78	2.26
4	15-C1	109.97	13	1429.61	1.81	2.19
5	15-C2	114.01	13	1482.13	1.71	2.14
6	15-C3	112.54	13	1463.02	1.8	2.19
7	15-R1	102.14	13	1327.82	1.81	2.2
8	15-R2	107.11	13	1392.43	1.82	2.19
9	15-R3	97.75	13	1270.75	1.76	2.18
10	16-B1	100	10	1000	2.03	2.08
11	16-B2	100	10	1000	2.03	2.08
12	16-B3	100	10	1000	2.03	2.08
13	16-C1	100	10	1000	2.03	2.13
14	16-C2	100	10	1000	2.03	2.13
15	16-C3	100	10	1000	2.03	2.13
16	16-R1	100	10	1000	1.98	1.84
17	16-R2	100	10	1000	1.98	1.84
18	16-R3	100	10	1000	1.98	1.84
19	P7-1	168	10	1680	1.97	2.34
20	P7-2	219.1	10	2191	1.99	2.36
21	P7-3	211.3	10	2113	1.97	2.33
22	R7-1	197.8	10	1978	1.93	2.34
23	R7-2	209	10	2090	1.96	2.38
24	R7-3	210.5	10	2105	1.93	2.34

Table 2.6: Table of sample labelling for submission to CGS for Genome-Wide microarray analysis.

18 samples were submitted to CGS for microarray analysis. The number is indicated next to its sample name. The KCL22 clone number, either 15 or 16, or EM2 clone, P7 or R7, are indicated, followed by treatment type in the case of KCL22: B for siBCR and C for siControl. The number at the end indicates number of repeats. Concentration of RNA, sample volume and total quantity, as well as quality assessment by Nanodrop are indicated in the following columns, respectively.

2.15 Microarray sample and raw data processing

Submission of RNA samples from the siBCR/siControl treated KCL22 single-cells and EM2 P7 and R7 cells was sent to Cambridge Genomic Services (CGS), University of Cambridge via postal service. Microarray was performed using a Clariom S Human HT Array Plate (Affymetrix) in conjunction with a WT PLUS amplification kit (Affymetrix). Data processing was done using a GeneTitan instrument and a Command Console viewer (Affymetrix). CEL file generated by the scanner are submitted into the R studio (RStudio, Inc.) package, Bioconductor. In here, the raw data was processed using the RMA method for sample normalisation.

2.16 Linearization algorithm and expression fold-change calculation

The data was received from CGS as a Robust Multichip Analysis (RMA) format, which is the most frequently used method as it allows for data normalization, background correction and summarization [176]. In this data set, the expression levels of the gene probe are shown as Log_2 base values, and therefore, for analysis simplicity, linearization algorithms were performed in order to acquire natural base values. Such was performed by following the equation: $y=2^x$; where x is the RMA raw value and y is the linearized form of x. Then, for fold-change calculation, average of triplicates per sample was calculated and ratio was obtained by: $\frac{siBCR\ or\ EM2\ resistant}{siControl\ or\ EM2\ parental}$ After the fold change was obtained, we selected the genes with a 2-fold change above or below the control and considered them as differentially regulated upon treatment or resistance.

2.17 Gene Set Enrichment Analysis (GSEA)

For identification of pathway-related gene sets that could be compared to our microarray data (http://software.broadinstitute.org/gsea/downloads.jsp) was utilised with the assistance of Bioinformatician Dr. Dapeng Wang.

The RMA data set was uploaded as an expression data set and a permutation type Phenotype was selected. The program was instructed to run up to 1000 gene set permutations. The C2 curated collection gene set, which belongs to the Molecular Signatures Database (MSigDB), was selected for comparison to our microarray gene sets as it utilizes publication-based gene sets and microarray data. In here, other pathway-related gene set pathways are contained as BIOCARTA, KEGG and REACTOME. First, the cut-off statistical values necessary to discriminate relevant pathways across all the generated datasets were selected. It is of general recommendation by the GSEA to select pathways with Nominal p-value and False Discovery Rate (FDR)<0.05, which would provide enough stringency to identify important differentially expressed pathways and diminish the rates of false positives, respectively.

2.18 Plasmid transformation and isolation

For plasmid extraction, DH5α *E. coli* (New England Biolabs) bacteria were transformed with the plasmid or ligation of interest by the heat shock method. Next, bacterial cells were cultured onto plates with LB-Agar broth supplemented

with Ampicillin (100μg/ml) or Kanamycin (50μg/ml) Then, plates were left incubating for 10 hrs at 37°C. After incubation, individual colonies were selected and placed in 200ml LB and incubated for 10 hrs at 37°C under a 200rpm shaking. Cells were collected for plasmid purification using the Plasmid Maxi Kit (QIAGEN) following the manufacturer's instructions. Vector quality was assessed by Nanodrop measurements, considering 260/280 and 260/230 ratios of approximately 2.0 to be optimal.

2.19 Transfection of KCL22 cells with siRNA

In order to generate a knock-down of either SOX4 or siBCR in 16-IMr cells, 40 million cells were electroporated at the conditions previously mentioned with 10μM siSOX4 or siBCR and siControl (Ambion®) (**Figure 2.1**).

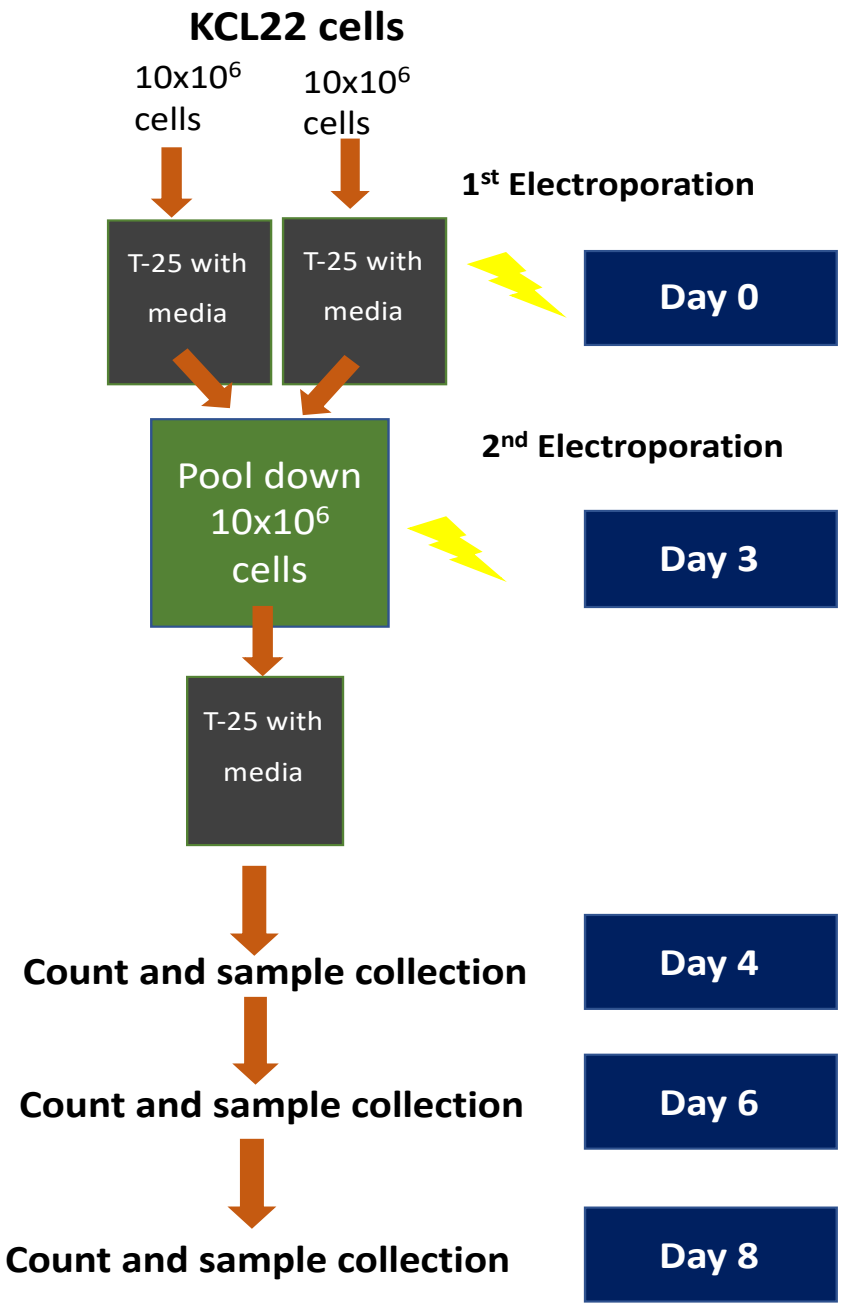


Figure 2.1: Electroporation protocol for double-transfection.

The electroporation protocol consists of electroporating two sets of $10x10^6$ cells with an siRNA of interest. These cells are taken into two separate T-25 flasks with media. The expected viability efficiency is around 50%, and hence, it is considered that ~$5x10^6$ cells will be alive after the transfection. Then, at day 3, cells are pooled into a single cuvette and electroporated again with the siRNA in order to prolong the depletion of the target. At day 4, cell numbers are normalized and left for day 6 and day 8 counts and sample retrieval as necessary. Such protocol was previously optimised by MSc. student A. Baral for maintaining a constant abolishment of SOX4 in 16IMr cells.

2.20 Wright-Giemsa staining

Approximately $1x10^5$ cells were spun onto slides at 750rpm for 3 minutes in a Cytospin™-4 Cytocentrifuge. Cells were fixed using absolute methanol for 30 seconds, stained in undiluted Wright stain for 2 minutes and in diluted Wright buffer for 5 minutes. Excessive stain was removed in distain buffer for 2 minutes. Slides were washed three times in deionized water, left to dry and cover-slipped for 30 minutes. Slides were visualised and pictures were taken from a Nikon® Eclipse E1000 coupled with a Film Camera System.

2.21 Statistical data analysis

MTS assays were performed in triplicates, the data presented as mean values and error bars indicate standard deviations. Statistical analysis was carried out using Prism for Mac. For cell growth analysis using Trypan Blue exclusion method, a Two-Way Anova was used for significance analysis, using a p-value<0.05. Experiments were carried out in three independent experiments unless otherwise is indicated.

Chapter 3: The role of the BCR-ABL1 protein in kinase-independent drug resistant CML.

3.1 Previous work and Aim

CML cells are 'oncogene addicted' being solely dependent upon BCR-ABL1 signalling for survival. Inhibition of BCR-ABL1 kinase activity within these cells induces apoptosis [66]. As modelled by the KCL22 cells, previous work in the lab recapitulated clinical observations in demonstrating that upon drug resistance the CML cells can become BCR-ABL1 kinase-independent.

Noting that BCR-ABL1 is a large protein (210 kDa) and contains several protein domains, the lab then addressed whether the oncogene itself is functionally required in drug resistant KCL22 cells.

In studies conducted by another PhD student in the lab (G. Bheesmachar), the effective, and specific, shRNA targeting of BCR-ABL1 could not be achieved; despite several attempts. To overcome this obstacle, two independent siRNAs targeting different regions of the BCR sequence (s1947 and s1948; separated by 1492bp) were used to deplete the oncogene within the KCL22 clones 16P and 16IMr respectively (**Figure 1.7a**). As predicted, transfection of the 16P cells with either siBCR sequence resulted in apoptosis with the rapid decline of a viable cell population (at d5 post-transfection) as determined by trypan blue exclusion (**Figure 1.7b, s1947 data presented**). As control, 16P cells transfected with the siControl sequence grew at an exponential rate. Strikingly, under identical conditions depletion of BCR-ABL1 protein within the 16IMr cells failed to show any signs of apoptosis and demonstrated a continued growth rate similar to that seen with siControl (**Figure 1.7b**). This result was observed within the 15P and 15IMr cells respectively (data not shown).

Collectively, these observations suggested that the drug resistant KCL22 cells have transformed to become independent of the BCR-ABL1 oncogene. Other regulators, such as deregulated transcription factors, likely play a significant role in maintaining cell growth and survival and thus compensate for the loss of BCR-ABL1.

As cautioned at the time, these results stem from the transient depletion of BCR-ABL1 and in establishing the primary aim of this chapter, such 'oncogene

independent' findings should be confirmed by the stable, and long-term, depletion of BCR-ABL1 in the drug resistant KCL22 cells.

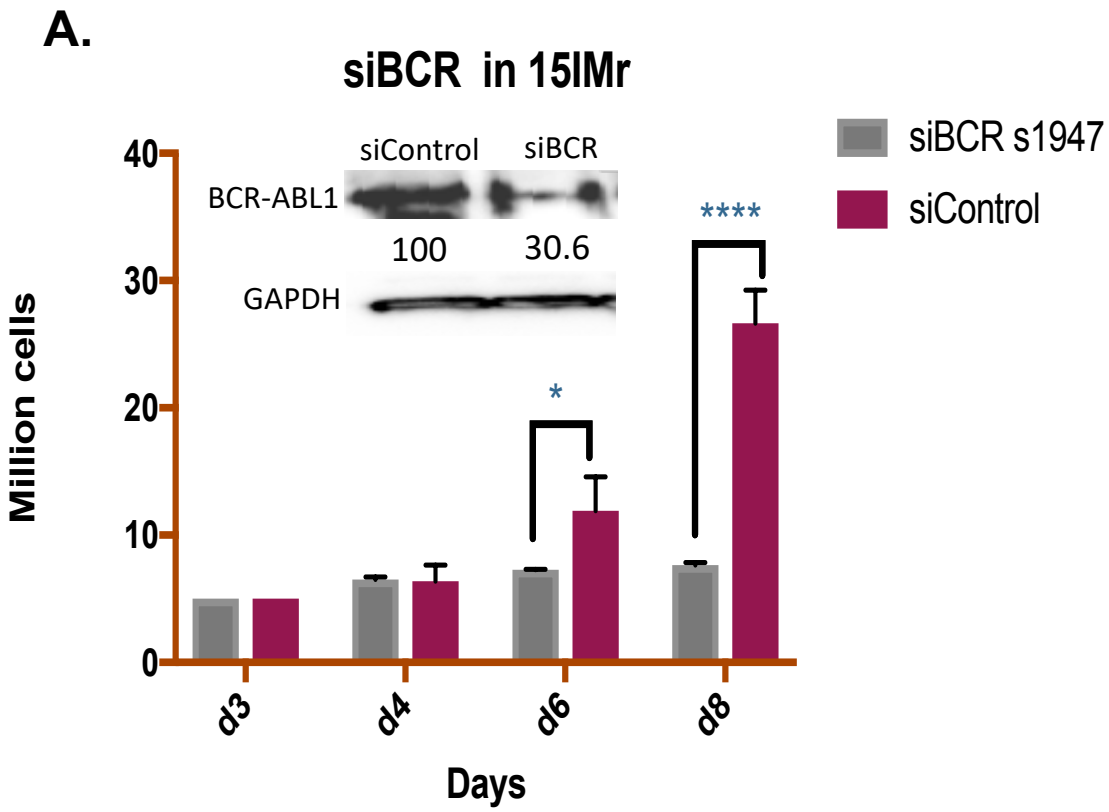
3.2 Generation of KCL22-IMr cells stably depleted of the BCR-ABL1-oncogene.

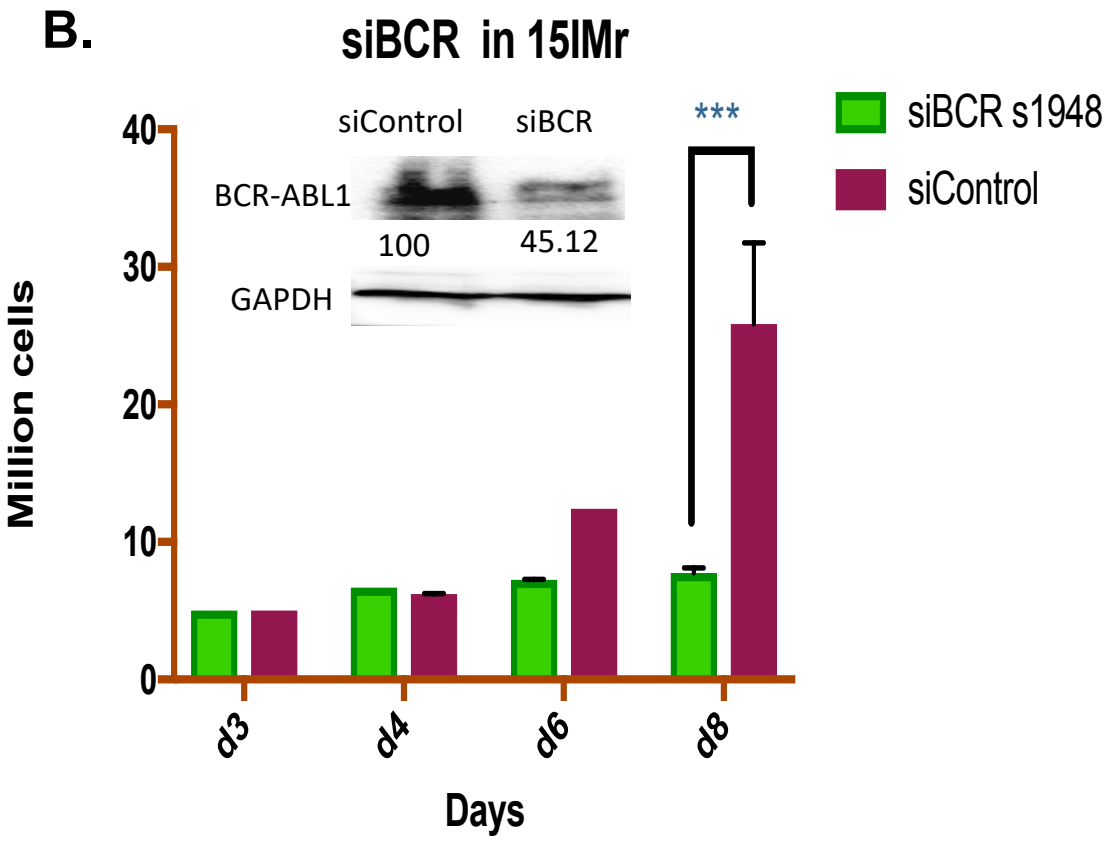
Prior to the generation of any stable cell lines, the previous observations for the independency of the KCL22 drug resistant cells to BCR-ABL1 was first confirmed. It is noted that slight modifications were made to the siRNA transfection protocol in order to improve the length of time in which the BCR-ABL1 protein is depleted within the cells. Extending from the previous protocol which consisted of transfection of the cells by a single transfection of the siRNA, the modified technique consists of a double-transfection with a 3-day rest period in between.

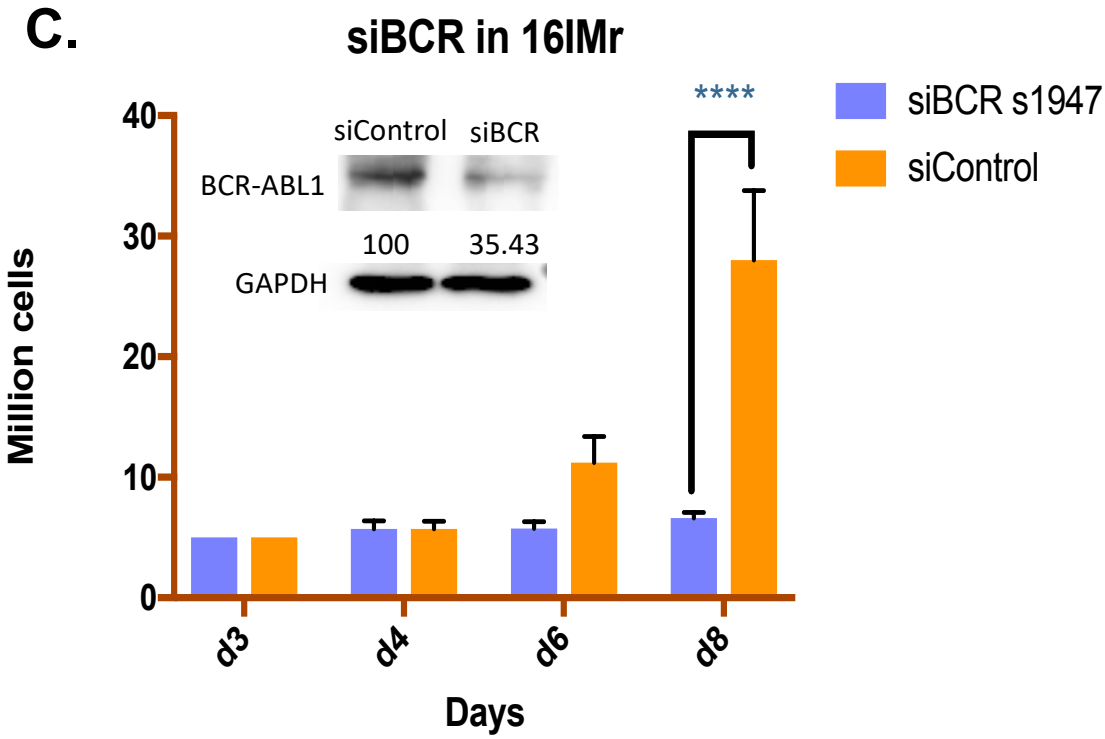
Briefly, at d0, 20 million KCL22 cells (10 million per cuvette) were electroporated (250V and 950uF) with 10μM siRNA (siControl or siBCR) and allowed to recover within two T25cm flasks respectively. On d3, the flasks were pooled and 10-million cells were re-electroporated (identical conditions) with the respective siRNA and allowed to recover in a T25 flask. Pilot studies utilising this modified protocol demonstrated that the BCR-ABL1 protein continued to be depleted within KCL22 drug-resistant cells for at least 5-days following the second electroporation (data not shown).

Having established optimal conditions for the sustained depletion of BCR-ABL1 protein, the consequences of such loss within the drug-resistant KCL22 cells was determined. Here, cell viability was measured by trypan blue cell counts. The 15IMr cells were double-electroporated (d0 and d3 respectively) with siControl or siBCR respectively. At d4, cell counts demonstrated that both siControl and siBCR treated cells had comparable viable cells of approximately 6 million cells. The siControl cells rapidly expanded reaching 25 million cells at d8 (**Figure 3.1a**). However, in contrast to the previous observations, the siBCR cells failed to grow throughout the experiment maintaining a count of 6 million viable cells at d8. The findings from this experiment were readily validated in biological repeats (three repeats) and additionally reproducible when using both siBCR sequences (**Figure 3.1b**). Moreover, similar results were displayed in 16IMR cells with several biological repeats performed as well as the use of both siBCR sequences (**Figure 3.1c and 3.1d**). In each experiment, the efficiency of BCR-ABL1 protein

depletion was determined by western analysis at the completion of the study. By densitometric analysis, and using GAPDH as a loading-control, a range of BCR-ABL1 knockdown efficiencies was achieved with the maximum protein loss of 83% and a minimum of 55%.




B.
siBCR in 15lMr
siControl
siBCR
BCR-ABL1
100
45.12
GAPDH
***
siBCR s1948
siControl
Million cells
40
30
20
10
0
d3
d4
d6
d8
Days


C.
siBCR in 16lMr
siControl
siBCR
BCR-ABL1
100
35.43
GAPDH
****
siBCR s1947
siControl
Million cells
40
30
20
10
0
d3
d4
d6
d8
Days

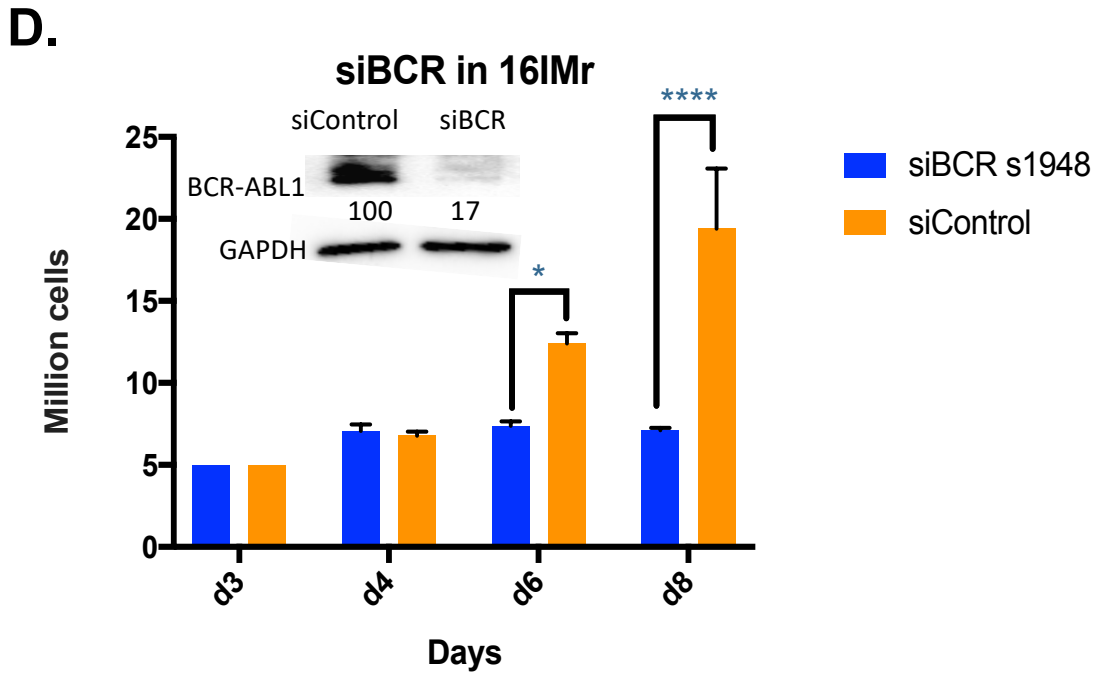


Figure 3.1: Viable cell counts of siBCR treated 15IMr and 16IMr cells.

Cells were treated with two sequences of siRNA against siBCR in order to deplete BCR-ABL1. (**A**) and (**B**) Shows cell counts of 15IMr and 16IMr cells when using siBCR sequence s1947 and (**C**) and (**D**) cell counts for both clones using the sequence s1948. A second transfection was carried out on day 3 and countings were done from day 3 up to day 8. Western Blots are shown above with the densitometry analysis of the total BCR-ABL1 (tBCR-ABL1) expression levels normalised its corresponding loading control GAPDH. Error bars represent SD. A 2-way ANOVA for multiple comparisons was used as statistic test, with * representing a $p<0.05$ and *** a $p<0.0001$. Data represents the statistical data of three independent experimental assays.

Although in direct contrast with the lab's earlier data, the findings presented herein with (i) multiple biological repeats, (ii) quantifiable protein loss by western analysis and (iii) presentation of a consistent phenotype within both KCL22 drug-resistant clones, clearly demonstrate that the KCL22-IMr cells undergo growth arrest upon BCR-ABL1 depletion and acquire a non-proliferating behaviour.

To clarify these conflicting results, the consequence of BCR-ABL1 depletion within drug resistant KCL22 cells was validated, using another 'independent' KCL22 model. This additional model was previously generated within the lab (G. Bheesmachar) which utilized the original heterogenous KCL22 cells to generate a kinase-independent drug resistant derivative line (termed hetKCL22-IMr). For clarity, the heterogenous KCL22 cells was the first established within the lab for generating drug-resistance cells. The same protocol of exposing the cells to

weekly-increments of titrating IM concentrations was used and the resulting KCL22-IMr line is considered a heterogenous population. Indeed, once the lab had determined the ability of the heterogenous KCL22 cells to become kinase-independent upon drug resistance, the decision was then made to re-generate these derivatives but starting with single cell clones (15P and 16P).

As performed for the 15IMr and 16IMr clones, the BCR-ABL1 protein was depleted within the hetKCL22-IMr cells by double-electroporation with the siBCR 1948 molecule. Western analysis confirmed knock-down of BCR-ABL1, at d8, with a 72% protein loss (**Figure 3.2**). In agreement with the findings presented herein, loss of BCR-ABL1 protein within the hetKCL22-IMr cells resulted in growth-arrest with the failure of these cells to proliferate. As expected, cells treated with the siControl readily expanded throughout the experiment **(Figure 3.2)**.

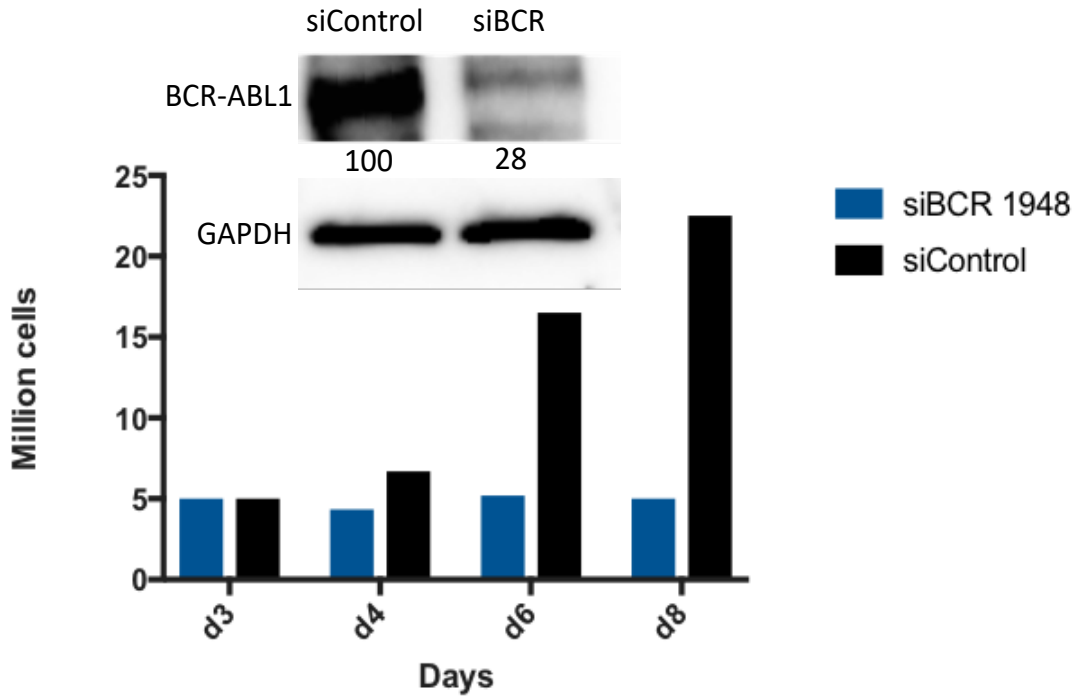


Figure 3.2: Depletion of BCR-ABL1 within hetKCL22-IMr cells.

Viable cell counts of hetKCL22-IMr cells treated with either siBCR s1948 or siControl. Cells counts are represented as million cells. Western Blot showing the knockdown efficiency of the siBCR s1948 (d8). The GAPDH antibody was used as a loading control. Data represents the result of one pilot experimental assay.

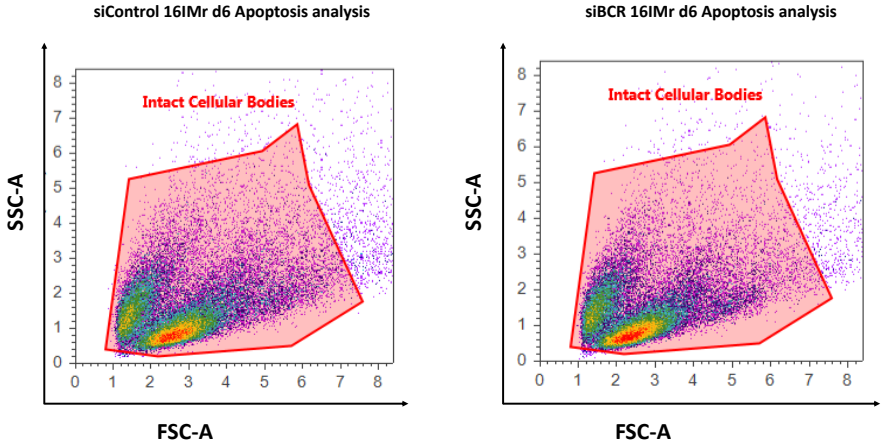
The depletion of BCR-ABL1 protein within several KCL22 drug resistant models (single cell clones and heterogenous population) induces growth arrest. Although independent from the SH1 kinase domain, these cells are still dependent upon the oncogene for growth. Based on these findings the initial aim of generating stable KCL22 drug resistant cells stably depleted of BCR-ABL1 was abandoned as this is not feasible.

3.3 Depletion of BCR-ABL1 protein within the KCL22-IMr cells does not promote apoptosis.

The depletion of BCR-ABL1 protein within kinase-independent drug resistant KCL22 cells induces growth arrest. However, this phenotype was determined by viable cell counts of the bulk population. It is plausible that individual cells will have varying degree in the loss of BCR-ABL1 and this could differentially result in either proliferation or apoptosis respectively. Specifically, a proportion of the cells could continue to proliferate while others are concurrently dying. This simultaneous, but counteracting, effects on growth could result in the 'net cell viability' seen at all time-points and give the impression that the population has growth arrested.

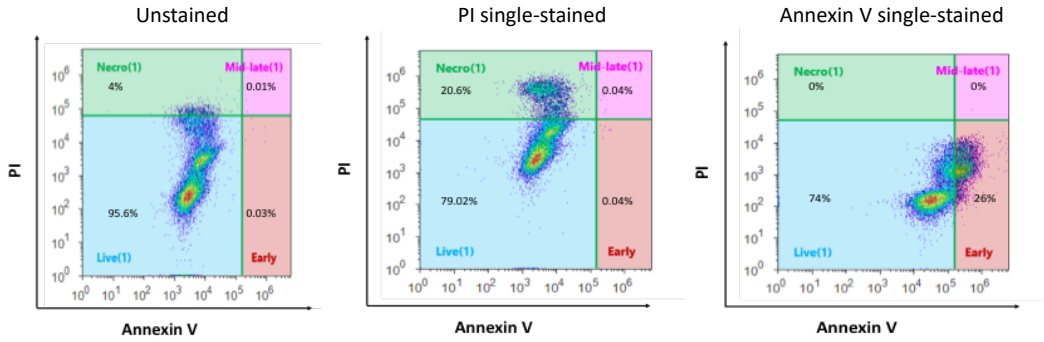
Taking this into consideration, the quantification of apoptosis was performed at a single-cell level on 16IMr cells that have been depleted of BCR-ABL1 protein with the siBCR s1948 molecule. As control, cells were transfected with the siControl molecule and samples were collected for analysis at d6 (2d post second-electroporation). Cell viability counts confirmed the non-proliferating phenotype of the BCR-ABL1 depleted cells (data not shown). For quantification of apoptosis, $5x10^5$ cells were washed twice with 1x PBS and incubated at room temperature in the dark with Annexin V for 30 minutes and subsequently incubated with Propidium Iodide (PI) for 15 minutes. The cell population was identified by forward (FSC-A) and side (SSC-A) scatter plots (Figure 3.3A) and the gating strategy to define Annexin V and PI positive staining is presented (Figure 3.3B). Double staining of the cells enabled the quantification of early- (Annexin V+, PI-), mid- (Annexin V+, PI+) and necrotic (Annexin V-, PI+) cells respectively. No overt differences in the apoptotic profile was identified between cells treated with siBCR compared to those transfected with the siControl (**Figure 3.3C**). These results were reproduced upon biological repeat as well as within the 15IMr cells (data not shown).

A.

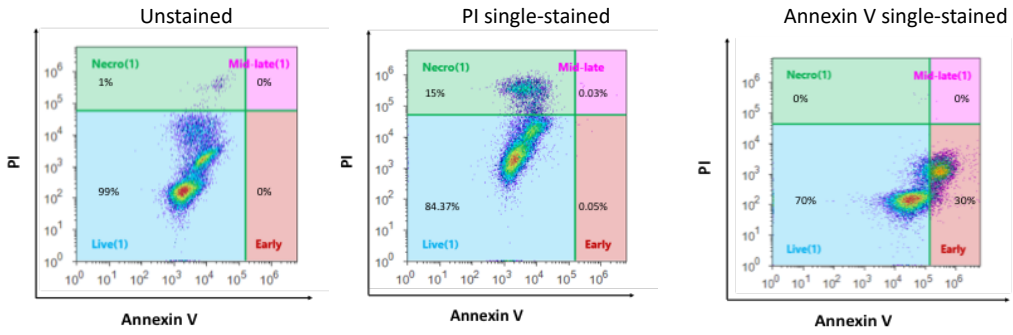


B.

- siC 16IMr d6 Apoptosis analysis



- siBCR 16IMr d6 Apoptosis analysis



C.

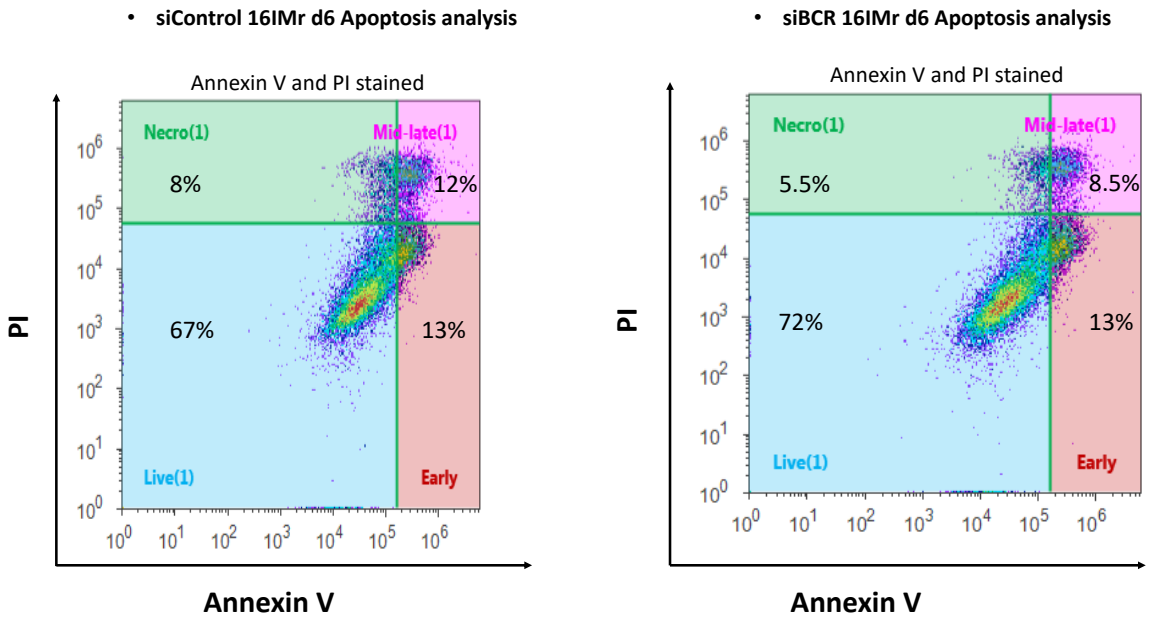
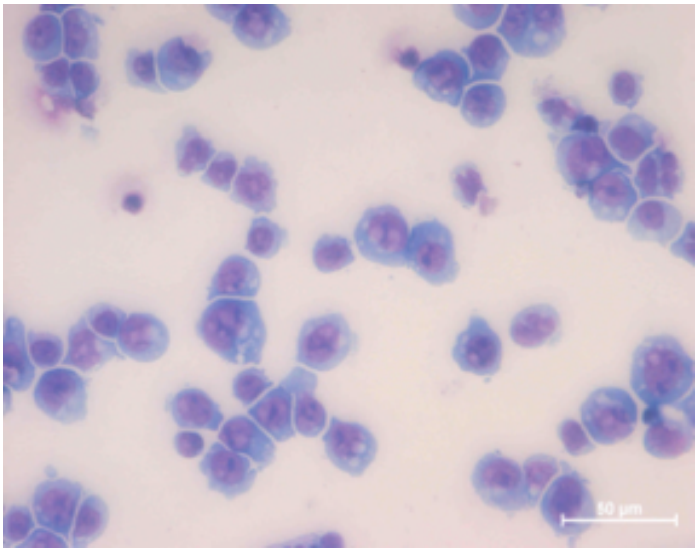


Figure 3.3: Annexin V and Propidium Iodide (PI) staining on drug resistant KCL22 cells depleted of BCR-ABL1.

Apoptosis assay by flow cytometry performed on 16IMr cells treated with siControl and siBCR at day 6. **(A)** SSC-A (y-axis) vs. FSC-A (x-axis) plots of siC and siBCR treated cells for viable cell detection. (**B**) Density plots of unstained, PI single-stained (y-axis) and Annexin V (x-axis) single-stained siC and siBCR treated cells as staining and gating controls. (**C**) Double staining consisting of Annexin V and PI. Percentages are shown inside the corresponding plot area. Figure shows representative data of three independent experimental assays.

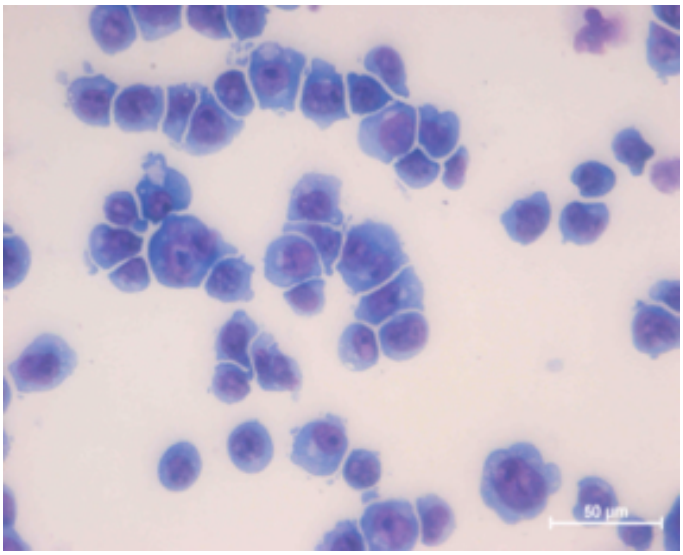
It is well established that haematopoietic progenitors will undergo growth arrest when induced to terminally differentiation [177]. This developmental process could account for why the drug resistant KCL22 cells become non-proliferating upon depletion of BCR-ABL1. To assay for differentiation, the cellular morphology of the siBCR and siControl cells was examined by Wright-Giemsa staining of cytospins from 16IMr siBCR and siControl treated cells (d5) respectively. The stained cells were visualised using a Nikon® Eclipse E1000 microscope at 40X magnification. No pronounced changes to cell morphology that were indicative of differentiation was observed with the siBCR cells having similar shape and size to the siControl treated cells (**Figure 3.4**).

A.



siBCR 80x10^5 cells (40X) 16IMr d5

B.



siControl 80x10^5 (40X) 16IMr d5

Figure 3.4: Wright-Giemsa staining of 16IMr cells depleted for BCR-ABL1.

Wright-Giemsa staining carried out in 16IMr cells treated with either (**A**) siBCR or (**B**) siControl sequences for 5 days. Magnification was done at 40-X, with the scale bar at the bottom right of the picture to measure cell size. Figure shows representative pictures of three independent experimental assays.

Collectively, these results suggest that the failure of the drug resistant KCL22 cells to expand upon loss of BCR-ABL1 is not due to (i) concomitant growth-death of the whole population or (ii) differentiation of the cells, and thus can only be accounted for by the onset of growth arrest.

3.4 BCR does not contribute to the cell proliferation of KCL22-IMr cells

As noted earlier, depletion of BCR-ABL1 protein was achieved using a siRNA sequence that targeted the 5'-end of the BCR transcript which results in the additional loss of the endogenous wild-type BCR protein. Although unavoidable, using a siRNA molecule that targets two transcripts confounds the interpretation of the current findings, whereby the observed cellular cell cycle blockage could be due to (i) continued dependency of the cells upon BCR-ABL1 or (ii) resulting directly from the loss of endogenous BCR protein.

Given this potential dilemma, it was important to determine the phenotype of depleting endogenous BCR within drug resistant KCL22 cells and subsequently assay whether it induces growth arrest. To achieve this, the 3' end of the BCR transcript was targeted in identifying unique sequence(s) that are present only in BCR but not in the BCR-ABL1 transcript respectively. In review of the Ambion® website (https://www.thermofisher.com/uk/en/home/life-science/rnai/), 3 different siBCR sequences were commercially available but all cross-reacted with the BCR-ABL1 transcript. A similar finding was noted when reviewing the available siBCR molecules at a different company source (Dharmacon™, https://dharmacon.horizondiscovery.com/rnai/).

Previous work in the lab had generated a mammalian expression vector harbouring a shRNA-sequence that uniquely targets the 3'-end of BCR (shBCRendo, **Figure 3.5**). Transient expression of this construct within 16P cells demonstrated a >90% efficiency of BCR protein loss (data not shown). Unlike siRNA whose knockdown effect is transient, shRNA molecules can be used to generate stable cell lines as the hair-pin structure can be expressed from a plasmid and thus capable of being integrated into the genome. To explore the functional requirement of BCR within the drug resistant KCL22 cells, a line stably depleted of its expression was attempted. Here, the 16IMr cells were chosen with the simple logic that if the cells do not require BCR for growth and expansion then a stable knock-down line should be readily generated.

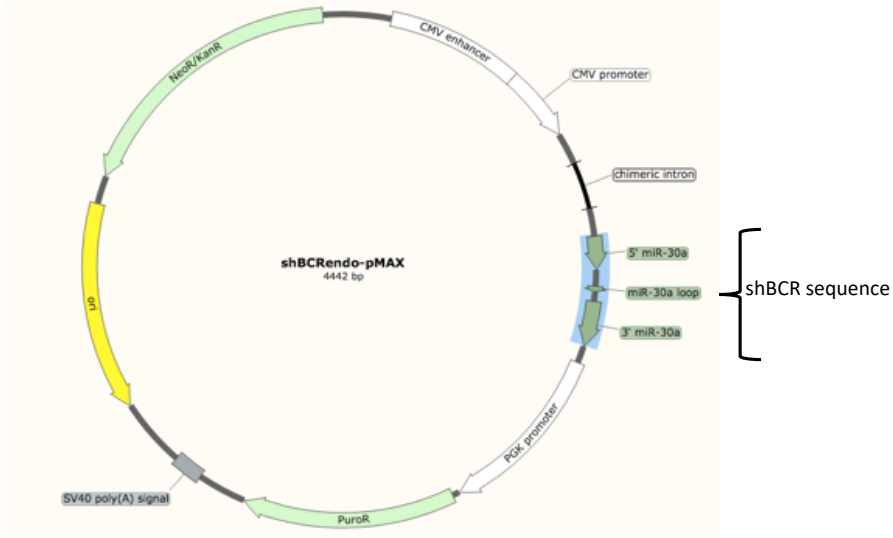


Figure 3.5: Map of the plasmid expressing a BCR-specific shRNA.

The shRNA vector that was designed to target the endogenous BCR in the KCL22-IMr cells was generated from the shBCRendo sequence 2, previously tested and generated by G.Bheesmachar. The shBCRendo sequence was subcloned into a pMAX vector (AMAXA) backbone.

The shBCRendo plasmid backbone is a simple mammalian-expression vector and does not contain any drug-resistant expression cassette (pMax vector, Amaxa Biosystems®). To enable the generation of a stable line, the shBCRendo plasmid was co-transfected with the pCDNA3-GFP vector which harbours a Neomycin resistance (G418) cassette and expresses the GFP protein. As control, the 16IMr cells were also transfected with the control shLuciferase-pMAX vector (shLuc) which expresses a shRNA targeting the luciferase transcript; a gene that is unique to insects and absent within mammalian cells. As with the shBCRendo vector, transfection of the 16IMr cells with the shLuc plasmid was in combination with pCDNA3-GFP vector. Approximately 10 million 16IMr cells were co-transfected by electroporation with the described vectors and after a 24hr period of recovery the recombinant cells were selected by the addition of 1.5mg/ml G418. Drug selection was maintained with the refreshment of G418 every 3-4 days and the growth of recombinant cells monitored by the visualisation of GFP expression.

After 6 weeks of continuous G418 selection, stable GFP^{+ve} 16IMr lines expressing either shLuc or shBCRendo were generated. Depletion of BCR protein was validated by western blot analysis where, in comparison to the shLuc cells, the BCR protein is absent within shBCRendo transfected cells (**Figure**

3.6a). Of note, expression of BCR-ABL1 protein is unaffected within the shBCRendo cells demonstrating the specificity of the shRNA sequence in targeting only the BCR transcript.

The ability to generate 16IMr cells that are stably deficient of the BCR protein clearly demonstrates that they can continue to proliferate and expand as a population in the absence of BCR. However, whether depletion of BCR had any impact upon the growth kinetics (e.g. doubling time) is unknown. To address this, the growth curve of the shBCRendo 16IMr cells was examined and compared to that of the corresponding shLuc population (**Figure 3.6b**). Both lines had a similar growth rate indicating the loss of BCR within the drug resistant KCL22 cells does not perturb the growth dynamics.

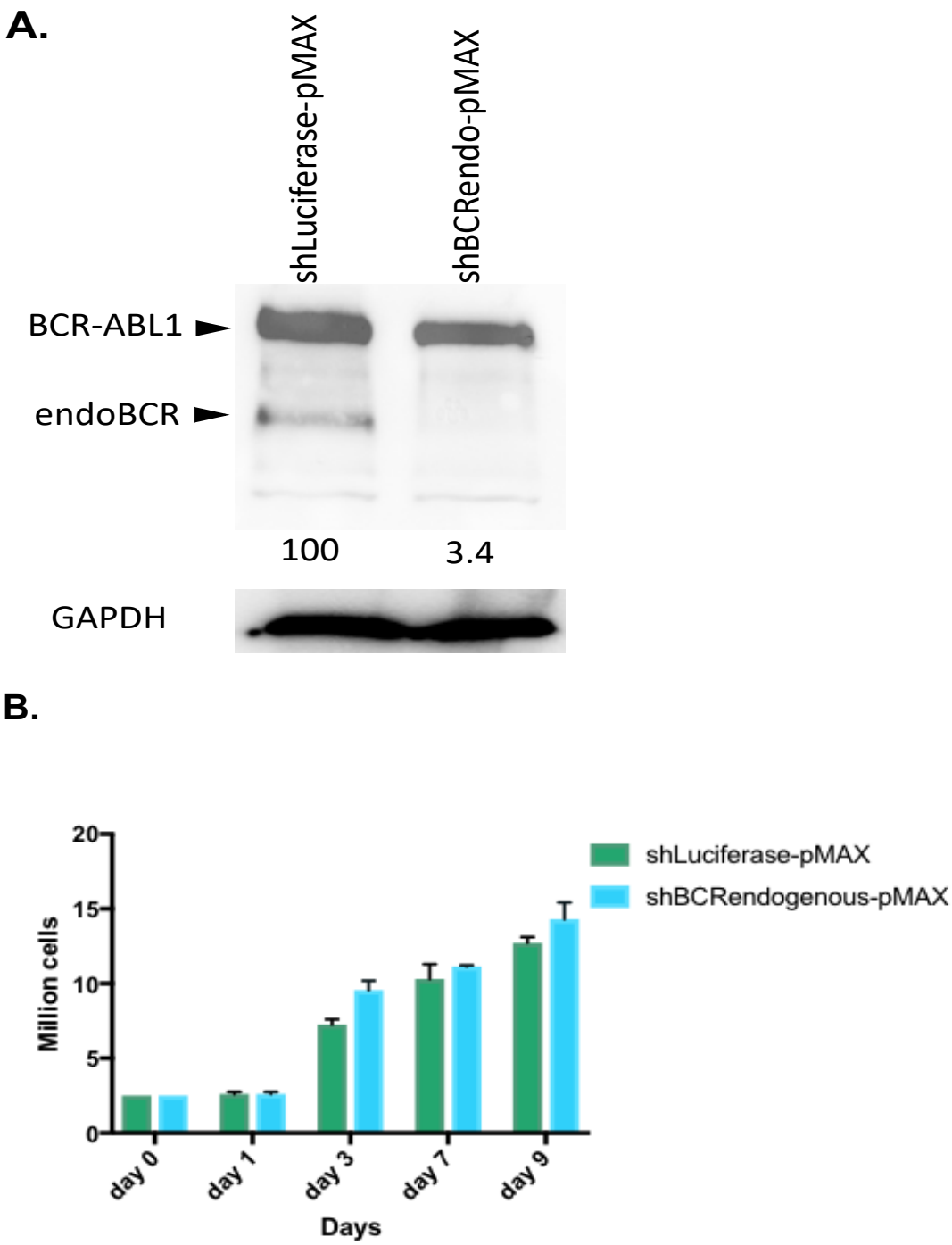


Figure 3.6: Generation of a stable 16IMr depleted of BCR.

(A) Western blot analysis analysis Luciferase-pMAX-16IMr cells and shBCRendo-pMAX-16IMr cells respectively. Densitometric analysis normalized to GAPDH control is indicated below each lane. (**B**) Trypan blue exclusion of shBCRendogenous-pMAX against shLuciferase-pMAX 16IMr cells. Mean and SD error bars are representative of 2 independent experiments.

These observations resolve the confounding interpretation of the siBCR results and strongly suggest that while both BCR-ALB1 and BCR transcripts were targeted, the previously described induced cellular cell cycle arrest can only result from the loss of the BCR-ABL1 oncogene. Moreover, it further supports, and reinforces, the functional role-requirement of the other protein domain(s) of BCR-ABL1 in regulating cell growth in the drug resistant KCL22 cells.

3.5 BCR-ABL1 is not required to maintain the expression of the deregulated transcription factors

The current observations identify a functional requirement for the other protein domain(s) of BCR-ABL1 within SH1-kinase independent drug resistant KCL22 cells. Indeed, the work of others support this notion whereby BCR-ABL1 can activate the expression of *Alox5* even though the activity of the SH1-kinase has been deactivated by the presence of IM [178].

In conjunction with the previous findings within the lab, the current model of kinase independent CML drug resistance identifies two features that are actively acquired during this transformation: (i) activation of other domain(s) of BCR-ABL1 protein and (ii) deregulated expression of transcription factors. As the other domains of BCR-ABL1 has previously been reported to regulate gene expression [179, 180], it was tempting to speculate that in addition to regulating cell proliferation, these other BCR-ABL1 domain(s) could (de)regulate the expression of certain transcription factors.

To explore this relationship, the expression of the previously identified transcription factors was profiled by semi-quantitative RT-PCR within 15IMr and 16IMr cells treated with siBCR (1948) and siControl for 8 days respectively (**Figure 3.7**). Although some minor fluctuations in the expression of a few transcription factors was identified, none were reproduced upon biological repeat nor replicated within the other clone respectively. The relative expression of each transcription factors is maintained in the absence of BCR-ABL1 and therefore the activity of the other protein domain(s) of the oncogene is unlikely to maintain the expression of this gene-set. However, this observation does not exclude the possibility that the other BCR-ABL1 domain(s) are required to initially regulate the expression of these factors but is not required to maintain expression; the so-called ‘hit and run’ mechanism of gene expression [181].

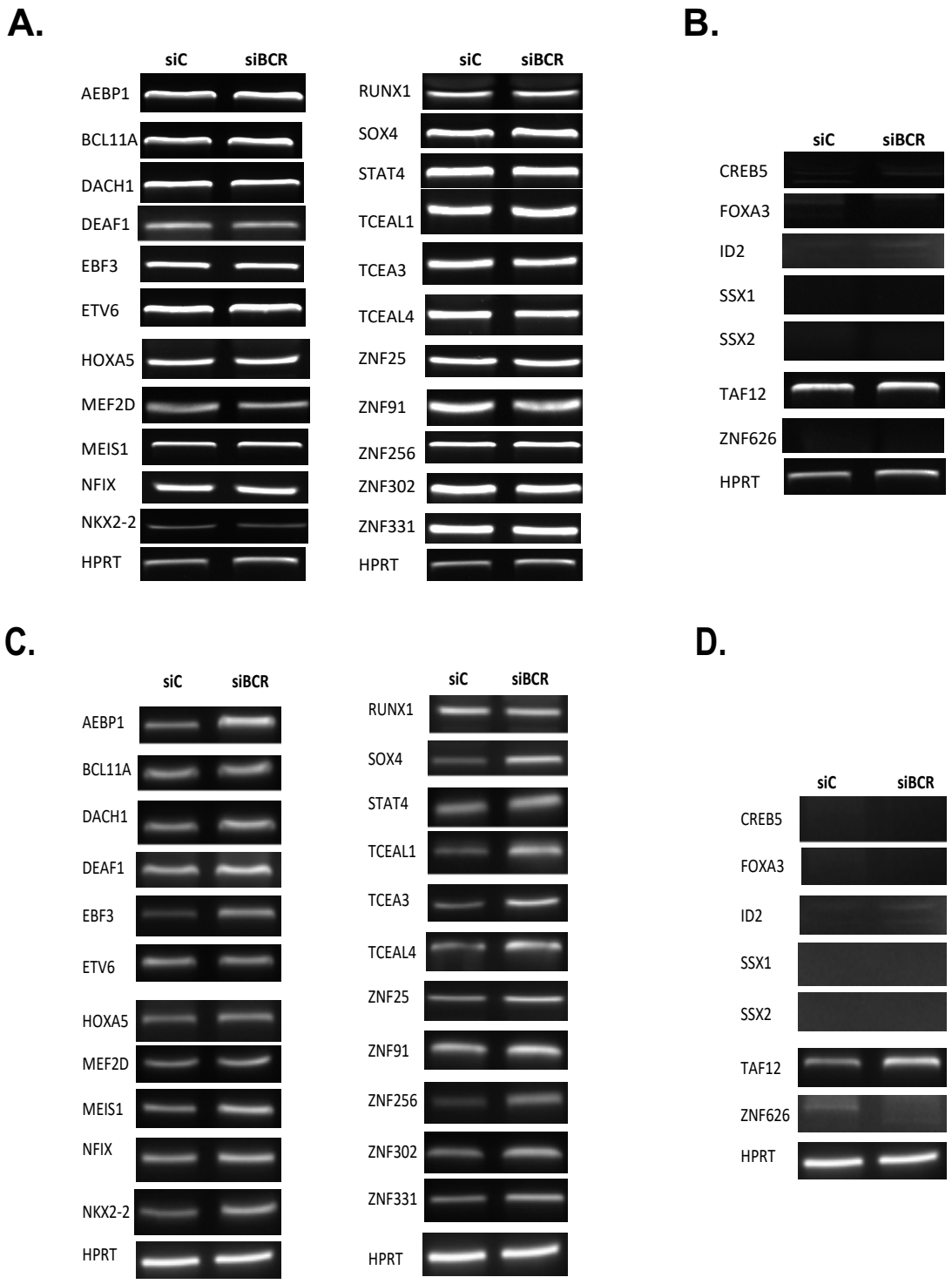


Figure 3.7: Transcription factor profiling in the 15IMr and 16IMr cells treated with siBCR s1948 day 8.

Relative expression of transcription factors within 15IMr and 16IMr cells after depletion of BCR-ABL1 via siBCR s1948 sequence (d8). Profile for 15IMr cells; (**A**) upregulated and (**B**) downregulated genes upon-drug resistance. Profile for 16IMr cells; (**C**) upregulated and (**D**) downregulated upon drug-resistance. Figure shows representative data of three independent experimental assays.

3.6 Genome-wide expression analysis of drug resistant KCL22 cells depleted of BCR-ABL1

To further understand the functional properties, if any, of the other domain(s) of BCR-ABL1 (e.g. metabolism), genome-wide expression analysis was performed on the KCL22-IMr single-cell clones treated with the siBCR and siControl respectively.

The interpretation of the siBCR dataset has to be cautioned with the caveat that the endogenous BCR protein is also depleted in these experiments and this itself may contribute to changes in gene expression. At the time of these studies, the shBCRendo line had not been generated; but can be employed in future work to readily determine whether the loss of BCR is contributing to any changes in gene expression. For simplicity, from herein the transcriptome analysis is discussed in terms of the genome response to loss of BCR-ABL1.

Briefly, both 15IMr and 16IMr cells were treated with siBCR s1948 and siControl sequences as previously described and total RNA collected at day 8. Samples was analysed as technical triplicates and the microarray was performed by Cambridge Genomic Services, University of Cambridge. The platform used was the Clariom S Human HT Array Plate (Affymetrix, Wooburn Green, UK) and processed on the Gene Titan instrument (Affymetrix). Quality Controls and basic bioinformatic analysis was performed by the genomic service.

Differentially regulated genes were defined, and identified, having a >2-fold change in relative expression to the respective siControl samples. Upon depletion of BCR-ABL1, the 15IMr cells induced 308 genes while 402 genes were repressed. Similarly, the 16IMr cells activated 863 genes with 655 repressed. Genes that were similarly regulated upon BCR-ABL1 depletion within both clones was identified; here a common set consisting of 60 induced and 102 repressed genes respectively (**Figure 3.8**).

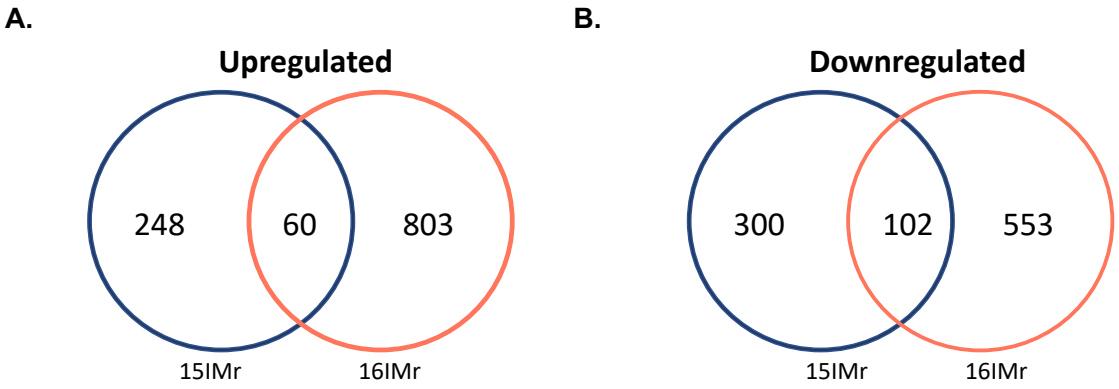


Figure 3.8: Venn diagrams summarising the genome-wide expression of drug resistant KCL22 cells depleted of BCR-ABL1.

Common (**A**) upregulated and (**B**) downregulated genes in 15IMr and 16IMr cells depleted of BCR-ABL1.

It is noted that there was a substantial number of genes that were regulated in a clone-dependent manner and this likely reflects genetic heterogeneity among single cell lines. Similar clone-specific variation has been previously observed in the lab upon analysing the transcriptome changes that occurred upon drug resistance for the 15P and 16P cells respectively. Such heterogeneity among single cell lines highlights the importance of performing these studies on more than one clone and focussing on the commonly regulated gene-set. Tables representing the expression profile of the top 20 upregulated (**Table 3.1a**) and downregulated (**Table 3.1b**) genes are presented respectively.

Gene name	Gene definition	Fold change 15IMr	Fold change 16IMr
EPB42	Erythrocyte Membrane Protein Band 4.2	2.33	7.56
RAB28	RAB28, Member RAS Oncogene Family	2.49	6.53
C1orf186	Regulator Of Hemoglobinization And Erythroid Cell Expansion	3.71	6.49
ZNF711	Zinc Finger Protein 711	2.26	6.24
TMEM244	Transmembrane Protein 244	3.27	5.98
GPR85	G Protein-Coupled Receptor 85	2.05	4.57
TNFAIP8	TNF Alpha Induced Protein 8	2.38	4.36
RAB7B	RAB7B, Member RAS Oncogene Family	2.69	4.15
IKBIP	IKBKB Interacting Protein	2.16	4.05
SLC40A1	Solute Carrier Family 40 Member 1	2.05	3.62
ANXA8	Annexin A8	2.06	3.52
COMMD4	COMM Domain Containing	2.21	3.30
SLC38A9	Solute Carrier Family 38 Member 9	3.53	3.26
HLTF	Helicase Like Transcription Factor	2.16	3.22
ITGA4	Integrin Subunit Alpha 4	2.25	3.11
FABP5	Fatty Acid Binding Protein 5	2.46	3.08
FABP4	Fatty Acid Binding Protein 4	2.26	3.03
ANKRD36	Ankyrin Repeat Domain 36	2.07	3.02
PHACTR2	Phosphatase And Actin Regulator 2	2.23	2.92
IL1B	Interleukin 1 Beta	8.01	2.81

Gene name	Gene definition	Fold change 15IMrs	Fold change 16IMrs
TRIB3	Tribbles Pseudokinase 3	0.45	0.06
ATF5	Activating Transcription Factor 5	0.39	0.07
TNF	Tumor Necrosis Factor	0.44	0.10
UBASH3B	Ubiquitin Associated And SH3 Domain Containing B	0.40	0.11
MUC4	Mucin 4, Cell Surface Associated	0.43	0.12
BHLHE40	Basic Helix-Loop-Helix Family Member E40	0.13	0.13
CDH15	Cadherin 15	0.29	0.14
BTS2	EBF Transcription Factor 1	0.28	0.15
EGR1	Early Growth Response 1	0.17	0.15
CISH	Cytokine Inducible SH2 Containing Protein	0.43	0.17
BCL	BCL2 Apoptosis Regulator	0.18	0.17
BLVRB	Biliverdin Reductase B	0.27	0.18
CD9	CD9 Molecule	0.23	0.19
SIGLEC6	Sialic Acid Binding Ig Like Lectin 6	0.48	0.20
ALOX5AP	Arachidonate 5-Lipoxygenase Activating Protein	0.33	0.20
BNIP3	BCL2 Interacting Protein 3	0.09	0.20
IL21R	Interleukin 21 Receptor	0.21	0.21
PYGM	Glycogen Phosphorylase, Muscle Associated	0.31	0.22
TRIM16L	Tripartite Motif Containing 16 Like	0.20	0.23
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1	0.23	0.23

Table 3.1: Table of 20-top upregulated and downregulated genes within siBCR and siControl treated cells.

Table listing the top 20 (**A**) upregulated and (**B**) downregulated genes in siBCR and siControl 15IMr and 16IMr treated cells.

As quality control of the microarray dataset, expression profile of randomly-selected genes was validated by semi-quantitative RT-PCR. Here, the clonal-specific variation in the differentially expressed genes was exploited and 3 genes were profiled that were exclusively induced, upon depletion of BCR-ABL1, in either the 15IMr or 16IMr cells respectively. Notably, this gene-profiling was performed on cDNA template that was independent from the samples used in the microarray. Such analysis would validate (i) the accuracy, and sensitivity, of the array in reporting the respective pattern of gene regulation, (ii) the changes in gene expression within a biological repeat sample and (iii) that the RNA samples were correctly handled throughout the procedure from initial lysate collection to end-point of bioinformatic analysis of the microarray itself.

Of the genes chosen that are selectively induced in 15IMr cells upon depletion of BCR-ABL1 (ARRDC4, TDRD9 and TXNIP) as well as those induced only in the 16IMr cells (AZU1, HGF and TFRC) all displayed the clone-specific pattern of gene regulation as predicted by the microarray (**Figure 3.9**). This data indicated that the microarray dataset is accurate in identifying differentially regulated genes and that such changes are reproducible upon repeat of the experiment.

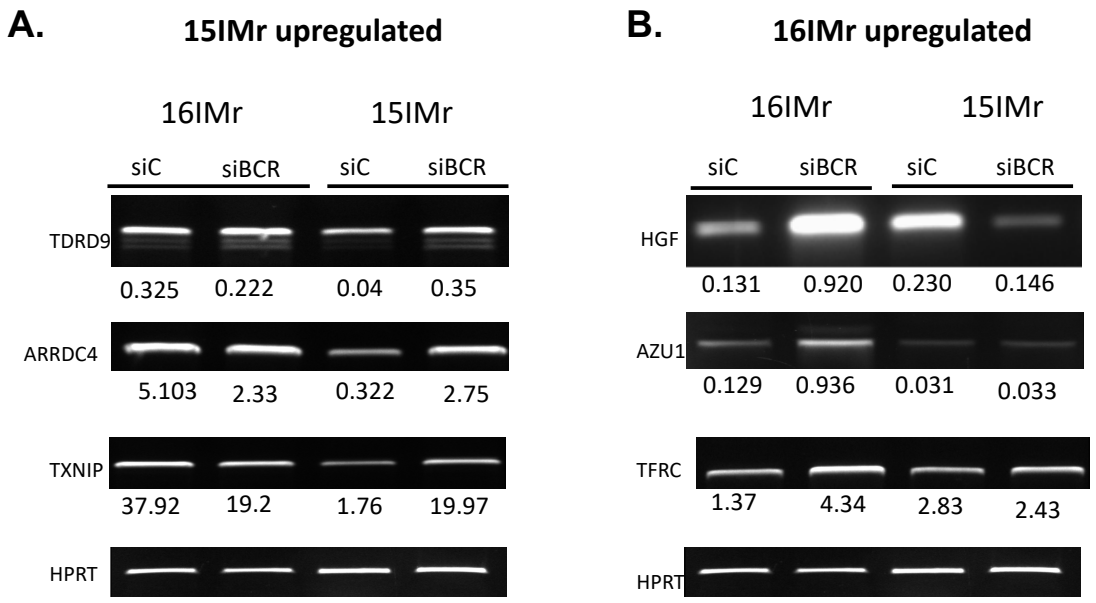


Figure 3.9: Semi-quantitative RT-PCR validation of selected genes from the microarray.

Genes were selected in order to verify the quality of the microarray dataset. **(A**) Genes that were upregulated in the 15IMr only and (**B**) genes that were upregulated in 16IMr cells only. HPRT was used as a loading control. Numbers reflect the relative expression as dictated from the microarray. Figure shows representative data of three independent experimental assays.

Having validated the quality of microarray dataset, a Gene-Set Enrichment Analysis (GSEA), was performed in order to identify the most enriched biological pathways regulated in the drug resistant clones upon loss of BCR-ABL1. This was performed in collaboration with Dr. Dapeng Wang (Bioinformatician, University of Leeds).

The GSEA analysis (nominal p-value <0.05 and FDR q-value <0.05) identified 90 enriched pathways that are common to both 15IMr and 16IMr cells respectively (**Table 3.2**).

Name of Set (PART 1)
ACINAR_DEVELOPMENT_LATE_2
ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS
ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX
AGING_OLD_DN
B_LYMPHOCYTE_MATURATION_BY_TACI_DN
BLADDER_CANCER_CLUSTER_3_UP
BOUND_BY_E2F
BRCA_CENTERED_NETWORK
BRCA2_PCC_NETWORK
BREAST_CANCER_GRADE_1_VS_3_UP
BREAST_CANCER_WITH_BRCA1_MUTATED_UP
CANCER_HEAD_AND_NECK_VS_CERVICAL_UP
CD5_TARGETS_DN
CELL_CYCLE
CELL_CYCLE_CHECKPOINTS
CELL_CYCLE_GENES_IN_IR_RESPONSE_6HR
CELL_CYCLE_LITERATURE
CELL_CYCLE_MIR192_TARGETS
CELL_CYCLE_MITOTIC
CELL_CYCLE_RB1_TARGETS
CELL_CYCLE_RB1_TARGETS
CELL_CYCLE_S
CERVICAL_CANCER_PROLIFERATION_CLUSTER
CML_DIVIDING_VS_NORMAL_QUIESCENT_UP
CML_QUIESCENT_VS_NORMAL_QUIESCENT_UP
CORE_SERUM_RESPONSE_UP
CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_
CYCLING_GENES
DNA_REPAIR_GENES
DNA_REPLICATION
E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION
E2F_TARGETS
E2F3_TARGETS
EARLY_T_LYMPHOCYTE_UP
EMBRYONIC_STEM_CELL_CORE
EPITHELIAL_MESENCHYMAL_TRANSITION_UP
ES_1
ESR1_TARGETS
ESRRA_TARGETS_RESPONSIVE_TO_ESTROGEN_DN
EWINGS_SARCOMA_UNSTABLE_VS_STABLE_UP
EXTENSION_OF_TELOMERES
EZH2_TARGETS
FANCONI_PATHWAY
G1_S_CELL_CYCLE
G1_S_SPECIFIC_TRANSCRIPTION

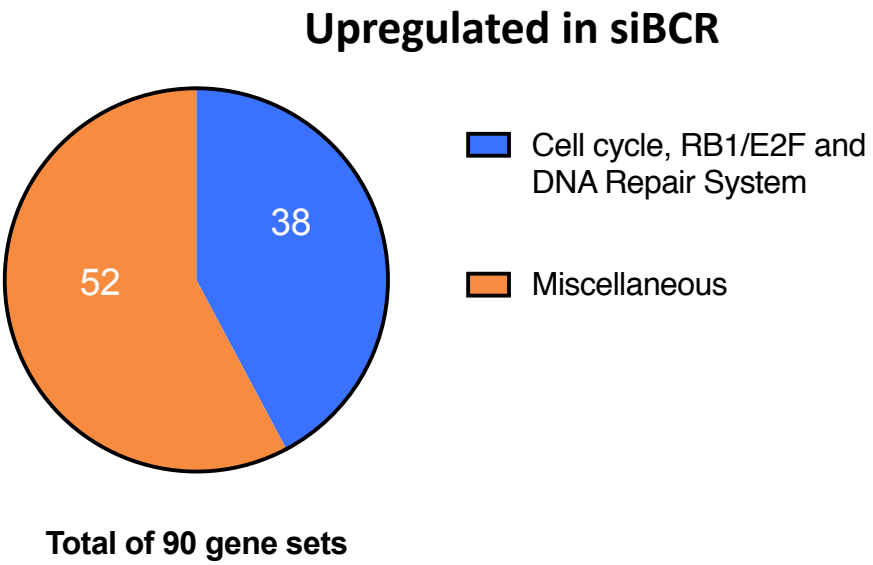
Name of Set (PART 2)
G1_S_TRANSITION
G2_M_CHECKPOINTS
HAVE_ZNF143_BINDING_SITES
HEAD_AND_NECK_CANCER_WITH_HPV_UP
HPV_POSITIVE_TUMORS_UP
HYPOXIA_BY_DMOG_DN
HYPOXIA_DN
IL6_DEPRIVATION_DN
INTERACT_WITH_AIRE
INTESTINE_PROBIOTICS_24HR_UP
LARGE_PRE_BII_LYMPHOCYTE_UP
LARGE_TO_SMALL_PRE_BII_LYMPHOCYTE_UP
LIVER_CANCER_SUBCLASS_G123_UP
M_G1_TRANSITION
MCM_PATHWAY
MELANOMA_METASTASIS_UP
MELANOMA_RELAPSE_UP
MITOTIC_G1_G1_S_PHASES
MITOTIC_M_M_G1_PHASES
MRNA_PROCESSING
MRNA_SPLICING_MINOR_PATHWAY
NASOPHARYNGEAL_CARCINOMA_UP
NEOCORTEX_BASAL_RADIAL_GLIA_DN
NORMAL_QUIESCENT_VS_NORMAL_DIVIDING_DN
NUCLEOTIDE_EXCISION_REPAIR
ORC1_REMOVAL_FROM_CHROMATIN
PEDIATRIC_CANCER_MARKERS
PLASMA_CELL_VS_PLASMABLAST_DN
PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_
PROGENITOR
PROLIFERATION
RB1_ACUTE_LOF_UP
RB1_TARGETS_GROWING
REGULATED_BY_METHYLATION_DN
RESPONSE_TO_APLIDIN_DN
RESPONSE_TO_SALIRASIB_DN
RESPONSE_TO_THC_DN
RETINOBLASTOMA_PATHWAY_UP
S_PHASE
TARGETS_OF_IE86_CMV_PROTEIN
TARGETS_OF_MIR34B_AND_MIR34C
TLX_TARGETS_DN
TRANSFORMED_BY_RHOA_UP
XPRSS_INT_NETWORK
YBX1_TARGETS_DN

Table 3.2: GSEA analysis of genes regulated in drug resistant KCL22 cells upon loss of BCR-ABL1

The selected gene sets were selected by using nominal p-Value and FDR q-value of >0.05. Gene sets correspond to the C2 curated data set from MSigDb. Gene signatures relating to the cell-cycle are highlighted.

In accord with the cell growth arrest phenotype, a recurring signature present in this analysis (38 of the 90 pathways) included those of the cell cycle, the RB1/E2F and DNA repair pathways respectively **(Figure 3.10a)**. The remaining 52 pathways were restricted to miscellaneous biological aspects such as T-lymphocyte and liver cancer pathology. Additionally, 103 enriched pathways were common for both clones treated with siControl (**Figure 3.10b**). The most dominant pathway being Hypoxia, with 13 enriched sets related, the rest was of diverse in nature, such as methylation and EGF response.

A.



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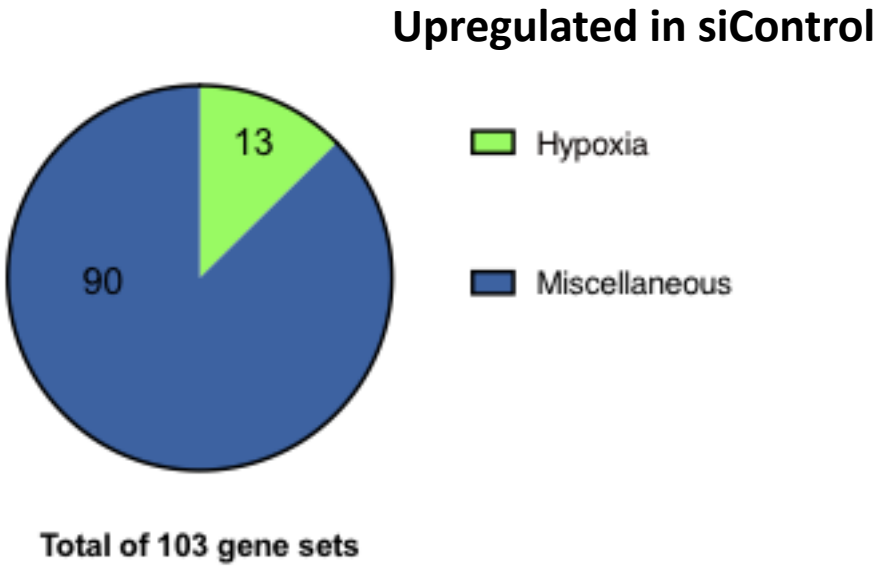


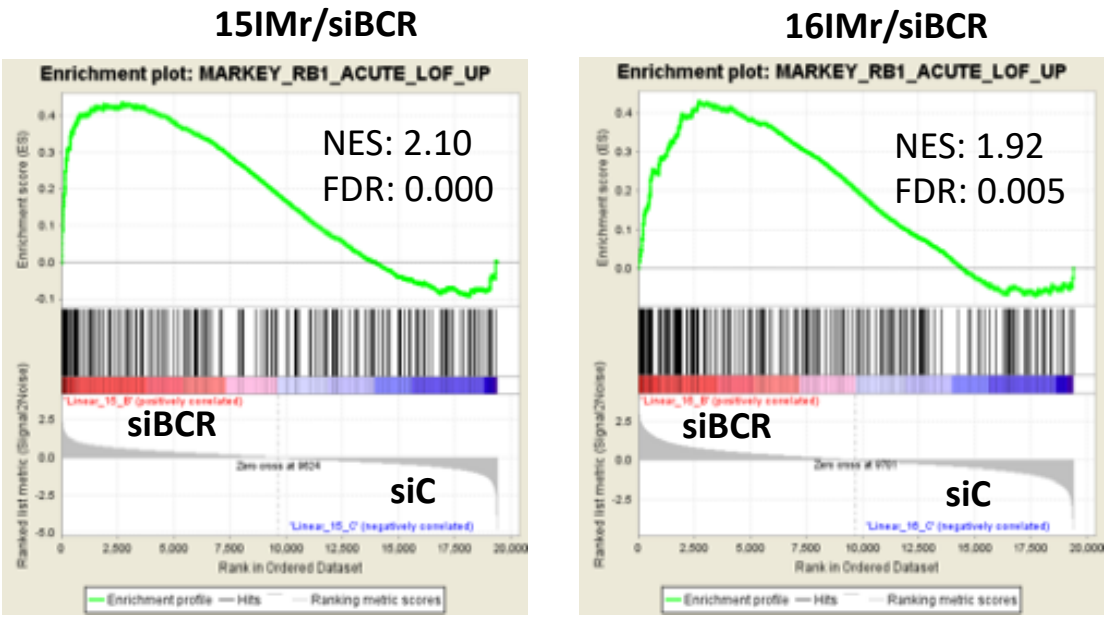
Figure 3.10: Pie charts of the enriched GSEA sets.

(**A**) Enriched gene-sets common to the 15IMr and 16IMr clones upon BCR-ABL1 depletion. (**B**) Relative enriched gene-sets common to the 15IMr and 16IMr clones treated with siControl.

In review of this GSEA analysis, three key findings are summarized. First, among the 90 enriched pathways, the most predominant (and frequent) signature is that of cell-cycle regulation (four representative GSEA enrichment plots is shown in (**Figure 3.11**). This was not a surprise given the cellular growth arrest observed upon depleting BCR-ABL1 within these cells (**Figure 1.7**). Second, and third, the GSEA analysis did not identify any signature(s) relating to either apoptosis or cellular differentiation. This was anticipated given the earlier Annexin V-PI (**Figure 3.3**) and cell morphology (**Figure 3.4**) analysis of these cells respectively.

The GSEA analysis was used in combination with the molecular signature database which is a collection of annotated gene-signatures (10,000+) and is divided into 8 major collections (H, C1, C2, C3, C4, C5, C6 and C7) [182]. In the current study the C2 (curated gene sets) category, which consists of 4,762 signatures, was selected. Searching through the C2 collection, over 60 signatures relating to either “apoptosis” or “differentiation-cell maturation” were readily identified. As such, the failure to identify any enriched pathways for either apoptosis or cellular differentiation is a direct reflection on the biology of the cells.

A.



B.

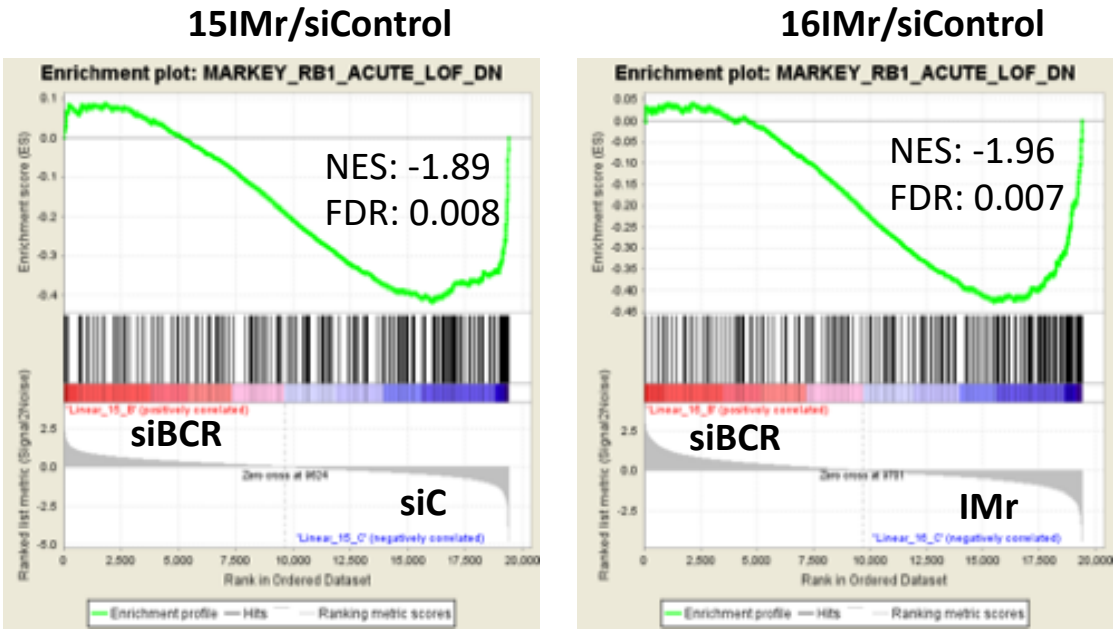


Figure 3.11: Example of GSEA enrichment score plots for the RB1 pathway.

Relative enrichment plot for the RB1 pathway within drug resistant KCL22 cells treated with (**A**) siBCR or (**B**) sicontrol respectively.

3.7 Proposed model of drug resistant CML

Collectively, the observations put forward a model of how the leukaemic phenotype is maintained in kinase-independent drug resistant CML. To place things into perspective, it is important to first appreciate that upon de-activation of the SH1 kinase domain by IM, the CML leukaemic cells must learn to maintain three basic hallmarks of cancer; cell proliferation, anti-apoptosis and block in differentiation. From the findings presented herein, it is hypothesized that upon drug resistance the primary function of the other domain(s) of BCR-ABL1 is to regulate the cell proliferation and is thus an oncogene-dependent mechanism. Based on the work of others, the regulation of anti-apoptosis is oncogene independent and is regulated by the acquired activation of the Src kinases [94, 107, 183-185]. The role of Src and apoptosis is discussed later in this chapter. Similarly, the block of cellular differentiation is proposed to be regulated in a BCR-ABL1 independent manner being maintained by the deregulation of transcription factors (previous work of the lab). This model is discussed in more detail, along with supporting data, in the Discussion.

3.8 The role of Src in kinase-independent drug resistant KCL22 cells

It is well established that the acquired activation of the Src Family Kinases (SFKs), namely the LYN kinase pathway, contributes to drug resistance in CML [183]. Specifically, the SFKs are overexpressed in drug resistant CML, as identified in cell model (kinase-independent K562) as well as patients, and targeting its activity induces apoptosis [186]. Indeed, based on these observations the second-generation of tyrosine kinase inhibitors was generated (Dasatinib [187]), which has a dual-target specificity capable of blocking the kinase activity of BCR-ABL1 as well as the SKFs family members.

From the current findings presented herein, it is proposed that upon drug resistance, the leukaemic cells will activate the SFK pathway to compensate for the loss of BCR-ABL1 function. Once activated, the SFK pathway functions autonomously in a BCR-ABL1 independent manner to block the onset of apoptosis. How the SFK pathway becomes activated is unknown, but it is tempting to speculate that it could be regulated by the other domain(s) of BCR-ABL1. Nevertheless, this model would predict that upon depletion of BCR-ABL1 that the SFK pathway remains intact and the cells fail to undergo apoptosis.

Indeed, a number of studies presented herein strongly support this model, namely the Annexin V and GSEA analysis respectively.

However, it is also recognised that the SFK pathway is complex and can regulate a multitude of biological properties including cell proliferation [90, 188]. Under such circumstances, an alternate model can be proposed whereby the other domain(s) of BCR-ABL1 can activate the SFK pathway and subsequently the regulation of cell proliferation. In this scenario, the activity of SFK pathway is continuously maintained by signalling from the other domain(s) of BCR-ABL1. As such, the loss of BCR-ABL1 in drug resistant cells would result in the collapse of the SFK pathway and subsequent onset of growth arrest. The observations presented herein also support this model, namely the induction of growth arrest upon the depletion of BCR-ABL1.

As the SFKs can regulate either apoptosis or cell proliferation the relationship between BCR-ABL1 and the SFKs pathways needed to be explored. The underlying question is what happens to the SFK pathway upon depletion of BCR-ABL1 within the drug resistant KCL22 cells. Does its activity collapse or remain autonomously active?

3.9 The SFKs are actively acquired within drug resistant KCL22 cells

As similarly reported by others using the K562 model [175, 189], upon kinase-independent drug resistance both 15IMr and 16IMr KCL22 cells overexpress a tyrosine-phosphorylated protein of 55k-60kDa in molecular weight (**Figure 3.12**).

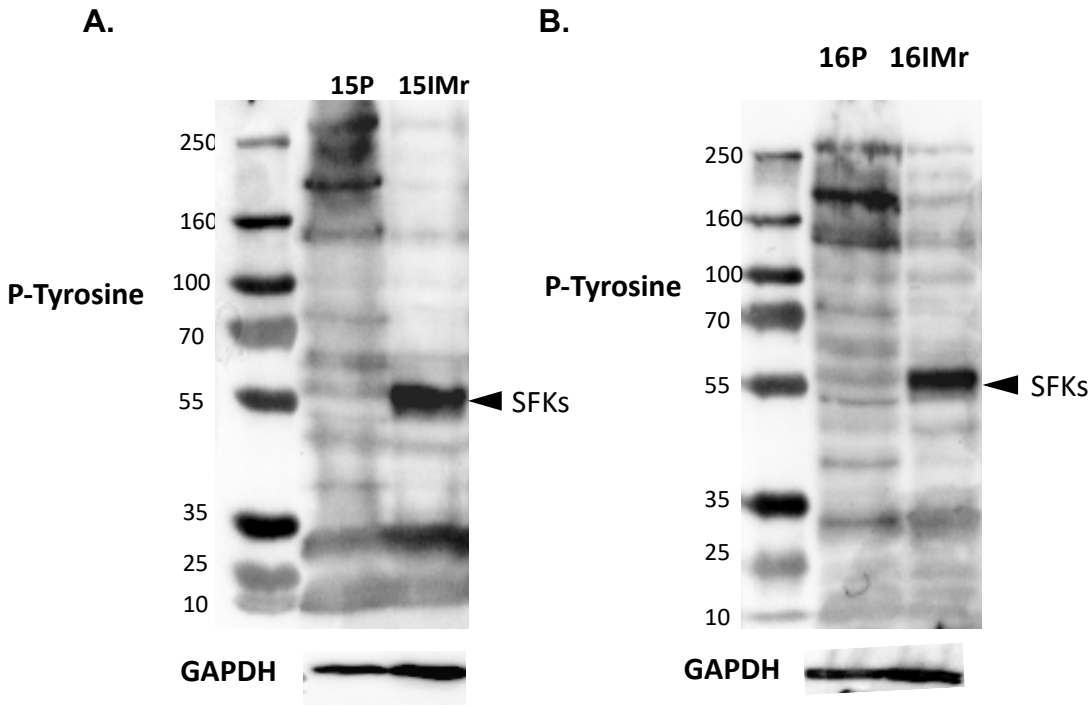


Figure 3.12: Induction of a unique phospho-protein upon drug resistant CML.

Western analysis of all proteins harbouring a phosphorylated tyrosine residue. Phospho-blot within (**A**) 15P and 15IMr as well as (**B**) 16P and 16IMr cells respectively. GAPDH was used as a loading control. The ladder lane is indicated at the left alongside the corresponding molecular weights in kilo-Daltons (kDa). Figure shows representative data of three independent experimental assays.

To validate that this protein(s) as a member of the SFKs, the 15IMr cells were treated with titrating doses of the SFK-inhibitor, Dasatinib [190]. After overnight treatment with Dasatinib (1, 2 and 10nM respectively), the samples were analysed by western blot which demonstrated that the tyrosine-phosphorylation of this ~60kDa protein was sensitive to drug treatment (**Figure 3.13**). Similar results were obtained within the 16IMr cells (data not shown).

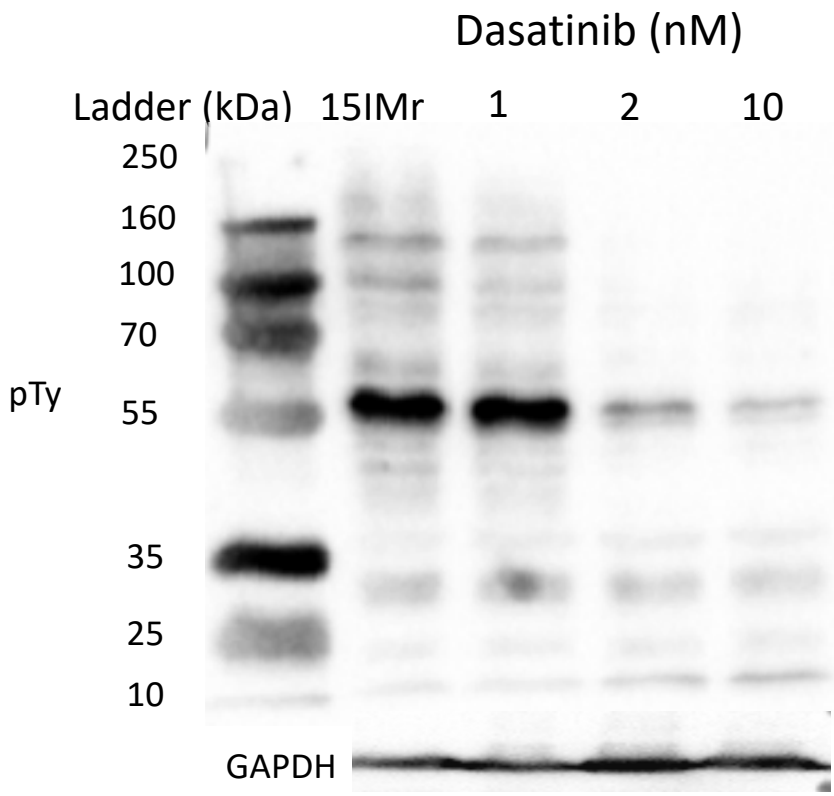


Figure 3.13: Drug resistant KCL22 cells express a phospho-protein that is sensitive to Dasatinib treatment.

Western analysis of all protein harbouring a phosphorylated tyrosine residue. Phospho-blot within 15IMr cells treated with titrating amounts of Dasatinib. GAPDH was used as a loading control. The ladder lane is indicated at the left alongside the corresponding molecular weights in kilo-Daltons (kDa). Figure shows representative data of three independent experimental assays.

A downstream target of the SFKs is the SKP2 protein, a member of the F-box family of subunits of the SCF ubiquitin-ligase complex. Upon SFK-mediated tyrosine-phosphorylation, the stability of the SKP2 protein is enhanced [191, 192]. Once stabilised, SKP2 promotes the degradation of the $p27^{kip1}$ protein [191, 193, 194]. A graphical description of the SFKs-SKP2-p27 pathway in the KCL22-IMr cells is presented (**Figure 3.14**).

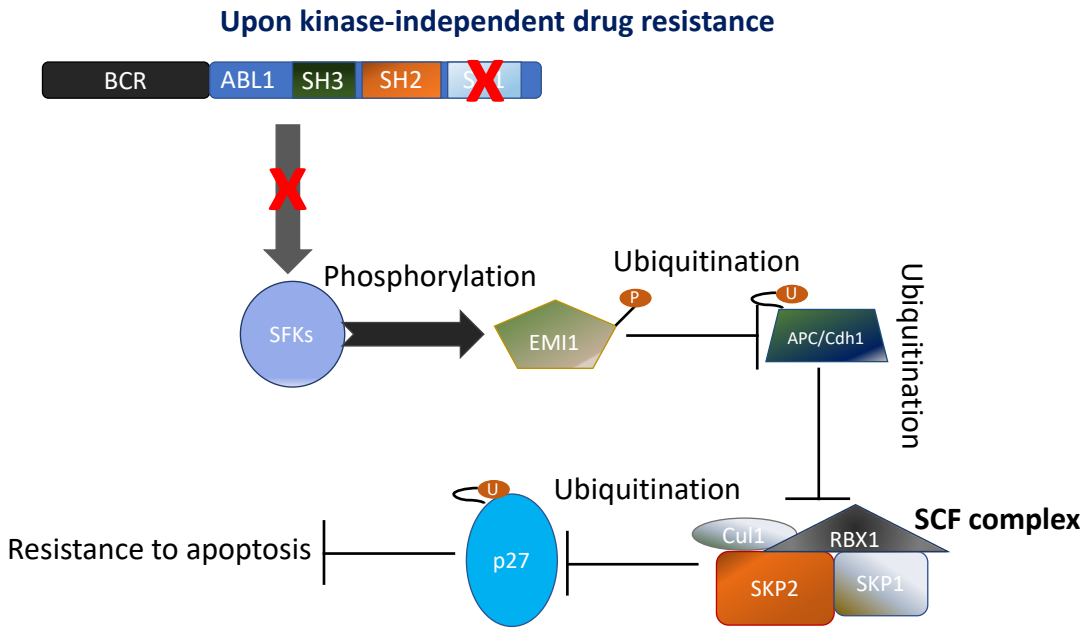


Figure 3.14: Diagramatic review of the SFK/SK2/p27 axis in drug resistant CML cells.

Upon activation, the SFKs are activated and promote the phosphorylation of the ubiquitin ligase EMI1, which marks the APC/Cdh1 ubiquitin ligase complex for degradation. This complex would additionally target the SCF complex (Cul1, RBX1, SKP1 and SKP2) for ubiquitination and subsequent degradation, however, under EMI1 activation, this is inhibited leaving the SCF complex active for ligating ubiquitin to the anti-tumoral protein p27, which is degraded and ineffective to promote apoptosis.

To further characterise the function of the SFKs within the drug-resistant KCL22 clones, the SFK-SKP2-p27 network was investigated. If this network is functional within the IMr clones, then the activity of the SFKs should result in high SKP2 protein expression and reciprocally low expression of p27. Upon kinase inhibition of the SFKs (Dasitinib treatment), this network should collapse leading to the degradation of an unstable SKP2 protein and subsequent rescue of high p27 expression. Indeed, the overnight-treatment of either 15IMr or 16IMr cells with 2nM Dasatinib displayed the predicted pattern of protein expression for each component of the SFKs-SKP2-p27 pathway (**Figure 3.15**).

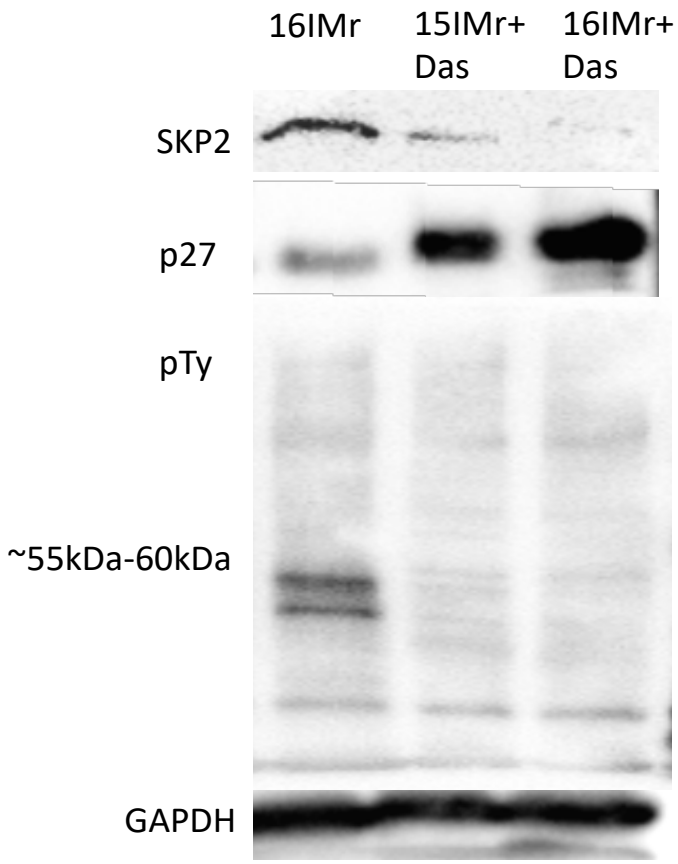


Figure 3. 15: Analysis on the SFK/SKP2/p27 axis in 15IMr and 16IMr cells treated with Dasatinib.

Western analysis of the SKP2 and p27 proteins as well as all proteins harbouring a phosphorylated tyrosine residue. The 15IMr and 16IMr cells were treated with 2nM Dasatinib, 16IMr cells were used as protein level controls. GAPDH was used as loading control. Figure shows representative data of three independent experimental assays.

These observations demonstrate that this network is functional within the drug resistant KCL22 clones and also establishes a reliable, and accurate, assay for SFKs activity**.**

It is of note that the 3-day treatment of drug-resistant KCL22 cells with Dasatinib results in apoptosis, as visualised under microscope examination and readily detected by the abnormal 'shrivelling' of the cells (**Figure 3.16a**). This is in clear contrast to cells that are depleted on BCR-ABL1 which maintains a uniform round shape and shiny reflection under the microscope (**Figure 3.16b**)**.** Here, it is evident that the loss of BCR-ABL1 within drug-resistant KCL22 cells induces a non-proliferating phenotype with the presence of healthy viable cells while the inhibition of Src activity results in the rapid onset of apoptosis and necrosis.

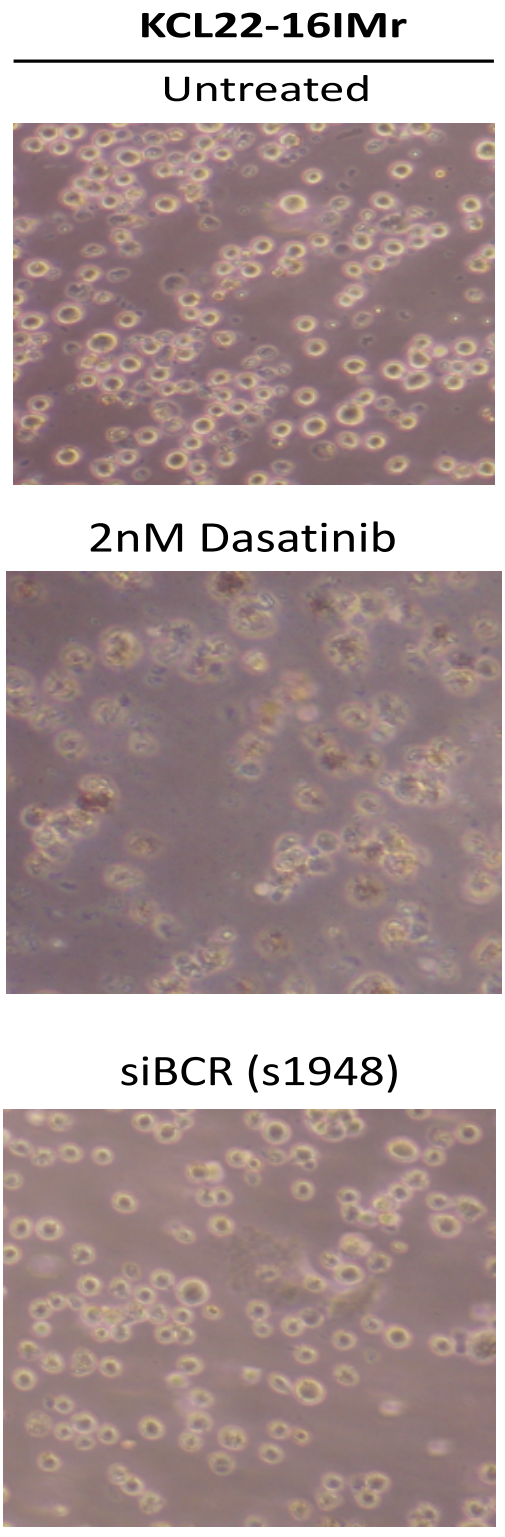


Figure 3.16: Light microscopy of 16IMr cells treated with Dasatinib or siBCR

Light microscope photographs of 16IMr cells under control, Dasatinib or siBCR treatments. Pictures taken using an Olympus C-7070 camera. Figure shows a representative picture of two independent experimental assays.

3.10 Regulation of the SFKs pathway is independent of BCR-ABL1 within the KCL22-IMr cells

How the SFK pathway becomes activated in drug resistant KCL22 cells in unclear. Yet, as the other domain(s) of BCR-ABL1 are functional, it was of interest to determine whether activity of the SFKs is dependent upon the oncogene in drug resistant cells. Here, the 16IMr cells were treated with siControl or siBCR for 8 days respectively. Protein lysates were collected and detection of activated-SFKs was determined by western analysis using an antibody that recognises all proteins harbouring a phosphorylated tyrosine residue. In comparison to siControl cells, there was no differences in the amount of phosphorylated SFKs protein upon depletion of BCR-ABL1 (**Figure 3.17a**). This observation suggests that the SFK activity is intact with further confirmation via the protein expression of both the SKP2 and p27 downstream targets which did not demonstrate any overt changes to that seen in siControl cells (**Figure 3.17b**). Similar results were obtained with the 15IMr cells (data not shown).

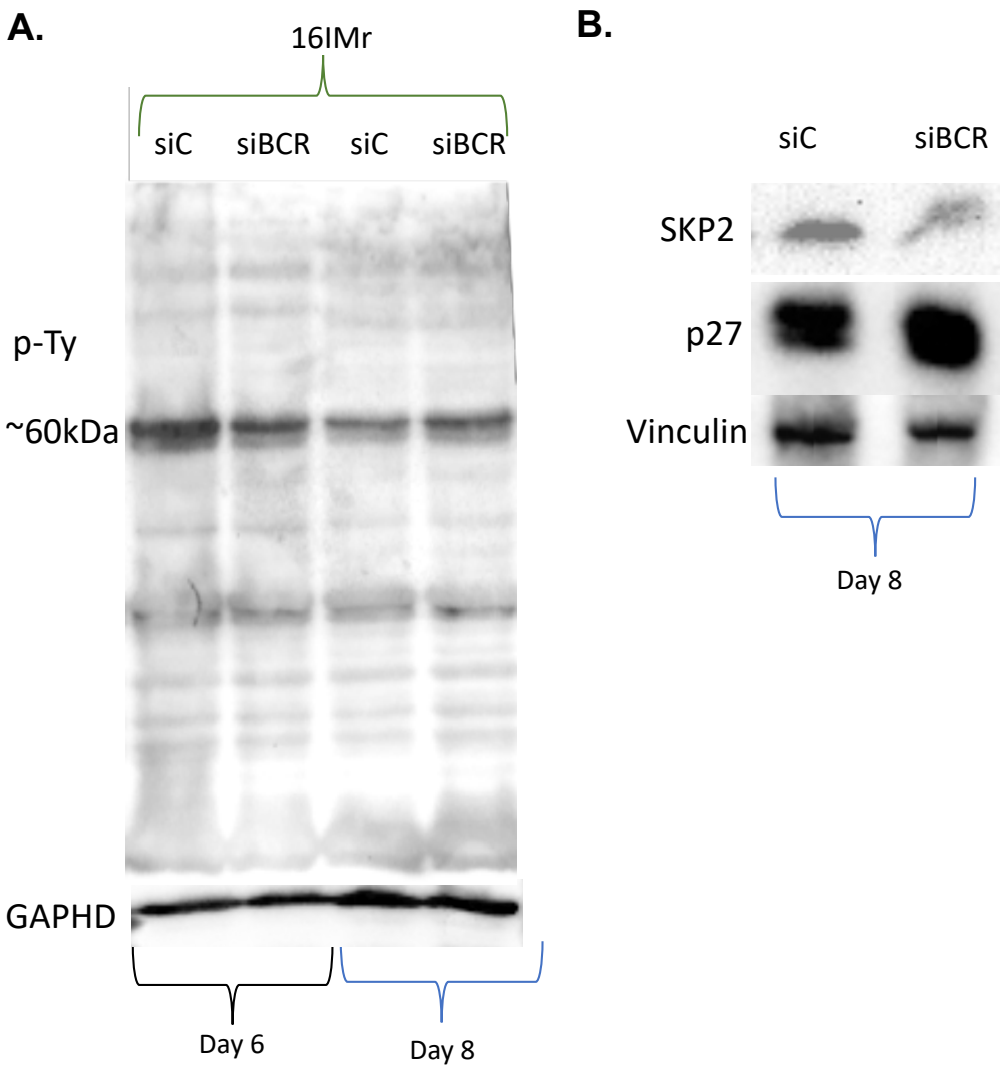


Figure 3.17: SFK activity and the SKP2/p27 axis in 16IMr cells upon depletion of BCR-ABL1.

The 16IMr cells were treated with the s1948 siBCR sequence for 8 days by following the double-electroporation transfection method. Cells were collected on day 8 and analysed by western blot (**A**) all proteins containing a phosphorylated tyrosine residue or (**B**) SKP2 and p27. GAPDH or Vinculin was used as a loading control. Figure shows representative data of three independent experimental assays.

In summary, upon depletion of BCR-ABL1 within the drug resistant KCL22 clones, the SFK-SKP2-p27 network is actively maintained suggesting that it is not accountable for the induced growth arrest of the cells and therefore has a more likely role in blocking apoptosis. Moreover, this further supports the direct relationship between the other domain(s) of BCR-ABL1 and cell proliferation.

3.11 The SFKs pathways does not maintain expression of the deregulated transcription factors.

Among the many functional roles of the SFKs, a lesser-known attribute is the ability of these proteins to regulate gene transcription. As example, the SFKs have been reported to negatively regulate the retinoic acid-induced gene expression of the C/EBPα and PU.1 transcription factors, as well as a myeloid differentiation of acute promyelocytic leukaemia cells [195, 196]. The KLC22 cells acquire both activation of the SFKs pathway as well as the (de)regulated expression of transcription factors upon drug resistance. Moreover, both of these aspects are independent of BCR-ABL1 where upon depletion of the oncogene neither the expression of the transcription factor gene-set (**Figure 3.7**) or activity of the SFKs pathway (**Figure 3.17**) are affected. Given these observations, and in concert with the ability of SFKs to regulate gene transcription, it was proposed that the SFK pathway functions to maintain expression of the (de)regulated set of transcription factors.

To address this, the 16IMr cells were treated with 2nM Dasatinib for 24hrs. Western blot analysis confirmed the collapse of the SFK-SKP2-p27 network (**Figure 3.18A**). Total RNA was collected and the relative expression of the transcription factor gene-set was profiled by semi-quantitative RT-PCR. In the absence of the SFK pathway, the relative expression of each transcription factor was maintained (**Figure 3.18B and C**). However, as discussed earlier regarding the ‘hit-run’ mechanism of gene activation, this observation does not exclude the possibility that the SFKs pathway is not required to initially regulate the expression of these factors [181].

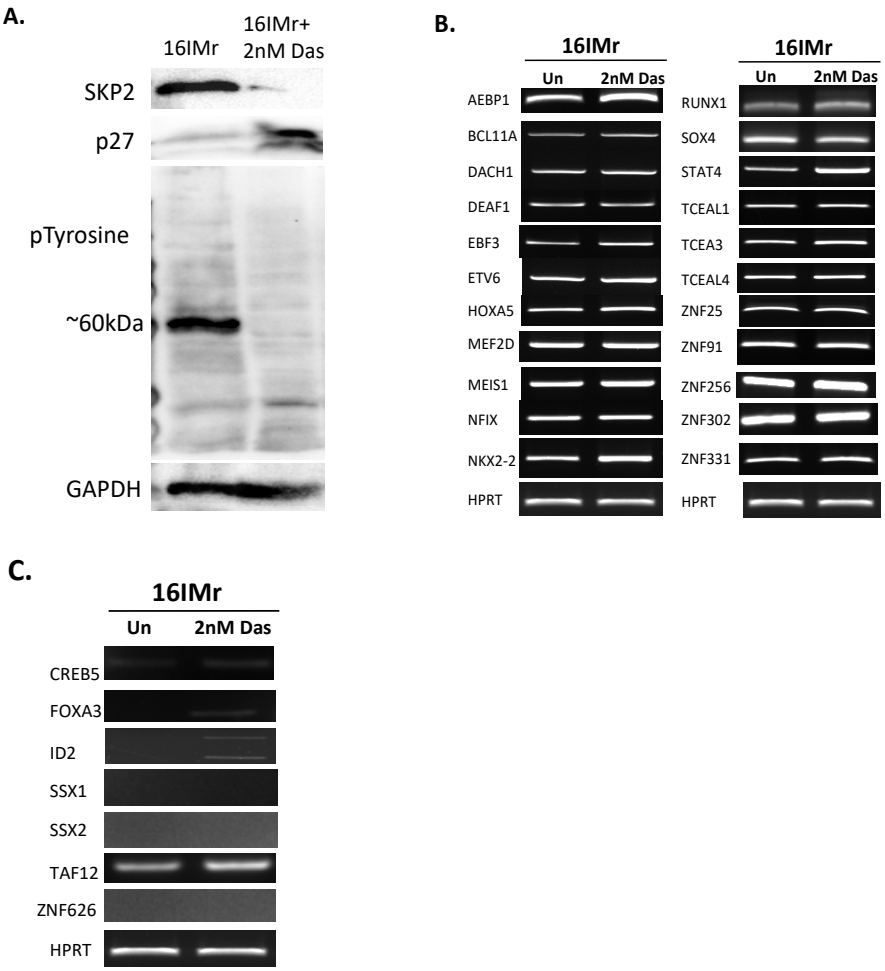


Figure 3.18: Semi-quantitative PCR analysis of the transcription factor gene-set within 16IMr cells treated with 2nM Dasatinib.

(A) Western blot of KCL22 16IMr cells that were treated with 2nM Dasatinib inhibitor for 24 hrs showing the SFK/SKP2/p27 axis collapse. RT-PCR analysis of transcription factors that are **(B)** induced and **(C)** repressed upon drug resistance was profiled by semi-quantitative PCR. Figure shows representative data of three independent experimental assays.

3.12 Discussion

Oncogene independent discrepancy

Previous work from the lab had demonstrated that the drug resistant KCL22 clones were capable of survival and growth in the absence of BCR-ABL1 protein. The primary emphasis of this chapter stemmed from these findings.

However, despite using the same cells (15IMr and 16IMr) and siBCR sequences (s1947 and s1948), this observation was unable to be reproduced.

A plausible explanation for this discrepancy could relate to the length of time in which the cells were actually devoid of the BCR-ABL1 protein. Specifically, previous work performed only a single transfection of the cells with the siBCR molecule(s) and then recorded cell viability for the next 5 days. In the modified protocol, as used herein, the cells underwent two rounds of transfections (3-days apart) prior to the monitoring of cell viability for the next 5 days. Under this condition, the production of BCR-ABL1 protein has already been inhibited within the cells for 3-days prior to the second-transfection. This 'second-hit' of siBCR transfection should maintain loss of the BCR-ABL1 protein for a longer period. It is therefore likely that the earlier observations failed to uncover the growth arrest phenotype as the cells weren't devoid of BCR-ABL1 protein for a sufficient period of time.

Nevertheless, the study herein conclusively demonstrated, based on the several biological repeats as well as the validation within another 'independent' KCL22 line (hetIMr cells), that depletion of BCR-ABL1 protein within drug resistant KCL22 cells results in the rapid onset of growth arrest. Moreover, this block in proliferation is not associated with onset of apoptosis nor any over cellular differentiation.

As discussed earlier, the depletion of BCR-ABL1 protein was achieved by the use of a siBCR molecule(s) that also targets the endogenous BCR protein. While BCR itself does not regulate proliferation within the drug resistant cells, it is recognised that the growth arrest mediated by siBCR could result from the simultaneous loss of both BCR-ABL1 and BCR proteins respectively. Simply put, the combined loss of both proteins is required to induce growth arrest. To address this concern, future studies should knock-down the expression of BCR-ABL1 with more accurate technique such as CRISPR-Cas9 in combination with a guide-RNA that is targeted against the fusion junction sequence respectively.

Collectively, these results demonstrate that upon inhibition of the SH1-kinase activity, the other protein domain(s) of BCR-ABL1 protein become functional and confer the ability for CML cells to proliferate. Furthermore, they now focus attention to the functional role, and clinical relevance, of these other domain(s) of BCR-ABL1 protein in drug resistant CML.

BCR-ABL1; switching from a tyrosine kinase into a scaffold protein

BCR-ABL1 is the driving mutation of CML and its constitutive tyrosine kinase (SH1 domain) is central for its pathology [6, 28, 80]. However, leukemic cells are dependent upon the SH1-kinase activity only within early-stages of the disease. Upon drug resistance, the majority of patients will relapse despite receiving IM therapy that continues to be effective in inhibiting the SH1-kinase activity [68, 82, 197].

This raises the question, and clinical concern, as to how these leukemic cells are capable of growth and survival in the absence of an active SH1 kinase. In this context, it is important to recognise that BCR-ABL1 is a large complex protein [28, 32, 198] consisting of many domains including (i) SH2 and SH3 Src-homology domains, (ii) Rho/GEF (Ras homolog gene family/guanine nucleotide exchange factor kinase domain), (iii) proline-rich domain, (iv) oligomizeration domain which also functions as a docking site for Grb2 (growth factor receptor bound protein 2) as well as an (v) actin binding site.

It is proposed that upon the loss of the SH1-kinase activity, the other domain(s) of the BCR-ABL1 partially compensate for its loss by functioning as a scaffold to facilitate new protein-protein interactions and the regulation of cell proliferation. The findings presented herein strongly support a model whereby the primary role of BCR-ABL1 has switched from an initial tyrosine kinase oncogene to that of a scaffold protein. In support of this notion, the work of others provided clear evidence of functional role for the other domains of BCR-ABL1 protein.

Other domains and gene regulation

It has long been established that BCR-ABL1 can regulate gene expression as mediated by the SH1-kinase phosphorylation of the STAT5 transcription factor [199]. Although poorly understood, other research has demonstrated that in the absence of SH1-kinase activity the BCR-ABL1 protein can still regulate gene expression; as exemplified by induction of Alox5 expression in the presence of

IM [178]. In this context, it is of interest that work presented herein demonstrate that upon depletion of BCR-ABL1, the expression of 162 genes are commonly regulated in both drug resistant KCL22 clones. In this scenario, the molecular connectivity between cytoplasmic BCR-ABL1 and the nucleus is unclear; particularly as STAT5 is deactivated. It is possible that upon the loss of SH1 kinase activity, the scaffold-function of BCR-ABL1 enables another signalling pathway (e.g. another STAT) to become active to subsequently regulate gene expression. However, the GSEA analysis failed to identify any such signalling signatures although target-genes for the RB1/E2F, cell cycle checkpoints and DNA repair were enriched respectively. How these gene-sets are deregulated upon the loss of BCR-ABL1 is unclear. In understanding this non-SH1 relationship between BCR-ABL1 and gene regulation, studies must first identify which other domain(s) is responsible.

Other domains and intra-protein regulation

In addition to the SH1 domain, BCR-ABL1 contains two other Src-homology regions; SH2 and SH3 respectively. The SH2 domain is a positive regulator of BCR-ABL1 function as mutations within this region result in reduced SH1-kinase activity and subsequent failure to induce leukaemia in mice [200]. Following on from these observations, small molecules (monobodies) specifically targeting the SH2-domain of ABL1 (and BCR-ABL1) have been generated [32, 54]. Here, use of the monobodies to block SH2 function resulted in the anticipated reduced kinase activity of BCR-ABL1 and the rapid induction of apoptosis within CML cell lines (K562) as well as primary CML patient samples respectively.

The SH3 domain of BCR-ABL1 recognizes proline-rich peptide sequences and functions primarily to facilitate protein-protein interactions including the interaction of the CrkL protein with the SH2-SH3 interface [33]. In contrast to the SH2 function, the SH3 domain negatively influences the SH1-kinase activity, whereby the deletion of this region results in an increased SH1-kinase activity of BCR-ABL1 and enhanced oncogenicity [201].

Other BCR-ABL1 domain(s) and activation of the Src pathway

As reported by others, the Src pathway is activated upon drug resistance in CML cells and contributes to the inhibition of apoptosis [202, 203]. Of great interest is that the SH1-kinase activity is not required for activation of Src and could

therefore be mediated by another protein domain(s) of BCR-ABL1 [204]. The interaction between BCR-ABL1 and the Src family is complicated and consists of cross-talk communication between the two. As example, the Src kinase Hck can interact with BCR-ABL1 in a SH1-domain independent manner. Specifically, Hck can bind to BCR-ABL1 at the BCR portion of the protein and promotes the protein-protein interaction between Grb2 with BCR-ABL1 respectively [184]. Furthermore, others have reported similar protein-protein interaction where Hck can also interact with the SH3 domain, although the consequence of this was not determined [185].

The functions of the other aforementioned protein domains of BCR-ABL1 is unclear; although the actin binding site enables BCR-ABL1 to interact with the cytoskeleton [205, 206].

Summary and Future Work

Despite the complex nature of BCR-ABL1, these observations support the notion that other, non-SH1, protein domain(s) of the oncogene account for how the leukaemic cells continue to proliferate upon drug resistance. Molecular targeting of these other domain(s) of BCR-ABL1 could be an attractive approach for therapeutic applications in drug resistance CML.

Future work should, however, first confirm the key findings of this chapter within a second CML model of kinase-independent drug resistance. This forms the basis of a future chapter herein.

It would be of keen interest to gain a greater understanding of the functional requirement of each domain(s) of BCR-ABL1 in regulating cell proliferation. Here, the use of small-molecules targeting each domain would be exploited. Indeed, the use of the SH2 monobody would be an easy approach to explore the role of SH2 in these cells. The lab is in current communication with the Hantschel lab (EPFL, Lausanne, France) for access to this molecule. While no other small molecules currently exist for the other domains (e.g. SH3 or Rho/GEF domain) these can certainly be achieved by the use of Affimer technology. In brief, Affimers are akin to monobodies being antibody-like engineered proteins capable of targeting, and interfering, with specific domains-epitopes[50]. The use of such technology is currently being explored within the lab. In preliminary discussion with the Tomlinson lab (University of Leeds), Affimers to the SH2

domain of ABL1 are available and these could be used in parallel to the published SH2 monobody.

Chapter Summary

Model of drug resistance CML

A key feature of the SH1 kinase of BCR-ABL1, as with other oncogenes, is the ability to confer various 'cancer-hallmark' properties, including the ability to self-renew and the inhibition of both apoptosis and cellular differentiation respectively [207, 208]. To maintain these minimum 'cancer-hallmark' properties upon drug resistance, new 'oncogenic drivers' must be acquired.

One such 'oncogenic driver' is achieved by the other domains of BCR-ABL1 which, functioning as a scaffold protein, regulates cell proliferation. However, this is only a partial compensation for the loss of SH1-kinase activity. The data presented herein demonstrate that even in the absence of BCR-ABL1, the drug resistant KCL22 cells still retain the additional 'cancer-hallmarks' of anti-apoptosis and block of cellular differentiation respectively.

How are drug resistant CML cells capable of maintaining these two hallmarks in the complete absence of BCR-ABL1? Previous work in our lab, as well as those of others, provide some possible answers.

Deregulated transcription factors and block in differentiation

The lab has previously identified a panel of 29 transcription factors that are commonly deregulated in expression upon drug resistant within the KCL22 clones. Transcription factors are frequently mutated in leukaemia and the consequence often leads to the block of cellular differentiation [70, 209].

It is proposed that upon the loss of SH1-kinase activity, the panel of 29 transcription factors are (de)regulated in the KCL22 cells to block differentiation and maintain the cells in a progenitor-like leukemic cell-state. Interestingly, as reported herein, the expression of this transcription factors panel is independent of BCR-ABL1.

Previous work in the lab has explored the role of the RUNX1 transcription factor, which is strongly induced in the KCL22 lines upon drug resistance (data not shown, G. Bheesmachar). Depletion of RUNX1 within 15IMr and 16IMR cells, respectively, resulted in the onset of growth arrest. However, unlike the observations seen with siBCR, this growth arrest coincided with distinct

morphological features associated with a macrophage-like phenotype (large cytoplasm, ruffling of cell membrane and off-centred nuclei). Moreover, expression profiling demonstrated that several myeloid-lineage specific genes were upregulated as a consequence of the RUNX1 loss including the CD14 and CD11b markers as well as transcripts for all three myeloid growth factor cytokines, G-, GM- and M-CSRF respectively.

In summary, the targeting of RUNX1 within drug resistant KCL22 cells resulted in the onset of growth arrest and acquisition of a macrophage-like phenotype. This data strongly supports the notion that a primary role of the deregulated transcription factor(s) in drug resistant CML cells is to block cellular differentiation. Being regulated in a BCR-ABL1 independent manner (likely due to changes in the epigenetic landscape), these transcription factors impart the second 'cancer-hallmark' function upon the drug resistant cells.

Src family of kinases and apoptosis

The SFKs family consist of nine non-receptor tyrosine kinases with pleiotropic functions being important in cellular proliferation, survival, plasticity and migration [88]. Previous reports have demonstrated that the SFKs pathway, mainly Lyn and Hck, becomes activated within CML cells upon drug resistance and function to confer anti-apoptosis properties [107, 184, 203, 210, 211]. The lab has previously characterised the Src pathway within the KCL22 model of drug resistance and as reported herein, the cells are sensitive to treatment with a Src-inhibitor, Dasatanib, which readily induces apoptosis.

Interestingly, the SFKs pathway remains active in the absence of BCR-ABL1 and accounts for why the siBCR treated cells failed to undergo apoptosis. Although likely activated by one of the other domain(s) of BCR-ABL1, how is the activity of the SFKs maintained in absence of the oncogene? It is possible that alternate signalling pathways have become activated to maintain SFKs activity. Additionally, the SFKs may have acquired gain-of-function mutation which confer constitutive activity of the proteins, as previously reported in other malignancies such as advanced colon cancer [212].

Collectively, these observations demonstrate that upon drug resistance the SFK pathway is activated and functions to inhibit apoptosis. Once activated, this pathway is regulated in a BCR-ABL1 independent, self-autonomous, manner

and confers the third, and final, ‘cancer-hallmark’ function upon the drug resistant cells (**Figure 3.19**).

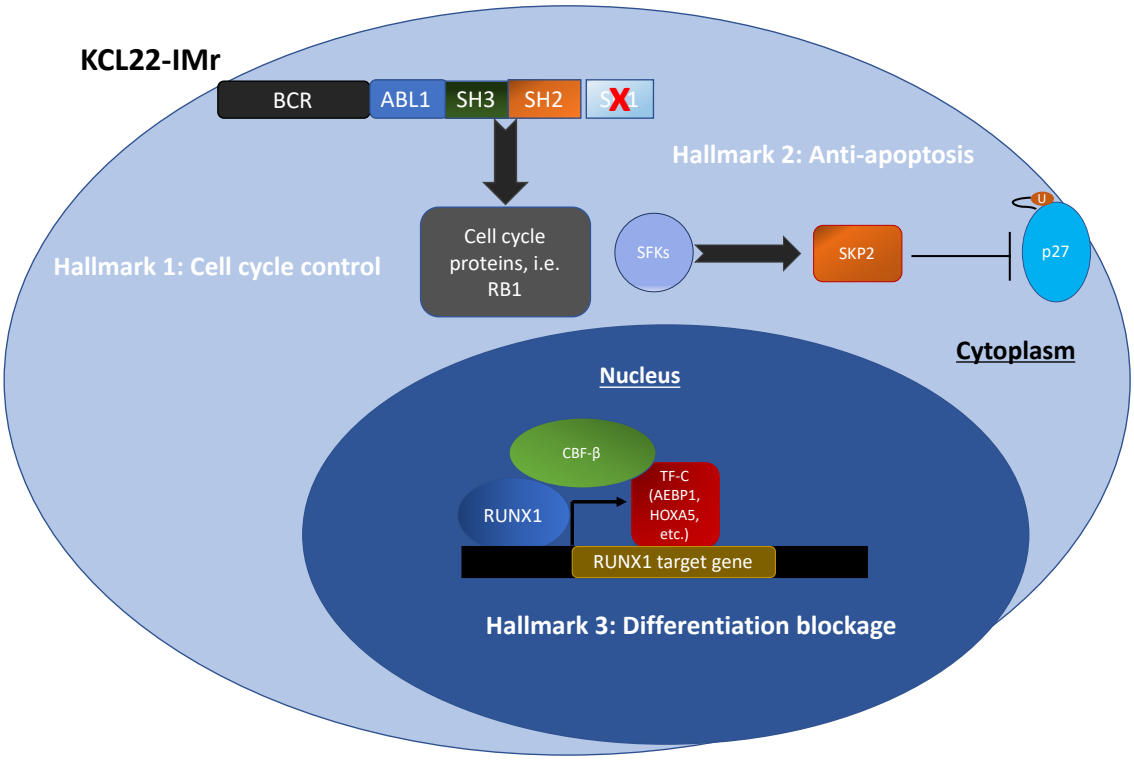


Figure 3.19: Proposed model of cell growth, survival and block of differentiation in BCR-ABL1 kinase independent drug resistance.

A proposed model for maintenance of key ‘hallmarks of cancer’ within a kinase independent CML. Upon loss of SH1 activity, other domain(s) of BCR-ABL1 regulate cell proliferation. Moreover, the Src pathway is independently activated to regulate apoptosis. Finally, deregulated expression of transcription factors functions to block cellular differentiation.

Chapter 4: Defining the transcription factor network within a second cell model of kinase-independent CML: The EM2 cell line

4.1 Introduction

Previous work in the research group has identified a novel mechanism for establishing drug resistance in CML; namely the ability of leukaemic cells to become BCR-ABL1 kinase independent by the dysregulation of transcription factors which, in turn, function as new oncogenic drivers. Despite the promise of these findings, this has only been demonstrated within the KCL22 cells and suffers from concerns that it may be a cell-line specific phenomenon. Whether this mechanism is employed in other CML-lineages is yet to be determined.

Establishing a second BCR-ABL1 kinase-independent cell line will also serve as a valuable screening-filtering tool to determine which, if any, of the deregulated transcription factors function as 'master regulators' (whether in combination or alone) in establishing drug resistance. As all CML lines are solely dependent upon the kinase activity of BCR-ABL1 for survival, it is hypothesized that in order to become kinase-independent they will each (de)regulate a common core network of transcription factors for continued survival. It is predicted, and highly anticipated, that among the dysregulated network of transcription factors identified within this second-model a subset will be shared among those previously identified from the KCL22 clones. If this hypothesis holds true, then this approach should define the minimal, core-network of transcription factors required for BCR-ABL1 kinase independence and, furthermore, identify gene candidates for future clinical therapy.

Additionally, based on previous work describe herein (**Chapter 3**), the discovery of a novel role for the other domain(s) of BCR-ABL1 in regulating the cell-cycle of the drug resistant leukemic cells can now be validated within this second model system.

The Aims of this chapter are:

1. Choose an appropriate second CML cell line and attempt to establish a kinase-independent drug-resistant derivative.
2. Determine whether there is a shared core-network of deregulated transcription factors by profiling the existing list of deregulated transcription factors established from the KCL22 model.
3. Validate the putative functional importance of the minimally defined core-network of transcription factors by depletion of candidate proteins by siRNA within both model systems.
4. Validate the role of the other domain(s) of BCR-ABL1 in regulating the cell-cycle within this second model system by siBCR depletion (including appropriate controls such as targeting the endogenous BCR protein).

4.2 Generation of a second kinase-independent CML cell line

CML is a heterogeneous disease with clinical presentation dependent on the origin of the lineage afflicted. While commonly viewed as a neoplasia of early myeloid progenitors, CML can also develop within erythroid, megakaryocyte or lymphoid lineages respectively [11, 213]. With a pathology afflicting multiple blood-lineages, the robust demonstration for the role of dysregulated transcription factors in establishing kinase-independence would require that this phenomenon be tested within every lineage-afflicted CML cell line. However, the technical scale required to achieve this goal is challenging. As kinase-independence has been demonstrated in the myeloid KCL22 cells, the primary goal of this chapter was to first validate this mechanism within another myeloid-afflicted CML cell.

To identify a suitable CML myeloid cell line, the DSMZ catalogue of cell cultures was reviewed. There are 47 established CML cell lines and unfortunately only 12 are commercially available. Of these 12 lines, 2 are of the lymphoid lineage (Nalm-1, BV173), 1 is T-cell (CML-T), 3 are myeloid (EM2, GDM-1 and KCL22), 1 is basophil (KU182), 2 are Ery/Meg (K562, LAMA84), 1 is erythroid (JK-1) and 2 are megakaryocytic (MEG-01, JURL-MK1) [128, 214].

Of the myeloid-afflicted CML lines both GDM-1 and EM2 cells were considered. A review of the literature revealed that GDM-1 is not commonly used among the scientific community (4 publications) while that of EM2 is more frequent (>10

citations). Moreover, a detailed review of the DSMZ catalogue characterised the GDM-1 cells as an AML-like model being a “myelocytic cell line derived from AML M4 following a CML-like myeloproliferative disorder” and do not harbour the Philadelphia chromosome. Based on these observations, the EM2 cells were chosen.

4.3 Generation of drug-resistant EM2 cells

Both KCL22 and EM2 cell lines correspond to a myelocytic BC-CML lineage [215]. EM2 cells were isolated from bone marrow of a 5-year old Caucasian female in 1982 [128]. The EM2 line is a heterogeneous population and it is therefore important to determine whether any proposed changes to the transcriptome occurring during drug resistance is the result of an acquired change in transcriptional regulation rather than clonal selection of a pre-existing population. Specifically, two alternate interpretations can account for the induction of mRNA expression of a candidate transcription factor (as detected by RT-PCR analysis) when comparing between drug-sensitive cells and their resistant derivate respectively:

A. **Clonal Selection**: This model proposes that of the heterogeneous EM2 population a very small proportion of cells already expresses the transcription factor and is considered a ‘pre-existing’ drug resistant clone. In this scenario, RT-PCR analysis of the bulk population would result in the failure to detect transcripts of the candidate factor and thus the cells are deemed negative (or very low level) in its expression respectively.

Upon exposing the heterogeneous cell population to IM the majority of the population will undergo apoptosis while this pre-existing IM-resistant population (i.e. already expressing the transcription factor) will be clonally selected. Transcriptome profiling would now readily detect expression of the candidate transcription factor. In comparison to the starting drug-sensitive population, this analysis could be mistakenly inferred that the transcription factor is actively induced during the process of drug-resistance.

B**. Acquired**: In this model, cells adapt to the loss of BCR-ABL1 kinase signalling by specifically regulating the expression of the transcription factor and is therefore acquired during the transition. To confirm such a model, it is necessary to have a pure population that was expanded from a single cell. Here, the RNA profiling of the cell population will accurately reflect the expression level of a

given transcription factor within this clonal line. As such, any changes in gene expression between the initial drug-sensitive cells to that of the drug-resistant derivative can be viewed as an acquired transcriptional change.

For the robust identification, and analysis, of any acquired changes in gene expression occurring upon drug resistance at least 2 independent EM2 clones should be used. It is noted that in generating the KCL22 drug-resistant model, only two single cell clones were used (15P and 16P respectively) with both lines utilising the kinase-independent mechanism to become drug resistant. Given this apparent high success rate in generating BCR-ABL1 kinase-independent derivatives, it was decided to generate drug resistant lines from 5 EM2 single-cell clones in the hopes ensuring that at least 2 of the clones would become BCR-ABL1 kinase-independent. Single cell clones from the heterogeneous EM2 population were generated by serial dilution and five randomly selected for analyses, termed P3, P4, P5, P6 and P7 respectively.

While the KCL22 model has been invaluable for the greater understanding of dysregulated transcription factors in establishing kinase-independent CML, the dynamic expression of each factor during the course of drug-resistance was not determined. Specifically, which factors are regulated at the early onset of drug resistance? Such analysis could identify so-called ‘master regulators’ of the drug resistant phenotype. To address this, a minor modification to the protocol for generating drug resistant cells was made whereby frozen stocks will be made at the end of each step-wise IM treatment respectively.

4.4 Identification of the EM2 IM killing dose.

The lab has previously generated drug resistant KCL22 clones by initially culturing the cells in IM at a concentration that is 10% of the pre-determined ‘killing’ concentration; with subsequent 10%-increments every week until they reached the ability to grow in 100% IM concentration. Although the EM2 line is akin to KCL22 in being solely dependent upon BCR-ABL1 for survival, the concentration of IM required to inhibit the kinase activity could differ between the two lines. Accordingly, the minimum IM concentration required to inhibit cell proliferation (a functional consequence of kinase inhibition) for each EM2 clone was determined.

In brief, $0.2x10^6$ cells were treated with titrating doses of IM ranging from 0μM to 1μM respectively for 72hrs. Cell viability was quantified by the MTS assay. For all 5 clones, a decline of cell viability is seen with ascending concentrations of IM with a maximum reduction seen at 0.3μM IM (**Figure 4.1**). Despite increasing concentrations of IM the cell viability did not decrease any further beyond treatment with 0.3μM IM. Based on these observations, the minimum IM-concentration required to induce maximum kinase inhibition was identified at 0.3μM IM and this concentration was used as the 'killing' dose for the EM2 drug-resistance protocol.

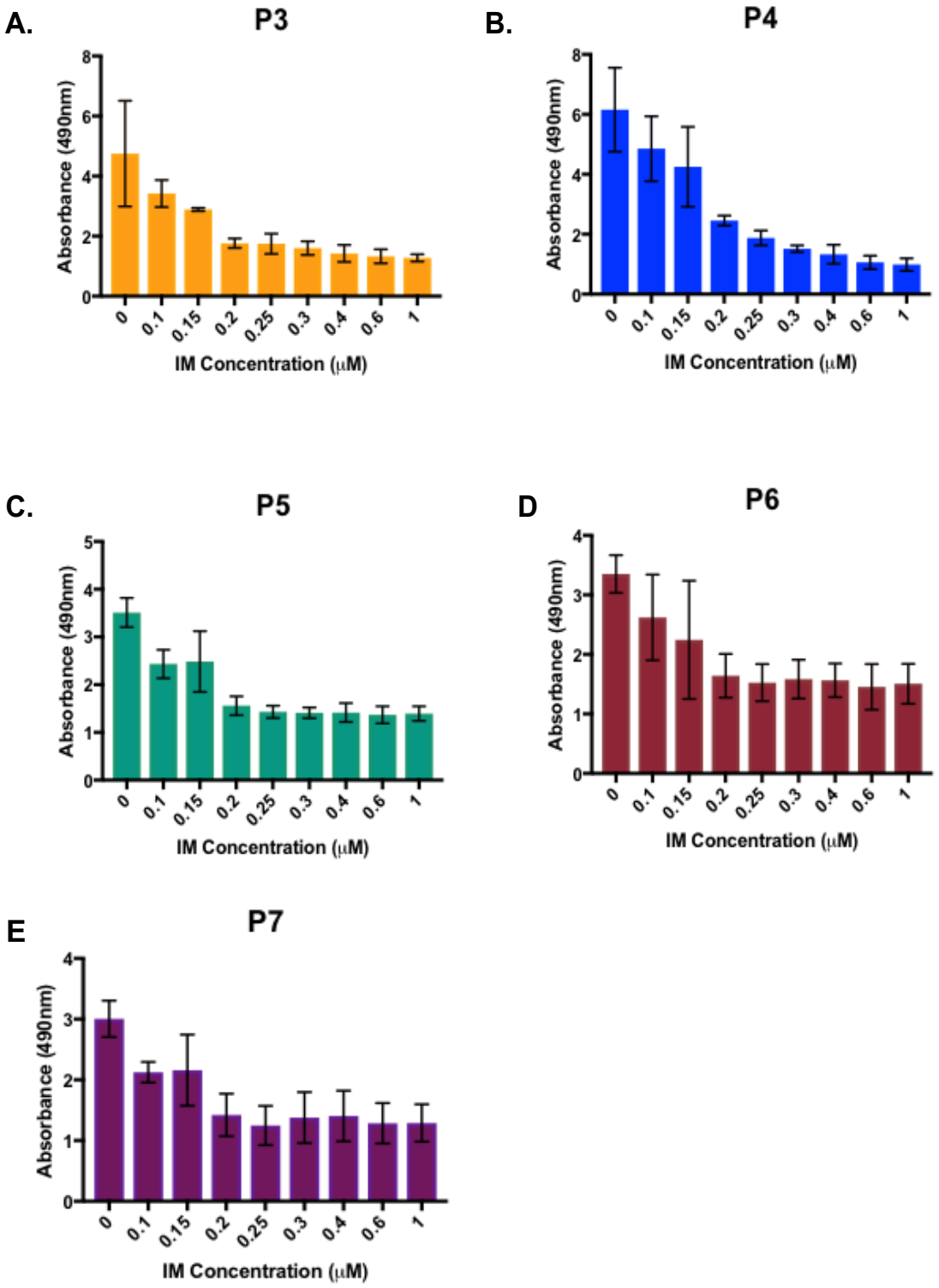


Figure 4.1: MTS viability assay on EM2 single-cell clones.

Cell viability analysis on the five single-cell clones (**A-E**). Cells were cultured under the indicated IM concentrations for 3 days and viability measured by MTS. Absorbance was used at 490nm. Absorbance values are relative to no-MTS control. Mean and SD (error bars) is presented from three independent experiments.

The EM2 clones were initially cultured in 0.03μM IM (10% of 'killing' concentration) with the refreshment of IM every 3-days. After a week in culture, or until such time that a healthy population was obtained, aliquots of the cells were frozen down, and the drug concentration increased by 10% increments until the final 0.3μM IM concentration was reached (**Figure 4.2**). Once established, the drug resistant cells were continuously cultured in 0.3M IM with drug replenished every 3-days.

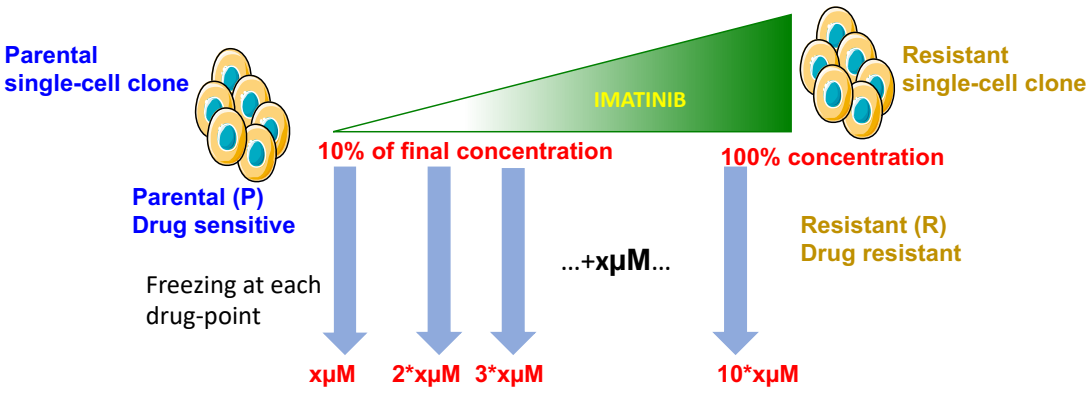


Figure 4.2: Schematic of generating IM-resistant EM2 clones.
EM2 clones were grown in increasing concentrations of Imatinib. Concentration ranged from 0.03μM to 0.3μM.

4.5 Characterisation of drug resistant EM2 clones

The drug-resistant EM2 derivatives were termed R3, R4, R5, R6 and R7 respectively. To identify which, if any, were BCR-ABL1 kinase-independent the activity of the oncogene was assessed by examining the protein phosphorylation levels of CrkL and STAT5 respectively. As this was a preliminary screen, lysates from both parental P3 and P7 clones were included as a positive control for baseline kinase activity of BCR-ABL1.

In comparison to the parental EM2 controls, there was no discernible reduction in pCrkL for both R6 and R7 respectively; suggesting that the kinase activity of BCR-ABL1 is still functional within these clones (**Figure 4.3**).

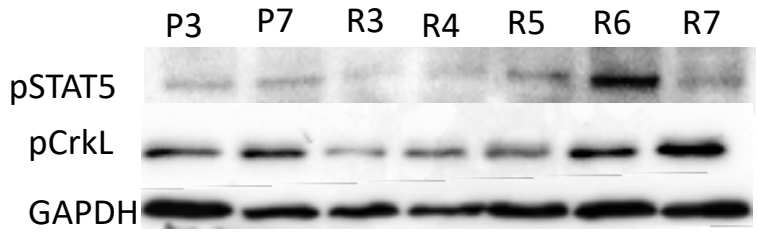


Figure 4.3: Phosphorylation status of BCR-ABL1 target proteins in drug resistant EM2 cells.

Western blot analysis for the phosphorylated STAT5 and CrkL proteins respectively within the EM2 drug resistant clones. GAPDH was used as a loading control. Figure shows representative data of three independent experimental assays.

Here, the likely mechanism for drug-resistance is the acquisition of a point-mutation within the SH1 kinase domain of BCR-ABL1. For the R5 clone there was a reduction in pCrkL but no change in pSTAT5 and therefore an inconclusive determination of the mechanism for drug resistance.

Interestingly, clones R3 and R4 had a visible reduction of both pCrkL and pSTAT5. Given this promising finding the BCR-ABL1 kinase activity was further analysed in these two clones by assessment of phosphorylated BCR-ABL1 and all proteins with a phosphorylated-tyrosine residue respectively (**Figure 4.4a**). As positive controls, lysates from the corresponding parental counterparts (P3 and P4 respectively) were included. In support of the earlier findings, a reduction in the phosphorylation of both BCR-ABL1 and all tyrosine-residue containing proteins was readily observed. Based on these findings, it is evident that IM is effectively blocking the SH1 domain of BCR-ABL1 within both R3 and R4 clones and thereby both lines have become kinase-independent for their survival and growth.

However, the reduction in kinase activity within the R3 and R4 lines failed to be reproduced upon the repeat biological analysis (**Figure 4.4b**). Given this contrasting result, the quality of protein lysates was questioned and subsequently re-assured by making fresh solution of all reagents and buffers. Moreover, as a clear and definitive positive control for the phospho-protein analysis the protein lysates from KCL22 model (15P and 15IMr cells) was generated in parallel with all EM2 sample preparations and additionally included in all subsequent western analysis. Finally, new aliquots of IM were made and

the refreshment of cells with drug was adhered to strictly; 24hr prior to isolation of protein lysate.

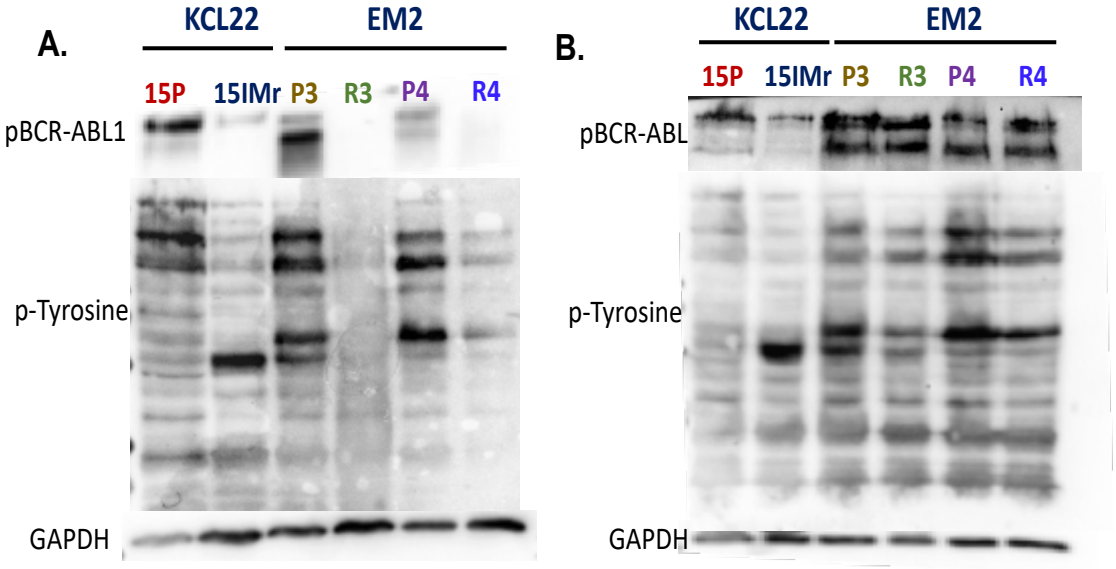


Figure 4.4: Kinase activity of BCR-ABL1 indirectly measured in drug resistant EM2 cells, biological repeats.

The phosphorylation of BCR-ABL1 as well as all proteins carrying a phosphorylated tyrosine residue was assessed in the drug resistant EM2 clones. Panel (**A**) and (**B**) represent independent biological repeats. GAPDH was used as loading control. Figure shows representative data of three independent experimental assays.

Surprisingly, in several repeat attempts of analysing the R3 and R4 clones, the phosphorylation pattern of the BCR-ABL1 downstream targets was inconsistent and confusing (data not shown). At times it was clear that the BCR-ABL1 kinase activity was reduced or still active. Yet, some analysis demonstrated a mixed phenotype where within the same protein lysate some target proteins demonstrated a reduction in phosphorylation while others did not change. In all cases, protein lysates from the KCL22 positive controls consistently demonstrated the expected results thus validating the robustness and sensitivity of the technical protocol.

After numerous repeats it is clear that the analysis of the R3 and R4 clones was inconclusive. Why different protein lysates gave such differing patterns of kinase activity is unknown. Given the failure to obtain reproducible and consistent analysis of these clones any further studies with these lines was abandoned. The next approach was to understand, and overcome, these inconsistencies so a stable, and reproducible, EM2 drug-resistant line can be generated.

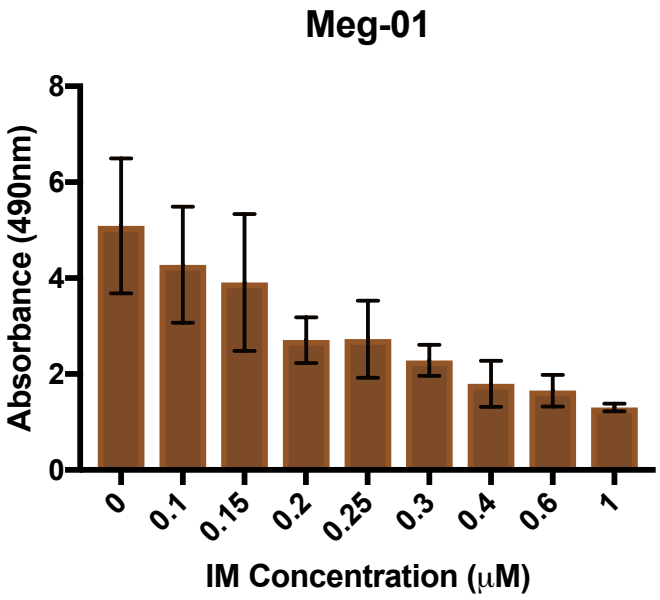
4.6 Summarising the BCR-ABL1 kinase (in)dependency in EM2 drug resistant clones.

In reviewing the kinase activity of BCR-ABL1 within the EM2 drug resistant clones, two striking, and certainly odd features, were evident. First, the phosphorylation-status of any given downstream BCR-ABL1 target protein was inconsistent across numerous biological repeats. For example, in one protein lysate there could be a clear reduction in the phosphorylation of STAT5 while upon second repeat this had disappeared (i.e. no change in comparison to the parental control) only to be reduced again upon a third repeat. Secondly, in some analysis it was noted that not all target proteins had a reduction in phosphorylation; exemplified where pCrkL was reduced yet normal pSTAT5. And again, this mixed-phenotype pattern of which downstream targets were dephosphorylated would differ between biological repeats.

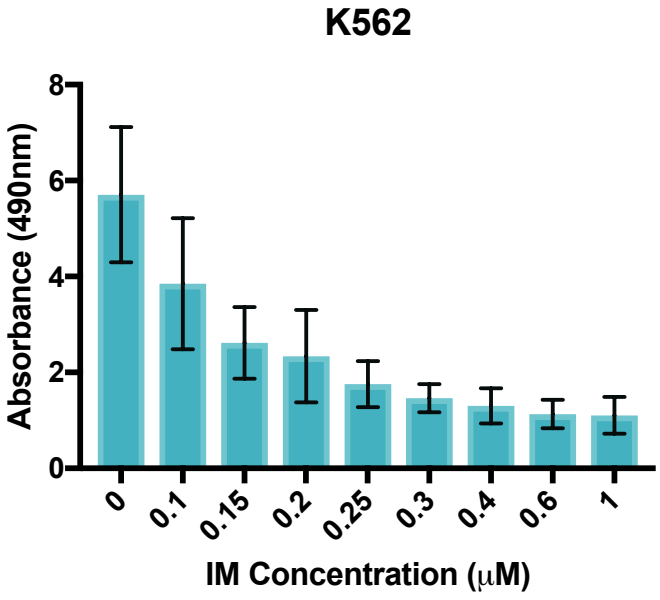
This unstable phenotype suggested a simultaneous tethering of the R3 and R4 clones between a kinase-independent and -dependent cell-state; with some undetermined stochastic factor determining which state (whether complete or partial) exists at the time of analysis. Given this observation, it was proposed that in contrast to the interpretation of the MTS assay, the kinase activity of BCR-ABL1 is only partially inhibited at 0.3μM IM and this itself could account for the apparent random nature of whether a given downstream target is dephosphorylated or not. To address this issue, a more detailed analysis of the pharmacological effect that 0.3μM IM had upon the EM2 cells was performed.

As a first-line response, the MTS assays of the EM2 cells upon IM treatment was reviewed and this was done in comparison with data from other CML lines, K562 and Meg-01 respectively (**Figure 4.5**). In all cases the pharmaco-relationship between IM treatment and the cell proliferation response was similar with a positive correlation and cells responding in a dose-dependent manner. Given the similar dynamics among the three different CML cell lines, there was no apparent reason to suspect that the MTS assay for the EM2 lines was incorrect.

A.


Meg-01
Absorbance (490nm)
0
2
4
6
8
0
0.1
0.15
0.2
0.25
0.3
0.4
0.6
1
IM Concentration (μM)

B.


K562
Absorbance (490nm)
0
2
4
6
8
0
0.1
0.15
0.2
0.25
0.3
0.4
0.6
1
IM Concentration (μM)

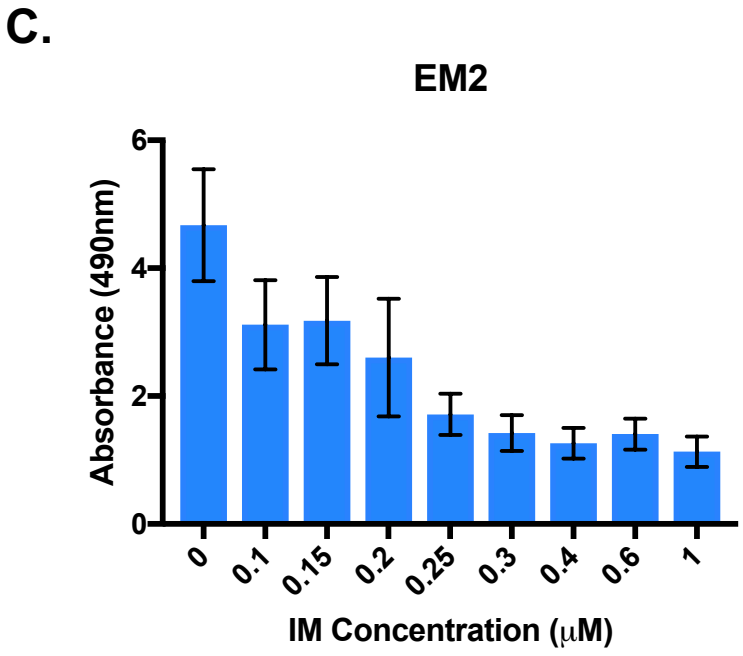


Figure 4.5: Dose response of CML cell lines to Imatinib treatment.

MTS analysis was performed on **(A)** Meg-01, **(B)** K562 and **(C)** EM2 lines under titrating Imatinib concentrations. Cells were cultured under the indicated IM concentrations for 3 days cell viability measured by MTS. Absorbance was used at 490nm. Absorbance values are relative to no-MTS control. Mean and SD (error bars) is presented from three independent experiments.

What was of interest is how, at the microscopic level, the different CML cells respond to IM shortly after treatment. During the course of this PhD, it was observed that the K562, Meg-01 and the KCL22 cells would display overt signs of apoptosis-necrosis (evident by cell shape abnormality and shrinkage) within 3d of IM treatment (data not shown). Yet, the EM2 cells would first become non-proliferating for a few days (up to 5d) before they presented any indicators of cell death. This is important to note with reference that the MTS assay as it is measured at 3d post-IM treatment; raising concerns that some of the MTS values could reflect a sub-optimal IM concentration whereby the EM2 cells could enter cell cycle arrest but not apoptosis. Specifically, the incomplete block of BCR-ABL1 activity would only induce growth arrest with the residual kinase activity still capable of an anti-apoptosis mechanism. It could therefore have been a mistake to assume that the induction of growth arrest was a result of complete inhibition of kinase activity. Clearly, the correlation of a functional consequence of IM treatment, such as the induction of growth arrest, may not correlate with a complete inhibition of BCR-ABL1. It is therefore plausible that the 0.3μM IM

treatment is inducing cell cycle arrest which is interpreted as maximal inhibition of kinase activity.

4.7 Re-validation of the EM2 response to Imatinib treatment.

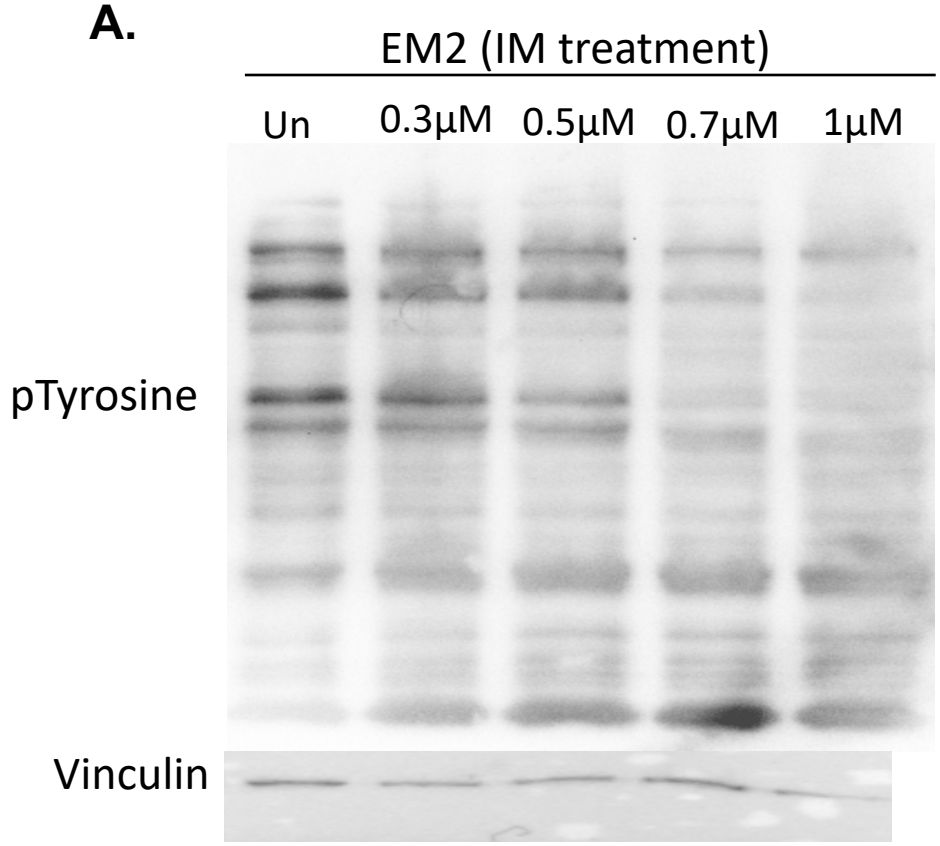
With the likelihood that the IM-mediated growth arrest of the EM2 cells does not correlate with a complete inhibition of BCR-ABL1, the relationship between drug treatment and kinase activity was further explored. In brief, the original heterogenous EM2 line was treated with various concentration of IM for 24hrs (0.3μM, 0.5μM, 0.7μM and 1μM IM) and the BCR-ABL1 kinase activity determined by western analysis for all proteins phosphorylated at a tyrosine residue (**Figure 4.6a**). As expected, a gradual reduction of kinase activity strongly correlated with increasing concentration of IM and all tyrosine residues were substantially dephosphorylated at 0.7μM and 1μM treatment respectively. In direct support of the concerns raised, treatment of the cells with 0.3μM IM resulted in only a slight reduction of BCR-ABL1 activity.

These observations confirm that 0.3μM IM does not completely block the BCR-ABL1 activity within EM2 cells but reduces it enough to induce growth arrest. This would clearly account for the often conflicting and confusing analysis of the R3 and R4 clones as BCR-ABL1 is still active albeit as a slightly reduced level.

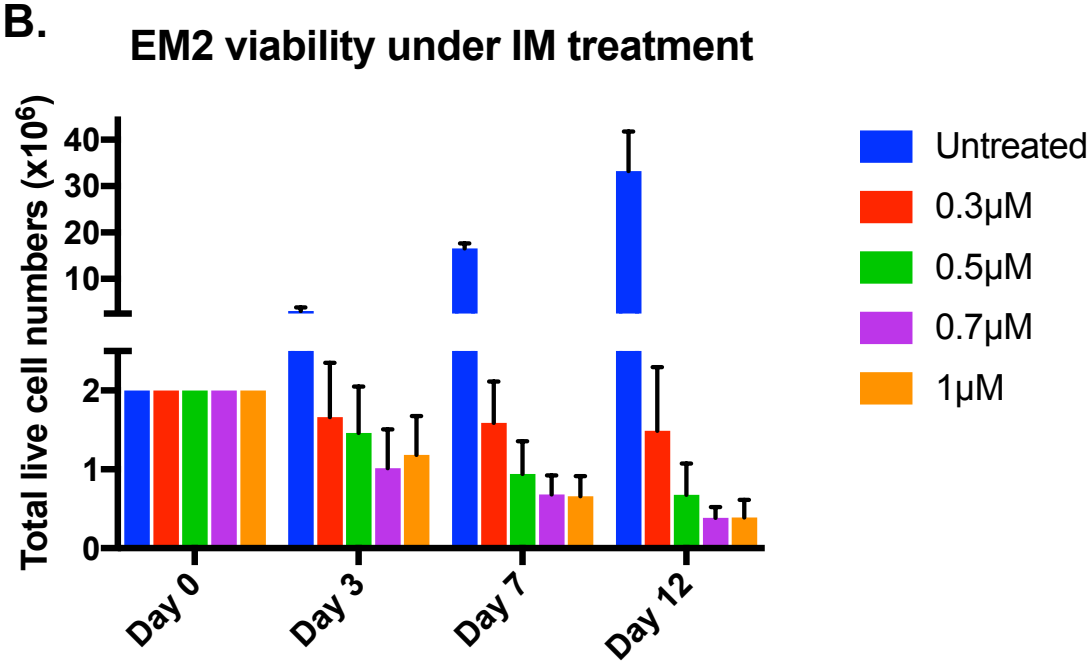
The re-analysis of IM and kinase activity now identified either 0.7μM or 1μM IM as the appropriate, and correct, concentration required to make kinase-independent drug resistant lines. However, to avoid any further misinterpretations, these findings were further corroborated by initially examining the cell viability as assessed by trypan blue exclusion. Here, the EM2 heterogeneous population was treated with titrating IM concentrations for 3, 7 and 12 days. As expected, the untreated EM2 cells grew in an exponential manner reaching a maximum of 33 million at the end of the 12d culture period (**Figure 4.6b**). Confirming the earlier suspicions, cells treated with 0.3μM IM became non-proliferating and failed to grow or demonstrate any loss in viability and maintained a steady viable count of 1.6 million at each time point. Treatment of cells with either 0.5μM, 0.7μM or 1μM IM resulted in a gradual and steady decline in cell viability with the latter two concentrations demonstrating the most potent effect with only 0.38 million cells at end of the experiment.

As the treatment of EM2 cells with either 0.7μM or 1μM IM resulted in a similar loss of kinase activity (as determined by phospho-tyrosine blots) as well as cell viability, the concentration of 0.7μM was selected for use in generating drug-resistant cells (**Figure 4.6c**).

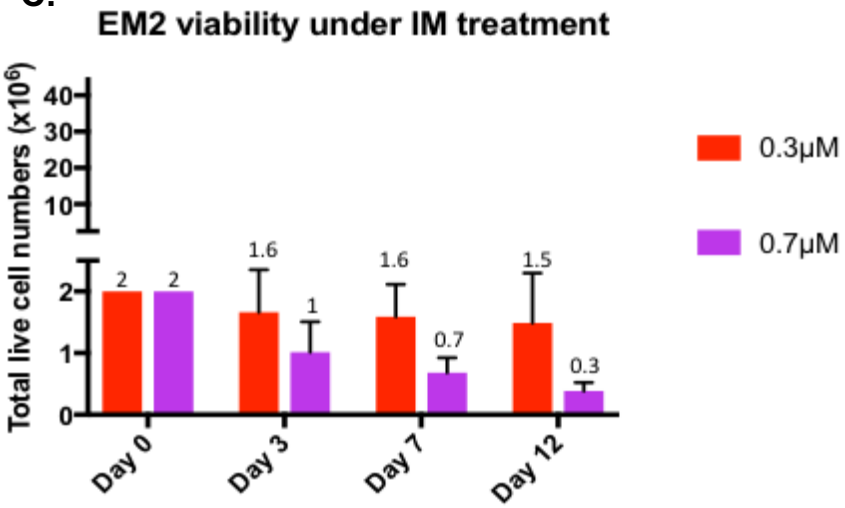
To further illustrate the differences in how the EM2 cells respond to either 0.3μM or 0.7μM IM treatment, the induction of apoptosis was visually confirmed by microscopy over the 12d period (**Figure 4.6d**). As control, the treatment of KCL22 cells with 1μM IM was included. As repeatedly seen and reported, apoptosis is readily visible for the KCL22 cells after 3-days post-treatment, while in contrast the EM2 cells treated with either IM concentrations show no signs of cell stress. It is only at d12 that EM2 cells treated with 0.7μM IM display gross necrosis while the 0.3μM treated cells show some apoptotic death but the majority of the cells retaining a healthy bright-shiny reflection albeit slightly abnormal in shape.



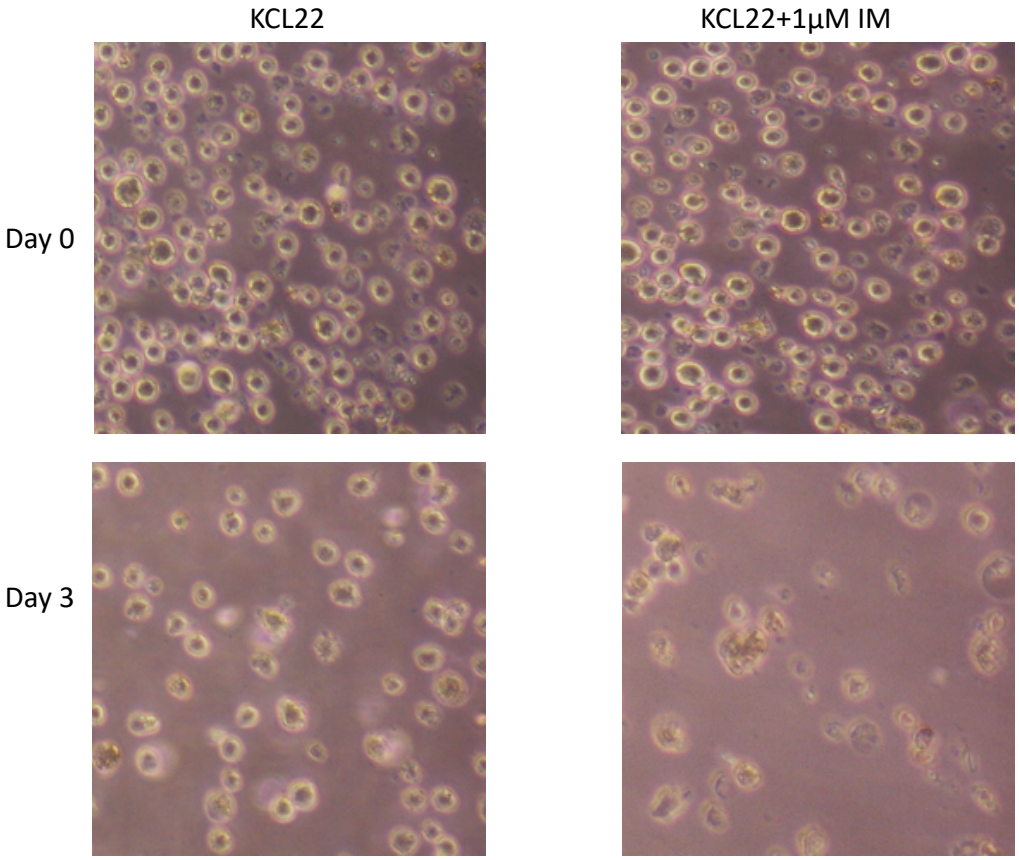
B.


EM2 viability under IM treatment
Total live cell numbers (x10^6)
40
30
20
10
2
1
0
Day 0
Day 3
Day 7
Day 12
Untreated
0.3μM
0.5μM
0.7μM
1μM

C.


EM2 viability under IM treatment
Total live cell numbers (x10^6)
40
30
20
10
2
1
0
2
2
1.6
1
1.6
0.7
1.5
0.3
Day 0
Day 3
Day 7
Day 12
0.3μM
0.7μM

D.


KCL22
KCL22+1μM IM
Day 0
Day 3

E.

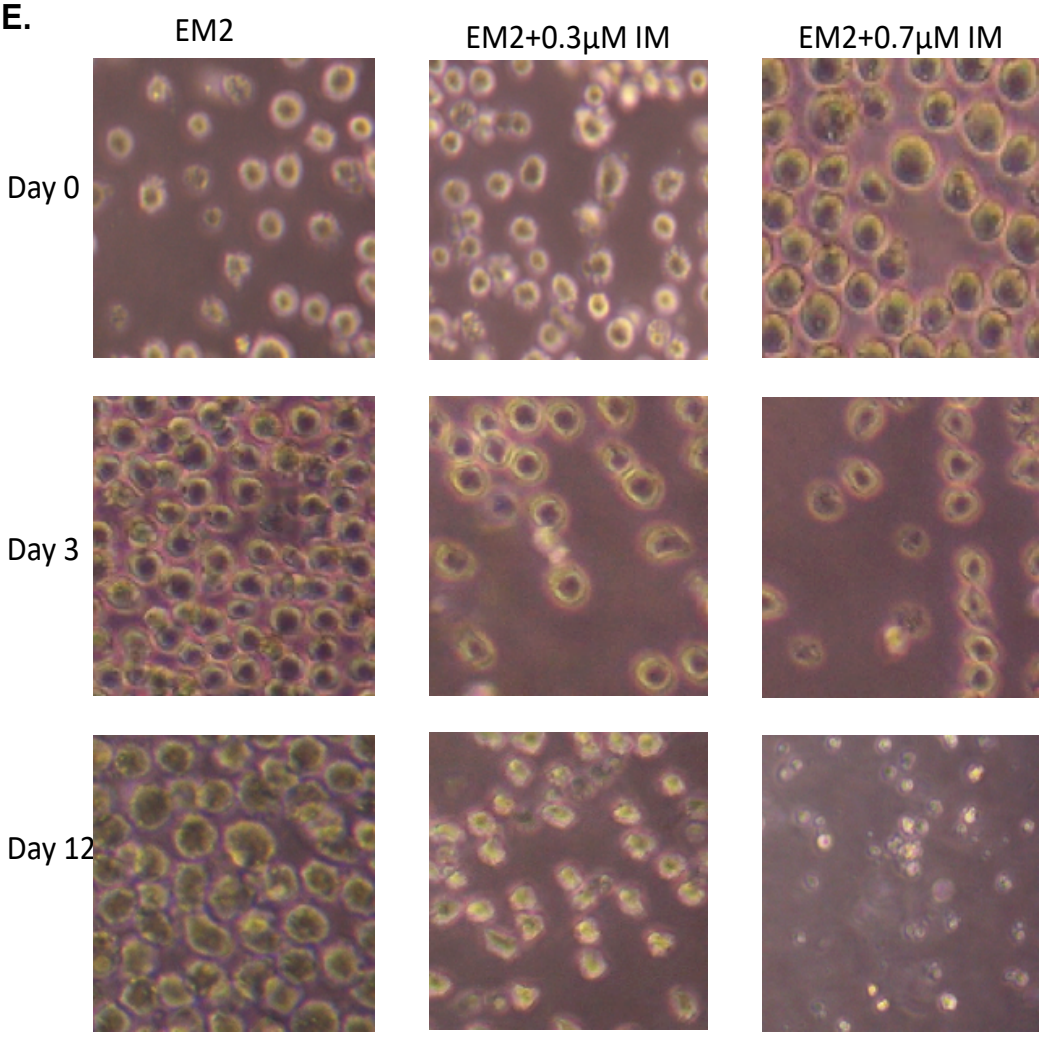

EM2
EM2+0.3μM IM
EM2+0.7μM IM
Day 0
Day 3
Day 12

Figure 4.6: Cell viability analysis of the EM2 line under increasing IM concentrations.

(**A**) Western analysis all proteins carrying a phosphorylated tyrosine residue on EM2 cells treated with titrating IM concentrations. Vinculin was used as a loading control. (**B**) Cell viability counts as determined by trypan blue exclusion cell count for EM2 cells under increasing concentrations of IM on days 0, 3 and 12. Values are in $1x10^6$ cells. Mean and SD (error bars are shown from three independent experiments. (**C**) Direct comparison of the results presented in (B), focusing on 0.3μM and 0.7μM. Mean count values (numbers in $1x10^6$ cells) are shown above each corresponding bar. (**D**) Light microscopy pictures of EM2 cells treated with 0.3μM or 0.7μM, under day 0, day 3 and day 12. Figures show representative data and pictures of three independent experimental assays.

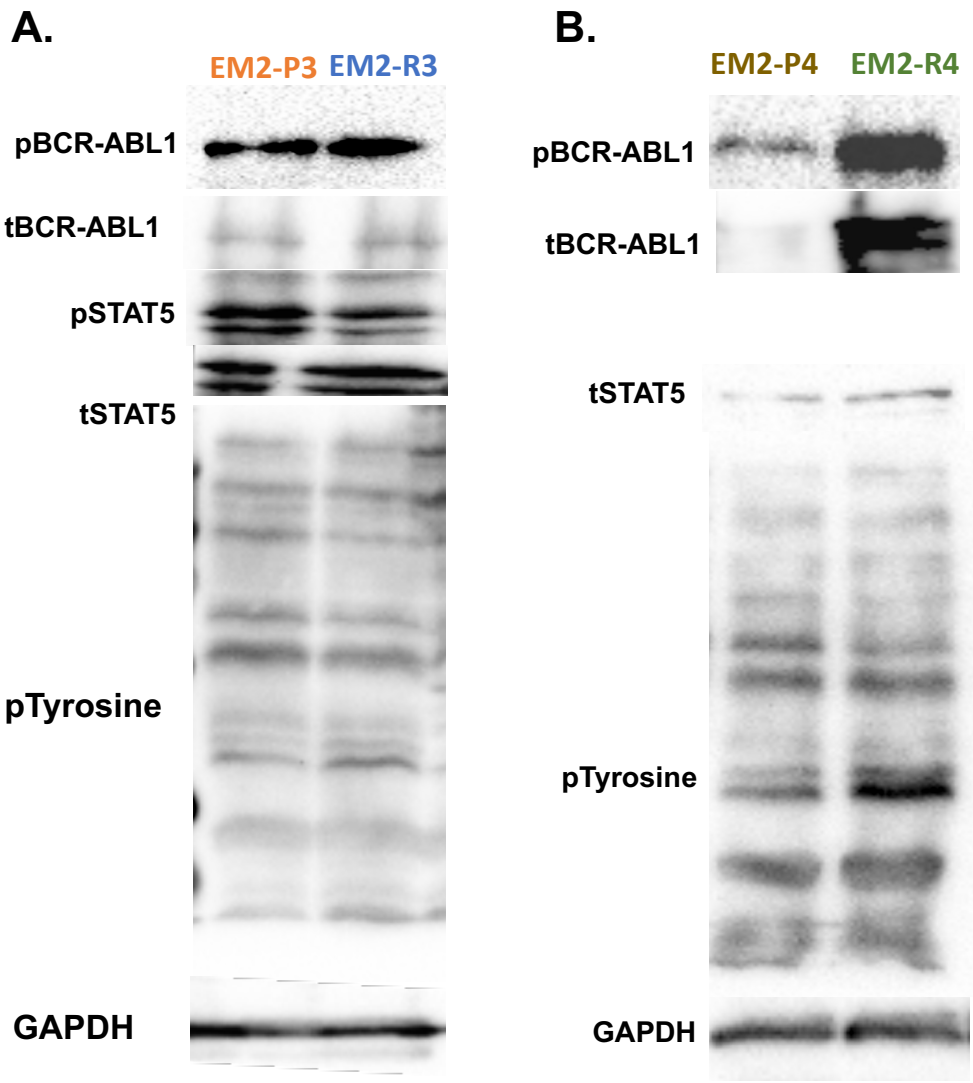
In summary, these observations clarify how the attempt to generate EM2 drug-resistant cells failed by an incorrect assumption that the induction of growth arrest reflects a complete block of BCR-ABL1 kinase activity. This aspect should be considered for other cell lines when attempting to understand their specific drug responses and killing doses. Particularly taking into consideration that the MTS assay is commonly used to determine drug pharmaco-kinetics; despite it working for the KCL22 model.

4.8 Re-generation and characterisation of drug-resistant EM2 clones.

As detailed above, drug resistant lines were re-generated from the 5 single-cell EM2 clones adapting 0.7μM IM as the newly defined 'killing' concentration. The new derivatives were termed R3, R4, R5, R6 and R7 with BCR-ABL1 kinase activity readily assessed within each clone respectively. As control, the respective parental lines were included as well as the protein expression of both total BCR-ABL1 and STAT5.

In comparison to the P3 cells, the R3 drug-resistant clone had similar phosphorylation levels of both BCR-ABL1 and STAT5 as well as all proteins carrying a phosphorylated tyrosine residue (**Figure 4.7a**). These cells clearly had active BCR-ABL1 kinase and as the total amount of BCR-ABL1 protein was not amplified the likely mechanism of drug-resistance is a point mutation within the SH1 domain or defects in the drug efflux pump system.

The analysis of clones P4, P5 and P6 demonstrated that the drug-resistant cells (R4, R5 and R6 respectively) had an active BCR-ABL1 kinase (**Figure 4.7b, 4.7c and 4.7d**). However, the level of total BCR-ABL1 protein was grossly over-amplified in the R4, R5 and R6 cells, respectively, demonstrating that a BCR-ABL1-dependent mechanism of drug resistance was achieved by overexpressing the oncogene.


A.
EM2-P3
EM2-R3
pBCR-ABL1
tBCR-ABL1
pSTAT5
tSTAT5
pTyrosine
GAPDH
B.
EM2-P4
EM2-R4
pBCR-ABL1
tBCR-ABL1
tSTAT5
pTyrosine
GAPDH

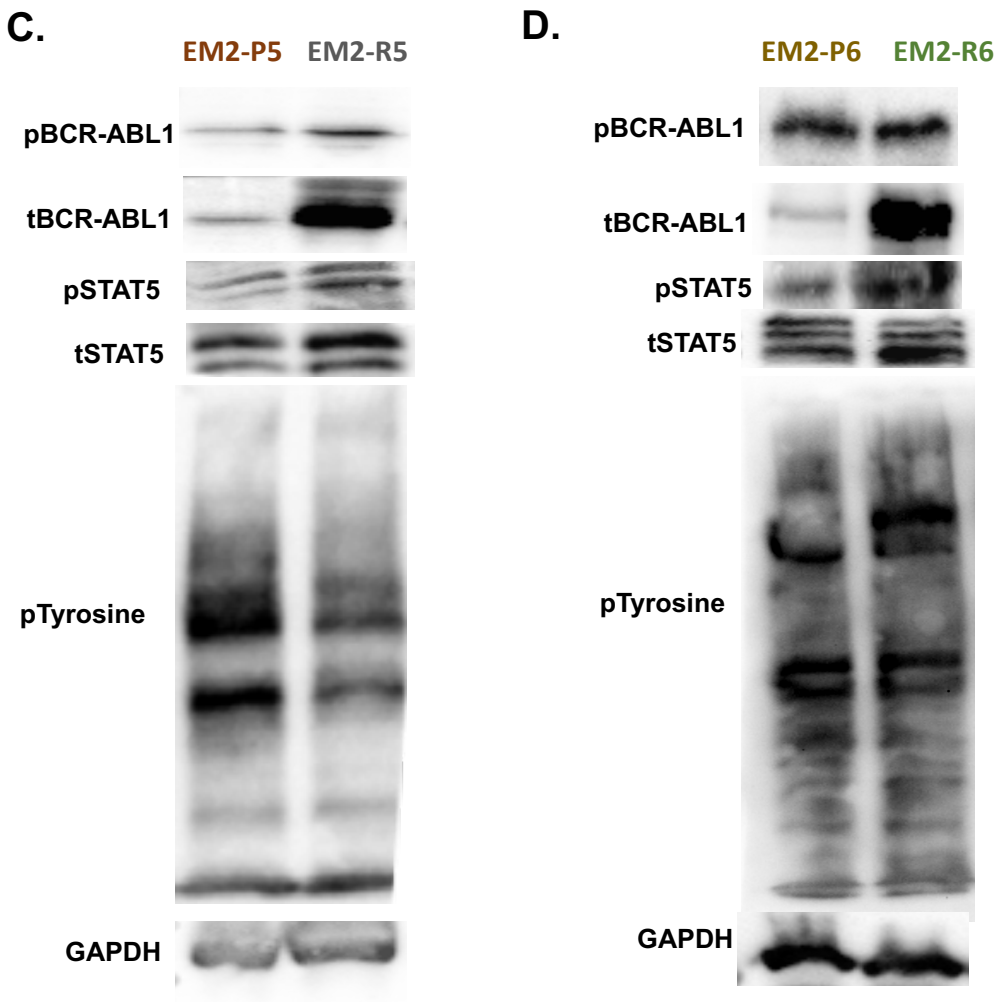


Figure 4.7: Western blot analysis of BCR-ABL1 kinase activity within EM2 drug resistant clones.

Western analysis of indicated proteins within appropriate paired EM2 clones of (**A**) clone 3, (**B**), clone 4, (**C**) clone 5 and (**D**) clone 6. GAPDH was used as a loading control. Figure shows representative data of three independent experimental assays.

As a mechanism of CML drug resistance, the over-amplification of BCR-ABL1 protein has been clinically observed and can occur by either (i) enhancement of protein stability, (ii) duplication of the Philadelphia chromosome (iii) overexpression of the transcript [210, 216, 217]. To determine the molecular understanding of the regulation of BCR-ABL1 within R4, R5 and R6 cells, expression of the BCR-ABL1 (b2-a3 isoform) mRNA was analysed by semi-quantitative PCR. As control, both P7 and R7 cells, which did not demonstrate any amplification of BCR-ABL1 protein, were included. An increase in BCR-ABL1

transcripts is readily observed in the R4, R5 and R6 cells respectively and, as expected, the expression levels was unperturbed in R7 (**Figure 4.8**). These observations demonstrate that the gross increase in BCR-ABL1 protein directly corresponds to the increase in transcript expression which could occur by either duplication of the *BCR-ABL1* loci or increased transcriptional activity mRNA production.

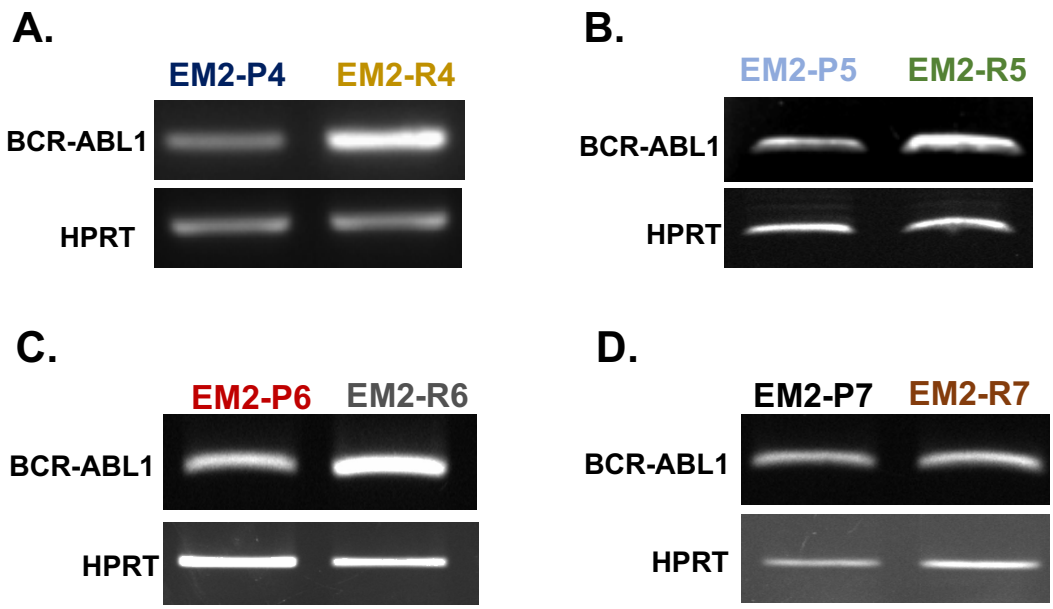


Figure 4.8: Semi-quantitative analysis of BCR-ABL1 expression within EM2 drug resistant clones.

Expression of BCR-ABL1 was examined within drug resistant EM2 clones that were predicted to have overexpression of oncogene; (**A**) clone 4, (**B**) clone 5 and (**C**) clone 6 as well as (**D**) clone 7 which is predicted to be kinase-independent. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

Interestingly, in comparison to P7, the R7 cells had a severely reduced BCR-ABL1 kinase profile, as demonstrated by the pan-phospho Tyrosine pattern, with both pBCR-ABL1 and pSTAT5 nearly depleted (**Figure 4.9**). The levels of total BCR-ABL1 and STAT5 proteins remained constant between the parental and resistant derivative respectively. These observations were reproduced upon several biological repeats and concluded that the mechanism of drug-resistance within R7 is BCR-ABL1 kinase-independent.

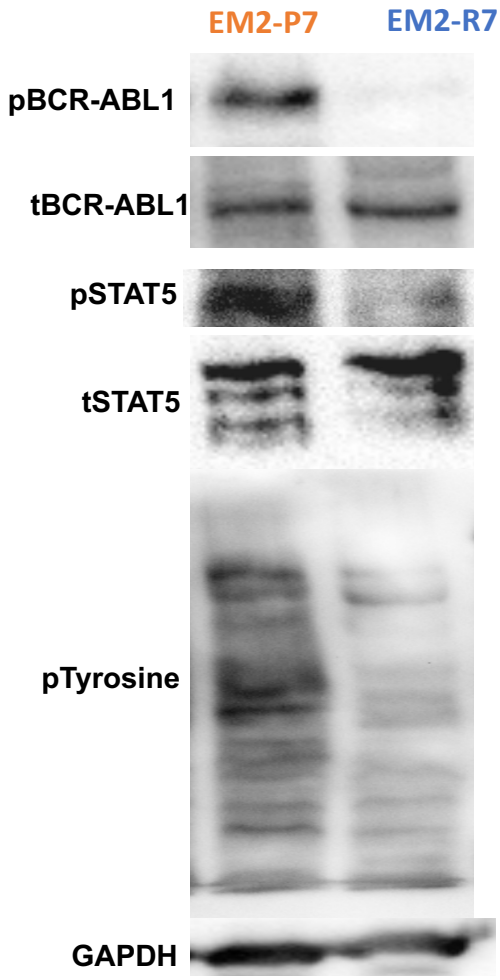


Figure 4.9: BCR-ABL1 kinase activity is diminished in EM2 drug resistant clone 7.

Western blot analysis of the indicated proteins within EM2 clone 7. GAPDH was used as loading control. Figure shows representative data of three independent experimental assays.

4.9 The role of deregulated transcription factors in EM2 kinase-independent drug resistance.

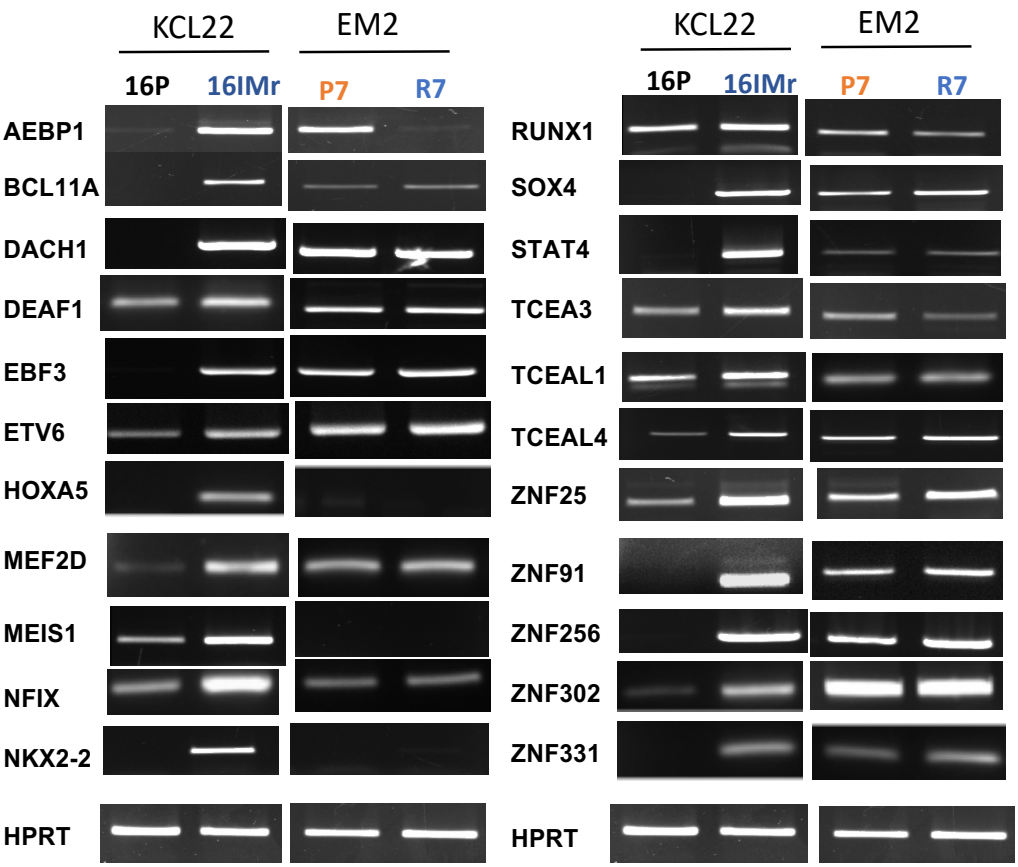
Given the evidence that, like the KCL22 IMr lines, the drug resistant EM2 clone 7 is BCR-ABL1 kinase independent; the pertinent question remains as to how are these cells surviving? As described earlier, the lab has previously identified a clinically relevant network of transcription factors within KCL22 cells proposed to function as new oncogenic drivers in compensating for the loss of BCR-ABL1 activity. Given the establishment of this second CML drug resistant model, it was

of interest to determine whether any of the factors (de)regulated in the KCL22 cells is mirrored within the EM2 counterpart.

Expression of the clinically relevant network of transcription factors was profiled within the P7 and R7 lines respectively by semi-quantitative RT-PCR (**Figure 4.10a and 4.10b**). As control, cDNA samples from the KCL22 model (16P and 16IMr) were included. As expected, each transcription factor was regulated within the KCL22 model, however, it was of great surprise that none of the 29 genes analysed had a similar pattern of expression within the EM2 model. This disparity between the KCL22 and EM2 models was reproducible upon several biological repeats (data not shown).

It is noted that expression of the transcription factor AEBP1 is downregulated upon drug resistance in the EM2 cells; albeit reciprocally induced within the KCL22 model. Nevertheless, to correlate the regulation of AEBP1 within the EM2 cells to the BCR-ABL1 kinase independent mechanism, its expression was investigated in the P6 and R6 cells respectively. As BCR-ABL1 is still active within the R6 cells, it is predicted that there would not be any necessary changes to the transcriptome and therefore these cells act as a presumptive negative control. Specifically, any transcriptome changes within the R6 cells certainly cannot be argued that these arise to compensate for the loss of BCR-ABL1 activity given that the kinase is still present. In support of this notion, the expression of AEBP1 did not change upon drug resistance in R6 and strongly suggests that deregulation of this gene is exclusive kinase-independent resistant cells (**Figure 4.10c**).

A.


KCL22
EM2
16P
16IMr
P7
R7
AEBP1
BCL11A
DACH1
DEAF1
EBF3
ETV6
HOXA5
MEF2D
MEIS1
NFIX
NKX2-2
HPRT
KCL22
EM2
16P
16IMr
P7
R7
RUNX1
SOX4
STAT4
TCEA3
TCEAL1
TCEAL4
ZNF25
ZNF91
ZNF256
ZNF302
ZNF331
HPRT

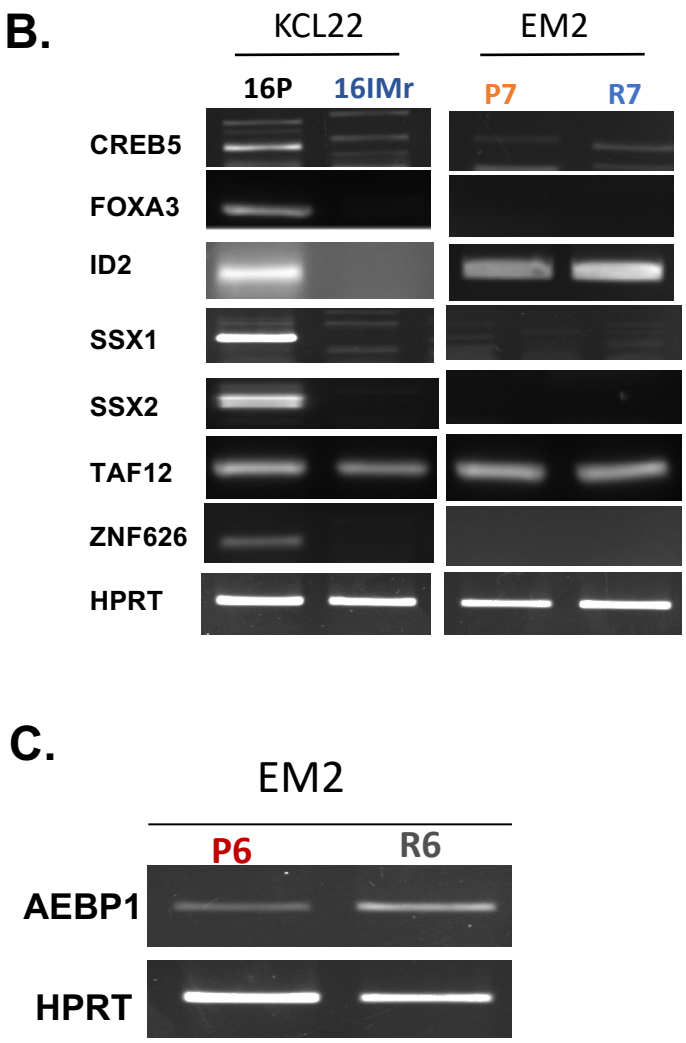


Figure 4.10: Semi-quantitative analysis of transcription factor expression within drug resistant EM2 and KCL22 cells respectively.

Expression profiling of the transcription factors identified within KCL22 cells within the EM2 P7-R7 model: (**A**) upregulated genes and (**B**) downregulated genes upon resistance in KCL22 cells analyzed. (**C**) Expression of the AEBP1 transcription factor within the EM2 P6-R6 model. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

4.10 The cell-of-origin concept and kinase-independent drug resistant CML

The failure to identify any commonly regulated transcription factor between the KCL22 and EM2 models was of concern and casted doubt on the proposed hypothesis for the role of deregulated transcription factors in kinase independent CML.

The KCL22 and EM2 cell lines were originally generated in 1983 and 1980, respectively [218, 219]. Initially, both lines were classified as Philadelphia chromosome-positive myeloid-lineage CML in BC, and they remained to be considered of myeloid origin [220]. Yet, in more recent years the KCL22 cells have been reported to have the potential to differentiate in erythroid cells [221], whilst the EM2 cell line characterised to have granulocytic-like features such as the production of α(2-3)-sialyltransferase [222]. The potential that the cell-of-origin differs between KCL22 and EM2 cells led to a revision of the current observations and proposal of a new hypothesis.

At the start of this study, the originating hypothesis was predicated on the basis that two cell lines of the same lineage (myeloid) and bearing the same oncogene (BCR-ABL1) would deregulate a common network of transcription factors to compensate for the loss of kinase activity. However, the broad definition, and usage, of the term ‘myeloid’ could be misleading and in this regards it should be noted that when these two cells lines were originally generated, early 1980s, the term ‘myeloid’ was often used to reference both GMP and MEP lineages. If KCL22 and EM2 cells do stem from different progenitors, erythroid and GMP respectively, it is highly likely that a “lineage-specific set of transcriptions factors” are utilized by each cell type for drug resistance respectively.

The revised hypothesis is based on the “Cell-of-Origin” concept whereby a given oncogene-driven disease can clinically present itself in different, and distinct, pathologies dependent on the origin of the cell type from which the tumour-initiating cell originate (**Figures 4.11**) [223]. A clear example of this “one oncogene – different diseases” is the comparison of CML and acute lymphocytic leukaemia (ALL) whereby both diseases are Philadelphia chromosome-positive and are dependent on the BCR-ABL1 oncogene but the pathology manifests within either GMP or lymphoid lineages depending on which specific progenitor is transformed; a.k.a “Cell-of-Origin”. Given the striking genetic differences between GMP and lymphoid progenitors respectively; it is easy to comprehend

how, and why, a different set of transcription factors would be deregulated in either leukemic cells in establishing drug-resistance in a BCR-ABL1 kinase independent manner. Analogous to this example, the data presented herein suggests a similar differential usage of the transcription factor network between erythroid (KCL22) and GMP-like (EM2) progenitors.

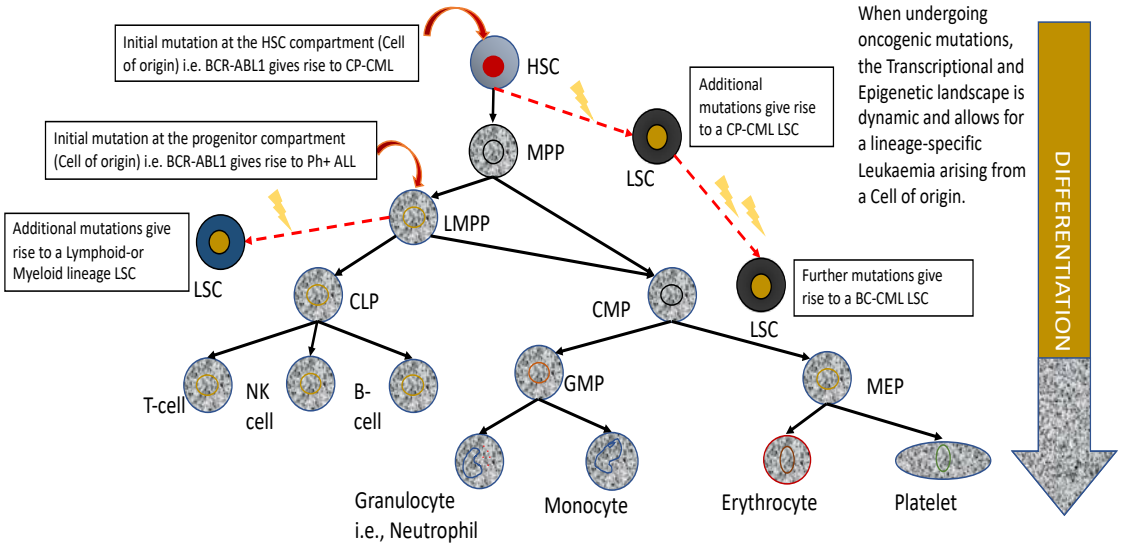


Figure 4.11: Cell of origin model in malignant haematopoiesis.

With reference to malignant haematopoiesis, the 'Cell of Origin' is the clone which suffers from the initial mutation and in collaboration with additional mutations the clone is transformed to a LSC. In the figure, grey-stone texture represents cells from the healthy compartment. Red-dotted arrow connects from the Cell of Origin to its respective LSC in differentiation. Thunder-bolts indicate additional mutations that would generate a LSC.

To determine the lineage identity of the KCL22 and EM2 cells, the parental populations were transcriptionally profiled, by semi-quantitative RT-PCR, using a gene-set comprising of well-known transcription factors (GATA1, NFE2, PU.1, Gfi1 and CEBPα) as well as cytokine receptors (EPO-R, GM-CSFR, G-CSFR and M-CSFR) that are preferentially expressed in a lineage-specific manner within erythroid and GMP networks respectively. By this comparison, it is clear that the KCL22 cells are erythroid-biased as determined by the relative higher transcript expression of every erythroid-specific gene (**Figure 4.12**). Notably, the expression of a number of the erythroid-genes was not readily detected in EM2 cells including GATA1, PKLR, HEMGN and ALAS2. Displaying a reciprocal pattern of expression, the EM2 cells can be readily delineated as a GMP-biased

progenitor noting the strong expression of the master myeloid transcription factors PU.1, Gfi1 and CEBPα (which are near absent in KCL22) as well as all 3 cytokine receptors (all undetected in KCL22).

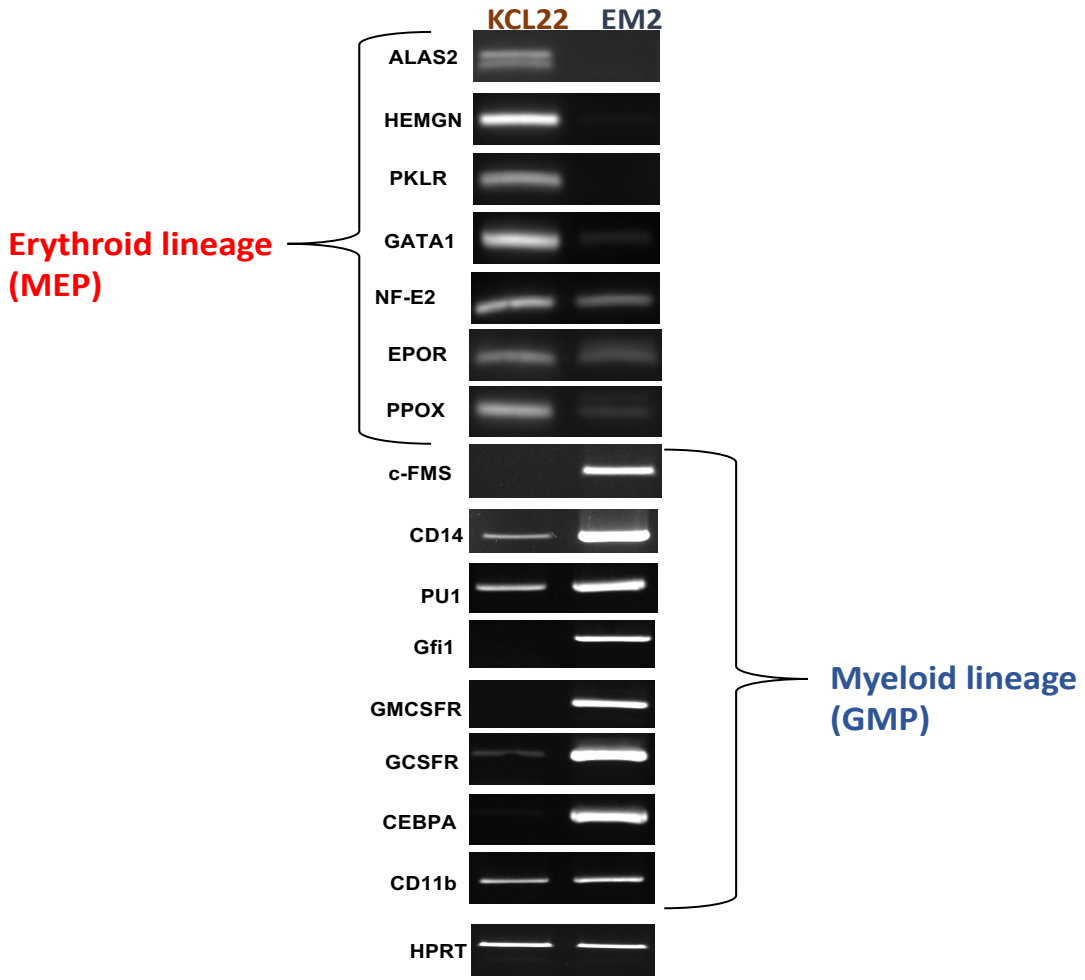


Figure 4.12: Lineage-specific transcript expression analysis of KCL22 and EM2 cells.

Semi-quantitative expression of erythroid and GMP specific transcripts within KCL22 and EM2 cells respectively. HPRT was used as loading control. Figure shows representative data of three independent experimental assays.

Transcript profiling segregated these two 'myeloid' cells along distinct lineages of the haematopoietic map and provides strong support in accounting for why the KCL22 and EM2 cells did not share a common set of deregulated transcription factors upon drug resistance.

4.11 The cell-of-origin model: comparison of erythroid CML.

Extending from the 'Cell-of-Origin' model it is predicted that if disease pathology is lineage-specific, then the molecular mechanism(s) exploited to become drug resistant (BCR-ABL1 kinase independent) should also be dependent on the identity of the transformed progenitor. If this holds true, then a common set of deregulated transcription factors should be identified within another erythroid- and/or GMP-biased drug-resistant line respectively. Specifically, in comparison of "like-for-like" progenitors, it is anticipated that another erythroid-biased CML line would share transcription factors with the drug resistant KCL22 cells.

The K562 cells are a widely-studied model of CML pathology and have the capacity to undergo chemical-induced erythroid development [224, 225]. Given the differentiation potential of K562 it makes them a suitable, and desirable, model to compare with KCL22 cells. Notably, work by others have generated drug-resistant BCR-ABL1 kinase independent K562 cells [189]. Although both K562 and KCL22 cells are closely-related with respect to progenitor origin, it is important to note the technical differences in the methodology in generating each model system respectively. First, the drug-resistant K562 cells are generated using the heterogenous population whereas single cells clones were used for KCL22. Second, the drug to which to deactivate BCR-ABL1 activity is different with KCL22 model using IM and K562 are resistant to the second-generation of kinase inhibitors Dasatinib.

In collaboration with the Dr. Auberger's lab the K562 cells were obtained, termed K562-SD for the parental (Das-sensitive) line and K562-DR for the Das-resistant derivates. Several quality controls were executed prior to the use of these cells. First, the lack of any significant BCR-ABL1 kinase activity within the K562-DR cells was successfully confirmed by western analysis of all phosphorylated tyrosine-residue containing proteins (**Figure 4.13**).

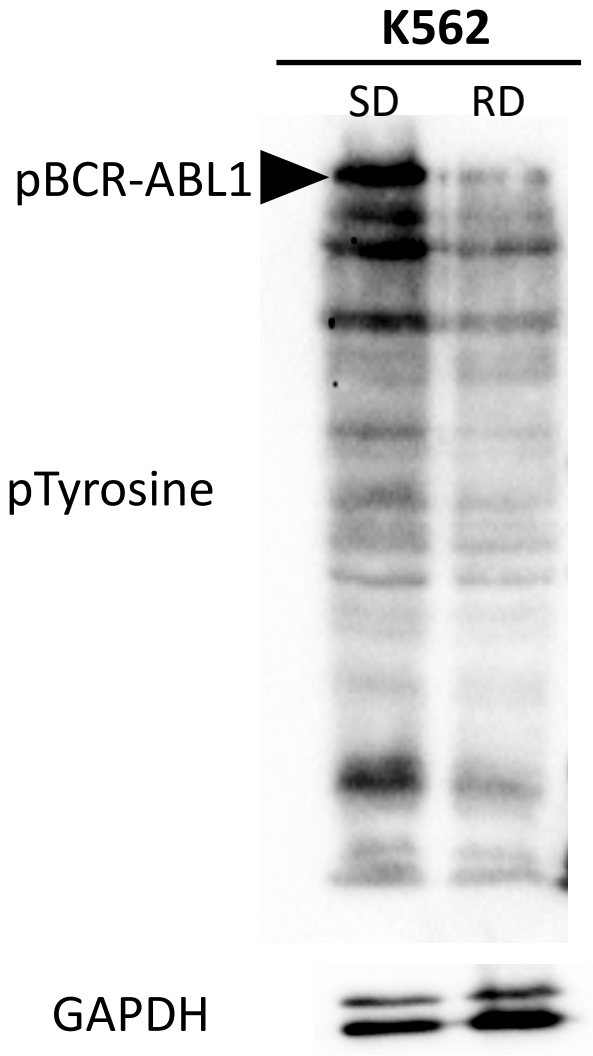


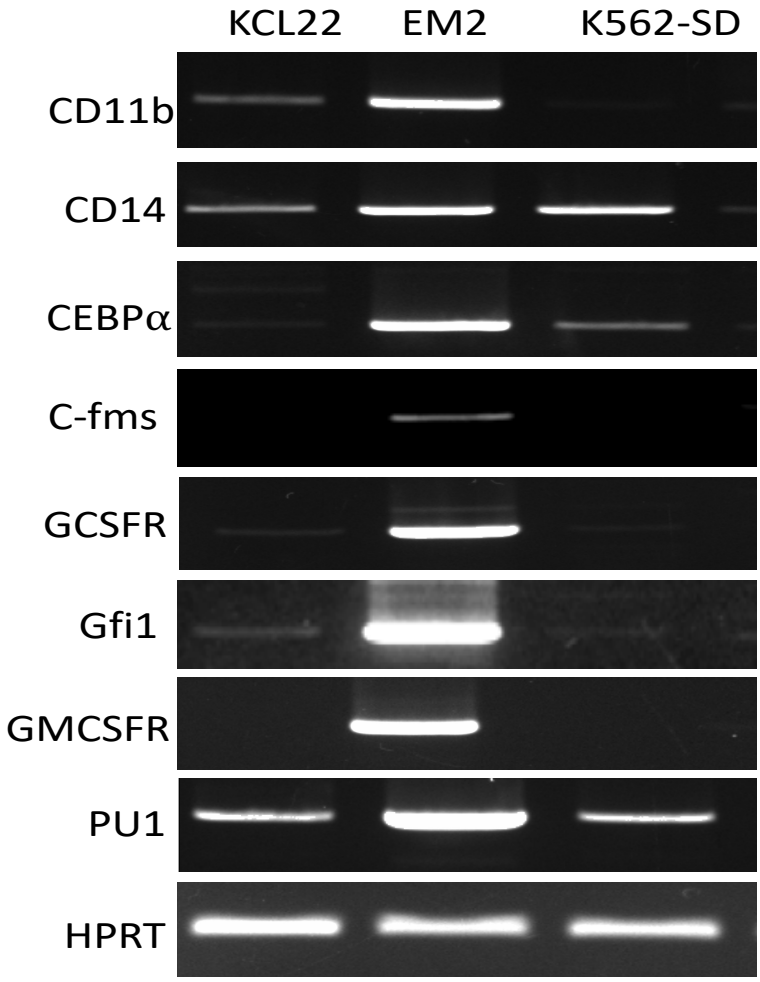
Figure 4.13: Western analysis of phosphorylated tyrosine proteins within the K562 drug resistant model.

Detection of all proteins containing a phosphorylated tyrosine residue within the K562-SD and K562-RD cells respectively. GAPDH was used as loading control. Figure shows representative data of two independent experimental assays.

Secondly, the erythroid-lineage identity of the K562-SD cells was validated by expression-profiling using the previously described gene-sets for GMP-specific and erythroid-specific lineages respectively (**Figure 4.14**). As control, both EM2 and KCL22 cDNA samples were included. Here, it is clear that the K562 cells have a very similar expression profile to the KCL22 cells namely that they strongly express all the erythroid genes and are absent for the majority of the GMP-specific genes.

A.

- Myeloid (Granulocyte/Monocyte) markers



B.

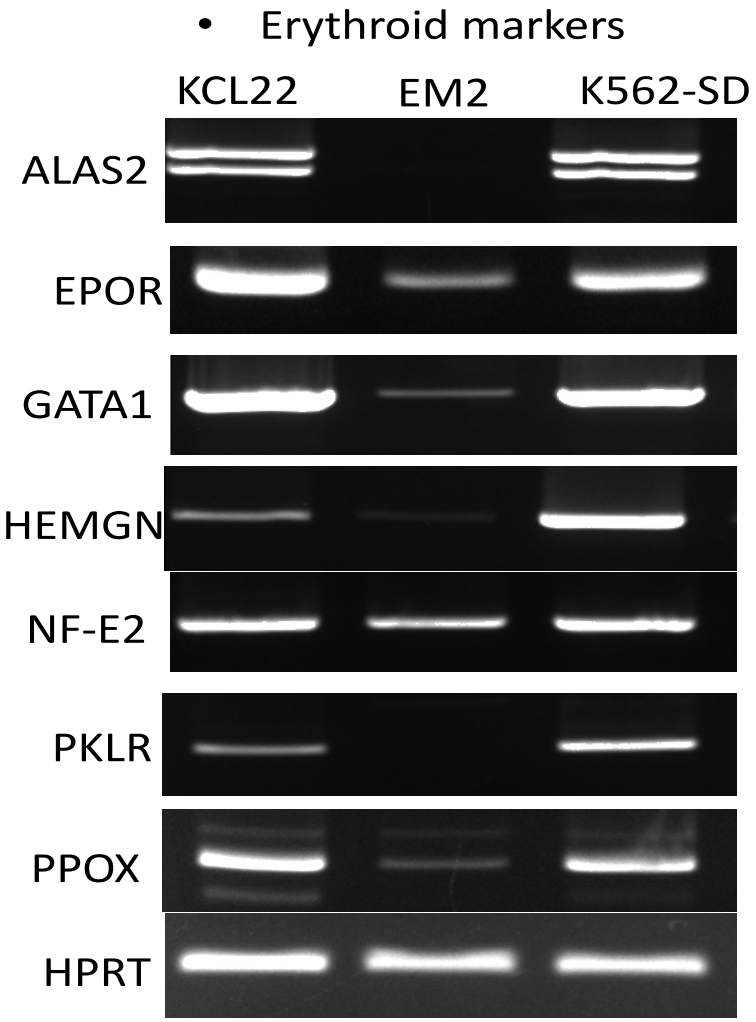


Figure 4.14: Transcript profiling of lineage-specific genes within several CML lines.

Semi-quantitative expression of erythroid and GMP-specific genes within KCL22, K562 and EM2 cells respectively. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

4.12 Comparison of deregulated transcription factors within erythroid drug-resistant CML

With all quality controls validated within the K562 model, the question of whether closely-related CML lines would use a common set of deregulated transcription factors to become kinase independent drug-resistant could finally be addressed. The expression of the drug-resistant network of transcriptions factors identified within the KCL22 model was screened within the K562-SD and K562-DR cells respectively by semi-quantitative RT-PCR. As control, cDNA templates from the KCL22 cells were included (16P and 16IMr).

Of the 30 transcription factors, it was of great interest, and excitement, to identify that 8 genes shared the same pattern of deregulated expression in both KCL22 and K562 cells upon drug-resistance. Specifically, 4 genes were upregulated upon drug-resistance being AEBP1, MEF2D, SOX4 and TCEAL1 (**Figure 4.15a**) with the remaining repressed; FOXA3, SSX1, SSX2 and ZNF626 (**Figure 4.15b**). Expression of the remaining 22 transcription factors had demonstrated that 3 other factors were also deregulated in K562 cells albeit in the opposite pattern of expression than that seen in KCL22 (DACH1, ZNF331 and BCL11A); the remaining 19 genes failed to show any change in expression within K562 cells upon drug-resistance; although, as expected, were deregulated within the KCL22 cells (data not shown). These results were biological reproducible upon several repeats (data not shown).

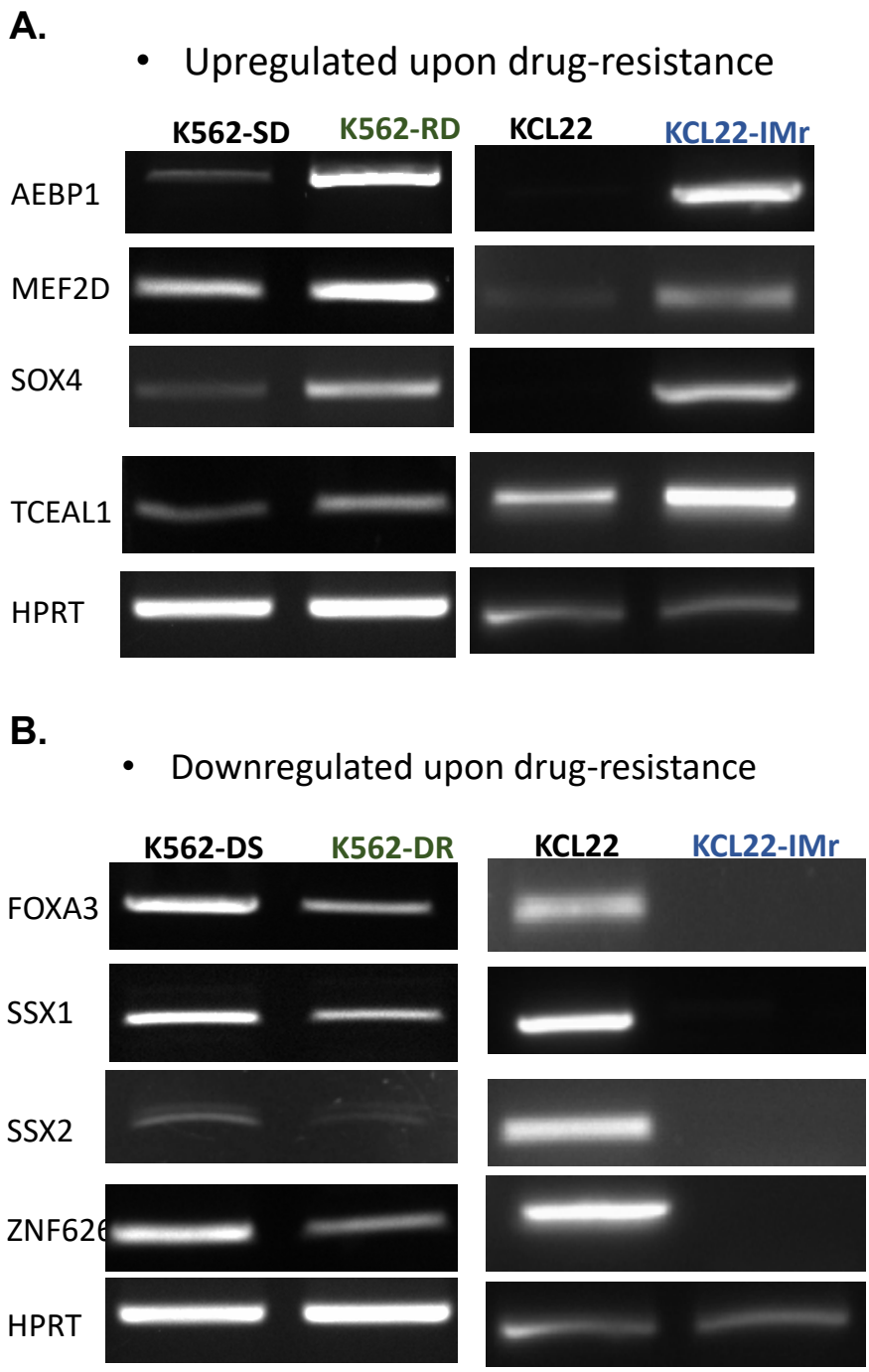


Figure 4.15: Common deregulated transcription factors within erythroid drug resistant CML.

Semi-quantitative analysis of commonly deregulated transcription factors that are (**A**) upregulated or (**B**) downregulated upon drug resistance in both KCL22 cells and K562 cells. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

In summary, these observations strongly support the notion that the lineage-identity of the progenitor transformed by BCR-ABL1 will dictate the transcription factor network utilized in order to compensate for the loss of kinase activity upon drug resistance. Moreover, such profiling of closely-related CML models now identify a proposed a core-network of transcription factors that are of potential significance for diagnostic and therapeutic purposes in drug-resistant CML.

4.13 Are transcription factors deregulated within the in GMP EM2 single-cell line model?

Having gained a better understanding of the erythroid CML lines, it was now of interest to further characterize the acquired changes to the transcriptome of the EM2 cells upon kinase-independent drug resistance. In brief, total RNA was isolated from biological triplicates of P7 and R7 cells, noting that the R7 cells were treated with 0.7μM IM 24hr prior to collection. The microarray was performed by Cambridge Genomic Services, University of Cambridge, using the GeneTitan (Affymetrix) platform. Basic bioinformatic analysis including sample QC and normalisation was performed by the genomic service.

Differentially regulated genes were defined, and identified, having a >2 fold-change in relative expression and consisted of a total of 420 genes with 274 induced and 146 repressed upon drug-resistance respectively. Tables representing the expression profile of the top 20 upregulated (**Table 4.1a**) and downregulated (**Table 4.1b**) genes are presented respectively.

Gene name	Gene definition	Fold change
CHAC1	ChaC, cation transport regulator homolog 1	19.86
SLC7A11	Solute carrier family 7 member 11	12.41
GAL	Galanin	12.04
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	10.76
S100A8	S100 calcium binding protein A8	6.69
SLC6A9	Solute carrier family 6 member 9	6.62
CARD9	Caspase recruitment domain family, member 9	6.19
SESN2	Sestrin 2	6.12
PPIC	Peptidylprolyl isomerase C (cyclophilin C)	5.15
DDIT4	DNA-damage-inducible transcript 4	4.92
PPM1B	Protein Phosphatase, Mg2+/Mn2+ Dependent 1B	4.46
CST3	Peptidylprolyl isomerase C (cyclophilin C)	4.42
MLLT4	myeloid/lymphoid or mixed-lineage leukemia; translocated to, 4	4.21
PCK	Phosphoenolpyruvate Carboxykinase 1	4.03
ASNS	Asparagine synthetase	4.00
PSAT1	Phosphoserine aminotransferase 1	3.96
GPR52	G Protein-Coupled Receptor 52	3.94
TMEM14EP	Transmembrane Protein 14E, Pseudogene	3.88
ABCA1	ATP Binding Cassette Subfamily A Member 1	3.87
CDA	Kinesin Family Member 23	3.74

Gene name	Gene definition	Fold change
CPA3	Carboxypeptidase A3	0.10
CHRNA6	Cholinergic Receptor Nicotinic Alpha 6 Subunit	0.11
FZD6	Frizzled Class Receptor 6	0.16
SERPINB10	Serpin Family B Member 10	0.18
AEBP1	AE Binding Protein 1	0.19
MAN1A1	Mannosidase Alpha Class 1A Member 1	0.21
SNAPC4	Small Nuclear RNA Activating Complex Polypeptide	0.26
IRAK2	Interleukin 1 Receptor Associated Kinase 2	0.27
HSPA1A	Heat Shock Protein Family A (Hsp70) Member 1A	0.28
PITX1	Paired Like Homeodomain 1	0.28
COL9A3	Collagen Type IX Alpha 3 Chain	0.29
PSRC1	Proline And Serine Rich Coiled-Coil 1	0.30
C11orf45	Chromosome 11 Open Reading Frame 45	0.30
EFHC2	EF-Hand Domain Containing 2	0.31
SLC7A8	Solute Carrier Family 7 Member 8	0.32
RUNX2	RUNX Family Transcription Factor 2	0.32
SSTR2	Somatostatin Receptor 2	0.32
GJB2	Gap Junction Protein Beta 2	0.32
MTMR7	Myotubularin Related Protein 7	0.33
GATSL2	Cytosolic Arginine Sensor For MTORC1 Subunit 2	0.33

Table 4. 1: Table of 20-top upregulated and downregulated genes within kinase-independent drug-resistance EM2 cells.

Table listing the top 20 (**A**) upregulated and (**B**) downregulated genes in EM2 cells (clone 7) upon drug resistance.

As quality control of this microarray data, the expression profile of randomly selected genes that were predicted to be (i) highly induced (>10-fold), (ii) low-level induced (approx. 3-fold) or (iii) repressed (approx. 3-fold) upon drug resistance was validated by semi-quantitative RT-PCR. Notably, this gene-profiling was performed on cDNA template that was independent from the samples used in the microarray. Of the 8-genes examined, all presented a pattern of expression (either induced or repressed) in correlation with the microarray prediction (**Figure 4.16**). Although a semi-quantitative PCR analysis was performed, the results nevertheless demonstrate a strong correlation, and confidence, between the pattern of gene expression identified from the microarray and the biological replicate.

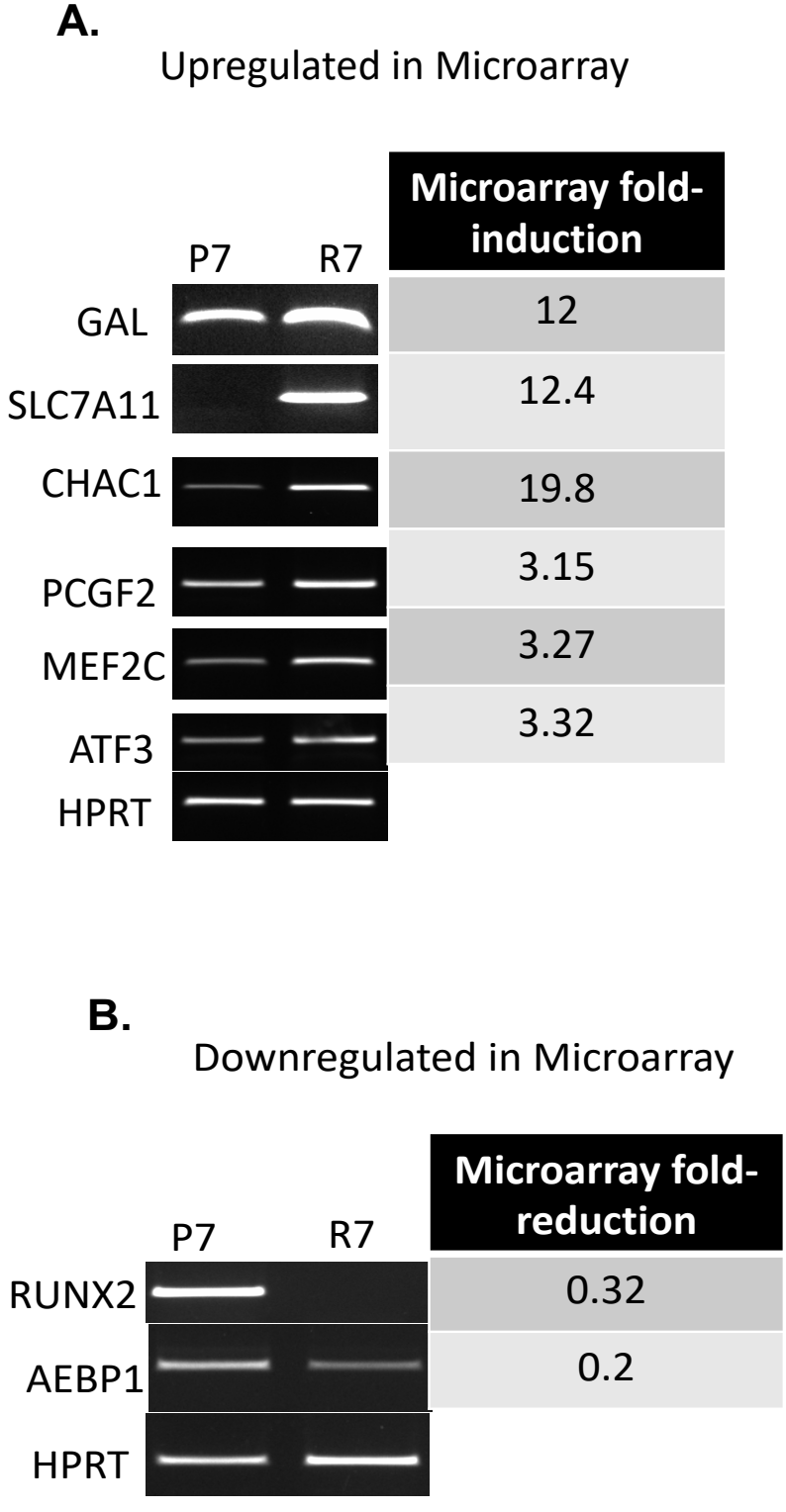


Figure 4.16: Validation of the EM2 microarray by semi-quantitative PCR profiling.

Semiquantitative expression analysis of (**A**) upregulated and (**B**) downregulated genes. The microarray predicted fold induction is presented to the right. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

In collaboration with Dr. Dapeng Wang (Bioinformatician, University of Leeds) a Gene-Set Enrichment Analysis (GSEA), in combination with the Molecular Signature Data Base, was performed in order to identify the most enriched biological pathways regulated in EM2 cells upon drug resistance. The GSEA analysis (nominal p-value <0.05 and FDR q-value <0.05) identified 68 enriched pathways which, of interest, included signatures for ATF4, FOXO3, IGF, FOXA2, HOXA1 and CEBPα gene-networks (**Table 4.2**).

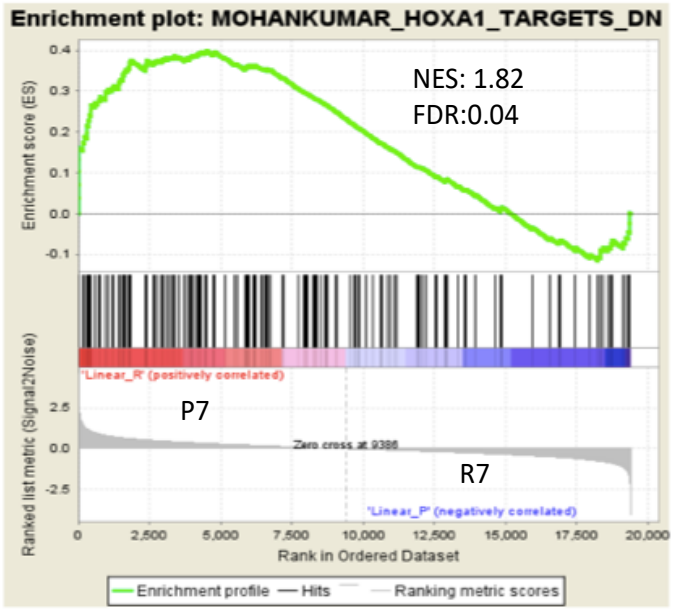
As examples, two enrichment score graphs of the GSEA data analysis are presented (**Figure 4.17 a and b**).

EM2-R7
PATHWAY NAME
ACTIVATION_OF_GENES_BY_ATF4
AMINO_ACID_DEPRIVATION
AMINO_ACID_SYNTHESIS_AND_INTERCONVERSION_TRANSAMINATION
AMINOACYL_TRNA_BIOSYNTHESIS
AMYLOIDS
AR_TARGETS_UP
B_CLL_WITH_6Q21_DELETION
BREAST_CANCER_RELAPSE_IN_PLEURA_DN
CANCER_MICROENVIRONMENT_DN
CD24_TARGETS_DN
CHROMOSOME_MAINTENANCE
CHRONIC_LYMPHOCYTIC_LEUKEMIA_UP
CYTOSOLIC_TRNA_AMINOACYLATION
DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE
E2A_TARGETS_UP
E2F3_TARGETS_DN
EMBRYONIC_CARCINOMA_VS_SEMINOMA_UP
EPITHELIAL_TO_MESENCHYMAL_TRANSITION_UP
FLUOROURACIL_RESISTANCE_DN
FOXA2_TARGETS_UP
FOXO3_TARGETS_DN
G2M_ARREST_BY_2METHOXYESTRADIOL_DN
GEMCITABINE_RESISTANCE_DN
GENOTOXIC_DAMAGE_24HR
HGF_SIGNALING_NOT_VIA_AKT1_48HR_UP
HOXA1_TARGETS_DN
HSC_MARKERS
INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION
INTEGRIN_CS_PATHWAY
LEUCINE_DEPRIVATION_UP
LIVER_CANCER_WITH_H3K27ME3_DN
LIVER_CANCER_WITH_H3K9ME3_DN
MEIOSIS
MEIOTIC_RECOMBINATION
MEIOTIC_SYNAPSIS
MITOTIC_ARREST_BY_DOCETAXEL_2_UP
MLL_AF4_FUSION_TARGETS_C_DN
MULTIPLE_MYELOMA_CD1_UP
MULTIPLE_MYELOMA_CD1_VS_CD2_UP
MYELODYSPLASTIC_SYNDROM_LOW_RISK_UP
MYELOID_CEBPA_NETWORK
OPN_TARGETS_CLUSTER_7
PACKAGING_OF_TELOMERE_ENDS
PEPTIDE_CHAIN_ELONGATION
PERK_REGULATED_GENE_EXPRESSION
RB1_TARGETS_LOW_SERUM
RESISTANCE_TO_BCL2_INHIBITOR_DN
RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_AND_HEAT_PRODUCTION
RESPONSE_TO_ADAPHOSTIN_UP
RESPONSE_TO_ARSENIC_TRIOXIDE
RESPONSE_TO_FENRETINIDE_UP
RESPONSE_TO_HD_MTX_UP
RESPONSE_TO_METHOXYESTRADIOL_UP
RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_RED_UP
RESPONSE_TO_SALIRASIB_UP
RESPONSE_TO_TRABECTEDIN_UP
RESPONSE_TO_UV_C7
RIBOSOME
RNA_POL_I_PROMOTER_OPENING
RNA_POL_I_RNA_POL_III_AND_MITOCHONDRIAL_TRANSCRIPTION
RNA_POL_I_TRANSCRIPTION
SILENCED_BY_METHYLATION_DN
SYSTEMIC_LUPUS_ERYTHEMATOSUS
TARGETS_OF_IGF1_AND_IGF2_UP
TELOMERE_MAINTENANCE
TRANSCRIPTION
TRNA_AMINOACYLATION
UV_RESPONSE_CLUSTER_G2

Table 4.2: GSEA enrichment analysis for the EM2-R7 GSEA analysis.

Gene-set enrichment analysis of the enriched pathways within drug resistant EM2 cells. Nominal p-value and FDR q-value <0.05.

A.



B.

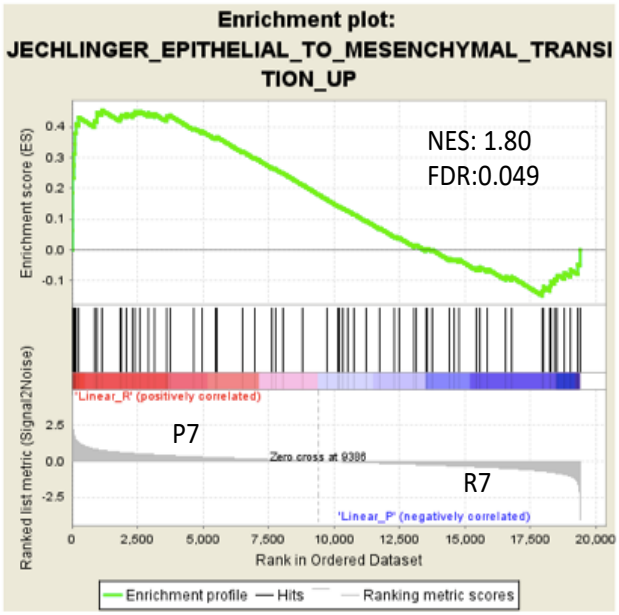


Figure 4.17: Example of two Enrichment Plots (A) and (B) for the EM2 R7 compared to the P7.

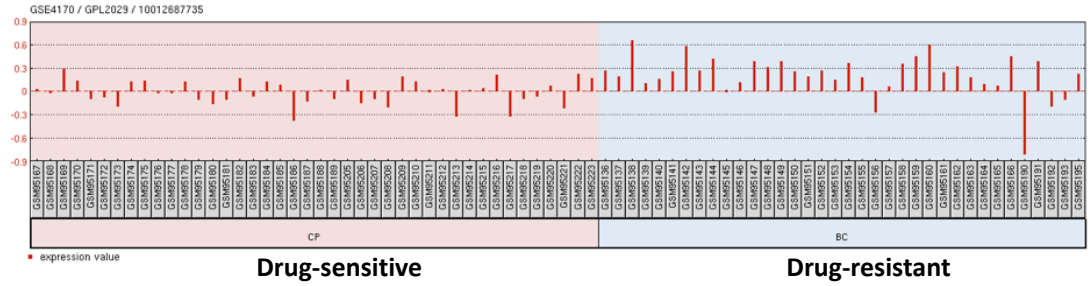
Given the focus of this study, it was of interest to note that of all the regulated genes upon drug-resistance (>2-fold), a total of 37 transcription factors were manually identified with 18 induced and 19 repressed respectively (data not shown). This list was blindly reviewed by another member of the lab and the same set of transcription factors were identified; thus, limiting the chances that any important factors were over-looked.

As previously performed for the KCL22 dataset, this list of 37 transcription factors was filtered based on the identification of a similar pattern of expression within a clinical cohort dataset comprising of drug-sensitive and -resistant patients respectively [69]. This process narrowed the list down to 7 'clinically-relevant' transcription factors with 5 induced (ELF1, ATF3, MEF2C, BCL7A and RFX3 and two repressed (PITX1 and FOXL2). The pattern of expression of each factor within the clinical cohort is presented (**Figure 4.18**) and summarized (**Table 4.3**).

A.

Upregulated Transcription Factors upon Drug-Resistance

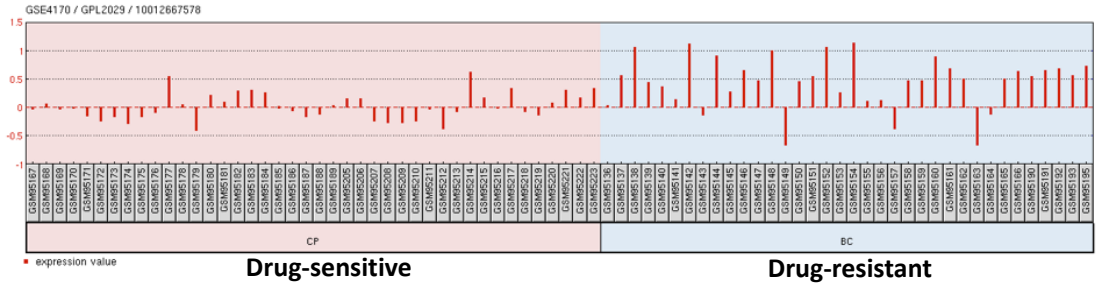
- ELF1



Drug-sensitive **Drug-resistant**

B.

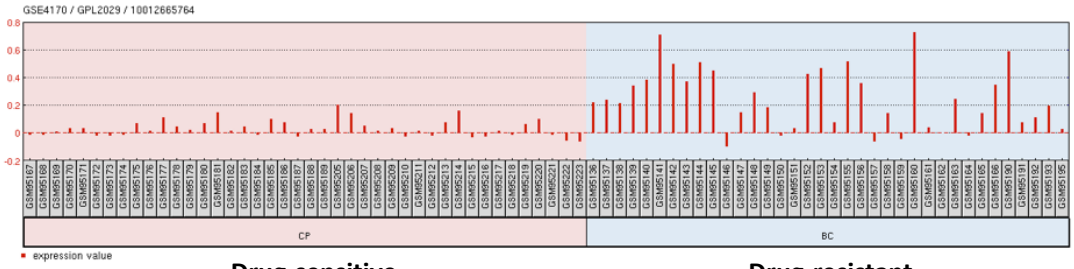
- ATF3



Drug-sensitive **Drug-resistant**

C.

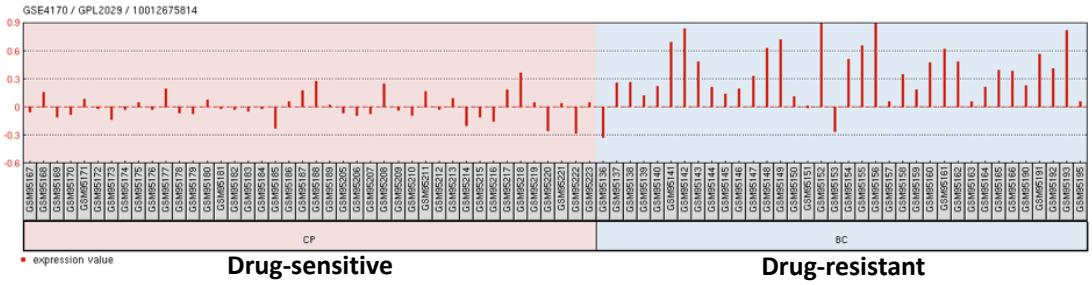
- MEF2C



Drug-sensitive **Drug-resistant**

D.

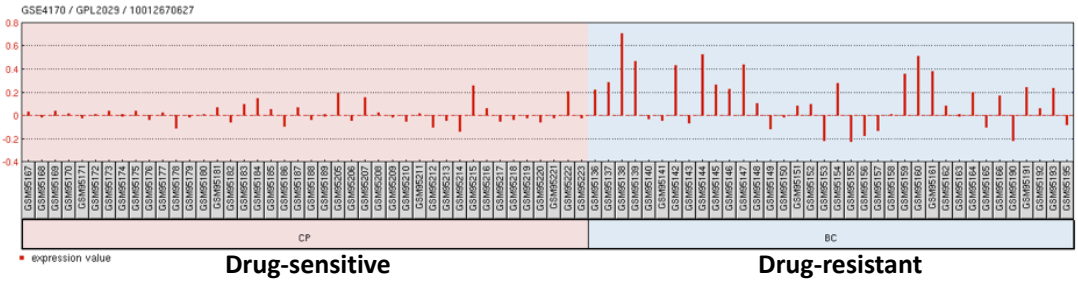
- BCL7A



Drug-sensitive **Drug-resistant**

E.

- RFX3

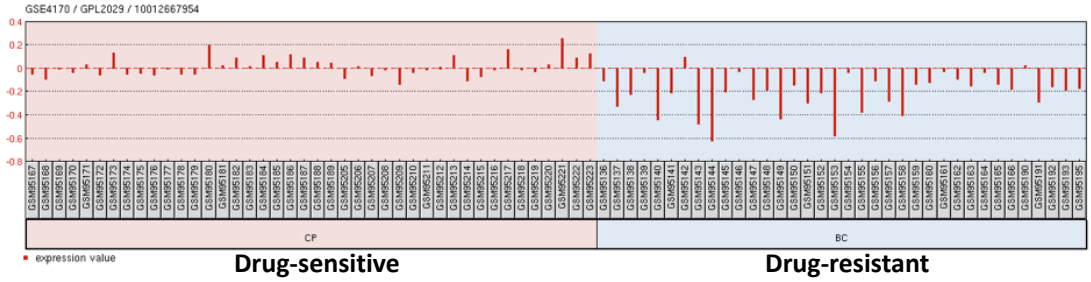


Drug-sensitive **Drug-resistant**

Downregulated Transcription Factors upon Drug-Resistance

F.

- PITX1



G.

- FOXL2

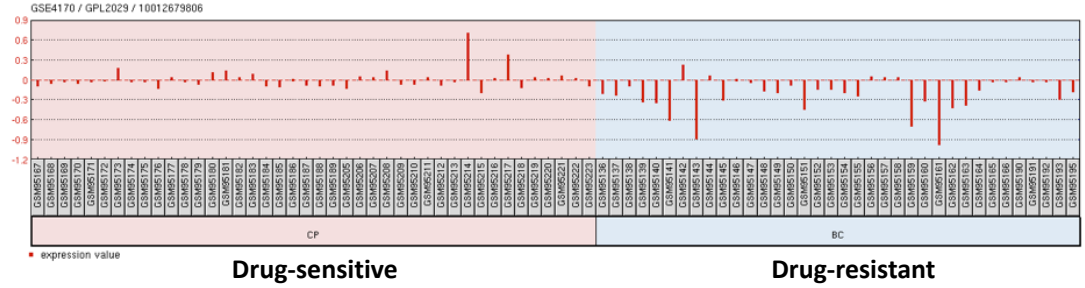


Figure 4.18: Transcription factor expression from microarray database of drug-sensitise (chronic phase) and drug resistant (blast crisis) CML patients.

Graphical representation of (**A**) ELF1, (**B**) ATF3, (**C**) MEF2C, (**D**) BCL7A, (**E**) RFX3, (**F**) PITX1 and (**G**) FOXL2 transcription factors from publicly available database of drug sensitive and resistant patients respectively. Patient numbers are indicated below. Red group are CP patients indicates chronic phase, whilst the blue group, BC stands for Blast Crisis.

TF	Microarray fold change	Clinical dataset adjusted p-value
ELF1	3.59	4.32E-05
ATF3	3.32	1.57E-06
MEF2C	3.28	1.10E-07
BCL7A	2.27	3.63E-09
RFX3	2.18	4.74E-03
PITX1	0.28	1.94E-10
FOXL2	0.45	2.74E-05

Table 4.3: Clinically relevant transcription factors deregulated in EM2 cells upon drug resistance.

Summary of the clinically relevant transcription factors that are deregulated within EM2, clone 7, upon drug resistance. Represented are the fold change in gene expression as identified from the microarray, this was compared to the clinical dataset adjusted p-value, which corresponds to the gene expression profile between CML CP (n=42) drug sensitive patients and BC (n=36) drug resistant patients for the dataset GSE4170 found in the GEO2R tool.

Future work will focus on the functional requirement of these factors in maintenance of the drug-resistant leukaemic phenotype.

4.14 Molecular mechanism of CML drug-resistance: predetermined or stochastic?

A key feature identified from the work presented herein is that various mechanisms were used by different EM2 single cell clones to become drug resistant. Indeed, of the 5 clones generated, 3 had gross over-amplification of BCR-ABL1 protein (R4, R5 and R6), one presumed to have a SH1-domain point mutation (R3) and another kinase independent (R7). Such diversity of mechanisms for CML drug resistance is well-recognized, in both cell line models as well as clinically, and has been reported by others [226].

The molecular basis for the aetiology of drug-resistance is not well understood. How does a clone decide which mechanism to employ in order to escape drug targeting? Is a given clone capable of exploiting all mechanisms and the one selected based on a random choice (stochastic)? Or is the clone genetically predisposed towards one mechanism (predetermined)? To gain a greater understanding, the respective EM2 clone(s) that each displayed a different mechanism for drug resistance was taken advantage of. Here, the generation of drug resistant cells was technically repeated, with 7 independent replicates, and

the mechanism subsequently determined in each of the sub-clones respectively. This analysis would directly address whether the same single-cell clone would, upon 7 technical replicate repeats, employ the same mechanism of drug-resistance (pre-determined) or different mechanisms are randomly used (stochastic).

For this analysis, both P5 and P6 clones were chosen as representative clones that become drug-resistant by overamplification of BCR-ABL1 and the P7 cells selected based on their previous ability to become kinase-independent. At the time of this experiment the characterization of the R3 clone was not complete and therefore the 'point-mutation' mechanism was not included.

Each of the three EM2 clones were expanded in culture and subsequently split into 7 independent flasks. This established 7 sub-clones for each of the P5, P6 and P7 cells respectively. Following the protocol previously described, drug resistant lines were subsequently generated from each sub-clone. The derivates were termed R5-1 to R5-7 for the sub-clones generated from P5; and similarly, for P6 (R6-1 to R6-7) and P7 (R7-1 to R7-7) sets (**Figure 4.19**).

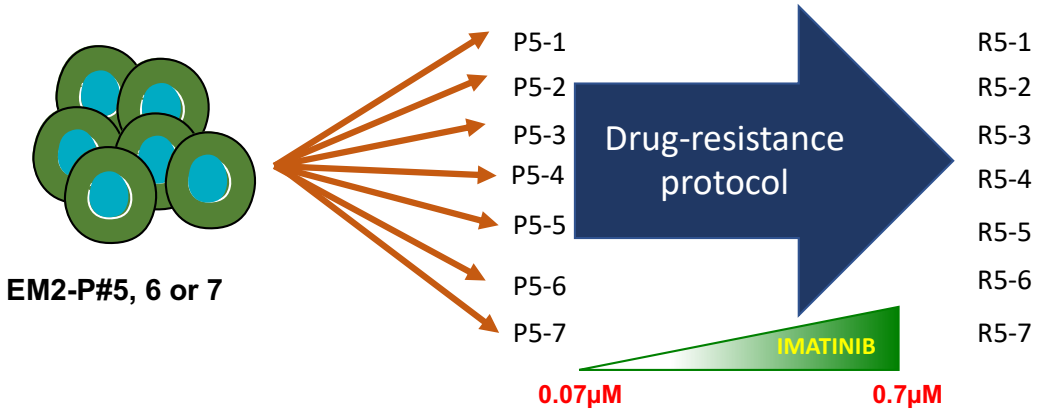


Figure 4.19: Schematic of the protocol of technical repeats of generating drug resistant derivates from the EM2 clones respectively.

The EM2 P5, P6 and P7 clones were aliquoted into 7 independent flasks and drug resistant lines subsequently made.

To address whether the mechanism of drug resistance is stochastic or pre-determined, the kinase activity of BCR-ABL1 of all the sub-clones was determined by western analysis. For the analysis of each sub-clone series, protein lysates from the respective parental line (P5, P6 or P7) as well as the original drug-resistant line (R5, R6 or R7) were included as control.

As detailed above, the P5 clone over-amplified the expression of BCR-ABL1 protein to become drug-resistant. To determine whether the same mechanism was used within the 7 independent replicates (R5-1 to R5-7) the protein expression of total-BCR-ABL1 was examined. In comparison to the P5 parental control, the BCR-ABL1 protein is clearly over-expressed within each sub-clone; as well as the previously generated R5 line (**Figure 4.20**).

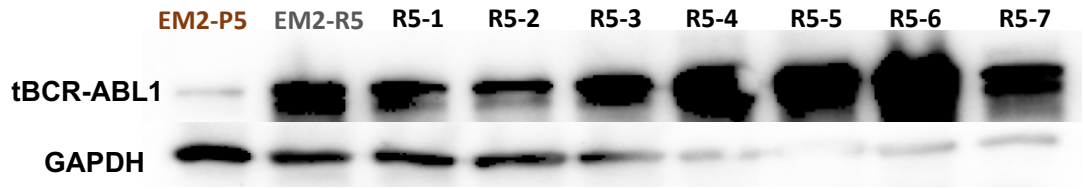


Figure 4.20: Protein expression of BCR-ABL1 protein within the technical repeat of drug resistance from the P5 EM2 clone.

Western blot analysis of total BCR-ABL1 protein within the technical repeat of generating drug resistance with the P5 EM2 clone. Original P5 and R5 lysates were used as controls. GAPDH was used as a loading control. Figure shows representative data of three independent experimental assays.

In a similar screening analysis, the re-generation of the 7-independent drug-resistant lines from the P6 clone (R6-1 to R6-7) also had over-expressed the BCR-ABL1 protein respectively (**Figure 4.21**).

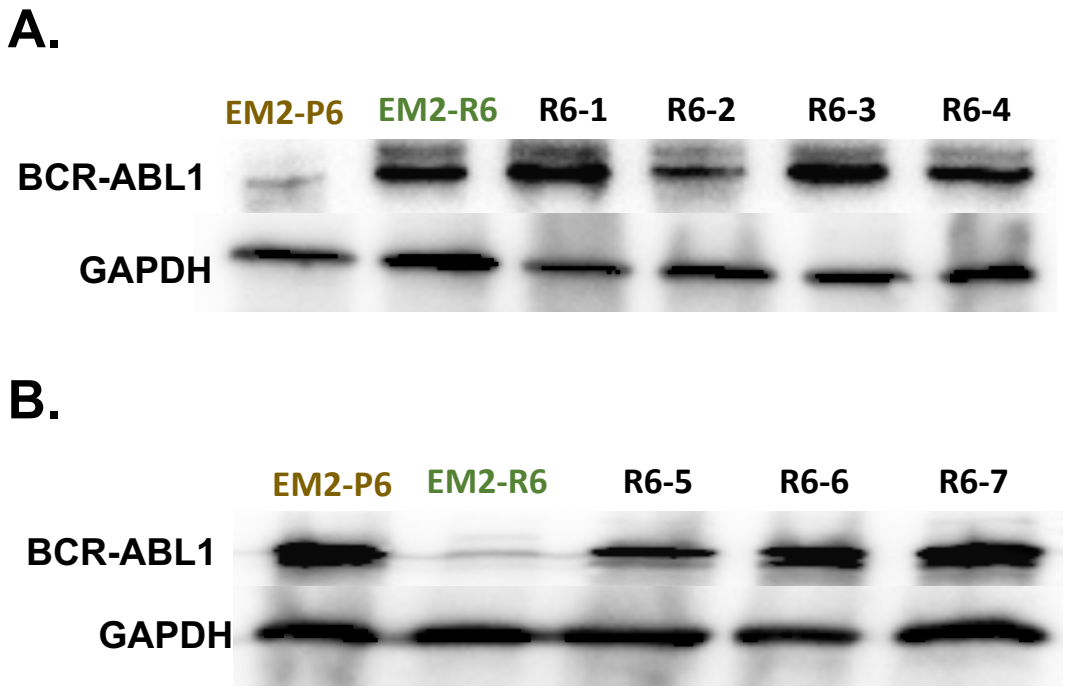


Figure 4.21: Expression of BCR-ABL1 protein from technical repeat of drug resistance from P6 EM2 clone.

Western blot analysis of total BCR-ABL1 protein within the technical repeats, (**A**) R6-1 to R6-4 and (**B**) R6-5 to R6-7, of drug resistance using the P6 EM2 clone. Original P6 and R6 lysates were used as control. GAPDH was used as a loading control. Figure shows representative data of three independent experimental assays.

Unlike the P5 and P6 lines, the P7 cells had previously become drug resistant by a BCR-ABL1 kinase independent mechanism. Accordingly, the rederived R7-1 to R7-7 lines were analysed for all phosphorylated-tyrosine residue containing proteins. In the control lysates, phosphorylated proteins are readily detected in P5 and this is severely reduced in the original R5 sample (**Figure 4.22**). There seemed to be a similar reduction of all phosphorylated-tyrosine proteins in each of the 7 replicate repeats, particularly in the loss of the higher-MW proteins. However, it is noted that unlike the original R7 cells, the R7-1 to R7-7 lines expressed a strong phosphorylated-tyrosine protein of 60kDa in size; the identity of which is unknown. While future studies need to clarify the phosphorylation status of the 7 replicates drug-resistant lines, the current observations suggest that they all have a reduced BCR-ABL1 kinase activity.

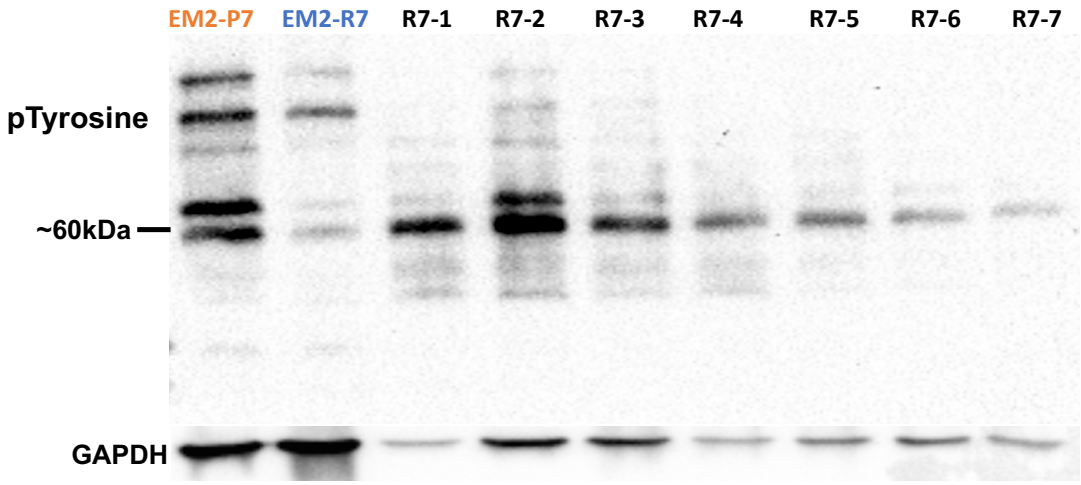


Figure 4.22: BCR-ABL1 kinase activity with the technical repeat of drug resistance using the P7 EM2 clone.

Western blot analysis of all proteins containing a phosphorylated tyrosine residue within the technical repeats of drug resistance using the P7 EM2 clone. Lysates from P7 and the original R7 are included as control. GAPDH was used as a loading control. Figure shows representative data of three independent experimental assays.

It is important to note that the analysis of all the sub-clones (21 in total) has only been analysed once as a preliminary study. Future work is clearly required to validate these findings as well as further investigation, and clarity, of the R7-1 to R7-7 lines. Nevertheless, this pilot data provides promising evidence in support of the hypothesis that the mechanism of how a given CML clone will become drug resistant is predetermined, by either genetic and-or epigenetic factor(s).

4.15: Discussion

Analysis of drug resistance at the single cell level

CML lines are widely used to model, and elucidate, the mechanisms of drug resistance, with the KCL22 [227-229] and K562 [230, 231] cells being the most studied respectively. Using these model systems, various molecular mechanisms of drug resistance have been identified and subsequently substantiated within clinical CML patient samples: (i) point mutations within the SH1 domain of BCR-ABL1, (ii) overamplification of oncogene, (iii) overexpression of efflux drug pumps and (4) BCR-ABL1 kinase-independent mechanisms [210].

CML is a clonal disorder [108, 232] and as such the leukaemic lines derived from patients (KCL22 etc.) are often considered a homogenous clone. As exemplified by the KCL22 model, all four mechanisms of drug resistance have been reported for the same cell line [227, 229, 233, 234]. Yet, how are studies achieving different mechanisms of drug resistance when all are using the same homogenous KCL22 line; noting that this has also been observed with the K562 model [111, 235]. There are several possibilities that could account for this observation (i) different labs having genetic variations of the same cell line, (ii) CML cells are capable of all 4 mechanisms and drug resistance is a stochastic event, or (iii) technical differences in the methodology used to generate drug resistance which may influence the mechanism employed.

However, the data presented herein details the heterogeneity of a given CML line (EM2 cells) and provides another explanation for how different mechanisms of drug resistance can arise from the same cell line (clonal competition, as discussed below).

First, it is important to distinguish how this study differs from those previously reported. Specifically, the work of others have generated drug resistant lines using the standard cell line (as obtained from ATCC) as their so-called parental line or ‘starting material’. In this chapter, single cell clones were first generated from the standard ATCC cell line which were then used as the respective ‘starting material’ for generating drug resistant derivatives. Although reports have published analysis of imatinib-resistant K562 cells at a single cell level; the ‘starting material’ was nevertheless the standard bulk K562 population [235].

By analysing drug resistance at the single cell level, from ‘beginning to end’, this study demonstrated that the EM2 population is heterogenous, consisting of different subclones each capable of utilizing distinct molecular mechanisms to escape drug targeting. From the 5 single-cell EM2 clones analysed, three different mechanisms were identified; likely point mutation, oncogene overexpression and kinase-independence respectively. Moreover, evidence is provided that strongly suggests that the execution of each respective mechanism is ‘predetermined’ and is innately established within the original starting (drug-sensitive) clone.

Although the work herein is limited to the analysis of the EM2 cell line, the preliminary findings suggest a heterogeneity to CML biology.

Heterogeneity and mixed-population of drug resistant cells

Since the EM2 line consists of various ‘pre-determined’ subclones, it would be anticipated that if this bulk population was used as the ‘starting material’ then a mixed-population of drug resistant cells would be generated with at least 3 mechanisms present. However, this study never tested this hypothesis; although this would be of great interest and is considered for future work. Unfortunately, no information can be extrapolated from the literate as drug-resistant EM2 cells have yet to be reported. Although the EM2 line was first established in 1980, the work herein is the first to describe the successful generation of such drug-resistant derivates.

As the EM2 line is heterogenous then it is highly likely that other CML lines, K562 and KCl22, are also. Under this assumption, it is curious that of the several K562 and KCL22 drug resistant models generated, none have ever reported a mixed-phenotype of the respective drug resistant line. Indeed, the studies that did analyse the drug resistant population at the single cell level did not identify any heterogeneity [235].

Collectively, these observations suggest that although the CML lines themselves are heterogenous, during the drug resistance process there is stochastic competition resulting in one dominant clone and the establishment of a homogenous drug resistant line.

The stochastic nature of this clonal-competition could account for why different mechanisms of drug resistance are obtained when using the same CML line. To further address this, future studies could explore the dynamics of a EM2

heterogenous population by taking advantage of the single cells clones and their inherent ‘pre-determined’ nature. Specifically, a heterogenous population could be reconstructed by mixing equal ratios of the P3 (point mutation), P5 (overexpression) and P7 (kinase independent) clones. Noting that each clone can be stably labelled with a fluorochrome such as GFP or RFP respectively. Under these conditions, the drug resistant process can be dynamically visualised over time (weekly periods, FACs analysis) and the presumed clonal competition recorded. If the hypothesis is correct, then the contrived ‘starting population’ will be an equal mix of GFP, RFP and unlabelled cells respectively while the resulting drug resistant line should be a ‘random’ uniform fluorochrome derived from the stochastic clonal competition.

Mechanism of drug resistance – a predetermined choice.

A surprising finding of this study was the reproducibility of the single cell EM2 clones to use the same mechanism for drug resistance upon biological repeats. Essentially, prior to any exposure to imatinib, the CML cells are preprogramed to employ a given molecular mechanism to escape drug targeting. Yet, what is it that makes the P5 clone to always overexpress the BCR-ABL1 oncogene upon drug resistance while that of P7 capable of surviving in the absence of SH1 kinase activity?

To understand this ‘predetermined’ genetics it would be necessary to investigate the molecular pathway(s) required for the successful completion of each mechanism respectively. For example, in understanding how BCR-ABL1 expression can be grossly overexpressed it would be necessary to determine how the *BCR* loci is transcriptionally regulated. Notably, the Myc transcription factor has been previously reported to directly regulate the *BCR* loci [237]. It would then follow that during the drug resistance process the clone must (A) express, or be able to activate, Myc and (B) the chromatin structure of *BCR* be in open-confirmation for Myc binding. Predisposition of these two factors could therefore bias the clone to consistently overexpress the BCR-ABL1 transcripts as the drug resistant mechanism.

Similar scenarios can be modelled for the kinase-independent mechanism which likely involves the association of epigenetic factors (e.g. loss of DMNT3A function) as well as for point-mutation within the SH1 domain mechanism relying on defects within the DNA-repair machinery.

The Cell of Origin model in drug resistance

Work presented in an earlier chapter proposes that the primary role of deregulated transcription factors in drug resistance CML is to maintain the leukaemic phenotype by inhibiting cellular differentiation. On the premise that both KCL22 and EM2 cells are of the same cellular lineage, myeloid, it was anticipated that a common set of factors would function to block each line respectively. Indeed, once the EM2 drug resistant cells was established this study readily, and eagerly, profiled the expression pattern of the transcription factors as previously identified within the KCL22 model. As noted, it was a surprise to not find any common overlap between these two models. However, lineage-specific profiling of these lines readily designated them as erythroid (KCL22) and GMP (EM2) respectively.

Given the current findings, why were KCL22 cells described as myeloid; as per the DSMZ catalogue? This likely stems from the historic context of the term ‘myeloid’ in relation to the haematopoietic system. Back in the 1980s, when the line was originally generated, lineage classification was based on morphology and basic biochemical properties and the understanding of the haematopoietic system broadly categorized myeloid cells as any lineage that was not T- or B-lymphoid. While the haematopoietic map has since been significantly reorganized (with the segregation of the myeloid and erythroid branches) the lineage characterization of such ‘old’ cell lines has not been updated. These observations heed a warning with all ‘myeloid-based’ cell lines as they could suffer the same misinterpretation-labelling.

Transcription factors have lineage-specific functions and can have opposing actions in different progenitors. Indeed, RUNX1 has a paradoxical role in haematological malignancies, capable of functioning as either a tumor-suppressor or dominant oncogene [238]. Inhibition of RUNX1 activity is a frequent event in the pathogenesis of several myeloid dysplasias, as demonstrated by its involvement with chromosome translocations in AML [239] as well as loss-of-function mutations found in both MDS [240] and cytogenetic normal AML patients [241]. Conversely, mouse genetic studies, using random retroviral genome insertions, identified that overexpression of RUNX1 leads to the onset of T- or B-cell lymphomas [242, 243].

Based on these observations, it is not surprising that transcription factors are deregulated in a lineage-specific manner (e.g. GMP versus erythroid) in order to maintain the block of differentiation upon CML drug resistance.

In support of our model, it was of great interest to define a minimal network of 8 transcription factors that are commonly deregulated upon drug resistance within two independent erythroid CML cells (K562 and KCL22). Below is a brief synopsis of this network with any relevant evidence in support of their role in lineage differentiation, if any, detailed.

AEBP1: Adipocyte enhancer binding protein-1 is a transcriptional repressor and overexpression can inhibit the differentiation of preadipocytes [244]. It is also associated with various human malignancies including ovarian, glioblastoma and gastric cancer respectively [245-248].

MEF2D: Myocyte enhancer factor 2D functions with MyoD and Myogenin to regulate the differentiation of skeletal muscle cells [249, 250]. Its overexpression contributes to the transformation of lymphocytes as seen in paediatric B-cell ALL [251].

SOX4: This transcription factor is detailed elsewhere in another chapter.

TCEAL1: Transcription Elongation Factor A-like 1 is a transcriptional repressor and its over-expression within 3T3 fibroblasts promotes tumour formation [252]. Additionally, TCEAL1 expression has been correlated to esophageal cancer [253].

FOXA3: Forkhead box A3 promotes adipocyte differentiation [254] (as opposed to AEBP1). It is overexpressed in lung adenocarcinomas as is correlated with patient overall survival [255].

SSX1 and SSX2: Synovial Sarcoma X family members of transcriptional repressors with homology to the Kruppel zinc-finger family [256]. Overexpression of either protein is associated with synovial sarcoma [257].

ZNF626: Zinc nuclear factor 626 is a protein coding gene with presumptive DNA-binding properties. It has been reported to protein-protein interact with both ID3 and FHL2 factors [258].

Based on their reported role in cellular differentiation, future work could explore the role of the AEBP1, MEF2D, TCEAL1 and FOXA3 factors in both the K562 and KCL22 drug resistant lines. Here, it is hypothesized that the respective loss-of-functions studies will promote differentiation of the drug resistant CML lines.

Analogous to the erythroid CML modelling with two independent lines, a second GMP-like CML line is required to identify, and validate, the predicted shared network of deregulated transcription factors with those identified in the EM2. Pilot studies considered the KYO-1 cells as such a candidate [259, 260]. However, lineage-specific profiling of these cells (as performed for KCL22, K562 and EM2) failed to categorize these cells as GMP but rather more erythroid-biased (data not shown). Unfortunately, based on the commercially available cohort of available CML cell lines, there are no more lines that are characterized as myeloid. In addition to EM2, the other 'myeloid' CML lines are the KCL22 cells which have now been disregarded and the GDM-1 which, as previously discussed, is not a bona-fide Philadelphia chromosome-positive CML.

In the absence of securing another GMP line to support the current observations, future studies should consider exploring the 'cell of origin' model within other lineages that have more flexibility in the number of lines readily available; either the lymphoid (Nalm-1, BV173) or megakaryocytic (MEG-01, JURL-MK1) CML lines respectively.

EM2 drug resistant cells: a novel tool for CML study

Only a handful of models for CML drug resistance have been generated; namely derived from the KCL22, K562, LAMA-84 and Meg01 lines respectively [183, 189, 226]. While the generation of the EM2 model is a new additional to this cohort, it also contributes a uniqueness that should further advance the understanding of this pathology. As discussed herein, the EM2 is the only GMP-based model of CML drug resistance. Of the other 4 models, three are erythroid (KCL22, K562 and LAMA-84) while that of Meg01 is a closely associated lineage being megakaryocytic. Moreover, as there is a likely need to stratify CML based on lineage identity, the introduction of this GMP-biased model will be an invaluable tool.

Similar to studies performed for the KCL22 model, the work presented herein identified, and defined, a clinically relevant set of 7 transcription factors that are deregulated within the EM2 cells upon drug resistance. Future studies should explore the relevance of this gene-set with the focus of their proposed role in blocking cellular differentiation. In prioritising which factor(s) to initially study, there are two approaches recommended.

The first is the ‘candidate gene’ approach which is guided by a review of the literature to indicate which transcription factors would likely have an important role within drug resistant CML. Based on the literature, the recommended list of genes to target are:

MEF2C: Myocyte enhancer factor 2C is of the MADS box family of transcription factors and primarily involved in the development of the muscle, skeleton and cardiac system [261]. MEF2C is an effector gene target of Scl/Tal1 factor during megakaryopoiesis. Knockout adult mice fail to develop megakaryocytes although erythroid differentiation is normal [262]. MEF2C also regulates the ability of leukaemic cells to home and localize within tissues such as spleen. Notably the loss of MEF2C within Mixed-lineage leuakemia tumour cells results in a peripheral blood leukaemia with no tissue invasion [263]. Finally, the phosphorylation of MEF2C (Ser222) in AML is associated with chemo-resistance [264].

ATF3: Activator of Transcription-3 is a member of the AP-1 family of transcription factors and is functions as a stress-induced responsive factor during the immune response [265]. ATF3 is highly expressed in AML patient samples and contributes to the block of myeloid differentiation in a CEBPα mediated manner [266].

ELF1: ETS-like Factor 1 is a member of the ETS family of transcription factors and its downregulation is necessary for erythroid differentiation [267]. Moreover, ELF1 is associated with the metastatic category of non-small cell lung cancer [268].

The second approach to prioritize the EM2 transcription factor network takes advantage of the methodology used in generating the drug resistant lines. Specifically, a stock of cells was frozen at every 10% increment of imatinib treatment. By capturing the cells in this step-wise manner enables the induction of this gene-set to be quantified in a dynamic manner; and those that are activated early in the process can be readily identified. These early-response genes would be considered ‘master’ regulators of drug resistance and candidate genes for targeting.

Summary and Future Prospective

First, the focus of this chapter developed from earlier findings demonstrating a novel role for the other domain(s) of BCR-ABL1 in regulating cell proliferation of

the drug resistant KCL22 cells. Future studies must investigate this hypothesis within the EM2 system in hopes of confirming such a role.

The work herein provides a novel perspective on the clonality of CML and pushes this pathology into the spotlight of personalised-medicine. The observed heterogeneity of CML cells opens a spectrum of future research into further understanding its clinical impact. While it will not be surprising to soon learn that single cell RNA-Seq of patient CML leukaemic cells will identify heterogeneity, the impact of this will need to be explored. Does cell heterogeneity within a given patient relate to the mechanism of drug resistance? Are subclones of a 'predetermined mechanism' present in patients and, if so, does such clonal-stochastic competition accounts for the failure to predict which mechanism a given patient will clinically present upon resistance?

The unexpected finding that prior to any exposure, or treatment, to drug, the CML cells are genetically pre-programmed towards a given mechanism of drug resistance offers hope to new therapies. If the molecular components of each mechanism can be understood, then these themselves could be targets for future therapies. If the mechanism can be 'pharmacologically sabotaged' from completing the task (whether overamplifying BCR-ABL1 or point mutating the SH1 domain), then it is foreseeable that the generation of a drug resistant clone can be inhibited. Preventative drug measures could then be used as a prophylaxis to stop the successful transformation of a clone into a drug resistant state.

Finally, future treatment of drug resistant CML could become more personalised where the cell (or lineage) of tumor-origin could direct the course of therapy treatment. If the hypothesis for the role of deregulated transcription factors holds true, then these could be used as targeted treatments for 'differentiation therapy' of drug resistant cells. Moreover, as the network of factors is dependent on the 'cell of origin' then patient stratification would be required.

Chapter 5: The Role of SOX4 in drug-resistant CML

5.1 Introduction and Aims

Ineffective haematopoiesis is a 'cancer-hallmark' of aggressive CML (BC) and is directly correlated with the onset of drug resistance [8]. BCR-ABL1 can incur a pathological interference of differentiation by disrupting the regulation of transcription factors including the master myeloid regulator C/EBPα [180]. Although BCR-ABL1 is required to inhibit cellular differentiation, drug resistant KCL22 cells nevertheless fail to differentiate in its absence (**Chapter 3**). As such, in the absence of BCR-ABL1, how are drug resistant cells able to maintain a progenitor-like leukaemic cell state?

It is proposed that upon CML drug resistance, the expression of transcription factor(s) is permanently deregulated (likely associated with epigenetic changes) and function to maintain the block of differentiation in compensation for the loss of BCR-ABL1. Indeed, transcriptome analysis identified a gene-set of transcription factors that are (de)regulated upon drug resistance within KCL22 cells and included SOX4.

Elevated levels of SOX4 mRNA and protein are associated with many malignancies such as breast and prostate cancers as well as several blood leukaemias including adult T-cell leukaemia/lymphoma and AML [134, 135, 269, 270]. Moreover, within a cohort of drug-sensitive or -resistant CML patients, high levels of SOX4 transcripts is strongly correlated with the drug resistant population [69].

The aim of this chapter is to investigate the proposed function of SOX4 in inhibiting cellular differentiation of drug resistant KCL22 cells.

5.2 Expression of SOX4 in drug resistant KCL22 cells

The microarray predicted induction of SOX4 mRNA within both 15IMr and 16IMr cells was confirmed by semi-quantitative RT-PCR (**Figure 5.1a**). Notably, the parental 15P and 16P cells had negligible expression of SOX4 while transcripts were readily detected in both drug resistant derivatives respectively. This analysis was performed on cDNA template that was generated from three independent 'pairs' (16P-16IMr and 15P-15IMr) of biological replicates. Data representative from these analyses is presented.

To confirm that the pattern of induced SOX4 transcripts held true at the protein level, western analysis was performed. As with the gene profiling, the protein

analysis was performed on lysates generated from three independent pairs of the KCL22 clones respectively. Data representative from these analyses is presented. In correlation with the SOX4 mRNA expression, the protein was undetected in 15P and 16P cells while in the drug resistant lines the predicted 74kDa band was readily present (**Fig. 5.1b**).

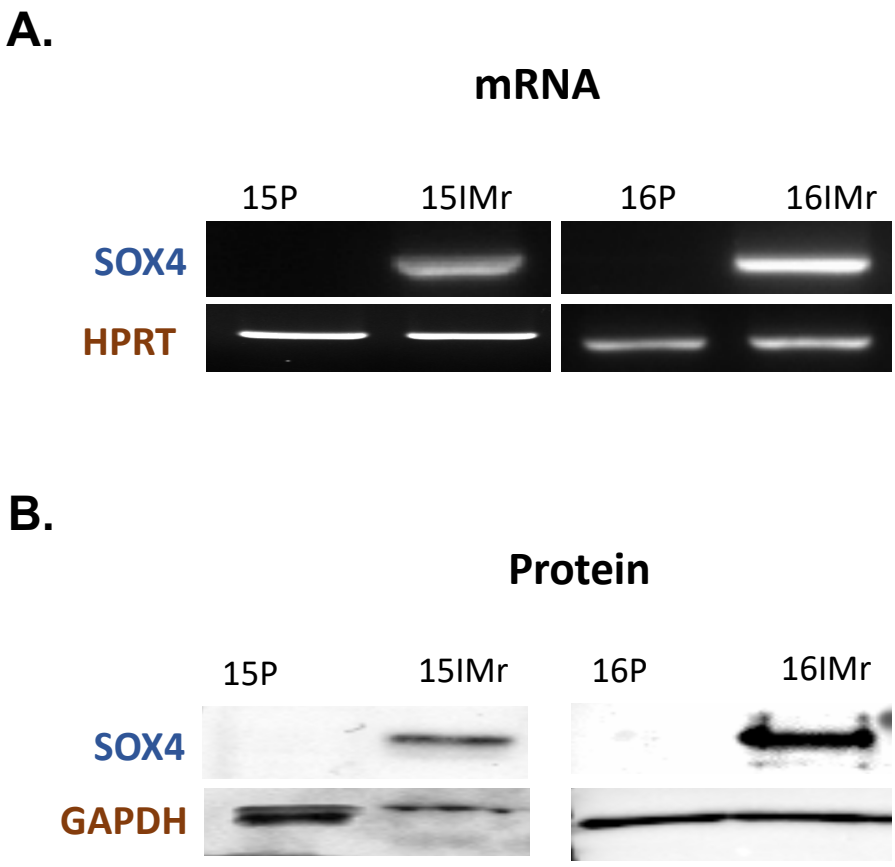


Figure 5.1: Expression levels of SOX4 in the 15IMr and 16IMr single-cell clones.

Semi-quantitative expression of SOX4 (**A**) transcripts and (**B**) protein in the KCL22 drug resistant model. HPRT and GAPDH were used as loading controls for the PCR and western respectively. Figure shows representative data of three independent experimental assays.

5.3 Does loss of SOX4 induce differentiation in drug resistant KCL22 cells?

It is postulated that upon drug resistance, CML cells will induce the expression of SOX4 to function as a new 'oncogenic driver' and inhibit cellular differentiation. Notably, overexpression of SOX4 has previously been reported to block differentiation of myeloid progenitors [145]. Based on these observations, the drug resistant KCL22 cells are postulated to be dependent upon the activity of SOX4 for maintenance of a 'transformed stem-cell progenitor'.

To directly test this hypothesis, the expression of SOX4 was targeted within drug resistant KCL22 cells and the induction, if any, of differentiation was assessed. Sustained depletion of SOX4 within 15IMr and 16IMr cells was performed by the double-transfection protocol, as previously described (**Material and methods**). Here, a siRNA (siSOX4-1, Ambion™ s13302) was used which targets the 1079bp of SOX4 CDS region; as depicted in **Figure 5.2**.

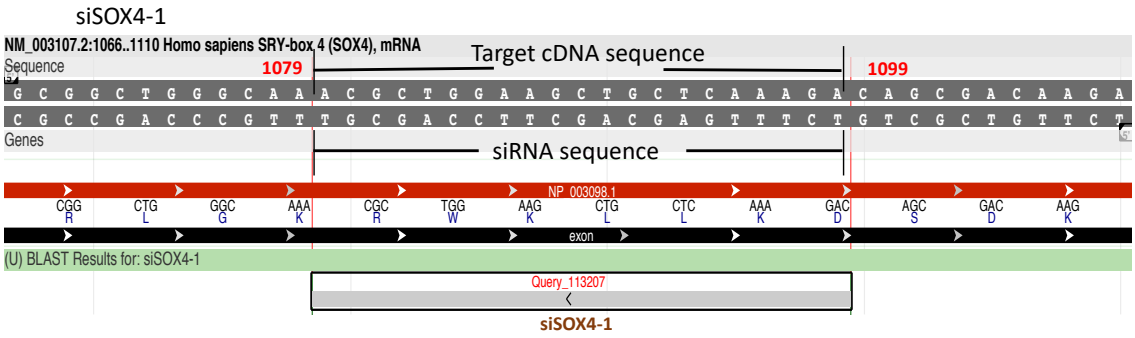


Figure 5.2: Nucleotide BLAST sequence alignment of siSOX4-1 to the target SOX4 transcript.

BLAST alignment of the siSOX4-1 sequence against the mRNA of SOX4 (NM_003107). The grey bars indicate the SOX4 cDNA sequence. The red bar indicates the amino-acid sequence with the corresponding codons below. The 3'-5' strand correspond to the antisense sequence of the SOX4 mRNA and same sequence for the siSOX4-1. The 5'-3' sense strand corresponds to the target sequence. The annealing region (base pair position) is highlighted from 1079 to 1099.

In brief, the 15IMr and 16IMr cells were double-electroporated with either siSOX4-1 or siControl sequences respectively. Viable cell numbers were assessed by trypan blue exclusion. For simplicity, the analysis of the 15IMr cells is detailed. At d4 (24 hours after the second electroporation), cell counts

demonstrated that both siControl and siSOX4 treated cells had comparable viable cells of approximately 5 million respectively (**Figure 5.3a**). Subsequent cell counts (day 6 and 8) demonstrated a rapid growth of siControl treated cells, reaching a maximum count of 21 million cells at d8. This equated to a 4.2-fold increase in cell growth in 4 days. However, the siSOX4 treated cells failed to demonstrate any substantial growth and underwent growth arrest having reached a maximum population of only 6 million cells at d8. This equated to a 1.2-fold increase in cell growth over 4 days. As control, lysates were collected at day 8 (i.e. completion of the experiment) and western blot analysis, in conjunction with densitometry, demonstrated 90% loss of SOX4 protein in the siSOX4 treated cells (**Figure 5.3a**).

Similar results were obtained for 16IMr cells (**Figure 5.3b**) and was repeatable upon biological repeats for both clones respectively.

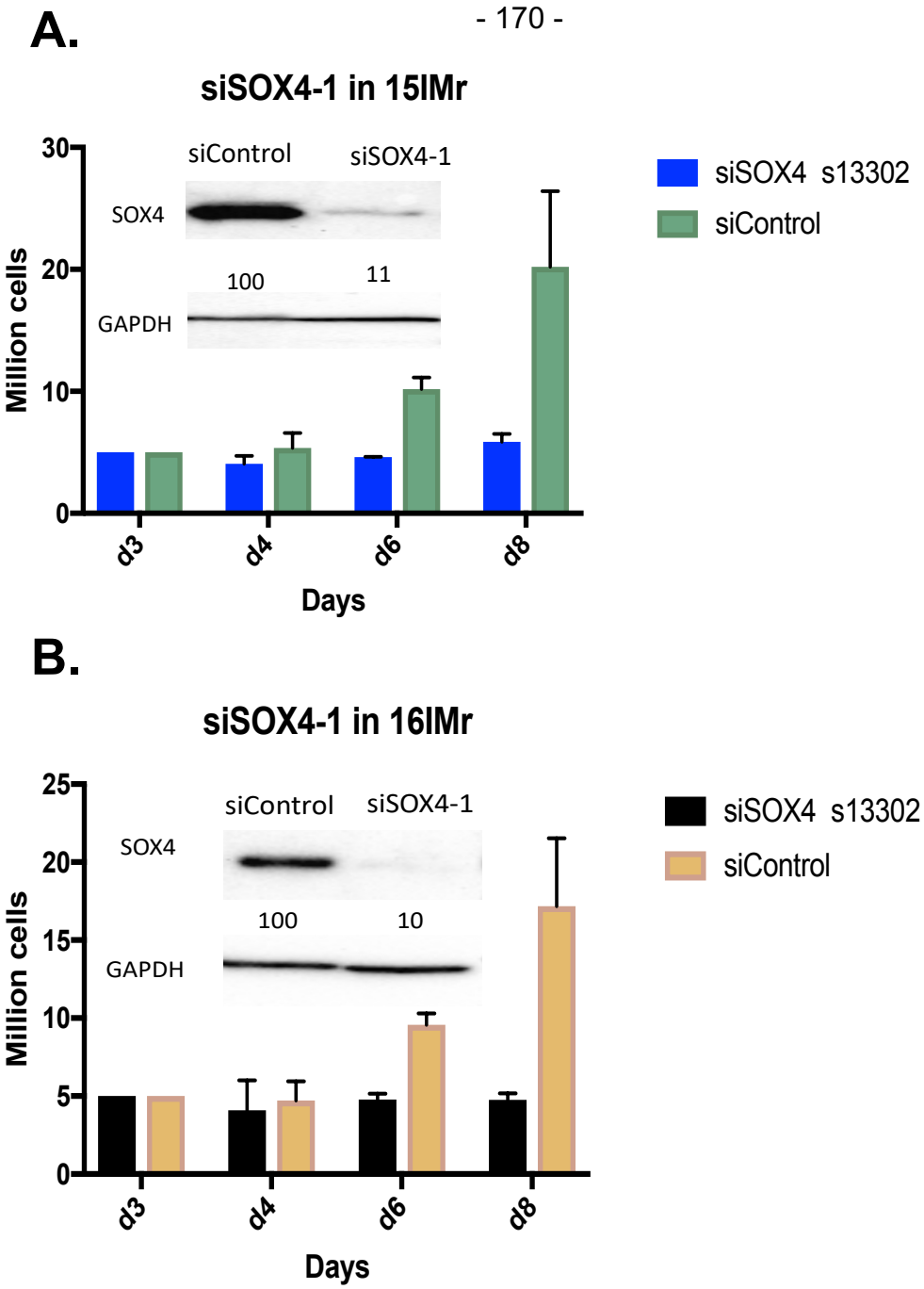


Figure 5.3: Depletion of SOX4 induces growth arrest within 15IMr and 16IMr KCL22 cells.

Viable cell counts (trypan blue exclusion) of (**A**) 15IMr and (**B**) 16IMr cells treated with siControl or siSOX4-1 respectively. In picture; western blot analysis of SOX4 and the densitometric analysis of the bands is presented as a percentage, which was normalized to the loading control. GAPDH was used as a loading control. Error bars indicate SD. N=3 for 16IMr, n=2 for 15IMr.

To further characterise the consequence of SOX4 depletion within drug resistant cells, the d8 morphology was assessed (**Figure 5.4**). Both siControl and siSOX4 treated cells had similar progenitor-like characteristics and any anticipated

changes to morphology (indicative of cellular differentiation) as a result of the loss of SOX4 was not readily apparent. It is noted that some siSOX4 cells developed larger cytoplasm with a less defined, ruffle-like, cell-membrane; characteristics associated with macrophages. However, this was not consistent throughout the population.

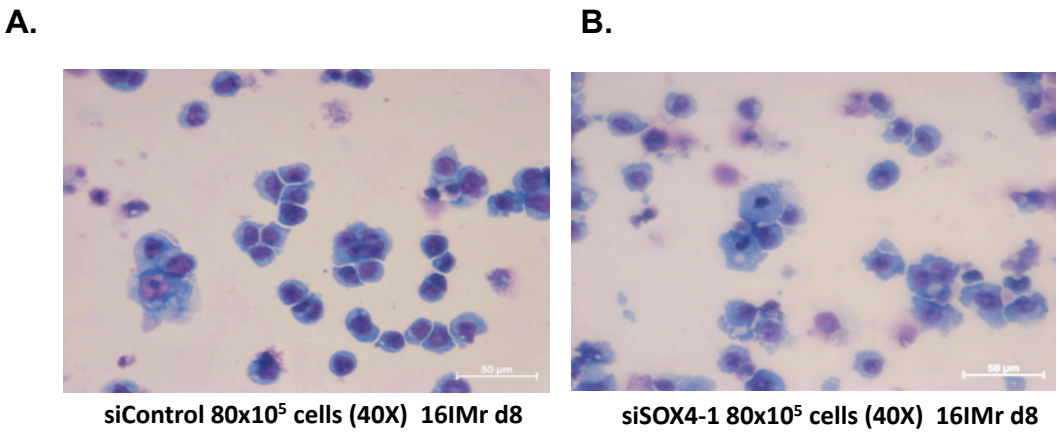


Figure 5.4: Cell morphology of drug resistant KCL22 cells depleted of SOX4 protein.

Micrographs (A) siControl (B) siSOX4-1 treated 16IMr cells. Wright stained cytopins of resistant cells were collected at day 8. Figure shows a representative picture of three independent experimental assays.

To identify any molecular differentiation of the siSOX4 treated cells, the expression of lineage-specific genes characteristic to macrophage, neutrophil and erythrocyte fates was profiled respectively. Specifically, the macrophage gene signature included CD11b, CD14, M-CSFR, PU.1, TLR2 and TLR4 while that of neutrophil was C/EBPα, Lactoferrin and Gfi1 genes. The erythroid lineage consisted of the CD235a, CD71, EPO-R, GATA1, and NF-E2 genes respectively. Expression of this gene-set was performed on cDNA generated from d8 siSOX4 treated cells with the siControl transfected cells included as control. For simplicity, data from the 16IMr treated cells is shown with similar results obtained in the 15IMr cells. No overt difference in the relative expression profile between the siSOX4 and siControl treated cells was observed (**Figure 5.5**).

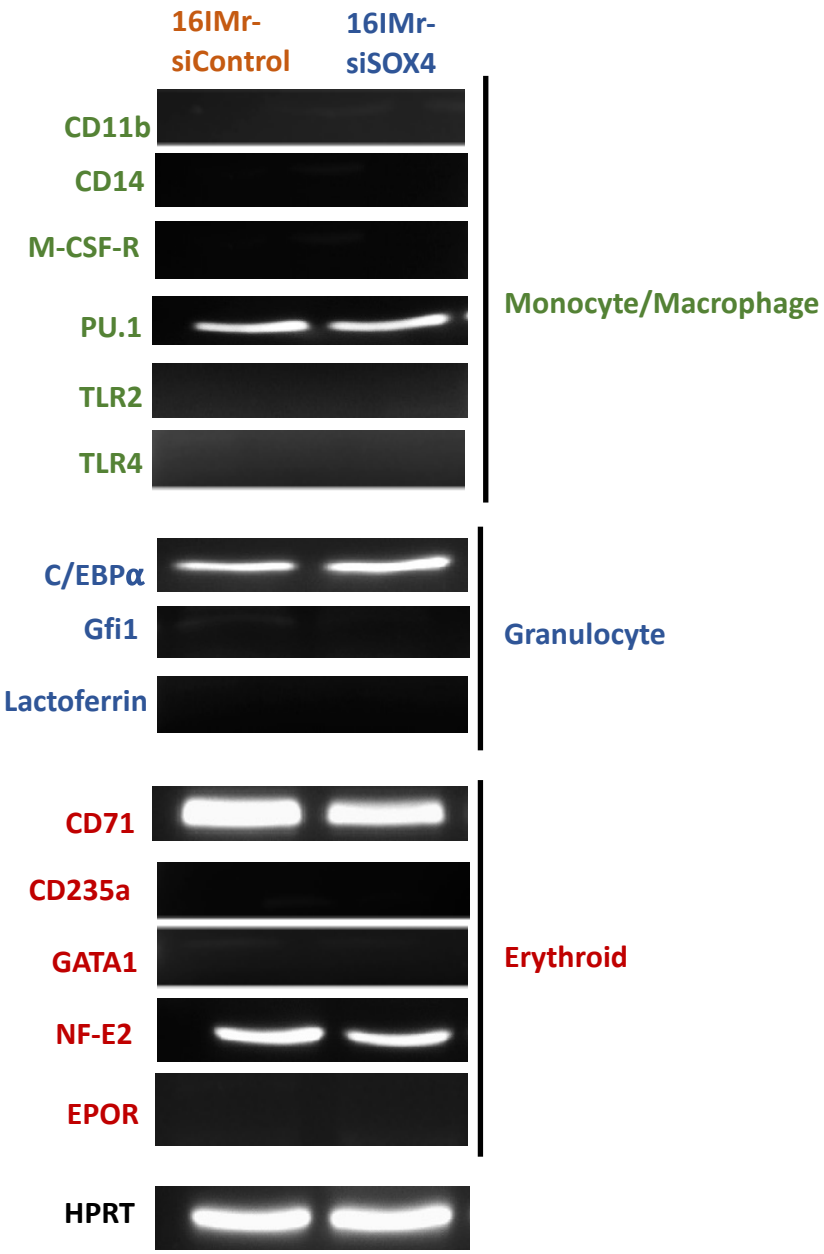


Figure 5.5: Expression profile of lineage-specific genes in 16IMr cells depleted of SOX4 protein.

Semi-quantitative analysis of macrophage-, neutrophil- and erythrocyte-specific genes within on siControl and siSOX4-1 treated 16IMr cells (d8). HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

Although a very limited number of genes were analysed, this observation suggests that depletion of SOX4 in drug resistant KCL22 cells does not promote differentiation along either the macrophage, neutrophil or erythroid lineages respectively. It is noted that some PCR reactions failed to amplify any DNA product, and this could either reflect the absence of any transcript expression or

a failed PCR amplification. Future studies with the inclusion of an appropriate positive control is required.

The findings herein fail to support the proposed function of SOX4 in blocking cellular differentiation. Nevertheless, they uncover a novel role for this transcription factor within drug resistant KCL22 cells with regards to regulation of cell proliferation. Interestingly, this phenotype is in accordance with reports by others that demonstrate SOX4 can regulates cell cycle progression directly by repressing *CDKN1A* gene activation [271].

5.4 KCL22 parental cells do not tolerate SOX4 ectopic overexpression

As a complimentary study to the loss-of-SOX4 function, the ability of SOX4 protein to confer drug resistance (gain-of-function) was investigated. Previous work in the lab had attempted to stably overexpress SOX4 within 16P cells (data not shown). In brief, 16P cells were transfected with a commercially available vector (hSOX4-pCMV6, Origene®) that expressed human SOX4 transgene as well as the neomycin-resistant gene for stable selection of recombinant cells. A stable recombinant line was generated by drug selection with G418. The levels of SOX4 expression within the recombinant population was assessed by western blotting. Although the cells continue to be resistant to G418, the expression of recombinant SOX4 was negligible. As control, 3T3 fibroblasts were transiently transfected the hSOX4-pCMV6 plasmid and following an overnight recovery, lysates demonstrated the robust expression of recombinant protein upon western blot analysis (data not shown).

Collectively, these observations validate that although the hSOX4-pCMV6 plasmid can express high-levels of recombinant protein, stable 16P derivatives overexpressing the SOX4 protein cannot be generated. It is postulated that unlike drug resistant KCL22 cells, which express high levels of SOX4 protein, the parental cells cannot tolerate SOX4 and its expression is toxic.

To address this issue, the SOX4 coding region from the hSOX4-pCMV6 plasmid was sub-cloned into an IRES-GFP vector (pcDNA3-IRES-GFP) where SOX4 is translated simultaneously from a single mRNA transcript along with GFP due to the IRES sequence [272]. Here, the expression of SOX4 is indirectly quantified by GFP intensity. The final vector was defined as hSOX4-IRES-GFP.

The 16P cells were transfected by electroporation with hSOX4-IRES-GFP or the vector control, pcDNA3-IRES-GFP, respectively. After an overnight incubation,

the GFP expression was visualised by fluorescent microscopy and recorded (**Figure 5.6a**, Week 0). The expression of GFP, from both plasmids, was heterogeneous within the respective population and ranged from relatively bright, medium and low intensities. Selection of stable recombinants was achieved by the addition of G418 with GFP visualised (**Figure 5.6a**, Week 6). Cells stably expressing the pcDNA3-IRES-GFP vector displayed a similar heterogeneous range of GFP intensities to that seen shortly after electroporation. This observation confirms that 16P cells can clearly express high levels of the GFP protein without causing any detriment to viability.

The stable 16P population expressing the hSOX4-IRES-GFP vector were negligible for GFP fluorescence (**Figure 5.6a**, Week 6) despite displaying bright levels shortly after transfection. These observations strongly suggest that the loss of the initial bright-GFP population is due to inability of the 16P cells to tolerate high levels of SOX4 expression. Due to this SOX4-mediated toxicity, only cells that have very low SOX4, and in turn low-GFP, expression can survive. This provides a clear explanation for the earlier observations as too why the previous stable line generated with the hSOX4-pCMV6 plasmid, despite being G418 resistant, failed to express any readily detectable SOX4.

5.5 Does transient expression of SOX4 confer drug resistance in CML cells?

As stable 16P derivatives overexpressing SOX4 could not be generated, the ability of SOX4 to confer drug resistance was tested in a transient manner. In brief, 16P cells were transfected with the hSOX4-IRES-EGFP vector and following an overnight recovery the GFP-positive population was purified by FACS-sorting. As control the pcDNA3-IRES-EGFP vector was used.

GFP-positive cells were subsequently treated with 0μM, 0.3μM, 0.5μM and 0.7μM of IM for 72hrs and cell growth-viability determined by MTS assay. The IM concentrations were chosen on the basis that they are sub-optimal doses for killing 16P cells (**Figure 5.6b**) and should provide the sensitivity to detect any potential drug resistance. Despite expectations, there was no overt differences in the cell response to titrating IM concentrations between SOX4-expressing cells and the control GFP population (**Figure 5.6b**). It is noted that this was a pilot experiment and additional biological replicates, as well as validation that the recombinant cells do express SOX4 protein (and at what levels) is required.

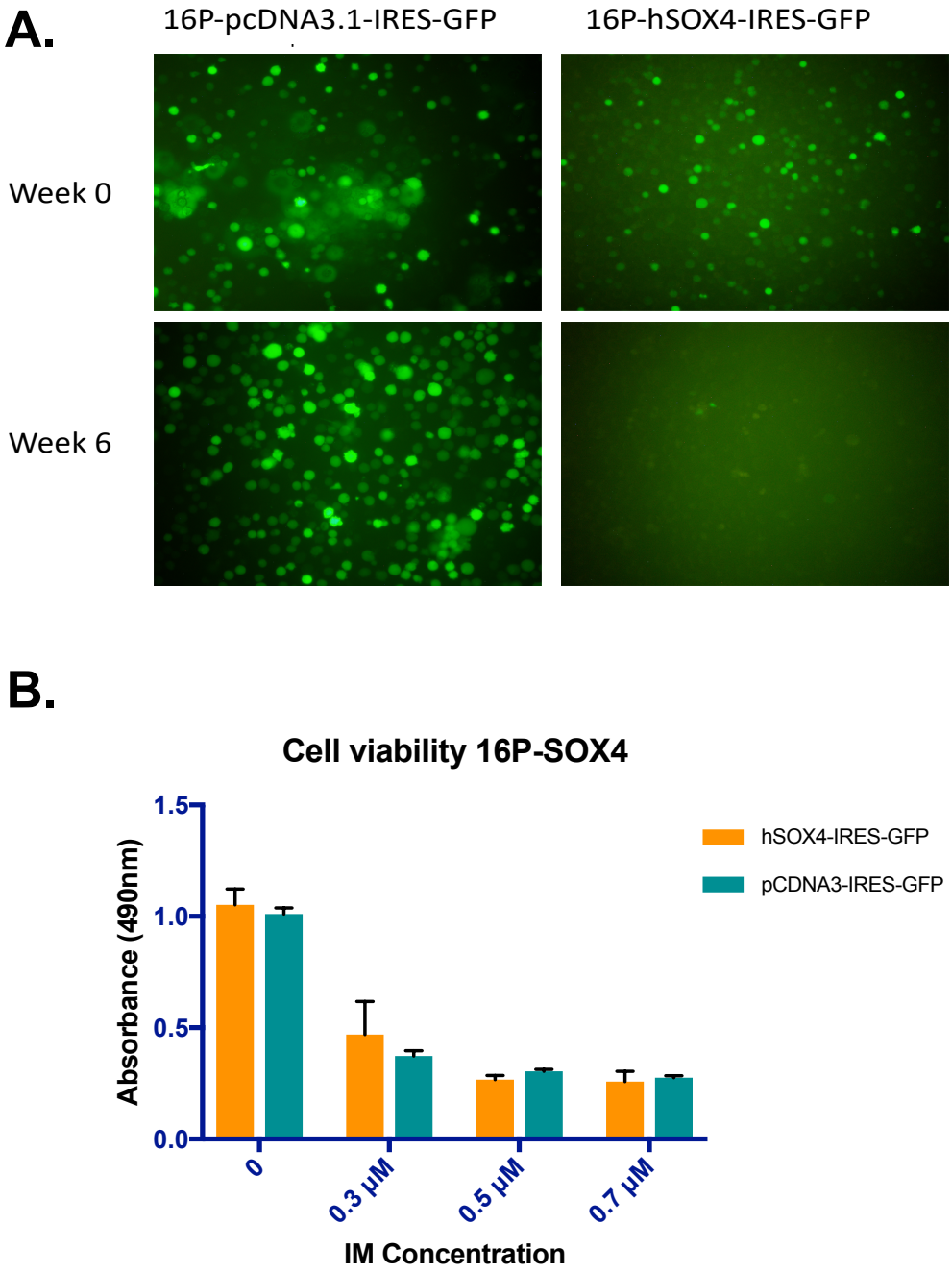


Figure 5.6: Gain-of-function of SOX4 within drug resistant KCL22 cells.

(**A**) Fluorescent cell pictures of 16P KCL22 cells transfected with either pcDNA3-IRES-GFP or hSOX4-IRES-GFP vectors. Pictures were taken at week 0 and at week 6. (**B**) Transient expression of SOX4 within 16P cells and response to titrating IM concentrations as measured by MTS assay. after drug administration. Wavelength absorbance detected was at 490nm. Panel (A) shows a representative picture of two independent experimental assays. Mean and SD (error bars) of 3 technical repeats.

5.6 Induction of growth arrest is not reproduced with second siSOX4 sequence

The preliminary observations, presented herein, identify a novel role for SOX4 in regulating cell proliferation of the drug resistant KCL22 cells. To validate these findings, the loss-of-function studies was repeated using a second independent siSOX4 sequence. This new sequence, termed siSOX4-2 (Ambion™, s13301), targeted the SOX4 CDS at position 1106bp (**Figure 5.7**). Notably, this binding region does not overlap with the previous siSOX4-1 sequence which complimented the CDS at position 1079bp to 1099bp.

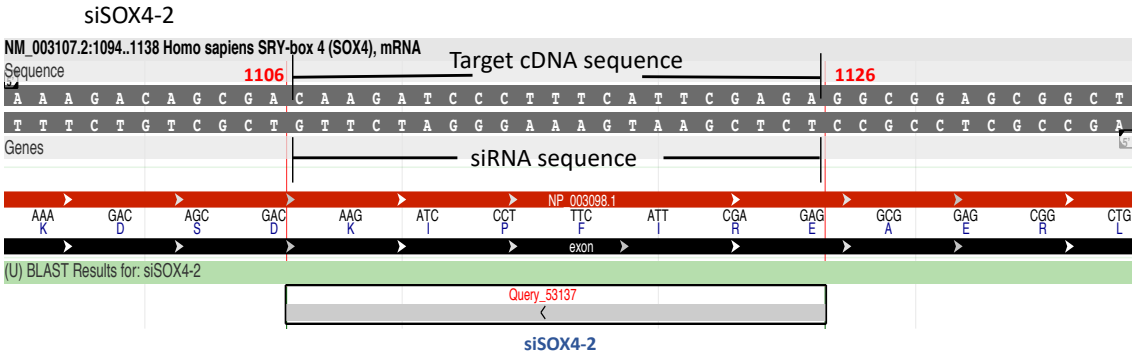


Figure 5.7: Nucleotide BLAST sequence alignment of siSOX4-2 to the target SOX4 mRNA.

Nucleotide BLAST alignment of the siSOX4-2 sequence against the mRNA of SOX4 (NM_003107). From bottom to top, the siSOX4-2 sequence (indicated as Query_53137) is anti-sense aligned to the mRNA sequence of the only exon (black) of SOX4. The grey bars indicate the SOX4 cDNA sequence in double strand. The red bar indicates the amino-acid sequence with the respective codons below. The annealing region (base pair position) is highlighted from 1106 to 1126.

Depletion of SOX4 protein with siSOX4-2 was performed within 15IMr and 16IMr cells respectively; as described earlier. As control, the siControl sequence was used. Surprisingly, cells treated with siSOX4-2 failed to undergo growth arrest and proliferated at similar rates to the siControl cells (**Figure 5.8**). The efficiency of the SOX4 knockdown was confirmed by western blot (at d8, end of experiment) and in conjunction with densitometry demonstrated a >83% loss of protein in either 15IMr (**Figure 5.8a**) or 16IMr (**Figure 5.8b**) cells respectively. These results were reproduced upon multiple biological repeats (data not shown).

Densitometric quantification of SOX4 protein expression demonstrated that both siSOX4 sequences depleted SOX4 with comparable efficiencies (>75%). Therefore, the observed discrepancy upon cell growth cannot be accounted for by differences in the level of SOX4 protein knockdown by each siRNA sequence respectively.

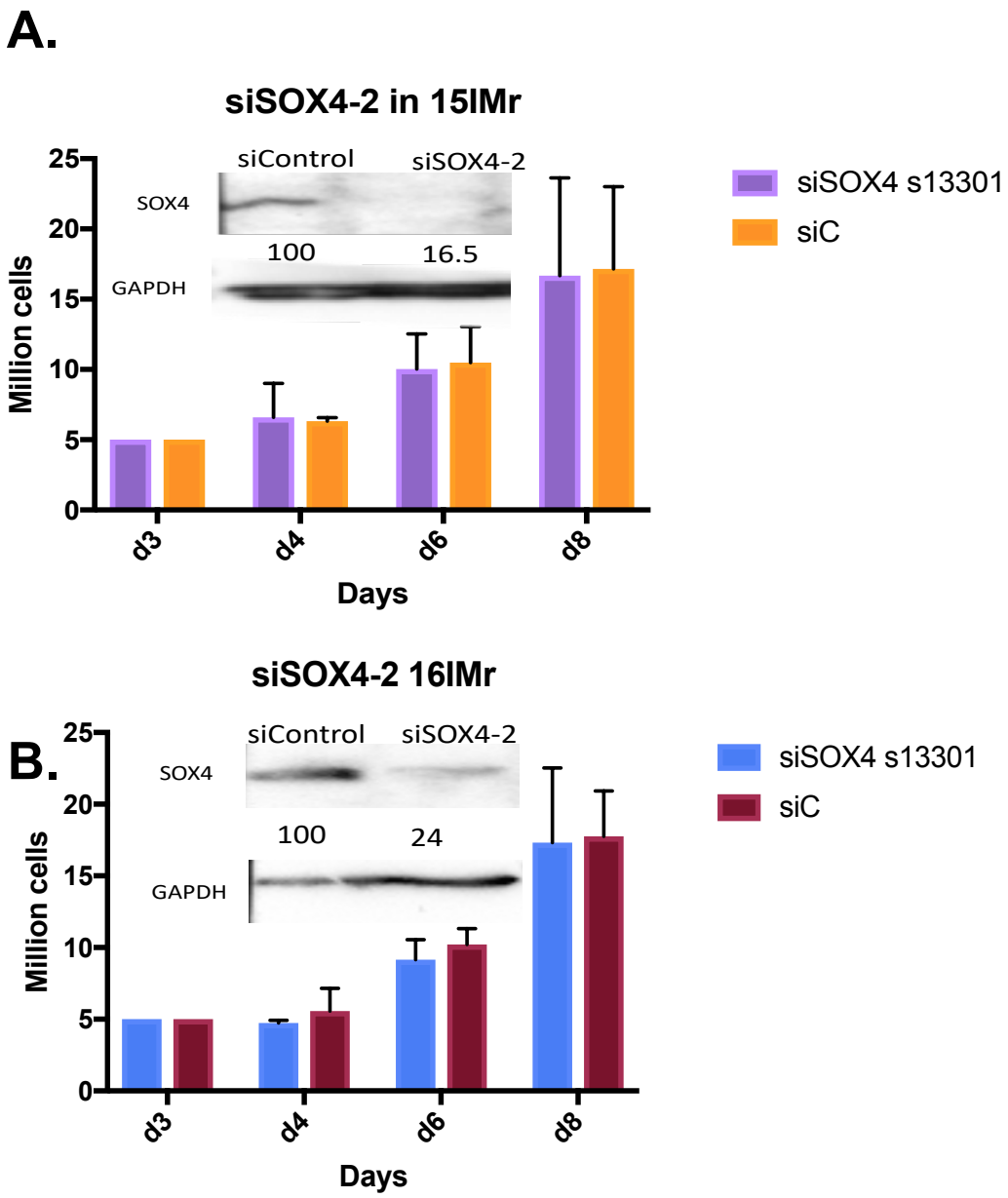


Figure 5.8: Depletion of SOX4 protein by siSOX4-2 in 15IMr and 16IMr KCL22 cells.

(**A**) 15IMr and (**B**) 16IMr cells were treated with siControl or siSOX4-2 sequences respectively. Cell viability was determined by trypan blue exclusion (d3, 4, 6 and 8). Inserts: western blot analysis of SOX4 protein with the densitometric analysis of the bands is presented as a percentage, which was normalized to the loading control. GAPDH was used as a loading control. Error bars indicate SD. n=2 for 15IMr and 16IMr clones.

As the studies relating to the siSOX4-2 sequence was performed several months after the siSOX4-1 experiments, there is a remote possibility that biological differences in the cell line(s) itself (different freezing batches) contributed to the discrepancy. To address this, the depletion of SOX4 was repeated within a freshly-thawed batch of 16IMr cells using both sequences in a side-by-side comparison. In line with the observations reported, both siSOX4 sequences gave the same respective result with siSOX4-1 inducing growth arrest while siSOX4-2 failed to impact upon cell proliferation (**Figure 5.9**). Analysis of cell lysates demonstrated the expected clear loss of SOX4 protein with both sequences (data not shown). Similar results were also seen in 15 IMr cells (data not shown).

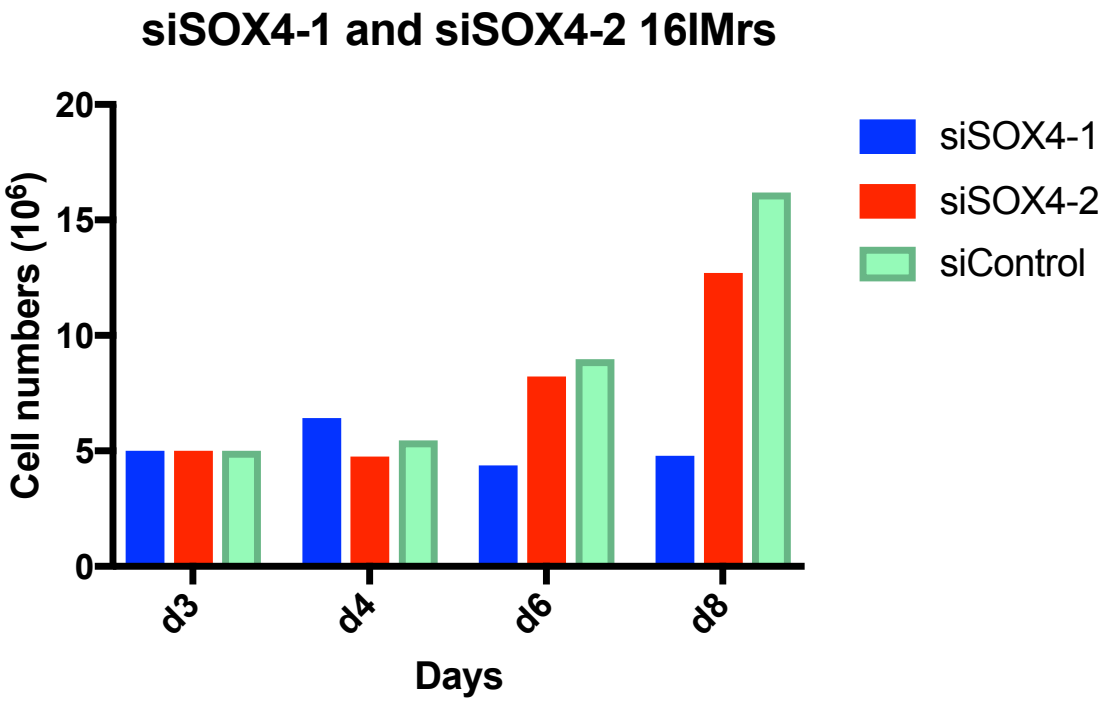


Figure 5.9: Depletion of SOX4 within 16IMr KCL22 cells using siSOX4-1 or siSOX4-2 sequences respectively.

Side-by-side comparison of SOX4 depletion with 16IMr cells using either siSOX4-1 (s13302) and siSOX4-2 (s13301) sequences. Cell viability was determined by trypan blue exclusion (d3, 4, 6 and 8). Data represents the results of one pilot experimental assay.

5.7 SOX4 and functional redundancy of the SOXC family

SOX4 is member of the SOXC family of transcription factors which consists of *SOX4*, *SOX11* and *SOX12* genes respectively. The family classification is based on the peptide sequence homology shared among each member and includes

the HMG-box (High Motility Group-box) DNA-binding region, (86% identity and 95% similarity), as well as the trans-activation domain located at the final 33 residues of the C-terminus (67% sequence identity and 94% sequence similarity) respectively [150, 152, 273] (**Figure 5.10**).

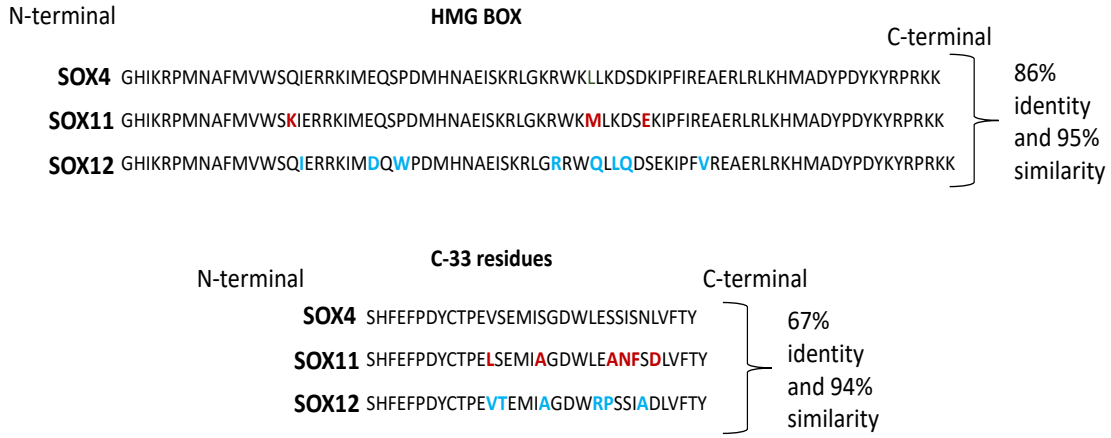


Figure 5.10: Protein sequence homology of the SOXC family members.

Peptide sequence comparison among the SOXC family member for the HMG Box (86% sequence identity and a 95% sequence similarity) and the 33 final residues of the transactivation domain (67% sequence identity and 94% sequence similarity). Red color letters indicate amino-acid changes in SOX11 in comparison to SOX4, blue color letters indicate amino-acid changes in SOX12 in comparison to SOX4. Redrawn and with information from Dy *et al.* 2008.[152]

Given the high degree of peptide homology among this family, it is not surprising to note that the SOXC genes have common gene-targets (via HMG-box). Depending on the cellular context they can functional redundantly among one another [274]; as exemplified by the *in vivo* generation of neural and mesenchymal progenitor cells and their subsequent development [152, 153, 275].

Based on these observations, it was hypothesized that functional redundancy among the SOXC genes could explain the contrasting phenotypes produced by the siSOX4 sequences. Under these circumstances, it is proposed that the siSOX4-1 sequence targets not only the SOX4 mRNA, but also affects SOX11 and-or SOX12 transcripts respectively. Here, the depletion of more than one family member results in the induction of growth arrest. On the other hand, the siSOX4-2 sequence has greater specificity and only targets the SOX4 mRNA

and its loss fails to result in growth arrest as it is being compensated by the functional redundant actions by SOX11 and/or SOX12 protein(s).

To explore this possibility, a nucleotide BLAST alignment of siSOX4-1 and siSOX4-2 was performed. However, there was no indication of cross-reactivity to either SOX11 or SOX12 (data not shown).

5.8 Expression of the SOXC family in clinical CML

If the SOXC family is functionally redundant in drug resistant CML, then additional support for this hypothesis should be evident from patient samples. To address this, a publicly available database (GSE4170) comprising of a microarray comparison between 42 chronic phase (CP) CML and 36 blast crisis (BC) CML bone marrow samples respectively was reviewed [69]. It is noted that this dataset is not of 'paired' samples with leukemic cells taken from the same patient at CP and subsequently upon relapse at BC. However, for simplicity the collective cohort of CP samples can be generalized as drug-sensitive (as IM therapy is working at this clinical stage) while BC can be considered a drug resistant population (patients fail to respond to IM treatment).

The database was viewed using the GEO2R tool in the GEO website for patient data acquisition. The expression profile of SOX4, SOX11 and SOX12 was extracted from the GPL2029 dataset, visualised in the GEO2R tool and the normalised expression values analysed on Prism 7® software for statistical purposes.

The relative, and normalised, expression of the SOXC family was compared between CP (sensitive) and BC (resistant) patient cohort. First, as previously performed, expression of SOX4 was significantly higher in BC (mean 0.31) compared to CP (mean 0) patients (**Figure 5.11a**). Of great interest, and in direct support of the hypothesis, the expression of both SOX11 and SOX12 was also significantly higher in BC patients compared to the CP counterpart. The expression of SOX11 had a mean of 0.02 within CP cohort and 0.34 in BC patients (**Figure 5.11b**). Similarly, expression of SOX12 had a mean of -0.04 in CP and 0.20 in BC population (**Figure 5.11c**).

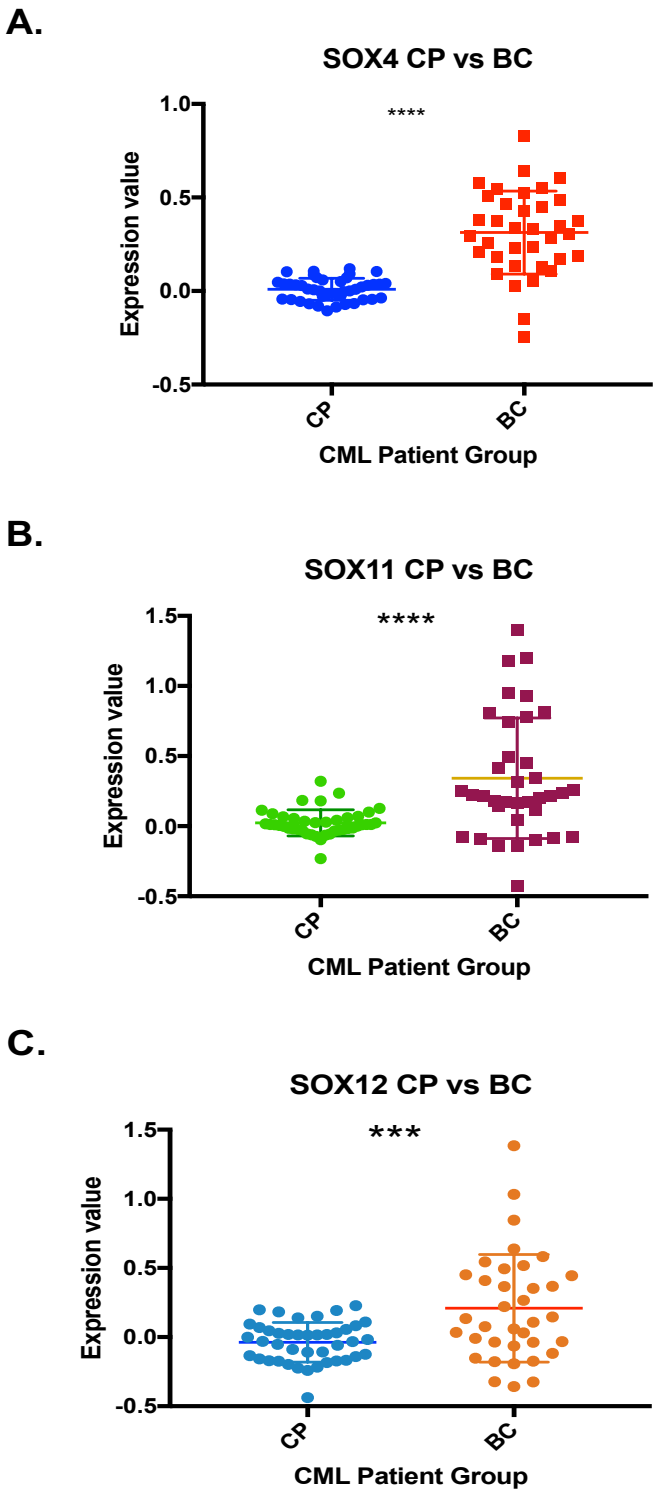


Figure 5.11: Profile of the SOXC expression in CP versus BC patients.

SOXC genes expression values in CP-CML vs BC-CML patients. (**A**) Comparison of expression of SOX4, (**B**) SOX11 and (**C**) SOX12. Horizontal lines indicate mean and error as SD. CP n=42, BC n=36. Unpaired t-test was used as a statistic. *** (p=0.0003), **** (p=0.0001).

In reviewing the relative expression of the SOXC members within a given patient group, it is interesting to note that (i) the spread of expression of each family member is relatively tight within the CP cohort while (i) within the BC population the range of SOX11 and SOX12 expression is quite broader to that of SOX4. This later observation may suggest that the expression of SOX11 and SOX12 within drug resistant patients is more inconsistent and their respective regulation could be defined by the relative expression of SOX4. In other words, within a given patient, whether the redundant SOX11 and SOX12 genes are induced (as compensation mechanism) could depend on the relative expression of SOX4.

To further test the functional redundant hypothesis, the analysis focused on BC patients that had low levels of SOX4 ($SOX4^{low}$). Here, a BC patient was designated $SOX4^{low}$ if the expression level was the same, or less than, that seen in the CP cohort (mean 0). From this analysis, 6 patients of the 36 BC cohort were considered to have $SOX4^{low}$ expression (range 0 to -0.25).

A prediction from the functional redundancy model is that if SOX4 is not induced in a BC patient (i.e. $SOX4^{low}$), then either SOX11 and-or SOX12 should be highly expressed as compensation. The expression of SOX11 and SOX12 was reviewed within the 6 BC patients with $SOX4^{low}$ expression (**Figure 5.12**). Of this defined, although limited, cohort, SOX11 had a broad range of expression but was nevertheless significantly higher that seen in the CP population. While expression levels of SOX12 was also similarly broad within the $SOX4^{low}$ patients, it was not significantly higher to that seen within the CP population.

In summary, and in strong support for the hypothesis of functional redundancy, the expression of all SOXC family members are significantly higher in BC drug resistant patients compared to CP drug-sensitive counterparts. Moreover, there is preliminary evidence to indicate that SOX11 is the likely redundant partner within drug resistant CML patients that fail to activate SOX4 during disease progression and transformation.

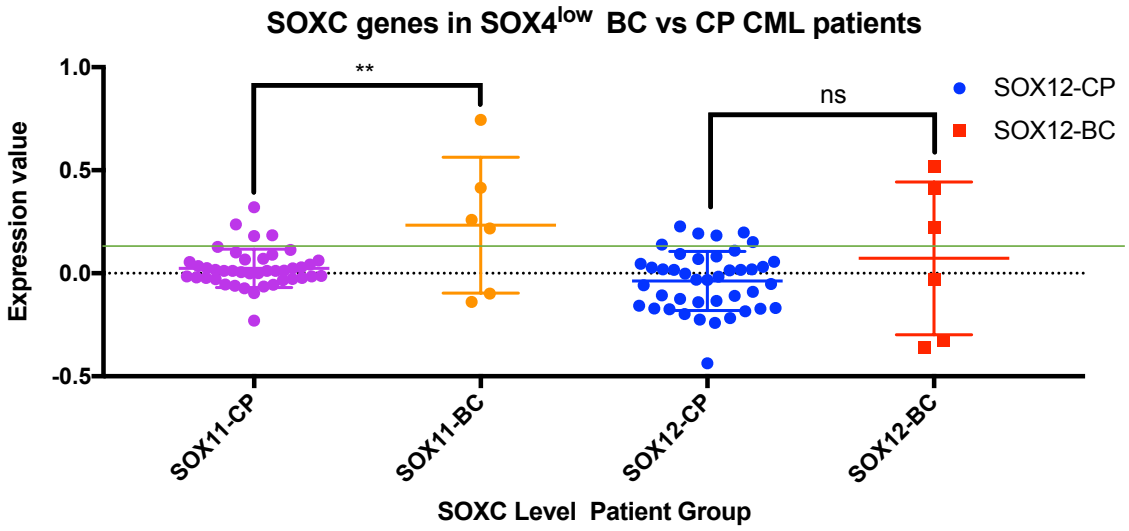


Figure 5.12: SOXC gene expression in SOX4^{low} BC patients.

SOXC genes expression of CP-CML vs SOX4^{low} BC-CML patients. SOX4^{low} BC patients were selected according to their expression levels similar or less to the highest expression in the CP cohort (expression value<0.12). For the populations, horizontal lines indicate mean and error as SD. Dotted black line indicates the 0 basal expression. The horizontal green line indicates the maximum expression of SOX4 (0.12) in the CP patient cohort. CP n=42, BC n=6. Unpaired t-test was used as a statistic. ** (p=0.0013).

5.9 Expression of SOX4 and SOX11, but not SOX12, is consistent in the KCL22-IMr phenotype

To investigate the potential redundant role of the SOXC family of transcription factors (SOX4, -11 and -12) in establishing drug resistance in CML, the expression profile of each member was examined within the KCL22 model. As previously described, the semi-quantitative PCR analysis demonstrated that upon drug resistance both KCL22 clones strongly induce the expression of SOX4. Interestingly, a similar pattern of gene expression was also seen for SOX11 (**Figure 5.13**). Notably, expression of SOX12 did not follow this pattern, and while it was induced in clone 15 upon drug resistance it was, however, repressed within clone 16. The inconsistent pattern of SOX12 regulation strongly suggests that it does not contribute to the proposed SoxC functional redundancy. Several biological repeats had replicated this pattern of gene expression of the SOXC family members (data not shown).

In summary, the profiled expression of the SoxC family identified both SOX4 and SOX11 as strong candidates for the development of tyrosine kinase independence in the KCL22 model.

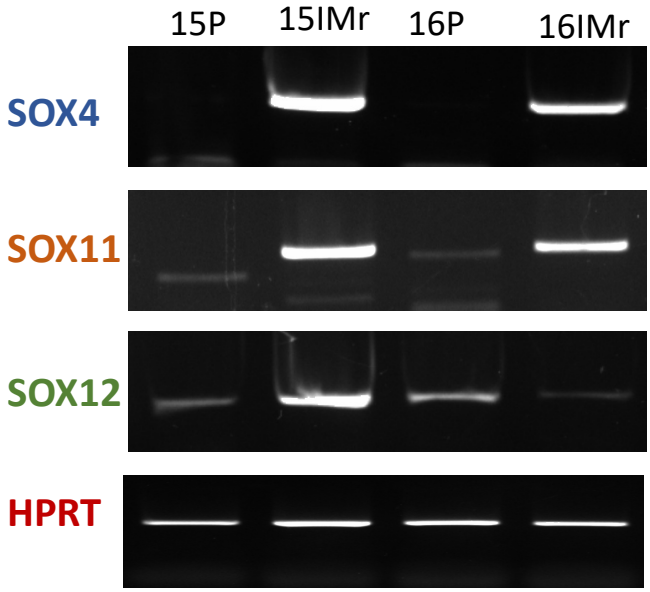


Figure 5.13: Expression profile of the SOXC genes within drug resistant KCL22 clones.

Semi-quantitative PCR analysis of the SOXC genes in the drug resistant KCL22 clones. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

5.10 Expression of the SOXC genes are regulated upon kinase-independent drug resistance in both the K562 and EM2 cell models.

The analysis of the (i) clinical CML dataset as well as (ii) profiling of the KCL22 model strongly support the notion that both SOX4 and-or SOX11 are regulated upon drug resistance and function in a redundant manner to regulate cell proliferation.

To further support this hypothesis, the expression profile of SOX4 and SOX11 transcripts was examined within the EM2 and K562 models of kinase-independent CML drug resistance. For the EM2 model (clone 7), expression of SOX11 was strongly induced upon drug resistance with no change to SOX4 and a reduction in SOX12 (**Figure 5.14**). As for the K562 cells, a different pattern of expression was noted with SOX4 induced upon drug resistance and no change to SOX12 with SOX11 being down-regulated.

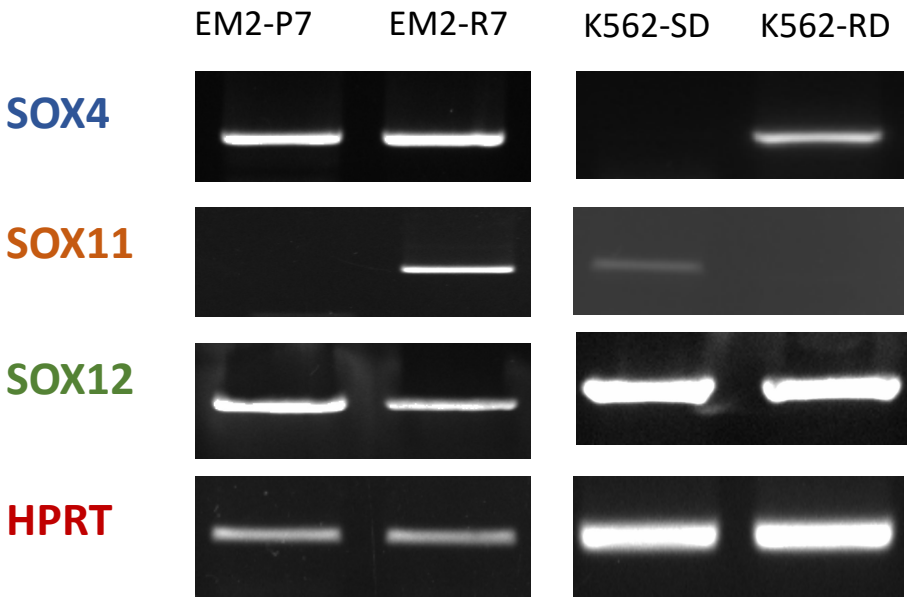


Figure 5.14: Expression of the SOXC genes in EM2 and K562 drug resistant lines.

Semi-quantitative PCR analysis of the SOXC genes in EM2 and K562 drug resistant models. HPRT was used as a loading control. Figure shows representative data of three independent experimental assays.

Although there was no consistency in the expression profile of any given SOXC among the three model systems (KCL22, K562 and EM2), it is noted that either SOX4 or SOX11 is consistently induced upon drug resistance respectively. Specifically, within the KCL22 both SOX4 and SOX11 are upregulated, with SOX11 activated in EM2 and SOX4 within K562 cells upon drug resistance respectively.

These observations support the proposed model of functional redundancy of the SOXC family with at least SOX4 or SOX11 being induced upon drug resistant CML. Further studies are required to clearly demonstrate the functional requirements of SOX4 and SOX11 within K562 and EM2 cells respectively with the model predicting growth arrest of the cells in loss-of-function studies.

5.11 Discussion

SOXC family and functional redundancy

The SOXC super-family of HMG-box transcription factors regulate various mammalian developmental processes [276, 277]. SOX4 has a primary role in haematopoiesis and is required for the development of the B-cell lineage [142, 278] and maintaining the population-pool of early multipotential progenitors (CD48+CD150-Lin-kit+Sca1+; MPPs) [141-143]. Deletion, or loss-of-function mutations, of SOX11 results in the onset of Coffin-Siris syndrome; a congenital disorder which is characterised by neurological defects including cranial malformation, developmental delay and limb malformations [158-161, 174]. Despite expression in the majority of tissues, SOX12-deficient mice develop normally with no overt phenotype reported to date [169].

By virtue of their shared DNA binding domain (HMG-box) all three family members recognise the cis-motif AACAAAG sequence and can regulate a common set of gene-targets [152]. Not surprisingly, genetic studies have demonstrated functional redundancy among the SOXC proteins [153]. The compound knockout of both SOX4 and SOX11 present with a more severe neurological defect than that seen in the SOX11 mutant alone [275]. Moreover, the triple knockout mice die at mid-gestation with a lack of facial and limb morphogenesis [153, 154, 174].

SOX4, SOX11 and Cancer

Given their fundamental role in development, it is not surprising that dysregulation of SOX4 and SOX11 are associated with cancer [279].

SOX4

Overexpression of SOX4 is associated with numerous human cancers including hepatocellular carcinoma, breast, and prostate as well as several blood cancers [135, 139, 280, 281]. Moreover, a meta-analysis performed on microarray profiles from 40 human cancers (consisting of 12 different cancer tissues) identified 67 genes that were frequently overexpressed in cancer [282]. SOX4 was included within this ‘cancer-gene signature’ suggesting a fundamental role of this factor in neoplastic transformation.

Although SOX4 is not required for normal myeloid development, its oncogenic role in the pathogenesis of myeloid leukemias has been reported. Increased expression of SOX4 transcripts is noted within a subset of human acute myeloid

leukaemia (AML) patients that harbor a mutated or silent *C/EBPα* oncogene, a mutation found in around 10% of AML patients [269]. Notably, this enhanced expression of SOX4 blocked myeloid differentiation and its downregulation resulted in an increase of self-renewal and restoration of granulocytic differentiation of the leukaemic AML cells *in vivo* [269].

Additional support for an oncogenic role of SOX4 in myeloid malignancies was demonstrated using mouse models whereby primary mouse bone marrow cells infected with a retroviral vector expressing SOX4 were transplanted into recipient mice resulting in the development of hematopoietic cancers such as lymphomas (B/T cell and splenic marginal zone) as well as myeloid leukemia [283-287]. In these studies, the development of myeloid leukaemia identified SOX4 to cooperate with other genes such as Spf1, Mef2C and CREB respectively.

SOX11

The role of SOX11 in cancer is contradictory – reports have identified both oncogenic and tumour-suppressor roles dependent on the type of malignancy. High levels of SOX11 expression is a poor prognostic indicator in basal-like breast cancers [288], gliomas [289] and haematopoietic malignancies such as Mantle cell lymphoma, Burkitt's lymphoma and B Cell Lymphoblastic Leukaemia [290]. On the other hand, increased SOX11 expression can promote neuronal differentiation in glioma cells and thus prevent the proliferation of undifferentiated tumour cells [291]. Similarly, overexpression of SOX11 can inhibit cell proliferation in B cell lymphomas [292] and is a good prognostic indicator in epithelial ovarian cancer [293].

Functional redundancy of the SOX4 and SOX11 genes within drug-resistant KCL22 cells

The data presented herein strongly suggest a (redundant) role of both SOX4 and SOX11 transcription factors in drug resistant CML. Analysis of three different, kinase-independent, drug resistance lines identify that expression of at least one of these genes is induced upon resistance (KCL22: SOX4 and SOX11, K562: SOX4 and EM2: SOX11). Moreover, this pattern of expression was corroborated in clinical patient (blast crisis versus chronic phase) samples.

Despite the overall findings, the discrepancy of the depletion of SOX4 within the KCL22 clones remains unresolved. On the presumption that the siSOX4-1

sequence had off-target effects and additionally targeting SOX11; two alternate models could account for the observed findings. First, the growth arrest induced by the siSOX4-1 sequence could results from the combined loss of both SOX4 and SOX11. Alternatively, the unintentional loss of SOX11 itself led to growth arrest.

Future studies should first determine whether siSOX4-1 does indeed result in the additional loss of SOX11 protein. The premise of SOXC functional redundancy within the KCL22 cells is based on this presumption; otherwise the observed growth arrest is simply an artefact of the siSOX4-1 sequence. Notwithstanding, on the basis that this presumption is correct, then the role of SOX11 itself should be explored. It would also be of keen interest to explore the individual role of SOX4 and SOX11 within K562 and EM2 drug resistant cells.

The focus of this chapter, SOX4 in drug resistant CML, originated from analysis of microarray dataset of the KCL22 clones. In context of the current findings, it is important to note that although semi-quantitative PCR analysis clearly establishes the induction of SOX11 transcripts within the drug resistant KCL22 cells (data presented herein), this pattern of expression was not identified within the array. While a probe for SOX11 is present within the array, it nevertheless failed detect any transcripts (data not shown). This highlights a common fault with the microarray platform where detection of transcript expression is dependent on probe hybridization. Depending on the probe-sequence and hybridisation conditions, false negatives (and positives) are common. Future work should avoid such concerns and use more sensitive and accurate techniques such RNA-Seq in all genome-wide expression analysis.

Epigenetic regulation of the SOX4 and SOX11 loci within drug-resistant KCL22 cells

While not a primary goal of this study, it is curious to consider how both SOX4 and SOX11 genes are regulated within the KCL22 cells during the transition from drug-sensitive to –resistant cell states. Previous work in the lab (G. Bheeshmachar) has analysed the 16P and 16IMr cells for epigenetic changes (i.e. genomic regions that have acquired or lost a chromatin mark) by chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq). The modifications examined were the H3K4me3 histone status (active chromatin mark) and CpG methylation, a repressive mark. Notably, changes in the

epigenome, including both CpG DNA methylation and histone modification, has been previously described in CML patients and are associated with disease severity, clinical onset of drug resistance and decreased patient survival [294, 295].

By interrogating the ChIP-Seq data (Integrated Genomics Viewer software; version 2.3.49), the chromatin profile of *SOX4* demonstrated epigenetic modification upon drug resistance (**Figure 5.15a**) In comparison to the chromatin status within the 16P cells, there was a loss of the repressive CpG methylation and gain of the active H3K4me3 mark over the *SOX4* loci within the 16IMr cells. These chromatin changes indicate that the *SOX4* gene is actively transcribed within the 16IMr cells and indeed correlate with the observed changes in SOX4 transcription.

Similarly, the profile of the *SOX11* loci identified the gain of H3K4me3 upon drug resistance with minor changes to the CpG status was identified **Figure 5.15b** As with the *SOX4* loci, these epigenetic changes predict the activation of SOX11 expression upon drug resistance and is in accordance with the observed semi-quantitative PCR analysis.

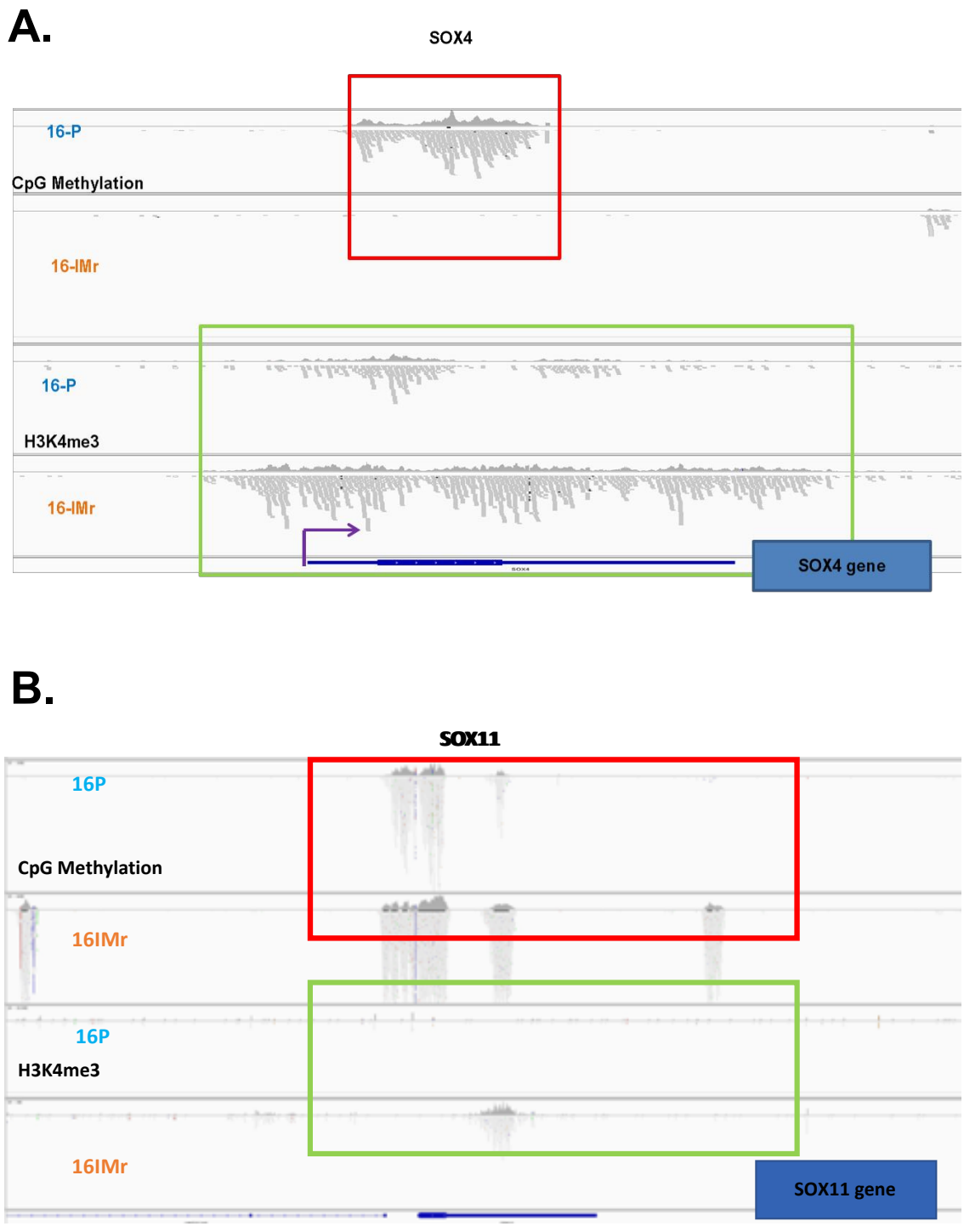


Figure 5.15: Epigenetic marks in the SOX4 and SOX11 loci in 16P and 16IMr cells.

ChIP-Seq analysis of the A) SOX4 and B) SOX11 loci in 16P and 16IMr cells respectively using IGV_2.4.6. Data was kindly provided by G. Bheeshmachar.

Collectively, these observations demonstrate that both *SOX4* and *SOX11* loci are epigenetically reprogrammed with these modifications acquired during the development of drug resistance. Moreover, the co-ordinated chromatin modification of these two loci provide additional support for the likely redundant role of these two transcription factors in the KCL22 model.

Why SOX4 can't be tolerated within KCL-22 cells?

Although SOX4 protein is clearly expressed in drug-resistant KCL22 cells, gain-of-function studies failed to generate over-expressing stables within parental 16P cells. This paradox has been previously encountered within the lab when attempting to stably over-express the RUNX1 protein within both 15P and 16P cells respectively. Similar to SOX4, the expression of RUNX1 is strongly induced within the drug resistant derivatives and its depletion also results in growth arrest associated with macrophage-like differentiation (data not shown). Additionally, like SOX4 protein expression, RUNX1 is essentially absent within the 15P and 16P cells respectively (data not shown). Yet, stable overexpression of RUNX1 within the parental KCL22 lines could not be accomplished despite several attempts, and the use of different vector-expression plasmids (data not shown).

The inability to expand 16P cells overexpressing SOX4 could be accounted for by two non-exclusive mechanisms; induction of apoptosis or deregulation of the cell-cycle machinery. While this was not directly examined in this study future work aims to address this issue. In brief, cells will be stained with Annexin-V shortly after transfection with the hSOX4-ires-GFP vector or the GFP-positive cells and assessed for apoptosis.

The pertinent question remains as too why SOX4 cannot be tolerated within the parental KCL22 cells. Given the parallel findings between SOX4 and RUNX1, a likely mechanism to account for these observations is dependent on how these transcription factors interact with the genome within the drug-sensitive (16P) and –resistant (16IMr) cells respectively. Noting that (i) both transcription factors are only expressed within the drug-resistant cells and (ii) the epigenetic landscape significantly changes upon drug resistance; it is proposed that the epigenome within the 16P cells is inaccessible for ectopically-expressed SOX4 (and RUNX1) to bind and activate the drug-resistant gene network. Moreover, this epigenetic state would direct these factors to occupy and regulate the apoptotic and-or cell-

cycle pathway(s) and thus account for the inability to expand when ectopically expressed within these cells. However, during the transition between drug-sensitive to –resistant the epigenome is modified accordingly whereby the promoters and enhancers of the drug-resistant network is now accessible. Reciprocally, the apoptotic and-or cell machinery pathways are in a closed-chromatin formation. Here, SOX4 (and RUNX1) can now interact with its respective target genes to regulate the drug-resistant gene network while concurrently hindered to occupy the apoptotic and-or cell-cycle pathway(s). Indeed, this model would account for why the transient expression of SOX4 failed to confer any drug-resistance.

Summary and future work

The work presented herein unravels a potential complexity for the redundant function of SOX4 and SOX11 within drug resistant KCL22 cells. While much work still needs to be done to validate this hypothesis, this notion is supported by several key findings. First, within two additional models of CML (K562 and EM2) the expression of either SOX4 or SOX11 is induced upon drug resistance. Secondly, the analysis of the clinical cohort provides clear evidence that both genes are expressed at higher levels in drug-resistant patients in comparison to drug-sensitive. Third, the toxicity of SOX4 within the drug-sensitive KCL22 cells suggests a unique function of this transcription factor within the resistant derivatives. Finally, both loci undergo epigenetic changes upon drug resistance with these acquired changes likely establishing the redundant function-expression of both factors.

Yet, despite the strength of these observations, the evidence is still correlative and future work must investigate the protein expression of SOX11 within the siSOX4 depletion studies. Specifically, the entire premise of the SOX4 and SOX11 functional redundancy is solely based on the presumption that SOX11 is also depleted when using the siSOX4-1 sequence. Failure to experimentally establish this would void all current speculations and validate the observation from the siSOX4-2 sequence whereby no overt phenotype is observed upon depletion of SOX4 protein within the drug resistant cells.

Conclusion and Summary

A long-standing challenge to CML pathology is the understanding of how leukemic cells are able to grow and survive in the absence of BCR-ABL1 kinase activity. How have the cells learnt to compensate for the loss of this once crucial kinase? The work presented herein puts forward a preliminary model which uncovers this complexity and focusses the attention onto the importance of acquired 'oncogenic drivers'. While early-haematopoietic progenitors are transformed by BCR-ABL1 and the oncogenic pathology of the clone is dependent on the master SH1-kinase activity (one oncogene 'rules all'), the establishment of drug resistance seems to be a highly co-ordinated response involving several independent oncogenic drivers (other BCR-ABL1 domains, Src and deregulated transcription factors) which work as a new gene-network in maintaining the leukaemic cell state. While more studies are required to confirm this hypothesis, these findings provide novel avenues of potential therapeutics for treatment of this pathology.

Leukaemic CML cells can utilise a variety of different molecular mechanisms to escape drug targeting. While cell models have been instrumental in deciphering these mechanisms, they nevertheless have taken the approach of bulk population analysis and little is known about drug resistance at the single-cell level. In addressing this, the view of CML as a 'clonal disorder' has come under scrutiny and evidence now suggests a greater heterogeneity to the disease as once thought. Such clonal heterogeneity spurs the concept of 'clonal-competition' and the establishment of a dominant drug resistant clone. These findings also provide some understanding as to the unpredictability as to which mechanism a given patient will clinically present. Moreover, the single cell modelling of drug resistance suggests a novel phenomenon that the choice of mechanism is genetically engrained within the leukaemic clone (even prior to exposure to any drug) and this provides another opportunity for therapeutic targeting. Finally, the greater understanding of the importance of the lineage-identity of the originally transformed clone and its impact upon drug resistance could stratify patient care, and understanding, and direct future therapy applications.

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