Chapter 3
Developing working siRNAs for members of the SCL erythroid complex in K562 cells

3.1 Introduction

3.1.1 The SCL erythroid complex and its downstream regulation

The SCL erythroid complex was first described in 1997 and contains at least 5 members (SCL, GATA1, E2A, LDB1 and LMO2) (Wadman et al., 1997). This complex recognises the consensus E-box and GATA motifs separated by approximately 9 nucleotides. SCL and E2A dimerise with each other and bind to the E-box motif while GATA1 binds to the GATA site. LDB1 and LMO2 act as bridges for the SCL/E2A heterodimer and GATA1 protein. Whereas the downstream regulation by some of the members of this complex has been widely studied, the regulation by SCL and its interacting partners in the SCL erythroid complex is poorly defined. Only three genes (glycophorin A, c-kit and α-globin) have been shown to be directly regulated by this complex in human erythroid cells (discussed in Chapter 1, Section 1.4.2.1 F). Therefore; the focus of this thesis is to further delineate and characterise additional transcriptional targets of the SCL erythroid complex in the erythroid lineage.

In previous studies, overexpression or targeted gene knockdown or knockout have been used to study the downstream targets of members of the SCL erythroid complex. Ectopic expression of SCL in transgenic mice has been shown to correlate with increase in c-kit expression (Lecuyer et al., 2002). GPA expression was shown to be dependent on the levels following ectopic expression of SCL in the human TF-1 cell line and in primary cells (Lahlil et al., 2004). siRNAs targeting SCL was used to study the requirement of SCL in endothelial differentiation and angiogenesis (Lazrak et al., 2004). The same RNAi sequence targeting SCL was used in a shRNA-expressing construct to study the regulation of some putative direct target genes of SCL in a T-ALL cell line Jurkat (Palomero et al., 2006). Global gene expression patterns were compared before and after the induction of GATA1 expression in the GATA1-null erythroblast cell line G1E-ER4 (Rylski et al., 2003; Welch et al., 2004) and between wild type and GATA1-deficient murine megakaryocytes (Muntean and Crispino, 2005). Ectopic expression of GATA1 in a non-erythroid cell line, U937, was shown to stimulate expression of its direct target GFI-1B (Huang et al., 2004). Conversely, siRNA knockdown of GATA1 was used to study its requirement for the auto-regulation of GFI-1B in K562 cells (Huang et al., 2005).

3.1.2 The RNA interference system

RNA interference (RNAi) is the endogenous pathway of suppression of gene expression at the post-transcriptional level. The use of RNA interference to silence transcription of genes is a powerful way to identify putative downstream target genes of transcription factors. Typically, the gene or transcription factor of interest is silenced by RNAi and the downstream effect is studied. Follow-up assays to study such downstream effects include conventional expression assays such as northern blotting and quantitative PCR and high-throughput genome-wide studies such as expression microarrays and sequencing. The type of assays chosen depends on what kind of information the researcher wants to generate.

3.1.2.1 Comparison between RNAi and traditional knockouts

Many different RNAi approaches have been used to silence genes in mammalian systems (discussed in Chapter 1, Section 1.3.1.2). Regardless of the approach of the RNAi trigger, RNAi has a number of advantages over traditional knockouts. Firstly, traditional knockouts usually require a number of rather complicated cloning steps for mammalian cells. In addition, stable integration and selection are also needed for the generation of knockouts. Secondly, complete elimination of a transcription factor may lead to lethality which makes subsequent analyses difficult. However, the major disadvantage of using the RNAi system is that 100% knockdown is hard to achieve and therefore, some putative downstream targets may not be detected.

3.1.2.2 Components of a good RNAi system

A number of factors should be taken into account when generating a good working RNAi system in mammalian cells. Some of the most important factors are discussed below.

(i) The RNAi system

Different types of RNAi triggers have been documented to generate efficient knockdowns in mammalian cells (Chapter 1, Section 1.3.1.2). These include the use of siRNA, shRNA and shRNA-mir. The choice of the RNAi trigger depends on the type of studies the researcher wants to perform and achieve. Typically, if a transient assay is sufficient, siRNA would be the best choice due to its ease of use and availability of validated siRNA sequences. If stable gene silencing is required for the study, shRNA and shRNA-mir would be required to generate stable transfectant.

During the design of siRNA or shRNA sequences, many considerations should be taken into account. These will be discussed in further details in Section 3.1.2.3. However, the target regions of the siRNA or shRNA should also be chosen with care. Some genes have a number of splice variants. Thus, when designing the regions to be targeted, a region common to all splice variants

(usually the 3' ends of the mRNA) would be more desirable so that all the splice variants are silenced in the assay. Conversely, if a particular splice variant is of interest, a region unique to the relevant variant should be used as the basis for designing the siRNA or shRNA.

(ii) The cell system and delivery strategy

To deliver siRNA or shRNA constructs into mammalian cells, various transfection methods can be employed dependent on the cell types (Chapter 1, Section 1.3.1.2). An optimal delivery strategy should be used for a specific cell type to achieve the highest possible knockdown efficiency. Regardless of the delivery strategy being used, the transfection efficiency should be monitored. This is because the transfection efficiency of siRNA or shRNA constructs directly affects the silencing level of the targeted gene. This can be done by transfecting a fluorescently-labelled siRNA or a shRNA construct expressing a fluorescent protein such as GFP and monitoring the fluorescence intensity. In addition, the effect of transfection on the cell should also be studied to identify any possible non-specific effects induced by the transfection method or the RNAi system. Studying the cell morphology or growth patterns of the cells are ways to determine such non-specific effects.

To trace the delivery of siRNAs into cells and test the transfection efficiency of the electroporation system, chemically synthesised siRNAs can be modified to have a fluorescent dye attached at the 3' or 5' end. Thus, when this labelled siRNA is transfected, cells containing the labelled siRNA will emit fluorescence which can be detected when the fluorophores bound to the siRNA molecules are excited by the appropriate laser. The choice of the fluorescent dye is crucial. Considering a siRNA duplex is a very small molecule, attaching a fluorescent dye with a high molecular weight may affect the uptake by the cells during electroporation and thus, such transfections may not truly mimic normal siRNA delivery. Because of the relatively low molecular weight of fluorescein (FITC), studies have demonstrated that attaching this fluorophore to the 3' end of siRNA duplexes will not reduce its efficacy in transfection (Holen et al., 2002). FITC labelled siRNAs have previously been used to monitor siRNA delivery by lipofectamine using fluorescence microscopy. It was demonstrated that delivery was the highest at 2-4 hours after transfection but diminished at later time points (Holen et al., 2002).

(iii) Minimizing non-specific effects

A number of non-specific effects have been described for RNAi (Chapter 1, Section 1.3.1.3). These non-specific effects should be minimised and monitored during the design of the RNAi experiment.

IFN response

To limit the IFN response, several considerations should be made during the design of RNAi experiments. Firstly, expression levels of IFN-stimulated genes (ISGs) should be monitored in any

RNAi experiments. IFIT1 is one of the most sensitive markers for ISG activation (Marques et al., 2006). Secondly, siRNAs should be of high purity and their concentration should be titrated to the lowest effective dose. Thirdly, earlier time points after induction of siRNA knockdown are preferable for studying their effects in perturbation experiments. This is because the hydrolysis of the 3' overhangs which leads to accumulation of blunt-ended siRNAs can trigger the IFN response, and these usually occur at later, rather than earlier, time points. Fourthly, certain sequence motifs such as UCUCU and GUCCUUCAA should be avoided in designing siRNAs as they have been shown to induce the IFN response in immune cells (Judge et al., 2005). Finally, for shRNAs expressed by a vector under the control of the H1 or U6 promoter, AA dinucleotide motifs near the transcription start sites should be avoided.

Off-target effects

Chemical modification of siRNAs where a 2'-O-methyl ribosyl substitution at position 2 of the guide strand has been demonstrated to reduce off-target effects (Jackson et al., 2006). This modification could also eliminate siRNA-induced undesirable and toxic phenotypes (Fedorov et al., 2006). In addition to siRNA design, other experimental parameters such as rescue and redundancy experiments should also be considered (Echeverri et al., 2006). Rescue experiments are performed by expressing a functional, though mutant, version of the target gene which is resistant to the siRNA trigger. If the phenotype can be rescued, one can be convinced that the phenotype is caused by a specific siRNA induction. Redundancy experiments involve the use to two or more siRNAs with different sequences raised against the same target gene. This can significantly reduce the probability that the resulted phenotype or gene expression changes are caused by off-target effects.

Saturation of RNAi pathway

Whereas saturation effects induced by siRNAs can be effectively suppressed by optimizing siRNA concentrations (Semizarov et al., 2003), expression of shRNAs in mammalian systems is more difficult to monitor as random integration of the expression construct can result in varied levels of shRNA expression. One solution to this problem would be to screen transfected cell lines for the copy number of integration events.

(iv) Functional validation

As different siRNA or shRNA generate different levels of gene knockdown, it is essential to evaluate the silencing capability before moving forward with large-scale experiments. Many different ways can be used to detect the expression changes in the gene being targeted at the mRNA and protein levels. These include northern blot, qRT-PCR, western blot, immuno-fluorescent (IF) studies, and fluorescence-activated cell sorting (FACS). These methods allow us to detect the expression of the endogenous gene and their advantages and disadvantages are summarised in Table

3.1. One of the biggest issues regarding the detection of changes in protein level is the availability of antibodies against the proteins of interest. Another option is to clone the targeted gene with an epitope tag (such as FLAG and MYC) so that the expression of level of the fusion protein can be detected using antibodies against the tag. This method circumvents the problem associated with requirement of good antibodies against the protein being targeted but requires the expression of an exogenous fusion protein.

Method	Detection level	Advantage	Disadvantage	Throughput
Northern blot	Endogenous mRNA	Easy	Non-quantitative RNA isolation	Low
qRT-PCR	Endogenous mRNA	Sensitive Quantitative	RNA isolation Primer design	High
Western blot, Immunofluorecence (IF), FACS etc	Endogenous protein	Easy	Antibody availability	Low
Western blot, Immunofluorecence (IF), FACS etc. on epitope tag	Exogenous fusion protein	Same antibody for detection	Cloning	High

Table 3.1. Commonly used assays for functional validation of mammalian RNAi.

qRT-PCR is particularly useful in the functional validation of knockdown as it is relatively fast and easy. Two major methods have been developed for qRT-PCR assays: the Taqman™ system and the SYBR Green system. Fluorescent reporter probes methods such as the Taqman™ system (AppliedBiosystems Inc.) is the most expensive but accurate way to quantify the PCR products. It involves the use of a probe which is specific to the amplified sequence and has a reporter fluorescent dye at the 5'end and a quencher dye at the 3'end. The probe, when unbound to any DNA, is non-fluorescent due to fluorescence quenching by the quencher dye when it is close to the fluorescent dye. However, when it binds to the target DNA during the PCR, the DNA polymerase (which has 5' to 3' exonuclease activity) cleaves the probe separating the fluorescent and quenching dyes leading to the emission of fluorescence.

Another type of probe is the DNA-binding dye such as SYBR Green (Morrison et al., 1998) which binds to double stranded DNA during the PCR amplification of the target DNA and emits fluorescence upon binding. The fluorescence can be detected with a real-time thermocycler. The intensity of fluorescence directly correlates with the exponential increase in the PCR products and can be determined by the threshold cycle (Ct). Thus, by comparing the experimental and reference samples, changes in expression level of genes can be detected. DNA-binding dyes are comparatively cheaper than the Taqman™ system but they are less accurate as they bind to any double-stranded DNAs including primer dimers.

(v) Knockdown effectiveness

The ">70% knockdown" threshold is considered to be a benchmark by which commercial providers of siRNAs assess whether a siRNA has been validated as a good working assay against a gene of interest. This threshold is also supported in the wider scientific community. In a RNAi screen of the PI3K pathway, a 70% knockdown efficiency was used as a cut-off for screening of effective siRNA assays (Hsieh et al., 2004). Subsequent proof-of-principle cell-based genetic screen showed that only siRNAs with >70% knockdown scored in a functional assay (Hsieh et al., 2004). However, even if a siRNA has a 70% knockdown efficiency at a specific time point after transfection, it may be possible to increase the efficiency by performing time-course experiments and identifying additional time points where the knockdown is at its highest level. However, one must also be aware, as mentioned in Chapter 1, that accumulation of blunt-ended siRNAs through time (as a result of the hydrolysis of the 3' overhang) will enhance the non-specific IFN response (Chapter 1, section 1.3.1.3 A). Therefore, it is also a common view that using an earlier, rather than a later, time point in perturbation studies reduces the likelihood of identifying non-specific effects at the level of gene expression. It has been shown, in at least one study, that a maximum knockdown was observed at the 24 hour time point for a human coagulation tissue factor (Holen et al., 2002), although this is likely to vary from gene to gene and between different siRNA assays for a given gene. Indeed, the time required for the maximum RNAi efficiency was shown to be proportional to the half-life of the target protein (Choi et al., 2005). Thus, all of these factors need to be considered when deciding on the appropriate experimental conditions to analyse the biological effects of siRNA knockdowns.

3.1.2.3 Advantages of using siRNA in the current study

In this thesis, transient knockdown of members of SCL eythroid complex by siRNAs was employed for a number of reasons, as follows:

(i) Rapid and easy of use: Transfection of small nucleic acid molecules, such as siRNAs, into mammalian cells is a relatively simple process. Delivery methods using lipofection and electroporation are well-developed and reagents and optimised protocols are available commercially.

(ii) Commercial siRNAs: Commercially- or custom- designed siRNAs are widely available (from companies such as Ambion, Dharmacon and Invitrogen) for most human and mouse genes. In some cases, functionally validated siRNAs can also be purchased which further facilitates the use of working siRNA assays for the gene of interest.

(iii) Optimisation of siRNA design: siRNA design is arguably the most critical step developing an effective knockdown assay for the gene of interest. One of the major advantages of using a siRNA

platform is that the experimenter can optimise the design of siRNAs. *In silico* design of siRNAs utilises different algorithms for sequence design and chemical modifications of the siRNA duplexes. Such optimisation can enhance their knockdown efficiency, reduce non-specific effects, improve their stability in culture systems and lower their cellular toxicity.

Many researchers have studied ways to optimise sequence design for generating effective siRNAs. Elbashir et al (2002) elaborated several guidelines for chemical synthesis of siRNAs (Elbashir et al., 2002) and these are summarised in Table 3.2. However, in addition to these rules, one should also take into account the secondary structure prediction of the mRNA and sequence comparison of the siRNA with the entire genome to reduce off-target effects. Some commercial companies, such as Ambion, also provide public resources for designing siRNA sequences (http://www.ambion.com/techlib/misc/siRNA_finder.html).

Length	19 nucleotides
GC content	30 to 70%
mRNA regions to target	Between 100 nucleotides from start codon and stop codon Avoiding 5' and 3' UTR
Overhangs	Two 3' 2-deoxythymidine residues

Table 3.2. Criteria for designing siRNAs (Elbashir et al., 2002).

Furthermore, chemical modifications have also been demonstrated to increase siRNA efficiency and stability, used either alone or in combination (Table 3.3).

Property	Modification
Increased thermal stability	2'-fluoro; 2'-O-methyl
Increased stability to digestion by nucleases	2'-fluoro pyrimidines; most chemically modified bases at the 3' and 5' termini
Reduced off-target effects	2'-O-methyl ribosyl substitution at position 2 of the guide strand

Table 3.3. siRNA properties that can be improved by the introduction of chemical modification (Corey, 2007).

(iv) Transient knockdown: Transient siRNA knockdown occurs from between 1 to 4-5 days after delivery of the siRNA to the cell where the mRNA level fully recovers after 4-5 days (Holen et al., 2002). This time interval is normally sufficient to experimentally observe transcriptional changes in downstream target genes. Such rapid changes in expression are often unobservable in stable knockdown cell lines, since a lot of selection and induction steps are required in order to obtain cell populations that display the knockdown phenotype.

3.1.3 The cell culture system under study

The human cell culture system used in this study is the erythroleukemic K562 cell line originally isolated from a chronic myeloid leukemia (CML) patient in blast crisis (Lozzio and Lozzio, 1977). It carries the BCR-ABL translocation and is thought to represent the common myeloid progenitor (CMP) stage of myeloid development which can give rise to both the megakaryocytic and erythroid lineages. It can be induced to erythroid differentiation by hemin and to megakaryocytic

differentiation by phorbol 12-myristate 13-acetate (PMA) (Huo et al., 2006). K562 cells have an approximate doubling time of 24 hours and it is well-characterised and studied for various aspects of haematopoietic function. Thus, its relevant biological characteristics, ease by which it can be differentiated and transfected makes K562 an excellent starting point from which to identify targets of the SCL erythroid TF complex.

K562 has been widely used in the study of erythroid development and transcriptional regulation of erythroid-specific genes. It was used in the study of GATA1 regulation of its target genes GFI1B and EKLF (Bose et al., 2006; Huang et al., 2005). This cell line was also used in the mapping of GATA1 binding sites along the β-globin locus (Horak et al., 2002) as well as in the study of histone modification and transcription factor binding in the α-globin locus (De Gobbi et al., 2007). These studies together demonstrated that K562 is a reliable system for the investigation of transcriptional regulation in erythroid cells.

3.2 Aims of this chapter

The aims of work presented in this chapter were:

1. To develop working siRNAs for five members of the SCL erythroid complex (SCL, GATA1, E2A, LMO2 and LDB1) in K562 cells.
2. To determine the effectiveness of the delivery strategy of the siRNAs into K562 cells.
3. To study the effect of these working siRNAs phenotypically as a function of the morphology and growth of K562 cells.
4. To characterise the efficacy of the siRNA knockdown in time-course experiments at the mRNA and protein level.

3.3 Overall strategy

Generating efficient knockdowns for each TF in the SCL erythroid complex were required for studying the transcriptional downstream target genes regulated by members of this complex (see Chapters 4, 5, and 6). To this end, a strategy was developed which allowed knockdown efficiencies to be monitored and studied in a variety of ways. The overall strategy is summarised in Figure 3.1. Firstly, commercially-designed custom-made siRNAs against each of five TFs (GATA1, E2A, LMO2, LDB1 and SCL) were transfected into cells by electroporation and knockdown efficiencies of the relevant mRNAs were measured by quantitative PCR (see section 3.4.1). At the same time, antibodies against the five TFs were tested and characterised in western blotting. Secondly, the efficiency of the delivery strategy was monitored by transfecting fluorescently-labelled siRNAs and

analysing the proportion of cells which showed fluorescence by FACS. Thirdly, physiological and morphological effects of siRNA-induced knockdown and transfections on cultured cells were also monitored to further characterise any gross phenotypic changes induced by the knockdowns. Finally, after identifying siRNAs which resulted in knockdowns by at least 70% of physiological mRNA levels, time-course experiments were performed over a period of 48 hours to study the changes in mRNA and protein expression during siRNA knockdown. This would allow timepoints to be identified at which the maximum knockdown efficiencies at the mRNA and protein levels were observed.

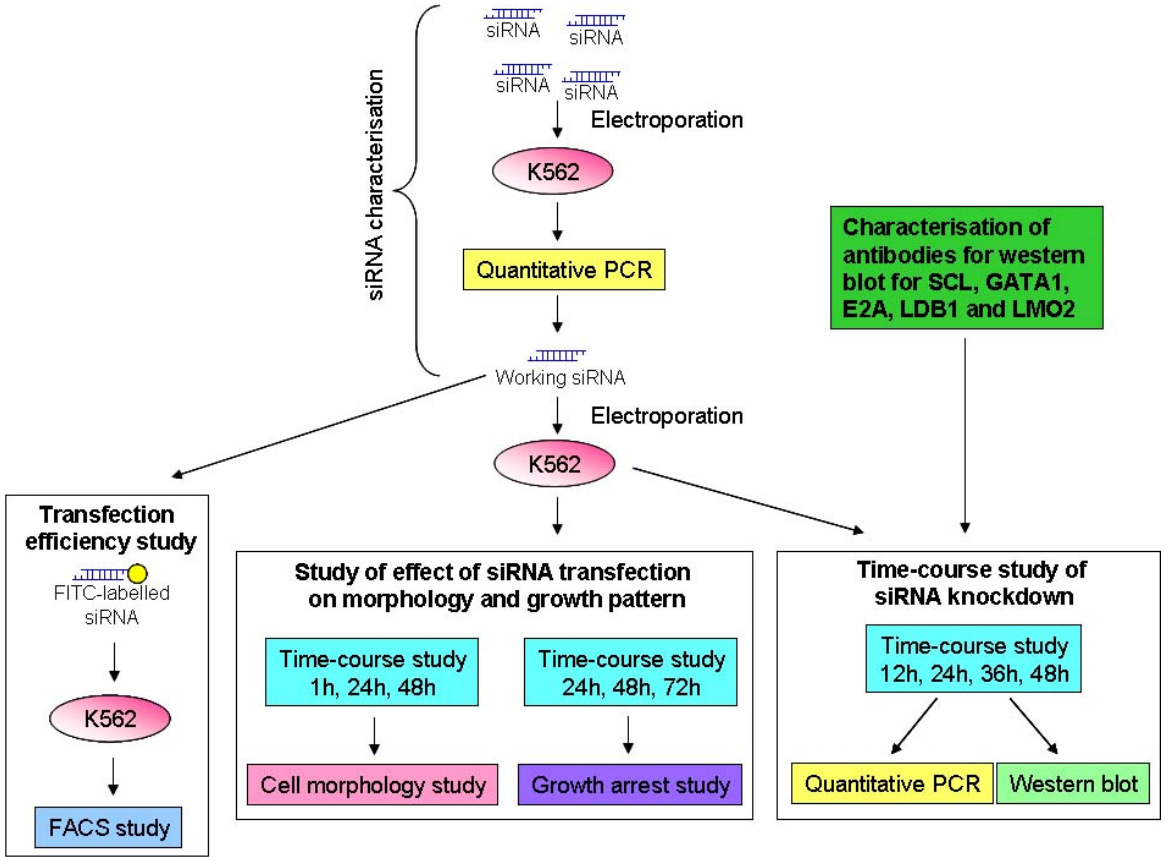


Figure 3.1. Overall strategy of siRNA knockdown analysis of TFs in the SCL erythroid complex. siRNAs and antibodies against each of five TFs were first characterised. The efficiency of the delivery strategy as well as the physiological and morphological effects of siRNA-induced knockdown were monitored. Time-course experiments were performed over a period of 48 hours to study the changes in mRNA and protein expression during siRNA knockdown.

3.4 Results

3.4.1 Developing working siRNA assays against TFs in the SCL erythroid complex (SEC)

A number of siRNAs were tested against each member of the SCL erythroid complex (SCL, GATA1, E2A, LMO2 and LDB1) to obtain siRNAs with high knockdown efficiencies. siRNAs were commercially designed and chemically synthesised by Eurogentec or Ambion without modifications. For all the five TFs under study, siRNAs were designed against a region common to

all transcript variants if applicable. Both of the commercial suppliers provided a guarantee service where replacements for custom siRNAs against any of the TFs tested were given when the siRNAs did not generate efficient knockdowns. For example, three siRNAs were supplied for each order and replacements were given so that at least two working siRNAs were obtained (please see below for criteria of working siRNAs). Transfections of siRNAs were performed using the Amaxa Nucleofector II. Amaxa has developed an optimised electroporation protocol for K562 cells which generates a transfection efficiency of more than 90% when monitored 24 hours after transfection (note: this efficiency is based on transfection of plasmids) (www.amaxa.com). To avoid any non-specific or stress responses (Semizarov et al., 2003), the siRNAs were transfected at final concentrations of 20 nM in the transfection media (although higher levels were shown in the Vetrie laboratory to result in similar knockdown efficiencies but with higher levels of non-specific effects; Philippe Couttet, unpublished observations) .

Knockdown at the mRNA level of each TF was monitored by quantitative real time PCR using SYBR assays at 24 hours after transfection. For each TF, two siRNAs targeting different regions of the gene with a knockdown efficiency of approximately 70% at 24 hours after transfection were chosen for further analyses. Using two siRNAs per TF lowers the likelihood of identifying off-or non-specific- targets in subsequent analyses (since the same off-targets are not normally found with two different siRNAs to the same gene; see also Chapter 1, Section 1.3.1.3 B). In addition, a siRNA against a gene which is not present in the human genome (firefly luciferase) was also tested and used as a negative control for all of the siRNA experiments performed for this thesis. Only one negative control siRNA was used as it does not target any regions in the human genome and should not elicit the off-target effect. Expression levels of the TF knockdown conditions were normalised against the expression levels found in the luciferase siRNA condition; this ensured that the changes in mRNA levels were due to the siRNAs targeting the gene of interest and not due to any generalised effects from electroporation or siRNA transfection. For a working siRNA to be chosen in the screening, less than approximately 30% of the mRNA level of the gene being targeted should remain 24 hours after transfection of the corresponding siRNAs at a concentration of 20 nM in the transfection media. Table 3.4 summarised the sequences, target exons and mRNA knockdown efficiencies of all the siRNAs tested. 16 siRNAs were tested in total and 11 of them passed the screening. Out of the 7 siRNA designed by Eurogentec (excluding the firefly pGL3 luciferase siRNA), 5 of them passed the siRNA screening. In contrast, out of the 6 siRNAs designed by Ambion, 3 of them passed. All the siRNAs which passed the screening (except those for LDB1) targeted the last exon. Among all the five TFs studied, the siRNAs for GATA1 gave the best knockdown efficiency with only 4 - 10% of the mRNA remaining after siRNA transfection. Figure 3.2 summarised the knockdown efficiency of all the siRNAs tested in the screening in one replicate.

Numbering for Figure 3.2	TF	Sources	Sense sequence (5' to 3')	Antisense sequence (5' to 3')	Exon	% of mRNA remained at 24 hour after transfection	Screening results	Designations
	Firefly Luciferase	Eurogentec	CUUACGCUGAGUACUUCGAtt	UCGAAGUACUCAGCGUAAGtt		---	---	LUC
1	SCL	D. Mathieu	GAAGCUCAGCAAGAAUGAGtt	CUCAUUCUUGCUGAGCUUCtt	4	29	Passed	SCLa
2	SCL	D. Mathieu	GGGAAUCACAUCUUUUAAGtt	CUUAAAAGAUGUGAUUCCCtt	4	31	Passed	SCLb
3	GATA1	Eurogentec	GGAUGGUAUUCAGACUCGAtt	UCGAGUCUGAAUACCAUCCtt	6	4	Passed	GATA1a
4	GATA1	Ambion	UGCGGAAGGAUGGUAUUCAtt	UGAAUACCAUCCUUCCGCAt	6	10	Passed	GATA1b
5	GATA1	Ambion	CAGGCCACUACCUACGCAAtt	UUGCAUAGGUAGUGGCCUGtc	6	71	Failed	---
6	E2A	Ambion	GGAAAAGGUGUCAGGUGUGtt	CACACCUGACACCUUUUCCtc	18	20	Passed	E2Aa
7	E2A	Eurogentec	CCUGGCUUAUUCUUCUAAAtt	UUUAGAAGAAUAAGCCAGGtt	18	16	Passed	E2Ab
8	E2A	Ambion	GCUCAAUGCCUGGUAUCUGtt	CAGAUACCAGGCAUUGAGCtg	18	100	Failed	---
9	E2A	Ambion	GCAGCCUGUUUGAAACGGCtt	GCCGUUUCAAACAGGCUGCtt	18	100	Failed	---
10	E2A	Ambion	GGUCUCCUUUUCUGGUCUUtt	AAGACCAGAAAAGGAGACCtg	18	30	Passed	---
11	E2A	Eurogentec	GUUCGGAGGUUCAGGUCUUtt	AAGACCUGAACCUCCGAACtt	2	39	Failed	---
12	LMO2	Eurogentec	CAAGCGGAUUCGUGCCUAUtt	AUAGGCACGAAUCCGCUUGtt	6	23	Passed	LMO2a
13	LMO2	D. Mathieu	GCAUCCAAGUGGCAUAAUUtt	AAUUAUGCCACUUGGAUGCtt	6	30	Passed	LMO2b
14	LDB1	Eurogentec	GGAUGGACCAAAGAGAUAUtt	AUAUCUCUUUGGUCCAUCCtt	5	16	Passed	LDB1a
15	LDB1	Eurogentec	CCUCCGACUCUGUGUGAUAtt	UAUCACACAGAGUCGGAGGtt	8-9	17	Passed	LDB1b
16	LDB1	Eurogentec	GGCAUUCCACAGCAACUUUtt	AAAGUUGCUGUGGAAUGCCtt	6	36	Failed	---

Table 3.4. Characterisation of siRNAs for knockdown of the SCL erythroid complex. The siRNA sequences, target exons and % of mRNA remaining at 24 hour after transfection are shown in the table. A siRNA passed the screening should be able to silence the target mRNA at a final concentration of 20 nM in the transfection media so that less than approximately 30% of the target mRNA remained 24 hour after transfection. The designation shows the symbols designed for each working siRNA which were used in subsequent analyses throughout this thesis.

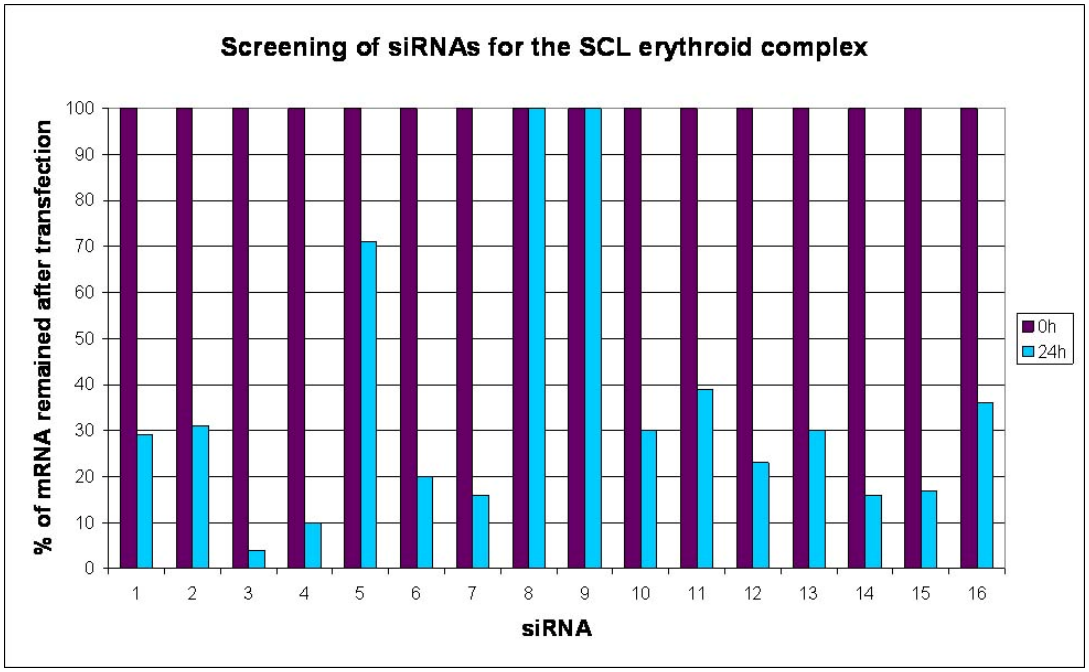


Figure 3.2. Screening of siRNAs for five members of the SCL erythroid complex. Y-axis: % of mRNA of the targeted gene remaining after transfection; x-axis: siRNA numbering as shown in Table 3.4. Figure shows results of one replicate in the initial screening

3.4.2 Characterisation of antibodies for western blotting analyses

The knockdown of specific mRNAs using siRNAs does not preclude that the effect is the same at the protein level. Thus, for the purposes of the work for this thesis, the time point after transfection when the maximum effects of a knockdown were observed were based on when the relevant proteins were reduced to their lowest levels in K562 cells. Therefore, it was necessary to develop western blot assays to quantify the protein levels for each of the TFs in the SCL erythroid complex before and after siRNA transfection in K562 cells. A number of commercially-available antibodies against each of the TFs were tested in western blotting assays with nuclear protein extracts of K562 cells. Antibody concentrations used in the initial western analyses were the highest recommended concentration by the manufacturers. However, it was also necessary to test some of the antibodies in appropriate dilution series to minimise background effects in the detection of the relevant proteins. All the antibodies tested and the how they performed in western assays were summarised in Appendix 3A.

For SCL, four antibodies were tested. The polyclonal antibody (Active Motif) detected a band on for the SCL protein which was approximately 45 kDa on western blots (Figure 3.3 B). The monoclonal anti-TAL1 3BTL73 antibody detected the appropriate band size (data not shown), whereas the unpurified sera generated a high background (Figure 3.3 C). The TAL1 Abcam antibody showed bands which were slightly higher in molecular weight than the expected SCL one

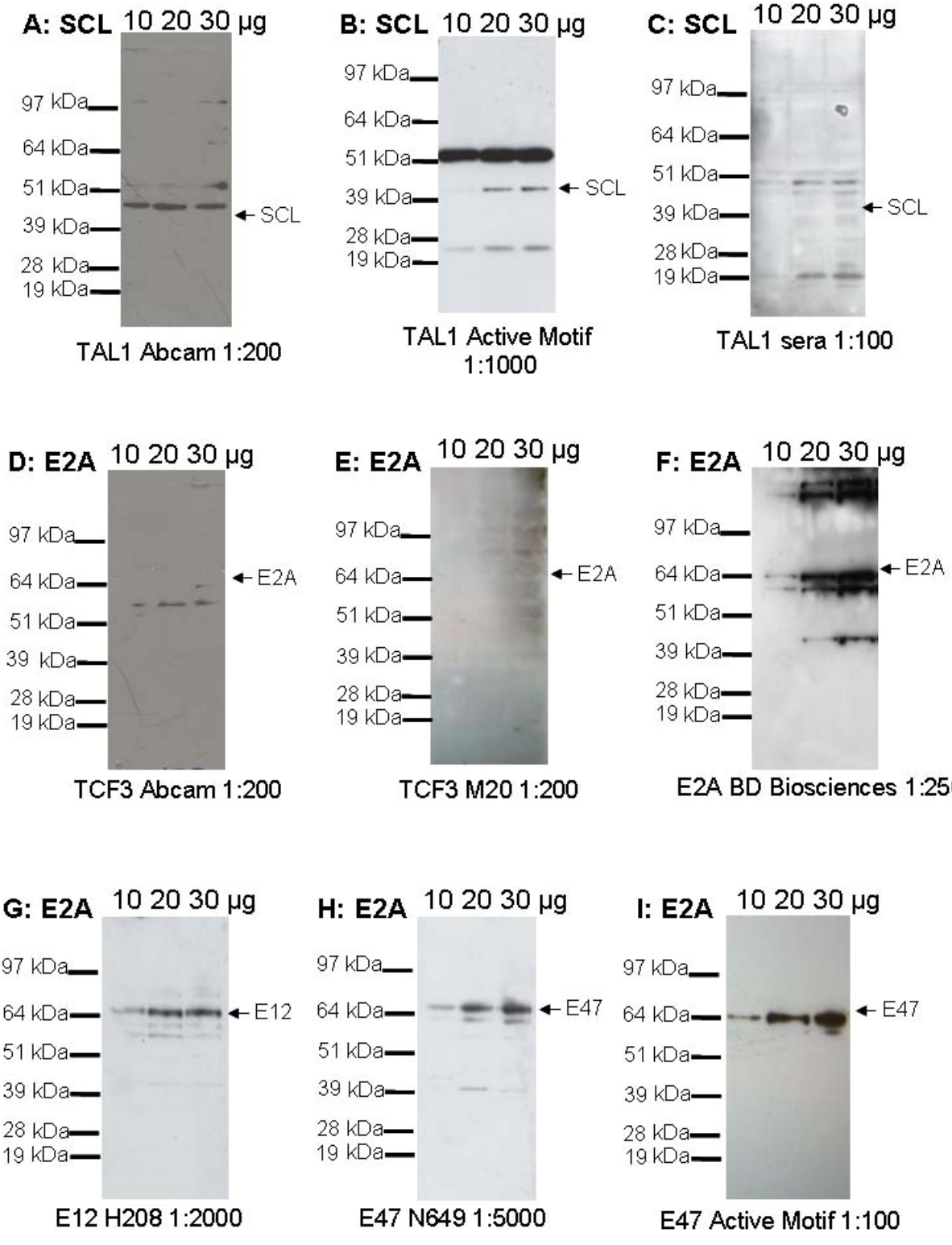
at 45 kDa (Figure 3.3 A). However, only the TAL1 Active Motif antibody showed a reduction in band intensity after knockdown by SCL siRNA (Section 3.4.5.1). Therefore, the Active Motif SCL antibody was used in subsequent analyses.

For E2A, seven antibodies were tested, including three which were raised against E2A (TCF3) and four raised against the splice variants E12 and E47. The TCF3 Abcam and TCF3 M20 Santa Cruz antibodies for E2A did not identify the predicted E2A protein band on western blots which was approximately 67 kDa in K562 cells (Figure 3.3 D and E). The E2A BD Biosciences antibody detected a band at approximately 64 kDa (Figure 3.3 F) and this band did not diminish in western analysis of E2A siRNA knockdown (Figure 3.4 A). Similarly, protein bands of approximately 64 kDa were observed for the E47 antibodies (Active Motif and Merck) (Figure 3.3 I and J respectively) but no reduction in band intensity was seen in siRNA knockdown (Figure 3.4 B and C respectively). Protein bands of the predicted size (67 kDa) for E12 and E47 protein were detected using the E12 H208 and E47 N649 antibodies (Figure 3.3 G and H respectively). The identities of these bands were confirmed in the knockdown experiment as they showed diminished intensities in E2A siRNA transfected cells (Section 3.4.5.3).

For GATA1, a polyclonal antibody from Santa Cruz gave a band of the predicted size of 45 kDa (Figure 3.3 K). This antibody generated very low background and did not cross-react with a closely related member of the GATA family, GATA2 (molecular mass of 51 kDa), which is also expressed in K562. The identity of the band observed in western analysis with this antibody was confirmed in the knockdown study (Section 3.4.5.2).

Similarly, an antibody for LDB1 from Santa Cruz was characterised which detected the LDB1 isoforms of the correct predicted size (43 kDa) (Figure 3.3 L). The identity of the band observed in this antibody was confirmed in the knockdown study where both isoforms were knocked down (Section 3.4.5.4.).

Three commercially available antibodies were tested for detection of the LMO2 protein which is approximately 18 kDa (Figure 3.3 M to O). However, none of them detected bands of the predicted size. Furthermore, high background and non-specific bands were detected even under both reducing and non-reducing electrophoresis conditions. One possible reason for the inability to detect this protein by western analysis is that the expression level of LMO2 in K562 cells may be below the limits of detection. However, it is equally likely to be an issue with the performance of the antibodies.


A: SCL 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
SCL
TAL1 Abcam 1:200
B: SCL 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
SCL
TAL1 Active Motif 1:1000
C: SCL 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
SCL
TAL1 sera 1:100
D: E2A 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
E2A
TCF3 Abcam 1:200
E: E2A 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
E2A
TCF3 M20 1:200
F: E2A 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
E2A
E2A BD Biosciences 1:250
G: E2A 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
E12
E12 H208 1:2000
H: E2A 10 20 30 µg
97 kDa
64 kDa
51 kDa
39 kDa
28 kDa
19 kDa
E47
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19 kDa
E47
E47 Active Motif 1:100

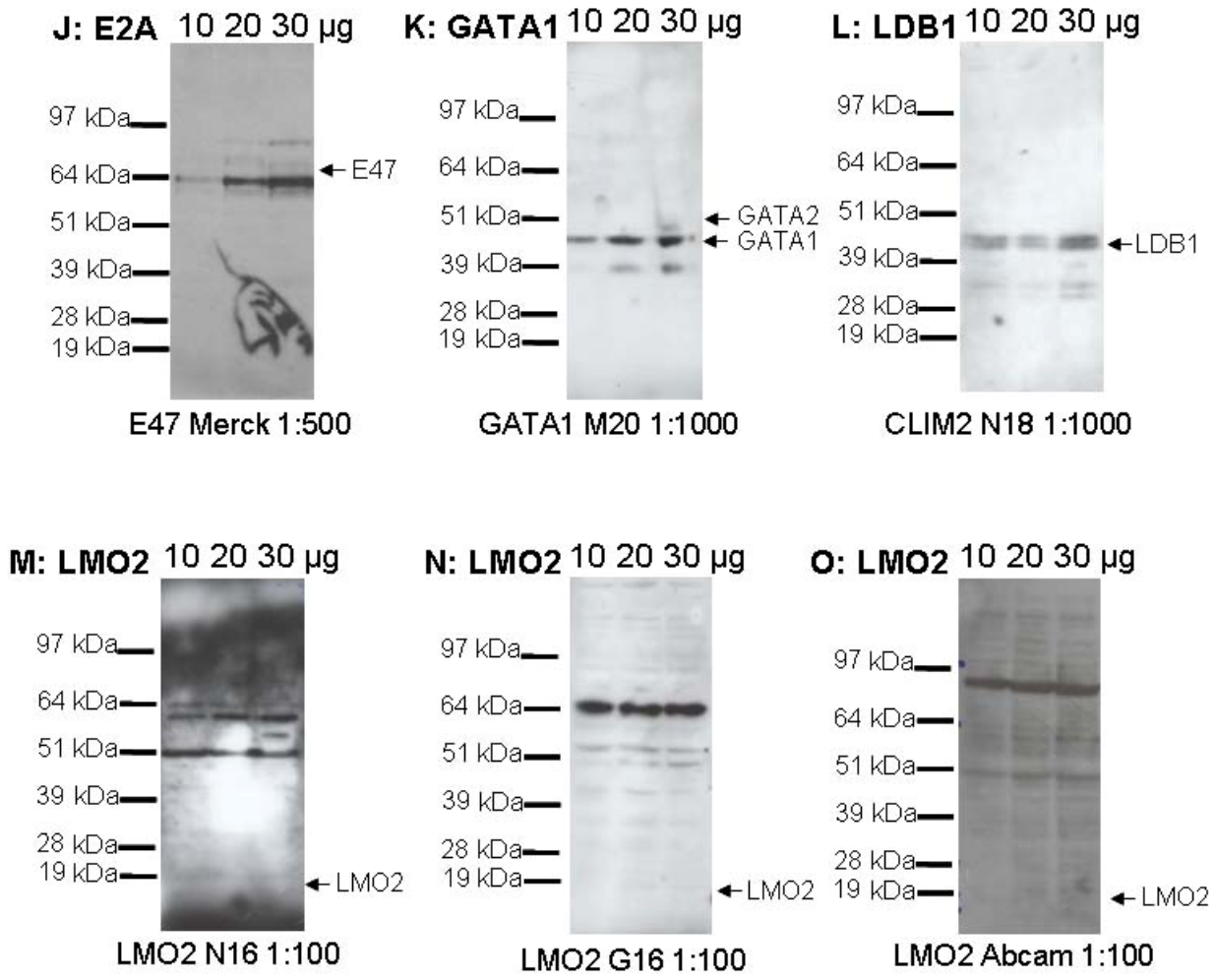


Figure 3.3. Western blotting analyses for the characterisation of antibodies against members of the SCL erythroid complex (SEC). 10, 20 and 30 µg of K562 nuclear protein extracts were used for western blot analyses to characterise the antibodies. SDS-PAGE was performed under denaturing and non-reducing conditions except for the results shown in panel B where reducing condition were used. In the analyses of LMO2 antibodies, both reducing and non-reducing conditions were tested. Each panel shows the x-ray films developed by chemiluminescence. The commercial names and dilutions of antibodies used are stated at the bottom of each panel. The dilutions used for western analyses were the recommended dilution from the company and they were titrated for optimisation in those working ones (panels B, G, H, K and L). The arrows on the right of each blot indicate the predicted protein size of the transcription factor under study. Size markers are shown on the left of each panel. A: TAL1 Abcam antibody; B: TAL1 Active Motif antibody; C: TAL1 2BTL73 anti-sera; D: TCF3 Abcam antibody; E: TCF3 M20 Santa Cruz antibody; F: E2A BD Biosciences antibody; G: E12 H208 Santa Cruz antibody; H: E47 N649 Santa Cruz antibody; I: E47 Active Motif antibody; J: E47 Merck antibody; K: GATA1 M20 Santa Cruz antibody; L: CLIM2 (LDB1) N18 Santa Cruz antibody; M: LMO2 N16 Santa Cruz antibody; N: LMO2 G16 Santa Cruz antibody; O: LMO2 Abcam antibody.

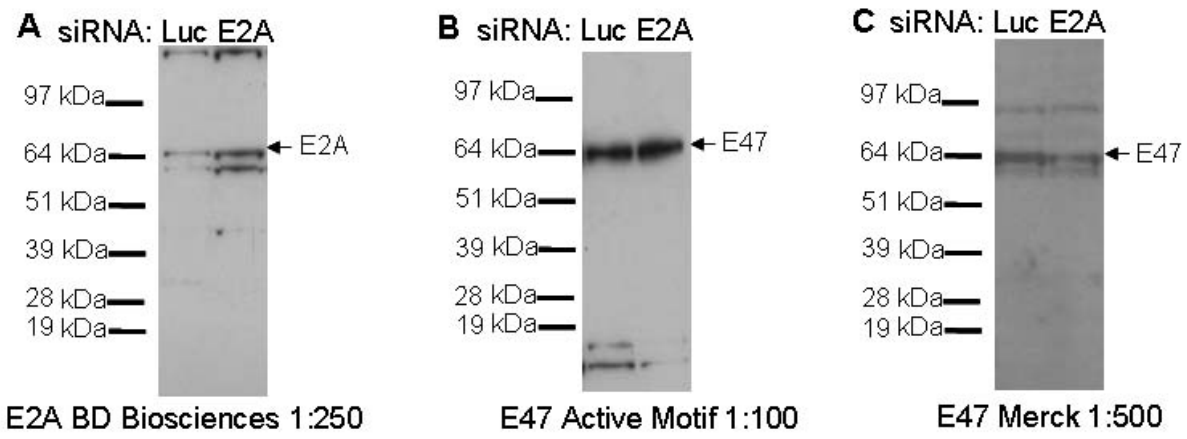
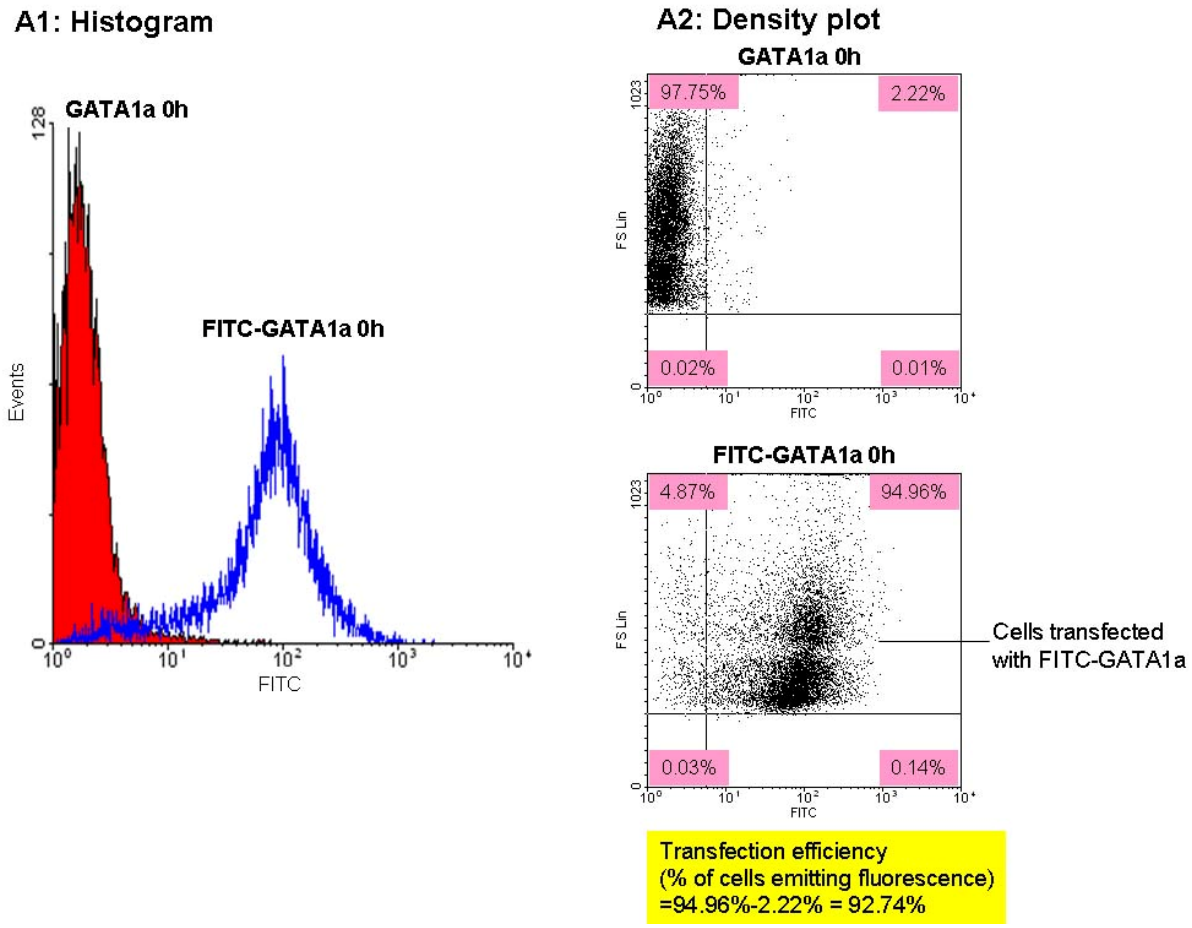


Figure 3.4. Western blot analyses for E2A knockdown. Nuclear proteins were extracted from firefly luciferase siRNA and E2Aa siRNA transfected K562 cells at 24 hour. 30 µg of nuclear protein extract were used for western blot analyses to characterise the E2A or E47 antibodies. SDS-PAGE was performed under denaturing and non-reducing conditions. Each panel shows the x-ray films developed by chemiluminescence. The commercial names and dilutions of antibodies used are stated at the bottom of each panel. The dilutions used for western analyses were the recommended dilution from the company. The arrows on the right of each blot indicated the predicted protein size of E2A or E47. Size markers are shown on the left of each panel.

3.4.3 Determination of the transfection efficiency of siRNAs

After working siRNAs were selected for each TF according to the criteria described above, the efficiency of delivery of the siRNAs into K562 cells by electroporation was studied. It was important to perform these experiments, since the K562 electroporation procedure used here was developed and validated by Amaxa using plasmids, not siRNAs. To this end, the efficiency of transfection was monitored using a 3' fluorescein (FITC)-labelled GATA1a siRNA (one of the GATA1 siRNAs which was used in further studies in this thesis). This experiment would be used as a model to provide evidence that the transfection efficiency was not a limiting factor in obtaining good knockdowns using the siRNAs for each TF. Fluorescein was used as a tag due its relatively low molecular weight compared to other fluorophores. Thus, the effect of the fluorescein tag on the transfection efficiency of the labelled siRNA could be minimised. FITC-labelled and unlabelled GATA1a siRNAs were transfected into K562 cells and aliquots of cells from both conditions were taken 24 hours after transfection. The cells were subjected to FACS and the proportions of cells carrying fluorescence were determined. The percentage of cells emitting fluorescence in the transfection of FITC-GATA1a siRNA was compared to the background of cells transfected with unlabelled GATA1a siRNA. There was a significant shift in the detection of fluorescence in the FITC-GATA1a siRNA transfected cells compared with GATA1a siRNA transfected cells at both 0 hour and 24 hour time points (Figure 3.5 A1 and B1). By setting the fluorescence intensity of the GATA1a siRNA transfected cells as the baseline in the density plot, percentages of cells which

carry FITC fluorescence were calculated for 0 hour and 24 hour time points (Figure 3.5 A2 and B2). The percentage of FITC-GATA1a cells emitting fluorescence was approximately 93% at the 0 hour time point and 84% at the 24 hour time point. It was also noted that the overall FITC fluorescence of the FITC-GATA1a transfected cells was higher at 0 hour than at 24 hour. The transfection efficiency of the FITC labelled siRNA (93%) is consistent with the knockdown efficiency of the GATA1a siRNA (approximately 4% of GATA1 mRNA remained after transfection) (Table 3.4). Taken together, the Amaxa Nucleofector II system provided a high transfection efficiency in K562 cells which was consistent with the knockdown efficiency of the corresponding siRNA. Therefore, transfection efficiency *per se* was not a limiting factor in obtaining knockdowns for the relevant TFs reported in this thesis.



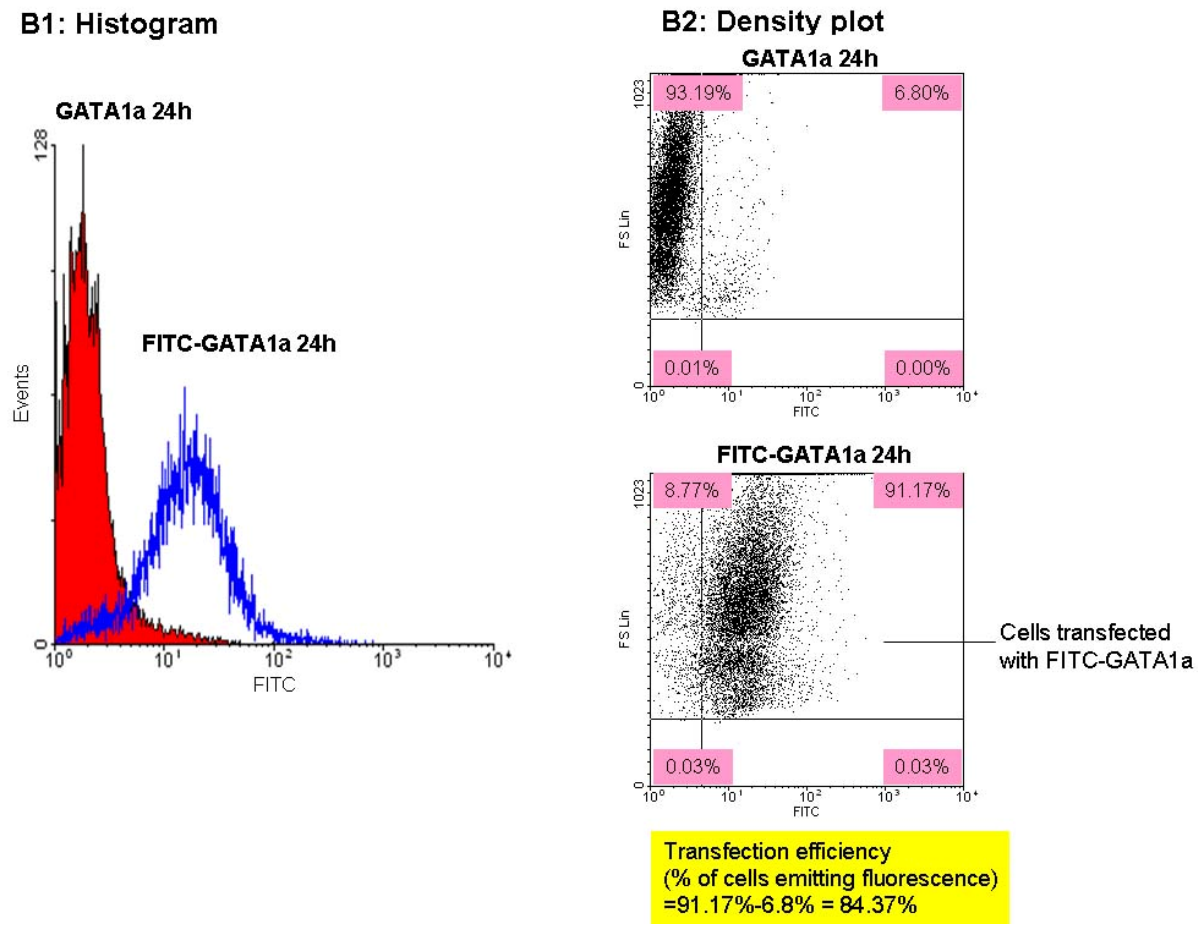


Figure 3.5. Flow cytometric analysis of the FITC labeled siRNA transfection into K562 cells. A) 0 hour and B) 24 hour. Panels A1 and B1 show histogram plots of GATA1a (red curve) and FITC-GATA1a (blue curve) transfections with the y-axis showing the number of cells (events) and x-axis showing FITC intensity. Panels A2 and B2 show density plots of GATA1a (top) and FITC-GATA1a (bottom) transfections with the y-axis showing the forward scatter (an indicator of cell size) and x-axis showing FITC intensity. Numbers shown in pink boxes show the percentage of cells in each of the quadrants. Top left quadrants contain cells emitting background fluorescence while top right quadrants contain cells emitting FITC fluorescence. Transfection efficiencies were calculated as described in the yellow boxes: transfection efficiency = % of cells in the top right quadrant in the FITC minus the control.

3.4.4 Changes in K562 cell growth and morphology induced by siRNA transfection

To determine whether electroporation of siRNAs had any physiological effects on K562 cells which were not specific to the knockdown of the TFs, changes in growth rates and morphology of K562 cells were assessed before and after transfection. For growth rates studies, the total number of K562 cells were determined at four time points (0, 24, 48 and 72 hours) after transfection with the same numbers of cells under each of the following conditions: a) no transfection; b) transfection with water; c) transfection with the luciferase siRNA and d) transfection with the GATA1a siRNA. In the case of the 0 hour time point, cells were harvested and counted immediately after transfection (or at the same time equivalent for cells that were not transfected). Transfection with water was included to investigate any effects solely due to electroporation itself rather than due to siRNA

transfection. In turn, transfection with luciferase siRNA was used to study any effects induced by the RNAi pathway which were not specific to the TF of interest (in this case, GATA1). As in section 3.4.3, GATA1a siRNA was used as the model for these studies. The number of viable cells at each of the 24, 48 and 72 hour time points (determined by trypan-blue staining) was determined relative to the 0 hour time point and plotted against time (Figure 3.6). In this way, cells which died as a result of electroporation were not included in the calculations. There were observable decreases in the growth rates of K562 cells at 24, 48 and 72 hour transfection conditions compared with the equivalent conditions for untransfected cells. The slope of the curve indicated how rapidly the cells were dividing. The effect on growth rate was the greatest between 0-24 hours as the slope was the smallest in the transfected cells compared to the wild type cells. After the 24 hour time point, the effect on growth rate diminished as the slopes were more similar between the wild type and transfected cells. Moreover, the growth patterns for transfections with water, luciferase siRNA and GATA1a siRNA were very similar at all time points. This indicates that the growth arrest observed in K562 cells was an effect of electroporation but not due to effects of siRNA transfection or induction of the RNAi pathway.

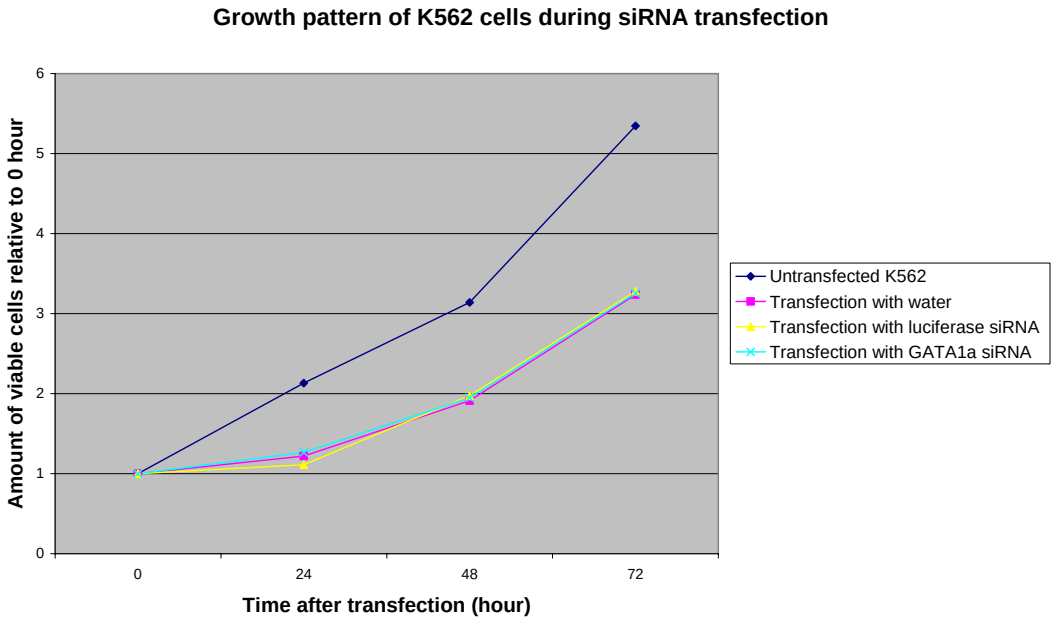
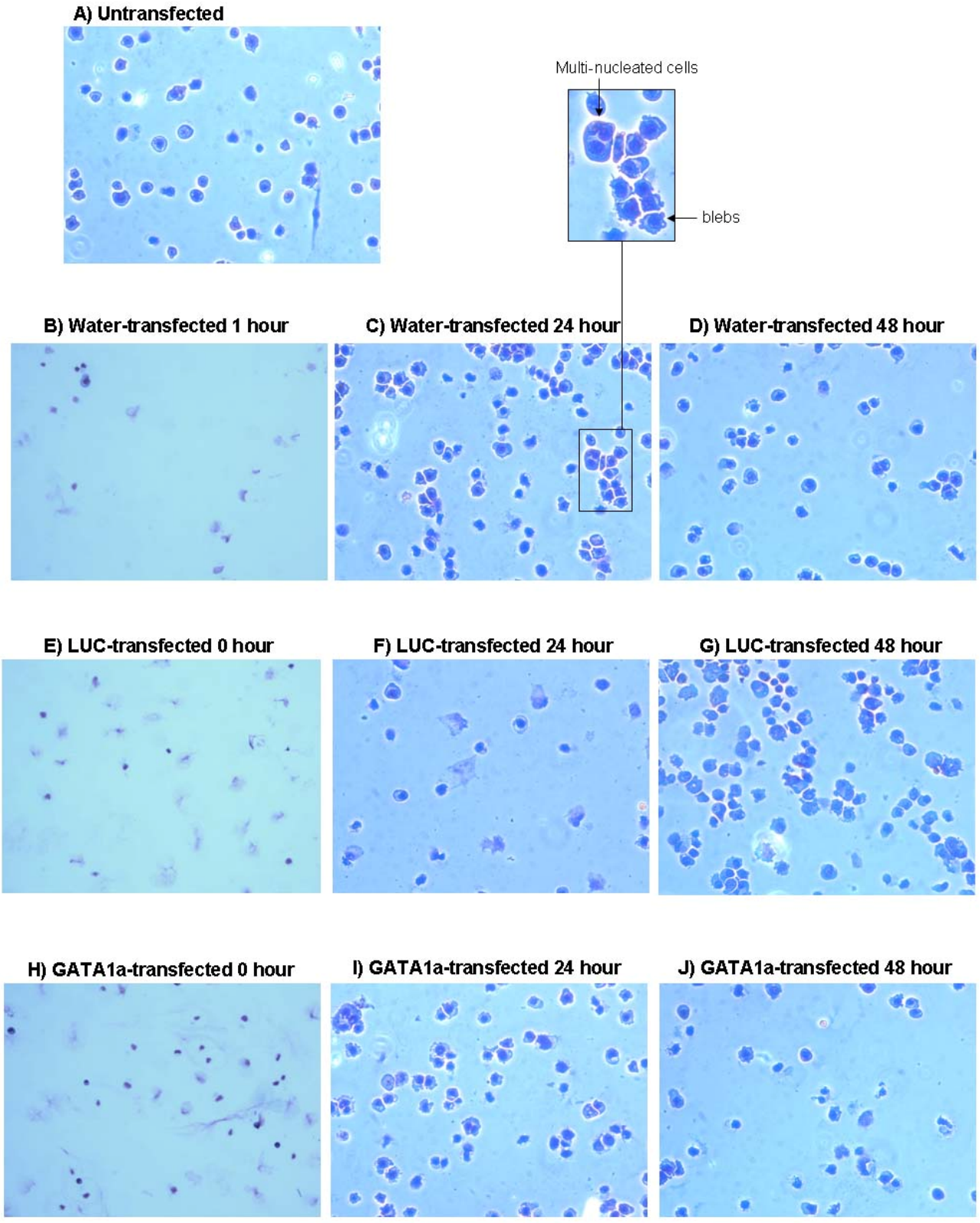


Figure 3.6. Growth pattern of K562 cells during siRNA transfections. The growth patterns of K562 cells under different transfection conditions were studied. Dark blue curve: untransfected K562 cells; pink curve: K562 cells transfected with water; yellow curve: K562 cells transfected with the luciferase siRNA; light blue curve: K562 cells transfected with the GATA1a siRNA. The graph shows the number of viable cells at different time points after treatment relative to the 0 hour time point. Results shown were average of two independent biological replicates.

Similarly, changes to cell morphologies of K562 cells under various transfection conditions were also studied. In addition to the various controls (no transfection, transfection with water, and

transfection with the luciferase siRNA), the effects of siRNAs against either GATA1 (GATA1a) and E2A (E2Ab) were monitored at three time points (1 hour, 24 hour and 48 hour) after transfection. As growth characteristics of cells were difficult to assess objectively, the experiment was performed blind and the E2Ab siRNA was also included as an additional control to monitor for any effects induced by siRNAs against specific TFs. Cells were harvested, spun down on a glass slide using a cytospin, stained with Stain Quick-Staining Kit (Lamb) and characterised under a light microscope. Untransfected cells were collected at the 0 hour time points, while transfected cells were allowed to recover from transfection and only collected at the 1 hour, 24 hour and 48 hour time points (the 1 hour time was used as cells at the 0 hour time point were considered to be fragile and often burst during preparation with the cytospin). Cells with different morphologies including cells with small blebs, large blebs, 2 nuclei and more than 2 nuclei were quantitated in a blind test. Wherever possible, 100 cells of different morphologies were counted by two independent scientists. The percentages of cells having different morphologies were calculated (Figure 3.8).

The untransfected cells were round, their cell membranes were smooth without any projections and were primarily mono-nucleated (Figure 3.7 A). The transfected cells, on the other hand, under all four transfection regimes, were smaller and had noticeable blebs (projections) of a variety of sizes on the cell membranes at both the 24 hour and 48 hour time points. For the transfected cells, more multi-nucleated cells were also observed (Figure 3.7 B-I). No differences were observed between the various transfection conditions. Thus, changes in the morphologies of K562 cells during transfection were mainly due to electroporation, and not as a result of siRNA transfection or induction of the RNAi pathway. This was further supported by the results of the blind test (Figure 3.8). Only less than 5% of wild type cells were shown to have blebs or be multi-nucleated whereas up to more than half of the cells were shown to possess these morphologies in the transfected cells, particularly at the 48 hour time point. It should be noted from Figure 3.8 that the blind counts at the 1 hour time point was not representative as the majority of the cells were dead after cytospin and only a small number of cells could be counted. These results, taken together with those described for the growth arrest studies, suggest that, at the level of gross morphology and growth, off-target or side-effects of the siRNA studies performed for this thesis could largely be attributed to the effects of electroporation.


A) Untransfected
Multi-nucleated cells
blebs
B) Water-transfected 1 hour
C) Water-transfected 24 hour
D) Water-transfected 48 hour
E) LUC-transfected 0 hour
F) LUC-transfected 24 hour
G) LUC-transfected 48 hour
H) GATA1a-transfected 0 hour
I) GATA1a-transfected 24 hour
J) GATA1a-transfected 48 hour

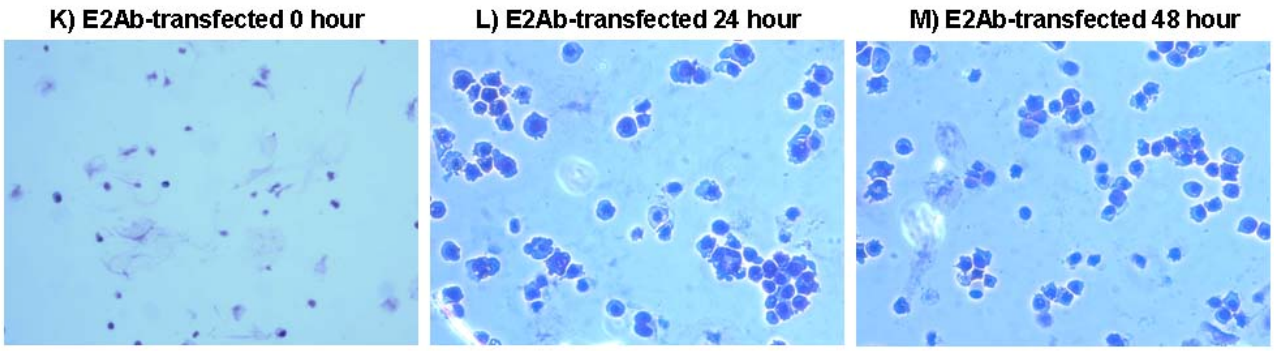


Figure 3.7. Cell morphological studies of K562 cells during siRNA transfection. K562 cells were untransfected or transfected with different conditions and collected at different time points. A: untransfected K562 collected at the beginning of experiment (0 hour); B, C and D: K562 cells transfected with water only and collected at 0 hour, 24 hour and 48 hour time points respectively; E, F and G: K562 cells transfected with luciferase siRNA and collected at 0 hour, 24 hour and 48 hour time points respectively; H, I and J: K562 cells transfected with GATA1a siRNA and collected at 0h, 24 hour and 48 hour time points respectively; K, L and M: K562 cells transfected with E2Ab siRNA and collected at 0 hour, 24 hour and 48 hour time points respectively. Multinucleated cells and cells with blebs are shown in the zoomed-in window for figure C.

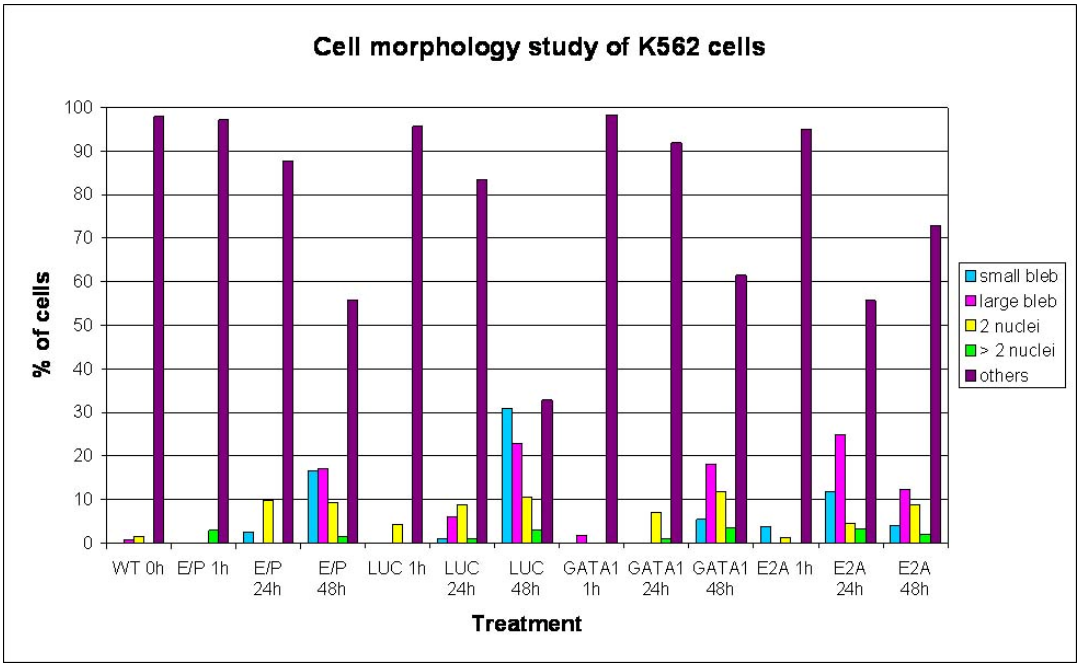


Figure 3.8. Quantitative analysis of cell morphology studies of K562 cells. Approximately 100 cells were counted randomly for different morphologies for each transfection at each time point. Y-axis: % of cells with different morphologies; x-axis: transfection of K562 cells. WT: wild type; E/P: electroporated with water; LUC: transfection with firefly pGL3 luciferase siRNA; GATA1: transfection with GATA1a siRNA; E2A: transfection with E2Ab siRNA. Key on the right shows the colour bars representing different morphologies.

3.4.5 Time-course study of siRNA knockdown

Studying the knockdown at one particular time point fails to provide a full picture of how the siRNAs are reacting within the cells and how the mRNAs or proteins are being silenced across

time. Therefore, time-course studies were required to investigate the changes in gene expression after siRNA transfections and to identify the time point where the best knockdown effects were induced. The following criteria were used in choosing the optimal time point in the time-course study:

1. The average remaining protein level of the targeted protein by the two independent siRNAs should be less than 30% compared to the firefly luciferase siRNA control. For each independent siRNA, the remaining targeted protein level should be 40% or less.
2. If protein level cannot be assessed due to the lack of a suitable antibody for western analysis, the average remaining mRNA level of the targeted gene by two independent siRNA should be less than 30%. For each independent siRNA, the remaining targeted mRNA level should be 40% or less.
3. The earliest time point where criteria 1 and 2 can be achieved should be chosen to reduce off-target effects.
4. The same time point should be used for both siRNAs to reduce discrepancies due to variations in the growth patterns of cells and induction of the RNAi pathway.
5. The earliest time point that can be chosen is 24 hours. The 12 hour time point should not be chosen as sufficient time is needed to allow the cells to be recovered after electroporation. As shown in the growth pattern study in section 3.4.4 (Figure 3.6), the growth rate of electroporated cells was lower in the first 24 hours and the cells slowly recovered after 24 hours. Also, the amount and quality of RNA extracted at the 12 hour time point were lower than other time points making subsequent analyses difficult.

To this end, time course experiments for GATA1, E2A, SCL, LMO2 and LDB1 were performed and the knockdowns were analysed at the mRNA and protein levels. mRNA and nuclear protein samples were collected at 12, 24, 36 and 48 hours after siRNA transfection and quantified by quantitative real time PCR and western blotting. For the mRNA quantification by real-time PCR, expression levels of the TF knockdown were normalised against the expression levels found in the luciferase siRNA condition for reasons described in section 3.4.1. The internal house-keeping controls, β-actin, GAPDH, β-tubulin and RPL19, were also included in the normalisation to minimise effects of variations of RNA concentration and quality across samples. For protein quantifications by western blotting, the nuclear protein extracts from transfected cells were first quantified and then equal amounts of protein were loaded into each well prior to electrophoresis and western analysis. The relevant protein bands detected from the western blots were quantified by densitometry and knockdown levels were determined relative to the luciferase control. Equal

loading of each lane of the westerns was verified by staining the membrane as described in Chapter 2 (Section 2.10.4). The results of each TF knockdown time course analysis are described in the following sections. Three biological replicates were performed for each siRNA time-course and the qRT-PCR and western blot data from one representative replicate time-course were reported in the following sections.

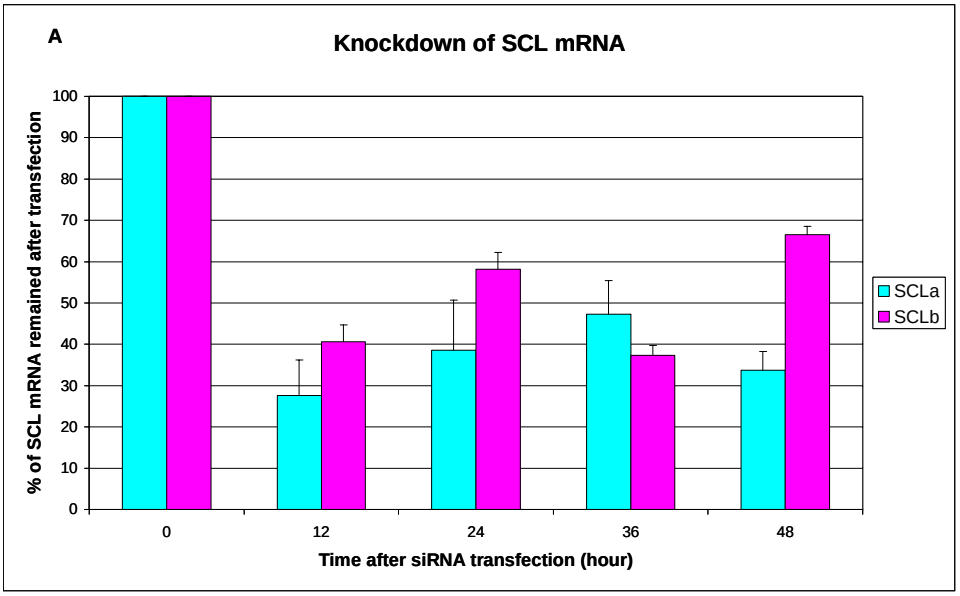
3.4.5.1 Knockdown of SCL

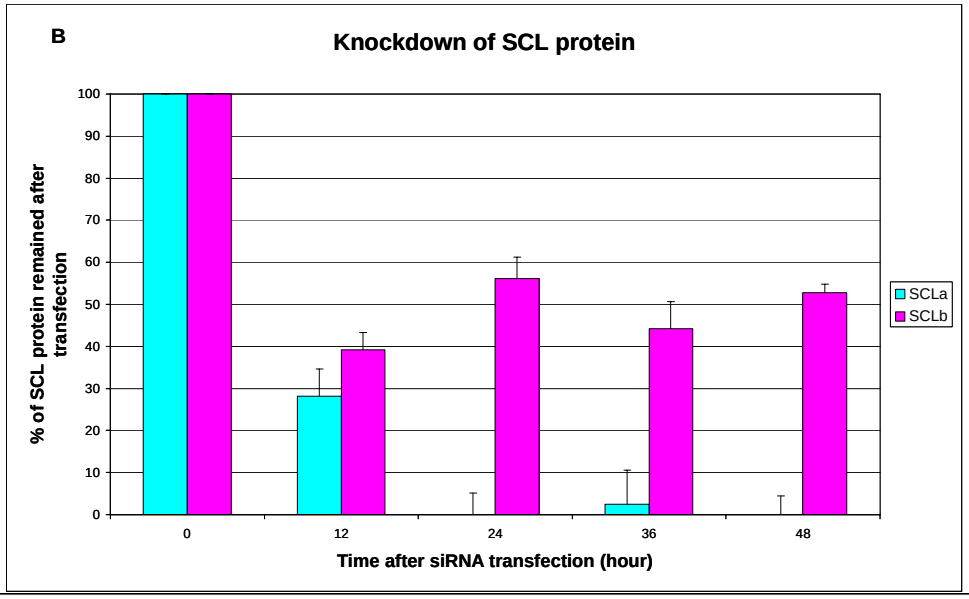
The knockdown of SCL with two independent siRNAs (SCLa and SCLb) was studied through a 48 hour time-course experiment as shown in Figure 3.9. SCL mRNA levels in the SCLa siRNA transfected cells decreased substantially to approximately 40% at 12 hour and fluctuated in the later time points at between 30-60% of its original physiological level. The knockdown at the protein level for siRNA SCLa was consistent with the mRNA level after 12 hours, but then showed a dramatic reduction to less than 10% its original level to nearly 100% knockdown at the 24 hour, 36 hour and 48 hour time points. These results were in marked contrast to the mRNA levels detected during these later time points. The SCLb siRNA, however, did not show reduction in the mRNA levels that had been anticipated based on the results of the initial screening described in section 3.4.1. The SCLb siRNA demonstrated a relatively weak knockdown after 24 hours with 60% of the mRNA remaining – this siRNA had previously given a knockdown to 31% of the physiological level of the SCL mRNA in the initial screens. However, SCLb reported better knockdowns at the 12 and 36 hour time points, where it achieved knockdowns with approximately 42% and 32% of the SCL mRNA remaining, respectively. SCL protein levels across the SCLb time-course showed the maximum knockdown after 12 hours (40% of protein remaining) which was consistent with its mRNA levels. However, whilst the maximum knockdown at the mRNA level was achieved at the 36 hour time point, this was not reflected at the protein level (maximum knockdown 43% protein remaining).

An optimal time point satisfying the four criteria listed above for both siRNAs against SCL could not be identified. The protein level of SCL was knocked down to less than 40% remaining at the 24 hour time point for SCLa but not for SCLb. The required remaining protein level (40%) for SCL was not reached for SCLb at all the time points tested (24 hour, 36 hour and 48 hour). Although less than 40% of the protein remained at 12 hour for SCLb, this time point was not considered according to the criteria set above. The 24 hour time point was chosen as the optimal time point for further study for the SCLa siRNA.

The time-course experiment for the SCLa siRNA suggested that the protein has a much shorter half-life than the mRNA because there was a lag in the mRNA levels being knocked down. However, from the data obtained for SCLb, the half-lives of mRNA and protein seem to be similar. Therefore,

it is difficult to draw any conclusion on the half-lives of mRNA and protein of SCL from these results.





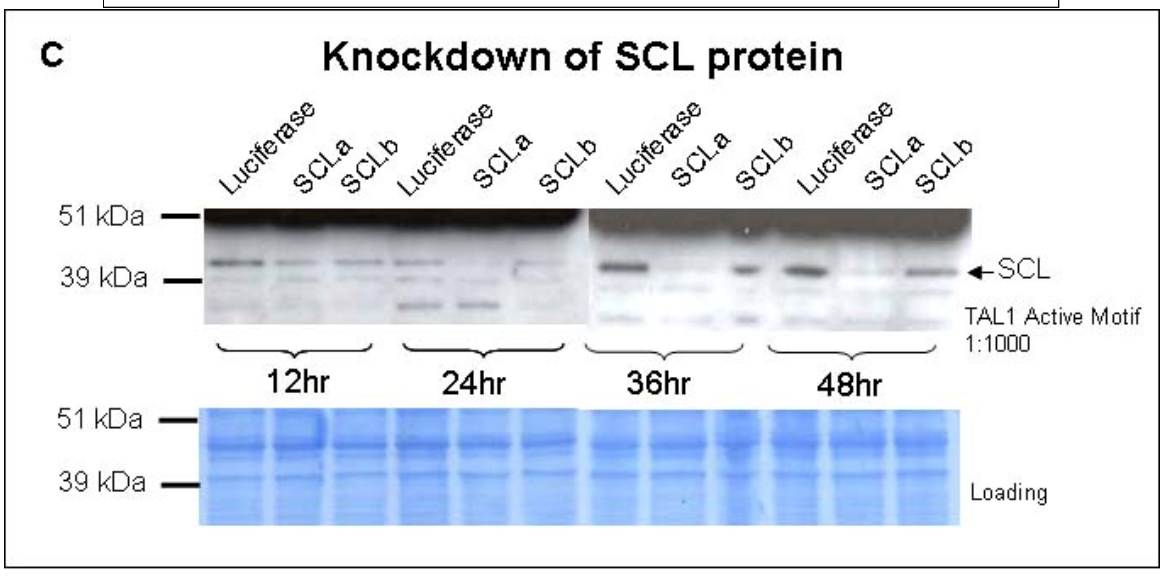


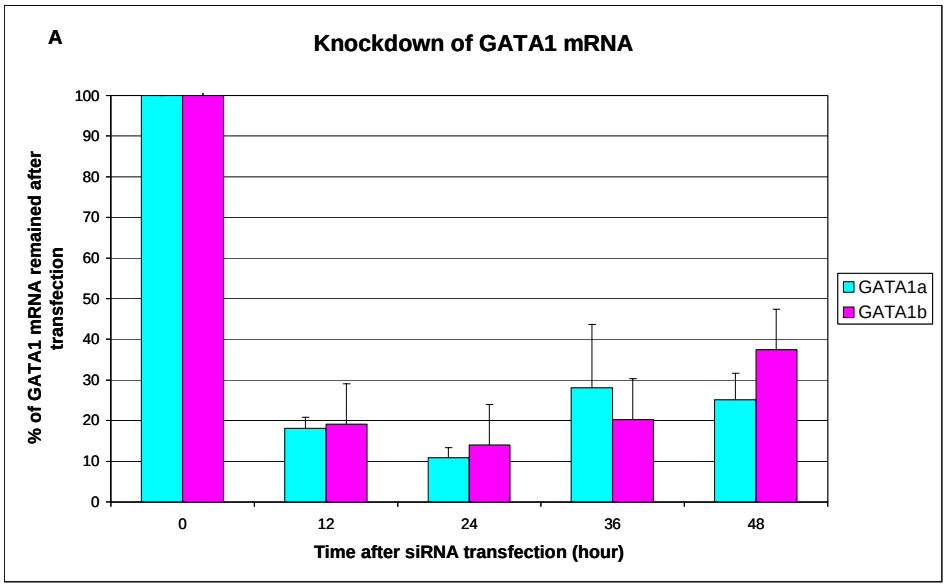
Figure 3.9. siRNA knockdown time-course study of SCL. Two siRNAs directed against SCL were used: SCLa and SCLb. A: Knockdown of SCL at the mRNA level was quantified by quantitative PCR as described in the text. Bar chart shows the mRNA level of SCL remaining (y-axis) after siRNA transfection relative to luciferase siRNA transfection across the time points (x-axis) Blue bars indicate mRNA levels in the SCLa condition while the pink bars indicate mRNA levels in the SCLb condition. The error bars show the standard error of the mean between the three independent biological replicates. B: Knockdown of SCL at the protein level by densitometry of bands determined by immuno-detection of the relevant protein band on western blots. Bar chart shows the protein level of SCL remaining after siRNA transfection relative to luciferase siRNA transfection. Blue bars indicate protein levels in the SCLa condition while pink bars indicate the protein levels remaining in the SCLb condition. The error bars show the standard error of the mean between the three independent biological replicates. C: Western blot analyses of SCL protein knockdowns. Upper panel shows the bands detected by immuno-detection of the western blot with the TAL1 Active Motif antibody. The lower panel shows the blot stained with Bradford reagent as a protein loading control. The arrow shows the predicted size of the SCL protein.

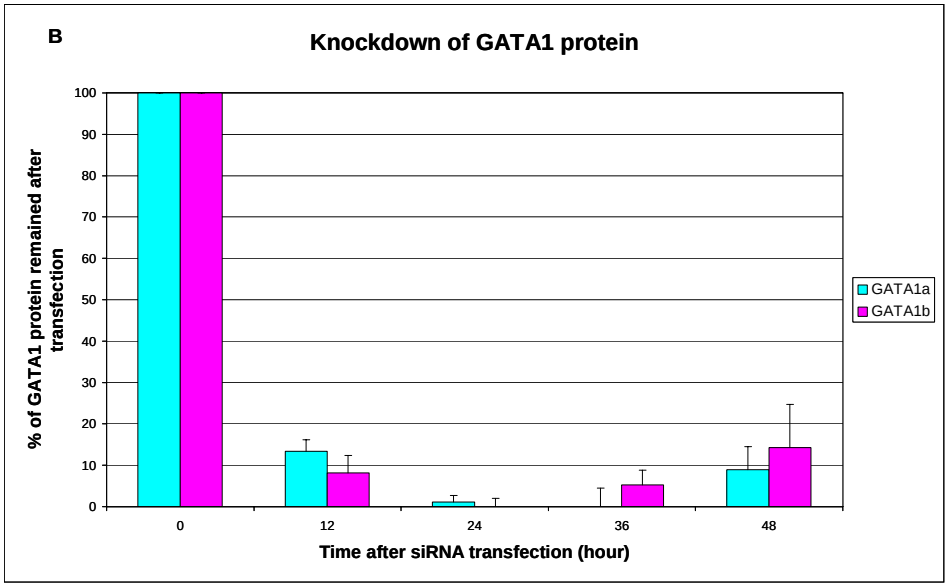
3.4.5.2 Knockdown of GATA1

The time-course analysis of the knockdown of GATA1 with two independent siRNAs (GATA1a and GATA1b) is shown in Figure 3.10. In general, both siRNAs generated substantial knockdowns at both mRNA and protein level. However, the GATA1a siRNA induced a marginally better knockdown effect at both mRNA and protein level at the majority of time points studied. GATA1 mRNA level in cells transfected with either of the siRNAs decreased dramatically to less than 20% of the original mRNA level at the 12 hour time point, further reducing to approximately 10% at 24 hour, then gradually increased in the later time points. The knockdown at the protein level had a similar trend with almost 0% of protein remaining at the 24 hour time point, and a gradual increase at the 36 hour and 48 hour time points.

Both siRNAs GATA1a and GATA1b gave the maximum knockdown at the mRNA and protein level at the 24 hour time point. This time point was the earliest time point where both siRNAs were able to knock down the GATA1 protein to less than 30% remaining on average. Although such knockdown was also observed at the 12 hour time point, this time point was not considered as the optimal time point for the reasons mentioned above. Therefore, the 24 hour time point was chosen for subsequent analysis.

The time-course experiment suggests that the half-lives of mRNA and protein of GATA1 were similar as a lag between the mRNA and protein being knocked down was not observed in both siRNAs. Also, the rapid reduction of both the mRNA and protein level at the 12 hour time point suggested that the half-lives of GATA1 mRNA and protein are relatively short.


A
Knockdown of GATA1 mRNA
% of GATA1 mRNA remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
GATA1a
GATA1b


B
Knockdown of GATA1 protein
% of GATA1 protein remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
GATA1a
GATA1b

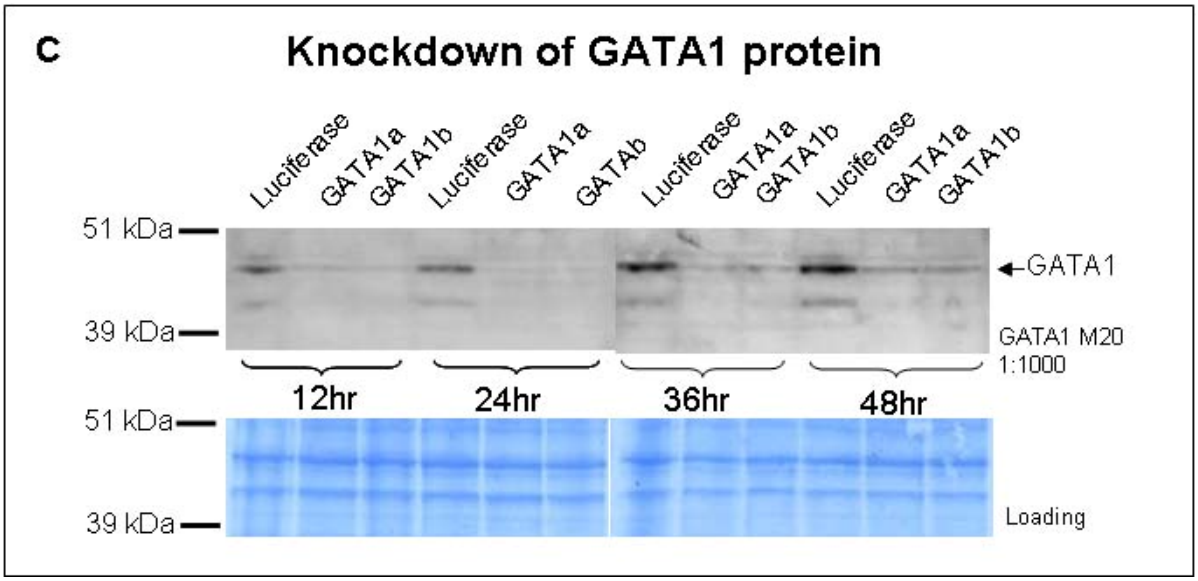

C
Knockdown of GATA1 protein
Luciferase
GATA1a
GATA1b
Luciferase
GATA1a
GATAb
Luciferase
GATA1a
GATA1b
Luciferase
GATA1a
GATA1b
51 kDa
39 kDa
GATA1
GATA1 M20
1:1000
12hr
24hr
36hr
48hr
51 kDa
39 kDa
Loading

Figure 3.10. siRNA knockdown time-course study of GATA1. Two siRNAs directed against GATA1 were used: GATA1a and GATA1b. A: Knockdown of GATA1 at the mRNA level was quantified by quantitative PCR as described in the text. Bar chart shows the mRNA level of GATA1 remaining (y-axis) after siRNA transfection relative to luciferase siRNA transfection across the time points (x-axis) Blue bars indicate mRNA levels in the GATA1a condition while the pink bars indicate mRNA levels in the GATA1b condition. The error bars show the standard error of the mean between the three independent biological replicates. B: Knockdown of GATA1 at the protein level by densitometry of bands determined by immuno-detection of the relevant protein band on western blots. Bar chart shows the protein level of GATA1 remaining after siRNA transfection relative to luciferase siRNA transfection. Blue bars indicate protein levels in the GATA1a condition while pink bars indicate the protein levels remaining in the GATA1b condition. The error bars show the standard error of the mean between the three independent biological replicates. C: Western blot analyses of GATA1 protein knockdowns. Upper panel shows the bands detected by immuno-detection of the western blot with the GATA1 M20 Santa Cruz antibody. The lower panel shows the blot stained with Bradford reagent as a protein loading control. The arrow shows the predicted size of the GATA1 protein.

3.4.5.3 Knockdown of E2A

The knockdown of E2A was performed using two independent siRNAs (E2Aa and E2Ab) targeting regions of the coding sequence found in both the E12 and E47 transcript variants. The results of the time-course analysis are shown in Figure 3.11. In general, both siRNAs generated similar and substantial knockdowns at both the mRNA and protein level for both transcript variants, but the effect on E12 was different from that on E47. E12 mRNA levels in cells transfected with either of the siRNAs decreased to their lowest levels of approximately 30% the original mRNA level at the 12 hour time point and remained at similar levels at all subsequent time points. In contrast, E47 mRNA levels achieved their lowest levels (< 30% of original mRNA levels) at the 36 hour time point and then began to increase again by 48 hours.

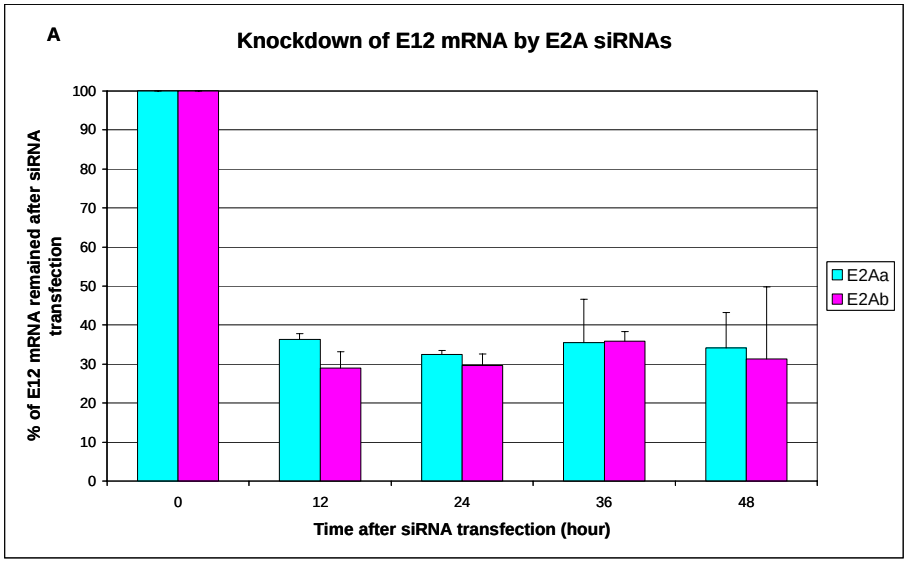
The knockdown at the protein level at the E12 and E47 isoforms could not be studied on the western blot due to issues associated with possible cross-reactivities of the E12 and E47 antibodies. There is no evidence from the antibody supplier showing that the two antibodies do not cross-react with both isoforms. The following interpretation is based on the assumption that the antibodies did not cross-react. The E2Ab siRNA produced a marked drop in E12 levels at the 12 hour time point, while the E2Aa siRNA had no effect after 12 hours. The largest knockdown effect of the E12 protein was achieved at the 24 hour time point for both siRNAs with only 15% of the protein remaining; subsequent time points showed a general increase in E12 protein levels. The E47 protein levels decreased gradually during the time course for both siRNAs and were at their lowest level (<30% of the original level) at 36 hours and had begun to increase substantially by 48 hours.

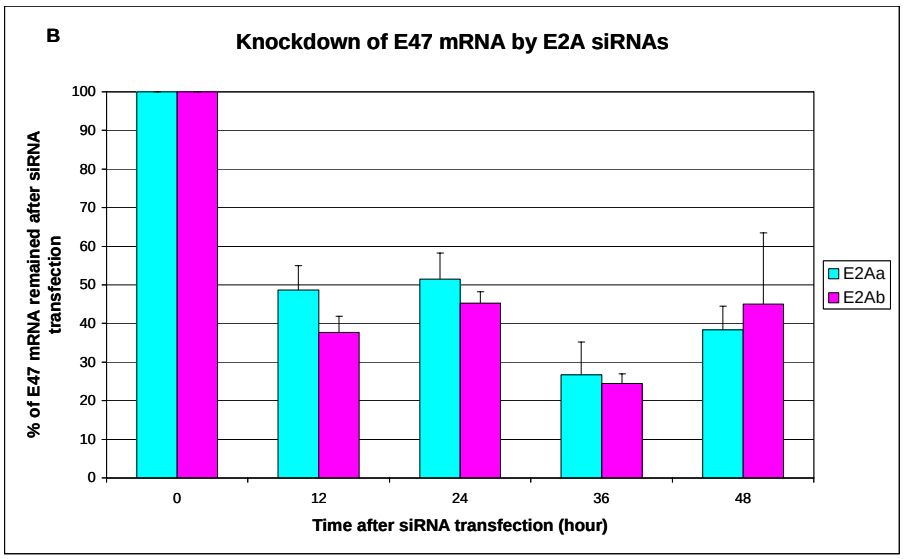
Assuming that the E12 and E47 antibodies did cross-react, western blots probed with either antibody would show the knockdown of E2A (the sum total of E12 and E47 isoforms). Based on this assumption, the greatest knockdown of E2A was achieved at the 24 hour time point for both

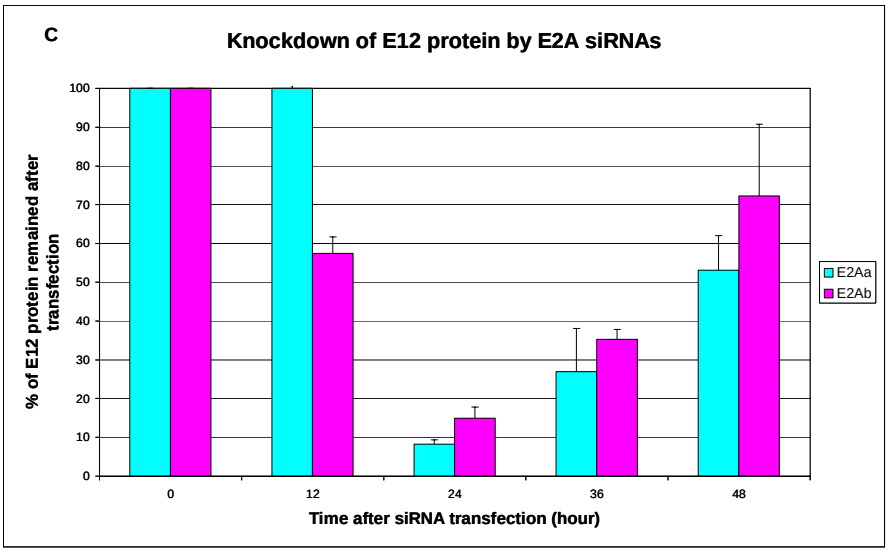
siRNAs with only 15% of the protein remaining using the E12 antibody. In contrast, the greatest knockdown of E2A was achieved at the 36 hour time point for both siRNAs with less than 30% of the protein remaining using the E47 antibody.

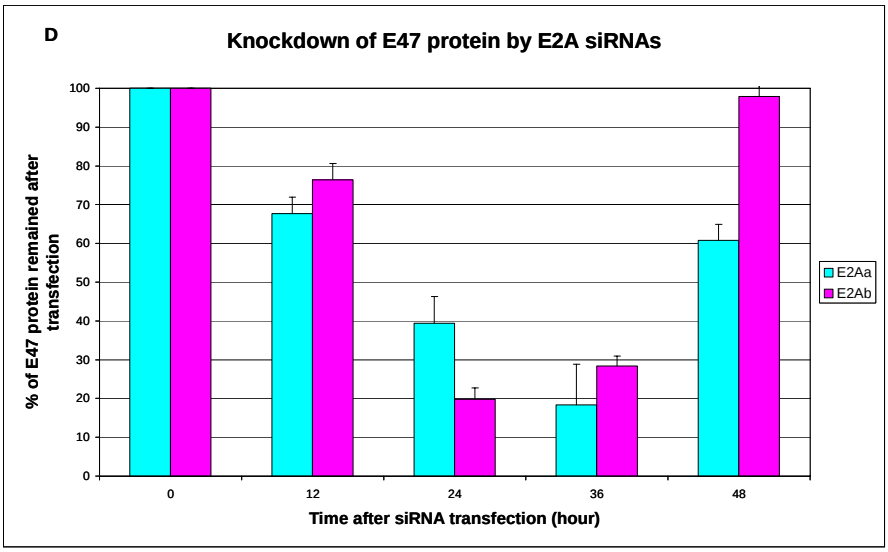
Both siRNAs E2Aa and E2Ab gave a knockdown of less than 30% protein remaining (on average of both siRNA) at the protein level at the 24 hour time point for the detection by both E12 and E47 antibodies. This time point was the earliest time point where both siRNAs were able to knock down the E2A protein to less than 30% remaining on average. Therefore, the 24 hour time point was chosen for subsequent analysis for both siRNAs.

The time-course experiment suggests that the half-life of the E2A mRNA was shorter than that of the E2A protein as a time lag between the mRNA and protein being knocked down was observed in both siRNAs. The mRNAs of E12 and E47 transcripts were reduced to less than 50% remaining at the 12 hour time point whereas the protein level of E2A remained at about 70% of its physiological level (on average). This suggests that the half-life of E2A mRNA appeared to be shorter than that of the protein.




B
Knockdown of E47 mRNA by E2A siRNAs
% of E47 mRNA remained after siRNA transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
E2Aa
E2Ab


C
Knockdown of E12 protein by E2A siRNAs
% of E12 protein remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
E2Aa
E2Ab


D
Knockdown of E47 protein by E2A siRNAs
% of E47 protein remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
E2Aa
E2Ab

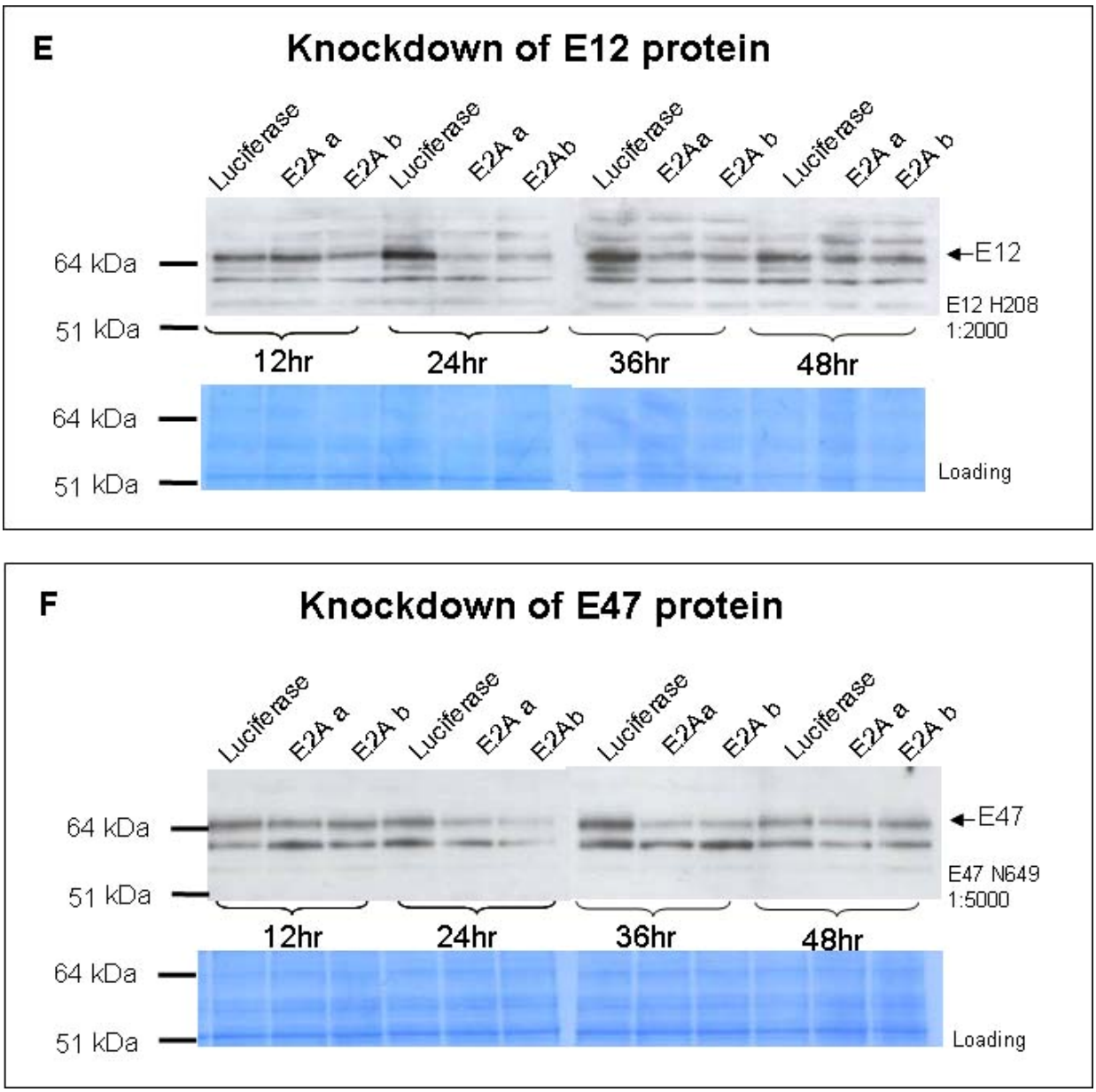


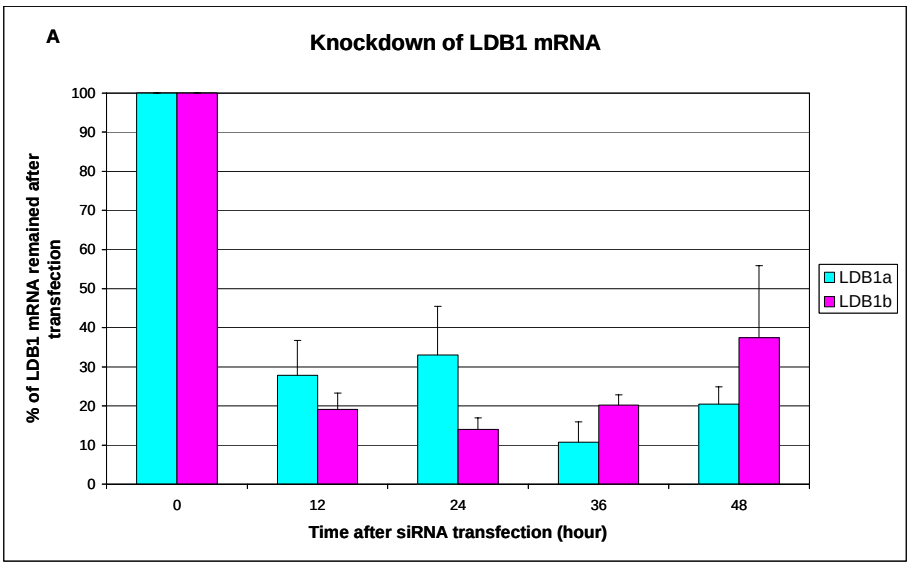
Figure 3.11. siRNA knockdown time-course study of E2A. Two siRNAs directed against E2A were used: E2Aa and E2Ab. A and B: Knockdown of E12 and E47 respectively at the mRNA level was quantified by quantitative PCR as described in the text. Bar chart shows the mRNA level of E12 or E47 remaining (y-axis) after siRNA transfection relative to luciferase siRNA transfection across the time points (x-axis) Blue bars indicate mRNA levels in the E2Aa condition while the pink bars indicate mRNA levels in the E2Ab condition. The error bars show the standard error of the mean between the three independent biological replicates. C and D: Knockdown of E12 and E47 respectively at the protein level by densitometry of bands determined by immuno-detection of the relevant protein band on western blots. Bar chart shows the protein level of E12 or E47 remaining after siRNA transfection relative to luciferase siRNA transfection. Blue bars indicate protein levels in the E2Aa condition while pink bars indicate the protein levels remaining in the E2Ab condition. The error bars show the standard error of the mean between the three independent biological replicates. E and F: Western blot analyses of E12 and E47 protein knockdowns respectively. Upper panel shows the bands detected by immuno-detection of the western blot with the E12 H208 and E47 N649 Santa Cruz antibody. The lower panel shows the blot stained with Bradford reagent as a protein loading control. The arrow shows the predicted size of the E2A protein.

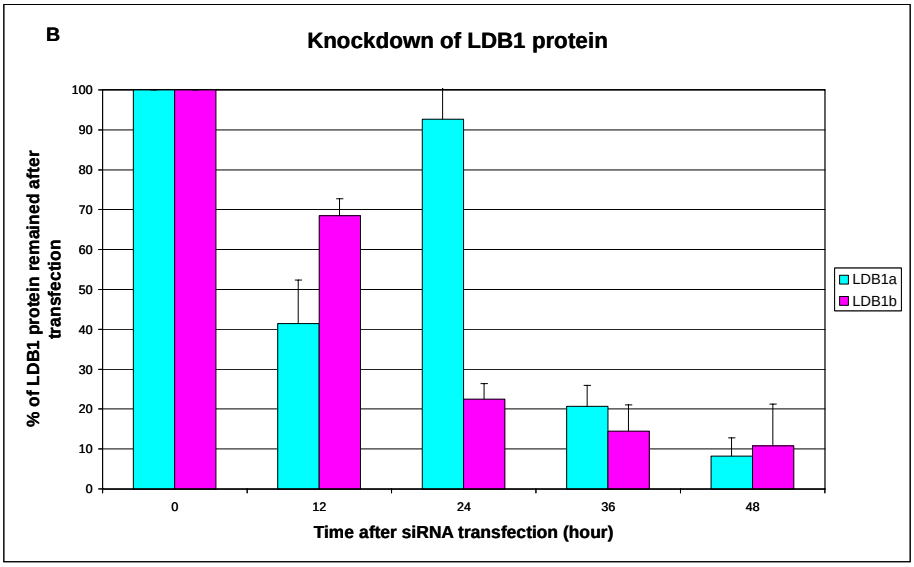
3.4.5.4 Knockdown of LDB1

The knockdown of LDB1 by two independent siRNAs (LDB1a and LDB1b) was studied through a 48 hour time-course experiment (Figure 3.12). Both siRNAs generated substantial knockdowns at both the mRNA and protein level. Overall, LDB1 mRNA levels in cells transfected with either of the siRNAs decreased dramatically to less than 20% of their original levels at the 12 hour time point. However, the mRNA levels in cells transfected with the LDB1a siRNA were at its lowest (only 20% of the original level of the mRNA remaining) at 36 hour and increased up to 30% by the 48 hour time point. The knockdown by the LDB1b siRNA was slightly different - approximately 35% of the original mRNA level was detected at the 24 hour time point and was further reduced to less than 10% at the 36 hour time point and increased up to 30% by the 48 hour time point. In contrast, the knockdown of both LDB1 isoforms was not significant at 12 hours in either siRNA condition (whereas only 20% of the original mRNA level was seen at the same time point). The protein knockdown was gradual for both siRNAs and reached a maximum knockdown to 10% of its original level at the 48 hour time point for both isoforms.

Both siRNAs LDB1a and LDB1b induced a substantial knockdown at the protein level with only 20% of the original remaining at the 36 hour time point. This time point was the earliest time point where both siRNAs were able to knock down the LDB1 protein to less than 30% remaining on average. Although a greater knockdown was achieved at the 48 hour time point, this time point was not considered as the optimal time point as an earlier time point is more desirable to reduce off-target effects. Therefore, the 36 hour time point was chosen for subsequent analysis.

Both LDB1 siRNAs were shown to induce knockdown to a similar level, however the maximum knockdown at the mRNA and protein levels was achieved at different time points. This suggests that the half-lives of mRNA and protein of LDB1 were different as a time lag between the mRNA and protein being knocked down was observed in both siRNAs. The mRNA of LDB1 was reduced to less than 20% remaining at the 12 hour time point whereas the protein level of LDB1 stayed at about 55% remaining on average for both siRNAs at the same time point. Also, the maximum knockdown at the mRNA level was achieved at the 36 hour time point whereas it was observed at the 48 hour time point at the protein level. This suggests that the half-life of the LDB1 mRNA was shorter than that of the protein.


A
Knockdown of LDB1 mRNA
% of LDB1 mRNA remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
LDB1a
LDB1b


B
Knockdown of LDB1 protein
% of LDB1 protein remained after transfection
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
Time after siRNA transfection (hour)
LDB1a
LDB1b

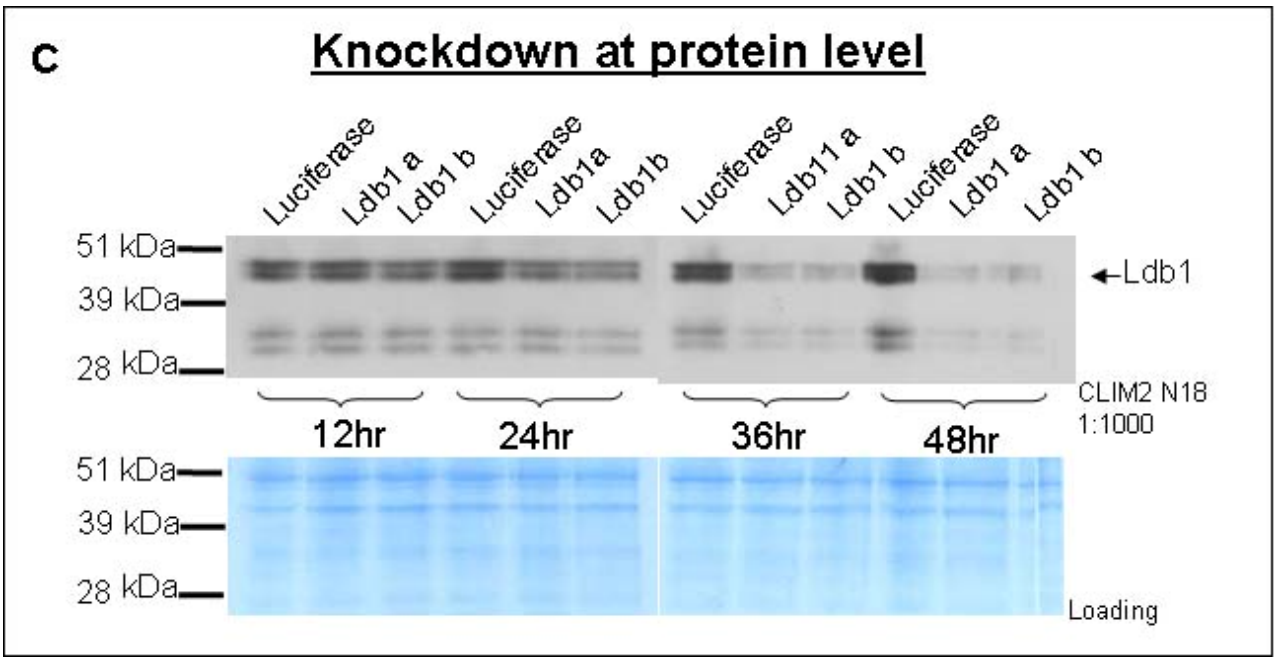

c
Knockdown at protein level
Luciferase
Ldb1 a
Ldb1 b
Luciferase
Ldb1a
Ldb1b
Luciferase
Ldb11 a
Ldb1 b
Luciferase
Ldb1 a
Ldb1 b
51 kDa
39 kDa
28 kDa
←Ldb1
CLIM2 N18
1:1000
12hr
24hr
36hr
48hr
51 kDa
39 kDa
28 kDa
Loading

Figure 3.12. siRNA knockdown time-course study of LDB1. Two siRNAs directed against LDB1 were used: LDB1a and LDB1b. A: Knockdown of LDB1 at the mRNA level was quantified by quantitative PCR as described in the text. Bar chart shows the mRNA level of LDB1 remaining (y-axis) after siRNA transfection relative to luciferase siRNA transfection across the time points (x-axis) Blue bars indicate mRNA levels in the LDB1a condition while the pink bars indicate mRNA levels in the LDB1b condition. The error bars show the standard error of the mean between the three independent biological replicates. B: Knockdown of LDB1 at the protein level by densitometry of bands determined by immuno-detection of the relevant protein band on western blots. Bar chart shows the protein level of LDB1 remaining after siRNA transfection relative to luciferase siRNA transfection. Blue bars indicate protein levels in the LDB1a condition while pink bars indicate the protein levels remaining in the LDB1b condition. The error bars show the standard error of the mean between the three independent biological replicates. C: Western blot analyses of LDB1 protein knockdowns. Upper panel shows the bands detected by immuno-detection of the western blot with the LDB1 CLIM2 N18 Santa Cruz antibody. The lower panel shows the blot stained with Bradford reagent as a protein loading control. The arrow shows the predicted size of the LDB1 protein.

3.4.5.5 Knockdown of LMO2

The knockdown of LMO2 by two independent siRNAs (LMO2a and LMO2b) was studied through a 48 hour time-course experiment (Figure 3.13). As no working antibodies were available (see section 3.4.4), only mRNA levels of LMO2 were monitored in the time-course experiments. Across the time-course, the two siRNAs behaved similarly although LMO2b siRNA generated a greater knockdown effect at all time points. The mRNA levels in cells transfected with LMO2b was similar (between 15-20% of the original mRNA level remained) at all the time points. The mRNA levels in cells transfected with LMO2a was slightly different with a similar mRNA level observed at the 12 hour, 24 hour and 36 hour time points (between 20 to 28%) and subsequently increasing to approximately 70% at the 48 hour time point.

Since the knockdown at the protein level could not be studied due to the lack of a working antibody, the choice of the optimal time point was based solely on the mRNA level. Both siRNAs LMO2a and LMO2b induced a significant knockdown at the mRNA level with only 20% of the original mRNA remaining on average at the 24 hour time point. This time point was the earliest time point where both siRNAs were able to knock down the LMO2 protein to less than 30% remaining on average. Although such knockdown was also observed at the 12 hour time point, this time point was not considered as the optimal time point for the reasons mentioned above. Therefore; the 24 hour time point was chosen for subsequent analysis.

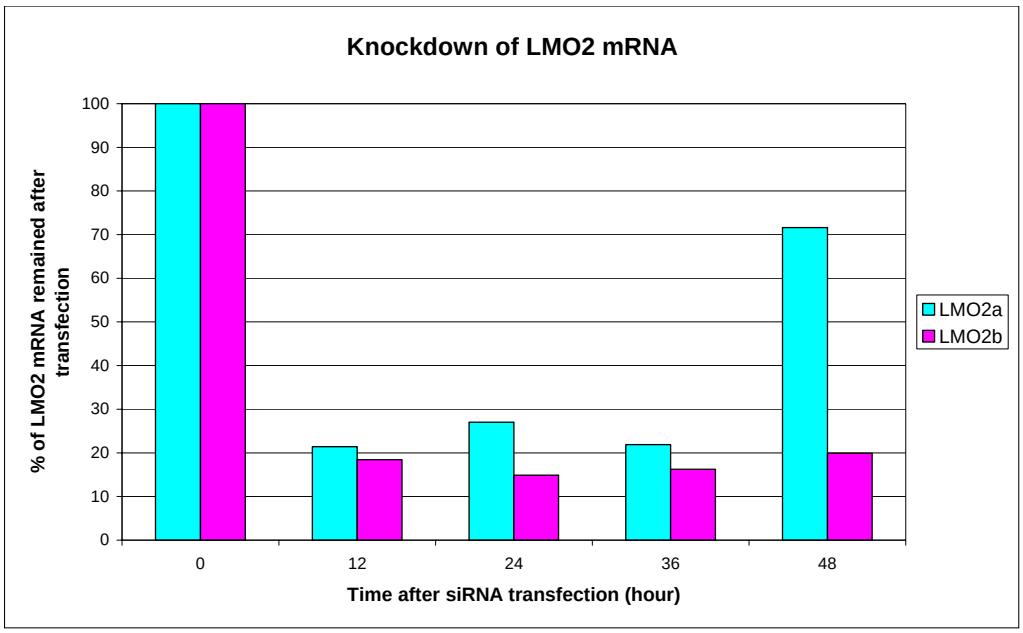


Figure 3.13. siRNA knockdown time-course study of LMO2. Two siRNAs directed against LMO2 were used: LMO2a and LMO2b. Knockdown of LMO2 at the mRNA level was quantified by quantitative PCR as described in the text. Bar chart shows the mRNA level of LMO2 remaining (y-axis) after siRNA transfection relative to luciferase siRNA transfection across the time points (x-axis) Blue bars indicate mRNA levels in the LMO2a condition while the pink bars indicate mRNA levels in the LMO2 condition. The error bars show the standard error of the mean between the three independent biological replicates.

3.5 Discussion

The work presented in this chapter describes the screening of working siRNA assays for five members of the SCL erythroid complex and the characterisation of these siRNAs in various studies. The siRNAs were shown to be delivered into K562 cells with high efficiency (>90% of cells contained the siRNA) by electroporation. Further characterisation of some of the siRNAs delivered to K562 cells demonstrated that effects on cell growth and morphology were as a result of electroporation and not due to the siRNA induction of the RNAi machinery or specific siRNA effects. Time-course knockdown experiments were performed to identify appropriate time points where the knockdown was greatest at the mRNA and proteins levels – these time points would serve as useful guides for further experiments aimed at using siRNA knockdowns to identify downstream targets of the SCL erythroid complex (SEC).

3.5.1 siRNA delivery

One of the most crucial factors affecting the efficacy of knockdown by siRNA is the efficiency of delivering siRNA into the cells. If the delivery efficiency is low, even a good siRNA cannot induce a high knockdown effect. Therefore; it is important that the delivery of the siRNAs is monitored to ensure that the strategy is optimised for subsequent experimental approaches which will use

knockdowns to address biological questions. Both electroporation and lipofection have been widely used in RNAi experiments, and electroporation by the Amaxa Nucleofector II system was used in this project due mainly to the high transfection efficiency which has been optimised by the supplier for K562 cells.

For similar reasons as discussed in the introduction to this chapter, fluorescein FITC-labelled siRNAs were used here to study the efficiency of delivery by the Amaxa Nucleofector II system. In contrast to fluorescence microscopy used in previously published studies, FACS analysis using an appropriate laser which can allow detection of FITC was used here. FACS was used in preference to microscopy because FITC emits a green fluorescence which is often difficult to distinguish from background fluorescence using microscopy. Furthermore, K562 cells, being a cell line which grows in suspension, requires the use of a cytospin to prepare microscope slides. Treatment of cells in this way can result in a high proportion of fragmented cells, making microscopic examination more difficult. Moreover, FACS analysis allows for the measurement of many more cells emitting fluorescence, when compared to microscopy, thus resulting in a more precise estimate of transfection efficiency.

Using FACS analysis, the transfection efficiency by a FITC-labelled siRNA, which showed a very high knockdown effect (GATA1a), was greater than 90% immediately after transfection. This efficiency diminished slightly after 24 hours - approx. 84% of cells carried the siRNA and the overall fluorescence levels emitted by the cells had also decreased. This is consistent with the transfection efficiency reported by Amaxa for K562 cells (www.amaxa.com) and reflects the knockdown efficiency of GATA1a, which is also over 90%. The reduction in % of cells emitting fluorescence after 24 hours was likely due to the presence of increased cell numbers in culture (due to on-going cell division), an ever-increasing proportion of which would not carry the labelled siRNA. The reduction in the fluorescence intensity emitted by the cells after 24 hours was due to the high photobleaching rate of FITC.

An alternative approach to study the transfection efficiencies of siRNAs would be to use quantum dots (QDs) which are highly photostable but relatively small fluorescent nanocrystals which are both brighter than conventional fluorescent dyes and easier to detect among *in vivo* background (Chan and Nie, 1998; Gao et al., 2004). QDs has been used to track RNAi by co-transfecting siRNA and QDs together into cells using lipofectamine (Chen et al., 2005). No chemical labelling with the QD is required for the siRNA during its synthesis, as QD/siRNA complexes are formed during transfection. This can be more cost efficient as well as preventing any undesirable effects of the chemical modification of siRNA on the delivery and induction of the RNAi pathways. To date, QD siRNAs have not been tested using delivery into cells by electroporation.

3.5.2 Effect of siRNA transfection on phenotypic changes

As mentioned in Chapter 1, induction of the RNAi pathway can induce non-specific effects including off-target effects, immune responses and saturation of the pathway itself (Chapter 1, section 1.3.1.3). Changes in growth patterns and cell morphologies upon transfection with siRNAs through time were also investigated to determine whether such changes were the result of specific knockdown effects of the protein of interest, or due to non-specific effects.

The cell morphology studies showed that electroporation of water, the control siRNA for luciferase and specific siRNA against GATA1 or E2A all induced changes in K562 cell morphology after 24 hours, resulting in the formation of small or big projections on the cell membranes and increased levels of multinucleated cells (the latter suggesting an increased rate of cell division may be occurring). These features were not present in cells which had not been electroporated. Furthermore, these features persisted at later time points (e.g. 48 hours after transfection). Nevertheless, it is clear from this data that all cells subjected to electroporation behaved in a similar way, suggesting that these morphological changes were not due to specific changes induced by the knockdown of the GATA1 or E2A mRNAs and proteins, or due to effects of introduction of siRNAs into K562 cells.

Similarly, the growth patterns of all cells which had been electroporated (with water, GATA1 and luciferase siRNAs) were different from the patterns observed with cells that had not been electroporated. The growth rate of the electroporated cells were significantly lower during the first 24 hours compared to their non-electroporated counterparts. Therefore, electroporation *per se* and not effects due to transfection with siRNA was the likely cause of such changes in growth rates. These growth rate effects appeared to diminish after the 24 hour time point, suggesting that the effect was transient, as one would expect given that the effect of electroporation would likely diminish as cells have had more time to recover in culture.

All of this data, taken together, suggests that transfection of siRNAs, regardless of whether they are against specific genes of interest or to luciferase controls, has no effect on cell morphology or growth pattern of K562 cells. Electroporation, however, does generate a stress response on the cells leading to visible changes in morphology and growth pattern. These results underlie the importance of comparing the effects of siRNA knockdowns with relevant controls for the cellular responses elicited by electroporation. Furthermore, by using a luciferase siRNA control, the effects of both electroporation and any generalised siRNA effects can also be taken into consideration when assessing the effects of siRNA knockdowns of specific genes of interest. The relevance of such controls are highlighted further in Chapters 4, 5 and 6, as they apply to our understanding of how

such effects can also be mediated at the molecular level and can have a bearing on our interpretation of data in both expression and ChIP-on-chip studies.

3.5.3 siRNA-induced knockdown of the SCL erythroid complex in time-course study

(i) Defining and quantitating knockdown levels for siRNA assays

During the screening of siRNAs for members of the SCL erythroid complex described in this Chapter, a knockdown efficiency of 70% (30% of original mRNA level) was considered to be the benchmark level (see introduction of this Chapter), above which siRNAs were considered to be working effectively enough to warrant their use in further aspects of this project. Two siRNAs were selected for each TF under study to reduce the chance of identifying off-targets in the subsequent expression analysis. In addition, time-course experiments monitoring the knockdown of each TF with the validated siRNAs were also performed. Determining the time points at which maximum knockdowns were achieved were crucial for the subsequent expression profiling analyses described in Chapter 4.

The knockdown levels for each siRNA assay were determined at both the mRNA and protein level for each TF using quantitative real time PCR (qPCR) and western blotting respectively. qPCR is a very sensitive and quantitative assay, although the SYBR green assays used in this study is less quantitative, but more economical, than Taqman assays. To circumvent any quantitation issues, accurate normalisation with internal housekeeping controls is required (Lupberger et al., 2002; Vandesompele et al., 2002). In this Chapter, β-actin, β-tubulin, GAPDH and RPL19 were used. These genes are normally highly and constitutively expressed in most tissues. Furthermore, by using more than one control, one can account for sample to sample variations in mRNA levels in some, but not all of the genes, which may affect accurate normalisation. Whilst other studies have used ribosomal RNAs (rRNAs) as normalisation controls, some researchers have demonstrated that rRNAs are not appropriate controls as there is an imbalance between rRNA and mRNA fractions and rRNA cannot truly reflect the mRNA levels (Vandesompele et al., 2002).

Although working antibodies were characterised for the majority of the TF under study, no antibodies were found to work in western blotting for LMO2. LMO2 is a very small protein (~18 kDa) which might be susceptible to degradation or denaturation during electrophoresis. In such cases, where it is not possible to use western blotting, immunofluorescence-based assays using microscopy or flow cytometry assays can be used to track the expression of the protein of interest. However, these assays are much more time-consuming and optimisation of assays is required – and this was beyond the scope of the work presented in this thesis. Also, accurate quantification cannot be easily performed, particularly for immunofluorescence-based assays.

The K562 cell line used in this study has the ability to spontaneously differentiate down the erythroid or megakaryocytic lineage in the absence of differentiating agents (Lozzio et al., 1981). Although the cell culture conditions were tightly monitored to avoid differentiation, it cannot be completely avoided. Thus, the siRNA knockdown efficiency across various replicates may differ due to self differentiation of K562 cells.

From the results obtained for all the time-course studies for the 5 TFs, it was demonstrated that the knockdown at the mRNA and protein levels do not necessarily agree with each other. For GATA1, the knockdown at both mRNA and protein levels were similar while the knockdown at the mRNA level was shown to be more rapid than that of the protein level for LDB1 and E2A. This suggests that mRNA and proteins half-lives may be different. Furthermore, the time points at which a maximum knockdown was obtained were not the same for all the siRNA assays. For instance, the maximum knockdown for LDB1 was obtained at a later time point from the other TFs, demonstrating that protein and mRNA half-lives also vary from gene to gene.

(i) The SCL knockdown

Two siRNAs were tested in the initial screening and both passed the criteria of selecting working siRNAs - i.e. reduction of mRNA level to 30% of its original level at the 24 hour time point. Both of the chosen siRNAs targeted exon 4 of the SCL gene. In the initial screening, SCLb only marginally passed the cut-off of 70% knockdown efficiency. Unfortunately, further characterisation in the time-course study showed that SCLb could not induce a sufficient knockdown at the protein level to 40% of its original level at 24 hour, 36 hour and 48 hour time points except at the 12 hour time point. One possible reason to explain this discrepancy is that the K562 cells may have undergone a degree of spontaneous differentiation that changed the expression level of SCL (which is known to vary during myeloid differentiation). This may have affected the degree to which SCL could be silenced by the SCLb siRNA. Whilst the 12 hour time point did show the appropriate level of knockdown for SCLb, it could not be chosen as the optimal time point due to the various reasons mentioned in section 3.4.5. Thus, no time points were shown to be suitable for further characterisation for this siRNA. One possible solution would have been to test more siRNAs against SCL and select another one which satisfied the required selection criteria. However, due to the time constraint for this project, this additional screening could not be performed. Thus, SCLb siRNA was not used in the gene expression profiling experiments described in Chapter 4.

An SCL knockdown to less than 10% of its original protein level was observed at 24 hours after SCLa siRNA transfection. For this reason, knockdown samples at this time point were chosen for

subsequent analysis by gene expression profiling studies described in Chapter 4. The knockdown at the mRNA level generated a reduction to approximately 30% of its original level at this time point which is higher than that at the protein level. This might be because the mRNA of SCL has considerably lower turn-over rate and less susceptible to degradation inside the cells than the SCL protein. This further illustrates the requirement to observe both mRNA and protein levels during knockdown, as the levels of one do not necessarily marry up with the levels of the other.

(ii) The GATA1 knockdown

The selection of optimal time point for GATA1 in the gene expression profiling experiment was comparatively simple. Both GATA1a and GATA1b siRNAs generated the maximum knockdown for both mRNA and protein levels at the 24 hour time points. The knockdown efficiency of these two siRNAs was high - with close to 0% of the protein remaining and 10% of the mRNA remaining at the 24 hour time point. Furthermore, the mRNA and protein knockdown levels correlated with each other for GATA1. The mRNA and protein levels were reduced to similar levels at all of the time points (within 10% of each other) and the maximum knockdown was observed at the same time point for mRNA and protein. This may be due to the fact that the mRNA and protein of GATA1 have similar stabilities.

(iii) The E2A knockdown

Studying the knockdown of E2A was particularly challenging for a number of reasons. Firstly, the E2A gene produces two mRNA transcript variants, E12 and E47, both of which encode functional proteins of similar molecular masses and which only differ in the amino acid content encoded by a single exon. Because of this, it was particularly difficult to choose siRNAs which could knockdown one variant but not the other. Secondly, monitoring knockdowns of the two protein isoforms was further complicated by the fact that polyclonal antibodies for these variants were most likely to cross-react.

For these reasons, two siRNAs against a region common to both variants were selected in the screening of working siRNA assays. This ensured that both variants were targeted. However, there was no guarantee that one may be silenced more than the other even with these siRNAs. In fact, from the time-course experiments, the knockdown of E12 and E47 appeared different across time. At the mRNA level, a maximum knockdown was achieved at the 24 hour time point for E12 and at the 36 hour time point for E47. The differences in the protein level of these two variants were shown to be hard to monitor due to the possible cross-reactivity of the antibodies. For subsequent gene expression profiling experiments, the 24 hour time point was chosen because a significant

knockdown at the protein level (with less than 30% protein remaining on average of both siRNAs) was induced as shown by the detection with both E12 and E47 antibodies. Although evidence was provided that E2A (or its isoform E47) showed a maximum knockdown for the protein at 36 hours, it could be argued that this time point would also satisfy the selection criteria for use in expression profiling. However, using the earlier 24 hour time point is always more desirable in such studies to avoid issues related to the induction of the innate immune response.

(iv) The LDB1 knockdown

The knockdown of LDB1 was another example where the mRNA and protein levels did not correlate. The maximum knockdowns at the mRNA and protein levels were achieved at different time points (36 hour time point for mRNA and 48 hour time point for protein). Furthermore, the reduction of mRNA levels was shown to be more rapid than at the protein level. The mRNA level dramatically reduced to 20% of its original level after 12 hours whereas the protein level only reduced to 50% of its original level initially and gradually reduced to less than 10% remaining at later time points. For subsequent expression profiling, the 36 hour time point was used as both siRNAs were able to knock down the LDB1 protein to less than 30% remaining on average. The 48 hour time point was not chosen even though the maximum protein knockdown was observed. This is again because an earlier time point with significant knockdown is more desirable to avoid non-specific effects.

(v) The LMO2 knockdown

Without a working antibody for LMO2 in western analysis, it was more difficult to identify a time point after siRNA transfection for subsequent expression profiling. From the time-course experiment described in this Chapter, it was assumed that a significant knockdown at the protein level was achieved at the optimal time point for mRNA knockdown. Therefore, the 24 hour time point after siRNA transfection was chosen. However, given that evidence was provided in this Chapter that mRNA and protein levels do not always correlate during knockdown, and that this effect can be gene-specific, there is no way of knowing whether the LMO2 protein was knocked down to appreciable levels at this time point. Thus, expression profiling data from the LMO2 knockdown experiments, described in Chapter 4, must be considered with this in mind. Further validation of LMO2 antibodies which perform well in western analysis would be required to resolve this issue.

3.6 Conclusions

The work presented in this Chapter demonstrated that RNAi is a relatively straightforward technique to knockdown gene expression of specific genes, provided that one is prepared to perform

the appropriate controls and develop assays to monitor both mRNA and protein levels across time courses. That said, the development of siRNA assays for members of the SCL erythroid complex has thus provided a means to elucidate the transcriptional targets of this complex in subsequent Chapters of this thesis.

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