Chapter 7

Summary and future work

7.1 Summary of work presented in this thesis

With the completion of the sequencing of the human genome, studying how gene expression is regulated at the transcriptional level is fundamental in order to delineate biological pathways. Whilst haematopoiesis is one of the most well studied biological systems, the transcriptional control of the genes expressed therein is not fully understood. SCL, also known as TAL1, is the master regulator of haematopoietic development. It forms a multiprotein complex with GATA1, E2A, LDB1 and LMO2 (SCL erythroid complex) which binds to sequence-specific motifs in regulatory elements to regulate expression of its target genes. However, only three human genes have been identified as target genes for this complex: glycophorin, c-kit and α-globin. Therefore, the aim of the work presented in this thesis investigated the downstream regulatory network controlled by this SCL erythroid complex in human haematopoiesis. This was addressed using a combination of two independent approaches: (i) expression profiling to determine the effects of siRNA-mediated knockdown of the members of the SCL erythroid complex, and (ii) ChIP-on-chip studies to identify promoters bound by the complex. As a conclusion to this thesis, summaries of the principle findings of this thesis and possible avenues of future work will be discussed.

7.1.1 Developing working siRNAs for members of the SCL erythroid complex in K562 (Chapter 3)

In Chapter 3, siRNAs were used to induce knockdown of each of five members of the SCL erythroid complex. The siRNA assays were first characterised in the following aspects:

- The transfection efficiency and the delivery of siRNAs to the cells by electroporation were monitored by flow analyses using a FITC-labelled siRNA. This was done to investigate whether the transfection method was efficient for siRNA and to determine the correlation with knockdown efficiency. Using GATA1 knockdown as a model, it was shown that the transfection efficiency was over 90%. This was consistent with the level of GATA1 knockdown at both the mRNA and protein level.
- The growth pattern and cell morphology of K562 cells were investigated to study non-specific effects of electroporation and siRNA-mediated knockdown. It was shown that electroporation, but not siRNA, induced growth arrest and cell morphology changes within 48 hours after transfection.

The siRNA knockdown for each of five members of the SCL erythroid complex (GATA1, SCL, E2A, LDB1, LMO2) was further optimised through time-course studies. At least two siRNAs were shown to induce a knockdown efficiency resulting in less than 30% of the mRNA remaining at the 24 hour time point for each gene. To identify the protein levels during knockdown, antibodies were also tested and characterised for all of the members of the complex except LMO2.

The kinetics of knockdown was shown to be different for the different siRNAs and/or different genes being targeted in time-course study. It was found that 24 hour was the most optimal time point for all knockdowns apart from LDB1 which had an optimal knockdown at 36 hour after transfection. It was also noted that the mRNAs and proteins had different stability in some cases as the time points at which a maximum level of knockdown was induced for the mRNA differed from that of the protein.

7.1.2 Expression profiling analyses of siRNA knockdowns of the SCL erythroid complex (Chapter 4)

Following the characterisation and optimisation of the siRNA assays, the effect of knockdown of members of the SCL erythroid complex on the expression of other genes in the genome was studied in Chapter 4 using a human Affymetrix expression GeneChips. This array covers all of the annotated protein-coding genes in the entire human genome.

Samples prepared from each siRNA-mediated knockdown of the members of the SCL eyrthroid complex were hybridised to the Affymetrix GeneChips. Published known target genes for most transcription factors were identified by expression changes in siRNA assays, including glycophorin A (one of the published targets of the SCL erythroid complex), which was shown to change its level of expression in assays for SCL, GATA1 and E2A. This proves that the siRNA-induced knockdown in combination with Affymetrix expression analyses was able to identify published target genes of the transcription factors. However, the validation rate of genes which showed expression changes on the Affymetrix GeneChips using qPCR was only 37%. This demonstrates that the Affymetrix GeneChips may not be a reliable way (at least for this study) of expression profiling for the knockdown assays used here.

A disproportionate number of DNA binding proteins and haematopoietic-specific genes were identified for each member of the complex being studied. The DNA binding proteins include both transcription factors and some chromatin remodelling/modifying factors. This implied that the SCL erythroid complex plays an important role in the transcriptional regulation of genes related to blood development possibly through the control of other transcription factors and chromatin remodelling complexes. A number of genes were found to show expression changes induced by siRNA-

mediated knockdown of more than one member of the complex. In particular, 102 probes (92 genes) were shown to be regulated by GATA1, SCL and E2A – these, again, included a number of transcription factors. Taken together, these data confirm the idea that members of the SCL erythroid complex are likely work co-operatively to control gene expression. In addition, an auto-regulatory role of the SCL erythroid complex was observed i.e. one member of the complex regulating another. For example, E2A was activated by both SCL and GATA1. This provided further insight into the regulation of the SCL erythroid complex during haematopoiesis.

7.1.3 ChIP-on-chip analyses of the SCL erythroid complex (Chapter 5)

As a complimentary approach to identify targets of the SCL erythroid complex, chromatin immunoprecipitation in combination with a human transcription factor promoter array was used to confirm and identify putative target genes. The human transcription factor promoter array contained PCR products which covered 1 kb regions around the transcription start sites of the majority of human transcription factors and chromatin modifying/remodelling proteins. Multiple biological and technical replicates of ChIP-on-chip experiments were performed using the antibodies against all five members of SCL erythroid complex in wild type K562 cells. A number of transcription factors and chromatin modifying/remodelling factors were shown to bind one or more members of the complex in their promoter regions and were thus considered putative targets of members of the complex. Furthermore, each member of the complex also detected enrichments at the promoter, -10 enhancer and the +51 erythroid enhancer of SCL (where the consensus E-box GATA motif of the SCL erythroid complex had previously been identified). Conserved GATA and E-box motifs were also identified in the promoter regions of some of the novel putative targets. Binding to most of these promoter regions, by various members of the complex, were confirmed by ChIP-qPCR in K562 cells. Many of these binding events were also confirmed by ChIP-qPCR in another human erythroid cell line HEL, validating the biological relevance of the K562 targets. Consistent with the data obtained in the Affymetrix expression analyses, an auto-regulatory role for members of the SCL complex was observed. It was shown that LMO2, SCL and ETO2 (the latter has also been shown to be a member of the complex), were bound at their promoters by at least one other member. Taken all together, the data from this Chapter strongly suggested that eight new targets of the SCL erythroid complex had been identified: ETO2, LYL1, SMARCA5, CTCFL, BRD2, SCL, ELKF1, EZH2.

7.1.4 Further characterisation of putative target genes of members of the SCL erythroid complex (Chapter 6)

The Affymetrix expression analyses and the ChIP-on-chip analyses provided different information regarding the transcriptional regulation of the SCL erythroid complex. These data were compared in

Chapter 6 and it was demonstrated that only a small proportion of genes were identified to be putative targets by members of the SCL eytrhoid complex in both datasets. Experiments were described in this Chapter which provided some evidence as to why ChIP-on-chip and Affymetrix analysis yielded mainly different sets of targets.

Expression time-course studies of the putative target genes (obtained from ChIP-on-chip analysis) during siRNA-induced knockdown of each member of the complex revealed that target genes are affected at the level of expression in complex ways. Out of the fourteen putative target genes, four are repressed and two are activated by at least one member of the complex. Five are either activated or repressed by different members and the remaining three showed no significant change in expression. Similarly, it was also confirmed in these time-course studies that all members of the SCL erythroid complex are affected at the level of expression, when any one of the members is knocked down. Both of these experiments highlighted issues that may mean that the Affymetrix datasets may be difficult to interpret. Another reason to explain the differences in the Affymetrix and ChIP-on-chip datatsets was revealed in the ChIP-on-chip study during GATA1 knockdown. Enrichments in promoters and enhancers were shown to be not affected substantially in some cases when GATA1 was silenced. Electroporation was also shown to affect the binding of GATA1 to promoters when compared to the ChIP-on-chip study in the wild type cells. Thus, siRNA-mediated knockdown may not be sufficient to remove bound transcription factors from the promoters of their target genes. This would suggest that the Affymetrix analysis of the siRNA knockdown samples may not reveal detectable expression changes in some of its direct targets, where the relevant TF was still bound to its regulatory elements. The actual proportion of targets affected in this way is not known.

One limitation of using a promoter-based array in ChIP-on-chip studies is that binding of members of the SCL erythroid complex mediated at locations outside of promoters are not likely to be detected (unless promoters and other regulatory elements are in contact within the nucleus). However, for one target gene of the SCL erythroid complex, LYL1, it was possible to deduce binding events which lay outside the promoter region. Using known structural and functional similarities between LYL1 and SCL, it was shown that the both loci are bound by the complex in similar locations in both K562 and HEL cells. It was confirmed that the complex bound to a region containing a consensus E-box/GATA motif lying 33 kilobases downstream of the LYL1 promoter. This resulted in the identification of a putative LYL1 enhancer (named +33) which corresponds structurally to the SCL +51 erythroid enhancer. This provides evidence that SCL, through its erythroid complex, regulates its structural and functional paralogue, LYL1 in a similar manner to which it regulates its own expression in the erythroid lineage.

Based on the data obtained from the previous Chapters of this thesis and the expression time-course studies, transcriptional networks describing the relationship between the putative target genes and the transcription factors regulating them were generated. These network diagrams integrated all the information together to simulate the downstream regulatory network by the SCL erythroid complex in human erythroid cells. Many network motifs were also identified in these networks which were linked to the biology of erythroid development. Thus, the results from this thesis presented, for the first time, a regulatory network for the erythroid lineage, which was based on interactions involving the SCL erythroid complex.

7.2 Future work

The data describing the regulatory interactions involving the SCL erythroid complex in this thesis provide a useful benchmark for the elucidation of more complex regulatory networks of erythroid development in human cells. Possible avenues of future work are discussed below.

7.2.1 Confirmation of putative target genes in primary cells

All the studies performed for this thesis centered around an analysis of the human erythroid cell line K562. K562 is a well-established cell line, which is relatively easy to culture and transfect *in vitro* and grows relatively quickly with an average doubling time of 24 hours. Despite these advantages, this cell line was developed from a chronic myeloid leukaemia (CML) patient in blast crisis (Lozzio and Lozzio, 1977). It carries the BCR-ABL translocation where the BCR-ABL fusion protein is constitutively expressed in these cells. The BCR-ABL fusion is implicated in the suppression of apoptosis by activating a number of cell cycle genes and altering signalling pathways [reviewed in (Mughal and Goldman, 2006)]. Such a translocation undoubtedly has a number of undesirable effects on the expression of other genes in the genome, making it, to a certain degree, unrepresentative of the normal erythroid lineage. Thus, studies carried out with K562 should be taken with care and confirmed in other erythroid cell lines or, ideally, primary erythroid cells. HEL, an erythroid cell line derived from an erythroleukaemic patient, which does not contain the BCR-ABL translocation, was used to confirm the enrichments of promoters in the ChIP-qPCR studies. However, the HEL cell line is still a cancerous cell line and may not reflect normal erythroid development.

To confirm the regulation of putative target genes in normal human cells, primary erythroid cells (CD71+/GPA+) from normal individuals should be used for siRNA-induced knockdown expression studies and ChIP-on-chip study. The isolation of sufficient amount of primary cells for ChIP-on-chip studies is one of the obstacles hindering such future analyses. Moreover, primary cells are not

easy to transfect (Marodon et al., 2003). However, with the rapid advances of ChIP protocols and RNAi delivery, this is becoming possible. As mentioned previously in Chapter 1, retroviruses and lentiviruses have been successfully employed to deliver shRNAs into primary cells (Barton and Medzhitov, 2002; Stewart et al., 2003). Obviously, optimisation of transfection is required for different types of primary cells and cloning of shRNA to viral cassettes is necessary. As working siRNAs were already validated in Chapter 3 for members of the complex, cloning of these sequences into shRNAs should be possible, although it is not known whether these sequences will work well in shRNA systems. shRNAs must be processed into siRNA *in vivo* using the endogenous RNAi system. The flanking sequences, together with the sequences complementary to the target gene, must be carefully designed so that the shRNA can be processed *in vivo*. New technologies of ChIP study such as Carrier ChIP and MicroChIP have been used to map histone modifications with a limited number of cells (Chapter 5, section 5.1.1). Indeed, a fast carrier ChIP protocol has been developed to study transcription factor-DNA interaction in brain tissues (Hao et al., 2008). Such protocols would be required to make it possible to use limited numbers of primary erythroid cells for ChIP-on-chip or ChIP-qPCR analyses.

7.2.2 Investigation of histone modifications associated with the regulatory pattern

Another component of a transcription regulatory network is the chromatin domains and modifications associated with the genes in the network. As mentioned in Chapter 1, chromatin structure affects the binding of both sequence-specific and general transcription factors to regulatory element and thus plays a crucial role in transcriptional regulation of target genes. As a result, integrating the information for both chromatin modification and transcription factor binding can facilitate a more complete understanding of transcription.

Histone lysine acetylation has been shown to be associated with active transcription whereas deacetylation of histone subunits correlates with repression of transcription (Tse et al., 1998). Methylation of histone H3 lysine 4 is linked to active genes while methylation of histone H3 lysine 9 is linked to a repressed state (Lachner and Jenuwein, 2002) (Chapter 1, section 1.1.2.5). ChIP in combination with qPCR or microarray analysis could be used to study the modification status at the promoters and other regulatory elements of some of the putative target genes. This will allow us to determine the role of histone modifications in the regulation as well as to investigate the activation or repression of the genes. Furthermore, ChIP studies of these histone modifications during the knockdown of transcription factors in the SCL erythroid complex could be performed to study the significance of the transcription factor binding on the modifications status of relevant regulatory regions. This is because in some cases, chromatin-modifying enzymes are recruited to the regulatory elements of genes by transcription factors and co-factors that bind to these regions

(Brehm et al., 1998) (Chapter 1, section 1.1.2.3). Thus, studying how the modification status changes during knockdown can reveal important information about the precise role that transcription factors play in recruiting chromatin modifying complexes and how they facilitate the open chromatin structures which are required for RNA polymerase complexes to bind and initiate transcription or how they facilitate repressive chromatin configurations.

7.2.3 Identification of all possible regulatory elements bound by the SCL erythroid complex

As demonstrated in Chapter 6 section 6.3.1.5, the SCL erythroid complex has been shown to bind to the SCL +51 enhancer and a putative enhancer for LYL1. However, the microarray used in this thesis only contains promoter regions of human transcription factors. Thus, many regulation and binding events may be missed out in this experiment. To facilitate the identification of all possible binding sites within or outside the promoter regions of each member of the SCL erythroid complex, alternative methods should be employed. Tiling arrays covering a broader region of a particular gene locus of interest or whole genome tiling arrays can be used to study binding outside the promoter region (Horak et al., 2002; Lee et al., 2006). With the recent advances in the next generation sequencing technology, ChIP-seq can be performed for whole genome Solexa or 454 sequencing to map the binding sites in an unbiased manner (Barski et al., 2007; Johnson et al., 2007; Schones et al., 2008).

7.2.4 Knockout and ChIP studies of interesting putative target genes

The studies of transcriptional regulation in this thesis only focused on the SCL erythroid complex itself but not other transcription factors which are involved in erythroid development. From the ChIP-on-chip study, only a handful of direct target genes were identified for this complex. On the other hand, the Affymetrix analyses possibly identified a large number of putative direct and indirect target genes. One obvious area of interest would be to begin to assemble networks which incorporate all the appropriate links between direct and indirect targets of the SCL erythroid complex. One solution to this issue is to further investigate the transcriptional targets of the direct target genes of the SCL eryrthroid complex and identify their targets (so called “targets of the targets”). This again could be done by using a combination of complete gene knockout in mouse, expression analysis and ChIP-on-chip or ChIP-sequencing. Given that many problems are associated with the siRNA knockdown studies presented in this thesis, a better method of studying mode of regulation would be to generate conditional homozygous gene knockouts in mouse.

EKLF, LYL1 and ETO2 are interesting target genes which could be prioritised for knockouts as well as for use in ChIP-on-chip or ChIP-seq studies. Evidence was provided in this thesis that all

three of these TFs are targets of the SCL erythroid complex and their expression is regulated by the complex. Thus understanding the targets of these three TFs would be important for our understanding of erythropoiesis and regulatory events downstream of the SCL erythroid complex. EKLF is known to be a transcriptional activator of β-globin (Miller and Bieker, 1993). Furthermore, perturbation of EKLF in mouse ES cells demonstrated that EKLF is required for the final stages of definitive haematopoiesis (Nuez et al., 1995). ETO2 was identified as an interacting partner of the SCL erythroid complex and was shown to repress the transcription activator activities of the complex (Goardon et al., 2006). This was suggested as a developmental switch for expression of regulators related to terminal erythroid differentiation. The repressive function of ETO2 has been suggested to link to histone deacetylation as ETO2 associates with HDAC family members (Gelmetti et al., 1998; Wang et al., 1998). Therefore; studying the genes regulated by ETO2 and comparing them with the target genes of the SCL erythroid complex will provide insights into the dynamic changes in gene expression and repression controlled by the SCL erythroid complex, and the role of ETO2 within this complex, during erythroid differentiation. As mentioned previously in Chapter 1, LYL1 has a highly similar expression pattern and function as SCL and is considered to be its functional and structural paralogue. The identification of the LYL1 putative +33 enhancer (which resembles the +51 enhancer of SCL) also revealed a possible similar mode of regulation between the two during erythroid development. Thus, studying LYL1 in details allows us to further assess the role of this gene during erythropoiesis and would shed further light on its regulatory relationship with SCL.

7.2.5 Identification of other interacting partners in the SCL erythroid complex

In order to further characterise the SCL erythroid complex, studying the DNA elements to which it binds tells only one part of the story. Another aspect of understanding the complex is to characterise all of the protein components in the complex. Using CASTing and gel shift assay, the five initial components were identified to recognise and bind to the consensus E-box and GATA motifs (Wadman et al., 1997). Previous protein interaction studies using GST pull-down assays and co-immunoprecipitation have been used to identify other interacting partners of this complex. These include Sp1 (Lecuyer et al., 2002), and pRb (Vitelli et al., 2000). A larger-scale analyses of SCL interacting partners by expression of a biotin-tagged SCL protein followed by pull-down assays and mass-spectrometry identified ETO2 as a novel component (Goardon et al., 2006). However, a large-scale study to identify all possible components which bind specifically to the E-box/GATA composite DNA motif is still lacking. Immobilised DNA probes containing TFBS motifs have been used in matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Nordhoff et al., 1999) to identify proteins which bind directly to DNA. Similar approaches

could be used to identify protein complexes which bind the E-box/GATA composite motifs or motifs nearby. However, this analysis would also allow us to examine protein components which do not directly bind DNA. Some known protein components of the SCL erythroid complex have been shown to recruit and interact with chromatin modifying or remodelling complexes which do not bind DNA directly. For example, SCL was shown to associate with HDACs (Goardon et al., 2006) whereas ETO2 was also shown to recruit HDAC family members (Gelmetti et al., 1998; Wang et al., 1998). In fact, the SCL complex has been demonstrated to associate with the SWI/SNF protein Brg1 which is a family of chromatin remodelling complexes which leads to transcriptional repression at the P4.2 protein in mouse (Xu et al., 2006). Thus, studying all the proteins by mass spectrometry would yield a better understanding of the role of all of the components of SCL erythroid complex and provide clues as to how this complex regulates gene transcription.

7.2.6 Functional assays for the putative LYL1 enhancer

Unlike SCL, where there have been many studies to identify the genomic sequences involved in its regulation (Chapter 1, section 1.4.2.1 E), little is known of the regulatory sequences which regulate LYL1 expression. Thus, the identification of a putative LYL1 enhancer (+33) which binds the SCL erythroid complex, gives one the opportunity to further characterise the regulation of LYL1. Previous analyses of the SCL +51 enhancer demonstrated it has enhancer activity in reporter assays (Dhami et al. submitted). To confirm the activity of the +33 region of LYL1, enhancer or promoter trap assays could be performed. This +33 region can be cloned into a reporter construct upstream of a luciferase reporter gene so that expression of the luciferase gene can be measured and compared to a control without the +33 region (Chapter 1, section 1.3.3.1 D). This will allow us to determine whether this region acts as an enhancer or promoter. However, similar to the situation with the +51 SCL enhancer, the +33 region of LYL1 lies closer to the promoter of another gene, NFIX than it does to the LYL1 promoter. Therefore, it would be necessary to determine whether this putative enhancer regulates expression of the LYL1 promoter and not the NFIX gene. One possible way would be to design the enhancer trap assays so that the reporter assay is driven under the control of either the LYL1 or NFIX promoters. However, given that enhancer traps are artificial constructs and do not take into account other regulatory aspects found *in vivo* or *ex vivo* (chromatin features, for example), this may not be sufficient to determine which gene the +33 region regulates *in vivo*. Therefore, one could generate mutations in the conserved E-box/GATA motif within the +33 element of an appropriate cell line (*ex vivo*) or as a mouse knockout (*in vivo*) and evaluate the effect this has on expression of NFIX and LYL1.

7.2.7 Studies of changes in expression or promoter binding of target genes at different stages of erythroid differentiation

As previously mentioned, the studies performed in this thesis focused on a cell line which may only reflect a specific timepoint in erythroid development. K562 cells can be differentiated into both erythroid and megakaryocytic lineages, suggesting that these cells are bipotential progenitors of both lineages. Thus, information obtained for this thesis, may not reflect aspects of early erythroid development alone, but also that of early megakaryocytic progenitors. The regulatory role of the SCL erythroid complex further downstream in erythroid development could be investigated by differentiating K562 cells. K562 can be differentiated into the erythroid lineage by the addition of hemin, and into the megakaryocytic lineage by the addition of phorbol myristate acetate (PMA), to culture media (Huo et al., 2006; Yi et al., 2004).

Indeed, hemin was used to demonstrate the differentiation of K562 in our laboratory (Figure 7.1). glycophorin A (GPA) is a cell surface marker of erythroid cells and a PE conjugated anti-GPA antibody was used to detect the cells expressing GPA in flow cytometry analysis. The highest GPA expression was observed at 72 hours after induction of differentiation. GPA expression diminished at 96 hour possibly due to the terminal differentiation of erythrocytes. This differentiation experiment demonstrated that K562 can be easily differentiated down the erythroid lineage and thus provides a useful platform for differentiation studies.

ChIP in combination with various analytical platforms (qPCR, arrays, or massively parallel sequencing) could be performed to study the changes in binding of DNA elements by the SCL complex during differentiation. This will allow us to determine which target genes are important at different stages of erythroid development.

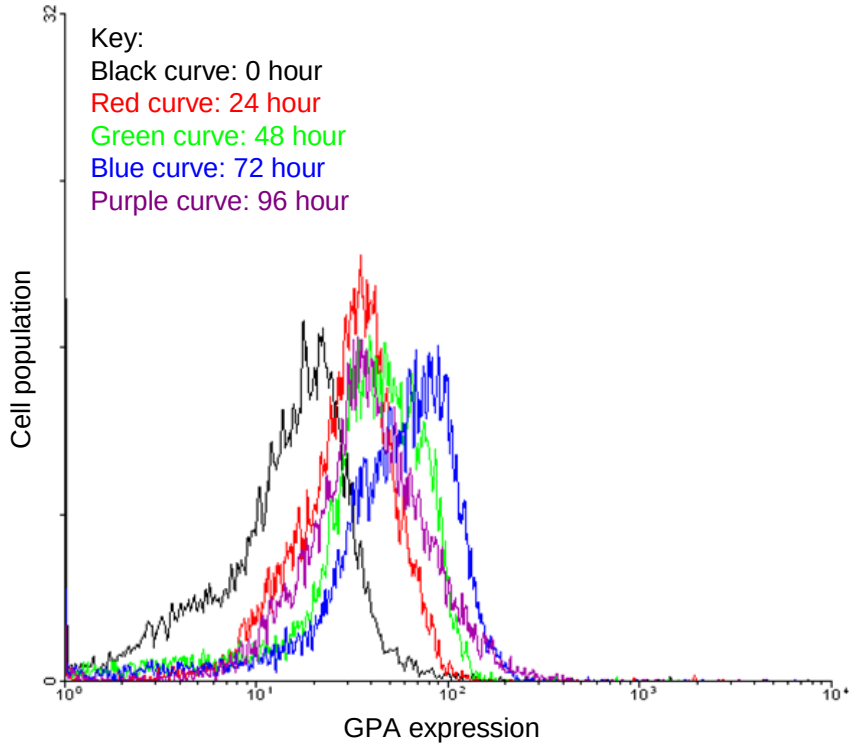

Figure 7.1. Differentiation of K562 cells by hemin. K562 cells were induced to differentiate down the erythroid lineage using hemin. The expression of the erythroid cell surface marker GPA was monitored by flow analysis using a PE conjugated anti-GPA antibody. Y-axis: number of cells in the population; x-axis: GPA expression. Black curve: 0 hour after induction of differentiation; red curve: 24 hour after induction of differentiation; green curve: 36 hour after induction of differentiation; blue curve: 48 hour after induction of differentiation; purple curve: 72 hour after induction of differentiation.

7.3 Final thoughts

The results presented in this thesis illustrated the downstream regulatory cascades of the SCL erythroid complex in the erythroid lineage. This is the initial effort of addressing its role in only one of the lineages in haematopoietic development. Given that there are more than 2000 transcription factors and more than 30000 human genes, understanding how various protein complexes control the expression of specific genes in haematopoietic development requires a huge effort. Advances in technology and computational tools are necessary to integrate all the data in a biologically meaningful way. The findings in this thesis provide a foundation and will make a valuable contribution towards this goal.

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