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Review Article

Identification and characterization of enhancer elements controlling cell type-specific and signalling dependent chromatin programming during hematopoietic development

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Abstract: The development of multi-cellular organisms from a single fertilized egg requires to differentially execute the information encoded in our DNA. This complex process is regulated by the interplay of transcription factors with a chromatin environment, both of which provide the epigenetic information maintaining cell-type specific gene expression patterns. Moreover, transcription factors and their target genes form vast interacting gene regulatory networks which can be exquisitely stable. However, all developmental processes originate from pluripotent precursor cell types. The production of terminally differentiated cells from such cells, therefore, requires successive changes of cell fates, meaning that genes relevant for the next stage of differentiation must be switched on and genes not relevant anymore must be switched off. The stimulus for the change of cell fate originates from extrinsic signals which set a cascade of intracellular processes in motion that eventually terminate at the genome leading to changes in gene expression and the development of alternate gene regulatory networks. How developmental trajectories are encoded in the genome and how the interplay between intrinsic and extrinsic processes regulates development is one of the major questions in developmental biology. The development of the hematopoietic system has long served as model to understand how changes in gene regulatory networks drive the differentiation of the various blood cell types. In this review, we highlight the main signals and transcription factors and how they are integrated at the level of chromatin programming and gene expression control. We also highlight recent studies identifying the *cis*-regulatory elements such as enhancers at the global level and explain how their developmental activity is regulated by the cooperation of cell-type specific and ubiquitous transcription factors with extrinsic signals.

Keywords: Hematopoiesis; gene regulatory networks (GRNs); signalling; RUNX1; embryonic stem cells (ESCs); identification of signalling responsive enhancers

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Introduction

Embryonic development is tightly controlled at the level of gene expression. The precise control of tissue-specificity of gene expression is essential for successful development and depends on distal *cis*-regulatory elements such as enhancers which interact with promoter elements in physical space. The activity of these elements is controlled by transcription factors (TFs) which bind to DNA wrapped into chromatin, leading to the modification of the chromatin landscape, and the assembly of the transcription machinery. Transcription

has been shown to occur within defined chromosomal locations termed topologically-associated domains (TADs) [reviewed in (1)] where TF complexes bring together control elements across large distances within the genome [reviewed in (2)].

In the developing embryo, TFs regulating the assembly/disassembly of transcriptional complexes and ultimately gene expression, are directed by complex extrinsic signalling processes which connect all cells within a multi-cellular organism to their environment. Cell-to-cell signalling is induced by specific ligands such as growth factors which activate their cognate receptor molecules. Upon binding of their respective ligands and activation, intracellular signalling cascades, often involving phophorylation are induced which eventually terminate at inducible TFs and regulate their activity. The regulation of cell growth and differentiation therefore involves the precise and coordinated interplay of cell extrinsic and intrinsic processes.

For many decades the development of the hematopoietic system has been used as a model for studying the molecular basis of cell fate decisions and gene regulation, and as such it is one of the best understood developmental pathways. In vertebrates, embryonic hematopoiesis is the process which generates hematopoietic stem cells (HSCs). These cells sit at the top of the hematopoietic hierarchy and have the ability to self-renew and give rise to all mature blood cell types in the adult organism (3). Furthermore, HSCs are maintained for life and replenish components of the blood system (4). Operationally, HSCs are defined as cells that provide long-term reconstitution of the entire hematopoietic system of an irradiated adult recipient (5).

An experimental model that has yielded important insights into the molecular details of hematopoietic specification is the differentiation of embryonic stem cells (ESCs) into blood (6,7). ESCs are derived from the inner cell mass (ICM) of the blastocyst (8-10). However, so far blood progenitor cells produced in such a system were unable to yield long-term hematopoietic reconstitution. The precise signals that control the formation of these cells and their correct gene expression patterns have been largely elusive. Understanding how signalling and the cellular environment direct the differentiation of ESCs to HSCs is therefore of great importance, as the ability to produce large quantities of HSCs capable of giving rise to any of the constituents of blood *in vitro* would be of significant therapeutic and biotechnological value [reviewed in (11,12)]. To achieve this aim, we need to know how HSC identity is established at the gene expression control level. We need to know the genomic location of *cis*-regulatory elements directing cell-type-specific control during the whole differentiation pathway, the TFs regulating their activity and which signalling pathways these TFs respond to. This review will cover (I) our current understanding of embryonic hematopoiesis, (II) the use of *in vitro* differentiation systems for study and production of HSCs and (III) the methods used for the identification and study of signalling responsive *cis*-elements and the TFs regulating these processes.

Embryonic hematopoietic development occurs in several waves

Pluripotent cells from the blastocyst give rise to any of the three germ layers: endoderm, mesoderm and ectoderm (13,14). In the mouse embryo primary germ-layer specification occurs between embryonic days (E) 4.5 and E7.5 (15). Hematopoietic specification occurs from the mesodermal germ layer in three waves. The first wave, known as 'primitive hematopoiesis', occurs at around E7 in the blood islands of the yolk sack producing mature primitive erythrocytes, macrophages and megakaryocytes (16-18). This wave does not produce HSCs, instead, it provides short-lasting hematopoietic cells required for the embryo's needs, such as oxygen supply, tissue remodelling and vascular maintenance (19,20). At E8.25 the second hematopoietic wave produces both erythroid-myeloid progenitors (EMPs) and long lasting late EMPs which can differentiate into cells displaying adult blood cell characteristics and functions (21,22). At this timepoint, embryonic lymphoid commitment begins through the emergence of immune-restricted and lymphoid-primed progenitors which contributes to the establishment of lymphoid and myeloid components of the immune system (23). However, no HSCs that fulfil the criteria above are produced.

In mice the final wave of blood cell development occurs at E10.5 [E27 to E40 in humans (24)] and gives rise to the definitive HSCs which emerge from the ventral section of the dorsal aorta in a region termed the aorta-gonad-mesonephros (AGM) which is derived from the mesodermal germ layer (5,25). It is these cells which are capable of reconstituting the entire adult hematopoietic system. In response to paracrine and autocrine signalling HSCs develop from a specialised hemogenic endothelium (HE) which overlays the dorsal mesenchyme within the AGM.

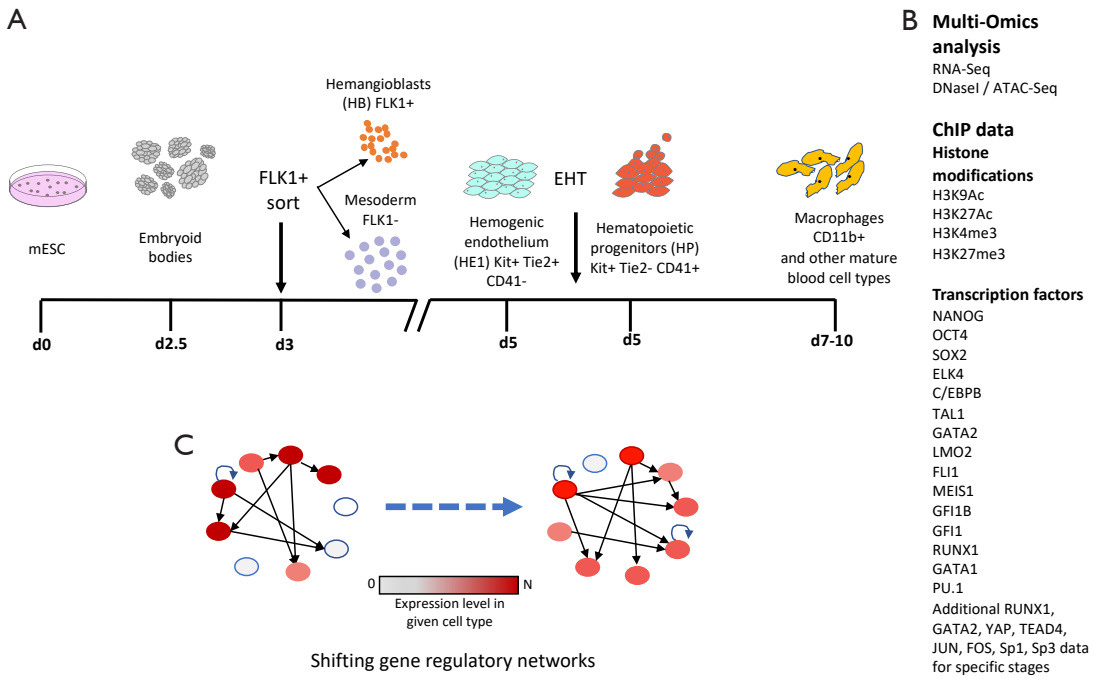

Figure 1 Using multi-omics analysis to identify gene regulatory networks driving blood cell development form mouse embryonic stem cells. (A) Schematic of the serum *in vitro* differentiation system used to differentiate mESCs through to macrophages as shown in Goode *et al.* (39). The timepoints and cell types generated are shown including the cell surface markers used for cell sorting. (B) Datasets generated by Goode *et al.* (39), Obier *et al.* (40), Gilmour *et al.* (41,42) and Kellaway *et al.* (43). (C) Schematic of gene regulatory networks consisting of TF encoding genes forming nodes (coloured circles) by virtue of their gene products (TFs) binding to other TF-encoding genes (edges drawn as arrows). The colour of the node represents the gene expression level in the given cell type with some genes being repressed or bound and not yet expressed (white circles). mESC, mouse embryonic stem cell; EHT, endothelial-to-hematopoietic transition; ATAC-seq, assay of transposase accessible chromatin sequencing; TF, transcription factor.

The HE then undergoes an endothelial-to-hematopoietic transition (EHT) (19,26-29) during which flat endothelial cells buldge upwards towards the intra-aortic lumen (30-32) by forming clusters, lose their endothelial transcriptomic signature and adopt a hematopoietic phenotype (33). Once formed and after maturation (34), HSCs bud off, enter the bloodstream and colonise the foetal liver and then subsequently the bone marrow where they generate all hematopoietic cell types (35). However, the dorsal aorta is not the only endothelial layer capable of generating blood cells, also the earlier waves of blood cell formation are formed by an EHT process (36).

Hematopoietic development from ESCs is regulated by dynamic gene regulatory networks (GRNs)

TFs and their respective targets, including genes that encode TFs themselves, form GRNs which define the identity of a cell (37,38). It therefore follows that in development, different cellular identities are established by changes from one GRN to another. To understand this process in molecular detail, it is necessary to (I) identify cell type-specific *cis*-regulatory elements, (II) how these impact on gene expression, (III) identify the transcriptional complexes which bind to them and (IV) to understand how these respond to external cues.

The *in vitro* differentiation of ESCs into blood cells which is representative of the second wave of blood cell development has proven instrumental in gaining a mechanistic understanding of this process. Goode *et al.* (39) employed a culture system in which mouse ES cells are replated into a culture medium that does not support pluripotency. Replating leads to the formation of embryoid bodies within which hematopoietic specification takes place, and from which the different cell types from this pathway can

be isolated by employing cell sorting (*Figure 1A*). Cell types include mesodermal cells and cells expressing the endothelial marker FLK1 (the receptor for the endothelial growth factor VEGFA encoded by the *Kdr* gene). These cells, which give rise to both endothelial and hematopoietic cells, are purified using cell sorting and replated in a culture medium that supports the formation of the hemogenic endothelium (HE1). From these cells, the first steps of hematopoietic commitment take place with the down-regulation of the endothelial marker *Tie2* and the up-regulation of the CD41 marker, generating HE2 cells which are still adherent. Thereafter, cells bud off and form hematopoietic progenitor (HP) cells through the EHT. When exposed to the right cytokines, HP cells are capable of forming both lymphoid and myeloid cells, exemplified here by macrophages (*Figure 1A*). In order to examine, how the interplay of TFs and chromatin components drives gene expression at the different stages of blood cell specification, Goode *et al.* (39) generated global multi-omics data on measuring TF binding, gene expression, histone modifications and open chromatin regions (listed in *Figure 1B*) during six sequential stages of hematopoietic differentiation from mouse ESCs to macrophages, including HE and HP. The work revealed the chromatin signature of potential regulatory elements and the TF binding patterns driving the differential gene expression required for hematopoietic lineage commitment. It allowed to group chromatin states into "inactive" (H3K27me3), "poised" ("H3K4me3/H3K27me3), "active" (H3K4me3 or H3K27Ac) and "unmarked". In addition, integrating TF binding, gene expression and chromatin structure, this work allowed to construct a comprehensive dynamic core regulatory network model for hematopoietic specification with network connections being rewired during differentiation (represented in *Figure 1C*). Moreover, beyond what could be measured by chromatin immunoprecipitation (ChIP) assays the work highlighted the relative importance of TFs with respect to their importance for the maintenance of specific cell types which is encoded in the binding motif composition of cell-type specific *cis*-regulatory elements (44). The analysis confirmed known roles of TFs involved in blood specification at key developmental stages but also identified signalling responsive TFs required for correct blood cell development, such as activator protein 1 (AP-1) and transcriptional enhanced associate domain (TEAD).

The hematopoietic cell fate is established by the interplay between signalling responsive and differentially expressed TFs

Becoming a blood cell requires the developmentally controlled expression of genes coding for TFs that are crucial for hematopoiesis, which interact with constitutively expressed and signalling responsive genes to control the transition from one GRN to another. In recent years, we have obtained significant insights into the mechanism of action of the most important hematopoietic TFs driving hematopoietic development and how they respond to signals.

The studies described above demonstrate that the final event in the generation of HSCs and multipotent progenitor cells is the EHT where blood cell fate is finally established. The EHT takes place in a defined cellular context. The site of the EHT in the midgestational mouse embryo is restricted to an endothelial cell layer at the ventral side of the dorsal aorta, demonstrating that the signalling environment plays an essential role in directing hematopoietic cell fate (5,45-48). These endothelial cells sit on the dorsal mesenchyme, which communicates through signalling to support and drive the commitment of endothelial cells towards the hematopoietic lineage between E8.5 and E10.5 (49-56). The TFs which direct this process can be broadly categorised into those that maintain an endothelial cell identity and those which drive the establishment of a hematopoietic cell fate. It is now clear from the study of the molecular events governing hematopoietic specification *in vivo* and *in vitro* that this process is highly complex and dynamic and is under tight transcriptional and signalling control (57). The next two paragraphs summarize the most important components.

The development and growth of endothelial cells and thus also the HE, requires the expression of the vascular endothelial growth factor receptor 2 (FLK-1) (28) which binds the vascular endothelial growth factor (VEGF) and induces signalling through the MAP kinase signalling pathway. TFs required for the development of these cells are SOX17, other members of the SOXF family (SOX7 and SOX18) and ETS TFs (ETV2) (58). At the same developmental stage, SOX17 is required for the repression of the hematopoietic genes *Runx1* and *Gata2* whose chromatin is already in a primed configuration

(39,59). ETV2 deficiency results in a complete block of endothelial and hematopoietic cell type formation in knockout mouse models (60). An important signalling pathway regulating the EHT is NOTCH1 signalling. Loss-of-function studies indicate that it is required for definitive hematopoiesis, while gain-of-function studies found that intra-aortic clusters failed to form in the AGM indicating that NOTCH1 signalling needs to be switched off after the EHT (61). ETV2 and SOX17 establish and maintain paracrine NOTCH1 signalling between endothelial cells by upregulating the expression of NOTCH1 and its ligand DLL4 (62). *Notch1* and *Dll4* expression requires the TFs FOXC1 and FOXC2 in response to VEGF/PI3K-mediated signalling (63-65). *FoxC2*-null mouse embryos show impaired definitive hematopoiesis while zebrafish *foxc1a/b* morphants have reduced expression of hematopoietic genes (*runx1*, *cmyb* and *rag1*) in the HE (66).

NOTCH signalling is directly linked to transcriptional control and provides an example of a tight feedback mechanism in the control of cell fate decisions. Binding of the NOTCH1 receptor to its ligand expressed on a neighbouring endothelial cell exposes the NOTCH1 cleavage site S2 to the metalloprotease ADAM10, leading to cleavage. ADAM-10 deficient mice die at E9.5 with defects in their central nervous system, somites and cardiovascular system. They show reduced expression of the NOTCH target gene *hes-5* and increased expression of the NOTCH ligand *Dll-1* (67-70). After cleavage, the NOTCH1 intracellular domain (NICD) translocates into the nucleus, interacts with the TF RBPJ and turns it from a repressor into an activator. NICD/RBPJ then recruit mastermind like transcription coactivator (MAML) and histone acetyl transferases which up-regulate the expression of NOTCH target genes (71). NOTCH1 signalling to RBPJκ controls the activation of hematopoietic genes, for example, RBPJκ binds to and activates the promoter of *Gata2* (72,73). In concordance with this result, RBPJκ mutant mouse embryos fail to form HSCs (72). However, NOTCH1 signalling also induces *Hes* genes encoding TFs that bind to and repress *Gata2*, thus preventing its overexpression once HES TFs reach a threshold level (72,74) thus maintaining endothelial identity.

Other important signalling pathways involved in the formation of endothelial cells and the HE are MAP Kinase signalling and HIPPO signalling, both of which regulate specific transcriptional programmes via signalling responsive TFs. MAP Kinase signalling terminates at the AP-1 family of TFs which consists of multiple JUN and FOS family proteins whereby FOS factors have to partner with JUN factors to be able to bind to DNA (75). The TF binding motif analysis performed by Goode *et al.*, showed a specific enrichment of AP-1 binding motifs in the HE suggesting an important role for these factors in regulating the hemogenic cell fate (39). It was indeed shown that c-JUN knockout mice die at mid- to late-gestation due to impaired hepatogenesis and foetal liver erythropoiesis (76,77). JUND and FOS are required for hematopoiesis in Xenopus embryos (78) and JUNB is an important regulator of the EHT in the HE derived from human ESCs (79). It has been shown that VEGFA signals via AP-1 (80). Experiments from our lab used ChIP experiments to determine the position of JUN and FOS within the GRN (40). Moreover, induction of a dominant-negative (dn)FOS peptide at different stages of murine ESC derived hematopoietic differentiation which blocked all AP-1 binding activity showed that expression affected the development of endothelial cells (40) and modulates the balance between vascular smooth muscle and hemogenic cell fate.

HIPPO signalling which involves a large number of different signals controlling cell shape and cell communication, such as integrin signalling, sheer stress and many others [reviewed in (81)] has only recently been shown to be important for hematopoietic specification (39,82). The central components of this signalling pathway are the TEAD TFs and their co-activators YAP/TAZ. When Hippo signalling is active, YAP/TAZ are phosphorylated by a variety of different kinases, bind 14-3-3 proteins and are targeted for degradation. If HIPPO signalling is off, YAP/TAZ translocate into the nucleus, partner with TEAD and regulate gene expression. We have shown that the interaction between TEAD and YAP is essential for the EHT to occur (39). In addition, experiments from the North lab (82) showed that YAP responds to shear stress as it would be observed in the aorta and is required to up-regulate *RUNX1* which initiates the hematopoietic program as explained in more detail below. Importantly, the dnFOS experiments (40) also revealed that TEAD and AP-1 cooperatively bind to specific *cis*-regulatory elements regulating endothelial and hematopoietic genes. Furthermore, they identified a sub-set of *cis*-regulatory elements where TEAD and AP-1 binding was interdependent to integrate MAP Kinase and HIPPO signalling at the genomic level (40). These experiments added to a growing body of literature that demonstrates that AP-1 is a common interaction partner driving a genomic response in the presence of multiple, but not individual

signals (83).

GATA2 is a member of the GATA family of TFs (84) and is considered essential for the EHT for both primitive and definitive hematopoiesis. In the dorsal aorta, *Gata2* deletion therefore results in a deficiency of intra-aortic clusters and HSCs in mouse embryos (85-88). Null mutations in *Scl* which encodes the TF SCL/TAL1 (89) cause a block of primitive erythropoiesis and homozygous mutant embryos die on E8.5 to E10.5. Furthermore, *Scl* (–/–) ESCs do not contribute to adult type hematopoiesis on chimeric analysis (90). However, the factor that truly drives the EHT is RUNX1 (91).

Runx1 is a master regulator of hematopoiesis and is essential for the EHT with *Runx1* knockout resulting in a complete failure in HSC production from the HE (91). RUNX1 requires its cofactor CBFβ to complete the EHT as demonstrated by knockout of *Cbfb* which caused a similar phenotype to that seen in *Runx1* knockout mouse embryos (92,93). The *RUNX1* gene is transcribed by two promoters with differential developmental activity. The proximal promoter drives low-level expression leading to the binding of RUNX1 to down-stream targets in the HE and resulting in the up-regulation of cell adhesion- and migration-associated genes (94,95). Expression in the HE correlates with the binding of GATA2 and SCL/TAL1 which are already expressed (39,96). RUNX1 binds to its own *cis*-regulatory elements which strongly up-regulates *Runx1* expression (97,98). Once RUNX1 levels pass a specific threshold, it reorganises the binding patterns of FLI-1 and SCL/TAL1 and the chromatin landscape of HE cells (97) and up-regulates other hematopoietic genes such as *Spi1* (PU.1) and *Cebpa* (99). In parallel, it induces the expression of the repressive TFs GFI1 and GFI1B which cooperate with the co-repressor LSD1 to shut down endothelial TF gene expression (100). RUNX1 also directly binds to and represses *Sox17* further shutting down endothelial-specific gene expression (97). In addition, RUNX1 expression changes the signalling environment by binding directly to the promoter of the *Flk-1* (*Kdr*) gene and down-regulating its expression (101). Furthermore, RUNX1 represses *Dll4* and *Notch1* transcription (102), resulting in the loss of the VEGF-NOTCH signalling axis which maintains an endothelial cell signature and represses a hematopoietic signature (100,103). In parallel, and in cooperation with PU.1, RUNX1 up-regulates genes for hematopoietic cytokines such as GM-CSF, IL-1 and CSF1R thus driving hematopoietic differentiation forward (104,105). RUNX1 therefore creates a feed forward loop which drives blood cells development and growth. However, as described above, it is under strong repressive control by NOTCH1 and SOX17 in the HE, and the question then remained of how the balance of *Runx1* activation and repression is regulated at the molecular level and which *cis*-regulatory elements are involved.

A genome-wide screen identifying developmentally regulated enhancer and promoter elements

To understand the mechanisms how dynamic GRNs control embryonic development processes we need to elucidate when and how *cis*-regulatory elements function at different developmental stages. For many decades, enhancer sequences were defined by transient or integrated reporter gene assays which show whether a specific sequence can stimulate the activity of a promoter independent of its position and distance (106). However, these assays do not inform on the dynamic behaviour of enhancers during development. In addition, enhancers were defined in a correlative way by the modification status of their flanking histones or by the fact that they are transcribed (107,108). The gold standard for experimentally studying the developmental activity of individual *cis*-regulatory elements are transgenic animal models which reveal the precise spatial and temporal activity of enhancers. However, these models are expensive and present challenges by integrating variable copy numbers of the transgene and displaying positional effects, requiring multiple transgenic lines to be produced to achieve a reproducible expression pattern (109,110). To study the role of individual enhancers within individual gene loci, they must be perturbed in their genomic environment using genome editing (107) but as with any candidate approach, such experiments only investigate one locus at the time.

To rigorously study developmentally controlled *cis*-element activity in a high-throughput fashion and in a chromatin environment, we adapted the enhancer reporter system developed by Wilkinson *et al.* (109,111) to perform a genome-wide enhancer screen (112). We first differentiated mouse ESCs in the serum containing culture system (*Figure 2A*) and sorted cells by fluorescence-activated cell sorting (FACS), which were then taken for assay of transposase accessible chromatin sequencing (ATAC-seq) to identify open chromatin regions highlighting potentially active *cis*-elements. ATAC fragments were sequenced directly or cloned into a targeting vector to generate a fragment library which was then integrated into a defined target site in the *HPRT* locus carrying a minimal promoter to drive

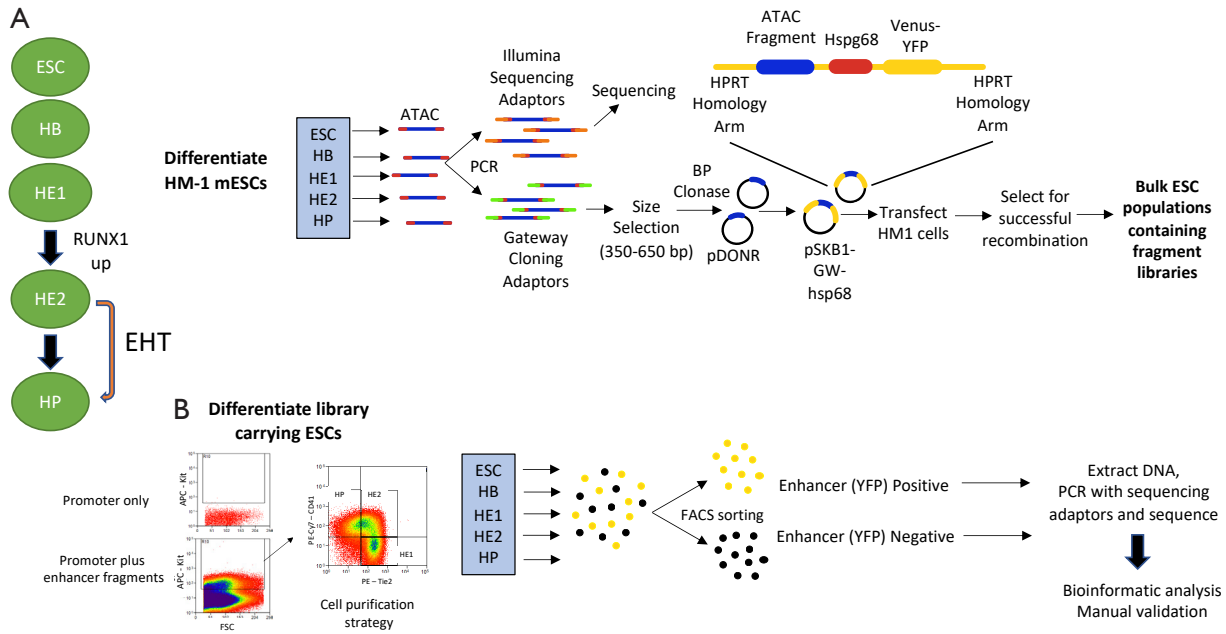

Figure 2 Diagram of the high-throughput enhancer screening method using ATAC-Seq fragments from ESCs, HB, HE1, HE2 and HP. (A) Schematic of producing baseline ATAC-Seq data by directly sequencing ATAC-fragments from cells representing different stages of hematopoietic differentiation as depicted on the left. Note that in contrast to Goode *et al*. (39) this differentiation scheme separates HE cells with a low (CD41–) and high (CD41+) level of RUNX1. In parallel isolated fragment libraries were inserted into Gateway cloning vectors, followed by integrating these libraries into HM-1 cells containing a modified HPRT locus by homologous recombination. The latter restores HPRT function, allowing to select for recombinant clones using HAT medium. (B) Enhancer identification. ESCs carrying fragment libraries were differentiated, sorted into the different cell populations with or without enhancer activity as shown in the FACS profile on the left and identification of stage-specifically active *cis*-elements by using bar-coded primers. ESC, embryonic stem cell; HB, hemangioblast; HE, hemogenic endothelium; HP, hematopoietic progenitor; EHT, endothelial-to-hematopoietic transition; mESC, mouse embryonic stem cell; PCR, polymerase chain reaction; ATAC, assay of transposase accessible chromatin; FACS, fluorescence-activated cell sorting.

a reporter gene (Venus-YFP). Bulk mouse ESCs were differentiated into hematopoietic cells and cells from each stage of development were purified using cell sorting to measure reporter activity by flow cytometry (*Figure 2B*). Our enhancer screen returned several hundred thousand fragments which could stimulate the reporter construct; 22–31% of fragments mapped to distal elements and covered >70,000 ATAC sites differentially active across five cell stages. The rest of the fragments were promoter sequences which were defined as being ± 1.5 kb from an annotated transcription start site. Most of our positively scoring sequences overlapped with open chromatin sites found in purified endothelial cells and HE from mouse embryos at E9.5 and E13.5 (113,114). In addition, our screen picked up multiple *cis*-regulatory elements which had been previously identified within endogenous loci. Between 10% and 20% of all the distal sites and between 15% and 50% of all promoter sites displayed cell type specific activity in our screen and directed cell-type specific expression of their associated genes. In concordance with this finding, enhancer sequences were enriched in TF binding motifs specific for this particular differentiation stage with HE-specific enhancers displaying a TEAD/SOX/AP-1 motif signature which was replaced by a GATA/RUNX/ETS (PU.1) motif signature in HP-specific enhancers after the EHT. Multiple studies associated the presence of an active enhancer with specific types of histone modifications, such as H3K27 acetylation (115,116) or RNA-Polymerase II binding (117,118). Only half of the enhancers identified in our study were flanked by modified histones or were bound by RNA-Polymerase II, indicating that the absence of such features did not indicate an absence of enhancer activity.

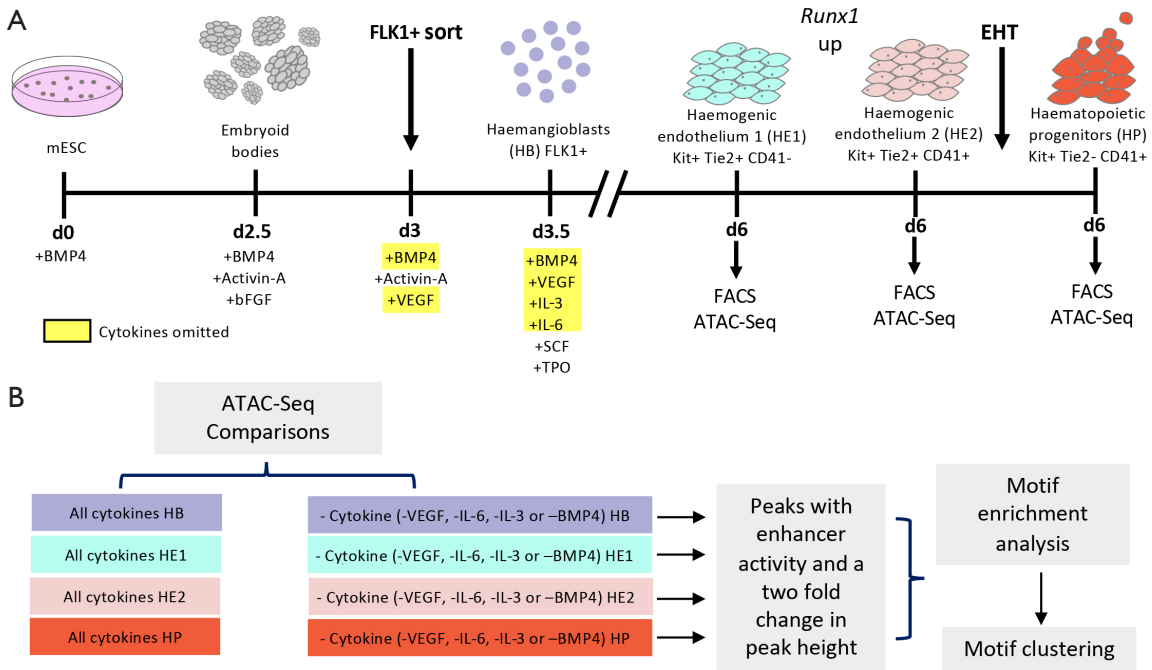

Figure 3 Identification of cytokine signalling responsive enhancer elements in four cell types. (A) Schematic of the serum-free *in vitro* differentiation system modified with permission from (119) and used to generate the indicated hematopoietic progenitors from mESCs. Individual cytokines were omitted at the start of blast culture (highlighted in yellow). (B) The enrichment analysis approach used to identify enriched motifs in ATAC peaks specific for each of the four cell types in the presence or absence of each cytokine (VEGF, IL-6, IL-3 and BMP4). mESC, mouse embryonic stem cell; EHT, endothelial-to-hematopoietic transition; FACS, fluorescence-activated cell sorting; ATAC-seq, assay of transposase accessible chromatin sequencing.

Hematopoietic specification is controlled by a relay of signalling responsive enhancers

We next asked the question which of these enhancer elements were signalling responsive. To this end, we modified a serum-free differentiation system which employed the sequential addition of different cytokines to generate blood cell precursors (119) and omitted specific cytokines as shown in *Figure 3* (112). We then used FACS to purify each cell type and measured chromatin accessibility in the presence and absence of these cytokines. These experiments showed that (I) thousands of *cis*-elements were cytokine responsive, (II) VEGF was the most important cytokine regulating the generation of blood cell precursor numbers and that (III) the presence of VEGF blocked the EHT. TF binding motif analysis of enhancer elements with and without cytokines demonstrated that in the presence of VEGF fewer cells containing open chromatin regions with motifs for hematopoietic TFs were formed. Cells were blocked at the endothelial stage as shown by an enriched endothelial TF motif signature. Interestingly, single cell RNA-Seq experiments showed that the actual differentiation pathway was not affected. Cells still underwent the correct succession of cell fate decisions from endothelial cells to HE to HP cells—what was different was the number of blood progenitors, suggesting that VEGF regulates a limiting factor operating at the EHT. This limiting factor turned out to be RUNX1 (112).

The Runx1 locus represents a signalling-responsive master switch driving the EHT

The single cell RNA-Seq experiments revealed that withdrawal of VEGF resulted in an up-regulation of *Runx1* mRNA in HP cells and that in the presence of VEGF signalling the endothelial TF gene *Sox17* was not efficiently down-regulated in these cells. In addition, the withdrawal of VEGF resulted in a significant decrease in the average mRNA expression values of *Notch1*. Moreover, the analysis of VEGF-responsive genes showed that the *Dlk1* gene, which encodes a membrane bound repressor of NOTCH1

activity (120), was strongly up-regulated in the absence of VEGF, and that this gene was differentially expressed between HE clusters in the absence of VEGF, compared with HE clusters cultured with VEGF. These data support previous findings that VEGF establishes and maintains NOTCH1 signalling, and that VEGF signalling maintains the HE via ETV2 and SOX17 (60,121). VEGF therefore is a truly instructive cytokine which has a profound influence on the balance of expression of endothelial and hematopoietic TFs, resulting in an alteration of both the gene regulatory and signalling network.

To obtain insights into the molecular mechanisms by which this balance is regulated, we used our new data resource of functionally characterised cell-type specific enhancer elements to examine individual gene loci (112). First, we studied the *Runx1* locus using our ATAC-seq data from cultures with and without VEGF. Our enhancer screen faithfully captured previously identified and validated *Runx1* enhancer elements: the +23 kb enhancer (122), the +3.7 kb enhancer (113), a –371 kb enhancer (113), the +204 kb enhancer (123), the +110 kb enhancer (123) and others (123). Strikingly, open chromatin sites overlapping these enhancer elements only formed in the absence of VEGF signalling, whereas under both conditions promoters existed as open chromatin regions. Moreover, our ChIP and motif enrichment data which we accumulated over several years showed that these enhancer elements were bound by endothelial and hematopoietic TFs in the HE and hematopoietic cells, respectively (39-43). One of the strengths of the enhancer screening system is that it allows to study the cell stage-specific activity of individual wild type enhancer elements together with versions where specific binding motifs were mutated in the presence and absence of cytokines. These experiments demonstrated that VEGF operates via binding motifs for TEAD and AP-1 and is counteracted by RUNX1. TEAD factors repress *Runx1* as, for example, at the *Runx1* +23 kb enhancer its activity was strongly up-regulated when the TEAD site which binds TEAD4 as measured by ChIP was mutated, and down-regulated once an essential RUNX1 site (122) was eliminated. Once RUNX1 reaches a high level after VEGF withdrawal, it binds to its own enhancers, represses endothelial genes including *Notch1* and activates hematopoietic genes.

The question now arises of the relevance of our *in vitro* data for mouse development. Recently Fadlullah *et al.* (33) used single cell (sc)-RNA-seq to capture the entire EHT process in mouse embryos focusing on the HE and dorsal aorta niche cells developing into intra-aortic hematopoietic clusters (IAHCs) which HP buds off from. Their data reveal a detailed HE differentiation continuum, which spanned the pre-HE and HE stages. When we mined the Fadlullah *et al.* data (33) we saw that, similar to what is seen in differentiating ESCs, *Kdr* (*Flk-1*) expression is highest in the endothelium and pre-EHT HE before expression is reduced dramatically in HE undergoing the EHT and IAHCs. *Vegfa*, which is expressed by the endothelium, also follows the same expression pattern. Conversely, *Runx1* expression is low in endothelium and pre-EHT HE before being up-regulated in HE undergoing the EHT and in IAHCs. NOTCH genes such as *Notch1*, *Dll1*, *Dll4*, *Jag1* and *Jag2* were also down-regulated in HE undergoing the EHT and IAHCs compared to the endothelium. These findings are consistent with those from a study profiling the gene expression and chromatin accessibility profile of the mouse AGM around the emergence of the HSC between 9.5 to 11.5 days post coitus, revealing that *Vegfa* and *Kdr* expression was highest in non-HE and was reduced in HE and lowest in intra-aortic clusters and mature HSCs (113). A single-cell transcriptome map of human hematopoietic tissues generated from three 4.5–5 weeks old embryos (124) also showed that the expression of VEGF and its receptor KDR was also reduced in HSC populations compared to endothelium, while RUNX1 expression was highest in the HSC cell type.

These findings support our observations that *Vegfa* and *Kdr* expression is highest when *Runx1* expression is low and this state switches in cells undergoing the EHT. Our studies of VEGF responsive enhancers now explain the molecular mechanisms governing how this occurs.

Outlook: using multi-omics data to understand developmental pathways

Our studies provide important mechanistic insights into the core regulatory and signalling network which regulates hematopoietic specification. They link extracellular signalling to the regulation of TF activity acting on specific *cis*-regulatory elements and shows that signalling has a profound impact on genomic events. Most importantly, they describe the dynamic activity of such elements within a developmental pathway adding an important dimension on our understanding of development. For example, we discovered multiple *cis*-regulatory elements which exist as open chromatin sites but lack enhancer activity in our assay [(39), Maytum, Edginton-White *et*

al., in preparation]. Some of these sites become functional once the next developmental stage is reached, others do not, thus adding to the increased list of elements which appear to function solely in chromatin opening, thus facilitating the establishment of enhancers activated later in development [reviewed in (125)]. Another example for the usefulness of such data is in the ability to use motif analyses to investigate TF cooperation as exemplified in the cooperation of AP-1 and TEAD (40), with many other combinations suggested by the co-localization of their motifs. Our data is a valuable source for the study of how TF motifs and their arrangements regulate enhancer function and coupled with recently published methods such as (126) will provide insight into these combinations. Our enhancer lists, linked to promoter regions, may also be valuable in revealing how enhancers and promoters are paired and to which type of *cis*-element they belong which has been reported for different transcriptional programs (127-129). Finally, our data which link the activity of individual enhancers to the activity of its rightful gene enable the construction of mathematical models to predict additional enhancer elements (130,131) and the GRN behaviour which they direct in the presence or absence of signals (130,132,133). We believe that such studies are of the essence if we want to understand developmental processes and recapitulate them *in vitro* for regenerative medicine purposes.

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Footnote

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