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Transcriptional hierarchies regulating early blood cell development

Victoria Moignard [a], Steven Woodhouse [a,b], Jasmin Fisher [b,c], Berthold Göttgens [a,*]

[a] University of Cambridge, Department of Haematology, Wellcome Trust and MRC Cambridge Stem Cell Institute and Cambridge Institute for Medical Research, Cambridge, CB2 0XY, UK
[b] Microsoft Research Cambridge, 21 Station Road, Cambridge CB1 2FB, UK
[c] Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, UK

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ABSTRACT

Hematopoiesis represents one of the paradigmatic systems for studying stem cell biology, but our understanding of how the hematopoietic system develops during embryogenesis is still incomplete. While many lessons have been learned from studying the mouse embryo, embryonic stem cells have come to the fore as an alternative and more tractable model to recapitulate hematopoietic development. Here we review what is known about the embryonic origin of blood from these complementary systems and how transcription factor networks regulate the emergence of hematopoietic tissue from the mesoderm. Furthermore, we have performed an integrated analysis of genome-wide microarray and ChIP-seq data sets from mouse embryos and embryonic stem (ES) cell lines deficient in key regulators and demonstrate how this type of analysis can be used to reconstruct regulatory hierarchies that both confirm existing regulatory linkages and suggest additional interactions.

Introduction

Hematopoietic stem cells (HSCs) are paradigmatic for studying stem cell biology as a result of their ability to maintain hematopoiesis throughout the life of an organism as well as the relative ease with which the hematopoietic system can be accessed for study [1]. However, while much is known about the function and regulation of HSCs in the adult, the mechanisms regulating their emergence during embryogenesis are less well understood.

The developing hematopoietic system has been the subject of over 100 years of study, since the appearance of hemoglobinised cells in the yolk sac was first noticed (reviewed by Refs. [2–4]). Hematopoietic cells develop from the embryonic mesoderm in two waves – the primitive wave that produces cells to sustain the developing embryo and the definitive wave that generates the HSCs that will populate the adult marrow – at multiple spatiotemporal sites within the embryo. As with adult hematopoiesis, transcription factors have emerged as key regulators of blood cell development. However, efforts to understand developmental hematopoiesis have been hampered by the difficulty in accessing the embryo during development. Here we will review what is known from our best-studied organism, the mouse, and discuss what has been learned from using the differentiation of embryonic stem cells (ES cells) as a model of development (see also [5]). We will focus particularly on the transcriptional hierarchies active during blood specification, including a meta-analysis of recently published gene expression profiles reporting loss of function analyses for the key transcriptional regulators, ETV2, SCL/TAL1 and RUNX1.

Overview of early blood cell development

In the mouse, hematopoiesis initiates at embryonic day 7.0–7.5 in the extra-embryonic yolk sac, where hematopoietic cells are first found neighboring endothelial cells within structures named blood islands [6,7]. The hemogenic potential of the yolk sac and its autonomous role in initiating the first stages of blood development was later demonstrated *in vitro* using explant cultures [8,9], confirming the yolk sac as the first site of hematopoiesis. Primitive erythrocytes are the first differentiated cells to be produced, with other cells of the myeloid lineages identified by E8.0 [10], indicating that multipotent progenitors are present at this stage. However, such progenitors do not yet have the ability to reconstitute the entire hematopoietic system of an irradiated mouse, the gold standard assay for hematopoietic stem cell activity, indicating that definitive HSCs are not produced during the earliest stages of developmental hematopoiesis [11–13].

The yolk sac remains the main site of hematopoiesis until around E10.0, when mammalian HSCs can first be detected within the embryo proper. It was originally thought that the mammalian hematopoietic system developed directly from yolk sac hematopoietic cells that migrated to the embryo after the initiation of the circulation at around E8.5. However, transplantation assays demonstrated that the yolk sac

Abbreviations: AGM, aorta-gonad-mesonephros; BL-CFC, blast colony-forming cells; E#, embryonic days post-fertilization; HSC, hematopoietic stem cell; VEGF, vascular endothelial growth factor.

* Corresponding author at: Department of Haematology, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, CB2 0XY, UK.
E-mail address: bg200@cam.ac.uk (B. Göttgens).

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does not contain transplantable adult-type definitive HSCs until E11.5 [11,12,14], which is later than can be detected within the embryo proper. The intra-embryonic origin of HSCs was first demonstrated in the avian system using chimeras in which quail donor embryos were transplanted onto chicken recipient yolk sacs. In this system, definitive hematopoietic cells were shown to be embryonic in origin as they always derived from the donor [15], and were identified as originating from a mesodermal region containing the dorsal aorta. A similar region was later identified as having hematopoietic activity in the mouse, with E10.0 cells from the aorta–gonads–mesonephros (AGM) region able to form spleen colonies at a higher rate than yolk sac cells when transplanted into lethally irradiated mice [12], and able to reconstitute the entire hematopoietic system [13]. Within the AGM, the dorsal aorta was then shown to be the site of hematopoiesis [11], with clusters of cells expressing hematopoietic markers appearing to bud out from the ventral aortic endothelium and be released into the circulation [16–18].

After the emergence of HSCs in the AGM, HSCs migrate to the fetal liver and undergo a period of expansion, finally colonizing the bone marrow shortly before birth where they remain throughout life (reviewed by Refs. [1–4]). Other auxiliary sites have been proposed for hematopoiesis, including the placenta, but they have been reviewed elsewhere and will not be discussed here [2–4]. Moreover, the yolk sac has long been suggested to contain precursor cells that can mature into fully competent and transplantable HSCs [19,20] and a very recent paper reported independent emergence of HSCs in the embryo head [21]. Taken together, therefore, a rather complex picture of developmental hematopoiesis is emerging.

In addition to the ongoing uncertainties regarding the anatomical sites of hematopoiesis, the cellular origin of hematopoietic cells has also been the source of some dispute. In the yolk sac, the observation that blood islands comprise inner hematopoietic cells surrounded by endothelium [22] lead to the hypothesis that these cell types emerge from a common precursor, the hemangioblast [6,7]. Furthermore, single-gene mutations have been shown to severely affect both endothelial and hematopoietic lineages, most notably those involving *Flk1* and *Etv2*, suggesting that they at least share common regulators, if not a common progenitor. The observation of hematopoietic clusters in the dorsal aorta of many species lead to an opposing theory by which HSCs develop from an endothelium with hematopoietic potential, the hemogenic endothelium. While it has been difficult to test these theories in the embryo, the use of ES cells as a model for development has provided evidence for both [2,3,5], and has recently allowed the two to be reconciled [23].

ES cells can be induced to differentiate, either in adherent culture on extracellular matrix or stromal cells, or through embryoid bodies – three-dimensional cystic structures reminiscent of the developing embryo - and can generate hematopoietic tissue with similar characteristics to the yolk sac including the development of hemoglobinised blood islands [5,24]. Detailed analyses have suggested that the kinetics of hematopoietic development, including expression of cell surface markers and hematopoietic genes, closely resemble that of the yolk sac (reviewed in [5]), indicating that ES cells provide an excellent model in which to study the developing hematopoietic system. When ES cells were plated in methylcellulose with vascular endothelial growth factor (VEGF) and Kit ligand, two cytokines important for hematopoietic development, they produced cells with blast morphology, called blast colony-forming cells (BL-CFCs). BL-CFCs could generate cells of multiple hematopoietic lineages and expressed the hemato-endothelial markers FLK1, CD34, *Scl* and *Gata1*, but not the pan-mesodermal marker *Brachyury* [25]. The same group later showed that in liquid culture these cells could form both adherent cells with endothelial characteristics, including expression of CD31, FLK1 and the ability to uptake low-density lipoproteins (LDLs), as well as non-adherent hemoglobinised cells that could produce multiple hematopoietic lineages in colony assays [26]. They were then able to use the same culture conditions to identify analogous cells from the posterior mesoderm of gastrulating embryos that could form blast colonies with both hematopoietic and vascular potential, the characteristics of the hemangioblast, and also the potential to give rise to vascular smooth muscle [27].

The first demonstration of the hemogenic endothelium came from studying the chicken where a subset of endothelial cells in the dorsal aorta was found to express the hematopoietic marker CD45. A lineage-tracing study demonstrated that DiI-LDL-labeled endothelium gives rise to hematopoietic cells bearing the same marker [17]. Hemogenic endothelium has been observed in both the dorsal aorta [18] and yolk sac [28–30], challenging the hemangioblast theory, and the first HSCs capable of repopulating the adult hematopoietic system in the mouse express endothelial cell surface markers [31,32] which are lost during fetal liver hematopoiesis [33]. The ES cell model has also been useful in demonstrating the hematopoietic potential of endothelium in the mouse. Isolation and culture of VE-cadherin+CD45− endothelial cells and VE-cadherin−CD45+ hematopoietic cells showed that both cell types could produce myeloid and lymphoid cells, but that the potential was higher in endothelial cells [30,34]. Live-imaging studies have attempted to visualize the so-called endothelial-to-hematopoietic transition where hematopoietic cells form clusters in the lumen of the dorsal aorta and bud off into the circulation. FLK1+E-cadherin+ mesodermal precursors have been isolated from ES cells and the development of these cells on an OP-9 stromal layer observed by time-lapse imaging [35]. Endothelial colonies were able to generate non-adherent proliferative hematopoietic cells expressing CD45 through a semi-adherent stage analogous to intra-aortic clusters in which cells expressed both CD41 and VE-cadherin. The adherent hemogenic endothelial cells were able to produce endothelium but not cardiac or smooth muscle, negating the possibility that they are an earlier mesodermal progenitor. Furthermore, the authors isolated equivalent cells from the E7.5 embryo and demonstrated similar hemogenic endothelial potential. Additionally, lineage tracing and live imaging of sections of Ly6a(Sca-1)-GFP mice at E10.5 indicated that rare GFP+ cells budded directly from CD31+GFP+ endothelial cells and emerged into the aortic lumen [36].

Recently, the two theories have been reconciled by showing that the development of hematopoietic cells proceeds sequentially from mesoderm through the hemangioblast to the hemogenic endothelium and hematopoietic progenitors [23] (Fig. 1). Hemangioblasts derived from ES cells gave rise to blast colonies that contained both endothelial and hematopoietic cells, and visualized the transition from adherent endothelial cells to free hematopoietic cells with concurrent up and down-regulation of markers for hematopoietic and endothelial cells, respectively. Again, analogous cells were identified within the embryo, indicating that the ES cell system faithfully recapitulates hematopoietic development in the embryo.

Efforts have also been made to identify key regulators of the above developmental stages and transitions, and the ability to use ES cells to introduce gene knockouts into embryos has been invaluable. These studies have opened the door to understanding the transcriptional regulatory hierarchies controlling development and have implicated three factors in particular in the regulation of key stages of early hematopoiesis: ETV2, SCL and RUNX1.

Transcriptional control of hemangioblast formation

While no single absolute marker has yet been identified for the hemangioblast, both blood and endothelium arise from cells expressing the VEGF receptor, FLK1 [37–39]. *Flk1*−/− embryos die between E8.5 and 9.5 due to defects in blood and endothelial development, including a lack of yolk sac blood islands, and *Flk1*−/− ES cells do not contribute to the hematopoietic system in chimeras [40], indicating a very early defect in hematopoiesis.

In zebrafish, the cloche mutant similarly affects the earliest stage of development of blood and endothelial cells, with a resultant loss of

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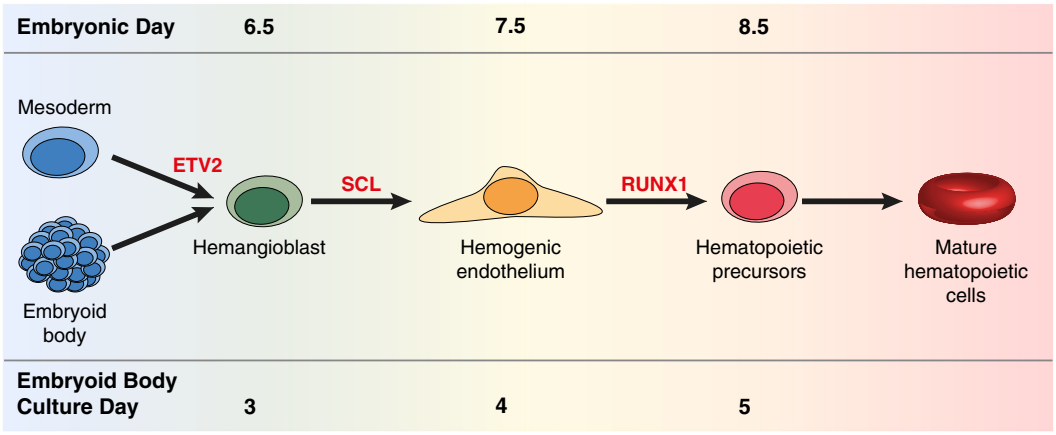

Fig. 1. Schematic of hematopoietic development *in vivo* in the developing mouse embryo and *in vitro* in embryonic stem cell cultures. Hematopoietic cells derive from the embryonic mesoderm through a hemangioblast intermediate, with the transcription factor ETV2 implicated in its emergence and/or commitment. Hemangioblast-like cells called BL-CFCs can also be identified when embryonic stem cells are induced to differentiate, either through embryoid bodies in suspension or in adherent culture. The transcription factor SCL then regulates the transition from the hemangioblast to the hemogenic endothelium, both *in vivo* and in ESC cultures. Clusters of hematopoietic cells form adjacent to the hemogenic endothelium from which hematopoietic precursor cells bud out into the blood vessels, in a process termed endothelial-to-hematopoietic transition, which is regulated by RUNX1.

expression of many known blood- and endothelial-specific genes. Study of this mutant identified a number of candidate regulators of hemangioblast formation including *Etsrp*, an Ets-family transcription factor expressed specifically in vascular endothelial cells [41]. The Ets family of transcription factors includes key hematopoietic regulators such as ETS1, FLI1 and PU.1 (*Sfpi1*). Knockdown of *Etsrp* resulted in the complete absence of circulation of zebrafish morphants, while over-expression could induce vascular endothelial markers in multiple cell types [42]. *Etsrp* was also a strong inducer of FLK1+ cells. The same group later studied the related mammalian Ets factor *Etv2/Er71*, which was able to expand the hemangioblast lineage when expressed ectopically in zebrafish [43]. *Etv2*−/− mouse embryos die during gestation with severe blood vessel defects similar to the *Flk1* phenotype, and *Flk1* expression is reduced. Over-expression leads to the induction of FLK1+ mesoderm and can rescue its formation when normal induction is blocked by the inhibition of bone morphogenetic protein, Notch and Wnt signaling [44].

Attempts have been made to define the temporal window of *Etv2* requirement during development. *Etv2* is expressed in early mesoderm in a subset of the FLK1+ population with enhanced hematopoietic and endothelial potential, but is down-regulated by E9.5 [45,46]. *Etv2*−/− ES cells, like embryos, do not make blood progenitors [45,47]. Using Flk1-Cre-mediated deletion of *Etv2*, Wareing et al. demonstrated that E9.5 embryos contained CD41+ cells and hematopoietic progenitors at comparable levels to wild type littermates, indicating that *Etv2* is no longer required for hematopoietic development once FLK1+ mesoderm has formed [47]. Additionally, re-expression of *Etv2* in *Etv2*−/− embryonic stem cells could initiate the formation of CD41+ cells in both FLK1+ and FLK1− cells isolated from embryoid bodies. Kataoka and colleagues showed that ETV2 is dispensable for generating FLK1+PDGFRa+ primitive mesoderm, but is required for its subsequent specification to FLK1+PDGFRa− hemogenic mesoderm. Comparison of FLK1+ mesoderm from wild type and *Etv2*−/− ES cells indicated that many key hemato-endothelial genes have greatly reduced expression in the absence of *Etv2*, including *Scl* and *Fli1* [45].

The above studies strongly implicate ETV2 as a key regulator of hemangioblast formation (Fig. 1). *Etv2* has also been shown to be critically dependent on PKA/CREB signaling [48]. While the data suggest that ETV2 acts downstream of multiple signaling pathways in development, how it may be functionally linked to VEGF/FLK1 signaling is not yet clear. However, these studies hold great potential for improving our understanding of how the hematopoietic transcriptional program is activated during development.

Transcriptional control of hemogenic endothelium

Like ETV2, the transcription factor SCL (also known as TAL1) is also vital for the development of the hematopoietic system. Identified in the human through chromosomal translocations in acute T-cell lymphoblastic leukemia [49–51], SCL is a basic helix–loop–helix transcription factor expressed from E7.5 in both embryonic and extra-embryonic mesoderm. It localizes to the blood islands of the yolk sac and later to the fetal liver and spleen, at the sites of hematopoiesis [52], as well as the dorsal aorta and endocardium between E10.5 and 13.5 and in multiple cell types of the adult hematopoietic system, including HSCs. *Scl* expression is highly conserved across vertebrates [53,54], and the *Scl* gene locus has become a paradigm for studying the conservation as well as evolution of transcriptional control mechanisms [53,55–60]. *Scl*−/− embryos die at around E9.5 with an absence of hematopoietic cells, while other mesodermal lineages are unaffected [61,62]. Complementary gain of function studies in zebrafish confirmed SCL's key role in early hematopoietic specification [63].

Morpholino-based down-regulation of *Scl* in the zebrafish had no significant effect on the expression of *Etsrp* in angioblasts [42]. *Scl* is not expressed in *Etv2*−/− or *Flk1*−/− embryos [45,47,64], but *Scl* expression in *Etv2*−/− mouse cells can restore hematopoiesis [45,47]. Furthermore ETV2 binds directly to the *Scl* locus [45], implicating *Scl* as a direct target downstream of *Etv2* in the developing hematopoietic system. When *Scl*−/− ES cells were induced to form hematopoietic tissues, BL-CFCs, the *in vitro* counterpart of hemangioblasts, were detected but were unable to generate endothelial and hematopoietic cells, instead forming colonies of vascular smooth muscle cells [65]. This places the function of SCL downstream of the specification of the hemangioblast.

In the ES cell system, several studies have demonstrated the requirement for *Scl* in the formation of the hemogenic endothelium from the hemangioblast [23,66]. FLK1+ cells from *Scl*−/− ESC lines cannot produce hematopoietic clusters in the aorta, blast colonies or CD41+ hematopoietic cells [23]. However, SCL, like ETV2, is required within a specific temporal window during development. When *Scl* was placed under the control of a tamoxifen-inducible Cre, it could rescue the generation of VE-cadherin+ hemogenic endothelial cells during a defined temporal window of differentiation 2–4 days after the initiation of differentiation of ES cells on OP9 stromal cells, but was ineffective at later stages [66]. When *Scl* was deleted using Tie2-Cre, which is expressed shortly after *Scl* in hemogenic and vasculogenic sites, fetal liver hematopoietic cells were still detected, indicating that by the

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time *Scl* is ablated it is no longer required for hematopoietic development, at least not in a non-redundant fashion [67–69].

As well as driving hematopoietic development directly by promoting the formation of the hemogenic endothelium, *Scl* may also have a role in inhibiting the potential of the hemangioblast to form vascular smooth muscle. Use of a doxycycline-inducible *Scl* transgene in ES cells showed that an early pulse of *Scl* expression expanded the number of hematopoietic colony forming cells while repressing the expression of cardiac markers and the formation of beating cardiomyocytes [70]. Similar results were found *in vivo* where *Scl*−/− embryos initiated a cardiac transcriptional program in the yolk sac, with CD31+PDGFRa+ cardiac progenitors emerging and generating beating cardiomyocytes [71].

These studies indicate that SCL is essential for the specification of the hemogenic endothelium from the hemangioblast (Fig. 1), but not for the subsequent specification of hematopoietic cells from the endothelium.

Transcriptional control of endothelial-to-hematopoietic transition

As outlined above, hematopoietic cells bud off from the hemogenic endothelium into the aortic lumen in a process termed endothelial-to-hematopoietic transition. The expression of the transcription factor *Runx1* in hematopoietic cells as well as their neighboring endothelial cells [72], both in the yolk sac and the embryo proper, suggested that this protein is involved in this transition. *Runx1*−/− embryos die at around E12.5 with severe hemorrhaging [73–75] and hematopoietic clusters fail to form in the dorsal aorta [72]. Conversely, overexpression of *Runx1* in a *Runx1*−/− ES cell line resulted in an increased number of CD41+ hematopoietic cells [23]. The level of *Runx1* expression is also important, as heterozygotes have reduced, but not lost, production of definitive HSCs in the AGM [76]. Interestingly, *Runx1* deficiency can be partially rescued by localized *Runx1* expression in explanted para-aortic splachnopleura, the mesodermal precursor of the AGM, adding weight to the argument that this region is capable of definitive hematopoiesis independently of the yolk sac [77].

To identify the temporal window of *Runx1* activity, Chen and colleagues used *VE-cadherin*− or *Vav1*-mediated Cre to ablate *Runx1* at different stages of hematopoietic development [78]. At E8.5, VE-cadherin is restricted to endothelial cells of the yolk sac, dorsal aorta and heart, and *Runx1* ablation at this stage caused severe defects in hematopoiesis, in line with the *Runx1* knockout [72,78]. However, when Cre was under the control of *Vav1*, which is restricted to hematopoietic cells and implicated as a RUNX1 target, fetal and adult viability were unaffected, demonstrating that *Runx1* expression is no longer required after the endothelial-to-hematopoietic transition [78]. This was further confirmed in the ES cell system where *Runx1* expression is critical for the formation of hematopoietic precursors from the hemogenic endothelium [23]. Time-lapse imaging identified the formation of clusters of tightly associated adherent cells in *Runx1*−/− ES cells. However, these cells expressed the endothelial marker CD31 but not CD41, and very few generated blast colonies.

The temporal window of RUNX1 activity focused attention on the identification of pathways downstream of RUNX1 that regulate the maturation of definitive HSCs from CD41+ cells. ChIP-seq for RUNX1 in early hematopoietic stages in the ES cell system identified multiple direct RUNX1 targets with expression correlated to *Runx1* and which were enriched for biological functions related to hematopoietic development [79]. Direct targets with negatively correlated expression were enriched for genes associated with non-hematopoietic tissue differentiation, indicating that RUNX1 may directly repress alternative fates in the hemogenic endothelium as well as promoting hematopoietic development. *Runx1* alone can completely reverse the endothelial program initiated by *HoxA3* in HE cells, while *HoxA3* can restrain or even reverse the differentiation of the earliest endothelial progenitors into CD41+ hematopoietic cells in the ES cell system, suggesting how the HE may balance the production of blood and endothelium [80].

In the hematopoietic lineage, *Gfi1* and *Gfi1b* are both able to partially rescue *Runx1* defects, triggering the down-regulation of endothelial markers and the formation of hematopoietic cells budding from the HE [81]. Expression of both factors increases when the hemogenic endothelium forms and is reduced in the absence of *Runx1*, but deletion of either alone does not phenocopy *Runx1*−/− embryos. However, in double knockout embryos, the cells of the hemogenic endothelium are unable to bud off into the yolk sac and embryo, highlighting the key regulatory role of these factors downstream of *Runx1*.

Revealing regulatory cascades from loss of function analyses

As outlined in the previous sections, a number of key regulatory transcription factors have been identified to function during the developmental progression from mesodermal cells to blood progenitors. In order to investigate the relationship between the three key developmental transcription factors, ETV2, RUNX1 and SCL, and the genes that they regulate, we performed an integrated analysis of five microarray knockout comparison studies and two ChIP-sequencing studies from ES cell-derived cultures. These data were analysed in the context of existing literature on the regulatory network of early hematopoiesis.

FLK1+ cells from *Etv2*-/- and wild-type cells were compared in two studies, revealing 170 [45] and 143 [47]differentially expressed genes, respectively. Together, these studies suggest that SCL, FLI1 and GATA2, which form a recursively wired gene-regulatory circuit in the hemangioblast [82], are key downstream targets of ETV2. Also, wild type CD31+ cells from the yolk sac were compared to those cells from *Scl*−/− embryos. In the latter circumstance, developmental hematopoiesis is arrested before the hemogenic endothelium stage and 2260 differentially expressed genes were identified. No equivalent study in the ES cell differentiation system has yet been described. Two studies have been described that compare *Runx1*−/− and *Runx1*+/− embryonic stem cell lines [79,83]. The genome-wide binding pattern of RUNX1 was also reported for these cells [79], which was combined with the gene expression data in an integrated analysis. A second RUNX1 ChIP-sequencing study has also been reported [84].

For each of the five microarray experiments the list of differentially expressed genes was downloaded and filtered for hematopoietic transcription factors, based upon annotation in the Mouse Genome Database [85] for the hematopoietic phenotype, and the RIKEN Transcription Factor Database [86] and GO annotation [87] for transcription factors. Hierarchical clustering was performed on the resulting merged matrix of discrete expression values (Fig. 2A). We next constructed putative network diagrams by considering the clustered expression data in combination with the two RUNX1 ChIP-seq experiments in hemogenic endothelium, and in light of what is already established in the literature. Binding by a transcription factor was taken as evidence of direct regulation.

Our analysis supports a model in which key hematopoietic transcription factors are activated sequentially and then later collaborate to control gene expression (Fig. 2B) [79,84]. This process begins with activation of *Etv2* by PKA/CREB signaling at an early differentiation stage [48]. ETV2 subsequently activates the core circuit comprised of *Scl*, *Gata2* and *Fli1* [82]. The small number of genes directly regulated

Fig. 2. Reconstructing regulatory hierarchies from genome-wide data. A. Hierarchical clustering of differentially expressed hematopoietic transcription factors in *Etv2*, *Scl*, and *Runx1* knockout comparison studies (data from Ferreras et al. [83] shown for *Runx1*). Red indicates that a gene is up-regulated and blue indicates that it is down-regulated. B. Reconstructed regulatory networks active in early blood development. The three network diagrams show the ETV2-activated genes in FLK1+ mesoderm, the genes regulated by the SCL-GATA2-FLI1 triad in the hemangioblast, and the RUNX1-dependent interactions active in the hemogenic endothelium. In the hemogenic endothelium, RUNX1 cooperates with SCL, FLI1 and GATA2 to activate some factors while independently regulating others.

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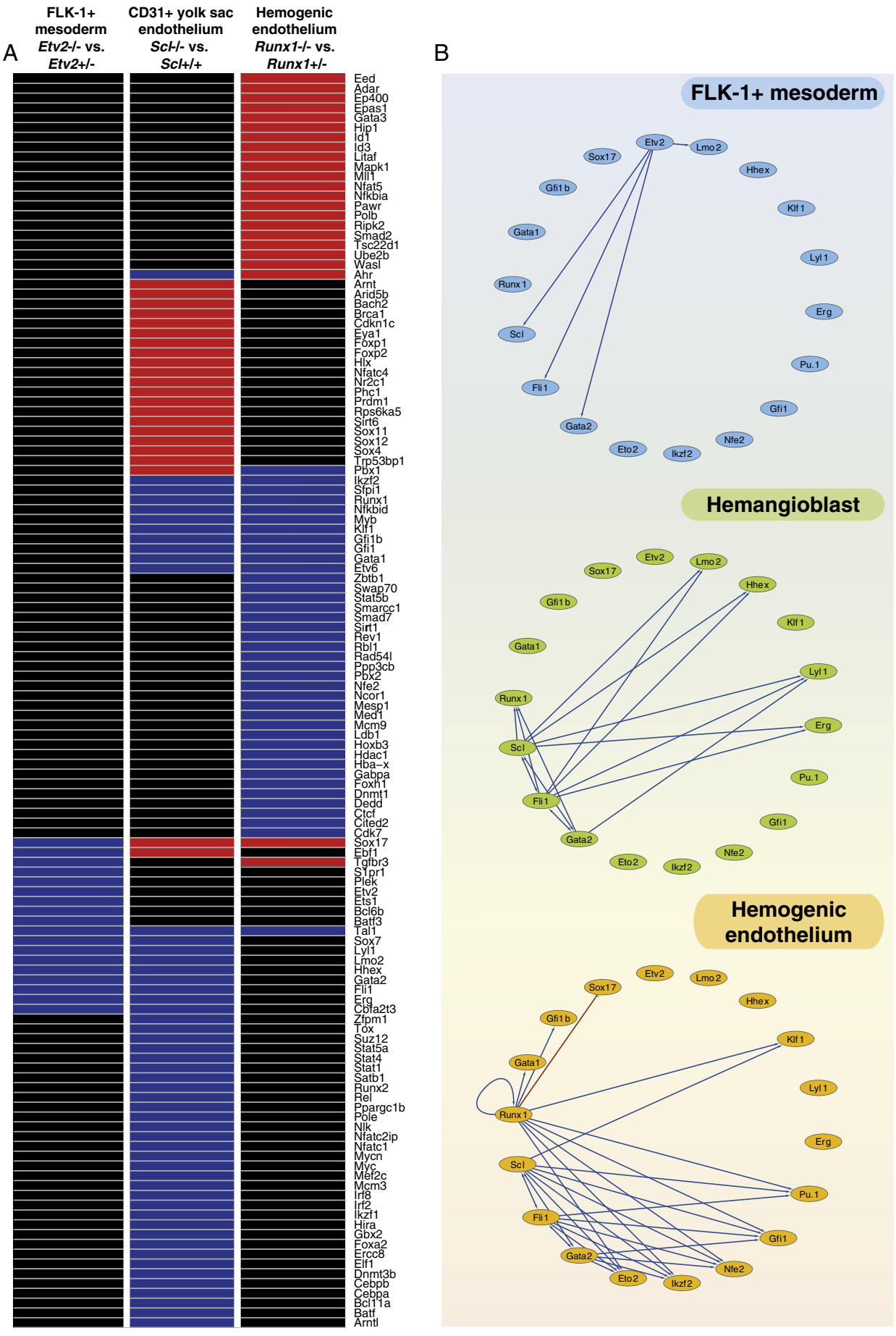
A
FLK-1+ mesoderm Etv2-/- vs. Etv2+/-
CD31+ yolk sac endothelium Scl-/- vs. Scl+/+
Hemogenic endothelium Runx1-/- vs. Runx1+/-
Eed
Adar
Ep400
Epas1
Gata3
Hip1
Id1
Id3
Litaf
Mapk1
Mll1
Nfat5
Nfkbia
Pawr
Polb
Ripk2
Smad2
Tsc22d1
Ube2b
Wasl
Ahr
Arnt
Arid5b
Bach2
Brca1
Cdkn1c
Eya1
Foxp1
Foxp2
Hlx
Nfatc4
Nr2c1
Phc1
Prdm1
Rps6ka5
Sirt6
Sox11
Sox12
Sox4
Trp53bp1
Pbx1
Ikzf2
Spi1
Runx1
Nfkbid
Myb
Klf1
Gfi1b
Gfi1
Gata1
Etv6
Zbtb1
Swap70
Stat5b
Smarcc1
Smad7
Sirt1
Rev1
Rbl1
Rad54l
Ppp3cb
Pbx2
Nfe2
Ncor1
Mesp1
Med1
Mcm9
Ldb1
Hoxb3
Hdac1
Hba-x
Gabpa
Foxh1
Dnmt1
Dedd
Ctcf
Cited2
Cdk7
Sox17
Ebf1
Tgfbr3
S1pr1
Plek
Etv2
Ets1
Bcl6b
Batf3
Tal1
Sox7
Lyl1
Lmo2
Hhex
Gata2
Fli1
Erg
Cbfa2t3
Zfpm1
Tox
Suz12
Stat5a
Stat4
Stat1
Satb1
Runx2
Rel
Ppargc1b
Pole
Nlk
Nfatc2ip
Nfatc1
Mycn
Myc
Mef2c
Mcm3
Irf8
Irf2
Ikzf1
Hira
Gbx2
Foxa2
Ercc8
Elf1
Dnmt3b
Cebpb
Cebpa
Bcl11a
Batf
Arntl
B
FLK-1+ mesoderm
Etv2
Lmo2
Sox17
Hhex
Gfi1b
Klf1
Gata1
Lyl1
Runx1
Erg
Scl
Pu.1
Fli1
Gfi1
Gata2
Nfe2
Eto2
Ikzf2
Hemangioblast
Etv2
Lmo2
Sox17
Hhex
Gfi1b
Klf1
Gata1
Lyl1
Runx1
Erg
Scl
Pu.1
Fli1
Gfi1
Gata2
Nfe2
Eto2
Ikzf2
Hemogenic endothelium
Etv2
Lmo2
Sox17
Hhex
Gfi1b
Klf1
Gata1
Lyl1
Runx1
Erg
Scl
Pu.1
Fli1
Gfi1
Gata2
Nfe2
Eto2
Ikzf2

by ETV2 suggests that its primary role is to activate this self-regulating triad, which controls further specification of differentiation. This is in agreement with the finding that expression of *Scl* alone is sufficient to restore hematopoietic and endothelial potential in *Etv2*−/− cells [45,47]. However, direct regulation of *Lmo2* by ETV2 has also recently been described [88].

At the hemangioblast stage, the SCL-GATA2-FLI1 triad forms a positive feedback loop that is independent of further activation by ETV2. The three transcription factors cooperate to activate *Lyl1*, *Hhex*, *Erg* and *Runx1*. In the hemogenic endothelium, we see a more densely connected network in which RUNX1 cooperates with SCL, FLI1 and GATA2 to activate some factors while independently regulating others. Among these factors are the transcriptional repressors GFI1 and GFI1B, which are key mediators of RUNX1's role in extinguishing the endothelial program during the transition to definitive hematopoietic progenitor cells [80,81].

It is illustrative to compare our predicted network links to well-established regulatory interactions active in HSCs. Notably, our analysis predicts the activation of *Gata1* by RUNX1, while existing models do not account for this activation. GATA1 can disable GATA2 auto-regulation by binding to the *Gata2* −3 kb regulatory region and mediating domain-wide chromatin remodeling. This mechanism may be involved in deactivating the autoregulatory loop of SCL, FLI1 and GATA2 in preparation for differentiation [82,89]. It is possible that RUNX1 activation of *Gata1* could be involved in priming early hematopoietic progenitors for an erythroid fate. Another noteworthy observation is the characterisation of *Sox17* as a direct target repressed by Runx1. Since *Sox17* is required for endodermal differentiation [90,91], this is consistent with RUNX1's role in extinguishing non-hematopoietic transcriptional programs [80,90].

Modeling hematopoietic regulatory networks

The previous section outlined how integrated analysis of a number of published datasets can be utilized to reconstruct likely regulatory hierarchies operating during the development of blood progenitors from mesoderm. Regulatory cascades are best understood in lower model organisms, where comprehensive functional studies in sea urchin for example have revealed in-built forward momentum, so that once a cell has embarked on a given developmental trajectory, this will be executed more or less automatically, and is largely dictated by the regulatory network wiring of feed forward loops between key regulators [92]. This forward momentum, however, needs to be halted at some stage, because at midgestation hematopoietic development entails the specification of blood stem cells, most of which do not automatically proceed to differentiate into mature blood cells because they are responsible for long-term maintenance of the blood system, both under homeostatic and stress conditions. When compared with intermediate developmental stages such as the hemangioblast or hemogenic endothelium, HSC regulatory network states have to show increased stability because HSCs not only need to have the ability to "move forward" and produce mature cell types, but also to "remain static" and contribute to stem cell self-renewal.

In the absence of comprehensive experimental approaches, several abstract models of hematopoietic stem and progenitor cell maintenance have been developed [93–96]. Models more explicitly anchored in molecular experimental data originally focussed on simple 2-factor or 3-factor models [97–103], which may capture some essential properties of core regulatory circuits but are clearly too simplistic to model complex network behavior. More recently, a qualitative model of the core transcription factor network active in common myeloid progenitors was constructed following a comprehensive literature survey [104] (Fig. 3). In their Boolean model, each transcription factor is represented by a variable which can take one of two values; 1, corresponding to an "on" or expressed state; and 0, corresponding to an "off" or unexpressed state. The complex combinatorial logic governing the interactions between transcription factors is encoded as Boolean update rules using the logical functions *And*, *Or* and *Not*. For example, GATA-2 positively regulates its own expression, and is inhibited by GATA-1 and FOG-1. As both GATA-1 and FOG-1 are required to fully repress GATA-2 expression, they are combined using *And* in the Boolean update rule for GATA-2.

Computational analysis of this model, beginning from an initial transcription factor expression state representing the common myeloid progenitor, revealed an acyclic, 232-element hierarchical state space which recapitulated the steps of myeloid differentiation. This state space contained four terminal steady states, which were found to be in good agreement with microarray expression profiles of megakaryocytes, erythrocytes, granulocytes and monocytes. Once it had been established that this model recapitulated myeloid differentiation, further analyses based upon perturbations to the network were conducted. These simulations demonstrated that *in silico* knockouts were able to reproduce known experimental lineage depletion results and that *in silico* overexpression reproduced known experimental reprogramming results.

However, the transcription factor network model constructed did not identify the multipotential progenitor cell as a stable state, and

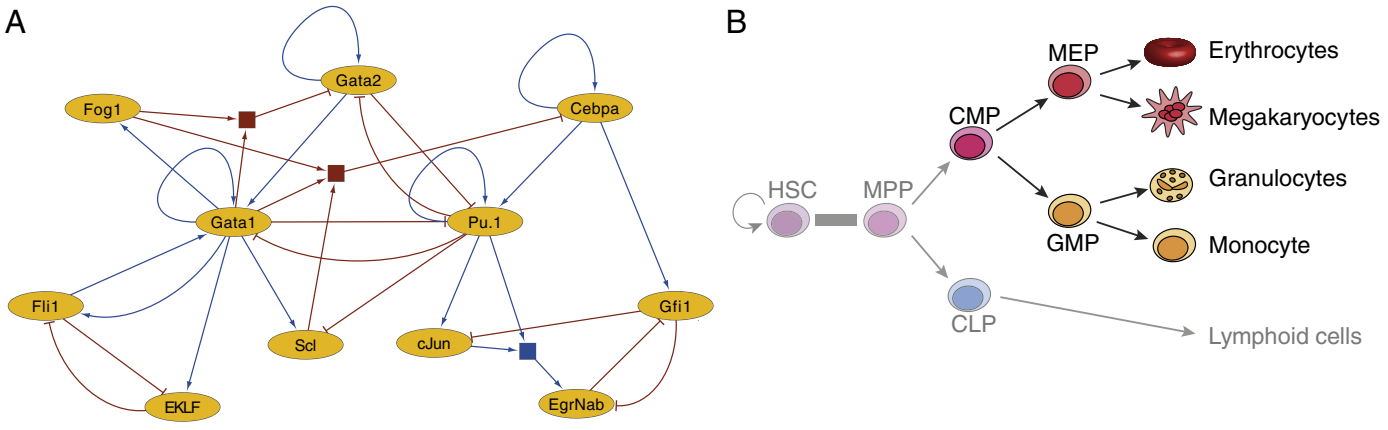

Fig. 3. A qualitative Boolean model of the core transcription factor network active in common myeloid progenitors. A. Visual representation of the common myeloid progenitor regulatory network model as encoded by the Boolean update rules from Krumsiek et al. [104]. Blue edges represent activation and red edges repression. Square boxes connecting edges represent AND operations. B. Schematic of the adult hematopoietic hierarchy in bone marrow. The CMP is regulated by the network in A to produce multiple outputs: the granulocyte–monocyte progenitor which gives rise to granulocytes, monocytes and other myeloid cells, and the megakaryocyte–erythroid progenitor, which produces erythrocytes and megakaryocytes. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte–erythroid progenitor; GMP, granulocyte–monocyte progenitor.

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therefore clearly did not capture the relative stability that one would expect to characterize blood stem cell network states [104]. Boolean modeling based on experimental knowledge of the connectivity between eleven HSC transcription factors by contrast did produce a stable attractor state reminiscent of the HSC [109], which indeed required simulation of external triggers to allow exit from this state. HSCs balance self-renewal and differentiation, which suggests that more sophisticated modeling approaches will be required to combine the faithful representation of differentiation reported [104] with an ability to simultaneously endow the multipotential stem cell with a degree of inherent stability. Given the types of experimental data that will be available, it is unlikely that the field will be able to employ, at least not in the short term, large-scale stochastic process modeling [105], which aims to capture the kinetics of molecules inside the cell but requires accurate quantitative measurements of many parameters. Qualitative modeling on the other hand has demonstrated its utility to drive the design of future experimentation in many other contexts [106–108], even though it relies only on the network topology, and therefore only makes qualitative statements about the network. It is likely therefore that close cooperation of experimental and theoretical biologists could result in an iterative workflow of model predictions followed by experimental validation, which in turn would advance our understanding of the underlying biological processes.

Concluding remarks

The field of developmental hematopoiesis is rapidly entering a new era where genome-scale experiments combined with downstream computational analysis are likely to provide unparalleled insights into early regulatory hierarchies. The speed of future progress will depend on both conceptual and technological parameters, such as (1) miniaturization of genome-wide profiling techniques to accommodate the small cell numbers present in early embryos, (2) continued improvement of our understanding of early developmental lineage relationships to ensure for example that snap-shot gene expression measurements can be viewed within an appropriate “developmental” sequence, (3) an enhanced emphasis on multi-disciplinary approaches where classical developmental and cell biologists will work in close collaboration with bioinformaticians, imaging experts and mathematical biologists. Finally, analysis of the more tractable ES cell differentiation systems alongside developing embryos will not only aid our understanding of developmental hematopoiesis but will also be essential for the production of true HSCs *in vitro* from ES cells or induced pluripotent stem cells, and therefore has not only fundamental but also translational relevance.

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