Decoding the regulatory network of early blood development from single-cell gene expression measurements

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Reconstruction of the molecular pathways controlling organ development has been hampered by a lack of methods to resolve embryonic progenitor cells. Here we describe a strategy to address this problem that combines gene expression profiling of large numbers of single cells with data analysis based on diffusion maps for dimensionality reduction and network synthesis from state transition graphs. Applying the approach to hematopoietic development in the mouse embryo, we map the progression of mesoderm toward blood using single-cell gene expression analysis of 3,934 cells with blood-forming potential captured at four time points between E7.0 and E8.5. Transitions between individual cellular states are then used as input to develop a single-cell network synthesis toolkit to generate a computationally executable transcriptional regulatory network model of blood development. Several model predictions concerning the roles of Sox and Hox factors are validated experimentally. Our results demonstrate that single-cell analysis of a developing organ coupled with computational approaches can reveal the transcriptional programs that underpin organogenesis.

Blood has long served as a model to study organ development owing to the accessibility of blood cells and the availability of markers for specific cell populations. Blood development initiates at gastrulation from multipotent Flk1+ (encoded by Flk1, also known as Kdr) mesodermal cells, which initially have the potential to form blood, endothelium and smooth muscle cells. Blood development represents one of the earliest stages of organogenesis, as the production of primitive erythrocytes is required to support the growing embryo. Single-cell gene expression analysis has already been successfully applied to study the earliest stages of preimplantation mouse and human development1–5; to identify lineage commitment6 and transcriptional regulatory7 events in blood, and, more recently, to probe the emergence of hematopoietic stem cells (HSCs) from the hemogenic endothelium of the dorsal aorta8.

Here we report in vivo gene expression analysis of early blood development at the single-cell level, focusing on transcription factors as regulators of cell fate. Using qRT-PCR, we analyzed >40 genes in 3,934 cells with blood and endothelial potential from five populations at four sequential stages of post-implantation mouse development between embryonic day (E)7.0 and E8.25. We adapted the diffusion plot methodology previously reported in nonbiological contexts9 for dimensional reduction of single-cell data, where pseudotemporal ordering of individual cells revealed a putative developmental hierarchy branching toward both blood and endothelial-like fates. To discover the underlying regulatory network, we developed a single-cell network synthesis (SCNS) toolkit to synthesizing executable Boolean network models from binary single-cell expression states, which correspond to the on and off patterns of transcription factor expression. Using this toolkit we identified a core network of 20 highly connected transcription factors, which could reach eight stable states representing blood and endothelium. We validated model predictions to demonstrate that Sox7 blocks primitive erythroid development, and Sox and Hox factors directly regulate expression of the HSC regulator, Erg. The SCNS toolkit therefore opens up network reconstruction for other systems without the requirement for prior knowledge of regulatory interactions.

RESULTS
Capturing cells with blood potential during gastrulation
The first wave of primitive hematopoiesis originates from Flk1+ mesoderm1,2,10, with all hematopoietic potential in the mouse contained within the Flk1+ population from E7.0 onwards. Although some blood

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progenitor cells lose Flk1 expression just before the onset of circulation\textsuperscript{11}, previous work using a LacZ reporter knocked into the Runx1 locus showed that hematopoietic potential remains confined to the Runx1\textsuperscript{+} fraction\textsuperscript{12}, which was confirmed with a GFP reporter driven by the Runx1 +23 enhancer, which reproduces Runx1 expression\textsuperscript{8}. Using Flk1 expression in combination with a Runx1-ires-GFP reporter mouse\textsuperscript{13} therefore allowed us to capture cells with blood potential at distinct anatomical stages across a time course of mouse development (Fig. 1a,b). Single Flk1\textsuperscript{+} cells were flow sorted at E7.0 (primitive streak, PS), E7.5 (neural plate, NP) and E7.75 (head fold, HF) stages. We subdivided E8.25 cells into putative blood and endothelial populations by isolating GFP\textsuperscript{+} cells (four somite, 4SG) and Flk1\textsuperscript{+}GFP\textsuperscript{−} cells (4SFG\textsuperscript{−}), respectively (Fig. 1b and Supplementary Fig. 1a). Cells were sorted from multiple embryos at each time point, with

Figure 1 Single-cell gene expression analysis of early blood development. (a) Flk1 and Runx1 staining in E7.5 mesoderm and blood band, respectively. Scale bar, 100 \(\mu\)m. (b) Single cells sorted from five populations at four anatomically distinct stages from E7.0–E8.25. (c) Quantification of cells sorted and retained for analysis after quality control. (d) Quantification of Flk1\textsuperscript{+}, GFP\textsuperscript{+} or Flk1\textsuperscript{+}GFP\textsuperscript{−} cells in embryos at each time point from FACS data (Supplementary Fig. 1a). Line indicates median. (e) Unsupervised hierarchical clustering was performed using the Spearman correlation and complete linkage for the normalized gene expression of the 33 transcription factors and 7 marker genes in all cells. Shown is the level of expression for each gene in every cell (see key). Rows represent genes and columns represent cells, with the top colored bar indicating the embryonic stage of origin for each cell (see b for color code). Major cell clusters I, II and III are indicated. ND, not detected.
3,934 cells going on to subsequent analysis (Fig. 1c). Total cell numbers (Supplementary Fig. 1b) and numbers of cells of appropriate phenotypes (Fig. 1d) present in each embryo were estimated from fluorescence-activated cell sorting (FACS) data, indicating that for the first three stages, more than one embryo equivalent of Flk1+ cells was collected.

We next quantified the expression of 33 transcription factors involved in endothelial and hematopoietic development14, nine marker genes, including the embryonic globin Hbb-bH1 and cell surface markers such as Cdh5 (VE-Cadherin) and Itga2b (CD41), as well as four reference housekeeping genes in all 3,934 cells using microfluidic qRT-PCR technology7 (Supplementary Table 1), which resulted in >150,000 quantitative expression scores.

**Development of blood progenitor cells is not synchronized**

Unsupervised hierarchical clustering of the 33 transcription factor and 9 marker genes across all 3,934 cells revealed three major clusters (Fig. 1e). Cluster I was small and comprised mostly PS and NP cells. It did not express blood-associated genes, but showed low expression of some endothelial genes and high expression of Cdh1 (E-cadherin), likely representing mesodermal cells at the primitive streak15. Cluster II contained the greatest number of cells and included most of the PS, NP, HF and 4SG- cells, was characterized by endothelial gene expression, and contained subclusters with elevated expression of hemogenic endothelial genes, such as Cdh5, or hematopoietic genes such as Gfi1, indicating that this cluster contains a continuum of cells maturing from mesodermal to hematopoietic and endothelial fates. Cluster III was formed by most of the E8.25 Runx1+ 4SG cells, and had robust expression of hematopoietic genes (including Hbb-bH1, Gata1, Nfe2, Gfi1b, Ikaros and Myb), and low expression of endothelial genes (Erg, Sox7, Sox17, Hoxb4, Cdh5). The mixing of cells from different anatomical stages by hierarchical clustering analysis therefore suggested that developmental maturation of single cells in early mesodermal cell populations is asynchronous, with cells at multiple stages expressing similar combinations of developmental regulators. This is consistent with the gradual ingress of cells through the primitive streak and lineage commitment during gastrulation.

Principal component analysis (PCA) of the expression values of all 3,934 cells confirmed the large-scale mixing of cells from different anatomical stages, with only 4SG cells forming a stage-specific group (Supplementary Fig. 2a). The PCA was retrospectively colored to show which embryo each cell belongs to (Supplementary Fig. 2b), to determine whether this mixing is the result of developmental asynchrony within embryos or differences in maturation between different embryos classified as being of the same anatomical stage. We quantified the percentage of cells from each embryo belonging to clusters I, II and III, as identified by hierarchical clustering (Fig. 1e and Supplementary Fig. 2c,d). This showed that cells collected from each embryo at the PS, NP and HF stages were distributed across clusters I and II, with the earlier stages showing a greater bias toward cluster I than later stages. These results are therefore consistent with a model whereby cells representing both early and later stages along the differentiation trajectory toward blood are present throughout the PS, NP and HF time points, captured as snapshot measurements in our high-throughput, single-cell expression profiling.

A proportion of Flk1+ cells will give rise to mesodermal lineages other than blood and endothelium, and the extent to which they emerge over time and contribute to the variability would need to be analyzed using different gene sets. Notably, however, >50% of PS, NP and HF cells expressed both Flk1 and Runx1 at the mRNA level, highlighting the presence of Flk1+ cells with hemogenic potential12 from the earliest time points (Supplementary Fig. 3). Analysis of 50-cell pools from the PS, NP and HF stages by RNA-seq showed graded expression increases of hematopoietic and endothelial genes from the E7.0 to the E7.5 and E7.75 samples. This is entirely consistent with the continuous emergence of blood-specified cells deduced from our single-cell data, as an increase in the proportion of cells expressing a given gene between stages will increase population-averaged expression measurements. Key mesodermal and cardiac genes, by contrast, showed graded downregulation in the pooled-cell RNA-seq (Supplementary Fig. 4). These graded expression changes over time are not consistent with a discrete on or off switch at a specific developmental time point, but could again be due to gradual changes in the proportion of cells expressing the marker genes, similar to our observations from single-cell analysis of blood and endothelial genes. Alternatively, quantitative changes in expression levels within a constant proportion of cardiac-specified cells would similarly result in a change in the overall expression level of a population and cannot
be excluded from the pooled-cell RNA-seq. Therefore, our results indicate, at least for cells destined to become blood and endothelium, that these cells arise at all stages of the analyzed time course rather than in a synchronized fashion at one precise time point, consistent with the gradual nature of gastrulation. Notably, only single-cell analysis over a developmental time-course has the power to reveal the contribution to cellular heterogeneity made by unsynchronized maturation of individual cells.

**Diffusion maps identify developmental trajectories**

To identify and visualize putative developmental trajectories from the PS to 4S stages in the single-cell gene expression data, we developed a computational approach for dimension reduction. Our method is based on the concept of diffusion distances, which can be interpreted as a metric for objects (here, cells) that are related to each other through a gradual but stochastic, diffusion-like process, such as cellular differentiation. In brief, similarities between all 3,934 cells are calculated based on their gene expression patterns, and then visualized globally in a three-dimensional (3D) map (Fig. 2 and Supplementary Fig. 5). The resulting components span a low-dimensional diffusion-space, in which distance reflects how similar cells are in terms of their diffusion distance, and can be inferred to represent developmental time.

Although there is extensive mixing between PS, NP, HF and 4SFG− populations in the diffusion plot, there is a general progression in the cell stages present in different regions of the plot from largely early E7.0 PS and E7.5 NP cells through the later HF cells to the E8.25 4SG cells that form a homogeneous cluster, in line with the expected
developmental progression of the blood system or 4SG- cells (see Supplementary Fig. 6 for projection of individual populations). Furthermore, we observed that whereas the E8.25 Flk1+Runx1-GFP- (4SG-) cells mostly mix with earlier Flk1+ cells, a subset that was not identified by clustering or PCA branches off. This branch expresses endothelial and hemogenic endothelial genes (Cd1, Erg, Itga2b, Pecam1 (CD31), Sox7, Fli1) with lower to absent expression of Ev2 and Runx1. This observation is consistent with the known bifurcation of blood and endothelium (reviewed in ref. 16) and the downregulation of Runx1 in more mature endothelial cells. This bifurcation was more apparent in the diffusion maps than by PCA, independent component analysis or t-SNE (Supplementary Fig. 7). Genes that mark early, intermediate and late stages of blood development showed dynamic expression across the diffusion map (Fig. 2), with Cd1 expressed first, followed by Cd5 and then the embryonic globin Hbb-BH1. The transcription factors Ev2, Tal1 (Scl), Runx1 and Gata1 were expressed in a pattern consistent with their known sequential roles during the development of hemangioblasts through to erythroid cells. Dynamic expression patterns were also observed for other transcription factors not previously recognized as major regulators of primitive hematopoiesis, including Erg, Sox7 and Hoxb4. The diffusion map method therefore represents an attractive approach for ordering cells in developmental time, identifying patterns of expression for key regulators and bifurcation events not readily found with standard algorithms.

Synthesis of a network model for early blood development

The correspondence between the diffusion map and known developmental timelines suggested that the measured expression changes reflect developmental trajectories and might be exploited to define the regulatory networks that drive mesodermal cells toward a hematopoietic fate. Cell fate decisions have been modeled successfully using state space analysis of asynchronous Boolean regulatory network models. In this approach, each gene is associated with a Boolean variable (1 or 0), which represents whether the gene is expressed or not expressed, respectively, in the cell. Each gene is also given a Boolean update rule that specifies how its expression value changes over time owing to regulation by other genes. Boolean network dynamics are then modeled by a series of asynchronous single-gene changes, and state space analysis reveals the final stable states of the model. We were interested in the inverse problem: if we think of the single-cell expression profiles as the state space of a Boolean network, can we identify the underlying gene regulatory logic? Although single-cell data have been used to refine static networks curated from the literature, to our knowledge Boolean rules have not been derived directly from single-cell expression data without a priori knowledge of the structure of the network. To tackle this complex question of revealing the molecular changes underpinning cell state transitions, we developed the SCNS toolkit to synthesize Boolean networks based on single-gene transitions in our data.

We first discretized all 3,934 single-cell expression profiles to binary states and connected those states that differ in the expression of only one gene. The threshold for binary discretization was determined as described in Online Methods. This yielded a connected state transition graph of 1,448 expression states, connected by single-gene transitions (Fig. 3a,b). The number of times each state occurs is indicated in Supplementary Figure 8. The probability of seeing even one repeated state or neighbor in the whole theoretical state space is negligible, illustrating the nonrandom nature of the data. Most states that correspond to the Runx1-GFP+ 4SG cells clustered together at one end of the state transition graph, whereas states corresponding to cells from other time points were dispersed between two additional clusters. Likely developmental transitions were revealed, with specific genes consistently switching on or off along all routes linking the major clusters. We therefore considered this state transition graph as a possible representation of developmental expression state changes based on single-gene switches, and next asked whether this could be used for regulatory network reconstruction. Notably, analysis of real and simulated populations of 20 cells showed that pools for the same stage clustered closely together, which masked variation and therefore
would not have provided the number of transcriptional states required for network synthesis (Supplementary Fig. 9).

The direction of movement between two states in the state transition graph is initially not defined. Our method assigns a direction to each connection based on overall movement from the early PS to the later 4SG states, and then finds Boolean update functions for each gene that are consistent with its expression changes across the entire transition graph. Unlike previous analyses of single-cell gene expression data, which have largely relied on statistical properties of the data viewed as a whole, our method can recover mechanistic logic and determine the direction of interactions. When the method was applied to our data set, we obtained a core network of 20 transcription factors with endothelial and blood-associated gene modules centered on Sox7, Hoxb4 and Erg, and on Gata1 and PU.1 (also known as Sp1), respectively. For some genes, there were multiple possible consistent update functions. For example, there are two solutions for Erg, both of which include activation by Hoxb4 and Sox17. In total there were 39 possible functions, an average of two per gene. This led to 46,656 possible models from the different combinations of the 39 update rules (Fig. 3c and Supplementary Table 2). Repeating the network synthesis with bootstrapping and a different discretization threshold demonstrated the robustness of our protocol (Supplementary Tables 3 and 4).

Network synthesis predicts direct regulation of Erg

We next asked whether links in our single-cell expression-derived network models can reveal direct regulatory interactions. To provide support for our model, we identified high-confidence gene regulatory regions in the gene loci of the 20 transcription factors in our network by interrogating a compendium of transcription factor ChIP-seq data from hematopoietic cell types39, followed by identification of binding sites for the 20 transcription factors within these regions (Supplementary Fig. 10). 27 of the 39 Boolean rules (70%) are supported by the presence of evolutionarily highly conserved motifs for the upstream regulators in the target gene locus (Supplementary Table 2), with support for at least one Boolean rule for 16/20 transcription factors. This finding suggested that many of the regulatory interactions proposed in our model may be direct upstream regulator/-downstream target gene relationships. To provide further validation, we focused on Erg, which our models predicted is activated by Sox17, or by Hoxb4 in combination with Ly11 or Scl (Tal1). By analyzing a Hoxb4 ChIP-seq data set39, we showed that Hoxb4 can bind to the Erg+85kb enhancer (Supplementary Fig. 11a), which we previously showed to be active in blood stem and progenitor cells31,32. Moreover, comparative sequence analysis revealed that the Erg+85kb contains highly conserved Hox and Sox binding sites (Fig. 4a).

Figure 5 In silico perturbations predict key regulators of blood development. (a) Network stable states for wild-type and Sox7 overexpression. Red indicates expressed; blue indicates not expressed. (b) Colony assays with or without doxycycline from genotyped E8.25 embryos from iSox7+rtTA+ mice crossed with wild types. (c) Quantification of primitive erythroid colonies after 4 days (mean and s.e.m. for the number of embryos indicated). P-value was determined using the student’s t-test for the number of embryos indicated.

To investigate regulation of Erg by Hox and Sox factors, we took advantage of a recently described embryonic stem cell–based reporter system in which single-copy enhancer transgenes linked to the Hsp68/Venus reporter are targeted to the Hprt locus33, allowing robust comparisons of wild-type and mutant enhancer activity during in vitro differentiation. We tracked enhancer activity during embryoid body differentiation, where cells transit from a Flk1+CD41+ mesoderm/hemangioblast state, through a Flk1+CD41+ intermediate, to a Flk1+CD41+ hematopoietic state33–36. Flow cytometric analysis revealed a dynamic pattern of YFP expression for the wild-type enhancer, peaking at days 4–5 and highest in the Flk1+CD41+ population (Fig. 4b and Supplementary Fig. 11b,c). Similar expression was seen in the Sox mutant, whereas mutation of the Hox motifs caused a reduction of YFP+ cells, and the combined Hox and Sox mutant reduced the proportion of YFP+ cells further still. We also saw similar patterns of expression in the other populations, which constitute a larger proportion of the embryoid body cells but have a lower percentage of YFP+ cells (Fig. 4b and Supplementary Fig. 11b,c). This suggests that Hox and Sox factors activate and maintain Erg expression largely independently and additively. When abstracted to the Boolean level, this result is therefore more consistent with the OR logic in our network than with the alternative AND logic, where single mutations would result in an effect as strong as the combined mutant.

Model execution reveals key switches during development

Next, we assessed whether our network models faithfully recapitulate blood and cardiovascular development, in which endothelial and primitive blood cells emerge from a common mesodermal progenitor. To do this, we determined the stable states of the network model that correspond to those expression patterns for the 20 transcription factors that satisfy all the Boolean network rules, and therefore can remain stable. We found that only eight stable states are reachable in total across all possible models, including “endothelial-like” (WT-S7) and “blood-like” expression states (WT-S2 to S6) (Fig. 5a). Of note, 432 models had both the endothelial-like state and at least one of the blood-like states (WT-6) as stable states, thus capturing the functionality of bipotential Flk1+ precursors.

Finally, we explored the consequences of in silico perturbation. Overexpression and knockout experiments were simulated for each transcription factor and the ability of the network to reach wild-type or new stable states was assessed (Supplementary Table 5). For a number of factors, stable states 6 or 7 were no longer reachable. Among these, enforced expression of Sox7, a factor normally downregulated when cells transit toward the 4SG state (Fig. 3b), resulted in the stabilization of the endothelial module and an inability.
to reach any of the blood-like states (Fig. 5a). Only two stable states were possible, among the lowest for all factors, and furthermore, Sox7 is predicted to regulate more targets than any other transcription factor, suggesting that perturbing its expression could have important downstream consequences (Supplementary Table 5). To validate this prediction, we crossed the previously reported iSox7^rtTA^ male mice with wild-type females, collected embryos at E8.25 and performed colony-forming assays (Fig. 5b). Embryos carrying both transgenes showed a 50% reduction of primitive erythroid colony formation and simultaneous appearance of undifferentiated hemangioblast-like colonies following doxycycline-induced Sox7 expression compared to controls (Fig. 5c and data not shown). This suggests, in agreement with modeling data and gene expression patterns, that downregulation of Sox7 is important for the specification of primitive erythroid cells.

DISCUSSION

Determining the structure and function of transcriptional regulatory networks is crucial to advancing our understanding of developmental and disease processes and is therefore a key aim of stem and developmental biology. However, studies to date have mainly used population-based data for network construction or have focused on statistical properties of populations of single cells for network inference.

Bayesian network methods provide a very computationally efficient approach to inferring causal relationships among a set of variables and have previously been applied to infer cellular signaling networks from single-cell data. However, these approaches infer a directed acyclic graph where there is no feedback between nodes, a limitation not shared by our approach. In addition, the inference of edges is reliant on network interventions in which many different cell populations are generated by experimentally perturbing genes, and the differences between these populations are used to infer causality. Generating such intervention data is very time consuming and cannot be done when studying wild-type in vivo development. Instead, researchers typically look at the pairwise correlation of genes across single-cell measurements. For example, partial correlation analysis measures the degree of association between two genes while controlling for potential effects of all other genes. We performed this analysis (Supplementary Fig. 12), and found agreement with many of the edges in our synthesized network; however, this analysis failed to predict the Sox/Hox regulation of Erg, which we validated experimentally. Moreover, connections do not specify which gene is the upstream regulator and which is the downstream target, and therefore do not reveal mechanistic logic.

To our knowledge no previous study has analyzed the development of an entire mammalian organ at single-cell resolution. Here we have studied the earliest stages of blood development from mesoderm through to the emergence of primitive erythroid cells, and demonstrate that single-cell expression profiling, coupled with computational approaches for network synthesis, can reveal molecular control mechanisms of mammalian organogenesis. Analysis of 46 genes in blood precursors across 1.25 days of post-implantation mouse embryonic development showed that cellular maturation may not be done when studying wild-type in vivo development. Instead, researchers typically look at the pairwise correlation of genes across single-cell measurements. For example, partial correlation analysis measures the degree of association between two genes while controlling for potential effects of all other genes. We performed this analysis (Supplementary Fig. 12), and found agreement with many of the edges in our synthesized network; however, this analysis failed to predict the Sox/Hox regulation of Erg, which we validated experimentally. Moreover, connections do not specify which gene is the upstream regulator and which is the downstream target, and therefore do not reveal mechanistic logic.

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ONLINE METHODS

Timed matings and embryo collection. Timed matings were set up between homozygous Runx1 reporter male and female mice using the Runx1-ires-GFP knock-in mouse previously described. Animals also contained a Gata1-mCherry reporter transgene not used in this study. All animal experiments were carried out in accordance with the RIKEN guidelines for animal and recombinant DNA experiments. Embryos were staged according to morphologic criteria. Suspensions of embryo cells were prepared as described previously and single-cell suspensions were stained with Flk-1-APC (AVAS12 at 1:100 dilution; BD Bioscience). Cells were sorted using a FACS Aria II (BD Bioscience) and 100 µm nozzle. 4SG cells were not sorted for Flk1 as its expression begins to be downregulated by this time. 4SG- cells were specifically Runx1-GFP at the protein level in order to exclude committed blood cells of the 4SG population, but could express Runx1 at the mRNA level.

Single-cell qRT-PCR. Single-cell qRT-PCR was carried out using the Fluidigm BioMark platform as described, with a limit of detection (LOD) of Ct 25. The LOD was determined according to Stahlberg et al. and manufacturer’s instructions. Briefly, standard curves were run on the BioMark with six repeats of each dilution. For each gene, the LOD was the average Ct value for the last dilution at which all six replicates had positive amplification. The overall LOD for the gene set was the median Ct value across all genes. TaqMan assays (Life Technologies) used are listed in Supplementary Table 1. Raw Ct values and normalized data can be found in Supplementary Table 7. Gene expression was subtracted from the limit of detection and normalized on a cell-wise basis to the mean expression of the four housekeeping genes (Ef2kb1, Mrpl19, Polr2a and Ubc) in each cell. Cells that did not express all four housekeeping genes were excluded from subsequent analysis, as were cells for which the mean of the four housekeepers was ±3 s.d. from the mean of all cells. A dCt value of −14 was then assigned where a gene was not detected. 85–90% of sorted cells were retained for further analysis. Gata2 did not amplify correctly and HoxB3 was not expressed in any cells, so these factors have been excluded from the analysis. Further analyses were done on the dCt values for all transcription factors and marker genes, but not housekeeping genes. Hierarchical clustering was performed in R (http://www.r-project.org/) using the hclust package and displayed with heatmap.2 from the gplots package. Complete linkage was used as recommended by Fluidigm for single-cell expression data, with the distance defined as 1 – Spearman rank correlation between samples or genes. Optimal ordering of leaves was determined using the cba package. Principal components analysis was done using the procmp function in R and PC scores for each cell are shown. Plots were colored retrodictively to indicate the anatomical stage of origin of each cell.

RNA sequencing. Cells were sorted into 2 µl of lysis buffer (0.2% (v/v) Triton X-100 and 2 U/µl RNase inhibitor (Clontech)) and stored at −80 °C. RNA-seq was carried out using the Smart-seq2 protocol according to Picelli et al. and sequenced on an Illumina HiSeq 2500. The reads for five biological replicates for each subtype were mapped using the RNA-seq aligner STAR version 2.3.0 (ref. 53). Parameters used to align with STAR were “–outFilterMultimapScoreRange 1–outSAMstrandField intronMotif–genomeLoad NoSharedMemory–outStd SAM”. Mus musculus Ensembl assembly GRCm38 (equivalent to UCSC mm10) was used to build the STAR index file, along with the GTF file (version GRCm38.74) from Ensembl. Samtools version 0.1.18 was used to sort the STAR SAM output file and convert it to BAM format, which would then be used as input for the HTSEQ-counts program. Counts were determined using the HTSeq-counts program version 0.6.1 (http://www.huber.embl.de/users/ anders/HTSeq/doc/overview.html) with the parameter “–stranded=no”. FPKM values were calculated using in-house scripts and are provided in Supplementary Table 8.

Diffusion plots. Our visualization approach is based on the diffusion map formalism. In brief, affinities between all cells based on their expression levels are calculated using a diffusion metric. Next, the cells are organized in 2D or 3D such that the Euclidean distance between the cells corresponds to the diffusion metric. We determined the cell-cell affinities using an isotropic Gaussian kernel

$$P(i,j) = \frac{1}{Z_i} \exp \left( -\frac{(x_i - x_j)^2}{\sigma} \right)$$

with $x_i$ and $x_j$ being the gene expression vector for cells i and j and Z, being a normalization constant such that

$$\sum_{j=1}^{N} P(i,j) = 1$$

The quantity $P(i,j)$ can then be interpreted as the transition probability of a diffusion process between cells. Consequently, it is particularly well-suited for representing the gradual change in the transcriptional landscape related to developmental trajectories. In contrast, other methods for dimensionality reduction and visualization of high-dimensional data such as t-SNE (ref. 34, 37), encourage a representation of the data as disjoint clusters, which is less meaningful for modeling continuous developmental trajectories.

In order to account for the nonuniform density $p$ of cells in the gene expression space (i.e., the potential presence of rare populations), we renormalize the affinities $P(i,j)$ between two cells i and j based on the local density $p(i)$ and $p(j)$ to $P(i,j)$. Furthermore, we encourage a better representation of local behavior by using only a subset of neighboring cells (20% nearest neighbors) for computing the affinities and by setting the diagonal of the affinity matrix $P$ to zero. We then calculate the eigenvectors of $P$ and retain the eigenvectors with the greatest eigenvalues, which we use for visualization.

Network analysis. In computer science, synthesis is a general term for the counterpart of verification. In verification, a hand-built model is given, along with a specification of how it ought to behave. Then the model is checked to ensure it satisfies the specification. In synthesis, a specification is given and a model is automatically generated that satisfies this specification. In biology, the specification is the experimental data that the model should reproduce. In our case, it is the state transition graph, which was derived from the single-cell gene expression data. Synthesis has recently been applied in the context of biology. In that work, state machine-like models were synthesized, which were consistent with known experimental mutation results, given in a genotype-phenotype table. Both the data and the type of model considered were different from those dealt with in the current work, which called for a different approach.

To synthesize a Boolean network model, we would like to orient the transitions in the state graph (previously every pair of states that differ in the expression of exactly one gene were connected by an undirected edge) such that a given set of final states will be reachable from a given set of initial states. We will allow edges to be directed in one direction, both directions, or in neither. We would then like to extract the Boolean update functions that give rise to these directed transitions. We try to get the best possible network by maximizing the number of states in which no transitions induced by the update functions are missing (condition 2, below).

We can state our synthesis problem formally as follows. We are given a set $N$ of vertices and a designated set $F$ of final states, along with a threshold $t_i$ for each variable $x_i \in V$.

$$P(i,j) = \frac{1}{Z_i} \exp \left( -\frac{(x_i - x_j)^2}{\sigma} \right)$$

with $x_i$ and $x_j$ being the gene expression vector for cells i and j and Z, being a normalization constant such that

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We can state our synthesis problem formally as follows. We are given a set $N$ of variables $x_i$, corresponding to genes, and an undirected graph $G = (N,E)$ where each node $x_i \in N$ is labeled with a state $x: N \rightarrow \{0,1\}$, and each edge $(x_i,x_j) \in E$ is labeled with the single variable that changes between state $x_i$ and $x_j$. Note that by $s$ we denote both a state and the node labeled by that state, unambiguously. We are also given a designated set $I \subseteq N$ of initial vertices and a designated set $F \subseteq N$ of final vertices, along with a threshold $t_i$ for each variable $x_i \in V$.

Our synthesis method searches for an orientation of $G$, along with an update function $u_i: \{0,1\}^I \rightarrow \{0,1\}$ for each variable $x_i \in V$, such that the following conditions hold:

1. For each edge $(x_i,x_j)$ labeled with variable $x_i$ in the oriented graph, the update function for $x_i$ takes state $x_i$ to state $x_j$: $u_i(x_i) = x_j$.
2. For every variable \( v_i \in V \), let \( N_i \) be the set of states without a \( v_i \)-labeled edge. For every \( i \) the number of states \( s \in N_i \) such that \( u(s) = s(i) \) is greater than or equal to \( t_i \).

3. Every final vertex \( f \in F \) is reachable from some initial vertex \( i \in I \) by a directed path in the orientated graph.

We restrict the update function \( u_i \) to have the form:

\[
f_1 \wedge \neg f_2
\]

where \( f_j \) is a Boolean formula that has and-nodes of indegree two and/or-nodes of arbitrary in-degree, and where \( f_1 \) has a maximum depth of \( N_i \) and \( f_2 \) has a maximum depth of \( M_i \), \( N_i \) and \( M_i \) are given as parameters to the method.

The search for edge orientations and associated Boolean update rules is encoded as a Boolean satisfiability (SAT) problem. The update functions of each variable can be sought after separately, giving rise to reasonably sized satisfiability queries. We then combine compatible single-gene update functions by restricting our attention to combinations that permit paths from initial to final nodes. Paths between initial and final nodes in orientated graphs are found using a breadth-first search for the shortest path between two nodes. We restrict our search to these shortest paths both for efficiency reasons and to eliminate routes that seem “unbiological,” for example, routes that cross a fate transition and then return to where they began. We exhaustively search for all satisfying solutions. The method is implemented in the F# programming language, and uses the Z3 solver to handle SAT queries.

After assessing the method’s capability to reconstruct literature-derived asynchronous Boolean networks from their own state spaces (Supplementary Note), we applied it to our biological data. From the resulting synthesized Boolean network models, we obtained a core network of 20 factors.

For our initial states, we took the set of the PS states in the earliest state cluster in the state transition graph. As the final states, we took a core of the 4SG states in the latest cluster. These states are listed Supplementary Table 9.

Note that owing to the intermixing between populations, there is no guarantee that a state measured on E7.75 is further ahead in development than a state measured on E7.5 (for example). We therefore only constrain reachability from start states to end states, and do not require that experimental measurement time is respected. To obtain the thresholds, \( t_i \), and the maximum sizes of the activating and repressing portions of update functions \( N \) and \( M \), we performed an optimization step for each gene independently, where the size of allowed update functions was steadily increased until \( N_i \) reached a maximum. \( t_i \) was then set to 0.66 \( N_i \) in order to allow the method room to find Boolean update rules that permit a path from initial to final states. To obtain the stable states of Boolean network models, the algorithm from Garg et al.37 was applied. Binary states can be found in Supplementary Table 10 and cells with equal cell states are listed in Supplementary Table 11.

Synthesis bootstrapping. To assess the robustness of the predictions of network synthesis, we performed bootstrapping. A random sample of 75% of the 3,934 gene expression profiles was retained, and a new state transition graph was built from this reduced data set. This state transition graph was then used as the basis to synthesize new Boolean rules, using the same parameters as the original analysis. The results of repeating this process five times are shown in Supplementary Table 3a–e. Bold entries indicate a rule is identical to a rule synthesized from the original data set. Underlined entries indicate that a rule is contained within a larger rule from the original synthesis. We see that in most cases the original rule or a closely related, underlined rule is synthesized. In general, the number of possible solutions for a gene’s update function grows as the amount of data used is decreased, and including the full data set narrows these possibilities.

Assessing sensitivity of synthesized rules to binary discretization threshold. In order to construct a state transition graph and apply our synthesis method, experimental data must first be discretized to binary values that indicate whether a gene is expressed or not expressed. The details of how we determine this threshold are covered in the section entitled “Single-cell q-RT-PCR.”

To assess sensitivity of results to the choice of threshold, we repeated our analysis with a more stringent cut off, increasing it by two cycles. This resulted in a state transition graph of 1,249 nodes (199 fewer nodes than the original state transition graph), which was then used as the basis to synthesize new Boolean rules, using the same parameters as the original analysis. The results are shown in Supplementary Table 4. Bold entries indicate a rule is identical to a rule synthesized from the original data set. Underlined entries indicate that a rule is contained within a larger rule from the original synthesis. We see that in most cases the original rule or a closely related, underlined rule is synthesized. In general, the number of possible solutions for a gene’s update function grows as the amount of data used is decreased, and including the full data set narrows these possibilities.

Embryonic stem (ES) cell gene targeting, differentiation and analysis. Erg+85kb/Hsp/Venus cassettes were targeted to the Hprt locus in Hprt-deficient mouse HM-1 ES cells58 to generate clonal lines that were differentiated into blood by embryoid body formation and analyzed at the stated time points by flow cytometry for Fik1 (as above) and CD41–PECy7 (eBiolMWR0030; 1:500, BioLegend), all as previously described39. Data are the combined average of three biological replicates from two ES cell clones. Two HM-1 lines carrying an enhancer-less Hsp/Venus cassette were used as a control, as described previously39.

Sox7 induction and colony assays. Timed matings were set up between transgenic male iSox7+/rtTA+ and wild-type ICR female mice55. The morning of vaginal plug detection was considered E0.5. All animal work was done under regulations governed by the Home Office Legislation under the Animal Scientific Procedures Act (ASPA) 1986. Cells from E8.25 embryos were tested in a clonogenic replating assay for hematopoietic progenitors with or without 1 μg/ml doxycycline as previously described37. For each embryo 1/10 of the cells was used for genotyping and the remaining cells were equally divided into −dox and +dox conditions. Primitive erythroid colonies were counted after 4 days in culture. Primers used for genotyping were rtTA-F ACAAGGTTTCTACTAGAAGACGG, rtTA-R AGATCGAATTCTGTCAGGGGCTCG, iSox7-F CTAAGATCTGAAGTTTTTCACTAGAGAACGCG, rtTA-R AGATCGAAATCGTATGACGCCAG GTTG, Erg+85attb1R GGGGACCACTTTTGATCAAGAAGAACGCTGGTGCC ATGAAATTCA. 

ACCTTTGGAAATTTGTCC; see Fig. 4a for sequences of mutated motifs.)
involved in blood differentiation, we collected all perturbations which retained the desired “endothelial-like” state, removed the undesired “blood-like” state, and then ranked these perturbations by the number of additional, undesired states that were introduced.

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