Review

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Primed and ready: understanding lineage commitment through single cell analysis

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Regulation of lineage commitment in multipotential cells is key to maintaining a balanced hematopoietic output throughout life while retaining the capacity to respond to stress and infection. Cell fate decisions are made by individual stem cells, but population-level analysis obscures the mechanics of cell fate choice by averaging the molecular and functional heterogeneity that exists even in the most highly purified stem cell populations. Therefore, single cell analysis of both molecular and cellular phenotypes is crucial to delineate and interrogate the process of lineage commitment. We review recent single cell expression profiling, imaging, and clonal tracking studies that have provided new insights into commitment, focusing on the hematopoietic system, and suggest how new technologies may illuminate our understanding of lineage commitment in the near future.

Understanding lineage commitment one cell at a time

Commitment is the point at which a cell becomes restricted irreversibly (under physiological conditions) to one particular fate and loses the potential to differentiate into other cell types. It can be discriminated from the earlier specification process in which the potential of a stem/progenitor cell is defined before committing to one particular lineage. In multipotent cells specification involves establishing the competence to differentiate down multiple different lineages. These processes are theoretically different and may be regulated by different albeit interlinked mechanisms.

Much attention has focused on the role of transcription factors (TFs) in specifying and committing cells to particular lineages. It has been suggested that intrinsic lineage choice is stochastic and depends upon the activation of lineage-specifying TFs at supra-threshold levels that then lock in cell fate through cross-antagonistic interactions with alternative lineage-specifying TFs [1]. However, understanding how multipotent cells are first specified, and subsequently make decisions about which fate to choose, is contingent upon the ability to measure gene expression at the single cell level. Averaging gene expression and cellular behavior by population-level analysis masks the presence of distinct expression patterns and functional potentials within single cells, which can help to reveal the molecular basis of cell fate decision-making. The development of a microfluidic qPCR platform for interrogation of a matrix of 96 genes in 96 individual cells has revolutionized single cell gene expression analysis [2]. Previous multiplex single cell reverse transcription and PCR (RT-PCR) methods were not quantitative and required manual handling and large reaction volumes [3]. The sensitivity and precision provided by microfluidic processing of small reaction volumes and qPCR analysis allows accurate quantification of coexpression of multiple genes in individual cells [4,5]. Hematopoietic stem and progenitor cells (HSPCs) are particularly amenable to single cell analysis because these cells can be readily isolated by fluorescence activated cell sorting (FACS) and have considerable clonogenic capacity both *in vitro* and *in vivo*. Single cell gene expression analysis was pioneered in this system and has been complemented by clonal tracking studies of HSPC behavior to link the molecular and cellular phenotypes.

We review here molecular and functional single cell studies performed in the hematopoietic system to interrogate gene expression patterns and cellular hierarchies during lineage specification and to infer mechanisms governing commitment (other cellular systems are briefly reviewed in Box 1). In addition, single cell studies have recently illuminated longstanding questions in the hematopoietic field regarding the role of extrinsic regulation in hematopoietic stem cell (HSC) commitment and the nature of HSC emergence in the embryo; these are outlined in Box 2.

Single cell approaches define cellular hierarchies and transcriptional networks regulating commitment

Multilineage priming in hematopoietic stem and progenitor cells

HSCs are multipotent cells that continuously replenish all classes of blood cells through a series of lineage-restricted steps, resulting in more differentiated and developmentally limited cells. Cell fate decisions in HSCs have been proposed to result from the activation and cross-antagonism of lineage-specifying TFs [1]. Early studies using single cell multiplex RT-PCR revealed that

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Box 1. Exploring commitment through single cell transcriptomics in non-hematopoietic tissues

In this review we focus on single cell studies informing on hematopoietic commitment, but this is not the only field being revolutionized by single cell transcriptomics. Some examples from the early mouse embryo, human ESCs (hESCs), as well as developing cerebral cortex and lung epithelium are summarized below.

In the early embryo single cell analyses have begun to reveal mechanisms driving the earliest stages of cell fate determination in the blastocyst. Microfluidic qPCR analysis in single cells isolated from embryos over the first 4 days of mouse development revealed that at the 16 cell stage cells coexpress high levels of TFs specific to different lineages [58]. The commitment of cells to different cell types during the morula to blastocyst transition is associated predominantly with downregulation of TFs associated with opposing lineages rather than upregulation of lineage-specific TFs. Multilineage priming is therefore apparent in blastomeres of the early embryo, similarly to that observed in HSPCs. This analysis also suggested that positional and signaling events precede the establishment of distinct transcriptional programs in the early embryo, suggesting a mechanism by which cell fates become specified from initially equivalent cells.

Global analysis of gene expression in single cells from the inner cell mass of 32–50 cell stage blastocysts using microarray analysis further revealed that initially variable stochastic expression is resolved during lineage segregation into correlated gene expression programs coincident with activation of lineage-specific signaling events [26]. This is similar to the model proposed based on single cell qPCR analysis of cells undergoing reprogramming in which early stochastic variable expression is followed by a hierarchical deterministic phase [27]. This analysis further predicted novel regulators of pluripotency from their correlated expression in this late phase of reprogramming.

In hESCs, microfluidic single cell qPCR analysis combined with isolation of functionally defined subsets of cells revealed a hierarchy of pluripotent cells. A population of highly self-renewing cells at the apex expressed high levels of pluripotency factors, but with little or no expression of lineage-specific genes consistent with a naïve groundstate [59]. However, a much larger set of hESCs exhibited pluripotency, and these were highly heterogeneous in their gene expression, featuring extensive priming of lineage-specific regulators, suggesting that lineage priming may poise hESCs for lineage specification, similarly to what has been proposed for HSCs.

Low coverage single cell RNA-seq has recently been validated as a useful tool for identifying cell types within complex tissues which are not amenable to purification by flow cytometry, such as neurons in the developing cerebral cortex [60]. Furthermore, single cell transcriptome analysis was recently used to delineate the cell lineage trees in distal lung epithelium and to identify lineage-specific regulatory factors [61].

In summary, the potential of single cell transcriptomics to reveal cellular and molecular pathways to commitment is only now beginning to be realized. In the coming years, with the advent of more precise and sensitive methods for transcriptome wide RNA-seq, this technology will revolutionize our understanding of the mechanisms underlying cell fate decision-making in a variety of tissues.

Box 2. Single cell analysis provides new answers to longstanding questions

Extrinsic versus intrinsic regulation of HSCs: cytokines can drive lineage commitment

A longstanding debate in the field has been whether intrinsic activation of TFs or extrinsic cytokine signaling instructs lineage commitment in multipotential cells [62]. Analysis of knockout mice revealed that cytokine signaling is not required for commitment, and in many cases cytokine receptor signaling domains are functionally interchangeable, leading to the idea that cytokines act on intrinsically committed progenitors to promote their growth and survival [62]. However, time-lapse imaging showed that cytokines drive lineage choice in individual granulocyte/monocyte progenitors (GMPs) [63], and recent studies have revealed an instructive effect of the cytokines erythropoietin (Epo) and macrophage colony stimulating factor (M-CSF) on the most primitive HSC compartment [64,65]. Continuous video imaging of the expression of a PU.1 reporter in individual cells in the presence of M-CSF showed that this cytokine rapidly induces PU.1 upregulation in HSCs without intervening cell division. Furthermore, single cell multiplex qPCR revealed that, before M-CSF exposure, most cells with PU.1 expression exhibit multilineage priming indicative an uncommitted state. A myeloid-restricted expression profile was only induced in PU.1^{+} cells after M-CSF exposure, suggesting that they are not pre-committed to the myeloid lineage. It therefore appears that cytokines can act to instruct lineage choice directly in HSCs and multipotent progenitors; however, the ability of the cells to respond depends on expression of the relevant receptor, which is likely to be intrinsically regulated. The action of cytokines, chemokines, and growth factors on HSCs has been implicated in ensuring robust and responsive hematopoiesis upon injury and infection [66]. The variable expression of receptors amongst individual HSPCs, either due to lineage priming or through other mechanisms such as ligand-induced receptor internalization, may act to ensure a mixture of responsive and non-responsive stem cells to prevent exhaustion of the stem cell pool. Lineage priming could therefore account for both extrinsic and intrinsic regulation of HSC commitment. Indeed, both may be required to ensure balanced and responsive hematopoiesis.

HSC emergence in embryos: single cell analysis begins to reveal the nature of the endothelial to hematopoietic transition

Another longstanding debate has surrounded the issue of how HSCs emerge in the embryo and whether they share a common origin with endothelial cells. It has been suggested, from imaging and genetic studies of early hematopoietic development, that HSCs arise from a specialized bipotential hemogenic endothelium in the dorsal aorta [13]. Hemogenic endothelium was originally defined as cells with endothelial morphology that can give rise to both endothelial and hematopoietic cells in culture [67]. However, isolation of single putative hemogenic endothelial cells using the Runx1 (runt-related transcription factor 1) +23 enhancer/GFP reporter (+23GFP) from embryonic (E) day E8.5–E10.5 embryos revealed that the generation of endothelial cells and hematopoietic cells is mutually exclusive, suggesting that cells rapidly transit from endothelial to hematopoietic committed cells with no stable bipotential intermediate [68]. Single cell qPCR analysis on purified +23GFP hemogenic endothelial cells from E8.5–E10.5 embryos revealed that a subset of these putative hemogenic endothelial cells begin to switch between endothelial and hematopoietic states very early at E8.5, before functional commitment at E9.5–10.5. Negative correlation between expression of the hematopoietic TF *Meis1* (Meis homeobox 1) and the endothelial Ets factor *Etv2* (Ets variant 2) in this early phase of hematopoietic specification occurred in the absence of Runx1 expression but was followed at E9.5 by a coherent hierarchical induction of a hematopoietic program in a subset of the cells that correlated with Runx1 upregulation. This study supports the observation that induction of Runx1 in ESC-derived hemogenic endothelium drives a hematopoietic-restricted pattern of TF binding [69], but it also suggests that the early specification of these cells is Runx-independent and may be regulated by an antagonistic effect of Meis1 on expression of the endothelial TF Etv2. Further functional analysis will be necessary to test this prediction, but this study illustrates how single cell expression analysis can be used to identify changes in cell identity that occur at the molecular level in rare cells in the embryo and thereby generate novel, testable hypotheses about the earliest stages of cell fate specification.

lineage-restricted genes are expressed at low levels in multipotential stem and progenitors, and were thus suggested to be 'primed' for expression later during differentiation [3,6]. Furthermore, significant numbers of multipotent hematopoietic cells coexpress genes associated with multiple different lineages (30–60% of cells in this study), suggesting that conflicting lineage programs are simultaneously primed in preparation for