Hematopoietic Stem Cells Focus

Analyzing signaling activity and function in hematopoietic cells

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Cells constantly sense their environment, allowing the adaption of cell behavior to changing needs. Fine-tuned responses to complex inputs are computed by signaling pathways, which are wired in complex connected networks. Their activity is highly context-dependent, dynamic, and heterogeneous even between closely related individual cells. Despite lots of progress, our understanding of the precise implementation, relevance, and possible manipulation of cellular signaling in health and disease therefore remains limited. Here, we discuss the requirements, potential, and limitations of the different current technologies for the analysis of hematopoietic stem and progenitor cell signaling and its effect on cell fates.

Cellular signaling
Cells must adapt their behavior to the needs of the organism. These needs are always changing, not only during development and aging, but throughout life even in seemingly homeostatic conditions. Cells constantly sense their environment through receptors for different chemical and physical components. These receptors activate signaling pathways, which activate molecular target programs initiating the cells’ responses (Fig. 1 A). Relevant environmental stimuli are highly diverse, complex, and dynamic, and cells must be able to compute finely tuned output responses, both as individual cells and coordinated, as a population, from these combined and changing inputs. This response computation happens at the level of signaling pathways, which translate the different combinations of inputs into output programs (Landry et al., 2015). While many receptors exist in our genomes, individual cells express far fewer receptors than the number of different environmental situations they can react to. In addition, an even more limited number of cell-intrinsic signaling pathways are available. As a result, although the activation of different receptors leads to different cellular responses, they often activate a highly overlapping set of pathways. To enable the required high number of fine-tuned responses with the limited set of existing pathways, these are wired in complex interconnected networks. Their activity is nonlinear, context-dependent, dynamic, and heterogeneous, even between closely related individual cells (Tay et al., 2010; Lane et al., 2017; Mitra et al., 2017; Wang et al., 2021), enabling a much-increased number of combinatoric and dynamic outputs (Fig. 1 B). A further increase in output possibilities is achieved by modulating the quantity and quality of the input ligands or receptors (Ho et al., 2017; Purvis et al., 2012). For example, a change in the topology of erythropoietin dimers by variation of distance and angle between monomers changes how the receiving pathways are activated (Kim et al., 2017; Moraga et al., 2015).

Inflammatory signaling in hematopoietic stem and progenitor cells (HSPCs)
Inflammatory signaling in HSPCs is activated upon detection of injury or infection and regulates the appropriate cellular responses. Various pathways, most prominently NF-κB, JAK/STAT, protein kinase B (AKT), and p38, are triggered by pathogen-associated molecular patterns or pro-inflammatory cytokines like TNF-α, IFNs, and ILs (Hemmati et al., 2017). Pathways lead to the expression of an array of target genes involved in pathogen elimination, immune cell recruitment, and a general adaptation of cell state and fate (Newton and Dixit, 2012). Inflammatory signaling controls HSPC fates (Endele et al., 2014; Rieger et al., 2009; Takizawa and Manz, 2017; Trumpp et al., 2010), and misregulation is associated with stem cell exhaustion, malignant transformation, and other disorders (Hemmati et al., 2017; Takizawa et al., 2012). For example, both IFN-γ and IFN-α activate quiescent hematopoietic stem cells (HSCs) and trigger cycling (Baldridge et al., 2010; Essers et al., 2009) and emergency mekaryopoesis (Chicha et al., 2004; Haas et al., 2015; Manz, 2008). LPS-induced NF-κB activation triggers apoptosis in T cells but proliferation in the common lymphoid progenitor population.
TNF-α promotes HSC survival but induces apoptosis in granulocyte monocyte progenitors (Yamashita and Passegué, 2019), and a correct NF-κB response seems crucial for ex vivo HSC expansion (Chagraoui et al., 2019). Inflammatory signaling is used as a target for therapeutic manipulation, most successfully by small molecules or peptides. For example, JAK2 inhibitors treat JAK2-induced myeloproliferative neoplasms (Kiladjian et al., 2016), and the p38 inhibitor ralimetinib reduced the proliferative effects of IL-1 in acute myeloid leukemia (AML) in preclinical studies (Carey et al., 2017). Inhibition of NF-κB signaling by parthenolide shows activity in AML stem cells (Guzman et al., 2005). Differentiation in myelodysplastic syndrome and AML can be induced by TLR agonists (Hemmati et al., 2017). For a more exhaustive discussion of the many effects of signaling, please refer to the other reviews of this series.

Technological requirements

Analyzing signaling networks and their functional implementation is notoriously difficult. As discussed above, individual activated receptors usually activate multiple parallel and interconnected pathways, each with complex regulation through cascades of biochemical layers and feed-forward and feedback loops (Fig. 1 A). This wiring leads to many possible dynamics and combinations, which massively increase the amount of information that can be encoded from the limited number of available signal transduction pathways (Fig. 1 B). Short- and long-range feedback signaling between cells of a tissue or even the whole organism makes this even more complex (Charest and Firtel, 2006). This increases both the signalings’ potential for accurately controlling biology and the experimental requirements for its comprehensive analysis. How information is encoded, how specificity is achieved (Behar and Hoffmann, 2010), and how it is possible to integrate many input combinations and their spatiotemporal dynamics over time to generate accurate spatiotemporal outputs remain some of the big central mysteries in life sciences. This is due not only to the limitations in mathematical and computational tools for understanding nonlinear dynamic control systems but also to the lack of technologies for the quantification of the many signaling components and associated cell fate choices with the required spatiotemporal resolution, dimensionality, and throughput.

Ideally, signaling is quantified (1) at the single-cell level, (2) continuously, (3) with spatial information of the cells in their environment, and (4) linked to past and future molecular and cellular behavior of the analyzed cells (Fig. 2).

Since signaling is usually heterogeneous between individual cells even in seemingly homogeneous and closely related cells, the analysis of population averages is insufficient (Etzrodt et al., 2014; Hoppe et al., 2014; Loeffler and Schroeder, 2019; Chandra et al., 2008; Welner et al., 2008).
Schroeder, 2005, 2008; Fig. 2). For example, there is a linear correlation between the TNF concentration used for stimulation and the NF-κB response amplitude of the whole stimulated 3T3 fibroblast population, suggesting an analogue NF-κB response. However, analysis at the single-cell level demonstrated an entirely different regulation: Individual cells respond in a digital all-or-nothing on/off fashion, and only the frequency of responding cells is correlating with the TNF concentrations (Tay et al., 2010). Furthermore, the current geometric state of individual cells can influence their transcriptional responses to TNF stimulation (Mitra et al., 2017).

In addition, signaling pathways are active not only in an on/off type fashion but also with different dynamics (e.g., sustained, transient, oscillatory; Fig. 1B; Behar and Hoffmann, 2010; Purvis and Lahav, 2013). If done only at individual time points, even single-cell analyses will miss this crucial information (Fig. 2; Hoppe et al., 2014; Loeffler and Schroeder, 2019; Purvis and Lahav, 2013; Schroeder, 2005, 2011). These signaling activity dynamics can be specific for the target cell type or stimulus used but are typically heterogeneous between individual cells. Importantly, these different dynamics seem to activate different molecular target programs (Hoffmann et al., 2002), and to cause specific cell fates, even though only one pathway is activated (Marshall, 1995; Nandagopal et al., 2018; Purvis et al., 2012). For inflammatory signaling, different ligands trigger NF-κB signaling, but with different dynamics, and different NF-κB activity dynamics are followed by the activation of distinct transcriptional target programs in the RAW 264.7 cell line and fibroblasts (Hoffmann et al., 2002; Lane et al., 2017; Werner et al., 2005). Even if all cells exhibited the same response dynamics, but in an asynchronous way, time course quantification of population averages would lead to wrong conclusions (Fig. 2).

Also, cellular responses are typically dependent on their location within a tissue or a culture dish (Battich et al., 2015), thus requiring analysis technologies to also provide that spatial information (Fig. 2). The generation and effects of spatiotemporally layered patterns of signaling activation and its coordinated propagation through 2D cultures and 3D tissues are even more complex, fascinating, crucial for normal biology, and ill-understood. The best in vivo examples come from embryology in simpler model organisms as well as vertebrates and mammals (Eldar et al., 2002; Golan et al., 2018; Hashimura et al., 2019; Mongera et al., 2019; Pohl and Bao, 2010; Schier, 2001; Chen and Schier, 2001).

In vitro assays, while providing better cell accessibility, throughput, and well-defined chemical conditions, only partly reflect conditions in vivo, and adjacent niche cells as well as surrounding factors typically differ substantially between the two (Juno et al., 2019). Therefore, in vitro findings have to be validated by in vivo analyses of cells in their native 3D environments. However, in vivo analyses are extremely complex, with many unknown and usually confounding influences on cells. Cell and molecular systems in vivo will be redundant and quickly adapt to, e.g., chemical or genetic manipulation, making
it almost impossible to analyze the precise immediate effect of a defined manipulation. The combination of simpler and chemically defined in vitro assays with in vivo studies will therefore often be required.

In vivo analysis is especially difficult for the hematopoietic system, where most tissues are (semi)liquid, are constantly mixing, and lack the obvious compartmentalized and stratified 3D organization of other tissues. The location and morphology of a cell in its tissue can reliably predict its type, environment, and signaling inputs and responses in many solid tissues (Barker et al., 2007; Quiroz et al., 2020; Sato et al., 2011). However, this is not the case, or not yet understood, for most of the highly motile hematopoietic cell types, which also share very similar in vivo morphologies. Indeed, the location of HSPCs in hematopoietic tissues, as well as the location and cellular and molecular composition of their niches, remain disputed in mice and unknown in humans (Acar et al., 2015; Hérault et al., 2017; Kokkaliaris et al., 2020).

Available technologies

**Snapshot analyses of population averages**
Quantitative PCR and RNA sequencing technologies enable detection of signaling target genes, but not of the activity of protein pathway components (Wu et al., 2014; Ziegenhain et al., 2017). While direct proteome analyses can analyze averages of large cell populations (Blagoev et al., 2003), specific signaling proteins are typically detected by antibodies (Abs). Western blotting identified expression and activity of signaling proteins (Janes, 2015; Renart et al., 1979), e.g., by detecting their phosphorylation (Li et al., 2006), and coimmunoprecipitation can detect their molecular interactions (Bonifacino et al., 1999; Laird et al., 2009; Lee, 2007). These approaches provided fundamental insights about signaling pathways (Danial et al., 1995; Zhang et al., 1997). However, while single-cell Western blot technology with limited sensitivity exists (Hughes et al., 2014), these analyses of bulk populations are not suitable for rare cell populations and miss crucial information about individual cells (Fig. 3).

**Snapshot single-cell analyses**
Flow cytometry is a core method for single-cell quantification, with high-throughput multiplexed protein detection. Fluorescent labels allow quantification of intracellular (cells are killed in the process; Firaguay and Nunez, 2009) and surface proteins, and sorting of living cells (Bonner et al., 1972; Herzenberg et al., 1976). Imaging flow cytometry adds useful information on cell morphology and subcellular molecule location (Doan et al., 2018; McGrath et al., 2008). However, fluorescent labels have limited multiplexing potential due to their spectral overlaps. Mass cytometry (cytometry by time of flight [CyTOF]) improves multiplexing by labeling Abs with heavy metal ions, distinguished by mass spectrometry with very high precision (Bandura et al., 2009; Forthun et al., 2019; Yao et al., 2014). CyTOF was used, for instance, to comprehensively quantify signaling behavior in different normal bone marrow HSPCs (Bendall et al., 2011), or to detect anti-tumor immune responses (Dempsey, 2017). However, CyTOF destroys cells and is thus blind to possible links between the cells’ current molecular state and future behavior.

Labeling Abs by DNA sequences and their detection by high-throughput sequencing increases multiplexing and throughput potential even more (Peterson et al., 2017; Stoeckius et al., 2017; Todorovic, 2017). These approaches have the potential to replace most of the current flow cytometry experiments for snapshot cell analysis, but not for live-cell sorting, since cells get destroyed in the process. All these analyses of dissociated cells lose the information on cells’ locations in their tissue or culture, which is crucial to understand signaling regulation and cell-cell communication (Fig. 3).

**Spatial single-cell analysis**
Imaging provides this spatial information including molecules’ subcellular location. Fluorescent Abs are the most used label for imaging signaling components (Coons et al., 1942; Coons and Kaplan, 1950). For HSPCs, much remains to be done for the in situ quantification of their locations and signaling activities induced by their microenvironments in vivo. Despite numerous publications, the exact location of HSCs and their niches in mouse bone marrow remains disputed (Acar et al., 2015; Boulaïs and Frenette, 2015; Kokkaliaris et al., 2020; Méndez-Ferrer et al., 2010; Morrison and Scadden, 2014). The locations and niches of other HSPC types have not yet been well analyzed in mice, and almost no studies exist for human hematopoietic organs. This is mostly due to lacking technology. 3D imaging usually only allows a very limited number of fluorescent channels to be distinguished. Together with the lack of single markers for the identification of specific HSPC types—combinations of several markers are usually required for their reliable identification—this prevents the simultaneous identification of specific cell types, their microenvironment, and signaling components required for understanding the spatiotemporal distributions of signaling activities in specific HSPCs in vivo. In addition, due to the acquisition time requirements on expensive machinery and the huge amounts of data resulting from imaging approaches, only small spatial volumes are typically imaged, limiting the required sample numbers for representative data acquisition and statistically sound conclusions. Recently, imaging approaches for large 3D volumes, such as whole mouse bones with up to 10 simultaneous fluorescent channels, and custom software pipelines for the quantitative analysis of the resulting huge data volumes have been developed (Coutu et al., 2017, 2018; Nombela-Arrieta et al., 2013). This indeed demonstrated that previous conclusions about HSC location bone marrow have to be taken with caution (Kokkaliaris et al., 2020). Recently, the quantification of 3D distributions of secreted regulatory proteins in large tissue volumes with even single-molecule sensitivity was developed (Kunz and Schroeder, 2019), based on proximity ligation assay amplification of fluorescent signals (Soderberg et al., 2006). This will now also allow quantifying where signaling ligands actually locate in hematopoietic tissues, greatly improving our understanding of the spatiotemporal concentrations and availability of these signals to HSPCs in their native in vivo environments.

Cyclic immunostainings with several rounds of Ab exposure increase multiplexing (Gut et al., 2018; Lin et al., 2015), and limited fluorescence multiplexing potential can be overcome by
mass cytometry detection of metal-labeled ABs. Stepwise scanning of tissue sections by sequential laser ablation of AB-stained tissue voxels with subsequent mass spectrometry and computational image reconstruction can generate 2D images with highly multiplexed protein quantification per pixel (Giesen et al., 2014). This approach was used to map the immune system changes in type 1 diabetes (Wang et al., 2019) and to phenotype breast cancer (Ali et al., 2020), and is continuously improving in scanning speed, multiplexing ability, and possible 3D imaging (Bodenmiller, 2016; Lun and Bodenmiller, 2020). For the detection of mRNAs, single-molecule RNA fluorescence in situ hybridization imaging (Raj et al., 2008) and spatial transcriptomics (Ståhl et al., 2016) provide spatial information with subcellular resolution. Cyclic fluorescence in situ hybridization staining and imaging enable very high multiplexing and allow absolute quantification of mRNA molecule numbers per cell (Lubeck and Cai, 2012; Xia et al., 2019). Spatial transcriptomics physically barcode 3D tissue positions with subcellular resolution before dissociation and later match these barcodes to single-cell sequencing results (Vickovic et al., 2019). This approach has enabled the identification of cells’ previous tissue location and helped to identify a plaque-induced gene network in Alzheimer’s disease (Chen et al., 2020). All of these technologies provide snapshot observations at single time points. However, for quantification of the important dynamic behavior of signaling networks, continuous analyses are required (Fig. 3).

Continuous long-term single live-cell quantification

Patch clamping (Neher and Sakmann, 1992) or impedance sensing (Xu et al., 2016) can measure cellular electrical properties over time and are therefore able to detect concentrations of second messengers like Ca2+ in real time (Franks et al., 2005; Frey et al., 2010). However, fluorescent live-cell imaging is the most abundantly used technology for continuous single live-cell analysis, even for up to weeks (Kokkaliaris et al., 2016; Schroeder, 2011). Genetically encoded biosensors can be used to quantify even very fast signaling activity dynamics. They typically report signaling activity either by changing fluorescence resonance energy transfer (Fritz et al., 2013), leading to a spectral change of signaling reporter emission (Krause et al., 2013; Tuleuova et al., 2010), or by changing the subcellular location of the biosensor. Fluorescence resonance energy transfer sensors can also be modified to detect the abundance of small molecule secondary signaling messengers, like cyclic di-GMP, and therefore expand the detection limit beyond proteins (Anderson et al., 2020; Dippel et al., 2018). Translocation-based reporters often have a higher dynamic range and require only one fluorescent channel, thus improving multiplexing with other reporters. They are usually generated by adding a fluorescence tag to a key signaling protein, thus changing its intracellular location upon pathway activation (e.g., NF-κB [De Lorenzi et al., 2009], AKT [Gross and Rotwein, 2015], and STAT3 [Herrmann et al., 2004; Watanabe et al., 2004]). They have provided important insights into the dynamical encoding of signal information. For example, a GFP-p65 reporter showed that different NF-κB dynamics correlate with the expression of specific target gene programs (Lane et al., 2017) and that NF-κB signaling can be entrained in cell populations, which are also regulated by noise (Heltberg et al., 2016). An AKT translocation sensor was employed to screen for the effect of growth factors on signaling dynamics (Gross and Rotwein, 2016). A new generation of translocation biosensors is based on a fusion between fluorescent proteins and a protein fragment containing nuclear export and localization signals that change activity upon phosphorylation by kinases of signaling pathways. This approach can be applied to report the activity of essentially any kinase and thus many signaling pathways by adjusting the kinase docking site in the protein complex (Regot et al., 2014).

Figure 3. Technologies for analyzing signaling at different levels of resolution. Technologies often used for analysis of signaling, categorized by their capacity for providing single-cell, temporal, and spatial information. Continuous single-cell technologies yield most relevant information but are usually lower in throughput and/or dimensionality than other methods. Blue text boxes labels technologies that additionally retain spatial information. q-rTPCR, quantitative RT-PCR; RNA-seq, RNA sequencing; scRNAseq, single-cell RNA sequencing; scWesternBlot, single-cell Western Blot.
Importantly, the relevance of signaling dynamics for controlling cell fates can only be evaluated if the future fates of the same cells are known. Only if specific signaling outputs reliably lead to specific future fates of the same cell or its progeny can their role in inducing this fate be demonstrated. However, due to the slow implementation of fate programs, especially in mammals, it can take up to days or weeks until the lineage choice of differentiating HSCs can be detected (Hoppe et al., 2016; Kokkaliaris et al., 2016; Strasser et al., 2018). The same cells (and all of their progeny) in which signaling had been quantified at the start of the experiments have to be tracked without losing their identity over these long periods of time. This is technically much more demanding than the relatively short-term live-cell imaging for the identification of signaling activity or dynamics, which only has to span minutes to hours (Endele et al., 2014; Endele and Schroeder, 2012; Schroeder, 2005, 2011). Long-term single-cell imaging and fate quantification of mammalian HSPCs has been accomplished in vitro, for instance demonstrating the production of blood cells from hemogenic endothelium (Eilken et al., 2009), the lineage instruction of hematopoietic progenitor cells by inflammatory cytokine signaling (Endele et al., 2017; Rieger et al., 2009), the asymmetric cell division of HSCs (Loeffler et al., 2019), or the direct regulation of the core hematopoietic transcription factor PU.1 by TNF and IL-1 in HSCs (Etzrodt et al., 2019; Pietras et al., 2016). This approach also enables the identification and quantification of signaling dynamics in HSPCs (Endele et al., 2014; Wang et al., 2021).

Combining protein analyses for detecting signaling activity with single-cell high-dimensional snapshot endpoint analysis methods like transcriptome sequencing (Wu et al., 2014; Ziegenhain et al., 2017) enables the comprehensive detection of molecular regulators and target programs of signaling pathways. It was used to show that specific NF-κB dynamics correlate with target gene expression (Lane et al., 2017). Detection of signaling differences even between closely related cells can further be used to distinguish cellular subpopulations that are not distinguishable by flow cytometry or other assays. In addition, it could contribute important insights to the ongoing discussion as to whether hematopoietic differentiation is a discrete process (with fast changes in relevant cell states) or a continuous one (with slow changes across the transcriptome and variable [de]activation sequences for individual genes), as suggested by recent transcriptome sequencing studies (Laurenti and Goettgens, 2018; Liggett and Sankaran, 2020).

While long-term single-cell imaging is possible in vitro, it remains even more demanding in vivo. In addition to the more difficult access to cells deeper in tissues, the required immobilization of animals or even patients typically prevents experiments longer than a day. Live HSPC in vivo imaging is possible (Lo Celso et al., 2009), but with much more limited duration and throughput, and signaling activity and links to future cell fates have not yet been analyzed. Due to its easier accessibility at the outside of the organism, single-cell imaging of skin (Rompolas et al., 2012; Sun et al., 2017) enables insights into the changing
signaling activities of differentiating primary skin stem and progenitor cells in vivo (Quiroz et al., 2020).

**Manipulation of cellular signaling**

For a full understanding of signal transduction mechanisms, it is usually not enough to only observe. Instead, defined perturbations are necessary to reveal signaling properties that are otherwise hidden. Classic transgenic manipulations for knockdown, knockout, and overexpression of pathway components in animal models provide important insights (Gao et al., 2019; Yamamoto et al., 2003). In humans, insights into signaling regulation and relevance came from “natural” pathway perturbations, due to disease-causing germline or somatic mutations that alter signaling (Katoh, 2007; Penick et al., 2016; Zhao et al., 2017). Signaling pathways can simply be stimulated with their ligands in vitro and, with much less control over concentrations, kinetics, and combinations, in vivo. In addition, pharmacologic activation or inhibition by small molecules, peptides, or ABs is an extremely successful addition, both for research and for clinical therapy (Fig. 4; Carey et al., 2017; Killadjian et al., 2016; Nelson et al., 2004; Purvis et al., 2012). Technologies for efficient, automated, and time-controlled (micro)fluidic handling can be required here to enable automated high-throughput manipulation of media compositions, in particular with defined fast changes over time. For NF-κB, it was shown that the pathway entrains to external periodic TNF-α stimulations, which were achieved with microfluidic cell culture devices (Heltberg et al., 2016; Kellogg and Tay, 2015). However, most microfluidic devices are not suitable for use with rare non-adherent cells like primary HSPCs, which require efficient cell capture and flow-free media changes with sufficient speed (Dettlinger et al., 2018, 2020). Optogenetic approaches are a very exciting approach allowing the experimental switching between signaling activity states by light exposure with single-cell resolution and extremely fast kinetics (Boyden et al., 2005; Bugaj et al., 2013). It has been used, e.g., to precisely control the activity of ERK and AKT pathways by light exposure (Johnson and Toettcher, 2019; Katsura et al., 2015), and will allow many important insights in the future.

**Data analysis**

While it cannot be covered in more depth here, it is important to point out that the analysis of the data generated by these technologies, in particular by high-throughput and time-resolved single-cell approaches, is critically dependent on computational data analysis, modeling, and curation (Hilsenbeck et al., 2016; Skylaki et al., 2016). This, and not the data acquisition, often is the relevant bottleneck for comprehensive insights, and the required software is typically not readily available. Researchers must therefore be able to develop or adjust the required software to their needs.

**Concluding remarks**

Cellular signaling is central for controlling cell fates. While we already understand a lot, and targeted manipulation of signaling is successfully used even for clinical therapy, much more remains to be analyzed and understood. Recent technological developments, in particular for single-cell analyses and dynamics quantification and manipulation, now enable improved insights into the regulation and function of cellular signaling in controlling cell fates. While often developed in easy-to-use model systems, they are increasingly applied to also analyze primary cells including HSPCs and will lead to important novel insights and the development of improved targeted therapeutic manipulations.

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