From cell to cell: Identification of actionable targets in bone marrow fibrosis using single-cell technologies

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Single-cell technologies have rapidly developed in recent years and have already had a significant impact on the research of myeloproliferative neoplasms. The increasing number of publicly available data sets allows characterization of the bone marrow niche in patients and mouse models at unprecedented resolution. Single-cell RNA sequencing has successfully been used to identify and characterize disease-driving cell populations and to identify the alarmin S100A8/A9 as an important mediator of myelofibrosis and potent therapeutic target. It is now possible to execute a streamlined set of experiments to specifically identify and validate actionable target genes functionally with the advance of reliable \textit{in vivo} models and the possibility of conducting single-cell analyses with a minimal amount of patient material. The advent of large-scale analyses of both hematopoietic and non-hematopoietic bone marrow cells will allow comprehensive network analyses guiding an increasingly detailed mapping of the MPN interactome. © 2021 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

**HIGHLIGHTS**

- Integration of murine and human single-cell data sets allows validation of disease models of MPN.
- Single-cell transcriptomics of mouse models and patients allows streamlined identification of druggable targets.
- Network analyses of single-cell data sets capture druggable interactions between hematopoietic and stromal cells.

**THE UNMET CLINICAL NEED IN THE TREATMENT OF BONE MARROW FIBROSIS IN MYELOPROLIFERATIVE NEOPLASMS**

BCR-ABL1-negative myeloproliferative neoplasms are a disease of the hematopoietic system with recurrent driver mutations in the JAK-STAT pathway: point mutations in the genes encoding Janus kinase 2 (JAK2; JAK2V617F), the thrombopoietin receptor (myeloproliferative leukemia protein, MPL; MPLW515L/K), or frameshift mutations in the calreticulin gene (CALR) [11]. The resulting constitutive activation of the JAK-STAT pathway leads to a complex expansion of myeloid cells in the hematopoietic system. Myeloproliferative neoplasm (MPN) hematopoietic stem and progenitor cells (HSPCs), as well as their mature progeny, can contribute individually to disease manifestation and well-known complications such as thrombosis [2–4]. They also add to the risk of fibrotic progression or leukemic transformation. Philadelphia-negative MPNs can follow a biphasic course: from a phase characterized by excess production of mature blood cells (myeloproliferation), they can progress to bone marrow (BM) fibrosis (myelofibrosis [MF]) resulting in hematopoietic insufficiency. While in the myeloproliferative phase, the median survival in patients with polycythemia vera (PV) and ET is 15 years, and dramatically drops once the disease progresses to MF. Patients living with MPNs, and MF in particular, frequently suffer from impeding constitutional symptoms, which significantly diminish their quality of life (QoL). Two key challenges still complicate current disease management of MPNs: (1) so far no biomarker has been identified that reliably predicts the progression from the myeloproliferative to the fibrotic phase, which is considered the turning point of disease control and is thus pivotal for individual treatment decisions [5]. Clinically based prognostic scoring systems, such as the dynamic international prognostic scoring system (DIPSS) among others, have recently been evolved into the genetically inspired prognostic scoring system (GIPSS) [6,7]. These scoring systems usually focus on leukemia-free survival or overall survival, however, and do not account for the onset of BM fibrosis as a turning point in the disease trajectory. Such a biomarker is urgently needed to continuously monitor the disease course and for clinical decision making. (2) No specific antifibrotic therapies are available [8]. Currently, the only potentially curative option remains an allogeneic stem cell transplantation (ASCT). However, because of age, debilitating disease symptoms, comorbidities, and availability of a suitable donor, the majority of patients are not eligible for this challenging procedure. So far, all approved therapeutic strategies focus on...
restraining the malignant hematopoietic clone but not on mechanisms related to the fibrosis-driving cells. In this review, we therefore focus on how the application of single-cell techniques to hematopoietic and nonhematopoietic cells in MPNs can guide the data-driven identification of druggable targets in the profibrotic BM niche.

SINGLE-CELL RNA SEQUENCING OF THE BM STROMA

Single-cell techniques and transcriptomics have radically changed our view of cellular identity and function [9]. The new and growing body of single-cell technologies has granted us insight into the clonal dynamics of MPN biology at an unprecedented level. Improved techniques, such as TARGET-Seq and genotyping of transcriptomes, now also allow reliable genotyping of the captured single cells to definitely distinguish malignant MPN progeny from (atypical) bystander hematopoietic cells and to unravel heterogeneity within the population of MPN cells itself [10,11]. A comprehensive overview of the accomplishments and potential future applications of single-cell approaches to the hematopoietic compartment in MPNs has recently been published by Royston et al. [12] and will thus not be discussed in more detail here.

Because of the importance of the microenvironment for the homeostasis and differentiation of hematopoietic cells, various research groups set out to map the composition of the nonhematopoietic elements in the BM. Baryawno et al. [13] isolated nonhematopoietic cells from whole BM and subsequently subjected them to 3’-scRNA-seq to reveal the complexity of the BM stroma. Tikhonova et al. [14] used reporter strains to enrich niche populations with the goal of generating a more detailed map of BM endothelial cells, osteoblasts, and mesenchymal stromal cells. These general findings are now increasingly being complemented by specific analyses of specific subniches and populations of the BM. For example, Yoshioka et al. [15] characterized BM osteolinesage cells in more detail by combining single-cell RNA sequencing and genetic fate tracing. Their analysis revealed the presence of four different osteoblastic subpopulations, one of which still retained strong features of mesenchymal precursors [15]. The possibility of mapping the BM stroma at an unprecedented resolution now allows the application of single-cell technologies to dissect the machinery of fibrosis-driving cells in MPNs at the single-cell level.

EMPLOYING MURINE MODELS AND PATIENT MATERIAL TO DISSECT THE SEQUENCE OF FIBROTIC TRANSFORMATION AT THE SINGLE-CELL LEVEL

Because myelofibrosis is associated with poor survival in patients with MPN and the most relevant risk factor for debilitating morbidity and leukemic transformation, we sought to identify the fibrosis-driving cells in the heterogeneous cell populations that constitute the hematopoietic niche of the BM in murine MPN models and patient samples [16]. A plethora of different mouse models have been developed to experimentally study MPN in vivo, each with a different severity of myelofibrosis, specific disease kinetics, and penetration [17]. All driver mutations of MPNs result in a constitutive activation of the JAK2 pathway. In particular the activation of this signal downstream of the thrombopoietin receptor, this model has been extensively used as proof of concept in mouse experiments of MPN. This very rapid phenotype, however, does not entirely reflect the phenotype observed in patients, in whom MF often progresses over the course of several years.

Transplanting HSPCs transduced with Jak2V617F into lethally irradiated mice induces an MF phenotype that exhibits slower disease kinetics. Mice develop progressive myelofibrosis over the course of approximately 20 weeks [21,22]. This model carries the exact mutation that is found in most patients with MF, while at the same time following the same experimental procedure as in the ThPO model, thereby making the two as comparable as possible. The Jak2 model induces a PV-like phenotype and at the same time thrombocytosis with increased frequency of the pathognomonic clustering, hyperlobulated megakaryocytes in atypical locations, reminiscent of ET.

Comparative single-cell transcriptomics of the hematopoietic niche revealed that ThPO-induced MF closely recapitulates Jak2V617F-induced MF and human PMF. Thus, we now have two distinct, validated models at our disposal: (1) ThPO-induced myelofibrosis with rapid progression to MF of all affected mice, allowing us to capture states of prefibrosis and manifest fibrosis [20]; (2) Jak2V617F-induced MF harboring the most frequent driver mutation of MPN exhibiting slower disease kinetics [22]. It will be crucial in the near future to extend these kinds of single-cell studies to other models of MPN and myelofibrosis, especially to those capturing mutations in the Mpl and Calr genes [23,24]. This will provide further validation of these models and enable us to unravel the disease heterogeneity conveyed by different driver mutations with new accuracy. However, both models do not yet entirely replicate the human disease phenotype. A better understanding of changes in BM of patients with MPN/MF might guide the generation of novel murine models. Obtaining primary material from patients is usually a challenge because of logistics and ethical considerations [25]. In the case of myelofibrosis, BM biopsies frequently result in a dry tap, where no BM can be aspirated because of extensive medullary scarring. This makes the already limited amount of material obtainable for single-cell studies even scarcer.

It has proven feasible to perform single-cell transcriptomics on minimal amounts of patient-derived biospecimens [26]. This makes it possible to conduct single-cell analyses without the need to obtain large amounts of additional material and, at the same time, without impeding routine diagnostics. Single-cell studies in BM of patients with PMF exhibited remarkable overlap with data from ThPO- and Jak2V617F-induced MF, thus further validating the power of these models [27]. So far, however, the sample sizes for human specimens have been limited. It will be vital to increase the number of available single-cell data sets in the future to achieve the same resolution as in the murine setting.

Importantly, single-cell studies of the BM niche in ThPO- and Jak2V617F-induced models of MF uncovered the very same stromal populations as those of human patients with PMF. In both, human and murine MF, mesenchymal stem cells (MSCs) were identified as the fibrosis-generating cell population in the BM stroma. The well-characterized ThPO models with distinct kinetics allow study of MF in the prefibrosis and manifest fibrosis phases. Transcriptome-based pathway analysis at both disease stages revealed a clear sequence of signals converging on fibrosis-driving MSCs. The prefibrotic stage is
governed primarily by extensive and heterogeneous inflammatory signals. In contrast, the signature of MSCs in manifest fibrosis is restricted to transforming growth factor (TGF)-β-related signals. The stroma is also increasingly recognized as an important therapeutic target in BM fibrosis, and our own previous data indicated that targeting alterations in the stroma during fibrotic transformation can ameliorate BM fibrosis [27]. Recent data on the single-cell level in MPN indicate three main approaches to affecting the main players in MPN, which could also be combined:

1. Target the MPN clone and its progeny as disease initiating cells.
2. Target stromal populations and prevent their fibrotic differentiation into extracellular matrix (ECM)-producing cells.
3. Intercept specific interactions between the hematopoietic clone and profibrotic stromal MSCs.

DETANGLING THE FATE OF FIBROSIS-GENERATING CELLS IN BM FIBROSIS IN MPN

Trajectory analysis is a powerful technique for ordering cells in a pseudotemporal space, allowing the characterization of cell differentiation and the detection of genes driving this process [28–30]. Trajectory methods usually work by finding a low-dimension representation of the cells, that is, diffusion techniques [31], followed by graph methods to find differentiation directions in this representation. These allow visual exploration, interpretation, and subsequent branch-specific differential gene expression analysis [28,29,31]. More recently, the methods have been integrated with RNA velocity analysis to indicate the differentiation direction by exploring the ratio of unspliced and spliced mRNA transcripts [30].

In our studies we could observe how MSCs experience a loss of the MSC-specific transcriptional signature, which is associated with trilineage differentiation and hematopoiesis support. At the same time, adipogenic and osteogenic MSCs exhibited a strong propensity to differentiate into osteolineage cells in MF, thereby contributing to osteosclerosis. A similar differentiation trajectory from MSC populations to osteogenic cells could also be verified employing other tools inferring differentiation capacities from single-cell data in the stromal compartment of healthy individuals [32]. Moreover, the differentiation trajectory also implied the possibility that MSCs could undergo transdifferentiation into Schwann cell progenitors [27]. The capacity of MSCs to undergo such a differentiation has been implied for a long time and underlines the neuroectodermal origin of a subset of MSCs and would not have been possible to detect by bulk sequencing approaches [33]. The exact origin and clinical implications of this finding will have to be explored and validated in future studies. Thus, aiming for analyses capturing the BM at single-cell resolution enables researchers to trace even rare, but disease- and treatment-relevant cell trajectories in MPN and MF.

TAKING THE FIBROSIS-INITIATING MPN CLONE INTO THE EQUATION

The discovery of driver mutations in malignant hematopoietic cells in MPN highlighted JAK-STAT signaling as an initiator of MF, supporting a strong rationale for pharmacologic JAK2 inhibition and resulting in more than one billion dollars in revenue. However, JAK inhibition alone seems insufficient to induce long-term remissions and has only a modest effect on BM fibrosis as the foremost life-limiting factor [34,35]. Alternative strategies focused on the hematopoietic compartment include immunotherapy (e.g., interferon α), epigenetic regulators (e.g., hypomethylating agents), and therapies affecting HSC maintenance (e.g., telomerase inhibitors) [8]. All of these, however, had poor response rates with respect to BM fibrosis. The only therapeutic approach focusing on fibrosis directly is the inhibition of TGF-β signals. Clinical studies in solid organ fibrosis have dampened the excitement over TGF inhibition as TGF signaling is also pivotal for various homeostatic functions, so that TGF inhibition is associated with severely adverse effects [36].

A large number of trials focused on compounds interfering with TGF-B1, among which megakaryocytes and megakaryocyte progenitors are the main source in the BM [37]. Megakaryocytes play a dual role in the pathogenesis of MPN. On the one hand, as hematopoietic cells, they are derived largely from the MPN clone. The megakaryocytes in PMF are often clustered together, hypolobulated, and they exhibit hypoploidy compared with their physiologically mature counterparts [38,39]. They have also been described as the sources of a plethora of fibrotic molecules including platelet-derived growth factors A and B (PDGF-A, PDGF-B) and TGF-B1 [40–42].

Single-cell transcriptomics have now been employed to elucidate the lineage commitment of megakaryocytes in patients [43]. Moreover, a single cell multiomics approach successfully dissected the megakaryocytically skewed hematopoiesis in PMF. Psaila et al. [44] identified the presence of nine different megakaryocyte progenitor subclusters in the BM of JAK2V617F- and CALRmut-associated myelofibrosis, all of which expressed profibrotic mediators such as TGFβ1 to varying degrees. On the basis of their data, they also managed to extract G6B as a distinguishing surface marker on JAK2V617F hematopoietic cells and, thus, a putative therapeutic target.

It has also been reported that monocyte-derived fibrocytes are expanded in the BM of PMF patients and MPLW515L mice. This collagen I-expressing cell population can originate from the neoplastic MPN stem cell and contribute to ECM production. Inhibiting the differentiation of monocytes into fibrocytes with pentraxin-2 prolongs the survival of mice in a xenograft model of myelofibrosis [45]. PRM-151, a recombinant pentraxin-2, has been reported to be well tolerated and to exhibit antifibrotic properties in a phase 2 trial [46]. The reductions in reticulin fibrosis observed were relatively mild after a duration of approximately 31 months, indicating that isolated inhibition of fibrocytes alone is insufficient to effectively treat manifest and extensive myelofibrosis. This population has not yet been identified or characterized on a single-cell level. Integrating single-cell data of fibrocytes into the profibrotic network will provide valuable insight into their role and position in the development and treatment of myelofibrosis.

In the context of MPN and myelofibrosis, megakaryocytes exhibit intricate contact and interactions with MSCs [47,48]. Most importantly, these MSCs have been identified as major driver cells of MF, a feature that is defined by excess deposition of fiber material in the extracellular space and is associated with an abysmal outcome for affected patients [49]. In particular, MSCs have been identified as the nonmutated fibrosis-driving cell population [47,50]. MSCs, however, have proven difficult to precisely define functionally because of their pluripotent nature and potential transition states. The exact surface marker composition for flow cytometry, for example, remains a matter of active research and debate [51]. Reliably isolating the exact cell
type responsible for the production of detrimental fibrotic material has important clinical implications, because healthy MSCs provide essential signals on which the already impaired physiological hematopoietic cells in the myelofibrotic BM depend [52]. In the sense of an ideal targeted therapy for MF it is pivotal to inhibit the profibrotic MSCs while leaving unaltered MSCs intact, thus creating a condition in which normal hematopoesis can expand and repopulate the BM.

With the power of novel single-cell tools, first and foremost single-cell RNA sequencing, the composition of the BM microenvironment has been characterized at an unprecedented resolution. It is now possible to distinguish functional subpopulations of these cells that constitute only about 0.5% of whole BM cells. Several single-cell studies of murine BM in homeostasis, as well as in the setting of myelofibrosis, revealed the presence of four distinct subpopulations in the MSC compartment [13,14,27]. Of these, only two, namely adipogenic and osteogenic-primed MSCs, actually acquire profibrotic properties in various models of murine MF and have to be considered the major targets of interest [27]. Single-cell RNA sequencing also allowed alignment of these two cell populations with other, broader MSC populations and provided clear evidence that the fibrosis-generating cells are a subpopulation of Gli1+ stromal cells, which have been described before to contribute to ECM production in MF [47].

**NETWORK ANALYSES OF SINGLE-CELL DATA CAPTURE THE COMPLEXITY OF INTERCELLULAR SIGNALS**

Novel bioinformatics algorithms have already successfully been used on single-cell data to infer ligand–receptor interactions between cell populations based on their expression profile [53]. Moreover, they can be now capture changes in the complex signaling network between populations of single cells, thus providing a more comprehensive picture of receptors, co-receptors, and endogenous agonists/antagonists involved [54]. Not only was it possible to thereby confirm the importance of an overactive TGFBR1–TGFBR3 axis between megakaryocytic cells and MSCs in PMF. They also provided evidence of a reduced antagonism by TGFBR3 and a signal modulation via the respective area in each section. It is a powerful addition to the network analyses described above as it allows integration of spatial distribution and physical interaction between cells. However, despite an ever increasing resolution, this approach does not allow transcriptomically informative tissue slides coated with spatially bar-coded immobilized oligonucleotides [59]. Fixed tissue slides are permeabilized, so intracellular mRNA can hybridize to the exposed poly(dT) residues. Reverse transcription takes place before removal of the tissue. Thus, the barcode carried by each cDNA molecule is determined by the spatial localization within the tissue. This method allows acquisition of morphologic or immunofluorescent images before RNA sequencing and, therefore, the association of transcripts with the respective area in each section. It is a powerful addition to the network analyses described above as it allows integration of spatial distribution and physical interaction between cells. However, despite an ever increasing resolution, this approach does not allow transcriptomic analyses at the level of the single cell yet. Additionally, BM is a particularly difficult material to work with and usually requires decalcification. Therefore, the eligibility of these methods for the study of the BM and the hematopoietic niche remains to be validated.

In contrast, Giladi et al. [60] recently presented a model to transcriptionally profile physically interacting cells. Here, the prior sorting and single-cell RNA sequencing of separate populations allows development of a background model of gene expression. This can be used to deconvolute and analyze the transcriptional features of directly interacting cells, purified by fluorescence-activated cell sorting (FACS). This powerful tool immediately verifies spatial proximity of the analyzed cells. A requirement that remains, however, is the availability of validated distinguishing surface markers to ensure clean

**Figure 1** Overview of cell populations and main mediators in the pathogenesis of myelofibrosis. Pharmacologic inhibitors of relevant pathways or interactions are highlighted in blue.
populations for the generation of valid background models. For many elements of the hematopoietic niche and its constituting cellular sub-populations, these are unfortunately still missing at this point.

SINGLE-CELL TRANSCRIPTOMICS DETECT ACTIONABLE DRUG TARGETS

Single-cell RNA sequencing of murine and human myelofibrotic BM revealed that profibrotic MSCs start expressing the alarmin S100A8/A9. Under homeostatic conditions, the stromal compartment does not exhibit this expression, indicating that MSCs themselves contribute to the proinflammatory milieu during fibrotic transformation. As a disease-specific alteration, this has to be considered a highly attractive therapeutic target. The S100A8/A9 heterocomplex exerts its effect on target cells via the toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE)[61]. The interaction between S100A8/A9 and its receptors can be competitively inhibited by the quinolone-3-carboxamide derivative tasquinimod [62].

Treating mice transplanted with Jak2\textsuperscript{V617F}-overexpressing HSPCs with tasquinimod leads to a significant reduction of the MPN phenotype and to an effective reduction of MF severity [27]. Thus, findings from a sequence of rigorous single-cell experiments and analyses in mice and patients could successfully be translated into a data-driven promising treatment option to tackle myelofibrosis. The TLR axis has been described to be involved in the development of MF before. Fibronectin isoforms can stimulate TLR4 and thus contribute to progenitor cell proliferation and dysmegakaryopoiesis. Intercepting this interaction also resulted in a striking reduction in MF severity in ThPO-induced myelofibrosis. These findings highlight the central role of TLR signaling mediated by danger-associated molecular patterns, but also the complexity of heterogeneous signals in the pathogenesis of MF[63].

Despite the fact that receptor interactions for S100A8/A9 and fibronectin have been described in the literature, these interactions are not yet fully represented in the curated databases of current ligand−receptor tools. It will therefore be important to conduct further research to dissect the exact mechanism by which TLR signaling

Figure 2 Overview over a streamlined translational process of single-cell analyses to identify and validate drug targets in MPN and MF. Future publicly available data sets—both murine and human—can be integrated as indicated on the left and the right. The most important steps still missing are highlighted in red.
can induce MF and, similarly, which effects of tasquinimod can ameliorate fibrotic transformation. These results can ultimately be incorporated into hallmark protein–protein interaction databases to allow more thorough network analyses in the future.

CONCLUSIONS AND TRANSLATIONAL OUTLOOK

With all models and techniques described at hand, we now have a unique translational framework: It is possible to continuously validate experimental findings from mouse models in human data sets and vice versa. On every level of single-cell analysis it is possible to correlate the findings between model and patient to extract actionable candidate targets for pharmacologic treatment. These can subsequently be tested and validated in vitro and in vivo. Employing this strategy, we were able to identify the S100A8/A9 heterocomplex as a major driver of myelofibrosis that can be targeted by treatment with tasquinimod to effectively reduce MF severity in vivo [27]. Tasquinimod has to be considered the most effective way to inhibit S100A8/A9 signaling in vivo at the moment. However, it is not a highly specific agent in this regard [64]. For instance, in vitro observations have indicated that tasquinimod also acts as an inhibitor of histone deacetylases and thereby influences the transcriptome as well [65,66]. It will be important to query these modes of action separately. Gathering transcriptional data on a single-cell level here under different biological conditions can aid in unraveling the various effects and consequences of tasquinimod treatment individually. This, in turn, can guide us in the development of novel and more specific drugs. In the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test the future, it will be essential to extend this data-driven experimental approach to translate the findings into the clinical research and test

REFERENCES


