The hematopoietic stem cells supportive megakaryocytes as shapers of the bone marrow niche

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ABSTRACT

The bone marrow (BM) niche plays a pivotal role in regulating the fate of hematopoietic stem cells (HSCs), and its integrity changes significantly during aging and in rare hematological disease, as in myelofibrosis (MF). In this study, we investigated how the localization and dynamics of HSCs are influenced under physiological and pathological conditions by a newly identified by HSC-supportive megakaryocytes (MKs) subpopulation. Using huCD34tTA/TetO-H2BGFP reporter mice, we analyzed HSCs distribution within the BM and quantified nuclear green fluorescent protein (GFP) intensity to assess the repopulating potential of aged controls and mutated Gata1^{low} mice for MF. In the control group of aged mice, cells with high levels of GFP are clustered, and adjacent to cells morphologically identifiable as supportive MKs. These clusters displayed homogeneous GFP intensity, indicating that HSCs with similar functional properties tend to co-localize in proximity to supportive MKs. By contrast, in aged huCD34/TET/Gata1^{low} mice, GFP cells were predominantly isolated and showed reduced fluorescence intensity. Although the frequency of MKs with a supportive phenotype was increased in MF mice, analyses of GFP revealed that the ability of these MKs to maintain the HSCs in their niche was significantly impaired. Our results provide new insights on the maladaptive remodeling of the BM niche. They highlight the supportive role of MKs as potential key regulators of HSCs homeostasis. Despite their numerical expansion in MF, these cells are functionally compromised, thereby contributing to altered HSCs localization, mobilization, and to hematopoietic failure.

Key words: bone marrow; hematopoietic stem cells; megakaryocyte; aging; myelofibrosis; hematopoiesis.

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Introduction

The bone marrow (BM) is a specialized microenvironment that supports hematopoiesis through two main anatomical and functional niches: the endosteal niche, which maintains hematopoietic stem cells (HSCs) in a quiescent state, and the vascular niche, which promotes their proliferation and differentiation.[1,2] Aging profoundly alters the BM, impairing niche function and reducing the regenerative capacity of HSCs. This contributes to hematological malignancies and immune-senescence.[3,4] A key underlying process of these changes is the "inflamm-aging",[5] a chronic low-grade inflammatory state, which affects the BM of aged individuals. Mesenchymal stem cells (MSCs) contribute to this process through the senescence-associated secretory phenotype (SASP). This process is characterized by the release of pro-inflammatory cytokines, reduced clonogenic potential, and an increase in adipogenesis at the expense of osteogenesis.[6] At the molecular level, aging of HSCs is driven by epigenetic, transcriptional, and metabolic alterations, as well as by exposure to pro-inflammatory signals within the aged niche.[7,8] Clonal hematopoiesis of indeterminate potential (CHIP) is a phenomenon closely linked to these changes and is characterized by the expansion of mutated HSC clones. The incidence of CHIP increases with age and represents a risk factor for several hematological cancers.[9-12]

Myelofibrosis (MF) is one of the most severe myeloproliferative neoplasms[13] and provides a paradigmatic example of how niche disruption can drive disease pathogenesis. This rare hematological disorder is characterized by progressive BM fibrosis, cytopenia, mobilization of hematopoietic progenitor cells, extramedullary hematopoiesis, and, in advanced stages, leukemic transformation [14].

Among the key cellular regulators of the HSC niche, megakaryocytes (MKs) play a dual role: a subtype of mature MKs negatively regulate HSC proliferation through the secretion of TGF-β, CXCL4, IGF-1, adhesion molecules, and WNT signaling,[15-18] while distinct transcriptionally defined MK subpopulations- termed HSC-supportive MKs -promote HSC maintenance and quiescence.[19] These supportive MKs display unique molecular signatures, such as high Myosin Light Chain Kinase Family Member 4 (MYLK4) expression and enriched adhesion-related pathways, and are strategically positioned within the BM to directly interact with HSCs.[20] The balance between supportive and non-supportive MKs, together with their spatial distribution, appear to be critical determinants of HSC behavior under steady-state, stress, and pathological conditions. Furthermore, HSC localization influences lineage output: lymphopoiesis occurs predominantly in the endosteal niche, while myelopoiesis, erythropoiesis, and megakaryopoiesis are enriched in central marrow regions near veins and arterioles.[21]

The BM perturbation is a typical feature of MF. The increased trafficking of stem progenitor cells and the ineffective BM hematopoiesis and fibrotic evolution in MF are related to the presence of abnormal MKs,[22] that secrete a number of factors involved in both BM remodeling[23,24] and HSC homeostasis.[15,16]

The cycling of HSCs has already been studied using a user-friendly confocal microscopy method that allows to track the cumulative division history of the HSCs throughout life, tracking can be achieved by analyzing the BM of hCD34tTA/TET-O-H2BGFP transgenic mice, in which only the HSCs are labelled by green fluorescent protein (GFP).[25-27] In this study, we investigated alterations of the HSCs niche during aging, under both physiological and pathological conditions, with particular focus on the role of HSC-supportive MKs in maintaining niche integrity and function, especially in the context of MF.

Materials and Methods

Animal model

We analyzed three aged (15-month-old) double *huCD34tTA/TetO-H2BGFP* mice carrying the *histone H2B* gene fused with GFP under the control of the regulatory sequences of human CD34, which in mice are active only in HSCs[25,27,30] (hereafter referred to as hCD34/TET) and three triple huCD34tTA/TetO-H2BGFP/Gata1^{low} mice (defined hCD34/TET/Gata1^{low}), generated using standard genetic approaches in the animal facility of the Italian National Institute of Health. All the mutations were present in the CD1 background for over 10 generations. Mice were euthanized by cranial cervical dislocation and femurs were collected and fixed in formalin (10% formaldehyde solution) for 24 h. All the experiments included single *TetO-H2BGFP* transgenic mice as control for possible leakage of the expression of the transgene as previously reported.[28] The experiments were performed according to the protocols D9997.121 approved by the Italian Ministry of Health on September 2nd, 2021, and according to the European Directive 86/609/EEC.

Immunofluorescence and image analysis

For BM analysis, femurs were collected from 15-month-old for all the experimental groups. Femurs samples were fixed in 10% formaldehyde solution with a neutral buffer, treated with decalcifying solution EDTA 10% (pH=7.4). Following decalcification, samples were immersed in a sucrose gradient (15% for 3 h and 30% overnight). After decalcification protocol, samples were processed and embedded in paraffin wax. For each experimental group, femurs samples were cut in 3 µm sections. Almost 3 consecutive sections were obtained for each mouse sample.

For immunofluorescent staining, antigen retrieval was performed using citrate buffer (pH=6) at 98°C for 30 min. The samples were stained with anti-CD61, 1:100 titre, ((B-7) mouse to mouse, sc-46655; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) conjugated with Alexa 488, and MYLK4, 1:100 titre, (polyclonal rabbit anti-mouse, 24309-1-AP; Proteintech Group, Inc., Rosemont, IL, USA); and with anti-von-Willebrand factor (vWF), 1:100 titre, (clone [RM1068] rabbit to mouse monoclonal, ab316979; Abcam, Cambridge, UK) for 2 h at room temperature. Control sections were incubated with 0.01M phosphate Buffer solution (PBS), without primary antibody. After 3 washes in 0.1M PBS, primary antibodies were visualized with the appropriate secondary antibody goat anti-rabbit Alexa Fluor 555, 1:200 titre, (cat. no. ab150078; Abcam), for 1h room temperature. Nuclear counterstaining was performed after 3 washes in 0.1M PBS for 10 min, using Hoechst 33342 trihydrochloride trihydrate (Invitrogen, Waltham, MA, USA), 1:1000 titre, for 5 min RT. The samples were then washed in 0.1M PBS, 3 times for 10 min each, and mounted with Fluor-shield histology mounting medium (catalog no. F6182-10MG; Sigma-Aldrich, St. Louis, MO, USA).

The slides were examined using a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to identify the fluorochromes employed. Images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01. The number of megakaryocytes was counted in 10 fields per section, and the mean value was calculated.

The HSC fluorescence intensity was analyzed in 5 µm BM sections after nuclear counterstaining. Images were analyzed with the confocal microscope Zeiss LSM 900 (Carl Zeiss GmbH, Jena, Germany) in the Airyscan mode. Excitation light was obtained by a Laser Dapi (405 nm) for Hoechst, the argon ion laser (488 nm) for GFP and argon ion laser MG 457-514 nm with a spectral detec-

tor for the simultaneous acquisition of 32-channel spectral images for TRICH (555 nm). The optical thickness varied from 0.50 µm for the 20× objective to 0.20 µm for the 63X objective. The images were processed and analyzed with the Zen Blue (3.2) software (Carl Zeiss GmbH, November 2021) and the ImageJ (version 1.53t) software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analyses were performed by using a parametric *t*-test or one-way ANOVA, as appropriate. For the confocal microscopy evaluation of GFP levels, each GFP positive single cell was grouped into classes as previously described.[28] The GFP levels between hCD34/TET and hCD34/TET/Gata1^{low} mice were compared by Student's *t*-test. Differences in GFP values among two mouse groups were analyzed using one-way analysis of variance (ANOVA). Continuous variables are presented as mean ±SD. All hypothesis testing was two-sided, with $p<0.05$ used as the threshold for statistical significance. Analyses were conducted using Prism 8 (version 8.0.2; GraphPad Software, La Jolla, CA, USA).

Results

HSC are localized in areas of the bone marrow surrounded by micro-vessels and megakaryocytes

Using confocal microscopy, we readily detected GFP-positive cells in the BM of both hCD34/TET and hCD34/TET/Gata1^{low} mice. Three-dimensional stacking analysis revealed that the majority of GFP-positive cells in both transgenic models were located in close proximity to micro-vessels as confirmed by vWF-positive endothelial cells (Figure1A). As the small GFP-tagged cells were found to be similarly localized under both physiological and patho-

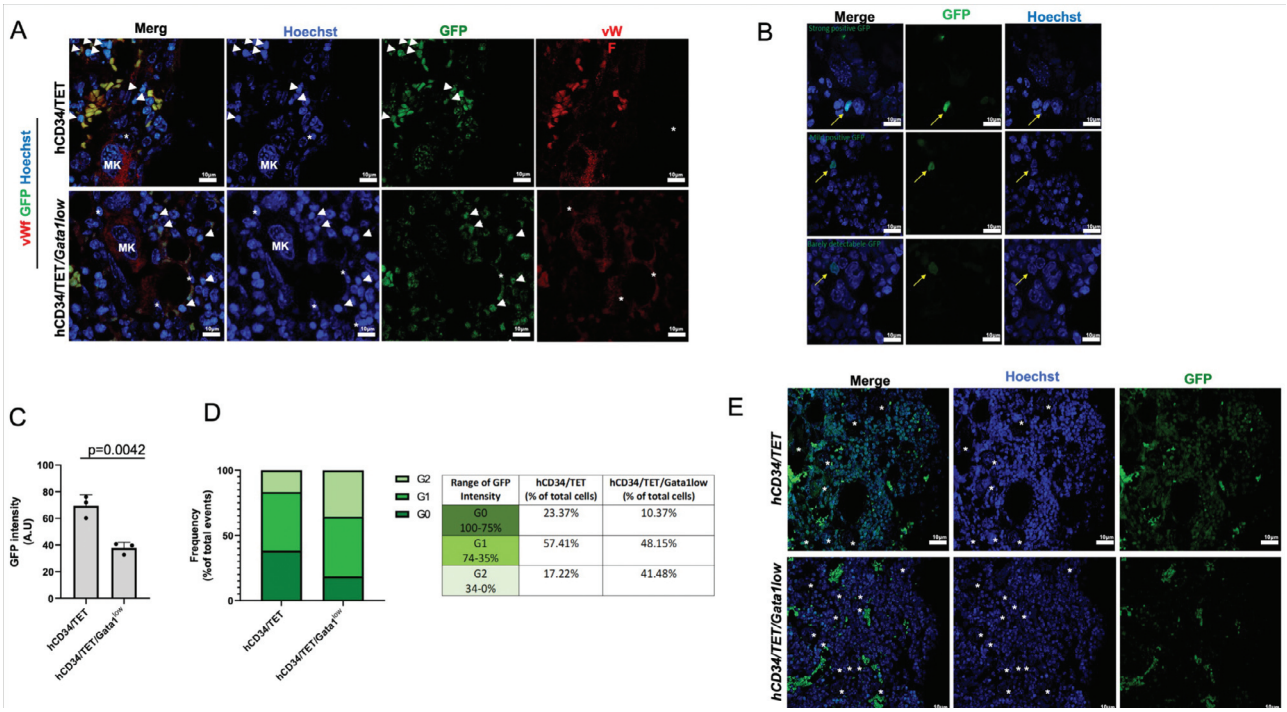

Figure 1. HSC are localized in areas surrounded by micro-vessels and megakaryocytes. **A**) Left panel showing vWF positive (red) endothelial cells (asterisk) in the femur of the mutated mouse models, which confirm that the recognizable GFP positive cells (white arrow) are mostly located near blood vessels; MK (megakaryocytes); original magnification 60x, acquired in zoom mode. **B**) The GFP positive cells within the bone marrow architecture with different fluorescence intensity, were defined as strong (G0), mild (G1) and barely (G2) positive; original magnification 60x. **C**) Overall GFP intensity expressed by single cells from the BM sections of the hCD34/TET and hCD34tTA/TET/Gata1^{low} mice, as indicated; data are presented as mean of those observed with three mice per group. **D**) Frequency distribution of the GFP levels divided into 3 classes according to the cumulative percentage of the values distribution and table with relative values in the BM of either hCD34/TET and hCD34tTA/TET/Gata1^{low}. **E**) Confocal microscopy with GFP and DAPI of a femur from hCD34/TET and hCD34tTA/TET/Gata1^{low} mice revealing that GFP positive cells are mostly near megakaryocyte (asterisk); original magnification 40x.

Table 1. Quantitative analysis of the strong positive GFP cells.

Mouse	FI mean	Standard deviation	*p*
hCD34/TET	75	1.25	0.0042
hCD34/TET/Gata1^{low}	35	1.00	

All the GFP positive cells present in one section were considered. The data with statistical analyses in the table format reported the mean GFP intensity for each group. FI, fluorescence intensity.

logical conditions, we next analyzed the intensity of the GFP signal to distinguish between long-term and short-term repopulating HSCs within the BM architecture, according to the intensity of the GFP fluorescence (Figure 1B). Nuclear GFP levels of single cells from the BM of >300 cells per group for both mutant mouse models were determined. To better define the HSCs dynamics, we considered both the nuclear GFP expression and the cell size in order to morphologically define the quiescent HSC and their immediate progeny. According to the following criteria, we scored the HSCs in three classes. The GFP intensity and hemi-decrement have been methodologically analyzed as previously described.[28] In femurs from aged control mice, GFP-positive cells were arranged either in clusters within the epiphysis or dispersed throughout the medullary region of the diaphysis. By contrast, in hCD34/TET/Gata1^{low} mice, the brightest GFP-positive cells predominantly appeared as isolated cells. Quantitative analysis showed that the number of GFP-high cells in hCD34/TET/Gata1^{low} bone marrow was markedly lower than in hCD34/TET controls (Figure 1 C,D and Table 1), consistent with previous evidence that the majority of SLAM cells in hCD34/TET/Gata1^{low} mice, are located in the spleen.[29]

Interestingly, cells within each cluster of old hCD34/TET mice, displayed a homogeneous GFP intensity (either uniformly high or uniformly low), suggesting that HSCs with similar functional potency tend to remain spatially associated. Furthermore, confocal imaging revealed that these clusters were often located next to MKs, which can be morphologically identified by their characteristic poly-lobulated nuclei (Figure 1 A,E).

The HSC-supportive MKs are unable to maintain the hematopoietic niches in MF

The presence of HSC-supportive MK subpopulations was analyzed using confocal microscopy and specific surface markers. The MKs with an HSC-supportive phenotype were identified in the BM of both aged transgenic mouse models and were predominantly located adjacent to or within sinusoids (Figure 2A). We then re-evaluated HSC dynamics in aged triple-mutant hCD34/TET/Gata1^{low} mice, comparing them with their respective controls *(hCD34tTA/TET)* based on their spatial distribution within the BM microenvironment. The BM of triple-mutant mice showed a significant increase in the number of supportive HSC-associated MKs (Figure 2B). However, notable differences in the expression of surface markers were observed: both CD61 and MYLK4 demonstrated a significantly higher fluorescence intensity in control mice (Figure 2C). Using confocal microscopy, we investigated the distribution of GFP positive cells within the BM architecture in relation to HSC supportive MKs. We subsequently quantified cells expressing robust- optically detectable- levels of GFP signal. Despite the significant increase of the HSC supportive MKs found in proximity to the GFP positive cells (Figure 2D), triple-mutant mice exhibited a different proportion of GFP-expressing cells compared with controls. According to the strong detectable GFP signal, a 68% reduction in the number of GFP positive cells was observed in the triple mutant mice (Figure 2E), accompanied by a marked decrease in GFP intensity, with an overall 50% reduction 68.33 hCD34tTA/TET *vs* 32.13 fluorescence intensity, in hCD34/TET/Gata1^{low} mice) (Figure 2F).

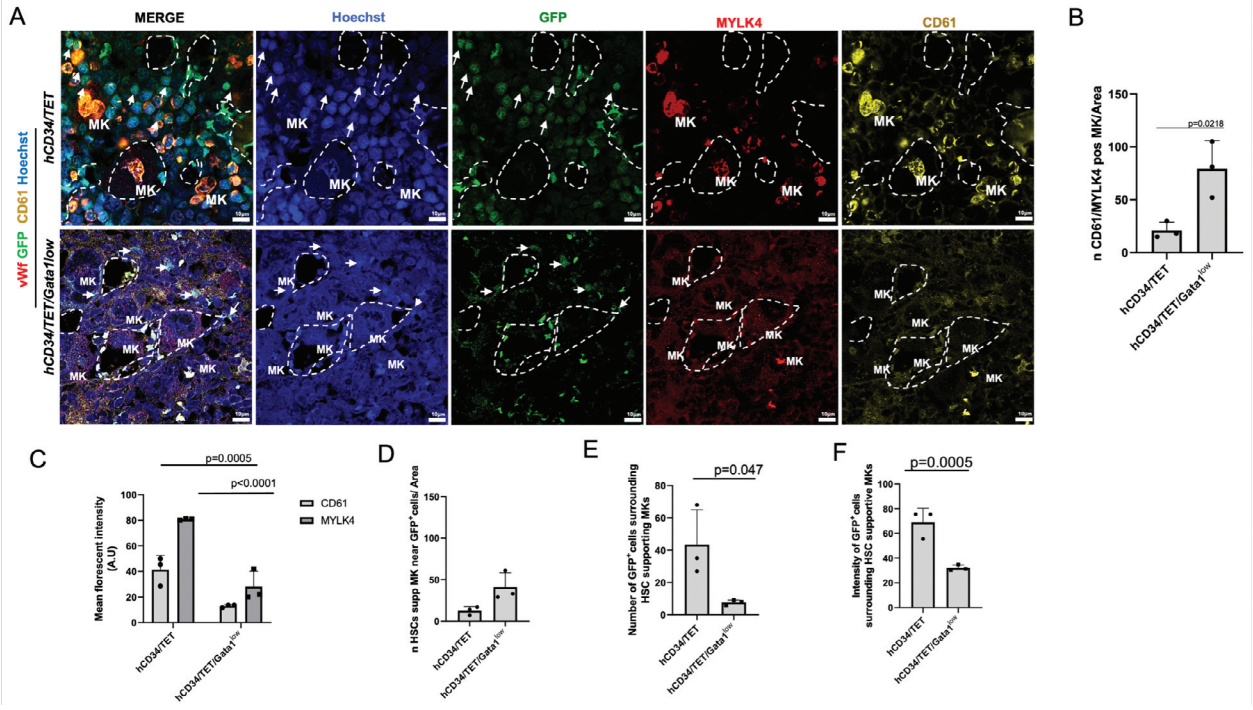

Figure 2. MKs with an HSC-supportive phenotype orchestrate a niche specific dynamic localization in MF. **A**) Confocal microscopy analyses with markers of HSC-supportive (CD61 green/MYLK4 red) MK in the BM from representative of either hCD34-TET and hCD34tTA/TET/Gata1^{low} 15-months (old) of age. **B)** Relative frequency of HSC-supporting MK is reported; original magnification 60x. **C**) Fluorescence intensity analysis of the HSC supportive MKS according with the MYLK4 and CD61 surface marker. **D**) Relative frequency of HSC-supporting MKs located near the GFP positive cells. **E**) Respective number GFP positive cells surrounding HSC supportive MKs in the triple mutant hCD34/TET/Gata1^{low} and hCD34/TET mice. **F**) Relative GFP protein intensity; the GFP positive cells were found in the diaphysis around structures resembling micro vessels and in proximity of CD61+/MYLK4+ MKs; white dotted lines indicate the contour of blood vessels; frequency of HSC-supporting and the number of GFP positive HSC near the supportive MK (D) are reported as mean ±SD and as value in individual subjects (each dot represents a single mouse). Data were analyzed using one-way ANOVA and significant *p*-values are indicated within the panels.

Discussion

Hematopoiesis is a process regulated by a wide variety of stimuli that can reshape the bone marrow niche by directly modulating the quiescence, self-renewal, and multi-lineage differentiation of hematopoietic stem and progenitor cells.[31] Direct cell-to-cell interactions and secretion of various factors, including cytokines and interferons, by different BM cells such as mesenchymal stem cells, endothelial cells, osteoblasts, and osteoclasts, are important regulators of the bone marrow niche.[32] The vascular and endosteal niche in the BM microenvironment have already been described as two distinct haemopoietic environments that can regulate the quiescence, proliferation, differentiation and mobilization of the stem cells.[16,33] In MF, the altered stem cell proliferation appears to be related to perturbations in the BM microenvironment, which directly affect both the endosteal and vascular niches.[34] BM microenvironment dysfunction in MF is driven by aberrant MKs,[35] which have been recently recognized as dynamic cells involved in heterogeneous functions in both physiological and in malignant hematopoiesis.[36]

This study aimed to investigate how aging and MF-associated alterations affect the HSCs niche, with a specific focus on HSC-supportive MKs. Our data demonstrate that while HSC-supportive MKs can be identified in both physiological and pathological conditions, their phenotype and functional properties are markedly altered in aged hCD34/TET/Gata1^{low} mice.

Using confocal microscopy and GFP intensity analyses, we observed that in control hCD34/TET mice, GFPhigh HSCs clustered within the epiphysis or medullary regions of the femur, often in close association with MKs displaying a supportive phenotype. Notably, within each cluster, cells exhibited homogeneous GFP intensity, suggesting that HSCs with similar repopulating potential preferentially co-localize. These clusters were consistently found adjacent to morphologically recognizable MKs, supporting the concept that MK spatial organization contributes to the maintenance of HSC quiescence and functional stability. By contrast, in *Gata1^{low}* triple mutant mice, the brightest GFP positive HSCs predominantly appeared as isolated cells, with overall fluorescence intensity significantly reduced compared to controls. Although the number of MKs with a supportive phenotype was increased in triple mutants, these cells showed reduced expression of CD61 and MYLK4. This result is consistent with previous gene expression data revealing a downregulation of genes that define the transcriptional signature of HSC-supportive MKs in aged *Gata1^{low}* mice.[37] This paradoxical increase in the number of supportive MKs, coupled with a decline in their function, suggests a maladaptive remodeling of the niche, possibly driven by the chronic pro-inflammatory[38-41] and fibrotic environment[42] characteristic of MF.

In accordance, our findings fit into a broader patho-physiological model in which the inflamm-aging milieu and aberrant MK development in MF lead to the expansion of phenotypically supportive MKs that are functionally impaired. As a consequence, the physiological cues required to maintain long-term HSC quiescence and self-renewal are lost, resulting in altered HSC localization and mobilization to extramedullary sites. This is consistent with reports that in MF, HSC/progenitor cells are prone to apoptosis,[29] increase in HSC trafficking and aberrant myeloproliferation, and that MK dysfunction contributes directly to hematopoietic failure. From a clinical perspective, our results suggest that MK-supportive subpopulations, identifiable by CD61 and MYLK4 expression, may serve as biomarkers of niche integrity. The observation that their molecular profile is altered in MF suggests that MK signaling pathways, such as TGF-β and CXCL4, could be targeted, or that the inflammatory microenvironment could be modulated, to restore HSC-supportive capacity. Since the present study is basically morphological analyses, some limitations should be acknowledged. First, it is based on a single murine model of MF; validation in other preclinical models and in patient-derived BM samples is required. Second, although our analyses revealed phenotypic and spatial alterations, functional assays, such as transplantation or competitive repopulation, are need to confirm the in vivo HSC-maintenance capacity. In conclusion, we demonstrate that MF significantly disrupt the HSC niche, leading to the development of phenotypically distinct but functionally impaired HSC-supportive MKs. This maladaptive remodeling likely contributes to the hematopoietic failure observed in MF. Future studies aimed at restoring MK-supportive function may represent a novel therapeutic strategy to preserve or re-establish niche integrity in hematologic disorders associated with fibrosis and inflammation.

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