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Fibrillin-1 microfibrils influence adult bone marrow hematopoiesis

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Abstract

We have recently demonstrated that fibrillin-1 assemblies regulate the fate of skeletal stem cells (aka, mesenchymal stem cells [MSCs]) by modulating TGF β activity within the microenvironment of adult bone marrow niches. Since MSCs can also influence hematopoietic stem cell (HSC) activities, here we investigated adult hematopoiesis in mice with Cre-mediated inactivation of the fibrillin-1 (*Fbn1*) gene in the mesenchyme of the forming limbs (*Fbn1^{Prx1}*^{-/-} mice). Analyses of 3-month-old *Fbn1^{Prx1}*^{-/-} mice revealed a statistically significant increase of circulating red blood cells, which a differentiation assay correlated with augmented erythropoiesis. This finding, together with evidence of fibrillin-1 deposition in erythroblastic niches, supported the notion that this extracellular matrix protein normally restricts differentiation of erythroid progenitors. Whereas flow cytometry measurements identified a decreased HSC frequency in mutant relative to wild type mice, no appreciable differences were noted with regard to the relative abundance and differentiation potential of myeloid progenitor cells. Together these findings implied that fibrillin-1 normally promotes HSC expansion but does not influence cell lineage commitment. Since local TGF β hyperactivity has been associated with abnormal osteogenesis in *Fbn1^{Prx1}*^{-/-} mice, 1-month-old mutant and wild type animals were systemically treated for 8 weeks with either a pan-TGF- β -neutralizing antibody or an antibody of the same IgG1 isotype. The distinct outcomes of these pharmacological interventions strongly suggest that fibrillin-1 differentially modulates TGF β activity in HSC vs. erythroid niches.

Keywords

erythropoiesis; fibrillin-1; hematopoiesis; marrow stem cells; TGF β

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Introduction

The adult bone marrow is the main source of bone, blood and immune cells [1, 2]. As result, identification of the extrinsic regulators of stem/progenitor cell niches in the adult bone marrow is a top research priority in tissue bioengineering and skeletal and hematopoietic regenerative medicine. The adult bone marrow contains mesenchymal stem cells (MSCs; aka, skeletal stem cells) capable of forming spindle-like colonies (colony-forming unit fibroblasts, CFU-Fs) that can be expanded and differentiated into osteoblasts, chondrocytes or adipocytes under appropriate cell culture conditions [3]. MSCs also support bone marrow hematopoiesis both *in vitro* and *in vivo*, thereby linking skeletal development with the assembly of hematopoietic stem cell (HSC) niches [2–7]. In contrast to the poorly defined nature of the MSC microenvironment [3], two intertwined niches are believed to support HSC maintenance and activation (the bone niche [8, 9]) and progenitor cell commitment and precursor cell differentiation (the vascular niche [10, 11]). Dynamic interactions among different marrow cell types, cell-bound molecules and soluble biochemical signal have all been shown to influence the activities of stem/progenitor cells residing within specific marrow niches [1,3,12–14]. By contrast, the role of the extracellular matrix (ECM) remains largely undefined with negative implications for our ability to develop more effective, evidence-based therapeutic strategies of skeletal tissue regeneration [15].

Studies of mouse models of Marfan syndrome (MFS), a connective tissue disease caused by mutations in fibrillin-1, have demonstrated the involvement of this structural component of the architectural matrix in regulating local bioavailability of TGF β and BMP signals that drive tissue formation, remodeling and regeneration [16]. A case in point is the skeleton where fibrillin-1 and the structurally related fibrillin-2 protein have been shown to differentially modulate TGF β and BMP signals during bone patterning and remodeling [17]. We have recently reported that fibrillin-1 influences MSC fate determination by controlling TGF β bioavailability within adult marrow niches [18]. Here, we investigated the role of fibrillin-1 in adult hematopoiesis in light of the functional relationship between MSC and HSC niches [2–7]. Our findings demonstrate that loss of fibrillin-1 in the mouse's marrow also causes significant hematopoietic abnormalities, such as HSC depletion and augmented erythropoiesis (polycythemia). Furthermore, the distinct outcomes of systemic TGF β neutralization in mutant mice strongly suggest that fibrillin-1 differentially modulates TGF β signaling within HSC and erythroid niches.

Results

Genetic inactivation of fibrillin-1 synthesis in the forming limbs of *Fbn1^{Prx1-/-}* mice perturbs MSC fate determination and leads to age-dependent bone loss associated with an unusual paucity of marrow adipocytes [18]. Consistent with the darker appearance of adult limb bones (Fig. 1a), progressive expansion of the hematopoietic marrow in 3- and 6-month-old *Fbn1^{Prx1-/-}* mice paralleled the age-dependent loss of trabecular bone and adipocytes recently described in these mutant mice (Fig. 1b) [18]. In light of this evidence and the functional crosstalk between MSCs and HSCs [2–7], we compared the status of hematopoiesis in 3-month-old wild type (WT) and *Fbn1^{Prx1-/-}* mice. Elevated hematocrits, hemoglobin content and circulating red blood cells in mutant relative to WT mice strongly

suggested dysfunctional hematopoiesis resulting in polycythemia (Fig. 1c). An in vitro assay designed to detect the number of colony-forming units of the erythroid lineage (CFU-E assay) implied enhanced marrow erythropoiesis (Fig. 1d). Consistent with this finding, we found that fibrillin-1 is deposited in the specialized, macrophage-containing marrow niches (aka, erythroblastic islands [19]) where erythroid progenitors proliferate and undergo terminal differentiation (Fig. 1e). Collectively, these findings support the novel notion that fibrillin-1 microfibrils normally restrict erythroid expansion.

Next, we performed flow cytometry analyses of cells flushed out from bone marrows so as to estimate the frequency of (CD34^{low}, Lin⁻, Sca-1⁺, c-Kit⁺ [LSK]) HSCs in 3-month-old *Fbn1^{Prx1}^{-/-}* and WT mice. The results of these analyses documented a statistically significant reduction of LSK-HSCs in MT relative to WT marrow samples (Fig. 2a). On the other hand, flow cytometry analyses showed no changes in the frequency of common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP) cells (Fig. 2b). Likewise and in contrast to the results of the CFU-E assay, no appreciable differences were noted between WT and mutant marrow-derived early progenitor cells differentiated into various hematopoietic cell types (Fig. 2c). Collectively, these findings demonstrated that fibrillin-1 is a novel ECM regulator of HSC maintenance, but not myeloid cell lineage specification.

TGF β hyperactivity in marrow niches of *Fbn1^{Prx1}^{-/-}* mice has been implicated in a premature depletion of MSCs and osteoprogenitor cells causing age-dependent bone loss [18]. We therefore explored if a similar mechanism may account for the hematopoietic defects noted in these mutant animals. To this end, WT and *Fbn1^{Prx1}^{-/-}* mice were treated with either the pan-TGF β -neutralizing antibody 1D11 or a control antibody of the same IgG1 isotype [18]. Treatment started at 1 month of age and ended 8 weeks later when mutant and WT mice were sacrificed to evaluate the impact of TGF β neutralization on hematopoiesis. Similar to the beneficial effect on bone mass and trabecular microarchitecture [18], chronic administration of 1D11 prevented polycythemia in *Fbn1^{Prx1}^{-/-}* mice (Fig. 3a). By contrast, HSC frequency in 1D11- vs. placebo-treated mutant animals did not change even though TGF β neutralization substantially reduced HSC abundance in WT mice (Fig. 3b). As explained in the Discussion, we interpreted these findings to indicate that fibrillin-1 differentially modulates TGF β signals within HSC and erythroid niches.

Discussion

In spite of much research effort, our current understanding of ECM's contribution to the structural and functional requirements of bone marrow niches remains limited. Previous immunohistological analyses have localized collagen, fibronectin and laminin to discrete areas of the mouse bone marrow believed to represent functionally distinct niches [20]. Additional studies have highlighted the importance of tenascin-C and agrin in modulating HSCs and progenitor cell proliferation and survival [21, 22]. The findings described here together with our previous characterization of osteopenia in MFS mice [18] are the first to identify a structural component of the ECM (fibrillin-1) that coordinates both adult osteogenesis and hematopoiesis.

Our studies also support the notion that dysregulated TGF β signaling secondary to fibrillin-1 deficiency in the marrow matrix is a prominent driver of bone loss and hematopoietic manifestations [18]. The finding that systemic TGF β neutralization rescued polycythemia in *Fbn1^{Prx1-/-}* mice suggests that fibrillin-1 normally restricts TGF β activity within the microenvironment of erythroblastic islands. This conclusion is consistent with prior cell culture evidence that TGF β 1 stimulates differentiation of non-cycling erythroid progenitors [23]. On the other hand, causal association between TGF β hyperactivity and stimulated erythropoiesis in *Fbn1^{Prx1-/-}* mice calls into question the physiological significance of prior *in vitro* evidence that TGF β is a potent inhibitor of erythropoiesis [19]. The impact of TGF β neutralization on HSC frequency in WT vs. *Fbn1^{Prx1-/-}* mice indicates that a different mechanism accounts for fibrillin-1 regulation of HSC niches. Whereas chronic administration of 1D11 halved HSC frequency in WT mice, TGF β neutralization had no appreciable effect on HSC abundance in *Fbn1^{Prx1-/-}* mice relative to placebo treatment (Fig. 3). One possibility is that fibrillin-1 deficiency somehow rendered HSCs unresponsive to TGF β neutralization. An alternative explanation is that fibrillin-1 deficiency inhibited TGF β activity within HSC niches to a level that could not be further decreased by 1D11 action. We favor the latter possibility based on recent evidence indicating that the initial consequence of fibrillin-1 deficiency in the aorta of MFS mice is to impair rather than stimulate TGF β activity [24]. In this respect, differential modulation of TGF β activity in HSC and erythroid niches is in line with prior evidence that fibrillin microfibrils provide contextual specificity to TGF β and BMP signals during bone patterning and remodeling [17]. We further argue that osteopenia-dependent expansion of hematopoietic tissue, as evidenced by increased marrow cellularity in fibrillin-1-deficient bones, may also contribute to HSC depletion independently of TGF β dysregulation. Ongoing investigations are testing this possibility along with additional aspects of marrow and extra-medullary erythropoiesis in mouse models of MFS.

Since macrophages are integral components of the erythroid niche [19], we speculate that TGF β over-activation secondary to fibrillin-1 deficiency may also influence differentiation of this hematopoietic cell lineage. Two indirect lines of evidence support our argument. First, we have previously demonstrated that increased osteoclastogenesis is a prominent determinant of progressive bone loss in MFS mice [18]. Second, clinical studies have reported that, irrespective of vessel diameter, more inflammatory cells were present in aortic specimens from MFS patients than individuals with non-syndromic aneurysms [25]. Hence, it is entirely plausible that sub-clinical impairment of marrow hematopoiesis might also contribute to aneurysm progression in MFS by exacerbating the immune-inflammatory response associated with maladaptive remodeling of aortic tissue [26]. In this view, our findings could potentially lead to a radically new paradigm of arterial disease in MFS with important implications for therapy.

Experimental procedures

Animals and treatments

WT and *Fbn1^{Prx1-/-}* male mice (C57BL/6-129SvEv genetic background) were used after approval of the studies by the institutional Animal Care and Use Committees of the Icahn

School of Medicine at Mount Sinai. Mice received intraperitoneal injections of pan-TGF- β -neutralizing antibody 1D11 (R&D Systems, Minneapolis, MN) diluted in PBS (pH 7.4) and administered at a dose of 10 mg/kg body weight thrice per week for 8 weeks starting at 1 month of age; an antibody of the same IgG1 isotype (clone 13C4) was used as placebo treatment [18]. Blood was collected from 1- and 3-month-old mice by retro-orbital bleeding and subsequent hematological assessments were performed with ProCyt Dx® Hematology Analyzer (IDEXX Bioresearch, Columbia, MO). For flow cytometry and cell culture assay, mice were sacrificed at the designed time points and bone marrow cells were isolated from femur and tibia. Contralateral limbs were fixed in 4% formalin for 24 h, decalcified in 14% EDTA for 2 weeks and processed for histological analysis.

Flow cytometry and immunohistological analyses

Marrow stromal cells were flushed out from femur and tibia of WT and *Fbn1^{Prx1-/-}* mice. For LSK-HSC analyses, approximately 10^6 bone marrow cells were labeled with antibodies anti-Lineage cocktail (APC), anti-CD34-FITC (clone RAM34), anti-Sca-1-PE-Cy7 (clone D7), anti-c-Kit-PE (clone 2B8) [27]. For CMP, MEP and GMP analyses, marrow cells were labeled with anti-FC γ R-eFluor®450 (clone 93), anti-IL-7R α -APC (clone A7R64), Lin-APC, anti-c-Kit-PE (clone 2B8), anti-CD34-FITC (clone RAM34) [28]. All antibodies were purchased from eBioscience (San Diego, Ca, USA). Samples were analyzed using a LSR II analyzer and FACSDIVA 6.1 software (BD Biosciences, San Jose, CA, USA); 3×10^5 live cell events were recorded to analyze HSC, CMP, MEP and GMP frequency. For fibrillin-1 and macrophage visualization paraffin sections of tibiae from 3-month-old WT and *Fbn1^{Prx1-/-}* mice were incubated with anti-fibrillin-1 (a kind gift of Dr. L. Sakai) and anti F4/80 (clone BM8, Biolegend, San Diego, Ca, USA) whereas Alexa Fluor® 568 and 488 anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA) were used for immunofluorescent labeling.

Hematopoietic colony-forming cell assays

For CFU-E assays, nucleated marrow cells were grown in MethoCult (StemCell Technologies, Vancouver, BC, Canada) in the presence of 50 ng/ml SCF (PeproTech, Rocky Hill, NY, USA) and 3U of EPO (Epogen®, Thousand Oaks, CA) [29]. Colonies were grown at 37°C with 5% CO₂ and 95% humidity and positive colonies were scored after 2 days. For burst-forming unit-erythroid (BFU-E) assays and CFU assays of granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM), GM, G and M, nucleated marrow cells were grown in MethoCult in presence of 50 ng/ml SCF, 10 ng/ml Hu-IL6, 10 ng/ml m-IL3 (PeproTech, Rocky Hill, NY, USA), and 3 U/ml of EPO. Colonies were grown at 37°C with 5% CO₂ and 95% humidity; differentiated BFU-E, CFU-GEMM, CFU-GM, CFU-G, and CFU-M colonies were counted after 7 – 12 days of culture [30].

Statistics

Two investigators blinded to genotype and treatment group examined all CFU colonies for comparative analyses performed on 5 or more independent samples in duplicate. Unpaired two-tailed *t* tests were used to determine the statistical significance of all experimental data between two groups, assuming a significance of $p < 0.05$. All values are graphically expressed as mean $\pm$ SD.

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Highlights

- Fibrillin-1 deficiency causes loss of hematopoietic stem cells (HSCs).
- Fibrillin-1 deficiency increases differentiation of erythroid progenitors.
- Fibrillin-1 differentially modulates TGF β activity within HSC and erythroid niches.

Fig. 1.

Polycythemia in *Fbn1^{Prx1}^{-/-}* mice. **(a)** Darker appearance of a 3-month-old mutant (MT) femur compared to a WT femur of the same age. **(b)** Number of nucleated bone marrow cells (expressed in millions) isolated from the femurs and tibias of 1-, 3-month and 6-month-old WT (white bars) and *Fbn1^{Prx1}^{-/-}* (gray bars) mice (n=6 per genotype and time point). **(c)** Bar graphs showing peripheral blood counts in 1- and 3-month old WT (white bars) and *Fbn1^{Prx1}^{-/-}* (gray bars) mice (n=16 per genotype and time points). **(d)** CFU-E assays of bone marrow cells isolated from 3-month old WT (white bars) and *Fbn1^{Prx1}^{-/-}* (gray bars) mice (n=5 per genotype). **(e)** Illustrative immunostaining of bone marrow tissue in a 3-month-old WT tibia showing fibrillin-1 (green) and a macrophage (F4/80; red) in an erythroblastic island (area inside the yellow dotted line) with nuclei stained in blue (DAPI). Note erythroblasts surrounding the central macrophage. Asterisks in relevant panels indicate statistically significant differences between samples of the same age ($p < 0.005$).

Fig. 2. HSC frequency and fate in *Fbn1^{Prx1}*^{-/-} mice. **(a)** Representative flow cytometry plots of lineage negative (Lin⁻), Sca-I and c-Kit double positive (LSK) HSCs flashed out from the bone marrows of 1- and 3-month-old (1M and 3M, respectively) WT and mutant (MT) mice. LSK cell populations are gated in the plots on the left (green cells in the rectangle gate) and then gated in the CD34-negative/dim histogram on the right (red arrows) to assess their frequency (Count). Bar graphs below summarize HSC frequency in the bone marrows of 1- and 3-month-old WT (white bars) and MT (gray bars) mice (n=5 per genotype and time points). **(b)** Representative flow cytometry plots and bar graph showing CMP, MEP and GMP frequency in 3-month old WT (white bars) and MT (gray bars) mice (n=5 per genotype and time points). **(c)** Hematopoietic BFU and CFU assays of bone marrow cells isolated from 3-month old WT and MT mice (n=5 per genotype and assay). Asterisks in

relevant panels indicate statistically significant differences between samples of the same age ($p < 0.005$).

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Fig. 3.

Systemic TGF β neutralization in *Fbn1^{Prx1}*^{-/-} mice. **(a)** Bar graphs showing peripheral blood counts in 1D11-treated 3-month old WT (white bars) and mutant (MT; gray bars) mice (n=8 per genotype and time points). **(b)** Representative flow cytometry plots of LSK-HSCs with bar graphs on the right summarizing HSC frequency in bone marrow aspirates from 3-month old WT (white bars) and MT (gray bars) mice treated with 1D11 or placebo (13C4) (n=5 per genotype and time points). Asterisks in relevant panels indicate statistically significant differences between samples of the same age ($p < 0.005$).