MicroRNA-23a mediates post-transcriptional regulation of CXCL12 in bone marrow stromal cells

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ABSTRACT

The chemokine CXCL12 regulates the interaction between hematopoietic stem and progenitor cells and bone marrow stromal cells. Although its relevance in the bone marrow niche is well recognized, the regulation of CXCL12 by microRNA is not completely understood. We transfected a library of 486 microRNA in the bone marrow stromal cell line SCP-1 and studied the expression of CXCL12. Twenty-seven microRNA were shown to downregulate expression of CXCL12. Eight microRNA (miR-23a, 130b, 135, 200b, 200c, 216, 222, and 602) interacted directly with the 3´UTR of CXCL12. Next, we determined that only miR-23a is predicted to bind to the 3´UTR and is strongly expressed in primary bone marrow stromal cells. Modulation of miR-23a changes the migratory potential of hematopoietic progenitor cells in co-culture experiments. We discovered that TGFB1 mediates its inhibitory effect on CXCL12 levels by upregulation of miR-23a. This process was partly reversed by miR-23a molecules. Finally, we determined an inverse expression of CXCL12 and miR-23a in stromal cells from patients with myelodysplastic syndrome indicating that the interaction has a pathophysiological role. Here, we show for the first time that CXCL12-targeting miR23a regulates the functional properties of the hematopoietic niche.

Introduction

Human hematopoiesis is a highly regulated process in which hematopoietic stem and progenitor cells (HSPC) give rise to all lineages of blood cells and a network of transcription factors is responsible for lineage development and proliferation of HSPC in response to external stimuli.[1,2]

In recent years, it has become evident that the microenvironment of HSPC plays an additional crucial role in HSPC proliferation, differentiation, engraftment, and mobilization.[3,4] The supporting components of the hematopoietic niche have not yet been completely identified, but evidence is accumulating that several types of cells may be responsible for the interaction between HSPC and the hematopoietic niche. Among such cells, spindle-shaped N-cadherin^{+} CD45^{-} osteoblastic cells (SNO cells) were found to be in contact with HSPC in the marrow, and ablation of these cells also reduced hematopoietic cells in some experiments.[5] Recently, Mendez-Ferrer *et al.* showed that Nestin positivity (Nes[+]) is not only associated with neural progenitors but that Nes^{+} cells are also found in the perivascular regions of the bone marrow. These cells were demonstrated to have the capacity for self-renewal, provide signals required for colony-forming unit activities in the bone marrow, and reside in close contact with HSPC. They also showed that Nes^{+} cells strongly express the chemokine CXC ligand 12 (CXCL12) [also known as stromal cell-derived factor-1 (SDF-1) or pre-B-cell-growth stimulating factor].[6] Other

cells in the bone marrow niche were originally identified by overexpression of CXCL12 and were thus termed CXCL12 abundant reticular (CAR) cells. The effects of ablating of CAR cells has suggested that these cells play an essential role in the maintenance of HPSC numbers and are also involved in maintaining HPSC in a proliferative and undifferentiated state.[4,7] Given the importance of stromal cells for the support of hematopoiesis and the observation that most if not all stromal cell compartments of the bone marrow express CXCL12, a functional role for CXCL12 in those cells is very likely. CXCL12 is a member of a large family of structurally related chemoattractive cytokines and was first characterized as a growth-stimulating factor for B-cell precursors.[8] The activity of CXCL12 is mediated by binding of the chemokine to its receptors (CXCR4 and CXCR7) on circulating hematopoietic cells.[9,10]

Experimental evidence has accumulated on the crucial role of the CXCL12/CXCR4 axis in the bone marrow niche. Sugijama *et al.* demonstrated that abrogation of CXCL12/CXCR4 signaling is associated with a reduction in HSPC numbers.[4]

Reduction of CXCL12 enables the mobilization of hematopoietic stem cell progenitors in the blood circulation. The disruption of CXCL12/CXCR4 signaling by mobilizing factors such as granulocyte colony-stimulating factor, fms-related tyrosine kinase 3 (FLT3) ligand, and stromal cell factor is thought to be mediated by the activation of bone marrow

proteases.[11,12] Furthermore, CXCL12 expression can be transcriptionally modulated by a variety of cytokines and growth factors, namely transforming growth factor-beta 1 (TGFB1). TGFB1 has a negative growth effect on HSPC which is mediated, in part, by the regulation of CXCL12 arising from stromal cells.[13] Thus, engraftment and response to cytotoxic drugs may vary according to CXCL12 levels provided by niche cells.[14,15]

Recently, Pillai *et al.* suggested that CXCL12 can also be regulated by microRNA (miRNA) in human marrow stromal cells.[16] There are more than 1000 miRNA which form 1–2% of the human genome. Approximately half of human structural genes are predicted to be under miRNA control. In animals, miRNA act by targeting the 3´ untranslated region (UTR) of genes with the consequence of repressing output of the respective protein. A classical switch interaction is used to avoid protein expression in a particular cell type, whereas tuning interactions are needed to supply the cell with the optimal level of protein.[17] In the hematopoietic system, CXCL12 must be fine-tuned in response to differing physiological requirements.

We, therefore, decided to study the interaction of CXCL12 and miRNA by applying a strategy of overexpression of a library to reveal the various miRNA responsible for CXCL12 regulation in primary human bone marrow stromal cells (hBMSC). Moreover, we mapped expression of candidate miRNA in primary hBMSC to understand their physiological function in fine tuning CXCL12 expression in the hematopoietic niche.

Methods

Cell lines

HS5, HS27, HL60, K562, KG1a, and HeLa cell lines were obtained from the German collection of micro-organisms and cell cultures (DSMZ, Braunschweig, Germany). The SCP-1 cell line, an hTERT immortalized human mesenchymal stromal cell line, was a kind gift from Prof. Schieker and Dr. Docheva (Ludwig Maximilians University of Munich, Germany).[18] Human BMSC and HSPC were isolated and cultured as described in the *Online Supplementary Methods*. All studies with human material were approved by the Institutional Review Board of the Medical Faculty of the Dresden University of Technology, and informed consent was obtained from the donors.

Transfection of human bone marrow stromal cells

The Amaxa Nucleofector kit (Lonza Ltd., Basel, Switzerland) was employed to transfect primary hBMSC with the respective oligonucleotides (*Online Supplementary Table S3*). After 24 h, cells were harvested for subsequent assays.

Luciferase reporter assay

The 3´UTR of the human *SDF1* gene (bp position 363–1935 from NM_199168.3) was amplified from cDNA from human macrophages (for sequences see *Online Supplementary Table S3*). The polymerase chain reaction product was cloned in the pMIRReporter vector (Ambion) via the *Spe*I and *Hind*III sites.

HeLa cells (1×10^5 cells/mL/well) were seeded out 1 day before transfection. Co-transfection of the respective Firefly-luciferase-containing pMIRReporter construct (500 ng/mL), the Renilla luciferase-containing plasmid [pRL-CMV (1:100 to pMIR Reporter)], and 30 pmole of the respective precursor miRNA molecule (*Online Supplementary Table S3*) was performed in duplicate using Lipofectamine 2000-based transfection (Invitrogen,

Carlsbad, CA, USA). After 24 h, cells were lysed in 1x lysis buffer (Promega, Madison, USA) and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Mitras LB 940 plate reader (Berthold Technologies GmbH, Germany). Data are presented as the ratio of Firefly/Renilla luciferase activity.

Migration assays

Primary hBMSC were transfected (as described above) with the precursor miRNA control (*Online Supplementary Table S3*). The conditioned medium obtained from the transfected hBMSC was added to the lower chamber of the transwell system (Chemotx, Neuro Probe, MD, USA). Then, 5×10^4 CD34+ HSPC were added to the upper chamber and allowed to migrate for 1 h at 37°C toward the conditioned medium through a 5-μM polycarbonate membrane. Subsequently, cells in the lower chamber were counted by flow cytometry. Recombinant human CXCL12 was used as a control.

Cytokine screening

Primary hBMSC were either untreated or treated with 20 ng/mL platelet-derived growth factor (PDGF; Biochrom, Germany), 20 ng/mL epidermal growth factor (EGF; Biochrom), 10 ng/mL basic fibroblast growth factor (bFGF; Biochrom) and 2 ng/mL of TGFB1 (Peprotech, Rock Hill, NJ, USA). Where indicated, the experiments with TGFB1 treatment (5 ng/mL) were performed in the presence or absence of 10 μM of the ALK-5 inhibitor, SB431542, (Sigma) for 24 h. Supernatants as well as the total-cell lysates were collected for further investigations.

Enzyme-linked immunosorbent assay for CXCL12 protein levels

Secreted CXCL12 levels were quantified using an enzyme-linked immunosorbent assay (ELISA) on conditioned media from hBMSC cultures (Duoset ELISA kit, RnD Systems, Minneapolis, USA) according to the manufacturer's instructions. Absorbance was measured on a Mitras LB 940 plate reader at 450 nm.

Statistical analysis

Results are presented as means ± standard error of the mean (± SEM). Statistical significance was calculated with a paired Student *t*-test using GraphPad Prism software (La Jolla, CA, USA). *P* values <0.05 were considered statistically significant. Heat maps were generated using R 2.15.2 with the gplots package.

Results

Screening and validation of microRNA regulating CXCL12

First, we established a system to prove the influence of miRNA overexpression on CXCL12 levels. There were two critical precautions for choosing a stromal cell line for miRNA experiments: (i) the cell line had to express considerable levels of CXCL12 to be measured in the supernatant, and (ii) the cell line had to be readily usable in transient transfection experiments with reliable targeting of CXCL12 as defined by a significant change of CXCL12 level after siCXCL12 transfection. A subset of cell lines (Hs5, Hs27, and SCP-1) was tested for the aforementioned criteria and SCP-1 was identified with respective precautions for further experimental work (*data not shown*). Next, we transfected a miRNA library comprising 468 pre-miRNA samples into the SCP-1 cells (Figure 1A, *Online*

Supplementary Table S1). Using a threshold of 30% change in protein level (determined by ELISA) after the transfection, we identified 27 miRNA that reduced CXCL12 protein levels (Table 1) and 33 miRNA that increased CXCL12 (*Online Supplementary Table S2*). Since the main focus of the study was to define directly interacting miRNA with suppressive activity, we further excluded miRNA that increased CXCL12 levels from our analyses. When comparing the effect of transfection of miRNA, we observed a correlation between protein levels as determined by ELISA and mRNA levels of CXCL12 in 18 miRNA (black font in Table 1), indicating a direct influence of miRNA on both the transcriptional and the translational level of CXCL12 expression. Next, we validated miRNA candidates in a reporter assay using the entire 3'UTR of the CXCL12 gene. Eight miRNA (miR-23a, miR-130b, miR-135a, miR-200b, miR-200c, miR-216, miR-222, and miR-602) showed a significant reduction in luciferase expression by a threshold of >30%, compared with control precursor miRNA (pre-miR co) and were regarded as potential regulators of CXCL12 3' UTR (Figure 1B).

Expression patterns and selection of the most relevant CXCL12-regulating microRNA in primary human bone marrow stromal cells

Next, we determined miRNA levels of the eight identified miRNA in primary hBMSC and compared these values to those in a subset of hBMSC cell lines and HSPC. Here, we found four miRNA (miR135, miR200b, miR216, and miR-602) that were not expressed or expressed at very low levels in primary hBMSC (C_T threshold >35 or $-\Delta C_T <$ −10) (Figure 2A). MiR-200c was expressed slightly in hBMSC and three miRNA (miR-23a, miR-130b, and miR-222) were expressed at readily detectable levels (Figure 2A). Of these, miR-130 and miR-222 were expressed to a similar degree in human CD34+ HSPC and in hBMSC. In contrast, HSPC did not express significant levels of miR-23a making it a BMSC-specific miRNA with regulatory potential on CXCL12 (Figure 2B).

An extended analysis showed that the expression of miR-23a in different body tissues was widely distributed, with the highest expression in adipose, placental, prostate, and bladder tissue (*Online Supplementary Figure S1*). Moreover, using the prediction algorithms (i.e. miRWalk which includes 10 prediction databases), we identified miR-23a as the only miRNA that was directly predicted to target CXCL12 by most (7 out of 10) algorithms (Table 1). Although we cannot completely rule out other miRNA with regulatory potential on CXCL12 (e.g. miR-222) we decided to focus on miR-23a. An overview of the workflow from the library to the selection of a candidate miRNA is depicted in *Online Supplementary Figure S2*.

Functional influence of microRNA-23a in primary human bone marrow stromal cells

To test the specificity of the candidate miRNA, the predicted binding sites of miR-23a on the 3' UTR of CXCL12 (Figure 3A) were cloned into the luciferase reporter vector. As controls, we used mutated variants of the binding sites (bs1 and bs2). As shown (Figure 3B), in comparison with

A

B
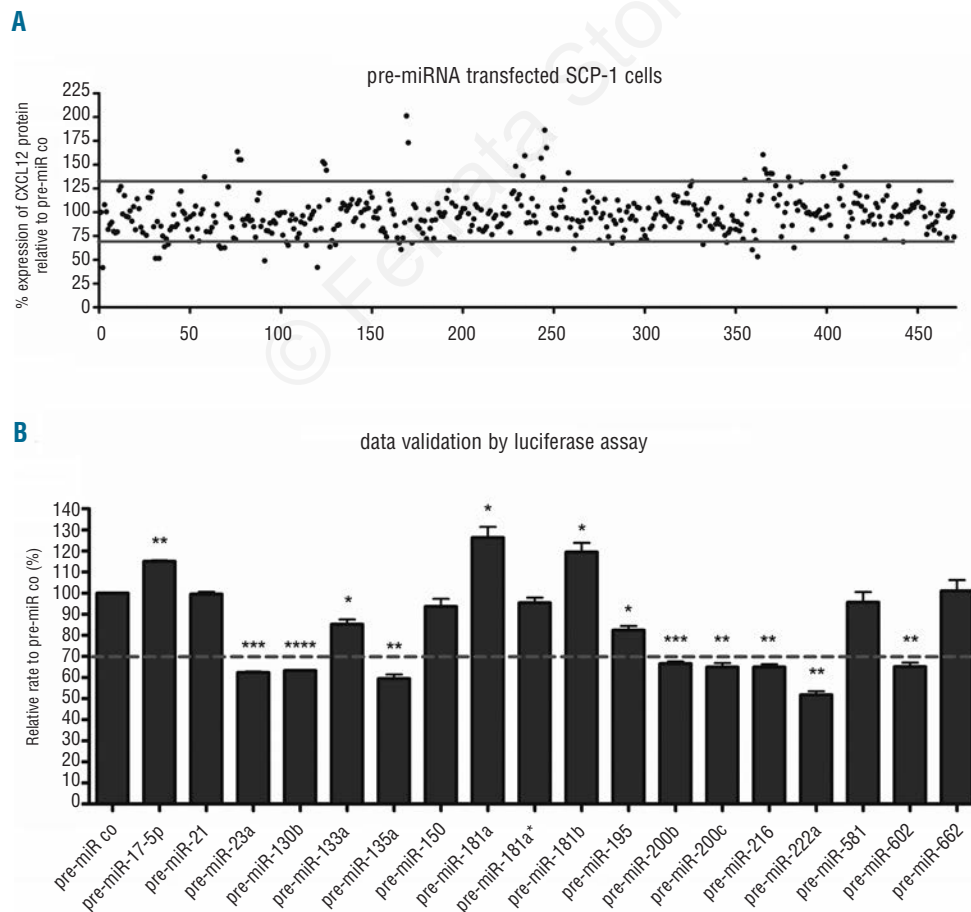
Figure 1. Validation and selection of candidate CXCL12-targeting miRNA (A) SCP-1 cells were transfected with a library of 468 pre-miRNA. The percentage of CXCL12 expression levels was determined in the supernatants of SCP-1 cells transfected with candidate miRNA relative to control precursor miRNA (pre-miR co). A gray line indicates the threshold for miRNA selection; values >130% represent CXCL12-upregulating miRNA and values <70% represent CXCL12-downregulating miRNA. (B) Selected CXCL12-downregulating miRNA (n=18) were validated by luciferase-reporter assays using the entire 3'UTR of the CXCL12 gene. A dashed line represents a threshold of 30% decrease. Results are representative of at least three independent experiments. The mean ± SEM are shown. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

the control pre-miRNA, pre-miR-23a significantly decreased the luciferase activity on the specific binding site-containing constructs. This effect was specific because the luciferase activity is not affected in the construct with the mutated binding site.

Next, we investigated the influence of miR-23a in primary hBMSC. A transient transfection strategy demonstrated that the expression of the mature pre-miR-23a was followed by a decrease in CXCL12 mRNA (Figure 3C) as well as protein (Figure 3D). In contrast, silencing of endogenous miR-23a with a specific anti-miRNA (anti-miR-23a) led to increased CXCL12 levels (mRNA and protein) in comparison to the controls (pre-miR and anti-miR) (Figure 3C,D). In these experiments, we also used precursor miRNA for let-7c (pre-miR-let7c) as an additional control because according to our library data (Figure 1A) and target-prediction algorithms it was not expected to affect CXCL12 expression. Transfection of pre-miR-let7c did not lead to significant changes in CXCL12 expression (*data not shown*).

The functional influence of miR-23a was further tested in transwell experiments. Confluent primary hBMSC layers were transfected with either pre- or anti-miR-23a molecules. After 24 h, hBMSC supernatant was collected and used for a migration assay of CD34⁺ HSPC. The transfection of pre-miR-23a molecules in hBMSC significantly reduced the number of migrating HSPC. Although not statistically significant, the anti-miR-23a transfection had a tendency to increase the number of migrating cells in the transwell assay (Figure 3E). Additionally, the same results were obtained by transfecting SCP-1 cells with the respective miRNA molecules and migratory assays using a bone marrow cell line, 32D cells (*data not shown*).

Physiological regulation of the CXCL12/microRNA-23a axis in primary human bone marrow stromal cells

Although hBMSC produce high levels of CXCL12, the expression of this chemokine is highly regulated by several factors. To reveal potential regulatory pathways that may influence the miRNA regulation of CXCL12, we treated primary hBMSC with a panel of growth factors that were previously shown to exert a biological influence on hBMSC (Figure 4A).[20,21] Among them TGFB1 and EGF, although to different degrees, were shown to reduce CXCL12 protein levels significantly, with TGFB1 being the strongest potent inhibitor (Figure 4A). The application of PDGF and bFGF did not lead to significant changes of CXCL12 in the hBMSC cultures. Next, we evaluated whether miR-23a varies in hBMSC in response to these growth factors. Only TGFB1 induced an increase of miR-23a, while EGF, PDGF, and bFGF did not significantly affect miR-23a levels in hBMSC (Figure 4B). Importantly, while TGFB1 decreased mRNA as well as protein levels of CXCL12 in a concentration-dependent manner (Figure 4C,D), miR-23a levels increased (Figure 4E). In consequence, we considered that miR-23a may be an intermediate in the signaling pathway of TGFB1 to reduce CXCL12 levels.

We therefore asked whether interference with the TGFB1 signaling pathway would change miR-23a expression. To address this question, we treated primary hBMSC cultures with TGFB1 and modulated the effect of TGFB1 with the ALK-5 inhibitor SB431542 (SB). Here we observed that while TGFB1 significantly reduced the expression of CXCL12 protein, co-application of SB

almost completely counteracted this effect (Figure 5A). We also detected an increased expression of miR-23a after stimulation with TGFB1 but not in the presence of SB, indicating a role for TGFB1 in miR-23a expression (Figure 5B).

Next, we determined whether direct interference with miR-23a would modify the response of primary hBMSC to TGFB1. An antagomir was used to neutralize miR-23a in TGFB1-treated hBMSC. Again, it was found that TGFB1 has a negative influence on CXCL12 expression and this was at least partly reverted by transfection of hBMSC with anti-miR-23a (Figure 5C).

Expression of microRNA-23a in human bone marrow stromal cells from patients with myelodysplastic syndrome

Next, we investigated a potential inverse correlation between CXCL-12 and miR-23 in human disease. We compared samples of *in vitro*-grown primary hBMSC from patients with myelodysplastic syndrome (MDS) and healthy donors. The hBMSC from MDS patients expressed significantly lower levels of CXCL12 while showing dramatically increased levels of miR-23a (Figure 6A,B). Since the TGFB1 pathway has recently been shown to influence the hematopoietic niche function in MDS patients and CXCL12 was found to be reduced in MDS

Table 1. CXCL12-downregulating miRNA.

miR-	CXCL12 protein (%)	CXCL12 mRNA	Targetscan prediction	Pictar prediction	Location
17-5p	64.5	0.64	no	no	Chr.13q
21	65.1	0.70	no	no	Chr.17q
23a	66.1	0.87	yes	yes	Chr. 19p
130b	67.1	0.72	no	no	Chr.22q
133a	51.6	0.71	yes	no	Chr. 18q
135a	63.8	0.73	no	yes	Chr. 3p
136	66.3	1.05	no	no	Chr.14q
150	69.5	0.75	yes	no	Chr.19
181a	62.2	0.84	no	no	Chr. 1q
181a*	66.4	0.80	no	no	Chr. 1q
181b	62.7	0.69	no	no	Chr. 1q
195	49.0	0.73	no	no	Chr. 17p
200b	68.7	0.60	no	no	Chr.1p
200c	65.3	0.66	no	no	Chr.12p
216	42.1	0.84	yes	no	Chr. 2p
222a	63.6	0.74	yes	no	Chr.Xp
324-5p	68.0	0.94	no	no	Chr.17p
325	60.7	1.02	no	no	Chr. Xq
331	67.8	1.11	no	no	Chr. 12q
500*	61.5	0.90	no	no	Chr.Xp
517a	67.6	1.20	no	no	Chr. 19q
551b	66.2	1.02	no	no	Chr. 3p
564	68.3	0.90	no	no	Chr. 3p
578	60.4	0.93	no	no	Chr.4q
581	53.2	0.86	no	no	Chr. 5q
602	62.7	0.85	no	no	Chr. 9q
662	68.9	0.74	no	no	Chr. 16p
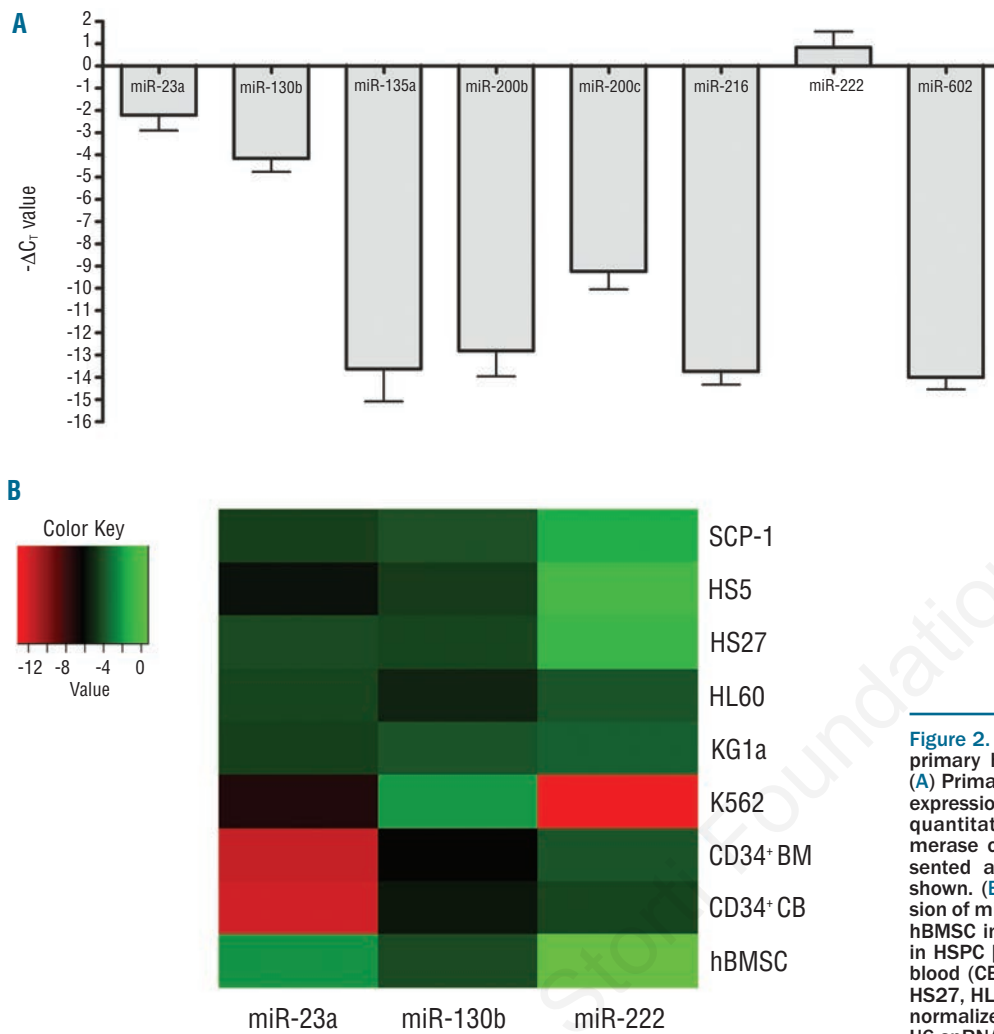
Figure 2. Expression of selected miRNA in primary hBMSC and hematopoietic cells (A) Primary hBMSC were screened for the expression of selected miRNA (n =5) by quantitative-reverse transcriptase polymerase chain reaction. Values are represented as $-\Delta C_T$. The mean ± SEM are shown. (B) Heatmap showing the expression of miR-23a, miR-130, and miR-222 in hBMSC in comparison to their expression in HSPC [from bone marrow (BM) or cord blood (CB)] and leukemic cell lines (HS5, HS27, HL60, KG1a, and K562). Values are normalized to those of the SCP-1 cell line. U6 snRNA served as the internal control.

patients[22,23] we additionally determined TGFB1 values in a subgroup of samples. We observed a substantial increase in the expression of TGFB1 in MDS samples (Figure 6C).
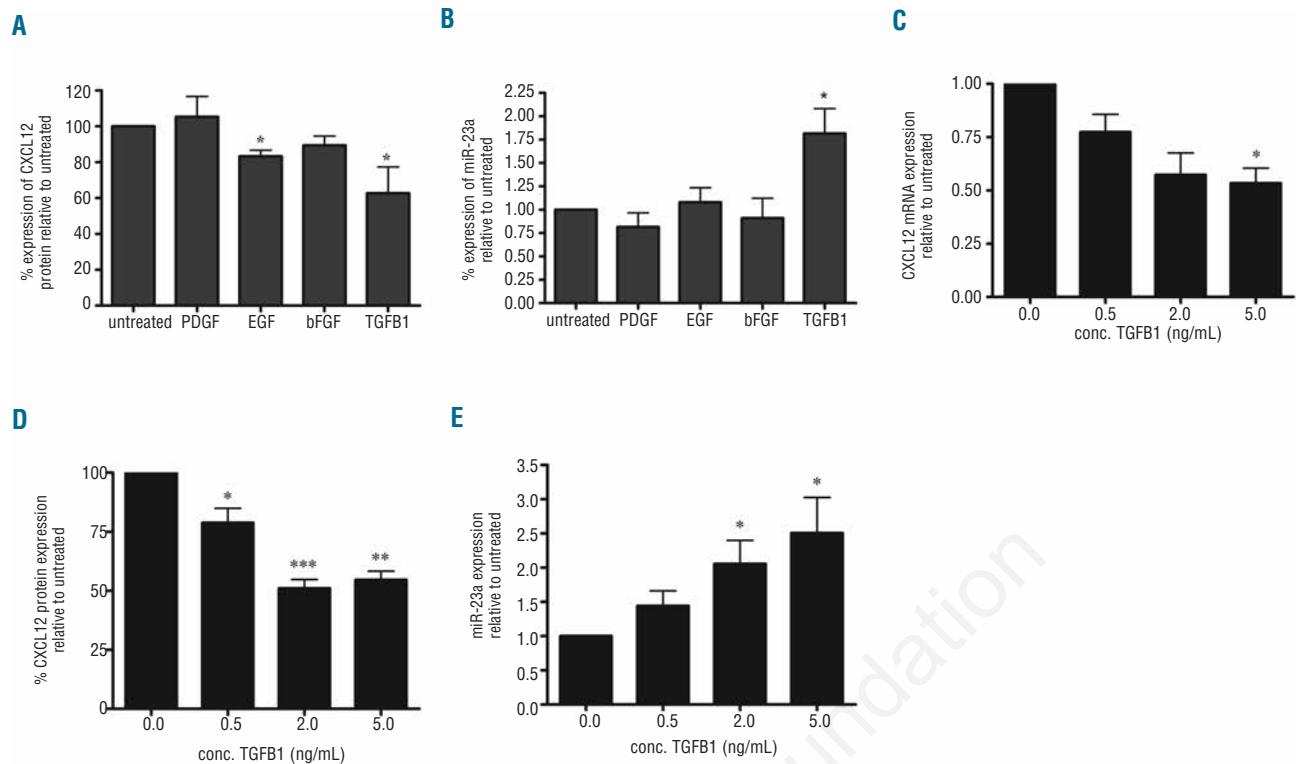
Discussion

Understanding the homing and mobilization of human HSPC is central for therapeutic interventions in hematologic diseases. Since it was shown that the interaction of human stromal cells with HSPC is critical for HSPC survival and homing, the signals emanating from hBMSC have been under intense research. The major cytokine of the niche, CXCL12, induces quiescence and retention of early HSPC.[4,24] In contrast, CXCL12 appears to trigger the entry of more mature HSPC into the cell cycle. Thus, the CXCL12-related system must be highly regulated to fulfill body requirements optimally. At the level of transcription, CXCL12 is regulated by HIF-1, which can be modified by metalloproteases such as MT1-MMP or by COMP-Ang1. Both stimuli activate HIF-1, which upregulates CXCL12.[25,26] In contrast, HSPC mobilization requires a reduction of CXCL12 in the bone marrow niche and thereby in hBMSC. Here, catecholaminergic signals modulate CXCL12 levels though β₃-adrenergic receptors, as

described by Mendez-Ferrer et al.[27] In the current study, a miRNA-mediated mechanism for CXCL12 reduction was proposed. Recently, Pillai et al. described the first miRNA, miR-886-3p, which effectively downregulates CXCL12.[16] The authors used a comparative gene expression study to identify regulating miRNA between CXCL12-expressing and non-expressing cell lines. We used another approach to conduct a library screen for CXCL12-modulating miRNA, and a subset of miRNA with regulatory potential was identified. Of the 27 candidate miRNA, eight interacted significantly with the 3´UTR of CXCL12, and three interacted and were expressed at detectable levels in primary hBMSC. We, therefore, consider those three miRNA (miR-23a, miR-130, and miR-222) to be functionally relevant in hBMSC. The algorithm employed here is based on the hypothesis of a tuning interaction, in which miRNA are present at the same time as their target to assure optimal target protein levels. The situation may vary in another cellular context e.g., the low expression of miR-23a in HSPC is awaiting further investigation.

In hBMSC, we determined miR-23a to be the most potent modulator of CXCL12 expression and function. This refers to the ability of miR-23a to change the migratory potential of HSPC in a co-culture with primary hBMSC and the changes in expression during differentia-

A

Predicted miRNA-mRNA duplex for miR-23a and CXCL12

1. binding site CXCL12:

3'-CCUUAGGGACCGUUACACUA -5'   miR-23a

5'- TATATGATATATAAATGTGAT -3'   CXCL12_bs1_WT
5'- TATATGATATATAAGTGTAAT -3'   CXCL12 _bs1_MT

2. binding site CXCL12:

3'-CCUUUAGGGACCGUUACACUA-5'   miR-23a

5'- TAAAATTGTATCAAATGTGAC -3'   CXCL12_bs2_WT
5'- TAAAATTGTATCAAGTGTAAC -3'   CXCL12_bs2_MT

B

C

D

E

Figure 3. MiR-23a has a putative binding site on the CXCL12 3´UTR, thereby regulating the expression and functionality of CXCL12 (A) The structure of miR-23a:CXCL12 mRNA predicted by the Pictar algorithm is shown ("|": Watson–Crick base pairs). The predicted binding sites are indicated as wild-type variants (bs1_WT, bs2_WT) as well as seed sequence (core region inversion) mutated variants (bs1_MT, bs2_MT). (B) The predicted binding sites were cloned as WT and MT downstream to the Firefly luciferase gene of pMIRReporter. Results represent Firefly luciferase activity after co-transfection with the Renilla luciferase-containing plasmid and the pre-miR control or pre-miR-23a. (C–D) hBMSC were transfected with control pre-miR (pre-miR co), pre-miR-23a, control anti-miR (anti-miR co), and anti-miR-23a. (C) CXCL12 mRNA expression 24 h post-transfection as determined by quantitative reverse transcriptase polymerase chain reaction. (D) CXCL12 protein levels in the supernatants detected after 24 h as quantified by ELISA. (E) HSPC migration in transwell assays in response to CXCL12 secreted in conditioned medium from transfected hBMSC. All data are representative of at least three independent experiments. The mean ± SEM are shown. *$P<0.05$ and **$P<0.01$.

tion of hBMSC towards adipocytes and osteoblasts. The latter data imply that upregulation of miR-23a is part of a differentiation program potentially involving several genes, which must be regulated during the process of osteogenesis. Hassan *et al.* recently demonstrated that miR-23a is upregulated during the osteogenic differentiation process and targets Runx-2 and SATB-2 to avoid premature osteocyte generation.[19]

Next, we attempted to determine the stimuli that could alter miR-23a expression. The promoter of the miR-23a-miR-24-miR27-cluster has been previously analyzed[28] and several groups have described binding of regulatory transcription factors such as NFATc3, CREB, and HIF to the promoter region.[29-31] We, therefore, tested a subset of potential stimuli (growth factors: mentioned earlier;

cytokines, chemotherapy, and hypoxia: *data not shown*) for miR-23a regulatory potential in hBMSC. The most consistent effect was achieved with the application of TGFB1. Here, cytokine application led to downregulation of CXCL12, which is in part explained by upregulation of miR-23a. The findings extend data that were reported by Wright *et al.* in the murine BMSC cell line MS-5. The authors described an early inhibitory effect of TGFB1 on the promoter of the CXCL12 gene which was counteracted by CXCL-12 mRNA increments after 12 h.[32] In the primary hBMSC culture, we observed a suppressive effect of TGFB1 on CXCL12 levels which was maintained for at least 24 h and was, therefore, long-lasting (*data not shown*). We speculated that the TGFB1-associated effect is pronounced by the activity of inhibitory miRNAs. Since in

Figure 4. TGFB1 regulation of the miR-23a/CXCL12 axis in primary hBMSC (A–B) Primary hBMSC were either left untreated or treated with PDGF (20 ng/mL), EGF (20ng/mL), bFGF (10 ng/mL) or TGFB1 (2 ng/mL) for 24 h (n = 6). (A) CXCL12 protein in supernatants of hBMSC after 24 h of treatment as analyzed by ELISA. The data are shown as % values relative to untreated control. (B) MiR-23a expression of hBMSC 24 h after treatment. Data are normalized to the untreated control. (C–E) Primary hBMSC were exposed to increasing concentrations (0.5, 2, and 5 ng/mL) of TGFB1 for 24 h. (C) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was conducted for CXCL12 mRNA expression. GAPDH served as the housekeeping gene. Values are normalized to the untreated control. (D) Subsequently, supernatants were analyzed with a specific ELISA for CXCL12 protein expression. Data are shown as percentages relative to untreated controls. (E) MiR-23a expression as determined by q-RT-PCR. U6 snRNA was used as the internal control. Rates are normalized to the untreated control. Error bars represent ± SEM from at least three independent experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.
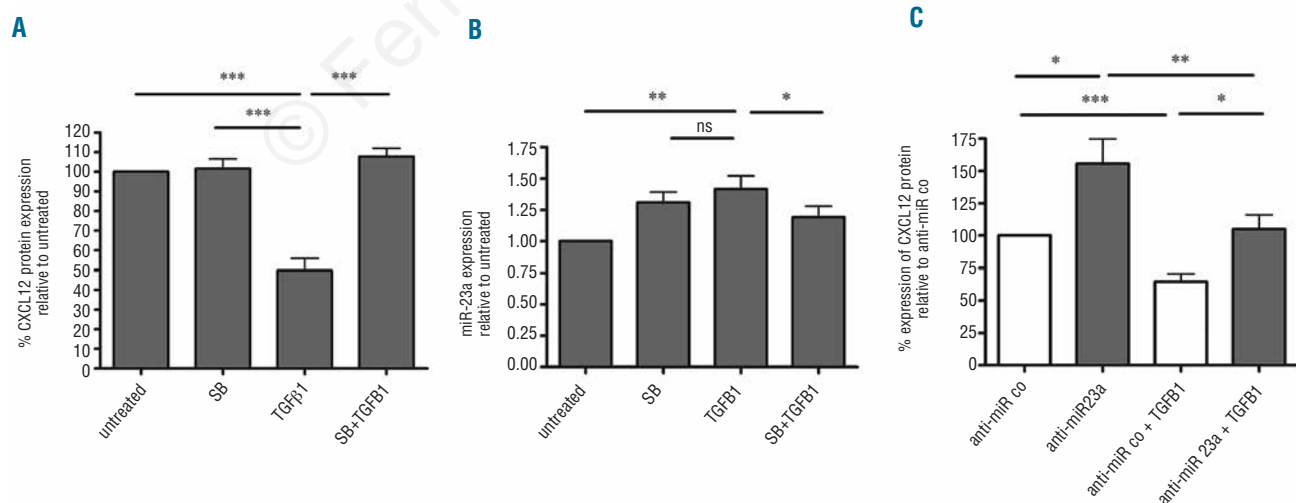
Figure 5. TGFB1-associated CXCL12 downregulation is partially mediated by anti-miR-23a (A–B) Primary hBMSC were either left untreated or treated with TGFB1 (5 ng/mL) in the presence or absence of SB (SB431542) (10 ng/mL) inhibitor for 24 h. (A) The percentages of CXCL12 protein relative to untreated cells were detected in the supernatants (n=12). (B) MiR-23a expression was evaluated by quantitative reverse transcriptase polymerase chain reaction relative to the untreated sample (n=10). U6 snRNA was applied as the internal control. (C) Primary hBMSC were transfected with anti-miR control (anti-miR co) and anti-miR-23a. Cells were left either untreated or treated with 5 ng/mL of TGFB1 for 24 h. The expression of CXCL12 protein in the supernatants of cells was determined by ELISA. Results are illustrated as percentage relative to anti-miR co (n = 12). U6 snRNA served as the internal control. The mean ± SEM are shown. *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

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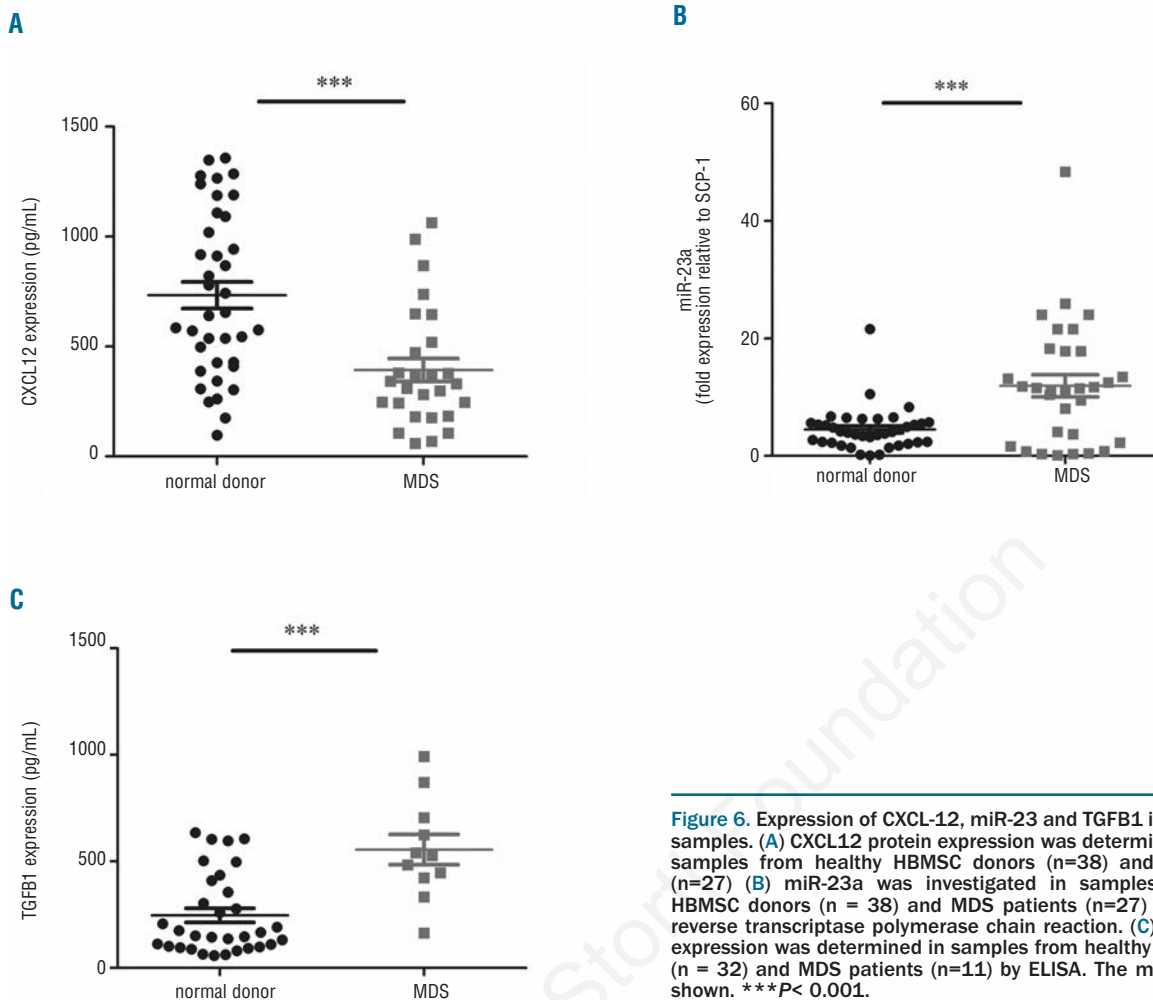
Figure 6. Expression of CXCL-12, miR-23 and TGFB1 in human BMSC samples. (**A**) CXCL12 protein expression was determined by ELISA in samples from healthy HBMSC donors (n=38) and MDS patients (n=27) (**B**) miR-23a was investigated in samples from healthy HBMSC donors (n = 38) and MDS patients (n=27) by quantitative reverse transcriptase polymerase chain reaction. (**C**) TGFB1 protein expression was determined in samples from healthy HBMSC donors (n = 32) and MDS patients (n=11) by ELISA. The mean ± SEM are shown. ***$P < 0.001$.

addition, the TGFB1-pathway is partially dependent on the activity of miR-23a, which was proven by an anti-miR23 strategy, we conclude that early TGFB1 inhibition of the promoter of CXCL12 is followed by a continuous effect, which is mediated at least in part by the upregulation of miR-23a.

The physiological consequences of the described pathway are not clear. However, the role of TGFB1 in HSPC surveillance and hibernation has recently been described in some detail. Mendez-Ferrer *et al.* reported that mobilization of HSPC is enforced via a catecholaminergic-dependent reduction of CXCL12 levels.[27] Simultaneously, the mature TGFB1 molecule is provided by the stromal component of the bone marrow via a catecholamine-dependent mechanism.[33] We, therefore, speculate that a catecholamine-dependent, TGFB1-mediated mechanism may be responsible for the reduction of CXCL12 and upregulation of miR-23a with subsequent stem cell release. We exclude a direct influence of catecholamines on miR-23a as we did not observe altered miR-23a expression after the application of adrenergic substances (*data not shown*).

The TGFB pathway may be critical for the development of a MDS phenotype since mice with constitutive stable expression of TGFB in the bone marrow develop progressive anemia as a hallmark of MDS.[34] Moreover, it has been

reported that hBMSC from patients with high-risk MDS show a significant increase of TGFB1.[35] However, Bhagat *et al.* recently reported that increased activity of the TGFB pathway is common in MDS patients and that loss of inhibitory function of SMAD-7 is responsible for the dysplastic phenotype.[22,23] In addition to these pathophysiological mechanisms exerted by TGFB we describe MDS patients with increased TGFB and miR-23a levels and decreased CXCL12 levels, which is in accordance with the regulatory effect of TGFB on CXCL-12 via miR-23 described here. Although there is currently no clear experimental evidence of how reduced CXCL12 levels could contribute to MDS, there are some data suggesting that loss of CXCL12/CXCR4 signaling could lead to increased HSPC proliferation,[4,24] which may be a precaution for MDS development.

In conclusion, the present study revealed a new mechanism for CXCL12 regulation. It also underlines the pivotal role of TGFB in the bone marrow niche. Because the majority of these data are from *in vitro* research, it is difficult to determine the physiological significance of miR-23a in the regulation of CXCL12 expression in bone marrow stromal cells *in vivo*. Further mechanistic studies are warranted to reveal the physiological consequences of miR-23a. Moreover, we believe that miR-23a acts in con-

cert with other miRNA to regulate TGFB/CXCL12 signaling in the hematopoietic niche.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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