Abnormal changes in the quantity and function of osteoblasts cultured *in vitro* in patients with myelodysplastic syndrome

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Abstract. Changes in bone marrow niches can lead to the occurrence of myelodysplastic syndrome (MDS). As an important part of the bone marrow niche, osteoblasts serve a key role in the progression of MDS. The present study investigated the quantity and function of osteoblasts and, through *in vitro* assays, detected changes in signaling pathways and the association with progression in MDS patients. The ratios of osteoprogenitors (CD34+OCN+) and OCN+CD34-Lin- osteoblasts in MDS patients were significantly less than those of normal controls. The results of this study demonstrated that the quantity and activity of osteoblasts in MDS patients were lower than those in normal controls. Furthermore the activity of osteoblasts in patients correlated with the severity of MDS. The quantity of osteoblasts cultured *in vitro* from high-risk and very high-risk MDS patients (WHO Classification-Based Prognostic Scoring System score 3-6) was decreased. The levels of T-cell immunoglobulin and mucin domain-containing 3 (TIM3) and Jagged 1 were also increased in the osteoblasts *in vitro*. These results indicated that osteoblasts are abnormally altered in MDS patients, and that there are associations between abnormal changes of osteoblasts and the severity of MDS.

Introduction

Myelodysplastic syndrome (MDS) represents clonal disorders primarily of the elderly, and is characterized by ineffective hematopoiesis and an increased risk of transformation into acute myeloid leukemia. Previous research has indicated that changes in bone marrow niches, such as abnormal interactions of hemopoietic stem cells (HSCs) and malignant clones, abnormal secretion of hematopoietic growth factors and cytokines, may contribute to the pathogenesis of MDS (1). Previous studies identified that mesenchymal cells in the bone marrow niche, including osteoblasts, osteoprogenitors, and reticular and fat cells, serve an important role in hematopoiesis regulation (2). Osteoblastic cells are critical for early differentiation of stem cells, and the destruction of osteoblasts can accelerate the progression of certain malignant hematological diseases (3).

Osteoblasts are bone-forming cells that secrete calcium and synthesize the bone matrix and typically cover the endosteal bone surface to form an interface between calcified bone and marrow cells, which are reported to provide signals required for HSC quiescence, long-term maintenance and bone marrow retention (4). The study Raaijmakers *et al* (5) provided evidence that disturbance of the endosteal niche can result in MDS. Deletion of Dicer 1, an RNase III endonuclease, in osteoprogenitors was demonstrated to impair osteoblastic differentiation *in vitro* and *in vivo*. These mice developed fatal neutropenia with hyperplastic bone marrow and dysmyelopoiesis, which is highly suggestive of MDS. Their research was the first to demonstrate that a change of the bone marrow niche caused by the destruction of osteoblasts led to the occurrence of MDS.

Osteocalcin (OCN) is a bone-specific protein that is closely related to the mineralization of osteoblasts, and reflects the osteoblast mineralization ability. OCN (also referred to as bone γ-carboxyglutamic acid-containing protein) is the most abundant non-collagenous protein found in bone (6). It is uniquely synthesized and secreted by mature osteoblasts and osteocytes, and is widely used as a marker for bone formation.

A number of studies have demonstrated that the Notch signaling pathway contributes to the occurrence and development of genetic diseases, autoimmune diseases and various types of cancer (7). Jagged 1 (JAG1) is the most highly expressed Notch ligand during skeletal development and healing, and participates in the regulation of bone metabolism in a compartment-dependent manner.

T-cell immunoglobulin and mucin domain-containing 3 (TIM3; also know as hepatitis A virus cellular receptor 2) is a negative regulator of T cells. TIM3+ HSCs in MDS display aberrant differentiation, over-proliferation and decreased apoptosis (8).

Research regarding osteoblasts in MDS patients is scarce. The present study aimed to address whether any changes in

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osteoblasts occurred in patients with MDS, and to assess their role in the bone marrow niche. The quantity and function of osteoblasts and osteoprogenitors were investigated; furthermore, the associations between changes in osteoblasts and the course of MDS, and the pathogenesis of the interactions between osteoblasts and bone marrow niches in MDS were explored.

Materials and methods

Patients. A total of 38 untreated patients (20 males and 18 females; median age, 63 years; age range, 27-77 years) with MDS, who were treated at the Hematology Department of Tianjin Medical University General Hospital (Tianjin, China) between September 2015 and September 2016 were enrolled in the present study (Table I). Patients were diagnosed according to the World Health Organization (WHO) classification (9) and were divided in two groups according to the WHO Classification-based Prognostic Scoring System (WPSS) score. Group 1 (18 patients) consisted of patients with a WPSS score of 0-2, and Group 2 (20 patients) consisted of patients with a WPSS score of 3-6 (10). In addition, 25 healthy donors (10 males and 15 females; median age, 56 years; age range, 19-72 years) were enrolled as controls. The ethics committee of Tianjin Medical University General Hospital approved this study. All patients provided written informed consent for the use of their clinical specimens for medical research.

Cell culture. A total of 5 ml fresh bone marrow specimens were collected in heparin-anticoagulant tubes from patients with MDS as specified previously. Specimens were added to Ficoll® PM 400 Histopaque®-1077 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 ml and centrifuged (700 x g) for 20 min at room temperature. Following centrifugation, there was obvious stratification: The upper layer was the plasma, the middle was the transparent separation liquid, and the buffy coat between the plasma and the separation liquid was the bone marrow mononuclear cells (BMMNC), collecting the buffy coat carefully with sterile pipettes. The BMMNC were cultured at a density of $3x10^5$ cells/cm^2 in 6-well culture plates with Dulbecco's modified Eagle's medium (DMEM), 15% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), $1x10^{-7}$ mmol/l dexamethasone, 0.01 mol/l β-glycerophosphate, 0.05 g/l vitamin C (all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/l penicillin and phytomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37°C with 5% CO_2. Cultures were refreshed by replacing the medium bi-weekly. Until confluence, the cultures were isolated with 0.5 ml 0.25% parenzyme (Gibco; Thermo Fisher Scientific, Inc.) each well for 3 min and subcultured at a density of $1x10^4$ cells/cm^2 in 6-well culture plates under identical conditions. Following two subcultures, osteoblasts differentiated from bone marrow were obtained. Osteoblasts were collected on the 6th day follwoing two subcultures. Osteoblasts from each well of the culture plates were placed into 1 ml buffer (PBS plus 2% FBS). The numbers of cells were determined under a light microscope (x100 magnification) (BX53; Olympus Corporation, Tokyo, Japan). Cultures were observed by inverted microscopy, with images captured daily. A cell growth curve was drawn to calculate the cell doubling time.

Immunohistochemical staining. Following two subcultures, osteoblasts were cultured at a density of $1x10^5$ cells/cm^2 in 6-well culture plates with cover slips for 3 days. Following washing with PBS, the osteoblasts on cover slips were identified by type-I collagen staining and intracellular alkaline phosphatase (ALP) activity (qualitatively and quantitatively assessed by fast violet staining). Osteoblasts showing mineralization (identified by von Kossa staining) were also measured.

Type-I collagen staining. Osteoblasts were fixed by 10% formaldehyde for 15 min at 4°C, washed with Hank's balanced salt solution (HBSS) for 5 min, stained at 4°C with rabbit anti-human collagen type I antibody (cat. no. BS-0578R; Bioss, Beijing, China; dilution, 1:100) for 30 min and developed with an SP kit, according to the manufacturer's protocol (cat. no. SP-0023; Zymed, Waltham, MA, USA). Under light microscopy, brown-yellow was considered to indicate a positive result.

ALP staining. Osteoblasts were fixed in 10% formaldehyde for 15 min at 4°C and washed with distilled water for 5 min. Staining was performed according to the ALP Kit (no. 507N; Sunbio, Beijing, China). Under light microscopy, brown-red was considered to indicate a positive result.

von Kossa staining. Following a 3-week culture, osteoblasts were fixed in 10% formaldehyde for 1 h, washed with distilled water for 5 min, and fixed in 10% formaldehyde for 1 h; 2% silver nitrate was then added and the cells were incubated in the dark at room temperature for 30 min. Finally, the cells were washed with distilled water for 5 min and exposure to sunlight and left to air-dry for 15 min. Under light microscopy, black nodules were considered to indicate a positive result.

Flow cytometry (FCM). Bone marrow samples (0.5 ml) were collected in heparin sodium-anticoagulant tubes then incubated with 1 ml erythrocyte lytic solution (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 10 min, then washed twice with PBS. Osteoblasts cultured *in vitro* were harvested. At harvesting, 0.5 ml 0.25% parenzyme (Gibco; Thermo Fisher Scientific, Inc.) was added to each well for 3 min, and then 1 ml PBS was added. Following gentle mixing with a plastic pipette. Cells were washed with PBS three times, the samples remained suspended in PBS and were incubated with 5 µg mouse anti-human direct-labeled immunoglobulin G antibodies. All antibodies were incubated at 4°C for 30 min in the dark. The dilution of all direct-antibodies were used at 1:10. No secondary antibody was used in this experiment. Anti-CD34-PerCP (cat. no. 555823 BD Biosciences), anti-Lin-FITC (cat. no. 562722 BD Biosciences) and anti-OCN-allophycocyanin (APC) (cat. no. 557833 BD Biosciences) staining was used to measure the normal osteogenic function by osteoblasts cultured *in vitro*. Anti-CD34-PerCP (cat. no. 555823 BD Biosciences) and anti-OCN-PE (cat. no. 564146, BD Biosciences) staining was used to identify osteoprogenitor cells in the bone marrow. The percentages of JAG1$^+$ osteoblasts and TIM3$^+$ HSCs were measured by FCM using anti-JAG1-PE (cat. no. 565495 BD Biosciences) and anti-TIM3-PE antibodies (cat. no. 563422, BD Biosciences). A minimum of 30,000 cells were acquired.

Data acquisition and analysis were performed using fluorescence-activated cell sorting analysis (FACS; FACScalibur™; BD Biosciences) and CellQuest™ software version 6.0 (BD Biosciences).

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation. Groups of data were compared by Student's t-test or one-way analysis of variance (ANOVA). Multi group comparisons of the means were carried out by one-way ANOVA test with post hoc contrasts by Student-Newman-Keuls test. Pearson's correlation analysis was used to assess the correlations between the activity of osteoblasts and severity of MDS $P<0.05$ was considered to indicate a statistically significant difference.

Results

Osteoprogenitors are significantly fewer in MDS patients than in normal controls. The phenotypes and growth characteristics of osteoblasts are shown in Fig. 1. The osteoblasts were characterized as large, fusiform and as growing in parallel or spiral patterns. Cells adhered in 24 h and the cell doubling time was ~36 h. After 2 weeks, the cells concentrated and formed nodes. The quantity of osteoblasts from normal controls were increased compared with that of osteoblasts from MDS patients (Fig. 1A and B). Type-I collagen staining (Fig. 1C), ALP staining (Fig. 1D) and von Kossa staining (indicating mineralization) (Fig. 1E) were positive, thereby identifying osteoblasts. The quantity of osteoblasts from patients with MDS ($5.11\pm0.61\times10^5$/ml) and Group 2 ($4.62\pm0.62\times10^5$/ml) was less than that of normal controls ($6.79\pm0.79\times10^5$/ml) ($P<0.05$), whereas that of osteoblasts from Group 1 ($5.75\pm1.16\times10^5$/ml) was close to that of normal controls ($P>0.05$) (Fig. 1F).

The percentage of osteoprogenitors (CD34$^+$OCN$^+$) out of all the CD34$^+$ cells in patients with MDS was also measured (Fig. 2). The ratio of osteoprogenitors in MDS patients ($6.29\pm1.27\%$) was less than that in normal controls ($13.01\pm1.26\%$; $P<0.05$). The percentage of osteoprogenitors in Group 1 ($7.59\pm2.26\%$) was not significantly different from that in normal controls ($P>0.05$); however, Group 2 exhibited a significantly decreased percentage ($5.39\pm1.49\%$) as compared with normal controls ($P<0.01$; Fig. 2).

Osteoblast activity is significantly downregulated in patients with MDS and significantly correlates with the severity of MDS. The ratio of OCN$^+$CD34$^-$Lin$^-$ osteoblasts of MDS patients ($3.61\pm0.47\%$) was less than that in normal controls ($6.55\pm1.46\%$; $P<0.05$). Group 1 ($4.67\pm0.85\%$) did not differ significantly from normal controls ($P>0.05$), whereas Group 2 ($2.83\pm0.43\%$) showed a significant decrease in OCN$^+$CD34$^-$Lin$^-$ osteoblasts compared with normal controls ($P<0.05$; Fig. 3).

This study also observed the association between the activity of osteoblasts and severity of MDS. The results of this study demonstrated that the ratio of OCN$^+$CD34$^-$Lin$^-$ osteoblasts in MDS patients was negatively correlated with

Table I. Characteristics of the patients with MDS enrolled in this study (n=38).

Characteristic	Value
Sex, n	
Male	20
Female	18
Age, years	
Median	63
Range	27-77
Cytogenetics	
+8	2
-7	1
20q-	1
Type of MDS, n (%)	
RCUD	1 (2.6)
RARS	2 (5.3)
RCMD	10 (26.3)
RAEB-1	16 (42.1)
RAEB-2	9 (23.7)
WPSS score, n (%)	
0	0
1	3 (7.9)
2	15 (39.5)
3	10 (26.3)
4	10 (26.3)
5-6	0

MDS, myelodysplastic syndrome; RCUD, refractory cytopenia with unilineage dysplasia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess blasts type 1; RAEB-2, refractory anemia with excess blasts type 2; WPSS, WHO prognostic scoring system (2011).

the blast count in the bone marrow smear ($r=-0.480$, $P<0.01$), and was positively correlated with hemoglobin level ($r=0.367$, $P<0.05$) and neutrophil count ($r=0.402$, $P<0.05$; Fig. 4) in the peripheral blood.

TIM3 and JAG1 are overexpressed on osteoblasts from patients with MDS. The percentage of JAG1$^+$OCN$^+$CD34$^-$Lin$^-$cells out of the total number of osteoblasts in patients with MDS ($24.34\pm3.55\%$) were higher than that of normal controls ($12.54\pm3.04\%$; $P<0.05$; Fig. 5). In addition, the percentage of TIM3$^+$CD34$^+$Lin$^-$ cells out of osteoblasts in patients with MDS ($0.87\pm0.23\%$) was higher than that of normal controls ($0.20\pm0.07\%$; $P<0.05$; Fig. 6).

Discussion

Approximately 30% of MDS patients develop acute leukemia, and the bone marrow niche is associated with the apoptosis, proliferation and migration of leukemia cells. Osteoblasts serve an important role in the stability of normal HSCs
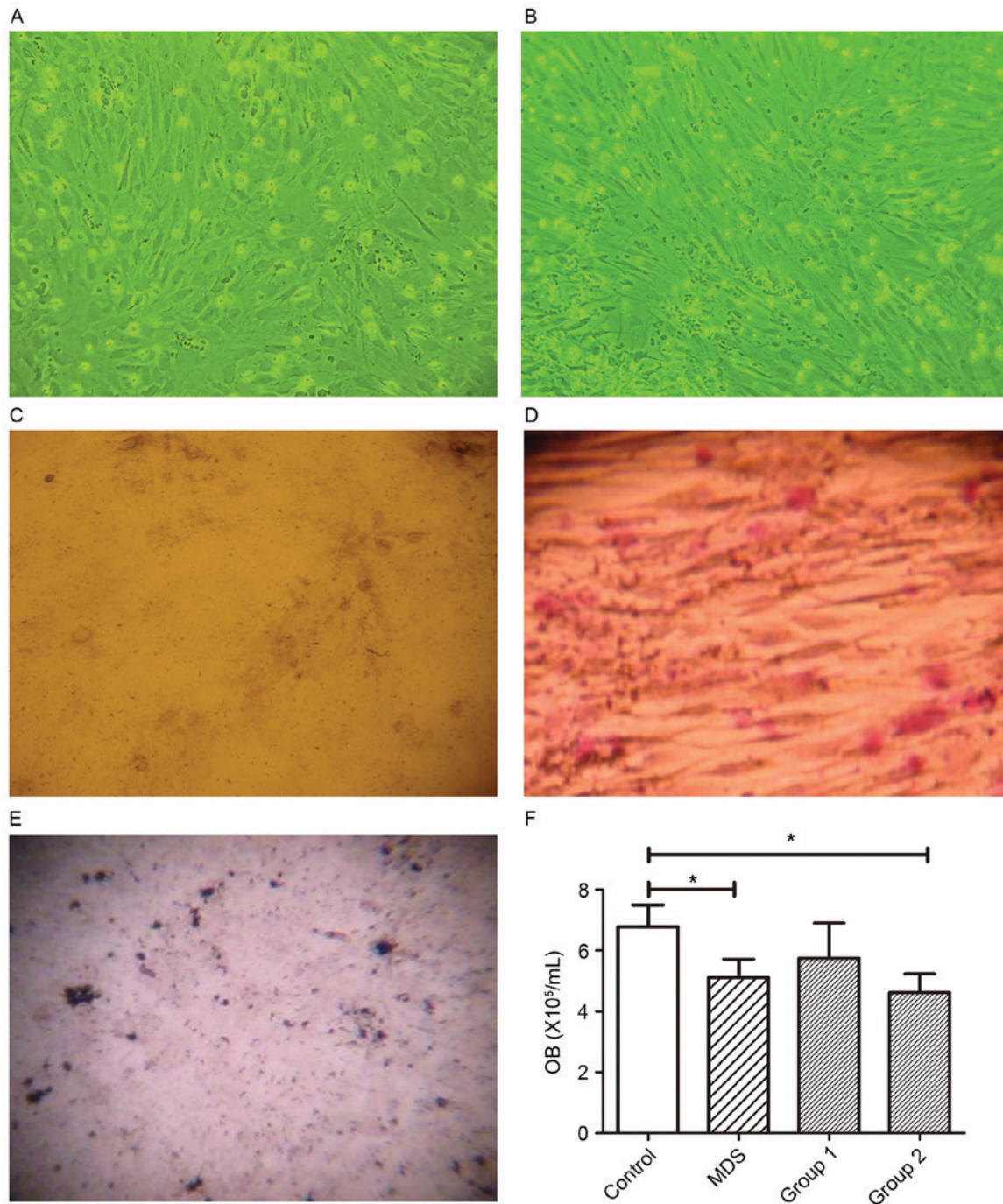
Figure 1. Osteoblasts in MDS patients and normal controls. The quantity of osteoblasts from (A) MDS patients was less than that of (B) normal controls under inverted microscope (magnification, x100). (C) Type-I collagen staining, (D) alkaline phosphatase staining and (E) von Kossa staining (indicating mineralization) were positive, which confirmed that the cultured cells were osteoblasts (magnification, x100). (F) The quantity of osteoblasts was detected on the 6th day after two subcultures. The quantity of osteoblasts from MDS patients and Group 2 was less than that of normal controls (*P<0.05), whereas that of osteoblasts from Group 1 was close to that of normal controls. Data are presented as the mean ± standard deviation. OB, osteoblasts; MDS, myelodysplastic syndrome.

via intracellular expression of JAG1 and other cytokines. Schepers *et al* (11) indicated that osteoblasts were remodeled in myeloproliferative neoplasia. Following abnormal remodeling, osteoblasts could better support tumor cell survival, but inhibited normal HSCs significantly. The present study used CD34^{+}OCN^{+} as a marker for osteoprogenitor cells (12,13), and identified that the percentage of osteoprogenitors was significantly decreased in MDS patients, thereby demonstrating that the differentiation pathway of HSC into osteoprogenitors may be abnormal in MDS.

A previous study demonstrated that dexamethasone, β-glycerophosphate and vitamin C could induce the differentiation of osteoblasts from bone marrow mesenchymal stem cells and their osteogenic function *in vitro* (14). In the present study, bone marrow cells in culture were induced to differentiate into osteoblasts by a nutrient solution with conditional factors such as dexamethasone, β-glycerophosphate and vitamin C *in vitro*. Patients in Group 1 were those with low- and intermediate-risk MDS (WPSS score 0-2), and patients in Group 2 had high- and very high-risk MDS (WPSS score 3-6). The results
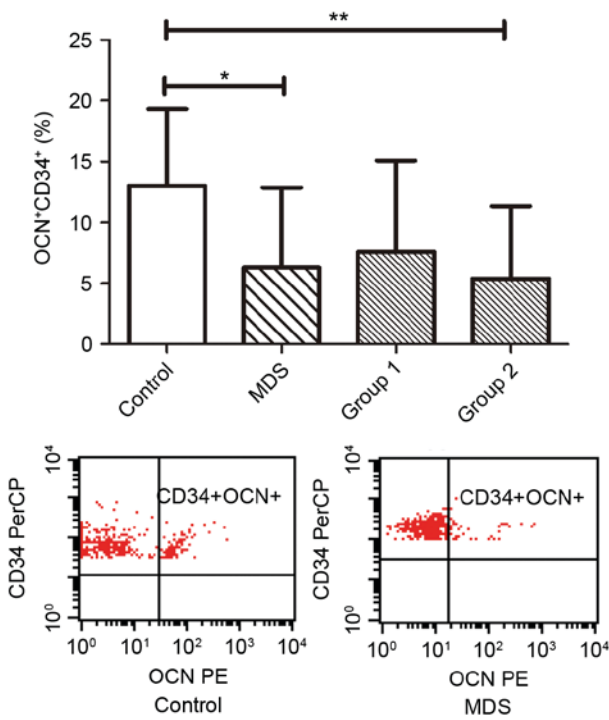
Figure 2. Osteoprogenitors in the bone marrow. The ratio of CD34+OCN+ cells from MDS patients was less than that of normal controls ([*]P<0.05), and those from Group 2 were great less than that of normal controls ([**]P<0.01). Data are presented as the mean ± standard deviation. MDS, myelodysplastic syndrome; OCN, osteocalcin; PE, phycoerythrin; PerCP, peridinin chlorophyll.
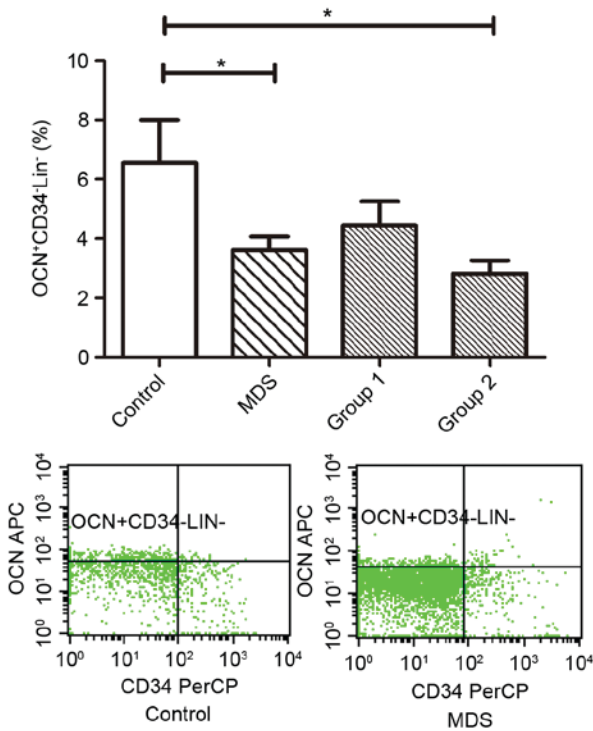

Figure 3. Activity of osteoblasts. The percentage of OCN+CD34-Lin- cells among osteoblasts from MDS patients was less than that of normal controls ([*]P<0.05). The percentage in Group 1 was close to that of normal controls (P>0.05), whereas the percentage in Group 2 was significantly less than that of normal controls ([*]P<0.05). Data are presented as the mean ± standard deviation. MDS, myelodysplastic syndrome; OCN, osteocalcin; APC, allophycocyanin; PerCP, peridinin chlorophyll.
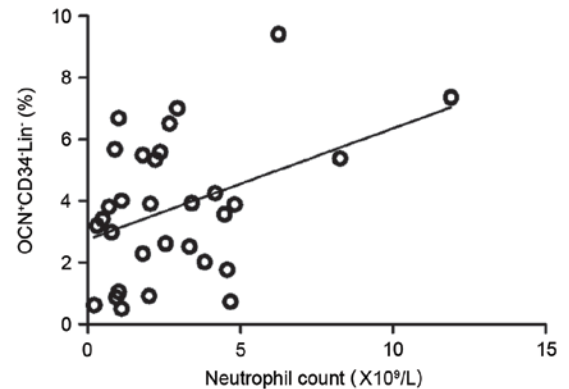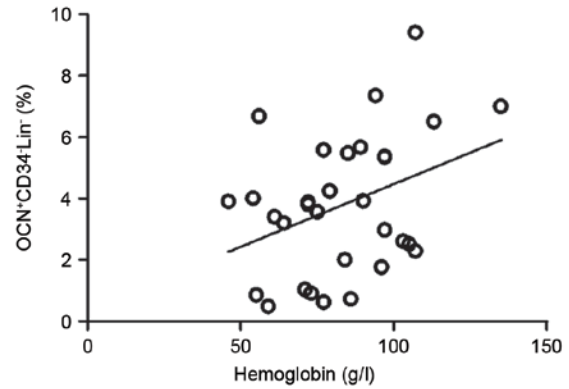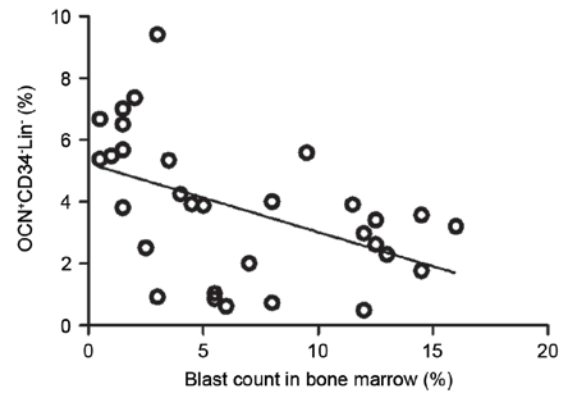

Figure 4. Correlation between the activity of osteoblasts from MDS patients and the severity of MDS. The percentage of OCN+CD34-Lin- cells among osteoblasts from MDS patients was negatively correlated with the blast count in bone marrow (P<0.01) and was positively correlated with hemoglobin level (P<0.05) and neutrophil count (P<0.05) in the peripheral blood of the MDS patients. MDS, myelodysplastic syndrome; OCN, osteocalcin.

demonstrated osteoblasts were decreased in patients with MDS especially in high-risk MDS, when compared with the normal control group.

OCN reflects the mineralization ability of osteoblasts. Carboxylated OCN, produced by bone-forming osteoblasts, has a high binding affinity for mineralized bone matrix (4,15). The present study used OCN+CD34-Lin- as a marker for mineralization and osteogenic potential of mature osteoblasts *in vitro*. The quantity and osteogenic potential of osteoblasts in MDS was lower, as indicated by a significantly decreased percentage of OCN+CD34-Lin- cells. The quantity and activity of osteoblasts in low-risk MDS patients was close to normal
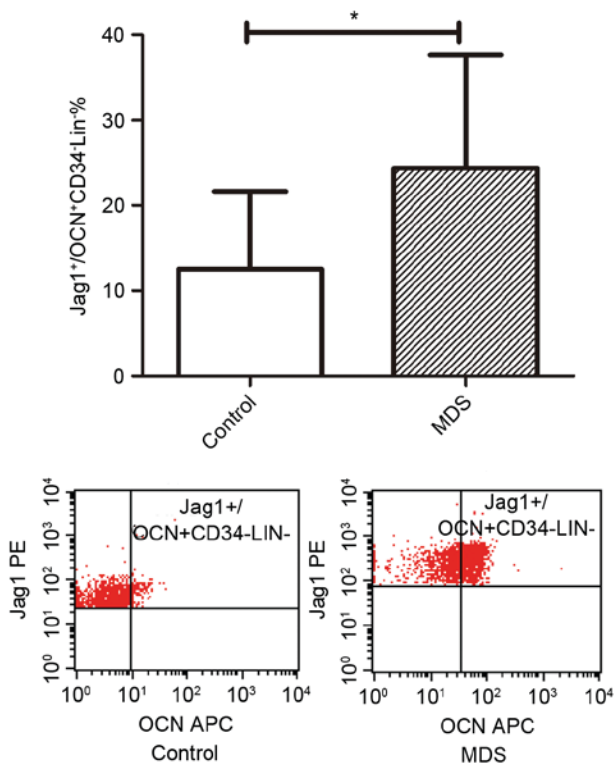
Figure 5. JAG1 expression on osteoblasts. The percentage of JAG1$^+$ cells out of the total number of OCN$^+$CD34$^-$Lin$^-$ osteoblasts was higher in MDS patients than in normal controls (*P<0.05). Data are presented as the mean ± standard deviation. MDS, myelodysplastic syndrome; OCN, osteocalcin; JAG1, Jagged 1.
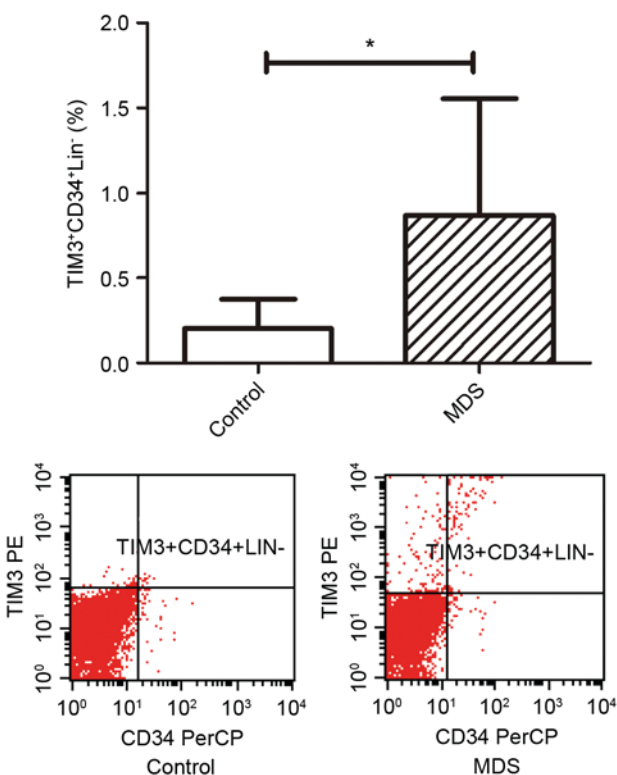

Figure 6. TIM3 expression on osteoblasts. The percentage of TIM3$^+$CD34$^+$Lin$^-$ cells among osteoblasts was higher in MDS patients than in normal controls (*P<0.05). Data are presented as the mean ± standard deviation. MDS, myelodysplastic syndrome; OCN, osteocalcin; TIM3, T-cell immunoglobulin and mucin domain-containing 3.

levels. Furthermore, the ratio of OCN$^+$CD34$^-$Lin$^-$ osteoblasts was negatively correlated with the blast count in the bone marrow and positively correlated with hemoglobin level and neutrophil count in the peripheral blood of the patients. The association between higher blast count in the bone marrow and decreased activity of osteoblasts demonstrated that there is a close association between the activity of osteoblasts in MDS patients and the severity of MDS.

In MDS, Notch signaling serves an important role in the development of drug resistance. Activation of this pathway occurs when a Notch ligand (JAG1 or 2, or Delta-like 1, 3 or 4) expressed on the surface of a signaling cell interacts with a Notch receptor (Notch 1-4) expressed on the surface of a receiving cell (16). Activating mutations of β-catenin in osteoblasts, which are commonly identified in patients with MDS, lead to increased synthesis of the Notch ligand JAG1, which in turn activates Notch signaling in HSCs, leading to alteration of the differentiation potential of hematopoietic progenitors and acute myeloid leukemia development (17). In the present study, the Notch ligand JAG1 was found to be increased in osteoblasts from MDS patients, indicating that the Notch pathway in osteoblasts was altered abnormally in MDS.

Bone formation and the immune system are closely associated through cellular and molecular interactions (18). The interaction of TIM3 with its ligand, Galectin-9, promotes apoptosis of T-helper 1 cells, and induces CD8$^+$ T cell exhaustion with decreased production of interferon γ; it also induces the expansion of myeloid-derived suppressor cells, which suppress immune responses indirectly. TIM3, as a negative regulator of anti-tumor immunity (19), is highly expressed on HSCs in MDS patients, and the high level of TIM3 expression on HSCs in MDS patients is closely associated with the WPSS score (20). In the present study, the percentage of TIM3$^+$CD34$^+$Lin$^-$ cells among the osteoblasts of MDS patients was higher than that of normal controls. The results showed the osteoblasts and HSC were homologous in bone marrow niche, and that the differentiation of osteoblasts is abnormal in MDS. The osteoblasts with abnormal immune expression may be one of the reasons the bone marrow niche is affected in patients with MDS.

In conclusion, the quantity of osteoprogenitors in the bone marrow of MDS patients was decreased when compared with that of normal controls. When cultured *in vitro*, the quantity of osteoblasts derived from high- and very high-risk MDS patients was decreased significantly. The osteogenic potential of osteoblasts was also decreased in MDS patients compared with normal controls, particularly those patients with high- and very high-risk MDS (WPSS score 3-6). The activity of osteoblasts from patients was correlated with the severity of MDS. JAG1 and TIM3 were highly expressed on the osteoblasts *in vitro*. Osteoblasts and HSCs are homologous in the bone marrow niche of MDS patients. These results indicated that, as an important part of the bone marrow niche, osteoblasts were abnormal in MDS. Certain abnormal changes were associated with the severity of MDS. Further study is required in order to determine whether the bone marrow niche can be altered by correcting the abnormal activity of osteoblasts, which can be used to assist the treatment of MDS.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its accompanying images.

Authors' contributions

SG carried out the cell culture and statistical analyses and drafted the manuscript. SG, HY, JT and CL conducted the flow cytometry. HW and HJ assisted in the design of the study and performed the statistical analysis. RF oversaw the collection of the data and performed the statistical analysis. ZS conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The ethics committee of Tianjin Medical University General Hospital approved this study. All patients provided written informed consent for the use of their clinical specimens for medical research.

Patient consent for publication

All of the patients in this study provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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