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RESEARCH

The TPO/c-MPL Pathway in the Bone Marrow may Protect Leukemia Cells from Chemotherapy in AML Patients

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Abstract Accumulating evidence indicates that the interaction of human LSCs (leukemic stem cells) with the hematopoietic microenvironment, mediated by the thrombopoietin (TPO)/c-MPL pathway, may be an underlying mechanism for resistance to cell cycle–dependent cytotoxic chemotherapy. However, the role of TPO/c-MPL signaling in AML (acute myelogenous leukemia) chemotherapy resistance hasn't been fully understood. The c-MPL and TPO levels in different AML samples were measured by flow cytometry and ELISA. We also assessed the TPO levels in the osteoblasts derived from bone mesenchymal stem cells (BMSCs). The survival rate of an AML cell line that had been co-cultured with different BMSC-derived osteoblasts was measured to determine the IC_{50} of an AML chemotherapy drug daunorubicin (DNR). The levels of TPO/c-MPL in the initial and relapse AML patients were significantly higher than that in the control ($P<0.05$). The osteoblasts derived from AML patients' BMSCs secreted more TPO than the osteoblasts derived from normal control BMSCs ($P<0.05$). A strong positive correlation between the TPO level and c-MPL expression was found in the bone marrow mononuclear cells of the relapse AML patients. More importantly, the IC_{50} of DNR in the HEL + AML-derived osteoblasts was the highest among all co-culture systems. High level of TPO/c-MPL signaling may protect LSCs from chemotherapy in AML. The effects of inhibition of the TPO/c-MPL pathway on enhancing the chemotherapy sensitivity of AML cells, and on their downstream effector molecules that direct the interactions between patient-derived blasts and leukemia repopulating cells need to be further studied.

Keywords Thrombopoietin · c-MPL · Acute myeloid leukemia · Chemotherapy resistance · Relapse

Introduction

Acute myelogenous leukemia (AML) is the most common acute leukemia affecting adults, characterized by clonal expansion of immature myeloblasts initiating from rare leukemic stem cells (LSCs). The long-term outlook for adult patients with AML remains dismal because a majority of patients will eventually relapse even after obtaining complete remission following front-line chemotherapy. Resistance to chemotherapy is still a challenge for the treatment of AML and increasing studies on its influencing factors and new therapeutic strategies become a hot spot of the leukemia research [1–3].

Available data indicate that LSCs, like normal hematopoietic stem cells (HSCs), have a hierarchical structure including a stem cell compartment with self-renewal capability, endowed in a neoplastic niche bearing resemblance to its normal hematopoietic counterpart [4, 5]. Moreover, Fumihiko Ishikawa et al. reported that LSCs homed to and engrafted within the osteoblast-rich area of the bone marrow where AML cells are protected from chemotherapy-induced apoptosis [6] . Accumulating evidence indicates that the interaction of human LSCs with the hematopoietic microenvironment, mediated by prosurvival cell adhesion process, may be a mechanism underlying resistance to cell cycle–dependent cytotoxic chemotherapy [2, 5, 7–10].

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Bone marrow microenvironment is composed of a three-dimensional structure containing stromal cells as the main tissue, some adhesion molecules, and growth chemokines. Stromal cells consist of osteoblasts, fibroblasts, endothelial cells and tissue cells. Recent studies have shown that the osteoblasts in marrow stroma secrete thrombopoietin (TPO). Its receptor c-MPL is not only expressed in different maturation stages of megakaryocyte, or early hematopoietic stem/progenitor cells, but also expressed in the primitive cells of AML, chronic myeloid leukemia and myelodysplastic syndrome patients [11, 12]. In addition to its role in thrombopoiesis, TPO has also been shown to play a role in maintaining HSC quiescence during hematopoiesis. So differentiation of HSCs can be balanced against self-renewal and quiescence to maintain the stem cell pool throughout life [13–15]. Yoshihara et al. have reported that MPL-expressing hematopoietic cells in the bone marrow are in close proximity to TPO-producing osteoblastic niche cells, suggesting that the TPO/MPL signal is highly enriched in that microenvironment to maintain HSC in their quiescent state [16].

Early studies have found that MPL mRNA expression is detectable in LSCs of AML and MPL^+ cells in response to exogenous TPO for proliferation in vitro [17, 18]. However, the correlation between TPO/cMPL expression levels and relapse of AML is not fully understood. It remains an open question as to how TPO/cMPL signaling pathway affects the resistance to chemotherapy of leukemia cells.

In our study, we collected the bone marrow samples from initial and relapse AML patients and determined the expression levels of TPO and c-MPL in the AML cells. We successfully induced bone marrow mesenchymal stem cells (BMSCs) into osteoblasts, which enabled us to evaluate TPO secretion of the osteoblasts and c-MPL expression of the AML cell line HEL cells. Furthermore, we co-cultured the induced osteoblasts and HEL cells in vitro in a two-dimensional co-culture system to investigate the role of TPO/c-MPL signaling pathway in osteoblast niche which protects the leukemia cells from chemotherapy. The sensitivity of HELs to daunorubicin (DNR), a chemotherapeutic drug of AML, in contexts of different interacting osteoblastic microenvironment were measured. We anticipate that our study could provide a new idea for the treatment of AML recurrence and resistance.

Materials and Methods

Case Collection

All clinical investigations detailed in the manuscript were conducted in accordance with the Declaration of Helsinki. These studies have been approved by the appropriate institutional human research committee. Sixty-eight bone marrow samples of AML patients were collected from Sep.2010 to Dec.2011. The median age is 40 years old (ranging from 8 to 72 years old). The median numbers of WBC (White Blood Cell), PLT (Platelet), HGB (hemoglobin) are 9.8×10^9/L (range: 0.6–221×10^9/L), 88×10^9/L (range:18–215×10^9/L), and 82 g/L (range: 11–130 g/L), respectively. The AML group included 33 initial AML patients (15 males and 18 females), among which 24 cases were completely relieved and 9 cases were refractory AML, and 35 relapse patients (16 males and 19 females). All the patients were classified by FAB type. Numbers of M_1, M_{2a}, M_3, M_4, M_5, M_6, M_7 between initial and relapse groups were 5 vs. 4, 6 vs. 8, 6 vs. 1, 4 vs. 7, 7 vs. 10, 3 vs. 4, and 2 vs. 1, respectively.

All the cases were also divided by prognostic classification according to chromosome nuclear type analysis. Among initial patients, there were 12 cases in low risk group with karyotype of t (15;17), inv (16), t (16;16), t (8;21), 10 cases in intermediate risk group with karyotype of normal nucleus, +21, +8, and 11 cases in high risk group with karyotype of −5, 5q-and complex karyotype. Among relapse patients, 8 cases in low risk group with karyotype of t (15;17), inv (16), t (8; 21), 11 cases in average risk group with karyotype of normal nucleus, t (9;11), +13, and 16 cases in high risk group with karyotype of −5, 7q-and complex karyotype were included in our study.

A total of 20 bone marrow samples from iron deficiency anemia patients or patients of WBC and platelets mildly abnormal and normal marrow were collected as the control. Their median age is 44-year old (ranging from 10 to 69 years old). All the participants signed informed consents.

Acquisition of Bone Marrow Mononuclear Cells and Supernatant

A volume of 2 ml of bone marrow was isolated from each patient of the initial AML group before chemotherapy, the remission group, the relapse group, and the control group, followed by incubation with heparin as anticoagulant. The blood was centrifuged at 1,000 rpm for 5 min. The resultant supernatant was recovered and frozen at −80 °C.

Red blood cell lysis buffer was added according at a ratio of 1:5. After standing for 5 min, the cells were centrifuged at 1,000 rpm for 5 min. The precipitated cells were washed twice with PBS. The bone marrow mononuclear cells were recovered for detection of the c-MPL levels.

Induction of Bone Mesenchymal Stem Cells (BMSCs) to Osteoblasts

A volume of 2–3 ml bone marrow from 6 of 33 initial AML patients with higher cMPL expression rate (>5 %) and 6 of 20 control patients was collected under sterile conditions and incubated with 1:50 heparin of anticoagulant. The same volume of PBS was mixed with the bone marrow. The mixture was centrifuged at 1,000 rpm for 5 min and the supernatant

was discarded, followed by addition of 4 ml of PBS and equal volume of Percoll separating lysis solution (1.073 g/L). After centrifuging at 2,000 rpm for 20 min, the white film layer of mononuclear cells was collected and washed twice with PBS. The cells were suspend in DMEM with 10 % FBS, seeded in 25-cm^2 plastic flasks at the density of 10^5–10^6/ml, and cultured under the condition of 37 °C, 5 % CO_2 and saturated humidity.

The second generation of bone mononuclear cells was incubated in the special medium which can differentiate them into osteoblasts or fat cells (Cyagen, Guangzhou, China). On the 21st day after induction, osteoblasts were identified.

Culture of hFOB1.19 and HEL Cell Lines

The ossification cell line hFOB1.19 and acute myeloid leukemia cell line HEL were purchased from Shanghai Cell Library of Chinese Academy of Sciences. The hFOB1.19 and HEL cells were resuscitated from the frozen stock, and cultured in DMEM/F_{12} containing 10 % FBS or RPMI1640 medium containing 10 % FBS, respectively.

Two-Dimensional Co-Culture System and Administration of Different Concentrations of Daunorubicin (DNR)

AML cells (HEL cells) were co-cultured with osteoblasts to build a two-dimensional system in vivo. Briefly, 1 ml of HEL cells was added into hFOB1.19 cells (group B), or into osteoblasts differentiated from normal BMSCs (group C), or into osteoblasts differentiated from AML patients' BMSCs (group D), respectively. HEL cells were cultured alone as the control (group A) (see Fig. 6). All the cells were cultured under the condition of 37 °C, 5 % CO_2 and saturated humidity with the cell concentration of 1×10^6/ml.

Different concentrations of daunorubicin (0.01, 0.1, 1, 10, and 100 μg/ml) were added into the groups A-D, respectively. After 24 h incubation, the survival rates of HEL cells under different co-culturing conditions were measured, and the corresponding IC_{50} values of DNR were calculated.

Detection of c-MPL Levels by Flow Cytometry

A total of 100 μl PBS was mixed with the cells. Twenty-five μl of the cell suspension were pipetted into two 1.5 ml tubes, respectively. PE (phycoerythrin)-labeled c-MPL monoclonal antibody (10 μl) and mouse IgG2A (as the blank control) were added and incubated at 4 °C for 30 min. The mixture was centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. A volume of 200 μl PBS was added to resuspend the cells, followed by detection of c-MPL expression by the flow cytometer (Beckman Coulter Inc., USA). The mean signal intensity of $cMPL^+$ cells is about 10 times more higher than c-MPL^- cells. The gating for c-MPL^+ cells in flow cytometry was determined by the fluorescent intensity of the PE and the blank control to exclude false positive.

Enzyme-Linked Immunosorbent Assay (ELISA)

TPO ELISA kits were purchased from Westang Inc., Shanghai. All the procedures were operated according to the kits' manual. TPO-capture antibody was pre-bound to the well surface. A volume of 100 μl antigen-containing standard substrate or samples was applied to each well of the plate and incubated at 37 °C for 120 min. The plate was washed with PBS to remove unbound antigen. A volume of 100 μl specific primary antibody was added. The plate was incubated at 37 °C for 60 min to allow the antigen-antibody reaction. The enzyme-linked antibody (100 μl) was applied incubated at 37 °C for 30 min. The plate was washed with PBS to remove the unbound antibodies, followed by the addition of 100 μl of peroxidase substrates. The plates were shaken and incubated at 37 °C for 15 min until the blue coloration had fully developed. Finally, 100 μl of sulphuric acid were added to stop the reaction. The absorbance at 450 nm wavelength (A450) was measured to determine the protein concentration. The assay was repeated for four times and the average values were used for the statistical analysis.

CCK-8 Detection in the Two-Dimensional Co-Culture System Treated with DNR

A volume of 100 μl cell suspension from group A to group D treated with different concentrations of DNR was mixed with 10 μl of CCK-8 solution in a 96-well plate. The wells containing no cells were set as blanks. The plate was incubated for 2 h before the A450 was measured.

The cell survival rate (%) was calculated as follows: (absorbance of DNR treated group - absorbance of blank)× 100 %/(absorbance of cell control- absorbance of blank). The measurement was repeated four times for statistical analysis.

Statistical Processing

The values are presented as means ± SD. Analysis of variance andLSD-*t* tests were performed where comparisons were indicated in the manuscript. There is statistical difference when $P < 0.05$.

Results

TPO/c-MPL Expression Levels in AML Bone Marrow

Flow cytometry showed that there was no significant difference in the c-MPL expression level between the remission

AML bone marrow mononuclear cells and the normal bone marrow mononuclear cells (2.81±1.306 % vs. 2.84±0.724 %, $P=0.922$). c-MPL level in bone marrow mononuclear cells from initial AML before chemotherapy was higher than that in cells not only from remission AML patients (6.04±4.072 % vs. 2.81 %±1.306 %, $P=0.027$), but also from the control group (6.04±4.072 % vs. 2.84±0.724 %, $P=0.013$). Relapse AML patients had a significantly higher c-MPL level in their bone marrow mononuclear cells than the control group (11.23 ±5.225 % vs. 2.84±0.724 %, $P=0.011$) and the initial AML group before chemotherapy (11.23±5.225 % vs. 6.04± 4.072 %, $P=0.016$).

ELISA showed that the TPO level in the bone marrow supernatant preparations of initial AML group before chemotherapy was significantly higher than that in the control group (162.21±39.92 ng/ml vs. 122.14±25.75 ng/ml, $P=0.025$). After chemotherapy, the level was decreased significantly (162.21±39.92 ng/ml vs. 115.82±22.32 ng/ml, $P=0.049$), and was comparable to the control ($P=0.351$). The TPO level in the relapse AML bone marrow samples was higher than that in the control group (213.19±48.34 ng/ml vs. 122.14± 25.75 ng/ml, $P=0.019$), and was even higher than that in the initial AML group before chemotherapy (213.19±48.34 ng/ml vs. 162.21±39.92 ng/ml, $P=0.014$, Table 1).

Relapse AML patients were predicted for their prognosis according to karyotype and were divided into low-risk, intermediate risk, and high-risk subgroups. The TPO and c-MPL expression levels were not different from each other among the 3 subgroups (Table 2). Furthermore, a positive linear correlation between the TPO in the bone marrow supernatant preparations and the c-MPL level in the bone marrow mononuclear cells was found in the relapse AML patients (Y=102.050+1084.670X, R=0.920, Fig. 1).

Successful Differentiation of Bone Mesenchymal Stem Cells from AML Patients or Normal Controls into Osteoblast and Culture of Osteoblast Cell Line hFOB1.19 In Vitro

Bone mesenchymal stem cells (BMSCs) were isolated from the AML patients' or the normal bone marrow. They appeared in a spindle, spiral shape or a radial arrangement (Fig. 2). Flow cytometry showed that the BMSCs were positive for CD44 (98.71 %) and CD29 (98.71 %), and negative for CD34 and CD45 (0.27 % and 0.00 %, respectively).

When the BMSCs were differentiated into fat cells by the special medium, the cells expanded increasingly in size. On the 7th day of induction, small lipid drops that had strong refraction were seen in a scattered distribution. With the progression of incubation time, the number of lipid drops increased. They fused together gradually, and pushed the nucleus to the other side. On the 14th day, the lipid drops were stained red by Oil Red O. When the BMSCs were cultured in the special medium which induced them into osteoblasts, their shape was changed to spindle patterns. From the 7th day of incubation, flocculent sediments were observed with high brightness, and increased gradually on the bottom of the holes. On the 21st day of incubation, a large number of calcium nodules were dried red by alizarin red (Fig. 3).

The hFOB1.19 cells grew well. At their early stage the cells appeared in the shapes of triangle or irregular polygon. Along the incubation, the cells integrated and shaped irregular polygon obviously. Moreover, plenty calcium salt nodules were visible. On the 10th day of incubation, cells grew into multilayer.

Table 1 Comparison of the TPO secretion level and c-MPL expressional level of bone marrow mononuclear cells among groups ($x \pm s$)

Groups	Number	TPO (ng/ml)	cMPL (%)
Before chemotherapy	33	162.21±39.92*	6.04±4.072*
Remission	24	115.82±22.32#	2.81±1.306#
Relapse	35	213.19±48.34*#	11.23±5.225*#
Control	20	122.14±25.75	2.84±0.724

* $P<0.05$, compared with the control

# $P<0.05$, compared with group of before chemotherapy

Table 2 Comparison of the TPO secretion level and c-MPL expressional level of bone marrow mononuclear cells among karyotype subgroups of relapse AML

Karyotype subgroup	Number	TPO (μg/ml)	cMPL (%)
Low-risk	8	208.64±46.223	10.69±5.036
Intermediate risk	11	211.30±55.20	10.62±5.596
High risk	16	214.90±48.91	12.10±5.373

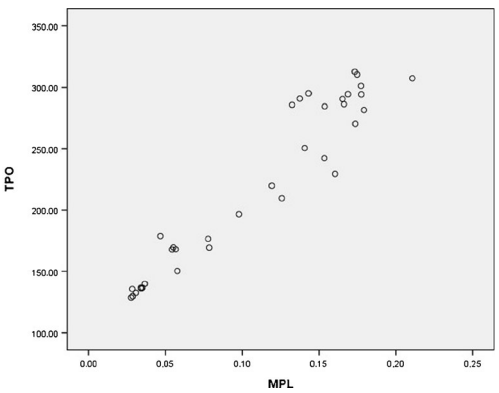

Fig. 1 A strong positive correlation is showed between c-MPL and TPO levels in the bone marrow mononuclear cells of relapse AML. Higher c-MPL level is correlated with stronger TPO expression. The model shown here is determined by the formula Y=102.050+1084.670X (R=0.920)

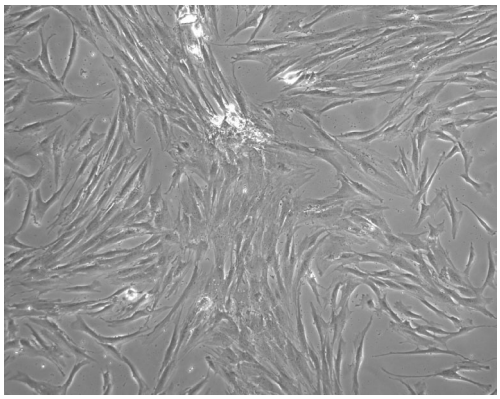

Fig. 2 The bone mesenchymal stem cells (BMSCs) were isolated from the bone marrow of AML patients or normal subjects. The primary culturing cells were observed with an optical microscope (×100) and were found to be in a spindle, spiral shape or radial arrangement

TPO Levels Secreted by BMSCs-Derived Osteoblasts and hFOB1.19

ELISA results showed that the AML BMSCs-derived osteoblasts secreted more TPO than the normal BMSCs-derived osteoblasts on 0th, 7th, 14th, 21st day of incubation ($P<0.05$, Table 3). hFOB 1.19 cells before passages secreted TPO at the same level as the normal BMSCs-derived osteoblasts cultured on 21st day (180.82±17.75 ng/ml vs. 178.84±21.17 ng/ml, $P>0.05$). The TPO level in the hFB1.19 cells was less than that in the AML BMSCs-derived osteoblasts on the 21st day.

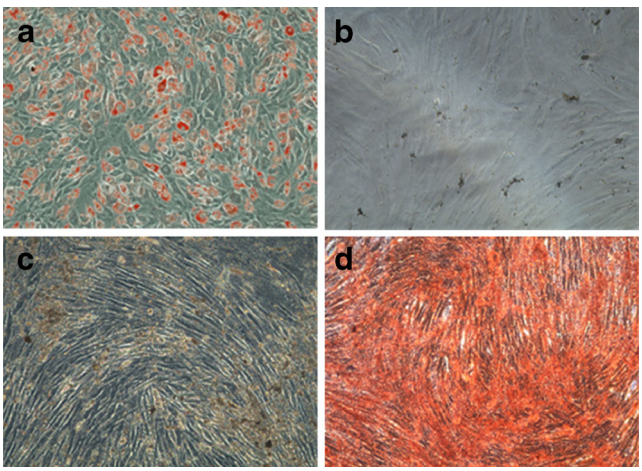

Fig. 3 Human BMSCs can be differentiated into fat cells and osteoblasts by the specific medium. On the 14th day of induction, fat cells were stained red by Oil Red. Large red fat granules were observed in the cytoplasm under the optical microscope (**a**). On the 14th day of induction, the BSMCs were spindle-shaped and radially arranged. Flocculent calcium salt deposit was faintly visible (**b**). On the 21st day of induction, the BMSCs were in an obvious vortex pattern. A large number of calcium salt deposits was formed in nodules (**c**) and stained red by alizarin red (**d**)

c-MPL Level in HEL Cells

The HEL cells were selected as a representative AML cell line. On the 1st day of culture, the cells attached to the bottom of the well, and grew in shapes of triangle or irregular polygon. On the 7th day, calcium salt deposit became faintly visible. The cells grew in multilayer on the 21st day (Fig. 4). Flow cytometry results showed that the c-MPL expression rate was 8.32 % (=9.19 %–0.87 %).

Co-Culture of HEL Cells with Osteoblasts Derived from Bone Marrow

HEL cells were co-cultured with the BMSC-derived osteoblasts (induced at the 21st day) from AML or normal bone marrow, or hFOB1.19, for 48 h, respectively. The osteoblasts grew by static adherence, and had an irregular shape. There was no difference in the morphology of the osteoblasts from different sources. HEL cell aggregates were glomerate and clingy on the osteoblast surface. More HEL cells attached to the AML BMCSs-derived osteoblasts than to the normal BMSCs-derived osteoblasts (Fig. 5).

Comparison of Survival Rate of HEL Cells and IC_{50} After Treatment of DNR for 24 h Among Different two-Dimensional Co-Culture Systems

The survival rate of HEL cells co-cultured with osteoblasts from various sources were measured after incubation for 24 h with different concentrations of DNR (0.01, 0.1, 1, 10, and 100 μg/ml). HEL cell survival rate became decreased along with increasing doses of DNR in all groups. The cell survival rate in the group of HEL + AML-derived osteoblasts was the highest, and that of the HEL-alone group was the lowest under each DNR concentration. Meanwhile, the cell survival rate was not different between the groups of HEL + normal bone marrow derived osteoblasts and HEL + hFOB1.19 (Fig. 6).

IC_{50} of DNR was calculated by SPSS 13.0 software. The IC_{50} in the group of HEL + AML-derived osteoblasts was higher than those of the other three groups. There was no difference in IC_{50} between the groups of HEL + AML-derived osteoblasts and HEL-alone. IC_{50} in the HEL group was the lowest (Table 4).

Discussion

Thrombopoietin is well-known to be the primary regulator of thrombopoiesis. A body of evidence has indicated the role of TPO/c-MPL in stimulating self-renewal and expansion of murine and human hematopoietic stem cells. Given that TPO/c-MPL pathway is active in the HSC compartment, it is reasonable to postulate that activation of the TPO/c-MPL

Table 3 The expression level of TPO secreted by differently derived osteoblasts (ng/ml)

Group	Time (day)			
	0	7	14	21
Induced osteoblasts of AML	163.44±23.31*	174.64±19.92*	191.53±17.83*	205.17±20.16*
Induced osteoblasts of normal	120.28±13.74	140.13±20.12	157.66±18.82	178.84±21.17

* $P<0.05$, compared with induced osteoblasts of normal group on the same day

pathway may play a role in leukemogenesis. An early study found that c-MPL mRNA expression was detectable by Northern blotting [17]. On the other hand, six out of 11 AML samples responded to exogenous TPO with either improved cell survival or proliferation. Serum TPO levels were significantly lower in patients with MPL-expressing AML when compared to AML without MPL expression or ALL, implicating uptake of TPO by the blast cells in the former group [18, 19]. Recently, Wu YX et al. explored the measurement and clinical significance of serum TPO level in patients with AML and healthy controls before chemotherapy, at the stage of bone marrow depression and bone marrow recovery [20–22], respectively. The results showed that the serum TPO level in AML before chemotherapy was significantly higher than that in healthy controls ($P<0.001$). Serum TPO level in the patients at the stages of bone marrow depression or bone marrow recovery was significantly lower than that in patients before treatment ($P<0.001$), and was not significantly different from the healthy controls [23]. Little is known regarding the changes and coherence of bone marrow TPO/c-MPL levels in initial AML before and after the chemotherapy, and in the relapse AML.

In our study, we found that TPO/c-MPL levels of myeloblasts in initial AML before chemotherapy were higher than those in the healthy controls and would reduce to the normal level in remission AML after chemotherapy. Furthermore, we found that TPO expression had a positive correlation with c-MPL levels in bone marrow not only from the healthy controls but also from AML. We conclude that there is a strong correlation between TPO/c-MPL levels of myeloblasts and the status of patients with AML, suggesting TPO/c-MPL levels of myeloblasts can be used for evaluation of therapeutic effect in AML. TPO/c-MPL levels were much higher in relapse AML patients' myeloblasts than those in both the initial AML and healthy controls. The results suggest that TPO/c-MPL levels may be related to AML recurrence, which is in line with Wetzler et al.'s conclusion that c-MPL(+) AML patients were more likely to have their cancer back [24]. Our data also indicate that there was no significant difference in TPO/c-MPL levels among 3 subgroups of the relapse AML patients which were classified by prognosis prediction based on karyotype.

It has been shown that the interaction of HSCs with their niches can regulate the quiescence of HSCs. HSC quiescence is critical to ensure lifelong hematopoiesis and to protect the HSCs pool from myelotoxic insult and premature exhaustion under conditions of hematopoietic stress. Arai F et al. determined that long-term (LT)-HSCs could express c-MPL. Exogenous TPO transiently increased the quiescent LT-HSC population [25]. Given the role of the TPO/c-MPL pathway in maintaining the repopulating ability of normal HSC, it would be interesting to investigate whether the TPO/c-MPL pathway plays a role in the regulation of leukemia initiating cells and whether blocking the pathway would aid on combating disease relapse. In our study, BMSCs-derived osteoblasts from initial AML and healthy controls and AML cell line hFOB1.19 were cultured in vitro. BMSCs-derived osteoblasts from initial AML secreted more TPO than the healthy controls, which was consistent with the results in the bone marrow from AML and healthy controls. BMSCs-derived osteoblasts and AML leukemia cell line HEL were co-cultured to mimic leukemia niche ex vivo. With the treatment of different concentrations of DNR, HEL cells had the most tenacious viability when they were co-cultured with BMSCs-derived osteoblasts from AML. The IC_{50} of DNR which caused 50 % of HEL lethality was the highest in HEL + BMSCs-derived osteoblasts from initial AML. We found that resistance to DNR is directly related to up-regulated TPO in the osteoblasts. It is possible that DNR treatment causes TPO level to be elevated in the osteoblasts, or on the contrary, DNR resistance

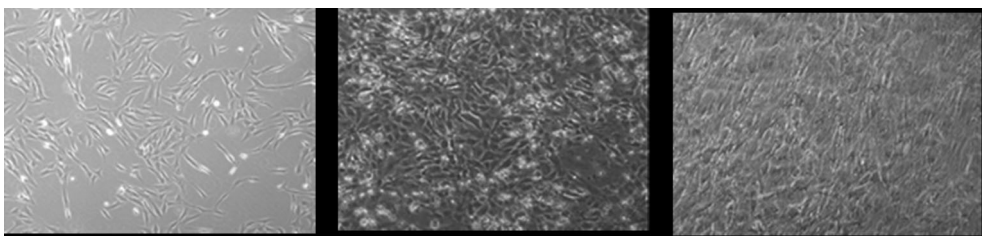

Fig. 4 hFOB1.19 cells were cultured in vitro. On the 1st day, the cells attached to the bottom of the well, and grew in shapes of triangle or irregular polygon (**a**). On the 7th day, calcium salt deposits were visible (**b**). The cells continued to grow in multilayer on the 21st day (**c**)

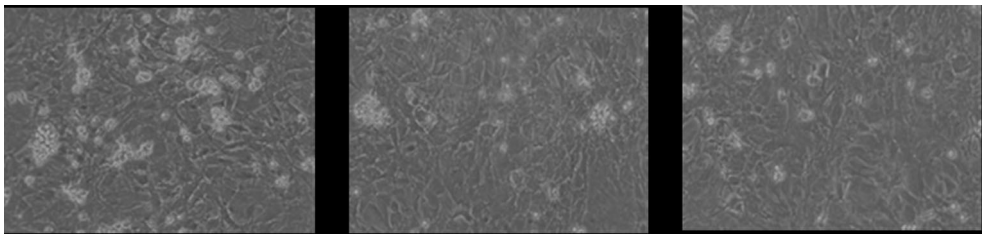

Fig. 5 HEL cells were co-cultured with the osteoblasts derived from different sources. **a** A representative image of AML-derived osteoblasts + HEL. **b** A representative image of normal bone marrow-derived osteoblasts + HEL. **c** A representative image of hFOB1.19+HEL. The osteoblasts grew by static adherence and had an irregular shape. There was no difference in the morphology of osteoblasts derived from different sources. The HEL cell aggregates were glomerate and clingy on the osteoblast surface. More HEL cells attached to the AML BMCSs-derived osteoblasts than to the normal BMSCs-derived osteoblasts. All images were taken at 200× magnification

is due to up-regulation of TPO. The exact mechanism could be explored in a further study. Satio Y et al. mentioned that leukemia stem cells (LSCs) were similar to HSCs. Some chemotherapy-resistant LSCs were thought to the cause of disease relapse [3]. Since LSC/HSC contributes to the c-MPL$^+$ cell population, we propose that up-regulated TPO/c-MPL signaling in premitive lukemia cells may be a potential mechanism to protect them against AML chemotherapy. Our study strongly indicates a possibility that high level of TPO/c-MPL signaling may protect LSCs from chemotherapy in AML.

Given the role of the TPO/c-MPL pathway in the regulation and repopulation ability of leukemia initiating cells, it would be interesting to investigate whether delivery of a c-MPL inhibitor could potentially enhance the chemotherapy sensitivity of AML cells. However, potential adverse effects such as thrombocytopenia or myelopathic anemia may hinder its clinical use. It would also be interesting to understand how the TPO/c-MPL pathway affects AML cells' maintenance and their resistance to chemotherapy. With the advancement and expansion of our knowledge regarding HSC/LSC biology in general, future studies should focus on a closer look at the effect of TPO-cMPL pathway on its downstream effector molecules that direct the interactions between patient-derived blast cells and leukemia repopulating cells.

It is known that not only different stages of megakaryocytes and early hematopoietic stem/progenitor cells, but the primitive cells of AML, chronic myeloid leukemia and myelodysplastic syndrome patients can express c-MPL [1]. More so, c-MPL expression can also be detected in the mononuclear cells of BM [2–4]. The nature of the c-MPL$^+$ BM cells is of great interest but can be addressed in a separate study where the characteritics of the c-MPL$^+$ cells, such as their dynamics and stem cell characteristics, can be fully investigated. Although it would be informative to identify the stem cell component responsible for c-MPL expression (or their dynamics in the c-MPL$^+$ population), we believe that determining the identity of the stem cell component would not further enhance the major conclusion of the current manuscript. Since LSC/HSC contributes to the c-MPL$^+$ cell population, we propose that up-regulated TPO/c-MPL signaling in premitive lukemia cells may be a potential mechanism to protect them against AML chemotherapy. The presented data can fully support such hypothesis and the study of the contribution of the stem cell component and early megakaryoblasts to c-MPL heterogeneity can be the basis of a separated,

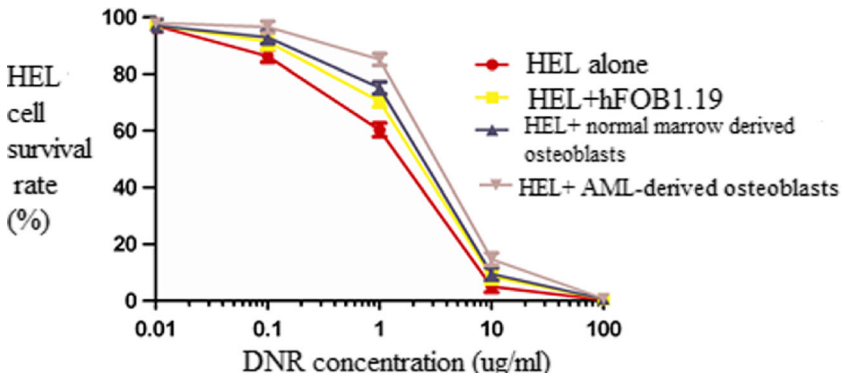

Fig. 6 Comparison of HEL cell survival rates at different concentrations of DNR (0.01, 0.1, 1, 10, and 100 μg/ml) with various two-dimensional co-culture systems. The HEL cell survival rate decreased with increasing doses of DNR in all the groups. The cell survival rate in the group of HEL + AML-derived osteoblasts was the highest, and that of the HEL-alone group was the lowest under each DNR concentration. Meanwhile, the cell survival rate was not different between the groups of HEL + normal bone marrow-derived osteoblasts and HEL + hFOB1.19

Table 4 Comparison of IC50 after treatment of DNR for 24 h among different two-dimension co-culture systems

Group	IC50(ug/ml)
HEL	1.643±0.108
HEL + hFOB1.19	2.254±0.111*#
HEL + Normal marrow derived osteoblasts	2.236±0.167*#
HEL + AML-derived osteoblasts	3.320±0.218*

* $P<0.05$, compared with HEL culture group

# $P<0.05$, compared with HEL + AML-derived osteoblasts co-culture group

independent project which can be further studied in another manuscript.

Clinical Practice Points

Given the role of the TPO/c-MPL pathway in the regulation and repopulation ability of leukemia initiating cells, it would be interesting to investigate whether delivery of a MPL inhibitor would be relatively enhance the chemotherapy sensitivity of AML cells. However, potential adverse effects such as thrombocytopenia or myelopathic anemia may hinder its clinical use. It would also be interesting to understand how the TPO/c-MPL pathway effects on AML cells' maintenance and their resistance to chemotherapy.

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Conflict of Interest All authors have no conflict of interest to state.

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