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ORIGINAL ARTICLE

Poor potential of proliferation and differentiation in bone marrow mesenchymal stem cells derived from children with severe aplastic anemia

Yu-Hua Chao · Ching-Tien Peng · Horng-Jyh Harn · Chin-Kan Chan · Kang-Hsi Wu

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Abstract The pathogenesis of severe aplastic anemia (SAA) has not been completely understood, and insufficiency of the hematopoietic microenvironment can be an important factor. Here, we compared the basic properties of mesenchymal stem cells (MSCs), a major component of bone marrow microenvironment, from five SAA children with those of MSCs from five controls. Although MSCs from SAA children and controls were similar in morphology and immunophenotypic profile, SAA MSCs had slower expansion rate and smaller cumulative population doubling (1.83±1.21 vs 3.36±0.87; p=0.046), indicating lower proliferative capacity. After osteogenic induction, SAA MSCs showed lower alkaline phosphatase activity (optical density, 1.46±0.04 vs 2.27±0.32; p=0.013), less intense von Kossa staining, and lower gene expression of core binding factor α1 (0.0015±0.0005 vs 0.0056±0.0017; p=0.013). Following adipogenic induction, SAA MSCs showed less intense Oil red O staining (optical density, 0.86±0.22 vs 1.73±0.42; p=0.013) and lower lipoprotein lipase expression (0.0105±0.0074 vs 0.0527±0.0254; p=0.013). These findings provided evidence that defects in bone marrow MSCs of SAA children do exist.

Keywords Aplastic anemia · Mesenchymal stem cells · Hematopoiesis · Bone marrow failure · Microenvironment

Y.-H. Chao
Department of Pediatrics, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan

Y.-H. Chao
Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan

C.-T. Peng · K.-H. Wu
Department of Pediatrics, China Medical University Hospital, Taichung, Taiwan

C.-T. Peng
Department of Biotechnology and Bioinformatics, Asia University, Taichung, Taiwan

H.-J. Harn
Center for Neuropsychiatry, China Medical University Hospital, Taichung, Taiwan

H.-J. Harn
Department of Pathology, China Medical University Hospital, Taichung, Taiwan

C.-K. Chan
Graduate Institute of Clinical Medical Science, Chang Gung University, Taoyuan, Taiwan

C.-K. Chan
Department of Pediatrics, Taoyuan General Hospital, Taoyuan, Taiwan

K.-H. Wu
Stem Cell Research Laboratory, Department of Medical Research, China Medical University Hospital, Taichung, Taiwan

K.-H. Wu (✉)
No. 2 Yuh-Der Road North District, Taichung 404, Taiwan
e-mail: m9683013@kimo.com

Introduction

Childhood acquired aplastic anemia, characterized by failure of hematopoiesis, is rare and potentially life-threatening with an annual incidence of one to six per million [1–3]. Severe aplastic anemia (SAA) is defined as profound bone marrow (BM) hypocellularity and marked peripheral blood pancytopenia. Despite of many putative etiologies, a specific cause cannot be identified in most children and is termed "idiopathic SAA." Significant advances have been made in the management of the disease, including allogeneic stem cell transplantation and immunosuppressive therapy [2–4]. However, the mechanism by which idiopathic SAA develops has not been completely elucidated. Although many studies demonstrated the association of immune-mediated pathogenesis, up to 30% of patients do not have detectable evidence for an underlying immune basis and not respond to immunosuppressive therapy [2]. Therefore, other mechanisms do exist.

Mesenchymal stem cells (MSCs), first described by Friedenstein et al. [5], play an important role in providing the specialized BM microenvironment for hematopoietic stem cell (HSC) survival and differentiation [6–11]. MSC dysfunction may result in the impairment of hematopoiesis, but data focusing on the role of MSCs in the pathophysiology of SAA are limited [12–16]. As the so-called stem cells, MSCs are capable of proliferating and differentiating into mesenchyme-lineage cells, and many studies used these properties as indicators to assess MSCs. Until now, no information about the basic characteristics of morphology, immunophenotyping, proliferative capacity, and differentiation potential of SAA MSCs has been reported. In order to clarify pathophysiology of SAA and to identify characteristic changes of SAA MSCs, we compared these basic properties of BM MSCs derived from SAA children and controls.

Design and methods

Materials

BM cells were obtained from iliac crest aspirates. Idiopathic SAA was defined as pancytopenia and hypocellular BM after excluding any other underlying diseases. To diagnose SAA, BM cellularity of less than 25% and at least two of the following criteria must be fulfilled: absolute neutrophil count less than 0.5×10^9/l, platelet count less than 20×10^9/l, and reticulocyte less than 1% [2, 3, 17]. Control subjects were patients who received BM examination for diseases other than hematological diseases with pathological proof of normal BM. All patients were previously untreated and aged less than 18 years old. The institutional review board of Tungs' Taichung MetroHarbor Hospital approved this protocol, and written informed consents were obtained from the parents or legal guardians of the patients.

Cell culture

Mononuclear cells were isolated from BM aspirates by Ficoll-Paque density centrifugation (1.077 g/ml; Amersham Biosciences, Uppsala, Sweden) and then seeded in low-glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic–antimycotic (Gibco). Cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere. After 48 h, medium with suspension of nonadherent cells was discarded, and fresh medium was added. Thereafter, medium was replaced twice a week. When reaching 80–90% confluence, cells were detached with 0.25% trypsin–EDTA (Gibco) and replated at a concentration of 8.5×10^3/cm^2 in 10-cm dishes.

Immunophenotypic analysis

Cultured MSCs (passage 4) were detached, washed, and resuspended in phosphate-buffered saline (Gibco). After fixing and blocking, the cells were immunolabeled with the following mouse antihuman antibodies: fluorescein isothiocyanate-conjugated CD45 (FITC-CD45; BD Biosciences, San Jose, CA, USA), phycoerythrin-conjugated CD14 (PE-CD14; BD Biosciences), FITC-CD34 (BD Biosciences), FITC-CD105 (Serotec, Oxford, UK), PE-CD73 (BD Pharmigen, San Diego, CA, USA), and FITC-CD44 (BD Pharmigen). The nonspecific mouse IgG (BD Biosciences) served as isotype control. Data were analyzed by flow cytometry (FACSCalibur; BD Biosciences) with CellQuest software.

Proliferative capacity

Yield of cells at each passage was enumerated using Trypan blue (Gibco) to exclude dead cells. The population doubling (PD) of cultured MSCs was calculated according to the equation: PD=$\log_2$ (the number of viable cells at harvest/the number of seeded cells). The cumulative PD was the sum of PD from passages 4 to 6.

Osteogenic and adipogenic potential

To evaluate differentiation potential, cultured MSCs (third passage, at 80–90% confluence) were subjected to osteogenic and adipogenic differentiation in vitro. Cells were detached from culture dishes and replated in 60-mm dishes for further studies.

To promote osteogenic differentiation, cells were incubated in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA), 0.1 μM dexamethasone (Sigma), and 0.2 mM ascorbic acid (Sigma) for 3 weeks. On day 21, cultures were stained for alkaline phosphatase (ALP; Sigma) activity and mineralized deposits were detected by von Kossa stain (Cedarlane, Ontario, Canada). To quantify ALP activity, 2 ml of 0.05 N NaOH in ethanol was added to each dish after ALP activity stain, and the extraction was measured by spectrophotometry (Ultrospec 1100 pro; Amersham Biosciences) at 550 nm.

For induction of adipogenic differentiation, cells were grown in DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 0.1 mM indomethacin (Sigma), and 10 μg/ml insulin (Novo Nordisk A/S, Bagsværd, Denmark) for 2 weeks. On day14, adipogenic differentiation was demonstrated by intracellular accumulation of lipid droplets stainable with oil red O (Sigma). The dye content was eluted by ethanol and quantified spectrophotometrically.

Differentiation was verified further by real-time polymerase chain reaction (Q-PCR) for the assessment of lineage-specific genes as core binding factor α1 (Cbfa1) for osteocytes and lipoprotein lipase for adipocytes. MSCs cultured in osteogenic and adipogenic induction medium were harvested on days21 and 14, respectively. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Concentration of the RNA samples was estimated spectrophotometrically at OD 260/280, and cDNA was synthesized using MMLV reverse transcriptase (Epicenter Biotechnologies, Madison, WI, USA) in the presence of oligo-dT primer (Promega, Madison, WI, USA). The sequences of PCR primers were as follows: Cbfa1 [18], sense 5′-CATGGCGGGTAACGATGAA-3′ and antisense 5′-CGGCCCACAAATCTCAGATC-3′; lipoprotein lipase [19], sense 5′-ATGGAGAGCAAAGCCCTGCTC-3′ and antisense 5′-TACAGGGCGGCCACAAGTTTT-3′. The expression of β-actin (sense 5′-TGTGGATCAGCAAGCAGGAGTA-3′ and antisense 5′-CAAGAAAGGGTGTAACGCAACTAAG-3′) was used as an internal control to normalize specific gene expression in each sample [18]. Q-PCR was performed using cDNA samples with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and carried out in the ABI 7300 Real-Time PCR system (Applied Biosystems).

Statistical analysis

Data analysis was performed using SPSS 14.0 for Windows. Kolmogorov–Smirnov Z test was used for comparison of the two groups. Statistical value of $p<0.05$ was considered significant.

Results

Morphology and immunophenotypic profile

A total of ten children, five SAA patients and five controls, were enrolled in the study. Clinical data of these children are outlined in Tables 1 and 2, and all BM aspirates were obtained at the time of diagnosis without concomitant medication. The average age was 11.9 and 11.6 years old, respectively. In vitro, MSCs of SAA and control group shared a similar spindle-shaped morphology (Fig. 1). Both revealed a consistent immunophenotypic profile which was negative for CD45, CD14, and CD34, and positive for CD105, CD73, and CD44 (Table 3). No significant difference was noted in the expression of any single surface marker between the two groups.

Proliferative capacity

To prevent hematopoietic cell contamination, which might be present in earlier passages, or the presence of senescent or differentiating MSCs in later passages, we used cells from passages 4 to 6 for the study of growth kinetics. SAA group had slower expansion rate than control group, shown as average PD of each passage (Fig. 2a). Two of five cultures of SAA group stopped proliferating at passages 5 and 6, respectively, whereas all cultures of control group continued to grow well into passage 9. Lower proliferation potential of SAA MSCs was also demonstrated by smaller cumulative PD from passages 4 to 6 (1.83 ± 1.21 vs 3.36 ± 0.87; $p=0.046$; Fig. 2b).

Differentiation potential

When exposed to osteogenic induction medium, MSCs from SAA children had less robust osteogenic differentiation than MSCs from controls as shown by lower ALP activity (Fig. 3a). Greater extent of mineralization in control group was also demonstrated by more intense von Kossa stain (Fig. 3b). Under adipogenic condition, SAA MSCs gave rise to less lipid-containing cells. The intracytoplasmic vacuoles of neutral fat can be identified by Oil red O stain (Fig. 3c). The droplets of fat were more and larger within a single adipocyte in control group. Quantitation of dye content of ALP activity stain in the osteogenic cultures (optical density, 1.46 ± 0.04 vs 2.27 ± 0.32; $p=0.013$) and Oil red O stain in the adipogenic cultures (optical density, 0.86 ± 0.22 vs 1.73 ± 0.42; $p=0.013$) confirmed the histochemical observations (Fig. 4).

Results of Q-PCR analysis presented in Fig. 5 confirmed the findings of histochemical stains further. After osteogenic induction, MSCs of SAA group expressed lower level of Cbfa1 than MSCs of control group (0.0015 ± 0.0005 vs

Table 1 Clinical characteristics of five children with severe aplastic anemia

Patient no.	Sex	Age (years)	Treatment	Outcome
A1	F	11.6	IST	Hematopoiesis recovery after IST
A2	F	11.3	IST; HSCT from MUD	No response to IST; hematopoiesis recovery after HSCT with mild chronic GVHD
A3	F	14.7	IST; HSCT from MUD	No response to IST; hematopoiesis recovery after HSCT
A4	M	10	IST	Hematopoiesis recovery after IST
A5	F	12.2	IST	Hematopoiesis recovery after IST

F female, *M* male, *IST* immunosuppressive therapy, *HSCT* hematopoietic stem cell transplantation, *MUD* matched unrelated donor, *GVHD* graft versus host disease

0.0056±0.0017; p=0.013). Following adipogenic induction, SAA MSCs showed lower lipoprotein lipase expression (0.0105±0.0074 vs 0.0527±0.0254; p=0.013). The results of Q-PCR analysis were consistent with histochemical findings. However, the proliferative capacity and differentiation potential of SAA MSCs from an individual patient did not decrease at the same degree. Probably, it is hard to draw conclusions with only five patients studied, but in our study there was no correlation between the in vitro proliferation or differentiation potential and the response to immunosuppressive therapy.

Discussion

MSCs can be characterized by a panel of surface markers, by their in vitro growth pattern and subsequent expansion, and by their multilineage differentiation potential [6, 8, 9]. Many studies used these basic properties as indicators to identify MSCs from origins other than BM [18–28]. We aimed at these properties of BM MSCs from SAA children and found that SAA MSCs had poor potential of proliferation and differentiation. The alterations may contribute to failure of hematopoiesis and lead to the development of the disease.

The immune-mediated HSC destruction for the pathogenesis of idiopathic SAA has been widely accepted, and many studies have been devoted to the role of T cells in this disease [2, 4, 29–31]. Dubey et al. found elevated levels of interferon gamma and tumor necrosis factor alpha (TNF-α) in BM plasma of SAA patients [29], and these cytokines can induce apoptosis of CD34+ BM cells [32]. Hara et al. demonstrated excessive production of TNF-α by BM T cells and higher sensitivity of HSCs to TNF-α in patients with SAA [30]. However, up to 30% of patients have no immune-associated evidence and respond poorly to immunosuppressive therapy [2]. Pathogenesis of SAA remains to be determined.

Primary HSC deficiency, including decrease in number and dysfunction, has also been proposed to account for the development of SAA [33–36]. In vitro long-term BM culture provided evidence for primary HSC dysfunction in the regenerative capacity and in the response to various cytokine stimuli [35, 36]. Abnormal telomere shortening of HSCs was found in some patients with SAA [34]. However, allogeneic HSC transplantation cannot cure all patients, suggesting that other mechanisms exist.

Another important concept proposed in the context of SAA is related to deficiency or dysfunction of BM microenvironment. Marrow stromal cells derived from MSCs, including fibroblasts, endothelial cells, and adipocytes, exert the regulatory role in hematopoiesis. They provide an appropriate scaffold and a complex network of cytokines, adhesion molecules, and extracellular matrix proteins [6–11]. Many studies have reported the promotive effect of MSCs for HSC expansion in vitro [10, 36–40]. In 2000, Koc et al. found rapid hematopoietic recovery after coinfusion of autologous MSCs at the time of HSC transplantation [41]. Accordingly, Lazarus et al. presented a multicenter trial of 46 patients receiving allogeneic HSCs and MSCs from HLA-

Table 2 Clinical characteristics of five controls

Patient no.	Sex	Age (years)	Diagnosis	Medication at time of bone marrow examination
C1	M	16.3	Rhabdomyosarcoma	Nil
C2	F	5.9	Ewing's sarcoma	Nil
C3	F	15	Rhabdomyosarcoma	Nil
C4	M	2.8	Hepatoblastoma	Nil
C5	M	17.8	Kikuchi disease	Nil

F female, *M* male

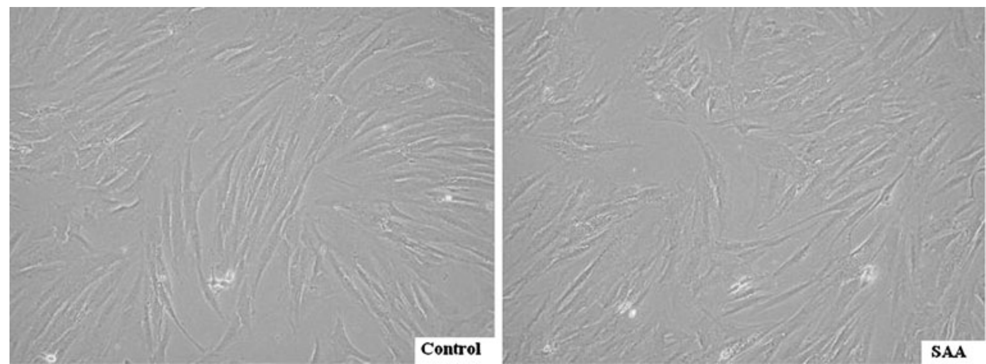


Fig. 1 Morphology. In vitro culture, MSCs of five controls and five SAA children shared a similar spindle-shaped morphology. Magnification of micrographs ×100

Table 3 Immunophenotypic analysis of mesenchymal stem cells from children with SAA and controls

	Control (n=5)		SAA (n=5)	
	Median (%)	Range (%)	Median (%)	Range (%)
$CD45^+$	1.47	0.84–2.44	1.92	0.60–3.22
$CD14^+$	1.09	0.64–1.42	0.94	0.38–2.42
$CD34^+$	1.17	1.07–1.46	1.63	0.13–2.60
$CD105^+$	94.46	92.57–95.29	92.86	84.66–96.66
$CD73^+$	96.55	92.74–97.84	94.59	90.42–95.65
$CD44^+$	90.86	85.74–97.11	87.21	82.40–94.39

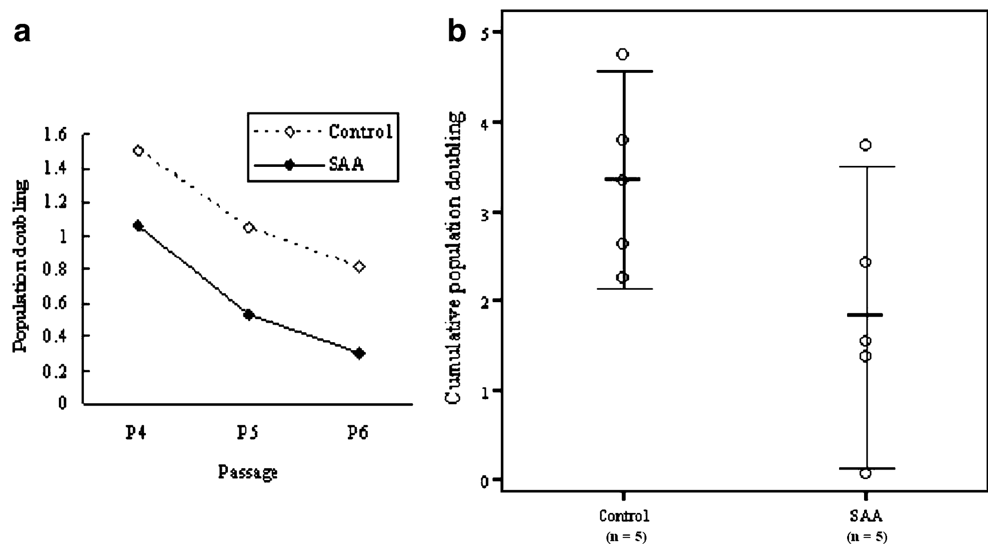
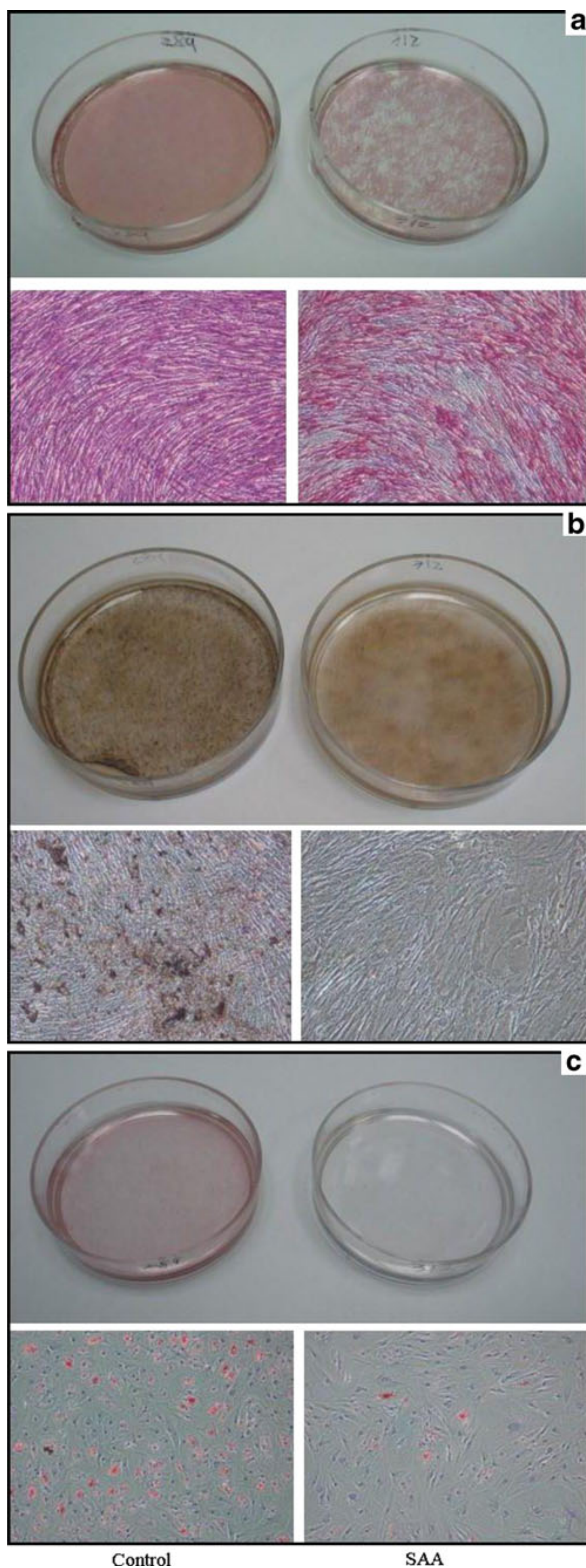


Fig. 2 Proliferative capacity. **a** Average PD of the two groups (n=5 for each group). **b** Comparison of cumulative PD from passages 4 to 6, shown as mean and 95% confidence interval. Each *circle* represents a subject studied



◀**Fig. 3** Osteogenic and adipogenic potential of MSCs from five controls and five SAA children. Osteogenic differentiation was demonstrated by ALP activity **a** and von Kossa stain **b** after 3-week induction. Adipogenic differentiation was demonstrated by Oil red O stain **c** after 2-week induction. Magnification of micrographs ×100

identical siblings, and found prompt hematopoietic recovery in most patients, suggesting that the beneficial effect of MSCs on engraftment may relate to their supportive role in the hematopoiesis [42].

In this study, we demonstrated defects of BM MSCs from SAA children. Although the morphology and surface marker expression of cultured MSCs did not change, SAA MSCs had slower expansion rate and smaller cumulative PD, indicating lower proliferation potential. Besides, only three of five cultures of SAA group retained the ability to grow confluent layers into passage 6, suggesting earlier senescence of SAA MSCs. The significant decrease in osteogenic and adipogenic potential of SAA MSCs was obvious. Even the three cultures which can proliferate beyond passage 6 showed less intense stains after differentiation under permissive conditions. The results of gene expression study confirmed the findings of histochemical stains.

Several studies have been conducted on the relationship between MSCs and SAA [12–16]. Hott et al. used long-term culture system to analyze the ability of BM stromal cells to support hematopoiesis and found that stromal layers from three of nine SAA patients failed to maintain normal HSCs [16]. Holmberg et al. evaluated stromal cell function in long-term marrow culture and observed that 6.8% of SAA marrow failed to grow any stromal cells and 42.5% failed to reach complete confluent stromal layers [15]. Bacigulupo et al. found that BM MSCs of SAA patients were deficient in their ability to suppress T cell proliferation and cytokine release, suggesting the lack of MSC immunoprotection in SAA BM [13]. Wu et al. investigated GATA gene expression of MSCs from chronic aplastic anemia patients and proposed that aberrant expression of these genes in BM MSCs may influence the BM microenvironment and lead to abnormal hematopoietic regulation [12]. In the current study, we examined the basic characteristics of MSCs and found poor potential of proliferation and differentiation in BM MSCs derived from SAA children. The above studies provide strong evidence for MSC defects in SAA BM.

Rubinstein et al. reported 562 recipients of cord blood HSC transplantation and observed successful engraftment reduced significantly among SAA patients [43]. It is of interest that cord blood MSCs exhibit higher proliferation capacity than bone marrow MSCs in vitro culture [22]. On the other hand, several investigations have found that MSCs cannot be acquired efficiently from umbilical cord blood, suggesting that MSCs are sparse in cord blood [20,

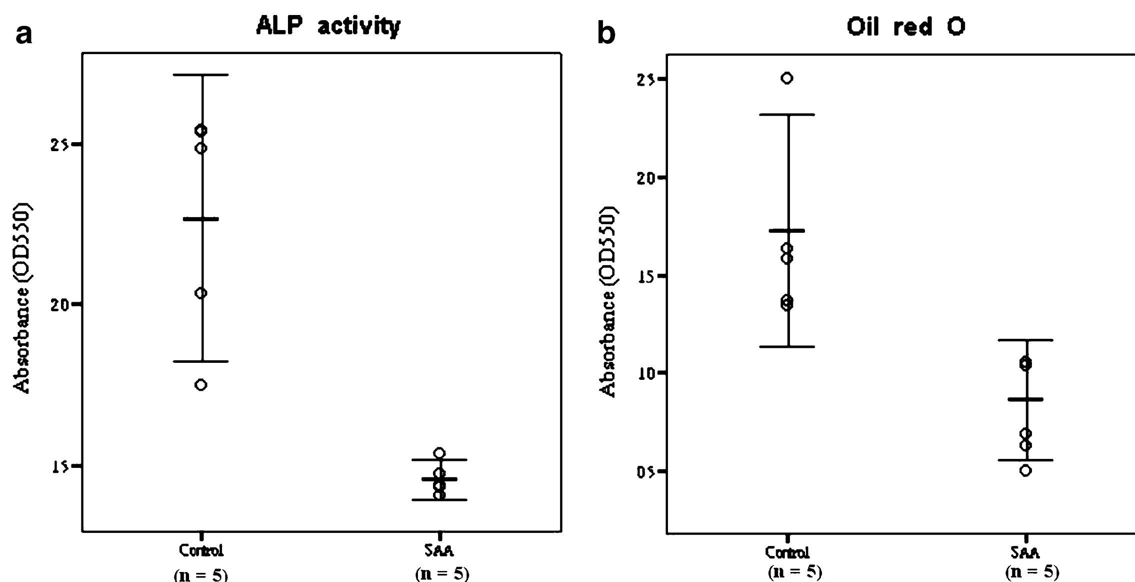


Fig. 4 Comparison of osteogenic and adipogenic potential by quantification of ALP activity (**a**) and Oil red O (**b**) stain spectrophotometrically, respectively. Mean and 95% confidence interval are illustrated. Each *circle* represents a subject studied

24, 26]. As we propose here, MSC defects in SAA patients lead to insufficiency of BM hematopoietic microenvironment. It can be assumed that the lower number of MSCs provided by cord blood during transplantation is the cause of engraftment failure in SAA patients despite of the higher proliferative capacity of cord blood MSCs. Therefore, cotransplant of MSCs and HSCs could be a potential strategy to treat SAA patients.

The present study was of course limited by the small number of patients and the diseases of controls. However, according to our results, BM MSCs derived from children with SAA had poor potential of proliferation and differentiation and these alterations may be important in the pathogenesis of the disease. Subsequently, we try to find genes that participate in proliferation or differentiation of MSCs and express aberrantly in SAA patients. Microarray will be used to find candidate genes, and their expression will be verified further by Q-PCR. Knockdown or knockout experiments will demonstrate their role in the development of the disease. Additional research is required for further understanding of the pathophysiology of SAA and leads to the development of novel treatment modalities.

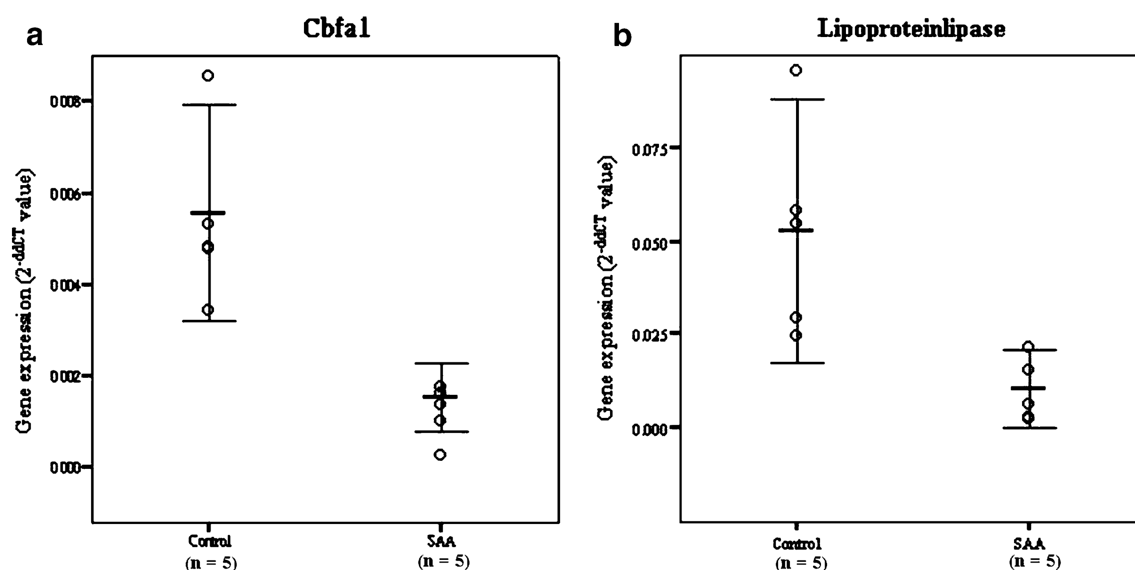


Fig. 5 Comparison of differentiation potential by lineage-specific gene expression, Cbfa1 for osteogenesis (**a**) and lipoprotein lipase for adipogenesis (**b**). Mean and 95% confidence interval are illustrated. Each *circle* represents a subject studied

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