

## Original Article

# Defective proliferative potential of MSCs from pediatric myelodysplastic syndrome patients is associated with cell senescence

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**Abstract:** Objectives: Aberrant MSC function was shown to contribute to the pathophysiology of myelodysplastic syndrome (MDS). In comparison to adult MDS, pediatric MDS displayed different features both in biologically and clinically. The mechanisms for adult MDS may not be applicable in pediatric MDS. However, understanding of the MSCs in pediatric MDS is lacking. In this study, we investigated the proliferation capacity of MSCs from pediatric MDS patients at clone cell level. Material and methods: Clone bone marrow MSCs were isolated from pediatric MDS patients and identified according to the criteria of the International Society for Cellular Therapy for MSCs. The proliferation capacity of pediatric MDS-derived MSCs was compared to healthy controls. Cell cycle was detected by flow cytometry following PI staining, as well as cell senescence was evaluated by  $\beta$ -galactosidase staining and telomere length. Results: Pediatric MDS-derived MSCs displayed similar basic biology characters as MSCs from healthy controls, including differentiation potential and surface markers. However, defective proliferative was displayed by pediatric MDS-derived MSCs. Pediatric MDS-derived MSCs were more prone to cellular senescence than healthy controls, and showed a decrease in the S phase. Conclusion: Pediatric MDS-derived MSCs possess the basic characteristics of normal MSCs, but display defective proliferation, which may be associated with cell senescence.

**Keywords:** Pediatric myelodysplastic syndromes, mesenchymal stem cells, bone marrow, proliferation, cell senescence

## Introduction

Pediatric myelodysplastic syndrome (MDS) comprises a group of heterogeneous clonal diseases derived from hematopoietic stem cells (HSCs) [1]. The incidence of pediatric MDS is 0.5-4 per 10<sup>6</sup>/year, which is lower compared to 20-40 per 10<sup>6</sup>/year in adults [2, 3]. There have been fewer studies focusing on pediatric MDS patients. Different features are apparent, both biologically and clinically, between pediatric and adult MDS patients [4]. Therefore, many researchers consider that pediatric MDS may be a group of diseases that differ to adult MDS, and that the mechanisms deciphered for adult MDS may not be comparable or applicable in pediatric MDS. So, it is important to understand the unique pathophysiology of pediatric MDS.

MDS pathogenesis was associated with a complex multistep alteration process, in which the

function of HSC was affected via regulated cell cycle, epigenetic machinery, signal transduction proteins and transcriptional factors [5]. The bone marrow (BM) microenvironment has also been considered to contribute to MDS pathogenesis [6]. However, the role of the bone marrow microenvironment in MDS remains obscure.

Multipotent mesenchymal stem cells (MSCs) are key components of the marrow microenvironment. According to the recent reports, bone marrow MSCs from adult MDS patients display functional and cytogenetic abnormalities, such as impaired proliferation potential, abnormal cytokine production and adhesion molecules expression, immunoregulatory capacity, and genetic aberrations [7-10]. However, existing data on the functional characteristics of bone marrow-derived MSCs in adult MDS are controversial [7, 8, 11-16]. Many factors could result in these inconsistent results, such as patient

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**Table 1.** Patient demographics

Patient NO.	sex	age	WHO	IPSS
1	F	3	RCMD	Intermediate-1
2	F	16	RCMD	Intermediate-1
3	F	12	RA	Low
4	F	14	RA	Intermediate-1
5	F	8	RA	Low
6	M	9	RCMD	Intermediate-2
7	M	14	RA	Low
8	M	5	RA	Intermediate-1

selection, MSC culturing methods, MSC heterogeneity, and study initiation at different MSC stages. To be able to compare between different studies regarding the characteristics of MDS-derived MSCs, consistency must be ensured to obtain relevant results. Our current study was designed to investigate the proliferation capacity of pediatric MDS bone marrow-derived MSCs at clone cell level. The data indicate that pediatric MDS-derived MSCs display defective proliferative properties, which may be associated with cell senescence.

## Materials and methods

### Patients

We studied eight pediatric patients with newly diagnosed MDS (median age: 10.1 years, range: 3-16 years) and ten age- and sex-matched hematologically healthy controls. Patients were enrolled in the study in accordance with the World Health Organization (WHO) and did not receive any medication after diagnosis. Patient demographics are displayed in **Table 1**. Informed consents were clearly explained to all patients and agreements were obtained. The study was performed in accordance with the Declaration of Helsinki and approved by our local institutional review board.

### MSC cultures

BM mononuclear cells were isolated by Ficoll-Paque gradient centrifugation from BM aspirates, and cultured in Dulbecco's modified Eagle's Medium with low-glucose (DMEM-LG; life technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; life technologies) and 100 IU/ml penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. Medium was replaced weekly and non-adherent cells were removed. Upon reaching more than 80% confluence, adherent cells were

detached using 0.25% trypsin/1 mM EDTA (life technologies), and sub-plated at a concentration of 1 cell/well in 96-well plates using the limited dilution method. After overnight culture, wells with single adherent cells were identified. Single colonies were harvested and thereafter expanded. All experiments were carried out using MSCs derived from identical passage.

### Differentiation potential of MSCs

OriCell™ Human Mesenchymal Stem Cell Adipogenic Differentiation Medium and OriCell™ Human Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cyagen Biosciences Inc, Guangzhou, China) were used to evaluate the differentiation potential of MSCs from MDS patients and healthy controls at passage 4. Adipogenesis was assessed by Oil Red O staining and osteogenesis was assessed by Alizarin Red and von Kossa staining.

### Analysis of surface marker expression of MSCs

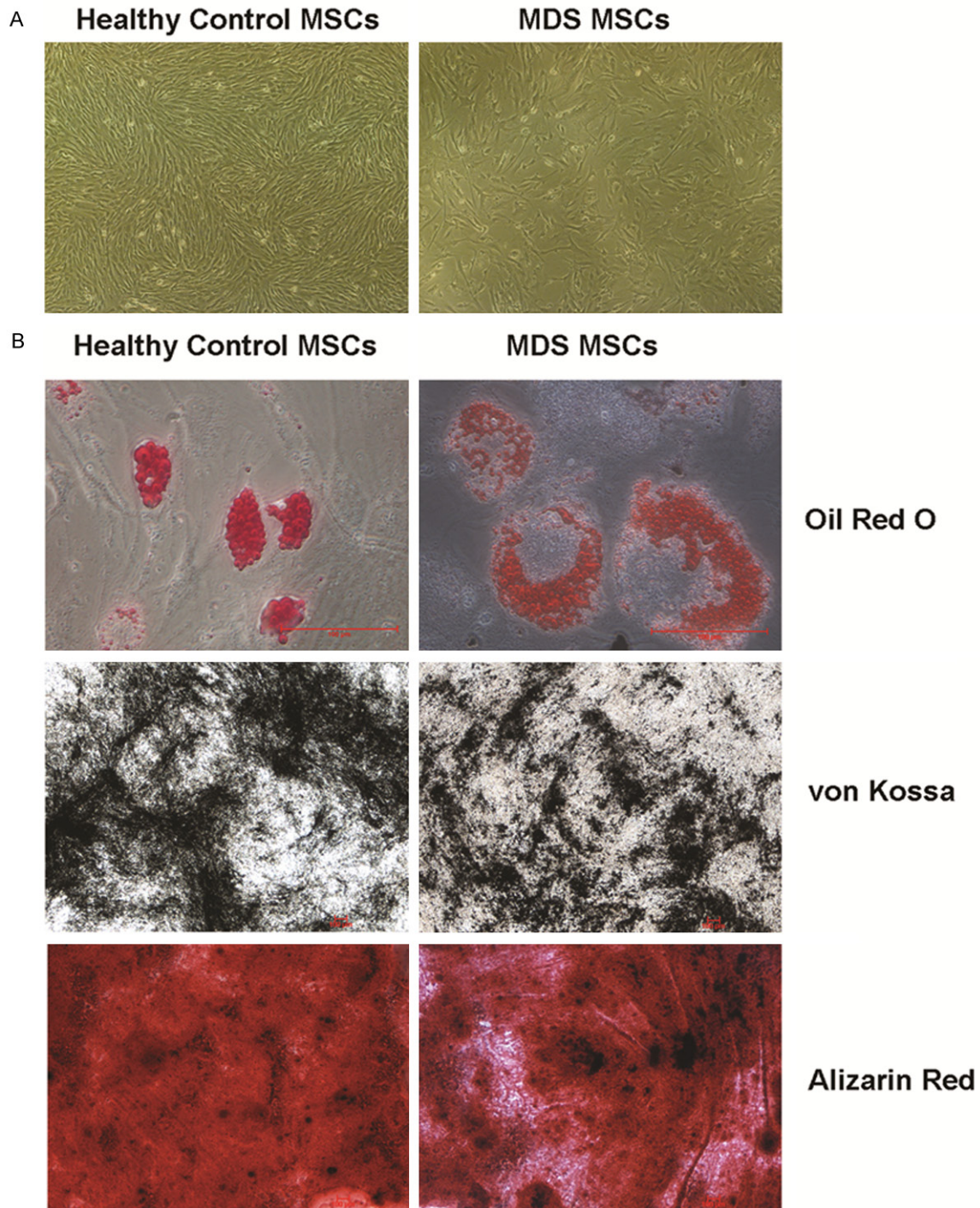
Cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: PE-Rat anti-human CD34, PE-Rat anti-human CD45, PE-Rat anti-human CD73, PE-Rat anti-human CD90, PE-Rat anti-human CD105, and PE-Rat anti-human HLA-DR (eBioscience, San Diego, CA, USA). Briefly, cells were incubated with the appropriate monoclonal antibodies for 30 min at 4°C. Appropriate isotype-matched control antibodies were used for all flow cytometric analyses. Flow cytometry was performed on a fluorescence-activated cell sorting (FACS) Cantoll (BD Biosciences, Franklin Lakes, NJ, USA) using FlowJo software (Tree Star, Ashland, OR, USA).

### Proliferative potential of MSCs

Population doubling (PD) was calculated at each passage by the formula  $\log N / \log 2$  as described by Stenderup et al [17], where N is the number of cells counted at the time of trypsinization divided by the initial number of cells plated. PD time was calculated by dividing the number of hours between the first and second passage by the cell expansion during the same period.

### Analysis of cell cycle in MSCs

MSCs were collected and then fixed with 70% methanol at 4°C overnight. The fixed cells were

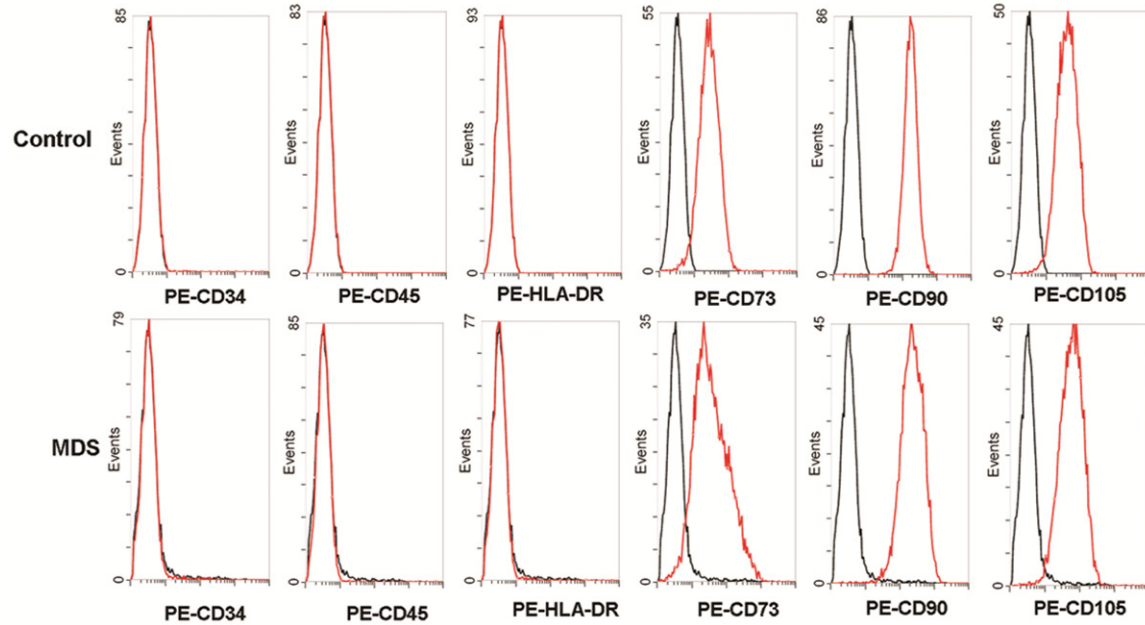


**Figure 1.** Morphology and differentiation potential of clonal pediatric MDS-derived MSCs. A: Morphology of clonal pediatric MDS-MSCs. MSCs was isolated from the bone marrow of pediatric MDS- and healthy control-patients. Clonal cells were obtained by limiting dilution. B: Adipocyte and osteoblast differentiation from clonal pediatric MDS-MSCs. MSCs were induced in adipogenic and osteogenic medium, respectively. Adipogenic differentiation was indicated by oil red O staining and osteoblast differentiation was indicated by Alizarin Red and von Kossa staining for calcium phosphate.

washed twice with PBS before being stained by incubation with 5 µg/ml propidium iodide

(Sigma, St. Gallen, Switzerland) and 1 mg/ml RNase A (Sigma) for 30 min in the dark at room

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**Figure 2.** Surface marker expression profile of clonal pediatric MDS-MSCs. Flow cytometry was performed to analyze surface marker expression of MSCs from pediatric MDS- and healthy control-derived MSCs. Blank line: isotype antibody; Red line: surface marker.

temperature. The cells were then analyzed on a BD FACS Canto II flow cytometer with ModFit LT 3.3 as the data analysis software.

### *Analysis of cellular senescence in MSCs by $\beta$ -galactosidase staining*

Senescent cells were detected using a cellular senescence assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. In the assay, senescence-associated  $\beta$ -galactosidase catalyzes the hydrolysis of X-gal, which results in the accumulation of a distinctive blue color in senescent cells. The number of  $\beta$ -gal-positive (blue) MSCs was evaluated under a phase-contrast microscope, per 100 consecutively counted MSCs. All experiments were performed in triplicate.

### *Analysis of telomere length in MSCs*

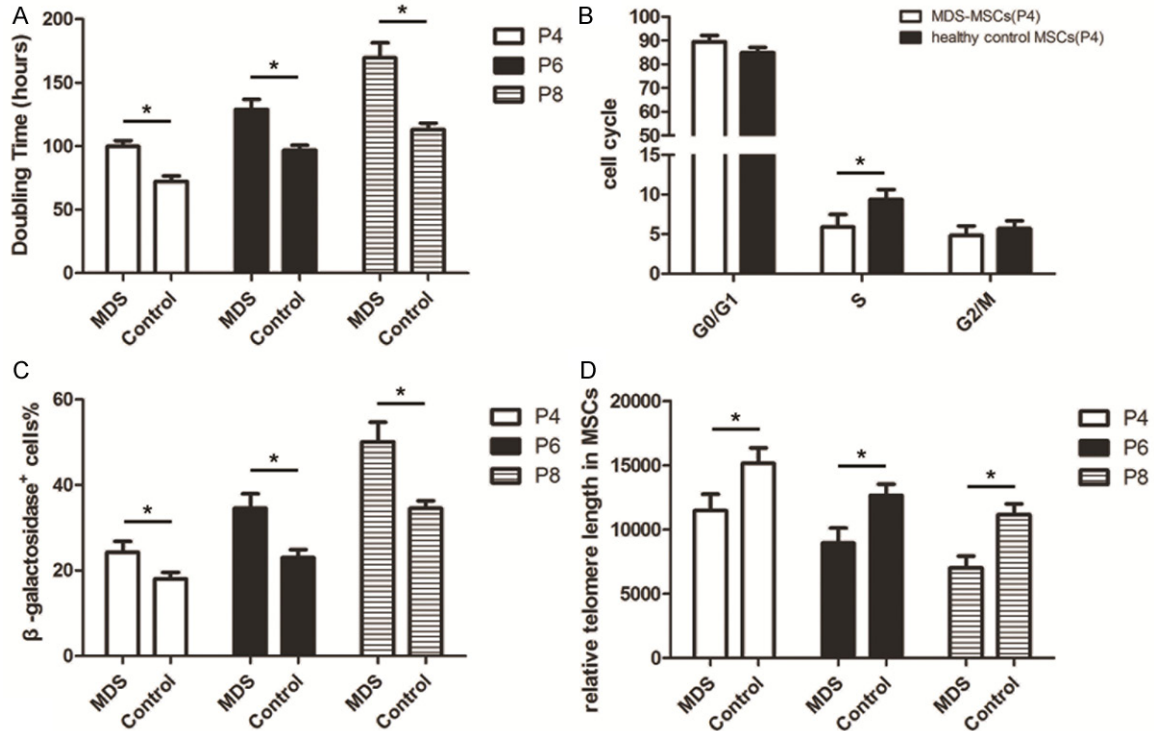
DNA was extracted from MSCs using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany).  $\beta$ -globin was used as a control single-copy gene. SYBR Green qPCR Supermix-UDG (Invitrogen life Technologies, Carlsbad, CA, USA) was used for the reactions. The forward and reverse primer sequences for telomere polymerase chain reaction (PCR) amplification were: 5'-GGTTTTTGGGGTGGGGTGA-

GGGTGAGGGTGGGGT-3' and 5'-CACCAACTTCATCCACGTTACC-3', respectively. For  $\beta$ -globin, the forward and reverse primer sequences were: 5'-GCTTCTGACAACTGTGTTCACTAGC-3' and 5'-CACCAACTTCATCCACGTTACC-3', respectively. For PCR amplification, reactions were incubated for 10 min at 95°C and then amplified over 40 cycles of 15 s at 95°C and 60 s at 56°C (for telomeres) or 58°C (for  $\beta$ -globin). Reactions were run on the Applied BioSystems 7900 Real-Time PCR System. All samples were processed in triplicate. A standard curve was generated using serially diluted genomic DNA. Telomere length was reflected by the relative telomere/single copy gene ratio (T/S) values:  $T/S = 2^{-\Delta Ct}$  ( $\Delta Ct = Ct^{telomere} - Ct^{\beta-globin}$ ).

### *Statistical analysis*

All statistical tests were performed with the use of PASW Statistics 18.0 (SAS Institute, Inc., Cary, NC) and were two-sided. Values of  $P < 0.05$  were considered statistically significant. Comparisons between groups were analyzed statistically using one way ANOVA and the unpaired Student's t test. Data were expressed as mean  $\pm$  standard error of the mean (SEM) and graphed using Prism 5.0 software (La Jolla, CA).

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**Figure 3.** Defective proliferative capacity of clonal pediatric MDS-derived MSCs might be associated with cell senescence. A: Population doubling (PD) was calculated at passage 4, 6 and 8 in MSCs from pediatric MDS patients and healthy controls. PD time was calculated by dividing the number of hours between the first and second passage by the cell expansion during the same period. B: MSCs cell cycle (passage 4) from pediatric MDS patients and healthy controls was analyzed by FACS. C: Cellular senescence was assessed by  $\beta$ -galactosidase staining in MSCs from both populations (passage 4, 6 and 8). All experiments were performed in triplicate. D: Telomere length was assessed by real-time PCR in MSCs (passage 4, 6 and 8). Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ .

## Results

### Biological characteristics of clonal MSCs from pediatric MDS patients and healthy controls

MSCs from healthy controls displayed a fibroblastic appearance, whereas pediatric MDS-derived MSCs were larger and appeared disorganized (**Figure 1A**). In accordance with previously reported data about adult MDS-derived MSCs, MSCs from pediatric MDS patients were able to differentiate into adipogenic and osteogenic lineages, similar to MSCs from healthy controls (**Figure 1B**) [12-14].

Purified MSCs from pediatric MDS patients and healthy controls were positive for CD73, CD90 and CD105 surface antigens and negative for the hematopoietic markers CD34, CD45 and HLA-DR, in accordance with the criteria of the International Society for Cellular Therapy for MSCs (**Figure 2**). There were no significant differences between MSCs from pediatric MDS patients and healthy controls.

### Pediatric MDS-derived MSCs displayed defective proliferative capacity compared to controls

Data showing the proliferative potential of MSCs in pediatric MDS patients and healthy controls are presented in **Figure 3A**. The PD time (mean  $\pm$  SEM) of MSCs during the P4-P8 culture period was significantly increased in pediatric MDS patients compared to healthy controls ( $P < 0.05$ ). More specifically, MSCs PD time ranged from  $99.75 \pm 13.22$  h (P4) to  $169.5 \pm 33.52$  h (P8) in MDS patients and from  $72.2 \pm 13.77$  h (P4) to  $113.0 \pm 16.44$  h (P8) in healthy controls.

### Pediatric MDS-derived MSCs were more prone to cellular senescence than healthy controls

Cell senescence and cell cycle were important for the proliferative capacity of cells. In this study, we assessed the cell senescence and cell cycle of MSCs from pediatric MDS patients in comparison to healthy controls for determining the underlying mechanism of defective proliferative capacity.

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In addition to defective proliferative capacity, MSCs (P4, P6 and P8) from pediatric patients with MDS were more prone to cellular senescence ( $24.23 \pm 7.32\%$ ,  $34.59 \pm 11.61\%$  and  $50.11 \pm 12.90\%$ , respectively) compared with healthy controls ( $18.03 \pm 4.87\%$ ,  $23.03 \pm 5.80\%$  and  $34.53 \pm 5.45\%$ , respectively), as shown by a significantly higher number of  $\beta$ -gal-positive cells (**Figure 3B**). This premature cellular replicative exhaustion might represent a mechanism responsible for the impaired growth of MSCs from pediatric MDS patients.

The relative telomere length of MSCs (P4, P6 and P8) was evaluated in pediatric MDS patients and healthy controls. Relative telomere length during the MSCs time-course was significantly lower in pediatric MDS patients compared with healthy controls (P4:  $11479.5 \pm 3568.5$  vs  $15139.8 \pm 3845.8$ ; P6:  $8948.6 \pm 3266.3$  vs  $12650.8 \pm 2782.1$ ; P8:  $7032.7 \pm 2515.2$  vs  $11166.4 \pm 2589.4$ , respectively,  $P < 0.05$ ), suggesting that defective MSC PD in MDS patients may be due to accelerated telomeric loss (**Figure 3C**).

Compared with MSCs from healthy controls, cell cycle analysis to pediatric MDS-derived MSCs showed a decrease in the S phase ( $P < 0.05$ ), whereas no difference was observed in the G0/G1 and G2/M phase. For pediatric MDS-derived MSCs (Passage 4),  $89.5 \pm 7.4\%$  cells were at G0/G1,  $5.9 \pm 4.4\%$  at S, and  $4.8 \pm 3.5\%$  at G2/M phases of cell cycle. The data from healthy controls was  $84.9 \pm 6.7\%$  (G0/G1 phases),  $9.4 \pm 3.9\%$  (S phases), and  $5.7 \pm 3.1\%$  (G2/M phases) of cell cycle, respectively (**Figure 3B**). The data for the comparison of MSCs in passages 6 and passage 8 was similar to MSCs in passage 4 (not show).

### Discussion

Myelodysplastic syndrome (MDS) comprises clonal disorders of HSCs characterized by ineffective hematopoiesis, and is responsible for one or several peripheral cytopenias [18]. Evidence accumulated in recent years demonstrates that, in addition to HSC defects, a particular role is also played by BM microenvironment dysfunctions, which mediate indirect and direct contact with hematopoietic precursor cells (HPCs) [6, 19]. As the important components of the BM microenvironment, MSCs from MDS patients have been the main focus.

However, conflicting results have also been reported with regard to the biological behavior of MDS BM-derived MSCs [7, 8, 11-16], which means that further studies are needed. To address this issue, this study aimed to investigate the proliferation capacity of BM-derived MSCs at clone cell level in pediatric MDS patients.

Our data revealed that the MSCs from pediatric MDS patients could be expanded and cloned *in vitro*, as well as normal MSCs. Moreover, flow cytometer analysis showed that pediatric MDS-derived MSCs displayed similar surface markers compared to MSCs from healthy controls, which were in accordance with the MSCs criteria established by the International Society for Cellular Therapy [20]. Furthermore, tests for the differentiation potential of MSCs showed pediatric MDS-derived MSCs could differentiate into adipocytes and osteoblasts *in vitro*. Herein, we conclude that pediatric MDS-derived MSCs possess the basic characters of MSCs, including plastic-adherence, multiple differentiation capacity and some non-specific surface markers.

In contrast to studies on their basic characteristics, the proliferation capacity of adult MDS-derived MSCs compared to normal MSCs is controversial, so too is data regarding the molecular mechanisms involved in their abnormal proliferation [7, 12, 13, 16]. Data from the current study showed that the PD time of pediatric MDS-derived MSCs was significantly increased compared with healthy controls in accordance with previous reports about adult MDS. Now our study limited in a featured cell population supply more solid evidence for the defective proliferation capacity of MDS-derived MSCs [13, 16]. The defective proliferation capacity may be associated with the process of MDS.

Regarding the defective proliferation, we found that the morphology of pediatric MDS-derived MSCs was slightly different to MSCs from healthy controls. Pediatric MDS-derived MSCs were larger and more irregular in shape, with the resemblance to normal MSCs undergoing senescence [21-24]. We therefore investigated further the senescence of MSCs from pediatric MDS patients and healthy controls using  $\beta$ -galactosidase activity as an indicator of senescence. A higher number of  $\beta$ -gal-positive

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cells in pediatric MDS-derived MSCs demonstrated these cells were more prone to cellular senescence compared with MSCs from healthy controls. Moreover, as the main indicator of cell senescence, MSCs telomere length was also determined. In line with the  $\beta$ -galactosidase activity assay, data from the telomere length assay showed that the mean relative telomere length of pediatric MDS-derived MSCs was significantly lower compared to healthy control-derived MSCs, implying accelerated telomere attrition in MDS-derived MSCs. Taken together, we can conclude that accelerated cell senescence might represent a mechanism responsible for the defective proliferation of pediatric MDS-derived MSCs.

In accordance with the analysis of cell senescence, pediatric MDS-derived MSCs displayed a significantly decreased proportion of cells occupying the S phase, which meant fewer cells were in proliferation phase. Since cell cycle is a series of events that occurs in a cell leading to its division and replication, and is a critical process for determining cell proliferation and senescence [25], so we think the abnormal cell cycle of pediatric MDS-derived MSCs might be associated with cell senescence.

However, due to the small sample number and different methodologies for MSC cultivation between our study and others, larger studies and standardized methodologies were necessary for the future research. In addition, the mechanism of cell senescence for MDS-derived MSCs remains unclear, further studies are warranted.

In conclusion, we have investigated for the first time the association between the defective proliferation capacity and cell senescence in MSCs from pediatric MDS patients at clone cell level. Our findings have demonstrated that pediatric MDS-derived MSCs possess the basic characteristics of normal MSCs, but display defective proliferation, which may be associated with cell senescence.

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### Disclosure of conflict of interest

None.

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