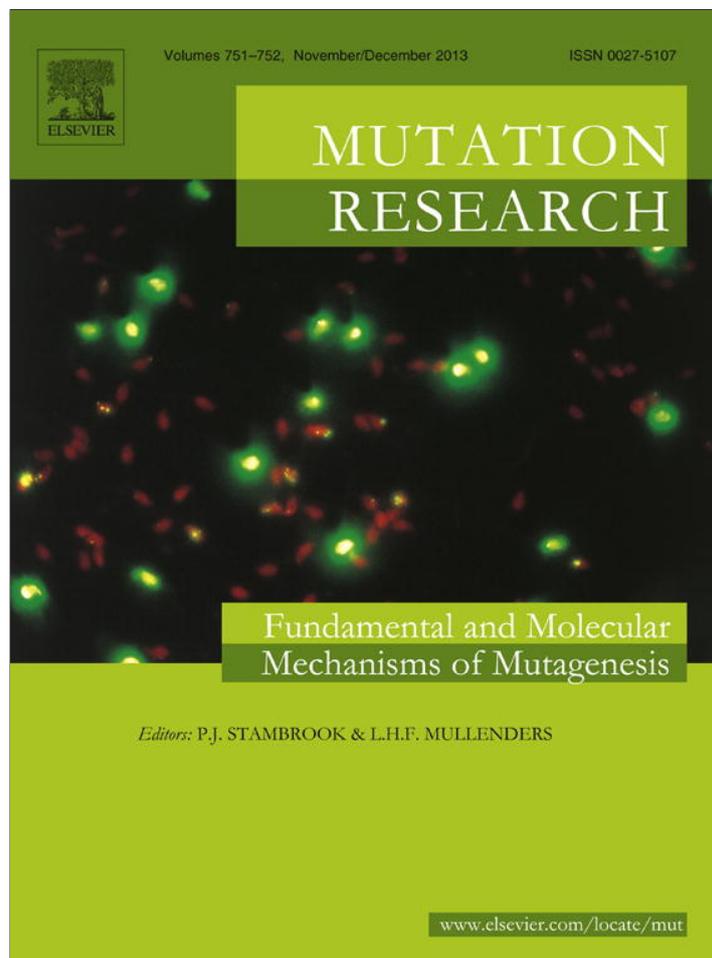


Provided for non-commercial research and education use.  
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/authorsrights>



Contents lists available at ScienceDirect

## Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: [www.elsevier.com/locate/molmut](http://www.elsevier.com/locate/molmut)  
 Community address: [www.elsevier.com/locate/mutres](http://www.elsevier.com/locate/mutres)



# Apparent mtDNA sequence heterogeneity in single human blood CD34<sup>+</sup> cells is markedly affected by storage and transport



Yong-Gang Yao<sup>a,b,\*</sup>, Sachiko Kajigaya<sup>b</sup>, Leigh Samsel<sup>b</sup>, J. Philip McCoy Jr.<sup>b</sup>,  
 Giuseppe Torelli<sup>c</sup>, Neal S. Young<sup>b</sup>

<sup>a</sup> Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan 650223, China

<sup>b</sup> Hematology Branch and Flow Cytometry Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

<sup>c</sup> Dipartimento di Oncologia ed Ematologia, Università di Modena e Reggio Emilia, Policlinico – Via del Pozzo 71, Modena 41100, Italy

### ARTICLE INFO

#### Article history:

Received 25 April 2013

Received in revised form 1 September 2013

Accepted 5 September 2013

Available online 14 September 2013

#### Keywords:

mtDNA

Single cell analysis

Mutation

Hematopoietic stem cell

Committed progenitors

### ABSTRACT

Single CD34<sup>+</sup> cells from adult human peripheral blood show mtDNA sequence heterogeneity. In this study, we compared mtDNA sequence variation in single CD34<sup>+</sup> cells from peripheral blood (PB) mononuclear cells (MNCs) from the same donors but under different conditions of storage and transport: group I, MNCs from heparinized PB that inadvertently required six days to be transported to the testing laboratory; group II, MNCs which were isolated from PB within a day of phlebotomy and frozen prior to transportation and storage. We observed more cell death for MNCs of group I than group II. Concordantly, group I CD34<sup>+</sup> cells had a very low potential for hematopoietic colony formation *in vitro* compared with group II cells. CD34<sup>+</sup> cells of group II showed an unexpectedly higher level of mtDNA sequence heterogeneity than was present in group I cells. These observations suggest that reduced mtDNA sequence heterogeneity in single CD34<sup>+</sup> cells of group I was likely due to elimination of cells harboring mutations. CD34<sup>+</sup> cells that survive stress *ex vivo* may be more enriched in quiescent primitive hematopoietic stem cells, with fewer mtDNA mutations than are present in committed progenitors. Technically, attention is required to conditions of preparation of human blood samples for single cell mtDNA analysis.

Published by Elsevier B.V.

## 1. Introduction

Human mtDNA is a circular double-strand molecule which encodes 2 rRNA, 22 tRNA, and 13 proteins essential for oxidative phosphorylation [1]. Because of the unique characteristics of this molecule, such as maternally inheritance, lack of recombination, a high mutation rate, and many copies per cell, mtDNA has been extensively used as a marker in the reconstruction of demographic history for ethnic populations and in forensic identification [2,3]. Germline and somatic mutations in mtDNA, which affect the transcription and translation of mtDNA genes or lead to defective protein production, cause mitochondrial dysfunction and contribute to a wide variety of disorders [4,5]. The cumulative burden of mtDNA mutations is central to the

“mitochondrial theory of aging” [6], which hypothesizes that aging is caused by a vicious cycle between reactive oxygen species generation and mtDNA mutation induction [7,8]. However, this theory has been challenged by recent data, and further there are common misconceptions and criticisms concerning the theory itself [9,10].

Over several years, we have systematically analyzed mtDNA mutation in single hematopoietic cells, with the intention to characterize potential roles of accumulated mtDNA mutations during the aging of human hematopoietic system and as they may relate to hematologic diseases [11–15]. We are also interested in whether somatic mtDNA mutations in single CD34<sup>+</sup> cells are stable and can be utilized as markers to trace the hematopoietic stem cell (HSC) and its progeny [16]. We detected aging-dependent accumulation of mtDNA mutations in single colonies of CD34<sup>+</sup> cells from cord blood, peripheral blood (PB), and bone marrow [12,13]. CD34<sup>+</sup> cells marked by certain mtDNA variant(s) are stable *in vivo* and repopulate recipients after HSC transplantation [16]. Moreover, some mtDNA variants in differentiated cells, such as T-cells, B-cells, and granulocytes, also are present in CD34<sup>+</sup> cells from that individual, suggesting common origin of these mature cells from the same HSC clone [11].

In subsequent experiments, we aimed to discern mtDNA sequence variations in single CD34<sup>+</sup> cells from multiple members of

**Abbreviations:** mtDNA, mitochondrial DNA; HSC, hematopoietic stem cell; PB, peripheral blood; MNC, mononuclear cell; PBS, phosphate buffered saline; BSA, bovine serum albumin.

\* Corresponding author at: Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China. Tel.: +86 871 65180085; fax: +86 871 65180085.

E-mail addresses: [ygyaozh@gmail.com](mailto:ygyaozh@gmail.com), [yaoyg@mail.kiz.ac.cn](mailto:yaoyg@mail.kiz.ac.cn) (Y.-G. Yao).

**Table 1**  
Number of mtDNA sequence haplotypes in the single CD34<sup>+</sup> cell population.

Donor <sup>a</sup>	Sample <sup>b</sup>	Age/sex	Live cells/gated cells (%) <sup>c</sup>	CD34 <sup>+</sup> cells/live cells (%) <sup>c</sup>	No. of cells for mtDNA analysis	No. of haplotypes	No. of haplotypes defined by nucleotide substitutions	No. of haplotypes/100 cells	No. of haplotypes by substitutions/100 cells
Donor #1	Sample 5 B-8	51/M	62.55	0.208	96	10	5	10.4	5.2
			91.92	0.371	94	35	28	37.2	29.8
Donor #2	Sample 10 B-3	89/F	70.29	0.079	95	22	14	23.2	14.7
			96.41	0.026	86	48	42	55.8	48.8
Donor #3	Sample 6 B-6	60/F	77.95	0.099	95	19	10	20.0	10.5
			89.71	0.025	95	47	38	49.5	40.0
Donor #4	Sample 9 B-5	87/F	71.24	0.019	95	13	8	13.7	8.4
			87.69	0.025	95	49	40	51.6	42.1

<sup>a</sup> These four donors were maternally related and belonged to family B described in our recent study [15]. Donors #1, #2, #3, and #4 refer to family members B-8, B-3, B-6, and B-5, respectively.

<sup>b</sup> Samples from the same donors that were included in group I and group II were marked by "Sample" and "B-", respectively. The mtDNA sequence data for samples of group II (B-8, B-3, B-6, and B-5) were taken from Yao et al. [15].

<sup>c</sup> The gating for living cells and CD34<sup>+</sup> cells was defined in Fig. 1.

a large pedigree whose ages varied widely [15]. Due to unexpected delay in the arrival of blood samples from Europe to the testing laboratory and apparently anomalous results, we were forced to analyze mtDNA sequence heterogeneity in CD34<sup>+</sup> cells isolated from peripheral blood (PB) mononuclear cells (MNCs) from the same donors twice. In the first round of sample collection (group I), PB was collected in heparin and shipped by express "overnight" service to the testing laboratory in Bethesda. However, the shipment was delayed, and MNCs were isolated upon receiving the samples six days after the initial phlebotomy (and subsequent isolation of cells and freezing in liquid nitrogen). Because of our concern for the integrity of the DNA ultimately extracted and anomalous results, we undertook a second round of sample collection from the same individuals (group II); MNCs were isolated from PB within 24 h of blood drawing, frozen in a local laboratory by the same procedure, and then cells were transported to the testing laboratory in dry ice and transferred to liquid nitrogen upon arrival. We observed markedly different levels of mtDNA sequence heterogeneity in single CD34<sup>+</sup> cells from the same donors' MNCs after these different collection, storage and transportation protocols. Our observation suggested that mtDNA sequence heterogeneity in single CD34<sup>+</sup> cells may unexpectedly alter *in vitro*, most likely due to changes in the composition of the CD34<sup>+</sup> cell population.

## 2. Materials and methods

### 2.1. Sample collection

PB was drawn from the maternally related healthy donors living in Italy and the United States (Table S1); these donors were from Family B described in our recent publication [15]. In group I, whole blood (8–10 mL) from each of ten donors was collected in a heparinized tube in Europe and mailed to Bethesda. MNCs were separated by Ficoll density gradient centrifugation upon arrival, but after an unexpected six days after blood collection, followed by storage in liquid nitrogen. In group II, a similar amount of PB was obtained from ten donors (eight of them had been sampled in group I) in a heparinized tube and processed in a proximate laboratory in Italy within 24 h of phlebotomy by sedimentation of MNCs using the same standard protocol, freezing, and storage in liquid nitrogen. Freezing medium was composed of 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS). The frozen MNCs were shipped to Bethesda on dry ice and then transferred to liquid nitrogen until they were thawed for sorting. MNCs of three healthy donors from the family living in the United States were processed within 24 h of blood collection and were stored promptly in liquid nitrogen. All donors gave informed consent according to a protocol for sample collection approved by the institutional review board of the National Heart, Lung, and Blood Institute.

### 2.2. Single CD34<sup>+</sup> cell sorting and culture

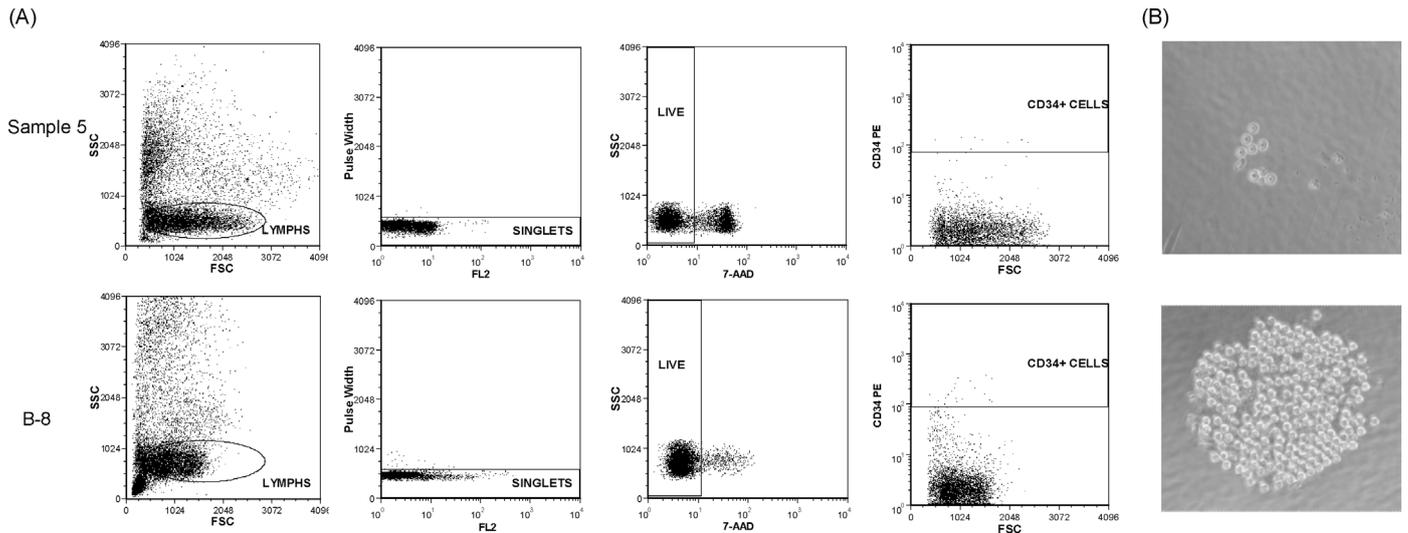
Frozen MNCs were thawed and washed with phosphate buffered saline (PBS), as described [11,15,16]. Briefly, about  $1 \times 10^6$  cells suspended in 100  $\mu$ L of PBS containing 0.5% bovine serum albumin (BSA) were incubated with anti-CD34 phycoerythrin

(PE)-conjugated monoclonal antibody (BD Bioscience, San Jose, CA) for 30 min at 4°C. Cells were then washed and resuspended in 600  $\mu$ L of PBS supplemented with 0.5% BSA. We added 4  $\mu$ L of 7-amino-actinomycin D (7-AAD) into the cell suspension and kept the cells on ice for approximately 20 min or until sorting on the MoFlo Cytometer (Dako-Cytomation, Ft. Collins, CO). Cell sorting was performed using 100 mW of the 488 nm line of an argon laser (I-90, Coherent, Inc., Palo Alto, CA) for excitation. Forward scatter was the triggering parameter. Single cell deposition was accomplished using the CyClone automated cloner (Dako-Cytomation) in the 0.5 single drop mode with gating based on forward scatter and fluorescence, as described in our previous studies [11,14,16]. Single CD34<sup>+</sup> cells from four donors, with ages of 51, 60, 87, and 89 years, respectively (Table 1), were collected in one or two 96-well plates (one cell/well) for mtDNA analysis, depending on the cell number. The remaining cells were then sorted into 96-well round-bottom cell culture plates (Corning, Inc., Corning, NY) in the same mode as used for culture. Each well contained 50  $\mu$ L of StemSpan<sup>®</sup> Serum-Free Expansion Medium supplemented with StemSpan<sup>®</sup> CC100 cytokine cocktail (StemCell Technologies, Inc., Vancouver, Canada). Single CD34<sup>+</sup> cells were cultured for a week in 5% CO<sub>2</sub> at 37°C. Colonies in wells of the culture plate were viewed using an inverted Olympus IX50 microscope. For the remaining six samples in group I and nine samples in group II (Table S1), we counted the ratio for CD34<sup>+</sup> cells/live cells of each sample, to show the relative number of CD34 positive staining cells.

### 2.3. Single cell PCR amplification and sequencing

For mtDNA analysis, we followed our previously described procedure [11,14–16]. In brief, single CD34<sup>+</sup> cells were sorted into each well of an optical 96-well reaction plate (MicroAmp; Applied Biosystems, Foster City, CA) containing 50  $\mu$ L of lysis buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 100  $\mu$ g/mL Proteinase K, 1% Triton X-100). The plate was incubated at 56°C for 30 min, followed by incubation at 96°C for 8 min to release the mtDNA molecules. Two-step nested PCR was used to amplify the entire mtDNA control region in single CD34<sup>+</sup> cells [14,16]. The first PCR was performed in 30  $\mu$ L of reaction mixture, which containing 5  $\mu$ L of cell lysate, 400  $\mu$ M of each dNTP, 1  $\times$  LA PCR<sup>™</sup> Buffer II (Mg<sup>2+</sup> plus), 1 unit of TaKaRa LA Taq<sup>™</sup> that with proof-reading activity (Takara Bio., Inc.), and 0.5  $\mu$ M of each primer (L15594: 5'-CGCCTACACAATTCTCCGATC-3' and H901: 5'-ACTTGGGTTAATCGTGTGACC-3'). PCR amplification was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) with the following cycles: one cycle of 94°C for 3 min; then 35 cycles of 94°C for 30 s, 50°C for 40 s and 72°C for 1 min with a 5 s increase per cycle; and ending with a full extension cycle of 72°C for 10 min. The second PCR was performed in 50  $\mu$ L of reaction mixture containing 400  $\mu$ M of each dNTP, 1  $\times$  LA PCR<sup>™</sup> Buffer II (Mg<sup>2+</sup> plus), 2 units of TaKaRa LA Taq<sup>™</sup>, 0.5  $\mu$ M of each (L15990: 5'-TTAACTCCACCATAGCACC-3' and H650: 5'-GAAAGGCTAGGACCAACCTA-3'), and 5  $\mu$ L of each first PCR product. Amplification cycles for the second PCR consisted of one cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 90 s; and ending with a full extension cycle of 72°C for 10 min.

Second PCR products were purified using the ExcelsaPure 96-Well UF PCR Purification Kit (EdgeBiosystems, Gaithersburg, MD) and sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3100 DNA sequencer (Applied Biosystems), according to the manufacturer's manual. We used the second PCR primers and the following primers that were described in our previous studies [14–16] to cover the entire mtDNA control region: L15996, 5'-CTCCACCATAGCACCACCAAGC-3'; L16209, 5'-CCCCATGCTTACAAGCAAGT-3'; L16517, 5'-CATCTGGTTCTACTTCCAGG-3'; H26, 5'-GCATGGAGAGCTCCCGTAGTGG-3'; L29, 5'-GGTCTATCACCTATTAACAC-3'; L332, 5'-CCCGCTTCTGGCCACAGCAC-3'.



**Fig. 1.** Sorting for CD34<sup>+</sup> cells using flow cytometry and culture assay for single CD34<sup>+</sup> cells. (A) Dead cells in the mononuclear cells from donor #1 were demonstrated by staining with 7-AAD. Cells from Sample 5 (group I) had a lower level of viability than cells from B-8 (group II). (B) Single CD34<sup>+</sup> cells from Sample 5 (group I) had less colony formation potential than those from B-5 (group II). A representative colony developed from individual CD34<sup>+</sup> cell of each group was shown.

2.4. Scoring mtDNA sequence variation and statistical analysis

Our approach to define sequence variations in single CD34<sup>+</sup> cells has been published [14–16]. In brief, sequences were aligned by SeqMan program in DNASTar package (DNASTAR, Inc.) and were proof-read by eye. Sequence variants were scored relative to the Cambridge Reference Sequence (rCRS) [1]. Cells with contamination were identified using the approach described before [17] and were excluded from the analysis. Due to the limits of sequencing, heteroplasmy (co-existence of wild-type and the mutant allele) of certain mtDNA variant was scored when a mutant allele was present at >10% level chromatography [14]. Because the four donors selected for mtDNA analysis were maternally related, their mtDNA sequences were essentially the same, and they contained a 16189T>C polymorphism that triggers heteroplasmy in multiple poly-C tracts in region 16184–16193; these length polymorphisms of poly-C tracts could be not reliably counted based on the sequencing electropherograms and were not analyzed in the current study. We scored the length variations of the C-tract in region 303–309 by direct counting of the base shift of T at site 310. The length mutation of the AC repeat in region 515–524 in the third hypervariable segment (HVS-III) was also based on sequencing electropherograms.

We counted the number of haplotypes in a population of cells from each sample and used it as an index to compare the level of mtDNA sequence heterogeneity between different samples. This index reflects the total number of mutations that have occurred or retained within a given number of cells, which in turn reflects the time over which the subclone has developed and the net mutation rate per unit time [14].

The unpaired *t* test was used to compare differences between the two groups of samples that had been processed differently. We used the Fisher exact test to quantify the difference of heterogeneity level in CD34<sup>+</sup> cells from the same donor. A value of *P* < 0.05 was regarded as statistically significant.

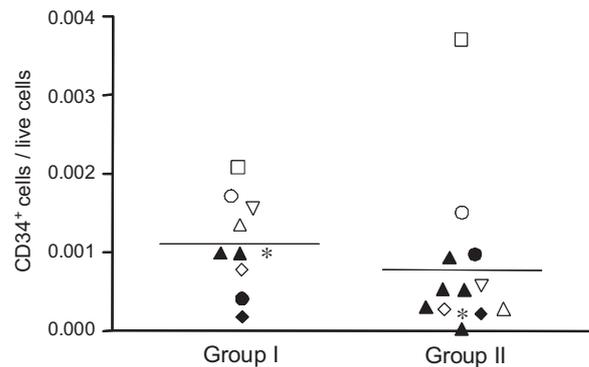
3. Results and discussion

3.1. Cell viability and colony formation

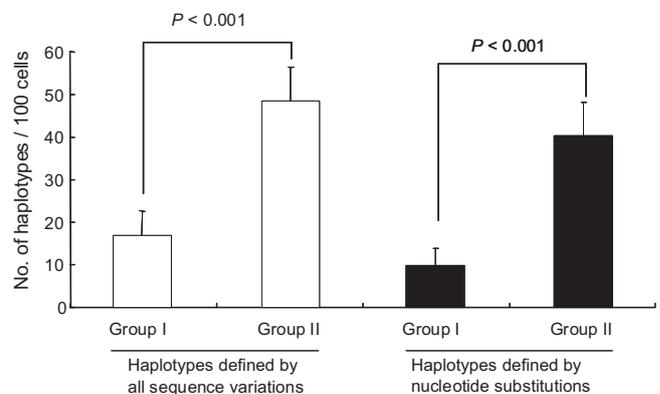
Upon receiving the heparinized blood shipped to Bethesda at day 6 after blood collection, we suspected hemolysis, as the serum was tinged red. After Ficoll density gradient centrifugation and washing in PBS, we discarded the cell clump and froze the remaining MNCs in freezing medium. Staining with trypan blue showed that 20–50% of suspended MNCs were dead cells among the samples.

After thawing and washing of the frozen MNCs, we observed cell debris clumps for the samples of group I but not for samples of group II, suggesting that many of the MNCs in the former were dead. Staining with 7-AAD during the sorting for the MNCs confirmed that a high proportion (22–58%) of MNCs of group I was dead cells, whereas of MNCs of group II, only 4–15% cells were positive for 7-AAD staining (Fig. 1A). However, we observed a generally higher

frequency of CD34<sup>+</sup> cells in the gated area for MNCs in samples of group I compared to group II (except for donor #1, which showed an inverse pattern; Tables 1 and S1 and Fig. 2). Since we removed the dead cells by 7-AAD staining during the sorting, we do not think the enriched CD34<sup>+</sup> cells in the MNCs of group I were artifacts of



**Fig. 2.** Enriched CD34<sup>+</sup> cells in mononuclear cells from group I but not in group II. The same donors that were sampled for both group I and group II studies were represented by the same icons. Donors sampled in either group I or group II study were demonstrated by filled triangles. The detailed information regarding samples was listed in Table S2.



**Fig. 3.** A lower level of mtDNA sequence variations in single CD34<sup>+</sup> cells from group I than group II.



and Table 1). The reduced level of mtDNA heterogeneity in group I CD34<sup>+</sup> cells was unexpected, as we had detected both global cell loss and deficiency of proliferating progenitor cells in these samples and anticipated DNA damage or stress might increase mtDNA mutations in these cells. Alternatively, these results are consistent with over representation of primitive and quiescent CD34<sup>+</sup> cells in group I and a lower level of mtDNA mutation than in the committed progenitor cells. Clonal expansion of certain CD34<sup>+</sup> cells in the heparinized blood during transport might also lead to a reduced level of mtDNA sequence heterogeneity, as we have observed for leukemic blasts [14], but would appear unlikely to occur under poor conditions for cell proliferation. Further experiments should be carried out to solidify this speculation.

### 3.3. Survival of CD34<sup>+</sup> cells marked by specific mtDNA variants

In our previous study, we found that mtDNA variants in CD34<sup>+</sup> cells can exist *in vivo* for years and be transplanted to a recipient [16]. Disregarding the differences in sample storage and transport, the analyzed CD34<sup>+</sup> cells from each donor represented two different samplings. It was of interest to compare the occurrence of clones marked by specific mtDNA variants in both batches of cells from the same donor in order to identify CD34<sup>+</sup> cells capable of survival over the 6 days of transport. As the length mutations of the C-stretch in regions 16183–16193 (due to the 16189T>C variation) and 303–309 constitute mutational hot spots, we only considered the haplotypes defined by nucleotide substitutions. For each donor, we identified at least one such haplotype in CD34<sup>+</sup> cells, e.g. a cell with 16131T>Y was observed in two batches of CD34<sup>+</sup> cells from donor #1; in donor #3, a cell clone with 182T>Y was observed in both groups (Fig. 4 and Table S1). This unique pattern suggested that these CD34<sup>+</sup> cells were presumably the primitive HSCs that had better survived storage and transport.

### 3.4. Implications for primitive CD34<sup>+</sup> cells and limitations of this study

For clinical reasons, the storage and transport of CD34<sup>+</sup> cells have been extensively studied [18–21], mainly because hematopoietic reconstitution following transplantation depends upon the number of infused CD34<sup>+</sup> cells [22]. In this study, we compared colony formation and the level of mtDNA sequence variations in CD34<sup>+</sup> cells from the same donors that were stored and transported differently. We speculate that surviving CD34<sup>+</sup> cells in group I were more primitive, based on two lines of evidence: (1) lower colony formation potential during short-term culture, and (2) low frequency of mtDNA sequence alterations. The persistence of certain CD34<sup>+</sup> cell clones marked by specific mtDNA mutations in two different samplings of the same donor further suggested that these clones were stable [16] and had good survival potential *in vitro*. Further study will be required to characterize functionally the primitive status of enriched CD34<sup>+</sup> cells as observed during the sorting for samples of group I and to elucidate why more primitive hematopoietic stem cells are more likely to survive stress. Regardless of the mechanism, caution in interpreting mtDNA mutation data in human blood samples is warranted, with attention to the details of collection, processing, and storage, especially in increasingly global studies of the human genomes and its variants.

The current study has two limitations. First, reduced mtDNA sequence heterogeneity in single CD34<sup>+</sup> cells of group I was based essentially on a single experiment and we were not able to repeat this experiment. Nonetheless, we examined four pairs of samples in the analysis and we obtained a similar pattern in all individuals, suggesting that this is unlikely to be a random finding. Second, except for the colony formation culture assay, we did not explore further characterization of the CD34<sup>+</sup> cells of group I and to confirm

that these cells are more primitive compared with the CD34<sup>+</sup> cells of group II. Staining with more markers to distinguish hematopoietic stem cells and progenitors would provide further evidence to clarify this issue.

### Conflict of interest statement

The authors declare no conflict of interest.

### Acknowledgements

This study was supported by NIH Research Program. Y.-G.Y. was supported by the National Natural Science Foundation of China (30925021 and 31171225), Yunnan Province (2009CI119) and the Chinese Academy of Sciences. Y.-G.Y. and N.S.Y. designed the research, Y.-G.Y., L.S., S.K. and J.P.M. performed the research, Y.-G.Y. and L.S. analyzed the data. Y.-G.Y., S.K. and N.S.Y. wrote the paper, G.T. contributed essential samples.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mrfmmm.2013.09.001>.

### References

- [1] S. Anderson, A.T. Bankier, B.G. Barrell, M.H. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J. Smith, R. Staden, I.G. Young, Sequence and organization of the human mitochondrial genome, *Nature* 290 (1981) 457–465.
- [2] Y.-G. Yao, Q.-P. Kong, H.-J. Bandelt, T. Kivisild, Y.-P. Zhang, Phylogeographic differentiation of mitochondrial DNA in Han Chinese, *Am. J. Hum. Genet.* 70 (2002) 635–651.
- [3] A. Salas, H.J. Bandelt, V. Macaulay, M.B. Richards, Phylogeographic investigations: the role of trees in forensic genetics, *Forensic Sci. Int.* 168 (2007) 1–13.
- [4] D.C. Wallace, W. Fan, The pathophysiology of mitochondrial disease as modeled in the mouse, *Genes Dev.* 23 (2009) 1714–1736.
- [5] R.W. Taylor, D.M. Turnbull, Mitochondrial DNA mutations in human disease, *Nat. Rev. Genet.* 6 (2005) 389–402.
- [6] D. Harman, The biologic clock: the mitochondria? *J. Am. Geriatr. Soc.* 20 (1972) 145–147.
- [7] A. Trifunovic, Mitochondrial DNA and ageing, *Biochim. Biophys. Acta* 1757 (2006) 611–617.
- [8] D. Harman, Free radical theory of aging: an update: increasing the functional life span, *Ann. N. Y. Acad. Sci.* 1067 (2006) 10–21.
- [9] R.M. Howes, The free radical fantasy: a panoply of paradoxes, *Ann. N. Y. Acad. Sci.* 1067 (2006) 22–26.
- [10] G. Barja, Updating the mitochondrial free radical theory of aging: an integrated view, key aspects, and confounding concepts, *Antioxid. Redox. Signal.* (2013), <http://dx.doi.org/10.1089/ars.2012.5148>.
- [11] Y. Ogasawara, K. Nakayama, M. Tarnowka, J.P. McCoy, S. Kajigaya Jr., B.C. Levin, N.S. Young, Mitochondrial DNA spectra of single human CD34<sup>+</sup> cells, T cells, B cells, and granulocytes, *Blood* 106 (2005) 3271–3284.
- [12] M.G. Shin, S. Kajigaya, J.P. McCoy, B.C. Levin Jr., N.S. Young, Marked mitochondrial DNA sequence heterogeneity in single CD34<sup>+</sup> cell clones from normal adult bone marrow, *Blood* 103 (2004) 553–561.
- [13] M.G. Shin, S. Kajigaya, M. Tarnowka, J.P. McCoy, B.C. Levin Jr., N.S. Young, Mitochondrial DNA sequence heterogeneity in circulating normal human CD34 cells and granulocytes, *Blood* 103 (2004) 4466–4477.
- [14] Y.-G. Yao, Y. Ogasawara, S. Kajigaya, J.J. Mouldrem, R.P. Falcão, M.-C. Pin-tão, J.P. McCoy, E.G. Rizzatti Jr., N.S. Young, Mitochondrial DNA sequence variation in single cells from leukemia patients, *Blood* 109 (2007) 756–762.
- [15] Y.-G. Yao, S. Kajigaya, X. Feng, L. Samsel, J.P. McCoy, G. Torelli Jr., N.S. Young, Accumulation of mtDNA variations in human single CD34<sup>+</sup> cells from maternally related individuals: effects of aging and family genetic background, *Stem Cell Res.* 10 (2013) 361–370.
- [16] Y.-G. Yao, R.W. Childs, S. Kajigaya, J.P. McCoy, N.S. Young Jr., Mitochondrial DNA sequence heterogeneity of single CD34<sup>+</sup> cells after nonmyeloablative allogeneic stem cell transplantation, *Stem Cells* 25 (2007) 2670–2676.
- [17] Y.-G. Yao, H.-J. Bandelt, N.S. Young, External contamination in single cell mtDNA analysis, *PLoS ONE* 2 (2007) e681.
- [18] A. Humpe, C. Beck, R. Schoch, M. Kneba, H.A. Horst, Establishment and optimization of a flow cytometric method for evaluation of viability of CD34<sup>+</sup> cells after cryopreservation and comparison with trypan blue exclusion staining, *Transfusion (Paris)* 45 (2005) 1208–1213.

- [19] R. Reich-Slotky, A.I. Colovai, M. Semidei-Pomales, N. Patel, M. Cairo, J. Jhang, J. Schwartz, Determining post-thaw CD34<sup>+</sup> cell dose of cryopreserved haematopoietic progenitor cells demonstrates high recovery and confirms their integrity, *Vox Sang.* 94 (2008) 351–357.
- [20] V. Antonenas, F. Garvin, M. Webb, M. Sartor, K.F. Bradstock, D. Gottlieb, Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport, *Cytotherapy* 8 (2006) 158–165.
- [21] H. Yang, J.P. Acker, M. Cabuhat, L.E. McGann, Effects of incubation temperature and time after thawing on viability assessment of peripheral hematopoietic progenitor cells cryopreserved for transplantation, *Bone Marrow Transpl.* 32 (2003) 1021–1026.
- [22] E.J. Shpall, R. Champlin, J.A. Glaspy, Effect of CD34<sup>+</sup> peripheral blood progenitor cell dose on hematopoietic recovery, *Biol. Blood Marrow Transpl.* 4 (1998) 84–92.

Table S1. Frequency of CD34<sup>+</sup> cells in living mononuclear cells isolated from samples of group I and group II

Donor	Age /Sex	Sample	Total number of cells	Number of gated cells	Number of live cells	Number of dead cells	Number of CD34 <sup>+</sup> cells	Live cells / gated cells (%)	CD34 <sup>+</sup> cells / gated cells (%)	CD34 <sup>+</sup> cells / live cells (%)
Donor #1	51/M	B-8	13511	5864	5390	474	20	91.9168	0.34106	0.37106
		Sample 5	10742	6141	3841	2300	8	62.5468	0.13027	0.20828
Donor #2	89/F	B-3	102366	47742	46026	1716	12	96.4057	0.02514	0.02607
		Sample 10	11323	3595	2527	1068	2	70.2921	0.05563	0.07915
Donor #3	60/F	B-6	102151	70343	63108	7235	16	89.7147	0.02275	0.02535
		Sample 6	11571	6472	5045	1427	5	77.9512	0.07726	0.09911
Donor #4	87/F	B-5	106871	59211	51925	7286	13	87.6949	0.02196	0.02504
		Sample 9	31612	14524	10347	4177	2	71.2407	0.01377	0.01933
Donor #5	57/F	B-10	101300	61689	54156	7533	28	87.7887	0.04539	0.0517
		—	—	—	—	—	—	—	—	—
Donor #6	79/F	B-4	311505	86398	80713	5685	47	93.42	0.0544	0.05823
		Sample 7	10702	4721	2563	2158	4	54.2893	0.08473	0.15607
Donor #7	28/F	B-16	59660	27212	24854	2358	7	91.3347	0.02572	0.02816
		—	—	—	—	—	—	—	—	—
Donor #8	48/M	B-7	12919	7853	7397	456	2	94.1933	0.02547	0.02704
		Sample 4	10711	5353	2217	3136	3	41.416	0.05604	0.13532
Donor #9	81/M	B-2	104184	45655	40752	4903	39	89.2608	0.08542	0.0957
		Sample 8	11806	6650	4809	1841	2	72.3158	0.03008	0.04159
Donor #10	27/M	B-11	152612	59207	49804	9403	76	84.1184	0.12836	0.1526
		Sample 1	10665	4156	2898	1258	5	69.7305	0.12031	0.17253
Donor #11	83/M	B-1	151859	80083	72857	7226	68	90.9769	0.08491	0.09333
		—	—	—	—	—	—	—	—	—
Donor #12	21/M	B-12	106046	57860	53145	4715	27	91.851	0.04666	0.0508

Donor	Age /Sex	Sample	Total number of cells	Number of gated cells	Number of live cells	Number of dead cells	Number of CD34 <sup>+</sup> cells	Live cells / gated cells (%)	CD34 <sup>+</sup> cells / gated cells (%)	CD34 <sup>+</sup> cells / live cells (%)
		—	—	—	—	—	—	—	—	—
Donor #13	32/F	B-15	114404	92898	87518	5380	4	94.2087	0.00431	0.00457
		—	—	—	—	—	—	—	—	—
Donor #14	37/M	Sample 2	11844	4466	3036	1430	3	67.9803	0.06717	0.09881
		—	—	—	—	—	—	—	—	—
Donor #15	42/F	Sample 3	5000000	120288	65831	54457	65	54.7278	0.05404	0.09874
		—	—	—	—	—	—	—	—	—

Note: Samples from the same donors that were included in group I and group II were represented marked by “Sample” and “B-”, respectively. These donors were maternally related and were from Family B described in Yao et al. [15]. The gating for living cells and CD34<sup>+</sup> cells was defined in Figure 1.

Table S2. mtDNA sequence variations in single CD34<sup>+</sup> cells isolated from group I samples

Sample	Sequence variation	No.	No. of haplotypes	No. of haplotypes defined by nucleotide substitutions
Sample 5	Aggregate sequence	36	1	
	+16086T>Y, 9C/10C	1	2	1
	+16131T>Y, 8C/9C/10C/11C	1	3	2
	+16150C>Y, 9C/10C/11C	1	4	3
	+16181A>A/C, 9C/10C/11C	1	5	4
	+16519T>Y, 9C/10C/11C	1	6	5
	8C/9C/10C/11C	1	7	
	8C/9C/10C/11C/12C	1	8	
	9C/10C	19	9	
9C/10C/11C/12C	34	10		
Sample 10	Aggregate sequence	34	1	
	+16034G>R, 9C/10C/11C/12C	1	2	1
	+16072C>Y, 9C/10C/11C	1	3	2
	+16081A>R, 16445T>Y, 9C/10C/11C/12C	1	4	3
	+16086T>Y, 9C/10C/11C	1	5	4
	+16124T>C, 9C/10C/11C	1	6	7
	+16129G>R, 9C/10C/11C/12C	1	7	6
	+16131T>C, 9C/10C	2	8	7
	+16131T>C, 9C/10C/11C	1	9	
	+16468T>Y, 9C/10C/11C/12C	1	10	8
	+204T>Y, 9C/10C/11C/12C	1	11	9
	+226T>Y, 9C/10C	1	12	10
	+302A>R, 9C/10C/11C/12C	1	13	11
	+303C>C/A, 9C/10C/11C/12C	1	14	12
	+303insA, 8C/9C/10C/11C	1	15	
	+405T>C, 466T>C, 9C/10C/11C/12C	1	16	13
	+405T>C, 9C/10C/11C/12C	1	17	14
	8C/9C/10C	1	18	
	8C/9C/10C/11C	3	19	
	8C/9C/10C/11C/12C	1	20	
	9C/10C	8	21	
	9C/10C/11C	31	22	
Sample 6	Aggregate sequence	40	1	
	+16018T>Y, 9C/10C/11C/12C	1	2	1
	+16250C>Y, 9C/10C	1	3	2

	+182C>Y, 9C/10C/11C	1	4	3
	+204T>Y, 9C/10C/11C	1	5	4
	+270A, 9C/10C	1	6	5
	+405T>C, 9C/10C/11C	1	7	6
	+418C>Y, 9C/10C/11C/12C	1	8	7
	+445C>Y, 9C/10C/11C/12C	1	9	8
	+513G>A, 9C/10C/11C/12C	1	10	9
	+573insC/non-ins, 9C/10C/11C/12C	1	11	
	+574A>R, 9C/10C/11C/12C	1	12	10
	+(523-524)delAC/non-del, 9C/10C/11C	1	13	
	+(523-524)delAC/non-del, 9C/10C/11C/12C	1	14	
	8C/9C/10C	1	15	
	8C/9C/10C/11C	1	16	
	8C/9C/10C/11C/12C	1	17	
	9C/10C	18	18	
	9C/10C/11C	21	19	
Sample 9	Aggregate sequence	44	1	
	+16095C>Y, 9C/10C/11C/12C	1	2	1
	+16129G>A, 9C/10C/11C/12C	1	3	2
	+16287C>Y, 308C>Y, 9C/10C/11C	1	4	3
	+16301C>Y, 9C/10C	1	5	4
	+217T>C, 9C/10C/11C	1	6	5
	+260G>R, 9C/10C/11C/12C	1	7	6
	+308C>Y, 9C/10C/11C	1	8	7
	+405T>C, 9C/10C/11C	1	9	8
	8C/9C/10C	1	10	
	8C/9C/10C/11C	5	11	
	9C/10C	8	12	
	9C/10C/11C	29	13	

Note: Sequence variation was scored relative to the Cambridge reference sequence [1]. A site heterogeneous for both thymidine (T) and cytidine (C) was abbreviated as Y, and heterogeneous for adenosine (A) and guanosine (G) was abbreviated as R. A site heterogeneous for a transversion or an indel (insertion and deletion) was highlighted by listing all status. We followed the approach in our previous studies [14, 16] to highlight the status of the C-tract length variations in region 303-309 and the dinucleotide AC repeat in region 515-524 in these cells differ from the aggregated sequence by listing all the status, e.g. 9C/10C means region 303-315 has heteroplasmy of CCCCCCCCCCTCCCCC and CCCCCCCCCCTCCCCC in the cell, 523-524delAC/non-del means co-existing of 4 and 5 repeats of AC in region 515-524 in the cell. +, extra mtDNA changes compared with the aggregate sequence. The aggregate sequence of Sample 5 contains 9C/10C/11C, whereas the other three samples all contain 9C/10C/11C/12C. The sequence variations in single CD34<sup>+</sup> cells isolated from group II samples (B-8, B-3, B-6 and B-5) were reported in Yao et al. [15].