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Chromosomal variability of human mesenchymal stem cells cultured under hypoxic conditions

Hanae Ueyama [a], Tomohisa Horibe [a], Shiro Hinotsu [a], Tomoaki Tanaka [b], Takeomi Inoue [b], Hisashi Urushihara [a], Akira Kitagawa [b], Koji Kawakami [a, *]

[a] *Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Yoshidakonoecho, Sakyo-ku, Kyoto, Japan*
[b] *ArBlast Co., Ltd., Kobe International Business Center, Minatojima-minamimachi, Chuo-ku, Kobe, Japan*

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Abstract

Bone marrow derived human mesenchymal stem cells (hMSCs) have attracted great interest from both bench and clinical researchers because of their pluripotency and ease of expansion *ex vivo*. However, these cells do finally reach a senescent stage and lose their multipotent potential. Proliferation of these cells is limited up to the time of their senescence, which limits their supply, and they may accumulate chromosomal changes through *ex vivo* culturing. The safe, rapid expansion of hMSCs is critical for their clinical application. Chromosomal aberration is known as one of the hallmarks of human cancer, and therefore it is important to understand the chromosomal stability and variability of *ex vivo* expanded hMSCs before they are used widely in clinical applications. In this study, we examined the effects of culturing under ambient (20%) or physiologic (5%) O_2 concentrations on the rate of cell proliferation and on the spontaneous transformation of hMSCs in primary culture and after expansion, because it has been reported that culturing under hypoxic conditions accelerates the propagation of hMSCs. Bone marrow samples were collected from 40 patients involved in clinical research. We found that hypoxic conditions promote cell proliferation more favourably than normoxic conditions. Chromosomal aberrations, including structural instability or aneuploidy, were detected in significantly earlier passages under hypoxic conditions than under normoxic culture conditions, suggesting that amplification of hMSCs in a low-oxygen environment facilitated chromosomal instability. Furthermore, smoothed hazard-function modelling of chromosomal aberrations showed increased hazard after the fourth passage under both sets of culture conditions, and showed a tendency to increase the detection rate of primary karyotypic abnormalities among donors aged 60 years and over. In conclusion, we propose that the continuous monitoring of hMSCs will be required before they are used in therapeutic applications in the clinic, especially when cells are cultured under hypoxic conditions.

Keywords: bone marrow derived mesenchymal stem cells • chromosomal aberration • hypoxia • karyotype • regenerative medicine

Introduction

Stem cells, such as embryonic stem cells (ES cells) and somatic stem cells, are characterized by their ability to both self-renew and differentiate into specific cell lineages. These cells may have potential use in regenerative medical therapies, and tissue engineering is a key component of regenerative products. In particular, bone marrow derived human mesenchymal stem cells (hMSCs) represent an appealing source of adult stem cells for cell therapy and tissue engineering. hMSCs have been isolated from multiple sources, including bone marrow, umbilical cord blood [1] and adipose tissue [2], and can be easily obtained and expanded *ex vivo* under appropriate culture conditions [3]. In addition, multipotent hMSCs can be selectively differentiated into various cell types such as osteocytes [4], chondrocytes [5], myocytes [6] and adipocytes [7]. Thus, the hMSCs have great potential for use in a variety of clinical applications. The beneficial effects of hMSCs have already been tested in the treatment of graft-*versus*-host disease [8], regenerative therapy such as improvement of organ function after injection of autologous and allogenic stem cells to

*Correspondence to: Dr. Koji KAWAKAMI,
Department of Pharmacoepidemiology,
Graduate School of Medicine and Public Health, Kyoto University,
Yoshidakonoecho, Sakyo-ku, Kyoto 606-8501, Japan.
Tel.: +81-75-753-4459
Fax: +81-75-753-4469
E-mail: kawakami.koji.4e@kyoto-u.ac.jp

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injured sites [9, 10] and treatment of osteogenesis imperfecta in children [11].

On the other hand, adult hMSCs have several problems that limit their usefulness for tissue engineering, such as their senescence and low abundance in human tissue. Because only a small number of hMSCs can be obtained from a donor's bone marrow at a time, these cells are usually expanded in culture before clinical application to yield an adequate number. For the initial expansion and further propagation of hMSCs, recombinant growth factors and other supplements are added to the culture medium, and the process is accompanied by the possibility of bacterial contamination and xenogeneic risk [12, 13]. Therefore, it is recommended to avoid unnecessary additional subculturing of these cells.

It was previously reported that culturing under hypoxic conditions enhances cell amplification [14–16], and culturing under hypoxia could be an alternative approach without the need for extra additives to stimulate primary culture and further expansion, yielding a sufficient supply of cells and avoiding multiple passages. However, one concern with this application is the accumulation of spontaneous mutations in the process of expansion, which probably occurs at a rate of 10^{-9} mutations per nucleotide [17]. Several lines of evidence have showed that *ex vivo* expanded stem cells may generate a population with tumorigenic potential, suggesting that such stem cells could become the origin of cancer [18, 19]. Therefore, it is important to evaluate the cytogenetic stability of *ex vivo* expanded hMSCs before they are widely used in clinical applications.

Here we present the results of continuous monitoring of chromosomal aberrations of hMSCs cultured under two distinct oxygen concentrations (5% and 20%), and discuss the importance of evaluating the cytogenetic stability of hMSCs to maximize their clinical therapeutic potential.

Materials and methods

hMSCs isolation and culture

Bone marrow (1–5 ml) was aspirated from the iliac crest of voluntary donors after obtaining written informed consent to participate in the research. The protocol has been approved by the Ethics Committee of each institutions within which sampling of bone marrow and the research work were undertaken. Of 40 patients aged between 26 and 77 years (median 61 years), 20 patients were admitted into the orthopaedic surgery ward and the others participated in the clinical research of dental osteogenesis employing autologous hMSC transplantation. The process for isolation and culture of hMSCs was conducted according to the method PCT/JP2006/309548. The isolated bone marrow was suspended in DMEM/10% foetal bovine serum (FBS)/4 mM L-glutamine/1% antibiotic/antimycotic (90–270 ml): when volume of the isolated bone marrow was more than 2 ml, 2 ml aspirate was diluted with 90 ml of the medium. In the case of 1 ml and less than 1 ml, it was diluted with 180 and 270 ml of the medium, respectively. The suspension (15 ml) was seeded in 75 cm^2 flasks and cultured in a 100% humidified incubator (MCO-18M; SANYO, Tokyo, Japan for hypoxic condition, BNS-110; Espec Co., Osaka, Japan for normoxic condition) at 37°C in 5% CO_2 in parallel under two distinct oxygen conditions (5% O_2 and 20% O_2). After non-adherent cells were removed by replacing the medium the next day, the medium was replaced once every 2 or 3 days. After 3–4 weeks these primary hMSCs (passage 0, P0) were harvested using 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) and subcultured in 75 cm^2 flasks at a density of 2×10^3 cells/cm^2; subsequently the passage of hMSCs was maintained once per week. The bone marrow was processed in the cell-processing centre (CPC), which is subject to good manufacturing practice (GMP) regulation. The materials used in this study included DMEM, FBS, 200 mM L-glutamine, antibiotic/antimycotic (100×) liquid (which contains amphotericin, streptomycin and penicillin G) and 0.5% trypsin/0.53 mM EDTA. Research-grade culture medium was purchased from Invitrogen (Carlsbad, CA, USA).

Karyotyping analysis

Cultures from bone marrow donors at different passages from primary culture to passage 7 were subjected to karyotyping analysis. To obtain chromosome preparations, actively dividing cells from subconfluent culture flasks were treated with 60 ng/ml colcemid (KaryoMAX-colcemid; Invitrogen) to block microtubular formation. After mitotic arrest, the cells were harvested using 0.05% trypsin/0.53 mM EDTA. The collected cells were immersed in 75 mM KCl for 30 min. at 37°C. Following cell culture, the cells were collected by centrifugation, the supernatant was replaced with fixative (methyl alcohol/acetic acid, 3:1) and the suspension was spread on slides. The karyotype was analysed by G-banding techniques and the number of chromosomes in 50 metaphases was counted in chromosome number analysis. All metaphases with aneuploidy were described in detail. Without aneuploidy, at least 10 metaphases with the normal number of chromosomes were analysed in detail. A chromosomal aberration was defined as clonal when at least two metaphases showed the same aberration; if the abnormality was a missing chromosome, the same change had to be present in at least three cells to be accepted as clonal, and described according to the recommendations from the International System for Human Cytogenetic Nomenclature 2005 [20].

Estimation of the number of propagating hMSCs

The amplification rate of hMSCs was assessed using cumulative population-doubling level (PDL), which is defined as total number of cell divisions. PDL at each subculture is calculated using the formula $\log_2(N_1/N_2)$, where N_1 is the number of cells in the confluent monolayer and N_2 is the initial number of cells seeded. Mesenchymal stem cell (MSC) culture is usually isolated by plastic-adherent growth and hence the initial number of MSCs can only be estimated by accounting for fibroblast colony-forming unit frequency based on the assumption that every colony has been derived from a single clonogenic MSC.

Statistical analysis

Comparison of cumulative PDL between two different culture conditions was made using multivariate regression analysis including culture condition and the number of passages as independent variables. Simple comparison of estimated proliferative cells was performed with Student's *t*-test. A survival

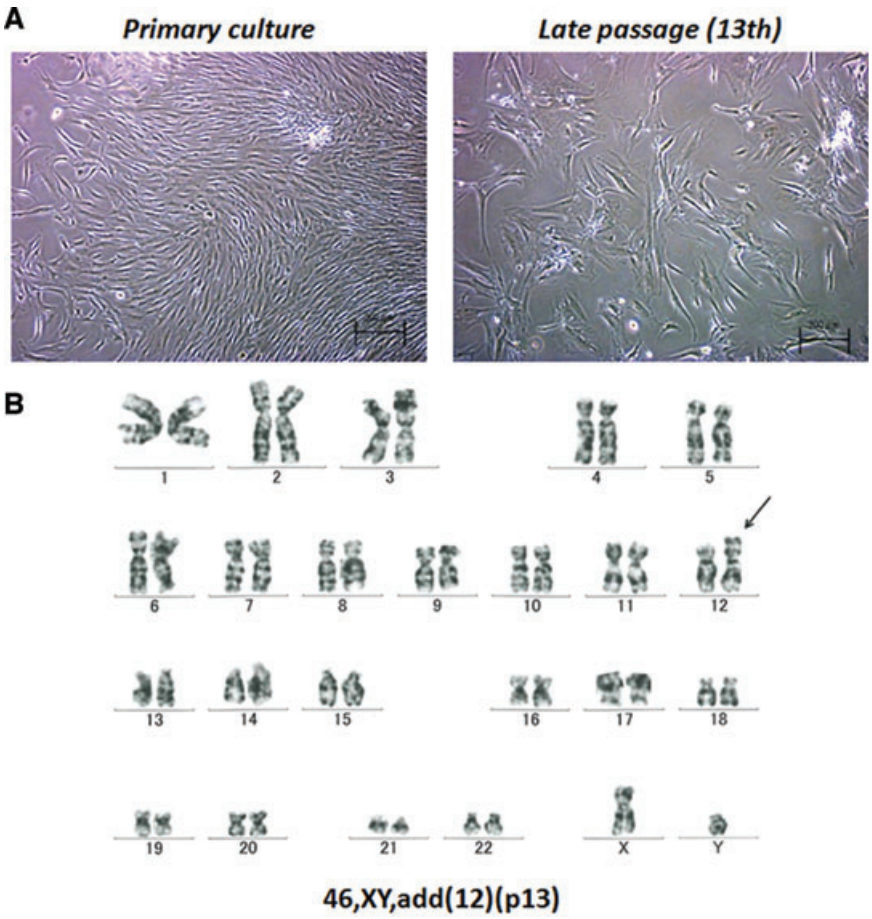

Fig. 1 Karyotyping analysis of hMSCs. (**A**) Morphology of cells in primary culture (left) and in late (13th) passage (right). The size of hMSCs in culture was enlarged with increasing passages. Scale bars, 200 μm. (**B**) G-banding karyotyping of cells with chromosomal anomalies (donor #100). Karyotyping analysis was performed as described in 'Materials and methods'. The karyotype shows an addition on chromosome 12 [46,XY,add(12)(p13)]. The chromosomal anomaly is indicated by an arrow.

curve was estimated using the Kaplan–Meier method, with the occurrence of spontaneous chromosomal aberrations as an end-point throughout *in vitro* expansion. Between-group comparison of survival was made using the log-rank test. To estimate the effects of PDL and donor age after controlling for the effect of culture condition, logistic regression analysis was used as appropriate and the results presented as odds ratios. All values are presented as means ± S.D. A smoothed hazard model of chromosomal aberrations was constructed as described previously [21] using the kernel function [22]. Calculations were performed with JMP® 8.0 Statistical Discovery software from SAS Institute (Cary, NC, USA). In all the cases analysed, *P*-values of 0.05 or less were considered to be statistically significant.

Results

Observation of *ex vivo* expanded hMSCs and karyotype analysis

Forty hMSC samples isolated from bone marrow of donors (16 men and 24 women) with a median age of 61 years (range 26–77 years) were analysed (Table S1). Bone marrow derived hMSCs attached to the culture surface and showed a fibroblast-like appearance in both normoxic and hypoxic cultures, and radial axons were enlarged with apparent directional arrangement with increasing passages (Fig. 1A). The size of hMSCs also increased in later passages. With a single counting of chromosome numbers following Giemsa staining, the normal human karyotype was observed, written as 46,XY or 46,XX. We next examined karyotypes to confirm that these cells maintained their chromosomal stability after *in vitro* expansion. Abnormal karyotypes from detailed karyotypic analysis are summarized in Table 1. Anomaly karyotypes were not detected on the other samples. For example, karyotypic analysis of hMSCs from donor #100 cultured under normoxic conditions was done at passages 1 (P1), 3 (P3) and 6 (P6), and abnormal karyotypes were detected at each passage. Some donors including #118 and #127, had chromosomal aberrations at early passage but not later passage. It is possible that abnormal karyotypes were detected at early stage and not at next stage, because 50 cells were sampled at random from each sample and karyotypic analysis was conducted on these cells in this study. When hMSCs with anomaly karyotypes are detected from

Table 1 Abnormal karyotypes observed on *ex vivo* expansion of hMSCs

Culture condition	ID	Age (years)	Sex	Normal karyotype	P	Abnormal karyotypes	% with numerical aberration	% with structural aberration
Normoxia	100	66	M	46,XY	1	46,XY,add(6)(q11)	0	17
					3	46,XY,add(6)(q11)	0	40
					6	46,XY,add(12)(p13)	0	45
	117	77	F	46,XX	0	Zero found	–	–
					3	Zero found	–	–-
					6	46,XX,add(11)(p15)*type1/ 46,XX,add(11)(p15)*type2	0	12
	119	61	M	46,XY	0	Zero found	–	–
					3	Zero found	–	–
					6	45,XY,-16	6	0
	127	74	F	46,XX	0	47,XX,+5	6	0
					3	Zero found	–	–
					5	Zero found	–	–
Hypoxia	100	66	M	46,XY	1	Zero found	–	–
					3	Zero found	–	–
					6	45,X,-Y/45,X,-Y,add(7)(q36)	88	31
	104	69	M	46,XY,inv(9) (p12q13)	0	44-45,XY,del(4)(q27), inv(9)(p12q13) [cp2]	0	7
					3	44-46,XY,add(7)(q11.2), inv(9)(p12q13) [cp2]	0	10
	109	57	M	46,XY	0	Zero found	–	–
					3	46,XY,t(1;9)(q24;p22)	0	12
					6	44-45,X,-Y [cp5]	–	–
	110	69	M	46,XY	0	Zero found	–	–
					3	47,XY,+10	8	0
					6	46,X,-Y,+8	62	0
	112	59	M	46,XY	0	Zero found	–	–
					3	Zero found	–	–
					6	45,X,-Y	14	0
	115	73	M	46,XY	0	Zero found	–	–
					3	Zero found	–	–
					6	47,XY,+8	4	0
	116	75	M	46,XY	1	Zero found	–	–
					3	Zero found	–	–
					6	46,XY,del(8)(q22), add(19)(q13.3)	0	28
	117	77	F	46,XX	0	Zero found	–	–
					3	Zero found	–	–
					6	46,XX,add(11)(p15)*type1	0	24
	118	32	M	46,XY	0	Zero found	–	–

Continued

Table 1 Continued.

Culture condition	ID	Age (years)	Sex	Normal karyotype	P	Abnormal karyotypes	% with numerical aberration	% with structural aberration
					3	46,XY,add(10)(p11.2)	0	4
					6	Zero found	–	–
	119	61	M	46,XY	0	Zero found	–	–
					3	Zero found	–	–
					6	46,XY,t(2;11)(q33;p15), add(8)(q24.1)	50	0
	130	56	F	46,XX	0	Zero found	–	–
					3	47,XX,+X	4	0
					5	47,XX,+X	6	0

P indicates the passage number at which karyotypic analysis was conducted.
The following aberrations indicate a structural abnormality: add: addition; inv: inversion; t: translocation; del: deletion.
cp: The composite number of cells in which the clonal changes were observed is given in square brackets after the karyotype.
F: female; M: male.

early to later stage, it is more likely that the hMSCs have abnormal karyotypes. Numerical aberrations (*e.g.* aneuploidy) and structural aberrations (*e.g.* additions, deletions, inversions and translocations) were detected under both culture conditions (Table 1). Whereas insertions and translocations are detected frequently, relatively fewer deletions, resulting in simple inactivation of the associated genes, were observed (Table 1). For instance, one of the observed anomalous karyotypes was an addition on chromosome 12 [46,XY, add(12)(p13)], indicating that a portion of another chromosome was added to the short-arm p13 site of the right-hand chromosome 12 shown in Figure 1B. In terms of aneuploidy, gain and loss of sex chromosomes was detected frequently. Donor #104 had the karyotype inv(9)(p12q13), which is the most common pericentric inversion in human beings, with a frequency of 1–2.5% in the general population [23], and is considered a normal polymorphism.

Hypoxic culture enhances the amplification of hMSCs

One example (donor #100) of hMSCs amplification curves in the two culture conditions is shown in Figure 2A. In almost all cases tested hMSCs cultured under hypoxic conditions reached a higher cumulative PDL than those under normoxic culture conditions at later passages (Fig. 2B). The mean cumulative PDL at passage 6 was 27 $\pm$ 4 in the hMSCs cultured under hypoxic conditions and the mean was 24 $\pm$ 4 under normoxic conditions (Table S2). As shown in Figure 2B, cumulative PDL was correlated with the number of passages ($P < 0.0001$), and there was a significant difference between the two culture conditions with regard to cell amplification ($P = 0.0005$) (Table S3). At passage 3, mean counts of proliferating hMSCs from all samples were $(4.37 \pm 6.22) \times 10^8$ under normoxic conditions and $(1.04 \pm 1.26) \times 10^9$ under hypoxic conditions ($P = 0.0082$, Fig. 2C).

More chromosomal aberrations were detected in hMSCs cultured under hypoxia

We compared spontaneous transformations in cells cultured under normal-oxygen (20%) and low-oxygen (5%) conditions. Figure 3A shows aberration-free survival curves for occurrence of spontaneous chromosomal aberrations as an end-point throughout *in vitro* expansion, which is estimated using the Kaplan–Meier method. In survival analysis, once abnormal karyotypes are detected or follow-up time is ended, the donor becomes not at risk. Under hypoxia, spontaneous transformations were detected in earlier passages than under normoxic conditions, which was statistically significant (log-rank test; $P = 0.032$). Overall increased risk of spontaneous transformations under hypoxic conditions was estimated at 2.70 (95% CI, 1.01–8.10) compared with normoxic conditions, presented as odds ratios in Table 2. Smoothed hazard modelling for occurrence of chromosomal aberrations illustrated the increased hazard risk after passage 4 in both sets of culture conditions (Fig. 3B). An increased hazard of primary karyotypic abnormality was detected when donors had hMSCs with an initial anomalous karyotype. We found significant effects of PDL and donor age on the risk of spontaneous transformation after controlling for culture conditions. Incremental risk associated with one increase in PDL was estimated at 1.14 (95% CI, 1.04–1.26) and the risk associated with overall expansion was estimated at 18.6 (95% CI, 2.61–158), as presented by the odds ratios in Table 2. Similarly, donor age range of 50s, 60s and 70s

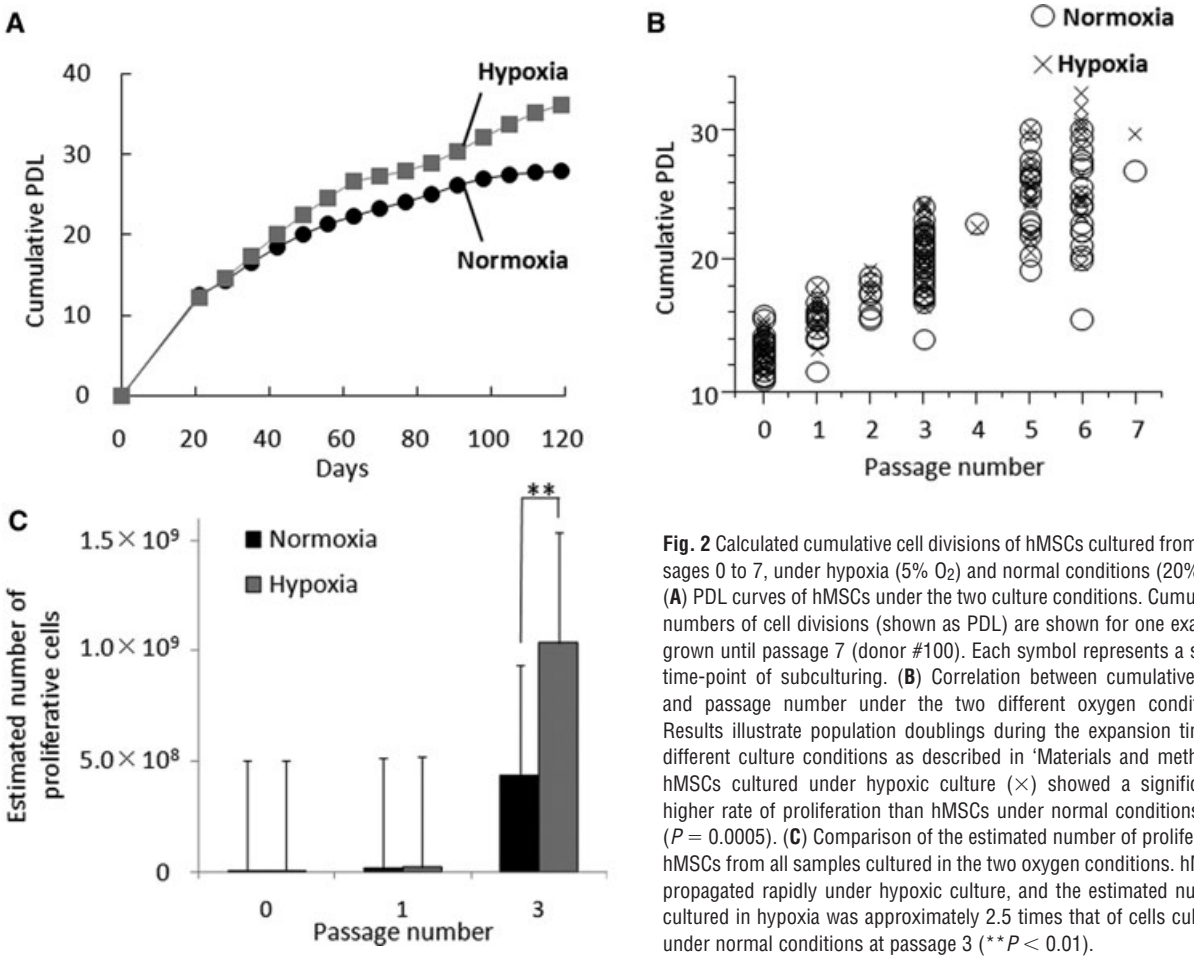

Fig. 2 Calculated cumulative cell divisions of hMSCs cultured from passages 0 to 7, under hypoxia (5% O_2) and normal conditions (20% O_2). (**A**) PDL curves of hMSCs under the two culture conditions. Cumulative numbers of cell divisions (shown as PDL) are shown for one example grown until passage 7 (donor #100). Each symbol represents a single time-point of subculturing. (**B**) Correlation between cumulative PDL and passage number under the two different oxygen conditions. Results illustrate population doublings during the expansion time in different culture conditions as described in 'Materials and methods'. hMSCs cultured under hypoxic culture (×) showed a significantly higher rate of proliferation than hMSCs under normal conditions (○) ($P = 0.0005$). (**C**) Comparison of the estimated number of proliferative hMSCs from all samples cultured in the two oxygen conditions. hMSCs propagated rapidly under hypoxic culture, and the estimated number cultured in hypoxia was approximately 2.5 times that of cells cultured under normal conditions at passage 3 ($^{**}P < 0.01$).

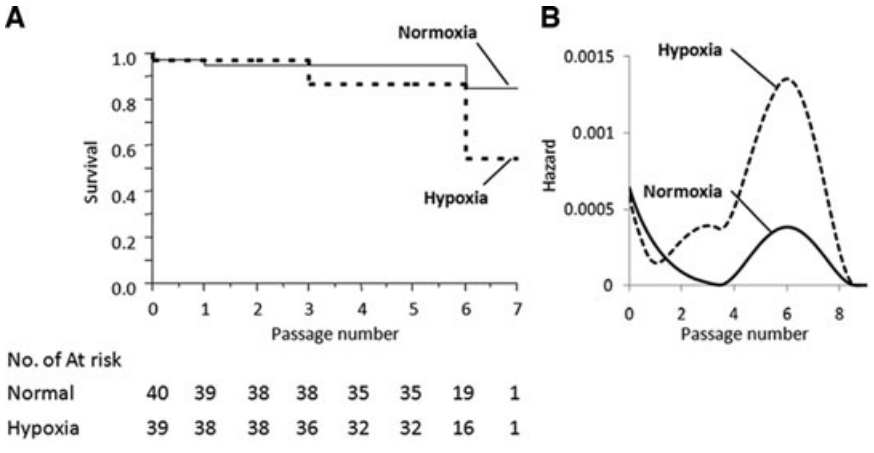

Fig. 3 Comparison of change in chromosomal aberrations under two different culture conditions. (**A**) Kaplan–Meier plot showing the aberration-free survival of hMSCs cultured under normoxic (solid line) and hypoxic (dotted line) conditions (log-rank test; $P = 0.032$). The end-point was defined as the occurrence of chromosomal aberrations. 'No. of At risk' below the figure indicates the number of samples which had never undergone chromosomal aberrations until the passage. (**B**) Estimated hazard curve for an anomalous karyotype during continuous passages from passage 0 to 7.

Table 2 Logistic model analysis for prognosis of spontaneous transformation of hMSCs cultured *ex vivo*

Explanatory variables		Odds ratio estimate (95% CI)	*P-value*
Culture condition	5% O_2 *versus* 20% O_2	2.70 (1.01–8.10)	0.058
PDL	One cell division	1.14 (1.04–1.26)	0.0048*
	Over all expansion	18.6 (2.61–158)	
Age (years)	50s *versus* 40s	6.59 (0.99–130)	0.094
	60s *versus* 40s	17.3 (3.00–329)	0.0087*
	70s *versus* 40s	7.33 (1.09–146)	0.078

40s includes donors aged 49 or under. PDL: population-doubling level; CI: confidence interval. *Statistically significant ($P < 0.05$).

elevated the risk of spontaneous transformations compared with donors in their 40s, at 6.59 (95% CI, 0.99–130), 17.3 (3.00–329) and 7.33 (1.09–146), respectively (Table 2). The confidence intervals of estimated odds ratio for donor age were wide because the sample size was limited in this research.

The proportion of cells with a chromosomal anomaly increased with increasing passage number, and chromosomal anomalies occurred more frequently under hypoxic culture conditions in later passages than under normoxic conditions (Fig. 4A). Estimated hazard risk curves for spontaneous transformations through *ex vivo* expansion by donor age group are shown for each culture condition in Figure 4B. Elevation of estimated hazard for spontaneous transformations were also observed after passage 4 in hMSCs derived from donors aged over 60 under normoxic culturing, and in hMSCs derived from all age groups except donors aged less than 50 under hypoxic culturing (Figs 4B, S1).

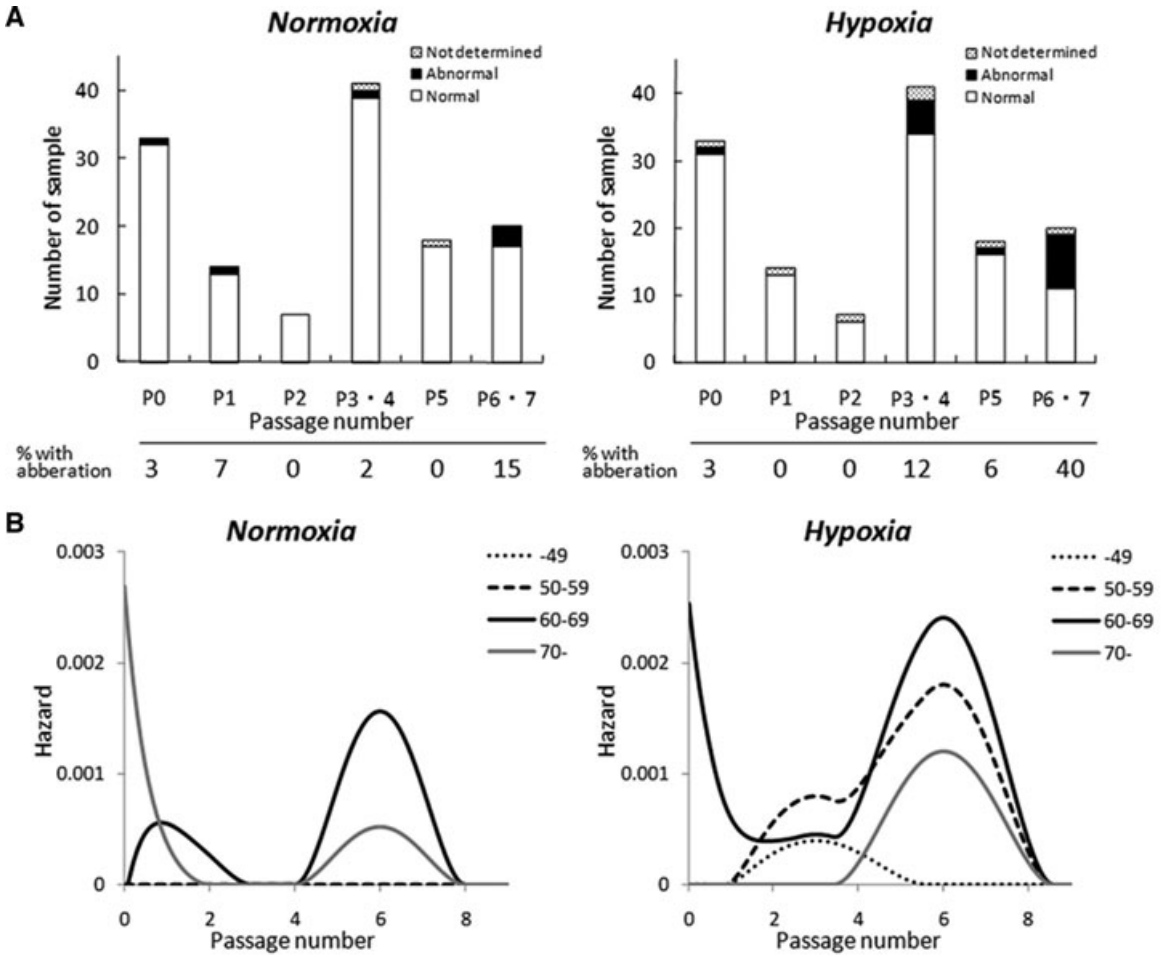

Fig. 4 Change in chromosome aberrations during *ex vivo* culture. (**A**) The proportion of aberrant karyotypes is illustrated at each culture passage during expansion. The vertical axis of figure indicates the total number of samples that were subjected to karyotyping analysis at each passage. The percentage of chromosome aberrations was as indicated below each graph. (**B**) Comparison of smoothed hazard modelling for anomalous karyotype by increasing donor age. Among hMSCs derived from donors in the age of over 60, the hazard risk increased under both conditions after passage 4 (P4). Especially under hypoxic conditions, the increase in hazard was observed at later passages in hMSCs derived from all age groups except donors aged 49 or under.

Discussion

Long-term *ex vivo* expansion under non-physiologic conditions evokes continuous changes in hMSCs: proliferation rate decays, cell size increases and chromosomal instabilities may arise. Requirements for quality control of stem-cell-based products have to be specified and standardized for their various therapeutic applications. Here we have described a continuous comparison of the effects of *ex vivo* culturing under physiologic oxygen (5%) or ambient oxygen (20%) on the hMSCs from clinical bone marrow samples, in particular focusing on chromosomal stability and proliferation rates at each expansion stage, and how many passages and population doublings are acceptable to grant the maintenance of genomic stability.

We analysed cellular expansion to compare the effect of differing oxygen concentration on their growth rates. It was confirmed that hypoxic culture conditions enhance the proliferation of hMSCs, which concurs with previous reports [14–16]. hMSCs grown under hypoxia exhibited higher cell cumulative PDL than hMSCs cultured under normoxic conditions at each passage (Fig. 2B, Table S3). On average an approximate 2.5-fold increase in cell expansion of hMSCs in hypoxia was observed at passage 3 compared with normoxia (1.04×10^9 cells under hypoxia compared with 4.37×10^8 cells under normoxia) (Fig. 2C). Generally, with repeated passages the proliferation rate decays and the cell size increases in all isolated cells: the so-called Hayflick limit is commonly observed [24]. Although enlarged cells were observed, it is unclear whether the hMSCs were shifting to a state of senescence (Fig. 1B). It was also previously reported that the number of cell divisions is closely related to donor age: donor age is negatively correlated with proliferative potential [25, 26]. We found a slight correlation between the cellular expansion potential of hMSCs and donor age under both sets of culture conditions (data not shown).

On the other hand, there have been some studies that have investigated the chromosomal stability of *ex vivo* expanded hMSCs. Some have reported that MSCs and ES cells could undergo frequent spontaneous transformations, with tumorigenic potential [27, 28], whereas some recent studies have found that hMSCs retained chromosomal stability following long-term culture *in vitro* [19, 29, 30]. In addition, it has been reported that the probability of spontaneous chromosomal aberrations in ES cells was decreased in low oxygen, or such aberrations were not detected at all [31, 32], whereas hypoxic environments have shown to increase mutation frequencies in cancer cell lines, and trigger genomic rearrangements [33, 34]. In this study, we confirmed that the frequency of karyotypic aberrations of hMSCs derived from the same donor throughout *ex vivo* expansion was significantly increased with hypoxic culturing (Fig. 3). We analysed some possible factors influencing genomic stability and estimated their impact using logistic regression analysis (Table 2). First of all, our results indicate that chromosomal instability is associated with repeated cell division (Table 2, Fig. S2). Because changes in growth rate correlated with culture conditions as described above, the influence of culture conditions and PDL on chromosomal stability was statistically underestimated (Table 2). It is also suggested that genomic stability may be influenced by complex factors inherent to hypoxic microenvironments. It was previously reported that most stem cells are in an environment with low oxygen *in vivo* [35], and oxygen concentration affects the function of stem cells in various ways. In *in vitro* culture, low oxygen enhances not only proliferation but also maintenance of self-renewal potential [14, 32, 36], and has a significant impact on differentiation in human ES cells and adult stem cells [37, 38]. Some of the mechanisms by which low oxygen influences stem-cell behaviour have been elucidated [35]. For example, hypoxia-inducible factor (HIF) has been shown to modulate specific stem-cell effectors that control stem-cell proliferation, differentiation and pluripotency [35, 39]. Therefore, it is suggested that oxygen concentration has a major impact on the amount of karyotypic aberration. Also, no clear correlation between donor age and the rate of spontaneous transformation was observed. It is generally known that chromosomal aberrations accumulate with age. Thus, we assume that the changes in chromosomal stability were related to age-associated changes in hMSCs from young compared with older donors.

Several types of chromosomal aberration, including translocations, deletions and insertions, were detected in our karyotypic analysis. Generally, chromosomes have fragile sites that are prone to exhibiting gaps and breaks during metaphase [40]. In cultured cells these hotspots can be where chromosome rearrangement occurs. Among the anomalous karyotypes observed in this study, most transformation sites corresponded to these fragile sites. Fragile sites are usually categorized into two main classes, common and rare, according to their frequency in the population [41]. A high frequency of chromosomal abnormality breakpoints in common fragile sites (CFSs) was detected by karyotypic analysis (*e.g.* 2q33, 7q11, 7q36, 8q22.1, 8q24.1, 11p15.1, 19q13) (Table 1, Fig. S3). We investigated all genes that are located in the abnormal karyotypic region detected in karyotypic analysis using Ensembl Genome Browser, and search for genes related to cell survival and cancer by referring to NCBI map viewer. Especially, on chromosome 11p15 which is included in CFSs and detected among distinct three samples repeatedly, we found that several genes which are involved in regulation of the cell cycle, transcription and cell adhesion map to that region with a frequency of 6%, 5% and 2%, respectively (data not shown). In particular, the gene domain of 11p15.5 is known as an important tumour-suppressor gene region; several genes, such as TSPAN32 (tetraspanin 32) and TSSC4 (tumour-suppressing subtransferable candidate 4), are located in this region. It is also known that alterations in this region have been associated with some neoplasia. Taken together with our results, it is suggested that the deletion of contiguous genes may induce a multisystem developmental disorder and that these alterations might influence normal functioning and cell survival.

In addition, there have been numerous studies on tumour genotyping and it is reported that genomic alteration is a hallmark of tumorigenesis [42, 43] and that these rearrangements occur frequently at CFSs [44, 45]. Hypoxia is known to be an important parameter of the tumour microenvironment, and it was previously reported that low oxygen enhances chromosomal instability at fragile sites [33, 34]. Chromosomal breaks in hMSCs cultured under

hypoxia occurred frequently at CFSs in our study (Table 1, Fig. S3). On the other hand, the most frequently observed aberrational karyotypes were sex-chromosome aneuploidies. It was previously reported that gain of a sex chromosome in cultured lymphocytes was significantly more frequent in females than in males, and that loss of Y chromosomes correlated with age in human bone marrow cells [46, 47]. These phenomena agree with our observations of abnormal karyotypes in this study, but only for hypoxic hMSCs. These results could provide insight into understanding the consequences of replication stress on DNA damage and genomic instability in hMSCs cultured under hypoxic conditions.

In summary, we confirmed that hypoxic culture conditions, donor age and multiple passages are factors influencing karyotypic stability: the frequency of karyotypic aberrations increased with passage number and hMSCs undergo spontaneous transformation with tumorigenic potential, especially in later passages under hypoxic culture conditions in hMSCs of elderly donors (Table 2, Fig. 4B). No increase in hazard was observed in hypoxic hMSCs from donors aged 49 or under and normoxic hMSCs derived from donors aged 59 or under, which it is difficult to generalize because of the underestimation of the hazards from limitation of sample size (Fig. 4B). Taken together with these results, it is suggested that monitoring of chromosomal stability in culture-expanded hMSCs is needed before administration to human beings in order to detect mutations and potentially immortalized clones and to prevent transplant-associated tumour formation. So far, some reports have shown that MSCs transplantation contributes to tumour formation *in vivo* [48–50], whereas Furlani *et al.* reported that cultured MSCs with spontaneous transformations had no functional effects after intracardiac transplantation [51]. Further investigations are necessary to evaluate the tumorigenicity and safety of the stem-cell-based products. Despite the great interest in the use of hMSCs for regenerative medicine, definitive standards for advanced medicinal products are still lacking because of the complexity of cell therapy, which is far beyond that for classical drug therapy [52]. Thus, especially in the field of regenerative medicine, concrete and specific standards, and governmental support systems, are necessary to promote their production [53]. We hope that our results will contribute to guidelines for regulating the manufacturing process of *ex vivo* cell expansion, and assist in the further application of stem-cell-based regenerative materials.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Comparison of chromosomal aberrations during *ex vivo* culture. The numbers of hMSCs with a normal karyotype, anomalous karyotype and unmeasurable samples subcategorized by donor age were compared at each passage stage. P0–P7 represent passage numbers.

Fig. S2 Comparison of cumulative PDL of hMSCs with anomalous karyotypes. The symbols represent cumulative PDL of hMSCs with an anomalous karyotype. Cumulative PDL indicates the total number of cell divisions.

Fig. S3 Common fragile sites (CFSs) and detected chromosome aberration sites. Arrows, bold arrows and dotted arrows indicate CFSs, detected abnormal sites including CFSs, and other abnormal sites detected by karyotypic analysis, respectively. Among the anomalous karyotypes detected, chromosome aberration sites frequently included CFSs.

Table S1 Characteristics of samples obtained in this study (n = 40)

Table S2 PDL at each passage under two culture conditions

Table S3 Multivariate regression model of change in cumulative PDL including interaction between culture conditions and passage number

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