potential than AMSCs (Hayashi *et al.*, 2008). Therefore, BMSCs may be appropriate stem cells for bone tissue regeneration, whereas AMSCs may be used for treatments that do not require bone formation, such as heart repair. Concerning the clinical application of MSCs, their proliferation and differentiation potentials are limited and drastically decrease after several passages, resulting in a restriction of their application in regenerative medicine.

Alternatively, embryonic stem cells (ESCs) have unlimited proliferation and differentiation potential. However, disruption of an embryo is required to establish ESCs and thus their uses in medical applications elicit ethical concerns. Furthermore, because ESCs cannot be established from adult cells, it is impossible to make patient-derived ESCs to be used as autogenous grafts, which avoid transplantation rejection. In this regard, induced pluripotent stem cells (iPSCs) with unlimited proliferation and differentiation potential equivalent to ESCs have recently been established (Takahashi *et al.*, 2006, 2007; Yu *et al.*, 2007). Because iPSCs can be generated from somatic cells even after their terminal differentiation, iPSCs have been attracting attention as a new type of possible patient-derived autogenous stem cells for regenerative medicine. Many sources of adult cells in various tissues have been used for the generation of iPSCs, including skin fibroblasts, keratinocytes, blood cells (Patel *et al.*, 2010) and MSCs. Because harvesting, depository and transport methods of the MSCs have been established, usage of the cells is feasible for clinical applications. Notably, MSCs can be frozen/stocked for a long period with high viability (Kotobuki *et al.*, 2005). Due to the well-established characteristic features of MSCs, they are targeted as an ideal cell source for iPSC generation. In fact, we have previously reported the generation of iPSCs from frozen AMSCs (Aoki *et al.*, 2010). However, there are no comparative studies of the efficiency and biological activities of the iPSCs that are derived from BMSCs vs. from AMSCs. In this study, we established several lines of iPSCs from frozen stocks of BMSCs and AMSCs, and compared the efficiency of the iPSC generation, as well as differentiation potentials both *in vitro* and *in vivo*.

2. Materials and methods

2.1. FACs analysis

Cell surface antigens of BMSCs and AMSCs were analysed by flow cytometric analysis (FACS Calibur, BD Biosciences, Le Pont de Claix, France). Mouse anti-human monoclonal antibodies of FITC-conjugated CD13, CD14 CD44, CD45 (BioCarta, CA, USA), CD29, CD56, CD90, CD105 (AbD Serotec, NC, USA), CD31, CD34, HLA-I (Invitrogen), PE-conjugated CD73 (BD Bioscience) and CD133 (Miltenyi Biotec, Gladbach, Germany) were used. FITC- and PE-conjugated mouse IgG (Beckman Coulter, CA, USA) were used as negative controls.

2.2. *In vitro* osteogenic differentiation of BMSCs and AMSCs

MSCs were seeded at a density of 5×10^3 cells/cm^2 in a 12-well culture plate in minimum essential medium-α (α-MEM; Invitrogen) containing 15% fetal bovine serum (FBS; JRH Biosciences, KS, USA) and cultured overnight. Next day, the medium was changed to osteogenic differentiation medium, which was supplemented with 10 mM β-glycerophosphate (Merck KGaA, Darmstadt, Germany), 0.07 mM L-ascorbic acid 2-phosphate magnesium salt *n*-hydrate (Wako) and 100 nM dexamethasone (Sigma). The medium was changed three times a week. As a control, the MSCs were also cultured in the medium without the ascorbic acid and dexamethasone.

After the osteogenic differentiation, the cells were used for assay for alkaline phosphatase (ALP) activity and ALP staining. For the ALP assay, the cells were washed with phosphate-buffered saline (PBS) and collected into a tube containing 500 µl TE buffer (pH 7.4, 1 mM EDTA and 100 mM NaCl). The cells in TE buffer were sonicated and 20 µl of the cell suspension was used to quantify DNA content, using Hoechst 33 258. Salmon sperm DNA (Life Technologies) was used as DNA standard. The sonicated cell suspension was centrifuged at 13 000 × *g* for 5 min at 4 °C, and 20 µl supernatant was used for ALP activity assay. *p*-Nitrophenylphosphate (pNPP; Zymed Laboratories, CA, USA) was used as the substrate, and the *p*-nitrophenol released during incubation for 30 min at 37 °C was measured. The ALP activity was normalized to DNA content (µmol/µg). ALP staining was done using an ALP Kit (Sigma) according to the manufacturer's instructions.

2.3. *In vitro* adipogenic differentiation of BMSCs and AMSCs

MSCs were seeded at a density of 2×10^4 cells/cm^2 in a 12-well culture plate in α-MEM containing 15% FBS. The adipogenic differentiation was performed using the hMSC Differentiation Bullet Kit®, Adipogenid (PT-3004, Takara) according to the manufacturer's instructions. After adipogenic differentiation, the cells were fixed with 10% formaldehyde for 10 min at room temperature. The fixed cells were washed with 60% isopropanol and stained with oil red O solution for 15 min. The stained cells were washed with 60% isopropanol and PBS. As a control, the MSCs were also cultured in the medium without dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine.

2.4. Plasmid construction

An open reading frame (ORF) cassette A (Invitrogen, Carlsbad, CA, USA) was introduced into the *Eco*RI site of the pMXs retroviral vector (Takahashi *et al.*, 2006). The ORFs of human *OCT3/4* (*POU5f1 isoform-1*), *SOX2, KLF4*

and *c-MYC* were amplified by RT–PCR and cloned into pENTR-D/TOPO (Invitrogen). All genes were transferred to the pMXs retroviral vector (kindly donated by Dr Kitamura), using Gateway Technology (Invitrogen) according to the manufacturer's instructions.

2.5. Cell culture

This study was approved by the ethics committee of the National Institute of Advanced Industrial Science and Technology. Culture expansion of BMSCs (0801TS33M A1/BMSC No. 1, P-2c AMS0422-PF57/BMSC No. 2 and 0702TS37M A2/BMSC No. 3) were carried out from frozen stocked BMSCs after informed consent of the donors had been obtained. Human AMSC lines (AMSC No. 1 and AMSC No. 3) were purchased from Invitrogen (lot numbers 1212 and 1199), and one line (AMSC No. 2) was from Lonza Biosciences (Gaithersburg, MD, USA; lot number 7F3890). The frozen BMSCs and AMSCs were thawed and used for the generation of iPSCs. Platinum-A (Plat-A) cells were purchased from Cell Biolabs (San Diego, CA, USA) (Takahashi *et al.*, 2007). SNL76/7 feeder cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). BMSCs and AMSCs were maintained in α-MEM containing 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Plat-A and SNL feeder cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The iPSCs were generated and maintained in human ESC medium (D-MEM/F-12 with GlutaMAX-I; Invitrogen), supplemented with 20% knockout serum replacement (KSR; Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and 5 ng/ml recombinant human basic fibroblast growth factor (bFGF; Wako, Osaka, Japan). MSCs, Plat-A and SNL76/7 feeder cells were passaged using 0.05% trypsin/0.53 mM EDTA (Invitrogen). The iPSCs were passaged using dissociation solution [0.25% trypsin (Invitrogen), 0.1 mg/ml collagenase type IV (Invitrogen), 10 mM $CaCl_2$ (Wako) and 20% KSR in distilled water]. The parental cells of these MSCs were free from bacterial, fungal and mycoplasma contamination because primary cultures of BMSCs were done in the clean room at our cell-processing centre and AMSC lines were obtained from the companies with certification. We also performed microbiological tests several times during the culture; furthermore, we have a system to avoid cross-contamination during iPSCs generation, evidenced by short tandem repeat (STR) profile analysis of genomic DNA (Aoki *et al.*, 2010).

2.6. Retroviral production

Plat-A packaging cells were seeded at 8×10^6 cells/100 mm dish and cultured overnight. The next day, pMXs retroviral vectors containing the ORFs of human *OCT3/4, SOX2, KLF4* and *c-MYC* were transfected into Plat-A cells using the FuGENE HD Transfection Reagent (Roche Diagnostics, Basel, Switzerland). Viral supernatants were collected 48 and 72 h post-transfection, then filtered through a 0.45 μm pore-size filter and supplemented with 4 mg/ml Polybrene (Sigma, St. Louis, MO, USA). The MSCs were transduced with a *OCT3/4:SOX2:KLF4:c-MYC* = 1:1:1:1 mixture of viral supernatant.

2.7. Generation of iPSCs

BMSCs and AMSCs were seeded at 5×10^4 cells/100 mm dish and cultured overnight. At this time, the passage numbers of BMSC nos 1, 2 and 3 and AMSC nos 1, 2 and 3 were P5, P7, P7, P6, P8 and P7, respectively. After the overnight culture, the culture medium was changed to viral supernatant and cultured for 24 h, then the viral supernatant was changed to fresh viral supernatant and cultured for an additional 24 h. The viral supernatant was changed to α-MEM containing 15% FBS with a daily medium change. After 3 days, the viral infected cells were seeded on SNL feeder cells at 5×10^3–5×10^5 cells/100 mm dish. The next day, the medium was changed to human ESC medium containing valproic acid (VPA; Wako). The medium was changed every other day for 2 weeks, then cultured without VPA. From 7 to 24 days post-infection, colonies were selected based on human ESC-like colony morphology. The selected colonies were subsequently expanded and maintained on SNL feeder cells in human ESC medium. Cells were always cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. Reprogramming efficiency was determined as the number of total human ESC-like colonies per total number of infected cells.

2.8. RNA isolation and reverse transcription

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with a TURBO DNA-free™ kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis, using a ReverTra Ace-α™ kit (Toyobo, Osaka, Japan) and oligo(dT) 20 primers. PCR was performed using an ExTaq HS™ kit (Takara Bio, Shiga, Japan). Primer sequences are shown in Table 3.

2.9. Alkaline phosphatase (ALP) staining and immunofluorescence microscopy

ALP staining was performed using a Leukocyte ALP Kit (Sigma) according to the manufacturer's instructions. For immunofluorescent microscopy, cells were fixed with PBS containing 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were treated with PBS containing 0.1% Triton X-100 for 10 min and then 1% bovine serum albumin (BSA, A2153, Sigma) for 10 min at room temperature. The cells were incubated

Table 1. Analyses of cell surface antigens on BMSCs and AMSCs

	Cell surface antigen (%)													
MSC line	CD13	CD14	CD29	CD31	CD34	CD44	CD45	CD56	CD73	CD90	CD105	CD133	HLA-DR	HLA-I
BMSC No.1	97.30	2.88	99.92	2.12	1.73	98.89	1.86	4.09	99.9	99.85	99.57	0.65	0.25	99.75
BMSC No.2	99.26	2.15	99.98	2.16	1.41	99.74	1.67	5.10	99.27	99.94	99.67	0.52	0.52	99.79
BMSC No.3	99.52	3.25	99.98	2.52	1.01	99.75	2.44	6.96	99.87	100.0	99.95	5.76	0.61	99.82
AMSC No.1	99.67	1.53	99.71	1.36	1.11	98.59	1.09	1.26	99.93	99.96	98.01	0.57	0.57	86.35
AMSC No.2	99.97	1.18	99.98	1.66	0.71	99.93	0.99	2.38	99.71	99.98	99.93	0.48	0.46	99.28
AMSC No.3	99.99	3.61	99.97	2.91	2.03	99.95	2.79	2.89	99.89	99.99	99.96	0.51	0.70	96.98

Table 2. Efficiency of iPSC generation from BMSCs and AMSCs

Parental cells	Passage No.	Number of colony	Reprogramming efficiency
BMSC No.1 (0801TS33MA1)	P5	8	0.0008
BMSC No.2 (P-2cAMS0422-PF57)	P7	1	0.0002
BMSC No.3 (0702TS37MA2)	P7	0	0
AMSC No.1 (Invitrogen #1212)	P6	88	0.0293
AMSC No.2 (Lonza #7F3890)	P8	31	0.0022
AMSC No.3 (Invitrogen #1199)	P7	5	0.0005

No statistically significant differences between BMSCs and AMSCs by Mann–Whitney U-test and χ^2 test, probably due to small sample size.

with a primary antibody overnight at 4 °C, washed and incubated with a secondary antibody for 30 min. The primary antibodies used were SSEA-3 (1:200, MAB4303; Millipore, Billerica, MA, USA), SSEA-4 (1:200, MAB4304; Millipore), TRA-1-60 (1:200, ab16288-200; Abcam, Cambridge, UK), TRA-1-81 (1:200, ab16289-200; Abcam), OCT4 (1:200, ab19857-100; Abcam), NANOG (1:50, ab21624; Abcam), SOX17 (1:200, AF1924; R&D Systems, Minneapolis, MN, USA), α-smooth muscle actin (pre-diluted, N1584; Dako, Glostrup, Denmark) and βIII-tubulin (1:200, CBL412; Millipore). Secondary antibodies used were from the Invitrogen AlexaFluor series (1:300). Nuclei were detected with 0.2 μg/ml Hoechst 33 342 (Molecular Probes, Eugene, OR, USA).

2.10. *In vitro* differentiation

For embryoid body (EB) formation, human ESC-like colonies were harvested by treatment with dissociation solution and transferred to a low attachment culture dish (Prime Surface; Sumitomo Bakelite, Tokyo, Japan) in human ESC medium without recombinant bFGF. The medium was changed every other day. After 9–12 days of floating culture, EBs were found and transferred onto gelatin-coated plates for an additional 10 days of culture in the same medium.

2.11. Teratoma formation

Clumps of ESC-like colonies from one 100 mm dish were suspended in 60 μl human ESC medium without human recombinant bFGF. The cell clump suspension (25 μl) was injected into each testis of a severe combined immunodeficient (SCID) mouse. Tumours were collected 8–12 weeks after injection and fixed with 10% paraformaldehyde. The paraffin-embedded tumours were sectioned and stained with haematoxylin and eosin (H&E).

2.12. Karyotype analysis

Chromosomal G-band analyses and multicolour Fluorescence in situ hybridization (FISH) were performed at the Nihon Gene Research Laboratories (Sendai, Japan). At this time, the passage numbers of BMSC nos 1 and 2 and AMSC nos1, 2 and 3 were P7, P12, P11, P9 and P9, respectively.

3. Results

3.1. FACs analysis of MSCs

Three lines of BMSCs and AMSC no. 2 were analysed by flow cytometry (Table 1). These numbers in Table 2 represent the percentages of marker-positive cells. All cell lines were positive for well-known mesencymal markers, such as CD13, CD29, CD44, CD73, CD90, CD105 and HLA-I. Meanwhile, they were negative for CD14, CD31, CD34, CD45, CD56 and CD133. These data indicate that these MSC lines were mesenchymal-type cells.

3.2. Differentiation analysis of MSCs

We examined the differentiation potentials of these MSCs from BMSCs and AMSCs. When three lines of BMSCs and

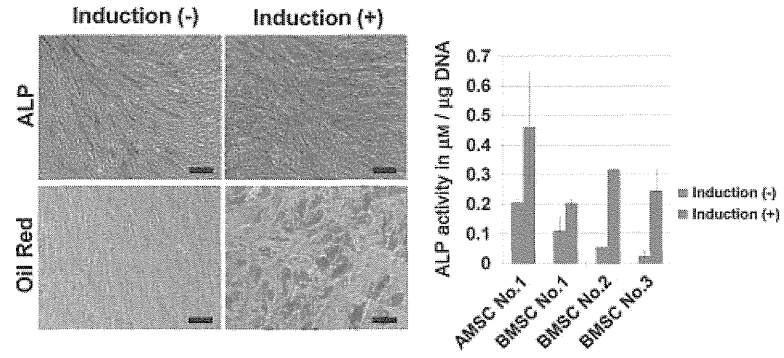

Figure 1. Osteogenic and adipogenic differentiation potential of BMSCs and AMSCs. MSCs were cultured with (induction^{+}) or without (induction^{-}) differentiation medium. ALP as a marker of osteogenic differentiation and oil red stain as a maker of adipogenic differentiation are seen in figures at left. Scale bars = 200 μm. ALP activity measurements are seen in figure at right

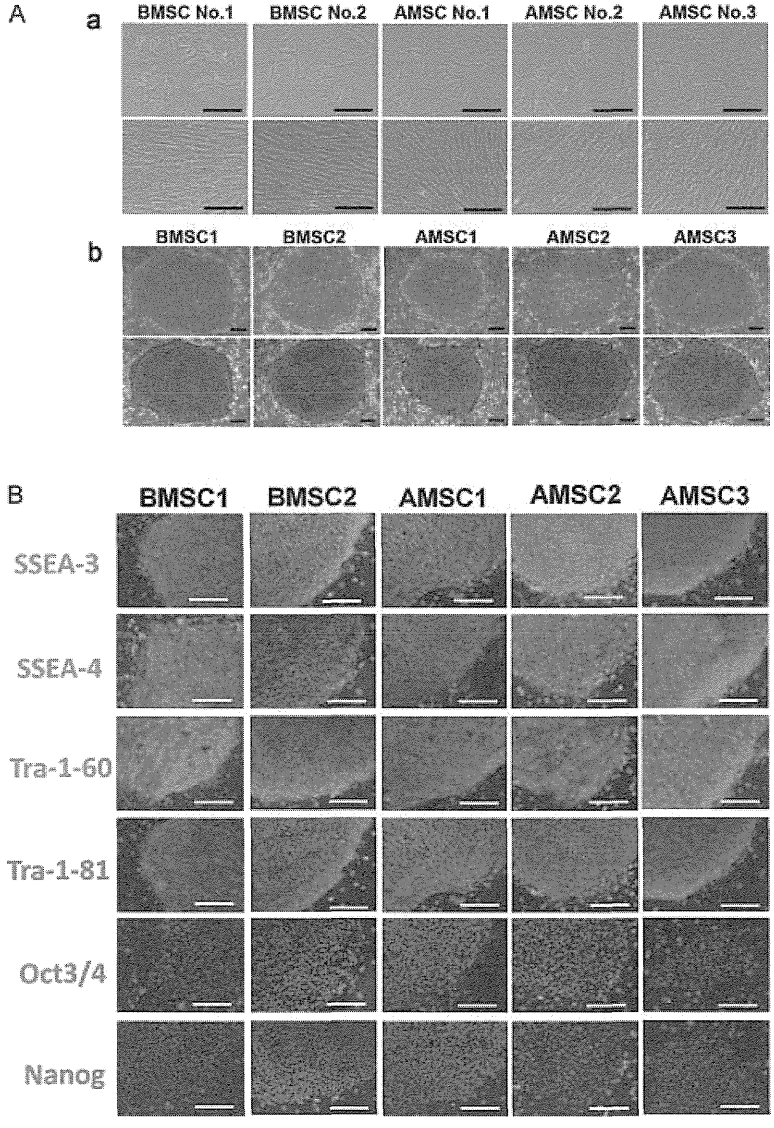

Figure 2. Morphology of the parental cells of BMSCs (BMSC nos 1 and 2) and AMSCs (AMSC nos 1–3) and ESC-like colonies (BMSC nos 1 and 2 and AMSC nos 1–3) from the corresponding parental cells. (A) (a) Typical microscopic view of parental cells at low density (upper) and high density (lower); (b) ESC-like colonies before (upper) and after (lower) ALP staining. Scale bar = 200 μm. (B) Immunofluorescent microscopy of ESC-like colonies for SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT3/4 and NANOG. Nuclei were stained with Hoechst 33342. Scale bar = 200 μm

AMSC no. 1 were cultured with osteogenic differentiation medium, all MSCs showed strong ALP staining and high ALP activity (upper left and right panels in Figure 1). Likewise, when cultured with adipogenic differentiation medium, these MSCs showed positive staining for oil red O (lower left panels in Figure 1). These data showed that all MSCs used for iPSCs generation had at least osteogenic and adipogenic differentiation potentials.

3.3. Generation of iPSCs from BMSCs and AMSCs

After approximately 22 days from the viral infection, we found several colonies displaying human ESC-like morphologies. The ratio of the number of colony formations (i.e. the reprogramming efficiency) in the BMSCs (nos 1, 2 and 3) and AMSCs (nos 1, 2 and 3) were 0.0008%, 0.0002%, 0%, 0.0293%, 0.0022% and 0.0005%, respectively (Table 2). Some of the human ESC-like colonies were selected, expanded for several passages (Figure 2Ab) and then stained for ALP activity. Almost all the colonies showed high ALP activity (Figure 2Ab). The data in the following sections were obtained from the putative ESC-like colonies derived from the corresponding culture-expanded MSCs. For example, the colonies named BMSC1 and AMSC3 were derived from BMSC no. 1 and AMSC no. 3, respectively.

3.4. Characterization of iPSCs from BMSCs and AMSCs

To confirm that the colonies derived from the BMSCs and AMSCs contained authentic iPSCs, we evaluated human ESC marker expressions, using immunofluorescent microscopy and RT–PCR. Immunofluorescent microscopy showed that the colonies expressed the following human ESC-specific surface antigens: stage-specific embryonic antigen (SSEA)-3; SSEA-4; tumour-related antigen (TRA)-1-60; and TRA-1-81. The cells also showed the ESC-specific transcription factors, *OCT3/4* and *NANOG* (Figure 2B).

RT–PCR analysis showed that the colonies expressed human ESC marker genes *OCT3/4, SOX2, NANOG,* reduced expression 1 (*REX1*), undifferentiated embryonic cell transcription factor 1 (*UTF1*), growth and differentiation factor 3 (*GDF3*), developmental pluripotency associated 2 (*DPPA2*), *DPPA4, DPPA5* and telomerase reverse transcriptase (*TERT*). The retroviral transgenes (Tg) of *Tg-OCT3/4, Tg-SOX2* and *Tg-MYC* were not expressed, and a trace of Tg-KLF4 was detected in the colonies (Figure 3).

3.5. EB formation and *in vitro* differentiation

To examine differentiation potential, we induced EB formation. After 9–12 days of floating culture of the dissociated cells from ESC-like colonies, the cells formed

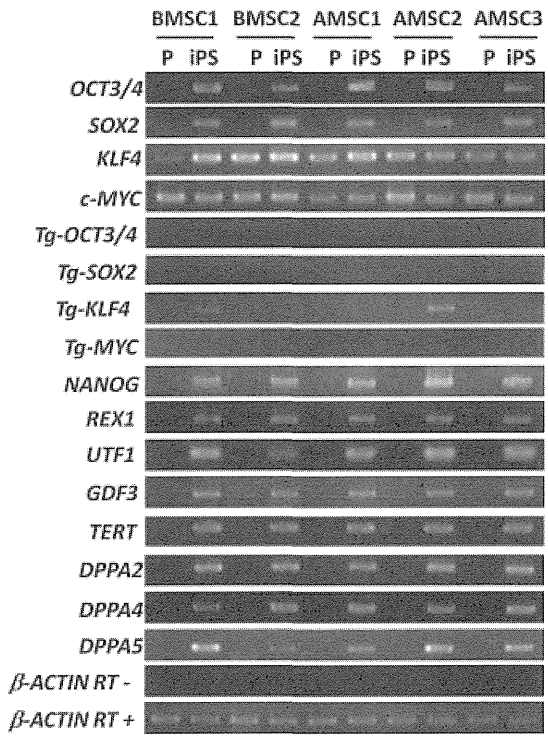

Figure 3. RT–PCR analysis of expressions of ESC marker genes, endogenous *KLF4* and *c-MYC*, and retroviral transgenes (Tg). P, parental cells

spherical structures (EBs; Figure 4A). The EBs were transferred onto gelatin-coated plates and cultured for an additional 9–12 days. Immunofluorescence microscopy showed that the cells were *βIII-tubulin-* (a marker of ectoderm), *α-smooth muscle actin-* (*α-SMA*, mesoderm), *Vimentin-* (mesoderm and parietal endoderm) and *SOX17* (endoderm)-positive (Figure 4A). RT–PCR analysis confirmed that the cells expressed *MAP2, PAX6* (endoderm), *TNTC, BRACHURY, FOXA2* (mesoderm), *SOX17* and *AFP* (endoderm) (Figure 4B). These data imply that the ESC-like colonies had the potential to differentiate into various cells of the three germ layers *in vitro*.

3.6. Teratoma formation

To identify *in vivo* pluripotency, we injected the colonies from the AMSCs and BMSCs into the testes of SCID mice. We used three or four mice for each MSC cell line. More than half of the testes showed tumour formations 8–12 weeks after injection. Histological examination of the tumours revealed tissues representative of the three germ layers: gut-like epithelium (endoderm), cartilage (mesoderm) and neuroepithelial rosettes (ectoderm) (Figure 5). These findings indicate that the tumours were teratoma formations. Thus, the colonies from the BMSCs and AMSCs had the potential to differentiate into the three germ layers *in vivo*.

Collectively, the *in vitro* and *in vivo* data confirmed that the ESC-like colonies obtained after retroviral transduction of *OCT3/4, SOX2, KLF4* and *c-MYC* into either AMSCs or BMSCs were indeed authentic iPSCs.

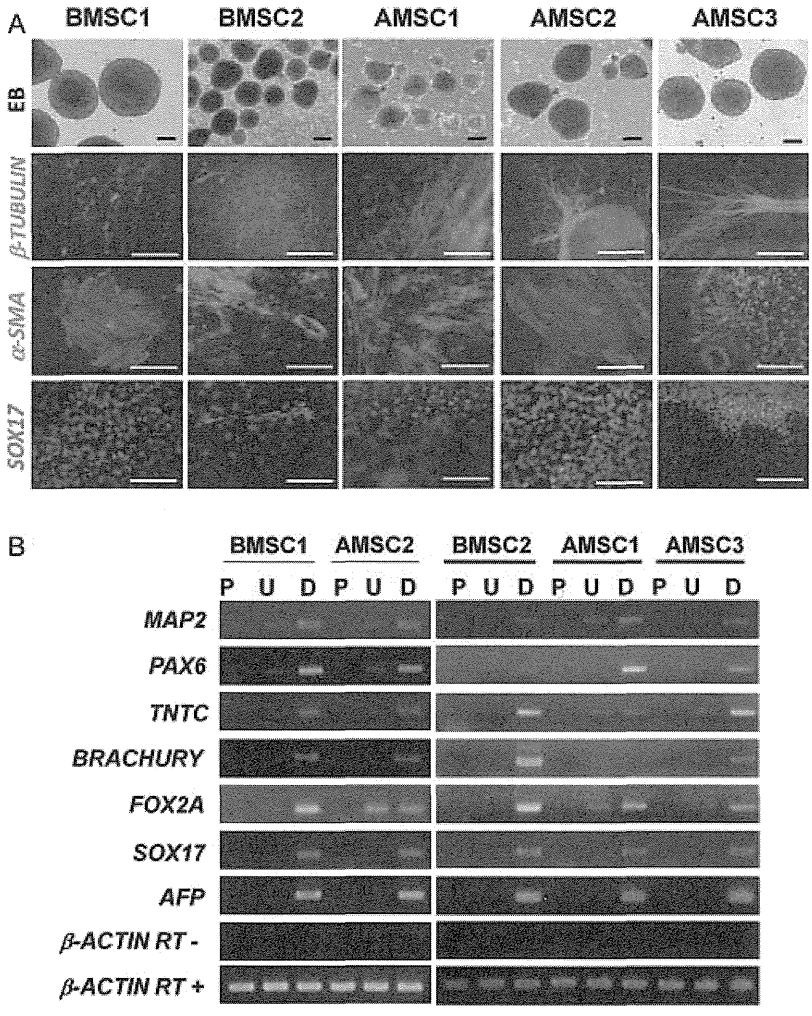

Figure 4. *In vitro* differentiation of ESC-like colonies from BMSCs and AMSCs. (A) EB formation. Immunofluorescent microscopy showing βIII-tubulin (green), α-SMA (green) and SOX17 (red). Nuclei were stained with Hoechst 33342. Scale bars = 200 μm. (B) RT–PCR analysis of expressions of differentiation marker genes: *MAP2, PAX6* (ectoderm), *TNTC, BRACHURY, FOXA2* (mesoderm), *SOX17* and *AFP* (endoderm). P, parental cells; U, undifferentiated cells; and D, differentiated cells via EB

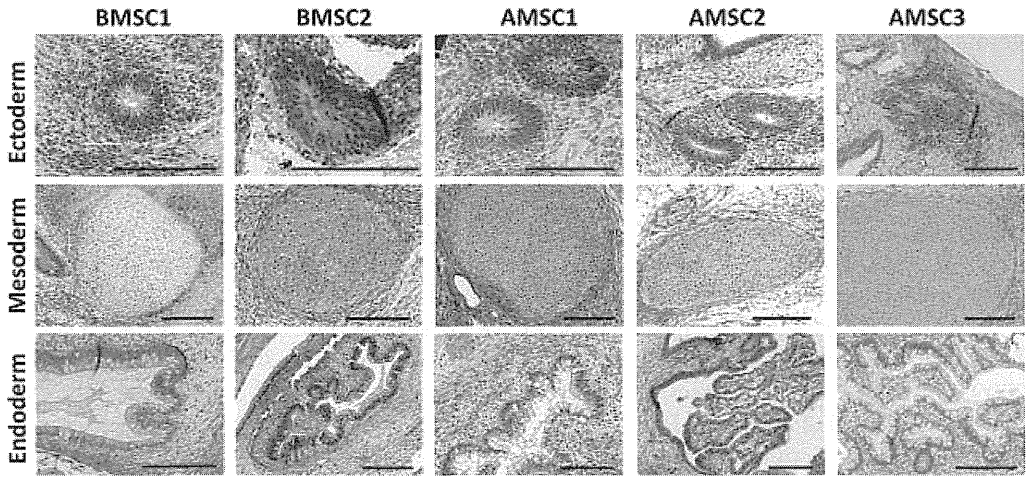

Figure 5. Teratoma formations of ESC-like colonies from BMSCs and AMSCs after their injection into mice testes. H&E staining showed neuroepithelial rosettes (ectoderm), cartilage (mesoderm) and gut-like epithelium (endoderm)

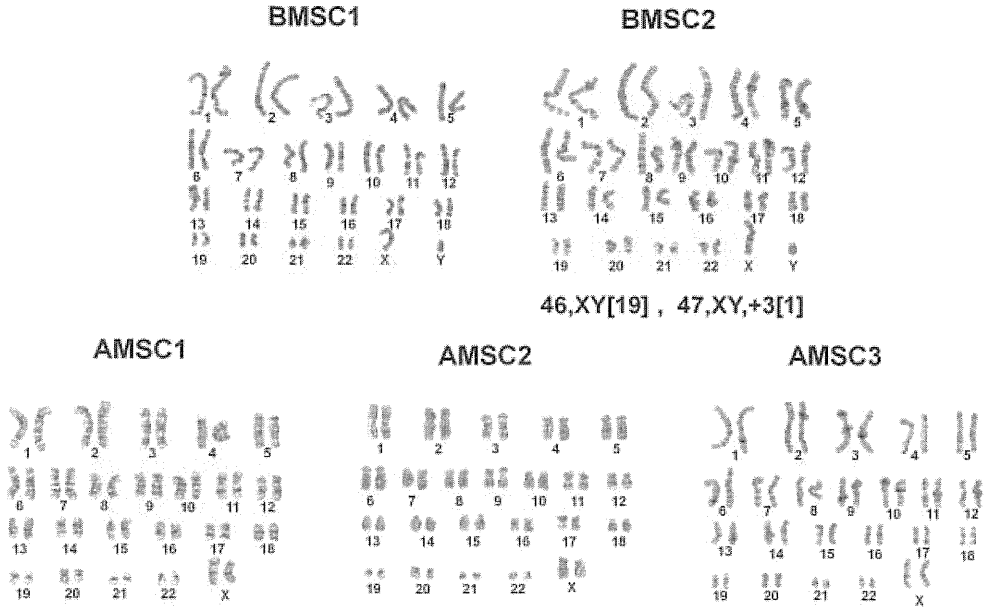

Figure 6. Karyotype (G-band) analyses of ESC-like colonies from BMSCs and AMSCs. BMSC1, AMSC1, AMSC2 and AMSC3 showed normal karyotypes. BMSC2 showed a partially abnormal karyotype

3.7. Karyotype analysis

We performed karyotype investigations by chromosomal G-band analysis (Figure 6) and multicolour FISH analysis (data not shown). Four lines of iPSCs of BMSC1, AMSC1, AMSC2 and AMSC3 showed normal karyotypes. BMSC2 showed chromosome 3 trisomy in only one cell out of 20 cells investigated.

4. Discussion

In the first report of mouse iPSC generation, the authors generated iPSCs using four transcription factors (*OCT3/4, SOX2, KLF4* and *c-MYC*) (Takahashi *et al.*, 2006). In 2007, they also generated human iPSCs using the same set of four factors (Takahashi *et al.*, 2007). Simultaneously, other investigators generated human iPSCs using another set of four factors (*OCT3/4, SOX2, NANOG* and *LIN28*) (Yu *et al.*, 2007). Since then, iPSC generation using three transcription factors (*OCT3/4, SOX2* and *KLF4*) without *c-MYC* (an oncogene) has been reported (Nakagawa *et al.*, 2008; Wernig *et al.*, 2008). Utilization of neural stem cells (NSCs) as a cell source resulted in successful iPSC generation using two transcription factors (*OCT3/4* and *KLF4* or *OCT3/4* and *c-MYC*) (Kim *et al.*, 2008). Furthermore, the same group also reported the generation of mouse, as well as human, iPSCs from MSCs using only one factor, *OCT3/4* (Kim *et al.*, 2009b, 2009c). Based on these reports, it is possible to decrease the number of transcription factors for iPSC generation by the selection of the parental cell source. In particular, cells having characteristic features of 'stemness' might be valuable for iPSC generation.

Various tissues in the human body contain MSCs. Although their proliferation/differentiation potentials are much less than those of ESCs and iPSCs, MSCs are adult cells and thus useful for clinical applications in regenerative medicine. As such, we have used BMSCs for bone/cartilage regeneration. We speculate that the proliferation/differentiation limitations of MSCs may be overcome by generation of iPSCs from MSCs. In our preliminary experiments, we transduced three transcription factors (*OCT3/4, SOX2* and *KLF4*) without *c-MYC* into human BMSCs; however it was extremely difficult to detect the iPSC-like colonies. Because MSCs also reside in adipose tissue, we then successfully generated iPSCs from AMSCs, using the same three factors (Aoki *et al.*, 2010). If BMSC-derived iPSCs have genuine iPSC properties, they could be useful for clinical applications despite their low efficiency of iPSC generation, because the iPSCs will have an infinite proliferative potential. In this study, we tried to generate iPSCs from both AMSCs and BMSCs using four transcription factors (*OCT3/4, SOX2, KLF4* and *c-MYC*), and we examined cellular biological characteristics by comparative analyses.

We determined the efficiency of iPSC generation from AMSCs and BMSCs using four factors. We found that the total number of ESC-like colonies in the AMSCs was more than that in the BMSCs, indicating a higher tendency of iPSC generation potential in AMSCs than in BMSCs (Table 2). We recently reported upregulation of some genes regarding DNA repair/histone conformational change in the high iPSC generation cells of mesenchymal types (Oda *et al.*, 2010). Difference of the gene expression profiles between AMSCS and BMSCs might be seen, although further extensive studies are needed to confirm this assumption. We also examined whether the obtained ESC-like colonies had sufficient pluripotent properties. Cells in the colonies from both BMSCs and AMSCs exhibited a human ESC-like morphology

Table 3. PCR primers

Primer	Sequence (5′ to 3′)	Applications	Tm
hOCT4–S842	CTGCAGCAGATCAGCCACATCGCCCAGCAG	OCT3/4 endo and transgene RT–PCR	73
hOCT4–AS1283	CTTCCCTCCAACCAGTTGCCCCAAAC	Endo OCT3/4 RT–PCR	65
hSOX2–S1430	GGGAAATGGGAGGGGTGCAAAAGAGG		65
		Endo SOX2 RT–PCR	
hSOX2–AS1555	TTGCGTGAGTGTGGATGGGATTGGTG		63
hSOX2–S1004	CAGATGCAGCCCATGCACCGCTACGACGTG	SOX2 transgene RT–PCR	73
hKLF4–S1457	ACGATCGTGGCCCCGGAAAAGGACC	KLF4 endo and transgene RT–PCR	67
hKLF4–AS1826	TGATTGTAGTGCTTTCTGGCTGGGCTCC	Endo KLF4 RT–PCR	65
pMXs–AS3201	TAAAATCTTTTATTTTATCGTCGACCACTG	Transgene RT–PCR	53
hMYC–S253	GCGTCCTGGGAAGGGAGATCCGGAGC		71
		c–MYC transgene RT–PCR	
hMYC–AS555	TTGAGGGGCATCGTCGCGGGAGGCTG		71
hMYC–S1580	CAACAACCGAAAATGCACCAGCCCCAG	c–MYC transgene RT–PCR	65
hNANOG–S968	CAGCCCCGATTCTTCCACCAGTCCC		67
		NANOG RT–PCR	
hNANOG–AS1334	CGGAAGATTCCCAGTCGGGTTCACC		65
hREX1–S	CAGATCCTAAACAGCTCGCAGAAT		57
		RET1 RT–PCR	
hREX1–AS	GCGTACGCAAATTAAAGTCCAGA		55
hUTF1–S832	CCGTCGCTGAACACCGCCCTGCTG		69
		UTF1 RT–PCR	
hUTF1–AS979	CGCGCTGCCCAGAATGAAGCCCAC		67
hGDF3–S243	CTTATGCTACGTAAAGGAGCTGGG		59
		GDF3 RT–PCR	
hGDF3–AS850	GTGCCAACCCAGGTCCCGGAAGTT		65
hDPPA2–S85	CCGTCCCCGCAATCTCCTTCCATC		65
		DPPA2 RT–PCR	
hDPPA2–AS667	ATGATGCCAACATGGCTCCCGGTG		63
hDPPA4–S532	GGAGCCGCCTGCCCTGGAAAATTC		65
		DPPA4 RT–PCR	
hDPPA4–AS916	TTTTTCCTGATATTCTATTCCCAT		49
hDPPA5–S40	ATATCCCGCCGTGGGTGAAAGTTC		61
		DPPA5 RT–PCR	
hDPPA5–AS259	ACTCAGCCATGGACTGGAGCATCC		63
hAFP–S948	GAATGCTGCAAACTGACCACGCTGGAAC		65
		AFP RT–PCR	
hAFP–AS1201	TGGCATTCAAGAGGGTTTTCAGTCTGGA		61
hSOX17–S423	CGCTTTCATGGTGTGGGCTAAGGACG		65
		SOX17 RT–PCR	
hSOX17–AS583	TAGTTGGGGTGGTCCTGCATGTGCTG		65
hFOXA2–S208	TGGGAGCGGTGAAGATGGAAGGGCAC		67
		FOXA2 RT–PCR	
hFOXA2–AS398	TCATGCCAGCGCCCACGTACGACGAC		69
hBRACHYURY–S1292	GCCCTCTCCCTCCCCTCCACGCACAG		73
		BRACHYURY RT–PCR	
hBRACHYURY–AS1540	CGGCGCCGTTGCTCACAGACCACAGG		71
hTNTC–S524	ATGAGCGGGAGAAGGAGCGGCAGAAC		67
		TNTC RT–PCR	
hTNTC–AS730	TCAATGGCCAGCACCTTCCTCCTCTC		65
hMAP2–S5401	CAGGTGGCGGACGTGTGAAAATTGAGAGTG		67
		MAP2 RT–PCR	
hMAP2–AS5587	CACGCTGGATCTGCCTGGGGACTGTG		69
hPAX6–S1206	ACCCATTATCCAGATGTGTTTGCCCGAG		63
		PAX6 RT–PCR	
hPAX6–AS1497	ATGGTGAAGCTGGGCATAGGCGGCAG		67
human beta–actin F	AGAAAATCTGGCACCACAC		53
		β–ACTIN RT–PCR	
human beta–actin R	CTCCTTAATGTCACGCACG		55

(Figure 2Ab), and the colonies exhibited high ALP activity (Figure 2Ab) as well as expressions of undifferentiated markers (Figures 2B, 3). Concerning the differentiation potentials of the ESC-like colonies, we made EBs from the colonies and demonstrated that the cells in the EBs differentiated well into three germ layer-derived cells. The *in vitro* differentiation potentials were also confirmed by *in vivo* transplantation of the ESC-like colonies into mouse testes. Histological findings of the transplants showed gut-like epithelium (endoderm), cartilage (mesoderm) and neuroepithelial rosettes (ectoderm) (Figure 5). The *in vitro*, as well as *in vivo*, differentiation potentials were well demonstrated using parental cells of both BMSCs and AMSCs. Collectively, these results indicate that the ESC-like colonies derived from both BMSCs and AMSCs were iPSCs, although there was a difference in the efficiency of iPSC generation.

The low generation efficiency of BMSCs may be improved by using other factors in the process of iPSC generation. It has been reported that small molecule compounds, such as histone deacetylase inhibitor (Huangfu *et al.*, 2008), GSK3 inhibitor (Li *et al.*, 2009), TGFβ signalling inhibitor (Lin *et al.*, 2009) and butyric acid (Mali *et al.*, 2010) enhance iPSC generation. We used *c-MYC* for iPSC generation, particularly for iPSC generation from BMSCs. *c-MYC* is a known oncogene, and Okita *et al.* (2007) reported that iPSCs can give rise to cancer by re-expression of the *c-MYC* transgene. This problem, which is crucial for clinical application, might be solved by using the Sendai virus method without insertion of transgenes into the genome (Fusaki *et al.*, 2009) or a protein transduction method without gene transfection (Kim *et al.*, 2009a; Zhou *et al.*, 2009).

One of the significant findings of this study was that we could generate iPSCs from frozen/stocked MSCs. According to our experience, MSCs can be stocked for several years, even at $-80\,^{\circ}C$, with high cell viability (Kotobuki *et al.*, 2005). In the future, we will provide regenerative medicine using patients' MSCs and at the same time we will stock some of the MSCs. If the MSC therapy is not effective by their proliferation/differentiation limitations, it will be possible to use iPSCs generated from the stocked MSCs. For clinical applications of iPSCs, we should consider possible complications, such as tumourigenicity of iPSCs, because iPSC transplantation can cause teratoma formation. Miura *et al.* (2009) reported that iPSC-derived secondary neurosphere transplantation to the striatum of a NOD/SCID mouse caused tumour formations. Interestingly, the frequency of the teratoma formation was related to the number of undifferentiated cells that resided in their preparation. Consequently, if undifferentiated cells can be removed, the tumourigenicity might be prevented. Indeed, there are methods to remove undifferentiated cells, and therefore clinical application of human iPSCs derived from MSCs might be possible in the future. The application could be done using differentiated cells of interest derived from the iPSCs and some have already reported differentiation methods towards neural cells (Vierbuchen *et al.*, 2010) and beating cardiomyocites (Ieda *et al.*, 2010). Interestingly, these differentiations from adult cells without iPS generation can be done. Vierbuchen *et al.* (2010) converted dermal fibroblasts to functional neurons using three factors (Ascl1, Brn2 and Myt1l) and Ieda *et al.* (2010) generated beating cardiomyocytes using three factors (Gata4, Mef2c and Tbx5). These direct reprogramming methods might be alternative methods to show functional differentiation from adult cells and circumvent the tumourigeneicity.

In conclusion, BMSCs tended to show a lower efficiency in the generation of iPSCs than AMSCs; however, iPSCs derived from both AMSCs and BMSCs exhibited equal differentiation potentials into cells of three germ layers. Therefore, we believe iPSCs generated from BMSCs or AMSCs can be used as patient-derived autogenous stem cells, although further studies are needed for consideration of clinical applications.

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5．間葉系幹細胞を用いた先天性骨代謝疾患の治療

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Summary　先天性骨系統疾患に対する確立した治療法はない。そこで，我々は低フォスファターゼ症で致死的な周産期型を対象に，骨髄由来の間葉系幹細胞（Mesenchymal Stem Cells：MSC）移植を行う「ヒト幹細胞臨床研究」に取り組んだ。今回，親をドナーとする骨髄移植を行った後，同じドナーからのMSCを複数回移植した。骨髄採取と細胞移植は島根大学，移植細胞のMSC培養は産業技術総合研究所が分担した。厳格な品質管理の下で培養されたMSCを移植した結果，骨が全く消失していた部位の骨石灰化も回復，生命予後を左右する呼吸障害も改善した。

はじめに

先天性骨系統疾患は450種類以上も存在するが，それぞれは非常に稀有な疾患であり，多くは骨・軟骨形成や骨代謝に関わる遺伝子異常により発症する。疾患によっては骨だけでなく，免疫系や中枢神経系などにも障害を引き起こす場合もある。根治療法が存在する疾患はほとんどなく，疾患によっては酵素補充療法や造血幹細胞移植を含む骨髄移植（Bone marrow transplantation：BMT）が行われているが，その効果は限定的である。そこで，我々は間葉系幹細胞（Mesenchymal Stem Cells：MSC）に注目した。MSCは多分化能を備えており，特に骨芽細胞や軟骨細胞に分化することから，これまで国内外で骨・軟骨再生治療に自己MSCが多用されている。それはMSCに関して，細胞源の採取が比較的容易で，その技術も確立していることと培養操作も容易であること，そして何よりも，多用されているにもかかわらず移植後の腫瘍発生が国内外で報告されていない安全な幹細胞であることが大きな理由である。実際，独立行政法人産業技術総合研究所（以下，産総研と記述）でもこれまで自己MSCを用いて，足関節症に代表される整形外科疾患の他，本稿で取り上げる先天性骨系統疾患や心疾患など約100症例もの臨床研究を行い，腫瘍発生も含め有害事象は全く生じていない。一方，先天性骨系統疾患のMSCは骨形成能の障害が想定されているため，骨再生治療を目的とした細胞治療に自己MSCを用いるのは困難と思われる。したがって，

MSC（Mesenchymal Stem Cells；間葉系幹細胞）　BMT（Bone marrow transplantation；骨髄移植）

本疾患の治療として採取するMSCは，自己ではなく同種のものが望ましいと考えられる。

1．疾患治療に向けて

我々が現在取り組んでいる臨床研究の対象は，この先天性骨系統疾患のうち，低フォスファターゼ症（Hypophosphatasia：HPP）である。この疾患は，骨の石灰化に関わる酵素であるアルカリフォスファターゼ（ALP）が，それをコードする*ALPL*（*TNSALP*）遺伝子変異によってその活性が低下して，正常な骨の石灰化が障害される疾患で，多くは常染色体劣性遺伝形式をとる。発症時期と症状によって，胎児期に発症する周産期型，生後半年以内に発症する乳児型，乳歯の早期脱落を特徴とする小児型の他，成人型，症状が骨には無く歯に限局する歯限局型の計５種類の病型に分類される。このうち，周産期型は10万出生に１人の頻度と言われている稀な疾患であるが，日本で最も頻度が高い病型で，かつ最も重症である。症状として全身骨の低石灰化・長管骨の変形・骨幹端不整などが顕著で，徐々に骨の石灰化が消失して呼吸不全などで致死的な経過をとる[1]。現在，Alexion Pharmaceuticals社（米国）によって周産期型・乳児型を対象とした酵素補充療法の治験が始まっているが，現在のところ本疾患に対しては確立した治療法はない[2]。細胞治療としては，これまで乳児型の患者に，健常人の骨髄および骨，骨をつくる骨芽細胞や骨芽細胞に分化するMSCを移植することにより，その提供者の細胞が患者の骨に到達して骨を作り，患者が救命されたことが報告されている[3, 4]。このことから，我々，島根大学医学部附属病院（以下，島根大と記述）と産総研は，2004年に骨髄（Bone marrow：BM），MSCならびに産総研が独自に開発した培養骨の移植を行い，周産期型の患者を救命することに世界で初めて成功した[5]。そこで，根治療法のないHPPの中でも生後６カ月以内に発症し，呼吸障害を伴うとりわけ重症の患者を救うために，全身骨の石灰化再生を目的として，厚生労働省「ヒト幹細胞を用いる臨床研究に関する指針（以下，ヒト幹指針と記述）」に従った臨床研究として同省に申請，省内の厚生科学審議会に諮問後，2010年に「ヒト幹細胞臨床研究（重症低ホスファターゼ症に対する骨髄移植併用同種間葉系幹細胞移植）」として許可された（図１）。BMTと同種間葉系幹細胞移植（Mesenchymal stem cell transplantation：MSCT）を併用する理由として，①HPPのMSCはALP活性が低く，骨形成能も著明に低下しているために自己（患者）MSCを治療に使用できず[6]，骨形成能が正常な同種MSCを使用，②同種BMT後の患者MSCは患者由来のままである[7]，③造血幹細胞移植を含む同種BMTでは骨は形成されない，④同種MSCTによる骨形成には免疫抑制剤が必要である[8]，⑤BMTを先行することにより同じドナーのMSCが拒絶されることを防ぐなどが挙げられる。

2．治療の実際

さて，実際に行った一連の治療について詳述する。

BMTの具体的な治療として，前処置にはブスルファン，シクロホスファミド，抗胸腺グロブリンの３剤を用い，移植片対宿主病（Graft versus host disease：GVHD）予防にはメトトレキサート（MTX）とタクロリムス（FK506）の２剤を使用した。MSCT用として，BMTの時に採取されたBM

HPP（Hypophosphatasia；低フォスファターゼ症）　ALP（アルカリフォスファターゼ）　BM（Bone marrow；骨髄）
MSCT（Mesenchymal stem cell transplantation；同種間葉系幹細胞移植）
GVHD（Graft versus host disease；移植片対宿主病）　MTX（メトトレキサート）　FK506（タクロリムス）

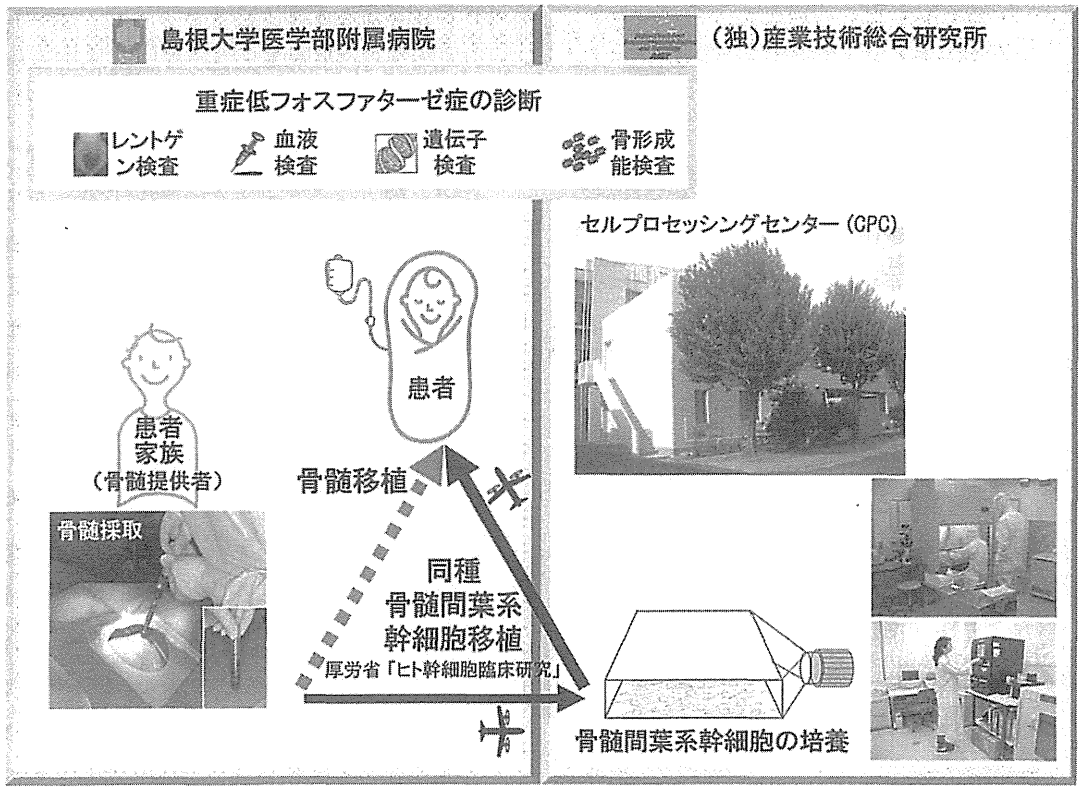

図1　重症低フォスファターゼ症に対する骨髄移植併用同種間葉系幹細胞移植治療の概要

重症低フォスファターゼ症の診断後，家族内で最適な骨髄提供者を決定する。前処置を行った後，骨髄移植を行う。骨髄移植に使用した骨髄の一部を用いて，セルプロセッシングセンターで間葉系幹細胞を培養増殖して患者に経静脈的に投与する。移植治療は島根大学で行い，間葉系幹細胞の培養増殖は産業技術総合研究所で行うため，骨髄および間葉系幹細胞は空輸している。　(筆者作成)

の一部を産総研の細胞製造施設（Cell Processing Center：CPC）へ搬送した。このとき，滅菌処理ができないBM（MSCも同様）は滅菌チューブによって二重梱包し，保冷剤を入れた運搬用クーラーボックス(症例の取り違え防止策として，BMあるいはMSCの搬送には単一症例ごとにそれぞれ1個の運搬用クーラーボックスをあてる)を用いた。島根大から産総研へのBMの搬送および産総研から島根大へのMSCの搬送中は，細胞の安全性および有効性に問題のないことを事前に確認した適温（10～30℃）を保つようにした。また，当初これらの搬送は，鉄道による陸路搬送を余儀なくされた。それは，空輸の場合，細胞を航空機内へ手荷物として持ち込む際，X線検査を免れることが不可能で，細胞への影響を危惧したからであった。搬送する内容物全ての詳細情報を航空会社へ事前に提供することで，最近になってようやくこのX線検査の免除について国土交通省の許可を得ることができた。こうして，空輸によって搬送時間の大幅な短縮化が可能となった。

このように，BMに含まれる幹細胞，すなわちMSCを採取する機関，そしてMSCを移植する機関はともに島根大であるが，前述の「ヒト幹指針」で実際に移植用細胞を培養すると定められた「調

CPC (Cell Processing Center；細胞製造施設)

整機関」は産総研である。産総研では10年程前に国内研究機関としてはいち早くCPCを設置した。この調整機関では，ヒト幹細胞の無菌的な調整および保存に必要な衛生上の管理がなされ，調整に関する十分な知識および技術を有する研究者を有していることがその要件として求められる。産総研CPCでも，一定陽圧の室圧を保ちながらフィルターを通した清浄な空気を流入させたグレードDからA(クラス100,000から100以下。クラスは米国Fed-Std-209E規格で1立方フィート中の基本粒子径0.5μm以上の粒子数)までのバイオクリーンルームを設置，作業従事者の動線も一方向にした作業空間を作っている。作業従事者は定期的な教育訓練に参加し，細胞製造に関わる製造管理部門，無菌試験に代表される品質検査に関わる品質管理部門をそれぞれ担当している。これら施設の維持，製造および品質管理，バリデーション業務の詳細について，標準作業手順書(Standard operating procedure：SOP)を作成，これまでの100症例程の豊富な実務経験を活かして，改訂を重ねながらより精緻なものにしてきた。作業従事者は高いスキルを身に付けているが，さらにこのSOPに従って毎回一定の作業を行うことで，作業ミスの防止・作業結果の再現性を高めることに役立ててきた。この産総研CPCにおいて，島根大から搬送されたBMからMSCの培養を行った。培養は15%牛胎児血清を含んでいる液体培地に採取した骨髄を混和し，培養容器を用いて炭酸ガス培養器(5%CO_2，37℃)内で行った。移植に必要な細胞数を得るために，培養容器底面に接着し増殖したMSCをプロテアーゼによって培養容器より剥がし，新たな培養容器で継代培養(2次培養)した。こうして2～3週間かけて培養し，移植当日にMSCを剥離し，PBSに浮遊させた状態で島根大へ搬送した。また，移植細胞の安全性検査として，まず骨髄採取に先立ちドナーのウイルス試験を行った。CPCでは，細胞培養に先立つ培養前検査として，培地に添加する薬剤や血清は各種微生物検査で陰性のものを購入するとともに，それらを含有する培地とBMを検体，培養工程内検査として工程ごとの検体，そして出荷検査(最終検査)用に最終培地交換時の検体として各検査を実施した。検査項目は，無菌検査，マイコプラズマ検査，エンドトキシン検査で，一部外部委託の検査もあるが，いずれの症例においても全て異常のないことを確認した。また，各工程で細胞の一部を凍結保存し，再検査・確認試験にも備えた。培養したMSCについては，同時に骨分化能を有していることも確認した。

産総研から搬送されたMSCは数時間後に島根大にて患者に移植した。MSCTは患者体重(kg)あたり1×10^6細胞以上，細胞生存率80%以上のMSCを経静脈的に約1時間かけて投与した。なお，これまでの報告および我々の経験から，単回のMSCTだけでは骨形成を十分に回復させることができなかったことから，MSCTを繰り返し行っている。特に後述の薬剤抵抗性のGVHDのように症状が悪化したり改善が見られなかったりした場合，短期間のうちにMSCTを繰り返した。

3．治療の効果

これまでの臨床研究の結果を示す。対象患者の条件として，①生後6カ月以内の発症，②呼吸障害を合併している，③ALP活性の低い*ALPL*遺伝子変異を有している，④*In vitro*で患者のMSCの骨形成能が低下しているという4条件を満たすこととした。これは，これまでの疫学調査から周産期に発症する患者の中に，徐々に自然回復する症

SOP (Standard operating procedure；標準作業手順書)

例が存在することが明らかとなったためである。ドナーの選択として，一般的な骨髄提供者の条件以外に，ALP 活性が正常であること，症状や検査（骨レントゲン・骨密度など）から骨形成が正常であることとした。また，MSCT を複数回行う度に BM を採取して MSC を培養する必要があることなどから，現実にはドナーとして両親のいずれかを選択せざるを得なかった。そこで，BM ドナーとして重要な条件である HLA に関しては，HLA の一致度は規定しないこととした。実際，全ての患者に対して，HLA 不一致ドナーである親からの BMT を行った。一方，MSCT は，BMT と同じドナーから複数回（症例ごとに異なるがこれまでに５～７回）行った。行った移植時期は乳児期であったが，前処置および移植後早期に重篤な有害事象は認めなかった。BM の生着は３週間前後で認められ，血球の回復も順調であった。骨の石灰化は，移植後６カ月ごろから徐々に改善し，骨が全く消失していた部位の石灰化も認められているまでに回復している（図２）。生命予後を左右する呼吸障害に関して，移植後１～２カ月で呼吸機能が改善して，１例は呼吸器からの短時間の離脱も可能となっている。骨の石灰化の改善に伴い筋肉量も増えて，発育発達も伸びている。キメリズム解析でドナー由来 MSC が骨髄および骨で生存していることから，この臨床的効果が MSC に起因すると思われた。また，この疾患に合併する難聴や精神発達障害などの中枢神経障害も改善を認めているが，MSC の中枢神経系への関与は明らかではない。なお，ドナー由来 MSC は有害事象なく投与できており腫瘍化も認めず，同種 MSC は乳児において安全に行える治療であると思われた。このように，本疾患に対して限定的な効果しか示さなかった BMT や酵素補充療法といった従来治療法に比べ，当初の予想をはるかに上回る効果を MSCT が示したことは特筆すべきである。さらに，１例では重症 GVHD（grade 4：皮膚 3，肝臓 1，腸管 4）を発症し，MTX・FK506 に加え，ステロイド・シクロスポリン・インフリキシマブも投与して全く改善されなかったが，MSC を投与したところ１週間で症状が改善し，その後再燃なく軽快した。GVHD に対する MSC の効果はこの号でも報告されているが，小児に対しても有効な治療法となり得ると思われる。

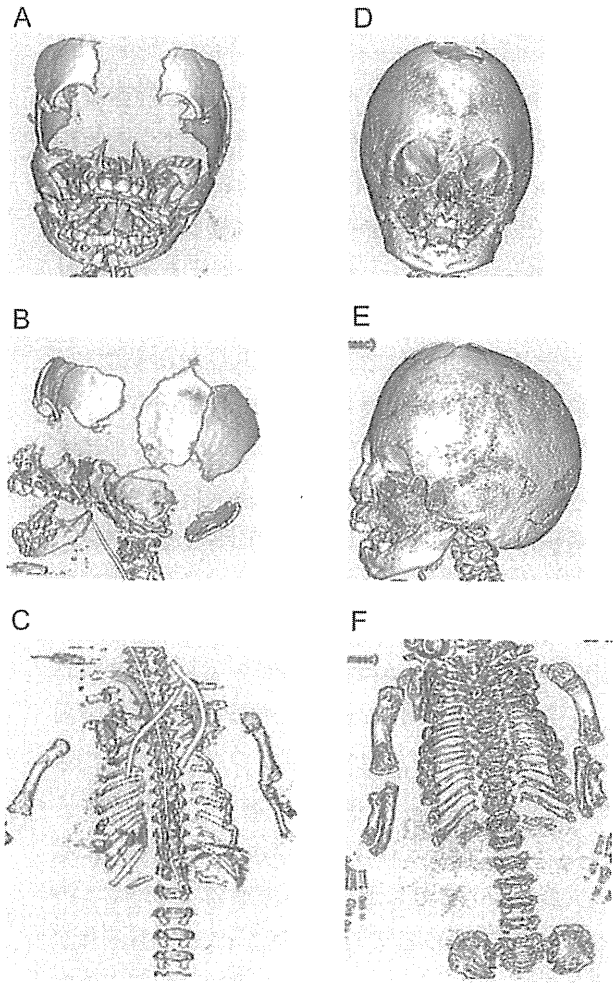

図２　骨髄と間葉系幹細胞の移植により骨の石灰化が改善する（骨 CT）

A，B，C：移植前（生後６カ月）。D，E，F：骨髄移植後１年＋間葉系幹細胞移植５回（生後１歳６カ月）。A，D：頭蓋骨（正面）。B，E：頭蓋骨（側面）。C，F：上半身。

（筆者提供）

おわりに

今後の課題として，今回用いるMSCは移植ごとにドナー骨髄を採取して分離増殖するものとして「ヒト幹細胞臨床研究」の申請を行った。しかしながら，ドナーには毎回採取で侵襲を加えることや，骨髄採取量などの条件が異なることで，細胞培養期間が異なる点でMSC搬出，すなわち移植日の設定も困難となる問題が浮上した。今後は，初回培養で生じた余剰MSCを凍結保存し，以後継続する移植には解凍した細胞を用いることが望まれる。実際，産総研では他の臨床用細胞の凍結に用いられている凍結保護剤でMSCを凍結保存し，解凍後の安定性（増殖・分化能）も確認している。また，ドナーの選択も今後の課題である。MSC自体，元々免疫原性が低いどころか，前述のGVHD治療に利用されるほど免疫抑制作用があるので，移植後短期間では生存することが期待できる。しかし，長期生着については，我々はFK506を投与しない限り，異系ラット由来MSCは生着しないことも動物実験で確認している[8]ので，HLA一致のドナーも考慮すべきであろう。我々のように，同じドナーのBMからその都度MSCを培養する方法では，骨髄バンクならびに臍帯血バンクからのHLA一致BMあるいは臍帯血を使用することはできない。そのために，ドナーとなった親もHLAが完全に一致したことはなかった。しかし，HLAを一致させた方がBMTおよびMSCTの成績が良いのは明らかである。HLAが適合した健常者のドナーを選択できれば，BMTをより安全に行うことができ，MSCの生着率が上がり，ひいては骨の石灰化の改善に寄与するものと思われるからである。そのためにも，前述の凍結MSCが頻回移植の必要性に対応できることも考え合わせると，最近iPS細胞の応用としてにわかに注目されるようになった臨床用細胞バンクをMSCについても整備することが，MSCTの可能性を拡げるものと思われる。さらに，その細胞バンク整備のための細胞源にも新たな展開が考えられる。この臨床用細胞バンクとして既に整備されているのが，前述の臍帯血バンクである。臍帯血は骨髄に較べて少ないものの造血幹細胞を含むので，これまで盛んに採取されてバンク化されてきたが，臍帯血採取後，破棄されていた臍帯の臍帯動静脈周囲にはMSCが豊富に存在することが知られている[9]。皮膚再生が対象ではあるが，CellResearch Corporation（シンガポール）も治験を進めているように，新たな細胞源として期待される。こうした医療廃棄物となる組織は，ドナーに細胞源採取を目的に新たに侵襲を加える必要がない点で注目に値する。ここで改めて触れておきたいのは，我々の臨床研究がBMTとMSCTを併用していることである。MSCTの効果は，初回のBMTに依存している可能性も考えられる。すなわち，元々ドナーBM中に存在していたMSCは，BMTによってもたらされるレシピエントのBMはもちろん，他の異所環境においても生着が促されていることが大いに考えられる。このことから，臍帯血とMSCが同じレシピエントから同時に採取できる臍帯は，移植細胞が少なくて済む小児であれば，我々の臨床研究と同様，臍帯血移植とMSCTの併用という新たな治療戦略を想起させる。対して，HPPには酵素補充療法の治験も進行している。先天性の代謝性疾患の多くは稀少疾患であり，HPPのようにオーファンドラッグ（稀少疾病用医薬品）が存在することは，患者にとって確かに福音である。ところが，継続的な投薬が必要であることに加え，その開発製造費と患者数を反映して薬価は高く，公費助成はあるものの，患者の経済的負担は甚大である。

骨髄および骨に生着しているドナーMSCが十分ではないこと，そして骨石灰化の程度が健常児のレベルに至っていないなど，根治に向けた課題はまだ残されている。しかしながら，本研究のさ

らなる進展によってこうした課題が解決されて，酵素補充療法しか治療法が存在しない疾患に対して，MSCTが医療経済的にも優れ，持続効果が期待できる治療として，さらにHPP以外の代謝性疾患への汎用的治療としても適応拡大ができればと願っている。

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Patient Report

Therapy-related Ph+ leukemia after both bone marrow and mesenchymal stem cell transplantation for hypophosphatasia

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Abstract Bone marrow (BM) transplantation (BMT) is one of the treatment strategies for congenital metabolic disease, but leukemia secondary to intensive cytoreductive treatment is a major concern. Besides BM cells, mesenchymal stem cells (MSC) are also used for transplantation. An 8-month-old girl with hypophosphatasia underwent transplantation of haploidentical BM cells followed by two transplants of MSC obtained from her father to facilitate osteogenesis. Fludarabine(Flu)/cyclophosphamide (CPA)/anti-thymocyte globulin were used for myeloablative conditioning, but the patient developed therapy-related leukemia harboring t(9;22)(q34;q11.2); minor *BCR-ABL* (t-leukemia with Ph) at the age of 32 months. At the age of 40 months she underwent a second BM and third MSC transplant from the same donor. Thereafter, she achieved complete histological and molecular remission. The present case suggests that the combination of cytotoxic agents (Flu/CPA) and MSC led to t-leukemia with Ph as a consequence of chromosome instability and suppression of host anti-tumor immunity.

Key words bone marrow transplantation, hypophosphatasia, mesenchymal stem cell transplantation, t(9;22)(q34;q11.2), therapy-related leukemia.

Hematopoietic stem cell transplantation (HSCT) is one of the treatment strategies for congenital metabolic disease, but myelodysplastic syndrome and acute myeloid leukemia (MDS/AML) secondary to intensive cytoreductive treatment is a major concern. In contrast, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and mixed phenotype acute leukemia (MPAL) associated with HSCT are seldom reported.[1] Cytotoxic agents implicated in therapy-related hematologic neoplasms are alkylating agents, DNA topoisomerase II inhibitors, ionizing radiation therapy, and other drugs such as antimetabolites and anti-tubulin agents.[2] Monosomy or deletions of the long arms of chromosome 5 and 7 and the rearrangement of the mixed-lineage leukemia (*MLL*) gene located on chromosome 11q23 are frequently detected in therapy-related hematological malignancies.[1] Translocation of (9;22)(q34;q11), however, is rare.[1] Besides hematopoietic stem cells (HSC), mesenchymal stem cells (MSC) that normally reside in bone marrow (BM) and other tissues are also used for transplantation. MSC differentiate into various mesenchymal lineages and several mesoderm lineages.[3,4] Their regenerative ability makes MSC an ideal source for transplantation in patients with osteogenesis imperfecta, inborn errors of metabolism and ischemic heart disease.[3,4] Furthermore, MSC help maintain hematopoietic stem cells, facilitate hematopoietic recovery and reduce graft versus host disease (GVHD) by modulating immune function of T cells, B cells, NK cells, monocyte, and dendritic cells.[3,4] Herein we describe a girl with therapy-related leukemia harboring t(9;22)(q34;q11.2) (t-leukemia with Ph) that had developed following transplantation of HSC and MSC for the treatment of lethal congenital hypophosphatasia (HPP).

Case report

A female infant suffering from severe respiratory distress was diagnosed with perinatal HPP based on low serum alkaline phosphatase and hypomineralization of bones soon after birth. At 8 months old, she underwent sequential transplantation of haploidentical BM cells and MSC obtained from her father to facilitate osteogenesis, given that perinatal HPP manifesting respiratory disturbance is lethal.[5] Fludarabine (Flu; 30 mg/m^2 per dose × 5 days)/cyclophosphamide (CPA; 50 mg/kg per dose × 2 days)/anti-thymocyte globulin (30 mg/kg per dose × 3 days) were used for non-myeloablative conditioning. Short-term methotrexate (sMTX)/cyclosporin A/mycophenolate mofetil was given for GVHD prophylaxis. Although donor-derived bone marrow did not engraft, her respiration improved.[5] GVHD was not observed. MSC from the same donor were re-infused for recurrence of respiratory failure at the age of 15 months.[5] Although the patient had no respiratory complications following the second MSC

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transplant (MSCT), she was found to have hepatosplenomegaly at 32 months of age, 24 months after initial transplantation. Peripheral blood (PB) examination showed an elevation of white blood cells (WBC) up to 48 990/uL with 40% blasts, 5.5% myelocytes, 2.5% metamyelocytes, 3.5% bands, 31% segments, 1% eosinophils, 7.5% monocytes and 9.5% lymphocytes. Hemoglobin and platelets were 14.6 g/dL and 163 000/uL, respectively. Marrow nuclear cell count was 156 000/μL with 23.4% lymphoblasts and myeloblasts, which were positive for myeloperoxidase (Fig. 1a,b). On flow cytometry, the blasts were positive for CD10, CD13, CD19, CD33, CD34, HLA-DR, TdT, cyMPO, cyCD22, and cyCD79a antigens. The data indicate that the leukemic clones expressed both myeloid and B lineage antigen and that these cells were biphenotypic, not biclonal (Fig. 1c). On G-banding chromosomal analysis, four out of 20 BM cells were 46 XX, t(9;22)(q34; q11) (Fig. 1d). No additional chromosomal abnormalities were detected. A minor *BCR-ABL* fusion gene, but not the major *BCR-ABL* fusion gene, was detected on quantitative reverse transcription–polymerase chain reaction (RT-PCR). MPAL does not involve myeloid differentiation. The patient presented with differentiated myeloid cells as well as leukemic blasts. Three weeks before onset, PB cells, including the leukocyte number and differential count, were normal and the minor *BCR-ABL* fusion was not detected on RT-PCR, resulting in no appearance of CML chronic and accelerated phase. The present patient, therefore, was diagnosed with undifferentiated leukemia. Daily imatinib at 230 mg/m^2 normalized the WBC count and eliminated the blasts in the PB. The blasts re-emerged, however, 3 months after imatinib treatment despite increasing of the dose (300 mg/m^2 per day). Neither mutations within *BCR-ABL* kinase domain nor *BCR-ABL* gene amplifications were found at this time. Irrespective of multiple chemotherapeutic drugs in addition to imatinib (prednisolone, vincristine, CPA, daunorubicin, L-asparaginase, cytarabine, and etoposide), the blasts never

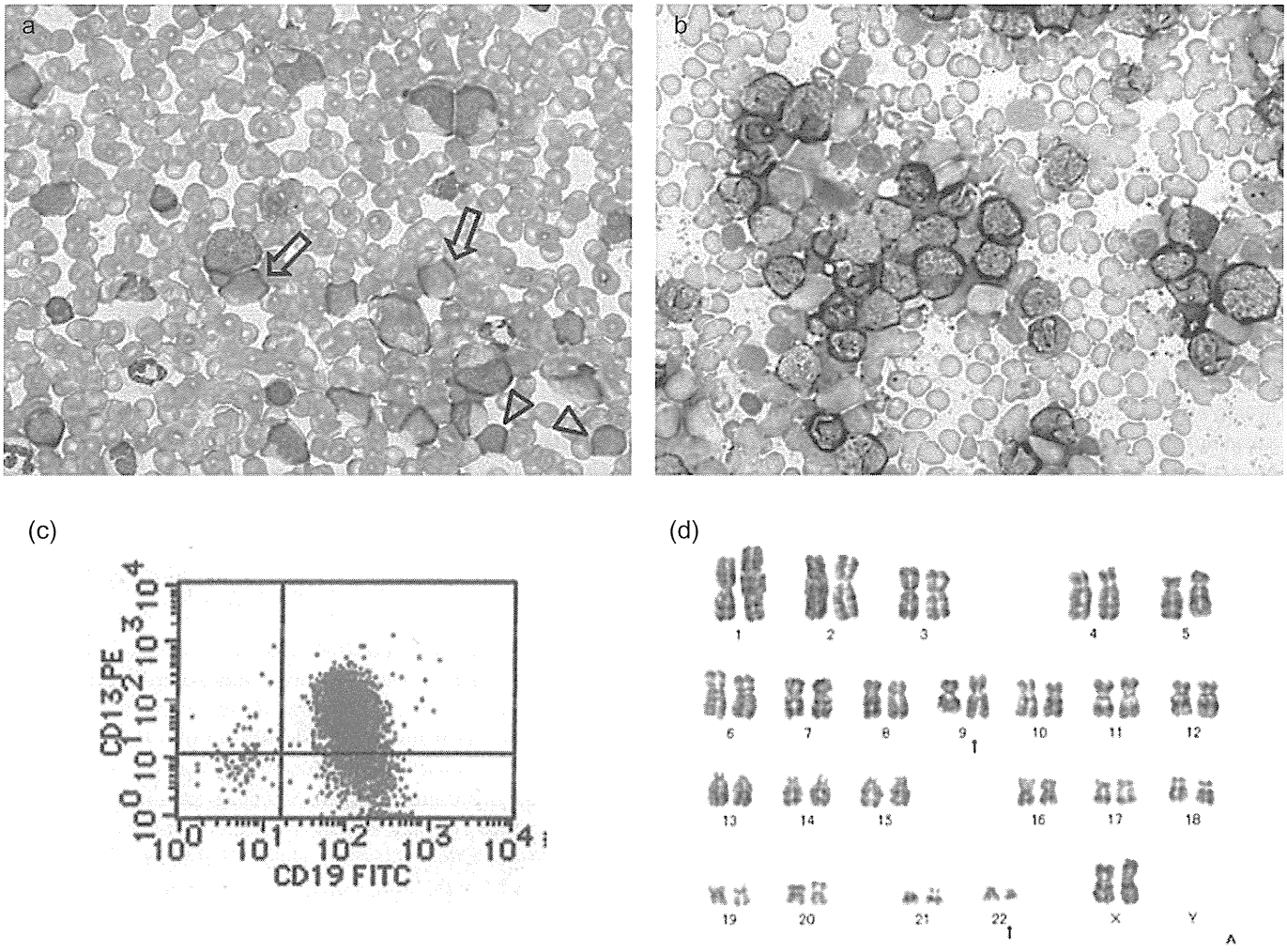

Fig. 1 Bone marrow examination. (a) Myeloid (arrows) and lymphoid (arrowheads) blasts exist simultaneously (May–Giemsa staining). (b) Myeloperoxidase staining. (c) Flow cytometry: marrow blasts expressing both CD19 (lymphoid antigen) and CD13 (myeloid antigen). (d). G-banding chromosome analysis.

disappeared. At the age of 40 months (32 months after the first BM transplant [BMT]), the patient underwent a second BMT from the same donor. VP16 (60 mg/kg/dose × 1 day)/CPA (60 mg/kg/dose × 2 days)/total body irradiation (13.4 Gy), and sMTX/tacrolimus (FK506) were given for myeloablative conditioning and prophylaxis of GVHD, respectively. A third MSCT was also performed to prevent severe GVHD because of HLA haploidentical BMT. Donor-derived marrow cells were engrafted 17 days after the second BMT. *BCR-ABL* fusion gene disappeared 1 month after the second BMT. Grade 1 acute GVHD was detected only in the skin but chronic GVHD was not observed. Although the patient maintained complete histological and molecular remission thereafter, she suddenly died due to unknown reasons at the age of 65 months.

Discussion

The present patient had t-leukemia with Ph. The frequency of therapy-related MDS/AML, ALL, MPAL, and CML with Ph, resulting from cytoreductive treatment is rare in adult and pediatric cases.[1,6,7] Previous reports indicate that therapy-related MDS/acute leukemia with Ph is predominant in female subjects and adults (the median age at diagnosis of therapy-related disease is 48 years; range, 25–69 years). The median interval from initial therapy to development of therapy-related diseases is 110 months and the median survival is 5 months,[6] indicating long latency and poor outcome. Exposure to dose-dependent ionizing radiation is reported as one of the mechanisms responsible for therapy-related CML with Ph.[1,7] Ph+ AML/ALL/CML is reported to be associated with DNA topoisomerase II inhibitors, but not the alkylating agents.[1,7,8] Treatment with radioactive ^{131}iodine, 5-fluorouracil, 5′-deoxy-5-fluorouridine or tegafur for some solid tumors is also reported to be associated with subsequent emergence of CML.[7,9] There is also increasing recognition of CML in patients with non-malignant diseases treated with long-term immunosuppressive drugs and intensive treatment for organ transplantation.[7] Both alkylating agent (CPA) and antimetabolites (Flu) were given in the present case but neither DNA topoisomerase II inhibitors nor irradiation were used. Immunosuppressant drugs were used for <2 months in the present case and the known evidence indicates that CPA was also unlikely to contribute to the onset of Ph+ leukemia, suggesting that the combination of the two cytotoxic agents, CPA and Flu, may be implicated in the present case of Ph+ leukemia. A number of clinical trials of transplantation of HSC plus MSC have been performed in order to prevent graft failure and/or GVHD.[3,4] Although the risk of recurrence of hematological malignancies is a matter of concern because of the immunosuppressive function of MSC, the frequency of recurrence of leukemia in the patients who received HSCT plus MSCT was similar to those treated with HSCT alone.[4] The MSC is considered to be one of the components of the leukemia stem cell niche in addition to normal HSC.[3,4] Soluble factors secreted from MSC and cell–cell interactions regulated by MSC can aggravate the tumor microenvironment.[2] The cellular components of the tumor stroma, such as blood vessels, connective tissue, and inflammatory cells, proliferate and survive in the presence of a variety of chemokines, cytokines and adhesion molecules derived from MSC.[3,4,10] Moreover, chemokines secreted by MSC can recruit tumor-associated macrophages and correlate with the progression of cancer cells by favoring tumor angiogenesis, proliferation, and metastasis.[3,4,10] MSC inhibit the function of cytotoxic T cells[10] and the proliferation of NK cells as well as dendritic cells,[10] suggesting that host anti-tumor immunity can be compromised by MSC.[10] These findings suggest that transplanted MSC likely supported tumor growth in the present case by inhibiting host anti-tumor immunity. Although a lack of phosphatase in general can increase phosphorylation, which likely affects intracellular signaling associated with cell proliferation, none of the patients with HPP reported so far developed any hematological malignancies. This may be attributed to lack of expression of alkaline phosphatase in primitive hematopoietic cells. Alternatively, the substrates for alkaline phosphatase may not be involved in cell proliferation, although this remains to be explored. These findings strongly suggest that deficiency of alkaline phosphatase itself was not the primary mechanism responsible for t-leukemia with Ph in the present patient. The present case instead suggests that a combination of cytotoxic drugs and MSC used for the treatment of HPP lead to t-leukemia with Ph as a consequence of chromosome instability and suppression of host anti-tumor immunity.

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