

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). This allowed for differences in the amount of total RNA added to each reaction to be taken into account.

For biochemical analysis, the ALP activity and osteocalcin contents of the implants were measured at 2 and 4 weeks post-implantation. After washing with PBS, the implants were homogenized in 1 ml 0.2% Triton-X 100 solution and sonicated for 5 min. After centrifuging, the supernatant was assayed for ALP activity as described above. To measure osteocalcin in the implants, osteocalcin was extracted from the sediment of the Triton-X 100 extract by decalcification, using a 20% formic acid solution for <24 h at 4 °C. After desalting, using a prepacked Sephadex G-25 column (NAP-5 column; GE Healthcare UK, Buckinghamshire, UK), the eluted protein fraction was lyophilized and subjected to a Rat Osteocalcin EIA Kit (Biomedical Technologies, MA, USA) according to the manufacturer's instructions.

2.7. Statistics

For multiple comparisons, the groups were compared by non-parametric Kruskal–Wallis test. When significant variance was demonstrated, differences between individual groups were determined using the Mann–Whitney U-test with Bonferroni correction. In all analysis, the significance level was set at $p < 0.05$.

3. Results

3.1. MicroCT analysis

GT sponges implanted subcutaneously into the backs of rats were retrieved for μ CT and histological analysis 2 and 4 weeks after implantation. Typical μ CT images of sagittal sections of the implants are shown in Figure 1. The GT sponges had a low intensity before implantation. μ CT images of some implant groups using GT sponges showed an increase in intensity at 2 weeks. Four weeks after implantation, the images of both the BMP–GT and BMP–MSC–GT implants showed high-intensity (white) areas. In the implants using gelatin sponge (Sp), a high-intensity area was only observed in the BMP–MSC–Sp group 2 weeks after implantation.

As described in our previous report (Nishikawa *et al.*, 2004), we could define the high-intensity areas as new bone formation. The average volumes of bone in the GT sponge implants were $0.0043 \pm 0.0010 \text{ mm}^3$ in GT, $0.0057 \pm 0.0503 \text{ mm}^3$ in MSC–GT, $0.16010 \pm 0.1142 \text{ mm}^3$ in BMP–GT, and $5.8690 \pm 1.5267 \text{ mm}^3$ in BMP–MSC–GT composites, respectively. The numbers of detections of bone were 0 in GT, 1 in MSC–GT, 4 in BMP–GT and 5 in BMP–MSC–GT composite, respectively (Figure 2A, C). The number of bone detections in the BMP–MSC–Sp group was only one out of five samples 2 weeks after implantation. After 4 weeks, we could not detect newly formed bone in any implants of the

Sp, MSC–Sp, BMP–Sp or BMP–MSC–Sp groups. We confirmed that control samples (GT-alone) showed a background level of X-ray absorbance (Figure 2B). β -TCP granules (2 μm average diameter) incorporated sponge did not appear in 3D imaging because the μ CT had a resolution of about 5 μm .

3.2. Histological analysis

Histological evaluation confirmed that the high-intensity areas seen in the μ CT scan corresponded to areas of newly formed bone. Two weeks after implantation, histological sections showed new bone formation with osteoblasts and osteocytes in both BMP–GT and BMP–MSC–GT implants (Figure 3A), although new bone formation was only observed in the peripheral areas of the BMP–GT implants, while bone was seen throughout the BMP–MSC–GT implants (Figure 3B). Newly formed bone was also detected in the BMP–MSC–Sp implants but not in Sp-alone, BMP–Sp or MSC–Sp implants 2 weeks after implantation. After 4 weeks, more extensive bone formation was observed in the BMP–GT and BMP–MSC–GT implants (Figure 3A, B). The bone tissue in both BMP–GT and BMP–MSC–GT implants showed many osteocytes together with abundant extracellular bone matrix. Some areas of the BMP–MSC–GT implants also showed the existence of bone marrow-like tissue. As described above, none of the Sp implants were detected in the implanted areas and therefore could not be harvested at the 4-week time point.

3.3. Alkaline phosphatase and osteocalcin expression of implants

The gene expression profiles of alkaline phosphatase (ALP) and osteocalcin in the implants were analysed by qPCR (Figure 4). The expression levels of *ALP* mRNA in the BMP–MSC–GT group were higher than in the other GT groups after 2 weeks. After 4 weeks, the expression levels of *ALP* mRNA in the MSC–GT, BMP–GT and BMP–MSC–GT groups increased. The expression levels of *osteocalcin* mRNA in the BMP–MSC–GT group were also higher than those of the other GT groups.

Biochemical measurement of ALP activity was significantly higher in BMP–MSC–GT than in the other GT groups at 2 weeks, but after 4 weeks the ALP activity levels decreased (Figure 4B). However, both the BMP–GT and BMP–MSC–GT groups had high ALP activity levels compared with the GT-alone and MSC–GT groups. ALP activity in the Sp implants was as low as the GT-alone group (data not shown). High osteocalcin levels were detected in the BMP–MSC–GT group 2 weeks after implantation and this increased five-fold after 4 weeks. The BMP–GT group also showed a similar increase between 2 and 4 weeks. The highest content was seen in the BMP–MSC–GT 4 weeks after implantation. These findings correlated well with the histological sections,

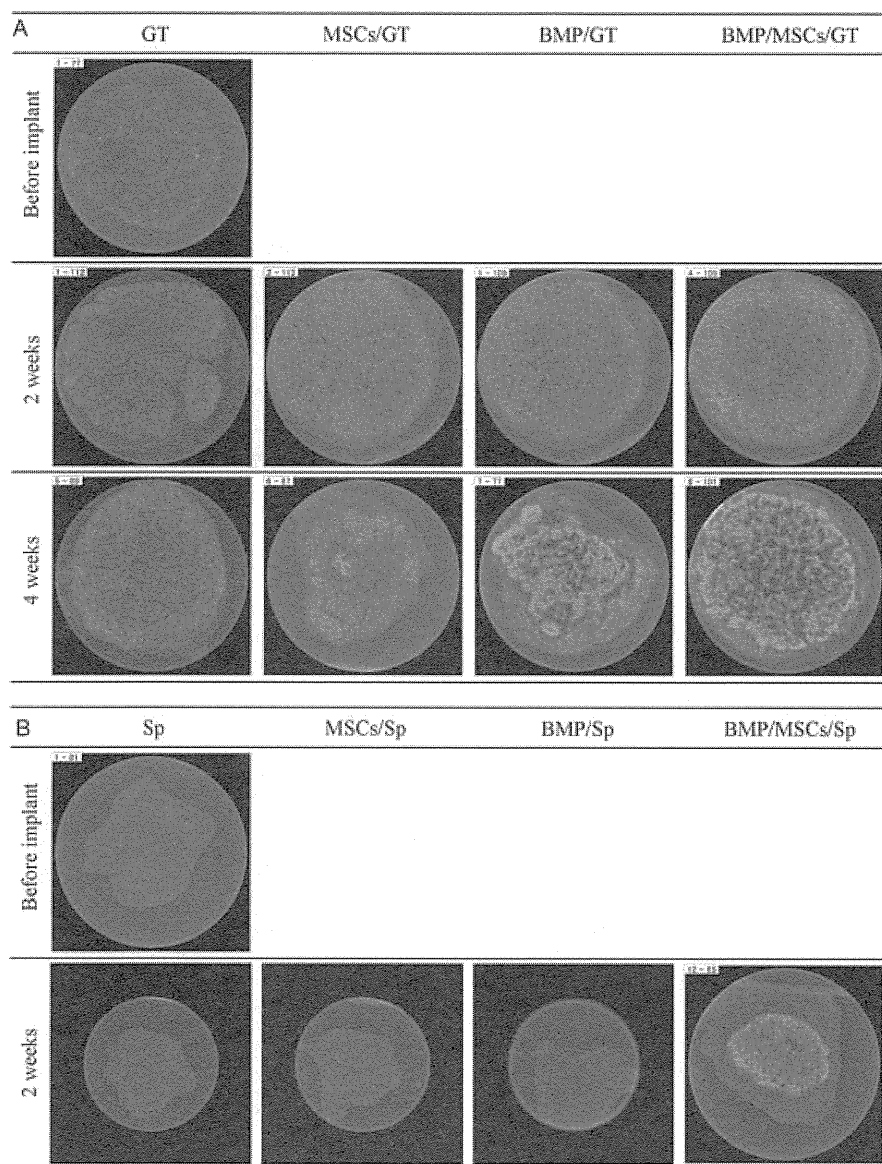


Figure 1. Typical micro-computed tomography (μ CT) images of the implants using gelatin- β -TCP (GT) and gelatin (Sp) sponges. Images of various implants using GT (A) and Sp (B) materials after *in vivo* implantation. The MSC-GT, BMP-GT and BMP-MSC-GT implants show high-intensity areas (white) as compared to the image of the GT-alone implant 4 weeks after implantation

which showed abundant bone formation in the BMP-GT and BMP-MSC-GT groups and the *osteocalcin* gene expression profiles.

4. Discussion

Bone marrow-derived MSCs are a well-accepted source of osteogenic cells that can be used in bone tissue engineering. However, it is still a challenge to enhance the proliferation and differentiation of the *in vitro* expanded MSCs for bone tissue engineering, a problem that can be solved by approaches using growth factors (Betz *et al.*, 2008; Chan *et al.*, 2005). A number of factors have been shown to play a role in the osteogenic differentiation of MSCs (Kodama *et al.*, 2009; Maegawa *et al.*, 2007; Raiche and Puleo, 2004; Shimaoka *et al.*, 2004). In this

study, we demonstrated that differentiation was greatly enhanced by BMP-2 *in vivo* using gelatin- β -TCP sponge incorporating BMP-2.

GT sponge could be fabricated to function as a release carrier of BMP-2. When BMP-2-incorporated GT sponges were implanted subcutaneously, the BMP-2 was retained in the sponges for at least 1 month and steadily released over that time. *In vivo* bone formation was observed in BMP-2-incorporated GT sponges 4 weeks after implantation in a subcutaneous site in rats (Takahashi *et al.*, 2005a). In this study, we aimed to demonstrate that early bone formation could be achieved by incorporating BMP-2 into GT sponges when combined with MSCs. As expected, we confirmed that new bone was observed in the BMP-MSC-GT and BMP-MSC-Sp groups 2 weeks after implantation. Furthermore, maturation of the new bone tissue was observed in the BMP-MSC-GT after

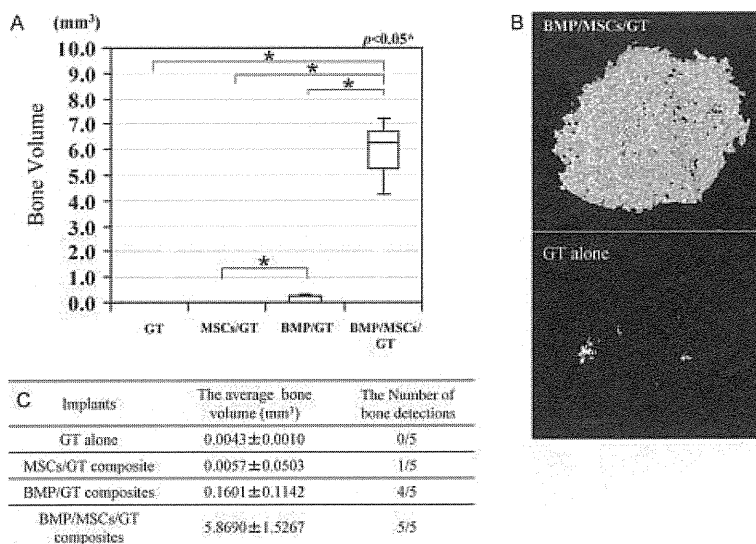


Figure 2. Bone volume and 3D CT images of implants 4 weeks after subcutaneous implantation ($n = 5$). Bone volume (A) and 3D images (B) were calculated from μ CT data. (C) Details of average bone volume and number of bone detections. $*p < 0.05$

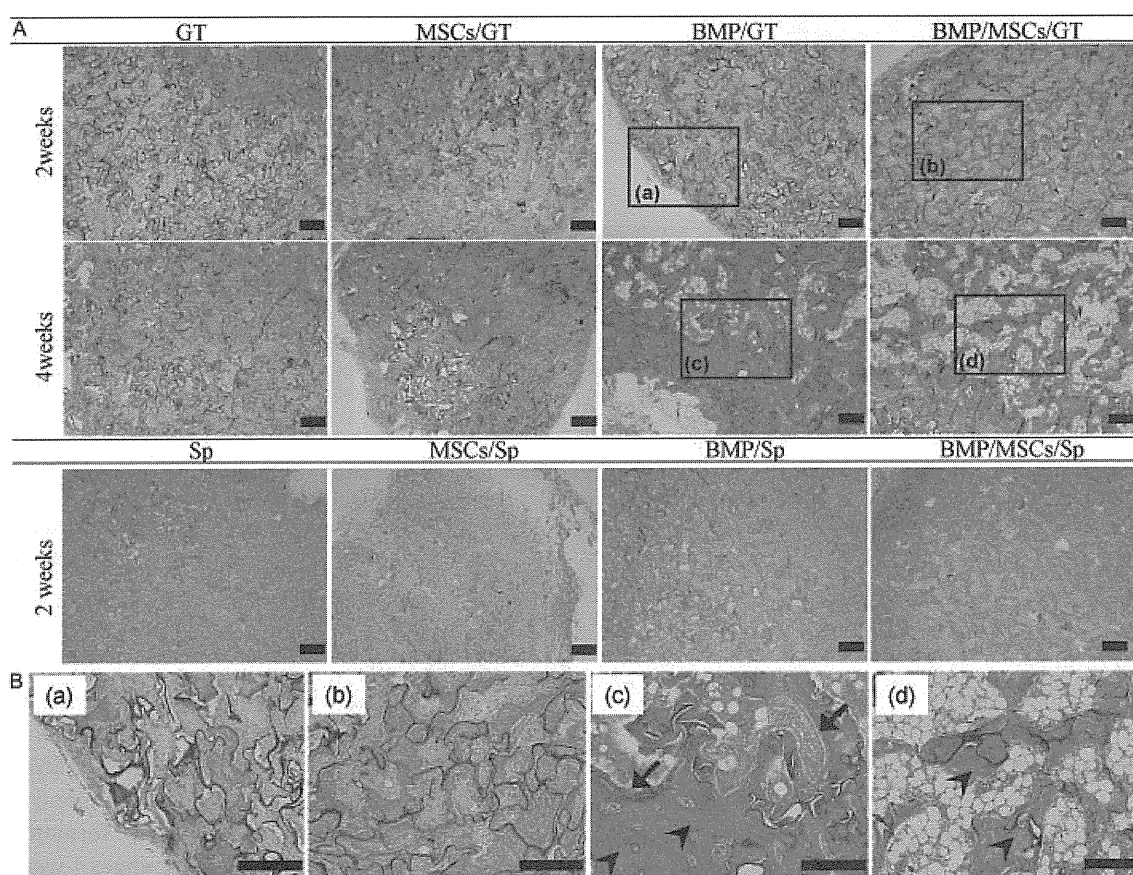


Figure 3. Histological findings of implants using gelatin- β -TCP (GT) and gelatin (Sp) sponges after implantation. (A) In implants using GT, new bone formation is observed in BMP-GT and BMP-MSC-GT implants 2 weeks after implantation. GT-alone and MSC-GT implants exhibit fibrous tissue invasion with no evidence of bone formation. Four weeks after implantation, massive bone formation is observed in the BMP-GT and BMP-MSC-GT implants. The GT-alone and MSC-GT implants exhibited fibrous tissue invasion but the bone formation was hard to detect. In Sp implants, new bone formation was observed in only BMP-MSC-Sp implants. The Sp alone, MSCs/Sp and BMS/Sp implants exhibited fibrous tissue invasion without bone formation. (B) High-magnification images of the rectangular areas of (a-c) and (e) in (A), respectively. Osteoblasts (arrows) and osteocytes (arrowheads) were observed within the newly formed bone. Scale bar = 200 μ m

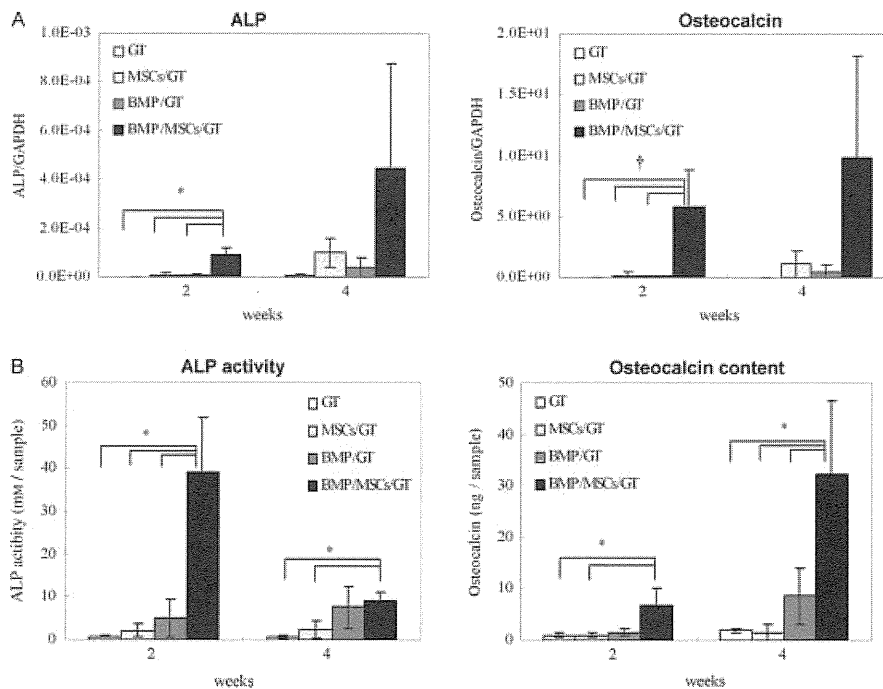


Figure 4. Gene expression and biochemical analysis of alkaline phosphatase (ALP) and osteocalcin in the implants. (A) The gene expression of ALP and osteocalcin were measured by quantitative real-time PCR 2 and 4 weeks after implantation. Values are shown as mean \pm SD ($n = 3$). * $p < 0.05$. † $p < 0.01$. (B) ALP activity and osteocalcin content of the implants were measured after 2 and 4 weeks. Implants are GT-alone, MSC-GT, BMP-GT and BMP-MS-GT. Values are shown as mean \pm SD ($n = 4$). * $p < 0.05$.

4 weeks. On the other hand, although new bone formation was observed in the BMP-GT group, which was free of MSCs, the volume of new bone was less than in the BMP-MS-GT and the BMP-MS-Sp groups. Biochemical analyses of the osteogenic differentiation at the levels of gene (Figure 4A) and protein expressions (Figure 4B) also demonstrated that the highest ALP and osteocalcin was in the BMP-MS-GT group. These results indicate that the new bone-forming capability of BMP-2 incorporated into GT sponges could be enhanced by supplementation with MSCs.

We previously demonstrated that new bone formation was observed following subcutaneous implantation of MSCs combined with calcium phosphate ceramics, such as HA (Matsushima *et al.*, 2009; Ohgushi and Caplan, 1999; Okamoto *et al.*, 2006). However, the amount of new bone was limited 4 weeks after implantation and could not even be detected after 2 weeks. By adding BMP-2 into the HA ceramics, new bone formation increased, but in the composites of HA and BMP-2 without MSCs it was hard to identify the new bone (Shimaoka *et al.*, 2004). In contrast, the results from the present study clearly demonstrated the bone-forming capability of the BMP-GT implants, indicating the excellent ability of the GT to carry BMP-2. Furthermore, obvious bone formation was detected only 2 weeks after implantation in the BMP-MS-GT group and extensive bone formation was observed in the composites after 4 weeks. With regard to β -TCP-containing sponge, β -TCP content did not change *in vivo* release behaviour of BMP-2 (Takahashi *et al.*, 2005b). However, in comparison with gelatin sponge incorporating other ceramics granules, such as

HA, α -TCP and alumina, gelatin- β -TCP sponges exhibited the highest ALP activity of MSCs among them (Takahashi *et al.* 2005b). These findings demonstrate the superior properties of GT sponge compared to HA ceramics.

One other disadvantage of the calcium phosphate ceramics is that absorption of the ceramics takes several months to years, and in the case of HA ceramics they are considered to be essentially non-absorbable. It is well known that the bone formation process followed by the absorptive capability of the implant, more specifically bone substitution, is an important factor for bone graft implantation in clinical situations. In this regard HA ceramics are not ideal materials, due to their poor absorption (Barrere *et al.*, 2006). However, too-early absorption hampers bone formation, as was seen in the BMP-MS-Sp group as a result of the rapid absorptive properties of gelatin. Only one sample of the BMP-MS-Sp group showed bone formation after 2 weeks, but none of the implants or bone tissue was detected after 4 weeks. We improved the absorption properties of the gelatin, which is known to have relatively rapid absorption properties compared with HA. Using the gelatin β -TCP sponge (GT), we successfully showed early bone formation with osteoblasts after 2 weeks and extensive bone tissue with many osteocytes in abundant bone matrix after 4 weeks.

5. Conclusion

Overall, the results presented here demonstrate the usefulness of the GT, BMP-2 and MSC composites as an

innovative osteoinductive bone graft substitute. Currently, gelatin, β -TCP, BMP-2 and MSCs have been used for various purposes in regenerative medicine. The ability of BMP-2 incorporated into GT sponges to form new bone could be enhanced by supplementation with MSCs. Moreover, the combination of GT sponge, BMP-2 and MSCs led to early bone formation. Due to the extensive *in vivo* bone formation together with reasonable GT absorption, the

composites could be applied in tissue engineering aimed at bone tissue reconstruction.

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Review Article

Forced Expression of Transcription Factors in Human Mesenchymal Cells to Promote Proliferation and Osteogenic Differentiation

Hiroe Ohnishi, Shunsuke Yuba, and Hajime Ohgushi

Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Amagasaki City, Hyogo 661-0974, Japan
Address correspondence to Hajime Ohgushi, hajime-ohgushi@aist.go.jp

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Abstract Mesenchymal stromal cells (MSCs) derived from human bone marrow have capability to differentiate into cells of mesenchymal lineage [7]. Especially, the differentiation capability towards osteogenic cells is very well known. We have already used the patient's MSCs for the treatments of osteoarthritis [8], bone necrosis [2] and bone tumor cases [5]. In most cases, the MSCs were culture-expanded from patient's fresh bone marrow cells, and then combined with porous ceramics. The MSCs/ceramics composites were further cultured in a medium containing dexamethasone to promote osteogenic differentiation of the MSCs. In this culture condition, we could detect bone forming osteoblasts together with mineralized bone matrix on the ceramics [7]; therefore, we could fabricate cultured bone using patient's bone marrow and ceramics. However, the proliferation and differentiation capability of the MSCs are variable and many lose their capabilities after several passages. With the aim of conferring higher capability on human bone marrow MSCs, some of transcription factors could be introduced into the MSCs. This review paper demonstrates the importance of the transcription factors to promote the osteogenesis as well as proliferation capabilities of human MSCs.

Keywords mesenchymal stem cells (MSCs); osteogenesis; differentiation; transcription factor; cell culture; induced pluripotent stem cells (iPS cells)

1 Introduction

Embryonic stem (ES) cells are cultured cells derived from the inner cell mass of blastocysts. ES cells have pluripotency in that they can differentiate into cells of all lineages. Murine ES cells are commonly maintained on primary mouse embryonic fibroblast feeder cells in culture medium supplemented with bovine serum and leukemia inhibitory factor (LIF). In the absence of LIF, murine ES cells differentiate spontaneously in serum containing culture medium [9]. In recent years, the mechanisms involved in maintaining the pluripotent state of human and mouse

embryonic stem cells have been shown to differ. Whilst mouse embryonic stem cells are dependent upon the LIF, human ES cells are dependent on basic fibroblast growth factor (bFGF) to maintain self renewal, pluripotency and prevent differentiation [3].

In addition to these factors, Oct4, Nanog and Sox2 are considered to form transcriptional regulatory circuitry for pluripotency and self-renewal of ES cells [4] (Figure 1). These observations demonstrate a possibility that forced expression of these transcription factors could render bone marrow mesenchymal stromal cells (MSCs) better growth and plasticity properties, because the MSCs have limited proliferation and differentiation capabilities (Figure 2). In this paper, I focused on transcription factors especially Sox2 and Nanog aiming to elucidate the role of human MSCs in bone tissue engineering strategy.

2 Expression of transcription factors in human MSCs

We used the construct in which IRES sequence was placed between the gene of interest and the Venus gene, a variant of GFP, so that expression of the construct was easily detectable during the cell culture. Sox2-expressing cells

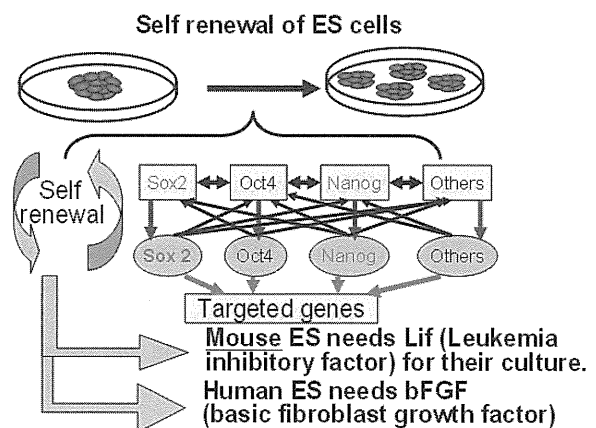


Figure 1: Transcription factors in ES cells.

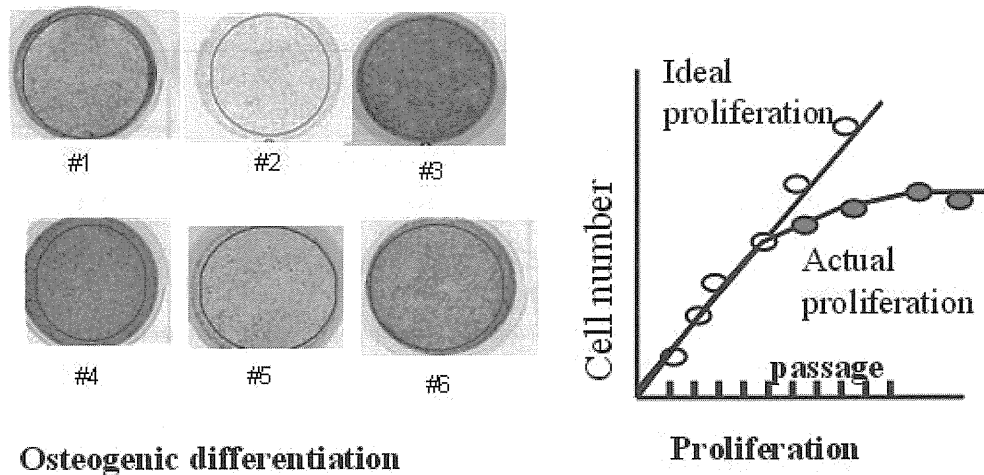


Figure 2: Proliferation and osteogenic differentiation capabilities among the patients.

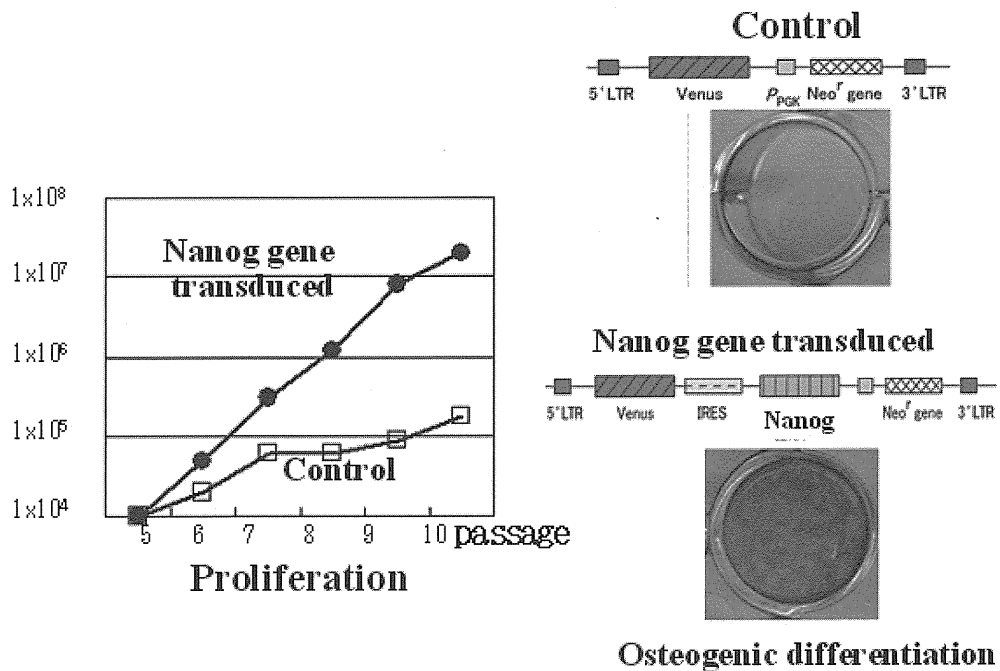


Figure 3: Nanog gene transduction into human mesenchymal cells.

showed distinct growth pattern in the presence of bFGF in culture media. In the presence of the bFGF protein in culture media, bone marrow MSCs show characteristic morphology changes, in which the cells become elongated in shape. In contrast, the Sox2-expressing MSCs responded to bFGF very differently, where the cells grew well as relatively round and small cells. The Sox2-expressing MSCs in the presence of bFGF had higher proliferation and osteogenic differentiation potential than control cells, in which only Venus was expressed [1].

We observed that Nanog-expressing MSCs were also relatively small and found that Nanog-expressing MSCs

showed significantly higher proliferation potential than control cells (Figure 3). We failed to observe significant effects of addition of bFGF in culture media in the case of Nanog-expressing cells in terms of both cell growth ability and cell morphology change. We also found that Nanog-expressing cells showed higher differentiation abilities for osteoblasts than control cells both in terms of both ALP activity and calcium deposition assayed by Alizarin Red staining (Figure 3) [1].

Recently Yamanaka et al. reported that pluripotent stem cells can be directly generated from mouse [11] and human fibroblasts [10] by the introduction of several defined

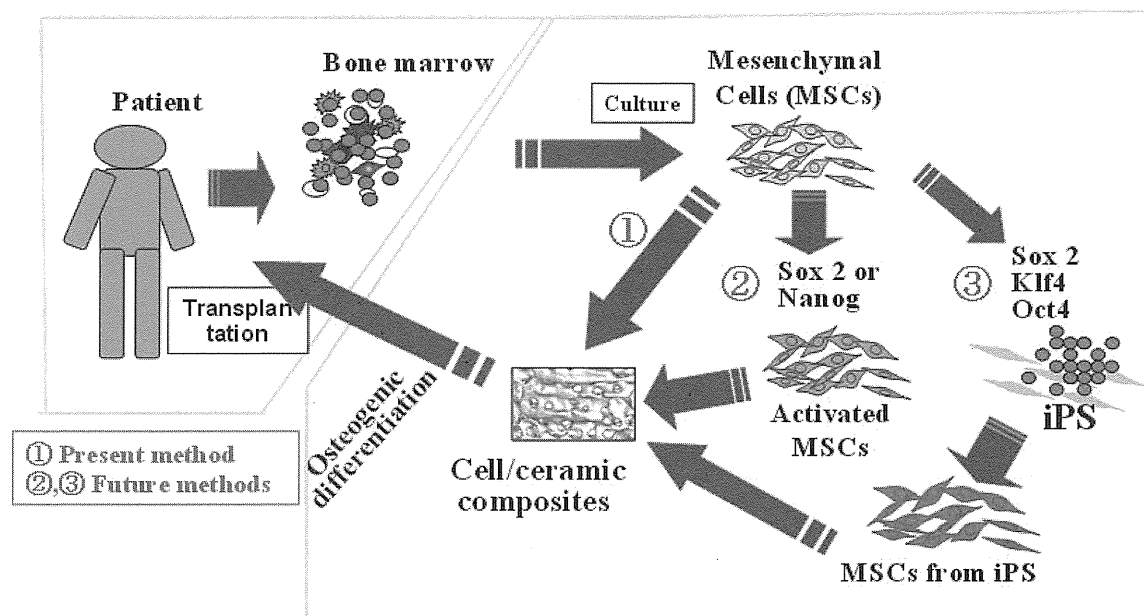


Figure 4: Current and future technologies for bone tissue engineering using MSCs.

genes, one of which was Sox2. Thomson et al. [12] also reported the generation of the induced pluripotent stem cells (iPS cells) by introduction of genes in which Nanog was included. These reports confirmed the importance of Sox2 and Nanog gene for the proliferation/differentiation capabilities of the stem cells. Though the single gene transduction is not sufficient to generate the iPS cells, the functional importance of Sox2 and Nanog for altering the cell status was clearly demonstrated.

3 Current and future technique for bone tissue engineering using MSCs

Our observations on the forced expression of Sox2 or Nanog in adult human MSCs are indeed consistent and succeeded to maintain the proliferation and osteogenic differentiation capabilities of otherwise senescent passaged cells by introducing single gene. We also experienced that these single gene expressing MSCs did not show teratoma formation, whereas the iPS cells have capability to show teratoma after their implantation. Based on our clinical experiences using patient's MSCs; serial passaged MSCs usually reduce their proliferation/differentiation capability (Figure 2). Therefore, our approach using single gene introduction could be an effective and realistic way of maintaining high quality of MSCs for regenerative medicine, especially for bone tissue regeneration. In addition, if we can solve the problem of teratoma formation after the iPS implantation, the iPS made from the patient cells could be available for bone tissue regeneration; especially in patients having severe bone diseases. Interestingly, we could generate the iPS cells from human MSCs and the iPS cells are indeed

pluripotent stem cells because they could differentiate into cell types of all three germ layers [6]. These strategies using single and multiple gene transduction could be available in a near future as seen in Figure 4.

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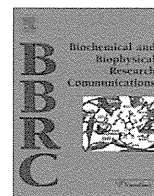
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Physical properties of mesenchymal stem cells are coordinated by the perinuclear actin cap

Takanori Kihara^{a,*}, Seyed Mohammad Ali Haghparast^a, Yuji Shimizu^a, Shunsuke Yuba^b, Jun Miyake^a

^aDepartment of Mechanical Science and Bioengineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan

^bHealth Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 3-11-46 Nakoji, Amagasaki, Hyogo 661-0974, Japan

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ABSTRACT

Mesenchymal stem cells (MSCs) have been extensively investigated for their applications in regenerative medicine. Successful use of MSCs in cell-based therapies will rely on the ability to effectively identify their properties and functions with a relatively non-destructive methodology.

In this study, we measured the surface stiffness and thickness of rat MSCs with atomic force microscopy and clarified their relation at a single-cell level. The role of the perinuclear actin cap in regulating the thickness, stiffness, and proliferative activity of these cells was also determined by using several actin cytoskeleton-modifying reagents. This study has helped elucidate a possible link between the physical properties and the physiological function of the MSCs, and the corresponding regulatory role of the actin cytoskeleton.

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1. Introduction

Mesenchymal stem cells (MSCs) are a heterogeneous population of stem/progenitor cells with the pluripotent capacity to differentiate into mesodermal and non-mesodermal cell lineages. They have generated a great deal of interest owing to their potential use in regenerative medicine and tissue engineering [1,2]. In order to maximize the potential of MSCs for biomedical applications, a more comprehensive characterization of MSCs is required. To achieve this goal, the development of a direct, relatively non-destructive method for measuring physical properties, which reflect the fate and physiological state of MSCs, is necessary. Population thickness (height) of adhered human MSCs may be related to various cell functions, such as proliferation activity and cell cycle [3,4]. However, a more comprehensive view of the physical properties of MSCs is required.

Mechanical properties such as cytoskeleton organization and elasticity, membrane tension, cell shape, and adhesion strength may play important roles in stem-cell fate and differentiation [5–7]. A change in mechanical properties, and in particular, in the stiffness (elasticity) of tissue cells, has been recognized as an indication of cancer [8]. Several techniques have been successfully

employed to study cell mechanical properties, including micropipette aspiration, magnetic twisting cytometry, optical traps, and atomic force microscopy (AFM) [9–11]. In particular, AFM can be used to analyze live cells [12,13] and to investigate their mechanical properties in physiological conditions in a relatively non-destructive manner [14].

Surface mechanical properties of a cell are dominantly defined by the actin cytoskeleton [15–18]. Stress fibers are specific determinants of cell mechanics [19], and cortical actin promotes cortical rigidity [20,21]. Dominant types of actin cytoskeleton differ by cell types as well as position. Therefore, the key determinants for cell mechanics probably vary across different cell types, and it is necessary to determine the character of cell mechanics in each cell type.

Previously, we reported that the elastic modulus of human MSCs decreased dramatically by actin de-polymerization, whereas the cell thickness increased [22]. The elasticity and the thickness of an actin de-polymerized MSC and a bare nucleus were almost the same. Thus, regulatory factors of nuclear thickness and cell elasticity are possibly the same, and these may be related to the actin cytoskeleton. Recently, Khatau et al. reported that a perinuclear actin cap, which is an actin filament structure that forms a cap or dome above the apical surface of the nucleus, tightly regulates the nuclear shape of adherent fibroblasts [23]. In this study, we examined the regulatory effect of the perinuclear actin cap on thickness and stiffness of adherent rat MSCs by using several actin cytoskeleton-modifying drugs.

Abbreviations: MSC, mesenchymal stem cell; AFM, atomic force microscopy; FBS, fetal bovine serum; CLSM, confocal laser scanning microscopy.

* Corresponding author. Fax: +81 6 6850 6557.

E-mail address: takanori.kihara@gmail.com (T. Kihara).

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2. Materials and methods

2.1. Materials

The AFM probe (ATEC-CONT; spring constant: 0.02–0.75 N/m) was purchased from Nanosensors (Neuchatel, Switzerland). Cell culture media was purchased from Nacalai Tesque (Kyoto, Japan), and fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Antibiotics were purchased from Sigma–Aldrich (St. Louis, MO). Fisher 344 male rats were purchased from Japan SLC (Shizuoka, Japan). ATP bioluminescence assay kit was purchased from Toyo Ink (Tokyo, Japan). Other reagents were purchased from Sigma–Aldrich, Wako Pure Chemical Industries Ltd. (Osaka, Japan), or Invitrogen (Carlsbad, CA).

2.2. Preparation and culture of rat MSCs

Rat MSCs were isolated and primarily cultured as described previously [24]. In brief, bone marrow cells were obtained from the femoral shafts of 7-week-old male Fisher 344 rats. The cells were obtained from at least 2 rats and mixed. The culture medium was Eagle's minimal essential medium (with Earle's Salt and L-glutamine) containing 15% FBS and antibiotics (100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B). The medium was renewed twice a week, and cells at passages 2–6 were used in this study.

Y-27632 (10 µM), blebbistatin (5 µM), cell permeable C3 transferase (20 ng/mL), calyculin A (0.15 nM), and wiskostatin (1 µM) were used to analyze inhibition or acceleration of the actin cyto-

skeleton. Cells were cultured in the medium containing these reagents for 2 days and then manipulated by AFM.

2.3. AFM measurements

Rat MSCs cultured on 35-mm culture dishes in the medium were manipulated by AFM (Nanowizard I; JPK Instruments AG, Berlin, Germany) at room temperature. The probe was indented into the cells up to 50 nN at 10 µm/s. The Young's modulus of the cell was calculated according to the Hertz model [25]. Although the Hertz model is accommodated in an elastic body, various kinds of cellular stiffness have been evaluated by this model as Young's modulus [26]. The force–distance curve at the region up to 400 nm of cell surface indentation was fitted to the model (Fig. 1A). Although the ATEC-CONT is a tetrahedral probe, the edge of the probe is conoidal. Thus, the following equation was used in the model of indentation:

$$F = \frac{E}{(1 - \nu^2)} \frac{2 \tan \alpha}{\pi} l^2$$

where F = force, l = depth of indentation, α = semi-opening angle of the cone (5°), ν = Poisson's ratio (0.5), and E = Young's modulus.

Cell thickness was derived from the length, from the cell contact point to the substrate (Fig. 1A). All experiments were performed in more than 10 cells, and each cell was examined at 9 different points within a size-defined grid on the nuclear region of the membrane. In this study, we assumed that the distribution of Young's moduli and the thickness of cells were in accordance with the

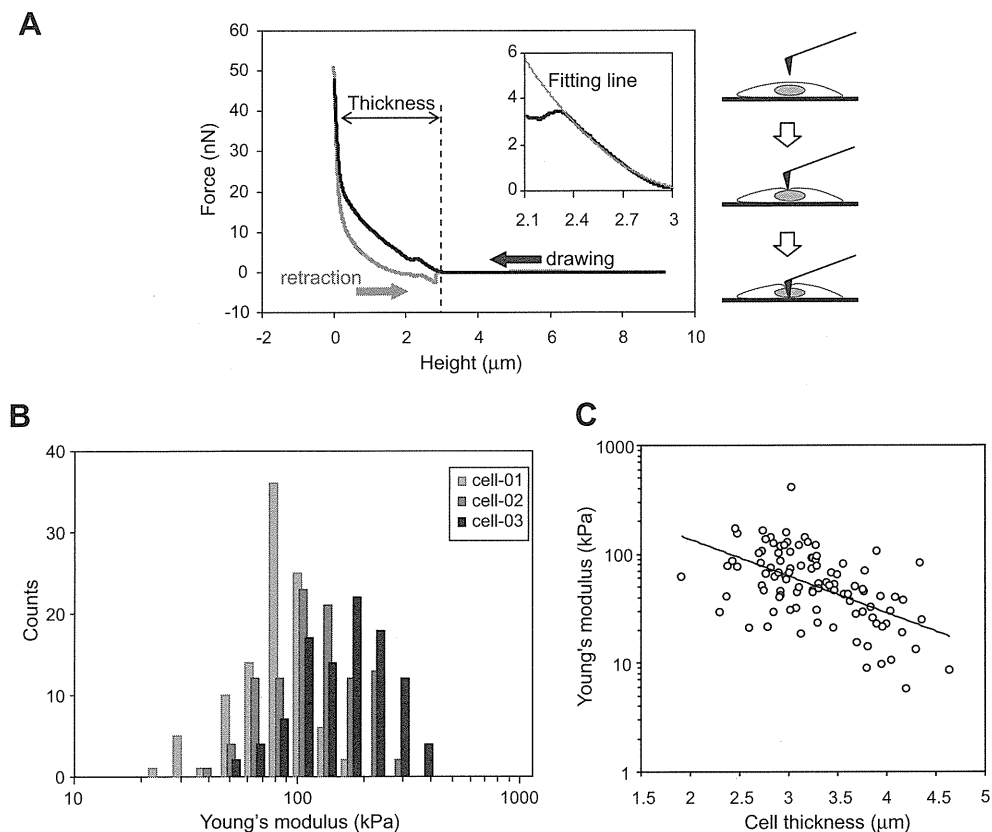


Fig. 1. Measurement of the cell stiffness and cell thickness of MSCs. (A) Details of the method of measuring cell stiffness and thickness by AFM. Typical force–distance curves obtained from the indentation of and pulling up from the surface of rat MSCs are shown at the left, and the schema for the AFM manipulation is shown at the right. Cell thickness is represented as the distance between the contact point of cell surface and substrate. Stiffness is calculated from the force curve at the region of indentation (up to 400 nm) by fitting it to the Hertz model (inset). (B) Distribution of Young's modulus of the same MSCs. Young's modulus of each cell (total of 3 cells) was measured 100 times repeatedly. The distribution pattern of Young's modulus of rat MSCs shows a log-normal pattern. (C) Relationship between the median value of Young's modulus and cell thickness in rat MSCs. Each data point is for one cell (total of 100 cells). The line shows approximated curve for the points ($R = 0.35$, $P = 0.0004$).

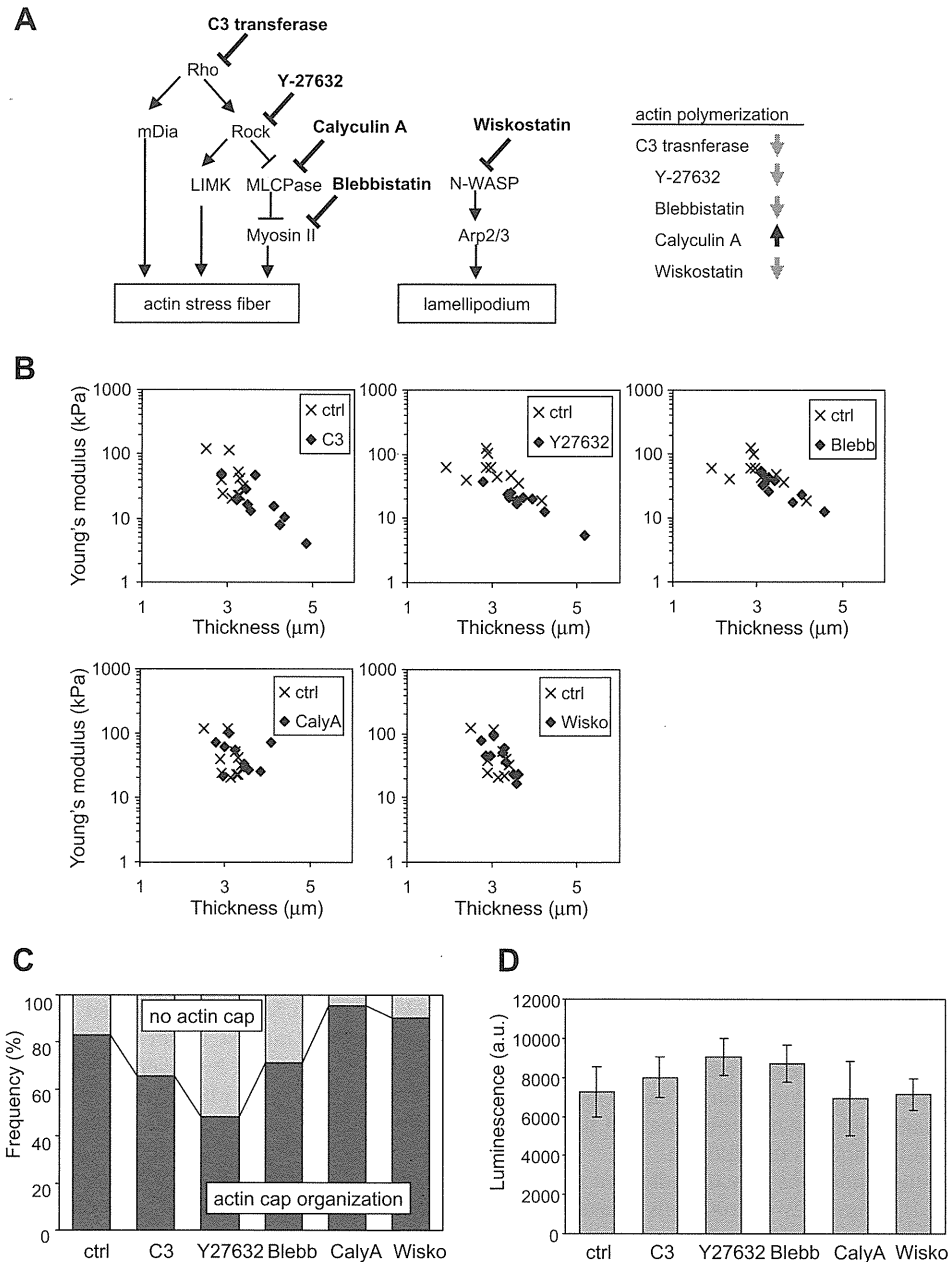


Fig. 2. Role of the actin cytoskeleton in regulating the cell properties of rat MSCs. (A) Schematics of effects of the used reagents on the actin cytoskeleton. C3 transferase, Y-27632, and blebbistatin inhibit actin polymerization and stress fiber formation. In contrast, calyculin A inhibits myosin light chain phosphatase and leads to actin polymerization, resulting in stress fiber and cortical actin formation. Wiskostatin inhibits lamellipodium formation. (B) Influence of the agents on Young's modulus and thickness of rat MSCs. Each point indicates a data point from each cell (a total of 10 cells for each condition). C3 transferase (C3), Y-27632 (Y27632), and blebbistatin (Blebb) decreased surface rigidity of rat MSCs and increased cell thickness. On the other hand, calyculin A (CalyA) and wiskostatin (Wisko) had little impact on these physical properties of MSCs. (C) Organization of perinuclear actin cap of rat MSCs following treatment with the agents. The frequencies of organization of perinuclear actin cap are shown. The presence of the perinuclear actin cap at each condition was determined from more than 23 cells. The typical images for organization and disruption or not of the actin cap are shown in Supplementary Fig. S1. C3 transferase (C3), Y-27632 (Y27632), and blebbistatin (Blebb) decreased actin cap organization; calyculin A (CalyA) and wiskostatin (Wisko) increased actin cap organization. (D) Proliferation activity of rat MSCs cultured with various reagents. The cell number at 6 days was evaluated by measuring the cellular ATP contents with chemiluminescence system. C3 transferase (C3), Y-27632 (Y27632), and blebbistatin (Blebb) slightly increased the cell numbers; on the other hand, calyculin A (CalyA) and wiskostatin (Wisko) did not influence the proliferation.

log-normal distribution, and a median value of 9 points was adopted for the Young's modulus and the thickness of each cell.

2.4. Cell proliferation assay

Approximate cell numbers were determined using an ATP bioluminescence assay kit according to the manufacturer's instructions. Rat MSCs were plated at a density of 1×10^3 cells/well into white 96-well culture plates and cultured in the culture medium containing the cytoskeleton-modifying reagent for 6 days. The

ATP bioluminescence reagent was added to the wells, and the cultured cells were shaken for 1 min and then incubated for another 10 min at 25 °C. Luminescence was measured with Synergy HT (BioTek, Winooski, VT).

2.5. Evaluation of perinuclear actin cap

The perinuclear actin cap was evaluated by staining the actin filaments of rat MSCs. Cells cultured for 2 days with the cytoskeleton-modifying reagent were fixed with 4% paraformaldehyde,

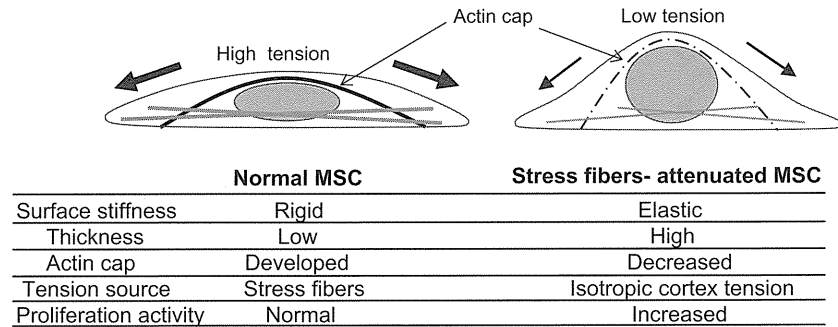


Fig. 3. Overview of the physical properties of MSCs. In normal MSCs, developed perinuclear actin caps generate highly surface tension and press tightly against the nucleus, giving the cells a rigid surface stiffness and flat thickness. In contrast, in stress fibers-attenuated MSCs, the surface tension was decreased and the cell surface was boosted by the nucleus, resulting in the cells with elastic surfaces and a high thickness. Furthermore, elastic and tall cells tended to also have high proliferation activities.

permeabilized with 0.5% Triton X-100, and then stained with FITC-labeled phalloidin for actin filaments and propidium iodide for nucleus. Specimens were observed by confocal laser scanning microscopy (CLSM; FV-1000; Olympus, Tokyo, Japan) in 0.5 μm serial sections. The presence of the perinuclear actin cap was determined from the status of actin filaments over the nucleus in more than 23 cells for each condition (Supplementary Fig. S1).

3. Results

3.1. Relationship between stiffness and thickness of rat MSCs

First, we examined the relation between every AFM-measured Young's modulus and the thickness of each rat MSC (Supplementary Fig. S2). Compared with the thickness, the measured Young's modulus varied widely for each AFM manipulation (Supplementary Fig. S2). This was due to the fact that cellular mechanical properties are local and can change dramatically based on the location being probed [27]. Since it was difficult to determine the cell stiffness from these scattered Young's moduli, we examined the distribution of Young's moduli of rat MSCs in single cells and found that the distribution of Young's moduli corresponded to a log-normal pattern of distribution (Fig. 1B). Therefore, we selected the median value of the widely spread Young's moduli of each cell in this study, because the median provides a better estimate of the target value than the mean in a log-normal distribution.

Thickness versus Young's modulus of each adherent rat MSC was then plotted (Fig. 1C). Analyses of 100 cells revealed that Young's modulus of various adherent rat MSCs varied largely, and that it had a weak inverse correlation with cell thickness in each cell (Fig. 1C). The correlation coefficient R between these 2 factors was 0.35 ($P = 0.0004$). In short, flat, adherent MSCs tended to be rigid, whereas tall MSCs showed a relatively elastic property.

3.2. Regulation of cell stiffness and thickness of rat MSCs by actin cytoskeleton

An intact actin cytoskeleton contributes a major part of cell stiffness, yet there are several forms of actin cytoskeleton in a cell, e.g., stress fibers, lamellipodia, filopodia, and cortical actin. Here, we examined the effect of actin stress fibers and lamellipodia on stiffness and thickness of adherent rat MSCs, using several pharmacological agents that affect the actin cytoskeleton (Fig. 2A). We used gentle concentrations of all agents to avoid inducing obvious morphological changes (Supplementary Fig. S3).

Rho inhibitor C3 transferase, ROCK inhibitor Y-27632, and myosin II ATPase inhibitor blebbistatin prevent and attenuate stress fiber formation [28–30]. The Young's moduli of the cells treated with these agents decreased relatively while their thickness increased (Fig. 2B). On the other hand, the properties of the cells treated with

myosin light chain phosphatase inhibitor calyculin A [31], which activates actomyosin formation and enhances actin polymerization in stress fibers, lamellipodia formation, and cortical actin, were hardly altered (Fig. 2B). Moreover, cells treated with the N-WASP inhibitor wiskostatin, which inhibits lamellipodia formation [32], showed no changes in either the Young's modulus or thickness (Fig. 2B). Thus, the cell stiffness and thickness of adherent rat MSCs were strongly affected by attenuated actin stress fibers, but were barely influenced by activation of actomyosin formation or inhibition of lamellipodia formation.

3.3. Perinuclear actin cap correlation with cell thickness and stiffness of rat MSCs

Actin filament structures that form a cap or dome located above the apical surface of the nucleus, referred to as the perinuclear actin cap, regulate the nuclear shapes of adherent fibroblasts [23]. The perinuclear actin cap is specifically disorganized or eliminated by the inhibition of actomyosin contractility. To identify a possible role of the perinuclear actin cap in regulation of stiffness and thickness of rat MSC, we examined the changes in the actin cap organization in rat MSC under the presence of each actin cytoskeleton-modifying agent by observing with CLSM.

The majority of rat MSCs had a well-developed perinuclear actin cap under normal culture conditions (Fig. 2C and Supplementary Fig. S1), rendering a flat shape to rat MSCs. Analysis of collected images demonstrated that treatment with actin stress fiber-formation inhibitors, namely, C3 transferase, blebbistatin, and particularly Y-27362, led to an increase in the number of cells with no actin cap as compared to the control cells (Fig. 2C and Supplementary Fig. S1). In contrast, more MSCs possessed an organized actin cap after treatment with calyculin A and wiskostatin (Fig. 2C). Attenuation of actin stress fibers results in reduction of perinuclear actin cap organization, which corresponds to the data of the cell thickness and stiffness of rat MSCs (Fig. 2B, C). On the other hand, augmentation of actin cap organization had little impact on the cell thickness and stiffness of rat MSCs (Fig. 2B, C).

3.4. Actin cytoskeleton affects proliferation activity of rat MSCs

Population thickness of adhered human MSCs may be related to proliferation activity at the donor level [3]. We thus examined the possible affect of actin cytoskeleton on proliferation activity of rat MSC population to determine whether cell stiffness relates with proliferation. Adding the agents to the cell culture media revealed that Y-27632, C3 transferase, and blebbistatin, which attenuate the actin stress fibers and the perinuclear actin cap, slightly increased the growth activity of rat MSCs (Fig. 2D). On the other hand, upregulation of perinuclear actin cap formation, as caused by treating the MSCs with wiskostatin or calyculin A, had little effect on cell

proliferation (Fig. 2D). These data consist with the results of cell thickness and stiffness. We therefore propose that actin cap organization regulates the proliferation activity and cell thickness and stiffness of rat MSC.

4. Discussion

In this study, we experimentally demonstrated two major points relevant to surface stiffness of substrate-adhering rat MSCs. One is that cell stiffness and cell thickness showed an inverse correlation at a single-cell level (Fig. 1C). The second is that the perinuclear actin cap organization regulates the cell stiffness, thickness, and possibly proliferation activity of rat MSCs (Fig. 2). A schematic diagram of our findings is shown in Fig. 3. Originally, MSCs adhere flat on a given substrate, and their actin stress fibers and perinuclear actin cap are well developed. A developed actin cap flattens out the nucleus and increases surface stiffness. On the other hand, cells with diminished stress fibers also lose their actin cap, with a resulting decrease in surface stiffness, and the nucleus boosts the cell height according to the plasticity of nuclear lamin A [23]. Thus, although surface stiffness and cell thickness are essentially different physical properties of cells, the perinuclear actin cap coordinates these properties in MSCs. However, these cell properties were not affected by augmentation of the actin cap organization after treatment with either calyculin A or wiskostatin (Fig. 2). In particular, calyculin A is an activator for actomyosin formation, and it increases surface stiffness in *Drosophila* embryonic S2R+ cells by accelerating cortical actin formation [21]. Cortical actin determines isotropic cortical tension of non-adherent or mitotic cell [33], and the surface stiffness of some cells is mainly determined by this cortical actin formation [21]. Thus, it is believed that anisotropic surface tension that arises from the perinuclear actin cap is higher than the isotropic cortical tension in MSCs, which would explain why behavior of the stiffness of rat MSCs differed from the above cell. In addition, further-developed stress fibers and actin cap of MSCs may induce partial buckling of the actin cytoskeleton [34] or fluidization of cell [35]; the physical properties are unaffected by augmentation of the actin cap formation. Interestingly, the stress fiber-attenuated MSCs tended to show a high proliferation activity (Fig. 2). This result corresponds with the previous study relating cell thickness with proliferation activity in human MSCs [3,4]. Our results additionally show that the states of the actin cap and actin stress fibers in MSCs are potent regulators of the proliferation activity of each cell.

It is well known that mechanical properties of cell environments control cell life [36,37]. In particular, commitment of differentiation lineage of MSCs is specified by matrix elasticity which is sensed by actomyosin contraction [38]. Furthermore, the surface stiffness of MSCs changes depending on the substrate elasticity. The surface stiffness of MSCs also changes during cell differentiation [39]. Our present study reveals that the surface stiffness of MSCs is regulated by actin stress fibers, including the perinuclear actin cap, and is related with proliferation activity. Thus, the mechanical properties of MSCs are potent indicators for their cell behavior and physiological functions. The evaluation of cell quality and estimation of cell activities of MSCs are essential cytotechnology for applications in regenerative medicine and tissue engineering. We predict that in the future non-destructive and high-speed methods of measuring mechanical properties of MSCs will become an essential part of the cytotechnology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.04.022.

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II. 基礎研究の進歩と展望

間葉系幹細胞研究(臨床研究からみた
同種間葉系幹細胞移植)大串 始^{1,2} 有馬 靖佳³ 竹谷 健⁴

Regenerative therapy using allogeneic mesenchymal stem cells

^{1,2}Hajime Ohgushi, ³Nobuyoshi Arima, ⁴Takeshi Taketani¹Department of Orthopedics, Ookuma Hospital²Research Institute for Cell Engineering,

National Institute of Advanced Industrial Science and Technology (AIST)

³Department of Hematology, Kitano Hospital⁴Division of Blood Transfusion, Shimane University Hospital

Abstract

The use of mesenchymal stem cells(MSC) for tissue and organ regeneration offers advantages because the MSC contain multipotent progenitor cells and reported to be immunoprivileged as well as immunosuppressive. Therefore, cell therapy with allogeneic MSC has been reported as a promising treatment for severe acute graft versus host disease(GVHD). We reported a pilot study for GVHD treatments using a small number of allogeneic MSC. We also reported that MSC can show osteogenic differentiation capability when implanted *in vivo* as well as cultured *in vitro*. Based on these findings, we attempted to use allogeneic MSC for the treatment of genetic disorder of hypophosphatasia patient. Present paper summarizes our clinical experiences of allogeneic MSC for the purpose of regenerative medicine.

Key words: mesenchymal stem cells(MSC), allogeneic cell, graft versus host disease (GVHD), hypophosphatasia, bone tissue engineering

はじめに

骨内に存在する骨髄組織を骨外の場に移植すること(heterotopic implantation)により, 移植されたドナー細胞が増殖・骨分化を引き起こして新生骨形成を示すことは古くより知られ, ロシアのFriedensteinらの先駆的な業績がある¹⁾. 興味深いことに, この骨組織には造血組

織も形成されるが, 造血組織はレシピエント由来である. すわち, 骨髄には造血幹細胞以外の幹細胞が含まれ, Friedensteinらはこの幹細胞をstromal mechanocyteと称していたが, 現在では間葉系幹細胞(mesenchymal stem cells: MSC)という呼称が一般的となっている²⁾. このMSCは骨・軟骨に分化するのみならず, 心筋(中胚葉), 神経(外胚葉), 肝臓(内胚葉)などへ

¹医療法人大隈病院 整形外科 ²産業技術総合研究所 健康工学研究部門 ³財団法人田附興風会北野病院 血液内科 ⁴島根大学医学部附属病院 輸血部

の分化能も有することが報告され、これらの分化能を利用して、患者自身のMSCを用いた種々の再生医療が開始されている。また、MSCは骨髄のみならず脂肪を含む他の種々組織にもその存在が知られるとともに、免疫反応を抑制する作用を有することも報告され、患者自身のMSCでなく同種(他人)MSCを用いた再生医療の可能生も報告されている。

本稿では著者らの臨床経験を中心にして同種MSCを用いた再生医療、特に免疫反応や骨再生について論じる。

1. 自己MSCを用いる再生医療

MSCを用いる再生医療において、多量の同種(他人由来)MSCが保存・利用可能であれば、患者自身から細胞を採取する必要もなく、また随時に移植することが可能であり、再生医療は容易となる。この点において、著者らは増殖・分化能を有するヒトMSCが長期にわたって冷凍保存できうことを確認している³⁾。しかし、同種細胞を用いるには拒絶反応や患者には存在しないドナー細胞由来の感染症の危険性がある。そのため、患者自身の自己細胞を用いるのが現実的で安全である。実際、著者らは患者の骨髄からMSCを培養増殖させ、このMSCを用いて骨・軟骨再生のみならず⁴⁻⁶⁾、心再生にも応用している⁷⁾。特に、奈良県立医科大学整形外科とともに2001年より世界に先駆けて、患者由来MSCが組み込まれた人工関節を用いる関節再生を行っている。これらの症例において感染はみられず、更に人工関節そのものに起因する痛みもなく順調に経過し、自己MSCを用いることの安全性を証明している。また、この自己MSCを用いての安全性について、骨・関節領域(整形外科、歯科、口腔外科)で実際に再生医療を行っている研究者が集まり、これまでの経緯などについて議論し合った結果を最近報告した⁸⁾。この報告書にみられるように、これまで国内において、自己MSCを用いて治療を行った骨腫瘍、関節症、歯周病、軟骨欠損などの患者は総計約200症例であり、すべての症例で移植細胞に起因する感染や腫瘍発生はみられな

かった。

以上、これら自己MSCを用いる再生医療は安全に行えるものであり、今後も幅広く行われると思われる。しかし、自己MSCは当然ながら患者自身に由来するものであり、増殖能や分化能には個人差がある。また、患者自身の遺伝子異常に起因する疾患には当然ながら自己細胞を用いることができない。これらの問題点の克服には同種MSCを用いる可能性についても考慮する必要がある。

2. MSCによる免疫抑制効果

著者らが臨床に用いている種々の患者MSCの細胞表面抗原は血球・血管系細胞のマーカーであるCD14, CD31, CD34, CD45は陰性でCD13, CD29, CD44, CD71, CD90, CD105は陽性である。このように、いわゆる間葉系の性質を有する。更に、主要組織適合抗原に関して多くの細胞に発現するといわれるHLA class Iは陽性であるが樹状細胞、活性化T細胞、B細胞などの抗原提示細胞などに発現しているHLA-DRは陰性である^{3,4)}。また、他の報告ではMSCはT細胞やB細胞、NK細胞、抗原提示細胞といった免疫担当細胞の活性を抑制することや、リンパ球の増殖やサイトカイン産生を抑制し、プロスタグランジンやIL-10などの抗炎症物質を産生するとされている⁹⁾。著者らもリンパ球の増殖培養系において、stimulatorとして同種のリンパ球を添加することによりその増殖を刺激したが、同種MSCは増殖刺激がないことを報告している¹⁰⁾。これらより、MSCは免疫反応を抑制することが期待される。例えば、移植ドナーがレシピエントの体を攻撃する移植片対宿主病(graft versus host disease: GVHD)は免疫を担う組織が含まれる造血幹細胞移植(骨髄移植)後に起こることが知られている。このGVHDによる免疫反応をMSCが抑制することが報告され¹¹⁾、既にアメリカのオサイリス社が同種MSCを用いてのGVHD治療の臨床第III相試験を行っている¹²⁾。日本ケミカルリサーチ社も、オサイリス社の技術を用いての臨床試験を日本国内において行っている¹³⁾。なお、上記の

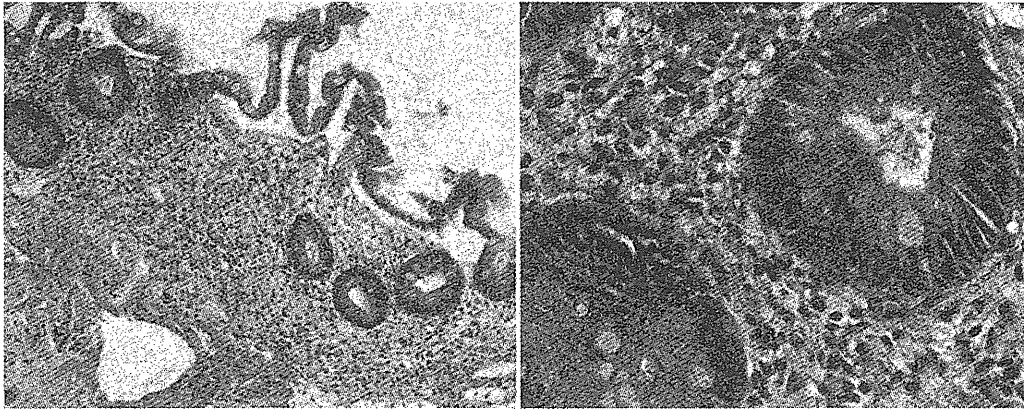


図1 同種 MSC 投与後 17 日目の腸管のバイオプシー(右図は左図の強拡大)
アポトーシスを示すリンパ球浸潤が著減している。

オサイリス社は予備結果ではステロイド抵抗性の肝 GVHD, 消化管 GVHD において反応率に明らかな改善が示されたものの主要評価項目は達成できなかつたと発表した¹²⁾。これらの GVHD の治療は同種 MSC を患者へ全身投与することであり, 多量の MSC を必要とする。著者らは, 少数のドナー細胞による GVHD 治療の可能性を探るべく, 数例の患者に対して同種 MSC を経動脈的に投与した¹⁴⁾。この投与は安全に行われ, 腸管においてアポトーシスを示す腺管周囲へのリンパ球浸潤が著減しており, GVHD の改善が示唆された(図 1)。しかし有効であったのは一時的で, 長期にわたる臨床効果に課題を残した。また, Sudres らは *in vitro* で同種抗原による T 細胞の増殖を抑制することを確認できた MSC が GVHD に対しては治療効果のないことを報告している¹⁵⁾。以上より, GVHD への MSC 投与による効果については今後も検討を要する。

3. 同種 MSC による骨再生の基礎研究

以上, MSC は少なくとも *in vitro* において免疫反応を modulate する可能性を示している。また, 同種 MSC を用いての骨再生が報告されている。例えば, Arinze らはイヌの骨欠損モデルにおいて同種 MSC が欠損部を治癒することを報告している¹⁶⁾。また, *in vitro* でのリンパ球増殖を抑制することの確認を行ったラットの同種 MSC がラット脊椎固定に有用であること

も報告されている¹⁷⁾。これらの報告は移植された同種 MSC による新生骨形成の可能生を示している。しかし, これらのモデルでは MSC が移植された部位には既存の骨形成細胞である骨芽細胞が存在し, ドナー MSC が生着して骨形成を営んだのか, あるいはレシピエントの細胞による骨形成か不明の点がある。

そこで著者らは既存の骨の影響を受けないように, 種々のドナー MSC を細胞担体であるハイドロキシアパタイトと混和(複合体形成)して Fisher344(F344)ラットの皮下へ移植する実験を行った¹⁸⁾。ドナーのラットとして, F344 ラットに加えて, F344 ラットと同種で遺伝的に近い(minor mismatch) Lewis ラットと遺伝的に遠い(major mismatch) ACI ラットを使用した。F344 ラット同士は同系(syngeneic)であるので, F344 ラット由来のドナー MSC はレシピエントの F344 ラットにとって自己細胞と同等として認識される。一方, Lewis ラット, ACI ラットの MSC はレシピエントである F344 ラットにとっては同種(他家)細胞として認識される。なお, レシピエントへの移植条件をそろえるために, 3 種の異なった MSC は同一レシピエントラットに移植した。また, 免疫抑制剤(tacrolimus)を与える群と与えない群も作製した。移植後 4 週間でこれら MSC/ハイドロキシアパタイト複合体を摘出し, 新生骨の形成について評価を行った。tacrolimus 投与群において, F344, ACI および Lewis の MSC を用いたすべての群で新

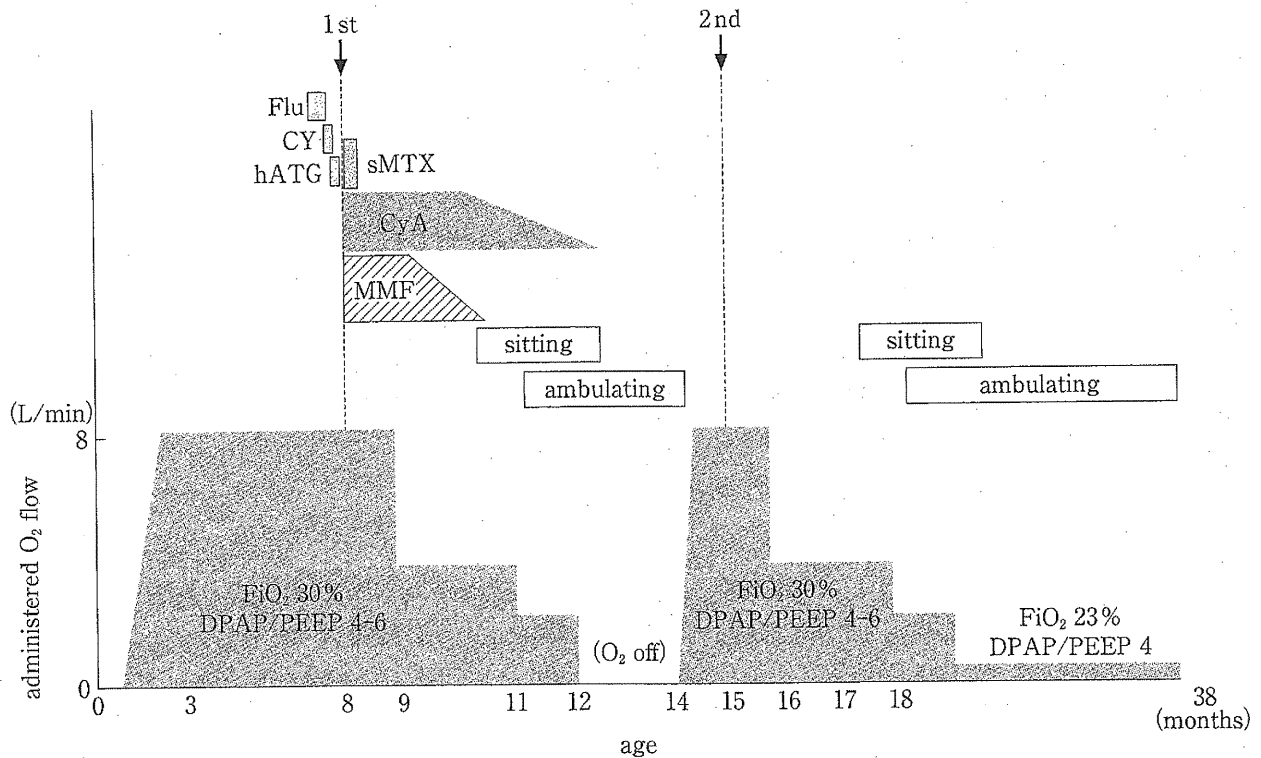


図2 低ホスファターゼ症に対する同種間葉系幹細胞移植治療

呼吸不全のため、生後3カ月でDPAP(呼吸気変換方式陽圧呼吸)を行った。8カ月で同種の骨髄移植と培養骨ならびにMSC移植を行った。その後呼吸状態は改善し、酸素投与を必要としなくなった。14カ月より再度呼吸状態が悪化し、15カ月でMSCのみの移植を行い呼吸状態は改善した。

(文献¹⁸⁾より転載)

生骨の形成が示されたのに対し、tacrolimus非投与群では同系移植であるF344のMSCを用いた群にのみ新生骨が確認された¹⁰⁾。LewisはACIに比べ、F344ラットの移植抗原に関して遺伝的に近縁である。しかし、Lewisの細胞でも骨形成を示さないことは、同種MSCを用いた骨再生において、たとえminor mismatchのドナー細胞を用いる場合においても免疫抑制剤の必要性があることが示唆された。すなわち、MSCが免疫反応を抑制するとしても、同種MSCが移植により単純に生着するとは考えられない。

4. 同種MSCによる骨再生の臨床研究 (遺伝子疾患患者に対する同種細胞を用いた再生治療)

上記に述べたことは、免疫抑制剤を使用して移植免疫反応を調整することにより、同種MSCを用いた再生治療が可能であることを示唆している。例えば、まれな疾患である低ホ

スファターゼ症(hypophosphatasia)は、組織非特異的アルカリホスファターゼ(tissue non-specific alkaline phosphatase: TNSALP)遺伝子の異常による骨の形成不全に起因する遺伝子疾患である。そのため、患者自身の細胞を用いての治療はできない。また、著者らはMSCの増殖技術のみならず、培養MSCから*in vitro*で骨形成(再生培養骨)を生じる系を確立している。そこで、低ホスファターゼ症に対して同種MSCと培養骨を用いての治療を試みた¹⁸⁾。なお、過去に数例骨髄移植を併用したMSCの治療を低ホスファターゼ症に対して行った例があり¹⁹⁾、著者らも骨髄移植を併用した。患者の父親をドナーとして骨髄を採取し、MSCおよび骨芽細胞の培養ならびに再生培養骨の作製を行った。患者の父親は本遺伝子変異のキャリアであるが、血清ALP値は正常値を示すのみならず、骨分化能を有することも事前に確認した。

図2にみられるように、生後8カ月の患者に

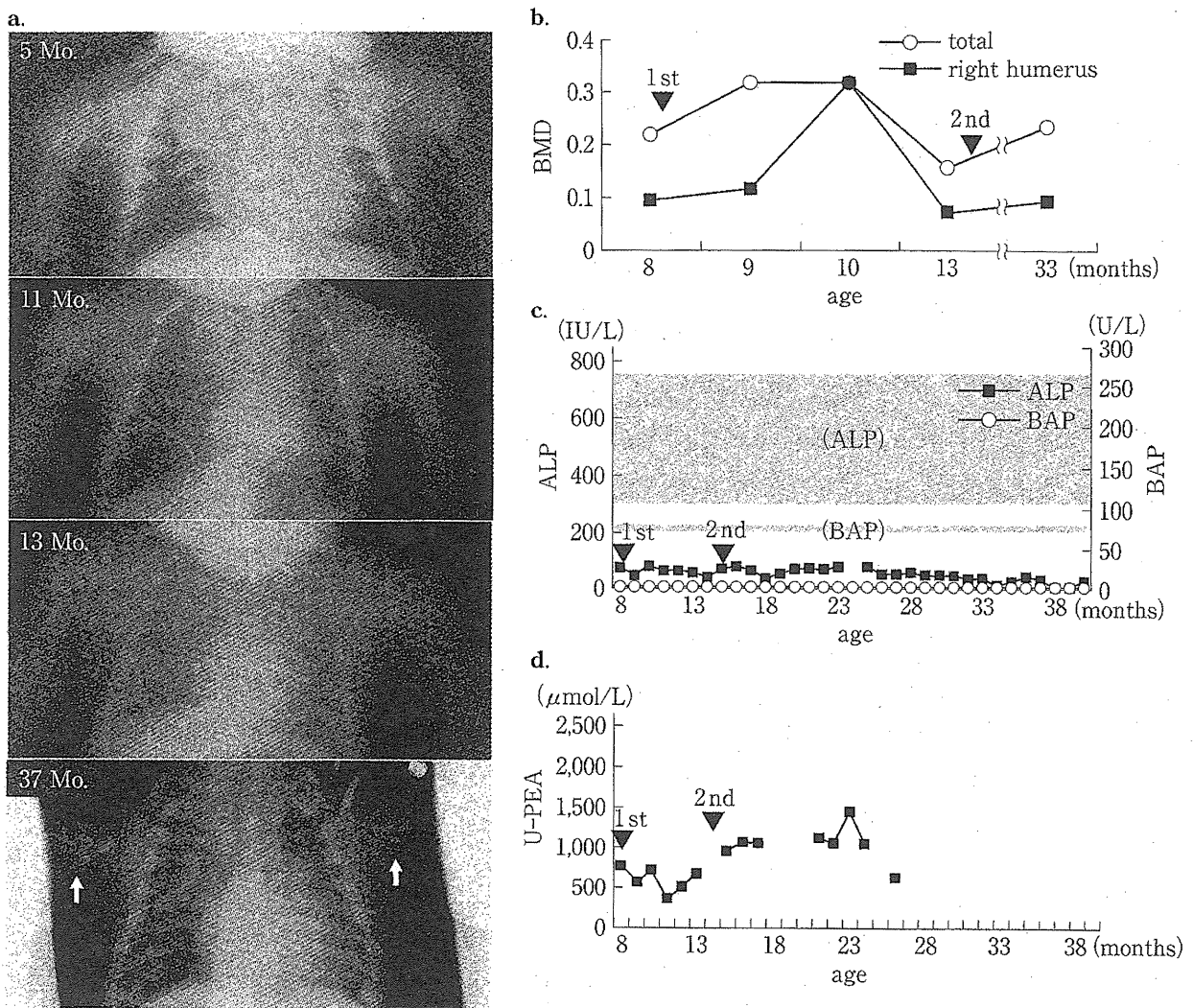


図3-a 生後5カ月から37カ月までの経時的な胸部X線像
 5カ月のX線で肋骨の変形、脆弱性ならびに上腕骨骨幹端にくる病様変化がみられる。37カ月ではこの骨幹端に骨化像がみられる。
-b 全身と上腕の骨密度測定
-c 血中のアルカリホスファターゼ(ALP)活性と骨特異的アルカリホスファターゼ(BAP)活性
 灰色部分は正常値を示す。
-d 尿中 phosphoethanol amine (PEA)
 (文献¹⁸⁾より転載)

対し、免疫抑制剤投与下でドナー骨髄移植、MSCの全身投与、および培養骨芽細胞、再生培養骨の移植を行った。術後1カ月後、上腕骨ならびに全身の骨密度も軽度上昇し(図3)、本症にみられる呼吸不全も改善した。しかしながら術後7カ月後には再度呼吸状態が悪化したため、生後15カ月でドナーMSCのみを経静脈的に追加投与した。1回目の投与と同様、投与

の1カ月後に呼吸状態の改善がみられた。つまり、MSCのみの投与で1回目の投与と同等の効果がみられた。再手術時に得られた再生培養骨の組織解析を行ったところ、ドナー細胞が骨を形成し、移植後7カ月においても患者体内で生着していることが証明できた。更に、Fluorescence *in situ* hybridization (FISH)法で新生骨基質に含まれる骨細胞がドナー細胞と患者細胞の